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Differential Adaptation of Candida albicans In Vivo Modulates Immune Recognition by Dectin-1


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Abstract

The β-glucan receptor Dectin-1 is a member of the C-type lectin family and functions as an innate pattern recognition receptor in antifungal immunity. In both mouse and man, Dectin-1 has been found to play an essential role in controlling infections with Candida albicans, a normally commensal fungus in man which can cause superficial mucocutaneous infections as well as life-threatening invasive diseases. Here, using in vivo models of infection, we show that the requirement for Dectin-1 in the control of systemic Candida albicans infections is fungal strain-specific; a phenotype that only becomes apparent during infection and cannot be recapitulated in vitro. Transcript analysis revealed that this differential requirement for Dectin-1 is due to variable adaptation of C. albicans strains in vivo, and that this results in substantial differences in the composition and nature of their cell walls. In particular, we established that differences in the levels of cell-wall chitin influence the role of Dectin-1, and that these effects can be modulated by antifungal drug treatment. Our results therefore provide substantial new insights into the interaction between C. albicans and the immune system and have significant implications for our understanding of susceptibility and treatment of human infections with this pathogen.

Introduction

The immune system of healthy individuals has effective mechanisms for preventing fungal infection, yet immunosuppressive infections, such as HIV/AIDS, and modern immunosuppressive and invasive medical interventions can substantially increase the risk of infection with numerous fungal pathogens. Candida albicans is one such microorganism, which is normally found as a commensal on host epithelial surfaces, colonizing more than 50% of individuals [1]. However, in severely immunocompromised patients, those enduring invasive clinical procedures or those requiring extended stays in intensive care units, Candida species are the most important etiological agent of life-threatening, invasive systemic and bloodstream fungal infections [1].

In addition to life-threatening invasive infections, superficial mucocutaneous infections with Candida species are also common, even in immunocompetent individuals. While the underlying mechanisms are still incompletely understood, a link to defects in Th17 immunity have been identified in some patients [2]. Indeed, mutations in STAT1, STAT3, IL-17 and IL17RA all result in susceptibility, especially to mucocutaneous candidiasis [2]. Furthermore, C-type lectin receptors (CLR) and their intracellular signalling pathways, particularly the CARD9 pathway, are now recognized to play a predominant role in driving these and other protective antifungal immune responses [3]. CLRs are required for the recognition and ingestion of fungi by phagocytes, the induction of antimicrobial effector mechanisms and inflammatory mediators, and they direct and modulate adaptive immunity, including Th17 responses [3].

One such CLR is the β-glucan receptor, Dectin-1, which can mediate multiple cellular functions through its cytoplasmic signalling domain including phagocytosis, the respiratory burst,

Strain-Specific Recognition by Dectin-1

Author Summary

Dectin-1 is a pattern recognition receptor recognizing the fungal cell-wall component, β-glucan, and plays an essential role in controlling C. albicans infections in both mouse and man. Candida albicans is part of the normal human microflora, yet is capable of causing superficial mucosal infections as well as life-threatening invasive diseases, particularly in patients whose immune function is compromised. Here we found that the contribution of Dectin-1 is limited to specific strains of C. albicans; effects which are due to the differential adaptation of these pathogens during infection. Importantly, C. albicans strains showed variations in both the composition and nature of their cell walls, and it was these differences which influenced the role of Dectin-1. Crucially, we found that we could alter the fungal cell wall, and subsequent interactions with the host, using antifungal drugs. These findings have substantial implications for our understanding of the factors contributing to human susceptibility to infections with C. albicans, but also treatment strategies.

and the production of soluble factors, such as cytokines, chemokines and eicosanoids [3]. The importance of Dectin-1 has been exemplified in our recent studies that have implicated it in the control of antifungal immunity in humans [4,5]. An essential role for Dectin-1 has also been demonstrated in mouse models by several groups, where loss of Dectin-1 resulted in a failure to mount protective inflammatory responses and an inability to control fungal growth [6,7,8,9,10]. During infection with C. albicans, these defects resulted in systemic and mucosal susceptibility [6,7,8,9]. Surprisingly another group found no role for Dectin-1 in immunity to systemic infections with this pathogen, however, this study was performed using a different strain of C. albicans and a different mouse genetic background [11]. As innate recognition of C. albicans can be influenced by both fungal and mouse strain [9,12,13,14], we have explored the contribution of these variables to Dectin-1-mediated recognition of this pathogen.

Results

Dectin-1 dependency is Candida albicans strain specific

Using 129Sv mice, we had found that Dectin-1 was essential for controlling systemic infection with C. albicans strain SC5314 [6]. In contrast, Iwakura’s group had found that Dectin-1 was not required for the control of this pathogen; their experiments being performed using C57BL/6 mice and the C. albicans strain ATCC18804 [11]. To explore the contribution of mouse and/or fungal strain to the Dectin-1-mediated recognition of this pathogen, we backcrossed our 129/Sv background knockout mice for nine generations onto the C57BL/6 background (data not shown), and then assessed the role of Dectin-1 in both mouse backgrounds with both strains of C. albicans. Consistent with our earlier results [6], Dectin-1−/− mice (cle7A−/−) on a 129/Sv background showed significantly enhanced susceptibility to infection with C. albicans SC5314 (Figure 1A). Moreover, similarly enhanced susceptibility to this strain of C. albicans was also observed in the knockout mice on a C57BL/6 background. In contrast, loss of Dectin-1 had no effect on susceptibility to C. albicans ATCC18804 on either murine background (Figure 1B). Thus Dectin-1-mediated control of systemic C. albicans infection is not dependent on the genetic background of the mice.

Strains of C. albicans have been subdivided into 17 clades based on multilocus sequence typing [15] and as the two strains used in these experiments (SC5314 and ATCC18804) come from different clades (1 and 3, respectively), we explored the possibility that Dectin-1 dependency is associated with different clades of C. albicans. Wild type and Dectin-1−/− mice were infected with representative isolates from several clades (listed in Table S1), as well as two additional isolates from clade 1 (i.e. the same clade as SC5314), and survival of these animals was assessed over time. Dectin-1 was required to control infection with roughly half of the strains tested (Figure 2A), as evidenced by significantly increased mortality in the knockout mice. However, this receptor was not required to control infection with other strains from clade 1 (Figure 2B). Thus we conclude that Dectin-1 dependency is C. albicans strain-specific, but does not correlate with particular clades of this pathogen.

Strain-specific Dectin-1-dependency corresponds with enhanced fungal burdens and dysregulated cytokine responses during systemic infection

To gain further insight into the mechanisms underlying the difference in Dectin-1 dependency between C. albicans SC5314 and ATCC18804, we examined fungal burdens and cytokine responses in the kidneys of infected wild-type and Dectin-1−/− mice. These analyses were performed on day 9, a time point chosen when the animals were just starting to succumb to the infection (see Figure 1). In line with our survival analysis, we observed significantly higher fungal burdens in the Dectin-1−/− animals infected with C. albicans SC5314, using both high-dose (Figure 3A) and low-dose inocula (Figure 3B). Moreover, these mice also had substantially altered levels of cytokines known to be critical for controlling C. albicans infection, as we reported previously [6] (Figure 3C). In contrast, loss of Dectin-1 had no effect on fungal burdens or cytokine responses in mice infected with C. albicans ATCC18804 (Figure 3A–C).

We also assessed the possibility that these in vivo differences may be related to the virulence of the individual strains. Indeed, a 4- to 10-fold higher inoculum of ATCC18804 was required to induce a level of mortality in mice that was roughly equivalent to that of SC5314 (Figure 1 and Table S1). We therefore examined kidney fungal burdens and cytokine responses with the Dectin-1-independent s20175.016 C. albicans strain, which was roughly equivalent in virulence to SC5314 (Figure 2 and Table S1). However, as we observed for ATCC18804, neither fungal burdens nor cytokine responses were affected by Dectin-1 deficiency during infection with s20175.016 (Figure 3A–C). Taken together, these results demonstrate that the absence of Dectin-1 results in substantially increased fungal burdens, dysregulated cytokine responses and enhanced susceptibility to infection, but only with specific strains of C. albicans.

Dectin-1 dependency cannot be recapitulated in vitro

Given the clear-cut difference in Dectin-1 dependency between infections with SC5314 and ATCC18804 in vivo, we reasoned that there might be substantial differences in the cell wall β-glucan content of these fungal strains. However, the cell walls of these strains grown in vitro revealed nearly identical levels of glucosamine, glucose and mannose, reflecting equivalent amounts of chitin, β-glucan and mannan, respectively (Figure 4A). To confirm this crude biochemical analysis and verify that the cell walls of SC5314 and ATCC18804 were also similar from an immunological perspective, we compared recognition of these fungal strains using wild type and Dectin-1−/− thiglycollate-elicted macrophages in vitro. Using fluorescently labelled live yeast cells, and zymosan particles as a positive control [6], we demonstrated that...
Figure 1. Dectin-1 dependence is related to fungal strain and not mouse background. Survival analysis of 129/Sv and C57BL/6 wild-type and Dectin-1<sup>−/−</sup> mice following infection with (A) C. albicans SC5314 or (B) ATCC18804. The data shown are representative of at least two independent experiments. Also shown are number of animals per group (n) and dosage used for infection. *; p<0.05. See also Table S1. doi:10.1371/journal.ppat.1003315.g001

Figure 2. Dectin-1 dependence is not related to the clade of C. albicans. Survival analysis of 129/Sv wild-type and Dectin-1<sup>−/−</sup> mice following infection with strains from (A) several different clades of C. albicans, or (B) two additional isolates from clade 1 as indicated. The data shown are representative of at least two independent experiments. Also shown are number of animals per group (n) and dosage used for infection. *; p<0.05. See also Table S1. doi:10.1371/journal.ppat.1003315.g002
they bound to macrophages at equivalent levels. Importantly, the binding of both strains and zymosan was Dectin-1 dependent (Figure 4B). Consistent with these observations, both strains induced inflammatory cytokine responses, determined by measuring TNF, to similar levels and in a Dectin-1 dependent manner (Figure 4C). Moreover, we demonstrated the Dectin-1 dependent nature of recognition of these strains using human monocytes deficient in Dectin-1, isolated from individuals homozygous for the Y238X polymorphism of the receptor [5] (Figure 4D). Thus the yeast forms of these strains demonstrate an equivalent requirement for Dectin-1 recognition in vitro.

The exposure of β-glucans on C. albicans, and hence recognition by Dectin-1, may be restricted to the yeast form of this fungus [16], although this is controversial [17]. Nevertheless, we explored the possibility that the strain-linked dependence for Dectin-1 may stem from differential recognition of fungal hyphae, particularly as this morphological form is abundant in kidney lesions in vivo [6]. However, as we found for yeast cells, co-culture of thioglycollate-elicited macrophages with pre-formed live C. albicans hyphae of both strains induced similar levels of TNF (Figure 4E). Importantly, these responses were equivalently Dectin-1 dependent. Similar results were also obtained when the hyphae were killed using UV-irradiation (see Figure 5B). Thus the exposure of β-glucans on in vitro generated hyphae, and subsequent recognition by Dectin-1, does not differ between the two strains of C. albicans.

We then considered the possibility that differential exposure of β-glucans by these C. albicans strains may be occurring under conditions of hypoxia, mimicking the conditions in vivo and which we have shown to alter cell wall β-glucan content and immune recognition of Aspergillus [18]. We therefore first examined the growth of several C. albicans strains from different clades and observed no obvious effects of hypoxia on the growth of these organisms, when compared to cells grown under normoxic conditions (Figure 5A). Subsequently, we examined the inflammatory response of wild type and Dectin-1−/− thioglycollate-elicited macrophages to UV-irradiated SC5314 and ATCC18804

Figure 3. Loss of Dectin-1 is associated with increased fungal burdens and dysregulated cytokine responses with specific strains of C. albicans. Fungal burdens in the kidneys of 129/Sv wild-type and Dectin-1−/− mice at day 9 post-infection with a (A) high or a (B) low dose of various C. albicans strains, as indicated. (C) Characterisation of cytokine levels in the kidneys of 129/Sv wild-type and Dectin-1−/− mice at day 9 post-infection with a high dose (as in A) of various C. albicans strains, as indicated. Shown are data from two pooled (A, B) and one representative (C) experiment. Each experiment involved 7–10 mice per group. Bar indicates the mean.*; p<0.05.
doi:10.1371/journal.ppat.1003315.g003
Figure 4. The dependence of Dectin-1 cannot be recapitulated in vitro. (A) Cell wall biochemical composition of in vitro grown *C. albicans* SC5314 and ATCC18804 yeast cells, as indicated. (B) Relative binding of fluorescently-labelled live *C. albicans* yeast cells or zymosan (Zy) to C57BL/6 wild-type and Clec7A<sup>−/−</sup> thioglycollate-elicited peritoneal macrophages, as indicated. (C) Measurement of TNF in culture supernatants from C57BL/6 wild type versus Clec7A<sup>−/−</sup> peritoneal macrophages after stimulation with live *C. albicans* yeast cells or zymosan (Zy), as indicated. (D) Measurement of TNF and IL-6 responses from homozygous Y238X patients after stimulation with *C. albicans* yeast cells or heat-killed yeast (HK; strain ATCC MYA-3573), as indicated. Results were normalized to the treated cells from normal individuals. (E) Measurement of TNF responses from 129S/Sv wild type versus Clec7A<sup>−/−</sup> peritoneal macrophages after stimulation with live *C. albicans* hyphae, as indicated. Data shown are means ± SEM of pooled data from at least two independent experiments, except for (E), which is the mean ± SD of a representative experiment.

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Figure 5. Dectin-1-responsiveness but not dependence is induced by hypoxia in vitro. (A) Growth of various strains of *C. albicans* under normoxia or hypoxia, as indicated. (B) Measurement of TNF responses from C57BL/6 wild type versus Clec7A<sup>−/−</sup> peritoneal macrophages after stimulation with UV-irradiated *C. albicans* yeast or hyphae, grown under normoxic or hypoxic conditions, as indicated. (C) Quantitation of β-glucan exposure on UV-irradiated *C. albicans* hyphae, grown in vitro under conditions of normoxia or hypoxia, as indicated, and stained with soluble Dectin-1. (D) Fold change in FKS gene expression in *C. albicans* hyphae under normoxic versus hypoxic conditions, as indicated. Data shown (mean ± SD) are from one representative experiment.

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Dectin-1

C. albicans fungal cells isolated from the infected kidneys of C57BL/6 mice at day 9 and stained with (Figure 6). In all cases, there were no substantial differences in TNF production between SC5314 and ATCC18804, and these responses all required Dectin-1. Interestingly, yeast and hyphal cells from both strains that were grown under hypoxia were more inflammatory, inducing much higher levels of TNF and displaying a greater Dectin-1 dependency (Figure 5B). Consistent with these observations, when grown under hypoxia, we detected higher levels of exposed β-glucans on cells from both strains under these conditions (Figure 5C), which correlated with an increased expression of genes involved in β-glucan synthesis (Figure 5D). Thus, we conclude that the strain dependency for Dectin-1 is not recapitulated in vitro, at least using the parameters examined here.

Dectin-1 dependency is not related to the level of fungal β-glucan exposure in vivo

Following infection of mice, C. albicans cell-wall β-glucans are thought to be initially masked and only become exposed after several days [17]. Indeed, early during infection, at day 3, we observed that fungal burdens (Figure 6A) and cytokine responses (Figure 6B) were not influenced by the loss of Dectin-1, irrespective of the C. albicans strain. This suggested that the fungal-strain dependency for Dectin-1 could be related to differential unmasking of β-glucans at later time points in vivo. To explore this, we examined the exposure of β-glucans in vivo at day 9 after infection, a time point when these carbohydrates become exposed [17], and where we had observed significant differences in fungal burdens and cytokine responses (see Figure 2). Surprisingly, when staining with an anti-β-glucan antibody, we observed equivalent exposure of β-glucans in both strains (Figure 6C). To verify that this result reflected immunological recognition by Dectin-1, we also examined β-glucan exposure using a soluble version of this CLR as a probe (Figure 6D). In fact, using this approach, we found that the ATCC18804 strain exposed significantly more β-glucan than SC5314. Thus, these results paradoxically suggest that the strain-linked Dectin-1 dependence observed in vivo does not correlate with the level of exposed β-glucan.

Dectin-1-mediated recognition is linked to cell wall composition and architecture in vivo

Interactions of C. albicans with the innate immune system involve many different fungal cell wall pathogen associated molecular patterns (PAMPs) [19]. We therefore tested whether induced modifications in the cell wall structure, and hence PAMP exposure, would alter innate recognition of the ATCC18804 strain. For these experiments we examined the effects of caspofungin, an echinocandin antifungal drug whose actions significantly change cell wall architecture and have previously been shown to influence β-glucan exposure [17,20]. As before, equivalent fungal burdens were observed in the kidneys of wild type and Dectin-1−/− mice infected with ATCC18804 (Figure 7A). However, while treatment with caspofungin significantly reduced fungal burdens in the kidneys of wild-type mice, it had no effect on colonization in the kidneys of the Dectin-1−/− animals; i.e. the clearance of this organism following treatment with caspofungin was now Dectin-1-dependent (Figure 7A). Thus changes in cell wall architecture can alter the dependency on Dectin-1, following infection with an otherwise Dectin-1-independent strain of C. albicans.

To gain insight into the cell wall components that contribute to the Dectin-1-independence of ATCC18804, we compared the expression profiles of a range of selected genes in both ATCC18804 and SC5314 cells isolated from the kidneys of infected wild-type and Dectin-1-knockout mice. The genes

![Figure 6](https://journal.ppat.1003315.g006)

**Figure 6. Dectin-1 dependence is not related to β-glucan exposure in vivo.** (A) Fungal burdens in the kidneys of 129/Sv wild-type and Dectin-1−/− mice at day 3 post-infection with various C. albicans strains, as indicated. (B) Characterisation of cytokine levels in the kidneys of 129/Sv wild-type and Dectin-1−/− mice at day 3 post-infection of various C. albicans strains, as indicated. Images and quantitation of β-glucan expression on fungal cells isolated from the infected kidneys of C57BL/6 mice at day 9 and stained with (C) anti-β-glucan antibodies or (D) soluble Dectin-1 (FcDectin-1). Control cells were stained with secondary antibody only. Data shown are from a representative experiment. Bar indicates the mean; *p<0.05, ns, not significant.

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examined encoded many cell-wall-associated and secreted functions, including genes involved in chitin and β-glucan biosynthesis, cell wall remodelling, adhesion, secreted proteases, iron assimilation, and transcription factors that regulate cell wall biogenesis (Table S2). We found that the expression of many of these genes did not differ significantly between these strains, or during infection in wild-type versus Dectin-1−/− mice. However, several cell-wall-associated genes were differentially expressed under these conditions. Interestingly, the ATCC18804 strain appeared more adaptable to the immunological status of the host, as suggested by the relatively large subset of ATCC18804 genes (60%) that displayed significant differences in expression in the wild-type versus the Dectin-1−/− animals (Figure 7B, right panel). Notably, the virulence related genes ALS3, CAE2, and SAP5 were expressed at lower levels in ATCC18804 versus SC5314, in both wild-type and Dectin-1−/− animals, possibly reflecting the differences in virulence of these strains (Figure 7B and see Figure 1).

Of particular interest was the chitin synthase gene CHS3 (Figure 7B, red arrow), which was strongly and reproducibly up-regulated in ATCC18804, versus SC5314, but only in wild type mice. Furthermore, two other genes implicated in chitin regulation (PGA31 and CHT2, [21]) also displayed significant differential expression between strains. This suggested that there may be differences in chitin levels in ATCC18804 and SC5314 in vivo. To explore this possibility, we determined the levels of this cell-wall component in fungal cells isolated directly from infected mouse kidneys using Calcoflour White. Consistent with the increased expression of these chitin-related genes described above, we detected significantly elevated chitin levels in ATCC18804 cells compared to SC5314 cells (Figure 7G). Moreover, TEM analysis revealed substantial differences in the architecture of their cell walls (Figure 7D).

These data indicate that the Dectin-1-dependent differences in pathogenicity of SC5314 and ATCC18804 correlate with differences in chitin levels during infection in vivo. To test whether differences in chitin levels cause differential Dectin-1 dependence, we exploited our previous observation that in vitro treatment of C. albicans with Calcoflour White and CaCl2 induces high-chitin levels (Figure 7E) which can be maintained in vivo by treating mice with caspofungin [22]. Mirroring our in vivo findings (see Figure 6), we observed increased β-glucan exposure on high-chitin containing cells generated in vitro (Figure 7F). Using these in vitro treated fungal cells, we observed significantly increased resistance of Dectin-1−/− mice following infection with high-chitin SC5314 (Figure 7G), compared to normal-chitin containing cells. In other words, the control of infection with high-chitin cells showed a reduced dependence on Dectin-1. Furthermore, using a model of peritoneal infection, we found that the inflammatory response to high-chitin SC5314 cells was Dectin-1 independent (Figure 7H).

In contrast, the peritoneal inflammatory response to SC5314 cells with normal chitin levels required Dectin-1, as we had shown previously [6]. We conclude that variation in adaptability of individual strains of C. albicans in vivo results in substantial differences in cell wall architecture and exposure of PAMPs, particularly chitin, which significantly influences the innate recognition pathways utilized by the host during infection.

Discussion

Fungal strain-dependent requirement for Dectin-1 in protective anti-Candida immunity

Direct comparisons of previous studies on the role of Dectin-1 have been complicated by the use of different strains of C. albicans and different mouse genetic backgrounds [6,11]. While mouse background can influence the immune response to Candida [23,24], we have found that the discrepancies between these earlier studies on Dectin-1 stems from the use of different strains of C. albicans. Indeed, in complete agreement with the study by Iwakura [11], we could demonstrate that Dectin-1 was not involved in immunity to C. albicans ATCC18804, in terms of cytokine responses, fungal burdens or resistance to infection. In contrast, and consistent with our previous observations [6,7], we found an absolute requirement for Dectin-1 for all these parameters in the control of infections with C. albicans SC5314. Notably, in both cases, these phenotypes were obtained irrespective of the mouse background tested. However, it is important to note that mouse strains do express different isoforms of Dectin-1 [25,26], these isoform differences can directly relate to resistance or susceptibility during systemic infection [25].

We also explored the possibility that Dectin-1 dependency correlated with the clade of C. albicans, but did not find any such association. This result is not particularly surprising, as the multilocus sequence typing used to assign strains to their various clades relies on sequence differences in just seven genes, none of which are related to fungal morphogenesis or cell wall architecture [15]. Furthermore, previous analyses have also not revealed any association between clade and fungal virulence or type of infection [26,27].

Differential adaptation of C. albicans strains in vivo and effect on immune recognition

Despite significant differences in the strain-specific Dectin-1 dependency in vivo, this phenotype could not be recapitulated in vitro. Unexpectedly, the composition of the cell walls of in vitro grown C. albicans ATCC18804 and SC5314 were nearly identical. Indeed, we found that macrophages could recognize and respond similarly to yeast and hyphal cells from both strains in a Dectin-1-
dependent manner. We also studied the effect of hypoxia in vitro, which although understudied in this fungal species in the context of infection, is increasingly being appreciated to have a major influence on fungal adaptation and pathogenesis in vivo, including alterations in the cell wall [28,29,30]. In fact, in Aspergillus, we have recently shown that hypoxia enhances total and surface exposed β-glucan, leading to enhanced Dectin-1-dependent leukocyte inflammatory responses [18]. Consistent with these observations, we observed that hypoxia augments Dectin-1-dependent inflammatory responses to C. albicans, yet there was no difference in the leukocyte responses in vitro to each of the fungal strains tested. Interestingly, different strains of C. albicans have also been observed to have a variable dependency on TLR4 in vivo [31,32,33], but for this pattern recognition receptor these differences could be recapitulated in vitro and are thought to be related to differences in exposure of TLR4 ligands on the fungi [12]. Never-the-less, our observations highlight an inadequacy of studying Candida-host interactions in vitro.

We then examined the possibility that the dependency for Dectin-1 was related to the differential display of β-glucans in vivo, which occurs later during infection [17]. Consistent with this notion, we detected a substantial requirement for Dectin-1 at late stages of infection (day 9), with the appropriate fungal strain, whereas the control of all strains in the kidney was largely Dectin-1 independent at an earlier time point (day 3). Surprisingly, more β-glucan was exposed on the surface of the Dectin-1-independent strain (ATCC18804) in vivo, than the Dectin-1-dependent strain (SC5314) later during infection. However, altering the cell-wall architecture of ATCC18804 with caspofungin, converted this organism into a Dectin-1-dependent strain. Caspofungin inhibits the catalytic subunit of β-(1,3)-glucan synthase, which produces the β-1,3-glucan ligands that are recognised by Dectin-1 [34,35]. Treatment with this echinocandin markedly alters the cell wall, inducing substantial changes in the β-glucan and chitin levels, which both become exposed at the cell surface [17,20,36]. Intriguingly, these alterations are thought to enhance Dectin-1 mediated recognition by unmasking cell-wall β-glucans [17]. However, ATCC18804 already possessed high levels of exposed β-glucans in untreated mice, hence other alterations in the cell wall were probably responsible for the conversion to a Dectin-1-dependent phenotype.

To identify factors that were contributing to the in vivo differences between these C. albicans strains, we analysed the expression of a number of selected cell-wall-associated genes in fungal cells isolated directly from infected kidneys. C. albicans is known to undergo substantial transcriptional reprogramming during infection, relating to changes in metabolism, stress responses, morphology and virulence [37,38,39,40,41]. Our analyses also revealed the dynamic regulation of cell wall-related genes upon infection, but we also observed significant differences in gene expression patterns between the two C. albicans strains during infection. This highlights strain-specific differences in host adaptation in vivo, and is consistent with previous observations [39,40]. Remarkably, we also found striking differences in gene expression within the same strain, when comparing cells isolated from wild-type versus Dectin-1−/− mice. For example, CRH11 and UTR2 encode GPI-anchored chitin-glucan cross-linking enzymes that were more highly expressed during infections of wild-type mice compared to the Dectin-1−/− animals. These proteins are known to be antigenic [42] and the genes are up-regulated in response to caspofungin [43]. In S. cerevisiae, orthologues of these genes are included in a subset that is a signature for cell wall stress and a hallmark of activation of cell wall integrity pathways [44]. Thus, these data indicate that substantial changes in the C. albicans cell wall occur during infection, that there are strain-specific differences in host adaptation in vivo, and that this adaptation can be influenced by the immunological status of the host.

Of particular interest was the differential regulation of genes involved in regulating chitin biosynthesis, including CHS3, PGK31 and CHT2. Notably, CHS3 encodes Chs3p, a class IV chitin synthase which is responsible for the synthesis of short chitin-rodlets which make up the majority of the cell wall chitin in both yeast and hyphae [45]. These transcriptional analyses therefore suggested that there were differences in the amounts of chitin between these fungal strains in vivo, a conclusion we confirmed by demonstrating elevated chitin levels in ATCC18804 cells isolated from infected kidneys. Moreover, TEM analysis also demonstrated substantial differences in the architecture of their cell walls. We have previously shown that increased cell wall chitin in C. albicans can modulate inflammatory leukocyte responses in vitro [20]. Furthermore, we demonstrated here that high-chitin levels reduce the dependence on Dectin-1 in vivo, despite the presence of exposed β-glucans on the fungal cells. Thus variations in chitin content and differences in cell wall structure provide a rational explanation, at least in part, for the strain-specific differences in Dectin-1 dependency in our mouse models. While the mechanisms underlying these effects await further elucidation, our data indicate that detection of exposed β-glucans on fungal cells does not necessarily correlate with recognition by Dectin-1 in vivo.

Implications for human infections

These observations have significant implications for our understanding of C. albicans infection in humans. We previously identified a polymorphism (Y238X) which could render individuals susceptible to infections with C. albicans and other fungi [5]. This polymorphism is common in many populations, yet its clinical penetrance is low, indicating that other factors are influencing susceptibility to infection [5]. Our data suggest that the effect of this polymorphism may only become apparent in individuals if they are infected with a Dectin-1-dependent strain of C. albicans. This link between host genotype and fungal strain needs to be explored in greater detail if we are to understand the contribution of polymorphisms of Dectin-1 and other PRRs to human disease susceptibility.

Our results also have implications for anti-fungal drug therapy. We have demonstrated, for example, that caspofungin treatment of ATCC18804 infections alters cell wall architecture and enables enhanced clearance in vivo. However, this treatment was not effective under conditions of Dectin-1 deficiency. Moreover, enhanced cell wall chitin levels can confer enhanced resistance to echinocandins [22,36,46], and reduce the dependency on Dectin-1 (Figure 4). These results underpin our previous in vitro-based observations suggesting that antifungal therapy should be targeted at more than one cell wall component [36].

In conclusion, our results provide substantial new insights into the interaction between C. albicans and its host and have significant implications for our understanding of anti-Candida immunity and drug treatment in humans.

Materials and Methods

Animals

Eight to 12-week old 129/SvJ [6] and C57BL/6 (backcrossed for at least nine generations) ClecTa−/− (Dectin-1−/−) and wild-type (wt) mice were obtained from the specific pathogen free facilities of the University of Cape Town and University of Aberdeen. All animal experimentation was replicated at least twice using groups
of 5–10 animals, unless otherwise stated. Mice were housed in groups in individually ventilated cages and provided with food and water ad libitum. Peritoneal thioglycollate-elicited macrophages were prepared as described previously [6]. All animal experimentation was conducted in animal care and welfare protocols approved by the Universities of Aberdeen (project license numbers 60/4007 and 06/4135) and Cape Town (license number: 06/035) and in strict accordance with the guidelines for the usage of animals in laboratory research of the South African Association for Laboratory Animal Science and the UK home office.

C. albicans strains and growth conditions

The C. albicans strains used in these experiments are listed in Table S1. C. albicans was maintained on YPD (Sigma-Aldrich) or Sabouraud (Oxoid) agar plates. For high chitin cells, cells were grown in YPD containing 0.2 M calcium chloride (CaCl₂) and 100 µg/ml of Calcofluor White. For yeast cell preparation, cultures were incubated in Sabouraud broth (Oxoid) at 30°C for 24 h with shaking. To obtain hypoxic yeast cells, cultures were incubated under 1% O₂ and 5% CO₂ for the indicated times. Before use, the cells were washed in phosphate-buffered saline (PBS), and the cell density adjusted to the desired level with PBS. For inoculations, dosage was confirmed by viable cell counts on agar plates. To generate hyphae, a defined number of yeast cells were cultured at 37°C overnight in RPMI containing 10% heat-inactivated FCS. To obtain hypoxic hyphae, hyphae were cultured overnight under hypoxia, and washed as described above. Fungal cells were used either live or killed, by heat (100°C for 30 min) or UV-irradiation (6000 Joules), as indicated.

In vivo inoculations and analyses

Mice were inoculated intravenously with the indicated doses of the various C. albicans strains in 100 µl sterile PBS. Mice were monitored daily and sacrificed at the indicated time points, or when judged to be moribund. In some experiments, mice were treated with 33 µg/kg caspofungin daily i.p., starting one day post-infection. Experiments were continued for a maximum of 21 days. Fungal burdens and cytokines in lysates from infected kidneys were determined as described previously [6]. Cytokines were normalised to lysate protein concentrations (BCA protein assay kit, Pierce).

Intraperitoneal infections were performed as described previously [6]. In brief, 1 x 10⁵ CFU in 100 µl PBS were injected i.p. into wt and Dectin-1⁻/⁻ mice and four hours post-infection, peritoneal inflammatory cells were harvested in PBS containing 5 mM EDTA. Cells were stained with CD11b-PEcy7 and Ly6G-PE (both from BD Biosciences) with neutrophils defined as CD11b⁺ Ly6G⁻. Data was acquired on FACScalibur and analysed using FlowJo.

Cell wall analysis

Cell wall mannán, β-glucan and chitin contents were determined by hydrolysis of these oligosaccharides and quantification by high-performance anion-exchange chromatography, as described previously [47]. To detect chitin, ex vivo isolated C. albicans cells were stained and quantified using Calcofluor White, as previously described [22]. TEM analysis was performed as previously described [36].

To detect exposed β-glucan, C57BL/6j mice were injected in the tail vein with 5.2 x 10⁷ CFU of either SC5314-GFP or ATCC18800-GFP. SC5314-GFP and ATCC18800-GFP strains were created by transformation with the pEN01-yEGFP-NAT plasmid and verified by PCR as described previously [17]. After nine days, mice were sacrificed and the kidneys were harvested, homogenized, and processed as described [17]. Homogenates were stained with anti-β-glucan antibody (Biosupply, Inc., Australia) at a concentration of 1.7 µg/ml, then stained with goat anti-mouse Cy3 antibody (Jackson ImmunoResearch) at a concentration of 3.8 µg/ml. For soluble Dectin-1-Fc staining, homogenates were instead stained with Alexa488-labelled Dectin-1-Fc [48] at a concentration of 17 µg/ml and then with donkey anti-rabbit IgG Cy3 antibody (Jackson ImmunoResearch) at a concentration of 0.8 µg/ml. Cells were visualized by optical sectioning fluorescence microscopy using a Zeiss Axiosio Vivomte microscope (Carl Zeiss Microscopy, LLC). Live cells were identified based on characteristic EGFP fluorescence. Maximum projection images were quantified using Cellprofiler (www.cellprofiler.org) as described [17]. Briefly, EGFP fluorescence was used to manually define individual cell segments and average fluorescence intensity of β-glucan or Dectin-1-CRD fluorescence was measured for the whole cell segment. Cells labelled without primary antibody or Dectin-1-CRD were used as negative controls. In vitro grown cells were stained with soluble Dectin-1 at 5 µg/ml and then with anti-human IgG antibody (used at 1:200) (Jackson ImmunoResearch). Controls were stained with secondary antibody only.

C. albicans gene expression analysis from normoxic and hypoxic in vitro cultures

C. albicans normoxic and hypoxic grown hyphae were resuspended in Trizol reagent and chloroform to extract RNA. Tubes were centrifuged at 16,000 x g for 15 min at 4°C. The clear upper layer was further extracted with an equal volume of 80% EtOH. Samples were applied to RNeasy spin columns (Qiagen RNA kit) following manufacturer’s instructions. RNA was eluted with RNase free water.

RNA was DNase treated with DNA-free kit (Ambion) and reverse transcribed with QuantiTect reverse transcription kit (Qiagen, USA). Primers for all genes of interest were designed with PrimerQuest (IDT) and manufactured by IDT, USA. Sequences are: Fks1, Fwd-5'TGATACTGTTAATCATGACAAAA-3', Rev- 5'AACCTGAATGTATTGTAATAGG3', Fks2, Fwd- 5'ACTTGTGACCTGCGCAAT-3', Rev- 5'ACCACATGAGCGGTAGAC-3', Fks3, Fwd- 5'ACCTCAATATCCAGCTTGCCGCC-3', Rev- 5'GGACACATTCCGACTTGAGCG-3', and Efb1, Fwd- 5'CATTGATGGTACTACTGCCC-3', Rev- 5'TTTACCGCTGTCAG-3'. All reactions were performed on BioRad MyIQ real-time PCR detection system with IQ SYBR green supermix (BioRad, Hercules, CA). The ΔAC method was used to assess changes in mRNA abundance, using Efb1 as the housekeeping gene. Relative transcript abundances for FKS1/2/3 are reported as means plus/minus SEM that are normalized to EFB1 and presented as relative to the normoxic values for each strain.

C. albicans gene expression analysis from infected tissue

C. albicans-enriched cortical shavings from two kidneys of the same animal were fixed in RNAlater according to manufacturer’s instructions (Qiagen, Crawley, UK) and combined into a single sample for further processing. Approximately 3 mm³ of tissue were transferred to 600 µl QiAzoI reagent (Qiagen, Crawley, UK), an equal volume of acid-washed glass beads added and the material homogenised using FastPrep-24 bead mill (10×20 sec bursts at 6.0 m/sec setting, with 4 min intervals on ice) (MP Biomedicals, Luton, UK). RNA extraction was carried out according to standard procedures. Nucleic acids were precipitated with RNA grade glycogen solution (Fermentas, Loughborough, UK). The final RNA pellet was suspended in 100 µl DEPC-
treated water (DEPC-H₂O) and further purified using NucleoSpin RNA Clean-up XS columns (Macherey-Nagel, Loughborough, UK). After repeated DNase I (Invitrogen, Paisley, UK) treatment the isolated RNA was assayed with NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Loughborough, UK). Up to 40 ng isolated RNA was used in qRT-PCR reactions with ACT1 reverse primers to verify complete removal of genomic DNA (see below, and Table S2).

Total RNA (4.5 μg) was used in a cDNA synthesis reaction primed with a cocktail of all the qRT-PCR reverse primers (0.125 μM final primer concentration for each primer) (Table S2) and SuperScript II Reverse Transcriptase (Invitrogen, Paisley, UK). Single stranded cDNA was purified with QIAquick PCR Purification Kit (Qiagen, Crawley, UK), and eluted in 50 μl DEPC-H₂O. qRT-PCR reactions were prepared in 10 μl volumes containing 2 μL of at least 10× diluted cDNA templates and the appropriate Universal Probes (Table S2) as per manufacturer’s instructions, and run on a LightCycler 480 machine (Roche Applied Science, Burgess Hill, UK) using a Monocolour Hydrolysis Probe programme. All reactions were run at least in duplicate, with in-run ACT1 gene standard. The relative transcript abundances normalised to ACT1 were calculated based on the individually determined primer pair efficiencies with LightCycler 480 Software release 1.5.0.

In vitro analyses

To measure binding and cytokine production, live C. albicans yeast cells were labelled with Rhodamine Green-X (Invitrogen) as described [6]. Labelled yeast cells were then added (MOI 5:1 or 10:1) to thioglycollate-elicited macrophages, which had been seeded the previous day at a density of 1–2.5×10⁵ cells/well in 24-well plates in RPMI media. After incubation for 30 min at 4°C, to allow particles to settle, followed by 30 min at 37°C, unbound particles were removed by washing. Cells were then cultured for a further 3 h at 37°C for analysis of proinflammatory cytokine production. After incubation, supernatants were stored at −80°C for subsequent TNF analysis. Cells were then lysed in 3% Triton X-100 and fluorescence measured using a Titer-Tek Fluoroskan II (Labsystems). TNF concentrations in the supernatants were measured by ELISA (OptEIA TNF kit; BD Pharmingen). Fluorescein isothiocyanate–labelled zymosan (Invitrogen) was used as a control in these experiments. In some experiments, as indicated, unlabelled UV-killed yeasts were used, the cells were not washed after addition of the particles, and TNF responses were assayed after overnight incubation at 37°C. The inflammatory response to hyphae was measured similarly, except that the thioglycollate-elicited peritoneal macrophages were directly added to live or heat-killed hyphae and supernatant samples taken after overnight incubation at 37°C. Isolation and stimulation of human PMBCs was performed as described previously [5].

Statistics

All data were plotted using GraphPad Prism software. A two-tailed Student’s t-test was used to analyse differences between two groups. The Mann-Whitney U test was used to determine statistical significance of differences between relative transcript abundances in each of the in vivo experimental groups. The reported values are arithmetical means of abundances normalised to ACT1, with the appropriate p-values from two-tailed t-test in pair-wise comparisons. Survival data were analyzed with the log rank test. Results were considered statistically significant with p values of less than 0.05.

Supporting Information

Table S1 Details of C. albicans strains used in this study. (DOCX)

Table S2 qRT-PCR primers and Universal Probes. (DOCX)

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Author Contributions

Conceived and designed the experiments: RW CAM NARG RAC GDB. Performed the experiments: MJM SV JP LAW KMS AH HMM NARG. Analyzed the data: MGN GIM DMM RW CAM NARG RAC GDB. Performed the experiments: MJM SV JP LAW KMS AH HMM NARG. Analyzed the data: MGN GIM DMM RW CAM NARG RAC GDB. Contributed reagents/materials/analysis tools: GIM NARG GDB. Wrote the paper: GDB.

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