MODULATION OF REACTOGENICITY OF GENERALIZED MODULES FOR MEMBRANE ANTIGENS (GMMA) BY GENETIC LIPID A MODIFICATION

Omar Rossi

Dottorato in Scienze Biotecnologiche – XXVI ciclo
Indirizzo Biotecnologie Industriali e Molecolari
Università di Napoli Federico II

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Dottorando:	Omar Rossi
Relatore:	Prof. Giovanni Sannia, Dr. Christiane Gerke
Coordinatore:	Prof. Giovanni Sannia

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Summary

Introduction

Infectious diseases are still a main cause of mortality and morbidity in developing countries, especially in children under the age of 5 years. For many of these diseases no vaccines are available. An ideal vaccine should be easy to administer, well tolerated, protect against multiple pathogens, easy to manufacture, available at low cost (ideally < 1 $ per dose), and able to induce long-term protection.

NVGH's mission is to develop effective and affordable vaccines for neglected diseases of impoverished communities. Towards this goal, NVGH is developing platform technologies that can be applied to the development of vaccines for several pathogens. One major platform is based on outer membrane particles, called Generalized Modules for Membrane Antigens (GMMA). This platform is used in three major programs of NVGH: *Shigella,* a major cause of dysentery with more than 125 million cases and 100,000 deaths per year, invasive non-Typhoidal *Salmonella* (iNTS), with estimated more than 100,000 deaths per year, and *Neisseria menigitidis,* responsible for epidemic outbreaks causing more than 50,000 deaths per year and permanent sequelae.

The GMMA platform takes advantage of the ability of Gram-negative bacteria to naturally shed small particles from the outer membrane into the environment. The naturally shed particles, called native outer membrane vesicles (NOMV), consist of outer membrane lipids, outer membrane proteins, and soluble periplasmic components. They present surface antigen in their natural environment and orientation and thus have been proposed for use as vaccines. However, the yield is usually too low for a practical production. NVGH has developed a high yield production process suitable for industrial production using genetic modification of bacteria to induce high level shedding of outer membrane particles. To differentiate these particles that are released from the genetically modified bacteria from NOMV and other types of particles produces by bacteria, they were named GMMA.

GMMA are highly immunogenic. In part, this is related to strong self-adjuvanticity. Since GMMA are derived from the outer membrane of Gram-negative bacteria, they naturally contain high levels of Pathogen-Associated Molecular Patterns (PAMPs). PAMPs are present only in bacteria and are sensed by Pattern Recognition Receptors (PRRs), including Toll-like receptors (TLRs), expressed on a wide range of mammalian cells. The receptors of particular importance for recognition of Gram-negative bacteria are TLR4 and TLR2. TLR4 is the receptor involved in the recognition of lipopolysaccharide (LPS). TLR2 is involved in the recognition of a wide range of molecules, including lipoproteins which, together with LPS, are major constituents for the outer membrane of Gram-negative bacteria.

The single most abundant and highly potent immune-stimulatory component in GMMA is the LPS. Depending on the dose of GMMA to be used in a vaccine, strong activation of the innate immunity may lead to unacceptable reactions in human subjects, e.g. a febrile response, or in extreme cases, septic shock, especially if parenterally administered. Thus, the reactogenicity of GMMA, especially the LPS endotoxicity, might need to be modified.

LPS consists of three main regions, i.e. the glycolipid lipid A, a core oligosaccharide, and an outer polysaccharide chain (O antigen). The lipid A is the reactogenic part interacting with TLR4. The most reactogenic form of lipid A is a hexa-acylated glucosamine disaccharide phosphorylated at the 1 and 4' positions. This structure is

common to *E. coli* and *Shigella*. During the lipid A synthesis first a tetra-acylated core structure composed of the phosphorylated glucosamine disaccharide acylated at 2, 3, 2' and 3' position with R-3-hydroxymyristate is generated. Subsequently, the late acyltransferases HtrB (also called LpxL) and MsbB (also called LpxM) transfer a lauroyl fatty acid (HtrB) and a myristoyl fatty acid (MsbB) to the hydroxymyristate in the 2' and 3' positions, respectively.
The total number and the length of acyl chains are critical factors for full lipid A activation of human TLR4. Changes to the structure of the lipid A, either by removal of critical components or by replacement e.g. of one of the acyl chains by a different acyl residue, effects the binding and recognition by TLR4 and results in a lower endotoxicity *in vitro*. Thus, changes in the acylation status of lipid A presents an attractive way for reducing the TLR4-mediated endotoxicity of GMMA. The effects of genetic lipid A modification needs to be evaluated in the context of the relative contribution of TLR2 and TLR4 pathways to the activation of human cells to define the impact on the overall reduction of GMMA reactogenicity.

Aims of the project

The broad aim of the project was to identify ways of reducing the reactogenicity of GMMA. This broad aim was divided into three specific goals:

1. Proof of feasibility of reduction of GMMA endotoxicity by lipid A modification using *Shigella*.
2. Assessment of relative contribution of TLR4 and TLR2 pathways to stimulation of human cells by GMMA with different lipid A modifications
3. Demonstration of general applicability of the identified methods for the GMMA platform.

The work for goals 1 and 2 has been part of the NVGH program to develop a *Shigella* GMMA vaccine. The work for goal 3 has been involved in the development of GMMA-based non-Typhoidal *Salmonella* and *Neisseria meningitidis* vaccines for Africa.

1. Proof of feasibility of reduction of GMMA endotoxicity by genetic lipid A modification

Optimization of GMMA quantification

Establishing a consistent and reproducible method for the quantification of GMMA is important in order to use them as vaccine and for comparing them in functional assays. GMMA are conveniently quantified based on protein amount. The gold standard for protein quantification is quantitative amino acid (AA) hydrolysis. It is a primary method as it is not affected by protein composition and does not need a calibration curve with a standard protein. However, analysis based on AA content is slow and for this purpose secondary colorimetric assays using a standard protein are usually preferred.
I compared different routinely used color-based protein assays for quantifying GMMA samples: Bradford assay, Lowry assay, and Non-Interfering protein assay (NI), a method that involves a protein precipitation step. I examined several factors to determine the most useful secondary assay for measuring the protein concentration of GMMA: the ease of performing the assay, the reproducibility of the assay, the linearity of the assay, and the comparability of the results for GMMA from multiple different bacteria. By these criteria, the Lowry assay was superior. It was by far the simplest assay to perform, gave substantially lower inter- and intra-assay variation than the other assays, and most importantly showed the most consistent results for

different types of GMMA. The Lowry assay consistently overestimated the GMMA protein concentration by about 35% as the color yield of BSA differed from the color yield of GMMA. In contrast, the Bradford assay underestimated the GMMA protein concentration by 50 % to 80%, and the NI assay varied from giving a very similar result as the AA quantification to 40% underestimation based on the type of GMMA.
The results of the Bradford and NI assays varied even for GMMA from isogenic strains differing by a single mutation making them less useful for comparing different GMMA than the Lowry assay. These results also highlighted the need for caution when comparing results obtained with NOMV by various laboratories if different protein assays were used.

Genetic lipid A modification in S. sonnei *and* S. flexneri *and MALDI-TOF analysis*

The *htrB* or *msbB* genes encoding for the late acyl transferases were inactivated in GMMA-producing *S. sonnei* and *S. flexneri* strains. All of the lipid A mutants were able to reach high optical densities in the medium optimized for industrial production and produced GMMA with similar yields. Electron microscopy confirmed that lipid A modification did not alter the size or organization of GMMA. In addition, SDS-PAGE showed that the overall protein composition remained similar. Four protein bands were found to be up-regulated in GMMA from *S. flexneri* Δ*htrB*. As these were identified by peptide mass fingerprinting to be predicted cytoplasmic proteins, no effect on the reactogenicity studies was expected.
To analyze the lipid A composition resulting from the different genetic modifications, the lipid A was purified from GMMA and analyzed by MALDI-TOF. The main peaks in the mass spectra of lipid A from *S. sonnei* and *S. flexneri* GMMA with wild-type lipid A had an *m/z* corresponding to the theoretical mass of the hexa-acylated lipid A of 1798 Da. The results of deleting the *msbB* gene from both *S. sonnei* and *S. flexneri* as well as by deleting the *htrB* gene from *S. sonnei* were as expected: conversion of a hexa-acylated lipid A to a penta-acylated lipid A through loss of a myristic acid (a C_{14} fatty acid chain, 210 *m/z* shift in Δ*msbB*) or lauric acid (a C_{12} fatty acid chain, 182 *m/z* shift, Δ*htrB*). In contrast, the spectra obtained from *S. flexneri* Δ*htrB* GMMA, showed a peak at 1851 *m/z* as dominant species, corresponding to a hexa-acylated lipid A but with different composition compared to the wild-type lipid A. The *m/z* is in accordance with the exchange of a C_{12} chain at the HtrB site for a $C_{16:1}$ fatty acid chain (*m/z* shift of 54 to WT, m/z shift of 236 to *S. sonnei* Δ*htrB*). The presence of a palmitoleoyl chain in lipid A from *S. flexneri* ∆*htrB* GMMA was confirmed by Collision Induced Decay MS/MS analysis. The palmitoleoylated hexa-acylated form was also the main form in lipid A preparations from of GMMA from *htrB* mutants of other *S. flexneri* strains, e.g. serotypes 3a and 6, suggesting a strong selection for palmitoleoylation after *htrB* knock out in *S. flexneri*. To confirm that the lipid A modifications observed in the *htrB* mutants were linked to the *htrB* deletion, the mutants were complemented with a low copy plasmid encoding *htrB*. As expected, the respective MALDI-TOF spectra showed a lipid A with an *m/z* of wild-type hexa-acylated lipid A. No hepta-acylated lipid A was observed, suggesting that the palmitoleoyl chain peak was attached to the HtrB site or is dependent on the HtrB site being vacant.
It is unclear why the palmitoleoylation was not found in *S. sonnei* Δ*htrB.* In addition to our findings with *S. flexneri* ∆*htrB*, an *E. coli htrB* mutant has previously been described to produce palmitoleoylated lipid A, but as a minor species. Palmitoleoylation at the HtrB site is known to occur as cold response in *E. coli*

catalyzed by LpxP. After growth of *S. sonnei* Δ*htrB* at 12℃ to induce the cold response, small amounts of palmitoleoylated lipid A were detected by MALDI-TOF, demonstrating that LpxP is functional. By quantitative real time PCR we observed a 7-fold higher expression of the *lpxP* gene at 30℃ in *S. flexneri* Δ*htrB* compared to *S. sonnei* Δ*htrB*, most likely accounting for the difference in the palmitoleoylation.

TLR4 stimulation by GMMA after lipid A modification

In order to assess the ability of the different lipid A mutants to activate TLR4 a reporter cell line expressing luciferase under control of human TLR4 was used.
GMMA from *S. flexneri* Δ*msbB* and *S. sonnei* Δ*msbB* showed an approximately 600-fold decrease in their ability to stimulated TLR4 compared to the parent GMMA with wild-type lipid A. A substantial further decrease of stimulatory activity was observed with GMMA from *S. sonnei* Δ*htrB*. Luciferase induction was only detectable at a high concentration, requiring a 60,000-fold higher amount of GMMA than the parent GMMA with wild-type lipid A and a 100-fold higher amount than Δ*msbB* GMMA to induce the same level of induction.
In contrast, GMMA from *S. flexneri* Δ*htrB* with palmitoleoylated hexa-acylated lipid A retained higher TLR4 stimulation than Δ*msbB* GMMA requiring 10-fold less GMMA to result in similar induction of luciferase. Accordingly they showed a relatively small (50-fold) decrease of stimulatory activity compared to GMMA with wild-type lipid A. This suggested that the palmitoleoylated hexa-acylated lipid A retained TLR4 stimulatory activity but that the substitution of the lauroyl chain by the palmitoleoyl chain decreased TLR4 stimulation compared to wild-type lipid A.

2. Assessment of relative contribution of TLR4 and TLR2 pathways to stimulation of human cells by GMMA with different lipid A modifications

In order to evaluate the endotoxin activity of GMMA in a more natural context, GMMA purified form different mutants were used to stimulate human Peripheral Blood Mononuclear Cells (PBMC) in a Monocyte Activation Test (MAT).
GMMA purified from *Shigella s*trains carrying lipid A modification (both *htrB* and *msbB*) showed a reduction in their activity to stimulate cytokine production (Interleukin 6 [IL-6], IL-8, IL-1β, Tumor Necrosis Factor α) in comparison to GMMA from strains without LPS modifications, with a similar trend as observed in TLR4-specific assay. GMMA purified from Δ*msbB* strains required an approximately 300-fold higher amount, GMMA from *S. flexneri* Δ*htrB* an approximately 50-fold higher amount compared to GMMA with wild-type lipid A to induce similar IL-6 release. In contrast, GMMA from *S. sonnei* Δ*htrB* showed a 800-fold decreased ability to stimulate IL-6 release from PBMC and thus a lower decrease in activity than expected from the 60,000-fold reduction of TLR4 stimulation, indicating that additional TLR pathways contribute to the stimulation of PBMC, most likely the TLR2 pathway.
In order to determine the relative contribution of the TLR4 and TLR2 pathway to the stimulation of human PBMC by GMMA, PBMC were incubated with anti-TLR4 or anti-TLR2 antibodies to selectively block TLR4 or TLR2 activity prior to stimulation by GMMA. No remaining TLR4 activity of GMMA from *S. sonnei* Δ*htrB* with penta-acylated lipid A was detected. GMMA with penta-acylated lipid A from Δ*msbB* strains retained some ability to activate human TLR4, but the induction of IL-6 via the lipid A dependent TLR4 in these GMMA was smaller than the induction via TLR2. This showed that the residual reactogenicity in GMMA with penta-acylated lipid A was

mainly mediated through TLR2. In contrast, a substantial decrease of activation by GMMA from *S. flexneri* Δ*htrB* was achieved using anti-TLR4 antibody but not with anti-TLR2 antibody demonstrated that palmitoleoylated hexa-acylated lipid A retained strong TLR4 activation.
To confirm that the differences in the relative contribution of TLR4 and TLR2 to activation observed in the blocking experiments were solely dependent on the differential TLR4 activation by the different GMMA the ability of the GMMA to activated TLR2 was tested using a human TLR2 reporter cell line. All four Δ*msbB* or Δ*htrB* GMMA showed similar TLR2 activation.

Conclusion: Feasibility of reduction of reactogenicity by lipid A modification

Proof of concept of reduction of reactogenicity of GMMA by genetic lipid A modification has been obtained.
GMMA with penta-acylated lipid A showed a marked reduction in the induction of inflammatory cytokines from human PMBC. Deleting *htrB* gene from *S. sonnei* resulted in a 800-fold reduction of cytokine stimulatory activity and the remaining activity was TLR2-mediated. GMMA from *msbB* deletion strains showed a 300-fold reduction. They retained detectible TLR4 activity, however, the major activated pathway was TLR2, similar as for *S. sonnei* Δ*htrB* GMMA.
In contrast, compensatory palmitoleoylation in the *S. flexneri* Δ*htrB* GMMA resulted in retained TLR4 activation by the hexa-acylated lipid A and thus higher reactogenicity suggesting that modifications yielding penta-acylated lipid A will be preferred.

3. Demonstration of general applicability of the identified methods for the GMMA platform

Salmonella

Salmonella enterica is a species within the *Enterobacteriaceae* closely related to *Shigella* and shares the same hexa-acylated lipid A. Thus, as in *Shigella*, lipid A modification by deletion of *htrB* and *msbB* of GMMA-producing strains of *Salmonella enterica* serovars Typhimurium (S. Tm) and Enteritidis (S. En) was investigated.
However, the situation in *Salmonella* is more complex as the lipid A is, at least in part, hepta-acylated through the PagP-catalyzed addition of a palmitoyl chain (a C_{16} fatty acid chain) to hydroxymyristate in position 2. The relative amounts of hexa- and hepta-acylated lipid A vary in different strains from more than 95% hexa-acylated to more than 30% hepta-acylated. In line with the presence of a mixture of hexa- and hepta-acylated lipid A in GMMA before lipid A modifications, the removal of a single acyl chain by deletion of *msbB* or *htrB* resulted a mixture of penta- and hexa-acylated lipid A lacking the myristoyl or a lauroyl chain, respectively. Interestingly, after *htrB* deletion in S. En, we observed the same compensatory palmitoleoylation as observed in *S. flexneri* Δ*htrB* resulting in a mixture of hexa- and hepta-acylated lipid A with the replacement of the lauroyl chain by the palmitoleoyl chain (*m/z* shift =54).
S. Tm Δ*msbB* GMMA contained more than 95% penta-acylated lipid A and showed similar level of IL-6 stimulation from human PBMC as *Shigella* GMMA with penta-acylated lipid A. All other tested STm and SEn GMMA with lipid A modifications contained a higher percentage of hexa-acylated lipid A. In accordance to the results obtained with *S. flexneri* Δ*htrB* GMMA with hexa-acylated palmitoleoylated lipid A, S. Tm and S. En GMMA with hexa-acylated lipid A retained higher TLR4 activity and are less likely to result in a useful vaccines.

In conclusion, S. Tm GMMA with penta-acylated lipid A showed a similarly reduced reactogenicity profile as *Shigella* GMMA with penta-acylated lipid A. However, to obtain *Salmonella* GMMA with predominantly penta-acylated lipid A and without hexa-acylated lipid A, additional mutations to *htrB* or *msbB* might be needed.

Neisseria

The reduction of GMMA endotoxicity by genetic lipid A modification was also assessed in *Neisseria meningitidis*, a species less genetically related to *Shigella*. Lipid A from *Neisseria* is hexa-acylated with a different fatty acid composition and distribution compared to *Shigella* lipid A. In addition, the phosphate in 4' position carries a phosphoethanolamine. Despite the differences, similar amounts of *Neisseria* and *Shigella* GMMA with wild-type lipid A induce similar levels of IL-6 release from human PBMCs. To obtain penta-acylated lipid A we deleted the gene *lpxL1,* as was previously reported, encoding an acyl transferase that adds a lauroyl chain to the hydroxymyristoyl in 2' position. Penta-acylation of the resulting lipid A was confirmed by MALDI-TOF showing an *m/z* shift of 182 Dalton to WT lipid A, consistent with the absence of the lauroyl chain.
The resulting GMMA from the Neisseria Δ*lpxL1* mutant showed a marked reduction of TLR4 activation and stimulation of IL-6 from human PBMC, at similar levels to *S. sonnei* Δ*htrB* GMMA. This result emphasized that despite differences in lipid A composition, penta-acylated lipid A retains little TLR4 activation and results in substantial reduction of the reactogenicity of GMMA.

Conclusions

The broad aim of the thesis was to examine ways of reducing the reactogenicity of GMMA to make them suitable for use as human vaccine. It resulted in surprisingly complex outcome.
I demonstrated the feasibility to reduce GMMA reactogenicity by lipid A modification. I found a clear relation between the composition of the lipid A species and reactogenicity. Our analysis of GMMA from isogenic lines and from different species highlighted the importance of determining the composition of the lipid A after the genetic modification as the same genetic modification gave different outcomes even in closely related *Shigella* species.
I demonstrated in *Shigella*, *Salmonella*, and *Neisseria* that GMMA with penta-acylated lipid A have a marked reduction of cytokine stimulation ability. Using *Shigella* GMMA we showed that the residual reactogenicity is predominantly TLR2 mediated. In contrast, GMMA with hexa-acylated lipid A, either through palmitoleoylation after *htrB* knock-out, and/or due to additional palmitoylation in *Salmonella*, retain TLR4 stimulatory activity and are less likely to be useful as vaccine.
Based on the results presented in this thesis, genetic modification of the lipid A biosynthesis pathway resulting in penta-acylated lipid A is a promising strategy for reducing the reactogenicity of GMMA. If further decrease of reactogenicity was required, modifications of TLR2 activating components would be necessary. However, the level of reduction required for an acceptable vaccine will depend on the dose necessary to give a strong immune response and that can only be determined by clinical trials. Clinical trials underway now using the *S. sonnei* Δ*htrB* GMMA should give an important indication of the tolerability of these constructs.

Riassunto

Introduzione

Le malattie infettive sono ancora oggi una delle cause principali di mortalità e morbidità nei paesi in via di sviluppo, specialmente nei bambini di età inferiore ai 5 anni e per molte di queste malattie non sono disponibili vaccini. Un vaccino ideale dovrebbe essere facile da amministrare, ben tollerato, conferire protezione contro molteplici patogeni, facile da fabbricare, disponibile a basso costo (idealmente < 1 $ per dose) ed essere in grado di indurre una protezione a lungo termine.
La missione di NVGH è quella di sviluppare vaccini efficaci e accessibili per le malattie trascurate che affliggono i paesi poveri. Per perseguire questo obiettivo, NVGH sta sviluppando piattaforme tecnologiche che possono essere applicate allo sviluppo di vaccini per diversi agenti patogeni. Una importante piattaforma si basa su particelle membrana esterna, nominate Moduli Generalizzati per Antigeni di Membrana (Generalized Modules for membrane Antigens, GMMA). Questa piattaforma è utilizzata nei tre maggiori programmi di NVGH: *Shigella*, una delle principali cause di dissenteria, che causa più di 125 milioni di casi e 100,000 morti all'anno, *Salmonella* non-Tifoidea invasiva (iNTS), con circa più di 100,000 morti all'anno, e *Neisseria menigitidis*, responsabile di epidemie che causano più di 50,000 morti all'anno e sequele permanenti.
La piattaforma GMMA sfrutta la capacità dei batteri Gram-negativi di espellere naturalmente piccole particelle dalla membrana esterna e rilasciarle nell'ambiente. Queste particelle naturalmente rilasciate, chiamate anche vescicole di membrana esterna native (Native Outer Membrane Vesicles, NOMV), sono costituite da lipidi e proteine della membrana esterna e componenti periplasmatiche solubili. Esse presentano antigeni di superficie nel loro orientamento e conformazione naturale, quindi sono stati proposti per l'uso come vaccini. Tuttavia, la resa è di solito troppo bassa per un utilizzo pratico. NVGH ha sviluppato un processo di produzione ad alta resa adatto per la produzione industriale, mediante modificazione genetica dei batteri al fine di indurre un elevato livello di rilascio. Per differenziare le particelle rilasciate dai batteri geneticamente modificati dalle NOMV e da altri tipi di particelle prodotte dai batteri, sono stati chiamate GMMA .
GMMA sono altamente immunogeniche ed in parte questo è legato alla forte capacità auto-adiuvante. Poiché GMMA derivano dalla membrana esterna dei batteri Gram-negativi, queste contengono alti livelli di molecole chiamate Pathogen Associated Molecular Patterns (PAMPs). I PAMPs sono molecole presenti solo nei batteri e sono riconosciute da Pattern Recognition receptors (PRR), tra i quali i Toll-like receptors (TLR), che sono espressi su una vasta gamma di cellule di mammifero. I recettori di particolare importanza per il riconoscimento dei batteri Gram-negativi sono il TLR4 ed il TLR2. Il TLR4 è il recettore coinvolto nel riconoscimento del lipopolisaccaride (LPS), mentre il TLR2 è coinvolto nel riconoscimento di una vasta gamma di molecole, tra cui lipoproteine che, insieme all'LPS, rappresentano i costituenti essenziali della membrana esterna dei batteri Gram-negativi.
Il singolo componente immuno-stimolante più abbondante e più potente presente nelle GMMA è rappresentato dall'LPS. A seconda della dose di GMMA che debba essere utilizzata in un vaccino, una massiccia attivazione dell'immunità innata può provocare reazioni inaccettabili in soggetti umani, ad esempio una risposta febbrile o, in casi estremi, shock settico, soprattutto se somministrato per via parenterale. Per

questo motivo, la reattogenicità delle GMMA, ed in particolare l'endotossicità dell'LPS, potrebbe dover essere modificata.
L'LPS è costituito da tre regioni principali: il glicolipide "lipide A", un "core" oligosaccaridico, ed una catena polisaccaridica esterna (l'antigene O). Il lipide A è la parte reattogenica della molecola che interagisce con il TLR4. La forma più reattogenica di lipide A è rappresentata da un disaccaride di glucosamina esa-acilato e fosforilato nelle posizioni 1 e 4'. Questa struttura è la forma comune presente in *E. coli* e *Shigella*. Durante la biosintesi del lipide A viene prima generata una struttura di base tetra-acilata, composta da un disaccaride di glucosamina fosforilato ed acilato nelle posizioni 2, 3, 2' e 3' con R-3-idrossimiristoile. Successivamente le acilasi tardive HtrB (chiamata anche LpxL) ed MsbB (chiamata anche LpxM) trasferiscono l'acido grasso lauroile (HtrB) e l'acido grasso miristoile (MsbB) sugli idrossimiristati nelle posizioni 2' e 3' rispettivamente.
Il numero totale e la lunghezza delle catene aciliche sono fattori critici per la piena attivazione del lipide A TLR4 umano. Modifiche nella struttura del lipide A, sia per quanto riguarda la rimozione che per quanto riguada la sostituzione, ad esempio, di una delle catene aciliche con un diverso residuo acilico, hanno effetti sul legame e sul riconoscimento da parte del TLR4, che si traduce in una minore endotossicità *in vitro*. Per cui i cambiamenti nello stato acilazione del lipide A rappresentano un modo interessante per ridurre l'endotossicità TLR4-mediata delle GMMA. Al fine di valutare l'effetto di tali modificazioni genetiche sulla reattogenicità complessiva delle GMMA e di individuare ulteriori strategie per ridurre la reattogenicità di quest'ultime, è importante determinare il contributo relativo dei pathways TLR2 e TLR4 nell'attivazione delle cellule umane da parte di GMMA.

Obiettivi del progetto

L'obiettivo generale del progetto è quello di individuare i metodi per ridurre la reattogenicità delle GMMA. Questo obiettivo principale è stato diviso in tre obiettivi specifici:

1. La prova di fattibilità della riduzione della reattogenicità delle GMMA mediante modifica genetica del lipide A utilizzando *Shigella*.
2. La valutazione del contributo relativo dei pathways TLR4 e TLR2 nella stimolazione delle cellule umane mediante GMMA con differenti modificazioni del lipide A.
3. La dimostrazione di applicabilità generale dei metodi individuati per la piattaforma GMMA.

Il lavoro per obiettivi 1 e 2 è parte del programma NVGH per sviluppare un vaccino per *Shigella* utlizzando le GMMA. L'obiettivo 3 è coinvolto nello sviluppo vaccini basati su GMMA contro *Salmonellae* invasive non-Tifoidee e *Neisseria meningitidis* per un loro utilizzo in principalmente in Africa.

1. Prova di fattibilità della riduzione dell'endotossicità delle GMMA mediante modificazione genetica del lipide A

Ottimizzazione dei motodi di quantificazione delle GMMA

Stabilire un metodo coerente e riproducibile per la quantificazione dei campioni GMMA è importante al fine di utilizzarli come vaccino e per confrontarle in saggi funzionali. Le GMMA sono convenientemente quantificate sulla base del loro contenuto proteico. Lo standard per la quantificazione delle proteine è l'idrolisi di aminoacidi (AA) quantitativa. Si tratta di un metodo primario, in quanto non è

influenzato dalla composizione proteica del campione di partenza, e non necessita di una curva di calibrazione con una proteina standard. Tuttavia, l'analisi basata sul contenuto AA è lenta e per questo scopo saggi colorimetrici secondari che utilizzando una proteina standard sono normalmente preferiti. Ho, quindi, confrontato differenti dosaggi colorimetrici di proteine utilizzati abitualmente, nella quantificazione di campioni di GMMA: il saggio Bradford, il saggio Lowry, e il saggio Non-Interfeering (NI), un metodo che prevede una fase di precipitazione delle proteine. Ho esaminato diversi fattori per determinare il saggio secondario più utile per misurare la concentrazione delle proteine nelle GMMA: la facilità di esecuzione del test, la riproducibilità del dosaggio, la linearità del dosaggio e la comparabilità dei risultati per GMMA provenienti da batteri diversi. Valutando questi criteri, il test Lowry si è dimostrato migliore. È di gran lunga il saggio più semplice da eseguire, presenta inferiore variazione inter- e intra-saggio rispetto agli altri metodi, e soprattutto ha mostrato i risultati più coerenti per i diversi tipi di GMMA. Il saggio di Lowry costantemente sovrastima la concentrazione proteica nelle GMMA di circa il 35% utilizzando la BSA come standard. Al contrario, il saggio Bradford sottostimava la concentrazione proteica delle GMMA dal 50% al 80%, mentre il saggio NI variava dal dare un risultato molto simile a quello della quantificazione AA, alla sottostima del 40% in base al tipo di GMMA testato. Il fatto che i risultati di questo saggio varino, quindi, anche per GMMA provenienti da ceppi isogenetici che differiscono per una singola mutazione, lo rende meno utile per confrontare diverse GMMA, soprattutto in saggi funzionali.
I risultati hanno anche evidenziato la necessità di cautela quando si confrontano i risultati ottenuti con NOMV da vari laboratori se sono stati usati diversi dosaggi di proteine .

Modifica genetica del lipide A in* S. sonnei *e* S. flexneri *ed analisi MALDI -TOF

I geni *htrB* o *msbB,* codificanti per aciltransferasi tardive, sono stati inattivati in ceppi di *S. sonnei* e *S.flexneri* produttori di GMMA. Tutti i ceppi mutanti nel lipide A erano in grado di raggiungere elevate densità ottiche nel terreno ottimizzato per la produzione industriale ed hanno prodotto GMMA con rese analoghe. Mediante microscopia elettronica è stato confermato che modifiche nel lipide A non hanno alterato la dimensione o l'organizzazione delle GMMA. Inoltre, mediante SDS - PAGE è stato osservato che la composizione proteica compessiva rimaneva simile. Solo quattro bande proteiche sono state osservate come sovraregolate in GMMA da *S. flexneri* Δ*htrB;* queste sono state identificate mediante Peptide Mass Fingerprinting e sono state predette come proteine citoplasmatiche, per cui nessun alcun effetto sugli studi di reattogenicità è stato predetto.
Per analizzare la composizione del lipide A risultante dalle diverse modificazioni genetiche, quest ultimo è stato purificato da GMMA ed analizzato tramite spettrometria MALDI- TOF. I principali picchi osservati negli spettri di massa di lipide A da GMMA provenienti da *S. sonnei* e *S.flexneri* con lipide A wild-type hanno un rapporto massa/carica (*m/z*) corrispondente alla massa teorica del lipide A esa-acilato di 1798 Da. I risultati della delezione del gene *msbB,* sia da *S. sonnei* che da *S.flexneri*, nonché quelli dell'eliminazione del gene *htrB* da *S. sonnei* sono stati quelli previsti: la conversione di un lipide A esa-acilato ad uno penta-acilato dovuto alla mancanza di un acido miristico (una catena di acido grasso C_{14}, shift *m/z* di 210 nel caso di Δ*msbB*) o di un acido laurico (una catena di acido grasso C_{12}, shift *m/z* di 182 nel caso di Δ*msbB*). Al contrario, lo spettro ottenuto da GMMA di *S. flexneri* Δ*htrB*, ha mostrato un picco a 1851 *m/z* come specie dominante, corrispondente ad un

lipide A esa-acilato ma con composizione differente rispetto al lipide A wild-type (WT). Il *m/z* è conforme allo scambio di una catena C_{12} nel sito HtrB con una catena $C_{16:1}$ di acido grasso (spostamento *m/z* di 54 rispetto a WT, 236 *m/z* rispeto a GMMA da *S. sonnei* Δ*htrB*). La presenza di una catena di palmitoleoile nel lipide A proveniente da GMMA di *S. flexneri* Δ*htrB* è stata confermata dall'analisi mediante spettrometria massa/massa avvalendosi del Collision Induced Decay. La forma esa-acilata palmitoleoilata è anche la principale forma di lipidi A presente in GMMA da mutanti *htrB* con diverso background genetico e da altri ceppi di *S. flexneri*, ad esempio sierotipi 3a e 6, suggerendo una forte selezione per la palmitoleoilazione dopo delezione di *htrB* in *S. flexneri*. Per confermare che le modifiche osservate nei mutanti nei lipidi A Δ*htrB* fossero direttamente legate alla delezione di *htrB*, le mutazioni sono state complementate utilizzando un plasmide a basso numero di copie codificante il gene *htrB*. Come previsto, i rispettivi spettri MALDI-TOF hanno mostrato un lipide A esa-acilato con *m/z* di tipo WT; inoltre non è stato osservato lipide A epta-acilato A, suggerendo che la catena palmitoilica venga attaccata al sito HtrB o sia dipendente dal fatto che il sito HtrB sia libero.
Non è chiaro il motivo per cui la palmitoleoilazione non sia stata osservata in *S. sonnei ΔhtrB*. In aggiunta ai nostri risultati, un mutante di *E. coli* Δ*htrB* è stato precedentemente descritto come capace di produrre lipide A palmitoleoilato, ma come specie minore. La palmitoleoilazione nel sito *htrB* è riportata come risposta da freddo in *E. coli* catalizzata da LpxP. Dopo una crescita di *S. sonnei* Δ*htrB* a 12 °C per indurre la risposta da freddo, piccole quantità di lipide A palmitoleoilato sono state rilevate mediante MALDI-TOF, dimostrando che il gene *lpxP* è funzionale. Molto probabilmente la differenza nella palmitoleoilazione nei due mutanti è riconducibile ad un livello di espressione 7 volte più elevato del gene *lpxP* a 30 °C in *S. flexneri* Δ*htrB* rispetto a *S. sonnei* Δ*htrB* come osservato mediante PCR quantitativa in tempo reale.

Stimolazione del TLR4 da parte di GMMA dopo modifica genetica del lipide A

Per valutare la capacità dei diversi mutanti di lipide A di attivare TLR4 è stata utilizzata una linea cellulare che esprime un gene reporter di luciferasi sotto il controllo del TLR4 umano.
GMMA purificate da mutanti Δ*msbB* hanno mostrato una diminuzione di circa 600 volte nella loro capacità di stimolare il TLR4 rispetto a GMMA contenenti lipide A wild-type. Un'ulteriore sostanziale diminuzione di attività stimolante è stata osservata con GMMA da *S. sonnei* Δ*htrB*. L'induzione della luciferasi è stata rilevabile solo ad una concentrazione elevata di GMMA, richiedendo una quantità 60,000 volte più elevata rispetto a quella necessaria nel caso di GMMA contenenti lipide A WT e 100 volte superiore rispetto a GMMA Δ*msbB* nell'indurre lo stesso livello di attivazione. Al contrario, GMMA da *S. flexneri* Δ*htrB,* contenenti lipide A esa-acilato palmitoleoilato, hanno manetenuto una superiore capacità di stimolare il TLR4 umano rispetto alle GMMA Δ*msbB*, richiedendo una quantità di GMMA 10 volte inferiore per provocare una simile induzione di luciferasi. Di conseguenza queste GMMA hanno mostrato una relativamente piccola (50 volte) diminuzione di attività stimolante rispetto al GMMA contenenti lipide A WT. Questo suggerisce che lil lipide A esa-acilato palmitoleoilato mantiene attività stimolante del TLR4, ma che la sostituzione della catena di lauroile con quella di palmitoleoile provoca una diminuizie della stimolazione del TLR4 rispetto al lipide A WT.

2. Valutazione del contributo relativo dei pathways TLR4 e TLR2 nella stimolazione delle cellule umane mediante GMMA contenenti differenti modificazioni dei lipide A

Al fine di valutare l'attività di endotossica delle GMMA in un contesto più naturale, GMMA purificate dai diversi mutanti sono state usate per stimolare cellule umane mononucleate da sangue periferico (PBMC) in un test di attivazione dei monociti (MAT). .

GMMA purificate da ceppi di *Shigella* contenenti lipide A modificato (sia Δ*htrB* che Δ*msbB*) hanno mostrato una diminuzione della capacità di stimolare la produzione di citochine (interleuchina 6 [IL- 6], IL-8, IL-1β, Tumor Necrosis Factor-α) rispetto a GMMA purificate da ceppi senza modifiche nell'LPS, con una tendenza simile a quella osservata nel test TLR4-specifico. GMMA purificate da ceppi Δ*msbB* necessitano di una quantità circa 300 volte superiore rispetto alle GMMA con lipide A WT per indurre un simile rilascio di IL-6, mentre GMMA da *S. flexneri* Δ*htrB* di circa 50 volte maggiore. Al contrario, GMMA da *S. sonnei* Δ*htrB* hanno mostrato un capacità 800 volte inferiore nella capacità di stimolare il rilascio di IL-6 da PBMC, per cui una diminuzione minore in attività rispetto a quanto previsto dalla riduzione di 60,000 volte osservata nella stimolazione di TLR4 (rispetto a GMMA contenenti lipide A WT), ad indicare che ulteriori PRR contribuiscono alla stimolazione dei PBMC, molto probabilmente la via TLR2.

Al fine di determinare il contributo relativo dei pathways TLR4 e TLR2 nella stimolazione mediante GMMA di PBMC umani, quest'ultimi sono state incubati con anticorpi anti-TLR4 o anti-TLR2 per bloccare selettivamente l'attività di TLR4 o TLR2 prima della stimolazione con GMMA. Non è stata rilevata alcuna attività residua di TLR4 in GMMA da *S. sonnei* Δ*htrB* con lipide A penta-acilato A. GMMA con lipide A penta-acilato da ceppi Δ*msbB*, invece, hanno mantenuto una certa capacità di attivare il TLR4 umano, ma l'induzione di IL- 6 TLR4-dipendente in queste GMMA era più piccola rispetto all'induzione TLR2-dipendente. Questo ha dimostrato che la reattogenicità residua nella GMMA contenenti lipide A penta-acilato è mediata principalmente da TLR2. Al contrario, una sostanziale diminuzione nell'attivazione di GMMA da *S. flexneri* Δ*htrB* è stata ottenuta utilizzando anticorpi anti-TLR4 ma non con anticorpi anti-TLR2, dimostrando che il lipide A esa-acilato palmitoleoilato mantiene una forte attività stimolante nei confronti del TLR4.

Per confermare che le differenze nel contributo relativo di TLR4 e TLR2 all'attivazione osservato negli esperimenti di blocco erano dipendenti esclusivamente dalla differenziale attivazione di TLR4 da parte delle differenti GMMA, la capacità delle GMMA di attivare il TLR2 è stata testata utilizzando una linea cellulare che esprime solo il TLR2 umano Tutti e quattro le GMMA Δ*msbB* o Δ*htrB* hanno mostrato in questo saggio simile attivazione del TLR2.

Conclusione: fattibilità della riduzione di reattogenicità mediante modifica del lipide A

La prova concenttuale della riduzione della reattogenicità delle GMMA mediante modificazione genetica del lipide A è stata ottenuta.

GMMA con lipide A penta-acilato hanno mostrato una marcata riduzione nella capacità di induzione di citochine infiammatorie da PMBC umani. La delezione del gene *htrB* da *S. sonnei* ha comportato una riduzione di 800 volte dell'attività stimolante di citochine e l'attività residua è TLR2-mediata. GMMA da mutanti con delezione di *msbB* hanno mostrato, invece, una riduzione di 300 volte, mantenendo

un'attività TLR4-mediata misurabile; tuttavia, la principale via attivata era quella TLR2-mediata, similmente a quanto osservato per GMMA da *S. sonnei* Δ*htrB*.
Al contrario, una compensazione mediante palmitoleoilazione in GMMA da *S. flexneri* Δ*htrB* ha comportato il mantenimento dell'attivazione TLR4-mediata da parte del lipide A esa-acilato, e quindi una superiore reattogenicità, suggerendo che modificazioni che risultino in lipidi A penta-acilati siano da preferirsi.

3. Dimostrazione di applicabilità generale dei metodi individuati per la piattaforma GMMA

Salmonella

Salmonella enterica è una specie di *Enterobacteriaceae* strettamente correlata a *Shigella*, con la quale condivide lo stesso lipide A esa-acilato. Per cui, come per *Shigella*, è stato indagatao l'effetto di una modifica del lipide A, mediante delezione di *htrB* e *msbB,* in ceppi GMMA-produttori di *Salmonella enterica* sierotipi Typhimurium (S. Tm) e Enteritidis (S. En).
Tuttavia, la situazione in *Salmonella* è più complessa rispetto a *Shigella*, in quanto il lipide A è, almeno in parte, epta-acilato a causa del'aggiunta, catalizzata da PagP, di una catena di palmitoleoile (una catena di acido grasso C_{16}) a livello dell'idrossimiristato in posizione 2. Le quantità relative di lipide A esa- ed epta-acilato variano in ceppi diversi, da più del 95% esa-acilato ad oltre il 30 % epta-acilato. In accordo con la presenza di una miscela di lipide A esa- ed epta-acilato nel lipide A prima delle modifiche genetiche del lipide A, l'eliminazione di una singola catena acilica dovuta alla delezione di *msbB* o *htrB* hanno comportato l'ottenimento di una miscela di lipide A penta- ed esa-acilato mancante di una catena miristoilica o lauroilica rispettivamente. È interessante notare come, dopo delezione di *htrB* in SEn, sia stata osservata la stessa compensazione mediante palmitoleoilazione osservata in *S. flexneri* Δ*htrB*, risultante in una miscela di lipide A esa- ed epta-acilato con la sostituzione della catena di lauroile con quella di palmitoleoile (spostamento $m/z = 54$).
GMMA da S. Tm Δ*msbB,* contenenti oltre il 95% di lipide A penta-acilato, hanno mostrato un analogo livello di rilascio di IL-6 da PBMC rispetto a GMMA di *Shigella* contenenti lipide A penta-acilato. Tutte le altre GMMA da STm e SEn con modificazioni del lipide A che sono state testate, contenevano una percentuale più elevata di lipide A esa-acilato e, in linea con i risultati ottenuti con GMMA da *S. flexneri* Δ*htrB* contenneti lipide A esa-acilato palmitoleoilato, GMMA di S. Tm e S. En con lipide A esa-acilato hanno mantenuto attività stimolante TLR4 superiore rispetto a quelle con lipide A penta-acilato, risultando in una minore probabilità di tradursi in un vaccini utili.
In conclusione, GMMA da S. Tm con lipide A penta-acilato A hanno mostrato un profilo di reattogenicità ridotto in maniera simile a quanto osservato in GMMA di *Shigella* contenenti lipide A penta-acilato. Tuttavia, per ottenere GMMA di *Salmonella* con lipide A prevalentemente penta-acilato e senza lipide A esa-acilato, ulteriori mutazioni potrebbero essere necessarie oltre ad *htrB* o *msbB*.

Neisseria

La riduzione di endotossicità delle GMMA mediante modificazione genetica del lipide A è stata valutata anche in *Neisseria meningitidis*, una specie meno geneticamente correlate a *Shigella*.

Il lipide A di *N. meningitidis* è esa-acilato, ma con una composizione e distribuzione degli acidi grassi differente rispetto al lipide A di *Shigella*. Inoltre, sul fosfato in posizione 4' è presente una fosfoetanolamina. Nonostante le differenze, simili quantità di GMMA da *Neisseria* e *Shigella* contenenti lipide A WT, hanno indotto simili livelli di rilascio di IL-6 da PBMC umani. Per ottenere un lipide A penta-acilato è stato deleto il gene *lpxL1* che, come precedentemente riportato, codifica per una aciltransferasi che aggiunge una catena di lauroile all'idrossimiristoile in posizione 2'. La penta-acilazione del lipide A risultante è stata confermata tramite MALDI-TOF con uno spostamento *m/z* di 182 Dalton rispetto al lipide A WT, coerente con l'assenza della catena lauroilica.
Le GMMA provenienti dal mutante *Neisseria* Δ*lpxL1* hanno mostrato una marcata riduzione dell'attivazione di TLR4 e di stimolazione di IL-6 da PBMC umani, a livelli simili a quelli osservati in *S. sonnei* Δ*htrB* GMMA. Questo risultato ha sottolineato che, nonostante le differenze di composizione, il lipide A penta-acilato conserva poca capicità di attivazione del TLR4, e questo si traduce in una sostanziale riduzione della reattogenicità delle GMMA.

Conclusioni

L'obiettivo generale della tesi è stato quello di esaminare i metodi per ridurre la reattogenicità di GMMA in modo da renderle idonee ad essere utilizzate come vaccino ad uso umano. Il risultato è stato sorprendentemente complesso. Ho dimostrato la possibilità di ridurre la reattogenicità delle GMMA mediante modificazione del lipide A. Ho trovato, inoltre, una chiara relazione tra la composizione delle varie specie di lipide A e la reattogenicità. La nostra analisi di GMMA purificate da linee isogeniche e da specie diverse ha evidenziato l'importanza di determinare la composizione del lipide A dopo la modificazione genetica, dato che la stessa modificazione genetica ha dato risultati diversi anche in specie di *Shigella* strettamente correlate.
Ho dimostrato in *Shigella*, *Salmonella* e *Neisseria* che GMMA con lipide A penta-acilato presentano una marcata riduzione della capacità di stimolazione di citochine e, utilizzando GMMA di *Shigella,* che la reattogenicità residua in è prevalentemente TLR2-mediata. Al contrario, GMMA con lipide A esa-acilato, sia attraverso palmitoleoilazione dopo delezione di *htrB*, che a causa di una ulteriore palmitoilazione in *Salmonella*, mantengono una residua attività stimolante sul TLR4, rendendo meno probabile il loro utilizzo come vaccino.
Sulla base dei risultati presentati in questa tesi, quella della modificazione genetica del lipide A risultante in un lipide A penta-acilato rappresenta una strategia promettente per ridurre la reattogenicità delle GMMA. Se si renderà necessaria un'ulteriore riduzione della reattogenicità, modifiche nelle molecole responsabile dell'attivazione del TLR2 sarebbero necessarie. Tuttavia, il livello di riduzione richiesto per un vaccino accettabile dipende dalla dose necessaria per dare una forte risposta immunitaria, e questa può essere determinata solo da studi clinici. Studi clinici in corso utilizzando la GMMA di *S. sonnei* Δ*htrB* dovrebbero dare un'indicazione importante della tollerabilità di questi costrutti.

Introduction

Neglected disease and need of vaccines

Globally diarrheal disease, especially during the first 5 years of life, results in about 760,000 deaths annually, mostly in sub-Saharan Africa and south Asia (1). In those areas the poverty of people, the low sanitary and hygienic conditions, the difficult access to hospitalization, malnutrition and concomitant diseases that immunocompromise people, strongly facilitates the spread of enteric pathogens (2).
Enteric diseases are also a significant cause of morbidity among people from industrialized countries, mostly as traveler's diarrhea and during military operations. In addition, there are long-term consequences of early childhood diarrheal disease on growth and physical and cognitive development (3).
Gram-negative bacteria are the major causes of neonatal sepsis in developing countries, causing a wide range of diarrheal, respiratory and invasive diseases (4); between them the most frequent Gram-negative isolates are *E. coli*, *Salmonella* spp. and *Shigella* spp., as well as other non-invasive pathogens, like *Vibrio cholerae* and enterotoxigenic *E. coli* (ETEC) (5). The wide diversity of bacterial and viral infections that may cause diarrhea complicates accurate surveillance and diagnosis, especially in developing countries with little or no access to modern laboratory procedures. In fact, it must be considered not only that several different pathogens may cause diarrheal diseases, but also the antigenic and strain diversity among these pathogens, raising severe obstacles for proper etiological diagnostic and the development of effective vaccines (3). In fact, besides the progressive improvement of basic hygienic and sanitary conditions, access to clean water and better nutrition obtained in the last years (4), that resulted in the decrease the deaths caused by enteric infections, immunization still represent the most cost effective and efficient way to control and possibly defeat infectious disease. Unfortunately, vaccines are not yet available to prevent many major bacterial infections especially affecting developing countries, for example, *Shigella* and non-Thypoidal *Salmonella* infections.
An ideal vaccine should be easy to administer, i.e. not requiring high professional skills for being administered, well tolerated, formulated for protecting against multiple pathogens (both for reducing cost and also for reducing the number of injections per child)), easy to manufacture, has low cost (ideally lower than 1 $ per dose), and able to induce long-term protection.
The mission of NVGH is to develop effective and affordable vaccines for neglected diseases of impoverished communities. Thus, NVGH is developing platform technologies that can be applied to the development of vaccines for several pathogens. One major platform is based on outer membrane particles called Generalized Modules for Membrane Antigens (GMMA). This platform is used in three major programs of NVGH, *Shigella,* non-Typhoidal *Salmonella* and *Neisseria meningitidis.*

Shigella

Shigella are a non-motile, facultative anaerobic, Gram-negative bacteria that belong to the family *Enterobacteriaceae*. *Shigella* are the causative agents of shigellosis, a global human health problem, especially in developing countries and in children younger than 5 years (6), with more than 125 million cases (7) and 100,000 deaths

(8) per year. This estimate indicates a significant decrease in deaths in the last 10 years (9) assumed to be due to non-specific intervention as improved nutrition, vitamin A supplementation and measles vaccination (7). However, the incidence rate remained high. Shigellosis is also a frequent cause of traveler's diarrhea (10).
Humans and non-human primates are the only hosts for *Shigella,* in which they can cause diarrheal disease. The bacteria multiply within colonic epithelial cells, causing mucosal ulceration, inflammation, and bleeding. Transmission usually occurs by fecal-oral contact and thus is promoted by substandard hygienic conditions. As few as 10-100 microorganisms may be sufficient to start infection, indicating that *Shigella* is highly contagious (11). The disease caused by *Shigella* can range from mild diarrhea to a severe form of dysentery with frequent mucoid bloody stools, abdominal cramps and tenesmus. The severity of disease depends on the infecting species and also on the condition of the patient, especially the nutritional status and immune status (12).
Starting from the first *Shigella* strain isolated in 1896, now called *Shigella dysenteriae* type 1, today the genus *Shigella* includes 50 different serotypes characterized by the carbohydrate composition of the O antigen (OAg) of the LPS. These serotypes are grouped into 4 serogroups that historically were classified as species, based on biochemical and serological differences: *Shigella dysenteriae* (group A, 15 serotypes), *Shigella flexneri* (group B, 15 serotypes), *Shigella boydii* (group C, 20 serotypes) and *Shigella sonnei* (group D, 1 serotype). In addition, more than a dozen putative new serotype or subtype strains are being considered for possible official classification (13). *S. flexneri* and *S. sonnei* are endemic and cause most of the infections. *S. dysenteriae* type I is epidemic and *Shigella boydii* is currently not regarded of global importance (10).
Shigella is genetically closely related to *Escherichia coli*. The sequence divergence between *S. flexneri* and *E. coli* K12 is only 1.5%, and biochemically, *Shigella* is very similar to enteroinvasive *Escherichia coli* (12).
Due to the global emergence of drug resistance, the choice of antimicrobial agents for treatment of shigellosis is limited. In addition, the costs of treatment could be inhibitory, considering that this disease is endemic in the poorest area of the world. Thus, there is remerging interest in developing a vaccination against *Shigella*. Currently, no vaccine is available. Natural immunity to *Shigella* is protective but serotype-specific. Thus, the large number of serotypes (10) and constant changes of the serotypes present in a given region has been a challenge for vaccine development (10).

Shigella *vaccine*

As mentioned above, shigellosis is mainly a disease of the poorest people in the world and results in a high number of deaths. Thus, vaccination offers the greatest hope as an effective and sustainable strategy against that disease.
Considering that immunity in *Shigella* is serotype-specific and that multiple serotypes are present at same time, (10) a broadly protective vaccine will be needed to target multiple serotypes. This could be achieved using a multivalent O antigen vaccine or a protein-based vaccine targeting proteins that are conserved in the different *Shigella* strains.
For developing a broadly protective*Shigella* vaccine based on serotypes, the Global Enteric Multicenter Study (GEMS) suggest that a quadravalent vaccine, containing strains or antigens from *S. sonnei* and *S. flexneri* 2a, 3a and 6, would directly cover

approximately 65% of current circulating strains. With cross protection based on shared *S. flexneri* group antigens, such a quadravalent vaccine could cover more than 85% of currently circulating *Shigella* strains. Many argue for including *S. dysenteriae* 1 coverage in a serotype-based vaccine in the expectation that pandemic *Shigella* dysentery will return and a vaccine could constitute an important public health tool (13).
Different types of vaccines candidates against *Shigella* have been developed and tested so far, but all presented some problems. The main approaches have been based on live attenuated *Shigella* vaccine candidates and O antigen conjugates which both raise serotype-specific responses. Live attenuated strains or killed whole-cell vaccines have the advantage of presenting almost all the antigenic repertoire of the bacteria to the host immune system and are easy to manufacture, but it is very difficult to obtain strains that are sufficiently attenuated to avoid reactogenicity and maintaining good immunogenicity (14,15). O antigen conjugates, i.e. vaccines in which polysaccharide antigens are covalently linked to carrier proteins, like recombinant *Pseudomonas* exoprotein A (rEPA), have been shown to induce protection against homologous strains (16) but are not able to generate cross-protection against other strains of *Shigella* (17,18).An interesting bioconjugate vaccine technology developed at GlycoVaxyn, in Switzerland, utilizes recombinant DNA technology to catalyze the *in vivo* synthesis of conjugate vaccines by using the glycosylation machinery from *Campylobacter* cloned in an *E. coli* production strain for generating a protein carrier glycosylated with a specific *Shigella* OAg, then purified as a conjugate vaccine (19); a *S. dysenteriae* 1 OAg–rEPA bioconjugate vaccine produced in this system was found safe and immunogenic after two doses. Other strategies involved the use of proteosomes (20) and a nuclear protein-ribosomal vaccine (21), but these also raised O antigen-specific responses.
To achieve cross protection the ideal approach could be the use of a protein based vaccine containing proteins conserved in the different strains and thus could give broad spectrum protection. Protein vaccine candidates against shigellosis were developed by purifying outer membrane proteins (22) or Ipa proteins (23,24), but they were not protective against all of the tested strains, e.g the Invaplex vaccine, formulated from a bacterial extract composed of Ipa proteins, which are highly conserved among all *Shigella* serotypes and have an essential role in *Shigella* pathogenesis, and LPS. In animals, serotype-specific protection has been demonstrated and has also been shown to be safe and immunogenic after intranasal delivery in healthy volunteers (25). Despite all these efforts, only one *Shigella* vaccine candidate, the *S. sonnei* OAg-conjugate has so far been tested in Phase III clinical trials and has been shown to elicit approximately 70% protection in adults with one immunization and in children older than 3 years of age with two immunizations (16,26).

Salmonella

The genus *Salmonella* causes a large global burden of morbidity and mortality and it is composed of two distinct species: *Salmonella bongori* and *Salmonella enterica*, which has been divided into six subspecies. The subspecies have been classified into more than 50 serogroups based on the OAg, and are divided into 2,400 serovars based on the flagellar antigen (27).
Salmonella infections in humans can be divided into typhoid fever, caused by *Salmonella enterica* serovar Typhi (*S.* Typhi) and Paratyphi (*S.* Paratyphi), and a

range of diarrheal diseases, caused by a large number of non-typhoidal *Salmonella* serovars (NTS) (27).
Typhoid fever is a global problem, that causes every year more than 27 million cases worldwide resulting in an estimated 217,000 deaths (28). This disease is most common among children, especially in areas of Asia and Africa that lack clean water and adequate sanitation, and is also an important travel-associated disease (29). *S.* Typhi is an exclusively human pathogen, and typhoid fever is characterized by high fever, severe headache, nausea, abdominal pains, and loss of appetite, constipation or sometimes diarrhea that can last for several weeks. The most frequent complications, that can results in death in 1-4% of the cases, are intestinal perforation and peritonitis or severe toxic encephalopathy (30). Paratyphoid fever, which is caused by any of three serotypes of *S.* Paratyphi A, B and C, is similar in its symptoms to typhoid fever, but tends to be milder, with a lower fatality rate (28).
NTS serovars, such as *S.* enterica serovar Typhimurium and Enteriditis, also cause a significant disease burden, with an estimated 100,000 deaths/year (31). The host range of non-Typhoidal *Salmonella* serovars is broad. In industrialized countries, NTS infection is commonly due to food contaminations. NTS disease cause usually self-limiting diarrhea associated with nausea, vomiting and abdominal pain in healthy individuals and bacteremia occurring in less than 5% of patients with mortality rate lower than 1%. In contrast, in sub-Saharan Africa, NTS serovars cause high rates of bacteremia in the immune-compromised patients and in children younger than 5 years, and where often associated with HIV infection (32,33). In addition to sepsis, invasive NTS (iNTS) can seed to the meninges, especially in children. Also antimicrobial resistance is widely spread and increases the problems in treatment of disease (30).

Salmonella *in Africa*

Salmonella Typhi represent the most common form of invasive *Salmonella* disease in South-East Asia, but it is non common in sub-Saharan Africa. In sub-Saharan Africa, instead, invasive NTS are among the most common isolates from febrile presentations in adults and children, and *Salmonella* Typhimurium and *Salmonella* Enteritidis represent the most common serovars.
In sub-Saharan Africa, high-risk hosts for iNTS were represented by young children and HIV-infected individuals, with case fatality rates around 20–25%. In contrast HIV infection does not appear to predispose to infection with *Salmonella* Typhi in parts of Africa where typhoid fever has been reported (34). iNTS infections in sub-Saharan Africa were often associated also with malaria, severe anemia and malnutrition, as these are also risk factors for iNTS (35). iNTS disease, nevertheless, is not only the responsible of deaths in presence of coinfentions, it remains a problem in locations where malaria is almost absent (36), or where HIV prevalence is low (37), and continues to be a problem in children not infected with HIV.
Due to the absence of a clear clinical presentation (fever is the most common clinical feature and gastrointestinal symptoms occur in less than half of the patients) and high incidence of confections, iNTS can be the cause of deaths without being diagnosed, and a lot of deaths due to iNTS will be recorded as due to HIV, malaria and malnutrition (35). Infact, a diagnosis is only possible where blood culture is available, and that is, unfortunately, not so common in sub-Saharan Africa. Thus, it is difficult to quantify with precision the overall incidence of iNTS disease in sub-

Saharan Africa. Data obtained in 11 study sites across sub-Saharan Africa give rates of around 500/100,000 children/year (38).
The disease is highly seasonal (39) with peaks of infection during the rainy season in both adults and children coincide with increased incidences of malaria and malnutrition. Invasive NTS disease has also been present in epidemics that last several years and are caused by sequential single serotypes among adults and children. These epidemics have been linked to the emergence of resistance to commonly used antimicrobial drugs (39). In general increasing antibiotic resistance strongly complicated the treatment of salmonellosis. iNTS disease in Africa, unlike NTS disease in industrialized nations, does not have an animal reservoir(35).

Salmonella *vaccine*

Several vaccines are available for *S.* Typhi and Paratyphi, instead the situation is less developed for NTS. For which concerns *S.* Typhi, the live attenuated oral vaccine Ty21a, obtained by chemical mutagenesis from wild type is immunogenic and protective above the age of 5 years (40), like the Vi capsular polysaccharide and several other live attenuated strains (41), but requires multiple doses s (42). Because of the limitations of the currently available vaccines, considerable interest remains in developing new vaccines that are immunogenic and well tolerated in infants and offer greater protection to older children and adults. The most promising candidates are a new generation of live-attenuated vaccine strains and especially Vi-conjugate vaccines (the immunogenicity of polysaccharide antigens in infants is greatly enhanced when they are conjugated to a carrier protein and antigens became T-cell dependent antigens) (43). Several Vi-conjugate vaccines are in late stages of development, like the Vi-rEPA (44), using the recombinant *Pseudomonas aeruginosa* exotoxin A as the protein carrier, and the Vi-CRM_{197}, a conjugate vaccine developed by NVGH for infant immunization; immunogenicity and safety were assessed in two phase 2 trials conducted in endemic countries (Pakistan, India and Philippines) in adults and children younger than 2 years. The vaccination was conducted in concomitance with vaccines of the Expanded Programme on Immunization (EPI), according to WHO schedule, showing a promise for potential inclusion of Vi-CRM_{197} in EPI schedules of countries endemic for typhoid (45).
There are currently no vaccines directed against *S.* Paratyphi. Several candidate vaccines against *S.* Paratyphi are under investigation. Also for that disease live attenuated strain were developed and the NVGH O:2 polysaccharide conjugated to Vi-CRM_{197} with the ultimate aim of manufacturing a bivalent enteric fever vaccine (43).
The diversity of nontyphoidal Salmonella serovars makes the development of a vaccine a big challenge; however *S.* Enteritidis and *S.* Typhimurium are the most common serovars in sub-Saharan Africa and account for nearly all iNTS reported (46). The development of vaccines against iNTS for Africa is in progress with O polysaccharide-flagellin conjugate vaccine (47) and live attenuated (48).

Neisseria meningitidis

Neisseria meningitidis is a Gram-negative encapsulated, aerobic diplococcus, which colonizes the nasopharynx of humans and has no other known environmental niche (49). It is one of the major causes of bacterial meningitis and sepsis, and is the only form of bacterial meningitis responsible for epidemic levels of meningitis infection

worldwide (50). Bacterial meningitis is a serious threat to global health, accounting for an estimated 170,000 death/year (50). Up to 25% of survivors develop permanent sequelae, such as epilepsy, mental retardation, or sensorineural deafness (50). The disease still remains one of the most feared infections due to its rapid progression and the tendency to cause outbreaks and epidemics. Clinical syndromes caused by *N. meningitidis* include meningitis, with or without meningococcemia, relatively mild bacteremia, fulminant meningococcemia, meningoencephalitis, pneumonia, and septic arthritis, as well as other presentations. Even when the disease is diagnosed early and adequate therapy is instituted, 5–10% of patients die (50). Together with *Haemophilus influenzae* and *Streptococcus pneumoniae*, *Neisseria meningitidis* is one of the major causes of bacterial meningitis and sepsis, causing at least 500,000 cases and more than 50,000 deaths every year (50).
The bacteria are commonly carried asymptomatically in the upper respiratory tract of between 8–20% of healthy individuals (51) and more frequently in adolescents and other populations with close contact. Only occasionally *N. meningitidis* invades the bloodstream and meninges to cause disease. The disease incidence varies in human populations from rare to over 1,000 per 100,000 people/year in localized outbreaks, epidemy and pandemy (52).
A polysaccharide capsule, which covers the outer membrane of the bacteria, is a major virulence factor for *N. meningitidis* and although capsular-deficient strains are commonly isolated, these are non-infectious (53). Capsule mediates resistance to phagocytosis, complement mediated lysis and protects against environmental insults. *Neisseria meningitidis* has been divided in 13 serogroups based on different capsular polysaccharide structure, but only 6 serogroups are associated with significant pathogenic potential: A, B, C, W-135, Y and X (54). *Neisseria meningitidis* has been further classified into serotypes and serosubtypes, basing on antigenic differences of the major outer membrane proteins PorB and PorA. *N. meningitidis* presents a different distribution in different areas of the world (50). *N. meningitidis* B is a major cause of sporadic or endemic meningitis in developed countries, accounting for up to 80% in certain European countries, with most of the remaining cases being caused by serogroup C strains (55) (and Y in North America), instead the type of strains presents in Africa is quite different (56).

Neisseria *in Africa*

Neisseria meningitidis is a major cause of epidemics in sub-Saharan Africa (56). These were mainly caused by strains belonging to capsular group A, that is responsible of epidemics. Outbreaks occur during the dry season and diminish with the onset of the rainy season (57). It's also has been observed an increasing contribution of serogroups W and X strains with epidemic potential in the last two decades (58,59).
A polysaccharide conjugate vaccine (MenAfriVac) has been developed for preventive mass immunization in the meningitis belt against serogroup A. Where the vaccine has been implemented, the number of reported cases caused by serogroup A strains has decreased dramatically, but group W and X strains remain a persistent problem. This observation underlines the need for an affordable vaccine that can provide broad protection against all three main serogroups causing meningitis in Africa and potentially against new disease-causing serogroups that may emerge in the region in the future (Koeberling at al., 2014 submitted).

Neisseria *vaccine*

The rapid onset of invasive meningococcal disease, the high incidence in childhood, the severity of the sequellae and the high mortality rate clearly indicate the importance of vaccine development against *N. meningitidis*. An ideal meningococcal vaccine should be able to protect against all disease-causing meningococci, developing protective immunity at the mucosal surface and in the circulation (50).
Several polysaccharide vaccines, which have been available for over 30 years, exist against serogroups A, C, Y, W-135 in various combinations. A major advance in the prevention of meningococcal disease has been the development and introduction of meningococcal polysaccharide-protein conjugate vaccines (60). These vaccines are immunogenic, particularly for children under 2 years of age whereas polysaccharide vaccines are not. All these vaccines have been proven to be safe and effective with infrequent and mild side effects. The development of vaccines for serogroup B *Neisseria meningitidis* remains a challenge (61), due to the fact that the serogroup B capsule has an identical structure to polysialic structures expressed in fetal neural tissue, and does not induce a protective IgG response, rendering needed the development of a different class of antigens to obtaining an efficient vaccine.
The most promising candidate protein vaccine antigen against meningococcus to date is factor H binding protein (fHbp). The fHbp gene is present in most invasive meningococcal isolates independent of serogroup. fHbp has been divided into three antigenic variants (v. 1, 2 or 3) (62). Within each variant, the peptides differ by a few amino acids and are identified by a unique peptide ID. The outer membrane protein, PorA, is highly immunogenic but antibodies to PorA tend to provide subtype-specific protection, especially in young children (63).
Bexsero, a vaccine developed by Novartis Vaccines and Diagnostics, has been recently approved and is the first vaccine against *N. meningitidis* B strain. It is a multi-component meningococcal B vaccine (also called 4CMenB) containing three surface-exposed recombinant proteins (fHbp, NadA and NHBA) and New Zealand strain detergent extracted outer membrane vesicles (NZ OMV) with PorA 1.4 antigenicity. A four-dose schedule for infants (three primary doses and one booster dose) and a two-dose schedule for adolescents provided an estimated coverage between 66 – 91% against meningococcal serogroup B strains worldwide (64).
Research is continuously developing and several promising and innovative vaccines against meningitidis are based on Native Outer Membrane Vesicles (NOMV) (65).

Native Outer Membrane Vesicles (NOMV) NVGH vaccine platform: Generalized Modules for Membrane Antigens (GMMA)

One of the platform technologies developed by NVGH to develop effective and affordable vaccines is based on Generalized Modules for Membrane Antigens. This platform technology takes advantage of the ability of Gram-negative bacteria to naturally shed small particles from the outer membrane into the environment (native outer membrane vesicles, NOMV). NVGH has developed a high yield production process using genetic modification of bacteria to induce high level shedding of these outer membrane particles. To differentiate these particles that are released from the genetically modified bacteria from NOMV and other types of particles produced by bacteria, they were named GMMA.

Native Outer Membrane Vesicles (NOMV)

Composition and biological role

Gram-negative bacteria naturally release blebs from the outer membrane usually called Outer Membrane Vesicles (OMV) (66) or more specifically native Outer Membrane Vesicles (NOMV). These are small spherical structures, 30-250 nm in diameter, which protrude from the outer membrane and then are released into the environment (Fig. 1) (67). The term OMV has also been used for vesicles derived by detergent-extraction of homogenized bacteria which have a very different composition (68) and have been used as vaccines (69). Thus, to avoid confusion the term NOMV is used for particles naturally shed from the surface.
The two-layered outer membrane of Gram-negative organisms is an asymmetric membrane composed of an outer layer containing surface proteins and lipopolisaccaride (LPS). The inner leaflet consists of phospholipids and lipoproteins that link the outer membrane to a thin layer of peptidoglycan. Between the outer membrane and peptidoglycan lies a gelatinous material called periplasm, which also separates the peptidoglycan and the cytoplasmic membrane (70). NOMV, in general, maintain the structure, composition, and orientation of the outer membrane, including the presence of LPS, glycerophospholipids, outer membrane proteins and also contain soluble periplasmic proteins entrapped in the lumen (71,72).
Gram-negative bacteria, both pathogenic and non-pathogenic, secrete NOMV during constitutive growth, including *E. coli*, *N. meningitidis*, *V. cholerae*, *Pseudomonas aeruginosa*, *Brucella melitensis*, and *Helicobacter pylori* especially during the end of log phase growth and at sites of cell division (73). Proposed models for vesicle formation conclude that an intact peptidoglycan layer is not contained within NOMV and that vesicle production probably originates at sites where the outer membrane-peptidoglycan linkages are disrupted**.**

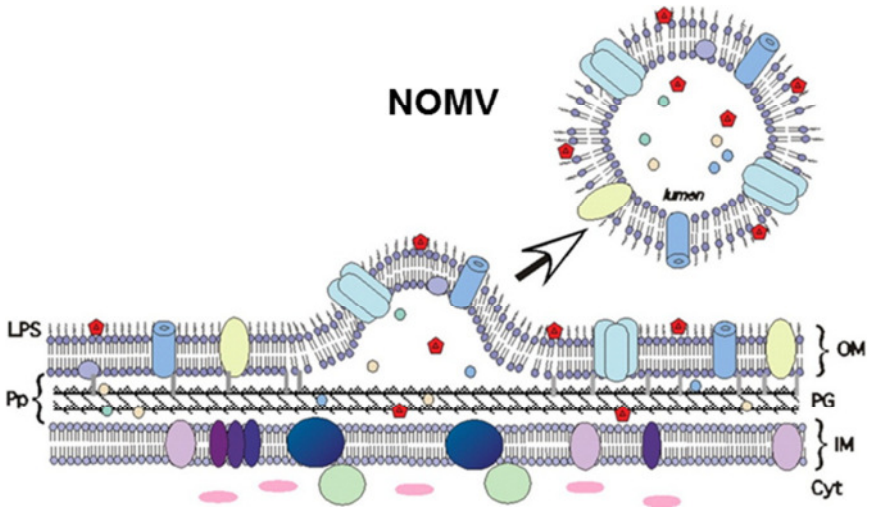

Fig. 1 Native Outer Membrane Vesicles (NOMV) generation. Modified from (67)

In the literature this particles have usually been called Outer Membrane Vesicles (OMV).
NOMV are very similar to but not identical to that in the outer membranes. For the most part, NOMV are devoid of inner membrane and cytosolic constituents. Some proteins and lipids are consistently detected in NOMV while others are always

excluded. Proteomic analyses of the protein content of NOMV from several different organisms have shown that vesicles enclose a wide variety of envelope components (70).
NOMV have been discussed to have different biological roles, e.g. as delivery vehicles for active components to host cells, as nucleators in the formation of bacterial communities (biofilms) and as contributors to bacterial survival and virulence (72).
The composition of NOMV from pathogens should reflect the state of the bacterial outer membrane, and were also dependent on the site of culturing of the bacteria. Active toxins are associated with several pathogen-derived NOMV and some of the toxins appear to be enriched in NOMV. In some cases, NOMV-associated toxins are more active than the toxins alone, and some toxins are associated with the exterior surface of NOMV (74).

Potential of NOMV as vaccine

As NOMV represent the envelope of Gram-negative bacteria and, thus, most of the components that might be recognized by the immune system (75), they have been proposed for use as vaccines. Also, NOMV combine both adjuvant and carrier activity, increasing the low immunogenic properties of some protein antigens (70). It was also demonstrated that proteins from a foreign source can be localized in the outer membrane and periplasm of the parent cell and successively internalized into OMVs (74).
NOMV have been shown to induce protection in mice against multiple pathogens, including *Salmonella enterica* serovar Typhimurium (76), *Helicobacter pylori* (77), *Vibrio cholera* (78,79) and *Shigella bodyii* (80) or to elicit antibodies with in vitro bactericidal activity in mice, e.g. for *Neisseria meningitidis* (81,82). In addition, NOMV have been tested as veterinary vaccine candidate, e.g. against *Edwardisiella tarda* implicated in the death of see water fish (83).
The yield of NOMV is usually too low for a practical production. The application of NOMV as vaccines requires high production and efficient purification. Various methods have been employed to induce and increase blebbing of the outer membrane of Gram-negative microbes. These techniques include inhibiting protein synthesis, autolytic cell wall degradation by lysine starvation, increasing growth temperature (even to 55^{o}C), and the addition of sub-lethal doses of antibiotics (70,72). However, all of these conditions alter the composition of OMVs so that they are no longer “native” and have not resulted in true high yield production.

High yield GMMA production process for use as vaccines

NVGH has developed a high yield NVGH has developed a high yield production process suitable for industrial production using genetic modification of bacteria to induce high level shedding of outer membrane particles.
As mentioned above, NOMV preferentially form where the link between the inner and outer membrane is weakened. In the case of *Shigella* and *Salmonella*, this linkage can be genetically disregulated by deletion of *tolR*, a gene of the Tol-Pal pathway (66,84). The Tol-Pal system is present in most of Gram-negative bacteria and is required to maintain outer membrane integrity. Thus, a mutation in any of *tol-pal* genes, and especially on *tolR*, confers a defect in other membrane integrity, resulting in a major release of GMMA (84). In the case of *N. meningitidis*, which do not have a

homologue of *tolR*, deletion of *gna33*, encoding a membrane bound lipoprotein with murein hydrolase activity, has been shown to impair the integrity of the cell envelope and increase the release of GMMA (68).
No growth defects or obvious loss of membrane integrity have been detected in production strain lacking *tolR* (66,84). However, deletion of *gna33* (in *Neisseria*) resulted in slightly slower growth than the wild-type.
Another challenge for GMMA production at industrial scale was the identification of an efficient purification method. A widely used methods at research scale is the separation of GMMA from the bacterial cells by low speed centrifugation, in which the outer membrane particles remain in the culture supernatant, followed by collection of the particles by using ultrafiltration (85). Also alternative methods have been tested, e.g. purification over a sucrose density gradient or gel filtration chromatography, resulting in vesicles of high purity and being relatively homogeneous. Those systems were based on differential diffusion and pore size exclusion, but the use of ammonium sulfate in the precipitation of OMVs before density gradient centrifugation can lead to artifacts (72).
NVGH has developed a new method to collect GMMA from bacterial suspensions, by using two consecutive Tangential Flow Filtration steps: the first one with 0.22 µm to separate the biomass from the GMMA, and the second one with a smaller cut-off for puryfing and concentrating GMMA (66), thus resulting in an high yield process.

Reactogenicity

Innate immune stimulation, Pathogen Associated Molecular Patterns and Toll-like receptors

Usually, when occurs infections or tissue damages, the activation of innate immunity takes place within minutes and lasts for several days with the purpose of recognizing and clearing most of the microbes or damaged tissue. The adaptative immune response, instead, follows the innate ones and peaks within the following weeks, resulting in an immunological memory that can last for the life of the individual. Induction of adaptive immune responses and especialy the immunological the memory is the response desired from vaccination.
sion of pathogenic infectious agents are generally initiated via host recognition of conserved molecular structures, known as Pathogen-Associated Molecular Patterns (PAMPs). PAMPs include molecules generally present in bacteria, including lipoproteins and the LPS of Gram-negative bacteria, but also components, necessary for the survival of specific pathogens in the host, e.g. flagella (86). These PAMPs are sensed by receptors, called Pattern Recognition Receptors (PRRs), which are predominantly expressed on innate immune cells such as dendritic cells, macrophages and neutrophils. The interactions of PAMPs with PRRs rapidly induces host immune responses via the activation of complex signaling pathways that culminate in the induction of inflammatory responses mediated by various cytokines and chemokines, which subsequently facilitate the eradication of the pathogen (87).
There are several classes of PRRs, such as Toll-Like Receptors (TLRs), RIG-I-Like Receptors (RLRs), NOD-Like Receptors (NLRs) and DNA receptors (cytosolic sensors for DNA), that sense various classes of pathogen's molecules including proteins, lipids, carbohydrates and nucleic acids (88).
TLRs are the most widely studied PRRs, and were first discovered in *Drosophila* as proteins involved embryonic development. Since then, many homologous proteins

have been discovered in different organisms and, today, in humans, 10 TLR family members have been identified and 12 in mice (TLR1 to 9 are conserved in both humans and mice). TLR 1, 2, 4, 5 and 6 are primarily expressed on the cell surface and recognize PAMPs derived from bacteria, fungi and protozoa (Fig.2), whereas TLR 3, 7, 8 and 9 are exclusively expressed within endocytic compartments and primarily recognize nucleic acid PAMPs derived from various viruses and bacteria (87).

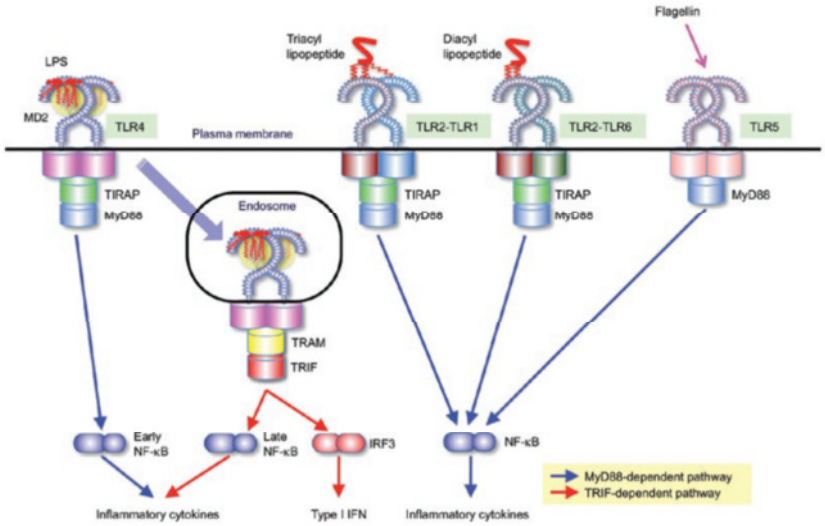

Fig. 2 Interaction between various PAMPs and TLRs exposed on cell surface. Picture from (87)

TLRs are type I membrane glycoproteins that consist of an extracellular Leucine Rich Repeats (LRRs), required for PAMP recognition, and a cytoplasmic TIR domain (that consist in 150 aminoacids and show homology with Interleukin-1 receptor), required for downstream signaling. TLR signaling is primarily meditated via the recruitment of different TIR domain containing adaptor molecules such as MyD88, TRIF, TIRAP, and TRAM to the TIR domains of the different TLRs. Recruitment of these adaptor molecules activates various transcription factors such as NF-κB, IRF3/7, and MAP kinases to induce the production of pro-inflammatory cytokines (for example IL-6) and type I interferons (86). Each TLR recognizes only specific PAMPs.

Needs of reduction of endotoxicity for using GMMA in vaccination

As GMMA are derived from the outer membrane of Gram-negative bacteria, they naturally contain high levels of PAMPs, for example lipopolysaccharide (LPS), and could trigger a strong stimulation of the innate immune response that could potentially result in unacceptable reactions in human subjects, e.g. a febrile response, or in extreme cases, septic shock (89), especially if parenterally administered. Thus, the reactogenicity of GMMA, especially the LPS endotoxicity, might need to be modified. However, limited activation of the innate immune response will aid the immune response to the vaccine and thus, a balance of triggering immune stimulation and reactogenicity is desired in a vaccine (90). Considering the composition of GMMA, the main TLRs activated in the host are likely TLR4 and TLR2 (91). TLR4 is the receptor involved in the recognition of LPS with MD-2, CD14 and LBP (87,92). TLR2 is involved in the recognition of a wide range of PAMPs that include lipoproteins (di- or tri-acetylated) (87).
LPS very likely is the major reactogenic component that is present in outer membrane of Gram-negative bacteria and thus in GMMA. Its structure is amenable to

genetic modification to reduce the activation of the TLR4 pathway. Thus, to asses the feasibility of reduction of GMMA reactogenicity, at NVGH, reduction of LPS endotoxicity was addressed first.
Shigella and *Salmonella* has previously been reported to stimulate TLR2 responses; e.g. porins, lipoproteins, and major outer membrane proteins from *Shigella flexneri* and *Shigella dysenteriae* have been found to stimulate TLR2 (93-97).

LPS

LPS consists of three main regions: the glycolipid lipid A, a core oligosaccharide, and the outer polysaccharide chain (O antigen) consisting of 20-40 repeating units of usually 2-8 sugar molecules (Fig. 3). The OAg, if present (for example is not present in *N. meningitidis*), is the most variable part of LPS and shows even a high degree of variability between different strains of the same species (98). The variation of OAg lies in the sugar composition, the arrangement of the sugars, and the linkages between the sugars within the O unit as well as the linkages between O units (99). OAg is an important component in the resistance to serum-mediated killing, phagocytosis, and to killing by cationic peptides. OAg is highly immunogenic, thus widely used as main antigen in various vacines.

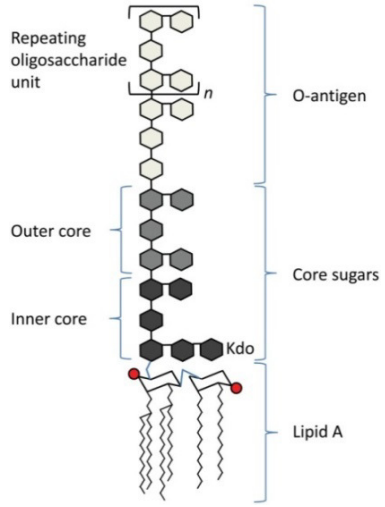

Fig. 3 LPS structure. Picture from (100)

The lipid A, that anchors the LPS to outer membrane of bacteria, is the reactogenic part of LPS and thus the part that can be modified to detoxify the LPS. Lipid A "core structure" is composed by a β-1',6-linked disaccharide of glucosamine phosphorylated at the 1 and 4' positions and acylated at 2, 3, 2' and 3' position with R-3-hydroxymyristate (structure usually called lipid IV A), that is a conserved structure in most of Gram-negative bacteria (101,102). Subsequently, the late acyltransferases HtrB (also called LpxL) and MsbB (also called LpxM) transfer a lauroyl fatty acid to the 3' position (HtrB, (103)) and a myristoyl fatty acid at position 2' (MsbB, (104)). This structure is common to *E. coli* and *Shigella* (101,102) (Fig. 4A).
Exceptions to this this hexa-acylated lipid A structure are present, for example, in the case of *Salmonella enterica,* with the addition of a seventh fatty acid chain to hexa-acylated lipid A by a 2-O-palmitoylation catalized by PagP (105), expressed under certain conditions modulates the bacterial pathogenesis (106) (fig. 4A). Further modifications at the *E. coli* wild type lipid A structure were induced upon cold-shock with the addition of a palmitoleoyl chain in place of lauroyl chain catalyzed by LpxP

(107,108) (Fig. 4A). Other differences in lipid A structure are observed in different species, especially regarding types of fatty acid chains attached on lipid A and addition of phospatidiletanolamine and/or arabinose to 1 and 4' phosphate groups (101). It is the case, for example, of the *Neisseria meningitidis* lipid A, constituted of a β-1',6-linked disaccharide of glucosamine phosphorylated at the 1 and 4' positions and acylated at 2, 2' with R-3-hydroxymyristate and at 3, and 3' position with a R-3-hydroxylauroylate with the presence of secondary lauroyl chains in position 2' and 2' to the hydroximyristate chains. Furthermore the phosphate in 1 position is piro-phosporilated by a phospoetanolamine (Fig. 4B).

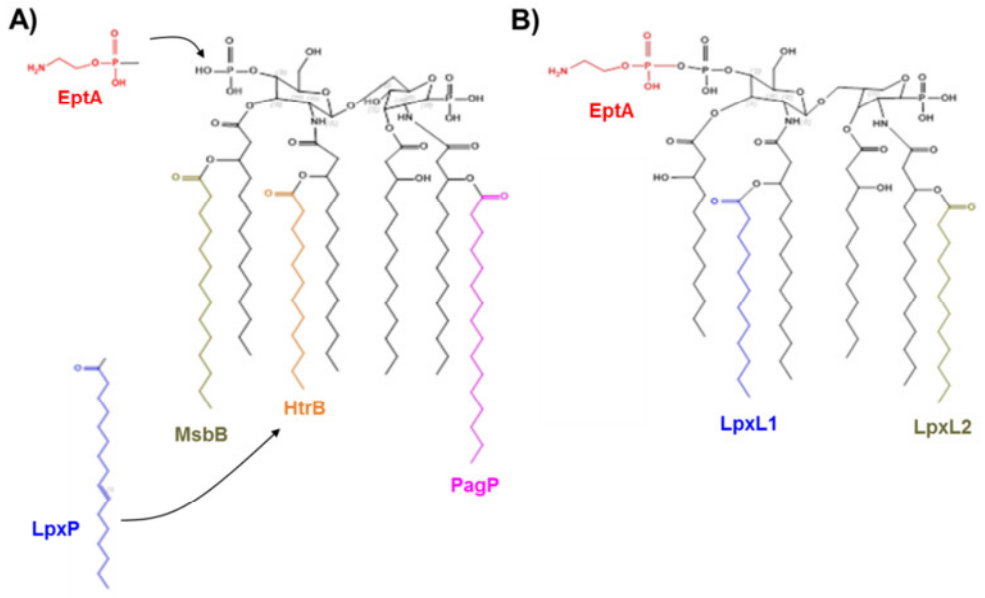

Fig. 4 A) *Shigella* and *Salmonella* type lipid A. B) *N. meningitidis* type lipid A. Lipid A structures usually found and some of enzymes involved in last steps of lipid A biosynthesis.

Strategy for reduction of reactogenicity used

Changes to the structure of the lipid A effect the binding and recognition by TLR4. 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. 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 (109-111). Alteration to this structures, both in terms of lacking or presence of a different, even if chemically related, constituent of the previous structures, results in a lower endotoxicity *in vitro* (109,110).

Modifications in lipid A structure in terms of numbers/types of fatty acid chains present and in terms of changes in type/presence of substituent in the phosphates regions, represents systems used quite extensively by different species of bacteria in response to changes in environmental conditions and are modulated in pathogenesis, helping on the evasion the recognition by TLR4 (109,110,112,113); e.g. a palmitoleoyl fatty acid is incorporated at the 3' position instead of the lauryl chain, catalyzed by LpxP in *E. coli* under cold shock conditions (107,108). Especially pathogenic bacteria use modifications of the lipid A structure as a strategy to evade host recognition (101,114). E.g. induced by the temperature increase from 25℃ in the environment to 37℃ in the host, *Yersinia pestis* produces a tetra-acylated lipid A,

that is poorly recognized by the host's TLR4 (115). Similar strategies have been used naturally by *Helicobacter pylori, Legionella pneumophila* and *Francisella* (101), that naturally possess lipid A moiety that are poorly recognized by human TLR4 helping them to evade host immune response (114). In the case of *Salmonella enterica* has been observed the addition of a seventh fatty acid chain to hexa-acylated lipid A by a 2-O-palmitoylation catalized by PagP in certain environmental conditions (e.g. like different magnesium concentration). The palmitoleoylation has been used also by a wide range of bacteria and is correlated to modulating bacterial pathogenesis (106). Other differences in lipid A, besides the changes of type and/or number of fatty acid chains, have been observed in different bugs, especially regarding addition of phospatidyletanolamine and/or arabinose to 1 and 4' phosphate groups (101).

Similar strategies as observed in pathogenic bacteria have been used with the objective of decreasing LPS reactogenicity. A primary focus has been the modification of the acylation of the lipid A by inactivation of the genes encoding the late acyltransferases HtrB (103) and MsbB (104), resulting in predominantly penta-acylated LPS. As humans are very sensitive to LPS usually the level of reduction of endotoxicity achieved by the modifications has been assessed in *in vitro* assays measuring cytokine release from human peripheral blood monocytes (PBMC), mainly looking at TNF-α and IL-6. It's hard a correlation between results of reactogenicity observed in mice and in humans due to the fact that penta-acylated lipid A acts as antagonist for human TLR4 but as agonist for mouse TLR4 (116), and pure pirogenicity test in rabbits were too expensive and ethically not good for being performed as routine use. Thus, is important to study cytokine response directly in human cells or using cell lines that mimics the human TLR4 recognition system. A strong decrease of lipid A endotoxicity has been observed for both *htrB* and *msbB* inactivation using human cell lines. E.g. inactivation of *htrB* results in 40-fold decrease of TNF-α production in *Haemophilus* (117), whereas inactivation of *msbB* resulted in 10,000-fold decrease of TNF-α production in *E. coli* (118,119).

This approach of generating a lipid A molecule with decreased acylation has been used for the reduction of endotoxicity of live attenuated vaccine strains, e.g. *Shigella flexneri* 2a (120), and has also been tested with NOMV from *Salmonella enterica* (121), *Neisseria meningitidis* by *lpxL1* or *lpxL2* knock-outs (65,81,122-125), and in and *Vibrio cholera* (79) with promising results both in terms of reactogenicity than immunogenicity in mice. Experience with outer membrane particles generated with modified LPS in humans has been obtained in a Phase I clinical trial with NOMV from Δ*lpxL1* (126) and Δ*lpxL2* (127) *Neisserria meningitidis* mutants. The NOMV with penta-acylated lipid A showed 100-fold reduced by in vitro TNF-α, 200-fold reduced by pyrogenicity, good adjuvanticity (65) and was well tolerated and immunogenic in a Phase I clinical trial (126). Those clinical studies confirm the good correlation between *in vitro* results (65) about reduction of endotoxicity and safety in humans (126,127).

We have described the high yield production process for GMMA from *Shigella* based on *tolR* knock out and demonstrated the feasibility of using this process with a modification of the lipopolysaccharide structure (128). After lipid A modifications usually the ability to grow of bacteria in rich media (129) and at 37°C is reduced (128,130), with also other modifications like suppressor mutations (129,131) have been observed and a general increased sensitivity to antibiotics (108) and the induction of sigma E regulon (129,132).

Other modifications of lipid A, besides the deletion of genes that encodes the late acyltrasferases LpxL and LpxM, were analyzed with the objective of decreasing

reactogenicity; some of them included the dephosphorylation of lipid A by knocking-in the dephosphorylases encoded by *lpxE* from *Francisella tularensis* in *Salmonella enterica* (133), or by overexpressing the deacylases enchoded by *lpxR,* causing the cleavage of two fatty acid chains (134,135), or PagL, that acts as 3-O-deacylases (134). Unfortunately both those results didn't gave unsatisfactory results or in terms of decreasing reactogenicity or not complete de-acylation and so presence of lipid A molecules not deacylated in the mixture

Aims of the project

The broad aim of the project is to identify ways of reducing the reactogenicity of GMMA. This broad aim was divided into three specific goals:

1. Proof of feasibility of reduction of GMMA endotoxicity by lipid A modification using *Shigella*.

2. Assessment of relative contribution of TLR4 and TLR2 pathways to stimulation of human cells by GMMA with different lipid A modifications

3. Demonstration of general applicability of the identified methods for the GMMA platform.

The work for goals 1 and 2 is part of the NVGH program to develop a *Shigella* GMMA vaccine. The goal 3 is involved in the development of GMMA-based non-Typhoidal *Salmonella* and *Neisseria meningitidis* vaccines for Africa.

Material and methods

Strains and generation of mutations

Shigella *strains*

Shigella sonnei 53G (136) and *Shigella flexneri* 2a 2457T (137) were chosen as parent strains. The list of *Shigella* mutants strains used in this study and their abbreviated identifications are listed in Tab. 1. As all strains used in this study are GMMA-producing strains (thus lackying tolR gene, substituted by Kn resistance cassette as reported in (66)) the abbreviated names only refer to the additional mutations and characteristics. The *tolR* gene knock-out in both *S. flexneri* and *S. sonnei* strains was achieved as previously described on (128). The curing of virulence plasmid (-p) in *S. sonnei* was achieved as described in (128). 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 gene *ospD3* using PCR. The primers are listed in Tab 2. To generate the *tolR* deletion in *S. flexneri* 2a and plasmid-cured *S. flexneri* 2a –pINV the same strategy and primers as previously described for the generation of the *S. sonnei* Δ*tolR* mutant (138) were used.
The null mutation of *msbB1*(120), *htrB* (103) and *rfbG* (essential for O antigen biosynthesis in *S. flexneri*, (139)) were obtained by replacing the gene of interest (*gene)* with an antibiotic resistance cassette using the following strategy. The upstream and downstream regions of *gene* of interest 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 upstream flanking region and the 3'end of the downstream flanking region of *gene* (see Tab. 2) and used to transformed recombination prone *tolR*-deletion strains of *S. sonnei* or *S. flexneri* as previously described (128). In *S. sonnei*, the *htrB* gene was replaced by the chloramphenicol resistance gene *cat* (140). *S. sonnei* -p Δ*tolR* Δ*msbB* was obtained as described in (128). In *S. flexneri*, *msbB1* and *htrB* were replaced by *cat* and *rfbG* was replaced by the erythromycin resistance gene *erm* (141). In the *rfbG* knockout, also the flanking genes *rfbF* and *rfc* (139) 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*, (120)). For simplicity, the mutant is referred to an Δ*msbB*.
To complement strains carrying the *htrB* deletion, the *htrB* gene was amplified from *Shigella sonnei* 53G including 239 bp upstream and 172 bp downstream using primers P1.htrBcompl-EcoRI and P2.htrBcompl-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* Δ*htrB* cells.

Primer name	Sequence 5' → 3'
htrB-U1 Xba Sma	CTAGTCTAGAAACCCGGGCAATTGTATGTATTGTCG
htrB-soU2 SacI	ACTCGAGCTCCCGTCATCATCCAACGC
htrB-flexU2 SacI	ACTCGAGCTCATCCGATATACGTTCGCCC
*htrB-so*D1 SalI	ACGCGTCGACCTCAGTAATCAGGGTTCTTTG
*htrB-so*D2 SmaI	CTAACCCGGGTAAATCTCCCCTGCCGGATG
htrB-flexD1 SalI	ACGCGTCGACCCTGTAATCTCAGGTCAAATG
htrB-flexD2 SmaI	CTAACCCGGGTAAATCTCCCATGCCGGATG
*msbB*flex-U5 Sma	CTAGTCTAGAAACCCGGGTGATAGTGTAGCGGCACA
*msbB*flex-U3 Sac	ACTCGAGCTCGTGAGCAAAGCCAGCTG
*msbB*flex-D5 SalI	ACGCGTCGACCTCGGTGTGGAAATTGG
*msb*Bflex-D3 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
virGup -5 Sac	ACTCGAGCTCTGTAGTTGATTTGACAGTTGACATCC
virGup-3 Sma	CTAACCCGGGCACTATATTATCAGTAAGTGGTTGATAAACC
virGdown-5 Sma	CTAACCCGGGCGTGTTGATGTCCTGC
virGdown-3 Sal	ACGCGTCGACAGTTCAGTTCAGGCTGTACGC
wzz-5 Sac	ACTCGAGCTCGGCAGACTCAGCGCAAG
wzz-3 Sma	CTAACCCGGGCATTGACACAACAATACGTAACCCAG
wgbZ-5 Sma	CTAACCCGGGTGCGATTTGGTAATGTACTCGG
wgbZ-3 SalI	ACGCGTCGACATTGCTCGCTTGTGATAACAGC
nadA-5 Sma	CTAACCCGGGCAAGCAACTCTATGTCGGTGGAAT
nadA-3 Hind	TATCAAGCTTGGCAAGGCCAATACACAGC
nadB-5 Hind	TATCAAGCTTAGGGTTAGAGTGTCTCGTTTTTG
nadB-3 Sma	CTAACCCGGGCCAGACCAGAACTATTCC
Ori-1	CGGCATCAGAATAATACAAGCAGC
Ori-2	AGGTGTACCGTGCTCTGGG
ospD3-1	GTTTTGCCTCATTCAAGATATCACC
ospD3-2	TGACGATGGTTTGTCAGGATTGC
lysP.F	CCGAAACTAAAACTACAGAAGCGC
lysP.R	CCTTCTTCAACATAGTTCTGACCG
msbB.F	CGCCAAAGTTCCGTGATCCCATT
msbB.R	CTCTTCGATGATCTCCAGCCCTT
lpxP.F	GGCTTTGGGTACAGCTTCCTTA
lpxP.R	CCAACCCTTCAACATCAAACC

Tab. 1 List of primers used.

During cultivation *in vitro*, *S. sonnei* spontaneously loses the virulence plasmid giving rise to phase II OAg deficient colonies (142). In order to avoid the loss of the pSS and consequently of the ability to synthetize the OAg repeating units, a mutant selectable for the presence of the pSS was produced by replacement of the plasmid-encoded virulence gene *virG* (143) in *S. sonnei* Δ*tolR* with the genes *nadA* and *nabB*

from *E. coli* (144). These genes encode intermediate steps in the nicotinic acid biosynthesis that are defective in wild-type *Shigella sonnei*. Inserting *nadA* and *nadB* into the virulence plasmid allows the strain to grow without exogenous nicotinic acid. Thus, growth in chemically defined media without nicotinic acid serves as selection for bacteria containing the plasmid. Similarly, a *S. sonnei* O antigen deficient strain still carrying the virulence plasmid was obtained by replacement of the plasmid-borne gene cluster encoding the biosynthesis of the O repeating units from gene *wzz* to *wbgZ* with the genes *nadA* and *nabB* from *E. coli* in *S. sonnei* Δ*tolR* to yield *S. sonnei* Δ*tolR* Δ*wbg*.

Strain name abbreviation	Genotype	OAg	LPS mod*	Ref
Ss$_{-p\text{-}OAg}$	*S. sonnei –pSS* Δ*tolR*	-	-	(128)
Ss$_{-p\text{-}OAg}$ Δ*msbB*	*S. sonnei* –pSS Δ*tolR* Δ*msbB*	-	+	(128)
Ss$_{-p\text{-}OAg}$ Δ*htrB*	*S. sonnei –pSS* Δ*tolR* Δ*htrB*	-	+	This study
Sf$_{-p\text{-}OAg}$	*S. flexneri* 2a –pINV Δ*tolR* Δ*rfbG*	-	-	This study
Sf$_{-p\text{-}OAg}$ Δ*msbB*	*S. flexneri* 2a –pINV Δ*tolR* Δ*rfbG* Δ*msbB*	-	+	This study
Sf$_{-p\text{-}OAg}$ Δ*htrB*	*S. flexneri* 2a –pSS Δ*tolR* Δ*rfbG* Δ*htrB*	-	+	This study
Ss$_{-p\text{-}OAg}$ Δ*htrB* (pACYC*htrB*)	*S. sonnei* –pSS Δ*tolR* Δ*htrB* (pACYC*htrB*)	-	-	This study
Sf$_{-p\text{-}OAg}$ Δ*htrB* (pACYC*htrB*)	*S. flexneri* 2a –pINV Δ*tolR* Δ*rfbG* Δ*htrB* (pACYC*htrB*)	-	-	This study
Sf$_{+p\text{-}OAg}$	*S. flexneri* 2a +pINV Δ*tolR* Δ*rfbG*	-	-	This study
Sf$_{+p+OAg}$	*S. flexneri* 2a +pINV Δ*tolR*	+	-	This study
Sf$_{-p+OAg}$	*S. flexneri* 2a –pINV Δ*tolR*	+	-	This study
Sf$_{+p+OAg}$ Δ*htrB*	*S. flexneri* 2a +pINV Δ*tolR* Δ*htrB*	+	+	This study
Sf$_{-p+OAg}$ Δ*htrB*	*S. flexneri* 2a –pINV Δ*tolR* Δ*htrB*	+	+	This study
Sf$_{+p+OAg}$ Δ*htrB* (pACYC*htrB*)	*S. flexneri* 2a +pINV Δ*tolR* Δ*htrB* (pACYC*htrB*)	+	+	This study
Sf$_{-p+OAg}$ Δ*htrB* (pACYC*htrB*)	*S. flexneri* 2a –pINV Δ*tolR* Δ*htrB* (pACYC*htrB*)	+	+	This study
Ss$_{+p\text{-}OAg}$	*S. sonnei* +pSS Δ*tolR* Δ*wbg*	-	-	This study
Ss$_{+p+OAg}$	*S. sonnei* +pSS Δ*tolR* Δ*virG*	+	-	This study
Ss$_{+p+OAg}$ Δ*htrB*	*S. sonnei* 2a +pINV Δ*tolR* Δ*virG* Δ*htrB*	+	+	This study
Ss$_{+p+OAg}$ Δ*htrB* (pACYC*htrB*)	*S. sonnei* 2a +pINV Δ*tolR* Δ*virG* Δ*htrB* (pACYC*htrB*)	+	+	This study
Sf$_{-p+OAg}$ Δ*msbB*	*S. flexneri* 2a -pINV Δ*tolR* Δ*msbB*	+	+	This study
Sf$_{3a}$	*S. flexneri* 3a +pINV Δ*tolR*	+	-	This study
Sf$_{3a}$ Δ*htrB*	*S. flexneri* 3a +pINV Δ*tolR* Δ*htrB*	+	+	This study
Sf$_{6}$	*S. flexneri* 6 +pINV Δ*tolR*	+	-	This study
Sf$_{6}$ Δ*htrB*	*S. flexneri* 6 +pINV Δ*tolR* Δ*htrB*	+	+	This study

Tab. 2 List of *Shigella* strains used in this study and their abbreviations. + means presence and – means absence of the characteristic. *LPS mod, lipid A of LPS modified; -pSS, -pINV, cured of virulence plasmid; +pSS, +pINV, virulence plasmid present

The mutations were generated using the same strategy as above with the execption that instead of the antibiotic resitance cassette the genes *nadA* and *nadB* (144) from *E. coli*, were used to replace the gene of interest. The upstream and downstream regions of *virG* were amplified using primer pairs virGup-5/virGup-3 and virGdown-5/virGdown-3. For the OAg knock out, internal fragments of the genes *wzz* and *wbgZ* were amplified by PCR using primers wzz-5_Sac, wzz-3_Sam, and wbgZ-5_Sma and wgbZ-3_Sal, respectively. The "*nadAB*" cassette was generated by amplifying *nadA* and *nadB* from *E. coli* using primers nadA-5/nadA-3 and nadB-5/nadB-3. The fragments were inserted into pBluescript (Stratagene) so that *nadA* and *nadB* were linked and interposed the flanking regions of the gene of interest.

Salmonella *strains*

Salmonella enterica serovar Typhimurium D23580 is an invasive strain isolated in Malawi in 2004 (145). The double mutant Δ*tolR* Δ*wbaP* of D23580, lacking the O-Ag repeating unit (O:4,5), was used in this study. First the rough Δ*wbaP* mutant of D23580 was constructed by homologous recombination as described by Datsenko and Wanner (146), replacing *wbaP* coding sequence with a kanamycin resistance (Km^r) cassette. Briefly, we used one-step PCR protocol to fuse the 50 bp *wbaP* upstream and downstream regions to the Km^r gene using the primer pair *wbaP1/wbaP2* (Tab.2) and the plasmid pKD4 as template. The linear fragment was then used to transform the recombination-prone D23580 strain (carrying the helper plasmid pSIM18) (147) and *wbaP* mutants were selected by plating transformed bacteria on Luria-Bertani (LB) plates containing 25 μg/ml kanamycin. Afterwards, tolR knock-out gene was performed following the same procedure. *tolR* coding sequence was replaced with a chloramphenicol resistance (Cmr) cassette using the plasmid pKD3 as template and the primer pair tolRP1/tolRP2 (Tab. 3). *tolR* mutants were selected by plating transformed bacteria on LB plates containing 20 μg/mL chloramphenicol.

Identification	Strains genotypes	Lip.A modified	OAg	Reference
S.Tm	*S.* Typhimurium 1418 Δ*tolR*	-	+	This study
S.Tm Δ*htrB*	*S.* Typhimutium 1418 Δ*tolR* Δ*htrB*	+	+	This study
S.Tm Δ*msbB*	*S.* Typhimutium 1418 Δ*tolR* Δ*htrB*	+	+	This study
S.En	*S.* Enteritidis 618 Δ*tolR*	-	+	This study
S.En Δ*htrB*	*S.* Enteritidis 618 Δ*tolR* Δ*htrB*	+	+	This study
S.En Δ*msbB*	*S.* Enteritidis 618 Δ*tolR* Δ*htrB*	+	+	This study
Salmonella	*S.* Typhimurium D23580	-	-	This study

Tab. 3 List of *Salmonella* strains used in this study and their abbreviations. + means presence and – means absence of the characteristic.

Salmonella enterica serovar Typhimurium 1418 and *Salmonella enterica* serovar Enteritidis 618 are invasive strains isolated from animal source. Different *Salmonella* mutants were obtained by homologous recombination as described by Datsenko and Wanner (146), replacing target gene coding sequence with an antibiotic resistance cassette. *Salmonella enteritidis* spp. mutants with Δ*tolR* and Δ*htrB or* Δ*msbB* in different combinations were generated (stains and abbreviations are listed in Tab. 3). To generate the knock-out mutants, transformants were selected on LB agar plates

containing 30 µg/mL kanamycin (*tolR* mutant), 20 µg/mL tetracyclin (*msbB* mutant) and 20 µg/mL chloramphenicol (*htrB* mutant).

Neisseria *strains*

Different *Neisseria meningitidis* W-135 mutants carrying Δ*cps,* Δ*lpxL1,* Δ*gna33,* or overespressinf factor H binding protein (OE fHbp) in different combinations were generated (mutants and their abbreviations are listed in Tab. 4). Deletion of *lpxL1* and *gna33* (68,148) was performed by insertional inactivation as described previously (149). Deletion of the capsular biosynthesis was performed by replacement of a DNA fragment containing *synX*, *ctrA* and the intergenic promoter that drives transcription of the capsular biosynthesis and transport operon with a chloramphenicol resistance gene (149). OE fHbp was obtained by insertion of fHbp gene (150) under the regulation of a strong syntetic promoter. To generate the knock-out mutants, *N. meningitidis* was transformed with 0.5 to 1 ug linearized plasmid DNA and transformants were selected on GC agar plates containing 80 ug/mL kanamycin (*lpxL1* mutant), 5 ug/mL erythromycin (*gna33* mutant) and 5 ug/mL chloramphenicol (capsular KO mutant).

Identification	Strains genotypes	Lip. A modified	Reference
Nm	*N. meningitidis* Δ*cps*	-	This study
Nm Δ*fHbp*	*N. meningitidis* Δ*cps* Δ*fHbp*	-	This study
Nm Δ*fHbp* OE fHbp	*N. meningitidis* Δ*cps* Δ*fHbp* OE fHbp	-	This study
Nm Δ*fHbp* Δ*lpxL1*	*N. meningitidis* Δ*cps* Δ*fHbp* Δ*lpxL1*	+	This study
Nm Δ*fHbp* Δ*lpxL1* OE fHbp	*N. meningitidis* Δ*cps* Δ*fHbp* Δ*lpxL1*OE fHbp	+	This study
Neisseria	*N. meningitidis* Δ*fHbp* Δ*lpxL1* Δ*gna33*	+	This study

Tab. 4 List of *Neisseria* strains used in this study and their abbreviations. + means presence and – means absence of the characteristic.

Growth conditions

Shigella bacterial strains were routinely grown at 30°C in liquid or on solid M9 medium supplemented with nicotinic acid (Na_2HPO_4 7 g/L, KH_2PO_4 3 g/L, NaCl 0,5 g/L, NH_4Cl 1 g/L, $MgSO_4$ 1M 2mL/L, $CaCl_2$ 1M 0,1 mL/L, glucose 0,4%, nicotinic acid 0,01 g/L) or in chemically defined medium (SDM), with the same composition to the previously described SSDM (128) with the exception of the carbon source: KH_2PO_4 13,3 g/L, $(NH_4)_2HPO_4$ 4 g/L, Citric acid 1,7 g/L, L-aspartic acid 2,5 g/L, D-Glucose 15 g/L, $CoCl_2$ $6H_2O$ 0.0025 g/L, $MnCl_2$ $4H_2O$ 0.015 g/L, $CuCl_2$ $2H_2O$ 0.0015 g/L, H_3BO_3 0.003 g/L, Na_2MoO_4 $2H_2O$ 0.0025 g/L, $Zn(CH_3COO)_2$ $2H_2O$ 0.0025 g/L, Ferric citrate 2 µM, $MgSO_4$ 2 mM, Thiamine 0.05 g/L, Nicotinic acid 0.01 g/L, 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.
Salmonella bacterial strains were grown at 30/37°C in Lauria Bertani (LB) liquid coltures with the addition in the pre-coltures of the required antibiotics.
Neisseria bacterial strains were grown at 37°C in liquid coltures in meningococcus chemically-defined medium (MCDMI) with the addition in the pre-coltures of the required antibiotics.

Production and purification of GMMA

Preparation of GMMA from Shigella *strains*

For GMMA production overnight cultures were grown in 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℃ and 200 rpm overnight. Culture supernatants were collected by 10 min centrifugation at 5,000g followed by 0.22 µm filtration. GMMA were concentrated using an Amicon stirrer cell with a regenerated cellulose filter with a nominal molecular weight limit of 100 kDa (Amicon Ultracell) under nitrogen flow. The retentate was collected in 70 mL ultracentrifuge propylene tube (Beckman Coulter) and ultracentrifuged at 186,000 g using 45Ti rotor (Beckman Coulter) for 2 hours at 4℃. Pellets were resuspended in 4 mL of PBS followed by 0.22 µm filtration. GMMA were stored at 4℃.

Preparation of GMMA from Salmonella *strains*

5 mL of LB was inoculated with single colonies of Salmonella enterica serovar Typhymurium ΔOAg and incubated at 30℃ over-night in presence of selective antibiotic. The starter culture was used to inoculate 70 mL LB to OD=0.05 and the culture was incubated at 30℃ over-night (approximately 15 hours). The culture was centrifuged and the supernatant was sterilized by filtration through a 0.22 µm filter. To collect the GMMA the sterile culture supernatant was centrifuged (186,000 g, 2 hours, 4℃), the membrane pellet was washed once with PBS and after a second centrifugation step the GMMA pellet was resuspended in 1X PBS and was sterilized by filtration through a 0.22 µm filter.

Preparation of GMMA from N. meningitidis *strains*

7 mL of Meningitis chemically defined medium I (MCDMI) was inoculated with single colonies to OD = 0.15 to 0.17 and incubated at 37℃, 5% CO_2 to mid log phase (OD=0.6). The starter culture was used to inoculate 50 mL MCDMI to OD=0.05 to 0.1 and the culture was incubated at 37℃, 5% CO2, 185 rpm until stationary phase (approx. 9 hours). The culture was centrifuged and the supernatant was sterilized by filtration through a 0.22 µm filter. To collect the GMMA the sterile culture supernatant was centrifuged (186,000 g, 2 hours, 4 ℃), the membrane pellet was washed once with PBS and after a second centrifugation step the GMMA pellet was resuspended in 1X PBS and was sterilized by filtration through a 0.22 µm filter.

Protein quantification of GMMA

Standard curve for the assays were prepared with bovine serum albumin (Pierce) in the range 1-10 µg/assay for Bradford assay and 4-50 µg/assay in both Lowry and Non-Interfeering assays.

Bradford assay

Bradford Protein Assay (Bio-Rad) kit is used following the producers specifications. Prior quantification GMMA were diluted 1:2 with 6.0 M guanidine hydrochloride pH 7.8 (vol/vol) and boiled for 10 minutes vortexing occasionally. Different quantities of

samples were adjusted to a final volume of 200 µL with water. 800 µL of Bradford reagent concentrate (5X) diluted with water were added to each sample. Absorbances were measured at 595 nm after 30 minutes.

Lowry assay

Detergent Compatible (DC) Protein assay (Bio-Rad) is used following the producers specifications. Different quantities of GMMA were adjusted to a final volume of 25 µL with water. 125 µL of Reagent A' (prepared adding 20 µL of reagent S to every mL of reagent A) were added and samples were vortexed. 1 mL of reagent B was added and absorbances were read at 750 nm after 15 minutes.

Non-Interfering protein assay (NI assay)

For the assay use the standard procedure reported in the NI manual (G-bioscience, USA).
Different quantities of GMMA were adjusted to a final volume of 25 µL with water. 500 uL of Universal Protein Precipitation Agent (UPPA) I was added, incubated for 2 minutes and vortexed. Afterwards 500 µL of UPPA II was added and vortexed. Samples were centrifuged for 5 minutes at 10,000 g and supernatants were discarded by inverting the tube. Sample were centrifuged a second time for 5 minutes at 10,000 g and supernatant was discarded by using a pipette. 500 µL of reagent 1 (prepared adding 400 µL of water to 100 µL of Copper solution) were added to each sample and resuspend vortexing. 1 mL of reagent 2 was added (prepared adding 1 part of Color reagent B every 100 part of Color reagent A) and Abs was read at 480 nm after 15 minutes (using water as blank).

Quantitative aminoacid hydrolysis analysis

Aminoacid hydrolysis analysis of GMMA was performed by AltaBioscience (Birmingham, UK) using an ion exchange separation of aminoacid followed by post column detection with ninidrine [1;50].

SDS-PAGE

GMMA were denatured for 3 min at 95°C in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% (wt/vol) SDS. GMMA were loaded onto 12% (wt/vol) polyacrylamide gels (BioRad, Hercules, U.S.A.). Gels were run in 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (BioRad) and were stained with Coomassie Blue Stain (Sigma-Aldrich) following the data sheet's procedures for proteins.

Negative Staining Trasmission Electron Microscopy

A drop of 5 µL of GMMA suspension prediluted 100 µg/mL was placed onto 300 mesh copper formvar/carbon-coated grids and adsorbed for 5 min. Grids were then washed with few drops of distilled water and blotted with a Whatman filter paper. For negative staining, grids were treated with 2% uranyl acetate in ddH_2O for 1 min, blotted with Whatman filter paper and air-dried and observeded 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 87,000 – 105,000 X.
The non-parametric Kruskal-Wallis test has been used to statistically evaluate GMMA dimensions (with n=30 per strain).
For bacteria 5 µL of liquid colture were prediluted to O.D.=0,1 in PBS and were placed on copper formvar/carbon-coated grids and adsorbed for 5 min. Afterwards the drop was dried using a Whatman filter paper and 5 µL of formaldeyde (sigma Aldrich) 2% (in PBS) were added and incubated 5 min. The residual drop were dried using Whatman filter paper and observeded with a CM100 transmission electron microscope (Philips, Eindoven, the Netherlands) operating at 80 kV.

Monocyte Activation Test (MAT)

PBMC isolation and freezing

Buffy coat from different donors were used to isolate PBMC by Ficoll (Amersham Pharmacia Biotec, cat N° 17-1440-03) density centrifugation and following protocol reported in (123).
PBMC were frozen resuspending in freezing medium (heat-inactivated FBS (Fetal Bovine Serum), 10%DMSO) at a consentration of $5x10^7$ cells/cryovial. Cryovials were prepared dispensing 1 mL aliquots of cell suspension in pre-cooled 2 mL cryovials marked with subject's code and were immediately transferred into the Cell Freezing Box (Nalgene) and stored at –80°C for 24-48h and then transfered in Liquid Nitrogen tank.

PBMC thawing and stimulation

For thawing, each cryovial conserved in liquid nitrogen was warmed at 37°C in water bath gently shaking until a small, pea-sized pellet of ice remained. 1 mL of thawing medium (PBS without Ca^{2+} and Mg^{2+}, with 2.5 mM EDTA and 20 µg/mL DNAse (Sigma, 20 mg/mL stock) warmed at 37°C was added directly into each cryovial; thus, the cell suspension was added to a 40 mL of warm thawing medium. Each cryovial was rinsed with 1 mL of fresh thawing medium to collect residual cells and samples were centrifuged at 320 g for 10 minutes at room temperature. Supernatants were discarded by gently inverting the tube and the pellet of cells were washed with 40 mL of pre-warmed thawing medium and centrifugation and discarding of supernatants were repeated. Pellet cointaining PBMCs was diluted in adequate volume of medium RPMI-1640 complete (with 25 mM HEPES, glutammine, 10% Fetal Bovine Serum (FBS) + 1% Antibiotics Penicilline-Streptomicine (InvitroGen)).
For the stimulation PBMC were cultured at a density of 2×10^5 cells/well with 180 µL of medium RPMI-1640 complemented with 25 mM HEPES, 2 mM glutamine, 10% FBS, 1% Pen-Strep (InvitroGen) in 96-well round bottom plates. After incubation at 37°C for 30 min, 20 µL of 10-fold serial dilutions of in PBS (0.0001-1,000 ng/mL final concentration in the assay) were added. In blocking experiments, 2.5 µg/mL of TLR4 blocking antibody (e-Bioscience) and/or 1.5 µg/mL of TLR2 blocking antibody (e-Bioscience) in RPMI were added for 30 minutes before the addition of GMMA. Cells were incubated for 4 hours at 37°C and supernatants were recovered after centrifugation of the plates at 400 g and stored at -80°C until analysis.

Cytokine analysis by ELISA and 7-plex Mesoscale

For IL-6 detection by ELISA Nunc MaxiSorp 96-well plates were coated overnight at 4℃ with 2 µg/mL human IL-6 capture antibody (eBioscience 14-7069) in PBS, subsequently washed with PBS with 0.05% Tween 20 (PBS-T), blocked for 1 h with PBS with 1% BSA at room temperature (RT) and washed 3-times with PBS-T. 50 µL of supernatants from PBMC experiments, diluted 1:4 with PBS, were incubated for 2 h at RT. A two-fold dilution series of recombinant human IL-6 (eBioscience 39-8069) of 31.24 pg/mL to 4,000 pg/mL in RPMI with 10% FBS was included as standard curve on each plate (in duplicate). Plates were washed with PBS-T. Bound IL-6 was detected using 2 µg/mL of biotin-conjugated anti-human IL-6 (e-Bioscience 13-7068) in PBST with 0.1% BSA for 2 h at RT, followed by 3 washes with PBS-T, 20 min incubation at RT with streptavidin-horse radish peroxidase (R&D Systems, DY998) diluted 1:200 in PBS-T with 0.1% BSA, 3 washes with PBS-T, and a color reaction with 100 µL/well of substrate (R&D Systems, DY999) for 8 min at RT in the dark. The reaction was stopped by adding 50 µL/well of 12.5% sulfuric acid. The plates were read at 450 nm and 630 nm and the $OD_{450nm-630nm}$ was determined. IL-6 concentrations in the samples were calculated in comparison to the IL-6 standard using a four parameter logistic curve and plotted against the amount of GMMA in the sample. Results below the detection limit were assigned half of the detection limit. The average of the IL-6 release at the lowest concentration of GMMA that did not trigger activation was defined as background level. For comparison of the stimulatory activity of GMMA, the amounts of GMMA needed to obtain a 10-fold increase of IL-6 release over background were determined from the generated curves.

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 to the preloaded standard in the plates. The average of the cytokine release at the lowest concentration of GMMA that did not trigger activation was defined as background level. For comparison of the stimulatory activity of GMMA, the amounts of GMMA needed to obtain a 10-fold increase of cytokines released over background were determined from the generated curves.

The average cytokine release at the lowest concentration of GMMA that did not trigger activation was defined as background level. For comparison of the stimulatory activity of GMMA, the amounts of GMMA needed to obtain a 10-fold increase of IL-6 release over background were determined from the generated curves. The non-parametric 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, was calculated as to normalize between the different experiments using PBMC from different donors. The ratio was determined for each replicate in the experiments. The non-parametric Wilcoxon signed rank test was used to assess if the obtained ratios were significantly different form 1 (no effect by blocking).

NF-kB luciferase reporter assay

TLR-specific activation assays were performed using Human Embryonic Kidney 293 (HEK293) cells expressing luciferase under control of the NF-kB promoter and stably transfected with either TLR4, MD2, and CD14 (TLR4-HEK293) or TLR2 (TLR2-HEK293) (151). HEK293 transfected cells were maintained in DMEM medium complemented with 4,5 g/L of glucose and HEPES (Invitrogen), 10% fetal bovine serum (FBS) 1% penicillin-streptomycin solution (Pen-Strep, 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-kB luciferase assay 25,000 cells/well were seeded in 90 µL of complete DMEM medium without antibiotics in 96-well microclear luciferase plates (PBI international) and incubated for 24 hours at 37℃. 10 µL of serial 5-fold dilutions of GMMA in PBS (0.0001-1,000 ng/mL final concentration in the assay) were added. After incubation for 5 h at 37℃ supernatants were aspired from each well and cells were lysed for 20 minutes at room temperature using 20 µL/well of 1:5 diluted 'passive lysis buffer' (Promega). Produced luciferase was detected using 100 µL/well of luciferase assay reagent (Promega) and emitted light was immediately quantified using a luminometer Lmax II^{384} (Molecular Devices). NF-κB actiation of cells stimulated with GMMA is expressed as fold-increase of emitted light over the average result of PBS-stimulated control cells. Values were plotted as average of duplicates ± SD (NF-kB fold induction). Data were analyzed by Excel and GraphPad Prism. For comparison of the stimulatory activity of GMMA, the amounts of GMMA needed to obtain a 3-fold (TLR4 experiments) or 10-fold (TLR2 experiments) induction of NF-κB were determined from the generated curves. The non-parametric Mann-Whitney test was used to statistically evaluate the results obtained with different GMMA.

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℃ (152). Samples were centrifuged at 14,000 g for 15 min, the pellets 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-Aldrich). 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 (CID) and 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 to the Peptide Standard.

Peptide Mass Fingerprinting (PMF)

Protein spots were excised from the gel and processed as previously described (128). 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 PMF with flexAnalysis (flexAnalysis version 2.4, Bruker Daltonics). Monoisotopic 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 used in (66).

KDO quantitation by semi-charbazide method

Core reducing end KDO (2-keto-3-deoxyoctonate) was quantified using the semicarbazide/High Performance Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC) method as we previously reported (153). 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 g. Supernatants were collected and dried in a speedvac and the pellets were resuspended in water. Samples and a standard of 4 µg/mL to 40 µg/mL KDO ammonium solution (Sigma Aldrich, K2755) were derivatized using semicarbazide and analyzed by HPLC-SEC 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.

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 (OD) of 1 with on-column DNase purification step. Purity of RNA was evaluated by gel and NanoDrop profile. Retrotrascription was performed using Superscript II; for each reaction 2 µg of RNA, 1 µL of 10 mM sNTP mix, 1 µL of random primers (50 ng/µL) and DEPC-treated water to 10 µL were added and incubated at 65°C for 5 min.
9 µL of reaction mix (composed by 2 µL of 10X RT buffer, 4 µL 25 mM $MgCl_2$, 2 µL 0,1M DTT and 1 µL of RNase OUT 40 U/µL) were added to each RNA/primers mixture and incubated at room temperature for 2 min. 1 µL of SuperScript II RT were added to each tube (Negative Control: 1 µL of DEPC-treated water) and incubated at room temperature for 10 min, afterwards at 42°C for 50 min. and reaction was terminated incubating at 70°C for 15 min and then chilling on ice. 1 µL RNase H was added to each tube and incubated for 20 minutes at 37°C. The reaction was purified with QIAquick (Quiagen) PCR purification kit following manufacturer instructions. Quantitative RT-PCR for genes *lysP*, *lpxP*, and *msbB* was performed using 10 ng cDNA, SYBR green kit (Lifetechnologies), and 0.4 µM primers *lysP*.F/*lysP*.R, *lpxP*.F/*lpxP*.R, *msbB*.F/*msbB*.R (sequences of primers reported in Tab. 1) in thermocylcer MX3005P (Stratagene) with 40 cycles (95°C, 15 sec; 60°C, 60 sec). Fold induction was calculated as $2^{-\Delta(\Delta Ct)}$, were ΔCt is the difference between the number of cycle of amplification needed to reach the threshold (fluorescence dRn=0,018) for *lpxP* or *msbB* versus the cycle of amplification of the house-keeping gene *lysP* (154) in same strain or versus the cycle of amplification of the late acyl transferase gene *msbB* of the constitutive lipid A biosynthesis pathway(155) in same strain. Δ(ΔCt) represent the difference in the ΔCt obtained for the same gene in the different strains.

Results

Comparison of different color-based methods to AA hydrolysis for measuring GMMA protein concentration

Establishing a consistent and reproducible method for the quantification of GMMA samples is important in order to use them as vaccine and for comparing them in functional assays. GMMA are conveniently quantified based on protein amount, although other assays, e.g. sugar or lipid content, may be appropriate for specific vaccines. Even in these cases, since protein assays are usually simpler to perform, for in process assays, e.g. during purification or formulation, measuring protein content is preferred.
The quantitative amino acid (AA) hydrolysis is considered a primary method for protein quantification (156), as it is not affected by protein composition and does not need a calibration curve with a standard protein. Routine AA analysis slightly underestimates the actual concentration due to the loss of cysteine and tryptophan during the analysis, but this difference is usually marginal and can be corrected in a more detailed analysis (157). However, analysis based on AA content is slow and therefore not suitable for in-process assays, and for this purpose secondary colorimetric assays using a standard protein are usually preferred and have been used by different groups (66,68,83). Thus, to establish the best method for quantifying GMMA samples, we have compared different commercially available color based protein assays: Bradford assay (158), Lowry assay (159) and Non-Interfering protein assay (NI), a method that involves a protein precipitation step that allows to discard interfering agents already been used for the quantification of GMMA samples (83); for all of them GMMA protein concentration has been evaluated by using their amino acid content as the primary standard compared to the color yield of bovine serum albumin (BSA) as a secondary standard.
The criteria for evaluating the colorimetric assays were: the ease of performing the assay, the reproducibility of the assay, the linearity of the assay, and the comparability of results for GMMA produced from different bacterial lines (*Shigella*, *Salmonella* and *Neisseria*).

Color yield and reproducibility

The quantities of the different GMMA as measured using Bradford, Lowry and NI assays using a BSA standard curve and the reproducibility of the results are listed in Tab. 5. The concentrations of GMMA shown in this table were determined by comparison of the color yield obtained with the GMMA to the color yield of the BSA standard.

	AA quantification [µg/mL]	Measurements on different days by tested assay (average of 3 repetitions) [µg/mL]					Mean [µg/mL]	Standard deviation [µg/mL]	CV % Inter-assay	CV % mean intra-assay	% of AA quantification obtained by tested assay
		1	2	3	4	5					
Bradford assay											
S. sonnei –p ΔOAg	**14200**	3700	3190	3747	4858	4795	**4058**	735	**18,1**	12,7	**28,6**
S. sonnei +p ΔOAg	**5080**	1250	1177	1445	1554	1893	**1464**	283	**19,3**	13,9	**28,8**
S. sonnei +p	**7910**	1650	2086	2129	2375	1951	**2038**	266	**13,0**	11,7	**25,8**
S. flexneri +p ΔOAg	**8310**	3247	2691	2969	2790	3413	**3022**	304	**10,1**	7,6	**36,4**
S. flexneri +p	**5090**	2690	2432	2455	2631	2344	**2510**	145	**5,8**	14,2	**49,3**
S. flexneri –p ΔOAg	**8510**	1545	1298	1718	1880	2258	**1740**	361	**20,8**	14,2	**20,4**
Salmonella	**8170**	1805	2097	2186			**2029**	200	**9,8**	7,0	**24,8**
Neisseria	**656**	338	399	476			**404**	69	**17,1**	11,3	**61,6**
Lowry assay											
S. sonnei –p ΔOAg	**14200**	17024	16309	20844	18043	20630	**18570**	2073	**11,2**	11,2	**130,8**
S. sonnei +p ΔOAg	**5080**	6774	5973	7872	6861	6759	**6848**	676	**9,9**	9,9	**134,8**
S. sonnei +p	**7910**	9758	10650	11405	11911	11681	**11081**	879	**7,9**	5,3	**140,1**
S. flexneri +p ΔOAg	**8310**	10748	10546	12182	10278	12113	**11173**	905	**8,1**	6,5	**134,5**
S. flexneri +p	**5090**	7667	7488	8101	6387	7555	**7440**	635	**8,5**	5,9	**146,2**
S. flexneri –p ΔOAg	**8510**	11350	12490	12221	10385	11681	**11625**	825	**7,1**	8,6	**136,6**
Salmonella	**8170**	11415	10500	10360			**10758**	573	**5,3**	7,2	**131,7**
Neisseria	**656**	1136	1265	1205			**1202**	65	**5,4**	11,4	**183,2**
NI assay											
S. sonnei –p ΔOAg	**14200**	17580	19615	20297	19942	17863	**19059**	1249	**6,6**	7,1	**134,2**
S. sonnei +p ΔOAg	**5080**	3090	5448	3463	7015	6159	**5035**	1704	**33,8**	20,4	**99,1**
S. sonnei +p	**7910**	7950	9755	9911	10995	10648	**9852**	1180	**12,0**	9,9	**124,5**
S. flexneri +p ΔOAg	**8310**	9690	11454	11610	11172	11254	**11036**	772	**7,0**	10,0	**132,8**
S. flexneri +p	**5090**	3400	4627	5412	5221	4849	**4702**	790	**16,8**	18,5	**92,4**
S. flexneri –p ΔOAg	**8510**	10930	12692	11577	11453	10805	**11491**	748	**6,5**	7,2	**135,0**
Salmonella	**8170**	8287	9182	9060			**8843**	485	**5,5**	12,7	**108,2**
Neisseria	**656**	901	880	837			**873**	33	**3,7**	9,8	**133,0**

Tab.5 – Results and analysis of concentrations obtained by different assays

With all of the GMMA, the Bradford assay gave a substantially higher color yield than the equivalent amount of BSA and, without applying a color yield factor, underestimated the protein content by up to 80% (Fig. 5). In addition, the difference between the Bradford results and the quantitative AA hydrolysis results varied for GMMA produced from different species and with different genetic modifications without a clear correlation to phenotypic background tested. For example, in multiple assays, the *S. flexneri* +p ΔOAg consistently gave nearly twice the BSA equivalent as *S. flexneri* –p ΔOAg although they have similar amino acid compositions (Tab. 5) and were tested at similar concentrations.

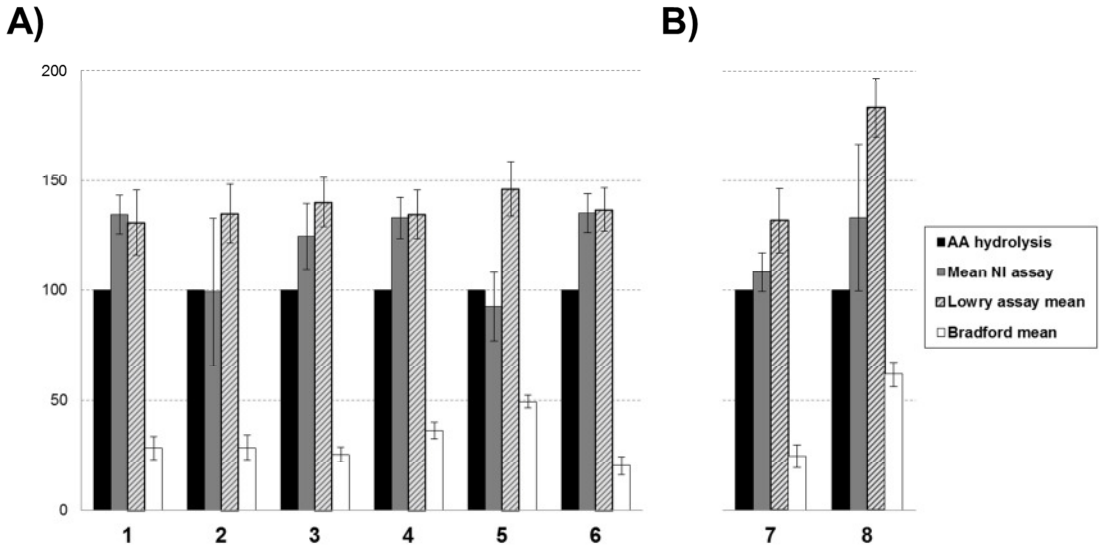

Fig. 5 – Results obtained quantifying GMMA by different assays – related to Tab.5. Values and standard deviations were normalized to percentages measured in comparison to AA hydrolysis. **A)**

GMMA were respectively obtained from: 1. *Ss* –p ΔOAg; 2. *Ss* +p ΔOAg, 3. *Ss* +p; 4. *Sf* +p ΔOAg; 5. *Sf* +p; 6. *Sf* -p ΔOAg. **B)** GMMA were obtained from 7. *Salmonella*; 8. *Neisseria*

By contrast, GMMA assayed by Lowry gave a consistently lower color yield than BSA and in the absence of a color yield factor for the *Shigella* and *Salmonella* GMMA underestimated the protein content of the GMMA by about 35% (Fig. 5). For both the Bradford and Lowry assays, *Neisseria* GMMA gave a lower color yield (i.e. a higher BSA equivalent) than the *Shigella* and *Salmonella* GMMA. The *Neisseria* GMMA also have a substantially different amino acid composition compared to the *Shigella* and *Salmonella* GMMA (Tab. 6).

	***S.flex -p* ΔOAg**		***S.flex +p***		***S.flex +p* ΔOAg**		***S.so -p***		***S.so +p* ΔOAg**		***S.so +p***		***Salmonella***		***Neisseria***	
	nmol/mL	%	nmol/mL	%	nmol/mL	%	nmol/mL	%	nmol/mL	%	nmol/mL	%	nmol/mL	%	nmol/mL	%
Cysteic acid	-		-		-		-		-		-		-		-	
Hydroxyproline	-		-		-		-		-		-		-		-	
Aspartic acid	13600	17,53	7970	16,99	12800	16,64	20900	15,95	7210	15,31	11500	15,84	11500	14,92	704	11,52
Threonine	4860	6,26	2810	5,99	4550	5,92	8960	6,84	3100	6,58	4910	6,76	5190	6,73	349	5,71
Serine	4910	6,33	3270	6,97	5440	7,07	8000	6,11	2900	6,16	4420	6,09	4840	6,28	457	7,48
Glutamic acid	6960	8,97	4620	9,85	7330	9,53	12300	9,39	4050	8,60	6350	8,75	6800	8,82	663	10,85
Proline	1750	2,26	1270	2,71	2080	2,70	3940	3,01	1260	2,68	2130	2,93	2500	3,24	157	2,57
Glycine	7280	9,38	4950	10,55	8110	10,55	16000	12,21	5980	12,70	7890	10,87	11500	14,92	695	11,37
Alanine	7290	9,39	4290	9,15	7410	9,64	11200	8,55	3970	8,43	6280	8,65	6820	8,85	656	10,74
Cystine	50,5	0,07	0	0,00	49,3	0,06	119	0,09	34	0,07	58,8	0,08	0	0,00	0	0,00
Valine	5090	6,56	2690	5,74	4730	6,15	8440	6,44	2970	6,31	4710	6,49	4440	5,76	511	8,36
Methionine	624	0,80	504	1,07	582	0,76	775	0,59	228	0,48	790	1,09	575	0,75	27,7	0,45
Isoleucine	2530	3,26	1470	3,13	2620	3,41	4820	3,68	1720	3,65	2660	3,66	2490	3,23	194	3,18
Leucine	5470	7,05	3470	7,40	5370	6,98	8760	6,69	3120	6,62	4710	6,49	4640	6,02	313	5,12
Tyrosine	3870	4,99	2150	4,58	3410	4,43	6650	5,08	2330	4,95	3630	5,00	4100	5,32	277	4,53
Phenylalanine	2810	3,62	1550	3,30	2410	3,13	4800	3,66	1700	3,61	2660	3,66	2800	3,63	213	3,49
Histidine	2420	3,12	1310	2,79	1890	2,46	2910	2,22	1940	4,12	3080	4,24	1160	1,50	163	2,67
Tryptophan	-		-		-		-		-		-		-		-	
Lysine	4490	5,79	2550	5,44	4510	5,86	7560	5,77	2660	5,65	4110	5,66	4990	6,47	450	7,36
Arginine	3600	4,64	2040	4,35	3540	4,60	5250	4,01	1920	4,08	2760	3,80	2840	3,68	285	4,66
Total	77600	100,01	46900	100,00	76900	100,00	131000	100,00	47100	100,00	72600	100,00	77100	100,00	6110	100,00

Tab. 6 – AA hydrolysis analysis results

On average, with the NI assay, the GMMA gave approximately a 20% lower color yield than BSA (Fig. 5). However there was variation between closely related GMMA e.g., the *S. flexneri* +p ΔOAg gave 40% higher BSA equivalent as *S. flexneri +p.*
When using plastic cuvettes, the Lowry assay gave a lower average inter- and intra-assay variation than the other methods (Tab. 5). With quartz cuvettes, the reproducibility of the Lowry assay was further improved: the intra-assay variation decreased to less than 4% for each sample (Tab. 7).

	AA quantification [µg/mL]	**Concentration measured on single [µg/mL]**				**Mean [µg/mL]**	**Standard deviation [µg/mL]**	**CV % Intra-assay**	**% of AA quantification obtained by tested assay**
		1	2	3	4				
S. sonnei –p ΔOAg	**14200**	21064	20001	19353	19885	**19965**	2073	**3.6**	**141**
S. sonnei +p ΔOAg	**5080**	6657	6976	6826	6454	**6728**	676	**3.3**	**132**
S. flexneri +p ΔOAg	**8310**	11449	11272	10845	10491	**11014**	905	**3.9**	**133**
S. flexneri –p ΔOAg	**8510**	11256	11522	11293	11718	**11477**	825	**1.9**	**135**

Tab. 7 – Results and analysis of intra-assay variations obtained by Lowry assay using quartz cuvettes

For all the assays the measurements of GMMA concentration were performed in the range in which the quantification's methods are linear accordingly to BSA standard curve. The differences in protein concentrations obtained by different methods are due to GMMA properties, and not to different methods themselves. In fact,

quantifying by different assays purified proteins (ex. CRM_{197} [48]), we gained similar concentrations using Lowry, Bradford and NI assay (data not shown).

Linearity of measurements

S. sonnei –p ΔOAg GMMA were assayed at 5 to 20 µg, in terms of absolute amount of proteins as quantified by AA hydrolysis, for Bradford and in the range of 6.25-21.5 µg for Lowry and NI assay to measure the linearity of each assay. Graphs obtained plotting directly BSA equivalents obtained from the assay in comparison to absolute amount as measured by AA hydrolysis (Fig. 6A), or BSA equivalents "normalized" using colorimetric correlation factors obtained for each assay (Fig. 6B), were produced to compare different colorimetric assays in terms of linearity. The criteria for evaluating the linearity were the analysis of the slope and the intercept of the trendlines obtained for each colorimetric assay: the linearity is better the closer intercept is to 0 and the closer the slope is to 1. Both Lowry and NI assay fits better than Bradford to the previous parameters and Lowry is the closest ones to the ideal fits (formulas reported in Fig. 6a and Fig. 6B). Furthermore both Lowry and NI assay have shown an R^2 more closed to 1 that Bradford assay (Fig. 6).

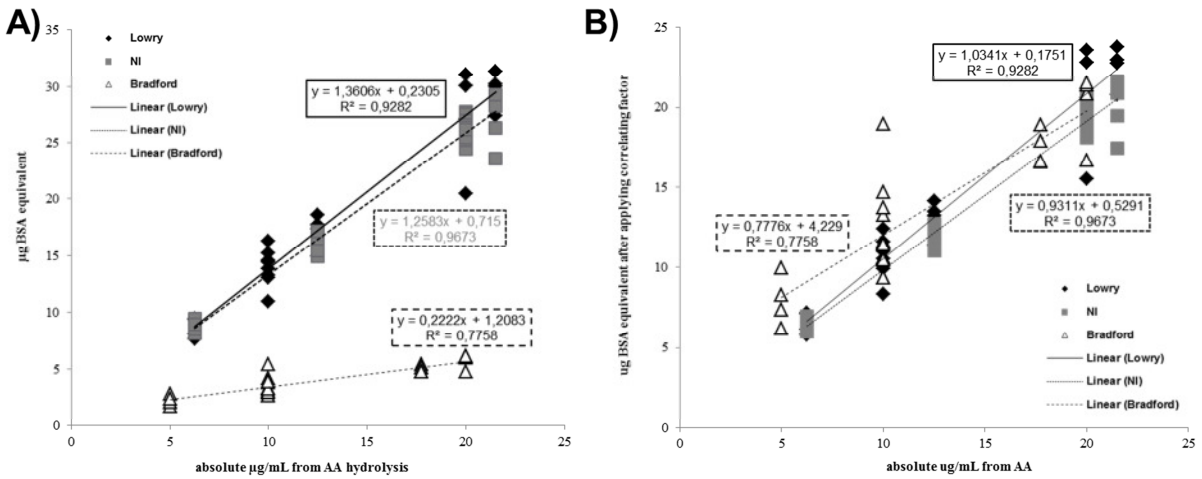

Fig. 6 Linearity of assays. GMMA purified from Ss -p ΔOAg were assayed using different colorimetric assays starting from different absolute amounts as obtained by AA hydrolysis; values obtained from different colorimetric assays were plotted against quantities of GMMA as measured by AA hydrolysis **A) as BSA equivalents B) applying a colorimetric correlation factor to BSA equivalents obtained by each colorimetric assay prior plotting**. The colorimetric factors used were 3.5, 0.74 and 0.76 respectively for Bradford, NI and Lowry assay basing on results shown in tab.2. Trendlines for each assay were afterwards obtained and formulas were shown on graphs.

Evaluation of proportionality by SDS-PAGE

The results obtained by different methods of protein quantification were also evaluated by analyzing the same amount of proteins (as quantified by the different assays) from different types of GMMA onto SDS-PAGE (Fig. 7). Visually, samples loaded according to concentration measured by AA hydrolysis (Fig. 7A) and by Lowry assay (Fig. 7B) gives a similar protein pattern, indicating a good correlation between these two methods even without application of a colorimetric correlating factor. In SDS-PAGE profile displayed in Fig. 7A and Fig. 7B are detectable, in fact, only small differences in terms of absolute amounts of GMMA in each lane, due to the general

overestimation by Lowry method. Anyway the proportionality between different samples is maintained. Due to the fact that we established an approximately 4-fold underestimation by Bradford method loaded on gel 2.5 µg of GMMA according to concentration measured by Bradford assay (Fig. 7C). Although this adjustment, we observed that supposedly same amounts of different types of GMMA as quantified by Bradford method, clearly differed to the ones loaded accordingly to AA hydrolysis (Fig. 7A). This behavior is most critical comparing GMMA obtained from *S. sonnei* and *S. flexneri* strains than between GMMA obtained from different *S. sonnei* mutants compared in previous studies (66).

The same types of assays' comparison for GMMA protein quantifications were performed for GMMA obtained from *Salmonella* and *Neisseria,* and results are reported in Tab. 5 and Fig. 5B. Gels after SDS-PAGE loading 10 µg of GMMA from those strains accordingly to AA hydrolysis and Lowry assay were also shown in Fig. 7A and Fig. 7B; SDS-PAGE loading 2.5 µg of GMMA accordingly to Bradford assay were, instead, shown in Fig. 7C. Also for *Salmonella* and *Neisseria* GMMA Bradford assay underestimate the concentrations obtained by quantitative AA hydrolysis; instead the results obtained by Lowry and NI assay generally over estimates the concentration obtained by AA hydrolysis, following the trend observed for *Shigella* GMMA.

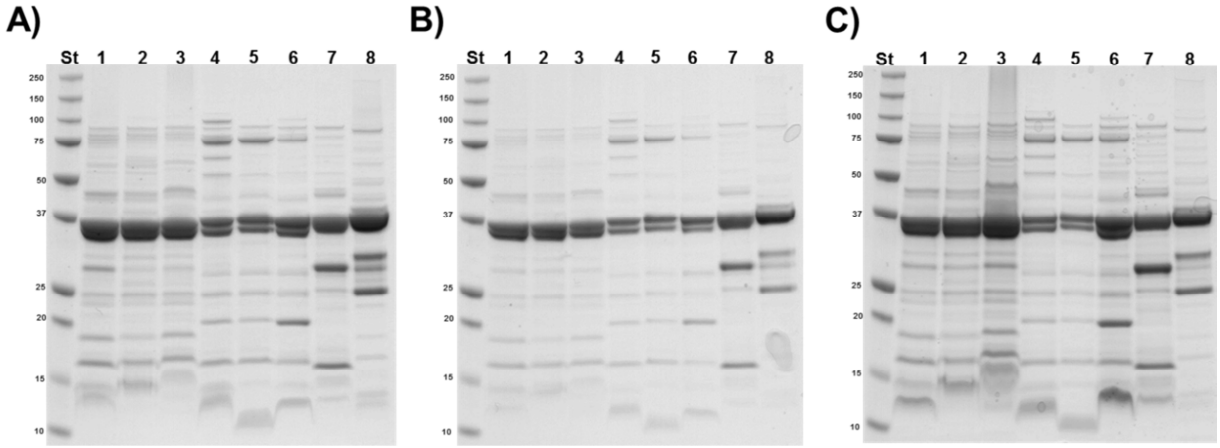

Fig. 7 SDS-PAGE 12% - AA hydrolysis (A) vs Lowry (B) vs Bradford (C). 10 µg of GMMA/well loaded accordingly to AA hydrolysis quantification (A) or Lowry assay (B) in a 12% SDS-PAGE, Comassie stained. In (C) 2.5 µg of GMMA/well loaded accordingly to Bradford quantification in a 12% SDS-PAGE, Comassie stained. GMMA were respectively obtained from: 1. Ss –p ΔOAg; 2. Ss +p ΔOAg, 3. Ss +p; 4. Sf +p ΔOAg; 5. Sf +p; 6. Sf -p ΔOAg; 7. Salmonella; 8. Neisseria

Reduction of GMMA endotoxicity

General strategy of engeenerization and electron microscopy of bacteria

In order to obtain GMMA-producing strains, *Shigella* and iNTS strains (*S.* Enteritidis and *S.* Typhimurium) were genetically engineered by Δ*tolR* deletion (66). In case of *N. meningitidis*, hyper-blebbing strains have been obtained by the knock-out of *gna33*. Bacteria carrying *tolR* or *gna33* kock outs were viewed by electron microscopy. Blebbing could be observed with all strains (Fig. 8). However, the difference between the electron micrographs obtained with the *Neisseria gna33* mutant compared to the *Shigella* and *Salmonella tolR* mutants is striking. *Neisseria* appears to 'accumulate' GMMA at the cell surface, whereas in *Shigella* and *Salmonella* few GMMA were detected at the cell surface but GMMA were visible in the surrounding milieu (see *S. sonnei* panel in Fig. 8).

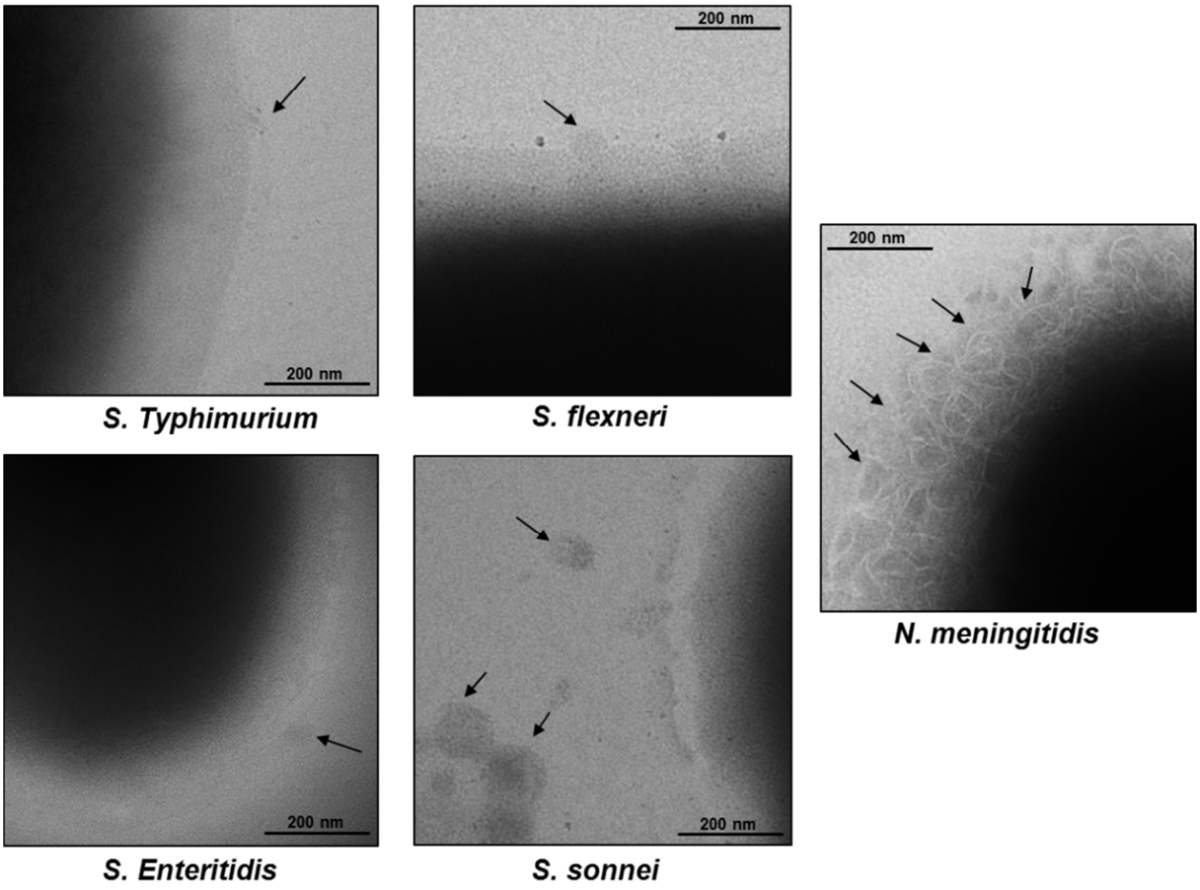

Fig. 8 Electron microscopy on GMMA-prucing bacteria bacteria (*S. sonnei* Δ*tolR*, *S. flexneri* Δ*tolR*, S. enteritidis Δ*tolR*, S. typhimurium Δ*tolR, N. meningitides Δgna33). Arrows indicate GMMA/GMMA formation.

Starting from these hyper-blebbing strains, the feasibility of reducing lipid A reactogenicity of GMMA was assessed using *htrB* and *msbB* knock outs for *Shigella* and iNTS or *lpxL1* for *Neisseria*.

Shigella

Mutants production and set up conditions for GMMA production

Shigella strains with comparable genetic background were generated in order to better characterize and compare GMMA from different strains. Initially GMMA without major virulence plasmid and lacking OAg were compared, as these were used to set up the industrial production process (66).
As reported in (128) mutants lacking the major virulence plasmid grow similarly to wild type strains, as well after *tolR* knock out, both at 30 than at 37℃, both in terms of duplication time than of optical density reached after over-night colture that was comparable both in Lauria Broth (LB) median than in SDM (data not shown). Strains carrying *msbB* knock out, besides maintained the ability to grow at 37℃, reached a higher optical density at 30℃ and in chemical defined media (128). In contrast, Δ*htrB* strains only grew in chemical defined or minimal media and only at 30℃. The duplication time of the Δ*htrB* or Δ*msbB* strains increased from 30 min to approximately 2 hours, but all the strains were able to reach high ODs (OD 10) after overnight incubation in chemically defined medium (SDM) at 30℃. Thus 30℃ in SDM was chosen as the standard growth condition. All strains with lipid A modifications yielded more than 50 mg/L GMMA protein.

Characterization of GMMA from different mutants: Electromicroscopy and Protein pattern

The shape of the GMMA from *Shigella* strains carrying different mutations was characterized by electronmicroscopy. GMMA from *Shigella* strains carrying different mutations were sishowed similar morphology by electron microscopy (fig. 9A) with average sizes of 30-32 nm in all 6 strains and a size distribution of 17-53 nm. A comparison of the GMMA sizes form the all strains gave no significant difference (p=0.90).

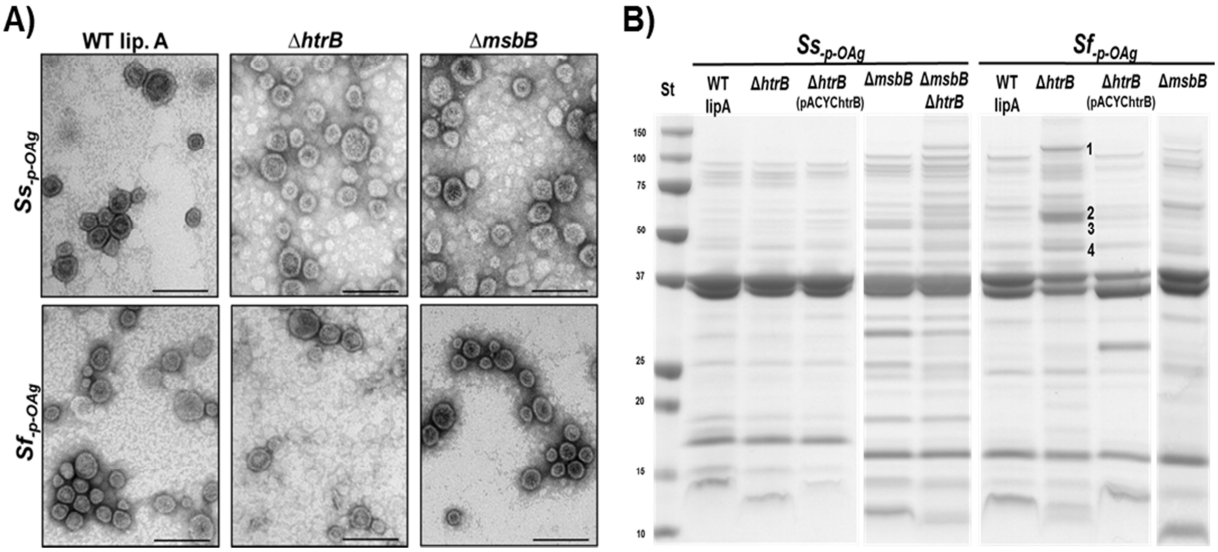

Fig. 9 A) Electron microscopy of GMMA from different strains. GMMA were purified from *Ss*$_{\text{-p-OAg}}$ and *Sf*$_{\text{-p-OAg}}$ containing wild-type lipid A (*Ss*$_{\text{-p-OAg}}$ WT lip. A, *Sf*$_{\text{-p-OAg}}$ WT lipA), *Ss*$_{\text{-p-OAg}}$ Δ*msbB*, *Sf*$_{\text{-p-OAg}}$ Δ*msbB*, *Ss*$_{\text{-p-OAg}}$ Δ*htrB*, *Sf*$_{\text{-p-OAg}}$ Δ*htrB*, negatively stained and viewed by electron microscopy (105,000-

fold magnification) revealing the presence of well-organized membrane particles with diameter of 17-53 nm in each preparation. Bar length = 100 nm. **B) SDS-PAGE**. 10 µg of protein of GMMA shown in A) and GMMA from *Ss*$_{\text{-p-OAg}}$ Δ*htrB* Δ*msbB, Ss*$_{\text{-p-OAg}}$ Δ*htrB* (pACYC*htrB*) and *Sf*$_{\text{-p-OAg}}$ Δ*htrB* (pACYC*htrB*) produced under the same growth conditions were separated by SDS-PAGE (12% polyacrylamide) and Comassie stained. Four protein bands which were more abundant in GMMA from *Sf*$_{\text{-p-OAg}}$ Δ*htrB* than in GMMA from other *Sf*$_{\text{-p-OAg}}$ strains were identified by Peptide Mass Fingerprinting: 1, pyruvate dehydrogenase; 2, glutamine synthetase; 3, ketol-acid reductoisomerase; 4, D-3-phosphoglycerate dehydrogenase.

To characterize if the genetic lipid A modifications might alter the protein composition of GMMA (it has been reported that the relative quantity of some of the proteins was affected in presence of different lipid A modifications due to sigma E-dependent response (132), the protein pattern of GMMA from the different mutants was evaluated by SDS-PAGE (fig. 9B). While the overall pattern remained similar, 4 protein bands, identified pyruvate dehydrogenase, glutamine synthetase, ketol-acid recutoisomerase and D-3-phosphoglycerate dehydrogenase (fig. 9B) by Peptide Mass Fingerprinting were found to be upregulated in GMMA from *Sf*$_{\text{-p-OAg}}$ Δ*htrB*. As these proteins are cytoplasmic proteins, no effect on the reactogenicity studies is expected.

Characterization of lipid A by MALDI-TOF and MALDI-TOF/TOF

In order to characterize the lipid A of LPS of the mutants produced, a lipid A extraction was performed followed by a MALDI-TOF analysis. The spectra obtained were reported in fig. 10 and correlation about the species of lipid A correspondent to the main peaks were also displayed basing on data obtained from similar mutants of *Escherichia coli* (160); the structure correspondent to main peaks were also displayed in fig. 10L.
The main peaks in the mass spectra obtained by MALDI-TOF from lipid A purified from GMMA from *Shigella sonnei* and *S. flexneri* strains with wild type (WT) LPS (fig. 10, *Ss*$_{\text{-p-OAg}}$ (A), *Sf*$_{\text{-p-OAg}}$ (B)) has an *m/z* corresponding to the theoretical mass of the hexa-acylated lipid A of 1798 Da.
The main peaks obtained by mass spectrometry from the *Ss*$_{\text{-p-OAg}}$ Δ*msbB* GMMA (fig. 10C) and *Sf*$_{\text{-p-OAg}}$ Δ*msbB* (fig. 10D) GMMA correspond, in both strains, to a penta-acylated lipid A lacking a miristoyl chain (a C_{14} fatty acid chain, 210 *m/z* shift) in comparison to the hexa-acylated WT forms (fig. 10A and 10B respectively), consistent with *msbB* knock outs.
The mass spectrum of lipid A from *Ss*$_{\text{-p-OAg}}$ Δ*htrB* GMMA (fig. 10E) showed a main peak at 1615 *m/z*, corresponding to a penta-acylated lipid A lacking a lauroyl chain (a C_{12} fatty acid chain) in comparison to strain with unmodified lipid A (182 *m/z* shift, fig. 10A), consistent with an *htrB* knock out. By contrast, the spectra obtained from GMMA Δ*htrB* of *Sf*$_{\text{-p-OAg}}$ (fig. 10F), besides showing the presence of the penta-acylated lipid A species with the deletion of a lauroyl chain, already observed in the *Ss*$_{\text{-p-OAg}}$ Δ*htrB* mutant (peak at 1615 *m/z*), in addition, showed also a main peak at 1850 *m/z* (fig. 10F), most likely corresponding to a hexa-acylated lipid A species with a *m/z* different to WT lipid A. The mass of this lipid A species corresponds to a lipid A containing a palmitoleoyl chain (a $C_{16:1}$ fatty acid chain, 236 *m/z* shift) as a sixth acyl chain in penta-acylated background, most likely in the position where normally the lauroyl chain is attached by the *htrB* gene product. A further confirmation about palmitoleoylation has also been obtained by analyzing in MS/MS spectrometry the chemical species obtained fragmenting by Collision Induced Decay lipid A species presents in main peaks observed in first dimention MS for GMMA from *Ss*$_{\text{-p-OAg}}$ Δ*htrB*

(*m/z* 1615 fig.10E) and *Sf*$_{p\text{-}OAg}$ Δ*htrB* (*m/z* 1850 fig.10F): the main difference observed when overlaying the MS/MS spectra is represented by a peak with a *m/z* correspondent to a palmitoleoyl chain(252 Da, highlighted in fig.10G) in *Sf*$_{p\text{-}OAg}$ Δ*htrB*.

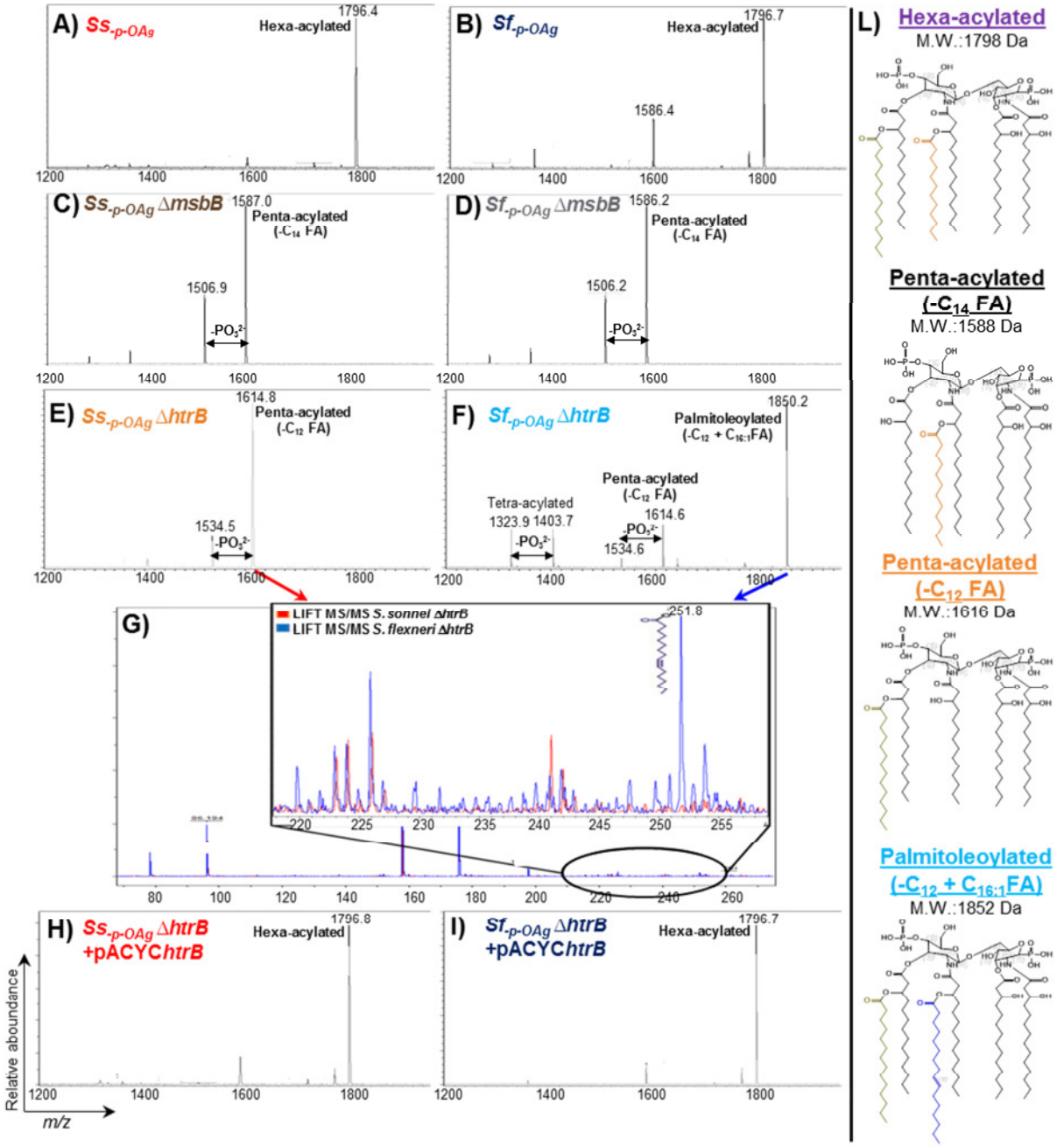

Fig. 10 MALDI-TOF spectra obtained analyzing lipid A precipitated from GMMA in refletron ion-negative mode. GMMA from: **A)** *Ss*$_{p\text{-}OAg}$ **B)** *Sf*$_{p\text{-}OAg}$ **C)** *Ss*$_{p\text{-}OAg}$ Δ*msbB* **D)** *Sf*$_{p\text{-}OAg}$ Δ*msbB* **E)** *Ss*$_{p\text{-}OAg}$ Δ*htrB* **F)** *Sf*$_{p\text{-}OAg}$ Δ*htrB* **H)** *Ss*$_{p\text{-}OAg}$ Δ*htrB* + pACYChtrB **I)** *Sf*$_{p\text{-}OAg}$ Δ*htrB* + pACYChtrB. **G) MS/MS spectra obtained in ion negative mode**: Overlay of negative ion LIFT MALDI-TOF/TOF spectra in the low *m/z* range of the dominant species in lipid A from *Ss*$_{p\text{-}OAg}$ Δ*htrB* (E) and *Sf*$_{p\text{-}OAg}$ Δ*htrB* (F) after Collision Induced Dissociation. **L)** Lipid A structures with molecular weights corresponding to the observed main peaks.

A further indirect confirmation about the location of the sixth compensatory fatty acid chain, has been obtained testing by MALDI-TOF spectra of GMMA from the *Ss*$_{p\text{-}OAg}$ an *Sf*$_{p\text{-}OAg}$ Δ*htrB* strains complemented with pACYC*htrB* (fig. 10 H for *Ss*$_{p\text{-}OAg}$ and fig.

10 I for *Sf*$_{\text{-p-OAg}}$) resulting in both cases in lipid A hexa-acylated WT as main peak (1797 *m/z*). The inability to detect a hepta-acetylated lipid A in either the *S. flexneri* 2a GMMA or the *S. flexneri* 2a Δ*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.
The MALDI-TOF profile observed in *Sf*$_{\text{-p-OAg}}$ Δ*htrB* has been obtained also in *Sf*$_{\text{+p+OAg}}$ Δ*htrB*, in *Sf*$_{\text{-p+OAg}}$ Δ*htrB* and the palmitoleoylated hexa-acylated form has been confirmed to be the main form also in GMMA purified from *S. flexneri* 3a and 6 Δ*tolR* after *htrB* knock out (data not shown).
In order to obtain a tetra-acylated lipid A a *Ss*$_{\text{-p-OAg}}$ Δ*htrB* Δ*msbB* mutants has been produced and the lipid A from its GMMA has been purified and analyzed by MALDI-TOF (fig. 11). The MALDI profile present a main population correspondent to a tetra-acylated lipid A (peak at *m/z*=1404 and his respectively peak lacking phosphate group lost during analysis at *m/z*=1324), consistent with the deletion of both a lauroyl and a miristoyl chain. Also in that genetic background, GMMA from *Ss*$_{\text{-p-OAg}}$ Δ*htrB* Δ*msbB* grown at 30°C doesn't showed the palmitoleoylation observed in various *S. flexneri* strains after *htrB* knock-out.

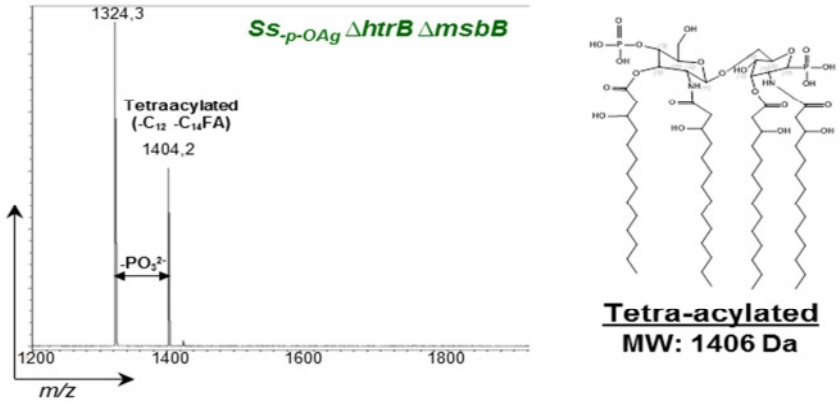

Fig. 11 MALDI-TOF of lipid A from *Ss*$_{\text{-p-OAg}}$ Δ*htrB* Δ*msbB*

Similar results in terms of lipid A populations and relative abundance of different species obtained with GMMA have been obtained also from bacteria (data not shown), demonstrating that the lipid A composition of GMMA and bacteria is similar.

Lipid A changes at different temperatures

The palmitoleoylation observed in GMMA from *Sf*$_{\text{-p-OAg}}$ Δ*htrB* is consistent with the activity by the late acyl transferase LpxP (108). *lpxP* has been reported to be overexpressed and more active than *htrB* as cold response (108). In order to test if the production of the palmitoleoylated hexa-acylated lipid A species (*m/z* 1851) could be induced in *S. sonnei* under stress conditions, *Ss*$_{\text{-p-OAg}}$ Δ*htrB* was grown at 12°C to induce a cold stress response. In the corresponding lipid A analyzed by MALDI-TOF a small amount (below 5% of the total amount, fig. 12) of the palmitoleoylated lipid A species was identified. At 12°C a long duplication time, up to 7-8 hours, was needed and a long incubation, up to 1.5 days was necessary to reach O.D.=1 starting from O.D.=0,1.
By this experiment we have demonstrated that "forcing the system", as cold response, LpxP is "functional" also in *Ss*$_{\text{-p-OAg}}$ Δ*htrB* strain, suggesting that differences observed were related to differential expression level of *lpxP* gene at 30°C in the two *Shigella* strains.

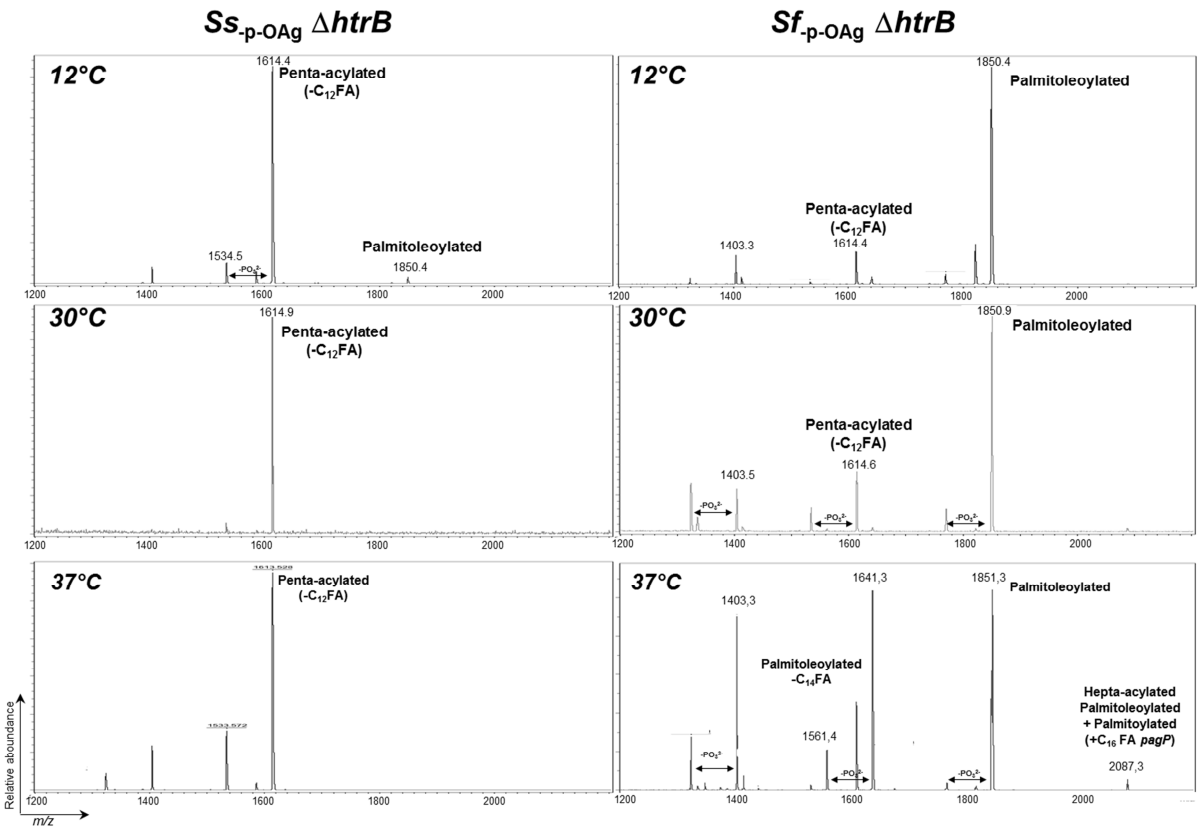

Fig. 12 MALDI-TOF spectra obtained analyzing lipid A obtained from GMMA Δ*htrB* produced at different temperatures. GMMA from of *S. flexneri* Δ*htrB* and *S. sonnei* Δ*htrB* were produced at 12, 30 and 37°C and tested by MALDI-TOF in reflectron ion-negative mode

Lipid A from GMMA obtained from *Ss*$_{\text{-p-OAg}}$ Δ*htrB* and *Sf*$_{\text{-p-OAg}}$ Δ*htrB* grown at 37°C. In that case the colture of bacteria was possile only after an adaptation in SDM at 30°C, by restarting the colture from O.D.=0,1 at 37°C; the OD reached was anyway lower than the ones obtained at 30°C after overnight, around 2. Lipid A from GMMA obtained at 37°C have been analyzed by MALDI-TOF (fig.12) and we have observed that lipid A from GMMA of *Ss*$_{\text{-p-OAg}}$ Δ*htrB* is only penta-acylated, like at 30°C. Instead, for *Sf*$_{\text{-p-OAg}}$ Δ*htrB*, besides the main peak is still represented by the palmitoleoylated lipid A, also another small peak, most likely correspondent to an hepta-acylated lipid A at *m/z*=2087, has been observed, correspondent to the activation of *pagP* and the attach of a palmitoleoyl chain (a C_{16} fatty acid chain) on the compensated hexa-acylated lipid A (fig.12).

Characterization of differences in *lpxP* between *S. flexneri* and *S. sonnei*

The *lpxP* gene is present in both *Shigella sonnei* 53G and *Shigella flexneri* 2a and, by translating the two coding regions, only one amino acid might be different in the two strains (Leu^{116}→ Pro in *S. sonnei*).
As palmitoleoylation is consistent with the activity by the late acyl transferase LpxP, the expression of *lpxP* was tested in *Ss*$_{\text{-p-OAg}}$ Δ*htrB* and *Sf*$_{\text{-p-OAg}}$ Δ*htrB* by quantitative real time PCR (fig. 13). When grown at 30°C, the level of transcript of *lpxP* was 11-fold higher in *Sf*$_{\text{-p-OAg}}$ Δ*htrB* compared to *Ss*$_{\text{-p-OAg}}$ Δ*htrB*, whereas level of transcript of *msbB* was 1.5-fold higher in S. flexneri compared to S. sonnei.

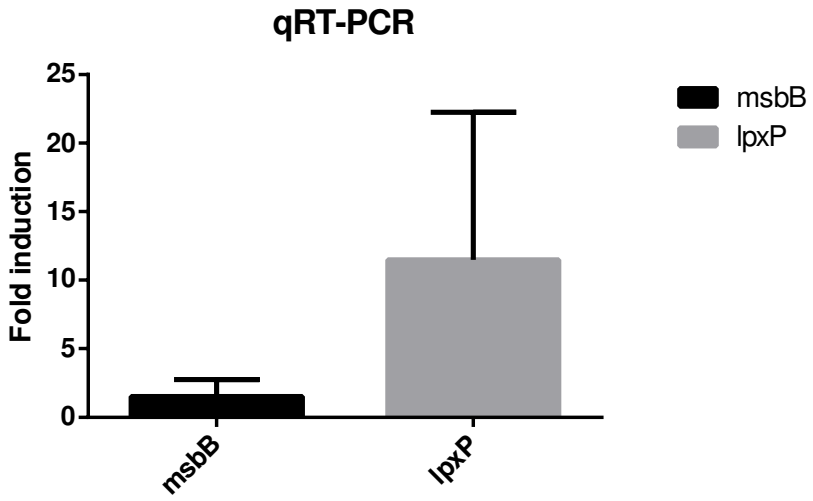

Fig. 13 qRT-PCR relative expression in comparison to *lysP* housekeeping of *msbB* and *lpxP* genes in *Sf*$_{\text{p-OAg}}$ Δ*htrB* in comparison to *Ss*$_{\text{p-OAg}}$ Δ*htrB.* The mean of 3 indipendent experiment (each run in duplicate) have been plotted. Error bars represent the standard deviation.

If we performed the analysis by comparing to the *msbB* gene which is part of the constitutive lipid A pathway. In three independent experiments, the level of transcript of *lpxP* to *msbB* was on average 7.3-fold higher (standard deviation 0.9) in *Sf*$_{\text{p-OAg}}$ Δ*htrB* compared to *Ss*$_{\text{p-OAg}}$ Δ*htrB*.

Characterization of endotoxin activity after lipid A modifications

TLR4 - NF-kB luciferase reporter assay

Human Embrionic Kidney cells in fact naturally don't express TLR. Thus, these cells were stably transfected in order to express only one type of human Toll-Like Receptor and an NF-κB-inducible luciferase reporter gene. Thus, activation of the TLR, results in the activation of a signaling cascade that finally activates NF-kB. The stronger is the activation of the specific TLR, the higher is the activation of NF-kB. NF-kB acts as the specific activator of the promoter of the luciferase reporter gene.

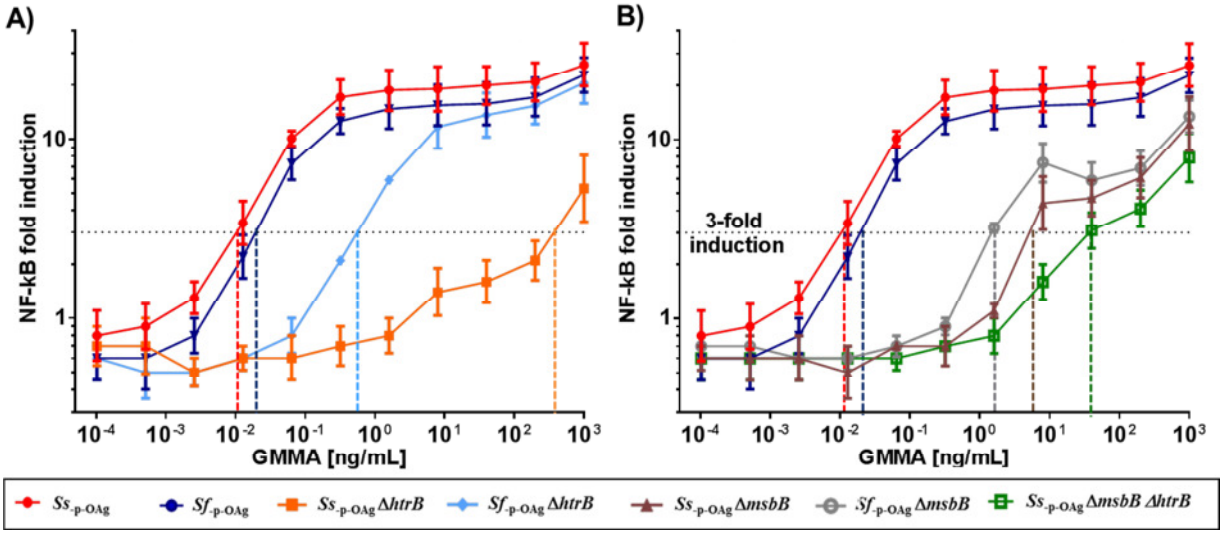

Fig. 14 Activation of TLR4 reporter cell line by different GMMA. 25,000 of TLR4-HEK293 cells/well were stimulated with 1000 – 0.0001 ng/mL (5-fold steps) of GMMA obtained from **A)** *Ss*$_{\text{p-OAg}}$, *Sf*$_{\text{p-OAg}}$,

Ss-p-OAg Δ*htrB*, *Sf*-p-OAg Δ*htrB* and **B)** *Ss*-p-OAg, *Sf*-p-OAg, *Ss*-p-OAg Δ*msbB*, *Sf*-p-OAg Δ*msbB and Ss*-p-OAg Δ*htrB* Δ*msbB*. After 5 h, luciferase expression was measured and expressed as fold-induction compared to cell incubated with PBS (averages of duplicates with standard deviation). The GMMA concentrations that resulted in 3-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). Representative results of three independent experiments are shown.

The measurement of luciferase activity by lumenometer gives us the measurements of the activation of the specific TLR (151). We stimulated Human Embryonic Kidney cells (HEK293) stably transfected to express only human TLR4 recognition complex, the PRR involved in the recognition of lipid A (116) and an NF-κB-inducible luciferase reporter gene to characterize the NF-kB specific activity associated to each lipid A. The results obtained with different concentrations of GMMA were displayed in fig. 14 and analysis has been reported in Tab. 8.
GMMA from *Sf*-p-OAg Δ*msbB* and *Ss*-p-OAg Δ*msbB* stimulated similar levels of NF-κB expression in the HEK293 TLR4 transfectant cells (fig. 14A) and required approximately 600-fold more GMMA than the parent GMMA (fig. 14A) to give a 3 fold increase in activity. In contrast, GMMA from *Sf*-p-OAg Δ*htrB* and *Ss*-p-OAg Δ*htrB* gave very different results. *Ss*-p-OAg Δ*htrB* GMMA resulted in substantially lower stimulation than *Ss*-p-OAg Δ*msbB* GMMA (fig. 14B), and the induction of NF-κB was only detectable at a high concentration of GMMA and required 60,000-fold more GMMA than the GMMA with wild-type lipid A to result in 3x NF-κB induction. However, *Sf*-p-OAg Δ*htrB* GMMA (fig. 14B) gave substantially more stimulation than *Sf*-p-OAg Δ*msbB* GMMA ($p = 0.0286$) in the HEK293 TLR4 transfectant cells (fig. 14B), and required only 50x more GMMA than the GMMA with wild-type lipid A to result in 3x NF-κB induction.
Testing GMMA from *Ss*-p-OAg Δ*htrB* Δ*msbB* by that assay the luciferase activity measured is similar to the ones observed for *Ss*-p-OAg Δ*htrB* (fig.14B).

GMMA from strain:	GMMA concentration at 3-fold NF-kB induction [ng/mL]				
	1	2	3	4	Mean
Ss-p-OAg	0.003	0.003	0.010	0.005	**0.005**
Sf-p-OAg	0.004	0.004	0.021	0.010	**0.010**
Ss-p-OAg Δ*msbB*	-	-	5.17	4.16	**4,67**
Sf-p-OAg Δ*msbB*	-	-	1.56	7.71	**4,64**
Ss-p-OAg Δ*htrB*	418	324	362	897	**500**
Sf-p-OAg Δ*htrB*	0.39	0.16	0.59	0.44	**0.39**

GMMA containing "lipid A type":	Mean [ng/mL]	Fold difference to GMMA containing WT lipid A	Fold difference to Δ*msbB* GMMA
Wild-type	**0.008**	-	**581**
Δ*msbB*	**4.65**	**581**	-
Ss-p-OAg Δ*htrB*	**500**	**62,500**	**0,01**
Sf-p-OAg Δ*htrB*	**0.40**	**50**	**12**

Tab. 8 NF-kB specific TLR4 results analysis. Concentrations of each type of GMMA resulting in 3-fold increased activation of NF-κB were determined using TLR4/MD2/CD14 HEK293 trasfectant cells as shown in Fig.14. Fold difference of the respective concentration of GMMA from lipid A mutants to GMMA with wild-type lipid A (obtained as mean of average concentrations resulting in 3-fold NF-kB induction by *Ss*-p-OAg and *Sf*-p-OAg GMMA), to GMMA from Δ*msbB* (obtained as average of mean concentrations resulting in 3-fold NF-kB induction by *Ss*-p-OAg Δ*msbB* and *Sf*-p-OAg Δ*msbB* GMMA) and to GMMA from *Ss*-p-OAg Δ*htrB* have been displayed

It has been reported that mouse and human TLR4 binds lipid A differently (161,162). In addition, it is know that mice are less sensitive to LPS than humans. And in mouse

immunization studies we observed that GMMA with and without lipid A modification were equally well tolerated and elicited similar antibody responses (data not shown). Thus, we characterized the TLR4 stimulation of mouse TLR4 by GMMA with and without lipid A modification using HEK293 trasfectant cells that expressed mouse TLR4/MD2/CD14.

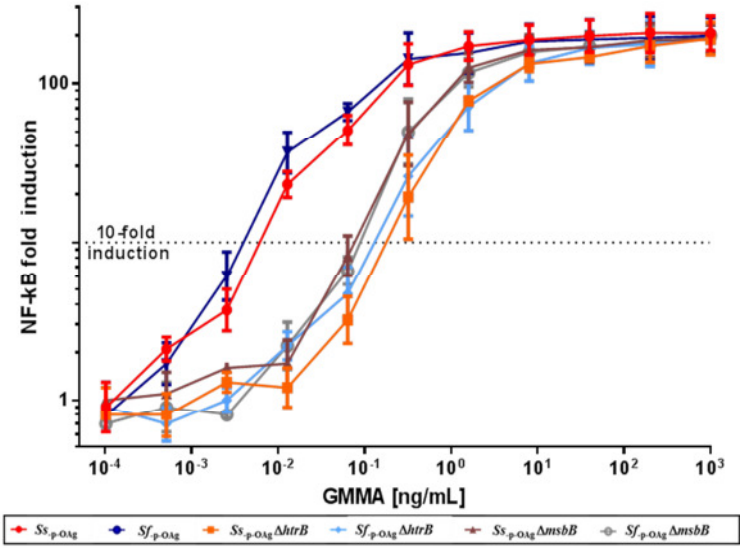

Fig. 15 NF-kB specific assay. TLR4 specific assay: 25000 of mouse HEK293 TLR4, MD2, CD14/well were stimulated for 5 hours with serial dilutions of GMMA obtained from different mutants.

By that approach we might appreciate (fig. 15 and tab. 9) that differences between GMMA from lipid A mutants and GMMA with wild type lipid A in terms of TLR4 specific activaction for mice (as measured to cause a 10-fold NF-kB induction) are smaller than what observed by using humans receptors and GMMA carrying *htrB* or *msbB* knock outs gave a similar activation and without showing the difference observed using human cells between $Sf_{\text{-p-OAg}}$ Δ*htrB* and $Sf_{\text{-p-OAg}}$ Δ*htrB* GMMA.

	GMMA to have 10 fold induction [ng/mL]	Fold difference to undetoxified GMMA
$Ss_{\text{-p-OAg}}$	0,006	**1**
$Ss_{\text{-p-OAg}}$ **Δ*htrB***	0.181	**30**
$Ss_{\text{-p-OAg}}$ **Δ*msbB***	0.074	**12**
$Sf_{\text{-p-OAg}}$	0.004	**0.8**
$Sf_{\text{-p-OAg}}$ **Δ*htrB***	0.139	**23**
$Sf_{\text{-p-OAg}}$ **Δ*msbB***	0.090	**15**

Tab. 9 Fold difference in NF-kB specific assays calculated as ratio between concentration of GMMA needed to cause a 10-fold induction in the case of mouse TLR4 HEK293 cells

Lipid A quantities in GMMA from different mutants (KDO quantitation)

In order to ensure that the observed differences are the results of difference of the stimulatory activity of the lipid A and is not caused by different amounts of lipid A present in GMMA the molar amount of lipid A per mg/protein was determined by quantified the LPS core sugar KDO. The amounts of lipid A were similar in all GMMA. In comparison to GMMA from $Ss_{\text{-p-OAg}}$ Δ*htrB* with the lowest activity, GMMA from the

Ss$_{\text{-p-OAg}}$ contains 1.1-fold, *Sf*$_{\text{-p-OAg}}$ 2.0-fold, *Sf*$_{\text{-p-OAg}}$ Δ*htrB* 0.3-fold, *Ss*$_{\text{-p-OAg}}$ Δ*msbB* 0.8-fold, and *Sf*$_{\text{-p-OAg}}$ Δ*msbB* 1.1-fold the amount of lipid A (Tab. 10).

	Retention time (min)	Area	Carbonyl reactive Groups	Fold difference to S. sonnei ΔhtrB GMMA in Kdo quantitation	Fold difference to *S. sonnei* ΔhtrB GMMA in TLR4	Fold difference to *S. sonnei* ΔhtrB GMMA in MAT
S. sonnei	17,49432	3,67E+06	**81,9** nmol/mL	**1,1**	**62,500**	**831**
	17,49422	3,94E+06	5% Std. Dev.			
S. flexneri	17,58197	7,72E+06	**158,9** nmol/mL	**2,0**		
	17,58202	7,74E+06	0% Std. Dev.			
S. sonnei Δ*htrB*	17,48383	3,85E+06	**77,6** nmol/mL	-	-	-
	17,48518	3,82E+06	0% Std. Dev.			
S. flexneri Δ*htrB*	17,50834	1,92E+06	**26,3** nmol/mL	**0,3**	**1250**	**16**
	17,49077	2,11E+06	7% Std. Dev.			
S. sonnei Δ*msbB*	17,49582	3,82E+06	**62,6** nmol/mL	**0,8**	**107**	**3**
	17,49056	3,67E+06	3% Std. Dev.			
S. flexneri Δ*msbB*	17,49897	5,91E+06	**86,9** nmol/mL	**1,1**		
	17,4996	5,53E+06	5% Std. Dev.			

Tab. 10 KDO quantitation using semicarbazide derivatization. Samples were normalized as starting volum based on protin amount and threated simulataneusly. Each sample has been run in duplicate. For comparison, the fold-difference in TLR4 stimulatory activity and in IL-6 stimuation is shown.

Cytokine release from human PBMC

In order measure understand the level of reactogenicity of GMMA in a more natural contest, GMMA purified form different mutants were used to stimulate human PBMC in MAT. Upon stimulation with GMMA, Pattern Recognition Receptors (PRRs) present in PBMC were stimulated, and cytokines would be released in a proportion way to the PRR's stimulation. The GMMA concentration required to give a 10-fold increase over background was arbitrarily used for comparing relative activity (124) since that is in the middle of the linear part of the sigmoidal curves. The same calculations performed at other points within the linear part of curve (3-fold and 30-fold over background, respectively) yielded similar the results (data not shown).
So, with the objective of better characterizing the differences in cytokine stimulation between lipid A unmodified and modified GMMA, we have measured the release of a panel of cytokines simultaneously by human pro-inflammatory 7-plex MesoScale. GMMA purified from *Shigella* strains (both *Ss*$_{\text{-p-OAg}}$ and *Sf*$_{\text{-p-OAg}}$) without lipid A modifications stimulated a strong and similar cytokine response from PBMC (fig. 16) and whole blood (data not shown).

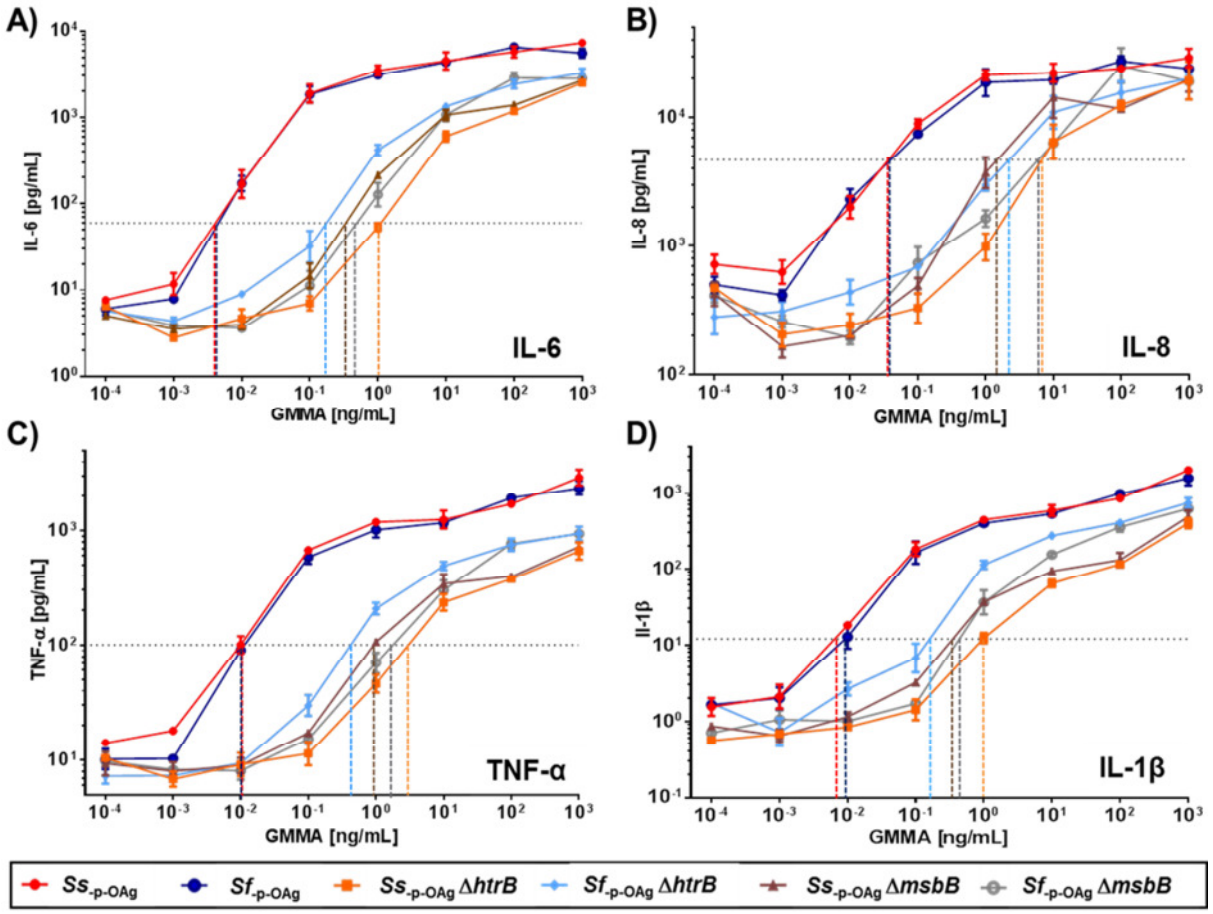

Fig. 16 Cytokine release by human PBMC after stimulation with different types of GMMA. 200,000 human PBMC cells were stimulated for 4 hours with 1000 – 0.0001 ng/mL (10-fold steps) of GMMA from *Ss*$_{\text{-p-OAg}}$, *Sf*$_{\text{-p-OAg}}$, *Ss*$_{\text{-p-OAg}}$ Δ*msbB*, *Sf*$_{\text{-p-OAg}}$ Δ*msbB*, *Ss*$_{\text{-p-OAg}}$ Δ*htrB*, *Sf*$_{\text{-p-OAg}}$ Δ*htrB*.Release of IL-6 **(A)**, IL-8 **(B)**, TNF-α **(C)**, IL-1β **(D)**, were measured by human pro-inflammatory 7-plex mesoscale and plotted as averages of duplicates with standard deviations. The GMMA concentrations that resulted in 10-fold increase of cytokine release over background are shown as x axis intercepts (colored dashed lines). Only small amounts of IFN-γ, IL-10 and IL-12 p70 were produced with similar differences in the induction by different GMMA (data not shown).

GMMA without lipid A modification stimulated the release of high amounts of Interleukin 6 (IL-6, fig. 16A), IL-8 (fig. 16B), Tumor Necrosis Factor TNF-α (fig. 16C), and IL-1β (fig. 16D). Instead, we did not observe a strong production of interferon gamma, IL-10 and IL-12 (data not shown). All the GMMA purified from *Shigella s*trains carrying lipid A modification resulted in strong reduction of IL-6 (fig. 16A), IL-8 (fig. 16B), TNF-α (fig. 16C) and IL-1β (fig. 16D) release upon stimulation in comparison to lipid A unmodified parent strains (fig. 16), with a similar trend to what observed in TLR4 specific assay (fig. 14).
The MesoScale experiments demonstrated that comparative quantification of IL-6 release between GMMA with wild type and GMMA with modified lipid A can be a marker of reduction of GMMA endotoxicity, representing the stimulation pattern of the other inflammatory cytokines, indicating that analysis of IL-6 stimulation is sufficient to compare the reactogenic potential of different GMMA; thus, for confirm and better fix the level of reduction of endotoxicity of GMMA from different lipid A mutants we have performed MAT using cells from 4 different donors and results obtained were summarized in tab. 11 (and a representative experiment analyzed by ELISA is shown in Fig. 17).

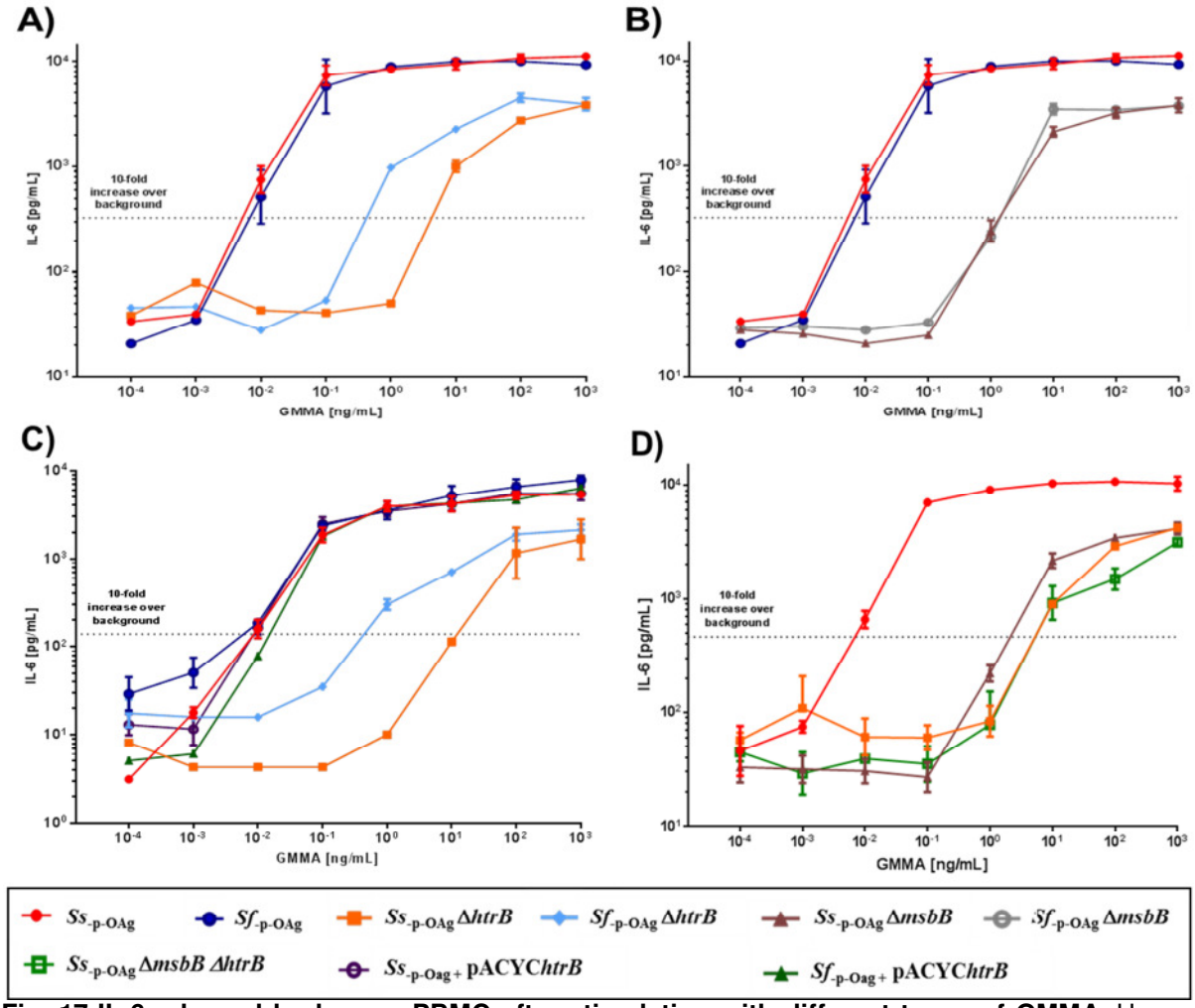

Fig. 17 IL-6 released by human PBMC after stimulation with different types of GMMA. Human PBMC were isolated using a Ficoll density gradient and 200,000 cells were plated in each well; PBMC were stimulated for 4 hours with different concentration of GMMA, supernatants were recovered and tested by IL6 ELISA. Dashed line represent the IL6 released 10-fold over the background (mean of minimum point detected). Colored dashed lines represent the GMMA concentration to reach the 10-fold increase of cytokine over background. Data shown is representative of the ones obtained from 4 different donors, each ones measured in duplicates. **A)** MAT assay obtained testing GMMA from *Ss*$_{\text{-p-OAg}}$, *Sf*$_{\text{-p-OAg}}$ and their relative Δ*htrB* mutant strains **B)** MAT assay obtained testing uGMMA from *Ss*$_{\text{-p-OAg}}$, *Sf*$_{\text{-p-OAg}}$, their relative Δ*msbB* mutant strains and *Ss*$_{\text{-p-OAg}}$ Δ*htrB* mutant (the ones that showed the best reduction of endotoxicity). **C)** MAT assay obtained testing lipid A unmodified GMMA from *S. sonnei* and *S. flexneri*, their relative Δ*htrB* mutant strains and from strains Δ*htrB* complemented with pACYChtrB **D)** MAT assay obtained testing GMMA from *S. sonnei* Δ*htrB*, Δ*msbB* or Δ*htrB* and Δ*msbB* mutant strains.

The maximum cytokine release stimulated by all the GMMA obtained from all lipid A mutants (both *htrB* and *msbB*) was 2-3-fold lower for the different GMMA than the maximum cytokine production induced by GMMA with wild-type lipid A (Fig. 16 and Fig. 17).

Furthermore is immediately appreciable in general an higher concentration of GMMA needed by lipid A mutants before reaching the same IL-6 release than lipid A unmodified strains (as measured at 10-fold increase over background, but similar analysis have been performed at 3-fold and 30-fold over background, and results obtained were similar, data not shown).

In particular GMMA that lack the *htrB* gene stimulated significantly lower levels of cytokine release in comparison to lipid A unmodified strains for *Ss*$_{\text{-p-OAg}}$ (fig. 17A).

The reduction of endotoxicity is closed to 800-fold comparing *Ss*$_{\text{-p-OAg}}$ Δ*htrB* and *Ss*$_{\text{-p-OAg}}$ (Tab. 11). GMMA lacking *htrB* gene in *Sf*$_{\text{-p-OAg}}$, although showing a strong reduction of reactogenicity in comparison to strain with WT lipid A (around 50-fold lower), were about 15-fold more reactogenic than *Ss*$_{\text{-p-OAg}}$ Δ*htrB* strains (fig. 17A).
In order to test if the different behavior in reduction of reactogenicity is due to *htrB* knock out itself, mutation was complemented. Thus, complementing the knock out by the low copy plasmid pACYC184 that carries the *htrB* gene of *Ss*$_{\text{-p-OAg}}$ (pACYChtrB) in Δ*htrB* background, the level of endotoxicity of GMMA produced from both *Ss*$_{\text{-p-OAg}}$ Δ*htrB* and *Sf*$_{\text{-p-OAg}}$ Δ*htrB* strains comes back to the level of lipid A unmodified strain (Fig. 17C).

GMMA from strain:	GMMA concentration at 10-fold increase of IL-6 over background [ng/mL]				
	1	2	3	4	Mean
Ss$_{\text{-p-OAg}}$	0.007	0.006	0.003	0.016	**0-008**
Sf$_{\text{-p-OAg}}$	0.010	0.003	0.003	0.018	**0.009**
Ss$_{\text{-p-OAg}}$ Δ*msbB*	2.05	2.81	0.62	2.55	**2.01**
Sf$_{\text{-p-OAg}}$ Δ*msbB*	1.98	2.44	1.31	3.79	**2.38**
Ss$_{\text{-p-OAg}}$ Δ*htrB*	5.38	12.5	4.53	4.18	**6.65**
Sf$_{\text{-p-OAg}}$ Δ*htrB*	0.52	0,31	0,29	0.56	**0.42**

GMMA from strains:	Mean combined [ng/mL]	Fold difference to GMMA containing WT lipid A	Fold difference to *Ss*$_{\text{-p-OAg}}$ Δ*htrB* GMMA	Fold difference to Δ*msbB* GMMA
WT lipid A	0.008	-	**831**	**274**
Δ*msbB*	4.65	**274**	**3**	-
Ss$_{\text{-p-OAg}}$ Δ*htrB*	6,65	**831**	-	**0.33**
Sf$_{\text{-p-OAg}}$ Δ*htrB*	0,42	**53**	**16**	**5**

Tab. 11 MAT assays 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 4 different donors as shown in Fig.4. Fold differences were calculated for each donor of the respective concentration of GMMA from lipid A mutants to GMMA with wild-type lipid A (obtained as mean of average concentrations resulting in 10-fold increased release of IL-6 by *Ss*$_{\text{-p-OAg}}$ and *Sf*$_{\text{-p-OAg}}$ GMMA), to GMMA from *Ss*$_{\text{-p-OAg}}$ Δ*htrB* and to GMMA from Δ*msbB* (obtained as average of mean concentrations resulting in 10-fold increased release of IL-6 by *Ss*$_{\text{-p-OAg}}$ Δ*msbB* and *Sf*$_{\text{-p-OAg}}$ Δ*msbB* GMMA) have been displayed.

GMMA purified from *Ss*$_{\text{-p-OAg}}$ or *Sf*$_{\text{-p-OAg}}$ strains that carry the *msbB* knock-out produced, instead, similar level of cytokines (fig. 16 and 17 A). The level of reduction of endotoxicity gained is around 300-fold in comparison to the GMMA purified from the respective lipid A WT strains (tab. 11), and they were around 3-fold more toxic compared to GMMA from *Ss*$_{\text{-p-OAg}}$ Δ*htrB*, the strain that showed the best reduction of reactogenicity (fig 17 B and tab. 11).
No substantial differences were observed between the differently lipid A modifiyed *Ss*$_{\text{-p-OAg}}$ GMMA Δ*htrB* mutant and lipid A Δ*htrB* and Δ*msbB* double mutant (fig. 17D), suggesting a strong reduction of reactogenicity already achieved with the *htrB* deletion (not additive).

Characterization of residual toxicity in GMMA purified from lipid A mutants

TLR blocking

With the objective of identifying the TLRs that contributes to the residual activation observed by the lipid A modifyed GMMA, PBMC were incubated with TLR2 and/or TLR4 blocking antibodies, before stimulating with 1 and 10 ng/mL of GMMA, concentrations chosen to give a significant but not saturating increase of IL-6 (Fig.18).

Targeted blocking of the two TLRs (TLR4 and TLR2) highlighted which type of TLR receptor is important for IL-6 release.
The three GMMA with penta-acylated lipid A (*Ss*$_{\text{-p-OAg}}$ Δ*htrB* (fig.18D), *Ss*$_{\text{-p-OAg}}$ Δ*msbB* (fig.18B), *Sf*$_{\text{-p-OAg}}$ Δ*msbB* (fig.18C)) gave similar results: IL-6 production gave substantial reduction following incubation with TLR2 blocking antibody (70-90%) but either no or minimal reduction with TLR4 blocking antibody (10-30%) suggesting residual activity was principally due to TLR2 activation. With the *Ss*$_{\text{-p-OAg}}$ Δ*msbB* and *Sf*$_{\text{-p-OAg}}$ *ΔmsbB* GMMA, a small reduction was observed with the TLR4 blocking antibody alone (*Ss*$_{\text{-p-OAg}}$ Δ*msbB*, p = 0.0234; *Sf*$_{\text{-p-OAg}}$ *ΔmsbB*, p = 0.0078 in 4 experiments) suggesting that there is still residual TLR4 activation. Further reduction was obtained with the combination of TLR2 and TLR4 blocking antibodies compared to TLR2 blocking antibody alone. With the *Ss*$_{\text{-p-OAg}}$ Δ*htrB* GMMA, no effect from TLR4 blocking was observed.

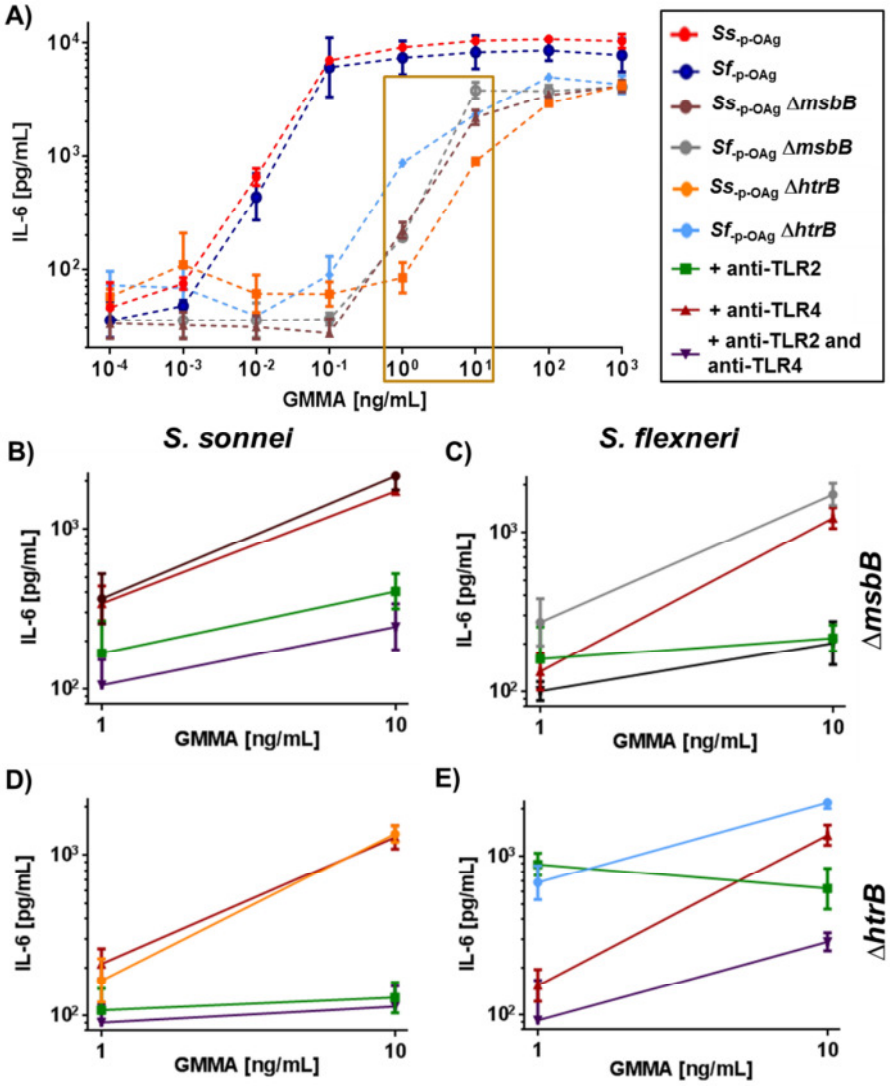

Fig. 18 TLR blocking experiments. 200,000 human PBMC cells were stimulated with GMMA from different strains. After 4 h incubation, IL-6 release was measured by ELISA and plotted as average of duplicates with standard deviation. **A)** IL-6 release after stimulation with 1000 – 0.0001 ng/mL (10-fold steps) of GMMA from different strains. The rectangle highlights the concentration of GMMA used in blocking experiments. **B-E)** In blocking experiments, cells were incubated with 2.5 µg/mL anti-TLR4 (dark red graphs), 1.5 µg/mL of anti-TLR2 (green graphs), or both (violet graphs) for 30 min before exposure to GMMA from *Ss*$_{\text{-p-OAg}}$ Δ*msbB* (B), *Sf*$_{\text{-p-OAg}}$ Δ*msbB* (C), *Ss*$_{\text{-p-OAg}}$ Δ*htrB* (D), and *Sf*$_{\text{-p-OAg}}$ Δ*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 GMMA with mostly hexa-acylated GMMA (*Sf*$_{p\text{-}OAg}$ Δ*htrB*, fig. 18E) gave a substantial reduction with a TLR4 blocking antibody, i.e. 80% reduction at 1 ng/mL, 40% at 10 ng/mL GMMA concentration, but not with a TLR2 blocking antibody. Incubation with both TLR2 and TLR4 blocking antibodies gave lower IL-6 production suggesting that the TLR4 activation was dominant, but both were still active.
As controls for specificity of TLR blockings experiments with GMMA, several experiments have been performed. In order to prove the possibility of block specific TLR, we started optimizing the antibodies concentration needed for blocking, to what needed to avoid the stimulation due to the specific agonist (LPS from *E. coli* or PAM3CSK4 respectively) after TLR blocking (TLR4 and TLR2 respectively) (fig. 19A and data not shown). The optimal concentration of blocking antibodies (in order to block and do not increase the cytokine release aspecifically) was determined to be 2.5 µg/mL to block TLR4, and 1.5 µg/mL to block TLR2. The decoration of GMMA with TLR blocking antibodies doesn't give significant unspecific cytokine release, as tested stimulating PBMC with GMMA in presence of isotype control antibodies (data not shown).
To further investigate if the depletion of IL-6 release observed in TLR blocking experiments with GMMA is not due only to the presence of Anti-TLR antibodies that mask the other TLRs unblocked, we have tried to stimulate with soluble LPS after blocking the TLR2 by Anti-TLR2 Ab. Following that approach, if the shielding effect was not unspecific and the molecules might reach their respective receptor, we should appreciate the same activation of TLR4, so the same IL-6 release. With that approach soluble LPS should stimulate GMMA also after blocking of TLR2, so it's not present a steric hindrance effect (fig. 19 B).

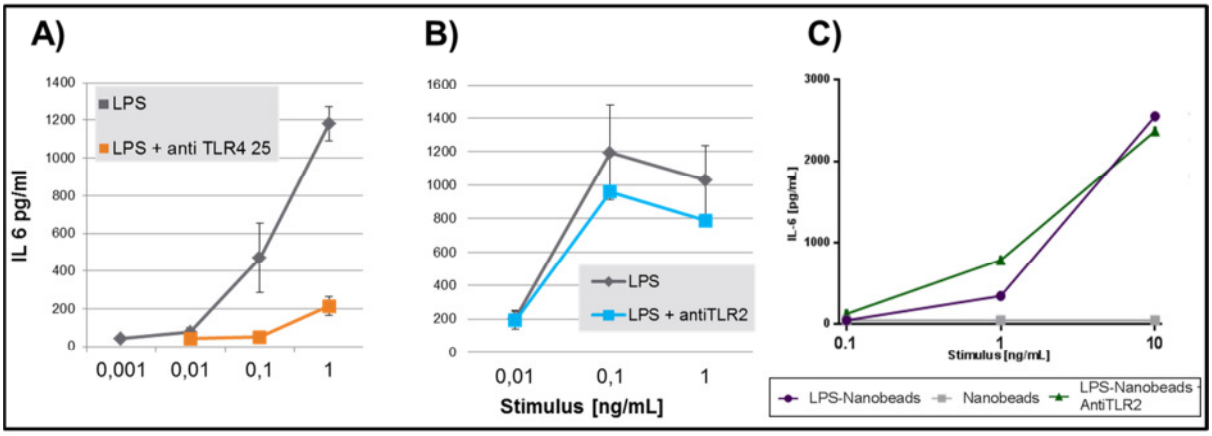

Fig. 19 IL-6 release obtained stimulating PBMC in presence or absence of Anti-TLR antibodies **A)** Stimulation with LPS (grey line) and stimulation with LPS after blocking of TLR4 with 25 µg/mL of anti-TLR4 antibodies (orange line) **B)** Stimulation with LPS (grey line) and stimulation with LPS after blocking of TLR2 with 15 µg/mL of anti-TLR2 antibodies (blue line) **C)** Stimulation with 30 nm polistrene nanobeads conjugated with LPS (violet line) and stimulation with 30 nm nanobeads conjugated with LPS after blocking of TLR2 with 15 µg/mL of anti-TLR2 antibodies (dark green line) and stimulation with 30 nm polistrene nanobeads (light grey line).

To further investigate if the depletion of IL-6 release observed in TLR blocking experiments with GMMA is due only to a steric hindrance effect given by the physical presence of Anti-TLR antibodies on the surface of PBMC that mask the other TLRs unblocked, we have tested polystyrene nanoparticles with 30 nm of average diameter (similar to the GMMA size), conjugated/not conjugated with LPS, before and after blocking with Anti-TLR2 Ab. Nanobeads conjugated with LPS should stimulate PBMC after blocking of TLR2 receptors (fig. 19C). Nanobeads unconjugated, instead, did

not activate at all the IL-6 release by PBMC, meaning no steric hindrance effect observed for TLR not blocked.

Characterization differences in GMMA between *S. sonnei / S. flexneri*

TLR2-NF-kB luciferase reporter assay

To confirm that the differences in the relative contribution of TLR4 and TLR2 to activation observed in the blocking experiments were solely dependent on the differential TLR4 activation by the different GMMA, the ability of the GMMA to activated TLR2 was tested by stimulating HEK293-TLR2 transfectant cells (Fig. 20).

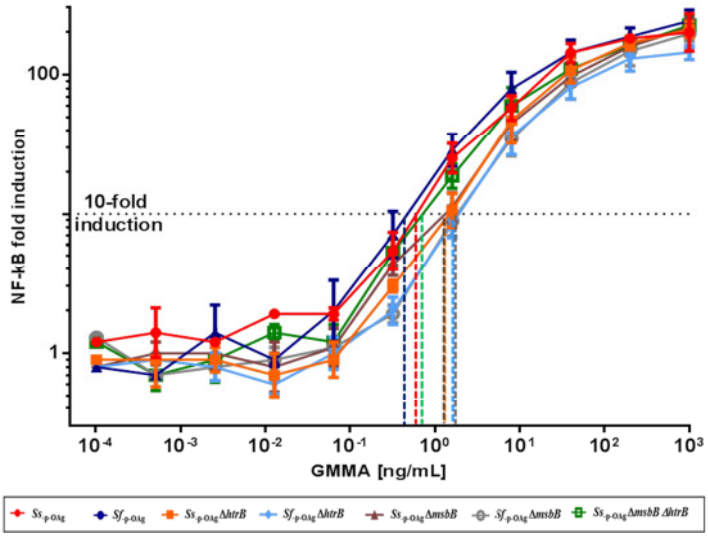

Fig. 20 Activation of TLR2 reporter cell line by different GMMA. 25,000 of TLR2-HEK293 cells/well were stimulated with 1000 – 0.0001 ng/mL (5-fold steps) of GMMA from *Ss*$_{-p\text{-}OAg}$, *Sf*$_{-p\text{-}OAg}$, *Ss*$_{-p\text{-}OAg}$ Δ*msbB*, *Sf*$_{-p\text{-}OAg}$ Δ*msbB*, *Ss*$_{-p\text{-}OAg}$ Δ*htrB*, *Sf*$_{-p\text{-}OAg}$ Δ*htrB*, *Ss*$_{-p\text{-}OAg}$ Δ*htrB* Δ*msbB*. After 5 h, luciferase expression was measured and expressed as fold-induction compared to 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 three independent experiments is shown.

All 5 Δ*msbB* and/or Δ*htrB* GMMA required similar GMMA concentrations (2.6 – 4.9 ng/mL as obtained in 4 independent experiments) to give a 10-fold NF-kB induction (Fig. 20), further confirming that differences observed in MAT assay reflect only differences in terms of TLR4 activation, so lipid A-related, and furthermore that the quantity of TLR2 activating molecules were similar in GMMA from all the strains tested; thus, TLR2 activating components were responsible of almost all the residual endotoxin activity of GMMA after lipid A modifications, in a comparable level.

Effect of lipid A modification in *Shigella* strains with different phenotypic backgrounds

Endotoxin activity

So far all of the data shown have been performed with GMMA without OAg. However, we have previously observed that the OAg can shield surface epitopes in *in vitro* grown *Shigella* (data not shown) and thus potential TLR activators. In order to test if the presence of OAg has an effect on the reactogenicity of GMMA or on the reduction of endotoxicity achieved with the *htrB* and *msbB* mutation, we inserted similar lipid A modifications in OAg-containing strains.
First of all the effect of the presence/absence of major virulence plasmid and the presence/absence of OAg were evaluated in lipid A WT background.
By MAT (fig. 21) we might appreciate that there aren't difference bot in *S. sonnei* than in *S. flexneri* given by the presence of major virulence plasmid or OAg.

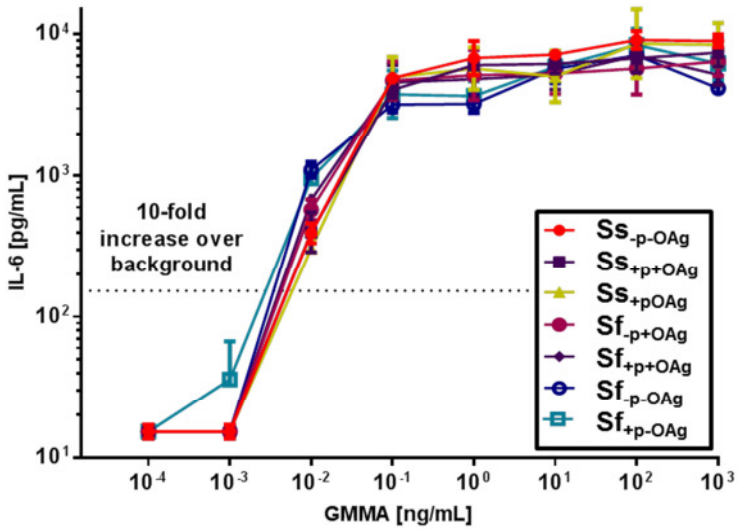

Fig. 21 Cytokine release by human PBMC after stimulation with different types of GMMA. A) GMMA with different genetic background were compared (+/- OAg or +/- virulence plasmid). Strains used for purifying GMMA were listed in material and method section. Human PBMC were isolated using a Ficoll density gradient and 200,000 cells were plated in each well; PBMC were stimulated for 4 hours with different concentration of GMMA, supernatants were recovered and tested by IL6 ELISA. Dashed line represent the IL6 released 10-fold over the background (mean of minimum point detected).

The *htrB* or *msbB* knock out were also introduced into strains that have the intact OAg synthesis gene cluster. In particular *S. flexneri* Δ*htrB* mutants with OAg and with or without virulence plasmid were produced, and also a Δ*msbB* mutant without the virulence plasmid. For *S. sonnei* a Δ*htrB* mutant with OAg and with the major virulence plasmid was generated. In *S. sonnei*, the OAg synthesis gene cluster is located on the virulence plasmid (163). The plasmid also carries a second copy of the msbB gene. Thus, we've chosen OAg-containing *S. flexneri* as parent strain for *msbB* knock out.
As shown in Fig. 22, the level of reactogenicity and endotoxin activity are not affected by the presence of OAg as resulted by comparing the IL-6 release obtained upon stimulating PBMC with GMMA that carries similar mutations from OAg deficient strains (compare Fig. 22 with Fig. 17). Nevertheless, also in OAg positive background, in *S. flexneri* we observed the compensation due to *lpxP* product, not

present in Ss_{+p+OAg} Δ*htrB* that carry the same mutation. Again, by complementing the knock out in *S. flexneri* by pACYC*htrB*, we observed the restorying of reactogenicity and lipid A type to wt level (by MALDi-TOF, data not shown).

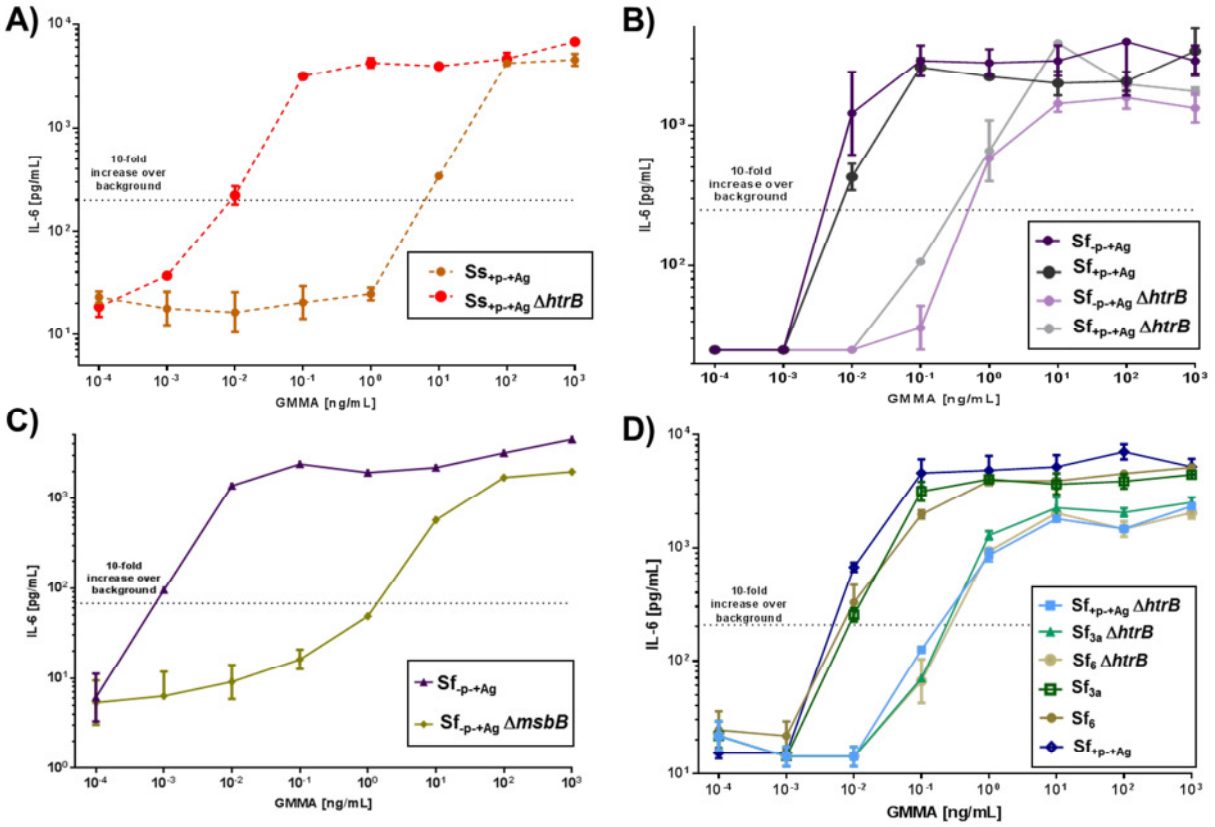

Fig. 22 MAT assay obtained with GMMA from different *Shigella* +OAg Δ*tolR* strains. A) *S. sonnei +p* and relative Δ*htrB* mutants B) *S. flexneri + and - p* and relative Δ*htrB* mutants C) *S. flexneri -p* and relative Δ*msbB* mutants D) Different *S. flexneri* and relative htrB mutant strains. Human PBMC were isolated using a Ficoll density gradient and 200,000 cells were plated in each well; PBMC were stimulated for 4 hours with different concentration of GMMA, supernatants were recovered and tested by IL6 ELISA. Dashed line represent the IL-6 released 10-fold over the background (mean of minimum point detected).

Salmonella

GMMA generation from different mutants

Salmonella enterica serovar Typhimurium and Enteritidis (animal clinical isolates) were chosen as parent strains and engineered to have overproduction of GMMA by *tolR* knock-out. The mutants were generated and characterized in respect to growth kinetics and GMMA yield by the *Salmonella* group at NVGH. Similar to what observed for *Shigella* GMMA, after *tolR* knock-out, both at 30 than at 37°C, the growing charateristics were similar; in fact, both duplication time and optical density reached after over-night colture were comparable in Lauria Broth (LB) media and in chemical defined media (data not shown). Strains carrying *msbB* and *htrB* knockout, instead were able to reach a higher optical density only at 30°C, both in chemical defined media and in LB. The duplication time in general increased after *htrB* or *msbB* deletion to up to 2 hours (data not shown) and a long lag phase (higher than 4 hours), but all the strains were able to reach high, even if lower than parent strains with wild type lipid A, and comparable O.D. after incubation over night in LB at 30°C (O.D. reached around 10 for all of them). 30°C in LB media was chosen as growing condition. GMMA yield from the different lipid A mutant strains, was high, in line to what observed for *Shigella* GMMA producing strains caring similar gene knock-outs (data not shown).

Shape (electron microscopy)

The morphology of GMMA from different *Salmonella* mutants was also evaluated by electron microscopy (fig. 23). Electron micrographs showed differences, especially between Typhimurium and Enteritidis GMMA.

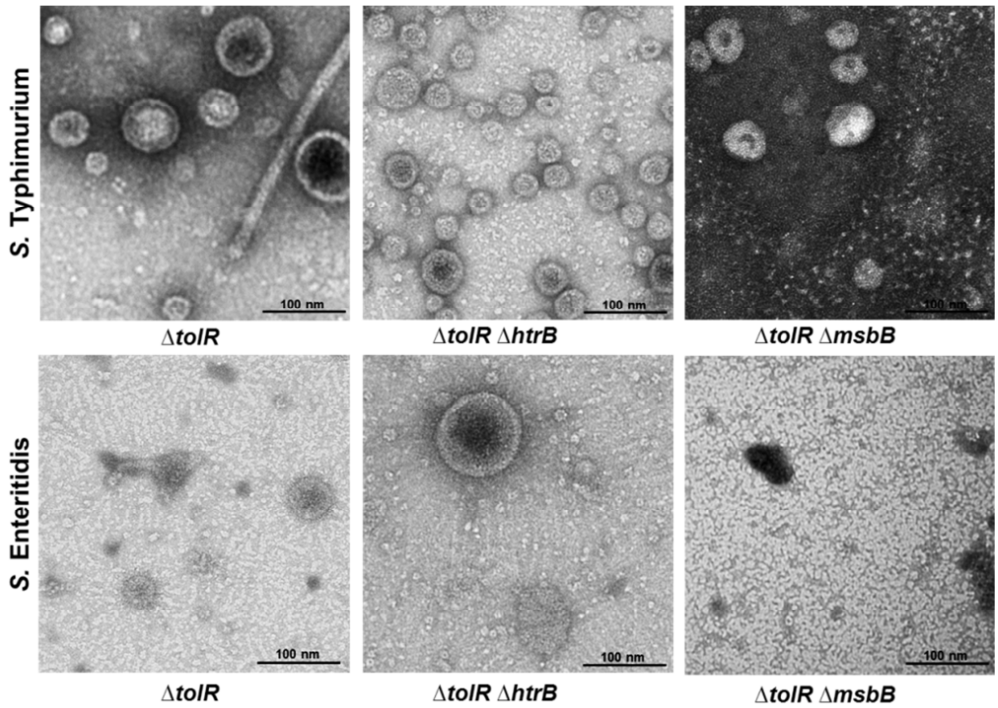

Fig. 23 – Electron microscopy of GMMA obtained from different strains. GMMA were prepared for negative staining, and viewed by electron microscopy (87 000 fold magnification). Scale bar represent 100 nm.

In general, GMMA from *S.* Typhimurium strains, before and after lipid A modifications, showed a similar shape with dimensions between 20 and 80-100 nm in diameter, and resulted to be well organized and broken GMMA were not observed at all in all the different field analyzed. Furthermore, in S. Tm GMMA we observed the presence of some flagella (in line with the process of purification and concentration; in fact the GMMA purification was obtained by ultracentrifugation directly of the supernatants, filtere at 0,22 µm, after growth).
In GMMA from *S.* Enteritidis strains we observed a general smaller organization, with the presence of two populations of GMMA, one really small (around 10 nm) and few bigger (even more than 100 nm, especially in Δ*htrB* mutants); this data was also confirmed by dynamic light scattering (data not shown).

Lipid A analysis by MALDI-TOF

A lipid A precipitation followed by mass spectrometry analysis by MALDI-TOF was performed (fig. 24) in order to evaluate the lipid A populations obtained after the *htrB* and *msbB* knock outs.
By that approach we might appreciate that the starting situation for lipid A unmodifid GMMA is correspondent to a mixture of hexa-acylated lipid A (peak at 1797 *m/z*, same as observed in *Shigella* GMMA with unmodified lipid A) but also an hepta-acylated lipid A (peak at 2035 *m/z*) due to the presence of a palmitoyl chain (a C_{16} fatty acid chain) attached to the hydroxil group of the myristoyl chain in position 2 and due to the action of PagP acyltransferases. Furthermore we observed, that, in lipid A unmodified background, the palmitoyl chain is present in all the *S.* Enteritidis strains tested and that only GMMA from S. Typhimurium strain didn't showed by MALDI-TOF the seventh chain to start with, even if PagP product is present in all the GMMA from lipid A mutant strains as demonstrated by the peak observed in Δ*htrB* and Δ*msbB* strains. Furthermore the presence of palmitoyl chan was not due to Δ*tolR* knock out itselves, because it has been observed directly on wild type bacteria, and the MALDI-TOF profile is similar both before and after that gene knock out.

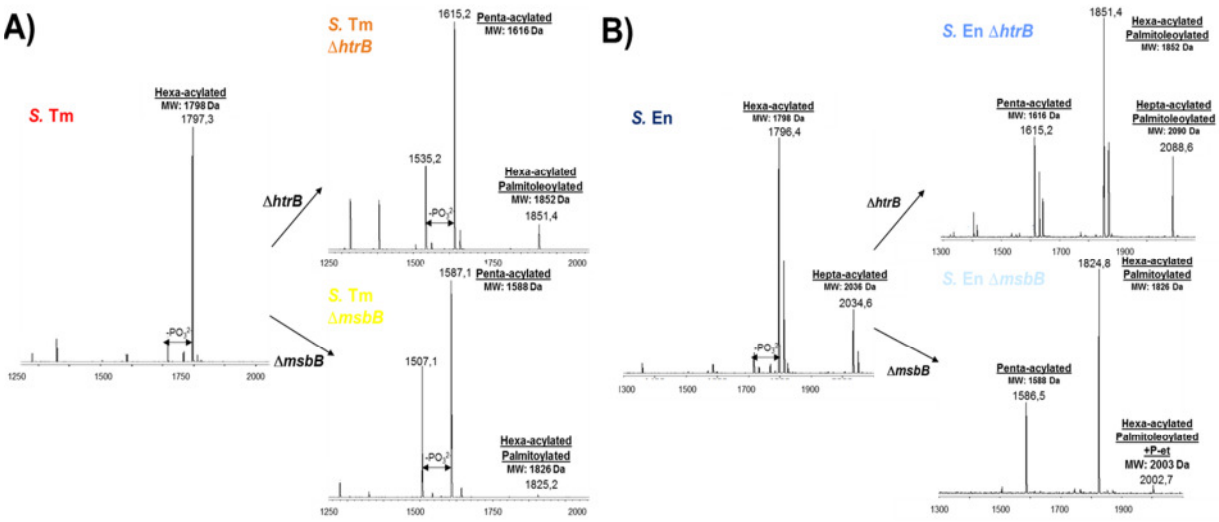

Fig. 24 MALDI-TOF spectra obtained analyzing lipid A obtained from *Salmonella* GMMA. Spectra were acquired in reflectron ion-negative mode

S. Tm Δ*msbB* GMMA, showed a lipid A mainly penta-acylated (peak at 1587 *m/z*) and only a small peak at 1825 *m/z* has been detected, most likely corresponding to a hexa-acylated lipid A species with a *m/z* different to "wild type" lipid A. The mass of this lipid A species corresponds to a lipid A containing a palmitoyl chain (a C_{16} fatty acid chain, 238 *m/z* shift) in the penta-acylated lipid A (1587 *m/z*), most likely due to activation of *pagP* after *msbB* knock out.
The same situation in terms of peaks observed was present also in S. En Δ*msbB* GMMA (fig. 24 B) but in that case the magnitude of the peak is different with a predominant presence of hexa-acylated palmitoylated lipid A (*m/z*=1825) and most likely is due to the knock-out of a miristoyl chain in the hepta-acylated lipid A species observed in lipid A unmodified background. Furthermore, in S. En Δ*msbB* we observed a small peak at *m/z* 2003, most likely correspondent to an hexa-acylated palmitoleoylated lipid A with the attach of a phospoetanolamine to the phosphate in position 1'; the shift of 177 Da is due to the shift of 54 m/z due to difference between $C_{16:1}$ fatty acid chain attached by LpxP and the C_{12} fatty acid chain attached by HtrB, plus 123 Da due to the attach of phospho-etanolamine at one of the phosphate group in position 1 or 4', catalyzed by EptA in the hexa-acylated palmitoylated background (*m/z*=1825).
In line with previous observation, we observed that *S.* Tm Δ*htrB* GMMA showed mainly two population of lipid A, coming from the hepta-acylated and hexa-acylated unmodified lipid A or to the action of PagP after knock out of *htrB* in the penta-acylated lipid A, that are correspondent to an hexa-acylated palmitoylated lipid A and a pure penta-acylated lipid A (peaks at *m/z* 1851 and 1615 respectively).
S. En Δ*htrB* GMMA showed the same two peaks at m/z 1615 and 1851 observed in S. Tm Δ*htrB* GMMA, but also a peak at m/z 2088 (shift of 236 Da) that is most likely due to the addition of the palmitoleoyl chain (a $C_{16:1}$ fatty acid chain) in hexa-acylated palmitoylated background. This attach is most likely due to LpxP and is the same compensatory effect observed in *S. flexneri* after *htrB* knock out.

Endotoxin activity of *Salmonella* GMMA

TLR4-NF-kB reporter assay

In order to evaluate the ability in activating TLR4 of the different "species" of lipid A molecules observed in GMMA purified from different lipid A mutants, experiments using TLR4-HEK293 cells have been performed (fig. 25).
In terms of TLR4 specific assay, the concentration of GMMA from S. Tm Δ*msbB* with mostly penta-acylated lipid A was around 10-fold higher than concentration of S. En Δ*msbB* GMMA and the GMMA from Δ*htrB* mutants with a mixture of hexa- (not wild type) and/or hepta- acylated form of lipid A to stimulate the same level of NF-kB induction. All the GMMA with unmodified WT lipid A, retained an at least 10-fold higher ability than GMMA with modified lipid A to activate TLR4 (fig. 25). The observations were similar to the observations in *Shigella*. Activation of TLR4 was retained in GMMA from Δ*htrB* mutants and S. En Δ*msbB*, similar to GMMA from *S. flexneri* Δ*htrB* with palmitoleoylated hexa-acylated lipid A. Only GMMA from S. Tm Δ*msbB* showed a similar behaviout to GMMA from *Shigella* Δ*msbB* mutants in terms of TLR4 specific activation (compare fig. 25 and fig 14).

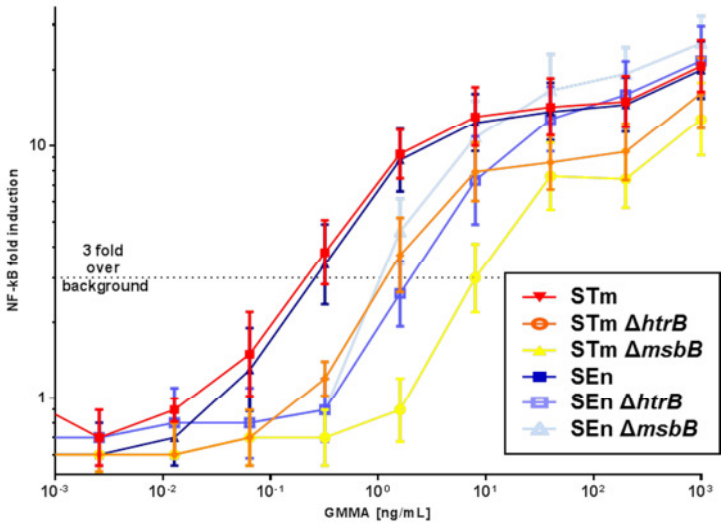

Fig. 25 TLR4-NF-kB specific assay. 25000 of HEK293 TLR4, MD2, CD14/well were stimulated for 5 hours with serial dilutions of GMMA obtained from different mutants.

It's important, to notice that the GMMA concentration to cause 3-fold NF-kB induction is around 10-fold lower in the *Salmonella* GMMA with WT lipidA in comparison to GMMA with WT lipid A from *Shigella* (compare tab. 12 and tab. 8).

GMMA from strains	GMMA conc. resulting in 3-fold NF-kB induction	Fold difference to GMMA with unmodified lip.A	Fold difference to *S.* Tm Δ*msbB GMMA*
S. Tm	0.23	-	36
S. Tm Δ*htrB*	1.16	5	7
S. Tm Δ*msbB*	8.18	36	-
S. En	0.27	-	30
S. En Δ*htrB*	2.02	7,5	4
S. En Δ*msbB*	1.06	4	8

Tab. 12 NF-kB specific TLR4 results analysis. Table showing concentrations of each type of GMMA resulting in 3-fold increased activation of NF-κB and fold difference of the respective concentration of GMMA from lipid A mutants to GMMA with wild-type lipid A (*S.Tm* and *S.* En respectively) and of all GMMA to GMMA from *S.* Tm Δ*msbB*

Comparison of endotoxicity of GMMA from different *Shigella* and *Salmonella* strains in MAT

GMMA from different *Salmonella* mutants were also tested in MAT by using human PBMC for evaluating their endotoxin activity in a more natural context where more PRRs might be activated simultaneusly. In order to compare the level of IL-6 stimulation by GMMA with lipid A modification from *Shigella* and *Salmonella*, they were tested together in the same PBMC experiment (fig. 26).

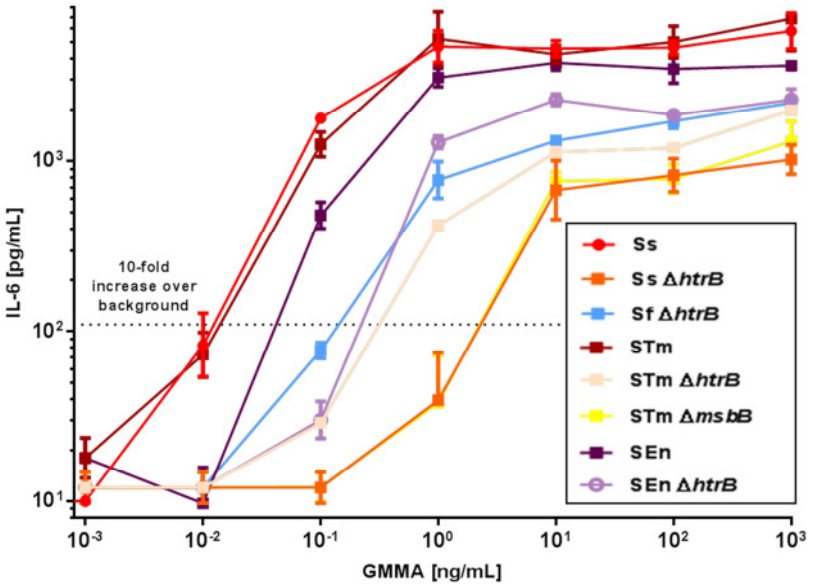

Fig. 26 Cytokine release by human PBMC after stimulation with different types of GMMA. 200,000 human PBMC cells were stimulated for 4 hours with 0.001-1000 ng/mL (10-fold steps) of GMMA from *Ss*$_{+p+OAg}$, *Ss*$_{+p+OAg}$ Δ*htrB*, *Sf*$_{-p-OAg}$ Δ*htrB*, *S*Tm, *S*Tm Δ*htrB*, *S*Tm Δ*msbB*, *S*En, *S*En Δ*htrB*, *S*En Δ*msbB*. Release of IL-6 was measured by specific ELISA and plotted as averages of duplicates with standard deviations. The black dashed line represents a 10-fold increase of cytokine release over the average release at the lowest concentration of GMMA.

By that approach we might appreciate that the 'starting situation' (with WT lipid A) is similar for GMMA produced from *Shigella* and *S* Tm. (tab. 13) which both contain hexa-acylated lipid A. In contrast, GMMA from *S.* Enteritidis were less stimulatory and required about 10-fold the amount of GMMA compared to S. Tm or *Shigella* GMMA to stimulate the same level of IL-6 release. This correlates to the fact that GMMA from *S.* Enteritidis contain a mixture of hexa-acylated and hepta-acylated lipid A.
Lipid A modifications due to *htrB* or *msbB* knock-out in S. Tm and S. En resulted in reduction of reactogenicity in MAT, in comparison to GMMA containing WT lipid A with a similar trend as in the TLR4 assay. In addition, the plateau of IL-6 release is lower for all GMMA with lipid A modification in comparison to GMMA from lipid A unmodified strains.

GMMA from strains	GMMA conc. resulting in 10-fold IL6 release	Fold difference to GMMA with unmodified lip.A	Fold difference to *Ss*$_{+p+OAg}$ Δ*htrBB GMMA*
Ss$_{+p+OAg}$	0,01	-	235
Ss$_{+p+OAg}$ Δ*htrB*	2,35	235	-
Sf$_{+p+OAg}$ Δ*htrB*	0,15	15	16
S. Tm	0,01	-	235
S. Tm Δ*htrB*	0,32	32	7
S. Tm Δ*msbB*	2,33	233	1
S. En	0,04	-	58
S. En Δ*htrB*	0,23	6	10

Tab. 13 MAT assays results from different blood donors. Concentrations of each type of GMMA resulting in 10-fold increased release of IL-6 were determined using PMBC. Fold differences were

calculated of the concentration of GMMA from lipid A mutants to GMMA with wild-type lipid A (*Ss*$_{+p+OAg}$, *S.Tm* and *S.En* respectively) and to GMMA from *Ss*$_{+p+OAg}$ Δ*htrB*

In particular, GMMA from S. En Δ*msbB* with predominantly penta-acylated lipid A showed a similarly low level of endotoxicity as GMMA from Ss$_{+p+OAg}$ Δ*htrB* containing penta-acylated lipid A that did not show detectable TLR4 activation in the PMBC assay (fig.18). Instead, GMMA containing palmitoleoylated or palmitoylated hexa-acylated lipid A or hepta-acylated lipid A retained approximately 10-fold higher IL-6 stimulation (tab. 14), similar to the observation with GMMA from Sf ΔhtrB with palmitoleoylated hexa-acylated lipid A.

Characterization of GMMA from *Salmonella* strains besides lipid A modifications

TLR2 – NF-kB reporter assay

Also the TLR2 activation from different mutants has been analyzed by using HEK293 trasfectant cells expressing human TLR2 receptors, with the objective of looking if something other is responsible of the endotoxicity in *Salmonella* GMMA besides TLR4 activator (lipid A) and especially if the activator of TLR2 from different mutants will be drastically different.
In terms of TLR2 activation all GMMA are similar (differences observed are below 5-fold), even if seems that all the lipid A mutants activated slightly more TLR2 than GMMA from strains with lipid A unmodified (most likely changes sigma E related) (fig.27).

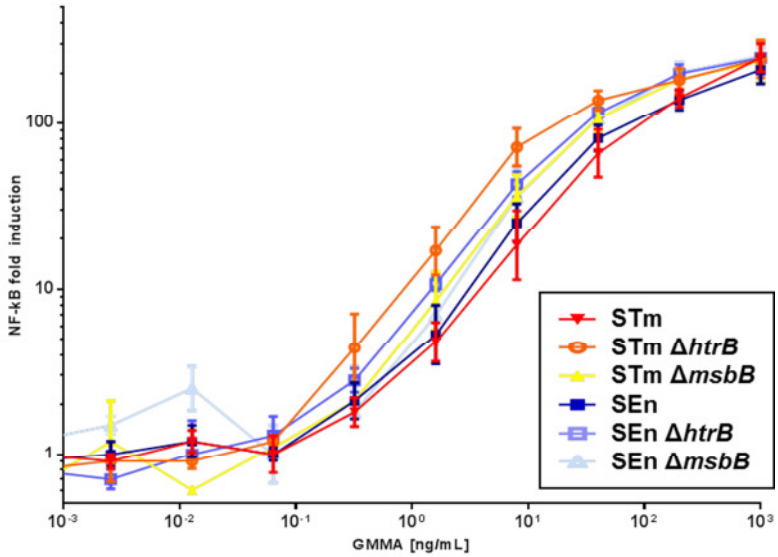

Fig. 27 TLR2 - NF-kB specific assay. 25000 of HEK293 TLR2 /well were stimulated for 5 hours with serial dilutions of GMMA obtained from different mutants.

Neisseria meningitidis

Characterization of GMMA from different mutants

GMMA tested were generated by strains carrying various combinations of mutations generated by the *Neisseria* group. The mutants (and GMMA generated from them) were engineered with the objective of obtaining a highly immunogenic and safe vaccine, with protection against multiple serogroups of *N. meningitidis* in order to use them in Africa. The deletion of the capsule (Δ*cps*) allows obtaining strains that are safer. In fact, strains without capsule were not infectious (53) and allow to present better antigens known to be protective (PorA, that gave subtype-specific protection) and fHbp (81,124). In addition, to increase the coverage of the vaccine, fHbp, that is divided into three antigenic variants (v. 1, 2 or 3) (164), was deleted from parent strain and substituted with fHbp from a more frequent variant and over-expressed (150). Furthermore, the deletion of *lpxL1* (81) should give strains with a reduced endotoxin activity, whereas the *gna33* knock out (148) allowed to increase the release of GMMA for increasing the yield and so reducing the final cost of the vaccine.

Shape of GMMA (Electron Microscopy)

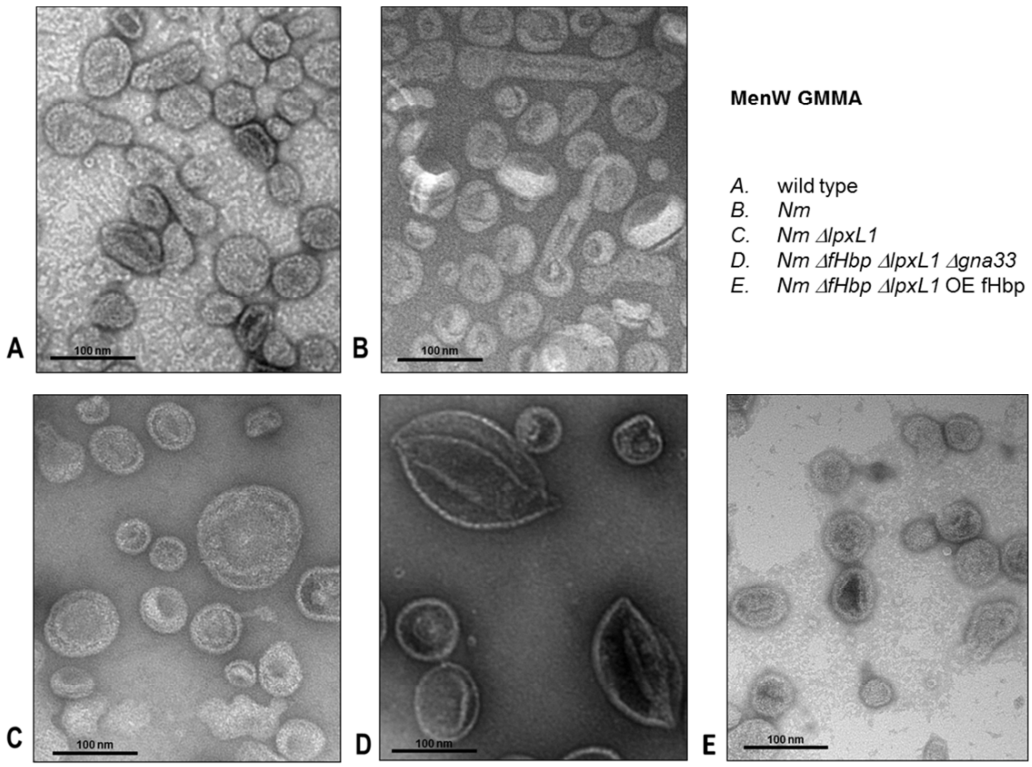

Fig. 28 – Electron microscopy of GMMA obtained from different strains. GMMA were prepared for negative staining, and viewed by electron microscopy (105,000-fold magnification). Scale bar represent 100 nm.

The shape of GMMA from different strains were also observed in electron microscopy (fig. 28) in order to see if differences in lipid A acylation (due to *lpxL1* knock out) or deletion and/or over-expression of a lipoprotein like fHbp gave significant differences

in terms of dimensions. We observed generally that the shape of GMMA is generally circular, but also different shapes have been observed and can be attributed to the random blebbing of *Neisseria* that is a high GMMA producer even in absence of *gna33* knock out. After the gna33 knock out we observed generally an increase in terms of the dimension of GMMA. In any case all the GMMA seem well organized and only few of them were broken and bigger than *Shigella* or *Salmonella* GMMA (around 100 nm in diameter).

MALDI-TOF analysis of lipid A

Lipid A from the various mutants has been extracted from GMMA and analyzed by MALDI-TOF. The obtained spectra and *Neisseria* lipid A species corresponding to the observed m/z (160) are reported in fig. 29.
The main peaks in the mass spectra obtained by MALDI-TOF in ion-negative reflectron mode from GMMA purified from lipid A unmodified strains of *Neisseria* (fig. 29) present two main peaks; the first one has an *m/z* correspondent to the theoretical mass (1838 Da, 1837 *m/z*) of an hexa-acylated lipid A that present a phospoetanolamine attached on the phosphate 1' due to the action of transferases encoded by *eptA,* instead the second ones is correspondent to an hexa-acylated lipid A without the phosphoetanolamine (1715 Da, 1714 *m/z*). We observed also the two peaks correspondent to the same two lipid A molecules described before but that have lost a phosphate in 4' position, most likely during the analysis.

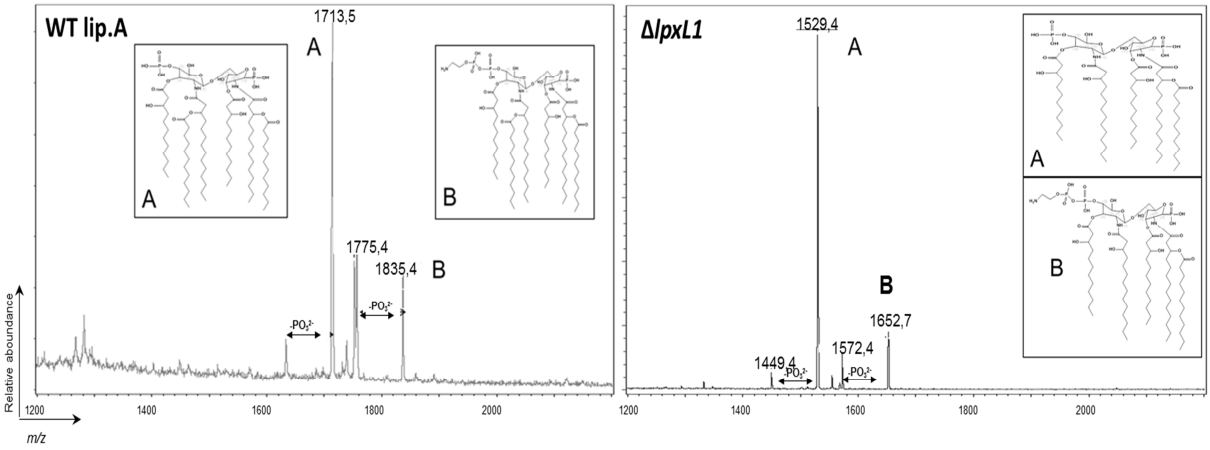

Fig. 29 MALDI-TOF spectra obtained analyzing lipid A obtained from *N. meningitidis* GMMA. Spectra were acquired in reflectron ion-negative mode

In the lipid A purified from GMMA from *lpxL*1 mutant strains we observed two populations, with and without phosphoetanolamine, with a shift of exactly 182 Dalton correspondent to the lack of a lauroyl (-C_{12} fatty acid chain), from the two lipid A species observed in GMMA purified from lipid A WT strain, consistent with an *lpxL1* knock out. The discrepancy between the theoretical *m/z* and the observed ones is only 1 Dalton and it's due to calibration (the discrepancy is the same for all the peaks observed). In fact, the *m/z* shift is perfect at Dalton level in chemical shift due to lack of fatty acid chains and/or phospoetanolamine.

Evaluation of endotoxin activity of *Neisseria* GMMA after genetic manipulation

Evaluation of reactogenicity by MAT and NF-kB specific assays (TLR2 and TLR4)

We investigated the reduction of endotoxicity of GMMA after lipid A modification (*lpxL1* knock out) by MAT (IL-6 release) and TLR4-specific assay. As shown in fig. 30 A, we observed a reduction of around 1,000 fold of ability to release IL-6 after *lpxL1* deletion compared to GMMA with wild-typd lipid A. In the TLR4-speicifc assay, the large decrease of TLR4 stimulating acitivity in the Δ*lpxL1* GMMA was further emphasized. They required 10,000-fold the amount of GMMA with unmodified lipid A to reach the same level of TLR4 activation. These results were in line with results obtained with *S. sonnei* Δ*htrB* GMMA.

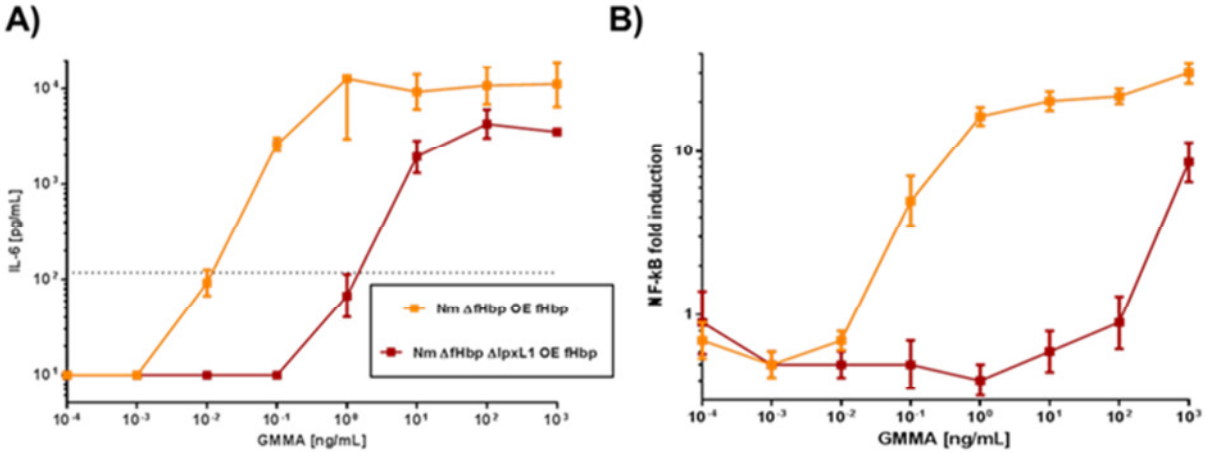

Fig. 30 Evaluation of endotoxin activity in *Neisseria* GMMA. A) IL-6 release by human PBMC: PBMC were stimulated for 4 hours with different concentration of GMMA (0,001-1000 bg/mL, 10-fold steps dilutions). **B) TLR4 specific assay**: 25000 of HEK293 TLR4, MD2, CD14/well were stimulated for 5 hours with serial dilutions of GMMA obtained from different mutants.

Comparison of endotoxicity of GMMA from different Shigella *and* Neisseria *strains in MAT*

In order to directly compare the reduction of reactogenicity after lipid A modifications in *Neisseria* and *Shigella*, GMMA from strains with WT and modified lipid A were testedin the same experiment (fig. 31).

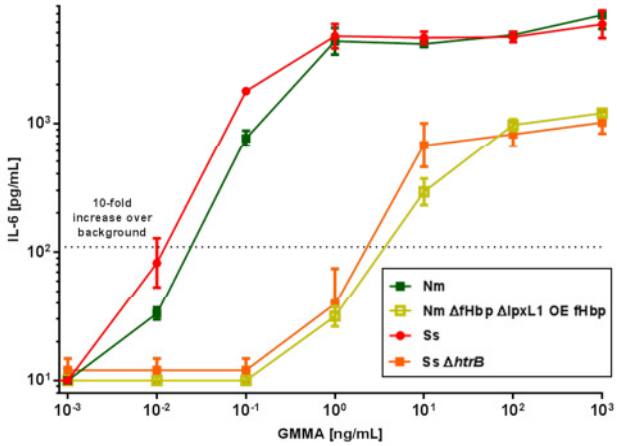

Fig. 31 IL-6 release by human PBMC. PBMC were stimulated for 4 hours with different concentrations of GMMA and IL-6 released in the supernatants was measured by specific ELISA.

By that approach we might appreciate that the starting (with WT lipid A) situation is similar for GMMA produced from *Shigella* and *Neisseria* (tab. 14). GMMA from both, Ss_{+p+OAg} Δ*htrB* and Nm Δ*fHbp* Δ*lpxL1* OEfHbp, gave similarly low IL-6 release results. They required approximately 1000-fold less amount compared to GMMA wtihout lipid A to stimulate the same level of IL-6 release. In addition, the plateau of maximum release is lower for both in comparison to lipid A WT GMMA.
GMMA from Ss_{+p+OAg} Δ*htrB* and Nm Δ*fHbp* Δ*lpxL1* OEfHbp both contain penta-acylated lipid A. The result indicate that the different fatty acid composition and the presence of phospoetanolamine in *Neisseria* lipid A does not alter the activation of TLR4. That results further suggest that a fully pent-acylated lipid A is required for drastically abolish TLR4 stimulation and that reflects in a big reduction on human PCMC).

GMMA from strains	GMMA conc. resulting in 10-fold IL6 release	Fold difference to GMMA with unmodified lip.A	Fold difference to *Ss+p+OAg ΔhtrBB GMMA*
Ss+p+OAg	0,01	-	235
Ss+p+OAg ΔhtrB	2,35	235	-
Nm	0,02	-	118
Nm ΔlpxL1 ΔfHbp OE *fHbp*	3,70	185	1,6

Tab. 14 MAT assays results from different blood donors. Concentrations of each type of GMMA resulting in 10-fold increased release of IL-6 were determined using PMBC. Fold differences were calculated of the concentration of GMMA from lipid A mutants to GMMA with wild-type lipid A (*Ss*+p+OAg, *Nm*) and to GMMA from *Ss*+p+OAg Δ*htrB*

Discussion

GMMA are attractive candidates for vaccines as the present surface antigens in their natural environment and confirmation. For use as vaccines, depending on the dose, the reactogenicity might need to be reduced as GMMA contain LPS and other TLR stimulatory components, e.g. lipoproteins. We have developed a high yield production process for GMMA (128). The goal of this thesis was to demonstrate the feasibility of reduction of reactogenicity of GMMA by genetic modification of the lipid A component of LPS.

We started by examining several factors to determine the most useful in-process assay for measuring the protein concentration of GMMA from multiple different bacteria, that is essential for comparing GMMA in functional assay. Those factors are: the ease of performing the assay, the reproducibility of the assay and the linearity of the assay. By comparing Bradford, Lowry and NI assays using a BSA standard calibrated against GMMA concentrations determined by amino acid analysis. By the above criteria, the Lowry assay was superior. First, it was by far the simplest assay to perform, requiring neither a strong chaiotropic agent (guanidine) and boiling (Bradford) or multiple centrifugations (NI Assay) and took significantly shorter time to perform the assay (approximately 15 minutes compared to approximately 45 minutes (Bradford) and 35 minutes (NI)). Second, and most important, the Lowry also gave substantially lower inter- and intra-assay variations. All three assays gave good linearity over a range of protein concentrations useful for vaccine production and testing (around 5 to 25 µg). For all three assays, and especially the Bradford assay, the color yield was substantially different to the color yield of BSA in the same assay and highlights the necessity to calibrate a secondary standard such as BSA against a primary GMMA standard determined by amino acid concentration or some other methods. Without this calibration, the Bradford assay substantially under estimated the amount of protein present and the Lowry gave a modest over-estimate. While generally true of all GMMA tested, the details did vary between GMMA with the *Neisseria* GMMA showing lower color yields for both the Bradford and Lowry assays. The NI assay relies on depletion of copper ions in solution when they bind to peptide bonds and thus generally has minimal protein to protein variation. In our studies it gave color yields of the GMMA protein closest on average to the color yield of BSA. However, there was substantial variation between different GMMA, greater intra- and inter-assay variation and it was a more complicated assay to perform that the Lowry assay. In the absence of a primary GMMA standard, for many applications these considerations are likely to outweigh the small underestimation in GMMA protein concentration that would result from the use of the Lowry assay calibrated only against a BSA standard.
Another key aspect, especially for the work performed in this thesis, was the comparability of the results for GMMA from multiple different bacteria. Also by this criterion, the Lowry assay was superior and showed the most consistent results for different types of GMMA. In general Lowry assay has been reported to show better results than other assays for membrane containing fractions (165), further showing a low inter-protein variability, and thus is beneficial for analyses when the standard is a different protein from the measured sample (166). Finally these results highlight the need for caution when comparing results from different laboratories if different protein assays are used and if they have not been calibrated against a primary GMMA

standard. In the absence of such calibration, GMMA assayed by Bradford and Lowry, two commonly used methods would have given more than a 5-fold difference in the apparent protein concentrations: an error sufficiently large to impact on interpretation of reactogenicity and immunogenicity of GMMA based vaccines.

The feasibility of reduction of GMMA reactogenicity by genetic lipid A modification was first assessed using *Shigella*.
Various *Shigella* strains lacking *htrB* or *msbB* have been succesefully obtained and GMMA from them have been characterized. All the lipid A mutants produced were able to grow under same conditions, optimized to 30°C as growing temperature and by using chemical defined or minimal medias; all lipid A mutants, besides growing slower than wild type, were able to reach high O.D. after over-night under those conditions, maintaining the utility of the process from an industrial point of view (128). The preferential for minimal or not reach media by lipid A mutants and lower growing temperature might be explained by the fact that membrane of bacteria with modified lipid A growing fast, as in conditions with high nutrient, have not enough time to rearrange and create a shape of outer membrane compatible with life.
The results of deleting the *msbB* gene from both *S. sonnei* Δ*tolR* and *S. flexneri* 2a Δ*tolR* and by deleting the *htrB* gene from *S. sonnei* Δ*tolR* were as expected: conversion of a hexa-acylated lipid A to a penta-acylated lipid A through loss of a myristic acid (Δ*msbB*) or lauric acid (Δ*htrB*). However, we have demonstrated the presence of a palmitoleoyl chain in lipid A purified from $Sf_{\text{-p-OAg}}$ Δ*htrB* GMMA by MALDI-TOF and MALDI TOF-TOF.
The inability to detect a hepta-acetylated Lipid A in either the *S. flexneri* 2a GMMA or the $Sf_{\text{-p-OAg}}$ Δ*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. This would also be consistent with palmitoleoylation catalyzed by the action of LpxP, a late acyltransferases that acts at the HtrB site, described as cold response in *E. coli* (108). Thus, the palmitoleoylation could be part of a stress response. Similarly, in the *E. coli* Δ*lpxL* mutant the abundance of the palmitoeoylated lipid A increased with stress: it was a medium abundant species at 30°C and the dominant species at 37°C (167). In *S. flexneri* 2a Δ*htrB* the palmitoleoylated lipid A was the dominant species at 30°C, and very small amount of penta-acylated lipid A has been found. In Δ*tolR* mutant strains, the *tolR* mutation could provide stress, and, thus, result in higher abundance of palmitoleoylated lipid A at 30°C, although why this was not found in the $Ss_{\text{-p-OAg}}$ Δ*htrB* strain examined is not clear. All Δ*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 ($Sf_{\text{-p+OAg}}$ Δ*htrB*, $Sf_{\text{+p+OAg}}$ Δ*htrB*). For these, palmitoleoylation might be a strongly selected compensation after the *htrB* knock out. Consistent with this hypothesis, all the attempts to obtain an *lpxP* knock out in $Sf_{\text{-p-OAg}}$ Δ*htrB* failed, besides the possibility of knocking out the gene in $Ss_{\text{-p-OAg}}$ Δ*htrB.*
The reason for the differential presence of palmitoleic acid on lipid A between $Ss_{\text{-p-OAg}}$ Δ*htrB* and $Sf_{\text{-p-OAg}}$ Δ*htrB* is not clear. We were able to observe a small amount of palmitoleoylated lipid A in this *S. sonnei* grown at 12°C, indicating that there was a functional LpxP present. However, by using quantitative real time PCR, we found a 7-fold higher gene expression of *lpxP* compared to the constitutive *msbB* in $Sf_{\text{-p-OAg}}$ Δ*htrB* compared to in $Ss_{\text{-p-OAg}}$ Δ*htrB* grown at 30°C, possibly resulting in a higher level of LpxP protein present at 30°C in the *S. flexneri* Δ*htrB* strain. Furthermore, LpxP

carries a Leu^{116}→ Pro substitution in *S. sonnei* which could affects the activity of the enzyme.
Interestingly, when growing bacteria at 37°C, the lipid A profile of *Ss*$_{\text{-p-OAg}}$ Δ*htrB* remain similar to the one at 30°C (penta-acylated), instead in *Sf*$_{\text{-p-OAg}}$ Δ*htrB*, besides the main peak is still represented by the palmitoleoylated ones, appeared also one peak correspondent at the addition of a palmitoyl chain, most likely due to the action of PagP (fig. 32). This might indicated a further stress response and again a difference of *S. flexneri* to *S. sonnei* besides a similar genetic background.

Although the impact of lipid A modifications on TLR4 activation has been widely studied (117,121,168) the availability of GMMA from isogenic *Shigella* lines all with similar concentrations of lipid A enabled us to examine in more detail, the residual ability of modified lipid A to activate human TLR4 receptors either in HEK293-TLR4 transfectant cells or in human PBMC in the presence or absence of TLR2 and TLR4 blocking antibodies. We could detect no residual TLR4 activity of the 1616 Da penta-acylated lipid A resulting from the loss of a lauroyl chain as a result of the Δ*htrB* mutation. This was accompanied by a 60,000x increase in the amount of GMMA required to give similar activation of TLR4 compared to the parent GMMA (500 ng/mL for a 3x activation measured by NF-κB luciferase induction). GMMA from both *S. sonnei* and *S. flexneri* with the 1588 Da penta-acyl lipid A resulting from a loss of a myristoyl chain as a result of the Δ*msbB* mutation, gave activation at similar GMMA concentrations (4.7 ng/mL and 4.6 ng/mL, respectively, for a 3x NF-κB luciferase induction). Although this required substantially more GMMA than ones needed from the parent strains (0.008 ng/mL) it was still much less than the *S. sonnei* Δ*htrB* penta-acylated lipid A, showing that this penta-acylated lipid A retains some residual ability to activate human TLR4.
In *S. flexneri* 2a GMMA, the substitution of a lauroyl chain with the longer palmitoleoyl chain in hexa-acylated lipid A also led to approximately a 30x decrease in the ability to simulate human TLR4 as judged by the concentration of GMMA (0.42 ng/mL) required to give a 3x NF-κB luciferase induction. Besides the fact that the mass spectrum is represented by a mixture of hexa-, penta- and tetra-acylated lipid A, the hexa-acylated peak was by far the dominant peak present, and while the relatively heights of the peaks in this experiment is not necessarily strictly proportional to the abundance in the GMMA, it seems highly unlikely that a 50x reduction in activation could be due to the decrease in percentage of the hexa-acylated by the presence of these lower sized peaks.

In order to evaluate the endotoxin activity of GMMA in a more natural context and in particular to examine if the GMMA could simulate PRRs rather than TLR4, GMMA purified form different mutants were used to stimulate human PBMC in MAT. In general, the differences in the reactogenicity measured on PBMC were smaller than what observed looking at the specific TLR4 response using HEK293 transfectants. This observation is in line with the complexity of the material tested. As GMMA are derived from the outer membrane of Gram-negative bacteria they also contain lipoproteins and thus likely stimulated TLR2 in addition to TLR4.
The *S. sonnei* Δ*htrB* GMMA showed the biggest decrease in cytokine release in comparison to lipid A unmodified GMMA, approximately 800-fold on average. This was a 20-fold larger decrease than the decrease observed for *S. flexneri* Δ*htrB*. GMMA purified from *S. sonnei* or *S. flexneri* Δ*msbB* strains produced similar level of cytokines with a decrease around 300-fold to lipid A unmodified GMMA and 3-fold

lower than GMMA from *S. sonnei* Δ*htrB*. GMMA from *S. sonnei* Δ*htrB* Δ*msbB* (containing only tetra-acylated lipid A) gave similar results, both in MAT and TLR4 specific assay, as *S. sonnei* Δ*htrB* GMMA. This showed that the Δ*htrB* deletion reduces the lipid A endotoxicity to a level that cannot be further reduced by deletion of an additional acyl chain.

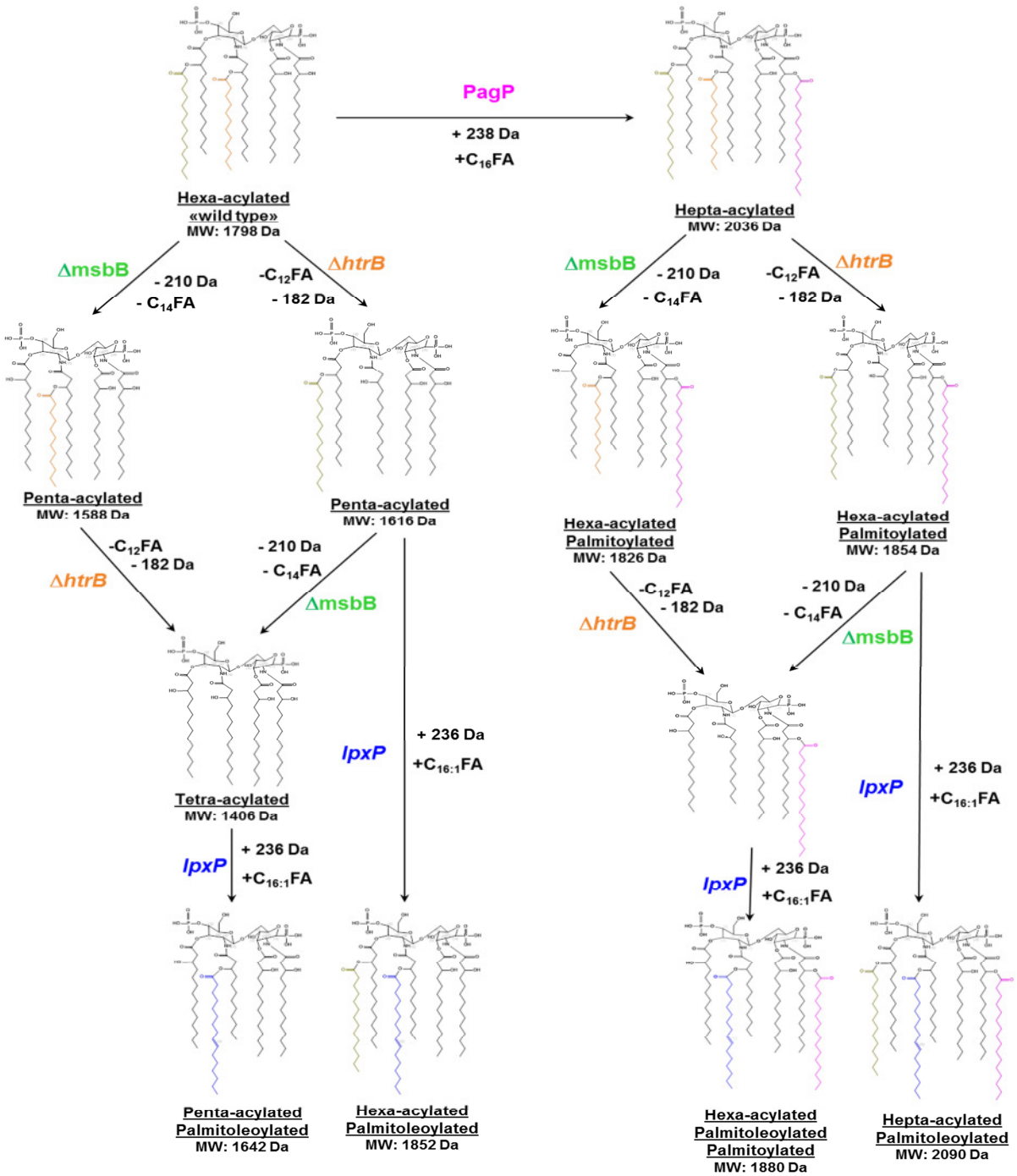

Fig. 32 *Shigella* and *Salmonella* lipid A acylation after lipid A mpodifications. Presence of Phispoetanolomine due to action of EptAin phosphate in position 1 is theoretically possible in all the lipid A structures (not reported)

As previously described, the different acylation status of lipid A, and the phosphorylation status of the molecule, affects the tilt angle of di-glucosammine blackbone and accordingly the recognition by TLR4 (109,110,113). Thus, the

differences observed between GMMA that carry *htrB* or *msbB* knock out are likely linked to the fact that HtrB and MsbB act in different position in lipid A, resulting in different binding to TLR4. The palmitoleoylated hexa-acylated lipid A is likely more similar in structure to the wild-type lipid A explaining the higher TLR4 activation by *S. flexneri* Δ*htrB* in comparison to *S. sonnei* Δ*htrB* GMMA.

The similar strategy for reducing the endotoxin activity as used for *Shigella* GMMA has subsequently been applied to *Salmonella*. In these bacteria the lipid A composition is complicated by the constitutive presence of PagP resulting in the addition of a palmitoyl chain. Thus, in GMMA cointaing unmodified lipid A we observed a mixture of hexa-acylated lipid A and hepta-acylated lipid A by MALDI-TOF (fig. 32 right side). In line with that observation, *Salmonella* Δ*msbB* GMMA showed a penta-acylated and an hexa-acylated lipid A species different to WT, consistent with an *msbB* knock-out. The ratio between penta- and hexa-acylated lipid A is different in S. Typhimurium and S. Enteritidis Δ*msbB* GMMA, with the latter containing mainly penta-acylated lipid A. In a similar way *S.* Typhimurium Δ*htrB* GMMA showed two populations of lipid A coming from the hepta-acylated and hexa-acylated WT lipid A lacking a lauroyl chain. Furthermore, S. Enteritidis Δ*htrB* GMMA, besides showing the presence of the same two peaks observed in S. Typhimurium Δ*htrB* GMMA, showed also a hepta-acylated peak given by the addition of the palmitoleoyl chain in hexa-acylated palmitoylated background (same compensatory effect observed in *S. flexneri* after *htrB* knock out).

The different types of lipid A observed by MALDI in GMMA from different *Salmonella* mutants results in differences in reactogenicity, as observed by HEK-TLR4 cells and MAT assay. In fact, by TLR4-specific assay, the GMMA with mostly penta-acylated lipid A derived from S. Typhimurium Δ*msbB* were around 10-fold less stimulatory than the mixture of hexa- (not wild type) and/or hepta-acylated lipid A species observed in the GMMA from S. Enteritidis Δ*msbB* and from both Δ*htrB* strains. Those GMMA resulted in 10-fold lower TLR4-stimulatory activity than GMMA containing unmodified lipid A.
S. Typhimurium Δ*msbB* GMMA were also the least stimulatory of all tested *Salmonella* GMMA with modified lipid A by MAT, in accordance to be the only ones showing mainly penta-acylated lipid A (more than 95%). The resulting difference to GMMA from the respective wild type was approximately 100-fold. In addition, the level of reactogencity was similar to GMMA from *Shigella* mutants cointaing penta-acylated lipid A. The reduction of endotoxin activity observed, especially for the mostly penta-acylated GMMA from *S.* Tm Δ*msbB*, is in line to what was observed by other groups testing heat-killed bacteria (169) and rise also the problem of the needs of a different strategy for lipid A modifications in *Salmonella* in order to gain a fully penta-acylated lipid A.The finding that in the other analyzed *S.* Tm and *S.* En Δ*htrB* and Δ*msbB* mutants hexa-acylated lipid A was found (due the plamitoleoylation through LpxP or plamitoylation through PagP) shows that additional genetic modifications to *htrB* and *msbB* will be needed to obtain predominantly penta-acylated lipid A in *Salmonella,* for examples a Δ*msbB* Δ*pagP* double mutation (170).

Also the reduction of endotoxin activity in species less related to *Shigella* has been evaluated by testing GMMA from *Neisseria meningitidis* lipid A mutants that carries *lpxL1* knock out (122). This gene is functionally similar to Δ*htrB* and the mutation has

been widely studied for reducing -reactogenicity on GMMA (81) and NOMV vaccines (126).
Lipid A extracted from GMMA purified from non-modified lipid A *Neisseria* stains showed two main peaks correspondent to an hexa-acylated lipid A (with a different lipid A composition in comparison to *Shigella* GMMA) with and without the presence of a phospoetanolamine (fig. 33) as indicated by a shift of 182 Dalton, consistent with an *lpxL1* knock out.

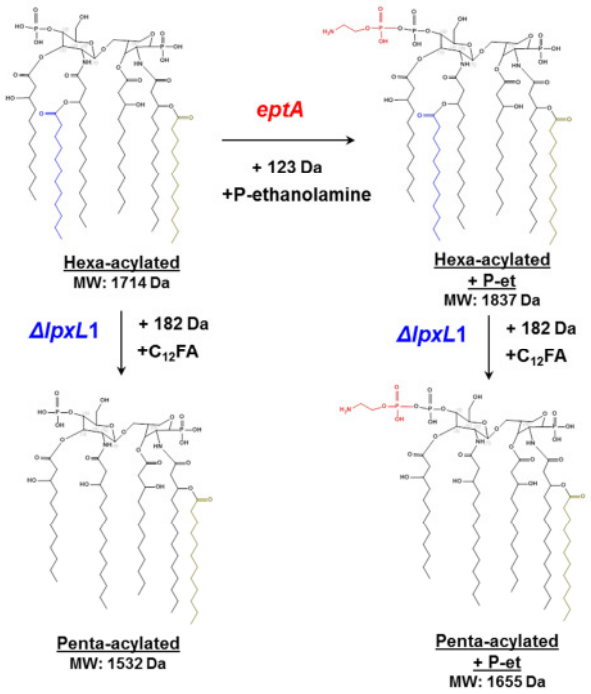

Fig. 33 *Neisseria* lipid A acylation pattern in lipid A mutants

In TLR4 specific assay *lpxL1* knock-out resulted in an almost complete reduction of endotoxicity of *Neisseria* GMMA, like what observed with *S. sonnei* Δ*htrB*, meaning no effect due to presence of phospoetanolamine in lipid A and due to the different fatty acid composition in terms of TLR4 activation.
By MAT that translates in a reduction of 1,000 fold after *lpxL1* deletion (in line to what has been observed with *S. sonnei* Δ*htrB* GMMA).

Besides the fact that the main activator of innate immune response contained in GMMA is LPS, we have also evaluated other characteristics that might have been changed in GMMA after lipid A modification. The objective was to characterize and understand the relative contribution of other activators besides LPS present in GMMA to the activation of other pathways besides TLR4.
By using Anti-TLRs antibodies we demonstrated that the residual reactogenicity in GMMA after lipid A modification is mainly mediated through TLR2, except the residual TLR4 activation present in *S. flexneri* Δ*htrB* with palmitoleoylated hexa-acylated lipid A. Furthermore, by blocking with both anti-TLR4 and anti-TLR2 antibodies, we demonstarted that the stimulation caused by GMMA is almost completely abolished (more than 90%), suggesting that, in the conditions used in the assay, the relative contribution of other pathways is marginal (lower than 10%) in the activation of PBMC.

By using HEK293-TLR2 cells we observed that TLR2 stimulation is similar for all the GMMA from *Shigella* (and *Salmonella*) mutants, confirming that differences observed in MAT assay reflects differences in terms of TLR4 activation; furthermore, we demonstrated that TLR2 activating components were the main responsible of the residual endotoxicity of GMMA after lipid A modifications, in a comparable level; thus, TLR2 activating components represents the target if a further reduction of reactogenicity of GMMA will be needed.
Protein composition was evaluated by SDS-PAGE and the overall pattern resulted to be maintained in GMMA purified from different mutants from same bacteria; only small differences have been observed and, by identifying using Peptide Mass Fingerprinting the proteins correspondent to most different bands, those resulted to be metabolism-related and not reported as PRR activators.
Also the shape of the GMMA from different strains was evaluated by electron-microscopy, and GMMAresulted to be composed mainly by an homogenous population with no differences related to lipid A modifications. The possible presence of flagellin in *Salmonella* GMMA preparations (as impurities that remains stacked to GMMA during purification, or purified with them as small fragments released during growth of bacteria) might be evaluated in the final development of a vaccine strain, because the presence of flagella might mediate innate immune stimulation through TLR5 activation, and can be the responsible of smaller differences observed in MAT.

In conclusion, the broad aim of the thesis was to examine ways of reducing the reactogenicity of GMMA to make them suitable for use as human vaccine. It resulted in surprisingly complex outcome.
We demonstrated the feasibility to reduce GMMA reactogenicity by lipid A modification. We found a clear relation between the composition of the lipid A species and reactogenicity. Our analysis of GMMA from isogenic lines and from different species highlighted the importance of determining the composition of the lipid A after the genetic modification as the same genetic modification gave different outcomes even in closely related *Shigella* species.
We demonstrated in *Shigella*, *Salmonella*, and *Neisseria* that GMMA with penta-acylated lipid A have a marked reduction of cytokine stimulation ability. Using *Shigella* GMMA we showed that the residual reactogenicity is predominantly TLR2 mediated. In contrast, GMMA with hexa-acylated lipid A, either through palmitoleoylation after *htrB* knock-out, and/or due to additional palmitoylation in *Salmonella*, retain TLR4 stimulatory activity and are less likely to be useful as vaccine. Thus, that compensatory effect must be evaluated in bacteria that possesses *lpxP* and may undergo on same compensatory effect
Based on the results presented in this thesis, genetic modification of the lipid A biosynthesis pathway resulting in penta-acylated lipid A is a promising strategy for reducing the reactogenicity of GMMA. If further decrease of reactogenicity was required, modifications of TLR2 activating components would be necessary. However, the level of reduction required for an acceptable vaccine will depend on the dose necessary to give a strong immune response and that can only be determined by clinical trials. Clinical trials underway now using the *S. sonnei* Δ*htrB* GMMA should give an important indication of the tolerability of these constructs.

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Appendix

Period abroad:

28th January – 1st March 2014, visiting scientist at Wellcome Trust Sanger Institute, Cambridge (UK), working on Pathogen Genomic group under the supervision of Prof. Nicholas Thomson

Publications:

1) F. Berlanda Scorza, A. M. Colucci, L. Maggiore, S. Sanzone, **O. Rossi**, I. Ferlenghi, I. Pesce, M. Caboni, N. Norais, V. Di Cioccio, A. Saul, C. Gerke - High yield production process for *Shigella* Outer Membrane Particles – 2012 - PlosOne, volume 7 (6) e35616
2) O. Koeberling, E. Ispasanie, J. Hauser, **O. Rossi**, G. Pluschke, D. Caugant, A. Saul, C.A. MacLennan - A broadly protective vaccine based on generalized modules for membrane antigens (GMMA) from recombinant serogroup W strains against meningococcal disease in sub-Saharan Africa - submitted – Vaccine
3) **O. Rossi**, I. Pesce, C. Giannelli, S. Aprea, M. Caboni, F. Citiulo, S. Valentini, I. Ferlenghi, C.A. MacLennan, U. D'Oro, A. Saul, C. Gerke – Modulation of endotoxicity of *Shigella* Generalized Modules for Membrane Antigens (GMMA) by genetic lipid A modification: relative activation of TLR4 and TLR2 pathways in different mutants – submitted – Journal of Biological Chemistry
4) **O. Rossi**, L. Maggiore, O. Koeberling, F. Necchi, C.A. MacLennan, A. Saul, C. Gerke – Comparison of colorimetric assays with quantitative amino acid hydrolysis for quantifying proteins in GMMA - Analytical biochemistry - in preparation (aimed submission April 2014)

Conferences and advanced courses attended:

- Microbial pathogenesis and host response, Cold Spring Harbor Laboratory meeting (New York, USA), 17th-21st Sep. 2013
- Workshop "Working with pathogen genomes" – Wellcome Trust Sanger Institute (UK), 9th- 14th Dec. 2012
- Novartis-Harvard meeting, Minisimposium in vaccines and host pathogen interaction – Novartis (SI) – 09th Jun. 2012

Communication at conferences (underlined the presenter):

- **O. Rossi** - Detoxification of *Shigella* GMMA by genetic lipid A modification: two strains, one mutation, two outcomes – Novartis Ph.D. workshop – Novartis (SI) – 20-21 Nov. 2013 – Oral presentation
- A. Marini, **O. Rossi**, S. Guadagnuolo, I. Delany, I. Ferlenghi, A. Saul, C.A. MacLennan, O. Koeberling - GMMA from genetically-engineered meningococcus

for an affordable vaccine against *N. meningitidis* in sub-Saharan Africa – Novartis Ph.D. workshop – Novartis (SI) – Nov. 2013 - Poster presentation

- S. Sanzone, A. M. Colucci, C. Giannelli, L. Sollai, **O. Rossi**, O. Vassallo, E. Cannizzaro, A. Podda, K. Bloink, A. Saul, V. Di Cioccio, C. Gerke - A *Shigella sonnei* vaccine based on GMMA – towards proof-of-concept for a novel vaccine platform – Vaccine for Enteric Disease 2013 (Bangkok, Thailand) – Nov. 2013 - Oral presentation
- **O. Rossi**, I. Pesce, C. Giannelli, S. Aprea, S. Valentini, F. Citiulo, M. Caboni, I. Ferlenghi, U.D'Oro, A. Saul, C. MacLennan, C. Gerke – Detoxification of *Shigella* GMMA by genetic lipid A modification: two strains, one mutation, two outcomes - Microbial pathogenesis and host response, Cold Spring Harbor Laboratory meeting (New York, USA) - 17th-21st Sep. 2013 – Poster presentation
- O. Koeberling, E. Ispasanie, **O. Rossi**, J. Hauser, G. Pluschke, D. Caugant, C.A. MacLennan - A broadly protective vaccine based on GMMA from recombinant serogroup W strains against meningococcal disease in sub-Saharan Africa - EMGM conference 2013 – Sep. 2013 – Poster presentation
- M. Caboni, T. Pedron, **O. Rossi**, D. Goulding, D. Pickard, T. Connor, N. Thomson, C. A. MacLennan, A. Saul, G. Dougan, P. J. Sansonetti, C. Gerke - A deep rough mutant of *Shigella sonnei* reveals the expression of an O antigen capsule important for bacterial pathogenesis - Gordon Research Conference 'Microbial Adhesion and Signal Transduction – Jul. 2013 - Poster presentation
- **O. Rossi**, I. Pesce, F. Citiulo, M. Caboni, C. Giannelli, S. Aprea, S. Valentini, U. D'Oro, A. Saul, C. MacLennan, C. Gerke - Detoxification of *Shigella* GMMA by genetic lipid A modification: two strains, one mutation, two outcomes - Research days 2013 – Novartis (SI) – Jul. 2013 – Poster presentation
- C. Gerke, F. Berlanda Scorza, A.M. Colucci, L. Maggiore, I. Pesce, T.A. Schrandt, R. Cole, J. Vandenhoek, H. Casanova, M. Caboni, S. Sanzone, **O. Rossi**, I. Ferlenghi, N. Norais, V. Di Cioccio, K. Bloink, A. Saul. - Development a *Shigella* vaccine based on GMMA. Poster – ASTMH meeting (Atlanta, USA) – Nov. 2012 – Oral presentation
- **O. Rossi** - Reactogenicity and composition of *Shigella* GMMA from lipid A mutants – Novartis Ph.D. workshop – Novartis (SI) – 22-23 Nov. 2012 – Oral presentation
- Gerke C., Berlanda Scorza F., Colucci A.M., Cole R., Schrandt T.A., Maggiore L., **Rossi O.**, Pesce I., Caboni M., Vandenhoek J., Casanova H., Norais N., Di Cioccio V., Bloink K., Saul A. – Development a *Shigella* vaccine based on GMMA – Phacilitate Vaccine Forum 2012 (Washington DC) – Jan. 2012 – Oral presentation

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High Yield Production Process for *Shigella* Outer Membrane Particles

Francesco Berlanda Scorza[1¤], Anna Maria Colucci[1], Luana Maggiore[1], Silvia Sanzone[1], Omar Rossi[1], Ilaria Ferlenghi[2], Isabella Pesce[1], Mariaelena Caboni[1], Nathalie Norais[2], Vito Di Cioccio[1], Allan Saul[1], Christiane Gerke[1]*

1 Novartis Vaccines Institute for Global Health, Siena, Italy, **2** Novartis Vaccines and Diagnostics, Siena, Italy

Abstract

Gram-negative bacteria naturally shed particles that consist of outer membrane lipids, outer membrane proteins, and soluble periplasmic components. These particles have been proposed for use as vaccines but the yield has been problematic. We developed a high yielding production process of genetically derived outer membrane particles from the human pathogen *Shigella sonnei*. Yields of approximately 100 milligrams of membrane-associated proteins per liter of fermentation were obtained from cultures of *S. sonnei* Δ*tolR* Δ*galU* at optical densities of 30–45 in a 5 L fermenter. Proteomic analysis of the purified particles showed the preparation to primarily contain predicted outer membrane and periplasmic proteins. These were highly immunogenic in mice. The production of these outer membrane particles from high density cultivation of bacteria supports the feasibility of scaling up this approach as an affordable manufacturing process. Furthermore, we demonstrate the feasibility of using this process with other genetic manipulations e.g. abolition of O antigen synthesis and modification of the lipopolysaccharide structure in order to modify the immunogenicity or reactogenicity of the particles. This work provides the basis for a large scale manufacturing process of Generalized Modules of Membrane Antigens (GMMA) for production of vaccines from Gram-negative bacteria.

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* E-mail: christiane.gerke@novartis.com

¤ Current address: Novartis Vaccines and Diagnostics, Siena, Italy

Introduction

Shigella spp. are Gram-negative bacteria that infect the intestinal epithelium and cause dysentery. In 1999 the World Health Organization estimated an annual burden of 164.7 million shigellosis cases throughout the year of which 163.2 occur in developing countries, including 1.1 million deaths, mostly in children younger than 5 years of age [1]. Four serogroups have been identified: *S. dysenteriae* (15 serotypes), *S. boydii* (20 serotypes), *S. flexneri* (14 serotypes) and *S. sonnei* (1 serotype) [2]. No vaccine is currently available. So far, vaccine candidates based on O antigen conjugates and live attenuated strains have been shown in clinical trials to protect against homologous strains [2–6]. Vaccines using inactivated bacteria or subcellular components are at various stages of development [3,6].

Gram-negative bacteria naturally shed outer membrane particles consisting of outer membrane lipids, outer membrane proteins, and enclosed periplasmic proteins [7–9]. Unlike most unilamellar biological vesicles, outer membrane particles are formed by blebbing and not by invagination of the membrane. Thus, the orientation of components in the membrane of the outer membrane particles is the same as in the bacterial outer membrane and the components in the outer face of the bacterial outer membrane are also in the outer face of the outer membrane particles [7]. Outer membrane particles are naturally shed at low concentration. Mutations such as the deletion of gene *gna33* in *Neisseria meningitidis* [10] or modifications of the *tol-pal* pathway of *Escherichia coli*, *Shigella flexneri*, and *Salmonella enterica* serovar Typhimurium [11,12] can increase the level of shedding. Especially, deletion of the *tolR* gene in *E. coli* has been shown to result in substantial overproduction of outer membrane particles without loss of membrane integrity [11,13]. Studies have characterized the protein content of these outer membrane particles [10,13], and unlike conventional detergent-extracted outer membrane vesicles derived from homogenized bacteria they are almost free of cytoplasmic and inner membrane components and maintain lipoproteins. The outer membrane particles used for those proteomic studies have been derived in small quantities from cells grown to low cell density.

It has been previously proposed that outer membrane particles could be exploited for use as vaccines [10,12]. The immunogenicity of outer membrane particles from a variety of Gram-negative bacteria has been studied. Consistent with their high content of stimulators of the innate immune system, e.g. lipopolysaccharide (LPS) [7] and Toll-like receptor 2 (TLR2) agonists [14], they are strongly immunogenic in the absence of adjuvant. They have been shown to induce protection in mice against multiple pathogens, including *Salmonella enterica* serovar Typhimurium [15], *Helicobacter pylori* [16], *Vibrio cholera* [17,18], or to elicit antibodies in mice with *in vitro* bactericidal activity, e.g. for *Neisseria meningitidis* [19]. Recently, outer membrane particles from *Shigella flexneri* 2a have been shown to confer protection in mice after mucosal immunization [20]. Although these studies suggest that outer membrane particles may form the basis of vaccines [15,17,18], there remain several problems: their reactogenicity and the difficulty of purifying them in the quantity and at costs that would make them attractive as vaccines for the public sector most impacted by diseases such as shigellosis.

The problem of reactogenicity is amenable to genetic manipulation. A variety of strategies has been examined to attenuate the pyrogenicity of LPS by modifying genes involved in lipid A biosynthesis, e.g. *msbB* and *htrB* in *Shigella* and *E. coli* or *lpxL* in *Neisseria* that are required for complete acylation and thereby pyrogenicity of lipid A [21–24]. However, a major remaining difficulty is developing a scalable method for the high volume and low unit cost production of vaccines based on this method.

In this paper we show that high purity outer membrane particles from *Shigella sonnei* mutant strains can be produced from fermentation in chemically defined medium with high yield using a simple purification process thus making production of inexpensive vaccines feasible. We believe that this process will be widely applicable for production of Gram-negative membrane antigens and thus call it the 'Generalized Modules for Membrane Antigens (GMMA)' process. In the literature, outer membrane particles that are either naturally released or produced by genetically modified strains are usually referred to as outer membrane vesicles (OMV). The same term has also been used for the vesicles derived by detergent-extraction of homogenized bacteria currently used as vaccines, e.g. MeNZB, an outer membrane vesicle vaccine used to control *Neisseria meningitidis* type B infections in New Zealand. In order to differentiate the two substantially different types of OMV [10] we chose the term GMMA to specify the particles released from the surface of intact cells used in this study.

Methods

Construction of Shigella Sonnei 53G Mutants

Shigella sonnei 53G [25] was chosen as parent strain. The null mutants *tolR* [13], *galU* [26], and *msbB1* [21] were obtained by replacing the *gene* coding sequence with a resistance cassette [27]. Kanamycin was used for *tolR*, chloramphenicol for *galU* and erythromycin for *msbB1*. To achieve this, we used a three step PCR protocol to fuse the *gene* upstream and downstream regions to the resistance gene. Briefly, the upstream and downstream regions of the gene were amplified from *Shigella sonnei* 53G genomic DNA with the primer pairs gene.AB.500-5/gene.ABL-3 and gene.AB.L-5/gene.AB.500-3, respectively (details of target 'gene', antibiotic cassette 'AB' and sequence are reported in Table 1). The kanamycin cassette was amplified from pUC4K [28] and the *cat* gene from pKOBEG [29] using the primers ampli.AB-5/ampliAB-3 (Table 1). Finally the three amplified fragments were fused together by mixing 100 ng of each in a PCR reaction containing the gene.AB.500-5/gene.AB.500-3 primers. The linear fragment to delete *tolR* was used to transform recombination-prone *Shigella sonnei* 53G carrying pAJD434 to obtain the respective deletion mutant *S. sonnei* Δ*tolR*. Recombination-prone *S. sonnei* Δ*tolR* was then transformed with the linear fragment for the deletion of *galU*, resulting in mutant strain *S. sonnei* Δ*tolR* Δ*galU*. A clone of *S. sonnei* Δ*tolR* lacking the virulence plasmid, *S. sonnei* –pSS Δ*tolR*, was selected by white appearance on congo red agar. The curing of the virulence plasmid (pSS) was confirmed by the absence of the origin of replication and the plasmid encoded gene *wzy* using primers pS.so53G.oriF/pS.so53G.oriR and pS.so53G.wzyF/pS.so53G.wzyR respectively (Table 1). Two functional *msbB* genes are present in *Shigella* [21]. In the Δ*tolR* background, the copy located on the virulence plasmid (*msbB2*) was removed by curing the plasmid and the plasmid pΔ*msbB*ko::ery was constructed to delete the gene *msbB1* on the chromosome. Upstream and downstream flanking regions of the *msbB1* gene were amplified by PCR with the XbaI.*msbB*.5′.F/EcoRV.*msbB*.5′.R and EcoRV.*msbB*.3′.F/XhoI.*msbB*.3′.R primers, respectively. Both products were cloned into the pBluescript (Stratagene) vector in Max Efficiency® *E. coli* DH5α^{TM-}T1^{R} (Invitrogen). The *erm* erythromycin resistance gene [30] was amplified with primers EcoRV.Ery.F/EcoRV.Ery.R and was inserted into the EcoRV site between the flanking regions generating pΔ*msbB*ko::ery. Primers XbaI.*msbB*.5′.F/XhoI.*msbB*.3′.R were used to amplify by PCR a linear fragment from pΔ*msbB*ko::ery plasmid, containing the resistance cassette flanked by *msbB1* flanking regions that was used to transform the recombination-prone plasmid-cured *Shigella sonnei* 53G Δ*tolR* strain to generate the *msbB* knockout mutant. Recombination-prone

Table 1. Primers used in this study.

tolR.Kan.500-5	TCTGGAATCGAACTCTCTCG
tolR.Kan.L3	ATTTTGAGACACAACGTGGCTTTCATGGCTTACCCCTTGTTG
tolR.Kan.L5	TTCACGAGGCAGACCTCATAAACATCTGCGTTTCCCTTG
tolR.Kan.500-3	TTGCTTCTGCTTTAACTCGG
ampli.Kan-5	ATGAGCCATATTCAACGGGAAAC
ampli.Kan-3	TTAGAAAAACTCATCGAGCATCAAA
galU.Cm.500-5	AAAATCAACGGTTGCCAGAG
galU.Cm.L-3	CGAAGTGATCTTTCCGTCACATTAAATTCTCCTGGACTGTTC
galU.ext-5	GCCTGGTGCTTGATATTGC
galU.Cm.500-3	GCGCAGGCAAGAGAATGTA
galU.Cm.L-5	CAGTTATTGGTGCCCCATCCGTATCGGTGTTATCC
galU.ext-3	TCCTGGCTATTGCACAACT
ampli.Cm-5	TGTGACGGAAAGATCACTTCG
ampli.Cm-3	GGGCACCAATAACTGCCTTA
XbaI.*msbB*.5′.F	CTAGTCTAGAAGTGCTTTCAGTGGGTGACG
EcoRV.*msbB*.5′.R	AGCTTGATATCCCATGCTTTTCCAGTTTCGG
EcoRV.*msbB*.3′.F	AGCTTGATATCGGCGAAATCCAACCGTATAAG
XhoI.*msbB*.3′.R	CCGCTCGAGGGGGAAGTTGTTAAGACAGAC
EcoRV.Ery.F	AGCTTGATATCAGAGTGTGTTGATAGTGCAGTATC
EcoRV.Ery.R	AGCTTGATATCACCTCTTTAGCTTCTTGGAAGCT
pS.so53G.oriF	CGTAACCGTAATTACAGCCG
pS.so53G.oriR	GATTTCACCTTACCCATCCC
pS.so53G.wzyF	CGTTGAGGTTTCACGTTTCT
pS.so53G.wzyR	TTACCAATATACCCTCCGCA

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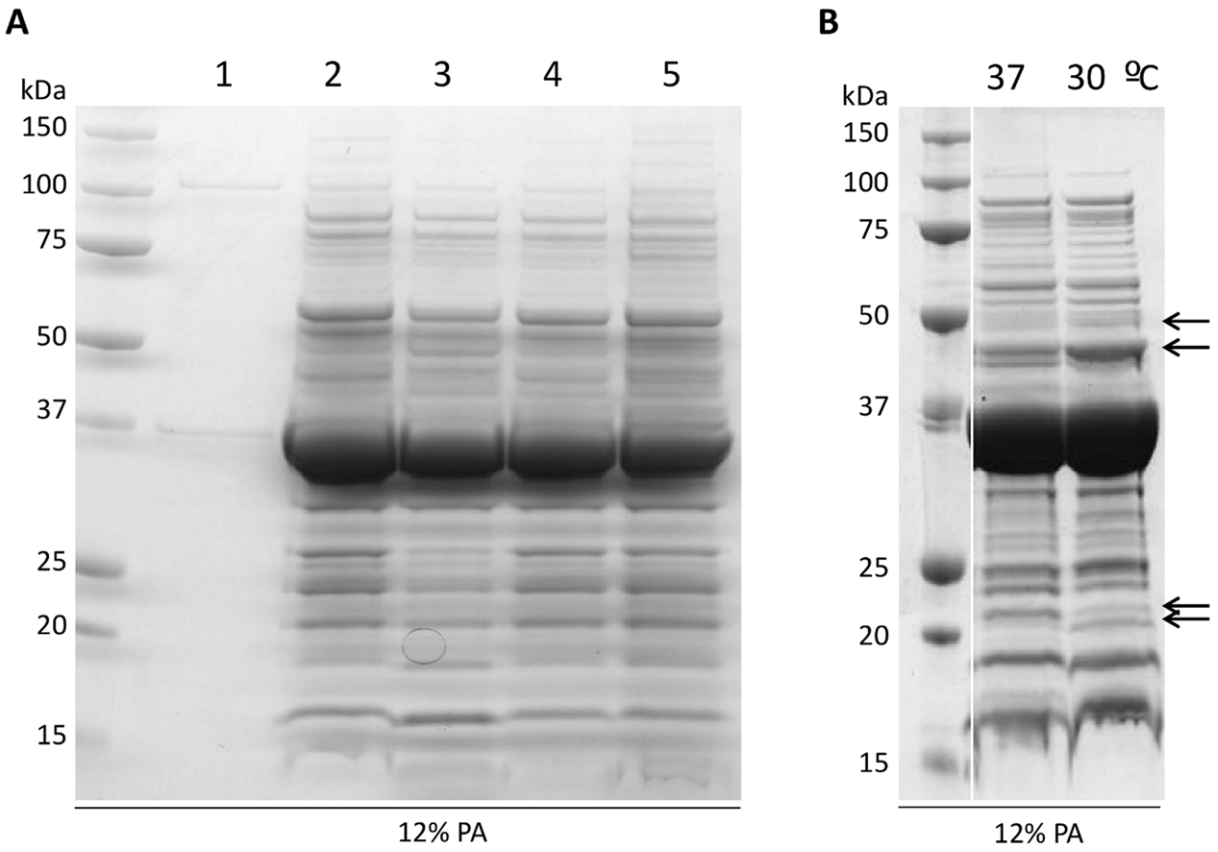
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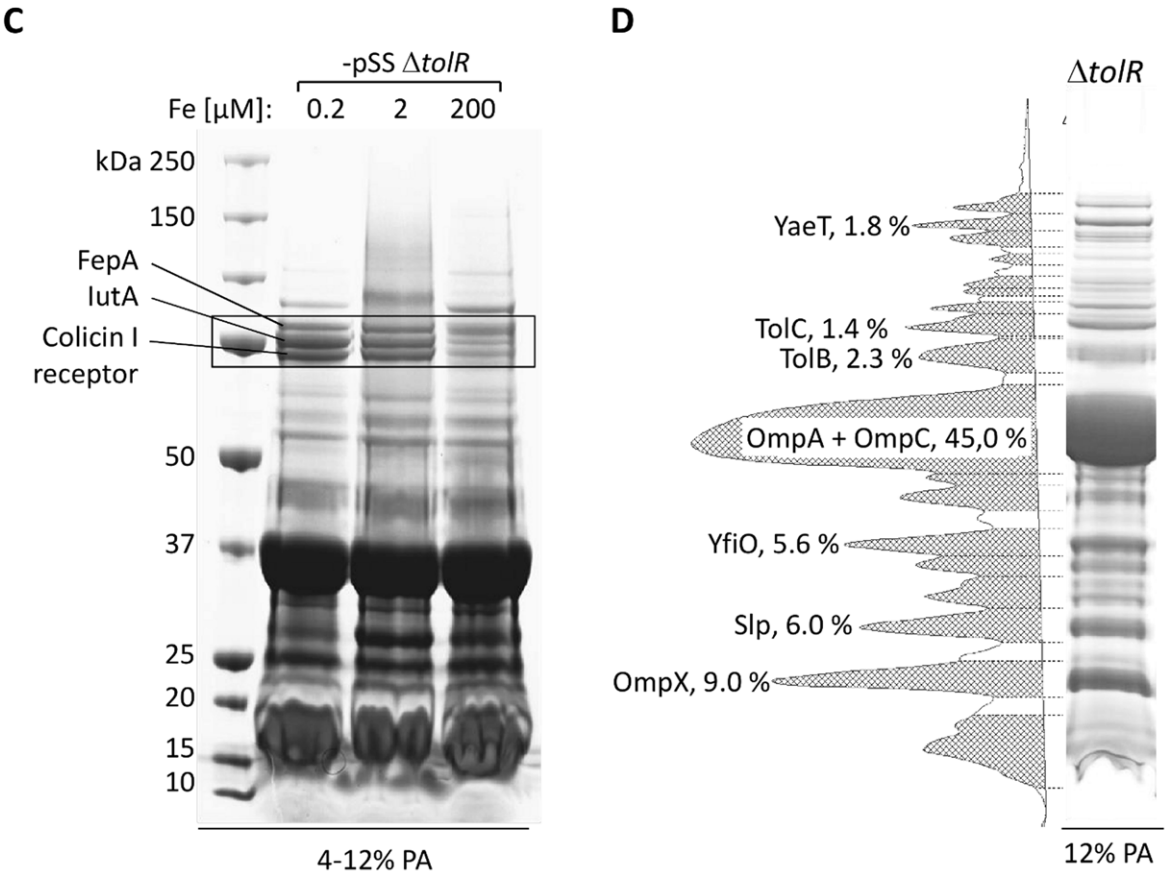
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ΔtolR
YaeT, 1.8 %
TolC, 1.4 %
TolB, 2.3 %
OmpA + OmpC, 45,0 %
YfiO, 5.6 %
Slp, 6.0 %
OmpX, 9.0 %
12% PA

Figure 1. Comparison of S*higella sonnei* GMMA from different strains and different conditions. A)25 ml of culture supernatants were collected from (1) wild type *S. sonnei* 53G, (2) *S. sonnei* Δ*tolR* (3) *S. sonnei* Δ*tolR* Δ*galU*, (4) *S. sonnei* –pSS Δ*tolR*, and (5) *S. sonnei* –pSS Δ*tolR* Δ*msbB* grown in flasks in chemically defined medium at 30°C. Proteins were precipitated from the supernatants and quantified using Bradford assay. 10 μg of samples 2–5, respectively, and the total quantity of sample 1 obtained from 25 mL of supernatant were separated by SDS-PAGE (12% polyacrylamide (PA)). All strains with deletion of the *tolR* gene show an extensive protein profile in the supernatant compared to wild type. B) GMMA were purified by ultracentrifugation from flask cultures of *S. sonnei* –pSS Δ*tolR* grown in chemically defined medium with 100 μM iron at 37°C and 30°C. 10 μg of protein were separated by SDS-PAGE (12% PA). The protein pattern of GMMA obtained at the different temperatures is similar. Visible differences are marked by arrows. C) *S. sonnei* 53G –pSS Δ*tolR* was grown in flasks in chemically defined medium with defined iron concentrations. GMMA were purified by ultracentrifugation and GMMA proteins were separated by SDS-PAGE (4–12% PA). Three bands identified as FepA, IutA, and colicin I receptor were shown to be repressed by high iron concentration. D) Densitometry analysis of GMMA preparation from strain *S. sonnei* Δ*tolR* Δ*galU* grown in a 5 L fermenter to OD 45. The most abundant proteins were identified by protein mass fingerprint and relative amounts were determined by densitometry analysis. Of the highlighted proteins, all proteins with exception of TolB are predicted to be associated with the outer membrane, indicating that approximately 69% of the total protein amount in GMMA is derived from abundant proteins linked to the outer membrane.
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Shigella sonnei 53G cells were produced by using the highly proficient homologous recombination system as previously described (*red* operon) [31] encoded on pAJD434 [32]. pAJD434 was subsequently removed from the mutant strains.

Bacterial Strain Growth Conditions and Media

Shigella sonnei and *E. coli* strains were routinely cultured in Luria-Bertani (LB) medium. When required, kanamycin (30 μg/mL), chloramphenicol (20 μg/mL), trimethoprim (100 μg/mL), or ampicillin (100 μg/mL) were added. Tryptic soy agar (30 g/L tryptic soy broth, 15 g/L agar) supplemented with 150 mg/L congo red was used to evaluate the presence of the virulence plasmid in *Shigella*. GMMA were prepared from cultures grown in flasks or in a 5 L fermenter (Applikon) in yeast extract medium (HTMC) or *Shigella sonnei* defined medium (SSDM). HTMC was prepared as follows: yeast extract 30 g/L, KH_2PO_4 5 g/L, K_2HPO_4 20 g/L, $MgSO_4*7H_2O$ 1.2 g/L, glycerol 15 g/L, polypropylene glycol (PPG) 0.25 g/L. SSDM was prepared as follows: glycerol 30 g/L, KH_2PO_4 13.3 g/L, $(NH_4)_2HPO_4$ 4 g/L, $MgSO_4*7H_2O$ 1.2 g/L, citric acid 1.7 g/L, $CoCl_2*6H_2O$ 2.5 mg/L, $MnCl_2*4H_2O$ 15 mg/L, $CuCl_2*2H_2O$ 1.5 mg/L, H_3BO_3 3 mg/L, $Na_2MoO_4*2H_2O$ 2.5 mg/L, $Zn(CH_3COO)_2*2H_2O$ 13 mg/L, ferric citrate 2 μM (unless specified differently in text), thiamine 50 mg/L, nicotinic acid 10 mg/L, L-aspartic acid 2.5 g/L, PPG 0.25 g/L. For fermentation, starter cultures were grown from glycerol stocks to OD 0.8 and subsequently transferred to the 5 L fermenter to reach a starting OD of 0.02. Dissolved oxygen was maintained at 30% saturation by controlling agitation and setting maximum aeration. The pH was maintained at 7.2 in HTMC or at 6.7 in SSDM, with 4 M ammonium hydroxide by a pH controller and temperature was kept constant either at 37°C or at 30°C. From flask cultures, supernatants were collected by 10 min centrifugation at 4000 g followed by 0.22 μm filtration or by tangential flow filtration. The optical density (OD) of cultures was measured at 600 nm wavelength.

Tangential Flow Filtration Purification

A 2-step tangential flow filtration (TFF) process was used to purify GMMA. During the first TFF step, the culture supernatant which contains the GMMA was separated from the bacteria using a 0.2 μm pore size cassette (Sartocon HYDROSART 0.2 μm, Sartorius). When approximately 80% of the starting feed was recovered as filtrate, the remaining biomass (retentate) was washed in five diafiltration steps with phosphate buffered saline (PBS). The GMMA-containing culture supernatant and the GMMA-containing filtrate of the diafiltration steps were combined. In a modified process the diafiltration of the biomass was omitted. Experiments performed without this diafiltration step are specified in the text. In the second step, the combined filtrate was micro-filtered using a 0.1 μm pore size membrane (Sartocon SLICE 200 0.1 μm, Sartorius) in order to separate GMMA that remain in the retentate from soluble proteins (filtrate). After five diafiltration steps using PBS, the retentate containing the GMMA was collected and sterile filtered using a 0.22 μm Express^{TM} PLUS stericup (Millipore).

Protein Quantification

Proteins were quantified by Bradford method, using bovine serum albumin as standard. GMMA were boiled for 10 minutes in 3.0 M guanidine hydrochloride prior quantification.

Negative Staining Electron Microscopy

A drop of 5 μL of GMMA suspension was placed on copper formvar/carbon-coated grids and adsorbed for 5 min. Grids were then washed with few drops of distilled water and blotted with a Whatman filter paper. For negative staining, grids were treated with 2% uranyl acetate in ddH_2O for 1 min, air-dried and viewed with a CM100 transmission electron microscope (Philips, Eindoven, the Netherlands) operating at 80 kV. Electron micrographs were recorded at a nominal magnification of 60000×.

Denaturing Mono-Dimensional Electrophoresis

GMMA were denatured for 3 min at 95°C in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% (wt/vol) SDS. 20 μg of proteins were loaded onto 12% (wt/vol) or 4–12% (wt/vol) polyacrylamide gels (BioRad, Hercules, U.S.A.). Gels were run in 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (BioRad) and were stained with Coomassie Blue R-250.

Two-Dimensional Electrophoresis

Two hundred micrograms of GMMA were separated by 2-dimentional electrophoresis (2-DE) as previously described [10]. Briefly, proteins were separated in the first dimension on a non linear pH 3–11 gradient and in the second dimension on a linear 4–12% polyacrylamide gradient unless specified in text. Gels were stained with colloidal Coomassie G-250 [33].

Densitometry Analysis

SDS-PAGE and 2-DE gels were scanned with an Image Quant 400 (GE Healthcare). Images were analyzed with the software Image master 2D Platinum 6.0 (Amersham Biosciences).

In-Gel Protein Digestion and MALDI-TOF Analysis

Protein spots were excised from the gels and processed as previously described [13]. Mass spectra were acquired on a Ultraflex MALDI TOF-TOF mass spectrometer (Bruker Dal-

tonics) 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 Protein Mass Fingerprint (PMF) with flexAnalysis (flexAnalysis version 2.4, Bruker Daltonics). Monoisotopic 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 seven sequenced *Shigella* genomes, downloaded from NCBInr or from the Wellcome Trust Sanger Institute database. Genomes used were from strains *Shigella sonnei* 53G, *Shigella flexneri* 2a str. 301, *Shigella flexneri* 2a str. 2457T, *Shigella sonnei* Ss046, *Shigella* boydii Sb227, *Shigella flexneri* 5 str. 8401, *Shigella boydii* CDC 3083-94. Search parameters, mass tolerance, known contaminant ions, validation and handling of multiple matches were performed as described previously [13].

Protein Precipitation and In-solution Protein Digestion

Proteins from supernatants or purified GMMA were precipitated by adding TCA and deoxycholate to a final concentration of 10% and 0.04%, respectively. The precipitation was allowed to proceed for 30 min at 4°C. The precipitate was recovered by 10 min centrifugation at 20,000×g at 4°C. The pellet was washed once with 10% TCA (wt/vol) and twice with absolute ethanol, dried with Speedvac (Labconco, Kansas City, U.S.A). For analysis by SDS-PAGE, the precipitates were resuspended with 200 mM Tris-HCl, pH 8.8, and quantified. For LC-MS/MS analysis 20 µg of GMMA were precipitated and resuspended in 50 µL, 6 M guanidinium chloride, 5 mM DTT, 200 mM Tris-HCl, pH 8.0. Denaturation proceeded for 60 min at 60°C. Prior to digestion, the solution was diluted 1:8 with a solution of 100 mM Tris-HCl, pH 8.0, 5 mM DTT and 5 µg of trypsin (Promega) were added to the diluted solution. Digestion was carried out over night at 37°C. The reaction was stopped by adding formic acid to 0.1%. Peptides were extracted using Oasis extraction cartridges (HLB 1cc (30 mg) extraction cartridges, Waters, Milford, MA, USA) and analyzed by LC-*MS/MS*.

Protein Identification by Nano-LC-MS/MS

Peptides were separated by nano-LC on a NanoAcquity UPLC system (Waters) connected to a Q-ToF Premier ESI mass spectrometer equipped with a nanospray source (Waters). Samples were loaded onto a NanoAcquity 1.7 µm BEH130 C18 column (75 µm×25 mm; Waters) through a NanoAcquity 5 µm Symmetry C18 trap column (180 µm×20 mm; Waters). Peptides were eluted with a 120 min gradient of 2–40% acetonitrile (98%), 0.1% formic acid solution at a flow rate of 250 nL/min. The eluted peptides were subjected to an automated data-dependent acquisition using the MassLynx software, version 4.1 (Waters) where an MS survey scan was used to automatically select multicharged peptides over the m/z ratio range of 300–2000 for further MS/MS fragmentation. Up to eight different peptides were individually subjected to MS/MS fragmentation following each MS survey scan. After data acquisition, individual MS/MS spectra were combined, smoothed, and centroided using ProteinLynx, version 3.5 (Waters) to obtain the peak list file. The Mascot Daemon application (Matrix Science Ltd., London, UK) was used for the automatic submission of data files to in-house licensed Mascot, version 2.2.1, running on a local server. The Mascot search parameters were set to (i) 2 as the number of allowed missed cleavages (only for trypsin digestion), (ii) methionine oxidation as variable modifications, (iii) 0.05 Da as the peptide tolerance, and (iv) 0.05 Da as the MS/MS tolerance. Only significant hits were considered as defined by the Mascot scoring and probability system.

Bioinformatics

Prediction of protein localization was carried out using PSORTb v3.0 [34] and Lipo program [35].

Mouse Immunizations

Outbred CD1 mice (female, 4 to 6 weeks of age) received three injections of GMMA via the subcutaneous route on days 0, 21, and 35. Each injection contained GMMA normalized to 0.2 µg or 2 µg of protein and formulated in PBS only, with Freund's adjuvant (FA), or adsorbed onto aluminum hydroxide (alum), 2 mg/mL, in a final volume of 100 µL. If Freund's adjuvant was used, Freund's complete adjuvant (FCA) was used for the first immunization, Freund's incomplete adjuvant (ICFA) was used for the second and third immunization. Control mice received either adjuvant or PBS alone. Blood samples were collected before immunization and 14 days after the second and third injection. The animal experiments complied with the relevant guidelines of Italy and the institutional policies of Novartis. The animal protocol was approved by the Animal Welfare Body of Novartis Vaccines and Diagnostics, Siena, Italy, approval number AEC 2009-05.

Western Blot

GMMA were boiled in loading buffer and loaded on 12% (wt/vol) polyacrylamide-SDS gels (BioRad) or on 2D gels as described. Gels were run in MOPS buffer (BioRad) and protein were subsequently transferred onto nitrocellulose membrane using Trans-blot transfer medium (BioRad). The membranes were blocked in PBS containing 3% (wt/vol) powdered milk, then incubated with mouse polyclonal antisera diluted (1:1000) in PBS containing 3% (wt/vol) milk for 90 min at 37°C. Membranes were washed three times with PBS containing Tween 20, 0.1% (vol/vol) and then incubated with sheep anti-mouse horseradish peroxidase-conjugated IgG (GE Healthcare, UK Limited), diluted (1:7500) in PBS containing 3% (wt/vol) milk. Colorimetric staining was performed, after washing the membranes, with SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, U.S.A.) as described by the manufacturer. Positive signals were related to the corresponding proteins by comparing the Western blot membrane to the gel using Ponceau staining of the membrane as a reference and aligning the images with Image master 2D Platinum 6.0.

Enzyme-linked Immunosorbent Assay (ELISA)

To measure *Shigella sonnei* GMMA-specific immunoglobulin G (IgG) in mice serum, Nunc Maxisorb 96-well plates were coated over night at 2 to 8°C with 100 µL/well of a 0.5 µg/mL suspension of *Shigella sonnei* 53G –pSS Δ*tolR* GMMA, purified from defined medium with 2 µM ferric citrate in the same way as the GMMA in the vaccine, diluted in phosphate-buffered saline (PBS). Plates were then washed three times with 300 µL/well of phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (PBST) and blocked with PBS containing 1% (wt/vol) BSA for 60 min at 37°C. Serial dilutions of reference and sample sera were prepared in PBST, 1% (wt/vol) BSA in a separate dilution plate, and 100 µL/well of each serial dilution was transferred to the coated plate, incubated for 2 hours at 37°C, and then washed as described above. Bound antibody was detected using a goat anti-mouse IgG conjugated to alkaline phosphatase, diluted in PBST,

Table 2. *Shigella sonnei ΔtolR ΔgalU* GMMA-associated proteins identified by proteomics.

A	B	C	D	E
Outer membrane				
1	*3*	outer membrane channel protein [S. flexneri 2a str. 301]	*tolC*	gi|56480244
2	*3*	outer membrane porin protein C [S. sonnei Ss046]	*ompC*	gi|74312736
3	*3*	outer membrane protein A [S. sonnei Ss046]	*ompA*	gi|74311514
4	*3*	outer membrane protein induced after carbon starvation [S. flexneri 5 str. 8401]	*slp*	gi|110616891
5	*3*	outer membrane protein X [S. flexneri 2a str. 301]	*ompX*	gi|56479734
6	*2*	outer membrane protein assembly factor YaeT [S. Flexneri 2a str. 301]	*yaeT*	gi|24111612
7	*2*	outer membrane protein C [S. boydii CDC 3083-94]	*ompC*	gi|187733369
8	*2*	outer membrane receptor FepA [Shigella sonnei Ss046]	*fepA*	gi|74311118
9	*2*	ferrichrome outer membrane transporter [Shigella sonnei Ss046]	*fhuA*	gi|74310771
10	*2*	colicin I receptor [Shigella sonnei Ss046]	*cirA*	gi|74312677
11	*2*	maltoporin [Shigella flexneri 2a str. 301]	*lamB*	gi|56480532
12	*4*	putative ferric siderophore receptor [S. sonnei Ss046]	*iutA*	gi|74313972
13	*2*	outer membrane protein W [Shigella sonnei Ss046]	*yciD*	gi|74312394
14	*2*	serine protease [S. flexneri 2a str. 301]	*sigA*	gi|24114232
Outer membrane Lipoproteins				
15	*3*	murein lipoprotein [S. flexneri 2a str. 301]	*lpp*	gi|24113066
16	*2*	outer membrane lipoprotein LolB [S. flexneri 2a str. 301]	*lolB*	gi|24112608
17	*3*	peptidoglycan-associated outer membrane lipoprotein [S. flexneri 2a str. 301]	*pal*	gi|56479690
18	*1*	entericidin B membrane lipoprotein [S. flexneri 2a str. 301]	*ecnB*	gi|24115506
19	*1*	hypothetical protein S2067 [S. flexneri 2a str. 2457T]	*yedD*	gi|30063370
20	*1*	hypothetical protein S4565 [S. flexneri 2a str. 2457T]	*yjeI*	gi|30065519
21	*1*	hypothetical protein SF0398 [S. flexneri 2a str. 301]	*ybaY*	gi|24111837
22	*1*	RpoE-regulated lipoprotein [S. flexneri 2a str. 301]	*SF2485*	gi|24113773
23	*1*	hypothetical protein SSON_2966 [S. sonnei Ss046]	*SSON_2966*	gi|74313380
24	*1*	lipoprotein [S. flexneri 2a str. 2457T]	*nlpB*	gi|30063856
25	*2*	entry exclusion protein 2 [S. sonnei Ss046]	*exc*	gi|145294038
26	*2*	LPS-assembly lipoprotein RplB [S. dysenteriae Sd197]	*rplB*	gi|82775909
27	*2*	putative pectinesterase [S. sonnei Ss046]	*ybhC*	gi|74311310
28	*4*	outer membrane protein assembly complex subunit YfiO [Shigella sonnei Ss046]	*SSON_2721*	gi|74313154
29	*3*	outer membrane lipoprotein [S. flexneri 2a str. 301]	*yraP*	gi|24114441
30	*3*	DNA-binding transcriptional activator OsmE [S. flexneri 2a str. 301] lipo	*osmE*	gi|24112862
31	*1*	outer membrane protein [S. flexneri 2a str. 301]	*slyB*	gi|24113033
Periplasmic				
32	*3*	FKBP-type peptidyl-prolyl cis-trans isomerase [S. Flexneri 2a str. 301]	*fkpA*	gi|24114611
33	*3*	histidine-binding periplasmic protein of high-affinity histidine transport system [S. sonnei Ss046]	*hisJ*	gi|74312826
34	*3*	serine endoprotease [S. flexneri 2a str. 301]	*htrA*	gi|24111599
35	*3*	translocation protein TolB [S. flexneri 2a str. 2457T]	*tolB*	gi|30062097
36	*1*	molybdate transporter periplasmic protein [S. flexneri 2a str. 301]	*modA*	gi|24111968
37	*1*	peptidyl-prolyl cis-trans isomerase A (rotamase A) [S. flexneri 2a str. 301]	*ppiA*	gi|24114628
38	*1*	peptidyl-prolyl cis-trans isomerase SurA [S. flexneri 2a str. 301]	*surA*	gi|24111499
39	*1*	periplasmic oligopeptide binding protein [S. flexneri 2a str. 2457T]	*oppA*	gi|30062764
40	*1*	periplasmic protein [S. flexneri 2a str. 2457T]	*osmY*	gi|30065614
41	*2*	arginine 3rd transport system periplasmic binding protein [S. sonnei Ss046]	*artJ*	gi|74311404
42	*2*	bifunctional UDP-sugar hydrolase/5′-nucleotidase [S. sonnei Ss046]	*ushA*	gi|74311061
43	*2*	cystine transporter subunit [S. sonnei Ss046]	*fliY*	gi|74311733
44	*2*	glucan biosynthesis protein G [S. flexneri 5 str. 8401]	*mdoG*	gi|110805056
45	*2*	thiosulfate transporter subunit [S. sonnei Ss046]	*cysP*	gi|74312961
46	*2*	hypothetical protein SBO_2040 [Shigella boydii Sb227]	*ycdO*	gi|82544504

Table 2. Cont.

A	B	C	D	E
47	*2*	hypothetical protein SFV_2968 [S. flexneri 5 str. 8401]	*yggE*	gi|110806822
Cytoplasmic				
48	*3*	chaperonin GroEL [S. flexneri 2a str. 301]	*groEL*	gi|24115498
49	*3*	dihydrolipoamide dehydrogenase [S. flexneri 2a str. 301]	*lpdA*	gi|56479605
50	*1*	purine nucleoside phosphorylase [S. flexneri 2a str. 2457T]	*deoD*	gi|30065622
51	*1*	succinyl-CoA synthetase subunit beta [S. flexneri 2a str. 301]	*sucC*	gi|24111996
52	*2*	PTS system glucose-specific transporter subunit [S. flexneri 2a str. 301]	*crr*	gi|24113762
53	*2*	molecular chaperone DnaK [S. flexneri 2a str. 301]	*dnaK*	gi|24111463
54	*1*	pyrroline-5-carboxylate reductase [S. flexneri 2a str. 301]	*proC*	gi|24111764
55	*2*	hypothetical protein SF1022 [S. flexneri 2a str. 301]	*SF1022*	gi|24112431
Inner membrane				
56	*1*	hypothetical protein SSON_1546 [S. sonnei Ss046]	*ydgA*	gi|74312061
Unknown				
57	*3*	putative receptor [S. sonnei Ss046]	*SSON_1681*	gi|74312191
58	*2*	hypothetical protein SSON_1556 [S. sonnei Ss046]	*ydgH*	gi|74312071
59	*2*	hypothetical protein SSON_3340 [S.sonnei Ss046]	*yrbC*	gi|74313729
60	*2*	putative lipoprotein [Shigella dysenteriae Sd197]	*ybjP*	gi|82777619
61	*1*	hypothetical protein S3269 [S. flexneri 2a str. 2457T]	*ygiW*	gi|30064374

GMMA were purified by 2-step TFF from *S. sonnei* Δ*tolR* Δ*galU* grown in HTMC at 37°C to an OD of 45. GMMA-associated proteins were separated by SDS-PAGE or nano-LC and identified by mass spectrometry. 61 GMMA-associated proteins were identified. The columns show: A) position in list, B) method used to identify each protein ('1': identified from total digestion LC/MS-MS, '2': identified from 2D SDS-PAGE PMF, '3' identified from LC/MS-MS and 2D SDS-PAGE PMF, '4' identified from 1D SDS-PAGE PMF), C) annotation, D) gene name, E) accession number. The entries are divided by predicted location. All proteins were analyzed by PSORTb 3.0 and Lipo. If a prediction as lipoprotein was obtained, the protein is listed as lipoprotein irrespective of its PSORTb prediction. Only lipoproteins predicted to be located in the outer membrane have been identified. The other proteins are listed in sections corresponding to their location predicted by PSORTb. For 5 proteins no prediction was obtained by PSORTb or Lipo. These are listed as 'unknown'.

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1% (wt/vol) BSA to 1:5000 and incubated for 2 hours at 37°C. After a wash with PBST, 100 µL/well of *p*-nitrophenyl phosphate substrate dissolved in diethanolamine buffer (1 M, pH 9.8) was added, and after 20 minutes optical densities were measured with a plate reader (ELx800, BioTek) at 405 and 490 nm wavelength. Absorbance at 490 nm was subtracted from the absorbance at 405 nm. Results are expressed in arbitrary ELISA units relative to a standard serum raised against GMMA from *S. sonnei* 53G Δ*tolR* Δ*galU*. One unit equals the reciprocal of the dilution of the standard serum giving an $OD_{405-490}$ of 1 in the assay. All samples were measured in duplicate.

Statistical Analysis

Antibody levels (ELISA units) in different groups after the third immunization were compared by non-parametric Kruskal-Wallis and Mann-Whitney tests. A p value of 0.05 was considered to be significant. For multiple comparisons the p value considered to be significant in each of the comparisons was adjusted according to the number of analyses.

Results

Generation of a Shigella Sonnei 53G Strain Capable of Overproducing Modified GMMA

The first aim of the study was to investigate if *Shigella sonnei* 53G could be developed as a strain suitable to overproduce GMMA through modification of the Tol-Pal system. A null mutation of the *tolR* gene was introduced as this has previously been demonstrated to result in overproduction of GMMA in *E. coli* [11,13]. The mutation in the *tolR* gene led to the release of large amounts of GMMA from the surface of *S. sonnei* 53G as assessed by SDS page (Fig. 1A). The deletion of *tolR* had no detectable influence on bacterial growth (data not shown). In addition, to test if GMMA overproduction is also feasible in strains with additional genetic modifications we removed the O antigen of the LPS, either by deletion of *galU* [26] or by curing the virulence plasmid from strain *S. sonnei* 53G Δ*tolR* as the biosynthesis genes for the O antigen in *Shigella sonnei* are encoded on the plasmid [36]. GMMA obtained from *S. sonnei* Δ*tolR* Δ*galU* showed a similar protein profile to GMMA obtained from *S. sonnei* Δ*tolR* with minor differences in the 37 kDa to 50 kDa range and proteins smaller than 30 kDa appeared to be less abundant in *S. sonnei* Δ*tolR* Δ*galU* (Fig. 1A). Also GMMA obtained from the plasmid-cured *S. sonnei* Δ*tolR* mutant (*S. sonnei* –pSS Δ*tolR*) showed a nearly identical protein pattern to GMMA from *S. sonnei* Δ*tolR* (Fig. 1A).

Furthermore, the genes *msbB1* and *msbB2* involved in lipid A biosynthesis were deleted since these deletions have previously been reported to decrease LPS toxicity in *Shigella* [21]. As the gene *msbB2* is encoded on the virulence plasmid and thus absent in *S. sonnei* –pSS Δ*tolR* we deleted the chromosomal gene *msbB1* in this strain to generate a mutant strain lacking *msbB1* and *msbB2*. For simplicity the Δ*msbB1*Δ*msbB2* mutant is referred to as the Δ*msbB* mutant. The Δ*msbB* mutant was selected at 37°C on LB plates and grew in LB and yeast extract at 37°C with a duplication time of about 55 min compared to a duplication time of about 28 min for the single Δ*tolR* mutant. In the defined medium developed for fermentation, the plasmid-cured Δ*tolR* Δ*msbB* mutant strain (*S. sonnei* –pSS Δ*tolR* Δ*msbB*) was able to grow to high optical density (OD) at 30°C, but grew poorly at 37°C. Thus,

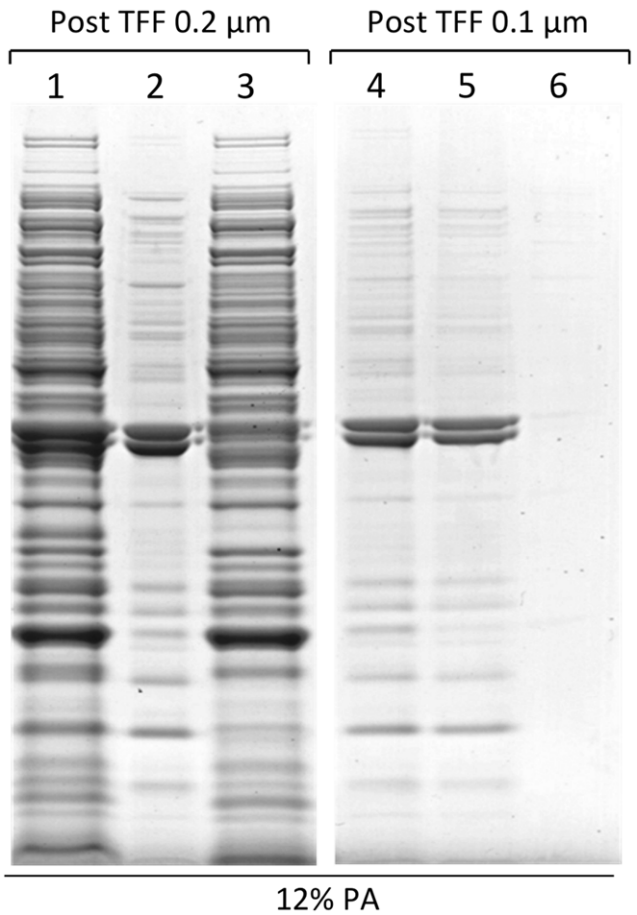

Figure 2. GMMA enrichment and purity after TFF. GMMA were purified from a 5 L fermentation culture of *S. sonnei* Δ*tolR* Δ*galU* grown in HTMC at 37°C to OD 45 using 2-step TFF. In the first step, the culture supernatant which contains the GMMA was separated from the bacteria using a 0.2 µm filter. The biomass was subjected to 5 diafiltration steps and all filtrates were combined with the initial supernatant to obtain the total permeate. To determine the amount of GMMA in the permeate, GMMA were separated from soluble proteins by ultracentrifugation. After ultracentrifugation, the pellet (GMMA) was resuspended in the initial volume of the centrifuged material to normalize all samples to fermentation volume. Equivalent volumes of the 0.2 µm filtrate before ultracentrifugation (1), the resuspended GMMA pellet (2), and the supernatant of the ultracentrifugation (3) were separated by SDS-PAGE (12% PA) and showed a large amount of soluble proteins (3) in comparison to GMMA-associated proteins (2) to be present in the post 0.2 µm TFF permeate. In the second TFF step, GMMA were separated from soluble proteins using a 0.1 µm filter. The retentate (4) was analyzed by ultracentrifugation as described above and was found to contain almost exclusively GMMA (5) as determined by the strong reduction of soluble proteins (6).
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for generation of GMMA from *S. sonnei* –pSS Δ*tolR* Δ*msbB* cultivation in chemically defined medium at a growth temperature of 30°C was chosen. GMMA from *S. sonnei* –pSS Δ*tolR* Δ*msbB* produced under these conditions showed a similar protein pattern to GMMA generated by *S. sonnei* –pSS Δ*tolR* and *S. sonnei* Δ*tolR* with only minor variation in relative amounts of proteins visible by SDS-PAGE in the 45–75 kDa range (Fig. 1A). In order to test if the lower temperature would change the GMMA composition we compared GMMA derived from *S. sonnei* –pSS Δ*tolR* at 30°C or 37°C. Only few differences were detected as highlighted in Fig. 1B, indicating that GMMA can be generated at 30°C without major effects on the composition. In conclusion, deletion of *tolR* greatly enhanced GMMA release while additional genetic modification of the strain or a change in growth temperature only had minor effects on the protein composition visible by SDS-PAGE.

High Density Cultivation of Shigella Sonnei

To investigate the feasibility to produce GMMA at large scale, *S. sonnei* 53G Δ*tolR* Δ*galU*, *S. sonnei* 53G –pSS Δ*tolR*, and *S. sonnei* 53G –pSS Δ*tolR* Δ*msbB* were tested for their capacity to grow to high densities in a 5 liter reactor. Starter cultures were grown in flasks to OD 0.8 and were then transferred to the 5 L fermenter to reach a starting OD of 0.02. Dissolved oxygen was maintained at 30% saturation. The pH was maintained at 7.2 in HTMC or at 6.7 in SSDM and the temperature was kept constant either at 37°C or at 30°C when *S. sonnei* 53G –pSS Δ*tolR* Δ*msbB* was used. Under these conditions, cultures with optical densities of 45 to 80 were obtained.

Iron-regulated proteins have previously been shown to be important in vaccine formulations against *Pasteurella* and *Salmonella* [37,38]. Thus, we evaluated if the GMMA process would allow the upregulation of iron-regulated proteins. Growth of *S. sonnei* 53G –pSS Δ*tolR* with 0.2 µM iron concentration in chemically defined medium led to the induction of iron-regulated proteins but hindered high density cultivation of bacteria. The addition of 2 µM iron to the medium was sufficient to allow optimal growth and the induction of three iron-regulated proteins visible by SDS-PAGE (Fig. 1C), identified by protein mass fingerprint as FepA (gi|74311118), IutA (gi|74313972) and Colicin I receptor (gi|74312677). The expression of these proteins was reduced when bacteria were grown in 200 µM iron (Fig. 1C). In bacteria grown in HTMC the iron-regulated proteins are expressed to a similar level as in chemically defined medium with 200 µM iron (data not shown). Their presence was confirmed by protein mass fingerprint analysis of GMMA generated from *S. sonnei* Δ*tolR* Δ*galU* grown in HTMC (Table 2, proteins 8, 10, 12). Growth of *S. sonnei* 53G –pSS Δ*tolR* Δ*msbB* at 30°C in defined medium with 2 µM iron also enhanced expression of FepA and IutA. Colicin I receptor (marked in Fig. 1C) was less expressed than in GMMA from *S. sonnei* 53G –pSS Δ*tolR* prepared from cultures grown at 37°C (data not shown).

Purification of GMMA from High Density Culture Supernatant

So far, GMMA have always been purified from flask cultures by ultracentrifugation [13]. Cultures were centrifuged at low speed (4000 g) to separate biomass from supernatant which was subsequently filtered through a 0.22 µm filter. GMMA present in the supernatant were collected by ultracentrifugation, washed, and then resuspended and stored in PBS [10,13]. Since this technique is not suitable for large volumes we developed a scalable purification method to purify GMMA from high density cultures using tangential flow filtration (TFF). In TFF, also known as crossflow filtration, the feed stream is pumped tangentially across the surface of the membrane rather than into the filter as in conventional 'dead-end' filtration. A proportion of the soluble components and particles smaller than the membrane's pores penetrates the filter (filtrate/permeate). The remainder (retentate) is circulated back to the reservoir and over the filter again. In this way, the larger particles do not build up at the surface of the filter but are swept away by the tangential flow allowing smaller molecules to continuously reach and pass through the membrane. This feature makes TFF an efficient process for size separation, concentration and diafiltration.

Table 3. Yield, purity, and recovery rate of GMMA by the high yield production process.

	Fermentation A OD 45	Fermentation B1 OD 30	Fermentation B2 OD 39
Protein content [mg/L fermentation]			
0.2 μm TFF permeate			
Total protein*	1465	1237	797
GMMA-associated protein	214	143	138
Soluble protein	1251	1094	659
0.1 μm TFF retentate			
Total protein#	108	144	118
GMMA-associated protein	120	127	114
Soluble protein	14	5	3
GMMA-associated protein per OD	2.7 mg/L/OD	4.2 mg/L/OD	2.9 mg/L/OD
Purity of GMMA after 0.1 μm TFF [%]			
GMMA (GMMA-protein/total protein)	90	88	97
Soluble protein (sol. protein/total protein)	10	3	3
Recovery of GMMA by 0.1 μm TFF [%]			
GMMA-protein after 0.1 μm TFF/0.2 μm	56	89	83

*Total protein amount calculated as sum of GMMA-associated protein and soluble protein.
#Total protein amount measured directly by Bradford assay.
S. sonnei Δ*tolR* Δ*galU* was grown in HTMC in a 5 L fermenter to high densities of OD 45 (A), OD 30 (B1) and OD 39 (B2) and GMMA were purified using 2-step TFF. Purification from fermentation A was performed including 5 diafiltration steps of the biomass, for GMMA purification from fermentations B1 and B2 the biomass was not subjected to diafiltration. The GMMA content in the permeate of the 0.2 TFF step (culture supernatant) and the retentate of the 0.1 μm TFF (purified GMMA) were determined by separation of GMMA from soluble protein by ultracentrifugation. Protein was quantified using Bradford assay. All samples were normalized to amount per liter fermentation broth. To compare the yields from different ODs, yields are also expressed as amount per liter fermentation per OD.
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GMMA were purified from fermentation cultures in a 2-step TFF process. In the first step, the culture supernatant that contains the GMMA was separated from the bacteria using a 0.2 μm filter. In this step, the bacteria remained in the retentate and GMMA transferred into the filtrate. In the second filtration step using a 0.1 μm filter, GMMA were separated from soluble components present in the culture supernatant, including proteins secreted by the bacteria or released by lysis. In this step, GMMA were retained by the filter and collected and concentrated in the retentate whereas soluble proteins passed through the filter. We tested this purification process under two slightly different conditions. Firstly, when the fermentation culture of *S. sonnei* Δ*tolR* Δ*galU* reached OD 45, the culture was transferred directly from the fermenter to the first TFF and the culture supernatant containing the GMMA was collected. The retained biomass was washed with 5 volumes of PBS buffer (diafiltration) to recover remaining GMMA and the filtrate containing these GMMA was combined with the culture supernatant. In the slightly modified purification process tested, this diafiltration step was omitted. Proteins were quantified by Bradford method. In the purification performed with diafiltration of the biomass the total protein content of the TFF 0.2 μm filtrate was approximately 1.5 g/L of fermentation culture of which 15% was GMMA-associated as determined by separation of the soluble components from the high molecular weight portion (GMMA) via an ultracentrifuge step (Fig. 2 and Table 3). In the second TFF step, GMMA were concentrated in the retentate and washed with five volumes of PBS to remove remaining soluble proteins. As TFF is usually performed under non-sterile conditions, the final retentate was sterilized by filtration through a 0.22 μm filter. An aliquot of the sterilized retentate was subjected to ultracentrifugation to determine the content of GMMA as above. As shown in Fig. 2 most of the proteins in the retentate are GMMA-associated. Protein quantification of the retentate, of the GMMA fraction, and of the supernatant of the ultracentrifugation step (soluble proteins) determined that 90% of all protein present in the retentate was GMMA-associated (Table 3). Thus, soluble proteins were efficiently removed in this step (Fig. 2). GMMA recovery after the 0.1 μm TFF step was 56% of the quantity present after the 0.2 μm cassette. The final yield of GMMA was 120 milligrams of proteins per liter of fermentation (Table 3). In two subsequent tests of the purification method with *S. sonnei* Δ*tolR* Δ*galU* without diafiltration of the biomass, a lower amount of GMMA was obtained in the 0.2 μm TFF filtrate (Table 3). However, recovery of GMMA in the second TFF step (0.1 μm) was enhanced resulting in an overall similar yield of GMMA with equivalent purity (Table 3 and suppl. figures Fig. S1, Fig. S2). This suggests, firstly, that washing of the biomass increases the recovery GMMA from the fermentation culture, and secondly, that a higher starting concentration might be beneficial for the second TFF step. The 0.1 μm TFF step can likely be optimized to take advantage of the larger amounts of GMMA obtained by diafiltration of the biomass. Fermentations of *S. sonnei* –pSS Δ*tolR* Δ*msbB* resulted in yields of 140 mg/L from a culture at OD 65 (2.2 mg/L/OD) and 230 mg/L from a culture at OD 80 (2.9 mg/L/OD), demonstrating that the yield of GMMA can be further improved by growing the culture to a higher OD.

The preparation of GMMA generated from *S. sonnei* –pSS Δ*tolR* Δ*msbB* obtained after the second TFF step was subjected to electron microscopy analysis, revealing the presence of well-organized membrane vesicles with a diameter of about 30–60 nm (Fig. 3) which is consistent with the reported average size of

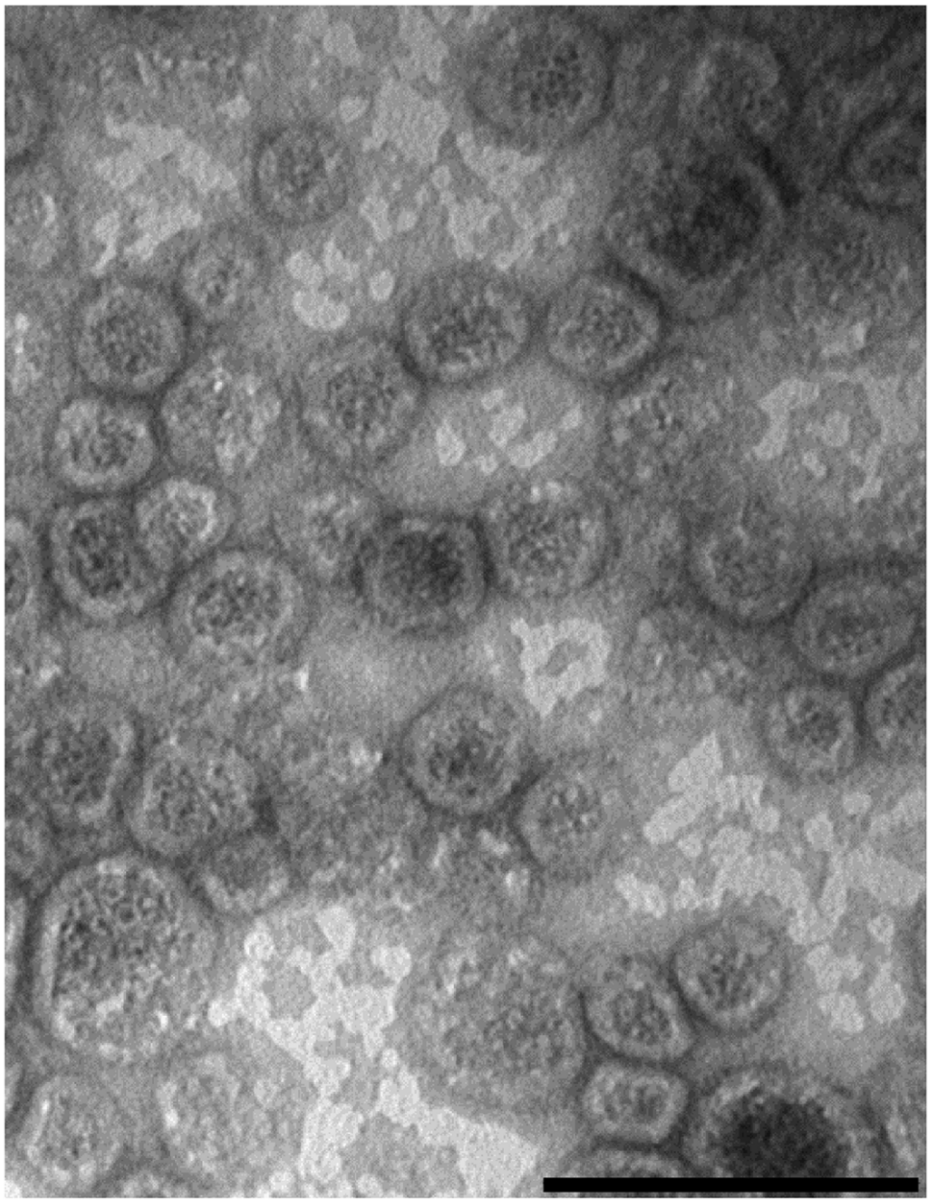

Figure 3. Electron microscopy of *Shigella sonnei* Δ*tolR* Δ*galU* GMMA. GMMA were isolated from the culture supernatant of *S. sonnei* –pSS Δ*tolR* Δ*msbB* by TFF, prepared for negative staining, and viewed by electron microscopy revealing the presence of well-organized membrane vesicles with a diameter of about 30–60 nm. Bar length = 100 nm.
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40±20 nm of outer membrane particles produced by *E. coli tol-pal* mutants [11].

Characterization of GMMA Protein Content

GMMA purified by TFF from *S. sonnei* 53G Δ*tolR* Δ*galU* grown in high density culture were characterized to confirm their integrity and to analyze their protein content. One- and two-dimensional SDS-PAGE of GMMA and densitometry analysis (Fig. 1D and Fig. 4) were used to determine the protein profile and to study relative protein quantities of the most abundant proteins. Most of the Coomassie blue-stained bands and spots were identified using peptide mass fingerprint (Table 2). OmpA and OmpC are known to be among the most abundant proteins present in the outer membrane. In fact, densitometry analysis of GMMA from *S. sonnei* Δ*tolR* Δ*galU* grown in HTMC and analyzed by 1D SDS-PAGE indicated that OmpA and OmpC together contribute for 45% of the total protein; OmpX, 9%; Slp, 6%; YfiO, 5.6%; TolB, 2.3%; TolC 1.4%; and YaeT, 1.8% (Fig. 1D). With the exception of the predicted periplasmic protein TolB, all of these proteins are predicted to be associated with the outer membrane. YfiO is predicted to be an outer membrane lipoprotein. OmpA, OmpC, OmpX, Slp, TolC, and YaeT are predicted to be outer membrane proteins. Thus, the seven most abundant outer membrane-associated proteins account for approximately 69% of the protein amount in GMMA. Further densitometry analysis after 2D SDS-PAGE determined that there are approximately equal quantities of OmpA and OmpC (OmpA:OmpC is 1:0.83 by densitometry of a Coomassie blue-stained gel). In order to identify the diverse and less expressed proteins, GMMA were studied by proteolytic digestion and reverse

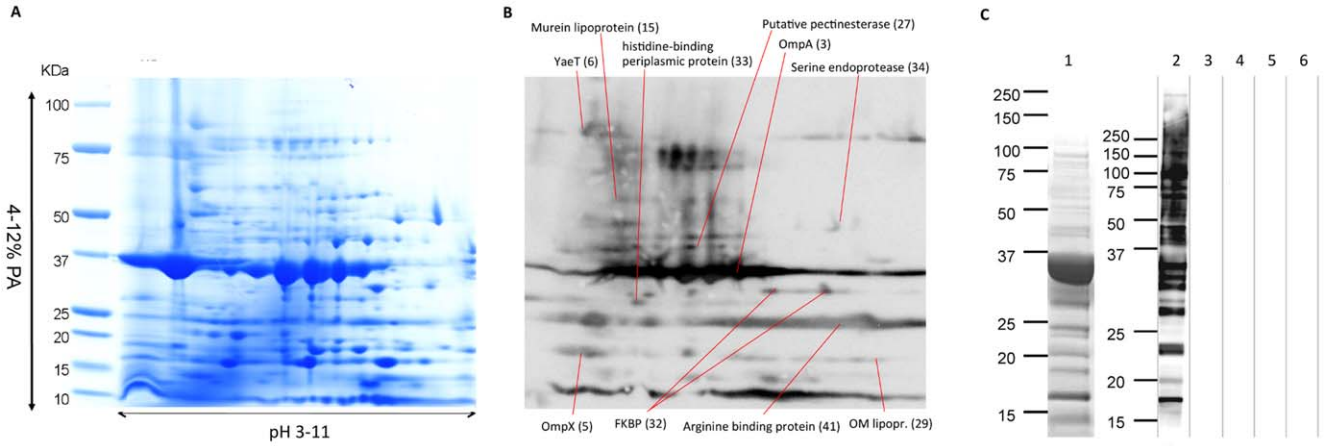

Figure 4. 2D gel electrophoresis of *Shigella sonnei* Δ*tolR* Δ*galU* GMMA and immunoblot. A)200 μg of proteins from *S. sonnei* Δ*tolR* Δ*galU* GMMA were separated in the first dimension on a non linear pH 3–11 gradient, and in the second dimension on a 4–12% polyacrylamide gradient. Visible bands were identified by protein mass fingerprint. OmpA and OmpC were quantified with Image master 2D Platinum 6.0. B) Sera from mice immunized with GMMA from *S. sonnei* Δ*tolR* Δ*galU* were used to study the subset of proteins present in GMMA that are able to raise antibodies. A 2D gel containing 20 μg of GMMA protein from *S. sonnei* Δ*tolR* Δ*galU* was blotted and the membrane was incubated with sera from immunized mice with GMMA from *S. sonnei* Δ*tolR* Δ*galU* in combination with Freund's adjuvant. Several reactive proteins were identified. The numbers behind the names refer to the position of the proteins in Table 2. C) To verify that the signal observed in the 2D Western blot was due exclusively to antibody raised upon immunization with GMMA, 10 μg of GMMA were separated by 1D SDS-PAGE (12% PA) and stained with Coomassie (1) or transferred to a membrane. Western blots were developed using (2) sera raised against GMMA from *S. sonnei* Δ*tolR* Δ*galU* as used for the 2D Western blot in B, (3) preimmune serum, (4) sera raised in mice immunized with Freund's adjuvant or (5) PBS, or (6) secondary antibody only. A signal could only be observed when sera raised against GMMA were used (2).
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phase liquid chromatography coupled to MS/MS. 61 proteins were identified in total (LC-MS/MS, 1D and 2D SDS-PAGE PMF) (Table 2), with 31 of these proteins predicted to be associated with the outer membrane (Fig. 5). Of these, 14 proteins were predicted to be outer membrane proteins and 17 to be outer membrane lipoproteins. In addition, 16 proteins were predicted to be periplasmic, 8 to be cytoplasmic, 1 to be located in the inner membrane, and for 5 proteins no prediction could be obtained. No inner membrane lipoproteins were predicted. Thus, GMMA generated by the high yield production process are mostly composed of outer membrane-associated and periplasmic proteins as previously seen for outer membrane particles release from cultures at the early logarithmic phase [10,13].

GMMA Immunogenicity

Groups of 8 CD1 mice were immunized 3 times with GMMA (2 μg of total protein) obtained from *S. sonnei* 53G –pSS Δ*tolR* and *S. sonnei* 53G –pSS Δ*tolR* Δ*msbB*, both grown in defined medium with 2 μM iron, and *S. sonnei* 53G Δ*tolR* Δ*galU* grown in HTMC. GMMA from *S. sonnei* 53G –pSS Δ*tolR* Δ*galU*, *S. sonnei* 53G –pSS Δ*tolR* and *S. sonnei* 53G –pSS Δ*tolR* Δ*msbB* were also administered in combination with Freund's adjuvant (FA). Freund's complete adjuvant was used in the first immunization and Freund's incomplete adjuvant was used in the second and third immunization. In addition, a lower dosage of 0.2 μg of GMMA from *S. sonnei* 53G –pSS Δ*tolR* Δ*msbB* was tested. Serum samples were obtained 2 weeks after the second and third doses and analyzed individually. Mice immunized with GMMA showed very high IgG responses to all 3 types of GMMA that were tested. No difference was found between groups immunized with different GMMA or between groups receiving the same GMMA with or without FA (Fig. 6). Adsorption of GMMA onto alum as adjuvant also did not have an effect on the IgG response (data not shown). Control mice immunized with PBS or FA alone had very low levels of anti-GMMA antibodies (Fig. 6). The 10-fold lower dosage of GMMA from *S. sonnei* 53G –pSS Δ*tolR* Δ*msbB* (0.2 μg) resulted in a statistically significant, approximately 3-fold reduction in the IgG response compared to the group immunized with 2 μg of the same GMMA. However, the IgG response to the lower dosage still showed an approximately 8000-fold increase compared to preimmune sera (Fig. 6).

To investigate which components of GMMA were responsible for the reactivity of the sera, 2D Western blots were performed. As GMMA from *S. sonnei* 53G Δ*tolR* Δ*galU* were characterized best in respect to their protein content, sera from mice immunized with the GMMA from *S. sonnei* Δ*tolR* Δ*galU* were used to probe blots of 2D SDS-PAGE of GMMA from the same strain. Reactive proteins were identified by protein mass fingerprint. Several proteins were detected by the sera (Fig. 4 B) of which OmpA gave the strongest response. OmpC which is as abundant in GMMA as OmpA was not detected. Not all of the visible reactive proteins could be identified.

Discussion

Recent advances in genomics and reverse vaccinology have identified promising protein targets for vaccines [39]. In many cases, suitable candidate antigens for Gram-negative bacterial vaccines are outer membrane proteins and these pose particular challenges in their expression and purification and in serotype variability. An ideal delivery system especially for bacterial vaccines for developing countries will encompass multiple antigens and enable vaccines to be rapidly tailored to local and changing antigenic serotypes. Ideally, it will also be inexpensive to manufacture. We propose a platform for rapid development and delivery of vaccines against Gram-negative bacteria. The approach is based on the production of outer membrane particles we have named GMMA by genetically modified bacteria. Using genetic manipulation, it is possible to increase their yield, to remove immunodominant structures, to overexpress certain

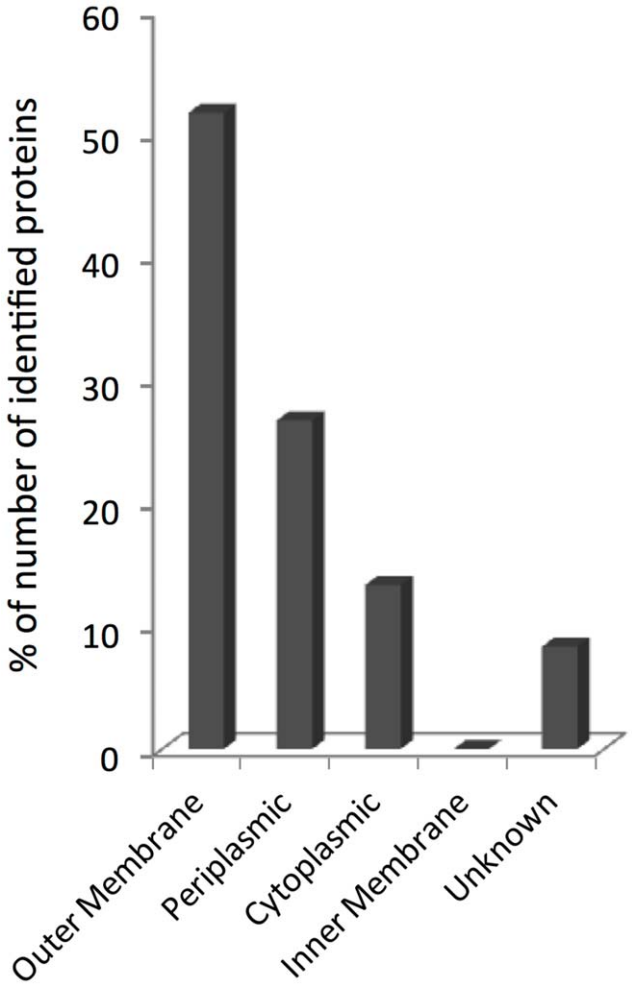

Figure 5. *Shigella sonnei* Δ*tolR* Δ*galU* GMMA proteome. The 61 GMMA-associated proteins that were identified are grouped into families based on their predicted cellular location, according to bioinformatic prediction by PSORTb v3.0 [34] and Lipo program [35]. The 'outer membrane' column comprises outer membrane proteins (identified by PSORTb) and lipoproteins predicted to be located in the outer membrane (identified by Lipo). No lipoproteins associated with the inner membrane were identified. The distribution shown is based on the number of identified proteins predicted to be located in a certain compartment. It does not reflect the protein amount. As analyzed by densitometry (Fig. 1D), the outer membrane fraction contains at least 69% of the total protein present in GMMA.
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antigens, and to reduce the endotoxic activity [10,13,19,21,26,40,41]. GMMA could potentially be a safe, effective and low cost vaccine but need a practical way of manufacture at scale.

Shigella sonnei 53G was chosen for a first approach to develop a scalable process and a null mutation of the *tolR* gene was introduced to overproduce GMMA as previously described for *E. coli* [13]. To verify that the process is applicable to produce GMMA harboring modified lipid A, which would be more suitable for use as vaccine, and/or lacking the O antigen of the LPS we grew high density cultures of *S. sonnei* Δ*tolR* Δ*galU*, *S. sonnei* –pSS Δ*tolR* (cured of the virulence plasmid pSS), and *S. sonnei* –pSS Δ*tolR* Δ*msbB* in a 5 L fermenter in complex (HTMC) or chemically defined medium. Chemically defined medium was used to avoid contamination from proteins present in complex media and to have the possibility to regulate iron concentration.

Bacteria were removed from the culture supernatant by a tangential flow filtration step using a 0.2 μm membrane. A second tangential flow filtration step with a 0.1 μm membrane was used to concentrate GMMA and to remove soluble proteins. This choice of appropriate molecular weight membranes allowed the purification of GMMA in an easy, efficient, and scalable process. After purification, approximately 90% of all protein was consistently GMMA-associated with reproducible yields of more than 100 mg of GMMA-associated protein per liter fermentation volume from OD 30–45 cultures of *S. sonnei* Δ*tolR* Δ*galU*. The integrity of GMMA obtained by this process was confirmed using electron microscopy. The purity and yield can likely be increased as indicated by fermentations with *S. sonnei* –pSS Δ*tolR* Δ*msbB* to densities of 65 and 80. Furthermore, first results obtained by quantitative amino acid analysis of different types of GMMA indicated an at least two-fold higher protein amount in the GMMA preparations than determined by the Bradford assay used in this study (data not shown). Still, assuming an average yield of 100 mg/L fermentation and a dosage of 25 μg as used for the MeNZB outer membrane vesicle meningococcal vaccine, at least 400,000 doses could be obtained from a 100 L fermenter.

A proteomic approach confirmed that *Shigella sonnei* 53G Δ*tolR* Δ*galU*-derived GMMA are composed mostly of outer membrane and periplasmic components. They conserve lipophilic polypeptides. Only a small number of cytoplasmic components and one inner membrane protein were predicted. Thus, the proteomic analysis of GMMA obtained from an OD 45 culture revealed a similar composition as previously seen in proteomic analyses of outer membrane particles that were obtained from cultures at early logarithmic phase to avoid impurities by cytoplasmic proteins [10,13].

In accordance with previous reports [15,17] GMMA were highly immunogenic in mice with titers around 1:100,000 after administration of 2 μg of GMMA with and without adjuvant. A 10-fold lower dosage of GMMA (without adjuvant) resulted in only a 3-fold reduction and still very high antibody titers suggesting that low amounts of GMMA might be sufficient for vaccination. GMMA from the *msbB* mutant *S. sonnei* strain did not show a difference in immunogenicity which was expected due to a recent report that the resulting lipid A modification does not affect LPS recognition in mice [42]. Immunoblots confirmed that antibodies to proteins, including outer membrane proteins OmpA, OmpX, and YaeT, strongly contributed to the reactivity of the sera. Interestingly, the outer membrane protein OmpC which represents about 20% of protein in GMMA was not detected by sera raised against GMMA. Previously, an immunoproteomic analysis of isolated outer membrane proteins of *Shigella flexneri* 2a [43] also failed to detect OmpC as immunogenic protein. This could suggest that either OmpC is not immunogenic or that epitopes potentially recognized by antibodies are not maintained after SDS-PAGE. This might also apply to other membrane proteins that were not found by the Western blot analysis even though not all reactive proteins could be identified.

The *msbB* mutant strain of *Shigella* lacking the genes *msbB1* and *msbB2* [21] was generated to investigate if the production process was applicable to GMMA with modified lipid A. A previous report [21] had shown that these deletions result in the synthesis of a penta-acylated lipid A instead of a hexa-acylated lipid A in *Shigella* [21]. While the *S. sonnei* –pSS Δ*tolR* Δ*msbB* mutant grows in rich media at 37°C temperature, its growth is impaired in the chemically defined medium developed for fermentation at 37°C but shows a normal growth in this medium at 30°C. Previously,

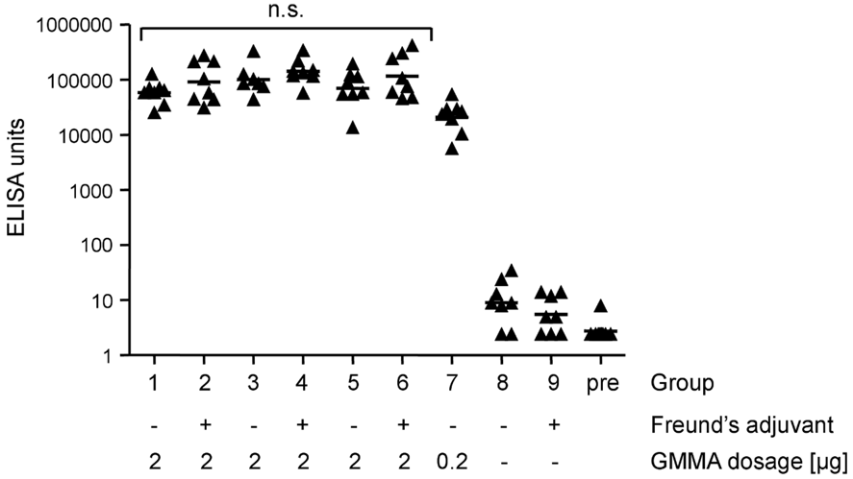

Figure 6. ELISA analysis of sera reactivity against GMMA. Groups 1–6 received 2 μg of GMMA with or without Freund's adjuvant (FA), group 1) GMMA from *S. sonnei* Δ*tolR* Δ*galU* (grown in HTMC, 37°C), 2) GMMA of group 1 plus FA, 3) GMMA *S. sonnei* –pSS Δ*tolR* (defined medium, 37°C), 4) GMMA of group 3 plus FA, 5) GMMA from *S. sonnei* –pSS Δ*tolR* Δ*msbB* (defined medium, 30°C), 6) GMMA of group 5 plus FA. Group 7 received 0.2 μg of GMMA from *S. sonnei* –pSS Δ*tolR* Δ*msbB*. Control groups were immunized with PBS alone (group 8) or FA alone (group 9). Sera from individual mice obtained 14 days after the third immunization and pooled preimmune sera from each group respectively were assayed in dilutions of 1:1000, 1:10,000, and 1:100,000 on GMMA from *S. sonnei* 53G –pSS Δ*tolR* as coating and arbitrary units were calculated. Data are presented as scatter plots of ELISA units determined in individual mice (groups 1–9) or of the pooled preimmune sera (pre). The horizontal lines represent the geometric mean. ELISA units of groups 1–6 receiving 2 μg of GMMA were analyzed using the non-parametric Kruskal-Wallis test to compare the immunogenicity of the different GMMA to each other and with and without FA. No statistically significant differences were found (n.s.). Reduction of the immunization dosage of *S. sonnei* –pSS Δ*tolR* Δ*msbB* GMMA to 0.2 μg (group 7) resulted in statistically significant reduction of ELISA units in the sera of the immunized animals compared to sera of mice immunized with 2 μg of the same GMMA (group 5) as determined by Mann-Whitney test ($p = 0.0047$). All groups receiving GMMA showed higher *S. sonnei* –pSS Δ*tolR*-specific antibody responses than groups immunized with PBS or FA alone (Mann-Whitney, $p \leq 0.003$). For all comparisons a p value smaller than 0.05 was considered to be significant.
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a *Shigella flexneri* 5a *msbB* mutant and an *E. coli msbB* mutant in the K-12 background were reported not to show any growth defects [21,23]. In contrast, an *msbB* mutant of the clinical isolate *E. coli* H16 formed filaments when grown at 37°C but not at 30°C or when functionally complemented by the cloned *msbB* gene [44]. The *S. sonnei* –pSS Δ*tolR* Δ*msbB* mutant strain used in this study does not form filaments. The reason for the slower growth at 37°C, especially in defined medium, is not clear and could be a result of the background of the strain, the combination of the *tolR* and *msbB* mutation, or a suboptimal composition of the defined medium that can likely be optimized. Importantly, a comparison of the protein pattern of GMMA generated from *S. sonnei* –pSS Δ*tolR* at 37°C and 30°C showed only minor differences in the protein profile visible by SDS-PAGE indicating that the change in temperature does not have major effects on GMMA composition.

In summary, we have identified an easy process to produce large quantities of GMMA from high density culture. GMMA purified from fermentation are extremely pure particles composed almost exclusively of outer membrane and periplasmic components. The simplicity and high yield of the process support its applicability for large scale manufacturing. We have also shown that this process can be used with strains genetically modified to reduce reactogenicity or to remove immunodominant antigens, e.g. the O antigen. While this work focused on *Shigella sonnei*, we believe that this technology is an innovative platform for efficient vaccine manufacturing for Gram-negative bacteria.

Supporting Information

Figure S1 GMMA enrichment and purity after TFF without diafiltration of the biomass. GMMA were purified from a 5 L fermentation culture of *S. sonnei* Δ*tolR* Δ*galU* grown in HTMC at 37°C to OD 39 (fermentation B2 in Table 3) using 2-step TFF. In the first step, the culture supernatant which contains the GMMA was separated from the bacteria using a 0.2 μm filter without further diafiltration of the biomass. To determine the amount of GMMA in the permeate GMMA were separated from soluble proteins by ultracentrifugation. After ultracentrifugation, the pellet (GMMA) was resuspended in the initial volume of the centrifuged material to normalize all samples to fermentation volume. Equivalent volumes of the 0.2 μm filtrate before ultracentrifugation (1), the resuspended GMMA pellet (2), and the supernatant of the ultracentrifugation (3) were separated by SDS-PAGE (12% PA) and showed a large amount of soluble proteins (3) in comparison to GMMA-associated proteins (2) to be present in the post 0.2 μm TFF permeate. In the second TFF step, GMMA were separated from soluble proteins using a 0.1 μm filter. The retentate (4) was analyzed by ultracentrifugation as described above and was found to contain almost exclusively GMMA (5) as determined by the strong reduction of soluble proteins (6). The high recovery rate of 83% in this process (see Table 3) is reflected in the similar strength of the visible protein bands in lane 2 (GMMA in the 0.2 μm TFF filtrate) and lane 5 (GMMA in the 0.1 μm retentate).
(TIF)

Figure S2 Reproducibility of purity and protein composition of GMMA obtained by the high yield production process. *S. sonnei* Δ*tolR* Δ*galU* was grown in HTMC at 37°C in a 5 L fermenter to high densities of OD 30 (B1) and OD 39 (B2) and GMMA were purified using 2-step TFF. To determine the amount of GMMA in the retentate of the 0.1 μm TFF (purified GMMA) GMMA were separated from soluble proteins by ultracentrifugation. After ultracentrifugation, the pellets (GMMA) were resuspended in the initial volume of the centrifuged material to

normalize all samples to fermentation volume. Equivalent volumes of the retentate before ultracentrifugation (1), the resuspended GMMA pellet (2), and the supernatant of the ultracentrifugation (3) were separated by SDS-PAGE (12% PA). The retentates were found to contain almost exclusively GMMA (2) as determined by the strong reduction of soluble proteins (3). In addition, the protein pattern in GMMA from the 2 fermentations was very similar suggesting good reproducibility of the process. Minor differences in the visible amount of proteins are highlighted by arrows.
(TIF)

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Author Contributions

Conceived and designed the experiments: FBS AMC SS NN VDC AS CG. Performed the experiments: FBS AMC LM SS OR IF IP MC VDC. Analyzed the data: FBS LM SS OR IF IP CG. Contributed reagents/materials/analysis tools: IF NN. Wrote the paper: FBS CG.

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