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Skin receptors agonists as novel adjuvants for Meningococcal glycoconjugate vaccines: the impact of antigen-agonist conjugation and evaluation of intradermal vaccine delivery as an alternative to the intramuscular route

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Summary

Summary

Vaccines are complex multi-component products, in which an interdependent relationship among constituents exists: the concomitant inclusion of antigens, adjuvants, and excipients is essential to guarantee the efficacy of the final product. The selection of adjuvants and formulation criteria is a fundamental aspect, having implications towards efficacy, safety and stability of vaccines. In this field, the work was aimed at the design, formulation and characterization of novel vaccines suitable for intradermal delivery and able to induce earlier onset of immune response as well as more potent and longer lasting immune responses in comparison to conventional vaccines. With this purpose two different model antigens were used to evaluate the intradermal route as immunological target for vaccination: MenC-CRM_{197} glycoconjugate and CRM_{197} protein (detoxified diphtheria toxin). Moreover different adjuvants were tested to potentiate and modulate the immune responses towards a desired quality profile. The impact of adjuvant-antigen conjugation on immune response against the antigen was investigated in comparison to the adjuvant/antigen co-delivery. For the first time, the intradermal delivery was evaluated as route for the administration of a MenC-CRM_{197} glycoconjugate vaccine. LT mutant (LTK63), MPL (TLR4a), Toll like receptor 7 agonist (TLR7a), αGalCer analog, Dectin-1 receptor agonist (β-(1-3)glucan hexasaccharide) and an oil in water (o/w) emulsion have been chosen to target the skin-resident antigen-presenting cells and to enhance the immune response against the antigen. The *in vivo* evaluation of such novel formulations provided encouraging results with respect to quantity, quality and functionality of the induced immune responses. Notably, the intradermal delivery of MenC-CRM_{197} glycoconjugate showed superiority in term of immunogenicity and serum bactericidal titers compared to the intramuscular administration, highlighting the power of the intradermal route for glycoconjugate vaccine delivery. Moreover, the addition of LTK63, TLR4a, TLR7a, αGalCer analog adjuvants allowed the reduction of the number of doses administered and especially LTK63, TLR4a, TLR7a adjuvants were able to modulate the quality of the immune response towards a more beneficial, for the model antigen used, Th1 response. No adjuvant effect was observed when formulations were combined with (β-(1-3) glucan hexasaccharide. When MenC-CRM_{197} +TLR4a formulation was intradermally delivered in combination with the o/w

emulsion adjuvant no additional improvements in immunogenicity were observed in comparison to the single adjuvant effect. The immune system activation was also investigated using a protein model antigen (CRM_{197}) intradermally; however no improvements in term of immunogenity were observed in comparison to the intramuscular administration. This finding has specified the interest in selecting a glycoconjugate antigen to exploit the advantages of the intradermal route in terms of immunogenicity. To further investigate the role of adjuvant and to ensure codelivery of adjuvant/antigen, the conjugations of TLR7a to the glycoconjugate model antigen have been investigated. Two different approaches were developed: the conjugation of TLR7a to MenC polysaccharide and to CRM_{197} carrier protein. When the conjugation of TLR7a to CRM_{197} carrier protein was tested *in vivo*, results clearly showed that conjugation of the TLR7a enhances the anti-carbohydrate response. This effect was less pronounced than after co-administration of a commercial glycoconjugate with a standard dose of TLR7a adsorbed on AlumOH. Conjugation of the small immunopotentiator molecule was particularly suited for vaccination via intradermal delivery, where insoluble salts of aluminum cannot be used because of their reactogenicity. These findings have opened the path to the rational design of improved adjuvanted glycoconjugate vaccines for intradermal routes. Regarding the conjugation of TLR7a to MenC polysaccharide tested *in vivo*, the obtained results have shown a negative impact of the conjugation to MenC polysaccharide receptor recognition and consequently a reduction of the anti-MenC carbohydrate response. In order to investigate the potential use of a short β-glucan chain as adjuvant for intradermal vaccination toward the CRM_{197} protein model antigen, in comparison to the previously presented glycoconjugate model antigen, the results obtained by the *in vitro* and *in vivo* evaluation of the β-glucan conjugation to the CRM_{197} protein were reported. The results have demonstrated that receptor activation was significantly impacted by the presentation of the glucan conjugated to the protein antigen. Considering that glycoconjugate vaccines are some of the safest and most effective vaccines for reducing, even eradicating, infectious diseases, the conjugation of well-defined synthetic glucans could represent a useful strategy for developing new glycoconjugate vaccines for intradermal delivery, with inherent adjuvant properties. The combination of antigens and adjuvants needs to be rationally defined to develop stable formulations and to give the safest and most efficient response with respect to

the considered pathogen and route of administration. In the present work, formulation science has been proposed as a key element of novel vaccine development suitable for intradermal delivery.

Riassunto

I vaccini sono prodotti complessi costituiti da più componenti, nei quali esiste un rapporto di interdipendenza tra i costituenti: la concomitante presenza di antigeni, adiuvanti, ed eccipienti è essenziale per garantire l'efficacia del prodotto finale. La selezione degli adiuvanti e i criteri di formulazione sono un aspetto fondamentale, avendo implicazioni su efficacia, sicurezza e stabilità dei vaccini. In questo ambito, il lavoro è stato finalizzato alla progettazione, formulazione e caratterizzazione di nuovi vaccini atti alla consegna per via intradermica e capaci di indurre uno sviluppo precoce, più intenso e più duraturo della risposta immunitaria rispetto ai vaccini convenzionali. Con questo scopo due diversi antigeni sono stati utilizzati come modello per valutare la via di somministrazione intradermica per la vaccinazione: il glicoconiugato MenC-CRM_{197} e la proteina CRM_{197} (tossina difterica detossificata). Inoltre diversi adiuvanti sono stati usati per potenziare e modulare le risposte immunitarie verso un profilo di qualità desiderata. In aggiunta è stato valutato l'impatto che la coniugazione dell'adiuvante all'antigene ha sulla risposta immunitaria contro l'antigene in confronto alla semplice miscelazione. La via di immunizzazione intradermica è stata valutata, per la prima volta, per la somministrazione del glicoconiugato MenC-CRM_{197}. Per dirigere l'antigene verso le cellule APC presenti nella pelle e migliorare la risposta immunitaria verso l'antigene, sono stati testati diversi adiuvanti, quali la tossina enterica detossificata di Escherichia coli (LTK63), un agonista del recettore Toll Like 4 (TLR4a) il monofosfolipide A (MPL), un agonista del recettore Toll like 7 (TLR7a), un analogo dell'αGalCer, un agonista del recettore Dectin-1 (β- (1-3) hexasaccharide glucano) e l'emulsione olio/acqua (o/w). La valutazione *in vivo* di tali nuove formulazioni ha fornito alcuni risultati incoraggianti riguardanti la quantità e la qualità delle risposte immunitarie indotte. In particolare, i risultati immunologici ottenuti hanno dimostrato una netta superiorità della via di immunizzazione intradermica rispetto a quella intramuscolare quando è utilizzato il glicoconiugato MenC-CRM_{197}. Inoltre, l'aggiunta degli adiuvanti LTK63,

TLR4a, TLR7a, l'analogo dell'αGalCer ha consentito la riduzione del numero di dosi somministrate e soprattutto le formulazioni adiuvantate con LTK63, TLR4a e TLR7a sono state in grado di modulare la qualità della risposta immunitaria verso una più vantaggiosa risposta Th1. Nessun effetto adiuvante è stato osservato utilizzando il glucano esasaccarico come adiuvante miscelato all'antigene. Il glicoconigato MenC-CRM_{197} adiuvantato con il TLR4a è stato consegnato per via intradermica anche in combinazione con l'emulsione o/w, ma non è stato osservato alcun effetto adiuvante aggiuntivo della miscela in confronto all'effetto dei singoli immunopotenziatori. In aggiunta è stata valutata l'immunogenicità della proteina CRM_{197} a seguito della consegna intradermica ma non è stato osservato alcun miglioramento in termini di immunogenicità in confronto alla somministrazione intramuscolare. Questo risultato ha messo in evidenza l'importanza dell'adeguata scelta dell'antigene per sfruttare i vantaggi della via di immunizzazione intradermica: un antigene glicoconiugato è preferibile ad un antigene proteico. Per meglio definire l'effetto adiuvante e garantire la concomitante somministrazione di antigene e adiuvante, sono stati svilippati e studiati due approcci di coniugazione del TLR7a all'antigene modello MenC-CRM_{197}: la coniugazione del TLR7a prima al polisaccaride MenC e poi alla proteina CRM_{197}. I risultati ottenuti dai tests *in vivo* del coniugato TLR7a alla proteina CRM_{197} hanno dimostrato che la coniugazione del TLR7a migliora la risposta anti-MenC. L'effetto adiuvante del TLR7a coniugato si è rivelato meno pronunciato rispetto alla somministrazione di una dose standard di TLR7a adsorbito su AlumOH. Bisogna però sottolineare che il sale di alluminio non può essere consegnato per via intradermica, dato la sua reattogenicità, perciò ne risulta che la coniugazione del TLR7a alla proteina del glicoconiugato MenC-CRM_{197} può essere molto utile per la vaccinazione intradermica. I risultati ottenuti costituiscono la base per una razionale progettazione di vaccini glicoconiugati adiuvantati da somministrare per via intradermica. Per quanto riguarda l'approccio di coniugazione del TLR7a al MenC, i risultati ottenuti *in vivo* hanno portato alla luce un impatto negativo della coniugazione sul riconoscimento recettoriale del polisaccaride e di conseguenza una riduzione della risposta anti-MenC. Il glucano esasaccaridico è stato utilizzato per studiare il suo effetto adiuvante quando coniugato ad un antigene proteico a seguito di una somministrazione intradermica. I risultati ottenuti dalla sperimentazione *in vitro* ed *in vivo* dell'esasaccaride coniugato alla proteina CRM_{197} hanno dimostrato che l'attivazione del recettore Dectin-1 è

significativamente influenzata dalla presenza del glucano. Considerando che i vaccini glicoconiugati sono alcuni dei vaccini più sicuri e più efficaci per ridurre e sradicare le malattie infettive, la coniugazione di glucani sintetici potrebbe rappresentare una strategia utile per lo sviluppo di nuovi vaccini glicoconiugati adatti alla somministrazione intradermica, con intrinseche proprietà adiuvanti. La combinazione appropriata di antigeni e adiuvanti deve guidare la formulazione di vaccini stabili che forniscano una risposta più sicura ed efficace contro il patogeno desiderato mediante la via di somministrazione scelta. Nel presente lavoro, la scienza di formulazione è stata proposta come un elemento chiave dello sviluppo di nuovi vaccini adatti alla consegna intradermica.

Chapter 1

General introduction and objective of the thesis

Vaccines

Vaccines represent one of the most important and effective public-health interventions to control infectious diseases. Impact of vaccination is clearly proven by the eradication of smallpox and the extraordinary reduction of the morbidity and mortality due to various diseases in the world (1-2). During the last three centuries vaccinology has enabled the development of numerous vaccines, and modern approaches appear promising for the selection and the design of novel protective antigens that will increase the number of preventable diseases in the future.

- ***Vaccine formulations***

However vaccine formulations are complex biopharmaceutical products, in which the appropriate combination of antigen(s) and adjuvant(s) is a crucial aspect for the efficacy and safety. All these active components need to be stable in a wide range of storage conditions, so different techniques for vaccine formulation characterization are required in order to define their reproducibility and robustness. Different regulatory organizations clarify the requirements for vaccine development (3-4). The modern approach of vaccine formulation development to transform the discovery of an immunogen into a usable vaccine includes:

- physical and chemical characterization of the antigenic component;
- development of stability/potency indicating assays;
- evaluation and optimization of the route of administration and adjuvants
- appropriate formulation design to maximize the candidate vaccine's stability, shelf life, and immunogenic potential.

In the setup process of vaccine formulations, stabilizers and protectors cover an important role for the stability in storage and transportation. In particular, vaccine development for developing countries must include consideration of the difficulty to ensure the maintenance of cold chain across the entire commercial distribution and storage infrastructure. The World Health Organization (WHO) and PATH have developed strategies to better implement improvements in vaccine stability and the vaccine cold chain worldwide (5-6). Some intradermal delivery devices such as microneedle patches are likely to occupy less volume than vials or prefilled

syringes, thereby reducing demands on cold-chain capacity. However the development of these new devices imply the development of new formulation dosages and compositions in order to deliver the same amount of antigen in a smaller volume per dose, while maintaining integrity and immunogenicity after assembly. The rational selection of vaccine components, route and device of delivery allows for choosing the best formulation strategy in order to obtain the desired immune response against the target-diseases.

Intradermal immunization

The skin is the most peripheral tissue that covers all of the body. One of its major functions is to protect the interior of the body from pathogens and chemical agents, but at the same time acts as a niche for commensal skin flora which is mainly composed of bacteria (7). The concept of 'skin as immunological organ' was launched in the 1980s, describing the prominent presence of immune cells and molecules in equilibrium with the systemic immune system (8). The skin is composed of three different layers (Figure 2) with specific functions:

1. epidermis, consisting of stratified squamous epithelium;
2. dermis, rich of blood capillaries, nerves, lymph vessels, fibroblasts, mast cells, macrophages, DCs, Langerhans cells (LCs), natural killer cell and natural killer T-cells;
3. hypodermis, comprising connective tissue, blood vessels, nerve tissue, fibroblasts and macrophages (9-11).

It is important to highlight the fundamental role of skin DCs for the efficacy of dermal vaccination, thanks to their essential activity in initiating the immunization, with the consequent activation of the immune system (12). The skin is an easily accessible organ with optimal connection to the regional lymphatic tissues. In fact, after intradermal immunization, besides the targeting of the DCs in the site of injection, the vaccine rapidly diffuses into the surrounding tissue and is drained via the lymph vessel, reaching the lymph nodes and targeting the lymph nodes resident DCs. For these reasons it is possible to deliver vaccines intradermally with superior

immune response in comparison to the classical intramuscular routes (12-13) or to deliver a smaller quantity of vaccine antigen while obtaining the equivalent immune response (14). It is still not known how long the vaccine persists in the site of injection after intradermal immunization but viscosity of the injected formulation and size of a particle-based vaccine can influence its drainage. To better reach DCs and facilitate antigens uptake it is possible to direct the vaccine to specific receptors present on the cell surface (15-16). Different C-type lectins, highly expressed, were investigated as candidate for antigen targeting to DCs, such as DEC-205, CLEC9A, CLEC4A and CLEC12 (16-18).

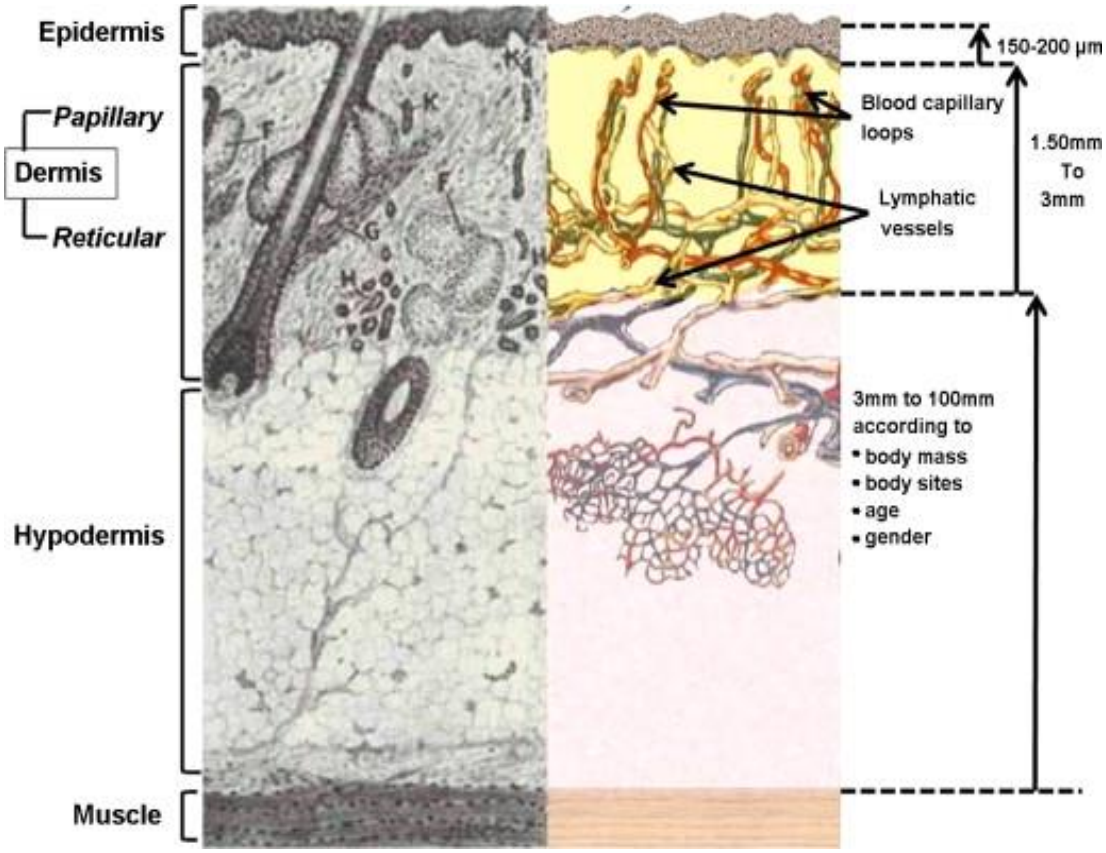

Figure 2| Skin anatomy. *Three different layers compose the skin: epidermis, dermis and hypodermis. Age, gender, body mass and body sites influence the hypodermis's thickness. (Adapted from Lambert PH, Laurent EP. Intradermal vaccine delivery: Will new delivery systems transform vaccine administration? Vaccine 2008;26:3197-208)*(13).

Several benefits of intradermal vaccine delivery can be counted, such as dose-sparing, particularly important in resource-poor settings, by potentially reducing the

per-injection cost (including transport and storage) of vaccines because more doses might be obtained from the existing vaccine presentation. Dose-sparing might also be a solution for the availability of vaccines in cases where supply is limited by manufacturing capacity. It is also necessary to ensure adequate immunogenicity across the whole target population and potency, of the reduced dose, sufficient to induce protection at the end of the vaccine shelf life. The use of needle-free devices, such as for example microneedle patches, drives a reduction in the health-care costs resulting from self-administration, simplicity of use and small-packaged volumes. Moreover the use of needle-free disposable devices allows the reduction of needle stick injuries. However, some challenges need to be addressed regarding the vaccine manufacture, device development and approval by regulatory authorities for intradermal vaccination. For instance, smaller administration volumes are typically requested compared to other routes, thereby the antigen might need to be concentrated in order to ensure adequate amount of antigen delivery. Moreover, without the development of successful methods to coat sufficient antigen onto microneedles, or new devices, and controlling the reproducibility of antigen delivery, all the upstream research work becomes in vain (19).

Vaccine adjuvants

The discovery of the immune-enhancing effects of adding an adjuvant to a vaccine was an important milestone in the vaccine history. Most of the licensed vaccines are based on highly purified recombinant antigens or on subunits of pathogens, which significantly decrease the risk of vaccine toxicity but, as a consequence, reduce their immunostimulatory activity.

The importance of combing such antigens with compounds able to potentiate, modulate and boost the antigen-specific immune response becomes clear. These immunostimulatory molecules are known as adjuvants (from the Latin word adiuvare meaning 'to help') (20) and play a key role in modern vaccines.

Vaccine adjuvants can be classified according to their source (natural, synthetic or endogenous), mechanism of action, or physical or chemical properties. However,

due to their great diversity, an accurate separation is difficult to achieve. The most appropriate existing classification divides adjuvants in two main groups on the basis of their main mechanism(s) of action (21). The first considers adjuvants as "delivery systems", according to the key function to target and localize antigens to antigen-presenting cells (APCs); the second takes into account the role of adjuvants as immunopotentiators. In the latter group molecules able to directly activate the cells of the innate immune system through specific receptors, contributing to the inflammatory context required for optimal immune responses are included.

- ***Adjuvants impact on immune mechanisms***
  - Help to make antigens more visible or reactive to the immune system
    - Creation of an antigen depot at the injection site
    - Improved antigen delivery and enhanced uptake by APCs
  - Direct activation of innate immune cells, including APCs
    -Mimic natural defensive molecules
    - Release of cytokines and chemokines
  - Potent stimulation of targeted adaptive immune responses

- ***Expected benefits of adjuvants***
  - Stronger immune priming
    - Faster immune response
    - Enhanced immune response
    - Broader and cross-protective immunity
    - Longer lasting immune response, fewer boosters needed
  - ***Enhanced and effective immune responses in specific low-responding populations (newborns, elderly, immunocompromised patients)***
  - ***Antigen sparing (use of smaller amounts of the antigen)***
    - Reduction of costs
    - Face pandemic events
  - ***Overcoming antigenic competition in complex vaccine combinations***

- ***Adjuvants requisites***
  - Easy manufacture
  - Stability
  - Acceptable safety and tolerability profile

- ***Adjuvants suitable for intradermal immunization***

Various adjuvants have been used for intradermal immunization and their selection could be based on whether a Th1 or Th2, or mixed Th1/Th2, immune response is preferred. A first group of adjuvants are represented by the bacterial adenosine diphosphate (ADP)-ribosylating exotoxins as Cholera toxin (CT), Escherichia coli heat labile toxin (LT), or subunits of these proteins have commonly been utilized to elicit or enhance the response toward a vaccine given via the cutaneous route (22-25). It is not yet clear how bacterial ADP-ribosylating toxins influence the type of immune reaction but several studies report the induction of Th2-biased immunity (22) and CTL proliferation, indicating activation of epidermal LCs (26). A second group of adjuvants for intradermal immunization are specific ligands of toll-like receptors (TLR). These receptors play a major role in the detection of pathogens and are therefore an ideal target for enhancing the immune reaction (27). In table 1 are reported a wide variety of adjuvants that have been utilized in cutaneous vaccination studies (28). A suitable experimental model to investigate immediate and long-term skin reactions after intradermal adjuvants injection remains to be developed. For example, adverse reactions were observed after aluminum hydroxide intradermal injection, in particular the development of persistent intradermal granulomas (29). Different studies have evaluated a-Galactosylceramide (a-GalCer), a synthetic glycolipid, as a potential adjuvant, because it selectively and potently activates natural killer T (NKT) cells, which are among the most effective innate immune modulators for inducing activation and maturation of dendritic cells (DC) that in turn induce CD4 and CD8 T-cell mediated adaptive immune responses (30-32). This glycolipid seems to be suitable for intradermal vaccination due to the high presence of DCs in the skin layer.

Table 1|Overview of adjuvants and their effects on the immune response upon cutaneous vaccination compared to non-adjuvanted (w/o) studies. *(Adapted from Engelke L, Winter G, Hook S, Engert J. Recent insights into cutaneous immunization: How to vaccinate via the skin. Vaccine 2015;33:4663-74)*(28).

Adjuvant	Antigen	Type of immunity
w/o	OVA, TT, DT	Th2
	OVA	Th2
	DT	Th2
	DT, bGal	Th2
ADP-ribosylating toxins		
Whole protein, subunits, or recombinant subunits of Cholera toxin (CT), E. coli heat-labile toxin (LT)	TT, LT, DT	Th2
	pDNA	Th2, Cellular
	pDNA, OVA	Th1
	DT, influenza	Th1
	Influenza	Th1, Mucosal
TLR agonists		
CpG (TLR9)	Phl p 5, bGal	Th1
	SIINFEKL	Cellular
	Influenza	Th1, Mucosal, Cellular
Imiquimod (TLR7)	SIINFEKL + anti-CD40 mAb (i.v.)/UVB	Cellular
Poly (I:C) (TLR3)	OVA	Th1, Cellular
Quillaja saponins		
Quil-A	Influenza	n.d.
QS-21	Influenza	Mucosal
Alum	bGal	n.d.
	DT	Th2
Other adjuvants		
c-di-AMP	OVA	n.d.
Mannan-coated liposomes	pDNA	Th1
Deformable liposomes	pDNA	Mucosal
Poly[di(carboxylate-phenoxy)phosphazene]	HbsAg	n.d.

Immune responses and vaccination

The deliberate stimulation of an immune response by immunization was originated in 1796 with E. Jenner's experiments, in which the cowpox virus was inoculated into the skin to render individuals resistant to the subsequent infection with lethal smallpox virus. This practice, called vaccination, takes advantage of the immune system's capacity to develop an adaptive immune response after an exposure to a small sample of disease-causing microorganism - or to a part or portion of it (Figure 1). The main actors of the adaptive immune response are antibodies secreted by B lymphocytes. These antibodies can act in different ways to protect against the pathogens: they can neutralize the pathogens by blocking their access to the binding sites on the host cell surface, a mode of action that is very important for protection against viruses; antibodies can coat the bacteria in order to make them attractive for phagocytes, which have receptors able to recognize the stems of the antibodies coating, also known as opsonization process; antibodies can manage the activation of the complement system once they have coated the pathogen surface, with the results of lysis and ingestion by phagocytes (33). Other important effectors of adaptive immune response are the $CD8^+$ cytotoxic T lymphocytes (CTL) that have a role in the eradication of viruses and tumors. In effect, immunotherapies that promote antitumor CTL responses showed significant evidence of clinical success in the melanoma, renal and lung cancers treatments (34). The activation of B-cells to produce antibodies, their class switching and affinity is supported by growing factors and signals provided by $CD4^+$ T helper cells (Th). The protective efficacy of a vaccine is often conferred to the production of antigen-specific antibodies but this is not always sufficient. T-cells play an important role in developing an effective and long lasting immune response. The antigen-specific T-cell activation is driven by the specific antigen presenting cells (APC), of wich the dendritic cells (DC) are the most professional. They can perform uptake of microorganisms in different ways (phagocytosis, endocytosis and pinocytosis), with the consequent loading on MHC class II of processed molecules and secretion of specific cytokines.

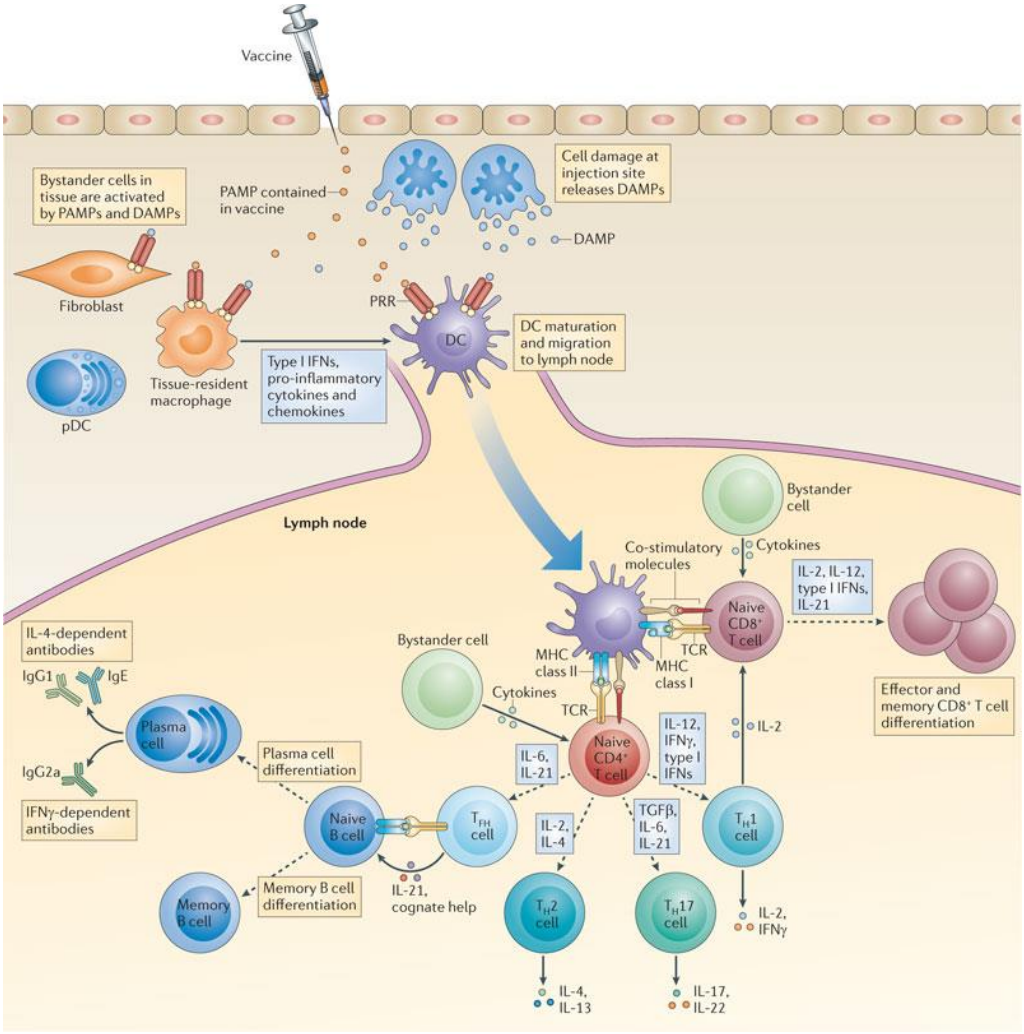

Figure 1| Induction of adaptive immune responses. *Pattern-recognition receptors (PRRs), expressed by dendritic cells (DCs), recognize and bind the pathogen-associated molecular patterns (PAMPs,) contained in vaccine formulation after the delivery. This connection leads to DC activation, maturation and migration to the lymph nodes. The activated DCs present antigens to naïve T-cells and stimulate their differentiation by specific cytokines secretion. (Adapted from Desmet CJ, Ishii KJ. Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nat Rev Immunol. 2012;12:479-49)*(35).

DCs, monocytes and neutrophils constitute the first line of defense against invading microbial pathogens and rely on a large family of pattern recognition receptors (PRRs), which detect distinct evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs). The potential to activate DCs targeting their surface receptors can be a good strategy to manipulate the first line of defense against invading pathogens.

Outline of the thesis

The PhD work was aimed at the design, formulation and characterization of a novel vaccine suitable for intradermal delivery and able to induce earlier onset of immune response as well as more potent and longer lasting immune responses compared to conventional vaccines. In this context adjuvants are used to potentiate the immune responses towards specific antigens and to modulate the immune response towards a desired quality profile. Conjugation of adjuvants to a model antigen is investigated for its potential to increase antigen immune response when compared to co-delivery of adjuvant and antigen by simple mixture in respective formulations. The thesis reports obtained results through the *in vitro* and *in vivo* testing of different adjuvants in physical mixture or conjugated to model antigens.

- *Chapter 2*: **Evaluation of receptors agonists as immunopotentiators for glycoconjugate vaccines –** is focused on the hypothesis that by targeting the superficial receptors present on skin-resident antigen-presenting cells, using receptor agonists as adjuvants, it is possible to enhance the immune response against the model antigen. In particular, were investigated:
  - a small molecule as toll like receptor 7 agonist;
  - Monophosphoryl Lipid A (MPL) as toll like receptor 4 agonist;
  - the heat-labile enterotoxin from Escherichia coli (LT) mutant as GM1 receptor agonist;
  - a short β-glucan as Dectin-1 (CLEC9A) receptor agonist;
  - a α-GalCer analog as CD-1d receptor agonists;

  The MPL immunopotentiator was also studied in combination with an oil-in-water emulsion (o/w) adjuvant.

  This work finds its justification in the discovery of novel adjuvants suitable for intradermal vaccination since the classical adjuvant, aluminum salt, cannot be used for intradermal immunization due its local reactogenicity. This work is part of a broader vision of development of new devices for intradermal vaccination, such as microneedles patches, in which the optimized formulation can elicit an efficient and long lasting immune

response. The Meningococcal C glycoconjugate vaccine was used as a model antigen for intradermal and intramuscular investigations.

- *Chapter 3:* **The adjuvant effect and impact of conjugation of TLR7 agonist in a tricomponent anti serogroup C meningococcal glycoconjugate vaccine** – is focused on the TLR7 agonist adjuvanticity investigation after intramuscular delivery; where the TLR7 agonist is either co-delivered or conjugated to the Meningococcal C glycoconjugate vaccine. Two different approaches of agonist conjugation were investigated *in vivo*:
  - TLR7a conjugation to CRM_{197} carrier protein;
  - TLR7a conjugation to MenC polysaccharide;

  The *in vitro* receptor activation and *in vivo* immunological evaluations were reported. The TLR7a adjuvanticity was compared to the classical aluminum salts adjuvant suitable for intramuscular vaccine delivery.

- *Chapter 4:* **Conjugation of a short β-glucan chain, as Dectin-1 agonist, to the detoxified diphtheria toxin (CRM_{197}) as model antigen, to enhance the immune response after intradermal vaccination –** describes the conjugation of a β-glucan hexasaccharide to the CRM_{197} model antigen as strategy to enhance the immune response toward a protein antigen.

Chapter 2

Evaluation of receptors agonists as immunopotentiators for glycoconjugate vaccines

Selection of the model antigen

The Meningococcal C glycoconjugate was used as a model vaccine for the investigation of intradermal vaccine delivery as an alternative to the intramuscular route. The recommended vaccination schedule of the licensed group C meningococcal vaccines is composed by a first intramuscular dose in infancy followed by a boost in the adolescence or a single intramuscular dose at 11 years. Therefore, the possible elimination of pain at the injection site and the easier administration with the possibility of self-administration might increase the vaccine acceptance and the numbers of vaccinated people especially in adolescents, which often are reluctant to vaccination.

Neisseria meningitidis: disease and vaccines

Meningococcal meningitis and sepsis are devastating diseases which still affect people with an incidence varying between 0.5 and 1000 cases/100 000 depending on the epidemiological areas (36). *N. meningitidis* is a gram-negative β-proteobacterium and member of the bacterial family Neisseriaceae (Figure 1) (37). There are 13 serogroups of *N. meningitidis* based on different capsular polysaccharide structures, but only six serogroups (A, B, C, W-135, X, and Y) cause most life-threatening disease (38). Meningococci of serogroups B, C, Y, and W-135 express capsules composed entirely of polysialic acid or sialic acid linked to glucose or galactose (39), while the capsule of group A *N. meningitidis* is composed of *N*-acetyl mannosamine-1-phosphate (39). Complement-dependent bactericidal activity is an established correlate of protection against meningococcal disease. Studies by Goldschneider and colleagues firmly established the role of Ab in protection against invasive meningococcal infection (40). Over the past several years, much attention has been focused on the role of membrane proteins in complement evasion, in particular the interactions between factor H (FH), an inhibitor of the alternative pathway of complement, and meningococcal factor H binding protein (41-43). Other meningococcal surface molecules that inhibit the alternative pathway include Neisseria surface protein A (NspA) and PorB2, which

directly bind to FH. Meningococcal infection is a global but not uniform problem occurring as sporadic, hyper-sporadic, and epidemic disease. Disease patterns vary widely over time and between geographical areas, age groups, and bacterial serogroups. Of the five common serogroups (A, B, C, Y and W135) responsible for about 90% of infections caused by *N. meningitides*, serogroups A, B, and C account for most cases of meningococcal disease throughout the world, with serogroups B and C responsible for the majority of cases in Europe and the Americas and serogroups A and C predominating throughout Asia and Africa (44-46). Israel and Sweden are the only countries, other than the United States, that have reported an increase in serogroup Y disease (47).

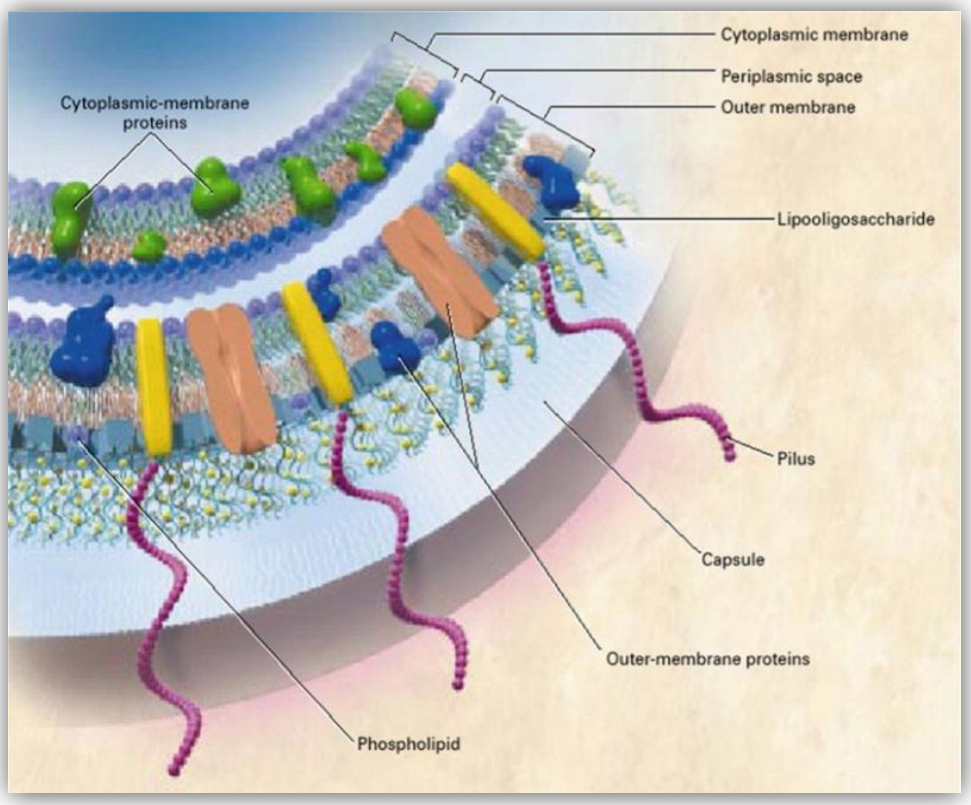

Figure 1*| *Neisseria meningitidis. *Cross-sectional view of the meningococcal cell membrane. (Adapted from Rouphael NG, Stephan DS. Neisseria meningitidis: Biology, Microbiology, and Epidemiology. Methods Mol Biol. 2012; 799: 1–20 (48)).*

Table 1*| *Licensed Meningococcal conjugate vaccine products with used schedules *(Adapted from Ali A, Zehra Jafri R, Messonnier N, Tevi-Benissan C, Durrheim D, Eskola J et al. Global practices of meningococcal vaccine use and impact on invasive disease. Pathogens and Global Health 2014;108:11-20 (49)).*

Vaccine	Manufacturer	Serogroups	Protein Conjugate	Licensed schedule
Menveo™	Novartis Vaccines	A, C, Y, W	Diphtheria cross	In US, one dose at the age of 2 years. 2nd dose 2 months after 1st dose in high-risk children aged 2–5 years
			Reactive material 197 (CRM_{197})	In Europe, approved for use from 2 months of age
Menactra™	Sanofi Pasteur	A, C, Y, W	Diphtheria toxoid	1st dose in children aged 9–23 months. 2nd dose after 3 months in the age 2–55 years.
Meningitec™	Neuron Biotech	C	CRM_{197}	4 doses for children <1 year at 2, 4, 6, and 12 months. One dose for children over 12 months, teenagers and adults
Menjugate®	Novartis Vaccines	C	CRM_{197}	Age 2–4 months – 3 doses 8 weeks apart, 4–11 months – 2 doses 8 weeks apart, ≧12 months – 1 dose
NeisVac-C™	Baxter Bioscience	C	Tetanus toxoid	Age 2–12 months – 2 doses 2 months apart. ≥12 months – 1 dose
Menitorix™	GlaxoSmithKline	C	Tetanus toxoid	Age 2–12 months – 3 doses, 1 month apart. Booster from 12 months – 2 years
MenAfriVac™	Serum Institute of India	A	Tetanus toxoid	To be defined
MenHibrix™	GlaxoSmithKline	C & Y	Tetanus toxoid	Age 2–15 months – four doses

Different vaccines were developed against the meningococcal A, C, W, and Y serogroups, including polysaccharide vaccines and polysaccharide–protein conjugate vaccines based on the meningococcal capsule. For serogroup B, vaccine development has included protein vaccines based on meningococcal outer membrane vesicle (OMV) and, more recently, a range of conserved proteins including Factor H Binding Protein (fHBP), Neisseria Adhesin A (nadA), and

Neisseria Heparin Binding Antigen (NHba) have been used as vaccine components. The major disadvantage of polysaccharide vaccines is their inability to produce memory cells leading to poor response to boosters and short duration of protection. For this reason Meningococcal conjugate vaccines were introduced in 1999 and since then, conjugate quadrivalent (A, C, Y, W) and monovalent MenA and MenC vaccines (Table 1) have been licensed in various countries. Conjugate vaccines use a carrier protein to present the polysaccharide antigen to the immune system, in a manner that induces a T-cell immune response.

Selection of adjuvants

As previously stated, different immunopotentiators were tested *in vivo* in order to investigate their ability to target the superficial skin-resident antigen-presenting cells receptors. This strategy was based on the idea that the use of receptors agonists can better direct the antigen toward the APCs and enhance the immune response against the antigen.

1. Toll-like receptors and their agonists

Toll-like receptors (TLRs), named pattern recognition receptors (PRRs), are expressed in dendritic cells and macrophages, the sentinel cells of the innate immunity, and recognize highly conserved structural pathogen components (50).

It is well known that the TLRs are able to stimulate the innate immune system and guide the magnitude and quality of an adaptive response (51). For this reason different molecules were selected in order to mimic the natural TLRs ligands and act as vaccine adjuvants (52). In the current study, Monophosphoryl Lipid A (MPLA) (53), a TLR4 agonist (TLR4a), and a synthetic compound, as TLR7 agonist (TLR7a), were evaluated for a Meningococcal C glycoconjugate vaccine. These agonists were also formulated, and *in vivo* evaluated, with an o/w emulsion adjuvant. MPLA is a derivative of the lipid A portion of *Salmonella Minnesota* Re595 lipopolysaccharide (LPS) which retains the immuno-stimulatory properties, while lacking in toxicity. The TLR7a compound belongs to the imidazoquinolinamines class (54), generically known as SMIPs (small molecule

immune-potentiators), since they are able to modulate the immune response through immunoglobulin production (55), interferon and cytokine synthesis (56).

2. *Heat-labile enterotoxin from Escherichia coli (LT) mutant as GM1 receptor agonist*

Bacterial adenosine diphosphate (ADP)-ribosylating exotoxins are a group of adjuvants able to elicit or enhance the response toward a vaccine given via the cutaneous route (22-25). In particular, the heat-labile enterotoxin from Escherichia coli (LT) mutant, LTK63, is composed of two subunits coded by an operon; subunit A (LTA) is a 28-kDa enzyme and subunit B (LTB) is a 60-kDa protein, composed of five identical polypeptides (11.6 kDa) (57).

The B pentamer structure contains the cellular receptor binding function. The principal receptor for LTB is the ganglioside GM1, a glycosphingolipid found ubiquitously on the cell surface of mammalian cells. The receptor binding activity of LTB is required for the uptake and internalization of the AB_5 complex by host cells. The A subunit is the toxic portion of LT and is internalized and subsequently stimulates the cellular adenylate cyclase-cyclic AMP system, leading to dramatic and unregulated elevation of intracellular cAMP. In intoxicated gut epithelial cells, cAMP elevation results in massive secretion of electrolytes and water into the gut lumen, clinically manifesting as diarrhea. Therefore, the A subunit, responsible for the toxicity of LT, was modified in the active site, (serine-to-lysine substitution at position 63) and lacks total ADP-ribosylating activity, generating the LTK63 mutant. This molecule has been reported to retain the potent adjuvant properties of LT (58). In particular, immunomodulatory effects of LTK63 appear to vary with vaccination route. For example, subcutaneous administration of LTK63 with a meningococcal serogroup C polysaccharide conjugate induced a dominant Th1 response whereas intranasal administration induced a mixed Th1/Th2 response (58). Regarding intranasal and intramuscular delivery, LTK63 has reportedly enhanced APC activation and maturation, increased the proportion and total number of B-cells as well as $CD4^+$ and $CD8^+$ T-cells, and both stimulated and enhanced both Th1-type cytokine responses (including IFN-γ and TNF-α) as well as Th2 responses

(59). In the current study the LTK63 was evaluated for a Meningococcal C glycoconjugate vaccine.

3. β-glucan hexasaccharide as Dectin-1 receptor agonist

β-(1-3)-glucans, which are the most abundant components of the fungal surface, are known to activate the innate immune response by interaction with the C-type lectin-like Dectin-1 receptor (60-61). β-(1-3)-glucans binding to Dectin-1 induces the phosphorylation of a tyrosine of the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail that elicits the secretion of inflammatory cytokines, such as tumor-necrosis factor (TNF) and IL-12 (62). β-glucans activated-DCs are able to stimulate antigen specific CD8+ T-cell responses, inducing enhancement of antigen presentation, priming and expansion of antigen specific CD8+ T-cells (63-65). The interaction of β-(1-3)-glucans, which are triplic helix forming polysaccharides, with Dectin-1 has been detailed by diverse studies (66-69). Linear structures have been found to bind best to the receptor in comparison to branched ones (70). The minimal length required for interaction is 6-7 repeating units, although the binding becomes tighter as the length increases (69-70). In the current study the β-glucans hexasaccharide was evaluated for a Meningococcal C glycoconjugate vaccine.

4. α-GalCer analog as CD-1d receptor agonists

α-GalactosylCeramide (α-GalCer), a potent lipidic agonist, found in the marine sponge *Agelas mauritianus* (71), strongly activates the invariant natural killer T (iNKT) cells, binding the lysosomal CD1d molecules. Upon activation, iNKT-cells modulate the activity of CD1d-expressing cells, such as DC and B-cells, via the costimulatory molecule CD40 ligand, and induce IFN-γ-dependent activation of NK cells, ultimately enhancing adaptive immune responses (72-73). By influencing the activation state of an APC, α-GalCer plays an important role in the control of bacterial, parasitic, viral infections, as well as natural immunosurveillance, contributing to the regulation of a variety of processes, such as self-tolerance, tumor surveillance, and antimicrobial responses. It has been also observed that α-GalCer triggers enhanced antibody production in mice lacking MHC II, suggesting that

iNKT-cells can substitute for conventional $CD4^+$ T-cells and provide assistance for maximal activation of B-cells (74). A α-GalCer analog was evaluated, in the current study, as agonist in co-delivery with the Meningococcal C glycoconjugate vaccine.

5. Oil-in-water emulsion (o/w) adjuvant

The oil-in-water nanoemulsion (o/w) adjuvant used in the present work is based on squalene droplets dispersed in an aqueous phase containing two non-ionic surfactants, Tween 80 and Span 85 (75). A similar nanoemulsion adjuvants namely the MF59 was the second adjuvant to be licensed in Europe for seasonal and pandemic influenza adjuvanted vaccines, demonstrating superior immunogenicity in comparison to aluminum salts (76-78) and excellent safety and tolerability (79) even after intradermal administration (80). The adjuvant effect of such nanoemulsion adjuvants was shown to be closely dependent on its formulation since none of the individual components induced comparable adjuvanticity (81). In the current study the nanoemulsion was tested in combination with the Meningococcal C glycoconjugate vaccine and further adjuvanted with the MPL immunopotentiator mentioned before, in order to improve the vaccine immunogenicity and evaluate the quality of the immune response.

Intradermal evaluation of receptors agonists as immunopotentiators for glycoconjugate vaccine delivery

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Abstract

Intradermal vaccine delivery is a promising alternative to the conventional intramuscular route. The skin layer is immunologically supported by a densely connected network of antigen presenting cells (APCs), while the skeletal muscle is loaded with a relatively sparse population of APCs. Thanks to this immunological machinery intradermal delivery induced a higher immunogenicity and a prolonged persistence of immune response for several antigens when compared to the intramuscular route. Moreover reduced pain, the potential for self-administration, speedier vaccine delivery and the prospective for the elimination of the "cold chain" increase the interest in intradermal vaccination. The scope of this study is to evaluate for the first time intradermal delivery as the route for the administration of the MenC glycoconjugate vaccine. A vaccine formulation suitable for intradermal delivery was prepared and its immunogenicity with or without adjuvants was tested in comparison to the classical intramuscular route. As adjuvants several Toll like receptor agonists, an LT mutant, a α-GalactosylCeramide analog and an oil in water emulsion were investigated in order to target skin-resident antigen-presenting cells. The intradermal delivery of MenC glycoconjugate vaccine showed superiority in terms of immunogenicity and serum bactericidal titers compared to the intramuscular administration even in absence of adjuvant. Importantly, the use of adjuvants allowed to reduce the number of doses administered and to modulate the quality of the immune response towards a more beneficial Th1 response. This approach has the potential to be extended to other meningococcal serogroups representing a promising strategy for the development of dermally administered multivalent glycoconjugate vaccines.

Introduction

The dermal skin layer, that lies beneath the stratum corneum, is characterized by a densely network of immune modulating antigen presenting cells (APCs). While in the epidermis and dermis APCs are most significantly represented by Langerhans cells and dermal dendritic cells, the skeletal muscle is only loaded with a relatively sparse population of APCs. Importantly for several antigens intradermal delivery demonstrated an induction of a higher immunity and a prolonged persistence of immune response compared to the intramuscular route (82-89). Recently, enhanced anti-PCV immunogenicity was demonstrated, for a licensed pneumococcal-conjugate vaccine when delivered with a skin patch and compared to the intramuscular injection of the same vaccine (90). Intradermal delivery might also be explored in order to increase patient compliance and so the volume of people vaccinated, increasing the reduction of the disease (91). Moreover certain formulations of intradermal vaccine delivery, such as patches, may abolish the necessity of the "cold chain," which should decrease the cost of vaccine delivery and increase and facilitate distribution in developing countries (92). To further increase the benefits induced by intradermal delivery, adjuvants could be included in respective vaccine formulations. Commonly used adjuvants for transcutaneous immunization are the bacterial ADP-ribosylating exotoxins, including the heat-labile enterotoxin from Escherichia coli (LT), and its mutant LTK63 (22-25, 58). In addition several Toll like receptor agonists (52), the oil in water emulsion (76-79, 80) and a α-GalactosylCeramide analog (72-73) were evaluated in order to target skin-resident antigen-presenting cells (93-94) and enhance the immune response. Moreover the adjuvants might allow for the reduction of the number of doses administered and the modulation of the quality of the immune response (95). For all these reasons the purpose of this study was to evaluate intradermal delivery as a route for the administration of a MenC glycoconjugate vaccine. The capsular polysaccharides of the five meningococcal serogroups, A, B, C, W135, and Y, represent the major antigen for the conjugate vaccines (96-98). All the licensed meningococcal vaccines are administered by intramuscular injection. For MenC and

multivalent Men A, C, W135, and Y a first dose is recommended in infancy followed by a boost in adolescence or a single dose at the age of 11 years. The reduction of pain at the injection site and the ease of administration with the possibility of self-administration might increase the acceptance and the numbers of vaccinated people, especially adolescents, which for the first time must receive the meningococcus vaccines and have been generally reluctant to vaccination.

To achieve our goal the MenC glycoconjugate vaccine was used as a model with the intent of extending this approach to further glycoconjugate vaccines. A formulation suitable for intradermal delivery was prepared and its immunogenicity with or without adjuvants was tested and compared to the classical intramuscular route.

Materials and methods

Tangential Flow Filtration (TFF)

The MenC-CRM$_{197}$ conjugate (component of Menveo®, Menjugate® vaccines) was concentrated by tangential flow filtration (Tandem Model 1082 - Sartorius) with a 30 kDa MW cut-off, 200 cm^2 membrane (Hydrosart Sartocon Slice 200 - Sartorius). The collected concentrate materials were then characterized for protein and polysaccharide content.

High Performance Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC)

The chromatographic method was performed as previously described (99). Data was processed using Chromeleon^{TM} 6.7 software. Protein and conjugate peaks were detected at 214 and 280 nm and by fluorescence (emission spectrum at 336 nm, with excitation wavelength at 280 nm).

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The total saccharide content of MenC-CRM$_{197}$ conjugate was determined by HPAEC-PAD as previously described (99). All chromatographic data was integrated and processed using Chromeleon^{TM} 6.8 software.

Determination of CRM_{197} protein content by BCA assay

The CRM_{197} protein content in the sample was determined by colorimetric estimation using bicinchoninic acid (BCA) (Pierce™ BCA Protein Assay Kit) reagent with a bovine serum albumin (BSA) standard. The light absorbance at 562 nm was measured using the Gen5^{TM} software and a tunable micro plate reader (BioTek).

Production and size characterization of self-emulsifying oil-in-water emulsion adjuvant

The oil-in-water (o/w) emulsion adjuvant was manufactured as previously described (75). The droplet size was measured on Malver Zetasizer (Malvern Instruments, Malvern, UK) by dynamic light after filtration through membrane filters.

Immunogenicity testing in mice

Protocols were approved by the Italian Ministry of Health. All mice were housed under specific pathogen-free conditions at the Novartis Vaccines Animal Resource Center. Groups of eight 6-week-old female BALB/c mice were immunized twice, four weeks apart, intramuscularly (i.m.)/intradermally (i.d.). The MenC-CRM_{197} conjugate (1 µg/mouse of MenC) adjuvanted with alum hydroxide (AlumOH – internal compound) (0.2 mg/mouse) and delivered intramuscularly was used as a positive control. For i.m. immunization, 100 µl of CRM_{197} alone (2 µg/mouse) and MenC-CRM_{197} (1 µg/mouse of MenC) alone or co-delivered with 5 µg/mouse of the TLR4 agonist (TLR4a) Monophosphoryl lipid A (MPL - Avanti Polar), 25 µg/mouse of a TLR7 agonist (TLR7a) (internal compound), 5 µg/mouse of heat labile enterotoxin (LTK63) and 5 µg/mouse of α-GalactosylCeramide analog (α-GalAnalog) were injected. The same antigen/adjuvant dose was delivered intradermally in a 20 µL formulation using a 0.3 mL BD Micro-Fine syringe (30Gx8mm) inserted at a 45 degree angle. The vaccinations were dispensed in the back after shaving. In addition MPL adjuvant was tested in combination with 50% v/v o/w emulsion for the intradermal delivery. The appearance of nodules, erosion/scab, erythema and redness at the site of administration was monitored.

Serum samples were collected on days 14 and 42 and stored at -20 °C before analysis.

Determination of MenC-specific antibody titers by ELISA

Determination of anti-MenC specific total IgG and subclasses IgG1 and IgG2a titers was performed by ELISA using individual serum samples. Maxisorp plates (Nunc) were coated overnight at +4°C with 0.5 µg/well of MenC polysaccharide in PBS pH 8,2 and blocked for 1 h at 37°C with 200 µL of 1% BSA (Sigma), in PBS. Serum samples were initially diluted in PBS, 0.1% BSA and 0.05% Tween-20 (Sigma), and serially diluted into coated and blocked plates. Bound MenC-specific IgG, IgG1 and IgG2a were detected with alkaline phosphatase-conjugated goat anti-mouse IgG subclasses respectively (SouthernBiotech). Antibody titers, normalized with respect to the reference serum assayed in parallel, were obtained using IrisLab software. GraphPad Prism v.6.04 (GraphPad Software, La Jolla, CA) was used to graph and analyze data for statistical significance. Intergroup comparison was analyzed using the Mann-Whitney test.

Determination of functional antibodies titers against Neisseria meningitidis

Functional antibodies induced by vaccine immunization were analyzed by measuring the complement-mediated lysis of *Neisseria meningitidis* with an *in vitro* bactericidal assay using commercial pooled baby rabbit serum as a complement source (rSBA) (41).

CRM_{197}-specific T-cell intracellular cytokine responses

Spleens were taken two weeks after the second immunization. Single cell suspensions were obtained from spleen grinding through a 70 µm nylon mesh (BD Falcon). Red blood cells were lysed and cells cultured in complete medium as previously described (100) For T-cell cytokine responses, 2 x 10^6 cells/well were stimulated, overnight (ON) at 37°C, in the presence of anti-CD28/anti-CD49 Abs (4 µg/mL each) (Pharmingen/eBioscience) and CRM_{197} (10 µg/mL), or with anti-CD28/anti-CD49 alone (4 µg/mL each, unstimulated), or with anti-CD28/anti-CD49 (4 µg/mL each) plus anti-CD3 (0.1 µg/mL) (Pharmingen). After stimulation overnight, 5 µg/mL Brefeldin A (Sigma Aldrich) was added and for an additional 4

h incubated at 37°C. Cells were washed with 1x PBS (Gibco), and stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit for 635 nm excitation (Life Technologies). Cells were fixed, permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with the following mAbs: anti-TNFα (BD Pharmingen), anti-IFN-γ (BioLegend), anti-IL-2 (BioLegend), anti-IL-4 (eBioscience), anti-IL-13 (eBioscience) and anti-IL-17 (eBioscience). Stained cells were acquired using an LSR II special order system (Becton-Dickinson) and analyzed using FlowJo software (Tree Star).

Results

MenC-CRM_{197} glycoconjugate retains its integrity after the concentration process

To obtain an appropriate MenC-CRM_{197} concentration, for the delivery of the established antigen dose in 20 µL of formulation volume, required for the intradermal immunization, the glycoconjugate was concentrated using the TFF process. The obtained material was characterized in order to define the total saccharide content of MenC-CRM_{197} conjugate by HPAEC-PAD (Table 2). The yield of the process, approximately total, is an indication of the optimal applicability of the process to the MenC-CRM_{197} glycoconjugate. To prove the absence of material degradation after the concentration process, the glycoconjugate was analyzed through HPLC-SEC (Figure 2a) and SDS-Page (Figure 2b). The chromatographic profile was comparable with the untreated material. Around 20 fold more concentrated material was obtained and no impact on glycoconjugate integrity was evidenced by the weight ratio between MenC and CRM_{197} before and after the concentration process.

Table 2| Total saccharide content of MenC-CRM$_{197}$ concentrated glycoconjugate

Sample	Total saccharide mg/mL[a]	Protein mg/mL[b]	Saccharide/Protein w/w[c]	Free Saccharide %[d]	Process yield %[e]
MenC-CRM$_{197}$	0.69	1.21	0.57	< 4.4	-
MenC-CRM$_{197}$ concentrated	10.81	19.35	0.56	< 4,5	97

a: Recovered conjugate saccharide as determined by HPAED-PAD.

b: Recovered protein as determined by microBCA.

c: Weight ratio between MenC/MenA and CRM$_{197}$ in the glycoconjugate.

d: Recovered free saccharide as determined by HPAED-PAD.

e: Yield obtained on protein content before and after concentration process as determined by microBCA.

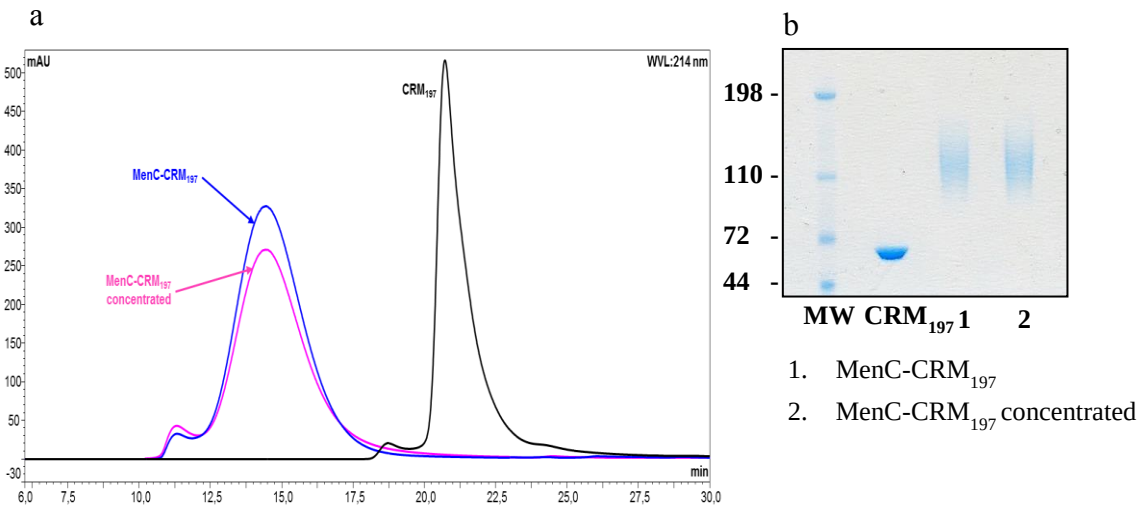

Figure 2| Concentrated material characterization. *(a) HPLC-SEC chromatographic profiles and (b) SDS page gel electrophoresis of respectively Neisseria meningitides capsular polysaccharides serogroup C and A, conjugated to the CRM$_{197}$ carrier protein, before and after the concentration processes. Void volume 10.97 min, bed volume 24.79 min.*

MenC-CRM_{197} glycoconjugate shows superiority after intradermal immunization when compared to the intramuscular route

In order to evaluate intradermal delivery as an alternative route for the administration of meningococcal vaccine an *in vivo* study was designed (Figure 3). Non-adjuvanted MenC-CRM_{197} was administered either intramuscularly or intradermally; whereas the glycoconjugate adjuvanted with AlumOH was used as a positive control for intramuscular administration only, given that AlumOH should not be used for intradermal delivery (29). Interestingly only the vaccine delivered intradermally and the AlumOH adjuvanted vaccine given intramuscular were able to elicit an anti-MenC immune response after the first immunization. For the non-adjuvanted vaccine given intramuscularly two vaccine administrations were required to induce detectable antibody titers; moreover these titers were not superior to titers induced after a single injection with the vaccine given intradermally or the AlumOH adjuvanted vaccine given intramuscular. As seen after the first immunization also total IgG titers in sera from mice immunized with two doses of MenC-CRM_{197} intradermally showed comparable antibody titers to AlumOH adjuvanted vaccines given by the intramuscular route (Figure 3a). These data clearly show the superiority of the intradermal route when compared to the intramuscular administration and confirm the ability of a quicker onset of immune responses by either using a different route of administration or the use of adjuvants.

For the protein alone anti-CRM_{197} IgG titers elicited after intradermal delivery resulted significant higher than that of the respective intramuscular titer, although the interquartile range bar associated with the intradermal response is very high (Figure 3b). While when comparing anti-CRM_{197} antibody titers induced by the MenC-CRM_{197} glycoconjugate vaccine the immune response was higher after intradermal delivery. Thus it seems that the presence of the polysaccharide component in respective glycoconjugate has an adjuvant effect versus the protein when intradermally administered. Importantly the elicited CRM_{197} specific IgG titers resulted in lower values than the AlumOH adjuvanted formulation given intramuscular. No adverse reactions such as nodules, erosion/scab, erythema or redness at the site of administration after intradermal immunization, were detected.

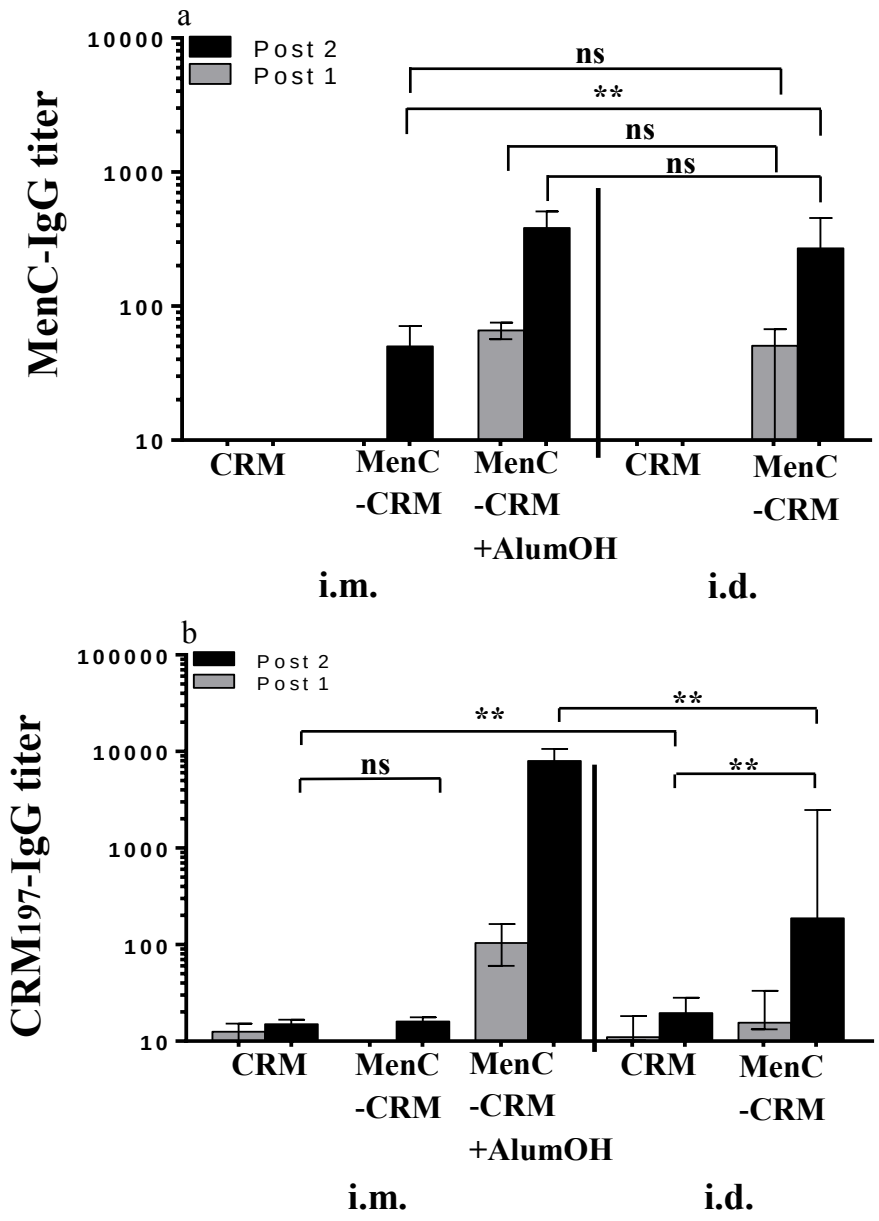

Figure 3| Systemic humoral responses to i.m. and i.d. administration of MenC-CRM$_{197}$ alone, adjuvanted with alum hydroxide (AlumOH) and CRM$_{197}$. *(a) MenC-specific and (b) CRM$_{197}$-specific IgG titers were analyzed after each immunization. Intergroup comparisons were analyzed using the Mann-Whitney statistical assay. Medians of MenC/CRM$_{197}$-specific IgG titers for i.m. and i.d. immunizations are reported.*

Table 3| pValues (a) associated to groups in figure 3a (b) associated to groups in figure 3b.

a	***Groups***		***pValue***
MenC-CRM_{197} i.m.	***vs***	MenC-CRM_{197} i.d.	0,03
b	***Groups***		***pValue***
CRM_{197} i.m.	***vs***	CRM_{197} i.d.	0,0093
MenC-CRM_{197}+AlumOH i.m.	***vs***	MenC-CRM_{197} i.d.	0,0019
CRM_{197} i.d.	***vs***	MenC-CRM_{197} i.d.	0,0017

Oil in water emulsion and TLR4a increase the immunogenicity meningococcal glycoconjugate vaccine

To evaluate if immunogenicity induced after intradermal immunization can be increased by the use of adjuvants two different adjuvants, an oil in water emulsion and a TLR4a, were evaluated either alone or in combination (Figure 4a). After a single immunization all adjuvanted groups induced antibody titers comparable or superior to titers induced by two immunizations with the non-adjuvanted vaccine given intramuscular. After two immunizations both adjuvants resulted in significant enhancement of the immune response against MenC polysaccharide in comparison with the glycoconjugate alone delivered i.d. and the AlumOH adjuvanted group given i.m. In order to evaluate if the immune response against MenC can be further maximized after intradermal vaccination, the TLR4a adjuvant was co-delivered with the oil in water emulsion; and significantly improved immune response was detected in comparison to the o/w adjuvanted formulations however no improved immunogenicity compared to the TLR4a single adjuvanted formulation was observed. With respect to anti-carrier immune responses all adjuvanted groups induced higher titers than the MenC-CRM_{197} alone, however comparable to the AlumOH adjuvanted formulation intramuscular delivered. No differences for anti-CRM_{197} IgG titers were observed for the double adjuvanted formulation in comparison to both single adjuvanted formulations (Figure 4b).

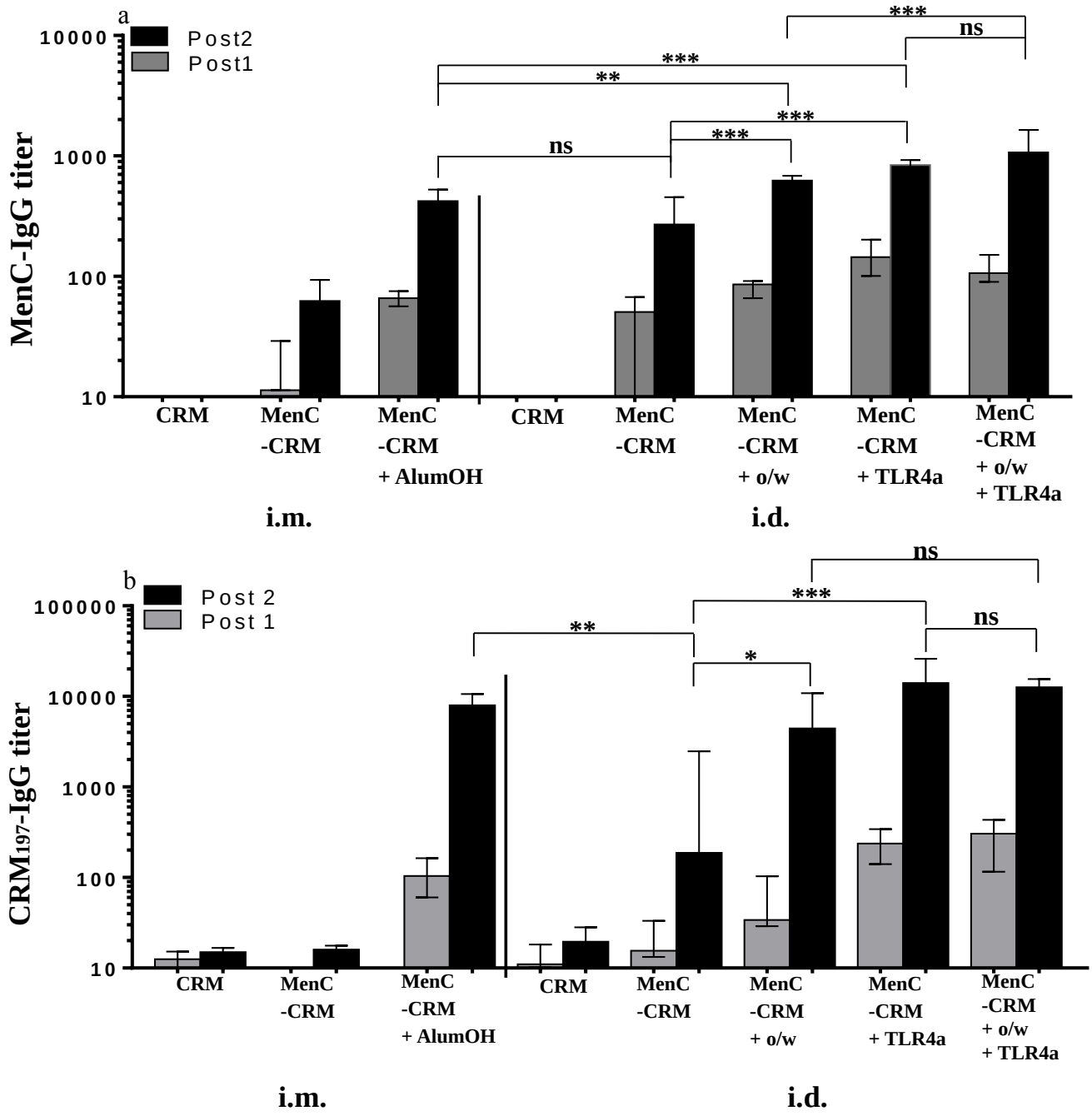

Figure 4| Systemic humoral responses to i.m. and i.d. administration of MenC-CRM_{197} alone, adjuvanted with oil in water emulsion (o/w) and TLR4 agonist (TLR4a) and CRM_{197}. *(a) MenC-specific and (b) CRM_{197}-specific IgG titers were analyzed after each immunization. Intergroup comparison was analyzed using the Mann-Whitney statistical assay. Medians of MenC-specific and CRM_{197}-specific IgG titers for i.m. and i.d. immunizations are reported.*

Table 4| pValues (a) associated to groups in figure 4a (b) associated to groups in figure 4b.

a			***pValue***
MenC-CRM_{197} i.d.	***vs***	MenC-CRM_{197}+o/w i.d.	0,0002
MenC-CRM_{197} i.d.	***vs***	MenC-CRM_{197}+TLR4a i.d.	0,0002
MenC-CRM_{197}+o/w i.d.	***vs***	MenC-CRM_{197}+o/w+TLR4a i.d.	0,0002
MenC-CRM_{197}+o/w i.d.	***vs***	MenC-CRM_{197}+AlumOH i.m.	0,0021
MenC-CRM_{197}+TLR4a i.d.	***vs***	MenC-CRM_{197}+AlumOH i.m.	0,0003
b	***Groups***		***pValue***
MenC-CRM_{197} i.d.	***vs***	MenC-CRM_{197}+o/w i.d.	0,0104
MenC-CRM_{197} i.d.	***vs***	MenC-CRM_{197}+TLR4a i.d.	0,0006
MenC-CRM_{197}+AlumOH i.m.	***vs***	MenC-CRM_{197} i.d.	0,0019

Bactericidal titers correlate with the obtained ELISA titers

The functionality of the elicited antibody titers post 2 was assessed by rabbit complement serum bactericidal assay (rSBA) (Table 4), a model that is considered a surrogate of protection for meningococcal antigens (101-102). In agreement with the total IgG titers the MenC-CRM_{197} associated rSBA titer after intradermal delivery was comparable to the AlumOH adjuvanted after intramuscular injection (Table 3); and a significant increase of rSBA titer was measured when TLR4a and/or emulsion adjuvanted formulations were delivered intradermally and compared to the respective titers elicited by non-adjuvanted CRM_{197} and MenC-CRM_{197} formulations. As expected no bactericidal activity was elicited by the CRM_{197} for both immunization routes.

Table 5| Bactericidal titers for mice sera post 2 analyzed by measuring the complement-mediated lysis of Neisseria meningitidis.

Vaccine			rSBA titer MenC - C11strain	
			i.m.	i.d.
CRM_{197}		-	<16	<16
MenC-CRM_{197}	+	-	128	8192
		AlumOH	8192	-
		o/w	/	32768
		TLR4a		131072
		o/w+TLR4a		65536

Immunopotentiators for intradermal delivery MenC-CRM_{197} glycoconjugate vaccine

In addition to the tested TLR4a and the o/w emulsion adjuvant, MenC-CRM_{197} glycoconjugate vaccine was adjuvanted with three alternative adjuvants: TLR7a, LTK63 and αGalAnalog, in order to evaluate novel adjuvants able to target skin-resident antigen-presenting cells. Intramuscular and intradermal routes were investigated (Figure 5). Data obtained confirmed that in absence of adjuvant immunogenicity of MenC glycoconjugate vaccine when delivered intradermally not only showed a quicker on-set but was also significant higher in comparison to respective intramuscular administration. For both immunization routes, -intradermal and intramuscular - and after both - the first and the second - immunizations, total IgG titers induced in presence of immunopotentiators were higher than the titer of the non-adjuvanted formulation. Interestingly for the intramuscular route the presence of adjuvants seem to be mandatory in order to elicit an immune response after a single immunization only; whereas non-adjuvanted vaccine given intradermally is able to induce already detectable immune response after the first immunization. Moreover the MenC-specific IgG titers, obtained in sera post1, associated to all adjuvants tested and delivered intradermally are comparable to the titer of the antigen alone delivered intramuscularly after two immunizations.

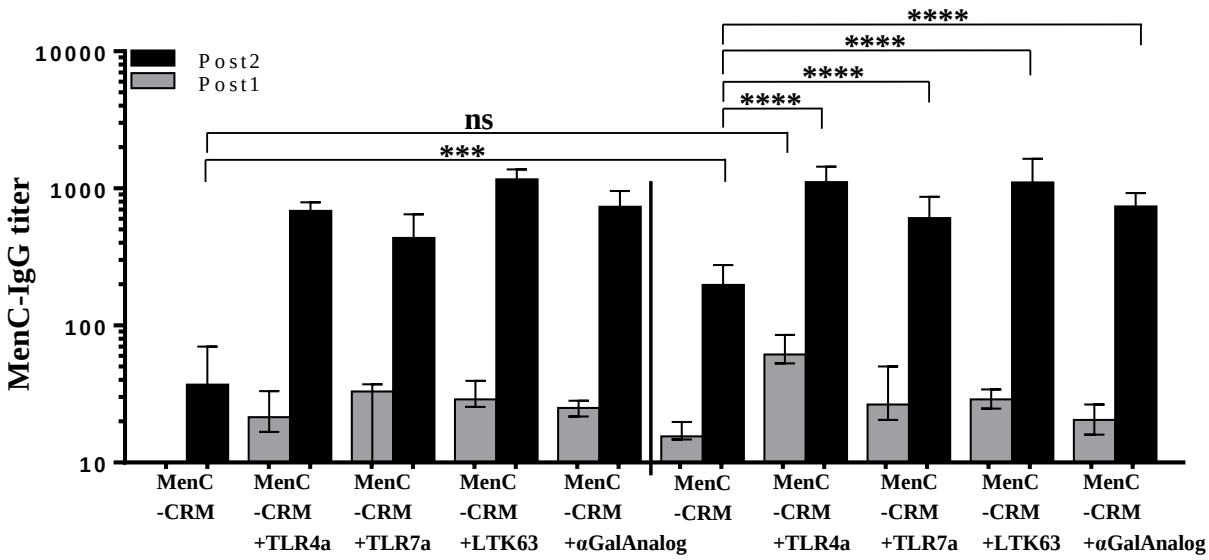

Figure 5| Systemic humoral responses to i.m. and i.d. administration of MenC-CRM$_{197}$ alone and adjuvanted with TLR4/TLR7 agonists (TLR7a – TLR4a), LTK63 and αGalAnalog. *MenC-specific IgG titers were analyzed after the first and the second immunization. Intergroup comparison was analyzed using the Mann-Whitney statistical assay. Medians of MenC-specific IgG titers for i.m. and i.d. immunizations are reported.*

Table 6| pValues associated to groups in figure 5

Groups			***pValue***
MenC-CRM$_{197}$ i.m.	***vs***	MenC-CRM$_{197}$ i.d	0,0003
MenC-CRM$_{197}$ i.d.	***vs***	MenC-CRM$_{197}$+TLR4a i.d.	<0,0001
		MenC-CRM$_{197}$+TLR7a i.d.	
		MenC-CRM$_{197}$+LTK63 i.d.	
		MenC-CRM$_{197}$+αGalAnalog i.d.	

- ***Bactericidal titers correlate with the obtained ELISA titers***

In agreement with the total IgG titers a significant increase of bactericidal activity was measured when MenC-CRM$_{197}$ glycoconjugate was intradermally administered and compared to the intramuscular route. For both immunization routes all the adjuvants tested increased serum bactericidal titers and TLR4a and LTK63 adjuvanted formulations seem to induce higher titers when delivered intradermally in comparison to the two respective formulations given through the intramuscular

route. Whereas no differences were observed between the two immunization routes in presence of the TLR7a and αGalAnalog adjuvanted formulations.

Table 7| Bactericidal titers for mice sera post 2 analyzed by measuring the complement-mediated lysis of Neisseria meningitidis.

Vaccine		rSBA titer MenC - C11strain	
		i.m.	**i.d.**
	-	**256**	**4096**
	TLR4a	**65536**	**131072**
MenC-CRM_{197} +	**TLR7a**	**16384**	**16384**
	LTK63	**32768**	**65536**
	αGalAnalog	**16384**	**16384**

Immunopotentiators induces a Th1 shift

To determine the quality of the immune response, post 2 mice sera were analyzed for anti-MenC-specific IgG1 and IgG2a titers (Figure 6). The presence of TLR4a and TLR7a elicited higher IgG2a antibody titers than the LTK63 adjuvanted vaccine formulations and the Th1 isotype shift was induced for both i.m. and i.d. immunization routes. On the contrary, the αGalAnalog adjuvant enhances the Th2 isotype antibody response. Remarkably LTK63 induced a Th1 shift in a more predominant way after intradermal delivery, confirming previously reported data obtained after the mucosal administration of Influenza vaccine (100), where it seems that the ability of the LTK63 adjuvant to shift immune responses to a more dominant Th1 profile is immunization route dependent. This result was also confirmed by the CRM_{197}-specific T-cell responses in spleens evaluated by intracellular staining and flow cytometry. The percentage of $CD4^+$ $CD44^{high}$ T-cells producing IL-2, IL-4/IL-13, IL-17, IFN-ɣ and TNFα were analyzed (Figure 7). CRM_{197} -specific T-cell responses in spleens, after LTK63 administration, showed that IFN-ɣ production was elicited only after intradermal delivery and higher TNFα and IL-2 cytokines production occurred after intradermal delivery in comparison to

the intramuscular route. No particular differences were observed for the other presented cytokines between i.m. and i.d. delivery (data not shown).

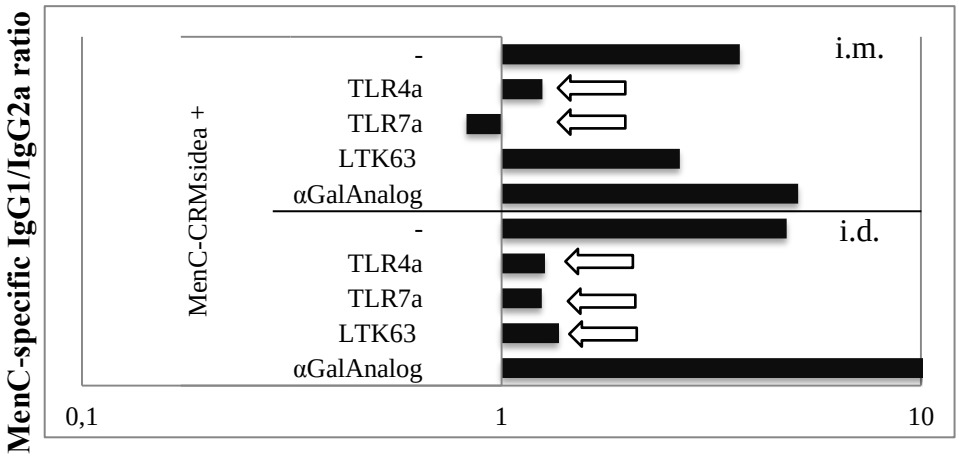

Figure 6| *Systemic humoral responses to i.m. and i.d. administration of MenC-CRM_{197} (**Ag**) alone and adjuvanted with TLR7/TLR4 agonist (**TLR7a/TLR4a**), **LTK63** and **αGalAnalog** are reported. MenC-specific IgG1/IgG2a titers ratio of mice sera post 2 are reported.*

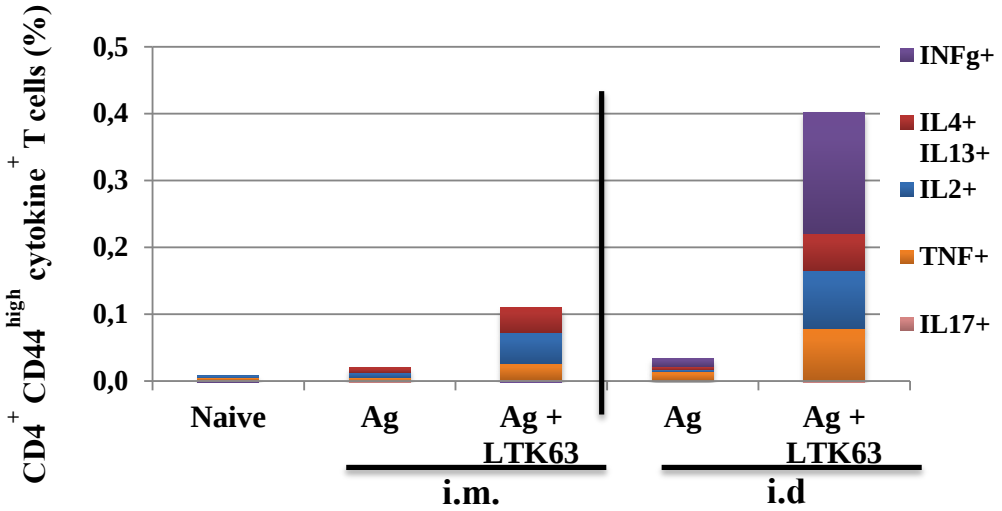

Figure 7| *Intramuscular and intradermal immunizations elicited CRM_{197} -specific T-cell responses. Mice were vaccinated i.m. or i.d. with MenC-CRM_{197} (**Ag**) alone or in combination with **LTK63**. $CD4^+$ T-cell cytokine responses to CRM_{197} were evaluated in spleen two weeks after the second immunization. T-cell responses were calculated by subtracting fluorescence percentages of untreated samples from the CRM_{197}-stimulated samples. Untreated mice (naïve) were used as negative controls*

Discussion

In the present study it has been shown that by using Tangential Flow Filtration (TFF), a scalable and robust process, it is possible to prepare highly concentrated MenC glycoconjugate vaccines for subsequent intradermal delivery. Importantly, it was demonstrated that the process does not affect the integrity and the immunogenicity of the antigen. The characterization data generated, along with the systemic immune response in mice, clearly establish TFF as a suitable process for the concentration of MenC glycoconjugate vaccine. Moreover this scalable technique might help to process large batches of MenC glycoconjugate vaccine and could be easily adapted to further glycoconjugate vaccines as have already been tested for MenA-CRM_{197} glycoconjugate (data not shown). The concentrated material was used to evaluate the administration of the MenC glycoconjugate vaccine through the intradermal route in comparison to intramuscular delivery. Superiority, in term of immunogenicity and protection, of the intradermal delivery was demonstrated. Considering that the non-adjuvanted vaccine given intradermally is able to induce a detectable immune response after the first immunization and that comparable antibody titers to AlumOH adjuvanted formulations injected intramuscularly, after one and two doses, are achieved the intradermal route has the potential to allow the elimination of AlumOH from the vaccine formulation.

The good intradermal immunogenicity of the MenC glycoconjugate vaccine might be explained by the presence of several receptors on the surface of Langerhans cells, the primary antigen presenting cell in the skin, which allow a better cell targeting and activation and consequently, a more efficient antigen presentation than other dendritic cell subpopulations. Whereas conventional intramuscular route of vaccine administration may limit the magnitude and the quality of the immune response by targeting and activating less immunologically active sites.

It was demonstrated that the use of immunopotentiators can further enhance the immunogenicity and increase bactericidal activity after intradermal delivery; depending on the adjuvant used an evident shift in the quality of the immune response is observed. For meningococcus, there are indications that the induction of

the IgG2a subclass is highly protective (103). Interestingly the TLR4 agonist and TLR7 agonist induced increased IgG2a antibody titers – thus a Th1 shift - independently from the route of immunization, while LTK63 induced the shift toward a Th1 response in a more predominant way after intradermal delivery, therefore confirm results obtained after mucosal delivery and published elsewhere (100). Moreover LTK63 induced INFɣ production from CD4+ T-cells after intradermal but not after intramuscular delivery confirming respective evidence. Regarding the αGalCer analog, the enhanced IgG1 antibody production indicates this adjuvant as more convenient to be used to treat diseases that need a predominant Th2 response. It also has been demonstrated that the oil in water emulsion adjuvant can be used for the intradermal administration of a glycoconjugate vaccine, increasing its immunogenicity. In the present studies the pre-clinical observation revealed that the tested adjuvants were well-tolerated, and that any evident local reaction was evidenced. A more in depth analysis is required to better investigate the safety profile of these compounds after intradermal delivery.

In the context of anti-carrier immunity, the administration of the CRM_{197} protein alone did not show a good immune response against the protein for both routes of immunization; at the same time the elicited CRM_{197} –specific IgG titers after MenC-CRM_{197} intradermal administration resulted in higher levels than the same formulation administered intramuscularly. In previous studies (18, 104-105) it was demonstrated how polysaccharides that are weak receptor binders can be turned into more potent agonists by their multivalent presentation through conjugation to a protein core. In that case the MenC, as saccharide, might have acquired an immune stimulant role for skin dendritic cell after its conjugation to the CRM_{197} carrier protein. Nevertheless anti-CRM induced antibody titers after intradermal vaccine delivery remained significant lower when compared to anti-CRM titers induced with the Alum adjuvanted formulation given intramuscular. Results published by Pecetta et al indicate that anti-CRM immunity might even improve the anti-saccharide response for corresponding glycoconjugates vaccines (106). Therefore enhanced CRM_{197} –specific IgG titers after intradermal delivery of MenC-CRM_{197}

should not negatively impact the anti-saccharide response of a subsequent CRM_{197} glycoconjugate vaccination.

All the above findings demonstrate that the modulation of vaccine formulation through careful selection of antigen, adjuvants and the route of administration, is an important approach for the development of effective vaccines. Thus promising adjuvants combined into new vaccine formulations and devises as for example microneedle technologies might lead to the development of multivalent meningococcal glycoconjugate vaccines given by the dermal route. Importantly, recent studies among volunteers have demonstrated a clear preference for intradermal route of vaccine delivery over the conventional i.m. route (107). Several factors might contribute to this preference, such as needle phobia, lack of pain, and the potential for self-administration.

Supplementary data

- ***β-glucan hexasaccharide as adjuvant: co-delivered and conjugated to MenC-CRM_{197} glycoconjugate***

Introduction

To better evaluate the β-(1-3) hexasaccharide as adjuvant for MenC-CRM_{197}, in addition to the previous tested adjuvants, it was delivered in physical mix as well as conjugated to CRM_{197} carrier protein and *in vivo* evaluated after intramuscular, intradermal and subcutaneous vaccinations. As already reported, β-(1-3)-glucans and are able to activate innate immunity by the interaction with the Dectin-1 receptor (66-69). In chapter 4 the conjugation of β-glucan hexasaccharide to the CRM_{197} model antigen is proposed as strategy to test the capability to enhance the immune response toward a protein model antigen in comparison to the here presented glycoconjugate model antigen.

Materials and methods

Immunogenicity testing in mice

Protocols were approved by the Italian Ministry of Health. All mice were housed under specific pathogen-free conditions at the Novartis Vaccines Animal Resource Center. For intramuscular (i.m.) and intradermal (i.d.) vaccinations, groups of eight 6-week-old female BALB/c mice were immunized twice, four weeks apart. 100 µl of MenC-CRM_{197} (1 µg/mouse of MenC) alone or co-delivered with 25 µg/mouse of β-glucan hexasaccharide were injected intramuscularly. The same antigen/adjuvant dose was delivered intradermally in a 20 µL formulation using a 0.3 mL BD Micro-Fine syringe (30Gx8mm) inserted at 45 degree angle. The vaccinations were dispensed in the back after shaving. The appearance of nodules, erosion/scab, erythema, redness at the site of administration was monitored. Serum samples were collected on days 14 and 42 and stored at -20 °C before analysis. For subcutaneous (s.c.). vaccination, groups of eight 6-week-old female BALB/c mice were immunized three times, three weeks apart. 200 µl of MenC-CRM_{197} (1

µg/mouse of MenC) and MenC-CRM_{197}-hexa (1 µg/mouse of MenC, 0,25 µg/mouse of hexasaccharide) were injected. Serum samples were collected on days 20, 35 and 56 and stored at -20 °C before analysis

Determination of MenC-specific antibody titers by ELISA

See manuscript above.

Results and conclusions

- ***β-glucan hexasaccharide co-delivered with MenC-CRM_{197} glycoconjugate doesn't increase the immune response to MenC polysaccharide***

After two immunizations, β-glucan hexasaccharide co-delivered with the antigen didn't show an adjuvant effect as revealed by the absence of a statistical significance between the adjuvanted and non-adjuvanted formulations for both immunization routes (Figure 8). In the presented study we showed the lack of β-(1-3)-glucan hexasaccharide adjuvant effect when co-delivered with the antigen.

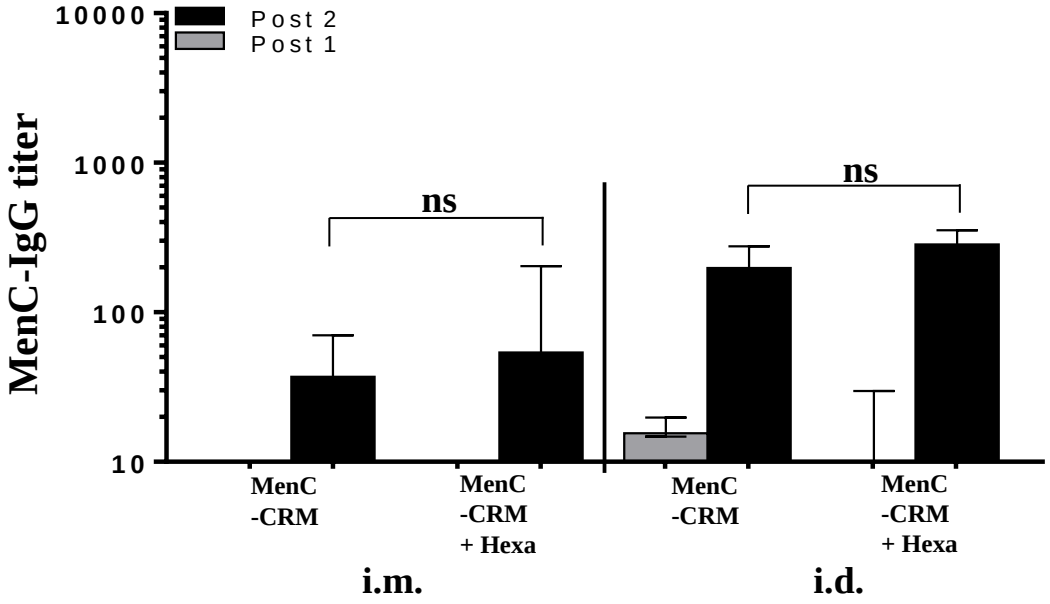

Figure 8| Systemic humoral responses to i.m. and i.d. administration of MenC-CRM_{197} alone and adjuvanted with β-glucan hexasaccharide (Hexa). *MenC-specific IgG titers were analyzed after the second immunization. Intergroup comparison was analyzed using the Mann-Whitney statistical assay. Medians of MenC-specific IgG titers for i.m. and i.d. immunizations are reported.*

- ***Conjugation of hexasaccharide to CRM_{197} carrier doesn't increase the immune response to MenC polysaccharide***

After three immunizations, total IgG titer induced by MenC-CRM_{197}-hexa conjugate is comparable to the MenC-CRM_{197} control after subcutaneous immunization. B-glucan hexasaccharide conjugated with the antigen didn't show an adjuvant effect as revealed by the absence of a statistical significance between the two tested formulations (Figure 9). This result can be explained by the steric conformation of the conjugate. The long MenC polysaccharide chain eventually interfered with the accessibility of the short hexasaccharide chain to the Dectin-1 receptor and consequently no adjuvant effect was observed.

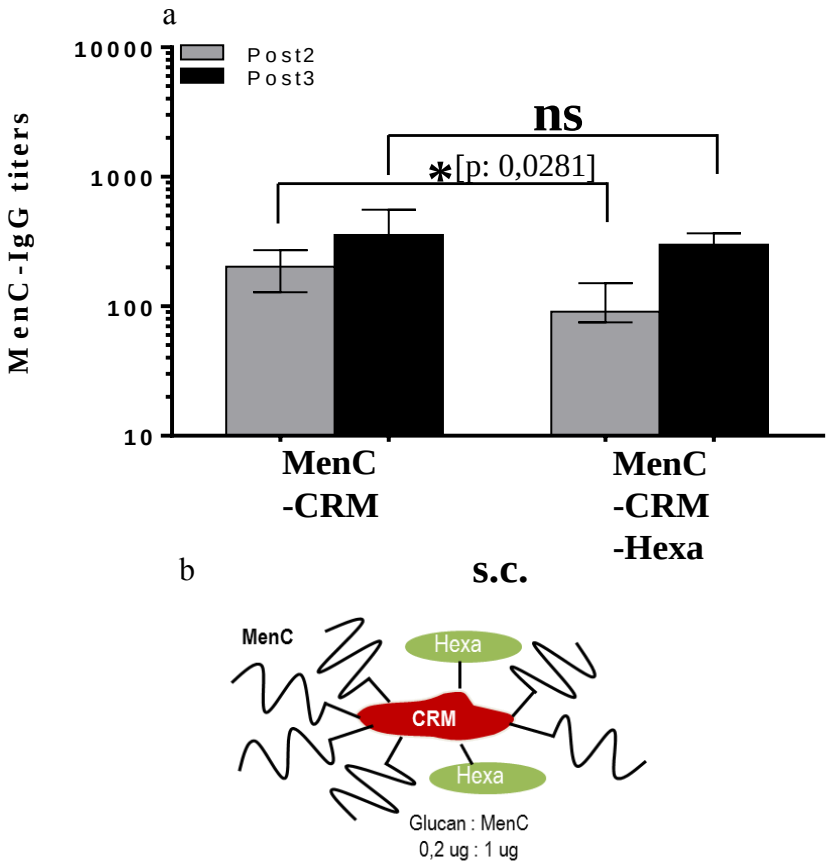

Figure 9| (a) Systemic humoral responses to s.c. administration of MenC-CRM_{197} alone and conjugated with ß-glucan hexasaccharide (MenC-CRM_{197}-hexa). *MenC-specific IgG titers were analyzed after the second and third immunization. Intergroup comparison was analyzed using the Mann-Whitney statistical assay. Medians of MenC-specific IgG titers for s.c. immunizations are reported.* ***(b) Graphic representation of MenC-CRM_{197}-hexa compound.***

Chapter 3

The impact of TLR7 agonist conjugation to anti serogroup C meningococcal glycoconjugate vaccine

Two different approaches of agonist conjugation

As previously stated in chapter 2, agonists of Toll like receptor 7 (TLR7a) can activate innate immunity and initiate a cascade of immune responses that can impact the magnitude and the persistence of the immune response. These features render TLR7a a very attractive class of small molecules for the development of adjuvants for vaccination (108-109). Recently, Vecchi et al has proven that conjugation of TLR7a to an anti-Streptococcus pneumoniae protein antigen increases the immunogenicity and reduced the vaccine dose required to elicit protective immunity (125). In this chapter the conjugation of TLR7a to the antigen is proposed as a viable strategy to ensure codelivery of TLR7a/antigen and to transport the ligand to the receptor, to promote its intracellular accumulation and to favor the receptor activation. Two different approaches of TLR7 agonist conjugation to MenC-CRM_{197} glycoconjugate vaccine were investigated *in vivo*:

1. TLR7a conjugation to CRM_{197} carrier protein (Figure 1a);
2. TLR7a conjugation to MenC polysaccharide (Figure 1b);

The obtained findings for the TLR7a conjugation to CRM_{197} carrier protein approach were reported in a manuscript submitted to the European Journal of Pharmaceutics and Biopharmaceutics, instead the results obtained for the TLR7a conjugation to MenC polysaccharide approach were organized as supplementary data.

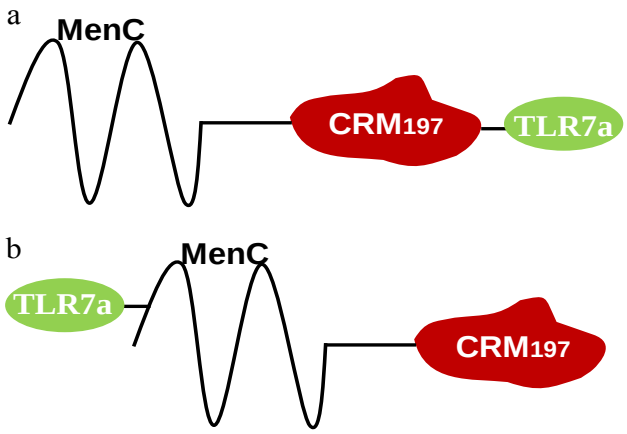

Figure 1| Graphic representation of the two different approaches of TLR7 agonist conjugation to MenC-CRM_{197} glycoconjugate vaccine.

Adjuvant effect of TLR7 agonist in a tricomponent anti serogroup C meningococcal glycoconjugate vaccine

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Abstract

Agonists of Toll like receptor 7 (TLR7a) have emerged as important targets to develop novel adjuvants for vaccination. Conjugation of a small immunopotentiator molecule has been proposed to vehicle the ligand to the receptor, localize its action and reduce toxicity. However, the effect of conjugation on the immunogenicity of carbohydrate-based vaccines is unknown. In this study we synthesized a tricomponent anti-*Neisseria meningitidis* serogroup C (MenC) glycoconjugate vaccine composed of MenC oligosaccharide antigens covalently linked to the carrier protein CRM_{197}, to which a TLR7a has been in turn conjugated. The tricomponent vaccine demonstrated *in vitro* to activate the TLR7 comparably to the unconjugated ligand. The impact of the adjuvant conjugation in the anti-carbohydrate immune response was evaluated in mice by comparing the tricomponent vaccine to a MenC-CRM_{197} conjugate, prepared through the same conjugation chemistry and co-administered with the unconjugated TLR7a. A commercialized anti-MenC glycoconjugate was used as further control to determine the influence of the coupling approach and level of carbohydrate incorporation on the anti-MenC immune response. The possible additive effect of co-administration with Alum hydroxide (AlumOH) was also examined. Results clearly showed that conjugation of the TLR7a enhances the anti-carbohydrate response. This effect was less pronounced than the co-administration of a commercial glycoconjugate with a standard dose of TLR7a-phosphonate adsorbed on AlumOH. The reduction of sugar incorporation which is needed for the conjugation of the TLR7a lessens the anti-carbohydrate response, and is therefore a parameter which counteracts the enhancement of the immune response produced by the adjuvant conjugation. While absorption on AlumOH offers more flexibility in the administered dose of TLR7a, conjugation of the small immunopotentiator molecule could be particularly suited for vaccination via skin delivery, where insoluble salts of aluminum cannot be used because of their reactogenicity. These findings pave the ground for the rational design of improved adjuvanted glycoconjugate vaccines and for establishing novel and safer vaccination routes.

Introduction

Agonists of Toll like receptor 7 (TLR7a) can activate innate immunity and initiate a cascade of immune responses that can impact the magnitude and the persistence of the immune response. These features render TLR7a a very attractive class of small molecules for the development of adjuvants for vaccination (108-109). Different potent synthetic ligands for TLR7, based a variety of chemical scaffolds (8-oxoguanosines, 8-oxo-3-deazapurines, 6-amino-9-benzyladenine, imidazoquinolinamines and benzonaphtyridine), have been reported (110-115). Initial studies have proven the adjuvant effect of the imidazoquilinone imiquimod formulated as cream for dermal application (Aldara®) (116), and administered locally in mice after subcutaneous immunization with ovalbumin (OVA). Likewise, topical treatment with Aldara®, following to subcutaneous immunization of mice with Plasmodium falciparum circumsporozoite peptides, enhanced the anti-peptide response (117). The rapid production of cytokines induced by TLR7a is responsible for a proinflammatory response which besides the desired adjuvant effect can lead to toxicity (118). For instance, both oral and topical preparations of imiquimod and resiquimod and other small-molecule TLR7/8 agonists can exhibit serious side effects, including emesis and liver disfunction (119). Reduction of toxicity can be achieved by lower doses of administered TLR7a. Conjugations to lipids (113) or to polysaccharides (120) have been proposed to improve immunostimulatory potency and pharmacodynamics, while reducing the administered dose. Localization of the small molecules in the administration site appears as an important prerequisite to modulate the inflammatory effect and reduce possible toxicity in the use of TLR7a. In addition, pH-dependent concentration of the TLR7a in the MHC class II loading compartment (MHCII) has been proven important for activation of dendritic cells (121). Therefore co-localization of antigen and TLR7a create a favorable synergy in the activation of the immune system. Different strategies have been pursued for co-administration of peptide or protein antigens while achieving temporal and spatial restriction of TLR7a induced inflammation. Encapsulation of resiquimod (R-848) into liposomes showed to increase the anti-OVA antibody response more

effectively than the simple administration of OVA and R-848 (119). Wu et al showed that absorption onto AlumOH of TLR7a phosphonates can remarkably enhance the immune response (antibody titers and functional activity) of a recombinant protein antigen directed to *Neisseria meningitidis* serogroup B and of the anti-B. Anthracis recombinant protective antigen (115). Conjugation of TLR7a to the antigen has been proposed as a viable strategy to ensure codelivery of TLR7a and antigen. The protein mouse serum albumin (MSA) was tested as delivery system for intranasal or intratracheal administration to increase the potency of the small molecule, while reducing the systemic cytokine syndrome (114). Covalent linkage of the TLR7/8a to the HIV-1 gag protein was demonstrated crucial to generate CD4+ and CD8+ T-cell responses in both mouse and non-human primates (122-123). This would result from robust accumulation of DCs in regional lymph nodes, compared to administration of unconjugated TLR7 ligand (124).

Recently, Vecchi et al has proven that conjugation of TLR7a to an anti-Streptococcus pneumoniae protein antigen increases the immunogenicity and reduced the vaccine dose required to elicit protective immunity (125). In the present study we exploited the conjugation of TLR7 to an anti-meningococcal serogroup C glycoconjugate vaccine as a strategy to enhance the anti-carbohydrate immune response. Different glycoconjugates are currently used in the prevention of different microbial infections, including meningitis and pneumonia (126). The capability of carbohydrates, which are per se T-cell independent antigens, to engage a T-cell memory response is strictly dependent to the covalent bond with a protein carrier (127). This linkage ensures uptake of the glycoconjugate by antigen processing cells and re-exposition of the generated peptides or glycopeptides in complex with the MHCII. We anticipated that the covalent linkage of the TLR7a to the glycoconjugate could vehicle the ligand to the receptor, promote its intracellular accumulation and favor the receptor activation. To test this hypothesis we conjugated the TLR7a to the protein component of a glycoconjugate composed of the MenC oligosaccharide andCRM_{197}. This serogroup causes one third of meningococcal cases reported with US, with a significant high case fatality ratio (14.6%) (128). Outbreaks of meningococcal disease related to serogroup C have

been identified also in Brazil and Australia (129-130). Monovalent and multivalent vaccines are currently available for the prevention of this infection (131), therefore an anti-MenC conjugate appeared an attractive target for comparison of the immune response towards the novel constructs with an existing vaccine. After developing a synthetic approach for the preparation of the tricomponent MenC-CRM_{197}-TRL7a conjugate **(1)**, composed of the MenC (B-cell epitope), CRM197 as (T-cell epitope) and the small immunopotentiator molecule, the construct was first evaluated *in vitro* to ascertain the effective ligand-receptor binding. Then the impact of the adjuvant conjugation in the anti-carbohydrate immune response was evaluated *in vivo* by comparison to the MenC-CRM197 conjugate **(2)**, prepared through the same conjugation chemistry and co-administered with the unconjugated TLR7a. A commercialized anti-MenC glycoconjugate **(3)** (component of Menveo®, Menjugate® vaccines) was used as further control to determine the influence of the coupling approach and level of carbohydrate incorporation on the anti-MenC immune response. The possible additive effect of co-administration with AlumOH was also examined. In the animal studies, co-administration of MenC glycoconjugate **(3)** and a small molecule-phosphonate adsorbed on AlumOH, previously reported to confer a strong adjuvant effect towards protein antigens, was used a benchmark. Evaluation of antibody levels and bactericidal activity of the elicited sera enabled to ascertain the adjuvant effect of the TLR7a covalently linked to MenC-CRM197 conjugate.

Materials and methods

Preparation of CRMemcs (4)

The protein was modified as previously described (132). Briefly, 1.52 mg of EMCS linker (Pierce, Thermo) were dissolved in 50 µl of DMSO, and 44 µl of the prepared mixture (10 equiv.) were added to a solution of CRM_{197} (obtained from Novartis Vaccines Manufacturing) (1 ml of 32.5 mg/ml stock solution) in 400 µl of 100 mM NaPi, 1 mM EDTA pH 8.1. The solution was stirred for 3 h. CRMemcs **(4)** was purified by 30 kDa Vivaspin filter, dialyzing against 10 mM NaPi, 1 mM EDTA

pH 7.2 (x 5 dialysis cycles), and its content was quantified by colorimetric assay (MicroBCA assay, Pierce, Thermo). The linker/protein molar ratio was determined by MALDI-TOF mass spectrometry analysis run in an UltraFlex III MALDI-TOF/TOF instrument, (Bruker Daltonics) in linear mode and with positive ion detection. The samples for analysis were prepared by mixing 2.5 µl of product and 2.5 µl of sinapinic acid matrix (Sigma); 2.5 µl of each mixture was deposited on a samples plate, dried at room temperature for 10 min, and subjected to the spectrometer. An average of 6.5 linkers was introduced.

Conjugation of TLR7a to CRMemcs (4)

TLR7a (5 mg, 0.085 µmol, 500 µ of a 8.75 mg/ml stock solution) was diluted with 500 µl of 350 mM NaPi, then TLR7a was added (20 equiv. dissolved in 20 µl of DMSO). After dialyzing against 150 mM NaCl, 10 mM NaPi pH 7, the protein was recovered in 10 mM NaPi and the protein content was estimated by microBCA (Pierce, Thermo). Conjugate **(5)** was recovered in 90% yield.

Preparation of MenC-SH (8)

MenCcysteamine oligomer **(7)** was first obtained by reaction of MenCsidea **(6)** oligosaccharide with average degree of polymerization (avDP) of 20 and 2-Mercaptoethylamine-2'-thiopyridine disulfide (prepared as reported in literature (133)) (5 equiv) at 20 mg/ml concentration in 1:9 H2O:DMSO (5 ml) containing 10% v/v of triethylamine. After stirring for 3 h at room temperature, the oligosaccharide was precipitated by addition of 9 volumes of ethyl acetate. The solid was purified on a G15 Sephadex column (GE Healthcare), eluting with 20 mM NaCl. Fractions containing the oligomer **(7)** were combined and concentrated to obtain a concentration (sialic acid based) of 20 mg/ml in 10 mM sodium phosphate (NaPi) pH 7.2. To release the free thiol groups, compound **(7)** was treated with 3 equiv. of 0.5 M TCEP and stirred for 3 h. After purification on a G15 Sephadex column (GE Healthcare), the thiol groups of MenC-SH **(8)** were estimated before conjugation.

Conjugation of MenC-SH (8) to CRMemcs-TRL7a (5) and CRMemcs (4)

Glycoconjugate **(1)** was prepared by incubating MenC-SH **(8)** with CRMemcs-TLR7a **(5)** (30:1 mol saccharide/protein) in 10 mM NaPi pH 7.2, 1 mM EDTA. The control MenC-CRMemcs 2 was prepared as previously reported (133). For purification, the crude mixtures were precipitated from aqueous ammonium sulfate, reconstituted in 3 M NaCl and purified on a High Trap Phenyl sepharose column (GE Healthcare) with a gradient 3 M NaCl to remove the unconjugated oligomers. Fractions containing the glycoconjugate were pooled and concentrated on a 30 kDa Vivaspin filter (Sartorius) to be reconstituted with 10 mM NaPi pH 7.2. The protein content of final glycoconjugates was determined by colorimetric assay micro-BCA, while the saccharide content and free saccharide % by HPAEC-PAD analysis (100). Endotoxin levels were measured for all the prepared glycoconjugates and resulted below 4 EU/µg.

NF-κB Luciferase Reporter Assay

TLR-specific activation assays were performed using human embryonic kidney 293 (HEK293) cells expressing luciferase under control of the NF-κB promoter and stably transfected with human TLR7. HEK293-transfected cells were maintained in DMEM complemented with 4.5 g/l glucose and HEPES (Invitrogen), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin solution (Invitrogen), puromycin (5 µg/ml), and blasticidin (5 µg/ml).

For the NF-κB luciferase assay, 25000 cells/well were seeded in 90 µl of complete DMEM without antibiotics in 96-well µClear® luciferase plates (PBI International) and incubated for 24 h at 37 °C. 10 µl of serial 5-fold dilutions of TLR7a in PBS+10% DMSO (0.0001–10 µM in 1% DMSO final concentration in the assay) were added. All compound concentrations were tested in duplicate. After incubation for 6 h at 37 °C, supernatants were discarded from each well, and cells were lysed for 20 min at room temperature using 20 µl/well of 1:5 diluted 'passive lysis buffer' (Promega). Luciferase assay reagent (100 µl/well) (Promega) was added, and emitted light was immediately quantified using a luminometer Lmax II384 (Molecular Devices). NF-κB activation of cells stimulated with TLR7a is expressed

as fold-increase of emitted light over the average result of PBS+1% DMSO-stimulated control cells.

Immunization studies

Protocols were approved by the Italian Ministry of Health. All mice were housed under specific pathogen-free conditions at the GSK Vaccines Animal Resource Center. For both *in vivo* studies, groups of ten 6-week-old female BALB/c mice were immunized by intramuscular injection (i.m.) of 0.5 µg/mouse carbohydrate-based dose of glycoconjugates on days 1 and 14. TLR7a was used as immunopotentiator at a dose of 0.5 µg/mouse, either conjugated or co-delivered with compound **(2)**. Coadministration of glycoconjugate **(3)** and SMIP7,10 (5 µg) adsorbed on AlumOH was the positive control. For i.m. immunization with AlumOH, a dose of 200 µg/mouse physically mixed with the conjugates was used (134). PBS and AlumOH were the negative controls. Serum samples were collected on days 0, 14 and 28 and analyzed by ELISA for their anti-MenC IgG levels.

Determination of anti-MenC antibody titers by ELISA

Determination of anti-MenC specific total IgG and subclasses IgG1 and IgG2a titers was performed by ELISA using individual serum samples. Maxisorp plates (Nunc) were coated overnight at +4°C with 5 µg/well of MenC polysaccharide solution in pH 8.2 PBS buffer, and blocked for 1 h at 37°C with 200 µl of 1% BSA (Sigma), in PBS. For the IgG ELISA assay, serum samples were initially diluted in PBS (1:25 for pre immune, 1:200 for a reference serum, 1:50–1:100 for test sera), 0.1% BSA and 0.05% Tween-20 (Sigma), transferred into coated and blocked plates and serially diluted. Bound MenC-specific IgG, IgG1 and IgG2a were detected with alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1 and IgG2a respectively (SouthernBiotech). Antibody titers, normalized respect to the reference serum assayed in parallel, were obtained using IrisLab software. GraphPad Prism v.6.04 (GraphPad Software, La Jolla, CA) was used to graph and analyze data for statistical significance. Intergroup comparison was analyzed using the Mann-Whitney test.

Rabbit serum bactericidal assay (rSBA)

Functional antibodies of pooled sera induced by vaccine immunization were analyzed by measuring the complement-mediated lysis of *N. meningitidis* with an *in vitro* bactericidal assay described in the literature (135). SBA titers were conventionally assigned when two successive sera dilutions lead to at least to 90% bacteria killing and were expressed as the reciprocal serum dilution which results in 50% of bacteria killing. MenC11 was used as reference strain.

Results and Discussion

Glycoconjugate assembly

MenC CPSs consists of (2→9)-linked polysialic acids partially acetylated at 7- or 8-OH, respectively (Figure 2) (136). A well-established strategy to produce an anti-MenC glycoconjugate vaccine consists of the hydrolysis of the native polysaccharide, followed by sizing of medium length oligomers (136) and reductive amination to provide the terminal amine group for final conjugation with the lysine residues of the protein via active ester chemistry (100).

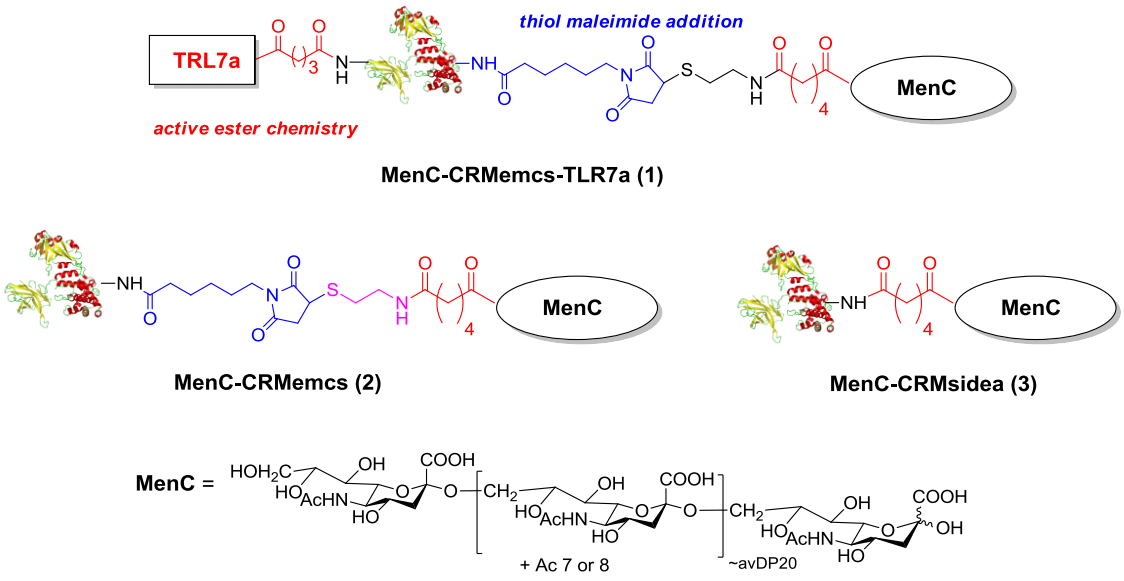
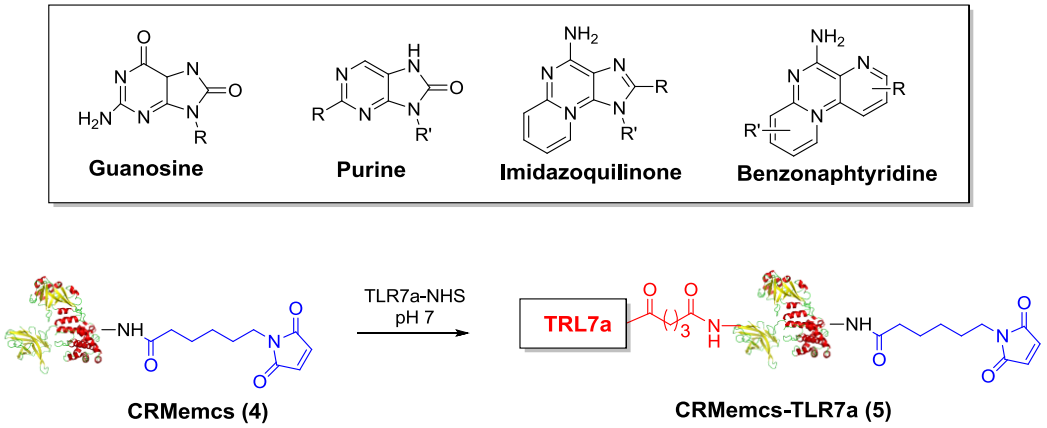


Figure 2| Glycoconjugates prepared for evaluating the effect of covalently linked TLR7a to CRM_{197}; *emcs = N-(ε-maleimido) caproyl group.*

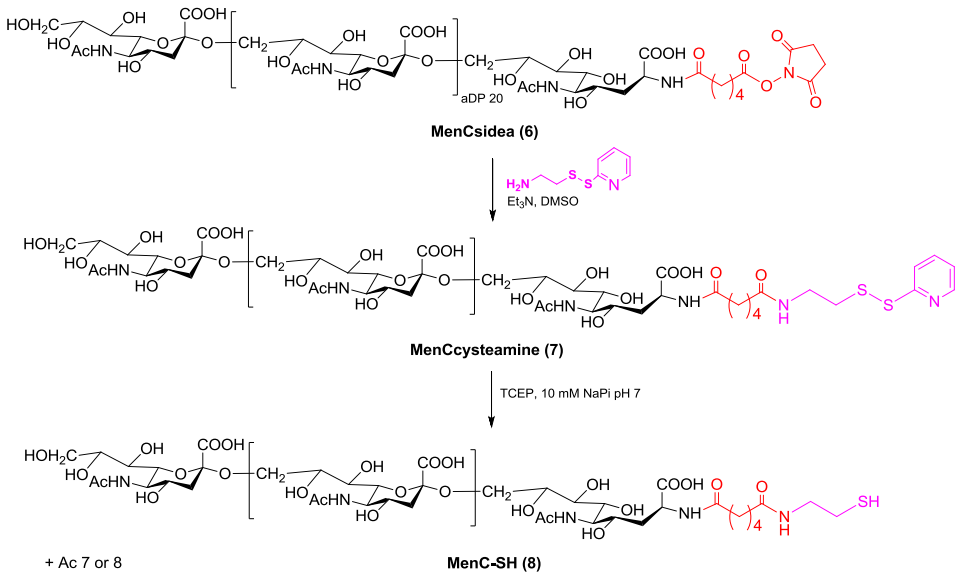
TLR7a are typically based on a guanidine, purine, imidazoquinolines or benzonapthyridine scaffolds (Scheme 1) (110-115). In our studies we employed a guanidine analogue bearing a spacer with an N-hydroxysuccinimidyl ester for conjugation to the protein. This requested the development of a coupling strategy for the meningococcal antigen that would preserve the amine group of the TLR7a, which is fundamental for the activity of the small molecule. We have recently shown that two different carbohydrate haptens can be conjugated to the same molecule of carrier protein by means of active ester chemistry and thiol-maleimide addition as orthogonal reactions (132). CRM_{197} was used as protein carrier, since it is the component of a number of approved glycoconjugate vaccines (131). This protein possesses 39 lysine residues, of which 19 are known to be sufficiently available conjugation and 6 are highly exposed on the protein surface (100,134-137).



***Scheme 1*| *Conjugation of TLR7a to CRMemcs*.**

To apply a similar strategy we first inserted maleimide groups onto CRM_{197} by reaction of the protein with the commercial EMCS linker. An incorporation of 6.5 maleimides was found by MALDI TOF MS analysis of the modified protein. After a second condensation with the NHS ester of the TLR7a, the small molecule was inserted into the protein at a level of 6.6 mol/mol of protein, as estimated by MS analysis (Scheme 1). It is worth of note that, due to the lipophilic nature of the TLR7a we employed, insertion of higher loadings was not achievable due to protein

aggregation. In addition this would imply insertion of a lower number of maleimides, rendering more challenging the incorporation of the MenC ligand. The meningococcal oligomers for condensation with CRMemcs-TLR7a conjugate were prepared by reaction of the known MenCsidea (sidea = di-N-hydroxysuccinimidyl) active ester **(6)** with 2-Mercaptoethylamine-2'-thiopyridine to give the oligomer **(7)** (Scheme 2) (109). After removal of thiopyridone by treatment with TCEP, the sugar hapten **(8)** bearing the sulfhydryl groups was incubated with CRMemcs-TLR7a **(5)** to give addition to the maleimides exposed onto the protein surface.



Scheme 2| Reactions to modify MenC antigens for coupling to the protein.

SDS-page electrophoresis and MALDI TOF MS clearly showed conjugation of the MenC antigen to the modified protein (Figure 3a and Figure 3b). An average ratio of ten TLR7a molecules was incorporated for each MenC chain in the final product. A MenC-CRMemcs conjugate **(2)**, needed as control for the *in vivo* studies, was prepared as control by direct reaction of CRMemcs with MenC-SH, as previously reported. A second control **(3)** was prepared by reaction of MenCsidea with CRM197 to compare our novel construct to a well-known glycoconjugate vaccine. The characteristics of the conjugate in comparison to the controls are summarized in Table 1.

Table 1| Physicochemical characteristics of conjugate (1) and controls.

Glycoconjugate	Saccharide /protein (w/w)	MenC/CRM_{emcs} (mol/mol)	Saccharide/TLR7a/protein (w/w/w)	MenC/TLR7a (mol/mol)
MenC-CRMemcs-TLR7a **(1)**	0.06	0.6	1:1.1:35	1:10
MenC-CRMemcs **(2)**	0.18	1.8	-	-
MenC-CRMsidea **(3)**	0.57	5.6	-	-

a. The sugar content was estimated by HPAEC-PAD;

b. The number of conjugated small molecules was determined by MALDI TOF MS.

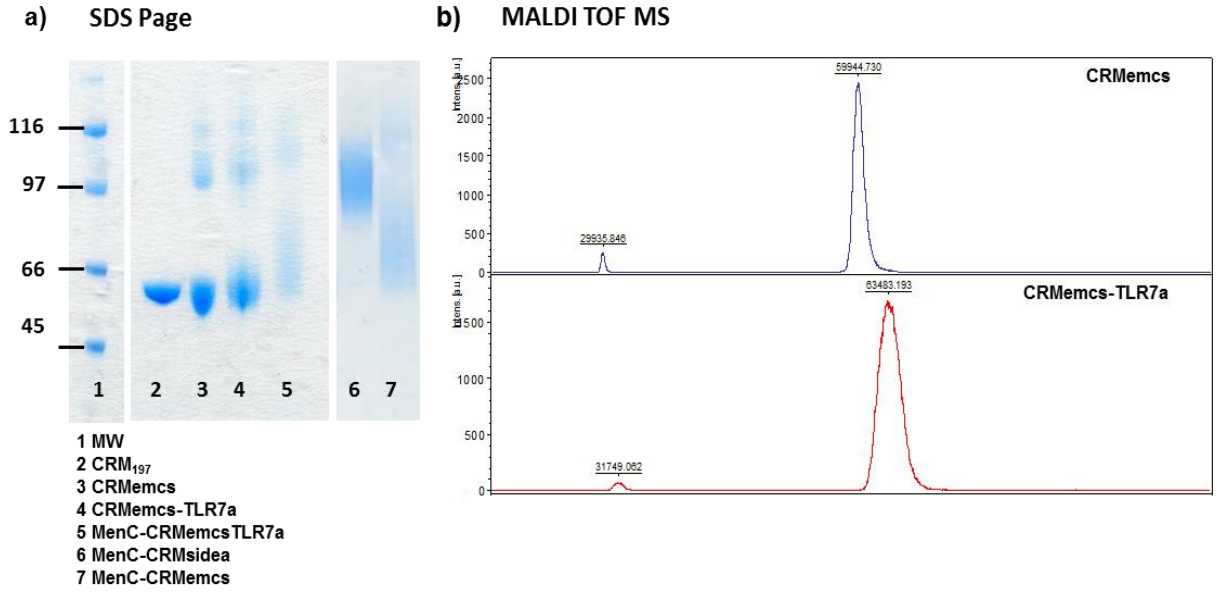


Figure 3| Characterization of the formed MenC-CRMemcs-TLR7a conjugate*. (a) SDS-page electrophoresis of synthetic intermediates and final glycoconjugate in comparison to MenC-CRMemcs and MenC-CRMsidea; (b) MALDI TOF MS analysis of incorporation of TLR7a (MW of TLR7a = 534 Da) on CRMemcs, prepared as reported in ref (132).*

In vitro testing

The capacity of the tricomponent glycoconjugate to engage TLR7 receptor was first evaluated *in vitro*, using human embryonic kidney 293 (HEK293) cells expressing luciferase under control of the NF-κB promoter and stably transfected with human TLR7 (Figure 4). In this assay, NF-κB activation is measured by monitoring the levels of luciferase expression following stimulation of cells with serial dilutions of

TLR7 agonists. Resiquimod (R-848) was used as control. The conjugated TLR7a showed activation of the receptor comparable to that induced by the unconjugated form. Furthermore, the small molecule was an agonist ligand as good as the SMIP7,10-phosphonate that was recently shown to behave as excellent adjuvant for vaccine formulation when adsorbed on AlumOH (115). This experiment indicated that conjugation was not impacting the activity of the TLR7a molecule when covalently linked to CRM_{197} in presence of the carbohydrate hapten.

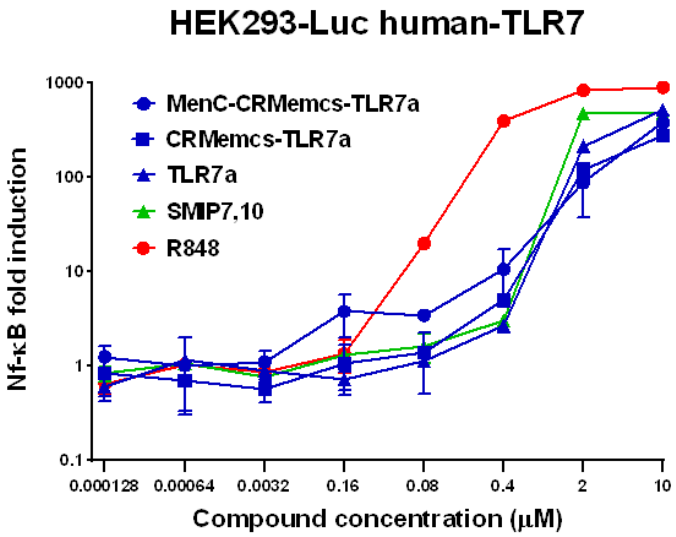


Figure 4*| *Activation of TLR7 reporter cell line by different MenC-TLR7a constructs. *25000 TLR7-HEK293 cells/well were stimulated with 0.0001–10 µM (5-fold steps) of TLR7 agonists. After 6 h, luciferase expression was measured and expressed as fold-induction compared to cells incubated with PBS+1% DMSO and plotted as averages of duplicates with standard deviations.*

In vivo immunological evaluation

The effect of conjugation of TLR7a to MenC-CRM_{197} was evaluated by i.m. immunization of groups of ten BALB/c mice with two 0.5 µg carbohydrate-based doses of vaccine, 2 weeks apart, corresponding to the administration of 0.5 µg of TLR7a. The constructs was compared to the conjugate MenC-CRMemcs prepared with the same conjugation chemistry and a low saccharide/protein molar ratio, co-delivered with the same amount of TLR7a. The MenC-CRMsidea, prepared by the active ester chemistry and with a higher saccharide/protein molar ratio, was used as

control to study the effect of the conjugation chemistry and different level of sugar incorporation compared to MenC-CRMemcs. Co-administration of MenC-CRMsidea with a standard dose (5 µg) of SMIP7,10 adsorbed onto AlumOH was the benchmark (115). PBS was the negative control. While anti carbohydrate IgG titers measured after the first dose were very low, high levels of anti MenC IgG were measured following the boost (Figure 5a). The MenC-CRMemcs-TLR7a conjugate elicited IgG titers significantly higher that MenC-CRMemcs or its physical mixture with the unconjugated form. The immune response induced by the tricomponent vaccine was also higher than the one given by the MenC-CRMsidea control. The adjuvant effect imparted by the conjugated TLR7a was remarkably lower than that achieved by co-administration of MenC-CRMemcs and a ten-fold higher dose (5 µg) of SMIP7,10/AlumOH. This suggests that a threshold level of TLR7a accumulation is needed to observe an optimal adjuvant effect.

When a commonly used dose of AlumOH (200 µg) was mixed to the formulation of MenC-CRMemcs-TLR7a conjugate no additive effect was observed in the anti MenC IgG levels (Figure 5b). The anti-carbohydrate IgG titers were increased for the groups immunized with MenC-CRMemcs alone or with TLR7a, and with MenC-CRMsidea, indicating that the adjuvant effect of the TLR7a was overwhelmed by the presence of AlumOH. The additive effect of the small molecule and AlumOH was only detectable in the group immunized with 5 µg of SMIP7,10/AlumOH (Figure 5b), further proving that absorption of the phosphonate-small molecule onto AlumOH is needed to enhance of the adjuvant activity of the TLR7a.

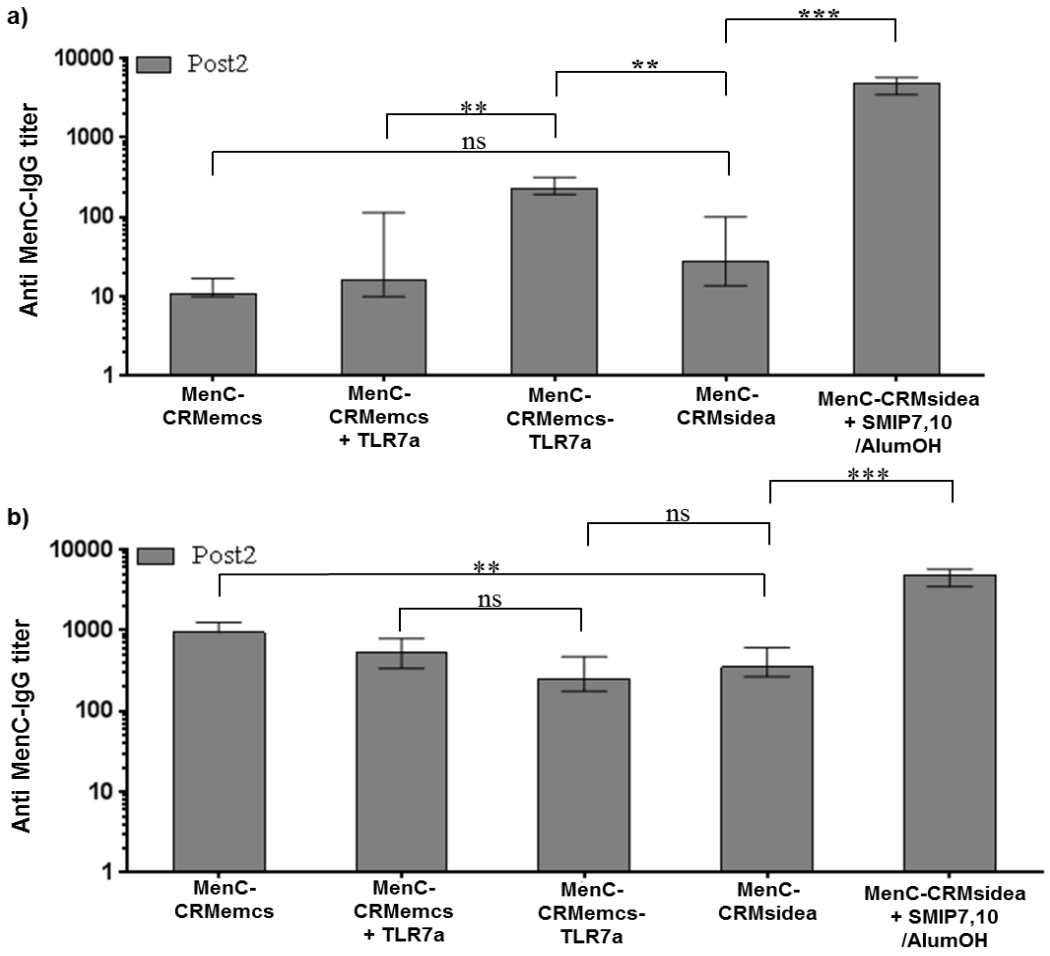


Figure 5| *(a) Systemic humoral responses to i.m. administration of MenC-CRMemcs alone, conjugated or physically mixed with TLR7a. MenC-CRMsidea+SMIP7,10/AlumOH were the controls. MenC-specific IgG titers were analyzed after the second immunization. Intergroup comparison was assessed using the Mann-Whitney statistical assay. Medians of MenC-specific IgG titers for i.m. immunizations are reported. pValues: MenC-CRMemcs vs. MenC-CRMsidea (p 0.14), MenC-CRMemc_s+TLR7a vs. MenC-CRMemcs-TLR7a (p 0.003), MenC-CRMemcs-TLR7a vs. MenC-CRM_{sidea} (p 0.002), MenC-CRMsidea vs. MenC-CRMsidea+SMIP7,10/AlumOH (p 0.0002).*

(b) Systemic humoral responses to i.m. administration of same samples reported in Fig. 4a in mixture with AlumOH. MenC-specific IgG titers were analyzed after the second immunization. Intergroup comparison was assessed using the Mann-Whitney statistical assay. Medians of MenC-specific IgG titers for i.m. immunizations are reported. pValues: MenC-CRMemcs+AlumOH vs. MenC-CRMsidea+AlumOH (p 0.002), MenC-CRMsidea+TLR7a+AlumOH vs. MenC-CRMemcs-TLR7a+AlumOH (p 0.065), MenC-CRMemcs-TLR7a+ AlumOH vs. MenC-CRMsidea+ AlumOH (p 0.19), MenC-CRMsidea+ AlumOH vs. MenC-CRMsidea+ SMIP7,10/AlumOH (p 0.0002).

The predominance of Th1 and Th2 T helper cell subsets is known to result in distinct patterns of cytokine secretion, and therefore response to bacterial infections. TLR ligands can shift this balance to a higher IgG2a production (138). An increase of this isotype levels with Th1-biased response has been associated to the protection conferred by different vaccines (139-140). To determine the impact of the conjugated TLR7 ligand on the Th1/Th2 balance, the levels of antibody subclasses were analyzed by ELISA (Table 2). The co-administration of TRL7 ligand resulted in augmented amount of both IgG1 and IgG2a levels, with a stronger effect on the latter isotype. The highest level of IgG isotypes were measured for the group immunized with MenC-CRMsidea in mixture with SMIP7,10/AlumOH. Importantly, the level of the IgG2a was more remarkably increased when TLR7a was conjugated to MenC-CRMemcs, as it can be seen from the IgG1/IgG2a ratio in Figure 6. This suggests that covalent linkage of the TLR7a is important to determine a shift to a Th1 type response. By contrast, when AlumOH was used in the formulations, the response was characterized by a prevalence of IgG1 with respect to IgG2a also for the tricomponent MenC-CRMemcs-TLR7a vaccine, indicating a Th2 driven response which is typical of AlumOH formulated antigens (141).

Table 2| Anti-MenC IgG1 and IgG2a median titers

	IgG1 Median (IQR)[a]	**IgG2a Median (IQR)[a]**
MenC-CRMemcs	2 (2-14)	2 (2-2)
MenC-CRMemcs + TLR7a	27 (2-175)	2 (2-49)
MenC-CRMemcs-TLR7a	188 (144-228)	280 (254-356)
MenC-CRMsidea	24 (2-119)	31 (2-67)
MenC-CRMsidea + SMIP7,10[b]	2110 64-2326)	983 (81-1063)

a. Medians with interquartile range (IQR);

b. pValues: IgG1 MenC-CRMemcs-TLR7a vs MenC-CRMsidea + SMIP7,10 p = 0.0002, IgG2a: MenC-CRMemcs-TLR7a vs MenC-CRMsidea + SMIP7,10 p = 0.0002.

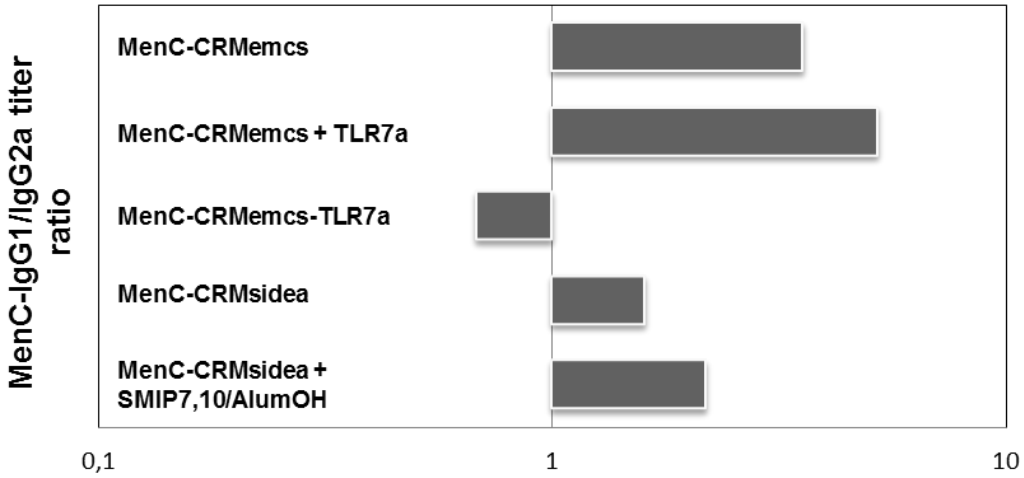


Figure 6| *IgG1/IgG2a ratio of anti MenC IgG titers (shown in Table 2) elicited by i.m. administration of MenC-CRMemcs alone, conjugated or physically mixed with TLR7a, after the first boost. MenC-CRMsidea alone and adjuvanted with SMIP7,10/AlumOH was used as control.*

The functionality of the elicited murine sera in the first immunization schedule (Figure 5a) was assessed by rabbit complement serum bactericidal (rSBA), a model that is considered a surrogate of protection for meningococcal antigens (Table 3) (104-105). In agreement with the total IgG titers and the increment of IgG2a subclass production, a significant increase of bactericidal activity was measured when the TLR7a was conjugated to MenC-CRMemcs in comparison to the use of the plain MenC-CRMemcs conjugate or its physical mixture with the TLR7a. Murine sera from the control MenC-CRMsidea were endowed with higher bactericidal activity than MenC-CRMemcs, likely, as result of the higher saccharide/protein ratio. This is a parameter which is known to affect the immunological activity of glycoconjugate vaccines. The reduced saccharide/protein ratio in the tricomponent vaccine, therefore, acts in opposition to the adjuvant effect of the TLR7a. The adjuvant effect achieved by conjugation of the small molecule was 8-fold inferior to the administration of MenC-CRMsidea and a standard dose of SMIP7,10 adsorbed on AlumOH. This is due to the combination of the higher level of carbohydrate incorporation of MenC-CRMsidea in combination with the use of a

10-fold higher dose of small immunopotentiating molecule/AlumOH in respect to conjugation to MenC-CRMemcs.

Table 3| rSBA titers (MenC C11 strain) measured after the boost

Vaccine	**rSBA titer** MenC - C11strain
PBS	<16
MenC-CRMemcs	256
MenC-CRMemcs + TLR7a	512
MenC-CRMemcs-TLR7a	8192
MenC-CRMsidea	2048
MenC-CRMsidea + SMIP7,10/AlumOH	>65536

Conclusions

TLR7as have emerged as important targets to develop novel adjuvants for vaccination. Conjugation of a small immunopotentiator molecule could vehicle the ligand to the receptor, localize its action and reduce toxicity. We designed a tricomponent anti serogroup C *Neisseria meningitidis* glycoconjugate vaccine composed of MenC oligosaccharide antigens covalently linked to the carrier protein CRM_{197}, to which a TLR7a has been in turn conjugated. The assembly of this complex construct required the development of a synthetic strategy for orthogonal coupling of two different ligands to the same protein molecule. The construct was first evaluated *in vitro* demonstrating that the presence of the oligosaccharides was not impairing the interaction of the TLR7a with the receptor. Next, the tricomponent vaccine was used to immunize mice in order to determine the effect of the adjuvant conjugation in comparison to the co-administration of the conjugate and the TLR7a. The influence of the conjugation chemistry and carbohydrate/protein molar ratio in the controls was taken into consideration. Co-administration of a small molecule-phosphonate adsorbed on AlumOH, previously reported to confer a strong adjuvant effect towards protein antigens, was used a benchmark. Our results unambiguously indicated that conjugation of the TLR7a increased the anti-carbohydrate response. This effect was less pronounced than the

co-administration of a commercial glycoconjugate with a standard dose of SMIP-phosphonate adsorbed on AlumOH. The reduction of sugar incorporation which is needed for the conjugation of the TLR7a lessens the anti-carbohydrate response, and is therefore a parameter which counteracts the enhancement of the immune response produced by the adjuvant conjugation. An additive effect of the conjugable form of TLR7a and AlumOH was not achieved when the small molecule was administered either coupled or physically mixed with the glyconjugate. On the contrary, it was manifested when a 10-fold higher dose of TLR7a was adsorbed onto the inorganic salt via a phosphonate group. This indicated that a threshold amount of TLR7a adsorbed onto AlumOH was necessary to further potentiate its adjuvant effect. Absorption on AlumOH offers a greater flexibility in the administered dose of TLR7a when compared to the adjuvant conjugation to the vaccine antigen. However, insoluble salts of aluminum such as AlumOH are known to be reactogenic and not usable for transdermal routes (29). The skin is an attractive route for delivery of vaccines since it contains a number of immunocompetent cells and is accessible without the use of needles and syringes (142). Transcutaneous delivery of small-molecule drugs and skin-permeable vaccine adjuvants, for example through microneedle patches, has been shown the key to modulate the duration and of the type of immunogenic response to vaccine antigens administered by this alternative route (143). Hence, conjugation of the small immunopotentiator molecules to glycoconjugates could be particularly beneficial for the development of vaccination via skin delivery. We expect that our findings will pave the ground for the rational design of improved adjuvanted glycoconjugate vaccines and aid establishing novel and safer vaccination routes.

Acknowledgments

We acknowledge Dr. Tom Y.-H. Wu and Dr. Alex Cortez, from the Genomics Institute of Novartis Foundation (10675 John Jay Hopkins Drive, San Diego, CA 92121, USA) for kindly providing the TLR7a molecule.

Supplementary data

- ***TLR7a conjugation to MenC polysaccharide***

Introduction

Here are reported the results obtained for the conjugation of TLR7 agonist to the polysaccharide chain of anti-meningococcal serogroup C glycoconjugate vaccine as a strategy to enhance the anti-carbohydrate immune response. This covalent linkage could transport the ligand to the receptor, promote its intracellular accumulation and favor the receptor activation following the same receptor targeting concept already discussed before.

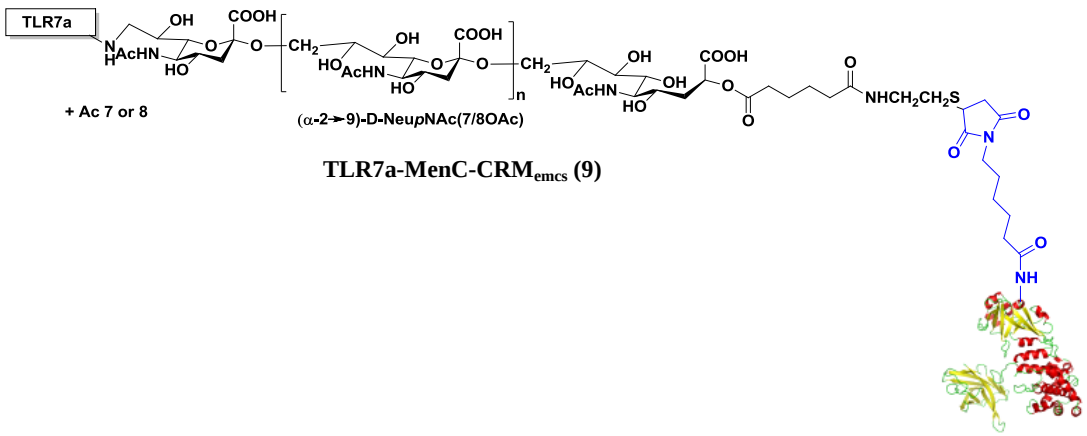


Figure 7| Glycoconjugates prepared for evaluating the effect of covalently linked TLR7a to MenC*; emcs = N-(ε-maleimido)caproyl group.*

After developing a synthetic approach for the preparation of the tricomponent TLR7a-MenC-CRM_{emcs} conjugate **(9)**, the construct was evaluated *in vivo* for the impact of the adjuvant conjugation in the anti-carbohydrate immune response by comparison to the MenC-CRM_{emcs} conjugate **(2)**, prepared through the same conjugation chemistry and co-administered with the unconjugated TLR7a. A commercialized anti-MenC glycoconjugate **(3)** was used as a further control to determine the influence of the coupling approach and level of carbohydrate incorporation on the anti-MenC immune response. In the animal studies, co-

administration of MenC glycoconjugate **(3)** and a small molecule-phosphonate adsorbed on AlumOH, was used a benchmark.

Materials and Methods

Conjugation of TLR7a to MenC

The oligosaccharide **(7)** was oxidized with NaIO4 (3 equiv) in 10 mM NaPi pH 7 at 10 mg/ml sugar based concentration. After stirring at ambient temperature for 3 h, the mixture was purified using a G25 Sephadex column. Fractions containing the sugar were combined and concentrated. The sugar content was estimated by sialic acid assay. The oligomer (10 mmol) was dissolved in 500 ul of NaOAc 100 mM containing 3.3 mg of NH4OAc and then 0.5 mg of NaCNBH3 was added. After incubation for 48 h at 37°C, the crude mixture was purified using a G25 Sephadex column, eluting with 20 mM NaCl. Combined fractions containing the oligomer were quantified by sialic acid assay and concentrated. The residues were dissolved in DMSO-H20 9:1, then TLR7a (5 equiv) and trimethylamine (20 equiv) were added. The mixture was stirred overnight at room temperature, then again purified by size exclusion chromatography. Conjugation was carried out after treatment with TCEP as described for compound **(8)**.

Immunization study

For *in vivo* study, groups of ten 6-week-old female BALB/c mice were immunized by intramuscular injection (i.m.) of 0.5 µg/mouse carbohydrate-based dose of glycoconjugates on days 1 and 28. TLR7a was used as immunopotentiator at a dose of 0.05 µg/mouse, either conjugated or co-delivered with compound **(2)**. Coadministration of glycoconjugate **(3)** and SMIP7,10 (5 µg) adsorbed on AlumOH was the positive control. Serum samples were collected on days 0, 14 and 42 and analyzed by ELISA for their anti-MenC IgG levels.

Determination of anti-MenC antibody titers by ELISA

See above manuscript

Results and Discussion

In vivo immunological evaluation

The effect of conjugation of TLR7a to MenC-CRM_{197} was evaluated by i.m. immunization of groups of 10 BALB/c mice with two 0.5 µg carbohydrate-based doses of vaccine, 2 weeks apart, corresponding to the administration of 0.05 µg of TLR7a. The constructs were compared to the conjugate MenC-CRMemcs prepared with the same conjugation chemistry and a low saccharide/protein molar ratio, co-delivered with the same amount of TLR7a. The MenC-CRMsidea, prepared by the active ester chemistry and with a higher saccharide/protein molar ratio, was used as a control to study the effect of the conjugation chemistry and different levels of sugar incorporation compared to MenC-CRMemcs. Co-administration of MenC-CRMsidea with a standard dose (5 µg) of SMIP7,10 adsorbed onto AlumOH was the benchmark (115). PBS was the negative control. While the MenC-CRMemcs-TLR7a conjugate elicited IgG titers significantly higher than MenC-CRMemcs or its physical mixture with the unconjugated form as previously described, no adjuvant effect is shown when TLR7a is conjugated to the polysaccharide in the MenC-CRM_{197} glycoconjugate (Figure 8). The immune response induced by the tricomponent vaccine was also lower than the one given by the MenC-CRMsidea and MenC-CRMemcs controls. These results are probably the consequence of the very low amount of TLR7a that can be delivered when conjugated to MenC polysaccharide, approximately ten-fold lower than the TLR7a amount delivered when conjugated to CRM_{197} carrier. The adjuvant effect imparted by the conjugated TLR7a was remarkably lower than that achieved by the co-administration of MenC-CRMemcs with a hundred time higher dose (5 µg) of SMIP7,10/AlumOH.

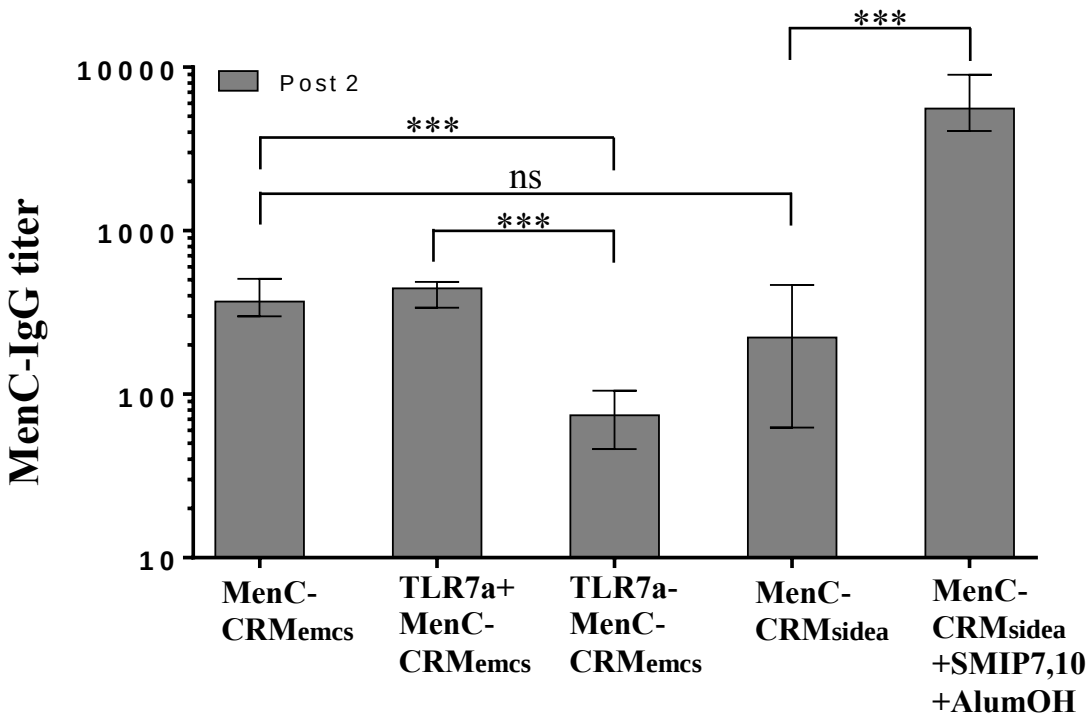


Figure 8| *Systemic humoral responses to i.m. administration of MenC-CRMemcs alone, conjugated or physically mixed with TLR7a. MenC-CRMsidea+SMIP7,10/AlumOH was the control. MenC-specific IgG titers were analyzed after the second immunization. Intergroup comparison was assessed using the Mann-Whitney statistical assay. Medians of MenC-specific IgG titers for i.m. immunizations are reported. pValues: MenC-CRMemcs vs. MenC-CRMsidea (p 0,2239), MenC-CRMemc$_s$+TLR7a vs. MenC-CRMemcs-TLR7a (p 0,0002), MenC-CRMemcs vs MenC-CRMemcs-TLR7a (p 0,0002), MenC-CRMsidea vs. MenC-CRMsidea+SMIP7,10/AlumOH (p 0,0002).*

Conclusions

A tricomponent anti serogroup C *Neisseria meningitidis* glycoconjugate vaccine, composed of TLR7a conjugated to MenC oligosaccharide antigen covalently linked to the carrier protein CRM_{197}, was designed. The tricomponent vaccine was used to immunize mice in order to determine the effect of the adjuvant conjugation in comparison to the co-administration of the TLR7a. Co-administration of a small molecule-phosphonate adsorbed on AlumOH was used as benchmark. The obtained results indicate that conjugation of TLR7a to MenC polysaccharide has a negative impact on the recognition of MenC polysaccharide antigen and consequently a reduction of the anti-carbohydrate response is observed. Moreover the TLR7a

amount conjugated to MenC polysaccharide, with this chemical approach, is too low to appreciate an adjuvant effect in comparison with the dose of small molecule adsorbed onto the inorganic salt via a phosphonate group. The same formulations were also tested in combination with AlumOH (200 μg/dose) but no differences were observed across the groups indicating that the presence of AlumOH obscures all others immune responses.

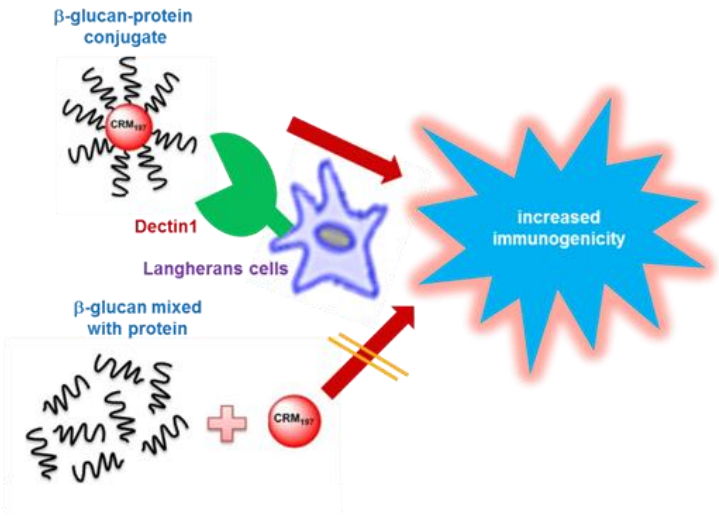
Chapter 4

Conjugation of a short β-glucan chain, as Dectin-1 agonist, to the detoxified diphtheria toxin, (CRM197), as model antigen, to enhance the immune response after intradermal vaccination

Conjugation vs physical mixture

As previously stated, β-(1-3)-glucans are good activators of the innate immune response by interaction with the C-type lectin-like Dectin-1 receptor (60-61). In chapter 2 it was shown that the absence of adjuvant effect when the β-glucan hexasaccharide was delivered in physical mixture or conjugated with the Meningococcal C glycoconjugate vaccine. In this study the conjugation of β-glucan hexasaccharide to the CRM_{197} model antigen is proposed as strategy to test the capability to enhance the immune response toward a protein model antigen in comparison to the previously presented glycoconjugate model antigen. The obtained findings are organized in the following manuscript published on Molecular Pharmaceutics.

Graphical abstract



Rational design of adjuvant for skin delivery: conjugation of synthetic β-glucan Dectin-1 agonist to protein antigen

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Abstract

The potential benefits of skin delivery of vaccines derive from the presence of a densely connected network of antigen presenting cells in the skin layer, most significantly represented by Langerhans cells and dermal dendritic cells. Targeting these cells by adjuvant conjugated to an antigen should result in enhanced immunogenicity of a vaccine. Since one of the most widely used adjuvants is an insoluble salt of aluminium (aluminum hydroxide) which cannot be used for skin delivery due to reactogenicity, we focused our attention on agonists of receptors present on skin dendritic cells, including the Dectin-1 receptor. β-(1-3)-glucans, which are the most abundant components of the fungal surface, are known to activate the innate immune response by interaction with the C-type lectin-like Dectin-1 receptor. In this work we identified by rational design a well-defined synthetic β-(1-3) glucan hexasaccharide as a Dectin-1 agonist and chemically conjugated it to the genetically detoxified diphtheria toxin (CRM_{197}) protein antigen, as a means to increase the binding to Dectin-1 receptor and to target to skin dendritic cells. We demonstrated that the *in vitro* activation of the receptor was significantly impacted by the presentation of the glucan on the protein carrier. *In vivo* results in mice showed that the conjugation of the synthetic β-(1-3) glucan when delivered intradermally resulted in higher antibody titers in comparison to intramuscular (i.m.) immunization, and was not different from subcutaneous (s.c.) delivery. These findings suggest that weak receptor binders can be turned into more potent agonists by the multivalent presentation of many ligands covalently conjugated to the protein core. Moreover, this approach is particularly valuable to increase the immunogenicity of antigens administered via skin delivery.

Keywords: β-glucans, Dectin-1 targeting, glycoconjugates, adjuvant conjugation, Skin delivery

Introduction

There are many potential benefits of vaccine skin delivery, which result from the presence of a densely connected network of antigen presenting cells (APCs) in the skin layer that lies beneath the stratum corneum. This cellular population is most significantly represented by Langerhans cells and dermal dendritic cells in the epidermis and dermis of the skin (92,144). Using conventional intramuscular immunization a large dose of antigen is required to induce a significant immune response, due to the relatively sparse population of APCs present in the muscle. Hence skin delivery for vaccination offers a promising alternative potentially allowing immunization with a lower amount of antigens (145-148). The advantages of skin immunization predicted from animal studies have stimulated sufficient interest to initiate a numbers of clinical trials (149-152). To further increase the systemic and mucosal immune response after skin vaccination a number of additional approaches might be used (153). Because the cornified outer layer and tight junctions limit vaccine penetration of skin, different devices such as microneedles have been used to disrupt the stratum corneum and deposit vaccine in the epidermal/dermal space (154). The most promising systems combine barrier disruption with the addition of an adjuvant that further increases the vaccine potency (75). Adjuvants represent an important component of many modern vaccines as they increase the immunogenicity of co-administered antigens such as purified, soluble recombinant proteins, which are less immunogenic than whole, split, killed or attenuated pathogens. Very few adjuvants are included in licensed human vaccines, for example aluminum salts (alum) have been widely used for more than 70 years and until recently represented the only adjuvant approved in the United States (155). Unfortunately, alum is not useable for skin immunization due to the formation of persistent intradermal granulomas at the injection-site (29). Adjuvants can enhance the immune responses by “targeting” antigens to receptors on dendritic cells. Moreover the physical linking of adjuvant to antigen potentially enhances antigen uptake and targets the immunopotentiator properties of adjuvant to the antigen-presenting cell (156). Starting from this basic concept, we identified

the C-type lectin-like Dectin-1 as an uptake receptor widely expressed by a variety of cells present in the skin layers, including monocytes, macrophage, neutrophils, Langerhans and dermal dendritic cells, both in humans and the mouse (63,157). Hence, the idea was to use a dectin-1 agonists as an adjuvant suitable for administration into the skin. The capability of β-(1-3)-glucans, which are the most abundant component of the fungal surface, to activate the innate immune response by interaction with the Dectin-1 receptor is well documented (61-62). The β-(1-3)-glucans binding to Dectin-1 induces the phosphorylation of a tyrosine of the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail, that elicits the secretion of inflammatory cytokines, such as tumor-necrosis factor (TNF) and IL-12 (63). β-glucans activated-DCs are able to stimulate antigen specific CD8+ T-cell responses, inducing enhancement of antigen presentation, priming and expansion of antigen specific CD8+ T-cells (64-65). To increase the adjuvanticity and to better target the dermal dendritic cells, the chemical conjugation of β-glucans to the antigen was investigated. Recently it has been demonstrated that the covalent linkage of Laminarin (Fig. 1) to a β-mannan-Tetanus Toxoid conjugate enhanced the immune response against the mannan antigen in a tricomponent vaccine against *Candida albicans* (66). The interaction of β-(1-3)-glucans, which are triplic helix forming polysaccharides, with Dectin-1 has been detailed by diverse studies (67-69,158). Linear structures have been found to bind best to the receptor in comparison to branched ones (70). The minimal length required for interaction is 6-7 repeating units, although the binding becomes tighter as the length increases (69-70). Therefore, we were interested in understanding whether conjugation of glucan hexamer, which can be synthesized as a well-defined pure molecule, to the well-known protein antigen genetically detoxified diphtheria toxin (CRM_{197}) could increase the immune response against the protein antigen compared to its administered in unconjugated form. CRM_{197} is widely used as protein carrier in many glycoconjugate vaccines (71) and detailed knowledge of the chemistry of its conjugation to polysaccharides facilitated its use as an antigen for co-administration with the glucan hexamer. The linear and long chain naturally extracted Curdlan polysaccharide, considered a potent agonist of Dectin-1 receptor,

was used as positive control for comparison. To determine *in vitro* the optimal sugar amount to be loaded onto CRM_{197}, we synthesized a set of β-(1-3) glucan hexasaccharides conjugated to CRM_{197} at different carbohydrate/protein ratios to be assayed for Dectin-1 receptor activation. It is known that the binding of weak ligands, such as carbohydrates, to specific receptors can be increased by their multivalent presentation as glycoclusters or glycodentrimers (159). Also in the case of Dectin-1, an important pre-requisite for its signaling has been identified in the presentation mode of the β-glucans. For example, an immobilized form of β-(1-3)-glucans, on the surface of a phagocytosable particle, such as a yeast cell, would favor the binding to the receptor (160). These previous findings strengthen our choice to conjugate the β-(1-3) glucan hexasaccharide to antigen to better present β-glucans to the receptor surface. Next, the properties of the molecule as an adjuvant after administration by skin delivery were compared to the intramuscular route following *in vivo* by immunization of mice. Antigen-specific (anti-CRM_{197},) antibody titers induced in the presence of β-glucan, either conjugated or co-delivered with the antigen, were analyzed and the capability of the short well-defined β-glucan oligosaccharide as an effective adjuvant for skin delivery was evaluated.

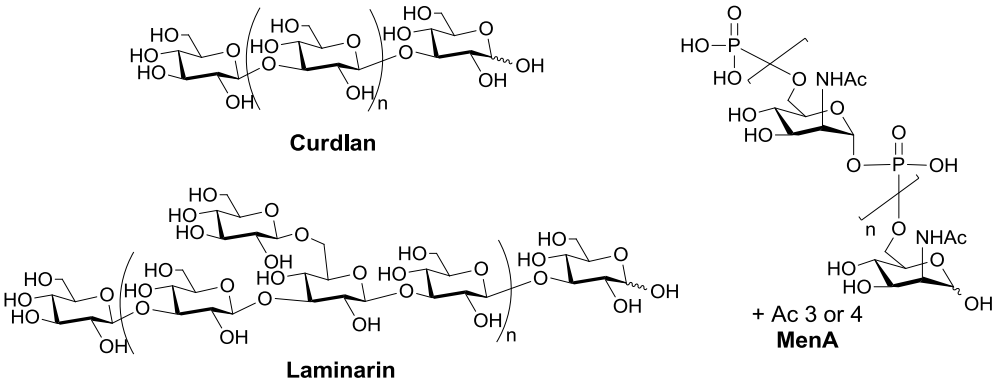


Figure 1| Chemical structures of the polysaccharides employed in the study*: Curdlan, Laminarin and Meningococcal serogroup A (MenA) capsular polysaccharide (71,143,159-163).*

Materials and methods

Synthesis of glycoconjugates

Activated hexasaccharide **(2)**, prepared as previously reported (161) was incubated with CRM_{197} at a molar ratio of 20:1, 30:1 or 50:1, respectively (moles of active ester per moles of protein), in 100 mM phosphate buffer at pH 7.0. CRM_{197} contains 40 lysine residues, of which 19 are available for glycoconjugation (162). After overnight reaction at RT, the conjugate was purified by ammonium sulfate precipitation (500 mg/ml). The pellet was dissolved in 10 mM phosphate buffer at pH 7.0 and again precipitated with ammonium sulfate (500 mg/ml). After repeating this operation twice, the solid was washed twice with a solution of ammonium sulfate (500 mg/ml) in 10 mM phosphate buffer at pH 7.0, and finally reconstituted with 10 mM phosphate buffer at pH 7.0. The amount of carbohydrate covalently linked to the protein was estimated by MALDI TOF MS, using an UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics) in linear mode and with positive ion detection. The samples for analysis were prepared by mixing 2.5 µl of product and 2.5 µl of sinapinic acid matrix; 2.5 µl of each mixture was deposited on a samples plate, dried at RT for 10 min, and subjected to the spectrometer. The formation of glycoconjugates was monitored by SDS-page: samples (5 µg) were electrophoresed on a 7% Tris Acetate gel or 4-12% Bis-Tris gel (NuPage, Invitrogen) and stained with Coomassie blue. Curdlan polysaccharide, with an average polymerization degree (aDP) of 25 repeating units which is typically considered agonist of Dectin-1 receptor (positive control), was conjugated to CRM_{197}, at an average loading of 9.4 carbohydrate moieties, using a procedure reported in literature (143) CRM_{197}-Curdlan conjugate was prepared from hydrolyzed Curdlan aDP 25 (143).

Determination of β-glucan - receptor binding in vitro

An ELISA assay was performed to test the direct binding of different agonists to Dectin-1 receptor. Maxisorp plates (Nunc) were coated overnight at 4°C with 0.2 µg/well of CRM_{197}-Curdlan (normalized for CRM_{197} content) in phosphate buffered saline (PBS), and blocked for 1 h at 37°C with 200 µl of 1% bovine serum albumin,

BSA (Sigma), in PBS. Three different Fc-mDectin-1 (soluble murine Dectin-1 receptor fused to the C-terminus of human IgG1 Fc domain, InvivoGen) concentrations, 1 µg/ml - 0.1 µg/ml - 0.01 µg/ml, were diluted in PBS, 0.1% BSA and 0.05% Tween-20 (Sigma) and incubated with 2 µg/ml (sugar content) of each analyzed agonist for 10 minutes at RT. 100 µl/well of each mixture (Fc-mDectin-1 at different concentration plus agonists) were consequently added to the blocked ELISA plates and incubated for 2 h at 37°C. The specific binding of agonist to Fc-mDectin-1 and consequent decrease of coated Curdlan recognition was detected with alkaline phosphatase-conjugated goat anti-human IgG (γ chain specific, SouthernBiotech). The different Fc-mDectin-1 concentrations not pre-incubated with agonists were used as negative control of the assay. The optical density was read at 405 nm using an ELISA reader.

Cell culture

RAW-Blue™ cells (InvivoGen), derived from RAW 264.7 macrophages, were used as a Dectin-1 reporter cell line as they express high levels of endogenous Dectin-1. RAW-Blue™ cells were grown in T-75 culture flasks (Iwaki) in Dulbecco's Modified Eagle Medium, DMEM (Gibco), 4.5 g/l glucose, 10% heat-inactivated Fetal Bovine Serum, FBS (Gibco) (30 min at 56°C), 100 mg/ml Normocin™ (InvivoGen), 2 mM L-glutamine (Gibco), 200 mg/ml Zeocin™ (InvivoGen) incubated at 37°C in presence of 0.5% CO_2.

Dectin-1 receptor activation: QUANTI-Blue™ assay

The QUANTI-Blue™ assay allows detection of NF-kB/AP-1 activation, consequent to the Dectin-1 agonist binding, by measuring secreted embryonic alkaline phosphatase (SEAP) production, using QUANTI-Blue™ (InvivoGen), SEAP detection medium. A cell suspension at ~550,000 cells/ml of growth medium was prepared. 180 µl of cell suspension plus 20 µl of different Dectin-1 agonists, positive (CRM_{197}-Curdlan) and negative controls (CRM_{197} and CRM_{197}-MenA (71,163)) were distributed in a flat-bottom 96-well plate (Corning). The plate was incubated at 37°C in a 5% CO_2 incubator for 18 h. Subsequently 150 µl of

suspended QUANTI-Blue™ plus 50 µl of induced RAW-Blue™ cells supernatant were added per well and the plate was incubated at 37°C for 30 min to 6 h. SEAP (secreted embryonic alkaline phosphatase) levels were revealed at 620-655 nm using a spectrophotometer.

Immunogenicity testing in mice

Protocols were approved by the Italian Ministry of Health. All mice were housed under specific pathogen-free conditions at the Novartis Vaccines Animal Resource Center. For both *in vivo* studies, groups of eight 6-week-old female BALB/c mice were immunized. Subcutaneous (s.c.)/intradermal (i.d.) immunization on days 1, 21 and 35 for the first *in vivo* study and intramuscular (i.m.)/intradermal (i.d.) immunization on days 1 and 21 for the second were conducted. For s.c. immunization, 100 µl of CRM_{197} (4 µg/mouse) conjugated or co-delivered with β-glucan hexasaccharide (1 µg/mouse) and Curdlan (2,7 µg/mouse) were injected. The same antigen/adjuvant dose was delivered intradermally in a 20 µl formulation. S.c. immunization with alum hydroxide adjuvanted formulation (0.2 mg/mouse) was used as a positive control. Alum hydroxide is an adjuvant widely used for carbohydrate-based vaccines (164-165). For i.m. immunization, 100 µl of CRM_{197} (50 µl for each muscle - 4 µg/mouse) conjugated or physically mixed with β-glucan hexasaccharide (1 µg/mouse) was injected. The same antigen/adjuvant doses were delivered intradermally in 20 µl formulations. I.m. immunization with alum hydroxide adjuvanted formulation (0.2 mg/mouse) was used as positive control. Serum samples were collected on days 20, 34 and 49 for the first *in vivo* experiment and on days 20 and 34 for the second study and analyzed by ELISA for their anti-CRM_{197} IgG levels.

Determination of CRM_{197}-specific antibody titers by ELISA

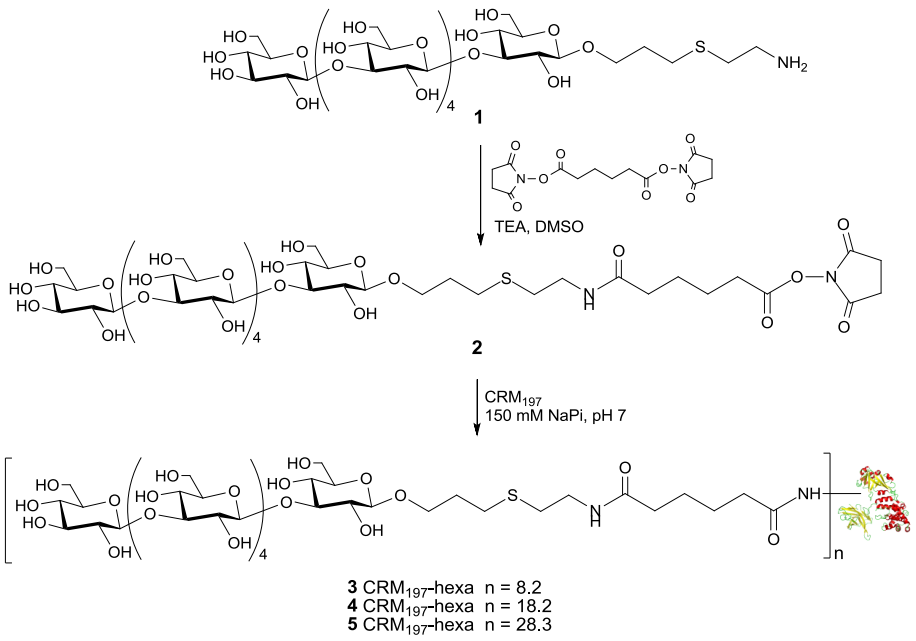
Determination of anti-CRM_{197} specific total IgG and subclasses IgG1 and IgG2a titers was performed by ELISA using individual serum samples. Maxisorp plates (Nunc) were coated overnight at +4°C with 0.2 µg/well of CRM_{197} in PBS, and blocked for 1 h at 37°C with 200 µl of 1% BSA (Sigma), in PBS. For the IgG

ELISA assay, serum samples were initially diluted in PBS, 0.1% BSA and 0.05% Tween-20 (Sigma), transferred into coated and blocked plates and serially diluted. Bound CRM_{197}-specific IgG, IgG1 and IgG2a were detected with alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1 and IgG2a respectively (SouthernBiotech). Antibody titers, normalized respect to the reference serum assayed in parallel, were obtained using IrisLab software. GraphPad Prism v.6.04 (GraphPad Software, La Jolla, CA) was used to graph and analyze data for statistical significance. Intergroup comparison was analyzed using the Mann-Whitney test.

Results

Synthesis of glycoconjugates with different degrees of glycosylation

CRM_{197}-Hexasaccharide (CRM_{197}-Hexa) **(3)**, **(4)**, and **(5)** at different carbohydrate loading were prepared as described in Scheme 1.



Scheme 1| Preparation of the CRM_{197}-Hexa glycoconjugate at different carbohydrate loading (161).

As described above three conjugates with carbohydrate: protein ratios of 8.2, 18.2 and 28.3, respectively, were obtained. The amount of carbohydrates covalently linked to the protein was estimated by MALDI TOF MS (Fig. 2 and Table 1).

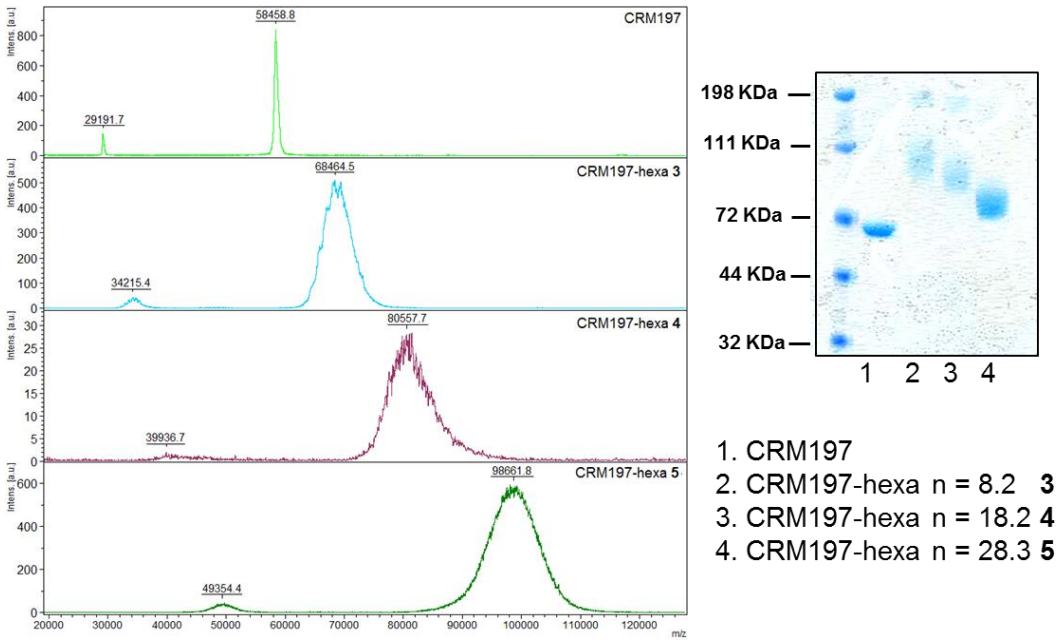


Figure 2| SDS page gel electrophoresis and MALDI TOF MS spectra of the glucan hexamer conjugated to the model protein CRM_{197}.

Table 1| Characteristics of protein-adjuvant conjugates.

Compound	MW	Sugar loading	Yield %[d]	Conjugation efficiency %[e]
CRM_{197}-hexa **3**	68864	8.2[a]	89	41
CRM_{197}-hexa **4**	80577	18.2[a]	94	62
CRM_{197}-hexa **5**	98662	28.3[a]	81	56
CRM_{197}-Curdlan	nd	9.4[b]	nd	31
CRM_{197}-MenA[c]	nd	5.2[b]	nd	20

a. The sugar loading was determined by MALDI TOF MS.
b. The amount of conjugated carbohydrate was determined as described in literature (143).
c. This conjugate was prepared as reported in literature (71,163)
d. Recovered protein after purification as determined by microBCA.
e. Mol of linker sugar/mol reacted sugar.

Conjugation of Hexasaccharide improves Dectin-1 receptor activation

A preliminary study was performed to evaluate the *in vitro* binding of different agonists to Dectin1 receptor. First, the specificity of the binding of the constructs presenting the β-(1-3) glucans for the Dectin-1 receptor was assessed (Fig. 3A). To this end, by a competitive ELISA, the capability of the different biomolecules to compete with the binding of the receptor pre-incubated with CRM_{197}-Curdlan to the same conjugate coated on the plate was measured. The receptor, either pre-incubated or not pre-incubated with the conjugated CRM_{197}-Curdlan, was used as control. As additional negative control, we used the CRM_{197} conjugate of Meningococcal serogroup A capsular polysaccharide (MenA), that is known not to be a Dectin-1 receptor agonist. As expected, CRM_{197}-MenA conjugate did not interact with Dectin-1, indicating that β-(1-3) glucans exhibited specific binding to the receptor (Fig. 3A). The unconjugated Curdlan was a weaker competitor in comparison to the conjugates, while the hexasaccharide showed almost no binding to the receptor, similarly to CRM_{197}-MenA.

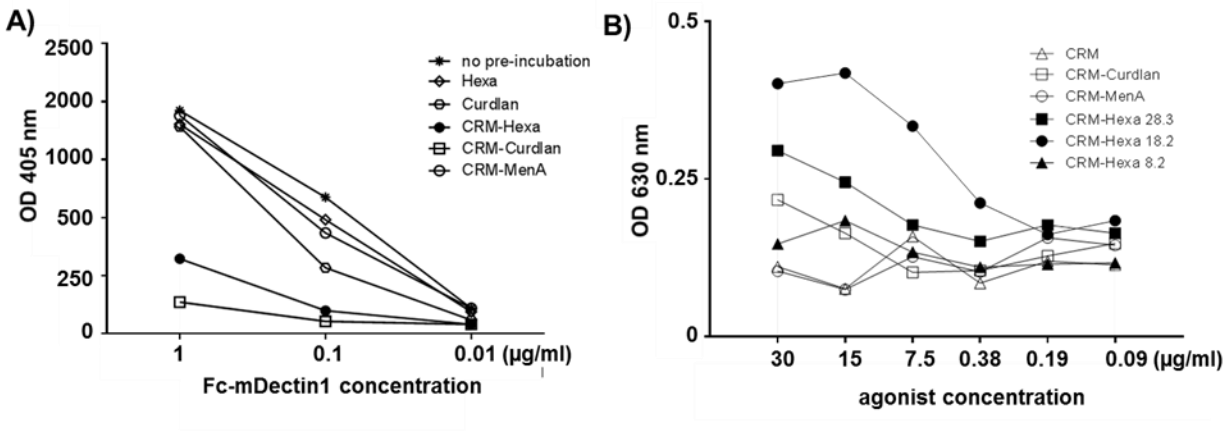


Figure 3| Dectin-1 receptor activation. *(A) Competition for Dectin-1 receptor between conjugated and not conjugated β-glucans pre-incubated with CRM_{197}-Curdlan on plate coated with the same conjugate. The not pre-incubation of the receptor was used as blank test of assay. (B) RAW-Blue™ cells treated with the three different CRM_{197}-Hexa glycoconjugates* ***(3)(4)(5)*** *with different loaded sugar chains. CRM_{197}-Curdlan was the positive control and Hexa* ***(1)****, CRM_{197} and CRM_{197}-MenA were the negative controls. The experiments were run in duplicates.*

Having ascertained that the conjugated glucans were recognizing the Dectin-1 receptor specifically, an *in vitro* test was performed to evaluate the ability of the different CRM_{197}-Hexa glycoconjugates **(3)(4)(5)** to bind Dectin-1 in cells which highly express the receptor and activate the intracellular pathway (Fig. 3B). In order to test the effect of the different numbers of sugar chains present in each glycoconjugate, all biomolecules were analyzed at the same amount of sugar in work solution. CRM_{197} alone and CRM_{197}-MenA glycoconjugate were used as negative controls. CRM_{197}-Curdlan, with an average loading of 9.4 carbohydrate moieties, was the positive control. As it appeared from the absorbance values at 630 nm depicted in Fig. 3B, all the glucan conjugates exhibited activation of the Dectin-1 pathway, while almost no absorbance was associated to the negative controls, CRM_{197} and CRM_{197}-MenA, respectively. This result suggested that conjugated β-(1-3) glucans are better agonists than the unconjugated counterparts. Therefore, the multivalent exposition of the carbohydrate moieties linked to the protein core in form of glycoclusters appeared to potentiate the binding to the receptor (159,166) The Dectin-1 activation was found to be influenced by two interconnected parameters, which are the number of attached sugar moieties and the relative distance onto the protein core. When the number of sugar moieties on the protein was similar, as in the Curdlan and hexasaccharide conjugates at loading 9.4 and 8.2 respectively, the product with the longest sugar chain resulted in a better activation. On the other hand, the conjugates **(4)** and **(5)** with highest sugar density increased the SEAP production in comparison to **(3)**, indicating that this was another important feature for the receptor activation. Unexpectedly, conjugate **(4)** resulted in a higher SEAP production than **(5)**, suggesting that a too dense distribution of the sugars around the core might not be beneficial for the Dectin-1 stimulation. This could be due to the irregular distribution of the glycans around the protein core in comparison to the use of synthetically defined clusters (167), however this aspect was not further explored. Following the main purpose of this work, to investigate the adjuvant effect of the conjugated hexasaccharide compared to its unconjugated form for skin delivery, CRM_{197}-Hexa conjugate **(4)** was selected for the *in vivo* studies.

Conjugation of the glucan hexasaccharide to the antigen is essential for adjuvant effect in vivo

To evaluate the effect of conjugation of the synthetic β-(1-3) glucan hexamer on its ability to adjuvant the anti-protein response, a set of *in vivo* experiments was designed. In the first *in vivo* study, immune responses induced by conjugated and unconjugated hexasaccharide and Curdlan against the protein CRM_{197} through subcutaneously route (s.c.) and intradermal immunization (i.d.) were investigated (Figure 4A and 4B). CRM_{197} adjuvanted with AlumOH was used as positive control for the subcutaneous administration only, since this adjuvant cannot be used for intradermal delivery (29), and CRM_{197} alone was the benchmark. Importantly, CRM_{197}-Hexa 18.2 and CRM_{197}-Curdlan conjugates induced anti-CRM_{197} IgG titers significantly higher than the CRM_{197} alone and corresponding formulations of CRM_{197} physically co-delivered with hexamer or Curdlan, independently of the route of administration. Thus the conjugation was essential to induce an adjuvant effect (Fig. 4B and Table 2). When the subcutaneous route was evaluated (Fig. 4A), anti-CRM_{197} IgGs titers of sera from mice immunized with two doses of CRM_{197}-Hexa 18.2 or CRM_{197}-Curdlan were comparable to the sera from mice immunized with the positive control - AlumOH adjuvanted formulation. After the third dose CRM_{197}-Hexa 18.2 was able to further increase anti-CRM_{197} IgGs titers while CRM_{197} adjuvanted with CRM_{197}-Curdlan and AlumOH seemed to plateau. Thus conjugation of the synthetic hexamer induces a higher immune response compared to Curdlan. In contrast, anti-CRM_{197} IgGs titers of mice immunized with unconjugated saccharide mixed with the protein showed no increase in titers compared to CRM_{197} alone (Fig. 4A).

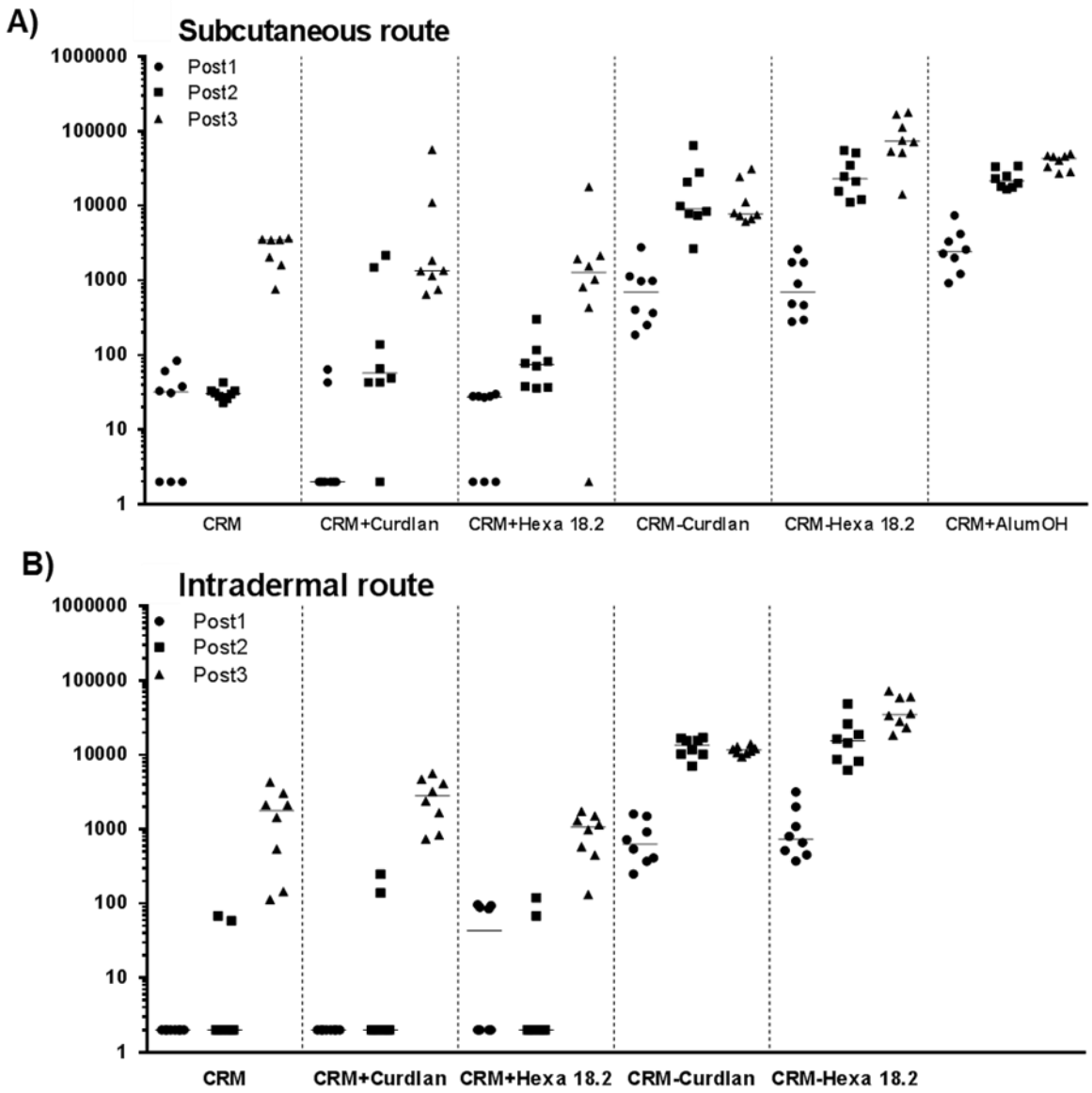


Figure 4| Systemic humoral responses to s.c. and i.d. administration of Hexa and Curdlan conjugated to or physical mixture with CRM_{197} protein. *CRM_{197}-specific IgG titers were analyzed after first, second and third immunization. Intergroup comparison was analyzed using the Mann-Whitney statistical assay. Statistically significant differences are summarized in Table 2. (A) Medians of CRM_{197}-specific IgG titers for s.c. immunizations are reported. (B) Medians of CRM_{197}-specific IgG titers for i.d. immunizations are reported.*

Table 2| Comparison of IgG levels, expressed as medians with interquartile range (IQR), of groups showing statistically significant differences (p value) in Figure 4.

Immunization Groups	Post 1		Post 2		Post 3	
	median (IQR)	p[a]	median (IQR)	p[a]	median (IQR)	p[a]
CRM_{197}+Curdlan SC *vs* **CRM_{197}**-Curdlan SC	2 (2-33) *vs* 695 (281-1100)	***	57.5 (43-1158) *vs* 9198 (7517-26174)	***	1349 (856-8724) *vs* 7794 (6846-21323)	*
CRM_{197}+Hexa 18.2 SC *vs* **CRM_{197}**- Hexa 18.2 SC	28 (2-28) *vs* 695 (339-1753)	***	75 (37-108) *vs* 23037(13055-47355)	***	1288 (528-2104) *vs* 74055 (52153-154580)	***
CRM_{197}+Curdlan ID *vs* **CRM_{197}**-Curdlan ID	2 (2-2) *vs* 635 (381-1355)	***	2 (2-105) *vs* 13643 (10212-16484)	***	2813 (1043-4569) *vs* 11624 (10476-12764)	***
CRM_{197}+Hexa 18.2 ID *vs* **CRM_{197}**- Hexa 18.2 ID	44 (2-92.75) *vs* 732 (470-1777)	***	2 (2-51.5) *vs* 15488 (8342-24319)	***	1068 (483-1456) *vs* 34832 (24604-59682)	***
CRM_{197}-Curdlan SC *vs* **CRM_{197}**+ AlumOH SC	695 (281-1100) *vs* 2446 (1419-3997)	*	9198 (7517-26174) *vs* 21680 (17907-31449)	ns	7794 (6846-21323) *vs* 43195 (29770-46856)	***
CRM_{197}- Hexa 18.2 SC *vs* **CRM_{197}**+ AlumOH SC	695(339-1753) *vs* 2446 (1419-3997)	*	23037(13055-47355) *vs* 21680 (17907-31449)	ns	74055 (52153-154580) *vs* 43195 (29770-46856)	*
CRM_{197}-Curdlan ID *vs* **CRM_{197}**+ AlumOH SC	635 (380.5-1355) *vs* 2446 (1419-3997)	**	13643 (10212-16484) *vs* 21680 (17907-31449)	***	11624 (10476-12764) *vs* 43195 (29770-46856)	***
CRM_{197}- Hexa 18.2 ID *vs* **CRM_{197}**+ AlumOH SC	732 (470-1777) *vs* 2446 (1419-3997)	*	15488 (8342-24319) *vs* 21680 (17907-31449)	ns	34832 (24604-59682) *vs* 43195 (29770-46856)	ns
CRM_{197}-Hexa 18.2 SC *vs* **CRM_{197}**- Hexa 18.2 ID	695 (339-1753) *vs* 732 (470-1777)	ns	23037 (13055-47355) *vs* 15488 (8342-24319)	ns	74055 (52153-154580) *vs* 34832 (24604-59682)	**

a. p values are indicated as follows: * $0.05 \leq p < 0.01$, ** $0.01 \leq p < 0.001$, and *** $p < 0.001$; ns = not significant.

Following intradermal delivery, the anti-CRM_{197} IgG titer elicited by CRM_{197}-Hexa 18.2 was comparable to the positive control (CRM_{197}+AlumOH by s.c. route, Fig. 4B and Table 2), whereas sera from the CRM_{197}-Curdlan conjugate exhibited anti-CRM_{197} IgG titers significantly lower than the positive control (CRM_{197}+AlumOH by s.c. route, Fig. 4B and Table 2). This indicated that conjugation of the synthetic hexamer, but not Curdlan, provided an adjuvant effect comparable with that of AlumOH (subcutaneously administered) for intradermal immunization. This effect was in agreement with the *in vitro* Dectin1 receptor activation assay, which showed CRM_{197}-Hexa 18.2 as a stronger activator than CRM_{197}-Curdlan. We can, therefore, assume that the Dectin-1 recognition was better affected by the sugar exposition around the protein core rather the repetition along the polysaccharide chain. Notably, for both the immunization routes (s.c. and i.d.), antibody titers detected after the first immunization using a conjugated form of the antigen highlighted a quicker on-set of immune response compared to antigen administered alone that was not able to induce any response. Moreover independently from the routes of immunization, antibody titers detected after two doses using the conjugated forms were higher than titers induced after three immunizations using non-adjuvanted formulations or physical mixtures of protein-adjuvant (Fig. 4A and Fig. 4B, Table 2). This result confirmed the capability of the hexamer **(1)**, when conjugated to the protein, to exert an adjuvant effect. To further assess this effect and compare the intradermal *versus* the intramuscular route, a second *in vivo* experiment was conducted. CRM_{197} co-delivered either with the unconjugated hexamer or AlOH was used as control. In the first study no relevant increase in the anti-CRM_{197} responses could be observed after the second boost with CRM_{197}-Hexa 18.2. Therefore, in the second *in vivo* study, only two immunizations were evaluated. As depicted in Figure 5A and 5B, ELISA titers of sera from first and second vaccination confirmed that the conjugation of the sugar to the antigen was crucial to achieve an adjuvant effect.

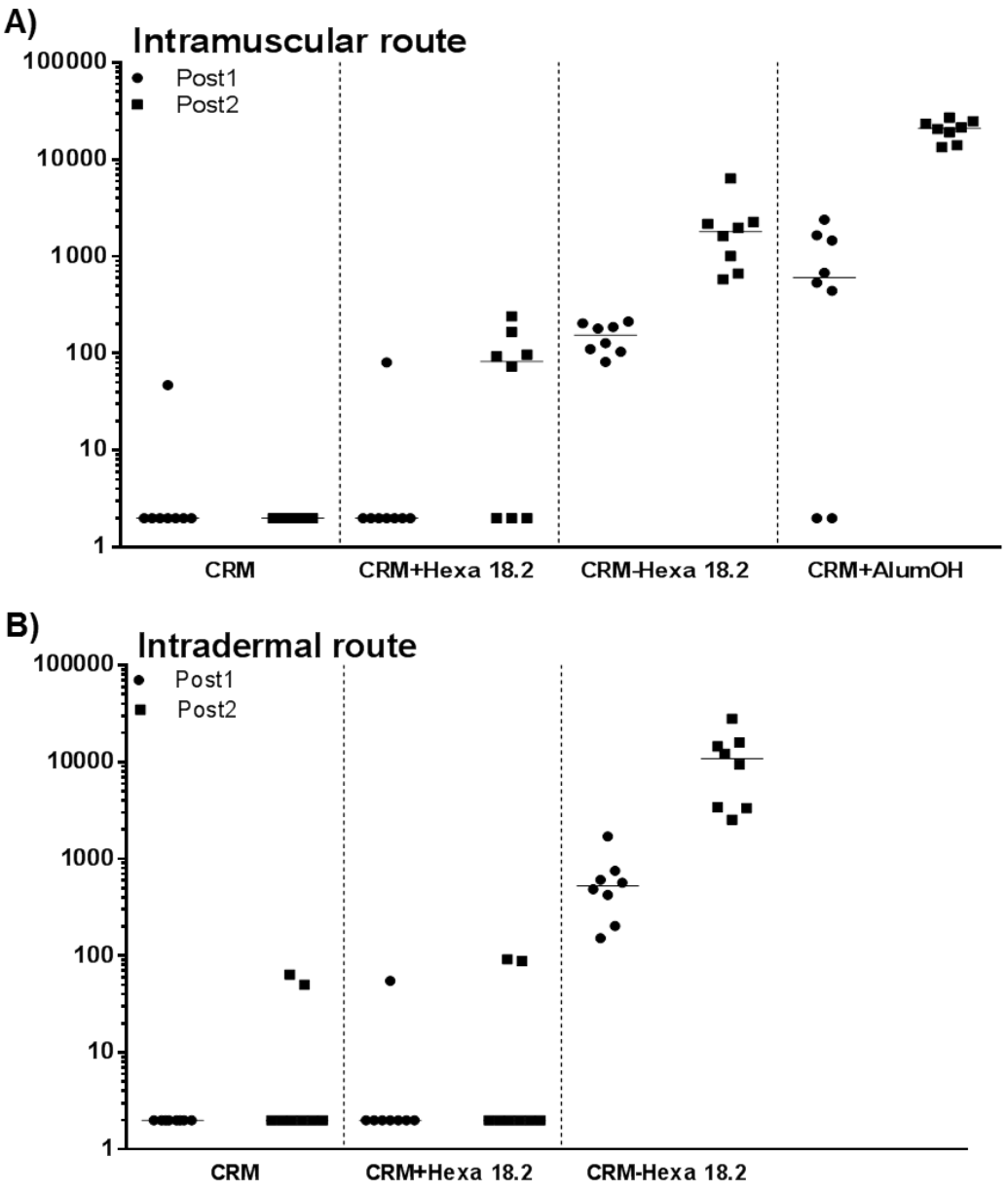


Figure 5| Systemic humoral responses to i.m. and i.d. administration of Hexa conjugated to or physical mixture with CRM_{197} protein. *CRM_{197}-specific IgG titers were analyzed after first and second immunization. Intergroup comparison was analyzed using the Mann-Whitney statistical assay. Statistically significant differences are summarized in Table 3. (A) Medians of CRM_{197}-specific IgG titers for i.m. immunizations are reported. (B) Medians of CRM_{197}-specific IgG titers for i.d. immunizations are reported.*

After the first immunization respective titers were comparable to the AlumOH adjuvanted formulation (Table 3, p for post 1 sera). After the second immunization, anti-CRM_{197} IgG titers of sera of CRM_{197}-Hexa 18.2 immunized mice both i.m. and i.d. were lower in comparison to the AlOH formulated control (Table 3, p post 2 sera). Interestingly CRM_{197}-Hexa 18.2 conjugate delivered by i.d. induced significantly higher antibody titers when compared to the i.m. route both after the first and second immunization. This result confirmed the potential benefit of using the synthetic β-(1-3) glucan hexamer as adjuvant for skin delivery.

Table 3| Comparison of IgG levels, expressed as medians with interquartile range (IQR), of groups showing statistically significant differences in Figure 5.

Immunization Groups	Post 1		Post 2	
	Median (IQR)	**p[a]**	**median (IQR)**	**p[a]**
CRM_{197}- Hexa 18.2 IM	155 (106-201)		1804 (752-2246)	
vs	*vs*	ns	vs	***
CRM_{197}+ AlumOH IM	609 (113-1610)		21069 (15326-24496)	
CRM_{197}- Hexa 18.2 ID	526 (258-717)		10861 (3365-15576)	
vs	*vs*	ns	*vs*	*
CRM_{197}+ AlumOH IM	609 (112.3-1610)		21069 (15326-24496)	
CRM_{197}- Hexa 18.2 IM	155 (105.8-200.5)		1804 (752-2246)	
vs	*vs*	**	*vs*	**
CRM_{197}-Hexa 18.2 ID	526 (258-717)		10861 (3365-15576)	

a. p values are indicated as follows: * $0.05 \leq p < 0.01$, ** $0.01 \leq p < 0.001$, and *** $p < 0.001$; ns = not significant.

Dectin-1 agonist elicits the same IgG subclass CRM_{197}-specific response as Alum hydroxide

To characterize the quality of the immune response, serum samples of the second *in vivo* study were analyzed for anti-CRM_{197}-specific IgG1 and IgG2a titers (168). Alum is known to induce a Th2 response, which results in the predominant segregation of IgG1 subclass (144). Conjugation of the β-(1-3)-(1-6) glucan Laminarin to OVA peptide has also been reported to result in a prominent production of IgG1 antibodies (147). No difference between AlumOH adjuvanted formulation and CRM_{197}-Hexa 18.2 was found for either IgG1 or IgG2a subclasses, independently of the route of administration (Fig. 6). Interestingly, a prevailing

IgG1 production was found both for Alum and the conjugated glucan hexamer, in agreement with what observed for peptide-bound Laminarin (147). Therefore, conjugation of the Dectin-1 agonist appeared to enhance the immune response in terms of raised IgG levels, while no shift in the Th1/Th2 balance was observed. Unconjugated Curdlan-like polysaccharides have been demonstrated to be capable of inducing Th17 activity (169). Whether this type of immune response could be also evoked by conjugated Curdlan was not explored in the present study, however this point may merit further investigation.

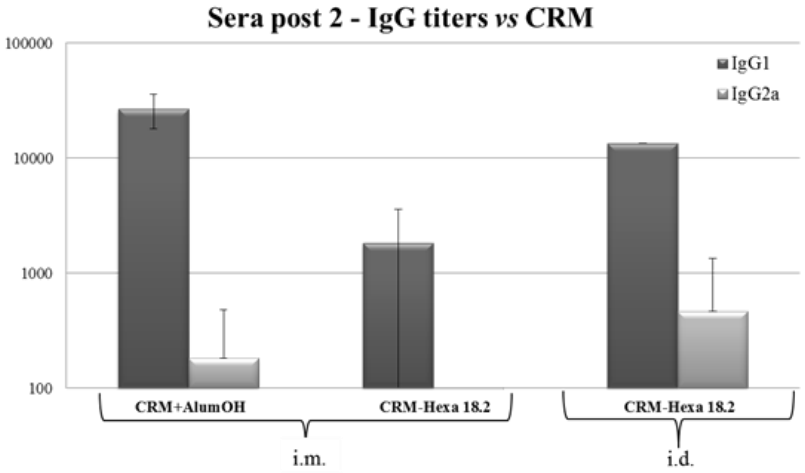


Figure 6| CRM$_{197}$-Hexa 18.2 glycoconjugate elicits both IgG1 and IgG2a. *Post 2 CRM$_{197}$-specific IgG1 and IgG2a titers of mice sera reported in figure 5 are reported. Mean titers with standard deviations bars are reported.*

Discussion

Different routes for administration of vaccine antigens have been proposed on many occasions (170). Intranasal administration, for example, has been shown to represent a viable alternative to classic administration routes, particularly when mucosal immunity needs to be stimulated (171). However, safety concerns have been reported for some intranasal vaccines, possibly as a consequence of the redirection of antigens and/or adjuvants to the central nervous system through the olfactory epithelium (172-173). In the present study, Dectin-1 agonists were investigated as potent adjuvants for skin delivery of protein antigens. Since

Langerhans cells highly express the Dectin-1 receptor on their surfaces, skin delivery should benefit more than the classical routes of administration of the effect of β-glucans as immunopotentiators. By comparison of three different administration routes (s.c., i.d. and i.m.), we observed that conjugation of the synthetic β-(1-3) glucan in combination with intradermal delivery resulted in higher antibody titers compared to intramuscular and subcutaneous delivery. Our finding highlights that covalent linkage of the synthetic agonist to proteins is an approach particularly suited for increasing the immunogenicity of antigens administered intradermally. This is a relevant benefit since classical alum based adjuvants are not suitable for administration by this route (29). Analysis of CRM_{197}-specific IgG subclasses indicated that conjugation of the glucan hexamer resulted in a prevailing IgG1 production. This outcome is in agreement with recent publications, in which cooperation between Dectin-1 and TLR4 induces mainly IgG1 production by mouse B-cells (174-175). AlumOH used for intramuscular immunization elicited IgG1 and IgG2a subclasses in the same ratio as the conjugated glucan hexamer administered intradermally. These results were achieved due to a rational study design that allowed us to identify a well-defined synthetic β-(1-3) glucan hexasaccharide as a suitable Dectin-1 agonist, that was chemically conjugation to a protein antigen to increase binding to the Dectin-1 receptor and targeting to the Dendritic cells (DCs).

It was previously reported that Laminarin, a heterogeneous and variable polysaccharide from algae could be used in a tricomponent glycoconjugate vaccine to enhance the immune response against a β-mannan antigen (158). The structural requirements of β-glucans for Dectin-1 binding have been thoroughly explored. Measurements of competition between oligosaccharides for binding a soluble form of Dectin-1 revealed that both branched and linear hexadecasaccharides are capable of Dectin-1 receptor binding (158). This finding suggests that linear portions of the glucans are recognized by the receptor (158). In particular, it has been shown using a glycan-array that 9 to 10 β-(1-3) repeating units are the minimal requirement for efficient receptor binding (67). Glycan-arrays are usually affected by the exposition of the sugar on the chip surface. Analysis by titration NMR techniques of a set of short β-(1-3)-glucans and Laminarin has revealed that binding to the receptor takes

place starting from six repeating units and becomes stronger for longer glucans (70). Moreover, it has been observed that short β-(1-3)-glucan oligomers, although they are not able to adopt any helical arrangements, are able to increase the ratio of granulocytes in peripheral blood, potentiation of phagocytosis and production of interleukin-2 after intra-peritoneal injection in murine models (62). Using *in vitro* assays we demonstrated that whilst the short hexamer was unable to efficiently bind to the receptor, conjugation dramatically increased binding. This result confirmed that the mode of presentation is important for activation of the receptor (160) and, most importantly, highlighted that receptor binding of weak ligands of short length can be increased by multivalent presentation. Binding to receptor was found to increase for longer and more densely distributed sugars around a protein core. However, above a certain threshold, further increases in density had no beneficial effect. The optimal carbohydrate-protein ratio to achieve the Dectin-1 activation was determined and the selected CRM_{197} conjugate was utilized in *in vivo* studies. Following immunization of mice, sera were tested to detect CRM_{197}-specific antibody titers. Notably, conjugation of the synthetic β-(1-3) glucan hexasaccharide to CRM_{197} exhibited an adjuvant effect, comparable to AlumOH adjuvanted formulations, thereby demonstrating that conjugation is fundamental for use of the hexasaccharide as an adjuvant. This finding was confirmed by the negligible or very limited antibody titers elicited by the hexamer administered in physical mixture with the antigen. Importantly, after two doses of the hexasaccharide conjugate, the anti-protein immune response was higher than that seen after three doses of the antigen-hexasaccharide physical mixture. Thus, adjuvant-antigen conjugation facilitates vaccine dose sparing. Besides Dectin-1, other lectins like the murine leukocyte CR3 (CD11b/CD18) have been shown to function as a receptor for β-glucans, promoting activation of cytotoxic responses against tumor cells. Within the scope of the present study, interaction of the conjugated hexamer with other β-glucan receptors cannot be excluded (176). CRM_{197} conjugated to Laminarin (177) or to synthetic or natural linear β-(1-3) glucans has also been reported to confer protection against *C. albicans* infections (143,177). Specific anti β-glucans antibodies have been also found to recognize glycan structures on the surface of

Aspergillus fumigatus (178). In the present study we ascertained the induction of anti β-glucan IgGs (see SI, Figure 7). Therefore, the short synthetic glucan hexamer could behave simultaneously as both antigen and adjuvant to target *C. albicans* and *A. fumigatus* infections. Considering that glycoconjugate vaccines are some of the safest and most effective vaccines for reducing, even eradicating, infectious diseases (179), conjugation of well-defined synthetic glucans could represent a useful strategy for developing new glycoconjugate vaccines for skin delivery, with inherent adjuvant properties. We expect that our findings will open new perspectives in the use of well-defined carbohydrates as vaccine adjuvants for targeted delivery of protein or polysaccharide antigens to dendritic cells helping to establish intradermal delivery as a safe vaccination route.

Acknowledgments

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Supplementary data

- ***Hexa-specific IgG titers***

Introduction

In the present study the induction of anti β-glucan IgGs were considered in order to evaluate the immunogenity against the hexasaccharide.

Materials and Methods

Immunization study

See above manuscript

Determination of anti-MenC antibody titers by ELISA

See above manuscript

Results and conclusions

Anti β-glucan IgGs were detected after each immunization for the conjugated hexasaccharide. A simultaneous behavior as both antigen and adjuvant can be associated to the hexasaccharide. For more details see above manuscript.

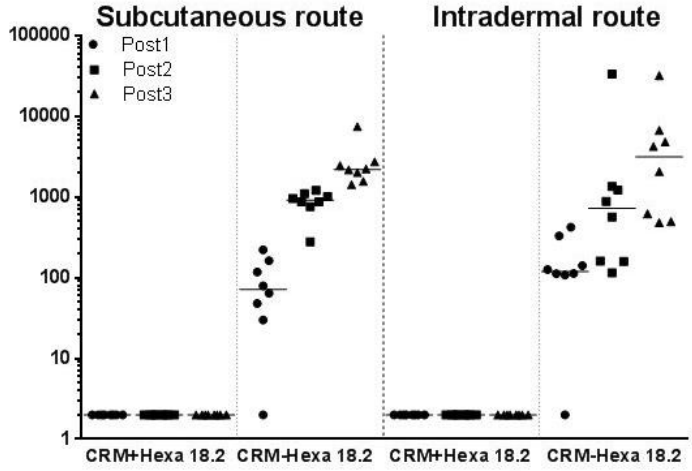

Figure 7| Systemic humoral responses to s.c. and i.d. administration of Hexa conjugated to CRM_{197} protein. *The immunization dose was based on protein content (4 µg). Hexa-specific IgG titers were analyzed after first, second and third immunization. Medians of Hexa-specific IgG titers for s.c. and i.d. immunizations are reported. Unconjugated glucan hexamer was used as coating reagent.*

Chapter 5

Conclusions

Conclusions

The current work is aimed at the design, formulation and characterization of novel vaccines suitable for intradermal delivery and able to induce earlier onset of immune response as well as more potent and longer lasting immune responses compared to conventional vaccines. With this purpose, two different model antigens were used to evaluate the intradermal route as immunological target for vaccination: MenC-CRM_{197} glycoconjugate and CRM_{197} protein. Moreover different adjuvants were used to potentiate the immune responses to antigens and to modulate the immune response towards a desired quality profile. Conjugation of adjuvants to model antigens was investigated for potential to increase antigen immune response when compared to co-delivery of adjuvants and antigens by simple mixture in respective formulations. Chapter 2 described the evaluation of the intradermal delivery route for the administration of a glycoconjugate vaccine and the use of immunopotentiators to further enhance the immunogenicity and to increase bactericidal activity after intradermal delivery. Notably, the intradermal delivery of MenC-CRM_{197} glycoconjugate showed superiority in term of immunogenicity and bactericidal titer compared to the intramuscular administration, highlighting the power of intradermal route for glycoconjugate vaccine delivery. Moreover, the addition of adjuvants, in particular TLR4a, LTK63 and TLR7a, allowed for a reduced number of doses administered and to modulate the quality of the immune response towards a more beneficial, for the model antigen used, Th1 response. The improvement of immunogenicity was not appreciable when CRM_{197} carrier protein was intradermally administered. This finding has specified the rational of choosing a glycoconjugate antigen to exploit the intradermal route advantages in term of immunogenicity.

To further investigate the role of adjuvant and to ensure codelivery of adjuvant/antigen, the conjugation of TLR7a to the glycoconjugate model antigen has been investigated in chapter 3. Of the two different conjugation approaches developed, the conjugation of TLR7a to CRM_{197} carrier protein rresulted the only one able to enhance the anti-carbohydrate response. In fact, the conjugation of

TLR7a to MenC polysaccharide, has showed a negative impact on MenC polysaccharide receptor recognition and consequently a reduction of the anti-carbohydrate response. These findings have opened the path to the rational design of improved adjuvanted glycoconjugate vaccines for intradermal route.

In order to enhance the immune response toward a protein model antigen in comparison to the glycoconjugate model antigen, as described in chapter 2, the conjugation of β-glucan hexasaccharide to the CRM_{197} model antigen was presented in chapter 4. The results have demonstrated that receptor activation was significantly impacted by the presentation of the glucan conjugated to the protein carrier. Considering that glycoconjugate vaccines are some of the safest and most effective vaccines for reducing, even eradicating, infectious diseases, the conjugation of well-defined synthetic glucans could represent a useful strategy for developing new glycoconjugate vaccines for intradermal delivery, with inherent adjuvant properties.

The entire picture of reported findings highligthed the importance of the rational combination of antigens and adjuvants to develop stable formulations and efficient immune responses considering the pathogen and the route of administration. For this reason, the formulation science has been proposed as key element of novel vaccine development suitable for intradermal delivery.

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