Endothelin receptor antagonists attenuate the inflammatory response of human pulmonary vascular smooth muscle cells to bacterial endotoxin

Jürgen Knobloch, Maria Feldmann, Chiara Wahl, David Jungck, Jürgen Behr, Erich Stoelben, Andrea Koch

Department of Internal Medicine III for Pneumology, Allergology, Sleep- and Respiratory Medicine, University Hospital Bergmannsheil, Bochum, Germany (J.K., M.F., C.W., D.J., A.K.), Department Internal Medicine V, Comprehensive Pneumology Center, Member of the DLZ, University of Munich, Germany (J.B.) and Department of Thoracic Surgery, Lungenklinik Merheim, Kliniken der Stadt Köln, Cologne, Germany (E.S.)
Running Title Page

Running title: Endothelin receptor antagonists suppress LPS responses

Corresponding author: Priv.-Doz. Dr. med. Andrea Koch
Department of Internal Medicine III for Pneumology, Allergology, Sleep- and Respiratory Medicine, University Hospital Bergmannsheil
Bürkle-de-la-Camp-Platz 1; D-44789; Bochum; Germany
phone: +49-234-3023530
e-mail: andrea.koch@bergmannsheil.de

number of text pages: 39
number of tables: 1
number of figures: 6
number of references: 51
number of words in the abstract: 250
number of words in the introduction: 750
number of words in the discussion: 1833

Section assignment: Inflammation, Immunopharmacology, and Asthma
Abbreviations:

COPD; chronic obstructive pulmonary disease

ET-1; endothelin-1

ETAR; endothelin A receptor

ETBR; endothelin B receptor

ERAs; endothelin receptors antagonists

LPS; lipopolysaccharide

mCD14; membrane-bound CD14

M-LPS; LPS with an undefined mixture of long and short LPS forms

PH, pulmonary hypertension

PVSMCs; pulmonary vascular smooth muscle cells

R-LPS, rough LPS

Re-LPS; shortest form of rough LPS

sCD14; soluble CD14

S-LPS; smooth LPS

SMA; α-smooth muscle actin

SMC; smooth muscle cell

SMMHC; smooth muscle myosin heavy chain

TLR4; toll-like receptor 4
TNFα; tumor necrosis factor α

VSMCs; vascular smooth muscle cells
Abstract

Bacterial infections induce exacerbations in chronic lung diseases, e.g. COPD, by enhancing airway inflammation. Exacerbations are frequently associated with right heart decompensation and accelerate disease progression. Endothelin receptor antagonists (ERAs) might have therapeutic potential as pulmonary vasodilators and anti-inflammatory agents, but utility in exacerbations of chronic lung diseases is unknown. We hypothesized that cytokine releases induced by lipopolysaccharide (LPS), the major bacterial trigger of inflammation, are reduced by ERAs in pulmonary vascular smooth muscle cells (PVSMCs). Ex-vivo cultivated human PVSMCs were pre-incubated with the endothelin-A-receptor selective inhibitor ambrisentan, with the endothelin-B-receptor selective inhibitor BQ788 or with the dual blocker bosentan before stimulation with smooth LPS (S-LPS), rough LPS (Re-LPS) or a mixture of long and short forms (M-LPS). Expression of cytokines and LPS receptors (TLR4, CD14) were analyzed via ELISA and/or qRT-PCR. All LPS forms induced IL-6-, IL-8- and GM-CSF-release. Bosentan and BQ788 inhibited M-LPS-induced release of all cytokines and soluble CD14 (sCD14) but not TLR4 expression. Ambrisentan blocked M-LPS-induced IL-6 release but not IL-8, GM-CSF or LPS receptors. IL-8 release induced by S-LPS, which requires CD14 to activate TLR4, was blocked by bosentan and BQ788. IL-8 release induced by Re-LPS, which does not require CD14 to activate TLR4, was insensitive to both, bosentan and BQ788. In conclusion, PVSMCs contribute to inflammation in bacteria-induced exacerbations of chronic lung diseases. Inhibition of the endothelin-B-receptor suppresses cytokine release induced by long/smooth LPS due to sCD14 down-regulation. ERAs, particularly when targeting the endothelin-B-receptor, might have therapeutic utility in exacerbations of chronic lung diseases.
Introduction

Airway inflammation drives progression of chronic inflammatory lung diseases, like chronic obstructive pulmonary disease (COPD) (Barnes 2008a; Stockley, 2009). Airway infections with gram-negative bacteria induce exacerbations in COPD and other chronic lung diseases (Jackson et al., 2011; Decramer et al., 2012). This aggravates inflammation and inflammation-induced airway remodeling leading to accelerated disease progression including the development of comorbidities (Barnes 2008a; Decramer et al., 2012; Stockley, 2009). COPD patients with pulmonary hypertension (PH) have more frequent exacerbations, and inflammation contributes to PH (Price et al. 2012; Wells et al., 2012). Therefore, inflammation might contribute to the pathogenesis of right heart failure in acute exacerbations of COPD. As therapies for exacerbations in chronic lung diseases are insufficient, alternative therapeutic strategies are required (Adcock and Barnes, 2008).

Pulmonary vascular smooth muscle cells (PVSMCs) are involved in airway remodeling in chronic inflammatory lung diseases and drive vascular remodeling in PH (Al-Muhsen et al., 2009; Barbera and Blanco, 2009; Dupuis and Hoeper, 2008). They contribute to inflammation during bacterial infection by releasing cytokines in response to lipopolysaccharide (LPS), which is the major inflammatory trigger synthesized by gram-negative bacteria (Gatheral et al., 2012). Therefore, PVSMCs might contribute to the link between infection-induced inflammation and vascular remodeling associated with the development of PH as a comorbidity in chronic inflammatory lung diseases like COPD. This renders PVSMCs interesting therapeutic targets in early disease stages regarding the reduction of inflammation-induced airway vascular remodeling in consequence of infection-caused exacerbations.

PVSMCs express both subtypes of endothelin receptors: ET\(_A\)R and ET\(_B\)R (Henno et al., 2009). Two types of ERAs are approved in therapy of PH: ET\(_A\)R-
selective blockers, namely ambrisentan, and non-selective/dual blockers, like bosentan. However, studies comparing the efficacy of ET<sub>A</sub>R-selective versus dual blockers are missing. Endothelin-1 (ET-1) does not only trigger remodeling but also inflammation (Dupuis and Hoeper, 2008). ERAs have anti-inflammatory and anti-fibrotic potential in bronchial smooth muscle cells: they reduce the TNF<sub>α</sub>-induced release of cytokines/chemokines and remodeling proteins (Knobloch et al., 2009; Knobloch et al., 2013). This was first evidence for roles of ET<sub>A</sub>R and ET<sub>B</sub>R in inflammation-induced remodeling processes. From this, we hypothesized that the anti-inflammatory properties of ERAs could have a utility in early stages of chronic lung diseases with regard to attenuating inflammation-induced remodeling processes and the consequential development of comorbidities like PH.

Here, we aimed to support this hypothesis by means of clinical basic research. We focused on the question whether ERAs might suppress the inflammatory response of PVSMCs to bacterial infection, which is critical in disease exacerbations and promotes remodeling. Given the importance of LPS in infection-induced inflammation, LPS-stimulated PVSMCs are a suitable cell culture model for addressing this question. We further aimed to investigate the underlying mechanisms for putative anti-inflammatory effects of ERAs. This requires consideration of the specific characteristics of the response of PVSMCs to LPS and of the receptors involved.

LPS sensitivity is inconsistent among different human arterial VSMC preparations (Yang et al., 2005): VSMCs from coronary arteries recognize LPS via the TLR4/CD14 receptor complex on their surface. However, PVSMCs do not express membrane-bound CD14 (mCD14) although CD14 mRNA is detectable. Nonetheless, LPS induces cytokine release in PVSMCs (Gatheral et al.; 2012) suggesting that soluble CD14 (sCD14) might compensate for the lack of mCD14, as
it has been shown for other cell types including VSMCs of venous origin (Bäckhed et al., 2002; Loppnow et al., 1995).

Complete LPS (smooth LPS, S-LPS) consists of lipid A, a core oligosaccharide and an O-polysaccharide (King et al., 2009). Some bacteria that are relevant for exacerbations in chronic lung diseases synthesize truncated forms (rough LPS, R-LPS) with oligosaccharide structures in different degrees of completion. Re-LPS, the shortest form, lacks the O-polysaccharide and has the most incomplete core oligosaccharide (Huber et al., 2006). For example, *Pseudomonas aeruginosa* strains, which are frequently found in COPD patients, synthesize a broad spectrum from S-LPS to shortest R-LPS forms (Bantroch et al., 1994; King et al., 2009). This suggests that the whole LPS spectrum has clinical relevance. Whereas S-LPS requires mCD14 or sCD14 to activate TLR4, this CD14-dependence diminishes with increasing degrees of LPS truncation. Re-LPS activates TLR4 completely independent from CD14 (Huber et al., 2006).

We hypothesized that PVSMCs differentially respond to both S- and Re-LPS with the release of IL-6, IL-8 and GM-CSF, all of which are important for inflammation-induced remodeling. We further hypothesized that selective and dual ERAs differentially suppress LPS-induced cytokine release from PVSMCs by interfering with TLR4 signaling and, therefore, could be considered candidate drugs for attenuating bacteria-induced inflammation in exacerbations of chronic lung diseases.
Methods

Human PVSMC isolation and cultivation

Human PVSMCs were dissected from main pulmonary artery explants from patients (four females, two males) undergoing lung resection for carcinoma of the bronchus as described previously (Peng et al., 1996). For PVSCM isolation, healthy tumor-free tissue was used. Briefly, the arteries were separated from their adventitia and endothelium and then cut into 1- to 3-mm² pieces with sterile scalpel blades. These tissue pieces were incubated for 30 min at 37° C and 5% CO2 in HBSS (Sigma, St Louis, MO, USA, cat#-H6648) containing 10 mg/ml bovine serum albumin (BSA; Sigma, cat#-A0281) and the enzymes collagenase (type XI, 1 mg/ml; Sigma, cat#-C9407) and elastase (type I, 3.3 u/ml; Sigma, cat#-E7885). Tissue pieces were then incubated for a further 30-60 min in the enzyme solution outlined above with elastase content increased to 15 u/ml. In order to separate the dispersed cells from the enzyme solution they were centrifuged (100 g, 5 min) at 4°C and then resuspended in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany; cat#-31885-023) with 10% FCS (Sigma; cat#-N-4637), sodium pyruvate (1 mM; Invitrogen; cat#-11360-039), L-glutamine (2 mM; Sigma cat#-G-7513), non-essential amino acids (1%; Invitrogen; cat#-11140-035), penicillin (100 U/ml), streptomycin (100 μg/ml; Sigma; cat#-P-4333), and amphotericin B (1.5 μg/ml; Sigma cat#-A-2942). The PVSMC cellular suspension was placed in a tissue culture flask (25 cm²; Nunc, Roskilde, Denmark, cat#-156367) with 8 ml of supplemented DMEM and incubated in a humidified atmosphere at 37° C in 5% CO2. The culture medium was replaced after 4-5 days and subsequently every 2-3 days. After 4 to 8 weeks the cells reach confluence. Subconfluent cells were passaged with trypsin (1% in HBSS; Sigma; cat#-T-4674). This study has been approved by the ethics committee of the
University of Bochum (4257-12), Germany, and all patients have given their written consent.

**PVSMC characterization**

PVSMCs were characterized by positive immunostaining for smooth muscle $\alpha$-actin (SMA) and myosin heavy chain (MHC). Fluorescence-based immunostaining was done as described before (Knobloch et al, 2007). Briefly, cells were seeded on coverslips in 6-wells plates (Sarstedt, Nümbrecht, Germany, cat#-83.1839) in DMEM with supplements as described above. After 16 hours, the medium was removed and the cells were washed in phosphate-buffered saline (PBS; Sigma, cat#-P4417) and fixed on the coverslips with 4% paraformaldehyde in PBS for 60 min at $4^\circ$ C. Cells were washed three times in PBS (3 min at room temperature, RT), incubated for 10 min in PBS + 0.5% Triton X-100 (Merck, Darmstadt, Germany, cat#-112298) at RT and then washed three times in PBS + 0.1% Triton X-100 (PBT; 3 min, RT). Blocking was done with 10% FCS in PBT for 30 min at RT. Cells were then incubated with primary antibodies against $\alpha$-smooth muscle actin (SMA) (mouse anti-human SMA, clone 1A4; Sigma cat#-A5228) or smooth muscle myosin heavy chain (SMMHC) (mouse anti-human SM-1 and SM-2, clone h-SM-V; Sigma, cat#-M7786) diluted 1:100 or 1:400, respectively, in blocking solution for 2 hours. Cells were washed five times in PBT (2 min, RT) and then incubated with an goat anti-mouse IgG Cy3-coupled secondary antibody (Dianova, Hamburg, Germany, cat#-115-165-003) diluted 1:100 in blocking solution for 30 min at RT in the dark. Cells were then washed five times in PBT (3 min, RT). Counterstaining with 4',6-diamidino-2-phenylindole (DAPI; Sigma, cat#-D9542; 0.1 µg/ml) was done for 15 min at RT in the dark. Cells were washed three times with PBT and one time with PBS (each 3 min at RT in the dark). Cells were mounted in Dako fluorescent mounting medium (Dako,
Hamburg, Germany, cat#-S3023) and analyzed by fluorescence microscopy. Cells were >95% positive for SMA and MHC. Cell viability was assessed by counting cells after staining with trypan blue (Invitrogen, cat#-15250) in a Neubauer haemocytometer. Cell viability was found to be greater than 95%.

**PVSMC stimulation**

Before stimulation, subconfluent cell monolayers (approximately 80% confluence) in six-well cell culture plates at passages 3 to 6 were deprived of serum for 24 h in serum-free and low glucose (1 g/l) DMEM (Invitrogen; cat#-41966-029) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5 μg/ml amphotericin B, 1 mM insulin (Sigma, cat#-I1882), 5 mg/ml apo-transferrin (Sigma, cat#-T1147) and 100 μM ascorbic acid (Sigma, cat#-A4403). Subsequent stimulation with different LPS forms and ERAs (see below) was done in serum-free and low glucose (1 g/l) DMEM supplemented with 1mM sodium pyruvate, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1.5 μg/ml amphotericin B for 4, 16 (for RNA analyses) or 72 hours (for cytokine measurements in cell culture supernatants).

Mixed LPS (M-LPS) preparations (containing undefined amounts of S-LPS and short-chain LPS variants) from *Salmonella enteritidis* (Sigma; Munich, Germany; cat#-L7770) contained < 1% protein and 10-20% nucleic acids. Preparations of S-LPS from *Salmonella minnesota* (ALEXIS/EnzoLifeSciences GmbH, Lörrach, Germany cat#-581-020) and Re-LPS from *Salmonella minnesota R595* (ALEXIS; cat#-581-008) did not contain detectable protein or DNA contaminants with agonistic TLR activity. According to previous studies (Knobloch et al., 2009; Knobloch et al., 2013) PVSMCs were pre-incubated for two hours with ambrisentan ((2S)-2-[(4,6-
dimethyl(pyrimidin-2-yl)oxy]-3-methoxy-3,3-diphenylpropanoic acid; kindly provided by GSK, Munich, Germany), bosentan (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide; Actelion Pharmaceuticals, Freiburg, Germany), or BQ788 (Sodium (2R)-2-[[2S]-2-([(2R,6S)-2,6-dimethyl-1-piperidinyl]carbonyl]amino)-4,4-dimethylpentanoyl][1-(methoxycarbonyl)-D-trypophyl]amino]hexanoate; Sigma-Aldrich, cat#-B157) (each at 0.1-10 µM) before stimulation with LPS. According to previous studies (Knobloch et al., 2011a) PVSMCs were pre-incubated with polymyxin B (N-[4-amino-1-[1-[4-amino-1-oxo-1-[(6,9,18-tris(2-aminoethyl)-15-benzyl-3-(1-hydroxyethyl)-12-(2-methylpropyl)-2,5,8,11,14,17,20-heptaoxo-1,4,7,10,13,16,19-heptazacyclotricos-21-y1]amino]butan-2-yl]amino]-3-hydroxy-1-oxobutan-2-yl]amino]-1-oxobutan-2-yl]-6-methyloctanamide; 1-10 µg/ml; Invivogen, cat#-trl-pmb), with CLI-095 (TAK-242, Ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate; 1 µM; Invivogen, cat#-trl-cli95), with blocking antibodies for TLR2 (10 µg/ml; Invivogen, San Diego, CA, cat#-pab-hstlr2) or CD14 (1-10 µg/ml; clone biG14; biometec, Greifswald, Germany, cat#-021-3c.2), with an isotype control (R&D Systems, Wiesbaden, Germany; cat#-MAB003) or with the TLR9-specific inhibitory nucleotide ODN TTAGGG (2.5 µM; Invivogen, cat#-hinhodn; 5’-ttt agg gtt agg gtt agg gtt agg g-3’) 30-60 min before stimulation with LPS.

RNA Isolation and Quantitative RT-PCR

DNA-free total RNA was extracted from 80% subconfluent PVSMCs with the chromatography-based RNaseasy technique (QIAGEN GmbH, Hilden, Germany, cat#-74106). Quantitative RT-PCR (qRT-PCR) was done as described previously (Knobloch et al., 2011a). Briefly, after cDNA synthesis (Kit: QIAGEN, cat#-205113) with random primers (Promega, Mannheim, Germany, cat#-C1181), quantitative
PCR amplifications (PCR Kit: QIAGEN, cat#-203205) were performed with gene-specific and intron-spanning primers. The housekeeping gene EF1α was used as a reference. PCR conditions were established for each primer pair in the exponential range allowing quantification of signal intensities after ethidium bromide staining and standard agarose gel electrophoresis by densitometry using Alpha Innotech (San Leandro, CA) software, version 1.3.0.7. As a prerequisite for reliable quantification of signal intensities, Alpha Innotech software recognizes oversaturation (e.g., due to excess loading of the agarose gel) and non-linear gamma or contrast correction of signals and disables densitometry tools in those cases. Values for TLR4 and CD14 under the different stimulation conditions were normalized to EF1α reference signals in order to correct for putative differences in RNA/cDNA load. Primer sequences have been published previously (Knobloch et al., 2011a).

**ELISA**

IL-6, IL-8, GM-CSF and sCD14 concentrations in cell culture supernatants were measured via ELISA (Duo Sets or Quantikine, R&D systems, Wiesbaden, Germany, cat#-DY206, DY208, DY215, DC140) according to standard protocols (Knobloch et al., 2011b; Koch et al., 2007).

**Statistical Analysis of qRT-PCR and ELISA data**

Drug effects on cytokine expression in LPS-stimulated PVSMCs were expressed as % inhibition versus stimulation with LPS alone. According to previous studies (Knobloch et al., 2010; Knobloch et al., 2011b) Gaussian distributions of the data describing LPS and drug effects on cytokine expression in primary cells were expected and were confirmed for all data sets by Kolmogorov-Smirnov tests.
Therefore, the data were expressed as mean ± SEM, and parametric tests (with 95% confidence intervals) were used for analyses. \( \alpha < 0.05 \) was considered as statistically significant. All calculations were done with GraphPad Prism.

The effects of LPS on cytokine release investigated in concentration-response experiments were analyzed by one way repeated measures ANOVA tests. If \( \alpha < 0.05 \) was reached, post hoc bonferroni-holm tests were used to analyze for differences between the data for individual LPS concentrations versus unstimulated controls.

The % inhibition data for Polymyxin B, CLI-095, \( \alpha \)TLR2AB, IgG control antibody and ODN TTAGGG regarding IL-8 release in M-LPS-treated cells were analyzed for differences to a hypothetical value 0, which stands for no inhibition or stimulation with M-LPS in the absence of inhibitors, respectively, with one sample t-tests and bonferroni correction for multiple testing.

Concentration response curves showing the reducing effects of ERAs on cytokine release in M-LPS-treated cells were calculated by non-linear (sigmoidal) regression with variable parameters. The % inhibition data for each ERA at 10 \( \mu M \) were analyzed for differences to a hypothetical value 0, which stands for no reduction or stimulation with M-LPS in the absence of ERAs, respectively, with one sample t-tests and bonferroni correction for multiple testing. If \( \alpha < 0.05 \) was reached for an ERA, the same analysis was done with the % inhibition data for this ERA at 1 \( \mu M \). Additionally, the % inhibition data were analyzed for differences between the three ERAs at the same concentration. This was done by one way repeated measures ANOVA tests. If \( \alpha < 0.05 \) was reached, post hoc bonferroni-holm tests were performed.

The effects of ERAs on sCD14 release as well as on TLR4 and CD14 mRNA levels in the presence and absence of LPS were analyzed by one way repeated
measures ANOVA. If $\alpha<0.05$ was reached, post hoc bonferroni-holm tests were performed to analyze for drug effects versus unstimulated and LPS-stimulated cells.

The effects of different concentrations of the CD14 blocking antibody on cytokine release in S-LPS-exposed cells were analyzed by one way repeated measures ANOVA tests and, if $\alpha<0.05$ was reached, post hoc bonferroni-holm tests.

The % inhibition data for each ERA regarding IL-8 release in S-LPS- and Re-LPS-treated cells were analyzed for differences to a hypothetical value 0, which stands for no reduction or stimulation with S-LPS/Re-LPS in the absence of ERAs, respectively, with one sample t-tests and bonferroni correction for multiple testing. If $\alpha<0.05$ was reached for an ERA, its % inhibition data regarding S-LPS-induced IL-8 release were compared to those regarding Re-LPS-induced IL-8 release with a two-tailed, paired student’s t-test.
Results

M-LPS, S-LPS and Re-LPS induce cytokine release from PVSMCs

There is considerable similarity between LPS forms and spectra of *Salmonella* spp and *Pseudomonas aeruginosa*, which is frequently isolated in exacerbations of chronic lung diseases (King et al., 2009). As it is easily available, LPS from *Salmonella* has been established as a model LPS in experimental basic research regarding exacerbations of chronic inflammatory lung diseases (Knobloch et al., 2011a). Thus, we have used LPS from *Salmonella* spp. in order to experimentally investigate the responses of PVSMCs to different LPS forms and the effects of ERAs. M-LPS is an undefined mixture of smooth LPS and multiple short-chain LPS forms of *Salmonella enteritidis*. S-LPS and Re-LPS are pure and defined preparations of smooth LPS or short-chain LPS, respectively, both from *S. minnesota*. All LPS forms induce a robust release of IL-8, IL-6 and GM-CSF from PVSMCs in a concentration-dependent manner (Figure 1). There were no statistically significant differences between the inductive effects of S-LPS and Re-LPS on all cytokines analyzed (Figure 1, data not shown) suggesting that PVSMCs almost equally respond to LPS forms of different length. In contrast to S-LPS and Re-LPS, which are highly purified, M-LPS preparations might contain small amounts of bacterial lipopeptides and nucleic acids, which are putative TLR2 or TLR9 agonists (see methods). M-LPS-induced IL-8 release from PVSMCs was completely blocked by polymyxin B, which neutralizes LPS (Morrison et al., 1976), and by CLI-095 (TAK-242), a specific inhibitor for TLR4 that binds to its intracellular TIR domain (Kawamoto et al., 2008), but was not affected by a TLR2 blocking antibody and by ODN TTAGGG (Figure 2), a TLR9-specific inhibitory nucleotide (Gursel et al., 2003). All blocking reagents did not modulate baseline IL-8 release (data not shown). These data confirm that we have
measured true M-LPS effects and demonstrate that LPS signals via TLR4 to induce cytokine release in PVSMCs.

**Endothelin receptor antagonists attenuate LPS-induced cytokine release from PVSMCs**

The ETAR-selective blocker ambrisentan, the ETBR-specific blocker BQ788 and the dual blocker bosentan did not modulate baseline releases of IL-8, IL-6 or GM-CSF from PVSMCs (data not shown). In the presence of M-LPS, all three ERAs reduced IL-6 release from PVSMCs almost equally (Figure 3A, Table 1). Bosentan and BQ788 reduced IL-8 and GM-CSF release from M-LPS-stimulated PVSMCs almost equally. In contrast, ambrisentan did not modulate IL-8 and GM-CSF in M-LPS-stimulated PVSMCs in a statistically significant manner but showed a trend towards up-regulating these two cytokines (Figure 3B,C). This suggests that blocking ETBR might be more effective in reducing LPS-induced cytokine release in PVSMCs than blocking ETAR.

**Inhibition of ETBR reduces CD14 mRNA levels and sCD14 release**

PVSMCs express TLR4 and CD14 mRNA (Figure 4 A-C; Yang et al., 2005) as well as functional TLR4 but are mCD14-negative (Yang et al., 2005). As PVSMCs responded to S-LPS (Figure 1B, E, H), which requires CD14 to activate TLR4 (Huber et al., 2006), we speculated that sCD14 might replace mCD14. However, PVSMCs were stimulated with S-LPS in serum-free medium, which did not contain detectable levels of sCD14 in the absence of PVSMCs (data not shown). This suggests that sCD14 might be endogenously released from PVSMCs. Indeed, we were able to detect sCD14 in PVSMC culture supernatants (Figure 4D).
Neutralization of sCD14 with a specific blocking antibody (biG14; Dziarski et al., 1998) concentration-dependently reduced S-LPS-induced IL-6, IL-8 and GM-CSF release (Figure 5). This demonstrates that the induction of cytokine release from PVSMCs by long LPS forms depends on the constitutive release of sCD14, which functionally replaces mCD14.

Regulation of cytokine gene expression by LPS/TLR4 signaling does not directly involve endothelin receptors. In order to elucidate the molecular mechanism responsible for ETₐR-blockade to reduce LPS-induced cytokine expression, we investigated the effects of ERAs on the expression of TLR4 and sCD14. M-LPS did not modulate TLR4 and CD14 mRNA levels after 4 and 16 hours of stimulation in PVSMCs (Figure 4A-C). All three ERAs did not modulate TLR4 mRNA levels at both time points (Figure 4A, B) and did not modulate CD14 mRNA after 4 hours. However, Bosentan and BQ788 but not Ambrisentan clearly reduced CD14 mRNA levels after 16 hours (Figure 4A, C). According to the mRNA data, M-LPS did not modulate sCD14 release, and bosentan and BQ788 but not ambrisentan suppressed sCD14 release from PVSMCs (Figure 4D).

**ETₐR blockade reduces S-LPS-induced IL-8 release but not Re-LPS-induced IL-8 release**

In contrast to S-LPS, Re-LPS activates TLR4 independent from CD14 (Huber et al., 2006). M-LPS contains long and short LPS forms suggesting that the cytokine response to M-LPS is partially but not completely dependent on CD14 (Knobloch et al., 2011a). Thus, if sCD14 repression by ETₐR blockade is a major mechanism explaining the reduction of LPS-induced cytokine expression by bosentan and BQ788, S-LPS-induced but not Re-LPS-induced cytokine expression should be sensitive to these ERAs. We investigated this hypothesis exemplary for IL-8: indeed,
bosentan and BQ788 but not ambrisentan suppressed S-LPS-induced IL-8 release, whereas all three ERAs did not significantly modulate Re-LPS-induced IL-8 release (Figure 6). These data provide evidence that sCD14 suppression caused by ET₃R blockade explains the reductive effects of bosentan and BQ788 on LPS-induced cytokine expression in PVSMCs.
Discussion

IL-6 and IL-8 become up-regulated in the airways in response to bacterial infections in chronic lung diseases, particularly in COPD, and are believed to have key roles in infection-induced exacerbations (Barnes, 2008a; Wedzicha and Donaldson, 2003). GM-CSF is involved in the pathogenesis of chronic inflammatory lung diseases and might link inflammation with remodeling processes (Barnes 2008a; Knobloch et al., 2009). Our data suggest that PVSMCs contribute to the enhancement of inflammation during exacerbations induced by bacteria, as they respond to LPS by releasing these cytokines. The PVSMC response to bacterial infections might contribute to the development of PH in chronic inflammatory lung diseases, which is associated with IL-6 excess in the lung (Mathew, 2010, Pulamsetti et al., 20011). Therefore, PVSMCs might represent suitable targets in alternative therapies for infection-induced exacerbations of chronic inflammatory lung diseases, for example, in order to reduce the risk of developing PH.

Although PVSMCs do not express mCD14 (Yang et al. 2005) and were stimulated in sCD14-free medium, they responded almost equally to S-LPS and Re-LPS. As CD14 mRNA is detectable in PVSMCs (Yang et al.; 2005; this study) we hypothesized that endogenously released sCD14 might functionally replace mCD14. This was confirmed by data showing that sCD14 is constitutively released by PVSMCs and that sCD14 neutralization abolishes S-LPS-induced cytokine release. There are two ways how cells can produce sCD14: first, by shedding from mCD14, which is attached via a GPI-anchor to the outside of the plasma membrane (Bufler at al., 1995). This protease-dependent mechanism is primarily found on activated cells and does probably not apply to PVSMCs as they do not carry mCD14. Second, by protease-independent mechanisms: these are based on intracellular post-translational mechanisms allowing CD14 to escape from the GPI-anchoring
mechanism and to become directly secreted (Bufler et al., 1995). This mechanism is suggested to primarily trigger constitutive sCD14 release and, therefore, might apply to the constitutive and LPS-independent sCD14 release from PVSMCs.

ERAs have been approved for therapy in PH but are also being considered for chronic inflammatory lung diseases. As endothelin has potent inflammatory potential, ERAs are believed to have anti-inflammatory properties. Experimental research supported this hypothesis suggesting that ERAs could block the link between inflammation and remodeling (Knobloch et al., 2009; Knobloch et al., 2013; Teder and Noble, 2000). Recent clinical trials, however, failed to provide evidence for a utility of ERAs in inflammation-associated chronic lung diseases, like idiopathic pulmonary fibrosis, severe asthma or severe COPD, when administered at late disease stages with extensive tissue remodeling (King et al., 2011; Stolz et al., 2008). However, we believe that ERAs would have to be administered at early disease stages, characterized by airway inflammation but without extensive fibrosis or emphysema, in order to utilize a putative interference of ERAs with inflammation-induced remodeling. Here, we investigated by the use of ex vivo cultivated primary PVSMCs, whether ERAs have potential to counteract the amplification of inflammation induced by bacterial infections during exacerbations of chronic lung diseases. We showed that ERAs reduce cytokine release from PVSMCs activated with the bacterial endotoxin LPS. We used ambrisentan and bosentan at concentrations of 0.1-10 µM in PVSMC culture. This resembles peak plasma concentrations of bosentan (about 1-2 µM) in vivo when taken twice daily at dosages of 62.5 or 125 mg per tablet (Treiber et al., 2007).

The ET_A-selective inhibitor ambrisentan only suppressed IL-6, whereas specific ET_B as well as dual blockade additionally suppressed IL-8 and GM-CSF. This indicates divergent roles for ET_A and ET_B in the up-regulation of cytokines by
LPS and might render ET<sub>A</sub>R antagonism interesting for therapy requiring selective IL-6 downregulation. IL-6 produced by airway cells is currently discussed as a major inflammatory mediator triggering the development of co-morbidities in COPD (Barnes and Celli, 2009). Our cell culture data provide first indication that ET<sub>A</sub>R antagonism might be useful in this context. However, at high concentrations ambrisentan non-significantly up-regulated IL-8 and GM-CSF by trend. Translated to putative therapy, this indicates for an increase in associated inflammatory processes at high ambrisentan dosages, which might promote chronic inflammation. This would have to be balanced with a possible benefit of IL-6 down-regulation.

In order to therapeutically reduce chronic inflammation, e.g. in COPD, the development of approaches targeting a broad spectrum of cytokines might be more auspicious, since therapeutic neutralization of single mediators has been proven to be ineffective due to functional redundancy of inflammatory proteins (Barnes, 2008b). In this context, selective ET<sub>B</sub>R or a combined ET<sub>A</sub>R/ET<sub>B</sub>R blockade might have more putative therapeutic potential because BQ788 as well as bosentan were both able to reduce not only IL-6 but also IL-8 and GM-CSF. IL-6, IL-8 and GM-CSF are believed to be important for the link between airway inflammation and remodeling (Knobloch et al., 2009; Mathew, 2010, Pulamsetti et al., 20011). Therefore, targeting the ET<sub>B</sub>R might not only have broader anti-inflammatory properties but also more potential to attenuate remodeling than selective ET<sub>A</sub>R blockade. Remodeling processes in part are mediated by PVSMCs, for example, when chronic inflammatory lung diseases become associated with PH (Al-Muhsen et al., 2009; Barbera and Blanco, 2009). ET<sub>B</sub>R-selective inhibitors have not yet been successfully developed for therapy but our data indicate that dual blockers might have similar anti-inflammatory properties.

A direct molecular link between endothelin receptors and LPS/TLR4 signaling has not yet been reported. This raises the question of mechanisms, by which ERAs
could interfere with LPS-induced cytokine release in PVSMCs. In bronchial smooth muscle cells ERAs suppress TNFα-induced GM-CSF release by an indirect mechanism: ERAs suppress an ET-1 autoregulatory feedback mechanism, which is activated by TNFα and is crucial for maintenance but not for initiation of TNFα-induced GM-CSF transcription. Thus, ERAs reduce TNFα-induced long-time but not short-time GM-CSF expression (Knobloch et al., 2009). We expected a similar kind of indirect mechanism to be responsible for cytokine suppression by ERAs in LPS-exposed PVSMCs. Therefore, we performed long-time cell culture experiments with 72 hours of stimulation for the investigation of drug effects. We have provided evidence that inhibition of ETBR activity leads to the downregulation of constitutive sCD14 secretion by PVSMCs. Cytokine expression induced by long LPS forms depend on sCD14 in PVSMCs. Therefore, sCD14 reduction caused by ETBR inactivation is a reasonable mechanistic explanation for the suppression of LPS-induced cytokine release by dual and ETBR-specific inhibitors. In support of this, we demonstrated clear effects of bosentan and BQ788 on IL-8 release induced by S-LPS, but did not find significant effects of these ERAs on IL-8 release induced by Re-LPS, which activates TLR4 independent from CD14 (Huber et al., 2006). Thus, a putative utility of ERAs in infection-induced exacerbations could depend on the bacteria involved: for example, ERAs could be useful for infections with Pseudomonas aeruginosa, which synthesizes long LPS forms (Bantroch et al., 1994; King et al., 2009), rather than for infections with nontypeable Haemophilus influenzae, which exclusively produces short LPS forms (Schweda et al., 2007). However, how ETAR inactivation is coupled to IL-6 suppression and possible IL-8 and GM-CSF up-regulation mechanistically, remains to be explained.
PVSMCs release sCD14 at concentrations of about 700 pg/ml after 72 hours of cultivation. This might appear as a rather low sCD14 concentration in terms of adopting mCD14 functions in LPS/CD14/TLR4 signaling, considering that S-LPS induces a robust IL-6 and IL-8 production at about 4.5 or 6 ng/ml, respectively, in PVSMCs after 72 hours of stimulation. For example, the addition of 5 ng/ml recombinant sCD14 was required to facilitate an LPS-induced release of IL-6 at about 3 ng/ml in VSMCs of venous origin, which do not express CD14 mRNA, sCD14 and mCD14 but TLR4 (Loppnow et al., 1995). Therefore, the low sCD14 amounts produced by PVSMCs might be close to the minimum threshold concentration required for detectable responses to S-LPS. However, considering that bosentan and BQ788 only slightly reduce sCD14 by about 25-29%, just the fact that PVSMCs secrete low sCD14 amounts might be a prerequisite for the clear suppressive effects of bosentan and BQ788 on cytokine production. Otherwise, if sCD14 would be produced in excess, these low reductive effects of ERAs on sCD14 would likely not result in significant consequences on LPS-induced cytokine expression. Nonetheless, this suggests that PVSMCs might have increased cytokine responses to long LPS forms in vivo, where additional exogenous sources of sCD14 are available like activated monocytes and macrophages that enter the lung tissue during acute bacterial infections. Therefore, future studies might investigate ERA effects on sCD14 production by monocytes/macrophages in order to provide more arguments for a utility of ERAs in infection-induced exacerbations of chronic lung diseases.

Limitations of the study: PVSMC preparations might contain fibroblasts and myofibroblasts, which also express endothelin receptors and cytokines and respond to LPS (Ahmedat et al., 2013; Zhang et al., 2011). We used SMA as a marker to exclude the presence of fibroblasts in our cell culture. SMA is expressed in a filamentous pattern in smooth muscle cells (SMCs) and myofibroblasts but, if any, in
a different, diffuse pattern in some fibroblast populations (Hinz et al., 2007; Singh and Hall, 2008). The discrimination between PVSMCs and myofibroblasts in culture is difficult because there is a lack of suitable markers. Smoothelin is not expressed in myofibroblasts, definitely identifies terminally differentiated SMCs in tissue but gets immediately lost when SMCs start to proliferate in culture (van der Loop et al., 1996). Myofibroblasts express other canonical SMC markers like calponin, h-caldesmon and desmin, and SMCs can express canonical fibroblast markers like vimentin, Thy-1 and D7-FIB (Gabbiani, 1992; Singh and Hall, 2008; Skalli et al., 1989). Although not completely unequivocal, SMMHC might be the best marker currently available for discrimination. Myofibroblasts associated with normal wound healing do not express SMMHC (Benzonana et al., 1988; Eddy et al., 1988; Gabbiani, 1992) but a chronic pathological environment might induce SMMHC expression in myofibroblasts (Chiavegato et al., 1995). As we isolate PVSMCs from healthy, tumor-free tissue, it is unlikely to have SMMHC-positive myofibroblasts in our preparations. Moreover, if myofibroblasts are SMMHC-positive, the staining is rather weak, but we observed strong staining in our cell cultures, which is characteristic for SMCs (Singh and Hall, 2008). Finally, cultured SMCs might de-differentiate and gain characteristics of myofibroblasts (Singh and Hall, 2008). We have exclusively used early passages and have not observed significant changes in cytokine responses to our stimulants when comparing different passages of one donor (unpublished observation). We conclude that by phenotyping with SMA and SMMHC and by using early passages, we have done the best what is currently possible to exclude the presence of significant amounts of (myo-)fibroblasts in our PVSMC cultures.

In summary, we have shown that ET_{B}R blockade reduces LPS-induced cytokine production in PVSMCs due to sCD14 suppression. Therefore, we provide a first evidence by means of data obtained from primary cell culture experiments that
specific ET_{B}R blockade or dual blockers might attenuate inflammation in bacterial infection-induced exacerbations of chronic lung diseases. However, before this idea might be considered for clinical trials, it requires further experimental support, e.g. by investigating the sensitivity of additional cytokines to ERAs in different cell culture and animal models of bacterial infection and chronic lung diseases.
Acknowledgements

We thank Carmen Meinig and Sandra Körber for excellent technical assistance.
Ambrisentan was kindly provided by GlaxoSmithKline, Munich, Germany.

Authorship Contributions

Participated in research design: Knobloch, Koch

Conducted experiments: Knobloch, Feldmann, Wahl

Contributed new reagents or analytic tools (primary PVSMCs): Stoelben

Performed data analysis: Knobloch, Feldmann, Wahl, Koch

Wrote or contributed to the writing of the manuscript: Knobloch, Jungck, Behr, Koch
References


activation of an ET-1 autoregulatory positive feedback mechanism. *Thorax* 64:1044-52.


Zhang J, Wu L, Qu JM (2011) Inhibited proliferation of human lung fibroblasts by LPS is through IL-6 and IL-8 release. *Cytokine* 54:289-295.
Footnotes

Financial support: This work was funded by Actelion Pharmaceuticals, Germany. Actelion Pharmaceuticals were not involved in study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

Conflict of interest: J.K. has received travel grants from Actelion Pharmaceuticals. D.J. has received travel grants from Actelion Pharmaceuticals and from GlaxoSmithKline. J.B. has received travel grants, fees for speaking and research grants from Actelion Pharmaceuticals, fees for speaking from GlaxoSmithKline, and has served as consultant/advisor for Actelion Pharmaceuticals and GlaxoSmithKline. A.K. has received travel grants from Actelion Pharmaceuticals and from GlaxoSmithKline and research funding from Actelion Pharmaceuticals.
Legends for Figures

**Figure 1. Different forms of LPS induce cytokine release in PVSMCs.** Cultivated PVSMCs of 4-6 donors were stimulated with M-LPS from *Salmonella enteritidis*, S-LPS or Re-LPS both from *Salmonella minnesota* at concentrations as indicated. After 72 hours, cytokine concentrations in the supernatant were measured by ELISA. Data are presented as mean ± SEM. Data were analyzed with one-way repeated measures ANOVA (all p<0.02) and post hoc bonferroni-holm tests: * p<0.05; **, p<0.01; ***p<0.001 compared to unstimulated controls.

**Figure 2. LPS induces IL-8 release via TLR4.** Cultivated PVSMCs were pre-incubated with the LPS-neutralizing drug polymyxin B (PB), with the specific TLR4 inhibitor CLI-095 (1 µM), with a neutralizing antibody against TLR2 (αTLR2AB) or a control antibody (both at 10 µg/ml) or with the TLR9 inhibitor ODN TTAGGG (2.5 µM) 30 min (drugs and nucleotides) or 60 min (antibodies) before stimulation with M-LPS (1 µg/ml). After 72 hours, IL-8 was measured in the supernatant via ELISA. Data were calculated as percent reduction versus stimulation with M-LPS alone (LPSmax). Data of PVSMCs from n=4 donors are presented as mean ± SEM. One-sample t-tests versus a hypothetical value 0 (= no inhibition/LPSmax) with bonferroni correction for multiple testing: *, p<0.05; **, p<0.01; ***p<0.001.

**Figure 3. ERAs reduce cytokine release in M-LPS-stimulated PVSMCs.** Cultivated PVSMCs were pre-incubated with ambrisentan, bosentan or BQ788 at 100 nM - 10 µM two hours before stimulation with M-LPS at 1 µg/ml. After 72 hours, cytokines were measured in the supernatant via ELISA. Data were calculated as % reduction (negative values) versus stimulation with M-LPS alone (LPSmax). Data (n=5
for IL-6 and GM-CSF; n=6 for IL-8) are presented as mean ± SEM. Curves were created by non-linear regression analyses with sigmoidal curve fit using variable slopes. \( r^2 \) (goodness of fit): (A) ambrisentan, 0.67; bosentan, 0.60; BQ788, 0.53; (B) ambrisentan, 0.22; bosentan, 0.42; BQ788, 0.41; (C) ambrisentan, 0.39; bosentan, 0.53; BQ788, 0.34. One-sample t-tests versus a hypothetical value 0 (= no inhibition/LPS\(_{\text{max}}\)) with bonferroni correction for multiple testing: M-LPS + ambrisentan vs. LPS\(_{\text{max}}\): §§§, p<0.001; M-LPS + bosentan vs. LPS\(_{\text{max}}\): *, p<0.005; **, p<0.01; ***, p<0.001; M-LPS + BQ788 vs. LPS\(_{\text{max}}\): +, p<0.05; ++, p<0.01. One way repeated measures ANOVA (in each case p<0.001 for comparisons of drug effects at 10\(^{-6}\) M or 10\(^{-5}\) M, respectively, in panels B and C) and post hoc bonferroni-holm tests: M-LPS + ambrisentan vs. M-LPS + bosentan: ##, p<0.01; ###, p<0.001; M-LPS + ambrisentan vs. M-LPS + BQ788: ƒƒ, p<0.01; ƒƒƒ, p<0.001.

**Figure 4. Bosentan and BQ788 reduce sCD14 in PVSMCs.** Cultured PVSMCs were pre-incubated for two hours with ambrisentan, bosentan or BQ788 each at 1 µM before stimulation with M-LPS at 1 µg/ml. (A-C) After 4 or 16 hours of incubation RNA was isolated and subjected to quantitative RT-PCR (see methods) with specific primers for TLR4, CD14 and EF1\(\alpha\). A representative set of RT-PCRs after agarose gel electrophoresis is shown in panel A. (B, C) Densitometric analysis of PCR signals after gel electrophoresis. Values for TLR4 and CD14 were, first, normalized to the house keeping gene and reference EF1\(\alpha\) and, second, to the unstimulated control, which was set to 1. (D) After 72 hours of incubation, sCD14 concentrations in supernatants were measured by ELISA. Data from n=4 (B, C) or n=5 (D) donors are presented as mean ± SEM. One-way repeated measures ANOVA (B, p>0.05; C, p<0.001; D, p=0.0042) with post hoc bonferroni-holm tests: *, p<0.05; ***, p<0.001.
vs. unstimulated controls; +, p<0.05; ++, p<0.01; +++, p<0.001 vs. stimulations with M-LPS alone.

**Figure 5. CD14 neutralization reduces S-LPS-induced cytokine release from PVSMCs.** Cultivated PVSMCs were pre-incubated with a CD14 blocking antibody (clone biG14) or an isotype control one hour before stimulation with S-LPS (1 µg/ml) for 72 hours. Cytokines were measured in culture supernatants via ELISA. Data (n=4) are presented as mean ± SEM. Data were analyzed with one-way repeated measures ANOVA (A, p=0.0018; B, C, p<0.0001) and post hoc bonferroni-holm tests: * p<0.05; **, p<0.01; ***p<0.001.

**Figure 6. Bosentan and BQ788 reduce S-LPS- but not Re-LPS-induced IL-8 release from PVSMCs.** Cultivated PVSMCs were pre-incubated with ambrisentan, bosentan or BQ788 (all at 1 µM) two hours before stimulation with S-LPS (1 µg/ml) or Re-LPS (0.1 µg/ml). After 72 hours, IL-8 was measured in supernatants via ELISA. Data were calculated as percent reduction versus stimulation with S-LPS/Re-LPS alone. Data of PVSMCs from n=6 donors are presented as mean ± SEM. One sample t-tests versus a hypothetical value 0 (= no inhibition/stimulation with S-LPS/Re-LPS alone) with bonferroni correction for multiple testing: **, p<0.01 (symbols are placed on top of bars); two-tailed, paired student’s t-tests: *, p<0.05 related to values as indicated.
**Tables**

<table>
<thead>
<tr>
<th></th>
<th>EC\textsubscript{50} (M)</th>
<th>logEC\textsubscript{50} ± S.E.</th>
<th>E\textsubscript{max} ± SEM (% reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambrisant</td>
<td>1.39 ( \times 10^6 )</td>
<td>-5.86 ± 0.39</td>
<td>-50.06 ± 9.45</td>
</tr>
<tr>
<td>Bosentan</td>
<td>2.30 ( \times 10^6 )</td>
<td>-5.64 ± 0.53</td>
<td>-49.71 ± 12.85</td>
</tr>
<tr>
<td>BQ788</td>
<td>1.46 ( \times 10^6 )</td>
<td>-5.83 ± 0.52</td>
<td>-47.50 ± 11.82</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambrisant</td>
<td>5.44 ( \times 10^7 )</td>
<td>-6.27 ± 0.99</td>
<td>18.92 ± 10.84</td>
</tr>
<tr>
<td>Bosentan</td>
<td>1.21 ( \times 10^6 )</td>
<td>-5.92 ± 0.56</td>
<td>-43.75 ± 9.07</td>
</tr>
<tr>
<td>BQ788</td>
<td>9.50 ( \times 10^7 )</td>
<td>-6.02 ± 0.57</td>
<td>-41.77 ± 9.09</td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambrisant</td>
<td>3.34 ( \times 10^7 )</td>
<td>-6.48 ± 0.94</td>
<td>28.42 ± 13.99</td>
</tr>
<tr>
<td>Bosentan</td>
<td>4.71 ( \times 10^7 )</td>
<td>-6.33 ± 0.59</td>
<td>-55.25 ± 11.42</td>
</tr>
<tr>
<td>BQ788</td>
<td>4.30 ( \times 10^7 )</td>
<td>-6.37 ± 0.91</td>
<td>-56.28 ± 14.71</td>
</tr>
</tbody>
</table>

**Table 1.** EC\textsubscript{50} and E\textsubscript{max} values of ERA effects on M-LPS-induced cytokine production in PVSMCs. Values were calculated from non-linear regression analyses with sigmoidal curve fit of the data presented in Figure 3. ERA effects were calculated as % reduction vs. stimulation with M-LPS alone. Reductive effects are expressed as negative percent values. EC\textsubscript{50}: effective concentration 50%; E\textsubscript{max}: maximum possible effect; S.E. standard error; SEM, standard error of the mean.
Figure 2

M-LPS (1 μg/ml)

IL-8 [% Inhibition]

-10 0 10 20 30 40 50 60 70 80 90 100 110

PB (1 μg/ml)  PB (10 μg/ml)  CLI-095  αTLR2AB  IgG control  ODN TTAGGG

*  ***  **
Figure 5

(A) IL-6 [pg/mL]

(B) IL-8 [pg/mL]

(C) GM-CSF [pg/mL]
Figure 6

[Graph showing IL-8 inhibition levels under different conditions with Ambrisentan, Bosentan, and BQ788 treatments for S-LPS and Re-LPS]

- **Ambrisentan**
  - S-LPS: +
  - Re-LPS: +

- **Bosentan**
  - S-LPS: +
  - Re-LPS: -

- **BQ788**
  - S-LPS: -
  - Re-LPS: +

The graph indicates a significant reduction in IL-8 levels with Ambrisentan treatment compared to Bosentan and BQ788. Statistical significance is marked with * for P < 0.05 and ** for P < 0.01.