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A novel mechanism for glycoconjugate vaccine activation of the adaptive immune system

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Abstract

Although glycoconjugate vaccines have provided enormous health benefits globally, they have been less successful in significant high-risk populations. Exploring novel approaches to the enhancement of glycoconjugate effectiveness, we investigated molecular and cellular mechanisms governing the immune response to a prototypical glycoconjugate vaccine. In antigen-presenting cells, a carbohydrate epitope is generated upon endolysosomal processing of group B streptococcal type III polysaccharide coupled to a carrier protein. In conjunction with a carrier protein-derived peptide, this carbohydrate epitope binds to major histocompatibility class II (MHCII) and stimulates carbohydrate-specific CD4+ T-cell clones to produce interleukins 2 and 4 —cytokines essential for providing T-cell help to antibody-producing B cells. An archetypical glycoconjugate vaccine constructed to maximize the presentation of carbohydrate epitopes recognized by T cells is 50–100 times more potent and significantly more protective in an animal model of infection than is a currently used vaccine construct.

Pathogenic extracellular bacteria often express large-molecular-weight capsular polysaccharides (CPSs), which coat the microbial surface. CPSs have been considered T cell–independent antigens[1–5] primarily because, when used as vaccines, they induce specific IgM responses in wild-type and T cell–deficient mice without inducing significant IgM-to-IgG switching[3]; fail to induce a booster response (i.e., a secondary antibody response after recall immunization); and fail to induce sustained T-cell memory[4].

The advantages of glycoconjugate vaccines over pure glycans in inducing immune responses are well documented[5]. Covalent coupling of a T cell–independent CPS to a carrier protein yields a glycoconjugate that, when used to immunize mammals, elicits T-cell help for B cells that produce IgG antibodies to the polysaccharide (PS) component[5–11]. Thus glycoconjugates induce PS-specific IgM-to-IgG switching, memory B-cell development, and long-lived T-cell memory. Glycoconjugate vaccines have played an enormous role in preventing infectious diseases caused by virulent pathogens such as *Haemophilus influenzae, Streptococcus pneumoniae,* and *Neisseria meningitidis*[9,12]. However, the

immunogenicity of these glycoconjugates has been variable, and this variability has been attributed to the structure of the particular PS in a given construct[13,14]. In addition, in some high-risk populations, immunogenicity has been relatively poor[5,9]. The current hypothesis— i.e., that, in the context of major histocompatibility complex class II (MHCII), a peptide generated from glycoconjugates can be presented to and recognized by T cells[15]—overlooks the strong covalent linkage of carbohydrates to proteins in glycoconjugate vaccines that is unlikely to be broken within the endosome[3,5]. The current hypothesis of peptide-only presentation has been promulgated mainly because proteins have generally been viewed as the only antigens presented by MHCII molecules to T cells. We considered whether T cells can recognize "T cell–independent" carbohydrates covalently linked to another molecule (e.g., a peptide) whose binding to MHCII allows presentation of the hydrophilic carbohydrate on the antigen-presenting cell (APC) surface. We hypothesized that T-cell failure to respond to carbohydrates (e.g., bacterial CPSs) is due to failure of these molecules to bind to MHCII, not to T-cell inability to recognize presented glycans. We tested this hypothesis to gain insight into the mechanisms involved in carbohydrate processing and presentation by MHCII and in subsequent T-cell recognition of glycoconjugate vaccines. An understanding of the immune mechanisms involved in glycoconjugate immunization is of paramount importance in the rational design of new-generation vaccines against emerging infections.

We investigated the mechanisms underlying APC processing and presentation of glycoconjugates consisting of the type III PS of group B *Streptococcus* (GBSIII)—a typical T cell–independent PS—coupled to a carrier protein/peptide such as ovalbumin (OVA), tetanus toxoid (TT), or ovalbumin peptide (OVAp).

RESULTS

MHCII-presented carbohydrate epitopes elicit T-cell help

The adaptive immune response to glycoconjugates (Fig. S1) was first examined by priming mice with OVA and boosting them 2 weeks later with GBSIII conjugated to OVA (III-OVA). We compared PS-specific IgG levels in the sera of these mice with levels in the sera of mice both primed and boosted with the conjugate (Fig. 1a). Priming of naïve animals with the carrier alone did not support a robust secondary antibody response to the PS upon boosting with the glycoconjugate. However, mice primed and boosted with the glycoconjugate had strong IgG responses after recall vaccination. To determine whether the inability of OVA to induce a priming response for glycoconjugate boosting is due to a failure of T-cell or B-cell priming, we immunized mice with an unconjugated mixture of GBSIII and OVA (GBSIII+OVA), thereby providing B cells that had recent experience with GBSIII and T cells that had experience with presentation of the peptides derived from the OVA protein, and then boosted these mice with the glycoconjugate (Fig. 1a). After III-OVA recall immune stimulation, mice primed with GBSIII+OVA—unlike III-OVA-primed mice —had essentially no secondary antibody response to the glycan (Fig. 1a). We measured OVA-specific IgG titers and GBSIII-specific IgG and IgM titers after only a priming dose of either GBSIII+OVA or III-OVA. GBSIII-specific IgG levels were detectable only after priming of mice with III-OVA (Fig. S2a). Whether the glycan was conjugated or not, serum levels of IgM antibody to GBSIII were similar in both groups of immunized mice (Fig. S2b), an observation suggesting equivalent levels of carbohydrate-specific B-cell priming. After priming, approximately the same level of OVA-specific IgG was measured in serum from both groups; this result suggested that OVA-specific T-cell help was recruited after priming with either the GBSIII+OVA mixture or the III-OVA glycoconjugate (data not shown). Additional control groups for this experiment involved mice primed with unconjugated GBSIII or with no antigen (PBS+ alum) and boosted with III-OVA (Figs. 1a, S2a, and S2b).

In experiments examining whether CD4+ T-cell recognition of a carbohydrate is a major factor in induction of the humoral immune response to glycoconjugates, BALB/c mice were primed with III-OVA and boosted with a conjugate comprising GBSIII and TT (III-TT), and serum levels of GBSIII-specific IgG were measured (Fig. 1b). Control groups included mice primed and boosted with III-TT, primed and boosted with III-OVA, primed with GBSIII (unconjugated) and boosted with III-TT, primed with III-OVA and boosted with GBSIII (unconjugated), primed with III-OVA and boosted with GBSIII+TT, and primed with III-OVA and boosted with TT. Boosting of III-OVA-primed mice with III-TT induced GBSIII-specific IgG levels similar to those after priming and boosting with III-OVA (Fig. 1b). These results strongly support recruitment of T-cell help for induction of GBSIII carbohydrate-specific secondary immune responses via carbohydrate recognition.

Another possible explanation is that activated B cells respond to III-TT without T-cell help. We tested this possibility by boosting III-OVA-primed mice with III-TT after treatment with antibody to CD4 during the interval between priming and boosting. The excellent booster response observed in isotype control antibody–treated mice was abolished in anti-CD4-treated mice (Fig. 1b). Using flow cytometry, we demonstrated the complete depletion of CD4+ T cells by anti-CD4 treatment of splenic mononuclear cells from anti-CD4-treated mice before secondary vaccination (Fig. S2c). In addition, mice primed with III-OVA and boosted with GBSIII, TT, or GBSIII+TT had no booster response. These results led to further examination of the mechanisms by which CD4+ T-cell recognition of GBSIII glycoconjugate vaccines could be mediated by the carbohydrate portion.

Glycoconjugate carbohydrate is processed into smaller glycans

To investigate the molecular and cellular mechanisms involved in immunization with GBSIII-containing glycoconjugates, we first examined glycoconjugate processing and presentation by APCs (e.g., B cells, dendritic cells). Some CPSs are taken up by APC endosomes and depolymerized into smaller carbohydrates by oxidative agents such as ROS and reactive nitrogen species[16,17]. We assessed whether pure GBSIII (>100 kDa) is depolymerized within the APC endolysosome, as reported for *Bacteroides fragilis* PS A[17]. Radiolabeled GBSIII (Fig. S3) was incubated with Raji B cells for 18 h, endolysosomes were isolated and lysed, and GBSIII within the endolysosome was determined by molecular sieve chromatography to be significantly depolymerized, with a major peak at ~10 kDa (Fig. 2a). Western blot analysis showed that endolysosome preparations contained both the endosomal marker Rab5 and the lysosomal marker LAMP-1 (Fig. S4). Comparison of the band density of CD19 (cell surface protein)–labeled endolysosomal fractions with the band density of CD19-labeled, serially diluted cell surface fractions showed that endolysosomal fractions were essentially free (5%) of cell surface content (data not shown).

Since GBSIII and proteins are processed by different mechanisms in the endolysosome, we wondered whether the carbohydrate in the context of a glycoconjugate is also processed. We incubated Raji B cells with III-OVA, selectively radiolabeling only the PS with ^{3}H (Fig. S3). After 18 h of uptake and processing, [^{3}H]III-OVA was degraded to a molecular size similar to that of pure unconjugated GBSIII after depolymerization [processed glycan (glycan$_p$) ~10 kDa; Fig. 2b]. To identify the oxidative agent(s) responsible for PS processing in III-OVA, we incubated Raji B cells with [^{3}H]III-OVA in the presence of ROS inhibitors, detecting inhibition of PS processing by superoxide and hydroxyl radical inhibitors (Figs. S5a, S5b, respectively) but not by a hydroperoxide inhibitor (Fig. S5c).

Processed carbohydrates are presented on the APC surface

To determine whether MHCII-associated processed carbohydrates (glycans$_p$) are presented on the APC surface, we conducted co-immunoprecipitation (co-IP), flow cytometry, and

western blot experiments. First, Raji B cells were incubated with unconjugated pure GBSIII. Although [³H]GBSIII was endocytosed and processed to a smaller molecular size (Fig. 2a), co-IP of Raji B-cell surface-membrane fractions with a monoclonal antibody (mAb) to MHCII revealed no GBSIII on the cell surface in the context of MHCII (Fig. 2a). However, co-IP of APC surface membranes with mAb to MHCII after incubation of [³H]III-OVA with Raji B cells (Fig. S6a) or mouse splenic mononuclear cells (Fig. 2c) demonstrated surface-associated, endosomally processed [³H]GBSIII (glycan$_p$). Co-IP of Raji B cells after incubation with either [³H]III-OVA or [³H]III-TT showed the carbohydrate epitope on the cell surface in the context of HLA-DR but not on MHCII-deficient Raji-derived RJ2.2.5 cells (Fig. S6b). Western blot analyses of cell-surface and endolysosome fractions revealed that the Raji cell surface was essentially free of endosomal and lysosomal content (Fig. S4). As controls for anti-HLA-DR, cell surface membrane–solubilized extracts of Raji B cells were immunoprecipitated with antibodies to LAMP-1 (lysosomal protein) and CD19 (cell surface protein); no radiolabeled carbohydrate was detected in immunoprecipitates (Fig. S6a). Co-IP with mAb to HLA-DQ or HLA-DP molecules expressed by Raji B cells[18] did not significantly enhance radioactivity (p > 0.05) over that seen with mAb to LAMP-1 (not shown). By co-IP with [³H]III-OVA as antigen, surface-associated GBSIII was sought with anti-IA/IE on splenocytes from C57Bl/6 wild-type mice and various knockout strains (Fig. 2c). Splenocytes from wild-type and MHCI-deficient (*B2m^{tm1Jae}*) mice had surface GBSIII (~10 kDa); cells from MHCII-deficient (*H2-Ab1^{tm1Gru}*) mice did not.

For flow cytometry, bone marrow–derived dendritic cells (BMDCs) from wild-type and MHCII-deficient mice were incubated with GBSIII or III-OVA for 18 h and then labeled at 4 C with a GBSIII-specific mAb (IgG2a) followed by fluorophore-conjugated anti-mouse secondary antibody. Only membranes of III-OVA-incubated wild-type BMDCs were labeled with GBSIII-specific mAb (Figs. 2d, 2e).

Endosomally processed carbohydrates are presented by the MHCII pathway only when covalently conjugated to carrier proteins. One possible explanation is that processed carbohydrate epitopes (glycans$_p$) are presented by MHCII only when linked to an MHCII-binding peptide formed in the endolysosome by proteolytic digestion of the carrier protein (as in a glycan$_p$-peptide conjugate). Perhaps the peptide portion of a glycan$_p$-peptide (an MHCII-binding peptide covalently linked to a carbohydrate T-cell epitope) binds to MHCII, which carries the covalently linked carbohydrate to the APC surface. To assess whether glycan$_p$-peptides created from the complex glycoconjugate vaccine are presented by MHCII, we conducted a western blot experiment with a glycoconjugate containing a single peptide epitope as the carrier (Figs. S7a, S7b). In this vaccine, ovalbumin peptide epitope OVA323–339 (a T-cell epitope of OVA[19]) was conjugated to GBSIII to form III-OVAp. The peptide was N-acetylated at its N terminus and extended with four amino acids at the C terminus to permit controlled conjugation to the PS. (OVAp can react with only one aldehyde group on the sugar chain through its free amino group at the C-terminus lysine residue.) In preliminary experiments (Figs. S7b, S7c), this peptide bound readily to MHCII on Raji B cells. Modifications of the peptide's amino acid composition did not affect its activation of the αβ T-cell receptor (αβTCR); the modified peptide and the OVA323–339 peptide gave similar MHCII-restricted (e.g., T-cell activation blocked by mAb to I-A^d) CD4+ T-cell proliferative responses in a mouse assay (not shown).

III-OVAp, pure GBSIII, and pure OVAp were each incubated with Raji B cells; cell surface–associated contents were examined on western blots. Membranes were incubated with antibody to HLA-DR, antibody to GBSIII (capable of high-affinity reactions with PSs as small as 6 repeating units), or antibody to OVAp. Immune complexes containing HLA-DR, GBSIII, and peptide appeared in a band at ~82 kDa in membrane extracts from cells incubated with III-OVAp but not in those from cells incubated with unconjugated GBSIII

(Fig. S7a). HLA-DR αβ dimers (self peptide–loaded or empty) were identified with mAb to HLA-DR at ~64 kDa[20,21] (Fig. S7a). The ~18-kDa difference in size (determined by protein markers) between unloaded HLA-DR and the glycan$_p$-peptide–HLA-DR complex represents the approximate molecular size of the predicted glycan$_p$-peptide, as carbohydrates mobilize more slowly than proteins in gels[22]. However, the mAb to GBSIII bound nonspecifically to the over-expressed free MHCII at ~64 kDa (Fig. S7a). To validate the nonspecificity of this interaction, we stimulated lysed naïve Raji cells with OVAp and stained the transferred gel with mAb to GBSIII. A light band similar in intensity to that in lanes 5 and 6 (Fig. S7a) at 64 kDa was observed (Fig. S7b). Human MHCII molecules (e.g., HLA-DR10) have been shown to present OVAp[23]. We tested whether Raji B cells (whose HLA-DRB1*100101 allele encodes for HLA-DR10[18,24]) can present OVAp in the context of MHCII. OVAp was detected on a western blot by antibody to OVAp (Fig. S7b), and an OVAp-biotin conjugate was detected when Raji cells (but not RJ2.2.5 cells) incubated with OVAp-biotin were labeled with NeutrAvidin-fluorescein conjugate in a flow cytometry experiment (Fig. S7c).

The co-IP and flow cytometry experiments (Figs. 2c–e) demonstrate that GBSIII glycan$_p$ is presented on the cell surface in the context of MHCII only when conjugated to a carrier protein/peptide. Although the exact structural features of this complex must be defined by crystallography studies, western blot analysis (Fig. S7a) suggests the possibility that a peptide portion of a glycan$_p$-peptide binds to MHCII, presenting glycan$_p$ on the APC surface in the context of MHCII.

MHCII carbohydrate presentation inhibits TCR OVAp recognition

We constructed a glycoconjugate in which the T-cell response could be directed only toward a single carrier peptide (III-OVAp). III-OVAp, which contains a single-peptide T-cell epitope, proved useful in this situation. Lymphocytes were obtained from OVAp-specific TCR transgenic mice (DO11.10) and wild-type mice (BALB/c) after immunization with III-OVAp or OVAp (2 doses, 2 weeks apart). In T-cell proliferation experiments, irradiated splenic mononuclear cells (iAPCs) from wild-type naïve BALB/c mice were co-incubated with CD4+ T cells from either immune BALB/c mice (Figs. 3a, 3c) or immune DO11.10 mice (Figs. 3b, 3d). The iAPC/CD4+ T-cell mixtures were stimulated in vitro with III-OVAp (50 μg/ml), GBSIII (37.5 μg/ml; equivalent GBSIII content to that in 50 μg/ml of III-OVAp), or OVAp (12.5 μg/ml; equivalent OVAp content to that in 50 μg/ml of III-OVAp). As expected, unconjugated GBSIII did not stimulate proliferation of CD4+ T cells from immunized BALB/c or DO11.10 mice. However, CD4+ T cells from III-OVAp-immunized BALB/c mice responded better to the glycoconjugate than to the peptide alone (Fig. 3a).

With DO11.10 CD4+ T cells, OVAp induced a strong proliferative response in III-OVAp-immunized mice [stimulation index (SI) = 284.6; Fig. 3b] and OVAp-immunized mice (SI = 277.6; Fig. 3d). As anticipated, CD4+ T cells from naïve DO11.10 mice proliferated similarly strongly in response to OVAp (data not shown). DO11.10 CD4+ T cells (in the presence of iAPCs) were stimulated only minimally after co-culture with III-OVAp. Proliferation was 36-fold lower in DO11.10 T cells stimulated with III-OVAp (SI = 7.9; Fig. 3b) than in those stimulated with OVAp (SI = 284.6; Fig. 3b). These results support the hypothesis that a prominent glycan$_p$ epitope is presented by iAPCs incubated with III-OVAp and that this carbohydrate is not recognized by the TCR of DO11.10 T cells, which is, of course, specific for OVAp. Moreover, these findings suggest that the carbohydrate is masking presentation of OVAp on the APC surface. We hypothesize that the hydrophilic carbohydrate epitope is placed between the MHCII-bound peptide and the TCR (see Fig. 6 below). Further characterization of MHCII-bound glycan$_p$-peptide interactions with the TCR necessitates crystallography studies.

αβTCR glycan_p recognition mediates PS-specific isotype switch

To determine whether the PS-specific IgG response to glycoconjugate immunization is due to carbohydrate recognition, we measured GBSIII-specific IgG titers in DO11.10 and wild-type BALB/c mice immunized with III-OVAp. DO11.10 mice produced no GBSIII-specific IgG; wild-type mice developed high titers (Fig. 3e). DO11.10 and wild-type mice both developed GBSIII-specific IgM, a result indicating that lack of IgG production in DO11.10 mice was not due to lack of GBSIII-specific B cells (Fig. S8). These results support the hypothesis that GBSIII recognition by the receptor of helper T cells induces PS-specific IgG secretion by B cells. Since DO11.10 mice lack the T-cell repertoire recognizing the carbohydrate epitope of glycoconjugates, DO11.10 T cells could not induce PS-specific IgG secretion. In addition, DO11.10 mice produced higher titers of OVAp-specific IgG than wild-type mice (Fig. S8), an observation indicating that a global inability to produce IgG in DO11.10 mice does not account for their failure to produce GBSIII-specific IgG antibodies.

CD4+ clones recognize and react with carbohydrate in MHCII context only

Investigating whether CD4+ T cells can recognize and react with glycan_p presented by MHCII, we generated carbohydrate-specific murine CD4+ T-cell clones. Splenocytes were collected from III-OVA-immunized BALB/c mice, enriched for CD4+ T cells, and co-cultured with iAPCs from spleens of naïve BALB/c mice in the presence of III-TT for 6 days. After purification of CD4+ T cells from these co-cultured mixed cells, immune CD4+ T cells were cloned by limiting dilution. Cloned cells were restimulated at 12-day intervals with III-OVA-pulsed iAPCs in the medium containing the T-cell culture supplement. Interleukin 2 (IL-2), IL-4, and interferon γ (IFN-γ) ELISpot assays were then conducted to verify whether the clones recognized carbohydrate epitopes (Fig. 4). Two distinct CD4+ T-cell clones secreted both IL-2 and IL-4 upon stimulation with GBSIII conjugated to any of three carrier proteins: OVA, TT, or hen egg lysozyme (HEL). Neither clone responded to unconjugated carrier proteins alone, and neither produced IFN-γ in the presence of GBSIII-containing glycoconjugates (not shown). These data indicate the existence among T cells expanded with III-OVA and III-TT of cloned CD4+ T cells recognizing only the carbohydrate portion of the glycoconjugate.

To identify the restriction elements of these carbohydrate-specific clones, we added mAbs to MHCII proteins I-A^d and I-E^d to the ELISpot assays. These antibodies completely inhibited T-cell responses to both III-OVA and III-TT (Fig. 4). Most strikingly, mAb to I-E^d inhibited IL-2/IL-4 secretion by CD4+ T-cell clone #1 (Figs. 4a, 4b), whereas mAb to I-A^d inhibited the responses of CD4+ T-cell clone #2 (Figs. 4c, 4d). These results indicate that T-cell clones #1 and #2 recognize carbohydrate epitopes in the context of I-E^d and I-A^d, respectively.

In an anti-TCR blocking experiment (Fig. S9), we preincubated CD4+ T-cell clones with various concentrations of Fab fragments of a mAb to either αβTCR or TCR[25]. We incubated these cells with iAPCs in the presence of each antigen in culture medium and performed IL-2 ELISpot assays 24 h later. The mAb to αβTCR, but not that to γδTCR, inhibited activation of both T-cell clones in a dose-dependent manner (Fig. S9). Thus stimulation of T-cell clones by carbohydrate epitopes is accomplished through the TCRs.

An archetypical knowledge-based glycoconjugate construct

We considered whether our insights into the mechanisms involved in activation of the adaptive immune system by glycoconjugate vaccines might guide the design and synthesis of a future generation of knowledge-based vaccines. The III-OVAp vaccine we designed for some of our mechanistic studies was of interest in this regard. We calculated that a glycan_p-peptide with ~1 peptide per 8 repeating units of PS could be created by depolymerization of

GBSIII. This ratio would be likely to translate as a several-fold increase in the number of glycan$_p$-peptides that could be presented from each processed PS molecule over the number presented with standard glycoconjugates (Fig. S10). The presentation of more carbohydrate epitopes per unit of vaccine could in turn enhance immunogenicity. We compared the immune response to this vaccine designed to maximize presentation of glycan$_p$ epitopes (III-OVAp) with that to a glycoconjugate (III-OVA) constructed by the technology currently used in the industrial manufacture of several vaccines, including the GBS vaccines now in clinical trials (Fig. 5). Although neither vaccine has been optimized for carrier protein or peptide, immunization of mice with III-OVAp (carbohydrate content, 0.2 μg/dose) yielded GBSIII-specific IgG titers of 66 μg/ml—i.e., ~80-fold higher than those following immunization with III-OVA at the same carbohydrate dose (Fig. 5a). In fact, the IgG titer evoked by III-OVAp immunization with a carbohydrate dose of 0.2 μg/mouse was higher than that (53 μg/ml) elicited in III-OVA-immunized mice by a 100-fold greater carbohydrate dose (20 μg/mouse; Fig. 5a). Despite the dramatic difference in GBSIII-specific IgG titers (~11-fold at the 2-μg dose), GBSIII IgM titers were identical in III-OVAp-immunized and III-OVA-immunized mice (not shown).

Higher IgG levels are not necessarily the only factor to consider in potential vaccine efficacy. Other factors include epitope specificity, affinity, avidity, subclass, and functionality. Ultimately, however, the bottom-line predictor of vaccine efficacy is prevention of disease. These two vaccines were compared in a neonatal mouse protection assay that is the current benchmark for GBS vaccine efficacy[26,27]. Because severe GBS infection occurs primarily in the immediate postnatal period, vaccine-induced IgG antibodies must cross the placenta and protect infants during this period. Simulating human vaccine delivery, we immunized adult female mice with III-OVA or III-OVAp (2 μg of carbohydrate/dose); after vaccination, female mice were housed with males and impregnated. Pups were challenged with 5 LD$_{50}$ of live type III group B *Streptococcus*. All pups of III-OVAp-immunized mice survived, whereas only 65% of pups of III-OVA-immunized mice survived (Fig. 5b). Thus the antibody levels achieved with this archetypical new-generation vaccine are correlated with efficacy in an animal model. This experiment suggests that knowledge of the mechanisms responsible for glycoconjugate processing and presentation permits the design of more efficacious vaccines.

DISCUSSION

The insights reported herein raise the possibility of novel glycoconjugate vaccines with chemical and physical properties specifically designed in light of information on antigen presentation. Until now, glycoconjugate construction has been a random process of linking two molecules (carbohydrate and protein) without due consideration of optimal design based on scientific principles. The focus on achieving a chemical link between PS and protein by trial and error has necessitated lengthy, complex development efforts. The findings detailed here, which offer a rational explanation for how conjugates work, may well render vaccine development a more straightforward, directed process.

Interactions of the mammalian immune system with protein antigens have been investigated for many years. More information has recently become available on significant interactions of the adaptive immune system with nonprotein antigens. For instance, lipids and glycolipids are presented to T cells by MHCI-like CD1 family molecules (e.g., CD1b, CD1d)[28–31]. Glycopeptides containing monosaccharides or small oligosaccharides generated by processing of natural glycoproteins and their synthetic derivatives (e.g., type II collagen, HEL, or viral nucleoprotein–derived glycopeptides)[32–37] are recognized by CD4+ or CD8+ T cells. Moreover, synthetic and natural glycopeptides containing tumor-associated mono- or oligosaccharides are recognized by T cells[38,39]. A number of synthetic vaccines

comprising tumor-associated glycopeptides elicit humoral immune responses against cancer cells expressing tumor-associated carbohydrates.[40] One class of complex carbohydrates (zwitterionic PSs) activates T cells[5,16]—an unexpected observation, given the immunologic paradigm categorizing carbohydrates as strictly T cell–independent antigens. The original hypothesis for glycoconjugate action[3,15] was based on the failure of most pure PSs to elicit IgG memory in mice. This paradigm presumes that elicitation of T-cell help by glycoconjugates is attributable to MHCII presentation of peptides (derived by protein processing) to the TCR. In general, carbohydrates can be processed to smaller size in APC endolysosomes[17] but fail to bind directly to MHCII, are not presented to T cells, and consequently are indeed "T cell–independent"[2,5].

Several reports have suggested that attributing all of the T-cell activation in glycoconjugate immunization to peptide presentation may reflect incomplete information. Greenberg et al.[41] showed that infants vaccinated with *H. influenzae* type b (Hib) PS conjugated to CRM_{197} were primed for a secondary anti-Hib response to an Hib-TT conjugate. This observation suggested the possibility of carbohydrate presentation to T cells since the use of heterologous carriers did not interfere with the booster response. Colino et al.[11] explored how a purified pneumococcal glycoconjugate induces PS-specific memory, whereas intact pneumococci do not. These investigators suggested that covalent protein–PS bonding in a glycoconjugate—rather than a peptide alone—may be responsible for the T cell–dependent humoral immune response to the PS. This finding is consistent with our data showing that carbohydrate presentation to CD4+ T cells in the context of a covalently bound peptide can induce PS-specific adaptive immune responses. Supporting our findings, a recent report based on confocal microscopy images suggests the interesting possibility of localization of the carbohydrate component of a pneumococcal glycoconjugate to the APC surface[42].

The specific conditions used here (single carrier, conjugate, conjugation chemistry, and one well-defined MHC-restricted $glycan_p$-peptide tested in inbred mice) will need to be expanded upon to generate relevant vaccines for clinical use. Other facets of the immune response to glycoconjugates that require study include the role of B-cell subsets with different functions (e.g., B1 and marginal-zone B cells) and the role of B-cell maturation. A key issue is whether the same adaptive immune response to these PSs can be expected in humans.

In our new working model (Fig. 6), whose ultimate design and understanding will require structural studies, we discovered that there are T-cell populations that recognize carbohydrate epitopes derived by APC processing of conjugate vaccines and that, when presented by MHCII, these epitopes recruit T-cell help for the induction of adaptive immune responses to these vaccines. Understanding interactions between $glycan_p$-peptide, MHCII and TCR at the structural level is of very high importance. Data provided in this manuscript map out a conceptual framework upon which to base future investigations of these interactions.

In short, this study demonstrates that some of the variable immunogenicity previously considered PS specific may actually be related to efficiency of carbohydrate presentation. New-generation vaccines with optimal, high-density presentation of carbohydrate epitopes could play a significant role in prevention and control of many diseases. Glycans conjugated to proteins and lipids were recently cited as possibly "the most abundant structurally diverse class of molecules in nature"[43]. An understanding of the basic mechanisms governing glycoconjugate processing and presentation may be crucial to an understanding of immunity to microbial infections.

METHODS

Mice, cell lines

We purchased wild-type (C57BL/6NTac), MHCI-deficient (*B2m^tm1Jae^*), and MHCII-deficient (*H2-Ab1^tm1Gru^*) mice (female, 6–8 weeks old) from Taconic Farms. We used Raji B cells and MHCII-deficient Raji cells (RJ2.2.5) for antigen presentation studies. We used wild-type BALB/c mice (either from Taconic Farms or from Jackson Laboratories) and OVAp-specific TCR transgenic mice [C-Cg-Tg(DO11.10)10Dl0/J] from Jackson Laboratories for immunization, and we used primary cells from the immunized mice in T-cell assays. All animal experiments were approved by Harvard Medical Area Standing Committee on Animals (Animal protocol # 866).

Antigens

We isolated and purified GBSIII from type III group B *Streptococcus* strain M781[44]. GBSIII was allowed to react with amine-containing peptides (e.g., lysine) of OVA (Sigma), TT (North American Vaccine Inc.), or OVAp (N-acetyl-ISQAVHAAHAEINEAGRESGK; Genscript) forming PS-protein/peptide conjugates[44].

Immunizations

We immunized groups of 4–6 mice i.p. on days 0 and 14 with the antigen of interest mixed with 0.5 mg of $Al(OH)_3$ gel adjuvant. Mice receiving injections of $Al(OH)_3$ only served as negative controls.

Measurement of specific serum antibodies

We bled mice from the tail vein (~4 drops of blood from each) on days 0, 14, and 21 of the immunization protocols. We determined levels of GBSIII-specific or carrier-specific antibodies in dilutions of sera by solid-phase ELISAs as described previously[3].

Co-immunoprecipitation

We incubated Raji B cells (10^8), RJ2.2.5 cells (10^8), or splenic mononuclear cells from wild-type, MHCI-deficient, or MHCII-deficient mice (2×10^8) with 0.4 mg of [^{3}H]III-OVA for 15–18 h. We isolated cell membrane fractions by differential centrifugation and solubilized at 4 C with lysis buffer[16]. Supernatants obtained from Raji and RJ2.2.5 cells were incubated overnight with mixing at 4°C in the presence of protein A agarose beads containing 25 μg of mAb to HLA-DR (clone L243, BioLegend), HLA-DQ (clone HLADQ1, BioLegend), HLA-DP (clone HI43, BioLegend), or LAMP-1 (clone 25, BD Biosciences) or a polyclonal Ab to CD19 (Cell Signaling Technology). Cell membrane fractions obtained from mouse splenic mononuclear cells were incubated with protein G agarose beads containing 25 μg of mouse I-A/I-E antibody (clone M5/114.15.12, BioLegend). After overnight incubation, we washed the beads and then boiled for 20 min in 10% SDS-3 M NaCl and analyzed the supernatants by Superose 12 chromatography.

GBSIII presentation by BMDCs

We incubated BMDCs from wild-type or MHCII-deficient mice for 18 h at 37 C with either GBSIII or III-OVA. After incubation, we washed cells 5 times with 1X PBS and then labeled at 4 C (to ensure cell surface labeling) with a GBSIII-specific mAb (IgG2a) followed by AlexaFluor647-conjugated anti-mouse IgG secondary antibody (Invitrogen). We tested surface staining by flow cytometry (FACSCalibur System).

In vitro T-cell proliferation assays

We conducted mouse T-cell proliferation assays as described previously[17]. In brief, irradiated splenic mononuclear cells (iAPCs; 10^5/well) from naïve mice were co-cultured for 4 days with CD4+ T cells (10^5/well) purified from the spleens of each immunized mouse strain (Mouse CD4 Subset Column Kit, R&D Systems) and were stimulated *in vitro* with the specified antigens (Fig. 3). Proliferation was measured by [^{3}H]thymidine incorporation 8 h before harvesting.

T-cell clone generation

We primed groups of BALB/c mice subcutaneously (s.c.) with 4 µg of III-OVA emulsified in complete Freund's adjuvant and boosted 3 weeks later by s.c. injection of 4 µg of III-OVA emulsified with incomplete Freund's adjuvant. One week after boosting, we isolated lymphocytes from draining lymph nodes and cultured in vitro in the presence of III-TT (100 µg/ml) for 6 days. After this incubation, we purified CD4+ T cells with Lympholyte M (Cedarline Laboratories) and incubated for an additional 6 days in cDMEM, 10% FCS, and 10% T-cell culture supplement. Meanwhile, splenocytes from III-OVA-immunized mice (2 doses) were isolated and depleted of CD3+ T cells by anti-CD3 beads (Miltenyi) for use as APCs. These splenocytes (3×10^6) were irradiated and then cultured with 1×10^6 in vitro–expanded CD4+ T cells (see above) in the presence of III-TT (100 µg/ml) for 6 days. We then cloned these highly enriched CD4+ T cells by limiting dilution as described previously[45].

ELISpot assays

We co-cultured CD4+ T-cell clones (5×10^4) with irradiated syngeneic splenocytes (5×10^5) in the presence of specified antigens (Fig. 4). In some cases, we added a mAb (50µg/ml) to I-A^d (BD Bioscience) or I-E^d (Biolegend) or an isotype control to the wells. Finally, we determined the relative numbers of antigen-specific CD4+ T cells secreting IL-2, IL-4, or IFN-γ by ELISpot assays[46]. For the anti-TCR blocking experiment (Fig. S9), we preincubated cells from each CD4+ T-cell clone for 30 min with the indicated concentrations of Fab fragments of mAb to either αβTCR (H57-597) or γδTCR (GL3), as previously performed[25].

Neonatal protection assay

We used a maternal immunization–neonatal challenge model of GBS infection in mice to assess the protective efficacy of GBS vaccines[26,27]. We vaccinated female BALB/c adult mice (Taconic) with GBSIII, III-OVA, or III-OVAp (i.p. immunizations on days 0, 14, and 28). Mice were bred 1 week after receiving the third dose of vaccine. GBSIII cultures (strain M781) were injected i.p. (in a volume of 0.05 ml) into neonatal mouse pups (<36 h old). We assessed survival of pups 24, 48, and 72 h after challenge.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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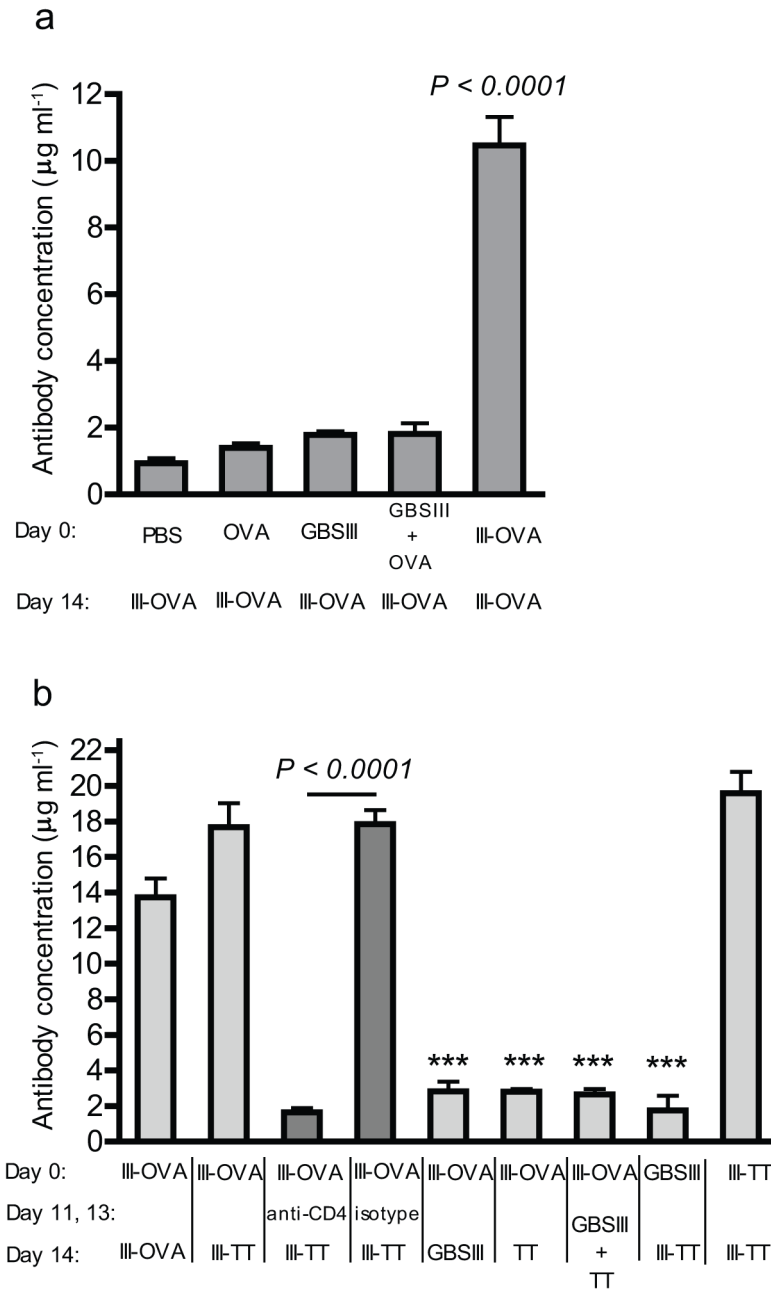
**Figure 1. GBSIII-specific IgG secretion can be stimulated by CD4+ T cells recognizing carbohydrate epitopes**

(**a** and **b**) Concentration of IgG antibody to GBSIII in BALB/c mice primed (day 0) and boosted (day 14) with different antigen combinations, as measured by ELISA in serum obtained on day 21. *** As shown in panel **b**, mice primed with III-OVA and boosted with unconjugated GBSIII and mice primed with unconjugated GBSIII and boosted with III-TT had significantly lower specific IgG levels than either mice primed and boosted with III-OVA or mice primed with III-OVA and boosted with III-TT (p < 0.0001). None of the mice had detectable antibodies to either GBSIII or OVA before immunization (data not shown).
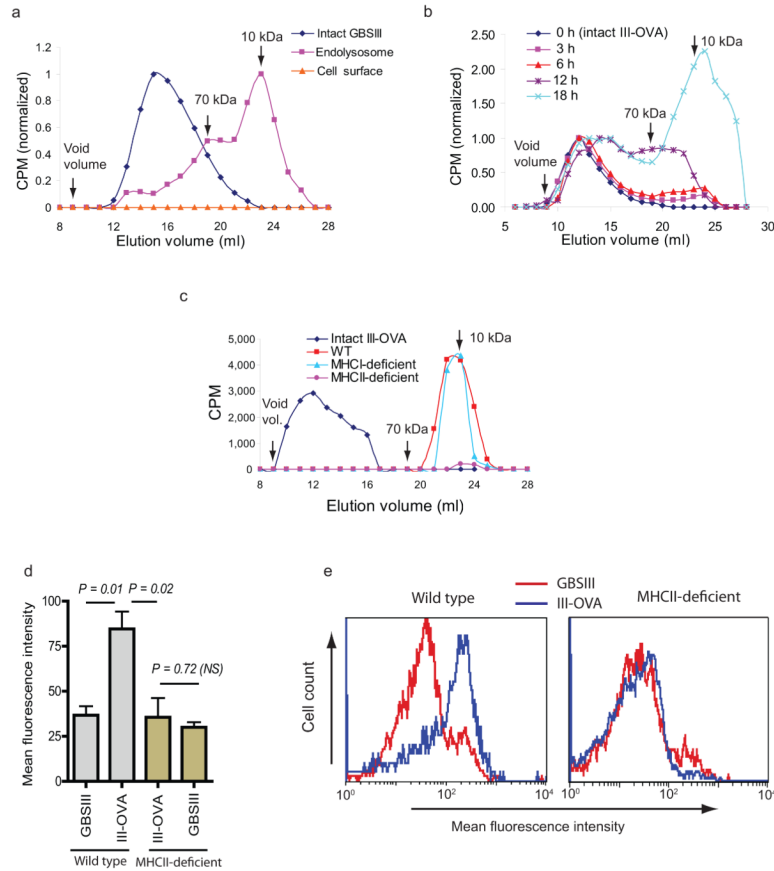
Figure 2. Processing and presentation of a glycoconjugate vaccine. (a) Endosomal processing and failure of presentation of unconjugated GBSIII
Lysates of Raji B-cell endosomes after 18 h of incubation with unconjugated [³H]GBSIII were analyzed by chromatography and found to be processed to smaller molecular size. Cell membranes were co-immunoprecipitated with HLA-DR mAbs, and no [³H]GBSIII was found in the co-IP product. **(b) Processing kinetics of GBSIII conjugated to ovalbumin (III-OVA).** [³H]III-OVA was analyzed from Raji B-cell endolysosomes (after incubation for 3, 6, 12, and 18 h), and molecular size distribution of the processed polysaccharide was determined. **(c) Requirement for MHCII in the presentation of GBSIII epitopes in the III-OVA conjugate vaccine.** The elution profile of [³H]III-OVA was obtained from surface extracts of mouse splenic mononuclear cells (2x10⁸) after incubation with [³H]III-OVA and co-immunoprecipitated with anti-I-A^b antibody. **(d, e) GBSIII epitopes on the surface of Raji cells incubated with III-OVA.** Flow cytometric analysis of wild-type and MHCII-deficient bone marrow dendritic cells after incubation (18 h) with unconjugated GBSIII or III-OVA was followed by surface staining of the cells with mAb to GBSIII. WT, wild-type.
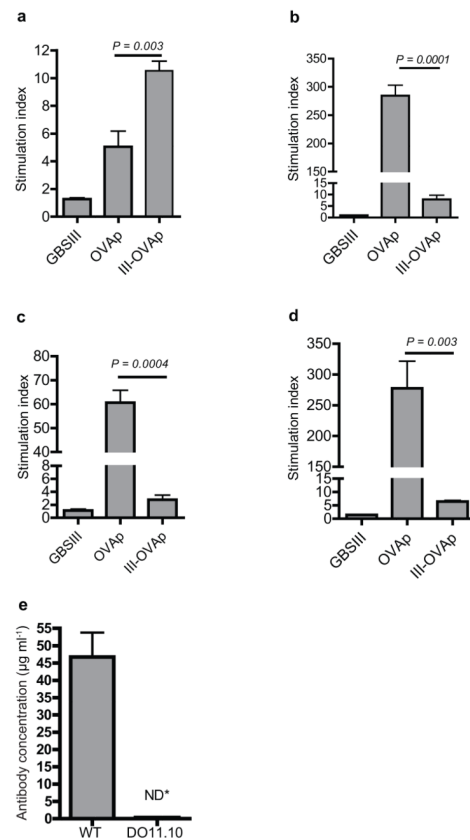
Figure 3. T cells distinguish OVAp from III-OVAp, as presented by APCs
(a–d) Wild-type (WT) BALB/c (**a**) and DO11.10 (**b**) mice were immunized with III-OVAp.
Wild-type (WT) BALB/c (**c**) and DO11.10 (**d**) mice were immunized with OVAp. Naïve
irradiated splenic mononuclear cells (iAPCs; 10^5/well) were co-cultured for 4 days with
CD4+ T cells (10^5/well) from each mouse strain and stimulated with III-OVAp, OVAp, or
GBSIII. Proliferation was measured by [^{3}H]thymidine incorporation. Data are expressed as
the mean stimulation index. Controls included stimulation by GBSIII or no antigen; all
negative controls had a stimulation index of ~1. **(e)** GBSIII-specific IgG concentrations on
day 21 after immunization with III-OVAp in DO11.10 and WT BALB/c mice (same mice as
in Figs. 3a-d). *ND, not detectable. Neither WT nor DO11.10 mice had detectable
antibodies to either GBSIII or OVAp before immunization (not shown).
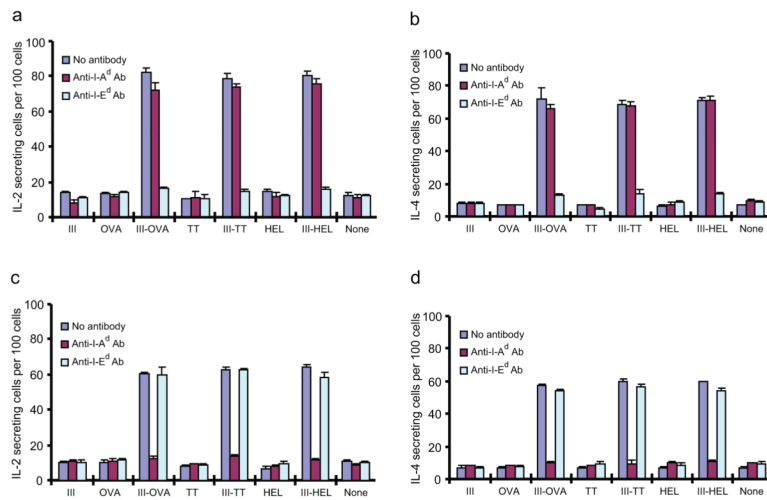
Figure 4. Two CD4+ T-cell clones specifically recognize the carbohydrate portion of a glycoconjugate vaccine in the context of MHCII on APCs
(a–d) ELISpot assays for the detection of CD4+ T-cell clones (clone #1 (a, b) and clone # 2 (c, d))secreting IL-2 (a, c) and IL-4 (b, d) were conducted with several antigens, first in the absence and then in the presence of mAb to I-A^d or I-E^d. Isotype controls for mAb to I-A^d (IgG2b isotype) or mAb to I-E^d (IgG2a isotype) did not inhibit IL-2 or IL-4 production in either T cell clone (data not shown). Irradiated naïve mouse splenocytes were used as APCs.
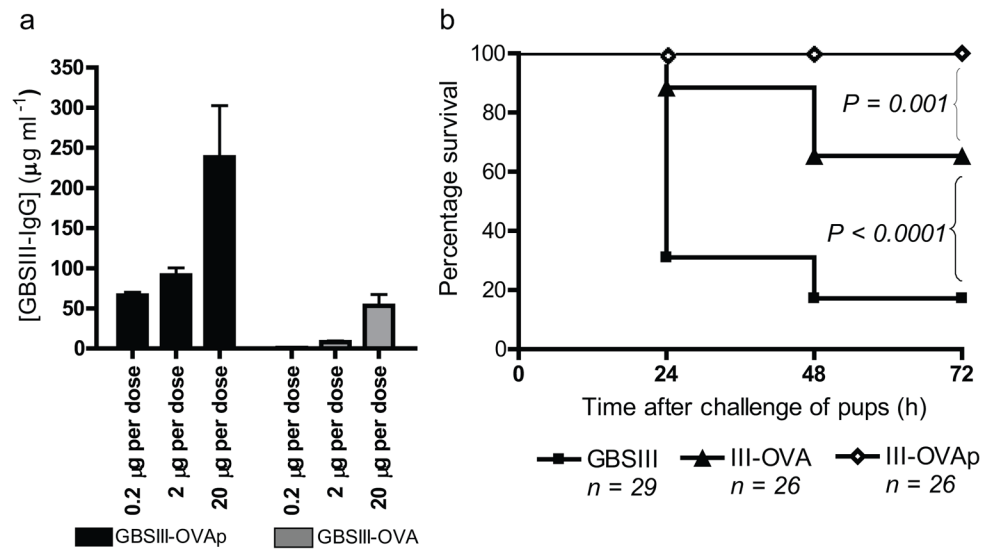
Figure 5. Immunization with III-OVAp induces a significantly stronger humoral immune response and greater protection than immunization with III-OVA

(a) Groups of BALB/c mice (6 mice per group) were vaccinated three times (days 0, 14, and 28) with three different doses (0.2 μg, 2 μg, or 20 μg; as carbohydrate) of either III-OVA or III-OVAp. GBSIII-specific IgG titers were measured in serum obtained on day 35. **(b)** Survival of pups born to III-OVAp-, III-OVA-, or unconjugated GBSIII–immunized dams and challenged with type III group B *Streptococcus* (*n*: number of challenged pups in each group).
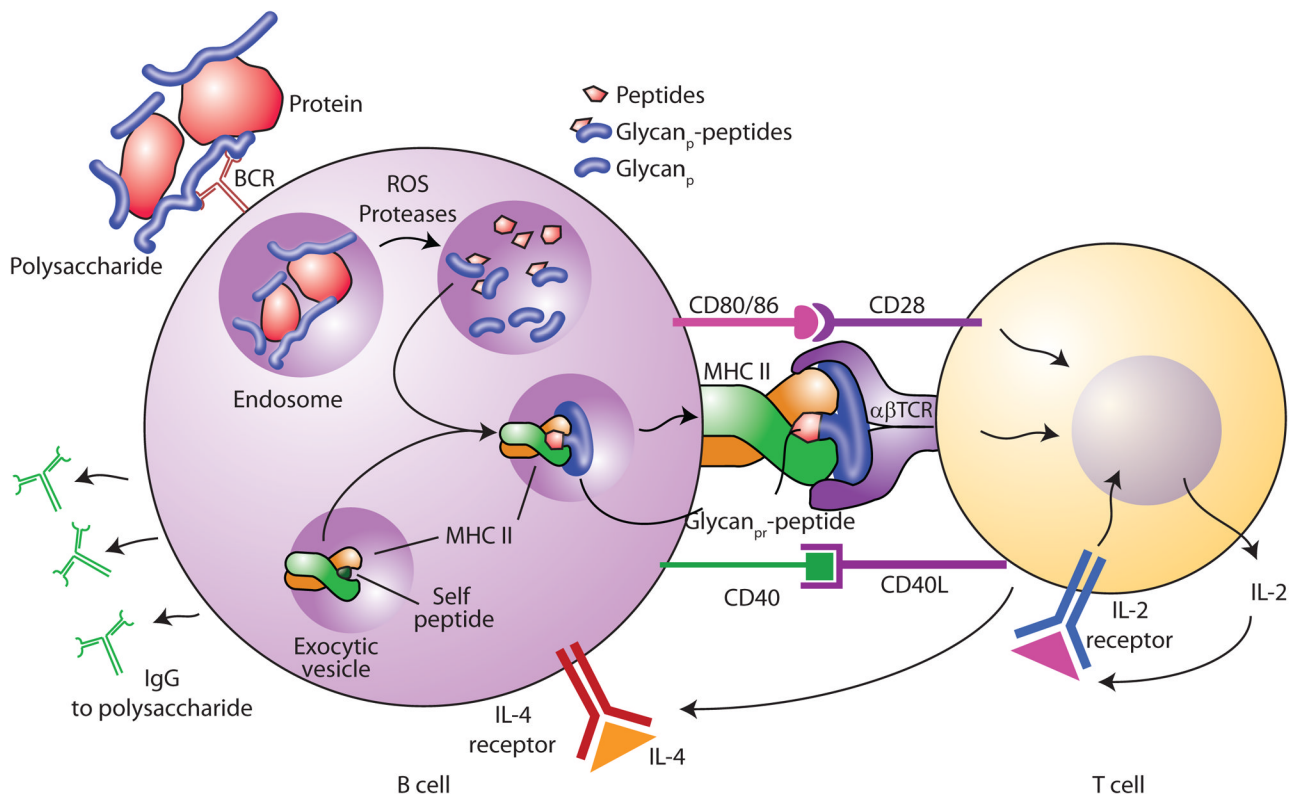
Figure 6. Mechanism of T-cell activation by glycoconjugate vaccines: a new working model
Schematic representation shows the steps in antigen processing and presentation of
glycoconjugate vaccines resulting in helper CD4+ T-cell induction of B-cell production of
IgG antibodies to the polysaccharide. (1) The carbohydrate portion of the glycoconjugate
binds to and cross-links the receptor of a B cell (BCR) whose specific destiny is to produce
antibodies to the polysaccharide. (2) The glycoconjugate is internalized into an endosome of
the B cell. (3) The carbohydrate portion of this GBSIII glycoconjugate is processed in the
endolysosome by ROS into saccharides composed of smaller numbers of repeating units
than the full-length polysaccharide used in construction of the vaccine. The protein portion
is processed by acidic proteases into peptides. Processing of both the protein and the
carbohydrate portions of glycoconjugates generates $glycan_p$-peptides with a molecular size
of ~10 kDa. (4) MHCII binding of the peptide portion of the $glycan_p$-peptide allows the
presentation by MHCII of the more hydrophilic carbohydrate to the $\alpha\beta$ receptor of CD4+ T
cells ($\alpha\beta$TCR). (5) The $\alpha\beta$ receptor of CD4+ T helper cells recognizes and responds to the
non-zwitterionic saccharide presented in the context of MHCII. (6) Activation of the T cell
by the carbohydrate/MHCII, along with co-stimulation, results in T-cell production of
cytokines such as IL-4 and IL-2, which in turn induces maturation of the cognate B cell to
become a memory B cell, with consequent production of carbohydrate-specific IgG
antibodies.