Cryptococcus neoformans evades pulmonary immunity by modulating xylose transport

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Running Head: Xylose and cryptococcal immunity

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ABSTRACT

Cryptococcus neoformans is a fungal pathogen that kills almost 200,000 people each year and is distinguished by abundant and unique surface glycan structures that are rich in xylose. A mutant strain of *C. neoformans* that cannot transport xylose precursors into the secretory compartment is severely attenuated in virulence in mice, yet surprisingly is not cleared. We found that this strain failed to induce the non-protective T helper cell type 2 (Th2) responses characteristic of wild type infection, instead promoting sustained Interleukin (IL)-12p40 induction and increased IL-17A (IL-17) production. It also stimulated dendritic cells to release high levels of pro-inflammatory cytokines, a behavior we linked to xylose expression. Finally, we discovered that inducible bronchus associated lymphoid tissue (iBALT) forms in response to cryptococcal infection. Although iBALT formation is delayed upon infection with xylose-deficient cryptococci, it subsequently restricts infection. These studies demonstrate that cryptococcal infection triggers iBALT formation and elucidate the role of xylose in the modulation of host response to this fungal pathogen.

16 **INTRODUCTION**

17 *Cryptococcus neoformans* is a ubiquitous environmental fungus that causes pneumonia
18 and meningitis. This opportunistic pathogen infects over a million individuals each year,
19 with overall mortality exceeding 20% (1-3). Patient immune status is the main determinant
20 of infection outcome, highlighting the importance of efficient host immune responses in
21 the control of cryptococcosis.

22 *C. neoformans* infection begins when the organism is inhaled, followed by its proliferation
23 in the lungs. This pulmonary infection may then disseminate to the brain, where it causes
24 a frequently lethal meningoencephalitis. In the lungs, *C. neoformans* first interacts with
25 host macrophages and dendritic cells (DC). These can engulf the fungus and migrate to
26 draining lymph nodes, where they present antigen to T cells to initiate the adaptive im-
27 mune response. The protective immune response to *C. neoformans* is primarily mediated
28 by T cells. Although T helper cell type 1 (Th1) responses are protective against *C. neofor-*
29 *mans* (4), it is likely that T helper type 17 (Th17) responses participate in pathogen clear-
30 ance at mucosal surfaces (5-7). In contrast, T helper cell type 2 (Th2) responses are
31 considered detrimental for protective immunity, leading to fungal growth and facilitating
32 dissemination to the CNS (8, 9). The induction of these non-protective responses is not
33 well understood, but cryptococcal glycans may contribute to this process (10).

34 Glycan structures participate in antigen recognition, immune activation, or immune regu-
35 lation in multiple organisms (11). In *C. neoformans* the major virulence factor, a polysac-
36 charide capsule, modulates the immune response via multiple mechanisms (12, 13). The
37 capsule is primarily composed of two large polysaccharides: glucuronoxylomannan

38 (GXM) and glucuronoxylomannogalactan (GXMGal) (14, 15). One major component of
39 both polymers is xylose (Xyl), which comprises almost one fourth of the polysaccharide
40 capsule mass (16). Xylose also occurs in cryptococcal glycolipids (17) and as both Xyl
41 and Xyl-phosphate modifications of protein-linked glycans (18-20). We demonstrate here
42 that Xyl plays a critical role in the immune recognition of, and response to, *C. neoformans.*

43 The incorporation of Xyl into cryptococcal glycan structures occurs in the lumen of the
44 secretory pathway, via enzymatic reactions that use the substrate molecule UDP-Xyl.
45 This xylose donor is imported into the synthetic compartment by two transporters, Uxt1
46 and Uxt2 (21). A mutant strain that lacks both transporters (*uxt1*Δ *uxt2*Δ) exhibits defects
47 in glycosylation, including incomplete synthesis of capsule polysaccharides, and consid-
48 erable attenuation of virulence in mouse models of infection. Surprisingly, despite its com-
49 promised virulence, this attenuated strain persists in the lungs of infected mice. In pursu-
50 ing the mechanism of this persistence, we observed the formation of inducible bronchus
51 associated lymphoid tissue (iBALT) in the lungs of both wild-type (WT) and *uxt1*Δ *uxt2*Δ
52 infected mice. In the lungs of mice infected with the mutant strain, iBALT formation was
53 significantly delayed. However, once formed, it was better organized than the iBALT
54 formed when mice were infected with fully xylosylated WT *C. neoformans.* Furthermore,
55 animals infected with the mutant experienced a long-term asymptomatic infection rather
56 than the rapid and lethal disease observed upon WT infection. Together, these results
57 suggest that luminal Xyl modifications of cryptococcal glycoconjugates interfere with im-
58 mune recognition and activation of immune cells, events that are required for efficient
59 control of the pulmonary infection.
60

61 **RESULTS**

62 *UDP-Xyl transport is required for cryptococcal virulence and dissemination*

63 We previously observed that a *C. neoformans* strain lacking UDP-Xyl transporters (*uxt1*Δ
64 *uxt2*Δ) was highly attenuated in virulence compared to the WT parental strain KN99α, yet
65 persisted in the lungs of asymptomatic mice for at least 100 days after infection (21). To
66 investigate the development of this chronic infection, we first examined the kinetics of
67 fungal burden in mice after intranasal inoculation with WT and mutant strains. In A/JCr
68 mice infected intranasally with WT fungi, which typically succumb to infection roughly 18
69 days post-infection (dpi), we observed a rapid and significant increase in pulmonary fun-
70 gal burden (Fig 1A) along with significant dissemination to the spleen (Fig 1B) and brain
71 (Fig 1C). In contrast, the pulmonary burden in *uxt1*Δ *uxt2*Δ-infected mice increased only
72 gradually from initial inoculum levels to a modest peak at 63 dpi (Fig 1A). Colony-forming
73 units in the lung then gradually declined, with complete clearance occurring in 30% of the
74 mice by 189 dpi (Fig 1A). Although this infection was not limited to the lungs, only a frac-
75 tion of the mice had measurable *uxt1*Δ *uxt2*Δ cells in the spleen (Fig 1B) and brain (Fig
76 1C) at 63 dpi. Furthermore, this limited dissemination was only observed at the times of
77 highest lung burden (63 and 126 dpi), with no fungi detected at distal sites by 189 dpi (Fig
78 1B, Fig 1C). These results suggest that the A/JCr mice would eventually clear the *uxt1*Δ
79 *uxt2*Δ infection.

80

81 *Cryptococcal xylosylation influences differential production of pulmonary cytokines.*

82 We next sought to identify the mechanism(s) responsible for the protracted host clearance
83 of *uxt1*Δ *uxt2*Δ infection. As summarized above, protection against *C. neoformans* is gen-
84 erally associated with production of type 1 cytokines like interleukin (IL)-2, IL-12, inter-
85 feron gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α). In contrast, a Th2 re-
86 sponse to cryptococcal infection (characterized by the production of IL-4, IL-5, IL-10, and
87 IL-13) compromises control of pulmonary fungal burden (22) and facilitates dissemination
88 to the central nervous system (4, 22).
89
90 We hypothesized that the increased survival of mice infected with the mutant cryptococci
91 reflected an alteration in the immune response elicited by pulmonary infection. To test
92 this hypothesis, we analyzed cytokine levels over time in lung homogenates from mice
93 infected with WT or *uxt1*Δ *uxt2*Δ strains (Fig 1). A strong Th2-type response with signifi-
94 cant induction of IL-4 and IL-5 over the 15 days prior to sacrifice was detected in mice
95 infected with WT cryptococci (Fig 1D, Fig 1E). In contrast, *uxt1*Δ *uxt2*Δ cells elicited a
96 more quiescent cytokine response at early stages of infection. Furthermore, at the peak
97 of fungal burden in *uxt1*Δ *uxt2*Δ-infected mice (day 63, Fig. 1A), there was a significant
98 increase in the Th1 polarizing cytokine IL-12p40 compared to the levels observed in naïve
99 lungs (Fig 1F). Subsequently, IL-12p40 levels slowly declined, along with fungal burden
100 (Fig 1F). Although levels of IFNγ and TNFα (Fig 1G, Fig 1H) were similar to those in the
101 lungs of naïve mice, IL-17 trended higher at the peak of infection (Fig 1I). Overall, while
102 WT infection rapidly induced non-protective Th2 responses, infection with *uxt1*Δ *uxt2*Δ
103 failed to do so (even at the peak of fungal burden) and instead promoted the sustained
104 induction of IL-12p40 and increased production of IL-17.

105

106 *Inducible bronchus-associated lymphoid tissue develops following cryptococcal infection*

107 The differences that we observed in fungal burden and cytokine levels between mutant
108 and WT infection suggested alterations in the immune response at the cellular level. To
109 test this, we performed blinded morphometric analysis of inflammatory cell infiltration in
110 the lungs at 15 dpi in mice infected with WT fungi and at 15 and 189 dpi in *uxt1*Δ *uxt2*Δ-
111 infected mice. The animals infected with the mutant strain showed significantly reduced
112 lung inflammation at both time points (Fig 2A), consistent with the more quiescent cyto-
113 kine response reported above (Fig 1). We also noted distinctive lymphocytic accumula-
114 tions proximal to the basal side of the bronchial epithelium in both groups of mice (Fig
115 2B). The lymphoid populations within these structures were organized into B cell follicles
116 surrounded by $CD3^+$ T cell cuffs (Fig 2C). The cellular composition of these inflammatory
117 foci suggested inducible bronchus-associated lymphoid tissue (iBALT) (23).

118

119 iBALT is a tertiary lymphoid tissue that forms in the lung. It resembles secondary lymphoid
120 organs (lymph nodes) in cellular composition and organization, with two distinctive zones:
121 the B cell follicle and the T cell zone (24). The B cell follicle contains mainly follicular B
122 cells and its organization is maintained by the continuous production of CXCL13. It is
123 surrounded by a T cell zone that contains CD4 and CD8 T cells as well as dendritic cells
124 (DCs) (25). At 15 dpi with WT cryptococci, we observed numerous iBALT structures, with
125 compact B cell follicles surrounded by T cells (Fig 2C, Fig 2D). In contrast, we found
126 considerably fewer and less organized B cell follicles at 15 dpi in mice infected with the
127 *uxt1*Δ *uxt2*Δ strain (Fig 2C, Fig 2D). By late in infection (189 dpi for *uxt1*Δ *uxt2*Δ and 15

128 dpi for WT), although iBALT structures in mice infected with the mutant strain were still
129 less abundant than those found in lungs of WT (Fig. 2D), their average size was signifi-
130 cantly larger (Fig. 2E). Additionally, the iBALT was more organized and even contained
131 IgG^+ plasma cells (Fig 2C). Early in *uxt1*Δ *uxt2*Δ infection, the compromised formation of
132 iBALT structures was accompanied by increased peri-vascular accumulation of T cells
133 (Fig. 2F) and a significantly decreased production of CXCL13 (Fig 2G, Fig2H), a chemo-
134 kine essential for iBALT formation and organization (23). In contrast, at late time points
135 in this protracted developmental process, T cell cuffing was significantly reduced (Fig 2F)
136 and CXCL13 expression was increased (Fig 2G, Fig 1H). While infection with WT crypto-
137 cocci induced early and robust formation of iBALT, *uxt1*Δ *uxt2*Δ infection induced delayed
138 but ultimately larger iBALT.

139

140 To quantify the host adaptive immune response to *uxt1*Δ *uxt2*Δ, we used flow cytometry
141 to enumerate lymphocytes in the lungs of mice infected with WT or *uxt1*Δ *uxt2*Δ cells. By
142 12-15 dpi, the fraction of $CD19^+$ B cells (Fig 3A) or T cells ($CD4^+$ and $CD8^+$; Fig 3B, Fig
143 3C) in *uxt1*Δ *uxt2*Δ-infected mice significantly exceeded those of WT-infected mice, and
144 continued to accumulate as infection progressed. The total numbers of cells in these pop-
145 ulations remained elevated through 126 dpi (Fig. 3A-C, right), although they subsequently
146 declined, consistent with the decreased average size in T cell cuffing observed at 189
147 versus 15 dpi (Fig 2F).

148

149 *Survival of uxt1Δ uxt2Δ-infected mice is dependent on T cells*

150 To test the protective role of T and B cells in the prolonged survival of *uxt1*Δ *uxt2*Δ-in-
151 fected mice, we examined whether ablation of these cell types altered the course of in-
152 fection. Because the mouse strains used for these studies were engineered in the
153 C57BL/6 background, we included infections of this strain as controls. C57BL/6 mice are
154 less resistant to *C. neoformans* than A/JCr mice used above (26), as shown by their even-
155 tual susceptibility to *uxt1*Δ *uxt2*Δ infection (Fig. 3D, solid black line). In contrast, *Rag1*-/-
156 mice were significantly more susceptible to this strain, and succumbed by 55 dpi (Fig 3D;
157 solid blue line).
158
159 Since *Rag1*-/- mice lack both T and B cells, we also infected mice deficient in either T
160 cells (*TCRβ* -/-) or B cells (*μMT*) with *uxt1*Δ *uxt2*Δ cryptococci. B cell deficient mice ex-
161 hibited a more protracted course of disease than *Rag1*-/-, succumbing over an 85-day
162 period (Fig 3D, solid green line), while mice lacking only T cells succumbed to infection
163 with kinetics similar to those of the *Rag1*-/- mice (Fig 3D, solid red line). In contrast, all
164 mice inoculated with WT *C. neoformans* succumbed by 20 dpi (Fig 3D; dashed lines). Of
165 this group, *Rag1*-/- and *TCRβ*-/- mice still succumbed slightly faster than C57BL/6 and
166 μMT mice (Fig 3D). Together, these data show that T cells are the prominent cell type
167 responsible for increased survival of *uxt1*Δ *uxt2*Δ-infected mice, although B cells may also
168 play a role in protection.
169
170 *Lack of luminal xylose modification in C. neoformans stimulates DC activation*
171 DCs are critical for induction of protective immunity against *C. neoformans* (27), and their
172 activation and cytokine production are required for iBALT formation and maintenance (25,

173 28). During iBALT formation, antigen-driven activation of DCs and their consequent cyto-
174 kine and chemokine production are likely responsible for T and B cell recruitment (25),
175 which could potentially both induce the observed increase in these cell populations and
176 contribute to the protective effect of T cells during cryptococcal infection. For these rea-
177 sons, we wished to specifically measure DC responses. To do this we tested DC interac-
178 tions with WT cryptococcal cells, which exhibit antigens modified with Xyl, and *uxt1*Δ
179 *uxt2*Δ cryptococci, which are impaired in these modifications. We also tested single *uxt*Δ
180 mutants: *uxt1*Δ shows an intermediate level of Xyl utilization while *uxt2*Δ is similar to WT
181 (21). In these assays, we measured the ability of heat-killed fungi to stimulate the produc-
182 tion of pro-inflammatory cytokines by DC, comparing WT, *uxt1*Δ *uxt2*Δ, single *uxt* mu-
183 tants, *uxs1*Δ (which lacks all Xyl modification because it cannot synthesize UDP-Xyl), and
184 an acapsular control strain (*cap59*Δ) that induces a potent DC response (29). We found
185 that bone marrow derived DCs (BMDCs) cultured with *uxt1*Δ *uxt2*Δ cells released high
186 levels of IL-1β, IL-6, and TNF-α, similar to the levels observed following exposure to a
187 completely acapsular control strain, *cap59*Δ (Fig 4A-C). In contrast, individual *uxt* mutants
188 and *uxs1*Δ induced IL-1β and IL-6 levels similar to those induced by WT fungi (close to
189 background levels; Fig 4A, Fig 4B). TNF-α similarly showed the greatest response upon
190 challenge with *uxt1*Δ *uxt2*Δ, with levels like those induced by an acapsular strain, alt-
191 hough some increase was also noted with other xylose-deficient strains (*uxt1*Δ and
192 *uxs1*Δ). These results suggest that early interaction with DCs and consequent induction
193 of proinflammatory cytokines are regulated by Xyl expression in Cryptococcus.
194
195 **DISCUSSION**

196 In this study, we report for the first time that *C. neoformans* infection triggers iBALT for-
197 mation. Notably, the iBALT detected in our experimental infections resembles subpleural
198 nodules that have been observed in cryptococcosis patients (30). To our knowledge, the
199 only other fungus reported to induce iBALT is *Pneumocystis* (31). Further examination
200 will be necessary to establish the similarities between histological features of inflamma-
201 tory cell infiltration in mice and humans infected with *C. neoformans* and to delineate the
202 molecular host-pathogen interactions that drive iBALT formation following *C. neoformans*
203 infection.
204
205 When we infected mice with mutant fungi unable to use Xyl for luminal glycoconjugate
206 synthesis (*uxt1*Δ *uxt2*Δ), we observed a delay in fungal accumulation and in the organi-
207 zation of the iBALT structures. When *uxt1*Δ *uxt2*Δ burden did peak, it was accompanied
208 by a rise in IL-12p40 (Fig 1F), a cytokine normally required for inducing a protective re-
209 sponse against *C. neoformans* (32), which may have facilitated subsequent resolution
210 of the *uxt1*Δ *uxt2*Δ infection. A change in IL-12p40 level might reflect an increase in ei-
211 ther IL-23 or IL-12 (33), which stimulate a Th17 or Th1 type response, respectively; the
212 former is required for iBALT formation(31). Since we saw no difference in IFN-γ levels,
213 IL-12p40 is more likely associated with a Th17 response. We did observe a trend to-
214 wards increased IL-17 levels when *uxt1*Δ *uxt2*Δ burden was the highest, which was ab-
215 sent in WT infection. Our results show that cryptococcal infection can induce iBALT
216 structures, although this does not prevent mice from succumbing to the disease. We fur-
217 ther show that the kinetics of iBALT formation and its organization are dependent on vir-
218 ulence factors of the pathogen. Future studies should futher define the functional role of

219 iBALT in mediating immunity to Cryptococcus and determine whether the iBALT is me-
220 diating early and local immune protection in the lung.

221

222 DCs reacted more strongly to *uxt1*Δ *uxt2*Δ than to WT *in vitro*, releasing greater amounts
223 of pro-inflammatory cytokines (Fig 4A-C), even though the global cytokine response to
224 the mutant is less perturbed (Fig 1D-I). These cytokines may directly or indirectly lead to
225 the increased recruitment of T and B cells in *uxt1*Δ *uxt2*Δ infection (Fig 3A-C) and perhaps
226 accelerate compartmentalization into compact B cell follicles and discrete T cell areas in
227 iBALT (Fig 2). Such differences in cellular composition and organization, coupled with the
228 differences in the local cytokine production, may confer distinct properties on the iBALT
229 induced by WT and *uxt1*Δ *uxt2*Δ, influencing their ability to protect against *C. neoformans*.

230

231 We expected that the highly immunostimulatory behavior of *uxt1*Δ *uxt2*Δ *in vitro* reflected
232 the lack of Xyl modifications on its surface glycoconjugates, a consequence of the ab-
233 sence of UDP-Xyl transport into the secretory compartment. We were surprised, there-
234 fore, that mutants unable to synthesize UDP-Xyl (*uxs1*Δ), which similarly lack Xyl modifi-
235 cations on secreted glycoconjugates, did not phenocopy this broad and robust DC re-
236 sponse. This suggests that Uxs1 itself is required for the strong response seen in the
237 absence of xylosylation in the secretory pathway. It may be that this protein plays addi-
238 tional roles unrelated to glycosylation, or that the stimulatory component is dependent on
239 cytosolic UDP-Xyl, rather than UDP-Xyl that has been transported into the secretory path-
240 way.

241

242 Unlike encapsulated strains, *C. neoformans* mutants lacking capsule induce the upregu-
243 lation of multiple genes involved in cytokine responses and enhance antigen processing
244 and presentation by DCs (34). Interestingly, the cytokine levels induced in DC by the
245 *uxt1*Δ *uxt2*Δ mutant were similar to those induced by the acapsular mutant *cap59*Δ (Fig
246 4A-C). Xyl may thus be critical for the immune suppressive effects normally exerted by
247 capsule material. The mechanisms behind the robust DC activation seen in these two
248 mutants, including the cellular receptors and fungal components responsible, remain to
249 be determined. It is possible that the *uxt1*Δ *uxt2*Δ mutant lacks immune suppressive
250 mechanisms that are present in WT *C. neoformans* infection, and therefore induces ro-
251 bust cytokine responses and consequent recruitment of T and B cells to limit early infec-
252 tion. Further studies addressing the nature, type, and extent of the T and B cell responses
253 in the context of WT and *uxt1*Δ *uxt2*Δ infection should allow us to fully understand the the
254 initiation, formation, and organization of iBALT structures in the lung during *C. neofor-*
255 *mans* infection. Further, as iBALT is induced in WT *C. neoformans* infection where the
256 mice succumb to the disease, it is unclear if iBALT structures play a protective or patho-
257 logical role in immunity. Specific chemokine deficient mice, such as CXCL-13 deficient
258 animals that lack the ability to induce iBALT structures (35), will be useful in future studies
259 to define the function of iBALT in fungal infections.
260
261 Control of *uxt1*Δ *uxt2*Δ infection requires both T and B cells (Fig 3D), which suggests that
262 iBALT formation may play a key role in restricting disease. Even in the absence of T and

263 B cells, however, mutant-infected mice still survived more than twice as long as WT-in-
264 fected animals (Fig 3D). This may be due to the slightly slower growth of the mutant,
265 which we have previously observed (21).
266
267 We have found that *C. neoformans* infection induces iBALT formation, although the for-
268 mation of iBALT by itself is insufficient to protect against wild-type fungal infection. Inoc-
269 ulation with a *uxt1*Δ *uxt2*Δ strain, however, induced similar lymphoid structures, which do
270 appear to restrict infection despite their delayed organization. *In vitro*, this mutant induced
271 DCs to release much higher levels of proinflammatory cytokines than WT fungi. This is
272 presumably a consequence of the lack of surface Xyl modification, suggesting a role for
273 this structure in the immunosuppressive features of capsule polysaccharides or other sur-
274 face exposed or secreted glycoconjugates, although the full mechanism remains to be
275 defined. By influencing the dynamics of the inflammatory process, we may be able to
276 improve pulmonary responses to help control infection and use this information to help
277 guide novel therapies that stimulate the induction of protective lung fungal immunity.
278
279 **MATERIALS AND METHODS**
280 *Fungal strains*
281 *C. neoformans* strains (Table S1) were grown at 30 °C in YPD medium (1% [wt/vol] yeast
282 extract, 2% [wt/vol] peptone, 2% [wt/vol] dextrose) with shaking (230 rpm) or on YPD agar
283 plates (YPD medium with 2% [wt/vol] agar) supplemented with the following antibiotics as
284 appropriate: 100 µg/ml nourseothricin (NAT; Werner BioAgents) or Geneticin (G418; Invi-
285 trogen).

286

287 *Mice*

288 C57BL/6J (RRID: IMSR_JAX:000664) and A/JCr (RRID: IMSR_CRL:563) mice were ob-
289 tained from Jackson Laboratory. *Rag1*-/- (RRID: IMSR_JAX:002216), *TCRβ*-/- (RRID:
290 IMSR_JAX:002118), and μMT mice (RRID: IMSR_JAX:002288) (all on C57BL/6 back-
291 ground) were generously provided by Dr. Michael Diamond and Dr. Wayne Yokoyama
292 (Washington University School of Medicine); breeders were originally purchased from
293 Jackson Laboratory. All mice were 6 to 8 weeks old at the time of infection. A/JCr mice
294 were female; male and female mice were used for the other strains with gender and age
295 matched C57BL/6 controls.

296

297 *Ethics statement*

298 All animal protocols were reviewed and approved by the Animal Studies Committee of
299 the Washington University School of Medicine and conducted according to National Insti-
300 tutes of Health guidelines for housing and care of laboratory animals.

301

302 *C. neoformans infection*

303 Fungal strains were cultured overnight (O/N) and diluted to 10^6 cells/mL in sterile PBS.
304 Mice were intranasally inoculated with a 50 μL aliquot, and then weighed and monitored
305 daily. Infected mice were sacrificed if they lost >20% relative to peak weight or at specified
306 time points. At the time of sacrifice, mice were perfused intracardially with 10 mL sterile
307 PBS, and organs were processed as described below for fungal burden, histology, flow
308 cytometric analysis, and cytokine measurements.

309

310 *Organ burden and cytokines*

311 Brain and spleen homogenates were harvested and plated for CFU at the specified time
312 points. 50 μL aliquots of the left lung homogenates (V_T = 1 mL) were similarly plated for
313 CFUs. The remaining sample was assayed for pulmonary cytokine levels using the Bio-
314 Plex Protein Array System (Bio-Rad Laboratories). Briefly, the lung homogenates were
315 mixed with an equal volume of PBS/0.1% Triton 100x/2x protease inhibitor (Pierce EDTA-
316 free protease inhibitor; Thermo Scientific), vortexed for 3 seconds, and clarified by cen-
317 trifugation (2500 x g, 10 min). Supernatant fractions were then assayed using the Bio-
318 Plex Pro Mouse Cytokine 23-Plex (Bio-Rad Laboratories) for the presence of IL-1α, IL-
319 1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, granulo-
320 cyte colony stimulating factor (G-CSF), granulocyte monocyte colony stimulating factor
321 (GM-CSF), interferon-γ (IFN-γ), CXCL1/keratinocyte-derived chemokine (KC),
322 CCL2/monocyte chemotactic protein-1 (MCP-1), CCL3/macrophage inflammatory pro-
323 tein-1α (MIP-1α), CCL4/MIP-1β, CCL5/regulated upon activation normal T cell expressed
324 and secreted (RANTES), and tumor necrosis factor-α (TNF-α).

325

326 *Immunofluorescence staining and histologic analysis*

327 Mice were perfused intracardially with sterile PBS, and the lungs inflated with 10% forma-
328 lin. Lung tissue was then fixed O/N in 10% formalin, and submitted to the Washington
329 University Developmental Biology Histology Core for paraffin-embedding, sectioning, and
330 staining with H & E. Immunofluorescence staining for B cells (APC-conjugated rat anti-
331 mouse CD45R/B220, clone RA3-6B2, BD Biosciences, RRID: AB_627078), T cell cuffing

332 (CD3-ε, clone M-20, Santa Cruz Biotechnology, RRID: AB_631129), and CXCL13-pro-
333 ducing cells (goat α-mouse CXCL13, AF470, R&D Systems, RRID: AB_355378) was per-
334 formed as in Ref (31). Images were taken with a Zeiss Axioplan2 microscope and rec-
335 orded with a Hamamatsu camera. Blinded morphometric analysis of lung structures was
336 performed with the outline tool of Zeiss Axiovision software.
337
338 *Flow cytometric analysis*
339 The right lung of individual mice was enzymatically digested (in 5 mL RPMI with 1 mg/mL
340 collagenase type IV) at 37 °C with shaking (230 rpm) for 30 min, and then sequentially
341 passed through sterile 70 and 40 µm pore nylon strainers (BD Biosciences, San Jose,
342 CA). Red blood cells in the samples were lysed by treatment for 3 min on ice with 5 mL
343 ammonium-chloride-potassium lysing buffer (8.024 g/L NH_4Cl, 1.001 g/L $KHCO_3$, 2.722
344 mg/L EDTA•Na_2 $2H_2O$), followed by the addition of 2 volumes of PBS. The remaining cells
345 were pelleted (1000 x g, 5 min, 4 °C), washed twice with PBS, diluted to 10^6 cells/mL in
346 PBS, and stained with LIVE/DEAD fixable blue dead cell stain (1:1000; Thermo Scien-
347 tific). Following incubation in the dark for 30 min at 4 °C, cells were washed with PBS and
348 FACS buffer (2% fetal bovine serum in PBS) before resuspension in FACS buffer. Sam-
349 ples were then blocked with CD16/CD32 (1:500; Fc Block™; BD Biosciences, RRID:
350 AB_394656) for 5 min and incubated for 30 min with optimal concentrations of fluoro-
351 chrome-conjugated antibodies (Table S2) diluted in Brilliant Stain Buffer (BD Biosci-
352 ences). After three washes with FACS buffer, the cells were fixed in 2% formalde-
353 hyde/FACS buffer. For data acquisition, >50,000 events were collected on a BD LSRFor-
354 tessa X-20 flow cytometer (BD Biosciences), and the data were analyzed with FlowJo

355 V10 (Fig. S1; TreeStar). The absolute number of cells in each leukocyte subset was de-
356 termined by multiplying the absolute number of $CD45^+$ cells by the percentage of cells
357 stained by fluorochrome-labeled antibodies for each cell population analyzed.
358
359 *Isolation of bone marrow derived cells*
360 Bone marrow was flushed from the femurs and tibiae of C57BL/6 mice using RPMI. Cells
361 were collected (1000 x g, 5 min, 4°C), resuspended in RPMI, and counted using a hemo-
362 cytometer. To prepare bone marrow derived dendritic cells (BMDCs), 2 x 10^6 bone mar-
363 row cells were plated in 10 mL R10 medium (10% FBS, 0.4% Penicillin-Streptomycin, 2
364 mM L-glutamate, 50 μM 2-β-mercaptoethanol in RPMI) supplemented with 1 ng/mL GM-
365 CSF, and incubated at 37 °C and 5% CO_2. Medium was changed 3 and 6 days after
366 plating, and cells were harvested on day 8. BMDCs were enriched by depletion of BMMs
367 using biotinylated α-F4/80 antibody (eBioscience, RRID: AB_466657) and anti-biotin con-
368 jugated magnetic beads (Miltenyi Biotec). The BMDCs in the flow through were positively
369 selected using α-CD11c magnetic beads according to the manufacturer's protocol (Mil-
370 tenyi Biotec).
371
372 *Dendritic cell assays*
373 To assay the ability of fungal strains to activate BMDCs, *C. neoformans* strains of interest
374 were grown O/N, washed in PBS, and incubated at 65 °C for 15 min to heat kill (HK) the
375 fungi. BMDCs and HK fungi (10^6 cells of each) were then co-incubated for 24 h, sedi-
376 mented, and the supernatant fractions transferred to 1.5 mL centrifuge tubes containing

377 10 μL of 100x protease inhibitor (ThermoScientific). IL-1β, IL-6, and TNF-α levels in su-
378 pernatants were determined by ELISA according to the manufacturer's protocol (R&D
379 systems).
380
381 *Statistical analysis*
382 Each experiment was performed a minimum of two times. Statistical analyses were con-
383 ducted using GraphPad Prism version 6.0f (GraphPad Software). All studies comparing
384 two groups were analyzed with a Student's t-test. Those with three or more groups were
385 compared using an ordinary one-way ANOVA with Tukey's *post-hoc* test. $p < 0.05$ was
386 considered statistically significant.
387
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AUTHOR CONTRIBUTIONS

Conceptualization, LXL, CRH, SAK, and TLD; Methodology, LXL, CRH, JRM, SAK, TLD; Investigation, LXL, CRH, and JRM; Writing – Original Draft, LXL and CRH; Writing – Review & Editing, LXL, CRH, JRM, SAK, and TLD; Funding Acquisition, LXL, SAK and TLD; Resources, SAK and TLD; Supervision, SAK and TLD.

DECLARATION OF INTERESTS

LXL, CRH, JRM, SAK and TLD have no interests to declare.

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414

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528

FIGURES WITH LEGENDS

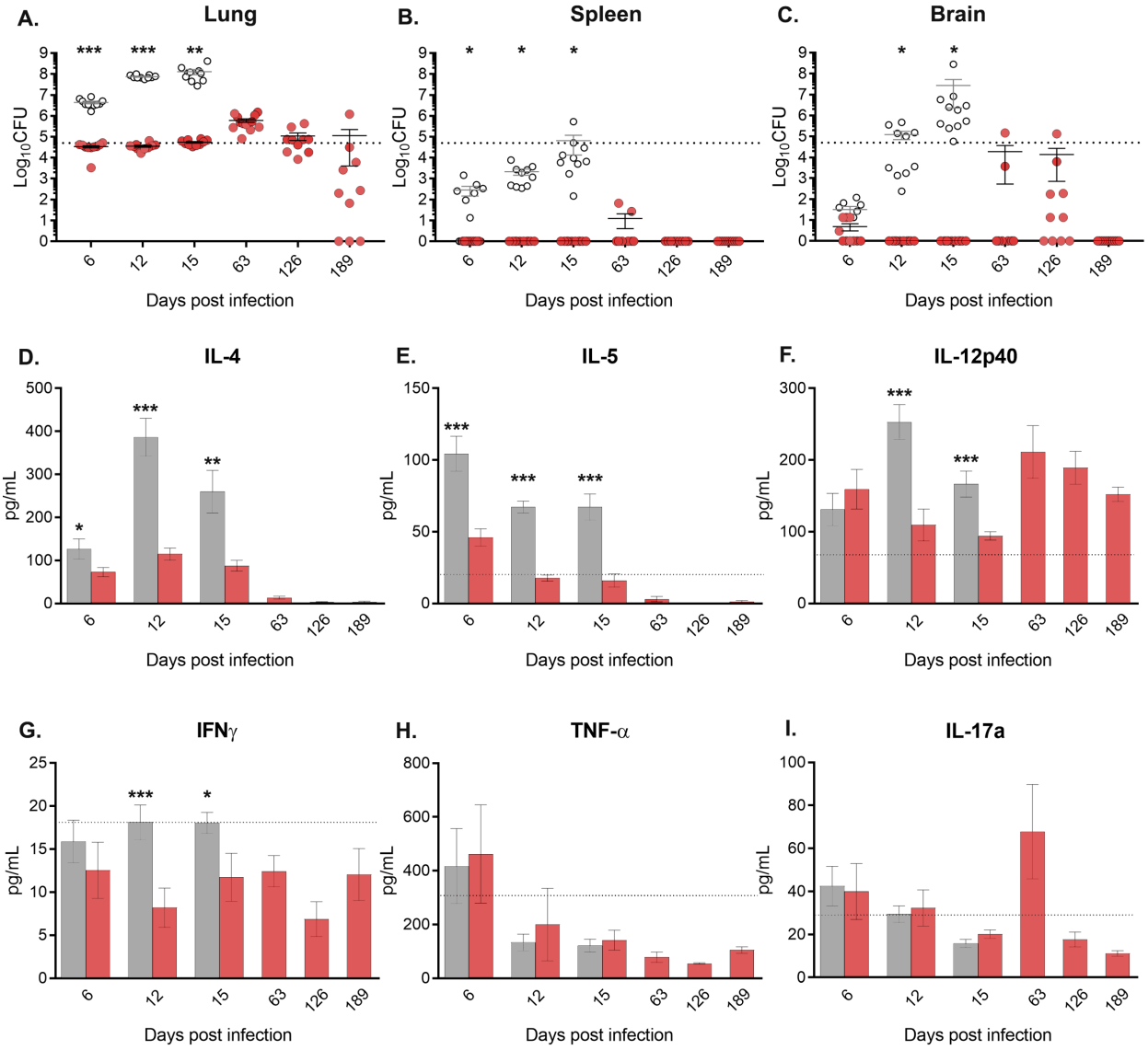


Figure 1. Fungal burden and pulmonary cytokine production in WT and *uxt1*Δ *uxt2*Δ mice. All panels show the combined results of two independent experiments (5 mice per group per experiment). (A-C) Tissue homogenates of infected A/JCr mice were plated for CFUs at the indicated dpi (open circles, WT; red circles, *uxt1*Δ *uxt2*Δ; dashed line, initial inoculum). Values from individual mice are plotted along with the mean ± SEM. (D-I) Levels of the cytokines indicated in lung homogenates over time after infection (gray bars, WT; red bars, *uxt1*Δ *uxt2*Δ; dashed line, naïve). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ for Student's t-test comparing wild type to mutant.

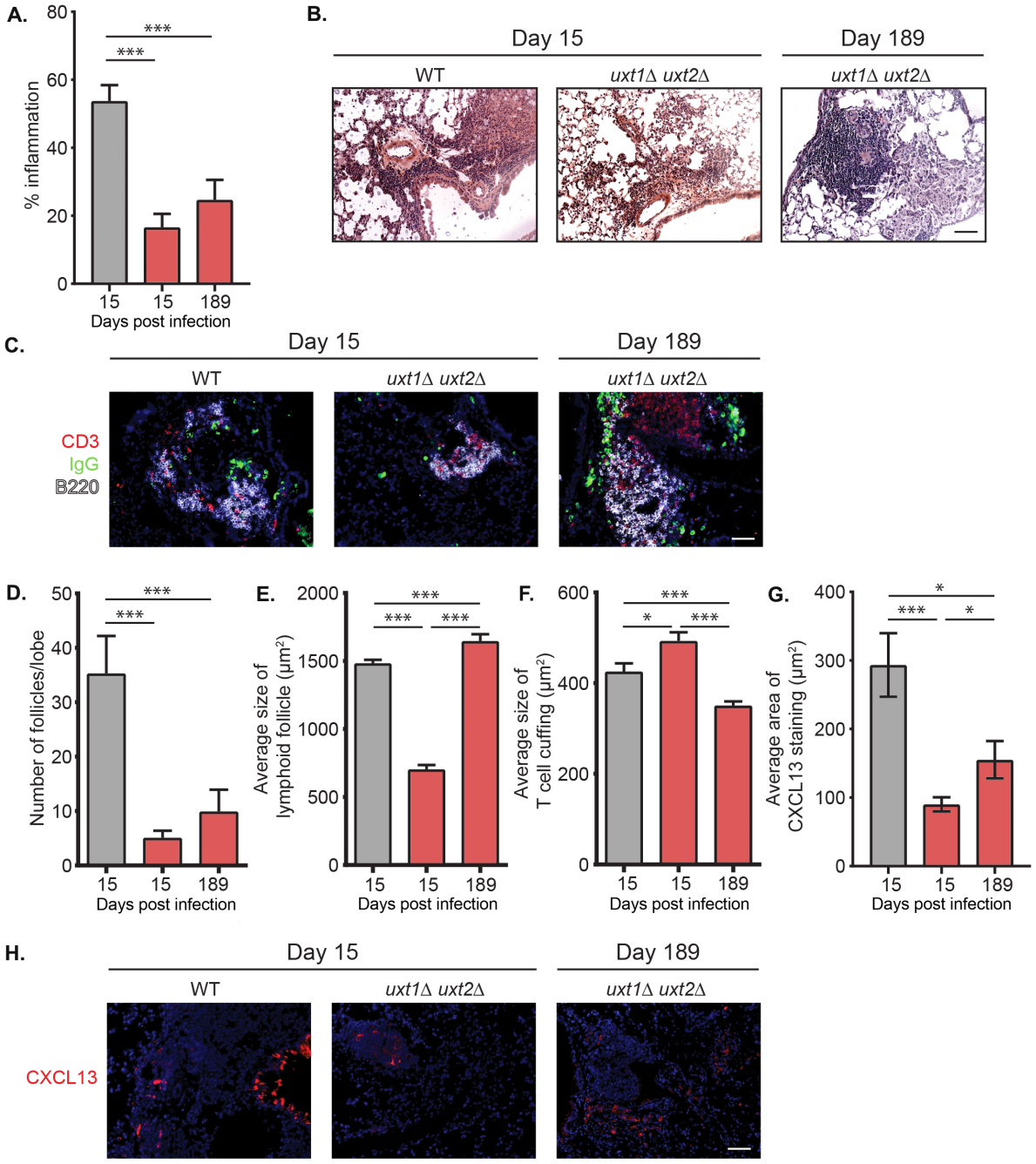


Figure 2. Inflammation and iBALT complexity in the context of *C. neoformans* infection. (A) Percent of area covered by inflammatory cells in individual lungs and (B) representative H&E micrographs from lungs collected at 15 and 189 dpi are shown. Scale bar = 100 μm. (C) Representative immunofluorescent staining of B cells ($B220^+$), T cells ($CD3^+$), and plasma cells (IgG^+) in the lungs of infected A/JCr mice. Scale bar = 100 μm. (D-F) Morphometric analysis of iBALT structures in A/JCr mice infected with WT and *uxt1*Δ *uxt2*Δ. (G,H) Quantification and examples of immunofluorescent staining of CXCL13 (red). Nuclei are stained with DAPI (blue) and scale bar = 100 μm. Plots show the combined mean ± SEM of two independent studies (4-5 mice per group per experiment). *, $p < 0.05$; ***, $p < 0.005$ by Student's t-test.

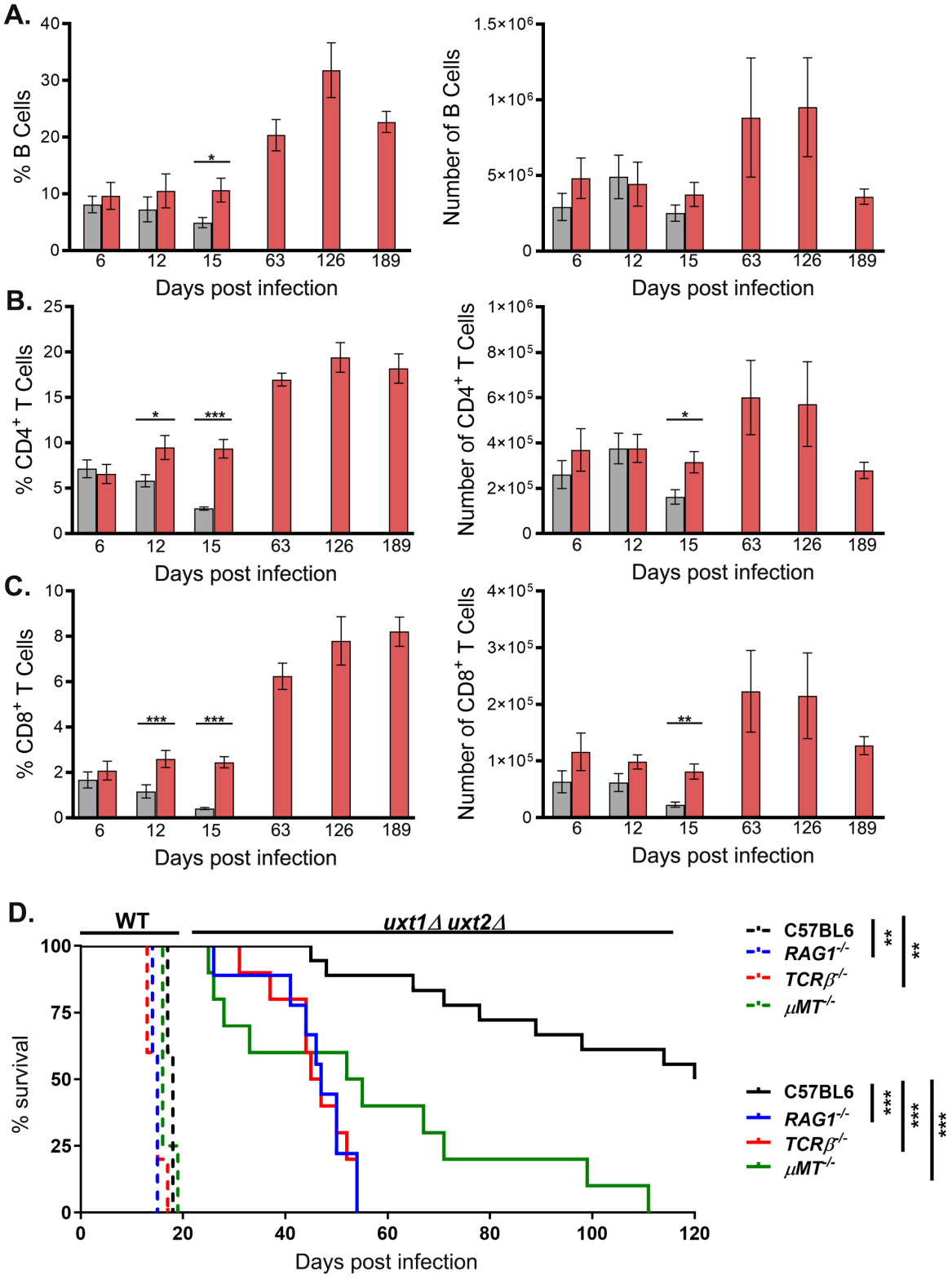


Figure 3. Accumulation of T and B cells in the lungs of mice infected with *uxt1*Δ *uxt2*Δ cells is required to prevent disease progression. (A) CD19⁺ B cells, (B) CD4⁺ T cells, and (C) CD8⁺ T cells were enumerated in the lungs of infected mice by flow cytometry (gray, WT; red, *uxt1*Δ *uxt2*Δ). Plotted are the combined mean ± SEM values from two independent experiments (5

mice per group per experiment). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ by Student's t-test. (D) Survival of mice after intranasal inoculation with 5×10^4 cells of WT (n = 5) or *uxt1*Δ *uxt2*Δ (n = 10). Results shown are combined from two independent experiments. **, $p < 0.01$; ***, $p < 0.005$ by the log-rank test.

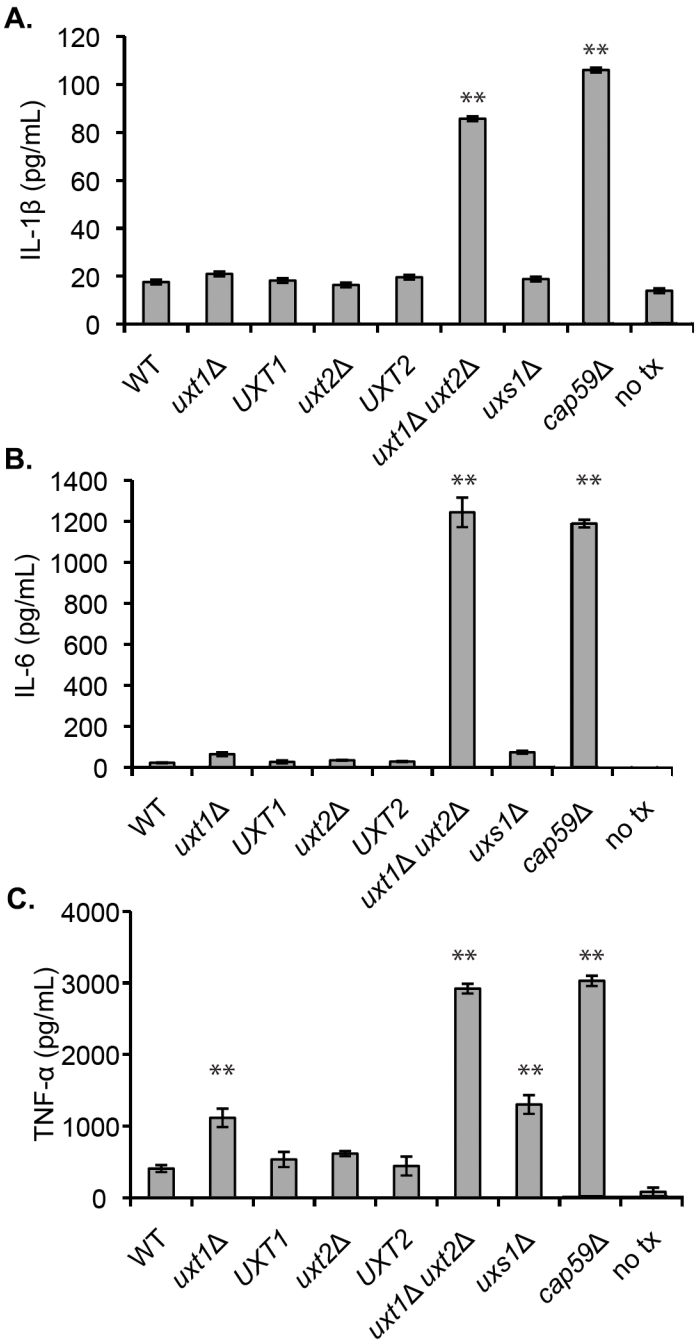
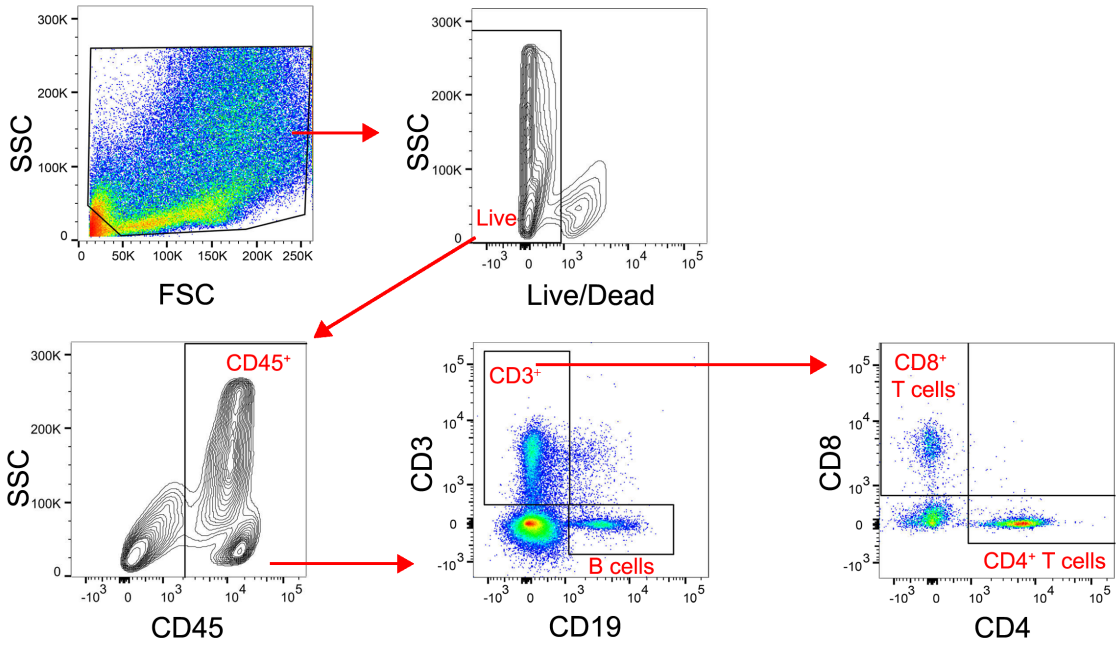


Figure 4. Cytokine production by dendritic cells stimulated with cryptococcal antigens. DCs were co-incubated for 24 h with heat-killed cells of the strains indicated or subjected to no treatment (no tx). Levels of (B) IL-1β, (C) IL-6, and (D) TNF-α in the supernatant were quantified by ELISA. Data shown is the mean ± SD (n = 3) of one representative experiment from five independent experiments that yielded similar results. **, $p < 0.01$ by one-way ANOVA with Tukey's post hoc test.

SUPPLEMENTARY MATERIAL



Supplemental Figure 1. Example of the multi-color flow cytometry gating strategy used to enumerate lung infiltrating immune cells.

Table S1. *C. neoformans* strains utilized in these studies.

C. neoformans strain[a]	Origin
KN99α	(Nielsen et al., 2003)
*uxt1*Δ	(Li et al., 2018)
UXT1	(Li et al., 2018)
*uxt2*Δ	(Li et al., 2018)
UXT2	(Li et al., 2018)
*uxt1*Δ *uxt2*Δ	(Li et al., 2018)
*uxs1*Δ	(Gish et al., 2016)
*cap59*Δ	(Moyrand and Janbon, 2004)

[a] All mutant strains are derived from KN99α

Table S2. Antibodies for flow analysis.

Antigen	Clone	Fluorophore	Dilution	Company	RRID
CD3	17-A2	APC-Cy7	1:250	BioLegend	AB_2242784
CD4	GK1.5	BUV737	1:500	BD Biosciences	AB_2738734
CD8a	53-6.7	BV650	1:125	BD Biosciences	AB_2738084
CD16/CD32 Fc Block	2.4G2	(not applicable)	1:500	BD Biosciences	AB_394656
CD19	1D3	BV786	1:125	BD Biosciences	AB_2738141
CD45	30-F11	Pacific Blue	1:250	BioLegend	AB_493535

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