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Isolation of carbohydrate-specific CD4⁺ T cell clones from mice after stimulation by two model glycoconjugate vaccines

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Abstract

Here we describe how to isolate carbohydrate-specific T cell clones (for which we propose the designation ‘Tcarbs’) after stimulation by two glycoconjugate vaccines. We describe how to prepare, purify and characterize two model glycoconjugate vaccines that can be used to generate Tcarbs. These glycoconjugate vaccines (GBSIII-OVA and GBSIII-TT) are synthesized by conjugation of type III group B streptococcal polysaccharide (GBSIII) to ovalbumin (OVA) or tetanus toxoid (TT). Upon immunization of mice with GBSIII-OVA, carbohydrate epitopes are presented to and recognized by CD4⁺ T cells. Subsequently, polysaccharide-recognizing CD4⁺ T cells are expanded *in vitro* by stimulating splenic CD4⁺ T cells with GBSIII-TT. The sequential use of two distinct glycoconjugate vaccines containing the same polysaccharide conjugated to heterologous carrier proteins selects for and expands carbohydrate-specific T cells. This protocol can readily be adapted to study the stimulation of the immune system by alternative glycoconjugate vaccines. This protocol takes 1–2 years to complete.

INTRODUCTION

Here we describe detailed protocols for the isolation of carbohydrate-specific CD4⁺ T cell clones (Tcarbs) derived from stimulation by two model glycoconjugate vaccines, whose generation was first described in ref. 1. Isolation of Tcarbs is essential to more fully understand the immune interactions between glycoconjugate vaccines and the immune system. Elucidation of the structural requirements for T cell stimulation will enable design and synthesis of knowledge-based vaccines that are highly protective against many different bacterial pathogens. This protocol can be adapted to study the presence and isolation of Tcarbs stimulated by epitopes generated from capsular polysaccharides (CPSs) of other

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pathogens such as *Haemophilus influenzae*, *Streptococcus pneumonia* and *Neisseria meningitidis*.

Most CPSs expressed by pathogenic bacteria have been considered T cell-independent antigens^{2–6} because they fail to induce T cell-mediated immune responses such as IgM-to-IgG class switching⁴, a booster antibody response (i.e., a secondary antibody response after recall immunization) and T cell memory⁵. T cell help for B cells that produce IgG antibodies to the polysaccharide component^{6–12} can be recruited by immunization with a glycoconjugate vaccine prepared by covalent coupling of a CPS to a carrier protein. CD4⁺ T cells recognize peptides in the context of major histocompatibility class II (MHCII) molecules through their variant T cell receptor (TCR). Therefore, the traditional view of the mechanism for induction of an IgG memory-type immune response by glycoconjugate vaccines has been that the peptides generated from the carrier-protein portion of the conjugate vaccine are presented to CD4⁺ T cells, with consequent induction of B cell secretion of IgG antibodies to the carbohydrate portion.

We recently showed that, upon uptake into the endolysosomes of antigen-presenting cells (APCs), the carbohydrate portion of a model glycoconjugate vaccine undergoes depolymerization along with digestion of the carrier protein to create carbohydrate T cell epitopes in the form of peptide-carbohydrate conjugates, which we have termed glycan_p peptides¹. These epitopes formed in the endolysosome bind to MHCII through the peptide portion and are subsequently presented on the APC surface with the carbohydrate epitope positioned for recognition by the TCR. Recognition of the carbohydrate in the presence of MHCII drives CD4⁺ T cell help for antibody-producing B cells. On the basis of these results, we have formulated a mechanism that revises classical teaching about antigen presentation of glycoconjugates and in which T cell recognition of carbohydrates is central.

Comparison with other methods

Several glycopeptide-specific T-hybridoma cell lines and T cell clones have been established by other investigators using an immunization regimen distinctly different from ours^{13–15}. In these studies, mice were immunized with either a natural glycopeptide/glycoprotein or a glycopeptide consisting of a monosaccharide or an oligosaccharide conjugated to a synthetic peptide that corresponded to a CD4⁺ T cell epitope of a model antigen. The same glycopeptide was used to stimulate T cells *in vitro*. CD4⁺ T cell recognition of glycopeptides containing a small number of sugars generated by the processing of natural glycoproteins (e.g., type II collagen or hen egg lysozyme glycopeptides)^{13,15} is dependent upon the presentation of a few glycosylated residues on a peptide. In these protocols, the majority of immune lymphocytes obtained might have been CD4⁺ T cells specific for the peptide portion of the glycopeptide used for both *in vivo* priming/boosting and *in vitro* re-stimulation, and the frequency of carbohydrate-specific T cells might have been diminished. In contrast, we opted to immunize mice and re-stimulate immune cells in culture with glycoconjugates containing the same polysaccharide but different carrier proteins—i.e., GBSIII-OVA and GBSIII-TT. Because immunization of mice with GBSIII-OVA enriches for both Tcarbs and OVA-specific T cells, we needed to eliminate OVA-specific T cells and selectively stimulate Tcarbs. Therefore, we conducted *in*

vitro experiments, stimulating splenic T cells from GBSIII-OVA-immunized mice with GBSIII-TT. By using this approach, we expanded T cell populations that recognized only GBSIII epitopes. We eliminated OVA-specific T cells in the splenic T cell pool because of the absence of OVA during *in vitro* expansion. In fact, after re-stimulation of immune lymphocytes with GBSIII-TT *in vitro*, we found that the frequency of carbohydrate-specific T cells was approximately one-third to one-half that of OVA-specific T cells.

In addition to the glycoconjugates used, the key steps involved in Tcarb isolation distinguish this protocol from others. To isolate Tcarbs, we had to optimize our protocol for many different variables through a number of steps, including the selection of *in vitro* stimulation procedures, the APC population and stimulation agents and incubation times that would maintain the highly sensitive Tcarbs. Below we list the crucial steps that we used to isolate Tcarbs, which would not have been isolated by traditional methods. An alternative method for Tcarb isolation is to immortalize T cells obtained from glycoconjugate-immunized mice by fusing them with a tumor cell line to obtain T cell hybridomas¹³. This approach has a major drawback, however: upon fusion of T cells with tumor cells, the T cells lose most of their *in vivo* and *in vitro* functional characteristics, and their utility for further characterization is thus limited.

This protocol is designed and optimized to isolate CD4⁺ T cells whose TCRs recognize only carbohydrate epitopes and not glycopeptides or peptides. This protocol is the first description of a method for the isolation of Tcarb clones. Several methodological steps differ from those used to isolate peptide-specific CD4⁺ T cell clones, as described in detail here.

Limitations

The limitations of our protocol for the preparation of glycoconjugate vaccines containing different polysaccharides may include the absence of a readily oxidizable terminal sialic acid in the carbohydrate to be conjugated. In such cases, carbohydrate moieties with 1,2-*cis*-diol functional groups can be sought for oxidation with periodate. Reaction conditions must be optimized for different carbohydrates. Alternative conjugation methods can also be sought^{6,16}. Limitations encountered in the isolation of Tcarbs may include different levels of viability of Tcarbs obtained from different polysaccharide antigens conjugated with carrier proteins. In our experience, Tcarbs are highly sensitive to incubation conditions and stimulation factors. Therefore, optimizing parts of the protocol (e.g., the duration of *in vitro* T cell stimulation, the concentration of antigens used for stimulation, the number and type of APCs used for stimulation) must be considered.

Applications of the method

We hope that this protocol will facilitate investigations into the interactions of glycoconjugate vaccines with Tcarbs, ultimately contributing to the development of novel, more immunogenic carbohydrate-based vaccines that are effective against many bacterial pathogens. Potential applications of this protocol include investigation of the roles of other bacterial carbohydrate antigens (e.g., those found in pathogens such as *Staphylococcus aureus*, *S. pneumoniae* and *N. meningitidis*) in the stimulation of the T cell-mediated

adaptive immune response¹⁰. In addition, other pathogenic microorganisms, including viruses, parasites and fungi, also contain carbohydrate virulence factors¹⁷. The discovery of novel approaches for enabling these carbohydrates to induce T cell activation may prove greatly beneficial in combating these disease-causing organisms.

Experimental design

The isolation of Tcarbs requires two glycoconjugate vaccines containing the same polysaccharide (e.g., GBSIII) and a different carrier protein (e.g., OVA or TT). In this protocol, we isolated Tcarb clones that are stimulated by carbohydrate epitopes generated from either GBSIII-OVA or GBSIII-TT. However, this protocol can be adapted to study Tcarbs stimulated by other carbohydrates. Depending on the structure of the polysaccharide of interest, conjugation reactions may differ. If reductive amination is suitable for the polysaccharide used (e.g., polysaccharide containing a sialic acid or a monosaccharide with vicinal diol group within its structure), then this protocol can readily be applied to the conjugation of the polysaccharide with a carrier protein. Isolation of the Tcarbs can also be adapted for use with other glycoconjugate vaccines. First, GBSIII oxidation with sodium periodate is followed by conjugation with OVA or TT via a reductive amination reaction to obtain GBSIII-OVA or GBSIII-TT. The conjugation reaction section (PROCEDURE Steps 10–16) describes the conjugation of GBSIII with OVA. In the protocol for the generation and isolation of Tcarbs, GBSIII-TT is also used as an antigen. The conjugation of GBSIII to TT is carried out in the same way as the conjugation of GBSIII to OVA.

Next, groups of BALB/c mice are immunized with GBSIII-OVA to enrich for carbohydrate-specific T cells in the lymphoid organs. Isolated lymphocytes from draining lymph nodes are then cultured *in vitro* in the presence of III-TT. After this incubation, CD4⁺ T cells are purified and incubated again, this time in medium containing 10% T cell culture supplement. Meanwhile, splenocytes from GBSIII-OVA-immunized mice are isolated, irradiated and then cultured with *in vitro*-expanded CD4⁺ T cells (as described above) in the presence of GBSIII-TT. This treatment results in the isolation of a Tcarb line. Finally, these highly enriched CD4⁺ T cells are cloned by limiting dilution. The specificity and function of T cells obtained after *in vitro* incubations and limiting dilutions can be evaluated by interleukin (IL)-2 and IL-4 ELISpot assays. Stimulations with GBSIII, OVA, TT or no antigen serve as controls in the ELISpot assays. Tcarbs can be assessed when T cells are stimulated by both GBSIII-OVA and GBSIII-TT and not stimulated by GBSIII, OVA, TT or no antigen. Stimulation of a T cell population by one of the two glycoconjugates (GBSIII-OVA or GBSIII-TT) but not by the other is interpreted as evidence of carrier-protein specificity in that T cell population. This interpretation can be validated by stimulation of the same T cell population by the carrier protein alone.

MATERIALS

REAGENTS

Conjugate vaccine preparation

- GBSIII: GBSIII is an abbreviation for the CPS isolated and purified from type III group B *Streptococcus*, which can be obtained from American Type Culture Collection (see ref. 18)
- Sodium periodate (Fisher Scientific, cat. no. AC41961)! **CAUTION** Sodium periodate is toxic upon ingestion; it is also an irritant.
- Sodium bicarbonate (Fisher Scientific, cat. no. S233)
- Sodium cyanoborohydride (Matreya, cat. no. SPL1804-100MG)
! **CAUTION** Sodium cyanoborohydride is a flammable solid; it is highly toxic upon inhalation, ingestion or skin absorption.
- OVA, grade VII (Sigma-Aldrich, cat. no. A7641)
- TT, monomeric form (North American Vaccine)
- Sodium borohydride (Sigma-Aldrich, cat. no. S9125) ! **CAUTION** Sodium borohydride emits flammable gases upon contact with water; it is toxic upon ingestion or skin absorption.
- Sodium acetate (Sigma-Aldrich, cat. no. S8750)
- Acetic acid solution, 0.1 M (Sigma-Aldrich, cat. no. 34254)
! **CAUTION** Acetic acid solution causes skin irritation; it may also cause allergic skin reactions and serious eye irritation.
- Ethylene glycol (Sigma-Aldrich, cat. no. 324558)
- Deionized water
- Dry ice ! **CAUTION** A carbon dioxide concentration of >1.5% may cause death. At higher concentrations, CO₂ displaces oxygen in air to a level below that necessary to support life.
- Bicinchoninic acid (BCA) protein assay kit (Pierce, cat. no. 23227)

Tcarb isolation

- Mice: We have purchased female BALB/c mice (6–8 weeks old) from Taconic Farms and maintained them in the Laboratory Animal Research Center at the Rockefeller University. After immunization, BALB/c mice have shown high titers of protective antibodies; therefore, we have been using this strain in our glycoconjugate vaccine studies. However, other inbred mice may be used. All our experiments involving mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Rockefeller University !

CAUTION Experiments involving rodents must conform to all relevant governmental and institutional ethics regulations.

- Inject Freund's complete adjuvant (CFA; Thermo Scientific, cat. no. 77140)
- Inject Freund's incomplete adjuvant (IFA; Thermo Scientific, cat. no. 77145)
- Lympholyte-M (Cedarline Labs, cat. no. CL5031)
- PBS (Cellgro, cat. no. 21-040-CV)
- Hank's balanced salt solution (HBSS; Gibco, cat. no. 14175)
- Fetal calf serum, heat inactivated (hiFCS; Thermo Scientific, cat. no. SH30071) ▲
CRITICAL It is important to inactivate FCS properly by heating at 56 °C for 30 min.
- Sterile filtered mouse serum, heat inactivated (hiNMS; Equitech-Bio, cat. no. SM-0100HI)
- RPMI-1640 (Cellgro, cat. no. 10-040-cv)
- Sodium pyruvate (Gibco, cat. no. 11036)
- MEM non-essential amino acids (Gibco, cat. no. 11040)
- L-Glutamine (Cellgro, cat. no. 25005238)
- MEM vitamin (Gibco, cat. no. 11120)
- 2-Mercaptoethanol (Gibco, cat. no. 21985-023)

! CAUTION 2-Mercaptoethanol is very hazardous in case of skin contact (permeator) and ingestion. It is also hazardous in case of skin contact (irritant), eye contact (irritant) and inhalation. Severe overexposure can result in death. The bottle containing this chemical should be opened and handled under a ventilation hood.

- Penicillin/streptomycin (Cellgro, cat. no. 30002201)
- Gentamicin (Gibco, cat. no. 15750-060)
- Sodium bicarbonate (Worthington, cat. no. P8N10891)
- ACK lysing buffer (Invitrogen, cat. no. A1049201)
- T cell culture supplement without ConA (TCCS, IL-2 culture supplement; BD Biosciences, cat. no. 354116)
- Multiscreen HA 96-well ELISpot plate (Millipore, cat. no. MAHAS4510)
- Anti-mouse IL-2 capture antibody, clone JES6-1A12 (Biolegend, cat. no. 503704)
- Biotin-labeled anti-mouse IL-2 antibody, clone JES6-5H4 (Biolegend, cat. no. 503804)
- Anti-mouse IL-4 capture antibody, clone 11B11 (Biolegend, cat. no. 504115)
- Biotin-labeled anti-mouse IL-4 antibody BVD6-24G2 (Biolegend, cat. no. 504202)

- Avidin-HRP (Biolegend, cat. no. 405103)
- AEC ELISpot substrate kit (BD Bioscience, cat. no. 551951)
- TruStain Fc α anti-mouse CD16/32 antibody (Biolegend, cat. no. 101320)
- FITC-labeled anti-mouse CD3 antibody, clone 145-2C11 (Biolegend, cat. no. 100306)
- APC anti-mouse CD3 antibody, clone 145-2C11 (Biolegend, cat. no. 100312)
- FITC anti-mouse CD4 antibody, clone GK1.5 (Biolegend, cat. no. 100406)
- PerCp anti-mouse CD8 antibody, clone 53.67 (Biolegend, cat. no. 100732)
- PE anti-mouse TCR β chain antibody, clone H57-597 (Biolegend, cat. no. 109208)
- Allophycocyanin-labeled anti-mouse CD4 antibody, clone GK1.5 (Biolegend, cat. no. 100412)
- LEAF purified anti-mouse CD4 antibody, clone GK1.5 (Biolegend, cat. no. 100435)
- LEAF purified anti-mouse CD8 α antibody, clone 53-6.7 (Biolegend, cat. no. 100735)
- Ethanol
- Trypan blue stain
- DMSO
- Tween-20

EQUIPMENT

Conjugate vaccine preparation

- Vial, 1 dram
- pH meter
- Magnetic stir bars
- Magnetic stir plate
- PD-10 columns (GE Healthcare, cat. no. 17-0851-01)
- Sterile syringe-driven filter unit (0.22 μ m)
- Freeze dryer (Freezemobile 12SL)
- Ultraviolet spectrophotometer (Beckman, cat. no. DU 640)
- BioLogic HR FPLC system (Bio-Rad)
- Superdex 200 HiLoad 16/60 prep grade column (GE Healthcare)
- Dialysis membrane (molecular weight cutoff (MWCO): 12,000–14,000 Da; Spectrum Labs)

- 96-Well plate, flat-bottomed
- PowerWave HT microplate reader (Bio-Tek)
- Criterion TGX any kD stain-free gel (Bio-Rad)
- Mini-PROTEAN Tetra Cell (Bio-Rad)
- ChemiDoc MP imager (Bio-Rad)

Tcarb isolation

- Perfektum Micro-Mate interchangeable syringes (Fisher Scientific, cat. no. 5014)
- Popper micro-emulsifying needles (Fisher Scientific, cat. no. 14-825-17E)
- BD Falcon cell strainer, 40 μ m, nylon (BD Biosciences, cat. no. 352340)
- Glass slides (Fisherbrand, cat. no. 12-550-016)
- Needles: 21-G, 1 inch (BD Biosciences, cat. no. 305165); 25-G, 5/8 inch (BD Biosciences, cat. no. 305122)
- Plastic tubes: 15 ml (Falcon, cat. no. 352196); 50 ml (Falcon, cat. no. 352070); 1.5 ml (Eppendorf, cat. no. 3810)
- Conical tubes
- Culture plates and dishes: 96-well (Falcon, cat. no. 353072); 24-well (Falcon, cat. no. 353047); 12-well (Falcon, cat. no. 353043); six-well (Falcon, cat. no. 353046); 60- $\times$ 15-mm polystyrene Petri dish (Falcon, cat. no. 351007)
- BD LSRII flow cytometer (BD Biosciences)
- ImmunoSpot reader (Cellular Technology)

REAGENT SETUP

Acetate buffer 0.1 M (pH 5) Adjust the pH to 5 by titrating sodium acetate solution with 0.1 M acetic acid. This solution can be stored at 4 °C.

We prepare fresh solution every 2 months.

GBSIII-OVA solution Reconstitute GBSIII-OVA with PBS at a concentration of 1 mg ml⁻¹ and sterilize by passage through a filter (pore size, 0.22 μ m). This solution can be stored at 4 °C for up to ~4 weeks under sterile conditions. Any sign of cloudiness or precipitation in the solution may indicate aggregation, degradation or contamination of the glycoconjugate; therefore, when these signs are observed, prepare a fresh solution.

GBSIII-TT solution Reconstitute GBSIII-TT with PBS at a concentration of 1 mg ml⁻¹ and sterilize by passage through a filter (pore size, 0.22 μ m). This solution can be stored at 4 °C for up to ~4 weeks under sterile conditions. Any sign of cloudiness or precipitation in the solution may indicate aggregation, degradation or contamination of the glycoconjugate; therefore, when these signs are observed, prepare a fresh solution.

GBSIII-OVA/CFA emulsion Mix a 400- μ l volume of GBSIII-OVA at 1 mg ml⁻¹ with 600 μ l of PBS; then, emulsify with 1 ml of CFA by interchangeable syringes and emulsifying needles. Use freshly made emulsion for immunizations.

▲ **CRITICAL** To ensure that the emulsion is well formed, centrifuge the solution in a 1.5-ml Eppendorf tube at 800g for 1 min and confirm the lack of oil separation.

GBSIII-OVA/IFA emulsion Mix a 400- μ l volume of GBSIII-OVA at a concentration of 1 mg ml⁻¹ with 600 μ l of PBS; next, emulsify with 1 ml of IFA by interchangeable syringes and emulsifying needles. Use freshly made emulsion for immunizations.

Complete RPMI medium (CRPMI) This medium contains RPMI-1640, sodium bicarbonate (2 g per liter), 2-mercaptoethanol (5×10^{-5} M), L-glutamine (100 $\times$), sodium pyruvate (100 $\times$), MEM non-essential amino acids (100 $\times$), MEM vitamin (100 $\times$), penicillin/streptomycin (100 $\times$), gentamicin (10 μ g ml⁻¹). Store this preparation at 4 °C under sterile conditions for up to 1 week.

Washing medium Washing medium is CRPMI-1640 supplemented with 5% (vol/vol) hiFCS. This medium can be stored at 4 °C. We prepare fresh medium every 2 weeks.

FACS buffer PBS plus 2% (vol/vol) hiFCS. This medium can be stored at 4 °C. We prepare fresh medium every 2 weeks.

PROCEDURE

GBSIII oxidation ● TIMING 1 d

- 1| Dissolve GBSIII (10 mg) to a concentration of 20 mg ml⁻¹ in 0.1 M acetate buffer (pH 5) in a 1-dram vial.

▲ **CRITICAL STEP** For this protocol, procedures are written for 10 mg of GBSIII as a starting material. However, various amounts of GBSIII (from ~1 mg to ~30 mg or more) can be used. The largest amount of GBSIII that we conjugated with OVA was 30 mg. The conjugation reaction can be scaled up.

- 2| Prepare 0.01 M sodium periodate solution in 0.1 M acetate buffer.
- 3| Calculate the amount of sodium periodate needed for oxidation of the side chain sialic acid residues located in each repeating unit of GBSIII (Fig. 1). Treatment of GBSIII with sodium periodate yields a reactive aldehyde at position C-8 of the terminal sialic acid of the repeating unit of GBSIII (ref. 18).

▲ **CRITICAL STEP** To achieve approximately equal weight contents of GBSIII and OVA in the final GBSIII-OVA product, the amount of sodium periodate is optimal at 0.5 molar equivalents of GBSIII. Therefore, per mole of each repeating unit of GBSIII, 0.5 mol of sodium periodate is needed for an oxidation reaction; that is, 10 mg of GBSIII (9.8 μ mol of repeating units) is reacted with 490 μ l of 0.01 M sodium periodate (4.9 μ mol).

- 4| While stirring the GBSIII solution on a stir plate at room temperature (RT, 25 °C), add sodium periodate (490 µl).
- 5| Incubate the reaction mixture in the dark for 90 min at RT.
▲ CRITICAL STEP Aldehydes are light sensitive. To prevent any decomposition of the aldehydes formed, the reaction should be performed in the dark.
- 6| Quench any unreacted sodium periodate by the addition of 100 µl of ethylene glycol.
- 7| Desalt and purify the oxidized GBSIII on a Sephadex G-25 M desalting column (PD-10 column) in deionized water according to the manufacturer's protocol. Collect the oxidized GBSIII fraction (3.5 ml) and pass it through a sterile filter (pore size, 0.22 µm).
- 8| Freeze the oxidized GBSIII fraction on dry ice and lyophilize it overnight by freeze drying.
- 9| Weigh the oxidized GBSIII to determine the amount recovered. The yield in this reaction is ~80% (~8 mg of oxidized GBSIII).
▲ CRITICAL STEP Although oxidized GBSIII can be stored in the dark at -20 °C as a powder, as aldehydes are highly sensitive functional groups and they are crucial for the conjugation reaction in the next step, we recommend continuing with the conjugation immediately after purification of oxidized GBSIII.

Conjugation reaction ● TIMING 2–4 d

- 10| Dissolve oxidized GBSIII (8 mg) to a concentration of 20 mg ml⁻¹ in 0.1 M sodium bicarbonate (pH 8.3) in a 1-dram vial.
- 11| Weigh OVA to obtain a 1.5 weight equivalent of GBSIII (i.e., 12 mg). Dissolve OVA to a concentration of 20 mg ml⁻¹ in 0.1 M sodium bicarbonate (pH 8.3) in a 1-dram vial.
▲ CRITICAL STEP The amount of OVA is optimized at a 1.5 weight equivalent of GBSIII to achieve a 40–60% OVA weight content in the final GBSIII-OVA product.
- 12| Transfer the OVA solution to the GBSIII solution while stirring the GBSIII solution on a stir plate at RT under a ventilation hood.
- 13| Quickly add a molar-excessive amount of sodium cyanoborohydride (~10 mg) to the oxidized-GBSIII–OVA reaction mixture while stirring.
▲ CRITICAL STEP Sodium cyanoborohydride is a hygroscopic chemical whose exposure to air before a conjugation reaction dampens its conjugation reactivity (reductive amination reactivity). We buy this chemical in 100-mg capsules prepared under nitrogen. We break each capsule and divide its contents

into ~10-mg portions in 1-dram vials filled with nitrogen gas under the ventilation hood. Furthermore, we store these vials in a desiccator at RT.

? TROUBLESHOOTING

- 14|** Incubate the vials at RT for 1 h. Next, transfer the reaction mixture to a 37 °C room (or place the vial in a 37 °C water bath on a stir plate) and continue stirring. Reaction yields are optimized at 37 °C and are higher than those at RT.

▲ CRITICAL STEP Gelling or precipitation of the reaction mixture takes place if multiple polysaccharide and protein molecules spontaneously interact to form matrix-like, cross-linked conjugates that are too large to remain in solution. Conjugation reactions involving GBSIII almost never yield precipitates, because GBSIII is relatively small (~100 kDa) in comparison with some other bacterial polysaccharides (e.g., >100 kDa) that are more susceptible to precipitation. Nevertheless, two actions will prohibit any precipitation of the conjugation product. First, keep the reaction vial at RT for the first hour before transferring it to 37 °C. Second, closely monitor the reaction vial for the first hour for signs of gelling or precipitation; if such signs are seen, remove the reaction vial immediately from the 37 °C environment to RT, add 1 ml of water while stirring, and then keep the mixture at RT for the remainder of the reaction.

? TROUBLESHOOTING

- 15|** Monitor the completion of the conjugation reaction at various intervals (e.g., 0, 24, 48 and 72 h). Remove 25-μl aliquots at the specified time points, add sodium bicarbonate (0.1 M) to a final volume of 500 μl and run the solution on a Superdex 200 gel permeation chromatography (GPC) column. Completion of the reaction is documented by monitoring the elution profile of OVA in the reaction mixture on the GPC column; unlike GBSIII, OVA absorbs ultraviolet light at 280 nm. OVA in its intact form elutes from the GPC column at 78–94 ml of the elution volume. GBSIII-bound OVA elutes at an earlier elution volume (46–62 ml); this result indicates a major shift in the molecular size of the OVA-containing conjugate (Fig. 2a). The conjugation reaction is complete when there is no more change in the intensity of the GBSIII-OVA peak at the GPC elution profile of the conjugation product (Fig. 2a). Conjugation of GBSIII and OVA reached completion in 48 h under the conditions specified here (Fig. 2a). GPC separation of the reaction product (GBSIII-OVA) is described in detail in Steps 17–26.
- 16|** Terminate the conjugation reaction by adding an excessive amount of sodium borohydride (~10 mg) to the reaction vial. Aldehyde groups on the sialic acids that have not reacted with OVA are reduced to alcohol groups by sodium borohydride. Incubate the reaction mixture at RT for 1 h.

Purification and characterization of GBSIII-OVA ● TIMING 1 week

- 17|** Add sodium bicarbonate (0.1 M) to a final volume of 2 ml and pass the reaction mixture through a sterile filter (pore size, 0.22 μm).

- 18| Load the filtered solution onto a Superdex 200 GPC column that is connected to a BioLogic HR FPLC system.
- 19| Run the sample through the column at a flow rate of 1.0 ml min^{-1} , with PBS as the eluent.
- 20| Assay 2-ml fractions for their absorbances at 280 nm to determine the elution profile based on OVA (Fig. 2a).
- 21| Collect fractions corresponding to an elution volume of 50–62 ml (fractions 25–31) and dialyze them against deionized water (2 liters) in a dialysis membrane (MWCO 12,000–14,000 Da) for 48 h at 4 °C, changing the water three times.
- 22| Lyophilize the purified GBSIII-OVA (2–3 d) by freeze drying.
- 23| Weigh the purified GBSIII-OVA to determine the amount recovered. Approximately 10 mg of GBSIII-OVA should be recovered after this reaction.
- 24| Prepare a 1 mg ml^{-1} solution of GBSIII-OVA in PBS for characterization.
- 25| Measure the protein content of GBSIII-OVA by using the microplate procedure with the BCA protein assay kit. Use 25- μl replicates of GBSIII-OVA (unknown: 1 mg ml^{-1}) or GBSIII (negative control: 1 mg ml^{-1}), or use varying concentrations of OVA (we used concentrations of 1, 0.5, 0.25, 0.125 and 0.06 mg ml^{-1}) to generate a standard curve for the identification of the OVA content in GBSIII-OVA. OVA weight content should be 45% for the conjugation product (GBSIII-OVA) described here.

▲ CRITICAL STEP In ruling out the possibility that Tcarbs are driven by unconjugated OVA, it is important to confirm the absence of unconjugated OVA in purified GBSIII-OVA. To this end, perform SDS-PAGE. Load the precast SDS-PAGE gel (Criterion TGX gel) with purified GBSIII-OVA (1 mg ml^{-1} , 10 μl), GBSIII (1 mg ml^{-1} , 10 μl), OVA (1 mg ml^{-1} , 10 μl) or a protein ladder standard and run the gel according to the manufacturer's guidelines. We visualize the protein-containing bands on the gel with the ChemiDoc MP imager. This visualization technique requires the specified precast gels used here and is a stain-free alternative to Coomassie blue staining of protein bands on a gel. As shown in Figure 2b, purified GBSIII-OVA should not show an OVA band at 45 kDa. GBSIII should be loaded as a control to demonstrate that visualization is protein specific. The smeared band detected for GBSIII-OVA is typical for carbohydrate-containing molecules, as, unlike proteins, polysaccharides are heterogeneous in size and do not elute uniformly in gels. If the GBSIII-OVA contains unconjugated OVA, reseparator it on a Superdex 200 GPC column.

? TROUBLESHOOTING

- 26| Perform conjugation of oxidized GBSIII with TT. Follow the same protocol as that used for the conjugation of oxidized GBSIII with OVA, but substitute TT for OVA (Steps 10–16). The Superdex 200 GPC elution profile for the GBSIII and TT conjugation reaction (performed in the same way as GBSIII and OVA

conjugation (Steps 17–20), with the same quantities of reagents) at 72 h is provided in Figure 2c. We collected fractions corresponding to an elution volume of 46–62 ml (fractions 23–31, Fig. 2c) for purification of GBSIII-TT and processed them as described above for GBSIII-OVA purification.

Mouse immunization ● TIMING 4 weeks

- 27| Immunize three BALB/c mice with 50 µl of GBSIII-OVA/CFA emulsion (containing 10 µg of GBSIII-OVA) by subcutaneous injection at the base of the tail. Return the mice to their normal housing conditions.
- 28| Four weeks later, intraperitoneally boost the primed mice with 50 µl of GBSIII-OVA/IFA emulsion (containing 10 µg of GBSIII-OVA) and then return the mice to their normal housing conditions.

Preparation of mouse lymphocytes ● TIMING 2 h

- 29| Euthanize primed and boosted mice by CO₂ inhalation 10–14 d after boosting.
- 30| Lay each mouse on its back on the dissecting board, fixing the forelegs upward and the hind legs downward with pins.
- 31| Surface-sterilize the skin with 70% (vol/vol) ethanol/H₂O or a proprietary compound.
- 32| Cut the skin along the left caudal side of the rib cage and the abdominal midline; next, expose the spleen with sterile surgical instruments (forceps and scissors).
- 33| Remove the spleen with sterile surgical instruments and trim away the fatty tissue.

▲ **CRITICAL STEP** Do not discard the rest of the mouse, as this is needed for the isolation of lymphocytes in Step 38.

- 34| Squeeze the spleen between the rough surfaces of two sterilized glass slides to prepare a single splenocyte suspension in a 60- × 15-mm polystyrene Petri dish containing 5 ml of cold washing medium. Transfer the splenocyte suspension into a 50-ml conical tube by passage through a cell strainer (pore size, 40 µm).
- 35| Wash splenocytes by adding 20 ml of washing medium for cells obtained from one spleen, centrifuging at 250g for 5 min at 4 °C and discarding the supernatant.
- 36| To separate erythrocytes from splenocytes, resuspend the cell pellet in 5 ml of ACK lysing buffer for 5 min at RT, and then dilute the mixture with 45 ml of washing medium. Centrifuge at 250g for 5 min and remove the supernatant.
- 37| Wash the cells twice more as described in Step 35 and then count the number of splenocytes by resuspending them in 20 ml of culture medium and diluting 10 µl of the cell suspension 1,000-fold in 10% (vol/vol) trypan blue. Follow this by filling a chamber of a hemocytometer with the diluted cell suspension and

counting the number of cells under the microscope. Keep splenocytes stored on ice while isolating the lymphocytes.

- 38| Take the rest of the mouse from which the spleen was isolated in Step 33 and cut the skin along the abdominal midline and quadriceps muscles; next, expose the popliteal lymph nodes with sterile surgical instruments (forceps and scissors).
- 39| Dissect out and place the lymph nodes into a 60- × 15-mm polystyrene Petri dish containing 5 ml of cold washing medium.
- 40| In one hand, hold each lymph node with a 21-G needle locked onto a 3-ml syringe; with the other hand, gently strip out the capsule membrane of the lymph node, using a 25-G needle locked onto a 3-ml syringe. By this method, lymphocytes can be teased out from the lymph nodes and released into the medium.

▲ CRITICAL STEP It is important to gently tease lymphocytes out from the lymph node capsule in order to avoid collecting cells other than lymphocytes.

- 41| Collect the cell suspension, pass it through a cell strainer (pore size, 40 µm) and transfer the filtered cell suspension into a 50-ml conical tube.
- 42| Wash lymphocytes with washing medium by resuspending them in 10 µl of washing medium, centrifuging at 250g for 5 min and discarding the supernatant. Repeat the wash twice.
- 43| Pass splenocytes and lymphocytes through separate 40-µm cell strainers in order to remove cell clumps.

▲ CRITICAL STEP Cell clumps will ultimately hamper lymphocytes from growing in culture.

- 44| Count the number of lymphocytes in the hemocytometer by microscopy as described in Step 37. Set aside the majority of the lymphocytes to generate GBSIII-specific T cell lines by *in vitro* expansion of CD4⁺ T cells as in Steps 46–71. Set aside several million lymphocytes, keeping them on ice, for an evaluation of specificity for the carbohydrate portion of the glycoconjugate antigens. This is determined by IL-2 and IL-4 ELISpot assays, which are performed on the same day (Fig. 3a; described in Step 72A).

▲ CRITICAL STEP It is important to ascertain the presence of Tcarbs in primary immune lymphocytes before proceeding to the next step.

Generation of Tcarb lines ● TIMING 4–6 weeks

- 45| Set aside the number of splenocytes (obtained in Step 37 above) necessary to coculture with lymphocytes and irradiate them at 3,000 rad for use as APCs. Freeze down the remaining splenocytes at a density of 5×10^6 per ml in a cryovial in CRPMI supplemented with 10% (vol/vol) hiFCS and 10% (vol/vol) DMSO and store them in a liquid nitrogen tank.

- 46| Resuspend lymphocytes and splenocytes at a concentration of 3×10^6 per ml each in CRPMI supplemented with 0.5% (vol/vol) hiNMS.
- ▲ **CRITICAL STEP** It is important to use hiNMS instead of hiFCS in order to prevent the growth of nonspecific T cells in culture.
- 47| Mix 1 ml of lymphocytes with 2 ml of irradiated splenocytes (both at 3×10^6 per ml) in CRPMI supplemented with 0.5% (vol/vol) hiNMS in the presence of GBSIII-TT antigen ($100 \mu\text{g ml}^{-1}$) and place the entire mixture into one well of a 12-well plate; culture the mixture at 37 °C in a 5% CO₂ incubator.
- 48| After incubation for 3 d, replace one-half of the volume of the culture medium with freshly made CRPMI supplemented with 10% (vol/vol) hiFCS and continue culturing the cells at 37 °C in a 5% CO₂ incubator.
- 49| Two days later, collect the cells and wash them once with washing medium by centrifugation at 250g for 5 min.
- 50| Resuspend the cells in 25 ml of washing medium, and then lay down the suspension on top of 15 ml of Lympholyte-M in a 50-ml conical tube.
- 51| Centrifuge this preparation at 500g for 20 min at RT.
- ▲ **CRITICAL STEP** This step should be performed at RT to yield a density gradient for the separation of live lymphocytes in good condition.
- 52| Collect cells from the interface between the washing medium and Lympholyte-M. Resuspend the cells in 40 ml of washing medium, and then centrifuge this preparation at 250g for 5 min at 4 °C.
- ▲ **CRITICAL STEP** Purification of lymphocytes with Lympholyte-M should make the lymphocytes more viable by removing toxic substances and dead cells.
- 53| Wash the cells twice with washing medium by centrifugation at 250g for 5 min.
- 54| Count the lymphocytes and confirm that the majority are alive by trypan blue staining; resuspend the lymphocytes at a density of 3×10^6 per ml in CRPMI supplemented with 10% (vol/vol) hiFCS and 10% (vol/vol) TCCS, place 1 ml of the suspension in one well of a 24-well plate and incubate the plate at 37 °C in a 5% CO₂ incubator for 3 d.
- ▲ **CRITICAL STEP** TCCS should be added to the culture 5–6 d after stimulation with the antigen (when the lymphocytes are well rested).
- 55| Three days later, replace one-half of the volume of the culture medium with freshly made CRPMI supplemented with 0% (vol/vol) hiFCS and 10% (vol/vol) TCCS. If the cells are overcrowded (more than a monolayer), split them into two or three wells.
- 56| Incubate the cells for another 2–3 d.
- 57| By removing a cryovial from a liquid nitrogen tank and holding it in a 37 °C water bath until the sides are thawed but the center remains frozen, thaw the

frozen splenocytes from GBSIII-OVA-immunized BALB/c mice stored in Step 45. Next, irradiate thawed splenocytes at 3,000 rad and use them as APCs, as described in Step 45.

- 58| Count the live lymphocytes (from Step 56 or 62) and APCs (from Step 57). Resuspend the lymphocytes at a density of 2×10^6 per ml and the APCs at a density of 8×10^6 per ml in CRPMI supplemented with 10% (vol/vol) hiFCS without TCCS.
- 59| Mix 0.5 ml of lymphocytes at a density of 2×10^6 per ml and 0.5 ml of APCs at a density of 8×10^6 per ml in the presence of the GBSIII-TT antigen ($100 \mu\text{g ml}^{-1}$). Place the entire mixture in a single well of a 24-well plate and incubate the plate at 37 °C in a 5% CO₂ incubator.
- 60| After 3 d, replace one-half of the volume of the culture medium with freshly made CRPMI supplemented with 10% (vol/vol) hiFCS.
- 61| Incubate the plate for another 2–3 d. Thereafter, collect the cells, purify them with Lympholyte-M as in Steps 50–53 and culture them as described in Steps 54–56.
- 62| Collect the cultured cells, set aside one-half of the cells and stimulate the other half of the cells for one more round as in Steps 57–60. At this stage, the growing cells should be enriched for Tcarbs, the result being a ‘Tcarb line.’ Put aside several hundred thousand Tcarb-line cells for testing of specificity for the carbohydrate portion of the glycoconjugate antigens as determined by an IL-2 ELISpot assay (Fig. 3b; Step 72A). Freeze these cells in a vial and store them in a liquid nitrogen tank.

Generation of Tcarb clones ● TIMING 2–3 months

- 63| Take the remaining cells from Step 62 and dilute them in CRPMI supplemented with 10% (vol/vol) hiFCS and 10% (vol/vol) TCCS at serial concentrations of 10 per ml, 100 per ml and 1,000 per ml. Next, place the diluted cells into the wells of a 96-well plate at concentrations of 1 cell per 100 μl , 10 cells per 100 μl and 100 cells per 100 μl .
- 64| Thaw frozen splenocytes from GBSIII-OVA-immunized BALB/c mice, irradiate the cells and use them as APCs (as described in Step 57); resuspend these cells at a density of 7×10^5 per 100 μl in CRPMI supplemented with 10% (vol/vol) hiFCS and 10% (vol/vol) TCCS in the presence of GBSIII-TT antigen ($200 \mu\text{g ml}^{-1}$). Place the APCs (at a concentration of 7×10^5 per 100 μl) into each well of the 96-well plate that contains the serial dilutions of T cells, as set up in Step 63.
- 65| Coculture the cloned T cells and APCs for 14 d at 37 °C in a 5% CO₂ incubator, replacing one-half of the volume of the culture medium with freshly made CRPMI supplemented with 10% (vol/vol) hiFCS and 10% (vol/vol) TCCS every 2–3 d.

- 66| Every 2 weeks, re-stimulate the cloned T cells by adding 7×10^5 cells per 100 μ l of APCs in the presence of the GBSIII-TT antigen ($100 \mu\text{g ml}^{-1}$) to each well until the growth of the cloned T cells becomes visible under the stereomicroscope. When the cloned T cells fully occupy the well (monolayer) of the 96-well plate, transfer the cells into a larger well (24-well plate) with a greater number of APCs (3×10^6 cells per 1 ml per well) in CRPMI supplemented with 10% (vol/vol) hiFCS and 10% (vol/vol) TCCS.
- 67| Count the cells and set aside a few hundred thousand cloned T cells for the determination of the surface phenotype (CD4 or CD8) by flow cytometric analysis (Fig. 4a; Step 72B). These cloned T cells should also be used in determining the specificity for the carbohydrate portion of the glycoconjugate antigens by IL-2 ELISpot assay (Fig. 4b; Step 72A). Continue to incubate the remainder of the cells as described in Step 68.

Maintenance of Tcarb clones ● TIMING up to 2–3 months

- 68| Incubate 2×10^6 cells from a Tcarb clone and 2×10^7 APCs in one well of a six-well plate in the presence of the GBSIII-TT antigen ($100 \mu\text{g ml}^{-1}$) in CRPMI supplemented with 10% (vol/vol) hiFCS and 10% (vol/vol) TCCS at 37°C in a 5% CO_2 incubator for 3 d.
- 69| After the 3-d incubation period, replace one-half of the volume of the medium with freshly made CRPMI supplemented with 10% (vol/vol) hiFCS and 10% (vol/vol) TCCS.
- 70| One week later, purify T cells with Lympholyte-M as described in Steps 50–53, and then place 2×10^6 cloned Tcarbs in one well of a six-well plate.
- 71| Incubate the cells for another week; next, re-stimulate cloned Tcarbs with APCs and GBSIII-TT antigen as described in Step 68.

Detection of primary Tcarbs and of Tcarb lines and clones

- 72| Confirm that Tcarbs have been isolated by performing the ELISpot assay (option A) and flow cytometric analysis (option B) as indicated on samples stored from Steps 44, 62 and 67, respectively.

(A) ELISpot assay ● TIMING 2–3 d

- i. Coat each well of a Multiscreen HA 96-well ELISpot plate with anti-mouse IL-2 capture antibody or anti-mouse IL-4 capture antibody ($1 \mu\text{g}$ per 100 μ l of either) and incubate overnight at RT.
- ii. Wash the wells three times with sterile PBS; next, block the wells with 200 μ l of CRPMI supplemented with 10% (vol/vol) hiFCS for 2–3 h at 37°C .

▲ **CRITICAL STEP** It is important to block the ELISpot wells sufficiently to reduce the background response.

- iii. After washing the wells once with CRPMI supplemented with 10% (vol/vol) hiFCS, place 5×10^5 T cells and 3×10^5 APCs in the ELISpot wells and coculture them in CRPMI supplemented with 10% (vol/vol) hiFCS in the presence of either $50 \mu\text{g ml}^{-1}$ GBSIII, OVA or TT antigen or $100 \mu\text{g ml}^{-1}$ GBSIII-OVA or GBSIII-TT antigen.
- iv. Incubate the plates either for 24 h (for IL-2 detection) or for 48 h (for IL-4 detection) at 37°C in 5% CO_2 .
- v. After washing the wells twice with PBS alone, wash them three times with PBS plus 0.05% (vol/vol) Tween-20 and add biotin-labeled anti-mouse IL-2 antibody or biotin-labeled anti-mouse IL-4 antibody to each well (at $0.2 \mu\text{g}$ per $100 \mu\text{l}$) for IL-2 and IL-4 detection, respectively.
- vi. After incubation at 4°C overnight, wash the plates three times with PBS plus 0.05% Tween-20. Next, add avidin-HRP to each well and incubate the plates again for 1 h at RT.
- vii. After washing the wells three times with PBS plus 0.05% (vol/vol) Tween-20 and twice with PBS alone, develop the color of each well by adding ACE substrate and waiting for 10–15 min. After 15–20 min, the color of the solution in the wells should be fully developed (dark brown). Wash the plate thoroughly with running water; distinct brown spots at the bottom of the wells should be visible. Count the number of spots using an ImmunoSpot reader.

(B) Flow cytometric analysis ● TIMING 2 h

- i. After observing a sufficient number of cloned Tcarbs growing in culture, collect 1×10^5 cloned Tcarbs from the culture plate and wash them once with FACS buffer.
- ii. Resuspend the cloned Tcarbs in $50 \mu\text{l}$ of FACS buffer containing $0.5 \mu\text{g}$ of anti-mouse CD16/CD32 antibody and incubate this preparation for 15 min at RT.
- iii. Wash the cloned Tcarbs twice with FACS buffer and resuspend them in $50 \mu\text{l}$ of FACS buffer containing $0.2 \mu\text{g}$ of allophycocyanin-labeled anti-CD3 antibody, $0.2 \mu\text{g}$ of phycoerythrin-labeled anti- $\alpha\beta\text{TCR}$ antibody, $0.5 \mu\text{g}$ of FITC-labeled anti-CD4 antibody and $0.2 \mu\text{g}$ of peridinin-chlorophyll protein-labeled anti-CD8 antibody. Incubate the mixture in the dark for 30 min at RT.
- iv. Wash the cloned Tcarbs twice with FACS buffer, resuspend them in $50 \mu\text{l}$ of FACS buffer and read the samples by BD LSRII flow cytometry.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Steps 1–9, GBSIII oxidation: 1 d

Steps 10–16, conjugation reaction: 2–4 d

Steps 17–26, purification and characterization of GBSIII-OVA: 1 week

Steps 27 and 28, mouse immunization: 4 weeks

Steps 29–44, preparation of mouse lymphocytes: 2 h

Steps 45–62, generation of Tcarb lines: 4–6 weeks

Steps 63–67, generation of Tcarb clones: 2–3 months

Steps 68–71, maintenance of Tcarb clones: up to 2–3 months

Step 72A, detection of primary Tcarbs and of Tcarb lines and clones by ELISpot assay: 2–3 d

Step 72B, flow cytometric analysis: 2 h

ANTICIPATED RESULTS

Detection of primary Tcarbs in GBSIII-OVA-immunized BALB/c mice by *ex vivo* ELISpot assay

Approximately 10–14 d after priming and boosting of BALB/c mice with GBSIII-OVA antigen emulsified in CFA and IFA, respectively, lymphocytes should be collected by draining lymph nodes of immunized mice, and the cells' antigen specificity determined by immediate *ex vivo* IL-2 and IL-4 ELISpot assays. As shown by the typical results shown in Figure 3a, there are certainly a number of lymphocytes—presumably T cells—that should secrete both IL-2 and IL-4 in response to OVA and GBSIII-OVA antigen. However, it is noteworthy that significantly more lymphocytes are able to respond to GBSIII-OVA than to OVA. Moreover, a considerable number of lymphocytes can respond to GBSIII-TT even though they are obtained from GBSIII-OVA-immunized mice. Finally, neither GBSIII nor TT alone should stimulate the lymphocytes. Thus, certain populations of lymphocytes derived from GBSIII-OVA-immunized mice can react with the GBSIII portion of GBSIII-OVA or GBSIII-TT, but not with GBSIII alone.

Detection of Tcarb lines by ELISpot assay

After three rounds of *in vitro* GBSIII-TT antigen stimulation and resting of the T cells for >10 d after the last stimulation, Tcarb lines should be collected and their antigenic specificities be determined by an IL-2 ELISpot assay. As shown in Figure 3b, the relative proportion of T cells that react with either GBSIII-OVA or GBSIII-TT should increase drastically—i.e., by >2,000-fold over the results of the immediate *ex vivo* ELISpot assays shown in Figure 3a. Because the number of T cells that react with OVA alone has almost completely diminished, most of the GBSIII-OVA- or GBSIII-TT-reactive T cells shown in Figure 3b should recognize the GBSIII portion of the respective glycoconjugate antigens. At this stage, these T cells approach equivalence to a T cell line in that >50% recognize the GBSIII portion of the glycoconjugates. Therefore, these T cells can be used to generate Tcarb clones by limiting dilution, as described in the Procedure section.

Detection of Tcarb clones by flow cytometric analysis and ELISpot assay

After successfully isolating cloned Tcarbs by limiting dilution and expanding them to sufficient numbers, subject cloned Tcarbs to flow cytometric analysis and ELISpot assays to determine their CD4/CD8 phenotype and antigen specificity, respectively. Figure 4a shows the staining profile of one representative Tcarb clone, 2B11, which expresses $\alpha\beta$ TCR chains together with CD3 to form a CD3- $\alpha\beta$ TCR complex. This also shows that clone 2B11 expresses CD4⁺ but not CD8⁺ molecules (Fig. 4a). Thus far, we have generated more than four Tcarb clones from GBSIII-immunized BALB/c mice, and all these clones are CD4⁺ CD8⁻ cells (data not shown). The antigen specificities of clone 2B11 against the GBSIII portion of the glycoconjugates have been confirmed by an IL-2 ELISpot assay (Fig. 4b). In addition, when anti-CD4 or anti-CD8 antibody is added to the ELISpot assay, only anti-CD4 antibody—and not anti-CD8 antibody—completely inhibits the response of cloned Tcarbs to GBSIII-OVA or GBSIII-TT. This result not only correlates with the surface phenotype of the Tcarb clone determined by flow cytometric analysis but also indicates that CD4 molecules expressed on the Tcarb clone contribute to the recognition of the GBSIII portion of the glycoconjugate antigens.

Antigen-specific T cell responses can also be evaluated by the traditional tritiated thymidine (³H]-TdR) incorporation method, which requires a radioactive scintillation counter. Fluorescence-based assays (nonradioactive) for cell proliferation are likewise readily available. The cell proliferation assay kit from Invitrogen (cat. no. C7026) or Biomedica Medizinprodukte (cat. no. BI5000) can be substituted for the [³H]-TdR assay.

Finally, we wish to emphasize that all the methods described in this study for GBSIII-OVA and GBSIII-TT, particularly the ELISpot assay and flow cytometric analysis, can be readily adapted for primary Tcarbs, Tcarb lines and clones that recognize other carbohydrates.

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Figure 1.

Reaction scheme for the preparation of GBSIII-OVA and GBSIII-TT. First, GBSIII is treated with sodium periodate to generate a reactive aldehyde at the C-8 position of the side-chain sialic acid residue. Oxidized GBSIII and OVA (or TT) are then reacted by a reductive amination mechanism: the amine group on a lysine residue reacts with the aldehyde group on the sialic acid in the presence of sodium cyanoborohydride to form a covalent linkage between the carbohydrate and protein.

Figure 2.

Purification and characterization of GBSIII-OVA and GBSIII-TT. **(a)** Superdex 200 elution profile of samples taken from the GBSIII and OVA conjugation reaction at different time points. The y axis is normalized to 1 on the basis of the absorbance of unconjugated OVA at 280 nm. **(b)** SDS-PAGE analysis of purified GBSIII-OVA (1 mg ml⁻¹), GBSIII (1 mg ml⁻¹) and OVA (1 mg ml⁻¹) on the basis of visualization of protein-containing bands. **(c)** Superdex 200 elution profile of GBSIII and TT conjugation reaction at 72 h.

Figure 3.

Antigen specificity of GBSIII-OVA-immune lymphocytes and their Tcarb line. **(a)** Antigen specificity of lymphocytes obtained from GBSIII-OVA (III-OVA)-immunized BALB/c mice. *Ex vivo* IL-2 and IL-4 ELISpot assays were performed to determine the proportions of lymphocytes (obtained from the GBSIII-OVA-immunized BALB/c mice) secreting IL-2 and IL-4, respectively, in response to a specific antigen. Lymphocytes (5×10^5) were cocultured in an ELISpot well with irradiated splenocytes in the presence of $50 \mu\text{g ml}^{-1}$ GBSIII, OVA or TT antigen or $100 \mu\text{g ml}^{-1}$ GBSIII-OVA or GBSIII-TT antigen. The results are expressed as the mean values for triplicate wells with standard deviation. Student's *t*-test was used to determine statistical significance. The data represent one of three similar experiments. **(b)** Antigen specificity of a Tcarb line derived from GBSIII-OVA-immune lymphocytes. After three rounds of *in vitro* GBSIII-TT antigen stimulation and resting of T cells for >10 d after the last stimulation, an IL-2 ELISpot assay was performed, in which 1×10^3 cells of the Tcarb line and 5×10^5 APCs were cocultured in a well in the presence of the same antigens listed in **a**. All experiments involving mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Rockefeller University.

Figure 4.

Phenotype and antigen specificity of a Tcarb clone. **(a)** Phenotype of a Tcarb clone, as determined by flow cytometric analysis. Cells (1×10^5) of Tcarb clone 2B11 were incubated with allophycocyanin-labeled antimouse CD3 antibody and phycoerythrin-labeled anti-mouse $\alpha\beta$ TCR antibody. The cells were also stained with FITC-labeled anti-mouse CD4 antibody and peridinin-chlorophyll protein-labeled anti-mouse CD8 antibody. The data represent one of three similar experiments. **(b)** Antigen specificity of a Tcarb clone. Cloned Tcarbs (1×10^3) and irradiated splenocytes (5×10^5) were cocultured in an ELISpot well with the same antigens listed in Figure 3, and an IL-2 ELISpot assay was performed to determine the proportions of cloned Tcarbs secreting IL-2 in response to the antigen. To determine the contribution of CD4 or CD8 molecules in mediating the Tcarb response, anti-CD4 or anti-CD8 antibody ($100 \mu\text{g ml}^{-1}$) was added to the ELISpot well before the addition of APCs and antigens. The results are expressed as the mean values for triplicate wells with s.d. Student's *t*-test was used to determine statistical significance. The data represent one of three similar experiments. All experiments involving mice were performed in accordance

with protocols approved by the Institutional Animal Care and Use Committee at the Rockefeller University.

Table 1

Troubleshooting table.

Step	Problem	Possible reason	Possible solution
13	No conjugation observed	Sodium cyanoborohydride contact with air before reaction	Restart the conjugation reaction, using freshly prepared oxidized GBSIII and dry sodium cyanoborohydride (e.g., from a freshly opened capsule)
	No conjugation observed	Problems pertaining to use of other polysaccharides as reactants	In such cases, each step in the conjugation reaction (e.g., percent oxidation, concentrations and amounts of reactants and reagents) needs to be optimized for the polysaccharide of interest
14	Gelling of the conjugation reaction	High-molecular-weight polysaccharides and/or proteins used in conjugation	Closely monitor the reaction, and follow the Critical Step in Step 14 of the conjugate reaction section. Alternatively (especially when a high-molecular-weight polysaccharide is used as reactant), use a lower molar equivalent of sodium periodate per oxidation site in the polysaccharide when performing the oxidation reaction (e.g., 0.4, 0.3 or 0.2 molar equivalents of periodate per each repeating unit of polysaccharide)
25	Unconjugated OVA in the conjugation reaction product	Poor column separation of the conjugation product	Rerun the conjugation product on the Superdex 200 GPC column and collect the fractions for the glycoconjugate peak, taking special care not to collect any fraction from the protein peak