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Characterization of O-antigen delivered by Generalized Modules for Membrane Antigens (GMMA) vaccine candidates against nontyphoidal *Salmonella*

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ABSTRACT

Invasive nontyphoidal *Salmonella* disease (iNTS) is a leading cause of death and morbidity in Africa. The most common pathogens are *Salmonella enterica* serovars Typhimurium and Enteritidis. The O-antigen portion of their lipopolysaccharide is a target of protective immunity and vaccines targeting O-antigen are currently in development. Here we investigate the use of Generalized Modules for Membrane Antigens (GMMA) as delivery system for *S.* Typhimurium and *S.* Enteritidis O-antigen. Gram-negative bacteria naturally shed outer membrane in a blebbing process. By deletion of the *tolR* gene, the level of shedding was greatly enhanced. Further genetic modifications were introduced into the GMMA-producing strains in order to reduce reactogenicity, by detoxifying the lipid A moiety of lipopolysaccharide. We found that genetic mutations can impact on expression of O-antigen chains. All *S.* Enteritidis GMMA characterized had an O-antigen to protein w/w ratio higher than 0.6, while the ratio was 0.7 for *S.* Typhimurium Δ*tolR* GMMA, but decreased to less than 0.1 when further mutations for lipid A detoxification were introduced. Changes were also observed in O-antigen chain length and level and/or position of O-acetylation. When tested in mice, the GMMA induced high levels of anti-O-antigen-specific IgG functional antibodies, despite variation in density and O-antigen structural modifications.

In conclusion, simplicity of manufacturing process and low costs of production, coupled with encouraging immunogenicity data, make GMMA an attractive strategy to further investigate for the development of a vaccine against iNTS.

1. Introduction

Salmonella enterica Typhimurium and Enteritidis are the most common serovars responsible for invasive nontyphoidal *Salmonella* disease (iNTS) in Africa [1–4], resulting in case-fatality rates of around 20% [5]. iNTS is closely associated with malaria and malnutrition among African infants and children, and with HIV infection in all age groups [6]. Antibiotics are not always available in rural African settings, and increasing levels of multidrug-resistance are limiting their effectiveness [7–9], making this disease a high priority for vaccine development. Currently, there are no licensed vaccines against iNTS and efforts are ongoing to identify protective antigens and best strategies for vaccine development [10,11].

Antibodies directed against the O-antigen (OAg) portion of *Salmonella* lipopolysaccharide (LPS) have been shown to be able to mediate killing [12–16] and protect against infection in animal models [14,15,17]. *S.* Typhimurium and *S.* Enteritidis OAg share a common backbone consisting of galactose (Gal), rhamnose (Rha), and mannose (Man), which serologically constitutes epitope O:12 [18]. A different 3,6-dideoxy-hexose residue is linked to Man in these two serovars: abequose (Abe), conferring O:4 specificity to *S.* Typhimurium, or tyvelose (Tyv), conferring O:9 specificity to *S.* Enteritidis (Fig. 1) [19,20]. *Salmonella* OAg can demonstrate high levels of heterogeneity in terms of chain length and variation in O-acetylation and glucosylation of the repeating units [14,21–24]. For *S.* Typhimurium, Abe may be O-acetylated at

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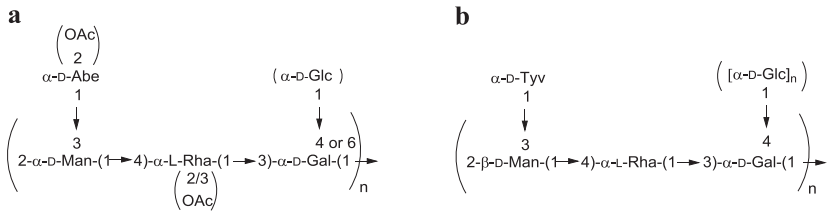


Fig. 1. OAg repeating unit structure of (a) *S.* Typhimurium (O:4,5) and (b) *S.* Enteritidis (O:9).

position C-2, which adds the O:5 specificity [25]. The additional presence of O-acetyl groups at C-2 and C-3 of Rha has also been reported [22,26]. OAg chains can also be variably glucosylated, with glucose (Glc) linked at C-4 (O:12_2 specificity) or C-6 (O:1 specificity) to Gal [21,27]. Studies in mice indicated that all these structural modifications can impact the immunogenicity of the corresponding glycoconjugate vaccines [14,23].

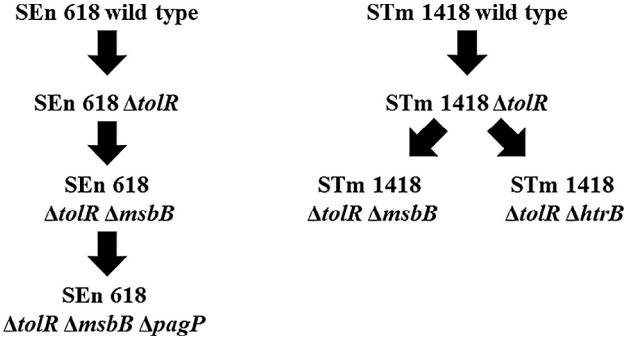
We are investigating a Generalized Modules for Membrane Antigens (GMMA) [28] approach to the development of a bivalent vaccine against *S.* Typhimurium and *S.* Enteritidis [29]. Gram-negative bacteria naturally shed outer membrane as blebs [30,31]. The release of blebs can be greatly increased by genetic manipulation of the bacteria resulting in GMMA. In *Salmonella*, deletion of the *tolR* gene affects the stability of the linkage between the inner and the outer membrane, and results in an enhanced shedding process [28,29,32,33]. GMMA derived from the surface of Gram-negative bacteria contain potent immunostimulatory components, such as lipoproteins and LPS, which can contribute to their immunogenicity, but also to reactogenicity [30,31]. In GMMA vaccine development, removal or modification of such components may alter the balance between reactogenicity and immunogenicity. One way to reduce the reactogenicity of LPS is to modify its acylation pathway, for example by deletion of *msbB*, *pagP* and *htrB* genes [29].

In this study, we report that mutations introduced to increase GMMA release and decrease reactogenicity are associated with changes in the structure of surface OAg. We investigate the impact of these changes on the antibody response to GMMA in mice and serum bactericidal activity of these antibodies *in vitro*.

2. Materials and methods

2.1. Strains

Salmonella enterica serovar Typhimurium isolate SGSC1418 (STm 1418) (LT-2 collection [34], University of Calgary) and Enteritidis SA618 (SEn 618) (CEESA EASSA II collection [35] of Quotient Bioresearch Limited), both isolated from animals, were chosen as parent strains [23]. Mutants were generated as previously described [29] (Scheme 1).



Scheme 1. GMMA of *S.* Enteritidis and *S.* Typhimurium mutated strains chosen for characterization [29].

2.2. GMMA production and characterization

GMMA were produced and purified as described [29]. Total protein content was estimated by Lowry assay [36,37]. OAg sugar content was quantified by High-Performance Anion-Exchange Chromatography coupled to Pulsed Amperometric Detector (HPAEC-PAD) as previously described [21], after performing acid hydrolysis directly on GMMA. GMMA components did not interfere in the quantification of the OAg sugar monomers. The amount of core reducing end KDO (2-keto-3-deoxy-octonate) was assumed equal to the amount of lipid A and quantified by semicarbazide/High Performance Liquid Chromatography - Size Exclusion Chromatography (HPLC-SEC) method after sugar extraction [21]. The percentage of OAg chains was calculated as the molar ratio of their KDO divided by total KDO, including LPS molecules with just core. Lipid A structures were investigated by Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS) [29]. Protein pattern profile was analyzed by SDS-PAGE analysis (SI).

2.3. OAg purification and characterization

OAg extraction and purification from wild type bacteria was performed as previously described [38]. For extraction of the OAg from GMMA, a similar procedure was used as detailed in SI. Gel filtration chromatography with differential refractive index (dRI) detection was used to fractionate the OAg chains obtained after extraction from GMMA. Samples were run on Sephacryl S300 column (90 cm × 1.6 cm i.d.). The mobile phase was $NaNO_3$ 0.05 M at the flow rate of 8 mL/h. Fractions from Sephacryl S300 were desalted by gel filtration chromatography on Biogel P2 column (90 cm × 1.6 cm i.d., flow rate 8 mL/h) or on a Bioline preparative chromatographic system equipped with Superdex G30 column (90 cm × 1.0 cm i.d., flow rate 1.5 mL/min). OAg structural analysis was performed as previously reported [21]. OAg peak molecular weight (MP) was calculated by HPLC-SEC analysis by using dextrans as standards on TSK gel G3000 PWXL column (30 cm × 7.8 mm; particle size 7 μm; Cat. N. 808021) with TSK gel PWXL guard column (4.0 cm × 6.0 mm, particle size 12 μm; Cat. N. 808033) (Tosoh Bioscience) and with in-line dRI detector. 80 μL of samples 100 μg/mL polysaccharide content were injected and eluted at the flow rate of 0.5 mL/min with 0.1 NaCl, 0.1 NaH_2PO_4, 5% CH_3CN, pH 7.2 as mobile phase.

2.4. High-Performance Liquid Chromatography - Size Exclusion Chromatography/Multi-Angle Light Scattering (HPLC-SEC/MALS)

GMMA samples were analyzed by HPLC-SEC with Sepax SRT-C 2000-1000 columns in series (Cat. N. 235980-7830; 235950-7830) equilibrated in PBS and with in-line UV, fluorescence emission, dRI and MALS detectors. 80 μL of samples 100 μg/mL protein content were injected and eluted with a flow rate of 0.5 mL/min.

MALS data were collected and analyzed using ASTRA 6 software (Wyatt Technology).

2.5. Immunogenicity of candidate Salmonella GMMA vaccines in mice and serological analysis

Five groups of eight 5-weeks old female CD1 mice were purchased from Charles River Laboratory and maintained at Novartis Vaccines Animal Care. Mice received two subcutaneous immunizations at 28 days interval with 200 μL/dose of 1 μg of OAg formulated with Alhydrogel (final concentration 0.7 mg/mL Al^{3+}). Mice were bled before the first immunization (sera pooled for each group at day 0) and at days 14, 28 and 42 after the first immunization (collected as single sera). All animal protocols were approved by the local animal ethical committee (approval N. AEC201309) and by the Italian Minister of Health in accordance with Italian law. Individual mouse sera, collected at each time point, were tested for anti-OAg IgG antibody titers by ELISA, as previously described [23]. For each group, equal volumes of sera collected at day 42 from each mouse were pooled, and tested for serum bactericidal activity (SBA) against *S.* Typhimurium D23580, an endemic clinical isolate from Malawi [8,39], obtained from the Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi, or against the laboratory strain *S.* Enteritidis CMCC4314, (corresponding to ATCC4931) obtained from the Novartis Master Culture Collection (NMCC), as previously described [23]. Bactericidal activity was determined as serum dilutions necessary to obtain 50% colony forming units (CFU) reduction after 3 h incubation at 37 °C compared with CFU counted at time 0. To evaluate possible nonspecific inhibitory effects of baby rabbit complement (BRC) or mouse serum, bacteria were also incubated with SBA buffer and active BRC as negative control, and with pooled sera from a control placebo group diluted at the same dilutions of test sera (starting from 1:100) with active BRC. For all negative controls we did not observe any killing effect up to the highest serum concentration tested of 1:100 (assay's baseline).

Statistical and graphical analysis was performed using GraphPad Prism 6 software. The non-parametric Mann-Whitney test (two-tailed) and Kruskal-Wallis analysis with Dunn's test for post hoc analysis were used, respectively, to compare two or multiple groups. Response at day 14 and 42 for each group was compared by Wilcoxon matched-pairs signed rank test (two-tailed).

3. Results

As part of our previous work, several different *S.* Typhimurium and *S.* Enteritidis isolates were screened as sources of OAg for use in a bivalent glycoconjugate vaccine against iNTS [23]. Based on the results obtained, SEn 618 and STm 1418 strains were also selected for use as GMMA-producing strains. They were genetically modified through deletion of *tolR* gene for GMMA overproduction and of further genes (Δ*msbB*, Δ*htrB* and Δ*pagP*) to reduce reactogenicity [29]. IL-6 release was used as an indicator for proinflammatory responses to toll like receptors (TLR) stimulation. The combination of *ΔmsbB* and *ΔpagP* mutations provoked the least stimulation of cytokine release from human PBMC compared to GMMA with wild type lipid A. We also found that the residual activity was largely due to TLR2 activation, although GMMA significantly signaled through TLR4 [29]. Some of the resulting GMMA (Scheme 1) were here fully characterized and tested in mice.

3.1. OAg characterization of SEn 618 GMMA

OAg from SEn 618 wild type bacteria was fully characterized, as detailed in SI (Tables S1, S2; Fig. S1), and used as a comparison with OAg present on GMMA obtained from genetically modified GMMA-producing strains, in order to examine how mutations impacted on structural features.

HPLC-SEC fluorescence emission profile of GMMA samples from all mutated strains revealed very low residual soluble proteins (<5%), indicating good sample purity (Fig. S2). The particle size of GMMA from all mutated strains was similar, as estimated by HPLC-SEC analysis coupled with MALS detector (Table 1). The w/w ratio of OAg chains per mg of total protein was high for all SEn 618 GMMA. OAg chains constituted a low percentage of total LPS molecules containing core oligosaccharide, indicating that, for all mutants, a large proportion of LPS molecules contain very few or no OAg repeating units (Table 1). Analysis by SDS-PAGE performed on SEn 618 Δ*tolR* Δ*msbB* Δ*pagP* GMMA (Fig. S3) showed no major changes in the protein pattern compared to GMMA with no lipid A modification (Δ*tolR*).

Following sugar extraction by acetic acid hydrolysis of GMMA suspensions, OAg populations were isolated by size exclusion chromatography and compared to the OAg population purified from the wild type strain (Fig. 2). OAg populations from all GMMA and wild type strain showed a similar average number of repeating units close to 25. GMMA produced from Δ*tolR* single-mutant SEn 618 expressed an additional OAg population of relatively higher molecular weight (MW), with an average of 70 repeating units. This population also appeared to be present as a shoulder in the HPLC-SEC profile of the OAg from the wild type strain. The two OAg populations at different MW from Δ*tolR* GMMA were quantified by the phenol-sulfuric acid assay. The higher MW (HMW) and lower MW (LMW) fractions represented 22% and 44% respectively of the total amount of sugar extracted. For GMMA derived from the other two 618 mutant strains, OAg chains accounted for 75% (Δ*tolR* Δ*msbB*) and 68% (Δ*tolR* Δ*msbB* Δ*pagP*) of total sugar extracted. These percentages are in agreement with the molar ratios of OAg chains to total LPS molecules found (Table 1).

All OAg populations derived from all mutated as well as wild type SEn 618 strains revealed similar sugar composition by HPAEC-PAD and NMR (Table 2), in agreement with that expected for *S.* Enteritidis OAg [19]. All samples were characterized by low glucosylation levels of 8–11%, compared to 19% for the wild type OAg (Table 2).

O-acetylation levels were low for all *S.* Enteritidis OAg samples and decreased with the addition of mutations into the

Table 1
Characterization of GMMA from SEn 618 and STm 1418 mutated strains.

GMMA	w/w OAg/GMMA protein ratio	molar % OAg chains/total LPS	nmol lipid A/mg GMMA protein	nm radius (MALS)
SEn 618 Δ*tolR*	0.6	14	156.6	22
SEn 618 Δ*tolR* Δ*msbB*	1.7	22	240.1	23
SEn618 Δ*tolR* Δ*msbB* Δ*pagP*	1.5	12	528.0	20
STm 1418 Δ*tolR*	0.66	10	172.8	22
STm 1418 Δ*tolR* Δ*msbB*	0.03	<1	108.2	15
STm 1418 Δ*tolR* Δ*htrB*	0.02	0.45	154.8	11

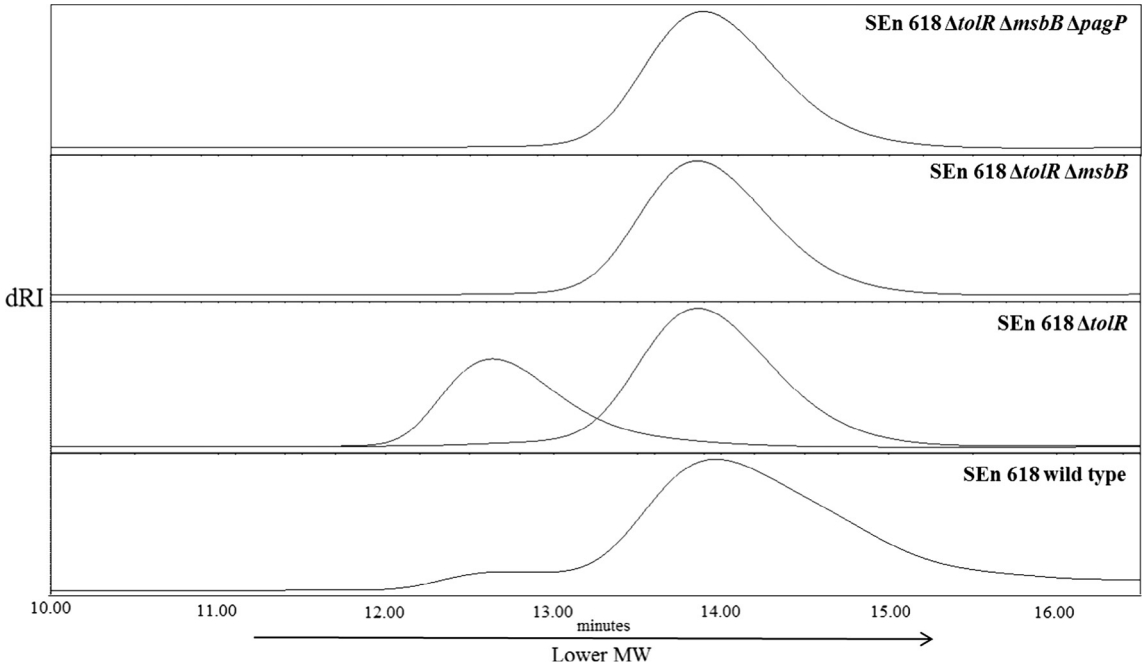


Fig. 2. HPLC-SEC chromatograms of OAg populations extracted from GMMA produced by mutated strains of SEn 618 and compared to OAg purified from the wild type SEn 618 isolate (dRI profiles; TSK gel 3000 PWXL column; NaPi 100 mM NaCl 100 mM 5% CH_3CN pH 7.2; 0.5 mL/min; V0 11.20 min; Vtot 23.29 min).

Table 2
Characterization of OAg populations extracted from GMMA produced by SEn 618 and STm 1418 mutated strains and comparison to the OAg from corresponding wild type strains. Sugar composition (molar ratio to Rha) and average number of repeating units (RU) calculated by HPAEC-PAD and HPLC-SEC/semicarbazide (assay for KDO quantification). O-acetylation (O-Ac) level calculated by ^{1}H NMR.

OAg	Tyv/Abe[a]	Rha	Man	Gal	Glc	Number RU (MP[b])	O-Ac %
SEn 618 wild type	1.00	1.00	1.00	1.08	0.19	28 (27.3)	16
SEn 618 Δ*tolR* (HMW OAg)	0.99	1.00	1.04	1.05	0.12	70 (90.3)	8
SEn 618 Δ*tolR* (LMW OAg)	0.93	1.00	0.99	1.07	0.11	23 (30.3)	4
SEn 618 Δ*tolR* Δ*mbB*	0.97	1.00	1.00	1.05	0.09	26 (30.4)	3
SEn 618 Δ*tolR* Δ*msbB* Δ*pagP*	0.97	1.00	0.99	1.06	0.08	27 (29.5)	2
STm 1418 wild type	1.00[a]	1.00	1.00	1.00	0.80	na	73
STm 1418 Δ*tolR* (HMW OAg)	0.91[a]	1.00	0.97	0.98	1.00	75 (104.7)	54
STm 1418 Δ*tolR* (LMW OAg)	0.93[a]	1.00	0.98	1.04	0.96	25 (32.9)	79

[a] Calculated by ^{1}H NMR; na: not applicable.
[b] MP: peak molecular weight in kDa calculated by HPLC-SEC analysis by using dextrans as standards.

GMMA-producing strain, from 16% found in the wild type (non-GMMA) OAg to 2% for the OAg purified from the 'triple-mutant' GMMA (Table 2 and Fig. S4).

3.2. OAg characterization of STm 1418 GMMA

OAg from STm 1418 wild type strain has been fully characterized previously [21] (Table 2, Figs. 3 and S5–S6). Table 1 details the main characteristics of *S.* Typhimurium GMMA from corresponding mutant GMMA-producing strains. As for SEn 618 GMMA, all preparations had low residual soluble proteins, as indicated by HPLC-SEC analysis (data not shown). After the introduction of the Δ*tolR* mutation, the w/w ratio of OAg chains per total protein amount was high (0.66), indicating maintenance of good levels of OAg expression. However, after the introduction of Δ*htrB* or Δ*msbB* mutations in the Δ*tolR* strain, OAg to GMMA protein ratio was greatly reduced, consistent with inhibition of OAg production (Table 1). At the same time, MALS analysis showed that GMMA producing lower amounts of OAg chains were characterized by reduced size with respect to Δ*tolR* GMMA (Table 1). As for SEn 618 GMMA, analysis of GMMA by SDS-PAGE analysis (Fig. S3) showed similar proteins profile after introduction of mutations to reduce reactogenicity compared to GMMA with no lipid A modification (Δ*tolR*).

For Δ*tolR* Δ*htrB* GMMA, the presence of an extra polysaccharide was revealed, identified as glycogen by ^{1}H NMR [40] (Fig. S5A). In Δ*tolR* Δ*msbB* GMMA, very high levels of Glc were quantified by HPAEC-PAD analysis and HPLC-SEC (dRI) chromatography revealed the presence of an additional peak, thus strongly suggesting glycogen production by this mutant as well (Fig. S5B).

OAg from Δ*tolR* GMMA showed two main populations with different average MW, consisting of 75 and 25 repeating units respectively, similar to those OAg populations from the corresponding wild type strain, but with different relative proportions (Table 2, Fig. 3). The two populations had the same sugar composition, in agreement with OAg from 1418 wild type strain, with glucosylation levels >80% (Table 2). Glycosidic linkage analysis confirmed that the main linkage for Glc was in position C-6 on the Gal residue (Table S4).

O-acetyl groups quantification by Gas Liquid Chromatography (GLC) and Gas Liquid Chromatography Coupled to Mass Spectrometry (GLC-MS) [21] performed on these populations detected the

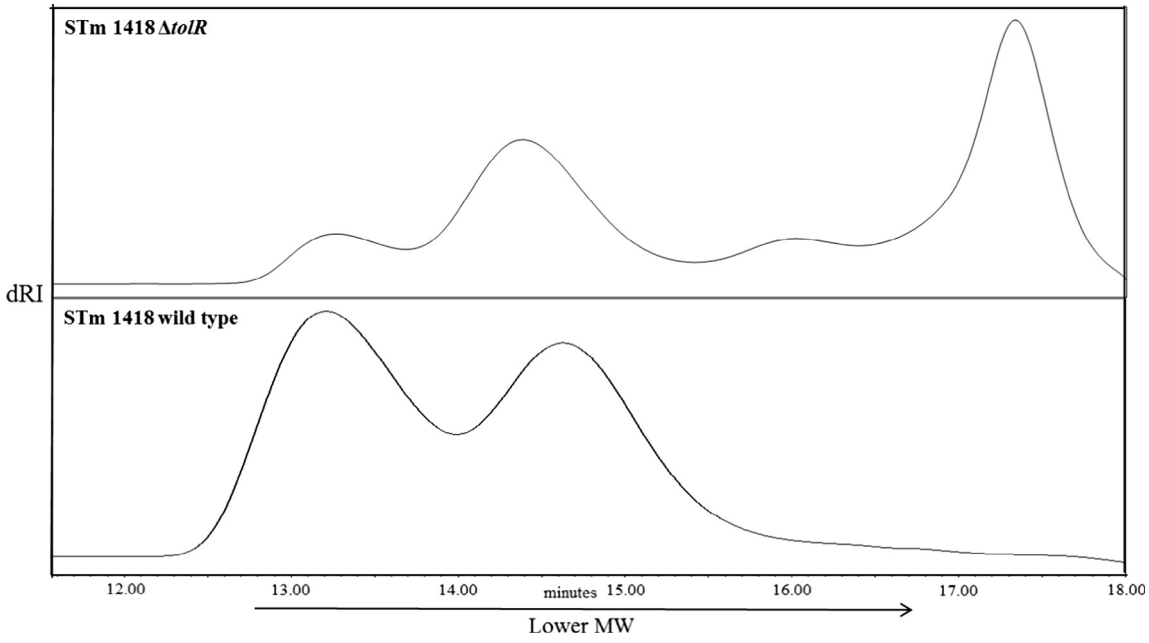


Fig. 3. HPLC-SEC chromatograms of the OAg populations extracted from STm GMMA of the Δ*tolR* mutant and compared to the OAg purified from the wild type strain (TSK gel 3000 PWXL column; NaPi 100 mM NaCl 100 mM 5% CH3CN pH 7.2; 0.5 mL/min; V0 11.20 min; Vtot 23.29 min). The component eluting at 17.30 min in the Δ*tolR* OAg refers to the core of the LPS. Core fractions for wild type OAg were lost in the purification process.

presence of O-acetyl groups only on C-2 of Abe, with O-acetylation levels of 39% for the OAg population at HMW and 32% for that at LMW, compared to 52% for the wild type OAg (Table S5). ^{1}H NMR spectroscopy analysis [21] confirmed the presence of O-acetyl groups on C-2 of Abe (O-acetyl signal at 2.10 ppm, H-2 of Abe2OAc at 5.10 ppm) in both samples (43% for HMW, 69% for LMW OAg populations), as well as in the OAg produced by the wild type strain (73%). An additional peak at 2.17 ppm (12% for HMW, 10% for LMW OAg populations) (Fig. S6) was tentatively attributed to the O-acetyl group on C-2 of Rha, by comparison with the O-acetylation pattern assigned to the OAg from *S.* Typhimurium D23580 strain [21].

The low amount of OAg chains on the double mutant GMMA impeded their detailed structural characterization.

3.3. Immunogenicity in mice

Groups of mice were immunized with SEn 618 Δ*tolR* and Δ*tolR* Δ*msbB* Δ*pagP* GMMA, and STm 1418 Δ*tolR*, Δ*tolR* Δ*msbB* and Δ*tolR* Δ*htrB* GMMA. All the GMMA candidate vaccines were compared at the same OAg dose of 1 μg. The main objective was to verify the ability of GMMA to induce an immune response and whether the observed OAg modifications, following the genetic manipulations required to generate GMMA-producing strains, affected immunogenicity in terms of anti-OAg responses. Fourteen days after the second injection, all GMMA were able to induce a serovar-specific anti-OAg IgG response, with no significant differences among GMMA of the same serovar (Mann-Whitney test for SEn 618 GMMA and Kruskal-Wallis for STm 1418 GMMA) (Fig. 4). However, more variability was observed in the anti-OAg IgG response induced in mice injected with SEn 618 Δ*tolR* Δ*msbB* Δ*pagP* GMMA (3 non-responders) compared to 618 Δ*tolR* GMMA, and with STm 1418 Δ*tolR* Δ*htrB* GMMA compared to 1418 Δ*tolR* *GMMA*. All GMMA were able to boost the anti-OAg IgG response (same p value of 0.0078 comparing day 14 and day 42 response for each group). Immune sera were also bactericidal *in vitro* against *S.* Typhimurium D23580 or *S.* Enteritidis CMCC4314 (Fig. 4).

4. Discussion

GMMA are nano-sized particles, displaying high-densities of antigens and containing bacterial pathogen associated molecular patterns (PAMPs), with the potential to trigger strong immune responses [30,31]. Furthermore, GMMA can be produced efficiently, economically and rapidly [28,32,33], making them an attractive vaccine candidate, particularly for low and middle income countries.

A comprehensive panel of analytical methods has been assembled for GMMA characterization with particular attention to their surface OAg, which is a key target of protective antibody responses. Such methods allowed verification of whether mutations introduced into GMMA-producing strains impact on OAg expression and structure, enabling the identification of optimal potential GMMA candidate for inclusion in a vaccine against iNTS. Furthermore, such methods are of fundamental importance in the process of vaccine development, to ensure consistency of production, vaccine efficacy and to monitor stability of GMMA vaccines.

The Δ*tolR* mutation introduced to increase GMMA production (approximately 300 mg OAg, obtained per liter of fermentation broth of SEn 618 Δ*tolR* and STm 1418 Δ*tolR* GMMA) was accompanied by changes in the OAg chain length distribution in both SEn 618 and STm 1418 strains. In contrast to what was observed for SEn 618 Δ*tolR* GMMA, the Δ*tolR* mutation in the STm 1418 strain selected caused a reduction of the OAg population at HMW compared to the wild type strain. In addition, mutations to modify lipid A and reduce toxicity need to be carefully monitored as they may inhibit OAg production, as observed in this study with STm 1418 clones.

With STm 1418, a reduction in the amount of OAg was associated with the production of glycogen as additional polysaccharide on GMMA. Production of such polysaccharide was not observed in the panel of nontyphoidal *Salmonella* wild type strains we previously characterized [22,23]. We have also observed this phenomenon in other mutants, derived both from *S.* Enteritidis and *S.* Typhimurium GMMA-producing strains, when genetic

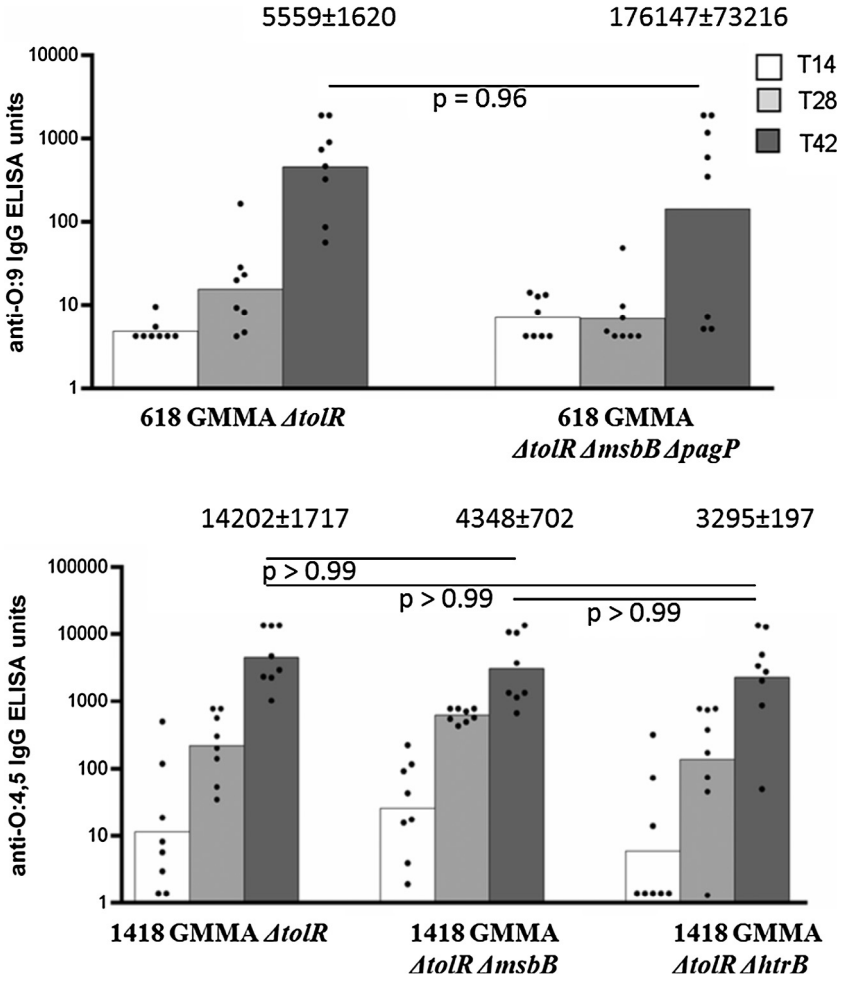


Fig. 4. Summary graphs of anti-OAg IgG geometric means (bars) and individual antibody levels (dots) induced in CD1 mice by GMMA vaccines injected at days 0 and 28 at 1 μg OAg dose. Mice were bled before the first immunization (pooled sera from each group at day 0) and at days 14, 28 and 42 after the first immunization. ELISA limit of detection was calculated as 2.9 and as 2.2 ELISA units/mL for anti-O:9 and anti-O:4,5 IgG, respectively. Numbers above day 42 bars represent SBA results of pooled sera collected at day 42 from each group against *S.* Enteritidis CMCC4314 or *S.* Typhimurium D23580. SBA titers were calculated as the serum dilution necessary to obtain 50% bacterial killing after 3 h incubation at 37 °C compared with bacteria counted at time 0. Each serum pool was tested in triplicate in three independent experiments, except for SEn 618 Δ*tolR* serum, which was tested in duplicate. Initial serum dilution was 1:100.

mutations have resulted in low OAg expression (data not shown). The reasons for this are not clear and it would be interesting to verify whether there is a correlation between lack of OAg production and glycogen formation and what functional implications this may have. Glycogen has been described to be produced in enteric bacteria for energy storage and to accumulate in cytoplasmic granules [41]. Most specifically, glycogen production in *S.* Enteritidis has previously been shown to be related to virulence, colonization and resistance mechanisms; it is usually produced under limiting growth conditions or environmental stresses [42].

OAg chain length and O-acetylation level are parameters that are known to affect the immunogenicity of OAg-based vaccines [14,17,23,25]. Mutations introduced into the wild type strains also affected the OAg O-acetylation pattern. When tested in mice at the same OAg dose, all GMMA were able to induce anti-OAg IgG specific antibodies, with functional activity, independent of the OAg density on GMMA and with no major impact of any OAg structural modification. The effective amount of OAg injected, rather than its density on GMMA, may determine the anti-OAg antibody response. The implication of this observation is that a higher OAg to GMMA ratio should permit the use of a lower amount of GMMA per vaccine dose for the same anti-OAg antibody response. This will have potential benefits in relation to cost and safety of the vaccine.

In parallel work, same GMMA characterized here were assessed for their reactogenicity. We showed that the combination of Δ*msbB* and Δ*pagP* mutations is the optimal approach to minimize reactogenicity of *S.* Enteritidis and *S.* Typhimurium GMMA, resulting in uniformly penta-acylated lipid A [29]. GMMA from this triple mutant strain provoked the least stimulation of cytokine release from human PBMC, compared to GMMA with wild type lipid A, and stimulatory potential similar to that of a *Shigella sonnei* GMMA vaccine tested in Phase I clinical trials [29,32]. *S.* Enteritidis and *S.* Typhimurium GMMA with reduced toxicity, without a major impact on OAg expression level, seem to be good components for a vaccine against iNTS. We have verified that mutations introduced to reduce GMMA reactogenicity do not have a major impact on the protein pattern profile. A more accurate analysis will be done on final selected strains in order to identify nature and amount of the most abundant proteins, considering that they may be additional key mediators of functional antibody. It will be important to verify batch to batch consistency, and to investigate the contribution that anti-protein antibodies can have on the overall immune response induced by GMMA vaccines.

Simplicity of manufacturing process and low costs of production, coupled with encouraging immunogenicity data, make the GMMA vaccine approach particularly attractive. The comparison

of OAg-GMMA with other candidate vaccines under development against iNTS, such as live-attenuated and glycoconjugate vaccines, will be of great interest for the development of a successful and efficacious intervention against iNTS.

Declaration of interests

RA, MC, LL, FN, AS, CAM, SR and FM were permanent employees of Novartis Vaccines Institute for Global Health (NVGH) at the time of the study. Following the acquisition of NVGH by the GSK group of companies in March 2015, RA, LL, FN, AS, SR and FM are now permanent employees of GSK Vaccines Institute for Global Health (GVGH), part of the GSK group of companies.

Contributorship

GDB, RA, PC, MC, AS, CAM, SR, FM were involved in the conception and design of the study. GDB, RA, MC, LL, FN, SR, FM acquired the data. GDB, RA, PC, MC, LL, FN, AS, CAM, SR, FM analyzed and interpreted the results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

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