

Complement plays a central role in *Candida albicans*-induced cytokine production by human PBMCs

Shih-Chin Cheng^{1,2}, Tom Sprong^{1,2}, Leo A.B. Joosten^{1,2},
Jos W. M. van der Meer^{1,2}, Bart-Jan Kullberg^{1,2}, Bernhard Hube^{3,4},
Lone Schejbel⁵, Peter Garred⁵, Marcel van Deuren^{1,2} and Mihai G. Netea^{1,2}

¹ Department of Medicine, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

² Nijmegen Institute for Infection, Inflammation and Immunity (N4i), Nijmegen, The Netherlands

³ Department of Microbial Pathogenicity Mechanism, Leibniz Institute for Natural Product Research and Infection Biology—Hans Knoell Institute, Jena, Germany

⁴ , Friedrich Schiller University, Jena, Germany

⁵ Department of Clinical Immunology, Laboratory of Molecular Medicine, Rigshospitalet, Copenhagen, Denmark

In experimental studies, the role of complement in antifungal host defense has been attributed to its opsonizing capability. In this study, we report that in humans an activated complement system mainly augments *Candida albicans*-induced host proinflammatory cytokine production via C5a-C5aR signaling, while phagocytosis and intracellular killing of *Candida* are not influenced. By blocking the C5a-C5aR signaling pathway, either with anti-C5a antagonist antibodies or with the C5aR antagonist W-54001, *C. albicans*-induced IL-6 and IL-1 β levels were significantly reduced. Recombinant C5a augmented cytokine production. In addition, using serum from patients with various complement deficiencies, we demonstrated a crucial role of C5, but not C6 or the membrane attack complex, in *C. albicans*-induced IL-6 and IL-1 β production in monocytes. These findings reveal a central role of anaphylatoxin C5a in augmenting host proinflammatory cytokine production upon contact with *C. albicans*, and define the role of the complement system in anti-*Candida* host defense in humans.

Keywords: *Candida* • Complement • C5a • IL-6 • IL-1 β



See accompanying article by Zipfel and Skerka



Supporting Information available online

Introduction

Candida albicans is the major cause of human fungal diseases. *Candida* colonizes the mucosae of more than 50% of healthy individuals, but this is usually asymptomatic. However, once host

immunity is compromised, such as in patients with inborn genetic defects [1] or neutropenia due to immune suppression [2], *C. albicans* can become invasive and spread systemically. The mortality due to disseminated candidiasis is very high, up to 30–50% [3].

The innate immune system is critical for clearing fungal invasion. The first step in the activation of innate immunity is the recognition of fungal structures, called pathogen associated molec-

Correspondence: Dr. Mihai G. Netea
e-mail: M.Netea@aig.umcn.nl

ular patterns, by pattern recognition receptors (PRRs) [4]. Among the known PRRs, Toll-like receptors (TLRs; TLR2 and TLR4) and C-type lectin receptors (CLRs, such as dectin-1, dectin-2, mannose receptor (MR)) have been identified to be involved in both the direct killing of *C. albicans* through phagocytosis [5], and in the induction of proinflammatory cytokines that recruit and activate phagocytes to clear and control the infection [6].

Another important arm of the innate immunity is the complement system. The complement system can be activated by three pathways: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP) that all converge on the activation of C3, leading to phagocytosis of the C3b-opsonized microorganisms [7]. It has been shown that all these three activation pathways can be activated by *C. albicans* [8–10], and experimental studies have suggested that this facilitates the phagocytosis of the fungus through the deposition of C3b [11]. Unlike for bacterial pathogens, the membrane attack complex (MAC), comprised of C5–C9, has not been convincingly demonstrated to lead to direct lysis of *C. albicans*, due to the thick fungal cell wall which presumably blocks the formation of the MAC [12, 13].

In addition to its ability to facilitate phagocytosis by opsonization, the activation of complement system also generates anaphylatoxins (C3a, C4a, and C5a), which mediate pleiotropic effects such as triggering degranulation, inducing smooth muscle contraction, chemotaxis, or inducing a local inflammatory response [14]. C3a has been reported to act as an antifungal peptide by perturbing membrane integrity [15]. C5a has also been shown to be crucial for the phagocytosis of Gram-negative bacteria and subsequent oxidative burst by upregulating CR3 [16, 17]. The role of anaphylatoxins in host defense against *C. albicans* and other *Candida* species is not known.

In this study, we aimed to explore the role of complement for the activation of innate host defense by *C. albicans*. By a combination of methodologies using blocking antibodies, recombinant C5a, and plasma from patients with complement deficiencies, we demonstrate that the main role of complement activation in human antifungal defense is to augment *C. albicans*-induced cytokine production by the anaphylatoxin C5a.

Results

Heat-inactivated and antimannan Ab-depleted serum reduce *C. albicans*-induced cytokine production

In the presence of intact human pooled serum (HPS), human peripheral blood mononuclear cells (PBMCs) induced significantly higher proinflammatory cytokines, such as IL-6, IL-1 β , and TNF- α (Fig. 1A) upon *C. albicans* stimulation. A clear reduction of cytokine production was observed after heat inactivation (56°C for 30 min) of HPS (Fig. 1B).

The presence of antimannan antibodies in the normal human serum has been reported previously [8]. Depletion of the antimannan antibodies by preabsorption of HPS with *C. albicans* at 4°C for 30 min resulted in the reduction of cytokine production

induced by the yeast (Fig. 1C). In addition, when antimannan antibodies were depleted at 37°C, IL-6, TNF- α , and IL-1 β production were comparable with the intact HPS (Supporting Information Fig. 1).

Cytokine production is downregulated by the addition of specific Abs against complement components

To further delineate which pathway is involved in augmenting *C. albicans*-induced cytokine production, specific antagonist antibodies were used to block the activation of individual complement factors. Previous reports showed that all the three complement activation pathways could be activated by *C. albicans* [8, 9, 18]. Our results demonstrate that cytokine production, as measured in our system, was significantly reduced when the classical/LP (by anti-C2 antibody) and the AP (by anti-Factor D) were inhibited (Fig. 2A).

All three complement activation pathways converge at the level of activation of C3, leading to downstream cleavage of C5 into C5a and C5b. C5a functions as anaphylatoxin and has pleiotropic effects in the inflammatory process [19]. Therefore, we investigated the role of C5a in augmenting *C. albicans*-induced cytokine production. Cytokine production was significantly downregulated by addition of a blocking anti-C5a antibody (Fig. 2B).

C5a receptor antagonist inhibits *C. albicans*-induced IL-6 and IL-1 β , but not TNF- α

C5a transduces signals through the G-protein coupled C5a receptor (C5aR) [20]. To delineate the role of C5a-C5aR signaling pathway in *C. albicans*-induced cytokine production, C5aR specific antagonist W-54001 was used to inhibit C5aR dependent signaling [21]. A clear dose-dependent inhibition of IL-6 and IL-1 β production was observed (Fig. 3). However, TNF- α production was not influenced, irrespective of the inhibition of C5aR signaling.

C5a augments *C. albicans*-induced IL-6 and IL-1 β production

To further demonstrate the role of C5a in augmenting *C. albicans*-induced cytokine production, recombinant human C5a was added simultaneously with *C. albicans* to PBMCs in serum free medium. In line with the previous finding, recombinant C5a by itself could not induce cytokine production. However, recombinant C5a significantly augmented *C. albicans*-induced IL-6 and IL-1 β production and TNF- α to a lesser extent (Fig. 4A).

C5a crosstalk with TLR2 and TLR4

We have previously demonstrated that fungal cell wall components such as mannan and β -glucan could be recognized by TLR2, TLR4, dectin-1, and MR and induce cytokine production [4]. To get

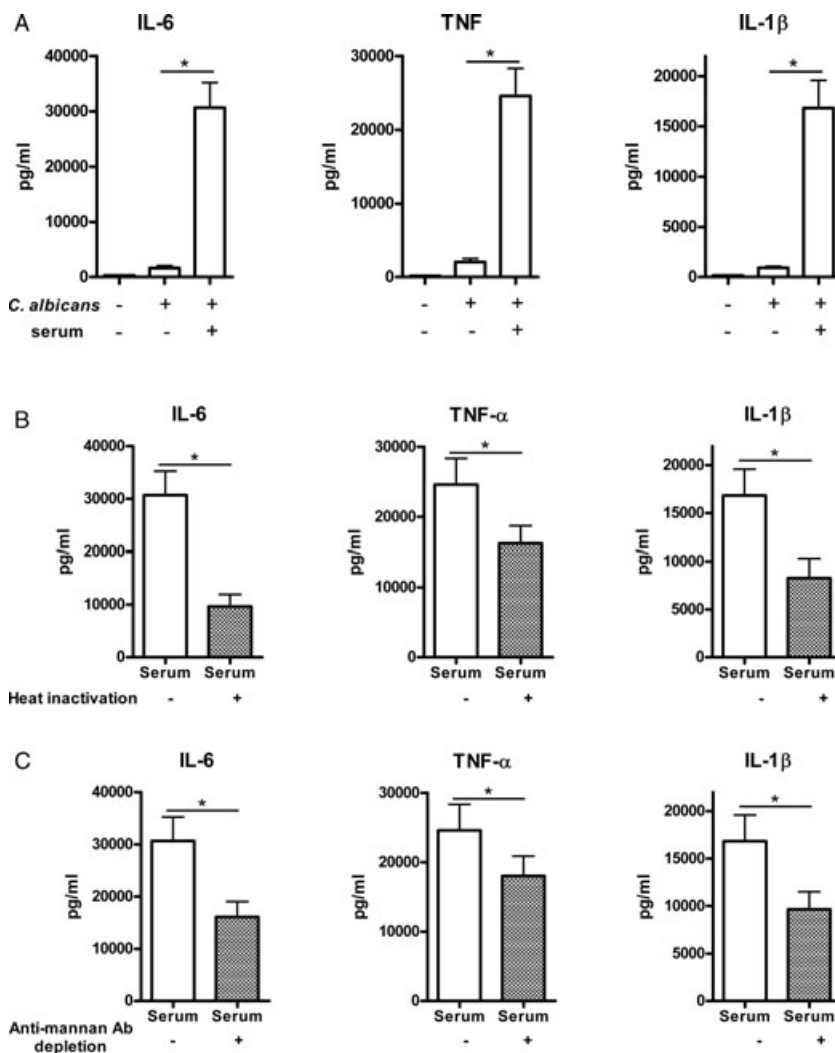


Figure 1. Intact serum potentiates *C. albicans*-induced cytokine production in PBMCs. Human PBMCs were stimulated with live *C. albicans* in the presence of (A) healthy human pooled serum, (B) heat-inactivated human pooled serum, or (C) anti-mannan antibody depleted human pooled serum for 24 h. Supernatant was collected for IL-6, IL-1β, and TNF-α measurement (pooled data of three separate experiments, $n = 6$ volunteers, means $\pm$ SD, * $p < 0.05$ determined by paired Wilcoxon test).

a better understanding of how C5a augments *C. albicans*-induced cytokine production, we stimulated PBMCs simultaneously with lipopolysaccharide (LPS) (TLR4 agonist), Pam3Cys (TLR2 agonist), β -glucan (dectin-1 ligand), or mannan (MR and dectin-2 ligand), alone or in combination with recombinant C5a. As shown in Fig. 4B, we observed that both LPS and Pam3Cys induced higher IL-6, TNF- α , and IL-1β in the presence of C5a.

C5-deficient serum induced lower cytokine production

To assess whether these findings may impact cytokine production in vivo, we investigated the effect of serum collected from patients with complement deficiencies for *C. albicans*-induced cytokine production. C5-deficient serum potentiated less strongly IL-6 (94% reduction), TNF- α (29% reduction), and IL-1β (75% reduction) production induced by *C. albicans*, compared with that by control serum. This effect however was not observed with serum from patients suffering from C6 and C7 deficiency (Fig. 5A). To further demonstrate that the observed cytokine reduction is not due to a defect of opsonization activity, we performed a

C. albicans phagocytosis and killing assay in the presence of HPS or C5-deficient serum: no significant difference was observed (Fig. 5B). This implies that C5-deficient serum possesses intact opsonization activity, while failing to support proper cytokine production.

In addition, by adding recombinant C5a to the C5-deficient serum, a clear augmentation of IL-6 and IL-1β production was observed (Fig. 5C). The augmentation effect could be blocked by preincubation with C5aR antagonist. As for the normal HPS (Fig. 5D) and other complement deficient sera (Supporting Information Fig. 2), only a marginal effect was observed by adding exogenous C5a.

C5a enhanced *C. albicans*-induced production of IL-10, but not IFN- γ and IL-17

In addition to its role on promoting proinflammatory cytokine in monocytes/macrophages, C5a can also modulate adaptive immunity by altering T-cell differentiation [19]. Therefore, we

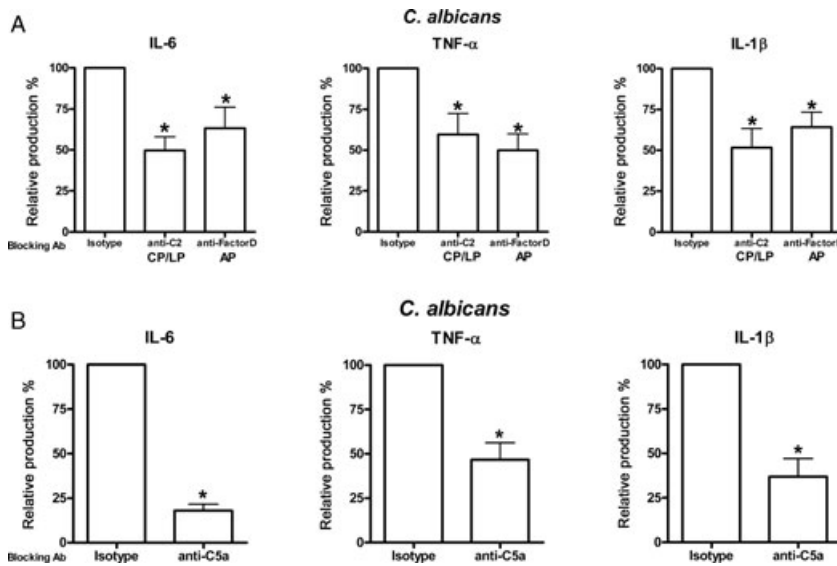


Figure 2. Specific complement antagonist Abs reduce *C. albicans*-induced cytokine production in PBMCs. Human PBMCs were stimulated with live *C. albicans* in the presence of healthy human pooled serum together with (A) anti-C2, anti-Factor D, or (B) anti-C5a and isotype (mouse IgG1) for 24 h. Supernatant was collected for IL-6, IL-1β, and TNF-α measurement (pooled data of three separate experiments, $n = 6$ volunteers, means + SD, * $p < 0.05$ determined by paired Wilcoxon test).

further examined the role of C5a on the production of the signature cytokines for Th1, Th2, and Th17 cells. No effect of C5a on the production of IFN-γ and IL-17 was observed, while IL-10 production was significantly upregulated by C5a (Fig. 6A). In a following set of experiments, we performed real-time polymerase chain reaction (RT-PCR) to examine whether the transcription factors specific for T-helper-cell differentiation are influenced by C5a. C5a did not alter the expression level of T-bet, GATA-3, and RORγt (Fig. 6B).

Candida albicans pH-regulated antigen 1 (Pra1) inhibits *C. albicans*-induced cytokine production

Pra1 is a glycoprotein located at the *C. albicans* cell surface that has been reported to inhibit complement activation. To study the role of Pra1, a recently identified surface protein of *C. albicans* that modulates complement activation [22, 23], during interaction with monocytes, we stimulated human PBMCs with a *pra1Δ* strain and its parental strain in the presence of serum. As expected, the *pra1Δ* strain induced significantly higher amounts of

TNF-α (1.7-fold increase) and IL-1β (2.1-fold increase) as compared with the parental strain. IL-6 production induced by *pra1Δ* strain was also higher, although it did not reach statistical significance (Fig. 7A). Next, a Pra1-overexpressing strain was used to stimulate the PBMCs. However, no differences in cytokine production were observed between the overexpressing strain and its corresponding parental strain (Fig. 7B), implying that the endogenous Pra1 expression is sufficient to inhibit complement activation.

Discussion

Innate immunity is the first line defense against *C. albicans* infection. Upon encountering *C. albicans*, phagocytes either kill *C. albicans* by phagocytosis and intracellular killing, or secrete chemokines and cytokines to recruit and activate effector cells. The present study has identified a previously unknown effect of C5a for augmenting *C. albicans*-induced cytokine production. Through the crosstalk between C5aR and TLR pathways, the complement system augments the proinflammatory cytokine production that

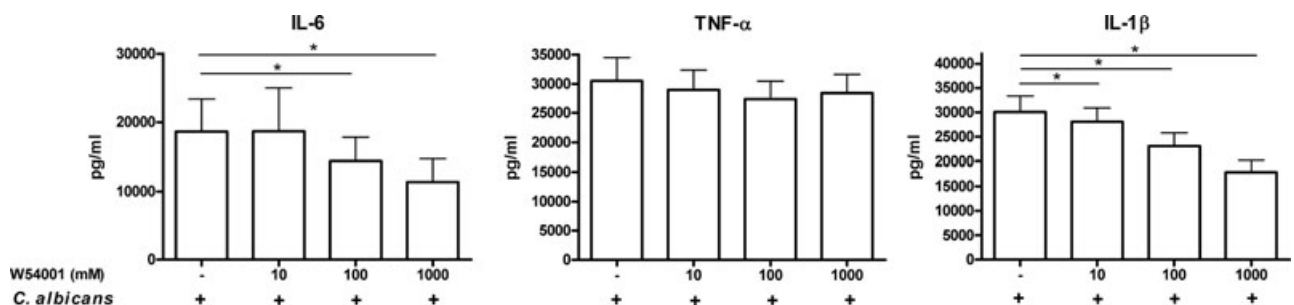


Figure 3. Blocking of C5a receptor with specific antagonist reduces *C. albicans*-induced cytokine production. Human PBMCs were stimulated with live *C. albicans* in the presence of healthy human pooled serum together with C5a receptor antagonist, W54001, in a dose-dependent manner for 24 h. Supernatant was collected for IL-6 (left), IL-1β (right), and TNF-α (middle) measurement (pooled data of three separate experiments, $n = 6$ volunteers, means + SD, * $p < 0.05$ determined by paired Wilcoxon test).

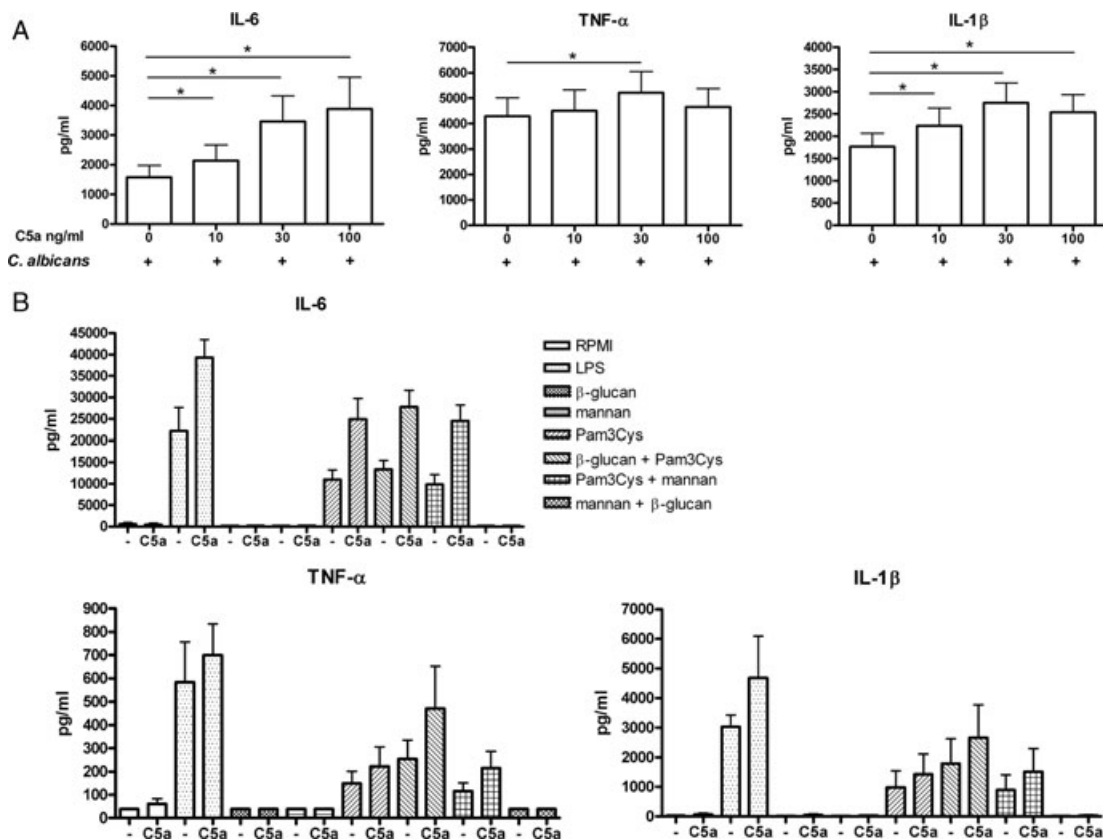


Figure 4. C5a interacts synergistically with TLR2 and TLR4 to induce cytokine production. (A) Human PBMCs were stimulated with live *C. albicans* in serum free medium together with recombinant C5a in a dose-dependent manner for 24 h. Supernatant was collected for IL-6, IL-1 β , and TNF- α measurement. (B) Human PBMCs were either left unstimulated or stimulated with LPS, β -glucan, mannan, Pam³Cys, alone or in combination, in the presence or absence of recombinant C5a for 24 h. Supernatant was collected for IL-6, IL-1 β , and TNF- α measurement (pooled data of three separate experiments, $n = 6$ volunteers, means + SD, * $p < 0.05$ determined by paired Wilcoxon test).

will contribute to fungal clearance. As phagocytosis and killing of *Candida* by human phagocytes was not significantly reduced when using complement-deficient plasma, it is likely that the augmentation of cytokine production is the main function of complement in human antifungal defense.

The complement system plays an important role in anti-*C. albicans* host defense. On the host side, much attention had been drawn to the opsonization activity of complement system against *C. albicans* infection [24–26]. On the pathogen side, it has been reported that *C. albicans* interferes with complement activation through several different strategies: first, by direct degradation of complement components by secreted aspartic proteases [27]; second, through binding of complement regulators on the cell wall protein, such as Pra1 [28–30] and Hgt1p [31] to inhibit complement activation; and third, by blocking the activation of complement by surface mannan or pH-regulated antigen 1 [22,32]. Surprisingly however, the role of the anaphylatoxins for antifungal host defense has been less well characterized. In this study, we demonstrate that despite the fact that *C. albicans* phagocytosis was normally induced by serum from C5-deficient patients, the capacity of C5-deficient serum to potentiate proinflammatory cytokine production induced by *C. albicans* was significantly reduced. This

highlights the role of the complement system in augmenting cytokine production upon *C. albicans* infection, and suggests this is its main role in human antifungal immunity. C5a is a small peptide generated upon complement activation. It induces various inflammatory responses such as vasodilation, smooth muscle contraction, and accumulation of neutrophils at the extravascular sites [14]. In addition, the crosstalk between C5a and TLR has also been reported to modulate TLR-mediated cytokine production in different T-cell types [33]. It has been suggested that C5a potentiated LPS-induced IL-6 production in neutrophils by activation of ERK1/2 and p38 mitogen-activated protein kinase (MAPK) signaling through C5aR [34]. C5a also enhanced LPS-induced TNF- α and IL-1 β production by monocytes and macrophages [35]. Moreover, synergistic induction of cAMP and suppression of nitric oxide-dependent bacterial killing between TLR2 and C5aR was demonstrated in an in vivo mouse model [36].

The activation of complement was needed for the augmentation of *C. albicans*-induced cytokine production, as shown by using heat inactivation of HPS (Fig. 1A). It is known that naturally occurring antimannan antibodies bind to *C. albicans* and initiate complement activation via the CP [37]. By depleting the antimannan antibodies from the serum, we also observed significant reduction of cytokine production, indicating that the classical

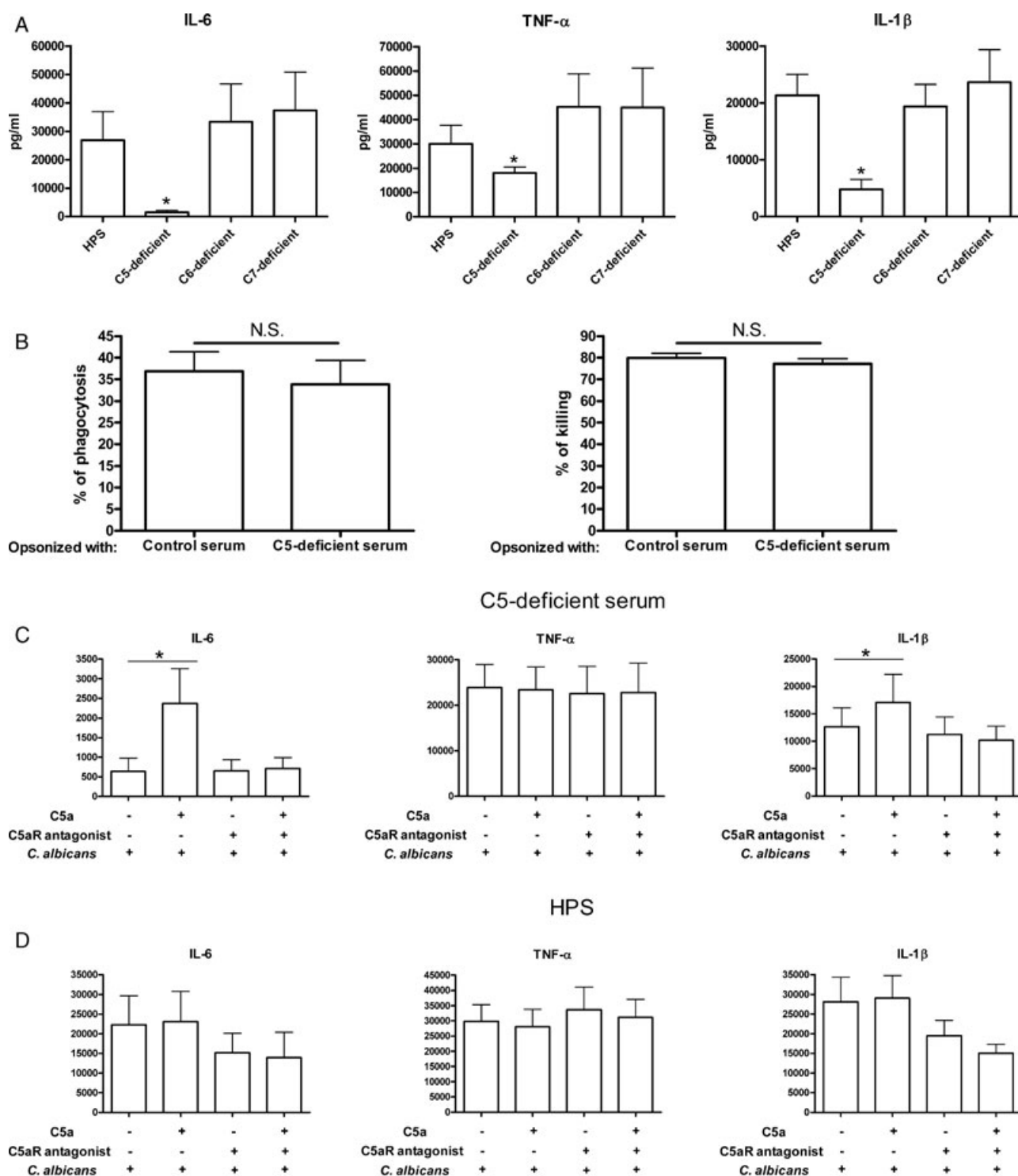


Figure 5. *Candida albicans*-induced cytokine production is severely impaired serum of C5-deficient patient. (A) Human PBMCs were stimulated with live *C. albicans* in the presence of serum from HPS, C5-, C6-, or C7-deficient patients for 24 h. Supernatant was collected for IL-6, IL-1 β , and TNF- α measurement. (B) Phagocytosis and killing ability after opsonization of live *C. albicans* with either control complement competent serum or C5-deficient serum was measured by incubating the opsonized *C. albicans* with human PBMCs at 37°C for 30 min. Human PBMCs were stimulated with live *C. albicans* in the presence of serum from (C) C5-deficient or (D) healthy pooled serum with recombinant C5a or C5a receptor antagonist, alone or in combination. Supernatant was collected for IL-6, IL-1 β , and TNF- α measurement (pooled data of three separate experiments, $n = 6$ volunteers, means $\pm$ SD, * $p < 0.05$ determined by paired Wilcoxon test).

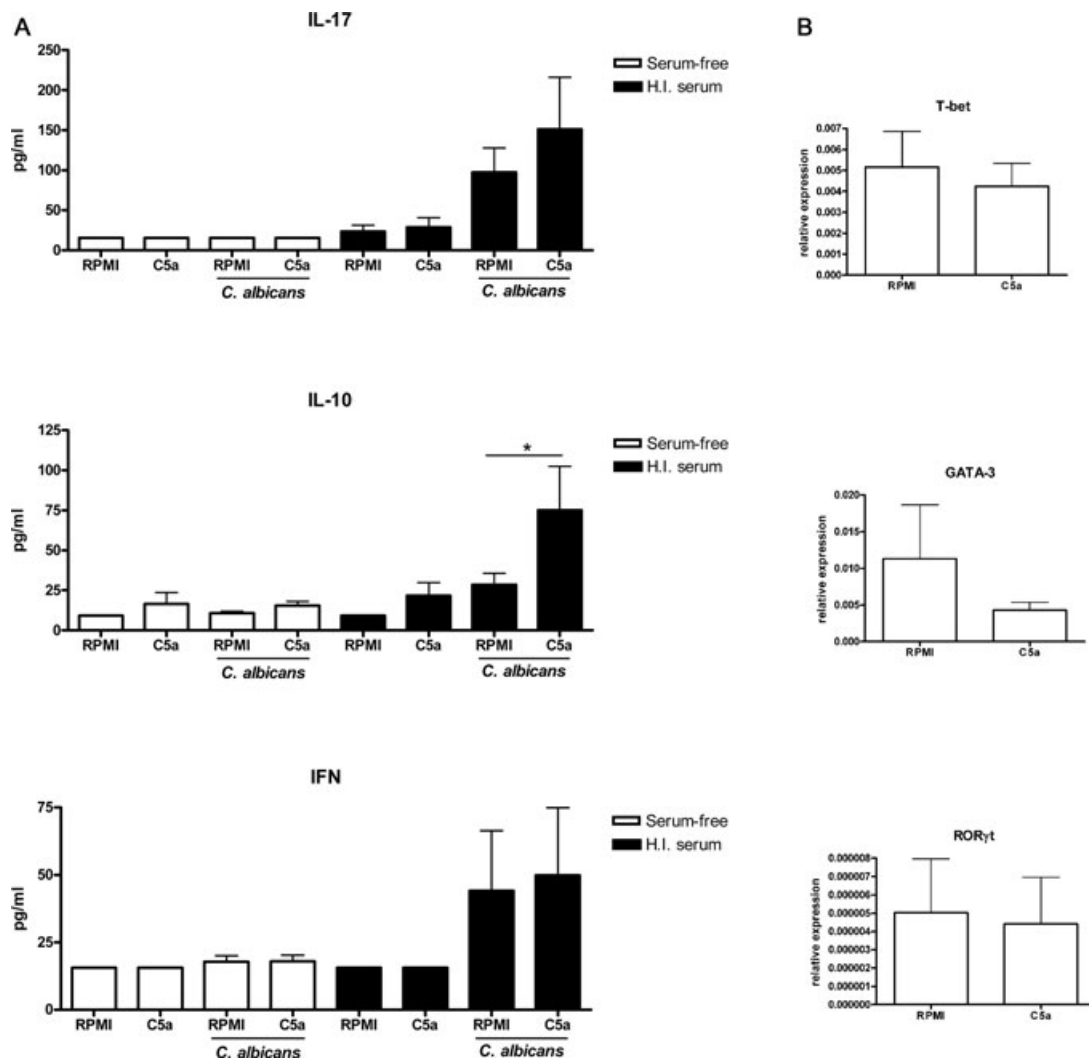


Figure 6. T helper-cell differentiation is not influenced by C5a. (A) Human PBMCs were stimulated with live *C. albicans* in the absence or presence of heat-inactivated human pooled serum for 48 h (IFN- γ and IL-10) or 7 days. Supernatant was harvested for cytokine measurement. (B) Human PBMCs were stimulated with live *C. albicans* in the absence or presence of heat-inactivated human pooled serum for 6 h. The relative expression of T-bet, GATA-3, and ROR γ t to β -2-microglobulin were determined by real-time PCR (pooled data of three separate experiments, $n = 6$ volunteers, means $\pm$ SD, * $p < 0.05$ determined by paired Wilcoxon test).

activation of complement system can stimulate cytokine production induced by *C. albicans*. Moreover, addition of antagonist antibodies against specific complement components to block either of the three complement activation pathways also leads to downregulation of cytokines, further strengthening the hypothesis that complement activation is needed for proper cytokine induction against *C. albicans* infection.

In a following set of experiments, we set to assess which complement factors are specifically needed for the effect on cytokine production. By using either an anti-C5a antagonist antibody or a C5aR antagonist, it was demonstrated that C5a is the key complement component responsible for the cytokine augmentation after stimulation of PBMCs with *C. albicans*. Both IL-6 and IL-1 β were significantly downregulated when anti-C5a antagonist antibody or C5aR antagonist were used. However, TNF- α production was reduced only when anti-C5a antagonist antibody was used,

but not C5aR antagonist. Similar cytokine induction pattern was observed when recombinant C5a was added simultaneously with *C. albicans*, with production of IL-6 and IL-1 β , but not TNF- α , being increased. This indicates that the C5a-C5aR signaling probably contributes directly to the augmentation IL-6 and IL-1 β production. A different receptor triggered by C5a, the recently described C5L2, might also be involved for the potentiation of TNF- α production. It has recently been demonstrated that the crosstalk between TLR and C5a is due to downregulation of C5L2 surface expression after TLR ligand exposure [38]. Therefore, future work is needed for elucidating the involvement of C5L2 in *C. albicans*-induced cytokine production. In line with our finding of the effect of C5a upon *C. albicans* infection, an acetylated C5a complementary peptide has been shown to reduce partially the lethal shock induced by *C. albicans* water-soluble mannoprotein- β -glucan complex in mice [39]. We also observed crosstalk between TLR4-C5aR and

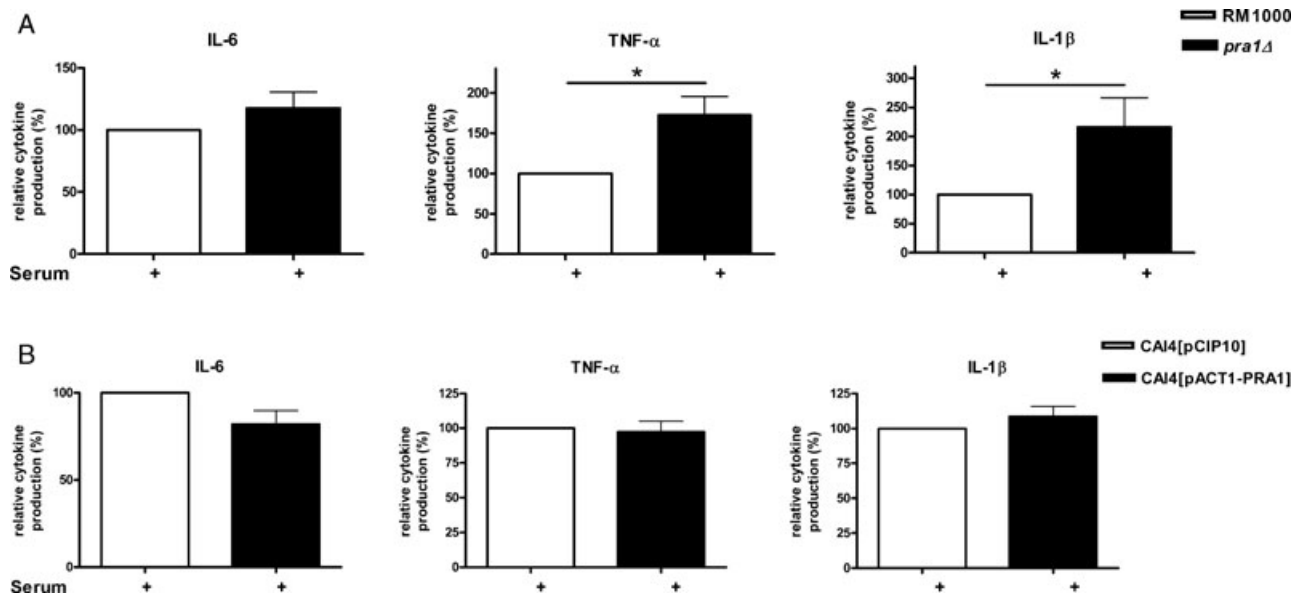


Figure 7. *Candida albicans* Pra1 partially inhibits cytokine production in PBMCs. (A) Human PBMCs were stimulated with live RM1000 (parental strain) or *pra1Δ* *C. albicans* strain in the presence of healthy human pooled serum for 24 h. Supernatant was collected for IL-6, IL-1β, and TNF-α measurement. (B) Human PBMCs were stimulated with live CAI4[pCIP10] (parental strain) or CAI4 [pACT-PRA1] overexpressing strain in the presence of normal human pooled serum for 24 h. Supernatant was collected for IL-6, IL-1β, and TNF-α measurement (pooled data of three separate experiments, $n = 6$ volunteers, means + SD, * $p < 0.05$ determined by paired Wilcoxon test).

TLR2-C5aR by using specific TLR agonists. It is noteworthy that on top of the synergism between dectin-1 and TLR2, the presence of C5a could further induce higher cytokine production, indicating that the crosstalk between TLR2 and C5a is independent from the crosstalk between TLR2 and dectin-1 (Fig. 4B). However, the effect of C5a seems to be mainly on monocyte-derived cytokines, such as IL-6 and IL-1β. In contrast, no effects have been observed on T-cell derived cytokines such as IFN-γ and IL-17, although there was a significant increase in IL-10 production in the presence of C5a, but the absolute concentrations were low (25 pg/mL (no C5a) versus 75 pg/mL (with C5a)). In addition, the T helper-cell specific transcription factors were not influenced by C5a.

In addition to these in vitro observations, C5-deficient mice were demonstrated to be more susceptible to systemic *C. albicans* infection [40,41]. Similar results were also observed in C3-deficient mouse [42]. Taken together, these in vivo data from both C3- and C5-deficient mice imply that the activation of C5 could render a protective effect against *C. albicans* infection. However, in contrast to our in vitro finding that demonstrates a role of C5a in augmenting IL-6 production, C5-deficient mice produced higher levels of circulating IL-6 in response to *C. albicans*, despite higher mortality [41]. The apparent contradiction between the in vivo and in vitro role of C5a could be attributed to the difference between humans and mice, or to the different *C. albicans* strains used in the experiments. However, this discrepancy might also be due to the nature of the experimental setting. One main characteristic of innate immunity is the prompt reaction to pathogen interaction: phagocytosis and proinflammatory cytokine/chemokine production are triggered very rapidly after encountering the pathogen. Therefore, in C5-deficient mouse,

C. albicans might have the chance to escape the host immune defense because of insufficient proinflammatory cytokine secretion due to the lack of C5a bioactivity, therefore leading to increased growth of *C. albicans*, resulting in cytokine storm due to the presence of excess amount of fungal burden [43,44]. A time-course experiment, especially during the early time point of *C. albicans* infection, in C5-deficient mice is needed to validate this hypothesis. In addition, C5a is a potent chemotactic agent for the recruitment of neutrophils [14]. Since neutropenia is one of the risk factors predisposing individuals to *C. albicans* infection [2], C5-deficiency might be detrimental.

The clinical significance of this finding has to be evaluated in future. Genetic C5 deficiency in humans is extremely rare and predisposes mainly to *Neisserial* infections. It might be anticipated that, with the increasing use of the anti-C5 antibody eculizumab, which prevents the cleavage of C5 by C5 convertase, in the treatment of paroxysmal nocturnal hemoglobinuria [45] and hemolytic uremic syndrome [46], acquired C5 deficiency will become more prevalent in the near future. Our results are an argument to monitor more carefully for the occurrence of mucosal *Candida* infections in these patients.

The Pra1 cell wall protein is released by both yeast and hyphae into the culture medium. Interestingly, soluble Pra1 could bind back to the fungal surface [47]. On the one hand, it has been demonstrated that *C. albicans* evades the complement system by binding Factor H [31], FHL-1 [47] and complement inhibitor C4b-binding protein [23] on the fungal surface. Pra1 is crucial for this effect. On the other hand, Pra1 could also block the activation and the conversion of complement C3 both in fluid and on the fungal surface [22]. This ability to inhibit complement activation might

be a strategy utilized by *C. albicans* to evade host complement activation [23, 47]. Subsequently, *C. albicans pra1*Δ strain induced higher TNF-α and IL-1β at the cellular level. However, as the Pra1 overexpressing strain failed to further inhibit host cytokine production, the endogenous expression Pra1 seems to be sufficient for executing its role in inhibiting complement activation. It might be interesting to compare the differential complement inhibition ability and the surface expression profile of Pra1 among clinical isolates from mucosal and systemic candidiasis patients. In addition, it might be worthwhile also to compare the virulence of *pra1*Δ and Pra1 overexpressing strains in an in vivo model in the future.

In conclusion, using complementary in vitro techniques and serum isolated from patients with various complement deficiencies, we demonstrate an important role of C5, specifically C5a, but not C6 or C7 and thus not the MAC, for the *C. albicans*-induced IL-6 and IL-1β in human monocytes.

Materials and methods

Reagents

Recombinant human C5a was purchased from R&D. C5a receptor antagonist (W-54001) was purchased from Santa Cruz Biotechnology (CA, USA). LPS (*Escherichia coli* serotype 055:B5) was purchased from Sigma (MO, USA). Synthetic Pam3Cys was purchased from EMC Microcollections (Tuebingen, Germany). *Candida* β-glucan and mannan were kindly provided by David Williams (East Tennessee State University, TN, USA).

Inhibitory antibodies

Mouse immunoglobulin G1 (IgG1) mAbs to human C2 (clone 175–26), factor D (clone 166–32), C5a (clone 137–26), and the isotype-matched control (clone G3–519, anti-HIV1 gp 120) were a kind gift from Michael Fung (Tanox Inc., TX, USA). The anti-C5a 137–26 antibody neutralizes C5a biologic effects by binding to the C5a moiety on native C5, without interfering with C5 cleavage. The anti-C5a antibody 137–26 is highly human specific, and no cross-reactivity has been observed with nonprimate species.

C. albicans strain

C. albicans ATCC MYA-3573 (UC 820) was used, unless otherwise indicated. *C. albicans* organisms were grown overnight in Sabouraud broth at 37°C, and cells were thereafter harvested by centrifugation, washed twice, and resuspended in culture medium (RPMI 1640; ICN Biomedicals, PA, USA) [48].

For overexpression of Pra1, the gene *PRA1* was cloned under the control of the *C. albicans ACT1* promoter and integrated into the *RPS10* locus of the *C. albicans* strain (Losse et al., unpublished data). Wild-type strain CAI4 [pCIP10] carrying pCIP10

[49] at the *RPS10* locus was used as a control for CAI4 (pACT1-PRA1) [50]. Overexpression was confirmed by RT-PCR under conditions when PRA1 was not expressed in the wild type. The mutant strain *pra1*Δ and the corresponding parental wild type strain RM1000 were kindly provided by Anne Marciel and Malcolm Whiteway, National Research Council of Canada, Montreal, Québec, Canada [51].

HPS and complement deficient serum

HPS was prepared from at least ten healthy individuals after informed consent, pooled and stored at –80°C. Antimannan antibody depleted serum was prepared as previously described [37]. In brief, antimannan antibody depleted serum was produced by two successive absorptions, each with 5×10^8 *C. albicans* yeast cells per mL serum for 30 min at 0°C. The absorbed serum was passed through a 0.45-μm filter and used immediately or stored at –80°C.

Complement factor C5-deficient serum was derived from a 25-year-old woman identified as C5-deficient because she and her brother both suffered from invasive meningococcal disease. In brief the brother, who was found to have the same genetic defect, died from fulminant meningococcal septic shock at 14 years of age. The 25-year-old woman suffered from serogroup C meningococcal meningitis at the age of 15 years. Functional analysis in the woman's plasma showed absent alternative and classical route induced haemolytic activity (AP50 < 15%; CH50 < 4%) that normalized after reconstitution with C5. Genetic analysis performed as previously described [52] revealed compound heterozygosity for a 4 bp deletion (del AAAC) causing a frameshift and resulting in a premature stop codon in Asp³⁹⁶ (NP_001726), and a nonsense mutation causing a premature stop codon in Gln¹⁶⁵⁸ of the C5 preprotein (NP_001726), inherited from the pair's father and mother, respectively. MBL levels were normal (3.22 mg/L).

C6-deficient serum was obtained from a 25-year-old man suffering from recurrent invasive meningococcal infections who was found to be C6 deficient restored after reconstitution with C6. Determination of MBL (MBL < 0.04 mg/L) further revealed complete MBL deficiency. C7-deficient serum was obtained from a 24-year-old woman with a history of chronic benign meningococcaemia at the age of 21 years. CH50 and AP50 in this patient were both undetectable, and normalized after reconstitution with C7. MBL in this patient was normal (3.78 mg/L).

Ethics statement

Patients and healthy volunteer gave written informed consent to participate in each of the studies under Institutional Review Board approval of Radboud University Nijmegen Medical Center.

Isolation and stimulation of PBMCs

Separation and stimulation of PBMCs was performed as described elsewhere [53], from buffy coats obtained from healthy blood

donors at the Bloodbank Nijmegen. Cells were adjusted to a concentration of 5×10^6 cells/mL, and thereafter incubated at 37°C in round-bottom 96-well plates (volume, 100 µL/well) with either live *C. albicans* (105/mL) or culture medium. After 24 h, 48 h, or 7 days, supernatants were collected and stored at –20°C until assay.

Cytokine measurements

IL-6, IL-10, IFN-γ (Pelikine Compact, CLB, Amsterdam, Netherlands), IL-1β, TNF-α, IL-17 (R&D, the Netherlands) concentrations from the culture supernatant were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

Phagocytosis and killing assay

The phagocytosis and killing assay are performed as described previously [54]. In short, human PBMCs (5×10^5) in 100 µL of RPMI-1640 were dispensed into the wells of a 96-well flat-bottom plate (Costar) and incubated at 37°C and 5% CO₂. Cells were incubated with 1×10^4 colony-forming unit (CFU) *C. albicans*, which was opsonized with 40 % of complement-competent control serum or C5-deficient serum in modified Eagle's medium (Gibco Life Technologies, Paisley, Scotland; minimal essential medium, MEM) for 45 min at 24°C. After 30 min of incubation, the non-phagocytosed cells were collected and the CFUs were quantified by plating on Sabouraud agar plates in a tenfold dilution manner and incubated at 37°C for 24 h. The percentage of yeast phagocytosed by the PBMCs was determined as follows: $(1 - (\text{CFU after incubation} / 1 \times 10^4)) \times 100$. Cell-free incubations of *C. albicans* yeast were included as a control.

After removal of the nonphagocytosed *C. albicans* 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 monocytes. 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 *C. albicans* yeast, 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 monocytes was determined as follows: $(1 - (\text{CFU after incubation} / \text{number of phagocytosed CFU})) \times 100$. Cell-free incubations of yeast were included as a control for yeast viability.

Quantitative PCR

The quantitative PCR (qPCR) experiment are performed as described previously [55]. PBMCs were stimulated as described above. After 6 h, the supernatant was removed, and the cells

were resuspended in 200 µL of RNeasy lysis RNA isolation solvent (Campco Scientific, The Netherlands) and stored at –80°C. mRNA was isolated according to the instruction of manufacturer. cDNA was synthesized from 1 µg of total RNA by use of SuperScript reverse transcriptase (Invitrogen, NY, USA). Relative mRNA levels were determined using the Bio-Rad i-Cycler and the SYBR Green method (Invitrogen). The following primers were used: Roryt forward primer 5'-CCGCTGAGAGGGCTTCAC-3' and reverse primer 5'-TGCAGGAGTAGGCCACATTACA-3', GATA-3 forward primer 5'-TCACAAAATGA ACGGACAGAACC-3' and reverse primer 5'-GGTGGTCTGACAGTTCGCAC-3', t-bet forward primer 5'-CAAGGGGGCGTCCAACAAT-3' and reverse primer 5'-TCTGGCTCTCCGTCGTTCA-3', and β2M forward primer 5'-ATGAGTATGCC TGCCGTGTG-3' and reverse primer 5'-CCAAATGCG GCATCTTCAAAC-3' (Biolegio, The Netherlands). Values are expressed as fold increases in mRNA levels, relative to those in unstimulated cells.

Statistical analysis

Results from at least three sets of experiments with minimum six volunteers were pooled and analyzed using GraphPad Prism software. Data are given as means ± SD and the paired Wilcoxon test was used to compare differences between groups. The level of significance was set at $p < 0.05$.

Acknowledgement: This work was supported by the grant from EU-FP7 FINSysB Marie Curie Initial Training Network (PITN-GA-2008-214004) to S. C. C., B. H., and M. G. N. M. G. N. was also supported by a Vici Grant of the Netherlands Organization for Scientific Research.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

- 1 Ferwerda, B., Ferwerda, G., Plantinga, T. S., Willment, J. A., van Spriel, A. B., Venselaar, H., Elbers, C. C. et al., Human dectin-1 deficiency and mucocutaneous fungal infections. *N. Engl. J. Med.* 2009. 361: 1760–1767.
- 2 Koh, A. Y., Kohler, J. R., Cogshall, K. T., Van, R. N. and Pier, G. B., Mucosal damage and neutropenia are required for *Candida albicans* dissemination. *PLoS Pathog.* 2008. 4: e35.
- 3 Perlroth, J., Choi, B. and Spellberg, B., Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med. Mycol.* 2007. 45: 321–346.
- 4 Netea, M. G., Gow, N. A., Munro, C. A., Bates, S., Collins, C., Ferwerda, G., Hobson, R. P. et al., Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest.* 2006. 116: 1642–1650.
- 5 Heinsbroek, S. E., Taylor, P. R., Martinez, F. O., Martinez-Pomares, L., Brown, G. D. and Gordon, S., Stage-specific sampling by pattern

- recognition receptors during *Candida albicans* phagocytosis. *PLoS Pathog.* 2008. 4: e1000218.
- 6 Netea, M. G., Brown, G. D., Kullberg, B. J. and Gow, N. A. R., An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat. Rev. Micro.* 2008. 6: 67–78.
 - 7 Ricklin, D., Hajishengallis, G., Yang, K. and Lambris, J. D., Complement: a key system for immune surveillance and homeostasis. *Nat. Immunol.* 2010. 11: 785–797.
 - 8 Zhang, M. X., Lupan, D. M. and Kozel, T. R., Mannan-specific immunoglobulin G antibodies in normal human serum mediate classical pathway initiation of C3 binding to *Candida albicans*. *Infect. Immun.* 1997. 65: 3822–3827.
 - 9 Zhang, M. X. and Kozel, T. R., Mannan-specific immunoglobulin G antibodies in normal human serum accelerate binding of C3 to *Candida albicans* via the alternative complement pathway. *Infect. Immun.* 1998. 66: 4845–4850.
 - 10 Sealy, P. I., Garner, B., Swiatlo, E., Chapman, S. W. and Cleary, J. D., The interaction of mannose binding lectin (MBL) with mannose containing glycopeptides and the resultant potential impact on invasive fungal infection. *Med. Mycol.* 2008. 1–9.
 - 11 Marodi, L., Korchak, H. M. and Johnston, R. B., Jr., Mechanisms of host defense against *Candida* species. I. Phagocytosis by monocytes and monocyte-derived macrophages. *J. Immunol.* 1991. 146: 2783–2789.
 - 12 Speth, C., Rambach, G., Wurzner, R. and Lass-Flörl, C., Complement and fungal pathogens: an update. *Mycoses* 2008. 51: 477–496.
 - 13 Kozel, T. R., Activation of the complement system by pathogenic fungi. *Clin. Microbiol. Rev.* 1996. 9: 34–46.
 - 14 Klos, A., Tenner, A. J., Johswich, K. O., Ager, R. R., Reis, E. S. and Kohl, J., The role of the anaphylatoxins in health and disease. *Mol. Immunol.* 2009. 46: 2753–2766.
 - 15 Sonesson, A., Ringstad, L., Nordahl, E. A., Malmsten, M., Morgelin, M. and Schmidtchen, A., Antifungal activity of C3a and C3a-derived peptides against *Candida*. *Biochim. Biophys. Acta* 2007. 1768: 346–353.
 - 16 Mollnes, T. E., Brekke, O. L., Fung, M., Fure, H., Christiansen, D., Bergseth, G., Videm, V. et al., Essential role of the C5a receptor in *E. coli*-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 2002. 100: 1869–1877.
 - 17 Sprong, T., Brandtzaeg, P., Fung, M., Pharo, A. M., Hoiby, E. A., Michaelsen, T. E., Aase, A. et al., Inhibition of C5a-induced inflammation with preserved C5b-9-mediated bactericidal activity in a human whole blood model of meningococcal sepsis. *Blood* 2003. 102: 3702–3710.
 - 18 Brouwer, N., Dolman, K. M., van, H. M., Sta, M., Roos, D. and Kuijpers, T. W., Mannose-binding lectin (MBL) facilitates opsonophagocytosis of yeasts but not of bacteria despite MBL binding. *J. Immunol.* 2008. 180: 4124–4132.
 - 19 Guo, R. F. and Ward, P. A., Role of C5a in inflammatory responses. *Annu. Rev. Immunol.* 2005. 23: 821–852.
 - 20 Wetsel, R. A., Structure, function and cellular expression of complement anaphylatoxin receptors. *Curr. Opin. Immunol.* 1995. 7: 48–53.
 - 21 Sumichika, H., Sakata, K., Sato, N., Takeshita, S., Ishibuchi, S., Nakamura, M., Kamahori, T. et al., Identification of a potent and orally active non-peptide C5a receptor antagonist. *J. Biol. Chem.* 2002. 277: 49403–49407.
 - 22 Luo, S., Hartmann, A., Dahse, H. M., Skerka, C. and Zipfel, P. F., Secreted pH-regulated antigen 1 of *Candida albicans* blocks activation and conversion of complement C3. *J. Immunol.* 2010. 185: 2164–2173.
 - 23 Luo, S., Blom, A. M., Rupp, S., Hipler, U. C., Hube, B., Skerka, C. and Zipfel, P. F., The pH-regulated antigen 1 of *Candida albicans* binds the human complement inhibitor C4b-binding protein and mediates fungal complement evasion. *J. Biol. Chem.* 2011. 286: 8021–8029.
 - 24 Ip, W. K. and Lau, Y. L., Role of mannose-binding lectin in the innate defense against *Candida albicans*: enhancement of complement activation, but lack of opsonic function, in phagocytosis by human dendritic cells. *J. Infect. Dis.* 2004. 190: 632–640.
 - 25 Kagaya, K. and Fukazawa, Y., Murine defense mechanism against *Candida albicans* infection. II. Opsonization, phagocytosis, and intracellular killing of *C. albicans*. *Microbiol. Immunol.* 1981. 25: 807–818.
 - 26 Solomkin, J. S., Mills, E. L., Giebink, G. S., Nelson, R. D., Simmons, R. L. and Quie, P. G., Phagocytosis of *Candida albicans* by human leukocytes: opsonic requirements. *J. Infect. Dis.* 1978. 137: 30–37.
 - 27 Gropp, K., Schild, L., Schindler, S., Hube, B., Zipfel, P. F. and Skerka, C., The yeast *Candida albicans* evades human complement attack by secretion of aspartic proteases. *Mol. Immunol.* 2009. 47: 465–475.
 - 28 Meri, T., Blom, A. M., Hartmann, A., Lenk, D., Meri, S. and Zipfel, P. F., The hyphal and yeast forms of *Candida albicans* bind the complement regulator C4b-binding protein. *Infect. Immun.* 2004. 72: 6633–6641.
 - 29 Meri, T., Hartmann, A., Lenk, D., Eck, R., Wurzner, R., Hellwage, J., Meri, S. et al., The yeast *Candida albicans* binds complement regulators factor H and FHL-1. *Infect. Immun.* 2002. 70: 5185–5192.
 - 30 Poltermann, S., Kunert, A., von der, H. M., Eck, R., Hartmann, A. and Zipfel, P. F., Gpm1p is a factor H-, FHL-1-, and plasminogen-binding surface protein of *Candida albicans*. *J. Biol. Chem.* 2007. 282: 37537–37544.
 - 31 Lesiak-Markowicz, I., Vogl, G., Schwarzmüller, T., Speth, C., Lass-Flörl, C., Dierich, M. P., Kuchler, K. et al., *Candida albicans* Hgt1p, a multifunctional evasion molecule: complement inhibitor, CR3 analogue, and human immunodeficiency virus-binding molecule. *J. Infect. Dis.* 2011. 204: 802–809.
 - 32 Boxx, G. M., Kozel, T. R., Nishiya, C. T. and Zhang, M. X., Influence of mannan and glucan on complement activation and C3 binding by *Candida albicans*. *Infect. Immun.* 2010. 78: 1250–1259.
 - 33 Hajishengallis, G. and Lambris, J. D., Crosstalk pathways between Toll-like receptors and the complement system. *Trends Immunol.* 2010. 31: 154–163.
 - 34 Riedemann, N. C., Guo, R. F., Bernacki, K. D., Reuben, J. S., Laudes, I. J., Neff, T. A., Gao, H. et al., Regulation by C5a of neutrophil activation during sepsis. *Immunity* 2003. 19: 193–202.
 - 35 Cavaillon, J. M., Fitting, C. and Haeflner-Cavaillon, N., Recombinant C5a enhances interleukin 1 and tumor necrosis factor release by lipopolysaccharide-stimulated monocytes and macrophages. *Eur. J. Immunol.* 1990. 20: 253–257.
 - 36 Wang, M., Krauss, J. L., Domon, H., Hosur, K. B., Liang, S., Magotti, P., Triantafyllou, M. et al., Microbial hijacking of complement-Toll-like receptor crosstalk. *Sci. Signal.* 2010. 3: ra11.
 - 37 Boxx, G. M., Nishiya, C. T., Kozel, T. R. and Zhang, M. X., Characteristics of Fc-independent human antimannan antibody-mediated alternative pathway initiation of C3 deposition to *Candida albicans*. *Mol. Immunol.* 2009. 46: 473–480.
 - 38 Raby, A. C., Holst, B., Davies, J., Colmont, C., Laumonnier, Y., Coles, B., Shah, S. et al., TLR activation enhances C5a-induced pro-inflammatory responses by negatively modulating the second C5a receptor, C5L2. *Eur. J. Immunol.* 2011. 41: 2741–2752.
 - 39 Okada, N., Asai, S., Hotta, A., Miura, N., Ohno, N., Farkas, I., Hau, L. et al., Increased inhibitory capacity of an anti-C5a complementary peptide following acetylation of N-terminal alanine. *Microbiol. Immunol.* 2007. 51: 439–443.
 - 40 Fulurija, A., Ashman, R. B. and Papadimitriou, J. M., Early inflammatory responses to *Candida albicans* infection in inbred and complement-deficient mice. *FEMS Immunol. Med. Microbiol.* 1996. 14: 83–94.

- 41 Mullick, A., Elias, M., Picard, S., Bourget, L., Jovceviski, O., Gauthier, S., Tuite, A. et al., Dysregulated inflammatory response to *Candida albicans* in a C5-deficient mouse strain. *Infect. Immun.* 2004. **72**: 5868–5876.
- 42 Tsoni, S. V., Kerrigan, A. M., Marakalala, M. J., Srinivasan, N., Duffield, M., Taylor, P. R., Botto, M. et al., Complement C3 plays an essential role in the control of opportunistic fungal infections. *Infect. Immun.* 2009. **77**: 3679–3685.
- 43 Netea, M. G., van der Meer, J. W., van, D. M. and Kullberg, B. J., Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol.* 2003. **24**: 254–258.
- 44 Gerard, C., Complement C5a in the sepsis syndrome—too much of a good thing? *N. Engl. J. Med.* 2003. **348**: 167–169.
- 45 Hillmen, P., Young, N. S., Schubert, J., Brodsky, R. A., Socie, G., Muus, P., Roth, A. et al., The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.* 2006. **355**: 1233–1243.
- 46 Gruppo, R. A. and Rother, R. P., Eculizumab for congenital atypical hemolytic-uremic syndrome. *N. Engl. J. Med.* 2009. **360**: 544–546.
- 47 Luo, S., Poltermann, S., Kunert, A., Rupp, S. and Zipfel, P. F., Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein. *Mol. Immunol.* 2009. **47**: 541–550.
- 48 van der Graaf, C. A., Netea, M. G., Verschuieren, I., Van der Meer, J. W. and Kullberg, B. J., Differential cytokine production and Toll-like receptor signaling pathways by *Candida albicans* blastoconidia and hyphae. *Infect. Immun.* 2005. **73**: 7458–7464.
- 49 Murad, A. M., Lee, P. R., Broadbent, I. D., Barelle, C. J. and Brown, A. J., Clp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* 2000. **16**: 325–327.
- 50 Fradin, C., de Groot, P., Maccallum, D., Schaller, M., Klis, F., Odds, F. C. and Hube, B., Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol. Microbiol.* 2005. **56**: 397–415.
- 51 Marcil, A., Gadoury, C., Ash, J., Zhang, J., Nantel, A. and Whiteway, M., Analysis of PRA1 and its relationship to *Candida albicans*-macrophage interactions. *Infect. Immun.* 2008. **76**: 4345–4358.
- 52 Lappegard, K. T., Christiansen, D., Pharo, A., Thorgersen, E. B., Hellerud, B. C., Lindstad, J., Nielsen, E. W. et al., Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. *Proc. Natl. Acad. Sci. U.S.A.* 2009. **106**: 15861–15866.
- 53 Netea, M. G., Gow, N. A., Munro, C. A., Bates, S., Collins, C., Ferwerda, G., Hobson, R. P. et al., Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest.* 2006. **116**: 1642–1650.
- 54 Netea, M. G., Vonk, A. G., van den, H. M., Verschuieren, I., Joosten, L. A., van Krieken, J. H., van den Berg, W. B. et al., Differential role of IL-18 and IL-12 in the host defense against disseminated *Candida albicans* infection. *Eur. J. Immunol.* 2003. **33**: 3409–3417.
- 55 Cheng, S. C., Chai, L. Y., Joosten, L. A., Vecchiarelli, A., Hube, B., van der Meer, J. W., Kullberg, B. J. et al., *Candida albicans* releases soluble factors that potentiate cytokine production by human cells through a protease-activated receptor 1- and 2-independent pathway. *Infect. Immun.* 2010. **78**: 393–399.

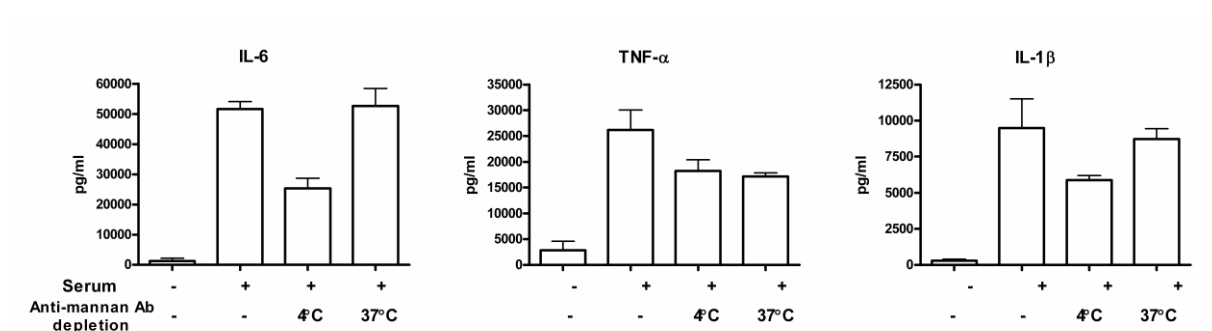
Abbreviations: C5aR: C5a receptor · HPS: human pooled serum · MAC: membrane attack complex · MR: mannose receptor

Full correspondence: Dr. Mihai G. Netea, Department of Medicine (463), Radboud University Nijmegen Medical Center, P.O. Box 9101, Geert Grootplein 8, 6525 GA Nijmegen, The Netherlands
 Fax: +31-243541734
 e-mail: M.Netea@aig.umcn.nl

See accompanying commentary:
<http://dx.doi.org/10.1002/eji.201242466>

Received: 23/8/2011
 Revised: 3/11/2011
 Accepted: 22/12/2011
 Accepted article online: 4/1/2012

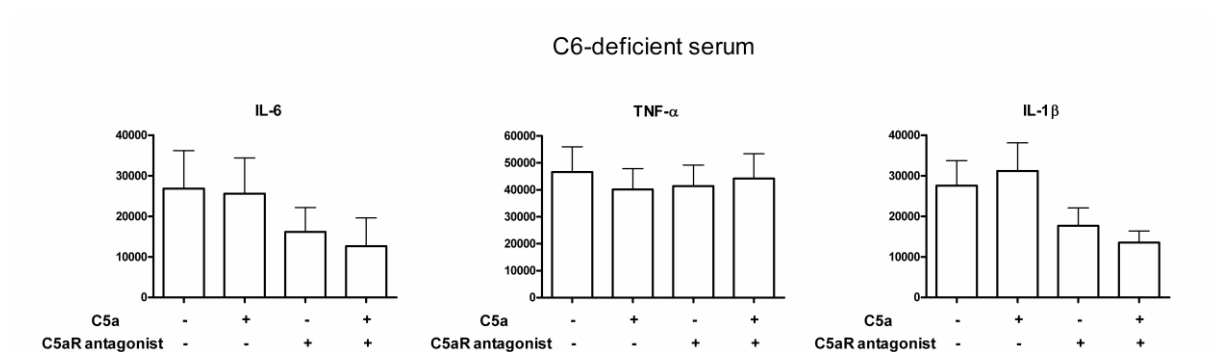
Supporting Information Figure 1



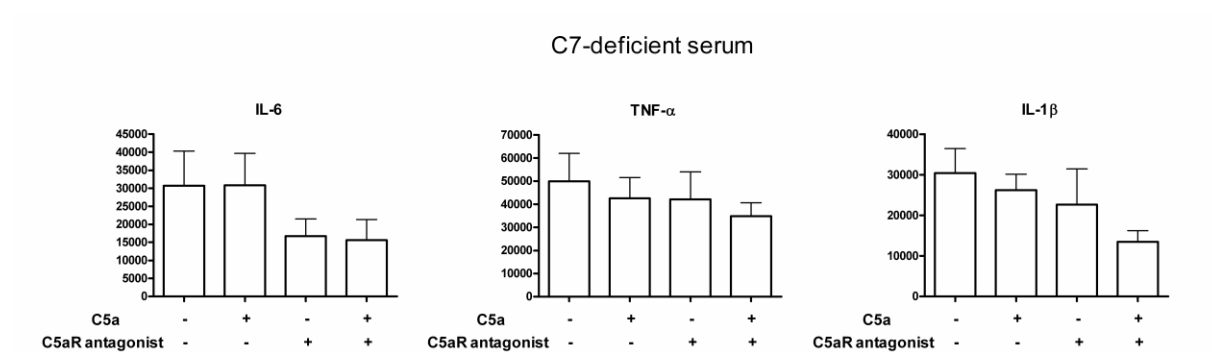
Human PBMCs were stimulated with live *C. albicans* in the presence of human pooled serum or anti-mannan antibody depleted human pooled serum, the depletion step was at either 4 °C or 37 °C, for 24 hours. Supernatant was collected for IL6, IL-1β and TNF-α measurement.

Supporting Information Figure 2

A



B



Human PBMCs were stimulated with live *C. albicans* in the presence of serum from (A) C6-deficient or (B) C7-deficient serum with recombinant C5a or C5a receptor antagonist, alone or in combination. Supernatant was collected for IL6, IL-1 β and TNF measurement.