PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher’s version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/19512

Please be advised that this information was generated on 2019-04-12 and may be subject to change.
HOST DEFENCE AGAINST DISSEMINATED AND INVASIVE

CANDIDA ALBICANS INFECTIONS

A.G. Vonk
HOST DEFENCE AGAINST DISSEMINATED AND INVASIVE

CANDIDA ALBICANS INFECTIONS

een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus prof. dr. C.W.P.M. Blom, volgens besluit
van het College van Decanen
in het openbaar te verkrijgen op maandag 18 oktober 2004
des namiddags om 3.30 uur precies
door

Alouise Gabrielle Vonk

geboren op 24 november 1969
te Rijswijk (ZH)
Promotores: Prof. dr. B.J. Kullberg
Prof. dr. J.W.M. van der Meer

Co-promotor: Dr. M.G. Netea

Manuscriptcommissie: Prof. dr. P.E. Verweij
Prof. dr. A. Voss
Dr. J.W. van’t Wout, LUMC

ISBN 90-9018415-5

© 2004 A.G. Vonk. All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means without written permission of the author, except for your own personal, non-commercial use, provided that you keep intact all copyright and other proprietary notices and due credit is given to the author.

Cover illustration: scanning electron microscopy showing Candida albicans morphologies. Photograph kindly provided by Dennis Kunkel (www.denniskunkel.com).

Printed by Ponsen & Looijen, Wageningen

The financial support from Pfizer bv is gratefully acknowledged
آن کس که بداند و بداند که بداند
اسب طلب خویش به افلاک رساند
آن کس که نداند و بداند که نداند
آو هم خرک لنگ به منزل برساند
آن کس که نداند و نداند که نداند
در جهل مركب ابدالدهر بماند

ابن يمين فري عمامدي

Iemand die weet en weet dat hij weet,
rijdt zijn paard van verlangen en wensen naar de hemel.
Iemand die niet weet en weet dat hij niet weet,
rijdt zijn manke ezel naar huis.
Iemand die niet weet en niet weet dat hij niet weet,
blijft voorgoed in absolute onwetendheid.

Ebnejamine Faryemady, omstreeks 1400 na Christus

براي فرزين
voor Farzin
Contents

Chapter 1
Introduction: host defence against disseminated and invasive candidiasis & outline of the thesis
Submitted for publication

Chapter 2
Phagocytosis and intracellular killing of Candida albicans blastoconidia by neutrophils and macrophages: a comparison of different microbiological test systems

Chapter 3
The influence of endogenous pro-inflammatory cytokines on neutrophil mediated damage of Candida albicans pseudohyphae; quantified in a modified tetrazolium dye assay
Submitted for publication

Chapter 4
Endogenous interleukin-1 alpha and interleukin-1 beta are essential to host defence against disseminated candidiasis
Submitted for publication

Chapter 5
Differential role of IL-18 and IL-12 in the host defence against disseminated Candida albicans infection
European Journal of Immunology 2003; 33: 3409-3417

Chapter 6
Apolipoprotein-E-deficient mice exhibit an increased susceptibility to disseminated candidiasis
Medical Mycology 2004; 42: 341-348
Chapter 7
Delayed clearance of intra-abdominal abscesses caused by *Candida albicans* in tumour necrosis factor-α and lymphotixin-α deficient mice
*Journal of Infectious Diseases* 2002; 186: 1815-1822

Chapter 8
Treatment of intra-abdominal abscesses caused by *Candida albicans* with anti-fungal agents and recombinant murine granulocyte colony stimulating factor
*Antimicrobial Agents and Chemotherapy* 2003; 47: 3688-3693

Chapter 9
Modulation of the pro- and anti-inflammatory cytokine balance by amphotericin B

Modulation of the pro- and anti-inflammatory cytokines by amphotericin B
*Journal of Infectious Diseases* 1999;180:1408–1409 (letter)

Chapter 10
Summary and discussion

Chapter 11
Samenvatting en discussie

Dankwoord

Curriculum vitae

Appendix A  List of publications

Appendix B  List of abbreviations
Introduction:
Host defence against disseminated and invasive 
\textit{C. albicans} infections

Alieke G. Vonk, Mihai G. Netea, Jos W.M. van der Meer, Bart-Jan Kullberg

Department of Medicine, Radboud University Medical Centre and
Nijmegen University Centre for Infectious Diseases
Nijmegen, the Netherlands

Submitted for publication
Introduction

The yeast *Candida* is a common colonizer of the human skin, as well as the oral and gastrointestinal mucous membranes. The genus *Candida* comprises more than 150 species, with only few species being considered as opportunistic fungal pathogens. These latter species are able to cause candidiasis. This is a disease which includes a broad spectrum of infections, ranging from superficial mucosal infections to life-threatening invasive disease in which virtually any deep organ can be involved. Discussion of the clinical manifestations of candidiasis is usually facilitated by dividing candidiasis into mucocutaneous and invasive infection. Examples of the various disease forms are shown in table 1.

<table>
<thead>
<tr>
<th>Table 1. Examples of disease forms of candidiasis.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muocutaneous infection</strong></td>
</tr>
<tr>
<td>- onychomycosis</td>
</tr>
<tr>
<td>- oropharyngeal candidiasis</td>
</tr>
<tr>
<td>- chronic mucocutaneous candidiasis</td>
</tr>
<tr>
<td>- <em>Candida</em> esophagitis</td>
</tr>
<tr>
<td>- intertrigo</td>
</tr>
<tr>
<td>- genital candidiasis</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

*Candida albicans* has been incriminated as the primary etiologic species of candidiasis. The most important risk factor for developing *C. albicans* infection is an impaired host defence system. Moreover, it has been known that the nature and extent of the impairment of the host defence dictates the manifestation and severity of the *Candida* infection. In other words, the clinical manifestations of *C. albicans* infection are contingent upon the immune response of the host. In this chapter, the host defence mechanisms that participate against disseminated and invasive *C. albicans* infection will be discussed.

**Definitions**

For this review *disseminated candidiasis* denotes the haematogenous spread and homing of *C. albicans* in distant organs during candidemia. *Invasive candidiasis* implies intraperitoneal *C. albicans* infection, which includes both *C. albicans* peritonitis and localized *C. albicans* abscesses. Description of host response against mucocutaneous *C. albicans* infections, as well as the other forms of invasive *C. albicans* infection goes beyond the scope of this review.
Host defence against disseminated candidiasis

After damage of the first line of defence against *Candida* infections, which includes the skin and the mucosa with its normal flora offering colonization resistance, *C. albicans* is able to enter the bloodstream and to spread to other parts of the body. Here, *C. albicans* is encountered with innate as well as acquired cell-mediated immune mechanisms.

**Innate immune system**

Blood-borne *C. albicans* blastoconidia that are not cleared by circulating polymorphonuclear phagocytes (PMNs), monocytes, and candidacidal factors (discussed below), are delivered to the target organs by adherence to endothelial cells or structures belonging to the extracellular matrix. Adhesion of *C. albicans* is mediated by mannoprotein receptors for iC3b and fibronectin [1-3]. In undamaged endothelium, the primary route of endothelial monolayer traversal consists of phagocytosis of *C. albicans* blastoconidia and germ tubes by endothelial cells, a process that does not require opsonisation [4-8].

After ingestion, *C. albicans* can escape from the endothelial cell by fusion of the phagosome with the abluminal plasma membrane, or damaging the endothelial cell by germination [5,6,8] (Fig.1). Endothelial contraction and damage expose the subendothelial extracellular matrix, which is normally only exposed in special anatomic locations such as the glomerulus. Additional *Candida* yeasts can adhere to the exposed subendothelial extracellular matrix and *C. albicans* binds more avidly to this structure than to endothelium [9,10] (Fig.1). The adherence is further enhanced by platelet aggregation on the exposed extracellular matrix, providing an extended surface area for adherence and the entrapment of *Candida* cells in the platelet-fibrin aggregate [11]. Platelets may also play an antagonistic role against *Candida* infection via the production of platelet microbicidal protein that has been shown to kill *Candida* blastoconidia [12-14].

The endothelial traversal of *C. albicans* also activates the endothelial cells. In response to the phagocytosis of live germinating *C. albicans*, endothelial cells have been reported to produce tumour necrosis factor-α (TNFα), interleukin (IL)-1α, IL-1β, IL-8, IL-6, granulocyte colony stimulating factor (G-CSF) and monocyte chemoattractant protein 1 (MCP-1). In addition, via (autocrine) effects of pro-inflammatory cytokines, endothelial cells respond to *Candida* invasion by expressing leukocyte adhesion molecules, such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular adhesion molecule 1 (VCAM-1) [8,15-18]. With the increased expression of adhesion molecules, endothelial cells provide phagocytes, that express the integrin counter-receptor following chemokine-
Introduction

stimulation, the opportunity to firmly adhere to their surface [19,20]. The magnitude and composition of leukocyte influx at the site of infection is regulated by the secretion of chemokines, such as MIP-1α, MIP-1β, MCP-1, IL-8 or its murine counterpart KC [19]. Subsequent migration of phagocytes upon a gradient of chemokines, released by various cell types, initiates a local inflammatory reaction. Pro-inflammatory cytokines released by macrophages, PMNs and NK-cells (discussed below) enhance their own function as well as the function of each other in anticandidal host response (Fig. 1).

Once Candida cells have passed the endothelium, traversal of the extracellular matrix has to ensue before tissue parenchyma can be infiltrated. During this process they are encountered by phagocytic cells.

Phagocytic cells

Phagocytes, i.e., PMNs and mononuclear phagocytes are essential components of the innate immune resistance. The contribution of PMNs and mononuclear phagocytes to innate resistance against disseminated candidiasis has been assessed in several in vivo experiments. Studies in which mice were rendered severely granulocytopenic have established that PMNs play a crucial role in host defence against disseminated candidiasis, as evidenced by an increased outgrowth of Candida blastoconidia from the organs and decreased survival rates compared to immunocompetent mice [21-25].

With respect to the role of macrophages in disseminated candidiasis however, evidence has been produced that both supports and refutes the importance of macrophages in host resistance against C. albicans. Selective elimination of macrophages from mice by liposomes containing dichloromethylene diphosphonate cleared C. albicans more slowly from the bloodstream and showed a significantly impaired survival of experimental disseminated candidiasis [26]. In agreement with these observations, depletion of macrophages with carrageenan, a cytotoxic agent for macrophages [27], significantly increased the number of Candida colony forming units (cfu) in the organs 24 h after intravenous injection of C. albicans into severe combined immunodeficiency (SCID) mice [28]. In contrast, monocytopenia in mice induced by etoposide did not have a detrimental influence on the course of disseminated candidiasis and confirmed that PMNs rather than macrophages play a dominant role in the early host response against systemic Candida infection [21].
Fig. 1. Cytokines are major orchestrators of the complex interplay between innate and acquired cell-mediated immunity against *Candida albicans* infection (φ: macrophage).
The contribution of PMNs and macrophages to host defence against *C. albicans* has been determined by assessing their capacity to internalise and kill *Candida* blastoconidia in vitro. Although these studies have yielded divergent results due to a number of substantial methodological variations, it can be concluded that both PMNs and macrophages ingest and kill internalised *Candida* blastoconidia [29-37], with PMNs showing a more potent capacity than macrophages to kill *Candida* blastoconidia intracellularly [29,33,38]. One explanation for the divergent results may be that resident macrophages require priming, for instance with interferon-γ (IFNγ), to activate their killing mechanisms [32,36,37,39]. Intracellular killing of *Candida* blastoconidia by PMNs in vitro is augmented by TNFα [34,40], IFNγ [29,34,41-43], and G-CSF (Fig.1) [44,45].

Because growth of *C. albicans* is polymorphic, i.e. it exits both as yeast and filamentous (pseudohyphal and hyphal) growth forms, phagocyte action against *Candida* hyphal morphologies is also of importance. Both PMNs and monocytes have been shown to kill large uningestible hyphal growth forms of *Candida* extracellularly [46].

Whereas the presence of fresh serum, which provides complement for opsonisation of *Candida* cells, is required for optimal phagocytosis [30,31,47-49], monocyte or PMN-mediated damage of *C. albicans* hyphae occurs both in the absence of serum and in the presence of heat-inactivated serum, indicating that for this process complement is not essential [46,50,51]. Furthermore, similar to the PMN-mediated intracellular killing of *Candida* blastoconidia, the extracellular antifungal activity of PMNs against *C. albicans* hyphal forms is enhanced by IFNγ, G-CSF and TNFα (Fig.1) [52,53].

Phagocytes kill *C. albicans* intracellularly or extracellularly by means of both oxidative and non-oxidative candidacidal mechanisms. Oxidative mechanisms of PMNs and monocytes can be divided into myeloperoxidase (MPO)-dependent and MPO-independent reactions (Table 2) [50,51,54-59].

During the transition of monocytes into macrophages, myeloperoxidase is lost, as a result of which macrophages are unable to oxidise a halide in the presence of hydrogen peroxide and to create a strong oxidant such as hypochlorous acid (HOCL). Instead, nitric oxide (NO) is produced by activated macrophages, which together with MPO-independent superoxide (O2-) generation by phagocyte NADPH oxidase gives rise to the formation of another strong oxidant, peroxinitrite (ONOO-). It has been shown that ONOO- exerts the candidacidal activity of activated macrophages [60]. Because ONOO- kills *C. albicans* blastoconidia in vitro and NO does not, it has been suggested that the enhanced resistance of mice to systemic candidiasis attributed to macrophage derived NO-production [61-63] is in fact mediated by ONOO- [60].
Non-oxidative candidacidal mechanisms of PMNs include granule-derived antimicrobial peptides such as lysozyme [50,54], calprotectin [64], lactoferrin [65], azurocidin [66], human β-defensin 3 [67], and cathelicidin LL-37 [68]. Macrophage non-oxidative *C. albicans* killing has been shown with lysosomal extracts from rabbit alveolar macrophages that contained the defensins microbicidal cationic protein-1 and 2 [69].

Table 2. Intra- and extracellular killing mechanisms of *C. albicans* by phagocytes.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Phagocytes involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative</td>
<td>PMNs, monocytes</td>
</tr>
<tr>
<td>MPO-dependent</td>
<td>PMNs, monocytes</td>
</tr>
<tr>
<td>MPO-independent</td>
<td>PMNs, macrophages</td>
</tr>
<tr>
<td>MPO-independent ONOO'</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Non-oxidative NO</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Antimicrobial peptides</td>
<td>PMNs, macrophages</td>
</tr>
</tbody>
</table>

**NK-cells**

Natural killer (NK) cells are large granular lymphocytes that arise from the same progenitor cell as T lymphocytes during differentiation. In contrast with T-cells, which require MHC class I expression by target cells to initiate lysis, NK cells preferentially kill cells lacking MHC class I expression [70]. NK-cells bind to *C. albicans* and one of the surface structures of NK-cells that mediates adhesion to *C. albicans* has been shown to be β2-integrin CD11b/CD18 [71]. Interleukin (IL)-2 activated natural killer cells (LAK) cells bind more extensively to *Candida* germ tubes than NK cells, but both are unable to kill or inhibit germ tube or hyphal growth forms of *C. albicans* [72-74]. These data suggest that NK-cells do not have an important direct antifungal effect. Furthermore, NK-depleted SCID mice did not show an enhanced susceptibility to candidiasis induced by i.p. *C. albicans* administration [75]. However, NK-cells are activated by *C. albicans* [76], and human LAK-cells respond to live or heat-killed *C. albicans* germ tubes with an increased expression of TNF-α, GM-CSF, and IFN-γ mRNA [73]. Hence, NK-cells are suggested to play a role in antifungal host defence through activation of phagocytes. Indeed, evidence has been provided that NK cells are the main inducers of phagocytic activity of splenic macrophages and that they mediate the protection against systemic *C. albicans* infection [77].
Soluble factors

Complement and mannose binding lectin

The complement system is a group of heat-labile proteins which can be activated in a cascade fashion to provide a humoral defence against various micro-organisms. Complement activation is initiated via several pathways that all converge at a single point, the third complement component (C3).

The role of complement in candidiasis is evidenced by several observations. First, *C. albicans* activates the complement cascade via both the classical (antibody-antigen) and alternative pathway (cell surface material with repeating chemical structure) and shows binding of C3 fragments [78,79]. Second, the presence of C3 promotes the ingestion of *C. albicans* by phagocytes and is prevented by anti-C3 [49]. Third, C5-deficient mice show an increased amount of *Candida* cfu in their organs and an increased mortality in disseminated candidiasis, probably due to lack of C5a, an important leukocyte chemoattractant [80].

Moreover, the classical pathway of the complement cascade can also be activated by mannose binding lectin (MBL). MBL is another important serum protein of innate immunity that is synthesized by the liver. MBL is able to recognize and bind to multiple sugar ligands expressed on the surface of microbes, such as mannose on the surface of *C. albicans* [81,82]. It has been shown that MBL-initiated complement activation may play an important role in anticandidal host defence of previously unexposed and non-immune hosts [81]. However, others have found no differences in the survival rates and fungal burdens of wild-type and MBL-A-deficient mice, indicating that MBL-A does not substantially contribute to host resistance against disseminated candidiasis [83]. Likewise, we were not able to find a role for MBL in *Candida*-induced cytokine production, and the MBL genotype of patients with candidemia did not differ from that of healthy individuals (C. van der Graaf et al., manuscript in preparation).

Lipoproteins

Major lipoprotein classes in plasma, such as very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) are able to detoxify lipopolysaccharide (LPS) [84,85]. The LPS-neutralising activity is mediated by both lipid-endotoxin and protein-endotoxin interactions. The lipid A domain of LPS is masked either by insertion of this domain in the phospholipid leaflet that covers the surface of lipoproteins, or it is bound and sheltered by the hydrophobic structures of apolipoproteins [84,86-88]. In addition, Apolipoprotein E (ApoE) directs LPS away from cytokine producing Kupffer cells to parenchymal liver cells [89], thus reducing cytokinemia.
Whereas hyperlipidemia and the presence of ApoE protect against LPS-induced lethality and Gram-negative bacterial infection [87,90-93], hyperlipidemia renders mice susceptible to disseminated candidiasis [94]. Infusion of reconstituted HDL into human volunteers resulted in increased *C. albicans* growth in the plasma of these subjects [95]. It is hypothesized that the excess of lipoproteins influences the growth of *C. albicans* blastoconidia either by serving as a nutrient [95-97], or by binding and inactivating plasma candidacidal factors, such as sphingosine [98], the calprotectin complex [64], or platelet microbicidal protein [12,13]. Whether ApoE plays an immunomodulatory role in the putative neutralisation of candidacidal factors by lipoproteins has yet to be established.

**Acquired immunity**

From the aforementioned data it has become apparent that professional phagocytes such as PMNs and macrophages play a crucial role in the innate immune defence against disseminated candidiasis. The innate immune system however, does not operate individually and isolated from acquired immunity. Phagocytes also serve afferent as well as efferent functions in the acquired immunity against infections by *Candida albicans*, as will be outlined in the following paragraphs.

**Humoral immunity**

The role of anti-*Candida* antibodies in host defence against disseminated candidiasis has remained an issue of debate since the finding that yeast-immunised mice showed resistance to disseminated candidiasis, which correlated with high anti-*C. albicans* antibody titres in their sera [99,100]. Since then, facts arguing in favour of, as well as facts militating against a protective function have accumulated [101-103].

Vaccination of mice with a liposomal mannan preparation of *Candida albicans* induced the production of antibodies that protect against disseminated candidiasis [104]. Protection was conferred by immunoglobulin M monoclonal antibody (MAb) B6.1 specific for a *C. albicans* cell surface β-1,2-mannotriose, or the IgG3 isotype of MAb C3.1 which is specific for the same B6.1 epitope on the yeast cell surface. Mice treated with Mab's had significantly reduced amounts of *Candida* cfu in their kidneys and extended survival times [104,105]. The mode of action appeared to be through activation of the classical complement pathway, and enhanced killing of *C. albicans* blastoconidia by neutrophils, which only takes place in the presence of complement [31,106,107].

In contrast, B-cell defective CBA/N or SCID mice deficient in both T and B cells are as resistant to systemic *Candida* infections as immunocompetent mice are [28,108,109]. Taken together, these data suggest that humoral immunity does not play a crucial role in the defence against disseminated candidiasis, but antibodies may aid innate host resistance...
against disseminated *C. albicans* infection. However, recently, a new mechanism through which antibodies may contribute to protective immunity has been provided [110]. *Candida* opsonizing antibodies were required for modulating the interaction of *Candida* and dendritic cells, in that opsonizing antibodies increased phagocytosis of *Candida* yeast through CR3 and IL-10 production by dendritic cells [110-112]. These events are important for T regulatory cell induction and generation of memory anticandidal immunity, as will be discussed below.

**Cell-mediated immunity**

Whereas some controversy exists with respect to the contribution of the humoral immune response to anticandidal host defence, the role of T-cell mediated immunity in host defence against disseminated candidiasis has been largely established. T-cell mediated immunity has been studied in several experimental models. Of these models, T-cell mediated immunity against acute disseminated candidiasis in naive mice (animals that encounter the antigen for the first time, i.e. primary infection) and T-cell mediated immunity against reinfection of mice that were vaccinated with a systemic infection using agerminative yeast cells of a *Candida* variant strain will be discussed.

After invasion of the host by *C. albicans*, activation of professional phagocytes is the main defence mechanism. Efficient activation of PMNs and macrophages depends on a CD4+ T helper (Th) 1 response, mainly IFN-γ.

Indeed, in the past, a correlation has been shown between the susceptibility of mice to *C. albicans* infection and the type of Th cytokine pattern that is produced. Resistance to primary systemic *C. albicans* infection is associated with a Th1 response, represented by the production of cytokines such as IFN-γ and IL-2. IL-12, mainly produced by macrophages, primes naïve T cells for IFN-γ production [113]. IFN-γ sustains the Th1 response, suppresses the induction of a Th2 response, and enhances phagocyte fungicidal effector function (Fig.1).

In contrast, susceptibility of mice to primary systemic *C. albicans* infection is associated with a Th2 response, characterized by the presence of anti-inflammatory cytokines such as IL-10 and IL-4 [114-119]. IL-10 and IL-4 sustain a Th2 response, suppress the induction of a Th1 response and down-regulate phagocyte candidacidal activity [120,121] (Fig.1). Furthermore, neutralization of IL-4 or IL-10 during primary systemic *C. albicans* infection has been shown to induce the development of protective Th1 responses. These Th1 responses positively influenced the course of primary infection and rendered susceptible mice resistant to primary *C. albicans* infection [116,118-120].
Next to T helper cells, the CD4+ T cell population also constitutes CD4+ T regulatory cells [122]. Different subsets of T regulatory cells have been described. Each subset may differ in the mode of generation and mechanism of action, although evidence for similarities between the different T regulatory subsets have been shown [122-124]. There are substantial data that regulatory T cells, specialized in controlling self-reactive T-cells, play a critical role in immune regulation. Therefore, it is suggested that immune responses driven by Th1 and Th2 cells are also influenced by T regulatory cells which are characterized by the production of IL-4, IL-10 and transforming growth factor-β (TGF-β) [122-124]. These cytokines are able to induce a Th2 polarization [16,116,125-130]. Their presence might explain why neutralization of IL-4 and IL-10 in primary systemic C. albicans infection enhances resistance of mice [116,118-120]. Moreover, mice deficient in T-cells, such as those found in CBA/N, SCID, and athymic mice are as resistant, or even more resistant to acute disseminated C. albicans infections as immunocompetent mice are [28,108,109,131-134]. The most likely explanation for these observations is that by depleting T-cells, T regulatory cells are depleted as well. By doing so, naïve mice are deprived of an early production of IL-4 and IL-10 directing the differentiation of naïve CD4+ T-cells into Th2 cells. Therefore, depletion of T regulatory cells during primary systemic C. albicans infection is suggested to be beneficial to host response.

However, it has to be taken into account that, after elimination of C. albicans, the pro-inflammatory reaction required in the early phase of the host response needs to be dampened, which is mediated through Th2 and T-regulatory cells.

Whereas early depletion of T regulatory cells in primary systemic C. albicans infection is suggested to be beneficial, T-regulatory cell depletion can be detrimental to T-cell mediated immunity of vaccinated mice that receive a second systemic C. albicans challenge. Indeed, although IL-4, IL-10, and TGF-β inhibit Th1 activation in the inductive phase of primary infection, all have been shown to be required for maintaining a long-lasting Th1 immunity to the fungus and protect against reinfection [118,121,135,136]. Also, the induction of a Th1 response required the combined effects of different cytokines, including IFN-γ [137], IL-12 [138], and TNF-α [139]. Thus, a finely regulated balance of Th1 and Th2 cytokines, rather than the absence of opposing Th2 cytokines, appears to be necessary for an optimal development and maintenance of Th1 responses in mice with a protracted C. albicans infection.

After systemic vaccination of mice with a low virulent C. albicans strain, T regulatory cells producing IL-10, IL-4 and TGF-β were indeed expanded in the mesenteric lymph nodes and thymuses of these mice and these mice were protected against reinfection [140]. Mice deficient in B7-2 or CD28 were not able to resist C. albicans reinfection and did not
show T regulatory cell expansion. This indicates that expansion of T regulatory cells required a costimulatory signal via engagement of CD28 by B7-2 which is expressed on antigen presenting cells. Moreover, T regulatory cells expanded in IL-4-deficient mice, but not in mice treated with anti-IL-10 Mab [140]. It appears that local levels of B7-2 expression, as well as IL-10 production for instance by dendritic cells, are both required for the generation of T regulatory cells during long-lasting *C. albicans* infection and T regulatory cells are an essential component of the memory-protective immunity against *C. albicans*. 
Chapter 1

Host defence against invasive candidiasis

Since colonization of the gastrointestinal tract with C. albicans is common, gastrointestinal perforation or surgery may be complicated by intraperitoneal Candida infection and formation of Candida abscesses. Invasive candidiasis that involves the abdominal cavity is as important as disseminated candidiasis and is responsible for considerable morbidity and high mortality rates [141-143].

Formation of Candida abscesses

Experimental studies have shown that, following peritoneal contamination, microbes are efficiently cleared within minutes via translymphatic absorption and subsequently exposed to systemic defence mechanisms [144,145]. Local resident peritoneal macrophages are the predominating phagocytic population and represent the first line of defence, and these are also cleared by the lymphatic system [146]. Because this clearance of invading micro-organisms is very efficient, the complex process of abscess formation in response to gastrointestinal perforation or following peritonitis induced by an experimental intraperitoneal microbial challenge occurs only when potentiating agents are present, such as autoclaved cecal contents (ACC) or bran [147,148]. These potentiating substances mechanically obstruct the lymphatics and serve as a niche for microbes to escape from phagocytosis. Furthermore, ACC and bran are opsonized [148,149], thus depleting the microenvironment of complement and subsequently inhibiting the opsonisation and intracellular killing of the invading microbial agent. With the influx of plasma proteins into the peritoneal cavity, which is normally a net fibrinolytic environment, fibrinogen is introduced as well, and fibrin is deposited after the release of procoagulant activity by activated macrophages [150]. The release of cytokines, chemokines (discussed above), other inflammatory mediators released by macrophages and mast cells, as well as activation of the alternative complement pathway by C. albicans [78] leads to recruitment and accumulation of PMNs at the site of infection.

Fibre and fibrin cloths provide a sequestered environment in which proliferation of C. albicans can prevail, and which impairs migration of phagocytes. These events together with fibroblast growth, collagen production, and the accumulation of monocytes and their subsequent transition into tissue macrophages are fundamental to the development of intra-abdominal abscess formation.

Functional activity of abscess-derived neutrophils

Abscess formation could presumably isolate the infecting Candida cells and prevent them from causing further damage to the host, provided that the organisms are kept within
the abscess cavity and killed. However, the cellular contents of abscesses consist primarily of viable and disintegrating PMNs but the killing function of PMNs is significantly suppressed within abscesses [151,152]. Bearing the last finding in mind, it is questionable whether abscess formation would be beneficial to the host. Fortunately, upon disruption or death of PMNs, cytoplasmic granules are released which contain an abundant amount of the protein calprotectin [153,154]. After lysis of PMNs, the calcium/zinc binding calprotectin accumulates in abscess fluids and competes for zinc with *C. albicans* which requires zinc for its growth [155-158]. The antifungal action of calprotectin is of a static nature and keeps *Candida* growth in an extended lag phase, an effect similar to that seen with zinc-deprived micro-organisms [159]. Furthermore, lysates of PMNs are able to prevent the formation of long, multicellular *Candida* hyphal forms and this might prevent *Candida* cells from penetrating the limits of the abscess cavity and disseminating into the surrounding tissue [154].

**Immunomodulatory activity of amphotericin B**

The polyene antifungal agent amphotericin B derives its beneficial effect mainly from direct candidacidal action. In addition, amphotericin B reportedly enhances host defence mechanisms against *Candida* infection. It has been shown that amphotericin B increases the oxidative burst of PMNs and macrophages [160-162]. Amphotericin B also stimulates the production of pro-inflammatory cytokines by mononuclear cells, such as TNF and IL-1β [163-167], and this amphotericin B-induced production seems to be mediated by Toll-like receptor 2 and CD14 [168]. As described above, pro-inflammatory cytokines such as TNF, IL-1β and IFNγ augment phagocytic effector functions. Therefore, next to accumulation of amphotericin B in phagocytes, it is likely that the amphotericin B-enhanced candidacidal functions of macrophages are mediated by the production of pro-inflammatory cytokines [169-174]. Furthermore, in vivo, amphotericin B did not increase survival of mice deficient for the IFNγ-receptor that were intravenously infected with *Candida albicans*, whereas a positive effect on survival was observed in immunocompetent mice. These results suggest an immunomodulatory effect of amphotericin B that is mediated through an IFNγ-dependent mechanism [175].
Outline of the thesis

The aim of the present thesis is to obtain further insight into antifungal host defence mechanisms against disseminated and invasive candidiasis. The contribution of PMNs and macrophages to host defence against *C. albicans* is usually determined by assessing their capacity to internalize and kill *Candida* blastoconidia in microbiological test systems. Due to a number of substantial methodological variations, these systems have yielded divergent results. In order to adequately and accurately define the phagocytosis and intracellular killing of *Candida* blastoconidia by murine exudate peritoneal macrophages and exudate peritoneal PMNs, several microbiological test systems are compared. This comparison has brought about a further refined laboratory method (Chapter 2). Both *C. albicans* blastoconidia and (pseudo)hyphae are present within lesions and the ability of *C. albicans* to undergo phenotypic switching between both growth forms is associated with virulence. Therefore, to further characterize the PMN-mediated host response against *C. albicans*, investigation of the extracellular antihyphal activity of PMNs, in addition to determination of the intracellular capacity of phagocytes to kill internalised *Candida* blastoconidia, is also required. To this purpose, a previously described colorimetric dye assay for assessment of pseudohyphal viability was optimized. The development of the assay and the influence of various pro-inflammatory cytokines on anti-pseudohyphal activity of PMNs are described in Chapter 3.

Exogenous administration of pro-inflammatory cytokines or administration of antibodies directed against pro-inflammatory cytokines has been shown to influence antifungal host defence. However, the mechanisms through which pro-inflammatory cytokines exert their effects, especially in physiologic amounts, remain to be established. Therefore, we assessed the influence of the endogenous pro-inflammatory cytokines IL-1, IL-18 and IL-12 in disseminated candidiasis. The role of these cytokines was studied mice in which the genes encoding for these cytokines had been disrupted. The influence of endogenous IL-1 in disseminated candidiasis is described in Chapter 4, and the influence of endogenous IL-18 and IL-12 in disseminated candidiasis is described in Chapter 5. Other factors, such as lipoproteins have also been shown to modulate host defence against candidiasis. In Chapter 6, the immunomodulating activity of hyperlipoproteinemia and apolipoprotein E in disseminated candidiasis was further investigated.

*Candida albicans* has also been shown to cause invasive candidiasis, i.e. *C. albicans* peritonitis and formation of *C. albicans* abscesses. There is some evidence that the risk of invasive candidiasis is increased with the systemic use of monoclonal antibodies against TNF for treatment of patients with an inflammatory bowel disease. The mechanisms through which neutralization of endogenous TNF increases the susceptibility to invasive
candidiasis are incompletely understood. Therefore, the significance of endogenous TNF and LT to the process of intra-abdominal *Candida* abscess formation and the subsequent clearance of the yeast was investigated in TNF/LT-deficient mice. The results are described in Chapter 7. In general, abscesses are difficult to treat with antimicrobial drugs only. Combinations of antifungal agents or the simultaneous administration of an immunomodulating substance that enhances host resistance, such as G-CSF, is suggested to yield better treatment results. The results of the investigation into the efficacy of combination therapy with an antifungal agent and recombinant murine G-CSF of intra-abdominal *Candida* abscesses is presented in Chapter 8.

The antifungal agent amphotericin B has both a direct antifungal effect and the ability to modulate host defence mechanisms via influencing pro-inflammatory cytokine production. The latter phenomenon is associated with side-effects such as fever and chills. The capacity of amphotericin B to modulate pro-and anti-inflammatory cytokine balance by peripheral blood mononuclear cells was determined and the results are described in Chapter 9.

**Chapter 10**: summary and discussion  
**Chapter 11**: samenvatting en discussie
References


Introduction


144. Dunn DL, Barke RA, Ewald DC, and Simmons RL. Macrophages and translymphatic absorption represent the first line of host defense of the peritoneal cavity. Arch Surg 1987; 122:105-10.


Phagocytosis and intracellular killing of *Candida albicans* blastoconidia by neutrophils and macrophages: a comparison of different microbiological test systems

Alieke G. Vonk, Catharina W. Wieland, Mihai G. Netea, Bart-Jan Kullberg

Department of Medicine, Division of General Internal Medicine, University Medical Centre Nijmegen, and Nijmegen University Centre for Infectious Diseases, Nijmegen, the Netherlands

*Journal of Microbiological Methods* 2002; 49: 55-62

© Elsevier Science B.V.
Phagocytosis and intracellular killing of C. albicans blastoconidia

Abstract

Polymorphonuclear neutrophils and mononuclear phagocytes represent an important first line and effector function in the control of Candida infections. Their relative contribution to host defence is frequently assessed by means of microbiological assays. However, reported results are divergent and might well be associated with study design-related issues. In the present study we compared frequently used microbiological candidacidal assays, in purpose to determine the most adequate method for assessment of phagocytosis and intracellular killing.

We concluded that microbiological assays using yeast-phagocyte suspensions are inappropriate for the assessment of intracellular killing of Candida blastoconidia by murine macrophages, due to adherence or clumping of cells. In contrast, an adherent monolayer of phagocytes can be applied as a single microbiological assay to independently study the process of phagocytosis and intracellular killing, by exudate peritoneal macrophages as well as exudate peritoneal PMN.
Introduction

Experimental models evaluating host defence against *Candida albicans* have shown that both innate resistance and acquired cell-mediated immunity are involved in anti-*Candida* response [1,16,19]. Essential components of both arms of the immune defence against infections by *Candida albicans* are phagocytic cells, i.e., polymorphonuclear neutrophils (PMN) and mononuclear phagocytes [9,15,20].

The precise contribution of phagocytes to host defence is usually assessed by determining phagocytosis and killing of *Candida* blastoconidia by means of the classical microbiological method of plate counting. However, divergent results are reported by investigators using these microbiological assays. To a large extent, the reported differences may be due to a number of substantial methodological variations. First, studies that have applied peritoneal phagocyte populations have used different eliciting agents, resulting in divergent cell populations [5,15,20]. Second, the microbiological method used has either been based on incubation of cells in suspension under slow rotation, using differential centrifugation to determine the amount of phagocytised *Candida* blastoconidia [11,13,14], or a monolayer of adherent cells [3,17,18]. Furthermore, it remained questionable whether assays based on the enumeration of *Candida* cells are not biased by clumping or adherence of cells resulting in erroneous conclusions regarding both the number of phagocytised and killed *Candida* blastoconidia.

In the present study we compared various frequently used microbiological candidacidal assays, to determine the most adequate method for assessment of phagocytosis and intracellular killing of *Candida* blastoconidia by murine peritoneal phagocytes.
Materials and methods

Mice
Specific pathogen-free CBA and C57Bl/6 female mice were used. Animals were housed under standard laboratory conditions and fed sterilised laboratory chow (Hope Farms, Woerden, The Netherlands) and water ad libitum.

Reagents
Sabouraud dextrose broth containing neopeptone 10 g x l⁻¹ and dextrose 20 g x l⁻¹ was obtained from Difco. Modified eagle’s medium (MEM), RPMI 1640 (RPMI), both obtained from Gibco Life Technologies, Paisley, Scotland, and RPMI 1640 Dutch modification with 20mM Hepes, without glutamine (RPMI-dm) purchased from ICN Biomedicals GmbH, Eschwege, Germany were supplemented with 1 % garamycin, 1 % glutamine and 1 % pyruvate.

Candida blastoconidia
Candida albicans (strain UC 820 or ATCC 10261) and C. parapsilosis (a clinical isolate from a patient with candidemia) were inoculated into 100 ml of Sabouraud broth and cultured for 24 h at 37°C. After three washes with sterile pyrogen-free PBS by centrifugation at 1500 g, the number of Candida blastoconidia was counted using a Bürker counting chamber. Candida blastoconidia were resuspended in culture medium, as indicated in the results section, and diluted to a final concentration of 1 x 10⁵ blastoconidia / ml medium.

Opsonins
For opsonisation of C.albicans, serum prepared from pooled blood of mice was used and stored in 1,5 ml aliquots at –80 °C. The percentage of serum varied per experiment as described below.

Recruitment of phagocytes
Peritoneal phagocytes were harvested by rinsing the peritoneal cavity with 4 ml of sterile ice-cold PBS containing 50 units / ml heparin and collected in separate sterile tubes. Exudate peritoneal granulocytes or exudate peritoneal macrophages were obtained 4 or 72 h after intraperitoneal (i.p.) injection of 1 ml of 10% proteose peptone (Difco Laboratories, Detroit, Michigan, USA), respectively. Exudate peritoneal lavage fluid was washed twice in sterile saline (1800 rpm, 10 min, 4 °C). The phagocytes were resuspended in culture medium, counted in a Bürker counting chamber, and diluted to a final concentration of 5 x
10^6 cells / ml of culture medium. The percentages of PMN, macrophages and lymphocytes were determined in Giemsa-stained cytocentrifuge preparations.

**Comparison of assays for the "over all" killing of Candida blastoconidia by murine resident peritoneal macrophages**

To assess the "over all" process of phagocytosis and killing, the non-ingested blastoconidia were not removed during the assay. Four types of assays of "over all" killing were compared. In all assays, resident murine peritoneal macrophages and Candida blastoconidia (effector to target ratio (E:T), 250:1) were incubated in RPMI-1640 medium with 10% fresh normal mouse serum at 37°C. After incubation of the macrophages with *C. albicans* UC820 or *C. parapsilosis*, the cell suspensions were collected. Serial tenfold dilutions were prepared and aliquots (100 µl) were spread in duplicate on Sabouraud dextrose agar plates. The numbers of CFU were counted after 24 h (*C. albicans*) or 48 h (*C. parapsilosis*) of incubation at 37°C.

1. **Adherent Cell Method.**

Peritoneal macrophages were allowed to adhere for 2 hours in 96-wells microtiter plates (Nunc, Roskilde, Denmark) before the addition of Candida blastoconidia. At various time points of incubation, cells were removed and lysed by scraping and 5 washes with distilled water containing 0.01% bovine serum albumin and 0.01% Tween-80 (lysis medium).

2. **Suspension Sampling Method.**

A 2 ml suspension of macrophages and Candida was incubated in silicone-coated 10 ml-glass tubes under rotation (4 rpm). At various time points of incubation, the suspension was vortexed, a 100 µl sample was taken, the cells were lysed with lysis medium, and plated on agar.

3. **Small Tube Method.**

To prevent variation over time by repeated sampling the suspension using the previous method, the Small Tube method applies a separate tube for each time point. Aliquots of 200 µl of macrophage/Candida suspension were incubated under rotation in small silicone-coated 1 ml-glass tubes, and at given times the entire contents of the tubes were lysed with lysis medium, and plated on agar. In order to assess whether Candida and macrophages were completely removed from the tubes, the tubes were subsequently washed 5 times with lysis medium by vortexing vigorously and the washout fluid was cultured separately.

In an attempt to circumvent adherence of cells to the tube, 200 µl of macrophage/Candida suspension was incubated under rotation in small silicone-coated 1 ml-glass tubes as described above. After incubation, the contents of the tube were spun down by centrifugation (5 min, 1500 x g) and the contents were collected. The tube was washed thrice with lysis medium by vortexing vigorously and the washout was pooled with the contents of the tube before plating on Sabouraud agar.

Phagocytosis and intracellular killing of C. albicans

To study the processes of phagocytosis and intracellular killing independently in a single assay, we applied the Adherent Monolayer Method, a modification of previously described assays [3,5]. Phagocytic cell suspensions were obtained from C57Bl/6 mice as described above, and resuspended in RPMI-dm containing 5 % heat-inactivated foetal calf serum (FCS). To create a monolayer of phagocytes, 5 x 10⁵ cells in 100 µl of RPMI-dm were dispensed into the wells of a 96-well flat bottom plate (Costar, Corning BV, the Netherlands) and incubated at 37 °C in air and 5 % CO₂. Granulocytes were allowed to adhere for 0.5 h and macrophages for 2 h before gently washing the monolayers with culture medium to remove non-adherent cells. The percentage of adherence was calculated as (1 – (number of non-adherent cells / 5 x 10⁵)) x 100. Subsequently, the cells were incubated with 1 x 10⁴ CFU C. albicans ATCC 10261, which were opsonised for 45 min at 24 ºC in MEM containing 2,5 % mouse serum (E:T ratio, approximately 50:1). After 15 min, supernatants were aspirated and monolayers were washed gently with MEM to remove uningested micro-organisms. The supernatant and well washings, containing the non-phagocytized Candida blastoconidia, were combined and plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytized micro-organisms was defined as (1 – (number of uningested CFU/ CFU at the start of incubation)) x 100.

Killing of C. albicans by phagocytes was assessed in the same monolayers. After removal of the non-phagocytized Candida blastoconidia, 200 µl of culture medium, consisting of Sabouraud in MEM (50 % v/v), was added to the monolayers. After 3 h of incubation at 37 °C in air and 5 % CO₂, the wells were gently scraped with a plastic paddle and washed with 200 µl distilled H₂O to achieve lysis of phagocytes. This procedure was repeated three times, after which the pooled washes were adjusted to a final volume of 1 ml with distilled water. Microscopic examination of the culture plates showed that there was a complete removal of phagocytes. To quantify the number of viable intracellular Candida blastoconidia, tenfold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37 °C for 24 h. The percentage of yeast killed by
the phagocytes was determined as follows: \(1 - \frac{\text{CFU after incubation}}{\text{number of phagocytized CFU}}\) x 100. Phagocyte-free incubations of blastoconidia were included as a control for yeast viability.

**Statistical analysis**

The results were analysed by the Mann Whitney U test. The numbers of samples are stated in the figure legends. The level of significance was set at \(p < 0.05\).

**Results**

**Comparison of assays for the “over all” killing of *Candida* blastoconidia by murine resident peritoneal macrophages**

As shown in figure 1A, the number of *C. albicans* rose to slightly above the initial number at the start of incubation when assessed with the Adherent Cell Method, as has also been described previously [3,10]. The results obtained with the Centrifugation Method were similar, indicating that adherent cells and cells in suspension do not differ in respect to their inability to kill ingested *C. albicans* blastoconidia.

In contrast, the Suspension Sampling Method and the Small Tube Method suggested that the macrophages were able to kill over 80% of the *C. albicans* blastoconidia in a 4.5 h period of time (Fig. 1a). However, when *C. albicans* blastoconidia were cultured in the absence of cells (Fig. 1b), similar differences between the various assays were observed, indicating that other factors than the killing capacity of the macrophages were responsible for these findings.

When the tubes were washed out vigorously after termination of the Small Tube Method, it was demonstrated that large numbers of CFU had remained adhered to the wall of the tubes, and this proportion rapidly rose with time. When these numbers were added to the numbers of CFU obtained during the assay, the totals indicated that no net killing of *C. albicans* had taken place (Fig. 1c). Similarly, when the numbers of CFU obtained from the Small Tube Method in the absence of macrophages (Fig. 1b) were added to the washout fluid counts from this assay, the total equalled the numbers of *Candida* obtained with microtiter plates, or the Centrifugation Method (data not shown), indicating that the majority of the blastoconidia had remained adhered to the wall of the silicone-coated tubes. When *C. parapsilosis* was used as the test organism, the Adherent Cell (Fig. 1d) and Centrifugation Methods (data not shown) showed that macrophages killed 48-52% of the organisms in 4 hours. Again, the numbers of CFU recovered from the Small Tube Method and from the washout fluid of the emptied tubes had to be added up to reflect the net killing of *C. parapsilosis* (Fig. 1d).
Figure 1. Over all killing of Candida blastoconidia by resident peritoneal macrophages according to different methods. (a) Numbers of C. albicans recovered after incubation with phagocytes according to the adherent (♦), centrifugation (●), suspension sampling (■), and small tube assay (▲). (b) Numbers of C. albicans obtained after incubation in RPMI-1640 without phagocytes. ((c) and (d) are depicted on the next page)
Figure 1. (c) Numbers of C. albicans incubated in the presence of resident peritoneal macrophages recovered from the small tube assay before (▲) and after (●) vigorous washing of the tubes, as well as the total of both (■). (d) Numbers of C. parapsilosis recovered after incubation with phagocytes according to the adherent assay (♦), and the small tube assay before (▲) and after (●) vigorously washing the tubes. Data are given as the mean ± SD of 3 separate experiments, with 4 samples per time point each.
Phagocytosis and killing: Adherent Monolayer Method

Since the data, obtained from the Adherent Cell Method, confirmed the inability of resident peritoneal macrophages to kill *C. albicans* blastoconidia as was shown previously [4,10], we investigated the capacity of proteose peptone elicited peritoneal macrophages and PMN to ingest and kill *C. albicans* blastoconidia. The constitution of the exudate peritoneal cell populations is depicted in table 1.

Table 1. Differential counts of intraperitoneal cell populations (% ± SD). Numbers were obtained from 8 mice at each time point in 2 separate experiments.

<table>
<thead>
<tr>
<th>Peritoneal cell population</th>
<th>Macrophages</th>
<th>PMNs</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resident</td>
<td>90 ± 6</td>
<td>1 ± 1</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>Exudate after 4 hours</td>
<td>29 ± 10</td>
<td>65 ± 11</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Exudate after 72 hours</td>
<td>84 ± 6</td>
<td>5 ± 4</td>
<td>11 ± 4</td>
</tr>
</tbody>
</table>

The average number of macrophages that adhered to the microtiter plates during 2 h of incubation at 37 °C, was in agreement with the data previously shown by Brummer and colleagues [3]. After 0.5 h of incubation, the amount of cells in the PMN monolayer exceeded the maximum adherence percentage of macrophages after 2 h of incubation (Fig. 2a). A time course conducted to determine PMN adherence showed that incubation for more than 30 min. did not substantially increase the number of adherent PMN (data not shown).

Both exudate peritoneal cell populations, PMN and exudate macrophages, were able to internalise and kill *C. albicans* blastoconidia. The capacity of exudate macrophages to ingest *C. albicans* blastoconidia significantly surpassed the ability of PMN (p < 0.005, Fig. 2b), whereas PMN showed significantly higher killing percentages compared with macrophages (p < 0.05, Fig. 2c).

To assess whether the established intracellular killing could be explained by decreased viability of *C. albicans* blastoconidia, *Candida* growth from phagocyte-free wells was quantified after 3 h of incubation. The numbers of CFU recovered from wells containing *C. albicans* alone were 150 % increased compared with baseline counts (data not shown). Microscopic evaluation of the wells showed no hyphal forms.
**Figure 2.** Adherence, phagocytosis and killing of *C. albicans* blastoconidia by exudate peritoneal phagocytes according to the adherent monolayer method.

(a) Percentage of proteose peptone-elicited PMN and macrophages, that adhered to microtiter plates at 37 °C in 0.5 h (PMN) or 2 h (macrophages).

(b) Percentage of *Candida* blastoconidia of the initial inoculum that was ingested by exudate peritoneal phagocytes after 15 min of phagocytosis (# p < 0.005).

(c) Percentage of phagocytized *Candida* blastoconidia that was killed by exudate peritoneal phagocytes after incubation at 37 °C for 3 h. Data are expressed as mean ± SD of at least 8 samples in 1-3 separate experiments (* p < 0.05).
Phagocytosis and intracellular killing of C. albicans blastoconidia

Discussion

Our results demonstrate that the process of phagocytosis and intracellular killing can independently be assessed by means of the Adherent Monolayer Method. Granulocytes are able to kill Candida yeast cells by internalisation and fusion of the phagosome with a lysosome within the cytoplasm, or pseudohyphae, and presumably also blastoconidia, by attachment and degranulation of the fungicidal content into the immediate microenvironment [6,7]. Therefore, it can not be assessed whether the observed killing by granulocytes in ‘over all killing’ assays is a result of intracellular or extracellular killing mechanisms. Furthermore, these assays measure ingestion and intracellular killing simultaneously, and apparent differences in "killing" of Candida may actually be due to differences in phagocytosis. To prevent a misinterpretation of the killing capacity due to these pitfalls, a system with adherent phagocytes is required, so that the supernatant containing the uningested yeast cells can be collected, thus assessing phagocytosis and intracellular killing independently.

We found that all Candida CFU that appeared to have been killed in the Suspension Sampling Method and the Small Tube Method could be recovered as viable organisms adhering to the tube, either by repeated washes or by centrifugation. The results obtained with C. parapsilosis illustrate that, whereas the killing of various Candida species by murine macrophages may differ (this study; [4,10]), the methodological issues of adherence or clumping of cells may be similar for different strains.

Using an assay with adherent cells in tissue culture plates, we have found that resident murine peritoneal macrophages are unable to kill ingested C. albicans in vitro. These findings are compatible with earlier reports that used adherent resident peritoneal macrophages in a microbiological assay [3,10]. However, researchers applying other assays have found divergent results [2,17].

Using the Adherent Monolayer Method, we have demonstrated that exudate peritoneal macrophages as well as peritoneal granulocytes were able to ingest and kill C. albicans blastoconidia. PMN showed significantly higher killing percentages compared with exudate macrophages, which is supported by other studies comparing candidacidal functions of phagocytes from different origin [2,10,21]. However, the killing percentages per phagocyte population may vary due to the various types of assays, mouse or Candida strains, or eliciting agents used. PMN elicited by injection of proteose peptone display significantly greater candidacidal activity than those elicited by thioglycollate [1]. Brewer
thioglycollate-elicited peritoneal macrophages showed an impaired killing capacity compared with other kinds of thioglycollate [12].

Incubations of phagocyte-free *C. albicans* suspensions showed that the observed killing by adherent peritoneal phagocytes could not be explained by loss of yeast viability. Microscopic evaluation of the wells showed no hyphal growth or residual cells after repeated well washings. Contaminating macrophages present in the peritoneal exudate granulocytes, obtained 4 h after injection of proteose peptone, were not likely to be responsible for the observed killing of *C. albicans* blastoconidia, since resident peritoneal macrophages were not able to kill *C. albicans* blastoconidia.

Another source of ample confusion is the definition of intracellular killing. Some authors express the number of yeast killed as (1 - (CFU after incubation / CFU of *Candida* cultured without cells)) x 100 [14]. By comparing the growth of intracellular *C. albicans* with the growth rate of *Candida* in the absence of cells, these authors actually describe the difference between the intra- and extracellular milieu. Even if the *Candida* replicates intra- and extracellularly, any retardation of the intracellular outgrowth is interpreted as killing, as long as the growth rate of the intracellular yeast cells is slower than that of the extracellular control. It may be more appropriate to describe this phenomenon as inhibition of intracellular outgrowth rather than killing [8]. Thus, the percentage killing of *C. albicans* should be defined as (1 - (CFU after incubation / CFU recovered at the start of incubation or numbers of CFU phagocytized)) x 100 [10].

In conclusion, comparing data from microbiological in vitro studies on the phagocytosis and killing of *C. albicans* blastoconidia by phagocytes is hampered by divergent methodologies. The results of some studies may be equivocal, and the reported differences may for a large part be due to the various assay conditions and methods that have been used in determining phagocyte candidacidal activity.

**Acknowledgements**

We wish to thank Ineke Verschueren, Margot van den Brink, Monique Bakker and Maichel van Riel for their help with the experiments.
References


The influence of endogenous pro-inflammatory cytokines on neutrophil-mediated damage of *Candida albicans* pseudohyphae, quantified in a modified tetrazolium dye assay

Alike G. Vonk¹⁴, Catharina W. Wieland¹⁴, Marieke Versteegen¹⁴, Ineke C. Verschueren¹⁴, Mihai G. Netea¹⁴, Leo A.B. Joosten²⁴, Paul E. Verweij³⁴, Bart-Jan Kullberg¹⁴

Departments of Medicine, Division of General Internal Medicine¹, Rheumatology², and Medical Microbiology³, University Medical Centre, Nijmegen, The Netherlands, and ‘Nijmegen University Centre for Infectious Diseases, Nijmegen, The Netherlands

Submitted for publication
Abstract

For quantitative assessment of polymorphonuclear granulocyte (PMN)-mediated pseudohyphal damage, an improved tetrazolium (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; XTT) dye assay was developed. The modified assay proved to be a reliable indicator of viable pseudohyphal inoculum sizes. In addition, the influence of various endogenous pro-inflammatory cytokines on the capacity of PMN to damage *C. albicans* pseudohyphae was investigated. PMN obtained from mice in which the genes encoding for tumour necrosis factor-α/lymphotoxin-α (TNF/LT), interferon-γ (IFNγ), interleukin (IL)-1α, or IL-1β were disrupted, showed a significantly reduced pseudohyphal damage capacity in comparison with control PMN. The reduction amounted 25 % for TNF−/−LT−/−, 11 % for IFNγ−/−, 21 % for IL-1α−/−, and 34 % for IL-1α−/−β−/− PMN. In contrast, deficiency of IL-12 or IL-18 did not result in a diminished capacity to damage pseudohyphae and the capacity of PMN to damage *Candida* pseudohyphae was even slightly increased by 10 % in IL-18−/− mice. These data demonstrate the importance of endogenous pro-inflammatory cytokines to antihyphal activity of PMN, the main effector cells against disseminated candidiasis by virtue of their capacity to kill both *Candida* blastoconidia and pseudohyphae.
Introduction

Candidiasis is an infectious complication in patients with various immunodeficiencies, of which neutropenia is recognized as the most important risk factor. Although the incidence of species other than *C. albicans* as a cause of invasive candidiasis is increasing, *C. albicans* remains the most common causative species [12]. *C. albicans* is a dimorphic yeast growing as blastoconidia or pseudohyphae, and both forms are present within lesions [19]. Polymorphonuclear granulocytes (PMN) are the predominant effector cells in host defence against both growth forms of *C. albicans* [2,3,28,31].

The contribution of PMN to host response against *C. albicans* is usually assessed by determination of the capacity to ingest and kill *Candida* blastoconidia. Whereas in vitro studies have shown that pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF) or interferon-γ (IFNγ), enhance PMN antifungal activity against *Candida* blastoconidia [7,8,21], few data are available regarding the immunomodulatory effect of pro-inflammatory cytokines on antihyphal activity of PMN. Moreover, although both *C. albicans* morphologies are found in lesions, the transition from a budding to a filamentous form is considered to be a virulence factor [17,22]. Therefore, further characterization of PMN-mediated host response against *C. albicans* warrants investigation of extracellular antihyphal activity of PMN in addition to determination of their intracellular capacity to kill phagocytized *Candida* blastoconidia.

The viability of pseudohyphae can be assessed using a colorimetric dye assay, which is based on the conversion of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) to an orange water-soluble formazan by fungal mitochondrial dehydrogenases in the presence of an electron-acceptor. This previously described tetrazolium dye assay [9,10,20] was further optimized to study the influence of endogenous pro-inflammatory cytokines on *C. albicans* antihyphal activity of PMN lacking the genes encoding for TNF/Lymphotoxin-α (LT), IFNγ, interleukin (IL) -12, IL-18, IL-1α, or IL-1β.

Materials and methods

Animals

TNF−LT− mice on a mixed 129/sv-C57BL/6 background in which the genes encoding for TNF-α and LT-α were replaced by a neo cassette (pMCI NeopA, Stratagene) and their wild-type littermates were used [1]. The DNA fragment encoding for IL-1α in C57BL/6 mice was replaced by the lacZ-pA-PGK-hph-pH cassette in IL-1α-deficient (IL-1α−/−) and IL-1α/β double deficient (IL-1α−/−β−/−) mice, and the genes encoding for IL-1β were replaced by the lacZ-pA-PGK-neo-pH cassette in IL-1β-deficient (IL-1β−/−) and IL-
Neutrophil-mediated damage of *C. albicans* pseudohyphae

1α⁺β⁻/⁻ mice. The IL-1-deficient mice were kindly provided by Dr. Y. Iwakura (Tokyo, Japan) [11]. Homozygous IL-18-deficient (IL-18⁻⁻) mice were generated by disruption of the IL-18 gene and the insertion of the MC-1-neo poly(A) gene, and these mice were kindly provided by Dr. S. Akira (Osaka, Japan) [25]. IL-12-deficient (IL-12 p40⁺⁻) chimeric mice on a C57Bl/6 background in which the IL-12 p40 gene was replaced by the insertion of the PGK-neo gene [18], and IFN-γ-deficient (IFNγ⁻⁻, B6.129S7-Ifngtm1Ts) were purchased from Jackson Laboratory (Bar Harbor, ME). C57Bl/6 mice were used as control mice for the IL-1, IL-18, IL-12 p40 and IFN-γ-deficient animals. All deficient and control mice were age-matched and were allowed to accustom to laboratory conditions for one week before experimental use at the Central Animal Laboratory and the Laboratory for Experimental Rheumatology of the Nijmegen University. The animals were housed under specific pathogen-free conditions and were fed standard laboratory food (Hope Farms, Woerden, the Netherlands), and water ad libitum. The ethics committee on animal experiments of the Catholic University Nijmegen had approved the experiments.

*Candida albicans*

*C. albicans* (strain UC820) was inoculated into 100 ml of Sabouraud broth and cultured for 24 h at 37°C. After three washes with pyrogen-free saline by centrifugation at 1500 x g, the number of yeast cells was counted in a haemocytometer; occasional strings of two or more yeast were counted as one cfu of *C. albicans*. Pyrogen-free saline was used to dilute the suspension to the appropriate concentration. The viability of the yeast was at least 98 %, as determined by plating serial dilutions on Sabouraud dextrose agar plates.

**Assessment of PMN-mediated pseudohyphal damage**

Exudate peritoneal PMN from mice were elicited by an i.p. injection of 10 % proteose peptone, as described earlier [13]. PMN were collected in sterile tubes by washing the peritoneal cavity with 4ml ice-cold PBS containing 50 U/ml heparin, 4h after injection of proteose peptone. The cells were centrifuged (2250 x g), counted in a Bürker counting chamber, and resuspended in RPMI 1640 without phenol red and L-glutamine (RPMI-wp; ICN Biomedicals, Eschwege, Germany). The percentage of PMN was determined in Giemsa-stained cytopsin preparations.

PMN-mediated damage of *Candida* pseudohyphae was determined by a modification of the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma Chemical, St. Louis, MO) dye assay, as described elsewhere [30]. *Candida* blastoconidia grown on Sabouraud agar plates were collected with a swab and suspended at a final concentration of 1 x 10⁶ cfu/ml in RPMI 1640 Dutch modification (with 20mM Hepes, without glutamine; ICN Biomedicals). The pH of the suspension was adjusted to
6.4 using hydrochloric acid. Pseudohyphae were obtained by incubating 10 ml of the suspension at 37°C for 24h. After incubation, the pseudohyphae were centrifuged (550 x g) and resuspended in RPMI-wp. 160 µl aliquots containing a pseudohyphal inoculum size that originated from 1 x 10^5 blastoconidia (henceforth referred to as 1 x 10^5 pseudohyphae) were then dispensed into a 24-well flat bottom plate (Costar, Corning, the Netherlands). 200 µl aliquots of the PMN suspension (8 x 10^5 PMN) were added to the wells containing pseudohyphae at a final E:T ratio of 8:1, in the presence of 10 % fresh serum (40 µl) obtained from the corresponding control mice. Control wells contained pseudohyphae or PMN only. After incubation for 2h, 800 µl of sterile distilled H2O was added to the wells and the plate was rocked at room temperature to achieve lysis of PMN. After 15 min., 800 µl sterile saline containing XTT, and the electron-coupling agent coenzyme Q0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) were added to each well at a final concentration of 400 µg/ml XTT and 50 µg/ml coenzyme Q0. After 1h of incubation at 37°C, the plate was centrifuged (770 x g) to deposit cells and cellular debris, and 150 µl of the supernatant of each well was transferred to a well of a 96-well microtiter plate. The absorbance of each well was measured in a spectrophotometer at 450 nm. The percentage of pseudohyphal damage was defined as 1– ((A450 hyphae + PMN – A450 PMN) / A450 hyphae) x 100.

Statistical analysis
The differences between 2 groups were analyzed by the Mann-Whitney U test. For these comparisons the level of significance was set at p < 0.05. For comparison of 3 or more groups, the data were analyzed using the Kruskal-Wallis one-way ANOVA. For post-test comparisons, the Bonferroni t method was used. To ascertain reproducibility, the experiments were performed at least twice, and the data represent the cumulative results of all experiments performed.

Results
Modification of the tetrazolium dye assay
In initial experiments in our laboratory using the tetrazolium dye assay, as described by Gaviria et al [9,10], or Meshulam et al [20], variability in assay outcome was observed. Several circumstances appeared to contribute to the observed variability. First, microscopic evaluation showed that pseudohyphae did not firmly attach to the bottom of the wells and could easily be removed during well washes, despite cautious aspiration of the supernatant. In order to obtain a better correlation between the number of undamaged pseudohyphae and amount of formazan products, the subsequent experimental steps were
performed without supernatant aspirations, thus excluding experimental bias due to hyphal loss. Second, it appeared that organic material (cells or cellular debris) interfered with accurate assessment of absorbance. Hence, prior to the transfer of well aliquots into corresponding wells of microtiter plates, the plates containing the cultures had to be centrifuged to spin down cells. In addition, since in our assay the plates were not rocked, centrifugation guaranteed homogenous distribution of formazan products throughout the supernatant, instead of mainly concentrating around hyphae at the bottom of the wells when centrifugation was not performed, as was observed macroscopically. Third, since the XTT conversion is not terminated during the course of the experiment, outcome variability was observed due to small variations in incubation time of the wells. Therefore, the addition, removal or transfer of well contents was performed according to a strict time schedule per well. After having obviated the aforementioned experimental biases, the assay described in the section materials and methods was tested for its ability to reliably indicate pseudohyphal inoculum size. To this purpose, it was assessed whether a linear relationship existed between increasing inoculum sizes of pseudohyphae that were exposed to 400 µg/ml XTT with 50 µg/ml coenzyme Q and the extinction. Indeed, the linearity of the relationship between formazan production with increasing amounts of pseudohyphae showed that the XTT assay was a reliable indicator of pseudohyphal inoculum size (Fig.1).

**Figure 1.** Relationship between the amount of formazan produced by increasing pseudohyphal inoculum sizes and the absorbance of light with a wavelength of 450 nm. The reduction of 400 µg/ml XTT in the presence of 50 µg/ml coenzyme Q by different inoculum sizes was measured in duplicate. Symbols represent the means. Linear regression was used to generate the regression lines of which the dotted lines delineate the 95% confidence intervals.
PMN-mediated pseudohyphal damage

The influence of endogenous pro-inflammatory cytokines on *C. albicans* antihyphal activity of PMN lacking the genes encoding for TNF/LT, IFNγ, IL-12, IL-18, IL-1α, or IL-1β, was studied.

The relative capacity of PMN from TNF⁻/⁻LT⁻/⁻ mice to damage of *C. albicans* pseudohyphae was significantly reduced by 25% compared to that of PMN from TNF⁺⁺LT⁺⁺ mice (Fig. 2; p < 0.001). Likewise, IFNγ⁻/⁻ PMN had a significantly impaired capacity to damage pseudohyphae relative to that of IFNγ⁺⁺ PMN (Fig. 2; p < 0.005).

![Figure 2](image)

**Figure 2.** Extracellular pseudohyphal damage of TNF⁻/⁻LT⁻/⁻ and IFNγ⁻/⁻ PMN relative to control PMN. *C. albicans* pseudohyphae were incubated in the presence of PMN for 2h. Horizontal bars indicate the means. Kruskal-Wallis one-way ANOVA,

- a p < 0.001 vs. TNF⁺⁺LT⁺⁺ PMN
- b p < 0.005 vs. IFN⁺⁺ PMN

Analogous to TNF and IFNγ, IL-1α is important for extracellular damage of *Candida* pseudohyphae, as was shown by the significantly reduced capacity to damage pseudohyphae of PMN from IL-1α⁻/⁻ mice or IL-1α⁻/⁻β⁻/⁻ mice compared to that of PMN from IL-1α⁺⁺β⁺⁺ mice (Fig. 3; p < 0.001). Deficiency of both IL-1β and IL-α showed a tendency to further impair PMN antipseudohyphal resistance compared to IL-1α deficiency alone. Deficiency of IL-1β alone did not diminish the capacity of PMN to damage *C. albicans* pseudohyphae.
Disruption of the genes encoding for IL-12 did not significantly affect PMN capacity to damage pseudohyphae compared to control PMN (C57Bl/6; Fig. 4; p > 0.05). In contrast to the diminished or unaltered PMN-mediated damage of Candida pseudohyphae in absence of the other pro-inflammatory cytokines tested, lack of endogenous IL-18 slightly but significantly increased the capacity to damage pseudohyphae compared to control PMN (C57Bl/6; Fig. 4; p < 0.01).
Discussion

Our experiments have further defined conditions for reproducible quantification of PMN-mediated damage of Candida pseudohyphae. The findings in the present study indicate that the modified tetrazolium dye assay is a reliable indicator of pseudohyphal inoculum size, and this is the first study to investigate the influence of several endogenous pro-inflammatory cytokines on anticandidal PMN activity against C. albicans pseudohyphae. It was shown that PMN-mediated damage of Candida pseudohyphae, the predominating growth form in invasive lesions of candidiasis, is significantly reduced in absence of endogenous TNF/LT, IFNγ, or IL-1α, whereas the PMN-mediated damage appeared to be unaltered or even slightly improved in mice deficient for endogenous IL-12 and IL-18, respectively.

Candida albicans is able to switch between yeast and filamentous growth, a strategy that may contribute to its pathogenicity in several ways. Disruption of genes essential for hyphal growth locks C. albicans in the yeast form, and these mutants had a reduced virulence in a mouse model [14]. Hyphal growth of C. albicans not only enables the pathogen to invade tissue cells; it has also been shown to enable it to escape from macrophages by piercing their cell membrane, thus avoiding destruction [17,22,29]. In addition to effects that are the direct consequence of differences in morphology of Candida, antigens and other properties may not be identical in blastoconidia and hyphae. Indeed, heat-killed and viable hyphae elicited different immunological responses from human monocytes compared with blastoconidia, as indicated by a reduced production of IL-12 and the chemokines MIP-1α, MIP-1β, IL-8 and MCP-1 in response to hyphae [16,26], which is detrimental to mounting a protective host response against C. albicans. PMN are the main effector cells against C. albicans by virtue of their capacity to kill Candida cells irrespective of their morphology. Priming of PMN with exogenous TNF or IFNγ has been shown to enhance the blastoconidia-induced respiratory burst and degranulation of PMN, and to increase PMN candidacidal activity against blastoconidia [7,8,13,24]. However, the possible application of immunotherapy requires insight into the influence of pro-inflammatory cytokines on PMN-mediated damage of Candida hyphal morphologies, in addition to their influence on PMN phagocytosis and intracellular killing of blastoconidia which is usually assessed. Hence, we investigated the influence of endogenous pro-inflammatory cytokines on C. albicans antihyphal activity of PMN. Our present data indicate that lack of endogenous TNF/LT or IFNγ significantly impaired PMN-mediated damage of C. albicans hyphae. This is in line with findings showing that
PMN pre-treated with exogenous recombinant TNF or IFNγ displayed an augmented fungicidal activity against *C. albicans* hyphae [4,9,23].

Since IL-12 is an important stimulant for the synthesis of IFNγ by lymphocytes and NK-cells [18,27], we also assessed the capacity of IL-12p40−/− PMN to damage pseudohyphae. However, unlike IFNγ−/− PMN, IL-12p40−/− PMN only showed a tendency towards diminished capacity to damage *Candida* pseudohyphae. Several mechanisms might explain this observation. First, this indicates that, in this model, the synthesis of IFNγ is not entirely dependent on IL-12. Second, the direct effect of IL-12 on antihyphal activity of PMN appeared to be limited.

The fact that IL-18 is also known to be a costimulator for IFNγ production [6], and that human neutrophils can be activated by IL-18 resulting in increased neutrophil granulation and respiratory burst [15], suggests that PMN from IL-18−/− mice might have an impaired antihyphal activity. However, unexpectedly, IL-18−/− PMN showed a slightly, but significantly increased capacity to damage *C. albicans* pseudohyphae compared to control PMN, although the magnitude of the effect is small and may be irrelevant in vivo. The mechanisms through which IL-18 exerted this effect are not clear as yet.

Like deprivation of endogenous IL-18, absence of IL-1β alone did not have a detrimental effect on PMN capacity to damage pseudohyphae. In contrast, absence of IL-1α alone significantly reduced the capacity of PMN to damage *Candida* pseudohyphae, and deprivation of both IL-1 molecules resulted in the strongest reduction. This suggests that both IL-1α and IL-1β beneficially influence PMN antihyphal response, with IL-1α inducing the strongest effect. The most likely explanation for this observation is the difference in amount of IL-1α and IL-1β produced in mice. Approximately 90% of IL-1 production in mice consists of IL-1α and the remaining 10% of IL-1β (Netea et al, unpublished observations). In addition, the stronger effect of IL-1α than IL-1β on PMN antihyphal response may also be due to the different affinity of the IL-1R type I and IL-1R type II for the IL-1 molecules [5]. In absence of IL-1α, most IL-1β probably binds to the IL-1R type II that has a high affinity for IL-1β but lacks a signal transducing cytoplasmic domain making it a functionally negative receptor. Subsequently, the resulting small amounts of IL-1β that do bind to the IL-1RI may not be enough to induce a positive effect on PMN capacity to damage *Candida* pseudohyphae.

The current study suggests that neutralization of pro-inflammatory cytokines could have detrimental effects on host response against *C. albicans* infection in which PMN play an essential role. In view of the expanding anti-inflammatory cytokine strategies in
humans, these exploring animal studies offer further insight into the clinical implications for the proposed use of these therapeutic options.

References

11. Horai R, Asano M, Sudo K et al. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med 1998; 187:1463-75.


Endogenous interleukin-1 alpha and interleukin-1 beta are essential to host defence against disseminated candidiasis

Arieke G. Vonk1,4, Mihai G. Netea1,4, Johan H. van Krieken2, Yoichiro Iwakura3, Jos W.M. van der Meer1,4, Bart-Jan Kullberg1,4

Departments of Medicine1 and Pathology2, Radboud University Medical Centre, and Nijmegen University Centre for Infectious Diseases4, Nijmegen, the Netherlands. 3Laboratory Animal Research Centre, Institute of Medical Science, University of Tokyo, Japan

Submitted for publication
**Abstract**

The effect of endogenous IL-1α and IL-1β on disseminated *C. albicans* infection was investigated in mice deficient in the genes encoding for IL-1α (IL-1α<sup>−/−</sup>), IL-1β (IL-1β<sup>−/−</sup>), or both IL-1α and IL-1β (IL-1α<sup>−/−</sup>β<sup>−/−</sup>). Both IL-1α and IL-1β were involved in the anticandidal host defense as shown by the significantly increased susceptibility of IL-1α<sup>−/−</sup> mice (59 % mortality) and IL-1β<sup>−/−</sup> mice (62 % mortality) versus control mice (28 % mortality; p < 0.05) to disseminated candidiasis. Moreover, mortality was even further increased in double-knockout IL-1α<sup>−/−</sup>β<sup>−/−</sup> mice (71 %; p < 0.001). Outgrowth of *Candida* in the kidneys was significantly increased in IL-1-deficient mice compared to immunocompetent mice. However, the mechanisms of host defense triggered by IL-1α and IL-1β differed. IL-1β<sup>−/−</sup> mice showed a decreased recruitment of granulocytes to the site of infection and generation of superoxide production. IL-1α<sup>−/−</sup> mice had a reduced capacity to damage *Candida* pseudohyphae extracellularly. Protective Th1 responses were deficient in both IL-1α<sup>−/−</sup> and IL-1β<sup>−/−</sup> mice, as assessed by production of interferon-γ in response to heat-killed *C. albicans* blastoconidia by splenic lymphocytes. In conclusion, whereas the modes of action differ, both IL-1α and IL-1β are essential for mounting a protective host response against invasive *C. albicans* infection.
**Introduction**

Interleukin-1 alpha (IL-1α) and interleukin-1 beta (IL-1β) are pro-inflammatory cytokines that exert similar biological activities after interaction with the IL-1 type I receptor (IL-1RI) and the IL-1R accessory protein [5]. Exogenous recombinant human IL-1α or IL-1β have been administered in disseminated murine candidiasis and these studies have clearly indicated a protective role for IL-1 in disseminated *C. albicans* infection [8,20]. The mechanisms for this beneficial effect have only partly been elucidated. It has been excluded that IL-1 has a direct antifungal effect, and the protective effect of IL-1 in host defence against *C. albicans* did not depend on the presence of granulocytes or humoral factors, such as acute phase proteins [8,20]. To characterize the role of endogenous IL-1α and IL-1β in disseminated candidiasis and to gain further insight into the mechanisms through which both IL-1 molecules confer protection against infection with *C. albicans*, mice in which the genes encoding for IL-1α (IL-1α−/−), IL-1β (IL-1β−/−), or both (IL-1α−/−β−/−) had been disrupted were used. The IL-1-deficient mice and their immunocompetent littermates were subjected to experimental *C. albicans* infection.

**Materials and methods**

**Animals**

The DNA fragment encoding for IL-1α was replaced by the lacZ-pA-PGK-hph-pH cassette in IL-1α−/− and IL-1α−/−β−/− mice, and the genes encoding for IL-1β were replaced by the lacZ-pA-PGK-neo-pH cassette in IL-1β−/− and IL-1α−/−β−/− mice on a C57Bl/6 background [6]. As control mice for the IL-1-deficient animals, age matched C57Bl/6 mice were used (IL-1α+/+β+/+ mice). The mice were allowed to accustom to laboratory conditions for one week before experimental use. The animals were housed under specific pathogen-free conditions and were fed standard laboratory chow (Hope Farms, Woerden, the Netherlands) and water ad libitum. The ethics committee on animal experiments of the Catholic University Nijmegen had approved the experiments.

**Candida albicans**

*C. albicans* (strains UC820 and ATCC 10261) was inoculated into 100 ml of Sabouraud broth and cultured for 24h at 37 °C. After three washes with pyrogen-free saline by centrifugation at 1500 x g, the number of yeast cells was counted in a haemocytometer; occasional strings of two or more yeast were counted as one cfu of *C. albicans*. Pyrogen-free saline was used to dilute the suspension to the appropriate concentration. The viability of the yeast was at least 98 %, as determined by plating serial dilutions on Sabouraud dextrose agar plates.
IL-1 in disseminated candidiasis

Infection model

Mice received an intravenous injection of 100 µl pyrogen-free PBS containing 1 x 10^5 live C. albicans UC820 blastoconidia into the retroorbital plexus. Survival was assessed daily for 21 days. For measurement of circulating granulocytes, cytokine concentrations and quantification of fungal outgrowth, subgroups of 5 mice were bled from the retroorbital plexus on day 1, 3 or 7 of infection, and blood was collected in EDTA tubes. The kidneys were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. Serial dilutions of the homogenates were plated on Sabouraud dextrose agar plates, as described elsewhere [8]. After incubation at 37°C for 48h, the numbers of cfu were counted and the results were expressed as log cfu per kidneys. For histology, kidneys obtained from subgroups of mice were fixed in buffered formaldehyde (4 %). Paraffin-embedded sections were stained with periodic acid-Schiff, or hematoxylin-eosin, and examined microscopically.

To investigate whether the role of endogenous IL-1α, and IL-1β in a model of disseminated candidiasis is mediated by polymorphonuclear neutrophils (PMN), subgroups of deficient and wild-type mice were rendered granulocytopenic by pretreatment with cyclophosphamide (Bristol-Myers Squibb, Weesp, The Netherlands), as described previously [8]. Cyclophosphamide 150 mg/kg was administered s.c. 4 days before infection, followed by 100 mg/kg 1 day before infection, as well as on day 2 after i.v. injection of 100 µl pyrogen-free PBS containing 1 x 10^4 viable C. albicans UC820. To compensate for the hyperuricemia-induced TNF production in cyclophosphamide treated mice, mice received sodium bicarbonate by gastric installation at 100 mg/kg b.i.d. daily, starting on day -4 until the end of the experiment [15]. Daily differential counts in peripheral blood smears confirmed that granulocytes remained < 100 x 10^6/L throughout the infection (data not shown). Outgrowth of Candida in the kidneys on day 1 or 3 of infection was determined as described above.

Recruitment

The recruitment of PMN to the site of infection was determined after injection of 10^7 cfu heat-killed C. albicans into the peritoneal cavity of uninfected subgroups of mice. Four hours after the injection of the heat-killed yeast, the mice were killed by CO2 asphyxia and the peritoneal cavities were rinsed with ice-cold PBS containing 50 U/ml heparin. After centrifugation of the peritoneal exudate, the cells were counted in a haemocytometer, and the percentage of PMN was assessed in Giemsa-stained cytocentrifuge preparations.
Superoxide production

Superoxide production was studied in a luminol-enhanced peroxidase-catalyzed chemiluminescence assay [9]. Exudate peritoneal PMN of IL-1α−/−, IL-1β−/−, and IL-1α+/β+/+ mice were collected 4 h after i.p. injection of 10 % proteose peptone. PMN were suspended at 2 x 10^6 cells/ml HBSS without phenol red (Life Technologies, Paisley, Scotland) supplemented with 0.25 % human serum albumin (Behringwerke, Marburg, Germany). Cells (2 x 10^5/well) were incubated in 96-well microtiter plates (Costar) with 50 µM luminol, 4.5 U/ml horseradish peroxidase (Sigma), and stimulated with Candida blastoconidia and phorbol 12-myristate 13-acetate (PMA; 50 ng/ml). Chemiluminescence was measured on a Victor^2 1420 multilabel counter (Wallac, Turku, Finland) at room temperature. Chemiluminescence was expressed as the total amount of superoxide produced in 35 min by integrating the area under the curve per PMN.

Phagocytosis and intracellular killing of C. albicans

Exudate peritoneal phagocytes from groups of five mice were elicited by an i.p. injection of 10 % proteose peptone. Phagocytes were collected in sterile tubes by washing the peritoneal cavity with 4ml ice-cold PBS containing 50 U/ml heparin 4h (PMN) or 72h (macrophages) after injection. The cells were centrifuged (2250 x g), counted in a Bürker counting chamber and resuspended in RPMI-dm supplemented with 5 % heat-inactivated foetal calf serum.

The processes of phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes, as described previously [23]. Briefly, phagocytes were allowed to adhere for 0.5h (PMN), or 2h (macrophages) before gently washing the monolayers with culture medium to remove non-adherent cells. Subsequently, the cells were incubated with 1 x 10^4 cfu C. albicans ATCC 10261, which were opsonized for 45 min at 24 ºC in Modified Eagle’s medium (Gibco Life technologies, Paisley, Scotland; MEM), containing 2,5 % fresh mouse serum (E:T ratio, 40:1). After 15 min, supernatants were aspirated and monolayers were gently washed with MEM to remove uningested blastoconidia. The supernatant and well washings, containing the non-ingested Candida blastoconidia, were combined and plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytized blastoconidia was defined as (1 – (number of uningested cfu/cfu at the start of incubation)) x 100.

Killing of C. albicans by PMN was assessed in the same monolayers. After removal of the non-phagocytized Candida blastoconidia, 200 µl of culture medium, consisting of Sabouraud in MEM (50 % v/v), was added to the monolayers. After 3h of incubation at 37 ºC and 5 % CO2, the wells were gently scraped with a plastic paddle and washed with 200 µl distilled H2O to achieve lysis of PMN. This procedure was repeated to a final volume of
Microscopic examination of the culture plates showed that there was a complete removal of phagocyes. The number viable intracellular Candida blastoconidia was quantified as described above. The percentage of yeast killed by the phagocytes was determined as follows: (1 – (cfu after incubation/ number of phagocytized cfu)) x 100. Phagocyte-free incubations of blastoconidia were included as a control for yeast viability.

**Assessment of PMN-mediated pseudohyphal damage**

The PMN-mediated damage of Candida pseudohyphae was determined by a modification of the XTT dye assay, as described previously [22]. Candida blastoconidia (strain UC820) grown on Sabouraud agar plates were collected with a swab and suspended at a final concentration of 1 x 10^6 cfu/ml in RPMI 1640 Dutch modification (with 20mM Hepes, without glutamine, ICN Biomedicals GmbH, Eschwege, Germany) supplemented with 1 % gentamicin, 1 % L-glutamine and 1 % pyruvate, henceforth to be called RPMI-dm. The pH of the suspension was adjusted to 6.4 using hydrochloric acid. Pseudohyphae were obtained by incubating 10 ml of the suspension at 37 ºC for 24h. After incubation, the pseudohyphae were centrifuged (550 x g) and resuspended in RPMI 1640 without phenol red and L-glutamine (RPMI-wp; ICN Biomedicals, Eschwege, Germany). 200µl aliquots of a suspension containing 1 x 10^5 pseudohyphae were dispensed into a 24-well flat bottom plate (Costar, Corning, the Netherlands). PMN were elicited by an i.p. injection of 10 % proteose peptone. After 4h, cells were collected and resuspended in 200 µl RPMI-wp, and added to the wells containing pseudohyphae at a final E:T ratio of 8:1, in the presence of 10 % fresh IL-1α+/β+ serum. Control wells contained pseudohyphae or PMN only. After incubation for 2h, 800 µl of sterile H2O was added to the wells and the plate was rocked at room temperature to achieve lysis of PMN. After 15 min., 800 µl sterile saline containing XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma Chemical, St. Louis, MO) and coenzyme Q0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) was added to each well at a final concentration of 400 µg/ml XTT and 50 µg/ml coenzyme Q0. After 1h of incubation at 37 ºC, the plate was centrifuged (770 x g) and 150 µl of the supernatant of each well was transferred to a well of a 96-well microtiter plate. The absorbance of each well was measured in a spectrophotometer at 450 nm. The percentage of fungal damage was defined as 1– ((A450 hyphae + PMN – A450 PMN) / A450 hyphae) x 100.

**Stimulation of peritoneal macrophages**

Resident peritoneal macrophages were obtained by rinsing the peritoneal cavity aseptically with ice-cold PBS containing 50 U/ml heparin. After centrifugation for 10 min, 1800 rpm, at 4ºC, the cells were resuspended in RPMI-dm and 10^5 cells/well were added
to round bottom 96-wells microtiter plate (Costar Corporation, Cambridge, MA). The cells were stimulated with heat-killed \textit{C. albicans} (UC 820) blastoconidia (10^7) or pseudohyphae (10^6). The supernatants were collected after 24 h of incubation and stored at -80 °C until cytokine determination. For measurement of cell-associated IL-1α, 200 µl RPMI 1640 was added to the remaining macrophages that were subsequently disrupted by three freeze-thaw cycles. The cell lysates were stored at -80 °C until assay.

\textbf{Stimulation of splenic lymphocytes}

Spleens were removed 14 days after an i.v. injection of 1 x 10^4 \textit{C. albicans}. Spleen cells were obtained by gently squeezing spleens in a sterile 200 µm filter chamber. Microscopic examination of Giemsa stained cytospin preparations showed that splenocytes consisted for 95 % of lymphocytes, 2 % monocytes and 3 % granulocytes. Splenocytes were washed and resuspended in RPMI-dm, counted in a Bürker counting chamber, and the number was adjusted to 5 x 10^6/ml. 1 ml of the cell suspension was stimulated with 1 x 10^7 heat-killed \textit{Candida albicans} UC820 blastoconidia (E:T ratio, 2:1). Measurement of IFNγ and IL-10 concentrations was performed in supernatants collected after 48h of incubation at 37 °C in 5 % CO₂ in 24-well plates (Greiner, Alphen a/d Rijn, The Netherlands).

\textbf{Cytokine assays}

The concentrations of TNFα were determined using specific radioimmunoassays as described previously [14]. The detection limit was 40 pg/ml for TNFα, and 20 pg/ml for IL-1α and IL-1β. IL-10, IFNγ and IL-6 concentrations were determined by a commercially available ELISA kit (Biosource, Europe), according to the guidelines of the manufacturer. The detection limits were 8, 15.6, or 150 pg/ml, respectively. Murine KC, macrophage inhibitory protein (MIP)-2, and monocyte chemotactic protein-1 (MCP-1) concentrations were measured by commercial ELISA kits, according to the instructions of the manufacturer (R&D Systems).

\textbf{Statistical analysis}

Values were expressed as means ± standard deviation (sd) or, in case of logarithmic data, as means and the 95 % confidence intervals. Since 3 groups or more were compared, the data were analyzed using the Kruskal-Wallis one-way ANOVA. For post-test comparisons, the Bonferroni \textit{t} method was applied. The data represent the average results of all experiments performed.
IL-1 in disseminated candidiasis

Results

Disseminated candidiasis in nonneutropenic mice

Whereas 72% of the IL-1$^{+/-}\beta^{+/-}$ mice survived the i.v. infection of $1 \times 10^5$ cfu *C. albicans*, only 41% of IL-1$^{+/-}$, and 38% of IL-1$^{+/-}$ mice did (p < 0.05; Fig. 1). Moreover, survival was even further impaired in mice lacking both IL-1$\alpha$ and IL-1$\beta$ and the difference was significant compared with IL-1$^{+/-}\beta^{+/-}$ mice (p < 0.001) or IL-1$^{+/-}$ mice (p < 0.05; Fig. 1).

**Figure 1.** The survival of IL-1-deficient mice after an i.v. injection of $1 \times 10^5$ *C. albicans* cfu. Survival of IL-1$^{+/-}$ or IL-1$^{+/-}$ mice was significantly impaired compared with IL-1$^{+/-}\beta^{+/-}$ mice (p < 0.05). IL-1$^{+/-}\beta^{+/-}$ mice showed a significantly impaired survival compared with IL-1$^{+/-}\beta^{+/-}$ mice (p < 0.001), or with IL-1$^{+/-}$ mice (p < 0.05). The data are the cumulative results of experiments performed in quadruplicate for at least 25 mice per group.

Since the kidneys are the main target organs in disseminated candidiasis [7,8], outgrowth of *C. albicans* in the kidneys was determined. Seven days after i.v. injection of $1 \times 10^5$ *C. albicans* blastoconidia, outgrowth of *C. albicans* was increased 51-fold in IL-1$^{+/-}$ mice (p < 0.01), 3-fold in IL-1$^{+/-}$ (p > 0.05), and 125-fold in IL-1$^{+/-}\beta^{+/-}$ (p < 0.001; Fig. 2) compared with IL-1$^{+/-}\beta^{+/-}$ mice. At this point in time, the number of *Candida* cfu in the kidneys from IL-1$^{+/-}$ mice was significantly higher than in those of IL-1$^{+/-}$ mice (p < 0.05; Fig. 2). To determine the difference in outgrowth between IL-1$^{+/-}$ and IL-1$^{+/-}$ mice on a later point in time during infection, subgroups of IL-1$^{+/-}$, IL-1$^{+/-}$, or IL-1$^{+/-}\beta^{+/-}$ mice received an i.v. injection of $5 \times 10^4$ *C. albicans* cfu. On day 14 of infection, no difference between the number of *Candida* cfu recovered from the kidneys of IL-1$^{+/-}$ (6.03 log cfu; 95%CI, 5.50, 6.57) or IL-1$^{+/-}$ mice (6.10 log cfu; 95%CI, 5.44, 6.77) was
observed, and outgrowth in both groups was 10-fold increased compared with IL-1α+/β+/ mice (4.25 log cfu; 95% CI, 2.54, 5.96; p < 0.05).

Figure 2. Outgrowth of *C. albicans* in the kidneys on day 7 of infection. Data represent the cumulative results of two experiments, and are expressed as the mean and 95% confidence interval for 5-10 mice per group. a p < 0.001 vs. IL-1α+/β+/+ mice; b p < 0.01 vs. IL-1β−/− mice; c p < 0.01 vs. IL-1α+/β+/+ mice; d p < 0.05 vs. IL-1β−/− mice.

**Circulating cytokine concentrations**

Determination of the circulating concentrations of TNFα, IFNγ, IL-10, and IL-6 in blood obtained from mice on day 7 of infection revealed no differences between groups. All data were around the detection limit (data not shown).

**Histopathological examination.**

Seven days after an i.v. injection of 1 x 10⁵ *C. albicans* blastoconidia, the inflammatory lesions in the kidneys of IL-1α+/β+/+ mice were healing, as evidenced by the presence of scar tissue with few PMN and fibroblasts (Fig. 3D). In the kidneys of IL-1β−/− and IL-1α−/β−/− mice, a granulomatous response was observed without the presence of yeast cells (Fig. 3B and C). In IL-1α−/− mice however, a large amount of *C. albicans* blastoconidia and pseudohyphae had accumulated in the collecting ducts, surrounded by PMN and lymphocytes (Fig. 3A).
IL-1 in disseminated candidiasis

Figure 3 (for colour printing, see cover). Histopathology of the kidneys after an i.v. injection of 1 x 10^5 cfu C. albicans on day 7 of infection (x 400; periodic acid-Schiff stained sections). (A) In the collecting duct of IL-1α^-/- mice a large number of C. albicans blastoconidia and pseudohyphae were observed surrounded by granulocytes and lymphocytes. In the kidney of IL-1β^-/- (B), as well as in that of IL-1α^-/-β^-/- mice (C) a granulomatous response with foreign body giant cells was observed. (D) The kidneys of IL-1α^+/+β^+/+ show scar tissue with fibroblasts and granulocytes in healing tissue.

Macrophage function and cytokine production.

Macrophage recruitment was determined by harvesting peritoneal exudate cells 72h after an i.p. injection of 10^7 cfu heat-killed C. albicans. No differences in the numbers of macrophages that were recruited into the peritoneal cavity were observed compared with control mice (IL-1α^+/+β^+/+, 1.01 ± 0.12 x 10^9; IL-1α^-/-, 1.10 ± 0.02 x 10^9; IL-1β^-/-, 0.96 ± 0.19 x 10^9; IL-1α^-/-β^-/- 1.18 ± 0.15 x 10^9). IL-1 neither influenced the capacity of exudate peritoneal macrophages to phagocytize C. albicans blastoconidia (IL-1α^+/+β^+/+, 41 ± 5%; IL-1α^-/-, 50 ± 4%; IL-1β^-/-, 56 ± 3%; IL-1α^-/-β^-/- 41 ± 16%10^7 cfu heat-killed C. albicans), nor their capacity to damage Candida pseudohyphae (data not shown).
IL-1-deficient resident peritoneal macrophages were stimulated in vitro with heat-killed *C. albicans* blastoconidia or pseudohyphae. Since stimulation with heat-killed blastoconidia or pseudohyphae yielded similar results, the differences between the groups were assessed using the cumulative results of both stimuli. IL-1-deficient macrophages showed a significantly impaired production of the chemokine KC compared with IL-1α+/β+/+ macrophages (p < 0.05; Table 1). The difference in IL-6 production was significant for IL-1α−/− or IL-1α−/β−− compared with IL-1α+/β+/+ macrophages (p < 0.05; Table 1).

**Table 1.** In vitro cytokine and chemokine production capacity of resident peritoneal macrophages stimulated with heat-killed *C. albicans*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>TNFα (ng/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>MCP-1 (pg/ml)</th>
<th>Mip-2 (pg/ml)</th>
<th>KC (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α+/β+/+</td>
<td>0.98 ± 0.71</td>
<td>959 ± 466</td>
<td>87 ± 45</td>
<td>2772 ± 2190</td>
<td>1881 ± 830</td>
</tr>
<tr>
<td>IL-1α−/−</td>
<td>0.92 ± 0.39</td>
<td>257 ± 177*</td>
<td>40 ± 1</td>
<td>2605 ± 1550</td>
<td>407 ± 188*</td>
</tr>
<tr>
<td>IL-1β−−</td>
<td>0.60 ± 0.29</td>
<td>373 ± 242</td>
<td>50 ± 22</td>
<td>2331 ± 1690</td>
<td>203 ± 258*</td>
</tr>
<tr>
<td>IL-1α−β−−</td>
<td>0.85 ± 0.40</td>
<td>195 ± 132*</td>
<td></td>
<td>1485 ± 638</td>
<td>284 ± 276*</td>
</tr>
</tbody>
</table>

Data are obtained from one experiment and expressed as means ± sd of at least 8 mice per group.
*p<0.05 v.s. IL-1α+/β+/+ mice

**Stimulation of splenic lymphocytes**

To assess whether IL-1 induces a T helper type 1 (Th1) or a Th2-type immune response, 5 x 10⁶ splenic lymphocytes obtained on day 14 of infection from IL-1α+/β+/+ mice and IL-1-deficient mice were stimulated with 1 x 10⁷ heat killed *C. albicans* cfu in-vitro. Whereas IL-1α+/β+/+ lymphocytes produced IFNγ (47.4 ± 30.7 pg/ml), no IFNγ production was detected by IL-1-deficient lymphocytes (< 15.6 pg/ml; p < 0.05). Both IL-1α+/β+/+ and IL-1α− lymphocytes produced IL-10 (respectively, 32.3 ± 8.8 pg/ml and 30.8 ± 12.2 pg/ml), whereas IL-1β−− as well as IL-1α−β−− lymphocytes did not (< 8 pg/ml; p < 0.05 vs IL-1α+/β+/+ or IL-1α−− lymphocytes). Data are 10⁷ cfu heat-killed *C. albicans* expressed as means ± sd for at least 4 mice per group.

**Circulating numbers of PMN and PMN recruitment**

The influence of IL-1 on the circulating numbers of PMN is depicted in table 2. The mean numbers of peripheral blood PMN were similar for all groups on day 7 of infection (data not shown). The numbers of PMN collected 4 h after an i.p. injection of 10⁷ cfu heat-killed *C. albicans* indicate that IL-1β itself is more important for the recruitment of PMN.
to the site of infection than IL-1α, and may partly explain the higher numbers of circulating PMN observed during the early days of infection in IL-1β−/− and IL-1α−/−β−/− mice compared with IL-1α−/− and IL-1α+/+β+/+ mice (Table 2).

**Table 2.** Number of circulating PMN prior to, or on day 3 of an infection with *Candida* blastoconidia (10⁵ i.v.), and recruitment of PMN 4h after injection of heat-killed *Candida* blastoconidia (10⁷ i.p.). The i.p. PMN recruitment is represented as the percentage of the total numbers of peritoneal exudate cells, as well as the absolute numbers of PMN.

<table>
<thead>
<tr>
<th>Strain</th>
<th>number of circulating PMN (x 10⁹/l)</th>
<th>i.p. PMN recruitment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>prior to infection</td>
<td>day 3 of infection</td>
</tr>
<tr>
<td>IL-1α+/+β+/+</td>
<td>0.89 ± 0.31</td>
<td>0.84 ± 0.71</td>
</tr>
<tr>
<td>IL-1α−/−</td>
<td>0.42 ± 0.10</td>
<td>0.86 ± 0.30</td>
</tr>
<tr>
<td>IL-1β−/−</td>
<td>1.38 ± 0.47</td>
<td>3.20 ± 0.99</td>
</tr>
<tr>
<td>IL-1α−/−β−/−</td>
<td>0.67 ± 0.35</td>
<td>2.95 ± 0.52</td>
</tr>
</tbody>
</table>

Data are obtained from 1 experiment and expressed as means ± sd of at least 3 mice per group.

a p < 0.005; b p < 0.01 vs. control or IL-1α−/− mice.

**Disseminated candidiasis in neutropenic mice**

To assess whether the protective effect of IL-1 is mediated through PMN, mice were repeatedly injected with cyclophosphamide to induce a profound and sustained granulocytopenia. At all time points tested, the largest quantity of *C. albicans* was recovered from the kidneys of IL-1α−/−β−/− mice, and the difference was significant compared with IL-1α−/−β−/− mice on day 3 of infection (5.23 log cfu; 95%CI, 4.85, 5.60 vs. 3.64 log cfu; 95%CI, 3.13, 4.14; p < 0.01). Outgrowth in the kidneys of IL-1α−/− mice (4.27 log cfu; 95%CI, 2.96, 5.58) was similar to that in the kidneys of IL-1β−/− mice (4.13 log cfu; 95%CI, 3.47, 4.78; data are the cumulative results of experiments performed twice for at least 6 mice per group).

**Anticandidal response of PMN against *C. albicans* blastoconidia and pseudohyphae**

To further characterize the influence of endogenous IL-1α and IL-1β on the activity of PMN against *C. albicans*, the fungicidal capacity of PMN was determined in vitro against both growth forms of *C. albicans*. Whereas PMN from IL-1α−/− only showed a tendency to reduced superoxide production (58.3 ± 15.4 x 10³ counts), IL-β−/− PMN produced significantly less superoxide than control PMN did (30.6 ± 15.9 x 10³ counts vs. 71.5 ± 10.5 x 10³ counts; p < 0.01). However, this observation was not accompanied by an altered capacity of IL-1-deficient PMN to phagocytize or kill internalized *C. albicans*.
blastoconidia (data not shown). In contrast, IL-1 modulated the capacity of PMN to damage C. albicans pseudohyphae extracellularly (Fig. 4). Pseudohyphal damage by PMN from IL-1α⁻/⁻ or IL-1α⁻/β⁻ mice was significantly less compared to that by PMN from IL-1α⁺/⁺ or IL-1β⁻/⁻ mice (Fig. 4; p < 0.001). Deficiency of both IL-1β and IL-α showed a tendency to further impair PMN antipseudohyphal resistance compared to IL-1α deficiency alone. Deficiency of IL-1β alone did not diminish the capacity of PMN to damage C. albicans pseudohyphae.

**Figure 4.** Percentage of exudate PMN-mediated pseudohyphal damage. 1 x 10⁵ pseudohyphae were incubated in the presence of 8 x 10⁵ PMN for 2h. Data are the cumulative results of two separate experiments. Horizontal bars indicate the means. a p < 0.001 vs. IL-1α⁺/⁺β⁺/⁺ or IL-1β⁻/⁻ mice.
Discussion

The results of the present study indicate that deficiency of endogenous IL-1 has deleterious effects on outcome of disseminated *C. albicans* infection. IL-1-deficient mice showed an increased mortality that was associated with an increased outgrowth of *C. albicans* in the kidneys. Presence of IL-1β proved to be important for recruitment of granulocytes to the site of infection and generation of superoxide production. IL-1α was essential for the capacity of granulocytes to damage *Candida* pseudohyphae and both IL-1α and IL-1β were elementary for the induction of protective Th1 responses.

One of the mechanisms through which IL-1 has been suggested to confer protection against infection is the enhancement of granulopoiesis and the influx of PMN to the site of infection. Although IL-1α−/− mice had 50% fewer circulating granulocytes compared to controls at the time of infection, their number in the circulation on day 3 of infection and the recruitment of PMN to the site of infection did not differ from that of control mice. In IL-1β−/− mice however, a trend towards an increased number of circulating granulocytes during infection was observed and this coincided with a significantly decreased migration of PMN to the site of infection. On the one hand, this indicates that both endogenous IL-1α and IL-1β are, either directly, or indirectly through induction of colony-stimulating factors, important for granulopoiesis and this is in keeping with earlier findings showing that exogenous administration of IL-1α or IL-1β induced peripheral blood granulocytosis [1,13]. On the other hand, although exogenous administration of either IL-1 molecule has been reported to induce PMN accumulation [4,18], our data of PMN recruitment in response to heat-killed *C. albicans* suggest an important role for endogenous IL-1β and not IL-1α in early PMN influx, an observation that was further corroborated by histopathological examination of the kidneys on day 3 of *C. albicans* infection, revealing PMN infiltration in IL-1α−/− mice whereas hardly any PMN were detected in IL-1β−/− mice (data not shown). In addition, PMN recruitment in IL-1-deficient mice may be further impaired due to decreased production of neutrophil chemoattractants. Therefore, production by macrophages of members of the CXC chemokine subfamily, such as MIP-2 and KC was determined. Only the production of KC by IL-1-deficient macrophages was significantly reduced, thus contributing to impaired granulocyte recruitment in IL-1β−/− mice. Transcription of the genes encoding for MIP-2 and KC is induced through signals mediated by Toll-like receptor 4 [16], and IL-1 has been shown to selectively stabilize KC mRNA [17]. Hence, it is hypothesized that the normal production of MIP-2 in contrast to the 80% reduced production of KC by IL-1-deficient macrophages is due to the selective effect of IL-1 on KC mRNA.
The effect of endogenous IL-1 on PMN function has not been investigated before. The results of the present study have indicated that IL-1 affects PMN function and that the modes of action differ for IL-1α and IL-1β. Whereas IL-1α−/− PMN showed a decreased capacity to damage *C. albicans* pseudohyphae, IL-1β−/− PMN had an impaired superoxide production. In addition, production of IL-6 that has been shown to stimulate PMN function [3,19], was diminished in IL-1α−/− mice. The impaired IL-6 production in IL-1α−/− mice may thus further diminish PMN function and anticandidal defence.

To test whether IL-1 exerts its beneficial effect mainly through PMN recruitment and function, the differences in outgrowth between the groups as observed in non-granulocytopenic mice, should disappear in granulocytopenic mice. However, the observed differences persisted, indicating that the effect of endogenous IL-1 is at least in part mediated through other mechanisms than modulation of PMN recruitment and function, which is in line with previous findings [8,20].

Macrophages are effector cells in anticandidal defence as well [2,21]. Although IL-1-deficient macrophages that were stimulated in vitro with heat-killed *C. albicans* responded with diminished IL-6 and KC production, macrophage recruitment and phagocytosis were not altered in IL-1-deficient mice. This is in line with previous research showing normal phagocytosis and NO production by IL-1-deficient macrophages [12,25]. However, activation of phagocyte functions strongly depends on the presence of the Th1 cytokine, interferon-γ [10,24]. Production of interferon-γ was virtually absent in IL-1-deficient lymphocytes, indicating that phagocyte activation and subsequent clearance of *Candida* cells will be impaired during infection. In addition, transition of macrophages to functionally inactive foreign body giant cells (FBGCs) is favoured in the presence of Th2 cytokines [11]. Therefore, the FBGCs that were observed in the kidneys of IL-1-deficient mice confirm the presence of an anti-inflammatory Th2 response, which is detrimental to anticandidal host defence.

In conclusion, the results of the present study further clarified the mechanisms through which IL-1 enhances host resistance to disseminated candidiasis. Although a functional difference between IL-1α and IL-1β was observed, both IL-1α and IL-1β have been shown to be essential to anticandidal host defence.
IL-1 in disseminated candidiasis

References


6. Horai R, Asano M, Sudo K et al. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med 1998; 187:1463-75.


Differential role of IL-18 and IL-12 in the host defence against disseminated *Candida albicans* infection

Mihai G. Netea¹,⁴, Alieke G. Vonk¹,⁴, Mabel van den Hoven¹,⁴, Ineke C. Verschueren¹,⁴, Leo A.B. Joosten², Johan H. van Krieken³, Wim B. van Den Berg², Jos W.M. van der Meer¹,⁴, Bart-Jan Kullberg¹,⁴.

Departments of Medicine, Division of General Internal Medicine¹, Rheumatology Research Laboratory and Advanced Therapeutics², and Pathology³, University Medical Centre St. Radboud Nijmegen, and Nijmegen University Centre for Infectious Diseases⁴, Nijmegen, the Netherlands

*European Journal of Immunology* 2003; 33: 3409-3417

© Wiley-VCH Verlag GmbH & Co. KGaA
Abstract

IFN-γ plays a crucial role in the defence against infection with Candida albicans. Since IL-18 and IL-12 are strong stimuli of IFN-γ production, we investigated whether endogenous IL-18 and IL-12 are involved in the host defence during disseminated candidiasis. IL-18 knockout (IL-18−/−) mice, but not IL-12−/− mice, displayed an increased mortality due to C. albicans infection, accompanied by a decreased clearance of the yeasts from the kidneys late during the course of infection. Histopathology of the organs, combined with phagocyte recruitment experiments, showed a decreased influx of monocytes at the sites of Candida infection, mainly in the IL-18−/− mice. Whereas production of the chemokine KC was decreased in both IL-18+/− and IL-12+/− mice, MIP-2 production was deficient only in IL-18−/− animals, which may explain the differences in phagocyte recruitment. In addition, although IFN-γ production capacity, as a parameter of the Th1-protective immunity, was reduced by 65 to 80% in the IL-12−/− mice, this defect was even more pronounced in the IL-18−/− mice (85 to 95% downmodulation). In conclusion, the anticandidal effects of endogenous IL-18 are mediated late during the infection by assuring a proper IFN-γ response and promoting the infiltration of the site of infection by monocytes.
Chapter 5

Introduction

Acute disseminated candidiasis is a life-threatening disease which occurs predominantly in immunocompromized patients. Despite development of new antifungal drugs, mortality associated with disseminated candidiasis remains high [2], and additional therapies directed towards the augmentation of host defence mechanisms would be a rational approach. A better understanding of the mechanisms responsible for the defence against an invasive Candida infection is therefore needed, in order to develop strategies aimed to enforce the antifungal actions of the immune system.

Candida albicans or mannoproteins derived from the yeast cell wall induce IFN-γ production by human mononuclear cells, and IFN-γ is a key cytokine for defence to candidiasis [15]. In vitro studies have demonstrated stimulatory effects of IFN-γ on the phagocytosis and killing of C. albicans by neutrophils and macrophages [5,22], whereas the central role of endogenous IFN-γ in the resistance against systemic candidiasis has been demonstrated in knock-out mice deficient in IFN-γ which are highly susceptible to C. albicans infection [1,18]. Moreover, administration of recombinant IFN-γ to wild-type mice infected with C. albicans improves the outcome of the infection [15].

Interleukin-18 (IL-18) and IL-12 are costimuli for IFN-γ production in the context of microbial stimulation [29,31]. When endogenous IL-18 is neutralized by administration of antibodies to IL-18 [7], there is little, if any, IFN-γ production after challenge with endotoxin. We have recently demonstrated that both endogenous IL-18 and IL-12 are involved in the production of IFN-γ production in human whole blood stimulated with C. albicans [28]. Administration of recombinant IL-18 restores anti-candidal defence in caspase-1-deficient mice, and challenge with IL-12 may have either protective or deleterious effects during systemic candidiasis [18]. It is therefore apparent that IL-18 and IL-12 are important for the modulation of the host defence against disseminated candidiasis, but a clear characterization of their respective effects is still missing.

The aim of the present study was to investigate whether endogenous IL-18 and IL-12 are involved in host defence against Candida infection. We have assessed susceptibility to a systemic C. albicans infection in IL-18 knockout (IL-18−/−) and IL-12−/− mice, and investigated the defence mechanisms responsible for the anti-Candida defence in these mice.
Materials and methods

Animals

Homozygous IL-18−/− mice on a C57BL/6 background were a kind gift from Dr. S. Akira (Osaka, Japan) [33]. The IL-18−/− knockout mice were initial on a C57BL/6J×Sv129 background, and they were backcrossed for six generations into a C57BL/6J background. Homozygous IL-12−/− mice on a C57BL/6 background were obtained from Jackson Labs [21]. Specific pathogen-free knockout mice and age and weight-matched C57BL/6 control mice (20-25 g, 6 to 8 weeks old) were used. Mice were fed sterilized laboratory chow (Hope Farms, Woerden, The Netherlands) and water ad libitum.

Candida albicans infection model

C. albicans UC 820, a clinical isolate well-described earlier [19] has been used in all experiments. Mice were injected intravenously with C. albicans (between 1 and 5 × 10^5 colony forming units (CFU)/mouse) in a 100-µl volume of sterile pyrogen-free phosphate-buffered saline (PBS), as indicated. Survival was assessed daily for 30 days in groups of at least 20 animals per group. At the end of the observation period, the surviving mice were anesthetized with ether and killed by cervical dislocation. In addition, subgroups of 5 animals were killed on days 1, 3, 7 or 14 of infection, and blood was collected on EDTA for plasma cytokine concentration measurements. To assess the outgrowth of the microorganisms, the liver, spleen and left kidneys of the sacrificed animals were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. The number of viable Candida cells in the tissues was determined by plating serial dilutions on Sabouraud dextrose agar plates as previously described [16]. The CFU were counted after 24 h of incubation at 37°C, and expressed as log CFU/g tissue. From the same animals, the right kidneys were fixed in formalin (4%), embedded in paraffin, and serial sections were examined microscopically after staining with a combination of periodic acid Schiff (PAS) and hematoxylin-eosin (HE).

Cytokine production capacity

Uninfected IL-18−/−, IL-12−/− mice and wild-type controls were killed and resident peritoneal macrophages were harvested by injecting 4 ml of sterile PBS containing 0.38% sodium citrate [15]. After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, 100 µg/ml gentamicin and 2% fresh mouse plasma (culture medium). Cells were cultured in 96-well microtiter plates (Costar, Corning, The Netherlands) at 10^5 cells/well, in a final volume of 200 µl. The cells were stimulated with heat-killed (1 h, 100°C) C. albicans, either blastospores or hyphae, at a
concentration of $10^7$ micro-organisms/ml. After 24 h of incubation at 37°C, the plates were centrifuged (500 x g, 10 min), and the supernatant was collected and stored at -80°C until cytokine assays were performed.

For the assessment of IFN-γ and IL-10 production capacity, splenic lymphocytes were stimulated in vitro with heat-killed *Candida*. Spleen cells were obtained by gently squeezing spleens in a sterile 200-µm filter chamber. Microscopic examination of Giemsa stained cytospin preparations showed that these cells consisted for 95 % of lymphocytes, 2 % monocytes and 3 % granulocytes. The cells were washed and resuspended in RPMI-dm, counted in a Bürker counting chamber, and the number was adjusted to $5 \times 10^6$/ml. 1 ml of the cell suspension was stimulated with $1 \times 10^7$ heat-killed *C. albicans* UC820 blastoconidia (E:T ratio, 2:1). Measurement of IFN-γ and IL-10 concentrations was performed in supernatants collected after 48 h of incubation at 37°C in 5 % CO₂ in 24-well plates (Greiner, Alphen a/d Rijn, The Netherlands).

In an additional experiment we have assessed the local cytokine levels in the kidneys of the infected mice by homogenization of the organs in 3 ml of sterile PBS. TNF, IFN-γ, IL-1β and IL-6 concentrations in the homogenates were measured as described below.

**Cytokine assays**

IL-1α, IL-1β and TNF-α were determined by specific radioimmunoassays (detection limit 20 pg/ml), as previously described [25]. IFN-γ concentrations were measured by a commercial ELISA (Biosource, Camarillo, CA; detection limit 16 pg/ml), according to the instructions of the manufacturer. MIP-2 and KC concentrations were measured by a commercial ELISA (R&D Systems, Abingdon, GB; detection limit 24 pg/ml), according to the instructions of the manufacturer.

**Extracellular killing of *C. albicans* hyphae by neutrophils (PMN)**

The PMN-mediated damage of *Candida* hyphae was determined by a modification of the XTT dye assay, which was previously described by Gaviria et al. [9]. *Candida* blastoconidia (strain UC820) grown on Sabouraud agar plates were collected with a swab and suspended at a final concentration of $1 \times 10^6$ cfu/ml in RPMI 1640 Dutch modification (with 20 mM Hepes, without glutamine, ICN Biomedicals, Eschwege, Germany) supplemented with 1 % gentamicin, 1 % L-glutamine and 1 % pyruvate. The pH of the suspension was adjusted to 6.4 using hydrochloric acid. Hyphae were obtained by incubating 10 ml of the suspension at 37°C for 24 h. After incubation, the hyphae were centrifuged (1800 rpm, 10 min) and resuspended in RPMI 1640 without phenol red and L-glutamine (RPMI-wp; ICN). Aliquots (160 µl) of a suspension containing $1 \times 10^5$ hyphae were dispensed into a 24-well flat-bottom plate (Costar). PMN were elicited by an i.p.
Injection of 10% proteose peptone. After 4 h, cells were collected and resuspended in RPMI, the number of PMN was determined in Giemsa stained cytospin preparations and 8 x 10^5 PMN (200 µl) were added to the wells containing hyphae at a final E:T ratio of 8:1, in the presence of 10% fresh mouse serum. Control wells contained either hyphae or PMN only. After incubation for 2 h, 800 µl of sterile H2O was added to the wells and the plate was rocked at room temperature to achieve lysis of PMN. After 15 min., 800 µl sterile saline-solution containing XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma Chemical, St. Louis, MO] and coenzyme Q0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) were added to each well at a final concentration of 400 µg/ml XTT and 50 µg/ml coenzyme Q0. After 1 h of incubation at 37°C, the plate was centrifuged (1800 rpm, 10 min) and 150 µl of the supernatant of each well was transferred to a well of a 96-well microtiter plate. The absorbance of each well was measured in a spectrophotometer at 450 nm. The percentage of fungal damage was defined as [1-((A450 hyphae + PMN - A450 PMN)/E450 hyphae)] x 100.

**Phagocytosis and intracellular killing of C. albicans blastoconidia by PMN and macrophages**

Exudate peritoneal macrophages from groups of five control, IL-12^−/− and IL-18^−/− mice were elicited by an i.p. injection of 10% proteose peptone. Phagocytes were collected in sterile tubes by washing the peritoneal cavity with 4 ml ice-cold PBS containing 50 U/ml heparin 72 h after injection. The cells were centrifuged (10 min; 3600 rpm; 2250 x g), counted in a Bürker counting chamber and resuspended in RPMI supplemented with 5% heat-inactivated foetal calf serum.

The processes of phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes, as described [34]. To create a monolayer of macrophages, 5 x 10^5 cells in 100 µl of RPMI-dm were dispensed into the wells of a 96-well flat-bottom plate (Costar) and incubated at 37°C and 5% CO₂. Macrophages were allowed to adhere or 2 h before gently washing the monolayers with culture medium to remove nonadherent cells. The percentage of adherence was calculated as [1-(number of nonadherent cells/5×10^5)] x 100. Subsequently, the cells were incubated with 1 x 10^4 CFU C. albicans, which were opsonised with 2.5% fresh mouse serum (E:T ratio, 40:1) in modified Eagle's medium (Gibco Life technologies, Paisley, Scotland; MEM), for 45 min at 24°C. After 15-min incubation, supernatants were aspirated and monolayers were gently washed with MEM to remove uningested blastoconidia. The supernatant and well washings, containing the non-ingested Candida blastoconidia, were combined and plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytized blastoconidia was defined as [1-(number of uningested CFU/CFU at the start of incubation)] x 100.
Killing of *C. albicans* by macrophages was assessed in the same monolayers. After removal of the non-phagocytized *Candida* blastoconidia, 200 µl of culture medium, consisting of Sabouraud in MEM (50 % v/v), was added to the monolayers. After 4 h of incubation at 37°C and 5 % CO₂, the wells were gently scraped with a plastic paddle and washed with 200 µl distilled H₂O to achieve lysis of macrophages. This procedure was repeated three times, after which the pooled washes were adjusted to a final volume of 1 ml with distilled water. Microscopic examination of the culture plates showed that there was a complete removal of phagocytes. To quantify the number of viable intracellular *Candida* blastoconidia, tenfold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37°C for 24 h. The percentage of yeast killed by the macrophages was determined as follows: \([1-(\text{CFU after incubation/number of phagocytized CFU})]\) x 100. Macrophage-free incubations of blastoconidia were included as a control for yeast viability.

**Superoxide production**

PMN from IL-18⁻/⁻, IL-12⁻/⁻ and control mice were collected 4 h after intraperitoneal injection of proteose peptone as described above. Luminol-enhanced chemiluminescence of proteose-peptone-elicited PMN was measured on a Victor 1420 multilabel counter (Wallac, Turku, Finland) at 20°C using white 96-well microplates (Costar Corporation, Cambridge, MA), as previously described [20]. Each well contained 2 x 10⁵ cells, 50 µM luminol, 4.5 U/ml horseradish peroxidase (HRP) and 50 ng/ml phorbol 12-myristate 13-acetate (PMA) in 200 µl of Hank's balanced salt solution without phenol red (Gibco Life Technologies) supplemented with 0.25 % human albumin (Behringwerke, Marburg, Germany). Reactions were started by adding PMA. Each experiment was performed in duplicate. HRP was added to the system in order to overcome peroxidase deficiency extracellularly. In previous experiments we found that the addition of extra peroxidase did not affect superoxide production (measured as reduction of cytochrome c) of human neutrophils stimulated with PMA, but increased luminol-enhanced chemiluminescence three- to fourfold. Hence, solely the detection of superoxide is enhanced in the presence of extra peroxidase. The chemiluminescence was expressed as the total amount of superoxide produced during the assay period by integrating the area under the curve (in mV.s) per PMN.

**NO production**

Resident peritoneal macrophages were collected as described above. Cells were counted and adjusted to 5 x 10⁵/ml, and stimulated with heat-killed *C. albicans* (10⁷ microorganisms/ml) and IFN-γ (100 U/ml) for 24 h at 37°C. Nitrite concentration in the
supernatants of cells from IL-18−/−, IL-12−/− and control mice was determined by the Griess reaction [4].

**Recruitment of neutrophils and monocytes**

To investigate the recruitment of PMN and monocytes at the site of *Candida* infection, groups of 5 IL-18−/−, IL-12−/− mice and wild-type controls were injected intraperitoneally with 10⁷ heat-killed *C. albicans* micro-organism. At 0 h (before *Candida*) and after 4 h (for neutrophils) and 72 h (for monocytes), peritoneal cells were collected in sterile saline containing 0.38 % sodium citrate, and the total cell number was counted in a hemacytometer. The percentage and the absolute numbers of neutrophils and monocytes were determined in Giemsa-stained cytocentrifuge preparations.

**Statistical analysis**

The differences between groups were analyzed by Mann-Whitney U-test, and where appropriate by Kruskal-Wallis ANOVA test. Survival curves were analyzed by the Kaplan-Meyer log-rank test. The level of significance between groups was set at *p*<0.05. All experiments were performed at least twice, and the data are presented as cumulative results of all experiments performed.
Results

*Candida albicans* infection model

After an infection with $5 \times 10^5$ CFU of *C. albicans*, 100% mortality was observed in all three groups, and the mean survival time did not differ between control, IL-12$^{-/-}$ and IL-18$^{-/-}$ mice (not shown). However, when mice were infected with $2.5 \times 10^5$ CFU *C. albicans*, a significantly greater mortality was recorded in the IL-18$^{-/-}$ mice (80%) compared with IL-12$^{-/-}$ (50%) and control (40%) mice (Fig. 1). In a model of infection of the mice with $1 \times 10^5$ CFU *C. albicans*, there was no mortality recorded in either of the groups.

![Figure 1.](image)

**Figure 1.** Survival of IL-18$^{-/-}$ and IL-12$^{-/-}$ after infection with *C. albicans*. Control mice (●), IL-18$^{-/-}$ mice (Δ) and IL-12$^{-/-}$ mice (○) were infected i.v. with $2.5 \times 10^5$ CFU *Candida/mouse*, and survival was assessed daily. IL-18$^{-/-}$ mice were significantly more susceptible to disseminated candidiasis compared with wild-type or IL-12$^{-/-}$ mice (ten mice/group, p < 0.05, log-rank test).

In further experiments, $1 \times 10^5$ CFU *C. albicans/mouse* was used, in order to avoid the bias induced by differential mortality at various time points in the three groups. After injection, the outgrowth of the yeasts in the kidneys was 2 to 5-fold higher than that in the brain, and 100-fold higher than that in the liver, which is in agreement with the notion that the kidneys are the target organ for *C. albicans* outgrowth in murine candidiasis (Fig. 2). IL-18$^{-/-}$ mice had an increased load of *C. albicans* in the kidneys on day 7 and 14, but not on day 1 and 3 post-infection, compared to IL-12$^{-/-}$ and control mice (Fig. 2A). In contrast, no difference between *Candida* growth in the brains (Fig. 2B) or livers (not shown) of IL12$^{-/-}$, IL-18$^{-/-}$ and control mice was apparent. Similar results were obtained in duplicate experiments with ten mice/group.
Figure 2. Growth of *C. albicans* in the organs. Outgrowth of *C. albicans* in the kidneys (A) and brain (B) of control mice (open bars), IL-12−/− mice (hatched bars) and IL-18−/− mice (dotted bars) after i.v. infection with 1 x 10⁵ CFU *Candida*/mouse. Data represent the mean ± SD for two experiments with a total of ten mice/group. Significant differences between wild-type and knockout mice are indicated (*p < 0.05).

Histopathology of the kidneys of control mice showed small PMN infiltrates on day 1, 3 and neutrophil/monocyte infiltrates on day 7 of infection with 1 x 10⁵ CFU *Candida*, with controlled growth of very small numbers of yeasts, without hyphal formation (Fig. 3). In contrast, the small neutrophil infiltrates present on day 1 and 3 after infection in the kidneys of the IL-12−/−, but particularly of IL-18−/− mice, were accompanied by the presence of extensive growth of *Candida*, which in the IL-18−/− mice had formed hyphae and pseudohyphae. On day 7, despite the presence of large granulocyte infiltrates of PMN, *Candida* was present in large numbers in the centers of the infiltrates in the kidneys of IL-12−/− and IL-18−/− mice (Fig. 3). There was relative paucity of monocytes in the infiltrates of IL-18−/− mice on day 7, compared with controls.
Figure 3 (for colour printing, see cover). Histopathology of the kidneys in the Candida-infected mice. In control mice (A), small PMN infiltrates can be seen on day 1 and neutrophil/monocyte infiltrates on day 7 after infection, with controlled growth of very small numbers of yeasts, without hyphal formation. In contrast, the small neutrophil infiltrates present on day 1 after infection in the kidneys of the IL-12^{−/−} (B), but particularly of IL-18^{−/−} mice (C), are accompanied by the presence of Candida growth, which had formed hyphae and pseudohyphae. On day 7, despite the presence of large granulocyte infiltrates, Candida is present in large numbers in the centres of the infiltrates in the kidneys of IL-12^{−/−} and IL-18^{−/−} mice. Periodic acid Schiff (PAS) combined with hematoxylin-eosin (HE) staining. Original magnification 800x.
Cytokine production capacity

Resident peritoneal macrophages from control, IL-12\textsuperscript{-/-} and IL-18\textsuperscript{-/-} mice were stimulated with 10\textsuperscript{7} heat-killed \textit{C. albicans} blastoconidia or hyphae \textit{in vitro} for 24 h. The production of TNF by macrophages from the three mouse strains was similar in response to \textit{Candida} stimulation (Fig. 4). IL-1\alpha and KC release after \textit{C. albicans} stimulation with both blastoconidia and hyphae was impaired in both IL-12\textsuperscript{-/-} and IL-18\textsuperscript{-/-} mice (Fig. 4). In contrast, MIP-2 production differed: macrophages of IL-18\textsuperscript{-/-}, but not of IL-12\textsuperscript{-/-}, exhibited an impaired production of MIP-2 upon stimulation with \textit{Candida} blastoconidia (Fig. 4). Stimulation of MIP-2 by \textit{Candida} hyphae was similar in the three mouse strains tested.

\textbf{Figure 4.} Cytokine production. Resident peritoneal macrophages from control (open bars), IL-12\textsuperscript{-/-} (hatched bars) and IL-18\textsuperscript{-/-} (dotted bars) mice stimulated \textit{in vitro} with either heat-killed \textit{C. albicans} blastoconidia or hyphae (10\textsuperscript{7} CFU/ml). Data represent the mean ± SD for two experiments (\textit{n} = 10/group). *\textit{p} < 0.05 compared to control mice.

Stimulation of spleen cells with \textit{C. albicans} blastoconidia resulted in normal IL-10 release by cells from IL-12\textsuperscript{-/-} mice (115 ± 31 pg/ml) compared to control mice (131 ± 38 pg/ml), whereas IL-18\textsuperscript{-/-} mice released lower IL-10 amounts (63 ± 22 pg/ml, \textit{p}<0.05).
Similar data were obtained when spleen cells were stimulated with *Candida* hyphae (not shown). Upon stimulation with both *Candida* hyphae and conidia, spleen cells from IL-12−/−, but in particular IL-18−/− mice, released significantly less IFN-γ compared to control mice (20 to 35% of normal production in IL-12−/− mice and 5 to 15% of normal production in the IL-18−/− mice; Fig. 5, *p* < 0.01).

In addition, we measured local cytokine production in the kidneys of infected mice. Production of IFN-γ and TNF in the kidneys was below detection limit, whereas the local release of IL-1α and IL-1β was low and varied between 30 and 100 pg/ml (no differences between the knockout mice and controls).

![Figure 5](image.png)

**Figure 5.** IFN-γ production after *Candida albicans* stimulation. Spleen cells from control (open bars), IL-12−/− (hatched bars) and IL-18−/− (dotted bars) mice were stimulated *in vitro* with either heat-killed *C. albicans* blastoconidia or hyphae (10^7 CFU/ml), and IFN-γ production was measured 48 h later. Data represent the mean ± SD for two experiments (*n*=10/group). *p* < 0.05 compared to control mice.

**Phagocytosis and killing of *C. albicans***

IFN-γ has important stimulatory effects on the fungicidal activities of phagocytes. The defective production of IFN-γ by cells of IL12−/− and IL-18−/− was accompanied by a tendency for a lower capacity of neutrophils from both knockout strains to kill *C. albicans* hyphae extracellularly, compared to neutrophils from control mice (58 and 61% in IL-18−/− and IL-12, respectively, whereas being 73% in wild-type mice). Similarly, the phagocytosis and intracellular killing of *Candida* blastoconidia was similar by the neutrophils or monocytes of the three mouse strains (not shown).

As superoxide radicals and NO are the two main candidacidal mechanisms employed by the phagocytes, we next assessed their release in the IL-12−/− and IL-18−/− mice. Both IL-12−/− and IL-18−/− neutrophils released less superoxide radicals compared to control cells: 0.13 ± 0.04 and 0.11 ± 0.03, respectively, vs. 0.17 ± 0.04 mV.s/PMN in control mice.
(p<0.05). In contrast, no differences between NO production in the three mouse strains were apparent: 33.2 ± 11.2 µg/ml in control mice, 40.2 ± 7.4 µg/ml in IL-12−/− mice and 31.0 ± 9.8 µg/ml in IL-18−/− mice (p > 0.05).

**Recruitment of neutrophils and monocytes**

To investigate the recruitment of neutrophils at the site of a *C. albicans* infection, groups of control, IL-12−/− and IL-18−/− mice were injected intraperitoneally with 10^7 heat-killed *C. albicans* micro-organisms, and exudate peritoneal neutrophils were harvested and counted 2 and 4 h later. The monocyte infiltration in the peritoneum was assessed 72 h after injection of *Candida*. As shown in Fig. 6, monocyte infiltration in the peritoneum increased 5-fold upon injection with *Candida* in the control and IL-12−/− mice. In contrast, there was only a 2.5-fold increase in the monocyte numbers in the peritoneal cavity of IL-18−/− injected with *C. albicans* (p<0.05, Fig. 6). There was no difference between the neutrophil infiltration in the peritoneal cavity of the three mouse strains at both 2 h and 4 h after injection of *Candida* (data not shown).

![Figure 6](image)

**Figure 6.** Recruitment of monocytes. Control mice (open bars), IL-12−/− mice (hatched bars) and IL-18−/− mice (dotted bars) were injected i.p. with 10^7 CFU *Candida*/mouse. 72 h later, fewer monocytes infiltrate the peritoneal cavity of IL-18−/− mice than of control and IL-12−/− mice. Data: mean ± sd for 2 experiments with 10 mice/group. *p < 0.05
Discussion

In the present study, it is shown that IL-18-/- mice have an increased susceptibility to a systemic infection with *C. albicans*, manifested as increased mortality and a 10- to 25-fold increased fungal outgrowth in their kidneys, the target organ of disseminated candidiasis in mice. In contrast, IL-12-/- mice demonstrated only a marginal increase in fungal load in their organs. A decreased Th1-type protective immunity, as revealed by lower IFN-γ production, and a reduced recruitment of monocytes at the site of infection, are likely responsible for the blunted host response against *Candida* in the IL-18-/- deficient mice.

Both IL-18 and IL-12 have been shown to be involved in the induction of IFN-γ by *C. albicans* [28], and IFN-γ is crucial for defence against disseminated candidiasis [1,18]. The results of the present study demonstrate that IL-18-/- mice have an increased susceptibility to *C. albicans* infection, and these data are sustained by the deleterious effects of a neutralizing anti-IL-18 antibody during systemic candidiasis [32]. In contrast, IL-12-/- mice had only a marginally increased outgrowth of *Candida* in their kidneys, which implies that an effective anti-candidal response can develop in the absence of IL-12. Our results are in line with the findings of Mencacci et al. [24], who reported that IL-12-/- mice are more susceptible to gastrointestinal, but not systemic *Candida* infection, underscoring the difference in pathogenesis between these two manifestations of candidal infection. Differences between the role of IL-18 and IL-12 have been also observed in other infectious models. It appears that IL-18-/-, but not IL-12-/- mice, are more susceptible to pneumococcal pneumonia, and this effect is mediated through differential neutrophil recruitment in the lungs [17]. This is in contrast with a model of experimental *Mycobacterium tuberculosis* infection, in which both functional IL-12 and IL-18 are necessary for the host defence, but IL-12p40-/- mice seem to be more susceptible than IL-18-/- mice in this infectious model [13].

There are marked effects of IL-18 and IL-12 in our study, and they are likely to be mediated at least in part by IFN-γ. Production of IFN-γ was decreased in IL-12-/- mice, and was even more strongly reduced in IL-18-/- mice. As IFN-γ is central to an effective antifungal defence, it is likely that this decreased response in the knock-out animals is involved in their susceptibility to candidiasis, which is also in line with our recent studies demonstrating that administration of anti-IL-18 antibodies to control animals, but not IFN-γ-/- mice, has deleterious effects during *Candida* infection [32]. A crucial role of IFN-γ in the host defence mechanisms activated by IL-18 was also reported in experimental infections with *Cryptococcus neoformans* [12], *Salmonella typhimurium* [23], *Toxoplasma*...
gondii [3], and Pseudomonas aeruginosa [10]. These mechanisms involve release of oxygen radicals [8] and NO [6], the two main antifungicidal mechanisms employed by phagocytes.

In addition to its role in the release of IFN-γ in the context of IL-12 co-stimulation, IL-18 has also pro-inflammatory effects that are independent of IFN-γ. IL-18 stimulates production of other pro-inflammatory cytokines and chemokines [30,27], directly induces expression of adhesion molecules [14], and is involved in the pathogenesis of lethal endotoxaemia and experimental arthritis through IFN-γ-independent pathways [11,26].

When the production of chemokines was investigated, only monocytes from IL-18−/− mice, but not IL-12−/− mice, had a decreased production of MIP-2 upon stimulation with C. albicans blastoconidia, and this was accompanied by a defective monocyte infiltration at the site of infection. KC production was decreased in both IL-18−/− and IL-12−/− mice. One should note that MIP-2 and KC are important chemoattractants for both monocytes and neutrophils, and only recruitment of the former is defective in the IL-18−/− mice. It is therefore conceivable that production of other monocyte-specific chemokines is also affected in IL-18−/− mice. In contrast, the phagocytosis and intracellular killing of Candida blastoconidia by monocytes of IL-12−/− and IL-18−/− mice was normal. It appears therefore that the intrinsic anti-candidal capacities of the monocytes are intact, but late during the infection the IL-18−/− mice fail to mount a proper monocytic infiltrate, which precludes normal clearance of the infection. Recruitment of neutrophils at the site of infection did not differ between control and knockout mice, suggesting therefore that the mice can mount proper neutrophil infiltrates.

The production of TNF by the peritoneal cells of IL-12−/− and IL-18−/− mice was normal. We have recently shown that synthesis of TNF is rapid and proximal to that of IL-18, and endogenous TNF is necessary for a proper release of IL-18 during C. albicans infection [32]. On the other hand, other pro-inflammatory cytokines such as IL-1α are released later after stimulation, and its production was significantly decreased in both knock-out strains. In view of the fact that no difference in the TNF or IL-1α could be observed between IL-12−/− and IL-18−/− mice, it is unlikely that their release can explain the differential roles of IL-12 and IL-18 in our model of systemic candidiasis.

In conclusion, endogenous IL-18 plays a protective role in the defence against disseminated infection with Candida albicans, and its effects are partly mediated by induction of a protective IFN-γ response, and partly through monocyte recruitment at the site of infection as a result of chemokine release. In turn, IL-12 has a less important role during invasive candidiasis in stimulating IFN-γ and chemokine release.
Chapter 5

References


IL-12 and IL-18 in disseminated candidiasis


Apolipoprotein-E-deficient mice exhibit an increased susceptibility to disseminated candidiasis
Abstract

The effect of hyperlipoproteinemia on systemic candidiasis was investigated by assessing the susceptibility of hyperlipoproteinemic apolipoprotein E deficient (ApoE -/-) mice to a systemic Candida albicans infection. The absence of ApoE in these mice resulted in an eightfold increase in plasma lipoprotein concentrations in the very low-density lipoprotein (VLDL) fraction, as compared with levels seen in ApoE +/+ mice. Mortality due to candidemia was significantly higher (86%) in ApoE -/- than in ApoE +/+ mice (52%), and in platings of homogenized kidney material in fungal culture medium, ApoE -/- mice yielded significantly higher levels of Candida outgrowth than did ApoE +/+ mice. C. albicans grew twofold better in ApoE -/- plasma in 4 h than in ApoE +/+ plasma, and depletion of lipoproteins from plasma resulted in a significant seven- to tenfold increase in C. albicans growth. Recombinant ApoE did not directly inhibit Candida growth. Our data indicate that the increased susceptibility of ApoE -/- mice to C. albicans is due both to increased growth of blastoconidia in ApoE -/- mice in response to the availability of lipids as nutrients, and to the neutralization of candidacidal factors by lipoproteins. This study suggests that lipoproteins play a significant role in host defence against candidiasis.
Introduction

Acute disseminated infections caused by Candida species are life-threatening diseases despite the availability of potent antifungal agents [21]. Patients receiving total parenteral nutrition (TPN) are at risk for disseminated candidiasis [6]. The growth of C. albicans is increased in lipid-rich formulations compared with growth levels seen in conventional TPN solutions [2,14]. Low-density lipoprotein (LDL) receptor-deficient mice, which have elevated plasma lipoprotein concentrations, are highly susceptible to C. albicans infection, and the proliferation of C. albicans blastoconidia in LDL-rich plasma is increased, as shown by studies on outgrowth of candidal colony forming units from this material [18]. In addition, plasma obtained from volunteers infused with high concentrations of recombinant high-density lipoprotein (rHDL) promotes the growth of C. albicans blastoconidia [17].

Apolipoprotein E (ApoE) is a major structural component of several plasma lipoproteins, such as chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL); it has an important role in the metabolism of these plasma lipoproteins. Furthermore, there is increasing evidence that ApoE has a beneficial role in modulating the pro-inflammatory immune response seen during exposure to nonspecific stimuli [9,15,20,23], as well as during bacterial infections [3,4,22]. Mice deficient in ApoE showed enhanced susceptibility to both lipopolysaccharide (LPS) [4], and Klebsiella pneumoniae [3,4]; this effect was brought about via down-modulation of the pro-inflammatory cytokine response. Indeed, a physiological increase of ApoE has been observed in response to LPS administration, and exogenous ApoE protects against LPS-induced death [24]. However, it is unclear whether ApoE exerts a protective effect during other infections, such as candidiasis.

In the present study, we assessed the influence of increased VLDL levels on host defences against disseminated candidiasis, and investigated whether this influence is mediated by ApoE. Mice lacking the gene encoding for ApoE (ApoE -/- mice) have elevated VLDL plasma concentrations. These mice and control ApoE +/+ mice were used in our experiments.
Materials and Methods

Animals and Reagents

For all experiments, we used homozygous apolipoprotein E-deficient mice with a C57Bl/6 genetic background. These mice (6-8 weeks old; 20-25 grams) were obtained from the Transgenic Facility of Leiden University Medical Centre, Leiden, The Netherlands [25]. Weight-matched C57BL/6 mice (ApoE +/+ mice) were selected as controls. The animals were kept under specific pathogen-free conditions and were fed standard laboratory chow. The experiments were approved by the ethics committee on animal experiments of the University Medical Centre Nijmegen. Human recombinant Apolipoprotein E4 was kindly provided by Dr. V.E.H. Dahlmans and Dr. L.M. Havekes (TNO-PG Leiden, The Netherlands).

Candida albicans

C. albicans blastoconidia (strains UC820 and ATCC 10261 [American Type Culture Collection, Manassas, VA, USA]), were inoculated in Sabouraud broth and cultured for 24h at 37°C. After 10 min centrifugation at 1650 x g, pellets were dissolved in sterile saline, and the number of colony forming units (cfu) was counted in a Bürker counting chamber.

Infection model

ApoE -/- and ApoE +/+ mice were infected with C. albicans strain UC820 by intravenous injection of 5 x 10⁵ blastoconidia in 100 µl of pyrogen-free phosphate-buffered saline (PBS) into the retroorbital plexus. Survival was assessed daily for 21 days in groups of at least 13 animals. For measurement of circulating cytokine concentrations and quantification of fungal outgrowth, subgroups of 6 mice were bled from the retroorbital plexus on day 1, 3 and 7 of infection, and blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes. The kidneys and liver were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. Serial dilutions of the homogenates were plated on Sabouraud dextrose agar plates as described earlier [10]. After incubation at 37°C for 48h, the number of cfu was counted and the results were expressed as the log cfu per gram of tissue.

Cytokine measurements

Concentrations of tumour necrosis factor (TNF)-α and interleukin-1α (IL-1) in EDTA plasma were measured using specific radioimmunoassays (RIA) developed in our laboratory, as described previously [26].
Recruitment of neutrophils

To investigate the recruitment of granulocytes (PMN) at the site of *Candida* infection, groups of 6 ApoE -/- mice and ApoE +/+ mice received an intraperitoneal (i.p.) injection of $10^7$ heat-killed *C. albicans* blastoconidia. Four hours after injection, cells were harvested by washing the peritoneal cavity with 4ml of ice-cold PBS, and the total number of cells was counted in a Bürker counting chamber. The percentage and absolute numbers of neutrophils were determined in Giemsa-stained cyt centrifuge preparations.

Phagocytosis and intracellular killing of *C. albicans*

The processes of phagocytosis and intracellular killing were studied using the Adherent Monolayer Method, as described [27]. Exudate-derived ‘exudate peritoneal granulocytes’ and ‘exudate peritoneal macrophages’ were obtained 4 or 72h after intraperitoneal (i.p.) injection of 1 ml of 10 % proteose peptone (Difco Laboratories, Detroit, MI), respectively. Peritoneal lavage cells from exudate were centrifuged (1800 rpm, 10 min, 4°C) and washed twice in sterile saline. The phagocytes were resuspended in RPMI 1640, Dutch modification (with 20 mM Heps, without glutamine; ICN Biomedicals, Eschwege, Germany) supplemented with 5 % heat-inactivated foetal calf serum (FCS) 1 % gentamicin, 1 % L-glutamine and 1 % pyruvate (RPMI-dm), counted in a Bürker counting chamber, and diluted to the required final concentration.

To create a monolayer of phagocytes, $5 \times 10^5$ cells in 100 µl RPMI-dm were dispensed into a 96-well flat bottom plate (Costar, Corning, the Netherlands) and incubated at 37°C in 5 % CO$_2$. Granulocytes were allowed to adhere for 0.5h and macrophages for 2h before gently the monolayers were gently washed with culture medium to remove non-adherent cells. The percentage of adherence was calculated as $(1 – \frac{\text{number of non-adherent cells}}{5 \times 10^5}) \times 100$. Subsequently, the cells were incubated with $1 \times 10^9$ cfu *C. albicans* strain ATCC 10261, cells of which had been opsonised at 24 °C with supplemented Modified Eagle’s Medium (MEM; Gibco Life Technologies, Paisley, Scotland) containing 2,5 % mouse serum (effector to target [E:T] ratio, approximately 40:1) for 45 min. After 15 min, supernatants were aspirated and monolayers were washed gently with MEM to remove uningested micro-organisms. The supernatant and washes, containing the non-phagocytized *C. albicans* blastoconidia, were combined and plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytized micro-organisms was defined as $(1 – \frac{\text{number of uningested cfu/cfu at the start of incubation}}{\text{cu at the start of incubation}}) \times 100$.

Killing of *C. albicans* by phagocytes was assessed in the same monolayers. After removal of the non-phagocytized *C. albicans* blastoconidia, 200 µl of culture medium, consisting of Sabouraud in MEM (50% v/v), was added to the monolayers. After 3h of
incubation at 37°C in air plus 5% CO₂, the wells were gently scraped with a plastic paddle and washed with 200 µl distilled H₂O to achieve lysis of phagocytes. This procedure was repeated three times, after which the pooled washes were adjusted to a final volume of 1 ml with distilled water. Microscopic examination of the culture plates showed that there was a complete removal of phagocytes. To quantify the number of viable intracellular Candida blastoconidia, tenfold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37°C for 24h. The percentage of yeast killed by the phagocytes was determined as: \((1 – [\text{cfu after incubation/ number of phagocytized cfu}]) \times 100\). Phagocyte-free incubations of blastoconidia were included as a control for yeast viability.

**Assessment of PMN-mediated hyphal damage**

PMN-mediated damage of hyphae was determined by a modification of the XTT (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) dye assay previously described by Gaviria et al [7]. *Candida* blastoconidia (strain UC820) grown on Sabouraud agar plates were collected with a swab and suspended at a final concentration of \(1 \times 10^6\) cfu/ml in RPMI-dm. The pH of the suspension was adjusted to 6.4 using hydrochloric acid. Pseudohyphae (henceforth to be called hyphae) were obtained by incubating 10 ml of the suspension at 37°C for 24h. After incubation, the hyphae were centrifuged (1800 rpm, 10 min) and resuspended in RPMI 1640 variant lacking phenol red and L-glutamine (RPMI-wp; ICN Biomedicals, Eschwege, Germany). 200µl aliquots of a suspension containing \(1 \times 10^5\) hyphae were dispensed into a 24-well flat bottom plate (Costar, Corning, the Netherlands). PMN, obtained as described above, were resuspended in 200 µl RPMI-wp, and added to the wells containing hyphae at a final E:T ratio of 10:1, in the presence of 20 % fresh ApoE +/+ serum. Control wells contained hyphae or PMN only.

After incubation for 2h, 800 µl of sterile H₂O was added to the wells and the plate was rocked at room temperature to achieve lysis of PMN. After 15 min., 800 µl sterile saline containing XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma Chemical, St. Louis, MO) and coenzyme Q₀ (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) were added to each well at a final concentration of 400 µg/ml XTT and 50 µg/ml coenzyme Q₀.

After 1h of incubation at 37°C, the plate was centrifuged (1800 rpm, 10 min) and 100 µl of the supernatant of each well was transferred to a well of a 96-well microtiter plate. The absorbance of each well was measured in a spectrophotometer at 450 nm. The percentage of fungal damage was defined as \(1 – ((A_{450} \text{hyphae + PMN} – A_{450} \text{PMN}) / A_{450} \text{hyphae}) \times 100\).
Superoxide production

To investigate whether the capacity to produce superoxide was altered in ApoE -/- mice, exudate peritoneal PMN were studied in a luminol-enhanced peroxidase-catalyzed chemiluminescence (CL) assay [13]. Exudate peritoneal PMN of 5 mice per group were obtained as described above and suspended at 2 x 10^6 cells/ml HBSS without phenol red (Life Technologies, Paisley, Scotland) supplemented with 0.25 % human serum albumin (Behringwerke, Marburg, Germany). Cells (2 x 10^5/well) were incubated in 96-well microtiter plates (Costar) with 50 µM luminol, 4.5 U/ml horseradish peroxidase (Sigma), and stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml). Chemiluminescence was measured on a Victor^2 1420 multilabel counter (Wallac, Turku, Finland) at room temperature.

Isolation of lipoprotein subclasses and lipoprotein-depleted plasma

For lipid measurement, VLDL (final cholesterol concentration 0.9 mmol/L), LDL (2.0 mmol/L cholesterol), and HDL (0.5 mmol/L cholesterol) subclasses, were isolated from fresh EDTA-plasma by sequential ultracentrifugation, as described earlier [5]. The triglycerides, total and lipoprotein specific cholesterol concentrations were determined by enzymatic methods on a Hitachi 747 analyser. For in vitro experiments, the density of the plasma samples was adjusted to 1.18 kg/L using a solution of KBr in 0.9 % pyrogen free saline. The LPS-free lipoprotein fraction (containing VLDL, IDL, LDL and HDL) as well as the lipoprotein-depleted plasma (<0.1 mmol/L cholesterol) were isolated after ultracentrifugation at 38.000 rpm, and 4ºC for 25h. Lipoproteins for lipid measurements and in vitro assays, as well as the lipoprotein depleted-plasmas were dialyzed against 0.05mM phosphate buffer, pH 7.4, containing 5 mM EDTA/L for 24h, with one exchange of the buffer.

C. albicans growth in vitro

Blood was collected from ApoE -/- and ApoE +/- mice by cardiac puncture. Pooled EDTA-plasma was obtained by centrifugation at 15000 x g for 5 minutes. C. albicans (UC820; 2 x 10^3 cfu/ml) was suspended in Sabouraud broth and diluted 1:1 in ApoE -/- or ApoE +/- plasma, or ApoE +/- plasma supplemented with the lipoprotein fraction obtained from ApoE -/- mice (supplemented ApoE +/- plasma). Likewise, in comparison trials, Candida blastoconidia were also incubated in the presence of ApoE -/- lipoprotein-depleted plasma, ApoE +/- lipoprotein-depleted plasma, and the lipoprotein-depleted plasma obtained from supplemented ApoE +/- plasma. As a control, Candida blastoconidia were grown in Sabouraud broth diluted 1:1 in PBS. Also, to assess whether ApoE could directly influence C. albicans growth, C. albicans blastoconidia were
incubated in the presence of 10, 1, or 0.1 µg/ml recombinant human ApoE4 in Sabouraud broth. The growth of *C. albicans* in the presence of the various factors was established after 2, 4, 8 and 24 h incubation in 96-well flat bottom plates (final volume 200 µl/well) at 37°C in 5 % CO₂. After incubation, the contents of the wells were harvested as described above, and serial dilutions were plated on Sabouraud agar. The colonies of *C. albicans* were enumerated after incubation at 37°C for 48h.

**Statistical analysis**

The Kaplan-Meyer log rank test was used to analyze survival data. Differences in cytokine concentrations, PMN recruitment, superoxide production, and outgrowth of micro-organisms in the organs or plasma were analyzed using the Mann-Whitney *U*-test. Differences in lipid concentrations were analyzed with the Students’ *t*-test. Differences between groups were considered significant at p < 0.05.
Results

Cholesterol and triglyceride concentrations

Plasma cholesterol concentrations were eight- to nine fold higher in uninfected ApoE -/- mice than in ApoE +/- animals (Table 1). These elevated concentrations were due to high cholesterol concentrations in the VLDL + IDL fraction and, to a lesser extent, increased LDL cholesterol concentrations. In contrast, HDL cholesterol concentrations were significantly lower in ApoE -/- mice than in controls. Plasma triglyceride concentrations were two-fold higher in ApoE -/- mice due to an elevation in the VLDL + IDL fraction (Table 1). The VLDL like particles of the ApoE -/- mice were cholesterol-rich and triglyceride-poor, in contrast to VLDL from ApoE +/- mice [4].

Table 1. Plasma and lipoprotein cholesterol and triglyceride concentrations in uninfected apolipoprotein E (ApoE) -/- mutant mice and ApoE +/- control mice.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ApoE -/-</td>
<td>ApoE +/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ApoE -/-</td>
</tr>
<tr>
<td>Plasma</td>
<td>16.14 ± 3.73</td>
<td>1.87 ± 0.15</td>
</tr>
<tr>
<td>VLDL</td>
<td>10.2 ± 1.14</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>LDL</td>
<td>0.60 ± 0.06</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>HDL</td>
<td>0.47 ± 0.10</td>
<td>1.04 ± 0.10</td>
</tr>
</tbody>
</table>

Results are given as mean ± SD for 30 animals per group. Significant differences between ApoE +/- and ApoE -/- mice are indicated as follows: *p<0.01, **p<0.001, ***p<0.0001. HDL, high-density lipoproteins; LDL low-density lipoproteins; VLDL very low-density lipoproteins.

C. albicans infection

Death occurred after day 5 of infection in mice administered 5 x 10^5 C. albicans blastoconidia/mouse. Mortality was significantly higher in ApoE -/- mice (86%) than in ApoE +/- mice (52%; p < 0.005; Fig. 1). Infection with a lower amount of C. albicans (2 x 10^3 blastoconidia/mouse) led to a similar difference in mortality between ApoE -/- and ApoE +/- mice (data not shown).

To examine whether the increased mortality in Apo E -/- mice could be explained by an increased outgrowth of C. albicans in the organs, animals were sacrificed on day 1, 3 and 7 after infection and the liver and kidneys were removed to assess fungal outgrowth. In both groups, the number of cfu recovered from the kidneys continued to increase from day 1 through day 7 of infection. On day 1 and 3, the outgrowth of C. albicans in the kidneys of ApoE -/- mice was significantly greater than in those of ApoE +/- mice (p <
0.05 and p < 0.001, respectively; Fig.2). On day 7, a similar trend was observed. No difference in outgrowth from the liver was observed between groups at any time (data not shown). Circulating cytokine concentrations were measured on day 1, 3 and 7. IL-1α was not detectable in the circulation at any time. TNFα concentrations were detectable on day 3 of infection only. The concentration observed in ApoE -/- mice (0.25 ± 0.21 ng/ml) did not differ significantly from that found in ApoE +/+ mice at the same time (0.18 ± 0.18 ng/ml; p > 0.05).

**Figure 1.** Survival of ApoE -/- (dashed line) and ApoE +/+ (solid line) mice after i.v. injection of 5 x 10⁵ cfu C. albicans per mouse. The figure shows pooled data from 2 experiments with at least 13 animals per group per experiment. p < 0.005 ApoE -/- vs. ApoE +/+ mice.

**Figure 2.** Outgrowth of C. albicans from the kidneys of ApoE -/- (■) and ApoE +/+ mice (▲) assessed on day 1, 3 or 7 after an i.v. injection of 5 x 10⁵ cfu of C. albicans. Results are pooled data of 3 experiments with 6 animals/group and presented as mean ± SD (a p < 0.05; b p < 0.005).

**Recruitment of PMN**

To assess whether the increased outgrowth of C. albicans in the kidneys of ApoE -/- mice could be due to differences in the numbers of granulocytes, which are the main effector cells in disseminated candidiasis, the recruitment of PMN to an injection of heat-killed C. albicans blastoconidia into the peritoneal cavity was investigated in ApoE -/- and
ApoE +/+ mice. No significant difference could be detected at any time between the numbers of PMN recovered from the peritoneal lavage fluid of the two mouse strains (at 2h counts were $5.91 \pm 1.97 \times 10^6/\text{ml}$ in ApoE -/- vs. $7.70 \pm 2.02 \times 10^6/\text{ml}$ in ApoE +/+; at 4h, counts were $11.98 \pm 2.67 \times 10^6/\text{ml}$ in ApoE -/- vs. $12.94 \pm 2.31 \times 10^6/\text{ml}$ in ApoE +/+ mice; $p > 0.05$).

**Phagocytosis and intracellular killing of *C. albicans* blastoconidia by murine phagocytes**

To characterize granulocyte function in the mouse strains, the capacity to internalize and kill *C. albicans* blastoconidia was quantified. PMN obtained from ApoE -/- mice phagocytosed numbers of *C. albicans* blastoconidia ($61.3 \pm 4.3 \%$) similar to the numbers taken up by PMN from ApoE +/+ mice ($60.7 \pm 5.7 \%$; $p > 0.05$). The number of *C. albicans* blastoconidia killed by ApoE -/- PMN ($84.0 \pm 4.1\%$) did not differ from the number killed by PMN of ApoE +/+ mice ($82.0 \pm 3.3\%$; $p > 0.05$). Exudate macrophages of ApoE -/- mice were able to phagocytose and kill *C. albicans* blastoconidia to the same extent as macrophages of ApoE +/+ mice did ($p > 0.05$; data not shown). To assess whether the established intracellular killing could be explained by decreased viability of *C. albicans* blastoconidia, *C. albicans* growth from phagocyte-free wells was quantified after 3 h of incubation. The numbers of cfu recovered from wells containing *C. albicans* alone remained unaltered when gauged against baseline counts (data not shown). Microscopic evaluation of the wells showed no hyphal growth.

**PMN-mediated hyphal damage**

In addition to making an assessment of the intracellular killing capacity of granulocytes, we also determined their extracellular killing capacity by means of a tetrazolium dye assay quantifying hyphal viability. The damage of *C. albicans* hyphae mediated by PMN from ApoE -/- mice ($45.9 \pm 4.4 \%$) did not differ significantly from that brought about by PMN from ApoE +/+ mice ($46.1 \pm 2.1 \%$; $p > 0.05$).

**Superoxide production**

The capacity to produce superoxide in response to PMA stimulation was similar for exudate peritoneal PMN of ApoE -/- mice ($12.9 \pm 0.86 \times 10^4 \text{ cps}$) and ApoE +/+ mice ($13.3 \pm 2.7 \times 10^4 \text{ cps}$, $p > 0.05$).
Growth of *Candida albicans* in various types of plasma or in the presence of ApoE4

To determine whether the increased susceptibility of the ApoE -/- mice could, at least in part, be explained by the hyperlipoproteinemia of ApoE -/- mice, growth of *C. albicans* in the presence of murine plasma was assessed in vitro. At 4 h of incubation, *C. albicans* outgrowth was significantly higher in ApoE -/- plasma than in ApoE +/+ plasma (Fig. 3; p < 0.05). However, plasma could be seen to function in the inhibition of candidal growth when *C. albicans* counts from plasma were compared with counts from a mixture of Sabouraud and PBS (Fig. 3; p < 0.05). In addition, enrichment of ApoE +/+ plasma by the isolated circulating lipoprotein fraction from ApoE -/- mice significantly increased outgrowth of *C. albicans* in ApoE +/+ plasma (Fig. 3).

![Figure 3](image_url)  
*Figure 3.* In vitro growth of *C. albicans* expressed as log cfu in Sabouraud broth supplemented with 50 % (v/v) various plasma sources after incubation at 37 °C for 4 hours (solid bars), as well as a control mixture of PBS in Sabouraud medium (50 % v/v; open bars). Data are expressed as mean ± SD of 4 samples obtained from 2 experiments. 'Lipoprotein-supplemented' stands for the addition of lipoproteins obtained from ApoE -/- plasma.

- p < 0.05 compared with growth in ApoE +/+ plasma.
- p < 0.05 lipoprotein-deleted plasma compared with non-lipoprotein-depleted plasma.
- p < 0.05 for comparison with Sabouraud medium mixed with PBS.

To determine whether this effect was caused by lipoproteins serving as nutrients or by the binding and inactivation of circulating candidacidal factors, growth of *C. albicans* was compared in lipoprotein-depleted plasma of ApoE -/- and ApoE +/+ mice, as well as ApoE +/+ plasma that had been supplemented with the ApoE -/- lipoprotein fraction before the depletion procedure was carried out. *C. albicans* growth was significantly enhanced in all
lipoprotein-depleted plasma samples, irrespective of the mouse strain involved. Supplementation of ApoE +/+ plasma with lipoproteins prior to their removal tended to lead to a further increase in C. albicans growth (Fig. 3). Similar results were observed after 8 and 24 h incubation (data not shown). This suggests that, in addition to lipids themselves serving as nutrients, extracellular candidacidal factors are bound to, and extracted together with lipoproteins during the depletion procedure.

To assess whether ApoE could directly inhibit C. albicans growth, blastoconidia were cultured in the presence of increasing amounts of recombinant human ApoE4 up to 10 µg/ml. No inhibitory effect was detected after 4 h incubation in 10 µg/ml ApoE4 (3.29 ± 0.06 x 10⁶ cfu vs. 3.26 ± 0.06 x 10⁶ cfu for control growth; p > 0.05). Similar results were obtained after 24 hours of incubation (data not shown).

Discussion

Our study showed that ApoE -/- mice exhibit a susceptibility to acute disseminated candidiasis that exceeds that of ApoE +/+ mice. Both mortality levels and outgrowth of C. albicans blastoconidia from kidney macerates became elevated in ApoE -/- mice. The observed increased susceptibility of ApoE -/- mice appears to be the result in part of an increased growth of C. albicans in plasma due to lipids serving as nutrients. In addition, the effect seen was caused in part by the neutralizing of candidacidal plasma factors by elevated lipoprotein levels. No parallel neutralizing effect was observed for ApoE itself.

The major reason for the enhanced susceptibility of ApoE -/- mice is the significantly increased growth of C. albicans blastoconidia in the hyperlipidemic plasma of these mice, as compared to growth in plasma of ApoE +/+ mice. Our results are similar to those obtained in LDL receptor-deficient (LDLr -/-) mice, which were also more susceptible to C. albicans infection than controls [18]. It is hypothesized that the excess of lipoproteins influences the growth of C. albicans blastoconidia either by serving as a nutrient [2,14,17] or by binding and inactivating plasma candidacidal factors, such as the calprotectin complex [16], sphingosine [19], or platelet microbicidal protein [29]. Fresh plasma inhibits fungal reproduction (Fig. 3), pointing to the presence of candidacidal factors in plasma, as has been described earlier [1,8]. An increased amount of lipoproteins in plasma enhanced the outgrowth of C. albicans, as was demonstrated by the increased outgrowth of C. albicans blastoconidia from ApoE -/- plasma. This is in agreement with our earlier findings that all lipoprotein fractions enhance growth of C. albicans in vitro [17]. Likewise, C. albicans growth was enhanced in ApoE +/+ plasma supplemented with the lipoprotein fraction obtained from ApoE -/- plasma. Lipoprotein depletion of plasma...
increased *C. albicans* growth even further. This suggests that candidacidal factors are neutralized by lipoproteins. When additional lipoproteins were added to ApoE +/- plasma before the depletion procedure, *C. albicans* growth was further enhanced, suggesting an augmented neutralization of candidacidal factors.

As the lipoproteins added were obtained from ApoE +/- mice and thus did not contain ApoE, the neutralization of candidacidal factors is not due to ApoE, and a direct candidacidal effect of ApoE can be excluded. In correlation with this, *C. albicans* growth was seen not to be affected by the addition of recombinant human ApoE4. Therefore, the reduced growth of *Candida* in ApoE +/- plasma is not due to a direct effect of ApoE. An earlier study has shown that *C. albicans* grew significantly better in lipoprotein-depleted plasma from mice lacking LDL receptors than in normal murine plasma [17]. In the present study, however, no significant difference in yeast outgrowth was observed in lipoprotein-depleted plasma from ApoE +/- and ApoE +/- animals, a finding that contrasted with the significant differences seen with non-depleted plasma samples from these mouse lines. In LDLr +/- mice, the major lipoprotein in the circulation is LDL, whereas VLDL is the main circulating lipoprotein in ApoE +/- mice. Again, this suggests that ApoE does not mediate the neutralizing of candidacidal factors. Lipoproteins other than VLDL appear to be able to bind and neutralize candidacidal factors in plasma, independent of ApoE availability.

Although ApoE +/- mice had already significantly elevated fungal burdens in their kidneys on day 1 of infection, differences in mortality between the two test groups were only observed after 1 week of infection. As has been described previously, the early differences in outgrowth during disseminated candidiasis subsequently lead to differences in mortality. Mortality is due to kidney failure [10,12].

Plasma factors and PMN are the most important host defence components in the initial stage of disseminated candidiasis. The outgrowth of *Listeria monocytogenes* was increased in ApoE +/- mice during the early stage of infection, presumably due to an impaired granulocyte defect [22]. Moreover, granulocytes pre-incubated with lipid emulsions showed an impaired intracellular killing capacity of *C. albicans* blastoconidia [28]. Therefore, the possibility was investigated that neutrophil function might be impaired in ApoE +/- mice, enhancing their susceptibility. However, the recruitment, as well as intra- and extracellular killing rates of PMN from ApoE +/- mice were similar to those of ApoE +/- mice. This excludes impaired granulocyte function as the cause of the increased susceptibility of ApoE +/- mice to acute disseminated candidiasis.
It has been shown that ApoE regulates cell-mediated immunity. This is evidenced by a decrease in the delayed-type hypersensitivity response of ApoE -/- mice [11], a phenomenon that may lead to impaired macrophage function at the site of infection. However, cell-mediated immunity does not play a significant role in the host defences to disseminated candidiasis [10]. Moreover, we have previously shown that the in vitro cytokine production capacity of ApoE -/- macrophages is not increased [4], and in the present study, the circulating pro-inflammatory cytokine concentrations were not significantly different in ApoE -/- mice from that in controls. Taken together, the results of the present study do not suggest a major immunomodulatory effect of ApoE mediated via cellular mechanisms.

In conclusion, this study demonstrates that increased (ApoE-deficient) VLDL levels have deleterious effects on the outcome of disseminated candidiasis. In addition to lipids themselves serving as nutrients for *C. albicans*, lipoproteins neutralize serum candidacidal factors. Hyperlipoproteinemia is deleterious for host defence against candidiasis.

**Acknowledgement**

The authors wish to thank Ineke Verschueren for cytokine determinations, Heidi Jacobs and Helga Toenhake for lipoprotein isolation, and Maichel van Riel and Bianca Lemmers for their assistance with the animal experiments.
References


26. Vogels MT, Mensink EJ, Ye K et al. Differential gene expression for IL-1 receptor antagonist, IL-1, and TNF receptors and IL-1 and TNF synthesis may explain IL-1-induced resistance to infection. J Immunol 1994; 153:5772-80.


Delayed clearance of intra-abdominal abscesses caused by *Candida albicans* in tumour necrosis factor-α and lymphotoxin-α deficient mice

Aline G. Vonk\textsuperscript{1,3}, Mihai G. Netea\textsuperscript{1,3}, Johan H. van Krieken\textsuperscript{2}, Jos W.M. van der Meer\textsuperscript{1,3}, Bart-Jan Kullberg\textsuperscript{1,3}

Departments of Medicine, Division of General Internal Medicine\textsuperscript{1} and Pathology\textsuperscript{2}, University Medical Centre Nijmegen, and Nijmegen University Centre for Infectious Diseases\textsuperscript{3} Nijmegen, the Netherlands

*Journal of Infectious Diseases* 2002; 186: 1815-1822
© The university of Chicago press
Abstract

The role of endogenous tumour necrosis factor-α (TNF) and lymphotoxin-α (LT) in a model of intra-abdominal Candida sepsis and abscess formation was investigated. A significantly higher number of abscesses was observed in TNF/LT double knockout (TNF\(^{-/-}\)LT\(^{-/-}\)) mice compared to wild-types (TNF\(^{+/+}\)LT\(^{+/+}\)). The outgrowth of Candida in abscesses of TNF\(^{-/-}\)LT\(^{-/-}\) mice was 10-fold increased on day 14, and 60-fold increased on day 21 of infection. The diminished host resistance was explained by an impaired extracellular killing capacity of granulocytes and a delayed development of a Th1 response in TNF\(^{-/-}\)LT\(^{-/-}\) mice. The IL-10/IFN\(\gamma\) ratio as measured in the supernatants of stimulated splenocytes, shifted from 131 for TNF\(^{-/-}\)LT\(^{-/-}\) mice and 13.9 for TNF\(^{+/+}\)LT\(^{+/+}\) mice on day 8, to 0.11 for TNF\(^{-/-}\)LT\(^{-/-}\) mice and 11.66 for TNF\(^{+/+}\)LT\(^{+/+}\) mice on day 14 of infection. In conclusion, TNF and LT are critical to the stimulation of effector cells consequently leading to elimination of Candida from abscesses.
Introduction

Tumour necrosis factor-α (TNF) and lymphotoxin-α (LT) are members of the TNF-superfamily [4]. Both ligands bind with similar affinity to the p55 and p75 TNF receptor, inducing the same biological responses [4,13]. Overproduction of TNF and the release of high amounts into the circulation induce a systemic inflammatory reaction resulting in systemic hypotension, multi organ failure, and eventually death [19]. In contrast, small amounts of TNF produced locally at the site of infection are important for mounting a proper host response to the invading micro-organism.

The role of TNF in systemic intravascular models of bacterial infection is significantly different from that in localized infections. Anti-TNF antibodies offer protection against a lethal intravenous bacterial challenge, whereas neutralization of endogenous TNF with anti-TNF antibodies has deleterious effects on the outcome of localized infection such as experimental peritonitis [3,21]. This is in agreement with the observation that administration of recombinant murine TNF, and its subsequent interaction with the p55TNF receptor, is required to mount an adequate host defence against bacterial peritonitis [7,8].

TNF and LT play a major role in host defence against disseminated candidiasis. Pharmacological agents and anti-TNF antibodies neutralizing endogenous TNF worsen the outcome of experimental disseminated candidiasis [16,20], and mice lacking the genes encoding for TNF and LT are highly susceptible to systemic Candida albicans infection [15,18]. Furthermore, evidence exists that the systemic use of monoclonal antibodies against TNF for treatment of patients with an inflammatory bowel disease might increase the risk of invasive fungal infections [25]. The mechanisms through which neutralization of endogenous TNF increases the susceptibility to fungal infections are incompletely understood.

To investigate the role of endogenous TNF and LT in a model of deep-seated localized Candida infection, we induced intra-abdominal abscess formation by an injection of C. albicans blastoconidia and sterile faecal content into the peritoneal cavity of TNF/LT double knockout (TNF^{-/-}LT^{-/-}) mice and their wild-type (TNF^{+/+}LT^{+/+}) littermates.
Influence of TNF on intra-abdominal C. albicans abscesses

Materials and methods

Animals
Female, 6-8 weeks old TNF$^{-/-}$LT$^{-/-}$ mice on a mixed 129/sv-C57BL/6 background in which the genes encoding for TNF-$\alpha$ and LT-$\alpha$ were replaced by a neo cassette (pMCI NeopA, Stratagene) and their wild-type littermates were used [1]. The mice were allowed to accustom to laboratory conditions for one week before experimental use. The animals were housed under specific pathogen-free conditions and were fed standard laboratory chow (Hope Farms, Woerden, the Netherlands) and water ad libitum. The experiments were approved by the ethics committee on animal experiments of the University Medical Centre Nijmegen.

Candida albicans
C. albicans (strains UC820 and ATCC 10261) was inoculated into 100 ml of Sabouraud broth and cultured for 24h at 37 °C. After three washes with pyrogen-free saline by centrifugation at 1500 x g, the number of yeast cells was counted in a hemacytometer; occasional strings of two or more yeast were counted as one cfu of C. albicans. Pyrogen-free saline was used to dilute the suspension to the appropriate concentration. The viability of the yeast was at least 98 %, as determined by plating serial dilutions on Sabouraud dextrose agar plates.

Abdominal abscess induction
Mouse faeces were ground in a tissue grinder and suspended in 0.9 % pyrogen-free saline to form a 5 % w/v mixture, and sterilized in a steam autoclave (15 min, 2 bar, 120 °C). The sterility of the preparation was confirmed by plating aliquots on blood agar plates. To induce abdominal abscess formation, mice received an intraperitoneal injection of 100 µl of sterile faecal suspension containing $5 \times 10^5$ live C. albicans cfu. Subgroups of animals were anesthetized with ether and bled from the retroorbital plexus on day 3, 8, 14 or 21 for measurement of circulating TNF$\alpha$, IL-1$\alpha$, IL-6, IFN$\gamma$ and IL-10 concentrations. At the same points in time, the abdominal cavity was explored for the presence and number of abscesses $\geq 1$ mm. All abscesses $\geq 1$ mm were removed, measured, rinsed with 70 % ethanol, and subsequently with saline to remove external micro-organisms.

Outgrowth of C. albicans
To assess the outgrowth of micro-organisms, the abscesses, left kidney, and a 10 x 10 mm sample of the peritoneum were weighed and homogenized in sterile saline in a tissue grinder. The number of viable Candida cells in the tissues was determined by plating
serial dilutions on Sabouraud dextrose agar plates, as described previously [14]. The cfu were counted after 24h of incubation at 37 °C, and expressed as log cfu/sample. To evaluate bacterial coinfection of the abscesses, aliquots were plated on blood agar. For histology, tissue samples were fixed in buffered formalin (4 %), and paraffin-embedded. Sections were stained with periodic acid-Schiff, hematoxylin-eosin or elastin von Gieson, and examined microscopically.

To assess the presence and severity of diffuse peritonitis, the number of Candida blastoconidia present in the peritoneal cavity was determined. Subgroups of mice were sacrificed on day 8, 10, 12 or 14, and the peritoneal contents were harvested by washing the peritoneal cavity with 4 ml ice-cold PBS. After centrifugation, the pellet was resuspended in 600 µl sterile water containing 0.01 % BSA. The undiluted suspension was subsequently plated on Sabouraud dextrose agar plates and the cfu were counted, as described above.

To investigate the role of polymorphonuclear neutrophils (PMN) in an abdominal Candida infection in TNF−/−LT−/− mice, subgroups of wild-type and deficient animals were rendered granulocytopenic by pre-treatment with cyclophosphamide (Bristol-Myers Squibb, Weesp, The Netherlands), as described previously [14]. Cyclophosphamide 150 mg/kg was administered s.c. 4 days before infection, followed by 100 mg/kg 1 day before infection, as well as on day 2 and 5 after i.p. injection of 100 µl sterile faecal suspension containing 5 x 10⁴ live C. albicans. Daily differential counts in peripheral blood smears confirmed that granulocytes remained < 100 x 10⁶/L throughout the infection (data not shown). The number of abscesses and outgrowth of the micro-organisms in the tissues on day 8 of infection were determined, as described above.

**Assessment of PMN-mediated hyphal damage**

Previous research conducted by our group revealed that the capacity to phagocytize Candida blastoconidia by PMN from TNF−/−LT−/− mice was significantly impaired compared with PMN from TNF+/+LT+/+ mice, whereas their capacity to kill the ingested Candida was similar [18]. The PMN-mediated damage of Candida pseudohyphae (henceforth to be referred to as hyphae) was determined by a modification of the XTT dye assay, which was previously described by Gaviria et al [11]. Candida blastoconidia (strain UC820) grown on Sabouraud agar plates were collected with a swab and suspended at a final concentration of 1 x10⁶ cfu/ml in RPMI 1640 Dutch modification (with 20mM Hepes, without glutamine, ICN Biomedicals GmbH, Eschwege, Germany) supplemented with 1 % gentamicin, 1 % L-glutamine and 1 % pyruvate, henceforth to be referred to as RPMI-dm. The pH of the suspension was adjusted to 6.4 using hydrochloric acid. Hyphae were obtained by incubating 10 ml of the suspension at 37°C for 24h. After incubation, the
hyphae were centrifuged (1800 rpm, 10 min) and resuspended in RPMI 1640 without phenol red and L-glutamine (RPMI-wp; ICN Biomedicals, Eschwege, Germany). 160µl aliquots of a suspension containing $1 \times 10^5$ hyphae were dispensed into a 24-well flat bottom plate (Costar, Corning, the Netherlands). PMN were elicited by an i.p. injection of 10 % proteose peptone. After 4h, cells were collected and resuspended in RPMI-wp, the number of PMN was determined in Giemsa stained cytospin preparations and $5 \times 10^5$ PMN (200 µl) were added to the wells containing hyphae at a final E:T ratio of 5:1, in the presence of 10 % fresh TNF+/+LT+/+ serum. Control wells contained either hyphae or PMN only. After incubation for 2h, 800 µl of sterile H2O was added to the wells and the plate was rocked at room temperature to achieve lysis of PMN. After 15 min., 800 µl sterile saline-solution containing XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma Chemical, St. Louis, MO) and coenzyme Q0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) were added to each well at a final concentration of 400 µg/ml XTT and 50 µg/ml coenzyme Q0. After 1h of incubation at 37ºC, the plate was centrifuged (1800 rpm, 10 min) and 150 µl of the supernatant of each well was transferred to a well of a 96-well microtiter plate. The absorbance of each well was measured in a spectrophotometer at 450 nm. The percentage of fungal damage was defined as $1 - ((A_{450} \text{hyphae + PMN} - A_{450} \text{PMN}) / E_{450} \text{hyphae}) \times 100$.

**Phagocytosis and intracellular killing of *C. albicans***

Exudate peritoneal macrophages from groups of five TNF-/-LT-/- and TNF+/+LT+/+ mice were elicited by an i.p. injection of 10 % proteose peptone. Phagocytes were collected in sterile tubes by washing the peritoneal cavity with 4ml ice-cold PBS containing 50 U/ml heparin 72h after injection. The cells were centrifuged (10 min; 3600 rpm; 2250 g), counted in a Bürker counting chamber and resuspended in RPMI-dm supplemented with 5 % heat-inactivated foetal calf serum.

The processes of phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes, as described previously [24]. To create a monolayer of macrophages, $5 \times 10^5$ cells in 100 µl of RPMI-dm were dispensed into the wells of a 96-well flat bottom plate (Costar, Corning BV, the Netherlands) and incubated at 37 ºC and 5 % CO2. Macrophages were allowed to adhere for 2h before gently washing the monolayers with culture medium to remove non-adherent cells. The percentage of adherence was calculated as $(1 - \text{(number of non-adherent cells} / 5 \times 10^5)) \times 100$. Subsequently, the cells were incubated with $1 \times 10^4$ cfu *C. albicans* ATCC 10261, which were opsonised for 45 min at 24 ºC in Modified Eagle’s medium (Gibco Life technologies, Paisley, Scotland; MEM), containing 2,5 % fresh mouse serum (E:T ratio, 40:1). After 15 min, supernatants were aspirated and monolayers were gently washed with
MEM to remove uningested blastoconidia. The supernatant and well washings, containing the non-ingested *Candida* blastoconidia, were combined and plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytized blastoconidia was defined as \(1 - \left(\frac{\text{number of uningested cfu}}{\text{cfu at the start of incubation}}\right)\) x 100. Killing of *C. albicans* by macrophages was assessed in the same monolayers. After removal of the non-phagocytized *Candida* blastoconidia, 200 µl of culture medium, consisting of Sabouraud in MEM (50 % v/v), was added to the monolayers. After 3h of incubation at 37 °C and 5 % CO₂, the wells were gently scraped with a plastic paddle and washed with 200 µl distilled H₂O to achieve lysis of macrophages. This procedure was repeated three times, after which the pooled washes were adjusted to a final volume of 1ml with distilled water. Microscopic examination of the culture plates showed that there was a complete removal of phagocytes. To quantify the number of viable intracellular Candida blastoconidia, tenfold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37 °C for 24h. The percentage of yeast killed by the macrophages was determined as follows: \(1 - \left(\frac{\text{cfu after incubation}}{\text{number of phagocytized cfu}}\right)\) x 100. Macrophage-free incubations of blastoconidia were included as a control for yeast viability.

**Stimulation of splenic lymphocytes**

Spleen cells were obtained by gently squeezing spleens in a sterile 200 µm filter chamber. Microscopic examination of Giemsa stained cytospin preparations showed that splenocytes consisted for 95 % of lymphocytes, 2 % monocytes and 3 % granulocytes. Splenocytes were washed and resuspended in RPMI-dm, counted in a Bürker counting chamber, and the number was adjusted to 5 x 106/ml. 1 ml of the cell suspension was stimulated with 1 x 107 heat killed Candida albicans UC820 blastoconidia (E:T ratio, 2:1). Measurement of IFNγ and IL-10 concentrations was performed in supernatants collected after 48h of incubation at 37 °C in 5 % CO₂ in 24-well plates (Greiner, Alphen a/d Rijn, The Netherlands).

**Cytokine assays**

The concentrations of TNFα and IL-1α were determined using specific radioimmunoassays as described previously [17]. The detection limit using a 100 µl sample was 40 pg/ml for TNFα and 20 pg/ml for IL-1α. IL-10, IFNγ and IL-6 concentrations were determined by a commercially available ELISA kit (Biosource Europe), according to the guidelines of the manufacturer. The detection limits were 8, 1.5, or 150 pg/ml, respectively.
Statistical analysis

Values were expressed as means ± sd. The differences between groups were analyzed by the Mann-Whitney U test. For all comparisons the level of significance was set at p < 0.05. All experiments were performed at least twice, and the data represent the average results of all experiments performed.

Results

Number of abscesses

Abdominal abscess formation was induced by the injection of 100 µl sterile faecal suspension containing 5 x 10^5 viable *C. albicans* blastoconidia into the peritoneal cavities of TNF^+/+LT^+/+ and TNF^-/-LT^-/- mice. All animals survived the acute period of infection and consistently developed abscesses, which became visible from day 3 of infection onwards. In TNF^+/+LT^+/+ mice, the maximum number of abscesses was reached on day 8 of infection, after which the number gradually decreased (Fig.1). During the first week of infection, similar numbers of abscesses were found in TNF^-/-LT^-/- mice. However, as opposed to the number of abscesses in the immunocompetent littermates, the numbers continued to increase to day 21 of infection in the TNF^-/-LT^-/- mice (TNF^-/-LT^-/- vs TNF^+/+LT^+/+ mice, p < 0.0001, Fig.1).

Histopathology of the inflammatory lesions recovered from the abdominal cavities of both groups of mice, showed that a small amount of the lesions were foreign body granulomas rather than abscesses, consisting of faecal contents surrounded by macrophages without *Candida* cells. No significant histopathological differences between abscesses recovered from both groups of mice on either day 8 or 14 of infection were detected (Fig.2).
To investigate whether the increasing number of abscesses after day 8 in TNF\(^{-/-}\)LT\(^{-/-}\) mice might be the result of local spread or due to disseminated candidiasis, the outgrowth of Candida cfu in the left kidney, peritoneal lavage fluid, and a standardized sample (1 cm\(^2\)) of the peritoneum was assessed. Only during the first week of infection the kidneys of both groups showed growth of Candida, but there was no difference between the groups (log cfu/kidney of controls vs. deficient mice on day 3: 2.68 ± 0.49 vs. 2.52 ± 0.50; day 8: 1.30 ± 1.22 vs. 0.93 ± 0.55; p > 0.05). The fungal load in the peritoneum of TNF\(^{-/-}\)LT\(^{-/-}\) mice was not significantly different compared with that of control mice (absolute
number of cfu/cm² peritoneum of controls vs. deficient mice on day 3: 149 ± 110 vs. 85 ± 35; day 8: 34 ± 29 vs. 22 ± 21; p > 0.05). On day 8 or 10 of infection, less than 25 *Candida* blastoconidia were recovered from the peritoneal lavage fluid in all mice of both groups. No cfu were recovered from peritoneal lavage fluid, kidney, or peritoneum after day 8 of infection.

**Number of *C. albicans* in abdominal abscesses**

To evaluate the fungal burden in the abscesses of TNF+/-LT+/- and TNF+/-LT-/- mice, all abscesses larger than 1 mm were homogenized and plated on Sabouraud dextrose agar plates. No other micro-organism than *C. albicans* was recovered from the abscesses.

The course of the number of *Candida* cfu in the abscesses is depicted in Fig. 3. During the first week of infection, the numbers of *Candida* cells in the abscesses decreased over time. No differences were found between the fungal load in TNF+/-LT+/- and TNF+/-LT-/- mice at day 3 and 8 of infection. From day 8 to 21 of infection, the outgrowth of *C. albicans* in the abscesses of TNF+/-LT+/- mice continued to decrease, whereas the numbers of cfu increased after an initial reduction in TNF-/-LT-/- mice. Quantification of yeast cells recovered from abscesses of TNF+/-LT-/- mice revealed significantly larger amounts of *Candida* blastoconidia, compared with those of TNF+/-LT+/- mice on day 14 and 21 of infection (P < 0.0001, Fig.3).

![Figure 3](image_url)

**Figure 3.** The number of *C. albicans* cfu in intra-abdominal abscesses per mouse. The amount of *Candida* cfu recovered from abscesses of TNF-/-LT-/- mice (○) was significantly increased compared to that from abscesses of TNF+/-LT+/- mice (●) on day 14 and 21 of infection **p < 0.0001.**
Circulating cytokine concentrations

During the entire experiment, circulating concentrations of TNF, IL-1α, or IFN-γ remained below the detection limit in TNF+/+LT+/+ mice. IL-6 appeared in the circulation on day 3 of infection only and the amounts were similar for both groups (197 ± 75 pg/ml in TNF−/−LT−/− vs. 180 ± 68 pg/ml in TNF+/+LT+/+ mice; p > 0.05). In TNF−/−LT−/− mice, IL-1α was undetectable at all time points tested. The systemic concentration of IFN-γ in TNF−/−LT−/− mice was significantly higher compared with TNF+/+LT+/+ mice on day 21 (Table 2.; p < 0.05). The circulating IL-10 concentration was significantly increased in TNF−/−LT−/− mice compared with TNF+/+LT+/+ mice on 8, 14 and 21 of infection (Table 1; p < 0.05).

Table 1. Circulating cytokine concentrations of TNF−/−LT−/− and TNF+/+LT+/+ mice at various time points after i.p. injection of 100µl sterile faecal suspension containing 5 × 10^4 viable C. albicans.

<table>
<thead>
<tr>
<th></th>
<th>IL-10 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF−/−LT−/−</td>
<td>TNF+/+LT+/+</td>
</tr>
<tr>
<td>day 3</td>
<td>47 ± 64</td>
<td>20 ± 25</td>
</tr>
<tr>
<td>day 8</td>
<td>195 ± 266 *</td>
<td>&lt; 8</td>
</tr>
<tr>
<td>day 14</td>
<td>49 ± 84 *</td>
<td>&lt; 8</td>
</tr>
<tr>
<td>day 21</td>
<td>141 ± 180 *</td>
<td>26 ± 22</td>
</tr>
</tbody>
</table>

Data are expressed as means ± sd of 10 mice per group obtained from 3 experiments. * p < 0.05 compared with circulating cytokine concentrations of TNF+/+LT+/+ mice.

Cyclophosphamide treatment

To investigate the role of TNF and LT on granulocytes in this model of intra-abdominal sepsis, subgroups of TNF+/+LT+/+ and TNF−/−LT−/− mice were rendered granulocytopenic by cyclophosphamide treatment and were injected i.p. with 100 µl sterile faecal suspension containing 5 × 10^4 viable C. albicans blastoconidia. Enumeration of Candida cfu recovered from abscesses on day 8 of infection showed that the absence of granulocytes resulted in significantly enhanced outgrowth (Table 2). Similar to the findings in nongranulocytopenic mice, the abscesses of neutropenic TNF−/−LT−/− mice contained significantly more Candida cfu than the abscesses of neutropenic TNF+/+LT+/+ mice did (p < 0.05; Table 2). Granulocytopenia resulted in dissemination of the infection, as shown by the presence of Candida cfu in the kidneys and the standardized sample of the peritoneum. At these sites, the numbers of Candida blastoconidia were also significantly greater in TNF−/−LT−/− mice (p < 0.05; Table 2).
Table 2. Outgrowth of *C. albicans* on day 8 of infection in abscesses of TNF<sup>+/+</sup>LT<sup>+/+</sup> and TNF<sup>-/-</sup>LT<sup>-/-</sup> mice after i.p. injection of 100µl sterile faecal suspension containing 5 x 10<sup>4</sup> viable *C. albicans*.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Pre-treatment</th>
<th>Number of abscesses</th>
<th>C. albicans outgrowth (log cfu in abscesses)</th>
<th>Number of cfu/kidney</th>
<th>Number of cfu/cm&lt;sup&gt;2&lt;/sup&gt; peritoneum</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF&lt;sup&gt;+/+&lt;/sup&gt;LT&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Saline</td>
<td>3 ± 1</td>
<td>2.29 ± 0.41</td>
<td>0</td>
<td>1.1 ± 1.5</td>
</tr>
<tr>
<td>TNF&lt;sup&gt;-/-&lt;/sup&gt;LT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Saline</td>
<td>4 ± 1</td>
<td>3.35 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>2.0 ± 1.3</td>
</tr>
<tr>
<td>TNF&lt;sup&gt;+/+&lt;/sup&gt;LT&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Cyclophosphamide</td>
<td>4 ± 1</td>
<td>4.69 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>127 ± 114&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF&lt;sup&gt;-/-&lt;/sup&gt;LT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Cyclophosphamide</td>
<td>5 ± 2</td>
<td>5.79 ± 0.33&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>712 ± 692&lt;sup&gt;b&lt;/sup&gt;</td>
<td>405 ± 266&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as means ± sd of 5 (TNF<sup>-/-</sup>LT<sup>-/-</sup>) or 4 (TNF<sup>+/+</sup>LT<sup>+/+</sup>) mice per group. <sup>a</sup> indicates a significant difference between TNF<sup>-/-</sup>LT<sup>-/-</sup> and TNF<sup>+/+</sup>LT<sup>+/+</sup> mice (p<0.05). <sup>b</sup> indicates a significant difference between granulocytopenic and non-granulocytopenic mice (p<0.05).
PMN-mediated hyphal damage

Since granulocytes contributed to host defence against *Candida* in this intra-abdominal model, their extracellular killing capacity was determined. The damage of *C. albicans* hyphae mediated by PMN from TNF<sup>+/+</sup>LT<sup>+/+</sup> mice was significantly less compared with that by PMN from TNF<sup>−/−</sup>LT<sup>−/−</sup> mice (Fig. 4; p < 0.0001).

**Figure 4.** Percentage of PMN-mediated hyphal damage. Exudate PMN obtained from TNF<sup>−/−</sup>LT<sup>−/−</sup> mice were significantly impaired in their capacity to damage *C. albicans* hyphae compared with PMN of TNF<sup>+/+</sup>LT<sup>+/+</sup> mice (p < 0.0001).

**Phagocytosis and killing of Candida blastoconidia**

To assess whether the capacity of macrophages deficient for TNF and LT to ingest and kill *Candida* blastoconidia was impaired, the percentage of phagocytosis and intracellular killing was determined in a monolayer of macrophages. No significant difference was found in either the capacity to ingest *Candida* by TNF<sup>+/+</sup>LT<sup>−/−</sup> macrophages (40 ± 17 %) compared with those of TNF<sup>+/+</sup>LT<sup>+/+</sup> mice (47 ± 16 %; p > 0.05), or to kill *Candida* by macrophages from TNF<sup>+/+</sup>LT<sup>−/−</sup> mice (82 ± 17 %) compared with TNF<sup>+/+</sup>LT<sup>+/+</sup> macrophages (86 ± 8 %; p > 0.05).
**Splenocyte stimulation**

To assess whether a dysbalance in Th1/Th2 cytokines may have led to an enhanced outgrowth of *Candida* blastoconidia from the abscesses of TNF<sup>−/−</sup>LT<sup>−/−</sup> mice, 5 x 10<sup>6</sup> splenic lymphocytes obtained on day 8 or 14 of infection were stimulated with 1 x 10<sup>7</sup> heat killed *C. albicans* cfu in vitro. On day 8 of infection, when the *Candida* load was similar in both groups of mice, lymphocytes of TNF<sup>−/−</sup>LT<sup>−/−</sup> mice produced hardly any IFNγ, whereas those of TNF<sup>+/+</sup>LT<sup>+/+</sup> mice produced a significantly larger amount (Fig. 5a, p < 0.0001). No significant differences in IL-10 production were observed. The IL-10/IFNγ ratio was 9-fold higher for TNF<sup>−/−</sup>LT<sup>−/−</sup> compared with TNF<sup>+/+</sup>LT<sup>+/+</sup> lymphocytes (131 vs. 13.9).

On day 14, when the *Candida* burden was significantly higher in TNF<sup>−/−</sup>LT<sup>−/−</sup> than in TNF<sup>+/+</sup>LT<sup>+/+</sup> mice, the opposite effect was found. At this time point, larger amounts of IFNγ were produced by TNF<sup>−/−</sup>LT<sup>−/−</sup> lymphocytes (Fig. 5c, p < 0.0001), whereas the IL-10 production was low in both mouse strains. A 100-fold higher IL-10/IFNγ ratio was found for TNF<sup>−/−</sup>LT<sup>−/−</sup> lymphocytes compared with TNF<sup>−/−</sup>LT<sup>−/−</sup> lymphocytes on day 14 (11.66 vs. 0.11). These results indicate that the development of a Th1 response is delayed in TNF<sup>−/−</sup>LT<sup>−/−</sup> mice during intra-abdominal *C. albicans* abscess formation.
Figure 5. In-vitro cytokine production in response to heat killed *Candida* blastoconidia (10^7/ ml) by splenic lymphocytes obtained on day 8 (upper panel) or 14 (lower panel) of infection.* indicates a significant difference between TNF^-/-LT^-/- and TNF^+/+LT^+/+ mice (p < 0.0001). No significant difference for IL-10 production between groups was observed on either day of infection. Results are expressed as mean ± sem from at least 8 samples per group.
Discussion

The main conclusion of the present study is that TNF and LT are necessary for host defence against intra-abdominal Candida abscesses. Two factors contributed to the reduced host resistance of TNF<sup>−/−</sup>LT<sup>−/−</sup> mice. First, the granulocytes of these mice had a significantly reduced extracellular killing capacity for C. albicans hyphae. Secondly, the absence of TNF and LT in TNF<sup>−/−</sup>LT<sup>−/−</sup> mice resulted in a delayed Th1 response, as was shown by the significantly increased IL-10/IFNγ ratio during the first week of infection. This delayed Th1 response is likely to lead to a deficient stimulation of effector cells, resulting in a significantly increased fungal burden in the abscesses.

The complex host response of abscess formation consists of various processes, such as recruitment and accumulation of granulocytes at the site of infection, deposition of fibrin, activation of the complement cascade and procoagulant activity, fibroblast growth, collagen production, and the accumulation of monocytes and their subsequent transition to tissue macrophages. An important function of TNF and LT is containment of the infection through activation of endothelial cells, resulting in expression of the vascular adhesion molecules, ICAM-1 and E-selectin, which are important for the recruitment of neutrophils and monocytes to the site of infection [6].

Previous studies have demonstrated that knock out mice lacking TNF, LT, or the p55TNF receptor, as well as mice treated with anti-TNF antibodies, have an impaired host defence against mycobacteria [5,9], bacterial peritonitis [3,7], or abscesses with B. fragilis [12]. However, our data as well as those from others [5,9,10], indicate that TNF and LT are not essential to abscess and granuloma formation, which also occurs in the absence of TNF, LT, or the p55TNF receptor. In contrast, TNF and LT play a major role in the subsequent stage of infection, i.e. the containment and clearance of abscesses through necrosis and killing of micro-organisms.

Although abscess formation was normal in TNF<sup>−/−</sup>LT<sup>−/−</sup> mice, i.e., granulocytes as well as macrophages accumulated at the site of infection, the question was raised whether the effect of TNF and LT was mediated through granulocytes in our model of intra-abdominal abscesses, as is the case in disseminated candidiasis [18]. Rendering mice granulocytopenic significantly enhanced Candida outgrowth from abscesses compared with non-granulocytopenic mice of the same group, and increased Candida dissemination and peritonitis. Likewise, it was shown that the capacity of PMN from TNF<sup>−/−</sup>LT<sup>−/−</sup> mice to damage C. albicans pseudohyphae was significantly impaired compared with PMN from TNF<sup>+/+</sup>LT<sup>+/+</sup> mice. In addition, our group has previously shown that PMN from TNF<sup>−/−</sup>LT<sup>−/−</sup> mice have a significantly reduced capacity of TNF<sup>+/+</sup>LT<sup>+/+</sup> PMN to phagocytize Candida blastoconidia compared with PMN from TNF<sup>+/+</sup>LT<sup>+/+</sup> mice [18]. Thus, the effector
function of PMN to both forms of the dimorphic yeast *C. albicans* is affected in TNF−/−LT−/− mice. In addition, the differences in outgrowth between TNF−/−LT−/− and TNF+/+LT+/+ mice occurring late during infection persisted during granulocytopenia and therefore are mediated through other cells than granulocytes. At this stage of infection, macrophages play the most important role in anticandidal defence, both by their afferent functions such as antigen presentation, and as effector cells killing *C. albicans* [2,23]. Macrophages obtained from uninfected TNF−/−LT−/− mice showed a similar capacity to ingest and kill *Candida* blastoconidia to that of TNF+/+LT+/+ macrophages, indicating a normal candidacidal function in the absence of infection. In contrast, during infection, the activation of TNF−/−LT−/− macrophages is likely to be deficient due to the delayed development of a Th1 response. TNF is an important co-stimulus for the induction of a protective Th1 response, primarily by its ability to induce IFNγ production in T cells (Netea M.G., unpublished data), and natural killer cells [22]. TNF−/−LT−/− mice are highly susceptible to disseminated candidiasis due to their incapability to mount a protective Th1-mediated immune response [15,18]. Similarly, TNF−/−LT−/− mice with *Candida* abscesses had a delayed Th1 response as shown by the IL-10/IFNγ ratio of 131 in TNF−/−LT−/− mice on day 8 of infection compared to a ratio of 13.9 in TNF+/+LT+/+ mice. This is in agreement with the delayed Th1 development observed in TNFR p55−/− mice accompanying the progression of granulomas in mycobacterial infection [9].

The delayed shift to a Th1-type of cytokine production in TNF−/−LT−/− mice leading to high IFNγ concentrations on day 14 of infection might merely be secondary to the increased outgrowth in the abscesses at later stages of the infection. Eventually, TNF−/−LT−/− mice also recovered from their infection, and on day 40 of infection hardly any abscesses were found (data not shown). Apparently, this response is mediated by cytokines other than TNF and LT (such as IL-12, IL-18, and IL-23).

In conclusion, intra-abdominal granuloma and abscess formation in response to *C. albicans* infection requires a Th1-type cytokine response. TNF and LT have a pivotal role in the elimination of *Candida* from abscesses both by directly influencing neutrophil functions and indirectly by the induction of a Th1-type response.

**Acknowledgements**

The authors wish to thank Ineke Verschueren for the determination of cytokine concentrations and Kay Poelen, Debby Smits and Maichel van Riel for their assistance with the animal experiments.
Influence of TNF on intra-abdominal C. albicans abscesses

References


Treatment of intra-abdominal abscesses caused by *Candida albicans* with antifungal agents and recombinant murine granulocyte colony stimulating factor
Abstract

The aim of the present study was to assess the influence of immunomodulation of host defence with recombinant murine granulocyte colony stimulating factor (rmG-CSF) on intra-abdominal abscesses caused by *Candida albicans*. Mice received prophylaxis or therapy with 1 µg of rmG-CSF/day in the presence or absence of antifungal treatment consisting of amphotericin B (0.75 mg/kg/d) or fluconazole (50 mg/kg/d).

The number of *Candida* cfu in abscesses was significantly reduced (p < 0.05) in mice receiving rmG-CSF prophylaxis (day –1, or day –1 through 2) compared with controls on day 8 of infection. Administration of rmG-CSF therapy alone (for 5 days starting on day 4 of infection) had no influence on the number of *Candida* cfu in abscesses. Amphotericin B treatment was significantly more effective than fluconazole treatment (3.41; 95%CI, 3.17, 3.65 vs. 3.90; 95%CI, 3.66, 4.16) log cfu/abscess; p <0.05). Therapeutic administration of rmG-CSF in conjunction with an antifungal agent showed a tendency towards a further reduction of *Candida* cfu in abscesses compared with antifungal treatment only.

In conclusion, in this experimental model of intra-abdominal *Candida* abscesses, rmG-CSF administration did not have a detrimental influence on the course of infection. Amphotericin B treatment was most effective and additional rmG-CSF therapy did not antagonize the effect of antifungal treatment. In contrast, addition of rmG-CSF therapy to antifungal treatment might further enhance the beneficial effect of the antifungal agent.
Introduction

During the past three decades, the incidence of *Candida* species as a cause of nosocomial infections has steadily increased. Colonization of the gastrointestinal tract with *Candida* is common, and gastrointestinal perforation or surgery may thus be complicated by abdominal candidiasis with abscess formation. The mortality rate among patients with abdominal candidiasis is high [4,22,23].

The polyene compound amphotericin B has been the mainstay of antifungal therapy for critically ill patients with invasive candidiasis. However, due to its severe and dose-limiting adverse effects, alternatives to amphotericin B are being used, either alone or as combination therapy. The triazole antifungal agent fluconazole has proven to be equally effective in treating candidemia as amphotericin B [19], and has become the drug of choice for treatment of candidemia and disseminated *C. albicans* infection [1,20]. Furthermore, fluconazole is considered the drug of choice in patients with peritoneal candidiasis [5], and fluconazole prophylaxis is able to prevent abdominal candidiasis in high-risk surgical patients [6]. However, despite antifungal treatment, mortality remains high and additional therapy with agents that augment host defence, such as granulocyte colony-stimulating factor (G-CSF) may be of potential therapeutic benefit.

Pretreatment with recombinant murine G-CSF (rmG-CSF) beneficially influences the course of acute disseminated candidiasis [11], or bacterial peritonitis [2,15] in mice. Combined therapy with an antifungal agent and rG-CSF has an additive effect against disseminated candidiasis in non-neutropenic mouse models, when compared with antifungal treatment alone [9,12]. However, recent findings from a randomized, double blind study have suggested that patients with intra-abdominal candidiasis treated with recombinant human G-CSF may have had a less favorable outcome compared to patients treated with fluconazole alone [14]. One of the explanations for the observed trend towards worse outcome of G-CSF treatment in intra-abdominal candidiasis may be that G-CSF down-regulates tumour necrosis factor-α production [7,11,15]. Previous research by our group has shown that the clearance of intra-abdominal abscesses caused by *C. albicans* is delayed in tumour necrosis factor-α (TNF) and lymphotoxin-α deficient mice, primarily by inducing a T helper (Th) 2 response [25].

To explore whether G-CSF application may indeed have detrimental consequences in intra-abdominal candidiasis, we assessed the effect of immunomodulation with rmG-CSF in the presence or absence of amphotericin B or fluconazole on intra-abdominal abscesses caused by *C. albicans* in mice.
Materials and methods

Animals

Female, 6-8 weeks old CBA mice were allowed to acclimatize to laboratory conditions for one week prior to infection. The animals were housed under specific pathogen-free conditions and were fed standard laboratory chow (Hope Farms, Woerden, The Netherlands) and water ad libitum. The ethics committee on animal experiments of the University Medical Centre Nijmegen had approved the experiments.

Compounds

Amphotericin B was obtained as Fungizone (Bristol-Myers Squibb, Woerden, The Netherlands), containing 50 mg of amphotericin B, 41 mg of sodium deoxycholate and 20.2 mg of sodium phosphate per vial, and was reconstituted with 10 ml of distilled water to obtain a standard solution of 5 mg/ml. Further dilutions were prepared in pyrogen-free dextrose 5%.

Fluconazole was purchased from Pfizer Nederland, as a stock solution containing 2 mg/ml. Recombinant murine G-CSF, provided by Amgen (Thousand Oaks, CA) was diluted in pyrogen-free saline to obtain a final concentration of 10 µg/ml.

Minimum inhibitory concentrations (MICs)

MICs were determined according to the M27-A microbroth dilution method as described by the National Committee for Clinical Laboratory Standards [16]. Briefly, C. albicans strain UC820 was cultured for 24 h at 35°C and resuspended in 0.9 % NaCl. The transmission was measured (530 nm) and was set between 75 % and 77 % (1-5 x 10^6 CFU/ml). The suspension was further diluted with distilled water (1:10) and RPMI-1640 containing 0.165 M 3-N-morpholinepropanesulfonic acid (MOPS; 1:100; pH, 7.0), to obtain a final suspension of 1-5 x 10^3 CFU/ml. 100 µl of the suspension were incubated for 48 h at 35°C in the presence or absence of the antifungal agents in various concentrations. The MIC of amphotericin B was defined as the lowest concentration of amphotericin B which resulted in an optically clear tube, and the MIC of fluconazole as the lowest concentration of fluconazole which resulted in a turbidity reduction of 50 % compared with that of the growth control as determined spectrophotometrically.

Abdominal abscess induction

C. albicans strain UC820 was inoculated into 100 ml of Sabouraud broth and cultured for 24h at 37°C. After three washes with pyrogen-free saline by centrifugation at 1500 x g, the number of yeast cells was counted in a hemacytometer; occasional strings of two or
more yeast cells were counted as 1 cfu of *C. albicans*. Pyrogen-free saline was used to dilute the suspension to the requested concentration. The viability of the yeast was at least 98%, as determined by plating serial dilutions on Sabouraud dextrose agar plates.

Mouse feces were ground in a tissue grinder and suspended in 0.9% pyrogen-free saline to produce a 5% w/v mixture, and sterilized in a steam autoclave (15 min, 2 bar, 120°C). The sterility of the preparation was confirmed by plating aliquots on blood agar plates. To induce abdominal abscess formation, mice received an intraperitoneal injection of 100 µl of sterile fecal suspension containing 5 × 10⁵ live *C. albicans* cfu.

**Treatment regimens**

Antifungal treatment started on day 3 of infection, using doses of fluconazole (50 mg/kg/d) or amphotericin B (0.75 mg/kg/d) that had proven to be equally effective in reducing the number of cfu in the kidneys in a series of pilot experiments of disseminated candidiasis (data not shown). Amphotericin B, fluconazole, or a combination of both agents was administered for 5 days. Amphotericin B was given as a subcutaneous (s.c.) injection once daily. Fluconazole was administered orally every 12 h (q12 h) via gavage, at a dosage of 25 mg/kg/dose. Control animals received 100 µl of sterile pyrogen-free dextrose 5% s.c., once daily.

In another set of experiments, the effect of rmG-CSF on *Candida* outgrowth from intra-abdominal abscesses was assessed. rmG-CSF was administered daily as a s.c. injection of 1000 ng/mouse (50 µg/kg) in 100 µl pyrogen-free saline. Injections of rmG-CSF were given according to one of the following three schedules: regimen A rmG-CSF prophylaxis was given once 24 h prior to infection, regimen B prolonged rmG-CSF prophylaxis was administered once daily for 4 days from day -1 through day 2 of infection, or regimen C rmG-CSF therapy was given once daily for 5 days commencing on day 4 of infection. For assessment of antifungal treatment combined with rmG-CSF on the number of *Candida* cfu in abscesses, rmG-CSF was administered according to regimen A, or C starting on day 3 of infection, either alone or in combination with amphotericin B or fluconazole, using the same doses and dosing schedules as described above.

**Outcome assessments**

The number of circulating granulocytes was determined in blood obtained from the retroorbital plexus from subgroups of mice prior to, or at different points in time during infection. On different days of infection, subgroups of animals were anesthetized with ether and bled from the retroorbital plexus for measurement of circulating TNFα, interleukin-1 alpha (IL-1α), IL-6, interferon gamma (IFNγ) and IL-10 concentrations. Thereafter, these mice were sacrificed and the abdominal cavity was explored for the
presence and number of abscesses ≥ 1 mm. All abscesses ≥ 1 mm were removed, measured, rinsed with 70 % ethanol, and subsequently with saline to remove external microorganisms.

Since it was hypothesized that rmG-CSF might alter the containment of Candida within the abscesses and lead to spread of the infection throughout the peritoneal cavity with subsequent haematogenous dissemination, we removed a 10 x 10 mm sample of the peritoneum for assessment of peritonitis, and the left kidney for assessment of Candida dissemination. The tissues were homogenized in sterile saline in a tissue grinder and Candida blastoconidia were enumerated by plating serial dilutions (abscesses, kidneys) or the complete homogenate (peritoneum) on Sabouraud dextrose agar plates, as described previously [13]. The cfu were counted after 24 h of incubation at 37°C, and expressed as log cfu/organ. Bacterial co-infection of the abscesses was evaluated by plating aliquots on blood agar. Cultures yielded no aerobic or anaerobic bacteria.

For histology, tissue samples were fixed in buffered formalin (4 %), and embedded in paraffin. Sections were stained with periodic acid-Schiff and hematoxylin-eosin and examined microscopically.

**Stimulation of splenic lymphocytes**

To determine the effect of rmG-CSF on Th cell responses, spleens of groups of 5 mice that received either rmG-CSF therapy (1000 ng once daily for 5 days) or control vehicle, were removed on day 8 of infection. Spleen cells were obtained by gently squeezing spleens in a sterile 200 µm filter chamber. Microscopic examination of Giemsa stained cytospin preparations showed that splenocytes consisted for 95 % of lymphocytes, 2 % monocytes and 3 % granulocytes. Splenocytes were washed and resuspended in RPMI-dm, counted in a Bürker counting chamber, and the number was adjusted to 5 x 10^6/ml. 1 ml of the cell suspension was stimulated with 1 x 10^7 heat killed C. albicans UC820 blastoconidia (E:T ratio, 2:1). Measurement of IFNγ and IL-10 concentrations was performed in supernatants collected after 48 h of incubation at 37 °C in 5 % CO₂ in 24-well plates (Greiner, Alphen a/d Rijn, The Netherlands).

**Cytokine assays**

The concentrations of TNFα and IL-1α were determined using specific radioimmunoassays, as described previously [17]. The detection limit using a 100 µl sample was 40 pg/ml for TNFα and 20 pg/ml for IL-1α. IL-10, IFNγ and IL-6 concentrations were determined by a commercially available ELISA kit (Biosource Europe), according to the guidelines of the manufacturer. The detection limits were 8, 1.5, or 150 pg/ml, respectively.
Statistical analysis

Values were expressed as means ± standard deviation (sd) or, in case of logarithmic data, as means and the 95 % confidence intervals. The differences between 2 groups were analyzed by the Mann-Whitney $U$ test. For these comparisons the level of significance was set at $p < 0.05$. For comparison of 3 or more groups, the data were analyzed using the Kruskal-Wallis one-way ANOVA. For post-test comparisons, the Bonferroni $t$ method was used. To ascertain reproducibility, most experiments were performed at least twice, and the data represent the average results of all experiments performed.

Results

In vitro susceptibilities and pharmacokinetics

The MIC of *C. albicans* strain UC820 was 0.25 mg/L for both amphotericin B and fluconazole. A dose of up to 1.5 mg/kg/d amphotericin B was well tolerated. No abnormalities of renal and hepatic function were detected after five days of amphotericin B administration, as measured by serum urea, creatinine, aspartate aminotransferase and alanine aminotransferase concentrations compared to mice receiving control vehicle (data not shown). Peak plasma concentrations of fluconazole (25 mg/kg) given by gavage were reached 0.5 h after administration (25.57 ± 1.36 mg/L), and trough concentrations measured before the next dose (12 h after administration) amounted 6.82 ± 0.21 mg/L. Thus, for fluconazole, time above the MIC in the circulation was 100 % of the dosing interval.

Intra-abdominal abscesses and antifungal therapy

All animals survived the acute phase of infection and consistently produced abscesses, which could be detected from day 3 of infection. No differences in the number of abscesses were observed between any groups on any day of infection (Table 1). On day 8 of infection, quantification of yeast cells recovered from intra-abdominal abscesses from amphotericin B-treated mice showed a significantly reduced outgrowth of *C. albicans* cfu compared to controls or fluconazole-treated mice ($p < 0.05$, Table 1). Fluconazole treatment did not reduce the number of cfu in the abscesses compared to controls. Combination therapy of amphotericin B with fluconazole did not further reduce outgrowth from abscesses compared to amphotericin B treatment alone. Seven days after the termination of antifungal treatment, on day 14 of infection, no differences in the number of *Candida* cfu in the abscesses were observed (Table 1).
<table>
<thead>
<tr>
<th>Agent</th>
<th>Day 3</th>
<th></th>
<th>Day 8</th>
<th></th>
<th>Day 14</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of abscesses/mouse</td>
<td>Log cfu in abscesses/mouse</td>
<td>Number of abscesses/mouse</td>
<td>Log cfu in abscesses/mouse</td>
<td>Number of abscesses/mouse</td>
<td>Log cfu in abscesses/mouse</td>
</tr>
<tr>
<td>control vehicle</td>
<td>5 ± 2</td>
<td>5.25 (95%CI, 4.67, 5.82)</td>
<td>13 ± 5</td>
<td>3.96 (95%CI, 3.70, 4.21)</td>
<td>8 ± 2</td>
<td>2.85 (95%CI, 2.61, 3.10)</td>
</tr>
<tr>
<td>amphotericin B</td>
<td>–</td>
<td>–</td>
<td>12 ± 4</td>
<td>3.41a (95%CI, 3.17, 3.65)</td>
<td>9 ± 1</td>
<td>2.75 (95%CI, 2.42, 3.09)</td>
</tr>
<tr>
<td>fluconazole</td>
<td>–</td>
<td>–</td>
<td>10 ± 4</td>
<td>3.90 (95%CI, 3.66, 4.16)</td>
<td>7 ± 2</td>
<td>2.71 (95%CI, 2.52, 2.89)</td>
</tr>
<tr>
<td>amphotericin B + fluconazole</td>
<td>–</td>
<td>–</td>
<td>10 ± 4</td>
<td>3.60 (95%CI, 3.43, 3.77)</td>
<td>6 ± 3</td>
<td>2.45 (95%CI, 2.12, 2.78)</td>
</tr>
</tbody>
</table>

The data are the cumulative results of experiments performed thrice, and expressed as means and 95% confidence intervals for at least 8 mice per group.

a p < 0.05 compared to controls or fluconazole treatment
**Immunomodulation with rmG-CSF**

At the time of intraperitoneal infection, the mean number of granulocytes in mice that received rm-GCSF 24 h earlier was $1.6 \pm 0.2 \times 10^9$/L, whereas in untreated mice the number amounted $0.8 \pm 0.1 \times 10^9$/L ($p < 0.05$). On day 3 of infection, the circulating numbers of granulocytes were unchanged in control mice ($0.8 \pm 0.1 \times 10^9$/L), whereas the numbers of granulocytes in mice that had received rmG-CSF prophylaxis ($2.2 \pm 0.6 \times 10^9$/L; $p < 0.05$), or prolonged rmG-CSF prophylaxis ($2.4 \pm 1.2 \times 10^9$/L; $p < 0.05$) were higher compared to those in controls.

On the third day of infection, mice that had received rmG-CSF prophylaxis had significantly fewer *Candida* cfu in their abscesses than untreated mice did ($p < 0.05$, Table 2). Both prophylaxis groups showed a reduced outgrowth of *Candida* cfu compared to controls on day 9 ($p < 0.05$, Table 2). Therapy with rmG-CSF that started on day 4 of infection did not influence the numbers of cfu in the abscesses on either day of infection compared to controls. The number of abscesses did not differ between the groups on any day of infection (Table 2).

Since administration of rmG-CSF might alter the containment of *Candida* cfu in the abscesses, the amount of peritonitis was determined. Although the amount of *Candida* in the peritoneum was around the detection limit, i.e. was negligible, mice that received prolonged rmG-CSF prophylaxis had a significantly larger number of *Candida* cfu/cm$^2$ peritoneum than did controls (day 3: $82 \pm 27$ vs. $37 \pm 22$, $p < 0.01$; day 9: $9 \pm 6$ vs. $2 \pm 2$ cfu/cm$^2$, $p < 0.05$; data are means ± sd of at least 10 mice per group, the experiment was performed thrice).

In addition, it was assessed whether rmG-CSF increases haematogenous dissemination of *Candida*. Again, the number of *Candida* cfu was around the detection limit at any point in time, and no differences in the number of *C. albicans* cfu, i.e. dissemination of *Candida*, in the kidneys were observed between groups (data not shown).

**Stimulation of splenic lymphocytes**

To assess whether rmG-CSF induces a Th1 or a Th2 type immune response, $5 \times 10^6$ splenic lymphocytes obtained on day 9 of infection from untreated mice and mice that received rmG-CSF therapy were stimulated with $1 \times 10^7$ heat killed *C. albicans* cfu in vitro. Lymphocytes of mice that were treated with rmG-CSF produced significantly more IL-10 ($901 \pm 304$ pg/ml) than control lymphocytes ($532 \pm 263$; $p < 0.05$). No significant differences in IFN$\gamma$ production between rmG-CSF-treated mice ($560 \pm 390$ pg/ml) and controls ($710 \pm 475$ pg/ml) was observed. The IL-10/IFN$\gamma$ ratio was 2-fold higher for mice treated with rmG-CSF ($1.9 \pm 0.58$) compared to the control group ($1.0 \pm 0.54$; $p < 0.05$).
Table 2. The effect of immunomodulation with rmG-CSF on the number of abscesses and outgrowth of *C. albicans* in abscesses on different days after i.p. injection of 100 µl sterile faecal suspension containing $5 \times 10^5$ viable *C. albicans* blastoconidia. rmG-CSF (1000 ng/d) was given as indicated.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Day 3</th>
<th>Day 9</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of abscesses/mouse</td>
<td>Log cfu in abscesses/mouse</td>
<td>Number of abscesses/mouse</td>
</tr>
<tr>
<td>control vehicle</td>
<td>4 ± 1</td>
<td>5.01</td>
<td>8 ± 3</td>
</tr>
<tr>
<td></td>
<td>(95%CI, 4.72, 5.29)</td>
<td>(95%CI, 3.64, 4.04)</td>
<td>(95%CI, 2.81, 3.56)</td>
</tr>
<tr>
<td>rmG-CSF prophylaxis (day -1)</td>
<td>6 ± 2</td>
<td>4.72^a</td>
<td>8 ± 5</td>
</tr>
<tr>
<td></td>
<td>(95%CI, 4.51, 4.93)</td>
<td>(95%CI, 3.19, 3.74)</td>
<td>(95%CI, 2.25, 3.40)</td>
</tr>
<tr>
<td>prolonged rmG-CSF prophylaxis</td>
<td>5 ± 2</td>
<td>5.01</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>(days -1 through +2)</td>
<td>(95%CI, 4.83, 5.18)</td>
<td>(95%CI, 3.15, 3.58)</td>
<td>(95%CI, 2.58, 3.32)</td>
</tr>
<tr>
<td>rmG-CSF therapy</td>
<td>–</td>
<td>–</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>(days +4 through +8)</td>
<td></td>
<td></td>
<td>(95%CI, 3.71, 4.12)</td>
</tr>
</tbody>
</table>

The data are the cumulative results of experiments performed thrice, and expressed as means and 95% confidence intervals for at least 7 mice per group. Treatment groups were compared to controls only. ^a p < 0.05, ^b p < 0.01.
**Immunomodulation combined with antifungal agents**

On day 8 of infection, amphotericin B treatment significantly reduced the number of cfu compared to controls (Fig. 1; \( p < 0.05 \)). Combination treatment of rmG-CSF therapy with fluconazole or amphotericin B was slightly more effective in reducing the number of cfu in abscesses, although the differences between mice treated with an antifungal agent alone and mice treated with the agent in combination with rmG-CSF therapy were not significant (Fig. 1B; \( p > 0.05 \)).

**Figure 1.** Effect of amphotericin B (AMB) or fluconazole (FCZ) combined with rmG-CSF on outgrowth of *C. albicans* in intra-abdominal abscesses per mouse on day 8 of infection. (A) mice received rmG-CSF on day -1 of infection. (B) mice received daily rmG-CSF on day 3 through 7 of infection. Horizontal bars indicate the means. \(^a\) \( p < 0.05 \) compared with control mice. \(^b\) \( p < 0.01 \) compared with control mice. The data are obtained from one experiment with 4 animals per group.

Histopathology of the abscesses on day 8 of infection showed that treatment with rmG-CSF either alone or in conjunction with antifungal treatment increased the number of granulocytes present in the abscesses. This was observed for both prophylactic (not shown) and to a larger extent for therapeutically administered rmG-CSF (Fig. 2). On day 15 of infection, no differences between groups were observed, neither by histopathological evaluation, nor in outgrowth (data not shown), although the combination of amphotericin B with rmG-CSF therapy (3.02; 95%CI, 2.43, 3.61 log cfu/abscesses) showed a tendency to reduce the outgrowth of *Candida* cfu from abscesses most compared to controls (3.39; 95%CI, 2.92 to 3.86); \( p > 0.05 \). No difference in the numbers of abscesses was observed between the groups at any point in time.
Mice that received rmG-CSF prophylaxis had significantly higher circulating IL-10 concentrations than controls (45.8 ± 33.6 pg/ml for rmG-CSF prophylaxis vs. 16.8 ± 1.5 for controls; p < 0.05) on day 3 of infection. No further differences in the concentrations of circulating cytokines on any day of infection were detected (data not shown).

Figure 2 (for colour printing, see cover). Histopathology of intra-abdominal Candida abscesses (x 400) in untreated mice and mice treated with rmG-CSF therapy (days 3 through 7), an antifungal agent or combination therapy on day 8 of infection. (A) Control mice showed an
inflammatory response to yeast cells with a low number of granulocytes bordered by a strong histiocytic response. (B) Mice treated with rmG-CSF had larger abscesses with an increased amount of granulocytes on a necrotic background. (C) Abscesses of amphotericin B-treated mice showed a necrotic area with disintegrating granulocytes. (D) The response of mice treated with rmG-CSF in combination with amphotericin B showed tissue necrosis, a histiocytic response, micro-abscess formation, and an increased number of granulocytes. (E) Abscesses of fluconazole treated mice revealed yeast cells in an area of necrosis with many granulocytes. (F) Abscesses of mice treated with rmG-CSF in combination with fluconazole had similar histopathologic features as those of mice treated with fluconazole alone, except for an increased number of granulocytes.

Discussion

The results of the present study indicate that modulation of host defence with rmG-CSF prophylaxis significantly reduces the number of *Candida* cfu in intra-abdominal abscesses, whereas rmG-CSF therapy (administered on days 4 through 8 of infection) had neither a beneficial nor an adverse effect. Antifungal treatment of intra-abdominal *Candida* abscesses with amphotericin B was significantly more effective than treatment with fluconazole. Addition of rmG-CSF therapy to conventional antifungal treatment did not antagonize the individual effect of the antifungal agents. In contrast, it showed a trend towards further reduction of *Candida* cfu in abscesses compared to antifungal treatment alone.

After injection of a sterile fecal suspension containing viable *Candida* blastoconidia, abscesses developed following a period of peritonitis. Administration of rmG-CSF during either of these periods had different effects on outgrowth from abscesses; rmG-CSF prophylaxis significantly reduced the amount of cfu in abscesses compared to that in abscesses of control mice, whereas administration rmG-CSF therapy induced a slight increase in the amount of cfu in abscesses compared with that in abscesses of control mice. Mice that had received rmG-CSF prophylaxis had large numbers of rmG-CSF-primed polymorphonuclear granulocytes (PMN) present at the time of infection and thus an enhanced capacity to clear the injected *Candida* blastoconidia from their abdominal cavities compared to controls, as was demonstrated by a reduced outgrowth from abscesses from these mice.

Although mice that had received prolonged rmG-GCSF prophylaxis showed an increased severity of diffuse peritonitis, the data were around the detection limit and do not seem to be of biological relevance, since it was not accompanied with an increased haematogenous dissemination of *Candida* into the kidneys. At the time of rmG-CSF therapy, which commenced not earlier than on day 3 of infection, abscesses were already
well-established, and at this point in time inflammatory cells other than granulocytes, such as macrophages and lymphocytes, gradually play a central role in host defence against *Candida* abscesses. G-CSF inhibits the TNF production by macrophages and monocytes [7,24], and has been shown to induce a Th2-type cytokine pattern via attenuation of monokine release [3,8]. Furthermore, we have previously shown that TNF is important for the clearance of *Candida* from intra-abdominal abscesses, primarily through induction of Th1 responses and enhancement of the extracellular killing capacity of granulocytes [25]. Hence, therapeutic administration of rmG-CSF was expected to have detrimental effects in this model of intra-abdominal *Candida* abscesses. However, rmG-CSF therapy had no adverse influence on the course of infection. Although the IL-10/IFNγ ratios of mice treated with rm-GCSF were significantly increased compared with controls, this may not have been of biological relevance. G-CSF not only increases the number of PMN, it also augments the expression of adhesion molecules, recruitment, and capacity to kill *Candida* blastoconidia [26-28]. These effects of G-CSF on anticandidal activity have probably compensated for the anti-inflammatory effect of G-CSF through its effect on macrophages and T-cells.

Intra-abdominal abscesses are difficult to treat, and therapy consists primarily of drainage of the infected cavity with or without antimicrobial treatment. To date, no guidelines for the use of antifungal agents for treatment of intra-abdominal candidiasis exist. In an international conference of investigators with extensive experience in the treatment of candidal infections, 60 % of the investigators would treat patients with peritoneal candidiasis with fluconazole alone and 5 % with amphotericin B lipid formulation [5]. In our model, fluconazole had no beneficial influence on the course of experimental intra-abdominal *Candida* abscesses in mice, whereas amphotericin B improved clearance of *Candida* blastoconidia from abscesses. This is in contradiction with the study of Sawyer et al., which showed that fluconazole was as effective as amphotericin B in reducing the number of *Candida* recovered from experimental intra-abdominal abscesses [21]. In that study however, fluconazole therapy was started at the time of infection, and thus represents the effect of fluconazole on *Candida* peritonitis rather than on well-established *Candida* abscesses. Indeed, fluconazole prophylaxis has proven to prevent development of abdominal candidiasis in high-risk surgical patients [6].

Simultaneous administration of combination therapy of fluconazole with amphotericin B was not as effective as amphotericin B treatment alone in reducing the number of cfu in abscesses in our experiments.
Since combination therapy of an antifungal agent with G-CSF has proven to be more efficacious than antifungal treatment alone against disseminated candidiasis [9,12] it was hypothesized that combination of rmG-CSF therapy with antifungal agents may have a synergistic effect, despite the observation that therapy with rmG-CSF alone was not effective in our model of intra-abdominal abscesses. Addition of rmG-CSF therapy to either amphotericin B or fluconazole did not significantly improve the outcome, although there was a slight tendency towards further reducing the number of *Candida* cfu per abscess. Both amphotericin B and fluconazole accumulate within PMN [18], and their concentration might have been increased by G-CSF, which has been shown to increase the intracellular concentration of antibiotics [10]. In addition, histopathological examination of abscesses obtained from mice treated with rmG-CSF showed a larger number of granulocytes than in control mice. Serving as secondary transport systems for amphotericin B or fluconazole, these cells might have contributed to achieving higher drug concentrations in the abscesses. In our experiments however, these putative effects have not led to significant synergistic effects of rmG-CSF and antifungal agents.

In conclusion, amphotericin B was effective in treating experimental intra-abdominal *Candida* abscesses, and concurrent administration of rG-CSF was suggested to further improve the outcome.

**Acknowledgements**

The authors thank Ineke Verschueren for the determination of cytokine concentrations and Geert Poelen, Theo van den Ing and Margot van den Brink for their assistance with the animal experiments.
References


Modulation of the pro- and anti-inflammatory cytokine balance by amphotericin B

Alieke G. Vonk, Mihai G. Netea, Nathalie E.J. Denecker, Ineke C.M.M. Verschueren,
Jos W.M. van der Meer, Bart-Jan Kullberg

Department of Medicine, Division of General Internal Medicine,
University Medical Centre, Nijmegen, the Netherlands

Journal of Antimicrobial Chemotherapy 1998; 42: 469-474
© Oxford university press
Abstract

Amphotericin B is an antifungal drug associated with side effects such as fever and chills, symptoms which may be mediated by pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNFα). We assessed the capacity of amphotericin B to modulate production of these pro-inflammatory cytokines as well as the anti-inflammatory IL-1 receptor antagonist (IL-1ra), induced by LPS, heat-killed Candida albicans, or Staphylococcus aureus.

The results of the present study show that amphotericin B slightly increased the production of pro-inflammatory cytokines by human mononuclear cells (PBMC), whereas the production of the anti-inflammatory cytokine IL-1ra was significantly inhibited. This results in a shift towards pro-inflammatory cytokine production, as indicated by a decreased IL-1ra/IL-1β ratio. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) indicated that levels of IL-1β and TNFα mRNA were increased.

In conclusion, amphotericin B is able to cause a shift towards pro-inflammatory cytokine production by human PBMC. This may explain the side effects, such as fever and chills, observed after treatment of patients with amphotericin B.
Introduction

The polyene compound amphotericin B is the cornerstone for antifungal therapy in critically ill patients with systemic fungal infections. Amphotericin B binds with a high affinity to ergosterol, the principal sterol of the fungal cell membrane. This binding alters membrane permeability with subsequent leakage of cations, eventually resulting in cell death [15]. Apart from this direct antifungal effect, amphotericin B can also increase the fungicidal properties of macrophages [15,17,21]. It is conceivable that the severe and dose-limiting adverse effects of amphotericin B, such as fever and chills, may be mediated at least in part through induction of pyrogenic pro-inflammatory cytokines [15].

Tumour necrosis factor (TNFα) and interleukin-1 (IL-1) are pro-inflammatory cytokines which are thought to mediate many pathophysiological events in severe infections, including fever, hypotension and multiple organ failure [16]. Contradictory data have been published regarding the capacity of amphotericin B to induce production of pro-inflammatory cytokines. Some authors have reported that amphotericin B can induce synthesis of pro-inflammatory cytokines in vitro, [4,5,7,13,24] but others have been unable to reproduce such findings [11,17,22].

There are counter-regulatory mechanisms, such as the production of anti-inflammatory cytokines, which can block the effects of pro-inflammatory cytokines. IL-1 receptor antagonist (IL-1ra) is the natural antagonist of IL-1β; its binding to IL-1 receptor blocks the effects of IL-1β stimulation. The capacity of amphotericin B to inhibit the production of this potent modulatory cytokine could be an important factor causing the above-mentioned adverse effects. An imbalance between pro-inflammatory and anti-inflammatory cytokines, expressed as the IL-1ra/IL-1β ratio, in favour of the pro-inflammatory cytokines, was recently found to be a key event in patients with type I diabetes, chronic inflammatory bowel diseases, rheumatoid arthritis and Lyme arthritis [3,6,18,20].

The aim of the present study was to investigate whether amphotericin B can stimulate pro-inflammatory cytokine production by human peripheral blood mononuclear cells (PBMC) or modulate cytokine production induced by various bacterial and fungal stimuli. We also assessed whether the balance between pro- and anti-inflammatory cytokines is modulated by amphotericin B.
Cytokine modulation by amphotericin B

Materials and methods

Reagents
Amphotericin B was purchased as Fungizone (Bristol-Myers Squibb, Woerden, The Netherlands) containing 50 mg of amphotericin B, 41 mg of sodium deoxycholate (DOC) and 20.2 mg of sodium phosphate and was dissolved in sterile, pyrogen-free dextrose 5%. The preparation contained < 0.06 endotoxin units of lipopolysaccharide (LPS) per ml as checked by a limulus amoebocyte lysate assay. LPS (Escherichia coli serotype O55:B5) was purchased from Sigma Chemical Co. (St.Louis, MO, USA). Culture medium (RPMI 1640 DM; ICN Biomedicals, Costa Mesa, CA, USA) was supplemented with 5% human serum, 1% gentamicin (50 µg/ml), 1% L-glutamine (2mM), and 1% pyruvate (1mM).

Stimulation of peripheral blood mononuclear cells
PBMC were isolated as described [9], with minor modifications. Briefly, venous blood, obtained from nine healthy volunteers, was drawn into 10 mL tubes containing 0.2 mg of EDTA (Monoject, ‘s-Hertogenbosch, The Netherlands). The PBMC fraction was obtained by density centrifugation of blood (diluted 1:1 in pyrogen free saline) over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). PBMC were containing washed twice in saline and resuspended in culture medium. The cells were counted in a Coulter counter (Coulter Electronics, Meijdrecht, the Netherlands) and the number was adjusted to 3.3 x 10^6 cells/ml.

The capacity of amphotericin B to stimulate or modulate cytokine production was assessed by incubating 150µL of the PBMC suspension (5 x 10^5 cells) for 24 h at 37 °C in 96-well plates (Greiner, Alphen a/d Rijn, The Netherlands) with amphotericin B in various concentrations (0, 0.125, 0.25, 0.5, 1 and 2 mg/L), or the corresponding vehicle, with or without LPS (1 ng/ml).

To investigate the capacity of amphotericin B to modulate cytokine production induced by other inflammatory stimuli, PBMC were incubated for 24 h at 37 °C with heat-killed (100°C, 30 min) Staphylococcus aureus (ATCC 25923), or heat-killed Candida albicans (strain UC820) at 2 x 10^6 cfu/well, in the presence or absence of amphotericin B (2 µg/ml).

Cytokine measurements
After 24 h of incubation at 37 °C in 5% CO_2 with the stimuli mentioned, supernatants were collected, and frozen (-80°C) until measurement of cytokines. The concentrations of TNFα, IL-1β and IL-1ra were determined by radioimmunoassay as described previously.
[8]. Detection limits were 20 pg/ml for TNFα, 40 pg/ml for IL-1β, and 600 pg/ml for IL-1ra.

mRNA measurement

After 4 h of incubation at 37°C in 5% CO₂ with the stimuli mentioned, the supernatants were discarded and 100 µL of RNAsol (Cinna/Biotecx Laboratories, Houston, TX, USA) were added to the adherent cells in order to isolate RNA. mRNA was isolated by the RNAsol method, as described by the manufacturer. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) (from a 0.5 µg sample RNA) was performed as described before [19]. The following primers used for the PCR reactions were: β2m, sense, 5'-CCAGCAGAGAATGGAAAGTC-3', and antisense, 5'-GATGCTGCTT-ACATGTCTCG-3'; TNFα, sense, 5'-ACAAGCCTG- TAGCCCATGTT-3', and antisense, 5'-AAAGTAGACCTGCCCAGACT-3'; IL-1β, sense, 5'GGATATGGAGCAACAAGTGG-3', and antisense, 5'-ATGTACCAG-TTGGGGAACTG-3' (Eurogenetic, Seraing, Belgium). PCR was performed (26 cycles for β2m; 30 cycles for IL-1β and TNFα) with each cycle comprising 92°C for 30 s, 55°C for 30 s, and 72°C for 90 s, using a Mastercycler 5330 (Eppendorf, Hamburg, Germany). PCR products were run on 2% agarose gels stained with ethidium bromide. The gels were scanned on a densitometer (GS-670, Bio-Rad, Veenendaal, The Netherlands) and analysed using Molecular Analyst™ software (Bio-Rad). The relative amount of TNFα and IL-1β mRNA in a sample was expressed as a ratio relative to the amount of mRNA for the housekeeping gene β2m.

Statistical analysis

Values were expressed as means ± S.E.M. Comparisons between groups were performed using the Kruskall-Wallis test. The Wilcoxon signed-rank test was used for paired values. For all comparisons the level of significance was set at P < 0.05.

Results

Modulation of cytokine production by amphotericin B

Stimulation of PBMC with amphotericin B did not influence IL-1β production, whereas TNFα production was marginally increased, and IL-1ra production slightly decreased when compared to unstimulated cells (Table I).
Table 1. Influence of amphotericin B (AmB) on cytokine production by human PBMC.

<table>
<thead>
<tr>
<th>AmB (mg/L)</th>
<th>TNFα (ng/ml)</th>
<th></th>
<th>IL-1β (ng/ml)</th>
<th></th>
<th>IL-1ra (ng/ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>lipopolysaccharide</td>
<td>control</td>
<td>lipopolysaccharide</td>
<td>control</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>0</td>
<td>0.1 ±0.1</td>
<td>3.4 ± 0.9</td>
<td>&lt; 0.04</td>
<td>1.8 ± 0.3</td>
<td>10.1 ± 0.9</td>
<td>11.0 ± 0.9</td>
</tr>
<tr>
<td>0.125</td>
<td>0.2 ±0.2</td>
<td>3.7 ± 0.9</td>
<td>&lt; 0.04</td>
<td>1.8 ± 0.3</td>
<td>9.2 ± 1.0</td>
<td>10.4 ± 1.0</td>
</tr>
<tr>
<td>0.25</td>
<td>0.2 ±0.1</td>
<td>3.6 ± 0.8</td>
<td>&lt; 0.04</td>
<td>1.7 ± 0.2</td>
<td>9.6 ± 1.8</td>
<td>11.0 ± 1.8</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2 ±0.1</td>
<td>4.4 ± 1.2</td>
<td>&lt; 0.04</td>
<td>1.9 ± 0.3</td>
<td>8.6 ± 1.0</td>
<td>9.6 ± 0.8a</td>
</tr>
<tr>
<td>1</td>
<td>0.2 ±0.1</td>
<td>3.8 ± 0.9</td>
<td>&lt; 0.04</td>
<td>2.2 ± 0.6</td>
<td>8.8 ± 1.1</td>
<td>9.6 ± 1.2</td>
</tr>
<tr>
<td>2</td>
<td>0.2 ±0.1</td>
<td>3.7 ± 1.2</td>
<td>&lt; 0.04</td>
<td>2.3 ± 0.7</td>
<td>8.5 ± 1.1</td>
<td>8.8 ± 1.3a</td>
</tr>
</tbody>
</table>

*p < 0.05 when compared with stimulation without amphotericin B (Wilcoxon signed-rank test). Data are mean ± S.E.M. (n = 9). PBMC were incubated with 0-2 mg/L amphotericin B in the presence or absence of LPS (1 ng/mL).
To assess the capacity of amphotericin B to modulate the LPS-induced production of cytokines, cells were incubated with LPS, or LPS plus amphotericin B. Amphotericin B slightly increased the LPS-induced IL-1\(\beta\) and TNF\(\alpha\) production, whereas IL-1ra production was significantly reduced at amphotericin B concentrations of > 0.25 \(\mu\)g/ml (Table I).

**Determination of cytokine mRNA**

The expression of IL-1\(\beta\) mRNA by PBMC stimulated with 2mg/L amphotericin B was not influenced (Figure 1A), whereas TNF\(\alpha\) mRNA was significantly higher than that in unstimulated cells (\(P < 0.05\), Figure 1B).

The combination of amphotericin B (2 mg/L) and LPS increased IL-1\(\beta\) mRNA (\(P <0.05\), Figure 1A) and slightly increased TNF\(\alpha\) mRNA when compared with LPS stimulation alone (Figure 1B). This strongly suggests that amphotericin B exerts its action at a transcriptional level.

**Figure 1.** (A) IL-1\(\beta\) and (B) TNF\(\alpha\) mRNA expression by human mononuclear cells incubated for 24h with dextrose, amphotericin B (AmB; 2mg/L), LPS (1 ng/mL) or LPS + amphotericin B. Data are mean ± S.E.M of nine values. Comparisons between groups were performed using the Wilcoxon signed-rank test. \(^aP < 0.05\) when compared with dextrose stimulation. \(^bP < 0.05\) when compared with LPS stimulation alone.
Because an imbalance between the pro-inflammatory IL-1β and the anti-inflammatory IL-1ra may play an important role in the development of the adverse effects of amphotericin B therapy, we calculated the IL-1ra/IL-1β ratios. The IL-1ra/IL-1β ratio was significantly reduced in the presence of amphotericin B (Figure 2).

In a separate experiment, we investigated whether the duration of stimulation was important for the effect of amphotericin B. Cells were stimulated with LPS in the presence or absence of 1 mg/L amphotericin B and supernatants were harvested after 2, 4, 8, 12 and 24 h of incubation. Cytokine production increased steadily as the duration of incubation increased. The production of TNFα an IL-1β was marginally increased, whereas IL-1ra production was decreased by amphotericin B with or without LPS stimulation (data not shown). These effects were similar at all time points tested and did not differ from those obtained with 24 h incubation.

**Table II.** Cytokine production by human PBMC after incubation with heat-killed *C. albicans* or *S. aureus* and 2 mg/L amphotericin B

<table>
<thead>
<tr>
<th></th>
<th>TNFα (ng/mL)</th>
<th>IL-1β (ng/mL)</th>
<th>IL-Ra (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>12.5 ± 7.1</td>
<td>6.4 ± 4.3</td>
<td>12.8 ± 3.8</td>
</tr>
<tr>
<td><em>C. albicans</em> + amphotericin B</td>
<td>11.5 ± 6.3</td>
<td>5.6 ± 3.4</td>
<td>10.3 ± 2.2</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>11.9 ± 6.3</td>
<td>5.1 ± 2.8</td>
<td>12.9 ± 2.3</td>
</tr>
<tr>
<td><em>S. aureus</em> + amphotericin B</td>
<td>11.1 ± 6.2</td>
<td>6.5 ± 3.9</td>
<td>10.0 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05 compared with *S. aureus* stimulation alone (Wilcoxon signed-rank test)
**Stimulation of PBMC with C. albicans or S. aureus**

To investigate whether amphotericin B can modulate cytokine production in response to inflammatory stimuli other than LPS, we assessed the cytokine production of PBMC induced by heat-killed *C. albicans* or *S. aureus* with or without amphotericin B (2 mg/L). After 24 h of incubation, no significant effect of amphotericin B on cytokine production induced by *C. albicans* was found (Table II). *S. aureus*-induced production of TNFα was not influenced by amphotericin B, whereas IL-1β was marginally increased (Table II). In contrast, IL-1ra production was significantly decreased by amphotericin B (P < 0.02, Table II). This resulted in a significantly reduced IL-1ra/IL-1β ratio (P < 0.02, Figure 3).

**Figure 3.** IL-1ra/IL-1β ratio (mean ± S.E.M.) in the supernatants of PBMC incubated for 24 h with heat-killed *S. aureus* or *C. albicans* with (hatched bars) or without (open bars) amphotericin B (2mg/L). Comparisons between groups were performed using the Wilcoxon signed-rank test. *P* < 0.05.
Discussion

In view of the side effects of intravenously administered amphotericin B, such as fever and chills, it is conceivable that the pathophysiological mechanism responsible for this phenomenon involves an imbalance in the production of pro-inflammatory and anti-inflammatory cytokines. This balance was recently found to play a key role in patients with type I diabetes, chronic inflammatory bowel diseases, rheumatoid arthritis or Lyme arthritis [3,6,18,20].

Until now, the reports on the capacity of amphotericin B to stimulate the production of cytokines in vivo have been contradictory. Several investigators have reported increased cytokine production upon amphotericin B stimulation of mononuclear cells in vitro [5,7,13,14,24], whereas others have been unable to find such an effect [11,17,22]. In order to explain this discrepancy, it is important to stress that, in the studies reporting induction of cytokines by amphotericin B, the stimulation was much lower than that obtained with a strong stimulus such as LPS [4,5,7,24]. An explanation that may account for the reported differences between studies is the source of the cells used in the studies: some studies were performed with human PBMC [11,17,22], whereas others used murine macrophages [5,7,13,14,24].

It has been reported that amphotericin B affects the production of pro-inflammatory cytokines in vivo after administration to mice [13,14]. Together with the clinical finding that amphotericin B causes fever accompanied by increased cytokine concentrations when injected into humans [2,17,17,23] and the moderate effect of amphotericin B on pro-inflammatory cytokine production by human PBMC in vitro, these findings suggest that pyrogens may be induced via an indirect mechanism in vivo after injection of amphotericin B. Interactions with plasma factors or induction of secondary mediators of cytokine induction could be involved.

Several authors have reported that amphotericin B can inhibit the cytokine production induced by C. albicans [11,14,22]. However, in two of these studies [11,14], live C. albicans with or without amphotericin B, or filtrates from live Candida pre-incubated with amphotericin B were added to cells. In both cases differential growth of the micro-organism in the presence of the antifungal drug could have been the cause of reduced cytokine production. In the study of Raponi and colleagues [22], C. albicans was pre-incubated with amphotericin B before being added to cells, and this pre-incubation might have affected the amounts of mannoproteins on the cell wall of the yeasts [1]. Since mannoprotein constituents are potent inducers of pro-inflammatory cytokines [25], it is probable that modification of the Candida cell wall by amphotericin B was responsible for
the decreased induction of cytokines. As we added heat-killed *C. albicans* and amphotericin B at the same time-point to PBMC, modulation of *Candida* growth by amphotericin B did not occur and the changes in the cell wall of the yeast may have been less significant. As a result, no effect of amphotericin B on *C. albicans*-induced cytokine production by PBMC was found.

Amphotericin B did not influence the *S. aureus*-induced production of TNFα. However, it marginally increased IL-1β production and significantly reduced IL-1ra production, resulting in a significantly reduced IL-1ra/IL-1β ratio. Unlike the cell wall of Gram-negative bacteria, the cell walls of Gram-positive bacteria like *S. aureus* do not contain LPS; instead they contain lipoteichoic acids (LTAs). The physiochemical properties of LTAs are similar to those of LPS [10,12]. Kusunoki and colleagues [12], showed that *S. aureus* LTA binds soluble CD14 and blocks binding of LPS to soluble CD14. The similarity between LPS and LTA may explain the resemblance of the PBMC response to LPS and *S. aureus* found in our study.

In conclusion, amphotericin B can cause a shift towards pro-inflammatory cytokine production by human PBMC. This may explain the side effects, such as fever and chills, observed after intravenous administration of amphotericin B to patients with systemic mycosis.

**Acknowledgement**

We thank Trees Verver and Liesbeth Jacobs for help with the cytokine assays, and Anneke Hijmans, Natasja de Bont and Louis van der Locht for helping with the mRNA assay. This work was supported in part by a grant from the Vesalius Foundation (Brussels).
Cytokine modulation by amphotericin B

References


Modulation of the Pro- and Anti-inflammatory Cytokines by Amphotericin B

Alieke G. Vonk, Mihai G. Netea, Jos W. M. van der Meer, Bart-Jan Kullberg
Division of General Internal Medicine, Department of Medicine,
University Hospital Nijmegen, the Netherlands

The Journal of Infectious Diseases 1999;180:1408–1409
© The University of Chicago press

To the Editor—We read with great interest the article by Rogers et al. [8] reporting the activation by amphotericin B of human genes encoding for the pro-inflammatory cytokines tumour necrosis factor (TNF) and interleukin (IL)–1β and for the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra). Amphotericin B is still a major component of antifungal therapy, but its use is limited by severe adverse effects such as fever, chills, and hypotension. It has been hypothesized that these adverse effects of amphotericin B treatment are mediated through induction of pyrogenic pro-inflammatory cytokines [1-3,9]. The increase of the mRNA expression of these genes reported by Rogers et al. represents a strong argument that amphotericin B induces production of cytokines through transcriptional mechanisms, and this information may prove valuable in finding strategies to combat this deleterious side effect of the drug. JID 1999;180 (October) Correspondence 1409 Recently, we also reported that amphotericin B increases the production of pro-inflammatory cytokines by human mononuclear cells, and this induction is mediated through activation of transcription of TNF and IL-1β genes [10], sustaining the results of Rogers et al. Our data not only corroborate the results of Rogers et al. that amphotericin B alone can induce TNF and IL-1β mRNA expression, but we extended these observations with data showing that amphotericin B potentiates lipopolysaccharide (LPS)–induced TNF and IL-1 mRNA expression. However, Candida albicans and Staphylococcus aureus–stimulated expression of cytokine genes were not influenced by amphotericin B [10], suggesting that induction of pro-inflammatory cytokines by different microbial stimuli is mediated through divergent pathways that are differentially modulated by amphotericin B.

Rogers et al. [6] also reported that the production of the anti-inflammatory cytokine IL-1Ra, as well as the expression of IL-1Ra mRNA, are significantly stimulated by
amphotericin B [6]. As IL-1Ra can block the IL-1 receptors and antagonize the pro-inflammatory action of IL-1, they hypothesized that this may represent a counter regulatory mechanism involved in the attenuation of the amphotericin B–related adverse effects. In contrast with the data of Rogers et al. obtained in a human monocytic cell line, we showed that amphotericin B under similar conditions causes a significant reduction of the spontaneous release of IL-1Ra by freshly isolated peripheral blood mononuclear cells (PBMC) of healthy volunteers [10]. Moreover, LPS and *S. aureus*–induced IL-1Ra production was also down-regulated by amphotericin B [10]. This inhibition results in a shift toward pro-inflammatory cytokine production, as indicated by a significantly decreased IL-1Ra/IL-1β ratio. This balance was recently found to play a key role in type I diabetes, chronic inflammatory bowel diseases, rheumatoid arthritis, and Lyme arthritis, and we propose that it also represents an important pathophysiologic mechanism mediating such adverse effects as fever and chills caused by amphotericin B treatment.

The cause of the differences in IL-1Ra induction between our study and those of Rogers et al. remains speculative. The cell types used in the experiments constitute the major difference between the two studies. In our study, we used freshly isolated PBMC, whereas Rogers et al. used the human mononuclear cell line THP-1, on which the effects of amphotericin B may be divergent. It may be hypothesized that the effects of amphotericin B on PBMC of human volunteers may closely mimic those in human subjects. The importance of the cell type used in this type of experiments is also underlined by the contradictory data in the literature regarding the capacity of amphotericin B to stimulate the production of cytokines. Several investigators have reported increased cytokine production upon amphotericin B stimulation of mononuclear cells in vitro [2,5,9], whereas others have been unable to find such an effect [4,6,7]. An explanation that may account for these differences is the source of the cells used; some studies have been done with human PBMC [3,4,6,7], and others have used murine macrophages [2,5,9]. It is also important to underline that even in the studies reporting induction of cytokines by amphotericin B, the stimulation was much lower than that obtained with a strong stimulus such as LPS [1–3,9]. Given the strong potency of amphotericin B to induce chills and fever in vivo, one may wonder whether the in vitro findings regarding cytokines explain these side effects.
Chapter 9

References


Summary and discussion
As it takes two to make a quarrel, so it takes two to make a disease, the microbe and its host [1].

The yeast Candida albicans is the primary etiologic agent of disseminated and invasive candidiasis. The incidence of disseminated and invasive candidiasis has paralleled the use of modern medical procedures that adversely affect the immune system, and highlights the difficulty of treating disseminated and invasive candidiasis. Overcoming these difficulties requires an improved understanding of host - C. albicans interactions. The most important aspect of these interactions is the host defence system. The nature and extent of the impairment of the host resistance determines the pattern of candidiasis. A better understanding of the mechanisms responsible for the defence against disseminated and invasive Candida infection is needed, in order to develop strategies aimed to enforce the anticandidal actions of the immune system. In this thesis, studies on host defence against disseminated and invasive candidiasis are described. Determination of the virulence attributes of C. albicans allowing commensalism or tissue invasion were not the subject of these studies.

The contribution of phagocytes to host defence can be assessed by determining phagocytosis and killing of Candida blastoconidia by means of the classical microbiological method of colony counting. However, these assays yield divergent results due to various methodological variations. To determine the most precise method for assessment of phagocytosis and intracellular killing of Candida blastoconidia by murine peritoneal phagocytes, microbiological candidacidal assays, such as yeast-phagocyte suspensions and an adherent monolayer of phagocytes were compared (Chapter 2). We concluded that yeast-phagocyte suspensions are inappropriate for the assessment of intracellular killing of Candida blastoconidia by murine macrophages, due to adherence of Candida blastoconidia to the wall of the tubes or clumping of cells. In contrast, the adherent monolayer of phagocytes could be applied to independently study the process of phagocytosis and intracellular killing by both exudate peritoneal macrophages and exudate peritoneal PMNs. As a result, this assay was implemented in our laboratory.

In line with previous reports, we found that resident murine peritoneal macrophages were unable to kill ingested C. albicans and demonstrated that exudate peritoneal macrophages as well as peritoneal PMNs were able to ingest and kill C. albicans blastoconidia. This suggests that macrophages need priming with an inflammatory stimulus for killing of C. albicans blastoconidia. Exudate peritoneal PMNs showed significantly higher killing percentages compared with exudate peritoneal macrophages.
Both *C. albicans* blastoconidia and hyphal morphologies are found in lesions and PMNs are the predominant effector cells in host defence against *C. albicans*, as described on page 12. Therefore, the extracellular antihyphal activity of PMNs, in addition to determination of their intracellular capacity to kill phagocytosed Candida blastoconidia, should be determined to further characterize the PMN-mediated host response against *C. albicans*. To this purpose, a tetrazolium dye assay was optimized (Chapter 3). This assay reliably assessed fungal viability by measuring the amount of orange water-soluble formazan that is produced by conversion of XTT by fungal mitochondrial dehydrogenases in the presence of an electron-acceptor. Similar to the phagocytosis and killing assay described in chapter 2, the assay to quantify the extracellular damage of *C. albicans* pseudohyphae was implemented in our laboratory.

Because previous studies have shown that recombinant Th1-type cytokines, such as TNF and IFN\(\gamma\), enhance anticandidal PMN-activity, the influence of Th1-type cytokines on *C. albicans* antihyphal activity of PMN was studied (Chapter 3). PMN obtained from mice lacking the genes encoding for TNF/LT, IFN\(\gamma\), IL-12, IL-18, IL-1\(\alpha\), or IL-1\(\beta\) were used. It was shown that PMN-mediated damage of Candida pseudohyphae, the predominating growth form in invasive lesions of candidiasis, is significantly reduced in absence of endogenous TNF/LT, IFN\(\gamma\), or IL-1\(\alpha\), whereas the PMN-mediated damage appeared to be unaltered or even slightly improved in mice deficient in endogenous IL-12 and IL-18, respectively.

Although the intracellular killing of *C. albicans* blastoconidia by TNF/LT\(^{-/-}\) PMNs appeared to be normal [2], the results of Chapter 3 showed that the extracellular pseudohyphal damage by TNF/LT\(^{-/-}\) PMNs was decreased. Therefore, it is concluded that for full characterisation of the PMN-mediated host response against *C. albicans* both intracellular and extracellular killing should indeed be determined.

**Disseminated candidiasis**

Exogenous administration of recombinant human IL-1\(\alpha\) or IL-1\(\beta\) in disseminated murine candidiasis has indicated a protective role for IL-1 in disseminated *C. albicans* infection. The mechanisms for this beneficial effect have not fully been elucidated. Interestingly, we found that IL-1\(\alpha\) and not IL-1\(\beta\) was important for PMN-mediated damage of Candida pseudohyphae (Chapter 3). To gain further insight into the mechanisms through which both endogenous molecules confer protection against infection with *C. albicans*, IL-1\(\alpha\)^\(-/-\), IL-1\(\beta\)^\(-/-\), IL-1\(\alpha\)^\(-/-\)IL-1\(\beta\)^\(-/-\) and control mice were subjected to experimental *C. albicans* infection (Chapter 4). The results of the present study show that endogenous IL-1 is required for host defence against disseminated *C. albicans* infection. IL-1-deficient mice showed an increased mortality, associated with an increased
outgrowth of *C. albicans* in the kidneys. Moreover, a functional difference between IL-1α and IL-1β was observed again. IL-1β, both by itself and by inducing the production of KC, proved to be important for recruitment of granulocytes to the site of infection, and IL-1β was important for the generation of superoxide production. IL-1α was essential for the capacity of granulocytes to damage *Candida* pseudohyphae and both IL-1α and IL-1β were required for the induction of protective Th1 responses.

The IL-1-receptor type I has been shown to be the only IL-1 receptor that leads to signal transduction after engagement of IL-1α or IL-1β. However, the results of Chapter 4 show that IL-1α and IL-1β are able to induce different responses. A similar result was obtained from studying the pathogenesis of rheumatoid arthritis, where IL-1β has been shown to be the pivotal cytokine in bone and cartilage destruction and not IL-1α [3]. Future studies into the mechanism of transduction of the IL-1 signal may be needed to elucidate the different results induced by IL-1α or IL-1β.

Histopathological examination of the kidneys of IL-1α−/− mice suggested that these mice had an increased number of *Candida* cfu in the collecting ducts of their kidneys compared with IL-1β−/− and control mice. It cannot be excluded that IL-1α is important for the production of a candidacidal substance that is excreted in the urine. This hypothesis may be tested in the future.

Administration of recombinant IL-18 protects mice against disseminated *C. albicans* infection [4], and administration of IL-12 may have either protective or deleterious effects during disseminated candidiasis. Thus, it is apparent that IL-18 and IL-12 play a significant role in the host defence against disseminated candidiasis, but a clear characterization of their modulating effects is still missing. To clarify the effects of endogenous IL-18 and IL-12 in host defence against candidiasis, IL-18−/−, IL-12−/−, and control mice were intravenously infected with *C. albicans* (Chapter 5). IL-18−/− mice, but not IL-12−/− mice, displayed an increased mortality due to *C. albicans* infection, accompanied by a decreased clearance of the yeasts from the kidneys late during the course of infection. Furthermore, histopathology of the organs, combined with phagocyte recruitment experiments, showed a decreased influx of monocytes at the site of *Candida* infection, mainly in the IL-18−/− mice. Whereas production of the chemokine KC was decreased in both IL-18−/− and IL-12−/− mice, MIP-2 production was deficient only in IL-18−/− animals, which may explain the differences in monocyte recruitment. Because IL-18 and IL-12 are strong stimuli of IFN-γ production, and IFN-γ plays a crucial role in the defence against infection with *Candida albicans*, production of IFN-γ was determined. Although IFN-γ production was reduced in IL-12−/− mice, this reduction was even more pronounced in IL-18−/− mice. In conclusion, the anticandidal effects of endogenous IL-18 are mediated
late during *Candida* infection by assuring a proper IFN-γ response and promoting the infiltration of the site of infection by monocytes. In addition, the results of *Chapter 3* and other studies from our group [4] did not show an effect of IL-18 on PMN function, explaining why there was no effect early during the course of disseminated candidiasis.

Lipoproteins have also been shown to modulate host defence against candidiasis. In *Chapter 6*, the immunomodulating activity of increased VLDL levels and apolipoprotein E in disseminated candidiasis was investigated. ApoE −/− and control mice were used in the experiments. ApoE −/− mice exhibited a susceptibility to acute disseminated candidiasis that exceeded that of ApoE +/+ mice; mortality due to candidemia was significantly higher in ApoE −/− mice, and outgrowth of *C. albicans* blastoconidia from the kidneys became elevated in ApoE −/− mice. These data indicate that lipoproteins play a significant role in host defence against candidiasis.

The increased susceptibility of ApoE −/− mice appeared to be the result of an increased growth of *C. albicans* in plasma due to lipids serving as nutrients, as well as the neutralization of candidacidal factors by lipoproteins. No neutralizing effect was observed for ApoE itself. However, the candidacidal factors have not been identified. Future studies are needed to extract these factors from serum (*Chapter 6*) or urine (as suggested to be induced by IL-1α, *Chapter 4*).

**Invasive candidiasis**

*Candida albicans* has also been shown to cause invasive candidiasis, i.e. *C. albicans* peritonitis and formation of *C. albicans* abscesses. TNF and LT play a major role in host defence against disseminated candidiasis. Furthermore, evidence exists that the systemic use of monoclonal antibodies against TNF for treatment of patients with an inflammatory bowel disease might increase the risk of invasive fungal infections. The mechanisms through which neutralization of endogenous TNF increases the susceptibility to fungal infections are incompletely understood. Therefore, the role of endogenous TNF and LT in a model of intra-abdominal *Candida* abscesses in TNF−/−LT−/− mice and their wild-type littermates was investigated (*Chapter 7*). The main conclusion of this study is that TNF and LT are necessary for host defence against intra-abdominal *Candida* abscesses. Two factors contributed to the reduced host resistance of TNF−/−LT−/− mice. First, TNF−/−LT−/− granulocytes were significantly impaired in their capacity to damage *C. albicans* pseudohyphae extracellularly. Secondly, TNF−/−LT−/− mice had a delayed Th1 response, as was shown by the significantly increased IL-10/IFNγ ratio during the first week of infection. This delayed Th1 response is likely to lead to a deficient stimulation of effector cells, resulting in a significantly increased fungal burden in the abscesses. Our data, as
well as those from others, indicate that TNF and LT are not essential to abscess and granuloma formation. TNF and LT play a major role in the subsequent stage of infection, i.e. the containment and clearance of abscesses through killing of micro-organisms.

In general, abdominal candidiasis and abscesses are difficult to treat with antimicrobial drugs. Despite antifungal treatment, mortality remains high and additional therapy with G-CSF that augments host defence may be of potential therapeutic benefit. However, treatment with G-CSF might also be detrimental to host defence, as G-CSF has been shown to down-regulate TNF production, which may worsen outcome of intra-abdominal *Candida* abscesses, as evidenced by the results of Chapter 7. Therefore, the influence of antifungal treatment with or without G-CSF administration in a model of intra-abdominal *Candida* abscesses was investigated (Chapter 8). The results indicated that modulation of host defence with rmG-CSF prophylaxis significantly reduces the number of *Candida* cfu in intra-abdominal abscesses, whereas rmG-CSF therapy (administered on days 4 through 8 of infection) had neither a beneficial nor an adverse effect. Antifungal treatment of intra-abdominal *Candida* abscesses with amphotericin B was significantly more effective than treatment with fluconazole. Addition of rmG-CSF therapy to conventional antifungal treatment did not antagonize the individual effect of the antifungal agents. In conclusion, amphotericin B was effective in treating experimental intra-abdominal *Candida* abscesses, and concurrent administration of rG-CSF was suggested to further improve the outcome.

Interestingly, fluconazole was not effective in treating intra-abdominal abscesses induced by a fluconazole-susceptible *C. albicans* strain. The plasma concentrations of fluconazole were adequate (trough plasma concentration of 24 times the MIC). Thus, there remain two possible explanations for the fact that fluconazole was not as effective as amphotericin B in treating intra-abdominal *Candida* abscesses as it was in treating candidemia. First, fluconazole may not penetrate into abscesses. Second, within pus fluconazole might lose its biological activity. Because Joiner et al. showed that most antibiotic agents freely diffuse into abscesses despite the lack of fenestrated vascular supply and the presence of a collagen wall [5], it is tempting to assume that fluconazole is inactivated by pus of *Candida* abscesses. This assumption may be tested by future investigations of the penetration of fluconazole into abscess fluid and the influence of abscess fluid on the antifungal activity of fluconazole.

Amphotericin B derives its beneficial effect mainly from direct candidacidal action. In addition, amphotericin B reportedly enhances host defence mechanisms by modulating the production of pro-inflammatory cytokines. The latter phenomenon may also be associated
with side-effects such as fever and chills. However, contradictory data have been published regarding the capacity of amphotericin B to induce production of pro-inflammatory cytokines. We investigated whether amphotericin B stimulates pro-inflammatory cytokine production by human PBMC or modulates cytokine production induced by different bacterial and fungal stimuli (Chapter 9). Amphotericin B alone induced TNF and IL-1β mRNA expression, and amphotericin B enhanced the lipopolysaccharide (LPS)–induced TNF and IL-1β mRNA expression. However, Candida albicans and Staphylococcus aureus–stimulated expression of pro-inflammatory cytokine genes were not enhanced by amphotericin B. In contrast, amphotericin B reduced the amount of IL-1ra released upon stimulation with LPS and heat-killed S. aureus, as reflected by significantly decreased IL-1ra/IL-1β ratios.

These data suggest that induction of cytokine production by different microbial stimuli is mediated through divergent pathways that are differentially modulated by amphotericin B. Such a pathway may be the TLR-dependent signal transduction. C. albicans possibly suppresses the pro-inflammatory response induced by amphotericin B by stimulating the TLR2-mediated production of IL-10 [6].

Given the moderate effect of amphotericin B on pro-inflammatory cytokine production by human PBMC in vitro and the strong potency of amphotericin B to induce fever and chills in vivo, these findings suggest that pyrogens may be induced via an indirect mechanism in vivo after injection of amphotericin B. Interactions with plasma factors or induction of secondary mediators of cytokine induction could be involved.

References
Samenvatting en discussie
De gist *Candida albicans* is de belangrijkste veroorzaker van gedissemineerde en invasieve infecties door *Candida* (candidiasis). De incidentie van gedissemineerde en invasieve candidiasis loopt parallel met het gebruik van moderne medische procedures die het immuunsysteem ondermijnen. Dit illustreert tevens dat de behandeling ervan gecompliceerd is. Verruiming van inzicht in de interacties tussen de gastheer en *C. albicans* zou deze situatie kunnen verbeteren. Het belangrijkste aspect van deze interacties is de gastheerweerstand. Het onderliggende defect in de gastheerweerstand bepaalt namelijk zowel de wijze waarop candidiasis zich manifesteert als de ernst ervan. Om in de toekomst behandelmethoden te ontwikkelen die erop gericht zijn de gastheerweerstand tegen gedissemineerde en invasieve candidiasis te versterken, is een beter inzicht in de afweermechanismen tegen *C. albicans* noodzakelijk. In dit proefschrift wordt onderzoek naar de afweermechanismen tegen gedissemineerde en invasieve candidiasis beschreven. Onderzoek naar virulentiefactoren die bepalen of *C. albicans* als onschuldige bewoner (commensaal) aanwezig zal zijn of tot invasieve groei overgaat zijn daarbij buiten beschouwing gelaten.

De bijdrage van fagocyten (witte bloedcellen die micro-organsimen kunnen opnemen en verteren) aan de gastheerweerstand wordt doorgaans vastgesteld door het bepalen van fagocytose en intracellulair doden van *Candida* blastoconidia. Dit geschiedt gewoonlijk door middel van klassieke microbiologische methoden waarbij het aantal *Candida* kolonies wordt gekwantificeerd. Omdat deze bepalingen methodologisch verschillen, variëren de resultaten. In *Hoofdstuk 2* zijn enkele van deze bepalingen die gebruik maken van gist-fagocyt suspensies en een adherente cellaag van fagocyten met elkaar vergeleken. Het doel van deze vergelijking was om de meest nauwgezette methode vast te stellen om het fagocyteren en doden van *Candida* blastoconidia door fagocyten te bepalen. Dit onderzoek heeft aangetoond dat gist-fagocyt suspensies ongeschikt zijn voor correcte determinatie van het intracellulair doden van *Candida* blastoconidia door macrofagen afkomstig van muizen. *Candida* blastoconidia blijken namelijk samen te klonten of aan de wand van testbuizen te kleven. De adherente cellaag van fagocyten bleek daarentegen juist wel geschikt om zowel de fagocytose als het doden van de gefagocyteerde *Candida* blastoconidia door exsudaat macrofagen en granulocyten (PMNs) te kwantificeren. Deze bepaling is in ons laboratorium in gebruik genomen.

In overeenstemming met voorgaand onderzoek heeft deze studie aangetoond dat residente peritoneal macrofagen niet in staat zijn gefagocyteerde *Candida* blastoconidia te doden. Exsudaat peritoneaal macrofagen doodden gefagocyteerde *Candida* blastoconidia echter wel. Dit suggereert dat macrofagen eerst aan een inflammatoire stimulus moeten zijn blootgesteld, c.q. zijn geactiveerd, alvorens zij tot het doden van gefagocyteerde
Candida blastoconidia over kunnen gaan. Bovendien doden exsudaat PMNs een groter aantal gefagocyteerde Candida blastoconidia dan exsudaat macrofagen.

Zowel Candida blastoconidia als (pseudo)hyfen zijn aanwezig in een infectiehaard. PMNs zijn de belangrijkste cellen in de afweer tegen Candida infecties, zoals beschreven op pagina 12. Om de PMN-gemedieerde gastheerweerstand volledig te karakteriseren zou derhalve naast de fagocytose en het intracellulair doden van Candida blastoconidia door PMNs, tevens de extracellulaire beschadiging van (pseudo)hyfen door PMNs moeten worden onderzocht. Hiervoor kan een XTT-assay gebruikt worden. In aanwezigheid van een electron-acceptor kunnen mitochondriale dehydrogenases van levende (pseudo)hyfen het substraat XTT omzetten in een oranje kleurstof. De hoeveelheid geproduceerde kleurstof is meetbaar en een maat voor de biomassa. Deze tetrazolium kleuring werd geoptimaliseerd en bleek de biomassa reproduceerbaar en betrouwbaar vast te kunnen stellen (Hoofdstuk 3). Ook deze bepaling werd in ons laboratorium in gebruik genomen.

Cytokinen zijn signaaleiwitten die van nature in het lichaam voorkomen en tijdens infectie mede de communicatie tussen de verschillende cellen van het afweersysteem verzorgen. Voorgaand onderzoek heeft uitgewezen dat recombinante type Th1 cytokinen, zoals TNFα en IFNγ, de anti-Candida werking van PMNs potentieren. Om deze reden werd de invloed van Th1 cytokinen op het vermogen van PMNs om Candida (pseudo)hyfen te beschadigen bestudeerd (Hoofdstuk 3). Hierbij werd gebruik gemaakt van PMNs afkomstig van muizen waarin de genen waren verwijderd coderend voor de cytokinen TNF/LT, IFNγ, IL-12, IL-18, IL-1α, of IL-1β. De resultaten toonden aan dat de PMN-gemedieerde beschadiging van Candida (pseudo)hyfen, de overheersende morfologie in invasieve candidiasis lesies, significant was gereduceerd in afwezigheid van endogeen TNF/LT, IFNγ, of IL-1α. De PMN-gemedieerde beschadiging van Candida (pseudo)hyfen was onveranderd tot licht verbeterd in afwezigheid van endogeen IL-12 of IL-18.

Terwijl de capaciteit van TNF/LT−/− PMNs om Candida blastoconidia intracellulair te doden normaal bleek te zijn [1], wijzen de resultaten van Hoofdstuk 3 uit dat de extracellulaire beschadiging van (pseudo)hyfen door TNF/LT−/− PMNs significant was afgenomen. Het is daarom gerechtvaardigd te concluderen dat voor volledige karakterisering van de PMN-gemedieerde gastheerweerstand tegen C. albicans zowel het intra- als extracellulaire doden van Candida groeivormen moet worden bepaald.

Gedissemeineerde candidiasis

Uit voorgaand onderzoek is gebleken dat toediening van recombinant humaan IL-1α of IL-1β een beschermend effect heeft op muizen met gedissemineerde candidiasis. De
mechanismen die aan dit positieve effect ten grondslag liggen zijn echter nog niet volledig opgehelderd. Daarnaast leerde Hoofdstuk 3 ons dat IL-1α en niet IL-1β van belang was voor de PMN-gemedieerde beschadiging van Candida (pseudo)hyphae. Om meer inzicht te verkrijgen in de mechanismen waardoor endogene IL-1α en IL-1β beschermen tegen gedissemineerde candidiasis werden IL-1α−/−, IL-1β−/−, IL-1α−β−/− en immunocompetente muizen intraveneus geïnfecteerd met C. albicans (Hoofdstuk 4). Uit deze studie blijkt dat endogene IL-1 noodzakelijk is voor de gastheerweerstand tegen een gedissemineerde C. albicans infectie. De mortaliteit onder IL-1-deficiënte muizen was toegenomen en ging gepaard met een groter aantal C. albicans kolonies in de nieren. Bovendien werd wederom een functioneel verschil tussen IL-1α en IL-1β waargenomen. IL-1β was van belang voor de influx van PMNs naar de plaats van infectie, zowel direct als indirect doordat het de productie van chemokine KC induceert. IL-1β was ook nodig voor de generatie van superoxide door PMNs. IL-1α was cruciaal voor de capaciteit van PMNs om Candida (pseudo)hyfen te beschadigen. Zowel IL-1α als IL-1β was noodzakelijk voor de inductie van een beschermende Th1 respons.

De IL-1-receptor type I is de enige IL-1 receptor die tot signaaltransductie leidt nadat IL-1α of IL-1β aan de receptor zijn gebonden. Echter, de resultaten van Hoofdstuk 4 hebben aangetoond dat IL-1α en IL-1β in staat zijn verschillende reacties te induceren. Een overeenkomstig resultaat werd gevonden bij bestudering van de pathogenese van rheumatoïde arthritis [2]. IL-1β heeft hierbij een centrale plaats in de destructie van bot en kraakbeen, en niet IL-1α. Verder onderzoek naar de mechanismen die betrokken zijn bij de IL-1 signaaltransductie kan nodig zijn om het verschillende effect van IL-1α en IL-1β te kunnen verklaren.

Histopathologie van de nieren van IL-1α−/− muizen suggereerde dat deze muizen een groter aantal Candida kolonies in de verzamelbuizen hadden in vergelijking met immunocompetente en IL-1β−/− muizen. Dat endogene IL-1α van belang is voor de productie van een candidacide factor die in de urine wordt uitgescheiden is een interessante hypothese die de moeite van nader onderzoek waard is.

Administratie van recombinant IL-18 beschermt muizen tegen gedissemineerde C. albicans infectie [3] en toediening van IL-12 kan zowel een beschermend als een nadelig effect hebben op het beloop van gedissemineerde candidiasis. IL-18 en IL-12 spelen dus een significante rol in de gastheerweerstand tegen gedissemineerde candidiasis, maar een goede karakterisatie van hun modulerend effect ontbreekt nog. Om de effecten van endogene IL-18 en IL-12 op de gastheerweerstand tegen candidiasis op te helderen werden IL-18−/−, IL-12−/−, en immunocompetente muizen intraveneus met C. albicans geïnfecteerd (Hoofdstuk 5). Alleen onder de IL-18−/− muizen was de mortaliteit toegenomen. Dit ging
gepaard met een verminderde klaring van *C. albicans* uit de nieren, laat tijdens het beloop van infectie. Bovendien bleek uit histopathologisch onderzoek van de nieren en uit fagocyten-influx experimenten dat de influx van monocyten naar de plaats van de *Candida* infectie verminderd was, voornamelijk in de IL-18<sup>−/−</sup> muizen. Terwijl de productie van KC was afgenomen in IL-18<sup>−/−</sup> en IL-12<sup>−/−</sup> muizen, was de productie van chemokine MIP-2 alleen verminderd in IL-18<sup>−/−</sup> muizen. Dit verklaart mogelijk het verschil in de influx van monocyten tussen IL-12<sup>−/−</sup> en IL-18<sup>−/−</sup> muizen. Omdat IL-18 en IL-12 de productie van IFN<sub>γ</sub> stimuleren, en IFN<sub>γ</sub> een cruciale rol in de afweer tegen infectie met *C. albicans* speelt, werd tevens de productie van IFN<sub>γ</sub> bepaald. Hoewel de IFN<sub>γ</sub> productie gereduceerd was in IL-12<sup>−/−</sup> muizen, was deze reductie meer uitgesproken in IL-18<sup>−/−</sup> muizen. Concluderend: het anti-*Candida* effect van endogeen IL-18 vindt pas laat plaats gedurende *Candida* infectie en IL-18 verzekert zowel de IFN<sub>γ</sub> respons als de influx van monocyten naar de plaats van infectie. De resultaten van **Hoofdstuk 3**, en overig onderzoek [3], hebben geen invloed van IL-18 op de PMN-functie aangetoond. Dit levert een aanvullende verklaring waarom IL-18 geen vroeg effect sorteert in het beloop van gedissemineerde candidiasis.

Lipoproteïnen zoals LDL kunnen de gastheerweerstand tegen candidiasis moduleren. In **Hoofdstuk 6** wordt het onderzoek naar de immunomodulerende werking van verhoogde VLDL concentraties en apolipoproteïne E tijdens gedissemineerde candidiasis beschreven. In deze experimenten werden ApoE<sup>−/−</sup> and immunocompetente (ApoE<sup>+/+</sup>) muizen gebruikt. ApoE<sup>−/−</sup> muizen waren gevoeliger voor acute gedissemineerde candidiasis dan ApoE<sup>+/+</sup> muizen. Dit uitte zich in een verhoogde sterfte onder ApoE<sup>−/−</sup> muizen en een groter aantal *C. albicans* blastoconidia in de nieren van ApoE<sup>−/−</sup> muizen. Lipoproteïnen spelen dus een belangrijke modulerende rol in de afweer tegen candidiasis.

De verhoogde gevoeligheid van ApoE<sup>−/−</sup> muizen was het resultaat van toegenomen *C. albicans* groei in plasma. Enerzijds kan de toegenomen groei worden verklaard doordat de lipiden dienst doen als nutrienten, anderzijds kunnen de lipoproteïnen candidacide factoren in het plasma neutraliseren. ApoE zelf had geen neutraliserend effect op candidacide plasma factoren. Echter, deze candidacide factoren zijn niet geïdentificeerd. In de toekomst zouden deze factoren uit plasma ([**Hoofdstuk 6**]) of urine (mogelijk geïnduceerd door IL-1α zoals gesuggereerd in [**Hoofdstuk 4**]) kunnen worden geïsoleerd.

**Invasieve candidiasis**

Naast gedissemineerde candidiasis kan *Candida albicans* tevens invasieve candidiasis veroorzaken, waarmee in dit proefschrift *C. albicans* peritonitis en intraperitoneale *C. albicans* abcessen wordt bedoeld. TNF en LT spelen een centrale rol in de

Twee factoren dragen bij aan de verminderde gastheerweerstand van TNF−/−LT−/− muizen. Ten eerste, TNF−/−LT−/− PMNs hebben een significant verminderde capaciteit om C. albicans pseudohyphen extracellulair te beschadigen. Ten tweede, TNF−/−LT−/− muizen hebben een vertraagde Th1 respons, zoals aangetoond door de significant verlaagde IL-10/IFNγ ratio gedurende de eerste week van infectie. De vertraagde Th1 respons leidt hoogstwaarschijnlijk tot onvoldoende stimulatie van effector cellen, waardoor de hoeveelheid Candida in abcessen significant toeneemt. Zowel uit onze gegevens, als uit die van anderen, kan geconcludeerd worden dat TNF en LT niet essentieel zijn voor het proces van abces- en granuloomvorming. TNF en LT spelen daarentegen wel een hoofdrol bij de expansiebeperking en klaring van C. albicans door het doden van de microorganismen.

Over het algemeen zijn abdominale candidiasis en abcessen moeilijk te behandelen met antifungale middelen. Ondanks antifungale therapie blijft de mortaliteit aanzienlijk hoog. Additionele behandeling met een middel zoals G-CSF dat de gastheerweerstand versterkt zou een potentieel gunstig effect op de behandeling kunnen uitoefenen. Echter, behandeling met G-CSF zou tevens een ongunstig effect op de gastheerweerstand kunnen hebben. Voorgaand onderzoek heeft namelijk aangetoond dat G-CSF de TNF productie kan verminderen en, zoals beschreven in Hoofdstuk 7, een verminderde TNF productie heeft een nadelige invloed op het beloop van intra-abdominale Candida abcessen. Om deze redenen werd de invloed van antifungale therapie al dan niet in combinatie met G-CSF behandeling bestudeerd in het model van intra-abdominale Candida abcessen (Hoofdstuk 8). De resultaten tonen aan dat modulatie van de gastheerweerstand door profylactisch toegediend G-CSF het aantal Candida cfu in de abcessen significant reduceert. Daarentegen heeft G-CSF therapie alleen (toegediend op dag 4 t/m 8 van infectie) noch een gunstig nog een ongunstig effect. Antifungale behandeling van intra-abdominale Candida abcessen met amfotericine B was significant effectiever dan fluconazol behandeling. Toevoeging van rmG-CSF therapie aan conventionele antifungale therapie antagooneerde niet het effect van behandeling met een antifungaal middel alleen.
Concluderend, experimentele intra-abdominale Candida abcessen kunnen effectief worden behandeld met amfotericine B, en simultane administratie van rmG-CSF kan leiden tot een beter resultaat.

Het was opvallend dat behandeling met fluconazol niet effectief was tegen intra-abdominale Candida abcessen. De C. albicans stam was gevoelig voor fluconazol en de plasma concentraties van fluconazol waren adequaat (dalconcentratie plasma 24 x MIC). Bovendien was fluconazol even effectief als amfotericine B ter behandeling van candidaemie. Er resten dus twee denkbare verklaringen voor de ineffectiviteit van fluconazol behandeling van intra-abdominale Candida abcessen. Ten eerste, fluconazol penetreert mogelijk niet in abcessen. Ten tweede, het is mogelijk dat fluconazol biologische activiteit verliest in pus. Omdat Joiner en anderen hebben aangetoond dat, ondanks de afwezigheid van gefenestreerde vascularisatie en de aanwezigheid van een collageen kapsel, de meeste antibiotica eenvoudig in abcessen penetreren [4], is het aanlokkelijk om aan te nemen dat fluconazol door de inhoud van Candida abcessen wordt geinactiveerd. Deze hypothese dient in toekomstige studies te worden getoetst.

Amfotericine B dankt het gunstige effect voornamelijk aan een direct candicide werking. Daarnaast is beschreven dat amfotericine B mogelijk de gastheerweerstand versterkt door de produktie van pro-inflammatoire cytokinen te induceren. Dit laatste fenomeen is mogelijk geassocieerd met het optreden van bijwerkingen als koorts en koude rillingen. Er zijn echter tegenstrijdige gegevens gepubliceerd met betrekking tot de capaciteit van amfotericine B om de produktie van pro-inflammatoire cytokinen te induceren. In Hoofdstuk 9 is het onderzoek beschreven naar de inducerende en modulerende werking van amfotericine B op de produktie van pro-inflammatoire cytokinen door humane mononucleaire cellen die geïsoleerd zijn uit perifeer bloed (PBMC). Amfotericine B induceerde de expressie van TNF en IL-1β mRNA, en amfotericin B versterkte de lipopolysaccharide (LPS)–geïnduceerde TNF and IL-1β mRNA expressie. De door Candida albicans en Staphylococcus aureus–gestimuleerde expressie van genen die coderen voor de pro-inflammatoire cytokinen werd niet versterkt door amfotericine B. Daarentegen reduceerde amfotericine B de hoeveelheid IL-1ra in respons op LPS en hitte-gedode S. aureus bacteriën, zoals blijkt uit de significant gereduceerde IL-1ra/IL-1β ratio’s.

Deze data suggereren dat de inductie van de cytokineproduktie door diverse microbiële stimuli via verschillende wegen wordt gemedieerd waardoor amfotericine B een verschillend effect sorteert. Een mogelijk voorbeeld is de TLR-gemedieerde signaal
transductie. *C. albicans* onderdrukt waarschijnlijk de amfotericine B-geïnduceerde pro-inflammatoire cytokinen respons door de TLR2-gemedieerde productie van IL-10 [5].

Omdat amfotericine B *in vitro* een geringe invloed heeft op de produktie van pro-inflammatoire cytokinen, en *in vivo* juist een hoge potentie heeft om koorts en koude rillingen te veroorzaken, kan gesuggereerd worden dat *in vivo* pyrogenen mogelijk via een indirect mechanisme na toediening van amfotericine B worden geproduceerd. Interacties met plasmifactoren of induktie van secondaire mediatoren van de cytokine produktie vormen daarbij een mogelijkheid.

Referenties


Dankwoord

Het schrijven van een proefschrift is nooit een doel op zich voor mij geweest. In feite is het een gevolg van een tijd waarin ik met plezier mijn werk heb mogen doen en mezelf wetenschappelijk en persoonlijk verder heb kunnen ontwikkelen. Ik ben er eigenlijk wel trots op dat deze ontwikkelingen hebben geresulteerd in dit proefschrift. Daar ben ik de volgende personen dank voor verschuldigd.

Jos van der Meer, mijn promotor, je hebt me de kans gegeven om als student onderzoek te doen en dat later als arts voort te zetten. Voornamelijk in die eerste periode heb je me intensief begeleid en zelfs nu nog voorzie je me zo nu en dan van advies waardoor ik aardig op koers blijf. Dank dat ik, ondanks alle drukte, altijd welkom ben met typische Vonk-zaken.

De goede basis voor dit proefschrift werd gelegd in de tijd dat ik als student onderzoek op het laboratorium van algemene interne geneeskunde deed. Ik ben niet vergeten dat Liesbeth de methode om PBMC’s te isoleren met de hand voor me opschreef. Zoiets had ik nog niet eerder meegemaakt en dat was duidelijk het begin van een hardnekkige labaffiniteit. Trees en Liesbeth, bedankt dat jullie me als het ware labtechnisch hebben leren lopen. Ik denk dat ik later door het klimmen met Natasja ook op de onderzoeksladder omhoog ben geklommen. Natasja bedankt voor je kameraadschap.

Een van de voornaamste lessen van deze onderzoeksperiode is dat een negatieve bevinding helemaal zo gek nog niet is, maar dat dit soms wel veel doorzettingsvermogen vraagt. Ineke, ik ben erg blij dat ik met je heb kunnen samenwerken. Voor mijn gevoel liep het als een goed geoliede machine. Ik heb enorm met je gelachen en de fijne praktische kneepjes van je geleerd. Ik bewonder je inzet. Je hebt er zonder twijfel aan bijgedragen dat mijn doorzettingsvermogen groter werd dan ik voor mogelijk hield.

Verreweg de meeste tijd heb ik doorgebracht op het dierenlab. Ik dank Debby, Kay, Geert, Theo, Maichel, Margot, Monique, Hennie, Helma en Bianca voor het aanleren van de benodigde technieken, jullie vakkundigheid en hulp bij mijn experimenten. Jullie waren altijd bereid om mee te denken, muizen te injektieren of materiaal op plaat te zetten. Dit allemaal volgens complexe schema’s op soms onmogelijke tijden. Dat “waar Vonk werkt, soms ook vuur is” zullen we maar niet meer letterlijk nemen, niet Kay? Dank allemaal voor al het plezier!

Cathrien Wieland, Yvonne Jonkers, Monique Joosten en Marieke Versteegen, bedankt voor de prettige samenwerking en de feedback die ik als begeleider van jullie heb ontvangen. Cathrien, ik vond het geweldig dat je als student binnen een mum van tijd met me in discussie kon treden. Onze bijzonder goede samenwerking heeft geresulteerd in een alom gehekelde fagocytose- en killingassay. Ik ben er trots op dat je onderzoek bent gaan doen. Nog is het jou niet welletjes.....

Naast het Candida-onderzoek heb ik tevens mogen proeven van onderzoek op andere gebieden. Ik dank Lorna Stearne, Inge Gyssens (UMC Rotterdam) en Maaike Dooper (Nuimico Research B.V., Wageningen) voor de plezierige samenwerking.

Toch is er ook een dip. Geen saus, maar Anna. Anna, dank dat je tijdens mijn ziekteproces een geweldige steun bent geweest. Ook als ik helemaal stuk zat gaf je me stof tot nadenken. Simpele puzzels. Lastige puzzels. Soms loste je ze aan de rand van mijn bed zelf maar even op. Ik lag er gewoon. Jij zat er gewoon.

Ik heb bijzonder genoten van de manier waarop we gepassioneerd over ons onderzoek en andere onderwerpen hebben gediscussieerd en van onze akties als A-team. Het spijt me dat ik dit niet in een formule kan uitdrukken, maar dat is immers jouw taak. Ik denk dat ik nu mijn vingers moet houden. Bedankt.

Gelukkig was er een man om het A-team op noodzakelijke momenten met beide benen op de grond te houden en ons, met gevaar voor eigen leven, voor serieus overgewicht te behoeden door regelmatig onze koekjes op te eten (ik schrijf dit uiteraard zonder toestemming van Anna). Reinout, bedankt voor de nodige humor en je uitstekende kamergenootschap.

Mihai Netea, mijn onnavolgbare copromotor, ik heb geprobeerd iets van jouw creatieve manier van kijken naar alles om me heen eigen te maken. Ergens een experiment in te zien. Wel, ik denk dat ik nog maar een rondje op de motor maak. Drie waarschijnlijk.
Tenslotte dank ik mijn directe begeleider en promotor Bart-Jan Kullberg. BJ, je hebt me geleerd kritischer dan kritisch onderzoek te doen. Je verfijnde steeds mijn schrijfwerk en tot op heden is het me niet gelukt iets zonder commentaar terug te krijgen. Met humor en geduld heb je *Candida* in RPMI laten groeien. Dat zal ik waarschijnlijk zelfs wel missen. De rest heb je inmiddels per mail ontvangen, denk ik. Ik dank je voor alles. Antoinette, bedankt dat ik vaak beslag op je tijd heb mogen leggen.


Mijn familie dank ik voor hun onvoorwaardelijke liefde en steun. Pap, zonder de orgametrilpraat 24/7 zou ik dit niet hebben kunnen schrijven. Lieve Farzin, zonder jou was dit simpelweg niet mogelijk geweest. Dank je voor je grenzeloze vertrouwen en geloof in mij.

فرزین من دوستت دارم و من به تو افتخار می‌کنم. الیکه
Curriculum Vitae

Appendix A

List of publications


List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmB</td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte-derived chemokine</td>
</tr>
<tr>
<td>LTα</td>
<td>Lymphotoxin-α</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose binding lectin</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein 2</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer (cell)</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>ONOO</td>
<td>Peroxinitrate</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMN(s)</td>
<td>Polymorphonuclear granulocyte(s)</td>
</tr>
<tr>
<td>RPMI</td>
<td>Acronym of Roswell Park Memorial Institute; cell culture medium</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency (T- and B-cell defect)</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour-necrosis factor-α</td>
</tr>
<tr>
<td>TPN</td>
<td>Total parenteral nutrition</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>XTT</td>
<td>tetrazolium dye: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide</td>
</tr>
</tbody>
</table>