Robust Stimulation of Humoral and Cellular Immune Responses following Vaccination with Antigen-Loaded beta-Glucan Particles

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Robust Stimulation of Humoral and Cellular Immune Responses following Vaccination with Antigen-Loaded β-Glucan Particles

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ABSTRACT

β-Glucan particles (GPs) are purified Saccharomyces cerevisiae cell walls treated so that they are primarily β1,3-d-glucans and free of mannans and proteins. GPs are phagocytosed by dendritic cells (DCs) via the Dectin-1 receptor, and this interaction stimulates proinflammatory cytokine secretion by DCs. As the hollow, porous GP structure allows for high antigen loading, we hypothesized that antigen-loaded GPs could be exploited as a receptor-targeted vaccine delivery system. Ovalbumin (OVA) was electrostatically complexed inside the hollow GP shells (GP-OVA). Incubation of C57BL/6J mouse bone marrow-derived DCs with GP-OVA resulted in phagocytosis, upregulation of maturation markers, and rapid proteolysis of OVA. Compared with free OVA, GP-OVA was >100-fold more potent at stimulating the proliferation of OVA-reactive transgenic CD8+ OT-1 and CD4+ OT-2 T cells, as measured by in vitro [3H]thymidine incorporation using DCs as antigen-presenting cells. Next, immune responses in C57BL/6J mice following subcutaneous immunizations with GP-OVA were compared with those in C57BL/6J mice following subcutaneous immunizations with OVA absorbed onto the adjuvant alum (Alum/OVA). Vaccination with GP-OVA stimulated substantially higher antigen-specific CD4+ T-cell lymphoproliferative and enzyme-linked immunospot (ELISPOT) responses than that with Alum/OVA. Moreover, the T-cell responses induced by GP-OVA were Th1 biased (determined by gamma interferon [IFN-γ] ELISPOT assay) and Th17 biased (determined by interleukin-17A [IL-17A] ELISPOT assay). Finally, both the GP-OVA and Alum/OVA formulations induced strong secretions of IgG1 subclass anti-OVA antibodies, although only GP-OVA induced secretion of Th1-associated IgG2c antibodies. Thus, the GP-based vaccine platform combines adjuvanticity and antigen delivery to induce strong humoral and Th1- and Th17-biased CD4+ T-cell responses.

IMPORTANCE

Most licensed vaccines work by promoting protective antibody responses. However, for many infectious diseases, antibody-mediated protection appears to play a relatively minor role, and vaccination has met with limited success. While live-attenuated organisms generally elicit T-cell responses, their use in vaccines is limited by the potential for causing disease. Thus, there is an urgent need for new vaccine platforms that deliver antigens in such a manner as to promote strong T-cell-mediated responses. Here we designed a novel vaccine platform consisting of yeast-derived β-glucan particles (GPs) that combines antigen delivery and adjuvant activity. GPs loaded with the model antigen ovalbumin (OVA) stimulated robust humoral and T-cell responses in mice. In addition, the cellular response was Th1 and Th17 biased. This work has implications for the design of vaccines that stimulate biased T-cell responses as well as for understanding how immunity to fungal pathogens develops.

β-Glucan particles (GPs) are purified Saccharomyces cerevisiae cell walls treated so that they are >85% β1,3-d-glucan polymers, ~2% chitin, and <1% lipids and protein, with the rest being mostly ash and moisture (1). β-Glucans are naturally found in fungi, algae, plants, and some bacteria. β-Glucans, especially β1,3-d-glucans, are key constituents of the cell walls of fungal and major fungal pathogen-associated molecular patterns (PAMPs) (2, 3). They are recognized by the pattern recognition receptor Dectin-1, a C-type lectin which has high levels of expression on phagocytes, including dendritic cells (DCs), macrophages, and neutrophils (4, 5). It has been previously demonstrated that GPs can be efficiently taken up by mouse bone marrow-derived DCs (BMDCs) in vitro (6). In addition, the hollow, porous GP structure allows for high antigen loading (7, 8). This suggests that GPs have the potential to be exploited as a targeted antigen delivery vehicle.

PAMPs have been extensively studied because of their importance in host defense against microbes. More recently, there has been a growing recognition of the potential role of PAMPs in vaccine development (9). PAMPs deliver a “danger” signal to DCs, resulting in DC activation and secretion of cytokines/chemokines, migration, maturation, antigen presentation, and costimulatory molecule expression. This, in turn, impacts B- and T-cell responses to antigens codelivered with PAMPs. Curdlan and yeast glucan particles have been shown to have this type of adjuvant activity (10, 11). We hypothesized that antigen-loaded GPs could serve as a
receptor-targeted vaccine delivery system that exploited the adjuvanticity of \( \beta_1,3 \)-d-glucan. The model antigen ovalbumin (OVA) was complexed in GPs and studied for its capacity to stimulate immunologic responses in mice in vitro and in vivo. We found that immunization with OVA complexed in GPs (GP-OVA) induced robust Th1- and Th17-biased T-cell responses and strong antibody responses.

RESULTS

Proteolysis of antigens loaded into GPs. We previously demonstrated that GPs are efficiently taken up by BMDCs in vitro (6). In order to demonstrate that antigens encapsulated in GPs can be efficiently proteolysed by DCs, we loaded GPs with DQ-OVA. DQ-OVA consists of OVA that is heavily conjugated with BODIPY FL, resulting in self-quenching. Upon proteolytic degradation of DQ-OVA to single dye-labeled peptides, bright green fluorescence is observed. Incubation of GP-DQ-OVA with BMDCs resulted in the uptake of the particles and subsequent proteolytic degradation of DQ-OVA (Fig. 1). This was evidenced by progressively increased fluorescence over the time period studied by both epifluorescence microscopy and fluorescence-activated cell sorting (FACS) analysis.

DC maturation. Optimal antigen presentation and subsequent CD4\(^+\) T-cell responses require DC maturation, a process that includes upregulation of costimulatory molecules and major histocompatibility complex class II (MHC-II) (12). Incubation of BMDCs with GP-OVA induced upregulation of two costimulatory molecules, CD40 and CD86, and MHC-II (Fig. 2). Lipopolysaccharide (LPS), a known DC maturation stimulus, also upregulated CD40, CD86, and MHC-II. The results suggest that GPs can have adjuvant activity when used to immunize animals.

Antigen delivery activity of GP-OVA particles. In order to demonstrate whether the antigens encapsulated in GPs can be efficiently presented by BMDCs, we performed in vitro T-cell proliferation assays, using OVA as the model antigen. CD8\(^+\) T cells obtained from “knock-in” transgenic OT-I mice (which recognize the OVA peptide in the context of MHC-I) and CD4\(^+\) T cells obtained from OT-II mice (which recognize the OVA peptide in the context of MHC-II) were stimulated with either free OVA or GP-OVA, using BMDCs as antigen-presenting cells (APCs). At concentrations from 0.03 to 0.5 \( \mu \)g/ml, free OVA failed to stimulate proliferation of either OT-I or OT-II T cells (Fig. 3A and B). In contrast, GP-OVA at those concentrations stimulated robust OT-I and OT-II T-cell proliferation (Fig. 3A and B). In order to achieve similar stimulation effects of GP-OVA, 100 times or higher concentrations of free OVA were required (Fig. 3A and B). These results demonstrate that antigens delivered in GPs were efficiently processed and presented by DCs.

Evaluation of GPs as an antigen delivery and adjuvant system. The in vitro T-cell proliferation and DC maturation data suggest that GPs have the potential to serve as both an antigen delivery system and an adjuvant system. Therefore, in the next set
of experiments, the immune responses in mice following immunization with GP-OVA were evaluated. Controls included OVA delivered in alum and unimmunized mice that received phosphate-buffered saline (PBS).

**T-cell proliferation after immunization.** Two weeks after the last boost, CD4\(^{+}\)/H11001 and CD8\(^{+}\)/H11001 T cells were purified from lymph nodes and spleens and restimulated with OVA (in the form of either free OVA or GP-OVA) in an *ex vivo* T-cell proliferation assay. The mitogen concanavalin A (ConA) served as a positive control. CD4\(^{+}\) T cells obtained from mice immunized with GP-OVA proliferated in response to free OVA and GP-OVA at much higher levels than those that had received OVA in alum or PBS (Fig. 4). GPs alone (without OVA) did not stimulate T cells from any of the groups to proliferate, thus demonstrating that the CD4\(^{+}\) T-cell lymphoproliferation was specifically in response to OVA. Lymphoproliferation of CD8\(^{+}\)/H11001 T cells from all immunization groups in response to stimulation with OVA was similar to that of unstimulated controls. This was not because the lymphocytes were incapable of proliferation, as the CD8\(^{+}\)/H11001 T cells did proliferate when stimulated with ConA (data not shown).

**Skewing of Th responses.** The *ex vivo* T-cell proliferation assay showed that GP-OVA induced strong T-cell responses in mice. To determine the Th bias of this response, gamma interferon (IFN-\(\gamma\); Th1 type cytokine), interleukin-4 (IL-4; Th2 cytokine), and IL-17a (Th17 cytokine) enzyme-linked immunospot (ELISPOT) assays were performed using CD4\(^{+}\)/H11001 T cells obtained from immunized mice. GP-OVA-immunized mice had considerably more IFN-\(\gamma\) and IL-17a ELISPOTs than IL-4 following *ex vivo* stimulation with OVA (Fig. 5). Consistent with the lymphoproliferation data, mice receiving OVA in alum had a near background number of spots. These data indicated that the CD4\(^{+}\) T-cell responses following immunization with GP-OVA were biased toward Th1 and Th17.

**Antibody responses.** The above-described results showed that GP-OVA was a strong inducer of T-cell responses. Measurement of the antibody response to vaccination is also important because for many infections, antibodies mediate protection. In addition, determination of relative IgG1 and IgG2c subclass titers can give clues as to the polarization of Th responses, as IgG2c titers are associated with Th1 responses (13). Serum samples were analyzed for OVA-specific IgG1 and IgG2c by enzyme-linked immunosorbent assay (ELISA). Both classes of antibodies were demonstrated after just one immunization with GP-OVA (Fig. 6), and their levels were elevated after the second and third immunization. In contrast, OVA in alum elicited only IgG1 antibodies. These data are consistent with the Th1 skewing results observed by ELISPOT assays.

**DISCUSSION**

DCs play central roles bridging innate and adaptive immunity and are the primary antigen-presenting cells initializing adaptive immunity. Targeted antigen delivery to DCs can result in enhanced antigen presentation by DCs. Both natural receptor ligands and receptor-specific antibodies have been used for targeted antigen delivery.
delivery (14–18). GPs are naturally occurring ligands for Dectin-1, which is highly expressed on DCs. GPs are efficiently phagocytosed by BMDCs in a Dectin-1-dependent manner in vitro. In addition, engagement of GPs with Dectin-1 leads to proinflammatory cytokine production (6) and upregulation of costimulatory molecules on DCs, part of the process of DC maturation, which is essential for antigen presentation and induction of adaptive immunity.

Mice vaccinated with GP-OVA had robust humoral and Th1/Th17-skewed T-cell immune responses. The GP-based platform combines targeted antigen delivery and adjuvant activity and has several advantages compared to other targeting systems. First, the antigens are complexed into GPs noncovalently through electrostatic interaction. This eliminates the requirement for a chemical synthesis step whereby the antigen and targeting molecule are chemically conjugated. Second, because GPs themselves serve as adjuvants, the need for additional adjuvants is obviated. Th1- and Th17-skewed cell-mediated immune responses appear to be important components of defenses against a wide range of fungal pathogens, including Cryptococcus neoformans, Aspergillus fumigatus, and Candida albicans (19–21). Recent studies suggest stimulation via Dectin-1 primes Th17, Th1, and cytotoxic T-cell responses (11, 22–25). Moreover, targeted delivery of antigen by conjugation to anti-Dectin-1 antibodies induced CD4+ and CD8+ T-cell responses at doses that antigen alone failed to produce a response (25). Taken together with the data presented herein, these results demonstrate the potential for β-glucan-based antigen delivery systems to stimulate robust T-cell-mediated immune responses.

We could not detect significant OVA-specific CD8+ T-cell proliferation in mice immunized with GP-OVA, GP-OVA absorbed onto the adjuvant alum (Alum/OVA), or PBS. GPs are thought to be taken up by DCs via phagocytosis, following which fusion with endolysosomal compartments occurs (6). Thus, OVA delivered in GPs would be expected to be preferentially presented by the MHC-II pathway rather than cross-presented by the MHC-I pathway (26–29). However, this explanation does not account for our finding that GP-OVA can stimulate CD8+ T cells obtained from OT-1 mice to proliferate ex vivo. It is possible that a low degree of cross-presentation may be sufficient to stimulate OT-1 CD8+ cells to proliferate, given that these cells are already OVA responsive. Alternatively, the capacity for BMDCs to cross-present OVA delivered via GPs may be significantly greater than that of the DC populations that phagocytose GP-OVA in vivo. Strategies are being investigated to improve the cross-presentation of antigens delivered by GPs in order to enhance the CD8+ response.

Elicitation of memory responses is essential if vaccines are to confer long-lasting protection. Studies are under way to determine if delivery of antigen via the GP platform stimulates central and effector memory responses, and if so, the skewing of those responses. Recent studies with models of listeriosis suggest that antigen-specific Th1 cells were much more likely to enter the memory cell pool than Th17 cells (30). However, delivery of antigen via Dectin-1 may prove more effective at eliciting long-lived Th17 T cells, given the observation that circulating human blood contains memory Th17 cells specific for fungal antigens (31–33).

In addition to T-cell responses, vigorous antibody responses were observed following vaccination with GP-OVA. Detectable titers occurred following the administration of the priming dose, and the titers increased with each subsequent boost. In addition, whereas mice that received OVA in alum mounted only an IgG1 response, mice vaccinated with GP-OVA had both IgG1 and

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FIG 4 OVA-specific ex vivo lymphoproliferative responses in vaccinated mice. CD4+ T cells were purified from lymph nodes and spleens of wild-type mice that received three injections of PBS (control), OVA admixed with alum (Alum/OVA), or OVA complexed in GPs (GP-OVA). Mitomycin C-treated BMDCs served as APCs. DCs and T cells were incubated for 4 days in the presence of no stimulants (Neg), 5 μg/ml of ConA, 50 μg/ml OVA, empty GPs (10:1 particle-to-BMDC ratio) or GP-OVA (10:1 particle-to-BMDC ratio; containing 0.5 μg/ml OVA [final concentration]). [3H]thymidine was added 24 h before harvesting. Results are the means ± SE from a representative experiment (out of three), with each sample tested in triplicate. *P < 0.0001, comparing the indicated groups.

FIG 5 Skewing of Th responses following vaccination with GP-OVA. CD4+ T cells purified from vaccinated mice were incubated with mitomycin C-treated BMDGs as APCs, with 50 μg/ml OVA as the antigenic stimulus (+OVA) or without stimulus (−OVA). Results for IFN-γ and IL-4 are the means ± SE from three independent experiments, and results for IL-17A are the means ± SE from two independent experiments, with 4 to 6 mice/group. *, P < 0.05, comparing GP-OVA to controls or Alum/OVA with (+OVA) or without (−OVA) stimulus. Note that the y axis scale differs among groups.
IgG2c responses. The latter subclass is associated with Th1 responses in the C57BL/6J mouse (13).

Data published by our group and others found undetectable IL-12p70 cytokine production following *in vitro* stimulation of BMDCs by β-glucans (6, 11, 34). IL-12p70 is a pivotal cytokine, guiding the differentiation of Th1 cells. Thus, the robust Th1 responses observed *in vivo*, following immunization with GP-OVA, and in other studies using OVA mixed with the β-glucan curdlan (11) would seem paradoxical. We speculate that several factors may contribute to this discrepancy. First, most of the *in vitro* studies used cultured BMDCs, which may not be fully representative of the heterogeneous DC populations that respond to β-glucans *in vivo*. Indeed, DC subsets vary greatly in their ability to process and present antigens (35–37). Second, cytokine secretion by macrophages responding to β-glucan stimulation could contribute to the Th skewing. Third, β-glucans are potent activators of the complement pathway (38), and responses to opsonized GPs likely differ from those to unopsonized GPs.

A low incidence of adverse events is critical for the advancement of vaccine candidates. β-Glucan preparations derived from fungi have a record of safety in both preclinical and human trials (39–41). In addition, GPs are derived from *S. cerevisiae*, which is regularly consumed by humans without ill effects. Although further testing is needed, these findings encourage the clinical development of GPs as a vaccine platform.

Finally, the structure of GPs permits loading of relatively large amounts of not only antigens but also other molecules with potential immunomodulatory activity, such as Toll-like receptor (TLR) ligands, small interfering RNA (siRNA), and cytokines (7, 8). This potentially allows manipulation of the magnitude and the qualitative nature of the vaccine response. For example, enhanced cytokine responses are seen when Dectin-1 and TLRs are cooperatively stimulated (6, 34). Thus, addition of a TLR ligand to GPs could lead to greater adjuvanticity. Similarly, siRNAs targeting negative regulators of proinflammatory pathways could potentially be added to the GP platform to inhibit the generation of regulatory T cells (42). Future studies will test these possibilities and determine the capacity of GPs loaded with microbial antigens to induce protective responses.

**MATERIALS AND METHODS**

**Chemicals and cell culture media.** Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. RPMI 1640 media were obtained from Invitrogen Life Technologies (Carlsbad, CA). R10 medium consists of RPMI 1640 containing 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Invitrogen), 0.5 μg/ml amphotericin B, and 55 μM 2-mercaptoethanol (2-ME; Invitrogen). Chicken OVA was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Incubation of cells was performed at 37°C in humidified air containing 5% CO₂.

**Mice.** Wild-type C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Transgenic OT-I and OT-II mice (Jackson Laboratory) were bred at the animal facility at the University of Massachusetts Medical School. Mice were specific pathogen free. All animal procedures were carried out under a protocol approved by the University of Massachusetts Medical School Institutional Use and Care of Animals Committee.

**Mouse BMDCs.** BMDCs were made as shown in previous studies, with a slight alteration (43, 44). Briefly, bone marrow cells were isolated from the tibiae and femurs of 8- to 12-week-old mice and then cultured in R10 medium supplemented with 10% conditioned medium from the murine granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting J558L cell line. On days 3 and 6, fresh GM-CSF-supplemented R10 medium was added. On day 8, nonadherent cells were harvested and purified with CD11c⁺ magnetic beads (magnetic cell separation system; Miltenyi Biotec, Auburn, CA), per the protocol supplied by the manufacturer. Positively selected BMDCs were washed with RPMI 1640 three times, treated with 50 μg/ml mitomycin C for 30 min at 37°C, and finally washed three times with R10 medium before used as APCs in lymphoproliferation and ELISPOT assays.

**GPs.** GPs were prepared from *S. cerevisiae* (Fleischmann’s baker’s yeast) using a series of alkaline and acidic extraction steps as previously described (1, 6, 8). Briefly, following centrifugation and washing in water, *S. cerevisiae* was subjected to two rounds of hot alkali extraction by heating for 1 h at 90°C in 1 M NaOH. The particles were suspended in water at pH 4.5, heated at 75°C for 1 h, and then successively washed with water (three times), isopropanol (four times), and acetone (two times). GPs were dried to yield a free-flowing light tan powder. To count the GPs or GP vaccine formulations, a 10⁻μg/ml suspension of particles in 0.9% saline was lightly sonicated, counted using a hemocytometer, and then kept in aliquots at −20°C until use. One microgram of GPs contains approximately 5 × 10⁵ particles.

**GP-OVA.** GPs containing complexed OVA (GP-OVA) were made as previously described, with a slight modification (8). Briefly, 10 mg of dry GPs were swollen with 50 μl of various concentrations of OVA dissolved in 0.9% saline for 2 h at 4°C to minimally hydrate the GPs, allowing OVA diffusion into the hollow GP cavity. The samples were then frozen at −80°C and lyophilized. To maximize OVA incorporation into the GP shells, the dry GP-OVA formulations were swollen, mixed with 50 μl of sterile water for 2 h at 4°C, and rehyphlized. To trap OVA inside the GPs, the dry GP-OVA formulations were heated to 50°C and swollen with 50 μl of 25 mg/ml RNA (derived from torula yeast, type VI) in 50 mM Tris-HCl, pH 8, 2 mM EDTA, and 0.15 M NaCl for 30 min, and then 500 μl of...
10 mg/ml tRNA was added for 1 h at 50°C to complete the complexation reaction, trapping OVA inside the GPs. The suspension was centrifuged and washed three times in 0.9% saline, and particles were resuspended in 70% ethanol for 30 min, washed three additional times in sterile 0.9% saline, resuspended, counted, diluted to 5 × 10^8 particles/mliliter in 0.9% saline, and stored at −20°C. To calculate the amount of OVA incorporated into the GPs, the unbound OVA protein in the saline washes was measured by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) against an OVA standard. In addition, fluorescein-labeled OVA was prepared by reaction with fluorescein isothiocyanate (FITC) and used as a fluorescent tracer to estimate OVA incorporated in the GP-OVA particles and unbound in the saline washes. Typically, OVA incorporation was in excess of 90%. DQ-OVA (Invitrogen) was loaded into GPs in an manner indicated times. BMDCs in glass-bottom dishes were washed, fixed, and examined by FACS analysis.

**Protein synthesis and OVA-specific ELISA.** Serum samples obtained from immunized mice were serially diluted starting at 1:40 and then progressively diluted 2-fold. Round-bottom 96-well plates with high binding (Corning, Corning, NY) were coated with 50 μl of 10 μg/ml OVA in 0.1 M NaHCO₃ overnight at 4°C, washed three times with PBS with 0.05% Tween 20, blocked with 100 μl 10% FBS in PBS for 1 h at room temperature, washed three times with PBS with 0.05% Tween 20, and incubated with 50 μl diluted mouse sera at room temperature for 2 h. Wells were then washed five times with PBS with 0.05% Tween 20, and incubated with biotin-conjugated rat anti-mouse IgG (1:50 dilution in 10% FBS in PBS) (BD Biosciences, San Jose, CA) or biotin-conjugated rat anti-mouse IgG2c (1:1,000 dilution in 10% FBS in PBS) (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 h. Then, the plates were washed five times with PBS-0.05% Tween 20, incubated with streptavidin-conjugated horseradish peroxidase (1:250 dilution in 10% FBS in PBS) (eBioscience) at room temperature for 1 h, washed five times with PBS-0.05% Tween 20, and then developed with tetramethylbenzidine solution (eBioscience). The optical density at 450 nm (OD₄₅₀) was measured on a plate reader (Molecular Devices, Sunnyvale, CA). Each serum dilution was tested in duplicate, and the antibody titer was defined as the dilution factor just above the inflection point.

**GraphPad Prism Software** was used for preparation of figures and statistical analyses. Comparisons of groups were performed using a one-way analysis of variance (ANOVA) with the Tukey multiple comparison test. Significance was defined as P values of <0.05.

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