The Role of *Haemophilus influenzae* Lipooligosaccharide in the Activation of Innate and Adaptive Immunity.

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by JOSHUA CHOI

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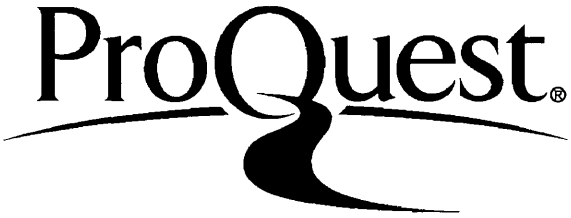

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Abbreviations

APRIL	A proliferation inducing ligand
APC	Antigen presenting cells
ASC	Apoptosis-associated speck-like protein containing a CARD
BAFF	B cell activating factor
BAFFR	B cell activating factor receptor
BCMA	B cell maturation antigen
BSA	Bovine serum albumin
COPD	Chronic Obstructive Pulmonary Disease
FBS	Fetal Bovine Serum
Hib	*Haemophilus influenzae* type b strain
IgG	Immunoglobulin G
IL	Interleukin
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption/ionization
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene 88
NAD	Nicotine adenine dinucleotide
NF-κB	Nuclear Factor Kappa B Transcription Factor
NLR	NOD-like receptors
NOD	nucleotide-binding oligomerization domain
NTHi	Non-typeable *Haemophilus influenzae*
OM	Otitis Media

PAFr	Platlet activating factor receptor
PAMP	Pattern association molecular patterns
PCho	Phosphorylcholine
PRR	Pattern recognition receptor
TACI	Transmembrane activator and calcium modulator
THP-1	Human monocyte derived from acute monocytic leukemia cell line
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
TRIF	TIR domain-containing adapter inducing IFN-β

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Chapter I: The innate immune response against LOS of *Haemophilus influenzae*

Abstract

A Gram negative human bacterial pathogen *Haemophilus influenzae* expresses a truncated endotoxin known as lipooligosaccharide (LOS). Recent studies on *H. influenzae* LOS have highlighted its structural and compositional implications on bacterial virulence; however, the role of LOS in the activation of innate and adaptive immunity is poorly understood.

THP-1 monocytes were stimulated with either lipopolysaccharide (LPS) from *E. coli* or LOS compounds derived from Eagan, Rd, and Rd *lic1lpsA H.influenzae* strains. Cell surface expression of key antigen-presenting, co-stimulatory, and adhesion molecules, as well as gene expression of some cytokines and pattern recognition receptors were studied.

Eagan and Rd LOS had a lower capacity to induce the expression of the pro-inflammatory molecules, ICAM-1, CD40, CD58, TNF-α, and IL-1β compared to LPS. In contrast, antigen presenting (HLA-ABC, HLA-DR) and co-stimulatory (CD86) molecules, and the pattern recognition receptor, NOD2, were similarly upregulated in response to LOS and LPS. LOS from a mutant Rd strain (Rd *lic1lpsA*) consistently induced higher expression of innate immune molecules than the wildtype LOS suggesting the importance of phosphorylcholine and/or oligosaccharide extension in cellular responses to LOS. An LOS compound with a strong ability to upregulate antigen-presenting and co-stimulatory molecules combined with a low pro-inflammatory activity may be considered as a vaccine candidate to immunize against *H. influenzae*.

1. Introduction

1.1 Innate Immune system

The innate immune system is often described as the first line of defence following microbial encounter by the host. Generally, the cells included in the innate immune repertoire are derived from myeloid progenitor stem cells in the bone marrow which then differentiate into various immune cells. These cells, among others, express a specific class of receptors known as pattern recognition receptors (PRR) which can recognize specific "non-self" patterns motifs on microorganisms. The types of motifs that are recognized are largely conserved patterns and therefore not subject to antigenic variability. In this regard PRRs can effectively distinguish between microbial and human patterns and initiate an immune response to combat infection. The limitation is that the repertoire of conserved motifs, also known as pattern association molecular patterns (PAMP), recognized by PRRs is narrow and finite.

One of these PRRs is Toll-like receptor 4 (TLR4), which has a critical role in activating the inflammatory response. It is the only known Toll-like receptor in humans to activate two signalling cascades (Lee and Kim 2007). These two pathways, the myeloid differentiation primary response gene 88 (MyD88) and the TIR domain-containing adapter inducing IFN-β (TRIF) pathway, activate the downstream transcription factor nuclear factor kappa B (NF-κB) and interferon regulatory factor 3/7 (IRF3/7), respectively (Lee and Kim 2007). NF-κB activation results in the secretion of pro-inflammatory cytokines whereas IRF3/7 induces type I interferon; these combined signals result in the recruitment of leukocytes to the site of infection and also in the priming of naïve T lymphocytes for the activation of the adaptive immune response.

Apart from outer-membrane PRRs, there are also intracellular PRRs; some of these include nucleotide-binding oligomerization domain (NOD)-like receptors (NLR). Among the identified NLRs, a select few have been found to be able to form larger protein complexes known as inflammasomes. The role of inflammasome in microbial infection has yet to be fully elucidated, however it has been established that inflammasome is required for the full maturation of cytokines IL-1β and IL-18 (Medzhitov 2007).

Antigen presentation is a key stage in activating T cells and requires the collaboration of several molecules (Fig. 1). HLA-ABC and HLA-DR are molecules directly involved in the presentation of peptide antigens to naïve T cells. HLA-ABC and HLA-DR are both part of larger molecular complexes known as major histocompatibility I (MHC I) and MHC II, respectively. The CD 86 molecule is also involved in antigen presentation and is recognized by its receptor on T cells, CD 28. Furthermore, CD86 provides a necessary co-stimulatory signal to activate naïve T cells. CD 40 is also a co-stimulatory molecule which binds to its ligand known as CD40L, on T cells. This interaction provides further internal signals in resting T cells allowing for the priming of the adaptive response. Finally, CD54 (ICAM-1), and CD 58 both act as cellular adhesion molecules by binding to their receptors, Lymphocyte function-associated antigen 1 (LFA-1) and Lymphocyte function-associated antigen 2 (LFA-2), respectively. Both of these interactions play integral roles in strengthening the adhesion between a resting T cell and antigen presenting cells (APC).

1.2 Biology of *Haemophilus influenzae*

Haemophilus influenzae is a Gram-negative, pleomorphic coccobacillus found in the respiratory tract of humans; its mode of transmission is through inhalation via aerosol droplets (Turk 1984).

H. influenzae acquires hemin and nicotine adenine dinucleotide (NAD) from the host environment, both of which are struict requirements for growth (Fink and GemeIII 2006). *H. influenzae* was first isolated during the influenza pandemic in 1889 and was thought to be the causative agent for the flu disease caused by influenza virus (Fink and GemeIII 2006). *H. influenzae* has a relatively small genome compared to other Gram-negative bacteria allowing the Rd KW-20 strain to be the first free living organism with its genome completely sequenced (Fleischmann, Adams et al. 1995).

H. influenzae strains have been classified into two classes contingent on the expression of a polysaccharide capsule. Strains expressing this capsule are known as encapsulated whereas unencapsulated strains are denoted as non-typeable (NTHi). There are six antigenically distinct polysaccharide capsules of the encapsulated serotypes designated as type a-f (Fink and GemeIII 2006). It has been well established that the type b encapsulated serotype (Hib), which contains a repeating unit of ribosyl-ribitol-phosphate (PRP) as its polysaccharide capsule, is significantly more virulent than the other serotypes (Moxon and Vaughn 1981, Turk 1984, Kimura and Hansen 1986, Zamze and Moxon 1987), and was one of the leading causes of meningitis and other invasive infections in young children (Turk 1984). Interestingly, more recent genomic studies have indicated that the Hib isolates show distinct genetic variability when compared to other capsule types (Power, Bentley et al. 2012). However, since the introduction of a PRP-conjugate vaccine in the late 1980s, invasive diseases caused by Hib have been virtually eliminated in developed countries (Kelly, Moxon et al. 2004). The dramatic decrease in the incidence rates of Hib infections has raised concerns of emerging infectious diseases caused by NTHi. Recent studies have documented increased rates of invasive NTHi disease in North America and Europe, especially among the elderly and immunocompromised individuals

(Dworkin, Park et al. 2007, Ladhani, Slack et al. 2010). Furthermore, there is a large body of work that corroborates the propensity of NTHi to cause disease (Swords, Buscher et al. 2000, Bouchet, Hood et al. 2003, Erwin and Smith 2007, Johnston, Zaleski et al. 2007, Schachern, Tsuprun et al. 2009, Hong, Peng et al. 2010).

The NTHi, as mentioned above, is the class of unencapsulated strains of *H. influenzae.* Although the polysaccharide capsule is a major virulence factor and seems to be a required component for invasive infections (Moxon and Vaughn 1981), NTHi has been implicated as the causative agent for respiratory tract infections. It is one of the leading causes of otitis media (OM) in children and exacerbations of chronic obstructive pulmonary disease (COPD) (Erwin and Smith 2007). Carriage rates of NTHi are up to 80% in human populations indicative of its commensal nature; however the specific mechanism behind the virulence factors that mediate the transition of NTHi from a commensal to pathogen is poorly understood. The exposed outer membrane of NTHi leaves the bacteria more susceptible to host clearance mechanisms; consequently, this causes a stronger selection for the expression of outer membrane moieties which are able to promote the bacteria's resistance to the host's defense pathways (Clark, Snow et al. 2012).

1.3 Lipooligosaccharide and its role in virulence

H. influenzae and a small subset of mucosal surface colonizing Gram-negative bacteria have been found to express lipooligosaccharide (LOS) rather than its more common counterpart lipopolysaccharide (LPS). LOS consists of a largely invariant tri-heptose oligosaccharide backbone covalently attached to a 3-deoxy-d-manno-oct-2-ulosonic acid moiety (KDO) otherwise known as the core region. Similar to LPS, the core region is covalently linked to the

endotoxin lipid A. The *H. influenzae* lipid A region is embedded in the outer membrane of Gram-negative bacteria which consits of an acylated glucosamine disaccharide backbone. LOS is a cognate ligand for TLR4 and plays a key role in the activation of the inflammatory response (Lee and Kim 2007). The main phenotypical difference with LPS, is that LOS lacks the repeating sugar additions on the distal end of the molecule, known as the "O-antigen," resulting in a rough, truncated phenotype. The truncated LOS of *H. influenzae* was first characterized by Flesher et al. (Flesher and Insel 1978); since then, the term lipooligosaccharide has been widely accepted in literature. However, despite the condensed structure, there is extensive inter- and intra-strain heterogeneity in the LOS glycoform populations. This heterogeneity is due to the variability of the oligosaccharide extensions that emanate from the tri-heptose core region which can be attributed to the molecular phenomenon known as phase variation (Swords, Jones et al. 2003, Schweda, Richards et al. 2007).

H. influenzae has several phase variable loci in its genome specific for LOS biosynthesis which results in a varying degree of expression from generation to generation. The common denominator sequence element among these loci is the presence of a tetranucleotide tandem repeat within the 5' end predisposing the open reading frames to slipped-strand mispairing during DNA replication (Maskell, Szabo et al. 1991, Swords, Jones et al. 2003). This simple mechanism removes the need for several regulatory factors that are normally expressed in larger genome bacteria whilst allowing for a varying expression of antigenic structures on the outer membrane (Swords, Jones et al. 2003).

The LOS of *H. influenzae* and its role in virulence has been of particular interest, especially for the NTHi strains, where the effect of LOS may be more prevalent than in the encapsulated

strains. Interestingly, an earlier study had shown LOS composition contributed to the pathogenicity of invasive infections caused by encapsulated *H. influenzae* (Zwahlen, Rubin et al. 1986), suggesting the virulence of LOS may not be limited to NTHi. Several studies have highlighted the role of LOS in bacterial virulence, i.e. in complement resistance (Griffin, Cox et al. 2005, Schweda, Richards et al. 2007, Langereis, Stol et al. 2012), adherence (Swords, Buscher et al. 2000), and host mimicry mechanisms (Bouchet, Hood et al. 2003, Johnston, Zaleski et al. 2007).

Adherence to the epithelial cells of the nasopharynx by *H. influenzae* is considered to be the initial interaction with the host to mediate colonization. Phosphorylcholine (PCho) expression on LOS has been implicated to mediate adherence and invasion by binding to the platelet activating factor receptor (PAFr) expressed on the epithelium (Swords, Buscher et al. 2000). Encapsulated strains seem to be far less efficient in their adherence which could be explained by their polysaccharide capsule essentially impeding the interactions between the bacteria and the host (Tomislav and Lena 2012). However NTHi strains, although much more susceptible to clearance by innate immune mechanisms, through various outer membrane structures, have evolved the ability to asymptomatically persist in the host without the protective coating of the polysaccharide which can be observed in the high carriage rates within the adult population. In addition to adherence, recent studies have looked into the role of PCho as a deterrent to bactericidal antibody activity (Clark, Snow et al. 2012). Although further investigation is warranted to assess the physiological implications in an *in vivo* environment, it is evident that PCho expression on LOS may be a multi-faceted component to virulence than initial thought.

It has been well established that sialic acid addition to bacterial outer membranes mediates host mimicry mechanisms. As *H. influenzae* cannot synthesize sialic acid, sialyation is contingent on the bacteria's capacity to scavenge N-acetyl-neuraminic acid (Neu5Ac) from the host. Similar to PCho addition, sialyation is a phase-variable occurrence encoded by lic1 and lic3 loci respectively (Weiser, Maskell et al. 1990, Weiser, Schchepetov et al. 1997). Sialyation has been found to confer serum resistance specifically with regards to the alternative complement pathway. Sialic acid binds to factor H (FH) which selectively inhibits C3b deposition (Hallstrom and Riesbeck 2010). Although this mechanism has not been completely elucidated, sialyation of LOS in *H. influenzae* has been found to act as a major virulence factor in experimental otitis media in chinchilla models (Bouchet, Hood et al. 2003).

1.3.1 LOS as a therapeutic target

As LOS is invariably present in all *H. influenzae* strains irrespective of their serotype, it can be considered as a potential vaccine candidate to prevent infections caused by various strains, including NTHi. It is noteworthy that there have been earlier studies that investigated the potential use of LOS based vaccines; however to our knowledge, clinical trials did not go past phase I (Gu, Rudy et al. 2003). Additionally, it is unknown whether LOS derived from different strains of *H. influenzae* have different immunostimulatory activity. The composition of LOS may have significant implications on its immunostimulatory capacity and therefore on the overall efficacy of a vaccine. The role of LOS as a potential vaccine adjuvant should also be addressed. The conserved region of lipid A is a TLR4 ligand and is responsible for the intensity of the inflammatory response. There have been several studies which have focused on utilizing the stimulatory properties of lipid A while minimizing the potential toxic side effects (Lewicky,

Ulanova et al. 2011). Although lipid A of LPS is a potent immunostimulator, it is extremely toxic and has been found to induce septic shock when injected systemically (Opal, Scannon et al. 1999), however this may not be the case for lipid A of LOS and is currently under investigation. In either case, there is a need for effective vaccine adjuvants that can boost the immune response to a specific antigen while minimizing potential exacerbations of systemic inflammation.

1.4 Rationale

The LOS of *H. influenzae* has been an area of significant research; however the fundamental question regarding the role of *H. influenzae* LOS as an immunostimulatory molecule has not been addressed and is poorly understood. An earlier study by Khair et al. looked at the expression of ICAM-1 and cytokines TNFα, IL-6, and IL-8 in human bronchial epithelial cells and found a significant increase in response to LOS stimulation (Khair, Devalia et al. 1994). Although this suggests that LOS has a role in the induction of the inflammatory response, it does not address the issue of whether LOS can act as an activator of the innate immune response and subsequent adaptive response. Another study found LOS of *Histophilus somni* to engage the NF-κB transcription factor, a potent activator of the innate immune response (Howard, Willis et al. 2011); however the capacity of LOS to induce key co-stimulatory molecules indicative of the innate response and essential for modulating the bridge to adaptive immunity is unknown.

2. Study Objectives

In this study we assessed the immunostimulatory capacities of three LOS compounds derived from 2 different strains of *H. influenzae* (Fig. 2) by measuring the expression of key co-stimulatory and antigen-presenting molecules in human monocytic cells. Furthermore, the effect of LOS on pro-inflammatory and anti-inflammatory cytokine responses was measured. The results were compared to the effects of *E. coli* LPS, which has been previously studied and served as a reference for data interpretation (Lewicky, Ulanova et al. 2011). A LOS compound with a strong ability to stimulate the expression of co-stimulatory and antigen-presenting molecules combined with a low potential to stimulate pro-inflammatory responses may be considered as a potential vaccine candidate.

3. Materials and Methods

2.1 Cell culture conditions

Human THP-1 monocytic leukemia cell line was obtained from ATCC (TIB-202) and stored in liquid nitrogen until thawed for culturing. Cells were maintained at 37°C, 5% CO_2 in RPMI-1640 media (Sigma-Aldrich, Oakville, ON, CAN) supplemented with 10% heat-inactivated fetal bovine serum (SAFC Biosciences, Lenexa, KS, USA) and 200 µl antibiotic–antimycotic (Life Technologies Inc. Burlington, ON, CAN). Cells were passaged when the culture density reached 1.0 x10^6 cells/ml; viability was determined by the trypan blue exclusion method using a ViCell XR Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA).

2.2 Purification of LOS compounds and analysis

The LOS compounds were donated by Dr. A. Cox of the National research Council (Ottawa, ON). The compounds were purified from *H. influenzae* strains Eagan (Hib), Rd (unencapsulated type d), and Rd *lic1lpsA* (mutant) (Fig. 1) as previously described (Zhou, Altman et al. 2010). Profiling of fatty acid additions to lipid A was done via Matrix-assisted laser desorption/ionization (MALDI) analysis in Dr. Cox's laboratory (Zhou, Altman et al. 2010). *E.coli* LPS 0111:B4 was obtained from Sigma-Aldrich (St. Louis, MO, USA). All compounds were solubilized in sterile distilled H_2O at a concentration of 1 mg/ml, aliquoted and stored at -20°C until use.

2.3 Cell stimulation and flow cytometry analysis

THP-1 cells were plated at 0.5 x 10^6 cells/2 ml/well in 12-well plates (Fisher Scientific, Fairlawn, NJ, USA), incubated in 37°C, 5% CO_2 for 24 hours and stimulated with LOS compounds at concentrations of 1, 5, 10, and 15 µg/ml for an additional 24 hours at 37°C, 5% CO_2. Following

stimulation, the cells were washed once with sterile PBS, centrifuged at 400 x g for 10 minutes and resuspended in PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich). Cells were immunostained with fluorochrome-conjugated antibodies against ICAM-1, CD40, CD86 (B7-2) (BD Biosciences, Mississauga, ON, CAN), CD58 (LFA-3) (Cedarlane, Burlington, ON, CAN), HLA-DR (MHC class II) (Biolegend, San Diego, CA, USA), or mouse IgG1 isotype control at a concentration of 2.5 µg/ml. Primary unconjugated HLA-ABC (MHC class I) antibody (BD Biosciences) was added to cell suspension at a concentration of 5 µg/ml. All samples were incubated in the dark for 1 hour at 4°C. Samples with directly conjugated primary antibodies were washed once with PBS, centrifuged at 1000 x g for 5 min and resuspended in 500 µl PBS for flow cytometry analysis. Samples with unconjugated primary antibodies were washed as above, resuspended in 1% BSA and incubated with secondary FITC-labelled rat anti-mouse IgG1 antibody (BD Biosciences) at a concentration of 2.5 µg/ml for 1 hour at 4°C. Following incubation with the secondary antibody, samples were washed with PBS and resuspended in 500 µl PBS for flow cytometry analysis. All samples were analyzed using a FACSCalibur flow cytometer with CELLQUEST PRO software (BD Biosciences), acquiring 15,000 total events. The results were expressed as the mean fluorescence intensity (MFI) on FL-1 and FL-2 channels for FITC and PE conjugated antibodies, respectively.

2.4 Gene expression analysis

RNA was isolated from THP-1 cells using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) following stimulation with LPS or LOS compounds for 4 hours. RNA integrity was determined using the Experion system (Bio-Rad); product with "RNA Quality Indicator" values greater than 9 were used for downstream applications. 500 ng of total RNA was reverse

transcribed using the First Strand cDNA Synthesis kit (Fisher Scientific) as per the manufacturer's instructions. 25 ng of the synthesized cDNA was used for each reaction well including 10 µl of RT^2 SYBR® Green Fluor qPCR Mastermix (Qiagen, Toronto, ON, CAN), and 1 µl of primer sets TNFα, IL-1β, IL-10, TLR4, NOD1, NOD2 (SA Biosciences, Mississauga, ON, CAN) for a total reaction volume of 20 µl. PCR was performed using the iQ5 real-time PCR detection systems (Bio-Rad); samples were pre-heated at 95°C for 10 min followed by 40 cycles of the two-step cycling program. The denaturing temperature was set for 15s at 95°C followed by 1 min at 60°C where annealing, fluorescence detection, and elongation occurred. The melt curve program immediately followed from 55°C to 95°C with plate reads every 0.5°C. The cycle threshold (Ct) values were used to compare relative amounts of measured transcripts, calculated as $\Delta Ct = 2^{-(Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}})}$. To account for variability in the starting template amount this expression was normalized to housekeeping gene Peptidylprolyl isomerase B (PPIB) and the relative gene expression was calculated as $\Delta\Delta Ct = 2^{-(\Delta Ct_{\text{experimental}} - \Delta Ct_{\text{control}})}$.

2.5 Statistical analysis

Data were expressed as mean of 3 independent experiments. Statistical significance was determined (GraphPad Prism 5.0, La Jolla, CA, USA) using one way ANOVA with Newman-Keuls Multiple Comparison post-hoc test. *P<0.05, **P<0.01, ***P<0.001 indicate significant difference compared to unstimulated control. $^{+}$P<0.05, ++P<0.01, +++P<0.001 indicate significant difference between Rd *lic1lpsA* and Rd wild type compounds. •P<0.05, ••P<0.01, •••P<0.001 indicate significant difference between LPS and LOS compounds.

4. Results

3.1 Characterization of lipid A of LOS isoforms by Matrix-Assisted Laser Desorption/Ionization analysis.

The Lipid A structure consists of an invariant di-phosphorylated glucosamine disaccharide with a varying number of acyl chains. The MALDI analysis was performed on the lipid A of each LOS compound and the relative intensity of acylation was determined (Table 1). The major ion with the highest intensity for each compound was given an arbitrary unit of 1.0; all other ions were expressed relative to that. For each compound lipid A structures acylated with 4 fatty acid chains exhibited the highest intensity. LOS from Eagan and Rd had comparable ion intensities for all fatty acid additions; however, Rd *lic1lpsA* LOS exhibited different intensities at both 3 and 6 fatty acid additions at 0.25 and 0.7, respectively. This suggests that there is a higher population of 6-acylated lipid A structures in LOS Rd *lic1lpsA* and a smaller population of 3-acylated lipid A compared to both Eagan and Rd LOS compounds.

3.2 *H. influenzae* LOS exhibits decreased immunostimulatory abilities compared to *E. coli* LPS

The capacity of LOS from *H. influenzae* to activate the innate immune response or induce co-stimulatory signals for adaptive immunity has not been studied. To elucidate this question, cell surface expression of ICAM-1, CD40 and CD58 (LFA-3) was measured on THP-1 monocytic cells in response to LOS stimulation using flow cytometry analysis. LPS from *E. coli* strain 0111:B4, a potent innate immune activator served as a positive control (Lewicky, Ulanova et al. 2011). Cell viability was not significantly affected by either LOS or LPS compounds (Table 2). Unstimulated THP-1 cells were used as a negative control.

The adhesion molecule ICAM-1 was found to be constitutively expressed on unstimulated control (MFI ≈ 10). ICAM-1 was significantly upregulated in response to stimulation with both Eagan and Rd *lic1lpsA* LOS compounds at 1 μg/ml (Fig. 3) ($P<0.01$ and $P<0.001$ respectively). LOS of the wild type Rd strain did not induce a significant increase over negative control at 1 μg/ml; however, a significant dose response was observed at higher concentrations (5-15 μg/ml) ($P<0.001$). Both the Rd and Eagan LOS compounds induced lower expression of ICAM-1 compared to *E. coli* LPS at 1 μg/ml ($P<0.001$); LOS of the Rd *lic1lpsA* induced ICAM-1 expression comparable with the effect of LPS. The effect of the Rd *lic1lpsA* LOS was consistently higher compared to the wildtype, Rd LOS (up to 10 μg/ml). Similar results were observed with regards to the expression of CD40 and CD58 (Fig. 4, 5), where LOS compounds from both Eagan and Rd strains induced significantly less expression compared to LPS whereas Rd *lic1lpsA* LOS induced comparable expression.

In summary, LOS from various strains of *H. influenzae* induced significant upregulation of all studied cell surface molecules on human monocytic cell line compared to unstimulated cells. Rd and Eagan LOS compounds induced significantly less expression of ICAM-1, CD40 and CD58 compared to LPS whereas Rd *lic1lpsA* induced expression comparable to the effect of LPS. The data suggest that LOS of *H. influenzae* exhibits a lower immunostimulatory potential that may be dependent on the LOS structure.

3.3 LOS significantly upregulates antigen presenting and co-stimulatory molecules

To address whether LOS would activate the adaptive immune response, the expression of antigen presenting molecules, HLA-ABC (MHC class I) and HLA-DR (MHC class II), as well as major

co-stimulatory molecule in the T-cell antigen presenting cells axis, CD86 (B7-2), were measured in THP-1 cells stimulated with either *H. influenzae* LOS or *E.coli* LPS.

The CD86 expression was significantly upregulated in response to Eagan and Rd *lic1lpsA* LOS compounds at a concentration of 1 μg/ml ($P<0.01$ and $P<0.001$, respectively). For all compounds, a significant positive dose response was observed (Fig. 6) ($P<0.001$). With exception to Rd LOS, there was no observed difference in expression between LPS and LOS compounds at 1 μg/ml; furthermore, LOS from Rd *lic1lpsA* did not confer an increased expression of CD86 compared to then wildtype, even at higher concentrations.

LOS compounds from Eagan, Rd and Rd *lic1lpsA* induced significant expression of HLA-ABC and HLA-DR that was comparable to the positive control, LPS at 1 μg/ml (Fig. 7, 8) ($P<0.001$). At higher concentrations of all the compounds (5-15 μg/ml), a plateau effect was observed.

Our findings show that LOS of *H. influenzae* is able to significantly increase expression of both the antigen presenting molecules and the co-stimulatory molecule, B7-2, that is similar to the effect of LPS suggesting that LOS can act as a potent activator of the adaptive immune response.

3.4 *H. influenzae* LOS has decreased capacity to activate pro-inflammatory response

To further address the immunostimulatory capacity of LOS, gene expression of pro-inflammatory cytokines TNF-α and IL-1β were measured following 4 hours stimulation at 0.1 and 1 μg/ml. LPS from *E.coli* was used as a positive control. Gene expression of anti-inflammatory cytokine IL-10 was also measured via real-time PCR. Gene expression was normalized to housekeeping gene PPIB, and presented in fold change relative to unstimulated control.

All LOS compounds induced significant upregulation of pro-inflammatory cytokines TNF-α and IL-1β (Fig. 9, 10) ($P<0.001$). However, similar to the effect on ICAM-1, CD40 and CD58, LOS of Eagan and Rd strains induced significantly less expression of both TNF-α and IL-1β when compared to LPS. In contrast, Rd *lic1lpsA* LOS stimulation resulted in a 5-fold increase in IL-1β expression compared to LPS at a concentration of 1 μg/ml. Furthermore, Rd *lic1lpsA* LOS stimulation at the concentrations of 0.1 and 1 μg/ml resulted in consistently higher gene expression of both pro-inflammatory cytokines compared to the wildtype.

Out of all of the LOS compounds, only Rd *lic1lpsA* LOS was able to induce a significant upregulation of the anti-inflammatory cytokine IL-10 gene expression ($P<0.05$) (Fig 11). Both Eagan and Rd LOS induced less expression of IL-10 compared to LPS, however only the effect of Eagan LOS was statistically significant ($P<0.05$).

Our findings show Eagan and Rd LOS compounds were able to elicit transcriptional upregulation of key pro-inflammatory cytokines TNF-α and IL-1β, however the response was significantly attenuated when compared to LPS of *E.coli*. Rd *lic1lpsA*LOS was shown to consistently induce higher cytokine gene expression when compared to the wildtype or LPS at 1 μg/ml.

3.5 *H. influenzae* LOS induces upregulation of intracellular pattern recognition receptors

In addition to inflammatory markers, we wanted to address whether LOS of *H. influenzae* would affect the genetic regulation of pattern recognition receptors (PRRs) such as TLR4, the cognate LOS receptor and intracellular PRRs such as NOD1 and NOD2. Gene expression was determined as described above.

TLR4 gene expression in THP-1 cells did not significantly change upon stimulation with any compound except for Rd *lic1lpsA* at the concentrations of 0.1 and 1 μg/ml ($P<0.05$) (Fig 12). The gene expression of NOD1 was not affected by any of the compounds (Fig.13), whereas NOD2 expression was significantly upregulated in response to all compounds at both concentrations ($P<0.001$) (Fig. 14).

Our findings show that NOD2 expression was increased by 4- to 6-fold with no observed differences between LPS or LOS stimulation. This suggests a possible relationship between the recognition of LOS and further downstream activation of the inflammasome complex.

5. Discussion

Although invasive disease caused by *H. influenzae* has decreased considerably since the introduction of Hib conjugate vaccines, there is a concern for emerging infections caused by non-type b serotypes. This can partly be explained by the serotype replacement phenomenon where there is less competition for non-type b strains to colonize the ecological niche once occupied by Hib (Berndsen, Erlendsdottir et al. 2012). Interestingly during the last decade, there has been an increase in reported invasive disease caused by NTHi, which was traditionally considered as a mucosal pathogen (Bamberger, Ben-Shimol et al. 2014). Whatever the cause behind the emergence of invasive non-type b serotypes, there is a pressing need for novel therapeutic treatments that would target all strains of *H. influenzae* regardless of serotype.

The *H. influenzae* LOS has several roles in bacterial virulence and has been the subject of significant research in the past decade; however its potential role in vaccine development has been of less interest. Although LOS is invariably present in all strains of *H. influenzae,* it shows extensive inter and intra-strain heterogeneity (Swords, Jones et al. 2003). Consequently, the implementation of LOS as a vaccine target has been difficult. Nonetheless, some recent findings have been promising, for instance, protection against otitis media, caused by NTHi, was observed in chinchillas that were immunized with LOS conjugate vaccines (Hong, Peng et al. 2010). Moreover, lipid A of LOS had been shown to be able to activate the TLR4 signalling cascade, namely the MyD88 pathway (Khair, Devalia et al. 1994). Therefore, LOS can potentially act as its own vaccine adjuvant as well as a general adjuvant which may be a more suitable candidate as an adjuvant than current monophosphoryl lipid A (MPL) adjuvant. Studies have shown the monophosphorylated nature of the MPL compound selectively activates the

TRIF pathway of the TLR4 signalling cascade (Mata-Haro, Cekic et al. 2007, Bowen, Minns et al. 2012). However, it may be more beneficial for a lipid A compound to activate both TRIF and MyD88 pathways without the toxic pro-inflammatory side effects.

It is important to note that LPS was not tested at concentrations greater than 1 μg/ml and that all statistical comparisons between LOS and LPS were performed at this concentration. To address whether the observed effect of LOS stimulation was indeed dose dependent, higher concentrations from 5-15 μg/ml were tested for only LOS compounds. A dose response was not tested for LPS because higher doses have been previously implicated in septic shock models and would have been clinically irrelevant for our purposes (Priest, Schlievert et al. 1989). Thus a concentration of 1 μg/ml LPS was used as a reference which had been optimized in several studies (Maaetoft-Udsen, Vynne et al. 2013, Nebel, Arvidsson et al. 2013).

Our findings show that LOS derived from Eagan and Rd strains of *H. influenzae* were able to elicit an *in vitro* innate immune response resulting in the upregulation of co-stimulatory and antigen presenting molecules, HLA-ABC, HLA-DR and CD86, comparable to that of LPS. These surface molecules play critical roles in the priming of naïve T cells essential for the activation of the adaptive immune response.

We also found that Eagan and Rd LOS had a decreased capacity to stimulate the surface expression of CD40, CD58 and ICAM-1, as well as gene expression of TNF-α and IL-1β, compared to LPS. ICAM-1, TNF-α, and IL-1β are critical mediators of inflammation where high expression has been correlated with potent pro-inflammatory responses via the recruitment of immune cells and the propagation of inflammatory cytokines (Lisby, Ralfkiaer et al. 1989, Cromwell, Hamid et al. 1992). This suggests that Eagan and Rd LOS elicits a decreased pro-

inflammatory effect compared to LPS making them attractive candidates for vaccine development.

All compounds were found to significantly upregulate NOD2 which has been found to directly interact with NLRP1, NLRP3 and NLRP12 inflammasomes (Moreira and Zamboni 2012). In contrast, NOD1 was unaffected by either LOS or LPS; indeed, no relationship between the inflammasome complexes and NOD1 have been previously identified (Moreira and Zamboni 2012). The addition of NOD2 activation widens the scope of our model, aside from inflammasome activation, NOD2 has been shown to interact with several different signalling pathways (Moreira and Zamboni 2012). Although the exact role of NOD2 in bacterial infection has yet to be fully elucidated, the activation of NOD2 has been implicated in T cell differentiation and host response to pulmonary pathogens such as *Chlamydophila pneumonia* (Shimada, Chen et al. 2009). This suggests that the role of LOS during infection may not be limited to just the inflammatory response.

To address whether LOS stimulation had a toxic effect on the cells at various concentrations, cell viability was measured. It was found that LOS had no effect on viability with exception of the highest concentration, i.e. 15 μg/ml (Table 2). However, in our experiments, this concentration did not have any significant biological impact; a plateau effect was observed for the majority of surface molecules at 10 μg/ml.

Rd *lic1lpsA*LOS was found to consistently induce a stronger effect than the wildtype on the expression of surface molecules ICAM-1, CD40, CD58 and pro-inflammatory cytokines TNF-α and IL-1β. The main compositional differences between Rd *lic1lpsA* and Rd are the deletions of phosphorycholine (PCho) at Heptose I and oligosaccharide extension at Heptose III (Fig. 1).

Previous work has shown that the loss of this oligosaccharide extension in this strain resulted in a decrease in complement resistance (Griffin, Cox et al. 2005). Our findings add to this by suggesting that the oligosaccharide extension may also have a role in attenuating the expression of aforementioned inflammatory markers as can be observed by the wildtype, Rd LOS. Moreover, PCho has been implicated in numerous studies as a virulence factor and adhesion molecule that enhances immune evasion by *H. influenzae* (Swords, Buscher et al. 2000, Clark, Snow et al. 2012). Whether PCho also has a role in reducing the inflammatory response *in vivo* has yet to be elucidated.

The number of acyl chains on the lipid A of each compound is of interest and should be addressed. Rd *lic1lpsA* LOS was found to have relatively less tri-acylated lipid A isoforms and considerably higher amounts of hexa-acylated lipid A compared to both Eagan and Rd lipid A (Table 1). Previous studies have shown that the immunostimulatory properties of LPS molecules are determined by the structure of lipid A (Schromm, Brandenburg et al. 2000). Furthermore, a correlation can be found with acylation of lipid A and an inflammatory response, where increased acylation is generally associated with more potent inflammatory responses and low acylation is associated with a weaker response (Maaetoft-Udsen, Vynne et al. 2013). This suggests that the pro-inflammatory capacity of Rd *lic1lpsA* LOS observed in our experiments may also be due to the acylation state of its lipid A. A recent study shows another Gram-negative pathogen, *Shigella flexneri* is able to modify the acylation state of its lipid A following invasion, thereby enhancing its immune evasion (Paciello, Silipo et al. 2013). This finding has not yet been observed in *H. influenzae* LOS and therefore further research is warranted to determine how much of the observed immunostimulatory effect is attributed to acylation and/or compositional motifs.

The balance between inflammatory and anti-inflammatory cytokines has been well described. It has been found that a strong inflammatory response is correlated with a strong anti-inflammatory response in order to maintain equilibrium (Ostrowski, Rohde et al. 1999). Unlike other LOS compounds, Rd *lic1lpsA* LOS alone was able to significantly increase IL-10 expression, which was expected since it also had the largest effect on the pro-inflammatory markers compared to other LOS compounds.

In summary, we have shown that LOS of *H. influenzae* strains, Eagan and Rd, are able to induce the upregulation of key co-stimulatory and antigen presenting molecules in human monocytic cells, as well as the production of inflammatory cytokines. Although these compounds were able to stimulate the expression of pro-inflammatory markers, their effect was markedly diminished in comparison to the toxic LPS compound of *E. coli.* Furthermore, all LOS compounds were found to upregulate NOD2 which has further downstream implications for the inflammasome complexes. Rd *lic1lpsA* LOS was found to consistently induce a stronger response than the wildtype on surface molecules and cytokines indicative of inflammation. However this was not observed for antigen presenting and co-stimulatory molecule expression. Whether this effect is due to the PCho or oligosaccharide deletion on Rd *lic1lpsA* LOS compound is yet to be determined. As LOS is invariably present in all *H. influenzae* strains it makes it an attractive candidate for vaccine development. Furthermore, the moderate immunostimulatory ability of LOS may be of consideration for potential adjuvant development and may offer a more robust response than current TLR4 agonist adjuvants.

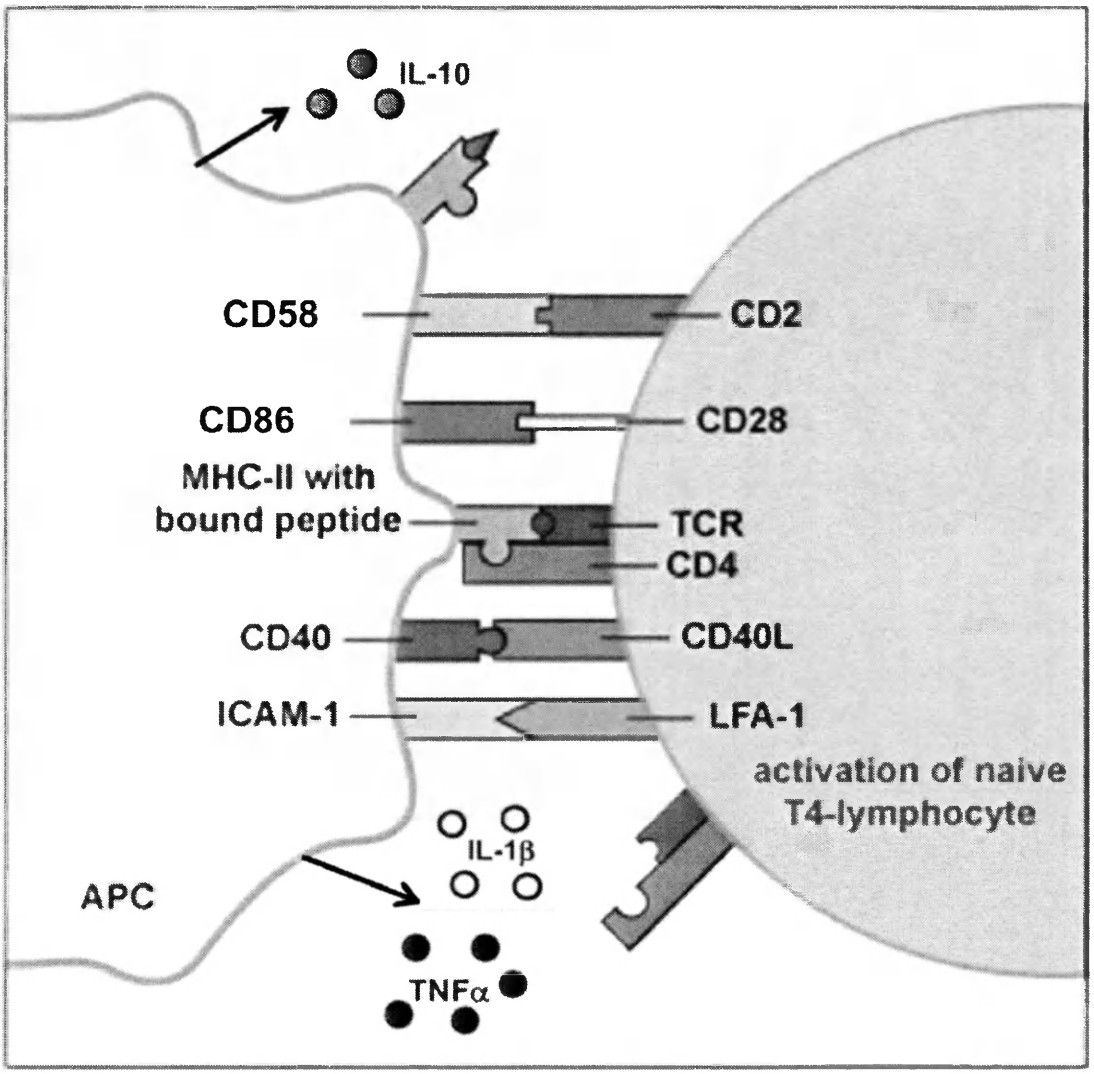
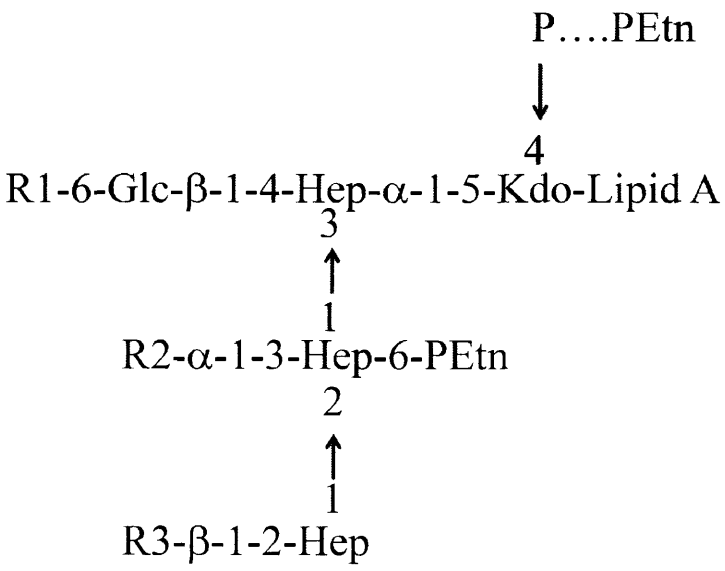


Fig 1. A schematic diagram of the various molecules involved in antigen presentation. Molecules of interest include: ICAM-1, CD40, MHC I, MHC II, CD 58, CD86 and cytokines, TNF-α, IL-1β, IL-10. This figure was taken and modified from The Community College of Baltimore County lecture slides available online.



For Rd: R1=PCho, R2=H & R3=GalNAc-β-1-3-Gal-α-1-4-Gal-β-1-4-Glc

For Rd *lic1lpsA*: R1=H, R2=H & R3=H

For Eagan: R1=H, R2=Gal-α-1-4-Gal-β-1-4-Glc-β-1-4-Glc & R3=Gal

Figure 2. A schematic representation of LOS from *H. influenzae* strains Eagan, Rd, and Rd *lic1lpsA* as determined by MALDI. Represented in the LOS structure: (Kdo) 2-keto-3-deoxyoctulosonic acid; (Hep) heptose; (Glc) glucose; (Gal) galactose; (GalNAc) N-acetylgalactosamine; (P) phosphate; (PCho) phosphorylcholine; (PEtn) phosphoethanolamine.

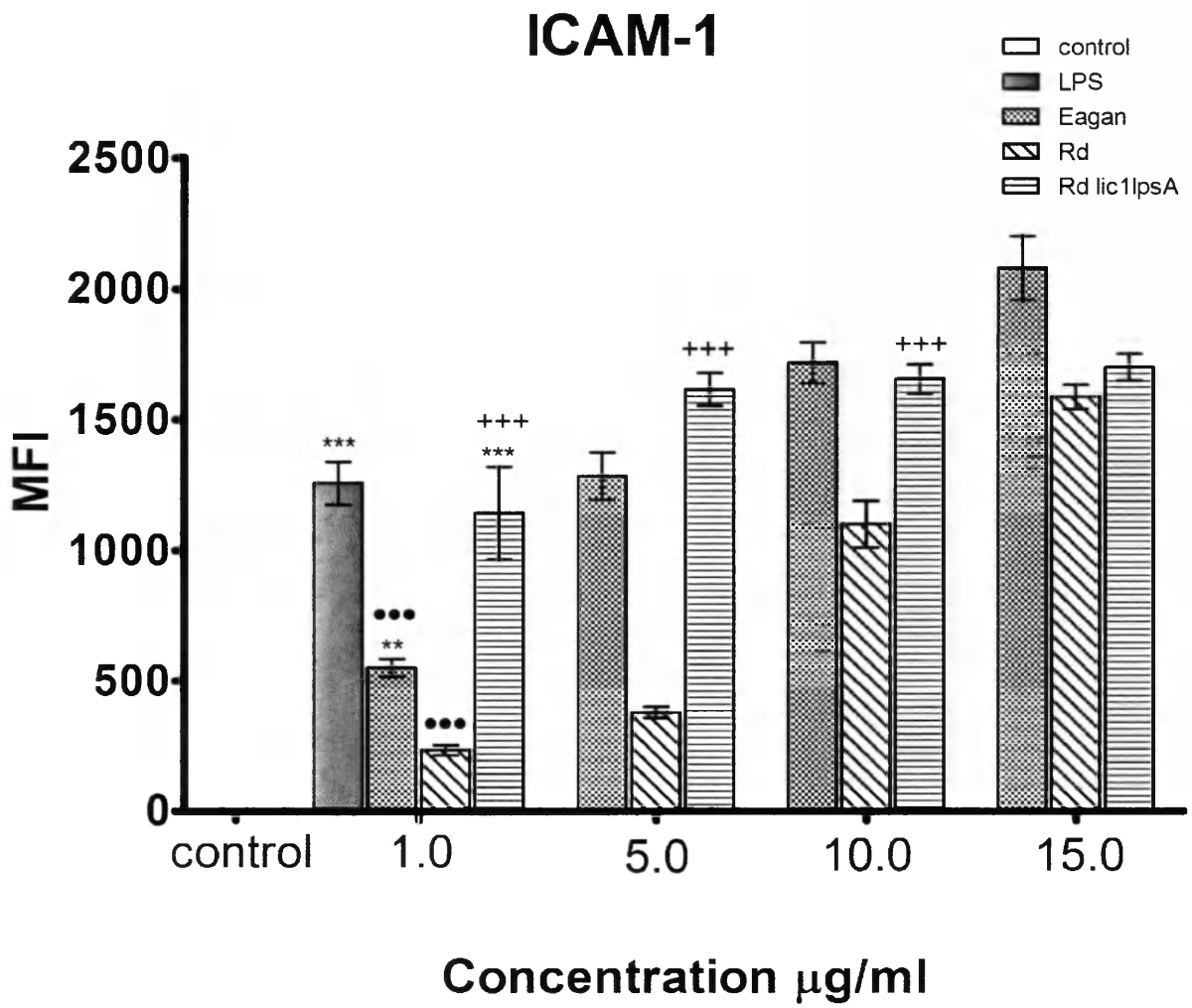


Figure 3. Flow cytometry analysis of ICAM-1 expression in response to LOS stimulation on THP-1 cells. Negative control was untreated, medium alone where LPS stimulation at 1 μg/ml was the positive control. THP-1 cells were stimulated with LOS compounds for 24 hours at concentrations 1, 5, 10, and 15 μg/ml and cell expression was measured. Data represents the mean fluorescence intensity (MFI) ± SEM (n=3).

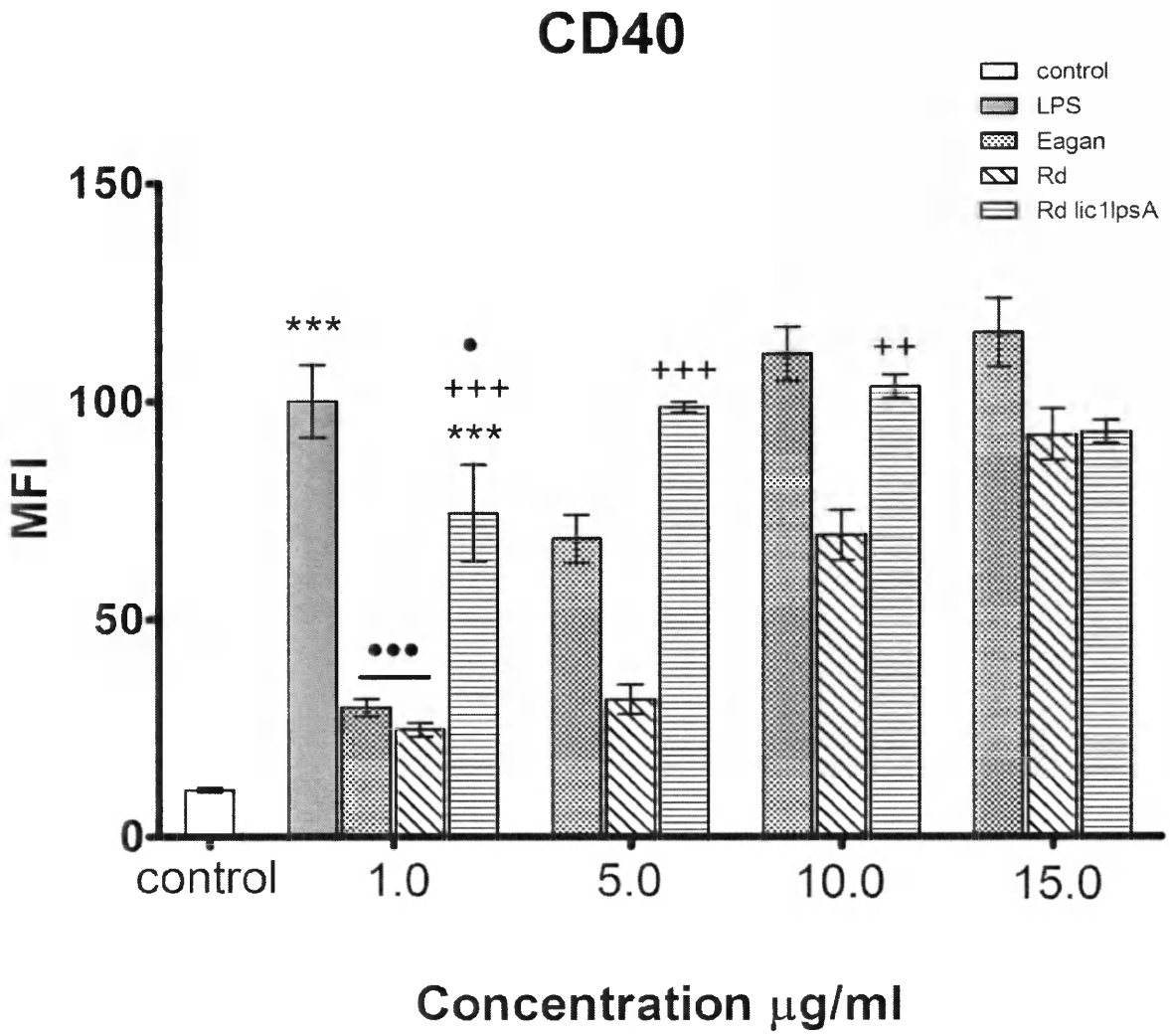


Figure 4. Flow cytometry analysis of CD40 expression in response to LOS stimulation on THP-1 cells. Negative control was untreated, medium alone where LPS stimulation at 1 μg/ml was the positive control. THP-1 cells were stimulated with LOS compounds for 24 hours at concentrations 1, 5, 10, and 15 μg/ml and cell expression was measured. Data represents the mean fluorescence intensity (MFI) ± SEM (n=3).

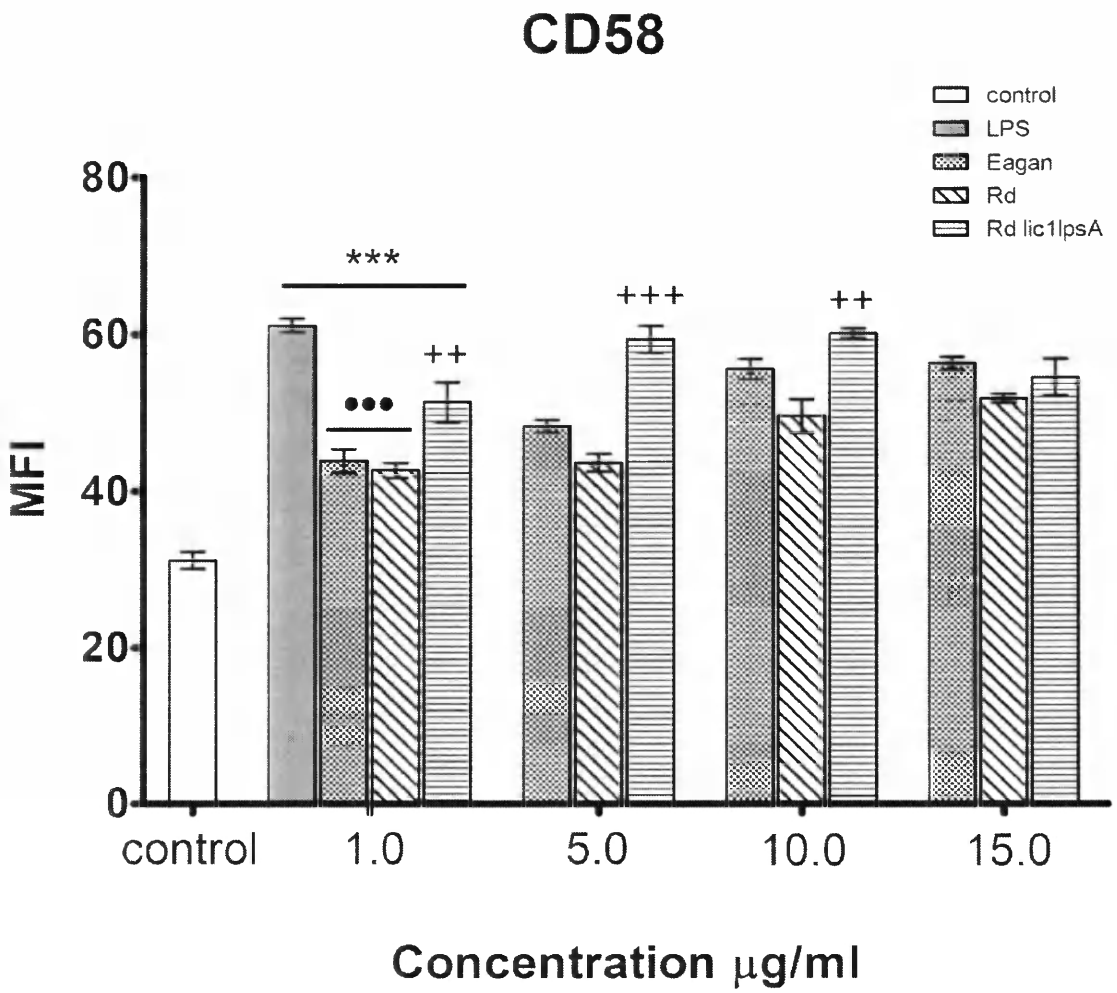


Figure 5. Flow cytometry analysis of CD58 expression in response to LOS stimulation on THP-1 cells. Negative control was untreated, medium alone where LPS stimulation at 1 µg/ml was the positive control. THP-1 cells were stimulated with LOS compounds for 24 hours at concentrations 1, 5, 10, and 15 µg/ml and cell expression was measured. Data represents the mean fluorescence intensity (MFI) ± SEM (n=3).

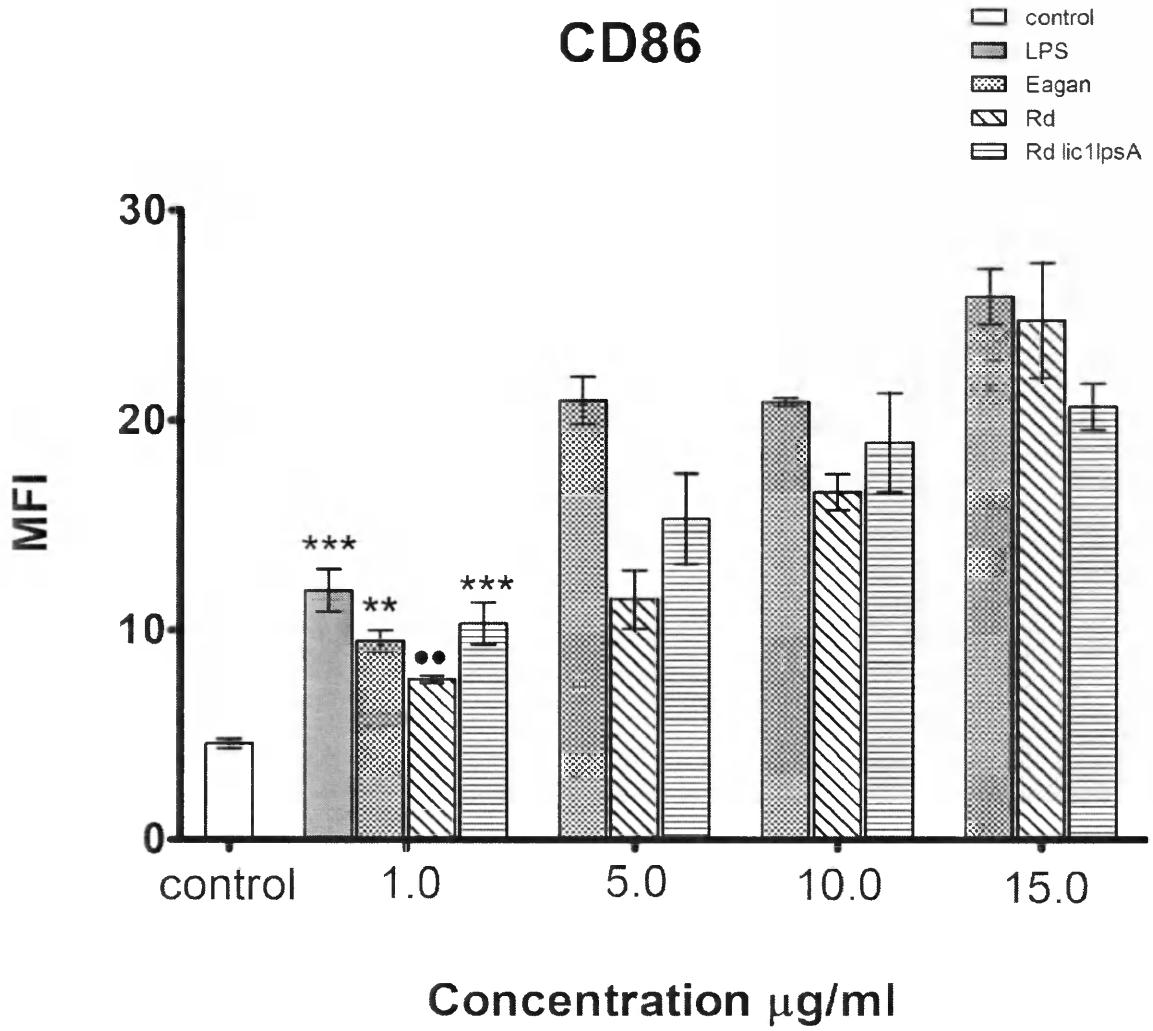


Figure 6. Flow cytometry analysis of CD86 expression in response to LOS stimulation on THP-1 cells. Negative control was untreated, medium alone where LPS stimulation at 1 μg/ml was the positive control. THP-1 cells were stimulated with LOS compounds for 24 hours at concentrations 1, 5, 10, and 15 μg/ml and cell expression was measured. Data represents the mean fluorescence intensity (MFI) ± SEM (n=3).

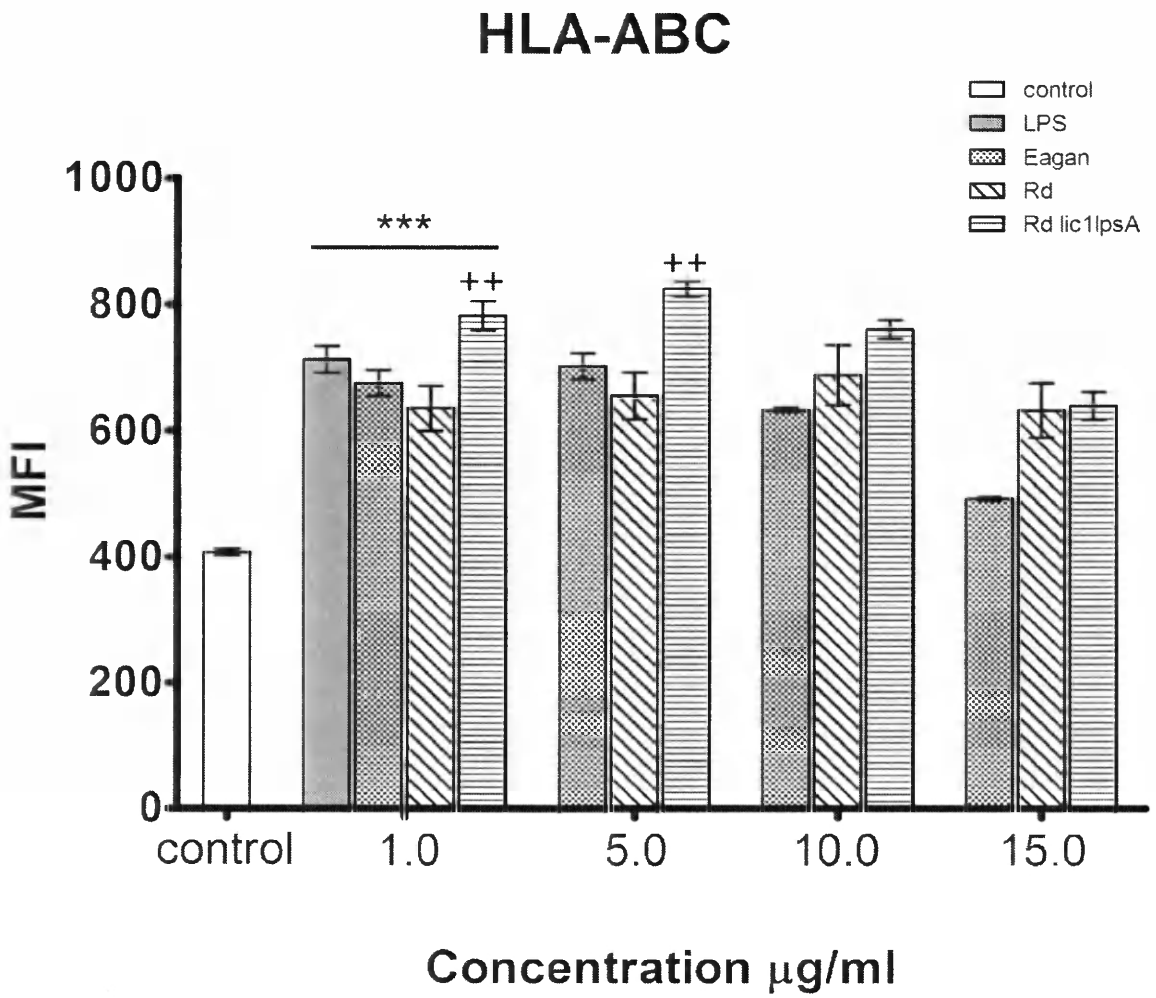


Figure 7. Flow cytometry analysis of HLA-ABC expression in response to LOS stimulation on THP-1 cells. Negative control was untreated, medium alone where LPS stimulation at 1 μg/ml was the positive control. THP-1 cells were stimulated with LOS compounds for 24 hours at concentrations 1, 5, 10, and 15 μg/ml and cell expression was measured. Data represents the mean fluorescence intensity (MFI) ± SEM (n=3).

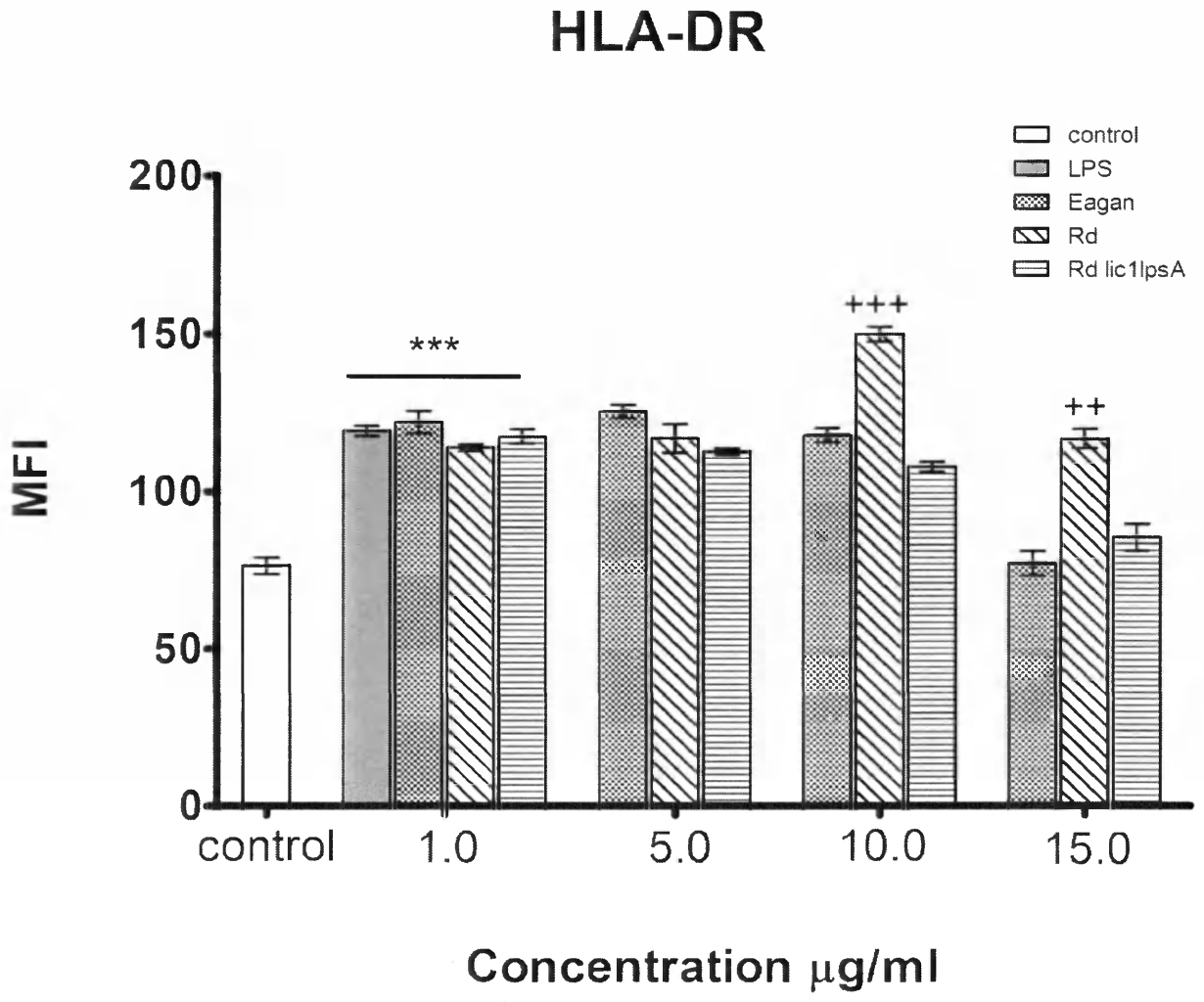


Figure 8. Flow cytometry analysis of HLA-DR expression in response to LOS stimulation on THP-1 cells. Negative control was untreated, medium alone where LPS stimulation at 1 µg/ml was the positive control. THP-1 cells were stimulated with LOS compounds for 24 hours at concentrations 1, 5, 10, and 15 µg/ml and cell expression was measured. Data represents the mean fluorescence intensity (MFI) ± SEM (n=3).

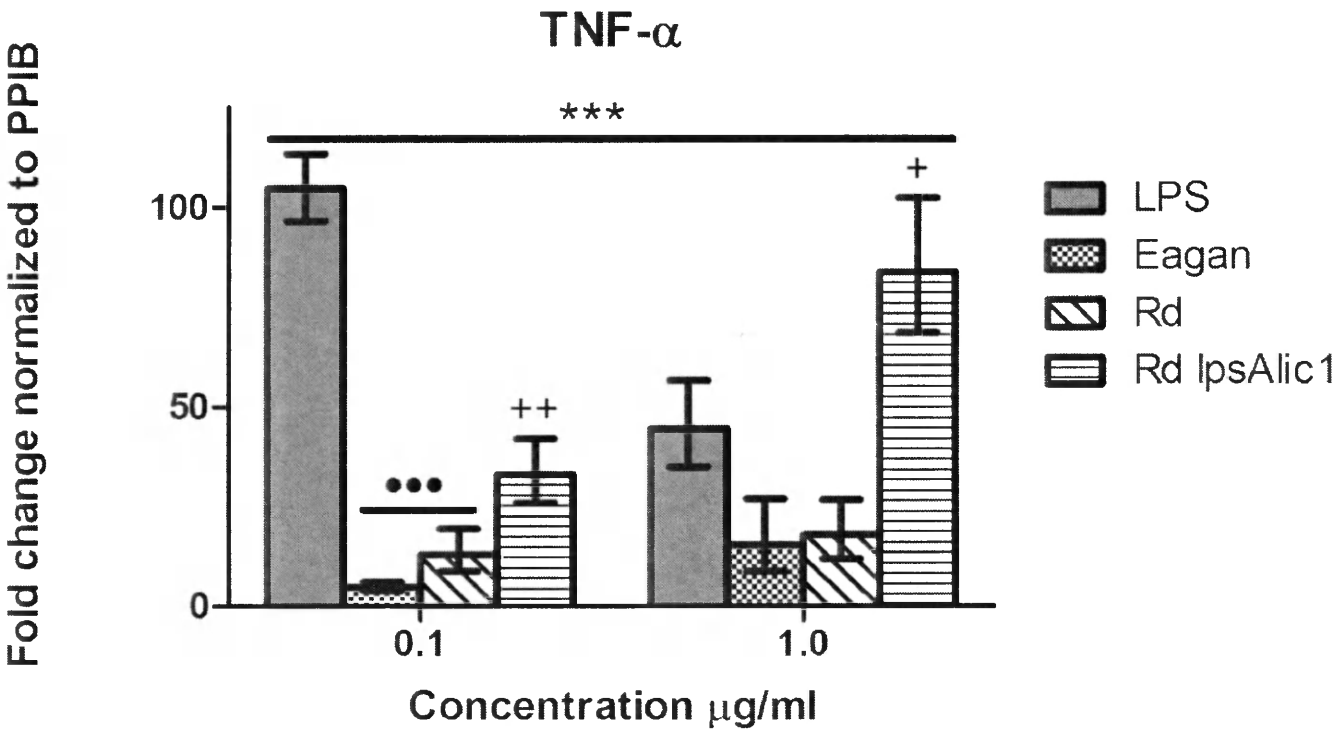


Figure 9. Relative gene expression of TNF-α in response to LOS stimulation. THP-1 cells were stimulated with LOS compounds for 4 hours at concentrations 0.1 and 1 μg/ml. RNA was extracted and genetic expression was measured using real-time PCR and presented as fold change relative to unstimulated control. Data represents mean ± SEM (n=3).

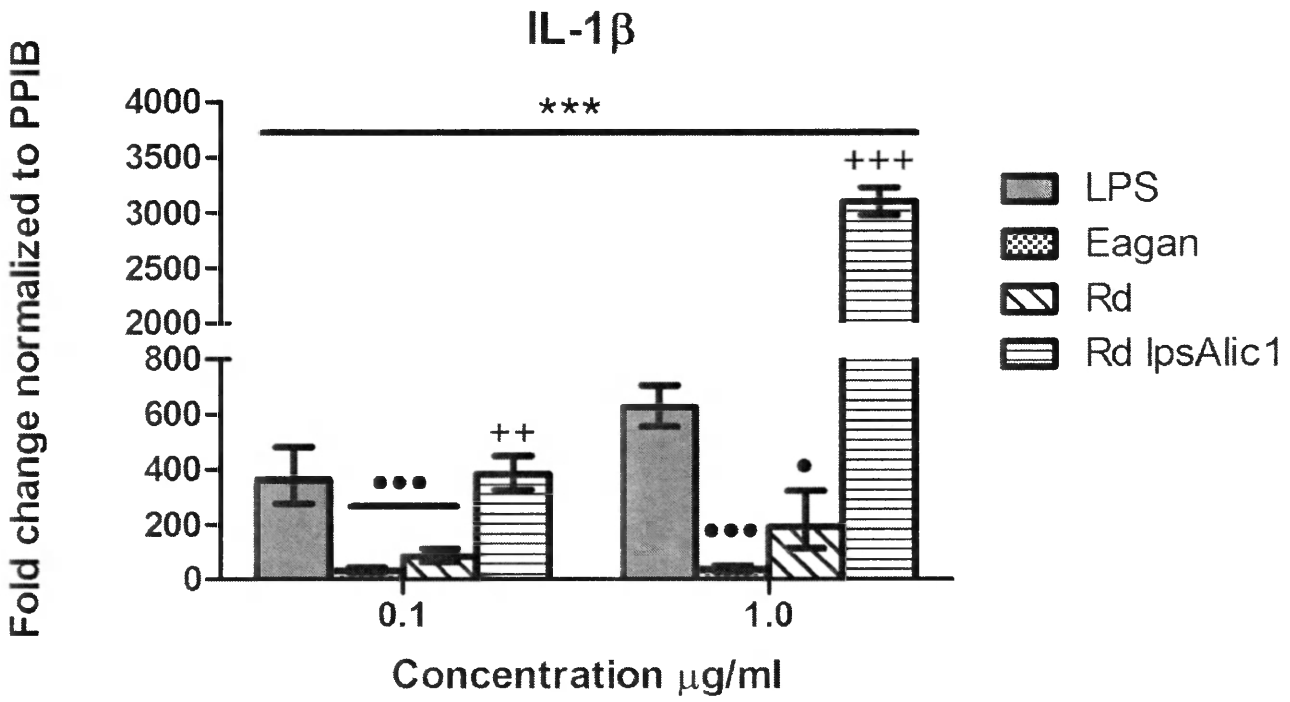


Figure 10. Relative gene expression of IL-1β in response to LOS stimulation. THP-1 cells were stimulated with LOS compounds for 4 hours at concentrations 0.1 and 1 μg/ml. RNA was extracted and genetic expression was measured using real-time PCR and presented as fold change relative to unstimulated control. Data represents mean ± SEM (n=3).

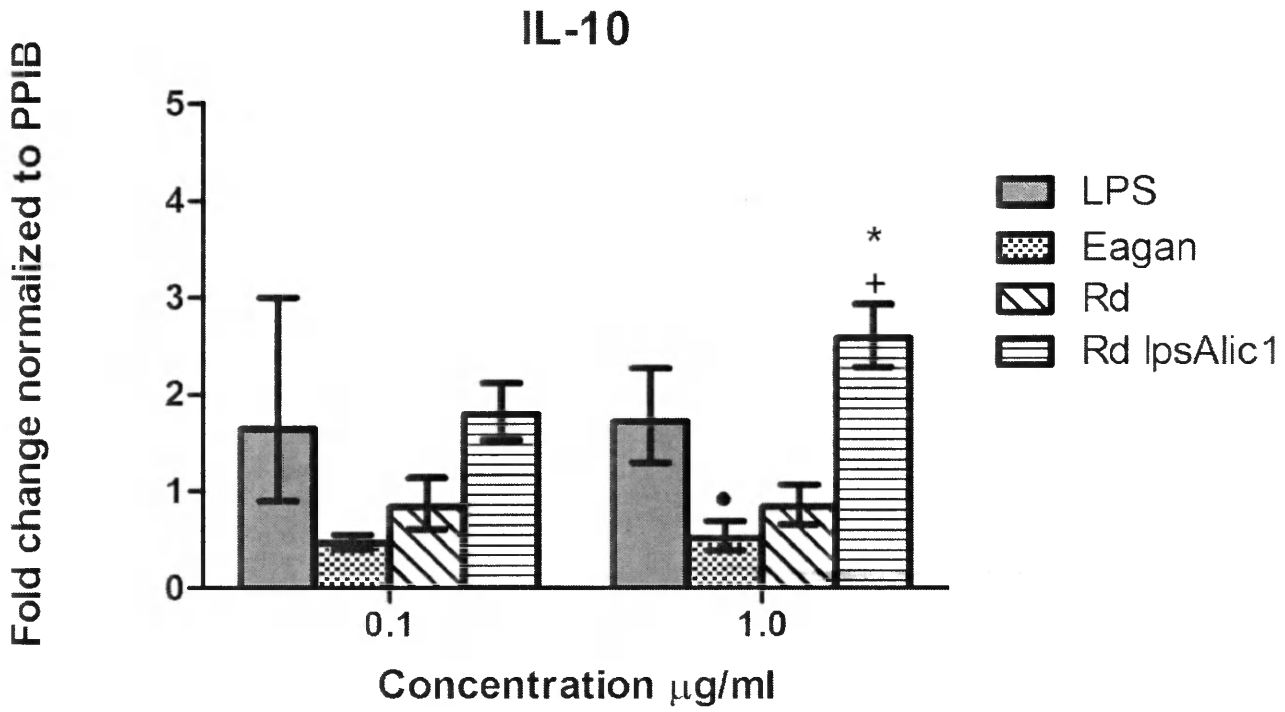


Figure 11. Relative gene expression of IL-10 in response to LOS stimulation. THP-1 cells were stimulated with LOS compounds for 4 hours at concentrations 0.1 and 1 μg/ml. RNA was extracted and genetic expression was measured using real-time PCR and presented as fold change relative to unstimulated control. Data represents mean ± SEM (n=3).

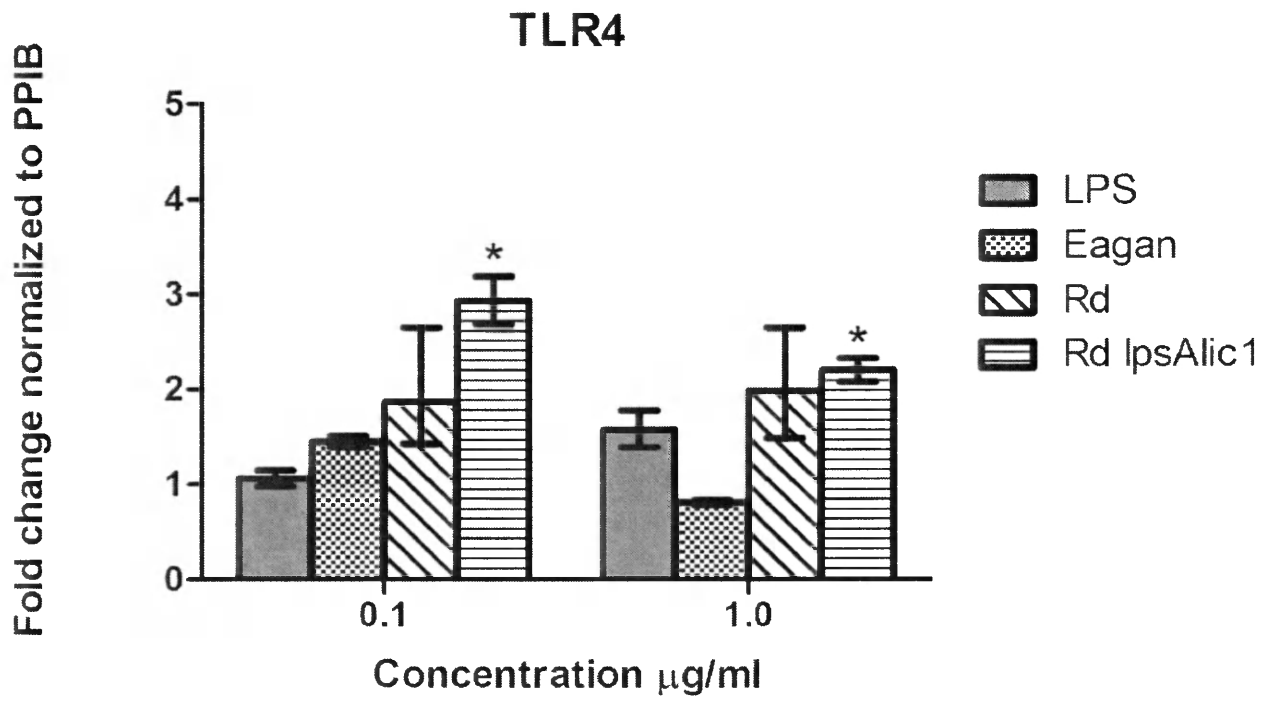


Figure 12. Relative gene expression of TLR4 in response to LOS stimulation. THP-1 cells were stimulated with LOS compounds for 4 hours at concentrations 0.1 and 1 μg/ml. RNA was extracted and genetic expression was measured using real-time PCR and presented as fold change relative to unstimulated control. Data represents mean ± SEM (n=3).

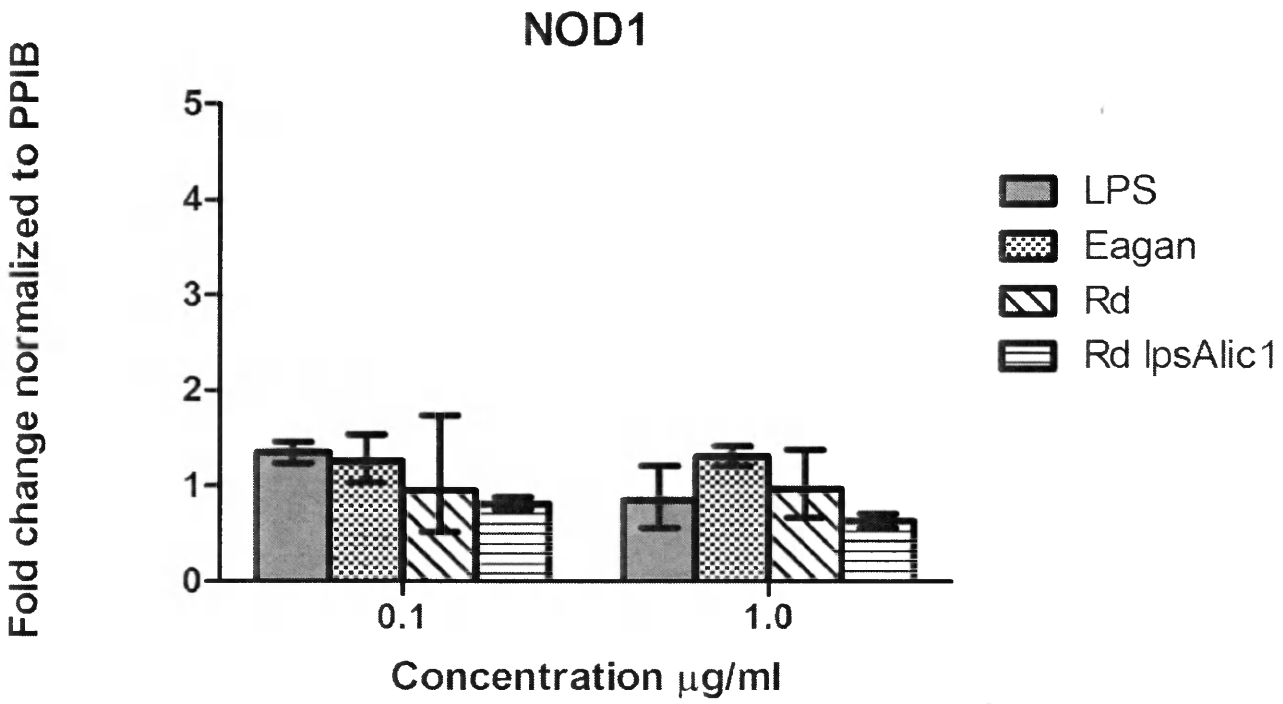


Figure 13. Relative gene expression of NOD1 in response to LOS stimulation. THP-1 cells were stimulated with LOS compounds for 4 hours at concentrations 0.1 and 1 μg/ml. RNA was extracted and genetic expression was measured using real-time PCR and presented as fold change relative to unstimulated control. Data represents mean ± SEM (n=3).

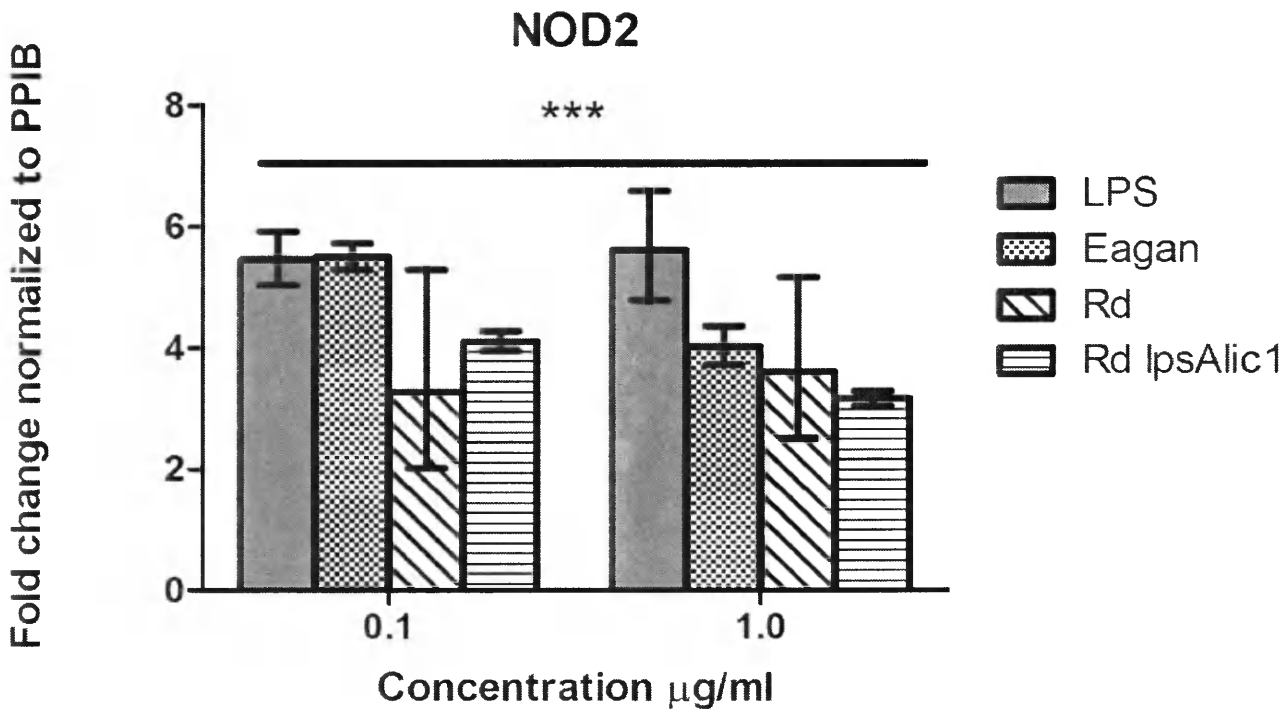


Figure 14. Relative gene expression of NOD2 in response to LOS stimulation. THP-1 cells were stimulated with LOS compounds for 4 hours at concentrations 0.1 and 1 μg/ml. RNA was extracted and genetic expression was measured using real-time PCR and presented as fold change relative to unstimulated control. Data represents mean ± SEM (n=3).

Table 1. MALDI data expressed in relative intensity of lipid A molecule from LOS compounds derived from Eagan, Rd and Rd *lic1lpsA* strains.

Strain	$[M-H]^-$ Singly charged ions	Relative Intensity	Proposed Composition	Number of acylations
Eagan	1161.7	.9	2 GlcN, 2 P, 2 C-14:0-3-OH, 1 C-14:0	3
	1387.9	1	2 GlcN, 2 P, 3 C-14:0-3-OH, 1 C-14:0	4
	1598.1	.2	2 GlcN, 2 P, 3 C-14:0-3-OH, 2 C-14:0	5
	1824.3	.3	2 GlcN, 2 P, 4 C-14:0-3-OH, 2 C-14:0	6
Rd	1161.7	0.7	2 GlcN, 2 P, 2 C-14:0-3-OH, 1 C-14:0	3
	1387.9	1	2 GlcN, 2 P, 3 C-14:0-3-OH, 1 C-14:0	4
	1598.1	0.1	2 GlcN, 2 P, 3 C-14:0-3-OH, 2 C-14:0	5
	1824.3	.2	2 GlcN, 2 P, 4 C-14:0-3-OH, 2 C-14:0	6
Rd *lic1lpsA*	1161.7	.25	2 GlcN, 2 P, 2 C-14:0-3-OH, 1 C-14:0	3
	1387.9	1	2 GlcN, 2 P, 3 C-14:0-3-OH, 1 C-14:0	4
	1598.1	.1	2 GlcN, 2 P, 3 C-14:0-3-OH, 2 C-14:0	5
	1824.3	.7	2 GlcN, 2 P, 4 C-14:0-3-OH, 2 C-14:0	6

Represented in lipid A: (GlcN) Glucosamine; (P) Phosphate; (C-14:O-3-OH), (C-14:0) Fatty acid chains;

Table 2. Cell viability (% viable cells). Trypan Blue exclusion test, mean of n=3 ± SD, following treatment with Eagan, Rd and Rd *lic1lpsA* LOS after 24 hours.

Concentration	Control	LPS	Eagan	Rd	Rd *lic1lpsA*
0 µg/ml	97.2 ± 1.5	-	-	-	-
1 µg/ml	-	97.5 ± 1.4	97.2 ± 0.6	97.9 ± 0.5	97.6 ± 1.5
5 µg/ml	-	-	96.7 ± 1.6	97.3 ± 1.7	97.27 ± 1.6
10 µg/ml	-	-	94.6 ± 2.6	97.3 ± 0.6	93.3 ± 0.6
15 µg/ml	-	-	81.47 ± 6.4	84.9 ± 3.2	81.6 ± 5.4

Chapter II- The adaptive immune response against LOS of *Haemophilus influenzae*

Abstract

The lipooligosaccharide (LOS) of *Haemophilus influenzae* is a truncated polysaccharide antigen expressed on the outer membrane. The structure and composition of LOS is highly heterogeneous among strains and it is unclear whether it can induce a natural antigen-specific antibody response. Serum was acquired from consenting healthy individuals and antibodies specific for Eagan and Rd LOS compounds of *H. influenzae* were measured using an indirect ELISA.

Out of 72 serum samples measured, 71 were positive for Eagan LOS specific IgG antibodies, however antibody titers were significantly different among age groups. All 72 samples were positive for Rd LOS specific IgG with no differences among age groups. Futhermore, Rd LOS was found to induce significantly higher IgG levels when compared to Eagan LOS.

These findings make a strong case for the natural antigenicity of *H. influenzae* LOS compounds which has further medical implications. If used as a potential vaccine candidate, LOS would act as an effective boost to an already existing natural immune response against this antigen.

1. Introduction

1.1 The adaptive immune response

Adaptive immunity is considered to be a higher order of immune responses compared to innate immunity. As innate immune response can be found in all multi-cellular organisms, adaptive immunity can only be found in vertebrates (Medzhitov and Janeway 1997). Although innate and adaptive immune responses are usually identified as the two distinct arms of immunity, their roles are not independent, or mutually exclusive. In fact, innate immune activation is a necessary prerequisite for the development of an adaptive immune response. The innate immune system also has a critical role in making initial contact with microorganisms and functions to keep the bacterial population at bay. During pathogenic infection however, the innate immune response is overwhelmed and cannot contain the invasion. It is at this point where adaptive immunity plays an essential role in neutralizing the foreign microbial invasion.

The adaptive immune response can be described as a clonal expansion of a germline encoded antigen-specific directed response (Medzhitov and Janeway 1997). The process of hypermutation in lymphocytes that results in the recombination of genes allow for literally billions upon billions of different sequences that antibodies can recognize (Alt, Oltz et al. 1992). Adaptive immunity is largely mediated by B and T lymphocytes. Regulated by selection, only the lymphocytes with the highest affinity for the target antigen become activated effector cells (Berek and Ziegner 1993). Within the adaptive immunity paradigm, there are two types of responses which are defined as cell-mediated and humoral. Cell mediated adaptive responses are dependent on effector T lymphocytes, known as cytotoxic T cells which destroy pathogen infected cells upon binding. Cell-mediated immunity is the most significant in defence against intracellular pathogens. In the

case of humoral immunity, both T and B lymphocytes are needed for the clonal expansion of effector cells to produce an excess of antigen specific antibodies which travel to the site of infection. The antibodies assist in pathogen opsonization, neutralization, and complement activation eventually leading to the elimination of the microorganism. The whole process is highly regulated to prevent the activation of lymphocytes that recognize "self" antigens which can result in various auto-immune diseases. The recognition repertoire of antibodies is practically unlimited; however, its limitation lies in its initiation. The processes involved to mount an effective adaptive immune response are long and energy costly which can take up to as many as 7 days. Thus an integrated response of innate and adaptive immunity is necessary for successful host defence against microbial infections.

1.2 The classical antibody production pathway

The classical antibody production pathway is a series of biochemical events that have been extensively studied over the last 30 years. Simply put, the cumulative contributions of both innate and adaptive immunity are realized in the production of antigen specific antibodies. The major point of inference is that APCs, such as dendritic cells or macrophages of the innate immune cell repertoire, invoke the co-operation of the T lymphocytes (Jawa, Cousens et al. 2013). This occurs via the MHC molecules which physically present foreign peptide sequences to naïve T cells, in the presence of additional co-stimulatory signals, resulting in the activation of the T cell. The activated T cell's main effector function, with respect to antibody production, is providing the activating and co-stimulatory signals to resting B cells. Subsequently, activated B cells undergo morphological changes to become plasma cells which are responsible for the mass production of antigen-specific antibodies (Jawa, Cousens et al. 2013). This classical antigen

presentation pathway has also been called the T-cell dependent pathway, due to the involvement of T cells and the necessary signals it provides for B cell activation. The drawback of this pathway lies within the antigen presentation. T cell receptors can only recognize MHC class molecules which are limited in their ability to present only peptide fragments. This means antigenic sugars, lipids, and nucleic acid motifs would not induce production of antibodies directed against them through the activation of T cells (Buus 1992). Thus, an alternative pathway is necessary for non-protein antigens.

1.3 T-cell independent pathway of antibody production against LOS

Non-protein antigens, as mentioned above, do not induce the classical pathway of antibody production; however, antibodies specific for non-protein antigens can still be found in the serum. This is due to the activation of the T-cell independent pathway. It was traditionally thought that the host immune system could produce antibodies against non-protein antigens such as the polysaccharide capsule of encapsulated bacteria, or glycolipids such as LOS, only by way of crosslinking of B cell receptors (Schreiber 2012). Although T cell receptors are limited to recognizing peptide antigens, B cell receptors can recognize a range of different three dimensional antigenic motifs (Buus 1992). Thus when two or more B cells would bind to a non-protein antigen, this would provide a sufficient internal signal to activate the B cell without any influence from T cells (Schreiber 2012). However this type of response was not comparable with a T-cell dependent pathway which produces a very robust, high affinity antibody response with a high degree of adaptability to possible antigen mutations. In contrast, crosslinking resulted in a

response with low affinity, low somatic hypermuation, virtually no isotype switching and poor memory (Mond, Vos et al. 1995).

Recent studies have shown that there are other factors that affect the T-cell independent paradigm. A specialized group of tumour necrosis family (TNF) receptors are expressed on B cells known as the B cell activating factor receptor (BAFFR), the B cell maturation antigen (BCMA) and the transmembrane activator and calcium modulator (TACI) which recognize specialized cytokines secreted by innate immune cells such as monocytes and dendritic cells. B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) are two such cytokines found to provide additional signals in activating the B cell, resulting in a stronger T-cell independent antibody response (Schreiber 2012). Interestingly, TACI KO mice were able to produce a normal antibody response against T-cell dependent antigens, however responses against a T-cell independent antigen were virtually abolished (von Bulow, van Deursen et al. 2001). Moreover engagement of TLR4 receptors was found to increase BAFF and APRIL secretion, which is integral to the antibody response against non-protein antigens (Ng, Ng et al. 2006). Whether LOS of *H. influenzae* has a role in inducing BAFF and APRIL mediated antibody secretion is not part of the scope of this study, however it remains a question that still needs to be investigated.

1.4 Rationale

In a post Hib vaccine era, invasive infections have been largely attributed to NTHi; there is a growing concern to address the increased incidence rates (Tanaka, Kurosaki et al. 2013, Bamberger, Ben-Shimol et al. 2014). There have been several studies which have measured the antibody response against whole cell NTHi; research indicates both healthy and

immunocompromised subjects have detectable levels of anti-NTHi antibody, however immunocompromised subjects presented a significantly different Immunoglobulin G (IgG) subclass population, CD40L expression, and changes in the cytokine profile (King, Hutchinson et al. 2003, King, Ngui et al. 2008). The question of whether there are naturally acquired antibodies against LOS has not been fully elucidated. Recent work by *Clark et al.* found the presence of anti-LOS antibodies in a single normal human serum sample (Clark, Snow et al. 2012), however whether this finding applies to a larger sample population is unknown. Furthermore, whether these naturally acquired antibodies against LOS are functional with regards to bactericidal activity is also unknown and is currently being investigated.

2. Study Objectives

In this study, we measured the presence of naturally acquired anti-LOS antibodies in serum samples from healthy individuals against LOS of Eagan and Rd strains of *H. influenzae.* The serum samples were divided into three age groups corresponding to age dependent changes in the adaptive immune system (Ligthart, Corberand et al. 1984). As LOS is present on all strains of *H. influenzae*, a comprehensive understanding of whether LOS can induce naturally acquired anti-LOS antibodies could provide novel insights in vaccine development against all serotypes of *H. influenzae.*

3. Materials and Methods

2.1 Serum samples

Serum samples from adults without any significant medical conditions residing in Thunder Bay and surrounding areas were obtained with informed consent by a registered phlebotomist. A total of 72 serum samples were measured; the donor ranged from 18-80 years. Blood was drawn into serum separator tubes, which were centrifuged at 1500 x g for 10 min. Serum was decanted and aliquoted into sterile eppendorf tubes, followed by immediate storage at -80 °C. Age groups were determined according to the SENIEUR protocol criterion (Ligthart, Corberand et al. 1984) with modifications as described (Plackett, Boehmer et al. 2004). The study was approved by the Research Ethics Board of Lakehead University.

2.2 Antibody Analysis

Serum antibody concentrations were measured using an indirect ELISA where purified LOS compounds derived from Eagan and Rd strains of *H. influenzae* were used for coating of Polystyrene 96-well Immuno Plate (Cedarlane) at 2.5 µg/ml/50 µl in 0.05 M carbonate buffer containing 0.02 M $MgCl_2$, pH 9.8 at 37°C for 3 hours followed by overnight incubation at 4°C. The wells were blocked with 1% BSA-PBS for 1 hour at room temperature and washed with PBS-0.05% Tween 20 (PBST). Sera was serially diluted and added to the wells for 1 hour at room temperature followed by washing with PBST. Mouse anti-human IgG horseradish peroxidase (HRP) conjugated antibody was added for an additional hour followed by washing and incubation with the substrate 3,3',5,5'-Tetramethylbenzidine (TMB) (Mandell Scientific, Guelph, ON, CAN) for 30 minutes. Then, 1 N HCl was added as a stop solution and absorbance ($A_{450\ nm-630\ nm}$) was determined with an automated microplate reader (Bio Tek Powerwave XS).

2.3 Relative antibody unit calculation

Serum samples were run at dilutions of 1:400, 1:800, and 1:1600 in duplicates. Duplicates with coefficient of variation (CV) greater than 15% were excluded from calculations. The mean of each optical density (O.D.) at 1:800 and 1:1600 was back-calculated to 1:400 where outliers were excluded if the CV of the 3 values was greater than 15%. One serum sample was chosen arbitrarily to be the relative standard to compare all other samples. All samples were normalized to the standard and expressed in relative units.

2.4 Statistical analysis

Data were expressed as the geometric mean with 95% confidence intervals. Statistical significance was determined (GraphPad Prism) using the Kruskal-Wallis test with Dunns post-hoc test, unless otherwise specified. *$P<0.05$, **$P<0.01$ were considered significant.

4. Results

3.1 Eagan LOS induces natural immunity which may be age sensitive

The question of whether LOS of *H. influenzae* can induce an antibody specific response has not been fully elucidated. Furthermore, there have been no studies, to our knowledge, that have addressed the antibody response against LOS in a human population. Thus, in order to detect specific antibodies against Eagan LOS an indirect ELISA was performed using serum samples collected from 72 healthy individuals that were divided into three age groups of 18-34, 35-59, and 60-80. The IgG isotype was measured which indicates a secondary response, i.e. the host had previously encountered LOS resulting in clonal expansion and isotype switching from IgM to IgG.

Data were expressed in relative units due to the non-existence of an LOS standard to derive absolute concentrations. Blank control wells were measured at less than 0.1 O.D. and were used as the negative control. With exception to one sample in the 18-34 age group, all samples were well above 0.1 O.D. indicating the presence of anti-Eagan LOS IgG antibodies (Fig. 15). Furthermore, the presence of Eagan LOS specific antibodies seemed to be age sensitive as well; the mid-age group (35-59) had significantly more LOS specific IgG antibodies than the younger age group ($P<0.05$). The older age group also had lower antibody levels than the mid age group however this difference was not significant.

3.2 Rd LOS induces natural immunity which may not be age sensitive

To address whether the observed presence of Eagan LOS specific antibodies in healthy individuals would be analogous for Rd LOS, ELISA plates were coated with Rd LOS overnight

at the same concentration as Eagan LOS and specific IgG were measured in serum samples using the same methodology described for Eagan LOS.

All serum samples in all age groups exhibited presence of Rd LOS specific IgG antibody, however no statistical differences were observed between the age groups (Fig. 16). Whether this effect is attributed to the age-dependent changes in the B cell memory repertoire or the antigenic nature of the Rd LOS is unknown and is warranted further investigation.

3.3 Rd LOS induces higher IgG concentrations in the sample population

To assess if there were any differences in IgG levels between LOS compounds, the raw O.D. values were used for comparison. It is important to note that relative units were not used in this case as there was no mutual point of reference for both Eagan and Rd values. As each value was a paired observation, statistical significance was determined using a two tailed Wilcoxon matched pairs test.

Our findings indicate that Rd LOS to be able to induce significantly higher levels of IgG in our healthy individual sample population (Fig. 17) ($P<0.01$). As these results are paired observations, differences due to serum variability are minimized. Therefore, we can safely conclude this observed difference in our sample population accurately reflects the true antibody concentrations of the members included in our population.

5. Discussion

Adaptive immunity is a critical component in microbial clearance following infection. As such, with regards to *Haemophilus influenzae,* its effect in the stimulation and/or evasion of the adaptive immune response has been of great interest. However, the role of the conserved glycoplipid LOS of *H. influenzae* in the adaptive immune paradigm is an area which has not been fully elucidated. It has been shown that LOS can act as both a direct and indirect virulence factor; the presence of LOS directly enhances evasion of host mechanism while acting as a scaffold which can bind to several different virulence factors which also enhance bacterial evasion (Swords, Jones et al. 2003, Hallstrom and Riesbeck 2010, Clark, Snow et al. 2012). Interestingly, a recent study had shown the LOS structure allowed for decreased binding by IgM thereby decreasing IgM-mediated complement killing (Langereis, Stol et al. 2012). These studies have highlighted the overall effect of LOS in the adaptive immune response; however a more fundamental question has not been fully addressed in the literature. It is clear that the LOS structure has several roles in enhancing the overall virulence of *H. influenzae*; however, it is unclear whether this structure is antigenic enough to elicit a natural LOS specific antibody response and whether this can be observed in human populations.

In this study we have shown that both Eagan and Rd LOS structures are sufficiently antigenic to induce a natural LOS specific antibody response. The therapeutic implications of this finding would mean novel targets for vaccine development, however further investigation into whether these apparent LOS specific antibodies are capable of bactericidal activity would be required. We also found, in the case of Eagan LOS, the antibody response suggested age dependence

whereas this effect was not observed, in the case of antibodies against Rd LOS. Extrapolation from a closed *ex vivo* sample population to the entire population should always be made conservatively. Having said that, a possible explanation for the higher anti-Eagan LOS IgG levels observed in the mid age group could be due to the correlation between the expansion of B-memory cell repertoire and increasing age (Aberle and Puchhammer-Stockl 2012). Older individuals have been exposed to more antigenic compounds than younger individuals leading to a constitution of a broader more robust immune memory repertoire. However, with elderly individuals, generally 60 years and older, this repertoire typically succumbs to degradation; this decrease in IgG was also observed with the old age group although the difference was not statistically significant (Fig. 15).

It is important to address the intentional exclusion of Rd *lic1lpsA* from the assays performed in this chapter. As these mutations were created in a laboratory setting, i.e. for the purpose of testing compositional effect of the wildtype, it did not exist in the natural world. Consequently, using this compound as an antigen of interest for serum samples was considered irrelevant.

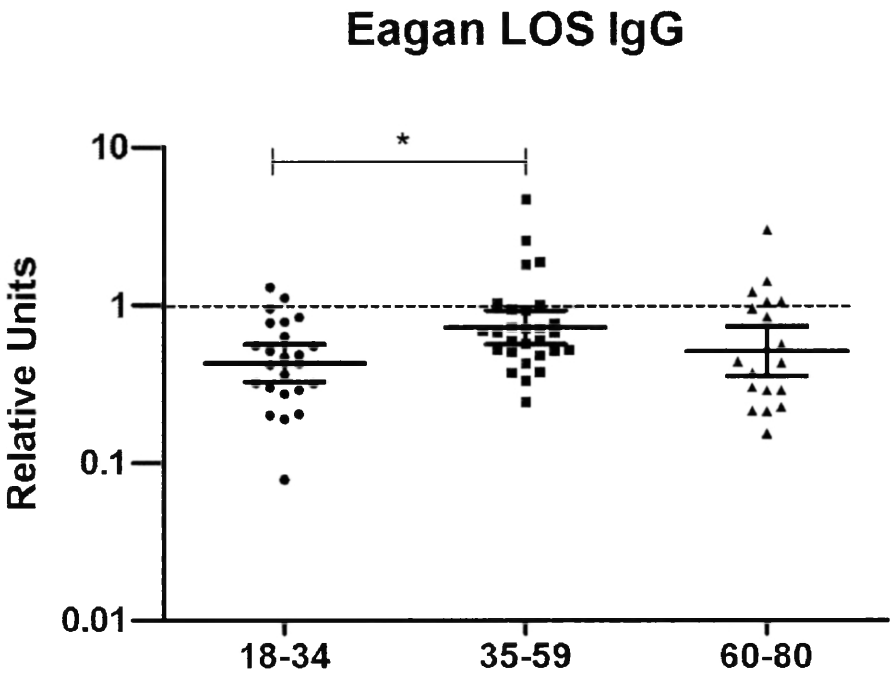
Our results also suggest a disparity in the immunogenicity between Eagan and Rd LOS compounds. Within our population of 72 samples, there were significantly higher IgG levels found against Rd than Eagan LOS (Fig. 17). According to our statistical analysis, we can infer, for the rest of our geographical region, a higher IgG level against Rd LOS to be present than Eagan LOS. This may be due to multiple factors; the Rd LOS structure may simply be more antigenic than the Eagan LOS, however this explanation is not completely satisfactory. The Eagan strain belongs to the serotype b, which was historically the most virulent serotype of *H. influenzae*. Thus it is difficult to reproach this disparity on the basis of antigenicity. Another

possible explanation could be epidemiological; this region may have been in more frequent contact with the Rd serotype and consequently developed more antibodies against that specific LOS. Moreover, with the introduction of the Hib conjugate vaccine, invasive infection caused by Hib has been virtually eliminated in developed countries (Kelly, Moxon et al. 2004). As the Hib vaccine is not selective for the Rd serotype d strain, this type of ecological environment could promote colonization by the Rd strain. This explains why the younger age group showed significantly less anti-Eagan LOS IgG, since they would have grown up in a post-Hib vaccine era, and thereby was subjected to a herd effect of immunization. In contrast, in the absence of this herd effect, the mid and older age groups would have been in frequent contact with Hib resulting in immunological memory against its antigens. This explanation does seem plausible however , further investigation into various epidemiological factors would be warranted.

If we attempt to create a comprehensive picture of the effect of LOS on both innate and adaptive immunity, we must first understand the scope of these two chapters and their limitations. There exists a disconnect between the results of the immunostimulatory nature of LOS shown in chapter I and the antibody production capacity of LOS shown in chapter II. As LOS is a non-protein antigen, this implies that the co-stimulatory and antigen presenting molecules measured in chapter I would not have a direct effect on the production of antibodies against LOS. In other words there is no direct relationship between the upregulation of co-stimulatory and antigen presenting molecules and the production of antibodies for non-protein antigens. Consequently, conclusions cannot be made in the same way as if LOS had been a peptide antigen; however this does not mean the exhibited immunostimulatory nature of LOS does not play a role in antibody production. It has been shown that TLR4 activation is crucial for the *in vivo* generation of long-

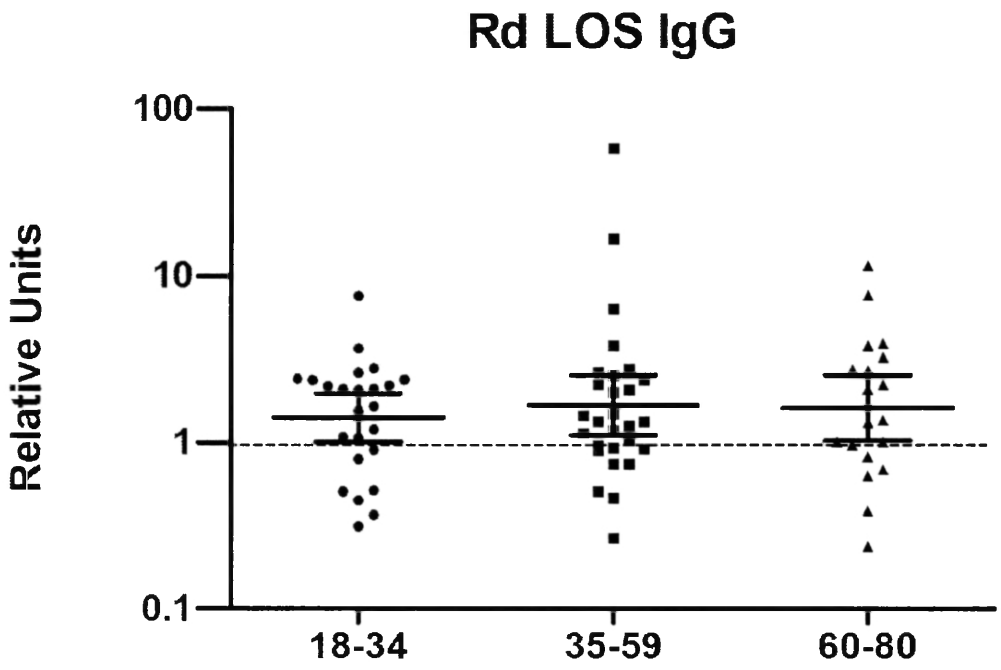
lived antibody secreting cells (Komegae, Grund et al. 2013), which suggests that there is undoubtedly an essential role for LOS in the production of antibodies.

In summary, we have shown both Eagan and Rd LOS compounds are capable in eliciting LOS specific IgG in healthy individuals. In addition, higher IgG levels were found against Rd LOS when compared with Eagan LOS. This is possibly due to epidemiological factors influencing our sample population, specifically the *H. influenzae* Rd strain may be more geographically prevalent than the Eagan strain. However these findings do support the possible benefits of focusing on Eagan and Rd as novel targets for vaccine development.



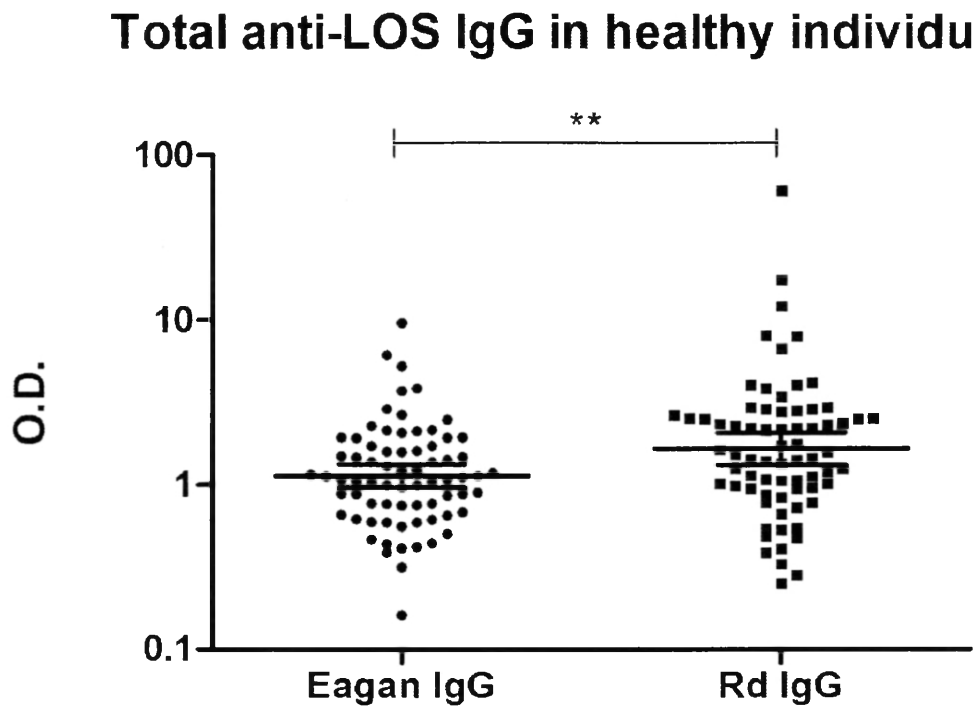
GMC:	0.431	0.727	0.512
CI 95%:	0.328 - 0.567	0.568 -0.931	0.356 -0.737
Median:	0.4519	0.6752	0.4368
n=	24	28	20

Figure 15. The presence of naturally acquired IgG against Eagan LOS in healthy individuals. Serum samples were run in an indirect ELISA, using microplates coated with Eagan LOS. One serum sample was chosen arbitrarily to be the relative standard to compare all other samples. All samples were normalized to the standard and the results were expressed in relative units. Age groups were determined by established SENIEUR protocol with modifications. Data shows GMC ± CI 95% where *P<0.05. (GMC) geometric mean; (CI) confidence intervals.



GMC:	1.42	1.68	1.62
CI 95%:	1.01 - 1.98	1.11 - 2.54	1.03 - 2.54
Median:	1.86	1.35	1.53
n=	24	28	20

Figure 16. The presence of naturally acquired IgG against Rd LOS in healthy individuals. Serum samples were run in an indirect ELISA, using microplates coated with Rd LOS. One serum sample was chosen arbitrarily to be the relative standard to compare all other samples. All samples were normalized to the standard and the results were expressed in relative units. Age groups were determined by established SENIEUR protocol with modifications. Data shows GMC ± CI 95% where *P<0.05. (GMC) geometric mean; (CI) confidence intervals.



GMC:	1.107	1.636
CI 95%:	0.9335 - 1.312	1.312 - 2.040
Median:	1.076	1.537
n=	72	72

Figure 17. A comparative analysis of total IgG levels in sample population. Serum samples were run in an indirect ELISA, using microplates coated with LOS of interest and results were expressed in raw O.D. values. Data shows GMC ± CI 95%. Statistical significance was determined by a two tailed Wilcoxon matched pairs test where *P<0.05. (GMC) geometric mean; (CI) confidence intervals.

Conclusion

The aim of these studies was to address the fundamental properties of the conserved endotoxin, LOS, of *Haemophilus influenzae*. Although LOS had been the focus of many studies, we felt that there were two questions that were not fully addressed in the literature. First, it had not been adequately shown whether LOS of *H. influenzae* could activate the innate and inflammatory responses. Second, it was also uncertain whether a natural immune response to LOS can result in an antigen-specific antibody response; furthermore, this had not been shown using a human sample population.

Our findings show that the LOS compounds derived from Eagan and Rd serotypes were able to induce an inflammatory response, however, this response was significantly lower when compared with the toxic LPS compound from *E. coli*. It was also shown that Rd *lic1lpsA* LOS induced consistently higher expression of inflammatory cytokines and adhesion molecules than the wildtype, suggesting a role for phosphorylcholine and oligosaccharide additions in suppressing the immune system.

We found that healthy individuals are capable of producing LOS specific antibodies against Eagan and Rd strains. The IgG isotype was measured because its presence would indicate a secondary response since people are generally asymptomatically colonized by *H. influenzae* and therefore a primary immune encounter should have already occurred. Interestingly, we observed significantly higher IgG levels against Rd LOS than Eagan LOS in the same serum samples. We suggest that there may be epidemiological factors that may explain the observed results.

These findings help provide some of the missing pieces of our collective understanding of host-pathogen interactions with *Haemophilus influenzae.* The role of LOS with regards to inducing the innate immune response and how this effect had translated into antibody production gives us insight into the larger scope of the human immune response in its entirety. Granted, there are still many questions with regards to the specific mechanisms behind the observed responses, however the overall immunological effect of LOS was shown.

The clinical applications of these findings highlight both Eagan and Rd LOS structures as potential candidates for vaccine and/or adjuvant development. As disease caused by non-type b *H. influenzae* strains are of growing concern, a LOS structure that has a low immunostimulatory potential but that is antigenic enough to elicit antibody production warrant serious consideration as a possible prophylactic treatment option.

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