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Enhanced Immunogenicity of a Tricomponent Mannan Tetanus Toxoid Conjugate Vaccine Targeted to Dendritic Cells via Dectin-1 by Incorporating β-Glucan

Tomasz Lipinski,*1,2 Amira Fitieh,1,2 Joëlle St. Pierre,§ Hanne L. Ostergaard,§ David R. Bundle,* and Nicolas Touret†

In a previous attempt to generate a protective vaccine against Candida albicans, a β-mannan tetanus toxoid conjugate showed poor immunogenicity in mice. To improve the specific activation toward the fungal pathogen, we aimed to target Dectin-1, a pattern-recognition receptor expressed on monocytes, macrophages, and dendritic cells. Laminarin, a β-glucan ligand of Dectin-1, was incorporated into the original β-mannan tetanus toxoid conjugate providing a tricomponent conjugate vaccine. A macrophage cell line expressing Dectin-1 was employed to show binding and activation of Dectin-1 signal transduction pathway by the β-glucan-containing vaccine. Ligand binding to Dectin-1 resulted in the following: 1) activation of Src family kinases and Syk revealed by their recruitment and phosphorylation in the vicinity of bound conjugate and 2) translocation of NF-κB to the nucleus. Treatment of immature bone marrow–derived dendritic cells (BMDCs) with tricomponent or control vaccine confirmed that the β-glucan-containing vaccine exerted its enhanced activity by virtue of dendritic cell targeting and uptake. Immature primary cells stimulated by the tricomponent vaccine, but not the β-mannan tetanus toxoid vaccine, showed activation of BMDCs. Moreover, treated BMDCs secreted increased levels of several cytokines, including TGF-β and IL-6, which are known activators of Th17 cells. Immunization of mice with the novel type of vaccine resulted in improved immune response manifested by high titers of Ab targeting C. albicans cells. Immunization of mice with the novel type of vaccine resulted in improved immune response manifested by high titers of Ab targeting C. albicans cells.

Candidiasis is one of the leading causes of hospital-acquired bloodstream infections. Despite antifungal therapy, at least 40% of affected individuals die of this disease (1, 2). The poor outcome of almost half the patients treated with appropriate antifungal therapy has spurred the development of preventative measures such as active immunization strategies (3–5). Although a few existing candidate vaccines show promise (6–9), others require aggressive immunization protocols and remain less satisfactory (10). We envisaged a conjugate vaccine based on the protective β-mannan epitope of Candida albicans conjugated to tetanus toxoid (11), a licensed carrier protein, with a third component, a β-glucan, that engages Dectin-1, a C-type lectin receptor of the dendritic cell (DC) and targets the vaccine to these professional APCs.

Invasion of opportunistic fungal pathogen, especially in the case of Candida—a human commensal organism—usually results from skin and mucosal interface exposure or following clearance of bacterial flora by antibiotic treatment. Susceptibility to fungal infection is exacerbated in patients who are immunocompromised, such as those who had elective abdominal or other surgical procedures (2) or chronically immunosuppressed in the case of HIV-positive patients (12). A major challenge in developing an efficacious anti-Candida vaccine is whether vaccination would be effective in immunosuppressed patients. Given that most of the therapies used to prevent xenograft rejection lower the acquired branch of the immune system, we hypothesized that developing a vaccine that targets the innate immune system would be useful in fighting Candida infections.

The first line of defense against fungal pathogens involves innate immune cells equipped with an arsenal of pattern-recognition receptors that recognize unique pathogen-associated molecular patterns. DCs, neutrophils, and macrophages are the main players in fungal recognition through several receptors that bind yeast cell wall components. Following binding and internalization of the pathogen, innate immune cells produce and secrete cytokines, such as IL-6 and TGF-β, which promote differentiation of naïve T cells into Th17 (13–15). The latter subclass of Th cells is involved in evoking a cell-mediated response against the invasive fungi, which is key to defending against the infection (16, 17).
Dectin-1 is a pattern-recognition receptor that has been implicated in defending against invasions by \textit{C. albicans} (18–20), \textit{Aspergillus} (21), \textit{Coccidioides} (22), and \textit{Pneumocystis} (23). This receptor is a single transmembrane protein with a carbohydrate recognition domain in its extracellular region that binds β,1,3-glucan (a major component of the cell wall of a variety of fungal pathogens), a transmembrane segment, and an N terminus cytosolic domain. Upon binding to β-glucans, the Src family-dependent tyrosine phosphorylation of the ITAM-like motif (abbreviated hemITAM) in the cytoplasmic tail recruits and activates the adaptor protein Syk (24). Syk, in turn, triggers downstream effectors, including NF-κB, which translocates to the nucleus to turn on mRNA translation of cytokines. In particular, synthesis and secretion of IL-6 and TGF-β are induced in a Dectin-1–dependent manner in DCs and macrophages and stimulate Th17 cells.

Two \textit{C. albicans} candidate vaccines based on carbohydrate Ags have received attention. One vaccine, which is based on β-mannan conjugates, showed potential in eliciting active and passive protective responses by targeting vaccines to DCs and macrophages and stimulate Th17 cells.

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β-glucans and vaccine conjugates

Propargylated laminarin preparation. Laminarin (100 mg, soluble β-glucan from \textit{Laminaria digitata}) and curdlan (from \textit{Alcaligenes faecalis}) were both from Sigma-Aldrich.

\begin{itemize}
  \item \textbf{Materials and Methods} Reagents and Abs
  
  \textbf{Rabbit anti-phospho Syk} (Tyr525/526), and rabbit anti-phospho Syk (Tyr552) were purchased from Cell Signaling Technology. Alexa Fluor 488 conjugated rabbit anti-phospho Syk (Tyr418) was from Life Technologies (Grand Island, NY). Monoclonal anti-p65 NF-κB was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse anti-actin was from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, and fluorescently conjugated secondary Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescently labeled Abs used for flow cytometry were from eBioscience (San Diego, CA) and included those specific for CD11c, CD11b, CD80, CD86, and F4/80. Anti-human Dectin-1 was from R&D Systems (Minneapolis, MN), and anti-mouse Dectin-1 was from AbD Serotec. Ultrapure LPS (LPS from \textit{Escherichia coli} 0111: B4, γ-irradiated) was purchased from Sigma-Aldrich. Mouse IL-4 was from eBioScience. Laminarin (soluble β-glucan from \textit{Laminaria digitata}) and curdlan (from \textit{Alcaligenes faecalis}) were both from Sigma-Aldrich.

  \item \textbf{Propargylated laminarin preparation.} Laminarin (100 mg, soluble β-glucan from \textit{L. digitata}; Sigma-Aldrich) was dissolved in 0.2 M phosphate buffer (pH 6.0; 1 ml) in a 6-ml Kimball glass vial with magnetic stirring bar. Propargylamine hydrochloride (120 mg) was added, followed by sodium cyanoborohydride (5 mg), and stirred in an incubator at 37°C for 7 d. Additional portions of sodium cyanoborohydride (5 mg each) were added on days 2 and 4. The reaction mixture was then diluted with water to 10 ml and precipitated with 4 vol of ethanol. After centrifugation (7000 × g, 20 min), the precipitate was dissolved in a minimal amount of water and passed through a Sephadex G-25 column (2.5 × 25 cm). The column was eluted with water, and fractions containing sugar were collected and lyophilized. Analysis led to the conclusion that 15% of laminarin molecules were substituted with a propargyl group. According to previously published results, only ~20% of laminarin molecules in commercial preparations are available for derivatization via the reducing end because the majority of chains are capped by mannitol (35, 36). In this study, we found that 15% of laminarin molecules were substituted with a propargyl group, which was not shown.

  \item \textbf{Tetanus toxoid conjugate vaccines} (Fig. 1). Tetanus toxoid (36 mg; State Serum Institute, Copenhagen) was azidinated (its amino groups were converted to azide groups) in 0.5 M carbonate/bicarbonate buffer (pH 9.8) at a protein concentration ~20 mg/ml with 1.2 M excess of the azo transfer reagent, imidazole-1-sulfonyl azide hydrochloride [Note: This reagent must be treated with extreme care. There has been one report of a serious explosion during its preparation alternates (35, 36)]., and 2 mM CuSO4 as catalyst. The reaction was passed through a Sephadex G-25 column (2.5 × 25 cm). The column was eluted with water containing 0.1% Tween-20 (to prevent protein aggregation) and washed by ultradialysis using a tangential flow membrane (Pellon XL 50; 10-kDa cutoff) with EDTA to remove copper, then with 0.15 M NaCl 100 mM 4-methylmorpholine, and concentrated to 18 mg/ml.

  \item The protein solution was transferred to a 4 ml Kimball vial, and 5.9 mg trisaccharide (26 molar equivalents) was added, followed by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (16 mg). The progress of conjugation was monitored by TLC for consumption of trisaccharide. After ~5 h, another portion of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (8 mg) was added, and the pH was adjusted to >8.0 by addition of 4-methylmorpholine. The reaction was complete when only trace amounts of unconjugated trisaccharide were detected by TLC. The data was diazilysed against 20 mg Tris HCl (pH 8.5) and loaded on a DEAE Sepharose CL column equilibrated with the same buffer. Conjugate was eluted with 0.1 M Tris HCl (pH 7.2) and 1 M NaCl. This step was performed to remove Tween-20 present in the conjugate. The conjugate was fractionated on Superdex S-200 column (2 × 100 cm, PBS), and material corresponding to a monomeric fraction was collected, concentrated, and divided into two equal portions. The first portion constituted the reference vaccine, TS-TT.

  \item The second portion of TS-TT (10 mg protein) was conjugated with propargylated laminarin. Briefly, propargylated laminarin (12.5 mg) was dissolved in 0.2 M Tris HCl buffer (pH 8.0) containing conjugate (10 mg by protein content) in a 4 ml Kimball vial; copper powder (~20 mg) and isobutanol (50 μl) were added. The vial was closed with a septa and purged with argon. Reaction was started with addition of bathophenanthroline/Fe2+ (50 μl) and 4-methylmorpholinium chloride (16 mg). The reaction mixture was dialyzed, purified by gel filtration, and concentrated as described for the Superdex S-200 column. This tricomponent conjugate vaccine, TS-TT with covalently attached laminarin, was designated TS-TT–laminarin (TS-TT-Lam) vaccine.

  \item The azide groups of both conjugates (TS-TT-Lam and TS-TT) were reduced back to amines by reaction with trimethylphosphine. Conjugates (10 mg/ml) in 0.5 M sodium carbonate were reacted with trimethylphosphine (50 μl of a 1 M solution in tetrahydrofuran) in 4-ml Kimball glass vials closed with a septa at room temperature, 18 h. Conjugates were then dialyzed against PBS, concentrated, and sterile filtered through 0.22-μm filters to yield solutions of TS-TT-Lam (2.7 mg/ml) and TS-TT (4.1 mg/ml).

  \item \textbf{Labeling conjugates with Alexa Fluor 546}. To the solutions of the tetanus toxoid conjugate vaccines in PBS (TS-TT and TS-TT-Lam) containing 400 μg protein, 1/10 vol 1 M sodium bicarbonate solution was added, followed by Alexa Fluor 546 NHS ester (Molecular Probes, Life Technologies, Grand Island, NY) (20 μg) dissolved in DMSO (~30 μl). The tubes were
wrapped in aluminum foil and left on an inverting mixer for 18 h. Purification was performed on PD-10 desalting columns (GE Healthcare) equilibrated with PBS. Fractions containing labeled conjugates were collected and concentrated on Amicon Ultra-4 Centrifugal Filter Units (10 kDa) to a final concentration of 2.5 mg/ml.

Phosphorylation of curdlan. Curdlan (from Alcaligenes faecalis; Sigma-Aldrich) (1 G), urea (18 G), and formamide (50 ml) were placed in a 500-ml round-bottom flask with magnetic stirring bar mounted in a temperature-controlled oil bath. The mixture was first heated to 130°C to aid dissolution of curdlan. After complete dissolution, the temperature was reduced to 100°C. Phosphoric acid (85%, 4 ml) was added in small portions over a 2-h period to avoid excessive foaming while maintaining rapid mixing. The reaction mixture was heated for 6 h after addition of the last portion of phosphoric acid. To the cooled mixture an equal volume of water was added and the solution was transferred to a dialysis bag. Dialysis was first performed against running tap water and then against milliQ water. A small amount of insoluble material was removed by centrifugation (20 min, 10,000 × g), and purified phospho-curdlan (P-curdlan) was lyophilized.

These β-glycans were solubilized in sterile PBS or RPMI 1640 medium (Life Technologies, Grand Island, NY), sterile filtered, and stored at 10 mg/ml stock solutions until use. All β-glycan preparations were endotoxin free and were used at a working concentration of 100 μg/ml, unless otherwise stated.

Cell culture and constructs

The mouse macrophage cell line RAW 264.7 cells (ref. TIB-71; American Type Culture Collection, Manassas, VA) were maintained in α-MEM (Life Technologies) with 10% heat-inactivated serum (Wisent Bioproducts, St. Bruno, QC, Canada). To generate RAW 264.7 cells stably expressing human resting or recombinant human Dectin-1, mouse anti-Dectin-1 Ab (GenBank NM_197947) was subcloned into the pFB-Neo retroviral vector (Strategene, Agilent Technologies, Santa Clara, CA) containing the neomycin-resistance gene (neo) for selection by G418. The pFB-Neo Dectin-1 vector was transfected together with the pVPack-GP and pVPack-VSV-G vectors in HEK 293T cells (ATCC CRL-11268), allowing virus that is next incubated with RAW 264.7 cells for 24 h before addition of growing media containing 1 mg/ml G418. After 2 wk of selection, Dectin-1 expression was confirmed by immunofluorescence and Western blot analysis and were further designated as RAW Dectin-1 cells.

Generation of bone marrow–derived DCs

Bone marrow–derived DCs (BMDCs) were prepared from bone marrow collected from wild-type (WT) C57BL/6 mice maintained at the Faculty of Medicine and Dentistry animal facility, University of Alberta (Edmonton, AB, Canada). BMDCs were prepared previously described (38), with minor modifications. Briefly, bone marrow cells (BMCs) were harvested from femurs and tibias of normal C57BL/6 mice and washed with PBS. To generate DCs, BMCs were resuspended in complete medium: RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS (Wisent Bioproducts), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 0.1 mM nonessential amino acids (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 50 μM 2-ME (BioShop Canada), and 20% v/v/superantigen from Chinese hamster ovary cells transfected with the plasmid pCDNA3 containing the mouse rGM-CSF gene (transduced Chinese hamster ovary cells secreting mouse rGM-CSF were provided by K. Kane, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada). Cell suspension (10 ml) containing 2 × 105 BMCs was cultured in 100-mm–diameter dishes (day 0) and incubated for 10 d at 37°C in a humidified 5% (v/v) CO2 atmosphere. During the incubation period, cells were fed with fresh medium containing 20% GM-CSF supernatant on days 3 and 5. A total of 10 ng/ml mouse rIL-4 (EbioSciences) was added to the medium on day 6. On day 10 of culture, suspension and loosely adherent cells (immature DCs) were harvested and resuspended in complete medium containing 10% (v/v) mouse rGM-CSF supernatant. Cell suspension was used for in vitro phenotypic analysis of immature DCs and for coculture for an additional 24 h with vaccine conjugates, or LPS.

Flow cytometry analysis of BMDC marker expression

On day 10 of culture, analysis of surface marker expression (surface phenotype analysis) on immature DCs was performed before stimulation with 10 μg/ml LPS. Cells were pelleted by centrifugation and resuspended at a concentration of 2 × 107 cells/ml in cold FACS buffer (PBS containing 2% FCS). Cell suspensions were then incubated on ice for 10 min with anti-FeRcII/III Ab (anti-mouse CD16/32; eBioscience) for FeRc blocking. Aliquots of 1 × 106 cells in 50 μl were then stained with a master mix of fluorochrome-conjugated Abs to stain for the various DC markers described in Table 1, or with a mix of corresponding IgG isotype controls. Abs were used from eBioscience (Table 1) and are as follows: PE-Cy7-conjugated anti-CD11c, FITC-conjugated anti-CD11b (B7-1), FITC-conjugated anti-F4/80, PE-conjugated anti-CD80 (B7-2), and eFluor 450-conjugated anti-MHC II (I-A/E). Isotype controls were done with similarly conjugated IgG. One aliquot of cells was left unstained for use as a control. After incubation with the Ab mix for 30–40 min at 4°C in the dark, cells were washed twice and resuspended in cold FACS buffer, and samples were acquired on a LSR II cytometer (BD Biosciences, San Jose, CA). Compensation was performed before each data collection using BD Biosciences CompBeads anti-rat/hamster Ig, k-negative control compensation particles set, according to manufacturer’s recommendations. Gating was performed for elimination of dead cells. Software analysis of flow cytometric data was performed on FSC3 Express from De Novo software (Los Angeles, CA).

Cytokine quantification in culture supernatants using ELISAs

For analysis of cytokine production, immature BMDCs were harvested on day 10 of culture and resuspended in complete RPMI 1640 medium containing 10% (v/v) mouse rGM-CSF supernatant. Cells were plated at 1 × 106 cells/well in a 12-well plate and stimulated with 100 μg/ml vaccine conjugates (TS-TT or TS-TT-Lam conjugates), or with 100 ng/ml LPS. Cell culture supernatants were collected after 24-h incubation. Concentration of the cytokines IL-4, IL-6, IL-12p70, TNF-α, and TGF-β1 in supernatant was assayed by precoated sandwich ELISA kits (R&D Systems, Minneapolis, MN). All of the samples were measured in triplicate, according to the manufacturer’s instructions. Statistical analysis was performed with Prism software using Student t test (GraphPad Software).

Immunofluorescence and Western blotting

Phosphorylated Syk was analyzed by immunofluorescence or by immunoblotting after 10-min stimulation at 37°C with 100 μg/ml vaccine conjugates or P-curdlan. Src activation was detected by immunofluorescence after 5-min stimulation. For immunofluorescence, cells were grown overnight on coverslips at 40% confluency, and the following day cells were serum starved for 4–5 h in serum-free α-MEM prior to stimulation. After stimulation, cells were washed three times with ice-cold PBS, fixed on ice in 4% paraformaldehyde, and blocked in PBS containing 5% BSA plus 3% fish skin gelatin (Sigma-Aldrich). To stain for surface dectin-1, cells were incubated before permeabilization with mouse anti-human Dectin-1, followed by a dye-coupled anti-mouse secondary Ab. Next, cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS, and stained for 30 min with either rabbit anti-phospho Syk (Tyr352) or rabbit anti-phospho Src (Tyr416) primary Ab (Cell Signaling Technology). Cells were incubated with Alexa Fluor 488–conjugated goat anti-rabbit IgG (Invitrogen), and DAPI was used to highlight cell nuclei. Following staining, coverslips were washed, mounted with DAKO mounting medium (DAKO, Agilent Technologies, Santa Clara, CA) and viewed on a spinning-disk confocal microscope (WaveFx from Quorum Technologies, Guelph, ON, Canada).

SDS-PAGE and immunoblotting

For analysis of Syk phosphorylation by immunoblotting, cells were cultured overnight in 6-well plates. The following day, cells at 80% confluency were serum starved for 5 h prior to stimulation. After incubation at 37°C with 100 μg/ml P-curdlan or vaccine conjugates for 10 min, cells were chilled on ice, washed with cold PBS, and lysed by addition of phosphorylation lysis buffer (20 mM MOPS, 1% Triton X-100, 2 mM EGTA, 5 mM EDTA, 1 mM sodium orthovanadate, 2 mM EGTA, 5 mM EDTA [pH 7.0]) containing phosphatase inhibitor mixture (PhosSTOP, Roche Applied Science, Laval, QC, Canada) and protease inhibitor mixture (Sigma-Aldrich). Cells were scraped and cell debris were removed by centrifugation at 14,000 rpm for 20 min at 4°C. The protein content of lysates was quantified using the Bio-Rad bicinchoninic acid protein assay. For Western blot analysis, cleared lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies for phospho Syk (Tyr525/526), rabbit anti-phospho Syk (Tyr525), rabbit anti-Syk, or mouse anti-actin for loading control. This was followed by incubation with appropriate HRP-conjugated secondary Abs and detection by an ECL kit from Roche.

NF-κB nuclear localization

RAW-Dectin-1 cells were plated overnight onto glass coverslips in a 12-well plate. The following day, cells were stimulated at 37°C for 10 min with

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vaccine conjugates or ligands in HEPES containing RPMI 1640 medium (Wisent Bioproducts). After stimulation, cells were washed three times with PBS, and fresh media was added for another 20 min to allow NF-κB nuclear translocation. Cells were then fixed with 4% paraformaldehyde, blocked, and permeabilized with PBS containing 0.5% Triton X-100. Cells were stained with anti-p65 Ab, followed with Alexa Fluor 488–conjugated goat anti-mouse IgG (Molecular Probes), and DAPI was added to detect cell nuclei. Following staining, coverslips were washed and mounted, and p65 nuclear localization was examined by confocal microscopy.

**Dectin-1 ligand-binding assay**

RAW 264.7, referred to as RAW WT and RAW Dectin-1 cells, were plated overnight on glass coverslips in a 12-well plate. The following day, cells at 60% confluency were incubated on ice for 10 min with Alexa Fluor 456–labeled vaccine conjugates. Cells were then washed four times with ice-cold PBS to remove unbound particles, fixed with 4% paraformaldehyde, and blocked with PBS containing 3% BSA plus 3% fish skin gelatin (Sigma) for dilution. For detection of surface Dectin-1 on the plasma membrane, nonpermeabilized cells were incubated with mouse anti-human Dectin-1 Abs, followed by incubation with Alexa Fluor 488–conjugated anti-mouse IgG and DAPI. Coverslips were mounted with DAKO and examined using confocal microscopy.

**Confocal microscopy**

Confocal microscopy was performed on a spinning disk microscope (WaveFx from Quorum Technologies, Guelph, ON, Canada) set up on an Olympus IX-81 inverted stand (Olympus Canada, Richmond Hill, ON, Canada). Images were acquired through a ×60 objective (N.A. 1.42) with an electron multiplying charge-coupled device camera (Hamamatsu, Japan). Z-slices of 0.3 μm were acquired through the cells using Velocity (Improvision, Perkin-Elmer, Waltham, MA).

**Mice vaccination**

The effect of vaccination with the TS-TT-Lam conjugate was studied in three mouse strains, as follows: BALB/c, C57BL/6, and CD1 (outbred strain). Groups of 10 mice from each strain were immunized with TS-TT conjugates. Control groups (10 mice each) were given the same conjugates that lacks the laminarin substitution. Both Abs were given as emulsion in mineral oil (IFA). Each dose contained 20 μg conjugate (equivalent of 1.25 μg trisaccharide) in 300 μl to the formulation administered i.p. (200 μl) and s.c. (100 μl). C57BL/6 and CD1 mice were immunized four times at 3-wk intervals, and BALB/c mice received four injections at 2-wk intervals. Sera were collected 10 d after each immunization. Ten days after the fourth immunization, mice were bled and examined.

**β-mannan–specific Ab titration**

Polyisoyl 96-well plates were coated overnight with trisaccharide-BSA conjugate (11) at a concentration of 5 μg/ml in PBS or with C. albicans phosphomannan complex (39) at the same concentration in 0.05 M carbonate buffer (pH 9.8). After washing with PBS containing 0.1% Tween-20 (PBST), 100 μl of serial 1/10 dilutions of sera (starting from 10^−5) were added to each well. A solution of 0.1% skim milk (Difco) in PBST was used for blocking to prevent nonspecific binding. Plates were sealed and incubated for 2 h at room temperature. After washing with PBST, a reporter Ab, anti-mouse IgG, HRP conjugate from Kirkegaard & Perry Laboratories, in 0.1% skim-milk PBST, at a dilution 1/2000, was applied and plates were incubated for 1 h at room temperature. Plates were washed again with PBST and color developed with HRP substrate system (Kirkegaard & Perry Laboratories) for 15 min. The reaction was stopped with 1 M phosphoric acid, and absorbance was measured. End point titres were recorded for dilutions that gave an OD reading of 0.2, which corresponded to an OD at least 0.1 U above background.

**Ab isotyping and titration**

ELISA plates were coated with a trisaccharide-BSA conjugate, as described above. Sera dilutions were chosen to yield optimal OD signal based on the total IgG titration. Subclasses were detected for duplicate samples with a subclass typing kit (Bio-Rad) and expressed as a percent of the OD signals read for individual subclasses. The change in subclass distribution was analyzed for statistical significance. A frequency of each four Ab subclasses was treated as a point on a simplex; the Bhattacharyya metric was defined as $B_h(\pi, \pi') = \sum_{i=1}^{4} \sqrt{\pi_i \pi'_i}$. Distribution of this statistic was estimated with permutation procedure. Differences between two vaccines were considered at $\alpha = 0.05$. Differences between effect sizes of two mouse strains were tested with Mahalanobis distance estimated based on bootstrap samples. Means’ SEs were computed with bootstrap approach.

**Statistical analysis of sera titers**

To test differences between two response curves after three vaccinations with TS-TT-Lam and TS-TT, the least squares method was used to derive a best-fitting line for each mouse. Thus, the set of vectors of three boost measurements $X_i = [b_{i1}, b_{i2}, b_{i3}, b_{i4}]$, where $b_{ij}$ was the first boost of the $i$th mouse in the $j$th group and so on, was replaced with a set of vectors $Y_i = (a_{ij}, b_{ij})$, where $a_{ij}$ and $b_{ij}$ were the intercept and slope of the regression line for the $j$th mouse in the $i$th treatment group. Next, Hotellings’s $T^2$ statistic was calculated to determine whether the responses after vaccination with two vaccines were similar. In the case of multivariate ANOVA, Pillai-Bartlett statistic was computed. Because of small samples, size permutation tests were applied. Means, SD, and SEMs were calculated. Cohen’s d was used as the measure of the effect size. All data were analyzed with R platform version 2.11.1. Results inferior to $\alpha = 0.05$ were considered statistically significant. Outliers were detected with Grubb’s test.

**Results**

We generated a tetanus-toxoid–bearing β-mannan vaccine, as initially described in Lipinski et al. (37) and conjugated additional laminarin molecules (Fig. 1). Laminarin is a β-glucan recognized by Dectin-1 (34, 41), a surface receptor that is highly expressed in DCs and macrophages. Tetanus toxoid was reacted with the diazo transfer reagent, imidazole-1-sulfonyl azide hydrochloride, to convert amino groups into azide groups. Transformation of aspartic and glutamine side chain carboxylic acids for coupling with the amino-terminated trisaccharide hapten. The resulting conjugate was divided into two portions; one was conjugated to propargylated laminarin using click chemistry, and the other was used as the control vaccine. The residual azide groups in each conjugate were reduced back to the original amino forms to give two conjugates designated TS-TT and TS-TT-Lam (Fig. 1).

We first decided to validate the Dectin-1–specific targeting of this trifunctional vaccine. We generated a stable murine macrophage cell line expressing Dectin-1 at noticeable level on the cell surface by viral transduction of RAW 264.7 cells (called RAW Dectin-1 thereafter) and neonym selection. As shown in Fig. 2A, the human Dectin-1 A isoform is readily detectable at the cell surface upon immunostaining of nonpermeabilized cells. Similarly, immunoblotting (Fig. 2B) of whole-cell lysates prepared from cells expressing Dectin-1 demonstrates the β-glucan receptor expression at a molecular mass of ~40–50 kDa. This m.w. corresponds to the complex glycosylated form of the transmembrane protein confirming the appropriate folding, targeting, and surface expression of Dectin-1 in the RAW macrophages.

We next investigated whether the presence of the laminarin polysaccharide on the tetanus toxoid (TS-TT-Lam) would allow for a better binding of the vaccine by macrophages expressing Dectin-1 compared with the TS-TT compound (tetanus toxoid with β-mannan only). To visualize the binding of the vaccine conjugate to RAW Dectin-1 cells, these compounds were coupled to AF546 (see Materials and Methods). Binding was achieved by incubation of the RAW Dectin-1 cells with the fluorescent compounds, TS-TT-AF546 or TS-TT-Lam-AF546, for 10 min, followed by washes with PBS and fixation with 4% paraformaldehyde (PFA). Dectin-1 immunostaining was performed in parallel, and, as shown in Fig. 3A, only the vaccine coupled to laminarin was able to bind RAW Dectin-1 cells. We confirmed that the binding of the TS-TT-Lam-AF546 was specific to the cells expressing Dectin-1, as RAW WT were not capable of recognizing the laminarin conjugate (data not shown).
Dectin-1 signaling requires phosphorylation of its cytosol hemITAM motif on tyrosine 15 by a Src family kinase (SFK), allowing the phospho-ITAM–like domain to recruit the Syk to mediate further downstream signaling (39, 41). To verify that TS-TT-Lam glycoconjugate was indeed able to activate SFK, we tested the appearance and colocalization of the active form of SFK with...

FIGURE 1. Conjugation scheme for synthesis of the two conjugates, TS-TT and TS-TT-Lam.
Dectin-1 following binding of fluorescent TS-TT-Lam. Following stimulation with TS-TT-AF546 or TS-TT-Lam-AF546, cells were fixed, permeabilized, and immunostained with an anti-phospho (Y418) Src Ab that reports SFK activation. Cells stimulated with TS-TT compound did not show significant staining for the phosphorylated form of Src (Fig. 3B). In contrast, the laminarin-carrying vaccine showed a pronounced increase in phosphorylated SFK (Fig. 3B).

We next confirmed the recruitment and activation of Syk by immunostaining and immunoblotting (Fig. 3C, 3E). Cells incubated with TS-TT or TS-TT-Lam for 10 min were either fixed and immunostained for Dectin-1 and phospho-Syk or lysed and analyzed by Western blotting with anti-phospho Syk Abs. By immunostaining (Fig. 3C), activation of Syk could be detected only upon stimulation with TS-TT-Lam, but not with TS-TT. This result was confirmed by the Western blot experiment showing that TS-TT-Lam activates Syk in a Dectin-1-dependent manner. In RAW cells that do not express Dectin-1, addition of either TS-TT or TS-TT-Lam did not increase Syk phosphorylation on either Y525/526 or Y352 (Fig. 3E). Similarly, Syk was not activated in the Dectin-1–expressing cells upon addition of TS-TT. However, Syk phosphorylation of Y525/526 and Y352 could only be detected when the laminarin conjugate was used on Dectin-1–expressing cells (Fig. 3E). This result indicates that, following binding to the β-glucan receptor, the oligovalent TS-TT-Lam molecules are capable of triggering Dectin-1 activation and, in particular, the Syk pathways. These results confirm that the laminarin conjugate is able to activate SFK and the Syk adaptor proteins, which are two crucial effectors of the Dectin-1 signaling pathway. Finally, we tested the activation and translocation of the NF-κB in fixed RAW Dectin-1 macrophages by immunostaining for the p65 subunit of NF-κB in parallel with DAPI following 10 min of incubation with either TS-TT or TS-TT-Lam. Although nuclear translocation of NF-κB was not seen in cells treated with TS-TT, TS-TT-Lam led to its pronounced translocation (Fig. 3D). Activation of NF-κB by the laminarin-containing vaccine further illustrates the capacity of this compound to stimulate innate immune cells in a Dectin-1–dependent fashion.

Using macrophages stably expressing Dectin-1 at the cell surface, we have validated that the laminarin-containing vaccine, TS-TT-Lam, was able to specifically interact with the β-glucan receptor and activate well-characterized downstream effectors. Before testing the vaccines directly in animals and to better define the immune response triggered by these compounds, we examined in vitro activity of the TS-TT and TS-TT-Lam conjugates.

Dectin-1 is expressed on cells from the monocytic lineage, especially on DCs (42), which are responsible for promoting antifungal immunity during pathogenic challenges (18, 20). To determine the potential of TS-TT-Lam conjugates in stimulating immune responses, we analyzed the response of primary BMDCs to β-glucans and the vaccine conjugates.

Preparation of DCs was performed from bone marrow lavages of C57BL/6 mice and differentiation in presence of GM-CSF and IL-4. DC surface marker characterization was performed by flow cytometry (Fig. 4A) with a series of Abs described in Table I. As expected for BMDCs, the majority of cells showed expression of CD11b and CD11c surface proteins. A small proportion (~9%) of the culture expressed the macrophage marker F4/80, but the majority of the cells presented an immature DC phenotype with a moderate expression of MHC-II and CD86 and high expression of CD80. Additionally, Dectin-1 immunofluorescence staining further confirmed the expression of the β-glucan receptor in these immature BMDCs. Cells plated on glass coverslips were fixed and permeabilized before addition of a rat anti-mouse Dectin-1 Ab (Fig. 4B). The expression of Dectin-1 in primary dendritic has been shown to promote several cell responses upon binding of particulate β-glucans (18, 43). We performed a series of experiments to test the responsiveness of immature DCs to Dectin-1 ligands. BMDCs were incubated for 10 min with P-curdlan, and activation of Syk and nuclear translocation of NF-κB were examined. Using the anti-phospho Syk Ab, we verified that β-glucans trigger Syk activation immediately following incubation with the ligand. Incubation of BMDCs with P-curdlan for 10 min resulted in the appearance and recruitment of a phosphorylated form of the kinase, as shown by the strong near-membrane localization of phospho-Syk (as shown in the middle panel of Fig. 4B). NF-κB translocation was analyzed after 10 min of ligand treatment, followed by another 20 min of incubation. Immunofluorescence staining of NF-κB in BMDCs untreated or treated with a soluble β-glucan demonstrated the responsiveness of DCs.
to Dectin-1 ligand (Fig. 4C). In unstimulated cells, NF-κB is dispersed throughout the cytosol, whereas cells incubated with β-glucans showed a strong relocalization to the nucleus, as demonstrated by the DNA-binding fluorophore DAPI (Fig. 4C). We have therefore shown that immature DCs express Dectin-1, and they respond to treatment with pure β-glucan (P-curdlan) by triggering Syk activation and NF-κB translocation in the nucleus.

We next tested the action of our vaccine conjugates on primary DCs. Secretion of specific cytokines and appearance of surface markers specific to DCs maturation were examined (Fig. 5). Immature BMDCs were treated with TS-TT, TS-TT-Lam, or LPS for 24 h before they were harvested. Cell supernatant was used for the determination of the secreted cytokines by specific ELISAs, and the cells were gently scraped, stained, and analyzed by flow cytometry for markers of maturation such as MHC-II and CD86.

Fig. 5A shows representative traces for the expression of CD86 and MHC-II and the quantification of the higher intensity section (M1) of the signals indicative of the upregulation of these markers in treated cells compared with unstimulated, TS-TT, TS-TT-Lam, or LPS. Fig. 5B shows the ELISA quantification of the following five cytokines: IL-4, IL-12, TNF-α, TGF-β, and IL-6. Stimulation with the TLR4 ligand LPS led to secretion of larger amount of cytokines, especially of IL-4, IL-12, TNF-α, and IL-6. Although TS-TT conjugate vaccine did not result in higher cytokine production when compared with untreated cells, TS-TT-Lam augmented the secretion of IL-4, IL-6, and TGF-β. In summary, we show that conjugating β-glucans to the TS-TT vaccine boosted its effects and especially raised the secretion of IL-4, IL-6, and TGF-β. Taken together, our results to date confirmed that TS-TT-Lam binds to and activates the β-glucan receptor in RAW macrophages expressing Dectin-1 and also in BMDCs. In the primary cells, this activation promotes the differentiation of DC and more significantly induces the production and secretion of cytokines that will act to stimulate the immune system.

Validation of the immunization potential of the TS-TT-Lam vaccine was next carried out in animal model. Groups of 10 mice from
three different mouse strains (BALB/c, C57BL/6, and outbred CD1) were vaccinated with either TS-TT-Lam or TS-TT conjugates four times (prime injection and three boosts). Serum samples were taken 10 d after each booster injection and analyzed using ELISA for titers against the trisaccharide hapten attached to a heterologous carrier protein (TS-BSA) and against *C. albicans* cell wall phosphomannan. Results of the sera titers for the three mouse strains after each boost are shown on Fig. 6A and demonstrate that Ab titers increased

Table I. List of Ags for the characterization of BMDCs used for flow cytometry

<table>
<thead>
<tr>
<th>Ag</th>
<th>CD11c</th>
<th>CD11b</th>
<th>CD80 (B7-1)</th>
<th>CD86 (B7-2)</th>
<th>F4/80</th>
<th>MHC-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorophore</td>
<td>PE-Cy7</td>
<td>Alexa Fluor 700</td>
<td>PE</td>
<td>FITC</td>
<td>FITC</td>
<td>eFluor 450</td>
</tr>
<tr>
<td>Final dilutions</td>
<td>1/1000</td>
<td>1/500</td>
<td>1/1000</td>
<td>1/800</td>
<td>1/500</td>
<td>1/1000</td>
</tr>
<tr>
<td>Isotype controls</td>
<td>Hamster IgG</td>
<td>Rat IgG2b, κ</td>
<td>Rat IgG2a, κ</td>
<td>Rat IgG2b, κ</td>
<td>Rat IgG2b, κ</td>
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following each challenge. Compared with TS-TT–vaccinated mice, TS-TT-Lam conjugate vaccination resulted in 5- to 10-fold higher titers against both synthetic Ag and cell wall phosphomannan (median values are indicated on the graph) in all tested mice strains (Fig. 6B). Because the vaccine was derived from a single batch, each conjugate had exactly the same number of trisaccharide epitopes. Ab IgG subisotype distribution (Fig. 6C) was also influenced by the conjugate used during the vaccination protocol. Vaccination with TS-TT-Lam resulted in an increased level of IgG1 (∼50%) and decrease of other subclasses, particularly IgG2a, in BALB/c and C57BL/6 strains. The changes in subclass distribution were significant in both BALB/c and C57BL/6 strains (*p = 0.04676 and 0.00375, respectively), but the results in CD1 mice were inconclusive due possibly to the low number of animals and high variation between individual mice.

In summary, we show that the novel laminarin-bearing vaccine is recognized by and activates Dectin-1 cells. In the case of primary DCs, this activation leads to the production of several regulatory cytokines, such as IL-4, IL-6, and TGF-β. Finally, animals challenged with TS-TT-Lam showed increased Ab responses, in the form of IgG isotypes, directed against the β-mannan trisaccharide and the tetanus toxoid compared with the TS-TT conjugate.

**Discussion**
We have previously shown that five of six synthetic β-mannotriose–peptide conjugates conferred good to excellent protection against *C. albicans* challenge if the glycopeptides were administered via Ag-pulsed DC vaccination (44). This was in sharp contrast to the poor immunogenicity of β-mannotriose–tetanus toxoid conjugate in mice (32, 33, 37). These observations prompted us to consider DC targeting as a means to enhance the antigenicity of the β-mannan–tetanus toxoid conjugate. Dectin-1 Ab-mediated targeting of the protein Ag OVA to DC has been reported to yield superior response to the protein Ag OVA, whereas targeting via DEC205 did not (45). In this study, we elected to target DCs by attaching β-glucan—a ligand for the C-type lectin receptor Dectin-1—to the β-mannan–tetanus toxoid conjugate. This approach has the potential for a dual outcome: by elevating the response to the β-mannan of *C. albicans* while taking advantage of the proven targeting and antifungal response of β-glucan conjugates.
As shown in Fig. 6B, DC targeting of the vaccine resulted in Ab titers that were 5- to 10-fold higher than those observed for a TT conjugate bearing only the β-mannan B cell epitope. The much higher Ab levels observed in BALB/c mice were also reflected in the production of protective β-mannan–specific mAbs, which is in sharp contrast to numerous unsuccessful attempts when mice were immunized with the TS-TT conjugate (T. Lipinski, X. Wu, J.M. Sadowska, and D.R. Bundle, unpublished observations; see also Refs. 32, 33).

The role of Dectin-1 in the binding and activation of innate immune cells was initially confirmed by in vitro experiments on a cell line of macrophages expressing a human isoform of Dectin-1. Stable Dectin-1–expressing cells were obtained after viral transduction of RAW 264.7 macrophages, and Dectin-1 was detected on their cell surface (Fig. 2A). This provided us with a system that allowed testing of ligand binding and activation of downstream Dectin-1 effectors in comparison with the WT cells (RAW WT). The laminarin-containing conjugate (TS-TT-Lam) could bind to RAW Dectin-1 cells only, whereas the β-mannan–only vaccine (TS-TT) did not show any significant interaction (Fig. 3A).

Laminarin, a small soluble ligand, has been reported to bind Dectin-1 (41), but it remains unclear whether the small β-glucan can produce specific Dectin-1–mediated responses. Goodridge et al. (46) recently reported that soluble β-glucans are also ineffective in triggering Dectin-1 activation. Because our TS-TT-Lam conjugate carries approximately three laminarin chains per vaccine molecule, we surmised that this oligovalent presentation, in contrast to the soluble form, could activate Dectin-1 by engaging a series of receptors. If so, this would suggest that even relatively short β-glucan sequences might be capable of activating Dectin-1, provided that they are presented in multivalent format. In accordance with this notion, we showed in this study that only the β-glucan–containing conjugate (TS-TT-Lam) was able to stimulate effectors of the Dectin-1 pathway (Fig. 3). Upon ligand binding, tyrosine 15 phosphorylation of Dectin-1 by Src family kinases enables the recruitment of Syk on the resulting phospho-hemITAM motif (18, 24). In RAW Dectin-1 macrophages, TS-TT-Lam was able to do the following: 1) increase phospho-SFK in the vicinity of bound conjugates (Fig. 3B), leading to 2) activation of Syk (Fig. 3C, 3E) and 3) inducing the translocation of NF-κB to the cell nucleus (Fig. 3D). Activation of Src family kinases, Syk...
and NF-κB, only occurred in Dectin-1–expressing cells with the lamarlin-containing conjugate demonstrating the specificity of this Dectin-1–dependent signaling pathway to β-glucans. Following binding of the β-glucan–conjugated vaccine TS-TT-Lam, it is taken up by RAW Dectin-1 macrophages, resulting in stimulation of the cells.

To further confirm the immune stimulation potential of these glycoconjugates, we tested their effects on immature BMDCs. Dectin-1 expression and activation by the conjugate vaccine TS-TT-Lam on BMDCs were confirmed (Fig. 4). The poor immunogenicity of the TS-TT conjugate can be explained by the inability of TS-TT to bind to macrophages or BMDCs, presumably resulting in poor uptake and processing for presentation by DCs. To validate the potency of TS-TT-Lam in inducing an immune response, we determined its effect on primary innate immune cells. We used BMDCs grown in medium containing IL-4 and GM-CSF. After 10 d of culture, BMDCs were mainly immature, as shown by the following flow cytometry profile: majority of CD11c+CD11b+CD80+ and moderate expression levels of MHC-II and CD86 (Fig. 4A). In response to 24-h incubation with the TS-TT-Lam conjugate, a significant fraction of cells expressed higher levels of MHC-II and CD86 (Fig. 5A) compared with unstimulated conditions, indicating an induction of the differentiation of the DCs toward a more mature phenotype. These results are in agreement with reports demonstrating that laminarin and curdlan can activate immature DCs (47). In addition, curdlan was shown to induce cytotoxic T cell priming through the stimulation of IFN-γ secretion by DCs (48). DCs are immune regulatory cells that can produce selective molecules that will influence the overall immune response (49–51).

Cytokines secreted by BMDCs stimulated with the TS-TT-Lam vaccine showed a modest increase in TNF-α and IL-12. Instead, we measured a significant augmentation of IL-4 secretion (p < 0.05) and a stronger induction of TGF-β and IL-6. IL-4 secretion favors the differentiation of type 2 Th cells, which in turn promotes Ab production by B cells and isotype class switching (52). TGF-β is an important regulatory signal for Foxp3+ regulatory T cells, which in turn promotes Ab and a stronger induction of TGF-β and IL-6. We conclude that our synthetic tripartite oligosaccharide conjugate vaccine equally promotes DC activation, the induction of the Th17 response, and potent immunization.

Several lines of evidence suggest that TS-TT-Lam will afford protection. The isotype shift that results form DC targeting of the vaccine is important. It has been shown by several indirect and direct measures that, in contrast to nonprotective mAbs, Abs that protect mice against C. albicans more efficiently bind complement factor C3 to the yeast cell, and that the mechanism of protection appears to be associated with enhanced phagocytosis and killing of the fungus (65). Our analysis of the IgG isotype distribution specific for the β-mannan trisaccharide, although variable across three strains of mice, shows a general shift toward IgG1 and significant levels of IgG2b with some IgG2a and IgG3. Literature data (66–68) indicate that IgG1 does not fix complement, but IgG2b does, as do the other two isotypes. Our own work (7) demonstrated that the glycopeptide of Ref. 10 (10), when conjugated to tetanus toxoid, yields anti-trisaccharide Abs with IgG2a and IgG3 subclass distribution and confers protection without adjuvant, presumably via the ability of the toxoid or its fragments to be presented by DCs. The literature also reports that monocyte responses to C. albicans are enhanced when C. albicans is opsonized by specific Abs (69).

Conjugate vaccines based on polysaccharides that combat pneumococcal pneumonia, bacterial meningitis caused by Neisseria meningitidis, or Hemophilus influenzae have been extremely successful in reducing disease (70). Whereas these polysaccharides conjugated to carrier protein are successful in raising protective Abs, smaller oligosaccharide epitopes are far less successful in conferring protection. Similar observations apply to tumor-associated carbohydrate Ags. A notable success in raising high titer of protective Abs has employed targeting of the innate immune system via TLRs. The approach we have adopted in this study does not require the activation of TLRs and is consistent with reports of DC targeting of protein Ags.

Aivi et al. (64) have identified how glycoconjugate vaccines are degraded after injection and then presented by cells of the immune system. These conclusions show that polysaccharide is degraded to ~10-kDa fragments with attached peptide fragments, and that these fragments are recognized by TCRs. When conjugates were created that maximized the presentation of these essential elements, a superior immune response was observed. This discovery raises the prospect of designing fully synthetic three-component conjugate vaccines composed of the optimum B cell epitope, a T cell peptide possibility replacing carrier protein with the third component, a glycan able to direct the vaccine to DCs for effective presentation. A number of glycan receptors displayed on the DC membrane are known to modulate immunological responses (71). Consequently, it might be expected that the most appropriate ligand for targeting may vary depending on the particular vaccine and B cell epitope.

Our combined approach of cellular immunology and in vivo vaccination challenges confirms the importance of DCs in the modulation of immune responses. By carefully defining the pathways stimulated by multicomponent vaccines, specific responses
could be induced and regulated. Whereas further characterization of the T cells activated by these compounds is required, we will first focus on optimizing vaccine conjugates. In the context of a practical C. albicans vaccine, we consider a trisaccharide B cell epitope (72) conjugated to the Fba 14 peptide (10) with attached multivalent ß-glucan may provide an even more potent immunogenic compound.

Disclosures
D.R.B. is a founder and shareholder in a University of Alberta spinoff company, TheraCarb Inc., to which the vaccine described in Ref. 63 has been licensed. The other authors have no financial conflicts of interest.

References


