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Final Report

Candida parapsilosis infection: How does the host respond?

Candida parapsilosis fertőzések által kiváltott immunválasz vizsgálata

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Although the inflammatory response is very important for the successful clearing of infection, it is a „double-edged sword" as excessive inflammation can also be harmful to the host. One of the most important pro-inflammatory cytokines is IL-1β that plays a unique role in host defense during systemic infections. We found previously, that *C. parapsilosis* induced the production of pro-IL-1β and secreted IL-1β in PBMC-DMs, although much less efficiently than *C. albicans*. To investigate the production of IL-1β upon *C. parapsilosis* infection, first we stimulated PMA-differentiated THP-1 macrophages with different doses of live *C. parapsilosis* cells. The THP-1 cell line is ideal and commonly used for the study of IL-1β induction and inflammasome activation. We found that *C. parapsilosis* induced the secretion of IL-1β in THP-1 macrophages after 24 hours when the MOI of 1 or 5 was used. In contrast, the more virulent species *C. albicans* induced the release of IL-1β even at a 100-times lower dose. Using different isolates of *C. parapsilosis* and *C. albicans*, we proved that this marked difference in IL-1β production is species-specific.
Next we aimed to examine how the synthesis of pro-IL-1β is regulated in macrophages following infection with *C. parapsilosis*. As a result of PMA-treatment, pro-IL-1β protein was already abundant in THP-1 cells, and we did not detect a further increase in its level upon *C. parapsilosis* stimulation (nor did we find a significant increase in the level of pro-IL-1β mRNA; data not shown). To assess whether *C. parapsilosis* can induce the synthesis of pro-IL-1β (and the release of the mature cytokine) in primary cells, we stimulated human PBMC-derived macrophages (PBMC-DMs) with *C. parapsilosis*. We found that *C. parapsilosis* induced the production of pro-IL-1β and secreted IL-1β in PBMC-DMs, although much less efficiently than *C. albicans*.

In the next set of experiments, we examined whether mature IL-1β is produced by a similar mechanism after *C. parapsilosis* stimulation as after *C. albicans* infection. In macrophages, the maturation of pro-IL-1β is mediated by the intracellular cysteine protease caspase-1, a component of the inflammasome complex. In addition to caspase-1, caspase-8 has also been shown to be required for the assembly of the NLRP3 inflammasome complex and the activation of caspase-1. To examine the role of caspases in *C. parapsilosis*-induced IL-1β release, we stimulated THP-1 cells with *C. parapsilosis* and *C. albicans* in the presence of caspase inhibitors. We found that in the case of both *C. parapsilosis* and *C. albicans* infection, all caspase inhibitors significantly reduced the amount of secreted IL-1β in cell culture supernatants, implicating the involvement of the inflammasome in pro-IL-1β processing (As *C. parapsilosis* only induces the secretion of IL-1β after longer incubation periods and at a relatively high MOI, but *C. albicans* would rapidly overgrow the cell culture under the same circumstances, a MOI of 5 was chosen for *C. parapsilosis* and a MOI of 0.04 for *C. albicans* during all 24-hour stimulation experiments.) To confirm that *C. parapsilosis* activates the NLRP3 inflammasome, we stimulated ASC and NLRP3 (inflammasomes components) deficient THP-1 macrophages with the two fungal species and measured their IL-1β production. IL-1β secretion was drastically decreased in these cell lines, indicating that similarly to *C. albicans*, *C. parapsilosis* also induces NLRP3 inflammasome-dependent IL-1β production.

After confirming that IL-1β production induced by *C. parapsilosis* is NLRP3-dependent, we wanted to examine the role of several molecules that have been associated with inflammasome activation. TLR4 and TLR2 are important pattern recognition receptors that have been implicated in the immune sensing of *C. albicans*, but their role in the recognition of *C. parapsilosis* has not yet been examined. The IL-

1 receptor associated kinase (IRAK) is important for TLR signaling and has been shown to link TLRs to rapid NLRP3 inflammasome activation. The spleen tyrosine kinase (Syk) also plays a crucial role in fungal recognition, as many C-type lectin receptors (such as dectin-1, dectin-2 or Mincle) associate with this kinase to induce signaling upon the sensing of fungal pathogens. Furthermore, Syk has been shown to control both pro-IL-1β synthesis and inflammasome activation upon *C. albicans* infection. To investigate the role of these molecules in *C. parapsilosis*-induced IL-1β production, we used chemical inhibitors in our experimental system, and the measured the concentration of released IL-1β by ELISA. We found that IL-1β release induced by *C. parapsilosis* and *C. albicans* is dependent on TLR4 and Syk, while TLR2 and IRAK seem to have a minor role.

As reactive oxygen species (ROS) production, phagocytosis, lysosomal cathepsin B release and K+-efflux have all been linked to inflammasome activation by *C. albicans*, we examined the role of these mechanisms in *C. parapsilosis*-induced IL-1β release in THP-1 macrophages. We found that inhibition of NADPH-oxidase significantly decreased the levels of both intracellular pro-IL-1β and secreted IL-1β in THP-1 cells following *C. parapsilosis* and *C. albicans* infection. We also measured the level of intracellular ROS in THP-1 cells in response to the two fungal species, and found that while *C. albicans* induced a significant increase in the level of ROS after 4 hours at MOI 1, *C. parapsilosis* only induced the generation of ROS after a longer incubation period. Furthermore, while inhibition of cathepsin B with CA-074 Me did not affect the production of IL-1β (data not shown), *C. albicans* induced higher cathepsin B release in THP-1 cells after 4 hours compared to *C. parapsilosis*. We also found that the secretion of IL-1β was dependent on K+-efflux and phagocytosis, and *C. albicans* cells were phagocytosed more rapidly by THP-1 macrophages than *C. parapsilosis* cells. Taken together, our results suggest that similar mechanisms are involved in inflammasome activation upon *C. parapsilosis* and *C. albicans* infection, but the slower production of “danger signals” leads to delayed inflammasome activation following *C. parapsilosis* stimulation. (we published these results in: Tóth et al. SCI REP 7, 2017).

A murine neonate model of *C. parapsilosis* systemic infection

In recent years, several studies have attempted to establish reliable animal models affecting the neonate's immune response to infections. In this project, we developed a neonatal model of disseminated candidiasis for *C. parapsilosis* infections (we published these results in: Pérez-García LA, Csonka K. et al. FRONT MICROBIOL 7 2016) In this research, we used a retro-orbital intravenous injection of 24 h old Balb/C mouse pups. We have previously shown that N-linked mannosyl residues play a significant role in the virulence of *C. parapsilosis* in *in vivo* mouse model. The neonatal mouse model was successfully used to study how this cell wall component effects the virulence of this pathogen; neonatal mice infected with the null mutant *och1*Δ/Δ *C. parapsilosis* showed significantly decreased fungal burdens in the spleen, kidneys, and liver at 2 or 7 days of post-infection compared to the wild-type strain. CFU data from the brain showed similar fungal loads in case of the wild-type, the *Cpoch1*Δ/Δ and the *OCH1* overexpression mutant strain infection. Interestingly, no significant clearance was detectable in the brain of the neonate pups at 7 days post-infection. Using histopathology analysis, we identified fungal cells of the null mutant Cpoch1Δ/Δ, the OCH1 overexpression and the wild-type *C. parapsilosis* strains in

brain, kidney, liver and spleen tissues 2 days after the infection. 7 days post-infection no detectable fungi were found in the harvested organs. This finding is in line with the CFU data, which showed decrease loads of fungal cells over time, indicating that the neonatal mice were able to effectively control the fungus in the spleen, kidney and liver. We also analyzed the cytokine response to *C. parapsilosis* infection from tissue homogenates (liver and kidney). At 2 days post infection, the *OCH1* overexpression mutant strain infected mice showed significantly higher production of TNFα and KC chemokine compared to the wild-type and the null mutant strains. At this time point, no detectable IL-1β was measured from the kidney. In the case of liver, we observed the highest production of TNFα and KC chemokine at 2 days post-infection. In contrast, increment in the IL-1β cytokine levels was observed at 7 days post-infection in this organ (we published these results in: Csonka et al. FRONT MICROBIOL 8, 2017).

The role of Dectin-1 receptor in the recognition of *Candida parapsilosis*

During infection, fungal cells are recognized via their outer cell wall by numerous pattern recognition receptors (PRRs) of the innate immune system. Antifungal immunity appears to be mediated primarily by members of the C-type lectin receptor (CLR) family. C-type lectin receptors (CLRs), play critical roles in host defense against *C. albicans* infections. For example, Dectin-1 recognizes β-1,3-glucans in fungal cell walls. *In vivo* studies in mice demonstrated the essential role of Dectin-1 in the protective immune response during systemic candidiasis: during *C. albicans* infection, Dectin-1 $^{-/-}$ mice fail to mount a protective inflammatory response, leading to an inability to control fungal growth and increased mortality, although this can be fungal strain dependent.

Previously, it has been shown that host defense mechanisms vary towards different *Candida* species. To date, our understanding of *C. parapsilosis* induced immune responses is limited, especially considering the role of CLRs in recognition. Therefore, in this project, we also aimed to investigate the role of Dectin-1 in *C. parapsilosis* recognition using an *in vivo* model of systemic candidiasis.

To examine if Dectin-1 plays a role in *C. parapsilosis* recognition, we intravenously (IV) infected wild-type and Dectin-1$^{-/-}$ mice with the *C. parapsilosis* CLIB 214 strain (2×10^7 cells/mouse). When comparing the fungal burden of the kidneys, liver and the spleen of wild type and Dectin-1 deficient mice, we found no significant differences neither 1, 3 nor 7 days after the infection (Fig. 1). These data suggests, that Dectin-1 does not influence the recognition of *C. parapsilosis* cells in the corresponding organs. Interestingly however, Dectin-1 might influence fungal recognition in the brain as significantly higher CFUs were recovered in the Dectin-1$^{-/-}$ mice compared to the wild type (Fig. 1).

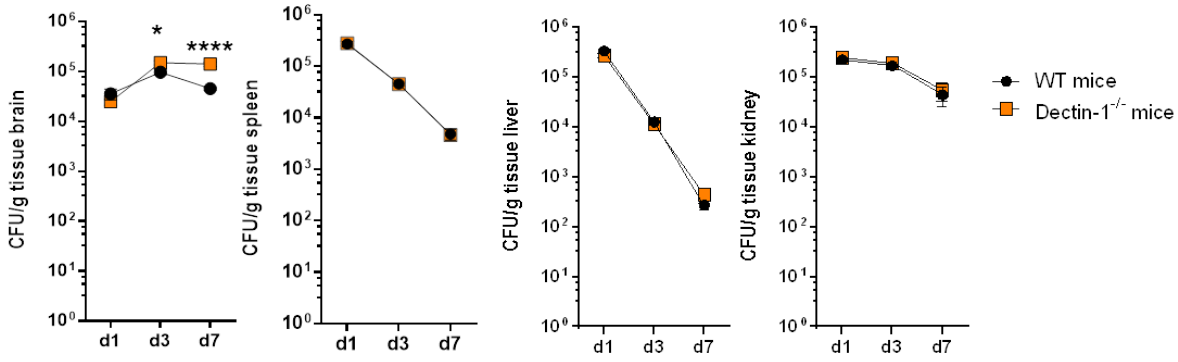

Figure 1. Fungal burden of the brain, kidneys, liver and the spleen of wild type and Dectin-1 deficient mice infected with *C. parapsilosis*.

Next, we aimed to examine the immune response towards a cell wall mutant *C. parapsilosis* strain (*och1*Δ/Δ) that was previously constructed in our laboratory. This mutant lacks the *N*-linked mannan outer chain in its cell wall. It has been shown that the *och1*Δ/Δ mutant's virulence significantly attenuated in a BALB/c mouse model of systemic infection (Pérez-García Luis A, Csonka K, Gacser A, Mora-Montes Role of Protein Glycosylation in *Candida parapsilosis* Cell Wall Integrity and Host Interaction Front. Microbiol. 2016.) During this project, our aim was to get a deeper insight into how the *N*-mannosilation in the cell wall effects the *C. parapsilosis*-induced immune response.

The *och1*Δ/Δ showed significantly reduced fungal loads in the kidney, liver and spleen of wt mice compared to the wild type *C. parapsilosis* strain. The only exception was the brain, where a higher number of colonies were detected at 1 and 3 days post-infection after challenging with the cell wall mutant (Fig.2).

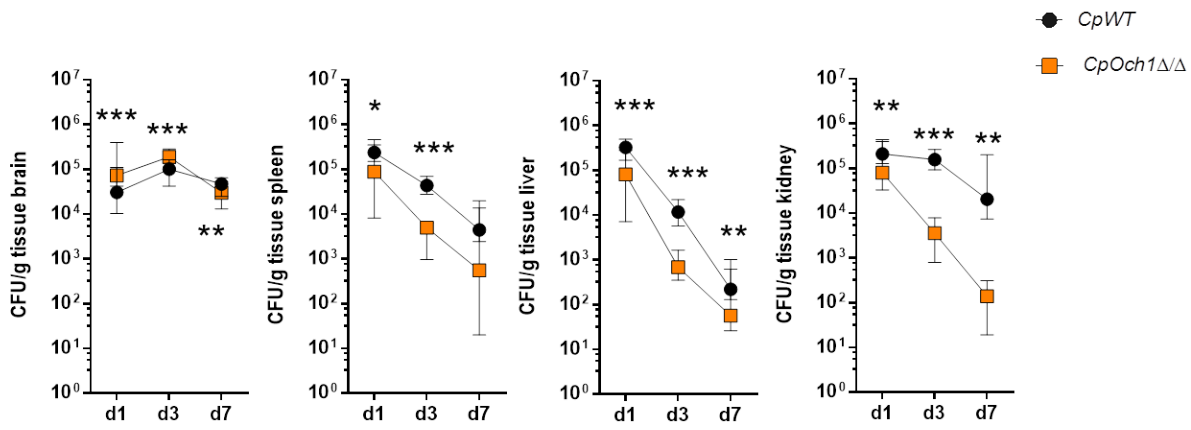

Figure 2. Fungal burden of the brain, kidneys, liver and the spleen of wild type mice infected with *C. parapsilosis* wild-type and *och1*Δ/Δ mutant strains.

In the wt mice model, the *och1*Δ/Δ mutant strain stimulated more effective recruitment of $Ly6G^{+}Cd11b^{+}$neutrophils, $Cd11b^{+}$ macrophages and $Cd11c^{+}$ dendritic

cells in the kidney compared to the reference strain (Fig. 3). These findings correlate with the reduced number of *och1*Δ/Δ colonies, as more effective immune activation resulted in a faster clearance of the mutant strain.

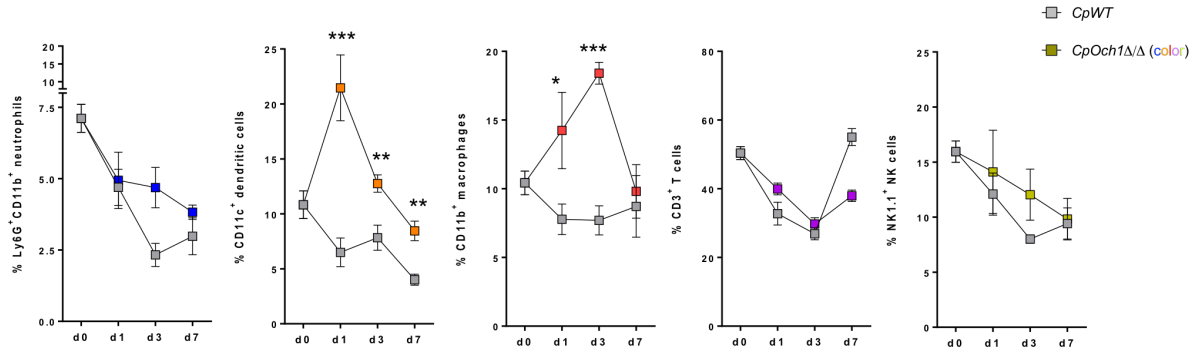

Figure 3. Recruitment of $Ly6G^{+}Cd11b^{+}$neutrophils, $Cd11b^{+}$ macrophages and $Cd11c^{+}$ dendritic cells in the kidney upon infection with the CpWT and *Cp och1*Δ/Δ strains.

Furthermore, cytokine analysis from the kidneys organ showed lower induction of TNFα, IL-1β, IL-6, and INF-γ levels after the *och1*Δ/Δ-challenge compared to the WT strain (Fig. 4). These data suggest, that the loss of the *N*-mannan layer in the cell wall induced lower activation of inflammatory cytokines in the host.

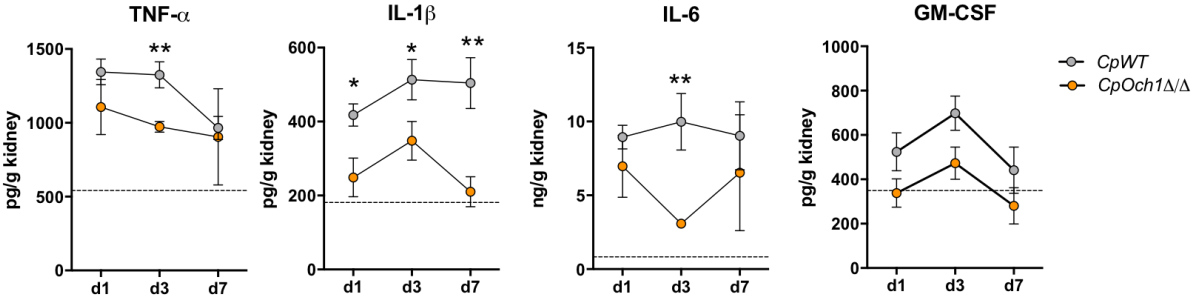

Figure 4. Induction of TNFα, IL-1β, IL-6, and INF-γ levels in the kidney, after challenge with *Cpoch1*Δ/Δ and CpWT strains.

Next, we examined, whether the decreased virulence of the mutant is dependent on the recognition of β-glycan by the Dectin-1 receptor. Therefore, we infected the wt and the Dectin-$1^{-/-}$ mice with the *och1*Δ/Δ strain. The absence of Dectin-1 did not influence fungal clearance in the spleen, kidney and liver after infection with the *och1*Δ/Δ strain (Fig. 5). Interestingly, in the brain of the Dectin-$1^{-/-}$ mice higher colonies of *och1*Δ/Δ were detected compared to wt mice.

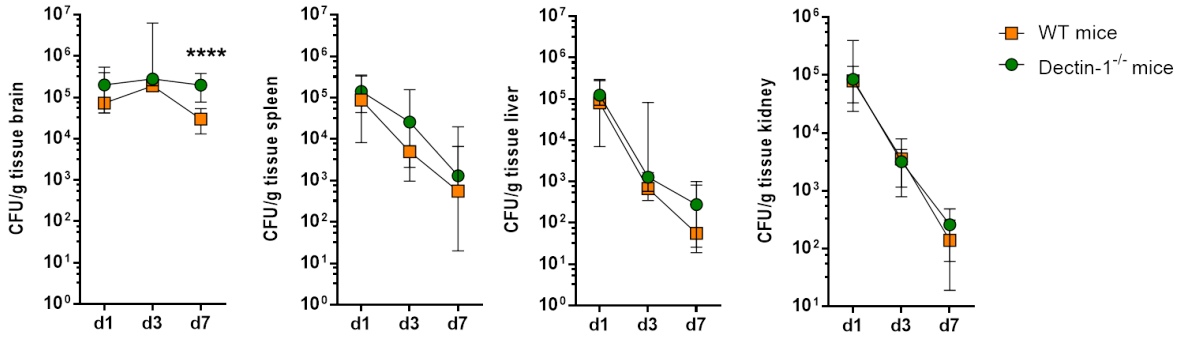

Figure 5. Fungal clearance in the spleen, kidney and liver of WT and Dectin-1 deficient mice after infection with the *och1*Δ/Δ strain.

However, we detected more efficient neutrophil activation and less effective dendritic cell infiltration in the kidney of Dectin-1$^{-/-}$ mice, but the change in the composition of these cells did not modulate the elimination of *och1*Δ/Δ cells from the kidney (Fig. 6).

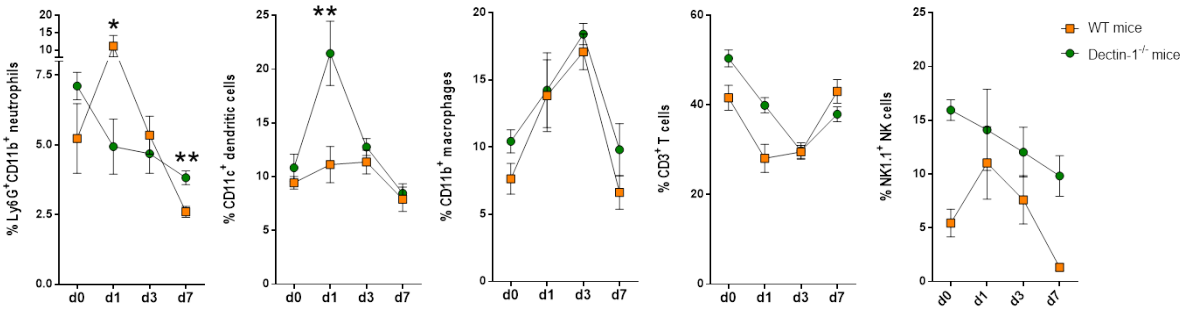

Figure 6. Netrophil, dendritic cell, macrophage, T cell and NK cell infiltration in the kidney of Dectin-1 deficient mice following *Cpoch1*Δ/Δ challenge.

Together these results lead us to the conclusion that, unlike in *C. albicans*, Dectin-1 is not involved in anti-*C. parapsilosis* immune responses. Furthermore, results with the *och1*Δ/Δ strain suggest that, the β-glucan in the cell wall and its recognition by the Dectin-1 receptor plays redundant role in the host defense against this pathogen.

The mini host: *Drosophila melanogaster*

The innate immune response against *Candida* species is widely studied in *Drosophila melanogaster* by using both receptor and signaling mutant strains. As an alternative, invertebrate model, *D. melanogaster* is a suitable tool to study the specific steps of innate immune responses against *C. albicans* and *C. glabrata:* fruit flies deficient in Toll signaling are extremely sensitive to fungal infections. This model is also suitable for the identification of novel virulence-associated genes in *Candida* species and also to investigate the role of antimicrobial peptides against *C. albicans*.

It has been shown, that in *D. melanogaster* the Toll pathway mediates both antifungal and antibacterial (Gram-positive) responses. A second NF-κB–like pathway (immune deficiency (IMD) is responsible for the immune protection against Gram-negative bacteria. The Toll receptor is activated by the Spätzle cytokine. During its proteolytic maturation lead by the Spätzle-processing enzyme, the Spätzle cytokine turns into an active ligand. Gram-negative binding protein 3 (GNBP3) binds to the fungal β-(1,3)-glucan and activates a proteolytic cascade that ultimately matures Spätzle through the Spätzle-processing enzyme. Furthermore, the host protease Persephone (psh) is able to sense pathogen associated molecular patterns and microbial enzymes and subsequently triggers the Toll pathway through the transcription factor Dorsal-related immunity factor (DIF).

In this study, we used *D. melanogaster* as a model to investigate the pathogenicity of *C. parapsilosis*. First, we infected the wt, the MyD88$^{-/-}$ and GNBP3$^{-/-}$ flies with *C. albicans*, *C. glabrata* and *C. parapsilosis* to identify the range of virulence of the *Candida* species. During the monitoring of survival, we found that, MyD88 and GNBP3 receptor mutation in flies led to sensitivity to *C. parapsilosis* infection. Furthermore, we observed that both MyD88$^{-/-}$ and GNBP3$^{-/-}$ flies were most susceptible to *C. albicans*, followed by *C. parapsilosis* and *C. glabrata* (Fig. 7). Therefore, we observed that *C. parapsilosis* cells are recognized by the fungal PRR of *Drosophila* and induced host response both by the transcription factor MyD88 and Toll-independent GNBP3 receptor.

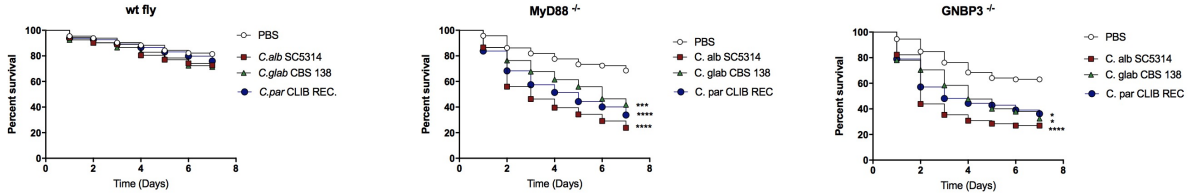

Figure 7. Susceptibility of WT, MyD88$^{-/-}$ and GNBP3$^{-/-}$ flies to *C. albicans*, *C. parapsilosis* and *C. glabrata.*

To determine if *Drosophila* could be used for *C. parapsilosis* virulence studies, the wt, MyD88$^{-/-}$, GNBP3$^{-/-}$ and the psh$^{-/-}$ flies were infected with the less virulent *och1*Δ/Δ *C. parapsilosis* strain and survival was compared to those injected with PBS and the reference *C. parapsilosis* strain. Although, the MyD88$^{-/-}$ and GNBP3$^{-/-}$ flies showed susceptibility to the *och1*Δ/Δ infection, the percentage of the survived flies were significantly higher compared to the wt *C. parapsilosis* infected group. The psh$^{-/-}$ mutant flies showed no susceptibility, as all flies were viable after injection with the wt or the *och1*Δ/Δ strain (Fig. 8).

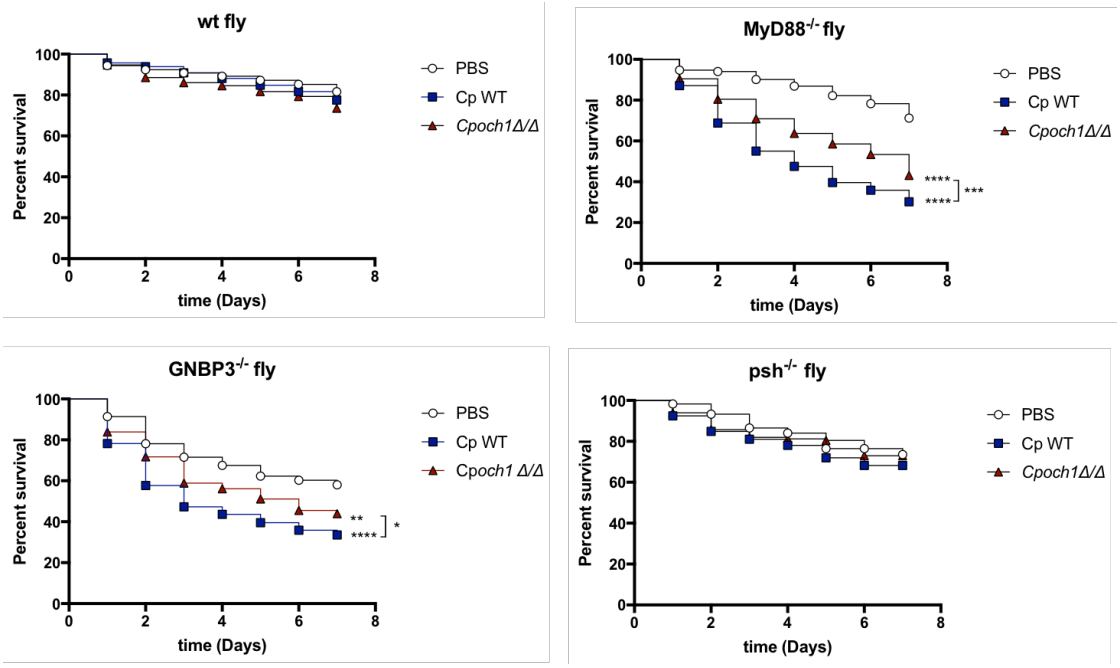

Figure 8. Susceptibility of WT, MyD88$^{-/-}$, GNBP3$^{-/-}$ and psh$^{-/-}$ flies to the *C. parapsilosis* wild-type and *OCH1* deficient strains.

The range of colonization by the *Candida* strains further supported the decreased virulence properties of *och1*Δ/Δ. The wt, MyD88$^{-/-}$, GNBP3$^{-/-}$ and psh$^{-/-}$ flies were able to control the growth of the *N*-mannan mutant strain more effectively than the wt *C. parapsilosis* strain (Fig. 9.).

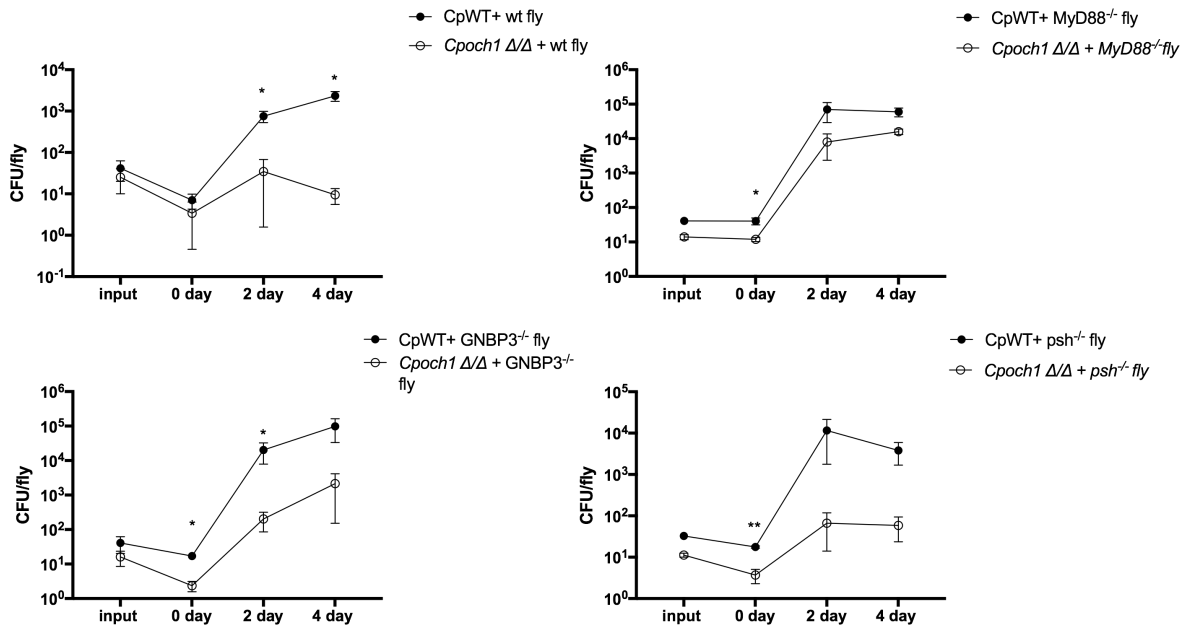

Figure 9. Fungal burden measured in colony forming units from wt, MyD88$^{-/-}$, GNBP3$^{-/-}$ and psh$^{-/-}$ flies after *Cp*WT and *Cpoch1*Δ/Δ challenge.

Taken together, our results demonstrate that the *Drosophila* Toll pathway restrains *C. parapsilosis* proliferation as the Toll pathway mutant MyD88 flies were more susceptible to the injected fungal cells. Previously, it has been shown, that both the Gram-negative binding protein 3 pattern recognition receptor and the Persephone protease-dependent detection pathways are required for Toll pathway activation during fungal infections. However, we found that solely $GNBP3^{-/-}$, and not psh mutants, are susceptible to the *C. parapsilosis* infection. During this study we demonstrated that using *D. melanogaster* as an alternative, invertebrate model is appropriate for the investigation of fungal virulence, as we were able to effectively reproduce our previous results: the *N*-mannosylation deficient *och1*Δ/Δ strain has decreased virulence *in vivo*.

References:

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