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THE IMMUNOGENICITY OF CHEMICALLY DERIVED ZWITTERIONIC POLYSACCHARIDES

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Index

Abstract 2

1 Introduction 4
1.1 Innate and adaptive immunity 4
1.2 Adjuvant and TLR 8
1.3 T-dependent and T-independent antigens 10
1.4 ZPS 13
1.5 GBS glycoconjugate vaccine 16

2 Aim of the study 19

3 Materials and Methods 21
3.1 *In vitro* 21
3.1.1 Purification of GBS capsular PS 21
3.1.2 Chemical modifications of GBS PS and conjugation to the carrier protein 23
3.1.3 Cell preparation and culture 24
3.1.4 Determination of cytokine and chemokine production 24
3.1.5 Plasmid production 26
3.1.6 HEK-293 stable transfectants 26
3.1.7 Proliferation assays 27
3.1.8 Enzymatic treatments 27
3.2 *In vivo* 28
3.2.1 Mice and Immunizations 28
3.2.2 Ag-specific T-cell cytokine responses 29
3.2.3 Determination of Ag-specific antibody by ELISA 29
3.2.4 Opsonophagocytosis assay 30
3.2.5 Statistical analysis 30

4 Results 31
4.1 ZPS immunogenicity in vitro 31
4.1.1 Chemical modifications of the native GBS PS 31
4.1.2 Chemically derived ZPS are able to activate APCs 33
4.1.3 The stimulatory activity of ZPS depends on the integrity of the zwitterionic motif and is not extracted by phenol 36
4.1.4 APC activation by ZPS is mediated by TLR2 39
4.1.5 ZPS induce T cells activation in APC - T cell co-culture 43
4.2 ZPS immunogenicity in vivo. 45
4.2.1 Chemical conjugation of the ZPS to carrier protein 45
4.2.2 ZPS-conjugates are more immunogenic than the corresponding PS-conjugates 46
4.2.3 ZPS-conjugates activate bone marrow derived DCs (BM-DCs) 54
4.2.4 ZPS-conjugates confer enhanced protection against GBS infection 56
4.2.5 TLR2 is critical for ZPS-conjugate adjuvant activity in vivo 59

5 Discussion 62
Acknoledgments 70
References 71

Abstract

Bacterial capsular polysaccharides (PS) which naturally contain zwitterionic charge motifs (ZPS) possess specific immunostimulatory activity, leading to direct activation of antigen-presenting cells (APCs) through Toll-like receptor 2 (TLR2) and of T cells in co-culture systems. When administered intraperitoneally, ZPS and bacteria expressing them are involved in the induction or regulation of T-cell dependent inflammatory processes such as intra-abdominal abscess formation. Moreover it has been published that ZPSs are processed to low molecular weight carbohydrates and presented to T cells through a pathway similar to that used for protein antigens. These findings were in contrast with the paradigm according to which polysaccharides are T-independent antigens unable to be presented in association with MHC class II molecules and unable to induce a protective immune response. For this reason in glycoconjugate vaccines polysaccharides often need to be conjugated to a carrier protein to induce protection. The aim of our work was to generate vaccine candidates with antigen and adjuvant properties in one molecule by the chemical introduction of a positive charge into naturally anionic PS from group B streptococcus (GBS). The resulting zwitterionic PS (ZPS) has the ability to activate human and mouse APCs, and in mixed co-cultures of monocytes and T cells, ZPS induce MHC II-dependent T-cell proliferation and up-regulation of activation markers. TLR2 transfectants show reporter gene transcription upon incubation with ZPS and these stimulatory qualities can be blocked by anti-TLR2 mAbs or by the destruction of the zwitterionic motif. However, in vivo, ZPS used alone as vaccine antigen failed to induce protection against GBS challenge, a

result which does not confirm the above mentioned postulate that ZPS are T-cell dependent Ags by virtue of their charge motif. Thus to make ZPS visible to the immune system we have conjugated ZPS with a carrier protein. ZPS-glycoconjugates induce higher T cell and Ab responses to carrier and PS, respectively, compared to control PS-glycoconjugates made with the native polysaccharide form. Moreover, protection of mothers or neonate offspring from lethal GBS challenge is better when mothers are immunized with ZPS-conjugates compared to immunization with PS-conjugates. In TLR2 knockout mice, ZPS-conjugates lose both their increased immunogenicity and protective effect after vaccination. When ZPS are co-administered as adjuvants with unconjugated tetanus toxoid (TT), they have the ability to increase the TT-specific antibody titer. In conclusion, glycoconjugates containing ZPS are potent vaccines. They target Ag to TLR2-expressing APCs and activate these APCs, leading to better T cell priming and ultimately to higher protective Ab titers. Thus, rational chemical design can generate potent novel PS-adjuvants with wide application, including glycoconjugates and co-administration with unrelated protein Ags.

1 Introduction

1.1 Innate and adaptive immunity

The mammalian immune system is comprised of two branches: innate and adaptive. The innate immune system is the first line of host defense against pathogens and is mediated, among others, by phagocytes including macrophages and dendritic cells (DCs). The adaptive immune system is involved in elimination of pathogens in the late phase of infection as well as the generation of immunological memory. The adaptive immune system detects non-self through recognition of antigens using antigen receptors expressed on the surface of B and T cells. In order to respond to a wide range of potential antigens, B and T cells rearrange their immunoglobulin and T cell receptor genes to generate over 10^{11} different species of antigen receptors. Engagement of antigen receptors by the cognate antigen triggers clonal expansion of the T lymphocyte that produces cytokines and gives help to the B lymphocyte in the production of antigen-specific antibodies. The innate immune response is not completely nonspecific, as was originally thought, but rather is able to discriminate between self and a variety of pathogens. The innate immune system recognizes microorganisms via a limited number of germline-encoded pattern- recognition receptors (PRRs). This is in contrast to the large repertoire of rearranged receptors utilized by the adaptive system (1). A class of PRRs called Toll-like receptors (TLRs) has the ability to recognize pathogens or pathogen-derived products and initiate signaling events leading to activation of innate host defenses. The subfamily of TLR1, TLR2, and TLR6 recognizes

lipopeptides, whereas TLR3, TLR7, TLR8, and TLR9, a group of tightly related TLRs, recognize nucleic acids. However, TLRs are unusual in that some can recognize several structurally unrelated ligands. TLRs are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells. Expression of TLRs is not static but rather is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stresses. Furthermore, TLRs may be expressed extra- or intracellularly. While certain TLRs (TLRs 1, 2, 4, 5, and 6) are expressed on the cell surface, others (TLRs 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes, and their ligands, mainly nucleic acids, require internalization to the endosome before signaling is possible. The transmembrane and membrane-proximal regions (TIR-domain) are important for the cellular compartmentalization of these receptors. Stimulation with their ligands recruits TIR-domain-containing adaptors including MyD88 to the receptor, leading to the formation of a complex of IRAKs, TRAF6, and IRF-5. This process induces the activation of NF-kB, a transcriptional factor, that translocates into the nucleus and initiates the expression of proinflammatory cytokine genes. Thus signaling by TLRs initiates acute inflammatory responses by induction of antimicrobial genes and inflammatory cytokines and chemokines. Subsequent events, such as recruitment of neutrophils and activation of macrophages, lead to direct killing of the microbes (2). The notion of TLRs being primary sensors of pathogens and responsible for orchestrating the innate responses is now widely accepted. In addition, there is accumulating evidence that TLRs contribute

significantly to activation of adaptive immune responses. Although T and B cells of the adaptive immune system express receptors of enormous diversity, activation of these cells depends on induction of co-stimulatory molecules and secretion of cytokines and chemokines by the cells of the innate immune system. In fact efficient priming of adaptive immune responses requires not only the presentation of antigen in the context of major histocompatibility complex (MHC) but also the induction of accessory signals (costimulators and cytokines) on antigen-presenting cells (APCs). TLRs expressed on APCs may regulate these accessory signals through their recognition of PAMPs and consequently control activation of antigen-specific adaptive immune responses (3, 4). Thus, we consider the innate and adaptive immune responses to be integrated in the vertebrate host as a single immune system, with the innate response preceding, and being necessary for, the adaptive immune response (Figure A) (5, 6).

Because of their capacity to bridge innate and adaptive immunity, Toll-like receptors (TLRs) have offered new opportunities for the development of immunostimulatory adjuvants (7, 8).

Figure A : TLRs link innate and adaptive immunity

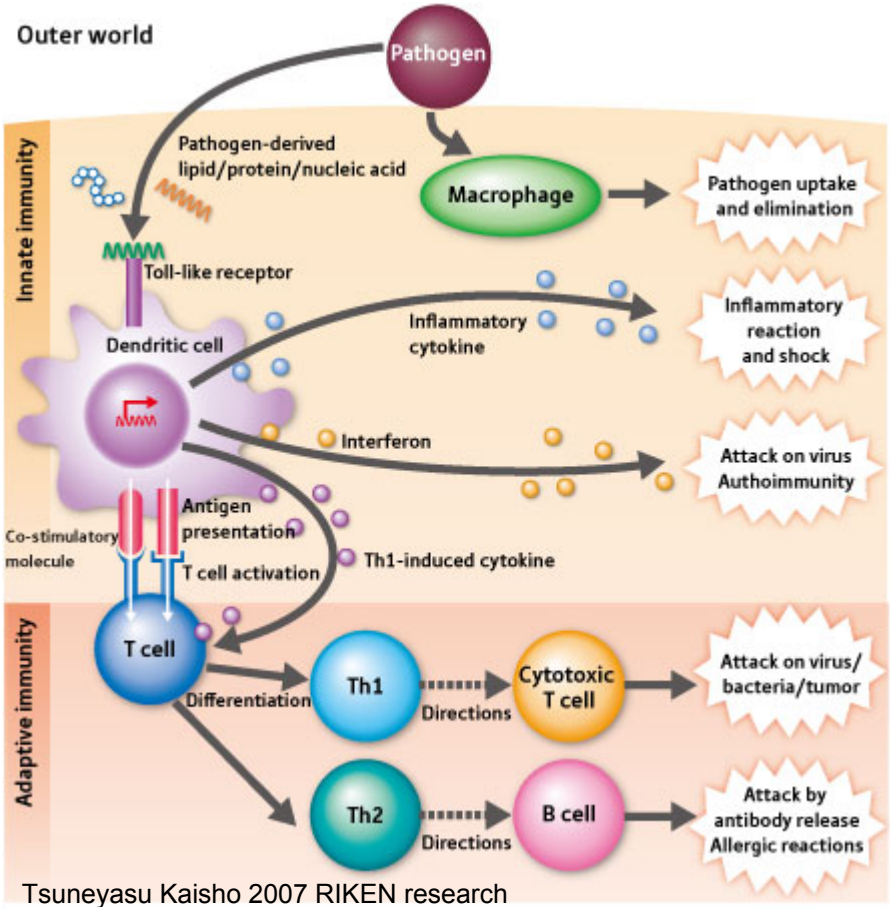


FIGURE A. APCs like DCs recognize pathogens by Toll-like receptors and produce a wide variety of cytokines. Cytokines serve to eliminate pathogens on one hand, and activate adaptive immunity on the other. Even when the same type of Toll-like receptor is involved, different signals are transmitted to cause different immune reactions depending on the type of dendritic cell in which the receptor is expressed.

1.2 Adjuvant and TLR

Adjuvants are molecules, compounds or macromolecular complexes that increase the potency and longevity of specific immune response to antigens, but cause minimal toxicity or long lasting immune effects on their own (9). The addition of adjuvants to vaccines enhances, sustains and directs the immunogenicity of antigens, effectively modulating appropriate immune responses, reducing the amount of antigen or number of immunizations required and improving the efficacy of vaccines in newborns, elderly or immuno compromised individuals (10). Traditional live vaccines based on attenuated pathogens typically do not require the addition of adjuvants. Likewise, vaccines based on inactivated viruses or bacteria are often sufficiently immunogenic without added adjuvants, although some of these can be formulated with adjuvants to further enhance the immune responses. By contrast, protein-based vaccines, although offering considerable advantages over traditional vaccines in terms of safety and cost of production, in most cases have limited immunogenicity and require the addition of adjuvants to induce a protective and long-lasting immune response. Adjuvants can be classified according to their component sources, physiochemical properties or mechanisms of action. Two classes of adjuvants commonly found in modern vaccines include: immunostimulants that directly act on the immune system to increase responses to antigens (Examples include: TLR ligands, cytokines, saponins and bacterial exotoxins that stimulate immune responses) and vehicles that present vaccine antigens to the immune system in an optimal manner, including controlled release and depot delivery systems, to increase the specific immune response to the

antigen. The vehicle can also serve to deliver the immunostimulants described in the previous point (Examples include: mineral salts, emulsions, liposomes, virosomes, biodegradable polymer, microspheres).

With few exceptions, aluminum salts (alum) are currently the only vaccine adjuvant approved for human use worldwide. Alum is effective at generating a strong antibody response to an antigen with a bias towards a Th2 type of immune response, and, as such, has been widely and effectively used in many vaccines, such as tetanus, diphtheria, pertussis and poliomyelitis vaccines (11, 12). The mechanism of immunopotentiation by alum involves inflammation and recruitment of antigen-presenting cells, retention of antigen at the injection site, uptake of antigen, dendritic cell maturation, T-cell activation and T-cell differentiation (13). Although alum adjuvants have proved their efficiency in a large number of applications, some limitations of alum have been reported. Thus, alum failed to confer satisfactory increase of the immune response in certain vaccines, such as typhoid fever and influenza vaccines. Reports have also demonstrated that alum displays limited ability to raise high antibody titers against small-size peptides. This calls for rational design of novel vaccine adjuvants that can establish protective immunity against different diseases. Advances in the design of efficient adjuvants based on the use of TLR agonists have been promising (although it should be noted that some of these were in development before the role of TLRs was identified) and some of these have reached advanced human trials and even registration. Thus we studied the chemical structure of TLR agonists in literature to

understand if we could find a new adjuvant, TLR agonist among in house compounds.

A very recent publication shows that the natural zwitterionic polysaccharide A (PSA) is a TLR2 agonist and able to activate a number of different APCs (14).

In house we had many polysaccharides extracted from the capsule of different bacteria used to make vaccine. It has been demonstrated that the induction of antibodies specific to the capsular PS confers protection against infection. This is the reason why a number of vaccines against bacterial infections aim exclusively at the induction of antibodies against capsular polysaccharides.

1.3 T-dependent and T-independent Antigens

Polysaccharide antigens are large molecules consisting of repeating epitopes which are not processed by antigen-presenting cells (APC) but interact directly with B cells, inducing antibody synthesis without the need of T cell help or in the absence of T cell help. In addition, pure PS do not contain structures that are assumed visible to T cells, since the antigen receptor of T cells (TCR) recognizes peptide fragments derived from protein antigens. This is why PS from bacteria are considered to be T-independent Ags (15) able to activate B cells without a contribution of help by CD4+ T cells. T-dependent antigens are proteins or peptides that require immune stimulation from helper T cells to elicit an immune response. Such antigens are presented to T cells in the context of MHC molecules on macrophages, B cells or dendritic cells following bacterial or viral infection. The subsequent activation of T cells induces cytokine production and a range of

immunologic effects. TD antigens are effective at inducing a lasting immune response, forming memory B and T cells, and producing high affinity antibodies of multiple isotypes. In contrast, T-independent responses are restricted in a number of ways. Most importantly, they fail to induce significant and sustained amounts of antibody in young children below the age of 18 months. While polysaccharides are immunogenic in older children and adults, the characteristics of the antibody responses are rather restricted. They are dominated by IgM and IgG2, are relatively short lived, and a booster response cannot be elicited on repeated exposure. This failure to induce immunological memory is also reflected in the absence of demonstrable affinity maturation. In contrast to polysaccharides, antibody responses to protein antigens have an absolute requirement for T cells. The consequence of this T cell help is that antibody responses to protein antigens can be elicited in the very young and immunity is long lived due the generation of immunological memory. Antibody responses to protein antigens are dominated by the IgG1 and IgG3 subclasses and affinity maturation can be demonstrated over time.

The ability to enhance the immunogenicity of polysaccharide antigens was first noted by Avery & Goebel in 1929, who demonstrated that the poor immunogenicity of purified S. pneumoniae type 3 polysaccharide in rabbits could be enhanced by conjugation of the polysaccharide to a protein carrier (16). Their observations have formed the foundation for the modern development of conjugate vaccines. It is hypothesized that PS-specific B cells internalize the PS-carrier complex. Proteolysis of the carrier protein produces peptides that bind to class II MHC

molecules and activate helper T cells (17). As a result, PS-specific B cells can then mature to antibody producing plasma cells or into memory cells (18-20).

The carrier protein provides T cell help and consequently immunological memory (21). The precise nature of the molecular events that permit polysaccharides conjugated to protein carriers to be processed as T-dependent antigens remains unclear and more research is required. In contrast to this paradigm, natural ZPS such as PSA may have the characteristics of a T-dependent antigen, despite the lack of a protein component. It has been published that ZPSs are processed to low molecular weight carbohydrates and presented to T cells through the MHCII endocytic pathway. Furthermore these carbohydrates bind to MHCII inside APCs for presentation to T cells (22). Therefore ZPS should not need the conjugation to a carrier protein to induce an immune response (Figure B).

Figure B: PSA mechanism of action as TLR2 agonist and

as ZPS T-dependent antigen.

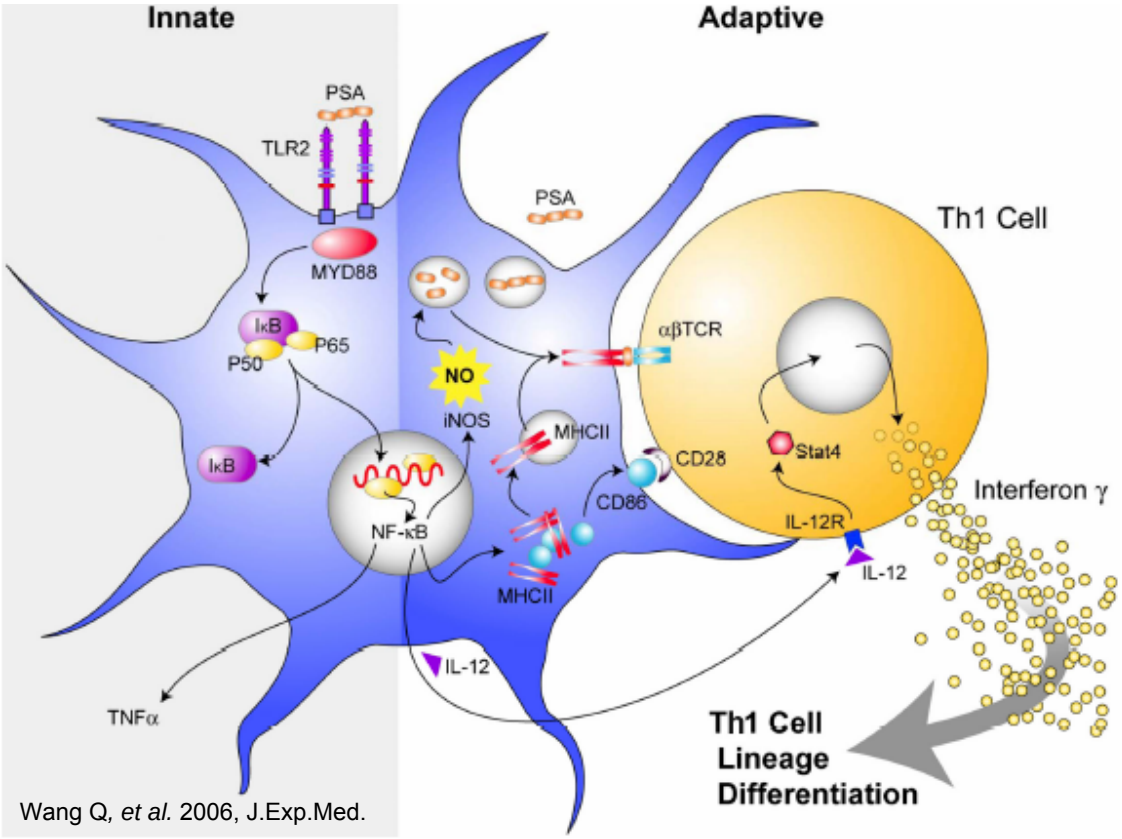


FIGURE B: The innate response begins with TLR2 recognition of PSA and the subsequent stimulation of the MyD88-mediated pathway inside the APC. Meanwhile, NF-κB translocation also leads to up-regulation of MHCII and CD86, thus facilitating PSA processing and presentation by MHCII proteins. Presentation of PSA on the cell surface by MHCII leads to adaptive CD4+ T cell activation and T cell secretion of cytokines like IFN-γ.

1.4 ZPS

PSA is a capsular polysaccharide (PS) from Bacteroides fragilis which naturally contains both positive and negative charges in its repeating structure, thus it is a zwitterionic polysaccharide (ZPS). A number of publications have shown in the past that PSA or the ZPS extracted from the capsule of Staphylococcus aureus and type 1 Streptococcus pneumoniae (Sp1) are able to activate T cells and APCs (23-25). The structures of the natural ZPS, PSA and Sp1, have been resolved (26, 27) (Figure C).

The initial findings demonstrated that abscess formation in a rat model was induced by PS containing zwitterionic charge motives (28) and that abscess formation was T cell dependent and transferable with ZPS-activated T cells (29, 30).

The integrity of the zwitterionic motif was essential for this biological activity, as removal of one of the two charges also removed the ability to induce abscesses (28). In vitro experiments with unfractionated splenocytes or with mixed populations showed that natural ZPS were able to induce T cell activation in these conditions, while the co-culture of fixed APCs with T cells was not sufficient to induce proliferation (31). The alternative, but not mutually exclusive hypotheses to explain these findings are that ZPS require processing to activate T cells directly through TCR recognition of MHC class II –ZPS complexes (32) or that ZPS activate APCs to up-regulate MHC class II, co-stimulatory molecules and cytokines and thus generate conditions that favor the activation of T cells (31). MHC class II

blocking antibodies inhibit T cell activation, and up-regulation on APCs of a number of molecules involved in T cell activation has been demonstrated (25).

To summarize, ZPS seem to be not only TLR2 agonists but also a T-dependent antigens. Thus, ZPS could be a perfect vaccine with the antigen and the adjuvant in a single molecule. Thus we explored the structure of in-house better available pure bacterial capsular polysaccharides and found that the polysaccharide extracted from Group B Streptococcus (GBS) that is naturally anionic could become zwitterionic through a chemical modification. Based on the results cited above, we hypothesized that ZPS obtained in this way should perform well as an efficient vaccine against GBS and may be used as alternative to the already existing glycoconjugate vaccine.

Figure C: PSA and Sp1 ZPS structure.

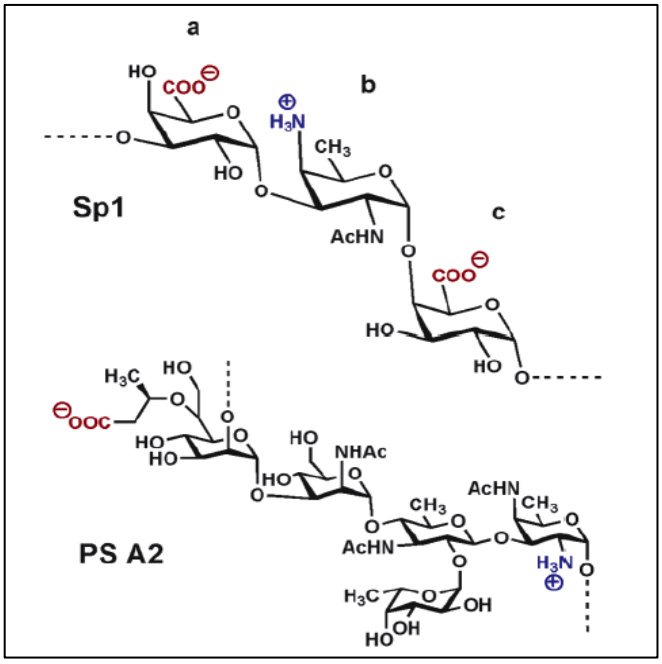


FIGURE C. Sp1 and PSA consist of a trisaccharide and a tetrasaccharide with free amino and carboxyl groups that confer zwitterionic characteristics to these polymers.

1.5 GBS Glycoconjugate vaccine

GBS is the foremost cause of life-threatening bacterial infections in newborns (33). In about 80% of cases, neonatal GBS infection is acquired during delivery by direct mother-to-baby transmission of the pathogen, which colonizes the anogenital

mucosa of 25 to 40% of healthy women (34). Despite the introduction of intrapartum antibiotic prophylaxis, in the United States GBS still causes 2500 cases of infection and 100 deaths annually among newborns in the first 3 months of life. About half of these cases occur in the first week after birth. Thus, it is commonly believed that effective vaccination will be the only way to reduce the incidence of GBS disease over the long term. GBS bacteria are encapsulated by complex branched polysaccharides, and variations in these sequences correspond to strain classifications. Each capsular polysaccharide (CPS) is derived generally from the same set of glycosyl residues; structural and immunological diversity arising from differences in linkage position and anomeric configuration (35). At present, nine serotypes of GBS (Ia, Ib, II–VIII) have been identified containing various arrangements of galactose, glucose, GlcNAc, and the most prevalent sialic acid (Sia) of humans, Nacetylneuraminic acid (Neu5Ac) (8–14). Neu5Ac residues of the GBS CPS are situated on the branching terminus of each repeating unit. These CPS are naturally anionic, as the otherwise neutral sugar backbone carries anionic groups such as carboxyls which are present as carboxylate ions at physiological pH.

The rationale for GBS vaccine development is supported by the observation that the risk of neonatal infection is inversely proportional to the maternal amounts of specific antibodies to the capsular polysaccharide (CPS) antigen that surrounds GBS (36, 37), implying that protective immunoglobulin G (IgG) antibodies are transferred from the mother to the baby through the placenta.

A glycoconjugate vaccine against GBS is currently being developed. This vaccine has been shown to confer serotype specific protection in mice and have been tested in clinical trials. In humans, addition of alum to the vaccine formulation did not further increase the immune response induced (38). In contrast, in animal models, adsorption to Al(OH)3 (Alum) enhances the immunogenicity of the glycoconjugate (39, 40), which may be explained by the possibility that animals are more naïve to GBS than humans. More generally, a number of commercially available glycoconjugate vaccines such as that against Meningococcus C, Haemophilus influenzae and the seven-valent Pneumococcus vaccine use Alum or aluminium phosphate as an adjuvant. Thus, it appears that the combination of glycoconjugates with adjuvants likely generates potent vaccines, able to activate APCs and induce strong Ag-specific T and B cell responses.

2 Aim of the study

The overall purpose of this study is to add, through rational chemical modification, biological function to vaccine Ags.

It has been described in the literature that bacterial capsular polysaccharides (PS) which naturally contain zwitterionic charge motifs (ZPS) possess specific immunostimulatory activity, leading to direct activation of antigen-presenting cells (APCs) through Toll-like receptor 2 (TLR2) and of T cells in co-culture systems. When administered intraperitoneally, ZPS and bacteria expressing them are involved in the induction or regulation of T-cell dependent inflammatory processes such as intra-abdominal abscess formation. Thus these natural polysaccharides have adjuvant properties, they are TLR-2 agonists, but they seem to be also T-dependent antigens. The majority of polysaccharides are naturally anionic and do not have these biological activities. To generate vaccine candidates with antigen and adjuvant properties in one molecule we have chemically introduced zwitterionic motifs into naturally anionic PS.

We chose the PS from type Ia, Ib and III of GBS, group B streptococcus. Positive charges were chemically introduced, and first of all the ability of these chemically derived ZPS to activate APCs and T cells were tested on a variety of human and mouse cell types. In a second step we asked if the in vitro activities of ZPS translate also into increased immunogenicity in vivo. Therefore, we tested the biological activities of ZPS in an animal model. The ZPS alone used as antigen to protect mice from GBS challenge showed little effect. This means that as T-dependent antigen the chemically modified ZPS was not efficient. Therefore, we

further esplored whether the ability to activate APCs shown in vitro translate into adjuvant activity in vivo. Thus, to render the ZPS visible to the immune system, we generated a glycoconjugate vaccine with ZPS in order to compare its immunogenicity with the glycoconjugate vaccine made with the native PS. The different glycoconjugates were injected into mice, and Ab titers, T cell responses, opsonophagocytosis and protection was measure. To test TLR dependence, immunogenicity and protection was compared in wt and TLR2 deficient mice.

This strategy may be applied to other polysaccharides and represents a new path for rational chemical design of novel adjuvants and glycoconjugate vaccines.

3 Materials and Methods

3.1 *In vitro*

3.1.1 Purification of GBS capsular PS

Capsular PS were prepared from *Streptococcus agalactie* bacteria using a modified version of the procedure previously published (41). Isolated PS were found to contain low concentrations of nucleic acids (<10 μg/mg), proteins (<10 μg/mg), Group B saccharides (<10 μg/mg), and LPS (< 0.001 UI/μg).

3.1.2 Chemical modifications of GBS PS and conjugation to the carrier protein

Cationic protonated forms of amines can be introduced into PS at (a) free N-acetyl groups, which are part of N-acetylneuraminic acid (NeuNAc) and Nacetylglucosamine residues, and at (b) the terminal aliphatic chain of NeuNAc (see Fig.1A). De-N-acetylation was achieved by basic hydrolysis (1 M NaOH for 60 min at 80°C) to make free amino groups available for further reaction (a in Fig.1A). Alternatively, chemical oxidation of the aliphatic chain from the terminal NeuNAc residue (b in Fig.1A) with sodium metaperiodate (Aldrich, 0.01 M NaIO4 for 90 min at room temperature) leaves an aldehyde group (42). Here, using NaIO4 as limiting (30%) or stoechiometric (100%) reagent of the reaction, the periodate oxidation selectively cleaves the C8-C9 bond between vicinal hydroxyl groups (-CHOHCH2OH) of NeuNAc residues, leaving an aldehyde group (-CHO) at C8. This group was converted to a cationic –NH3+ group by reductive amination using

300 mg/mL ammonium acetate (NH4Ac, Sigma) and 49 mg/mL sodium cyanoborohydride (NaBH3CN, Sigma) at pH 6.5 and T=37°C for 5 days (43). PS obtained by the reaction schemes a and b were treated with 37% formaldehyde (H2CO, Carlo Erba) in the presence of sodium cyanoborohydride in order to convert the generated free amino group to a tertiary dimethylamine such that it retained a positive charge (44). In order to remove the anionic charge on ZPS2 (Fig.1A, scheme c), the carboxyl group was reduced to alcoholic group by treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC – Sigma) and sodium borohydride (NaBH4, Aldrich). Where indicated, a phenol extraction of GBS PS was conducted as described by Sen et al. (45). As for the native PS, contamination with proteins, nucleic acids, and group B saccharide was also determined in all ZPS preparations and found to be below 10μg/mg. Since reagents used for the modifications contain undetectable level of these contaminants, the purity of ZPS products should be higher than the native PS due to additional purification steps performed after the chemical treatments.

The covalent attachment of the carrier protein CRM_{197} or HSA to the zwitterionic PS was performed according to the protocol used for the conjugation of the native CPS (41). The crucial step in the zwitterionization, first, and in the conjugation, in a second time, is the initial oxidation that has to occur only for a 10-30% of NeuNAc residues. Therefore, the final glycoconjugate has a 10-30% of NeuNAc residues modified with a positive charge, a 10-30% of NeuNAc residues implicated in the covalent binding with the carrier protein and the rest of NeuNAc residues unaltered. ZPS-conjugates were generated using ZPS from serotype Ib and V.

ZPS-conjugates were purified by gel filtration chromatography on the Sephacryl S-300 HR column. Polysaccharide content of ZPS and ZPS-conjugate preparations was estimated by the colorimetric detection of sialic acid residues with the Svennerholm method (46). The microBCA kit assay (Pierce) was used to estimate the protein content of ZPS-conjugate sample. The polysaccharide/protein ratio for all glycoconjugates used here was measured to be 1:1 (w:w).

Nuclear Magnetic Resonance (NMR) was used to assess first of all the structural identity of the purified PS and the chemically derived ZPS molecules then the structural identity of the native PS-conjugate and the chemically derived ZPS-conjugate. NMR spectra were recorded at 25°C on a Bruker DRX 600 MHz spectrometer using a 5-mm triple-resonance NMR probe (Bruker). For data acquisition and processing, XWINNMR 2.6 software package (Bruker) was used. NMR samples were prepared by dissolving lyophilized product in 0.75 ml of deuterium oxide (D_2O, Aldrich) to a uniform concentration and transferred to 5-mm NMR tubes (Wilmad). 1-D proton NMR spectra were collected using a standard one-pulse experiment and collecting 32 k data points over a spectral window of 6000 Hz. The complete relaxation of all nuclei was assured. The spectrum was Fourier-transformed after applying a 0.2 Hz line broadening function and referenced relative to mono-deuterated water (HDO) at 4.79 ppm.

3.1.3 Cell preparation and culture

PBMCs were collected by Ficoll Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation.from buffy coats of healthy donors who had given written informed consent Highly purified (>98%) monocytes were obtained from PBMCs by positive selection of CD14+ cells using anti-CD14 coated magnetic microbeads and MACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany).

Monocytes (2x105 per well) were cultured for 24h in RPMI 1640 (GIBCO Invitrogen) supplemented with Penicillin (100U/ml), Streptomycin (100 µg/ml), Glutamine (2mM) solution (Invitrogen Life Technologies) (RPMI-PSG) and 5% Human Serum (Sigma) using U-bottom 96-well plates. T cells (>98%) were prepared from PBMCs by MACS by negative selection using the Pan T Cell Isolation kit (Miltenyi Biotec). T cells (2x105 per well) were co-cultured with monocytes (1x105 per well) for 6 or 8 days in the same conditions as described above for monocytes.

Immature Mo-DCs were obtained culturing monocytes for 6 days in RPMI-PSG supplemented with 10% Fetal Calf Serum (Hyclone, Logan. Utah) (complete medium) with IL-4 (10% of supernatant from IL-4 secreting cell line, provided by A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland) and 50 ng/ml of GM-CSF (Gentaur, Brussels, Belgium). Immature dendritic cells were washed and cultured for the experiments in complete medium, using 96 well flatbottom cell culture plates. Human cells were stained with FITC-conjugated anti-CD14 or anti-CD83, PE-conjugated anti-CD80, allophycocyanin-conjugated anti-

CD86, PerCP-conjugated anti-HLA-DR (all Becton Dickinson). Rabbit serum was used as a blocking agent. After incubation for 20 min. on ice, cells were washed and analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

Mouse BM-DCs were generated culturing femoral bone marrow with recombinant murine GM-CSF (PeproTech) as described (47). At day 6, BM-DC were washed and cultured in complete medium with β-mercaptoethanol 50 μM (Sigma) and 100 U/ml mGM-CSF, using pro-bind U-bottom 96-well plates (Becton Dickinson).

Where indicated, cells were treated with LPS, macrophage-activating lipopeptide-2 (MALP-2) or N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysyl (Pam3CSK4) obtained from Alexis Biochemicals. The following purified mAbs were used in blocking experiments: anti-HLA-DR, DP, DQ (10 μg/ml) (BD Pharmingen) and anti-TLR2 clone T2.5 (50 μg/ml) (eBioscience). BM-DCs were stained with PE-conjugated anti-CD86, FITC-conjugated anti-MHC class II and allophycocyanin-conjugated anti-CD11c. Rabbit serum was used as a blocking agent. The acquisition was made on a LSR-II and data analyzed using DIVA software (BD).

3.1.4 Determination of cytokine and chemokine production

TNF-a production in culture supernatants was quantified by specific standard sandwich ELISA, using capture B154.9 and biotinylated B154.7 mouse monoclonal antibodies kindly provided by Dr. G. Trinchieri. Cytokine and chemokine secretion in supernatants was assessed by Bio-Plex analysis (Bio-Rad), according to the

manufacturer's instructions using the mouse 23-Plex panel. The following soluble proteins are assayed: IL-1α, IL1-β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, Macrophage Inflammatory Protein (MIP)-1α, MIP-1β, RANTES, TNF-α.

3.1.5 Plasmid production

A DNA fragment coding for human TLR2 with no signal peptide was obtained by RT-PCR from human PBMC cDNA using TLR2 specific primers and then cloned in the pFLAG-CMV-1 vector (Sigma) to attach an N-terminal FLAG epitope to TLR2; the FLAG-TLR2 sequence was then subcloned in pcDNA3 .1 (Invitrogen). The NF-κB-regulated promoter of the Igκ-luc plasmid (a gift of Dr. Antonio Leonardi, University of Naples, Italy) was subcloned in pd2EGFP-1 vector (Clontech) upstream to the EGFP coding sequence to obtain pNFkB-d2EGFP plasmid; the hygro gene with an upstream SV40 promoter, excised from the pTK-Hygro vector (Invitrogen), was then inserted in an un-influential region to obtain pNFkB-d2EGFP-Hygro.

3.1.6 HEK-293 stable transfectants

HEK-293 cells were grown in DMEM supplemented with glucose (4500g/l) glutamine (2mM), 10% FCS, penicillin and streptomycin. Cells were transfected using lipofectamine 2000 (Invitrogen) with the pcDNA-FLAG-TLR2 construct and a stable clone was derived by Geneticin (Invitrogen) selection. FLAG-TLR2 expression was verified by surface staining with FLAG-M2 monoclonal antibody

(Sigma) and FACS analysis. This clone was then transfected with pNFkB-d2EGFP-Hygro and a stable clone was derived by selection with Hygromycin B (Invitrogen). For experiments, cells were cultured for 24 hours with PS and controls, washed and analyzed by flow cytometry. The HEK-293 triple transfectants TLR4/MD-2/CD14 were obtained from Invivogen, and surface expression of CD14 was confirmed by FACS. In experiments comparing directly TLR2 and TLR4 agonist activity, the respective transfectant cell lines were incubated with PS for 20 hours, supernatant was obtained, and IL-8 content was determined using the FlexSet kit (Becton Dickinson) according to manufacturer's instructions. IL-8 produced by the transfectants was normalized to the amount of IL-8 present in the supernatant of the respective unstimulated transfectant line.

3.1.7 Proliferation assays

T cell proliferation was assessed by [3H] thymidine incorporation using 2x105 T cells and 1x105 γ-irradiated monocytes (3000 rad) per well in round-bottom 96-well plates. After 6 days of culture, cells were pulsed with 0,5 µCi/well of [3H] thymidine, incubated for 18 hours, harvested onto filter plates (Packard Instruments), and counts were analyzed using a Top Count NXT β counter (Packard Instruments).

3.1.8 Enzymatic treatments

Treatments were adapted from (Mattern 1999). Prior to addition to cells, ZPS and controls were incubated in PBS with 200 µg/ml lipoprotein lipase (LPL) from bovine milk or from pseudomonas sp. (Sigma-Aldrich) at 37°C for 7 hours. Results shown

were obtained using bovine milk-derived LPL. Alternatively, immobilized trypsin (Pierce) or Proteinase K-acrylic beads (Sigma-Aldrich) were incubated with samples at a 1:20 ratio at 37°C for 1 hour. Proteases were removed from the reaction by centrifugation. The enzymatic treatments were performed on ZPS and controls which were concentrated 10x compared to their final concentration in the in vitro experiments.

3.2 *In vivo*

3.2.1 Mice and Immunizations

Groups of 6-8 female 6-week-old Balb/C, C57BL/6, CD1 outbred mice (Charles River) or C57BL/6 TLR2$^{-/-}$ (48) (kindly provided by Giuseppe Teti, Messina, Italy) were used for experiments reviewed and approved by the institutional review committees. Animals were immunized intraperitoneally at days 0, 21 and 35 with 1 μg of glycoconjugates made as indicated. Where indicated, Alum was used at 0.4mg $AlOH_3$/dose. Serum and spleen samples were collected at 2 weeks following the third immunization. In adult mice, the challenges were performed injecting intraperitoneally strain H36B (serotype Ib) at 1 x 10^8 CFU at 2 weeks after the third immunization. For the neonatal challenge experiments, we first determined the 80% lethal doses (LD_{80}) by titration in both wt and ko mice. H36B was administered at 1 x LD_{80} to the pups subcutaneously between 24 and 48 h after birth. Mortality was recorded daily for the 2 days following challenge.

3.2.2 Ag-specific T-cell cytokine responses

Three mice per treatment were sacrificed, spleens were collected, and single cell suspensions were obtained. Red blood cells were lysed and splenocytes cultured in RPMI (Gibco) containing 2.5% FCS (Hyclone), beta-mercaptoethanol and antibiotics. Splenocytes were stimulated in the presence of anti-CD28 (1 µg/ml) (Becton–Dickinson [BD]) and the carrier protein CRM_{197} (30 µg/ml), or with anti-CD28 alone (unstimulated, <0.1% total cytokine-positive cells), or with anti-CD28 plus anti-CD3 (0.1 µg/ml) (BD). After 4 h of stimulation, Brefeldin A (2.5 µg/ml)(Sigma Aldrich) was added for additional 12 h. Cells were washed and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit for 405 nm excitation (Invitrogen). Cells were fixed, permeabilized and stained with the following mAbs: allophycocyanin-Alexa750-conjugated anti-CD4 (Caltag), Pacific Blue-conjugated anti-CD3, Alexa700-conjugated anti-TNF-α, Peridinin chlorophyll protein cyanine5.5-conjugated anti-IFNγ, PE-conjugated anti-IL-5, Alexa488-conjugated anti-IL-2 (BD). Cells were acquired on a LSR-II (BD) and analyzed using FlowJo software (Tree Star). For each individual mouse, percentages of unstimulated samples were subtracted from the Ag-stimulated sample.

3.2.3 Determination of Ag-specific antibody by ELISA

For titration of IgG specific for the native polysaccharides, Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 1 µg/ml (in PBS) of the glycoconjugate that

contains a different carrier protein to that used for the immunization, in order to detect only the antibodies specific for the polysaccharide. Antibody titers are those dilutions that gave an OD higher than the mean plus five times the SD of the average OD obtained in the pre-immune sera. The titers were normalized with respect to the reference serum assayed in parallel.

3.2.4 Opsonophagocytosis assay

Serum samples from mice immunized with serotype Ib glycoconjugates were tested for their in vitro ability to promote the opsonization of type Ib GBS strain H36B for phagocytosis and killing by differentiated HL60 cells in the presence of rabbit active complement. Results were expressed as the mean $\log_{10}$ reduction in GBS colony-forming units before and after 60 min of incubation at 37°C.

3.2.5 Statistical analysis

Statistical significance was determined using a two-tailed Student's T-test analysis or by Fisher's exact test. Significance was reconfirmed using nonparametric statistical analysis.

4 Results

4.1 ZPS immunogenicity in vitro

4.1.1 Chemical modifications of the native GBS PS

Figure 1A shows, as an example, the modifications introduced into the repeating unit of the capsular PS from GBS serotype III (GBS III). The same type of modifications was also introduced into GBS Ia and Ib. According to the annotations shown in Fig.1A, ZPS1 is the zwitterionic PS obtained from the chemical modification (a) that converts the N-acetyl groups to free amino groups. Chemical modification (b) generates the ZPS2 resulting from a periodate oxidation to generate an aldehyde group which is also converted to an amino group by a reductive amination reaction. Furthermore, all amino groups are converted to tertiary amines that retain their positive charges. Thus, in each repeating unit, ZPS1 molecules from all three serotypes contain two positive and one negative charge, while ZPS2 contain a balanced motif of one charge each. In order to remove the anionic charge from ZPS2, the carboxyl group of ZPS2 is reduced by a carbodiimide-mediated reaction with NaBH4 (modification c). All structural analyses of chemical modifications of the native PS were made using NMR spectroscopy (Fig.1B). In particular, the zwitterionic structure is confirmed by detecting the methyl group (CH3)2-N+H- which has been generated by the chemical modification scheme b (Fig.1A). All labeled signals have been assigned using bi-dimensional homo-nuclear (1H-1H) and hetero-nuclear 1(H-13C) NMR experiments (not shown). Additional saccharide contaminations (i.e. group B

polysaccharide) were also excluded by analyzing the NMR profiles. Only a little residual amount of ethanol used in the purification procedure has been detected.

Figure 1 : Chemical modifications of native GBS serotype III capsular PS A.

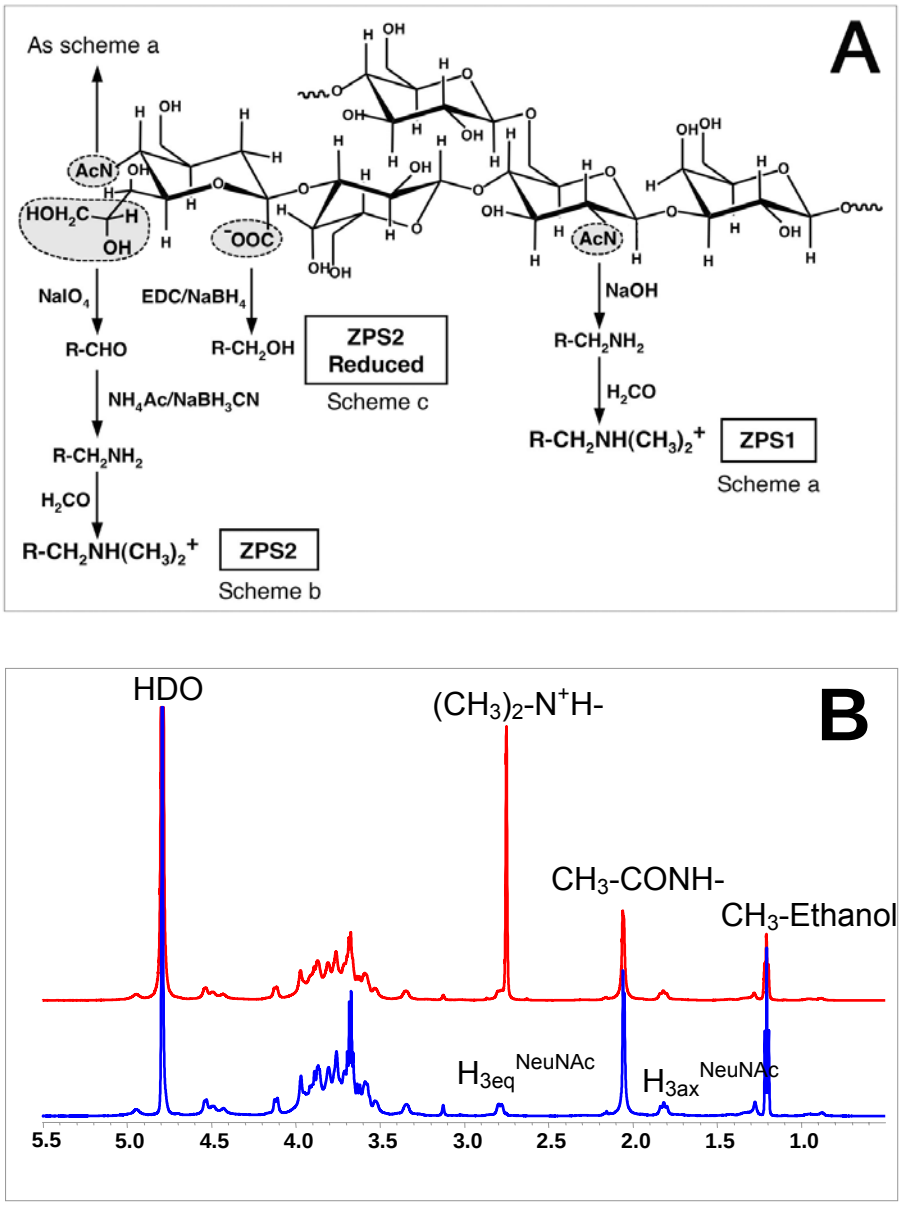


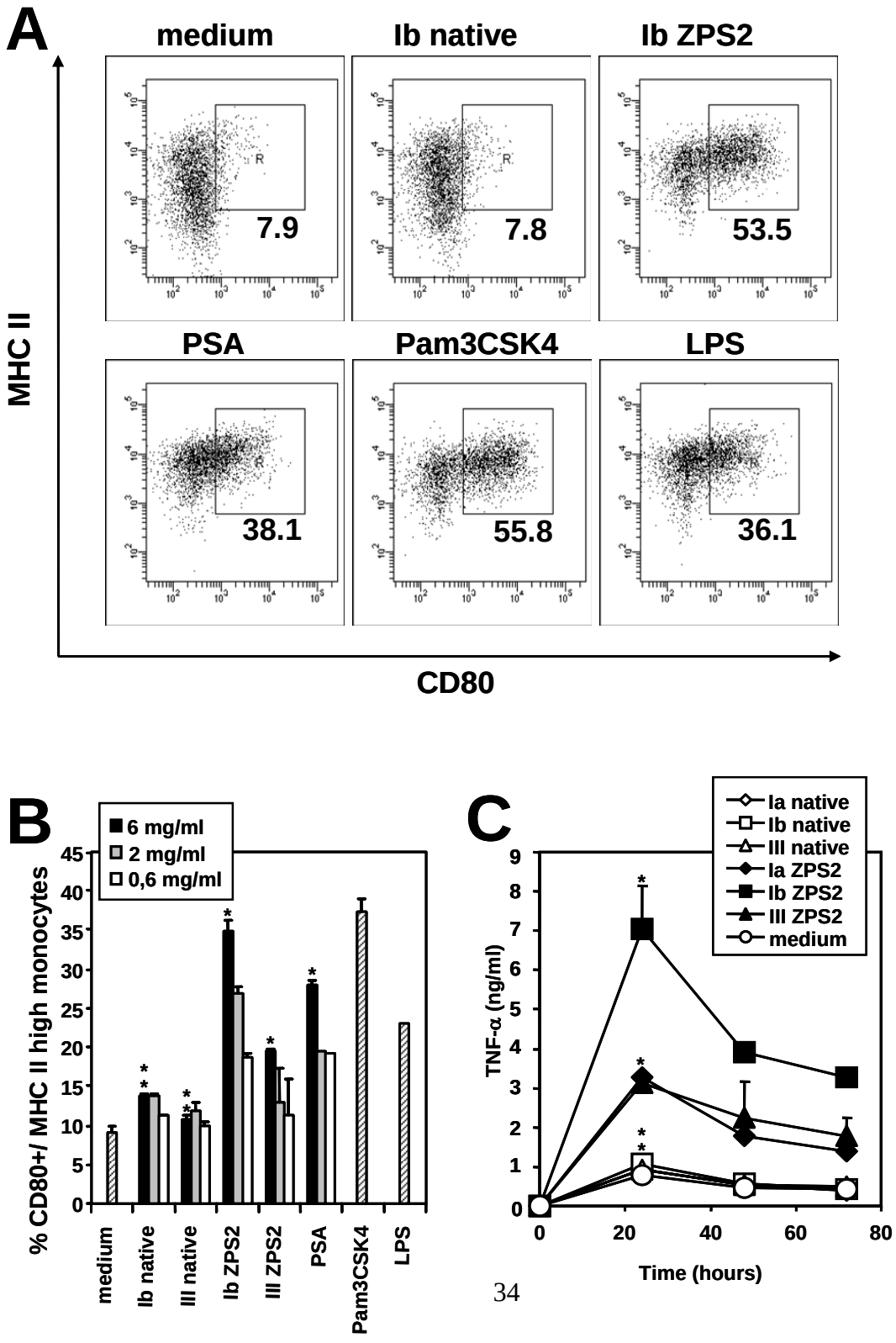
FIGURE 1. De-N-acetylation by basic treatment leading to ZPS1 (a), and periodate oxidation and reductive amination leading to ZPS2 (b), of one repeating unit of GBS serotype III PS. The additional reaction with formaldehyde to obtain tertiary amines is also shown. (c) Reduction of the carboxyl group leading to ZPS2 reduced. B, NMR spectra at 600 MHz and 25°C of the native form (top line) and the ZPS2 modification (bottom line) of serotype Ib PS from GBS. The peaks generated by the newly formed methyl group $(CH_3)_2$-N^+H- and by other groups are annotated.

4.1.2 Chemically derived ZPS are able to activate APCs

In order to test the ability of ZPS to activate APCs, purified human monocytes and Mo-DCs were incubated with either the natural ZPS purified from Bacteroides fragilis, the capsular PS A (PSA), or with the ZPS derived from GBS PS by the chemical modification a as indicated in figure 1A, leading to ZPS1, or by the modification b (ZPS2). The dot plots in figure 2A show that both PSA and the chemically derived ZPS2 are able to induce up-regulation of MHC class II and CD80 on human monocytes, while the native anionic GBS PS (Fig. 2A) or the ZPS1 derived by chemical modification a (not shown) do not activate monocytes. The dose response relation of this activation is shown in fig. 2B. Similarly, ZPS2 of all three GBS serotypes induce TNF-a production by purified human monocytes, while the native forms are inactive (Fig.2C). The amount of TNF-a induced in monocytes by PSA is comparable to that induced by serotype Ib ZPS2 (not shown). The production of TNFa is induced in human Mo-DC by the ZPS2 form but not the native or ZPS1 form of the GBS capsular PS, again indicating that the zwitterionic motif introduced is essential for the biological activities observed (Fig. 2D). Similar results were found for up-regulation of the maturation marker CD83 on human Mo-DCs (Fig. 3A). Finally, also mouse BM-DCs from Balb/C mice are activated by ZPS2 but not the native PS or ZPS1 (Fig. 2E). The dot plots and gates used for the determination of marker upregulation are shown in Fig. 2F. As previously reported (49), Balb/C mice are more responsive to TLR2 agonists than C57Bl/6 mice, and this is reflected in the stronger response of Balb/C BM-DCs to the positive control Pam3CSK4. In conclusion, these results indicate that the

chemical introduction of a zwitterionic charge motif into anionic PS confers the ability to activate a variety of human and mouse APCs.

Figure 2: ZPS activate human and mouse APCs.



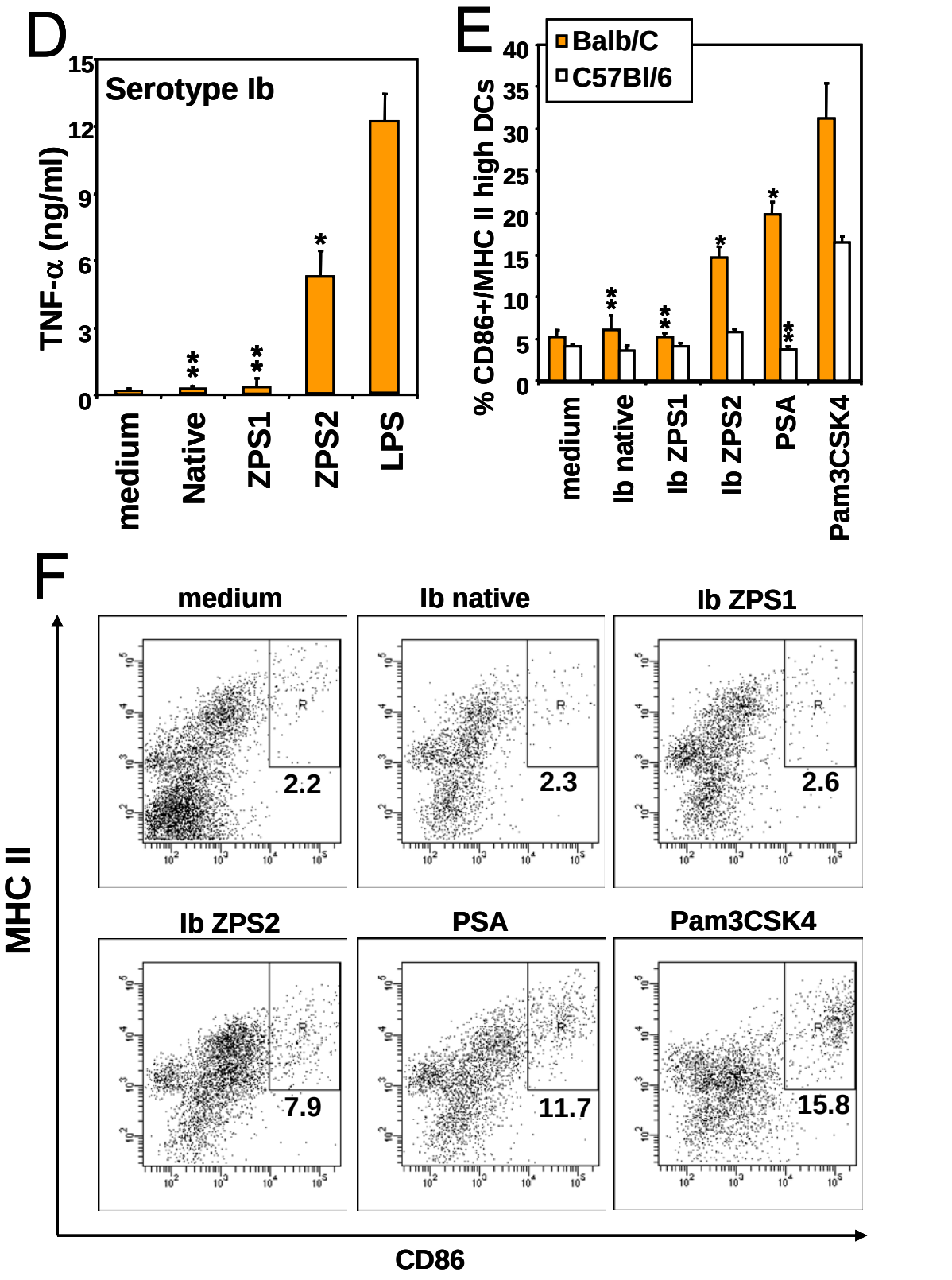


FIGURE 2. Human CD14+ monocytes (A,B,C), Mo-DCs (D) or mouse BM-DCs (E,F) were incubated with the indicated compounds for 24 hours (A,B,D,E,F) or as indicated (C), and up-regulation of the indicated surface markers was measured by flow cytometry (A,B,E,F) or TNF-α concentration in the culture supernatants was determined by ELISA (C,D). LPS 1μg/ml, Pam3CSK4 100 ng/ml; all PS in A,C-F 6μg/ml. Error bars indicate standard deviation of triplicate samples, results are representative of at least 3 experiments. *, $p<0.05$; **, $p>0.05$ compared to medium.

4.1.3 The stimulatory activity of ZPS depends on the integrity of the zwitterionic motif and is not extracted by phenol

The above results indicate that the chemical generation of ZPS leads to molecules with new biological activity. To confirm that the integrity of the zwitterionic motif is essential for the observed stimulatory abilities, we removed the negative charge from ZPS2 by modification C (see figure 1A) and tested whether the resulting cationic molecule can stimulate APCs. As shown in figure 3A, the ability to stimulate Mo-DCs disappears when the positive charge is removed from the molecules and thus the zwitterionic motif is destroyed. This result demonstrates that the zwitterionic motif is required for the ability of PS to stimulate APCs. A recent publication shows that the ability of Pneumococcus PS to induce strong immune responses in mice depends on associated TLR2 agonists which can be separated from the PS by phenol extraction (45). In order to ensure that the APC stimulatory abilities are not due to a lipophilic contamination, we subjected ZPS2 to phenol extraction and tested the residual biological activity. Figure 3B shows that the ability of ZPS2 to activate monocytes is not affected by phenol extraction. Similar results were obtained for the ability to stimulate Mo-DCs (not shown). In addition, limulus amoebocyte lysate tests performed on the native PS and on ZPS2 before and after phenol extraction resulted in extremely low endotoxin values, and no correlation was found between endotoxin content and the biological activity observed here (not shown). All subsequent experiments were done with ZPS2 that had undergone phenol extraction. As the native PS did not show any ability to activate APCs, it can be excluded that APC stimulation was due directly to

bacterial contaminants that remained after PS purification. In order to exclude also the possibility that contaminants were introduced during chemical modification, we subjected native PS to the whole chemical modification with the exception of the first step, thus not generating the ZPS molecule but allowing for all factors that may contribute to the introduction of contaminants (“no first step”). Fig. 3C shows that this preparation has no biological activity.

Figure 3: APC stimulatory capacity of ZPS depends on the zwitterionic motif and is not phenol extractable.

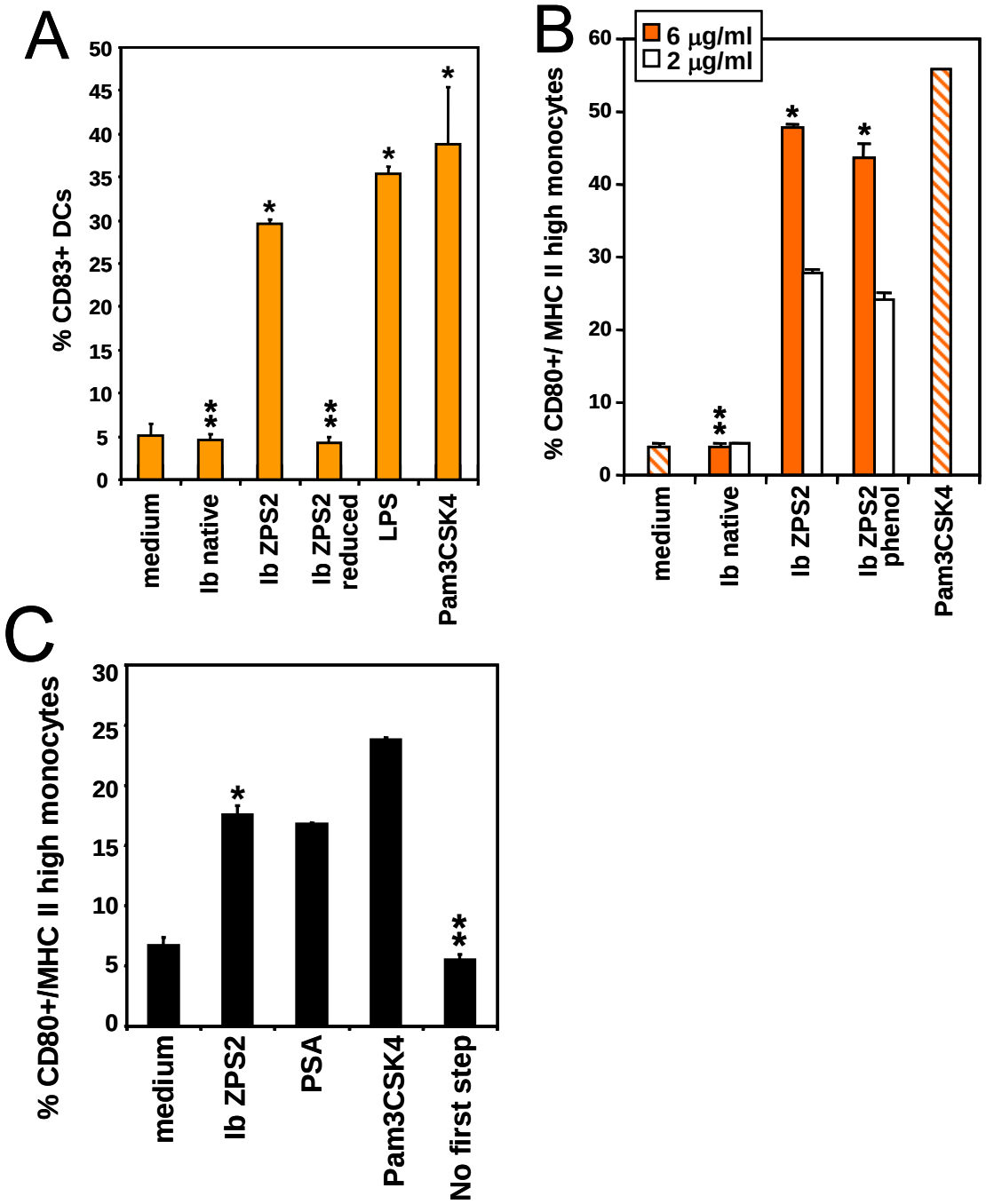


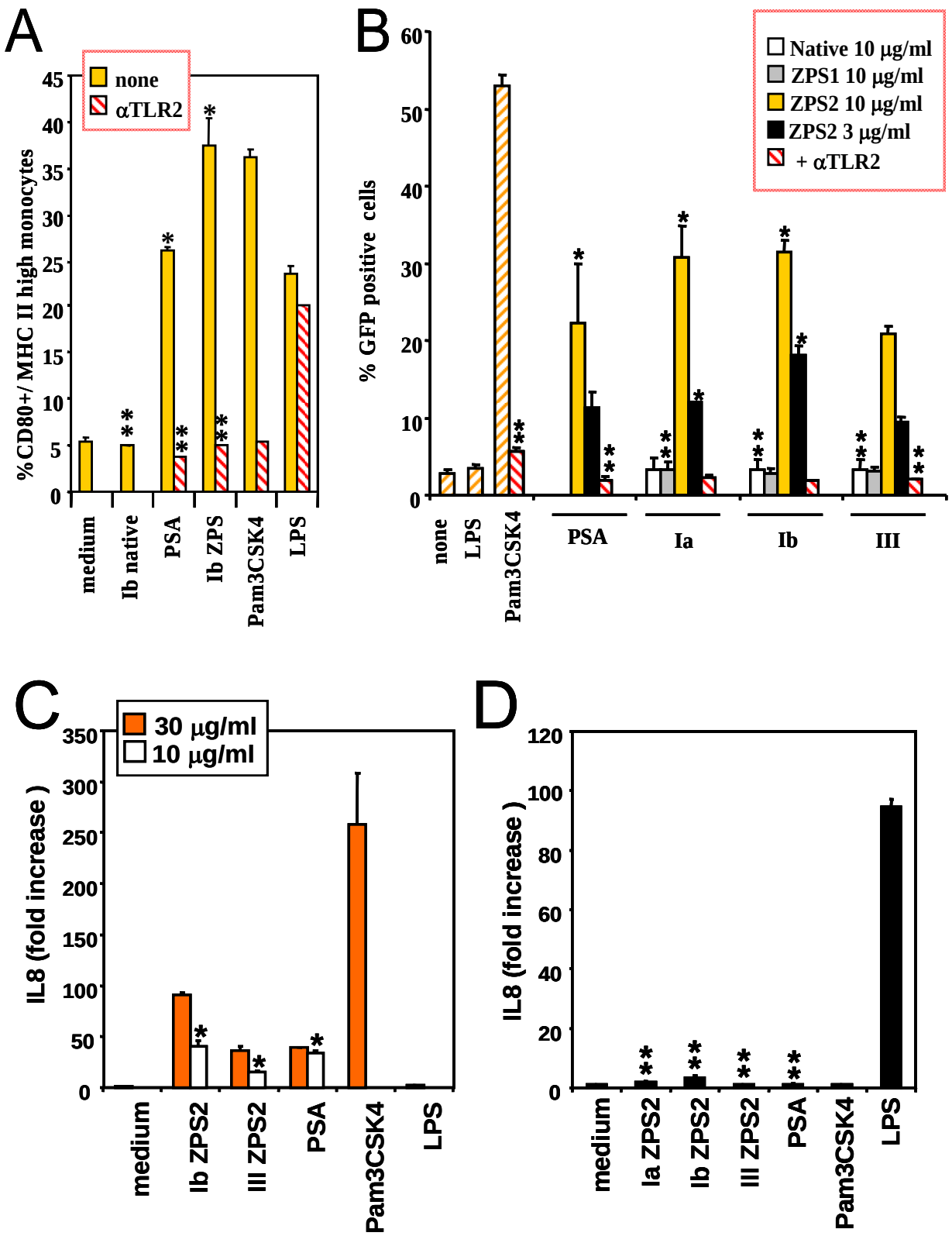
FIGURE 3. Human Mo-DCs (A) or purified monocytes (B,C) were incubated with the indicated compounds for 48 (A) or 24 (B,C) hours, and surface marker expression was measured by flow cytometry. (A) LPS 1µg/ml, Pam3CSK4 10 □g/ml; (B,C) LPS 1µg/ml, Pam3CSK4 100 ng/ml; All PS in A and C 6µg/ml. Error bars indicate standard deviation of triplicate culture, results are representative of at least 3 experiments. *, $p<0.05$; **, $p>0.05$ compared to medium.

4.1.4 APC activation by ZPS is mediated by TLR2

The results shown in figure 2E suggest an involvement of TLR2 in the APC activating properties of ZPS. In addition, it was recently reported that the natural ZPS, PSA, is able to activate APCs through TLR2 (14). To analyze in detail TLR2 involvement, we tested whether anti TLR2 blocking antibody can inhibit the observed effects. Figure 4A shows that anti-TLR2 mAbs block the induction on human monocytes of MHC II and co-stimulatory molecules by both the natural and the chemically derived ZPS. The canonical TLR2 agonist Pam3CSK4 induces the same effects on monocytes which can also be blocked by the anti-TLR2 mAb. This mAb did not block APC activation induced by TLR4 (Fig. 4A) or TLR7/8 agonists (not shown), thus confirming the specificity of the reagent. ZPS2- and PSA-induced TNF-a production by monocytes was also blocked by this mAbs (not shown). An isotype-matched control antibody was not able to block the effects described here (not shown). To reconfirm the specific interaction with TLR2, we used stable TLR2 transfectants and observed reporter gene transcription upon incubation with both natural and chemically derived ZPS but not with the native or the ZPS1 form of the GBS PS (Fig. 4B). GFP expression induced by Pam3CSK4 and the natural and chemically derived ZPS was blocked by the anti-TLR2 mAb, confirming the specificity of this induction. As a further reconfirmation of absence of LPS contamination or TLR4 agonist activity of the ZPS2, we performed in parallel experiments using TLR2 and TLR4/MD-2/CD14 transfectants and assayed for the same read out, namely IL-8 production (Fig. 4C, D). While all natural and chemically derived ZPS induce significant IL-8 production in TLR2 transfectants at

the two different doses tested, none of these molecules did so in the triple transfectants at any of the doses tested. Hence, this very sensitive assay confirms that TLR4-mediated activation does not play a significant role in the phenomena described here. TLR2 transfectants were also used to exclude a number of other contaminants (Fig. 4E, F). To exclude lipopeptide contaminants in the preparations, ZPS were treated with LPL. As shown in fig. 4F, pre-treatment with LPL does not reduce the TLR2 agonist activity of ZPS while Pam3CSK4 activity is greatly reduced. Similarly, pretreatment with proteases does not alter the biological activity of ZPS, while Pam3CSK4 activity is reduced to different degrees by these two proteases (Fig.4E). In conclusion, this set of experiments indicates that the biological activity of ZPS is mediated by TLR2 and that LPS, lipopeptide or protein contaminations do not play a role in this activity.

Figure 4: ZPS-induced APC activation is mediated by TLR2.



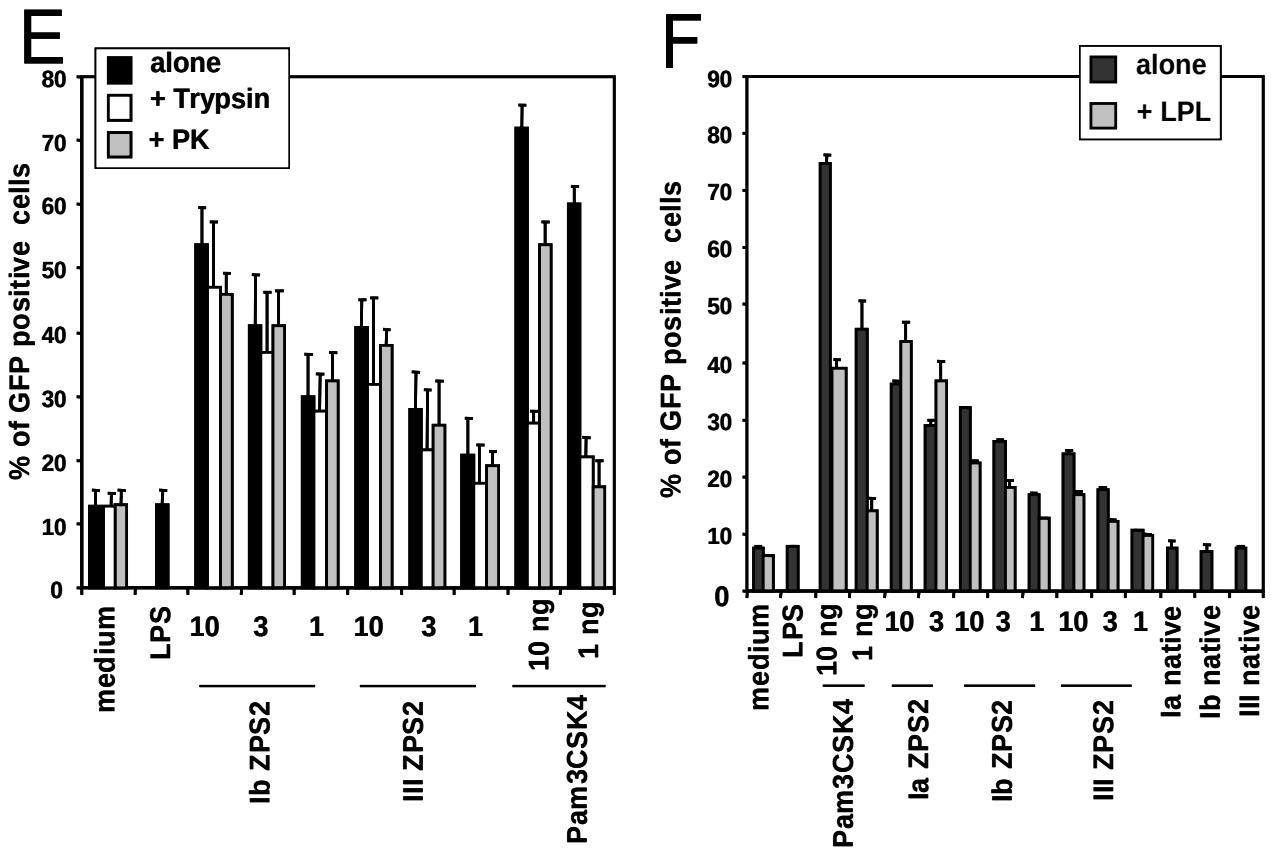


FIGURE 4. (A) Purified monocytes were incubated for 24 hours as indicated, and surface marker expression was measured on CD14+ gated cells by flow cytometry. (B,E,F) Stable transfectants for TLR2 and a GFP reporter gene under control of an NFkB-dependent promoter were incubated for 24 hours with the indicated compounds, and induction of GFP was measured by flow cytometry. (C-F) Stable transfectants for TLR2 (C,E,F) or TLR4/MD-2/CD14 (D) were incubated for 20 hours with the indicated compounds, and IL-8 concentration in the culture supernatants was measured. (A) LPS 1μg/ml, Pam3CSK4 100 ng/ml, PSA 20μg/ml, Ib native and ZPS2 6μg/ml, anti TLR2 50□g/ml. (B) LPS 1μg/ml, Pam3CSK4 10 ng/ml, anti TLR2 50□g/ml added to Pam3CSK4 10 ng/ml or to PSA or ZPS2 3□g/ml. (C,D) LPS 1μg/ml, Pam3CSK4 100 ng/ml, all ZPS in (D) 30 μg/ml. (E,F) LPS 1 □g/ml, native PS 10 □g/ml, ZPS as indicated (□g/ml). Error bars indicate standard deviation of triplicate culture, all experiments were performed at least 3 times with similar outcome. *, $p<0.05$; **, $p>0.05$ compared to medium/none.

4.1.5 ZPS induce T cells activation in APC - T cell co-culture

A series of previous publications show that natural ZPS can induce T cell proliferation in *in vitro* co-culture experiments (23, 24, 31, 44, 50). When co-

cultures of purified T cells and purified, γamma-irradiated syngeneic monocytes were incubated with PSA or ZPS2, both proliferation and up-regulation of activation markers on T cells were observed, while single populations of purified cells did not proliferate in the presence of PSA or ZPS2 (Fig. 5A and data not shown). Figure 5B shows that only ZPS2 but not ZPS1 are able to induce T cell proliferation. In order to test the relative contribution of MHC II and TLR2 to the T cell activation observed, blocking experiments with mAbs for both molecules were performed. Figure 5C shows that ZPS-induced T cell proliferation was consistently blocked by MHC II blocking mAbs, while anti TLR2 mAb significantly blocked proliferation induced by serotype Ib ZPS but not by serotype III ZPS or PSA. However, it is interesting to note that the all TLR agonists used as controls stimulate T cell proliferation that can be blocked by anti MHC II mAbs. As expected, LPS-induced T cell proliferation is not blocked by the TLR2 mAbs while TLR2 agonist-induced T cell proliferation is. In conclusion, these results confirm that the chemically derived ZPS show the same range of activities described for natural ZPS, and that APC activation is in some cases, but not always an essential component of the T cell stimulation induced by ZPS. It is also clear from these experiments that MHC II dependency of stimulation may not be sufficient to establish whether a molecule is an MHC II dependent T cell antigen or whether MHC II up regulation is part of the APC activation that eventually leads to T cell proliferation in this experimental set up.

Figure 5: T cells in co-culture with APCs are activated by ZPS.

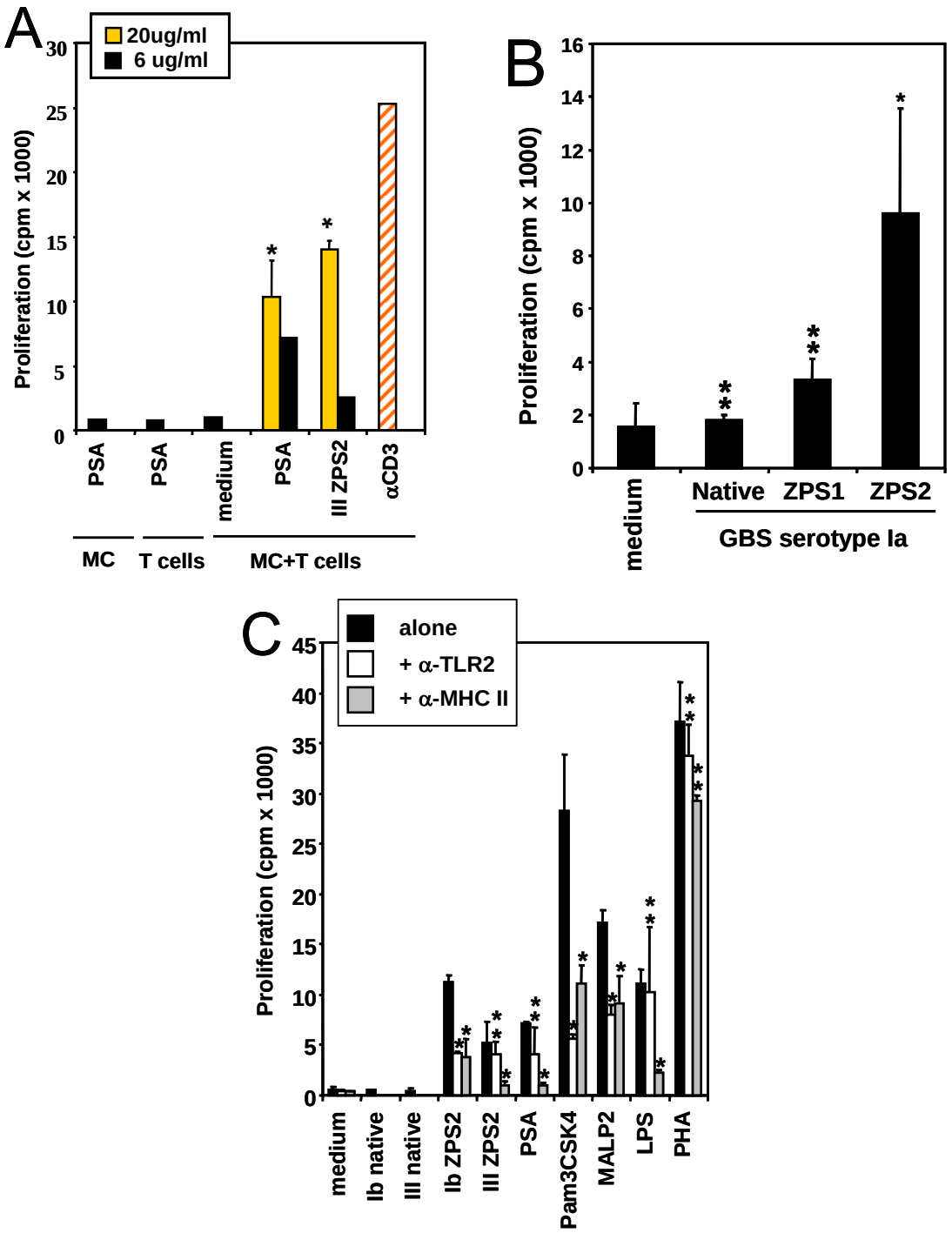


FIGURE 5. Purified T cells or pure, gamma-irradiated monocytes or both were incubated for 6 days with the indicated compounds, and proliferation was measured by thymidine incorporation. Where indicated, anti MHC class II (10□g/ml) or anti TLR2 (50□g/ml) mAbs were added throughout the culture. (B,C) All PS 6□g/ml. Error bars indicate standard deviation of triplicate culture, experiments were performed 3 times (A,B) or 5 times (C) with similar outcome. (A,B) *, $p<0.05$; **, $p>0.05$ compared to medium; (C) *, $p<0.05$; **, $p>0.05$ compared to the same treatment without blocking mAbs.

4.2 ZPS immunogenicity in vivo.

4.2.1 Chemical conjugation of the ZPS to carrier protein

Given the ability of chemically derived ZPS to activate APCs in vitro, we attempted to test whether this newly introduced biological activity would also confer adjuvant function in vivo. Since we demonstrated that the only ZPS having biological activity were that derived from the second chemical modification, we used only these to test their activity in vivo and for simplicity we called them ZPS and no longer ZPS2. We immunized mice with ZPS alone in order to see if they were T-dependent antigen as a protein Ag. After three immunizations, sera were tested in ELISAs using the native PS to coat the plate. By this method, we assessed for IgG antibody titers specific for the native PS, which is the relevant form present in the GBS capsule. ZPS used alone were not able to induce antibody titer against the native PS (data not shown). Thus the chemical modification was not able to turn the native polysaccharide in a T-dependent antigen. Therefore, to render ZPS visible to T cells, we conjugated ZPS with a carrier protein and compared them to the glycoconjugates containing the native form of the corresponding PS. The zwitterionic PS were obtained as previously described by the introduction of a positive charge in the aliphatic chain of the terminal N-acetylneuraminic acid (NeuNAc) residue. The covalent attachment of the carrier protein CRM_{197} or HSA to the zwitterionic PS was performed according to the protocol used for the conjugation of the native CPS (41). We conjugated ZPS to the widely used and highly immunogenic carrier protein called Cross Reactive Material of diphtheria toxoid (CRM_{197}) or to the protein human serum albumin (HSA). NMR spectra

confirmed the successful modification of the native serotype Ib polysaccharide which generated the zwitterionic motif (Fig. 6), and the integrity of Ib ZPS polysaccharide after conjugation to CRM_{197} (Fig. 6, Ib-ZPS-CRM) or HSA. The PS-to-protein ratio of all glycoconjugates is 1:1 (w:w).

Figure 6: Zwitterionic polysaccharide conjugation.

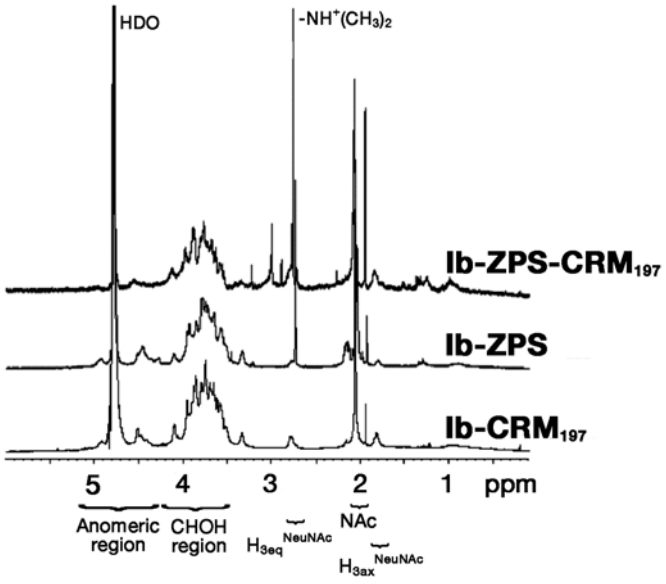


FIGURE 6. NMR 600 MHz spectra (spectral window from 0 to 6 ppm) recorded at 25°C of the Ib-CRM_{197} conjugate (bottom line), Ib-ZPS polysaccharide (central line) and the Ib-ZPS-CRM_{197} conjugate (top line). The peak of the methyl group $–NH^{+}(CH_3)_2$ which was chemically introduced in the ZPS before conjugation is present in the glycoconjugate as annotated. Other labels are indicated in order to facilitate the assignment of the peaks.

4.2.2 ZPS-conjugates are more immunogenic than the corresponding PS-conjugates

We immunized mice with ZPS-conjugates or those made with the native form of the PS and compared their immunogenicity. As positive control we used the PS-conjugate formulated with Alum as adjuvant. ZPS from both serotype Ib and V were used to generate glycoconjugates, and the results obtained after immunization were similar for serotype Ib (shown throughout this study) and V. After three immunizations, sera were tested in ELISAs using the native PS conjugated to an unrelated carrier for detection (PS-HSA for CRM_{197}-containing glycoconjugates and vice versa). By this method, we assessed for IgG antibody titers specific for the native PS, which is the relevant form present in the GBS capsule. As shown in Fig. 7A, the ZPS conjugated with CRM_{197} was considerably more efficient in inducing antibodies against the bacterial PS than the corresponding native PS-conjugate. The titers induced by ZPS-conjugate reached or exceeded those induced by the positive control, Alum-adjuvanted PS-conjugate. As expected, HSA turned out to be a less immunogenic carrier protein, since native PS conjugated to HSA did not induce antibodies significantly above background. In contrast, ZPS-HSA did induce a detectable titer which was comparable to that found after vaccination with the positive control containing Alum. We also tested if Ag-specific titers were induced more rapidly, as previously described for TLR2 agonists (51), and if the Ig subclass distribution was altered by the ZPS. ZPS-conjugates induced high titers already after two injections (Fig. 7B) and did not alter the relative contributions of different IgG subclasses (Fig. 7C). Thus we have generated glycoconjugates that show accelerated and increased immunogenicity in the absence of an additional adjuvant.

Figure 7: ZPS-conjugate immunogenicity.

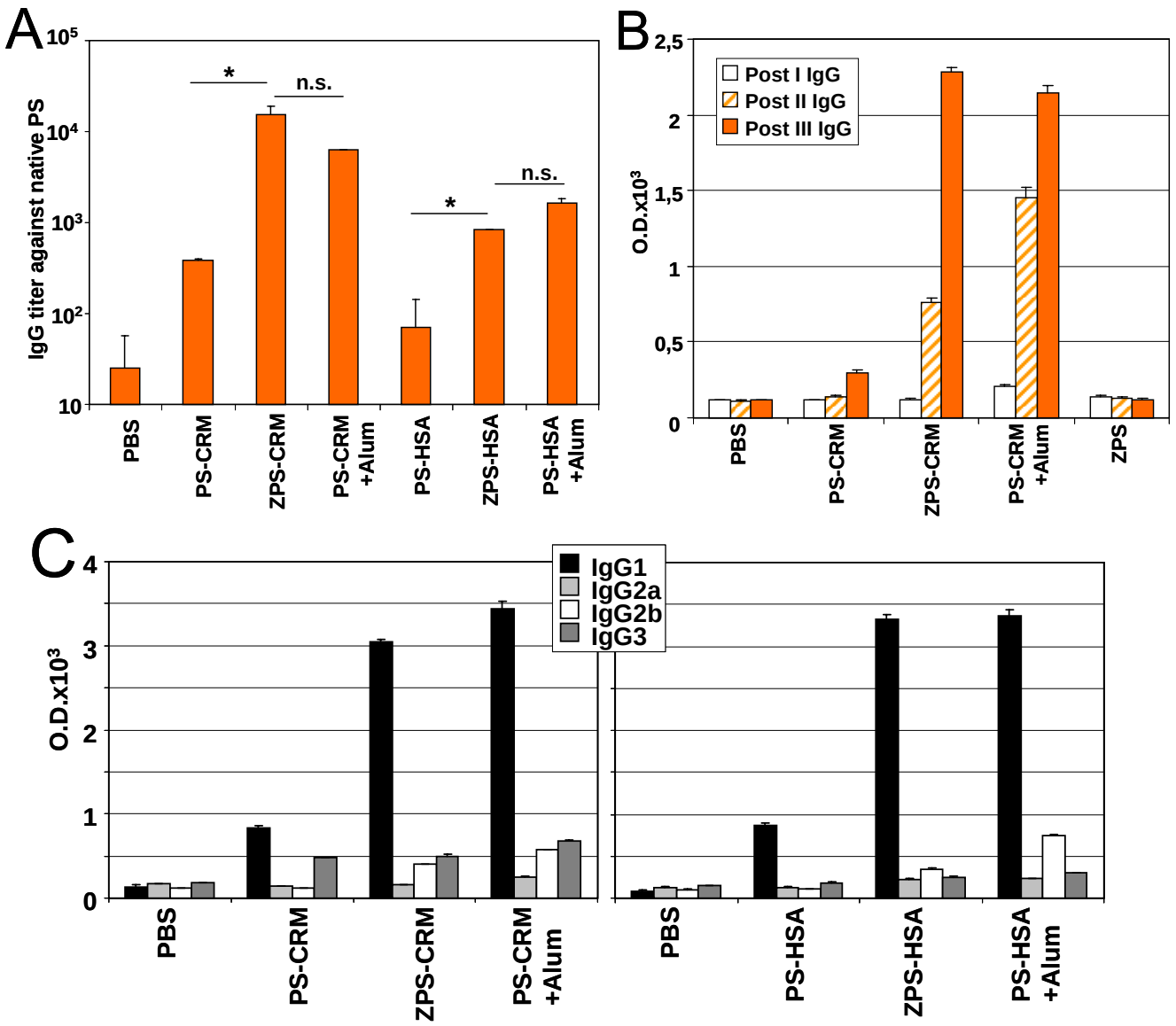


FIGURE 7. (A) Balb/C mice were immunized thrice as indicated. Two weeks after the third immunization, sera were tested by ELISA for PS-specific IgG titer. Results shown are pooled from two experiments using Balb/C mice out of a total of five experiments using Balb/C, CD1 or C57BL/6 strains with similar results. (B) Balb/C mice were immunized intraperitoneally with three doses of the PS-conjugates (1µg) with or without Alum (0.4mg), ZPS-conjugates or ZPS. PBS was used as negative control. Two weeks post first, second and third dose, sera were analyzed for PS-specific IgG. Results are mean of triplicates + SD. ZPS-CRM and alum-adjuvanted PS-CRM accelerate the induction of IgG compared to PS-CRM alone. Unconjugated ZPS do not induce any PS-specific IgG. (C) ZPS-conjugates and Alum increase the same PS-specific IgG subclasses that are above all IgG1.

To show that higher IgG production is due to a better anti-carrier T cell response, we evaluated the ex vivo T cell response in mice immunized with glycoconjugates. Splenocytes were cultured with CRM_{197}, and after four hours of stimulation, Brefeldin A was added overnight to block secretion and retain cytokines in the T cells, which allows detection of cytokines produced by individual CD4 positive T cells through intracellular staining. The total height of bars in Fig. 8 shows the overall percentage of T cells responding to CRM_{197} by cytokine production, and the color-coding indicates which cytokines or combinations are produced by individual T cells. We find that ZPS-CRM induce a higher overall percentage of cytokine-producing CRM_{197}-specific CD4 T cells than the corresponding PS-CRM ($P< 0.01$). In all groups, vaccinated with or without adjuvant, the dominant cytokines induced are IL-5, IL-2 and TNF-α, a combination that is expected for effector and memory T cell populations in the Th2 prone Balb/C mice used here. We conclude that the ZPS conjugation to CRM_{197} leads to enhanced CRM_{197}-specific T-cell responses compared to glycoconjugates containing native PS. In contrast, the cytokine profile is unaltered by ZPS-conjugates compared to that induced by PS-conjugates, suggesting that the adjuvant effect increases the magnitude but does not alter the quality of the specific T cell response. We also tested the T cell response to the whole glycoconjugate or single components of it and found that the response is directed against the protein, not the PS part of the glycoconjugate (Fig. 9). We conclude that ZPS act as adjuvants for increased Ab production through increased T cell responses to the protein part of the glycoconjugate.

Figure 8: ZPS induce a CD4+ CRM-specific T cell recall response.

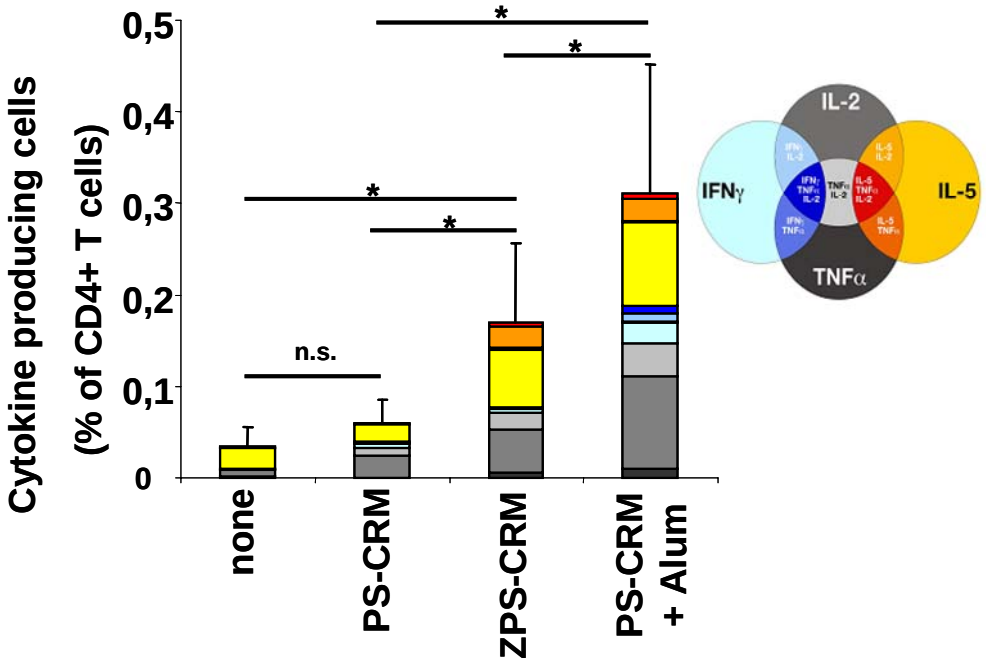


FIGURE 8. Balb/C mice were immunized thrice as indicated and spleen CD4+ T-cell cytokine responses to CRM_{197} at 2 weeks post third dose were evaluated. T cells producing one, two or three cytokines are represented as annotated and add up to the bars shown. Therefore, the bar height indicates the total of all cytokine positive CD4+ cells as percent of total CD4+ cells. Error bars indicate SD of total percentage of all cytokines of six mice. This experiment was performed at least three times with similar results. Statistical significance was analyzed using unpaired student's *t* test. *, $P < 0,01$; n.s., not significant.

Figure 9: The recall response after ZPS-CRM vaccination is directed against the CRM component, not the ZPS component

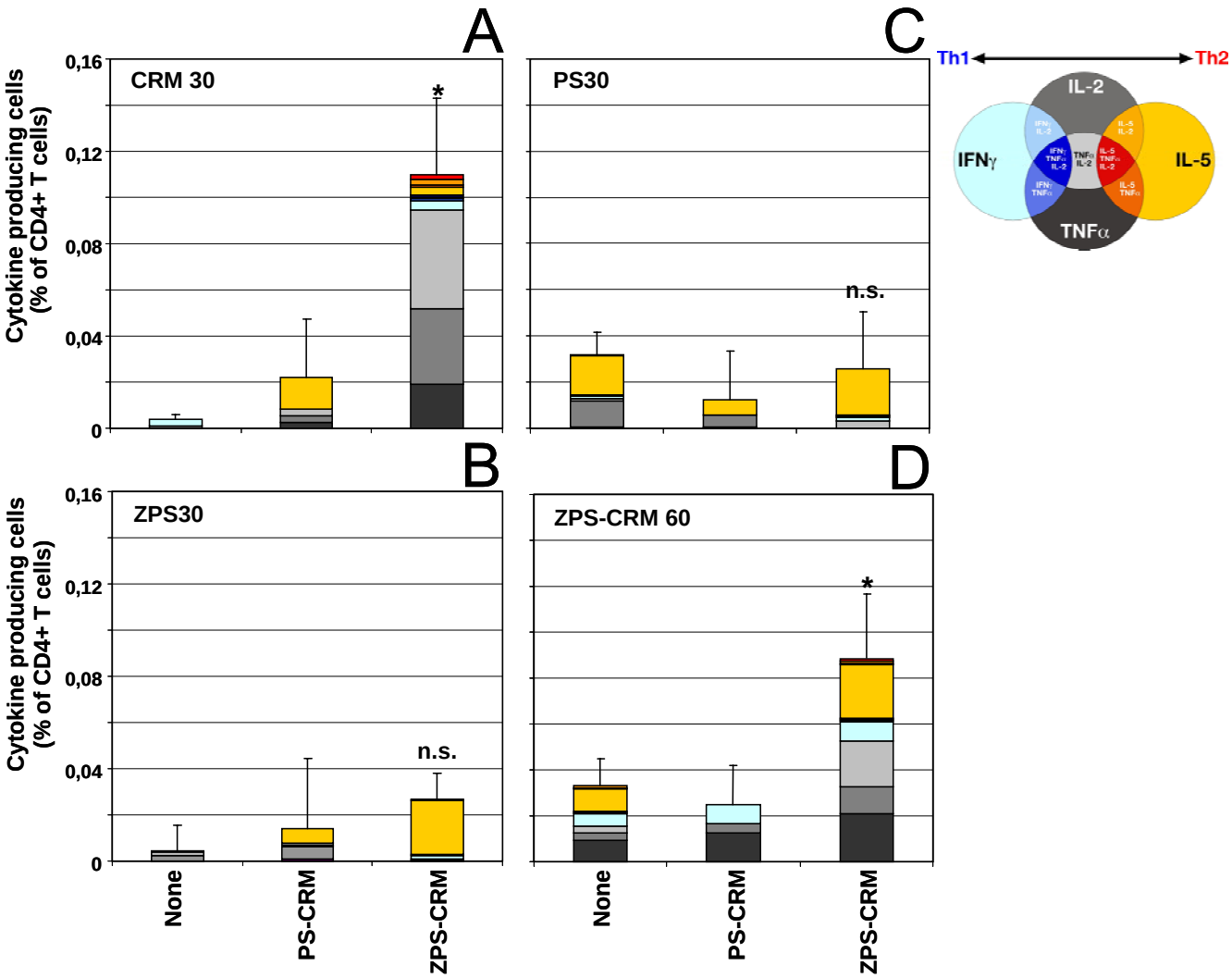


FIGURE 9. Balb/C mice were immunized intraperitoneally with three doses of the PS-conjugates (1µg), ZPS-conjugates (1µg) and PBS as negative control. Two weeks post third dose spleens were tested for CD4+ T-cell cytokine responses to CRM_{197}(30µg/ml), to ZPS (30µg/ml), to PS (30µg/ml) and to ZPS-CRM (60µg/ml).
ZPS enhance the T cell cytokine recall response to CRM197 (A), but they do not induce a significant T cell cytokine recall response to ZPS or PS (B, C). Thus the increase in CD4+ T cell response to ZPS-CRM (D) is exclusively to the CRM portion because ZPS do not induce a ZPS-specific T cell response (B). Error bars indicate SD of total percentage of all cytokines of three mice. This experiment was performed two times with similar results. Statistical significance was analyzed using student's *t* test. *, $P < 0,05$; n.s., not significant compared with PBS and PS-CRM.

We next tested whether the adjuvant effect of ZPS enhances the Ab responses to conjugated or unconjugated proteins. After injection of ZPS-CRM or ZPS-HSA as described above, we found the ELISA titers to these proteins were strongly enhanced compared to native PS-CRM or PS-HSA injection (Fig. 10A). We also coadministered ZPS with the unconjugated protein Ag TT and found strongly increased antiTT titers (Fig. 10B). In conclusion, ZPS are able to act as adjuvant both for conjugated and unconjugated proteins. In contrast, ZPS used alone, without carrier, are not able to induce PS-specific IgG antibody titers (Fig. 7B), suggesting that a protein component is required and that the combination of B cell epitopes and TLR2 agonist activity is not sufficient to increase immunogenicity of the PS part.

Figure 10: ZPS enhance responses both to conjugated and unconjugated proteins.

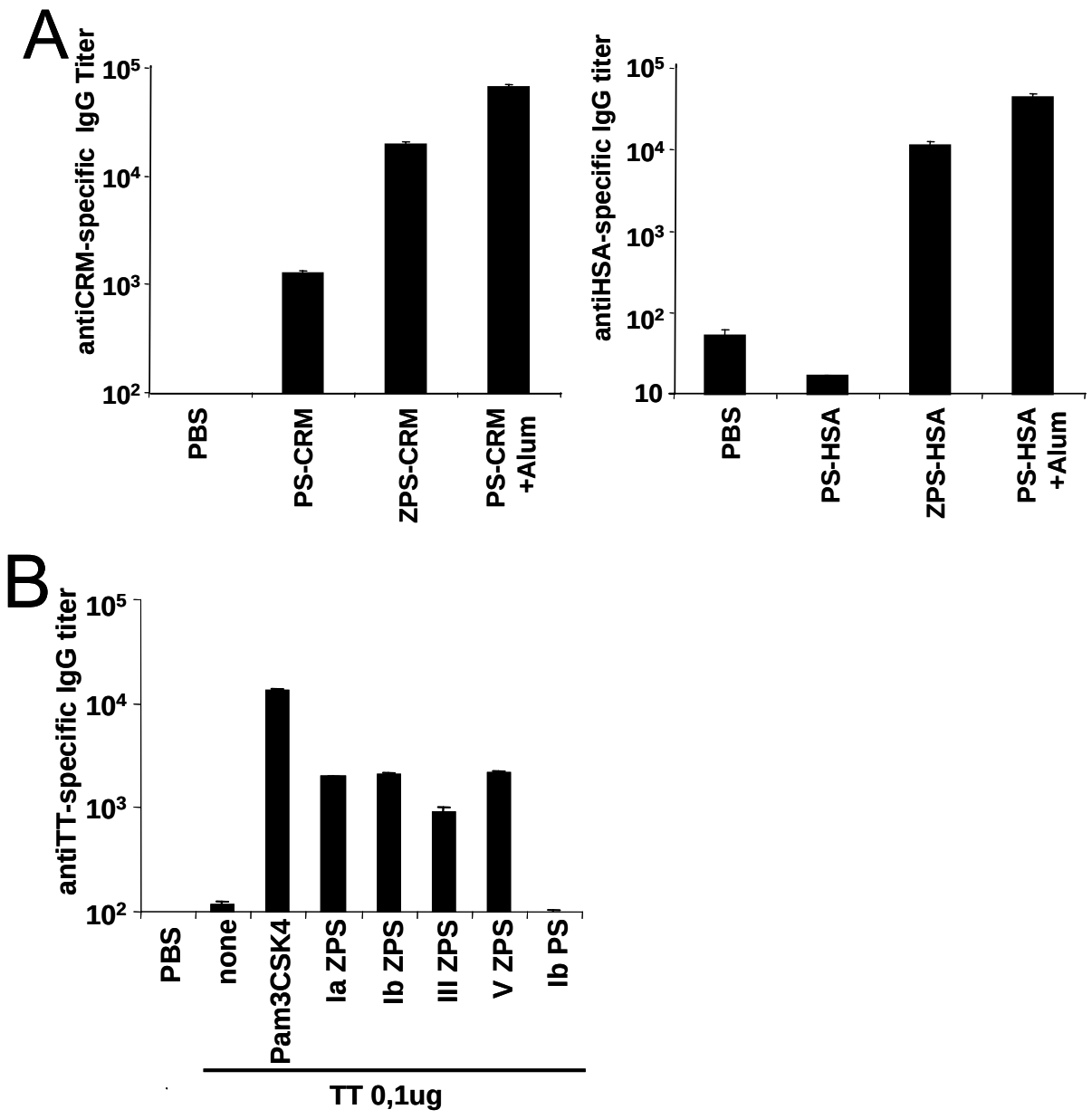


FIGURE 10. (A) Balb/C mice were immunized intraperitoneally with three doses of the PS-conjugates (1µg) with or without Alum (0.4mg), ZPS-conjugates (1µg) or PBS as negative control. Two weeks post third dose, sera were collected and analyzed for protein (CRM or HSA)-specific IgG titer. (B) Balb/C mice were immunized subcutaneously with Tetanus Toxoid (0,1µg) alone or in combination with admixed ZPS serotype Ia, Ib, III and V or the native PS serotype Ib. Pam_3CSK_4 was used as positive control and PBS as negative control. Mice received two doses at day 1 and 21, and two weeks after the second dose, sera were assessed by ELISA for TT-specific IgG antibody titers. Results are geometric means of triplicates + SD.

4.2.3 ZPS-conjugates activate bone marrow derived DCs (BM-DCs)

Since glycoconjugates containing ZPS are more efficient than the corresponding native ones at inducing Ab and T cell responses, we tested whether this correlates with their ability to activate APCs. The dot plots in Fig. 11A show that ZPS conjugated to HSA are able to induce the upregulation of MHC class II and the costimulatory molecule CD86, while HSA alone and PS-HSA do not activate BM-DCs. Similar results were obtained also with CRM_{197} as carrier protein (Fig. 11B). Pam_3CSK_4, a TLR2 agonist, and LPS, a TLR4 agonist, are used as positive controls. ZPS-conjugates induced in BM-DCs the production of cytokines like IL-6 and IL-12, chemokines like Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) and the stimulating factor G-CSF (Fig. 11C). Taken together, these results strongly suggest that the ability of ZPS to activate DCs will improve the priming of carrier-specific T cells and as a consequence the T cell help given to PS-specific B cells, ultimately leading to higher antibody titers. Thus we most likely have generated a glycoconjugate that contains B-cell epitopes, T-cell epitopes and adjuvant properties, leading to an overall better immunogenicity.

Figure 11: BM-DCs are activated by ZPS-conjugates.

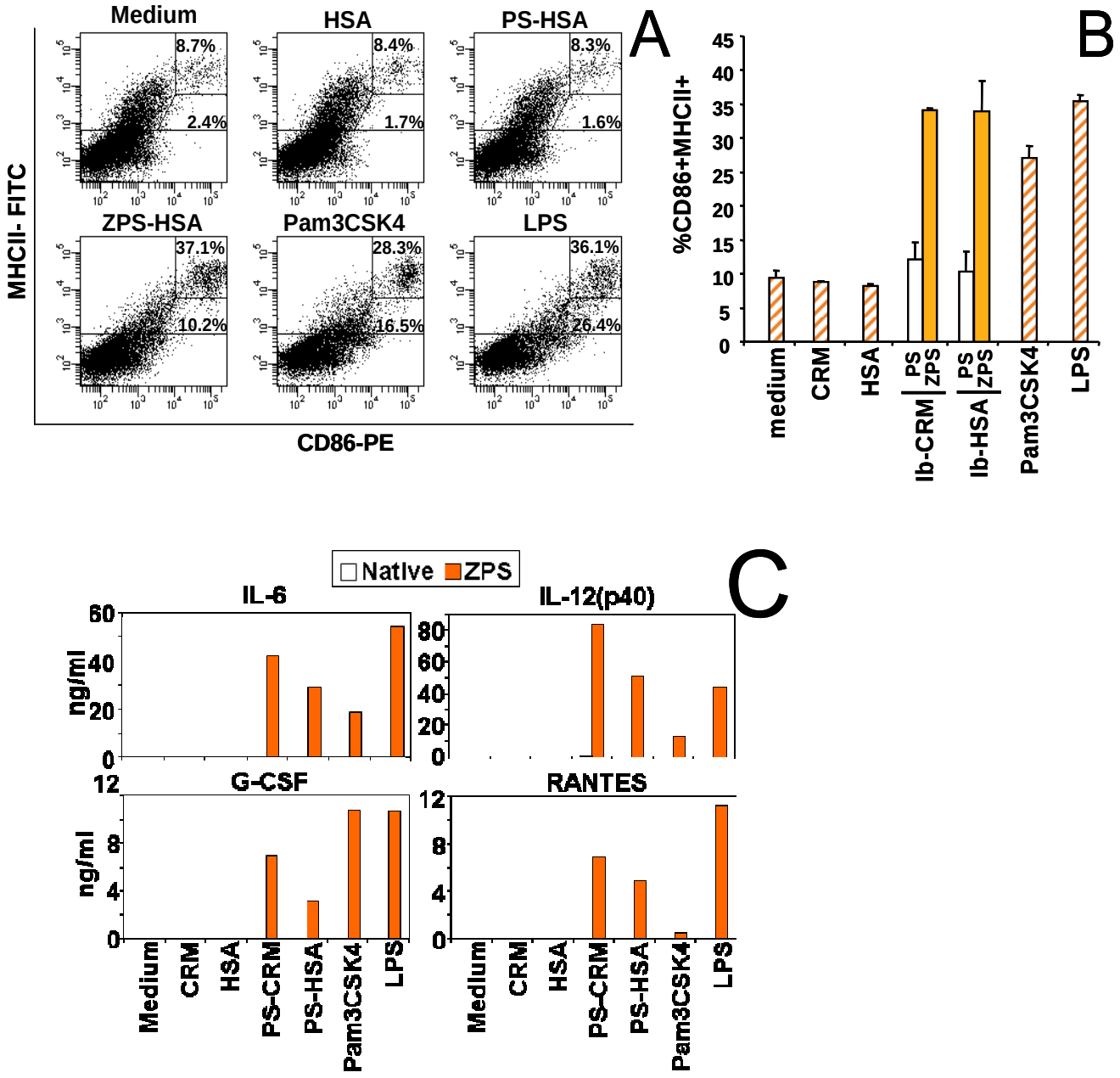


FIGURE 11. (A-B) Mouse BM-DCs were incubated for 20 hours with ZPS and PS conjugates (10µg/ml), CRM_{197} and HSA alone (10µg/ml), Pam_3CSK_4 or LPS (1µg/ml). The up-regulation of CD86 and MHC class II was evaluated by flow cytometry. Data represent mean + SD of triplicates and are representative of three experiments. (C) After 20h of incubation, cytokine and chemokine presence in the supernatants was tested through Bio-plex analysis. Data represent mean + SD of triplicates and are representative of two experiments with similar outcome.

4.2.4 ZPS-conjugates confer enhanced protection against GBS infection

To verify if antibodies induced by ZPS-conjugates were also protective against bacterial infection, we immunized CD1 or Balb/C mice, and after three immunizations we performed a challenge with GBS strain H36B. The percentage of survival after two days post challenge was evaluated and the statistical significance was estimated by Fisher's exact test. As shown in Fig. 12A, protection conferred by ZPS-conjugates is significantly increased compared to that given by PS-conjugates and similar to that induced by Alum-adjuvanted PS-conjugates, the positive control. We also used a neonatal mouse model of group B streptococcal infection (52) to evaluate the protection after challenge in the offspring of immunized mothers. Neonates from mothers immunized with ZPS-conjugates are better protected from GBS infection than those born from mice immunized with PS-conjugates. This is particularly visible in the context of the less immunogenic carrier HSA, and ZPS-HSA induce a level of protection in neonates that is similar to that of Alum-adjuvanted PS-HSA, as determined by Fisher's exact test (Fig. 12B). Using an opsonophagocytosis assay, we confirmed that sera from mice immunized with ZPS-conjugates were more efficient to promote killing of GBS by differentiated HL60 cells than sera from mice immunized with PS-conjugates (Fig. 12C), consistent with the above protection data. The half-maximal titers were also calculated from the opsonophagocytosis data (Fig. 12C bottom), and ZPS-CRM induces clearly higher titers than PS-CRM, reaching levels comparable to those of the Alum-adjuvanted positive control.

Figure 12: ZPS-conjugates confer protection against GBS challenge.

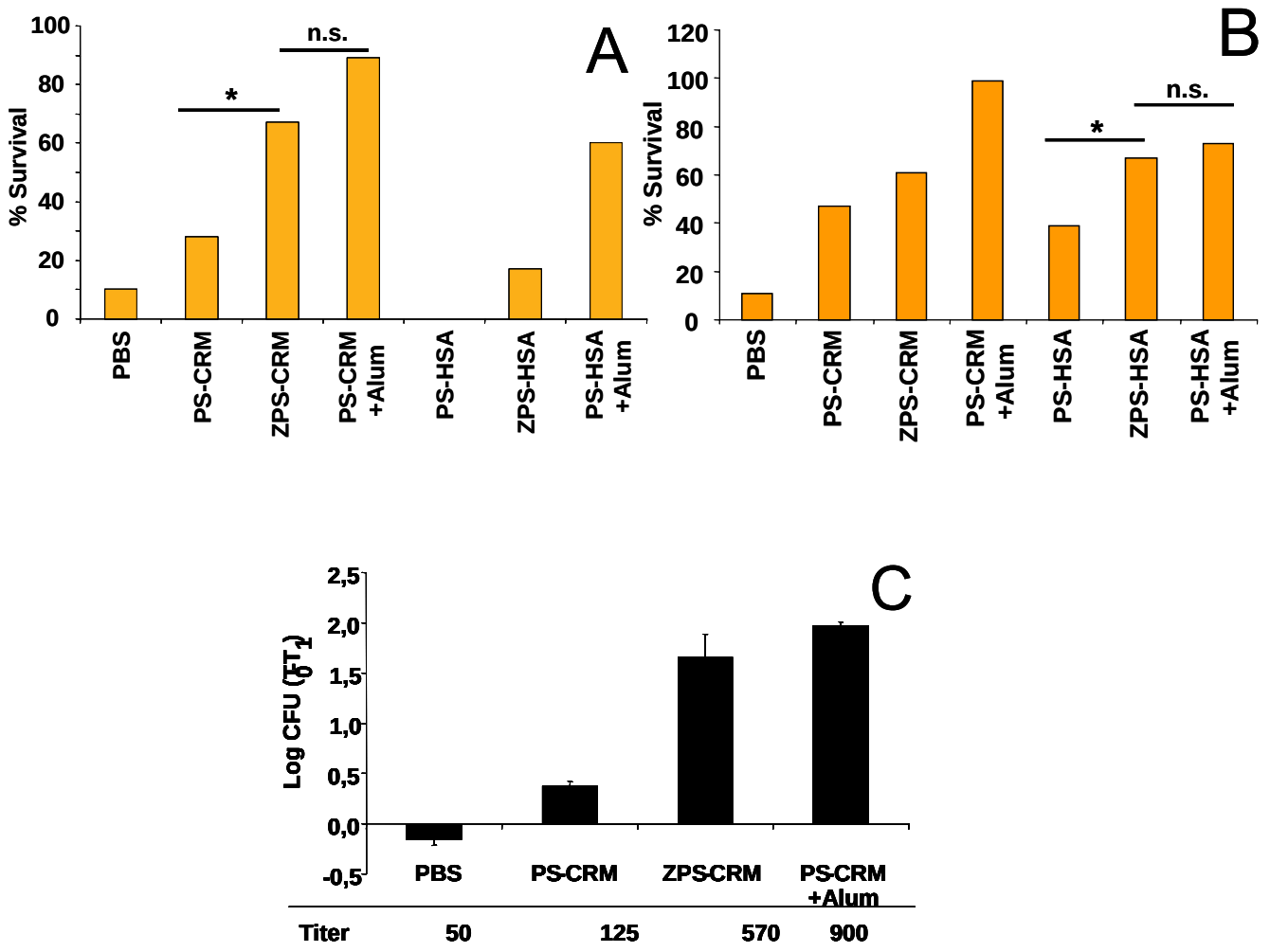


FIGURE 12. (A) CD1 mice were immunized intraperitoneally with ZPS and PS conjugates (1µg). PS-conjugate plus Alum was used as a positive and PBS as negative control. Two weeks after the third immunization, adult mice received a lethal challenge of GBS, and the percentage of survival two days after the injection was evaluated. Pooled data from two experiments are shown. (B) A lethal dose of GBS was administered subcutaneously to the offspring of mothers immunized as in (A). The challenge was performed within 48 hours after birth, and the percentage of survival was estimated two days post injection. Each experiment was repeated at least three times using CD1 or Balb/C strains with similar outcome. Statistical significance was calculated with Fisher's Exact Test. *, $P < 0,01$; n.s., not significant. (C) Opsonophagocytosis assays were performed to evaluate the capacity of sera from mice immunized as in (A) to induce GBS killing by differentiated HL60 cells in presence of rabbit complement. Triplicate results from sera diluted 1:100 were expressed as the mean $\log_{10}$ reduction in GBS colony-forming units before (T_0) and after (T_1) 60 min of incubation at 37°C. From the whole titration curves of the same assay, we also calculated opsonophagocytosis titers which are indicated below the graph.

We were also interested to see if Alum could be used in combination with the ZPS-conjugates and whether this combination would confer a further increase in immunogencity. Results in Fig. 13A and B show that the ZPS adjuvant activity synergizes with Alum to increase further the PS-specific IgG antibody titer and better CRM_{197}-specific CD4+ T cell response induced by the synergistic effect of ZPS and Alum.

Figure 13: ZPS-conjugates can be used in combination with Alum.

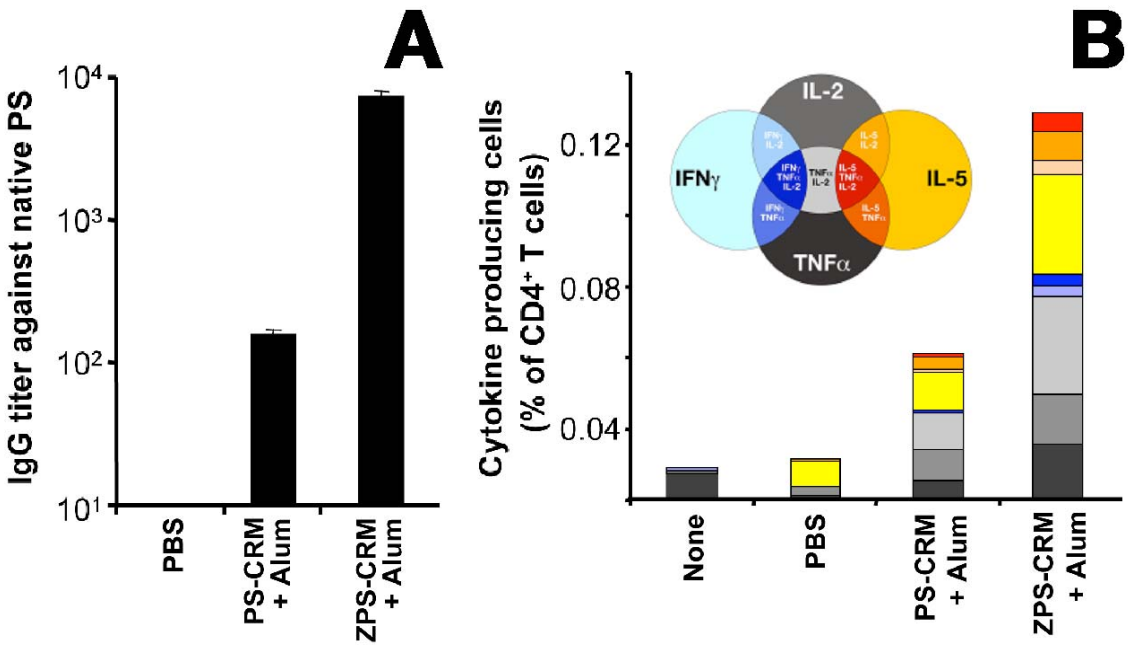


FIGURE 13. (A) C57BL/6 mice were immunized intraperitoneally with three doses of the PS-conjugates (1µg) plus Alum (0.4mg), ZPS-conjugates alone, or ZPS-conjugates (1µg) plus Alum (0.4mg). PBS was used as negative control. Two weeks post third dose, sera were analyzed for PS-specific IgG titers. Results are mean of triplicates + SD. (B) Spleens from the same mice were tested for CD4+ T-cell cytokine responses to CRM_{197}. Histograms show the total percentage of all cytokine positive live singlet CD3 + CD4+ cells. Results are obtained from a pool of six mice for each group. This experiment was performed three times with similar results.

4.2.5 TLR2 is critical for ZPS-conjugate adjuvant activity in vivo

We and others demonstrated previously that the ability of ZPS to activate APCs is TLR2 dependent (14). To test if this receptor is also required for the adjuvant activity of ZPS-conjugates in vivo, we immunized female TLR2$^{-/-}$ mice with glycoconjugates and paired them after three immunizations with wt males. The offspring received a challenge with a lethal dose of GBS and the percentage of survival was evaluated. Following this protocol, the offspring are genotypically heterozygous for TLR2 and therefore express this receptor, but they have acquired the antibody repertoire from mothers immunized in the absence of this receptor. In this experiment we compared the Alum-adjuvanted PS-conjugate, our positive control, to the ZPS-conjugate in wt versus ko mice. Fig. 14A shows that at challenge doses leading to 20-30% survival in the negative control groups, Alum-adjuvanted native glycoconjugate induces full protection in litters born from vaccinated wt and TLR2$^{-/-}$ mothers. In contrast, ZPS-conjugate induced protection is significantly reduced in litters from TLR2$^{-/-}$ mothers, clearly indicating that TLR2 is crucial for the protection induced by ZPS-conjugates. Similarly, the opsonophagocytosis titers induced by ZPS-conjugates were abolished in the absence of TLR2, while those induced by PS-conjugate plus Alum were not (Fig. 14B). In order to see whether the observed differences between wt and TLR2$^{-/-}$ mice are also reflected in differential DC activation by ZPS-conjugates, we assessed cytokine production by BM-DCs from both genotypes in response to ZPS-conjugates. As shown in Fig. 14C, the production of IL-6, IL-12, G-CSF and RANTES induced by ZPS-conjugate or Pam_3CSK_4 is strongly reduced when BM-

DCs were generated from TLR2$^{-/-}$ mice. No difference is observed when using the TLR4 agonist LPS.

Thus TLR2 has a crucial role in the in vivo activity of ZPS-conjugates and is required for the induction of higher functional Ab titers and consequently for higher protection. Since we observed in vitro that activation of BM-DCs by ZPS-conjugate is TLR2 dependent, we conclude that TLR2-dependent DC activation is the most likely mechanism of in vivo adjuvanticity of ZPS.

Figure 14: ZPS-conjugate activity in vivo is TLR2 dependent.

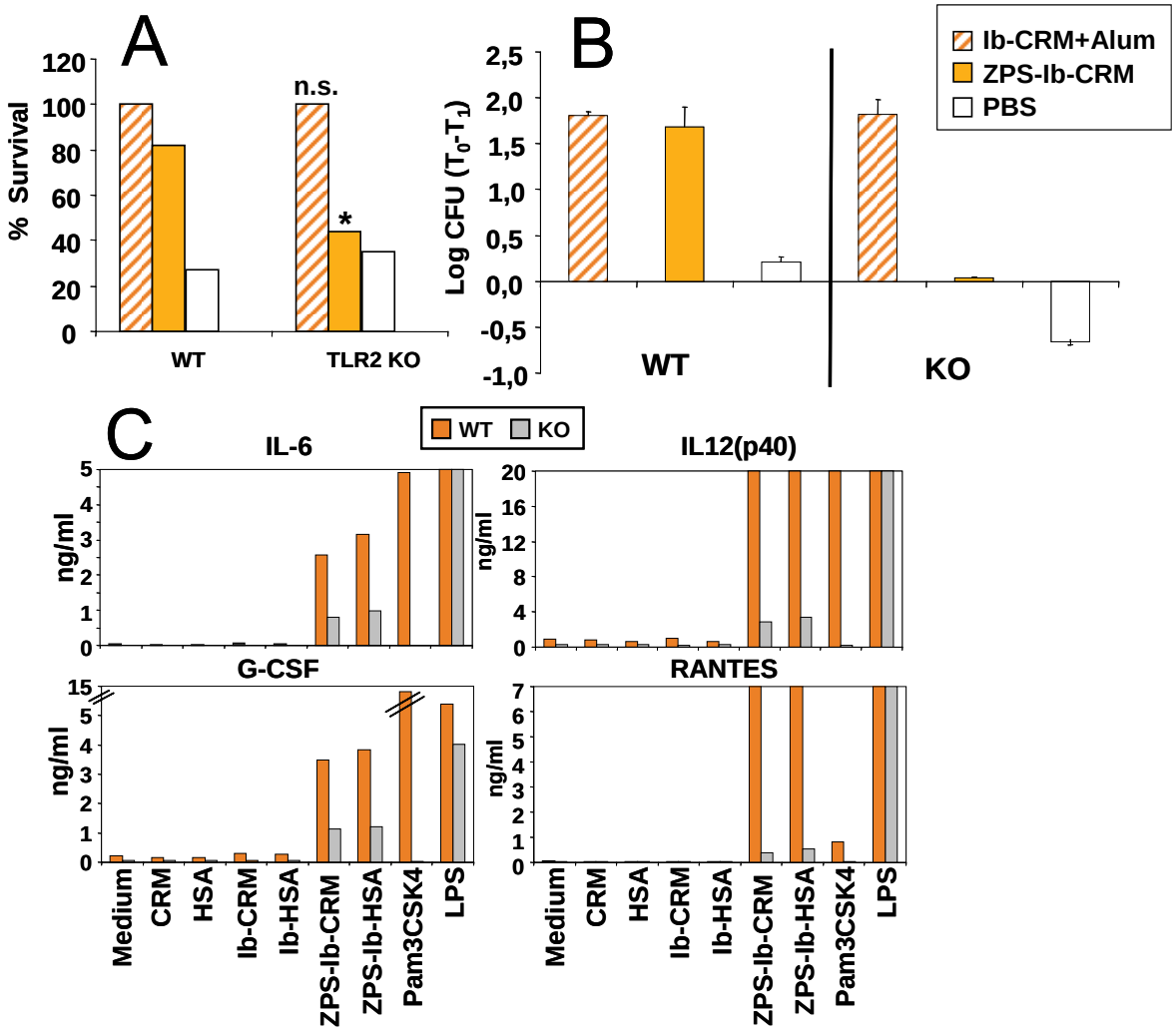


FIGURE 14. (A) C57BL/6 wt and TLR2$^{-/-}$ mice on a C57BL/6 background were treated intraperitoneally as indicated. WT and TLR2$^{-/-}$ female mice received three doses at day 1, 21, 35 and at day 38 all mice were coupled with wt males. The offspring from wt and TLR2$^{-/-}$ mice were injected subcutaneously with a lethal dose of GBS. The challenge doses were chosen in order to have the same percentage of survival for neonates born from wt or TLR2$^{-/-}$ mice treated with PBS. Significance was calculated using Fisher's Exact Test. *, $P < 0,05$; n.s., non significative compared with the respective wt group. Data pooled from two independent experiments are shown. (B) Opsonophagocytosis assays were performed using sera from wt and TLR2$^{-/-}$ mothers of (A). Triplicate results from sera diluted 1:100 were expressed as the mean $\log_{10}$ reduction in GBS colony-forming units before (T_0) and after (T_1) 60 min of incubation at 37°C. (C) BM-DCs generated from the bone-morrow of wt or TLR2$^{-/-}$ mice were incubated for 20 hours with ZPS and PS conjugates (10µg/ml), CRM_{197} and HSA alone (10µg/ml), Pam_3CSK_4 or LPS (1µg/ml). After 20h of incubation the supernatants were assessed by Bio-plex analysis for cytokine and chemokine content. The production of IL-6, IL-12, G-CSF and RANTES induced by ZPS-conjugates or Pam_3CSK_4 was considerably reduced in supernatants of BM-DCs generated from TLR2$^{-/-}$ mice. LPS induce the same cytokine and chemokine production in both genotypes. Data represent mean + SD of triplicates and are representative of two experiments.

5 Discussion

Adjuvants represent an important component of many modern vaccines as they increase the immunogenicity of co-administered Ags such as purified, soluble recombinant proteins, which are per se less immunogenic than whole or split, killed or attenuated pathogens used in the past. While a number of glycoconjugate vaccines induce high antibody titers without adjuvants, in other cases adjuvants are employed to induce a protective immune response. Here, we show for the first time that rational chemical modification can be used to produce a glycoconjugate vaccine in which the PS Ag has acquired additional adjuvant properties. This work is based on previous findings showing that natural ZPS, such as PSA, activate T cells and APCs (23-25). Since this biological activity depends on the zwitterionic structure of this capsular PS (28, 50, 53), we generated a similar charge motif in a vaccine candidate PS by the chemical introduction of positive charges into the naturally anionic capsular PS of GBS. The resulting ZPS activate APCs through a TLR2-dependent mechanism, and this effect depends on the integrity of the zwitterionic motif, similar to what was found for natural ZPS. Therefore ZPS represent a new class of agonists for these receptors. From our study, a clear structure-activity relationship emerges: The same PS repetitive unit shows biological activity in the presence of a zwitterionic motif of alternating charges, but not in the presence of either negative or positive charges only. It remains to be clarified why one of the two chemical modifications leading to ZPS did not yield biologically active molecules. Two possible explanations can be envisaged: either a specific spatial relationship between the positive and negative charges

is required which was met with one but not the other modification, or the fact that ZPS1 contain two positive versus one negative charge per repetitive unit and thus represent cationic molecules prevents their biological activity. Among the natural ZPS, some have a negative net charge and some are neutral but none of them is cationic.

It was shown previously that conversion of a primary amine to a tertiary amine on PSA was associated with a loss of T cell stimulatory activity (44). The chemically derived ZPS2 forms shown here all contain tertiary amines and are able both to activate APCs and to induce T cell proliferation. This indicates that the presence of primary amines is not mandatory for the ability to induce T cell proliferation. TLR2 expression on T cells has been observed (54), and both direct and indirect T cell co-stimulatory properties have been ascribed to TLR2 agonists (55, 56). In addition, it has been shown *in vitro* that TLR2 agonists alone are able to activate T cells (57). Similar observations were reported for the TLR4 agonist LPS. We confirm these findings in a co-culture system of human monocytes and T cells and show that in all cases, activation by TLR agonists is blocked by MHC II blocking mAbs, indicating that MHC II is essential for T cell activation in this set up, even when no T cell antigen is involved. This suggests that also ZPS-mediated T cell activation may be a consequence of APC activation leading to strongly enhanced T cell co-stimulation, possibly in combination with direct effects on TLR2-expressing T cells.

The alternative hypothesis is that ZPS represent both TLR2 agonists and true MHC class II-dependent T cell antigens. This second hypothesis implies that a separable set of structural requirements may apply for each of the two different biological activities, and that the two functions can be introduced separately by

different chemical modifications. A hint in this direction is that another natural ZPS, namely Sp1, was reported to be a T cell antigen but not a TLR2 agonist (14). In line with these observations, we find that among all our chemically derived ZPS2 forms, those most effective at activating T cells were not those inducing the strongest TLR2 dependent APC activation. In this context it is of note that the best APC activator, namely the serotype Ib ZPS, is the one whose T cell activating ability is completely abolished by the TLR2 blocking mAb. Importantly, while T cell activation induced by Pam3CSK4, macrophage-activating lipopeptide (MALP-2) and serotype Ib ZPS2 is abolished by TLR2 block, the same is not true for PSA and serotype III ZPS2, indeed suggesting that these latter molecules have an activity beyond the TLR2 agonist activity shown here.

In vivo, ZPS enhance IgG titers specific for a co-administered protein Ag, clearly demonstrating the adjuvant activity of the ZPS. We injected ZPS alone into mice but were not able to detect increased Ab titers to the ZPS or the maternal PS. This means that the chemically derived ZPS that we have generated is not a T-dependent antigen. Therefore, we conjugated ZPS prepared from GBS capsular PS with a carrier protein to provide a canonical T cell antigen. Adjuvanticity is maintained also when the ZPS is used in place of the native PS as Ag in a glycoconjugate vaccine against GBS. In fact, ZPS-conjugates are more immunogenic than the corresponding PS-conjugates, and mice immunized with ZPS-conjugates are better protected from GBS infection than mice immunized with the PS-conjugates. Increased induction of specific Ab titers is associated with increased T cell responses to the carrier protein. The fact that unconjugated ZPS are unable to induce Ab responses strongly suggests that T

cell help is required for ZPS adjuvanticity. The observation that the increased immunogenicity of ZPS-conjugates is TLR2 dependent further suggests that TLR2-expressing APCs may be involved. We find that ZPS-conjugates activate BM-DCs in vitro inducing surface molecules for T cell priming and production of cytokines, chemokines and growth factors. BM-DC activation induced by ZPS-conjugates is TLR2 dependent since BM-DCs generated from $TLR2^{-/-}$ mice are largely unresponsive. Taking together these data we hypothesize that the increased immunogenicity of ZPS-glycoconjugates is based on the TLR2 agonist properties of ZPS that allow these conjugates to target TLR2-expressing DCs and activate them. This in turn leads to better T cell priming, increased T cell help and ultimately to higher specific Ab titers (Fig. 15). This model also explains why conjugation to a protein carrier is still required for the immunogenicity of ZPS: the TLR2-dependent adjuvant effect on DCs can be transmitted to B cells only via T cell help, and in fact we find increased T cell responses to the carrier protein when conjugated to ZPS. In contrast, we were not able to find ZPS- or PS-specific T cell responses.

Compared to other Ag presenting cells, DCs are critical for the full activation of naïve T cells, and their maturation is essential for this (58, 59). DC maturation can be triggered by Toll-like receptor agonists which induce the increase in surface MHC class II and costimulatory molecules and thereby link innate to adaptive immune responses (60, 61). TLR2 has already been demonstrated to be the receptor implicated in the ability of PSA to link innate and adaptive immunity (14). Moreover the Haemophilus influenzae type b-outer membrane protein complex glycoconjugate has an optimal immunogenicity thanks to the TLR2 agonist properties of the carrier protein (51). Other examples of vaccines

owing their potency at least in part to the sometimes fortuitous presence of TLR2 agonists are the Yellow Fever vaccine (62) and PS vaccines against Streptococcus pneumoniae (45). Thus, TLR2 is a receptor implicated in the strong immunogenicity of natural ZPS but also of vaccines based on PS, attenuated virus or glycoconjugates.

Efforts have also been made to generate synthetic vaccines in which a peptide Ag was covalently linked to a TLR2 agonist (63-65). Such linked peptides are better internalized by DCs than the peptide alone or mixed with the TLR2 agonist, and this appears to depend on the TLR2 agonist internalization which carries along the linked peptide (66). Therefore we speculate that the ZPS as TLR2 agonist may increase T cell priming also through the internalization of ZPS-conjugates by DCs and targeting the carrier protein to endolysosomes. It has also been shown for vaccine formulations containing agonists to TLRs other than 2 that the physical coupling of Ag and agonist is far more effective than simple co-administration (67). We show here that ZPS are effective in both coupled and uncoupled form, and experiments are underway to compare directly the potency of these formulations.

When TLR2 deficient BM-DCs were stimulated with ZPS-conjugates, cytokine production was strongly reduced but not entirely abolished, at difference to the complete DC unresponsiveness to Pam_3CSK_4. This may be due to a different usage of the TLR2/1 or TLR2/6 heterodimers as compared to Pam_3CSK_4, or to an additional receptor. A synergy between TLRs and C-type lectin receptors recognizing PS structures has been described (68-70), and we speculate that similar mechanisms may contribute to the strong DC activation by ZPS.

It has been proposed that optimal vaccine formulations are able to target the Ag to DCs in order to allow more efficient Ag processing and presentation, and moreover provide the stimuli to induce DC maturation to enhance the adaptive immune response (71, 72). The ZPS-conjugates have both properties: a PS Ag that targets itself to TLR2-expressing DCs and activates, through TLR2, DC maturation, thereby increasing processing and presentation of the conjugated protein to naive T cells. Here, we have used this strategy firstly to provide increased T cell help to B cells recognizing the B cell epitopes of the PS, and secondly to increase the immune response to an unrelated protein, either conjugated or co-administered.

Over the years, chemical tools have proven essential to make progress in the generation of synthetic oligosaccharides and glycoconjugates (73). Chemical modifications in PS structure have been performed to enhance the PS-specific IgG antibodies or to eliminate epitopes that produce antibodies cross-reactive with host tissue (74, 75). More recently, as an example of rational design of the carbohydrate, the capsular PS serotype V GBS has been chemically desialylated to generate a glycoconjugate able to induce the IgM-to-IgG switching (8). In the present work, we have exploited existing structural knowledge of the charge motif in natural ZPS to generate chemically a similar structure in a vaccine PS and thereby to obtain the TLR2 agonist properties conferred by this peculiar charge motif. The modified PS acts as an adjuvant and has been used to generate a glycoconjugate vaccine that is more immunogenic and more protective. Our approach adds, through rational chemical modification, biological function to vaccine Ags. This strategy may be

applied to many other polysaccharides and represents a new path for rational chemical design of novel adjuvants and glycoconjugate vaccines.

Figure 15: Proposed mechanism of increased immunogenicity of ZPS-conjugates.

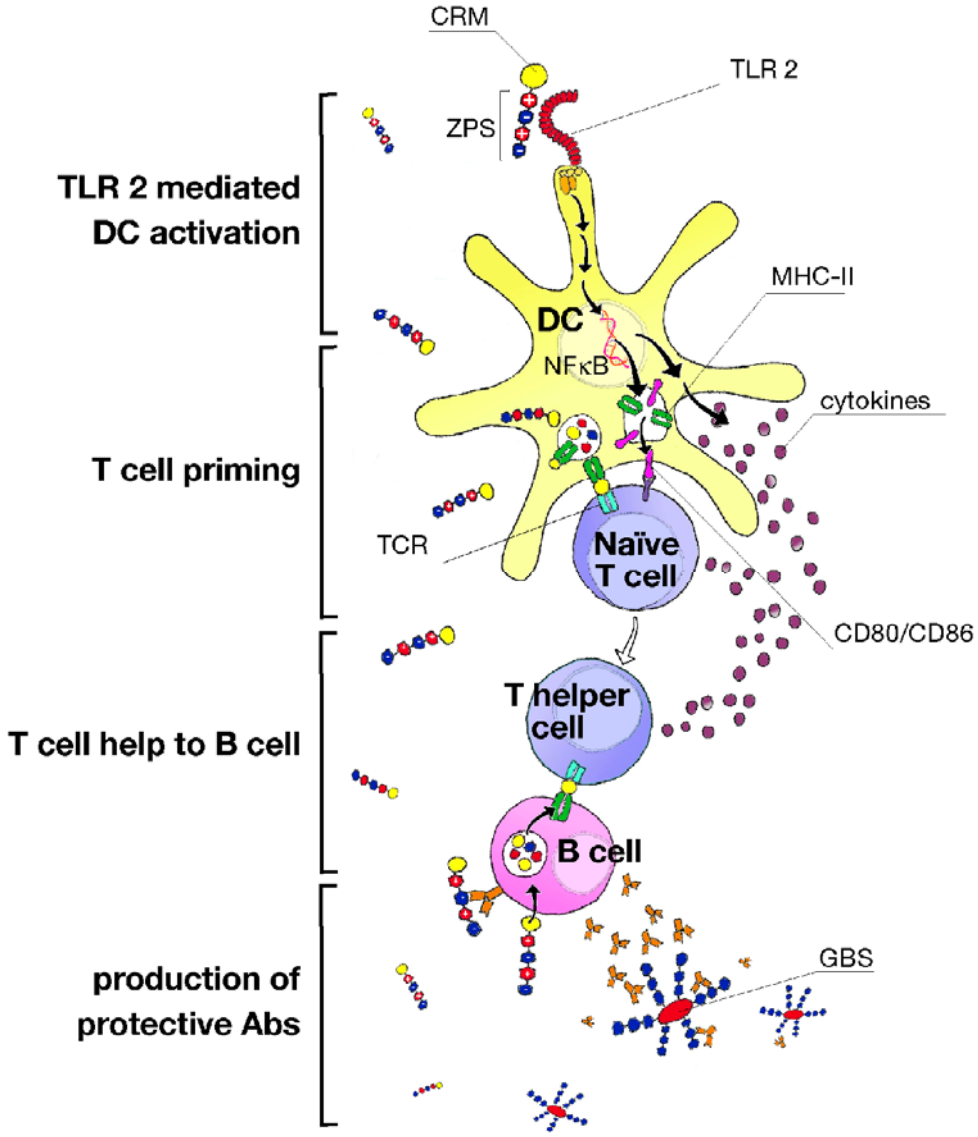


FIGURE 15. ZPS-conjugates are targeted to TLR2 positive DCs and activate them through receptor engagement. Increased DC activation leads to improved priming of naïve T cells specific for the conjugated protein. Enhanced T cell help to B cells will lead to higher Ab titers and thus improved protection against infection by GBS.

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