1. Extended Data

Figure #	Figure title One sentence only	Filename This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: *Smith_ED_Fig1.jpg*	Figure Legend If you are citing a reference for the first time in these legends, please include all new references in the Online Methods References section, and carry on the numbering from the main References section of the paper.
Extended Data Fig. 1	Transcriptomic profiling PBMCs stimulated with live *C. albicans* or *C. auris* and respective cell wall components β-glucans and mannans for 4 and 24 hours.	Ext_data_figure1.eps	**a**, Principal component analysis (PCA) performed on normalized count data (normTransform, DESeq2) demonstrates the main component introducing variance in the dataset is time (40%), as indicated by a clear split between the early 4-hour host induced response (left, triangle) and the late 24-hour response (right, circle). To a lower extent (15%), the second component introducing variance appears to be inherent to the stimulus (color). **b,** At 4 hours, PCA reveals a clear donor clustering (shape) irrespective of stimulus (color), indicating the main variance in the early host response reflects inter-individual differences (left). PCA on the late response, 24 hours, is predominantly influenced by the respective stimulus (38%, color), and to a lower extent by the donors (19%, shape), indicated by the scattering of stimuli together with a rough clustering amongst donors. **c**, Pathway enrichment plot displaying the top 20 enriched pathways for both *C. albicans* live and *C. auris* live (color) at 24 hours. Enrichment determined using Consensus PathDB, including pathways as defined by KEGG and Reactome (shape), considering a p-adjusted value < 0.01 (indicated as 'q-value') significant. Size of the geometric points indicates the amount of DEG in relation to the pathways' size. The exact q values and the data used to make this figure can be found in Source Data Extended Data Fig.1.
Extended Data Fig. 2	Comparative LDH secretion,	Ext_data_figure2.ep	**a**, Assessment of *Candida* induced cell death of PBMCs after 24 hours

	LDH cytokine gene expression and phagocytosis dynamics between *C. albicans* and *C. auris*.	S	stimulation without (RPMI; negative control) or with *C. albicans,* several *C. auris* strains originating from all five geographical clades or a positive control (dead cells). Lactate dehydrogenase (LDH) was detected as measure of cell death (Mean ± SEM, n=6, pooled from two independent experiments). **b**, Log_2Fold Change (Log_2FC) of *IL-6, IL-1β,* and *IL-1RN* (encoding for IL-1Ra) gene expression in PBMCs from 3 donors stimulated for 24 hours with *C. albicans* (1006110) and *C. auris* (KCTC17810, clade II) and their respective cell wall components, β-glucans (left) and mannans (right). Graphs represent Log_2FC from DEG analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 1-way ANOVA with correction for multiple comparison. **c**, The BMDM phagocytic capacity of Thimerosal-fixed *C. albicans* or *C. auris* strains in the course of 3-hours. BMDM engulfment depicted as the percentage of macrophages having phagocytosed at least one fungal cell (left), and the phagocytic index, here considered as the number of fungal cells engulfed per 100 macrophages (right); graphs represent mean, n=9, pooled from at least two independent experiments. **d**, BMDM phagocytic capacity of Thimerosal-fixed *C. albicans* or *C. auris* strains after 1 hour. Engulfment is depicted as the percentage of macrophages having phagocytosed at least one fungal cell; graphs represent mean ± SEM, n = 9, pooled from at least two independent experiments. **e**, BMDM phagocytic capacity of live *C. albicans* or *C. auris* strains after 1 hour. Engulfment is depicted as the percentage of macrophages having phagocytosed at least one fungal cell. Graphs represent mean ± SEM, n = 9 (n=7 for *C. auris* 10051893), pooled from at least two independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **d** 1-way ANOVA with a Holm-Sidak's multiple comparison test, **e** Kruskal Wallis test with two-sided Dunn's multiple comparison. **f**, Distribution of phagocytosed Thimerosal-fixed fungal cells per macrophage in a 3-hour period, n≥100 observations per condition. Data used to make this figure can be found in Source Data

			Extended Data Fig. 2.
Extended Data Fig. 3	Relative *C. auris* induced ROS production and heat-sensitivity of the cell wall components responsible for the *C. auris* induced cytokine production.	Ext_data_figure3.eps	**a**, Neutrophil ROS release after 1-hour stimulation without (RPMI; negative control) or with heat-killed *C. albicans*, *C. auris* strains or zymosan (positive control), depicted in relative light units (RLU) either as time-course (left) or as area under the curve (AUC, right), n=9. **b**, PBMC ROS release after 1-hour stimulation without (RPMI; negative control) or with heat-killed *C. albicans*, *C. auris* strains or zymosan (positive control), depicted in RLU either as time-course (left) or as AUC (right), n=6. **c**, TNF-α, IL-6, IL-1β, and IL-1Ra levels in the supernatant of PBMCs after stimulation without (RPMI; negative control) or with heat-killed *C. albicans* and *C. auris* from all five geographical clades for 24 hours, n=8. **d**, PBMC production of cytokines IFN-γ (n=10; n=7 for *C. auris* 10051895), IL-10 (n=6), IL-17 (n=6), and IL-22 (n=14; n=6 for *C. auris* 10051893; n=11 for *C. auris* 10051895) after stimulation without (RPMI; negative control) or with heat-killed *C. albicans* and *C. auris* for 7 days. Graphs represent mean ± SEM, data are pooled from at least two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p = 0.001$, **a-b** Time curves (left panels) were assessed for statistical differences between *C. auris* strains and *C. albicans* by a two-way ANOVA, Area Under curve (AUC) means (right panels) were compared using the two-sided Wilcoxon signed rank test, **c-d** two-sided Wilcoxon matched pairs signed-rank test comparing respective *C. auris* strains with *C. albicans* as control or reference species. Data used to make this figure can be found in Source Data Extended Data Fig. 3.
Extended Data Fig. 4	Transcriptional changes induced by purified cell wall components and their respective exposure on *C. albicans* and *C. auris*	Ext_data_figure4.eps	. **a**, Heatmap displaying the Log_2Fold change (color scale) of the top 50 DEG of *C. albicans* live, for both *Candida* species and their cell wall components, β-glucan and mannan, at 4 hour (left panel) and 24 hours (right panel). **b**, Flow cytometry plot based on forward scatter component (FSC) and side scatter component (SSC), demonstrating *C.*

	surface.		*auris* strains are slightly smaller and of higher complexity than *C. albicans.* **c**, Flow cytometry-based comparison of cell wall components of *C. albicans* and *C. auris* strains. Mean fluorescent intensity (MFI) of thimerosal-fixed *Candida* cells stained for Fc-Dectin-1, a marker for β-glucan (left), and ConA, a marker for mannans (right). Graphs represent mean ± SEM of the 3 means, each performed with three replicates in three independent measurements, * $p < 0.05$, Kruskall Wallis test with two-sided Dunn's multiple comparison test was performed comparing the respective *C. auris* strains with the two *C. albicans* reference strains. Data used to make this figure can be found in Source Data Extended Data Fig. 4.
Extended Data Fig. 5	Evaluation of cytokine production upon *C. albicans* and *C. auris* mannan stimulation.	Ext_data_figure5.eps	**a**, PBMC production of cytokines TNF-α, IL-6, IL-1β, and IL-1Ra after 24 hours stimulation without (RPMI; negative control) or with purified mannans from *C. albicans* and *C. auris* strains in the presence of 10% heat-inactivated human serum, n=7. **b**, PBMC production of cytokines IFN-γ (n=6), IL-17 (n=9), and IL-22 (n=9) after 7 days hours stimulation without (RPMI; negative control) or with purified mannans from *C. albicans* and *C. auris* strains in the presence of 10% human serum. Graphs represent mean ± SEM, data pooled from at least two independent experiments. * $p < 0.05$, two-sided Wilcoxon matched pairs signed-rank test, comparing respective *C. auris* strains with *C. albicans* as control or reference species. Data used to make this figure can be found in Source Data Extended Data Fig. 5.
Extended Data Fig. 6	Cytokine levels in plasma and organ homogenates from *C.albicans* and *C. auris*-infected mice.	Ext_data_figure6.jpg	. **a**, IL-6 production in plasma and supernatants from liver homogenates. **b**, IFN-γ production in supernatants from kidney and spleen homogenates. **c-e**, IL-1β (c), IL-17 (d), and IL-10 (e) production in plasma and supernatants from liver, kidney, and spleen homogenates. Mice have been infected i.v. with $1x10^6$ CFU of *C. albicans* or *C. auris.* Graphs represent mean ± SEM, n=6 per group per time-point pooled from two independent experiments. Data used to make this figure can be found in Source Data Extended Data Fig. 6.

Extended Data Fig. 7	Applied gating strategies across flow cytometry experiments.	Ext_data_figure7.eps	**a**, Gating strategy for FITC-labelled *Candida* in PBMCs (linked to Figure 2b). All events were plotted based on forward scatter (FS) and side scatter (SS) characteristics. In the upper plot (2.1) the region of cells positive for FITC-*Candida* was highlighted (green gate) while in the bottom plot (2.2) CD14 positive cells are represented (red gate) gated within the total PBMCs population (1). Within the CD14+ cells selection, the amount of phagocytosed FITC positive *Candida* was examined by plotting (3) the FITC signal against the CD14-PB450 signal (blue gate) and the percentage of cells and mean fluorescent intensity (MFI) were used for analysis. **b**, Gating strategy for Thimerosal-fixed *Candida* cells stained for either β-glucan using Fc-Dectin-1 or ConA as marker for mannans (linked to Figures S4c).

2. Supplementary Information:

A. Flat Files

Item	Present?	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. The extension must be .pdf	A brief, numerical description of file contents. i.e.: *Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.*
Supplementary Information	Yes	SI_Supplementary_tables.pdf	Supplementary Tables 1-4

Reporting Summary	Yes	Complete_Bruno_reporting_summary.pdf

B. Additional Supplementary Files

Type	Number If there are multiple files of the same type this should be the numerical indicator. i.e. "1" for Video 1, "2" for Video 2, etc.	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: *Smith_ Supplementary_Video_1.mov*	Legend or Descriptive Caption Describe the contents of the file
Supplementary Video	1	S1.mp4	*C. auris* is able to multiply within phagosomes.
Supplementary Video	2	S2.mp4	*C. auris* accumulates in high numbers within macrophages and does not induce macrophage lysis.
Supplementary Video	3	S3.mp4	*C. auris* cells are taken up extensively into a subpopulation of macrophages.
Supplementary Video	4	S4.mp4	Phagocytosis of *C. albicans* SC5314, macrophage lysis after 3 hours.

3. Source Data

Figure	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: *Smith_SourceData_Fig1.xls*, or *Smith_ Unmodified_Gels_Fig1.pdf*	Data description i.e.: Unprocessed Western Blots and/or gels, Statistical Source Data, etc.
Source Data Fig. 1	SD_Main_Fig1.xlsx	Supporting data for Fig. 1
Source Data Fig. 2	SD_Main_Fig2.xlsx	Supporting data for Fig. 2
Source Data Fig. 3	SD_Main_Fig3.xlsx	Supporting data for Fig. 3
Source Data Fig. 5	SD_Main_Fig5.xlsx	Supporting data for Fig. 5
Source Data Fig. 6	SD_Main_Fig6.xlsx	Supporting data for Fig. 6
Source Data Extended Data Fig. 1	SD_Ext_Data_Fig1.xlsx	Supporting data for Extended Data Fig. 1.
Source Data Extended Data Fig. 2	SD_Ext_Data_Fig2.xlsx	Supporting data for Extended Data Fig. 2.
Source Data Extended Data Fig. 3	SD_Ext_Data_Fig3.xlsx	Supporting data for Extended Data Fig. 3.
Source Data Extended Data Fig. 4	SD_Ext_Data_Fig4.xlsx	Supporting data for Extended Data Fig. 4.
Source Data Extended Data Fig. 5	SD_Ext_Data_Fig5.xlsx	Supporting data for Extended Data Fig. 5.
Source Data Extended Data Fig. 6	SD_Ext_Data_Fig6.xlsx	Supporting data for Extended Data Fig. 6.

Host immune response against the emerging fungal pathogen *Candida auris*: transcriptional and functional insights

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ABSTRACT (MAX 150 words)

Candida auris is amongst the most important emerging fungal pathogens, yet mechanistic insights in its immune recognition and control are lacking. Here, we integrate transcriptional and functional immune cell profiling to uncover innate anti-*C. auris* defense mechanisms. *C. auris* induces a specific transcriptome in human mononuclear cells, a stronger cytokine response compared to *C. albicans*, but a lower macrophage lysis capacity. *C. auris*-induced innate immune activation is mediated through recognition of C-type lectin receptors, mainly elicited by structurally unique *C. auris* mannoproteins. In *in-vivo* experimental models of disseminated candidiasis, *C. auris* was less virulent than *C. albicans*. Collectively, these results demonstrate that *C. auris* is a strong inducer of innate host defense and identify possible targets for adjuvant immunotherapy.

INTRODUCTION

Candida auris is an important emerging fungal pathogen that was first described in 2009, and has, since then, spread across six continents as a causative microorganism of hospital-acquired infections [1]. For several reasons, *C. auris* is among the most challenging of emerging human pathogens identified in the last decade. It is highly resistant to many of the commonly used antifungal drugs [1] and, within a few years, it has rapidly spread worldwide [2,3] through the nearly simultaneous (but independent) emergence of four distinct phylogeographical clades [4]. Recently, a potential fifth clade has been described in Iran [5]. Every major clade except for clades II and V has been linked to outbreaks with invasive infection [6]. On the contrary, clade II generally shows antifungal susceptibility and has a propensity for ear infections. Similar to clade II, clade V isolate was recovered from ear. Clade III is associated with bloodstream infections and, together with clade II, tends to form large cell aggregates [7]. This has been linked with a reduced virulence in a *Galleria mellonella* infection model [8].

C. auris poses difficulties in routine microbiological identification [9,10] and it is challenging to eradicate in healthcare settings [11-14]. This is due to its strong capacity to colonize skin, its transmittance *via* patient-to-patient route or contaminated fomites, and its high survival capacity on plastic surfaces and in the hospital environment [15]. The risk factors for *C. auris* infections are generally similar to those for other types of *Candida* infections, such as prolonged hospitalization, use of central venous catheters, abdominal surgery and exposure to broad-spectrum antibiotics or antifungals [16]. However, due to its acquired resistance to many antifungal drugs, the overall crude mortality rate of *C. auris* candidemia is high, ranging from 30% to 60%, with infections typically occurring several weeks (10-50 days) after admission [4,12,17,18]. Echinocandins are currently recommended by the CDC as empiric treatment of *C. auris* infections, although resistance has been reported. Several new therapeutic alternatives, such as fosmanogepix [19,20], ibrexafungerp [21-23] and rezafungin [24,25] are currently under clinical investigation.

Considering the importance of *C. auris* as an emerging human pathogen, it is imperative to understand the host defense mechanisms. This is particularly true given the high resistance of this

fungus to anti-mycotic drugs, which makes it a prime candidate for the development of host-directed therapy (i.e. immunotherapy). However, almost nothing is known regarding the host immune response against *C. auris*. Host defense against *Candida* species is dependent on a finely tuned interplay of innate and adaptive immune responses. A first physical barrier consists of the skin and mucosa. The second barrier, represented by the innate immune system, is largely dependent on the recognition of evolutionarily conserved fungal cell wall components (pathogen-associated molecular patterns, PAMPs) by innate immune cells such as monocytes, macrophages and neutrophils. In turn, the release of proinflammatory cytokines, combined with antigen-presentation activity of myeloid cells, is crucial for shaping the adaptive immunity, representing a third, long-term barrier against fungal infection [26].

The *Candida* cell wall is divided into an outer layer of highly mannosylated proteins (mannoproteins) and an inner layer, mainly comprised of β(1→3) and β(1→6)-glucans and chitin [27]. These PAMPs are recognized by various pattern recognition receptor (PRRs) on the surface of immune cells: C-type lectin receptors (CLRs) such as Dectin-1, Dectin-2, macrophage mannose receptor (MMR), Mincle (macrophage-inducible C-type lectin), DC-SIGN (dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin) and Toll-like Receptors (TLRs), especially TLR2 and TLR4 [28]. Coordinated engagement of PRRs results in the activation of innate immune effector mechanisms such as phagocytosis, reactive oxygen species (ROS) release and production of pro- and anti-inflammatory cytokines. In turn, together with the antigen-presentation activity of myeloid cells, the release of pro-inflammatory cytokines shapes the adaptive immune response [26].

While the antifungal host defense mechanisms have been extensively studied for *C. albicans,* little is known about the host immune response against *C. auris*. Almost all multi-drug resistant *C. auris* strains are susceptible to killing by the salivary antimicrobial peptide Histatin 5 (Hst-5) [29], while Johnson and colleagues showed that neutrophil recruitment and formation of neutrophils extracellular traps (NETs) were lower for *C. auris* than *C. albicans* [30]. Recently it was reported that *C. albicans, C. tropicalis, C. guilliermondii, C. krusei* and *C. auris* differentially stimulate cytokine

production in peripheral blood mononuclear cells (PBMCs) [31], but little is known regarding the particularities of these responses and the mechanisms mediating them. Considering the knowledge gap in our understanding of anti-*C. auris* host defense mechanisms, we set out to comprehensively assess the mechanisms through which innate immune cells recognize *C. auris,* initiate innate antifungal immune responses, and protect the host against *C. auris* infection. This mechanistic insight into *C. auris* host interactions is instrumental for the development of novel host-directed approaches for the treatment of severe *C. auris* infections and, thereby, improve patient outcomes.

RESULTS

Common and specific transcriptome signatures induced by *C. albicans* and *C. auris* in human immune cells

To gain a broad overview of the host immune response against *C. auris*, RNA sequencing was performed on PBMCs from three healthy donors that were exposed to either live *C. albicans* or *C. auris* for 4 or 24 hours. Due to the high genome-wide nucleotide identity across *C. auris* clades I-IV (98.7%) [32], the analysis of the *C. auris* reference strain KCTC171810 (clade II) is expected to provide valuable insight into generic *C. auris* induced host responses. This *C. auris* reference strain was compared to *C. albicans* 10061110, which, to date, remains the most common cause of mucosal and systemic candidiasis [33].

Principle component analysis (PCA) of the normalized PBMC RNAseq dataset revealed that the majority of the variance in the experiment as a whole was time-dependent, as demonstrated by a clear separation of the 4-hour and 24-hour stimulation time points (Extended Data Fig. 1a). Comparison of the stimulated and non-stimulated samples at the 4-hour time point indicates that a limited response was induced early on. Moreover, stimulus clustering at 4 hours suggests this short-term response was similar in *C. auris* and *C. albicans* (Extended Data Fig. 1a). At the 4-hour time point, clustering of the donors irrespective of stimulus indicates that inter-individual differences underpin

the observed variance (Extended Data Fig. 1b, left). As PBMC donors were considered biological replicates, comparison of the average PBMC response to their control condition revealed significant overlap in the 4-hour host response between *C. albicans* and *C. auris*. With 71 differentially expressed genes (DEG; fold change ≥2 and p-adjusted value <0.01) upregulated by both *Candida* species, the respective overlap ranges from 67% of the total number of DEG for *C. albicans* (71 / 109) to 95% of the total number of DEG for *C. auris* (71 / 79).

In contrast, at 24 hours, the response is primarily stimulus-driven (Extended Data Fig. 1b, right), as indicated by the scattering of donor responses dependent on pathogen exposure. After 24h of stimulation, the common *C. auris* and *C. albicans*-induced host response increased to 243 DEG (**Figure 1a**), in turn accounting for 55% of the total number of DEG for *C. albicans* (243 / 442) and 50% of the total number of DEG for *C. auris* (243 / 484). This late shared response between both *Candida* species was consistent with the observation that the 24-hour induced PBMC transcriptomes were more stimulus-specific (Extended Data Fig. 1b). Pathway enrichment analysis revealed that the 4-hour *Candida* intrinsic response was delineated by a common activation of the CC and CXC chemokines (Supplementary Table 1). In contrast, the 24-hour PBMC transcriptomic response was characterized by a broader upregulation of chemokines, interleukins (IL), tumor necrosis factor and their receptors (**Figure 1a** and Supplementary Table 2).

The substantial activation of glucose, fructose and mannose metabolism was unique to the *C. albicans*-induced transcriptional response of PBMCs at 24 hours. Conversely, the DEG that were more strongly induced upon PBMC exposure to *C. auris* appeared to be linked to type I and II interferons, as well as antiviral mechanisms triggered via IFN-stimulated genes, including the ISG15 immune mechanisms (**Figure 1a** and Supplementary Table 2). Collectively, these data show that *C. albicans* and *C. auris* are potent activators of the host immune system, and they are not only able to activate common transcriptional responses, but also induce pathways specific to each pathogen.

C. auris is a more potent inducer of host immune response compared to *C. albicans*

One-third of the top 15 enriched pathways, based on those DEG that were unique for *C. auris,* were also found to be enriched in the common *Candida* response (**Figure 1a**). This was not the case for *C. albicans*, indicating that *C. auris* has the ability to upregulate more genes in these pathways compared to *C. albicans*. Most pronounced within these unique DEG were distinct interleukins such as *IL1RN* (encoding for IL-1Ra), *IL10*, *IL19*, *IL26* and *IL27*, as well as interferon (IFN) associated genes, e.g. *STAT2, DDX58, EIF2AK2, OAS2, OAS3, IFIT2, IFIT3, IFIT35* and *IFITM1* (Supplementary Table 2). Furthermore, DEG were more potently induced in response to *C. auris* than *C. albicans* (Student's T-test, p-value of 0.003). An additional pathway enrichment analysis on all the upregulated DEG confirmed that the total number of DEG for mutually enriched pathways was higher when the PBMCs were stimulated with *C. auris* rather than with *C. albicans* (Extended Data Fig. 1c). Collectively, the broader and stronger induction of DEG by *C. auris* resulted in higher enrichment scores (q-value) for corresponding pathways in comparison to *C. albicans*, suggesting that *C. auris* is a more potent trigger of the host response.

With the transcriptomic analysis suggesting cytokine signaling to be at the core of the host response, we aimed to verify these observations at the protein level. For this, the cytokine production ability of PBMCs isolated from healthy volunteers was assessed following 24-hour exposure to three different live clinical isolates for each *Candida* species, all cultured under similar conditions. As a measure of *Candida* induced cytotoxicity, the detection of lactate dehydrogenase (LDH) release revealed that PBMC viability after 24 hours was not impacted (Extended Data Fig. 2a). With the exception of the anti-inflammatory cytokine IL-1Ra, PBMCs exposed for 24 hours to both clinical isolates of *C. auris* produced significantly higher amounts of the pro-inflammatory cytokines TNFα, IL-6, and IL-1β compared to the *C. albicans*-stimulated PBMCs (**Figure 1b**). Moreover, to test whether this observation is clade-dependent or reflects the general *C. auris* response, cytokine production was assessed upon 24-hour stimulation with different *C. auris* strains originating from the five different clades. While showing variation between *C. auris* strains in cytokine production (**Figure 1c**), a similar

pattern between pro-inflammatory cytokines was observed. Of note, *C. auris* clade V induced similar levels of pro-inflammatory cytokines and significantly lower IL-1Ra levels compared to *C. albicans. C. auris* clades II and III induced cytokine production moderately, but this was still significantly higher compared to *C. albicans. C. auris* clades I and IV present as extremely potent inducers of pro-inflammatory cytokines. Regardless of this clade dependent variability, all *C. auris* clades except for Clade V were observed to drive a significantly enhanced pro-inflammatory cytokine response compared to *C. albicans* (**Figure 1c**). However, at the transcriptional level, PBMCs stimulated with *C. auris* showed only a trend towards a stronger induction of *IL-6* (Log_2FC 8.41 ± lfcSE 1.4) and *IL-1β* (Log_2FC 6.45 ± lfcSE 1.29) expression levels than upon exposure to *C. albicans* (Log_2FC = 7.58 ± lfcSE 1.37 for *IL-6*; Log_2FC 5.59 ± lfcSE 1.31 for *IL-1β*) (ns, Extended Data Fig. 2b).

C. auris replicates faster than _C. albicans in vivo,_ leading to altered multiplicity of infection (MOI), but does not cause macrophage lysis

Killing of *Candida* by professional phagocytes of the innate immune system, such as monocytes, macrophages or dendritic cells, is an important line of defense at the site of infection. In order to study the differences in phagocytosis dynamics of professional phagocytes between *C. auris* and *C. albicans,* we employed live-cell video microscopy coupled with dynamic image analysis of bone marrow-derived macrophages (BMDMs). Phagocytosis capacity was assessed by combining BMDMs with live and thimerosal-killed *C. auris* and *C. albicans* strains at an intended MOI of 3:1, yeast cells per macrophage. By including fixed yeasts, we were able to assay phagocytosis in the absence of rapid adaptive changes in the composition of the *Candida* cell wall. Results are expressed as percentage of phagocytic BMDM (% uptake), indicating the percentage of macrophages having phagocytosed at least one fungal cell. In addition, we assayed the Phagocytic Index which is defined as the number of fungal cells engulfed and fully inside the phagosome per 100 macrophages (by excluding the fungal cells adhering but not internalized). No significant differences in *C. auris* and *C. albicans* phagocytosis (% uptake) were observed for fixed *Candida* (Extended Data Fig. 2c). There was a trend towards a

higher phagocytic index in BMDMs at later time points (after the second hour) for both of the fixed *C. auris* strains compared to *C. albicans* strains (Extended Data Fig. 2c), possibly because fungal cells tend to be phagocytosed in clusters. However, *C. auris* strain 10051893 had a lower phagocytosis efficiency after 1 hour compared to *C. albicans* SC5314 (Extended Data Fig. 2d). Focusing on live strains, we observed that the phagocytic index of both *C. auris* strains gradually increases in time, to a greater extent than the *C. albicans* strains (Figure 2a). In order to evaluate the phagocytosis dynamics using human cells, we incubated human PBMCs stimulated for 15, 30 and 120 minutes (2 hours) with thimerosal-fixed FITC-labeled *C. albicans* 10061110 and *C. auris* KCTC17810, and then calculated the percentage of FITC-positive cells as well as the mean fluorescence intensity (MFI) in the $CD14^+$ population. *C. auris* showed a significantly higher rate of phagocytosis and MFI at all the time points measured compared to *C. albicans* (Figure 2b).

Using real-time live cell microscopy, live *C. auris* cells were observed to bud repeatedly outside the macrophages, with a doubling time of ~1 hour. The *C. auris* budding rate decreased following phagocytic engulfment, although cells continued to multiply within phagosomes (Supplementary Video 1). Of interest, *C. auris* 10051895 accumulated in high numbers within macrophages, indicating that the starting MOI had exceeded the intended initial 3:1 ratio (Supplementary Video 2). This triggered our interest to estimate the actual MOI ratio at the start of image acquisition. Due to the time elapsed between the counting of *Candida* in each sample and the image acquisition, the starting 3:1 ratio for *C. auris* rose up to 7:1, presumably due to ongoing budding, despite the fact that samples were stored at 4°C in PBS until the live imaging commenced. In contrast, the MOIs for live *C. albicans* as well as for the fixed strains remained around the desired target of 3:1 yeast:macrophage.

The elevated MOIs for *C. auris* may be a contributing factor to the trend for higher phagocytic index achieved at 3 hours for live strain 10051895 (Figure 2a). However, an elevated starting MOI for *C. auris* 10051893 did not enhance phagocytosis, as the live form of this strain was poorly recognized by BMDMs, showing a significantly lower percentage of uptake as compared to *C. albicans* 10061110

(Extended Data Fig. 2e). From the representative movies recorded, we quantified the distribution of yeast per individual macrophage after 3 hours and found that for both *C. auris* strains, there was a tendency for some macrophages to engulf many fungal cells, yet for other macrophages to engulf none. This phenomenon was less surprising for the *Candida* experiments using live microorganisms (Figure 2c), because *C. auris* continues to divide prior to and during the phagocytosis experiment. However, the fixed strains also displayed this varied distribution, with both strain of *C. auris* being phagocytosed in large numbers by some macrophages (Extended Data Fig. 2f). Supplementary Video 3 shows that *C. auris* cells are taken up extensively into a subpopulation of macrophages, but despite the vast burden, these phagocytes continue to move around in pursuit of further fungal targets (Supplementary Video 3).

Finally, macrophage lysis was determined following engulfment of live *Candida* and it emerged that the *C. auris* strains examined were significantly less able to kill macrophages after 3 hours than *C. albicans* 10061110, but not compared to *C. albicans* SC5314 (Figure 2d, Supplementary Video 4), despite having a comparable (or greater, in the case of *C. auris* 10051895) phagocytic index. These findings demonstrate that *C. auris* is differentially recognized by phagocytic BMDMs and internalized with a higher phagocytic index compared to *C. albicans* but is not able to induce lysis of the phagocytic cells.

Host immune response upon *C. auris* exposure is mediated by heat-sensitive cell wall components.

Variability in the cell surface structures, such as differentially expressed mannoproteins or altered β-glucan exposure between the *C. auris* and *C. albicans* cell walls, could account for the differential cytokine responses triggered by these pathogens. To elucidate whether this might account for the observed differences in cytokine induction, the *C. auris* and *C. albicans* strains were subjected to heat-killing. This approach facilitates disruption of the outer layer of the *Candida* cell wall [34], in turn enhancing β-glucan exposure [35,36]. The heat-killed strains were used to stimulate PBMCs for 24 hours

and 7 days. Since the production of reactive oxygen species (ROS) can positively contribute to immune responses [37], in addition to PBMCs cytokine production, ROS release in both neutrophils and PBMCs was assessed during 1 hour stimulation with serum-opsonized heat-killed *C. auris* and *C. albicans* strains. The area under the curve (AUC) was calculated to examine quantitative differences in ROS release.

In neutrophils both the ROS release over time and the total amount of production (AUC) were significantly lower following *C. auris* stimulation, compared to *C. albicans* (Extended Data Fig. 3a). In PBMCs, although the time-course luminescence was significantly decreased in *C. auris*-stimulated cells, only *C. auris* 10051893 induced a significantly lower total ROS production than *C. albicans* (Extended Data Fig. 3b).

Unexpectedly, compared to *C. albicans*, the cytokine response was almost completely abrogated after PBMC stimulation with heat-killed *C. auris* for 24 hours (Figure 2e). This phenomenon was observed for all *C. auris* clades (Extended Data Fig. 3c), subsequently marking this characteristic for the general *C. auris* induced host immune response. In addition, after 7 days, the production of IFN-γ and IL-17 by PBMCs stimulated with heat-killed *C. auris* was significantly lower compared to heat-killed *C. albicans* (Extended Data Fig. 3d). Hence, we reasoned that a heat sensitive component of the cell wall might be responsible for most of the increased cytokine induction by *C. auris*.

Mannans drive the host response to *C. auris*

We attempted to unravel the contribution of the different fungal cell wall components to the activation of host responses by *C. auris,* compared to *C. albicans.* PBMCs were exposed to the purified cell wall components, β-glucans and mannans, from both *Candida* species, and the transcriptional responses of the stimulated immune cells were assessed by RNA sequencing. The species-specific cell wall contribution was assessed by comparison of the number of shared DEG upon exposure to the different cell wall components and expressed as proportion of the respective live stimulus.

307 The early 4-hour host response was predominantly induced by β-glucan, which was sufficient
308 to explain around 82% and 57% of the respective live responses of *C. albicans* (89 / 109) and *C. auris*
309 (45 / 79) (**Figure 3a**). The β-glucans from each species resulted in similar PBMC gene expression
310 profiles (Extended Data Fig. 4a). Although the relative contribution of *C. albicans* β-glucan decreases to
311 approximately 13% (55 / 442) in the late phase, 24 hours after stimulation, they remain able to
312 upregulate several *C. albicans*' top 50 DEG, leaving them a main contributor of the evoked response in
313 the live setting (**Figure 3a** and Extended Data Fig. 4a). In contrast, β-glucans from *C. auris* failed to elicit
314 a response analogous to the live *C. auris* exposure, explaining only a mere 2% (10 / 484) of the live *C.*
315 *auris*-induced response. Conversely, however, mannans from *C. auris* stimulated 28% (136 / 484) of
316 the evoked transcriptional response to live *C. auris* cells. Moreover, *C. auris* mannan seemed to
317 outperform *C. albicans* β-glucan in relation to the top 50 DEG of *C. albicans*, displaying an induction
318 pattern similar to its live setting (Extended Data Fig. 4a). Overall, these results indicate that the host
319 recognition and subsequent initiation of downstream responses against *C. albicans* is mainly
320 dependent on β-glucan. For *C. auris*, early 4-hour stimulation of PBMCs is mainly mediated by β-
321 glucan, whereas mannans are fundamental for orchestrating the *C. auris*-specific host response at
322 later time points (24 hours).
323 Our data suggest a stronger *C. auris* host immune response and a differential role in gene
324 expression between *C. albicans* and *C. auris* cell wall components. Therefore, to investigate the
325 differences in cell wall structure between *C. auris* and *C. albicans*, we first compared forward (FSC)
326 and side (SSC) light scatter of fungal cells using flow cytometry. In line with what has previously been
327 described [23], we found that the *C. auris* strains have a smaller average cell size compared to *C.*
328 *albicans*. Of the *C. auris* strains, strain 10051893 shows more complexity/granularity (higher SSC) than
329 strain 10051895 (Extended Data Fig. 4b).

330 Next, we measured β-glucan exposure on the fungal cell surface by flow cytometry on
331 thimerosal-fixed *Candida* cells stained with Fc-Dectin-1. *C. auris* 10051893 displayed significantly
332 reduced exposure of β-glucan as compared to *C. albicans* SC5314 (Extended Data Fig. 4c). At the gene

expression level, *C. auris* β-glucan (Log_2FC 2.9 ± lfcSE 0.6) tended to induce less effectively IL-1Ra as compared to β-glucan isolated from *C. albicans* (Log_2FC 4.5 ± lfcSE 0.6) (ns, Extended Data Fig. 2b). At the cytokine level, though large variation between different strains was observed, no significant differences in cytokine production of PBMCs stimulated with purified β-glucans from *C. auris* compared to *C. albicans* were found for TNFα and IL-1β, while there was significantly lower IL-6 production in response to β-glucan from *C. auris* 10031160, *C. auris* 10051256, *C. auris* 10051263, *C. auris* 10051522 and *C. auris* 10051252 when compared to *C. albicans* β-glucan. Interestingly, β-glucan from *C. auris* strains, except for *C. auris* 10051522, *C. auris* 10051244 and *C. auris* 10051252, induced a significantly lower IL-1Ra production compared to *C. albicans* β-glucan (**Figure 3b**). Moreover, similarly to *C. albicans* β-glucan, *C. auris* β-glucan synergistically boosted Pam3Cys (TLR2 agonist)-induced IL-1β production in PBMCs, as well as TNFα and IL-6 production (**Figure 3c**).

Having ruled out a major role for β-glucans in explaining the difference in cytokine stimulation induced by *C. auris* and *C. albicans*, we assessed the role of glycosylated mannoproteins from the fungal cell wall [34]. Examination of mannan exposure, by staining thimerosal-fixed *Candida* cells with Concanavalin A (ConA), revealed a relatively low level of exposure of surface mannans in *C. auris* strains. This difference was significant for *C. auris* 10051893 as compared with *C. albicans* 10061110 (Extended Data Fig. 4c). However, *C. auris* mannans were observed to significantly induce the gene expression of pro-inflammatory cytokines *IL-6* (padj = 0.0001) and *IL-1β* (padj = 0.0003) compared to those of *C. albicans* (Extended Data Fig. 2b).

In line with these observations, mannans from all eight *C. auris* strains induced a significantly higher cytokine production than *C. albicans* mannans 24 hours after stimulation of PBMCs, both pro-inflammatory and anti-inflammatory (**Figure 3d**). Except for IL-1Ra production, opsonization by human serum was necessary for mannan-induced production of cytokines (Extended Data Fig. 5a). After 7 days of stimulation, no significant differences between mannan from *C. auris* and *C. albicans* as well with the unstimulated cells have been observed (Extended Data Fig. 5b).

358

359 Unique structure of *C. auris mannans*

360 Nuclear magnetic resonance (NMR) spectroscopy analyses of respective cell wall components for both
361 *Candida* species revealed no structurally unique features in β-glucans isolated from *C. auris*. However,
362 the distance between side-chain branching points (average number of β-linked glucosyl repeat units),
363 was larger for *C. auris* β-glucans than for *C. albicans* β-glucans (Supplementary table 3). In contrast,
364 these side chains were much shorter for *C. auris* than for *C. albicans* β-glucans. For *C. auris* mannans,
365 the acid stable portion was similar across all clinical isolates, revealing long side chains with varying
366 lengths containing linked α-1,2-mannose, α-1,3-mannose and β-1,2-mannose in varying amounts
367 (**Figure 4)**. Strikingly, the acid labile portion of *C. auris* mannans revealed two distinct M-α-1-
368 phosphate side chains (**Figure 4**), marking a unique structural feature that has not been observed in
369 the fungal kingdom before. Subsequent multi-detector gel permeation chromatography highlighted *C.*
370 *auris* mannans as extremely small biopolymers with a molecular weight (MW) ranging from 6.1 to 16.1
371 x 10^3 Da among the clinical isolates. This represents a major difference from *C. albicans* mannan,
372 which has a MW of 500 x 10^3 D (Supplementary table 3).

373 We assessed the effects of these distinct and unique structural features of *C. auris* mannans
374 on their binding capacity with rhDectin-2 and rhMannose receptors, compared to *C. albicans*
375 mannans. Variability in binding affinities was observed amongst mannans from the different clinical *C.*
376 *auris* isolates, ranging from an equilibrium dissociation constant (K_D) of 1.0 to 6.0 μM for rhDectin-2
377 and 2.1 to 6.3 μM for rhMannose. Although differentially recognized by both receptors, their overall
378 binding affinities were an order of magnitude lower than those observed for *C. albicans* mannans
379 (Supplementary table 3). Moreover, of the examined structural features, the affinity of *C. auris*
380 mannans for the rhDectin-2 receptor was solely associated with a higher MW (r^2=0.4488, p=0.034). A
381 slightly lower association was observed for rhMannose (p=0.096) yet displaying a similar trend.
382 Collectively, our data suggest that the double M-α-1-phosphate side chains and small molecular size,

represent highly unique physicochemical properties of *C. auris* mannans that probably contribute to the decreased recognition efficiency by two important anti-fungal recognition receptors.

The CLRs complement receptor 3 (CR3) and MMR contribute to the *C. auris*-induced cytokine production

Next, we investigated the downstream effects of the reduced binding affinity of *C. auris* mannans on intracellular signaling pathways and the subsequent activation of the immune system. The spleen tyrosine kinase, Syk, is an important mediator downstream of several CLRs [38]. CLR signaling from Dectin-1 and other lectins also involves the serine/threonine-protein kinase Raf-1 [39]. Inhibition of Syk and Raf-1 decreased TNFα, IL-6, and IL-1 β production in response to *C. auris* stimulation (**Figure 5a**), indicating the involvement of these two signaling pathways in cytokine production.

Due to the importance of Syk and Raf-1 pathways in CLR pathway mediation, we subsequently hypothesized a role for these receptors in *C. auris* recognition. Therefore, we pre-incubated PBMCs with neutralizing antibodies against important *Candida* CLRs (e.g., Dectin-1, Dectin-2, Mincle, DC-SIGN, MMR, CR3, and their control isotypes), one hour prior to stimulation with live *C. albicans* or *C. auris*. As expected, blocking Dectin-1 significantly decreased TNFα production upon *C. albicans* stimulation but, surprisingly, increased TNFα upon *C. auris* stimulation. (**Figure 5b**). We observed a significant reduction in *C. auris*-induced IL-6 and IL-1Ra production upon blocking of MMR (**Figure 5b**). Interestingly, neutralization of Dectin-1 and DC-SIGN led to a significant increase of IL-6 induced by *C. auris* compared to IgG2b isotype control. Moreover, blockade of CR3 led to a significant reduction in IL-1β production and an increase in IL-1Ra production (**Figure 5b**). We conclude that CR3 and MMR signaling promotes cytokine production in response to *C. auris*, while blocking Dectin-1 functionality perturbs this cytokine production.

C. auris is less virulent than *C. albicans* in an experimental model of murine disseminated candidiasis

To evaluate the virulence of *C. auris in vivo*, immunocompetent C57BL/6J mice were injected

intravenously (i.v.) with either 10^7 Colony Forming Unit (CFU) of *C. auris* 10051895 (n=10) or *C. albicans* 10061110 (n=11). Their survival was monitored over the course of 14 days. Significantly more immunocompetent mice survived infection with *C. auris* than with *C. albicans* (3/11 deaths for *C. auris* and 11/11 deaths for *C. albicans* over 14 days; Chi square=21.42; p< 0.0001, Mantel-Cox test) (**Figure 6a**). In order to evaluate the differential organ invasion capacity between *C. auris* and *C. albicans* infection, we injected intravenously either 10^6 CFU of *C. auris* or *C. albicans* and sacrificed mice at day 3 (n=5 with *C. albicans*, n=5 with *C. auris*) and day 7 (n=4 with *C. albicans*, n=5 with *C. auris*) for CFU counting both in liver and kidneys. Although after day 3 there weren't any significant differences, a significantly lower fungal burden has been found at day 7 in the kidneys of *C. auris* infected mice compared to *C. albicans*-infected (**Figure 6b**). To confirm whether the *ex vivo* higher pro-inflammatory response towards *C. auris* found in human PBMCs holds *in vivo*, we measured myeloperoxidase (MPO) in organs and several cytokines both in plasma and organs of mice infected with either 10^6 CFU of *C. albicans* or *C. auris.* Except for a significantly lower Keratinocyte chemoattractant (KC) at day 7 (**Figure 6c**), no significant differences between cytokine levels have been found between *C. auris* and *C. albicans*-infected mice (**Figure 6d** and **Extended Data Fig 6a-e**). To understand the inflammatory cytokine induction after the same load of *Candida* CFU, we normalized the inflammatory cytokine production with the actual remaining CFU in organs by calculating the ratio of the mean MPO/CFU and KC/CFU. MPO and KC production per remaining *C. auris* CFU were higher than the remaining *C. albicans* CFU count (**Figure 6f**), supporting our in vitro findings in human PBMCs. In conclusion, improved survival and better clearance of invasive *C. auris* infection as compared to *C. albicans* is ensured by an adequate immune response in immunocompetent mice.

DISCUSSION

In the current study, we investigated the transcriptional and functional responses of human PBMCs and murine BMDMs to the rapidly emerging fungal pathogen *C. auris*. *C. auris*-induced host responses were compared to those elicited by *C. albicans*, as this species remains the most frequent cause of nosocomial fungal infections in humans to date [33]. A broad assessment of various clinical strains, as well as further verification amongst the five *C. auris* clades, revealed that, with the exception of clade V, *C. auris* induces a stronger immune response than *C. albicans in vitro*. Functional and structural assessment of β-glucans and mannans highlighted the presence of small and structurally unique *C. auris* mannans, which were crucial for immune recognition. Compared to *C. albicans, C.* auris isolates examined in this study were more efficiently phagocytosed by immune cells, induced lower levels of macrophage lysis, and displayed lower virulence in a murine model of disseminated infection.

C. auris induced robust transcriptional changes in human PBMCs. These included both common pathways induced by *C. albicans* as well, but also more robust specific IFN–dependent transcriptional programs and explicit cytokine responses. This conclusion is supported by a recent study by Mora-Montes and colleagues [31]. Secondly, *C. auris* appear to induce stimulation of immune cells by sequential engagement of different components of the cell wall. The early (4 hour) responses are mainly induced by β-glucans, and this initial phase of the response is largely similar to that induced by *C. albicans*. This is probably explained by the similar structure of *C. auris* and *C. albicans* β-glucans. In contrast, the late transcriptomic responses (24 hours) induced in PBMCs by *C. auris* display significant differences and broader upregulation of immune genes compared with those induced by *C. albicans*. These late responses are mainly mediated by *C. auris* mannoproteins with a specific structure that includes a unique M-α-1-phosphate side chain in the acid labile portion of C*. auris* mannans, which has not been observed in the fungal kingdom to date. In line with the results of Yan *et al.* reporting that *C. auris* mannans strongly bind to serum IgG and mannose-binding lectin (MBL) [40],

456 we showed that opsonization by human serum was necessary for *C. auris* mannan-induced cytokine
457 production.

458 By comparing *C. albicans* induced cytokine production with the different *C. auris* clades, we
459 observed variability that is probably linked to their distinct phenotypic, epidemiological, and drug
460 resistance properties. In particular, clade V was the least immunogenic, while clades I and IV were the
461 strongest inducers of cytokine production. The amount of cytokines induced by the various strains
462 might be correlated to clade-specific characteristics which, in turn, might influence the level of
463 colonization/persistence in the host. When a healthy host encounters *C. auris* from clade I or IV, it
464 responds with a prompt high pro-inflammatory cytokine response which is protective. Although it is
465 also linked with invasive infections, clade III isolates show a lower *in vitro* cytokine production
466 compared to isolates from other clades. This may be linked to their tendency to form aggregates,
467 which might make innate immune recognition challenging. Finally, the fact that Clade II has a relatively
468 lower cytokine production, despite a higher phagocytosis rate as compared with *C. albicans*. This
469 might be related to its simpler mannan structure compared to isolates from Clade I and Clade IV
470 (**Figure 4**).

471 An important question concerns the PRRs responsible for the recognition of *C. auris.* Our
472 experiments using neutralizing antibodies revealed a significant role for the CLRs, especially MMR and
473 CR3, in the induction of cytokines by *C. auris*. The role of these receptors in the recognition of
474 mannans is well known [41]. However, our binding affinity data show that r-MMR binds *C. auris* mannans
475 with a low affinity, and the *in vitro* neutralization of MMR led to only partial loss of cytokine
476 production, arguing that additional mannan-recognizing receptors contribute to anti-*C. auris* host
477 defense. On the other hand, the blocking of Dectin-1 significantly increased TNFα and IL-6 upon *C.*
478 *auris* stimulation for 24 hours. This interesting observation could be explained by differences in
479 relative β-glucan abundance. However, recently Navarro-Arias *et al.* [31] quantified the abundance of
480 different cell wall components and found that total β-glucan and mannans in *C. auris* were
481 comparable to *C. albicans*. We suggest that this phenomenon might be due to a combination of two

factors: i) the different exposure of *C. auris* β-glucan as compared to *C. albicans* (Extended data Fig. 4C); ii) the differential and variable cell wall adaptation of *C. auris* strains during the interaction with the host which determines Dectin-1 dependence of *C. auris* host response. Such phenomenon occurs *in vivo* during *C. albicans* infection in a strain-specific way and the differences in the levels of cell-wall chitin influence the role of Dectin-1 [42]. Interestingly, Navarro-Arias *et al.* have reported significantly more cell wall chitin in *C. auris* compared to *C. albicans* [31]. Since high chitin levels reduce the dependence on Dectin-1 recognition, we speculate that differences in *C. auris* cell wall adaptation to the host, variations in chitin content (higher chitin in *C. auris*) and differences in cell wall structure (less exposure of beta glucan in *C. auris* and structurally unique mannoproteins) might provide an explanation, at least in part, for the *C. auris* Dectin-1 (in)dependency.

Cytokine induction is important upon pathogen recognition, but the induction of phagocytosis is also crucial [43]. We observed a higher phagocytic index for *C. auris* compared to *C. albicans*. This is likely due to a better recognition of *C. auris* mannans by immune cells, as cell wall glycosylation is critically important for the recognition and ingestion of *C. albicans* by macrophages [44]. Therefore, to shed more light on these processes, future investigations might examine the phagocytosis dynamics of *C. auris* mutant strains that are defective in their cell wall architecture. Interestingly, when the fate of the fungus was assessed through video time-lapse microscopy, it was also clear that the continued cell division of *C. auris* leads to altered MOI that are greater than *C. albicans* and this may also contribute to the stronger stimulation of inflammation. However, this did not result in the death of the phagocytes, most likely due to the lack of hyphae formation by engulfed *C. auris* cells. In line with previous studies pointing to glucose competition as the main effector mechanism through which *C. albicans* induce macrophage death [45], a clear enrichment of glucose, fructose and mannose metabolism pathways in the host were observed unique for 24-hour *C. albicans* exposure (as seen in Figure 1A and Supplementary Table 2). Conversely, the respective absence of the induction of such a metabolic shift by *C. auris* suggests that this may be a likely mechanism to explain the lower macrophage lysis induced by phagocytosed *C. auris*, and subsequently may explain the lower

508 virulence. In addition, this could represent an important difference of the paths followed by different
509 *Candida* species as survival mechanisms.

510 The stronger induction of cytokines and lower macrophage lysis after 3 hours of phagocytosis
511 might have been expected to lead to lower virulence of *C. auris in vivo* compared with *C. albicans.* In
512 line with this hypothesis, experiments in a model of murine disseminated candidiasis demonstrate
513 that *C. auris* is less virulent compared to *C. albicans*, a conclusion supported by recent additional
514 studies [46,47]. Neutrophils are considered one of the most important host immune response to fungi
515 through phagocytosis and intracellular killing, or by releasing NETs [48]. In a recent study [30], human
516 neutrophils were poorly recruited to sites of *C. auris* infection, were less able to kill *C. auris* compared
517 to *C. albicans* and failed to form NETs in response *C. auris.* However, neutrophils are important
518 contributors to the host defense against *Candida* species [49]. Our *in vivo* results show that MPO
519 production in *C. auris*-infected mice is similar to *C. albicans*, indicating that neutrophil activation is
520 comparable. In addition, the comparable innate and adaptive cytokine production in mice infected
521 with *C. albicans* and *C. auris,* as well as the similar or even better organ clearance, suggest that the
522 immune response toward *C. auris* is fully functional in an immunocompetent host. When the cytokine
523 levels were linked to the remaining organ CFU count by a cytokine/CFU ratio, we observed that there
524 was a higher cytokine production per singular *C. auris* CFU cultured from the organ, as compared to *C.*
525 *albicans*. This is in line with the potent pro-inflammatory response observed in human PBMCs
526 stimulated with *C. auris*. Future studies are warranted to dissect the relative importance of
527 neutrophils and macrophages in the host defense against *C. auris*.

528 In conclusion, we performed a first comprehensive assessment of the innate host defense
529 mechanisms against the rapidly emerging human pathogen *C. auris*. The overall conclusion is that the
530 host defense mechanisms induced by *C. auris* are generally classical antifungal mechanisms, but
531 important specific responses are also triggered by unique *C. auris*-specific mannoprotein structures.
532 The ensuing immune responses are effective and lead to an effective elimination of the fungus. Our
533 study argues that the intrinsic virulence of *C. auris* is not higher than other *Candida* species circulating

534 in the patient population, but it is rather the infection control problem of this pathogen and its high
535 resistance to antifungal drugs that make it dangerous. The challenges that need to be pursued in the
536 coming years are to identify in even more detail the most effective components of the anti-*C. auris*
537 host defense, and to design and test novel host-directed therapies to enhance these pathways and
538 improve the outcome to the infection. In this respect, based on our results highlighting the peculiarity
539 of *C. auris* mannoprotein structures, one promising therapeutic possibility could be fosmanogepix
540 (APX001A), a novel agent that targets the fungal protein Gwt1 (glycosylphosphatidylinositol-anchored
541 wall transfer protein 1), thereby inhibiting the maturation and localization of GPI-anchored
542 mannoproteins in the cell wall [20]. Several studies reported higher survival rates and lower fungal
543 burden in *C. auris* infected mice treated with this novel drug [20,50]. One of the reasons for this higher
544 efficacy could be the crucial role of mannans for *C. auris* pathogenicity in the host. In addition to being
545 the first comprehensive study of the host immune response to *C. auris*, our data provide further
546 support from an immunological and microbiological perspective for the development of drugs
547 potentially targeting mannan synthesis as new and efficient anti-fungal drugs for *C. auris.*
548
549

METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement for ex vivo human PBMC stimulations

Ex vivo/in vitro human PBMC stimulations - Inclusion of healthy controls was approved by the local institutional review board (CMO region Arnhem-Nijmegen, #2299 2010/104) and conducted according to the principles of the International Conference on Harmonization–Good Clinical Practice guidelines. Buffy coats from healthy donors were obtained after written informed consent (Sanquin, Nijmegen, the Netherlands).

Ethics statement for in vivo mice studies

All animal experiments were conducted in the unit of animals for medical scientific purposes of University General Hospital 'Attikon' (Athens, Greece) according to EU Directive 2010/63/EU for animal experiments and to the Greek law 2015/2001, which incorporates the Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes of the Council of Europe (code of the facility EL 25BIO014, approval no. 1853/2015). All experiments were licensed from the Greek veterinary directorate under the protocol number 7467/24-12-2013. All animal experiments were reported using the ARRIVE guidelines.

PBMCs isolation and stimulation

Venous blood from the antecubital vein of healthy volunteers was drawn in EDTA tubes after obtaining written informed consent. PBMC isolation was performed as previously described [51]. Briefly, the PBMC fraction was obtained using density centrifugation in Ficoll-Paque (Pharmacia Biotech, Piscataway, USA). Cells were then washed twice in PBS and re-suspended in RPMI-1640+ medium (RPMI1640 Dutch modification supplemented with 50μg/mL gentamicin, 2mM L-glutamine and 1mM

pyruvate; Gibco, Invitrogen, Breda, The Netherlands). Afterwards, PBMCs were counted and re-suspended in a concentration of $5x10^6$ cells /mL. $5x10^5$ PBMCs were added in 100 µL to round-bottom 96-well plates (Greiner, Alphen Aan Den Rijn, NL) and incubated with 50 µL of stimulus (RPMI, live, 4% paraformaldehyde (PFA) or heat killed *Candida albicans* yeast $1x10^6$/mL or *Candida auris* $1x10^6$/mL; 100 µg/mL purified *C. albicans* or *C. auris* mannan; 10 µg/mL purified *C. albicans* or *C. auris* β-glucan) and 50 µL of eventual inhibitor or medium with or without 10% human serum. Serum was either complement active, if not otherwise indicated, or heat inactivated by incubation for 30 minutes at 56°C in a water bath according to a commonly used protocol. After 1 hour of pre-incubation with inhibitor or medium, stimuli or medium was added. In detail, for receptor blockade experiments, before stimulation with *C. albicans* or *C. auris*, PBMCs were pre-incubated for 1 hour with 5 µg/mL anti-DC SIGN antibody 10 µg/mL, anti-Dectin-1, 10 µg anti Mincle and 10 µg/mL control IgG2b; 10 µg/mL anti-Dectin-2 antibody and 10 µg/mL of its control IgG1]); 10 µg/mL anti-CR3 antibody and control IgG (R&D), 10 µg/mL MR-blocking antibody and 10 µg/mL Goat IgG isotype control. After 1 hour, cells were stimulated with 10^6 heat-killed *C. albicans* and *C. auris*. For the intracellular pathways blockade experiment 50 nM Syk inhibitor, 1 µM Raf-inhibitor or the same concentration of vehicle (DMSO) has been used. Concentrations of inhibitors were selected as being the highest non-cytotoxic concentrations. All supernatants were stored at -20°C until analyzed.

Antibodies		
Product	*Source*	*Article Number*
DC-SIGN Monoclonal Antibody (clone 120507)	Fisher Scientific	MA1-25615
Human Dectin-1/CLEC7A Allophycocyanin Mab (Clone 259931)	Bio-Techne/R&D	MAB1859
Anti-hMincle-IgG	Invivogen	mabg-hmcl
Mouse IgG2B Isotype Control	Bio-Techne/R&D	MAB004
Human Dectin-2/CLEC6A Antibody	Bio-Techne/R&D	MAB3114

IgG1 Isotype Control	Bio-Techne/R&D	MAB002
Human MMR/CD206 Antibody	Bio-Techne/R&D	AF2534
anti-hIntegrin beta2 - hIntegrin b2 Affinity Purified Goat IgG	Bio-Techne/R&D	AF1730

Cytokine and lactate measurements

All cytokine levels were measured in the cell culture supernatants using commercially available ELISA assays according to the protocol supplied by the manufacturer. For human cytokines, IL-1β, TNFα, IL-6, IL-1Ra, and IL-10 were measured after 24 hours, and IL-17, IL-22, and IFN-γ were measured after 7 days of stimulation. For mouse cytokines, KC, IL-1β, IL-6, IL-10, IL-17, and IFN-γ were measured after 3 and 7 days. Lactate was measured by a Lactate Fluorometric Assay Kit (Biovision, Milpitas, USA).

ELISA assays		
Product	*Source*	*Article Number*
Human IL-1β ELISA	R&D Systems	DY201
Human TNFα ELISA	R&D Systems	DY210
Human IL-17 ELISA	R&D Systems	D1700
Human IL-22 ELISA	R&D Systems	D2200
Human IL-6 ELISA	R&D Systems	DY206
Human IL-8 ELISA	Sanquin	M1918
Human IL-10 ELISA	R&D Systems	D1000B
Human IFN-γ ELISA	Sanquin	M1933
Mouse KC ELISA	R&D Systems	MKC00B
Mouse IL-1β ELISA	R&D Systems	MLB00C
Mouse IL-6 ELISA	R&D Systems	M6000B
Mouse IL-10 ELISA	R&D Systems	M1000B
Mouse IL-17 ELISA	R&D Systems	M1700
Mouse IFN-γ ELISA	R&D Systems	MIF00

Cytotoxicity assay

$5x10^5$ PBMCs were added in 100 µL to flat-bottom 96-well plates (Greiner, Alphen Aan Den Rijn, NL) and incubated with 50 µL of stimulus (RPMI, live *Candida albicans* yeast $1x10^6$ /mL or different strains of live *Candida auris* $1x10^{6/}$mL) for 24 hours. Cell viability was assessed using CytoTox 96 non-radioactive cytotoxicity assay (Promega, Leiden, the Netherlands) according to the manufacturers' instructions. Released lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis, was measured in the supernatant with a 30-minute coupled enzymatic assay. The color intensity, as a result of the conversion of tetrazolium salt (INT) into ref formazan, is proportional to the number of lysed cells. As positive control, cells were lysed with 0.5% Triton-X reflecting maximal LDH release.

Candida strains

C. auris strains from five clades have been used (clade I, South Asia; clade II, East Asia; clade III, South Africa; clade IV, South America; clade V, Iran). *C. albicans* and *C. auris* strains were prepared by growing cells for 24h in either a Sabouraud broth or on Sabouraud plates at 30°C. Unless otherwise indicated, experiments were performed using *C. albicans* CWZ10061110, *C. auris* KCTC17810 reference, *C. auris* CWZ10051893 and *C. auris* CWZ10051895. Stimulations were performed using either live, heat-killed (12 hours at 56°C) or 4% PFA-killed microorganisms. Heat killing caused the disruption of the outer layer of the *Candida* cell wall [34] in turn enhancing β-glucan exposure [35,36]. While *Candida* killing *via* heat treatment disrupts the outer layer causing exposure of β-glucan in the cell wall, with PFA fixation and thimerosal fixation the cell wall structure remains intact [34,52,53].

Fungal Strains	**from**	**name(s)**
C. albicans	ATCC	ATCC MYA-3573/UC820
C. albicans	ATCC	SC5314 / ATCC MYA-2876

C. albicans	J. Meis (Clinical blood isolate)	CWZ 10061110
C. albicans	J. Meis (Clinical blood isolate)	CWZ 10070679
C. auris (Clade I)	A. Chowdhary	CWZ 10051893 (Clade I)
C. auris (Clade I)	A. Chowdhary	CWZ 10051895 (Clade I)
C. auris (Clade II)	J. Meis	KCTC17810 (Clade II)
C. auris (Clade III)	J. Meis	CDC AR 383/B11221 (Clade III)
C. auris (Clade IV)	J. Meis	CWZ 10051522 (Clade IV)
C. auris (Clade V)	J. Meis	CDC AR 1097/ IFRC 2086/CWZ 10111018 (Clade V)

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629 β-glucan and mannan isolation from *C. auris* strains

630 For the cell wall experiments, a total of 8 different *C. auris* clinical strains were used for
631 glucan and mannan extraction, originating from three different clades (I, II and IV). Isolates
632 were grown in 25 mL YPD (1% yeast extract, 2% dextrose, 2% peptone) at 30˚C for 48 hours
633 before parallel isolation of respective cell wall components under identical conditions. Aiding
634 β-glucan and mannan collection, cell walls were disrupted by 3 cycles of freeze/thawing (-
635 20°C) and consecutive harvesting of cell pellets. Of each culture a small aliquot was plated on
636 YPD media to exclude the presence of viable cells, after which the cell pellets were suspended
637 in 10 mL of 0.75 N NaOH to a final concentration of ~3 mg/mL. Suspensions were heated to
638 105˚C for 15 minutes, cooled and separated by centrifugation (10 minutes x 863 g). The
639 supernatant, containing the mannans, were harvested and dialyzed against 300 volumes of 18
640 MOhm water (2000 MWCO). After subsequent harvesting, the pH was confirmed to neutral,
641 followed by freezing and lyophilization to dryness. For β-glucans, the centrifuged extract was

642 transferred, treated with 10 mL of 1.0N H_3PO_4 and heated 105˚C for 15 minutes. After cooling
643 down, pellets were collected by centrifugation (10 minutes x 863 g) and extracted once more
644 with 10 mL 100% ethanol containing 1% (v/v) H_3PO_4 at 90˚C for 15 minutes. β-glucans were
645 harvested by centrifugation and purified by washing 3x with 18 MOhm water, after which the
646 final pellet was frozen and lyophilized to dryness.
647
648 *C. albicans* mannan isolation
649 Mannan isolation was performed using a modification of the Fehling method as previously
650 described [54]. Briefly, yeast cells, grown O/N at 30˚C, were delipidated by suspending cell
651 pellets in 100 mL of acetone and incubation for 20 minutes. The supernatant was decanted
652 after 5-minute centrifugation at 500 rpm and pellets were air-dried for 30 minutes.
653 Facilitating mannan extraction, pellets were suspended in 200 mL of distilled H_2O (dH_2O) and
654 supplemented with an equivalent amount of glass beads 0.5µ to pellet weight and followed
655 by bead beating (3x 30 second pulses). Cell extracts were autoclaved for two hours and
656 subsequently centrifuged for 5 minutes at 5,000 rpm. Respective supernatant was split into
657 two; one half was left untreated while the other was treated with 500 mg of pronase for 16
658 hours at 37˚C to abolish glycosidic activity. Ensuring the suppression of bacterial growth
659 during pronase treatment sodium azide was added at a final concentration of 50 nM. The
660 pronase-treated and untreated samples were 1:1 diluted with freshly prepared Fehling
661 solution and allowed to mix for 1 hour at RT, followed by a 20-minutes incubation allowing
662 precipitation of the mannan-copper complexes. After decantation, complexes were treated
663 with 5 mL HCl. When dissolved, 100 mL of a 1:8 mixture of glacial acetic acid:methanol
664 (GAE:MEOH) was added for mannan precipitation. After 4 hours, samples were washed
665 repeatedly with GAE:MEOH until the remaining precipitate appeared colorless (lack of any

666 blue/green) and followed by 3x methanol washes. The final precipitate was dissolved in 100
667 mL of dH_2O and dialyzed against 300 volumes of dH_20 over 48 hours and for 12 hours against
668 200 volumes of ultrapure dH_2O to remove any salts, acid, methanol and other low molecular
669 weight contaminants remaining from the extraction protocol. Dialyzed mannans were frozen
670 and lyophilized for 48 – 72 hours and stored at -20˚C.

Cell wall components		
Name	**from**	**Fungal strains**
C. auris mannans 1	David Williams	KCTC17810 (Clade II)
C. auris mannans 2	David Williams	CWZ 10031160 (2012) (Clade I)
C. auris mannans 3	David Williams	CWZ 10031163 (2012) (Clade I)
C. auris mannans 4	David Williams	CWZ 10051256 (2013) (Clade I)
C. auris mannans 5	David Williams	CWZ 10051263 (2013) (Clade I)
C. auris mannans 6	David Williams	CWZ 10051522 (2014) (Clade IV)
C. auris mannans 7	David Williams	CWZ 10051244 (2014) (Clade I)
C. auris mannans 8	David Williams	CWZ 10051252 (2014) (Clade I)
C. albicans mannans	David Williams	SC5314
C. auris β-glucans 1	David Williams	KCTC17810 (Clade II)
C. auris β-glucans 2	David Williams	CWZ 10031160 (2012) (Clade I)
C. auris β glucans 3	David Williams	CWZ 10031163 (2012) (Clade I)
C. auris β -glucans 4	David Williams	CWZ 10051256 (2013) (Clade I)
C. auris β glucans 5	David Williams	CWZ10051263 (2013) (Clade I)
C. auris β glucans 6	David Williams	CWZ10051522 (2014) (Clade IV)
C. auris β glucans 7	David Williams	CWZ 10051244 (2014) (Clade I)
C. auris β glucans 8	David Williams	CWZ 10051252 (2014) (Clade I)
C. albicans β glucans	David Williams	SC5314

671

672 RNA purification

673 PBMCs from three healthy donors, with a concentration of 5 x 10^6 cells/mL, were stimulated
674 in flat-bottom 12-well plates (Corning, NY, USA) with freshly counted live *C. albicans* (1x10^6/mL)
675 and *C. auris* (1x10^6/mL), and purified cell wall components β-glucans (10 µg/mL) and mannans
676 (10 µg/mL) isolated from both *Candida* species as described above. Of note, the time between
677 *Candida* cell count and experiment start did not exceed 15 minutes. PBMCs were cultured in
678 the presence of 10% human pooled serum. At 4 and 24 hours cells were lysed with RLT

buffer. Prior to subjection to the RNeasy Mini Kit (Qiagen), lysates were homogenized using a 1 mL syringe with a 0.8 x 15 mm needle. RNA was subsequently extracted following manufacturers' protocol, including an on-column DNAse digestion using the RNAse-Free DNAse set (Qiagen). Quantification and quality assessment of extracted RNA was performed using the Qubit RNA HS assay (Thermo Fisher Scientific) and Agilent 2200 TapeStation (RNA HS Screentape, Agilent), respectively. The majority of samples subjected to quality assessment revealed an RNA integrity number (RIN^e) of ≥ 8.

QuantSeq 3' mRNA sequencing

Libraries were generated from the extracted RNA using the QuantSeq 3' mRNA-Seq Library Prep Kit-FWD from Lexogen (Lexogen, Vienna, Austria) in accordance to the manufacturers' protocol. Three separate preparations were performed, split by PBMC donor, in turn limiting the number of samples to 14 to 18 samples per prep. RNA input was normalized to 100 ng for donor A, and to 250 ng for donors B and C. An aliquot (1:10) of double stranded cDNA libraries was used for quantitative PCR, in turn indicating 17 – 18 cycles as optimal for endpoint PCR (17- donor B; 18 - donors A, C). Accurate quantification and assessment of quality of the generated libraries was performed using Qubit dsDNA HS assay (Thermo Fisher Scientific, Waltham, USA) and Agilent 2200 TapeStation (HS-D1000 ScreenTape, Agilent, Santa Clara, USA). The cDNA concentration and average fragment size were used to determine the molar concentration of the individual libraries. Consequently, libraries were pooled equimolar to 100 fmol. After a final dilution of the pool to a concentration of 4 nM, the libraries were sequenced on a NextSeq 500 instrument (Illumina, San Diego, USA), with 75 cycle (i.e. 75bp single-end sequence reads), high output kit with a 1.1 pM final loading concentration.

Differential gene expression analysis

Quality of the acquired sequencing data was controlled using FastQC tool v0.11.5 (Babraham Bioinformatics) and subsequently followed by the removal of adapter sequences and poly(A) tails with Trim Galore! v.0.4.4_dev (Babraham Bioinformatics) and Cutadapt v1.18 [55]. On average ~ 6 million reads per individual library were retrieved. Filtered and trimmed reads were mapped to the human reference genome (hg38/GRCh38) using the STAR aligner v2.6.0a (Supplementary Table 4) [56]. Less than 1% of all reads were comprised of overrepresented sequences and were uniquely mapped with a median of 4 million reads (74.1%). After generating gene level count data using the HTSeq-count tool v0.11.0 [57], an additional filtering step was performed ensuring the exclusion of several non-coding RNAs, i.e. mtRNA, lincRNA, snRNA, tRNA, miscRNA and snoRNA, in our dataset. Given the absence of sample replicates, PBMC donors were considered biological replicates. Hence, in the differential gene expression analysis using DESeq2 v1.22.0, including logFold Shrinkage and apeglm [58], the average PBMC donor response to the different stimuli were compared to their control condition, RPMI. Genes with a fold change of ≥2 and a p-adjusted value <0.01 were considered differentially expressed genes (DEG). Allowing identification of the main principle components underpinning the majority of the variance, a PCA analysis was performed on DESeq2 normalized counts (normTransform).

Pathway enrichment analysis

In order to distinguish between the responses triggered by both *Candida* species, DEG were compared between species for the analogous stimulations (live, mannan and β-glucan), and corresponding time-points. In turn resulting a group of DEG that overlap between the two species, and DEG that were uniquely attributed to either one of the *Candida* species.

Overrepresentation analysis were performed on all groups per stimulation (and time-point) using Consensus PathDB [59], including pathways as defined by pathway databases Kyoto Encyclopedia of Genes and Genomes KEGG [60] and Reactome [61]. Minimum overlap in input was set at 2, together with a p-value cut-off of 0.01. For downstream analysis, pathways were considered enriched with a corrected p-value <0.01 (indicated as 'q-value').

Structural characterization of mannans by NMR spectroscopy

To gain insight into the mannan structure of both *Candida* species, isolated mannans were subjected to solution-state 1D ^{31}P-coupled and ^{31}P-decoupled ^{1}H NMR spectroscopy and 2D COSY NMR spectroscopy. Data acquisition and subsequent analysis were based on methods described by Lowman et al. [62]. In short, ^{1}H NMR spectra were collected using a Bruker Avance III 600 NMR spectrometer operating at 331˚K (58˚C). Roughly 10 mg of mannan was dissolved in 600 mL of dH_2O. Chemical shift referencing was accomplished relative to TMSP at 0.0 ppm. Proton 1D NMR spectra were collected with 2 dummy scans, 256 scans, 65,536 data points, 20 ppm sweep width centered at 6.2 ppm, and 1 second pulse delay. For the 1D ^{31}P decoupled ^{1}H NMR experiment, spectra were collected at 333° K (60° C) with 2 dummy scans, 1024 scans, 65,536 data points, 21 ppm sweep width centered at 6.2 ppm and the ^{31}P decoupling pulse centered at 3.0 ppm. All 1D spectra were processed using exponential apodization with 0.3 Hz line broadening. COSY spectra were collected using 2048 by 128 data points, 8 dummy scans, 32 scans, and 6.0 ppm sweep width centered at 3.0 ppm and processed with sine apodization in both dimensions and zero-filled to 1024 data points in f1. Processing was accomplished with the JEOL DELTA (version 5.0.4.4) and Bruker TopSpin (version 4.0.6) software packages.

Molecular weight measurements

To determine the MW of mannans, isolates from *C. albicans* and *C. auris* strains were subjected to high performance gel permeation chromatography (GPC) as previously described [63]. Using a Viscotek/Malvern GPC system, consisting of a GPCMax autoinjector fitted to a TDA 305 detector (Viscotek/Malvern, Houston, TX). System calibration was achieved using Malvern pullulan and dextran standards. Mannan isolates, ranging between 3 to 6 mg/mL, dissolved in mobile phase (50 mM of sodium nitrate, pH 7.3) were subjected to a 60-minute incubation at 60˚C, followed by sterile filtration (0.2 µm) and injected into the GPC (100-200 µL). Samples were analyzed in duplicate or triplicate and data analysis performed with Viscotek OmniSec software (version 4.7.0.406).

Binding interaction of mannans with PRRs rhDectin-2 and rhMannose

Assessment of mannan binding interactions to the recombinant Dectin-2 and Mannose receptors (R&D systems) were carried out on an Octet K2 BLI instrument (ForteBio) in 10X Kinetics Buffer (pH 7.4) at 30°C and 1000 rpm. Increasing concentration of the respective ligands (3.125-400 µg/mL) were used to generate respective saturation curves, after which the binding affinities were calculated for mannans isolated from *C. albicans* and *C. auris* strains as previously described [64]. The Ni-NTA biosensor was subjected to a 3-minute equilibration prior to 10 minutes of exposure 0.1 ug/mL HIS-tagged rhDectin-2 or rhMannose receptor proteins and final a 10-minute dissociation in 10X kinetics buffer for measuring the BLI signal, consistently 20 seconds after transferring. Subsequently followed by a series 8 similar 5-minute exposure to an increasing concentration (2-fold) of carbohydrate. To control for receptor dissociation during the experiment, a parallel biosensor with the immobilized receptors was placed in the 10X kinetics buffer without respective carbohydrate exposure.

Data analysis was performed using the GraphPad Prism 7.0 software and the dissociation constant K_D is presented as mean value with a 95% confidence interval.

ROS assay

The induction of ROS was measured by oxidation of luminal (5-amino-2,3, dihydro-1,4-phtalazinedione) and determined in an automated LB96V Microlumat plus luminometer (EG & G Berthold, Bad Wildbad, Germany). Briefly, PBMCs (5×10^5 per well) or neutrophils (2.5×10^5) per well were seeded into white 96-well plates and incubated in medium containing either RPMI, Zymosan (100 µg/mL), heat-killed opsonized *C. albicans* or *C. auris* yeast (10^7 CFU/mL). 20 µL of 1 mM Luminol was added to each well in order to start the chemiluminescence reaction. Each measurement was carried out at least in duplicate. Chemiluminescence was determined every 145 seconds at 37°C for 1 hour. Luminescence was expressed as relative light units (RLU) per second. The RLU/sec within the area under the curve (AUC) were plotted against time and analyzed by using Graphpad Prism v.7.0.

FITC-labelling of *Candida*

To label cells with fluorescein isothiocyanate (FITC, CAS Number: 3326-32-7), $1x10^8$/mL of thimerosal fixed cells were sonicated and resuspended in 0.1 mg/mL of FITC in 0.1 M carbonate-bicarbonate buffer (pH 9.6). After incubation for 30 minutes on a tube roller at 4°C in the dark, unbound FITC was washed away by centrifugation at 3000 rpm 4°C for 10 minutes three times in PBS. Before use, cells were resuspended to a concentration of $1x10^7$/mL in PBS, aliquoted and stored in the dark at -20°C.

Phagocytosis assay in human cells

To test *Candida* strains uptake by human monocytes, 4×10^6 cells/mL of thimerosal-killed FITC-labelled *C. albicans* and *C. auris* were pre-opsonized with 20% human pooled serum for 1

hour at 37°C, 5% and subsequently incubated with 2 × 10^5 PBMCs/well (MOI 2:1, fungal:human cells) for either 30 minutes or 2 hours at 37°C, + 5% CO_2. After the incubation period, cells were gently washed with PBS (1% BSA) and then stained in a total volume of 50 µL using CD14 monoclonal antibody (Mouse-anti-Human CD14 Pacific Blue, Beckman coulter, clone RMO52, dilution 1:20) for 30 minutes at 4°C on ice in the dark. Afterwards, cells were washed, and the fluorescence signal of extracellular non-phagocytosed *Candida* was quenched with 0.1% Trypan blue solution (Sigma, St. Louis, USA; CAS Number 72-57-1). Cells were subsequently measured on a CytoFLEX flow cytometer (Beckman coulter, Pasadena, USA) and the data were analyzed using the Kaluza Analysis software version 2.1. To determine the uptake of *C. albicans* and *C. auris* by human monocytes, the percentage of CD14 positive cells which had phagocyted FITC positive *Candida* percentage of FITC-positive cells in the CD14-positive population) was calculated. For the detailed gating strategy see Extended data Fig. 7a.

Phagocytosis assay in BMDMs

Bone marrow was extracted from femurs and tibias of eight-week-old male C57BL/6 mice and differentiated for 7 days with RPMI Medium 1640 Glutamax (Gibco) supplemented with 10% heat-inactivated foetal calf serum, 100 U/mL Penicillin/Streptomycin and 15% L929 cell-conditioned medium at 37°C with 5% CO_2. BMDM were added to 8 well u-slide (ibidi, Gräfelfing, Germany) at 0.5 x10^5 cells per well to adhere overnight. *C. albicans* and *C. auris* strains were prepared by growing cells for 24 hours in Sabouraud broth at 30°C and followed by 3 washes in PBS. Fixed *Candida* cells were prepared by incubating the Sabouraud-grown yeast overnight at room temperature in 50 Mm thimerosal (Sigma, St. Louis, USA) followed by 5 wash steps in PBS. Phagocytosis dynamics were determined following the addition of 3:1;

yeast: BMDM. The intended Multiplicity of Infection (MOI) was calculated using cell count (hemocytometer) and the actual MOI was observed from videos. Live imaging of macrophage interactions with live or fixed *C. albicans* and *C. auris* were performed using a Nikon Ti Eclipse microscope with objective 20x magnification set to acquire images at 1-minute intervals using Volocity software (Version 6.3, PerkinElmer, Waltham, USA), with thanks to the University of Aberdeen Microscopy Core Facility. Movies generated from 3 hour interactions were analyzed to determine over time the proportion of macrophages phagocytosing yeast (% uptake), the number of yeast phagocytosed per 100 macrophages (phagocytic index), the proportion of macrophage death after 3 hours (macrophage lysis) and the distribution of yeast contained within individual macrophages. Experiments were performed on 3 occasions, with a total of 9 movies generated per condition. Phagocytic index data are based on yeasts fully inside macrophages. Yeasts adhering but not internalized would not be included in cell counts for phagocytic index. Statistical analyses were performed by ANOVA using GraphPad Prism (v 7.0).

Cell wall staining

Fixed *Candida* yeast were stained for exposed cell wall β-glucans using Fc-Dectin-1 (a gift from Gordon Brown, University of Aberdeen) and secondary F(ab')2 anti-human IgG AlexaFluor 488 conjugate (Life Technologies). ConA-Texas Red conjugate (Life Technologies, Carlsbad, USA) was used to detect cell wall mannans. Cells were counted, and 2.5 x 10^6 yeast were combined with FACS wash (1% bovine serum albumin and 5 mM EDTA in PBS) with either Fc-Dectin-1 at 1 μg/mL or ConA at 25 μg/mL. After a 30 min incubation on ice, cells were washed twice in FACS wash, then incubated with secondary F(ab')2 (for Fc-Dectin-1 only) on ice for 45 minutes, with a further 2 wash steps. Flow cytometry was performed on an LSR Fortessa

cytometer (BD, Macquarie Park, Australia) with thanks to the University of Aberdeen IFCC Core Facility. Data were analysed using FlowJo software (version 10.0.8). For the detailed gating strategy see Extended Data Fig. 7b.

In vivo experimental model of disseminated candidiasis

For the *in vivo* experiments *C. auris* strain 10051895 was selected as representative of Clade I, which was the first *C. auris* clade identified and associated with bloodstream infections and high mortality rates [17]. Experiments were conducted with a total of 200 C57Bl6 male mice, 7-8 weeks old, which were purchased from Pasteur Institute (Athens, Greece, EL 25 BIObr 011). Mice were allowed to acclimate for seven days before start of the experiments. Animals were housed in cages, with no more than 5 mice per cage, under constant temperature (21°C) and humidity with a 12-h light/dark cycle. All animals had ad libitum access to food and water. Analgesia was achieved with paracetamol suppositories. Other analgesics were avoided in order to avoid interactions with the immune system. Healthy mice were i.v. challenged via the tail vein with $1x10^7$ CFU/mouse log-phase inoculum [46,47] of *C. albicans* 10061110 (n=11) and *C. auris* 10051895 (n=10) following slight anesthesia with methoxyflurane (2, 2-dichloro-1,1 difluoroethyl methyl-ether in butylated hydroxytoluene 0.01% w/w). Mice were split into groups via a randomization table. Survival was recorded for 14 days; three- and seven-days post challenge mice were sacrificed by the intramuscular injection of ketamine. For evaluating the fungal burden at day 3 and day 7 from the inoculation of $1x10^6$ CFU/mice (n=4-5 mice per group per timepoint), we removed kidneys and livers were weighted and homogenized. The number of fungal counts were measured via serial dilutions 1:10 at 0.9% saline and expressed as log10 CFU/g. For collecting the organs homogenates, at day 3 and day 7 from the injection

of $1x10^6$ Candida CFU/mice (n=5-6 mice per group per timepoint), after a midline incision under aseptic conditions, the entire spleen was removed and segments of the right kidney and of the liver were cut and put into separate sterile containers.

Statistical analysis

Statistical analysis, except when otherwise indicated, was performed using the Graphpad Prism 7 software. All experiments were performed at least in duplicate. In experiments with a sample size <4, no statistical testing was performed due to a small sample size. Datasets with a sample size >8 were tested for normality via the D'Agostino-Pearson omnibus normality test, and when normally distributed subjected to a 1-way ANOVA including Holm-Sidak's multiple comparison test, as specified in corresponding method section and figure captions. Given the experimental set-up, where the same donors were used, yet exposed to different *Candida* species and strains (stimulations), measurements were considered paired/dependent. Hence, the Wilcoxon signed-rank test for non-parametric matched data was applied to non-normally distributed data. To enhance insight in the *C. auris'* induced host response, all statistical analysis was performed comparing respective *C. auris* strains with *C. albicans*, in the current study considered the control species. In the case of non-normally distributed data and comparison to multiple control strains, a Kruskall Wallis test including Dunn's multiple comparison was applied. For statistical testing of *IL-6*, *IL-1B,* and *IL-1RN* expression levels, an ordinary 1-way ANOVA was performed comparing the Log2 Fold Changes and corresponding lfcSEs between the respective *C. auris* and *C. albicans* (live, β-glucans and mannans) conditions. Moreover, *in vivo* mice data presents an exception, with a

Log rank-test for survival assessment and Mann Whitney U test to assess fungal burden due sample independence. In all cases, a p-value of <0.05 was considered significant.

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DATA AVAILABILITY

Requests for materials should be addressed to the corresponding autor (M.G.N.). The datasets generated from this study are accessible through GEO Series accession number

GSE154911 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154911). Source data is provided for main and extended data figures.

COMPETING INTERESTS

The authors declare no competing interests.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.F.M., D.L.W., M.G.N.; Methodology, M.B., S.K., J.M.B., M.J., D.R., M.D.K., D.W.L., M.Z., Y.N.J., A.C., A.H., N.A.R.G., A.J.P.B., J.F.M., D.L.W., M.G.N.; Investigation, M.B., S.K., J.M.B., M.J, D.R., D.W.L.,P.R., B.G., F.L.V., B.J.K., E.J.B., G.R., A.H, N.A.R.G, A.J.P.B., D.L.W.

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Main Figure Legends

Figure 1 | Comparative analysis of the general and clade-specific *C. auris* induced host response with *C. albicans* at 24 hours. a, Venn diagram representing the number of DEG of both *Candida* species and their relative overlap, reveals substantial overlap between the *C. albicans* (10061110) and *C. auris* (KCTC17810, clade II) live induced host-response at 24 hours. DEG were subjected to a pathway enrichment analysis, in turn revealing the top 15 *Candida* intrinsic (overlapping DEG, middle panel) and species specific (DEG unique for *C. albicans*, left panel; DEG unique for *C. auris*, right panel) pathways. Enrichment determined using Consensus PathDB, including pathways as defined by KEGG (red) and Reactome (pink), considering a p-adjusted value < 0.01 (indicated as 'q-value') significant. Size of the geometric points indicates the amount of DEG in relation to the pathways' size. The exact q values and DEG in pathways can be found in Supplementary Table 2. **b-c,** *C. auris* is a more potent inducer of the immune system in comparison to *C. albicans*. **b**, TNF-α, IL-6, IL-1β, and IL-1Ra levels in supernatants of PBMCs after stimulation without (RPMI; negative control) or with live *C. albicans* and *C. auris* for 24 hours (n=12). **c**, TNF-α, IL-6, IL-1β, and IL-1Ra levels in supernatants of PBMCs after stimulation without (RPMI; negative control) or with live *C. albicans* and *C. auris* from all five geographical clades for 24 hours (n=8). Graphs represent mean ± SEM, pooled from at least two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided Wilcoxon matched pairs signed-rank test was performed comparing respective *C. auris* strains with *C. albicans* as control or reference species. The data used to make this figure can be found in Source Data Fig.1.

Figure 2 | Evaluation of *C. auris* phagocytosis dynamics by human and murine host immune cells and heat-sensitivity of the cell wall component responsible for *C. auris* induced cytokine production. **a**, The BMDM phagocytic capacity of live *C. albicans* or *C. auris* strains in a 3-hour period. BMDM engulfment, depicted as the percentage of macrophages having phagocytosed at least one fungal cell (left). Phagocytic index was considered the number of fungal cells engulfed per 100 macrophages (right); graphs represent mean (n=9), pooled from at least two independent experiments. **b**, Phagocytosis assay in human PBMCs: percentage of FITC-positive cells in the $CD14^+$ population. Phagocytosis efficiency assessed as percentage of $CD14^+$ cells that engulfed FITC-labelled *Candida* (left) and corresponding mean fluorescence intensity (MFI) of the total $CD14^+$ population (right). Graphs represent mean ± SEM (n=6), pooled from two independent experiments, * $p < 0.05$, two-sided

Wilcoxon matched pairs signed-rank test, comparing respective *C. auris* strains with *C. albicans* as control or reference species. **c**, Distribution of phagocytosed live fungal cells per macrophage in a period of 3 hours, n≥100 observations per condition. **d**, Killing capacity of live *C. albicans* and *C. auris*, depicted as the percentage of lysed macrophages (BMDM) after 3 hours of exposure. Yeast:Macrophage ratio (MOI) was 3:1. Graphs represent mean ± SEM, n = 9 (n=10 for *C. auris* 10051895), pooled from at least two independent experiments, * $p < 0.05$, Kruskall Wallis test with two-sided Dunn's multiple comparison between the two *C. auris* strains and the two *C. albicans*. **e**, TNF-α (n=10), IL-6 (n=13), IL-1β (n=13), and IL-1Ra (n=9) levels in supernatants of PBMCs after stimulation without (RPMI; negative control) or with heat-killed *C. albicans* or *C. auris* for 24 hours. Graphs represent mean ± SEM, data are pooled from at least two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided Wilcoxon matched pairs signed-rank test, comparing respective *C. auris* strains with *C. albicans* as control or reference species. Data used to make this figure can be found in Source Data Fig. 2.

Figure 3 | Mannans are fundamental for orchestrating the *C. auris* induced late host response. **a**, Split Venn diagrams indicating the number of DEG upon *C. albicans* 10061110 (left) and *C. auris* KCTC17810 (clade II; right) live stimulation on the left, with its respective overlap between exposure to the purified cell wall components β-glucan and mannan. Left split Venn diagram visualizes the early, 4-hour response, and the right split Venn diagram reflects the late, 24-hour response. **b**, PBMC production of cytokines TNF-α, IL-6, IL-1β, and IL-1Ra after 24 hours stimulation without (RPMI; negative control) or with purified β-glucans from *C. albicans* and *C. auris* strains in the presence of 10% human serum, n=6 (n=3 for *C. auris* β-glucan 10051244). **c**, PBMC production of TNF-α, IL-6, IL-1β, and IL-1Ra after 24-hour stimulation without (RPMI; negative control) or with Pam3cys and/or purified β-glucans from different *C. albicans* and *C. auris* strains in the presence of 10% human serum, n=6. **d**, PBMC production of cytokines TNF-α, IL-6, IL-1β, and IL-1Ra after 24 hours stimulation without (RPMI; negative control) or with purified mannans from *C. albicans* and *C. auris* strains in the presence of 10% human serum, n=10. Graphs represent mean ± SEM, pooled from at least two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed Wilcoxon matched pairs signed-rank test and was performed comparing both cell wall components extracted from *C. auris* strains with *C. albicans* as control or reference species (SC5314). Data used to make this figure can be found in Source Data Fig. 3.

Figure 4 | Structural characterization of *C. auris* mannans. (**A**) 2D COSY Nuclear magnetic resonance (NMR) spectroscopy analysis of mannans purified from various *C. auris* clinical strains, originating from clades I, II and IV, and *C. albicans*. Although NMR reveals varying side-chain lengths containing α-1,2-mannose, α-1,3-mannose and β-1,2-mannose across clinical isolates, characteristic for *C. auris* mannans are the two distinct M-α-1-phosphate side chains.

Figure 5 | Exploration of PRR and signaling pathways involved in the *C. auris* induced host cytokine production.
a, PBMC production of cytokines TNF-α, IL-6, IL-1β, and IL-1Ra after 24 hours stimulation without (RPMI; negative control) or with PFA-fixed *C. albicans* and *C. auris* strains, subjected to vehicle (DMSO) (n=9) or a 1-hour pre-incubation with Syk (n=6) and Raf-1 (n=9) inhibitors. **b**, PBMC production of

cytokines TNF-α, IL-6, IL-1β, and IL-1Ra after 24 hours stimulation without (RPMI; negative control) or with live *C. albicans* and *C. auris* strains, subjected to a 1 hour pre-incubation with the isotype antibodies IgG2b, Goat IgG and IgG1, or DC-SIGN, Dectin-1, Mincle, MMR, CR3 and Dectin-2 blocking antibodies, n=6. Graphs represent mean ± SEM, data pooled from at least two independent experiments, * $p < 0.05$, ** $p < 0.01$, **a** two-sided Wilcoxon matched pairs signed-rank test comparing (within each *Candida* strain) the respective inhibitor with its vehicle, **b** two-sided Wilcoxon matched pairs signed-rank test comparing (within each *Candida* strain) the neutralizing antibodies with the correspondent isotype controls. Data used to make this figure can be found in Source Data Fig. 5.

Figure 6 | *C. auris* is less virulent than *C. albicans* in an experimental model of murine disseminated candidiasis. a, Survival curve of immunocompetent mice i.v. challenged with *C. albicans* (n=11) or *C. auris* (n=10). Mice were i.v. injected with $1x10^7$ CFU of the respective *Candida* strain and monitored daily. **b**, Fungal burden of immunocompetent mice i.v. challenged with $1x10^6$ CFU of *C. albicans* (day 3, n=5; day 7, n=4) or *C. auris* (day 3, n=5; day 7, n=5) in the liver and kidney at 3 (top) and 7 (bottom) days post injection. **c-d**, KC (**c**) and MPO (**d**) production in supernatants from liver, kidney and spleen homogenates (n=6 per group per time-point). **e,** KC production in plasma of mice (n=6 per group per time-point) infected i.v. with $1x10^6$ CFU of *C. albicans* or *C. auris*. Graphs represent mean ± SEM, data pooled from at least two independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **a**, Mantel–Cox two-sided log-rank test, **b-e** two-sided Mann-Whitney U test. **f**, Ratio of the mean MPO or KC production (log scale, data from Figure 6d, 6c) to the mean of fungal burden (log scale, data from Figure 6b) in kidney (blue bars) and liver (white bars) of mice infected with *C. albicans* or *C. auris*. Data are represented as the ratio of the mean log MPO and KC values (n=6 per group per time-point), to the mean log CFU (*C. albicans*: day 3, n=5; day 7, n=4. *C. auris:* day 3, n=5; day 7, n=5). Data used to make this figure can be found in Source Data Fig. 6.