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Species and mediator specific TLR4 antagonism in primary human and murine immune cells by βGlcN(1 ↔ 1)αGlc based lipid A mimetics

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ABSTRACT

Immune stimulatory pathogen associated molecular patterns (PAMPs) are major drivers of infection pathology. Infections with Gram-negative bacteria or negatively polar and single stranded RNA influenza virus are prominent causes of morbidity and mortality. Toll-like receptor (TLR) 4 is a major host sensor for both of the two infections. In order to inhibit TLR4 driven immune activation we recently developed synthetic tetra-acylated lipid A mimetics based on a conformationally restricted βGlcN(1 ↔ 1)αGlcN disaccharide scaffold (DA-compounds) that antagonized ectopically overexpressed human and murine TLR4/MD-2 complexes. Here we comparatively analyzed human peripheral blood mononuclear cell (hPBMC) and murine bone marrow derived macrophage (mBM) activation upon 30 min of preincubation in vitro with six variably acylated DA-compounds. 16 h subsequent to consequent LPS challenge, we sampled culture supernatants for cytokine and NO concentration analysis. Four compounds significantly inhibited release of both TNF and IL-6 by hPBMCs upon LPS challenge. In contrast, three compounds effectively inhibited mBM production of MIP-2 and KC, and even five of them inhibited IL-6 and NO production. LPS driven like other TLR ligand driven mBM TNF release was largely unimpaired. The inhibitory effect was specific in that Clo75 driven cytokine release by both hPBMCs and mBMs was unimpaired by the compounds analyzed. Our results indicate biological species specificity of LPS antagonism by variably tetraacylated lipid A mimetics and validate three out of six DA-antagonists as promising candidates for development of therapeutically applicable anti-inflammatory compounds.

1. Introduction

Pattern recognition receptor (PRR) activation upon binding of pathogen associated molecular patterns (PAMPs) is the prerequisite of appropriate immune activation aiming at quiet pathogen clearance but also elicits unwanted immune activity (Medzhitov and Janeway, 1997; Ulevitch and Tobias, 1995). For instance, it drives collateral host damage that outweighs pathogen arresting such as upon specific bacterial acute infection (Nathan, 2012; Stearns-Kurosawa et al., 2011). The first receptors implicated in the mediation of specific PAMP driven cell activation were members of the toll-like receptor (TLR) family, followed by cytoplasmic NOD like receptors and RIG I like RNA helicases as well as of further cell membrane spanning receptors such as C-type lectins (Takeuchi and Akira, 2010). A prototypic immune stimulatory PAMP is lipopolysaccharide (LPS, also known as endotoxin), which is a component of the outer membrane of the cell envelope of Gram-negative bacteria (Raetz and Whitfield, 2002). Early, TLR4 had been implicated as an exquisite immune stimulatory signal transducing cellular LPS receptor (Beutler and Rietschel, 2003). Aside from the TLR4 co-receptor named myeloid differentiation factor (MD)-2, other receptors such as TLR2 and/or a yet unknown cytoplasmic protein mediate LPS recognition (Hagar et al., 2013; Kayagaki et al., 2013; Shimazu et al., 1999; Spiller et al., 2007; Tan and Kagan, 2014; Werts et al., 2001). Moreover, numerous agonists, namely PAMPs (besides LPS) produced by various microbes, danger associated molecular patterns (DAMPs) of endogenous origin by definition, and allergens have been allocated to TLR4 (Bald et al., 2014; Biragyn et al., 2002; Hagar et al., 2013; Kayagaki et al., 2013; Kurt-Jones et al., 2000; Malley et al., 2003; Park and Lee, 2013; Qiang et al., 2013; Ryan et al., 2011; Schmidt et al., 2010; Termeer et al., 2002; Trompette et al., 2009; Vogl et al., 2007). Therefore, TLR4 confers immunity to microbes of different classes and mediates immune activation upon sterile host tissue insults including

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allergies. Anyhow, the TLR4/MD-2 complex together with the LPS binding protein (LBP) and cluster of differentiation (CD) 14 through which a monomeric LPS molecule is being transferred to MD-2 is most likely the forefront cellular sensor of Gram-negative bacteria (Beutler and Rietschel, 2003; Schumann, 2011; Spiller et al., 2008; Tan and Kagan, 2014). The lipid-containing terminus of LPS, namely hexaacylated bisphosphorylated lipid A is recognized by the TLR4/MD-2 complex and is bound by the hydrophobic binding pocket of MD-2. Since only five out of six lipid chains are accommodated within the deep binding groove of the co-receptor protein, the sixth acyl chain is exposed on the surface of MD-2 at the secondary dimerization interface, which drives the homodimerization of two TLR4/MD-2/LPS complexes and the ensuing cell activation as has been shown for *Escherichia coli* LPS. (Park and Lee, 2013; Park et al., 2009). In contrast, all four lipid chains of the tetraacylated bisphosphorylated lipid A structures such as biosynthetic precursor of *E. coli* lipid A, lipid IVa, or synthetic drug candidate Eritoran, are fully inserted in the hydrophobic cavity of hMD-2 which precludes the dimerization of the two receptor complexes and proclaim these compounds as hTLR4/hMD-2 antagonists (Kim et al., 2007; Ohto et al., 2007; Peri and Piazza, 2012; Thieblemont et al., 1998). Though lipid IVa exercises antagonistic properties on hTLR4, it was shown to exhibit agonistic activity on mTLR4 the structural basis of the so called "m"-shaped 2:2:2 complex of which has been first proposed and subsequently demonstrated (DeMarco and Woods, 2011; Ohto et al., 2012). We have previously disclosed that, in contrast to their natural counterpart lipid IVa, synthetic tetraacylated lipid A mimetics which are based on the conformationally restricted βGlcN(1 ↔ 1)αGlcN scaffold (DA-compounds) displayed species-independent antagonistic properties when applied to cells ectopically overexpressing human and murine TLR4/MD-2 complexes (Artner et al., 2013).

Herein we inhibited TLR4/MD-2 complex-mediated pro-inflammatory activity by applying novel synthetic antagonistic lipid A mimetics which compete with LPS for the binding to the same site on MD-2 on murine and human immune cells. Specifically, we analyzed six differently acylated DA-compounds for their capacities to inhibit LPS-induced pro-inflammatory activity of primary immune cells, namely murine bone marrow derived macrophages (mBMs) and human peripheral blood mononuclear cells (hPBMCs). DA254, DA255 and DA256- but not the other three DA-compounds impaired production of MIP-2 and KC by mBMs while IL-6 and NO production was abrogated by all compounds except for DA257. However, hPBMC TLR4/MD-2 driven TNF and IL-6 releases were most significantly inhibited by DA193, −253, and −256 upon LPS challenge. These results indicate the latter three DA-compounds – which are being validated through their additional murine cell inhibitory property – as prime anti-inflammatory therapeutic candidates.

2. Material and methods

2.1. Materials

TLR specific agonists applied were 10 ng/ml of LPS O111:B4 (treated by successive hydrolysis and purified by a triethylamine-deoxycholate sodium phenol–water procedure, Invivogen), 10 ng/ml of a tri-palmitoylated hexapeptide (Pam_3CSK_4, EMC Microcollections), and 10 μg/ml of Clo75 (Invivogen) to activate TLR4, TLR2, and TLR7/TLR8, respectively. For nitrite containment assaying 7.7 μM of *N*-((1-naphthyl) ethylenediamine dihydrochloride), 0.1 M of sulphanilamide, and 0.5 M of phosphoric acid (all Sigma–Aldrich) were applied. 50 μM of β-mercaptoethanol (Sigma–Aldrich), 50,000 U of Penicillin-Streptomycin and Antibiotic–Antimicotic mixture (both Gibco), Dulbecco's modified eagle medium (DMEM, Gibco), RPMI1640 medium (Gibco), and Ficoll (GE Health care) were applied. DA-compounds were synthesized as reported previously (Artner et al., 2013) (Fig. 1). The DA-compounds were stored as lyophilized powder. Samples were reconstituted in dimethyl sulphoxide (DMSO) (Sigma–Aldrich) to a final concentration of 1 mg/ml stock solution. Vials were vortexed for 10 min and incubated at room temperature for 20 min with occasional vortexing and were sonicated for 10 s. The stock solutions were further diluted with the RPMI for hPBMCs and DMEM for mBMs supplemented with 10% FCS to provide the tested concentrations and vortexed prior to challenging of cells.

2.2. mBM preparation

Mouse bone marrow was harvested from femurs and tibiae. Organ removals from animals were approved by the responsible local authorities, namely the state office of nature, environment, and consumerism of North Rhine Westphalia, Germany (G1230/11). Cells were washed with DMEM and resuspended in 100 ml DMEM supplemented with 10% FCS, 10% L929 conditioned medium, Penicillin-Streptomycin (100 U/ml, Gibco), Antibiotic-Antimicotic (amount as indicated by supplier Gibco) and 50 μM of β-mercapto ethanol (Sigma–Aldrich). Cells were incubated for one week under regular cell culture conditions prior to assaying.

2.3. Immunoblot analysis

MBM lysates were prepared upon treatments indicated and analyzed by SDS-PAGE and immuno blotting as described previously (Spiller et al., 2008). Antibodies toward IκBα (44D4), phospho-ERK and -p65 (Ser536, 93H1, Cell Signaling Technology) were applied according to manufacture instructions.

2.4. Reporter gene assay

NF-κB driven reporter gene activation was analyzed by transfection of two respective reporter gene constructs and controlled by cotransfection of a renilla luciferase construct in a 96-well format as described previously (Spiller et al., 2007). Briefly, NF-κB activity was analyzed by application of lysates of HEK293 (human embryonic kidney fibroblast) cells transfected with mouse TLR4, mouse MD2, and either with 6 × NF-κB promotor or ELAM promoter constructs and subsequently challenged with LPS.

2.5. hPBMC isolation

Total PBMCs were isolated from buffy coats from mixed and anonymized healthy blood donations and kindly provided by the local blood donation center (University Hospital Essen directed by P. Horn) upon approval by the local ethics committee (14-5804-B0, ibidem). 13 ml of blood was pipetted gently to the side of tubes filled with 20 ml of Ficoll (GE Health care). Tubes were spun 400 × *g* (Sorvall ST16 cell centrifuge) with accelerator set to 1 and decelerator set to 0 for 30 min, resulting in formation of three phases, namely a clear upper serum, a middle cloudy PBMC, and the lower debris and erythrocytes phase. Serum was collected into a fresh 50 ml falcon tube for later use (see below). The cloudy PBMC phase was suspended in PBS. The resulting suspension was centrifuged at 400 × *g* for 10 min at room temperature, the supernatant was discarded and the pellet was resuspended in 20 ml of PBS. Suspensions were spun again at 400 × *g* for 10 min. Pellets were resuspended in 10 ml of RPMI medium supplemented with 10% autologous serum.

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