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Whole glucan particles as a vaccine against systemic coccidioidomycosis

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Abstract:	We reported yeast-derived whole glucan particles (WGP), with or without conjugation to BSA, used as a vaccine protected against systemic aspergillosis in mice. Here, we examined their utility as a potential vaccine against coccidioidomycosis. WGP was prepared from Saccharomyces cerevisiae; conjugation with BSA (WGP-BSA) was done using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate-mediated conjugation. Heat killed S. cerevisiae (HKY) was used as a positive control vaccine. CD-1 mice were vaccinated with WGP or WGP-BSA, HKY or PBS once weekly beginning 21 days prior to infection. Mice were infected intravenously with arthroconidia of Coccidioides posadasii. In the low mortality study, 50% of PBS-treated controls died. Only WGP-BSA at 0.6 mg/dose induced significant protection compared to PBS treatment. All surviving mice were infected in all three organs examined. Those given WGP-BSA at 0.6 mg/dose had fewer CFU in liver and lungs ($P = 0.04$), and WGP at 6 mg/dose fewer in lungs ($P < 0.02$), compared to PBS. In the high mortality study, 90% of PBS mice died. Vaccination with HKY, and WGP or WGP-BSA at 6 or 12 mg/dose significantly prolonged survival ($P \leq 0.05$). No surviving mice were free of infection. HKY and WGP-BSA at 12 mg/dose reduced CFU in the liver and lungs ($P < 0.05$) and WGP-BSA 6 mg/dose reduced CFU in lungs ($P < 0.05$); unconjugated WGP did not reduce infection. WGP or WGP-BSA acted as a vaccine that protected against mortality caused by coccidioidomycosis. Thus, WGP protection against coccidioidomycosis and aspergillosis provides the basis for development of a panfungal vaccine.

Whole glucan particles as a vaccine against systemic coccidioidomycosis

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We reported yeast-derived whole glucan particles (WGP), with or without conjugation to BSA, used as a vaccine protected against systemic aspergillosis in mice. Here, we examined their utility as a potential vaccine against coccidioidomycosis.WGP was prepared from *Saccharomyces cerevisiae*; conjugation with BSA (WGP-BSA) was done using1-cyano-4-dimethylaminopyridinium tetrafluoroborate-mediated conjugation. Heat killed *S. cerevisiae* (HKY) was used as a positive control vaccine. CD-1 mice were vaccinated with WGP or WGP-BSA, HKY or PBS once weekly beginning 21 days prior to infection. Mice were infected intravenously with arthroconidia of*Coccidioides posadasii*. In the low mortality study, 50% of PBS-treated controls died. Only WGP-BSA at 0.6 mg/dose induced significant protection compared to PBS treatment. All surviving mice were infected in all three organs examined. Those given WGP-BSA at 0.6 mg/dose had fewer CFU in liver and lungs ($P = 0.04$), and WGP at 6 mg/dose fewer in lungs ($P< 0.02$), compared to PBS. In the high mortality study, 90% of PBS mice died. Vaccination with HKY, and WGP or WGP-BSA at 6 or 12 mg/dose significantly prolonged survival($P \leq 0.05$). No surviving mice were free of infection. HKY and WGP-BSA at 12 mg/dose reduced CFU in the liver and lungs ($P< 0.05$) and WGP-BSA 6 mg/dose reduced CFU in lungs ($P< 0.05$); unconjugated WGP did not reduce infection. WGP or WGP-BSA acted as a vaccine thatprotected against mortality caused by coccidioidomycosis. Thus, WGP protection against coccidioidomycosis and aspergillosis provides the basis for development of a panfungal vaccine.

Introduction

Coccidioidomycosis is endemic to parts of North America, Central and South America (Galgiani*et al.* 2005). Although effective therapy is available, the treatment duration is protracted and failures can occur while on therapy with azoles and some patients, such as those with meningitis, require life-long therapy (Dewsnup*et al.* 1996). Prevention of disease through the use of a vaccine is a highly desirable option, but one that is not currently available for coccidioidomycosis. Numerous studies have been done examining various vaccine preparations and a single preparation, formalin-killed spherules, was taken to clinical trial, but failed (Pappagianis 1993; Pappagianis 2001; Cole*et al.* 2004; Clemons & Stevens 2011).

During the course of studies on potential vaccines, we found that heat-killed yeast of *Saccharomyces cerevisiae* (HKY) provided protection against experimental systemic aspergillosis (Liu*et al.* 2011a; Stevens*et al.* 2011). Furthermore, we found that HKY induce protection against infection with *Candida albicans*(Liu *et al.* 2012a), *Cryptococcus grubii* (Majumder *et al.* 2014), *Mucor* (Luo *et al.* 2014),and*Coccidioides posadasii*(Capilla *et al.* 2009).To better understand the mechanism and components responsible for protection, we performed studies on cell wall glycans, showing mannans and glucans could induce protection against aspergillosis or coccidioidomycosis (Liu *et al.* 2010; Liu *et al.* 2012b). Most recently, we have demonstrated that highly pure particulate β-glucans alone or conjugated to bovine serum albumin (BSA) could induce protection against aspergillosis, whereas soluble preparations could not (Clemons*et al.* 2014a).

To further examine the specificity of protective nature of these particulate β-glucans, we have tested them against experimental systemic murine coccidioidomycosis. Our results demonstrate

the potential of particulate β-glucan preparations to induce protection and serve as a basis for the development of a pan-fungal vaccine.

Materials and Methods

Animals. Male,six-week-old, CD-1 mice from Charles River were acclimatized for 1 week prior to use in these studies. Micewere randomized to experimental groups, housed in microisolator cages and provided food and water *ad libitum*under Animal Biosafety Level 2 standards. All animal experiments were done under an approved protocol of the Institutional Animal Care and Use Committee of the California Institute for Medical Research. All guidelines for animal care and use from the Office of Laboratory Animal Welfare, National Institutes for Health, Washington, DC USA were followed(Council 2011).

Organism. *Coccidioides posadasii* strain Silveira (ATCC28868) was used in these studies. The organism was grown and arthroconidia for inoculation prepared as described previously(Clemons *et al.* 1990). All growth and handling of the organism were done under Biosafety Level 3 containment(Chosewood & Wilson 2009).

Vaccines. Heat killed yeast (HKY) of *Saccharomyces cerevisiae* strain 96-108 were prepared as described previously(Capilla *et al.* 2009; Liu *et al.* 2011a; Liu *et al.* 2011b) and served as a positive vaccine control in these studies. Whole glucan particles (WGP) and WGP conjugated to bovine serum albumin (BSA) were prepared from *S. cerevisiae* at Biothera as described previously(Clemons *et al.* 2014a). Other investigators have reported that immunization with BSA with or without adjuvant provides no protection against coccidioidomycosis in mice(Li *et al.* 2001). Thus, we did not feel that it was necessary to include a BSA only control.

Groups of 10 mice were vaccinated with PBS, HKY, WGP-BSA or WGP subcutaneously on days -21, -14, and -7 prior to infection. Vaccine doses were: HKY at 6 x 10^7 yeast per dose (2.5 mg/dose) in 0.15 ml(Capilla *et al.* 2009), WGP or WGP-BSA at 0.6, 6, or 12 mg/dose in 0.3 ml volumes(Clemons *et al.* 2014a)or 0.3 ml of PBS. All doses were given using volumes as a split dose in twodorsal sites based on previous studies with HKY, WGP and WGP-BSA. The vaccine regimens and schedules were the same in both studies. A single mouse in the group given PBS died from unknown causes prior to infection with *Coccidioides* and was not included in any analyses. Mice vaccinated with HKY and higher dosages of WGP or WGP-BSA often had small palpable granulomas at the site of injection. No other effects of vaccination were noted.

Infection model. The model of infection used in these studies was that of establishing systemic disease similar to previous investigations(Clemons *et al.* 1983; Clemons *et al.* 1985b; Clemons *et al.* 1985a; Clemons *et al.* 1990; Clemons *et al.* 1995; Clemons *et al.* 2000; Capilla *et al.* 2009). For the low mortality study, all mice were infected intravenously with 127 arthroconidia of *C. posadasii*in 0.25 ml volume; groups had 10 mice each, except for the PBS-treated group which had n = 9.For the high mortality study, all mice were infected intravenously with 275 arthroconidia of *C. posadasii*in 0.25 ml volume; all groups had 10 mice each. The group sizes were determined using StatMate ver 2 (GraphPad Software, Inc., La Jolla, CA) to have approximately 80% power to detect differences in survival at the 0.05 level. These group sizeshave been robust for determining differences in outcome using nonparametric statistics.

Mice were examined daily and deaths were tallied through day 28 postinfection. Mice found severely moribund or immobilized were euthanatized using CO_2 anoxia.On day 28 of infection all surviving mice were euthanatized using CO_2 and the CFU remaining in the lungs, liver, and

spleen were determined by quantitative plating of organ homogenates as described previously; these are the primary target organs of infection in this model(Clemons *et al.* 1983; Clemons *et al.* 1985b; Clemons *et al.* 1985a; Clemons *et al.* 1990; Clemons & Stevens 1992; Clemons & Stevens 1994; Clemons *et al.* 1995; Capilla *et al.* 2009).

Statistical analysis. Comparative survival was analyzed by log rank test and the residual burdens of *C. posadasii* in the organs was compared using a Mann-Whitney U test using GraphPad Prism (ver. 3.1). A $\log_{10}$ value of 7 was assigned to data points missing due to the death of an animal (Lachin 1999; Shih 2002). This value assures that death is assigned a worse outcome than issurvival with any fungal burden and is close to that found just prior to death (Clemons *et al.* 1983; Clemons *et al.* 1985b; Clemons *et al.* 1985a; Clemons *et al.* 1990; Clemons & Stevens 1992; Clemons & Stevens 1994; Clemons *et al.* 1995; Capilla *et al.* 2009)

Results

The aim of these studies was to determine whether vaccination with WGP or WGP-BSA could provide protection against experimental systemic coccidioidomycosis similar to the protection they afforded mice against systemic aspergillosis (Clemons *et al.* 2014a).

Low mortality study. In the initial study, 50% of PBS-treated controls succumbed to infection. Sixty percent or more of the mice vaccinated with HKY, WGP, or WGP-BSA survived through the 28 days of infection (Fig. 1). Statistically, only WGP-BSA 0.6 provided significant protection compared to PBS-treatment (P = 0.029) and WGP 6 approached significance (P = 0.052). HKY was not significantly protective in this study, but mice vaccinated with HKY did have longersurvival than did mice given PBS. Groups of mice givenany dose of WGP-BSA and

the two higher doses of WGP had longer survival than did the PBS-treated group or the HKY-treated group.

Vaccine effectiveness was also assessed by determination of the residual CFU burdens in the lungs, liver and spleenof surviving mice (Fig. 2). All mice had detectable infection in allthree organs. Mice vaccinated with WGP-BSA 0.6 had significantly fewer CFU in the liver and lungs than did PBS-treated mice (P = 0.04) and mice vaccinated with WGP 6 had significantly fewer CFU in lungs than did PBS-treated mice (P = 0.02) and in the spleen than did HKY-treated (P = 0.04). No other comparisons approached significance. It should be noted for WGP doses that the median burdens of the WGP 12 group were higher than those in the WGP 6 group (Fig. 2). Similarly, the median burdens recovered from mice given WGP-BSA 6 or 12 were higher than those given WGP-BSA 0.6. The CFU burden in the various organs corresponded with the survival data, with both supporting the lower vaccine doses (WGP-BSA 0.6 mg and WGP 6 mg) to be protective compared to the higher doses (WGP-BSA 6 mg, WGP-BSA 12 mg and WGP 6 mg). Thus, these data are suggestive of higher doses being less effective.

High mortality study. As shown in the initial study, WGP or WGP-BSA appeared to be potentially effective when used as a vaccine against coccidioidomycosis. We performed a replicate study to determine the reproducibility of this protection and to clarify the comparative protection. In addition, the replicate study was designed, through the use of a higher number of arthroconidia in the inoculum, to be a more rigorous challenge to the protective efficacy of these preparations as vaccines.

The infection proved highly lethal, with 90% of the PBS-treated control mice succumbing to infection by day 17 (Fig. 3). Comparatively, only 40% of HKY-treated mice died, this was significantly protective (P= 0.002), similar to our published studies(Capilla *et al.*

153 2009). WGP at 12, 6, or 0.6 mg/dose resulted in 50, 30 and 20% survival respectively. WGP at
154 12 or 6 mg/dose provided significant protection compared to PBS- treated (P = 0.0005), and
155 were equivalent to HKY. WGP-BSA at 12, 6, or 0.6 mg/dose resulted in 60, 60, and 30%
156 survival, respectively, suggestive of a dose-response, as was the case with WGP (Fig. 3). The
157 two higher dosages of WGP-BSA prolonged survival significantly compared to PBS-treated ($P \leq$
158 0.05) but were equivalent to HKY. Neither WGP nor WGP-BSA at the 0.6 mg dosage induced
159 significant protection.

160 The recovery of CFU of *C. posadasii* from the organs 28 dayspostinfection is shown in
161 Fig. 4. No animals in any vaccine regimen were cleared of infection in any organ. There were
162 no significant differences in burden in the spleen of the vaccinated groups compared to PBS
163 controls. However, HKY and WGP-BSA at 12 mg/dose both significantly reduced CFU in the
164 liver (P = 0.035) and in the lungs (P = 0.04 and 0.035, respectively). WGP-BSA at 6 mg/dose
165 significantly reduced CFU in the lungs compared to PBS (P = 0.035). In contrast, no dose of
166 the nonconjugated WGP vaccine resulted in a significant reduction in CFU in any of the three
167 organs. However, WGP-BSA and WGP were not significantly different at all doses and were
168 also equivalent to HKY.

169 Overall, the results of the replicate study corroborated those of the initial study showing that
170 WGP-BSA or WGP alone can provide some protection against experimental systemic
171 coccidioidomycosis, even when the model established was a rapidly lethal, and thus, a highly
172 rigorous test of protection. This severity of the infection in the replicate study may also
173 explainwhy the lowest dose of WGP-BSA tested was not effective, as it had been in the lower
174 mortality study.

175

Discussion

The results of our current studies demonstrate that WGP alone or conjugated with BSA can act as a vaccine against coccidioidomycosis. Protection was found to be dose-responsive, with some doses equivalent orsomewhat better than that provided by our positive control preparation of HKY depending on the severity of infection. Although survival was prolonged by vaccination with WGP or WGP-BSA in both studies, protection did not result in animals free of infection in the organs,and the severity of infection likely played a role in determining the effectiveness of a dosage. Interestingly, all the clinical evidence also indicates that successful resolution of natural coccidioidal infection results in persistence of viable organisms; to wit, reactivation of infection decades after moving from endemic areas, only when the individual is immunosuppressed(Deresinski & Stevens 1975).In addition,the literature suggests thus far that if a vaccine preparation protects against one species of *Coccidioides* it will also protect against the other, since these species are very closely related(Clemons & Stevens 2011). In conjunction with our previous results, it appears that WGP preparations are capable of inducingsome protection against coccidioidomycosis and aspergillosis (Clemons*et al.* 2014b).

We have addressed the question of mechanism of induced protection in part in previous studies. Examination of the cytokine profiles in serum and BAL of WGP vaccinated mice showed activation of an innate and adaptive immune profile (i.e., up-regulation of CSFs, INFγ, TNF-α, chemokines such as MCP-1, MIP-1α, RANTES, KC, and Th17- activating cytokines such as IL-6, IL-1β, IL-17) (Clemons *et al.* 2014b). Interestingly, only minimal rise in antibody to β-glucan was found, suggestive that antibodies play a minimal role in protection (Clemons *et al.* 2014b). The latter is in opposition to the studies using laminarin conjugated to diphtheria toxoid, which suggested antibody directed against β-glucan was a major mechanism of protection against

multiple fungal infections (Torosantucci*et al.* 2005; Torosantucci*et al.* 2009). As we noted previously, induction of the various proinflammatory cytokines and chemokines is suggestive of a priming of an active innate immune response, whereas increases in IFNγ and IL-17 suggest an induction of a cell-mediated immune response (Zelante *et al.* 2009; Clemons *et al.* 2014b; Whibley & Gaffen 2014). Additional studies are required to fully determine the nature of the protection induced by WGP or WGP-BSA.

It is possible that the conjugation of WGP to proteins specific for *Coccidioides* could improve the effectiveness of the vaccine preparation. Similarly, the use of multivalent epitope constructs specific to *Coccidioides* delivered in shells of β-glucan have been shown to induce protective cell-mediated responses (Hurtgen*et al.* 2012; Cole*et al.* 2013). These data also support the concept of using glycans as carriers of fungal proteins, similar to our own results and those of Torosantucci (Torosantucci *et al.* 2005), which take advantage of the immunostimulatory properties of β-glucan and reduce the need for an adjuvant such as aluminum hydroxide.

Overall, our results provide a basis for the development of a pan-fungal vaccine. That WGP or WGP-BSA has now been shown to induce a degree of protection against two different fungal infections in conjunction with our results showing HKY vaccination protects against five different fungi, would indicate that WGP is a candidate as a base preparation onto which specific proteins or protein constructs could be conjugated. Additional studies are needed to further this area of study.

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Conflict of interest: MED, KSM, and MAA are employed by Biothera.

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Figure Legends

Figure 1. Survival of mice vaccinated with PBS, HKY, WGP-BSA or WGP at various doses. All mice were vaccinated with three weekly doses and infected with a low inoculum of 127*C. posadasii* arthroconidia seven days after the last dose. Statistical analyses were done by log rank test. WGP-BSA 0.6 provided significant protection versus PBS (P = 0.029) and WGP 6 provided nearly significant protection (P = 0.052). No other comparisons were significant. There were 9 mice in the PBS-treated group and all other groups had 10 mice each.

Figure 2. CFU recovered from surviving mice. A value of $\log_{10}$ 7 was assigned to data points missing due to the death of the animal. Statistical comparisons were done by Mann-Whitney U test. In the spleen, the sole significant comparison was that WGP 6 had significantly lower CFU than HKY (P =0.043). For the liver, WGP-BSA 0.6 had significantly lower CFU than PBS (P = 0.043). For the lungs, WGP-BSA 0.6 and WGP 6 had significantly lower CFU than PBS (P = 0.043 and 0.022, respectively). No other comparisons were significant.There were 9 mice in the PBS-treated group and all other groups had 10 mice each. Horizontal bars represent the group median.

Figure 3. Cumulative survival of mice vaccinated with PBS, HKY, WGP or WGP-BSA at the doses (mg/dose) indicated and infected intravenously with 275 arthroconidia of *C. posadasii*.All groups consisted of 10 mice each.

348 **Figure 4**. Recovery of *C. posadasii* after 28 days of infection from the organs of mice vaccinated
349 with PBS, HKY, WGP, or WGP-BSA. A value of $\log_{10}$ 7 was assigned to data points missing
350 due to the death of the animal. Statistical comparisons were done by Mann-Whitney U test.
351 HKY and WGP-BSA at 12 mg/dose both significantly reduced CFU in the liver (P = 0.035) and
352 in the lungs (P = 0.04 and 0.035, respectively), and WGP-BSA at 6 mg/dose significantly
353 reduced CFU in the lungs compared to PBS controls (P = 0.035).All groups consisted of 10 mice
354 each. Horizontal bars represent the group median.

Figure 1

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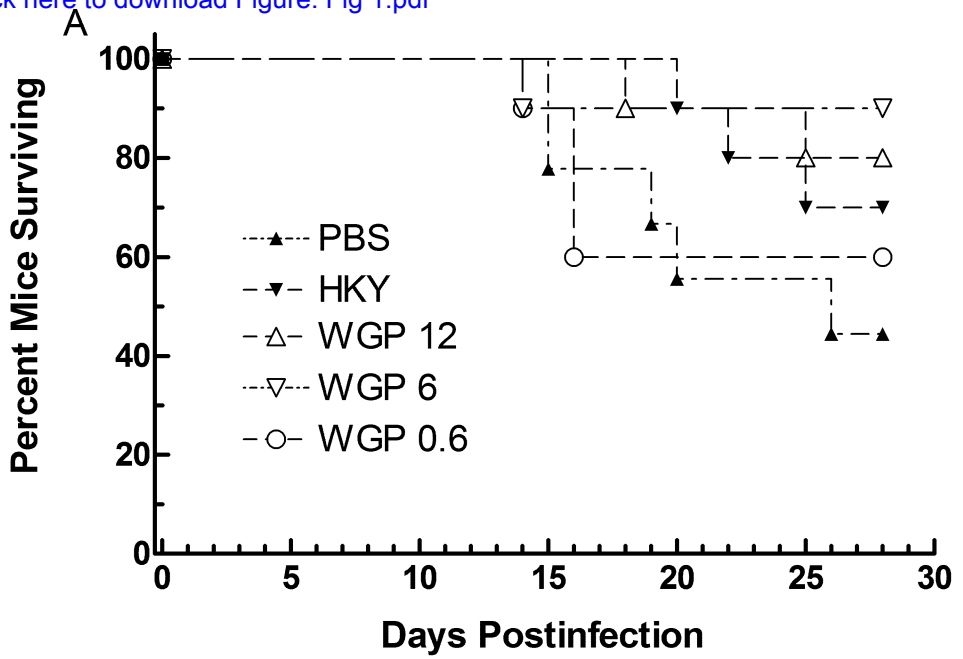

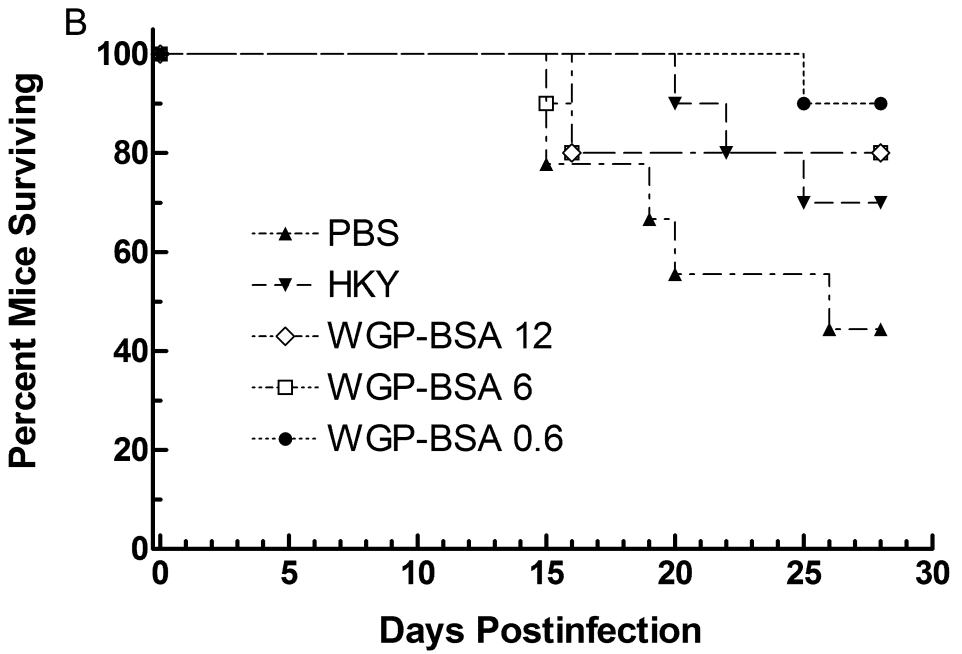

Figure 2

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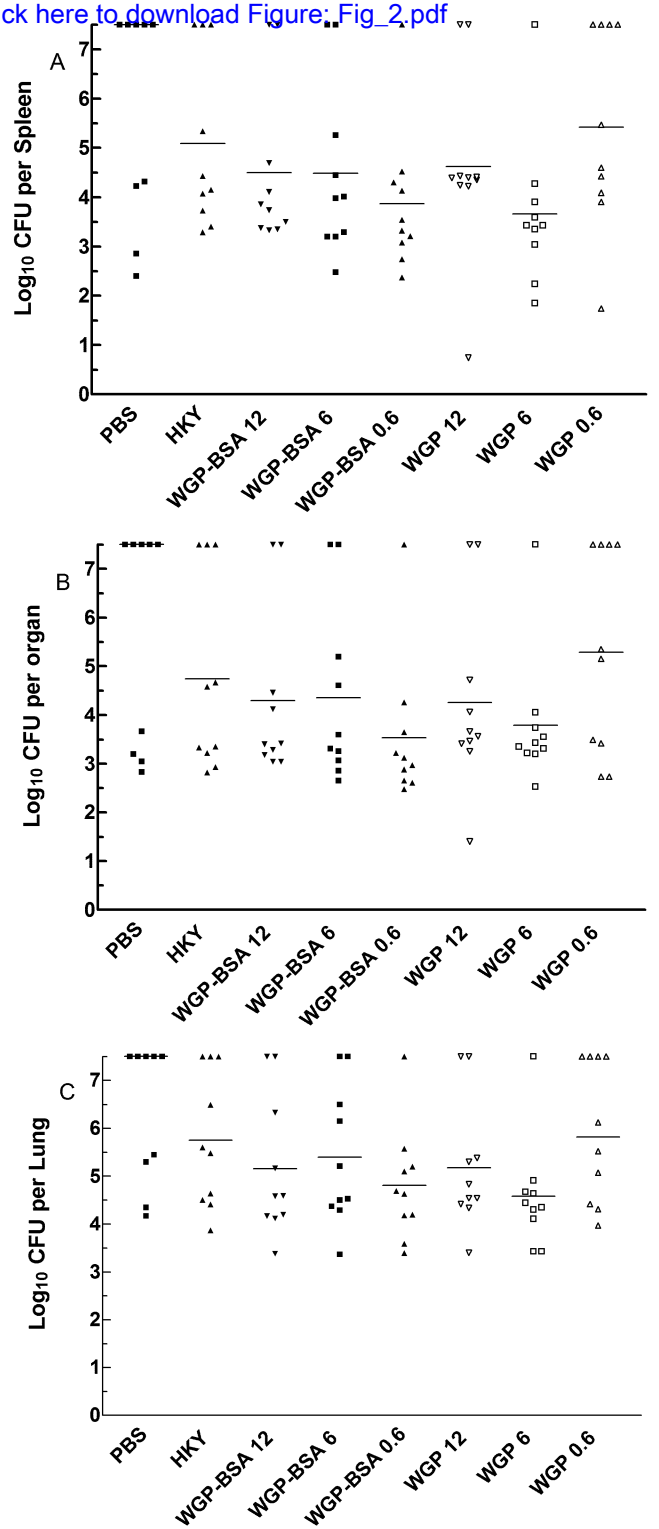

Figure 3
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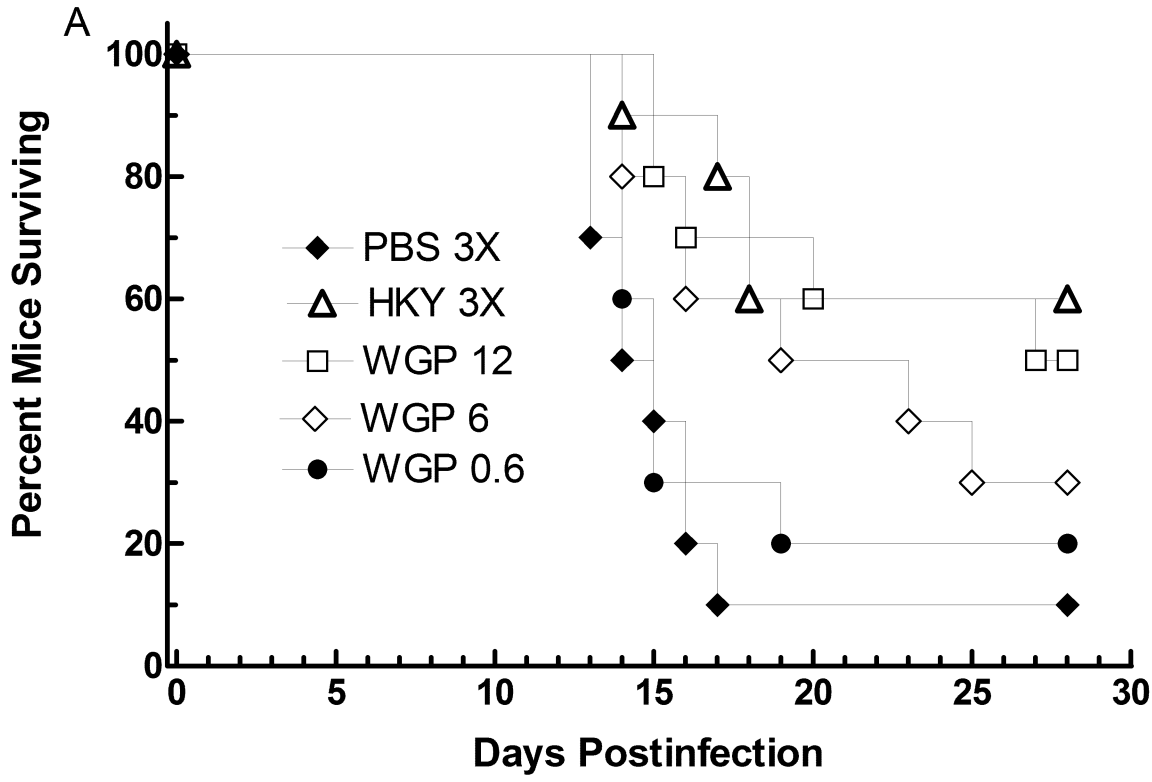

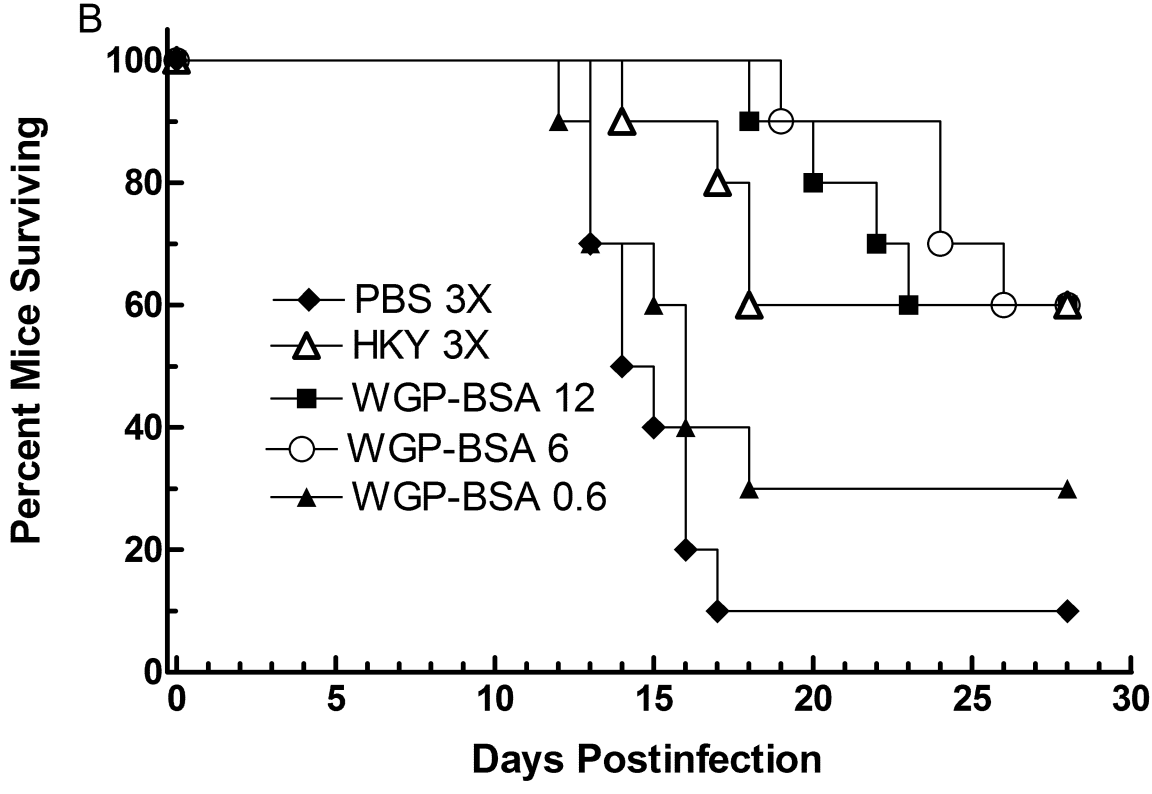

Figure 4

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