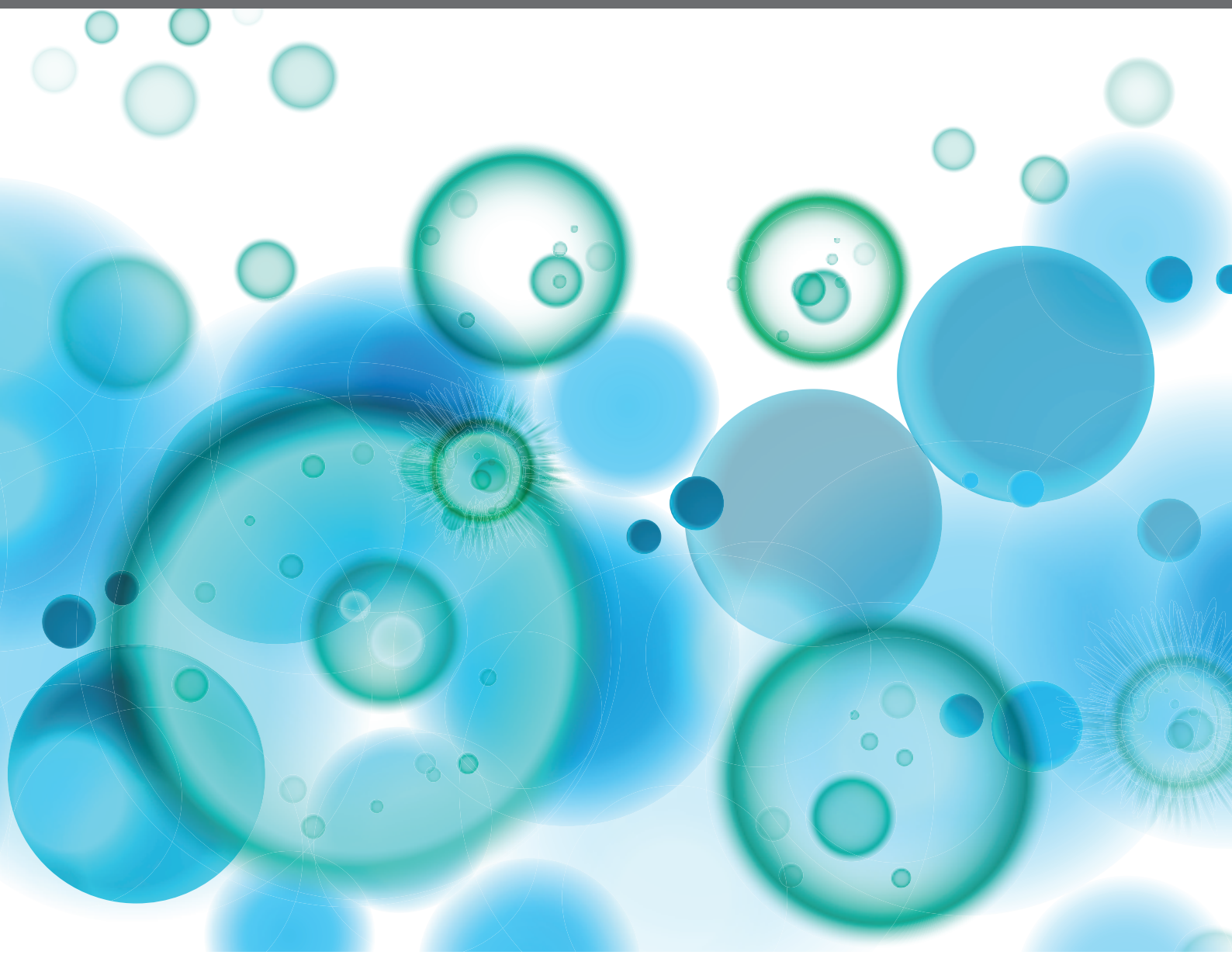
TLR4-DEPENDENT HOMEOSTASIS AND IMMUNOPATHOLOGY: FOCUS ON CROSS-TALK WITH OTHER SIGNALING PATHWAYS

EDITED BY: Francesco Peri and Jerrold Weiss
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-83250-512-0
DOI 10.3389/978-2-83250-512-0

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TLR4-DEPENDENT HOMEOSTASIS AND IMMUNOPATHOLOGY: FOCUS ON CROSS-TALK WITH OTHER SIGNALING PATHWAYS

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Citation: Peri, F., Weiss, J., eds. (2022). TLR4-Dependent Homeostasis and Immunopathology: Focus on Cross-talk With Other Signaling Pathways. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-512-0

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frontiers in Immunology

ORIGINAL RESEARCH
published: 16 April 2020
doi: 10.3389/fimmu.2020.00640

IRF-7 Mediates Type I IFN Responses in Endotoxin-Challenged Mice

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IRF-7 mediates robust production of type I IFN via MyD88 of the TLR9 pathway in plasmacytoid dendritic cells (pDCs). Previous *in vitro* studies using bone marrow-derived dendritic cells lacking either *Irf7* or *Irf3* have demonstrated that only IRF-3 is required for IFN-β production in the TLR4 pathway. Here, we show that IRF-7 is essential for both type I IFN induction and IL-1β responses via TLR4 in mice. Mice lacking *Irf7* were defective in production of both IFN-β and IL-1β, an IFN-β-induced pro-inflammatory cytokine, after LPS challenge. IFN-β production in response to LPS was impaired in IRF-7-deficient macrophages, but not dendritic cells. Unlike pDCs, IRF-7 is activated by the TRIF-, but not MyD88-, dependent pathway via TBK-1 in macrophages after LPS stimulation. Like pDCs, resting macrophages constitutively expressed IRF-7 protein. This basal IRF-7 protein was completely abolished in either *Ifnar1*$^{-/-}$ or *Stat1*$^{-/-}$ macrophages, which corresponded with the loss of LPS-stimulated IFN-β induction in these macrophages. These findings demonstrate that macrophage IRF-7 is critical for LPS-induced type I IFN responses, which in turn facilitate IL-1β production in mice.

Keywords: IRF-7, TLR4, IFN-β, IL-1β, macrophage, dendritic cell

OPEN ACCESS

Edited by:
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University of Milano-Bicocca, Italy

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *11 September 2019*
Accepted: *20 March 2020*
Published: *16 April 2020*

Citation:
Sin W-X, Yeong JP-S, Lim TJF, Su I-H, Connolly JE and Chin K-C (2020) IRF-7 Mediates Type I IFN Responses in Endotoxin-Challenged Mice. Front. Immunol. 11:640. doi: 10.3389/fimmu.2020.00640

INTRODUCTION

Sepsis is one of the leading causes of morbidity and mortality in hospital intensive care units worldwide (1). It is a systemic inflammatory response to severe microbial infections that is characterized by the excessive production of pro-inflammatory cytokines. Interleukin-1β (IL-1β) is one of the more studied pro-inflammatory cytokines, and is produced in response to the endotoxins from the outer membrane of the cell wall of Gram-negative bacteria. Excessive or inappropriate expression of IL-1β also occurs with tissue damage and various diseases, including autoimmune diseases, metabolic syndromes, and cryopyrin-associated periodic syndromes (2). The generation of active IL-1β from precursor IL-1β requires the assembly of multiple cytosolic proteins into a complex known as the inflammasome, which acts as a signaling platform to promote the activation of caspase-1 that cleaves pro-IL-1β into active mature IL-1β (3–5). The most extensively studied inflammasome complex to date is the NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome, which can be activated both in a canonical and in a non-canonical manner (6).

In the non-canonical NLRP3 inflammasome pathway, IL-1β induction in mice and humans after Gram-negative bacterial infections required interferon (IFN)-inducible caspase-11 in mice, or caspase-4/5 in humans (7–9). This response is mediated by Toll-like receptor 4 (TLR4), a receptor

that recognizes the lipopolysaccharide (LPS) component of Gram-negative bacteria. TLR4 is the only member in the TLR family that transduces signals via two distinct intracellular pathways, namely the myeloid differentiation primary response protein 88 (MyD88)- and Toll/interleukin-1 receptor (TIR) domain-containing adapter protein inducing interferon-β (TRIF)-dependent pathways. As in the canonical NLRP3 inflammasome pathway, the initial binding of LPS to TLR4 at the plasma membrane recruits the adaptor proteins MyD88 and MyD88 adapter-like (Mal), also termed TIRAP, which induce the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs), and thus promotes the expression of pro-inflammatory cytokine genes, including pro-IL-1β (10). Subsequently, endocytosis of TLR4 into endosomal compartments initiates a second signaling cascade mediated by the adaptor proteins TRIF and TRAM. This endosomal TRIF-TRAM axis activates TANK-binding kinase 1 (TBK-1) and I-κB kinase ε (IKK-ε), consequently inducing the phosphorylation and nuclear translocation of transcription factor interferon regulatory factor 3 (IRF-3) to promote the expression of type I IFN genes (11–18). In mice, this TRIF-dependent type I IFN production and signaling is required for non-canonical NLRP3 inflammasome activation via transcriptional induction of *Casp11*. Cytoplasmic LPS from Gram-negative bacteria binds to and activates caspase-11, thereby resulting in IL-1β processing and release in a NLRP3-dependent and caspase-1-dependent manner (9, 19–21). In agreement with this model, mice lacking TRIF or IFN-α/β receptor (IFNAR) exhibited defective IL-1β production in response to Gram-negative bacterial infection. In addition, neutralization of IFN-β decreased serum IL-1β levels after LPS challenge. These results support the notion that TRIF is required for LPS-induced IL-1β expression via type I IFN and IFN-induced caspase-11 *in vivo* (9, 22).

IRF-3 and IRF-7 are key transcriptional factors for type I IFN expression. Whilst IRF-3 is constitutively expressed in all cell types, IRF-7 is constitutively expressed only in plasmacytoid dendritic cells (pDCs), while in most of the other cell types it is expressed only after viral infection (23, 24). It was previously demonstrated that TRIF is able to interact with and activate both IRF-7 and IRF-3 (25, 26), which suggests that type I IFN induction in the TLR4-TRIF pathway may be mediated by both IRF-7 and IRF-3. However, it was reported that bone marrow-derived dendritic cells (BMDCs) from *Irf7*-deficient mice exhibited normal IFN-β induction by TLR4 stimulation, whereas IFN-β production was severely impaired in *Irf3*-deficient BMDCs (24). As macrophages and dendritic cells (DCs) originate from the same myeloid progenitors, and both cell types sense LPS via TLR4 to activate cytokine production via common MyD88 and TRIF pathways, the general consensus is that TLR4-induced IFN-β expression in macrophages is mediated by IRF-3 alone, as is the case in DCs (27). However, several reports have demonstrated that macrophages and DCs can display distinct effector functions in innate immune responses. While both MyD88- and TRIF-dependent pathways are required for sustained activation of NF-κB and pro-inflammatory cytokine production following LPS recognition by TLR4 in bone marrow-derived macrophages (BMDMs) (28), BMDC production of pro-inflammatory cytokines is dependent on MyD88, but independent of TRIF (29, 30). Furthermore, it has been shown that CD11b acts as a cell-type specific regulator to positively promote TLR4 signaling in DCs, but not in macrophages (31).

In this report, we used an established mouse model of LPS-induced acute septic shock to evaluate the role of IRF-7 in the activation of IL-1β and expression of type I IFN responses *in vivo*. According to our studies, mice lacking either IRF-7 or IRF-3 failed to produce IL-1β, and this correlated strongly with a severe defect in IFN-α/β production. From these findings, we conclude that IRF-7 and IRF-3 co-operate in the promotion of IFN-β and IL-1β production *in vivo*. Our studies using *in vitro* cultured bone marrow-derived macrophages and DCs allowed us to identify IRF-7 as a cell type-specific regulator in macrophages, but not in DCs. IRF-7, together with IRF-3, promotes type I IFN production in LPS-stimulated macrophages. Similar to pDCs, IRF-7 is constitutively expressed in resting macrophages, but not in DCs. This expression is dependent on basal IFN-β signaling that is present in macrophages, but not in DCs. In conclusion, our current study shows that IRF-7 is functionally important for the activation of type I IFN production in the TLR4 signaling pathway in macrophages, contrary to the previous conclusion that IRF-7 is completely dispensable in DCs.

MATERIALS AND METHODS

Mice

All mice were derived from a C57BL/6 genetic background. MyD88-deficient ($MyD88^{-/-}$) mice were from OrientalBioService, Inc. (Kyoto, Japan). TRIF-deficient ($Ticam1^{Lps2}$/J) mice were from The Jackson Laboratory (Bar Harbor, Maine, USA). IFNAR1-deficient ($Ifnar1^{tm1Agt}$/Mmjax) mice were from Mutant Mouse Regional Resource Centers (MMRRC), National Institutes of Health (NIH) (Bethesda, Maryland, USA). STAT1-deficient ($Stat1^{tm1Rds}$) mice were from Taconic Biosciences, Inc. (Hudson, NY, USA). IRF-3-deficient ($IRF\text{-}3^{-/-}$) and IRF-7-deficient ($IRF\text{-}7^{-/-}$) mice were from RIKEN BioResource Center (Ibaraki, Japan). IRF-3-IRF-7 double knockout mice were generated in-house by intercrossing $IRF\text{-}3^{-/-}$ and $IRF\text{-}7^{-/-}$ mice. Homozygous $IRF\text{-}3^{-/-}$-$IRF\text{-}7^{-/-}$ mice were generated by intercrossing heterozygous $IRF\text{-}3^{+/-}$-$IRF\text{-}7^{+/-}$ F1 mice, and were verified by genotyping tail biopsies. Bone marrow cells were obtained from STAT3 conditional knockout (MxCre-STAT3f/f) mice and control mice lacking the Mx-Cre transgene (STAT3f/f) (kind gift of Chien-Kuo Lee, National Taiwan University College of Medicine, Taiwan, Republic of China). All mice were bred and maintained at the A*STAR Biological Resource Center under specific pathogen-free conditions. All animal experimental procedures were conducted within the parameters of our Institutional Animal Care and Use Committee (IACUC)-approved protocol, in compliance with the National Advisory Committee for Laboratory Animal Research (NACLAR) Guidelines.

Preparation of Murine Bone Marrow Cells

Mice were euthanized using carbon dioxide followed by cervical dislocation to ensure death. After euthanasia, femurs and tibias

were dissected from each mouse using scissors and forceps, and the bones were placed into a petri dish containing DMEM complete medium. Both epiphyses were removed from each bone using scissors and forceps, and bone marrow cells were flushed into a 50-ml polypropylene tube using a 25-G needle and a 10-ml syringe containing DMEM complete medium. After centrifugation at 500 g for 10 min, the cell pellet was resuspended in 3 ml Red Blood Cell (RBC) lysis buffer for 3 min at room temperature. RBC lysis was stopped by adding 10 ml DMEM complete medium. After centrifugation at 500 g for 10 min, the cell pellet was resuspended in freezing medium (FBS + 10% DMSO). Bone marrow cells were aliquoted into cryogenic vials, and then frozen in liquid nitrogen.

Differentiation of Murine Bone Marrow-Derived Macrophages

Frozen bone marrow cells were thawed in a 37°C water bath and transferred to a 15-ml polypropylene tube containing 10 ml DMEM complete medium. After centrifugation at 500 g for 10 min, the cell pellet was resuspended in BMDM differentiation medium (50% DMEM + 4,500 mg/L glucose + 110 mg/L sodium pyruvate supplemented with 20% HyClone defined FBS and 30% L929 cell-conditioned medium, and 100 U/ml penicillin + 100 μg/ml streptomycin). Bone marrow cells were counted using trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) and a hemocytometer. For analysis of RNA and culture supernatants, 0.5×10^6 BM cells were cultured in each well of a 6-well plate containing 1.5 ml BMDM differentiation medium. For protein experiments, 1.5×10^6 BM cells were cultured in 60-mm dishes that contained 2.5 ml BMDM differentiation medium. For analysis of nuclear extracts, $6.0–7.0 \times 10^6$ BM cells were cultured in 100-mm dishes that contained 10.0 ml BMDM differentiation medium. For ChIP experiments, 20.0×10^6 BM cells were cultured in 150-mm dishes that contained 20.0 ml BMDM differentiation medium. On Day 3, an equivalent volume of fresh BMDM differentiation medium was added to the culture. On Day 5 and Day 6, the BMDM differentiation medium was aspirated and fresh BMDM differentiation medium was added to the adherent cells. On Day 7, BMDMs were used for experiments, and samples were harvested for downstream analysis. Differentiation of bone marrow progenitors into BMDMs was confirmed by flow cytometric analysis of F4/80 and CD11b surface marker expression.

Differentiation of Murine Bone Marrow-Derived Dendritic Cells

Frozen bone marrow cells were thawed in a 37°C water bath and transferred to a 15-ml polypropylene tube containing 10 ml RPMI complete medium. After centrifugation at 500 g for 10 min, the cell pellet was resuspended in BMDC differentiation medium (90% RPMI 1640 + 10 mM HEPES supplemented with 10% HyClone defined FBS and 20 ng/ml GM-CSF, and 100 U/ml penicillin + 100 μg/ml streptomycin). Bone marrow cells were counted using trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) and a hemocytometer. 1.5×10^6 BM cells were cultured in each well of a 24-well plate containing 1.0 ml BMDC differentiation medium. On Day 2, an equivalent volume of fresh BMDC differentiation medium was added to the culture. On Day 4, 1.0 ml BMDC differentiation medium was aspirated and 1.0 ml fresh BMDC differentiation medium was added to the culture. On Day 5, the non-adherent cells were collected and re-plated in suspension culture plates for experiments. For analysis of RNA and culture supernatants, 0.5×10^6 BMDCs were cultured in each well of a 24-well suspension culture plate containing 1.5 ml BMDC differentiation medium. For protein experiments, 1.5×10^6 BMDCs were cultured in each well of a 6-well suspension culture plate containing 2.5 ml BMDC differentiation medium. On Day 6, 1.0 ml BMDC differentiation medium was aspirated and 1.0 ml fresh BMDC differentiation medium was added to the culture. On Day 7, BMDCs were used for experiments, and samples were harvested for downstream analysis. Differentiation of bone marrow progenitors into BMDCs was confirmed by flow cytometric analysis of MHCII and CD11c surface marker expression.

Gene Expression Analysis by Real-Time Quantitative-PCR (qRT-PCR)

Total RNA was harvested using TRIzol Reagent (Ambion, Life Technologies, Carlsbad, California, USA) and isolated by acid guanidinium thiocyanate-phenol-chloroform extraction, followed by purification using the PureLink RNA Mini Kit (Ambion, Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg total RNA per sample by mRNA-specific reverse transcription using Oligo(dT)12-18 Primer and SuperScript III Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The cDNA was used as a template for amplification in qRT-PCR in duplicate. qRT-PCR analysis was performed by SYBR Green (Kapa Biosystems, Inc., Boston, MA, USA) detection using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA, USA). qRT-PCR primers for gene expression analysis are shown below.

m-Gapdh Forward → ATCTTCTTGTGCAGTGCCAGCCT CGTCCC
m-Gapdh Reverse → TTGACTGTGCCGTTGAATTTGCC GTGAGTG
m-Ifnb1 Forward → CCCTATGGAGATGACGGAGA
m-Ifnb1 Reverse → TCCCACGTCAATCTTTCCTC
m-Irf7 Forward → GCATTTCGGTCGTAGGGATCTGGA TGAAGA
m-Irf7 Reverse → CGTACACCTTATGCGGATCAACTGGA

Protein Expression Analysis by Western Blotting

Total cell lysates were harvested by lysing cells in Radio Immunoprecipitation Assay (RIPA) buffer (25 mM Tris-HCl, pH7.6, 150 mM NaCl (sodium chloride), 1% NP-40, 1% SDS (sodium dodecyl sulfate), 1% sodium deoxycholate) with protease and phosphatase inhibitors (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics,

Dubai, UAE; Pierce Phosphatase Inhibitor Tablets, Thermo Fisher Scientific Inc., Rockford, IL, USA) for 1 h at 4°C. Whole cell lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C. For nuclear and cytoplasmic lysates, cytoplasmic and nuclear protein fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Life Technologies, Thermo Fisher Scientific Inc., Rockford, IL, USA). Protein concentrations were determined by the Bradford assay using Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Tecan Infinite M200 Microplate Reader (Tecan Trading AG, Switzerland) according to the manufacturer's instructions. Protein concentrations were normalized, and sample lysates were denatured by addition of Sodium Dodecyl Sulfate (SDS) loading buffer with β-mercaptoethanol and then boiling for 5 min at 95°C. Equal amounts of sample lysates were separated by 9% Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) under reducing and denaturing conditions (Amersham, GE Healthcare Bio-Sciences, Sweden), and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, GE Healthcare Bio-Sciences, Sweden). Blots were blocked in 5% milk or BSA solution (for phospho-proteins) to prevent non-specific background binding, and probed with specific antibodies in 5% milk or BSA solution (for phospho-proteins) shown below.

Anti-Actin (MAB1501) was from Merck Millipore (Temecula, CA, USA), anti-α Tubulin (B-7) (sc-5286) and anti-IRF-3 (FL-425) (sc-9082) were from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA), anti-TATA binding protein TBP [1TBP18] (ab818) was from Abcam (Cambridge, MA, USA), anti-Stat1 (pY701) (612132) and anti-Stat1 (N-Terminus) (610115) were from BD Transduction Laboratories (Franklin Lakes, New Jersey, USA), anti-Phospho-IRF-3 (Ser396) (4947) was from Cell Signaling Technology, Inc. (Danvers, MA, USA), anti-IRF-7 (51-3300) was from Invitrogen (Life Technologies, Carlsbad, California, USA), AffiniPure Donkey anti-rabbit HRP (711-035-152), AffiniPure Donkey anti-mouse HRP (715-035-150), and AffiniPure Donkey anti-goat HRP (705-035-147) were from Jackson ImmunoResearch Inc. (West Grove, PA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine levels in culture supernatants were measured using VeriKine Mouse Interferon Beta ELISA Kit (PBL Assay Science, Piscataway, NJ, USA) according to the manufacturer's instructions.

Chromatin Immunoprecipitation (ChIP) Analysis

DNA and proteins in cells were cross-linked using 1% formaldehyde for 10 min at room temperature and quenched using 200 mM glycine for 1 min at room temperature to stop the cross-linking reaction. Cells were scraped and collected into a 50-ml polypropylene tube, and centrifuged at 3,000 rpm for 5 min at 4°C. Cells were lysed with FA cell lysis buffer with protease inhibitor, and nuclei were lysed with 1% SDS nuclear lysis buffer with protease inhibitor. Cross-linked chromatin and associated proteins were sonicated using the Bioruptor sonication device (Diagenode Inc., Denville, NJ, USA) to generate chromatin fragments with an average fragment size of 500 bp. Chromatin fragments were immunoprecipitated overnight at 4°C using control IgG or anti-STAT1 antibodies (sc-345, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) bound to Dynabeads Protein A/G magnetic beads (Life Technologies, Carlsbad, California, USA). Immunoprecipitated chromatin fragments were dissociated from the antibody-bound beads using ChIP elution buffer, cross-links were reversed by incubation with 20 mg/ml pronase for 2 h at 42°C followed by 6 h at 67°C, and DNA was purified using phenol-chloroform extraction followed by ethanol precipitation. Isolated DNA was analyzed to determine the fold enrichment of target DNA sequences relative to input chromatin. The isolated DNA was quantified by qRT-PCR analysis using SYBR Green (Kapa Biosystems, Inc., Boston, MA, USA) detection using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA, USA). qRT-PCR primers for ChIP analysis are: 5′- ccctaaaggtctacccactgc-3′ (m-Irf7 Enhancer Forward) and 5′-ctccacagtcaagggttgtgt-3′ (m-Irf7 Enhancer Reverse). ChIP data were normalized to and expressed as percent of input.

LPS Challenge Model of Septic Shock

Mice received intraperitoneal injections of LPS from *Escherichia coli* (0111:B4) in sterile PBS (30 μg/g body weight). Serum was obtained via retro-orbital bleeding 3 h after LPS administration, and stored at −80°C until analysis by ELISA. In separate experiments, survival was monitored for 72 h after LPS administration. Six to 8-week-old gender- and age-matched mice were used in all experiments.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, California, USA). Student's *t*-test one-way analysis of variance (ANOVA) or paired *t*-tests were used as indicated in the figure legends to calculate statistical differences in mean values between groups. Results are expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM), as indicated in the figure legends. Values of $P < 0.05$ were considered statistically significant.

RESULTS

IRF-7 Is Essential for IL-1β Production and Type I IFN Responses in a Mouse Model of Endotoxin-Induced Acute Septic Shock

To test the hypothesis that IRF-7 is involved in the TLR-4 pathway and is required to trigger the induction of type I IFN that, in turn, drives IL-1β production *in vivo*, we challenged wild-type, $Irf7^{-/-}$ and $Irf3^{-/-}$ mice with a lethal dose of LPS, and measured serum IL-1β levels in wild-type and knockout mice. In accordance with other studies, wild-type mice showed a marked induction of IL-1β after intra-peritoneal LPS administration, whereas mice lacking *Irf3* exhibited severely impaired production of IL-1β ($n = 6$, $P < 0.05$ compared with wild-type mice)

(**Figure 1A**). This is consistent with the requirement of IRF-3 for IL-1β responses to LPS administration. Interestingly, we found that serum IL-1β levels were significantly reduced in *Irf7*-knockout mice. The levels of IL-1β in *Irf7*-deficient mice were severely impaired to an extent that was similar to those in *Irf3*-knockout mice following intra-peritoneal LPS administration ($n = 6$, $P < 0.05$ compared with wild-type mice) (**Figure 1A**). These results suggest that TLR4-induced IL-1β production *in vivo* requires IRF-7, and is dependent on the co-operative action of both IRF-7 and IRF-3. Thus, IRF-7 is an essential factor for activation of the IL-1β response in the TLR4 pathway *in vivo*.

A recent report documented that TLR4-TRIF signaling and the IRF-3-mediated type I IFN response play important roles for *in vivo* IL-1β processing and production in response to Gram-negative bacterial infection (9). By investigating serum levels of IFN-α and IFN-β in wild-type and mutant mice following endotoxin exposure, we found that levels of type I IFN were positively correlated with levels of IL-1β in wild-type and knockout mice. As with the serum IL-1β levels (**Figure 1A**), wild-type mice exhibited increased type I IFN levels in serum after intra-peritoneal LPS administration, whereas serum levels of IFN-β cytokine ($n = 7$, $P < 0.05$ compared with wild-type mice) (**Figure 1B**) and IFN-α cytokine ($n = 4$, $P < 0.05$ compared with wild-type mice) (**Figure 1C**) were undetectable in both *Irf7*-knockout mice and *Irf3*-knockout mice. This suggests that, as is the case with IL-1β, IFN-β responses to LPS *in vivo* also require both IRF-7 and IRF-3.

Previous studies in mouse models of septic shock have shown that type I IFN and IL-1β contribute to LPS-induced lethality *in vivo* (27, 32–34). To assess the functional significance of attenuated type I IFN and IL-1β production in LPS-challenged $Irf7^{-/-}$ and $Irf3^{-/-}$ mice compared with wild-type mice, we measured the survival rate of these mice over 3 days after intra-peritoneal LPS challenge. We observed that both $Irf7^{-/-}$ and $Irf3^{-/-}$ mice exhibited improved survival compared with wild-type mice ($n = 21$, $P < 0.05$) (**Figure 1D**), thus demonstrating that both $Irf7^{-/-}$ and $Irf3^{-/-}$ mice exhibited increased resistance to LPS-induced endotoxin shock mortality *in vivo*. Taken together, our data demonstrate the *in vivo* physiological relevance of IRF-7 in the activation of IFN-β production by LPS, indicating that IRF-7 mediates IL-1β production *in vivo* via activation of type I IFN production, and that TLR4-induced type I IFN and IL-1β production in mice requires the combined action of both IRF-7 and IRF-3. These results provide evidence that IRF-7, which was previously shown to interact with TRIF, plays an active role in the TLR4-mediated TRIF-dependent signaling pathway.

Macrophages, but Not Dendritic Cells, Require IRF-7, Together With IRF-3, for LPS-Stimulated IFN-β Induction

The loss of type I IFN production in *Irf7*-deficient mice after LPS administration was intriguing. This is because previous studies by Honda et al. have, unequivocally, demonstrated that IFN-β mRNA transcription is largely retained in LPS-stimulated *Irf7*-deficient DCs, but is markedly abolished in *Irf3*-deficient DCs (24). We sought to confirm these findings by analyzing IFN-β mRNA induction and protein secretion in BMDCs from *Irf7*- and *Irf3*-knockout mice. As in the previous report by Honda et al. BMDCs from *Irf7*-knockout mice produced relatively normal amounts of IFN-β at both mRNA and protein levels, whereas IFN-β gene induction and protein secretion were inhibited in LPS-stimulated *Irf3*-knockout BMDCs (**Figures 2A–D**). Hence, our results are consistent with the previous study by Honda et al. which concluded that activation of the type I IFN response in LPS-stimulated DCs depended entirely on IRF-3 (24, 27).

Although both macrophages and DCs secrete IFN-β after LPS stimulation, BMDMs consistently produced significantly higher levels of IFN-β than DCs in response to a similar LPS stimulation (**Supplementary Figure 1**). Because IRF-7 has previously been shown to act together with IRF-3 to induce later-phase production of high levels of type I IFN in fibroblasts during viral infections (35), we hypothesized that in macrophages, which produced higher levels of IFN-β compared with DCs, IRF-7, in addition to IRF-3, induces IFN-β production after LPS stimulation. To test this hypothesis, we evaluated both IFN-β mRNA induction and protein secretion in BMDMs derived from *Irf7*- and *Irf3*-knockout mice. In line with previous studies, IFN-β production was impaired in BMDMs lacking *Irf3* (**Figures 2E,F**), indicating that IRF-3 is critical for type I IFN production in macrophages. Surprisingly, unlike in BMDCs, LPS-induced IFN-β expression in *Irf7*-deficient BMDMs was markedly inhibited (**Figures 2G,H**), suggesting that, unlike in BMDCs, IRF-7 is critical for TLR4-mediated IFN-β induction in macrophages. Therefore, our findings suggest that, as in viral-infected fibroblasts, induction of type I IFN in the TLR4 pathway in macrophages also depends on both IRF-7 and IRF-3 activities.

IRF-7 Is Constitutively Expressed in Resting Bone Marrow-Derived Macrophages, but Not in Dendritic Cells

IRF-7 is constitutively expressed in pDCs, where it is critical for rapid and robust type I IFN production during viral infections (24). The involvement of IRF-7 in the regulation of TLR4-induced IFN-β production in BMDMs led us to hypothesize that, as in pDCs, BMDMs may also constitutively express IRF-7 and this may be responsible for the robust activation of IFN-β production in these cells after LPS stimulation. To investigate this possibility, we analyzed the expression of IRF-7 protein in resting BMDMs and BMDCs by Western blotting. In line with the lack of IRF-7 function during induction of IFN-β production in DCs, we did not observe any IRF-7 protein in resting wild-type BMDCs (**Figure 3A**). On the contrary, resting wild-type BMDMs constitutively expressed IRF-7 protein (**Figure 3A**), as was also the case in pDCs. On the other hand, as expected, IRF-3 protein is constitutively expressed in both macrophages and DCs (**Figure 3B**). The kinetics of IRF-3 phosphorylation in response to LPS treatment was comparable between wild-type BMDMs and BMDCs (**Supplementary Figure 2**).

IRF-7 is an IFN-inducible protein, up-regulated by autocrine signaling through the IFN-α/β receptor (IFNAR), that promotes further production of type I IFN after viral infection in fibroblasts

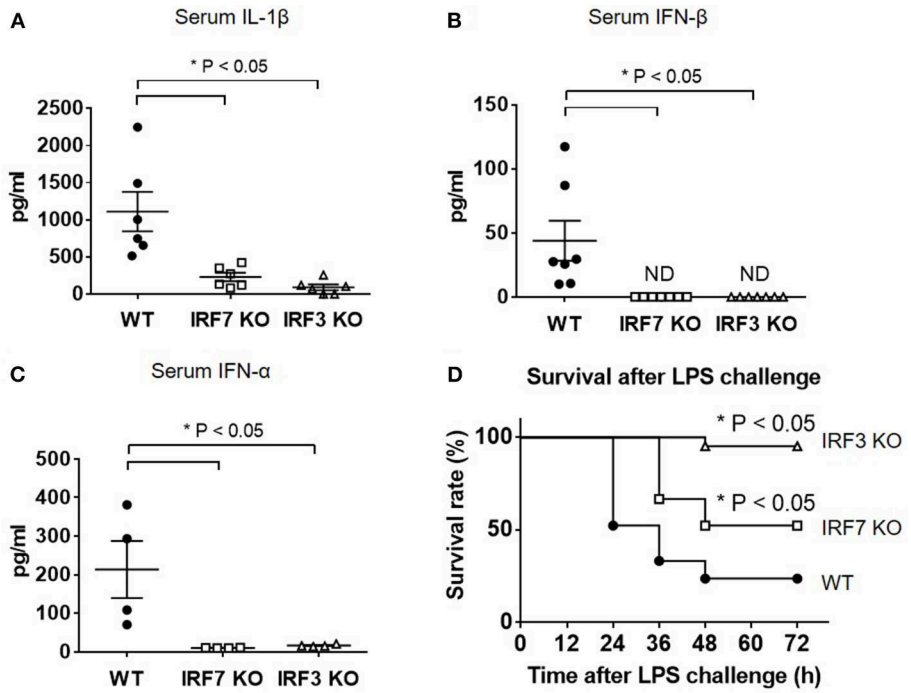

FIGURE 1 | IRF-7 facilitates IL-1β and type I IFN responses to LPS *in vivo*. **(A)** ELISA analysis of IL-1β cytokine levels in serum from IRF-7 or IRF-3 knockout mice ($n = 6$) compared to wild-type control littermates ($n = 6$), 3 h after I.P. injection of 30 μg/g LPS in sterile PBS. Data are presented as mean ± SEM. One-way ANOVA was used to calculate statistical differences (*$p < 0.05$). **(B)** ELISA analysis of IFN-β levels in serum from IRF-7 or IRF-3 knockout mice ($n = 7$) compared to wild-type control littermates ($n = 7$), 3 h after I.P. injection of 30 μg/g LPS in sterile PBS. Data are presented as mean ± SEM. One-way ANOVA was used to calculate statistical differences (*$p < 0.05$). **(C)** ELISA analysis of IFN-α levels in serum from IRF-7 or IRF-3 knockout mice ($n = 4$) compared to wild-type control littermates ($n = 4$), 3 h after I.P. injection of 30 μg/g LPS in sterile PBS. Data are presented as mean ± SEM. One-way ANOVA was used to calculate statistical differences (*$p < 0.05$). **(D)** IRF-7 knockout mice are protected from LPS-induced endotoxin shock mortality *in vivo*. Survival of IRF-7 and IRF-3 knockout mice, compared with wild-type control littermates, following i.p. injection of 30 μg/g LPS in sterile PBS ($n = 21$ mice, *p*-value * <0.05 compared with wild-type mice by log-rank test).

(24, 35–37). In contrast, we found that IRF-7 expression in macrophages remained constant up to 2 h after LPS stimulation, which corresponds with the peak in IFN-β transcriptional induction (**Figure 3A**). This suggests that pre-existing IRF-7 protein is responsible for the activation of type I IFN induction in macrophages. It is worth mentioning that IRF-7 expression in DCs remained undetectable within the first 2 h post-LPS stimulation. Altogether, these data suggest that, as observed in pDCs, resting BMDMs possess a pre-existing pool of constitutively expressed IRF-7 protein that is necessary for the activation of robust IFN-β responses in macrophages after LPS stimulation.

Basal Type I IFN Signaling Sustains Constitutive IRF-7 Expression, and Is Required for LPS-Stimulated IFN-β Induction in Macrophages

As IRF-7 is already constitutively expressed in macrophages, we hypothesized that this was due to the presence of a basal type I IFN production and signaling in macrophages, that is not present in DCs. To check this hypothesis, we analyzed the expression of the IRF-7 protein in BMDMs prepared from mice with defective type I IFN signaling components, namely *Ifnar1* and *Stat1*. We found that basal IRF-7 mRNA and protein levels were markedly inhibited in resting BMDMs lacking either *Ifnar1* or *Stat1* (**Figures 4A,B**), whereas IRF-3 protein levels remained unaffected (**Figure 4C**). However, constitutive IRF-7 expression at both mRNA and protein levels were found to be largely unaltered in BMDMs lacking other components of TLR signaling, namely *Myd88*, *Trif* and *Irf3* (**Figures 4A,B**). Thus, our data suggest that constitutive IRF-7 expression in resting BMDMs is mediated by basal type I IFN signaling in a STAT1-dependent manner.

Given that basal type I IFN signaling regulates constitutive IRF-7 expression in BMDMs, our next step was to check whether the absence of IRF-7 protein in resting BMDCs was due to an absence of basal type I IFN production and signaling in these cells. To do so, we analyzed the basal levels of IFN-β expression in resting BMDCs and BMDMs. Our results show that basal expression of IFN-β mRNA was significantly lower in wild-type BMDCs than in wild-type BMDMs (**Supplementary Figure 3A**), suggesting that DCs, intrinsically, lack basal type I IFN production and signaling, which explains the complete absence of IRF-7 protein in these cells. In line with the absence of type I IFN production and signaling, resting wild-type BMDCs were found to express minimal amounts of IRF-7 mRNA (over 200-fold lower than BMDMs), whereas BMDMs expressed high levels of *Irf7* transcripts (**Supplementary Figure 3B**). Overall, our data

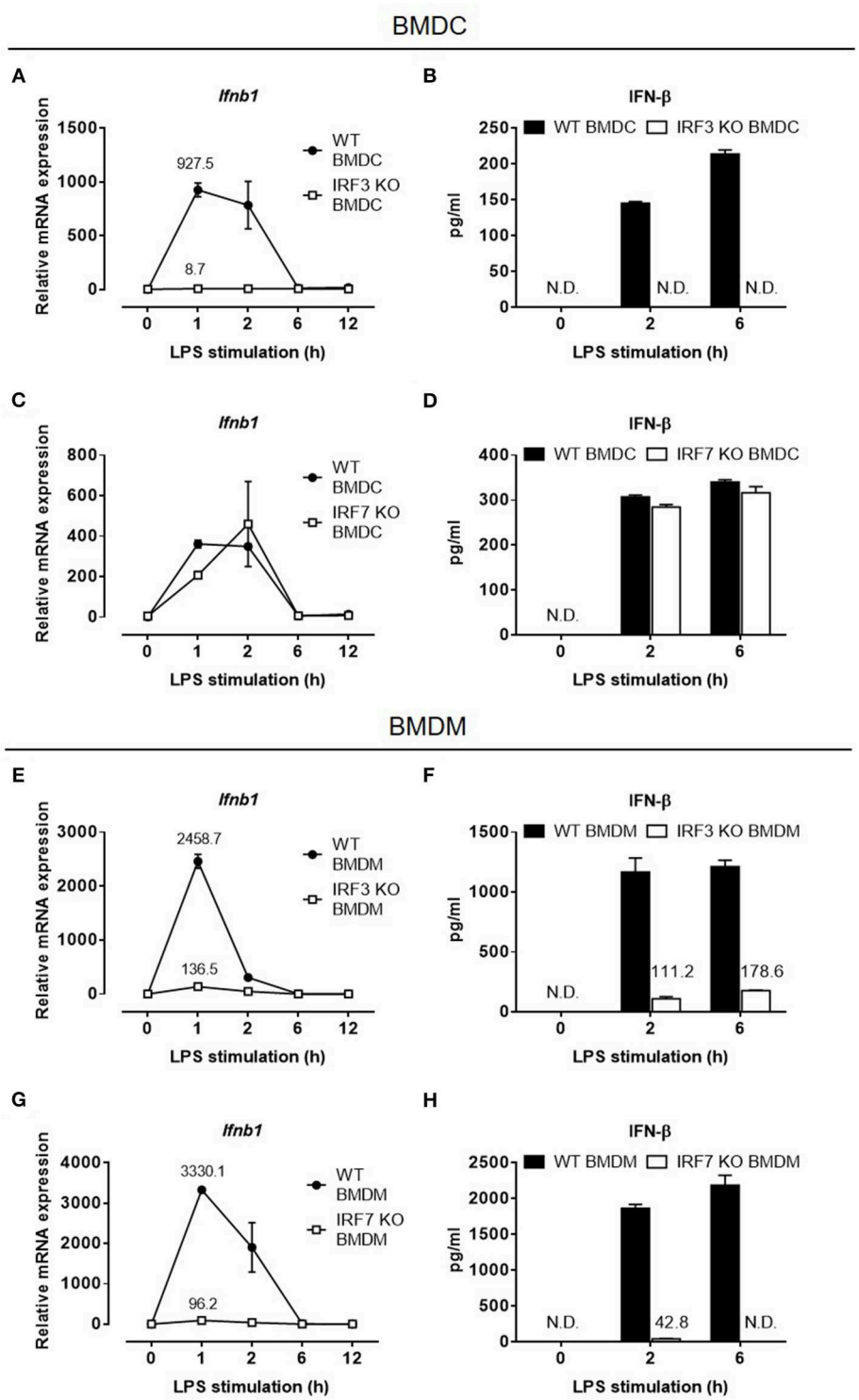

FIGURE 2 | IFN-β expression in LPS-challenged macrophages depends on both IRF-7 and IRF-3, whereas IFN-β expression in LPS-challenged DCs depends on IRF-3 but not IRF-7. **(A–D)** Real-time PCR and ELISA analysis of IFN-β gene and protein expression of BMDCs from IRF-3 knockout mice **(A,B)**, and IRF-7 knockout mice **(C,D)**, together with their respective wild-type control littermates, stimulated or not with 100 ng/ml LPS for 0–12 h. *Ifnb1* expression was normalized to *Gapdh,* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments (N.D.: not detected). **(E–H)** Real-time PCR and ELISA analysis of IFN-β gene and protein expression of BMDMs from IRF-3 knockout mice **(E,F)**, and IRF-7 knockout mice **(G,H)**, together with their respective wild-type control littermates, stimulated or not with 100 ng/ml LPS for 0–12 h. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments (N.D.: not detected).

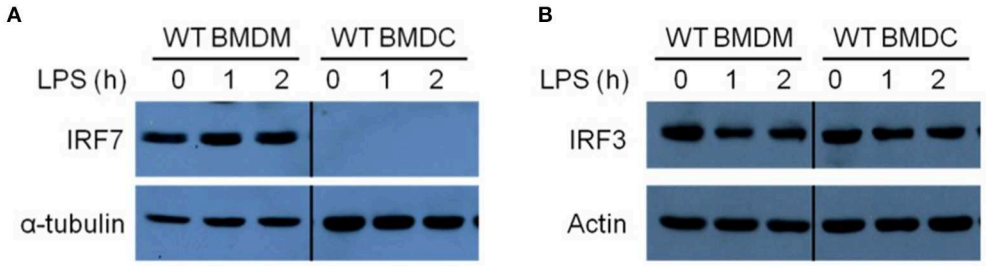

FIGURE 3 | Resting macrophages, but not DCs, constitutively express IRF-7 protein. Western immunoblot analysis of total IRF-7 **(A)** and total IRF-3 **(B)** protein expression in whole cell lysates of wild-type BMDMs and BMDCs, stimulated or not with 100 ng/ml LPS for 0–2 h. Data are representative of at least three independent experiments.

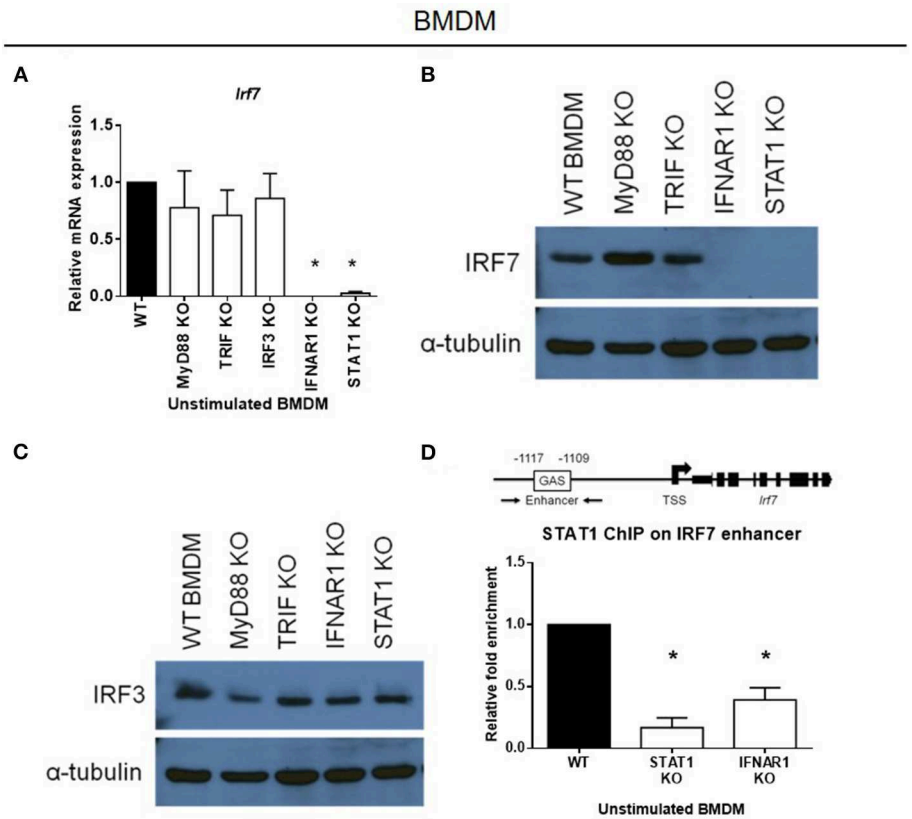

FIGURE 4 | Constitutive expression of IRF-7 in resting macrophages is sustained by constitutive IFNAR signaling and STAT1 binding to the *Irf7* enhancer. **(A)** Real-time PCR analysis of *Irf7* gene expression in resting BMDMs from MyD88, TRIF, IRF-3, IFNAR1, and STAT1 knockout mice, compared to wild-type control littermates. *Irf7* expression was normalized to *Gapdh*, and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SEM of at least three independent experiments. One-way ANOVA was used to calculate statistical differences (*$p < 0.05$). **(B,C)** Western immunoblot analysis of total IRF-7 **(B)** and total IRF-3 **(C)** protein expression in whole cell lysates of resting BMDMs from MyD88, TRIF, IFNAR1, and STAT1 knockout mice, compared to wild-type control littermates. Data are representative of at least two independent experiments. **(D)** ChIP analysis of STAT1 binding at the IRF-7 enhancer in resting BMDMs from STAT1 and IFNAR1 knockout mice compared to wild-type control littermates. ChIP-enriched DNA was normalized to input DNA and expressed relative to the levels observed in STAT1 ChIP in un-stimulated wild-type control cells. Data shown are presented as mean ± SEM of at least three independent experiments. One-way ANOVA was used to calculate statistical differences (*$p < 0.05$).

indicate that constitutive *Irf7* expression in macrophages is primarily regulated at the transcriptional level by basal IFN-β production and type I IFN signaling, and that this is not present in DCs.

Type I IFN signaling is mediated by STAT1 activation via the IFNAR. To understand how *Irf7* is constitutively regulated at the transcriptional level in macrophages, we explored whether STAT1 regulates the transcription of *Irf7* directly by basal

type I IFN signaling. The murine *Irf7* enhancer contains a IFN-γ-activated site (GAS) sequence, which binds STAT1 at a site 1.1 kb upstream of the transcription start site (TSS) (38). Chromatin immunoprecipitation (ChIP) experiments on STAT1 binding at this upstream GAS enhancer show significant constitutive binding of STAT1 to the *Irf7* enhancer region in resting wild-type macrophages. This constitutive binding of STAT1 to the *Irf7* enhancer region was completely abrogated in resting *Ifnar1*-deficient BMDMs that had disrupted basal type I IFN signaling (**Figure 4D**). These results indicate that basal IFN-β production and signaling in resting macrophages results in constitutive STAT1 binding to the *Irf7* enhancer region, and this sustains constitutive *Irf7* transcription and subsequent protein expression.

The absence of constitutive IRF-7 expression in resting macrophages lacking *Ifnar1* or *Stat1* implies that macrophages with defective type I IFN signaling would display defective induction of IFN-β in response to LPS. Consistent with our observations in *Irf7*-deficient macrophages, IFN-β mRNA induction and protein secretion were markedly abolished in LPS-stimulated macrophages that had defective type I IFN signaling, namely *Ifnar1*- and *Stat1*-deficient BMDMs (**Figures 5A–D**). Although type I IFN signaling following IFNAR engagement can also be mediated by STAT3 homodimers, we found that LPS-stimulated IFN-β expression was not affected in *Stat3*-knockout BMDMs, in contrast to *Ifnar1*- and *Stat1*-deficient BMDMs. On the contrary, IFN-β expression in response to LPS was elevated in *Stat3*-knockout BMDMs compared with wild-type BMDMs (**Supplementary Figure 4**). Therefore, our data indicate that STAT1, but not STAT3, downstream of basal type I IFN signaling, mediates constitutive IRF-7 expression in resting macrophages, which is in turn required for IFN-β responses in LPS-stimulated macrophages.

The absence of constitutive IRF-7 expression and the lack of basal type I IFN production and signaling in resting DCs prompted us to speculate that DCs with defective type I IFN signaling would display normal levels of IFN-β induction in response to LPS, similar to *Irf7*-deficient DCs. Indeed, in contrast to LPS-stimulated BMDMs, IFN-β induction in LPS-stimulated BMDCs lacking *Ifnar1* was not affected (**Supplementary Figure 5**). This confirms that TLR4-induced IFN-β production in DCs is independent of constitutive type I IFN signaling.

LPS-Induced IRF-3 Phosphorylation and Nuclear Translocation in Macrophages Is Not Affected by the Absence of IRF-7

IRF-3 phosphorylation and nuclear translocation is necessary for the activation of type I IFN production by macrophages after LPS stimulation (14, 39–41). Following our finding that both IRF-7 and IRF-3 are required for IFN-β induction in macrophages and in mice, we studied whether they could affect each other's phosphorylation and nuclear translocation in response to bacterial LPS. To do so, we performed biochemical analyses to determine the phosphorylation and nuclear translocation of IRF-3 in *Irf7*-deficient BMDMs before and after LPS stimulation. Our results demonstrated that IRF-3 phosphorylation in LPS-stimulated *Irf7*-knockout BMDMs was not much different from that in wild-type BMDMs (**Figure 6A**). The nuclear translocation of the phosphorylated form of IRF-3 is critical for the activation of *Ifnb* in LPS-challenged macrophages. Our analyses show that nuclear extracts from LPS-stimulated *Irf7*-deficient BMDMs and wild-type BMDMs contained a similar amount of IRF-3 (**Figure 6C**). We also found relatively normal levels of IRF-3 phosphorylation and nuclear translocation in LPS-stimulated BMDMs lacking *Ifnar1* when compared with that in wild-type BMDMs (**Figures 6B,C**). This supports the concept that constitutive type I IFN signaling is necessary for constitutive IRF-7 expression in macrophages, but is dispensable for IRF-3 phosphorylation and nuclear translocation in these cells. As expected, LPS-stimulated *Trif*-knockout BMDMs showed severe impairment in both phosphorylation and nuclear translocation of IRF-3 (**Figures 6A–C**). Due to the lack of a reliable antibody specific against the endogenous phosphorylated form of IRF-7, we were unable to investigate IRF-7 phosphorylation and nuclear translocation in LPS-stimulated *Irf3*-deficient macrophages. Thus, we concluded that TRIF-mediated phosphorylation and nuclear translocation of IRF-3 is completely independent of IRF-7 activity in LPS-stimulated macrophages. Taken together with our finding that IRF-7 levels are largely unaltered in IRF-3-null macrophages (**Supplementary Figure 6**), these data suggest that IRF-7 and IRF-3 are both required in combination to achieve optimal IFN-β production in endotoxin-challenged macrophages.

IRF-7-Mediated IFN-β Induction in LPS-Stimulated Macrophages Depends on TRIF and TBK-1

As IRF-3 alone can mediate type I IFN induction in BMDCs after LPS stimulation, and LPS-stimulated *Irf7*-deficient macrophages showed normal phosphorylation and nuclear translocation of IRF-3, we speculated that the presence of IRF-3 alone in *Irf7*-deficient macrophages might still retain some ability to mediate IFN-β induction, despite the absence of IRF-7 in these cells. Indeed, LPS-induced *Irf7*-deficient macrophages can still produce IFN-β, although its levels were low and attenuated compared with the ones found in wild-type BMDMs (**Figures 7A,B**). Our results indicated that *Irf3*-deficient macrophages also expressed low levels of *Ifnb* transcripts (**Figures 7A,B**). Consistent with these data, LPS-stimulated macrophages lacking either *Irf7* or *Irf3* exhibited low levels of STAT1 phosphorylation (**Figure 7C**). Thus, these results further support the premise that IRF-7 and IRF-3 activation and nuclear translocation in LPS-stimulated macrophages are independent processes. Our data also indicate that IRF-7 or IRF-3 alone can mediate IFN-β induction by LPS, but when IRF-7 and IRF-3 are simultaneously present in macrophages, transactivation of *Ifnb* is markedly enhanced, which, according to our results, is a requirement for robust IL-1β production in mice after LPS challenge.

The signaling adaptor MyD88 has been demonstrated to activate IRF-7 for induction of type I IFN by pDCs in response

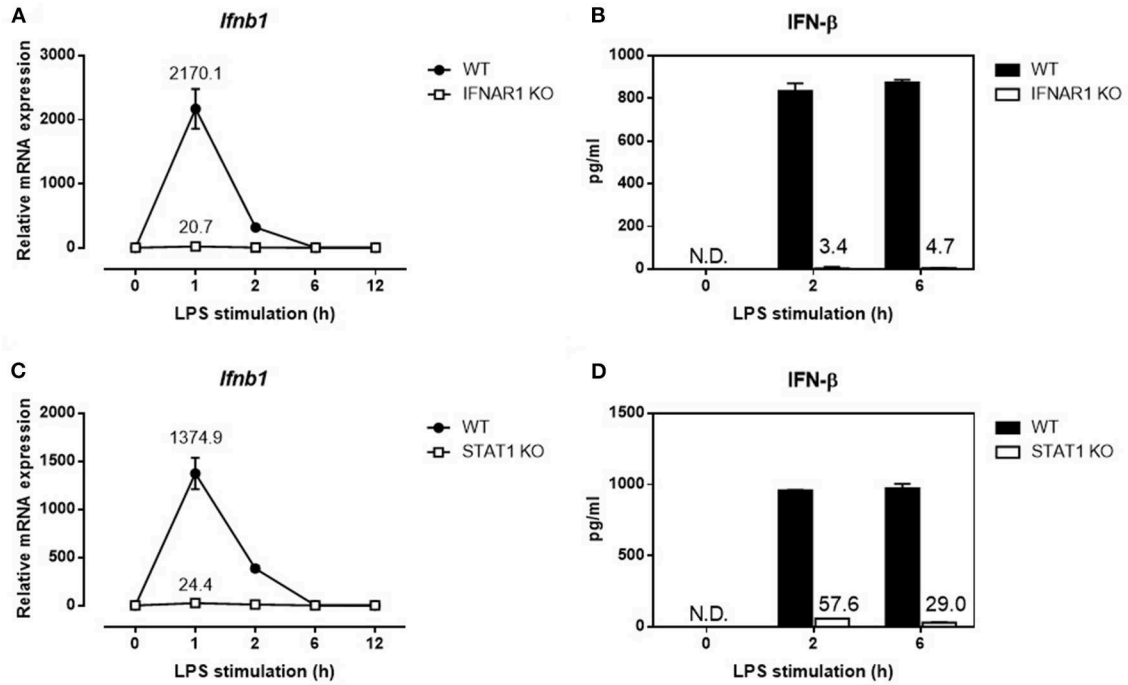

FIGURE 5 | Autocrine/paracrine IFNAR1-STAT1 signaling is required for IFN-β gene and protein expression in LPS-challenged macrophages. Real-time PCR and ELISA analysis of IFN-β gene and protein expression of BMDMs from IFNAR1 knockout mice **(A,B)**, and STAT1 knockout mice **(C,D)**, compared to wild-type control littermates, stimulated or not with 100 ng/ml LPS for 0–12 h. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least three independent experiments (N.D.: not detected).

to virus infection and TLR7/9 activation (24, 42). However, this and other studies have shown that IFN-β expression was not impaired in MyD88-deficient BMDMs compared with wild-type BMDMs after LPS stimulation (**Supplementary Figure 7**). These results indicate that, unlike the requirement for MyD88 and IRF-7 in TLR7/9-activated pDCs, IFN-β induction in TLR4-activated macrophages is dependent on IRF-7, but is independent of MyD88. Previous biochemical studies have demonstrated that TRIF can interact with and activate both IRF-7 and IRF-3 *in vitro* (25, 26). Hence, we predicted a complete loss of IFN-β induction by LPS in macrophages prepared from mice lacking *Trif* or both *Irf3* and *Irf7*, if it is true that TRIF is required for activation of both IRF-7 and IRF-3. As expected, LPS-stimulated IFN-β mRNA induction and protein secretion was completely abolished in macrophages lacking *Trif*, thereby suggesting that TRIF promotes the activation of both IRF-7 and IRF-3 in the TLR4 pathway (**Figures 7A–C**). Correspondingly, as in *Trif*-deficient macrophages, we also found that IFN-β transcription and secretion were entirely abrogated in *Irf3*/*Irf7* double deficient BMDMs (**Figures 7A–C**), supporting the hypothesis that TRIF mediates the activation of both IRF-7 and IRF-3 in LPS-stimulated macrophages.

Given that TRIF can interact with both IRF-7 and IRF-3, and *in vitro* kinase assays have shown that TBK-1 can mediate IRF-3 and IRF-7 phosphorylation (43–47), we hypothesized that TBK-1 can mediate IRF-7 activation in LPS-stimulated macrophages. To test this hypothesis, we used BX795, a small molecule inhibitor of TBK-1, to test whether IRF-7 activity and IRF-7-mediated type I IFN production are also dependent on TBK-1 activity (48, 49). Due to the lack of a reliable antibody specific against the endogenous phosphorylated form of IRF-7, we measured LPS-induced IFN-β gene expression in *Irf3* single knockout, *Irf7* single knockout, and *Irf3*/*Irf7* double knockout BMDMs in the absence or presence of BX795 (**Figure 7D** and **Supplementary Figure 8**). In line with previous studies, BX795 inhibited the IRF-3-mediated IFN-β transcription present in *Irf7*-knockout BMDMs. Interestingly, we found that BX795 also inhibited the IRF-7-mediated IFN-β transcription present in *Irf3*-knockout BMDMs, suggesting that TBK-1 also mediates IRF-7 activation and IRF-7-mediated type I IFN production in LPS-stimulated macrophages. Moreover, we also found that BX795 completely inhibited IFN-β transcription in wild-type BMDMs, similar to levels seen in *Irf3*/*Irf7* double knockout BMDMs, rather than to levels seen in the single knockout BMDMs, suggesting that TBK-1 is the kinase that mediates the phosphorylation of both IRF-3 and IRF-7 in TLR4 signaling in macrophages. In summary, we conclude that TRIF mediates activation

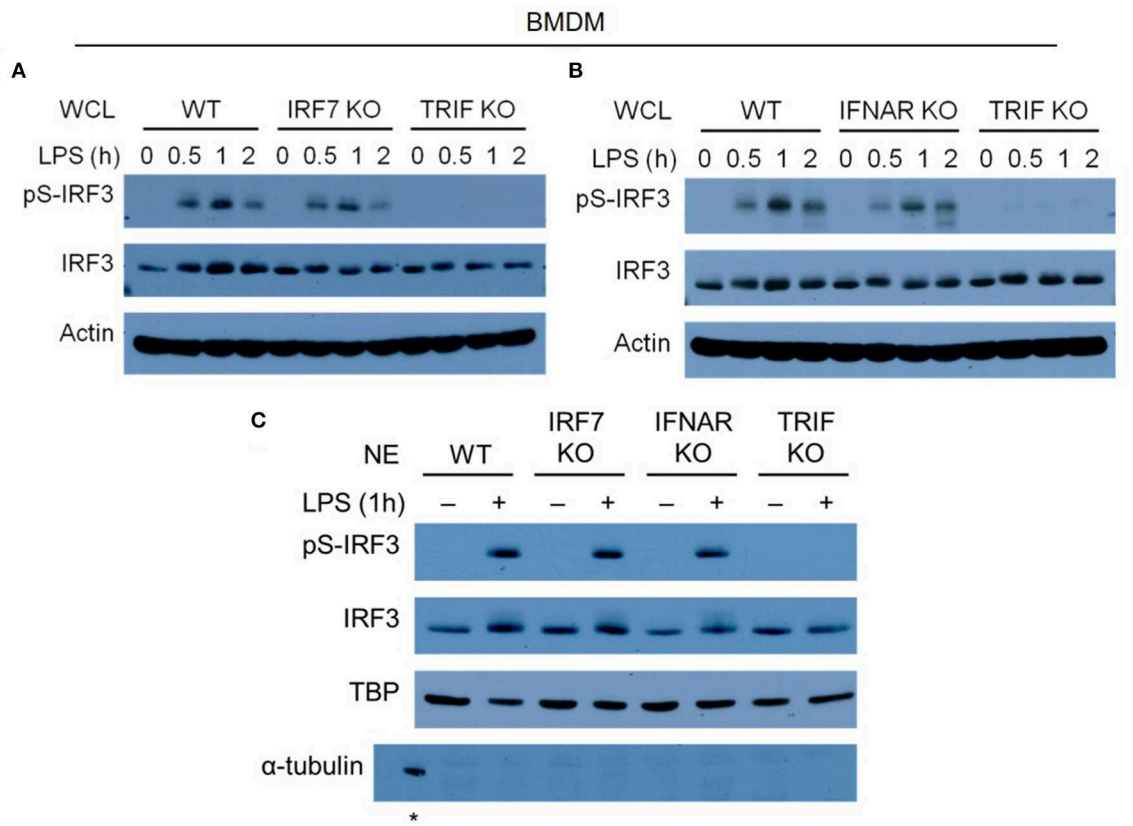

FIGURE 6 | IRF-7 and autocrine/paracrine IFNAR signaling regulate IFN-β expression independent of IRF-3 phosphorylation in LPS-challenged macrophages. **(A,B)** Western immunoblot analysis of phospho-IRF-3 and total IRF-3 protein expression in whole cell lysates (WCL) of IRF-7 knockout **(A)** or IFNAR1 knockout **(B)** BMDMs, compared to wild-type (WT) and TRIF knockout BMDMs, stimulated or not with 100 ng/ml LPS for 0–2 h. Data are representative of at least three independent experiments. Actin was used as a loading control. **(C)** Western immunoblot analysis of phospho-IRF-3 and total IRF-3 protein expression in nuclear extracts (NE) of wild-type (WT), IRF-7 knockout, IFNAR1 knockout, and TRIF knockout BMDMs, stimulated or not with 100 ng/ml LPS for 0–2 h. Data are representative of at least two independent experiments. Actin and TATA-binding protein (TBP) were used as loading controls. *indicates α-tubulin detected in WCL of un-stimulated WT BMDMs as a control.

of both IRF-7 and IRF-3 via TBK-1 in the macrophage TLR4 pathway.

DISCUSSION

Type I IFN is necessary for IL-1β production by the non-canonical NLRP3 inflammasome in response to Gram-negative bacterial infection. TRIF is essential for non-canonical NLRP3 inflammasome activation by LPS of Gram-negative bacteria through the activation of type I IFN induction (9). Previous studies have demonstrated that LPS induces type I IFN production via the TLR4-TRIF-TBK-1-IRF-3 pathway to promote the transcriptional induction of *Casp11*, which encodes caspase-11 as the key mediator of the non-canonical NLRP3 inflammasome (9). As TRIF was previously shown to interact with IRF-7 (25), and IRF-7 is known as a "master regulator" of type I IFN responses in viral infections (24, 35, 36), we hypothesized that IRF-7 is specifically involved in the TLR4 pathway and is required to trigger the induction of TRIF-dependent type I IFN that, in turn, drives IL-1β production. We tested this hypothesis in an established mouse model of endotoxin shock, in which it has been previously shown that the induction of IL-1β responses *in vivo* is dependent on the activation of type I IFN production by TLR4 in a TRIF- and IRF-3-dependent manner (7–9). By using this animal model, we identified IRF-7 as an essential regulator of IL-1β and type I IFN production in mice. We also demonstrated that the production of type I IFN and IL-1β in mice is dependent on the combined action of both IRF-7 and IRF-3, which have been shown to interact with TRIF in a yeast two-hybrid screening study (25). The low amounts of residual IL-1β present in the serum of IRF-7 knockout mice might be due to the activation of non-canonical inflammasome by "cytosolic" LPS (7, 8). Macrophages and DCs are key antigen-presenting cells that trigger both pro-inflammatory cytokine production and type I IFN production in the innate immune response to LPS. Our results show for the first time that macrophages, but not DCs, constitutively express IRF-7 and require IRF-7 to promote robust IFN-β induction following LPS stimulation. Macrophages lacking either *Irf7* or *Irf3* produce significantly lower levels of type I IFN in response

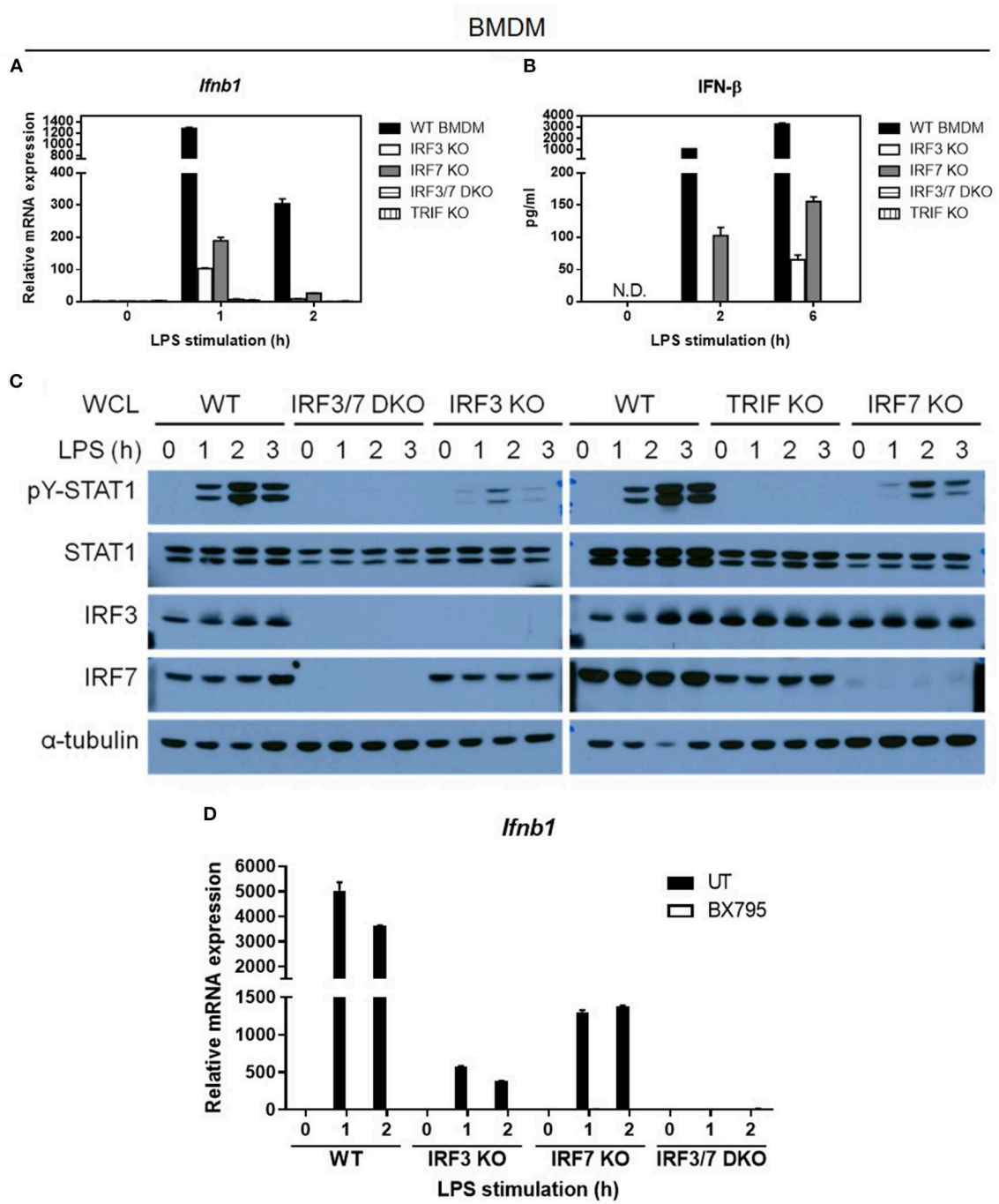

FIGURE 7 | TBK1 is required for the activation of both IRF-7 and IRF-3 downstream of TRIF for optimal IFN-β expression in LPS-challenged macrophages. **(A,B)** Real-time PCR and ELISA analysis of IFN-β gene and protein expression of BMDMs from IRF-3, IRF-7, and TRIF single knockout mice, and IRF-3-IRF-7 double knockout mice, compared to wild-type control littermates, stimulated or not with 100 ng/ml LPS for the indicated times. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments (N.D.: not detected). **(C)** Western immunoblot analysis of phospho-STAT1 and total STAT1 protein expression in whole cell lysates of BMDMs from IRF-3, IRF-7, and TRIF single knockout mice, and IRF-3-IRF-7 double knockout mice, compared to wild-type control littermates, stimulated or not with 100 ng/ml LPS for 0–6 h. Data are representative of at least two independent experiments. **(D)** Real-time PCR analysis of IFN-β gene expression in BMDMs from IRF-3 single knockout mice, IRF-7 single knockout mice, and IRF-3-IRF-7 double knockout mice, compared to wild-type control littermates, pre-treated or not with 2 μM BX795 (TBK1 inhibitor) for 1 h, and then stimulated or not with 100 ng/ml LPS for 0–2 h. *Ifnb1* expression was normalized to *Gapdh* and expressed relative to the levels observed in un-treated and un-stimulated wild-type control cells. Data are presented as mean ± SD of duplicate determinations from one representative of at least two independent experiments.

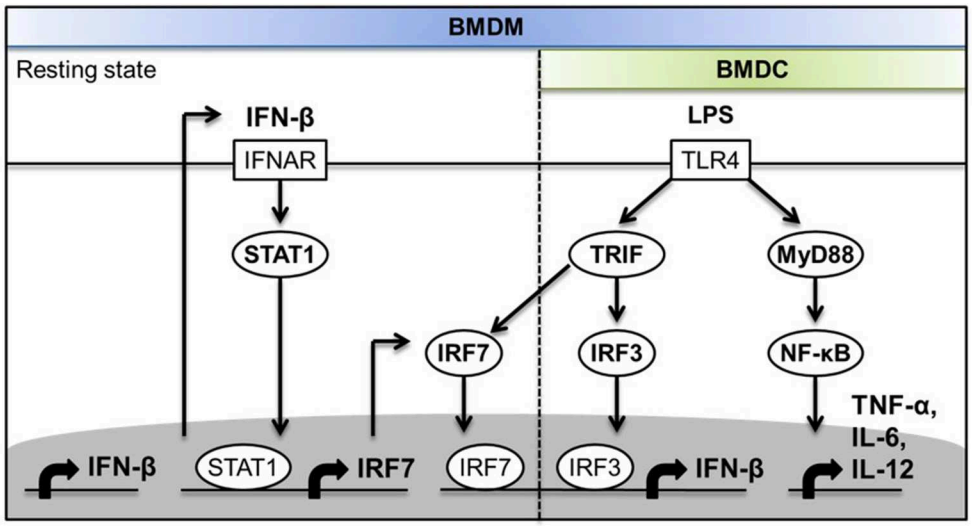

FIGURE 8 | Schematic diagram depicting the molecular mechanisms of the involvement of IRF-7 in TLR4-induced IFN-β expression in macrophages but not in DCs.

to LPS, which has led us to propose a new paradigm whereby both IRF-7 and IRF-3 are essential for TLR4-induced IFN-β production in macrophages. The activation of IRF-7 and IRF-7-mediated IFN-β induction in macrophages is dependent on TBK-1, which has been shown to be activated by TRIF in response to TLR4 ligation by LPS (43–46, 48–51). In contrast, we found that DCs lack constitutive IRF-7 expression, and are dependent on IRF-3, but not IRF-7, for IFN-β induction following LPS stimulation in the TLR4 pathway. Our results indicate that cell-type specific basal type I IFN production and signaling present in resting macrophages, but absent in DCs, is largely responsible for constitutive IRF-7 expression at both mRNA and protein levels, which, in turn, is required for IFN-β responses in LPS-stimulated macrophages, but not in DCs (**Figure 8**). Taken together, our *in vitro* studies in macrophages and DCs suggest that macrophages may represent a key cell type that contributes to type I IFN and IL-1β responses *in vivo*, since they depend on both IRF-7 and IRF-3 activities for activation of type I IFN responses after LPS stimulation.

Constitutively Expressed IRF-7 in Resting Macrophages act Together With IRF-3 to Confer Rapid and Robust IFN-β Induction in the TLR4 Pathway

In this study, we showed that IL-1β is rapidly induced in mice during LPS challenge. IL-1β has been shown to play an important role in early host defense against bacterial infections. Type I IFN is also rapidly induced in response to LPS and is essential for activation of IL-1β production by the non-canonical caspase 11-dependent inflammasome in mice. The timely and robust production of IFN-β may possibly contribute to the kinetics and amounts of IL-1β production by macrophages during Gram-negative bacteria infection. Type I IFN production differs in kinetics and magnitude between cell types (32, 52–55). We and others have previously reported that human monocytes produced IFN-β within 1–2 h of exposure to Sendai virus or LPS, whereas non-myeloid cell types, such as HeLa cells and fibroblasts, produced IFN-β after 6 h post-infection (52, 53, 56, 57). Maniatis et al. have used virus-infected human epithelial HeLa cells as a model to identify component transcription factors of the virus-induced IFN-β "enhanceosome," such as NF-κB RelA/p50, IRF-3/7, and ATF-2/c-Jun, that act at the IFN-β enhancer to induce IFN-β transcription (58). On the other hand, we showed that the rapid induction of IFN-β transcription in human monocytes was determined to some extent by the constitutive binding of the myeloid-specific transcription factors PU.1 and IRF-8 to the enhancer region of the *IFNB* promoter, which promoted the recruitment of IRF-3 to the *Ifnb* locus through direct physical interaction between IRF-8 and IRF-3 (52). We also noticed that, similar to human monocytes, murine BMDMs also rapidly expressed IFN-β mRNA within 1–2 h of LPS exposure. Since PU.1 and IRF-8 are present and functionally important in myeloid cells, it is highly plausible that in BMDMs, IRF-8 and PU.1 also constitutively bind to the *Ifnb* promoter and facilitate the recruitment of the transcription factors IRF-7 and IRF-3 to induce the rapid and robust LPS-induced IFN-β gene transcription in macrophages. The transcription factor IRF-7 is an IFN-inducible protein and is typically not endogenously expressed in most cell types except pDCs. The constitutive expression of IRF-7 protein in pDCs was previously shown to be responsible for high-level and rapid IFN-α production by these cells after stimulation with TLR7/8/9 ligands (24, 42). In contrast, the late inducible expression of IRF-7 in fibroblasts after virus infection was shown to be responsible for a delayed kinetics of type I IFN production (35, 36, 59–61). In the present study, when analyzing the expression of IRF-7 in BMDMs, we found, to our surprise, that resting BMDMs already expressed significant amounts of IRF-7 protein, a protein that is absent in resting BMDCs. Macrophages from mice lacking *Irf7* showed a

severe decrease in IFN-β production, suggesting that constitutive expression of IRF-7 in macrophages is responsible for the rapid and robust activation of the *Ifnb* promoter. This is consistent with defects in IFN-β production in BMDMs from mice lacking *Ifnar1* or *Stat1*, which lack constitutive expression of IRF-7. Altogether, our studies suggest that the rapid and robust activation of IFN-β production in macrophages is likely determined by a combination of both an already primed *Ifnb* promoter with constitutively bound PU.1 and IRF-8, and the constitutively expressed IRF-7 and IRF-3, which were rapidly activated and recruited to the primed enhancer region of the *Ifnb* promoter in macrophages after LPS stimulation.

Involvement of Both IRF-7 and IRF-3 in the Activation of Type I IFN Induction in Macrophages Suggests the Use of TRIF, Rather Than MyD88, in the TLR4 Signaling Pathway

Our discovery that IRF-7, in concert with IRF-3, regulates LPS-induced type I IFN production in mice and in macrophages, but not in DCs, provides a possible molecular explanation for the preferential usage of the TRIF rather than the MyD88 adaptor, in TLR4-induced type I IFN production. The transcription factors IRF-7 and IRF-3 are key master regulators of type I IFN production during viral infection or after activation by TLR ligands. Activation of these transcription factors in the TLR pathways is primarily mediated by two main adaptors: MyD88 and TRIF. Different adaptors engage different transcription factors that may dictate the kinetics, magnitude, and/or types of type I IFN genes expressed. MyD88 is utilized by all TLRs except TLR3, whereas TRIF is only used by TLR3 and TLR4 (62). TLR4 is the only TLR that can separately trigger pro-inflammatory cytokines and type I IFN responses, which have previously been demonstrated to be mediated by MyD88 and TRIF, respectively. Biochemical co-immunoprecipitation assays and FRET microscopy in live cells have been used to demonstrate that MyD88 directly interacts with IRF-7, but not with IRF-3 (42, 63). This MyD88-IRF-7 pathway was found to operate mainly in pDCs, and is largely responsible for the rapid induction of high levels of type I IFN, following the activation of TLR7/8/9 by nucleic acids during viral infection. The induction of type I IFN by TLR7/8/9 ligation was defective in splenic pDCs prepared from mice lacking *MyD88* or *Irf7*, but not *Irf3*, which was consistent with the ability of MyD88 to physically associate with IRF-7, but not with IRF-3 (24, 42). These studies clearly demonstrate that direct interactions between IRF-7 and MyD88 are essential for TLR7/8/9-induced type I IFN production in pDCs (63, 64).

While MyD88 forms a complex with only IRF-7, the adaptor protein TRIF, in contrast, has been shown to interact with and activate both IRF-7 and IRF-3 *in vitro* (25, 26), suggesting that the transcriptional activation of type I IFN genes, such as *Ifnb*, after TLR4 ligation by LPS may be regulated by both IRF-7 and IRF-3 via TRIF. However, in transient transfection studies, overexpression of IRF-3 alone was sufficient to induce the activation of the *Ifnb* promoter (65). Moreover, BMDCs from mice lacking *Irf7* displayed normal LPS-stimulated IFN-β transcription, whereas $Irf3^{-/-}$ BMDCs lacked LPS-stimulated IFN-β induction (24). Therefore, the general consensus is that the transcription factor IRF-3, rather than IRF-7, is the only mediator of IFN-β expression in the TLR4 pathway. There is also the general assumption that IRF-3 is the only factor that is responsible for the induction of IL-1β production in mice via type I IFN production in the non-canonical NLRP3 inflammasome pathway after *in vivo* Gram-negative bacteria infection. In our present study, we surprisingly found that in addition to IRF-3, the transcription factor IRF-7 is essential for type I IFN induction in mice and in macrophages. Moreover, we also demonstrated that, as with IRF-3, IRF-7 activation and IRF-7-mediated IFN-β production are also dependent on the adaptor TRIF in the TLR4 pathway. Our analysis of IRF-3 single knockout and IRF-7 single knockout BMDMs, together with IRF-3/IRF-7 double knockout and TRIF knockout BMDMs, showed that the absence of either IRF-3 or IRF-7 results in weak LPS-induced IFN-β responses, while the absence of both IRF-3 and IRF-7 phenocopies the complete shutdown of the LPS-induced IFN-β response as observed in TRIF knockout BMDMs. This suggests that both IRF-3 and IRF-7 need to act together downstream of TRIF to induce optimal IFN-β expression in LPS-challenged macrophages. Furthermore, we showed that macrophages, but not DCs, are dependent on both IRF-7 and IRF-3 to activate the *Ifnb* promoter. The requirement for both IRF-7 and IRF-3 in the activation of type I IFN production in macrophages by LPS could be a possible explanation for the preferential use of TRIF, rather than MyD88, in the TLR4 response to LPS, because of the ability of TRIF to interact with and activate both IRF-7 and IRF-3, whereas MyD88 can associate with IRF-7 but not with IRF-3. Thus, to the best of our knowledge, our study is the first to demonstrate that the physical interaction of TRIF with both IRF-7 and IRF-3 is functionally required for robust induction of type I IFN in macrophages. Additionally, mice lacking either *Irf7* or *Irf3* exhibited severely impaired IL-1β production *in vivo* after LPS challenge, indicating that IL-1β production in mice by LPS requires optimal type I IFN production, which is mediated by both IRF-7 and IRF-3 via the TRIF pathway.

TRIF has been shown to form a complex with TBK-1, a protein kinase that has been reported to directly phosphorylate IRF-3 and IRF-7 in response to viral infection or TLR3 and TLR4 stimulation based on *in vitro* kinase assays (43–47). Recent evidence has demonstrated that TRIF-dependent signaling cascades in LPS-stimulated macrophages involve the recruitment and phosphorylation of TBK1 at Ser172, of TRIF at the pLxIS motif, and of IRF-3 at Ser396 at the endosomal compartment (43–46, 48–51). Our finding that IRF-7 is critical for IFN-β induction in LPS-stimulated macrophages prompted us to investigate the contribution of TBK-1 to the activation of IRF-7 and IRF-7-mediated type I IFN production by LPS. Due to the lack of a reliable antibody specific against the endogenous phosphorylated form of IRF-7, we could only assess the involvement of TBK-1 on IRF-7 activity by measuring IFN-β expression in LPS-stimulated *Irf3*-deficient macrophages after treatment with BX-795, which is a specific

inhibitor of TBK-1. The BX-795 inhibition of TBK-1 in macrophages lacking either *Irf3* or *Irf7* can strongly abrogate the remaining type I IFN production in the single knockout BMDMs, suggesting that, similar to IRF-3, the transcription factor IRF-7 is also activated by TBK-1 and mediates type I production through a TRIF-induced TBK-1-dependent pathway in LPS-TLR4 signaling. Thus, our study further strengthened the concept that TRIF, rather than MyD88, is the preferred adaptor to mediate type I IFN induction in the TLR4 pathway in macrophages, because TRIF is endowed with the ability to activate both IRF-7 and IRF-3 via the recruitment of TBK-1, which was previously shown to phosphorylate both IRF-7 and IRF-3.

In summary, we have identified that IRF-7 plays an essential role in the production of type I IFN in the TLR4 signaling pathway. Importantly, we showed that IL-1β and IFN-β production in LPS-challenged mice requires the concerted activation of both IRF-7 and IRF-3 via a TRIF-TBK-1 signaling pathway. Furthermore, we demonstrated that macrophages, but not DCs, are dependent on both IRF-7 and IRF-3 for robust activation of IFN-β production. This suggests that macrophages are an important source of IFN-β that may contribute to the activation of IL-1β production by the non-canonical inflammasome pathway *in vivo* following LPS administration. These novel mechanistic insights into the molecular basis of the divergent roles played by macrophages and DCs in anti-microbial immunity will critically inform future studies of their disparate roles in host protection against bacterial pathogens.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Biological Resource Centre (BRC) Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

W-XS designed and performed experiments, acquired and analyzed data, and wrote the manuscript. JY designed and performed experiments, acquired and analyzed data, and helped in the writing of the manuscript. TL performed experiments and acquired data. I-HS designed experiments and provided materials. JC designed experiments and provided feedback on the study. K-CC contributed to the conception of the study, designed experiments, interpreted the data, and wrote the manuscript.

FUNDING

This work was supported by A*STAR Grant 06-001 to K-CC.

ACKNOWLEDGMENTS

We thank Prof. Chien-Kuo Lee (National Taiwan University College of Medicine, Taiwan, Republic of China) for kindly providing bone marrow cells from STAT3 conditional knockout (MxCre-STAT3f/f) mice and control mice lacking the Mx-Cre transgene (STAT3f/f).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00640/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 23 April 2020
doi: 10.3389/fimmu.2020.00724

TLR4 Cross-Talk With NLRP3 Inflammasome and Complement Signaling Pathways in Alzheimer's Disease

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Amyloid plaques, mainly composed of abnormally aggregated amyloid β-protein (Aβ) in the brain parenchyma, and neurofibrillary tangles (NFTs), consisting of hyperphosphorylated tau protein aggregates in neurons, are two pathological hallmarks of Alzheimer's disease (AD). Aβ fibrils and tau aggregates in the brain are closely associated with neuroinflammation and synapse loss, characterized by activated microglia and dystrophic neurites. Genome-wide genetic association studies revealed important roles of innate immune cells in the pathogenesis of late-onset AD by recognizing a dozen genetic risk loci that modulate innate immune activities. Furthermore, microglia, brain resident innate immune cells, have been increasingly recognized to play key, opposing roles in AD pathogenesis by either eliminating toxic Aβ aggregates and enhancing neuronal plasticity or producing proinflammatory cytokines, reactive oxygen species, and synaptotoxicity. Aggregated Aβ binds to toll-like receptor 4 (TLR4) and activates microglia, resulting in increased phagocytosis and cytokine production. Complement components are associated with amyloid plaques and NFTs. Aggregated Aβ can activate complement, leading to synapse pruning and loss by microglial phagocytosis. Systemic inflammation can activate microglial TLR4, NLRP3 inflammasome, and complement in the brain, leading to neuroinflammation, Aβ accumulation, synapse loss and neurodegeneration. The host immune response has been shown to function through complex crosstalk between the TLR, complement and inflammasome signaling pathways. Accordingly, targeting the molecular mechanisms underlying the TLR-complement-NLRP3 inflammasome signaling pathways can be a preventive and therapeutic approach for AD.

Keywords: TLR4, Alzheimer's disease, inflammasome, complement, amyloid, synapse

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *14 January 2020*
Accepted: *30 March 2020*
Published: *23 April 2020*

Citation:
Yang J, Wise L and Fukuchi K (2020) TLR4 Cross-Talk With NLRP3 Inflammasome and Complement Signaling Pathways in Alzheimer's Disease. Front. Immunol. 11:724. doi: 10.3389/fimmu.2020.00724

INTRODUCTION

Alzheimer's disease (AD) is characterized by two neuropathological hallmarks, extracellular amyloid β (Aβ) deposits in the brain parenchyma (amyloid plaques) and cerebral blood vessels (cerebral amyloid angiopathy, CAA) and abnormal aggregates of hyperphosphorylated tau protein in brain neurons (neurofibrillary tangles, NFTs). Amyloid plaques and NFTs are accompanied with neuroinflammation including activated microglia and increased levels of cytokines (1). Profound loss of neurons and synapses is also found in AD dementia. Except a small subset of

early-onset familial AD cases, the causes for the vast majority of AD cases are unknown and satisfactory therapeutic and preventive measures for AD are unavailable. Therefore, an urgent need exists to identify the molecular mechanisms that increase the risk for the vast majority of AD cases and to develop the preventive and therapeutic measures. Increasing lines of evidence indicate that central and systemic inflammation promotes AD progression and even initiates neurodegeneration (2–7). Indeed, recent genetic studies on late-onset AD have discovered about a dozen risk alleles that modulate innate immune activities and are highly expressed in brain-resident macrophages, microglia, highlighting the importance of immune responses and microglia in the pathogenesis of late-onset AD (8–10). Aging is the largest known risk factor for AD and represents chronic, systemic inflammation (inflamm-aging) (6, 11–13). Additionally, almost all highly ranked, modifiable risk factors for AD such as diabetes, obesity, hyperlipidemia, and hypertension are characterized by chronic, systemic inflammation (14–19). Inflammation caused by certain bacterial and viral infections is a risk factor of dementia (20–23). However, the precise molecular mechanisms by which inflammation increases the risk of AD remain to be elucidated. Here we discuss the impact of three innate immune signaling pathways including TLR4, NLRP3 inflammasome, and complement on the pathogenesis of AD.

TLRS AND ITS SIGNALING PATHWAYS

In responses to a variety of invading pathogens and tissue damages, the innate immune system initiates inflammatory responses through activation of pattern recognition receptors (PRRs) (24). PRRs recognize pathogen-associated molecular patterns (PAMPs), conserved structures commonly identified among different microorganisms, as well as damage-associated molecular patterns (DAMPS), molecules shed by injured cells. Currently identified classes of PRR families comprise the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the nucleotide-binding oligomerization domain (NOD)-Leucine Rich Repeats (LRR)-containing receptors (NLRs), and secreted proteins such as complement proteins (25, 26). TLRs are composed of an extracellular and cytoplasmic domain that belongs to a type I transmembrane receptor and recognize TLR ligands through the extracellular domain. TLR ligands can be either exogenous (PAMPs) or endogenous (DAMPs). At least 10 and 12 functional TLRs have been reported in human and mouse, respectively. The activation of TLRs by TLR ligands initiates both innate and adaptive immune responses (25, 27). TLR ligation initiates a signaling cascade that leads to activation of transcription factors that upregulate a number of target genes encoding cytokines, chemokines, growth factors, and other inflammatory mediators. Activation of TLR by pathogens and injured cells also induces phagocytic activities of macrophages/microglia and clears pathogens, damaged tissues and buildup wastes (28–31). The cytoplasmic domain of TLRs is termed Toll/interleukin-1 (IL-1) receptor (TIR) domain. TLR activation by TLR ligands initiates interaction of TLR's TIR domain with TIR domains of adaptors such as MyD88 and TRIF. Different TLRs utilize distinctive adaptor molecules, resulting in different signaling responses (**Figure 1**). TLR1, TLR2, TLR4, TLR5, and TLR6 are located on the cell surface membrane and recognize mostly bacterial products. TLR3, TLR7, TLR8, and TLR9 sense mostly bacterial and viral nucleic acids and are localized to intracellular vesicles including the endoplasmic reticulum, endosomes, lysosomes, and endolysosomes (32) All TLRs, with the exception of TLR3, use MyD88 as an adaptor. The ligation of TLR2 and TLR4 culminates in activation of transcription factors, NF-κB and AP1, through the MyD88-dependent pathway that is essential for expression of cytokines, chemokines and co-stimulatory molecules, such as TNF-α, IL-1β, IL-6, IL-8, IL-12, and MIP1α. TLR3 and TLR4 ligation can mediate signaling through the MyD88-indepenent (TRIF-dependent) pathway, leading to the activation of interferon regulatory factor 3 (IRF3). The activation of IRF3 induces expression of type I interferon (IFN) genes such as IFNβ and IFN-inducible genes (**Figure 1**). TLR3 and TLR4 ligation can activate NF-κB, also, via the TRIF-dependent pathway, resulting in induction of inflammatory cytokines (**Figure 1**). In TRIF-dependent signal transduction, the TLR4- lipopolysaccharide (LPS) complex on the plasma membrane is internalized to endosomes, where it triggers TRIF-dependent signal transduction (33). Importantly, although robust expression of inflammatory cytokines via MAP kinase and NF-kB activation is achieved by synergistic activation of both TRIF-dependent and MyD88-dependent pathways, TLR4 ligands can produce type I IFN solely through TRIF-dependent pathway activation (27, 34). TLR9 and TLR7 ligation can activate both IRF7 and NF-κB, leading to induction of type I IFNs and inflammatory cytokines, respectively [**Figure 1**; (25, 27)]. TLR signaling produce a number of genes involved in phagocytosis and inflammation through activation of transcription factors such as NF-κB, IRF3 and IRF7 (25, 35, 36).

Neurodegenerative diseases are characterized by progressive loss of specific synapses and neurons as well as abnormally aggregated proteins such as Aβ in AD (amyloid plaques) and α-synuclein in Parkinson's disease (Lewy bodies). Microglia are the principal innate immune cells in the CNS and modulate brain development, homeostasis and neuroinflammation in diseases and aging. Microglia express multiple classes of PRRs including all TLRs and respond to a variety of PAMPs and DAMPs through PRRs (37). DAMPs released from damaged or degenerating neurons and abnormally aggregated Aβ and α-synuclein (38, 39) activate microglia via PRRs, which may modulate progression of neurodegenerative diseases. Since aggregated Aβ has been shown to activate innate immune cells by interacting with several TLRs (see below), it may be possible to reduce Aβ load and neuronal injuries in the AD brain by regulating TLR signaling. However, it remains to be determined which TLR signaling pathways and effectors are involved in modulation of Aβ deposition, clearance and neuronal injuries in the brain.

ROLE OF TLR4 SIGNALING IN ALZHEIMER'S DISEASE BRAIN

Large-scale genome-wide association studies on late-onset AD have discovered a dozen genetic risk alleles that are

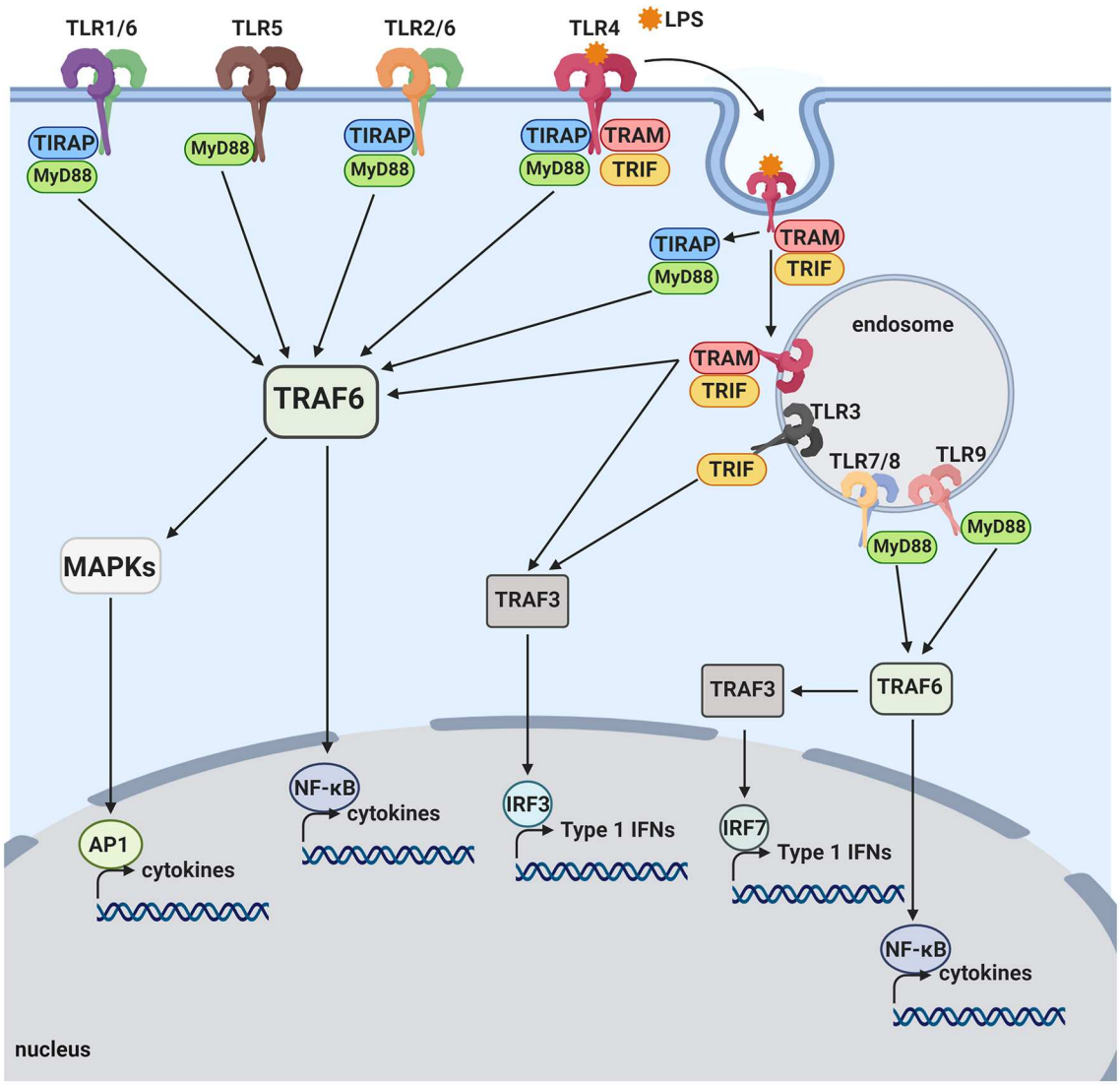

FIGURE 1 | Toll-like receptor pathways. TLR1, TLR2, TLR4, TLR5, and TLR6 are mostly expressed on the cell surface and bind to bacterial products. When activated by LPS, TLR4 is internalized onto an endosome surface. The internalization triggers the release of TIRAP/MyD88, activating the TRAF6 pathway and resulting in activation of transcription factors, NF-$_{\kappa}$B and AP-1. The release of TIRAP/MyD88 from TLR4 allows for the signaling by TRAM/TRIF to commence from the endosome, also activating NF-$_{\kappa}$B as well as the transcription factor, IRF3. TLR3, TLR7, TLR8, and TLR9 are located on internal vesicles and bind to bacterial and viral nucleic acids. TLR7, TLR8, and TLR9 each activate NF-$_{\kappa}$B, as well as the transcription factor, IRF7, through the MyD88 pathway. TLR3 is the only toll-like receptor that does not activate via the MyD88 pathway and instead activates NF-$_{\kappa}$B and IRF3 through the TRIF pathway.

involved in immune/inflammatory responses and highly expressed in microglia, highlighting the importance of microglial inflammatory responses in the pathogenesis of late-onset AD. Such risk loci include APOE, TREM2, CLU, CR1, MS4A6A, MS4A4E, CD33, ABCA7, EPHA1, HLA-DRB5 & DRB1, INPP5D, and MEF2C (8, 9). Their potential roles and functions in TLR4-complement-NLRP3 signaling, are summarized in **Table 1**. Particularly, APOE (43), CD33 (47), INPP5D (57), and TREM2 (66) have been shown to negatively regulate TLR4 signaling. CR1 can inhibit inflammasome activation by suppressing the complement activation pathways (52). However, activation of microglial CR1 induces neurotoxic cytokines and reactive oxygen species (53). Although TREM2 is found to upregulate complement components during aging (69), it can inhibit inflammasome activation (67). CD33 may induce NLRP3 inflammasome assembly (48). APOE (46) and CLU (50) inhibit complement activation and reduce inflammation.

Previously, a coding variant of TLR4 (rs4986790) was reported to increase longevity and reduce an AD risk in Italian cohorts (71, 72). Recently, this observation has been confirmed in independent cohorts (Quebec Founder Population and presymptomatic individuals with a parental history of

TABLE 1 | AD risk genes involved in inflammatory responses.

Genes	TLR4	References	Inflammasome	References	Complement	References	Functions
ABCA7	No		No		No		Involved in lipid homeostasis; enhances Aβ clearance by macrophages (40, 41)
APOE	Yes	(42–44)	Maybe	(45)	Maybe	(46)	Involved in lipid metabolism (42–44)
CD33	Yes	(47)	Maybe	(48)	Maybe	(49)	Inhibitory receptor exclusive to immune cells (47)
CLU	No		No		Yes	(50)	Inhibitor of complement receptors (50)
CR1	Yes	(51)	Yes	(52)	Yes	(53)	Influences complement cascade; binds C1q; inhibits formation of MAC (52, 54)
EPHA1	No		No		No		Promotes permeability of the blood-brain barrier (55, 56)
HLA-DRB1	No		No		No		Creates beta chain 1 of the MHC class II protein complex
HLA-DRB5	No		No		No		Creates beta chain 5 of the MHC class II protein complex
INPP5D	Yes	(57, 58)	No		No		Binds DAP12 which inactivates the TREM2-DAP12 signaling complex (59)
MEF2C	Maybe	(60)	No		No		Regulates apoptosis of T cells and is necessary for transcriptional activation of IL-2 (61, 62)
MS4A cluster (MS4A4E and MS4A6A)	Maybe	(63)	No		Maybe	(63)	Ligand binding promotes calcium conductance; may modulate TREM2 expression (and TLR/complement through TREM2) (63)
TLR4 variant (rs4986790)	Yes		Yes		No		Altered ability to recruit MyD88 and TRIF (64)
TREM2	Yes	(65, 66)	Maybe	(67)	Yes	(68, 69)	Found on myeloid cells and alters inflammatory functions (70)

AD), demonstrating the association of the TLR4 variant with a reduced AD risk, better visuospatial and constructional skills, an increased cortical thickness in visual cortices, and stable IL-1β levels in cerebrospinal fluid (CSF) over time (73). Additionally, certain TLR4 gene variants are associated with an increased risk of AD in the Chinese population (74–76). These associations of TLR4 with AD in different populations indicate an important role of TLR4 in the AD pathogenesis.

Microglia, brain resident phagocytes in the innate immune system, are thought to be macrophages in the central nervous system. Fibrillar Aβ deposits are closely associated with activated microglia in the brain (1). Microglia interact with fibrillary Aβ through their cell surface receptor complexes leading to Aβ phagocytosis and inflammation. Using cultured microglia, the receptor complexes of microglia, which recognize Aβ fibrils, have been shown to contain TLR2, TLR4 and their co-receptor, CD14, as indispensable constituents of the receptor (77–79). Activation of microglia by TLR2, TLR3, TLR4, TLR7, and TLR9 ligands boosts ingestion and/or clearance of Aβ by microglia *in vitro* (78, 80–84). In line with these *in vitro* experiments, an acute (one-time) injection of LPS, a TLR4 ligand, into the brains of AD mouse models activated microglia and decreased Aβ plaques (85–87). Additionally, activation of microglia by intracerebroventricular injection of CpG-oligodeoxynucleotides (ODN), a TLR9 ligand, reduced brain Aβ deposits and ameliorated cognitive deficits in Tg2576 mice (an AD mouse model) (80, 88–91). However, sustained brain injection of LPS induced premature cerebral Aβ deposits and cognitive impairments in AD mouse models (92–94).

APP/PS1 mice (an AD mouse model) homozygous for a loss-of-function mutation (Tlr^{Lps-d}/Tlr^{Lps-d}) of TLR4 had greater cerebral Aβ load and poorer spatial learning than APP/PS1 mice with TLR4 wild-type alleles (81, 95). AD mouse models show increases in brain cytokine levels including TNF-α, IL-1β, IL-17, and IL-10. Such increases in the brain cytokines were abolished in APP/PS1 mice with the TLR4 mutation, indicating TLR4-dependent upregulation of the cytokines in APP/PS1 mice (96). However, TLR4-dependent upregulation of cytokines and microglial activation were not observed in young APP/PS1 mice before Aβ deposition (95, 96). Additionally, TLR2 deficiency in an AD mouse model [APPSwe/PS1(A246E)] increased brain Aβ42 levels (toxic form of Aβ) and accelerated spatial and contextual memory impairments (97). These *in vivo* data suggest that activation of certain TLRs can be therapeutic option for AD. However, APP/PS1 mice defective for CD14 (CD14 gene knockout), a co-receptor for TLR4, showed a decrease in Aβ plaques (98). MyD88 deficiency decreased cerebral Aβ load and improved behavioral deficits in APP/PS1 mice (99). Additionally, transplantation of bone marrow cells with MyD88 deficiency in an AD mouse model ameliorated brain Aβ levels and cognitive deficits much better than MyD88-sufficient bone marrow cells (100). The latter experiments indicate that activation of certain TLRs can be detrimental to the AD progression. These experimental results also indicate that the *in vitro* data can be misleading perhaps due to oversimplification

of the *in vitro* systems as well as difficulties in mimicking chronic activation of TLRs in the *in vitro* systems. Accordingly, *in vivo* experiments in detail in TLR ligand treatment regimen, age, sex and genetic background of experimental animals are indispensable for a better understanding of the roles of the TLR signaling pathways in the AD pathogenesis.

ROLE OF TLR4 SIGNALING IN SYSTEMIC INFLAMMATION IN ALZHEIMER'S DISEASE (AD)

There are increasing lines of evidence that systemic inflammation promotes AD progression and initiates microglial activation and neurodegeneration (2–7). Aging is the largest known risk factor for AD and is characterized by chronic, systemic low-grade inflammation, referred to as "inflamm-aging" (11–13). Additionally, highly ranked, modifiable risk factors for AD such as depression, hypertension, diabetes, obesity, and hyperlipidemia are characterized by a chronic, systemic low-grade inflammation (14–19). For example, visceral adipose tissue of obese subjects contains innate and adaptive immune cells and shows low-grade chronic inflammation, which is identified as a major contributor to the advancement of metabolic diseases including type 2 diabetes mellitus and coronary heart diseases (101, 102). Indeed, when a diabetic AD mouse model was produced by crossing APP23 mice (an AD model) with leptin-deficient (ob/ob) mice, the onset of diabetes exacerbated cognitive deficits, cerebral amyloid angiopathy, and cerebrovascular inflammation (103). A high-fat diet increased insoluble cerebral Aβ and soluble tau in the brains of 3xTg-AD mice (an AD model) (104). Low-grade inflammation plays a pivotal role in the initiation, progression, and propagation of the atherosclerotic process (105, 106). Atherogenic diet exacerbated cognitive deficits and cerebral Aβ deposits in Tg2576 mice (an AD mouse model) and the aortic atherosclerotic lesion area positively correlated with cerebral Aβ deposits (107). Certain peripheral, as well as CSF inflammatory markers, such as IL-6 and C-reactive protein (CRP) have been reported to forecast dementia or decline in cognitive functions many years before their onset (106, 108–113). These AD risk factors have been shown to be associated with altered TLR4 signaling. The TLR4 +896A/G coding variant (rs4986790) is underrepresented in patients with myocardial infarction, Alzheimer's disease or prostate cancer, whereas it is more frequently found in centenarians in Italian and Canadian cohorts (71–73). Their blood samples produce less IL-6, TNF-α, and eicosanoids (PGE2 and LTB4) in response to LPS, compared to other TLR4 genotypes (114). Anti-aging effects of caloric restriction is associated with downregulation of the TLR4/MyD88/NF-κB pathway in rodents (115). Apolipoprotein E (ApoE)-deficient mice are prone to high-fat diet-induced atherosclerosis, which is reduced in additional TLR4-deficiency or MyD88-deficiency, indicating an important role of TLR4/MyD88 signaling in atherosclerosis (116). Activation of TLR4 contributes to insulin resistance by impairing insulin signal transduction via inhibitory phosphorylation on serine residues in insulin receptor substrate (IRS) (117). Therefore, these AD risk factors may contribute to the AD development via TLR4 signaling.

Systemic infections are also associated with AD although not all studies found such associations. Infection of certain bacteria including *Helicobacter pylori*, *Porphyromonas gingivalis*, *Chlamydia pneumonia*, and *Borrelia burgdorferi*, has been found to be risk factors for the development of dementia (20–22, 118, 119) In an AD mouse model (APP/PS1 mice), *Bordetella pertussis* respiratory challenge led to T cell infiltration into the brain and increased microglial activation and Aβ deposition (120). Peripheral injections of TLR ligands such as LPS and poly I:C, TLR4 and TLR3 ligand, into animals and humans have been commonly implemented to mimic bacterial and viral infections, respectively. Repeated peripheral LPS injection in wild type mice led to cognitive deficits and increases in cerebral Aβ levels and apoptotic cells (121, 122). A single intravenous poly I:C injection into 4-month-old 3xTg-AD mice increased cerebral Aβ deposits and altered tau phosphorylation at age 15 months. Additionally, systemic exposure to poly I:C during late gestation in wild type mice increased cerebral APP (Aβ precursor protein) levels, altered tau phosphorylation and cognitive function in old ages and these phenotypic alterations were exacerbated when the prenatal exposure was followed by a second challenge during their adulthood (123). Repeated systemic injection of LPS induced premature cerebral Aβ deposits and cognitive impairments in AD mouse models (92–94). Repeated intraperitoneal injection of LPS activated microglia and increased tau phosphorylation in an AD mouse model (3xTg-AD) (124). Daily intraperitoneal LPS injection in Kunming mice for 7 days induced microglia activation, upregulation of proinflammatory cytokines (both mRNA and protein) including IL-1β, TNF-α, and IL-6, synapse loss, and impairment of learning and memory (125). Acute intraperitoneal LPS injection also increased tau phosphorylation in the hippocampal neurons of C57BL/6 mice (126, 127). Furthermore, periodontitis evoked by inoculation of *Porphyromonas gingivalis* exacerbated brain Aβ deposition and cognitive deficits in an AD mouse model (J20 PDGF-APPSw-Ind mice) (128). Repeated intraperitoneal injection of LPS derived from *Porphyromonas gingivalis* induced cognitive deficits, intraneuronal Aβ accumulation, microglial activation, and increases in IL-1β in middle-aged (12 months) wild-type C57BL/6 mice but not in young (2 months) mice (129). These findings support the hypothesis that systemic inflammation promotes AD progression and even initiates AD-like pathological changes. Indeed, peripheral LPS administration has been widely used to model neuroinflammation and neurodegenerative diseases including AD in rodents and the lists of such experimental models are found in the following review papers (130–133). Importantly, TLR4 in brain-resident immune cells plays a predominant role in sustained neuroinflammation including IL-1β upregulation, which is induced by systemic LPS administration rather than TLR4 in peripheral immune cells (134). However, the precise mechanisms by which systemic inflammation contributes to AD initiation and progression remain to be elucidated.

So far, as we discussed above, almost all chronic, systemic inflammatory events predominantly exert pro-inflammatory

responses in brain microglia, leading to exacerbation of neurodegenerative diseases including AD. Recently, Wendeln et al. (135) reported that one-time peritoneal injection of LPS prior to brain Aβ deposition (at 3 months of age) in an AD mouse model primed microglia and exacerbated the brain Aβ load 6 months later while 4 consecutive peritoneal injections of LPS (0.5 mg/kg) induced tolerance and reduced the Aβ load. Additionally, chronic intraperitoneal administration of CpG-ODN (TLR9 ligand) and monophosphoryl lipid A (MPL, TLR4 ligand) reduced Aβ plaques and NFTs, and restored cognitive deficits in AD mouse models (80, 88–91). However, the precise mechanisms, by which the repeated TLR ligand treatments improve AD-like pathophysiology are unclear. One possible explanation is that the repeated TLR ligand treatments increase stress resistance or adaptation/tolerance of microglia, leading to reduced inflammatory responses of microglia, alleviation of AD-like pathology, and cognitive deficits (136). It is important to understand that systemic inflammatory events as well as peripheral treatment with TLR ligands can shape the phenotype of microglia in the CNS. These results suggest that modulation of brain microglial phenotype by peripheral treatment with certain TLR ligands at appropriate doses and treatment intervals can be therapeutic and/or preventive to AD.

NLRP3 INFLAMMASOME AND AGING

Inflammasomes consist of multimeric protein complexes in the cytoplasm, which mediate activation of IL-1β and IL-18 and induce pyroptosis, a programmed cell death. Inflammasomes are involved in initiation and sustainment of the innate immune response (137). The NLRP3 inflammasome consists of a sensor (NLRP3), and adaptor (ASC or PYARD) and an effector (caspase 1) (138). Activation of the NLRP3 inflammasome and the production of IL-1β are tightly regulated and require two triggering steps, a priming step and an activation step (**Figure 2**). In the priming step, expression of the inflammasome components (NLRP3, caspase 1 and pro-IL-1β) needs to be upregulated to their suitable expression levels for inflammasome activation. This upregulation can be induced by various PAMPs or DAMPs, including LPS or amyloid, respectively, through activation of PRRs and cytokine receptors, including TLRs and IL-1R, respectively (138). In the activation step, NLRP3 can be activated by a large number of stimuli such as endogenous DAMPs, PAMPs, efflux of potassium (K^+) or chloride (Cl^-) ions and flux of calcium ions (Ca^{2+}) (138).

The biggest risk factor for Alzheimer's disease is advanced age (139). Aging is characterized by systemic low-grade inflammation, referred to as "inflamm-aging" (11–13) and senescent cells are characterized by the senescence-associated secretory phenotype (SASP), indicating proinflammatory characteristics including increased secretion of IL-1β, IL-6, IL-8, TGF-β, and TNF-α (140). IL-1β production increases during aging in the mouse brain, which is exacerbated by intraperitoneal injection of LPS (1 mg/kg), (141, 142). IL-1β, IL-6, TGF-β, and TNF-α levels are elevated in AD brain tissue, as well as in AD patients' CSF and serum (143). Fibrillar Aβ induces more IL-1β production in microglia isolated from aged mice than those derived from young mice (144). NLRP3 deficiency ameliorates central and peripheral low-grade inflammation and SASP and improves cognitive function and motor performance in aged mice (141). IL-1R deficiency ($Il1r^{-/-}$) also ameliorates cognitive decline associated with aging in mice (141). Thus, inhibition of NLRP3 inflammasome can be a therapeutic and preventive target for age-related chronic diseases including AD.

ROLE OF NLRP3 INFLAMMASOMES IN ALZHEIMER'S DISEASE BRAIN

Fibrillary Aβ can induce IL-1β release from cultured microglia in an NLRP3-dependent and ASC-dependent manner, where NLRP3 serves as a sensor of aggregated Aβ for inflammasome activation (145). ASC deficiency decreases brain Aβ deposits and improves cognitive deficits in APP/PS1 mice. Injection of ASC specks induces spreading of Aβ deposits in APP/PS1 mice. However, this is not observed in ASC-deficient APP/PS1 mice, and co-administration of anti-ASC antibody blocks the spreading of Aβ pathology. Thus, ASC specks released from pyroptotic microglia induce seeding and spreading of Aβ oligomers and aggregates, leading to AD progression (146). NLRP3 or caspase-1 deficiency in APP/PS1 mice leads to reduced brain caspase-1 and IL-1β activation, increased microglial Aβ phagocytosis, reduced brain Aβ load, and protection of neuronal spine loss, long-term potentiation (LTP) decline, and cognitive deficits (147). However, the reduced Aβ load in NLRP3-deficient APP/PS1 mice is discernible at 16 months of age but not at 4 months of age (147). In patients with early AD or mild cognitive impairment due to AD, levels of IL-1β and caspase-1 activity are significantly increased (147, 148) and ASC-bound Aβ is found in AD patients' brains (146). These observations suggest that NLRP3 inflammasome activation represents an early pathogenic event in AD. Intrastriatal injection of fibrillar Aβ in mice causes microglial activation, which is inhibited in mice with MyD88 deficiency, ASC deficiency, caspase-1 deficiency, or IL-1R deficiency (145), suggesting that aggregated Aβ initiates a signaling cascade involving MyD88, NLRP3 inflammasome, and IL-1β. In line with these observations, MyD88-deficiency decreases microglial activation and cerebral Aβ load and improves behavioral deficits in APP/PS1 mice (99, 149). Moreover, MyD88 deficiency enhances Aβ phagocytosis by microglia/macrophages *in vitro* and bone marrow reconstitution by MyD88-deficient cells reduces Aβ load and improves cognitive functions more efficiently compared with MyD88-sufficient cells in AD mouse models including APP/PS1 and TgCRND8 mice (100). Expression levels of IL-1β mRNA and protein are upregulated in the brains of APP/PS1 mice compared to those in age-matched APP/PS1 mice with a loss-of-function TLR4 mutation at 9–15 months of age but not at 5 months (95, 96). These findings suggest that TLR4/MyD88 signaling is involved in the priming step of NLRP3 inflammasome activation in AD mouse models (**Figure 2**).

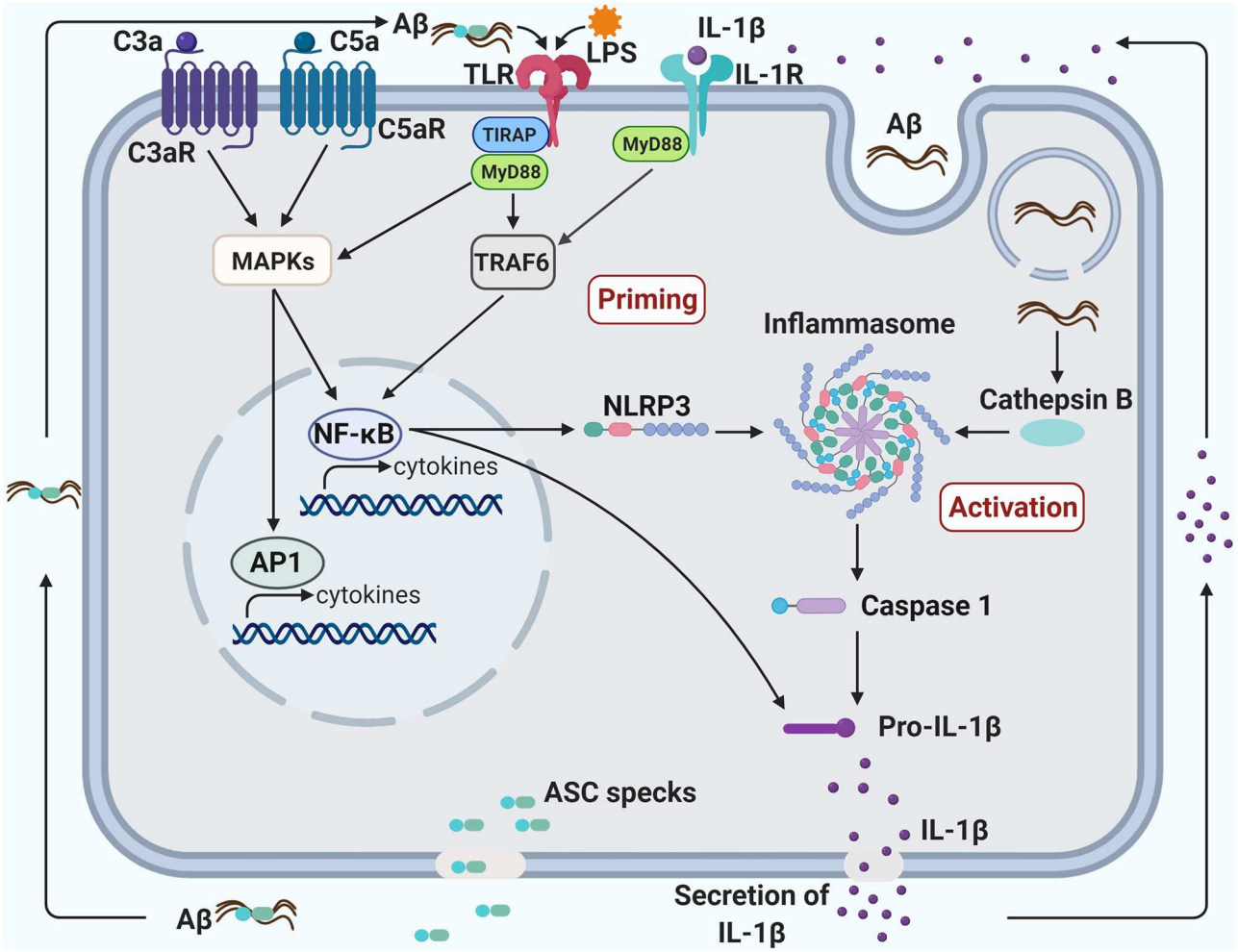

FIGURE 2 | Crosstalk between TLR4, NLRP3 inflammasome, and complement promotes neuroinflammation in Alzheimer's disease. Priming of the inflammasome occurs when the transcription factor, NF-$_{\kappa}$B, is activated, triggering the production of both NLRP3 and Pro-IL-1β. NF-$_{\kappa}$B can be activated via the TLR/IL-1R MyD88-dependent pathway and the C3/C5 MAPK pathway. The TLR pathway can be induced by a bacterial component, such as LPS, and the MAPK pathway can be induced by C3a/C5a binding to their respective receptors. The activation of NF-$_{\kappa}$B through complement, TLR and IL-1R pathways may create a synergistic increase in pro-inflammatory factors. The inflammasome can be activated in several ways, including an increase of endogenous damage-associated and pathogen-associated molecular patterns or an efflux of potassium or chloride ions. Additionally, aggregated fibrillary Aβ engulfed by the microglia can damage the lysosome and leak into the cytoplasm, also contributing to the activation of the inflammasome. Activation of the inflammasome can induce pyroptosis, leading to the secretion of IL-1β and ASC specks. ASC specks bind to Aβ and seed the surrounding parenchyma leading to further Aβ aggregation. Aggregated Aβ can also bind to TLR and induce activation of the MyD88 pathway. Likewise, IL-1β secreted from the pyroptotic microglia can bind to IL-1R and induce activation of the MyD88 pathway. The induction of the MyD88 pathway through the by-products of microglial pyroptosis may lead to a vicious cycle of inflammasome priming, inflammasome activation and pyropotosis that will exacerbate Aβ pathology.

In addition to a crucial role of the NLRP3 inflammasome in Aβ pathophysiology in AD, tau pathology is influenced by NLRP3 activation (150). NLRP3 or ASC deficiency decreases tau hyperphosphorylation and aggregation by regulating tau kinases (GSK-3β and CaMKII-α) and phosphatases (PP2A) in Tau22 mice that express tau mutations found in frontotemporal dementia. Intracerebral injection of fibrillar Aβ-containing brain homogenates enhances tau phosphorylation and aggregation in Tau22 mice, which is blocked by NLRP3 or ASC deficiency (150), suggesting that Aβ-induced NLRP3 inflammasome activation exacerbates tau pathology in AD and its animal models.

ROLE OF NLRP3 INFLAMMASOMES IN SYSTEMIC INFLAMMATION IN ALZHEIMER'S DISEASE

LPS is a potent TLR4 ligand and its systemic administration is widely used to model systemic inflammation. A list of animal models summarizing the effects of LPS treatment on NLRP3 inflammasome activation is found in an excellent review article by Heneka et al. (151). Several papers have reported microglial NLRP3 inflammasome activation after peripheral LPS injection. Single intraperitoneal injection of LPS (5 mg/kg) in

C57BL/6 (B6) mice induced microglial activation, upregulation of NLRP3, ASC, caspase-1p10, and IL-1β in the hippocampus, leading to behavioral alterations (depression like behavior and memory deficits) for 29 days after LPS injection, which were inhibited by a NLRP3 inhibitor (152). Intraperitoneal injection of LPS (3.5 mg/kg) in B6 mice induced activation of microglia and NLRP3 inflammasome, and increased IL-1β expression in CNS, which were exacerbated by microglia-specific A20 (NF-κB inhibitor) deficiency but not by deficiency in other cell types (neuron, astrocyte, and oligodendrocytes) (153). Intraperitoneal injection of LPS (0.5 mg/kg) in B6 mice induced activation of microglia, increases in NLRP3, ASC, caspase-1 and IL-1β in the hippocampus, and depressive behavior (154) and such effects by LPS (1 mg/kg) were inhibited in NLRP3-deficient mice (155). Intraperitoneal injection of LPS (1 mg/kg) in APP/PS1 mice at 15 months of age induced decreases in Aβ uptake by microglia, increases in the number and size of Aβ deposits and in peripheral myeloid cells that infiltrated into the brain but not at 5 months of age (156). Such changes by intraperitoneal LPS injection were blocked by NLRP3 deficiency. These results suggest that systemic LPS administration induces microglial NLRP3 inflammasome activation, increased brain Aβ load and brain infiltration of peripheral myeloid cells in an age dependent manner, leading to exacerbation of AD pathophysiology.

TLR/IL-1R/MYD88 SIGNALING IN SUSTAINED VICIOUS CIRCLE OF NLRP3 INFLAMMASOME ACTIVATION IN ALZHEIMER'S DISEASE

LPS is often used to prime NLRP3 inflammasome (157). LPS can induce canonical and non-canonical NLRP3 inflammasome activation (138). In canonical inflammasome priming, activated TLR4 by LPS signals through the adaptor protein, MyD88, culminating in activation of transcription factor, nuclear-factor-kB (NF-κB), that elevates pro-IL-1β and NLRP3 expression (158, 159). Toll-like receptors (TLRs) including TLR2, TLR4, TLR6, and their co-receptor, CD14, are indispensable constituents of the receptor complexes for microglial activation by Aβ, leading to cytokine and chemokine production (78, 79, 95). Extracellular fibrillary Aβ can prime the canonical inflammasome pathway by activating the TLR/MyD88/NF-κB signaling pathway [**Figure 2**; (160, 161)]. In the activation step, phagocytosed Aβ in microglia leads to lysosomal damage and liberation of cathepsin B and/or production of mitochondrial reactive oxygen species, which trigger formation of the NLRP3 inflammasome complex, causing caspase 1 activation, IL-1β production and pyroptosis (145, 162). Oligomeric and fibrillar Aβ can directly interact with NLRP3 and ASC, resulting in NLRP3 inflammasome activation, also (163). ASC specks released by microglial pyroptosis quickly bind to extracellular Aβ and induce seeding and spreading of Aβ oligomers and aggregates (146). Aggregated Aβ further promotes microglial inflammasome priming via TLR/MyD88 signaling. Additionally, secreted IL-1β also induces microglial inflammasome priming via IL-1R/MyD88 signaling (164). Thus, this vicious circle of NLRP3 inflammasome activation by TLR/IL-1R/MyD88 signaling may lead to chronic/sustained inflammation and neurodegeneration in AD (**Figure 2**).

COMPLEMENT IN AGING BRAIN

Complements belong to the pattern recognition receptors in the innate immune system and involved in recognition and clearance of pathogens, damaged tissues, aggregated proteins, and toxic wastes (165, 166). Additionally, complement proteins have been implicated in diverse processes during brain development, aging and neurological diseases (26). Virtually all complement components are locally expressed in the brain and microglia express almost all classical complement components and their receptors including C1qR, CR3, C3aR, and C5aR (167, 168). Particularly, complement and microglia play an important role in synaptic pruning, that is, complement-tagged synapse elimination by microglia, during neural development, aging, and neurodegenerative diseases (169). In the normally developing brain, opsonization of synapses by complement factors (tagged by C1q, C3b, and C4) triggers microglial phagocytosis, resulting in elimination of the tagged synapses.

During normal brain aging in human and mouse, C1q protein levels dramatically increase in certain regions of the brain, including the hippocampus, substantia nigra, and piriform cortex. Aged mice with C1q deficiency exhibit significantly less cognitive and memory decline compared with wild-type mice (170). Marked increases in C1q levels are found in dendritic spines at synapses in the aged rhesus macaque dorsolateral prefrontal cortex as well as glia ensheathed synapses, suggesting C1q-tagged synapse elimination by glial phagocytosis as a possible mechanism for age-related degeneration (171). C57BL/6J (B6) mice (at 16 months of age) show age-dependent neuron loss in hippocampal CA3 but not in CA1, which is not observed complement C3-deficient B6 mice. Additionally, aged C3-deficient B6 mice show better cognition and LTP than wild-type B6 mice, implying that C3 is also involved in age-dependent synapse loss and cognitive decline (172).

ROLE OF COMPLEMENT IN ALZHEIMER'S DISEASE BRAIN

In AD, the degree of region-specific synapse loss better correlates with cognitive decline than amyloid plaques, NFTs and neuron loss (173, 174) and genetic variants of complement receptor 1 (CR1) and clusterin (CLU, apolipoprotein J), which are parts of the complement system, are identified as AD risk factors by genome wide association studies (175). Certain components of complements including C1q, C3, C4, and C5b-C9 (membrane attack complex, MAC) accumulate in amyloid plaques and NFT in the brains of AD patients (176–179). A positive correlation is found between expression levels of C3 and C3a receptor (C3aR1) in the brain and cognitive decline and Braak staging in AD patients (180). Additionally, CD57 that prevents MAC assembly is decreased in AD brain (181). CSF and plasma levels of certain complement proteins

have been reported as promising biomarkers for AD diagnosis and progression (182–186). These observations suggest that activation of the complement system may contribute to the AD pathogenesis.

C1q deficiency decreases plaque-associated glial activation and mitigates progressive decreases in synaptic markers in Tg2576 mice without changes in brain Aβ load (187). In J20 mice (an AD mouse model), upregulation and deposition of C1q onto synapses precedes synaptic loss in the hippocampus before overt amyloid plaque formation (188). The toxic effects of Aβ oligomers on synapse loss and LTP inhibition are blocked by C1q deficiency or its inhibitor in mice (188). C1q tags tau-affected synapses and microglia eliminate C1q-tagged synapses by engulfment in PS19 mice (a frontotemporal dementia model). This process is inhibited by C1q-blocking antibodies (189). LPS and Aβ increases production of C3 in primary microglial cultures in a dose dependent manner (190). Aβ oligomer-induced synaptic engulfment by microglia is inhibited by CR3 deficiency in adult mice and inhibition of C3 or microglial CR3 decreases Aβ oligomer-induced synapse loss (188). C3 deficiency ameliorates age-dependent loss of synapses and neurons, and cognitive deficits in aged APP/PS1 mice although it increases cerebral Aβ deposits (191). C3 deficiency mitigates amyloid plaque-associated synapse loss in another AD model mice, PS2APP, and rescues neuron loss and LTP deficits in PS19 mice (192). Similarly, C3aR1 deficiency mitigates tau pathology, neuroinflammation, synaptic deficits and neurodegeneration in PS19 mice (180). Activation of microglia by LPS or Aβ increases sialidase activity and desialylation of the microglial surface, leading to stimulation of CR3-mediated phagocytosis of neurons by microglia in primary glial-neuronal co-cultures. This neuronal loss by microglial phagocytosis is inhibited by a blocking antibody against CD11b (a component of CR3) and a sialidase inhibitor (193). Oral administration of a C5a receptor antagonist (PMX205) decreases Aβ deposition and glial activation in Tg2576 and 3xTg mice, improves cognitive deficits in Tg2576 mice and reduces tau hyperphosphorylation in 3xTg mice (194). These observations support the hypotheses that complement activation exacerbates the AD progression and that the complement signaling pathway that regulates pruning of excess synapses by microglia during brain development is inadequately initiated and mediates synapse loss and neurodegeneration in AD.

In contrast with these hypotheses, the other investigators found beneficial effects of complement activation. C1q has been reported to have a protective effect against neurotoxic Aβ fibrils and oligomers by activating cAMP-response element-binding protein and AP-1, resulting in upregulation of LRP1B and G protein-coupled receptor 6(GPR6), in cultured neurons as well as 3xTg mice (195). Additionally, genetic deficiency of C3 increases Aβ deposition and induces neurodegeneration and alternative activation (M2) of microglia in aged J20 mice (17 months) (196). Inhibition of C3 by overexpressing soluble complement receptor related protein y (sCrry) increases Aβ deposition and neurodegeneration in J20 mice (197). These findings support the notion that activation of these complement components is neuroprotective.

ROLE OF COMPLEMENT IN SYSTEMIC INFLAMMATION IN NEURODEGENERATION

Intraperitoneal administration of LPS (10 mg/kg) for 7 days induces marked upregulation of C1q and C3 by activating the classical complement pathway, microglial activation, synapse loss in the hippocampus, and cognitive deficits in Kunming mice (125). Repeated intraperitoneal administration of LPS (1 mg/kg/day for 4 consecutive days) induces dopaminergic neuron loss in the substantia nigra in mice but a single LPS injection does not. This loss of dopaminergic neurons is prevented in C3-deficient mice and associated with increased expression of genes involved in the classical and alternative complement (Itgam of CR3, C4, C3, and HF1) and phagosome (Fcer2b, Fcgr3, Fcgr4, Tyrobp, and Fcer1 g) pathways in the brain, suggesting that repeated peripheral LPS administration induces complement-mediated elimination of dopaminergic neurons by microglial phagocytosis (198). Intraperitoneal injection of LPS (5 mg/kg) activates microglia and activated microglia induce A1 astrocytes by releasing TNFα, IL-1α, and C1q in B6 mice. A1 astrocytes can drive neurodegeneration by releasing a neurotoxin and multiple complement components including C1q and C3, leading to microglial CR3-mediated synapse pruning and loss (199). A1 astrocytes are abundantly observed in diverse neurodegenerative diseases including AD (199). These findings indicate that systemic inflammation can activate brain complement and microglia, leading to loss of synapses and neurons, cognitive deficits, and neurodegeneration.

POTENTIAL COMPLEMENT AND TLR CROSSTALK IN NEUROINFLAMMATION AND ALZHEIMER'S DISEASE

As parts of the host defense innate immune system, TLRs and complements engage in synergistic or antagonistic signaling crosstalk to orchestrate immune responses. Indeed, most pathogens activate both TLRs and complements. TLR4 activation upregulates expression of complement components, potentially leading to complement activation (200, 201). In responses to TLR ligands including LPS (TLR4), zymosan (TLR2/6), and CpG-ODN (TLR9), mice deficient in a major membrane complement inhibitor, decay-accelerating factor (DAF), show striking elevation of plasma IL-1β, IL-6, and TNF-α in a complement-dependent manner. This synergistic effect of complement on the cytokine production by TLRs in peripheral tissues has been attributed to activation of NF-κB and mitogen-activated protein kinases (ERK1/2 and c-Jun N-terminal kinase) through the C5a-C5aR1 and C3a-C3aR signal pathways in mice [**Figure 2**; (200)]. Indeed, co-stimulation of human monocytes (THP-1 cell line) with aggregated Aβ and C5a markedly enhances secretion of IL-1β and IL-6 through NF-κB activation *in vitro* (202). Therefore, it is possible that activation of C5aR and C3aR signaling by C5a and C3a, respectively, synergistically enhances proinflammatory responses initiated by aggregated Aβ-induced TLR4 activation in the

brain, leading to AD initiation and progression. Additionally, the formation of the complement membrane attack complex (MAC) triggers increased cytosolic Ca^{2+} concentration, resulting in mitochondrial dysfunction and NLRP3 activation that causes caspase 1 activation and IL-1β secretion *in vitro* (203), which may further promote a pathogenic cycle of the TLR4-complement-NLRP3 inflammasome interactions in AD.

In human monocytes, C5aR activation by C5a enhances LPS/TLR4-induced expression of IL-6 and TNF-α production while, in macrophages, C5a increases IL-10 secretion and inhibits LPS/TLR4-induced upregulation of IL-6 and TNF-α via C5aR/MEK/ERK signaling (204). This distinct regulation of LPS/TLR4 signaling by C5a in different cell types supports the concept that monocytes in circulation act as danger sensor and heighten inflammatory responses to PAMPs and DAMPs, while tissue macrophages restrain excess inflammation for host protection/tissue repair (204). Therefore, it is also possible that, in homeostatic/resting microglia, C5a and/or C3a synergistically enhance pro-inflammatory responses triggered by Aβ-TLR4 activation for removal of toxic Aβ aggregates while, in activated microglia, C5a and/or C3a antagonizes Aβ-TLR4-induced pro-inflammatory responses for neuroprotection. This host defense function of complement appears to be altered to host-offensive actions during aging (205). This detrimental alteration of complement-TLR signaling during aging may be exacerbated in AD.

CONCLUDING REMARKS

TLRs function as a host defense mechanism against pathogens and tissue damages. In peripheral tissues, complement and NLRP3 inflammasome modulate immune and inflammatory responses initiated by TLRs through crosstalk between their signaling pathways. TLR4 primes NLRP3 inflammasome in the peripheral tissues as well as in the central nervous system (CNS). As Aβ forms aggregates, a vicious cycle of Aβ-TLR4-NLRP3 inflammasome-IL-1β in microglia sustains neuroinflammation in AD. Systemic inflammation can exacerbate neuroinflammation and neurodegeneration in AD via TLR4 and complement activation. In the peripheral tissues, the crosstalk between TLR and complement is complex and contextual depending on cell type, tissue, species and disease models and complement seems to function as a molecular switch of TLR signaling (pro- or anti-inflammatory) and as a coordinator between innate and adaptive immune responses. However, such regulatory functions of complement have not been investigated in the CNS or brain-resident immune cells including microglia. One of the obstacles that hamper the investigation is that available microglial cell lines and primary microglia derived from the brain have characteristics different from brain resident microglia because microglia are sensitive to environmental changes. Such obstacles may be circumvented by use of new technologies such as the RiboTag and BacTRAP (Translating Ribosome Affinity Purification) methods (206, 207), single-nuclei or single cell RNAseq, genome editing tools, and iPSC-derived 3D co-culture brain models (208). Repeated failures of Aβ-targeted therapeutics indicate the need for a new approach for AD therapy and prevention based on disease mechanisms alternative to the amyloid cascade hypothesis. Inflammation and immune cells play a central role in the initiation and progression of AD. It is crucial to elucidate the molecular mechanisms by which inflammatory responses and immune cells drive the AD initiation and progression.

AUTHOR CONTRIBUTIONS

All authors are involved in writing and editing this manuscript and in intellectual contributions.

FUNDING

This work was supported in part by National Institutes of Health Grants including AG064811, AG062179, AG053719, AG056862, and AG054937.

ACKNOWLEDGMENTS

We thank Ms. Erika Sung for assistance in preparation of this manuscript. The figures in this review were created with BioRender.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 19 May 2020
doi: 10.3389/fimmu.2020.00807

Insight Into TLR4-Mediated Immunomodulation in Normal Pregnancy and Related Disorders

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Unlike organ transplants where an immunosuppressive environment is required, a successful pregnancy involves an extremely robust, dynamic, and responsive maternal immune system to maintain the development of the fetus. A specific set of hormones and cytokines are associated with a particular stage of pregnancy. Any disturbance that alters this fine balance could compromise the development and function of the placenta. Although there are numerous underlying causes of pregnancy-related complications, untimely activation of Toll-like receptors (TLR), primarily TLR4, by intrauterine microbes poses the greatest risk. TLR4 is an important Pattern Recognition Receptor (PRR), which activates both innate and adaptive immune cells. TLR4 activation by LPS or DAMPs leads to the production of pro-inflammatory cytokines via the MyD88 dependent or independent pathway. Immune cells modulate the materno–fetal interface by TLR4-mediated cytokine production, which changes at different stages of pregnancy. In most pregnancy disorders, such as PTB, PE, or placental malaria, the TLR4 expression is upregulated in immune cells or in maternal derived cells, leading to the aberrant production of pro-inflammatory cytokines at the materno–fetal interface. Lack of functional TLR4 in mice has reduced the pro-inflammatory responses, leading to an improved pregnancy, which further strengthens the fact that abnormal TLR4 activation creates a hostile environment for the developing fetus. A recent study proposed that endothelial and perivascular stromal cells should interact with each other in order to maintain a homeostatic balance during TLR4-mediated inflammation. It has been reported that depleting immune cells or supplying anti-inflammatory cytokines can prevent PTB, PE, or fetal death. Blocking TLR4 signaling or its downstream molecule by inhibitors or antagonists has proven to improve pregnancy-related complications to some extent in clinical and animal models. To date, there has been a lack of knowledge regarding whether TLR4 accessories such as CD14 and MD-2 are important in pregnancy and whether these accessory molecules could be promising drug targets for combinatorial treatment of various pregnancy disorders. This review mainly focuses on the activation of TLR4 during pregnancy, its immunomodulatory functions, and the upcoming advancement in this field regarding the improvement of pregnancy-related issues by various therapeutic approaches.

Keywords: TLR4, preterm birth, pregnancy, pro-inflammatory, innate immunity

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *15 January 2020*
Accepted: *08 April 2020*
Published: *19 May 2020*

Citation:
Firmal P, Shah VK and Chattopadhyay S (2020) Insight Into TLR4-Mediated Immunomodulation in Normal Pregnancy and Related Disorders. Front. Immunol. 11:807. doi: 10.3389/fimmu.2020.00807

INTRODUCTION

Pregnancy is an immunologically unique state owing to the fact that it requires the maternal immune system to be highly active so as to fight the upcoming intrauterine microbial challenges, but it is also simultaneously required to be immunosuppressed to maintain the semi-allogenic fetal development (1–3). A fine interplay between both phases ensures a healthy pregnancy. There are numerous reports that have suggested that any dysregulation in the immune status at the materno–fetal interface due to infections are the main cause of preterm delivery, preeclampsia, gestational diabetes, miscarriage, placental malaria, and other pregnancy-related disorders (4–7). There are multiple routes through which the infections can gain access to the placenta, maternal endometrium, and amniotic fluid; ascending through the genital tract and colonizing uterine cavity is the most preferred of all (8). Many of these microbial components act as a ligand for the pattern recognition receptors (PRRs). Pattern recognition receptors are an important element of the innate immune system since they act as a first line of defense against invading pathogens. Recognition of microorganism-originated pathogen-associated molecular patterns (PAMPs) or host-derived damage-associated molecular patterns (DAMPs) relays the signaling cascade, leading to an increase in the expression of cytokines, chemokines, and interferons (7, 9). The Toll-like receptor (TLR) family is one of the important subgroups of PRRs, and it acts as a bridge between innate and adaptive immunity. Expression of TLRs is not restricted to immune cells, but they are also present on variety of cell types, including fibroblasts, endothelial cells, and epithelial cells, and also on placental tissue (10, 11). Each TLR recognizes a specific microbial product and activates a defined signaling pathway leading to distinct immunological response. There are numerous studies that have reported that administration of a TLR4-specific ligand, lipopolysaccharide (LPS), stimulated the generation of pro-inflammatory cytokines and prostaglandins in gestational tissues that leads to preterm labor (12–14). This review emphasizes the role of TLR4 signaling in normal pregnancy and its dysregulation leading to adverse outcomes. We will also summarize promising therapeutic strategies that focus on targeting the TLR4 signaling pathway for the management of pregnancy-related disorders.

TOLL-LIKE RECEPTORS

The *Toll* gene was first discovered in Drosophila, where it plays a critical role in defining the dorso–ventral axis during embryonic development (15). A few key findings revealed that the Toll protein is involved in imparting an immune response against fungi and bacteria in adult fly (16, 17). Later, receptors similar to Toll were identified in humans, and the first one was mapped on chromosome 4 (18, 19). During that time, TLRs were believed to be important in the development process. Subsequently, however, human homologs of Drosophila Toll, TLRs, were also reported to be involved in activating innate and adaptive immune responses in vertebrates. There are a total of 10 homologs of TLR (TLR1-TLR10) that are known to be expressed by humans and that can specifically detect different surface and intracellular pathogen products.

Toll-like receptors (TLRs) comprise of an extracellular domain, including leucine-rich repeats and a Toll/interleukin-1 receptor (TIR) domain at the cytoplasmic end. Following ligand recognition, TLRs relay the signaling either via the intracellular signaling adapter protein, the myeloid differentiation factor 88 (MyD88)-dependent pathway, or the MyD88-independent pathway, which is also known as the TLR-mediated TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway. The MyD88-dependent pathway leads to the activation of early phase nuclear factor-κB (NF-κB), resulting in the production of pro-inflammatory cytokines, including IL-1β, IL-6, IL-12, and TNF-α. The TRIF-dependent pathway generates Type I IFNs (IFNα /β) through interferon regulatory factor (IRF-3) and via activation of late-phase NF-κB (20, 21).

Proper release of these cytokines by the activated leukocytes or uterine epithelial cells plays a key role in attaining a successful pregnancy by facilitating the fetus implantation. But there is increasing evidence to suggest that uncontrolled activation of TLRs—either on leukocytes or uterine epithelial and stromal cells, specifically TLR4—at the materno-uterine junction is associated with pregnancy-related problems (22–25).

Extracellular Receptor Complex

TLR4 in itself is unable to recognize LPS, and it therefore requires numerous other proteins for ligand recognition. The LPS-binding protein (LBP) is one such soluble plasma protein that first interacts with LPS and then transfers it to a cluster of differentiation 14 (either sCD14 or membrane bound). CD14 is a GPI-linked protein that is also one of the PRRs that can bind to the LPS-LBP complex; finally, it also chaperones the LPS molecule to MD-2/TLR4 signaling complex. Myeloid differentiation 2 (MD-2) is an adapter protein that directly recognizes and binds to the conserved lipid A moiety of LPS (26, 27). The intracellular signaling is triggered only when MD-2 interacts non-covalently on the extracellular domain of TLR4 to forms a heterodimeric complex $(LPS.MD\text{-}2.TLR4)_2$ (28).

TLR4 Signal Transduction

TLR4, the first identified human Toll-like receptor, is the only TLR that can signal via an MyD88-dependent as well as an MyD88-independent manner. It acts as a specific receptor for gram-negative bacterial lipopolysaccharide (LPS) and can also bind DAMPs, such as hyaluronic acid and β-defensin 2, fibrinogen, and heat shock proteins hsp60 and hsp70 (29, 30). The binding of the ligand to the receptor triggers the intracellular signaling pathway. Each TLR shares a similar cytoplasmic signaling domain, which is similar to the IL-1 receptor, the TIR domain. Numerous adaptor molecules that have a TIR domain, such as MyD88, TRIF, TIR domain-containing adaptor protein/MyD88 adapter-like protein (TIRAP/Mal), and TRIF-related adaptor molecule (TRAM), interact with the TIR domain of TLR4 and thus relay the downstream signal. Among all the TLRs, TLR3 is the only one that does not signal via the MyD88-dependent pathway. Furthermore, only TLR4 utilizes all of the

four adaptor molecules, namely, MyD88, TIRAP, TRIF, and TRAM, for signal transduction (9, 31) (**Figure 1**).

There are numerous reports that emphasizes the role of immune activation in the intestinal and respiratory tract, and a wealth of knowledge is currently focused on uterine epithelial cells of the female reproductive tract (FRT) being an essential immunological site (32–36). Several studies have shown that TLRs are expressed all through the pregnancy at different

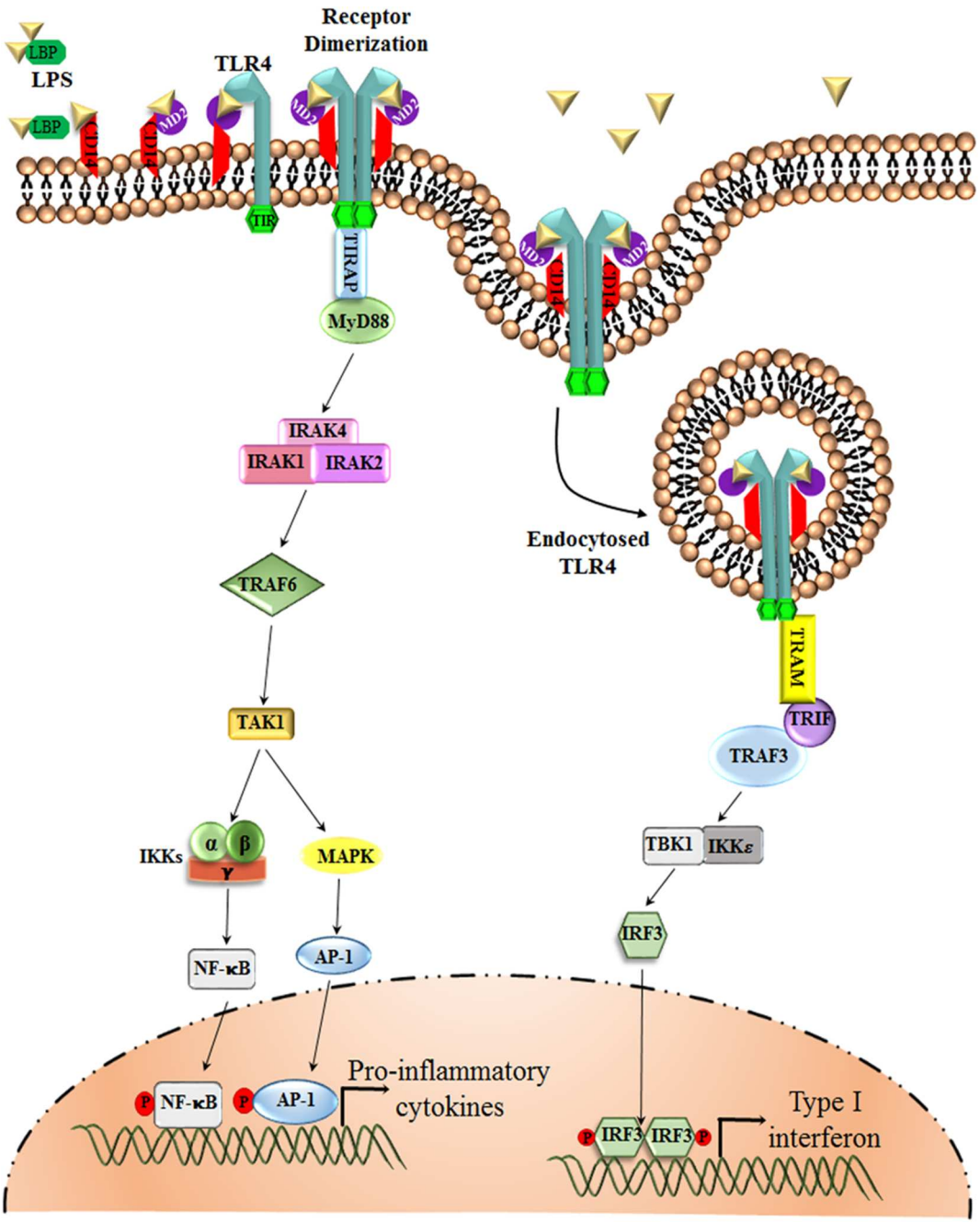

FIGURE 1 | TLR4 Signaling pathway. The LPS Binding Protein (LBP) binds to LPS and transfers it to CD14 or MD-2, which are the accessory proteins involved in the ligand recognition, dimerization, and endocytosis of TLR4. TLR4, upon dimerization, can signal via two separate pathways, the MyD88-dependent and the MyD88-independent pathway. The MyD88-dependent pathway involves the activation of IRAKs and TRAF6, which results in the phosphorylation of transcription factors, such as NF-κB and AP-1. These transcription factors upon phosphorylation translocate to the nucleus and are involved in triggering the transcription of pro-inflammatory cytokine genes. The MyD88-independent pathway, or the TRIF-dependent pathway, however, involves TRAF3 for the activation of transcription factor IRF-3, which favors the production of Type I interferons, such as IFN α, β.

locations in the FRT (37, 38). Expression of PRRs on the epithelial and stromal cells in the uterus helps in the recognition and timely response toward vaginal infections. Conversely, the uncontrolled activation of innate immune system may also result in poor pregnancy outcomes.

MyD88-Dependent Signaling

After the dimerization of TLR4 on ligand binding, MyD88 is recruited, and it interacts via its TIR domain to the cytoplasmic region of TLR4 through a homophilic interaction. Several other accessory molecules are also employed, including various IL-1 receptor-associated kinases (IRAKs), TRAFs, and mitogen-activated protein kinases (MAPKs). Next, NF-κB is activated and translocated to the nucleus via initiating the degradation of its inhibitory protein Iκ-Bα by inhibitory kappa B kinase (IKK). Activating protein-1 (AP-1) is one of the transcription factors that is activated by MAPKs (31). This pathway ultimately leads to the production of several pro-inflammatory cytokines and chemokines.

MyD88-Independent Signaling

TLR4/TRIF dependent signaling is only initiated after the receptor complex is internalized into the endosomes. Only TLR3 and TLR4 utilizes this pathway, involving the participation of TRIF and IRF-3 and resulting in the production of type I interferons (IFN) along with pro-inflammatory cytokines. They have the capability to stimulate IFN-β and Interferon-inducible genes in *MYD88* null cells owing to the fact that both the pathways need different accessory proteins to function (9). IRF-3 and IRF-7, upon phosphorylation, dimerize and translocate to the nucleus where they bind to the Interferon-Stimulated Response Elements (ISREs), giving rise to the expression of interferon-inducible genes. IRF-3 and IRF-7 are crucial among the IRF family, as Type I interferon production is severely hampered in *IRF-7* null mice and was completely abolished in *IRF-3* and *IRF-7* null cells (39). TLR4-induced type I IFN induction was highly compromised in *IRF-3* null mice emphasizing the importance of IRF-3 and IRF-7 in TLR signaling pathway (40). Interestingly, there are reports that have highlighted that CD14 plays a major role in supporting the internalization of $(LPS.MD\text{-}2.TLR4)_2$ receptor into the endosomes (41).

TLR4 Expression and Signaling at the Materno–Fetal Interface

Histological and functional changes of different parts of the female reproductive tract involving the perimetrium, myometrium, endometrium, cervix, and vagina take place throughout normal pregnancy. Several pregnancy-related tissues are also formed, including the amnion, chorion, and placenta, to support the development of the fetus. Any dysregulation in the usual scenario results in adverse pregnancy outcomes. Hence, in the current review, we have focused on the investigations that have been carried out to look into the function and expression profile of TLR4 during the course of pregnancy, exploring specific materno–fetal tissues of the female reproductive tract that have a close relationship with the developing embryo (**Figure 2**).

Placenta

The developing embryo is protected from the surrounding environment effectively by the placenta. Numerous PRRs are contemplated to take part in this interface, including Nod-like receptors (NLRs) and TLRs (42). All TLRs are found to be present in the normal-term placental tissue at the mRNA level, but only TLR2 and TLR4 are completely characterized at the protein level. The expression of these receptors is not continuous throughout the pregnancy but follows a definitive trend. In the first trimester placental tissues, trophoblast cells exhibit enhanced expression of TLR2 and TLR4. The villous cytotrophoblast along with extravillous trophoblast expresses TLR4 in first trimester trophoblast. The outer syncytiotrophoblast cells that directly interact with the maternal blood are found to lack TLR4 expression (43). Therefore, a pathogen can get access to the placenta by crossing the syncytiotrophoblast cell layer that is lacking in TLR4 and pose a threat to the inner placental compartments. The entrance of a pathogen into the trophoblast cell expressing TLR4, however, results in excessive chemokine secretion, which leads to enhanced chemotaxis of a monocyte and neutrophil to the site of infection (44).

The differential expression of TLRs persists till the end of the second trimester. There are various reports that suggest that TLR4 is expressed during the second and third trimester in human placentas obtained from normal and preterm pregnancies. The expression of TLR4 has also been found in the syncytiotrophoblast layer by the third trimester. These studies have signified that placental cells can effectively counter the intrauterine infections (45, 46). A recent study has now focused on the temporal changes of TLRs expression taking place throughout gestation, which can help in devising an effective clinical diagnostic marker by observing the TLR pattern shifts at the materno–fetal interface during pregnancy (11). Another study elucidated the mechanism that regulates IFN-β expression in the trophoblast through a negative feedback loop to ensure an effective response against invading pathogens (47).

Fetal Membranes

Chorioamnionitis is characterized by the inflammatory response generated in the amnion and chorion membranes by the invading pathogenic microbes, resulting in preterm labor (48). Fetal membrane infections are known to trigger pro-inflammatory cytokines, in particular IL-6, TNF-α, and IFN (α,β,γ), and chemokines in the amniotic sac (49, 50). There is much supporting evidence to suggest that fetal membranes do indeed respond to bacterial components and, in turn, generate cytokines (51, 52) along with many host defense peptides, which are anti-microbial in nature (53–55). During chorioamnionitis, the normal polarized distribution pattern of TLRs is completely lost, resulting in the overall upregulation of TLR2 and TLR4 expression (56).

A recent report demonstrated that human fetal membranes and neutrophils that interact directly, and LPS-stimulated factors originating from the fetal membrane, can effectively recruit, and trigger neutrophils to induce inflammatory cytokines and helps them build neutrophil extracellular traps. The effect of TLR activation in preterm infants has also been studied by checking

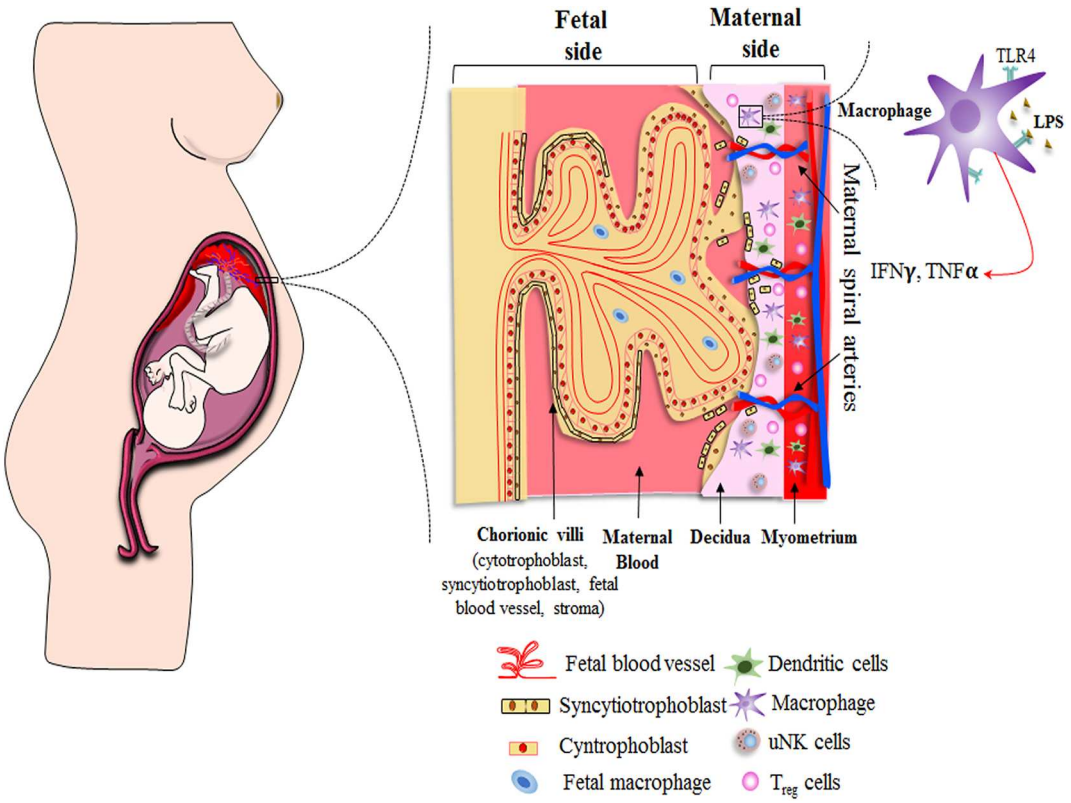

FIGURE 2 | Schematic showing distribution of immune cells across materno–fetal interface during early pregnancy. Macrophages, Treg cells, and dendritic cells are present in the myometrium as well as decidua, while uterine natural killer (uNK) cells are restricted to decidua. The effective crosstalk between various immune cells and extravillous trophoblast cells creates an immunosuppressive environment and helps in the formation of various pregnancy-related tissues, both of which are essential for a successful pregnancy. Extravillous cytotrophoblasts enter the decidua to reach out to maternal spiral arteries for obtaining required nutrient to support developing embryo. Other nutrients, gas, and waste exchange happens via placental villus, which interacts with the maternal blood directly. The villus has double layer of cells consisting of syncytiotrophoblasts and cytotrophoblasts. It encloses the fetal blood vessels along with fibroblasts and fetal macrophages (Hofbauer cells). Immune cells as well as placental cells protect the fetus by expressing PRRs, such as Toll-like receptors, on their surface throughout the pregnancy.

the level of the immunomodulatory factor, such as cAMP concentration in cord blood samples along with peripheral blood samples of preterm babies for the first month after delivery (57).

Decidua

Decidua harbors most of the immune cells, which have the capability to generate an instant immune response against invading pathogens. Immune cells, such as macrophages, dendritic cells, uterine Natural Killer (uNK) cells, and Regulatory T cells, present in decidua differentially express TLR2 and TLR4 on their surface during pregnancy (56). Additionally, resident cells in the decidua also express these pattern-recognition receptors. Transcripts of all TLRs have been found in the first and third trimester decidual cells, whereas only TLR2 and TLR4 have been found to be expressed in the first trimester decidual cells, and TLR1–TLR6 expression has been seen in the term decidua (58, 59). Furthermore, decidual cells, upon being stimulated with LPS, trigger the production of pro-inflammatory cytokines and many TLR4 pathway related downstream genes (60). These results have demonstrated the contribution of decidual stromal cells in fighting intrauterine infections and thereby act as a barrier between the developing fetus and invading microbes so as to ensure a safe environment for fetal development.

Immune Modulation During Pregnancy

The host graft model of pregnancy is an old paradigm that suggest that immune cells recognize the fetus as semi-allogenic and hence try to eliminate it. In the current school of thought, however, the immune cells facilitate the implantation, formation, and development of the blastocyst for the sustenance of the pregnancy. In the normal condition, there are three immunological stages: (i) the pro-inflammatory condition in the decidua that aids in implantation and placentation; (ii) the growth of the fetus occurs in an anti-inflammatory environment; and (iii) there is finally a change back to the pro-inflammatory state for parturition (25, 61, 62) (**Figure 3A**).

Fetus implantation in the early stages of pregnancy triggers the immune response at the junction of the decidual endometrium and extravillous trophoblast (EVT). Early contact of EVT with the maternal cells activates the immune system, primarily the innate immunity (63, 64). Innate immune cells, such

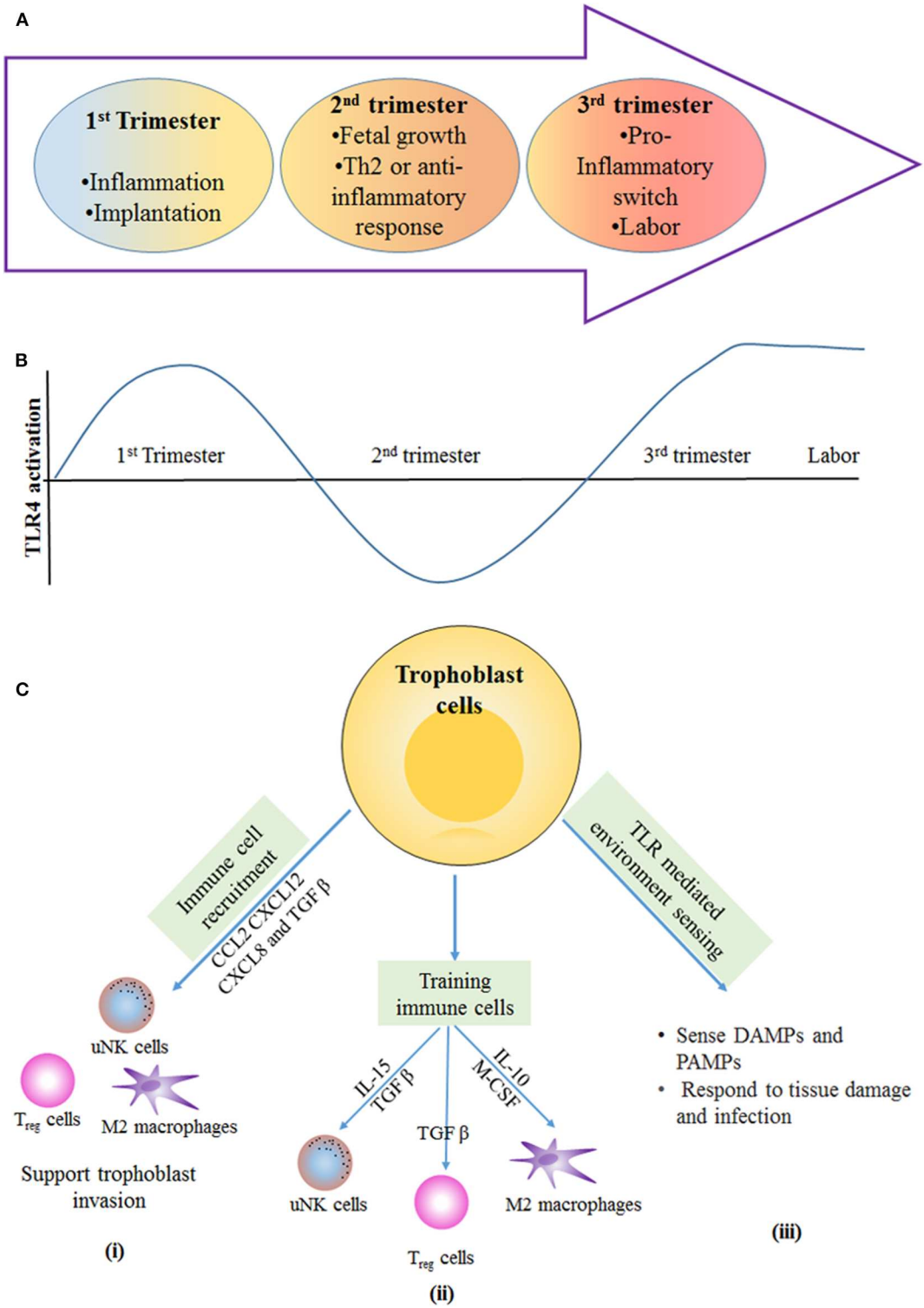

FIGURE 3 | Immunological stages of pregnancy: during first trimester of pregnancy, the inflammatory response is required for blastocyst implantation. **(A)** The second trimester is described by an anti-inflammatory and T-helper 2 (Th2)-type immune microenvironment that is necessary for fetal growth. In the third trimester, switching from anti-inflammatory to an inflammatory response happens, and this is essential for labor and delivery. **(B)** Different stages of pregnancy have altered the level of TLR4 activation the first and third trimester have more TLR4 activation in immune cells and trophoblast cells, which results in inflammation that is required for blastocyst implantation and term labor and delivery. In the second trimester, the lowered TLR4 activation supports the anti-inflammatory response for fetal growth.

(Continued)

FIGURE 3 | (C) Trophoblast-mediated immune regulation. (i) Trophoblast cells secrete number of cytokines and chemokines, such as CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 12 (CXCL12), CXCL8, and transforming growth factor-β (TGFβ), which are responsible for recruitment of immune cells to the materno–fetal interface. Immune cells provide support for invasion and implantation of trophoblast. (ii) Trophoblast cells secrete cytokines that help in training of uterine natural killer (uNK) cells and M2-like macrophages; in turn, these immune cells support the vascular and tissue remodeling that is necessary for trophoblast invasion and differentiation. TGFβ secreted by trophoblast cells induces the polarization of regulatory T (T_{reg}) cells, and these cells provide a feto-tolerant environment at the materno–fetal interface. (iii) Trophoblast cells express PRRs, such as TLRs, that allow them to sense and respond to DAMPs and PAMPs produced during tissue damage and infection. IL, interleukin; M-CSF, macrophage colony-stimulating factor.

as decidual macrophages, NK cells, dendritic cells, and T cells, are attracted toward the materno–fetal junction during the first trimester and remain there until parturition (65–67). These immune cells secrete different inflammatory cytokines, which are responsible for different states of the placenta. A distinct role is played by these immune cells for acceptance of the fetus and its protection from pathogens. The involvement of these immune cells and how TLR4 expression helps in pregnancy is described further (**Figures 3B,C**).

Uterine NK Cells

Natural Killer (NK) cells were initially derived by their cytotoxic activity against transformed cells. These cells have a unique ability to produce cytokines and perform cytotoxic functions other than T and B cells of lymphocyte origin.

Uterine NK cells are similar to systemic NK cells, but they do not express CD16 on their surfaces. They are translocated to the endometrium lining and placenta by the chemokine secreted from trophoblast cells. Uterine NK (uNK) cells are different in that they are highly granulated and are considered to play an essential role in maintaining a successful pregnancy by cytokine production in a temporal manner (68, 69). In addition to cytokine secretion, the crosstalk of uNK cells with dendritic cells supports the production of various growth and angiogenic factors, which helps in the implantation of trophoblast toward the vicinity of maternal blood (61, 63, 65, 70). These cells are dominant until mid-gestation, which helps in the implantation and acceptance of the fetus. uNK cells do so by getting activated or inhibited by ligands expressed in invading trophoblast (HLA-C) via the KIR receptor expressed on NK cells. uNK cells help in polarizing the Th2 subset of the CD4 T-cell subsets through the activation of KIR signaling in the second stage of pregnancy. The inhibitory KIR interaction with HLA C2 (in infants or trophoblast) is associated with preeclampsia (71, 72). *In-vitro* studies have shown that uNK cells have a high TLR expression (specifically TLR 2,3, and 4), which is stimulated to produce IFN-γ or IFN-β either by TLR agonist or through other cells in the endometrium (73, 74). TLR-induced cytokines and the effector function prevents the fetus from microbial infection and provides a feto-tolerant environment. The elevated inflammatory response is balanced by IL-10 and IL-1RA, and this downregulates the pro-inflammatory cytokines (75–77). The crucial role of IL-10 was elucidated in a mouse model, which resulted in frequent PTB upon TLR4 and TLR9 activation (78, 79). It is still unclear how TLR helps in shaping the uNK population in the materno–fetal interface.

Decidual Macrophages

In contrast to inflammatory cells, there is an abundant population of decidual macrophages, and these are critical to maintaining pregnancy after successful implantation. Decidual macrophages express CD206 and CD209 molecules on the surface along with CD11c hi/lo antigen. These cells act as antigen-presenting cells to innate (NK cells) and adaptive immune (T cells) cells at the materno–fetal interface during early pregnancy. Unlike circulating macrophages, decidual macrophages have a more M2-like phenotype and perform a "cleanup" function of apoptotic cells to prevent pro-inflammatory condition in the decidua (65, 80–83). Activation of the TLR pathway dictates the polarization of macrophages from anti-inflammatory to pro-inflammatory subsets in the uterus. Decidual macrophages have the potential to secrete cytokines like TNF-α and IL-1β along with IL-6, IL-8, and IL-10 as anti-inflammatory cytokines upon TLR agonist stimulation. TLR induced IL-10 by decidual macrophages inhibits excessive CD4 T-cell proliferation and activation (75, 84). Excessive administration of TLR ligand-like CpG or LPS modulates the macrophages to the M1 type, which leads to preterm birth or fetal reabsorption (79). Thus, the M1 phenotype of macrophages in uterus are harmful for normal pregnancy, which can be rescued either by depleting such macrophages or by administration of IL-10 cytokine (77, 85). Progesterone also prevent the NF-κB activation through TLR4 pathway in decidual macrophages, thus decreasing the production of inflammatory cytokines (86, 87).

Regulatory T Cells

Immunology during pregnancy is similar to tumor immunology. In cancer, the adaptive immunity plays a critical role in graft rejection, but cancer cells modulate the immune cells for its establishment. As opposed to rejection, maintaining pregnancy is also a kind of allograft tolerance (61, 88). In this scenario, a subset of adaptive immunity, i.e., regulatory T cells, plays a critical role in sustaining pregnancy. Amplification of these cells helps in restraining Th1 and Th17 responses and creates an immunosuppressive environment, thus protecting the fetal allograft from elimination. T_{regs} comes into play during the second stage of pregnancy where they crosstalk with other immune cells, such as uNK, dendritic cells, and decidual macrophages, to create a "tolerant" environment by reducing the Th1 and Th17 cytokines.

The temporal existence of T_{reg} cells is regulated by TLR4 expression, which is upregulated during early pregnancy in decidual stromal cells and thus decreases the T_{reg} population. This increased TLR4 signaling inhibits the transcription factor Foxp3, which in turn reduces regulatory T-cell polarization

(89). A reduced number of T_{reg} cells has been associated with preeclampsia and PTB. There is a report that, for the first time, has demonstrated the significance of regulatory T cells in a murine model, where depletion of these cells resulted in loss of pregnancy (90). $Rag^{-/-}$ mice were treated with a TLR4 ligand (LPS), causing preterm birth; however, the adoptive transfer of T_{reg} cells rescued these mice and ensured they were able to sustain the pregnancies to term (13, 91), and negatively regulated LPS induced fetal inflammation in a late pregnancy mouse model (92). Therefore, regulatory T cells are important in maintaining a tolerant environment, and their time of polarization decides the fate of pregnancy (93). During pregnancy, a pool of memory T_{reg} cells are differentiated against the paternal alloantigen, and they are responsible for inducing tolerance upon subsequent pregnancy with the same paternal alloantigen (94, 95).

ROLE OF TLR4 SIGNALING IN PREGNANCY

During normal pregnancy, a large number of cytokines and chemokines are secreted by trophoblasts, which helps in the proper implantation of the embryo on the uterine wall. These cytokines also help in the training of immune cells that are essential for the establishment of different stages of pregnancy (61, 70, 96).

TLR2 and TLR4 are widely expressed on various innate immune cells, including decidual macrophages and dendritic cells. Along with these immune cells, TLR4 is reported to express in decidual cells during the first trimester, EVTs, Villous cytotrophoblasts, and hofbauer cells, though not in syncytiotrophoblasts (70). These cells protect the fetus from various microbes and infectious agents, which indicates their critical role in placenta. There are many DAMPs, such as apoptotic cells or matrix component-like fibronectin and oligosaccharides, within the placenta that trigger TLR signaling via the MyD88-NF-κB pathway. This signaling results in the production of inflammatory cytokines by neighboring immune cells in the decidua.

TLR4 expression is found in various types of cells and at different time points. Any changes in this expression or perturbation in signaling causes pregnancy disorders like preterm birth, preeclampsia, and abortion. Recently, TLR4-mediated IFN-β production and its role in pregnancy has been widely elucidated. There is an increase in the production of IFN-β by trophoblast cells upon LPS-mediated TLR4 activation via the MyD88-independent (TRIF-TBK1-IRF-3 axis) pathway. Increased IFN-β induces downstream interferon stimulating genes and also triggers negative regulators of the TAM receptor, such as Mer and Axl. Absence of these negative regulators were found to be detrimental, as fetal rejection occur in the presence of increased IFN-β in the placenta (47).

Preterm Birth (PTB)

A major problem of neonatal mortality is due to preterm labor (gestation at < 37 weeks). PTB is marked by increased pro-inflammatory factors due to local or systemic infection or inflammation, such as infection in intra-amniotic (chorioamnionitis) or periodontitis, which interacts via maternal sera (97, 98) (**Figures 4A,B**). LPS-mediated TLR4 signaling is profound in PTB and IUFD (Intra Uterine Fetal Death) even with a low dose in LPS pre-treated mice (99). In the animal model, TLR4 knockout mice were unaffected by PTB, whereas a neutralizing antibody against TLR4 reduced fetal death in normal mice (98, 100). In chorioamnionitis, which leads to PTB, LPS-induced translocation of TLR4 toward the basal membrane is a protective mechanism to lower the immune response (101). Increased TLR4 expression on $CD14^{+}$ monocytes has been well-correlated in patients with PTB (102). Reports suggest that small doses of LPS (TLR4 agonist) treatment in *Il-10*$^{-/-}$ mice causes PTB, as opposed to in wild type mice (78). Also, upon LPS treatment, mice show increased uNK intrusion and placental cell death. But with depletion of uNK cells or deactivation of TNF-α, mice were rescued from PTB (103). During parturition or in preterm birth, it has been observed that TLR4 plays a critical role in developing inflammatory response by recruiting a number of monocytes and macrophages to the placenta. TLR4 and TREM-1 (triggering receptor expressed on myeloid cells 1) expression was found to be elevated in monocytes and neutrophils in patients diagnosed with PTB (104). *Tlr4*$^{-/-}$ mice showed delayed labor due to the absence of an inflammatory cytokine storm even after LPS treatment, suggesting that TLR4 indeed is necessary for timely labor. Inflammation-induced PTB can be delayed by small molecule-like (+) naloxone, which is specific to TLR4 receptor and has the ability to cross the placenta and delay labor (105, 106). As most of the studies were done under total TLR4 knockout conditions, involvement of TLR4 activation at the materno–fetal interface was still unclear. However, in a recent study, a decidua specific conditional TLR4 knockout was generated using the *Pgr*-Cre driver ($Pgr^{Cre/+}Tlr4^{f/f}$) to explore the physiological importance of TLR4 during pregnancy. Endothelial cells expressing TLR4 has reported to be important in sensing the inflammation in the decidua, which, in turn, activates STAT3 via IL-6 in perivascular stromal cells and hence regulates the anti-inflammatory IL-10 production. The homeostasis of TLR4 expression in endothelial cells determines the pregnancy outcome, as in case of PTB, and could be a probable therapeutic target in preventing PTB (107).

Preeclampsia (PE)

Preeclampsia is a heterogeneous disorder caused after the 20th week of pregnancy due to local or systemic abnormalities. There is much evidence to suggest that TLR signaling activation could cause PE in many ways (**Figures 4C,D**). An imbalance of the Th1 and Th2 response is a dominant immune response as a result of TLR4 activation which creates a pro-inflammatory environment leading to preeclampsia (108, 109). The abundance of TLR ligand could be linked to various pathogenic infection, such as *Chlamydia pneumoniae*, Cytomegalovirus, *Helicobacter pylori*, Malaria, *Toxoplasma gondii*, and Mycoplasma Hominis (110–112). Since PE is a multifactorial disorder, maternal health along with infectious load add up to the pathogenesis of this disorder. Pregnant women with urinary tract infection are also at a higher risk of this disorder (110, 113). Among all TLRs, TLR4 has

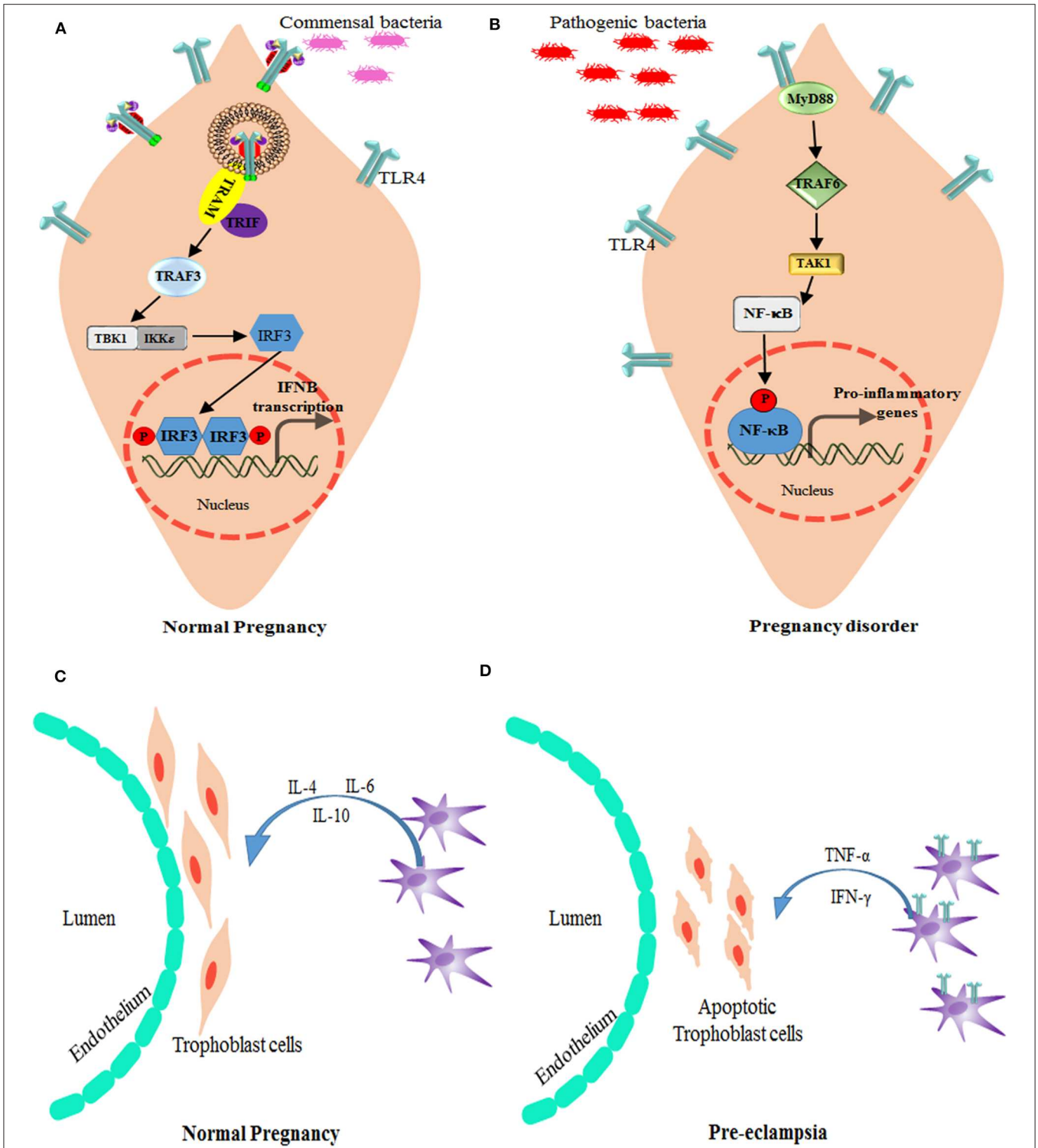

FIGURE 4 | Alteration of TLR4 signaling in pregnancy disorders. **(A)** Bacterial products, such as lipopolysaccharide, activate TLR4 and the signal TRIF-TBK1-IRF-3 pathway to induce the baseline expression of IFN-β (which is encoded by *IFNB*) in the placenta. The production of IFN-β by the placenta modulates the maternal immune system and promotes tolerance, while providing protection against viral and bacterial infections. **(B)** Bacterial products stimulate the production of type I IFNs by trophoblast cells and maintain an anti-inflammatory environment as well as active surveillance and protection against infections. However, in case higher antigen load or if pathogenic infection reaches the placenta, TLR4 expression increases and modulate the activation signals through MyD88-TRAF6-NF-κB leading to inhibition of type I IFNs, and promotion of pro-inflammatory response that is responsible for preterm labor. TRAM is the TRIF-related adapter molecule (also known as

(Continued)

FIGURE 4 | TICAM2). Role of macrophages and TLR4 in Pregnancy: **(C)** In normal pregnancy, M2-like macrophages are available around spiral arteries and the endothelium, which helps in the remodeling of these arteries by producing various factors associated with angiogenesis and tissue remodeling. They also play a role in immunomodulation, for instance by producing IL-10. **(D)** During preeclampsia, increased numbers of M1-like macrophages are found in the materno–fetal interface. These M1 type decidual macrophages have more TLR4 expression and signal via NF-κB pathway to produce pro-inflammatory cytokines, such as TNF-α and IFN-γ, which induces apoptosis of the trophoblast cells.

TABLE 1 | List of drugs targeting TLR4, and its downstream signaling molecules during pregnancy disorder.

Drugs	Role in pregnancy disorder	Disorder	References
TNF-α ANTAGONIST			
Hydroxyquinone	Reduces production of TNF and endothelin-1	PE	(127)
Asprin	Prevents endothelial dysfunction due to TNF	PE	(118)
TLR4 INHIBITOR			
Curcumin	Downregulates TLR4 expression and NF-κB mediated inflammatory response	PE	(108, 128)
Vitamin D	Calcitriol can modulate innate as well as adaptive response (pro to anti- inflammatory)	PTB, PE & spontaneous miscarriages	(129, 130)
	Decreases TLR4 expression	PE	(131, 132)
Rosiglitazone	Reduces TLR4 mediated inflammation Increases antioxidant response by NRF-2 and HO-1	PTB	(133)
Progesterone	Inhibit TLR4 expression in macrophages Promotes Th2 differentiation Induces tolerance at materno–fetal junction	PE	(86, 134) (135) (136, 137)
IMMUNOMODULATORS			
Inonotus obliquus polysaccharide	Maintain Th17/T_{reg} cell balance	Infection of *T.gondii*	(138)
IL-10	Maintains anti-inflammatory condition in decidua	PTB	(107)
IKK COMPLEX INHIBITOR			
NEMO-binding Domain Inhibitor	Reduces Prostaglandin E2 (PGE2) in LPS and *Ureaplasma parvum* stimulated *in-vitro* ovine gestational membrane model	PTB	(139)
Parthenolide	Reduces inflammatory gene expression in patient derived choriodecidual cells. Decreases TNF-α and COX-2 expression in human urothelial cell stimulated with TNF-α.	PTB	(140, 141)
TPCA-1	Similar effect as of parthenolide. Reduction in PGE2 level in LPS stimulated ovine pregnancy model	PTB	(139, 140, 142)

been found to be associated with preeclampsia. As reported by Mazouni et al. a patient with preeclampsia showed an imbalance of the pro-inflammatory form of monocytes due to TLR2 and TLR4 signaling (114). Another factor, which is predisposed to preeclampsia, is the genetics of TLR2 and TLR4 polymorphism. Single nucleotide polymorphisms in TLR2 (Arg753Gln) and TLR4 (Asp299Gly/Thr399Ile) have been associated with early onset of preeclampsia (115), with an exception in the Caucasian population (116).

Other than different maternal syndromes, which are associated with PE, serum TLR4 and NF-κB p65 could be used as a biomarker for predicting cytokine environment and its influence on the immune cells (117). Even microRNAs (miR-155, miR-335, and miR-584), which prevents free radicals (eNOS) in the endothelial cells, are associated with PE and can be upregulated by aspirin treatment that inhibits NF-κB mediated inflammation (118).

Placental Malaria

Parasitic infection caused by *Plasmodium* is known to stimulate various immune cells by activation of the TLR4–NF-κB axis. Placental malaria is marked by an increased innate immune response causing intra-uterine complications, decreased body weight of the fetus during birth, and susceptibility to recurrent infection in early life (119–121). The development of gestational malaria was studied in pregnant mice model infected with *P. berghei* NK65, where TLR2, TLR4, and TLR9 were identified to trigger the inflammatory pathway, leading to NF-κB activation. In this study, placental inflammation was associated with the TLR4 pathway because infection in TLR2 null and TLR9 null pregnant mice displayed no difference to that of wild-type pregnant mice. Moreover, a CD14/TLR4 blocker (IAXO-101) was successful in rescuing the malarial risk to both fetus and mother and helped in gaining the fetal body weight (122). As CD14 and lipoprotein can activate the TLR1/TLR2 pathway, inhibiting CD14 by IAXO-101 will cease the activation of TLR1/2/4 and hence affect cytokine balance, which can eventually lead to an adverse pregnancy outcome.

Under the same scenario, it was observed that the TLR4 receptor behaves differently on the maternal and fetal interface. Maternal TLR4 is involved in the pathogenesis of malaria severity, while fetal TLR4 has a protective response against placental parasite burden, which could be due to the paternal allele for *Tlr4*. Similarly, a decrease in maternal type 1 IFN

receptor 1 (IFNAR1) during the course of infection promotes the parasite burden by limiting the activation and accumulation of Helper T cells. Increased fetal IFNAR1, however, helps in eliciting an anti-parasite response, but fetal IFNAR1 is not sufficient enough to reduce the placental parasite burden and its harmful effect on the fetus (6). In placental malaria, the TLR4 downstream partner MyD88 has no significant role in pregnancy outcome irrespective of maternal or fetal genetic background

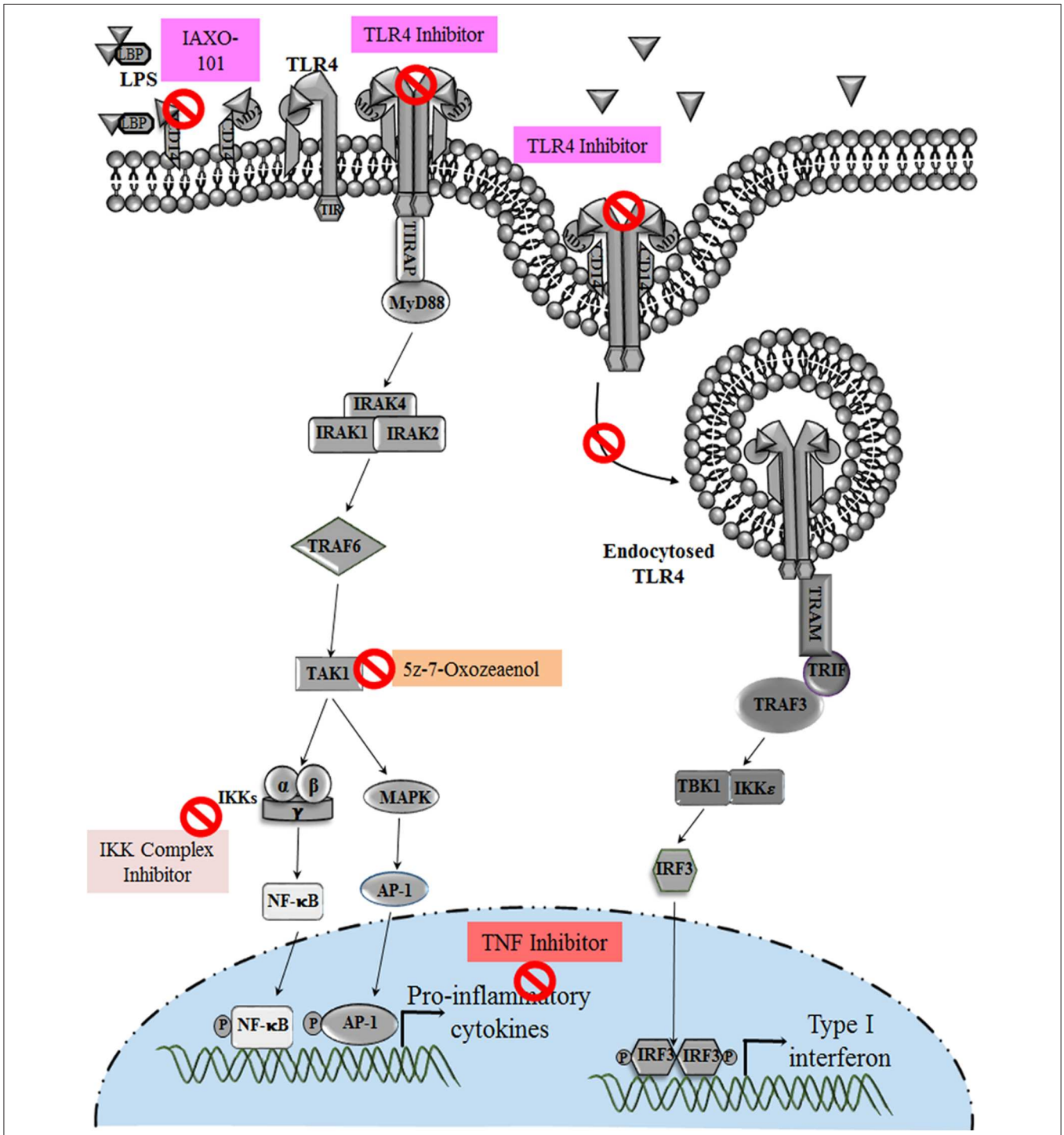

FIGURE 5 | Various Drugs that target TLR4 pathway in pregnancy disorders: drugs and anti-inflammatory agents that target TLR4 pathway and its downstream molecules during infection induced preterm birth. Hormones and drugs targeting TLR4 expression help in switching the pro-inflammatory environment to anti-inflammatory in various pregnancy disorders. TNF inhibitors reduce the increased TNF production during altered TLR4 activation in preeclampsia.

when infected with *P. berghei* NK65. The deletion mutant of MyD88 did not produce any abnormalities and affected growth in infected pregnant mice (123). An ideal vaccine approach against TLR4 could be formulated that can be specific to placental malaria and would provide protection against maternal anemia, PTB, and fetal growth retardation.

THERAPEUTIC MODALITIES FOR PREGNANCY RELATED DISORDER TARGETING TLR4 SIGNALING

Various TLR4 antagonist and inhibitors have been developed that are currently in different phases of clinical trial for diseases other than pregnancy. There are few options that are currently being studied for immune modulation and inhibition of TLR expression for pregnancy-related disorders. The association of TLR4 was studied in women with aPL (antiphospholipid antibodies), which activate the TLR4 pathway and the inflammatory response in trophoblasts leading to miscarriages, PE, and PTB (124). Recent studies have identified endothelial TLR4 to be a potential therapeutic target for PTB (107). Cytokines like IL-6 have been successful in delaying preterm birth by immunomodulation and regulating prostaglandin-related genes (125).

Cytokine-suppressive anti-inflammatory drugs (CSAID's) are a novel group that target the NF-κB and MAP Kinase pathways, making them more effective than Non-Steroidal Anti-inflammatory Drugs (NSAID). CSAIDs that can selectively inhibit TAK1 and the IKK complex are well-studied in animal models, which has resulted in the reduction of cytokines and prostaglandin levels (126) (**Table 1**). TAK1 inhibitor 5z-7-oxozeaenol (OxZnl), a resorcyclic acid lactone that is an excellent pharmacological target in CSAIDs, can effectively block the cytokine cascade to avoid preterm birth (143, 144) (**Figure 5**). Although these drugs can selectively target TLR-NF-κB pathway, there are some side effects associated with its use, such as how it may inhibit unwanted NF-κB activation, thus increasing the predisposition to opportunistic infection. To resolve such problems, these drugs can be administered in amniotic cavity to reduce the side effects and enhancing the efficacy of the drug. But the probable benefits and the risk assessment should be balanced, and such CSAID therapy should be given to women who can gain significant benefits.

CONCLUDING REMARKS

Detailed study of spatiotemporal expression of TLRs during normal pregnancy and related disorders using various model systems has increased our understanding of placental infections and furthered our development of strategies to overcome the adverse pregnancy outcomes. Activation of innate immune PRR through TLR4 at the materno–fetal interface ensures that the developing fetus is protected from invading pathogens at early stage of pregnancy. But uncontrolled activation of TLR4 has been proven to trigger chronic inflammation and to result in loss of pregnancy. Hence, increased levels of TLR4 on leucocytes or cells of maternal and fetal origin could be used as a biomarker for pregnancy disorders. Many studies have shown the involvement of innate immune cells for sustaining a successful pregnancy.

It is not yet clear how the TLR4 expression pattern alters during various stages of pregnancy and in what way its uncontrolled activation on immune or other decidual cells at the maternal–fetal interface leads to various pregnancy failures. Addressing this issue may help in developing certain clinical diagnostic markers as well as specific antagonists targeting either TLR4 specifically or its downstream effector molecules for improving pregnancy outcomes.

AUTHOR CONTRIBUTIONS

PF and VS contributed equally in writing the review. Conception of Idea was done by SC, PF, and VS. Manuscript writing and editing was done by all the authors.

FUNDING

We would also like to acknowledge Department of Biotechnology-Systems Medicine Cluster (DBT-SyMeC) grant and J C Bose fellowship-SERB (Science and Engineering Research Board) to SC, for the financial support.

ACKNOWLEDGMENTS

We are thankful to CSIR-Indian Institute of Chemical Biology, Jadavpur, Kolkata and National Centre for Cell Science (NCCS), Pune for providing infrastructure facilities.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 08 July 2020
doi: 10.3389/fimmu.2020.01455

Toll-Like Receptor 4 (TLR4)/Opioid Receptor Pathway Crosstalk and Impact on Opioid Analgesia, Immune Function, and Gastrointestinal Motility

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *23 January 2020*
Accepted: *04 June 2020*
Published: *08 July 2020*

Citation:
Zhang P, Yang M, Chen C, Liu L, Wei X and Zeng S (2020) Toll-Like Receptor 4 (TLR4)/Opioid Receptor Pathway Crosstalk and Impact on Opioid Analgesia, Immune Function, and Gastrointestinal Motility. Front. Immunol. 11:1455. doi: 10.3389/fimmu.2020.01455

Toll-like receptor 4 (TLR4) recognizes exogenous pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) and initiates the innate immune response. Opioid receptors (μ, δ, and κ) activate inhibitory G-proteins and relieve pain. This review summarizes the following types of TLR4/opioid receptor pathway crosstalk: (a) Opioid receptor agonists non-stereoselectively activate the TLR4 signaling pathway in the central nervous system (CNS), in the absence of lipopolysaccharide (LPS). Opioids bind to TLR4, in a manner parallel to LPS, activating TLR4 signaling, which leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) expression and the production of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. (b) Opioid receptor agonists inhibit the LPS-induced TLR4 signaling pathway in peripheral immune cells. Opioids operate as pro-inflammatory cytokines, resulting in neuroinflammation in the CNS, but they mediate immunosuppressive effects in the peripheral immune system. It is apparent that TLR4/opioid receptor pathway crosstalk varies dependent on the cell type and activating stimulus. (c) Both the TLR4 and opioid receptor pathways activate the mitogen-activated protein kinase (MAPK) pathway. This crosstalk is located downstream of the TLR4 and opioid receptor signaling pathways. Furthermore, the classic opioid receptor can also produce pro-inflammatory effects in the CNS via MAPK signaling and induce neuroinflammation. (d) Opioid receptor agonists induce the production of high mobility group box 1 (HMGB1), an endogenous TLR4 agonist, supporting intercellular (neuron-to-glia or glia-to-neuron) interactions. This review also summarizes the potential effects of TLR4/opioid receptor pathway crosstalk on opioid analgesia, immune function, and gastrointestinal motility. Opioids non-stereoselectively activate the TLR4 pathway, and together with the subsequent release of pro-inflammatory cytokines such as IL-1 by glia, this TLR4 signaling initiates the central immune signaling response and modifies opioid pharmacodynamics. The DAMP HMGB1 is associated with the development of neuropathic pain. To explain morphine-induced persistent sensitization, a positive

feedback loop has been proposed; this involves an initial morphine-induced amplified release of IL-1β and a subsequent exacerbated release of DAMPs, which increases the activation of TLR4 and the purinergic receptor P2X7R. Opioid receptor (μ, δ, and κ) agonists are involved in many aspects of immunosuppression. The intracellular TLR4/opioid receptor signaling pathway crosstalk induces the formation of the β-arrestin-2/TNF receptor-associated factor 6 (TRAF6) complex, which contributes to morphine-induced inhibition of LPS-induced TNF-α secretion in mast cells. A possible molecular mechanism is that the TLR4 pathway initially triggers the formation of the β-arrestin-2/TRAF6 complex, which is amplified by opioid receptor signaling, suggesting that β-arrestin-2 acts as a functional component of the TLR4 pathway.

Keywords: TLR4—Toll-like receptor 4, opioid receptor, opioid tolerance and dependence, hyperalgesia, crosstalk

INTRODUCTION

Toll-like receptor 4 (TLR4) is a pattern recognition receptor belonging to the Toll-like receptor (TLR) family that contains an extracellular domain and an intracellular domain (1). TLR4 activates the innate immune response by recognizing pathogen-associated molecular patterns (PAMPs, including bacteria, viruses, fungi, and protozoa) or danger-associated molecular patterns (DAMPs, mainly endogenous signals for cell death and tissue damage) (2). Lipopolysaccharide (LPS), an outer surface component of Gram-negative bacteria, is an exogenous TLR4 agonist, while high mobility group box 1 (HMGB1) and heat shock proteins (HSPs) are endogenous TLR4 agonists (3). TLR4 signaling is roughly divided into two distinct pathways depending on the usage of the distinct adaptor molecules, myeloid differentiation primary response gene 88 (MyD88) and Toll-interleukin receptor-domain-containing adapter-inducing interferon-β (TRIF): the MyD88-dependent and TRIF-dependent (also known as MyD88-independent) signaling pathways (2, 3).

Opioid receptors belong to the seven-transmembrane G protein-coupled receptor (GPCR) superfamily, the members of which use G proteins for signal transduction (4). Opioid receptors are expressed throughout the nervous system and peripheral tissues and play critical roles in antinociception and pain management. There are three major subtypes of opioid receptor: mu (μ), delta (δ), and kappa (κ) opioid receptors (also called MOR, DOR, and KOR, respectively), among which MOR plays a predominant role in analgesia (5, 6). Opioid receptors are activated both by endogenous opioid peptides (dynorphin and enkephalin) and exogenous synthetic opioid drugs (morphine, fentanyl, and remifentanil) (7). After activation by agonists, multiple intracellular effects are initiated, including inhibition of adenylyl cyclases and cyclic adenosine monophosphate (cAMP), suppression of Ca^{2+} channels, stimulation of K^{+} channels, and activation of phospholipase C (PLC) and protein kinase C (PKC), which together inhibit presynaptic neurotransmitter release, induce postsynaptic hyperpolarization, and decrease neuronal excitability (5, 8).

It is apparent that the classic functions of the TLR4 and opioid receptor signaling pathways are remarkably distinct. Additionally, the stereoselectivity of opioid action at TLR4 and the opioid receptor is also different. To be specific, the opioid receptor is stereoselective, only binding to (–)-opioid isomers but not (+)-isomers, while TLR4 is non-stereoselective, binding to both opioid isomers (9, 10). However, Zhang *et al* recently reported that (+)-norbinaltorphimine [formed by coupling two pharmacophores derived from (+)-naltrexone] inhibited the LPS-induced TLR4 signaling pathway in microglia, astrocytes, and macrophages, whereas (–)-norbinaltorphimine did not, indicating that some xenobiotics show stereoselectivity for TLR4 (11). An early opioid-binding experiment by Goldstein et al. in 1971 found that there are saturable but non-classic non-stereoselective opioid-binding sites, which are much more abundant (∼30-fold more abundant) than the classic stereoselective opioid-binding sites (12). This was the first evidence that opioids could non-stereoselectively bind to non-classic non-opioid receptors, although, for a long period of time, the findings of Goldstein et al. were considered to be experimental "noise" (13, 14).

In 1979, Wybran et al. reported that, based on active and total rosette tests, morphine inhibited human T lymphocytes, and this inhibition was completely reversed by the opioid receptor antagonist naloxone (15). This represents early evidence showing the immunosuppressive effects of opioids. Further evidence demonstrated that opioids suppress the immune system at various stages, starting from innate immune cells, encompassing antigen presentation, and ending with modulation of T lymphocyte activation and differentiation (16–18). The fact that MOR-knockout mice, unlike wildtype mice, did not show morphine-induced diminished natural killer (NK) cell activity indicated that MOR was implicated in immunosuppression (19). However, in 2005, Watkins et al. reported that spinal cord glia were activated and released neuroexcitatory substances in response to morphine, thereby inducing neuroinflammation and causing anti-analgesia effects, indicating a pro-inflammatory role for opioids in the central nervous system (CNS) (20). Further evidence collected during the last 10 years has confirmed that opioids also have pro-inflammatory effects in the CNS and induce the central immune response (21–23). Recognition of the involvement of TLR4 in opioid-induced central immune signaling arose from the early evidence that chronic intrathecal

(+)-methadone and (+)-morphine (which have no affinity for the opioid receptor) induced glial activation and increased the expression of chemokines and cytokines in isolated dorsal spinal cords from rats (24).

In this review, we discuss the potential crosstalk between the TLR4 and opioid receptor signaling pathways and the implications of the crosstalk for opioid analgesia, immune function, and intestinal motility. Firstly, four aspects of TLR4/opioid crosstalk are discussed: (a) Opioid receptor agonists directly activate the TLR4 signaling pathway in the absence of LPS, indicating crosstalk within the cell membrane. (b) Opioid receptor agonists inhibit the LPS-induced TLR4 signaling pathway, indicating negative intracellular crosstalk. (c) Both the TLR4 and opioid receptor pathways activate the mitogen-activated protein kinase (MAPK) pathway, representing downstream crosstalk between the TLR4 and opioid receptor pathways. (d) Opioid receptor agonists induce the production of HMGB1, an endogenous TLR4 agonist, supporting intercellular (neuron-to-glia or glia-to-neuron) interactions. Secondly, we summarize and update current knowledge on opioid-induced central immune signaling and the effect of non-stereoselective TLR4 activation in the CNS on opioid analgesia; findings on the role of HMGB1 in maintaining morphine-induced persistent sensitization are also discussed. Thirdly, we summarize the peripheral immunosuppressive effect of opioids on innate immune cells, involving modulation of the immune system related to TLR4 signaling and LPS-activated immune cells. Fourthly, the differential involvement of TLRs (in intact animals vs. isolated colon segments) regarding morphine-induced inhibition of gastrointestinal transit are discussed.

TLR4/OPIOID RECEPTOR PATHWAY CROSSTALK

Opioid Receptor Agonists Bind to TLR4 and Non-stereoselectively Activate TLR4

Many clinically relevant opioid receptor agonists, such as morphine, fentanyl, and oxycodone, bind to TLR4 by docking to the LPS-binding pocket of myeloid differentiation (MD)-2 (9, 21, 25, 26). Additionally, endogenous opioid peptides, for example, endomorphin (MOR), enkephalin (DOR), and dynorphin (KOR), and certain opioid metabolites are also TLR4 ligands (27–29). Morphine-3-glucuronide (M3G), an inactive metabolite of morphine, has little to no affinity for opioid receptors but enhances pain by activating the TLR4 signaling pathway (29). Naloxone and naltrexone are known as opioid receptor antagonists and are usually used to block the effects of opioids (30). Interestingly, acting as TLR4 antagonists, naloxone and naltrexone inhibit the opioid- or LPS-induced TLR4 signaling pathway (9, 25) and reverse TLR4-related neuropathic pain (31, 32).

Opioid receptor agonists bind to TLR4 and subsequently stimulate the TLR4 signaling pathway, which ultimately activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and releases pro-inflammatory cytokines (9, 21, 24, 25, 33). Wang et al. showed that, similar to LPS, morphine induced TLR4 dimerization and led to the formation of the (TLR4/MD-2)/(TLR4/MD-2) heterotetramer after docking with TLR4/MD-2 complexes (21). TLR4, MD-2, and MyD88 were found to be crucial for morphine-induced TLR4 pathway activation, as reduced production of NF-κB, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α and inhibition of morphine-induced neuroinflammation were observed when TLR4, MD-2, or MyD88 was either knocked out or knocked down in *in vivo* and *in vitro* experiments (21). Moreover, the p38 and extracellular signal-regulated kinase (ERK) MAPK pathways are also involved in morphine-induced TLR4 pathway activation (21). Taken together, these findings show that opioids, like LPS, bind to TLR4 and activate the TLR4/MyD88-dependent pathway (including MAPK signaling cascades) (21). This extracellular interaction between opioids and TLR4 has mostly been observed in the CNS, including astrocytes, microglia, and endothelial cells, and it produces a pro-inflammatory effect and mediates neuroinflammation (13, 14, 23). It remains unclear whether this kind of crosstalk between opioids and TLR4 exists beyond the CNS.

As they do not express opioid receptors, HEK-Blue^{TM}-hTLR4 cells (which are human embryonic kidney [HEK] 293 cells transfected with human TLR4 and related accessory proteins) are usually used to examine opioid effects targeted at TLR4 (25); TLR4 activity can be detected in these HEK-Blue^{TM} cells. Hutchinson et al. showed that, in the absence of LPS, nine opioids (morphine, methadone, M3G, etc.) at 10–100 μM non-stereoselectively activated the TLR4 signaling pathway, while naloxone and naltrexone did not (9). Moreover, the authors found that (–)-isomers (morphine and methadone) and (+)-isomers produced equivalent TLR4 activity, indicating that (+)-isomers and (–)-isomers have similar potency (9). Another two studies demonstrated that morphine at 3 and 10 μM, fentanyl at 0.3 μM, and M3G at 1–100 μM produced significant activation of the TLR4 pathway, while M6G (0.1–100 μM) did not (25, 33). Research has shown that LPS is the most potent agonist of TLR4 (9, 25, 33), while M3G is the second most potent. M3G is a consistent activator of TLR4 (M3G > 1 μM can activate the TLR4 pathway), while other opioid receptor agonists produce significant activation of the TLR4 pathway only at certain doses (9, 25, 33). Although these remaining opioid receptor agonists (including morphine, methadone, levorphanol, pethidine, buprenorphine, fentanyl, oxycodone, and dextrorphan) produced significant stimulation of TLR signaling (9), it is difficult to rank them in order, because of the limited data.

Opioid Receptor Agonists *per se* Activate TLR4 but Inhibit LPS-Induced TLR4 Signaling Pathway Activation

In 2013, Stevens et al. reported that co-treatment of HEK-Blue^{TM} cells with morphine (3–100 μM) or fentanyl (1–100 μM) plus LPS (100 ng/ml) led to significant inhibition of TLR4 signaling activation in a non-competitive fashion, compared with LPS alone (25). Moreover, this inhibition was not blocked by an LPS

antagonist (LPS-RS) or an opioid antagonist (naloxone or β-funaltrexamine [FNA]) (25). These findings are consistent with an *in vitro* experiment by Xie et al. (also using HEK-Blue^{TM} cells) and an *in vivo* experiment involving mice (33). The *in vitro* data showed that morphine and M3G (>1 μM) decreased LPS-induced TLR4 signaling activation (33). The *in vivo* data also supported this conclusion, as the plasma from morphine-treated mice inhibited LPS-induced TLR4 activation (33).

This phenomenon of opioids inhibiting LPS-induced TLR4 signaling activation is consistent with early studies (including on mast cells, human neutrophils, and human macrophages) (34–38) that showed that morphine and remifentanil inhibited LPS-induced production of TNF-α, IL-6, IL-8, IL-10, and IL-12 (34–38). Naloxone dose-dependently reversed the morphine-induced inhibition of LPS-induced TNF-α secretion in mice in a study by Bencsics et al. (36), while naltrexone did not prevent the decrease in LPS-induced IL-10 and IL-12 production in mice in a study by Limiroli et al. (35). In a study of human neutrophils, the p38 and ERK1/2 signaling pathways, but not c-jun N-terminal kinase (JNK) signaling, were implicated in remifentanil-induced inhibition of LPS-induced TLR4 signaling, and a KOR antagonist could reverse this inhibition (37). A further study by Madera-Salcedo et al. on bone marrow-derived mast cells proposed an underlying mechanism involving intercellular crosstalk between the TLR4 and opioid receptor pathways that induced the formation of an β-arrestin-2/TNF receptor-associated factor 6 (TRAF6) complex (34).

β-arrestins interact with certain TLR4 signaling molecules, such as IκB and TRAF6, and negatively regulate NF-κB activity (34, 39–41). In a study by Witherow et al., β-arrestin-1 and β-arrestin-2 bound to IκBα and subsequently attenuated NF-κB activity in transfected HeLa cells (39). Moreover, suppression of β-arrestin-1 expression using RNA interference led to a 3-fold increase in TNF-α-induced NF-κB activity (39). Gao et al. reported that activation of β_2-adrenergic receptors (a type of GPCR) induced β-arrestin-2/IκBα formation, which inhibited the LPS/NF-κB signaling pathway and decreased IL-8 and TNF-α production in HEK293T cells (41). TRAF6 is a critical mediator of TLR/IL-1 signaling. β-arrestins can interact with TRAF6 and prevent TRAF6 autoubiquitination or oligomerization, which subsequently inhibits NF-κB and AP-1 activity, as shown in *in vitro* and *in vivo* experiments (40).

In the study by Madera-Salcedo et al., morphine treatment of mast cells prevented the production of the LPS-induced pro-inflammatory cytokine TNF-α and the activation of the TLR4 signaling molecules ERK1/2 and IKK (both of which belong to the MyD88-dependent pathway) (34). There were also morphine-induced decreases in TRAF6 ubiquitination and TRAF-activated kinase 1 (TAK1) phosphorylation (34). Given that β-arrestin operates as a negative regulator of the TLR4 pathway, unsurprisingly, morphine and LPS co-treatment induced the formation of the β-arrestin-2/TRAF6 complex in the mast cells, which subsequently inhibited the TLR4 signaling pathway. Only the combination of MOR and DOR antagonists could reverse the morphine-induced inhibition of LPS-induced secretion of TNF-α in mast cells, indicating that MOR/DOR heterodimers may be implicated in this antagonism (34).

Unfortunately, there is currently no evidence regarding whether this intracellular negative crosstalk (opioid-induced inhibition of LPS-induced TLR4 pathway activation) exists in other cell types. Although TLR4 initiates the innate immune response, the extent to which this negative TLR4/opioid crosstalk participates in opioid-induced immunosuppression is also unclear. It is apparent that the phenotypes related to TLR4/opioid receptor pathway crosstalk are complicated and varied dependent on the cell type or cellular microenvironment. In the CNS, opioids non-stereoselectively activate TLR4 and operate as pro-inflammatory cytokines, thereby resulting in neuroinflammation (21–23). In contrast, in mast cells or other peripheral immune cells, opioids inhibit LPS-induced TLR4 pathway activation (34–38) and mediate peripheral immunosuppressive effects (18). We infer that the cell function and stimuli likely determines the phenotype that TLR4/opioid crosstalk will initiate. In the future, more studies are needed to investigate the precise mechanisms.

Both the TLR4 and Opioid Receptor Pathways Activate the MAPK Pathway

The MAPK pathway includes a range of proteins such as p38, ERK, and JNK, which are involved in many facets of cellular regulation, from gene expression to cell death (42). In the TLR4/MyD88-dependent signaling pathway, MyD88 activates TRAF6 and TAK1. Next, TAK1 activates p38, ERK, and JNK, which subsequently activate activator protein 1 (AP-1) and produce pro-inflammatory cytokines, thereby mediating the inflammatory response (2, 43–45).

The opioid receptor is primarily controlled by interactions with two proteins: G proteins and β-arrestins, which initiate G protein signaling and β-arrestin signaling, respectively (46–48). Evidence shows that both the G protein and β-arrestin pathways can activate MAPK (8, 49, 50). Acute ultra-low-dose morphine upregulated spinal phosphorylation of JNK1, JNK2, and c-Jun, and activated spinal astrocytes, which were inhibited by naloxone, MOR silencing, and a JNK inhibitor (51). The spinal JNK activated by PKC also contributed to morphine thermal hyperalgesia (51). Xie et al. showed that morphine-induced apoptosis of microglia was mediated by the GSK-3β and p38 MAPK pathways in an opioid receptor-dependent manner (52). In hippocampal neural progenitor cell lineages, ERK was also activated by morphine and fentanyl via the PKC-dependent and β-arrestin-dependent pathways, respectively (50).

The TLR4-induced MAPK pathway can initiate immune and inflammatory responses, defending against harmful stimuli (2, 43–45), while the opioid receptor-induced pathway is more complicated. Merighi et al. showed that, in activated mouse microglia, morphine acted as a pro-inflammatory mediator and induced the production of nitric oxide (NO), TNF-a, IL-1β, and IL-6 via the PKC-Akt-ERK1/2 signaling pathway in a MOR-dependent manner (53). Subsequently, the same group found that, in activated microglia treated with low-dose morphine, NF-κB was a downstream component of the PKC-Akt-ERK1/2 signaling pathway (54). As discussed above, spinal astrocytes were activated via the MOR-PKC-JNK signaling pathway and were involved in the contribution of morphine to thermal

hyperalgesia (51). The p38 MAPK pathway has also been linked to microglial activation and it contributed to postoperative thermal hyperalgesia and mechanical allodynia in rats (55) and morphine tolerance (56). Therefore, in glia, the intracellular TLR4/opioid receptor pathway crosstalk involves the MAPK pathway, which mediates the pro-inflammatory response and modifies the opioid analgesia effect (13, 14).

Opioids Induce HMGB1 Production

HMGB1 is a DNA-binding protein and is abundant in the cell nucleus (57). HMGB1 is an endogenous agonist of TLR4. During activation or cell death, HMGB1 translocates from the nucleus to the cytoplasm or extracellular space (57, 58). Extracellular HMGB1 binds to and stimulates a variety of receptors, including the receptor for advanced glycosylation end products (RAGE), TLR2, TLR4, TLR5, CD24, and other receptors (58–63). The HMGB1-RAGE signaling pathway was the first demonstrated pathway implicated in cell growth, migration, differentiation, and up-regulation of cell-surface receptors in endothelial and somatic cells (58). In addition, HMGB1-TLR4 signaling initiates the innate immune response, which activates NF-κB and produces cytokines such as TNF-α, IL-1β, and IL-6 in macrophages, monocytes, and glial cells (62, 63).

HMGB1 is passively released from necrotic or damaged cells or actively secreted by stimulated immune cells (57, 59). In macrophages and monocytes, HMGB1 was found to be released after stimulation with LPS, TNF-α, or IL-1β (64, 65). Notably, a study of chronic intrathecal injection of morphine showed that the expression of HMGB1, TLR4, and RAGE in the rat spinal dorsal horn increased (66), while another study of a neuropathic pain model showed that subcutaneous administration of morphine increased HMGB1 expression even at 5 weeks after morphine was ceased (67). In these two studies of morphine, only extracellular HMGB1, acting as a pro-inflammatory mediator, HMGB1 in the media also increased (66, 67). Taken together, these findings indicate that morphine increases the expression and release of HMGB (66, 67). Studies investigating the underlying mechanism demonstrated that TLR4, P2X7R, caspase-1 antagonists, and TLR4 siRNA inhibited the increased levels of HMGB1, while opioid receptor antagonists did not (66). Therefore, TLR4 may partially mediate morphine-induced HMGB1 production (66, 67).

TLR4/OPIOID RECEPTOR PATHWAY CROSSTALK, CENTRAL IMMUNE SIGNALING, AND OPIOID ANALGESIA

Opioids are used to treat severe pain, but they can also cause anti-analgesic effects, resulting in tolerance, hyperalgesia, or allodynia (68). Previous reviews highlighted that opioid-induced central immune signaling contributed to decreased opioid analgesic efficacy (13, 14, 23). In this section, we summarize the main opinions on opioid-induced central immune responses (13, 14, 23): (a) Non-neuronal immunocompetent cells (mainly astrocytes and microglia) in the CNS play a critical role in opioid-induced central immune signaling, modifying opioid pharmacodynamics by mediating pro-inflammatory reactivity. (b) The opioid-induced central immune signaling events include the release of a variety of immune molecules such as IL-1, TNF-α, IL-6, CCL2, CX3CL1, ATP, and NO, disruption of glutamate homeostasis, and increased neuronal excitability, which subsequently attenuate opioid analgesic efficacy. (c) Many intracellular signaling pathways are involved in opioid-induced neuroinflammation; the most prominently reported ones are the TLR4, MAPK, inositol trisphosphate (IP3)/Akt, and ceramide/sphingosine signaling pathways. Both classic opioid receptors and non-opioid receptors participate in this opioid-induced cellular adaptation. (d) *in vivo*, *in vitro*, and *in silico* approaches have demonstrated that opioids bind to TLR4 and non-stereoselectively activate the TLR4 signaling pathway. This non-stereoselective opioid activation of TLR4 triggers glial reactivity, which induces the release of neuroexcitatory immune mediators that play key roles in neuroinflammation.

The non-stereoselective response and opioid-induced hyperalgesia still observed in triple opioid receptor (MOR, DOR, and KOR)-knockout mice suggests that non-stereoselective non-classic opioid actions are implicated in opioid analgesia in these studies (10, 69, 70). At least some of these actions have been attributed to TLR4 (9, 13, 14, 21, 23). A diversity of clinically relevant opioids can bind to the TLR4/MD2 heterodimer, induce TLR4 oligomerization, and trigger a pro-inflammatory response, thereby resulting in neuroinflammation (21). Additionally, acute blockade (71, 72), genetic mutation (73), and knockout (74) of TLR4 each resulted in a significant potentiation of the magnitude and duration of opioid analgesia, compared with the observations in control animals.

However, evidence also shows that opioid tolerance and hyperalgesia were still retained in TLR4-mutant and -knockout mice (75–77). Nevertheless, findings from these mice, with regard to the influence of TLR4 on nociception, must be interpreted with caution and require further investigation. This is because of two findings: (a) some TLR4 agonists have been found to signal around TLR4 mutation (78) and (b) TLR4 is by no means the only receptor that mediates glial activation, and compensatory pathways may be activated in the absence of TLR4 (79). Hutchinson et al. believe that opioid-induced TLR4 signaling initially triggers opioid-induced central immune signaling (13); this does not mean that all opioid-induced neuronal activity depends on TLR4, but rather that this activity is complemented and facilitated by the TLR4 pathway (13).

HMGB1 is considered to be a pro-inflammatory cytokine and it is significantly expressed in rats with neuropathic pain caused by partial sciatic nerve ligation (80). Anti-HMGB1 monoclonal antibody significantly attenuated hind paw tactile hypersensitivity in these rats (80). Aside from neuropathic pain, increased HMGB1 has also been linked to other types of chronic pain including diabetic, arthritic, and cancer-induced pain (81–83). In a diabetic pain model, which involved the development of persistent mechanical allodynia, HMGB1 was significantly increased and anti-HMGB1 antibody inhibited mechanical allodynia (81). There is also other evidence demonstrating the critical role of HMGB1 in abnormal pain processing. Intrathecal, intraplantar, and perineural injection of HMGB1

produced mechanical hypersensitivity (62, 84, 85). HMGB1 is a multifunctional protein that interacts with a variety of receptors. Tolerance, hyperalgesia, and allodynia have been shown to involve HMGB1 activating the RAGE, TLR4, and TLR5 signaling pathways (60, 61, 84, 86).

In a mouse model of neuropathic pain, morphine has recently been reported to prolong the duration of mechanical allodynia for months after morphine treatment was ceased (87). The authors demonstrated that the prolonged neuropathic pain arose from activated spinal microglia, release of IL-1, and the NOD-like receptor protein 3 (NLRP3) inflammasome, a protein complex that activates IL-1β via caspase-1 (87). The amplification of spinal microglial activation may be explained by the "two-hit hypothesis," with nerve injury being the first "hit" and morphine treatment the second (87). However, the question is how spinal NLRP3 inflammasome signaling is continuously activated long after morphine treatment is stopped. In another study, Grace et al. concluded that morphine treatment leads to persistent release of DAMPs (including HMGB1 and biglycan) via TLR4, the purinergic receptor P2X7R, and caspase-1, and these DAMPs are involved in continuous NLRP3 inflammasome activation (67). There is a positive feedback loop that maintains the NLRP3 inflammasome activation, which begins with morphine-induced amplified release of IL-1β and ends with disruption of glutamate homeostasis and exacerbated release of DAMPs that increase the activation of TLR4 and P2X7R to maintain persistent NLRP3 inflammasome activation (67, 87). Opioid non-stereoselective activation of TLR4, together with the release of DAMPs that increase the activation of TLR4 and P2X7R signaling, may provide a critical initiating trigger for continuous NLRP3 inflammasome activation (13, 67).

TLR4/OPIOID RECEPTOR PATHWAY CROSSTALK AND THE IMMUNE RESPONSE

Opioid administration has been shown to inhibit the innate and adaptive immune systems at different stages, increasing the risk of opportunistic infection (16, 17, 88). Opioid-induced immunosuppression can be mediated directly via inhibition of immune cells and/or through indirect interaction with the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (16, 17, 88). However, the precise cellular mechanisms underlying the immunosuppressive effects of opioids are largely unknown.

In 1998, Gavériaux-Ruff et al. observed that in wildtype mice, but not MOR-deficient mice, morphine treatment led to compromised immune responses (lymphoid organ atrophy, a diminished ratio of $CD4^+/CD8^+$ cells in the thymus, and reduced natural killer activity) (19). Research using pharmacological antagonists and MOR-knockout mice confirmed that MOR participated in opioid-induced immunosuppression (18). TLR4 and opioid receptors are co-expressed in immune cells, and TLR4 has a key role in the innate immune response, so TLR4 may also be linked to opioid-induced immunosuppression. In this section, we summarize the opioid modulation of the immune system involving the TLR4 signaling pathway. As LPS acts solely through TLR4, research on LPS-activated immune cells is also included.

As shown in **Table 1**, MOR activation inhibited LPS-induced NF-κB DNA-binding in a NO-dependent mechanism in human neutrophils and monocytes (99). Additionally, MOR stimulation suppressed the LPS-induced p38 and ERK1/2 pathways in neutrophils (37). MOR agonists also inhibited the LPS-induced production of NO (13, 92) and prostaglandin E2 (PGE2) (94) and secretion of the pro-inflammatory cytokines IFN-α (94), TNF-α (92), and IL-8 (37, 98). Moreover, MOR agonists reduced LPS-induced macrophage viability (92), inhibited the capacity of macrophages and monocytes to respond to LPS (89, 94), and suppressed NK cell cytotoxicity (100) in both *in vitro* and *in vivo* studies. There have been some controversial studies on the MOR-induced expression of TLR4 mRNA and protein in macrophages (89, 90) and IL-6 production in neutrophils and NK cells (37, 100), as some studies indicated increases and other studies indicated decreases. Morphine, in the presence of LPS, has been shown to prevent macrophage and neutrophil recruitment to wound sites, which decreased wound closure and wound integrity and increased bacterial sepsis (93). In a study by Wan et al., although morphine facilitated macrophage autophagy initiation through the TLR4/p38 pathway, it also inhibited autophagolysosomal fusion, which decreased the bacterial clearance and increased the bacterial load (91).

In 2009, Li et al. showed that morphine-induced apoptosis was mediated via the TLR2 signaling pathway in HEK293 cells (110). Moreover, inhibition of MyD88 or overexpression of β-arrestin-2 attenuated morphine-induced apoptosis in TLR2-overexpressing HEK293 cells (110). The findings demonstrated that β-arrestin-2 negatively regulated morphine-induced TLR2-mediated apoptosis (110). However, the possible molecular mechanism was not explored in the study by Li *et al.* As previously mentioned, Madera-Salcedo et al. found that morphine-induced inhibition of LPS-induced TNF-α production was associated with the formation of β-arrestin-2/TRAF6 complex in bone marrow-derived mast cells (34). As a negative regulator of the TLR pathway (39–41), the findings indicated that β-arrestins also contribute to opioid-induced immunosuppression (34, 110). In the study by Madera-Salcedo et al., LPS stimulation led to the formation of the β-arrestin-2/TRAF6 complex, which was amplified by co-treatment with morphine (34). Furthermore, to some extent, this conclusion is consistent with a study published in 2006 showing that activation of the TLR/IL receptor increased β-arrestin-2/TRAF6 formation, but stimulation of the β_2-adrenergic receptor (a type of GPCR) did not, indicating that β-arrestins act as the intrinsic signaling molecules of the TLR/IL pathway (40). Therefore, β-arrestins, operating as a functional component of the TLR4 pathway, initiate the formation of the β-arrestin-2/TRAF6 complex; subsequently, the formation is amplified by opioid receptor signaling, which is thus implicated in the LPS-induced TLR4 signaling pathway.

On the other hand, NF-κB essential modulator (NEMO), acting as a regulatory subunit of the NF-κB complex, is also another important target site for regulating NF-κB

TABLE 1 | Effect of TLR4/opioid receptor pathway crosstalk in peripheral immune cells.

Opioid receptor	Cell type	*Vivo/vitro*	Pathway	Immunomodulatory effects	Inhibitor
MOR	Macrophages	BMDM, RAW 264.7, J774.1 cells; C57, TLR4/MOR knockout mice	Increase or decrease TLR4 mRNA and protein expression (89, 90). Potentiate autophagy initiation through TLR4/p38 pathway, but inhibit autophagosomal maturation though MOR pathway (91). Suppress LPS-activated NO and TNF-α production (92)	Compromise the capacity of macrophages to respond to LPS (89). Reduce the cell viability (92) and bacterial clearance (91). Increased bacterial load (91) and bacterial sepsis (93). Prevent macrophage recruitment to the wound site and decrease the wound closure and wound integrity (93)	Naltrexone (89), PTX (89)
	Monocytes	THP-1 and other cells	Suppress LPS-induced IFN-α and PGE2 production (94). Inhibit LPS-stimulated IL-10, IL-12 (95), and arachidonic acid, PGE2, ROI, and NO_2 production (96). Potentiate LPS-stimulated NF-κB DNA binding (95)	Decrease antiviral defense and inhibit their response to activating stimuli (94). Inhibit LPS-stimulated monocyte activation (95) and instauration of a hyporesponsive phenotype on DC development (96)	
	Mast cells	BMMCs cells, C57; MS deficient/reconstituted mice	Inhibit LPS-induced TNF-α (34, 38, 97) but not CCL2 release (38)	Resident mast cells mediate selective morphine immunosuppression (38)	
	Neutrophils	*vitro*	Inhibit LPS-induced p38, ERK1/2 pathway activation (37) and decrease TNF-α, IL-6 (37), and IL-8 production (37, 98). Inhibit LPS-induced NF-κB binding in a NO-dependent mechanism (99)	Reduce neutrophils recruitment to the wound site and decrease the wound closure and wound integrity and increase bacterial sepsis (93)	KOR antagonist (37), naloxone (98)
	NK cells	*vitro*	Increase IL-6 (naloxone) and granzymes A and B (TAK-242) production (100)	Decrease NK cell ability to induce apoptosis in K562 cells and suppress NK cell cytotoxic activity (100)	Naloxone (100), TAK-242 (100)
DOR	Macrophages	RAW 264.7 cells; sepsis rat model	Increase LPS-induced TNF-α and NO production (101). Suppress LPS-induced release of HMGB (102). DOR2: inhibit p38 MAPK activation and expression of TNF-α and MIP-2 (103)	Potentiate LPS-stimulated macrophage functions (101). Suppress LPS-induced cell death and protect rats from sepsis (102)	
KOR	Macrophages	J774 and other cells	Inhibit LPS-stimulated nitrite (104, 105), TNF-α (104, 105), IL-10 (104) and iNOS (104), IL-1 (105) and IL-6 production (105). Decrease NO release (106)	Moderate anti-inflammatory effects (104). Inhibit the cytotoxicity of macrophages (106)	Naloxone (104), naloxone (partially) (105), norBNI (104, 105)
	Monocytes	P388D1 and THP1 cells	Suppress LPS-stimulated IL-6 production (107). Inhibit LPS-induced NF-κB/p65 nuclear translocation and IL-1β, TNF-α release (108)	Anti-inflammatory effect (108)	nor-BNI (107), ML-190 (108)
	Neutrophils	Ischemia–reperfusion injured rat heart model	Attenuate the expressions of TLR4, NF-κB and TNF-α (109)	Inhibit neutrophil accumulation (109). Cardioprotective and anti-inflammatory effects (109)	nor-BNI (109)

MOR, μ opioid receptor; DOR, δ opioid receptor; KOR, κ opioid receptor; BMDM, bone marrow-derived macrophages; RAW264.7 cells, mouse leukemic monocyte macrophage cell line; BMMC, bone marrow–derived mast cells; K562 cells, a chronic myelogenous leukemia-derived; P388D1 cells, a mouse monocyte-like cell line; THP-1, human monocytic cell line; NK, natural killer cells, MOR agonists, morphine, fentanyl, remifentanil, DAMGO, and endomorphin 1/2; DOR agonists, DADLE, SNC 80, and Deltorphin-dvariant; KOR agonists, Salvinorin A, U50488H, and dynorphin 1–17; norBNI, nor-binaltorphimine (a KOR-selective antagonist); ML-190, a selective KOP receptor antagonist; TAK-242 (TLR4 signaling antagonist).

activity. Tripartite interaction motif 29 (TRIM29) is a key negative regulator of NF-κB activity, which functions via direct ubiquitination and proteolytic degradation of NEMO, which negatively regulates the production of type I interferons as well as pro-inflammatory cytokines in alveolar macrophages after infection (111). TRIM29 has been reported to inhibit the activation of the innate immune system (111, 112). Further studies are required to explore whether TRIM29 is involved in the opioid-induced inhibition of the LPS-induced TLR4 signaling pathway.

TLR4/OPIOID RECEPTOR PATHWAY CROSSTALK AND INTESTINAL FUNCTION

Constipation is the most common gastrointestinal side effect of opioids, occurring in 40–95% of patients (113). For 30 years, the opioid receptor was considered to exclusively mediate the morphine-induced inhibition of gastrointestinal transit. The supporting evidence was that MOR antagonist (naloxone) and MOR-knockout technology could abolish morphine-induced inhibition of gastrointestinal transit (114, 115). However,

TLR4 is widely expressed within the gastrointestinal tract and is associated with irritable bowel syndrome (116) and inflammatory bowel disease (117, 118), which are characterized by gut dysmotility. In 2015, using TAK-242 (a selective TLR4 antagonist), Farzi et al. demonstrated that TLR4 was also involved in morphine-induced depression of peristalsis in isolated guinea pig colons *in vitro* and it was also involved in inhibition of colorectal propulsion in mice *in vivo* (119).

However, the effects of TLR4 regarding opioid-induced inhibition of gastrointestinal transit are complicated. Farzi et al. found that TLR4 antagonism using TAK-242 failed to prevent morphine-induced inhibition of peristalsis in gastrointestinal regions besides the colorectum *in vivo* and *in vitro* (119). These findings indicated that TLR4/opioid receptor pathway crosstalk varies along the gastrointestinal tract. To some extent, the study by Farzi et al. was consistent with a study by Beckett et al. Using knockout technology, Beckett et al. showed that TLRs (TLR2 and TLR4) and the adaptor protein MyD88 participated in morphine-induced slowed movement of ingested content in mice, while *in vitro* results based on isolated colons did not support the involvement of TLRs (120); they hypothesized that TLR signaling pathways extrinsic to the colon may explain the differential involvement of TLRs (in intact animals vs. isolated colon segments) regarding the morphine-induced inhibition of the transit of ingested content (120). However, this hypothesis seems inconsistent with the well-accepted paradigm that the peripheral MOR expressed on intrinsic enteric neurons predominantly explains the phenomenon of opioid-induced constipation (121, 122), although there is still evidence supporting a central mechanism (123). Further studies are required to explore whether a peripheral mechanism vs. a central mechanism, or a combination of both, mediate the differential effects of morphine without TLR receptor signaling.

It is not an easy task to elucidate the mechanism underlying the inhibition of gastrointestinal transit. In the CNS, opioids have been demonstrated to directly bind to TLR4 and non-stereoselectively activate the TLR4 signaling pathway, which subsequently activates glial cells and initiates the immune response (13). Unfortunately, to date, not enough evidence has confirmed that non-stereoselective activation of TLR4 by opioids is also involved in gastrointestinal transit (119). Likewise, it is not wise to reject this possibility (119). Another explanation is that TLRs might be important functional components of the opioid receptor signaling pathway, and the two signaling events could interact with each other without direct binding of opioids to TLRs in the digestive system (119, 120, 124). The supporting evidence is that the opioid receptor pathway has been shown to synergize with the TLR4 pathway to impair the intestinal barrier function and increase bacterial translocation (124). In contrast, blocking the TLR pathway (either pharmacologically or using a genetic approach) elicits upon the actions of opioid agonists (119, 120). Further studies are needed to examine these hypotheses.

AUTHOR CONTRIBUTIONS

PZ and SZ designed and wrote the manuscript. MY and CC revised the manuscript. LL and XW generated the table. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by the following grants: Young Talents Foundation of Sichuan Provincial People's Hospital (2017QN10), Science Funds for Health and Family Planning Commission of Sichuan Province (18PJ157), and the Funds for Chengdu Science and Technology (2018-YF05-00332-SN).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 10 July 2020
doi: 10.3389/fimmu.2020.01210

Increasing the Chemical Variety of Small-Molecule-Based TLR4 Modulators: An Overview

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Toll-Like Receptor 4 (TLR4) is one of the receptors of innate immunity. It is activated by Pathogen- and Damage-Associated Molecular Patterns (PAMPs and DAMPs) and triggers pro-inflammatory responses that belong to the repertoire of innate immune responses, consequently protecting against infectious challenges and boosting adaptive immunity. Mild TLR4 stimulation by non-toxic molecules resembling its natural agonist (lipid A) provided efficient vaccine adjuvants. The non-toxic TLR4 agonist monophosphoryl lipid A (MPLA) has been approved for clinical use. This suggests the development of other TLR4 agonists as adjuvants or drugs for cancer immunotherapy. TLR4 excessive activation by a Gram-negative bacteria lipopolysaccharide (LPS) leads to sepsis, while TLR4 stimulation by DAMPs is a common mechanism in several inflammatory and autoimmune diseases. TLR4 inhibition by small molecules and antibodies could therefore provide access to innovative therapeutics targeting sepsis as well as acute and chronic inflammations. The potential use of TLR4 antagonists as anti-inflammatory drugs with unique selectivity and a new mechanism of action compared to corticosteroids or other non-steroid anti-inflammatory drugs fueled the search for compounds of natural or synthetic origin able to block or inhibit TLR4 activation and signaling. The wide spectrum of clinical settings to which TLR4 inhibitors can be applied include autoimmune diseases (rheumatoid arthritis, inflammatory bowel diseases), vascular inflammation, neuroinflammations, and neurodegenerative diseases. The last advances (from 2017) in TLR4 activation or inhibition by small molecules (molecular weight <2 kDa) are reviewed here. Studies on pre-clinical validation of new chemical entities (drug hits) on cellular or animal models as well as new clinical studies on previously developed TLR4 modulators are reported. Innovative TLR4 modulators discovered by computer-assisted drug design and an artificial intelligence approach are described. Some "old" TLR4 agonists or antagonists such as MPLA or Eritoran are under study for repositioning in different pharmacological contexts. The mechanism of action of the molecules and the level of TLR4 involvement in their biological activity are critically discussed.

Keywords: TLR4—Toll-like receptor 4, medicinal chemistry, inflammation, drug development, endotoxin

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *26 February 2020*
Accepted: *15 May 2020*
Published: *10 July 2020*

Citation:
Romerio A and Peri F (2020) Increasing the Chemical Variety of Small-Molecule-Based TLR4 Modulators: An Overview. Front. Immunol. 11:1210. doi: 10.3389/fimmu.2020.01210

INTRODUCTION

The immune system is a complex molecular and cellular machinery evolved to defend a multicellular organism from external pathogens and internal damages. It consists of innate immunity, based on the recognition of microbial pathogen-associated molecular patterns, PAMPs, and endogenous danger-associated molecular patterns, DAMPs, and adaptive immunity, mediated by the generation of a wide collection of antigenic sensors—the antibodies, produced by B cells (1).

Innate immunity is the first line of defense of a multicellular organism against internal or external threats. The molecular sensors of innate immunity are pattern recognition receptors (PRR), a large protein category comprising C-type Lectin Receptors, NOD-like receptors, RIG-I-Like Receptors and, most importantly, Toll-like Receptors. Toll-like Receptors (TLRs) are a family of proteins; in humans, 10 TLRs have been identified that recognize different molecular determinants or patterns from bacteria, viruses, and fungi (2).

TLR4, found in the plasma membrane of neutrophils, macrophages, dendritic and endothelial cells, selectively recognizes and responds to Gram-negative bacteria lipopolysaccharide (LPS) and lipooligosaccharide (LOS) (3, 4) (**Figure 1**).

LPS (**Figure 2A**) is the main chemical component of the Gram-negative bacteria outer membrane, and its chemical structure is characterized by a polysaccharide, the O-antigen, and a shorter oligosaccharide, the core region, bound to a glycolipid moiety called lipid A. Lipid A (**Figure 2B**) is the minimal LPS portion required to trigger immune activity through binding of CD14 and subsequent binding to the TLR4/MD-2 dimer on the plasma membrane (9, 10).

LPS is released from bacterial membrane as micelles or can be actively secreted via the formation of outer membrane vesicles (OMVs) (11). OMVs can directly deliver LPS in the cytosol of immune cells, where inflammatory caspases (caspase-4/5) serve as a specialized LPS receptor to induce the activation of the inflammasome and the production of bioactive interleukin-1β (IL-1β) and IL-18 (5).

In contrast to OMVs, the LPS contained in micelles requires the presence of accessory soluble proteins, such as LPS-binding protein (LBP), and, subsequently, CD14 and MD-2 must be recognized by TLR4.

LBP is required for transferring LPS monomers from micelles to TLR4-MD2 via the interaction with both soluble and membrane-anchored CD14 (6, 12, 13). The interactions between LBP and CD14 form a "capture and concentration module" upstream of TLR4-MD2 that regulates the ligand availability.

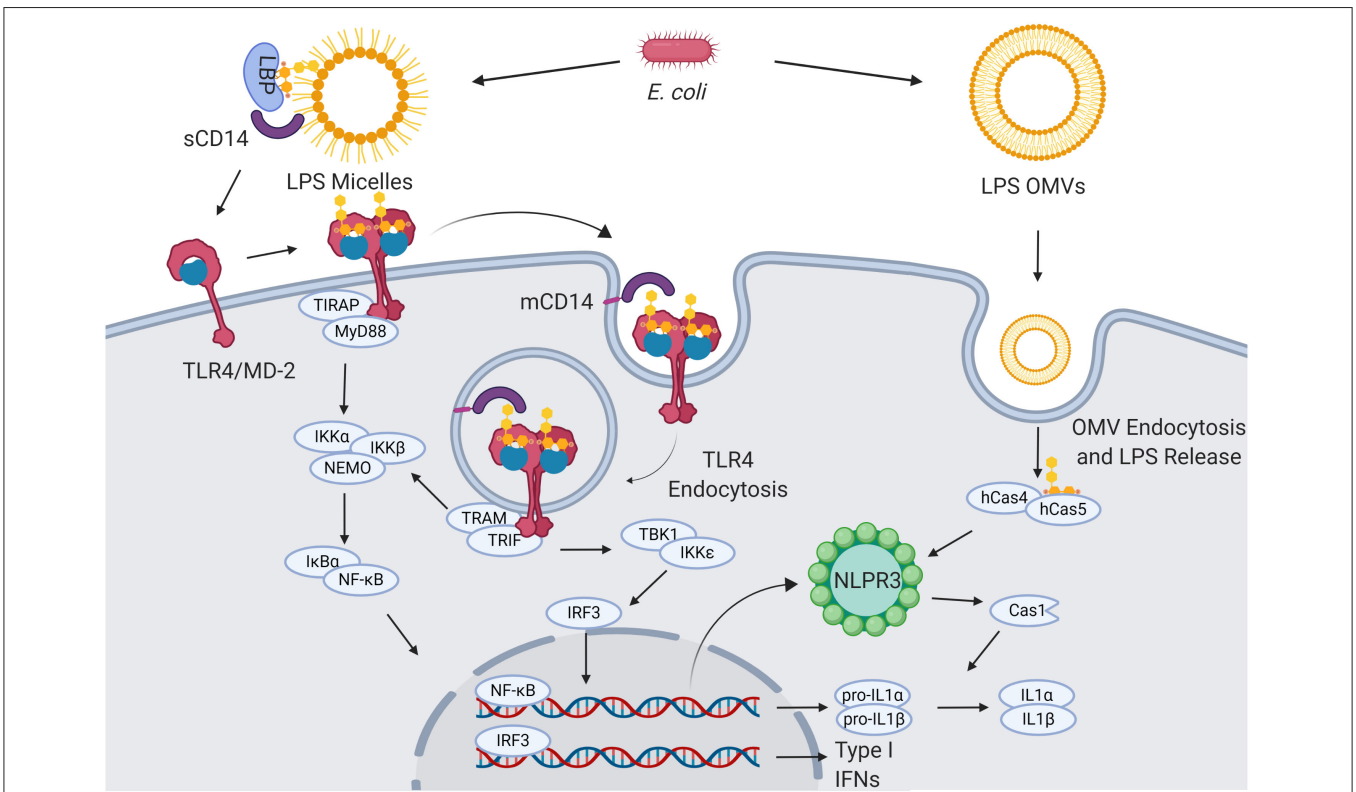

FIGURE 1 | LPS signaling. Extracellular gram-negative bacteria release LPS in the form of micelles or OMVs. OMVs and micelles containing LPS can be delivered intracellularly where LPS activates caspase-dependent responses (right). Soluble LPS-binding protein (LBP) allows CD14 to capture LPS monomers. CD14 increases the sensitivity of TLR4-MD2 for LPS and favors the re-location of the complex formed by LPS, CD14, and TLR4-MD2 in the plasma membrane lipids rafts. Once in the lipid rafts, TLR4-MD2 starts TIRAP-MyD88-dependent responses. CD14 also induces the endocytosis of LPS and TLR4-MD2. From endosomes TLR4-MD2 triggers the TRAM-TRIF pathway and thereby sustains the activation of NF-κB and the production of type I IFNs (5–8).

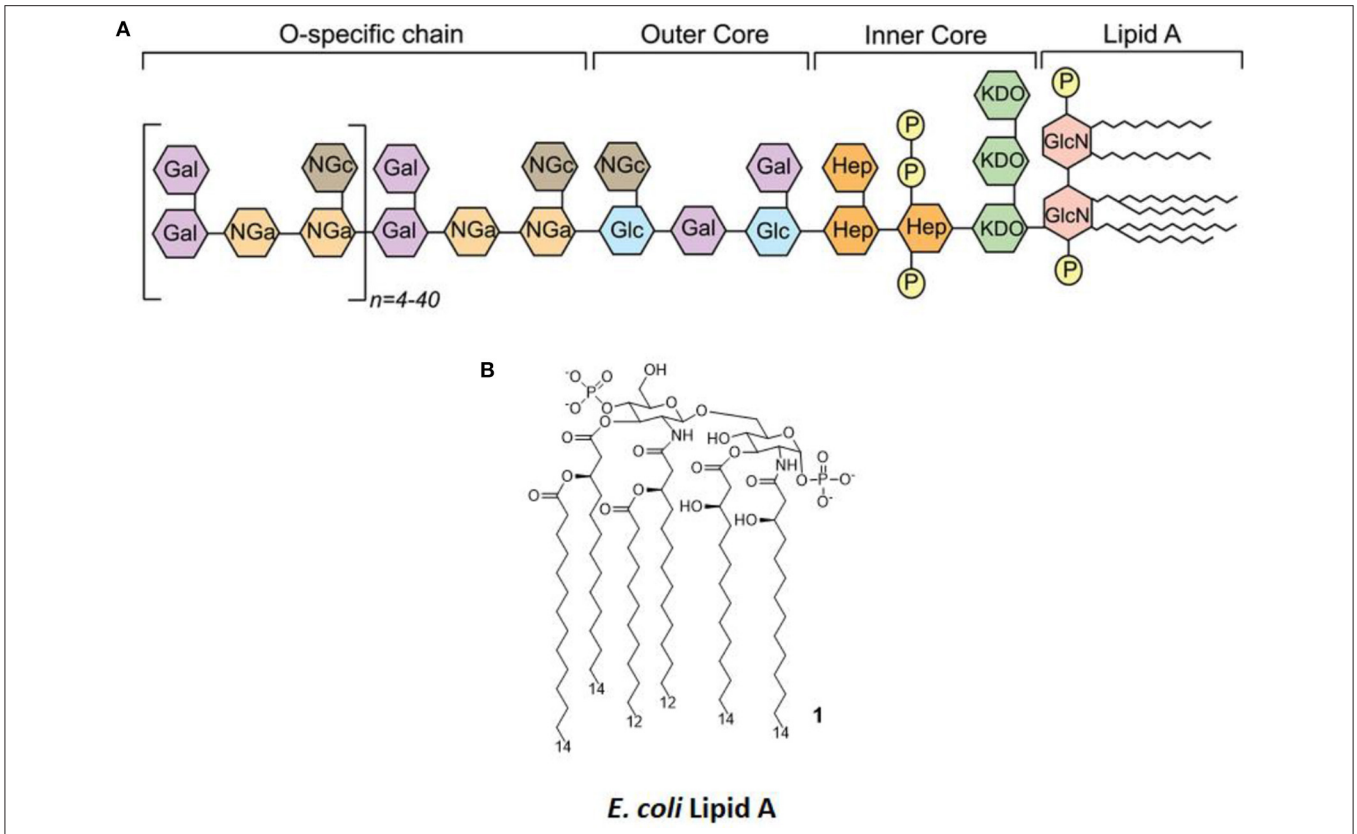

FIGURE 2 | LPS Structure. **(A)** General structure, highlighting the different regions of LPS (O-antigen, core, and lipid A) and the various sugars composing it. **(B)** The molecular structure of *Escherichia coli's* lipid A.

The process starts with the contact of LPS micelles with a soluble LPS-binding protein (LPB). CD14 is then recruited, and a transient ternary complex (LPS micelle-LBP-CD14) is formed. LPS transfer happens during this phase in which, via electrostatic interactions, LBP catalyzes multiple rounds of LPS monomer transfer to either soluble or membrane-bound CD14 (sCD14 and mCD14, respectively). Subsequently, s/mCD14 dissociates from the complex, and the single LPS molecule bound to the CD14 is then transferred to MD2 with the assistance of LRR13-LRR15 domains of TLR4 that trigger the dimerization of TLR4-MD2 and its activation (6, 13). Concomitantly with LPS presentation, mCD14 also facilitates the relocation of TLR4-MD2 in lipid rafts, where multiple signaling molecules are recruited to contribute to cell activation (14). Lipid rafts also favor the action of TLR4-independent effectors, such as specialized proteins for the internalization of the complex formed by LPS, mCD14, and TLR4-MD2 (7, 15). Once engaged by CD14, TLR4-MD2 undergoes an internalization process and moves into the endosomal compartment, where it triggers the TRIF-Related Adaptor Molecule (TRAM) and TIR-Domain-Containing Adapter-Inducing Interferon-β (TRIF)-dependent pathway, which sustains the activation of NF-κB and also induces the production of type I interferons (IFNs).

TLR4 excessive activation by LPS can lead to pathologies such as sepsis and septic shock, one of the leading death causes in western world, with a mortality rate between 20 and 50%; furthermore, it can induce the immune system to attack cells from its own organism, causing and array of autoimmune diseases (16, 17).

Modulating TLR4 activation and signaling is therefore of fundamental importance from a pharmacological and clinical point of view. On one hand, innate immunity stimulation is useful for the development of vaccine adjuvants or cancer immunotherapeutic drugs (1, 18). On the other hand, TLR4 inhibition is a therapeutic approach to Gram-negative and sterile sepsis as well as autoimmune inflammatory pathologies such as atherosclerosis, rheumatoid arthritis, or hemorrhagic shock (15, 19–21). Indeed, two compounds, Eritoran and Tak-242, reached phase III clinical trials as antisepsis agents, and both failed to meet their endpoints (21, 22).

In the perspective of developing new TLR4-directed drugs, the recent achievements (last 3 years, from 2017) on the discovery of synthetic and natural molecules that modulate TLR4 activity as agonists or antagonists are reviewed as a follow-up of our recent review on this topic (23). We focus on small molecules with drug-like properties, dividing them in two main categories according to their chemical structure, namely glycolipid- and non-glycolipid-based TLR4 modulators (**Tables 1**, **2**).

The validation of a new chemical entity as a selective TLR4 agonist or antagonist is a crucial step in the drug development

TABLE 1 | TLR4 agonists presented in this review, ranked by chemical structure (glycolipid or non-glycolipid) and stage of drug development.

Compound	Class	Drug Development Stage
MPLA (24)	Glycolipid based	Approved by FDA as vaccine adjuvant
BECC438 (25)	Glycolipid based	*In vitro*
GLA (26)	Glycolipid based	Clinical
LAM (27)	Glycolipid based	*In vitro*
E6020 (28)	Non-glycolipid	*In vivo*
1Z105 (29)	Non-glycolipid	*In vivo*
PTC (30)	Non-glycolipid	*In vitro*
LS-like (31)	Non-glycolipid	*In vitro*
VS1-like (32)	Non-glycolipid	*In vitro*
Saturated cardiolipins (33)	Non-glycolipid	*In vitro*

All agonists are validated according to the three postulates described in the text (34).

TABLE 2 | TLR4 antagonists presented in this review, ranked by chemical structure (glycolipid or non-glycolipid), stage of drug development, and mechanism of action (MOA).

Compound	Class	Drug Development Stage	MOA
FP7-like (35, 36)	Glycolipid based	*In vivo*	Competitive inhibition
LAM (37)	Glycolipid based	*In vitro*	Competitive inhibition
IAXO (38)	Glycolipid based	*In vivo*	Competitive inhibition; LPS sequestration
TAK-242 (39)	Non-glycolipid	Clinical	Non-competitive inhibition
Calixarenes (40)	Non-glycolipid	*In vitro*	Competitive inhibition
Opioid (41)	Non-glycolipid	*In vitro*	Competitive inhibition
Pip2 (42)	Non-glycolipid	*In vivo*	Competitive inhibition
Unsaturated cardiolipins (33)	Non-glycolipid	*In vitro*	Competitive inhibition
Alpinetin (43)	Non-glycolipid	*In vivo*	Down-regulation of TLR4 expression
Ferulic acid (44)	Non-glycolipid	*In vivo*	TLR4/MD-2 complex disruption

process. While TLR4 antagonist (inhibitor) validation is straightforward, as the TLR4 selectivity can be assessed through competition experiments with LPS -the natural TLR4 agonist-, TLR4 agonism assessment requires more careful investigation because it could be affected by false positive results due to endotoxin contamination. Therefore, three postulates have been proposed in order to ascertain and validate TLR4 agonists activity: (I) the requirement of bothTLR4 and MD-2 for the agonist effect; (II) the agonist or the active portion of it should be reproduced synthetically, and the synthetic derivative should preserve TLR4 activity; and (III) a specific molecular interaction between the agonist and TLR4/MD-2 must be identified (34).

Glycolipid-based compounds are Lipid A mimetics that can be obtained by (1) chemical modification of natural LPS/Lipid A, (2) direct extraction of lipid A variants after bacterial engineering, or (3) full chemical synthesis (45–47).

Some non-glycolipid compounds still reproduce the arrangement of lipid chains and phosphates found in the Lipid A but are devoid of the disaccharide scaffold (as in the case of Eisai's E6020). Others have a chemical structure totally unrelated to lipid A and have been developed by computer-assisted drug design (CADD) and a machine learning approach or have been selected from libraries of compounds (29, 32, 48).

The clinical and pharmacological potential of newly discovered, low-molecular weight (<2 kDa) compounds together with the preclinical and clinical validation level of known lead compounds is reviewed, paying special attention to validation of TLR4 targeting. **Tables 1**, **2** give a general picture of the state of the art in the clinical development of small-molecule-based TLR4 agonists and antagonists, respectively.

GLYCOLIPID-BASED TLR4 MODULATORS

Agonists

MPLA

Monophosphoryl Lipid A (MPLA, compound 2, **Figure 3**) is a well-characterized TLR4 agonist (45). MPLA is chemically derived from *Salmonella minnesota* LPS through treatment with mild acidic conditions, as this achieves the cleavage of the lipid A portion from the oligosaccharide core and the hydrolysis of the 1-phosphate group. TLR4 requirement for MPLA action has been thoroughly validated by numerous studies involving TLR4 $^{-/-}$ mice (45, 49).

MPLA is a potent TLR4 agonist, but it is weaker than LPS, as MPLA's affinity to TLR4/MD-2 is weaker than LPS. It has been also suggested that MPLA-activated TLR4 signal goes only or preferentially through TRIF-dependent and not through MyD88-dependent cascade. TRIF bias has been proposed to be related to the weaker inflammatory power and the reduced toxicity compared to LPS. TRIF bias also switches T-cell immunity to T_H1 helper, better suited for long-lasting immunization (50, 51).

MPLA is the only TLR4 agonist to be approved by the FDA for the use as a vaccine adjuvant on human (Cervarix®, Fendrix®) (52, 53).

Because of its immunostimulating activity and the lack of toxicity, the use of MPLA has been envisaged in a wide array of clinical settings. In a recent study it has been hypothesized that MPLA stimulatory activity on the innate immune system could mitigate the radiation injury provoked by ionizing radiation (IR) in cancer radiotherapy (24). Pre-treatment with MPLA prevented IR-provoked cell apoptosis *in vitro* and effectively attenuated tissue damage *in vivo*. Authors used siRNAs to knock down TRIF and MyD88 in wild type RAW264.7 cells. It was found that MPLA significantly inhibited apoptosis in TRIF knock-down cells, whereas, in MyD88 knock-down cells, MPLA had no effect on cell apoptosis induced by irradiation. These data point out that the MyD88 signaling pathway mainly accounts for the radioprotective effects of MPLA, which is in contrast to the TRIF-biased action of MPLA previously discussed.

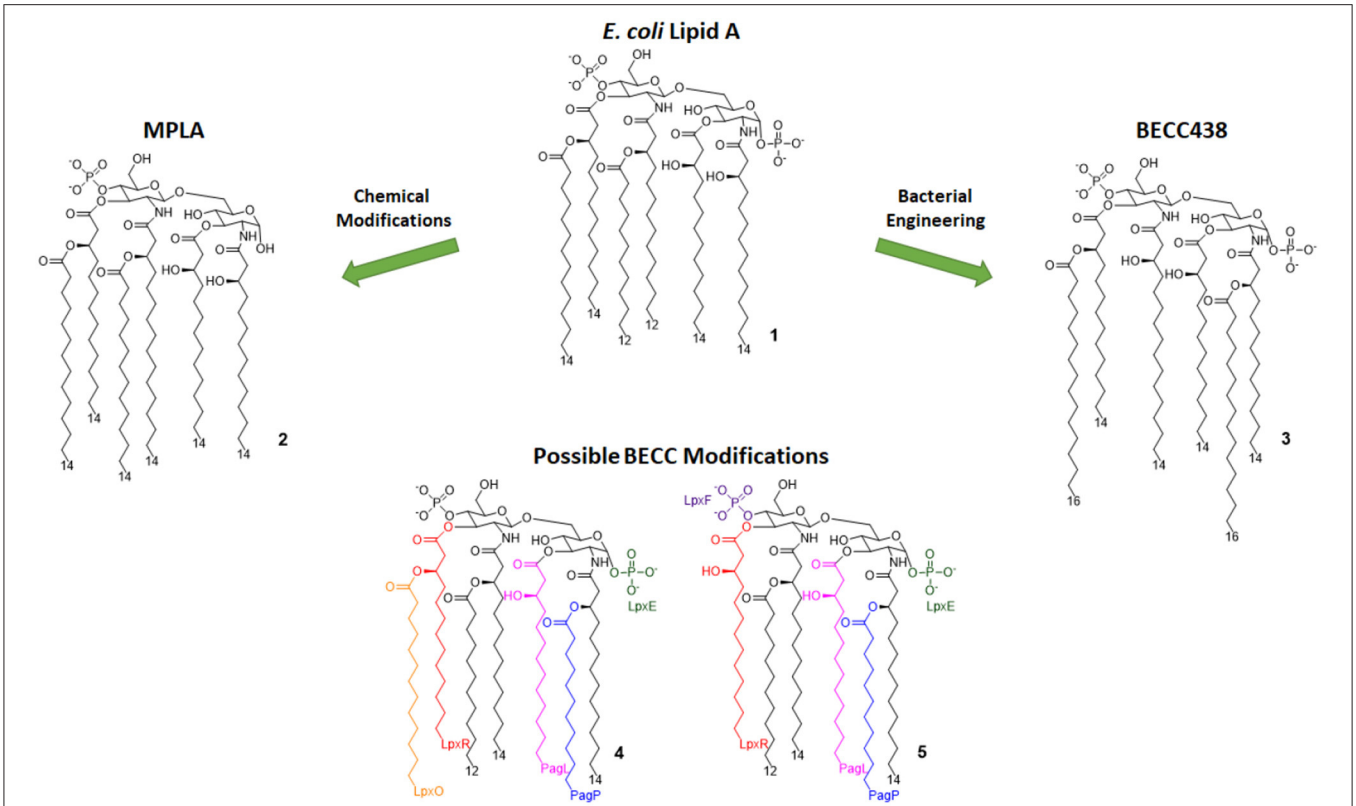

FIGURE 3 | Lipid A variants obtained by chemical and enzymatic modification. **MPLA** is obtained by chemical hydrolysis of the C-1 phosphate of the Salmonella Minnesota LPS. **BECC** (Bacterial Enzymatic Combinatorial Chemistry) allows for the selective modification of single or multiple fatty acid chains or phosphates (depicted by colors associated to involved enzymes) in order to obtain compounds such as **BECC438** with high purity.

Enzymatically Modified Lipid A

The approval of MPLA fostered the development of synthetic or semi-synthetic Lipid A variants as TLR4 modulator candidates for clinical use.

In 2013, Needham et al. developed a new technology to obtain naturally derived TLR4 agonist by using a technique that was recently named bacterial enzymatic combinatorial chemistry (BECC). BECC consists in bacterial gene engineering, removing or adding enzymes in LPS biosynthesis pathway, allowing the isolation of LPS/lipid A variants with non-natural modifications and their straightforward isolation from bacterial pellets without further purification (54).

In 2017, BECC was performed on an attenuated *Yersinia pestis* strain, consequently obtaining lipid A variants that were then screened *in vitro* and *ex vivo*, showing TLR4 activation levels comparable to those obtained with previously described MPLA (46).

Particularly, a compound named BECC438 (compound 3, **Figure 3**) showed good *in vitro* activity, which suggested a follow-up study *in vivo* to confirm its viability as a vaccine adjuvant and to compare its efficacy to other adjuvants (Alum and PHAD). All mice immunized using non-formulated BECC438 as an adjuvant survived after being challenged with *Y. pestis*: indeed, BECC438 group's survival rate (100%) was better than both Alum and Glucopyranosyl Lipid Adjuvant (GLA, see next paragraph) groups (both scored 80% survival rate), suggesting that properly formulated BECC438 could exceed GLA efficacy and encouraging follow-up studies on its use as a vaccine adjuvant (25).

Glucopyranosyl Lipid Adjuvant (GLA)

The Glucopyranosyl Lipid Adjuvant (GLA, **Figure 4**) has been developed by Avanti Polar Lipids Inc. as a fully synthetic MPLA analog with TLR4 agonistic activity (tradename: phosphorylated hexa-acyl disaccharide PHAD®) (55, 56). Being fully synthetic, the main advantage of this compound is its chemical homogeneity, which improves activity and safety with respect to MPLA, a semi-synthetic molecule. Moreover, LPS contamination is avoided (57).

In recent years, GLA has been formulated as a vaccine adjuvant both as aqueous formulation (GLA-AF) and as an oil-in-water stable emulsion (GLA-SE). When compared to MPLA in terms of activity, it showed an overall better response (57, 58).

GLA-AF was tested as a nasal vaccine adjuvant for HIV immunization *in vivo* on mice and rabbits, resulting in a good immunization profile with strong mucosal immune responses (59, 60). In 2018, Anderson et al. tested HIV immunization in humans following nasal administration of a vaccine containing GLA-AF as adjuvant and the HIV-1 CN54gp140 antigen. Early transcriptional signatures were investigated to identify

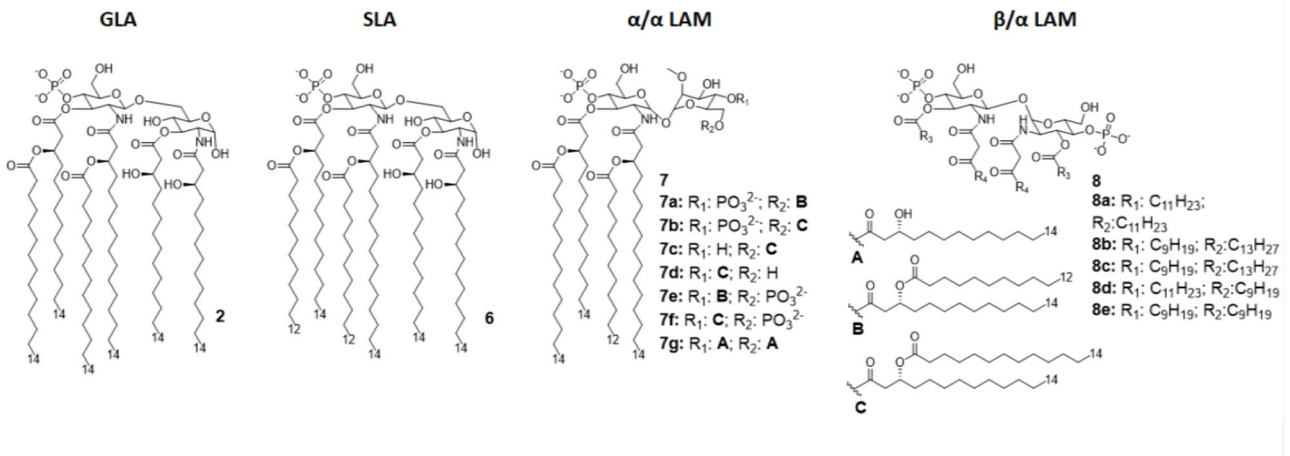

FIGURE 4 | Disaccharide-based synthetic Lipid A analogs. **GLA** is a fully synthetic MPLA. **SLA** is a second generation GLA optimized to be more compatible with MD-2 (by introducing C12 acyl chains). **LAMs** are trehalose-derived compound: changing the absolute configuration of the glycoside bond LAM allow the switch from TLR4 agonism to antagonism.

differentially expressed genes (DEG) and blood transcription modules (BTM) correlated with vaccination and successful immunization (26). Results were encouraging, indicating the activation of numerous vaccine related DEG and BTM, and this therefore suggests that immunization occurred. However, the small number of subjects involved and lack of analysis in the first 7 days suggest that additional studies are needed to validate data.

Recent advancement in cancer immunotherapy involving TLR to (re-)activate immune cells suggested the use of a GLA-SE (named G100) as a stand-alone cancer immunotherapeutic (61, 62).

Following a successful *in vivo* study on an A20 lymphoma murine tumor model in which half of the mice got regression in a dose dependent manner (63), a first clinical trial was started on a small number (n=10) of patients affected by merkel cell carcinoma (MCC), based on intratumoral injection of a low dose of G100. Out of the 10 patients, three presented local disease and were then treated with surgery (cohort A), while some presented a metastatic disease (cohort B). All patients in cohort A successfully completed surgery and radiotherapy after administration of G100, and two of them remained recurrence free; patients in cohort B received only G100 and two of them went in full remission (64).

The brilliant results obtained by GLA experimentations urged the development of an even better TLR4 glycolipid agonist, having higher efficiency and lower toxicity. In this way, Carter et al. recently developed a second-generation lipid adjuvant (SLA), reducing the length of two lipid chains from C14 to C12 (compound 6, **Figure 4**). Computational docking studies show that the reduction of the Hydrophobic part make this lipid A derivative better accommodate into the MD-2 hydrophobic pocket, allowing for and stronger interaction with TLR4/MD2 (65).

The activity of SLA and its TLR4 selectivity has been assessed both *in vitro*, *ex vivo,* and *in vivo* (65) SLA has been then formulated as an oil-in-water stable emulsion (SLA-SE) and tested *in vivo* as an adjuvant for nasal *Enterotoxigenic E. coli* vaccine in comparison with double-mutant LT (dmLT) adjuvant: results suggest that SLA-SE is at least as effective as dmLT, but it is able to further augment some of the specific immune responses (66).

Trehalose Derivatives (LAM)

While TLR4 plays a pivotal role in innate immunity, particularly protecting against infectious challenges and boosting adaptive immunity, it is not the only factor causing inflammation in the only LPS receptor. Indeed, caspase 4/5/11-mediated NLRP3 inflammasomes, activated by cytosolic LPS, is a crucial pathogenic factor in a variety of acute and chronic immune related diseases (67).

In order to obtain new agonists with increased TLR4/inflammasome selectivity, Zamyatina et al. aimed to design molecules capable of activating only the TLR4 pathway without activating NLRP3. To achieve this result, according to a computational structural analysis of the TLR4 dimerization process, two separate hydrophobic clusters are needed in the ligand to optimize the binding with the hydrophobic pocket of MD-2/TLR4, crosslinking the second MD-2*/TLR4* and consequently forming the activated $(TLR4/MD\text{-}2/ligand)_2$ complex. Seven novel trehalose-derived disaccharides were projected and synthesized based on an α,α-(1-1')-linked diglucosamine scaffold (Lipid A Mimetics, α/α LAMs, **Figure 4**). The conformational rigidity of the α,α glycosidic bond was exploited by rational design to obtain the two separate hydrophobic clusters for MD-2 binding and TLR4 activation (27, 37).

The activity of α/α LAM was tested on mononuclear cells (MNC), human airway epithelial cells (Calu-3) and human

monocytic cell line THP-1 and observed that, while 4'-6-diphosphate compounds (compounds 7a-b and 7e-f, **Figure 4**) induced both TLR4 and caspase 4/11 activity, monophosphate compounds 7c and 7d (**Figure 4**) effectively decoupled TLR4 and NLRP3, exclusively activating TLR4 without triggering a NLRP3-dependent response: these results open the way for future synthesis of safer TLR4 agonists and for clarifying the role of caspase 4/11 activation in inflammasome (27).

Interestingly, changing the stereochemistry of α/α glycosidic bond into β/α bond, a shift from TLR4 agonism to antagonism was observed (37). Indeed, five novel β(1-1')α linked diglucosamine LAMs, containing 2-N-, 2'-N-linked β-ketoacyl lipid chains were synthesized (α/β-LAMs, compounds 8, **Figure 4**). These new compounds were then tested for their antagonist activity *in vitro*, obtaining full inhibition of LPS-stimulated cytokine production at 1 μg/mL concentration. Surprisingly, concentrations higher than 10 μg/mL showed reduced antagonist activity, probably because the formation of aggregates. Finally, molecular dynamics simulations showed that MD-2 affinity of LAMs is higher than LPS. The keto-enolic tautomerism on acyl chains of LAMs very likely provides free hydroxyls that can be involved in additional interactions through hydrogen bonds with residues at the rim of MD-2 binding pocket (37).

Antagonists

Anionic Monosaccharide-Based TLR4 Antagonists

Synthetic monosaccharide mimetics of Lipid X, a monosaccharide biosynthetic precursor of lipid A, showed TLR4 antagonist activity in murine macrophages (68, 69).

A large panel of synthetic monosaccharide-based TLR4 modulators, named Gifu Lipid As, contain one or two phosphates groups and a variety of modifications in fatty chains length and nature as well as in their binding mode to glucosamine: esters, amide, ethers, and amines were used (70, 71).

Following this trend, monosaccharide-based pure TLR4 antagonists, called FP compounds, were developed, and they are active in inhibiting the LPS-stimulated TLR4-dependent cytokine production in human and murine macrophages in a dose-dependent manner (IC_{50} from 0.46 to 3.2 μM) (72).

FP compounds were tested as potential therapeutics in different clinical settings. The lead compound FP7, with two C-14 fatty acid chains, showed the ability to protect motoneurons from microglia activated by LPS in an *in vitro* motoneurons/microglia co-culture model of ALS (73).

The capacity of FP7 to protect mice from DAMP/TLR4 activation as a consequence of influenza virus pulmonary infection was evaluated (74). FP7 turned out to protect mice from acute lung injury (ALI), one of the most prominent influenza-related damages, and increase survival after viral infection with an efficiency similar to Eritoran, a well-established TLR4 antagonist developed by Eisai (75). In this model of infection, ALI would induce DAMP release from damaged tissues, likely HMGB1 and oxidized phospholipids, which, in turn, hyperactivate TLR4 with a subsequent cytokine storm and acute sepsis-like syndrome. An experiment on DCs activated by HMGB1 suggested that FP7 can block HMGB1-dependent TLR4 activation. Further data should be collected to assess the activity of this type of antagonist to block TLR4 activation by oxidized phospholipids (oxPL) and other DAMPs that highly likely are produced by ALI.

TLR4 gene deletion in hematopoietic and non-hematopoietic cells protects animals against cardiovascular diseases (CVD), suggesting a key role of the receptor in these pathologies (76). The potential of FP molecules to impact on inflammatory CVD was investigated *in vivo* on Angiotensin II-infused apolipoprotein E-deficient mice. After validating the capacity of FP7 to inhibit cytokine production *in vitro* on human umbilical vein endothelial cells (HUVEC), THP-1, and RAW 264.7 cells, *in vivo* experimentation demonstrated that Angiotensin II and FP7 co-administration prevented the initiation of sterile inflammation, protecting mice from consequent CVD (77).

Interestingly, in addition to inhibition of LPS-induced TLR4 signaling, FP7 negatively regulated TLR4 activation in response to ligands of sterile inflammation, namely, hydroperoxide-rich oxidized LDL (oxLDL) *in vitro* and Angiotensin II infusion *in vivo* (77).

Taken together, these studies suggest that FP molecules are able to contrast the action of structurally diverse DAMPs from HMGB1 (74) to oxLDL (77).

The synergistic action of FP monosaccharides with antibacterial peptides neutralizing LPS was recently investigated (78). After LPS stimulation, FP7 was co-administrated to cells together with two anti-microbial peptides: cecropin A–melittin (CA–M) or LL-37, a human cathelicidin that binds to and neutralize LPS (79, 80). A synergy between TLR4 antagonists and cationic peptides was observed in inhibiting TLR4-dependent cytokine production and NF-kB activation. Interestingly, the synergy was observed also in a case where TLR4 was activated with lectins. DOSY NMR experiments and TEM microscopy images suggest a change in the supramolecular aggregation state of peptides caused by the interaction with FP7 (78).

Two studies focused on the investigation of the structure-activity relationship (SAR) in FP monosaccharides (as depicted in **Figure 5**): one explored the effect of the length of saturated fatty on the TLR4 activity and the second investigated both the effect of unsaturated fatty chains and the suitability of succinate groups as bioisosteres of phosphate groups (35, 36).

In both studies, molecules were firstly designed *in silico* through docking with MD-2 receptor followed by and molecular dynamics simulation. Molecules were then synthetized and tested for their capacity to bind to MD-2 and to inhibit LPS-stimulated TLR4 activation in human and murine macrophages. The first study pointed out a very precise trend of activity on cells and MD-2 binding potency, indicating the compounds with C12 and C14 carbon chains (**Figure 5**) are the most active in inhibiting TLR4 activation and cytokine production (35). Interestingly, the compound with C16 was found to be totally inactive. The C12 and C14 compounds (compounds 11b and 9, respectively, **Figure 5**), named FP12 and FP7, respectively, were shown to form less tight aggregates with a higher fluidity of fatty acid chains than the C16 compound. As in the case of lipid A derivatives, it is very likely in this class of amphiphilic monosaccharides that the supramolecular structure

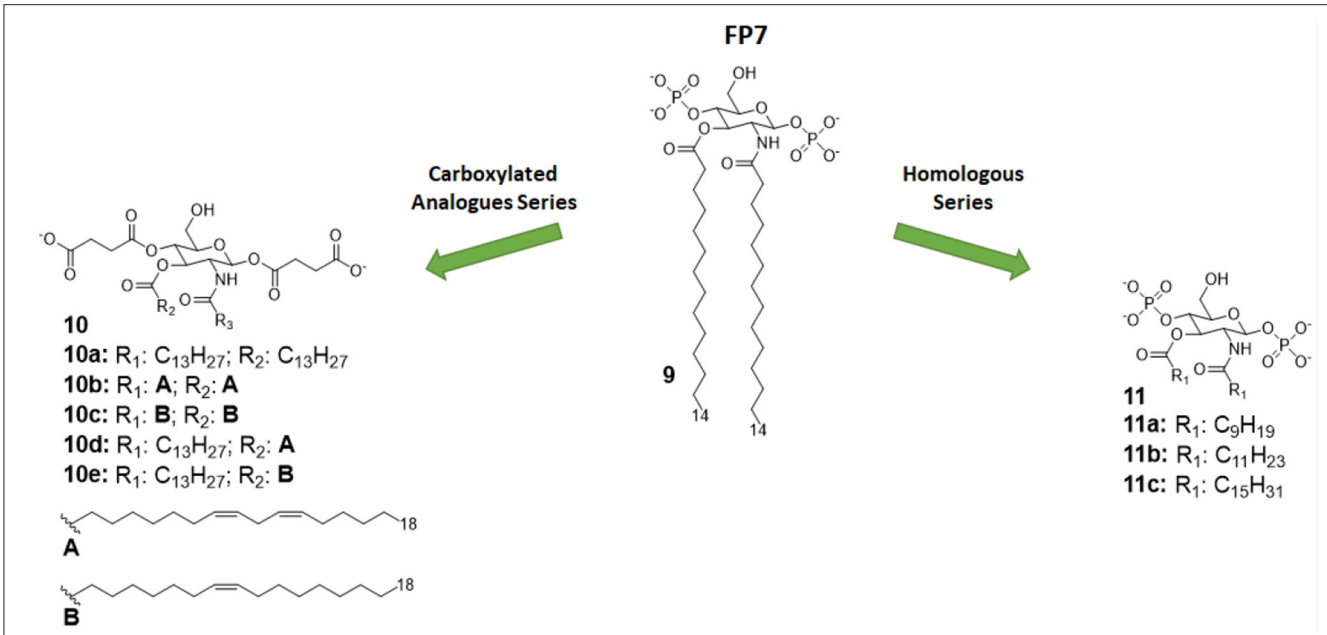

FIGURE 5 | Monosaccharide TLR4 antagonist of the FP series and their carboxylate analogs.

and the stability of aggregates influences the biological activity (8, 81).

The carboxylate analogs (Compounds 10, **Figure 5**) with two succinate esters units instead of phosphates retained TLR4 antagonist activity with an IC_{50} in the same range of lead compound FP7. Furthermore, the structure of the fatty acid chains turned out to be essential to TLR4 activity. Paralleling the SAR results in the FP family, the series of saturated fatty chains presented a maximum activity again around C12 and C14, while shorter (C10) and longer (C16) chains were unable to interact with MD-2 to inhibit TLR4-dependent cytokine production. On the other hand, unsaturated lipids retained activity even with longer chains (C18). It is known that unsaturated fatty acids are present in TLR4 antagonists such as the LPS synthesized by *Rhodobacter sphaeroides* (RS-LPS) or *Rhodobacter capsulatus* (RC-LPS) and the synthetic Eritoran. The results reported in this paper confirm that the presence of one unsaturation in the fatty acid chains favors the switch to antagonism (36).

Cationic Monosaccharide-Based TLR4 Antagonists

IAXO compounds are a class of cationic amphiphiles active as TLR4 antagonists. They are formed by a glucopyranose or a benzylamine core linked to two C14 lipid chains through ether bonds (compounds 12 and 13, **Figure 6**) (38).

IAXO's TLR4 antagonism is very likely the combination of two effects: in the form of cationic liposomes, these molecules form stable co-aggregates with LPS and make it less available for binding with CD14 and MD-2 (82). On the other hand, mechanism studies clearly show the ability of IAXOs to bind CD14 and MD-2, competing with LPS and displacing it from receptors (83–85).

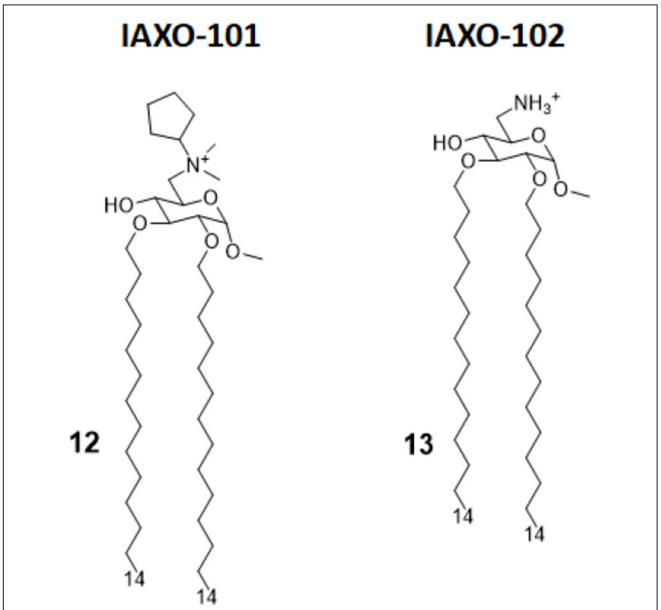

FIGURE 6 | IAXO-101 and IAXO-102 are glycolipid compounds bearing a positive charge. They form stable aggregates with LPS and compete with LPS for TLR4 binding.

In a new study on the role of TLRs in Placental Malaria (PM) by Barboza et al., IAXO 101 was used to assess the involvement of TLR4 in infant morbidity and mortality in a group of pregnant mice affected by *Plasmodium berghei* $NK65^{GFP}$, and its effect was

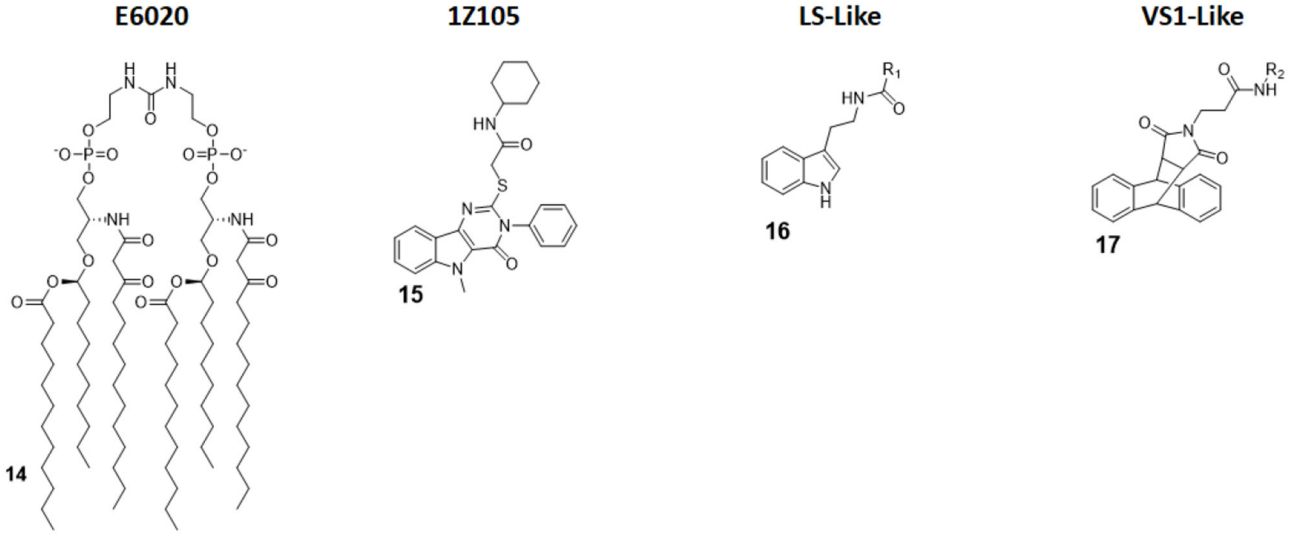

FIGURE 7 | Non-Glycolipid TLR4 agonists. **E6020** is a linear lipid A analog developed by Eisai Inc., currently in clinical trial in cancer immunotherapy. **1Z105**, a pyrimidoindole, is currently being tested *in vivo* as vaccine adjuvant. **LS-** and **VS1-**like compounds are novel TLR4 agonist structures obtained by computational approach.

compared with a group of TLR4$^{-/-}$ mice. While TLR4$^{-/-}$ mice did not show PM, and their fetuses did not show differences in body weight compared to non-infected WT mice, experiments demonstrated that mice treated with IAXO 101 2 weeks after infection showed a partial reverse in placental malaria, and their fetuses had an intermediate body weight between infected and non-infected WT mice. In addition to demonstrating the involvement of TLR4 in PM, this study also highlights the viability of IAXO 101 as a treatment for this pathology, which causes high neonatal mortality (86).

Another recent application of IAXOs has been the prevention of blood–brain barrier (BBB) disruption after subarachnoid hemorrhage (SAH). Okada et al. aimed to study the linkage between TLR4 activation and inflammatory BBB disruption (39). In an animal study, SAH was induced in C57BL/6 male mice, which were eventually treated with two different dosages of IAXO 102 (compound 13, **Figure 6**) after 30 min. This resulted in a significantly improved neurological score and in clear protection from BBB disruption. A control experiment was conducted involving TAK-242, a well-established TLR4 antagonist, providing similar results. Those experiments highlighted for the first time that BBB disruption after SAH is linked to TLR4 activation and can be efficiently reversed by administration of potent TLR4 antagonists as a treatment for post-SAH BBB disruption (39).

NON-GLYCOLIPID TLR4 MODULATORS

Agonists

Linear Lipid A Analogs (E6020)

E6020 (compound 14, **Figure 7**) is a synthetic agonist patented by Eisai Inc., which has been previously been experimented on as a vaccine adjuvant *in vivo,* and it turned out to be a viable alternative to traditional alum adjuvant both on boosting mucosal and systemic antibodies responses and in enhancing vaccine efficacy on a toxic shock syndrome model (48, 87, 88).

Following these successes, it has been recently assessed on the central nervous system (CNS) to test its activity in enhancing remyelination in spinal cord white matter following lysolecithin-induced demyelination. Remyelination is mediated by oligodendrocytes, which are vulnerable to a series of pathologies and infection: when their number is low, they can be replaced by oligodendrocyte progenitor cells (OPCs) after differentiation. However, myelin debris prevents OPCs differentiation, effectively hindering remyelination process. Indeed, it seems that the presence of E6020 stimulates macrophages to remove myelin debris, which is a vital step; this allows for and enhances remyelination in lysolecithin-induced demyelination animal models. The remyelination is therefore linked to TLR4 activation. This novel study opens the possibility to use TLR4 agonists to repair damages caused by aging or injury, and this prevents a series of CNS pathologies, including dementia (28).

Pyrimidoindoles

Pyrimido[5,4-b]indoles are a class of synthetic TLR4 agonists first identified by Cottam and coworkers through a high-throughput screening (HTS) approach (89). Subsequently, a structure-activity relationship (SAR) study allowed to select 1Z105 (compound 15, **Figure 7**) as the best agonist compound. 1Z105 has been tested as a vaccine adjuvant in combination with 1V270, a TLR7 agonist (89, 90).

As a follow-up of these studies, an influenza vaccine formulated with both 1Z105 and 1V270 was shown to function *in vivo* through TLR4 and TLR7 activation without any significant off-target effect, and it succeed in inducing protective immunity. The activation of TLR4 by 1Z105 mainly activated the MyD88 pathway. Furthermore, the TLR4 and TLR7 agonists worked

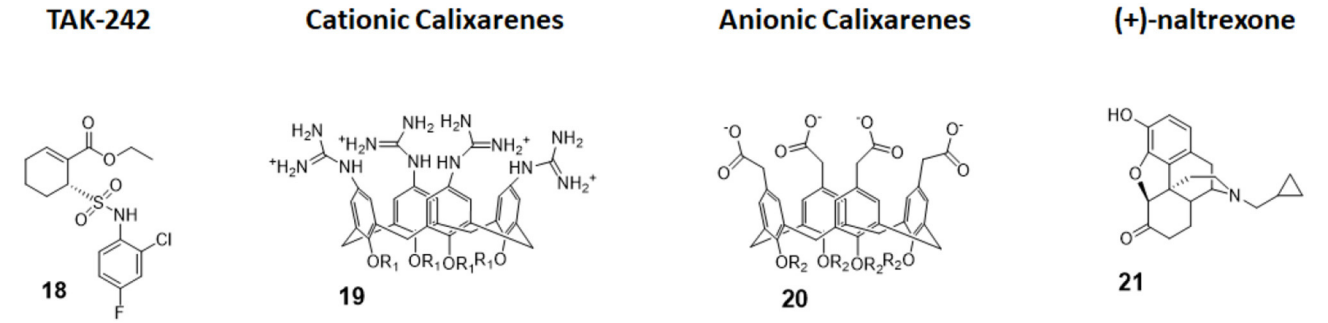

FIGURE 8 | Non-glycolipidic TLR4 antagonists. **TAK-242** is a potent TLR4 inhibitor non-competitive toward LPS. Cationic Calixarenes, were able to block TLR4 signaling while anionic calixarenes were inactive. **(+)-N17**-subsituted Naltrexone derivatives are the only known TLR4 antagonists able to cross the BBB to date and are drug candidate for neuropathic pain.

synergistically to reach a high adjuvant potency, allowing for a dose reduction of the antigen to achieve equivalent protection and enhancing the vaccine safety profile (29).

New Rationally Designed TLR4 Agonists

Michaeli et al. recently projected linear and cyclic peptides with the ability to bind MD-2/TLR4 and CD14/TLR4 by computer-assisted drug design (CADD). They used *ab initio* methods coupled with machine learning discovery software, which allowed the finding of a higher percentage of active molecule compared with an HTS approach. New cyclic peptide sequence containing also D-amino acids to increase conformational rigidity and drug-likeness were designed to dock with hMD-2 and the N-terminal region of h-CD14 using the CYCPEP program (30). Subsequently, *in silico* designed MD-2 and CD14 ligand peptides were synthesized and tested for their activity under physiologically relevant conditions by determining IL-1β release upon culture in human whole blood. Out of 27 linear and 26 cyclic peptides, two peptides (PTC-A-40 and PTC-A-83) were shown to be active in stimulating IL-1β production, validating the use of *ab initio* method to search for TLR4 ligands (30).

Honegr et al. investigated the advantages of *in silico* drug design in the search for TLR4 agonists, by using Ligand- or Structure-Based Virtual Screening (LBVS or SBVS). A large library of molecules (130,000) was screened *in silico* for their capacity to bind to a 3D model of hTLR4/MD2 heterodimer (PDB ID: 4G8A, RCSB Protein Data Bank). Two hit compounds were identified that optimized binding score: a N-(2-(1H-indol-3-yl)ethyl)benzamide (LS-like, compound 16 **Figure 7**) and a anthracene-succinimide hybrid (VS1-like, compound 17, **Figure 7**). Both compounds were then synthetized and chemically modified for SAR studies. While LS and LS-derived molecules didn't achieve a good activity profile (10% of MPLA activity), VS1 and VS1-derived molecules showed a much more promising efficacy when tested *in vitro* and *ex vivo*, scoring 50% of MPLA activation (31, 32).

TLR4 Antagonists

TAK-242

TAK-242 (compound 18, **Figure 8**) is a cyclohexene carboxylic ester derivative, produced by Takeda Pharmaceutical Company Ltd, that shows strong action (IC_{50} 1 to 11 nM) as a specific TLR4 non-competitive inhibitor (91). Indeed, studies performed by Takashima et al. (92) and Matsunaga et al. (93) demonstrated that TAK-242 binds intracellularly TLR4: it acts as a Michael acceptor for Cys747 residue present in the TIR domain of TLR4. Therefore, TAK-242 disrupts the TIR domain conformation and subsequent interaction with both TIRAP and TRIF, and this inhibits both MyD88-dependent and MyD88-independent pathways (92, 93).

TAK-242 was administered on sepsis patients in various intensive care units worldwide in a phase III clinical trial. Unfortunately, trials were terminated because TAK-242 was ineffective in reducing mortality and in suppressing cytokines production. Although the reasons for failure are unknown, a combination of individual differences in severity of illnesses and delay in administration of the drug are thought to be the main causes. Furthermore, enrolment in the study of patients without Gram-negative bacteria infections probably affected the results (22).

A new study by Wang et al. sought to investigate TAK-242 influence in coronary microembolization (CME)-caused myocardial apoptosis, starting by the fact that TLR4 had been demonstrated to be a promising target for atherosclerotic cardiovascular diseases treatment (94). In the study, the authors were able to reproduce i*n vivo* models of CME in mice. CME mice treated with TAK-242 showed a significant improvement in cardiac function and a decrease in micro-infarction area and in apoptotic index when compared with untreated mice, validating TLR4 as a target in this pathology and suggesting treatment with TLR4 inhibitors as an efficient therapeutic approach. However, authors claim the necessity of further studies, as they only experimented short-term effects of TAK-242 and the animal model of CME, obtained by plastic microspheres injection, does not completely mimic microembolization in patients (95).

Calixarene Amphiphiles

Calix[4]arenes are cup-shaped organic molecules formed by four or more phenol units linked together by methylene bridges. Calix[4]arenes possess a central hydrophobic conical cavity, and both cavity rims could be chemically functionalized to improve or modulate water solubility. The presence of a cavity and the

possibility to synthetically change their chemical structure and therefore modulate water solubility make calix[4]arenes, together with cyclodextrins and cucurbituryls, optimal hosts to carry small molecules and drugs. In recent years, there was a growing interest in calixarenes as drug carriers as they are biocompatible and show low cytotoxicity (96–99).

The capacity of amphiphilic calixarenes to modulate TLR4 signal was studied in cationic calix[4]arenes functionalized with guanidine groups on the upper rim (compounds 19, **Figure 8**) and anionic calix[4]arenes with carboxyl groups (compounds 20, **Figure 8**) (40).

Surprisingly enough, anionic calix[4]arenes, which should better mimic the negatively charged, amphiphilic lipid A, did not show any activity on TLR4. On the other hand, positively charged guanidinocalixarenes (compounds 19, **Figure 8**) successfully inhibited TLR4 activity in a dose-dependent manner, with an IC_{50} ranging from 0.7 to 63 μM. A previous report by Chen et al. (100) described the capacity of similar guanidino calix[4]arenes to neutralize the action of LPS by binding it. The authors sought therefore to verify if the activity of calixarenes in blocking TLR4 signals derived exclusively from LPS binding and neutralization or from a direct action on the TLR4/MD-2 complex or a combination of these two effects. Cells were treated with a plant lectin, which is known to activate TLR4 by a mechanism different than LPS, and then with different doses of guanidinocalixarenes. A dose-dependent TLR4 inhibition was still observed, and this is suggestive of a direct effect of calixarenes on the receptor (40).

Opioid Derivatives

The opioid inactive isomer (+)-naltrexone has emerged as the only known TLR4 antagonist having the required LogP to easily cross the blood–brain barrier, making it an interesting lead for the treatment of neuropathic pain and drug addiction (101). While a previous study by Wang et al. confirmed that (+)-naltrexone inhibits TRIF/TRAM pathway and binds to MD-2, the molecular mechanism of action and the precise binding to TLR4/MD-2 and/or CD14 interaction is still unclear (102).

Wang et al. recently investigated the interaction with MD-2 by molecular docking and experimentally validated the found binding affinity by *in vitro* fluorescence binding studies. Studying a variety of (+)-naltrexones derivatives substituted with different groups on nitrogen N-17, it turned out that the enhancement of the hydrophobic character of the molecules by the introduction of octyl, phenylethyl, or methylcyclopropyl groups (compound 21, **Figure 7**) improved MD-2 binding affinity. Adding a methyl group onto N-17 leads to quaternary ammonium cations, which showed poor MD-2 binding affinity ($K_d > 40$ μM) and lost the TLR4 antagonistic activity. Authors concluded that the binding of (+)-naltrexone and its derivatives to MD-2 are primarily driven by hydrophobic interactions. However, polar interactions, which includes both electrostatic interactions and polar solvation free energy, were negatively correlated with experimentally determined binding affinities (41).

Peptide Antagonists PIP2 and cPIP2

A phage display (PD) library of 12-mer peptides was constructed by enriching through six rounds of biopanning against hTLR4. One of the five selected peptides, PIP2, a rather hydrophobic 12-mer, inhibited LPS-stimulated TNF-α and IL-6 production in murine and human macrophages with an IC_{50} of 40 μM (42). Besides the relatively weak activity, PIP2 showed some out-of-target inhibitory effects on TLR2. In order to assess the PIP2 mechanism of action, fluorescence binding studies, surface plasmon resonance, confocal microscopy with both fluorescently labeled TLR4, and peptide and molecular dynamics experiments were run. All experiments pointed to a direct interaction between PIP2 and MD-2. Encouraged by these promising results, the authors cyclized PIP2 by a lactam bridge (cPIP2), a common strategy to force α-helix and rigidify small peptides, with the aim to enhance activity and drug-likeness. Indeed, cPIP2 showed better inhibitory profile on TLR4 (IC_{50} 25 μM) and was further tested *in vivo* in rheumatoid arthritis (RA) mice model. cPIP2 successfully alleviated RA symptoms in mice over a period of 6 weeks, improving histological scores, which suggests the use of cyclic PIP2 as drug lead in RA (42).

Cardiolipin

Cardiolipins (CLs) is a family of tetra-acylated diphosphatidylglycerols naturally produced by animals, plants, bacteria and yeasts, and they have with different fatty chains lengths and saturations (33). Unsaturated CLs showed activity as TLR4 antagonists although the precise molecular mechanism remains to be studied (103, 104). As already mentioned in this review, unsaturated fatty acids are present in natural and synthetic TLR4 antagonist. This suggests that the unsaturation of the acyl chains contributes to enhance TLR4 antagonist behavior. An exhaustive SAR study on CL variants was recently published, and it focused on the influence of chain lengths and saturation degree (105).

The activity on cells of a series of saturated and unsaturated derivatives (compounds 22 and 23, **Figure 9**) was tested. Results showed that all unsaturated CLs (compounds 23, **Figure 9**) are active as antagonists on human and murine TLR4 (in HEK-blue cells and murine macrophages), successfully inhibiting receptor signaling with IC_{50} ranging from high nM to low μM. Saturated CLs (compounds 22, **Figure 9**) can activate TLR4, inducing pro-inflammatory cytokines production (105). The only exception to this empirical rule is saturated C14:0 CL, which acted as agonist in murine cells but as antagonist in human cells, similarly to Lipid IVa (105, 106).

Alpinetin

Alpinetin (compound 24, **Figure 9**) is a natural flavonoid extracted from the plant *Alpinia katsumadai Hayata.* It has been demonstrated to possess anti-inflammatory activity, protecting against LPS-related damages both *in vitro* and *in vivo* (107). Subsequent studies clarified that alpinetin exerts its action as an agonist of PPAR-γ, which, in turn, downregulates TLR4 expression, effectively inhibiting receptor signaling (108): it is an indirect TLR4 antagonist. Recent studies proved alpinetin ability to protect mice against kidney damages and endometritis caused by LPS administration. Alpinetin-treated mice showed attenuated LPS-induced histopathological changes; furthermore,

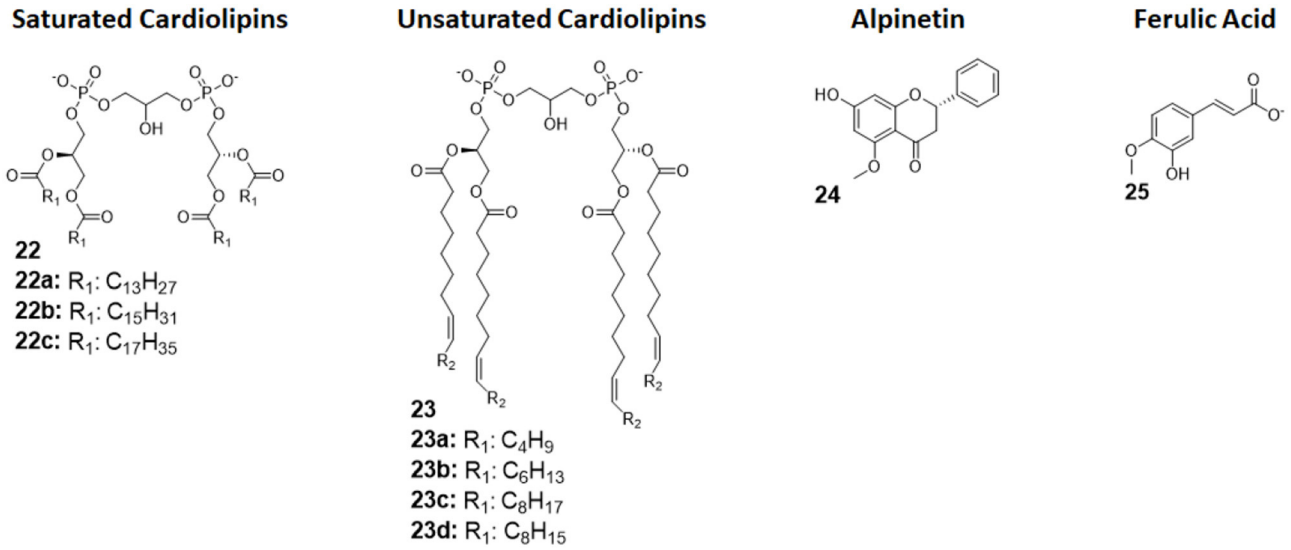

FIGURE 9 | Cardiolipin derivatives 21 and 22 modulate TLR4 activity as agonists or antagonists. The switch between agonism and antagonism seems to be related to the presence of unsaturation on fatty chains. **Alpinetin** (45) is a natural flavonoid which can modulate TLR4 by downregulating its expression by the cell. **Ferulic Acid** (46) showed to antagonize TLR4 by disrupting the TLR4/MD-2 complex.

alpinetin was showed to inhibit pro-inflammatory cytokines secretion in a dose-dependent manner (43, 109).

Ferulic Acid

Ferulic Acid (compound 25, **Figure 9**) is a phenolic compound abundant in various herbs, fruits, and vegetables, and it is extracted from *Ligusticum wallichii*. It has been recently shown to have various properties, among which antioxidant and anti-inflammatory effects in murine cells, but its exact mechanism remained unclear (110).

Two recent studies claim that FA can protect against LPS-induced bovine endometritis *in vitro* and against LPS-induced acute kidney injury *in vivo* by suppressing NF-κB and MAPK signaling, which strongly point toward a TLR4-related mechanism of action (111, 112).

Indeed, Rehman et al., in a recent study in which they demonstrate FA positive effects against LPS-induced neuroinflammation in mice, were able to elucidate FA Activity. By *in silico* molecular docking, the authors reported that FA action is exerted by interfering with MD-2 binding site on TLR4, effectively disrupting the TLR4-MD-2 complex and preventing LPS recognition and formation of the activated dimer $(TLR4/MD\text{-}2/LPS)_2$. However, the proposed mechanism, although intriguing, still lacks experimental proof, as it has only be postulated on the basis of molecular docking (44).

CONCLUSIONS

We presented here last advancements in the field of TLR4 modulators, focusing on small molecules of both synthetic and natural origin, as a follow-up of recent reviews on this topic (23, 113).

All TLR4 modulators described in this review have been validated or at least evaluated for their capacity to interact specifically with TLR4 and MD-2. For most of the molecules molecular docking calculations and experimental binding studies are available to assess their mechanism of action based on the binding of TLR4/MD-2.

While glycolipid-based TLR4 modulators present a high degree of similarity between them, as they mimic lipid A chemical structure, non-glycolipid TLR4 modulators can have a variety of structures, ranging from smaller bicyclic compounds, as the antagonist TAK-242 was approved for clinical use, to larger calix[4]arenes or peptides.

The structural diversity leads inevitably to a diversity in effects, potency, and mode of actions, which are reflected in different pharmacodynamics.

TLR4 is the only TLR that initiates two different signal pathways: the MyD88 and the TRAM/TRIF, ending up with the production of inflammatory cytokines or type-I interferons.

Interestingly, TLR4 modulators with different chemical structures can activate differentially the two different pathways.

Glycolipid TLR4 agonists, such as MPLA (and its synthetic form GLA)- or BECC-derived compounds, were found to preferentially activate the TRIF way, and this skews lymphocytes toward a T_H1 response, which is better suited to pathogens and pathogen-infected cells opsonization and elimination.

On the other hand, the pyrimidoindole derivative 1Z105 was found to activate TLR4 in a MyD88-biased fashion, leading to a T_H2 response, which is better in fighting parasites and extracellular pathogens infections. This difference in the mechanism of action is critical and can be exploited to optimize the rational design of vaccine adjuvants since they could be more

effectively formulated to elicit the most desirable response against a specific pathogen (25, 29, 45, 50, 57).

The structural diversity of TLR4 modulators leads therefore to different pharmacodynamics but also to different pharmacokinetic and targeting of different body districts.

Some glycolipid-based compounds, such as monosaccharidic FP7s and disaccharidic LAMs, can be effectively used systemically since they are water soluble and have a good distribution. On the other hand, highly hydrophobic non-glycolipid TLR4 antagonists, such as (+)-naltrexone, are better suited to target CNS diseases such as neuroinflammation, neuropathic pain, and neurodegenerative diseases including Alzheimer's disease (AD) (36–38, 41).

Another intriguing consequence of the structure diversity is the variety of the mechanism of actions. While agonists generally activate TLR4 by direct binding to MD-2, antagonists have much more possibilities to impact on TLR4 activity besides competitive inhibition.

Two interesting examples discussed within this review are TAK-242 and ferulic acid. Thanks to its cyclohexene carboxylic acid ester structure, TAK-242 acts as a good electrophilic Michael acceptor and is able to form a covalent bond with TLR4. This changes the TLR4 ectodomain conformation and modifies the conformation of TIR domain and the subsequent interaction with TRIFF and MyD88 (93). Ferulic acid binds in TLR4/MD-2 interaction interface. Consequently, this prevents TLR4/MD-2 complex formation, which, in turn, blocks the formation of the final activated complex $(TLR4/MD\text{-}2/LPS)_2$ (44).

Structure refinement and optimization of MD-2 binding *in silico* has proven to be a viable technique in this field, as recently shown by the works of Honegr, Michaeli, and Achek reviewed here (30–32, 42).

Novel anti-inflammatory mechanisms indirectly acting on TLR4 have emerged in last years: namely, alpinetin's ability to downregulate TLR4 gene expression by enhancing PPAR-γ activity and the activity of LAMs in regulating non-canonical NLRP3 inflammasomes at the level of caspases 4/11 (27, 108).

The presence of cross-talk between different inflammation pathways, in particular between TLR4 signaling and the non-canonical inflammasome initiated by cytosolic LPS, suggest the use of a combination of small molecules to simultaneously block several pathways or, alternatively, the development of dual ligands able to bind to TLR4 and caspases (27, 37).

AUTHOR CONTRIBUTIONS

FP mainly contributed to abstract, introduction and tables. AR mainly contributed to figures, main body of the review and conclusion.

FUNDING

We acknowledge the MSCA-ITN project BactiVax funded by European Union's Horizon 2020 grant agreement N° 860325.

ACKNOWLEDGMENTS

Figure 1 was Created with BioRender.com.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 23 July 2020
doi: 10.3389/fimmu.2020.01693

Chronic Exposure of Gingival Fibroblasts to TLR2 or TLR4 Agonist Inhibits Osteoclastogenesis but Does Not Affect Osteogenesis

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OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *30 April 2020*
Accepted: *25 June 2020*
Published: *23 July 2020*

Citation:
Karlis GD, Schöningh E, Jansen IDC, Schoenmaker T, Hogervorst JMA, van Veen HA, Moonen CGJ, Łagosz-Ćwik KB, Forouzanfar T and de Vries TJ (2020) Chronic Exposure of Gingival Fibroblasts to TLR2 or TLR4 Agonist Inhibits Osteoclastogenesis but Does Not Affect Osteogenesis. Front. Immunol. 11:1693. doi: 10.3389/fimmu.2020.01693

Chronic exposure to periodontopathogenic bacteria such as *Porphyromonas gingivalis* and the products of these bacteria that interact with the cells of the tooth surrounding tissues can ultimately result in periodontitis. This is a disease that is characterized by inflammation-related alveolar bone degradation by the bone-resorbing cells, the osteoclasts. Interactions of bacterial products with Toll-like receptors (TLRs), in particular TLR2 and TLR4, play a significant role in this chronic inflammatory reaction, which possibly affects osteoclastic activity and osteogenic capacity. Little is known about how chronic exposure to specific TLR activators affects these two antagonistic activities. Here, we studied the effect of TLR activation on gingival fibroblasts (GF), cells that are anatomically close to infiltrating bacterial products in the mouth. These were co-cultured with naive osteoclast precursor cells (i.e., monocytes), as part of the peripheral blood mononuclear cells (PBMCs). Activation of GF co-cultures (GF + PBMCs) with TLR2 or TLR4 agonists resulted in a weak reduction of the osteoclastogenic potential of these cultures, predominantly due to TLR2. Interestingly, chronic exposure, especially to TLR2 agonist, resulted in increased release of TNF-α at early time points. This effect, was reversed at later time points, thus suggesting an adaptation to chronic exposure. Monocyte cultures primed with M-CSF + RANKL, led to the formation of bone-resorbing osteoclasts, irrespective of being activated with TLR agonists. Late activation of these co-cultures with TLR2 and with TLR4 agonists led to a slight decrease in bone resorption. Activation of GF with TLR2 and TLR4 agonists did not affect the osteogenic capacity of the GF cells. In conclusion, chronic exposure leads to diverse reactions; inhibitory with naive osteoclast precursors, not effecting already formed (pre-)osteoclasts. We suggest that early encounter of naive monocytes with TLR agonists may result in

differentiation toward the macrophage lineage, desirable for clearing bacterial products. Once (pre-)osteoclasts are formed, these cells may be relatively insensitive for direct TLR stimulation. Possibly, TLR activation of periodontal cells indirectly stimulates osteoclasts, by secreting osteoclastogenesis stimulating inflammatory cytokines.

Keywords: chronic inflammation, toll-like receptors, periodontitis, TNF-α, bone resorption, osteoclasts, osteoblasts, innate immunity

INTRODUCTION

Periodontitis is a plaque-related inflammatory disease of the tooth-supporting tissues, leading to alveolar bone resorption which, eventually, can lead to tooth loss (1, 2). It is initiated by a disturbed balance between the host immune response and the bacterial load, modified by several factors such as lifestyle, genetics, and individual variation in the subgingival microbiome (3–5). Initially, the inflammatory response plays a protective role, orchestrated to eliminate the damaging stimulus and restore symbiosis (6). However, in patients with periodontitis, this inflammatory reaction is often chronic, leading to the irreversible alveolar bone resorption, which is mediated by bone-resorbing cells; the multinucleated osteoclasts (7).

The first line of host defense to micro-organisms or their products is initiated by the innate immune response. It is conceivable that gingival fibroblasts (GF), the predominant cell type of the alveolar bone-lining mucosa (gingiva), interact constantly with molecules from the oral microflora. These fibroblasts express receptors that sense the presence of microbes and substances released by these microbes. These receptors are referred to as "Pattern recognition receptors" (PRRs) since they recognize molecular patterns that are commonly present on many micro-organisms. One of the functions of the innate immune response is the recognition of pathogen-associated molecular patterns (PAMPs) by PRRs, including the Toll-like receptors (TLRs) (8). Up till now, ten TLRs (TLR 1-10) have been identified in humans which respond to these PAMPs (9, 10). Each TLR responds to specific PAMPs, however mainly a combination of them is required to be activated.

All of these TLRs are expressed in periodontal tissues (11, 12). TLR2 and TLR4 are the most extensively researched receptors of the TLR family in relation to periodontitis in mice and men (10, 13–16). This derives from the fact that TLR4 is stimulated by lipopolysaccharides (LPS) (17), the major glycolipid membrane component of the Gram-negative bacteria, such as the keystone periodontopathogenic bacterium *Porphyromonas gingivalis* (18, 19). TLR2 is involved in the recognition of cell-wall components of Gram-negative and Gram-positive bacteria. The participation of these two specific members of the TLR family in the triggering of the innate immune response in periodontitis patients is already established (10–12, 20, 21). Accordingly, higher expression of these receptors has been found in the periodontal tissues of periodontitis patients, in comparison with healthy controls (12, 22).

TLR2 and TLR4 are expressed in the periodontal tissues, and among them on GF (23, 24). GF play an important role in processes associated with bone remodeling such as the induction and inhibition of osteoclast formation (25, 26). Osteoclasts, the cells that are responsible for bone resorption, are derived from the monocyte lineage and express TLRs which respond to PAMPs (27). It has been shown that ligature and injection-induced periodontitis in mice is regulated through the activation of the TLR4 and TLR2 receptors (28, 29). However, there is also evidence that shows that the *in vitro* activation of human osteoclast precursors with TLR agonists results in the inhibition of osteoclastogenesis (30). Besides inhibition of osteoclasts, chronic TLR2 activation plays a significant role in T cell proliferation, mediated by GF or monocytes, resulting in the production of proinflammatory cytokines by human monocytes (24).

GF can also be stimulated into the osteogenic lineage (31). Little is known about the effect of TLR activation of these cells in the context of osteogenesis. It has been shown that TLR2 agonist (Pam3CSK4 or mutant *E. coli*) slightly enhances osteogenesis in human primary osteoblasts (32). However, the dose of the agonists was low (1 μg/mL and 1 ng/mL, respectively) and it was not clearly stated if the agonists were added only once or with every refreshment of the media. Others found that TLR4 has an inhibitory effect on osteogenesis in murine bone marrow mesenchymal stem cells (33). Recently, it was shown that *in vitro* TLR4 activation in high doses (10 μg/mL) inhibits the osteogenic potential of human periodontal ligament cells (34).

Although periodontitis is a chronic inflammation, and the expression of TLR2 and TLR4 is aberrant in the GF (24, 35), the effect of the activation of these specific TLRs on osteoclastogenesis and osteogenesis is only evaluated after short (<60 h) stimulation (36–40), and scarcely on cells derived from human periodontal tissues (34, 41–43).

To the best of our knowledge, this is the first study that evaluated the effect of chronic exposure of specific TLR2 and TLR4 agonists, molecules that activate TLR2 and TLR4, both on osteogenesis, in presence of human GF, and on osteoclastogenesis, in GF stimulated peripheral blood mononuclear cell (PBMC) cultures. Since TLR stimulators may also affect precursors of osteoclasts or multinucleated osteoclasts, we studied these effects on monocytes that were cultured with macrophage stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) for 1 week (pre-osteoclasts) and for 2 weeks (osteoclasts) followed by 2 vs. 1 week of TLR agonist exposure, to assess the effect on osteoclast differentiation and activity on bone slices. We hypothesized that the triggering of these TLRs would result in an induction of osteoclastogenesis and an inhibition of osteogenesis.

MATERIALS AND METHODS

Gingival Fibroblasts

GF were obtained from 6 systemically healthy individuals (age 22–38 years) who underwent extraction of a third molar (wisdom tooth). No overt signs of gingival inflammation and periodontitis were present (pockets $\leq$ 3 mm without bleeding). Sampling from the donors was conducted at VU University Hospital (Vrije Universiteit, Amsterdam, The Netherlands). All the individuals signed informed consent and samples were coded to guarantee the anonymity of the donors as required by Dutch law. Researchers handling the fibroblasts (G.D. Karlis and T.J. de Vries) could not retrieve the identity of the donors.

With the use of a scalpel-knife, free gingiva and part of the interdental gingiva were cut off the tooth. The tissue fragments were washed twice in culture medium (Dulbecco's minimal essential medium (DMEM, Gibco BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS, HyClone, Logan, USA), and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin, and 250 ng/mL amphotericin B [Antibiotic antimycotic solution, Sigma, St. Louis, MO, USA]) and cultured in a humified atmosphere of 5% CO_2 in air at 37°C. For the current study, GF of passages 4–6 were used.

Blood Cell Isolation

Buffy coats (Sanquin, Amsterdam, The Netherlands) of healthy donors were diluted 1:1 in 1% PBS-citrate (pH 7.4). Thereafter, 25 mm of diluted blood was carefully layered on 25 mL Lymphoprep (Axisshield Po CAS, Oslo, Norway) and centrifuged for 30 min at 800 x G without brake. The interphase containing the PBMCs was collected and washed three times in 1% PBS-citrate and finally recovered in culture medium.

Monocyte Isolation

CD14+ monocytes retrieved from peripheral blood were used in experiments where osteoclasts were grown using M-CSF and RANKL instead of fibroblasts and PBMCs. Here, CD14+ cells were isolated using CD14+ microbeads (Miltenyi, Bergisch Gladbach, Germany) according to a previously described method (44).

TLR Agonist Titrations

Optimal cell densities (ratio) of GF and PBMC were previously established by our group (25). GF (1.5 x 10^4 per well, $n = 3$) were seeded in duplicate and allowed to attach overnight in 48-well plates. 5 x 10^5 PBMCs were seeded in duplicate in co-culture with GF. To assess the optimal concentration of TLR agonists, co-cultures for osteoclastogenesis cultures as described above or GF monocultures for osteogenesis (3.0 x 10^4 cells per well) (31) were cultured and maintained in a humidified atmosphere of 5% CO_2 in ambient air at 37°C. Cultures were refreshed every 3–4 days. A titrated concentration of TLR2 ligand (10 ng/mL, PAM2CSK4, #14E14-MM, Invivogen, San Diego, CA, USA), TLR4 ligand (10 ng/mL, LPS- *Porphyromonas gingivalis*, Ultrapure, Version #14F18-MM, Invivogen), or a combination of both, was added to the culture media at the start of the experiment ($n = 6$) and with every subsequent culture media refreshment (every 3–4 days). For assessing the effect of TLR2 and TLR4 activation on TLR activation in general, a TLR2 and TLR4 targeting LPS from *Porphyromonas gingivalis* was used (Catalog number #tlrl-pglps, Invivogen). This LPS activates TLR2 at 10 ng ng/mL and TLR4 from 100 ng/mL. Both these concentrations were used in the relative experiments.

Control conditions contained culture media without TLR agonists but included similar additions of a vehicle (sterile water). PBMCs were also seeded in high-density cultures at 1 x 10^6 PBMCs per 96-well plate ($n = 4$).

CD14+ monocytes were cultured for 3 days in M-CSF (25 ng/mL), followed by 10 ng/mL M-CSF, and 10 ng/mL RANKL until 21 days. Pre-osteoclasts at 7 days, or early osteoclasts at 14 days, received TLR agonists for the remaining 14 or 7 days respectively.

TRAcP Staining

After 21 days, cells were fixed in 4% PBS-buffered formaldehyde for 10 min and washed with PBS. Cells were stained with a TRAcP staining (Sigma-Aldrich) according to the manufacturer's protocol. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. A combination of light and fluorescence microscopy (Leica DFC320; Leica Microsystems, Wetzlar, Germany) was used to count the TRAcP + multinucleated cells (MNCs) and cells were considered to be osteoclasts when TRAcP positive with at least three nuclei. Five standardized areas per well were analyzed at a magnification of 20 x to count the number for the number of MNCs containing at least three nuclei and are expressed as MNCs/ well.

Bone Resorption

Bone resorption was analyzed in cultures on bone after a culture period of 3 weeks. After this period, the cells present on the bovine cortical bone slices were removed with 0.25 M NH_4OH. The slices were washed in distilled water, incubated in a saturated alum solution, washed in distilled water, and stained with Coomassie Brilliant blue. The surface areas of individual resorption pits were measured using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Osteogenesis

Osteogenesis assays were performed as previously described (14, 31). Briefly, GF were seeded in 48-wells plates (3 x 10^4 cells/well). Culture medium (0.4 mL per well) was replaced twice per week. The culture medium contained 50 μg/mL ascorbic acid (Sigma) and 10 nM β-Glycerophosphate (Sigma), which are conductive to mineralization (further referred to as mineralization medium). Water as solvent control, TLR2 agonist, TLR4 agonist, or the combination of these agonists was added for 21 days. Cells were harvested for quantitative PCR analysis by adding RNA lysis buffer (Qiagen, Hilden Germany) containing 1% β-mercaptoethanol and were stored at −80°C until RNA extraction. Cells for alkaline phosphatase activity and DNA measurements were lysed in Milli-Q water and stored at −20°C. The cells for the mineralization assay were fixed with 4% PBS buffered formaldehyde for 10 min and were stored with PBS at 4°C.

In order to evaluate the osteogenic capabilities of the TLR agonists *in vitro*, we measured the calcium deposition (μg/mL), in the 4 different conditions (with TLR2, TLR4, TLR2 + TLR4, and without, respectively) and at 4 different time-points ($t = 0, 7, 14, 21$ days).

Alkaline Phosphatase

Alkaline phosphatase activity (ALP) was measured in lysates from cells that were cultured with mineralization medium. Cells were harvested at days 0 and 14 of culturing. Cells were washed with PBS and lysed with 200 μL Milli-Q water and were frozen in −20°C for storage. After three freeze-thaw cycles, samples were collected by scraping. ALP was measured according to the method described by Lowry (45), using 4-nitrophenyl phosphate disodium salt (Merck, Darmstadt, Germany) at pH 10.3, as a substrate for ALP. Absorbance was measured at 405 nm with a Synergy HT spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). DNA was measured in the same lysate using CyQuant Cell Proliferation Assay Kit (Molecular Probes, Leiden, The Netherlands). Fluorescence was measured at 485 nm excitation and 528 nm emission with a Synergy HT spectrophotometric microplate reader. Alkaline phosphatase was expressed as μmol/ng DNA.

Alizarin Red Staining

Mineral deposition, in triplicate wells per donor, was analyzed after 21 days of culturing. The cells were fixed for 10 min in 4% formaldehyde and rinsed with Milli-Q water before adding 300 μL of 2% Alizarin Red solution at pH 4.3 (Sigma-Aldrich, St. Louis, MO, USA). After incubation of 15 min at room temperature, the cells were washed with Milli-Q water and air-dried. Red nodules were a sign of mineral deposition.

Calcium Quantification

Samples for calcium deposition assay were collected on days 0, 14, 21. First, 1 mL of 0.5 M acetic acid was added. Secondly, calcium was extracted by shaking the samples overnight. Calcium content was measured in the extraction solution using the ortho-cresolphthalein complexone (OCPC) method (46). Absorbance was measured at 570 nm in a microplate reader (BioTek Synergy HT).

Scanning Electron Microscopy

Mineralization assays were performed on cells that were grown on glass insert slides, in the presence or absence of TLR agonists and mineralization medium. At 21 days, cells were washed in cacodylate buffer, fixed in MacDowells fixative, and dehydrated in steps of increasing percentage of ethanol. Gold-sputtered preparations were analyzed with a Zeiss Sigma 300 FESEM (Carl Zeiss Microscopy GmbH, Jena, Germany) scanning electron microscope.

ELISA

Conditioned medium was taken from mono- and co-cultures ($n = 6$) at 3, 7, and 21 days. Enzyme-linked immunosorbent assays (ELISA, R&D Systems, Minneapolis, MN, USA) were used for the detection of human tumor necrosis factor alpha (TNF-α) following the manufacturer's instructions.

Real-Time Quantitative PCR

After 7 and 21 days of culturing, RNA was extracted from samples using a commercial spin-column kit (RNeasy Mini kit, Qiagen, Düsseldorf, Germany) according to the manufacturer's protocol. RNA concentration was measured with Synergy HT spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). One hundred nanograms RNA was used in the reverse transcriptase reaction which was performed according to the manufacturer's instructions of the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers. The Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA) (**Table 1**) was used to design the Real-time PCR primers.

Real-time PCR was performed on the ABI PRISM 7000 (Applied Biosystems). The reactions were performed with 5 ng cDNA in a total volume of 25 mL containing SYBR Green PCR Master Mix, consisting of SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP instead of dTTP, passive reference and buffer (Applied Biosystems) and 300 nM of each primer. After an initial activation step of the AmpliTaq Gold DNA polymerase for 10 min at 94°C, 40 cycles were run of a two-step PCR consisting of a denaturation step at 94°C for 30 s and annealing and extension step at 60°C for 1 min. Subsequently, the PCR products were subjected to melting curve analysis to test if any unspecific PCR products were generated. The PCR reactions of the different amplicons had equal efficiencies. β2-microglobulin was used as the housekeeping gene. Expression of this gene was not affected by the experimental conditions. Samples were normalized for the expression of β2-microglobulin by calculating the ΔCt, ($Ct_{\text{gene of interest}}$ -$Ct_{\beta 2-\text{microglubulin}}$) and expression of the different genes is expressed as the mean relative fold expression $2^{-(\Delta Ct)}$.

Statistics

GraphPad Prism software (version 8.3.0, La Jolla, CA, USA) was used to analyze the data sets. Means and standard deviations (SD) were calculated and used for the presentation of the data in figures. All the data were analyzed with one-way ANOVA followed by Tukey's multiple comparison test. Tests were performed over the 4 (osteoclastogenesis) or 5 (osteogenesis) conditions per time point and per condition over time. Differences were considered significant at $p < 0.05$.

RESULTS

Chronic Exposure to TLR Agonists Decreases the Osteoclastogenic Capacity of GF-PBMC Co-cultures

In order to identify the most suitable concentration of TLR agonists for the experiment, various concentrations of TLR2 and TLR4 were tested (0.1, 1, 10, and 100 ng/mL). For osteoclastogenesis experiments, GF and PBMCs were co-cultured for 21 days with or without TLR agonists. In order to identify osteoclasts, TRAcP+ cells with more than 3 nuclei were counted and categorized into three different groups (3–5, 6–10, and ≥11 nuclei) (47). Because the vast majority of the multinucleated

cells had 3–5 nuclei, all three categories were merged into one category. The addition of TLR2 and TLR4 agonists appeared to be associated with the formation of fewer multinucleated cells (**Figures 1A,B**). This effect was statistically significant for all concentrations, except for the 0.1 ng/mL TLR2 condition (**Figure 1A**).

TLR2 Agonist Decreases the Number of Multinucleated Cells in GF-PBMC Co-cultures

Based on the results of the titration experiment (**Figure 1**), a concentration of 10 ng/mL of TLR agonists was chosen for further experiments. To analyze whether activation of both TLR2 and TLR4 would lead to increased sensitivity, a condition of 10 ng/mL of both TLR2 and TLR4 agonist was included in all further experiments. GF were co-cultured with PBMCs for 21 days. The cells were stained for TRAcP activity and cells with 3 or more nuclei were counted as multinucleated cells, in 5 standardized fields per well. The presence of TLR2 and TLR2 + 4 agonists was significantly associated with fewer multinucleated cells, in comparison with the control (**Figure 2A**), suggesting that TLR2 agonist decreased the number of MNCs in these co-cultures. Apart from GF-PBMC co-cultures, there is another way to culture multinucleated cells in the absence of cytokines. When PBMCs are plated at a high density, multinucleated cells will form (48). Here, T-cells seem important for providing the signals for the formation of multinucleated cells (49). Also, when applying this method, less multinucleated cells formed when TLR2 agonist was added (**Figure 2B**). Multinucleated cells were counted on bovine bone slices. The presence of these cells on the bone

TABLE 1 | Primer sequences used for RT-qPCR experiments.

Gene		Primer sequence	Ensembl gene ID
TRAcP	Forward	5′ CACAATCTGCAGTACCTGCAAGGAT 3′	ENSG00000102575
	Reverse	5′ CCCATAGTGGAAGCGCAGATA 3′	
NFATc1	Forward	5′ CATGCGAGCCATCATCGA 3′	ENSG00000206439
	Reverse	5′ TGGGATGTGAACTCGGAAGAC 3′	
TNF-α	Forward	5′ CCCAGGGACCTCTCTCTAATCA 3′	ENSG00000111956
	Reverse	5′ GCTTGAGGGTTTGCTACAACATG 3′	
RUNX2	Forward	5′ CCAGAAGGCACAGACAGAAGCT 3′	ENSG00000124813
	Reverse	5′ AGGAATGCGCCCTAAATCACT 3′	
ALP	Forward	5′ GCTTCAAACCGAGATACAAGCA 3′	ENSG00000162551
	Reverse	5′ GCTCGAAGAGACCCAATAGGTAGT 3′	
Osteonectin	Forward	5′ GCCCAGCGGTGCAGAGT 3′	ENSG00000196104
	Reverse	5′ GGCTCCCAGCCATTGATACA 3′	
TLR2	Forward	5′ GGCTTCTCTGTCTTGTGACCG 3′	ENSG00000137462
	Reverse	5′ GAGCCCTGAGGGAATGGAG 3′	
TLR4	Forward	5′ CTGCAATGGATCAAGGAACCAG 3′	ENSG00000136869
	Reverse	5′ CCATTCGTTCAACTTCCACCA 3′	
β2-microglobulin	Forward	5′ CGGGCATTCCTGAAGCTGA 3′	ENSG00000106927
	Reverse	3′ GGATGGATGAAACCCAGACACATAG 3′	

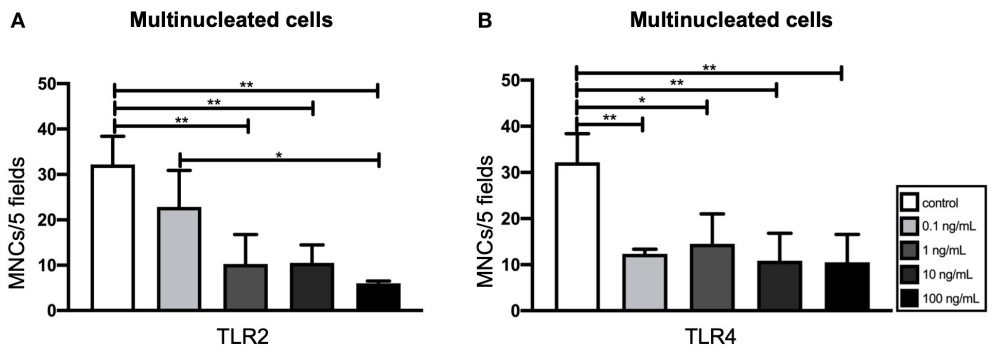

FIGURE 1 | Effect of Toll-like receptor (TLR) agonist concentrations on the osteoclastogenic and the osteogenic capacity of gingival fibroblasts (GF). **(A)** The number of multinucleated cells formed in the presence of TLR2 agonist (0.1–100 ng/ml). **(B)** The number of multinucleated cells formed in the presence of TLR4 agonist (0.1–100 ng/mL). Titration experiments were performed using three different GF donors in duplicates, average results ±SD are shown. Significant results for **(A)** and **(B)** are shown (black bars). *$p < 0.05$, **$p < 0.01$.

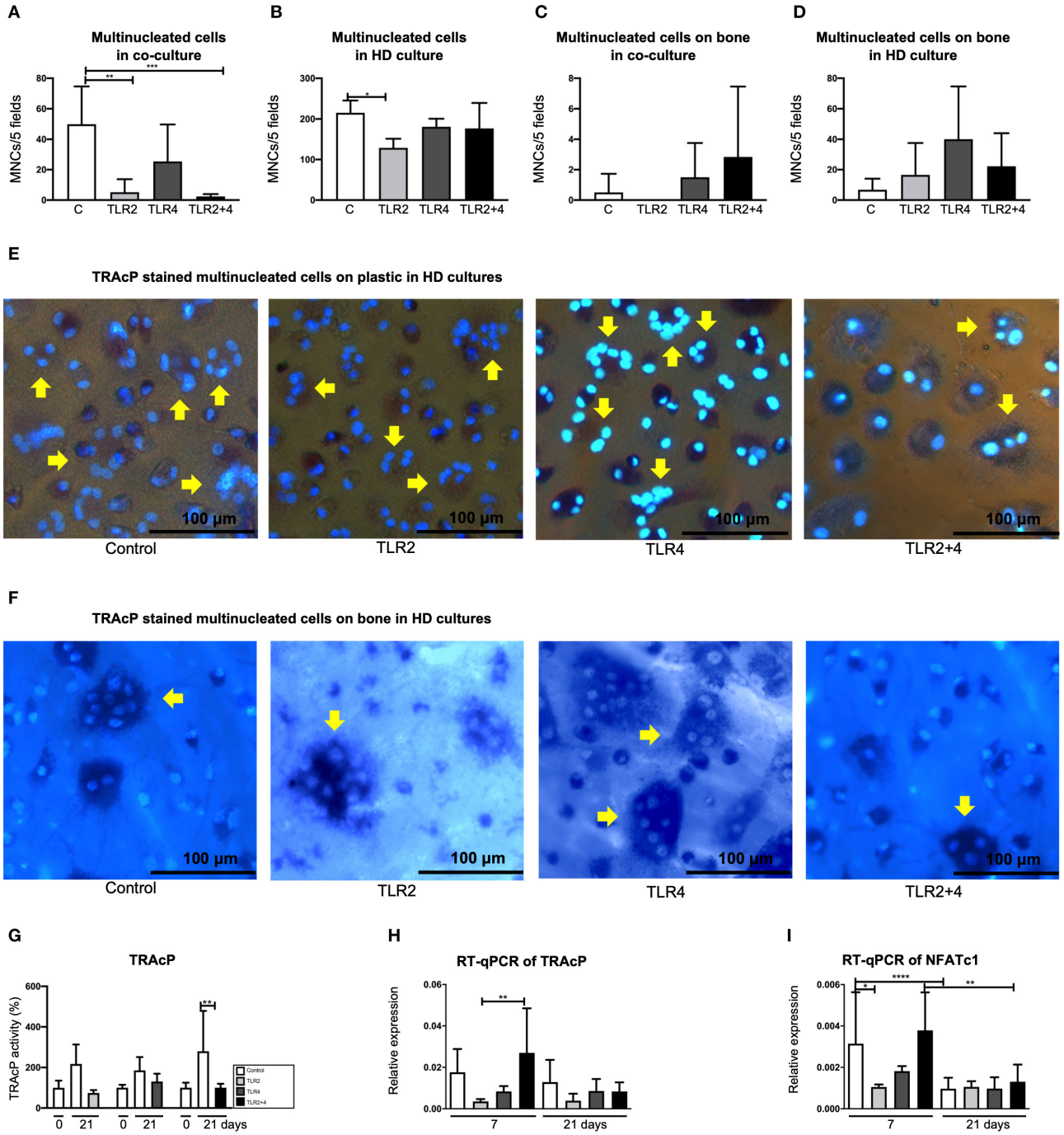

FIGURE 2 | TLR2 agonist decreases the number of multinucleated cells in GF-PBMC co-cultures and PBMC high-density cultures. The number of multinucleated cells is presented in **(A–D)**. The concertation of the agonists was 10 ng/mL. Osteoclast formation is particularly decreased in the presence of TLR2 (light gray bars, **A,B**) and TLR2+4 agonists (black bars, **A**). **(C)** Multinucleated cells were counted on bone slices but they were in very low numbers. **(D)** In high density cultures, multinucleated cells were a more common finding but without any difference between the conditions. Cells were stained for tartrate-resistant acid phosphatase (TRAcP, purple) and counterstained with DAPI (blue), in order to detect the multinucleated cells (depicted with yellow arrows); on plastic **(E)** and on bone **(F)**. All micrographs are representatives for three independent experiments with six different GF sources. Scale bar represents 100 µm. **(G)** TRAcP enzyme activity was measured by three different conditions, each with separate 0 days and a 21 days control samples. TRAcP enzyme activity is significantly decreased at day 21 at the TLR2+4 condition in comparison with the control. **(H,I)** Real-time quantitative PCR was performed for the genes of TRAcP and NFATc1). $n = 6$ GF cultures per condition in duplicates, average results ±SD are shown. For the HD cultures $n = 4$. Significant results for **(A–D)** and **(G–I)** are shown (black bars). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$. [a]High density culture.

was rare (**Figure 2C**). Multinucleated cells on bone found to be more often present in high-density cultures compared to normal density co-cultures on bone. No significant differences were found (**Figure 2D**) on bone slices, suggesting that the surface may modulate TLR agonist's activity. Representative micrographs of TRAcP+ cells on plastic and on bone are shown in **Figures 2E,F**, respectively. The TRAcP enzyme was also quantified at baseline (day 0) and day 21 (**Figure 2G**). TRAcP enzyme was statistically decreased at day 21 under the effect of TLR2+4 in comparison with the control. The expression of TRAcP mRNA was measured also with Real-Time qualitative PCR (**Figure 2H**). A trend of less expression in the conditions of TLR2 and TLR4 in comparison with the control was found without being statistically significant. The only difference was found between TLR2 and TLR2+4 at day 7, with the TLR2+4 being elevated compared to TLR2 (**Figure 2H**). The gene expression of NFATc1, a crucial transcription factor for osteoclast formation (50) was measured (**Figure 2I**). Expression of NFATc1 was significantly lower for TLR2 in comparison with the control, at day 7. At day 21, the expression of the gene was significantly lower in the conditions of control and TLR2 + 4 in comparison with day 7.

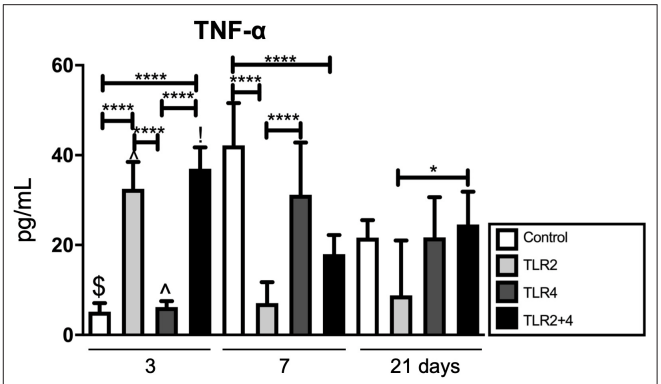

FIGURE 3 | TLR2 agonist has an evident effect on TNF-α; increasing in early timepoints, reversed later. TNF-α is significantly elevated at day 3 in all conditions containing TLR2 agonist (light gray and black); at day 7, these conditions are significantly decreased in comparison with the control. At day 21, the are no differences anymore between TLR conditions and control. $n = 6$ GF cultures per condition in duplicates, average results ±SD are shown. Significant results are shown (black bars). $^{*}p < 0.05$, $^{****}p < 0.001$, $^{\$}p < 0.01$ for the same condition between day 3 and day 7, day 3 and day 21 as well as day 7 and day 21, $\hat{}p < 0.001$ for the same condition between day 3 and day 7, as well as day 3 and day 21, $!p < 0.001$ for the same condition between day 3 and day 7.

Secretion of TNF-α: Early TLR2 Agonist Responses and Nullification Over Time

We previously described, that TLR activation results in the production of inflammatory cytokines (24). We next measured TNF-α in the supernatant of GF-PBMC co-cultures (**Figure 3**). Since little is known about the production of TNF-α in chronically TLR agonist exposed cultures, we measured TNF-α at 3 different time points; at 3, 7, and 21 days. At day 3, the levels of TNF-α were significantly elevated in the groups that contained TLR2 agonists (TLR2 and TLR2+4) in comparison with the control (**Figure 3**). At day 7, these results were reversed, where the levels of TNF-α of the control and TLR4 conditions were significantly higher than when TLR2 agonist was added. On day 21, secretion of TNF-α was significantly lower when TLR2 agonist was added compared to TLR2+4 agonists.

Effect of TLR Agonists on the Number of Multinucleated Cells and Bone Resorption in Monocyte Cultures Stimulated With M-CSF and RANKL

The above described osteoclastogenesis inhibitory effects by TLR2 and TLR4 agonists that were performed on cultures with naive monocytes, present in PBMCs that were stimulated with TLR agonists right after isolation. Although the GF-PBMC co-culture and the high-density PBMC cultures are good models to investigate the formation of multinucleated cells under the influence of GF or T-cells respectively, resorption by these multinucleated cells has never been observed (48, 51). In fact, the addition of M-CSF and RANKL is essential to achieve bone resorption (25). To further investigate TLR activation on osteoclast precursors of various stages of differentiation, TLR2 and TLR4 agonists were added to monocyte cultures that were stimulated with M-CSF and RANKL on day 7 (early stage osteoclast differentiation), day 14 (late stage, just before resorption takes place), or not at all (control condition) (**Figure 4A**). Cultures were terminated after 21 days. The cells were stained for TRAcP activity and DAPI and cells with 3 or more nuclei were counted (depicted with yellow arrows in **Figure 4D**). The addition of TLRs on day 7 and day 14 was not associated with a change in the number of multinucleated cells (**Figures 4B,D** which depicts condition II). For the cultures that were cultured on bovine bone slices (**Figure 4E**), bone resorption was quantified (**Figure 4C**). Bone slices were stained with Coomassie Brilliant blue and resorption pits were identified and their surface was measured using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Bone resorption was hardly affected when TLR agonists were added, both when added at 7 or at 14 days. The only conditions that differed statistically with the control were TLR2 and TLR4, both when added on day 14. These experiments show that chronic exposure to TLR2 or TLR4 agonists did not affect osteoclast formation from osteoclast precursors or already formed osteoclasts. Furthermore, no increase in bone resorption was observed, rather slightly decreased bone resorption compared to conditions without TLR activation (**Figure 4C**).

GFs Express TLR2 and TLR4 but at a Lower Level Compared to Osteoclasts

In order to confirm the expression of TLR2 and TLR4 by the cells of interest, RT-qPCR was performed (**Figure 5**). Co-cultures and CD14+ cultures stimulated with M-CSF and RANKL were cultured with a Pg-LPS that targets both TLR2 and TLR4, depending on the concentration. TLR2 is activated from 10 ng/mL, TLR4 from 100 ng/mL. Both these concentrations in M-CSF and RANKL stimulated monocyte cultures were used.

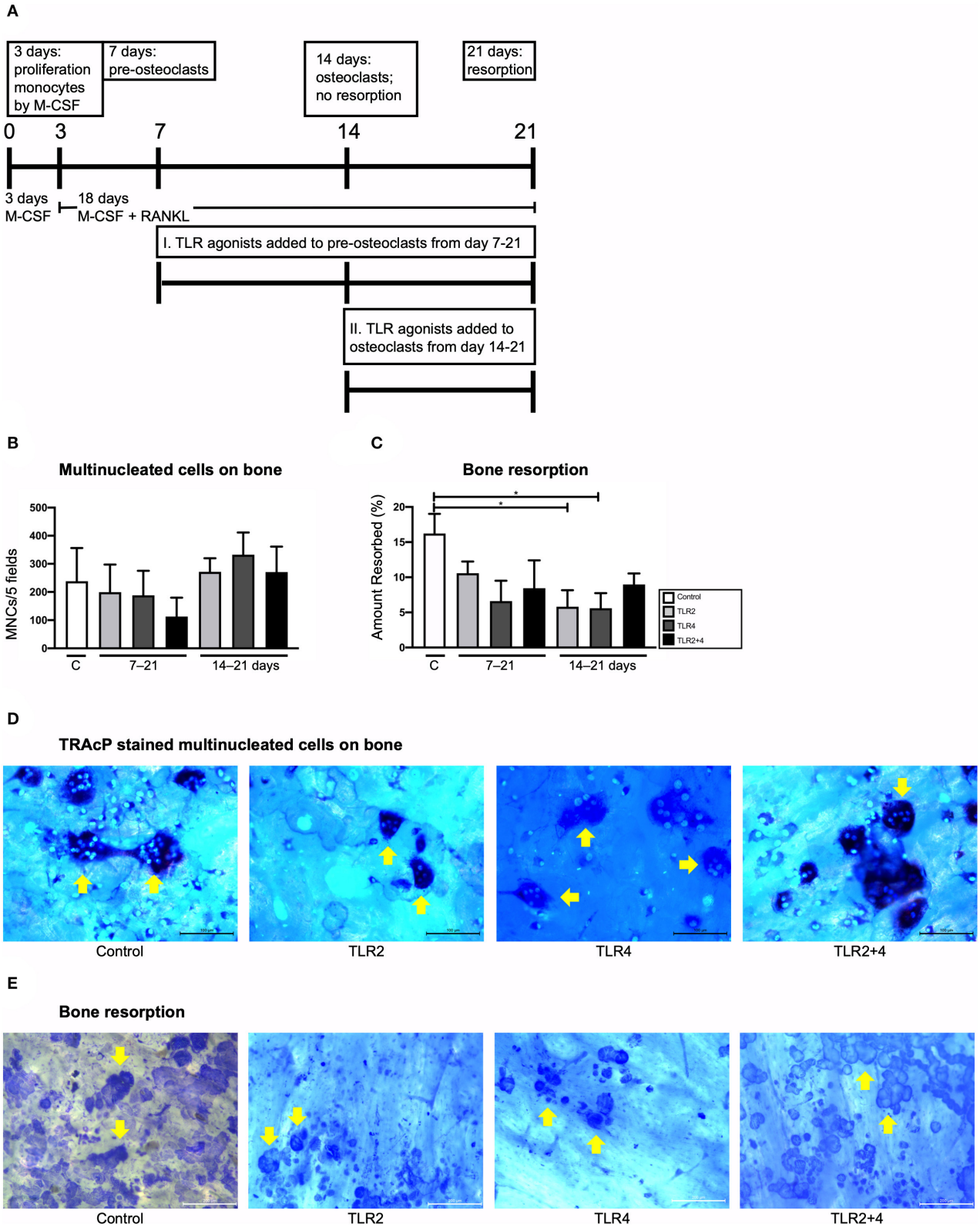

FIGURE 4 | Effect of TLR agonists on the formation of multinucleated cells and on bone resorption in CD14+ monocyte cultures stimulated with M-CSF and RANKL. **(A)** Flow chart of the conducted experiment. Day 0–3: monocytes were cultured with M-CSF. Day 3–7: M-CSF + RANKL were added to the monocyte cultures. Day

(Continued)

FIGURE 4 | 7: Under the effect of M-CSF + RANL, monocytes have formed pre-osteoclasts. Control condition: no TLR agonists were added to this culture; Condition I.: TLR agonists were added to pre-osteoclasts from day 7 to 21 (chronic exposure on pre-osteoclasts); Condition II.: TLR agonists were added to osteoclasts from day 14 to 21 (chronic exposure on osteoclasts before resorption). Cultures were terminated after 21 days. **(B)** Osteoclasts were counted. Condition I and condition II are shown in the x-axes (7–21 and 14–21 days, respectively). Osteoclast formation did not differ between the conditions. **(C)** Bone resorption was measured. Addition of TLR agonists did not affect bone resorption when added at day 7 and had a slightly inhibitory effect on bone resorption when added from day 14–21 for both TLR2 and TLR4 agonist. **(D)** Shows light microscopy micrographs of the TRAcP stained cells, and the DAPI counterstained nuclei. Traces of bone resorption were regularly noticeable, for instance in the condition with TLR2 agonist. **(E)** Resorption pits were quantified after staining with Coomassie brilliant blue. Examples are shown from TLR agonist exposures from 14 to 21 days. Bars in micrographs represent 100 μm **(D)** and 200 μm **(E)**. Data from 1 out of 2 experiments are shown, similar results were obtained in both experiments. $n = 4$ per condition, in quadruplicates; average results ±SD are shown. Significant results for **(B,C)** are shown (black bars). $^{*}p < 0.05$.

The expression of TLR2 and TLR4 in mono-cultures of GFs ($t = 0$) and in co-cutures of GF-PBMCs was measured at 3 different time points ($t = 7$, 14, and 21 days), after triggering with Pg-LPS that targets both TLRs at concertations of 10 ng/mL or 20 ng/mL and 100 ng/mL (**Figure 5A**). No differences were found between the conditions, indicating that TLR2 and TLR4 are expressed constantly over time, independent of the LPS concentration. The only significant difference was a reduction of the expression of TLR4 from day 7 to day 14, at the concentration of 10 ng/mL. The expression of TLR2 and TLR4 was measured also in CD14+ cultures that were stimulated with MCS-F and RANKL, at 21 days, the timepoint when all cells are in the osteoclast lineage, either as TRAcP mononucleated or as multinucleated cells (**Figures 5C,D**). Osteoclasts expressed both TLR2 and TLR4, at a much higher level than GF or GF-PBMC co-cultures.

TLR2 and TRL4 Agonists Do Not Affect the Osteogenic Capacity of the Gingival Fibroblasts

To establish the effect of TLR2 and TLR4 agonists on osteogenesis, different osteogenic assays were conducted. Alkaline phosphatase and DNA content were measured at baseline (day 0) and day 14, with and without osteogenic medium (**Figure 6A**). There were no significant differences between the control and the other conditions on day 14. However, the addition of especially TLR4 agonists seemed to reduce the number of cells on day 14 (**Figure 6B**). The calculated ALP/DNA, or alkaline phosphatase corrected per number of cells, was not significantly different, with a lot of variation between the conditions (**Figure 6C**). Deposited calcium was measured at three different time points ($t = 0$, 14, and 21 days, **Figure 6D**). On day 14 and 21, calcium was only measured in conditions cultured in osteogenic medium and the concentration of calcium increased between day 14 and 21. However, the addition of TLR agonists did not influence calcium deposition (**Figure 6D**). Alizarin red staining confirmed these findings; no effect of TLR activation was observed (**Figure 6E**). Mineralization was confirmed with scanning electron microscopy (SEM, **Figure 6F**). Mineralization was present on top of cells (**Figure 6F**i), as nodular structures, sometimes containing fibrillar structures, reminiscent of bone matrix proteins such as collagen I (**Figure 6F**ii).

Unlike the osteoclastogenesis experiment with co-cultures, TNF-α protein was undetectable in the supernatants of osteogenic cultures stimulated with TLR agonists (**Figure 6G**). However, at the mRNA level, low expression of TNF-α was detected, only significantly higher expression was found when TLR2 and TL4 agonists were added together at day 14 (**Figure 6G**). Early osteogenic marker RUNX2 was upregulated compared to t = 0 only in cultures with both TLR2 and TLR4, but no differences were found between conditions per time point, or between 7 and 14 days (**Figure 6H**). Intermediate marker ALP was upregulated at 7 days, especially in conditions where TLR4 agonist was added. As expected for ALP, the expression was lower at 14 days. No significant differences were observed between the conditions at 14 days (**Figure 6I**). Remarkably, late osteogenic marker osteonectin was significantly higher expressed at 7 days compared to 14 days (**Figure 6J**). Between conditions per time point, no significant differences were observed. Overall, influences of TLR agonists were limited in all gene expression analyses (**Figures 6G–J**).

DISCUSSION

Chronic diseases associated with bacterial pressure, such as periodontitis, are likely to experience phases of chronic exposure to bacterial products such as TLR activators. The effects of chronic exposure of cell cultures to TLR activators have been grossly neglected. In the present article, we describe the effects on osteoclast formation and activity on the one hand and on the osteogenic aspects on the other hand in cultures of GF that were chronically exposed to agonists of TLR2, TLR4, and their combination. TLR2 and TLR4 are the predominant TLRs activated in periodontitis (12, 22, 52).

A key finding of our study is that osteoclast formation is inhibited by TLR agonists when freshly isolated PBMCs are used. This was observed both in the co-culture's studies using GF and in the so-called high-density cultures. One could interpret these results in terms of the necessities of the inflamed periodontium, where relatively naive migrating monocytes may be triggered to differentiate into macrophages to nullify the effect of the bacterial products. The TNF-α ELISA results are in support of such a view: co-cultures produced high levels at early time points, especially in the presence of TLR2 agonists.

Intriguingly and relevant for our understanding of the immune reactions that take place during an infection is our finding that continuous exposure to TLR activators does not alter osteoclast differentiation when first primed with M-CSF and RANKL, both when added at the pre-osteoclast stage of 7 days and when added at the osteoclast stage of 14 days. This

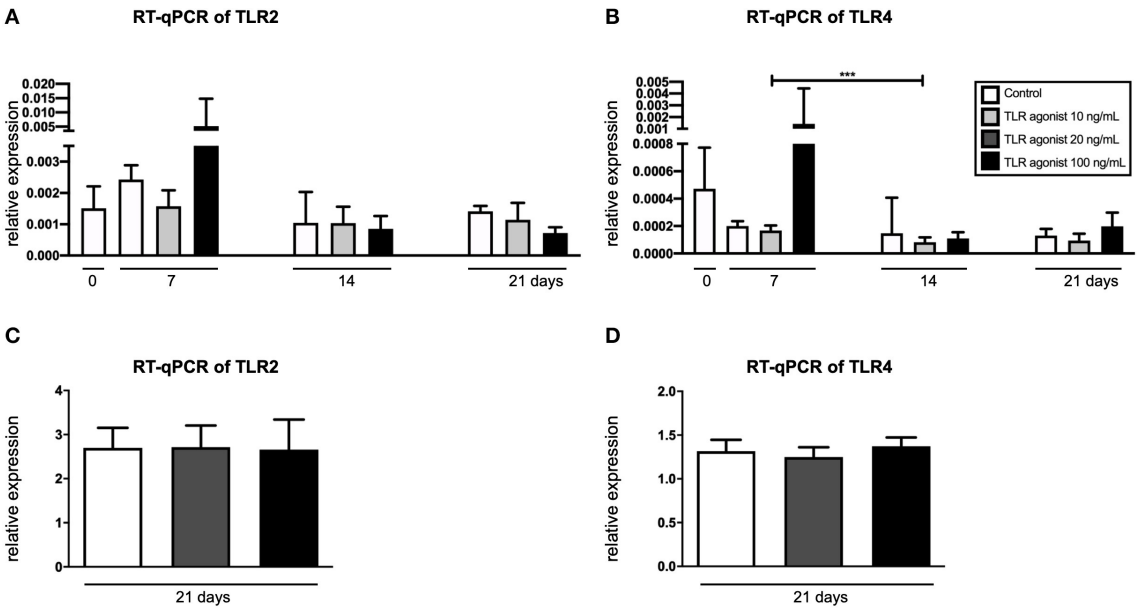

FIGURE 5 | GFs and monocytes express TLR2 and TLR4. **(A)** Expression of TLR2 and **(B)** TLR4 in GFs cultures ($t = 0$) and co-cultures of GFs and PBMCs ($t = 7$, 14, and 21). **(C)** Expression of TLR2 and **(D)** TLR4 in cultures of CD14+ cells, cultured with M-CSF and RANKL. LPS-PG (Invivogen, San Diego, CA, USA) was used as TLR2 and TLR4 agonist in concentrations of 10 ng/mL or 20 ng/mL and 100 ng/mL. $n = 6$ for the co-cultures and $n = 4$ for the CD14+ cells' culture; average results ±SD are shown. Significant results are shown (black bars). ***$p < 0.001$.

could indicate that the TLR-related induction of bone resorption *in vivo* (28, 29, 38), is due to the activation of the inflammatory milieu rather than directly through the osteoclast. In other words, the TLR reaction could elicit local stimulators of osteoclast differentiation such as IL-1β (53, 54) or TNF-α (55). Though not assessed, our results make it unlikely that osteoclasts or osteoclast precursors will express autocrine levels if osteoclast activate themselves after long-term exposure to TLR activators.

To the best of our knowledge, this is the first study that investigates the effect of TLR agonists in osteoclastogenesis and osteogenesis in a model of chronic exposure. Additionally, it is the only study that evaluated both osteoclastogenesis and osteogenesis on human periodontal cells, and more specifically GF. There are a few studies (34, 42, 43) that have studied the osteogenic potential of human periodontal ligament cells (hPDL) exposed to TLR agonists, sometimes with conflicting results. In two independent studies (42, 43), hPDL cells were infected with *E. coli* LPS (TLR4 agonist) and it was found that the osteogenic capacity of the cells was reduced significantly. In another study (34), the effect of TLR ligands was investigated on hPDL cells. High doses of TLR1, TLR3, and TLR6 ligands inhibited the osteogenic potential of these cells. On the contrary, Albiero et al. (41) infected hPDL cells with *Porphyromonas gingivalis* LPS (TLR2 agonist) and found no additional effect on the osteogenic differentiation potential of these cells. However, in this study was not clearly stated if the TLR agonists were added in the culture media only once or also in every refreshment. The osteogenic gene analyses of the above studies were limited to 2 weeks of cultures. For Alizarin red staining, the cells were cultured for 21–28 days. In these experiments, we unequivocally showed no effect of TLR2, TLR4, or the combination of the two when taking into account parameters like alkaline phosphatase, calcium deposition or Alizarin red staining. TLR4 could have a slight influence on the level of ALP at mRNA levels, or, in combination with TLR2 on the mRNA expression of TNF-α. Our results are in line with the findings of Albiero et al. (41), as we also found that the addition of TLR2, TLR4, and the combination of those agonists do not affect the osteogenic potential of the GF. Of special interest: the qPCR data from the osteogenesis were only partly in line with what is commonly seen in osteogenic differentiation. RUNX-2 was highest at an early timepoint, demonstrating its early osteogenic differentiation character. ALP expression was surprisingly highest at day 7 and significantly so in all conditions compared to day 0. Under normal circumstances, ALP protein expression peaks at 14 days (14), apparently the enhanced protein expression is prepared 1 week earlier. Osteonectin expression is believed to be a late marker of osteogenic differentiation, not seen in our results where expression lowered at day 14. When comparing gene expression of all genes, it is remarkable that addition of mineralization medium seemed not to influence gene expression of osteogenic genes. Apparently, the inevitable increased cell density seen in the wells might in part control gene expression. TLR2 and TLR4 agonists did not change gene expression, but interestingly the combination of the two altered TNF-α expression, the only assay where a synergistic effect was seen in our study. The apparent different-from-expected expression patterns of ALP and osteonectin, could be due to the fact that gingival fibroblasts are less suited for osteogenic differentiation compared to periodontal ligament fibroblasts (56). Scanning electron microscopy confirmed that mineral nodules

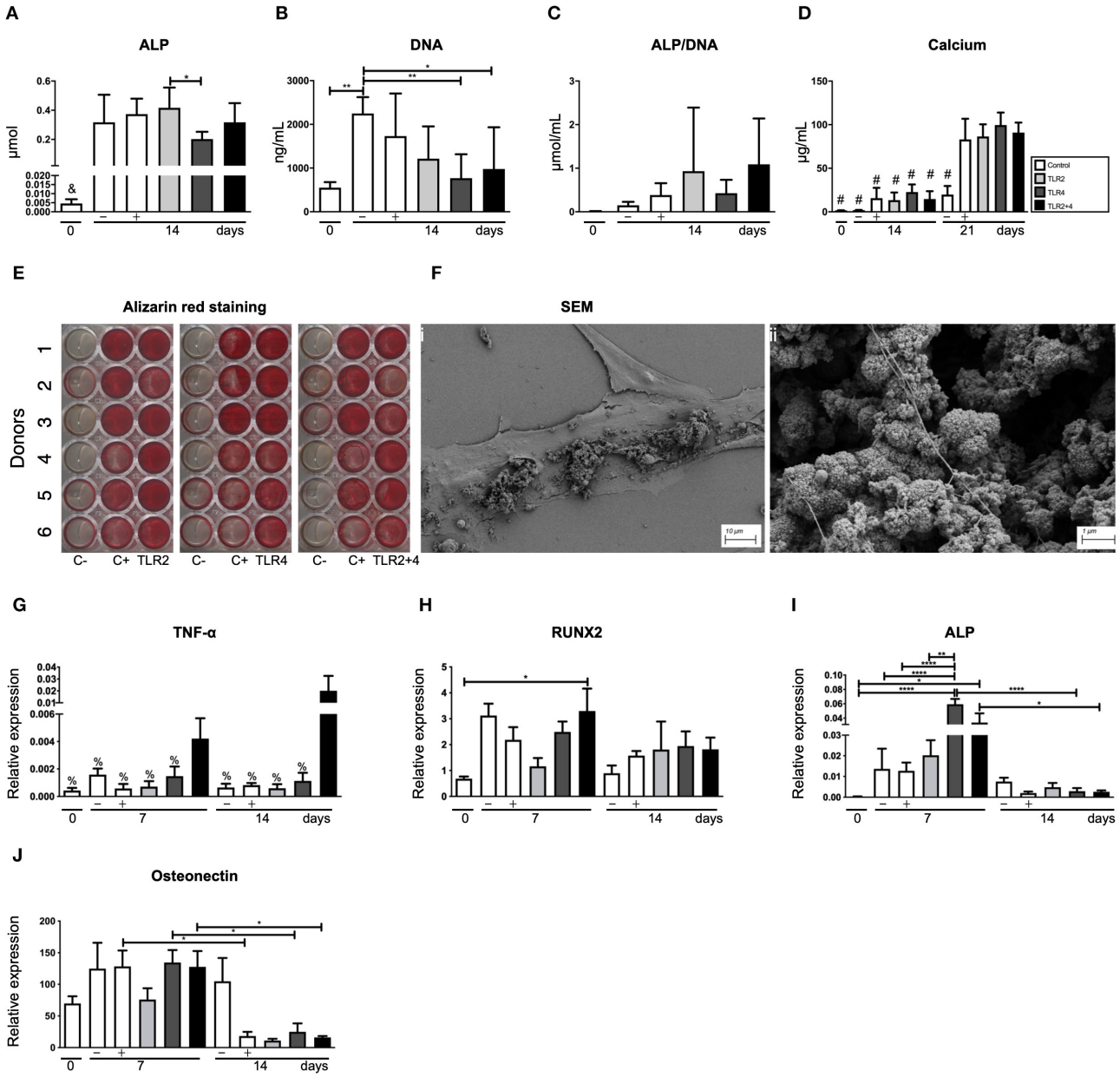

FIGURE 6 | TLR agonists do not affect the osteogenic capacity of gingival fibroblasts. **(A)** Alkaline phosphatase (ALP), **(B)** DNA, **(C)** alkaline phosphatase corrected per number of cells (ALP/DNA), and **(D)** calcium deposition were quantified. **(B)** Compared to control cultures, TLR4 agonist (alone or with TLR2) affected the proliferation of cells. Overall, no significant differences were observed between the control conditions and the TLR agonists for **(A)** ALP, **(C)** Calcium deposition, and **(E)** Alizarin red staining. Alizarin red staining was done using control conditions without (C–) or with (C+) mineralization medium. The staining showed heterogeneity between the 6 donors. **(F**i**)** Shows the osteogenic matrix under scan electron microscopy and **(F**ii**)**presents structures that resemble mineral nodules. **(G–J)** Present the results of the quantitative PCR. **(G)** TNF-α is positively mediated by the combination of TLR2+4 at early and late time points (day 7 and 14). **(H)** RUNX2 is only increased at day 7 by the combination of TLR2+4, in comparison with the baseline (day 0). **(I)** Expression of the ALP gene is elevated in all conditions at day 7 in comparison with the baseline (day 0). TLR4 is also elevated compared to the negative and positive controls at day 7 and compared to the TLR4 at day 14. TLR2+4 reduced significantly from day to day 14. **(J)** Osteonectin gene expression reduced significantly from day 7 to day 14 for the control, TLR4 and TLR2+4. $n = 6$ per condition in duplicates, average results ±SD are shown. Significant results for **(A–D)** and **(G–H)** are shown (black bars). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ in comparison to all the other conditions, $^{\#}p < 0.0001$ in comparison to the c (+), TLR2, TLR4, and TLR2+4 at day 21, $^{\%}p < 0.001$ in comparison to TLR2+4 at day 14.

were formed by the GF. A previous study (57) showed that it is possible to isolate and culture mesenchymal cells from human GF, which showed osteogenic differentiation capacity.

With respect to osteoclastogenesis, our results reject the hypothesis of this paper. Based on *in vivo* studies in mice (38, 39, 58–60) which Pg-LPS have shown that TLR activation induces

osteoclastogenesis and bone resorption, we hypothesized that the activation of the TLR ligands would lead to induction of osteoclasts formation and bone resorption. Our results present a mild inhibition of the formation of osteoclasts in the co-cultures and slight reduction of bone resorption. Ji et al. (30) studied osteoclastogenesis in monocytes cultures, primed with M-CSF, activated with TLRs or IFN-γ, and also in an *in vivo* murine model. They concluded that activation with TLR2 and TLR4 ligands results in inhibition of osteoclastogenesis via inhibition of RANK and CSF1R expression. In another study (61), they studied the formation of osteoclasts in murine bone marrow cells, activated with TLR2 and TLR4 ligands. These cells were primed with M-CSF + RANKL or only with M-CSF and the RANKL was added concomitantly with the TLR ligands (TLR2 and TRL4). They found that TLR ligands inhibited RANKL-mediated osteoclastogenesis when the ligand and RANKL were simultaneously added to the cultures. On the contrary, when the cells were primed with M-CSF and RANKL before the activation with the ligands, osteoclastogenesis was not arrested. In the recent review of Souza and Lerner (62), it is also supported that activation of TLR agonists in different stages affects differently the maturation of the osteoclasts. Concomitant addition of TLR agonists and RANKL at the stage of osteoclast progenitor cell leads to impaired osteoclastogenesis. On the contrary, osteoclast progenitor cells primed with RANKL and then activated with TLR agonists, in the absence of RANKL, differentiated into mature osteoclasts. Our results show an inhibition of the naive osteoclast precursor cells, whereas the already formed (pre-)osteoclasts were not affected, which is in line with these studies. As they state in the paper of Ji et al. (30),

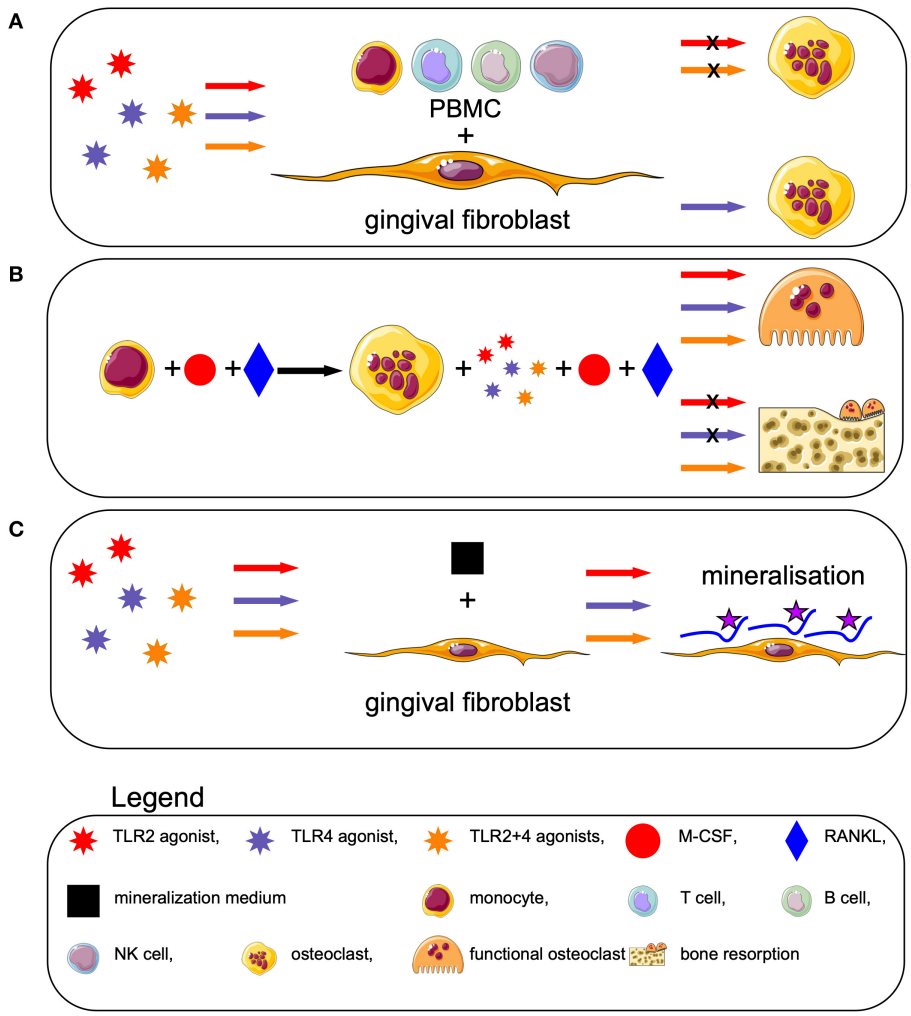

FIGURE 7 | TLR activation affects osteoclastogenesis differently, depending on the differentiation stage of the blood cells, but does not affect osteogenesis. **(A)** Activation of human peripheral blood mononuclear cells (PBMCs) with TLR2 and TLR2+4 agonists, in presence of GF, decreases the formation of osteoclasts. **(B)** Priming of monocytes with M-CSF + RANKL leads to formation of pre-osteoclast. Triggering of these cells with TLRs in an early stage (from day 7 to 21), concomitant with M-CSF and RANKL, induces formation of bone resorbing osteoclasts. When the TLRs are added on a later stage (from day 14 to 21), the bone resorption capacity of the osteoclasts is reduced. **(C)** Triggering of GF with TLRs, in presence of mineralization medium, precipitates osteogenesis.

the inhibition of the osteoclastogenesis can be explained as a homeostatic reaction against the inflammatory effects. Another possible explanation of our findings, as stated earlier in the discussion, could be that the activation of the monocytes with the TLR ligands (and more specifically the TLR2) leads to the formation of more macrophages instead of osteoclasts. This scenario could also be related to highly increased concentrations of TNF-α that we found on day 3 in the conditions of TLR2 and TLR2+4.

Another interesting implementation of our study was the 21 days duration of our experiments. The previously mentioned osteoblasts cultures (34, 41–43) were executed as well in a 21 days' timeframe, as this time is needed for the formation of osteoblasts. Regarding the osteoclastogenesis, this is the first experiment that evaluated the effect of the TLRs in a timeframe of 21 days. Most of the studies that were performed studied the formation of osteoclasts in a timeframe of 2–5 days, typical for mouse osteoclasts (36–40), and in the studies of Kassem et al. (38) and Liu et al. (37), the bone resorption experiments had a duration of 6–7 days. This long exposure on the TLR agonists shows an effect on the expression of TNF-α. Accordingly, we found a higher production of TNF-α on the conditions of TLR2 and TLR2+4 on day 3, in comparison with the control and the TLR4 condition. This finding was reversed on day 7, where TLR2 and TLR2+4 were significantly lower than the control. On day 21, the only remaining difference we observed, was between TLR2 and TLR4 conditions, with the former one being higher. Apparently, the co-culture cell system normalizes over time: at 3 days all cultures containing TLR2 agonists responded with increased TNF-α secretion, followed by the reverse on day 7 and no differences between the conditions at day 21.

In this study, we investigated the chronic effect of the TLR2 and TLR4 agonists on osteoclastogenesis and osteogenesis, measuring several parameters. When observing an effect, it was often TLR2 agonist-mediated. The ability of naive osteoclast precursor cells to form osteoclasts, for instance, was consistently inhibited by TLR2 agonists. Bone resorption also appeared to be reduced by the addition of TLR2 agonists when added at a late time point (day 14). TLR2 had also an evident effect on the production of TNF-α, playing an enchasing role on day 3, which was reversed at the later time points (days 14 and 21). At gene expression level, expression of NFATc1, a key transcription regulator of osteoclast differentiation (50), was downregulated on day 7 by TLR2 agonist. The only effect of TLR4 on osteoclastogenesis was a slight reduction of the bone resorption when it was added at a late time point (day 14). Furthermore, it affected ALP gene expression, with an enhanced expression at day 7. The combination of TLR2+4 agonists only enhanced TNF-α gene expression during osteogenesis, both at days 7 and 14.

In our previous study (24), we demonstrated that GF and monocytes can mediate the diversity of the cellular populations at the site of inflammation, by reducing the number of B-, T- and NK-cells. In this aforementioned study, we also showed that TLR2 activation is an important player in T cell proliferation in the presence of monocytes (20). In the current study, we showed that activation with TLR agonists of naive or M-CSF and RANKL primed human osteoclast precursors has differential effects. This could indicate that fresh monocytes that encounter bacterial products such as TLR2 or TLR4 agonists, may differentiate into macrophages that eradiate these inflammation activators (**Figure 7**). Osteoclasts could be the cells that are NOT activated by TLR directly, but rather indirectly, through the microenvironment's expression of inflammatory cytokines that are known activators of osteoclasts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by VUmc. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GK and TV designed the experiments. GK was involved in collecting most of the data, some of them were retrieved under the supervision of IJ, TS, JH, HV, and CM. GK and TV performed the TLR titration experiments together, results were analyzed by GK. TV pipetted the experiment with co-cultures and osteogenesis. ES and TV designed and performed the experiments with monocytes stimulated with M-CSF and RANKL and TLR agonists (**Figure 4**), analyzed by ES. KŁ-Ć and TV designed and performed the experiment of the expression of TLRs (**Figure 5**). Wisdom teeth, essential for all experiments involving GF, were collected by TF. GK initiated writing, first drafts were corrected by TV, and all authors have commented on the final version and agree with the present version.

FUNDING

This research was sponsored by Institutional Funds.

ACKNOWLEDGMENTS

We thank the class of 2018 of the Cell Biology and Physiology Lab course from Amsterdam University College for collecting some of the data under the supervision of co-authors (IJ, TS, TV, HV, and JH). The educational and pedagogical set-up of this particular 2018 class was recently published (16). Mick Roumen, a student from Amsterdam University College is acknowledged for performing the osteogenesis PCRs. We also acknowledge Petra Henning and Ali Kassem from the Department of Internal Medicine and Clinical Nutrition at Institute for Medicine, Sahlgrenska Academy at the University of Gothenburg, Sweden for their valuable advice regarding TLR2 and TLR4 agonists. KŁ-Ć received funding from Erasmus travel grant allowing experiments to be performed in Amsterdam (ACTA).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 26 August 2020
doi: 10.3389/fimmu.2020.01588

Role of Toll Like Receptor 4 in Alzheimer's Disease

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Long-term evidence has confirmed the involvement of an inflammatory component in neurodegenerative disorders including Alzheimer's disease (AD). This view is supported, in part, by data suggesting that selected non-steroidal anti-inflammatory drugs (NSAIDs) provide protection. Additionally, molecular players of the innate immune system have recently been proposed to contribute to these diseases. Toll-like receptors (TLRs) are transmembrane pattern-recognition receptors of the innate immune system that recognize different pathogen-derived and tissue damage-related ligands. TLR4 mediated signaling has been reported to contribute to the pathogenesis of age-related neurodegenerative diseases, including AD. Although the pathophysiology of AD is not clear, soluble aggregates (oligomers) of the amyloid β peptide (Aβo) have been proven to be key players in the pathology of AD. Among others, Aβo promote Ca^{2+} entry and mitochondrial Ca^{2+} overload leading to cell death in neurons. TLR4 has recently been found to be involved in AD but the mechanisms are unclear. Our group recently reported that lipopolysaccharide (LPS), a TLR4 receptor agonist, increases cytosolic Ca^{2+} concentration leading to apoptosis. Strikingly, this effect was only observed in long-term cultured primary neurons considered a model of aging neurons, but not in short-term cultured neurons resembling young neurons. These effects were significantly prevented by pharmacological blockade of TLR4 receptor signaling. Moreover, TLR4 expression in rat hippocampal neurons increased significantly in aged neurons *in vitro*. Therefore, molecular patterns associated with infection and/or brain cell damage may activate TLR4 and Ca^{2+} signaling, an effect exacerbated during neuronal aging. Here, we briefly review the data regarding the involvement of TLR4 in AD.

Keywords: TLR4, Alzheimer's disease, calcium, amyloid beta oligomers, aging, hippocampal neurons

OPEN ACCESS

Edited by:
Jerrold Weiss, The University of Iowa, United States

Reviewed by:
Alpdogan Kantarci, The Forsyth Institute, United States
John P. Vasilakos, 3M, United States

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *16 April 2020*
Accepted: *16 June 2020*
Published: *26 August 2020*

Citation:
Calvo-Rodriguez M, García-Rodríguez C, Villalobos C and Núñez L (2020) Role of Toll Like Receptor 4 in Alzheimer's Disease. Front. Immunol. 11:1588. doi: 10.3389/fimmu.2020.01588

TOLL LIKE RECEPTORS AND DISEASE

Toll-like receptors (TLRs) are innate immune receptors specialized in the detection of conserved molecular patterns present in pathogens, the so-called PAMP, and self-derived molecules released upon tissue damage, referred to as DAMP (1). The TLR family, which belongs to the type I membrane glycoproteins, is comprised of 10 members in humans, and 12 in mice (2). TLR4 was the first TLR identified in humans, which senses lipopolysaccharide (LPS)—a major component of the outer membrane of Gram-negative bacteria—which exhibits potent immuno-stimulatory activity (3). TLR4 also recognizes DAMPs released upon tissue injury, i.e., high-mobility

group box 1 (HMGB1), heat-shock proteins, reactive oxygen intermediates, and extracellular matrix breakdown products (4).

TLRs recognizing bacterial and fungal components (TLR 1, 2, 4, 5, 6) are expressed on the cell surface, while sensors of viral and nucleic acids (TLR 3, 7, 8, 9, 10, 11, 12, and 13) are localized within endosomal compartments, where TLR4 can also be translocated (1, 2). The association of TLRs with their specific ligands initiates intracellular signaling routes through the adaptor MyD88, except for TLR3 that signals *via* TRIF. This culminates in the induction of pro-inflammatory molecules *via* NF-κB activation or antiviral molecules *via* interferon regulatory factor routes (3, 5). Aberrant TLR activation has been associated with chronic inflammation and disease (6). A significant amount of evidence associates TLRs to several diseases, i.e., sepsis, asthma, autoimmune diseases, cancer, diabetes, intestinal disorders, cardiovascular diseases, and neurodegenerative disorders (2, 6, 7).

In the nervous system, TLRs are expressed in several cell types including neurons and glia (8), where they sense DAMPs released by undifferentiated or necrotic/injured cells. TLR activity has been associated with several neurodegenerative diseases, including stroke, amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (AD) (9).

TLR4 AND ALZHEIMER'S DISEASE

TLR4 is believed to mediate the neurotoxic actions of DAMPs associated with neuronal damage involved in AD. In fact, increasing evidence associates TLR4 with neuronal plasticity (10, 11) and AD (12). For instance, Fujita and colleagues have reported that HMGB1, a prototypic DAMP released from necrotic or hyperexcitatory neurons, induces neurite degeneration *via* TLR4 (13). The study showed that myristoylated alanine-rich C-kinase substrate (MARCKS), a submembrane protein involved in the actin network stability, is phosphorylated at Ser46 well before aggregation of the amyloid β peptide (Aβ), and this effect is sustained during the course of AD both in human and mouse models of the disease. HMGB1 released from necrotic or hyperexcitatory neurons binds to TLR4 and activates MAP kinases, inducing MARCKS phosphorylation leading to neurite degeneration, one of the classic hallmarks of AD pathology. Strikingly, subcutaneous injection of an antibody against HMGB1 prevented neurite degeneration and reversed cognitive loss, even in the presence of Aβ plaques (13). This study suggests a critical involvement of TLR4 in the effects of DAMPs like HMGB1, which acts as an essential pathogenic molecule in AD.

TLR4 is also considered to be one of the key receptors involved in the microglial innate immune system, since it could be involved in the production of pro-inflammatory cytokines in AD. Consistent with this concept, gene profile analysis of post-mortem human brains revealed an increased expression of TLR4, TNF, and IL-6 genes in the frontal cortex of AD patients relative to age-matched samples (14). Moreover, in the entorhinal cortex lesioned mouse, an experimental model of hippocampal deafferentation without amyloidosis, mimicking one of the first neuronal losses observed in AD, *tlr4* and *il-1b* genes were overexpressed during the deafferentation phase but not during the process of reinnervation. Therefore, TLR4 dependent modulation of cytokines could be differentially regulated by either Aβ plaques or by deafferentation processes (14).

TLR4 activation may also contribute to AD by blocking anti-inflammatory pathways. In particular, TLR4 and TREM2 (triggering receptor expressed on myeloid cells 2, which holds an anti-inflammatory role in the brain) may be a link between AD and systemic inflammation, a process generally having deleterious effects on AD progression (15). This view is supported by data obtained from APPswe/PSEN1ΔE9 (APP/PS1) mice, a mouse model of cerebral amyloidosis, which showed increased expression of both TLR4 and TREM2 in the cortex at the gene and protein levels (15). LPS treatment further aggravated cognitive impairment in these mice, implying that superimposition of systemic inflammation to familial AD may accelerate AD progression. Interestingly, after treatment of these mice with LPS, *tlr4* gene expression remained up-regulated, while *trem2* gene was down-regulated (15). These data suggest that the inhibitory effect of TREM2 on inflammation could be downregulated by TLR4 activation, resulting in inflammation and apoptosis in the cortex of APP/PS1 mice without changes in the Aβ levels.

TLR4 has also recently been linked to memory loss mediated by Aβ oligomers (Aβo) in AD. A single intracerebroventricular injection of Aβo in C57BL/6J naïve mice substantially impaired their recognition memory. Interestingly, it also activated glial cells, resulting in the enhanced expression of pro-inflammatory cytokines. Anti-inflammatory drugs prevented the memory impairment induced by the oligomers. In addition, cyanobacterial LPS (Cyp)—a specific TLR4 receptor antagonist—eliminated the deleterious effects of Aβo on memory. Aβo had no effect either on memory or glia activation in TLR4 knockout mice, supporting the involvement of TLR4 in the noxious effects (16). Collectively, these data suggest that Aβo may not only act directly on synapses, but may also impact the immune system, with TLR4 playing a major role.

A critical role of TLR4 in AD is also supported by recent data showing that LPS, the archetypal TLR4 agonist, was detected in brain lysates from the hippocampus and neocortex of post-mortem AD brains (17). LPS levels in AD brains were found to be two to three folds larger than age-matched control cases. Strikingly, in some cases of advanced AD, there was even a 26-fold larger level of LPS over control. The authors of the study suggest that LPS from microbiota and/or bacterial infections in the body may accumulate in the brain, contributing to AD. Consistently, it has been shown that LPS is able to induce memory impairment in rats. According to these data, Zakaria et al. have recently revised and proposed rats injected with LPS as a novel animal model of AD (18).

TLR4 AND CALCIUM SIGNALING IN ALZHEIMER'S DISEASE

Strong evidence supports the involvement of intracellular Ca^{2+} dyshomeostasis in aging and neurodegenerative disorders

including AD (19). Our group, and others, have recently shown that long-term cultures of rat hippocampal neurons display characteristics of aged neurons (20–22). These neurons exhibit enhanced susceptibility to neuronal cell death induced by either neurotoxins such as the glutamate receptor agonist NMDA (20, 23) or oligomers of the amyloid β peptide 1-42 (24). These effects are due to age-related changes in the expression of NMDA receptors resulting in an enhanced rise in the cytosolic Ca^{2+} levels induced by the neurotoxin, and in a subsequent mitochondrial Ca^{2+} overload and apoptosis (23–25). These changes have also been related to the remodeling of Ca^{2+} homeostasis associated with aging (26) and to the toxic effects of Aβo at a subcellular level (22, 27). This model of *in vitro* neuron aging has recently been used to investigate the effects of LPS on apoptosis and Ca^{2+} signaling on hippocampal neurons.

As described above, injection of LPS in rat brains promotes cognitive decline and brain damage and the rat injected with LPS has been indeed proposed as an animal model of AD (18). Interestingly, LPS induces apoptosis in rat hippocampal neurons in primary culture depending on the time of the culture. Specifically, short-term and long-term cultures of rat hippocampal neurons resembling young and aged neurons, respectively, were activated with LPS. In morphologically identified neurons, LPS treatment promoted apoptosis only in long-term cultures, but not in young ones, as analyzed using fluorescence imaging of annexin V. These effects were inhibited by the TLR4-antagonist CAY10614, indicating the involvement of TLR4 activation (28). These effects could be mediated by TLR4 expressed in glia and/or neurons. Fluorescence imaging followed by optical density analysis showed that identified neurons express TLR4 and the level of expression increases in the long-term cultured neurons. This result was further supported by double-staining immunofluorescence proving co-expression of neuronal specific markers and TLR4 (28). These data are consistent with increased levels of TLR4 expression reported in the aged human brain (10). Additionally, recent work by Hughes and collaborators (29) suggested that in a co-culture neurons-glia, the Aβo-driven neuronal cell death is mainly due to the Aβ-sensitized TLR4 signaling of glial cells (astrocytes and microglia in the co-culture) *via* autocrine/paracrine mechanism, thus proposing another mechanism for Aβ toxicity *via* TLR4.

The effects of LPS on Ca^{2+} signaling have also been investigated in cultured rat hippocampal neurons. LPS increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in rat hippocampal neurons loaded with the Ca^{2+} sensitive dye fura2. Consistently with TLR4 expression, LPS increased $[Ca^{2+}]_{cyt}$ only in long-term cultures of rat hippocampal neurons but not in the short-term cultured ones. In contrast, NMDA increased $[Ca^{2+}]_{cyt}$ both in young and aged neurons, although the effects in aged neurons were larger (28). Again, the effects of LPS on $[Ca^{2+}]_{cyt}$ were inhibited significantly by the TLR4-antagonist CAY10614, implying TLR4-mediated effects. Glial cells also displayed Ca^{2+} responses to LPS that were mostly in the form of Ca^{2+} oscillations in a small fraction (30%) of the glial cells (28).

On the contrary, glial cells did not display Ca^{2+} responses to NMDA, as previously reported (30). The effects of LPS on $[Ca^{2+}]_{cyt}$ are not mediated by NMDARs, since low concentrations of MK801, a NMDAR antagonist, prevented NMDA-induced but not LPS-mediated rises in $[Ca^{2+}]_{cyt}$. In addition, LPS treatment did not affect the expression of the NMDAR subunits, excluding the possibility that LPS may influence Ca^{2+} signaling and apoptosis by modulating expression of NMDARs (28). These data indicate that LPS promotes Ca^{2+} signaling and apoptosis in aged neurons and TLR4 contributes to these effects by NMDA receptor independent mechanisms. However, the ion channel involved in the effects remains unknown and additional studies are required to address this question.

The effects of amyloid oligomers on the LPS-mediated Ca^{2+} signaling and apoptosis have been addressed as well. In fact, both Ca^{2+} rises and apoptosis may be exacerbated in AD by the excess of Aβo formation. This view is supported by findings reported by Calvo-Rodriguez et al. (28) showing that co-treatment with Aβo and the TLR4 ligand LPS, potentiates Ca^{2+} responses and neuronal cell death in cultures of rat hippocampal neurons, particularly in aging. Specifically, studies performed with long-term cultured hippocampal neurons revealed that Aβo treatment potentiates the rise in $[Ca^{2+}]_{cyt}$ induced by LPS, suggesting a synergistic effect between TLR4 and Aβo involving Ca^{2+} signaling in aged neurons. Consistently, a 48 h exposure to either LPS or Aβo fails to induce apoptosis in young cultures. In contrast, the combination of LPS and Aβo significantly increased the neuronal apoptosis in young neurons, effects that were enhanced dramatically in aged neurons (28). These data indicate that Ca^{2+} signals induced by TLR4 activation and Aβo may crosstalk to enhance neuronal cell death, particularly in the aging scenario. This could be mediated by changes in the expression of TLR4 induced by Aβo. Supporting this, the treatment with Aβo induced changes in TLR4 expression in neurons depending on the time in culture. Specifically, TLR4 expression was low in young neurons and treatment with Aβo did not influence TLR4 expression, as shown by immunofluorescence. In contrast, after 2 weeks *in vitro*, when TLR4 expression is still not significantly different from young cultures, Aβo treatment increased significantly TLR4 expression. These effects were further exacerbated in neurons cultured for more than 3 weeks *in vitro*, corresponding to aged neurons. Therefore, evidence from this *in vitro* model of neuronal aging suggests that LPS promotes Ca^{2+} signaling and apoptosis in aged hippocampal neurons and that these effects are mediated by TLR4. More importantly, TLR4 expression is exacerbated by aging and the presence of Aβo (28) (**Figure 1**). As an *in vitro* model, the system used in this study has limitations: (i) as a model of aging, it might not reflect the complexity of *in vivo* aging in a living brain, since the effect and interaction with other cells in the brain and vessels is not considered in this model; (ii) as a model of neuroinflammation, LPS addition may be a simplistic model of recreating it, since neuroinflammation requires many other factors that are excluded here; (iii) as a model of AD, Aβ oligomers are the only element taken into account in this *in vitro* model, whereas the contribution of other factors involved in AD (tau, microglial activation, astrocytic reactivity, vessel dysfunction, etc.) remains to be explored. Nevertheless,

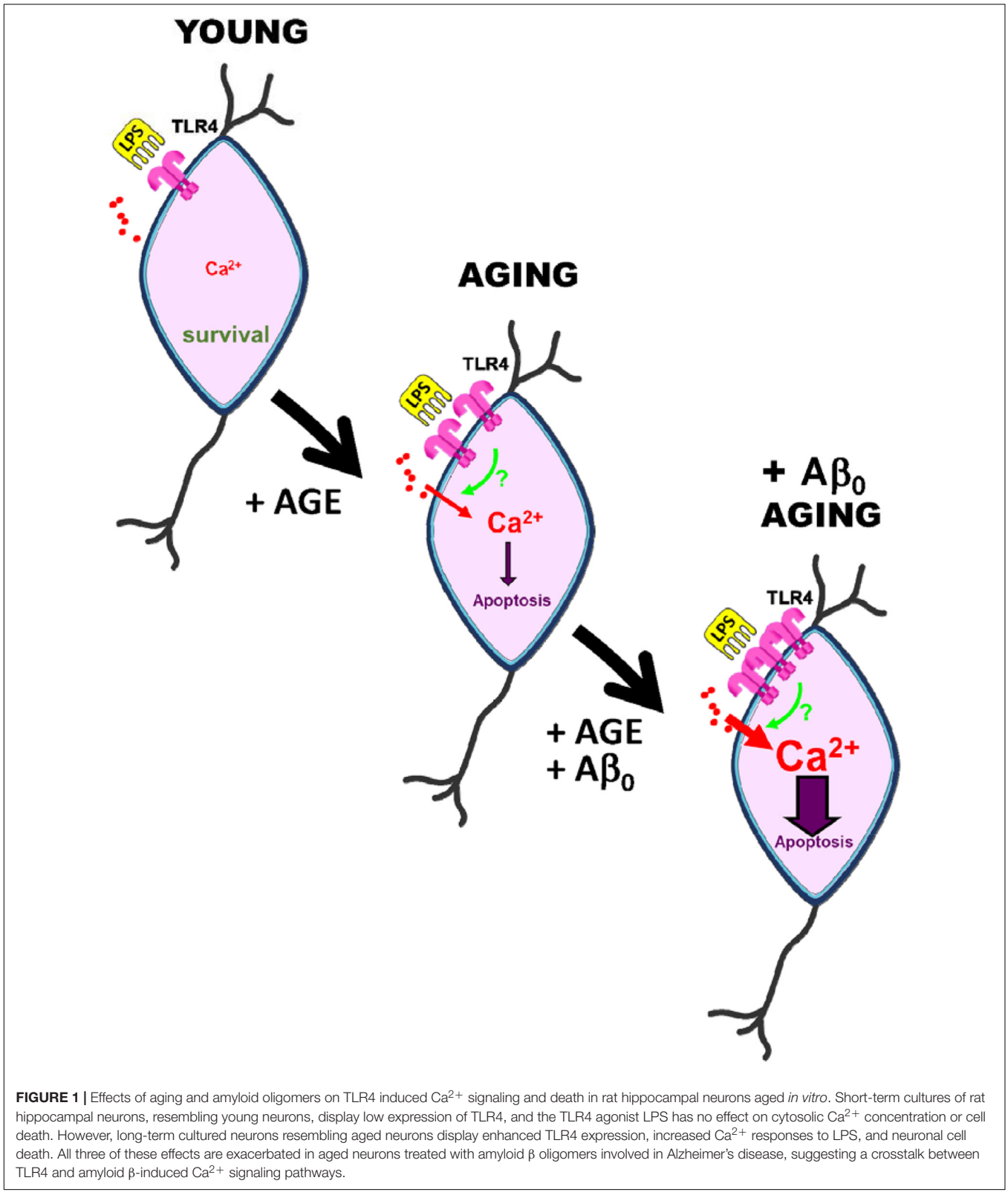

FIGURE 1 | Effects of aging and amyloid oligomers on TLR4 induced Ca^{2+} signaling and death in rat hippocampal neurons aged *in vitro*. Short-term cultures of rat hippocampal neurons, resembling young neurons, display low expression of TLR4, and the TLR4 agonist LPS has no effect on cytosolic Ca^{2+} concentration or cell death. However, long-term cultured neurons resembling aged neurons display enhanced TLR4 expression, increased Ca^{2+} responses to LPS, and neuronal cell death. All three of these effects are exacerbated in aged neurons treated with amyloid β oligomers involved in Alzheimer's disease, suggesting a crosstalk between TLR4 and amyloid β-induced Ca^{2+} signaling pathways.

this *in vitro* model is an excellent system to study certain precise mechanisms involving Ca^{2+} homeostasis and/or channel expression and helps in understanding the synergistic effects between Aβ and LPS on aged hippocampal neurons. Further studies *in vivo* animal models will be required to disentangle the pathology underlying this disease. Consistently, recent *in vivo*

data suggest the critical role of mitochondrial Ca^{2+} overload in AD models (31).

Additionally, inhibiting TLR4 activation in AD may suppress the neuroinflammatory process in the disease. The use of chemical TLR4 antagonists as a treatment for AD might not be of high specificity. However, a broad range of therapeutic compounds inhibiting TLR4 have proven evidence of efficacy in animal models of AD (32), suggesting that therapeutic blocking of TLR4 may be a candidate therapeutic approach for AD.

CONCLUDING REMARKS

Evidence provided in this mini review suggest the critical contribution of TLR4 in the pathogenesis of AD. TRL4 is expressed in the brain, and its expression increases with aging and the accumulation of amyloid β oligomers. DAMPs may also accumulate with aging and chronic inflammation. Therefore, the simultaneous accumulation of Aβ and DAMPs induced TLR4 activation may occur upon stress and/or brain damage, particularly in the context of aging. Increased serum levels of pro-inflammatory cytokines are often associated with aging, a chronic subclinical condition termed as inflammaging (33). Consequently, elevation in the levels of inflammatory cytokines induced by TLR4 activation may promote the accumulation of Aβ, which in turn may enhance expression of TLR4 levels, creating a damaging feedforward loop that may largely contribute to the progression of AD (34). This loop may also be further amplified by the age-associated increased expression of NMDA receptors, which could be simultaneously targeted by Aβo and perhaps by some other DAMPs. These processes may crosstalk at the level of Ca^{2+} signals induced by Aβo and TLR4 independently, particularly in the aging scenario, in which this crosstalk may contribute to brain damage during AD.

AUTHOR CONTRIBUTIONS

MC-R, CG-R, CV, and LN jointly wrote the mini review. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by grants RTI2018-099298-B-100 and BFU2015-70131R from the Ministry of Science, Innovation and Universities, Spain, as well as grant VA294-P18 from Junta de Castilla y León, Spain. MC-R holds a postdoctoral fellowship from the BrightFocus Foundation A2019488F, United States.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 August 2020
doi: 10.3389/fimmu.2020.01964

TLR4 Signaling by Heme and the Role of Heme-Binding Blood Proteins

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Toll-like receptors (TLRs), also known as pattern recognition receptors, respond to exogenous pathogens and to intrinsic danger signals released from damaged cells and tissues. The tetrapyrrole heme has been suggested to be an agonist for TLR4, the receptor for the pro-inflammatory bacterial component lipopolysaccharide (LPS), synonymous with endotoxin. Heme is a double-edged sword with contradictory functions. On the one hand, it has vital cellular functions as the prosthetic group of hemoproteins including hemoglobin, myoglobin, and cytochromes. On the other hand, if released from destabilized hemoproteins, non-protein bound or "free" heme can have pro-oxidant and pro-inflammatory effects, the mechanisms of which are not fully understood. In this review, the complex interactions between heme and TLR4 are discussed with a particular focus on the role of heme-binding serum proteins in handling extracellular heme and its impact on TLR4 signaling. Moreover, the role of heme as a direct and indirect trigger of TLR4 activation and species-specific differences in the regulation of heme-dependent TLR4 signaling are highlighted.

Keywords: heme, heme-binding proteins, hemopexin, hemolysis, inflammation, TLR4

OPEN ACCESS

Edited by:
Jerrold Weiss,
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *14 May 2020*
Accepted: *21 July 2020*
Published: *27 August 2020*

Citation:
Janciauskiene S, Vijayan V and Immenschuh S (2020) TLR4 Signaling by Heme and the Role of Heme-Binding Blood Proteins. Front. Immunol. 11:1964. doi: 10.3389/fimmu.2020.01964

INTRODUCTION

Toll-like receptors (TLRs) recognize invading pathogens and are essential sensors and regulators of the innate immune system (1, 2). Bacterial, fungal, and viral infections activate various TLRs that play a role in host defense but may also cause sepsis and tissue injury. Stimulation of TLRs by their respective specific ligands initiates signaling cascades that mediate activation of transcription factors and secretion of pro-inflammatory molecules (1, 2). For instance, TLR4 is stimulated by the prototypical pro-inflammatory bacterial wall compound lipopolysaccharide (LPS), also known as endotoxin (3). More recently, other compounds have been described to interact and stimulate TLR4 including hyaluronic acid, the dust mite protein Der p 2, nickel and various endogenous molecules released from injured cells, that are collectively termed danger-associated molecular patterns (DAMPs) (4–7). In particular, the red blood cell-derived product heme has been implicated in TLR4 signaling and has been proposed to be a DAMP that affects inflammatory responses in a variety of pathophysiological conditions (8–15). Heme is an iron-containing tetrapyrrole with important functions in various biological processes as a prosthetic moiety of hemoproteins in its covalent or non-covalent bound form (16, 17). For example, in hemoglobin and myoglobin, heme is used for oxygen transport and storage, whereas in cytochromes it is involved in electron transport, and generation of energy. Heme is also important for enzymes such as cyclooxygenase-2, nitric-oxide

synthase-1, NADPH oxidases, catalases, and peroxidases (16, 18). In contrast, non-protein bound heme, also termed "free" heme, can be harmful and cause pro-oxidant, pro-inflammatory, and cytotoxic effects as previously reviewed elsewhere (12, 13, 19, 20). Additionally, heme can mediate the recruitment of leukocytes, platelets, and red blood cells to the vascular endothelium. Many of the pro-inflammatory effects of heme have been associated with activation of TLR4 signaling, as initially demonstrated in macrophages (10). However, TLR4 signaling by heme appears to involve highly complex regulatory mechanisms, which are dependent on the applied models and experimental conditions (15, 21). For example, conflicting findings on potential heme-dependent pro-inflammatory effects have been reported in kidney injury models applying TAK-242, a specific inhibitor of TLR4 signaling, and TLR4 knockout mice (22–25). Hence, mechanistic details on how heme may mediate its pro-inflammatory regulation through direct or indirect interactions with TLR4 are not fully understood. In this review, the complex relationships between heme and TLR4 are discussed with a particular focus on the role of serum heme-binding proteins (HBPs).

DIRECT ACTIVATION OF TLR4 SIGNALING BY HEME

The mechanistic basis of how TLR4 signaling may be activated by heme has been primarily studied in mouse models with genetic TLR4 deficiency and with small molecule inhibitors of TLR4. For example, it has been demonstrated that treatment of TLR4-deficient macrophages with purified exogenous heme fails to induce expression of pro-inflammatory cytokines (10) and activation of the inflammasome (26). Moreover, inflammatory activation of the endothelium by heme has been found to be counter-acted in $TLR4^{-/-}$ mice and by administration of TAK-242 (27). Interestingly, in studies with human embryonic kidney 293 cells, heme, and LPS applied together expressed additive effects suggesting that they activate TLR4 by different mechanisms (28). Although such findings support a role of heme in direct TLR4 signaling, an activation site for heme-binding in this receptor is still elusive. As efficient TLR4-dependent cell activation by LPS requires the complex interplay of TLR4 with CD14, myeloid differentiation protein-2 (MD-2) and the serum protein lipopolysaccharide binding protein (LBP) (29) it is likely that cooperation of these proteins is also critically involved in heme-dependent TLR4 signaling (**Figure 1**). Notably, a heme activation site has recently been identified in human MD-2 which appears to play a critical regulatory role in TLR4 signaling by heme (30).

INDIRECT REGULATION OF TLR4 SIGNALING BY HEME

TLR4 ligands other than LPS can mediate TLR4 signaling independent of direct interactions with the receptor. For example, both, hyaluronic acid and the dust mite allergen Der p 2, have been demonstrated to induce TLR4 signaling indirectly (31, 32). Similarly, accumulating evidence indicates that certain pro-inflammatory heme effects may also be independent of direct heme-binding to TLR4.

Generation of Reactive Oxygen Species (ROS)

Pro-oxidant properties of free heme can cause the generation of ROS via the Fenton reaction of Fe(II) and H_2O_2 [reviewed elsewhere (14, 20, 33)]. As activation of TLRs and generation of ROS can be complementary in settings of so-called oxidative stress (34), it is likely that heme-induced ROS generation may also indirectly activate TLR4 signaling (**Figure 1**). It should be noted that ROS can rapidly oxidize phospholipids, which in turn initiate pro-inflammatory responses via TLR2 and/or TLR4. Independently, an inhibition of the oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC)-induced pro-inflammatory effects has been reported after down-regulation of TLR4 either by the antagonist eritoran or by antisense nucleotides (35–37).

Lipid Oxidation

Oxidized low-density lipoproteins reportedly cause activation of TLR4 (38) and binding of heme can rapidly bind to and oxidize lipoproteins in the serum (39, 40) (**Figure 1**). As binding of heme to lipoproteins occurs faster than that to serum HBPs such as hemopexin (Hx) and albumin, it is conceivable that oxidized lipoproteins can induce TLR4-mediated inflammatory signaling and expression of inflammatory cytokines. Yet, depending on the tissue, these inflammatory effects may contribute to arteriosclerosis, rheumatic diseases, and others (33, 40, 41).

Interaction With Lipid Raft-Associated Proteins

Due to its lipophilic nature, heme can form aggregates and interact with the hydrophobic phospholipid bilayer in lipid membranes affecting TLR4 signaling (42). Membrane lipid rafts are dynamic cellular assemblies of saturated sphingolipids, cholesterol, and selected proteins (43). There are some transmembrane proteins located in lipid rafts including CD44 and CD36, both of which are involved in TLR4 signaling. Ample data indicate that TLR4 and accessory proteins can associate with lipid rafts and that TLR4-raft association is stimulated by bacterial LPS (44). Depending on the TLR4 ligand, different co-receptors can be involved. For instance, the ability of LPS to activate TLR4 depends on CD14, a glycophosphatidylinositol-anchored protein and co-receptor of MD-2 for LPS recognition (45), which may also control internalization of heme via TLR4 (10). Interestingly, soluble TLR4 co-receptor CD14 has recently been reported to mediate pro-inflammatory effects of heme in a whole blood model (46).

Disruption of Lipid-Rafts

Extraction or sequestration of cholesterol with cyclodextrin or nystatin has been shown to disturb clustering of TLR4 and accessory proteins in rafts and to inhibit LPS-induced TNF-α production (47). According to recent reports, naturally high content of cholesterol in sickle and normal red blood cells provides protection against free heme-induced oxidative stress

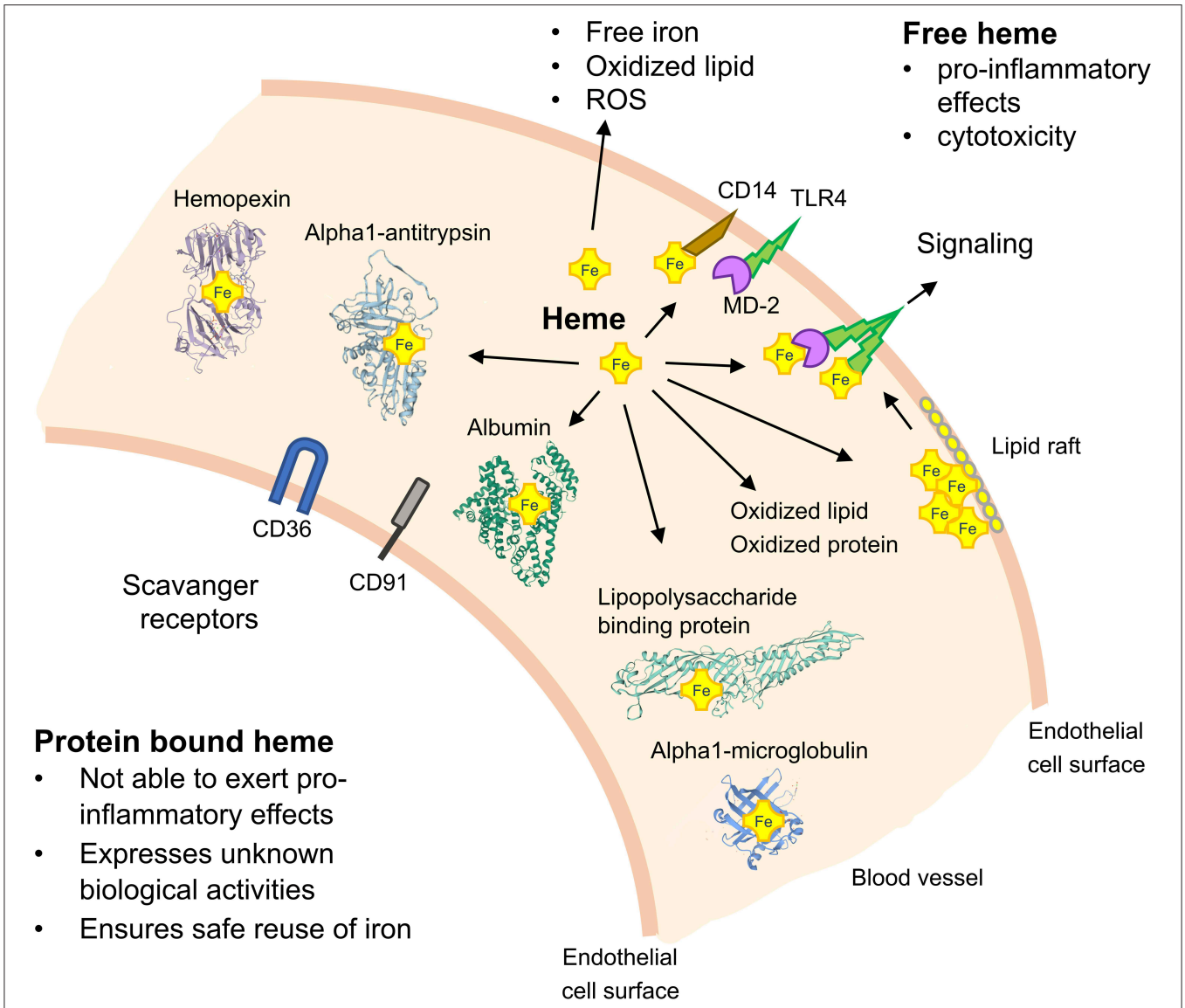

FIGURE 1 | Schematic on TLR4 signaling by heme and the role of heme-binding blood proteins. TLR4 activation depends on the serum protein lipopolysaccharide (LPS)-binding protein, the co-receptor CD14 and myeloid differentiation protein-2 (MD-2) for bacterial recognition. Free heme can activate TLR4-mediated signaling and soluble CD14 has been found to be critical for the regulatory effects of heme. Heme-binding proteins (HBPs) including hemopexin (Hx), albumin, alpha1-microglobulin (A1M), and alpha1-antitrysin (AAT) play critical roles for neutralization of heme and potential heme-dependent interactions with TLR4 to activate TLR4 signaling. The potential role of lipid rafts in TLR4 signaling and scavenger receptors, such as CD36 and CD91, in mediating cellular effects of heme are depicted. *ROS*, reactive oxygen species.

and membrane damage during normal and hemolytic conditions (48). Because cholesterol depletion affects lipid raft assembly, membrane trafficking, and TLR signaling, we speculate that free heme or specific heme-HBP complexes may have modulatory effects on TLR4 signaling via lipid rafts. Thus, we hypothesize that heme, depending on its conformational state, might be incorporated into rafts of the plasma membrane, affect lipid raft fluidity, polarity, thickness, and tension-properties, which, in turn, may influence recruitment (assembly) of TLRs and signaling. Thus, via unspecific hydrophobic interactions with lipid rafts, heme alone or in complex with HBPs may affect TLR4 signaling (**Figure 1**).

In summary, heme may mediate TLR4 activation via various indirect mechanisms including production of ROS, oxidation of lipoproteins, and modulation of lipid rafts in cell membranes.

HEME INTERACTIONS WITH SERUM HEME-BINDING PROTEINS AND ROLE IN TLR4 SIGNALING

Heme toxicity and its pro-inflammatory effects have been demonstrated in experimental disease models like sickle cell disease (SCD), malaria, sepsis, atypical hemolytic uremic

syndrome, arteriosclerosis, or ischemia-reperfusion injury (27, 41, 49–52). The damaging effects of free heme can be blocked by intracellular factors like heme oxygenases and ferritin, and extracellular factors such as various plasma proteins, respectively (**Figure 1**). Only if both intra- and extracellular defense mechanisms are overwhelmed, cellular toxicity arises (12, 33, 53). Independent reports have provided evidence that neutralization of free heme via Hx, the serum protein with the highest known heme-binding affinity (Kd $<10^{-12}$ in humans), counteracted the detrimental effects of heme (42, 54–57). However, serum concentrations of Hx are low (about 0.6–1.2 g/L), and in conditions of severe hemolysis (55) decreased systemic levels of Hx might not be sufficient to neutralize larger amounts of free heme. Therefore, other plasma proteins including albumin, alpha-1-microglobulin (A1M), and alpha1-antitrypin (AAT) appear to be also involved in binding and neutralization of free heme (12, 33, 58, 59). Although albumin binds heme with an affinity about 100-fold lower than Hx, the high concentration of albumin in serum (35–53 g/L) might compensate any potential deficiency in Hx. This, in part, may explain beneficial effects of albumin infusion to individuals with severe sepsis (60) and malaria (61, 62). Notably, albumin is a negative acute phase protein in humans and it is conceivable that during severe inflammatory conditions, when the heme-neutralizing capacity of albumin decreases, other acute-phase proteins such as AAT will participate. AAT is a HBP with binding affinity similar to albumin (59) and it has previously been demonstrated that AAT markedly reduces free heme neutrophil-activating effects, including the production of ROS (63). Serum HBPs not only bind and neutralize free heme with different binding affinities, but may also acquire novel biological activities via specific interactions with heme (64–66). For example, the HBPs Hx and A1M have recently been shown to exhibit differential heme transporter functions and are reciprocally regulated during SCD. While Hx directs heme to the liver and mediates its hepatic up-take via the scavenger receptor low-density lipoprotein receptor-related protein-1 (LRP1, synonymous with CD91) (67, 68), A1M directs heme to the kidney where it may cause detrimental effects including acute kidney injury (69). Finally, it has been found that the interplay of immunoglobulins with heme may alter their binding affinity for bacterial antigens (70).

The question, which form(s) of protein-associated heme is/are inert or biologically active *in vivo* remains open. For instance, high concentrations of albumin-associated heme in the presence of serum failed to induce inflammatory responses in endothelial cells and macrophages (21). Likewise, the local and systemic exposure to protein-associated heme did not induce inflammatory gene expression in mouse models. Heme-mediated signaling via NF-kB only occurred in serum-free conditions in cell cultures of macrophages (21). These findings imply that only the complete absence of serum proteins may allow TLR4 interactions of free heme or specific heme-HBP complexes which, in turn, activate pro-inflammatory pathways. Thus, direct heme-mediated TLR4 signaling appears to be unlikely in relevant clinical conditions, because levels of "free" heme *in vivo* appear to be orders of magnitude below those conditions applied *in vitro* to cause pro-inflammatory effects.

In conclusion, pro-inflammatory effects of heme are critically dependent on heme interactions with serum HBPs, which can largely vary in different pathophysiological settings.

HEME AS A SECOND HIT FOR TLR4 ACTIVATION

Cell-free hemoglobin and heme derived from lysed red blood cells have been reported to synergize with the pro-inflammatory effects of TLR4 agonists in culture models of mouse macrophages (11). These findings suggest that free heme may substantially aggravate inflammatory responses in settings of bacterial or viral infections with simultaneous intravascular hemolysis. Due to the difficulties in determining the biologically relevant concentrations of free heme, the mechanisms that mediate the synergism of heme with different TLR agonists are unclear. Independently, free heme has been demonstrated to synergistically activate the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in LPS-primed macrophages (26) and endothelial cells (71). The NLRP3 inflammasome is a multimeric protein complex comprising a sensor, an adaptor and the zymogen procaspase-1, which leads to activation of caspase-1 and release of the pro-inflammatory interleukins, IL-1β, and IL-18 (72). Heme activates the NLRP3 inflammasome leading to IL-1β production by peritoneal macrophages and in human endothelial cells, but this effect of heme is lost in NLRP3-deficient mice. Finally, free heme may contribute to the inflammatory activation of the endothelium via complement activation as demonstrated in various experimental models of intravascular hemolysis (51, 73). These studies have also provided experimental evidence that free heme may be an important second signal for pre-existing conditions of pro-inflammatory endothelial activation to further escalate the inflammatory vascular damage in disorders such as SCD and atypical hemolytic uremic syndrome (74).

In summary, heme may synergize with a variety of pro-inflammatory agonists to aggravate activation of TLR4 and inflammation.

SPECIES-SPECIFIC DIFFERENCES OF HEME-DEPENDENT TLR4 SIGNALING IN INFLAMMATION

Because heme interactions with TLR4 have largely been studied in rodent models, the extent to which these models apply to human conditions is very important. Due to the specific pathogens encountered by mice and humans, various aspects in the innate and adaptive immune systems are different between these two species (75). Thus, human and murine responses to TLR4 activation have some similarities, but also profound differences (76). For example, Akashi et al. reported that the lipid moiety of endotoxin, lipid A, acts agonistically on mouse, but not on human TLR4/MD-2 (77), which has

more recently also been confirmed in structural studies on the TLR4/MD-2 complex (78). It is also important to point out that murine and human TLR4 share 67–71 and 79–81% similarity at the nucleotide and amino acid levels, respectively (79, 80). Amino acid similarity between the mouse and human TLR4 sequences is 62% in the extracellular domain, 70% in the transmembrane domain, and 83% in the cytoplasmic domain (81). In mice, as in humans, cells of myeloid origin such as monocytes, macrophages, microglia, and granulocytes exhibit the highest levels of TLR4 expression. However, in sharp contrast to human macrophages and monocytes, which increase TLR4 expression in response to LPS, mouse peritoneal macrophages, and neutrophils decrease TLR4 expression after LPS challenge (82). Schroder et al. reported differences in the gene regulation of human and murine macrophages following LPS stimulation. Although various genes targeted by TLR4 signaling are more rapidly induced by LPS in human than in mouse macrophages, several negative feedback loops of the TLR4 pathway are differentially regulated in mouse macrophages (76). Existing knowledge suggests that rabbits and swine may be closer to humans than mice concerning TLR4 sequences and function. In fact, humans, swine, and rabbits are sensitive to LPS with physiological changes induced by a dose at nonograms per kilogram whereas mice are highly resistant to LPS with physiological changes induced by a dose at milligrams per kilogram (83, 84).

Given these above mentioned variations, it does not come as a surprise that mouse and human TLR4 signaling in response to free or HBP-bound heme appears to exhibit substantial differences (85). Moreover, TLR4 activation by LPS has also been found to cause opposing effects on the regulation of intracellular heme levels and heme oxygenase-1 expression in murine and human macrophages (86, 87). Furthermore, determinations of Hx in mouse models of endotoxemia, burn wound infections and peritonitis as compared to those in patients with sepsis and severe burns revealed that systemic levels of this HBP increased above baseline in each murine model, but decreased in comparable human inflammatory conditions (88). Hence, Hx is induced during the so-called acute phase response in rodents, but not in human (33, 89, 90). Another example is AAT (59), because plasma baseline concentrations of AAT in mice are about four times higher than in human plasma (normal levels in human plasma 1.3–2 g/L) (91), which may be important for neutralization and/or susceptibility to free heme toxicity. Thus, species-specific profiles of serum proteins may determine principle differences between mouse and human as shown for defense strategies against bacterial infections (92). Overall, mice have evolved in a different environment to humans, have a markedly lower body weight and have significantly shorter lifespans and, therefore, it is worth considering that the response to heme in mice may not occur in precisely the same way in humans (75, 93). Consequently, TLR4 activation in humans by heme is different from that in mouse models and such evolutionary differences need to be taken into account when translating findings from mouse disease models into human clinical applications.

In conclusion, species-specific differences between mouse and human appear to also apply to heme- and HBP-dependent pathways in TLR4 signaling.

CONCLUSIONS AND OUTLOOK

The regulatory role of heme in TLR4 signaling might be dependent on direct and indirect interactions. In particular, the interplay of heme with specific serum HBPs appears to play a major modulatory role in inflammatory conditions. Due to species-specific differences in heme-dependent TLR4 signaling findings from mouse models in experimental inflammatory diseases need to be carefully interpreted when translated to clinical settings. A major challenge will be to establish methods for determination of free heme in physiological and pathophysiological settings to allow a better understanding of the link between heme and the innate immune system.

AUTHOR CONTRIBUTIONS

SJ, VV, and SI planned and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

SI's work was supported by grants IM 20/4-1 of the Deutsche Forschungsgemeinschaft, Bonn (Germany) and grant EFRE ZW6-85007634 of the European Union and the state of Niedersachsen. SJ work supported by National Science Centre, Poland. Grant number 2015/17/B/NZ5/01370.

ACKNOWLEDGMENTS

We thank Dr. Sabine Wrenger for preparing the figure.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 18 September 2020
doi: 10.3389/fimmu.2020.02091

Macrophage TLR4 and PAR2 Signaling: Role in Regulating Vascular Inflammatory Injury and Repair

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Macrophages play a central role in dictating the tissue response to infection and orchestrating subsequent repair of the damage. In this context, macrophages residing in the lungs continuously sense and discriminate among a wide range of insults to initiate the immune responses important to host-defense. Inflammatory tissue injury also leads to activation of proteases, and thereby the coagulation pathway, to optimize injury and repair post-infection. However, long-lasting inflammatory triggers from macrophages can impair the lung's ability to recover from severe injury, leading to increased lung vascular permeability and neutrophilic injury, hallmarks of Acute Lung Injury (ALI). In this review, we discuss the roles of toll-like receptor 4 (TLR4) and protease activating receptor 2 (PAR2) expressed on the macrophage cell-surface in regulating lung vascular inflammatory signaling.

Keywords: macrophage, vascular permeability, acute lung injury, TLR4, PAR2, inflammation, alveolar macrophages

OPEN ACCESS

Edited by:
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Reviewed by:
Chaofeng Han,
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *13 April 2020*
Accepted: *31 July 2020*
Published: *18 September 2020*

Citation:
Rayees S, Rochford I, Joshi JC, Joshi B, Banerjee S and Mehta D (2020) Macrophage TLR4 and PAR2 Signaling: Role in Regulating Vascular Inflammatory Injury and Repair. Front. Immunol. 11:2091. doi: 10.3389/fimmu.2020.02091

INTRODUCTION

Macrophages (MΦ), initially classified as phagocytes by Metchnikoff in 1893 (1, 2), constitute a heterogeneous group of phenotypically and genetically distinct immune cells located within the lungs (3–9). Lung MΦ demonstrate high expression of pathogen recognition receptors (PRRs), such as Toll-like receptor 4, and play a dual role: initially, they trigger inflammatory signaling (10), but later signal removal of cellular debris and restoration of tissue homeostasis (11–13). Long lasting inflammatory signaling can impair the tissue repair process, leading to development of Acute Lung Injury (ALI). ALI frequently develops following sepsis, trauma or pneumonia, and if unresolved, may progress to Acute Respiratory Distress Syndrome (ARDS), resulting in high mortality and morbidity (14–18).

Alveolar macrophages (AMΦ) and interstitial macrophages (IMΦ) constitute the two key resident MΦ populations in the lungs. AMΦ, as the name suggests, are located within the airspace of the alveoli, juxtaposed to epithelial cells (19). Interstitial macrophages (IMΦ), on the other hand, have a more varied localization and have been shown to lie in the bronchi, airways, and interalveolar space shared by fibroblasts and other mesenchymal cells (5, 6, 20). A few studies have identified intravascular MΦ as a third resident population in the lung, but their existence remains questionable (21). Additionally, monocytes recruited to inflamed tissue differentiate into tissue macrophages (22). Macrophages can also "polarize"

along a continuum between two states designated M1 (pro-inflammatory) and M2 (anti-inflammatory) in response to different cytokines and tissue environments (23–25). However, the mechanism by which AMΦ, IMΦ, or recruited macrophages acquire pro-inflammatory or anti-inflammatory lineages and the signaling involved in their transition to these lineages during injury remains a topic of fierce debate.

Inflammation is also known to activate the coagulation cascade, which in turn affects inflammatory processes by generating a further suite of proteases such as trypsin, thrombin, elastases, FVIIa and FXa (26, 27). Protease activated receptors (PAR) such as PAR2, are known to ligate trypsin, tryptase, factor VIIa, factor Xa, and elastase (28, 29). Interestingly, recent studies suggest that thrombin also ligates PAR2 (30, 31). How then does PAR2 signaling affect TLR4-mediated inflammatory responses in lung MΦ. In this review, we focus on lung resident MΦ populations and the recently discovered coupling between TLR4 and protease activating receptor 2 (PAR2) signaling in regulating injury repair.

LUNG RESIDENT MACROPHAGES

Investigations into the ontogeny of the AMΦ and IMΦ populations have uncovered very distinct origins during their development (**Figure 1**). The Kosnav lab investigated the developmental origin of lung MΦ and showed that embryonic MΦ colonize the lung in three successive waves (32). In the first wave, F4/80^{+} embryonic MΦ from the yolk sac migrate into the lung bud around E10.5. These MΦ persist in the adult lung as "primitive interstitial MΦ" and localize peripherally and perivascularly. The second wave is initiated by Mac2^{+} embryonic monocytes at E12, most likely from the fetal liver (33), which enter the alveoli after birth and differentiate into AMΦ. The third wave, made up of F4/80^{+} bone marrow MΦ, arrives at the lung on E16 and expands to form "definitive" interstitial MΦ. Both F4/80 lineages cease to express F4/80 and begin expressing MHCII during the first 3 weeks of postnatal life. In humans, AMΦ can be detected in full term healthy infants as well as all infants who survive for 48 h after birth, irrespective of health (34). However, a study showed that AMΦ could be detected in a 20-week human fetus with congenital pneumonia (34), indicating that the lung niche may drive AMΦ generation prenatally.

The luminal surface area of adult human lungs ranges from 50 to 100 m^2 (35), larger than any other soft tissue, including the skin (2 m^2) (36) or the gut (10 m^2) (37). Because of their localization in the pulmonary epithelium, AMΦ are directly exposed to the external environment and so are the first immune cells to react to inhaled pathogens and pollutants. Additionally, AMΦ maintain the surfactant layer which prevents collapse of alveoli during respiration (38–40). On average, there is a single AMΦ for every three alveoli in mice (41). In humans, AMΦ constitute about ∼3–5% of all cells in a healthy lung (42). These AMΦ can be sessile or motile in nature. Westphalen et al. demonstrated that sessile AMΦ communicate directly with the alveolar epithelium to dampen immune responses (41). However, Paeo et al. described an AMΦ population that seems to move to-and-fro between alveoli through interalveolar fenestrae, the so-called Kohn pores (43). It is recognized that monocytes can also access the alveolar space and differentiate into AMΦ over the course of pulmonary disease, such as pulmonary fibrosis (44, 45). But whether this occurs during acute lung injury remains to be established.

IMΦ, initially referred to as septal cells (46), comprise a relatively small population of lung MΦ, ranging from a tenth to a half of the total number of AMΦ (8, 47–51). Many studies have defined IMΦ as precursors of AMΦ (44, 52). IMΦ contribute to tissue remodeling and maintenance as well as antigen presentation and thereby influence dendritic cell functions (38, 53–57). However, IMΦ have less phagocytic potential when compared to AMΦ (58).

Both AMΦ and IMΦ express conventional macrophage markers, such as CD64, CD68, MAC2, CD11b, CD11c, MERTK (59). Phenotypically, AMΦ are clearly separated from IMΦ and other non-alveolar MΦ through cell-surface expression of Itgax (CD11c), and Siglec 5 (Siglec F) (60) but lack Itgam (CD11b) expression. IMΦ can also be discriminated visually from AMΦ by their smaller size and smoother surface. Surface markers that specifically identify IMΦ remain to be established (61). However, CD11b, CX3CR1, MHCII, CD11c without SiglecF have all been used to identify IMΦ and other non-alveolar MΦ (6, 62). Recently, attempts have been made to categorize IMΦ into phenotypic and anatomical subsets such as Lyve1^{hi} and MHCII^{lo} IMΦ residing near blood vessels and Lyve1^{lo} MHCII^{hi} IMΦ residing near nerve fibers or endings. One study has suggested that Lyve1^{hi} IMΦ are responsible for exacerbated fibrosis and that both IMΦ populations are slowly replaced by Ly6C^{hi} monocytes over time (5). However, this notion is debated given that different subsets of monocytes are known to exist in adult non-diseased human lungs and naïve mice (62–68).

Transcriptional profiling of AMΦ indicated that GM-CSF secretion from alveolar epithelial type-II cells (ATII cells) along with MΦ-peroxisome proliferator-activated receptor γ (PPARγ) is required for differentiation and maintenance of the AMΦ phenotype from embryonic precursors (33, 69). This mechanism seems to be conserved in mice (33, 39, 69–71) and humans (72–75). Recent studies suggest that autocrine TGF-β signaling is also essential to maintain AMΦ lineage (76). Additionally, basophil imprinting of cytokines, such as IL-33 and GM-CSF (77), as well as L-plastin, an actin binding protein, were shown to contribute in generating AMΦ from fetal monocytes (78, 79). Transcription factors Bach1 and Bach2 have been shown to be involved in regulating AMΦ maintenance of lung surfactant homeostasis (80, 81). Moreover, once differentiated, resident AMΦ also self-proliferate to maintain their lineage (82), although it has been demonstrated that circulating monocytes contribute to this pool by differentiating into AMΦ following tissue injury or infection (83). Future studies will unravel additional transcriptional and signaling mechanisms by which monocytes, IMΦ or AMΦ themselves maintain the AMΦ pool during injury. Compared to AMΦ, transcriptional regulation of IMΦ is still in its infancy.

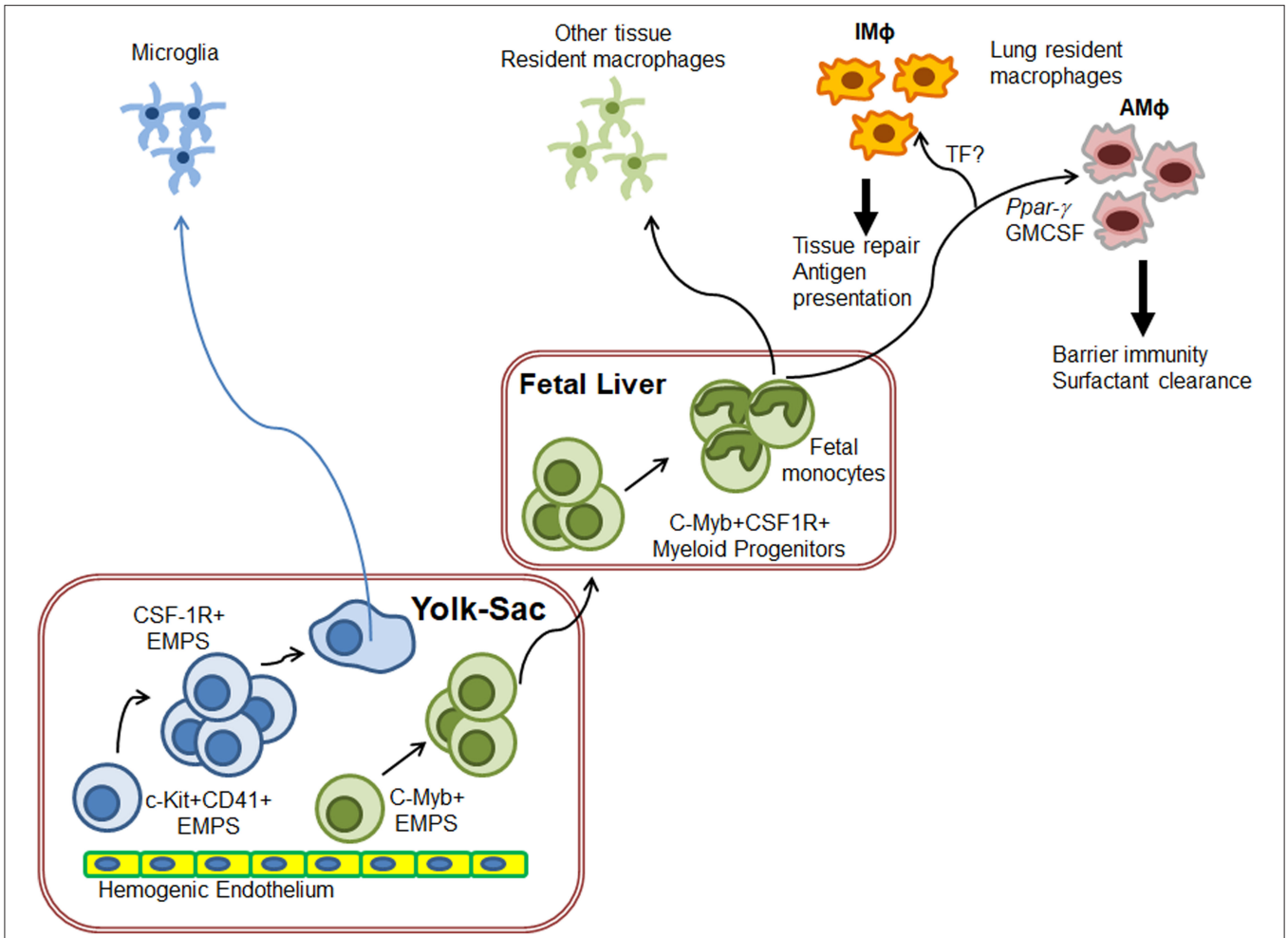

FIGURE 1 | Schematics of generation of lung resident macrophages. Based on the ontogeny of tissue resident macrophages (TRM), microglia originate directly from yolk-sac (YS) macrophages while other TRM originate from fetal liver monocytes. In the case of lungs, F4/80^{+} embryonic YS-MΦ seed the budding lung around E10.5 as primitive IMΦ. On E12, fetal liver monocytes enter the alveoli after birth and differentiate into AMΦ to regulate lung surfactant generation and host response F4/80^{+} bone marrow MΦ also arrive at the lung on E16 where they expand to form "definitive" IMΦ. IMΦ role needs to be defined but these are predicted to induce wound repair. Exact molecular control of IMΦ generation has not yet been fully deciphered.

MACROPHAGE TOLL-LIKE RECEPTOR 4 AND SIGNALING

Pattern or pathogen recognition receptors (PRRs) are a class of receptors that recognize pathogen-associated molecular patterns, PAMPs, of pathogenic organisms or endogenous signals from damaged cells, referred to as damage-associated molecular pattern or DAMPs. Upon binding with PAMPs or DAMPs, PRRs activate signaling cascades that lead to the production of pro-inflammatory cytokines and interferons, an important step in the initiation of adaptive immunity (84–86). Endocytic or phagocytic PRRs, such as mannose receptors, can aid in the recognition and intake of microbes by MΦ (87, 88).

TLRs contain 22–29 residue long leucine-rich repeats- (LRR-) N-terminal ectodomains and intracellular toll-interleukin-1 receptor (TIR) signaling domains. The LRR motif of TLRs play a key role in the protein-protein interactions involved in downstream signaling (89). MΦ have around 10 TLRs in humans and 13 in mice. Out of these, TLR 1, 2, 4, 5, and 6 are located on the cell membrane, while TLR 3, 7, 8, and 9 are intracellular (90–92). The TLR family recognizes a diverse range of DAMPs or PAMPs, such as lipoproteins, di- and triacyl lipopeptides, lipoteichoic acid, peptidoglycan, fungal zymosan, double-stranded RNA, flagellin, unmethylated CpG DNA, and LPS. A component of the cell wall from gram-negative bacteria, LPS, contains lipid A, a non-repeating "core" of oligosaccharide, and a distal polysaccharide. Lipid A has the endotoxic properties recognized by TLR4 (93, 94) and is a typical PAMP used in studies centered on TLR4 signaling.

TLR4 is unique among the various TLRs due to its ability to activate signaling from the cell-surface as well as intracellularly. Cell-surface TLR4 propagates signaling through both a MyD88-dependent and independent pathway, resulting in generation of proinflammatory cytokines and type I interferons,

respectively (95, 96). Upon binding LPS, cell-surface TLR4 recruits several adaptor proteins through its intracellular TIR domain (97). These adaptor proteins include MyD88, TRIF, MyD88 adapter-like (Mal/TIRAP), sterile and armadillo motif-containing protein (SARM), TRIF-related adaptor molecule (TRAM), tumor necrosis factor receptor associated factor6 (TRAF6) and the serine-threonine kinase, IL-1R-associated kinase (IRAK). TLR4 immune signaling is further accelerated by accessory molecules such as CD14, CD36, and myeloid differentiation2 (MD2) (98). TLR4-MyD88 signaling is mediated through complex formation between MyD88, phosphorylated IRAK, and TRAF6 which in turn activates the transcription factor, NFκB and MAPK to induce the generation of several pro-inflammatory cytokines, such as TNF-α and IL-6 (99, 100). In contrast, MyD88 independent TRIF-mediated TLR4 signaling occurs through activation of transcription factor, IFN regulatory factor 3 (IRF3) and STAT1, which leads to generation of IFN-β, IL-10, and RANTES, as well as late phase NFκB activation (100, 101). Both of these pathways propogate at the plasma membrane simultaneously, but recent studies suggest that TRAM-TRIF signaling can also be initiated following endocytosis of TLR4 (101, 102).

Endocytosed TLR4 can sense cytosolic LPS to induce NFκB and IRF3 mediated transcription, which is critical to full regulation of innate immunity during pathogenic insult (100, 102). Studies show that p120-catenin (p120), a member of a subfamily of armadillo repeat domain containing proteins, promotes the endocytosis of TLR4 in MΦ and stimulates TRIF, which in turn activates the transcription factor IRF3 to enhance the expression of type 1 interferons (92, 100).

Additionally, TLR4 activates the formation of inflammasomes, also known as inflammatory signaling platforms, by inducing the cytosolic innate immune sensor NLRP3, adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1 (103–105). Inflammasome activity requires both priming by TLR4-NFκB mediated production of pro-IL-1β and pro-IL-18 and an NLRP3-specific signal activated by either reactive oxygen species (ROS), extracellular ATP, alum, or pore-forming toxin nigericin. Upon activation, NLRP3 and ASC form a complex with pro-caspase-1 through homotypic domain interaction, leading to generation of active caspase-1, which cleaves pro-IL-1β and pro-IL-18 to the mature IL-1β and IL-18 forms. Evidence suggests that efflux of K^+ across the plasma membrane is a key factor regulating the activation of NLRP3. Di et al. recently showed that NLRP3 activation of K^+ efflux by two-pore domain weak inwardly rectifying K^+ channel 2 (TWIK2) played a critical role in regulating inflammasome formation in AMΦ (106).

Recent studies show that, in mice, caspase-11 (caspases-4 and 5 in humans) can bind cytosolic LPS and induces the NLRP3 pathway as well as gesdermin D to stimulate pyroptosis (105, 107) and the release of IL-1β. While pyroptosis, defined as gasdermin-mediated regulated necrosis, protects organisms from invading pathogens, it may cause local as well as systemic inflammation, including septic shock (108, 109).

Cell death leads to the generation of reactive species and activation of Z-DNA binding protein 1 (ZBP1). ZBP1 results in the release of mtDNA and/or dsDNA. Cyclic GMP-AMP synthase (cGAS) catalyzes generation of cyclic GMP-AMP (cGAMP) upon binding to dsDNA, which leads to the activation of STING/IFN-β signaling and lung injury (108, 110–112). Stimulator of interferon genes (STING), a transmembrane homodimer located in the endoplasmic reticulum (ER) membrane, has recently emerged as a potent inducer of MΦ inflammatory signaling following tissue injury (112). Joshi et al. recently showed that recruited MΦ were required to dampen AMΦ-STING signaling. They demonstrated that ER-localized sphingosine kinase-2 (SPHK2) generated sphingosine-1-phosphate (S1P), which prevented cGAMP activation of STING and thus attenuated lung vascular injury. Oxidized PAPC (oxPAPC) formed from phospholipid, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) and lipoproteins (113, 114) also modulated TLR4-induced inflammatory responses. At a very low concentration, oxPAPC antagonized TLR4-induced inflammation and injury, yet at higher doses enhanced the proinflammatory response to TLR4 signaling (113). While the exact mechanism of oxPAC anti-inflammatory function remains unclear, it was shown to inhibit NFκB transcription factor activity by generating cAMP (114) or by binding to CD14 and LPS binding protein (LBP), thereby reducing the sensitivity of TLR4 to LPS (114).

MACROPHAGE PROTEASE ACTIVATED RECEPTORS

Inflammation-induced injury releases a mélange of proteases, complements, chemokines, prostaglandins, and other inflammatory molecules, which activate several receptors, including G-protein coupled receptors (GPCRs) (115). Thus, in addition to expressing TLRs to detect pathogens, MΦ also express an array of GPCRs on their cell-surface, whose function is to optimize the inflammatory response and host-defense function (116, 117). Culture conditions, such as GM-CSF vs. M-CSF, seem to dictate the expression of different sets of GPCRs on MΦ (118). However, AMΦ heavily express complement receptors (C5R1; C3AR1), formyl peptidyl receptor 2 (FPRL2) and several chemokine receptors (CXCR6, CCR8, CCR4, CCR5 etc.) (119).

Protease activated receptors (PARs), PAR1, PAR2, and PAR3 encoded by the genes F2R, F2RL1, F2RL2, and F2RL3, respectively are also expressed on the MΦ cell-surface. As the name indicates, PARs are activated by several different proteases, including those involved in the coagulation pathway (26, 28, 120). While each of these receptors can be cleaved by their specific proteases, several common proteases can also cleave various PARs because of their sequence homology. For example, PAR1 is cleaved by proteases such as thrombin, factor Xa, plasmin, MMP1 and MMP13 (121). Originally, PAR2 was thought to be cleaved only by trypsin, tryptase, factor VIIa, factor Xa and elastase (28, 29). However, recent studies show that thrombin can also cleave PAR2, albeit at higher concentrations (30, 31). PAR3 is cleaved by thrombin only. These proteases cleave PARs at defined sites within the

N-terminus, unmasking new N-terminal peptides as tethered ligands. The tethered ligand then binds intramolecularly to a conserved second extracellular loop of cleaved receptor to trigger signaling through heterotrimeric G-proteins. However, subtle mechanistic differences exist among these proteases in initiating the activity of the relevant canonical pathway, depending on the PAR in question. Synthetic PAR peptides or activating peptides (APs) mimic the tethered ligand domains. These peptides directly activate their respective PARs, bypassing the proteolysis process (26, 122). Recent findings indicate that activation of PARs, specifically PAR2, expressed on AMΦ suppress TLR4 signaling, as we will discuss further.

MACROPHAGE PAR2 AND DOWNSTREAM SIGNALING

PAR2 couples to $G_{\alpha}s$, $G_{\alpha}q$, $G_{\alpha}i$, and $G_{\alpha}12/13$ and triggers several signaling cascades to mediate its diverse cellular functions (31, 123, 124). The canonical activation of PAR2 by its proteases occurs after hydrolysis at the R^{36}/S^{37} position. The exposed tethered ligand domain, SLIGRL (mouse) and SLIGKV (human), in turn binds to initiate PAR2 signaling. Other proteases, including thrombin, neutrophil elastase, cathepsin G, cathepsin S, proteinase-3, gingipain-R and kallikrein-14, cleave PAR2 at sites other than the tethered ligand site, leading to bias signaling (26, 122). Non-mammalian proteases such as LepA and elastase EPa, both secreted by *Pseudomonas aeruginosa,* also cleave PAR2 to either activate or deactivate its downstream signaling (125, 126). Activation of PAR2 by *Pseudomonas aeruginosa* has been shown to cause IFN-gamma production as a mechanism for stimulating bacterial clearance. Similarly, gingipain R produced by *Porphyromonas gingivalis,* Pen C secreted by *Penicillium citrinum* and supernatant from *Propionibacterium acnes* cultures can activate PAR2 (120, 122, 127). Additionally, several small molecule agonists of PAR2 have been synthesized, but their therapeutic efficacy remains uncertain (26, 128).

Classically, GPCR activation is followed by desensitization. GPCR phosphorylation uncouples it from its cognate G-protein and induces its binding to β-arrestin (129), facilitating receptor internalization by recruiting endocytic proteins (130, 131). PAR2 activation is associated with phosphorylation of its cytoplasmic tail, which is responsible for desensitization or internalization of PAR2 (132). The serine and threonine residues within the cytoplasmic tail of the receptor and third intracellular loop are the prime phosphorylation sites; however, it may occur at tyrosine residues as well (133). Ricks and Trejo showed that, compared to wild-type PAR2, desensitization was considerably reduced in PAR2 mutants in which all serine and threonine phosphorylation sites in the C-terminal tail were mutated to alanine (132). Moreover, wild-type phosphorylated PAR2 was internalized through a canonical dynamin, clathrin- or β-arrestin-dependent pathway, but the PAR2 mutant was internalized through a dynamin-dependent and clathrin- and β-arrestin-independent pathway.

PAR2 and Calcium Signaling

An increase in cytosolic Ca^{2+} is required for the regulation of several cellular processes (134). Agonist-induced increases in cytosolic Ca^{2+} occur by depletion of endoplasmic reticulum (ER) Ca^{2+} stores, followed by Ca^{2+} entry through plasmalemmal channels (135). PAR2 activation via its cognate agonists, such as trypsin, tryptase or agonist peptide, has been demonstrated to increase cytoplasmic Ca^{2+} levels via the phospholipase C-inositol trisphosphate ($PLC\text{-}IP_3$) axis (136–138). Ca^{2+} signaling by PAR2 is typically activated via Gαq/G11 and influences several intracellular targets, resembling PAR1 signaling. However, evidence shows that trypsin activation of PAR2 can also induce Ca^{2+} signaling by stimulating Gαi/Gαo (139, 140), indicating that coupling of PAR2 to G proteins may depend on variations in the density of cell-surface PAR2, availability of G proteins, or downstream effector protein interactions.

Transient receptor potential channels (TRP) are a group of Ca^{2+}-permeable non-selective cation channels involved in MΦ activation. Studies showed that TRPM2 and TRPV4 but not STIM-mediated store-operated calcium channels play an important role in mediating Ca^{2+} entry in MΦ (31, 106, 141). However, it appears that PAR2 was required to suppress TRPV4-mediated Ca^{2+}-entry in AMΦ (31). TRPV4 is a polymodally gated channel involved in several fundamental physiological functions of both sensory and non-sensory cells (142). It is also known to play a significant role in several pathophysiological processes, such as asthma, pulmonary fibrosis, cystic fibrosis, sepsis, and lung injury (143–146). TRPV4 is activated by several stimuli including mechanical stress, thermosensation or by intracellular metabolic products (147–149). Also, phospholipase A2 (PLA2)/arachidonic acid (AA) pathway signaling triggered by cell swelling can also activate TRPV4 (150–152).

Rayees et al. showed that thrombin-induced TRPV4 activity was markedly higher in PAR2-null bone-marrow derived macrophages (BMDM) compared to wild-type BMDM, indicating that PAR2 suppresses TRPV4 activity (31). Also, direct activation of TRPV4 with its agonist (GSK1016790A) (153) enhanced Ca^{2+} entry in PAR2-null BMDM more than in wild-type BMDM (31). Further studies will be required to determine whether thrombin activates TRPV4 in AMΦ by generating PLA2 products, cell shape change/swelling or pressure variation.

PAR2 and Cyclic Adenosine Monophosphate Generation

Cyclic AMP (cAMP) is a ubiquitous second messenger involved in numerous physiological processes in all domains of life. Adenylyl cyclases (AC) generate cAMP from ATP (154). AC have 10 isoforms, nine of which are transmembrane (tm-AC) and regulated by GPCRs, while the soluble form of adenylyl cyclase (sAC) acts as a bicarbonate/pH sensor (155) and is not regulated by G-proteins or forskolin, a direct activator of AC (29, 156). A family of enzymes called phosphodiesterases (PDEs) catabolize cAMP into AMP. There are 11 known PDEs, of which PDE4, 7, and 8 have a strong affinity for cAMP (157–159). cAMP is known to mediate its effects through three target proteins, protein kinase

A (PKA), cyclic nucleotide gated ion channels (CNGs and HCNs) and exchange proteins activated by cAMP (EPACs) (154, 160).

PAR2 is known to induce cAMP generation by coupling to Gαs (161, 162). Interestingly, LPS also induced cAMP in MΦ by generating thrombin and activation of PAR2. Forskolin induced a similar increase in cAMP in both wild-type and PAR2-null BMDM. Further, rolipram, a PDE inhibitor, alone or in combination with thrombin, did not induce any significant increase in intracellular cAMP in wild-type or PAR2-null BMDM, indicating that thrombin ligation of PAR2 is necessary for cAMP generation (31). Consistent with this finding, the cell permeable cAMP analog 8-Br-cAMP inhibited thrombin-induced Ca^{2+} entry in PAR2-null BMDM (163). Interestingly, 8-Br-cAMP inhibited TRPV4 induction by GSK1016790A. Additionally, cAMP is known to bind NRLP3 directly to dampen inflammasome generation (164), thus raising the possibility that cAMP generated through PAR2 can suppress both TRPV4 activity and inflammasome generation by TLR4. Though the mechanism by which cAMP inhibits TRPV4 is not yet clear, alignment of the TRPV4 sequence with cAMP PBC domain B, which is conserved in well-known cyclic AMP binding proteins, suggested that cAMP may inhibit the channel by binding to it directly (31). Nonetheless, these results identified PAR2 as a key switch in the control of Ca^{2+} entry in AMΦ through the generation of cAMP.

INTERPLAY BETWEEN TLR4 AND PAR2 SIGNALING

Role in Macrophage Polarization

As mentioned above, MΦ "polarize" into the M1 or M2 state through dynamic changes in cell response and phenotype, giving rise to the notion that the MΦ dichotomy is crucial for coordinating the initiation, progression, and ultimate resolution of inflammatory injury. However, this conclusion is mainly derived from *in-vitro* studies, using, for example, BMDM and RAW cells (165, 166). The M1 state, or "classically activated" MΦ, is considered pro-inflammatory, characterized by propagation of inflammatory signaling through the secretion of cytokines, such as IL-1β, TNF-α or interferons. LPS, a cell wall component of Gram- bacteria, and IFN-γ polarize MΦ to acquire a M1 state through activation of transcription factors, including NFκB, NFAT and STAT1 (121, 167–169). M2, or "alternatively activated MΦ," are considered anti-inflammatory, as they induce the arrest of inflammatory signaling and initiate wound healing and other regenerative processes (170). IL-4/IL-13 can program MΦ to adopt the M2 state by activating the STAT6 transcription factor. IL4-activated STAT6 can also compete with STAT1 to repress interferon-γ-mediated responses (168), indicating that M1-MΦ can themselves become M2 as inflammatory injury progresses from the acute phase to the resolution phase. However, recent studies suggest that, while M2 may transition to M1, the reverse is not true due to mitochondrial dysfunction induced by reactive oxygen species produced during M1-MΦ polarization (171).

Human monocytes primarily express PAR1, but upon differentiation into macrophages increase expression of PAR2 (172). PAR2 activation alone is able to skew macrophages into either the M1 or M2 phenotype (173–175). Stimulation of BMDM or RAW cells with the small molecule PAR2 agonist, 2-furoyl-LIGRLO-amide trifluoroacetate salt, skewed MΦ into M1-like cells due to activation of the forkhead box protein O1 (FOXO1) (173). Another study showed that PAR2 activating peptide shifted macrophages into the M1 or M2 phenotype depending on culture conditions. These authors showed that the PAR2 activating peptide SLIGKV, skewed GM-CSF-derived peripheral blood monocytes (PBMC)-MΦ into the M1 phenotype, while MCSF-derived PBMC-MΦ were skewed to the M2 phenotype (175). However, conjoint activation of PAR2 and TLR4 in peritoneal MΦ polarized them toward the M2 phenotype, since PAR2 peptide suppressed the LPS-mediated increase in M1 cytokines (TNFα, IL-6 and IL-12p40) (174). Similarly, other studies have shown that PAR2 null primary macrophages secreted less IL-4/IL-13 in response to LPS as compared to wild-type macrophages, and PAR2 activation was associated with greater M2 cytokine expression after LPS exposure (174, 176).

Role in Regulating Inflammatory Signaling

Inflammatory signaling induces the expression of tissue factor (TF) and elastase in leukocytes and monocytes, which facilitates activation of the coagulation pathway in part through the production of thrombin (31, 177). TF is also constitutively expressed by cells segregated from blood, mostly epithelial cells and macrophages (115, 178). However, a few studies have addressed the role of PAR2 activation by TF, elastase and thrombin in altering the TLR4-induced inflammatory cascade in alveolar macrophages *in vivo* (179–181). Rallabhandi et al. initially demonstrated, using a heterologous system, that TLR4 and PAR2 receptors physically interact, leading to receptor cooperativity and enhancement of pro-inflammatory signaling through NFκB. They showed that PAR2 activation of NFκB signaling occurred in an adaptor dependent manner. In the presence of TLR4, PAR2-activating peptide (PAR2-AP) enhanced NFκB signaling by recruiting MyD88. However, in the absence of TLR4, the PAR2-AP induced NFκB activity by recruiting the TRIF and TRAM adaptor proteins (181). This could be due to the presence of the TIR (Toll/IL-1 receptor/resistance protein) domain in the C-terminus of PAR2 (182). Thus, without TLR4, PAR2 signaled by recruiting TRIF/TRAM to the C-terminus of PAR2, but this interaction was dislodged by MyD88 in the presence of TLR4 (181).

Liang et al. followed up on the TLR4 and PAR2 receptor co-operativity concept discussed above and showed that TLR4 transactivated PAR2, which then enhanced TLR4 signaling (179) (**Figure 2A**). In this context, they showed that the endothelial cell protein C receptor (EPCR) serves as a bridge to engage PAR2 with TLR4 and induces pro-inflammatory genes in macrophages (179). Thus, they showed that LPS failed to induce interferon-regulated gene expression in several organs, including lungs, in mice lacking EPCR or PAR2 (179) At a mechanistic level, these authors used BMDM and monocytic RAW265.7 cells to show that LPS upregulated the expression of TF, which was followed by TF-VIIa-Xa complex formation. TF-VIIa-Xa assembly was

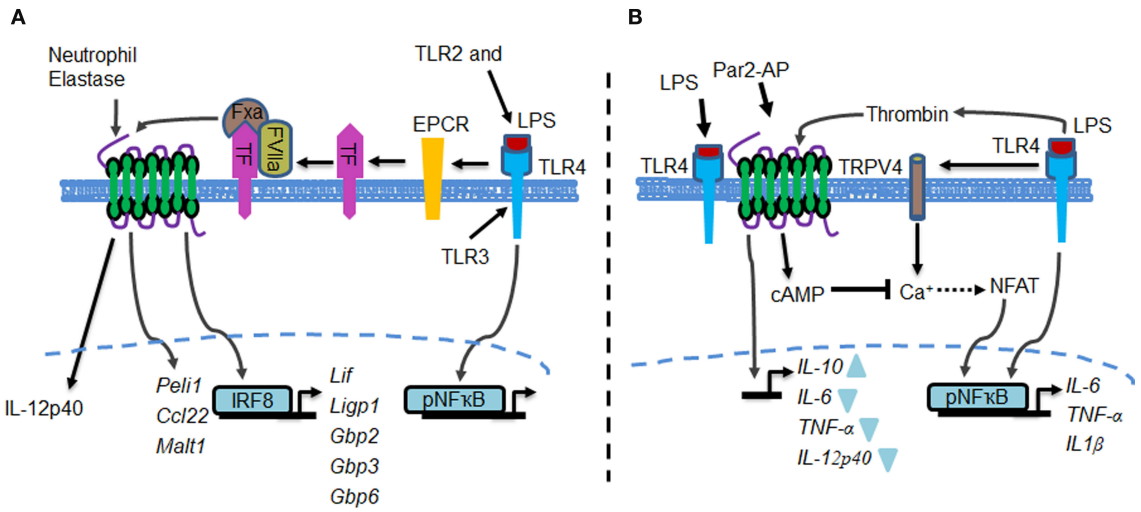

FIGURE 2 | Potential crosstalk models between TLR4 and PAR2. **(A)** PAR2 transactivation via EPCR and neutrophil elastase augments LPS-TLR4 inflammatory signaling. Tissue factor activates PAR2 via EPCR. TLR4-EPCR-mediated activation of PAR2 upregulated the expression of Peli1, Ccl22, and Malt1. Elastase secreted from neutrophils may cleave PAR2 to induce IL-12p40 generation in macrophages. Also, TLR2 and TLR3 may contribute to PAR2 regulation of TLR4 signaling. **(B)** PAR2 suppresses TLR4 inflammatory signaling, thereby facilitating resolution of lung injury. Thrombin secreted during TLR4-induced lung injury directly activates PAR2. PAR2 mediates the generation of cAMP, which suppresses TRPV4-induced Ca^{2+} entry. Deletion of PAR2, hence elimination of cAMP generation, fails to suppress Ca^{2+} entry via TRPV4, leading to protracted NFAT and NFκB activities, resulting in long-lasting inflammatory injury. Additionally, simultaneous activation of PAR2 and TLR4 in peritoneal macrophages enhanced IL-10 expression while the expression of TNF-α, IL-6, and IL-12p40 was decreased.

required for EPCR-mediated activation of PAR2, which resulted in induction of expression of Pellino-1 and IRF8 activity and thereby the full-blown, interferon-regulated, gene expression program (**Figure 2A**).

Another mechanism of transactivation of PAR2 by TLR4 in GM-CSF treated PBMC-MΦ was demonstrated by Yamaguchi et al. These authors showed that activated TLR4 induced the release of elastase from neutrophils, which cleaved PAR2, thereby producing IL-12p40 (183). IL-12p40, a common subunit of IL-12 and IL-23, is involved in several pathogenic inflammatory responses associated with MΦ and dendritic cells (184). However, neutrophil elastase *per se* failed to increase IL-12p40 production in MΦ without PAR2 expression (183) (**Figure 2A**). Nakayama et al. showed that IL-32γ, a pro-inflammatory cytokine, also stimulated PAR2 signaling in a THP-1 macrophage cell line by generating proteinase-3 (PR3) (185). They showed that PR3 activated PAR2, which engaged with TRIF via the TIR domain to augment TNF-α and IFNγ generation. Because bacterial infection may cause endotoxin tolerance, the IL-32-PAR2-TRIF axis may act as an alternative signaling pathway to the LPS-TLR4-TRIF axis in shaping adaptive immunity (185).

However, Nhu et al. demonstrated that interaction between TLR4 and PAR2 may not be that simple. They showed that cooperative signaling between PAR2, TLR2, TLR3, and TLR4 induced NFκB activity to upregulate IL-8 expression, a gene principally involved in neutrophil chemotaxis. Additionally, the activation of PAR2 by PAR2-AP reduced TLR3-mediated STAT1 activation and TLR3/IRF3-induced IFNβ expression. However, for optimal PAR2 signaling, the presence of TLR4 was required. This cross-cooperativity was validated by the authors in an influenza-induced lethality mouse model. Here, the authors found that the Influenza A virus, which is known to activate the TLR3 pathway, did not produce any lethality in PAR2-null or TLR4-null mice, while significant lethality was noted in wild-type mice. This receptor cooperativity was also demonstrated in a PAR2-AP induced footpad edema model, in which PAR2-AP was not able to induce edema in TLR4 null or PAR2 null mice (174, 186).

In contrast to the above studies, Rayees et al., by performing bone marrow transplantation and adoptive transfer of macrophages, showed that PAR2 expressed in AMΦ counteracted the TLR4-induced inflammatory response by modulating Ca^{2+} entry and cAMP generation (31) (**Figure 2B**). It is known that Ca^{2+} entry induces the activities of both NFκB as well as the transcription factor NFAT, but in a cell-context dependent manner (187, 188). NFAT is basally phosphorylated, but when dephosphorylated by calcineurin, a Ca^{2+}-dependent phosphatase, NFAT's transcriptional activity is turned on (188). Whereas, NFAT activity is known to regulate gene transcription in T cells, its role in MΦ remains understudied. Rayees et al. showed that PAR2 suppressed LPS-induced dephosphorylation of NFAT, i.e., activation of NFAT (31). These authors also showed mechanistically that PAR2 was required to suppress NFκB activity in part by blocking activation of NFAT. Thus, addition of 8-Br-cAMP, a membrane permeable cAMP-dependent protein kinase agonist, bypassed the requirement for PAR2 in diminishing TRPV4 activity and LPS-induced NFAT and NFκB activities as well as pro-inflammatory cytokine generation. These results, along with the findings listed above, identified the PAR2-cAMP cascade as a suppressor of TRPV4 activity and

NFAT-mediated cytokine generation, thus demonstrating that thrombin activation of PAR2 in AMΦ blocks TLR4-mediated inflammatory signaling to reinstate tissue integrity (**Figure 2B**) (31). Nhu et al. similarly showed that simultaneous activation of PAR2 and TLR4, by PAR2-AP and LPS respectively, led to a decrease in expression of TNF-α, IL-6 and IL-12 in peritoneal MΦ, and enhanced expression of IL-10 (**Figure 2B**). These results were confirmed in LPS-treated PAR2-null MΦ, which showed significantly decreased IL-10 expression and, interestingly, the expression of CXCL1/KC, a strong neutrophil chemokine, was increased (174). Further studies using macrophage specific PAR2-null mice are required to resolve the role PAR2 plays in regulating TLR4 signaling. Also, a fundamental question that remains to be answered is whether exaggerated coupling of PAR1-mediated signaling with TLR4 in AMΦ is responsible for altering inflammatory injury in PAR2-null mice, as discussed above.

CONCLUDING REMARKS

This review describes recent mechanistic developments in lung MΦ regulation of tissue-fluid homeostasis with an emphasis on PAR2-mediated signaling in AMΦ and its intersection with TLR4 signaling to modulate inflammation and lung vascular injury. We highlighted the subsets of lung resident MΦ and their dichotomous phenotypes, as regulated *in vitro* vs. *in vivo*. We also described recent advances in TLR4 signaling, such as the role of inflammasomes and STING in regulating AMΦ functions. We noted herein that generation of cAMP through PAR2 activity is critical in suppressing NFAT activity, thereby dampening AMΦ inflammatory signaling. Intriguingly, data also show the key role of cAMP in blocking TRPV4 activity in MΦ. However, several questions remain to be addressed: as for example

(1) Where does this cAMP comes from to bind TRPV4 in AMΦ and how does cAMP alter the affinity of TRPV4 for its agonists?
(2) Does PAR1 expression in AMΦ augment TLR4 activity in the absence of PAR2 expression?
(3) How does PAR2, or PAR1 expression, for that matter in IMΦ or monocyte-derived MΦ, which are known to be recruited to the lung during injury, regulate AMΦ inflammatory signaling?
(4) Are cAMP-induced transcription factors, such as CREB, involved in dictating AMΦ function? Further studies employing state of the art techniques such as macrophage imaging *in vivo* along with genetic mouse models will likely advance our understanding of lung MΦ subsets generation and function under normal conditions and during inflammation.

AUTHOR CONTRIBUTIONS

SR and DM: conceptualized the manuscript. IR and DM: edited the manuscript. SR, JJ, IR, BJ, SB, and DM wrote and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Institutes of Health grants HL 84153, PO1 HL60678, P01-HL077806, and HL137179-01.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 November 2020
doi: 10.3389/fimmu.2020.585146

Lipopolysaccharide Recognition in the Crossroads of TLR4 and Caspase-4/11 Mediated Inflammatory Pathways

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The innate immune response to lipopolysaccharide is essential for host defense against Gram-negative bacteria. In response to bacterial infection, the TLR4/MD-2 complex that is expressed on the surface of macrophages, monocytes, dendritic, and epithelial cells senses picomolar concentrations of endotoxic LPS and triggers the production of various pro-inflammatory mediators. In addition, LPS from extracellular bacteria which is either endocytosed or transfected into the cytosol of host cells or cytosolic LPS produced by intracellular bacteria is recognized by cytosolic proteases caspase-4/11 and hosts guanylate binding proteins that are involved in the assembly and activation of the NLRP3 inflammasome. All these events result in the initiation of pro-inflammatory signaling cascades directed at bacterial eradication. However, TLR4-mediated signaling and caspase-4/11-induced pyroptosis are largely involved in the pathogenesis of chronic and acute inflammation. Both extra- and intracellular LPS receptors—TLR4/MD-2 complex and caspase-4/11, respectively—are able to directly bind the lipid A motif of LPS. Whereas the structural basis of lipid A recognition by the TLR4 complex is profoundly studied and well understood, the atomic mechanism of LPS/lipid A interaction with caspase-4/11 is largely unknown. Here we describe the LPS-induced TLR4 and caspase-4/11 mediated signaling pathways and their cross-talk and scrutinize specific structural features of the lipid A motif of diverse LPS variants that have been reported to activate caspase-4/11 or to induce caspase-4/11 mediated activation of NLRP3 inflammasome (either upon transfection of LPS *in vitro* or upon infection of cell cultures with intracellular bacteria or by LPS as a component of the outer membrane vesicles). Generally, inflammatory caspases show rather similar structural requirements as the TLR4/MD-2 complex, so that a "basic" hexaacylated bisphosphorylated lipid A architecture is sufficient for activation. However, caspase-4/11 can sense and respond to much broader variety of lipid A variants compared to the very "narrow" specificity of TLR4/MD-2 complex as far as the number and the length of lipid chains attached at the diglucosamine backbone of lipid

OPEN ACCESS

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *19 July 2020*
Accepted: *23 October 2020*
Published: *27 November 2020*

Citation:
Zamyatina A and Heine H (2020) Lipopolysaccharide Recognition in the Crossroads of TLR4 and Caspase-4/11 Mediated Inflammatory Pathways. Front. Immunol. 11:585146. doi: 10.3389/fimmu.2020.585146

A is concerned. Besides, modification of the lipid A phosphate groups with positively charged appendages such as phosphoethanolamine or aminoarabinose could be essential for the interaction of lipid A/LPS with inflammatory caspases and related proteins.

Keywords: lipid A, inflammation, chemical structure, innate immunity, structural basis, molecular recognition, TLR4/MD-2, aminoarabinose

LIPOPOLYSACCHARIDE DETECTION BY THE INNATE IMMUNE SYSTEM

Early detection of pathogen-associated molecular patterns (PAMPs) universally shared by Gram-negative bacteria is a crucial element for the initiation of innate immune responses such as inflammation (1, 2). LPS is a glycan based Gram-negative PAMP that is either expressed on the bacterial cell surface or associated with intracellular or outer membrane vesicles (OMV). LPS prompts the induction of mammalian innate immune responses through a meticulously organized sequential event that starts with the binding of LPS to LPS-binding protein (LBP), transfer to cluster of differentiation-14 (CD14) and, finally, engagement of the germline-encoded pattern-recognition receptor (PRR) Toll-like receptor 4/myeloid differentiation-2 (MD-2) complex (3–6). TLR4 is a type I transmembrane protein entailing a leucine-rich repeats ectodomain, a transmembrane domain and a cytosolic Toll-IL-1 receptor (TIR) domain which is involved in induction of the downstream signaling cascades. MD-2 is a secreted accessory molecule which is physically associated with TLR4 and essential for LPS recognition and binding. LPS-induced homodimerization of ternary TLR4/MD-2/LPS complexes results in the assembly of particular intracellular adaptor protein complexes which leads to the activation of various transcription factors such as NF-κB, followed by induction of expression of cytokines and IFNs. Inadequate regulation of the TLR4 signaling contributes to the pathogenesis of a number of acute and chronic inflammatory as well as autoimmune diseases such as allergy, arthritis (7–9), asthma (10–12), cardiovascular disorders (13), Alzheimer disease-associated pathology (14) and systemic inflammatory response syndrome (SIRS) and septic shock (15, 16). Impressive research demonstrated that down-regulation of the TLR4 mediated signaling can be useful for therapeutic benefits and efficient for management of asthma (17, 18), arthritis, (8) viral infections [influenza (19) and Ebola virus (20)], cancer (21), and sepsis (22). Besides, TLR4-mediated signaling has been demonstrated to promote dendritic cells maturation thereby linking innate and adaptive immunity (23, 24) which features activation of the TLR4/MD-2 complex by TLR4-specific ligands of low toxicity as facile approach for development of novel vaccine adjuvants (25–27).

Whereas detection of extracellular LPS and ensuing immune responses through TLR4 signaling pathway plays a major role in the primary detection of LPS, the recognition of cytosolic LPS by intracellular proteases caspase-4/5 (and their mouse homologue caspase-11) is important at a later stage of severe bacterial infection (28–31). Inflammatory caspases are parts of the non-canonical inflammasome pathway involved in the initiation of a series of inflammatory effects such as endocytosis, autophagy and oxidative burst (28, 32, 33). Activated caspase-4/5/11 induces NLRP3 inflammasome activation and triggers the secretion of IL-1β and IL-18 *via* caspase-1 mediated processing of pro-IL-1β and pro-IL-18, and pyroptosis accounting for endotoxin-related pathology (34–36). Recent studies have underscored the significance of the non-canonical inflammasome signaling in acute and chronic inflammatory conditions including sepsis (37–39), diabetes (40), atherosclerosis (41), and Alzheimer's disease (42, 43). Although human caspase-5 has been shown to function similar to caspase-4, it is less studies in respect to LPS recognition. In this review we make emphasis on caspase-4/11 and mention caspase-5 whenever appropriate.

TLR4-Mediated Signaling Pathways

Upon engagement of MD-2 and TLR4 and the LPS-mediated generation of TLR4/MD-2/LPS homodimers, intracellular signaling is initiated by conformational changes of the Toll/IL-1R (TIR) domain of TLR4. Among all TLRs, TLR4 is unique since it is the only TLR that uses both major signaling adaptors, MyD88 (myeloid differentiation primary response 88) (44) and TIR (Toll IL-1R) -domain containing adaptor inducing Interferon-β [TRIF (45)], as well as the respective adaptor molecules MyD88-adaptor-like [MAL (46)], which is also known as TIR-domain containing adaptor protein [TIRAP (47)] and TRIF-related adaptor molecule [TRAM (48)]. This uniqueness enables TLR4 to induce two different sets of responses: the first set starts at the plasma membrane, depends on MyD88 and leads to a rapid induction of pro-inflammatory cytokines (**Figure 1**). The second set requires internalization, depends on TRIF and emanates signals from endosomal membranes which lead to the induction of a type I interferon response.

MyD88 is a 296 aa adaptor protein containing two major domains: a C-terminal TIR domain which associates with other TIR domain-containing proteins and a N-terminal death domain (DD) which mediates the interaction with the IRAK (IL-1R-associated kinase) family kinases (49–51). Whereas recruitment to TLR4 is facilitated by homotypic TIR domain interactions and requires the bridging adaptor MAL (52, 53), DDs are used to engage members of the IRAK family to the complex. The whole complex comprises of 14-16 MyD88 and IRAK1, -2, and -4 molecules and has been termed the myddosome (54). Such multi-molecular complexes that are functioning as signaling platforms have been termed supramolecular organizing centers (SMOCs) and are crucially important for innate immune

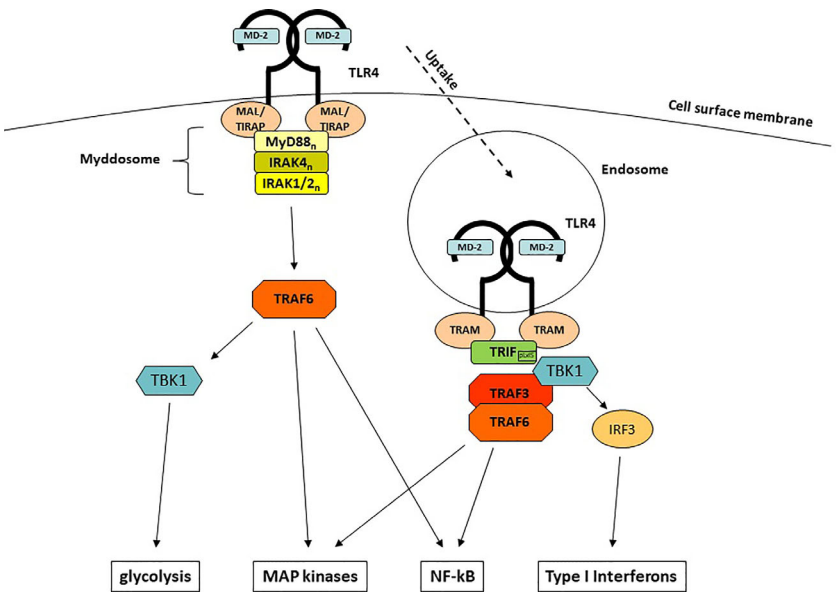

FIGURE 1 | LPS-induced TLR4-mediated signaling pathways.

signaling (55). The myddosome formation leads to autophosphorylation of the kinase domain (KD) of IRAK4 (56, 57), which in turn activates IRAK1/2. Subsequently, another central (but not exclusive) element of LPS-induced signaling, the E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) (58, 59) associates and gets activated. TRAF6 is central to immune activation (60), as it is able to induce further downstream major signaling pathways that end up in the activation of crucial innate immune transcription factors through TAK1 (NF-κB and AP-1) (61, 62) as well as the induction of glycolysis through TBK1 (63, 64).

Early investigations of MyD88-deficient animals and cells showed that not all LPS responses were completely abolished but rather delayed (44). The reason for the delayed response is the site of its origin: whereas the MyD88-dependent responses are initiated at the cell membrane, the MyD88-independent responses emanate from TLR4-harboring endosomes once MyD88 is discharged from TLR4 (65). The molecule responsible for this surprising effect is TRIF (45, 66). In contrast to TLR3 which can directly recruit TRIF, TLR4 needs assistance from the adaptor protein TRAM (48, 66). Upon binding of TRAM/TRIF to endosomal TLR4, the E3 Ub ligase TRAF3 (67) is recruited and subsequently activates TBK1. Although TBK1 is also part of the MyD88/TRAF6-dependent signaling, it only leads in conjunction with TRIF to the induction of IFNs, the hallmark of the TRIF-dependent immune response: a so-called pLxIS motif in TRIF becomes phosphorylated by TBK1 and can interact with the key interferon-regulatory factor IRF-3 (68), which by itself is another substrate for TBK1 (69, 70). In addition to TRAF3, TRIF can also recruit TRAF6, which explains the delayed NF-κB translocation and MAP kinase activation seen in MyD88-deficient cells.

Most of the LPS-induced cytokines, chemokines and interferons are regulated through the induction of mRNA expression. However, one of the major pro-inflammatory cytokine, IL-1β (as well as its related IL-1-superfamily member IL-18) which regulates a wide array of immune and physiological responses (71), requires an additional step of maturation/processing by caspase-1 (72). The multi-protein complexes facilitating this maturation are another example of SMOCs (s. above) and have been termed inflammasomes (73). The most important inflammasome responsible for TLR4-dependent IL-1β release consists of the processing protease caspase-1, the adaptor protein ASC and the NLR protein NLRP3 (74, 75). The overall production of IL-1β induced by LPS is controlled on several different levels: induction of NLRP3 and IL-1β mRNA (in part through translocation of NF-κB) (76, 77), phosphorylation and ubiquitination of NLRP3 on multiple sites (78–81). Eventually, IL-1β is released by the cells through a process called pyroptosis culminating in Gasdermin D-forming pores in the cell membrane (see also 1.2) (82–84). There are multiple pathways to activate inflammasomes, termed canonical and non-canonical inflammasome activation [reviewed in (85)] and interestingly, LPS shows another species-specific peculiarity: in human cells, LPS is able to induce IL-1β release through an additional inflammasome activating pathway, called alternative activation, which does not require potassium efflux and pyroptosis but uses the TLR4-TRIF axis to activate NLRP3 through caspase 8 (86).

Caspase-4/11 Mediated Signaling Pathways

Since its discovery in 1999, TLR4 was long believed to be the sole LPS receptor. So it was a surprising finding, when the first reports came out in 2013 showing that intracellular cytosolic

LPS—independent of TLR4—was able to trigger noncanonical caspase-11-dependent inflammasome activation that was accompanied by IL-1β release and pyroptosis (28, 34). Subsequently, it was revealed that it is actually caspase-11 and its human orthologs, caspases 4 and 5, that directly bind and get activated by LPS (30, 31, 87).

Binding of LPS by these caspases is mediated by their CARD domain and leads to oligomerization and proximity-induced activation (30, 88). Within this process, auto-proteolysis at Asp285 in the inter-subunit linker of caspase-11 is also required (87). The molecular mechanism by which pyroptosis as well as the release of IL-1β and IL-18 is facilitated was unidentified for many years, despite enormous efforts from multiple groups. Then, in 2015, the long-sought-after molecule was identified as Gasdermin D (82, 89). Gasdermin D belongs to a family of 6 members (based on sequence homology), all harboring an auto-inhibitory carboxy-terminal domain (CTD) linked to the membrane pore-forming amino-terminal domain (NTD). Proteolytically active caspase-11 then cleaves Gasdermin D within the linker region, effectively separating the two domains from each other. Since the NTD has a high affinity to the negatively charged membrane phospholipids such as phosphoinositides and cardiolipin, it localizes to the plasma membrane. Finally, the NTD self-assembles to form pores presumably out of 26-28 NTDs in the plasma membrane which rapidly induce pyroptosis and allow the release of IL-1β and IL-18 (82, 83, 89–94). Caspase-11 activation induced by intracellular LPS also drives the release of pro-inflammatory cytokines, interleukin (IL)-1β and IL-18 by triggering the activation of the NLRP3 inflammasome. But how is the activation of the NLRP3 inflammasome achieved? Neither caspase-11 nor Gasdermin D do activate the NLRP3 inflammasome directly, but it has been shown that Gasdermin D expression is absolutely required for the activation (89). Due to the size of the NTD pores, they are also non-selective ion channels and thus, enable the efflux of potassium which in itself is known driver of NLRP3 inflammasome activation (95).

Crosstalk of TLR4- and Caspase 4/11-Dependent Signaling Pathways

The TLR4- as well as the caspase 4/5/11-dependent signaling events induced by LPS are not independent from each other but rather cross-interact at different levels. For example, the expression of caspase-11 is very low under normal conditions, but significantly induced by LPS, whereas the expression of caspase-4 is relatively constant, even in the absence of a priming signal (30). How does LPS induce transcriptional expression of caspase-11? In 2012, the data that TRIF-dependent signaling is licensing caspase-11 for NLRP3 inflammasome activation were convincingly presented (33, 96). This licensing is not mediated by direct interaction of TRIF and caspase-11, but requires Type IFNs. As explained in earlier, TRIF initiates activation of IRF3/7 and the induction of Type I interferon release. The released Type I IFNs then activate in an autocrine/paracrine manner the cell *via* IFNAR1/2-dependent JAK/STAT signaling to initiate pro-caspase-11 expression. In addition, Type I IFNs also drive the expression of GBPs and IRGB10 that are required for caspase-11-dependent responses towards LPS (97–99). Another molecule involved in both the extracellular/endosomal and the cytosolic LPS response is caspase-8. Caspase-8 belongs to the pro-apoptotic caspases and takes part of the alternative inflammasome activation by LPS in human cells (86). However, caspase-8 also cooperates with caspase-11 in the tissues to execute the final steps of endotoxic shock, i.e., tissue injury and cell death (100). The activation of both caspases is cytokine driven, caspase-8 by TNF and caspase-11, as already mentioned, by Type I IFNs, implicating both the LPS/TLR4/MyD88-dependent pathway (TNF) and the LPS/TLR4/TRIF-dependent pathway (TRIF) in this process.

RECOGNITION OF LIPID A/LPS BY THE TLR4/MD-2 COMPLEX

Structural Determinants of Lipid A/LPS Guiding Activation of the TLR4/MD-2 Complex in Relation to Virulence

LPS is a micro-heterogeneous bacterial glycan which is constituted of three major motifs: the membrane-anchored lipid A, the conserved core oligosaccharide and the variable O-antigen, whereas the lipid A portion exemplifies an "endotoxic principle" of LPS (101–104). Glycolipid "lipid A"–a small (~ 2 kDa) amphiphilic terminal fragment of LPS–is responsible for the activation of the host innate immune response through engagement of two major LPS sensing platforms: transmembrane TLR4/MD-2 complex (105, 106) and cytosolic inflammatory caspases (30). Structurally, lipid A is composed of a polar "head group" and a bulky hydrophobic cluster entailing four to seven long chain 3-hydroxylated lipid residues (107, 108). The polar region of lipid A consists of a β(1→6)-linked diglucosamine backbone which is decorated by two phosphate groups - at position 4′ (P-4′) of a distal GlcN residue and at position 1 (P-1) of a proximal GlcN moiety (**Figure 2**). Positions 2,3 and 2′,3′ of the proximal and the distal glucosamines are usually acylated by the long chain (*R*)-3-hydroxyalkanoic and/or (*R*)-3-acyloxyalkanoic acids. The endotoxic activity of LPS generally relies on the number, length and distribution of lipid chains along the disaccharide backbone of lipid A as well as on the phosphorylation status of the sugar units. A canonical endotoxic lipid A of *E. coli* is hexa-acylated (the lipid chains entail 12 to 14 carbon atoms) and possesses two phosphate groups. The non-endotoxic lipid A variants are usually under-acylated, and/or possess longer (C_{16}-C_{18}) lipid chains and lack at least one of the phosphate groups. The TLR4/MD-2 receptor complex responds to very low concentrations (picomolar magnitudes) of LPS *via* recognition and binding of distinct structural motifs of lipid A through majorly hydrophobic, but also ionic interactions.

The lipid A binding site on MD-2 is remarkably large and consists of a deep hydrophobic Leu- and Phe- rich cavity, crowned on the top with a number of Arg and Lys residues. Hydrophobic groove of MD-2 can accommodate multiple

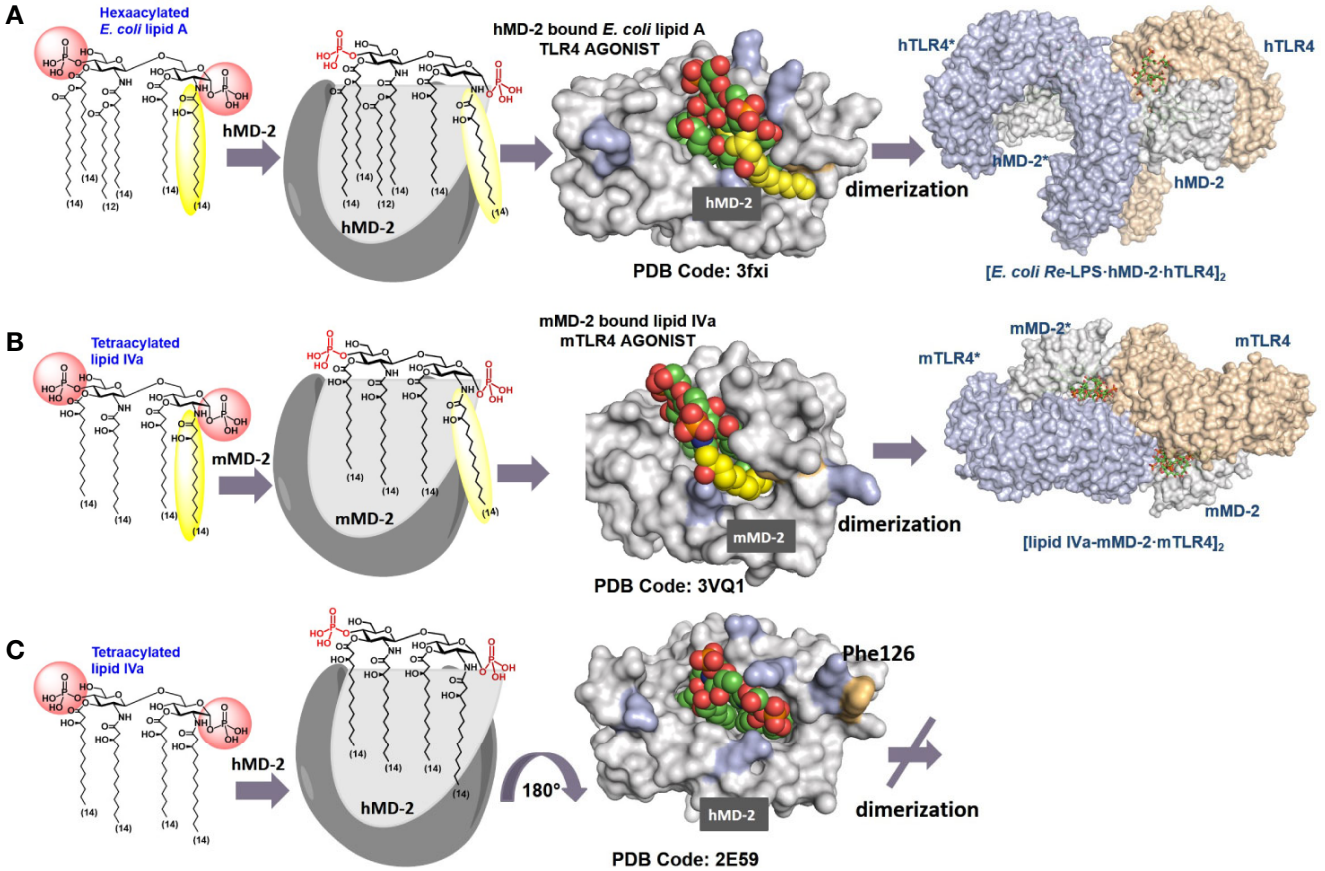

FIGURE 2 | Modes of interaction of agonist and antagonist lipid A variants with the TLR4/MD-2 complex. **(A)** Crystal structure of a binary TLR4·MD-2·LPS complex (PDB code: 3FXI). The 2N-acyl chain (yellow) of the TLR4·MD-2—bound *E. coli* lipid A is exposed on the surface of hMD-2. Phe126 (orange) is directed inward and supports the exterior positioning of the 6th (exposed) lipid chain *via* hydrophobic interactions. **(B)** Species-specific recognition of lipid IVa by mouse TLR4/MD-2 complex. **(C)** Crystal structure of hMD-2 bound lipid IVa, the orientation of the ligand is inverted by 180° compared to agonist ligands (PDB code: 2E59), Phe126 points outward which prevents dimerization with the second TLR4*·MD-2*-ligand complex. Images were generated with PyMol.

acyloxy- and acyloxyacyl lipid chains, whereas positively charged side chains at the rim of the binding pocket can establish ionic interactions with the lipid A phosphate groups (5). MD-2 is physically associated with TLR4 and the contact area is designated "primary dimerization interface". Binding of the lipid A motif of LPS by MD-2 initiates and supports the assembly of a hexameric ligand-receptor complex constituted of two copies of the TLR4/MD-2/LPS homodimer (**Figure 2A**). LPS-induced TLR4 complex dimerization is facilitated by hydrophobic interactions of a specific lipid chain of the MD-2–bound lipid A with the second TLR4* (designated as "secondary dimerization interface") (6). Upon formation of a $[TLR4/MD\text{-}2/LPS]_2$ complex, the intracellular TIR domains come into vicinity which triggers the recruitment of a number of adaptor proteins (109). The latter event eventually leads to the assembly of a large macromolecular signaling complex called "Myddosome" that, in turn, triggers diverse pro-inflammatory signaling pathways (110, 111).

Generally, binding of the bisphosphorylated hexaacylated lipid A from *E. coli*–a typical TLR4 agonist - results in the efficient TLR4 complex dimerization and robust activation of the pro-inflammatory signaling, whereas binding of tetraacylated lipid A variants blocks the binding pocket of MD-2 for the interaction with endotoxic LPS, thus rendering these lipid A types to potent TLR4 antagonists (**Figure 2C**) (112, 113). The principal differences in binding modes of agonist and antagonist lipid A include 1) the number of lipid chains (four to five) accommodated in the binding pocket of MD-2; 2) the orientation (binding pose +/- 180°) of the carbohydrate backbone of lipid A within the binding pocket of the co-receptor MD-2; 3) the deepness of insertion of the lipid A molecule into the binding cleft of MD-2 (TLR4 antagonists are accommodated deeper in the binding grove of MD-2 compared to agonist lipid A variants); and 4) ligand-induced rearrangement of MD-2 discriminated by different positioning Phe126 residue (located inward for agonist lipid A and outward for antagonist lipid A).

Accordingly, all four long-chain acyloxy residues of underacylated lipid A variants are entirely intercalated into the hydrophobic binding pocket of human MD-2, the lipid A molecule adopts "inverted" orientation with phosphate group

P-4′ facing secondary dimerization interface, and the whole molecule is inserted deeper into the binding pocket compared to agonist lipid A (**Figure 2C**) (112, 113). A number of natural and synthetic antagonist lipid As and analogues were shown to selectively bind to MD-2/TLR4 without triggering receptor complex dimerization (112–114). These compounds are extensively studied as candidates for potential therapeutic inhibition of harmful endotoxic effects induced by TLR4 activation (115–117).

In contrast, TLR4 activating lipid A variants are housed in the binding pocket of MD-2 with the glycosidic phosphate P-1 bordering secondary dimerization interface. Thus, the lipid A molecule is rotated by 180° in the binding pocket of MD-2 compared to antagonist binding mode (**Figures 2A, B**). The binding pocket of MD-2 can accommodate only five lipid chains of hexaacylated *E. coli* lipid A, while the 6th 2*N*-acyl lipid chain (linked to the proximal GlcN residue of the diglucosamine backbone) is excluded from the binding grove and presented on the surface of MD-2 at the site engaged in the dimerization with the second TLR4* (secondary dimerization interface). The expulsion of lipid chain out of the binding pocket and the resulting reorganization of the secondary structure of MD-2 is considered the major driving force of the dimerization process (5, 6). It is now well established that both ionic interactions of the lipid A phosphate groups with the Lys and Arg side chains as well as intermolecular hydrophobic interaction of the exposed 2*N*-acyl chain with the second TLR4* contribute to receptor complex dimerization and formation of the active $[TLR4/MD\text{-}2/LPS]_2$ hexamer (118–120).

The positioning of the ligand (+/-180°) within the binding cleft of MD-2 appears to be crucial for the expression of a particular biological activity. Thus, tetraacylated lipid IVa acts as antagonist at hTLR4 but performs as weak agonist at mouse (m-) TLR4 wherein it binds in an inverted by 180° orientation (similar to *E. coli* lipid A in the binding pocket of hMD-2) and exposes one lipid chain on the surface of the protein (**Figure 2B**) (6, 121). Species-specificity in ligand recognition by the TLR4 system, which is decisive for transition of *in vivo* data obtained in rodent or other animal models to clinical trials, is still not well understood. In addition to the length and number of lipid chains, the distribution pattern of acyl residues along the glucosamine backbone is decisive for lipid A recognition by the TLR4/MD-2. For instance, lipid A variants having four lipid chains attached at the distal GlcN ring and two lipid chains linked to the proximal GlcN (4 + 2 acylation pattern as in *E. coli*) as well as lipid A variants with the acylation pattern (3 + 3) as in *N. meningitidis* are the most powerful TLR4 activators; penta-acylated lipid As with (3 + 2) acylation pattern are inactive (or weakly active), whereas penta-acyl lipid A having (4+1) acylation pattern retains robust activating potential similar to *E. coli* lipid A (122, 123).

LPS Is Delivered to the TLR4/MD-2 Complex by the Proteins of the LPS Transfer Cascade

LPS is an amphiphilic molecule that contains a relatively small hydrophobic lipid region retaining LPS in the lipid (bi)layer (in the outer leaflet of the outer bacterial membrane or in the lipid layer of the endosomes/OMVs) and a large hydrophilic carbohydrate portion (inner and outer core, O-antigen) which is decorated by a number of negatively and positively charged appendages such as phosphates, phosphoethanolamines, or amino sugars. Despite its large size and complexity, LPS is recognized by the innate immune system through a fine-tuned molecular mechanism which is extraordinary sensitive to minor variations in the structure of lipid A. Regardless its relative heterogeneity in respect to acylation pattern, lipid A represents the most conserved fragment of LPS. Lipid A has an ability to establish high affinity interactions with a number of proteins involved in the LPS transfer and recognition cascades. Prior to interaction with TLR4, the LPS molecule must be "extracted" from the membrane surfaces and transferred to the binding pocket of MD-2 (124, 125) which requires a successive interaction of LPS with LPB (3), and the GPI-anchored differentiation antigen of monocytes CD14 (4, 126, 127). LBP binds sequentially to LPS micelles and to CD14 to form a dynamic intermediate LBP/LPS/CD14-complex, and accomplishes multiple rounds of LPS transfer to CD14. In turn, CD14/LPS rapidly dissociates from LPB-LPS complex and transfers a single LPS molecule to MD-2/TLR4 *via* a direct physical interaction between LRR13-LRR15 domains of TLR4 with CD14/LPS (4). In addition, CD14 mediates LPS internalization through LPS-induced endocytosis of TLR4/MD-2/LPS complexes which eventually leads to endosome-mediated TRIF-dependent signaling resulting in interferon production as well as in activation of NF-κB (128, 129). Although the crystal structures of LBP and CD14 are available (130–132), and the fine dynamics of the LPS transfer cascade by LBP and CD14 has been recently deciphered (4), the precise atomic mechanism and structural background of the LPS/lipid A recognition by LBP and CD14 are still not fully understood. Whereas LPS binding by LBP involves positively charged patches at the LBP N-terminal domain which could attract the phosphate groups/negative charges of LPS by ionic forces (131), CD14 possesses several hydrophobic cavities surrounded by positively charged side-chains which, most likely, bind LPS through majorly hydrophobic but also ionic interactions (132).

INTERACTION OF LIPID A/LPS WITH INFLAMMATORY CASPASES

In addition to the activation of a canonical (caspase-1–dependent) inflammasome, LPS mediates the noncanonical (caspase-4/11 –dependent) inflammasome activation when mammalian immune cells are challenged with intracellular bacteria including *Shigella flexneri*, *Salmonella enterica* serovar *Typhimurium* (*S. typhimurium*), *Legionella pneumophila*, *Francisella novicida*, several *Burkholderia* species, and *Chlamydia trachomatis* as well as extracellular bacteria such as enterohemorrhagic *E. coli* (EHEC), *Citrobacter rodentium*, and *Yersinia pseudotuberculosis*. For non-canonical inflammasome activation, the bacterial products such as LPS must be translocated into the host cytosol which can be achieved

via type III (T3SS) or type IV secretion system (T4SS) abundantly expressed in the infectious strains of several bacteria (31, 133).

Cytosolic Delivery of LPS for Noncanonical Inflammasome Activation

LPS is a relatively large 20 kDa glycan which cannot cross cellular membranes by itself, so that sophisticated molecular mechanisms are required to deliver or transfect LPS derived from non- cytosolic bacteria into the cytosol of the hosts' immune cells for non-canonical inflammasome activation. Also, many intracellular bacteria survive within vacuoles and use special protein complexes to let their PAMPs access the host cytosol. It has been proposed that LPS can enter the cytosol through multiple pathways.

Intracellular bacteria which reside and replicate within distinct cellular compartments evolved special secretion systems to allow LPS to access cytosol. For example, *Salmonella* uses type 3 secretion system (T3SS) to invade epithelial cells and to establish vacuolar compartments (SCV, *Salmonella*-containing vacuole), which helps bacteria to survive within phagocytes (133). LPS can gain access to the cytosol through lysis of bacteria-containing vacuoles formed by eukaryotic membranes of the host cells (134, 135). Also *L. pneumophila* usually survives within the vacuole, although certain mutants can atypically enter the cytosol (135). Since many Gram-negative pathogens known to activate caspase-4/11 are not cytosolic, a specific molecular mashinery which allows LPS from these bacteria to gain access to the cytosol for caspase-4/11 activation has been evolved.

One of the plausible mechanisms for LPS internalization and intracellular delivery involves LPS binding by high-mobility group box 1 (HMGB1) - an alarmin which can efficiently transport LPS into the cytoplasm through receptor for advanced glycation end products (RAGE)-mediated endocytosis (17, 136, 137). Through internalization of HMGB1-LPS complexes mediated by RAGE, HMGB1 induces destabilization of lysosomes for cytosolic LPS delivery. HMGB1 was demonstrated to bind LPS *via* LPS-binding domains (the A and B box), although the structural requirements for LPS recognition by HMGB1 are currently unknown (138). TLR4 activation by LPS was shown to induce HMGB1 release from hepatocytes followed by direct LPS binding, and the LPS translocation by induction of lysosomal rupture. Interestingly, HMGB1 has long been supposed to have high affinity to LPS and to interfere with TLR4/MD-2/CD14 signaling (138, 139). Another report describes elevated production of HMGB1 in hepatocytes in response to the LPS-induced TLR4 and caspase-11/Gasdermin D signaling (140) indicating that HMGB1 represents a danger molecule released in response to NLRP3 inflammasome activation.

It has been also suggested that outer membrane vesicles (OMVs)—the naturally secreted products of Gram-negative bacteria—can function as cytosolic LPS delivery vehicles (141). Generally, OMVs promote the induction of pro-inflammatory mediators *in vivo* during infection with Gram-negative pathogens such as *H. pylori*, *L. pneumophila*, *S. typhimurium* and other (142). The membrane composition of OMVs is rich with LPS required for OMV stability and is very similar to the content of extracellular vesicles formed by eukaryotic cells (143). Recent studies suggest that OMVs can directly transport membrane-associated PAMPs into the host cells where they can be taken up through endocytosis, or act as vehicle for the internalization of LPS into the cytosol (144–147). Furthermore, internalization of LPS-containing OMVs by guanylate-binding proteins (GBPs, interferon-inducible GTPases) promotes localization of LPS in the cytoplasm followed by caspase-4/11 mediated activation of NLRP3 (134, 148). GBPs associate with LPS-containing membrane surfaces and contribute to cytosolic immune detection of LPS by facilitating its interaction with caspase-4/11. GBPs were also shown to assist in disruption of pathogen-containing vacuoles thus allowing LPS of cytosolic bacteria to reach the cytosol (97, 149).

Guanylate-Binding Proteins as Co-Factors for Caspase-4/11 Mediated LPS Sensing

Caspase-4/11 was shown to directly bind to the lipid A motif of LPS, however, lipid A is hidden in the bacterial outer membrane or embedded within the lipid bilayer of liposomal aggregates spontaneously formed by LPS. Therefore, a central question on how the membrane-anchored LPS can interact by its lipid A motif with the CARD of the cytosolic protein caspase-4/11 had to be answered. Recently, guanylate-binding proteins (GBPs) were suggested to govern the recruitment of caspase-4/11 to LPS-rich membrane surfaces.

GBPs play a crucial role in antibacterial defense through modulation of both cell-autonomous and innate immunity against Gram-negative bacteria (148). The infection of mouse BMDMs with Gram-negative bacteria induces production of type-I IFNs which consequently upregulates mGBPs (134, 150). Activation of GBPs also contributes to secretion of IL-1β and IL-18, and the induction of pyroptosis through activation of the NLRP3 inflammasome and initiation of molecular mechanisms facilitating LPS release into the cytosol of host cells. GBPs were proposed to aid in the LPS uptake from membrane interfaces und thus, to be involved in the activation of caspase-11 and the assembly of noncanonical inflammasome (150).

Different roles and functions were suggested to explain GBPs involvement in the induction of proteolytic activity of caspase-4/11. For instance, in the gut infected with *S. Typhimurium*, GBPs are supposed to contribute to the death and expulsion of infected enterocytes into the lumen. It has been proposed that GBP2-dependent liberation of *S. typhimurium* LPS into the host cytosol through targeting *S. typhimurium* PCV and promoting its membrane lysis drives caspase-11- and NLRP3-dependent pyroptosis (134). Apparently, GBP2 contributes to induction of caspase-4/11 proteolytic activity and noncanonical inflammasome activation in response to infection with *F. novicida* (151), *L. pneumophila* (150) and other cytosolic bacteria (99). GBPs have also been shown to be recruited to cytosolic *S. flexneri* and to prevent spreading of intercellular bacteria by restricting its actin-driven motility (149). Interestingly, GBPs were degraded over time by *S. flexneri* bacterial proteasomes which were in turn activated by secreted bacterial effectors (152). It has been also reported that GBP recruitment to bacteria such as *Y. pseudotuberculosis* or *L.*

pneumophila or their PCVs is dependent on the bacterial type-3 or -4 secretion system, respectively (153, 154).

GBPs were demonstrated to be crucial in mediating caspase-11 activation in response to outer membrane vesicles (OMVs) from different Gram-negative bacteria such as *E. coli*, *S. typhimurium*, *S. flexneri* or *P. aeruginosa* (97, 98). GPBs were suggested to directly deliver LPS into the host cell cytosol after LPS had been internalized through endocytosis. GBPs could physically associate with cytosolic OMVs upon GBPs isoprenylation, and could govern the activation of caspase-11 and Gasdermin D *in vivo*. Optionally, OMVs could induce recruitment of GBPs through activation of the TLR4-TRIF pathway. In all circumstances, LPS was sufficient to initiate GBPs recruitment *in vitro* and GBP deficiency protected against OMV-induced lethal endotoxemia *in vivo* (98).

Thus, it is by now established that the function of guanylate-binding proteins is closely linked to their ability to interact with LPS; however, what part of LPS is recognized by GBPs was very long uncertain. Several studies reported on smooth LPS-induced GBP recruitment to intracellular bacteria which implied the major role of LPS O-antigen in GBPs sensing and the involvement of majorly ionic interactions in this process. Indeed, it was observed that the co-localization of hGBP1 with *S. flexneri* producing LPS-*Ra* mutants was reduced in relation to that of hGBP1 targeting wild-type bacteria, which insinuated that GBP1 recognizes LPS of *S. flexneri* by its O-antigen (149). Controversially, hGBP2 was shown to mediate caspase-4 activation in response to transfection with tetra-acylated LPS of *F. novicida* (151) which is known to lack the O-antigen and the core sugars (155, 156). This suggests variable sensitivity of different GBPs to particular structural features of LPS and that hGBP2 might contain specific lipid A recognizing motifs. In agreement with the latter observation, it was revealed that caspase-11 activation by transfected lipid A is fully GBP dependent (98) whereas GBPs were only partially required for caspase-11 activation induced by smooth or rough (*Re*-LPS) type *S. minnesota* LPS. Thus, not only the O-antigen but the lipid A region of LPS could be involved in recognition by GBPs.

Two recent cutting-edge studies independently postulated that the LPS-induced assembly of a GBP coat on the surface of cytosolic *Salmonella* (or on the LPS-rich membrane interface upon cytosolic delivery of LPS) is indispensable for caspase-4 activation (157, 158). Association of GBP1 with the LPS-rich surface of cytosolic *Salmonella* follows bacterial escape from the vacuole and initiates the recruitment of GBP2-4 to assemble a GBP-derived signaling platform. The LPS-induced GBP coating of bacterial surface promotes the recruitment of caspase-4 to the cytosolic face of the GBP coat followed by caspase-4 activation and pyroptosis. Indeed, caspase-4 can efficiently bind to purified LPS and lipid A by its CARD domain *in vitro* but does not bind LPS as a constituent of the bacterial outer membrane in cellular experiments in the absence of GBP. Thus, GBPs could make LPS available for the interaction with caspase-4 by disturbing the integrity of the outer bacterial membrane and making acyl chains of lipid A accessible to their ligand-binding CARD domain.

Further studies are required to understand the structural basis of GBP interaction with lipid A/LPS at the membrane interfaces. The latest findings, however, indicate that GBP1 can directly bind to the LPS coated surface (e.g. outer leaflet of the outer bacterial membrane) and that this interaction is driven by solely ionic forces, whereas the carbohydrate portion of lipid A and the glycan moiety comprising the inner core region are sufficient for LPS-GBP1 interaction (157, 158). All O-antigen and outer core lacking mutants of *E. coli* LPS (*Ra-, Rc-, Rd-, Re*-LPS) could associate with GBP1 and induce GBP1 oligomerization at the LPS-rich membrane interface. Negatively charged groups of the inner core sugars of LPS and the phosphate groups attached at the diglucosamine backbone of lipid A were shown to be crucial in promoting GBP1-LPS interaction (which proceeds presumably through involvement of positively charged surface patch of GBP1) and subsequent activation of the non-canonical inflammasome pathway (158). Remarkably, also LPS from *R. sphaeroides* that acts as caspase-11 antagonist could associate with GBP1 to form higher molecular weight aggregates, which assumes rather broad specificity of GBP1 in recognizing LPS motifs independently on their caspase-4 activity (158). Thus, GBP1 functions as a part of an upstream GBP1-4 complex and orchestrates the recruitment of GBP2-4 to initiate a formation of a signaling platform that is assembled on the LPS-containing membranes. GBP2 and GBP4 are involved in a subsequent recruitment of caspase-4, whereas GBP3 is thought to control its activity (157).

Two alternative modes for GBP-induced caspase-4 recruitment to membrane-embedded LPS have been proposed. A high molecular weight complex formed by GBP-LPS could promote the recruitment of caspase-4 and subsequently transfer LPS onto caspase-4 to trigger its activation. Otherwise, the assembly of GBP-LPS complex on the bacterial surface could disturb the integrity of bacterial outer membrane which would allow an access of caspase-4 to otherwise hidden acyl chains of membrane-anchored LPS (158).

Structural Features Characteristic to Lipid A of Bacterial Species Inducing Caspase-4/11 Activation: Is There Any Cross-Specificity With TLR4/MD-2?

It has been unambiguously shown that cytoplasmic LPS triggers caspase-4/11-dependent cell death in human 293T cells and mouse macrophages, respectively. Also, LPS–induced caspase-4/11 oligomerization was observed on the pore-limit native gel and the oligomerization was induced by the fully acylated (hexa- to heptaacylated) lipid A fragment of LPS from *S. typhimurium*, *C. rodentium*, *S. flexneri* and *E. coli* (**Figure 3**) (30). Notably, all LPS forms (LPS-*Ra, -Rc, -Rd*, and *-Re*) and *E. coli* lipid A alone could induce caspase-4/11 oligomerization and efficiently stimulated caspase-4/11 activation. Juxtapose, LPS variants bearing fewer lipid chains (LPS from *R. sphaeroides* and biosynthetic precursor of *E. coli* lipid A, lipid IVa) although being able to bind to caspase-11 CARD (caspase activation and recruitment domain) with the affinity similar to hexaacylated LPS, failed to induce caspase-4/11 oligomerization and activation *in vitro*. This was consistent with the reports on the inability of

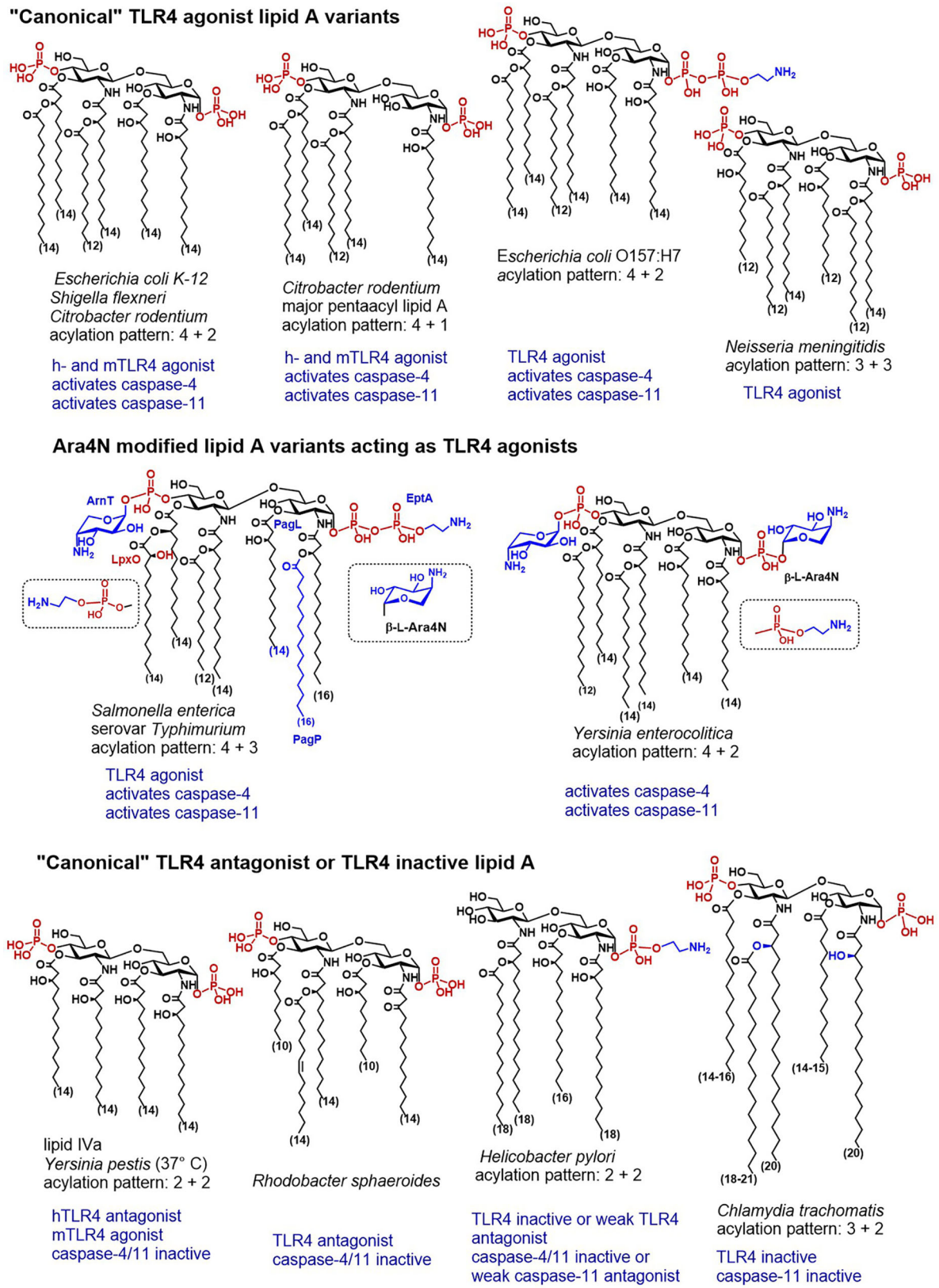

FIGURE 3 | Chemical structures of "canonical" TLR4 agonist and TLR4 antagonist lipid A variants and their caspase-4/11 specific activity (when known).

lipid IVa to activate the non-canonical inflammasome in mice (28, 34). Several positively charged residues at the lipid A binding site of CARD were identified indispensable for efficient lipid A—induced oligomerization (30). Interestingly, several lipid A–binding residues (K19, K52/R53, K62/K63/K64) are conserved in caspase-4 but not in caspase-11 which resembles species-specific differences between human and mouse MD-2. Whereas the rim of the binding pocket of human MD-2 is decorated by multiple Lys and Arg residues that are crucial for establishing ionic contacts with the lipid A phosphate groups, mouse MD-2 lacks most of these amino acids (6).

Along these lines, wt strain of *E. coli* induced expression of IL-1β and pyroptosis in wt and TLR4$^{-/-}$ BMDMs, whereas a mutant lipid IVa-producing strain induced TLR4-dependent production of IL-6 (which is consistent with weak agonist activity of lipid IVa at mTLR4), but not the release of IL-1β or pyroptosis in BMDMs (28). Activation of caspase-11 in BMDMs by transfected *E. coli* LPS was supressed when BMDMs were preloaded with underacylated *Helicobacter* lipid A (28) which is also known for its moderate antagonist activity at hTLR4 (159).

Another example of cytosolic bacteria producing LPS with canonical lipid A structure able to potently induce the TLR4-dependent immune responses is *S. flexneri*–a Gram negative pathogen responsible for invasion, disruption and inflammatory destruction of the intestinal mucosa. *S. flexneri* synthesises heterogeneous hexa- to tetraacylated LPS having a "canonical" endotoxin acylation pattern (4 + 2) (**Figure 3**) (160–162). Expectedly, hTLR4 was shown responsive to hexaacylated lipid A variants of *S. flexneri*, whereas mTLR4 was required to elicit TLR4-mediated NF-κB response to penta-acylated mutants (163). Caspase-11 was responsive to LPS introduced by *Shigella* into the cytosol after bacteria escaped the vacuole. *S. flexneri* LPS induced production of IL-1β and pyroptotic cell death resulting in lethal endotoxemia in mice (89). Similarly, infection with *S. flexneri* was responsible for caspase-4 driven inflammatory cell death in HaCaT keratinocytes and human colon adenocarcinoma HT29 cell line (164).

Juxtaposed, LPS from the obligatory intracellular pathogen *C. trachomatis* characterized by a "TLR4 inactive" acylation pattern (3 + 2) of its lipid A (165) failed to activate the non-canonical inflammasome (166). Three out of five acyl chains in *Chlamydia* lipid A exceed the length that is considered optimal (C_{12}-C_{14}) for the TLR4 activation (**Figure 3**). Both 2- and 2′-*N*-acyl chains as well as the secondary acyl chain at position 2′ of *C. trachomatis* lipid A have a length of up to 21 carbon atoms and the acyl chains that are ester-linked at positions 3- and 3′- are not hydroxylated. All these structural features confer low affinity to human TLR4/MD-2 complex (167, 168) and to the proteins of the LPS transfer cascade (169). Likewise, *Chlamydia* LPS failed to induce the dimerization of mouse TLR4/MD-2 complexes and to activate both NF-κB and caspase-11-mediated signaling in BMDMs (166).

Thus, structure-activity relationships for caspase-4/11 and TLR4 activation seem to be somewhat similar: LPS possessing a "canonical" lipid A structure-hexaacylated/bisphosphorylated-binds to CARD, promotes caspase-4 and caspase-11 oligomerization and induces caspase activation, whereas penta- and tetra-acylated lipid A variants fail to activate caspase-4/11 although can bind to CARD. Similar dependencies were recently demonstrated for synthetic lipid A mimetics close in structure to native lipid A molecules: tetraacylated disaccharide lipid A mimetics (DLAMs) acting as potent TLR4 antagonists did not induce caspase-4/11 proteolytic activity, while synthetic TLR4 agonists (DLAMs having picomolar affinity for TLR4/MD-2) were simultaneously very efficient in inducing oligomerization and proteolytic activity of caspase-4 *in vitro* (170). Intriguing results were obtained for the interaction of DLAMs with caspase-11: despite causing caspase-11 oligomerization, several synthetic lipid A mimicking molecules did not induce caspase-11 catalytic activity which correlated with their chemical structure (170). Thus, the CARD of both capase-4 and caspase-11 was extraordinary sensitive to variations in the primary chemical structure (acylation and phosphorylation pattern) of lipid A mimicking molecules.

All these findings match with a 1:1 ligand-receptor stoichiometry already postulated for the assembly of TLR4/MD-2/LPS complex, which insinuates a high affinity interaction of a single lipid A (or lipid A mimetic) molecule with the CARD. As far as the recognition process is concerned, both the primary chemical structure and the shape of aggregates formed by LPS/lipid A or lipid A mimetic could be involved. Indeed, the latter studies were performed *in vitro* using pore-limit native gel and relatively high lipid A/DLAMs concentrations. Since lipid A/LPS tend to form high molecular mass aggregates in a concentration-dependent manner (171, 172), DLAMs could also form aggregated structures which were, in turn, sensed by the CARDs of caspase-4/11. Such interpretation would be in line with a recent study showing that caspase-4 recognizes LPS-rich membrane interfaces. According to this study, caspase-4 could bind directly to LPS-rich OMVs formed by *N. meningitidis* as well as to the high molecular mass aggregates of purified metabolically radiolabeled LPS (173–175). Indeed, it has been demonstrated that purified caspase-4 (C258A) and CARD domain from *E. coli* could bind huge LPS micelles and disaggregate them to small complexes *in vitro* (176).

Taking into account substantial differences in the molecular and physical properties of monomeric and aggregated structures of lipid A (2 kDa amphiphilic glycolipid) and LPS (20 kDa heterogeneous glycan) and the fact, that lipid A alone could induce pyroptosis *in vivo* (30) and could bind to CARD *in vitro*, we assume that the recognition of particular chemical entities of lipid A is essential for caspase-4/11—LPS interaction. Considering that lipid A is buried within the lipid bilayer to anchor LPS in the membranes or other liposomal interfaces and, therefore, not freely available for the interaction with proteins, an intermediate step preceding lipid A/LPS-CARD interaction with involvement of additional proteins that can extract LPS from the membrane surfaces and deliver the lipid A fragment to CARD can be supposed. For example, TLR4/MD-2 complex "exploits" accessory proteins LBP and CD14 to let the lipid A portion of LPS being directly "delivered" to the binding pocket of MD-2. Recent studies disclosed a fine-tuned mechanism of LPS sensing by inflammatory caspases with involvement of GBPs as supplementary proteins having high affinity for LPS (98, 136, 157, 158). Similar to species-specific recognition of lipid A by the

TLR4 system, some not yet fully understood species-dependent differences in the activation of human caspase-4 and mouse caspase-11 by LPS have been observed.

WHAT ARE THE STRUCTURAL DETERMINANTS CRUCIAL FOR LPS/LIPID A RECOGNITION BY INFLAMMATORY CASPASES?

To explore structure-activity relationships and to establish primary molecular signatures recognised by caspase-4/11 and involved in the non-canonical inflammasome activation, we analyzed the relevant literature from the "chemical" perspective with a special emphasis on particular structural features of LPS/lipid A mentioned in the studies on caspase-4/11- and/or GBPs-mediated inflammasome activation. Remarkably, except for "canonical" hexaacylated lipid A of *E. coli*, *S. flexneri* and *C. rodentium*, the major lipid A species able to induce caspase-4/11 activation are characterized by specific lipid A modifications such as substitution of the phosphate groups by positively charged appendages (phosphoethanolamine or amino sugars) and by a specific acylation pattern (penta- to heptaacylated with fatty acids length up to 18 carbon atoms).

Covalent attachment of positively charged appendages to the phosphate groups of lipid A is considered a part of survival strategy of opportunistic Gram-negative bacteria. One of the most abundant phosphate group modifications-attachment of ethanolamine (*Helicobacter*) or phosphoethanolamine PNEt (*EHEC, Salmonella*)—is associated with bacterial resistance to cationic antimicrobial peptides (CAMPs) (177). In some species, the phosphate groups of lipid A are substituted by cationic amino sugars-4-amino-4-deoxy-β-L-arabinose (β-L-Ara4N) in *Burkholderia, Pseudomonas, Yersinia* or *Salmonella*, or by galactosamine (*Fransicella*) or glucosamine (*Bordetella*) (178–181). Inducible addition of β-L-Ara4N to the phosphate residues of lipid A is an adaptive mechanism that assists Gram-negative bacteria to oppose neutralization by CAMPs and to circumvent induction of the innate immune responses in the infected host. Despite rigorous research efforts, no explicit correlation between the presence of β-L-Ara4N as a lipid A phosphate group modification and the modulation of TLR4-dependent inflammation could be established (182, 183). To better comprehend the interrelation of caspase-4/11 activation and specific acylation and phosphorylation pattern of lipid A, we provide a short exposè on structural features of lipid A produced by bacterial species that are known to induce caspase-4/11-mediated inflammasome activation and/or TLR4 dependent signaling with special emphasis on LPS remodeling.

Activation of Inflammatory Caspases by Extracellular Bacteria That Produce TLR4-Agonist LPS Variants

Caspase-4 activating *E. coli* strain O157:H7 (enterohaemorrhagic *E. coli*, EHEC) produces 1-O-P-PNEt lipid A which differs from a "classic" lipid A (hexaacylated, bis-phosphorylated, to one-third substituted with pyrophosphate at position 1)synthesised by *E. coli* serotypes K12 and O111:B4. The occurrence of the phosphoethanolamine modification at the glycosidic phosphate group P-1 in EHEC has been distinctively confirmed (184). EHEC and EPEC (enteropathogenic *E. coli*) strains were shown to activate caspase-4 (133, 164), to induce caspase-4/11-mediated IL-1β and IL-18 secretion and inflammatory cell death, whereas specific T3SS effector protein could inhibit caspase-4/11-dependent inflammasome (185, 186). Whether 1-O-P-PEtN motif of the lipid A fragment of LPS is functionally involved in EHEC-induced caspase-4/11 dependent inflammasome activation remains for now unknown.

C. rodentium is a murine Gram-negative bacterium used as a surrogate to study human non-invasive gastrointestinal pathogens EPEC and EHEC since it causes similar transmissible diarrheal disease in mice. The lipid A acylation pattern of *Citrobacter* LPS is identical to that of *E. coli* with exception of relatively high proportion of the penta-acylated species having both 3+2 and 4 +1 acylation pattern, whereas the latter is more abundant (**Figure 3**) (187). In agreement with the known principle for "TLR4 agonist" acylation pattern of lipid A, *C. rodentium* induces rapid TLR4-dependent responses in intestinal epithelium, although TLR4-mediated pro-inflammatory signaling is not host-protective and contributes to pathology and morbidity during infection (188). TLR4 was demonstrated particularly important for NLRP3 inflammasome activation in *C. rodentium* and *E. coli* infected mouse macrophages. Importantly, the TLR4/TRIF axis—regulated expression of caspase-11 was indispensable for *E. coli*- and *C. rodentium*—induced NLRP3 inflammasome activation in macrophages (96). Thus, extracellular enteric bacteria must be recognized by both TLR4- and caspase-11 to induce the non-canonical inflammasome activation and pyroptosis. Like pathogenic human-specific *E. coli* strains EPEC and EHEC, *C. rodentium* modifies the phosphate groups of its lipid A with phosphoethanolamine. This covalent modification is primarily catalysed by specific transferases PmrC and CptA, the expression of which is regulated by PmrAB. Interestingly, PEtN modification contributed to maintenance of OMV integrity, but simultaneously negatively affected the rate of production of OMV by *C. rodentium* (189).

Yersinia species evolved many strategies to evade the recognition by the human innate immune system, including inducible LPS remodeling. To achieve a suppression of local and systemic inflammation, *Y. pestis* modifies the acylation degree of the diglucosamine backbone of its lipid A from hexaacylated (hTLR4 agonist) to tetraacylated (inactive or hTLR4 antagonist). Thus, the lipid A produced by *Yersiniae* in mammalian host at 37°C is underacylated and similar in structure to lipid IVa which deprives *Yersinia* LPS the hTLR4-mediated activity (**Figure 3**) (190). Mutants producing hexaacylated lipid A (normally synthesised by bacteria in a vector host at 25°C) have been shown to strongly activate the innate immune response in a TLR4-dependent manner (191). Recognition of lipid A by caspase-11 might follow similar structure-activity relationships: transfection of hexaacylated LPS from *Y. pestis* grown at 25°C

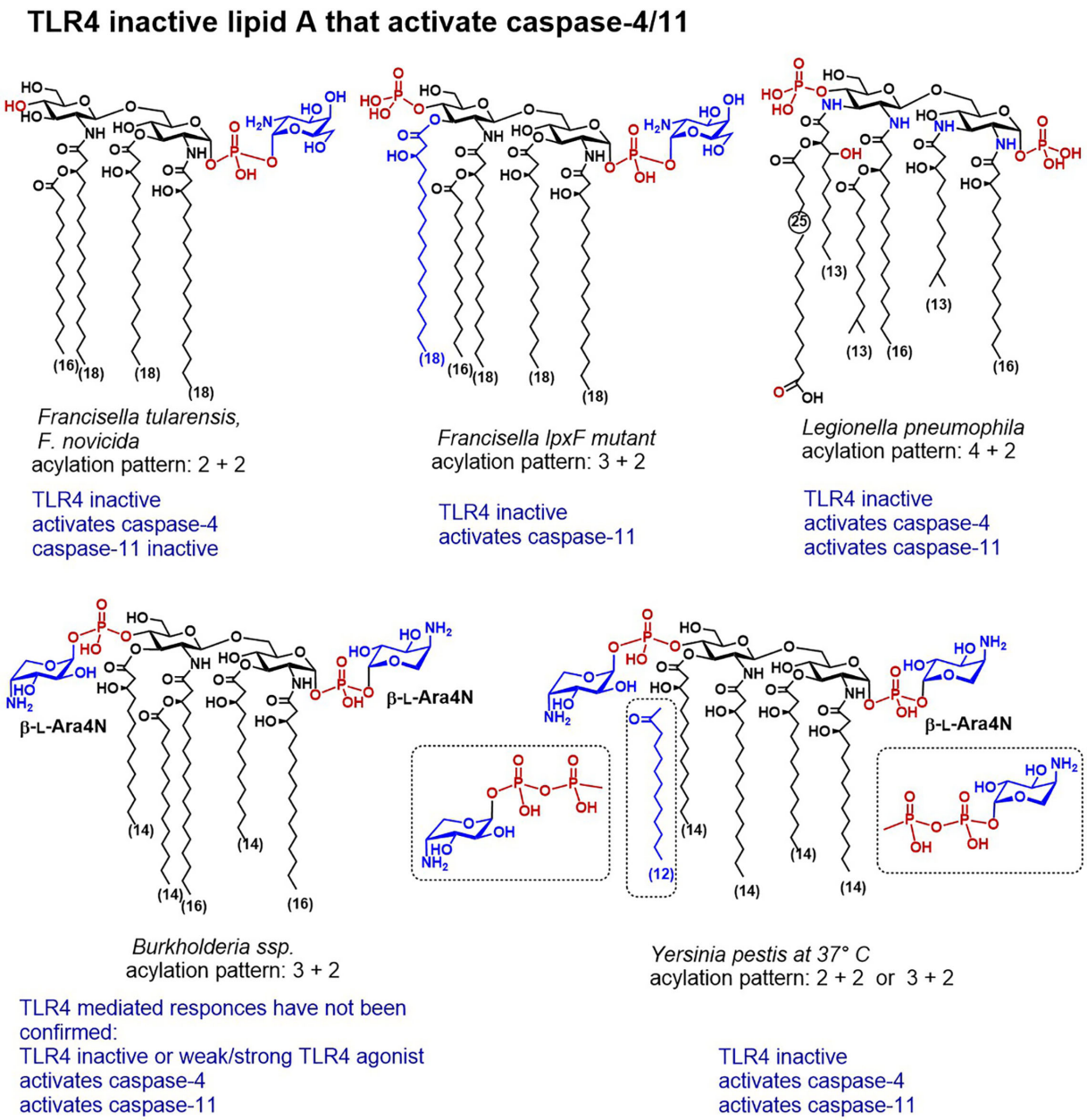

FIGURE 4 | Chemical structures of lipid A variants that do not activate TLR4/MD-2 but are distinguished by a confirmed caspase-4 or caspase-11 mediated activity.

induced caspase-11-mediated cytotoxicity in mouse macrophages whereas transfection of tetra-acylated LPS from bacteria grown at 37°C did not (34).

Apart from *Y. pestis*—a facultative intracellular Gram-negative bacterium, and causative agent of bubonic plague, two other *Yersinia* species: *Y. enterocolitis* and *Y. pseudotuberculosis* are pathogenic to humans and cause foodborne infections leading to gastroenteritis and septicemia. The structure of *Y. pseudotuberculosis* lipid A is the closest to *Y. pestis*, as far as the acylation pattern and Ara4N modification is concerned (192). The mechanism of Ara4N modification of lipid A *Y. pestis* is different and more complex than in other bacteria, and the Ara4N modification has been shown to play a crucial role in both transmission and survival of *Y. pestis* in its flea vector and in pathogenicity to human host (193, 194). Both or one phosphate groups of *Y. pseudotuberculosis* lipid A are covalently substituted by Ara4N, and the C_{14} acyloxyacyl chain at position 2 is esterified by palmitoylation (C_{16}) (**Figures 3**, **4**). *Y. enterocolitica* entails a shorter secondary acyl chain in position 2 (C_{12} or C_{14}). The functional role of inducible addition of Ara4N to the phosphate

residues of lipid A in *Yersinia* as well the propensity of its LPS to induce caspase-4/11-mediated pyroptosis has not yet been adequately studied. It has been recently reported that *Yersinia* infection induces caspase-8 mediated pyroptosis which proceeds through cleavage of Gasdermin D, although the involvement of LPS in this process has not been illustrated (195, 196). Temperature-regulated remodeling of lipid A in *Yersinia* substantially complicates studies of *Yersinia* LPS-induced pathogenicity *in vivo*, since tetraacylated lipid A variants produced by *Yersinia* at 37°C might act as TLR4 antagonists in human, simultaneously performing as weak TLR4 agonists in the mouse system. Indeed, the induction of the TLR4/MD-2/LPS—mediated protective responses in mice was responsible for reduced sensitivity of rodents to *Yersinia* infection and indicated a necessity for exploring *Yersinia* virulence factors in humanized mouse models (197).

LPS-Induced TLR4- and Caspase-4/11-Mediated Responses to Intracellular Gram-Negative Bacteria That Escape the Vacuole

Salmonella and *Legionella* are intracellular enteric pathogens known to cause gastroenteritis that can result in a systemic disease. These strains were shown to release specific bacterial antigens into the host cell cytosol and to trigger inflammasome activation within epithelial cells and macrophages (134, 198).

The disaccharide backbone of *L. pneumophila* lipid A contains two 2-diamino-2-dideoxyglucose (GlcN3N) residues instead of common 2-amino-2-deoxyglucose (GlcN) and the four amino groups are substituted by two long-chain (C_{16}) β-hydroxyacyl residues, one of which is further acylated by a branched C_{14} lipid chain, whereas the amino group in position 3′entails unusual long-chain (C_{25}) fatty acid (**Figure 4**) (199, 200). According to the well-establised structure-activity relationships, lipid A having acyl chains longer than C_{14} have usually much lower affinity to MD-2/TLR4 complex and to LBP than lipid As with a "classic" C_{12}-C_{14} acylation pattern. Indeed, the unusual structure of *L. pneumophila* lipid A accounted for the absence of TLR4-dependent endotoxic activity, due to a failure to interact with both TLR4/MD-2 complex and CD14 (201). Although *L. pneumophila* harbors a gene conferring resistance to cationic antimicrobial peptides, the modification of the phosphate groups of its lipid A with cationic residues has not yet been confirmed by structural analysis (202). Infection with *L. pneumophila* induced rapid caspase-11-mediated pyroptosis in mouse macrophages which has been accelerated by deletion of a specific effector supporting the integrity of *Legionella*-containing vacuole (135, 203).

Salmonella can alter the structure of its lipid A and remodel the content of the bacterial outer membrane by using several regulatory systems that govern phosphate group modifications crucial for resistance to CAMPs (204). Complex mechanisms which involve several regulatory proteins are implicated in the control of these processes. *S. enterica* serovar *Typhimurium* strains possess specific two-component systems that can add β-L-Ara4N to the lipid A phosphate groups or induce 3-*O*-deacylation at the proximal GlcN residue (**Figure 4**). The former modification promotes bacterial resistance to antimicrobial peptides, whereas the latter enhances the host recognition of lipid A by TLR4 (205). In particular, the PhoPQ two-component system regulates PagP-catalyses addition of a secondary palmitate residue at position 2 of the proximal GlcN moiety, and PagL-induces 3-*O*-deacylation, whereas PmrAB regulates the addition of Ara4N and phosphoethanolamine (206–208). Addition of Ara4N to lipid A inhibits the enzymatic activity of PagL which results in the synthesis of heptaacylated lipid A with Ara4N-modified phosphate groups (207). Heptaacylated *S. Typhimurium* lipid A has reduced hTLR4 activating potential, whereas its 3-*O*-deacylated counterpart (acylation pattern 4 + 2) belongs to the most powerful activators of hTLR4. Juxtaposed, in mouse TLR4 system heptaacylated *S. Typhimurium* LPS isolates induce robust IL-6 production in BMDMs (28).

Not surprisingly, *Salmonella* is able to activate the non-canonical capase-4 and caspase-11 dependent inflammasomes *via* intracellular LPS sensing (28, 133, 209). Whether and how the structure of *S. Typhimurium* lipid A and the presence of positively charged appendages at the phosphate groups influence caspase-4/11—LPS interaction remains to be determined.

Structural Peculiarities of *Francisella* LPS Accountable for Species-Specific Caspase-4/11 Activation and TLR4 Escape

The major lipid A of *Francisella* possesses a unique tetraacylated structure lacking the 4′-phosphate group and the 3′-acyl chain and containing an α-D-GalN residue at the glycosidically linked phosphate group (**Figure 4**) (210). All subspecies of genus *F. tularensis* (*Schu S4, holartica*, live vaccine strain LVS (attenuated type B strain), as well as a nonvirulent laboratory strain *F. novicida*) retain analogous lipid A modified with phosphodiester linked GalN at the glycosidic phosphate group P-1 (180, 211–213). The modification of *Francisella* lipid A phosphate residue with GalN is associated with augmented bacterial virulence and resistance to CAMPs, although the consequence of this modification for *Francisella* LPS/lipid A recognition by the innate immune system of the host has not yet been fully clarified. Mutants deficient in GalN modification were shown to induce activation of the innate immune responses in mice and to have weakened pathogenicity (214).

Francisella LPS escapes the recognition by both TLR4/MD-2 complex and LBP due to its hypoacylated structure which is assembled in a temperature-dependent manner (215), the inappropriate length of its four lipid chains (C_{18}-C_{16}) and the absence of a phosphate group at the distal GlcN moiety (P-4′) (216–218). Also, penta-acylated *Francisella lpxF* mutant failed to activate TLR4 which was explained by a non-optimal length of its acyloxy- and acyloxyacyl chains, although this mutant displayed attenuated virulence. Thus, the structural features of *Fransisella* lipid A do not comply with the well-establish requirements for the lipid A sensing by TLR4/MD-2. The lack of TLR4 stimulating potential of tetraacylated *Francisella* LPS is compensated by the

activation of NOD-like receptors (NLRs), and the adaptor molecule ASC which are involved in the regulation of caspase-1-mediated inflammasome activation (219, 220), as well as by the GBPs-promoted activation of AIM2 (Interferon-inducible protein AIM2 also known as "absent in melanoma 2") inflammasome in mice (221, 222). Thus, TLR4 signaling plays comparatively insignificant role in defence against *Francisella* infection or in protection after administration of live vaccine strain LVS in mice (223, 224).

The nonvirulent laboratory strain *F. novicida* as well as several other *Francisella* strains exhibit a truncated lipopolysaccharide form deprived of the polymeric O-antigen and the core sugars. A bifunctional Kdo-hydrolase, an LPS remodeling enzyme responsible for the synthesis of truncated LPS structures, has been identified in the inner membrane of *F. novicida* (155, 156, 225, 226). Thus, around 90% of *F. novicida* LPS consists of a solely tetraacylated lipid A modified with α-D-GalN at the glycosidic phosphate group P-1 (**Figure 4**) (227). A recent study postulated that tetra-acylated LPS/lipid A of *F. novicida* can be detected by caspase-4 upon LPS transfection in human monocyte-derived macrophages (151). Although transfected *F. novicida* LPS was 10-fold less potent compared to (transfected) *E. coli* LPS to induce activation of caspase-4, the innate immune responses to *F. novicida* LPS (i.e. IL-1β release and cell death) were fully caspase-4 driven. Importantly, these responses were essentially GBP2-dependent, highlighting a crucial role of guanylate binding proteins in facilitating recognition of cytosolic lipid A/LPS structures by inflammatory caspases. Similar to the TLR4/MD-2 complex, caspase-4 and caspase-11 exhibit species-specific differences in sensing underacylated lipid A which escapes caspase-11 recognition. The disparities in sensing structurally different lipid A molecules might be due to dissimilarities between the CARD domains of caspase-4 and caspase-11, which share 51% identity. Accordingly, tetraacylated *Fransicella* lipid A could not be detected by caspase-11 after *F. novicida* LPS transfection in mouse macrophages (34). However, transfection of penta-acylated *Francisella* LPS (*lpxF* mutant) that retains the phosphate moiety at position 4′ and the *N*-linked C_{16}-C_{18} fatty acid at position 3′ of the diglucosamine backbone (210, 228) resulted in a robust caspase-11 activation followed by pyroptosis. Thus, caspase-4 seem to be more receptive to the number of phosphate groups decorating the diglucosamine backbone of lipid A than to the acylation pattern, both in respect to the length and number of acyl chains. These data provide unequivocal evidence for the primary role of lipid A in driving the activation of caspase-4/11 and for apparently high affinity interaction of particular structural elements of lipid A with caspase-4/11 CARD.

Detection of Ara4N Modified *Burkholderia* LPS in the Cytosol of Mammalian Cells

The lipid A phosphate groups of clinical isolates of *Burkholderia* are substituted by an amino sugar β-L-Ara4N that is believed to reduce the net negative charge of the bacterial membrane and confer resistance to antibiotics. Host-adapted *Burkholderia* species cause severe pneumonia and systemic endotoxemia in cystic fibrosis and melioidosis patients which is linked to a potent cytokine-inducing capacity of *Burkholderia* LPS. Substitution of both phosphate groups with Ara4N was confirmed for ubiquitous environmental *Burkholderia* strain *B. pseudomallei*—an opportunistic facultative intracellular pathogen causing melioidosis in humans, as well as for a less virulent strain *B. thailandensis* (229–232). Notwithstanding its underacylated, heterogeneous tetra- and penta-acylated lipid A (233), LPS isolates from *B. cepacia, B. dolosa, B. cenocepacia, B. mallei*, and *B. multivorans* were reported to potently induce TLR4 mediated NF-κB signalling (234–237). The molecular background for a robust induction of the pro-inflammatory signaling by underacylated Ara4N-modified *Burkholderia* lipid A/LPS isolates is not yet clarified, particularly, because only hexaacylated lipid A patterns with fatty chain length 12-14 carbon atoms are known to elicit efficient TLR4-mediated responses (183). Whether the presence of Ara4N modification at both phosphate groups renders *Burkholderia* LPS to a strong TLR4 agonist is not yet proven, although mono-substitution of P-1 with Ara4N (1–O–P–β-L-Ara4N) did not significantly enhance the cytokine-inducing capacity of synthetic *Burkholderia* lipid A *in vitro* (182).

Since penta-acyl *Burkholderia* lipid A is structurally "unsuitable" to function as potent TLR4 agonist, it is rational to assume that other LPS sensing proteins could be responsible for recognition of *Burkholderia* LPS patterns. Indeed, it has been reported that caspase-11 activation by *B. thailandensis* and *B. pseudomallei* protected mice from lethal infection outcome (135). Newest reports demonstrated caspase-11 promoted cell death induced by wild type *B. thailandensis* (lipid A is penta-acylated, modified with Ara4N), whereas tetraacylated mutants lost the ability to activate TLR4 and had 30% lower capacity in induction of caspase-11 dependent pyroptosis (229). Infection with *B. thailandensis* triggered caspase-1 mediated release of IL-1β and IL-18 and caspase-11 induced activation of the NLRP3 inflammasome leading to death of infected lung epithelial cells by pyroptosis in mice (238, 239). Further studies revealed species-specific differences in the activation modalities of caspase-11 and caspase-4 by *B. thailandensis*. In rodents, the activation of caspase-1 provoked the release of IL-18 which, in turn, induced IFN-γ to prime caspase-11 activity, whereas caspase-4 transgenic mice did not necessitate IFN-γ priming upstream of caspase-4 to control the infection (240). The significance of caspase-4 activation implicated in the formation of autophagosomes was also confirmed for *B. cenocepacia* infected macrophages (241). Thus, TLR4 seem not to belong to the primary PRR able to sense penta-acylated *Burkholderia* LPS, rather this function is taken over by the cytosolic LPS receptors such as caspase-4/11 and GBP, or other not-yet-identified proteins.

CONCLUSION

The LPS induced TLR4-mediated signaling and caspase-4/11 activation drives the assembly of inflammasomes and contributes

to development of inflammation, thus, mounting a beneficial defensive host immune response against infectious challenge. Juxtapose, in the conditions of unresolved inflammation, TLR4 and caspase-4/11 activation can result in the amplified innate immune signaling, systemic overexpression of the pro-inflammatory mediators and pyroptosis which prompts the onset of sepsis syndrome and a fatal septic shock (15, 22, 38, 88).

Whereas LPS-induced TLR4 complex dimerization results in the expression and release of MyD88- and TRIF-dependent cytokines such as TNF-α and interleukins activation of inflammatory caspases-4/11 by LPS arbitrates the release of IL-1β and IL-18, Gasdermin-mediated pyroptosis and is associated with high lethality (242, 243). *In vivo*, activation of caspase-11 has been shown to provide protection against bacterial infections (135), but also to cause morbidity and mortality in a mouse model of endotoxemia (28, 34). Thus, inhibition of both TLR4 and caspase-4/11 activation could provide instruments to control acute inflammation and to reduce the LPS-induced toxic effects. Concurrently, coordinated induction of the pro-inflammatory signaling *via* TLR4 and/or caspase-4/11 pathways is believed to mount an advantageous immune activation aimed at protection from infection and management of chronic inflammation. Thus, modulation of the innate immune responses by application of TLR4 and/or caspase-4/11 agonists or partial agonists could be a promising therapeutic approach.

Although inflammatory caspases-4/11 can directly bind the lipid A moiety of LPS, the precise molecular mechanism and the structural basis for this recognition is not yet fully understood. Caspase-4/11 exhibit somewhat different requirements to the structure of LPS compared to the TLR4 complex and seem to be more receptive to the number of phosphate groups decorating the glucosamine backbone of lipid A than to the acylation pattern, especially in respect to number and length of acyl chains. LPS-remodeling resulting in decoration of the phosphate groups of lipid A with positively charged appendages has not yet been specifically addressed in biochemical and structural studies of caspase-4/11 ligand specificities, however, our analysis suggests that these modifications could be essential for LPS/lipid A sensing by inflammatory caspases and related proteins such as GBPs. Importantly, the well-known species-specific differences in sensing lipid A variants by human and mouse TLR4 also seem to apply for caspase-4/11. For instance, caspase-4 displays much broader reactivity in sensing underacylated LPS compared to caspase-11, which might have important consequences for translation *in vivo* studies to clinical trials. Although the structural basis of lipid A/LPS recognition by inflammatory caspases is not yet completely defined, and many questions still remain unanswered, further studies will certainly decipher particular molecular signatures conferring LPS responsiveness to caspase-4/11.

AUTHOR CONTRIBUTIONS

AZ and HH have written the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Financial support from Austrian Science Fund FWF grants P-28915 and P-32397 is gratefully acknowledged.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 30 November 2020
doi: 10.3389/fimmu.2020.573646

Hypoxia Shapes Autophagy in LPS-Activated Dendritic Cells

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During their lifespan, dendritic cells (DCs) are exposed to different pO_2 levels that affect their differentiation and functions. Autophagy is one of the adaptive responses to hypoxia with important implications for cell survival. While the autophagic machinery in DCs was shown to impact signaling of TLRs, its regulation by the MD-2/TLR4 ligand LPS is still unclear. The aim of this study was to evaluate whether LPS can induce autophagy in DCs exposed to either aerobic or hypoxic conditions. Using human monocyte-derived DCs and the combination of immunofluorescence confocal analysis, measure of mitochondrial membrane potential, Western blotting, and RT-qPCR, we showed that the ability of LPS to modulate autophagy was strictly dependent upon pO_2 levels. Indeed, LPS inhibited autophagy in aerobic conditions whereas the autophagic process was induced in a hypoxic environment. Under hypoxia, LPS treatment caused a significant increase of functional lysosomes, LC3B and Atg protein upregulation, and reduction of SQSTM1/p62 protein levels. This selective regulation was accompanied by activation of signalling pathways and expression of cytokines typically associated with DC survival. Bafilomycin A1 and chloroquine, which are recognized as autophagic inhibitors, confirmed the induction of autophagy by LPS under hypoxia and its impact on DC survival. In conclusion, our results show that autophagy represents one of the mechanisms by which the activation of the MD-2/TLR4 ligand LPS promotes DC survival under hypoxic conditions.

Keywords: hypoxia, dendritic cell, autophagy, (macroautophagy), hypoxia-inducible factor (HIF)-1α, lipopolysaccharide (LPS)

OPEN ACCESS

Edited by:
Francesco Peri, University of Milano-Bicocca, Italy

Reviewed by:
Marco A. Cassatella, University of Verona, Italy
Jerrold Weiss, The University of Iowa, United States

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *17 June 2020*
Accepted: *28 October 2020*
Published: *30 November 2020*

Citation:
Monaci S, Aldinucci C, Rossi D, Giuntini G, Filippi I, Ulivieri C, Marotta G, Sozzani S, Carraro F and Naldini A (2020) Hypoxia Shapes Autophagy in LPS-Activated Dendritic Cells. Front. Immunol. 11:573646. doi: 10.3389/fimmu.2020.573646

INTRODUCTION

Tissue hypoxia occurs in many physiological and pathological conditions, including lymphoid organs, inflammation, and cancer (1, 2). With regard to lymphoid tissues, oxygen tensions are lower than would be expected from the pO_2 of inspired air (159 mm Hg or a concentration of 21%, at sea level), of arterial and venous blood, being about 97–100 mmHg and 40 mm Hg, respectively (3). Indeed, the pO_2 is ∼10 mm Hg in the thymus (4), and the bone marrow and lymph nodes present

hypoxic areas, represented by immunological niches (5). Dendritic cells (DCs) are the most effective antigen presenting cells and, based on their differentiation and maturation states, they can be represented as immature and mature (6). Immature DCs circulate through tissues and lymphoid organs, while mature DCs are deputed to initiate the innate and adaptive immune response (7). Thus, DCs, as well as other immune cells, experience different physiological pO_2 levels ranging from 10 mmHg to 75–100 mmHg (8). When DCs patrol inflamed and cancerous tissues, they are exposed to further lower oxygen tensions, sometimes below 10 mm Hg or less (9), and their functions may be profoundly affected (10). Indeed, the pO_2 has been found to be important for DC differentiation and especially during final maturation by the MD-2/TLR4 ligand LPS (11, 12). Many of the adaptive responses to hypoxia are mediated by a family of transcription factors known as hypoxia inducible factors (HIFs) (13) and include modulation of glycolytic metabolism, cell survival and migration, pro-angiogenic cytokines, and pro- and anti-apoptotic molecules (14, 15). The HIF dimeric complex is comprised by the constitutively expressed HIF-1β subunit, which associates with one of two hypoxia inducible α subunits, HIF-1α or HIF-2α (16). HIF-1α is expressed ubiquitously and is involved in the inflammatory response in both hypoxic and normoxic conditions (3). Autophagy is one of the adaptive cellular responses to hypoxia. Specific HIF targets in autophagy include BNIP3, a Bcl-2 superfamily member, which modulates cell survival (17). Indeed, cells rely on autophagy to survive diverse cellular insults such as hypoxia, nutrient depletion, accumulation of protein aggregates, damaged mitochondria, or intracellular bacteria (18). Autophagy is a complex self-degradative process that involves several key steps (19).

During macroautophagy (hereafter referred to as autophagy), cytoplasmic material, including organelles, protein aggregates, or bacteria, is sequestered into double membrane–coated autophagosomes. The formation of phagophore is controlled by Beclin-1/VPS34 in response to various types of cellular stress stimuli. Subsequently, the Atg5–Atg12 conjugation is followed by its interaction with Atg16L and multimerization at the phagophore level. LC3B is then processed and inserted into the extending phagophore membrane. The lipidated form of LC3B, LC3B-II, interacts with SQSTM1/p62, a multi-functional adaptor molecule that promotes turnover of poly-ubiquitinated protein aggregates. This is followed by the fusion with endosomes and lysosomes to form autolysosomes where lysosomal degradation can occur (18). An increasing number of recent studies has characterized the involvement of autophagy in DC functions in various physiological and pathological contexts (20). Recently, it has been shown that signaling through TLRs can affect the autophagic process (21). However, while autophagy was shown to impact downstream signaling through some TLRs (TLR4, TLR7, and TLR8), its regulation by TLRs is less clear (20, 22). We have previously shown that hypoxia affected immune cell survival (23, 24). More interestingly, we have previously reported that hypoxia promoted a proapoptotic program in immature DCs (25). The aim of the present study was to investigate whether LPS may activate an autophagic program in hypoxic DCs. We here report that while under normoxia LPS inhibited autophagy, under hypoxia LPS induces increased functional lysosomes, along with modulation of the adapter protein SQSTM1/p62, of LC3B and the protein levels of Atgs, which are all known as markers of autophagy (26–28). All these observations, which were associated with the activation of pro-survival signaling pathways and cytokine expression, were abolished by treatment with two autophagy inhibitors: Bafilomycin (Baf A1) and chloroquine (CQ). The first one is a selective inhibitor of vacuolar-type H^+ ATPase (V-ATPase) that blocks the autophagic flux by inhibiting autolysosome acidification and autophagosome–lysosome fusion (29). The second one mainly inhibits autophagy by impairing autophagosome fusion with lysosomes rather than by affecting the acidity and/or degradative activity of this organelle (30). The overall results indicate that the ability of LPS to regulate DC autophagy is tightly related to tissue localization, physio-pathological conditions, and relative local oxygen tensions.

MATERIALS AND METHODS

Reagents

RPMI 1640, fetal bovine serum (FBS), penicillin/streptomycin, and L-Glutamine were purchased from Euroclone, Devon, UK. Fycoll was purchased from Cederlane Labs and Percoll from Amersham Bioscience, Pittsburgh, PA, USA. Recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-13 (IL-13) were purchased from ProSpec TechnoGene, East Brunswick, NJ, USA. All reagents contained <0.125 endotoxin units/ml, as checked by the Limulus Amebocyte Lysate assay (Cambrex, East Rutherford, NJ, USA). LPS from Escherichia coli strain 055:B5 was obtained from Sigma–Aldrich, Milano, Italy. Baf 1A was purchased from VWR Chemicals BDH Milano, Italy, and CQ was obtained by Enzo Life Sciences, Plymouth Meeting, PA, USA.

Human Monocyte-Derived DC Preparation and Culture Conditions

Human monocyte-derived DCs were generated as previously described (25). Briefly, highly enriched blood monocytes (>95% CD14) were obtained from anonymous buffy coats (through the courtesy of the South-East Tuscany Blood Establishment, AOUS, Siena) by Fycoll and Percoll gradient centrifugations. Monocytes were differentiated into immature DCs (>90% CD1a and < 5% CD14) upon 6 days culture (in RPMI 1640, supplemented with 10% FBS) with 50 ng/ml GM-CSF and 20 ng/ml IL-13, as previously reported (31). Immature DCs were then induced to terminal differentiation with LPS (100 ng/ml) for 24 h and cultured under either normoxia (atmospheric pO_2 levels: 21% O_2, 5% CO_2, and 74% N_2 corresponding to a pO_2 ~ 140 mmHg) or hypoxia (2% O_2, 5% CO_2, and 94% N_2, corresponding to a pO_2 ~ 14 mmHg) by the workstation InVIVO O_2 400 (Ruskinn, Pencoed, UK) as previously described (32). In some experiments, cells were treated with Baf A1 or CQ. Briefly, 100 nM Baf A1 or

100 μM CQ were added directly to the culture medium 6 h before the end of treatment (LPS under hypoxia). At the indicated times, cells were harvested for further analysis, as described below.

Cell Viability and Detection of Mitochondrial Membrane Potential

Cell viability was analyzed by Trypan Blue exclusion assay by Bio-Rad TC20™ automated cell counter (Biorad laboratories, Bio-Rad, Hercules, CA, USA), which provides a total cell count, and it assesses cell viability using a digital image analysis algorithm (33). Evaluation of mitochondrial membrane potential (ΔΨm) was performed by a fluorogenic lipophilic cation (JC-1; Sigma-Aldrich), according to the manufacturer's protocol as previously described (34). In cells with hyperpolarized mitochondrial membranes, JC-1 spontaneously forms complexes (J-aggregates) emitting red fluorescence. Fluorescence was detected by using microplate reader Fluoroskan Ascent (Thermolabsystem, Helsinki, Finland) protected from light. The ΔΨm was determined by the ratio between the red (~590 nm) and the green (~529 nm) fluorescent emission.

Immunofluorescence Staining and Confocal Microscope Analysis

DCs were plated on sterile chamber slides (Nunc Lab-Tek) cultured and treated as indicated above. At the indicated time, cells were fixed in cold methanol at −20°C for 10 min and permeabilized with HEPES/Triton for 3 min. Then they were washed with PBS-BSA 0.2% and blocked with 10% goat serum. Cells were incubated with the primary antibody diluted in PBS-BSA 2% anti-HIF-1α (Thermo scientific, Rockford, USA, 1:200 Cat.n° MA-516), LC3B (Cell Signaling Technologies, Danvers, MA, 1:200 Cat.n° 2775S), SQSTM1/p62 (Cell Signaling Technologies, Danvers, MA.1:100 Cat.n°7695), or Atg12 (GeneTex, USA,1:1000 Cat.n° GTX629815) overnight at 4°C in a humidified chamber. The following day, cells were incubated with Cy2 (green) (Jekson Laboratories, 1:5000 Cat.n°711-225-152) or Cy3 (red) (Jekson Laboratories, 1:5000 Cat.n°111-166-045) conjugated secondary antibodies for 1 h at room temperature. Nuclei were visualized by DAPI (Calbiochem, San Diego, CA 1:10000 Cat.n° D9542-1MG). Coverslips were mounted on slides and imaged with LSM-510 META confocal microscope (Carl Zeiss, Oberkochen, Germany). The fluorescence intensity was determined by ImageJ software as the mean pixel density of staining area in each cell. After subtraction of background, the intensity values were shown as arbitrary units relative to control: CTCF (corrected total cell fluorescence) = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

Lysotracker Staining

Cells were plated on 8-well coverglass slide (Sarstedt, Germany Cat.n° 94 6190802) and treated with LPS under normoxic or hypoxic conditions. For Baf A1 and CQ treatment, the compounds were added at a concentration of 100 nM and 100 μM, respectively, 6 h before the end of the experiment. After 24 h, cells were labeled by Lyso-ID Green Detection Kit (Enzo Life Sciences, Plymouth Meeting, PA, USA), and nuclear staining was performed by using DAPI. Cells were analyzed by confocal microscope and the fluorescence intensity was determined by ImageJ software, as described above.

Immunoblotting and Antibodies

DCs were lysed directly in tissue culture plates and processed, as previously described (33). Protein concentration was determined using Micro BCA Protein Assay Reagent kit (Rockford, USA) and equal amounts of total proteins were loaded onto SDS-PAGE gel. After transferring, PVDF membranes were incubated with the specific primary antibodies over night at 4°C: HIF-1α (BD Biosciences, San Jose, CA, 1:200 Cat.n° 610958), Bax (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n° 2772), Bcl-xl (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°2764), LC3B (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n° 2775), Beclin-1 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°3495), Atg3 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°3415), Atg5 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.8540), Atg7 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°8558), Atg12 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°4180), phNFKB (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°3033), phAkt (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°4058), php38 MAP Kinase (Thr180/tyr182) (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°9211), php44/42 MAP Kinase (Thr202/Tyr204) (Cell Signaling Technologies, Danvers, MA1:1000 Cat.n°9101), PARP (Cell Signaling Technologies, Danvers, MA1:1000 Cat.n°9542), and β-actin (Sigma-Aldrich, 1:50000 Cat.n° A3854). Anti-mouse IgG HRP (Cell Signaling Technologies, Danvers, MA, 1;2000 Cat.n°7076) and anti-rabbit IgG-HRP (Cell Signaling Technologies, Danvers, MA, 1;2000 Cat.n°7074) were used as secondary antibodies (Cell Signaling Technologies, Danvers, MA). Detection of images was performed by ChemiDoc™ MP System (Bio-Rad, Hercules, CA). The intensity of the band was quantified using Image Lab software (Bio-Rad).

RNA Extraction and RT-qPCR

Total RNA was extracted using EuroGOLD™Trifast reagent (Euroclone, Devon, UK) and cDNA was synthesized using iScript™cDNA Synthesis Kit (Bio-Rad Laboratories). RT-qPCR was performed using iTaq™SYBR Green Supermix (Bio-Rad Laboratories). mRNA levels of BNIP3, VEGF-A, IL-1β, IL-18, TNF-α, IL-6, IL-10, and TGF-β were determined by MiniOPTICON™ System (Bio-Rad Laboratories) and analyzed on an iQ5™ Optical System Software (Bio-Rad Laboratories). Relative quantification was done by using the $2^{-\Delta\Delta CT}$ method (35) and β-actin as housekeeping gene. Primers were validated as previously described (36).

Statistical Analysis

The data are presented as the mean ± SEM of at least 3 independent experiments. Statistical analyses were performed

with Graph-Pad Prism (San Diego, CA, USA). Analysis of variance (ANOVA) and unpaired two-tailed Student's t test were used to test for significant numerical differences among the group. Difference of $p \leq 0.05$ was considered to be statistically significant ($^*p \leq 0.05$; $^{**}p \leq 0.01$).

RESULTS

Hypoxia Affects Autophagy in DCs

In previous reports, we have shown that hypoxia affects DC cell death through the activation of a pro-apoptotic program that was antagonized by LPS (25). Thus, to investigate whether such a protection was associated with autophagy, we exposed human monocyte-derived DCs to a pO_2 of 140 mmHg (normoxia) or 14 mmHg (hypoxia), either in the presence or not of the MD-2/TLR4 ligand LPS. **Figure 1A** shows that hypoxia significantly enhanced HIF-1α at protein level, especially in the presence of LPS, as observed by confocal microscopy and Western blot analysis. This was paralleled by an increased expression of genes associated with hypoxia, including VEGF-A and BNIP3 (**Figure 1B**). The latter is strictly controlled at transcriptional level by HIF-1α and it is one of the main regulators of autophagy in several cell types upon exposure to hypoxia (17). Since the alterations of lysosome function and of their reformation process is tightly related to autophagy, we then analyzed the amount of acidic/functional lysosomes (37). As shown in **Figure 1C**, confocal analysis revealed a significant increase of acidic vesicles in LPS-treated DCs under hypoxic conditions, as compared to the relative normoxic treatment. We next investigated whether the promotion of autophagy was associated with a modulation of apoptosis. Specifically, we monitored the loss of mitochondrial transmembrane potential ($\Delta\Psi_M$), which is one of the major events associated with apoptosis (38). **Figure 1D** shows that, under hypoxia, LPS treatment resulted in a significantly higher $\Delta\Psi_M$. This was paralleled by an increased number of alive cells, as detected by cell viability assays, the reduction of the pro-apoptotic protein Bax, and enhancement of the antiapoptotic molecule Bcl-xl, thus confirming the protective role of LPS against apoptosis in hypoxic DCs.

Hypoxia Modulates Autophagy in DCs

To investigate more deeply how hypoxia may affect autophagy in LPS-treated DCs, we next analyzed the protein levels of two key autophagic markers, LC3B and SQSTM1/p62. The localization and aggregation of LC3B, after its conversion to LC3B-II, onto the membranes of autophagosomes is an index of the autophagic flux (39). In **Figure 2A**, confocal immunofluorescent analysis shows that under normoxic conditions the effects of LPS on LC3B-II was not significant. However, LC3B-II was significantly enhanced in LPS-treated DCs under hypoxia. Accordingly, the exposure to hypoxia of DCs in the presence of LPS resulted in a reduced protein level of SQSTM1/p62, indicating a significant increase in autophagy. Indeed, after delivering the autophagic substrates to autophagosomes, SQSTM1/p62 is degraded and its protein level is decreased when autophagy is induced (40). In contrast, LPS treatment under normoxia resulted in accumulation of SQSTM1/p62, suggesting that the induction of the autophagic process occurred only under hypoxic conditions. To corroborate the hypothesis that hypoxia induces autophagy in LPS-treated DCs we next analyzed LC3B-II/LC3B-I ratio by Western blot. **Figure 2B** shows that this ratio was significantly increased in hypoxic LPS-treated DCs, as compared with normoxia. Accordingly, with the immunofluorescent confocal analysis, the protein level of SQSTM1/p62 was significantly enhanced upon LPS treatment under normoxia, while it was reduced under hypoxia, indicating a pro-autophagic process only in the latter condition. The fact that LPS-treated DCs were more prone to autophagy under hypoxia was further confirmed by a significant increase of another marker of autophagy, Beclin-1, which is required for the autophagic flux induction (41).

Hypoxia Modulates the Atg Protein Levels in DCs

Since several reports indicate the involvement of Atgs in the functional aspects of DC maturation, we next analyzed the effect of hypoxia on the level of several Atg proteins in DCs treated with LPS. We first analyzed the protein level of Atg12 by confocal immunofluorescent analysis. Atg12, along with Atg5, upon binding to Atg16, is essential for autophagosome elongation and it is downstream of Beclin-1 (28). As shown in **Figure 3A**, Atg12 was apparently reduced upon LPS treatment under normoxic conditions. However, when DCs were exposed to hypoxia, LPS treatment resulted in a significant increase of Atg12 protein level, when compared with normoxic LPS-treated DCs. These results were confirmed by Western blot analysis (**Figure 3B**). Indeed, antibodies against Atg12-Atg5 complex and to Atg5 alone revealed a significant reduction in LPS-treated DCs under normoxia, while under hypoxia LPS treatment resulted in a significant increase in the protein levels of both Atgs. Similar results were obtained also for other Atg proteins, including Atg3 and Atg7, which are crucial for autophagosome formation (42). Indeed, under hypoxia, LPS treatment resulted in a significant increase in the protein levels of both Atgs, as compared with the relative normoxic controls. Even in these cases, when DCs were treated with LPS under normoxia, we observed a significant decrease in the Atgs that we had analyzed. Of note, in all cases Atg levels of LPS-treated cells were lower than in untreated cells. Indeed, we cannot exclude that, since autophagy is a degradative process, fewer levels of Atgs in hypoxic LPS-treated cells may be reduced by the turnover that is associated with autophagy.

Hypoxia Affects the Expression of Several Signaling Molecules and Cytokines Associated With DC Autophagy, Cell Survival, and Activation

To further analyze the impact of hypoxia on LPS-treated DCs, we next analyzed the activation of several signaling pathways associated with DC survival and, more recently, with autophagy (43). As shown in **Figure 4A**, LPS treatment

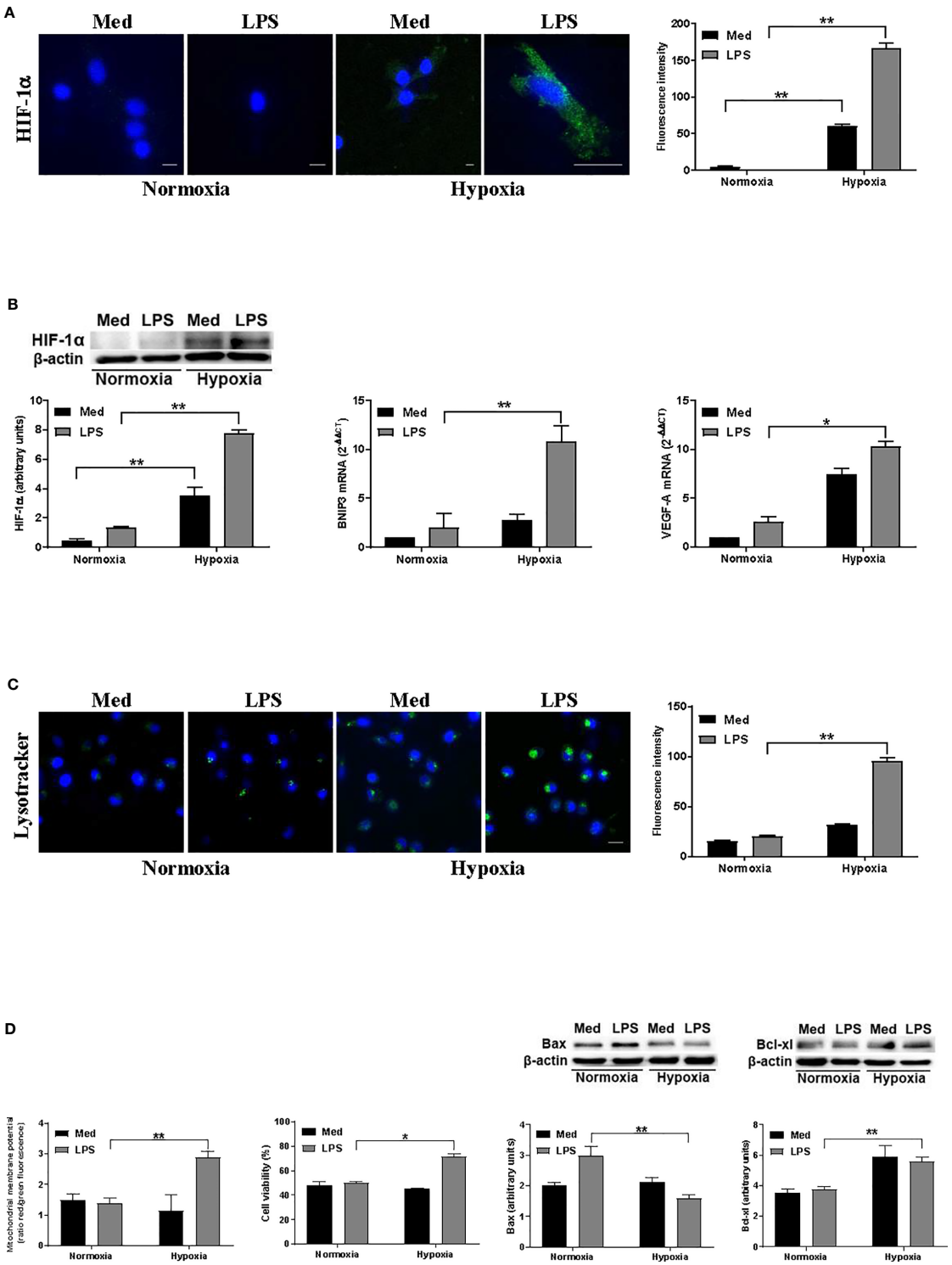

FIGURE 1 | Hypoxia affects autophagy and DC survival **(A)**. HIF-1α protein levels after 24 h exposure to normoxia and hypoxia as determined by confocal microscopy analysis (Scale bar: 8 μm; 15 μm only for LPS in hypoxia) **(B)**. HIF-1α protein levels as determined by Western blotting (blot shown is representative of four independent experiments and β-actin was used as loading control) and RT-qPCR analysis of BNIP3 and VEGF-A mRNA expression (β-actin was used as a housekeeping gene) **(C)**. Detection of acidic/lysosomal compartments by Lysotracker and confocal analysis (Scale bar: 15 μm) **(D)**. Mitochondrial membrane potential analysis by JC-1 dye, DC viability, and Western blot analysis of Bax and Bcl-xl protein levels, under normoxic or hypoxic conditions at 48 h. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively; $n = 4$).

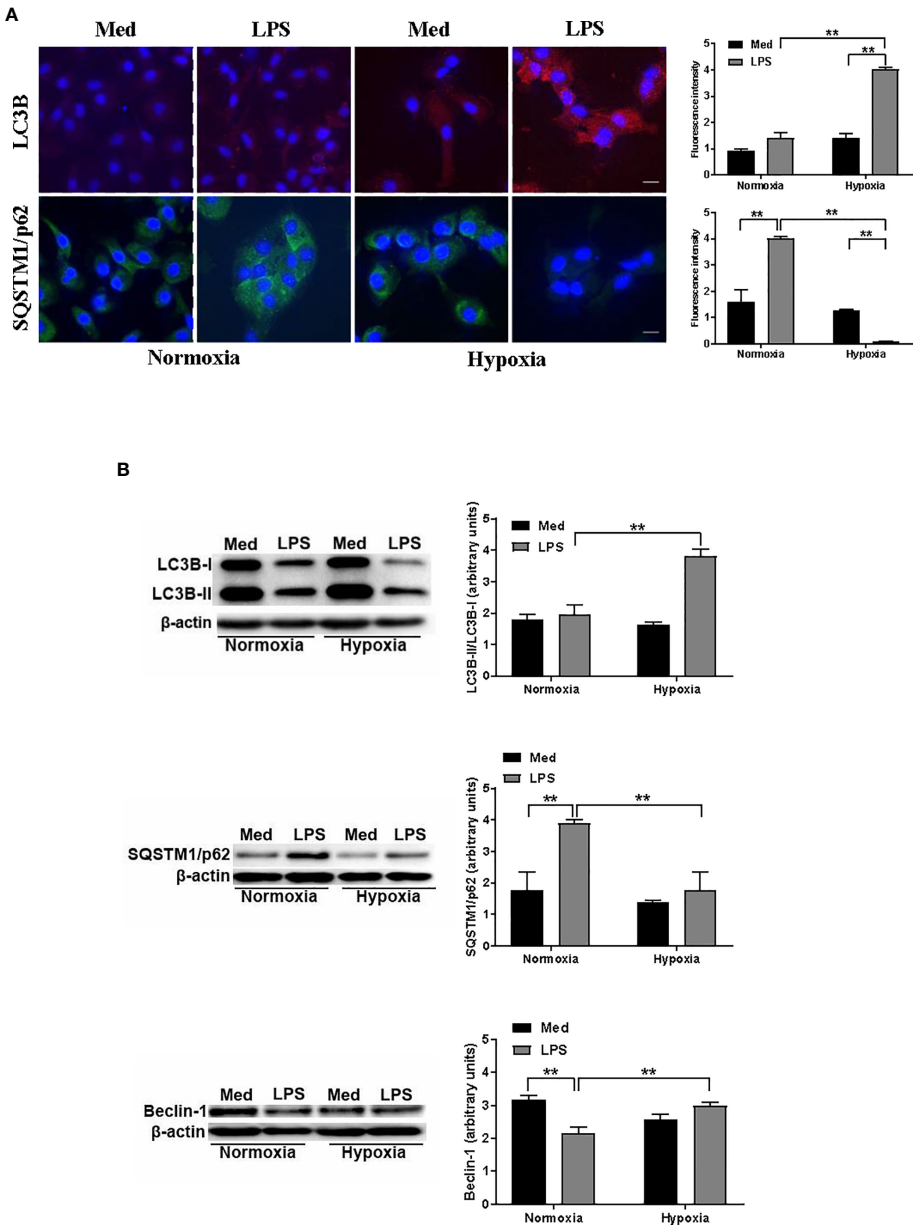

FIGURE 2 | Hypoxia modulates autophagy in DCs **(A)**. LC3B and SQSTM1/p62 protein levels after 48 h exposure to normoxia and hypoxia in DCs stimulated with LPS as determined by confocal microscopy analysis (Scale bar: 15 μm) **(B)**. LC3B-II/LC3B-I, SQSTM1/p62, and Beclin-1 protein levels as determined by western blotting (blot shown is representative of three independent experiments and β-actin was used as loading control). ** indicate statistically significant differences ($p \leq 0.01$; n = 4).

resulted in an increased phosphorylation of Erk in normoxic conditions. However, when DCs were treated with LPS under hypoxia, we observed a significant enhancement of Erk phosphorylation as compared with LPS-treated DCs in aerobic conditions. We observed a similar pattern also for Akt that, along with Erk, is essential to inhibit DC apoptosis and to promote DC survival (44). In addition, LPS treatment under hypoxia resulted in an increased phosphorylation of NFkB and p38. Both pathways are involved in DC maturation and activation, including the expression of several cytokines, which are released by DCs (45, 46). Indeed, LPS-treated DCs expressed significantly higher amounts of IL-1β, IL-18, TNF-α, IL-6, IL-10, and TGF-β mRNA, as measured by RT-qPCR, in both normoxic and hypoxic conditions (**Figure 4B**). However, when DCs were treated with LPS under hypoxic conditions, the expression of cytokine mRNA was significantly higher as compared with normoxia. Of interest, the pattern of expression was similar for all the cytokines that were analyzed, except for TGF-β. Indeed,

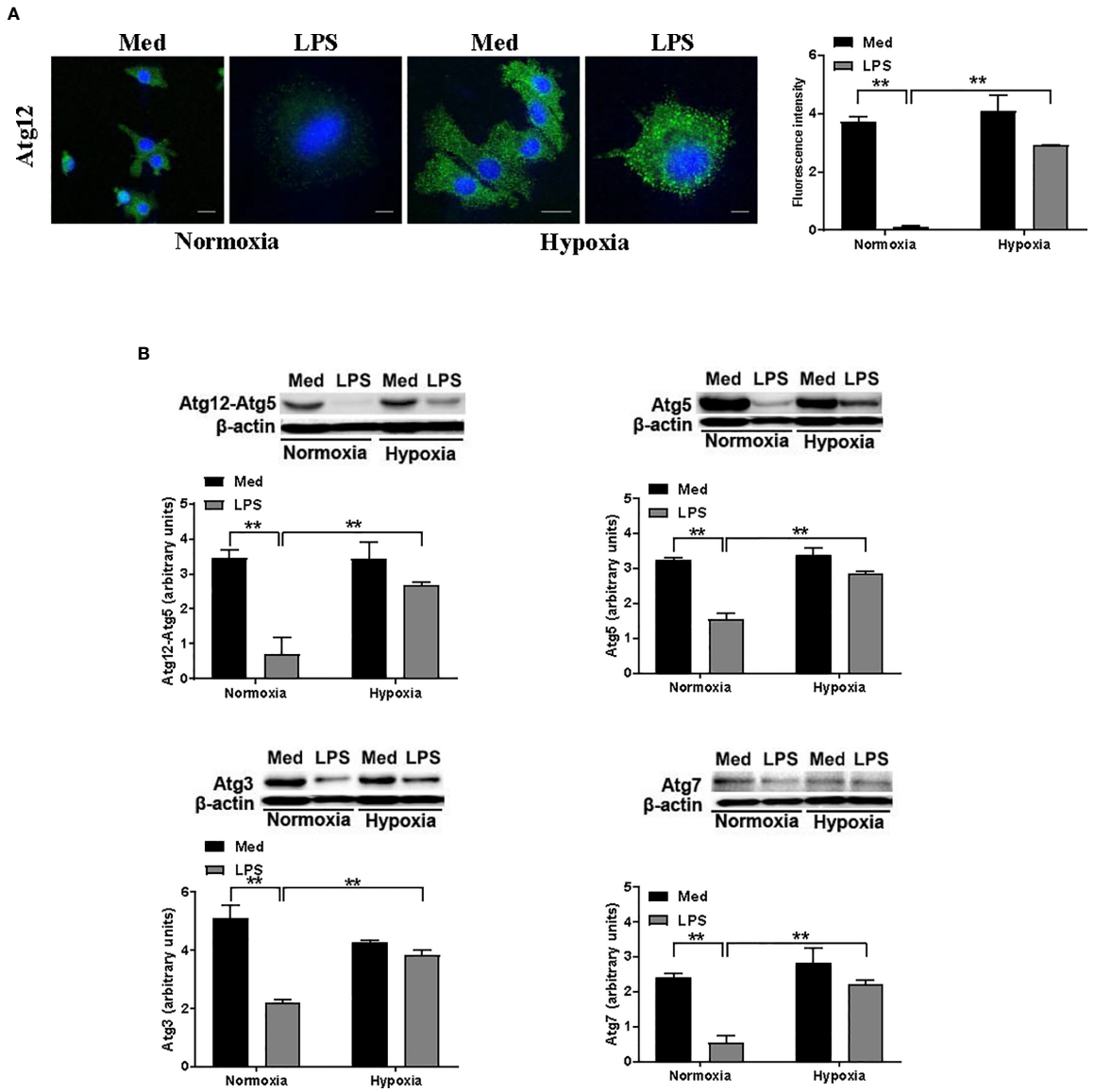

FIGURE 3 | Hypoxia modifies the levels of Atg proteins in DCs **(A)**. Atg12 protein levels after 24 h exposure to normoxia and hypoxia in the presence of LPS, as determined by confocal microscopy analysis (Scale bar: 15 μm for medium, normoxia, and hypoxia, and 4 μm for LPS, in normoxia and hypoxia) **(B)**. Atg12-Atg5, Atg5, Atg3, and Atg7 protein levels as determined by western blot analysis (β-actin was used as loading control). The blots are representative of three independent experiments. ** indicate statistically significant differences ($p \leq 0.01$; $n = 3$).

LPS treatment resulted in a significantly lower expression of TGF-β in both normoxic and hypoxic treatment. This observation, however, was in line with other previous reports (47). The overall results indicate that hypoxia positively regulates DC responses that are associated with their survival, maturation, and functional activation.

Autophagy Is Involved for LPS-Treated DC Survival

Due to the above observations, we decided to further investigate the potential mechanism by which hypoxia may affect LPS-treated DCs in terms of autophagy. To this end, we evaluated the effects of Baf A1 and CQ by confocal microscopy analysis using the pH-sensitive lysosomal dye LysoTracker in hypoxic LPS-treated DCs. As expected from an inhibitor of the vacuolar proton pump (29), Baf A1 treatment decreased the acidity of lysosomes as it led to a rapid decrease of fluorescence (**Figure 5A**). CQ, in contrast, but in agreement with previous reports, did not decrease LysoTracker-positive structures, which tended to be much larger after CQ treatment compared to control or Baf A1 treatment (30).

Autophagy inhibition by Baf A1 resulted in a higher protein level of SQSTM1/p62 and of LC3B-II/LC3B-I (**Figure 5B**). However, and in agreement with previous reports, the

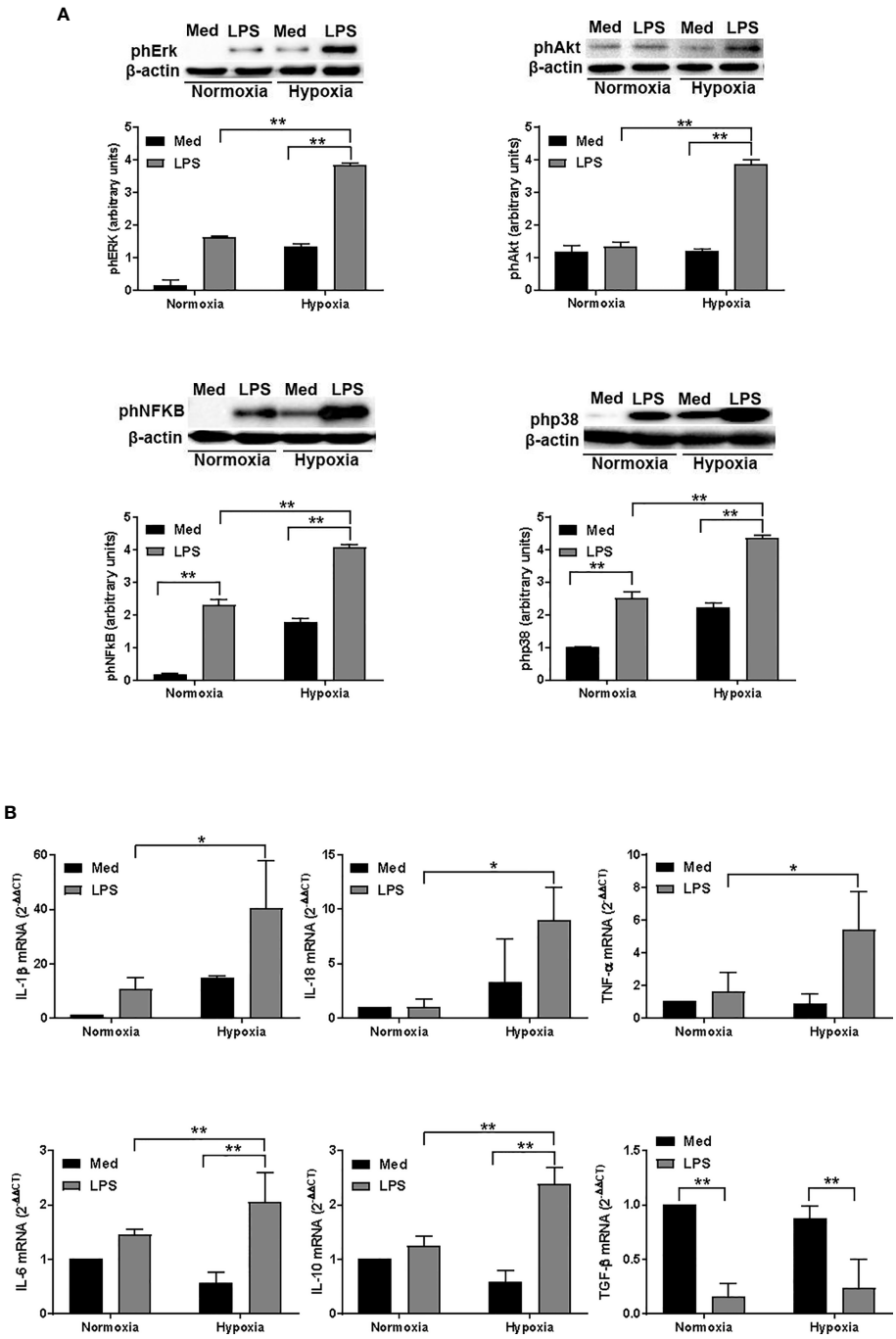

FIGURE 4 | Hypoxia affects signalling pathways and and cytokine expression in DCs **(A)**. Erk, Akt, NFκB, and p38 phosphorylation in DCs after a 24-hour exposure to normoxia and hypoxia with or without LPS, as determined by western blotting (the blots are representative of three independent experiments and β-actin was used as loading control) and **(B)** RT-qPCR analysis of IL-1β, IL-18, TNF-α, IL-6, IL-10, and TGF-β mRNA expression (β-actin was used as housekeeping gene) at the end of 24 h treatment. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively; $n = 4$).

increased amounts of LC3B-II can correlate with either an induction of autophagy or a block at the late steps of this pathway, i.e., autophagosome fusion with lysosomes and/or lysosomal degradation (30). Similarly, CQ treatment resulted in a significant enhancement of LC3B-II/LC3B-I ratio. However, CQ reduced the protein level of SQSTM1/p62, probably due to the fact that CQ does not substantially decrease lysosomal activity (30).

To further test whether Baf A1 and CQ affected the autophagic process in hypoxic LPS-activated DCs, we

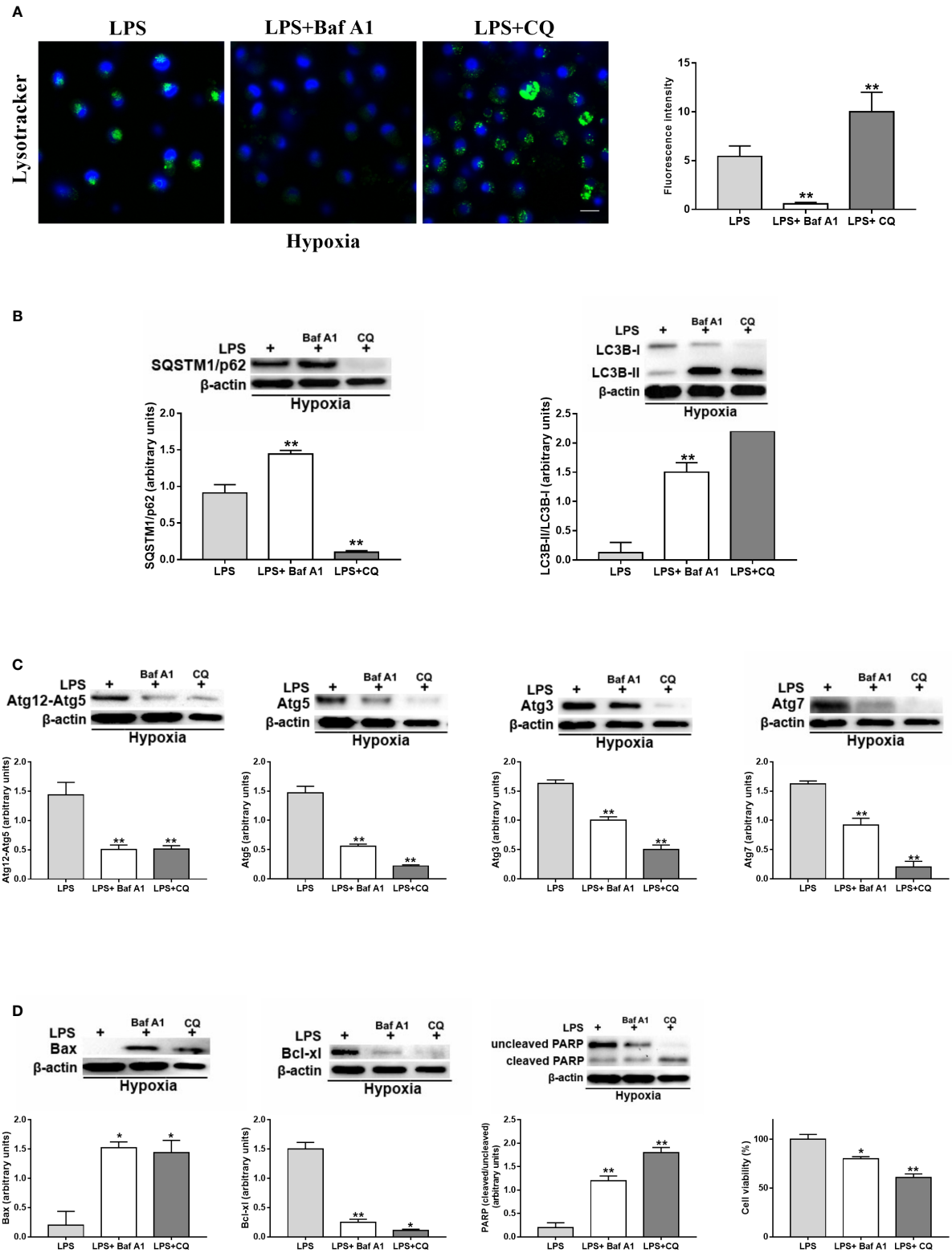

FIGURE 5 | Autophagy is involved in the survival of activated DCs **(A)**. Detection of acidic/lysosomal compartments by Lysotracker and confocal analysis (Scale bar: 15 μm) in DC stimulated with LPS under hypoxia for 24 h with Baf A1 or CQ **(B)**. SQSTM1/p62 and LC3B-II/LC3B-I **(C)**, Atg12, Atg5, Atg3, and Atg7 and **(D)** Bax, Bcl-xl, PARP protein levels as determined by western blotting (blot is representative of three independent experiments and β-actin was used as loading control) and cell viability. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively; n = 4).

evaluated the protein levels of several Atgs, which are involved in different steps of the autophagic process (20). **Figure 5C** clearly shows that both Baf A1 and CQ treatments reduced the protein levels of Atg12, Atg5, Atg7, and Atg3.

Finally, as shown in **Figure 5D**, Baf A1 and CQ enhanced the protein level of the pro-apoptotic protein Bax and reduced that of Bcl-xl, which is an anti-apoptotic and pro-survival protein (48). This was associated with a significant increase of PARP cleavage, which is another marker of apoptosis (49). More importantly, the inhibition of autophagy by Baf A1 and CQ resulted in a significant decrease of alive cells.

Thus, the overall results indicate that hypoxia enhanced autophagy in DCs activated by LPS, leading to the promotion of DC survival and activation.

DISCUSSION

In this paper we described for the first time how hypoxia may affect DC autophagy, with particular regard to DC final maturation induced by the MD-2/TLR4 ligand LPS (50).

We and others have previously shown that hypoxia significantly affects T cell and DC functions, with important physiological and pathological implications in the immune response (32, 51–53). Concerning DCs, we have previously shown that hypoxia promotes a pro-apoptotic program in immature DCs (25). However, in the same study, when hypoxic DCs were maturated with LPS, we did not observe an increase in cell death, while HIF-1α accumulation and BNIP3 expression were still significantly upregulated. The purpose of the present manuscript was to investigate whether LPS-treated DCs may undergo a pro-autophagic program. Accordingly, we here report that human derived DCs, treated with LPS, were more susceptible to autophagy under hypoxia (pO_2 = 14 mmHg, 2%O_2) as compared with the aerobic condition (pO_2 = 140 mmHg, 21%O_2). We should underline that the pO_2, which was employed in our study, is similar to the microenvironmental pO_2 present in lymphoid tissues (54), in inflammation (5), and in solid tumors (55). Such an effect was evident with regard to the number of acidic/functional lysosomes and to the protein levels of molecules associated with autophagy (18). Previous reports indicate that hypoxia promotes autophagy resulting in prolonged cell survival (56, 57). Indeed, the expression of BNIP3, which is transcriptionally regulated by HIF, is tightly related to autophagy (17). Accordingly, we here report that hypoxia enhances BNIP3 mRNA expression along with the protein level of Beclin-1, which is an important marker of the early autophagic program (41). Autophagy is a very complex process that has been associated with DC functions (20). While it is widely accepted that autophagy activates TLR4 downstream signaling, the effect of LPS activation on autophagy is still a matter of debate. Previous studies, which were all conducted under aerobic conditions, reported that TLRs stimulation either promoted (21) or reduced the autophagy flux, in particular, upon the stimulation of primary DCs by LPS (58). Our results are in line with the latter report, since we observed that under normoxic conditions the protein level of SQSTM1/p62 was significantly enhanced by LPS treatment. Furthermore, we did not observe a significant increase of LC3B-II, a phenomenon normally expected during the autophagic process. However, when LPS-treated DCs were cultured under hypoxic conditions, we observed a significant reduction of SQSTM1/p62 protein level, paralleled by an increase of LC3B-II protein level. Thus, the induction of the autophagic process in DCs is strictly related to their maturation state and to the microenvironment in which they localize. The fact that the differentiation and maturation stages are critical for DC autophagy was clearly suggested by several reports (59). Proteins implicated in the elongation and formation of autophagosomes are differently regulated during DC maturation steps (20). The elongation of the phagophore, along with autophagosome formation, is controlled by a series of ubiquitin-like conjugation reactions catalyzed by the E1-like enzyme Atg7 and E2-like enzyme Atg3 (28). Atg7 mediates the binding of Atg12 and Atg5. Of interest, Atg7, Atg5, and Atg3 are critical in DC autophagy and functional activities (22, 42). We here show that the protein levels of these and other Atgs, which were clearly inhibited by LPS under normoxic conditions, were significantly increased under hypoxia. DC survival and activation are commonly associated with several signaling pathways and molecules known to be involved in autophagy (25, 44, 60). Accordingly with the hypothesis that autophagy promotes cell survival and activation in LPS-treated DCs, we here show that hypoxia significantly upregulates phosphorylation of Akt, Erk, p38, and NFkB. While the first two molecules are part of signaling pathways associated with DC autophagy and survival, p38 and NFkB were also associated with DC activation, still in an autophagic context (46, 61). In addition, DC activation by LPS resulted in the expression of several cytokines, with some of them being considered pro-survival factors (62). It should be underlined that the expressions of some of these cytokines, such as IL-1β, IL-18, and TNF-α, are known to be regulated by autophagy (63). By showing the upregulation of these cytokines, this study further supports the hypothesis that hypoxia promotes survival and activation of TLR-activated DCs. However, we cannot exclude the possibility that changes in LPS-induced cytokine mRNA expression seen under hypoxic conditions may be linked to other pO_2-dependent cellular changes rather than to the observed effects on cellular autophagosomal machinery.

The concept that autophagy is induced by hypoxia was corroborated by two of the most commonly used inhibitors to study autophagy, Baf A1 and CQ (30, 64). Previous reports have shown that Baf A1 treatment severely affected autophagy in several cell types, including bone marrow–derived DCs (65). Accordingly, in our study Baf A1 inhibited the autophagic process in hypoxic LPS-treated DCs by reducing the number of functional lysosomes, upregulating SQSTM1/p62, and downregulating Atg protein levels. In contrast, Baf A1 enhanced the protein level of LC3B-II. This result was, however, in agreement with other studies, reporting an inhibition of autophagy even in the presence of enhanced LC3B-II (66).

Of interest, enhanced LC3B-II levels were also observed upon CQ treatment. It must be pointed out that increased LC3B-II levels can be associated with either enhanced autophagosome synthesis or reduced autophagosome turnover, probably due to delayed trafficking to the lysosomes, reduced fusion between compartments, or impaired lysosomal proteolytic activity (66). This also justifies the results obtained for functional lysosomes, where Lysotracker positive structures tended to be much larger after CQ treatment compared to control or Baf A1 treatment.

Furthermore, CQ treatment resulted also in SQSTM1/p62 reduction. This is in agreement with previous reports showing that the degradative capacity of the cells still remains intact especially upon exposure to CQ, and the lysosomes retain their capacity to degrade delivered material (67).

Keeping in line with the fact that Baf A1 and CQ inhibit autophagy, we observed that both compounds reduced the protein levels of all the Atgs that were analyzed. Finally, and in agreement with several studies showing that autophagy may promote a pro-survival program, Baf A1 and CQ treatments resulted in the modulation of pro- and anti-apoptotic Bcl-2 family proteins (68), in the increased cleavage of PARP, and, more importantly, in the reduction of alive cell numbers. However, further studies are required to better assess which autophagic marker may be involved in the observed effects by using either other inhibitors or specific siRNAs.

The apparent absence of CD14 in the mature DCs exposed to LPS and the critical role of CD14 in efficient delivery of activating LPS to MD-2/TLR4 leaves open the possibility of an alternative mechanism of LPS-induced DC responses that may be not TLR4-dependent. However, previous experimental evidence documents that soluble CD14 from plasma/serum contributes to LPS/TLR4 signalling in CD14-negative cells (69, 70). Still in line with the possibility of an alternative mechanism of LPS-induced DC responses in hypoxia, we should highlight that LPS enhances PI3K/Akt activation in hypoxic DCs and that its abrogation results in an enhanced DC cell death (25). Of interest, previous reports indicate that LPS-induced phosphorylation of Akt was TLR4-dependent (71). Thus, future studies are needed to further understand the possible involvement of PI3K/Akt for regulating DC autophagy under hypoxia.

In conclusion, our data indicate that under hypoxic conditions, LPS activation of DCs leads to a pro-autophagic program. Autophagy is crucial for DC orchestration of the immune response and hypoxia is a common feature in pathological conditions, such as inflammation, tumor microenvironment, and within the microenvironment of lymphoid tissues. Thus, this study contributes to the understanding on how DCs adapt to changes of pO2, typically associated with different immune responses, and provides the ground for new future therapeutic regulation of DC functions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Azienda Ospedaliera Universitaria Senese (AOUS) and University of Siena. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AN, FC, and SM designed the research. SM, CA, GG, IF, CU, and GM performed the experiments. SM, DR, FC, and AN analyzed the data. SM and FC produced the figures. SM, SS, and AN wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

MIUR: PRIN 2017NTK4HY_002 to AN and PRIN 20177J4E75 and AIRC: IG 2017 - ID. 20776 to SS.

ACKNOWLEDGMENTS

The authors thank Ms. Alida Quaranta, from AMBRIT International School in Rome, for checking and re-editing the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 16 September 2021
doi: 10.3389/fimmu.2021.727161

Mice Plasmacytoid Dendritic Cells Were Activated by Lipopolysaccharides Through Toll-Like Receptor 4/Myeloid Differentiation Factor 2

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *18 June 2021*
Accepted: *02 September 2021*
Published: *16 September 2021*

Citation:
Zhang W, An E-K, Hwang J and Jin J-O (2021) Mice Plasmacytoid Dendritic Cells Were Activated by Lipopolysaccharides Through Toll-Like Receptor 4/Myeloid Differentiation Factor 2. Front. Immunol. 12:727161. doi: 10.3389/fimmu.2021.727161

Plasmacytoid dendritic cells (pDCs) are known to respond to viral infections. However, the activation of pDCs by bacterial components such as lipopolysaccharides (LPS) has not been well studied. Here, we found that pDCs, conventional dendritic cells (cDCs), and B cells express high levels of toll-like receptor 4 (TLR4), a receptor for LPS. Moreover, LPS could effectively bind to not only cDCs but also pDCs and B cells. Intraperitoneal administration of LPS promoted activation of splenic pDCs and cDCs. LPS treatment led to upregulation of interferon regulatory factor 7 (IRF7) and induced production of interferon-alpha (IFN-α) in splenic pDCs. Furthermore, LPS-dependent upregulation of co-stimulatory molecules in pDCs did not require the assistance of other immune cells, such as cDCs. However, the production levels of IFN-α were decreased in cDC-depleted splenocytes, indicating that cDCs may contribute to the enhancement of IFN-α production in pDCs. Finally, we showed that activation of pDCs by LPS requires the TLR4 and myeloid differentiation factor 2 (MD2) signaling pathways. Thus, these results demonstrate that the gram-negative component LPS can directly stimulate pDCs *via* TLR4/MD2 stimulation in mice.

Keywords: lipopolysaccharide, plasmacytoid dendritic cell, conventional dendritic cell, toll-like receptor 4, myeloid differentiation factor 2

INTRODUCTION

Lipopolysaccharides (LPS) are lipid polysaccharides present in the outer membrane of gram-negative bacteria and are known to stimulate the immune system (1, 2). Amongst the three structural domains, lipid A (also known as the endotoxin) is primarily responsible for the immunostimulatory activity of LPS (3, 4). LPS are a classical pathogen-associated molecular pattern (PAMP) that can be recognized by innate immune cells through the toll-like receptor 4 (TLR4) (5). Upon interacting with LPS, TLR4 forms a heterodimer with an extracellular adaptor glycoprotein named myeloid differentiation factor 2 (MD2) and induces two distinct signaling cascades (6, 7). The first signaling pathway depends on myeloid differentiation primary response 88 (MyD88) and induces to the secretion of inflammatory cytokines by activating nuclear transcription factor κB (NF-κB) in innate immune cells, whereas the second pathway is independent of MyD88 and mediates interferon regulatory factor 3 (IRF3) activation to induce type-I interferon (IFN) responses (8, 9).

TLR4 is the crucial receptor of the mammalian innate immune system and can be expressed by various types of immune cells (10). Moreover, it is highly expressed by antigen-presenting cells (APCs) such as macrophages, dendritic cells (DCs), and B cells (11). Numerous studies have reported that stimulation with LPS induces the activation of these APCs. To elaborate, murine B cells show stronger cell proliferation, cytokine secretion, and class switch recombination in response to LPS stimulation (12, 13). Whereas in case of macrophages, the TLR4 stimulation promotes to the activation of these cells, which leads to the secretion of inflammatory cytokines in the macrophages (14, 15). Furthermore, after sensing LPS *via* TLR4, DCs not only undergo maturation and migration but also show improved regulation of the adaptive immune responses (16, 17).

DCs are professional APCs that capture antigens and then process and present them to T cells (18–21). They can be divided into two major subsets: plasmacytoid DCs (pDCs), which specialize in antiviral defense by producing interferon alpha (IFN-α), and conventional DCs (cDCs), which are essentially responsible for antigen-presentation and T-cell activation (22–24). Although it is still controversial, the pDCs may be more efficient at presenting endogenous antigens rather than exogenous antigens, such as viral proteins (25). By utilizing pattern-recognition receptors (PRRs), such as TLR7 and TLR9 that bind to viral nucleic acids, pDCs detect virus invasion and produce large amounts of IFN-α (26). However, fewer studies have focused on the response of pDCs against bacterial infection and the expression of TLR4 in the surface of pDCs, and on the effect of TLR4 ligands on pDC activation.

Our previous research showed that monophosphoryl lipid A (MPLA) induces the activation of pDCs and has a synergistic effect on anti-PD-L1-antibody-mediated anti-cancer immunity (27). MPLA is a detoxified form of LPS that stimulates TLR4 and leads to the activation of immune cells. However, the molecular details of the MPLA dependent activation of pDCs have not been studied thus far. Therefore, we hypothesized that pDCs may express considerable levels of TLR4 and that LPS may stimulate pDCs either directly or indirectly, as a result of the cytokines expressed by other immune cells. In the following study, we treated mice with LPS and characterized the molecules responsible for LPS-dependent activation of pDCs.

Abbreviations: LPS, lipopolysaccharide; cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell; TLR4, toll-like receptor 4; IFN-α, interferon-alpha; IL, interleukin; TNF, tumor necrosis factor; MD2, myeloid differentiation factor 2; IRF7, interferon regulatory factor 7; Ab, antibody; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear transcription factor-κB; PBS, phosphate buffered saline; APCs, antigen-presenting cells; MHC, major histocompatibilitycomplex; CTL, cytotoxic T lymphocyte; PRRs, pattern-recognition receptors; MPLA, monophosphoryl lipid A; KO, knockout.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (6 to 8 weeks) were obtained from Korea Orient Bio Inc. (Gyeonggi-do, Korea) and Shanghai Public Health Clinical Center (SPHCC, Shanghai, China). TLR2-knockout (KO), TLR4-KO, and B6.129P2-Ly96-KO (MD2-KO) mice were provided by SPHCC. The mice were maintained either in the Laboratory Animal Center of SPHCC or at Yeungnam University, under 50–60% humidity and at 20–22°C. This study was approved by the Ethics of Animal Experiments Committee of Yeungnam University (2020–015) and SPHCC (2018-A049-01).

Reagents and Antibodies

LPS (O111:B4) and FITC-conjugated LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). TLR4 Agonist-Ultrapure LPS (055:B5) and CpG-1826 were obtained from Invivogen (San Diego, CA, USA). The following fluorescence-conjugated antibodies (Abs) were provided by BioLegend (San Diego, CA, USA) and were used for flow cytometry analysis: anti-B220 (RA3-6B2), anti-CD11c (N418), anti-CD3 (17A2), anti-CD317 (927), anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-IRF7 (MNGPKL), and anti-TLR4 (SA15-21). Anti-IFN-α (RMMA-1) Ab was purchased from pbl Assay Science (Piscataway, NJ, USA). Anti-class I major histocompatibility complex (MHC) Abs (28–8–6) and anti-class II MHC (M5/114.15.2) Abs were purchased from eBioscience (San Diego, CA, USA).

Analysis of Mouse pDCs and cDCs

pDC and cDC activation was analyzed as described elsewhere (27, 28). The spleens were harvested after intraperitoneal (*i.p.*) administration of 0.1 mg/kg LPS or 10 mg/kg CpG to C57BL/6 mice and were then digested with 2% FBS, collagenase IV, and DNase containing digestion buffer for 20 min at 37°C. After filtering with 100-nm nylon mash, the cells were resuspended in 3 ml of Histopaque-1077 (Sigma-Aldrich) and layered over 3 ml of fresh Histopaque-1077, and 1 ml of FBS was then added above on the top. The cells were centrifuged at 1700 × g for 10 min to harvest the leukocytes (<1.077 g/cm^3). Leukocytes were

incubated with unlabeled isotype control Abs and Fc-block Abs for 15 min and then stained with anti-CD11c, anti-CD317, and lineage Abs such as anti-CD3 (17A2), anti-CD49b (DX5), anti-CD90.1 (OX-7), anti-B220 (RA3-6B2), anti-Gr-1 (RB68C5), and anti-TER-119 (TER-119). In addition, the cells were stained with anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-class I MHC (28–8–6), and anti-class II MHC (M5/114.15.2) Abs to determine cell activation. Following a second wash with PBS to remove the unbound Abs, the cells were resuspended in 50 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) containing PBS. The cells were analyzed using a Novocyte flow cytometer (ACEA Biosciences Inc., San Diego, CA, USA) after gating out DAPI-positive cells as dead cells. The cDCs and pDCs in splenocytes were identified in live leukocytes by flow cytometry and defined as lineage$^-$CD11c$^+$cells and CD317$^+$B220$^+$ cells, respectively.

Intracellular Cytokine Staining

Intracellular cytokine production was analyzed as described previously (29, 30). C57BL/6 mice were injected *i.p.* with PBS, 0.1 mg/kg LPS, and 10 mg/kg CpG. Twelve hours after the injection, splenocytes were harvested and incubated with 2 μM monensin solution (BioLegend) for 2 h. After washing with PBS, the cells were stained with surface Abs followed by labeling with the Zombie Violet Fixable Viability Kit (BioLegend) at 25°C for 20 min to remove dead cells. The cells were fixed with a fixation buffer (BioLegend) at 4°C for 20 min and then stained with intracellular staining Abs in permeabilization buffer (BioLegend) at 25°C for 15 min. After washing with PBS, the cells were analyzed using a Novocyte flow cytometer (ACEA Biosciences Inc.). IFN-α and IRF7 expression levels were analyzed in CD317$^+$B220$^+$ pDCs.

ELISA

The IFN-α concentration in serum or cultured media was measured in triplicates using ELISA kits from BioLegend. For the serum concentration of IFN-α, the mice received PBS, 0.1 mg/kg LPS, and 10 mg/kg CpG. Twelve hours after the injection, blood sera were harvested from the mice. IFN-α concentration in the cultured media was analyzed from LPS-stimulated enriched pDCs, splenocytes, or cDC-depleted splenocytes 12 h after LPS stimulation.

Isolation of pDCs

The pDCs were isolated from splenocytes using a pDC isolation kit (Miltenyi Biotec, Auburn, CA, USA). The pDC isolation purity was determined *via* flow cytometry, and the purity of CD317$^+$B220$^+$ pDCs was higher than 90%.

Depletion of cDCs

The cDCs in splenocytes were stained with an anti-CD11c-biotin Ab (BioLegend). The cells were then stained with a microbead-conjugated anti-biotin Ab (Miltenyi Biotec) for 15 min. The CD11c$^+$ cDCs were removed by negative selection using an LD column (Miltenyi Biotec). The efficacy of CD11c$^+$ cDC depletion was >98%.

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). One- or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and Mann-Whitney U-test were used for the analysis of datasets with the help of SPSS software (IBM, Armonk, NY, USA). $p < 0.05$ was considered to be statistically significant.

RESULTS

LPS Binds to pDCs

To identify TLR4-expressing APCs in splenocytes, we gated TLR4$^+$ and MHC class II$^+$ cells. The TLR4$^+$MHC class II$^+$ cell population included CD11c$^+$ cDCs, B220$^+$ B cells, and CD317$^+$B220$^+$ pDCs (**Figure 1A**). Although, the TLR4 expression levels in pDCs was lower than that in cDCs and B cells (**Figure 1B**). In addition, we observed that FITC-conjugated LPS could efficiently bind to pDCs, cDCs, and B cells (**Figure 1C**). Thus, our data indicate that pDCs express considerable levels of TLR4 on their surface, and that LPS can bind to pDCs in mouse splenocytes.

LPS Induces the Upregulation of Activation Markers in pDCs

After establishing that LPS is able to bind to pDCs, we next examined whether LPS can induce the *in vivo* activation of these cells. C57BL/6 mice were treated *i.p.* with PBS, LPS (0.1 mg/kg), and CpG (10 mg/kg), and the splenic pDCs and cDCs in the live leukocytes were defined as B220$^+$CD317$^+$ and lineage$^-$CD11c$^+$ cells, respectively (**Figure 2A**). LPS administration induced the upregulation of CD40, CD80, CD86, and class I and II MHC expression in both pDCs and cDCs, 12 h after injection (**Figures 2B, C**). LPS was able to upregulate the co-stimulatory molecules with a higher efficacy than CpG, a positive control for pDC activation (**Figures 2B, C**). In the mouse *in vitro* study, LPS exerted a considerably higher effect on the induction of pDC and cDC activation than CpG (**Figure S1**). The highest levels of co-stimulatory molecules in pDCs were recorded 12 h after LPS treatment, while those in cDCs peaked 18 h after LPS treatment (**Figure S2**). However, the expression of MHC class I and II in both pDCs and cDCs increased dramatically 3 h after LPS treatment, and there after decreased gradually (**Figure S2**). In addition, we examined whether LPS can induce the activation of liver and thymic pDCs and found that LPS treatment dramatically upregulated the expression levels of co-stimulatory molecules and class I and II MHC in both liver and thymic pDCs (**Figure S3**). In conclusion, our data suggest that treatment with LPS induces activation of pDCs in mice *in vivo*.

LPS Induces IFN-α Production in pDCs

Since it is well known that activated pDCs produce IFN-α (31–33), we studied IFN-α production in LPS activated pDCs and observed an increase in the levels of intracellular IFN-α (**Figure 3A**). The concentration of IFN-α in serum was also significantly increased in LPS-treated mice in comparison to the control mice (**Figure 3B**). In addition, LPS treatment also led to a

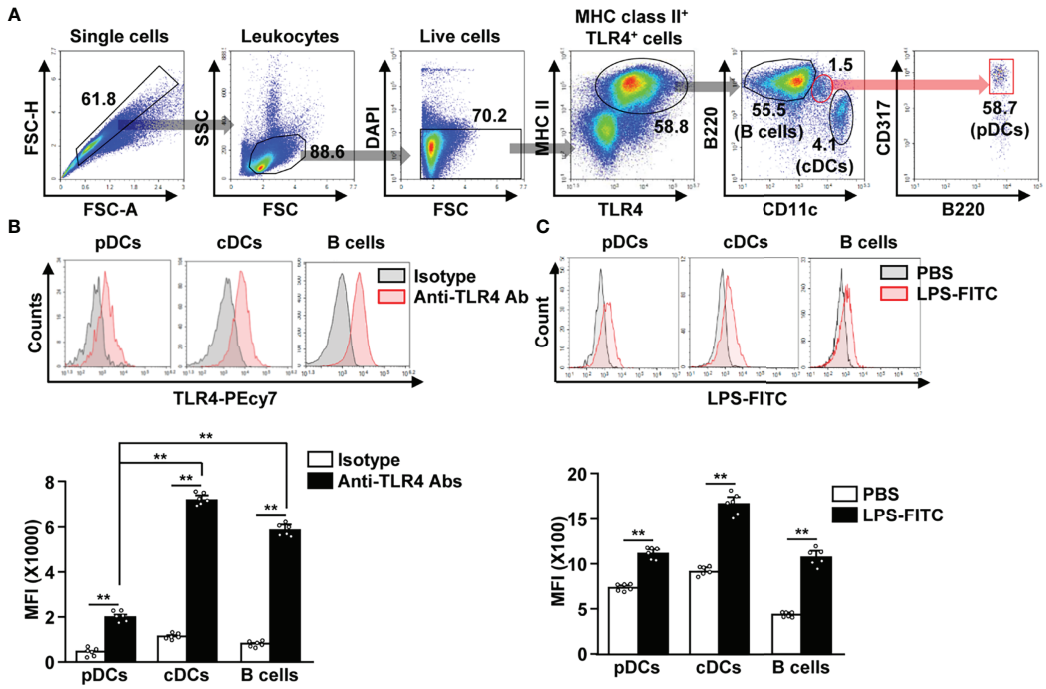

FIGURE 1 | Lipopolysaccharide (LPS) bound to the plasmacytoid dendritic cells (pDCs), conventional DCs (cDCs), and B cells in mice. **(A)** Toll-like receptor 4 (TLR4)-expressing major histocompatibility complex (MHC) class II positive cells were shown. **(B)** TLR4 expression in pDCs, cDCs, and B cells was analyzed by flow cytometry (upper panel). Mean fluorescence intensity (MFI) of TLR4 expression levels in pDCs, cDCs, and B cells is shown (lower panel) (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$). **(C)** Binding of FITC-conjugated LPS to pDCs, cDCs, and B cells was analyzed (upper panel). MFI of LPS-FITC binding to pDCs, cDCs, and B cells is shown (lower panel) (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$).

remarkable increase in IFN-α regulatory protein interferon regulatory factor 7 (IRF7) levels in pDCs (**Figure 3C**). Although the effect of LPS on IFN-α production was lower than that of CpG, the increase in IFN-α production in LPS-treated pDCs was significant (**Figures 3A, B**). These data suggest that LPS can promote IFN-α production in mice pDCs.

LPS Directly Upregulates the Surface Activation Markers in pDCs

Since cDCs can mediate the activation of other immune cells (18, 27, 34), we tried to ascertain if cDCs were required for the LPS-dependent activation of pDCs. The splenocytes were depleted of cDCs and then treated with 0.1 μg/ml LPS (**Figure S5**). In both total splenocytes (+cDCs) and cDC-depleted splenocytes (−cDCs), LPS treatment led to a significant increase in the expression of co-stimulatory molecules, and MHC class I and II (**Figure 4A**). Next, we also examined the effect of LPS on isolated pDCs (**Figure S6**) and found that LPS promoted their activation (**Figure 4B**). These data indicate that the increased expression of activation markers in pDCs by LPS does not require interaction with cDCs. In addition, LPS stimulation led to an increased IFN-α production in isolated pDCs, total splenocytes (+cDCs) and cDC-depleted splenocytes (−cDCs) (**Figure 4C**). Moreover, LPS-activated total splenocytes showed greater IFN-α production than isolated pDCs and cDC-depleted splenocytes (**Figure 4C**). Thus, these data suggest that LPS directly induces upregulation of co-stimulatory molecules in pDCs without interacting with other cells, especially cDCs. However, IFN-α production in pDCs in response to LPS may be influenced by the activation of cDCs.

LPS-Induced Activation of pDCs Require TLR4 and MD2

TLR4 and MD2 are the key receptors that are required in LPS-induced activation of cDCs (6, 7). To determine if this was also the case for LPS-stimulated pDCs, we *i.p.* injected 0.1 mg/kg LPS in C57BL/6, TLR4-KO, and MD2-KO mice. We observed that FITC-conjugated LPS was unable to bind to the pDCs in TLR4-KO and MD2-KO mice (**Figure 5A**). Moreover, LPS treatment did not lead to an increase in the serum concentration of IFN-α in TLR4-KO and MD2-KO mice (**Figure 5B**). The IRF7 expression levels were not increased in TLR4-KO and MD2-KO pDCs in response to LPS (**Figure 5C**). Furthermore, LPS did not affect the expression of co-stimulatory molecules and class I and II MHC in the pDCs of TLR4-KO and MD2-KO mice (**Figure 5D**). LPS from Sigma-Aldrich used in this study could stimulate TLR4 as well as TLR2. We confirmed this result using ultrapure LPS and data showed similar effects on the activation of pDCs by ultrapure LPS as well as that from Sigma-Aldrich (**Figure S4**). Moreover, LPS promoted the upregulation of these molecules in the pDCs of TLR2-KO mice (**Figure S7**). Therefore, these data suggest that LPS-induced pDC activation is dependent on the TLR4/MD2 pathway.

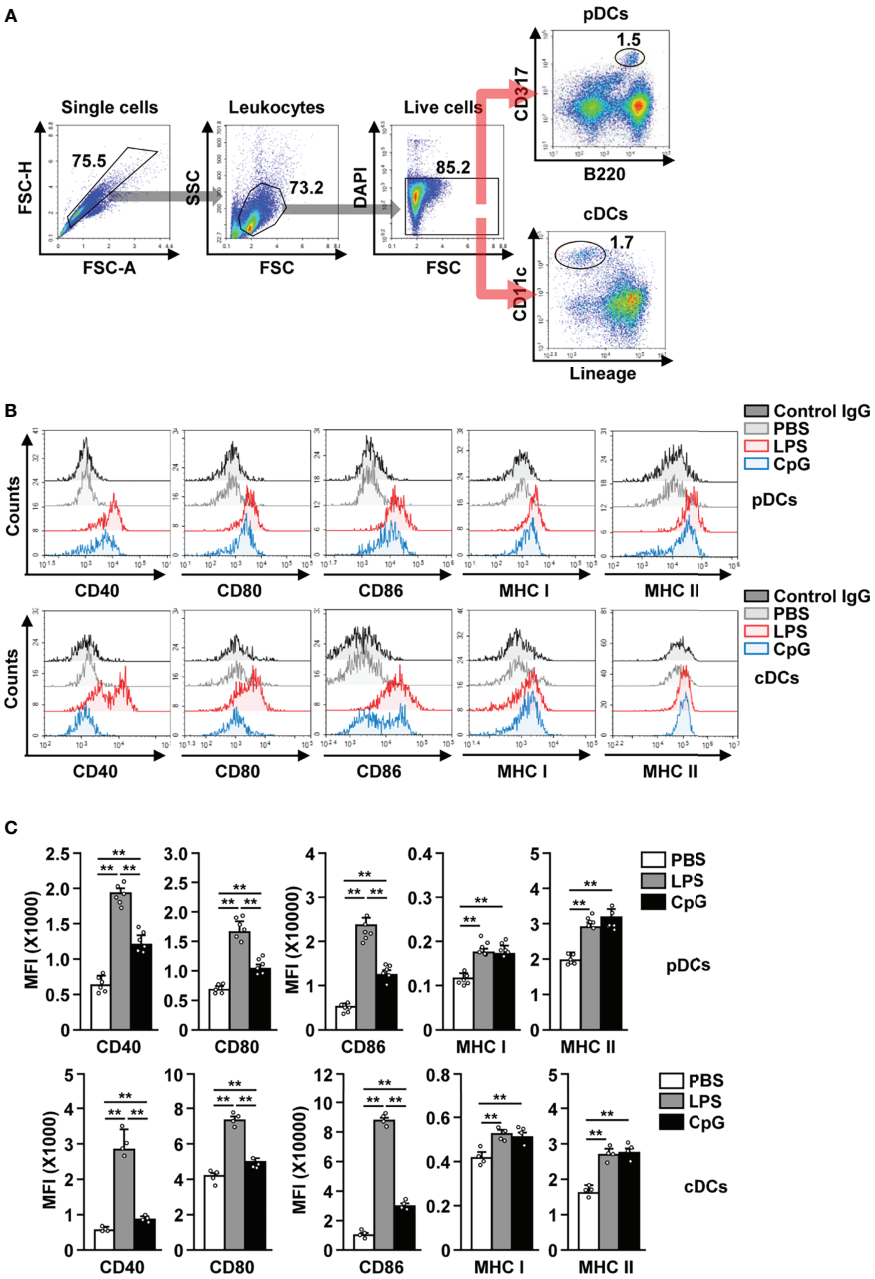

FIGURE 2 | LPS induced the activation of pDCs and cDCs in mice. C57BL/6 mice were treated intraperitoneally (*i.p.*) with 0.1 mg/kg LPS and 10 mg/kg CpG. The mice were sacrificed, and spleen was harvested 12 h after treatment. **(A)** Gating strategy for splenic pDCs and cDCs was shown. **(B)** CD40, CD80, CD86, and MHC class I and II expression levels in pDCs (upper panel) and cDCs (lower panel) were shown. **(C)** MFI of the indicated surface marker expression in pDCs (upper panel) and cDCs (lower panel) was shown (n = 6 mice, two-way ANOVA, mean ± SEM,$^{**}p < 0.01$).

DISCUSSION

Being a member of the DC family, pDCs can serve as a connecting link between the innate and adaptive immune system (35). Moreover, pDCs typically act as sensors of viral infections by producing large amounts of type I IFN and generating strong antiviral responses (24, 26). However, when compared to cDCs, due to the low expression of MHC and costimulatory molecules, pDCs are not efficient at presenting antigens and mediating T cell activation. They become potent APCs upon proper stimulation with TLR ligands, such as the TLR9 agonist CpG and TLR7 agonist imiquimod (36, 37). In addition, it has been demonstrated that human pDCs express TLR1/2. The TLR1 mechanism contributes to the upregulation

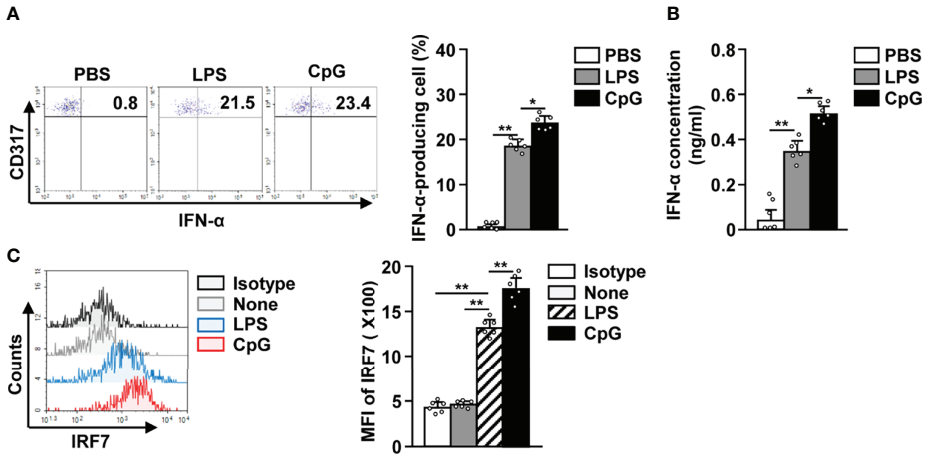

FIGURE 3 | LPS elicited interferon-alpha (IFN-α) production in pDCs. The LPS (0.1 mg/kg) and CpG (10 mg/kg) were *i.p.* injected in C57BL/6 mice. Twelve hours after treatment, spleens were harvested and the splenocytes cultured in 2 μM monensin solution for 2 h. **(A)** Intracellular production of IFN-α in pDCs was shown (left panel). Mean percentage of IFN-α-producing cells was shown (right panel) (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$, $^{*}p < 0.05$). **(B)** Serum concentration of IFN-α was measured by ELISA (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$, $^{*}p < 0.05$). **(C)** Intracellular expression levels of interferon regulatory factor 7 (IRF7) were measured in pDCs (left panel). MFI of IRF7 expression levels was shown (right panel) (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$).

of costimulatory molecules and pro-inflammatory cytokine production in response to gram-positive bacterial lipoproteins. In contrast, type I IFN production is controlled by TLR2 stimulation (38). However, it is still controversial whether LPS, the classical TLR4 ligand, can lead to pDC activation. Although it has been previously reported that pDCs do not respond to LPS due to a lack of corresponding TLRs (39), a study in mice demonstrated that LPS can enhance the expression of costimulatory molecules in pDCs (32). Another study in humans showed that LPS could upregulate IRF-7 expression and IFN-α production in pDCs (40). In this study, we found that pDCs expressed considerable levels of TLR4 on their surfaces, and that treatment with LPS induced upregulation of costimulatory molecules in pDCs. These data are consistent with those from our previous results, which suggest that MPLA enhanced pDC-mediated anti-cancer immunity in combination with anti-PD-L1 antibody treatment (27). Together, this indicates that pDCs can respond to LPS and suggests that these cells may exert protective effects during gram-negative bacterial infections.

Type I IFNs, a family of monomeric cytokines, are central players in the antiviral immune response of the host (26). Importantly, they have pleiotropic effects on many other immune cells, linking innate and adaptive immunity (41). IFN-α and IFN-β are the most well-studied members of the type I IFN family and have a broad degree of effects on the development of immune cells and on the regulation of immune response (33). While IFN-β can be produced by many types of cells, IFN-α is predominantly produced by pDCs against viral infection (42). In contrast, cDCs are non-professional IFN-α producers (42). However, TLR9 and TLR7 agonists are potent inducers of IFN-α production, and the well-known TLR4 agonist LPS can also upregulate IRF-7 expression and IFN-α production in human pDCs (40). In line with a study in humans, we found that LPS upregulated IRF-7 expression and induced the *in vivo* production of IFN-α in mice pDCs. Future studies should determine if human peripheral blood pDCs also express TLR4 and response to LPS treatment.

Although much remains unresolved about the interaction between pDCs and cDCs, it is known that this interaction plays an important role in immune defense (43). To elaborate, the CD40-CD40L interaction between pDCs and cDCs is necessary for IL-12 production in mouse cDCs during *Listeria monocytogenes* infection (44). In addition, co-culturing pDCs and cDCs had a synergistic effect on the optimal activation of both pDCs and cDCs in response to bacterial infections in human peripheral blood (45). In this study, we demonstrated that LPS induced the upregulation of co-stimulatory and class I and II MHC molecules in enriched pDCs as well as pDCs in cDC-depleted splenocytes, indicating that the upregulation of activation markers in pDCs was independent of cDCs. However, we found that LPS treatment caused a significant reduction in IFN-α levels in cDC-depleted splenocytes, indicating that cDCs may support IFN-α production in these cells. In addition, there is the possibility that cDCs can directly produce IFN-α in response to LPS (46). It is important to understand whether cDCs themselves produce IFN-α in response to LPS or indirectly promote the secretion of IFN-α by interacting with pDCs and cDCs. Therefore, a study on the interaction between cDCs and pDCs in response to LPS or other bacterial components is needed.

TLR4 has been established as a receptor for LPS (5). LPS is initially released from the outer membranes of gram-negative bacteria by the LPS binding protein (LBP). In serum, the LBP-

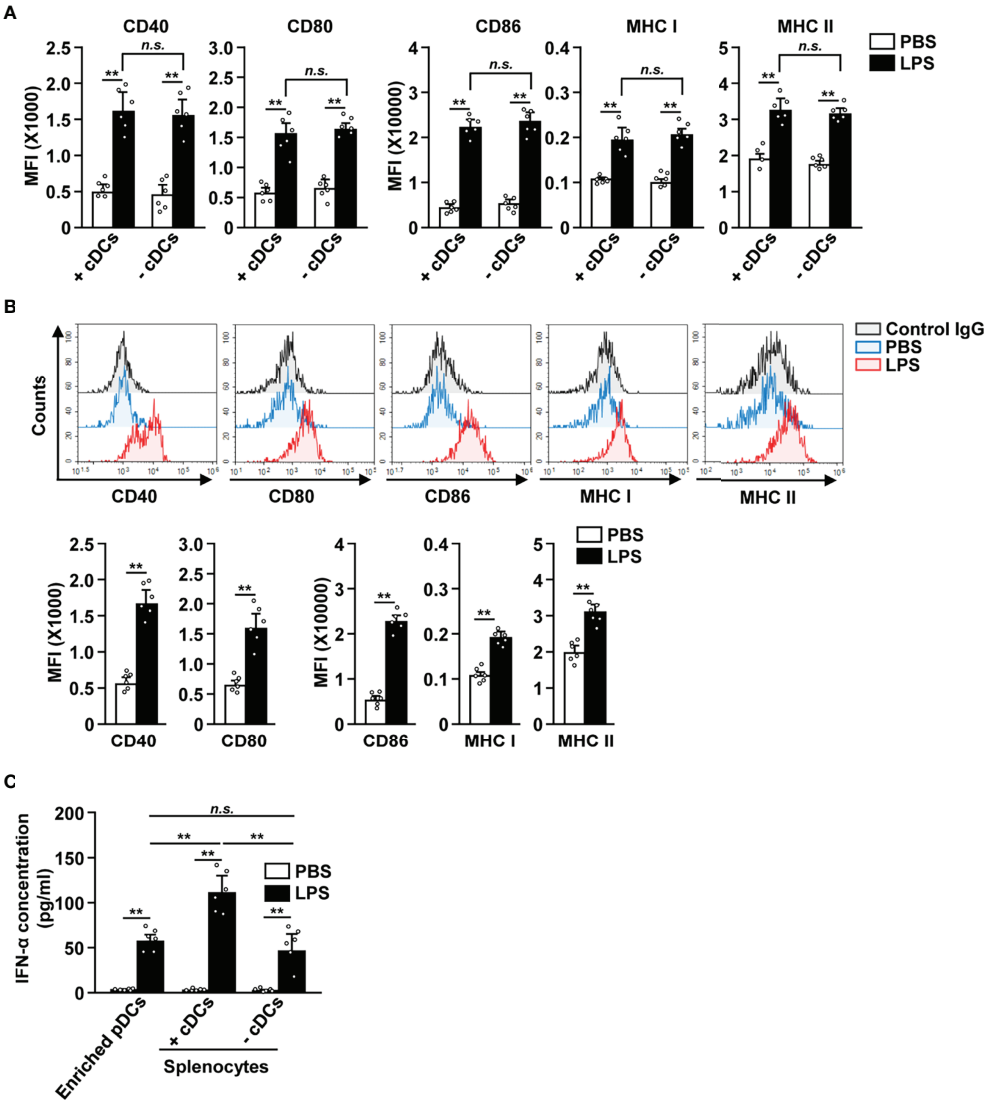

FIGURE 4 | LPS upregulated the surface activation markers of pDCs without interacting with cDCs. **(A)** Total splenocytes (+cDCs) and cDC-depleted splenocytes (–cDCs) were incubated with 0.1 μg/ml LPS. Expression of the markers indicated above (CD40, CD80, CD86, and MHC I and II) was measured in pDCs by flow cytometry, 12 h after LPS treatment (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$). **(B)** The expression levels of co-stimulatory molecules and class I and II MHC were measured in isolated pDCs 12 h after treatment with 0.1 μg/ml LPS (Upper panels). MFI of co-stimulatory molecules and class I and II MHC was shown (lower panels, n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$). **(C)** IFN-α concentration in cultured medium was measured by ELISA (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$, n.s., none significant).

LPS complex transfers LPS to $CD14^+$ cells. LBP and CD14 help in docking LPS to the TLR4 complex, which is composed of heterodimer with MD-2 (6). The binding of LPS to the TLR4–MD-2 complex leads to activation of cells by promoting cytokine production and induces the expression of activation markers (6, 47). In contrast to cDCs, pDCs are not derived from myeloid cells and therefore do not express CD14 on their surface. As mentioned above, CD14 is important for the transfer of LPS to the TLR4-MD2 complex (Park and Lee, 2013).Therefore, even though pDCs express TLR4, CD14 is essential for the transfer of LPS to the TLR4-MD2 complex. We speculate that the LPS-induced activation of pDCs may be due to the contribution of the soluble form of CD14. To elaborate, it has been shown that the soluble forms of CD14 can deliver LPS to the TLR4-MD2 complex and contribute to immune activation (48, 49).

In conclusion, we demonstrate that mouse pDCs not only express considerable levels of TLR4 but also respond to LPS. LPS treatment induced upregulation of co-stimulatory molecules and IFN-α production in the pDCs in a TLR4-MD2 dependent manner. Thus, these data suggest that pDCs can directly react

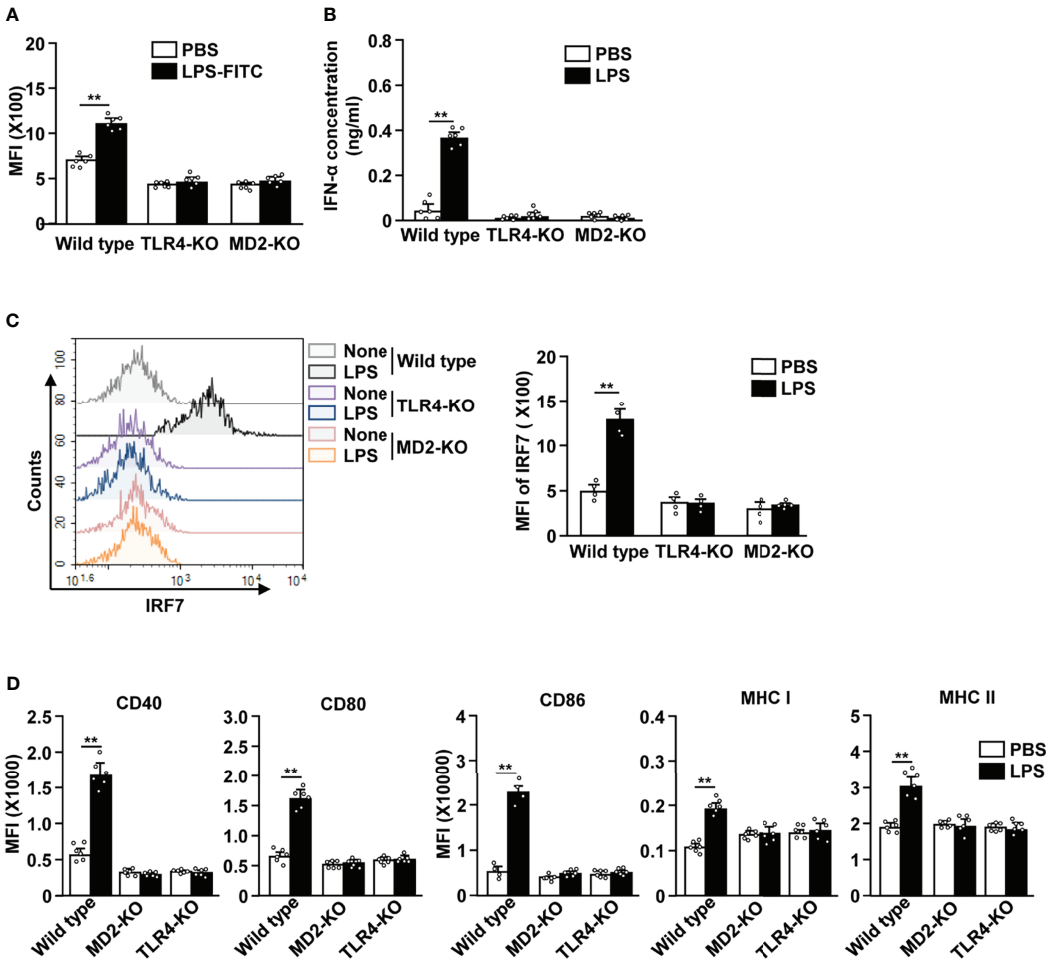

FIGURE 5 | LPS-induced pDC activation requires TLR4 and myeloid differentiation factor 2 (MD2). **(A)** LPS binding in pDCs was measured in C57BL/6 (wild type), TLR4-knockout (KO), and MD2-KO mice by flow cytometry (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$). (B to D) Wild type, TLR4-KO, and MD2-KO mice were injected *i.p.* with PBS and 0.1 mg/kg LPS. Twelve hours after LPS injection, spleen and serum were harvested. **(B)** Serum concentration of IFN-α was measured by ELISA (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$). **(C)** Expression levels of IRF7 in pDCs were analyzed by flow cytometry (left panel). MFI of IRF7 expression levels were shown (right panel, n = 4 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$). **(D)** MFI of costimulatory molecules, and class I and II MHC were shown (n = 6 mice, two-way ANOVA, mean ± SEM, $^{**}p < 0.01$).

against LPS and may play a role in shaping the immune response against gram-negative bacterial infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the Ethics of Animal Experiments Committee of Yeungnam University (2020–015) and Shanghai Public Health Clinical Center (2018-A049-01).

AUTHOR CONTRIBUTIONS

J-OJ designed the experiments and wrote the manuscript. WZ, E-KA, and JH participated in the experiments and data analysis. J-OJ and WZ reviewed the article. WZ and E-KA helped with manuscript writing and made important corrections to the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Research Foundation of Korea (NRF-2019R1C1C1003334 and NRF-2020R1A6A1A03044512) and the National Natural Science Foundation of China (81874164).

ACKNOWLEDGMENTS

We thank the animal facility of the SPHCC and Yeungnam University for housing the animals that were used in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.727161/full#supplementary-material

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