Modulation of Endotoxicity of *Shigella* Generalized Modules for Membrane Antigens (GMMA) by Genetic Lipid A Modifications

RELATIVE ACTIVATION OF TLR4 AND TLR2 PATHWAYS IN DIFFERENT MUTANTS[*]

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Background: GMMA from Gram-negative bacteria are an attractive vaccine technology, but lipopolysaccharide (LPS) reactogenicity limits use.
Results: Genetic LPS modification resulting in penta-acylation reduced *Shigella* GMMA reactogenicity to a TLR2-mediated limit. Modifications resulting in palmitoleoylated hexa-acylated LPS triggered higher TLR4-mediated reactogenicity.
Conclusion: Use of GMMA as vaccines will likely require LPS penta-acylation.
Significance: Understanding the relative contribution of TLR activation guides GMMA vaccine development.

Outer membrane particles from Gram-negative bacteria are attractive vaccine candidates as they present surface antigens in their natural context. We previously developed a high yield production process for genetically derived particles, called generalized modules for membrane antigens (GMMA), from *Shigella*. As GMMA are derived from the outer membrane, they contain immunostimulatory components, especially lipopolysaccharide (LPS). We examined ways of reducing their reactogenicity by modifying lipid A, the endotoxic part of LPS, through deletion of late acyltransferase genes, *msbB* or *htrB*, in GMMA-producing *Shigella sonnei* and *Shigella flexneri* strains. GMMA with resulting penta-acylated lipid A from the *msbB* mutants showed a 600-fold reduced ability, and GMMA from the *S. sonnei* ΔhtrB mutant showed a 60,000-fold reduced ability compared with GMMA with wild-type lipid A to stimulate human Toll-like receptor 4 (TLR4) in a reporter cell line. In human peripheral blood mononuclear cells, GMMA with penta-acylated lipid A showed a marked reduction in induction of inflammatory cytokines (*S. sonnei* ΔhtrB, 800-fold; ΔmsbB mutants, 300-fold). We found that the residual activity of these GMMA is largely due to non-lipid A-related TLR2 activation. In contrast, in the *S. flexneri* ΔhtrB mutant, a compensatory lipid A palmitoleoylation resulted in GMMA with hexa-acylated lipid A with ~10-fold higher activity to stimulate peripheral blood mononuclear cells than GMMA with penta-acylated lipid A, mostly due to retained TLR4 activity. Thus, for use as vaccines, GMMA will likely require lipid A penta-acylation. The results identify the relative contributions of TLR4 and TLR2 activation by GMMA, which need to be taken into consideration for GMMA vaccine development.

Gram-negative bacteria naturally shed particles that consist of outer membrane lipids, outer membrane proteins, and soluble periplasmic components. These particles, called native outer membrane vesicles, have been proposed for use as vaccines (1). However, the yield is usually too low for a practical vaccine production. We have developed genetic modification of bacteria to induce high level shedding of particles called generalized modules for membrane antigens (GMMA)[2] (2, 3) and the corresponding industrial processes required for a practical vaccine platform (2). In the case of *Shigella*, the required genetic modification is a deletion of the *tolR* gene whose corresponding protein is involved with linking the inner and outer membranes. This development is part of a program to develop an effective and affordable vaccine for the causative agents of shigellosis, a global human health problem, especially in developing countries and in children younger than 5 years (4), with more than 125 million cases (5) and 100,000 deaths per year (6). *Shigella* are Gram-negative bacteria divided into 50 different serotypes based on the carbohydrate composition of the O antigen of their lipopolysaccharide (LPS) (7). A limited number of serotypes contributes to the global burden of shigellosis, but the leading disease-causing serotypes vary between regions (8). The current globally dominant serotypes are *Shigella sonnei* and *Shigella flexneri* 2a, which account for more than 20% of shigellosis cases each (9, 10).

GMMA are highly immunogenic (2), in part, probably because of strong self-adjuvanticity. Because they are derived from the outer membrane of Gram-negative bacteria, they have high levels of LPS and lipoproteins, molecules that are strong activators of the innate immune response through recognition by different pattern recognition receptors, including Toll-like receptors (TLRs), a widely expressed sets of molecules present in mammalian cells (11). The receptors of particular impor-

[2] The abbreviations used are: GMMA, generalized modules for membrane antigen; PBMC, peripheral blood mononuclear cell; TLR, Toll-like receptor; KDO, 2-keto-3-deoxyoctonate; OAg, O antigen.

tance for recognition of Gram-negative bacteria are TLR2 and TLR4. TLR2 is involved in the recognition of a wide range of pathogen-associated molecular patterns that include lipoproteins (di- or tri-acetylated) (11). TLR4 is the receptor involved in the recognition of LPS with MD-2, CD14, and LPS-binding protein (11, 12).

Depending on the dose of GMMA to be used in a vaccine, this strong activation of innate immunity may lead to unacceptable reactions in human subjects, *e.g.* a febrile response or, in extreme cases, septic shock (13), especially if parenterally administered. Further genetic manipulations of the bacteria could be used to reduce this risk. The single most abundant and highly potent immunostimulatory component in GMMA is LPS (1). Its structure is amenable to genetic modification to reduce the activation of the TLR4 pathway. To assess the effect of such genetic modifications, it is important to determine the relative contribution of TLR2 and TLR4 pathways to the reactogenicity of GMMA. This needs to be tested in human cells or cell lines containing the human TLR recognition system because the cytokine response induced varies considerably between mammalian species. For example, penta-acylated lipid A is a poor inducer of cytokines from human cells but is a strong stimulator of mouse cells (14, 15).

LPS consists of three main regions as follows: the glycolipid, lipid A, a core oligosaccharide, and an oligosaccharide chain (O antigen, OAg) usually consisting of 20–40 repeating units comprising 2–8 sugar molecules (16). Lipid A, which anchors the LPS to the outer membrane of bacteria, is the endotoxic part of LPS. The most endotoxic form of lipid A consists of a hexa-acylated glucosamine disaccharide phosphorylated at the 1 and 4′ position with acyl chains from 12 to 14 carbons in length and an asymmetric (4/2) distribution (14, 16, 17). This structure is common to *Escherichia coli* and *Shigella* (18, 19). During the synthesis of the lipid A, first a tetra-acylated core structure carrying *R*-3-hydroxymyristate at the 2-, 3-, 2′-, and 3′-positions, called lipid IVA, is generated, which is conserved in most of the Gram-negative bacteria (18, 19). Subsequently, the late acyltransferases HtrB (also called LpxL) and MsbB (also called LpxM) transfer a lauroyl fatty acid to the 3′-position (HtrB (20)) and a myristoyl fatty acid at position 2′ (MsbB (21)). In some bacteria there are alternative pathways leading to a modified structure of lipid A, and these may depend on environmental conditions, *e.g.* a palmitoleic acid is incorporated at the 3′-position (palmitoleoylation) instead of the lauroyl chain, catalyzed by LpxP in *E. coli* under cold shock conditions (22, 23).

The total number and the length of acyl chains plus the presence of the two phosphates in positions 1 and 4′ are critical factors for full lipid A activation of human TLR4/MD-2 (14, 16, 17, 19). Changes to the structure of the lipid A, either by removal of critical components or by replacement of one of the acyl chains by a different acyl residue, affects the binding and recognition by TLR4 and results in a lower endotoxicity *in vitro* (14, 16). A primary focus for reducing the reactogenicity of lipid A has been the modification of the acylation by inactivation of the genes encoding the late acyltransferases HtrB (20) and MsbB (21). The resulting predominantly penta-acylated lipid A showed a strong decrease of reactogenicity in human cells. For example, an *htrB* mutant of *Haemophilus influenzae* elicited a 40-fold lower expression of tumor necrosis factor-α (TNF-α) than the wild type (24), and purified LPS from an *E. coli* Δ*msbB* mutant had a 10,000-fold decreased ability to induce TNF-α production (25). *Shigella* carries two copies of the *msbB* gene, one encoded on the chromosome (*msbB1*) and the other on the virulence plasmid (*msbB2*) (26). Mutant strains of *S. flexneri* 5 lacking both copies showed an 11–20-fold lower capability to induce TNF-α from adherent human monocytes (26). An *msbB* double mutant strain of *S. flexneri* 2a was shown to elicit decreased cytokine responses from a murine macrophage line (27), but it has not been reported to be tested with human cells.

Previously, we showed that genetic lipid A modification is compatible with the high yield production process using a *S. sonnei* Δ*msbB* GMMA-producing strain (2). However, as that study focused on production, reactogenicity tests with the resulting GMMA were not performed. In this study, we investigate the effects of *msbB* and *htrB* deletions on the lipid A structure in GMMA-producing strains of *S. sonnei* and *S. flexneri* 2a and the relative activation by the resulting GMMA of the TLR4 and TLR2 pathways in human peripheral blood mononuclear cells (PBMC) and in cells transfected with human TLR4 and TLR2. We show that the penta-acylated lipid A generated by the *msbB* mutation has a 600-fold lower ability to stimulate TLR4 than wild-type lipid A, whereas the penta-acylated lipid A from the *S. sonnei* Δ*htrB* mutant is 60,000-fold less active. We further demonstrate that the cytokine production in PBMC stimulated by GMMA with penta-acylated lipid A is substantially reduced and similar for GMMA from *S. sonnei* Δ*htrB* (800-fold) and *S. sonnei* and *S. flexneri* 2a Δ*msbB* mutants (300-fold) and that the residual cytokine production is largely due to TLR2 activation. We also show that, in contrast to *S. sonnei* Δ*htrB*, a compensatory palmitoleoylation occurs in the *S. flexneri* 2a Δ*htrB* mutant resulting in a high proportion of hexa-acylated lipid A with substantial ability to activate TLR4, albeit requiring approximately an order of magnitude higher concentration than the isogenic cell line.

EXPERIMENTAL PROCEDURES

Strains and Generation of Mutations—*S. sonnei* 53G (28) and *S. flexneri* 2a 2457T (29) were chosen as parent strains. The list of *Shigella* mutant strains used in this study and their abbreviated identifications are listed in Table 1. As all strains used in this study are GMMA-producing strains, the abbreviated names only refer to their additional mutations and characteristics. For generation of mutants from *S. flexneri* 2a without virulence plasmid, a white colony was selected by white appearance on Congo red agar before the start of the genetic modification. The curing of the virulence plasmid (pINV) was confirmed by the absence of the origin of replication (*ori*) and the plasmid-encoded genes, *virG* and *ospD3*, using PCR. The primers are listed in Table 2. To generate the *tolR* deletion in *S. flexneri* 2a and plasmid-cured *S. flexneri* 2a-pINV, the same strategy and primers as described previously for the generation of the *S. sonnei* Δ*tolR* mutant (2) were used.

The null mutation of *msbB1* (26), *htrB* (20), and *rfbG* (essential for OAg biosynthesis in *S. flexneri* 2a (30)) was obtained by replacing the gene of interest ("*gene*") with an antibiotic resistance cassette, using the following strategy. The upstream and

TABLE 1
Strains used in this study and their abbreviations

Strain name abbreviation	Genotype[a]	Modified lipid A	Ref.
Ss_{-p-OAg}	*S. sonnei*–pSS Δ*tolR*::*kan*	−	2
Ss_{-p-OAg} Δ*msbB*	*S. sonnei*–pSS Δ*tolR*::*kan* Δ*msbB*::*cat*	+	2
Ss_{-p-OAg} Δ*htrB*	*S. sonnei*–pSS Δ*tolR*::*kan* Δ*htrB*::*cat*	+	This study
$Sf2a_{-p-OAg}$	*S. flexneri* 2a–pINV Δ*tolR*::*kan* Δ*rfbG*::*erm*	−	This study
$Sf2a_{-p-OAg}$ Δ*msbB*	*S. flexneri* 2a–pINV Δ*tolR*::*kan* Δ*rfbG*::*erm* Δ*msbB*::*cat*	+	This study
$Sf2a_{-p-OAg}$ Δ*htrB*	*S. flexneri* 2a–pSS Δ*tolR*::*kan* Δ*rfbG*::*erm* Δ*htrB*::*cat*	+	This study
Ss_{-p-OAg} Δ*htrB* (pACYC*htrB*)	*S. sonnei*–pSS Δ*tolR*::*kat* Δ*htrB*::*cat* (pACYC*htrB*)	−	This study
$Sf2a_{-p-OAg}$ Δ*htrB* (pACYC*htrB*)	*S. flexneri* 2a–pINV Δ*tolR*::*kan* Δ*rfbG*::*erm* Δ*htrB*::*cat* (pACYC*htrB*)	−	This study
$Sf2a_{+p-OAg}$	*S. flexneri* 2a+pINV Δ*tolR*::*kan* Δ*rfbG*::*erm*	−	This study
$Sf2a_{+p+OAg}$	*S. flexneri* 2a+pINV Δ*tolR*::*kan*	−	This study
$Sf2a_{-p-OAg}$	*S. flexneri* 2a–pINV Δ*tolR*::*kan*	−	This study
$Sf2a_{+p+OAg}$ Δ*htrB*	*S. flexneri* 2a+pINV Δ*tolR*::*kan* Δ*htrB*::*cat*	+	This study
$Sf2a_{-p+OAg}$ Δ*htrB*	*S. flexneri* 2a–pINV Δ*tolR*::*kan* Δ*htrB*::*cat*	+	This study

[a] −pSS and −pINV, indicate that strain is cured of virulence plasmid; +pSS and +pINV, indicate that virulence plasmid is present.

TABLE 2
Primers used in this study

Primer name	Sequence 5′ → 3′
htrB-U1 Xba Sma	CTAGTCTAGAAACCCGGGCAATTGTATGTATTGTCG
htrB-soU2 SacI	ACTCGAGCTCATCATCCAACGC
htrB-flexU2 SacI	ACTCGAGCTCATCCGATATACGTTCGCCC
htrB-soD1 SalI	ACGCGTCGACCTCAGTAATCAGGGTTCTTTG
htrB-soD2 SmaI	CTAACCCGGGTAAATCTCCCCTGCCGGATG
htrB-flexD1 SalI	ACGCGTCGACCCTGTAATCTCAGGTCAAATG
htrB-flexD2 SmaI	CTAACCCGGGTAAATCTCCCATGCCGGATG
msbB-flexU5 Sma	CTAGTCTAGAAACCCGGGTGATAGTGTAGCGGCACA
msbB-flexU3 Sac	ACTCGAGCTCGTGAGCAAAGCCAGCTG
msbB-flexD5 SalI	ACGCGTCGACCTCGGTGTGGAAATTGG
msbB-flexD3 Xba Sma	CTAACCCGGGCAACGTACTTACTCTACCG
rfbF-1stop Sma	CTAACCCGGGCTAAGCATCTAAGACACCATTCTGTATC
rfbF-2 SalI	ACGCGTCGACAATATCCTGGAGCATACGTGT
rfc-1 SacI	ACTCGAGCTCACCAATAACGCCTGTTTTCTG
rfc-2 XbaSma3	CTAGTCTAGAAACCCGGGCTTCTTTGTCGGCTTATTAGC
P1.*htrB*compl-EcoRI	ACCGGAATTCGTGTAACACTGGCATGGTGTA
P2.*htrB*compl-NcoI	CATGCCATTGTAGCAATCCGCTGTTGGTGCG
EcoRV.Ery.F	AGCTTGATATCAGAGTGTGTTGATAGTGCAGTATC
EcoRV.Ery.R	AGCTTGATATCACCTCTTTAGCTTCTTGGAAGCT
EcoRV.Cm.F	AGCTTGATATCTGTGACGGAAGATCACTTCG
EcoRV.Cm.R	AGCTTGATATCGGGCACCAATAACTGCCTTA
Ori-1	CGGCATCAGAATAATACAAGCAGC
Ori-2	AGGTGTACCGTGCTCTGGG
virG-1	GTCACAGGTAACATGACTCTGGAG
virG-2	CCATGTGTGAATACTACCTTCACCC
ospD3-1	GTTTTGCCTCATTCAAGATATCACC
ospD3-2	TGACGATGGTTTGTCAGGATTGC
msbB.F	CGCCAAAGTTCCGTGATCCCATT
msbB.R	CTCTTCGATGATCTCCAGCCCTT
lpxP.F	GGCTTTGGGTACAGCTTCCTTA
lpxP.R	CCAACCCTTCAACATCAAACC

downstream regions of *gene* were amplified using the primer pairs *gene*-U and *gene*-D or *rfbF* and *rfc* (for the *rfG* knock out). The resistance cassette used to replace *gene* was amplified using primer pairs EcoRV.Ery.F/EcoRV.Ery.R or EcoRV.Cm.F/EcoRV. Cm.R. The fragments were inserted into pBluescript (Stratagene) so that the antibiotic resistance gene interposed the flanking regions of *gene*. The replacement construct (upstream region-resistance cassette-downstream region) was amplified using the primers binding to the 5′ end of the upstream flanking region and the 3′ end of the downstream flanking region of *gene* (see Table 2) and used to transform recombination-prone *tolR* deletion strains of *S. sonnei* or *S. flexneri* as described previously (2). In *S. sonnei*, the *htrB* gene was replaced by the chloramphenicol resistance gene *cat* (31). In *S. flexneri* 2a, *msbB1* and *htrB* were replaced by *cat,* and *rfbG* was replaced by the erythromycin resistance gene *erm* (32). In the *rfbG* knock out, also the flanking genes *rfbF* and *rfc* (30) were partially deleted. The *rfbG* deletion was introduced before the *msbB* or *htrB* deletion. The *msbB1* mutation was only introduced into the plasmid-cured strain as the plasmid carries a second copy of *msbB*

(*msbB2* (26)). For simplicity, the mutant is referred to as Δ*msbB*.

To complement strains carrying the *htrB* deletion, the *htrB* gene was amplified from *S. sonnei* 53G, including 239 bp upstream and 172 bp downstream using primers P1.*htrB*compl-EcoRI and P2.*htrB*compl-NcoI and inserted into low copy vector pACYC184 (New England Biolabs). The resulting plasmid pACYC*htrB* was introduced into electrocompetent *S. sonnei* or *S. flexneri* 2a Δ*htrB* cells.

GMMA Production and Purification—Bacterial strains were routinely grown at 30 °C in liquid or on solid M9 medium supplemented with nicotinic acid (Na_2HPO_4 7 g/liter, KH_2PO_4 3 g/liter, NaCl 0.5 g/liter, NH_4Cl 1 g/liter, 1 M $MgSO_4$ 2 ml/liter, 1 M $CaCl_2$ 0.1 ml/liter, glucose 0.4%, nicotinic acid 0.01 g/liter) or in chemically defined medium (SDM), with the same composition to the previously described SSDM (2) with the exception of the carbon source as follows: KH_2PO_4 13.3 g/liter, $(NH_4)_2HPO_4$ 4 g/liter, citric acid 1.7 g/liter, L-aspartic acid 2.5 g/liter, D-glucose 15 g/liter, $CoCl_2 \cdot 6H_2O$ 0.0025 g/liter, $MnCl_2 \cdot 4H_2O$ 0.015 g/liter, $CuCl_2 \cdot 2H_2O$ 0.0015 g/liter, H_3BO_3 0.003 g/liter, $Na_2MoO_4 \cdot 2H_2O$ 0.0025 g/liter, $Zn(CH_3COO)_2 \cdot 2H_2O$ 0.0025 g/liter, ferric citrate 2 μM, $MgSO_4$ 2 mM, thiamine 0.05 g/liter, nicotinic acid 0.01 g/liter, pH 6.7 (with NH_4OH). When required, kanamycin (30 μg/ml), chloramphenicol (20 μg/ml), erythromycin (100 μg/ml), or tetracycline (20 μg/ml) were added.

For GMMA production, overnight cultures were grown in the presence of the specific selective antibiotics and used to inoculate the production medium to an OD of 0.03– 0.05. Production cultures were incubated at 30 °C and 200 rpm overnight. Culture supernatants were collected by a 10-min centrifugation at 5,000 × g followed by a 0.22-μm filtration. GMMA were concentrated using an Amicon stirrer cell with a regenerated cellulose filter with a 100-kDa nominal molecular mass limit (Amicon Ultracell) under nitrogen flow. The retentate was collected in a 70-ml ultracentrifuge propylene tube (Beckman Coulter) and ultracentrifuged at 186,000 × g using 45Ti rotor (Beckman Coulter) for 2 h at 4 °C. Pellets were resuspended in 4 ml of PBS followed by 0.22-μm filtration. GMMA were stored at 4 °C.

GMMA Protein and KDO Quantification—GMMA quantities were expressed as total protein present in GMMA. The protein quantity was determined using the DC protein assay (Bio-Rad) kit (Lowry assay) according to the manufacturer's

instructions. Bovine serum albumin (Pierce) was used for the standard curve in the range 1–50 μg/assay. Measurements of GMMA were performed in two different dilutions, each in duplicate.

Core-reducing end 2-keto-3-deoxyoctonate (KDO) after lipid A cleavage was quantified using the semicarbazide/HPLC-size exclusion chromatography method as we reported previously (33). To apply the method to GMMA, 150 μg of GMMA were hydrolyzed in 1% acetic acid for 3 h at 100 °C and subsequently centrifuged for 15 min at $14,000 \times g$. Supernatants were collected and dried in a SpeedVac, and the pellets were dissolved in water. Samples and a standard of 4–40 μg/ml KDO ammonium solution (Sigma, K2755) were derivatized using semicarbazide and analyzed by HPLC-size exclusion chromatography using a TSKgel G3000 PW-XL column (TOSOH, 808021). The amount of core reducing end KDO was calculated using the calibration curve built with the peak areas of derivatized KDO standard at 252 nm.

Negative Staining Transmission Electron Microscopy—A drop of 5 μl of GMMA suspension at a concentration of 100 μg/ml in PBS was adsorbed onto 300 mesh copper Formvar/carbon-coated grids for 5 min. Grids were then washed with a few drops of distilled water and dried by blotting with Whatman filter paper. For negative staining, grids were treated with 2% uranyl acetate in double distilled H_2O for 1 min, blotted with Whatman filter paper, air-dried, and observed with a Tecnai 2 Spirit transmission electron microscope (FEI, Eindoven, The Netherlands) operating at 80 kV. Electron micrographs were recorded at a nominal magnification of $105,000\times$. GMMA diameters were measured manually on printed copies of the electron micrographs in comparison with the scale bar. The nonparametric Kruskal-Wallis test was used to statistically compare the sizes of GMMA obtained from different strains.

SDS-PAGE and Peptide Mass Fingerprinting—SDS-PAGE of GMMA was performed using 12% (w/v) polyacrylamide gels (Bio-Rad) as described previously (2). The gels were stained using Coomassie Blue stain (Sigma) according to the manufacturer's instructions for proteins.

Protein spots were excised from the gel and processed as described previously (34). Briefly, mass spectra were acquired on an Ultraflex MALDI TOF-TOF mass spectrometer (Bruker Daltonics) in reflectron, positive mode, in the mass range of 900 to 3,500 Da. Spectra were externally calibrated by using a combination of standards pre-spotted on the target (Bruker Daltonics). MS spectra were analyzed by peptide mass fingerprinting with FlexAnalysis (FlexAnalysis version 2.4, Bruker Daltonics). Peaks were annotated with FlexAnalysis default parameters and manually revised. Protein identification was carried from the generated peak list using the Mascot program (Mascot server version 2.2.01, Matrix Science). Mascot was run on a database containing protein sequences deduced from sequenced *Shigella* genomes (2).

MALDI-TOF and MS/MS Analysis on Lipid A—Lipid A was precipitated from GMMA using mild acid hydrolysis with 1% acetic acid for 2 h at 100 °C (35). Samples were centrifuged at $14,000 \times g$ for 15 min; the pellets were resuspended in water and washed twice with water. The pellets were dried overnight using a SpeedVac and resuspended in chloroform/methanol 4:1

and mixed with an equal volume of Super DHB solution (Sigma). 2 μl of the mixture were loaded to the target plate (MTP 384 target plate ground steel BC, Bruker Daltonics) and analyzed by Ultraflex MALDI-TOF (Bruker Daltonics) in reflectron ion-negative mode. A peptide calibration standard (Bruker Daltonics), mixed with the Super DHB solution, was included in each analysis. For MS/MS analysis of lipid A, main peaks from the linear mode analysis were selected for collision-induced dissociation, and the resulting fragments were detected by MALDI TOF-TOF in ion negative mode. For each sample, spectra represent the integration of the analysis of 20 different areas of the spot by 50 single laser shots. The m/z rations were determined by Flex Analysis software in comparison with the peptide standard.

Quantitative Real Time PCR and RNA Isolation—RNA was purified using RNeasy Plus mini kit (Qiagen) from 2 ml of bacteria grown at 30 °C to an optical density of 1. Reverse transcription (2 μg of RNA/reaction) was performed using Superscript II, and the product was purified by QIAquick PCR purification kit (Qiagen). Quantitative RT-PCR for genes *msbB* and *lpxP* and was performed using 10 ng of cDNA, SYBR Green kit (Invitrogen), and 0.4 μM primers *msbB*.F/*msbB*.R and *lpxP*.F/*lpxP*.R in thermocycler MX3005P (Stratagene) with 40 cycles (95 °C, 15 s; 60 °C, 60 s). Fold induction was calculated as $2^{-\Delta(\Delta Ct)}$, were ΔCt is the difference between the numbers of cycle of amplification needed to reach the threshold (fluorescence dRn = 0.018) for *lpxP* versus the cycle of amplification of the late acyltransferase gene *msbB* of the constitutive lipid A biosynthesis pathway (36) in same strain. $\Delta(\Delta Ct)$ represents the difference in the ΔCt of *lpxP* in $Sf2a_{-p-OAg} \Delta htrB$ compared with $Ss_{-p-OAg} \Delta htrB$.

NF-κB Luciferase Reporter Assay—TLR-specific activation assays were performed using human embryonic kidney 293 (HEK293) cells expressing luciferase under control of the NF-κB promoter and stably transfected with either TLR4, MD2, and CD14 (TLR4-HEK293) or TLR2 (TLR2-HEK293) (37). HEK293-transfected cells were maintained in DMEM complemented with 4.5 g/liter glucose and HEPES (Invitrogen), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin solution (Invitrogen), and specific antibiotics for the different cell lines: puromycin (5 μg/ml), blasticidin (10 μg/ml), and hygromycin (250 μg/ml) for TLR4-HEK293 cells, and puromycin and hygromycin for TLR2-HEK293 cells.

For the NF-κB luciferase assay, 25,000 cells/well were seeded in 90 μl of complete DMEM without antibiotics in 96-well μClear® luciferase plates (PBI International) and incubated for 24 h at 37 °C. 10 μl of serial 5-fold dilutions of GMMA in PBS (0.0001–1,000 ng/ml final concentration in the assay) were added. All GMMA concentrations were tested in duplicate. After incubation for 5 h at 37 °C, supernatants were aspirated from each well, and cells were lysed for 20 min at room temperature using 20 μl/well of 1:5 diluted "passive lysis buffer" (Promega). Produced luciferase was detected using 100 μl/well luciferase assay reagent (Promega), and emitted light was immediately quantified using a luminometer Lmax II[384] (Molecular Devices). NF-κB activation of cells stimulated with GMMA is expressed as fold-increase of emitted light over the average result of PBS-stimulated control cells. GMMA concen-

trations needed to obtain a 3-fold (TLR4 experiments) or 10-fold (TLR2 experiments) induction of NF-κB were arbitrarily used for comparing relative activity because that was in the middle of the linear part of the sigmoidal curves. They were determined as x axis intercepts from the generated curves. The nonparametric Mann-Whitney test was used to statistically evaluate the results obtained with different GMMA.

PBMC Isolation, TLR Blocking, and Stimulation (Monocyte Activation Test)—Buffy coats from three different donors were used to isolate PMBC using Ficoll density centrifugation as reported previously (38). PBMC were cultured at a density of 2×10^5 cells/well with 180 μl of RPMI 1640 medium complemented with 25 mM HEPES, 2 mM glutamine, 10% FBS, 1% penicillin/streptomycin (Invitrogen) in 96-well round bottom plates. After incubation at 37 °C for 30 min, 20 μl of 10-fold serial dilutions of GMMA in PBS (0.0001–1,000 ng/ml final concentration in the assay) were added. Each GMMA concentration was tested in duplicate. During experiment setup, PBMC were exposed to PBS as control to assess the baseline cytokine release and to determine the concentration of GMMA that did not trigger a significant activation of IL-6 release over baseline (0.0001 ng/ml). In further experiments, this GMMA concentration was used as background control. In blocking experiments, 25 μg/ml TLR4 blocking antibody (eBioscience) and/or 15 μg/ml TLR2 blocking antibody (eBioscience) in RPMI 1640 medium were added for 30 min before the addition of GMMA. Cells were incubated for 4 h at 37 °C, and supernatants were recovered after centrifugation of the plates at 400 $\times$ g and stored at −70 °C until analysis.

Cytokine Analysis by ELISA and 7-Plex Mesoscale—Nunc MaxiSorp 96-well plates were coated overnight at 4 °C with 2 μg/ml human IL-6 capture antibody (eBioscience 14-7069) in PBS, subsequently washed three times with PBS with 0.05% Tween 20 (PBST), blocked for 1 h with PBS with 1% BSA at room temperature, and washed three times with PBST. 50 μl of supernatants from PBMC experiments, diluted 1:4 with PBS, were incubated for 2 h at room temperature. A 2-fold dilution series of recombinant human IL-6 (eBioscience 39-8069) of 31.24 to 4,000 pg/ml in RPMI 1640 medium with 10% FBS was included as standard curve on each plate. Plates were washed three times with PBST. Bound IL-6 was detected using 2 μg/ml biotin-conjugated anti-human IL-6 (eBioscience 13-7068) in PBST with 0.1% BSA for 2 h at room temperature, followed by three washes with PBST, 20 min of incubation at room temperature with streptavidin/horseradish peroxidase (R&D Systems, DY998) diluted 1:200 in PBST with 0.1% BSA, three washes with PBST, and a color reaction with 100 μl/well substrate (R&D Systems, DY999) for 8 min at room temperature in the dark. The reaction was stopped by adding 50 μl/well of 12.5% sulfuric acid. The plates were read at 450 and 630 nm and the $A_{450 - 630 \text{ nm}}$ was determined. IL-6 concentrations in the samples were calculated in comparison with the standard. Results below the detection limit were assigned half of the detection limit.

Mesoscale 7-spot (MSD Technology) analysis for cytokines IL-6, IL-8, IL-1β, TNF-α, IL-10, IL-12, and IFN-γ was performed with 25 μl of supernatants from PBMC according to the manufacturer's instructions. Concentrations of the different cytokines in the samples were determined in comparison with the preloaded standard in the plates.

For the analysis of the cytokine release by PBMC, the average cytokine levels in the duplicate assays were plotted against the GMMA concentration. GMMA concentrations needed to obtain a 10-fold increase of IL-6 release over the average level obtained at the lowest GMMA concentration (background) were determined as x axis intercepts from the generated curves and used to compare the stimulatory activity of the different GMMA. The nonparametric Mann-Whitney test was used to statistically evaluate the results obtained with different GMMA.

For statistical analysis of the results of the TLR blocking experiments, the ratio of IL-6 produced by PBMC treated with anti-TLR4 or anti-TLR2 and the IL-6 produced by PBMC not treated with blocking antibodies, stimulated with the same concentration of GMMA, were calculated to normalize between the different experiments using PBMC from different donors. The ratio was determined for each replicate in the experiments. The nonparametric Wilcoxon signed rank test was used to assess whether the obtained ratios were significantly different from 1 (no effect by blocking).

RESULTS

Mutant Production and Conditions for GMMA Production—We previously reported the generation of a *S. sonnei*-pSS $\Delta tolR$ $\Delta msbB$ mutant ($Ss_{-p - OAg} \Delta msbB$) that was able to grown to high optical densities in chemically defined medium developed for fermentation at 30 °C. The $Ss_{-p - OAg} \Delta msbB$ strain was cured of the virulence plasmid (2) to remove a second copy of the *msbB* gene (*msbB2* (26)) and the OAg biosynthesis genes (39) encoded on the plasmid. Thus, we chose a plasmid-deficient background to generate the *htrB* mutant of *S. sonnei* ($Ss_{-p - OAg} \Delta htrB$) and also used a plasmid- and OAg-deficient background in *S. flexneri* 2a to compare GMMA from the respective $\Delta msbB$ and $\Delta htrB$ mutants to the GMMA from the *S. sonnei* strains.

Similar to the $Ss_{-p - OAg} \Delta msbB$ mutant (2), the $Sf2a_{-p - OAg}$ $\Delta msbB$ mutant retained the ability to grow in complex media at 37 °C but at a slower rate (2-fold duplication time) than a single $\Delta tolR$ mutant. In contrast, $\Delta htrB$ strains only grew in chemically defined or minimal media and only at 30 °C. The duplication time of the $\Delta htrB$ or $\Delta msbB$ strains increased from 30 min to ~2 h, but all the strains were able to reach high ODs (OD 10) after overnight incubation in chemically defined medium at 30 °C. Thus 30 °C and chemically defined medium were chosen as the standard growth conditions. All strains with lipid A modifications yielded more than 50 mg/liter GMMA protein.

GMMA from *Shigella* strains carrying different mutations showed similar morphology by electron microscopy (Fig. 1*A*) with average sizes of 30–32 nm in all six strains and a size distribution of 17–53 nm, measured with 30 GMMA per strain. A comparison of the GMMA sizes from all strains gave no significant difference ($p = 0.90$). To characterize whether the genetic lipid A modifications might alter the protein composition of GMMA, the protein pattern of GMMA from the different mutants was evaluated by SDS-PAGE (Fig. 1*B*). Although the overall pattern remained similar, four protein bands, iden-
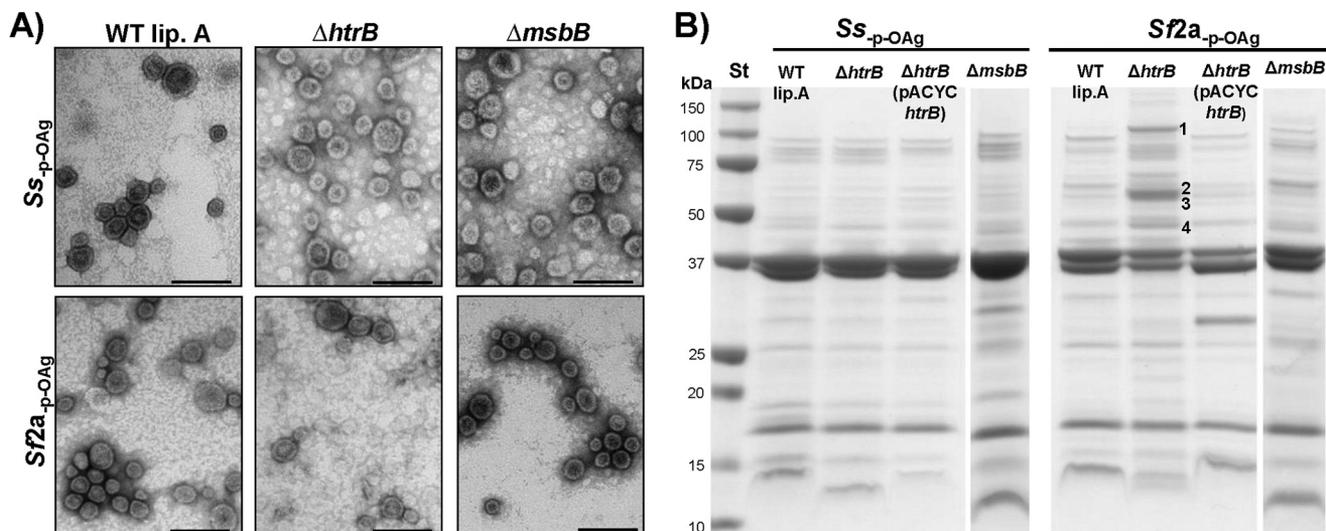
FIGURE 1. *A*, electron microscopy of GMMA from different strains. GMMA were purified from Ss_{-p-OAg} and $Sf2a_{-p-OAg}$ containing wild-type lipid A (Ss_{-p-OAg} *WT lip. A, Sf*2a$_{-p-OAg}$ *WT lip. A*), Ss_{-p-OAg} Δ*msbB*, $Sf2a_{-p-OAg}$ Δ*msbB*, Ss_{-p-OAg} Δ*htrB*, and $Sf2a_{-p-OAg}$ Δ*htrB*, negatively stained, and viewed by electron microscopy (105,000-fold magnification) revealing the presence of well organized membrane particles with diameters ranging between 17 and 53 nm in each preparation. *Bar length,* 100 nm. *B*, SDS-PAGE. 10 μg (protein) of the GMMA shown in *A* and GMMA from Ss_{-p-OAg} Δ*htrB* (pACYC*htrB*) and $Sf2a_{-p-OAg}$ Δ*htrB* (pACYC*htrB*) were separated by SDS-PAGE (12% polyacrylamide) and Coomassie stained. Four protein bands that were more abundant in GMMA from $Sf2a_{-p-OAg}$ Δ*htrB* than in GMMA from other $Sf2a_{-p-OAg}$ strains were identified by peptide mass fingerprinting: *1,* pyruvate dehydrogenase; *2,* glutamine synthetase; *3,* ketol-acid reductoisomerase; *4,* D-3-phosphoglycerate dehydrogenase.

tified as pyruvate dehydrogenase, glutamine synthetase, ketol-acid recutoisomerase, and D-3-phosphoglycerate dehydrogenase (Fig. 1*B*) by peptide mass fingerprinting, were found to be up-regulated in GMMA from $Sf2a_{-p-OAg}$ Δ*htrB*. As these proteins are cytoplasmic proteins, no effect on the reactogenicity studies was expected.

Characterization of Lipid A by MALDI-TOF and MALDI-TOF/TOF—The lipid A of LPS of the mutants was extracted and analyzed by MALDI-TOF. The spectra are reported in Fig. 2, and the structures of lipid A corresponding to the main peaks were assigned on the basis of mass and by comparison of results with similar mutants of *E. coli* (Fig. 2*L*) (40). The main peaks in the mass spectra obtained by MALDI-TOF from lipid A purified from GMMA from *S. sonnei* and *S. flexneri* 2a strains with wild-type (WT) LPS (Fig. 2, Ss_{-p-OAg} (*A*) and $Sf2a_{-p-OAg}$ (*B*)) had an *m/z* corresponding to the theoretical mass of the hexa-acylated lipid A of 1,798 Da. The main peaks obtained by mass spectrometry from the Ss_{-p-OAg} Δ*msbB* GMMA (Fig. 2*C*) and $Sf2a_{-p-OAg}$ Δ*msbB* (Fig. 2*D*) GMMA corresponded, in both strains, to a penta-acylated lipid A lacking a myristoyl chain (theoretical mass 1,588 Da, 210 *m/z* shift to WT lipid A due to the absence of a C_{14} fatty acid chain) consistent with *msbB* knock outs.

The mass spectrum of lipid A from Ss_{-p-OAg} Δ*htrB* GMMA (Fig. 2*E*) showed a main peak corresponding to a penta-acylated lipid A lacking a lauroyl chain (theoretical mass 1,616 Da, corresponding to the absence of a C_{12} fatty acid chain giving a *m/z* shift of 182), consistent with an *htrB* knock out. The spectra obtained from GMMA of $Sf2a_{-p-OAg}$ Δ*htrB* (Fig. 2*F*) also had the penta-acylated lipid A species with the deletion of a lauroyl chain but showed a new peak at *m/z* 1,850 (Fig. 2*F*), most likely corresponding to a hexa-acylated lipid A species with an *m/z* different from WT lipid A. The mass of this lipid A species corresponds to acylation by a palmitoleoyl chain (a $C_{16:1}$ fatty

acid chain, *m/z* shift of 236) of the penta-acylated lipid A (*m/z* 1,616) present in both the Ss_{-p-OAg} Δ*htrB* GMMA and of $Sf2a_{-p-OAg}$ Δ*htrB* to give a hexa-acylated lipid A. Confirmation for the palmitoleoylation was obtained by MS/MS analysis using collision-induced decay to fragment the lipid A species present in the main peaks of the first dimension MS for GMMA from Ss_{-p-OAg} Δ*htrB* (*m/z* 1,615, Fig. 2*E*) and $Sf2a_{-p-OAg}$ Δ*htrB* (*m/z* 1,850, Fig. 2*F*). The main difference observed when overlaying the MS/MS spectra was a peak with an *m/z* corresponding to a palmitoleoyl chain (*m/z* 252, highlighted in Fig. 2*G*) in $Sf2a_{-p-OAg}$ Δ*htrB*. MALDI-TOF spectra of GMMA from the Ss_{-p-OAg} Δ*htrB* and $Sf2a_{-p-OAg}$ Δ*htrB* strains complemented with pACYC*htrB* (Fig. 2, *H* and *I*) showed in both cases a hexa-acylated WT lipid A as the main peak (observed, *m/z* 1,797; theoretical, *m/z* 1,797), and no hepta-acylated peak (theoretical *m/z* 2,034 for a wild-type with extra palmitoleoyl chain) was observed.

The palmitoleoylated hexa-acylated form (*m/z* 1,852) was also the main form in MALDI-TOF spectra of lipid A purified from GMMA from $Sf2a_{-p+OAg}$ (Fig. 2*K*), $Sf2a_{+p+OAg}$, and *S. flexneri* 3a and 6 Δ*tolR* Δ*htrB* strains.[3]

To test whether the production of the palmitoleoylated hexa-acylated lipid A species (*m/z* 1,851) could be induced in *S. sonnei* under stress conditions, Ss_{-p-OAg} Δ*htrB* was grown at 12 °C to induce a cold stress response. In the corresponding lipid A analysis by MALDI-TOF, a small amount (signal intensity less than 5% of the main species) of the palmitoleoylated lipid A species was identified (Fig. 2*J*).

Palmitoleoylation of lipid A in the absence of the lauroyl chain is consistent with the activity by the late acyltransferase LpxP. Thus, the expression level of *lpxP* was quantified in
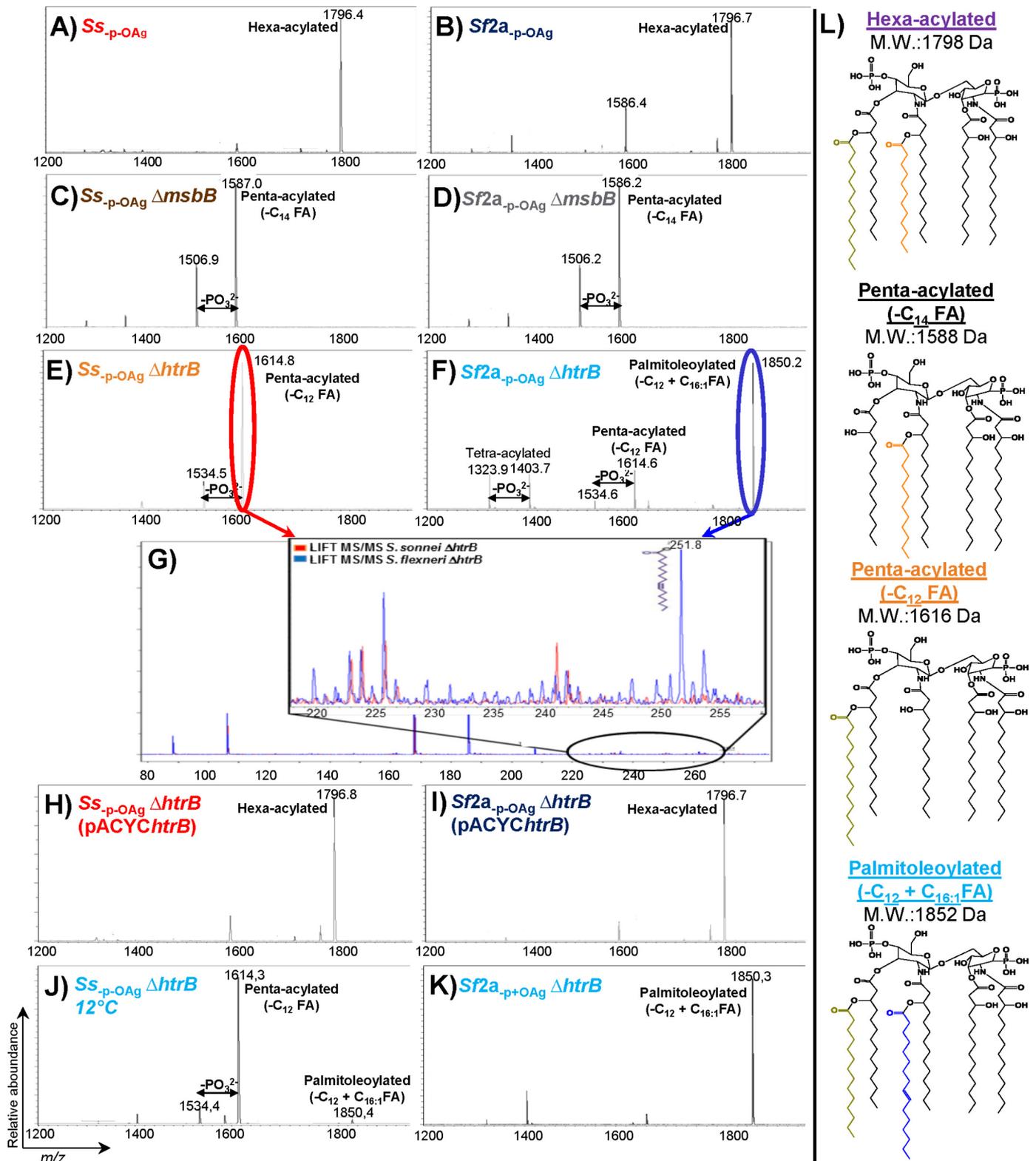
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[3] F. Citiulo, unpublished results.

FIGURE 2. **MALDI-TOF spectra of lipid A preparations in reflectron ion-negative mode.** Lipid A was extracted from GMMA from the following: *A, Ss*$_{-p-OAg}$; *B, Sf*2a$_{-p-OAg}$; *C, Ss*$_{-p-OAg}$ Δ*msbB*; *D, Sf*2a$_{-p-OAg}$ Δ*msbB*; *E, Ss*$_{-p-OAg}$ Δ*htrB*; *F, Sf*2a$_{-p+OAg}$ Δ*htrB*; *H, Ss*$_{-p-OAg}$ Δ*htrB* (pACYC*htrB*); *I, Sf*2a$_{-p-OAg}$ Δ*htrB* (pACYC*htrB*); *J, Ss*$_{-p-OAg}$ Δ*htrB* grown at 12 °C; and *K, Sf*2a$_{-p+OAg}$ Δ*htrB*. *G*, overlay of negative ion LIFT MALDI-TOF/TOF spectra in the low *m/z* range of the dominant species in lipid A from *Ss*$_{-p-OAg}$ Δ*htrB* (*E*) and *Sf*2a$_{-p-OAg}$ Δ*htrB* (*F*) after collision-induced dissociation. *L*, lipid A structures with molecular weights corresponding to the observed main peaks.

Ss$_{-p-OAg}$ Δ*htrB* and *Sf*2a$_{-p-OAg}$ Δ*htrB* grown at 30 °C in comparison with the *msbB* gene that is part of the constitutive lipid A pathway using real time PCR. In three independent experiments, the ratio of transcript of *lpxP* to *msbB* was on average 7.3-fold higher (standard deviation 0.9) in *Sf*2a$_{-p-OAg}$ Δ*htrB* compared with *Ss*$_{-p-OAg}$ Δ*htrB*.

TLR4-NF-κB Luciferase Reporter Assay—We stimulated HEK293 cells stably transfected to express only human TLR4 recognition complex and an NF-κB-inducible luciferase reporter gene (41) to characterize TLR4 stimulation by GMMA with the different lipid A species. The results obtained with different concentrations of GMMA are displayed in Fig. 3 and Table 3.
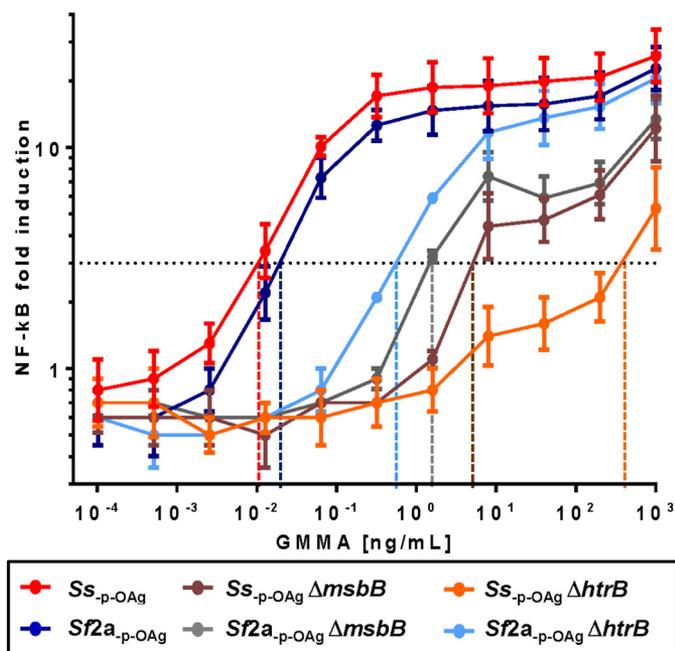

FIGURE 3. **Activation of TLR4 reporter cell line by different GMMA.** 25,000 TLR4-HEK293 cells/well were stimulated with 0.0001–1000 ng/ml (5-fold steps) of GMMA obtained from Ss_{-p-OAg}, $Sf2a_{-p-OAg}$, Ss_{-p-OAg} $\Delta msbB$, $Sf2a_{-p-OAg}$ $\Delta msbB$, Ss_{-p-OAg} $\Delta htrB$, and $Sf2a_{-p-OAg}$ $\Delta htrB$. After 5 h, luciferase expression was measured and expressed as fold-induction compared with cells incubated with PBS and plotted as averages of duplicates with standard deviations. GMMA concentrations that resulted in 3-fold increased activation of NF-κB (*black dashed line*) over background are shown as *x* axis intercepts (*colored dashed lines*). A representative result of four independent experiments is shown.

GMMA from $Sf2a_{-p-OAg}$ $\Delta msbB$ and Ss_{-p-OAg} $\Delta msbB$ stimulated similar levels of NF-κB expression in the HEK293 TLR4 transfectant cells (Fig. 3) and required ~600-fold more GMMA than the parent GMMA (Table 3) to give a 3-fold increase in NF-κB activity. In contrast, GMMA from Ss_{-p-OAg} $\Delta htrB$ and $Sf2a_{-p-OAg}$ $\Delta htrB$ gave very different results. NF-κB induction by Ss_{-p-OAg} $\Delta htrB$ GMMA was only detectable at a high concentration, requiring ~60,000-fold more GMMA than the GMMA with wild-type lipid A and 100-fold more than $\Delta msbB$ GMMA to stimulate the same level of NF-κB activity (Fig. 3 and Table 3). In contrast, $Sf2a_{-p-OAg}$ $\Delta htrB$ GMMA (Fig. 3 and Table 3) retained higher TLR4 stimulation and required 10-fold less GMMA ($p = 0.0286$) than $\Delta msbB$ GMMA to result in a similar induction of NF-κB. Accordingly, the decrease of TLR4 stimulation compared with GMMA with wild-type lipid A was the smallest (50-fold) of all tested GMMA with lipid A modifications.

To ensure that the observed differences are the results of differences of the stimulatory activity of the lipid A and not caused by different amounts of lipid A present in GMMA, the molar amount of lipid A per mg of protein was determined by quantifying the LPS core sugar KDO. The amounts of lipid A were similar in all GMMA. In comparison with GMMA from Ss_{-p-OAg} $\Delta htrB$ with the lowest activity, GMMA from Ss_{-p-OAg} contained 1.1-fold, $Sf2a_{-p-OAg}$ 2.0-fold, $Sf2a_{-p-OAg}$ $\Delta htrB$ 0.3-fold, Ss_{-p-OAg} $\Delta msbB$ 0.8-fold, and $Sf2a_{-p-OAg}$ $\Delta msbB$ 1.1-fold the amount of lipid A.

Cytokine Release from Human PBMC—In order measure the endotoxin level of GMMA in a more natural context, and in particular to examine whether the GMMA could stimulate additional pattern recognition receptors to TLR4, GMMA purified from different mutants were used to stimulate human PBMC in the monocyte activation test (Fig. 4). GMMA purified from *Shigella* strains without lipid A modification (both Ss_{-p-OAg} and $Sf2a_{-p-OAg}$) induced high levels of the pro-inflammatory cytokines interleukin 6 (IL-6), TNF-α, IL-1β, and

TABLE 3

TLR4 NF-κB-specific assay results

Concentrations of each type of GMMA resulting in 3-fold increased activation of NF-κB were determined in four independent experiments (wild-type and $\Delta htrB$ GMMA) or two independent experiments ($\Delta msbB$ GMMA) using TLR4/MD2/CD14 HEK293 transfectant cells as shown in Fig. 3. Results from GMMA from Ss_{-p-OAg} and $Sf2a_{-p-OAg}$ with wild-type lipid A and GMMA from Ss_{-p-OAg} $\Delta msbB$ and $Sf2a_{-p-OAg}$ $\Delta msbB$ ($\Delta msbB$ GMMA) were combined for further analysis. Fold differences of the mean concentration of the respective GMMA to stimulate 3-fold NF-κB induction were calculated in comparison with GMMA with wild-type lipid A and $\Delta msbB$ GMMA.

GMMA from strain:	GMMA concentration at 3-fold NF-κB induction [ng/mL]				
	1	2	3	4	Mean
Ss_{-p-OAg}	0.003	0.003	0.010	0.005	**0.005**
$Sf2a_{-p-OAg}$	0.004	0.004	0.021	0.010	**0.010**
Ss_{-p-OAg} $\Delta msbB$	-	-	5.17	4.16	**4.67**
$Sf2a_{-p-OAg}$ $\Delta msbB$	-	-	1.56	7.71	**4.64**
Ss_{-p-OAg} $\Delta htrB$	418	324	362	897	**500**
$Sf2a_{-p-OAg}$ $\Delta htrB$	0.39	0.16	0.59	0.44	**0.40**

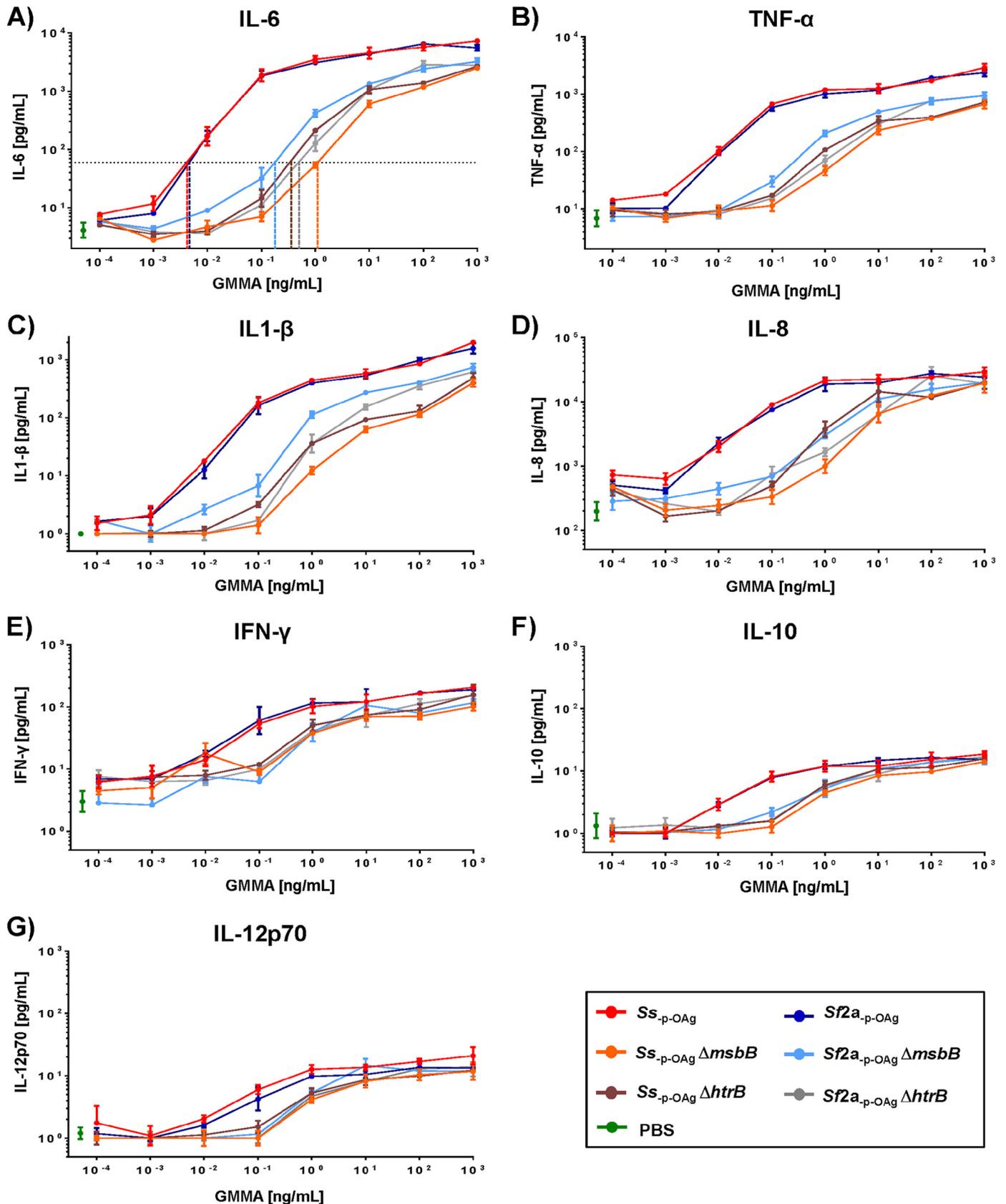| GMMA with lipid A type:	Mean [ng/mL]	Fold difference to GMMA with WT lipid A	Fold difference to $\Delta msbB$ GMMA
Wild-type	**0.008**	-	581
$\Delta msbB$	**4.65**	581	-
Ss_{-p-OAg} $\Delta htrB$	500	62500	0.01
$Sf2a_{-p-OAg}$ $\Delta htrB$	0.40	50	12

FIGURE 4. **Cytokine release by human PBMC after stimulation with different types of GMMA.** 200,000 human PBMC cells were stimulated for 4 h with 0.0001–1000 ng/ml (10-fold steps) of GMMA from Ss_{-p-OAg}, $Sf2a_{-p-OAg}$, Ss_{-p-OAg} $\Delta msbB$, $Sf2a_{-p-OAg}$ $\Delta msbB$, Ss_{-p-OAg} $\Delta htrB$, and $Sf2a_{-p-OAg}$ $\Delta htrB$. Release of the following: *A*, IL-6; *B*, TNF-α; *C*, IL-1β; *D*, IL-8; *E*, interferon-γ (IFN-γ); *F*, IL-10; and *G*, IL-12 p70, were measured by human pro-inflammatory 7-plex mesoscale and plotted as averages of duplicates with standard deviations. GMMA concentrations that resulted in 10-fold increase of IL-6 release over background are shown as *x* axis intercepts (*colored dashed lines*). Cytokine release by PBMC exposed to PBS was used as control.

TABLE 4

Monocyte activation test results from different blood donors

Concentrations of each type of GMMA resulting in 10-fold increased release of IL-6 were determined using PMBC from four different donors as shown in Fig. 4. Results for GMMA from Ss_{-p-OAg} and $Sf2a_{-p-OAg}$ with wild-type lipid A and GMMA from $Ss_{-p-OAg}\Delta msbB$ and $Sf2a_{-p-OAg}\Delta msbB$ ($\Delta msbB$ GMMA) were compared by the Mann-Whitney test ($p = 0.8824$ and $p = 1.000$, respectively) and combined for further analysis. Fold differences of the mean concentration of the respective GMMA to stimulate a 10-fold increase of IL-6 release were calculated in comparison of GMMA with wild-type lipid A and with $\Delta msbB$ GMMA.

GMMA from strain:	GMMA concentration at 10-fold increase of IL-6 over background [ng/mL]					GMMA with lipid A type:	Mean [ng/mL]	Fold difference to GMMA containing WT lipid A	Fold difference to $\Delta msbB$ GMMA
	1	2	3	4	Mean				
Ss_{-p-OAg}	0.007	0.006	0.003	0.016	**0.008**	Wild-type	0.008	-	274
$Sf2a_{--p-OAg}$	0.010	0.003	0.003	0.018	**0.009**				
$Ss_{-p-OAg}\Delta msbB$	2.05	2.81	0.62	2.55	**2.01**	$\Delta msbB$	2.19	274	-
$Sf2a_{-p-OAg}\Delta msbB$	1.98	2.44	1.31	3.79	**2.38**				
$Ss_{-p-OAg}\Delta htrB$	5.38	12.5	4.53	4.18	**6.65**	$Ss_{-p-OAg}\Delta htrB$	6.65	831	0.33
$Sf2a_{-p-OAg}\Delta htrB$	0.52	0.31	0.29	0.56	**0.42**	$Sf2a_{-p-OAg}\Delta htrB$	0.42	53	5

IL-8, intermediate levels of IFN-γ, and low levels of IL-12 and IL-10 (Fig. 4). All GMMA with lipid A modifications resulted in substantially lower cytokine release (Fig. 4).

The GMMA concentration required to give a 10-fold increase in IL-6 release over background (Table 4) was used for comparing relative activity (42). The same analysis performed at other points within the linear part of the curve (3- and 30-fold over background, respectively) gave similar results (data not shown).

GMMA purified from *Shigella* strains carrying lipid A modification gave a similar rank order in reduction of IL-6 release upon stimulation in comparison with the parent strains with wild-type lipid A (Fig. 4 and Table 4), as observed in the TLR4-specific assay (Fig. 3 and Table 3) but with smaller differences than in the TLR4-specific assay. The amount of GMMA required to give a 10-fold increase in IL-6 release was as follows: $Ss_{-p-OAg}\Delta htrB$ (800×) > $Ss_{-p-OAg}\Delta msbB \approx Sf2a_{-p-OAg}$ $\Delta msbB$ (300×) > $Sf2a_{-p-OAg}\Delta htrB$ (50×) the amount of GMMA wild-type lipid A (Table 4).

TLR Blocking—With the objective of identifying the TLRs that contribute to the residual activation by GMMA with lipid A modification, PBMC were incubated with TLR2 and/or TLR4 blocking antibodies before stimulation with 1 and 10 ng/ml of GMMA, concentrations chosen to give a significant but not saturating increase of IL-6 (Fig. 5). The three GMMA with penta-acylated lipid A, $Ss_{-p-OAg}\Delta htrB$ (Fig. 5D), Ss_{-p-OAg} $\Delta msbB$ (Fig. 5B), and $Sf2a_{-p-OAg}\Delta msbB$ (Fig. 5C), gave similar results as follows: IL-6 production was substantially reduced following incubation with TLR2 blocking antibody (70–90%) but either no reduction or minimal reduction was observed with TLR4 blocking antibody (10–30%) suggesting residual activity was principally due to TLR2 activation. With the $Ss_{-p-OAg}\Delta msbB$ and $Sf_{-p-OAg}\Delta msbB$ GMMA, a small reduction was obtained with the TLR4 blocking antibody alone ($Ss_{-p-OAg}\Delta msbB$, $p = 0.0234$; $Sf2a_{-p-OAg}\Delta msbB$, $p = 0.0078$ in four experiments) suggesting that there was still

residual TLR4 activation. Further reduction was achieved with the combination of TLR2 and TLR4 blocking antibodies compared with TLR2 blocking antibody alone. With the $Ss_{-p-OAg}\Delta htrB$ GMMA, no effect from TLR4 blocking was observed.

The GMMA with mostly hexa-acylated lipid A ($Sf2a_{-p-OAg}$ $\Delta htrB$, Fig. 5E) gave a substantial reduction with a TLR4 blocking antibody, *i.e.* 80% reduction at 1 ng/ml and 40% at 10 ng/ml GMMA concentration. TLR2 blocking showed no effect on the IL-6 release at 1 ng/ml GMMA concentration and resulted in ~35% reduction (average of three independent experiments) at the 10 ng/ml GMMA concentration. Incubation with both TLR2 and TLR4 blocking antibodies gave lower IL-6 production at 1 and 10 ng/ml GMMA suggesting that both TLR were still active but that the TLR4 activation was dominant especially at lower GMMA concentrations.

To confirm that the differences in the relative contribution of TLR4 and TLR2 to activation observed in the blocking experiments were primarily dependent on the differential TLR4 activation by the different GMMA, the ability of the GMMA to activate TLR2 was tested by stimulating HEK293-TLR2 transfectant cells. All four $\Delta msbB$ or $\Delta htrB$ GMMA required similar GMMA concentrations (2.6–4.9 ng/ml) to give a 10-fold NF-κB induction (Fig. 6).

DISCUSSION

GMMA are attractive candidates for vaccines as they present surface antigens in their natural environment and conformation. For use as vaccines, dependent on the dose, the reactogenicity needs to be reduced, as GMMA contain LPS and other TLR stimulatory components, *e.g.* lipoproteins. Previously, we described a high yield production process for GMMA (2). The goal of this study was to demonstrate the impact of reducing the endotoxin potential of *Shigella* GMMA by genetic modification of the lipid A component of the GMMA LPS.
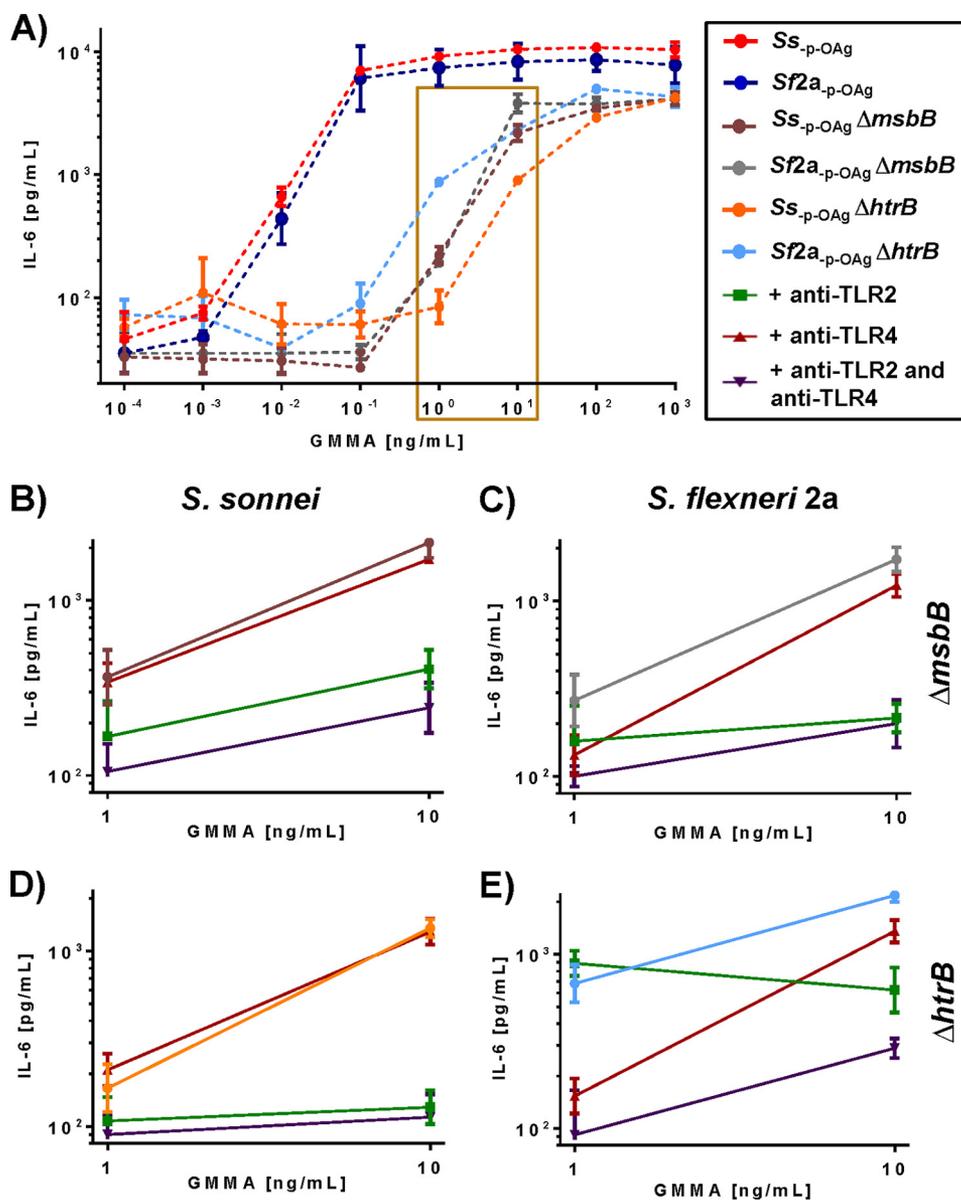
FIGURE 5. TLR blocking experiments. 200,000 human PBMC cells were stimulated with GMMA from different strains. After a 4-h incubation, IL-6 release was measured by ELISA and plotted as average of duplicates with standard deviation. *A*, IL-6 release after stimulation with 0.0001–1000 ng/ml (10-fold steps) of GMMA from different strains. The *rectangle* highlights the concentration of GMMA used in blocking experiments. *B–E*, cells were incubated with 25 μg/ml anti-TLR4 (*dark red graphs*), 15 μg/ml anti-TLR2 (*green graphs*), or both (*violet graphs*) for 30 min before exposure to 1 or 10 ng of GMMA from Ss_{-p-OAg} $\Delta msbB$ (*B*); $Sf2a_{-p-OAg}$ $\Delta msbB$ (*C*); Ss_{-p-OAg} $\Delta htrB$ (*D*); and $Sf2a_{-p-OAg}$ $\Delta htrB$ (*E*). The graphs from experiments without blocking are shown in the same color as in *A*. A representative result of three independent experiments is shown.

The results of deleting the *msbB* gene from both *S. sonnei* $\Delta tolR$ and *S. flexneri* 2a $\Delta tolR$ and by deleting the *htrB* gene from *S. sonnei* $\Delta tolR$ were as expected: conversion of a hexa-acylated lipid A to a penta-acylated lipid A through loss of a myristic acid ($\Delta msbB$) or lauric acid ($\Delta htrB$). However, we have demonstrated the presence of a palmitoleoyl chain in lipid A purified from Sf_{-p-OAg} $\Delta htrB$ GMMA.

Palmitoleoylation in *lpxL* (*htrB*) mutants has previously been reported in *E. coli*. It was shown to be a moderately abundant species at 30 °C and as the dominant species at 37 °C (43). In *S. flexneri* 2a $\Delta htrB$, the signal of palmitoleoylated lipid A was the dominant species at 30 °C, with very little signal due to the penta-acylated species present. Similar results were obtained for the *S. flexneri* 3 and *S. flexneri* 6 strains tested.

The inability to detect a hepta-acetylated lipid A in either the $Sf2a_{-p-OAg}$ GMMA or the $Sf2a_{-p-OAg}$ $\Delta htrB$ GMMA complemented with *htrB* expression from pACYC*htrB* suggests that the palmitoleoylation is on the same site occupied by lauroyl acid in the wild-type lipid A, although further studies would be required to prove this. This would also be consistent with palmitoleoylation catalyzed by the action of LpxP, a late acyltransferase that acts at the HtrB site, as part of what has been described as a cold response in *E. coli* (23). Thus, the palmitoleoylation could be part of a stress response. Similarly, in the *E. coli* $\Delta lpxL$ mutant, the abundance of the palmitoleoylated lipid A increased with stress (growth at 37 °C (43)). In $\Delta tolR$ mutant strains, the *tolR* mutation could provide stress and thus result in higher abundance of palmitoleoylated lipid A
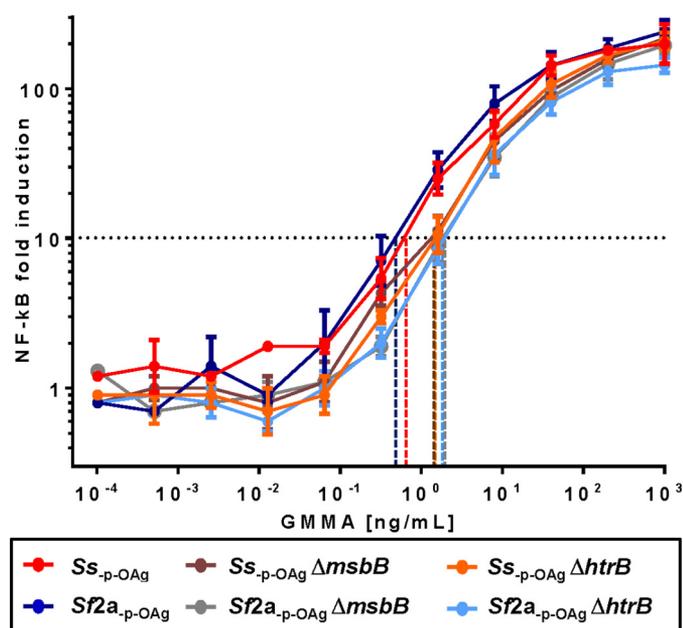
FIGURE 6. **Activation of TLR2 reporter cell line by different GMMA.** 25,000 TLR2-HEK293 cells/well were stimulated with 0.0001–1000 ng/ml (5-fold steps) of GMMA from Ss_{-p-OAg}, $Sf2a_{-p-OAg}$, Ss_{-p-OAg} $\Delta msbB$, $Sf2a_{-p-OAg}$ $\Delta msbB$, Ss_{-p-OAg} $\Delta htrB$, and $Sf2a_{-p-OAg}$ $\Delta htrB$. After 5 h, luciferase expression was measured and expressed as fold-induction compared with cells incubated with PBS. Average induction levels and standard deviations of duplicates are plotted. The GMMA concentrations that resulted in 10-fold increased activation of NF-κB (*black dashed line*) over the average induction at the lowest concentration of GMMA are shown as *x* axis intercepts (*colored dashed lines*). A representative result of four independent experiments is shown.

at 30 °C, although why this was not found in the Ss_{-p-OAg} $\Delta htrB$ strain examined is not clear. We were able to observe a small signal due to palmitoleoylated lipid A in this *S. sonnei* strain grown at 12 °C indicating that there was a functional LpxP present. However, using quantitative real time PCR, we found a 7-fold higher gene expression of *lpxP* in $Sf2a_{-p-OAg}$ $\Delta htrB$ compared with in Ss_{-p-OAg} $\Delta htrB$ grown at 30 °C, possibly resulting in a higher level of LpxP protein present at 30 °C in the *S. flexneri* 2a $\Delta htrB$ strain.

All $\Delta htrB$ *S. flexneri* isolates examined had the palmitoleoylation of the lipid A regardless of the presence or type of OAg on the LPS (*S. flexneri* 2a, 3, and 6 have different O antigens) or the presence of the virulence plasmid ($Sf2a_{-p+OAg}$ $\Delta htrB$, $Sf2a_{+p+OAg}$ $\Delta htrB$). For these, palmitoleoylation might be a strongly selected compensation after the *htrB* knock out. The reason for this is unclear. However, in line with this hypothesis, all of our attempts to introduce an *lpxP* knock out into *S. flexneri* 2a $\Delta htrB$ failed.

Although the impact of lipid A modifications on TLR4 activation has been widely studied (24–27, 44), the availability of GMMA from isogenic *Shigella* lines, all with similar concentrations of lipid A, enabled us to compare in detail the residual ability of modified lipid A to activate human TLR4 receptors either in HEK293-TLR4 transfectant cells or in human PBMC. The TLR4 reporter cell line was used to specifically assess the stimulatory activities of the different lipid A structures (45), whereas *in vitro* cytokine release from PBMC tests the stimulatory potential of a substance in the context of all TLRs and thus has been used to estimate human responses and to predict the safety of vaccines (38). In the TLR4 reporter cell line, we could

detect no residual TLR4 activity of the penta-acylated lipid A in *S. sonnei* $\Delta htrB$, indicated by a 60,000× increase in the amount of GMMA (500 ng/ml), required to give similar activation compared with GMMA with WT lipid A (0.008 ng/ml). GMMA from both *S. sonnei* and *S. flexneri* 2a $\Delta msbB$ mutants gave similar TLR4 activation at comparable GMMA concentrations (4.7 and 4.6 ng/ml). Although this required 600× more GMMA compared with GMMA with WT lipid A, the amount was 100× less compared with GMMA from the *S. sonnei* $\Delta htrB$ with penta-acyl lipid A, showing that the $\Delta msbB$ penta-acyl lipid A retains some residual ability to activate human TLR4. Although the magnitude of difference in TLR4 activation between the Ss_{-p-OAg} $\Delta htrB$ and the $\Delta msbB$ GMMA with penta-acylated lipid A was unexpected, minor modifications in penta-acylated lipid A have been shown to affect TLR4 stimulation (46). In $Sf2a_{-p-OAg}$ $\Delta htrB$ GMMA, the substitution of a lauroyl chain with the longer palmitoleoyl chain in hexa-acylated lipid A also led to approximately a 50× decrease in the ability to simulate human TLR4 (0.4 ng/ml) compared with GMMA with WT lipid A. The mass spectrum showed a mixture of hexa-, penta-, and tetra-acylated lipid A, of which the palmitoleoylated hexa-acylated peak was by far the dominant peak present. Although the relative height of the peaks in this experiment is not necessarily strictly proportional to the abundance in the GMMA, it seems highly unlikely that a 50× reduction in activation could be due to the decrease in percentage of the hexa-acylated by the presence of these lower sized peaks but that the reduction of TLR4 stimulation is rather linked to the difference of the acyl chain composition. In PBMC, GMMA without lipid A modification predominantly stimulated the pro-inflammatory cytokines IL-6, TNF-α, IL-1β, and IL-8 as observed previously for *Neisseria meningitidis* native outer membrane vesicles (47). All GMMA with lipid A modification showed a marked decrease of cytokine release compared with GMMA with WT lipid A. IL-6 release was chosen for detailed comparison of the relative ability of GMMA to elicit cytokine release due to its role in fever pathogenesis (48). In contrast to the TLR4-specific assay, the difference in IL-6 stimulation by *S. sonnei* $\Delta htrB$ GMMA and *S. sonnei* or *S. flexneri* 2a $\Delta msbB$ GMMA was very small, only ~3-fold (*S. sonnei* $\Delta htrB$ 800-fold and $\Delta msbB$ 300-fold), compared with the 100-fold difference in TLR4 activation (*S. sonnei* $\Delta htrB$ 60,000-fold and $\Delta msbB$ 600-fold). This small difference is in accordance with the presence of additional TLR being stimulated by GMMA. It indicates that the decrease of GMMA reactogenicity obtained by either form of penta-acylated lipid A is close to the maximum decrease of GMMA reactogenicity achievable by lipid A modification, when assayed in a complex system as PBMC that is more relevant for estimating responses in humans (38). Based on the large reduction of *in vitro* cytokine release from PBMC, an *S. sonnei* $\Delta htrB$ GMMA was chosen as a first candidate for vaccine development. Phase 1 clinical trials are currently underway and will give an important indication of the tolerability of GMMA from these constructs.

To characterize which TLR receptors contribute to the residual activity of GMMA, TLR blocking experiments in PBMC were performed. We demonstrated that the remaining activity of GMMA with penta-acylated lipid A was predominantly due to TLR2 activation. With GMMA from Ss_{-p-OAg} $\Delta htrB$, no

effect from TLR4 blocking was observed, whereas GMMA resulting from a *msbB* deletion retained detectable TLR4 activity in line with the results of the TLR4-specific assay. However, the induction of IL-6 via lipid A by the Δ*msbB* GMMA that was dependent on TLR4 was smaller than the induction via TLR2. In contrast, GMMA from *S. flexneri* 2a Δ*htrB* with palmitoleoylated hexa-acylated lipid A primarily stimulated TLR4. Further reduction of reactogenicity of GMMA from Ss_{-P-OAg} Δ*htrB* and the Δ*msbB* strains would require measures to reduce TLR2 activation. However, native outer membrane vesicles from *N. meningitidis* group B Δ*lpxL1* strain with penta-acylated lipid A (49) have been shown to be safe in Phase I clinical trials (50). Thus, although *N. meningitidis* and *Shigella* are not closely related and *e.g.* have different LPS structures (49) modification of TLR2 activators is not expected to be required.

The broad aim of this study was to examine ways of reducing the reactogenicity of GMMA to make them suitable for use as a human vaccine, but it resulted in a surprisingly complex outcome. Deleting the *htrB* gene from *S. sonnei* or the *msbB* gene from *S. sonnei* and *S. flexneri* 2a resulted in GMMA with a penta-acyl lipid A with a marked reduction in induction of inflammatory cytokines from human PMBC for which the residual activity is probably due to non-lipid A-related TLR2 activation. In contrast, compensatory palmitoleoylation in the *S. flexneri* 2a Δ*htrB* GMMA results in retained TLR4 activation. Although the level of reduction of GMMA reactogenicity required for an acceptable vaccine will depend on the dose required to give a strong immune response, which can only be determined in clinical trials, the data suggest that the use of GMMA as vaccines will likely require lipid A penta-acylation and that GMMA with palmitoleoylated hexa-acylated LPS are less likely to result in a useful vaccine.

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