This item is the archived peer-reviewed author-version of:

Human inflammatory response of endotoxin affected by particulate matter-bound transition metals

Reference:

Moretti Serena, Smets Wenke, Hofman Jelle, Mubiana Kayaw e Valentine, Oerlemans Eline, Vandenheuvel Dieter, Samson Roeland, Blust Ronny, Lebeer Sarah.-
Human inflammatory response of endotoxin affected by particulate matter-bound transition metals
Environmental pollution - ISSN 0269-7491 - 244(2019), p. 118-126
Full text (Publisher's DOI): <https://doi.org/10.1016/J.ENVPOL.2018.09.148>
To cite this reference: <https://hdl.handle.net/10067/1562120151162165141>

Accepted Manuscript

Human inflammatory response of endotoxin affected by particulate matter-bound transition metals

Serena Moretti, Wenke Smets, Jelle Hofman, Kayawe Valentine Mubiana, Eline Oerlemans, Dieter Vandenheuveel, Roeland Samson, Ronny Blust, Sarah Lebeer

PII: S0269-7491(18)32693-9

DOI: [10.1016/j.envpol.2018.09.148](https://doi.org/10.1016/j.envpol.2018.09.148)

Reference: ENPO 11725

To appear in: *Environmental Pollution*

Received Date: 15 June 2018

Revised Date: 11 September 2018

Accepted Date: 29 September 2018

Please cite this article as: Moretti, S., Smets, W., Hofman, J., Mubiana, K.V., Oerlemans, E., Vandenheuveel, D., Samson, R., Blust, R., Lebeer, S., Human inflammatory response of endotoxin affected by particulate matter-bound transition metals, *Environmental Pollution* (2018), doi: <https://doi.org/10.1016/j.envpol.2018.09.148>.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Human inflammatory response of endotoxin affected by particulate matter-bound transition metals

Serena Moretti^a, Wenke Smets^a, Jelle Hofman^a, Kayawe Valentine Mubiana^b, Eline Oerlemans^a, Dieter Vandenheuvel^a, Roeland Samson^a, Ronny Blust^b, Sarah Lebeer^{a,*}

^aEnvironmental Ecology and Applied Microbiology (ENdEMIC), Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

^bSystemic Physiological and Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

Running Head: endotoxins and transition metals

*Address correspondence to Sarah Lebeer, University of Antwerp, Dep. Bioscience Engineering, Groenenborgerlaan 171, B-2020: sarah.lebeer@uantwerpen.be

Keywords

Lipopolysaccharide; TLR4; particulate matter; inflammation; transition metals

Abstract

Bacterial endotoxins are a component of particulate matter (PM) with anticipated health implications, yet we know little about how host reception of endotoxin through toll-like receptor 4 (TLR4) is affected by its association with other PM components. Subsequently, we investigated the relationship between endotoxin concentration (recombinant Factor C (rFC) assay) and host recognition (HEK Blue-TLR4 NF- κ B reporter cell line based assay) in various compositions of urban PM, including road traffic, industrial and urban green land use classes. While the assays did not correlate strongly between each other, the TLR4 reporter cell line was found to be better correlated to the IL-8 response of PM. Furthermore, the ability of the quantified endotoxin (rFC assay) to stimulate the TLR4/MD-2 complex was significantly affected by the urban land use class, where traffic locations were found to be significantly higher in bioactive endotoxin than the industrial and green locations. We subsequently turned our attention to PM composition and characterized the samples based on transition metal content (through ICP-MS). The effect of nickel and cobalt – previously reported to activate the hTLR4/MD-2 complex – was found to be negligible in comparison to that of iron. Here, the addition of iron as a factor significantly improved the regression model between the two endotoxin assays, explaining 77% of the variation of the TLR4 stimulation and excluding the significant effect of land use class. Moreover, the effect of iron proved to be more than a correlation, since dosing LPS with Fe^{2+} led to an increase up to 64% in TLR4 stimulation, while Fe^{2+} without LPS was unable to stimulate a response. This study shows that endotoxin quantification assays (such as the rFC assay) may not always correspond to human biological recognition of endotoxin in urban PM, while its toxicity can be synergistically influenced by the associated PM composition.

24 **Capsule:** Although endotoxins are typically quantified in ambient air, its toxicity is
25 significantly influenced by the associated PM composition (e.g. as shown in this study with
26 iron, one of the most abundant transition metals found in urban PM).

27

1. Introduction

Numerous studies with varying populations, regions, and methodologies have associated an increase in respiratory and cardiovascular morbidity and mortality with a dose-dependent exposure to ambient particulate matter (PM) (Analitis et al., 2006; Baccini and Biggeri, 2012; Boldo et al., 2006; Dominici et al., 2003; Katsouyanni et al., 2001; Stafoggia et al., 2016). Despite the strong link, PM encompasses a highly diverse range of components varying in chemical composition (from natural to anthropogenic sources), phase (liquid/solid particles), and particle size, which makes it difficult to pinpoint the components responsible for these adverse effects. Furthermore, although regulatory standards and limits for PM size ranges, PM₁₀ (PM with aerodynamic diameter <10 µm) and PM_{2.5} (PM with aerodynamic diameter <2.5 µm), and specific particle constituents exist, there is still no threshold below which adverse health effects are no longer observed (WHO, 2016). This drives the urgent need to improve our understanding of the toxicological mechanisms – including synergist effects - of specific PM compounds to provide better air quality management.

There are many suggested mechanisms and outcomes of PM-induced toxicity (Anderson et al., 2012; Kelly, 2003; Valavanidis et al., 2008), but the exacerbation of inflammation and oxidative stress by PM on the respiratory system plays a major role, especially in susceptible individuals (Anderson et al., 2012; Li et al., 2008). Contributing towards this, bacterial endotoxins are suggested as apt candidates due to their ubiquitous and chemically-durable nature, their naturally high pro-inflammatory capacity, and their ability to prime immune responses resulting in a heightened immune response to other air pollutants (Imrich et al., 1999; Ning et al., 2000; Soukup and Becker, 2001). Subsequently, ambient endotoxins have been studied in several cities worldwide, typically collected using filter-based samplers and quantified with the LAL assay (Mueller-Anneling et al., 2004; Nilsson et al., 2011). Using

these methods, endotoxins have been spatiotemporally monitored in various PM size ranges, fluctuation of endotoxin content in PM was examined, the effects of meteorological factors or endotoxin sources were investigated, and correlations and contributions of endotoxins to inflammatory biomarkers have been explored (Heinrich et al., 2003; Tager et al., 2010; Traversi et al., 2011; Wang et al., 2016). Although higher levels of urban endotoxins have been related to higher levels of immune biomarkers, such as IL-6 and TNF α , direct proportionality is not often observed (Hetland et al., 2005; Steerenberg et al., 2004). What is currently lacking, is the link between quantified endotoxin – as a constituent of urban PM - and the host immune recognition through toll-like receptor 4 (TLR4). This relationship may vary depending on the different assays and their specificity of endotoxin recognition (Gutsmann et al., 2010), interfering or synergistic PM components or different LPS potencies from Gram-negative bacterial populations (Dehus et al., 2006; Erridge et al., 2002).

Pattern recognition receptors (PRRs) such as TLR4 can be found on the surface of barrier epithelial cells and many immune cells (such as macrophages) which represent the first line of innate immune defence against microorganisms entering the lungs. TLR4, together with MD-2, is responsible for the recognition of endotoxin. Stimulation of the TLR4/MD-2 complex sets off a cascade of reactions strongly activating the release of pro-inflammatory mediators such as interleukins IL-8, IL-1 β and IL-6, and tumor necrosis factor alpha (TNF α) (Becker et al., 2004; Carter et al., 1997). Recently it was also found that the transition metals nickel and cobalt could specifically activate the human TLR4/MD-2 receptor complex (initially considered to be limited to endotoxin) through conserved histidine residues in the TLR4 ectodomain of humans and primates (Oblak et al., 2015; Rachmawati et al., 2013;

Schmidt et al., 2010). Furthermore, nickel was shown to act synergistically with endotoxin in the production of hTNF α (Oblak et al., 2015).

This study aimed to bridge the gap between endotoxin quantification and the inflammatory response by investigating the biological recognition of endotoxin via the human TLR4/MD-2 complex. Endotoxin concentrations were quantified from a diverse representation of outdoor urban PM samples to determine whether their associations with different sources - and possibly other PM components, such as transition metals - are relevant for their biological recognition by the TLR4/MD-2 complex and subsequent inflammatory response.

2. Materials and Methods

2.1 Sample collection

Samples were collected from six monitoring sites (Fig. S1) within Antwerp (Belgium), a city of approximately half a million inhabitants and accommodating the second largest harbour in Europe. Antwerp provides an excellent European urban model due to its diverse environmental elements, such as heavily trafficked roads in densely-built locations, tram and train lines, urban green elements such as parks and tree linings, and an industrial harbour region. The city, including the harbour region, is also well monitored for its air quality by the Flanders Environment Agency (VMM). For 2015, the VMM reported the modelled city centre background for Antwerp, calculating annual pollutant levels ranging from 21-25 $\mu\text{g m}^{-3}$ for PM₁₀, 11-13 $\mu\text{g m}^{-3}$ for PM_{2.5}, 31-40 $\mu\text{g m}^{-3}$ for NO₂ (VMM, 2016a). More information on the specific sampling sites is available in the supplementary materials (Fig. S1).

In this study, sampling occurred during the day (09:00 to 17:00) from July until September 2015 (n=50) with real-time air temperatures ranging from 18-33 °C (see more details in supplementary data sheet). All sampling sites were distributed spatially within Antwerp, not more than 15 km apart. To account for local emission sources, samples were categorized as urban traffic (A, B), urban green (C, D), and industrial (E, F) based on the expected local dominant pollutant and bioaerosol source (Fig. S1). Typically, three samples were collected per day, of which each represented a different area (traffic, green and industrial) to account for confounding day-to-day variation. Samples were collected at a median height of 1.6 m using the Coriolis® μ air sampler (Bertin Technologies, France), where air was drawn in at a flow rate of 300 L min⁻¹ (for 40 min; 12 m³ air), creating a vortex by which the particles were deposited into a pyrogen-free, polycarbonate cone containing 15 mL of ultra-pure water. The Coriolis sampler technology has been validated by the Health Protection Agency (HPA; Porton Down, UK) and ISO 14698-1 certified for biological/physical efficiency with a d₅₀ < 0.5 μ m (i.e. at a particle diameter of 0.5 μ m and above, the sampler efficiency is 50% or more). To test for contaminants, both the sampling water and filled cones were regularly tested in all analyses.

After collection, samples were transported on ice back to the laboratory. The volume of every sample was normalized to 15 mL with ultra-pure water to compensate for evaporation losses of the collection liquid during sampling. The samples were vortexed (10 sec), aliquoted accordingly for the various assays, and stored in glass vials at -20 °C (typically within 12 hours after sampling) until analysis.

2.2 Endotoxin concentration

Recombinant Factor C assay. All samples were thawed only once and endotoxin quantification was determined in triplicate using the recombinant Factor C (rFC) assay according to manufacturer's instructions (Lonza Walkersville Inc., MD, USA; lot 0000 416 097) with further details provided in the supplementary materials . For rFC assay analysis, a blank and a five-point standard curve (10, 5, 1, 0.1, 0.02 Endotoxin Units (EU) mL⁻¹) was set up in glass vials using endotoxin standards (*Escherichia coli* O55:B5 lot 0000 441 186; Lonza Walkersville Inc., MD, USA). The endotoxin concentration for a sample was calculated from the arithmetic mean of those dilutions that fell within range of the standard curve and expressed as EU per m³ of air based on the sampling conditions. Samples were analysed at a 1:2 dilution and a single lot (0000 475 024) of rFC was used for all analyses. Endotoxin distribution plots were constructed in GraphPad Prism v6.05 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

TLR4 stimulation in HEK293 cells. HEK-BlueTM hTLR4 cells are stably transfected with human TLR4/CD14/MD2 (Invivogen). The cells were cultured in DMEM (Gibco, Life Technologies), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), normocin, and HEK-Blue Selection (Invivogen) and maintained at 37 °C in a humidified incubator containing 5% CO₂. Cells (100 µL) were seeded into 96 well plates at a concentration of 0.5 x 10⁶ cells mL⁻¹ and incubated for 48 hours until approximately 90% confluent. Media was removed from adherent cells and replaced with 100 µL concentrated (x2) DMEM (with no FCS, normocin, or selective marker) which was diluted with 100 µL sample/control. A standard curve was generated by stimulating the cells with a six-point serial dilution (0.5 - 75 EU mL⁻¹) of the same endotoxin standard as the rFC assay (*Escherichia coli* O55:B5, lot 0000 441 186; Lonza Walkersville Inc., MD, USA). After 20 h incubation, 50 µL-supernatant was transferred in

duplicate to a new 96 well plate, upon adding 100 μL of freshly prepared substrate solution (final concentration of 1 mg mL^{-1} pNPP in 100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl_2 , pH 9.5 buffer). After a 20 min incubation at 23 $^{\circ}\text{C}$, the absorbance was measured at 405 nm. From the standard curve and sampling efficiency, the predicted endotoxin concentrations (EU m^{-3}) could be calculated. Cell viability was determined by the CellTiter-Glo 2.0 Assay (Promega).

2.3 Particle count

Particle size distribution and count were determined by Coulter counter analysis (Beckman Coulter Inc.). Samples were diluted 1:4 in Isoton solution and 500 μL of sample analyte was analysed in duplicate using the Coulter Counter containing a 50 μm aperture (with counting limits of 0.5 - 50 μm). Using the Multisizer 3 software, insoluble particles were counted for the coarse fraction (aerodynamic diameters of 2.5 - 10 μm), which were then calculated to be expressed as particles m^{-3} .

2.4 Transition metal analysis

From each of the collected air samples, 2 mL was transferred into polypropylene vials and immediately acidified to 2% using highly purified nitric acid so as to prevent metal adsorption. Corresponding preparation and reagent blanks were always included. Samples were stored at -20 $^{\circ}\text{C}$ until elemental analysis. High resolution sector field ICP-MS (Element XR, Thermofisher Scientific, Bremen, Germany) was used to determine elemental concentrations in the samples. The following metals were measured in the Low Resolution (LR), silver (Ag), cadmium (Cd), and lead (Pb), while aluminium (Al), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn) were

measured in the medium resolution (MR). Arsenic (As), which is typically more problematic in matrices containing calcium and chloride, was measured in the highest resolution mode (HR). The three mass resolutions correspond to 300, 4000, and 10,000 as defined by the 10% valley, equivalent to 5% peak height. A NIST standard (SRM 1640a) was used as analytical quality control and the recoveries were consistently between 97 - 103% of the certified values for all the measured elements. Metal concentrations (ng m^{-3}) of the blank samples (collection liquid in cone; $n=5$) were all below the detection limit except for very low traces of Ag (<0.013), Mn (<0.061), Cu (<0.086), and Zn (<0.068).

In addition, nine transition metals (As, Cd, Pb, Zn, Cu, As, Ni, Cd) from the Traffic 1 and Metal recycling plant locations were reported by the VMM during the sampling period. These samples were collected (24 h) using a SEQ 47/50 sequential gravimetric sampler (Leckel, Germany) with PM_{10} inlet and $2.3 \text{ m}^3 \text{ h}^{-1}$ flow rate, after which ED-XRF analysis was performed (following the EN14902 reference).

2.5 Monitoring of pro-inflammatory response genes at mRNA level

Samples were exposed for 3 h to the PMA-differentiated human macrophage-like cell line U937 (ATCC® CRL-1593.2™) as more extensively discussed in the supplementary materials. After co-incubation, total RNA was isolated with the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and stored at -80°C . RNA integrity was verified on a bleach gel as described by Aranda et al. (2012). One μg of RNA (quantified with Qubit 3.0; Thermo Fisher, Waltham, Massachusetts, USA) was used for cDNA synthesis with the ReadyScript® cDNA Synthesis Mix (Sigma Aldrich). The expression of three selected pro-

inflammatory markers (IL-8, IL-1 β and TNF α) and two reference genes (M=0.258, CV=0.09) was quantified by RT-qPCR on a StepOne Plus Real-Time PCR System (v.2.0; Applied Biosystems, Foster City, California, United States). *Guanine nucleotide binding protein beta polypeptide 2-like 1 (GNB2L1)* and *cytochrome c-1 (CYC1)* were selected as reference genes, following the geNorm analysis in qbase⁺ (Vandesompele et al., 2002). All primers were tested for efficiencies between 90-110% and their sequences can be found in the supplementary information (Table S1). Every cDNA sample was used in duplicate, each 20 μ L reaction consisting of Power SYBR[®] Green PCR Master Mix (Applied Biosystems), 0.15 μ M of each primer, 25 ng of diluted cDNA, and nuclease-free water. The resulting data were analyzed using the qbase⁺ software package (Hellemans et al., 2007) for calculating relative expression levels of the cytokines as compared to the reference genes and statistical analysis for comparison amongst groups (One-way ANOVA, corrected for multiple testing).

2.6 Statistical analyses

In GraphPad Prism v6.05 the logarithm-transformed data that were normally distributed were analyzed for significance by one-way ANOVA followed by the Tukey test to correct for multiple comparisons, unless otherwise stated. Welch's t-test was used for unequal variance. $P < 0.05$ was judged to be statistically significant.

3. Results

3.1 Atmospheric endotoxin concentration

First, we monitored the airborne endotoxin concentrations (Fig. 1) over the different urban land use classes (all within 15 km distance), categorized as green, traffic, and industrial, based on the expected dominant local emission source (Fig. SA1). As measured by the rFC

assay, endotoxin concentrations ranged from 0.65 to 11.72 EU m⁻³. Although greener urban areas have many microbial sources (from plants, soil, and animals), the green areas studied here did not show significantly higher concentrations of airborne endotoxins (geomeans (GM) 2.79 EU m⁻³; 95% confidence interval 1.93-4.04) than the industrial (GM 1.92 EU m⁻³; 95% confidence interval: 1.21-3.02) or the urban traffic locations (3.68 EU m⁻³; 95% confidence interval 2.59-5.23). In fact, the traffic locations showed the highest GM (Fig. 1).

3.2 Biological recognition of endotoxin through human TLR4/MD-2 complex

Samples were tested for their biological recognition of endotoxin to human TLR4 using the HEK293 hTLR4 reporter cell. A standard curve was generated from the same endotoxin stock as used in the rFC assay to predict the endotoxin units per mL (EU mL⁻¹). In terms of this TLR4 bio-assay, the biological recognition of endotoxin differed significantly between the land use classes. Furthermore, the traffic areas studied here showed the highest response. Moreover, the methods for endotoxin quantification differed significantly for the green locations, where recognition of endotoxin by TLR4 stimulation was significantly lower than the rFC-determined endotoxin concentrations ($p=0.0023$). Subsequently, the TLR4 bioassay showed the green areas to have the lowest concentrations of endotoxins, significantly lower than the traffic sites.

3.3 Relationship between the rFC assay and TLR4 recognition.

Although endotoxin has typically been quantified with the LAL assay, we choose the rFC assay to analyse these environmental samples since its simplified reaction pathway offers reduced likelihood of interference from other PM components, such as the false activation by yeast glucans (Roslansky and Novitsky, 1991; Vassallo and Limper, 1999). Despite this,

the rFC assay still displayed higher endotoxin concentrations than the TLR4 assay for 34 out of 41 samples (Fig. 2B), suggesting the difference between Factor-C based assays and the TLR4 bioassay to be attributed to more than yeast glucans as suggested by Peters et al. (2012). The relationship between these assays was investigated using linear regression analysis and initially showed a moderate correlation ($R^2=0.55$, $n=41$, $p<0.001$) (Fig. 2A). However, when both land use class and the interaction effect between the latter and rFC measurements (i.e. change in gradients) were included in the model, the fit significantly improved ($R^2=0.73$, $n=41$, $p<0.0001$). The land use class of the collected air sample therefore seemed to affect the bioactivity of the quantified endotoxin to the human host receptor, with endotoxin from the traffic areas being most bioactive and the green areas showing a generally low response ratio (Fig. 2B).

While the HEK hTLR4 reporter assay offered a more accurate model for predicting the inflammatory potential of PM associated endotoxin – compared to the rFC assay which is used to quantify endotoxin based on its activation of Factor C, an immune glycoprotein of the horseshoe crab – the bioassay is not without its limitations. While TLR4 is not biologically sensitive to fungal glucans (as with the LAL assay), fungal overgrowth of an environmental sample during co-incubation is a problem for the cell line viability, and certain samples may thus need to be excluded. Here, the cell viability of 3/50 samples was severely affected in all three technical repeats and results were excluded. When fungal contamination was encountered (in 8 samples in only one of the technical repeats), the samples were repeated in another independent experiment. While this problem may be minimized by a shorter incubation period, it is an important consideration to keep in mind for future studies.

3.4 Relation of endotoxin to particle concentration

To investigate the difference in TLR4 recognition of endotoxin from the urban land use classes, we turned our attention to the concentration and composition of particles from these environments. Since endotoxins are predominantly associated with the coarse (2.5-10 μm) PM fraction (Heinrich et al., 2003; Traversi et al., 2011), we first investigated the concentration of these particles in the different land use classes using a Coulter counter, and secondly determined if the biological activity of endotoxin may be correlated to PM particle count (Fig. S2).

The traffic areas showed a trend to contain the highest number of coarse particles per cubic meter of air (GM: 5.56×10^4) compared to the industrial (GM: 4.83×10^4) and urban green locations (GM: 3.87×10^4), however, not significantly so ($p=0.1899$; Fig. S2A). Furthermore, the biological recognition of endotoxin was only significantly correlated to the coarse particle count from the traffic locations ($R^2=0.51$, $n=17$, $p=0.0014$) (Fig. S2C). This correlation could not be significantly found for green and industrial locations.

3.5 Atmospheric transition metals concentrations

Transition metals are important atmospheric contaminants which may not only confound responses to airborne microbial endotoxins through oxidative stress (Degobbi et al., 2011; Guastadisegni et al., 2010; Kelly, 2003), but transition metals such as nickel and cobalt are known to directly activate TLR4 (Oblak et al., 2015; Rachmawati et al., 2013). We subsequently quantified 12 major transition metals in each sample by ICP-MS and grouped the data per urban land use class described above. The concentrations of metals collected over 40 minutes with the Coriolis were found to be comparable with the 24 hour-averaged

concentrations reported by the VMM for the corresponding days and locations (Traffic 1 and the metal recycling plant; Table S3).

From Fig. 3 and S3, there are clear differences in metal concentrations between the traffic, urban green, and metal recycling locations. The two locations representing the urban green land use class showed similar and relatively low concentrations for all the corresponding transition metals (Table S2). In contrast, the traffic locations had significantly elevated concentrations of Fe, Cr, Zn, and Cu than the other land use classes (Kruskal-Wallis test with Dunn's test for multiple comparisons). Furthermore, in comparison to Traffic site 1 (vehicular traffic), Traffic site 2 (which had vehicular and tram traffic) had significantly higher concentrations of Cr, Mn, and Fe, which are relevant indicators for railway abrasion (Gehrig et al., 2007). Lastly, the two industrial locations showed to be the most distinct from each other in terms of their atmospheric metal profiles. The metal recycling plant (non-ferrous based) displayed an entirely different profile with overall high and variable metal concentrations, dominated by cadmium (Cd), cobalt (Co), silver (Ag), lead (Pb), and arsenic (As) – metals commonly recycled at this plant and often measured to be in exceedance of the European target values by the VMM (VMM, 2016b). Further information of the correlation of the twelve metals for the specific locations may be found in the supplementary material (Fig. S4).

3.6 Immune response of collected urban air samples

After quantifying microbial endotoxins concentration (Fig. 1A), insoluble particles (Fig. S2) and transition metals (Fig. 3) in the closely-located urban land use classes, we profiled the relative inflammatory responses of the different samples. Messenger RNA levels of the major pro-inflammatory markers IL-8, IL-1 β , and TNF α were measured with real-time qPCR

after a three hour co-incubation of a sample with the model human monocytic U937 cells. Monitoring of mRNA levels, instead of protein levels, was preferred here in order to detect early-response signals and to avoid cross-reaction with unknown contaminants in these environmental samples (Seagrave et al., 2004).

In general, the urban traffic and industrial areas showed a heightened and varied pro-inflammatory response for the three markers, in contrast to the urban green area samples which showed relatively low and stable responses (Fig. 4). The two industrial locations differed clearly for the IL-1 β and TNF α mRNA responses, with the harbour location showing a significantly higher immune response for TNF α mRNA, while no difference was seen for IL-8. Despite both being industrial sites, this suggests the activation of different inflammatory pathways based on the unique composition of the samples, for which the metal profiles were previously seen to differ significantly (Fig. 3). Consequently, for all the locations, IL-1 β and TNF α are better correlated to each other ($R^2 = 0.84$, $n=42$, $p<0.0001$) than to IL-8 ($R^2 = 0.61$; $R^2 = 0.41$, $p<0.0001$, respectively).

3.7 Associations between source composition and immune response

Consequently, we explored the correlations between the endotoxins, metals and inflammation markers. Typically, isolated LPS is very strongly correlated to the IL-8 mRNA response in the U937 cell model (Fig. S5). However, this is more complicated for environmental samples where many other components are capable of contributing to the IL-8 response and where different forms and thus potencies of LPS are present. Here, endotoxin concentrations as determined by the rFC assay were only weakly correlated with the IL-8 response ($R^2=0.2$, $p=0.003$). Although, when taking the land use classes into account, we found a positive correlation originating for the industrial ($R^2=0.15$, $p=0.1775$)

and traffic locations ($R^2=0.35$, $p=0.017$). Despite a wide range of endotoxin concentrations found in the green locations, the IL-8 response remained relatively low (below 2.9 relative expression, Fig. 5). However, endotoxin concentrations as measured by the rFC assay - specifically in the green locations - were observed (section 3.1) to be less capable of stimulating TLR4 (Fig. 1A), likely also explaining the lack of activation of the pro-inflammatory response compared to the other locations. Subsequently, by rather using the bioassay of TLR4 stimulation as a proxy for bioactive endotoxin concentration, the correlation significantly improved for the prediction of the IL-8 response ($R^2 = 0.38$, $p<0.0001$), no longer with significant effects or interactions from the areas. In fact, of the measured factors, IL-8 was best correlated to the TLR4 bioassay, in addition to Mn, Fe and Cr ($r=0.64$, 0.63 , 0.57 ; $p\leq 0.0001$).

Although nickel and cobalt have previously been shown to directly activate hTLR4/MD-2, their effect in this study was negligible in comparison to the surprisingly strong correlation of iron with the stimulation of TLR4 in the HEK293 cell line ($R^2=0.50$; $n=41$; $p<0.0001$), with no significant interaction effect of the considered monitoring locations/land use classes. Subsequently, we found that by adding iron values together with rFC assay values as factors in the linear regression model predicting TLR4 stimulation (HEK assay), the model was significantly improved ($R^2=0.77$, $n=41$; $p<0.0001$).

Using multivariate analysis, the IL-1 β response was best correlated with Mn ($r=0.55$; $p=0.0002$), especially in the traffic locations - Traffic 1 ($r=0.88$, $p<0.0001$) and Traffic 2 ($r=0.94$, $p<0.0001$) - and also the TLR4 bioassay ($r=0.52$, $p=0.0005$). Furthermore, the metals Mn, Fe and Cr were often very strongly correlated with each other (Fig. S4). Particle

concentration was also best correlated to Mn for the various locations (Fig. S6). Lastly, the TNF α response was better associated to the TLR4 bioassay ($r=0.5$, $p=0.0007$).

3.8 Influence of iron on TLR4 stimulation

Although iron may originate from different sources and occur in different forms/speciations in the various land use classes, location had no significant effect on the correlation of iron with the stimulation of TLR4. However compelling this association, studies have repeatedly shown that iron cations are not capable of independently stimulating the TLR4/MD-2 complex, unlike nickel and cobalt (Oblak et al., 2015; Rachmawati et al., 2013). However, the ability of iron to act synergistically with LPS to stimulate the TLR4/MD-2 complex has not yet been investigated. In this study, HEK-Blue hTLR4 cells treated with 5 EU mL⁻¹ LPS and increasing concentrations of FeCl₂ showed a significant increase in SEAP production (reporter of TLR4 activation) at iron concentrations of 0.1 and 0.2 mM (60, 64% respectively in terms of EU mL⁻¹), while the iron itself (in absence of LPS) did not stimulate a response significantly higher than the negative control (Fig. 6). Dosing LPS with another divalent cation, MgCl₂ (0.1 μ M -1mM), showed no significant effect on LPS stimulation from the negative control (Fig. S7). Cell viability was tested with the CellTiter-Glo 2.0 assay and a drop in viability was observed when the iron concentrations (in the absence and presence of LPS) exceeded 0.5 mM (Fig. S8), as similarly seen in the study of Rachmawati et al. (2013). A corresponding drop in TLR4 stimulation was observed beyond 0.5 mM FeCl₂ with LPS. Fortunately, the environmental samples did not reach these concentrations. Furthermore, since alkaline phosphatases depend on Zn²⁺ and Mg²⁺ divalent cations for their enzymatic activity, we established that the increasing concentrations of Fe²⁺ had no significant effect

on the activity of the secreted embryonic alkaline phosphatase (SEAP) reporter enzyme in this study.

4. Discussion and Conclusions

In this study, we not only quantified the concentration of airborne endotoxin for different urban land use classes, but we also determined the ability of endotoxin – as part of PM - to be recognised by human host cells *in vitro* through the key innate immune receptor TLR4. This TLR4 interaction was found to be a better indicator for the IL-8 response than endotoxin concentrations measured by the rFC assay. This may be partly explained by the decreased sensitivity of the rFC assay in discriminating between different degrees of biologically active endotoxin. Here, endotoxin concentrations calculated from a standard curve in the HEK hTLR4 assay generally gave lower values than those determined with the rFC assay. This suggests that LPS from the environmental samples is simply not as potent as the *E.coli* LPS used to construct the standard curve in the HEK hTLR4 bioassay, thus underestimating the endotoxin concentration of the environmental sample. This is highly plausible, as the hexa-acylated LPS structure of *E.coli* is considered to be close to that optimally recognised by human cellular LPS receptors such as TLR4, while many environmental gram-negative bacteria other than *E. coli* may have other LPS structures which are less stimulatory for TLR4 (e.g. penta-acylated and 1'-phosphorylated LPS), yet still exhibit high LAL activity (Schroemm et al., 2000).

Nevertheless, even when considering that ambient environmental LPS might be less pro-inflammatory than pathogenic LPS from bacteria such as *E. coli*, synergistic effects between environmental LPS and other PM components likely also affected the hTLR4 interactions in our assays. This was apparent by the correlation of the endotoxin assays being significantly

dependent on the land use class. In the urban locations studied here, the traffic locations were found to be significantly higher in bioactive endotoxin than the industrial and green locations, despite all areas having similar rFC-determined endotoxin concentrations.

We subsequently turned our attention to the chemical composition of PM to explain the disparity between the rFC assay and TLR4 stimulation for the different urban land use classes. We initially hypothesized that Co and Ni could improve the regression model since they were previously shown to directly activate the TLR4/MD-2 complex (Oblak et al., 2015; Rachmawati et al., 2013; Schmidt et al., 2010). Surprisingly, the effects of Co and Ni were negligible in comparison to that of Fe, improving the model significantly by explaining 77% of the variation of the TLR4 stimulation and no longer dependent on the urban land use classes. The effect of iron proved to be more than a correlation, since dosing endotoxin with iron chloride led to an increase in TLR4 stimulation in the HEK293 cell line while iron itself was unable to stimulate a response. Interestingly in a study by Becker et al. (2005), iron concentrations quantified from coarse PM fractions correlated well with the IL-6 release in alveolar macrophages (from normal individuals through bronchial brushings), while no correlation was found in ultrafine particles. Although endotoxin was not quantified in their study, it is interesting to note that the inflammatory potential of iron was best correlated in the coarse PM fraction where endotoxin is predominantly found.

Iron is typically one of the most abundant transition metals found in urban PM (Michael et al., 2013), as confirmed in this study, and may originate from crustal matter, iron industries, and traffic and railway related sources (Schauer et al., 2006). In terms of health effects, iron is commonly known as a redox-active metal capable of causing oxidative stress by generating reactive oxygen species (ROS) through Fenton reactions (Hofman et al., 2017;

Valavanidis et al., 2005). This study showed another way in which iron may contribute to the inflammatory response and confirms the importance of PM composition from different urban land use classes on their resulting synergistic and inflammatory potential. It also raises interesting further research questions regarding its mechanism, the chemical forms of iron or other potential metals involved, and whether the effect of iron and LPS on the TLR4/MD-2 complex is conserved within the human species (as with nickel and cobalt ions) or also evident in animal models and cell lines.

Acknowledgements

We acknowledge the valuable help of Karin Van den Bergh (SPHERE, University Antwerp, Belgium) for her assistance with the Coulter counter measurements. This research was financially supported by the University of Antwerp (BOF), EUROSAs, and the Research Foundation of Flanders (KaN research grant number 1507114N; ProCure project number IWT150052, J. Hofman postdoctoral grant number 1214816N, S. Moretti Ph.D fellowship 1120118N). The authors declare they have no competing interests

References

- Analitis, A., Katsouyanni, K., Dimakopoulou, K., Samoli, E., Nikoloulopoulos, A.K., Petasakis, Y., Touloumi, G., Schwartz, J., Anderson, H.R., Cambra, K., 2006. Short-term effects of ambient particles on cardiovascular and respiratory mortality. *Epidemiology* 17, 230-233.
- Anderson, J.O., Thundiyil, J.G., Stolbach, A., 2012. Clearing the air: a review of the effects of particulate matter air pollution on human health. *Journal of Medical Toxicology* 8, 166-175.
- Aranda, P.S., LaJoie, D.M., Jorcyk, C.L., 2012. Bleach gel: a simple agarose gel for analyzing RNA quality. *Electrophoresis* 33, 366-369.

- 436 Baccini, M., Biggeri, A., 2012. Short-term impact of air pollution among Italian cities covered
437 by the EpiAir2 project. *Epidemiol. Prev.* 37, 252-262.
- 438 Becker, S., Dailey, L.A., Soukup, J.M., Grambow, S.C., Devlin, R.B., Huang, Y.-C.T., 2005.
439 Seasonal variations in air pollution particle-induced inflammatory mediator release and
440 oxidative stress. *Environmental Health Perspectives* 113, 1032.
- 441 Becker, S., Soukup, J.M., Sioutas, C., Cassee, F.R., 2004. Response of human alveolar
442 macrophages to ultrafine, fine, and coarse urban air pollution particles. *Experimental*
443 *Lung Research* 29, 29-44.
- 444 Boldo, E., Medina, S., Le Tertre, A., Hurley, F., Mücke, H.-G., Ballester, F., Aguilera, I., 2006.
445 Apheis: Health impact assessment of long-term exposure to PM_{2.5} in 23 European cities.
446 *Eur. J Epidemiol.* 21, 449-458.
- 447 Carter, J.D., Ghio, A.J., Samet, J.M., Devlin, R.B., 1997. Cytokine production by human airway
448 epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicology*
449 *and Applied Pharmacology* 146, 180-188.
- 450 Degobbi, C., Saldiva, P.H.N., Rogers, C., 2011. Endotoxin as modifier of particulate matter
451 toxicity: a review of the literature. *Aerobiologia* 27, 97-105.
- 452 Dehus, O., Hartung, T., Hermann, C., 2006. Endotoxin evaluation of eleven
453 lipopolysaccharides by whole blood assay does not always correlate with Limulus
454 amoebocyte lysate assay. *Journal of endotoxin research* 12, 171-180.
- 455 Dominici, F., McDermott, A., Zeger, S.L., Samet, J.M., 2003. National maps of the effects of
456 particulate matter on mortality: exploring geographical variation. *Environ. Health*
457 *Perspect.* 111, 39.
- 458 Erridge, C., Bennett-Guerrero, E., Poxton, I.R., 2002. Structure and function of
459 lipopolysaccharides. *Microbes and infection* 4, 837-851.

- 460 Gehrig, R., Hill, M., Lienemann, P., Zwicky, C.N., Bukowiecki, N., Weingartner, E.,
461 Baltensperger, U., Buchmann, B., 2007. Contribution of railway traffic to local PM₁₀
462 concentrations in Switzerland. *Atmospheric Environment* 41, 923-933.
- 463 Guastadisegni, C., Kelly, F.J., Cassee, F.R., Gerlofs-Nijland, M.E., Janssen, N.A., Pozzi, R.,
464 Brunekreef, B., Sandström, T., Mudway, I., 2010. Determinants of the proinflammatory
465 action of ambient particulate matter in immortalized murine macrophages.
466 *Environmental health perspectives* 118, 1728.
- 467 Gutschmann, T., Howe, J., Zähringer, U., Garidel, P., Schromm, A., Koch, M., Fujimoto, Y.,
468 Fukase, K., Moriyon, I., Martínez-de-Tejada, G., Brandenburg, K., 2010. Structural
469 prerequisites for endotoxic activity in the Limulus test as compared to cytokine
470 production in mononuclear cells. *Innate immunity* 16, 39-47.
- 471 Heinrich, J., Pitz, M., Bischof, W., Krug, N., Borm, P.J.A., 2003. Endotoxin in fine (PM_{2.5}) and
472 coarse (PM_{2.5-10}) particle mass of ambient aerosols. A temporo-spatial analysis.
473 *Atmospheric Environment* 37, 3659-3667.
- 474 Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase
475 relative quantification framework and software for management and automated analysis
476 of real-time quantitative PCR data. *Genome biology* 8, R19.
- 477 Hetland, R.B., Cassee, F.R., Låg, M., Refsnes, M., Dybing, E., Schwarze, P.E., 2005. Cytokine
478 release from alveolar macrophages exposed to ambient particulate matter:
479 heterogeneity in relation to size, city and season. *Particle and fibre toxicology* 2, 4.
- 480 Hofman, J., Maher, B.A., Muxworthy, A.R., Wuyts, K., Castanheiro, A., Samson, R., 2017.
481 Biomagnetic monitoring of atmospheric pollution: a review of magnetic signatures from
482 biological sensors. *Environmental science & technology* 51, 6648-6664.

- 483 Imrich, A., Ning, Y.Y., Koziel, H., Coull, B., Kobzik, L., 1999. Lipopolysaccharide priming
484 amplifies lung macrophage tumor necrosis factor production in response to air particles.
485 Toxicology and applied pharmacology 159, 117-124.
- 486 Katsouyanni, K., Touloumi, G., Samoli, E., Gryparis, A., Le Tertre, A., Monopolis, Y., Rossi, G.,
487 Zmirou, D., Ballester, F., Boumghar, A., 2001. Confounding and effect modification in the
488 short-term effects of ambient particles on total mortality: results from 29 European cities
489 within the APHEA2 project. Epidemiology 12, 521-531.
- 490 Kelly, F.J., 2003. Oxidative stress: its role in air pollution and adverse health effects.
491 Occupational and environmental medicine 60, 612-616.
- 492 Li, N., Xia, T., Nel, A.E., 2008. The role of oxidative stress in ambient particulate matter-
493 induced lung diseases and its implications in the toxicity of engineered nanoparticles.
494 Free Radical Biology and Medicine 44, 1689-1699.
- 495 Michael, S., Montag, M., Dott, W., 2013. Pro-inflammatory effects and oxidative stress in
496 lung macrophages and epithelial cells induced by ambient particulate matter.
497 Environmental pollution 183, 19-29.
- 498 Mueller-Anneling, L., Avol, E., Peters, J.M., Thorne, P.S., 2004. Ambient endotoxin
499 concentrations in PM10 from Southern California. Environmental Health Perspectives
500 112, 583-588.
- 501 Nilsson, S., Merritt, A., Bellander, T., 2011. Endotoxins in urban air in Stockholm, Sweden.
502 Atmospheric Environment 45, 266-270.
- 503 Ning, Y., Imrich, A., Goldsmith, C.-A., Qin, G., Kobzik, L., 2000. Alveolar macrophage cytokine
504 production in response to air particles in vitro: role of endotoxin. Journal of Toxicology
505 and Environmental Health Part A 59, 165-180.

- 506 Oblak, A., Pohar, J., Jerala, R., 2015. MD-2 determinants of nickel and cobalt-mediated
507 activation of human TLR4. *PloS one* 10, e0120583.
- 508 Peters, M., Fritz, P., Bufe, A., 2012. A bioassay for determination of lipopolysaccharide in
509 environmental samples. *Innate immunity* 18, 694-699.
- 510 Rachmawati, D., Bontkes, H.J., Verstege, M.I., Muris, J., von Blomberg, B.M.E., Scheper, R.J.,
511 van Hoogstraten, I.M., 2013. Transition metal sensing by Toll-like receptor-4: next to
512 nickel, cobalt and palladium are potent human dendritic cell stimulators. *Contact*
513 *Dermatitis* 68, 331-338.
- 514 Roslansky, P.F., Novitsky, T.J., 1991. Sensitivity of *Limulus* amoebocyte lysate (LAL) to LAL-
515 reactive glucans. *Journal of clinical microbiology* 29, 2477-2483.
- 516 Schauer, J.J., Lough, G.C., Shafer, M.M., Christensen, W.F., Arndt, M.F., DeMinter, J.T., Park,
517 J., 2006. Characterization of metals emitted from motor vehicles. Research report (Health
518 Effects Institute), 1-76; discussion 77-88.
- 519 Schmidt, M., Raghavan, B., Müller, V., Vogl, T., Fejer, G., Tchaptchet, S., Keck, S., Kalis, C.,
520 Nielsen, P.J., Galanos, C., 2010. Crucial role for human Toll-like receptor 4 in the
521 development of contact allergy to nickel. *Nature immunology* 11, 814-819.
- 522 Schromm, A.B., Brandenburg, K., Loppnow, H., Moran, A.P., Koch, M.H., Rietschel, E.T.,
523 Seydel, U., 2000. Biological activities of lipopolysaccharides are determined by the shape
524 of their lipid A portion. *Eur J Biochem* 267, 2008-2013.
- 525 Seagrave, J., Knall, C., McDonald, J.D., Mauderly, J.L., 2004. Diesel particulate material binds
526 and concentrates a proinflammatory cytokine that causes neutrophil migration.
527 *Inhalation Toxicology* 16, 93-98.

- 528 Soukup, J.M., Becker, S., 2001. Human alveolar macrophage responses to air pollution
529 particulates are associated with insoluble components of coarse material, including
530 particulate endotoxin. *Toxicology and applied pharmacology* 171, 20-26.
- 531 Stafoggia, M., Zauli-Sajani, S., Pey, J., Samoli, E., Alessandrini, E., Basagaña, X., Cernigliaro,
532 A., Chiusolo, M., Demaria, M., Díaz, J., 2016. Desert dust outbreaks in Southern Europe:
533 contribution to daily PM₁₀ concentrations and short-term associations with mortality
534 and hospital admissions. *Environ. Health Perspect.* 124, 413.
- 535 Steerenberg, P., Withagen, C., Van Dalen, W., Dormans, J., Cassee, F., Heisterkamp, S., Van
536 Loveren, H., 2004. Adjuvant activity of ambient particulate matter of different sites, sizes,
537 and seasons in a respiratory allergy mouse model. *Toxicology and applied pharmacology*
538 200, 186-200.
- 539 Tager, I.B., Lurmann, F.W., Haight, T., Alcorn, S., Penfold, B., Hammond, S.K., 2010. Temporal
540 and spatial patterns of ambient endotoxin concentrations in Fresno, California.
541 *Environmental Health Perspectives* 118, 1491-1496.
- 542 Traversi, D., Alessandria, L., Schilirò, T., Gilli, G., 2011. Size-fractionated PM₁₀ monitoring in
543 relation to the contribution of endotoxins in different polluted areas. *Atmospheric*
544 *Environment* 45, 3515-3521.
- 545 Valavanidis, A., Fiotakis, K., Vlachogianni, T., 2008. Airborne particulate matter and human
546 health: toxicological assessment and importance of size and composition of particles for
547 oxidative damage and carcinogenic mechanisms. *Journal of Environmental Science and*
548 *Health, Part C* 26, 339-362.
- 549 Valavanidis, A., Vlahoyianni, T., Fiotakis, K., 2005. Comparative study of the formation of
550 oxidative damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) adduct from the

nucleoside 2'-deoxyguanosine by transition metals and suspensions of particulate matter
in relation to metal content and redox reactivity. Free radical research 39, 1071-1081.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology 3, research0034. 0031.

Vassallo, R., Limper, A.H., 1999. Fungal β -glucan can yield false-positive results with the limulus amebocyte lysate endotoxin assay. Chest 116, 583-584.

VMM, 2016a. Luchtkwaliteit in de Antwerpse haven en de Antwerpse agglomeratie in 2015-jaarrapport 2015, D/2016/6871/067 ed.

VMM, 2016b. Luchtkwaliteit in het Vlaamse Gewest. Jaarverslag Immissiemeetnetten – 2015 D/2016/6871/052 ed.

Wang, B., Li, N., Deng, F., Buglak, N., Park, G., Su, S., Ren, A., Shen, G., Tao, S., Guo, X., 2016. Human bronchial epithelial cell injuries induced by fine particulate matter from sandstorm and non-sandstorm periods: Association with particle constituents. Journal of Environmental Sciences 47, 201-210.

567

568

569 **Fig. 1: Comparison of endotoxin quantification assays**

570 Endotoxin quantification by the rFC assay and via TLR4 stimulation through the HEK-Blue™
 571 hTLR4 cell line, both using the same endotoxin stock for their standard curves. Endotoxin
 572 concentrations from the separate locations within each land use class were not significantly
 573 different and further comparisons were thus made between the three land use classes
 574 (traffic, green and industrial) with each sample represented by a black dot. Statistical
 575 significance indicated by * $p < 0.05$, ** $p < 0.01$

576

577

578 **Fig. 2 (A): Relation of endotoxin assays**

579 The correlation between the rFC assay and the HEK-BlueTM hTLR4 cells for samples in the
 580 land use classes: traffic ●, urban green ▲, and industrial ●. (B): The response ratio of the
 581 hTLR4 stimulation to the rFC assay for the considered urban land use classes.

Fig. 3: Transition metal analysis

Transition metal concentrations (ng m^{-3}) measured with ICP-MS, with each dot represented by a sample from the six sampling sites within the city of Antwerp for urban green, traffic and industrial locations. Black dots represent Traffic 1, Park, and Harbour; grey dots represent Traffic 2, Campus, and Metal recycling plant respectively. Note the differences in the vertical axes. Points on dotted horizontal line were samples below the minimum detection limit.

Fig. 4: Relative pro-inflammatory response of urban air samples

mRNA levels of the pro-inflammatory markers IL-8, IL-1β, and TNFα measured in human macrophage-like U937 cells after 3 h exposure with collected air samples. Values are expressed relative to the negative control, represented by an expression of one, and the geometric means of the locations are shown in dotted lines, while each sample is represented as a dot. The land use classes: traffic, green and industrial each comprise two different sampling locations. The relative expressions for these pro-inflammatory

biomarkers were significantly higher than the negative control for all locations (Welch's t-test for unequal variance). Statistical significance indicated by * $p < 0.05$, ** $p < 0.01$

Fig. 5: Relation of endotoxin concentration to IL-8 expression

Scatter plot of endotoxin concentration as determined with the rFC assay (EU m⁻³) vs the relative expression in mRNA IL-8 expression, with each dot representing a sample from either the green, industrial and traffic locations. Grey points represent Campus, Metal plant, and Traffic 2, respectively.

Fig. 6: Effect of ferrous iron on LPS activity

HEK-Blue hTLR4 cells were stimulated in triplicate with LPS (5 EU mL⁻¹) and/or increasing iron concentrations (2 μM to 0.75 mM) for 20 hours. Results shown are from one

610 experiment (standard deviation shown for three technical repeats) representative of three
611 independent experiments. The resulting SEAP activity was converted to EU mL⁻¹ with a
612 standard curve. The “no LPS” control tested the response of the iron solutions in the
613 absence of LPS. For statistical analysis, the Fe doses were compared to the negative control
614 (* p<0.05, ** p<0.01).

615

Highlights

- Quantified endotoxin not strongly correlated to host recognition through hTLR4
- HEK TLR4 cell line better assay for IL8 response than rFC-quantified endotoxin
- Bioactivity of atmospheric endotoxin influenced by land-use class
- Ferrous iron modulates host endotoxin response