Maize-Produced Ag2 as a Subunit Vaccine for Valley Fever

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Coccidioides is the causative agent of San Joaquin Valley fever, a fungal disease prevalent in the semiarid regions of the Americas. Efforts to develop a fungal vaccine over the last 2 decades were unsuccessful. A candidate antigen, Antigen 2 (Ag2), is notoriously difficult to express in *Escherichia coli*, and this study sought to accumulate the antigen at high levels in maize. Transformed maize lines accumulated recombinant Ag2 at levels >1 g/kg. Mice immunized with this antigen and challenged with live *Coccidioides* arthroconidia showed a reduction in the fungal load when Ag2 derived from either *E. coli* or maize was loaded into glucan chitin particles. A fusion of Ag2 to dendritic cell carrier peptide (DCpep) induced a T-helper type 17 response in the spleen when orally delivered, indicative of a protective immune response. The maize production platform and the glucan chitin particle adjuvant system show promise for development of a *Coccidioides* vaccine, but further testing is needed to fully assess the optimal method of administration.

Keywords. Valley fever; *Coccidioides*; subunit vaccine; maize-produced vaccine; plant vaccine; glucan chitin particles; bioencapsulation.

The proline-rich Antigen 2 (Ag2; also abbreviated as “Ag2/PRA”) is a promising antigen candidate for a coccidioidomycosis vaccine. It is a major immunoreactive component of the fungal cell wall in both the mycelial and spherule phases, and a recombinant form of the protein has shown protective efficacy in a murine model of pulmonary coccidioidomycosis [1, 2].

Ag2 is a glycosylphosphatidylinositol (GPI)-anchored protein that is difficult to express in traditional recombinant systems. The maize-based expression system is a cost-effective and high-yielding host for recombinant proteins, with expression levels of >2 g/kg in whole grain [3, 4], and is particularly suited for antigens as vaccines [5, 6].

One approach to enhance the immune response is the use of carrier to target the antigen. LT-B, a subunit of the heat-labile LT protein from *Escherichia coli*, acts as an enhancer of the immune response upon mucosal administration [7, 8]. A second approach uses DCpep, a dendritic cell (DC)-targeting 12-mer peptide that, when fused to the anthrax PA antigen and orally delivered, provides enhanced survival to lethal challenge [9]. DCs play a pivotal role in antigen presentation and lymphocyte activation in response to coccidioidal infection [10, 11]. Intranasal vaccination of mice with DCs transfected with Ag2

complementary DNA induced protective T-cell-mediated immunity and reduced the fungal burden following both pulmonary and systemic coccidioidal challenge [12]. A third approach has been to use glucan-chitin particles (GCPs) [13, 14], which are an effective adjuvant-delivery system to stimulate a protective T-helper type 1 (Th1) and Th17 mixed response against *Coccidioides* [15]. Increased interferon γ (IFN- γ) production is thought to be associated with vaccine-mediated protection against coccidioidomycosis [16]. However, recent studies suggest that Th17 immunity may be even more critical for vaccine protection against pulmonary coccidioidomycosis in mice [15, 17, 18]. While IFN- γ was dispensable, Th17A and Th17Ra were essential for ΔT (an attenuated coccidioidal triple-gene-knockout strain) vaccination against coccidioidomycosis [18]. Use of Th17 immunotargeting to provide vaccine protection was further demonstrated by formulation of a multivalent antigen (rCpa1) with GCPs to enhance the lung Th17 immune response upon pulmonary coccidioidal challenge [15]. In this study, GCPs with purified Ag2 and orally administered Ag2 in maize grain was able to elicit an immune response indicative of protection.

MATERIALS AND METHODS

Constructs

The full-length Ag2 sequence (GenBank accession U39835) was codon optimized for maize, predicted splice sites and instability elements were removed, and a valine was placed directly preceding the Ag2 sequence to improve stability. Specific constructs were as follows (Figure 1): VFA, a promoter for the gene encoding globulin 1 [19] and a barley aleurain vacuolar

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Figure 1. Schematic representations of recombinant Antigen 2 (rAg2) expression constructs. Nine plasmid constructs were designed to express rAg2 in maize. Tissue-preferential maize promoters (Pr25:seed embryo, Pr44:seed embryo, and Pr39:endosperm) were used to drive Ag2 gene transcription and produce a subsequently high yield of protein. In various constructs, cellular/organelle-targeting sequences (a barley aleurain vacuolar targeting signal [Vac] and a barley α -amylase [BAASS] cell wall-targeting signal) and a C-terminus (a KDEL C-terminal endoplasmic reticulum (ER)-targeting sequence) were added to the codon-optimized Ag2 (Ag2opt). Immunomodulators, a heat-labile enterotoxin-B subunit (LT-B), and a dendritic cell-targeting sequence (DC3) were fused to the C-terminus of Ag2opt as indicated for VFF and VFG constructs, respectively. The 3' untranslated region of the gene encoding potato protease inhibitor II (*pinII*) was added to all constructs as a transcriptional termination element to enhance messenger RNA stability.

targeting signal [20]; VFB, a promoter for the gene encoding globulin 1 and a barley α -amylase (BAASS) cell wall-targeting signal; VFC, a BAASS cell wall-targeting signal at the N-terminal region and a KDEL C-terminal endoplasmic reticulum (ER)-targeting sequence; VFD, an enhanced promoter for the gene encoding globulin 1 [21]; VFE, a promoter for the gene encoding globulin 1 and a BAASS cell wall-targeting signal with 2 copies of the transcription unit in tandem in a head-to-tail orientation; VFF, a promoter for the gene encoding globulin 1 and a BAASS cell wall-targeting signal (a linker sequence [VDPRVPSSG] connects the Ag2 C-terminal sequence to the LT-B N-terminal sequence [GenBank accession M17874] and begins after the signal sequence, starting with amino acids APQS); VFG, a promoter for the gene encoding globulin 1 and a BAASS cell wall-targeting signal, with an additional C-terminal DC-targeting sequence (DC3) but no linker [9]; VFH, an embryo-preferred promoter [22] and a BAASS cell wall-targeting signal; and VFJ, a promoter for the gene encoding globulin 1 and a fungal endogenous Ag2-targeting signal.

All coding sequences were followed by a potato protease inhibitor II (*pinII*) 3' untranslated region [23] and a glufosinate resistance gene [5], for selection of putative plant transformants. Ag2 transgenic constructs used for *E. coli* expression and purification were created as described previously [1].

Maize Transformation and Seed Propagation

Constructs were transformed into *Agrobacterium tumefaciens* and maize, as described previously [24]. Transformed lines were

selected and propagated as described previously [5, 19]. T1 seeds were generated by crossing the transformed ears with pollen from maize HiII germplasm. T2 seeds were self-pollinated.

Purification of Ag2 From Bacteria and Antibody Production

E. coli-derived Ag2 was purified as previously described [1], with modifications. Briefly, *E. coli* containing a thioredoxin-His(6x)-Ag2 fusion protein with a thrombin cleavage site was grown overnight in LB-antibiotic medium. The seed culture was inoculated into two 1-L flasks containing 250 mL of MagicMedia with antibiotic and was grown overnight. The cell pellets were harvested, ground with liquid nitrogen, resuspended in phosphate-buffered saline (PBS), and treated with DNaseI. The pellet was resuspended in 8 M urea and centrifuged; the supernatant was then adjusted to 2 M urea, and the extract was loaded onto a Ni-NTA His-bind resin. After being washed with the same buffer, the column was treated with thrombin to release Ag2 in the eluate, confirmed by Coomassie gel electrophoresis. This material was sent to Pacific Immunology to make rabbit polyclonal antibodies. The final blood specimen collected from rabbits was used for all analyses.

Quantitation of Ag2 From Maize

Protein was extracted from either single T1 seeds or 100 mg of ground 50-seed T2-bulk material, using 1 mL of PBS plus 1% Triton X-100 extraction buffer. Six single T1 seeds were assayed individually from each ear, while 50-seed T2-bulk material was assayed in duplicate. A minimum of 5 independent

transformation events were analyzed for all T1 constructs, with the exception of VFC, VFD, and VFH (1 or 2 events each). The percent total soluble protein in T1 seeds was determined using the Bradford assay. Levels of soluble protein (in milligrams/kilograms) were estimated by weighing the 6 T1 seeds together and calculating the mean weight per seed. Antigen in the extract was detected using rabbit polyclonal anti-Ag2 antibodies [1]. An enzyme-linked immunosorbent assay (ELISA) using antibody-coated plates was blocked with PBS plus 3% bovine serum albumin and washed with PBS, and detection antibody was applied in PBS plus 3% bovine serum albumin. Detection antibody was generated by purifying the serum antibody on a protein A column and biotinylating the purified antibody. Streptavidin-AP and pNPP tablets were used to detect antibody binding to Ag2. The recombinant *E. coli*-purified protein was used as a standard on ELISAs.

Purification of Maize-Derived Ag2

Ag2 was extracted from ground maize from VFG lines, using PBS plus 1% Triton X-100. After extraction for 30 minutes on ice, the suspension was centrifuged and filtered to remove cell debris. Extracts underwent affinity purification using custom polyclonal antibodies bound to AminoLink resin, Ag2 protein was eluted with glycine buffer (pH 3), and neutralization with buffer was immediately performed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% gels run with Tris/glycine/SDS buffer. For Western blotting, 2 gels were run simultaneously. One was stained using Coomassie blue, for molecular weight analysis; the other was transferred to a nitrocellulose membrane, using the iBlot-2 system. Custom rabbit polyclonal anti-Ag2 primary antibody was applied, followed by AP-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody and BCIP/ NBT liquid substrate, for Ag2 band visualization.

Vaccine Candidate Preparation

Maize grain was ground into flour and formed into wafer-like tablets, as described previously [25].

GCPs were loaded with *E. coli*-purified Ag2, as described previously [5, 26]. Each dose consisted of 200 µg of GCPs, 1 µg of Ag2, and 25 µg of mouse serum albumin. Ovalbumin (1 µg) was the positive control for loading the particles. Maize-produced Ag2-loaded GCPs were made using affinity-purified material from VFG seed. The concentration of maize-produced Ag2 was lower (approximately 10% of bacterially produced Ag2), owing to limitations in the amount of seed available at the time. Additional maize-produced Ag2 would have required another maize growing cycle. A 10% deviation in the concentration was deemed acceptable to complete the project within time and budget constraints. The efficiency of protein loading was confirmed by SDS-PAGE.

Mouse Studies

Immune Recall Study Using Formalin-Killed *Coccidioides Spherules* (FKSs)

Ten BALB/c mice, 5 males and 5 females, were assigned to each treatment group. Mice fasted overnight before the first day of each dose. Treatment 1 comprised subcutaneous injection of 1 µg of *E. coli*-derived Ag2 loaded into 200 µg of GCPs on day 0, and 15 g of oral VFE wafers for boosting doses on days 14, 28, and 42. Mice fed ad libitum on wafers that did or did not contain antigen, and wafers were administered for 3 consecutive days at 5 g/day/mouse. Treatments 2 and 3 were the same as treatment 1 except that VFF and VFG wafers were fed to mice, respectively. Treatment 4 consisted of Ag2-GCP injections on days 0, 14, 28, and 42. Treatment 5 was a combination of injected and oral VFG doses on all dosing days. Treatment 6 used control GCPs and control wafers administered on all dosing days. Treatment 7 comprised injection with saline on all dosing days.

After treatment, mice were treated intranasally with 5×10^4 FKSs on day 63 and euthanized on day 70 to evaluate the T-cell response in lung and spleen. Blood specimens were collected on days 0, 28, 54, and 70 to test for antibody production.

Live *Arthroconidia* Challenge Study

Treatment group size, mouse breed, and sex distribution were the same as described for the immune recall study. All doses were initiated on days 0 and 14 (1 primary dose and 1 boosting dose). Challenge was with intranasal delivery of approximately 100 viable arthroconidia per mouse on day 42. Lung colony-forming units (CFUs) were analyzed on day 56.

Anti-Ag2 Antibody Detection

Induction of anti-Ag2-specific serum IgG and immunoglobulin (IgA) following vaccination was assessed using indirect ELISA as previously described [27]. Briefly, 0.1 µg of recombinant Ag2 antigen in 50 mM NaC₂H₃O₂ buffer (pH 4.2) was coated on each well of a plate. Serum was applied at a dilution of 1:100 for IgG detection and a dilution of 1:30 for IgA detection. AP-conjugated anti-mouse IgG or AP-conjugated anti-mouse IgA were used to detect IgG and IgA, respectively. A pNPP solution was used to visualize results read at OD₄₀₅.

IL-17A-Secreting Splenocyte Recall Enzyme-Linked Immunospot (ELISPOT) Assay

Following collection of terminal blood specimens, spleens were dissected from 8 mice in each treatment group and analyzed for cytokine production by using a T-cell recall assay, as described previously [26, 28]. Briefly, splenocytes (1×10^6 splenocytes/well) were added to 96-well ELISPOT plates precoated with anti-mouse IL-17A monoclonal antibody and stimulated with Ag2b (100 nM and 200 nM), anti-CD3 antibody (positive control), or medium (negative control) for 24 hours. IL-17A-secreting splenocytes were detected by addition of biotinylated anti-mouse IL-17 monoclonal antibody, followed by incubation

with streptavidin-conjugated alkaline phosphatase and BCIP/NBT phosphatase substrate. Spot numbers were analyzed with an Immunospot S6 Micro Analyzer. Statistically significant differences between cytokine production in parenterally and orally vaccinated mice were determined using a Student *t* test, as previously described [26].

Intracellular Cytokine Staining

Intracellular cytokine staining assays were conducted to determine the numbers of IFN- γ - and IL-17-producing CD4⁺ T cells in lungs 9 and 14 days after challenge. Pulmonary leukocytes were isolated as previously reported [29]. The leukocytes were gated for CD4⁺ T cells, and their levels of cytokine expression were determined. Absolute numbers of specific cytokine-producing CD4⁺ T cells relative to the total population of lung-infiltrated leukocytes per lung homogenate were calculated by multiplying the percentage of each gated population by the total number of viable pulmonary leukocytes determined by hemocytometer counts, as previously reported [29]. The Student-Newman-Keuls test, a type of analysis of variance for all pair-wise comparisons, was used to analyze percentages and numbers of specific cytokine-producing T cells in lungs of mice.

Quantification of CFUs

The lung fungal burden was assessed at day 56, 2 weeks after challenge, the number of CFUs/lung was evaluated as previously described [30], and the Mann-Whitney *U* test was performed for statistical analysis, as previously described [1].

RESULTS

Accumulation of Ag2 in Maize

Sixty-three transformation events representing various constructs were evaluated for Ag2, using ELISA. The 10% of seeds with the greatest Ag2 levels were used to rank the potential for each construct to overproduce the antigen [3]. The highest Ag2 level was yielded by the VFF construct (Ag2-LT-B), with a mean percent total soluble protein (%TSP) yield of 1.95% (475 mg/kg) and the highest-producing seed yielding 3.99% (1388 mg/kg; Figure 2). The second highest mean level was produced by the VFG construct (Ag2-DCpep), with a mean %TSP yield of 1.09% (256 mg/kg) and the highest-producing seed yielding 3.07% (711 mg/kg). Subcellular localization affected accumulation, as demonstrated when comparing constructs VFA (containing a plant vacuolar signal), VFB (containing a plant cell wall signal), and VFC (containing a plant ER signal). A construct with the native fungal Ag2 targeting signal was also compared to the plant signal sequence. VFA and VFJ seed demonstrated the highest mean Ag2 accumulations, with TSP yields of 0.11% and 0.10%, respectively; VFB seed having a mean yield of 0.07%; and VFC seed had the lowest mean yield, at 0.04%. Using an enhanced promoter in construct

Figure 2. Accumulation of Antigen 2 (Ag2) proteins in the 10% of maize seeds with the greatest Ag2 levels from 9 maize lines that underwent transformation with an Ag2 gene construct. Data show the mean percent ($\pm$ standard error) of the total soluble protein content.

VFD, higher levels of Ag2 (%TSP yield, 0.20%) were obtained as compared to VFB with the same subcellular location. Two transcription units in tandem (VFE) expressed somewhat more (%TSP yield, 0.10%) than the equivalent single transcription unit (VFB). Use of an endosperm promoter (VFH) as compared to an embryo promoter (VFB) resulted in similar accumulation of recombinant antigen, based on whole seed. Constructs VFF and VFG resulted in a clearly superior accumulation of Ag2, compared with all other constructs.

Western blot analysis was used to determine the protein integrity and size of recombinant Ag2. Figure 3A shows the results for representative seed extracts from select constructs. Maize-derived Ag2 displayed a major band slightly smaller than that of *E. coli*-derived Ag2, with the exception of VFF with the Ag2-LT-B fusion, which accounted for a much larger size.

Formulation of Ag2-Based Vaccines

VFE, VFF, and VFG grain were formed into wafers for evaluation of oral delivery and encapsulation of soluble Ag2 with GCPs for subcutaneous injection. No antigen was lost during wafer production (Table 1).

Ag2-DCpep was isolated from VFG seed extracts by anti-Ag2 affinity chromatography. Figure 3B shows a Western blot of seed extract and eluate from the antibody column. Purified maize Ag2-DCpep (approximately 0.1 μ g) was mixed with 25 μ g of mouse serum albumin and encapsulated into GCPs (200 μ g) to make a each dose of GCP-Ag2m vaccine. Similarly, a GCP-Ag2b vaccine was generated using bacterium-expressed Ag2 (1 μ g/dose) and a control GCP-OVA (1 μ g ovalbumin/dose) without Ag2.

Maize-Derived Ag2-Induced Cell-Mediated Immune Responses in Mice

Seven groups of BALB/c mice were vaccinated with various vaccine formulations/regimens and then intranasally treated with formalin-killed *Coccidioides* spherules. The spleens were collected to make a splenocyte suspension for T-cell recall

Figure 3. Western blot analysis of maize-derived recombinant Antigen 2 (Ag2) proteins. Proteins were separated by gel electrophoresis, transferred to a nitrocellulose membrane, and probed with an anti-Ag2 antibody. *A*, Protein samples were from *Escherichia coli*-expressed rAg2 (1 µg, lane 1; 5 µg, lane 2) or crude seed extracts from maize lines transformed with VFE (lane 3), VFF (lane 4), VFG (lane 5), and VFH (lane 6) constructs. *B*, Proteins were isolated from untransformed (lane 2) or VFG-transformed (lane 3) maize seeds by anti-Ag2 affinity chromatography. Molecular weight markers are shown in lane 1. The detection of Ag2-DCpep (molecular weight, approximately 35 kDa) isolated from VFG seeds is indicated with an arrow.

assessment by IL-17A-specific ELISPOT, and the lungs were used for immune T-cell recruitment/activation assessment by flow cytometry. All mice received a prime vaccination followed by 3 booster doses 2 weeks apart. The first 4 groups of mice were primed with GCP-Ag2b by subcutaneous injection and boosted with oral delivery of VFE (Ag2; group 1), VFF (Ag2-LTB; group 2), or VFG (Ag2-DCpep; group 3) wafers or subcutaneous injection of GCP-Ag2b (group 4). Mice in groups 5–7 received the same vaccine materials for their priming and boosting doses, which consist of a combination of GCP-Ag2b (delivered subcutaneously) and VFG wafers (delivered orally; group 5), a combination of GCP-OVA (delivered subcutaneously) and control wafers without Ag2 (delivered orally; group 6), and PBS (delivered subcutaneously; group 7). Mice fed on the wafers ad libitum and were estimated to ingest 0.1 mg of VFE Ag2, 1.5 mg of VFF, and 1.5 mg of VFG per dose, based on wafer Ag2 concentrations and consumption estimates.

To assess whether these vaccine candidates induced protective immunity, we intranasally inoculated mice with FKS to examine immune recall by measuring IL-17-producing T cells in spleens (Figure 4) and lung T-cell recruitment (Figure 5) for all vaccinated groups of mice inside an animal biosafety level 2 (ABSL2) laboratory. The vaccine protective efficacy test was later conducted with live arthroconidia challenge, for

GCP-Ag2m vaccine derived from maize under ABSL3 confinement (Figure 6). For immune response assessment, mice in all treatments were exposed to FKS 3 weeks after the last vaccination. Naïve mice (group 8) without vaccination or FKS exposure were used as a control for immunoassays. Splenocytes collected from each group of mice 1 week after FKS exposure were stimulated with Ag2b (100 nM and 200 nM; antigen specific) and anti-CD3 (mitogen-positive control) or untreated (medium; negative control). Numbers of IL-17A-secreting cells were quantified by an ELISPOT assay. As shown in Figure 4, minimal numbers of IL-17A-secreting splenocytes were present in all untreated samples; however, the numbers increased significantly in all groups upon anti-CD3 antibody treatment, suggesting that the obtained splenocytes were receptive to antigen stimulation. Vaccination with 4 doses of GCP-Ag2b induced the highest Th17 cellular immune response (groups 4 and 5; Figure 4). The conditions for the assay were at saturation for groups 4 and 5; therefore, the extent of the additive effect of oral VFG vaccination on inducing Th17 immunity remains unclear. Mice receiving ingested booster doses of Ag2-DCpep (VFG material; group 3) showed an increased response over Ag2 alone or Ag2-LTB (VFE and VFF; groups 1 and 2, respectively).

Similarly, priming and boosting with GCP-Ag2b (group 4) enhanced the recruitment of IFN-γ (Th1) and IL-17A-producing T cells (Figure 5). There was a slight increase in the response when combined with VFG oral vaccine (group 5), but this was not statistically significant. The assay was at or near maximum for both groups, therefore it was not possible to resolve the effect conclusively.

A progressive increase in the serum IgG titer was observed in mice from groups 1–5, with titers of 10^3 for groups 1–3 and 10^4 for groups 4 and 5 (data not shown). A significant increase in the serum IgA titer was only detected in group 4 and 5 mice.

Table 1. Antigen 2 Concentrations in Wafers Used For Oral Vaccine Delivery

Wafer	Parent Material, mg/kg	Wafers, mg/kg in Flour
VFE	7	10
VFF	165	173
VFG	158	153
Control	Below detection	Below detection

Figure 4. Vaccination with Antigen 2 (Ag2) induced a cell-mediated T-helper type 17 (Th17) immune response. *A*, Groups of BALB/c mice received 1 priming (p) and 3 boosting (b) doses of vaccine formulations. Immunogenic vaccines included bacterium-expressed recombinant Ag2 encapsulated with glucan-chitin particles (GCP-Ag2b) and 3 types of maize-derived Ag2 in the form of edible wafers (VFE, VFF, and VFG). GCP-OVA, wafer containing no Ag2 (corn control), and phosphate-buffered saline (PBS) alone were used for mock vaccination. Vaccines were administered by oral delivery, for wafers, and subcutaneous injection, for GCP formulations. Vaccinated mice (groups 1–7) were exposed to formalin-killed spherules (FKSs) of *Coccidioides posadasii*, and spleens were removed for interleukin 17A (IL-17A)-specific enzyme-linked immunosorbent assay in a 96-well plate. Splenic cells were stimulated with Ag2b (100 nM and 200 nM) or anti-CD3 antibody (α CD3), or they were left untreated (Neg). Splenic cells that secreted IL-17A were visualized (*A*) and quantified as spot-forming units (*B*), with a maximum count of 3000 cells/well. TNTC, too numerous to count.

Purified Ag2m Reduced Fungal Burden Following Pulmonary *Coccidioides posadasii* Challenge

To further assess the protective efficacy of Ag2-DCpep, we purified the antigen from VFG maize seeds by anti-Ag2 affinity chromatography and formulated a GCP-Ag2m vaccine. Groups of mice (10/group) were vaccinated twice with GCP-Ag2m, GCP-Ag2b, or GCP without Ag2 by subcutaneous injection and challenged intranasally with a lethal dose of *C. posadasii* 2 weeks after the booster. The fungal burden in the lungs was assessed at day 14 after challenge. Compared with GCP-alone vaccination, GCP-Ag2b and GCP-Ag2m vaccinations significantly reduced the fungal burden in the lungs by an average of 92% and 82%, respectively (Figure 6). Note that GCP-Ag2m

contains approximately 0.1 μ g of Ag2-DCpep, while GCP-Ag2b consists of 1 μ g of Ag2 per vaccine dose. There was no statistically significant difference in efficacy between maize- and bacterium-produced Ag2.

DISCUSSION

Maize shows promise for the accumulation of many recombinant proteins using intracellular targeting signals [19, 21, 27, 31, 32]. We established that the vacuolar targeting sequence produced the highest levels of Ag2. However, fusion at the C-terminal end of Ag2 with LT-B or DCpep increased accumulation >10-fold, presumably due to stabilization of the protein. Analysis showed that most constructs had a cross-reacting

A

B

C

Figure 5. After stimulation with formalin-killed spherules (FKSs), the T-helper type 17 (Th17) cell count increased in the lungs of Antigen 2 (Ag2)-vaccinated mice. Flow cytometry of interferon γ (IFN- γ)- and interleukin 17A (IL-17A)-expressing Th1 and Th17 cells, respectively, in lungs of BALB/c mice that were vaccinated with various Ag2 formulations and stimulated with FKSs of *Coccidioides posadasii*. Bacterium-expressed recombinant Ag2 encapsulated with glucan-chitin particles (GCP-Ag2b) and 3 types of maize-derived Ag2 in the form of edible wafers (VFE, VFF, and VFG) were used for vaccine priming (p) and/or boosting (b) 3 times at 2-week intervals. GCP-OVA, wafer containing no Ag2 (corn control), and phosphate-buffered saline (PBS) alone were used for mock vaccination. The percentages of gated IL-17A-producing cells per lung (A) and numbers of Th1 cells (ie, IFN- γ -expressing CD4⁺ T cells; B) and Th17 cells (ie, IL-17A-expressing CD4⁺ T cells; C) were determined by intracellular cytokine staining 7 days after FKS exposure.

band slightly smaller than Ag2 produced in bacteria. Although these bands indicate a molecular weight bigger than that of Ag2, they are consistent with previous reports of *E. coli*-derived Ag2. The source of the discrepancy is not clear but may reflect the

relatively high proportion of proline that may increase protein bulk and slow its migration during SDS-PAGE or may be due to glycosylation. VFE displayed a doublet (Figure 3A), with the presumed uncleaved and unanchored protein as the top band and the cleaved (C-terminal 23 amino acids) and GPI-anchored protein as the smaller band. This presumption is supported by the fact that the VFG lines (Ag2:DCpep) showed a more intense top band. The GPI anchor prediction program, big PI Plant Predictor (version 2.1), fails to predict cleavage of the 23 C-terminal amino acids once DCpep is added to the C-terminus of Ag2. The endosperm-targeted Ag2 seems to undergo degradation (Figure 3).

Some VFF first-generation single seeds demonstrated levels of >1000 mg/kg for the recombinant antigen, with the potential for >10-fold greater accumulation using breeding programs. Recombinant protein concentration can be increased a further 7-fold by fractionation of the seed and retention in the embryo fraction. These levels of improvement by breeding and fractionation have been obtained for other recombinant proteins [21, 33]. This holds great promise for a low-cost subunit vaccine because levels should be >100-fold higher than when produced in *E. coli*.

To investigate whether the immune response elicited with maize-produced material is comparable to that of Ag2 made in microbes, purified maize-produced Ag2m was compared

Figure 6. Immunization with bacterium- and maize-expressed Antigen 2 (Ag2) protected pulmonary coccidioidomycosis. BALB/c mice (10 per group) were vaccinated with GCP-Ag2b, GCP-Ag2m, or GCP alone and challenged intranasally with a lethal dose of *Coccidioides posadasii*. The lung fungal burden was measured 14 days after challenge. CFU, colony-forming unit. * $P < .05$ and ** $P < .005$, by the Mann-Whitney U test.

to bacterially produced Ag2b. Both showed a reduction in the fungal burden after challenge when administered in GCPs subcutaneously. A more detailed study is required to understand whether there are quantitative differences; however, the maize-derived material appears to be effective.

In another study, GCP-Ag2b vaccination induced a weak Th1 response but a robust Th17 response (group 4; Figure 5). This observation supports the critical role of Th17 immunity in the control of coccidioid infection [15].

Finally, combinations of oral and injected GCP candidates were tested to determine whether a strong protective immune response could be obtained [34, 35]. The FKS recall assays showed that oral wafers using DCpep provided a better Th17 response in splenocytes than the other wafers. This was not likely due to concentration alone, because the VFF material had the same concentration of Ag2; therefore, DCpep may enhance the immune response. These studies also showed that the oral vaccines induced significant protective immune responses, without any indication of oral tolerance. It is unclear what determines whether an organism mounts a tolerogenic or immunogenic response to antigens; however, further studies will need to be undertaken to resolve this issue.

The combination of GCPs and oral wafers may have shown an improvement over injected doses alone. Because the immune response detection methods were saturated in the FKS exposure study, it was not possible to determine whether this was significant. In summary, the level of recombinant Ag2 produced in maize was ≥ 1 g per kg of grain, an economically feasible production system for a commercial vaccine. For comparison, the amount obtained from bacteria is <10 mg/L and only when fused to a second protein that requires protease cleavage. Promising data from mouse models indicate that the maize-produced Ag2m protein can provide immune protection. Additional studies are required to (1) optimize Ag2 accumulation in maize, (2) develop an efficient purification process, (3) characterize Ag2 from maize, and (4) develop the optimal vaccination regimen.

Notes

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consider relevant to the content of the manuscript have been disclosed.

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