Candida albicans: Mechanisms of biofilm resistance unraveled?

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Summary

Candida albicans is currently the fourth most common bloodstream infection in the world. It is the most widespread fungal pathogen and is associated with a wide range of clinical symptoms. *C. albicans* can grow in a variety of distinct morphological forms, ranging from unicellular budding yeast to hyphae. This fungus can be found as a free floating planktonic *C. albicans* or as a biofilm, as happens in disease states. Candidiasis is associated with biofilm formation on a wide range of implanted medical devices. Our immune system is not capable to defeat a *C. albicans* biofilm, therefore removal of the medical device is necessary. Knowing this, it is interesting to understand through which mechanisms a *C. albicans* biofilm is more resistant than its planktonic form. *C. albicans* biofilms are made of yeast, a large amount of hyphae and an extracellular matrix.

The cell wall of *C. albicans* consists of a skeletal component containing β-glucans and chitin, and a matrix component consisting out of glycosylated proteins with mannan and carbodhydrates. These can be recognized through opsonisation by antibodies, complement and Mannose binding protein. But also directly by pattern recognition receptors of mononuclear phagocytic cells. These consist out of Toll-like receptors, mannose receptors which recognize mannan, CR3 and dectin-1 receptors that recognize β-glucans. After recognition *C. albicans* can be phagocytised, where the dectin-1 receptor plays an important role. Inside the phagolysosome, the fungus can be killed by oxygen-dependant pathways like superoxide anion and hydrogen peroxide mediated killing and non-oxygen dependant pathways. Most likely *C. albicans* will go into apoptosis after oxidative stress.

But *C. albicans* is capable of resisting the different steps described above. It can cover the β-glucans in hyphae by mannans and α-glucans, thereby preventing detection by dectin-1. Other mechanisms are the inhibition of oxygen radicals by enzymes like catalase and even preventing the release of an oxidative burst by mononuclear cells. In biofilms it is even more specific, as they are less susceptible to these reactive oxygen species. But the immune system also helps *C. albicans* through these mononuclear cells as they raise the ability of *C. albicans* to form a hypha-rich biofilm. One of the ways is through the release of specific cytokines. But *C. albicans* also produces a metabolic product, farnesol, which prevents the formation of biofilms and upregulates the epithelial cell defense.

In conclusion, there are three very important players in the resistance of *C. albicans* to our immune system. First of all, the extracellular matrix, which makes it more difficult for immune cells to infiltrate the biofilm. It also makes the *C. albicans* biofilm less susceptible to molecules released by the mononuclear immune cells. Secondly, biofilms contain a lot of hyphae, dectin-1 recognition is prevented and therefore phagocytosis does rarely occur. The last way, is the ability of *C. albicans* to recruit mononuclear immune cells to build a hyphae-rich biofilm. In order to defeat *C. albicans,* these resistance factors have to be minimized. Thereby giving the advance in our body, back to the immune system.

Candida albicans: mechanisms of biofilm resistance unraveled?

Candida albicans is the most widespread systemic fungal pathogen and is associated with a range of clinical symptoms. Normally, *C. albicans* is part of the microbial flora and colonizes mucocutaneous surfaces of the oral and vaginal mucosa of numerous mammals and birds *(Rogers et al, 1980)*. However, they can cause a wide range of painful superficial and bloodstream infections *(Sudbery et al, 2004)*. Therefore in immunocompromised patients, this might lead to life-threatening systemic diseases *(Odds, 1988)*. Candidemia is currently the fourth most common bloodstream infection in the world *(Banerjee et al, 1991)*. *C. albicans* has many virulence factors, which enable the fungus to survive and grow in unfavorable conditions. The most important virulence factor is the cell wall; it provides rigidity, protection against osmotic lysis, immunosuppressive properties and supports the interaction between host-cell receptors and *C. albicans* adhesins (as reviewed in Vázques-Torres et al, 1997). These virulence factors contribute to the survival of *C. albicans*, but colonization of immunocompetent hosts, does not lead to clinical candidiasis *(Odds, 1979)*. The problem with *C. albicans* infections is the resistance to antimycotic therapies such as amphotericin, which is rising *(Mah et al, 2001)*.

One of the characteristics of *C. albicans* is its ability to grow in variety of distinct morphological forms. Which ranges from unicellular budding yeast to hyphae, although in between these extremes there are a variety of growth forms called pseudohyphae. The ability of *C. albicans* to switch between these morphologies is believed to be necessary for virulence. The hyphae and pseudohyphae morphological forms promote tissue penetration in the early stages and therefore are invasive. But they also play a role in succeeding stages, like the invasion of organs as kidneys. Whereas the yeast form is implemented for dissemination in the bloodstream *(Sudbery et al, 2004)* (Figure 1).

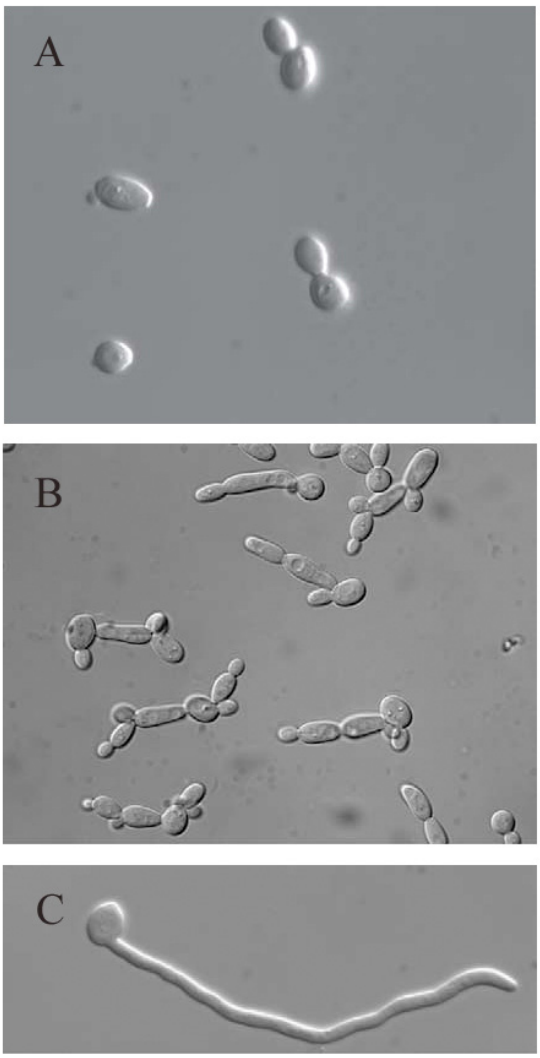

Figure 1. Yeast, pseudohyphal and hyphal morphologies. (A) Budding yeast cells. (B) Pseudohyphal yeast. (C) Hyphal forming yeast. From Subery et al, 2004.

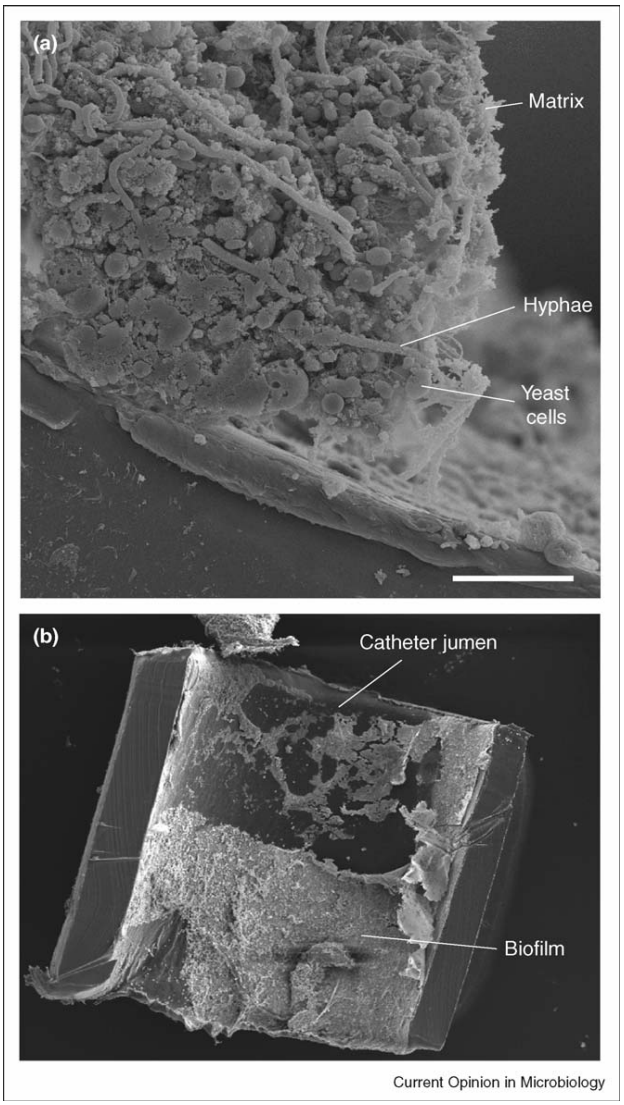

Figure 2. Scanning electron micrographs of a C. albicans biofilm on the inside lumen of a vascular catheter from a rat central venous catheter model. (a) Cross section of a biofilm. Yeast and filamentous cells are seen encased in matrix material. Magnification 1000 times. (b) Image of section of venous catheter. Majority of catheter lumen is coated with biofilm. Magnification 50 times. From Nett et al. 2006.

The morphological switching from unicellular budding yeast to hyphae can be induced by environmental changes *(Odds, 1988)*. Hyphae are induced by a culture temperature of 37 ^{o}C and addition of serum. This process starts at neutral pH and a temperature above 35 ^{o}C, which induces pseudohyphae to hyphae. For this morphological switching to occur, different signaling pathways have been studied. These pathways transduce environmental signals to internal signals. One example is the CaEfg1 transcription factor, which is based on the cAMP and protein kinase A signaling pathway. Another is a mitogen-activated protein MAP-kinase which targets the CaCph1 transcription factor *(Whiteway, 2000)*. These various morphological forms play an important role in the survival of *C. albicans* and are most likely, highly regulated.

Primarily, *C. albicans* has been studied while in "liquid" growth, but in the disease states it usually associates to surfaces. Candidiasis is associated with biofilm formation on a wide range of implanted medical devices. These devices are essential for patients and therefore this is a significant problem *(Kumamoto et al, 2005)*. Around 20% of the infections on medical devices are ascribed to *C. albicans (Nett et al, 2006)*. It is known, that *C. albicans* can form biofilms on almost any medical device. Mostly, systemic devices are involved like vascular and urinary catheters, cardiac valves, pacemakers and joint prostheses *(Kojic et al, 2004)* (Figure 2).

Biofilms are microbial communities with a certain structure, where cells bind tightly to a surface. They become enclosed in a matrix of extracellular polymeric materials produced by the cells in a biofilm *(Ramage et al, 2005)*. The communities formed on these devices have a characteristic phenotypic property and architecture, which are different than the planktonic form. One of the differences is the reduced

susceptibility to the host immune system in biofilms in comparison to the planktonic form. Thus, removal of the medical device is necessary. Biofilms consist of a highly heterogeneous architecture, a cell mixture of host cells, yeast, hyphae and the extracellular matrix (as reviewed in *Nett et al, 2006).*

The immune system is the bodies' defense system against pathogens. It consists out of an innate and adaptive component. Where the innate immune system is the first barrier, the adaptive part is needed for specificity. Phagocytes like macrophages and neutrophiles, complement and natural killer cells are considered the innate immunity. The adaptive immune system includes the T-lymfocytes and antibody producing B-lymfocytes. In order to defeat *C. albicans,* the innate and the adaptive component have to work together. But *C. albicans* manages to evade the immune system in biofilms, this is a poorly understood phenomenon. Therefore it is interesting to compare the planktonic and biofilm interaction with the immune system, focusing mostly on the innate immunity. By these means, trying to understand through which mechanisms this might happen.

Different steps in the formation of a C. albicans biofilm

A *C. albicans* biofilm is formed as a step-wise process. It all begins with the adherence to foreign substrate. This adherence is both to solid substrates and cells, as this is needed for biofilm formation. For this step special adherence factors are needed, one example are the Als (agglutinin-like sequence) proteins. During biofilm formation the expression of *ALS* genes is upregulated *(Green et al, 2004)*. Also other transcription factors contribute to biofilm formation like Bcr1 (a cell wall regulator) and Tec1 (regulator of hyphae-specific genes). Tec1 regulates the transcription of the *BCR1* gene, which regulates the genes important in hyphal differentiation. Bcr1 controls the expression of certain Als proteins like Als1 and Als3, but also the hyphal wall protein (Hwp1) *(Nobile et al, 2005)*. New genes are identified as players in the process of biofilm formation and our understanding of the role of adherence factors in biofilm formation is increasing *(Blankenschip et al, 2006)*.

The next step is the proliferation of *C. albicans* across the surface followed by hyphal development *(Chandra et al, 2001)*. As mentioned before Tec1 is important for adherence, but it also has a Bcr1-independent role. As over expression of *BCR1* in a *tec1* deficient mutant, only incompletely restores the biofilm formation. The biofilm still lacks hyphea *(Nobile et al, 2006).* Therefore Tec1 must have another pathway to influence biofilms. Other genes have been found, that are required for hyphal differentiation. As with Tec1, mutants with knock-outs of these genes lack correct hyphal formation. This data supports the hypothesis that hyphal formation is needed for the formation of a strong biofilm *(Blankenschip et al, 2006).*

Finally, the last step is called the maturation step, budding yeast growth is suppressed, hyphal growth is elevated and an extracellular matrix encloses the biofilm. The extracellular matrix is one of the most important characteristics of both fungal and bacterial biofilms. One of the functions might be the defense against the host immune cells, in particular the phagocytic cells. Next to this, other functions are maintaining the state of the biofilm and the limitation of toxic substances diffusion into the biofilm. The matrix consists of carbohydrates, phosphorusm, proteins, glucose and hexosamines. But still the full consistency of the matrix is not identified *(Baillie et al, 2000)*.

An important factor in biofilm formation is quorum sensing, a broad set of occurrences in which secreted molecules, through signaling orchestrate cell density-dependent responses *(Keller et al, 2006)*. It has functions as diverse as virulence and bioluminescence. One of those molecules in *C. albicans* is farnesol, this will be discussed later.

Interaction between immune system and C. albicans

In order for the immune system to successfully eradicate *C. albicans,* first the recognition step is important. Recognition can occur through opsonization and non-opsonization. This is studied mostly when *C. albicans* is in its planktonic form. After recognition *C. albicans* is mostly phagocytised by mononuclear phagocytic cells from the innate immunity.

Invasion of epithelia by C. albicans

In order for *C. albicans* to cause an infection it has to attach to epithelial surfaces. After adhesion it can invade the host cell. Overall, this can happen through three ways: by phagocytosis (only host is active), invasion (only pathogen is active) or induced endocytosis (both host and pathogen are active). After contact with epithelial cells, *C. albicans* switches to a hyphal form of growth. The hyphae, not yeast cells, causes a host response mechanism like formation of epithelial cell protrusions surrounding the hyphae and endocytosis. The adhesion occurs through the already discussed Als3 and Hwp1, they both also play a role by the endocytosis and active penetration (as reviewed by *Zakikhany et al, 2008).*

The cell wall of C. albicans

In order to understand the process of recognition, first the structure of the fungal cell wall must be explained. The viability of *C. albicans* depends on the cell wall, therefore it is an essential organelle. It consists of skeletal and matrix components, which are both recognized by the innate immune system. The skeletal component consists of a core structure of β-(1,3)-glucan covalently linked to β-(1,6)-glucan and chitin. The polymers form hydrogen bonds between adjacent polysaccharide chains, thereby forming a three-dimensional network of microfibrils. Glucan is mostly located in the inner layers of the cell wall of *C. albicans*, its function is to provide rigidity *(Calderone et al, 1994).* The cell wall of *C. albicans* consists of 60% of β-glucan *(Klis et al, 2001).* These β-glucans are usually exposed on the cell surface, but sometimes limited to certain regions. During a systemic fungal infection β-glucan are released into the circulation. *(Netea et al, 2008).* After separation of the bud a scar is left on the mother cell in budding yeast cells. At this site, parts of the cell wall, such as β-(1,3)-glucan and chitin, might get exposed at the surface *(Gantner et al, 2005).* The other part of the cell wall, the matrix is composed of glycosylated proteins. The major class of cell wall proteins are glycosylphosphatidylinositol (GPI)-anchor-dependent cell wall proteins (CWPs). They are normally highly glycosylated with mannose-containing polysaccharides (also called mannan) and carbohydrates (90%) (Figure 3) *(as reviewed by Netea et al, 2008).*

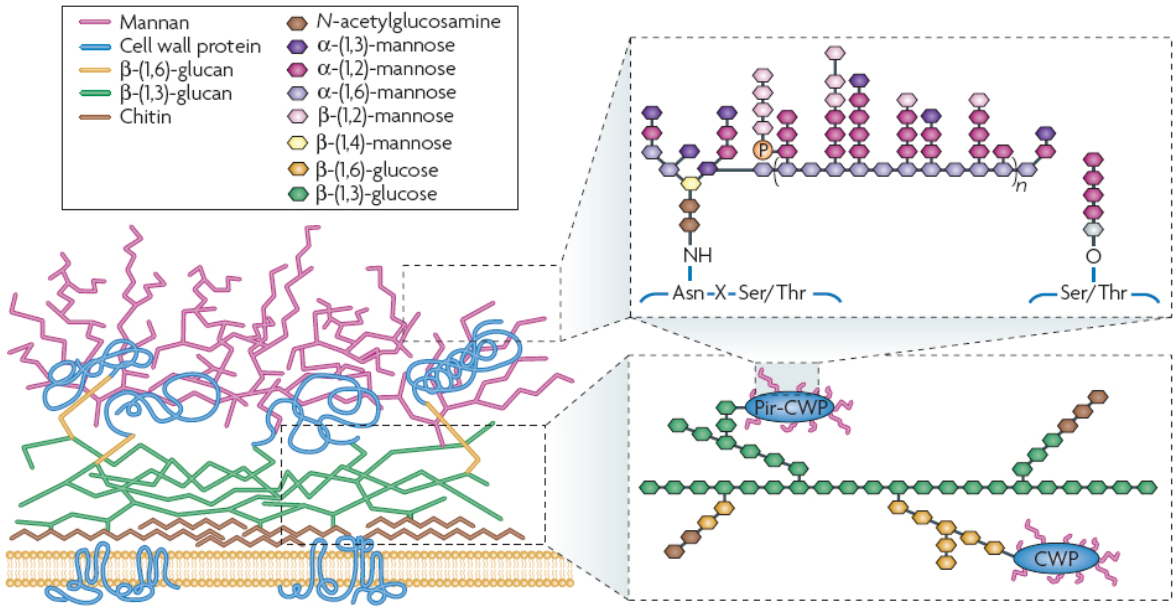

Figure 3. The structure of the Candida albicans cell wall. The schematic shows the major components of the cell wall and their distributions. β-(1,3)-glucan and chitin (poly-β-(1,4)-N-acetylglucosamine) are the main structural components, and these are located towards the inside of the cell wall. The outer layer is enriched with cell wall proteins (CWP) that are attached to this skeleton mainly via glycosylphosphatidylinositol remnants to β-(1,6)-glucan or, for mannoproteins with internal repeat domains (Pir-CWP), via alkali-sensitive linkages to β-(1,3)-glucan. From Netea et al, 2008.

Recognition of C. albicans

a. Opsonized C. albicans

One of the important opsonizing factors, are *C. albicans* specific antibodies (part of the adaptive immune system). *Cline et al, 1968* found, that human monocytes phagocytized *C. albicans* better when in the medium human serum was present. The opsonic properties were ascribed to IgG. Although the addition of anti- *C. albicans* specific antibodies does not increase the phagocytic capabilities of murine macrophages *(Kagaya et al, 1981)*. This might be because of the huge variety of mononuclear phagocytic cells, whereas only one was used in the experiment *(Vázques-Torres et al, 1997)*. Although the most convincing evidence comes from *Wagner et al, 1996*, where B-lymfocyte deficient mice were more susceptible to acute systemic candidiasis. B- lymfocytes produce antibodies, so they play a role in the reaction against *C. albicans*, more specifically in the opsonization. Antibodies can most likely recognize all of the surface proteins from *C. albicans* ranging from the recognition of hypha-specific surface proteins encoded by genes like *ALS3, HWP1 (Staab et al, 1998)* to mannan and β-glucan deep within the fungal cell wall *(Kondori et al, 2008)*. These IgG (and IgM) are mostly focused against native cell wall fragments and the phosphopeptidomannan fraction of the cell wall of *C. albicans (Kondori et al, 2004)*.

Another opsonizing factor is the complement system. It's a cascade of proteins, belonging to the innate immunity, which binds on the surface of pathogens. Thereby,

making it possible for antibodies and phagocytizing cells to bind. One study conducted by *Leijh et al, 1977* found that heat inactivated serum is less efficient than normal serum in enhancing the phagocytosis of *C. albicans* by human monocytes. In another study, murine macrophages could only reach their phagocytizing optimum when the complement system is intact *(Kagaya et al, 1981)*. Thus, the complement system plays a role, but is only player in a big field of players.

In addition the Mannose binding protein (MBP), an innate immunity molecule, plays a role. This is a C-type lectin secreted by the liver and has double function. *Kitz et al, 1992* showed that MBP inhibits the phagocytosis of *C. albicans* by murine macrophages. Thereby, interfering the macrophage-*C. albicans* interaction. But MBP also hinders the adherence of *C. albicans* to host tissue, because this is partly regulated by the mannan-mannose receptor interactions. Next to this, MBP can also activate the complement cascade, by that means promoting the release of opsonins. In summary, MBP can enhance and inhibit the effector mechanisms of the immune system.

b. Nonopsonized C. albicans

Although mononuclear phagocytic cells can recognize pathogens through opsonization, direct recognition of a diverse amount of antigens expressed on microorganisms also occurs. Phagocytosis can take place in the absence of opsonins *(Ofek et al, 1992)*. This task is accomplished by pattern recognition receptors (PRRs), they recognize conserved microbial chemical signatures, also called pathogen-associated molecular patterns (PAMPs) *(Netea et al, 2008)*. Direct recognition by mononuclear phagocytic cells is important in tissues which contain only a low amount of opsonins. For example, at the start of a microbial infection, when the concentration of antibodies is low *(Vázques-Torres et al, 1997)*. Different receptor groups will be discussed.

One of the main cells of the innate immunity are the monocytes/ macrophages and neutrophils. Macrophages express Toll-like receptors (TLRs) and lectin receptors (LRs) on their cell membranes. Neutrophils have a mild expression of TLRs, but strongly express receptors as complement receptor 3 (CR3) and Fcγ receptors (FcγRs). The different PRRs expressed by each cell, determines the type of response against *C. albicans* that follows *(Netea et al, 2008)* (Figure 4).

The C-type-lectin mannose receptor, a mannan receptor, is expressed on the surface on phagocytic cells like macrophages *(Stephenson et al, 1987)*. It takes part in the phagocytosis of microbes with a mannan-rich cell surface *(Ofek et al, 1992)*. This way of phagocytosis is in particular important for *C. albicans*, because the outer layer of the yeast cell wall mainly contains mannan *(Calderone et al, 1994)*. The mannose receptor actually recognizes oligosaccharides that end in mannose, but also others like fucose. This binding is acted through carbohydrate-recognition domains *(Linehan et al, 2000)*. The mannose receptor mostly recognizes linked oligomannoses with branched structures, this in contrast to TLRs, especially TLR4 which recognizes short linear oligomannose structures bound to mannan (this results in cytokine production) *(Netea et al, 2006)*.
The expression of the mannose receptor is an early marker in the differentiation of macrophages from monocytes *(Kataoka et al, 1985)*.

Another receptor, which is also from the lectin family, is dectin-2. It is mainly expressed on myeloid cells and maturing monocytes *(Taylor et al, 2005)*. Dectin-2

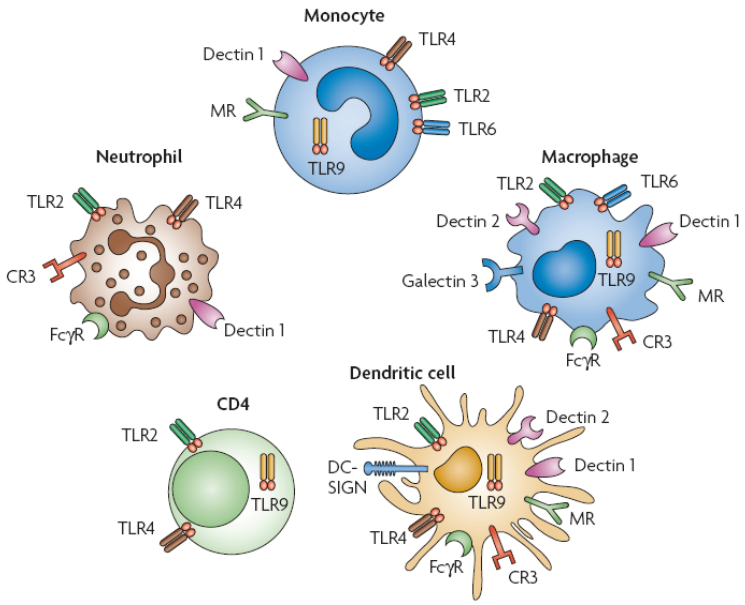

Figure 4. Cell populations and pattern-recognition receptors involved in Candida albicans recognition. The main populations involved in the recognition of C. albicans during the innate immune response are the monocytes, neutrophils and macrophages. Dendritic cells process antigen and present in to T cells (CD4). The diverse expression of pattern recognition receptors by these cell types is shown. CR3: complement receptor 3; FcγR: Fcγ receptor; MR: mannose receptor; TLR: Toll-like receptors. From Netea et al, 2008.

interacts with the FcγR, thereby inducing intracellular signals and plays a role in the recognition of *C. albicans* hyphae *(Netea et al, 2008).*

Furthermore there is the β-glucan receptor group. β-glucans are recognized by two receptors, CR3 and dectin-1, although there is evidence that others might be involved. CR3 is a β_2-integrin, which recognizes various endogenous and exogenous ligands, carbohydrates like β-glucans and pathogens opsonized by iC3b (the inactivated form of complement component C3b). The recognition of carbohydrates is performed by a lectin domain *(Diamond et al, 1993)*. Overall the recognition by CR3 does not trigger host responses like a respiratory burst, instead it can even repress pro-inflammatory signals *(Wright et al, 1982)*.

Dectin-1 is a transmembrane receptor and has a single extracellular, nonclassical C type-lectin-like domain that specifically recognizes β-(1,3)-glucans. It recognizes *C. albican*s yeast, but not the hyphae form. The tail of dectin-1, situated in the cytoplasm, consists out of an immunoreceptor tyrosine-based activation-like motif (ITAM). This motif can lead to the stimulation of interleukin-2, IL-10, IL-6 and IL-17 production through different pathways, like the Syk dependent signaling pathway (*as reviewed by Netea et al, 2008).*

The immune system can also recognize other parts from *C. albicans*. For example, chitin, a β-(1,4)-linked homopolymer of GlcNAc that forms antiparallel hydrogen-bonded chains, also called microfibrils *(Minke et al, 1978)*. Chitin recruits immune cells that release IL-4 and IL-13. Nothing is known about the pathways or recognition receptors involved *(van de Graaf et al, 2006).*

All these recognition steps most likely also occur in biofilms, but with a slower tempo. Because of the extracellulair matrix, it is more difficult for immune cells to infiltrate. Without this vital step, the immune reaction will not be sufficient to eradicate *C. albicans* in biofilms.

After recognition: C. albicans phagocytosis

After recognition *C. albicans* is phagocytized by nonopsonic and opsonic receptors. Opsonized *C. albicans* with complement is mostly important for chemotaxis and not for the lysis of *C. albicans,* because of the thick and complex cell wall *(Kozel, 1996)*. The receptors playing a role in the phagocytosis of *C. albicans* are the dectin-1 and mannose receptor. Although the phagocytosic abilities of the mannose receptor are not completely clear *(Le Cabec et al, 2005).* As for TLRs, they most likely play a role in directing the maturation of the phagosome and presenting antigens. Thus, not the fungal uptake, but this is still controversial *(Blander et al, 2006)*.

C. albicans killing inside phagolysosome

Inside phagocytes, *C. albicans* can be killed through oxygen-dependent or independent mechanisms. In order to kill ingested *C. albicans*, macrophages need to release an oxidative burst.

The first way is by killing through superoxide anion-mediated killing. This is one of the products from the reactive oxygen intermediate metabolism and is essential for oxidative killing *(Marodi et al, 1991).* There is a direct correlation between the capacity to generate superoxide anions and the killing efficiency. Killing of *C. albicans* by human phagocytic cells, mostly involves superoxide anions *(Sasada et al, 1980)*. Although this is not completely proven *(Brummer et al, 1989)*. Superoxide anions lead to *C. albicans* death, though the exact mechanism has not been fully elucidated. One of the possible mechanisms will be discussed further on.

Another molecule, hydrogen peroxide, has been implicated in the killing of *C. albicans* by macrophages *(Sasada et al, 1980)*. But the importance is not fully understood, as the peaks of hydrogen peroxide production did not correlate with the maximum macrophage killing activity. But hydrogen peroxide might be used as a substrate of myeloperoxidase (MPO), neutrophils most likely use MPO to kill *C. albicans (Vázques-Torres et al, 1997)*.

The last discussed way of killing is through reactive nitrogen. Nitrogen oxide (NO) is synthesized by NO synthases, enzymes that use NADPH, arginine and oxygen as substrates *(Nathan et al, 1994).* NO plays a role in the killing of pathogens like *Mycobacterium leprae* and *M. tuberculosis* by macrophages. *C. albicans* is also susceptible to NO, when produced by macrophages *(Nathan et al, 1991)*. Although the exact effector mechanism of NO is still not clear. Most probably NO in the form of nitrosonium is effective, as described *in vitro* by *Merendino et al, 1992*. Inside macrophages, not only NO is produced but also superoxide anions. It is hypothesized that superoxide anions by itself are not sufficient enough to kill *C. albicans*. Therefore coupling with NO is necessary, which forms peroxynitrate, a strong candidacidal molecule *(Vázques-Torres et al, 1997)*.

There are also oxygen-independent killing mechanisms. The liver is one of the organs that can resist *C. albicans*. Kupffer cells in the liver can ingest and kill *C. albicans*, most likely without the usage of oxidative stress. As the synthesis of these reactive oxygen species is independent of the killing capacity *(Sawyer et al, 1976)*. Hence, other mechanisms should be involved. There is also evidence that lysosomal

enzymes from macrophages may be important, next to reactive oxygen species. The ability of *C. albicans* to incorporate methionine, valine, lysine, phenylaline and others is reduced when lysosomal extracts from rabbit alveolar macrophages are added *(Peterson et al, 1978)*. The activity of these lysosomal extracts has been associated with two highly cationic proteins, defensins MCP-1 and MCP-2. They play a role in the damage process of the cytoplasmic membrane of *C. albicans* and suppress the consumption of oxygen by the fungus *(Patterson-Delafield et al, 1981)*.

Probably other nonoxidative mechanisms also play a role, but they have not been studied intensively yet. By combining oxidative and nonoxidative mechanisms, macrophages can kill *C. albicans*. Most likely, all of these mechanisms have to be used in order to defeat *C. albicans*.

C. albicans goes into apoptosis

After being exposed to oxidative stress, *C. albicans* goes into apoptosis. *Phillips et al, 2003* found that after exposure to low dosis of acetic acid and hydrogen peroxide C. *albicans* demonstrated chromatin margination and condensation, nuclear fragmentation and nuclear envelope separation. When *C. albicans* is treated with proapoptotic doses of acetic acid and hydrogen peroxide the yeast form switched to hyphae. It is hypothesized that the pathways involved for apoptosis and morphogenetic switching are closely linked *(Phillips et al, 2003)*. A study by the author and others (unpublished) found indications that *C. albicans* co-cultured and ingested by macrophages most likely goes into apoptosis. Also one of the apoptotic markers, the Mammalian caspase homologue 1, tends to be upregulated in *C. albicans*. Oxidative stress might play a role as superoxide dismutase (enzyme that neutralizes ROS) deficient mutants depict a higher apoptosis rate. Thus, mononuclear immune cells probably are capable of inducing apoptosis in *C. albicans*.

Resistance to the immune system from C. albicans

Mostly studied in the planktonic form

As *C. albicans* and the human body live next to each other, the fungus has developed mechanisms to evade the immune system. This phenomenon is seen as well in the planktonic as in the biofilm form. Overall it all starts with evading the recognition step.
While, it hasn't been studied extensively in *C. albicans*, in other organism evading of recognition is observed. For example, *Drosophila melanogaster* who has a Gram-negative binding protein 3 (like dectin-1 in humans) for detecting glucans from fungi *(Gottar et al, 2006)*. This protein 3 can be degraded by proteases from certain fungi, thereby avoiding being detected. But in the case of *D. melanogaster*, this protease activates a Toll pathway, after which recognition still occurs. This is most likely an example of co-evolution *(Netea et al, 2008)*. In our body, the recognition of β-glucans, like on *C. albicans*, by CR3 does not evoke a protective host response, but it can even repress pro-inflammatory signals *(Wright et al, 1982)*. There is also a difference in receptor activation between yeast and hyphae. Dendritic cells recognize yeast through the mannose receptor and dectin-1, while hyphae are recognized by dectin-2, FcγRII, FcγRIII

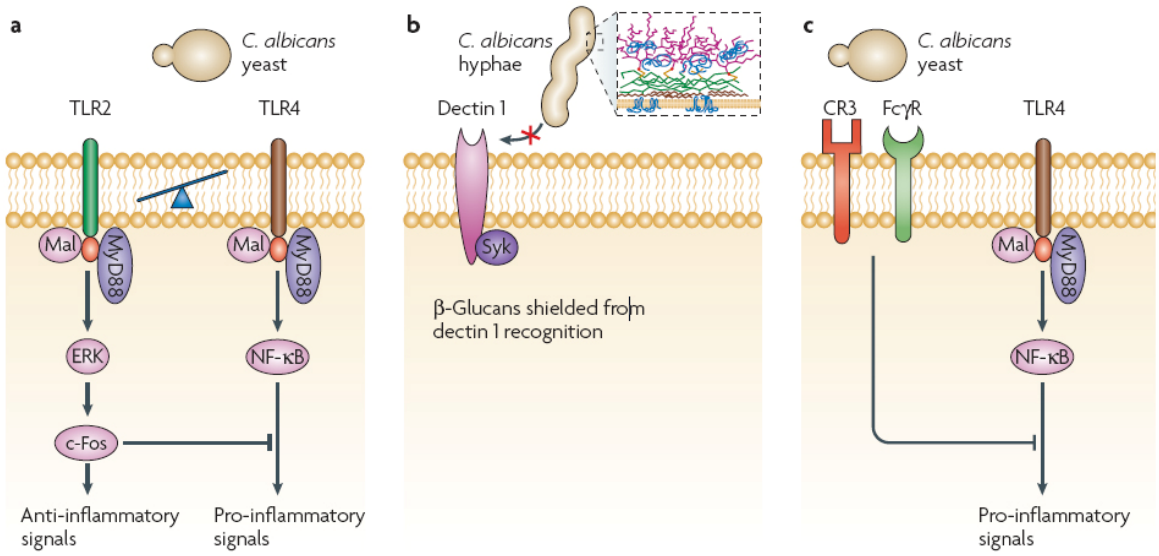

Figure 5. Candida albicans mechanisms to escape the innate response using pattern-recognition receptors. ***a*** *The balance between the signals that are induced by Toll-like receptor 2 (TLR2) and TLR4 seems to have a crucial role in the regulation of the immune response. TLR2-mediated signals are mainly anti-inflammatory and TLR4-mediated signals are mainly pro-inflammatory.* ***b*** *Shielding of the β-glucans by mannans in hyphae prevents activation of the dectin 1 signaling pathways.* ***c*** *Inhibitory signals from complement receptor 3 (CR3) and Fcγ receptor II (FcγRII)/FcγRIII have inhibitory effects on the activation of the immune system via TLR4. ERK: extracellular signal related kinase; MyD88: myeloid differentiation primary response gene 88; NF-κB: nuclear factor κB; Syk: spleen tyrosine kinase, TLR: Toll-like receptor. From Netea et al, 2008.*

and CR3 *(as reviewed in Netea et al, 2008)* (Figure 5). This lead to the hypothesis, that the morphogenetic switch of *C. albican*s from yeast to hyphae, is a mechanism by which the fungi avoids recognition of β-glucans *(d'Ostiani et al, 2000)*. Because hyphal filaments lack β-glucan exposed on the surface.

There are cell-surface modifications involved that might influence immune detection *(Gantner et al, 2005)*. Possible mechanisms are covering of the β-glucan layer of the cell wall by a mannoprotein layer, consequently blocking the interaction of dectin-1 with β-glucan. Then dectin-1 can't induce a pro-inflammatory response. β-glucan can also be protected by another cell wall component, α-(1,3)-glucan, so both mannans and α-glucans can act as a protective layer against dectin-1 interaction *(Rappleye et al, 2007)*. But mannans are also immunostimulatory, therefore they also lead to a response. But the stimulation of the β-glucan-dectin-1 and mannan-TLR pathway has a synergistic effect, amplifying the immune response. If the interaction with dectin-1 is shielded by mannans, the synergism might be less. Then the host immune system is less activated, although mannans are recognized *(Netea et al, 2008)*.

C. albicans also uses the environment. Apart from the usage of superoxide dismutase, catalase and thiourea enzymes to inhibit the oxygen radicals released by mononuclear cells from the host, *C. albicans* also can prevent the production of these. In anaerobic condition, the killing of *C. albicans* is much lower than observed in aerobic

conditions. In this situation the non-oxygen-dependent pathways kills *C. albicans*, but oxygen-dependent pathways are needed to reach the maximum killing capacity. Thus, most likely the virulence of *C. albicans* is higher in wounds or tissues with low oxygen concentration and poor blood supply *(Thompson et al, 1992)*.

There is also an indication, that *C. albicans* can inhibit the production of oxygen radicals by phagocytic cells. The suppression of reactive oxygen species production seems to override stimulatory signals from the cell wall, like β-(1,3)-glucan. Therefore *C. albicans* seems to have the ability to control host production of ROS and this might be an important advance in the *C. albicans*-host interaction. Although, up till now there is no concluding evidence *(Wellington et al, 2009)*.

Resistance in biofilms

Different studies concluded that *C. albicans* biofilms are more resistant to therapies. Substances like ethanol, H_2O_2 and sodium dodecyl sulfate reduce the metabolic activities of a *C. albicans* biofilm, at concentrations normally used for disinfection. But the concentrations of the biocides needed to inhibit growth were higher for biofilms than for the planktonic form, when containing the same amount of cells. *(Nett et al, 2008)*. Overall, the concentrations were 2 to 10-fold higher in order to inhibit a biofilm cell than for planktonic cell inhibition. Although the concentrations required of H_2O_2 were lower. *(Nett et al, 2008)*. Ethanol and H_2O_2 also positively affect the activity of fluconazole against *C. albicans* biofilms. As these cell wall disturbing agents act on the cell wall of *C. albicans* and have a positive impact on fluconazole, therefore in biofilm resistance a potential role for cell wall integrity is suggested *(Levin, 2005)*. The concentration of biocides needed to affect *C. albicans* is greater than for killing of planktonic cells, thus biofilms are less susceptible *(Nett et al, 2008)*.

C. albicans also can influence the immune system itself. *Chandra et al, 2007* found that a co-culture of *C. albicans* with adherent peripheral blood mononuclear cells (PBMCs) (like macrophages and monocytes) raises the ability to form a *C. albicans* hyphae-rich biofilm. This is most likely achieved by a soluble factor released by *C. albicans.* In fungal biofilms PBMCs are unable to phagocytise, in contrast, to when *C. albicans* is in its planktonic form. The PBMCs are also localized to the basal layers, where they can't escape the biofilm anymore. They also showed that the supernatant from biofilms co-cultered with PBMC is responsible for the biofilm enhancement. This supernatant has elevated levels of proinflammatory cytokine IL-1β, however decreased concentrations of IL-6, IL-10 (both anti-inflammatory), TNF-α (proinflammatory) and others. The cytokine profile of PBMCs co-cultured with the planktonic and biofilm *C. albicans* differs. When compared with the planktonic form IL-1β and IL-10 are upregulated in the biofilm. PBMCs evoke a strong inflammatory response when they are exposed to a biofilm. Most likely, anti-inflammatory cytokines (like IL-10) are induced through autoregulatory pathways. They contribute and regulate the inflammatory response. Therefore, biofilm formation might be a defense mechanism to protect against immune cells. *(Chandra et al, 2007).*

C. albicans helps the host immune reaction

Farnesol is a metabolic product of the sterol/mevaronate synthesis in eukaryotes *(Karst et al, 1977)*. In eukaryotes it overall functions endogenously, but in *C. albicans* this molecule acts through an autocrine pathway and regulates virulence and morphogenesis. When farnesol accumulates, it blocks the morphological shift from yeast to hyphal form at high cell densities *(Hornby et al, 2001)*. Farnesol produced in situ by *C. albicans* in the planktonic form, prevents biofilm formation *(Ramage et al, 2005). Décanis et al, 2009* found an involvement of gingival epithelial cells in an innate immunity response against *C. albicans*. This happens through TLRs, in particular TLR2. TLR2 is upregulated by farnesol, therefore this molecule supports and promotes the cell defense. In addition, farnesol downregulates TLR4 and TLR6 expression, thereby acting as an anti-inflammatory molecule. TLR4 is normally activated by a *C. albicans* infection, but farnesol suppressed this and thus most likely has also anti-inflammatory properties. TLR6 is another receptor playing a role in pathogen recognition and host defense. Farnesol has no effect on TLR6 expression, but when the cells were cultured with *C. albicans* farnesol inhibited TLR6 expression *(Décanis et al, 2009)*. TLRs play a critical role in the host's recognition and defense against pathogens, therefore farnesol may impair the response of the host *(Trinchieri et al, 2007)*. Farnesol can also modulate pro-inflammatory cytokines like IL-6 and IL-8. IL-6 is upregulated and to a lesser extent IL-8. Also, epithelial cells produce more antimicrobial peptides like hBDs, in particular, farnesol promotes hBD2 production. Hence, farnesol promotes epithelial cell defense against *C. albicans* through upregulation of hBD2, IL-6 and TLR2 *(Décanis et al, 2009)*.

Conclusion

C. albicans is a fungal pathogen, with rather a complex interaction with our immune system. This review compared the planktonic and biofilm form and its interaction with our immune cells, whereas there has not been a lot of research on *C. albicans* biofilms. In order to understand through which mechanisms *C. albicans* biofilms are more resistant to our immune system. It was important to first clearly define how this interaction is with the planktonic form, in order to understand what is different in *C. albicans* biofilms. From this review three main arguments can be deduced, where the *C. albicans* biofilm and the planktonic *C. albicans* clearly differ.

Firstly, *C. albicans* biofilms have an important characteristic extracellular matrix, which protects the cells. It is most likely more difficult for immune cells to infiltrate. But even when they do, they are localized to the basal layers and usually can not leave anymore. The extracellular matrix also restricts the diffusion of toxic substances; therefore any substances released by immune cells might not reach *C. albicans* inside the biofilm. Hence, the extracellular matrix is a strong defense for *C. albicans*, which the planktonic form does not have and explains part of the lower susceptibility of biofilms.

Secondly, the cell wall plays a role. The cell wall consists of a skeletal and matrix component. The skeletal component consists mostly of β-glucans and the matrix of glycosylated proteins like mannan and carbohydrates. Biofilms consist of a large amount of hyphae and the recognition of these hyphae plays a vital role. Antibodies most likely recognize all of the cell wall components, also hyphae-specific surface proteins encoded

by genes like *ALS3* and *HWP1*. As for others like the mannose receptor, TLRs and the hyphae specific dectin-2 receptor, they all recognize hyphae. But the β-glucan receptor group on immune cells is the one that differs. This group contains the CR3 and dectin-1 receptor, with the last one to be the most interesting one. Dectin-1 can recognize *C. albicans* yeast, but not hyphae. Because hyphae do not have β-glucans exposed on their surface as they are protected by mannans or α-glucans. Then dectin-1 can not induce a pro-inflammatory response. Dectin-1 is an important player, apart from the stimulation of IL-2, IL-6 and IL-10 production it also plays a vital role in phagocytosis. If the recognition of *C. albicans* by this receptor does not occur, then it will be much easier for *C. albicans* to evade our immune system. Especially if you keep in mind, biofilms have an extracellular matrix which already makes recognition and infiltration for immune cells much more difficult. Therefore the inability of dectin-1 to recognize gives *C. albicans* a significant advantage.

Finally, *C. albicans* has the ability to influence the immune system itself. Co-culture with PBMCs (like macrophages) raised the ability of *C. albicans* to form a hyphae-rich biofilm. Most likely this happens after release of a soluble factor by *C. albicans*. These PBMCs are unable to phagocytise (maybe because of no dectin-1 recognition) and the release of IL-1β and IL-10 is upregulated. IL-10 is an anti-inflammatory interleukin and therefore probably helps *C. albicans* by inhibiting the immune respons. As can be concluded from this, our immune system most likely helps *C. albicans* to survive and form a biofilm.

There are of course also other mechanisms which help *C. albicans* biofilms to evade the immune system. Different enzymes like catalase can inhibit oxygen radicals and therefore probably prevent apoptosis of *C. albicans*. Biofilms most likely have a lower oxygen concentration which makes it more difficult for phagocytic cells to kill *C. albicans* through oxygen-dependent pathways. Thus, the maximum killing capacity will never be reached. But also the cell wall might play a role in the defense against these oxygen radicals. Apart from this, there are indications that *C. albicans* can inhibit the production of oxygen radicals by phagocytic cells. As follows, other pathways adjacent to the most important ones discussed above are also most likely involved.

Although *C. albicans* tries to prevent destruction and killing by the human body, it also helps the immune system. Farnesol is produced in eukaryotes and regulates virulence and morphogenesis in *C. albicans*. When it accumulates in *C. albicans*, it prevents the morphological switch from yeast to hyphae at high cell densities. Also, it prevents biofilm formation and promotes epithelial cell defense against *C. albicans* through upregulation of IL-6, hBD2 and TLR2. As a result, farnesol probably is not a beneficial molecule for *C. albicans.*

In conclusion, *C. albicans* has a wide range of possibilities in order to evade our immune system and form strong biofilms. The three key players are the extracellular matric, cell wall and the interaction with PBMCs, but also other smaller mechanisms play a role. In order to defeat *C. albicans*, al least one of those key players must be removed to transfer the advance back to our immune system as it normally has in our body.

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