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Structural variants of *Salmonella* Typhimurium lipopolysaccharide induce less dimerization of TLR4/MD-2 and reduced pro-inflammatory cytokine production in human monocytes

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ABSTRACT

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) changes the structure of its lipopolysaccharide (LPS) in response to the environment. The two main LPS variants found in *S.* Typhimurium correspond to LPS with a hepta-acylated lipid A (LPS 430) and LPS with modified phosphate groups on its lipid A (LPS 435). We have previously shown that these modified LPS have a lower capacity than wild type (WT) LPS to induce the production of pro-inflammatory cytokines in mice. Nevertheless, it is not know if LPS 430 and LPS 435 could also subvert the innate immune responses in human cells. In this study, we found that LPS 430 and LPS 435 were less efficient than WT LPS to induce the production of pro-inflammatory cytokines by human monocytes, in addition we found a decreased dimerization of the TLR4/MD-2 complex in response to LPS 430, suggesting that structurally modified LPS are sensed differently than WT LPS by this receptor; however, LPS 430 and 435 induced similar activation of the transcription factors NF-κB p65, IRF3, p38 and ERK1/2 than WT LPS. Microarray analysis of LPS 430- and LPS 435-activated monocytes revealed a gene transcription profile with differences only in the expression levels of microRNA genes compared to the profile induced by WT LPS, suggesting that the lipid A modifications present in LPS 430 and LPS 435 have a moderate effect on the activation of the human TLR4/

Abbreviations: AP-1, activator protein 1; ERK, extracellular signal regulated kinase; IFN, interferon; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; MPL, monophosphoryl lipid A; MAP kinase, mitogen-activated protein kinase; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR4, toll-like receptor 4; TNF, tumour necrosis factor; TRIF, toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β

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MD-2 complex. Our results are relevant to understand LPS modulation of immune responses and this knowledge could be useful for the development of novel adjuvants and immunomodulators.

1. Introduction

Lipopolysaccharide (LPS) is the main component of the outer membrane of Gram-negative bacteria; it is a strong activator of the innate immune response and plays an important role in host-pathogen interactions (Needham and Trent, 2013). In mammals, high LPS concentrations can cause a systemic inflammatory response that leads to endotoxic shock and death (Morrison and Ryan, 1987). Most naturally occurring LPS have a lipid A portion with two phosphate groups and six acyl chains attached to a glucosamine disaccharide backbone. Several pathogenic bacteria have the capacity to modify the number of phosphate groups or acyl chains of the lipid A portion of their LPS as part of their immune evasion mechanisms (Steimle et al., 2016).

The recognition of hexa-acylated LPS by the innate immune system involves several proteins. LPS binding protein (LBP) extracts LPS from bacterial membranes and transfers it to CD14, which, in turn, transfers monomeric LPS to the TLR4/MD-2 complex in the plasma membrane of innate immune cells (Tobias et al., 1986; Wright et al., 1990). Five of the acyl chains (R3, R2', R3', R2'' and R3'') of the lipid A portion of LPS are inserted into an hydrophobic pocket of MD-2, while the sixth acyl chain (R2) is partially exposed and interacts with an hydrophobic portion of a second TLR4 (TLR4*). In addition, the two phosphate groups of lipid A interact with positively charged amino acid residues in TLR4, MD-2 and TLR4*. These interactions lead to the formation of a dimer that contains two TLR4/MD-2/LPS complexes (Park et al., 2009). After this receptor dimerization, two signalling pathways are activated: the MyD88 pathway and the TRIF pathway. The MyD88 pathway is activated at the plasma membrane and leads to p65 phosphorylation and to the nuclear translocation of the transcription factor NF-κB (p65/p50) (Medzhitov and Horng, 2009), while the TRIF pathway is activated after CD14-mediated internalization of the TLR4/MD-2/LPS dimer into endosomes, and leads to the nuclear translocation of IRF3 (Kagan et al., 2008; Zanoni et al., 2011); the MyD88 pathway also leads to the activation of MAP kinases, including p38, ERK1 and ERK2, which induce the nuclear translocation of AP-1 (Pandey et al., 2014). The end result of these signalling pathways is the production of pro-inflammatory cytokines (such as TNF-α, IL-1β and IL-6) and type I interferons (such as IFN-α and IFN-β), respectively (Yang and Seki, 2012).

The activation of the MyD88 and TRIF pathways begins with the binding of LPS (specifically, its lipid A portion) to the TLR4/MD-2 complex; the phosphate groups and the acyl chains of lipid A are essential for this process (Park et al., 2009), since lipid A variants with different numbers of acyl chains or phosphate groups have reduced or null capacity to induce the production of pro-inflammatory cytokines (Maeshima and Fernandez, 2013). Monophosphoryl lipid A (MPL), a lipid A variant that is 100-fold less toxic than LPS, induces a deficient dimerization of the TLR4/MD-2 complex and a reduced activation of the MyD88 pathway, but its activation of the TRIF pathway is comparable to that of biphosphoryl lipid A (Mata-Haro et al., 2007; Tanimura et al., 2014), which indicates that modifications in the number of phosphate groups or acyl chains of lipid A alter the binding of LPS to the TLR4/MD-2 complex, and can lead to a differential activation of the MyD88 and TRIF signalling pathways.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) modifies its lipid A through its PhoP/PhoQ and PmrA/PmrB two-component regulatory systems; these modifications confer resistance to antimicrobial peptides and thus increase bacterial virulence (Gunn et al., 1998; Gunn and Miller, 1996). The two main variants of LPS found in *S.* Typhimurium correspond to LPS with a hepta-acylated lipid A (LPS 430) and LPS with phosphoethanolamine and aminoarabinose attached to each of the phosphate groups of its lipid A (LPS 435) (Fig. 1). In a previous study, we reported that LPS 430 and LPS 435 induced lower levels of pro-inflammatory cytokines, compared to wild type (WT) *S.* Typhimurium LPS (which has two un-modified phosphate groups and six acyl chains), both in mice (in vivo) and in mouse macrophages (Pastelin-Palacios et al., 2011). Another study found that LPS 430 induced lower levels of TNF-α production by human monocytes, compared to WT LPS (Guo et al., 1997). However, the molecular mechanisms that explain the differential levels of cytokine induction by WT LPS, LPS 430 and LPS 435 are not known. In this study, we evaluated the effects of these LPS on human monocytes, analysing TLR4/MD-2 dimerization, signalling pathways activation, the transcriptional profile and the cytokines production induced.

2. Methods

2.1. Ethical approval

This study was approved by the Local Research and Ethics in Research Committee of National Medical Centre "Siglo XXI", Mexican

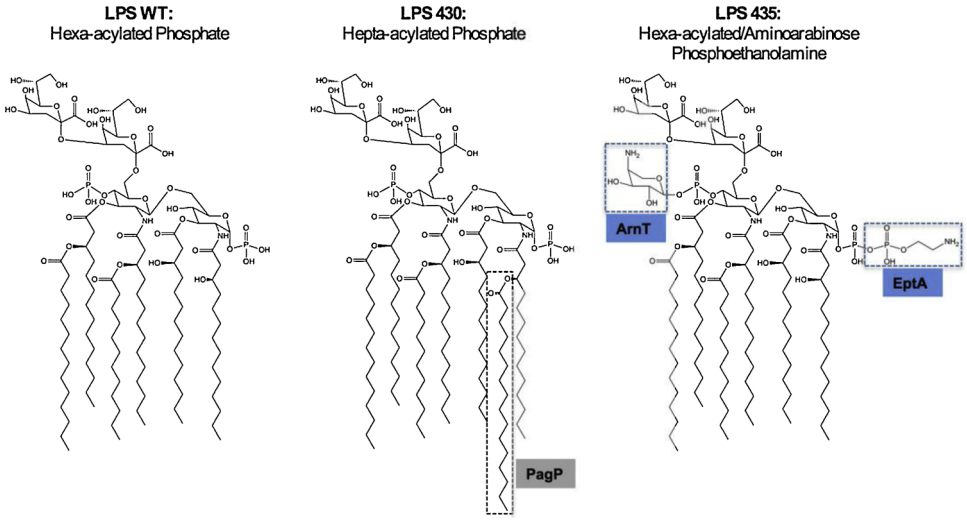

Fig. 1. Main lipid A structural modifications present in the lipopolysaccharides from *Salmonella enterica* serovar Typhimurium. The enzymes responsible for the structural modifications on LPS 430 and LPS 435 are controlled by the PhoP/PhoQ two-component regulatory system (PagP) or by the PmrA/PmrB two-component regulatory system (ArnT and EptA). The modifications of LPS 430 confer resistance to antimicrobial peptides (Gunn and Miller, 1996) and induce a decreased TLR4 activation, compared to WT LPS (Pastelin-Palacios et al., 2011; Guo et al., 1997); the modifications of LPS 435 also confer resistance to antimicrobial peptides (Gunn et al., 1998) and induce a decreased TLR4 activation, compared to WT LPS (Pastelin-Palacios et al., 2011). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Social Security Institute, Mexico City, Mexico (No. R-2015-3601-58). All the healthy volunteers signed an informed consent form, in accordance with the Good Clinical Practice.

2.2. Purification and characterization of Salmonella enterica serovar Typhimurium lipopolysaccharides

The LPS used in this study were purified from *Salmonella enterica* serovar Typhimurium WT (STWT, ATCC 14028s), 430 (ST430, CS022 pmrA::Tn10d) and 435 (ST435, ATCC 14028s pmrA505 zjd::Tn10dcam), using the hot phenol method followed by Folch extraction to remove lipid contamination (Tirsoaga et al., 2007). The LPS were lyophilized, weighed and diluted to the desired concentration in pyrogen-free water. One batch of LPS from each strain was used for all the experiments. LPS purity was evaluated with a colloidal gold protein stain (Blum, 1987), and LPS biological activity was assessed in TLR4/MD-2/CD14-transfected HEK293 cells, as previously reported (Pastelin-Palacios et al., 2011).

2.3. Separation and enrichment of peripheral blood human monocytes

Peripheral blood was drawn from healthy volunteers using EDTA Vacutainer tubes (Becton Dickinson, Plymouth, UK). Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation with Lymphoprep (Axis Shield, Oslo, Norway) and re-suspended in complete RPMI 1640 medium (BioWest, Nuaille, France) with 5% foetal calf serum (BioWest), 100 I.U./ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies, Carlsbad, CA). The PBMCs were analysed by flow cytometry (Accuri Flow Cytometer, BD Biosciences, San José, CA) to determine the percentage of monocytes (according to FSC and SCC parameters) present in each cell suspension, and the cell suspension was adjusted to the cell concentration required to plate 2.5×10^5 monocytes per well in 24-well culture plates, or 2×10^6 monocytes per well in 6-well culture plates (Costar, Sigma-Aldrich, St. Louis, MO). For the immunofluorescence experiments, pyrogen-free coverslips (Madesa, Mexico City, Mexico) were placed in the culture plates. Cells were incubated for 2 h at 37 °C and 5% CO_2, and washed three times with complete RPMI to remove non-adherent cells. After this process, the adherent cells were left at 37 °C and 5% CO_2 for at least 2 h in complete RPMI medium before stimulation. At least 90% of the adherent cells were $CD3^-CD19^-CD14^{++}CD16^-$ monocytes, as assessed by flow cytometry (Accuri Flow Cytometer).

2.4. Monocyte activation and cytokine quantification

For intracellular TNF-α determination, whole blood (300 µl) was stimulated with 1 µg/ml of WT LPS, LPS 430 or LPS 435 in the presence of brefeldin A (5 µg/ml) (BioLegend, San Diego, CA) for 2 h at 37 °C. The erythrocytes were lysed and the leukocytes were fixed with Lyse/Fix buffer (BD Biosciences); the cells were permeabilized with Perm/Wash buffer (BD Biosciences), and the corresponding antibodies were added: anti-TNF-α-APC (BioLegend), anti-CD14-PECy7, anti-CD16-Pacific blue, anti-CD19 PerCP-Cy5 and/or anti-CD3-FITC (all from BD Biosciences). After 30 min, the cells were washed and acquired in a FACS Canto flow cytometer (BD Biosciences). For each sample, at least 10,000 events were acquired in the monocyte ($CD14^{++}CD16^-$) gate. Data were analysed with FlowJo Software 10 (Tree Star, Ashland, OR).

Quantification of pro-inflammatory cytokines was performed after stimulating peripheral blood monocytes (2.5×10^5) with 1 µg/ml, or with 0.1 or 0.01 µg/ml, of WT LPS, LPS 430 or LPS 435 for 6. The plate was centrifuged at 1500 rpm for 5 min, and the supernatants were collected and stored at -70 °C until use. TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12 p70 were quantified with a Cytometric Bead Array (BD Biosciences). For each sample, at least 3000 events were acquired in an Accuri Flow Cytometer, and data were analysed with FCAP Array software 3.0 (BD Biosciences).

To determine the viability of LPS-activated monocytes, peripheral blood monocytes were activated with 1 µg/ml of WT LPS, LPS 430 or LPS 435 for 6 h. As a positive control, cell death was induced with 10% or 20% DMSO. The monocytes were stained with Annexin V-APC (BioLegend) and 7-amino-actinomycin D (7-AAD) (BD Biosciences); after 30 min, the cells were washed and acquired in a FACS Canto flow cytometer (BD Biosciences). For each sample, at least 10,000 events were acquired. Data were analysed with FlowJo Software 10 (Tree Star).

2.5. TLR4/MD-2 in vitro dimerization assay

The assay was performed as previously described (Yamakawa et al., 2013), with minor modifications. Briefly, 1 µg of the recombinant human TLR4/MD-2 complex [TLR4 Glu24-Lys631 (70.6 kDa, extracellular domain) and MD-2 Glu17-Asn160 (19.2 kDa), both with polyhistidine tags at the carboxyl-terminus] (R&D Systems, Minneapolis, MN,) dissolved in 30 µl of buffer [10 mM Tris-HCl (pH 8.0), 0.15 M NaCl and 0.02% Triton X-100], were mixed with 0.2, 1 or 2.5 µg of WT LPS, LPS 430, LPS 435 or LPS from *Escherichia coli* O111:B4 and incubated for 2 h at 37 °C. After this incubation, loading buffer [30% glycerol, 62.5 mM Tris-HCl (pH 8.0) and 0.001% bromophenol blue] was added, and the samples were separated by electrophoresis in native 10% polyacrylamide gels. Proteins were visualized with silver staining, and band density (after background subtraction) was determined with ImageJ software 1.50i (National Institutes of Health, Bethesda, MD). For each sample, the sum of the densities of the TLR4/MD-2 dimer and monomer bands were considered 100%, and the percentage corresponding to the band density of the TLR4/MD-2 dimer was calculated.

2.6. Determination of NF-κB, IRF3, p38 and ERK1/2 activation

2.6.1. Nuclear translocation

Peripheral blood monocytes, plated on coverslips, were stimulated with 1 µg/ml of WT LPS, LPS 430 or LPS 435 for 30 or 60 min. Non-stimulated monocytes were used as controls. The cells were then fixed for 20 min at room temperature with 4% paraformaldehyde, washed 3 times with PBS 1x and left for 1 h at room temperature with blocking buffer [PBS 1x, 5% foetal calf serum and 0.3% Triton X-100]. The blocking buffer was removed, and cells were left overnight with anti-NF-κB p65 (Cell Signalling Technology, Danvers, MA) or anti-IRF3 (Cell Signalling Technology) antibodies, diluted 1:400 in binding buffer [PBS 1x, 1% albumin and 0.3% Triton X-100]. The next day, the cells were washed 3 times with PBS 1x, and anti-rabbit IgG-Alexa Fluor 488 (Cell Signalling Technology) was added (1:500 dilution in binding buffer). After 2 h, the cells were washed 3 times with PBS 1x, and incubated for 1 h with phalloidin (Sigma-Aldrich), diluted 1:400 in PBS 1x, for actin staining. After 3 washes with PBS 1x, Hoechst stain (Invitrogen, Carlsbad, CA), diluted to 5 µg/ml in PBS 1x, was added for nuclear staining. The coverslips were mounted with Vectashield (Vector, Burlingame, CA) and analysed in a Nikon Ti Eclipse inverted confocal microscope equipped with an A1 imaging system and NIS Elements 5.0 software (Nikon Instruments, Tokyo, Japan. Images were analysed with ImageJ software 1.50i. Data are represented as the mean fluorescence intensity (MFI) of the transcription factor in the nucleus divided by the MFI of the transcription factor in the cytosol per cell, for each transcription factor.

2.6.2. Phosphorylation

One million PBMCs were stimulated with 0.1 µg/ml of WT LPS, LPS 430 or LPS 435 for 15 min. The cells were then fixed with Lyse/Fix Buffer (BD Phosflow, BD Biosciences), and permeabilized with cold methanol (70%) for 10 min at 4 °C. The cells were washed and the following antibodies were added: anti-CD14-PerCP-Cy5.5, anti-TLR-2-Alexa Fluor 647, anti-pp65-PE-Cy7 (pS529), anti-pp38 MAPK (pT180/pY182)-PE and anti-pERK1/2 (pT202/pY204)-Alexa Fluor 488 (all from

BD Biosciences). After 30 min at room temperature, the samples were washed and acquired in a FACS Aria III flow cytometer (BD Biosciences), and data were analysed with DIVA software 6.1.3 (BD Biosciences). For each sample, at least 10,000 events were acquired in the monocyte ($CD14^+TLR2^+$) gate, and the MFI of phosphorylated p65, p38 and ERK1/2 were measured on gated monocytes.

Alternatively, one million peripheral blood monocytes were stimulated with 1 μg/ml of WT LPS, LPS 430 or LPS 435 for 30 or 60 min. Non-stimulated monocytes were used as controls. The cells were lysed with RIPA buffer (Santa Cruz, Dallas, TX) and a cocktail of protease and phosphatase inhibitors (Roche, Basel, Switzerland). The supernatants were collected after centrifugation at 10,000 × *g* for 10 min at 4 °C, and protein concentrations were determined with a commercial kit based on the Bradford assay (Bio-Rad, Hercules, CA). Twenty micrograms of proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad). The membrane was blocked in TBS-T [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20] with 5% BSA for 1 h, and then incubated with the primary antibody in TBS-T with 5% BSA at 4 °C overnight. The membrane was rinsed three times with TBS-T and incubated with the secondary antibody in TBS-T with 0.5% BSA at room temperature for 1 h. The membrane was rinsed three times; Clarity Western ECL Substrate (Bio-Rad) was added, and chemiluminescence was detected with a C-DiGit Blot Scanner (LI-COR Biotechnology, Lincoln, NE). Images were analysed with Image Studio software 5.0 (LI-COR Biotechnology); lane normalization factors and normalized signals for each lane were used for the relative quantification of the target proteins. The primary antibodies were rabbit anti-NF-κB p65 (1:1000), rabbit anti-pNF-κB p65 (pS536) (1:1000), rabbit anti-IRF3 (1:1000) and rabbit anti-pIRF3 (pS396) (1:1000) from Cell Signalling Technology, and goat anti-GAPDH (1:5000) and mouse anti-β-actin (1:1000), kindly donated by Patricio Gariglio (CINVESTAV, IPN). The secondary antibodies were HRP-conjugated anti-rabbit IgG (1:1500) (Cell Signalling Technology), HRP-conjugated anti-mouse IgG (1:10,000) (Bio-Rad) and HRP-conjugated anti-goat IgG (1:20,000) (Santa Cruz).

2.7. Microarray analysis of the transcriptome of lipopolysaccharide-activated monocytes

Peripheral blood monocytes were stimulated with 1 μg/ml of WT LPS, LPS 430 or LPS 435 for 2 or 5 h. Non-stimulated monocytes were used as controls. RNA was extracted with an RNA Blood Mini kit, with on-column DNA digestion (Qiagen, Hilden, Germany). RNA purity and integrity were evaluated with an Agilent RNA Screen Tape in a Tape Station 2200 (Agilent, Santa Clara, CA); only samples with an RNA integrity number (RIN) of at least 9 were used. The RNA samples were processed and hybridized to a Human Gene 2.0 ST GeneChip (RNA from WT LPS- and LPS 430-activated monocytes, and from non-stimulated monocytes), or to a Human Transcriptome Array (HTA) 2.0 GeneChip (RNA from WT LPS- and LPS 435-activated monocytes, and from non-stimulated monocytes). Both arrays were from Affymetrix (Santa Clara, CA). The GeneChips were scanned with a GeneChip Scanner 3000 7G (Affymetrix).

Affymetrix GeneChip Command Console (AGCC) software was used to transform DAT files into CEL files. Affymetrix Expression Console software was then used to process the CEL files with the Robust Multi-chip Analysis (RMA) algorithm, including background correction, probeset signal integration and quantile normalization, to produce CHP files. CHP files were analysed with Affymetrix Transcriptome Analysis Console software 2.0 to detect differentially expressed genes between WT LPS, LPS 430 or LPS 435 vs. non-stimulated monocytes and LPS 430 vs. WT LPS or LPS 435 vs. WT LPS.

Gene Set Enrichment Analysis (GSEA) was performed with GSEA software 3.0 (Broad Institute, Cambridge, MA), using the RMA data from the gene expression profiles induced by WT LPS, LPS 430 or LPS 435, compared to non-stimulated monocytes (in the case of HTA GeneChips, only coding transcripts were considered). The gene sets database was derived from the Reactome pathway database (Molecular Signatures Database 5.2). The statistical significance of the enrichment score was analysed using gene set permutations (1000 permutations), and genes were ranked using ratio of classes (fold change) analysis. Gene sets with a false discovery rate (FDR) < 25% and nominal *p* values < 1% were considered significant. Heat maps were constructed with Morpheus software (Broad Institute, Cambridge, MA), using the RMA data from the genes differentially induced by LPS 430 vs. WT LPS.

To confirm the relative expression of the TNF-α, IL-6 and IL-1β mRNAs, peripheral blood monocytes were stimulated for 2 h with 1 μg/ml of WT LPS, LPS 430 or LPS 435, and RNA was extracted as described above. cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and the genes

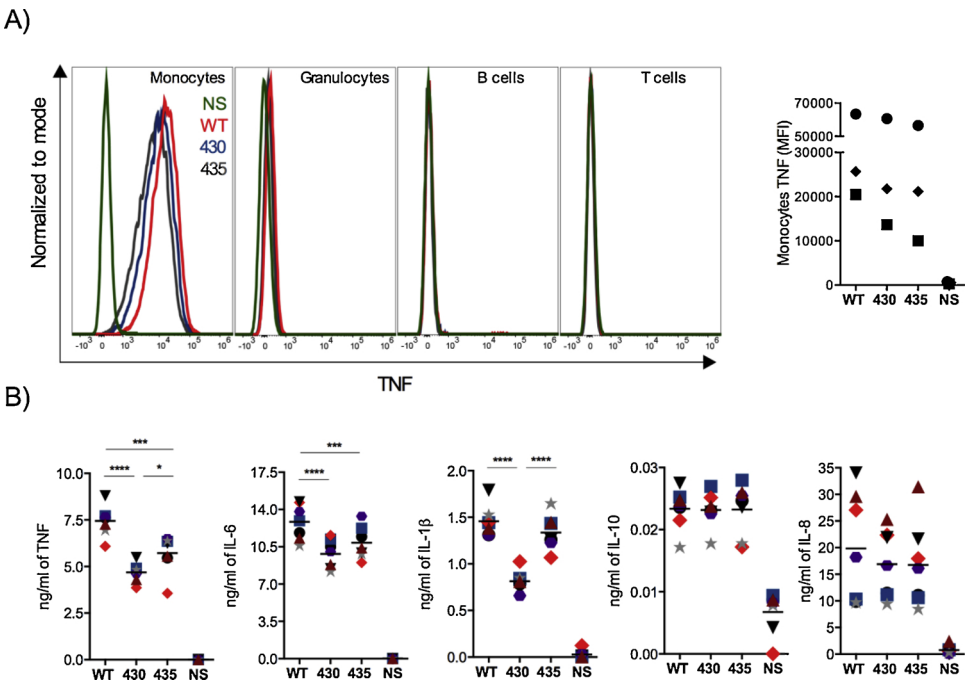

Fig. 2. LPS 430 induces the lowest production of pro-inflammatory cytokines by human monocytes. (A) Whole blood from three healthy volunteers was stimulated with 1 μg/ml of WT LPS, LPS 430 or LPS 435 in the presence of brefeldin A for 2 h, and the intracellular production of TNF-α by different cell populations was analysed by flow cytometry. The left panel shows the representative histograms obtained from one healthy volunteer; the right panel shows the mean fluorescence intensity (MFI) in monocytes ($CD14^{++}CD16^-$) from three healthy volunteers. (B) Peripheral blood monocytes from seven healthy volunteers (each symbol represents the results from one volunteer, the horizontal line is the mean) were stimulated with 1 μg/ml of WT LPS, LPS 430 or LPS 435. Supernatants were collected after 6 h and TNF-α, IL-6, IL-1β, IL-10, IL-8 and IL-12 p70 were quantified. One-way ANOVA with Tukey's multiple comparison test: $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$. WT, wild type. NS, not stimulated.

were amplified with the FastStart Universal SYBR Green master mix (Sigma-Aldrich), using the following primers: TNF-α, F: 5'-CTG-CTG-CAC-TTT-GGA-GTG-AT and R: 5'-AGA-TGA-TCT-GAC-TGC-CTG-GG; IL-6, F: 5'-AGC-CAC-TCA-CCT-CTT-CAG-AAC and R: 5'-GCC-TCT-TTG-CTG-CTT-TCA-CAC; IL-1β, F: 5'-GTG-GCA-ATG-AGG-ATG-ACT-TGT-TC and R: 5'-TAG-TGG-TGG-TCG-GAG-ATT-CGT-A; and GAPDH, F: 5'-GCA-CCG-TCA-AGG-CTG-AGA-AC and R: 5'-TGG-TGA-AGA-CGC-CAG-TGG-A. The PCR was performed in a StepOne Real-Time PCR System (Applied Biosystems), and the relative expression of TNF-α, IL-6 and IL-1β was determined with the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the reference gene, and non-stimulated monocytes as the control condition.

2.8. Statistical analysis

Data were analysed with GraphPad Prism software 6.0c (GraphPad Software, La Jolla, CA). The significance between three or more groups was determined with one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Differences with p values < 0.05 were considered significant.

3. Results

3.1. LPS 430 and LPS 435 induce lower levels of pro-inflammatory cytokines than WT LPS

Previous studies indicate that LPS 430 induces lower levels of TNF-α than WT LPS in human monocytes (Guo et al., 1997), while the effect of LPS 435 on these cells remains unknown. To evaluate the effect of LPS 430 and LPS 435 on the production of TNF-α and other cytokines by human monocytes, we performed an *ex vivo* experiment, stimulating whole blood from healthy volunteers (n = 3) with 1 μg/ml WT LPS, LPS 430 and LPS 435. After 2 h, monocytes ($CD14^{++}CD16^{-}$) were the main producers of TNF-α; granulocytes, T cells and B cells did not produce significant amounts of this cytokine (Fig. 2A). Monocytes where then enriched from peripheral blood and stimulated with 1 μg/ml WT LPS, LPS 430 and LPS 435 for 6 h. At this time point, LPS treatment did not cause significant monocyte apoptosis or dead (Supplementary Fig. 1). LPS 430 induced significantly lower levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β than WT LPS. LPS 435 induced significantly lower levels of TNF-α and IL-6 than WT LPS, but similar levels of IL-1β than WT LPS. In addition, LPS 430 induced significantly lower levels of TNF-α and IL-1β than LPS 435. No differences were found in the levels of IL-8 and IL-10 (Fig. 2B), and IL-12 p70 was undetectable at the analysed time (data not shown). Monocyte stimulation with lower concentrations of the LPS (0.1 or 0.01 μg/ml) for 6 h induced cytokine production; both LPS 430 and LPS 435 induced lower amounts of TNF-α and IL-6 than WT LPS, and LPS 430 induced lower amounts of IL-1β than WT LPS (Supplementary Fig. 2).

3.2. LPS 430 induces decreased dimerization of the TLR4/MD-2 complex

To elucidate the mechanism that leads to the reduced pro-inflammatory cytokine production in response to LPS 430 and LPS 435, we evaluated the capacity of the three LPS to induce the dimerization of the TLR4/MD-2 complex, which is the first step in the signalling pathway that leads to pro-inflammatory cytokine production. Recombinant human TLR4/MD-2 complex was mixed and incubated with each of the three LPS and analysed by native polyacrylamide gel electrophoresis. Formation of the 2:2:2 TLR4/MD-2/LPS dimer is weak with 0.2 μg of WT LPS, and the amount of dimer increases with 1 and 2.5 μg of WT LPS (Fig. 3A and B). Formation of the TLR4/MD-2/LPS dimer is very low with 0.2 μg of LPS 430 or LPS 435, and while dimer formation increases with 1 and 2.5 μg of LPS 430, it does not reach the dimer levels induced with 1 and 2.5 μg of WT LPS, respectively (the difference is statistically significant with 1 μg of each LPS). There is no significant difference in the amount of TLR4/MD-2/LPS dimer induced with 1 and 2.5 μg of LPS 435 and the amount of dimer induced with 1 and 2.5 μg of WT LPS (Fig. 3A and B).

3.3. LPS 430 and LPS 435 induce similar activation of the transcription factors NF-κB, IRF3, p38 and ERK1/2

MPL, the low-toxicity variant of LPS, induces a deficient dimerization of the TLR4/MD-2 complex, which leads to hypo-activation of the MyD88 pathway, while the activation of the TRIF pathway remains unaffected (Mata-Haro et al., 2007; Tanimura et al., 2014). Since we found that LPS 430 induced decreased dimerization of the TLR4/MD-2 complex, we investigated if this decreased dimerization was associated with reduced activation of the main transcription factors of the MyD88 and TRIF pathways: NF-κB p65 and IRF3, respectively; we focused on p65 because this is the only NF-κB family member that is associated with the transcription of the TNF-α mRNA (Falvo et al., 2010). Activation of monocytes with WT LPS, LPS 430 or LPS 435 significantly increased the phosphorylation of the NF-κB subunit p65 and of IRF3, compared to non-stimulated monocytes, but LPS 430 and LPS 435 induced the phosphorylation of these transcription factors to the same extents as WT LPS (Fig. 4A, B and C). The phosphorylation of the MAP kinases p38 and ERK1/2 were not significantly different in response to LPS 430 or LPS 435, compared to WT LPS (Fig. 4C). Immunofluorescence analysis indicated that monocytes activated with WT LPS, LPS 430 or LPS 435 significantly increased their nuclear translocation of NF-κB p65 and IRF3, compared to non-stimulated monocytes, but LPS 430 and LPS 435 induced nuclear translocation of these transcription factors to the same extents as WT LPS (Fig. 4D and E).

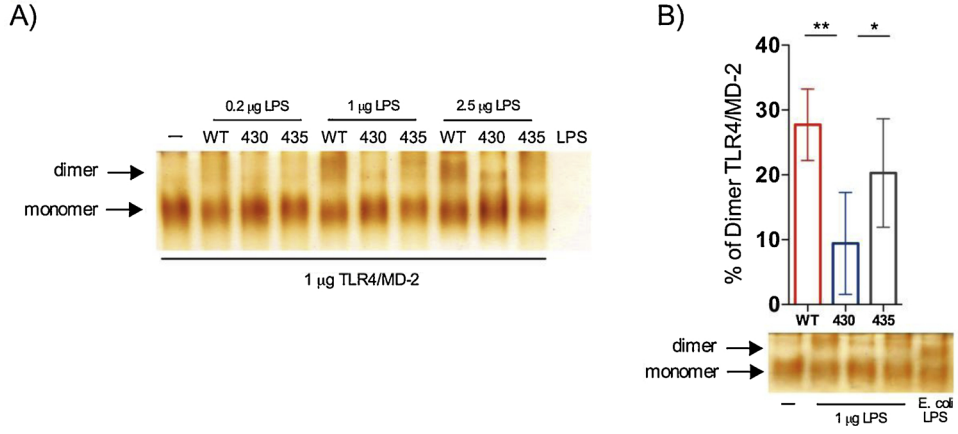

Fig. 3. LPS 430 induces decreased dimerization of the TLR4/MD-2 complex. Recombinant human TLR4/MD-2 complex was incubated with the indicated amounts of WT LPS, LPS 430, LPS 435 or LPS from *Escherichia coli* and subjected to electrophoresis in native 10% polyacrylamide gels. (A) Proteins were visualized with silver staining; "monomer" indicates 1:1:1 TLR4/MD-2/LPS, and "dimer" indicates 2:2:2 TLR4/MD-2/LPS. (-) indicates TLR4/MD-2 without any LPS. (B) Densitometry analysis. The data are represented as the percentage of the dimer band density compared to the sum of the monomer and dimer band densities, for each condition (3 independent experiments). The graph represents mean ± SD. One-way ANOVA with Tukey's multiple comparison test: $^{*}p < 0.05$, $^{**}p < 0.001$. WT, wild type.

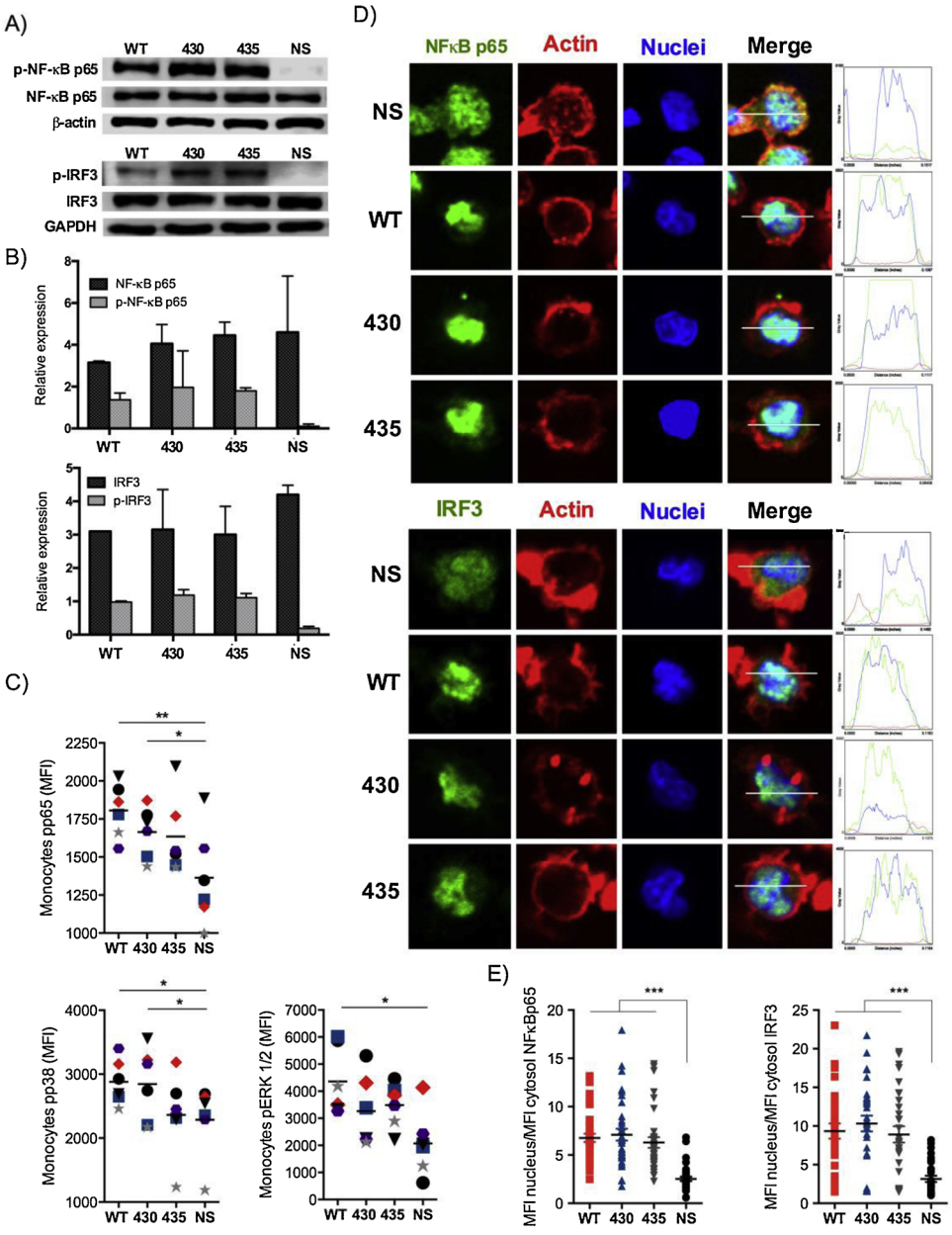

Fig. 4. LPS 430 and LPS 435 induce similar NF-κB, IRF3, p38 and ERK1/2 activation than WT LPS. (A) Peripheral blood monocytes were stimulated with 1 μg/ml of WT LPS, LPS 430 or LPS 435 for 30 min (for NF-κB detection) or 60 min (for IRF3 detection), and phosphorylation of NF-κB p65 and IRF3 were evaluated in the cell lysates, with β-actin or GAPDH as loading control (the images are representative of three independent experiments). (B) Densitometry analysis of the bands in (A), the graph represents mean ± SD. (C) Peripheral blood monocytes from six healthy volunteers were stimulated with 0.1 μg/ml of WT LPS, LPS 430 or LPS 435 for 15 min, and phosphorylated p65, p38 and ERK1/2 were analysed by flow cytometry. Each symbol represents the results from one volunteer, the horizontal line is the mean. (D) Monocytes stimulated as indicated in (A) were stained for NF-κB p65 or IRF3 in the presence of phalloidin (for actin staining) and Hoechst (for nuclear staining), and analysed by confocal microscopy (20x magnification). Histograms represent the intensity of each fluorophore across the cellular section indicated in the "Merge" panels. (E) Quantification of NF-κB p65 and IRF3 in the images in (D). Data are represented as the mean fluorescence intensity (MFI) of the transcription factor in the nucleus divided by the MFI of the transcription factor in the cytosol per cell, for each transcription factor. The graphs represent data from individual cells, from three independent experiments. Total n = 30 for NF-κB p65 and n = 30 for IRF3. The horizontal line is the mean. One-way ANOVA with Tukey's multiple comparison test: $*p < 0.05$, $**p < 0.001$, $***p < 0.001$. WT, wild type. NS, not stimulated.

3.4. The monocyte transcription profiles induced by LPS 430, LPS 435 and WT LPS are similar

Since LPS 430 induced similar nuclear translocation of NF-κB p65 and IRF3 than WT LPS, we investigated if the lower production of pro-inflammatory cytokines was caused by a different transcription profile in LPS 430-activated monocytes, compared to WT LPS -activated monocytes. We used microarrays to evaluate the transcription profiles of monocytes activated with LPS 430 (Supplementary Tables 1 and 2) or WT LPS (Supplementary Tables 3 and 4), compared to non-stimulated monocytes; we first focussed on the genes that are regulated specifically by the MyD88 and the TRIF pathways. In accordance with the nuclear translocation of NF-κB p65 and IRF3, both LPS induced the expression of MyD88- and TRIF-regulated genes after 2 and 5 h of activation (Fig. 5A). Gene Set Enrichment Analysis (GSEA) of the microarray data indicated that the biological processes and signalling pathways that were up- or down-regulated by LPS 430, compared to non-stimulated monocytes, were similar to those up- or down-regulated by WT LPS (Fig. 5B). Most of the biological processes and signalling pathways up-regulated by both LPS were related to the immune response, and both LPS down-regulated genes associated with the metabolism of lipids and lipoproteins (Fig. 5B). We then compared the transcription profile of LPS 430-activated monocytes with the transcription profile of WT LPS-activated monocytes (Supplementary Tables 5 and 6). We found that LPS 430 induced the differential expression of more than 100 transcripts with a fold change of at least ± 1.2 and $p < 0.05$, compared to WT LPS, after 2 and 5 h of activation (Fig. 5C); these included genes for microRNAs 323a (fold change 2.45), 4301 (fold change −2.35), 553 (fold change −2.34), 548a-1 (fold change 1.9), 548b (fold change 1.85) and 1263 (fold change 1.61), as well as several genes and pseudo-genes that were not enriched to a specific biological process or signalling pathway, since no significant differences were found by GSEA. Analysis by quantitative PCR indicated that the relative expression of TNF-α mRNA was significantly lower in monocytes activated for 2 h with LPS 430 than in monocytes activated with WT LPS, but no significant differences were found in the expression levels of IL-6 and IL-1β mRNAs (Supplementary Fig. 3).

Additionally, we evaluated the transcription profiles of monocytes

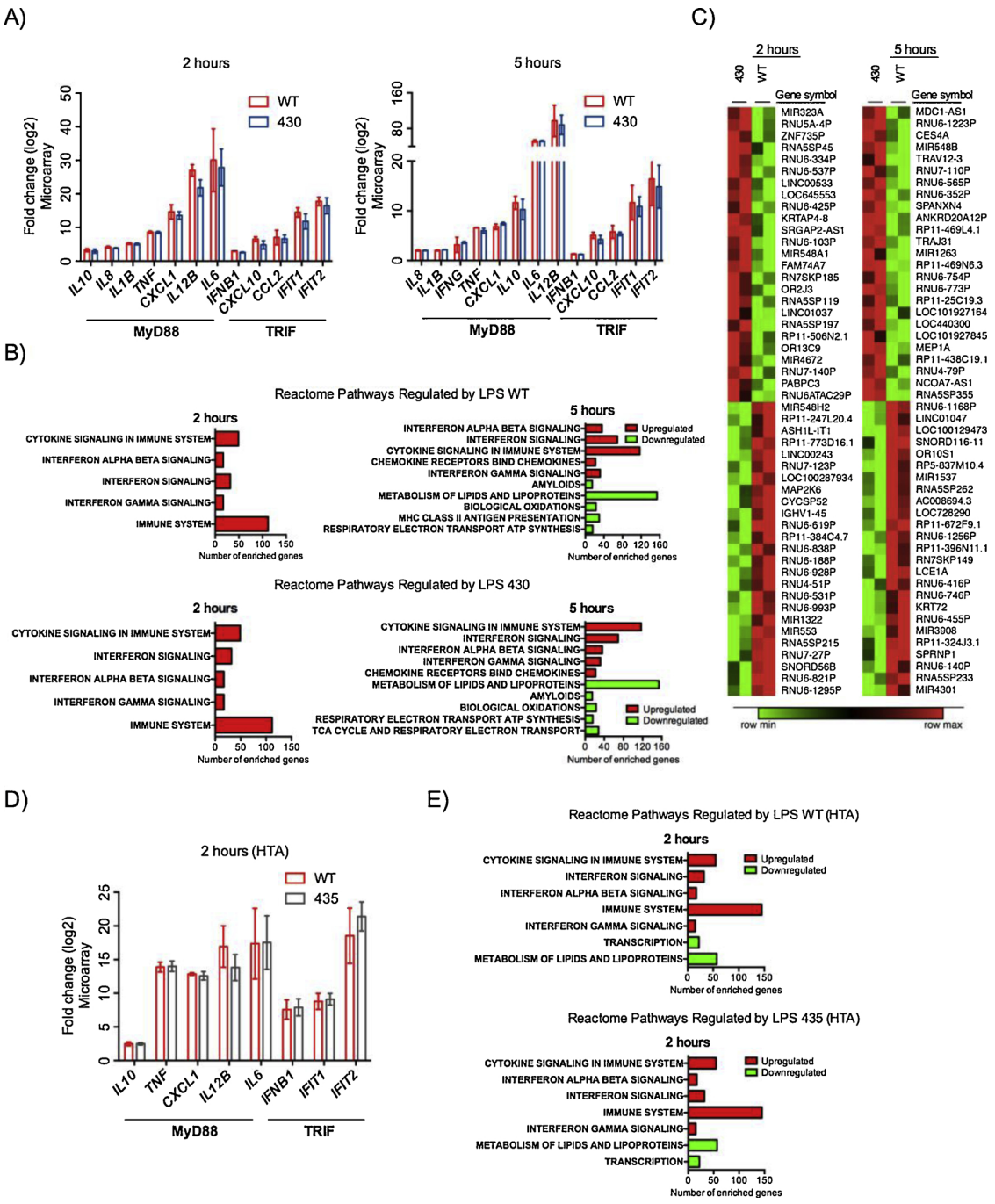

Fig. 5. LPS 430 and WT LPS induce similar transcription profiles in human monocytes. Peripheral blood monocytes were stimulated with 1 μg/ml of WT LPS, LPS 430 or LPS 435 for 2 or 5 h, and RNA was extracted. Microarray analysis was performed to identify the genes whose transcription was induced by WT LPS, LPS 430 or LPS 435, compared to non-stimulated (NS) monocytes. (A) and (D) Fold changes in the expression levels of genes regulated by the MyD88 and the TRIF pathways are represented (n = 2). The graph represents mean ± SD. (B) and (E) Robust Multi-chip Analysis (RMA)-corrected data from the microarrays in (A) were subjected to Gene Set Enrichment Analysis (GSEA) to define the biological processes and signalling pathways (as defined in the Reactome Pathway Database) that were up- or down-regulated in WT LPS-, LPS 430- or LPS 435-activated monocytes, compared to non-stimulated (NS) monocytes. The biological processes and signalling pathways are arranged according to significance; those with the highest significance are at the top of each graph. (C) Heat map showing the top 50 features of LPS 430-activated monocytes, compared to WT LPS-activated monocytes, at the indicated times.

activated with LPS 435 (Supplementary Table 7) or WT LPS (Supplementary Table 8), compared to non-stimulated monocytes. We found that both LPS 435 and WT LPS induced the expression of MyD88- and TRIF-regulated genes after 2 h of activation (Fig. 5D), and the two LPS activated similar biological processes and signalling pathways (Fig. 5E), but LPS 435 did not induce the differential expression of any transcript with a fold change of at least ± 1.5 and $p < 0.05$, compared to WT LPS (Supplementary Table 9).

4. Discussion

Pathogenic bacteria have virulence factors that allow them to colonize the host and survive; some of these virulence factors modify the LPS structure, which confers resistance to antimicrobial peptides and leads to decreased activation of TLR4. However, little is know about the mechanism of recognition and signalling of these modified LPS in human cells. In this study, we investigated the recognition and

signalling through human TLR4/MD-2 that are induced by *S.* Typhimurium LPS 430 (with a hepta-acylated lipid A) and *S.* Typhimurium LPS 435 (with a lipid A with modified phosphate groups), compared to WT LPS. We report that LPS 430 and LPS 435 induced the production of lower levels of the pro-inflammatory cytokines TNF-α and IL-6 by human peripheral blood monocytes, compared to WT LPS, in addition, we found decreased dimerization of the TLR4/MD-2 complex in response to LPS 430. Unexpectedly the activation of the transcription factors NF-κB p65, IRF3, p38 and ERK1/2 were similar between LPS 430 and WT LPS. The transcriptional analysis of the genes activated by LPS 430 and LPS 435 showed differences mainly in the expression of microRNAs and several genes and pseudogenes compared to WT LPS.

A previous study reported that LPS 430 induced a lower expression of E-selectin on human umbilical vein endothelial cells, and a lower production of TNF-α by human peripheral blood monocytes, compared to WT LPS (Guo et al., 1997). Here we report that, in addition, LPS 430 induced a lower production of IL-6 and IL-1β by human peripheral blood monocytes, compared to WT LPS. The additional acyl group of LPS 430 has a moderate effect on cytokine production, since the levels of TNF-α, IL-6 and IL-1β induced by LPS 430 were reduced by no more than 35%, compared to the levels induced by WT LPS. This moderate reduction in cytokine production is in contrast with the sharp reduction in cytokine production that is observed in the case of other hypo-acylated variants of LPS (such as the LPS of *Helicobacter pylori* and *Yersina pestis*), where the levels of pro-inflammatory cytokines are reduced by as much as 100–10,000 fold, compared to the levels induced by hexa-acylated LPS (Mandell et al., 2004; Matsuura et al., 2010; Stead et al., 2008). Our findings are in accordance with those reported for *S.* Minnesota hepta-acylated LPS, which induce the production of moderately lower levels of pro-inflammatory cytokines by human peripheral blood mononuclear cells, compared to *Escherichia coli* hexa-acylated LPS (Janusch et al., 2002).

With respect to the effects of the phosphoethanolamine and aminoarabinose groups, which are attached to the phosphate groups of the lipid A portion of LPS 435, we report that LPS 435 induced a decreased production of TNF-α and IL-6, compared to WT LPS, but similar levels of IL-1β than WT LPS. The reduction in the levels of these pro-inflammatory cytokines was not as large as the reduction caused by LPS 430; in fact, LPS 430 induced significantly lower levels of TNF-α and IL-1β than LPS 435. This is in accordance with a report of *Pseudomonas aeruginosa* hexa-acylated and aminoarabinose-containing LPS, which indicates that its capacity to activate THP-1 cells, or HEK cells transfected with human TLR4/MD-2, is similar to that of hexa-acylated LPS (Hajjar et al., 2002). Our results indicate that LPS 430 induced the production of lower levels of pro-inflammatory cytokines by human monocytes, compared to LPS 435. However, in mice, LPS 435 induces the production of lower levels of pro-inflammatory cytokines than LPS 430 (Pastelin-Palacios et al., 2011); this could reflect the species-specific recognition of the lipid A portion of LPS, as occurs with the hypo-acylated (tetra- and tri-acylated) *Yersinia pestis* LPS, which are poorly recognized by human TLR4 but efficiently activate mouse TLR4 (Matsuura et al., 2010).

The decreased capacity of LPS 430 to induce pro-inflammatory cytokines correlated with its decreased capacity to induce the dimerization of the TLR4/MD-2 complex, compared to WT LPS. There were no statistically significant differences in the amount of TLR4/MD-2 dimer induced by LPS 435 and WT LPS, although the amounts of dimer tend to be lower with LPS 435; this could explain the significantly higher levels of TNF-α and IL-1β produced by LPS 435-activated monocytes, compared to LPS 430-activated monocytes. X-ray crystallography of the TLR4/MD-2/LPS complex indicates that after LPS interacts with the TLR4/MD-2 complex, the 1-phosphate group and the R2 acyl chain of the lipid A portion of LPS remain exposed and are able to interact with another TLR4/MD-2/LPS complex; this interaction allows the formation of the TLR4/MD-2/LPS dimer (Park et al., 2009). In LPS 430, the additional acyl chain is bound to the R2 acyl chain (Fig. 1), and this additional acyl chain could interfere with the interaction of LPS with the second TLR4/MD-2 complex, thus reducing the efficiency of dimer formation. However, although the capacity of LPS 430 to dimerize the TLR4/MD-2 complex in vitro is reduced, the capacity of LPS 430 to induce the production of pro-inflammatory cytokines by human monocytes is not drastically reduced. This can be explained by the presence of LBP and CD14, which increase the efficiency of LPS binding to the TLR4/MD-2 complex (Heumann et al., 2003).

MPL induces an impaired dimerization of the TLR4/MD2 complex that causes reduced signalling through the MyD88 pathway, but normal signalling through the TRIF pathway (Mata-Haro et al., 2007). We found that LPS 430 also induced an impaired dimerization of the TLR4/MD-2/LPS complex, compared to WT LPS, but this reduced dimerization was not associated with a preferential activation of the MyD88 or the TRIF pathways. In fact, LPS 430 and LPS 435 induced the nuclear translocation of NF-κB p65 and IRF3 to a similar extent than WT LPS, and equivalent phosphorylation of the NF-κB subunit p65, of IRF3 and of the MAP kinases p38 and ERK1/2. The signalling pathway genes that were up-regulated or down-regulated by LPS 430 and LPS 435 in human monocytes were similar to the induced by WT LPS. LPS 430 and WT LPS increased the expression of genes associated with the immune response (including cytokine and chemokine genes) and reduced the expression of genes associated with lipid metabolism, with the tricarboxylic acid cycle and with respiratory electron transport and ATP synthesis. Likewise, LPS 435 induced the expression of genes associated with the immune response and reduced the expression of genes associated with lipid metabolism. These findings are in accordance with a previous report, which showed that stimulation of human monocytes with hexa-acylated LPS induces a metabolic reprogramming involving the down-regulation of genes related to lipid metabolism, to the tricarboxylic acid and to oxidative phosphorylation, while genes related to glycolysis are up-regulated. As a result, glycolysis becomes the main source of ATP in these cells (Lachmandas et al., 2016).

We report differential production of TNF-α, IL-6 and IL-1β by monocytes in response to WT LPS, LPS 430 and LPS 435, but differences in the amount of cytokines were not reflected at the mRNA level: no differences in the expression levels of the TNF-α, IL-6 and IL-1β mRNAs were detected by microarray analysis, and RT-PCR analysis only detected a decrease in the TNF-α mRNA produced in response to LPS 430, compared to the TNF-α mRNA produced in response to WT LPS. Differences in protein concentration, but not in mRNA levels, can be explained by post-transcriptional regulation, which refers to several pathways that control mRNA splicing, nuclear export and stability, as well as mRNA translation efficiency (Stamou and Kontoyiannis, 2010), and this is an important way in which gene expression is induced in activated macrophages (Schott et al., 2014).

Several studies indicate that the production of pro-inflammatory cytokines is regulated post-transcriptionally (Carballo et al., 1998; Espel et al., 1996; MacKenzie et al., 2002; Schott et al., 2014), and microRNAs contribute to this process: the production of TNF-α, IL-6, and IL-12 p40 is negatively regulated by microRNA 187 in TLR4-stimulated monocytes (Rossato et al., 2012), and microRNA 155 enhances the stability and translation of the TNF-α mRNA in LPS-activated macrophages, while microRNA 125 decreases its translation (Tili et al., 2007). We found that LPS 430 induced the differential expression of microRNAs 323a, 4301, 553, 548a-1, 548b and 1263 after 2 and 5 h of monocyte activation, compared to WT LPS; microRNA 323a is a negative regulator of IL-22 production in T cells (Karner et al., 2017), but there are no reports of the effects of microRNAs 4301, 553, 548a-1, 548b and 1263 on cytokine production by human immune cells. Our results suggest that these microRNAs could be involved in the transcriptional regulation of TNF-α and IL-6 in human monocytes. When we compared the transcription profiles induced by LPS 430 and WT LPS we found that, although some genes (including the microRNA genes) were differentially regulated after activation with these LPS, these genes

overall were not enriched to a determined signalling pathway, as determined by GSEA. Comparison between the transcription profiles induced by LPS 435 and WT LPS showed that some genes were differentially regulated, but not beyond 1.5 fold change. These results indicate that LPS 430, LPS 435 and LPS WT induced similar gene expression profiles in human monocytes, which differ mainly in the expression levels of microRNA genes.

In addition to post-transcriptional regulation, altered cytokine secretion could also explain the lower levels of TNF-α, IL-6 and IL-1β produced by monocytes in response to LPS 430 and LPS 435, compared to WT LPS. Since TNF-α and IL-6 contain an endoplasmic reticulum (ER)-targeting signal peptide, these cytokines are inserted into vesicles in the ER and delivered to the Golgi complex; they reach the cell surface via recycling endosomes, in a transit that is tightly regulated by several proteins and lipids (Murray and Stow, 2014). In contrast, the pro-IL-1β protein lacks a signal peptide and requires a proteolytic cleavage to gain its full biological activity; this cleavage is made by caspase-1 or caspase-11, which are activated via inflammasomes. Active IL-1β is released from the cell by many processes, including micro-vesicle shedding, necrosis and pyroptosis (Mayer-Barber and Yan, 2017). It remains to be determined if LPS 430 and LPS 435 can modulate these secretory pathways.

Our results showed that two structural modifications of lipid A (an extra acyl chain in LPS 430, or phosphoethanolamine and aminoarabinose attached to the phosphate groups in LPS 435) induced a reduced pro-inflammatory cytokine production in human monocytes, these might be due to a reduced capacity of these LPS to dimerize TLR4/MD-2 complex and post-transcriptional regulation of cytokine expression by microRNA. Identifying how modifications on the structure of lipid A affect its capacity to activate TLR4 is important to understand one of the mechanism of immune evasion that is used by pathogenic bacteria, and it could also be relevant to develop less toxic lipid A variants that retain their adjuvant capacities, as is the case of MPL. Since the modifications of LPS 430 and LPS 435 have a moderate effect on cytokine production, it would be possible to perform additional modifications on their structure, in order to reduce their toxicity and use them as vaccine adjuvants or immunomodulators for human use.

In conclusion, our results showed that *S*. Typhimurium structurally modified LPS are sensed differently than WT LPS by human monocytes, generating a reduced pro-inflammatory cytokine response.

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Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.03.003.

References

Blum, H., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8.
Carballo, E., Lai, W.S., Blackshear, P.J., 1998. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science 281, 1001–1005.
Espel, E., Garcia-Sanz, J.A., Aubert, V., Menoud, V., Sperisen, P., Fernandez, N., Spertini, F., 1996. Transcriptional and translational control of TNF-alpha gene expression in human monocytes by major histocompatibility complex class II ligands. Eur. J. Immunol. 26, 2417–2424.
Falvo, J.V., Tsytsykova, A.V., Goldfeld, A.E., 2010. Transcriptional control of the TNF gene. Curr. Dir. Autoimmun. 11, 27–60.
Gunn, J.S., Miller, S.I., 1996. PhoP-PhoQ activates transcription of pmrAB, encoding a two-component regulatory system involved in Salmonella typhimurium antimicrobial peptide resistance. J. Bacteriol. 178, 6857–6864.
Gunn, J.S., Lim, K.B., Krueger, J., Kim, K., Guo, L., Hackett, M., Miller, S.I., 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol. Microbiol. 27, 1171–1182.
Guo, L., Lim, K.B., Gunn, J.S., Bainbridge, B., Darveau, R.P., Hackett, M., Miller, S.I., 1997. Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. Science 276, 250–253.
Hajjar, A.M., Ernst, R.K., Tsai, J.H., Wilson, C.B., Miller, S.I., 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat. Immunol. 3, 354–359.
Janusch, H., Brecker, L., Lindner, B., Alexander, C., Gronow, S., Heine, H., Ulmer, A.J., Rietschel, E.T., Zahringer, U., 2002. Structural and biological characterization of highly purified hepta-acyl lipid A present in the lipopolysaccharide of the Salmonella enterica sv. Minnesota Re deep rough mutant strain R595. J. Endotoxin Res. 8, 343–356.
Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S., Medzhitov, R., 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat. Immunol. 9, 361–368.
Karner, J., Wawrzyniak, M., Tankov, S., Runnel, T., Aints, A., Kisand, K., Altraja, A., Kingo, K., Akdis, C.A., Akdis, M., Rebane, A., 2017. Increased microRNA-323-3p in IL-22/IL-17-producing T cells and asthma: a role in the regulation of the TGF-b pathway and IL-22 production. Allergy 72, 55–65.
Lachmandas, E., Boutens, L., Ratter, J.M., Hijmans, A., Hooiveld, G.J., Joosten, L.A., Rodenburg, R.J., Fransen, J.A., Houtkooper, R.H., van Crevel, R., Netea, M.G., Stienstra, R., 2016. Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. Nat. Microbiol. 2, 16246.
MacKenzie, S., Fernandez-Troy, N., Espel, E., 2002. Post-transcriptional regulation of TNF-alpha during in vitro differentiation of human monocytes/macrophages in primary culture. J. Leukoc. Biol. 71, 1026–1032.
Maeshima, N., Fernandez, R.C., 2013. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front. Cell. Infect. Microbiol. 3, 3.
Mandell, L., Moran, A.P., Cocchiarella, A., Houghton, J., Taylor, N., Fox, J.G., Wang, T.C., Kurt-Jones, E.A., 2004. Intact gram-negative Helicobacter pylori, Helicobacter felis, and Helicobacter hepaticus bacteria activate innate immunity via toll-like receptor 2 but not toll-like receptor 4. Infect. Immun. 72, 6446–6454.
Mata-Haro, V., Cekic, C., Martin, M., Chilton, P.M., Casella, C.R., Mitchell, T.C., 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 316, 1628–1632.
Matsuura, M., Takahashi, H., Watanabe, H., Saito, S., Kawahara, K., 2010. Immunomodulatory effects of Yersinia pestis lipopolysaccharides on human macrophages. Clin. Vaccine Immunol. 17, 49–55.
Mayer-Barber, K.D., Yan, B., 2017. Clash of the Cytokine Titans: counter-regulation of interleukin-1 and type I interferon-mediated inflammatory responses. Cell. Mol. Immunol. 14, 22–35.
Medzhitov, R., Horng, T., 2009. Transcriptional control of the inflammatory response. Nat. Rev. Immunol. 9, 692–703.
Morrison, D.C., Ryan, J.L., 1987. Endotoxins and disease mechanisms. Annu. Rev. Med. 38, 417–432.
Murray, R.Z., Stow, J.L., 2014. Cytokine Secretion in Macrophages: SNAREs, Rabs, and Membrane Trafficking. Front. Immunol. 5, 538.
Needham, B.D., Trent, M.S., 2013. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat. Rev. Microbiol. 11, 467–481.
Pandey, S., Kawai, T., Akira, S., 2014. Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors. Cold Spring Harb. Perspect. Biol. 7, a016246.

Park, B.S., Song, D.H., Kim, H.M., Choi, B.S., Lee, H., Lee, J.O., 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458, 1191–1195.

Pastelin-Palacios, R., Gil-Cruz, C., Perez-Shibayama, C.I., Moreno-Eutimio, M.A., Cervantes-Barragan, L., Arriaga-Pizano, L., Ludewig, B., Cunningham, A.F., Garcia-Zepeda, E.A., Becker, I., Alpuche-Aranda, C., Bonifaz, L., Gunn, J.S., Isibasi, A., Lopez-Macias, C., 2011. Subversion of innate and adaptive immune activation induced by structurally modified lipopolysaccharide from Salmonella typhimurium. Immunology 133, 469–481.

Rossato, M., Curtale, G., Tamassia, N., Castellucci, M., Mori, L., Gasperini, S., Mariotti, B., De Luca, M., Mirolo, M., Cassatella, M.A., Locati, M., Bazzoni, F., 2012. IL-10-induced microRNA-187 negatively regulates TNF-alpha, IL-6, and IL-12p40 production in TLR4-stimulated monocytes. Proc. Natl. Acad. Sci. U.S.A. 109, E3101–3110.

Schott, J., Reitter, S., Philipp, J., Haneke, K., Schafer, H., Stoecklin, G., 2014. Translational regulation of specific mRNAs controls feedback inhibition and survival during macrophage activation. PLoS Genet. 10, e1004368.

Stamou, P., Kontoyiannis, D.L., 2010. Posttranscriptional regulation of TNF mRNA: a paradigm of signal-dependent mRNA utilization and its relevance to pathology. Curr. Dir. Autoimmun. 11, 61–79.

Stead, C.M., Beasley, A., Cotter, R.J., Trent, M.S., 2008. Deciphering the unusual acylation pattern of Helicobacter pylori lipid A. J. Bacteriol. 190, 7012–7021.

Steimle, A., Autenrieth, I.B., Frick, J.S., 2016. Structure and function: lipid A modifications in commensals and pathogens. Int. J. Med. Microbiol. 306, 290–301.

Tanimura, N., Saitoh, S., Ohto, U., Akashi-Takamura, S., Fujimoto, Y., Fukase, K., Shimizu, T., Miyake, K., 2014. The attenuated inflammation of MPL is due to the lack of CD14-dependent tight dimerization of the TLR4/MD2 complex at the plasma membrane. Int. Immunol. 26, 307–314.

Tili, E., Michaille, J.J., Cimino, A., Costinean, S., Dumitru, C.D., Adair, B., Fabbri, M., Alder, H., Liu, C.G., Calin, G.A., Croce, C.M., 2007. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J. Immunol. 179, 5082–5089.

Tirsoaga, A., Novikov, A., Adib-Conquy, M., Werts, C., Fitting, C., Cavaillon, J.M., Caroff, M., 2007. Simple method for repurification of endotoxins for biological use. Appl. Environ. Microbiol. 73, 1803–1808.

Tobias, P.S., Soldau, K., Ulevitch, R.J., 1986. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. J. Exp. Med. 164, 777–793.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C., 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431–1433.

Yamakawa, N., Ohto, U., Akashi-Takamura, S., Takahashi, K., Saitoh, S., Tanimura, N., Suganami, T., Ogawa, Y., Shibata, T., Shimizu, T., Miyake, K., 2013. Human TLR4 polymorphism D299G/T399I alters TLR4/MD-2 conformation and response to a weak ligand monophosphoryl lipid A. Int. Immunol. 25, 45–52.

Yang, L., Seki, E., 2012. Toll-like receptors in liver fibrosis: cellular crosstalk and mechanisms. Front. Physiol. 3, 138.

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., Kagan, J.C., 2011. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell 147, 868–880.