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1 Protection of mice against experimental cryptococcosis by synthesized peptides delivered in
2 glucan particles

3

4 Running Title: *Cryptococcus* Peptide vaccines

5

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20 **Abstract**

21 The high global burden of cryptococcosis has made development of a protective vaccine a
22 public health priority. We previously demonstrated that a vaccine composed of recombinant
23 *Cryptococcus neoformans* chitin deacetylase 2 (Cda2) delivered in glucan particles (GPs)
24 protects BALB/c and C57BL/6 mice from an otherwise lethal challenge with a highly virulent *C.*
25 *neoformans* strain. An immunoinformatic analysis of Cda2 revealed a peptide sequence
26 predicted to have strong binding to the MHC Class II (MHC II) H2-IA_d allele found in BALB/c
27 mice. BALB/c mice vaccinated with GPs containing a 32 amino acid peptide (Cda2-Pep1) that
28 included this strong binding region were protected from cryptococcosis. Protection was lost with
29 GP-based vaccines containing versions of recombinant Cda2 protein and Cda2-Pep1 with
30 mutations predicted to greatly diminish MHC II binding. Cda2 has homology to the three other
31 *C. neoformans* chitin deacetylases, Cda1, Cda3 and Fpd1, in the high MHC II binding region.
32 GPs loaded with homologous peptides of Cda1, Cda3 and Fpd1 protected BALB/c mice from
33 experimental cryptococcosis, albeit not as robustly as the Cda2-Pep1 vaccine. Finally, seven
34 other peptides were synthesized based on regions in Cda2 predicted to contain promising CD4⁺
35 T cell epitopes in BALB/c or C57BL/6 mice. While five peptide vaccines significantly protected
36 BALB/c mice, only one protected C57BL/6 mice. Thus, GP-based vaccines containing a single
37 peptide can protect mice against cryptococcosis. However, given the diversity of human MHC II
38 alleles, a peptide-based *Cryptococcus* vaccine for use in humans would be challenging and
39 likely need to contain multiple peptide sequences.

40 **Importance**

41 Cryptococcosis, due to infection by fungi of the *Cryptococcus neoformans* species complex, is
42 responsible for substantial morbidity and mortality in immunocompromised persons, particularly
43 those with AIDS. Cryptococcal vaccines are a public health priority yet are not available for
44 human use. We previously demonstrated mice could be protected from experimental
45 cryptococcosis with vaccines composed of recombinant cryptococcal proteins encased in hollow
46 highly purified yeast cell walls (glucan particles). Here, we examined one such protective
47 protein, Cda2, and using bioinformatics, identified a region predicted to stimulate strong T cell
48 responses. A peptide containing this region formulated in glucan particle-based vaccines
49 protected mice as well as the recombinant protein. Other peptide vaccines also protected,
50 including peptides containing sequences from proteins homologous to Cda2. These preclinical
51 mouse studies provide a proof of principle that peptides can be effective as vaccines to protect
52 against cryptococcosis and that bioinformatic approaches can guide peptide selection.

53

54 **Introduction**

55 Virtually all cases of cryptococcosis are caused by *C. neoformans* and the closely related
56 species, *C. gattii* (1, 2). The global burden of cryptococcal meningitis has been estimated at
57 223,100 incident cases per year, with 181,100 deaths (3). The vast majority of patients with
58 cryptococcosis have qualitative or quantitative defects in CD4⁺ T cell function. Cryptococcal
59 meningitis accounts for ~15% of AIDS-related deaths (3). Other immunosuppressed persons
60 are also at high risk; e.g., solid organ transplant recipients have ~1 - 5% lifetime risk of
61 developing cryptococcosis (4). In mouse models of infection, CD4⁺ T cells are also critical for
62 protection, although other arms of the immune system may contribute (5).

63 Given the public health significance of cryptococcosis, vaccines to protect high risk individuals
64 are a high priority. While heretofore none has reached human clinical trials, promising results
65 have been obtained in animal models (reviewed in (6, 7)). Protection against experimental
66 cryptococcosis can be obtained by immunization with cryptococcal strains missing virulence
67 factors such as capsule, chitosan, serylglucosidase, and F-box protein (6, 8-11), or genetically
68 engineered to express interferon- γ (12, 13). Whole organism vaccines are relatively easy to
69 manufacture and contain a broad range of antigens. However, they may have difficulty reaching
70 clinical trials due to concerns regarding reactogenicity, autoimmunity and, if administered live,
71 the possibility of causing infection in immunosuppressed persons (14). To circumvent these
72 potential drawbacks, we have focused on identifying candidate antigens, adjuvants, and delivery
73 systems for use in subunit vaccines. We have manufactured vaccines consisting of antigens
74 that are recombinantly expressed in *E. coli* and then encapsulated in glucan particles (GPs) (15,
75 16). When administered as a prime followed by two boosts, 11 different individual GP-delivered
76 antigens protected BALB/c and/or C57BL/6 mice from pulmonary challenge with the highly
77 virulent KN99 α *C. neoformans* strain (15, 16).

78 Among the most promising of the protective vaccine antigens is chitin deacetylase 2 (Cda2,
79 originally named MP98) (15-17). The immunoreactivity of Cda2 was first demonstrated when it
80 was shown to stimulate a CD4⁺ T cell hybridoma clone created by fusing T cells from
81 immunized mice with a thymoma cell line (17). Subsequently, Weisner *et al.* synthesized a
82 recombinant peptide-MHC II tetramer containing a 13 amino acid peptide from Cda2 (18). Two
83 weeks following pulmonary infection of C57BL/6 mice with the KN99 α strain, up to 6.5% of the
84 lung helper T cell population was recognized by the tetramer, thus establishing Cda2 as a major
85 stimulatory antigen. Cda2 belongs to a homologous family which includes Cda1, Cda3, and
86 Fpd1 (also known as Cda4). Cda1, Cda2 and Cda3 have chitin deacetylase activity as shown
87 by their ability to deacetylate cell wall chitin to chitosan in *C. neoformans* (19). Fpd1, which
88 prefers partially deacetylated chitosan as a substrate, may be more properly referred to as a
89 chitosan deacetylase (20). None of the members of the Cda family have significant homology to
90 human proteins (15, 16).

91 Use of full length recombinant proteins in T cell vaccine studies has the advantage that all
92 epitopes are included in the antigen. However, there is a strong rationale for defining the
93 protective peptides contained within vaccine antigens. First, identifying immunodominant
94 peptide regions of the protein allows elimination of regions of the protein that could drive non-
95 essential, antagonistic, immune suppressive, or autoimmune responses. Second, using
96 synthesized peptides as vaccine antigens minimizes potentially confounding effects of
97 extraneous vector (e.g., *E. coli*)-derived products, such as lipopolysaccharides, lipoproteins, and
98 purification tags. Third, immunoprotective peptides could be combined into a chimeric
99 recombinant protein which would simplify manufacturing and testing of a vaccine in clinical
100 studies (21).

101 In the present study, we performed an immunoinformatic analysis (16, 22) of Cda2 with the goal
102 of defining CD4⁺ T cell epitopes for use in a *Cryptococcus* vaccine. Peptides within Cda2 were

103 selected based on their predicted binding to the MHC Class II alleles of BALB/c and C57BL/6
104 mice. Mutated peptides were then created to test the impact of MHC Class II binding on vaccine
105 efficacy

106

107 **Results**

108 Protection with the GP-Cda2 protein vaccine varies as a function of mouse strain.

109 Our published studies (15, 16) and new confirmatory data demonstrate that a GP-based vaccine
110 containing recombinant *E. coli*-derived Cda2 protect BALB/c mice more robustly than C57BL/6
111 mice (Figure 1). Moreover, DR4 mice, which contain a humanized MHC II allele (DRB1*04:01)
112 on a C57BL/6 genetic background are not significantly protected by the GP-Cda2 vaccine. This
113 led us to hypothesize that the disparities in how well the GP-Cda2 vaccine protected the
114 different mouse strains could be at least partially explained by differences in the MHC II
115 molecules expressed. Our initial focus was on BALB/c mice, given the potent protection
116 mediated by the GP-Cda2 vaccine in that mouse strain.

117 Mutations in a predicted high binding region of Cda2 result in substantial loss of vaccine- 118 mediated protection in BALB/c mice.

119 We previously identified a region of Cda2 predicted to have 15 amino acid peptides with strong
120 binding to H2-IA_d, the MHC II allele expressed by BALB/c [Figure S3 in (16)]. This region also
121 contains the amino acid sequence used to make a tetramer to identify Cda2-specific CD4 T
122 cells following infection of C57BL/6 mice (18). We therefore created mutations in this region of
123 Cda2 spanning amino acids 203-234 of Cda2 (Figure 2A) so that on immunoinformatic analysis,
124 the predicted H2-IA_d binding (three positions designated by asterisks in Figure 2B) was greatly
125 diminished or lost entirely. Two such mutated regions, designated M1 and M2 were selected. *E.*
126 *coli*-derived proteins comprising these mutated sequences were then synthesized, and GP-

127 based vaccines were manufactured. BALB/c mice were vaccinated, challenged via the
128 pulmonary route with the KN99 strain of *Cryptococcus*, and followed for survival over a 70d
129 observation period. Vaccine-mediated protection was robust with recombinant “wild-type” Cda2
130 protein but was mostly lost when Cda2 proteins (Cda2-M1 and Cda2-M2) containing mutated
131 sequences were used (Figure 2C).

132 Protection mediated by peptide vaccines containing the predicted high binding region of Cda2.

133 We next synthesized 32-mer peptides spanning amino acids 203-234 from the predicted high
134 binding region of Cda2, along with the corresponding regions of M1 and M2 mutants. These
135 peptides were named Cda2-Pep1, Cda2-Pep1-M1, and Cda2-Pep1-M2, respectively.

136 Remarkably, mice that received GP-based vaccines containing Cda2-Pep1 were protected from
137 experimental cryptococcosis (Figure 2D). In contrast, protection was diminished, albeit not
138 eliminated, with the vaccines containing Cda2-Pep1-M1 and Cda2-Pep1-M2.

139 Protection mediated by vaccines containing peptides homologous to Cda2-Pep1 that are
140 present in other cryptococcal chitin deacetylases.

141 Cda2 has homology to Cda1, Cda3 and Fpd1, including in the predicted MHC II H2-IA_d high
142 binding region of Cda2 (Figure 3A). We synthesized 32 amino acid peptides, termed Cda1-
143 Pep1, Cda3-Pep1 and Fdp1-Pep1 based on sequences homologous to Cda2-Pep1. The
144 peptides were loaded into GPs and used to vaccinate mice. Compared with unvaccinated mice,
145 mice vaccinated with any of the GP-Pep1 vaccines were protected against an otherwise lethal
146 pulmonary challenge with *C. neoformans* (Figure 3B). Protection was greatest for vaccines
147 containing Cda2-Pep1, followed by Cda1-Pep1, Cda3-Pep1, and Fpd1-Pep1.

148 Protection of BALB/c and C57BL/6 mice mediated by GP-based vaccines containing other
149 peptide sequences of Cda2.

150 In the last set of experiments, we synthesized eight 31-35 amino acid peptides based on
151 sequences in Cda2 (Table 1), loaded them into GPs, and tested the GP-peptide vaccines in
152 BALB/c and C57BL/6 mouse models of cryptococcosis. The eight peptides were chosen to
153 overlap with Cda2-Pep1 or based on regions in Cda2 deemed to contain strong CD4⁺ T cell
154 epitopes based on predicted binding to the H2-IA_d allele in BALB/c (Figure 4A) and/or the H2-
155 IA_b allele in C57BL/6 mice (Figure 4C). Regarding the GP-peptide vaccines, compared with
156 unvaccinated mice, in BALB/c mice, significant protection was seen in five of the eight vaccines
157 (Figure 4B). In contrast, of the eight peptide-based vaccines, only Cda2-Pep5 significantly
158 protected C57BL/6 mice (Figure 4D). Cda2-Pep5 includes what was predicted to be the
159 strongest H2-IA_b in the Cda2 recombinant protein (Figure 4C).

160

161 **Discussion**

162 CD4⁺ T cells are the most critical component of the adaptive protective immune response to
163 naturally acquired cryptococcal infection. A challenge in developing cryptococcal vaccines has
164 been the identification of antigens that induce protective CD4⁺ T cell responses, particularly
165 given the diversity of MHC II in the human population (23). Herein, we performed an in-depth
166 study of an immunodominant protective protein antigen, Cda2, identifying regions of the protein
167 contributing to vaccine-mediated protection in mice.

168 Our GP-based Cda2-derived peptide vaccines protected BALB/c more robustly than C57BL/6
169 mice. This is despite the protection afforded both mouse strains by the GP-based vaccine
170 containing the *E. coli*-derived Cda2 protein. Cda2-Pep1, which was only protective in BALB/c
171 mice as part of a vaccine, contains an epitope which is recognized by a sizable fraction of CD4⁺
172 T cells from infected C57BL/6 mice (18). This emphasizes that immunogenicity does not
173 necessarily result in protection. BALB/c mice are relatively resistant to cryptococcal infection

174 compared with C57BL/6 mice (24, 25). This effect has been attributed in part to a protective Th1
175 response developing in BALB/c, whereas C57BL/6 mice develop a Th2-biased response (24,
176 25). While the GP vaccine platform skews towards Th1- and Th17-type responses (26, 27), a
177 response that is broader than just to a single peptide may be required to protect C57BL/6 mice.
178 In addition, the possible contributions of antibody and CD8⁺ T cell immunity must be considered.
179 An alternate but not mutually exclusive possible explanation is the site in Cda2, which
180 comprises the BALB/c MHC II H2-IA_d binding site has a functional role that is targeted by the
181 immune response. Of note, Cda2-Pep1 contains two conserved histidine residues required for
182 metal-binding in the catalytic domain of fungal chitin deacetylases and bacterial peptidoglycan
183 deacetylases (28, 29).

184 DR4 mice are not significantly protected by the GP-Cda2 vaccine. As DR4 mice and C57BL/6
185 mice each express a different MHC II allele, it is tempting to attribute the disparate protection in
186 the two strains of mice to MHC II binding of processed peptides. However, there is evidence
187 that the CD4-MHC II interaction is impaired in DR4 mice (30, 31). Regardless, the disparate
188 results obtained using different strains of inbred mice emphasize that a successful human T cell
189 vaccine will likely need to contain multiple epitopes given the heterogeneity of the MHC II loci
190 within the human population. Differences in vaccine efficacy comparing mouse strains have also
191 been found using whole organism *Cryptococcus* vaccines (11).

192 Cda1, Cda2, Cda3, and Fpd1 define a family of homologous chitin deacetylases responsible for
193 deacetylating chitin to chitosan in the cryptococcal cell wall (19). Homology is particularly high in
194 the region contained within Cda2-Pep1, our most protective peptide. GP-based peptide
195 vaccines containing regions in Cda1, Cda2, and Fdp1 homologous to Cda2-Pep1 protected
196 BALB/c mice against cryptococcal challenge. Interestingly, in our previous studies with GP-
197 based vaccines containing recombinant *E. coli*-derived proteins, the same order of protection of
198 BALB/c mice against lethal cryptococcal challenge was observed (i.e., GP-Cda2 was the most

199 protective followed by GP-Cda1, GP-Cda3, and GP-Fpd1) (15, 16). What is unclear though is
200 the extent to which each vaccine elicits cross-protective responses to their homologous family
201 members. For example, is part of the protection mediated by the GP-Cda2-Pep1 vaccine due to
202 recall T cell responses stimulated, for example by Cda1 of *C. neoformans*?

203 MHC binding predictions focus on the flanking regions of the T cell epitopes, also called the
204 pocket positions. The binding affinity indicates the quantitative relationship of a potential epitope
205 with the cognate T cells, based on the on-off rate of the peptide in the MHC II molecular groove
206 and hence the frequency of interactions between the T cell and epitope. Conversely, the amino
207 acid motifs actually engaging a T cell receptor (the non-pocket residues or T cell-exposed motif)
208 are a qualitative interaction. The T cell-exposed motifs are comprised of the central amino acids
209 of any of the 15 amino acid peptides, typically a discontinuous pentamer comprising positions
210 2,3,5,7,8 of the central 9 amino acid core (22). While there is considerable homology between
211 the sequences from the four proteins we have examined, there is also sufficient sequence
212 diversity that the T cell-exposed motifs are different between the four proteins. Only Cda2-Pep1
213 and Cda3-Pep1 share exact identity in just 2 out of the 18 T cell-exposed MHC II motifs present
214 for each of the -Pep1 peptides. Any cross-reactivity among the proteins/peptides would depend
215 mostly on “near neighbor” binding of T cells to similar, but non-identical, motifs (32).

216 Our previous GP-based subunit vaccine studies used recombinant *E. coli*-expressed proteins as
217 the antigens. Although the proteins were His-tagged and affinity-purified on a nickel column, it is
218 possible that LPS and other bacterial pathogen-associated molecular patterns contaminated the
219 preparations and contributed adjuvanticity to the vaccines. The use of synthesized peptides
220 mitigates this concern. Other advantages of peptide vaccines are reviewed in the Introduction
221 and include elimination of regions of the protein that could drive undesirable responses.
222 However, as seen herein comparing the GP-Cda2-Pep1 vaccine in BALB/c and C57BL/6 mice,
223 a drawback of peptide vaccines is they may be MHC II allele-dependent. A chimeric

224 recombinant protein (21) containing multiple peptide antigens could be designed so that it
225 stimulated Th-dependent responses among a broad range of MHC Class II alleles found in the
226 human population. However, care would need to be taken to avoid creating spurious
227 neoepitopes at peptide junctions and/or dominance hierarchy issues (33, 34).

228 Our studies add to the growing literature regarding the power of bioinformatics to predict T cell
229 epitopes and inform vaccine development (35, 36). Nevertheless, *in silico* immunoinformatic
230 approaches have limitations because they cannot fully account for posttranslational
231 modifications. Furthermore, modeling has been done only those alleles for which there are
232 adequately large training sets. Relevant to our studies, native *C. neoformans* Cda2 is heavily
233 mannosylated (17, 37, 38), and glycosylation can interfere with antigen processing and
234 presentation (39). While the precise mannosylation sites are not known, each of the peptides we
235 tested contains multiple serines and threonines which are potential sites of O-linked
236 glycosylation. Moreover, peptides Cda2-Pep3, Cda2-Pep5, Cda2-Pep6, Cda2-Pep7, and Cda2-
237 Pep8 contain one or more consensus sequences (Asn-X-Ser or Asn-X-Thr) required for N-
238 linked glycosylation. Ultimately, antigen-specific CD4⁺ T cell-mediated protection is
239 multifactorial; additional determinants may include epitope combinations, binding MHC alleles, T
240 cell repertoires (prior exposure to same or similar epitopes creating a responsive clonal
241 population), and cathepsin and endosomal processing.

242 Our data serve as a proof of principle that peptide vaccines engineered to stimulate CD4⁺ T cell
243 responses can protect mice against a highly virulent *C. neoformans* strain. The vaccines were
244 adjuvanted and delivered using the GP platform, which biases towards strong Th1 and Th17
245 responses (26, 40, 41); future studies will be needed to determine whether other adjuvants can
246 be substituted. Given the diversity of MHC II alleles in the human population, a peptide-based
247 vaccine designed for use in humans would likely require multiple peptides. An additional
248 challenge to translate our findings to humans is the impairments in CD4⁺ T cell function present

249 in most individuals at risk for cryptococcosis. Individuals would likely need to be vaccinated
250 when their T cell function was relatively intact, such as early in HIV infection or prior to solid
251 organ transplantation. Moreover, combining a T cell vaccine with one that elicits protective
252 antibodies (42) merits testing.

253

254 **Methods**

255 Reagents, Peptides, and *C. neoformans*. Except where noted, chemical reagents were obtained
256 from Thermo Fisher Scientific. Peptides of >75% purity were synthesized by GenScript and
257 provided as lyophilized material in measured amounts of peptide. Each peptide was analyzed
258 by GenScript for purity using HPLC, Mass spectrometry and nitrogen analysis. Depending on
259 their solubility, peptides were dissolved in water, 50% DMSO, or 100% DMSO. Stock solutions
260 of each peptide were adjusted to 5 mg/ml based on their calculated extinction coefficient ($E^{0.1\%}$
261 at 280 nm; ProtParam tool at Expasy.org) and stored at -80°C. *C. neoformans* serotype A strain
262 KN99 α (43) was stored in glycerol stocks at -80°C and grown for in vivo infection studies as
263 described (15, 16). Briefly, following an initial culture on YPD (Difco Yeast Extract, Bacto
264 Peptone, Dextrose) with 2% agar, yeast cells were grown in liquid YPD at 30°C with shaking for
265 18h. Yeast cells were then harvested by centrifugation, washed with PBS, counted, and
266 suspended in PBS at 2-4x10⁵ cells/ml.

267 Recombinant *E. coli*-expressed proteins. National Center for Biotechnology Information file for
268 *C. neoformans* var. *grubii* H99 strain (taxid:235443) served as the source for cDNA and protein
269 sequences of Cda1 (CNAG_05799), Cda2 (CNAG_01230), Cda3 (CNAG_01239), and Fpd1
270 (CNAG_06291). cDNAs for these proteins and the mutated versions of Cda2 (Cda2-M1 and
271 Cda2-M2) were synthesized and cloned in pET19b (GenScript) so that the vector-encoded His
272 tag was integrated with the N-terminus of the cDNA. Recombinant protein was made in *E. coli*

273 strain BL21(DE3) (New England BioLabs) using Overnite Express™ TB medium (Novagen) and
274 purified on His-Bind resin (EMD Millipore) in the presence of 6M urea, as described (16).
275 Following elution with imidazole, proteins were dialyzed against 6M urea/20 mM Tris-HCl, pH7.9
276 and concentrated to 10 mg/ml using Amicon Ultra-15 centrifugal filters (10 kDA cutoff, Merck
277 Millipore). The protein concentration was determined by the bicinchoninic acid (BCA) assay. To
278 assess purity, the recombinant proteins were resolved on SDS-PAGE and stained with
279 Coomassie InstantBlue (Expedeon, Ltd.).

280 GP-based vaccines. Recombinant *E. coli*-derived proteins were co-trapped with mouse serum
281 albumin (MSA) complexed with yeast RNA (yRNA) in GPs as described (15, 16). Peptides that
282 were water-soluble were loaded in an identical manner. Peptides in DMSO (5 mg/ml) were
283 loaded by mixing 5 µl peptide per mg hydrated GPs, followed by lyophilization. DMSO (2.5
284 µl/mg GPs) was added to then "push" the peptides into the core of the GPs, followed by
285 lyophilization. A second "push" with 2.5 µl of water/mg GPs followed by lyophilization completed
286 the loading of peptide. Subsequent steps were the same as was done for protein: MSA was
287 loaded in 0.9% saline and the peptide/MSA inside the GPs were co-trapped with yRNA.
288 Following the yRNA trapping step, peptide vaccines were sonicated to single particles,
289 aliquoted, sonicated again and flash frozen. Protein vaccines were washed three times with
290 saline before sonication. Vaccines were stored at -80°C. A vaccine dose consisted of 100 µl of
291 200 µg GPs (approximately 10⁸ particles) containing 10 µg of recombinant protein or 5 µg of
292 synthesized peptide and 25 µg of MSA complexed with yRNA in 0.9% sterile saline. A control
293 preparation, designated GP-MSA, contained MSA and yRNA without the antigen.

294 Mouse studies. C57BL/6, BALB/c, and Abb Knockout/Transgenic, HLA-DR4 (DR4) mice of both
295 sexes were obtained from Charles River Laboratories, The Jackson Laboratory, and Taconic
296 Biosciences. Mice were bred and housed in a specific pathogen-free environment in the animal
297 facilities at the University of Massachusetts Chan Medical School (UMCMS). All animal

298 procedures were carried out under a protocol approved by the UMCMS Institutional Use and
299 Care of Animals Committee.

300 The vaccination and infection protocols were as described (15, 16). Briefly, vaccinations were
301 administered subcutaneously three times at biweekly intervals. Mice received their first dose of
302 vaccine when 6-10 weeks old. Two weeks following the last booster, the mice were
303 anesthetized with isoflurane and challenged orotracheally with *C. neoformans* strain KN99 α .
304 The inoculum for DR4 and C57BL/6 mice was 1×10^4 CFU while for BALB/c mice it was 2×10^4
305 CFU. Mice were observed twice daily; humane endpoints prompting euthanasia included ataxia,
306 listlessness, weight loss, and failure to groom. The experiment was terminated on day 70 post-
307 infection, at which time all survivors were euthanized.

308 Statistics. Kaplan-Meier survival curves were compared using the Mantel-Cox, log-rank test.
309 The Bonferroni correction was applied in instances where multiple comparisons were made,
310 with a P value of <0.05 considered significant after corrections were made. The software
311 program GraphPad Prism Version 9.2.0 was used for all statistical analyses and to generate
312 graphs.

313 Immunoinformatics. The immunoinformatics platform used has been described elsewhere (44,
314 45). Briefly, the mean and SD of natural log of ic50 MHC II allele binding for each sequential 15
315 amino acid peptide in the protein is predicted by artificial neural network ensembles using
316 algorithms based on vectors derived from the principal components of the physical and
317 chemical characteristics of each amino acid. Mean predicted binding is then standardized to a
318 zero mean unit variance (normal) distribution within the protein to provide a relative competitive
319 index of predicted binding for each peptide in the protein. This places binding predictions of all
320 MHC alleles on the same scale. This metric is expressed in SD units relative to the mean for
321 that protein. Comparison with other prediction systems indicates a predicted binding affinity of
322 <-1 SD units below the mean is a probable epitope (46). The platform also evaluates cathepsin

323 cleavage probability, and the frequency of any T cell exposed (non-pocket) motif relative to
324 reference databases of the human proteome and bacteria of the gastrointestinal microbiome
325 (47-49).

326 Alterations in the sequence of Cda2-Pep1 were designed to generate sequences with reduced
327 H2-IAd binding affinity. This was done by generating 50,000 random iterations of Cda2-Pep1,
328 progressively replacing 2-8 designated amino acids, and re-evaluating predicted binding of each
329 constituent 15 amino acid peptide. A subset of peptides was then subjected to closer
330 examination to select M1 and M2, each of which has diminished binding to H2-IAd at positions
331 203, 208 and/or 217. While the T cell exposed motifs in the region of interest are changed, there
332 were no significant differences in the frequency of the exposed motifs relative to the reference
333 databases, indicating that no obvious changes in T cell precursor frequency for the mutant
334 peptides were created. Figure 2 shows the differences in predicted binding of sequential 15
335 amino acid peptides within -M1 and -M2 compared to the original Cda2-Pep1 peptide.

336

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342 submit the work for publication.

343

344

345 **Table 1.**

346

<u>Peptide</u>	<u>Sequence</u>	<u>Amino Acid</u> <u>Position</u>	<u>Index</u> <u>Position</u>
Cda2-Pep1	RAHDEGHEICVHTWSHQYMTALSNEVVFAELY	203-234	203-220
Cda2-Pep2	ENNQKATMFFIFGSNVLDWPLQAMRAHDEGHE	180-210	180-196
Cda2-Pep3	TWGLGFDDGPNCSHNALYDLLLENNQKATMFF	158-189	158-175
Cda2-Pep4	EVVFAELYYTQKAIKAVLGVTQPCRPPYGDVDN	227-260	227-246
Cda2-Pep5	TAYSYPVTELISSFPTIWQTASIPSNDEAQ	60-91	60-77
Cda2-Pep6	MSVFMTMFPKIKSAFNIVPICTAYNITQPYAES	328-361	328-347
Cda2-Pep7	DKAGNGTYTTHGPVVLNHELNTNYTMSVFMTMFPKI	304-338	304-324
Cda2-Pep8	KINSTLNTKIPNDVPHGTPDGTGVDVYNSNDPDC	96-130	96-116

347

348 Table legend: Sequences of Cda2 peptides used in vaccines shown in Figure 4. Protein
349 sequence of Cda2 that was used is from GenBank Accession XP_012049402.1. The index
350 (start) position refers to the first amino acid in a 15 amino acid peptide. The index position is
351 used to identify the vertical bars that depict relative binding of the peptide to a MHCII allele
352 shown in Figures 2B, 4A, and 4C. Thus, in Figure 2B, the vertical bar for Cda2-Pep1 at amino
353 acid 203 refers to the peptide sequence spanning amino acids 203-217.

354 **Figure legends**

355

356 **Figure 1. Protection with the GP-Cda2 protein vaccine as a function of inbred mouse**

357 **strain.** BALB/c (n=15), C57BL/6 (n=25), and DR4 (n=28) mice were vaccinated thrice with GP-
358 Cda2 protein and then challenged with *C. neoformans*, as described in *Methods*. Mice were
359 followed daily for survival until day 70 post infection. The figure includes mice previously
360 published (16), as well as confirmatory new experiments. $P \leq 0.0001$ comparing any two groups.
361 Not shown, survival of unvaccinated mice ranged from 20-32 days post infection for each of the
362 mouse strains.

363

364 **Figure 2. The effect of mutations in a predicted high binding region of Cda2 on GP**

365 **vaccine-mediated protection in BALB/c mice.**

366 A: The sequences of amino acids 203-232 of the wild-type (Cda2-WT) and the Cda2-M1 and
367 Cda2-M2 mutants. Mutated amino acids in Cda2-M1 and Cda2-M2 are indicated by arrows and
368 shown in red. Mutations were designed as in *Methods*.

369 B: Immunoinformatic analysis showing predicted binding of sequential 15 amino acid peptides
370 spanning amino acids 203-232 of WT-Cda2, Cda2-M1 and M2. Predicted binding of each
371 sequential 15mer peptides in CDA2 is shown by index (start) position of the peptide (X axis).
372 The Y axis shows the predicted binding in standard deviation units relative to a mean of zero. A
373 lower Standard Deviation (Z-Score) indicates greater predicted binding, as described in
374 *Methods*.

375 C: Survival studies in mice vaccinated with *E. coli*-expressed Cda2 (wild type), Cda2-M1, and
376 Cda2-M2 protein loaded into GPs. Control mice received GPs containing mouse serum albumin

377 (MSA). The number of mice in each group: Cda2, n=10; Cda2-M1, n=10; Cda2-M2, n=10; MSA,
378 n=9.

379 D: As in C except synthesized 32 amino acid peptides were loaded into GPs rather than *E. coli*-
380 expressed protein. The number of mice in each group: Cda2-Pep1, n=10; Cda2-M1, n=10;
381 Cda2-M2, n=10; MSA, n=5.

382 **Figure 3. Protection mediated by GP-based vaccines containing peptides synthesized**
383 **based on cryptococcal chitin deacetylases sequences with homology to the Cda2**
384 **predicted high binding region.** A. Sequences of the 32 amino acid peptides, Cda1-Pep1,
385 Cda2-Pep1, Cda3-Pep1, and Fpd1-Pep1. Amino acids with identity to the corresponding amino
386 acid in Cda2-Pep1 are shown in red.
387 B. Survival studies in BALB/c mice vaccinated with Cda1-Pep1, Cda2-Pep1, Cda3-Pep1, and
388 Fpd1-Pep1 loaded into GPs and then challenged with *C. neoformans*. UnVac, unvaccinated *C.*
389 *neoformans*-challenged mice. *, $p=0.04$ (not significant after Bonferroni correction). **,
390 $p=0.009$. Not marked on the figure, $p<0.0001$ comparing UnVac with any of the four vaccinated
391 groups. There were 10 mice in each of the vaccinated groups and 15 mice in the unvaccinated
392 group.

393

394 **Figure 4. Protection of BALB/c and C57BL/6 mice mediated by GP-based vaccines**
395 **containing Cda2 peptide sequences.**

396 A: Immunoinformatic analysis showing predicted binding to the MHC II IAd allele (present in
397 BALB/c mice) of each sequential 15 amino acid peptide in Cda2 based on the index (start)
398 position of the peptide. The peptides that were tested are colored in purple. The y-axis shows
399 the predicted binding in standard deviation units relative to a mean of zero. A lower Standard
400 Deviation (Z-Score) indicates greater predicted binding, as described in *Methods*.

401 B: BALB/c mice received a prime and two biweekly boosts with the indicated peptide encased in
402 GPs and then challenged with *C. neoformans*, as described in *Methods*. Mice were followed
403 daily for survival until day 70 post infection. Each black dot represents one mouse, shown on
404 the day post infection the mouse succumbed. Each vaccinated group had 10 mice. The
405 experiment was terminated 70 days post infection; survivors were assigned to day 70. The red

406 bars denote the geometric mean survival. UnVac = unvaccinated controls; n=16 mice. Peptide
407 vaccines that afforded significant protection are shown in bold. $P < 0.0001$ for Cda2-Pep1, Cda2-
408 Pep2, Cda2-Pep3, and Cda2-Pep4. $P = 0.0003$ for Cda2-Pep5.

409 C: As in (A), expect the MHC II IAb allele (present in C57BL/6 mice) was interrogated.

410 D: As in (B) except C57BL/6 mice were studied. The number of mice in each group: -Pep1,
411 n=15; -Pep2, n=15; -Pep3, n=15; -Pep4, n=15; -Pep5, n=14; -Pep6, n=10; -Pep7, n=6; -Pep8,
412 n=9; UnVac, n=15. $P = 0.0005$ for Cda2-Pep5.

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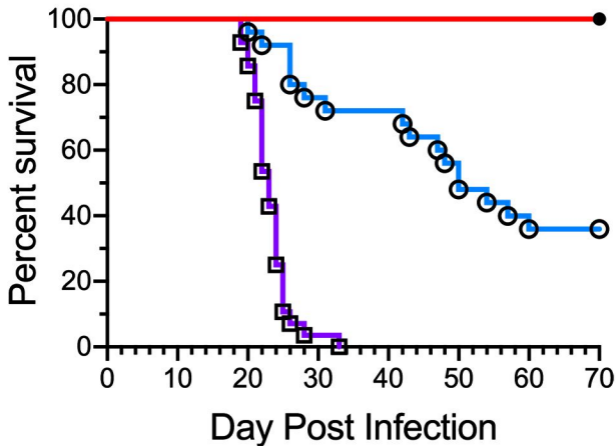
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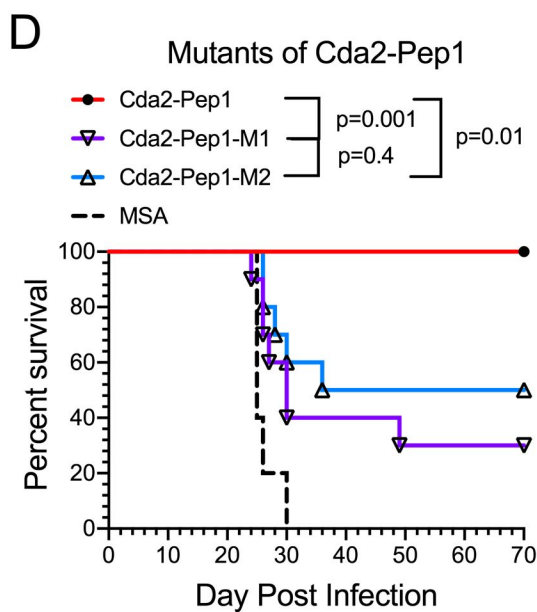
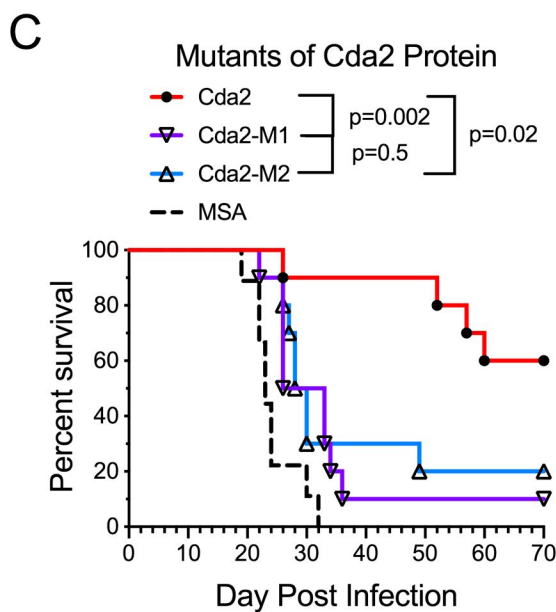
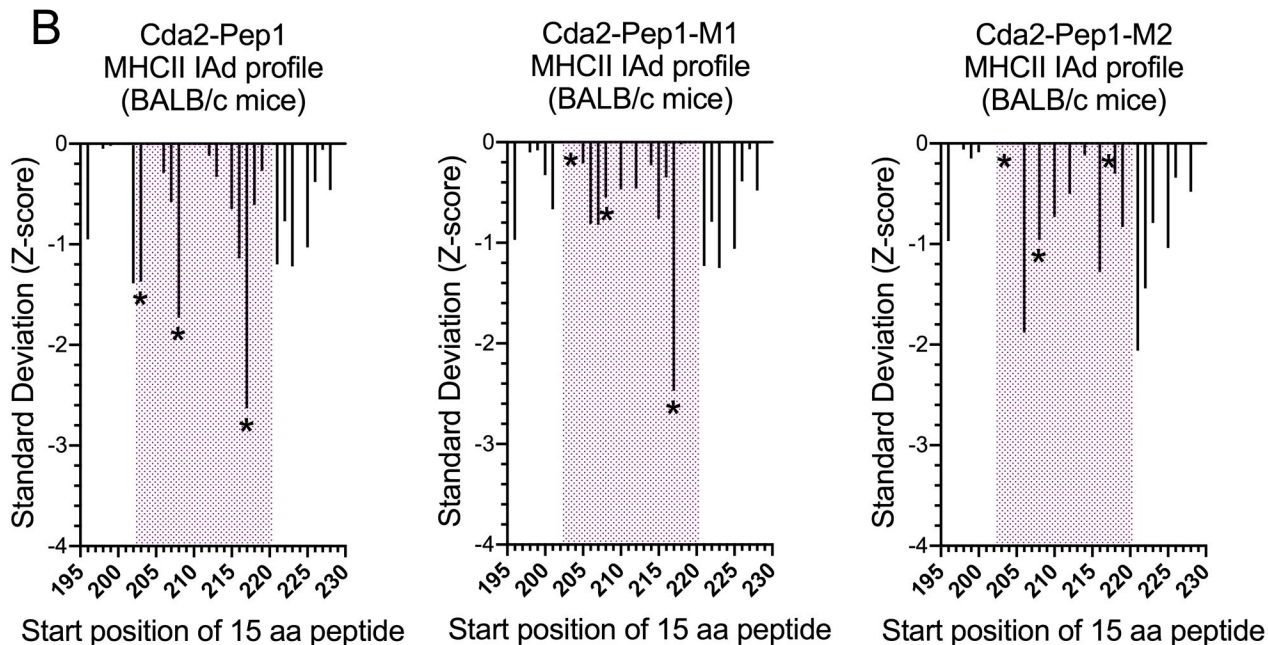
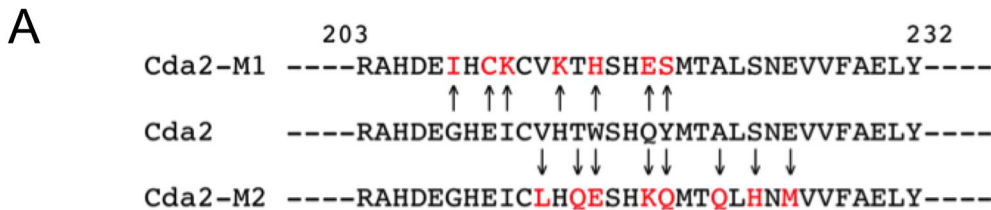
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550

Cda2 Protein

- BALB/c
- C57BL/6
- DR4 (C57BL/6)





A**Cda-Pep1 Peptides**

Cda1-Pep1 TEYMSGHQISIH**T**W**S**H**P**AL**T**TL**T**NEEIV**A**ELG
Cda2-Pep1 RAHDE**G**HEIC**V**H**T**W**S**HQ**Y**M**T**AL**S**NE**V**V**F**AEL**Y**
 Cda3-Pep1 VAVKA**G**GHLAV**H**T**W**S**H**P**Y**M**T**TL**T**NEQ**V**V**G**EL**G**
 Fpd1-Pep1 ALYDA**G**H**T**LG**S**H**T**W**S**H**A**DL**T**Q**L**DE**S**GIN**D**EL**S**

B**Cda-Pep1 Vaccines**

- **Cda2-Pep1** **
- Cda1-Pep1
- ▲ Cda3-Pep1
- ▼ Fpd1-Pep1 *
- UnVac

