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A Second Stimulus Required for Enhanced Antifungal Activity of Human Neutrophils in Blood Is Provided by Anaphylatoxin C5a

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Polymorphonuclear neutrophilic granulocytes (PMN) as cellular components of innate immunity play a crucial role in the defense against systemic *Candida albicans* infection. To analyze stimuli that are required for PMN activity during *C. albicans* infection in a situation similar to in vivo, we used a human whole-blood infection model. In this model, PMN activation 10 min after *C. albicans* infection was largely dependent on the anaphylatoxin C5a. Most importantly, C5a enabled blood PMN to overcome filament-restricted recognition of *C. albicans* and allowed efficient elimination of nonfilamentous *C. albicans cph1Δlfq1Δ* from blood. Major PMN effector mechanisms, including oxidative burst, release of secondary granule contents and initial fungal phagocytosis could be prevented by blocking C5a receptor signaling. Identical effects were achieved using a humanized Ab specifically targeting human C5a. Phagocytosis of *C. albicans* 10 min postinfection was mediated by C5a-dependent enhancement of CD11b surface expression on PMN, thus establishing the C5a-C5aR-CD11b axis as a major modulator of early anti-*Candida* immune responses in human blood. In contrast, phagocytosis of *C. albicans* by PMN 60 min postinfection occurred almost independently of C5a and mainly contributed to activation of phagocytically active PMN at later time points. Our results show that C5a is a critical mediator in human blood during *C. albicans* infection. *The Journal of Immunology*, 2015, 194: 1199–1210.

Sepsis is defined as a clinical syndrome resulting from infection-triggered uncontrolled and systemic dysregulation of the immune system. In recent years, the spectrum of pathogens associated with the development of sepsis has changed and fungal pathogens, mostly *Candida albicans* and other *Candida spp.*, have increased dramatically (1). Initial activation of the immune system in sepsis is dominated by innate mechanisms. Among those, neutrophilic granulocytes (polymorphonuclear neutrophilic granulocytes [PMN]) have been found to play a central role in the defense against invasive candidiasis and neutropenia is an independent risk factor (2). Isolated human neutrophils respond to *C. albicans* in a morphotype-specific manner: only filamentous forms, which have been associated with tissue invasion and destruction, trigger an activation program that leads to targeted motility, degranulation, formation of reactive oxygen intermediates (ROI), and secretion of the chemotactic factor IL-8 (3). Rapid engulfment of filamentous *C. albicans* by activated neutrophils results in efficient killing of the pathogen. Furthermore, PMN are the only known host cell type that strongly inhibits filamentation of *C. albicans*. Among all components of blood, PMN have been shown to dominate the transcriptional adaptation of *C. albicans* and mediate elimination of the yeast from human blood (4, 5). PMN antifungal activity is triggered by activation of pattern recognition receptors as well as various opsonin receptors (6). Opsonisation by complement promotes phagocytosis through complement receptors CR1, CR3, and CR4 (7). Among these, complement receptor 3 (CR3, also known as Mac-1; CD11b/CD18; integrin αβ₂) has been shown to play a crucial role for uptake of *C. albicans*, recognizing a range of potential ligands (8). CR3 is the major receptor for complement product C3b and its cleavage product iC3b and can therefore recognize fungi opsonized by complement. Furthermore, the *C. albicans* surface protein Pra1 as well as other cell-wall components can directly bind to CR3 (9). Finally, *C. albicans* harbors a set of proteins that can recruit the complement regulator factor H to its surface. Factor H is by itself a ligand for CR3 and has been shown to mediate binding of pathogenic microorganisms to this receptor (10). In sepsis, complement activation is of central importance for pathological immune activation. *C. albicans* activates all three pathways of the complement system (11), resulting in generation of chemotactic cleavage fragments (12) and subsequent fungal opsonization by C3b which facilitates phagocytosis (13). In parallel, *C. albicans* has been shown to regulate complement activation at multiple stages and is able to cleave complement components and recruit factor H to its surface resulting in conversion of C3b to iC3b (8). In contrast to bacterial pathogens, it is...
unlikely that formation of the membrane-attack complex C5b-C9 contributes to *C. albicans* killing (11). Among complement activation products, the anaphylatoxin C5a, generated by cleavage of C5 by a C5 convertase mediates a broad range of proinflammatory effects: it is a strong chemoattractant for neutrophils as well as monocytes and increases PMN effector functions (oxidative burst, phagocytosis, and degranulation) (14–16). Elevated plasma levels of C5a are found in sepsis patients. However, several studies demonstrate that an excessive C5a production during sepsis is correlated with harmful effects for the poor outcome (17–19), and a blockade of either C5a or the C5aR has been shown to be protective in experimental sepsis (20, 21). Consequently, C5a has been proposed as a promising therapeutic target in sepsis. Recently published data also provide evidence for a role of C5a in human antifungal defense as C5a increases the *C. albicans*-induced inflammatory cytokine release from PBMCs (22).

To investigate the complex inflammatory network activated in response to systemic *C. albicans* infection in a situation similar to in vivo, we previously established a human whole-blood infection model. In this study, the model was used to analyze mechanisms that contribute to antifungal activity of human blood with regard to cellular and humoral components (23). Our data show that at 10 min postinfection (p.i.) activation of complement and generation of its activation product C5a predominantly contribute to *C. albicans*-induced PMN effector mechanisms, including phagocytosis, oxidative burst, and degranulation. In contrast, 60 min p.i.,

FIGURE 1. Different activation patterns of primary PMN and blood PMN in response to *C. albicans*. PMN activation is shown 10 min (A) and 60 min (B) after inoculation of *C. albicans* yeasts into primary PMN confrontation assay or human whole blood by detection of intracellular generated reactive oxygen intermediates and by changes in surface expression levels of the activation markers CD66b and CD16. Gray filled histograms indicate basal expression on PMN from mock-infected samples. Black open histograms indicate surface levels following *C. albicans* infection and differentiate between the entire PMN population (solid line), PMN without fungal contact (dashed line), and PMN that interact with *C. albicans* (dotted line). Data from one of at least three independent experiments with virtually identical results are shown.
phagocytosis of *C. albicans* resulted in PMN activation independent of C5a. However, PMN without fungal contact, which formed the majority of the PMN population, were still affected by C5a and induced to release antimicrobial effector proteins. Most importantly, C5a enabled human PMN to effectively eliminate *C. albicans* yeast cells from the circulation, despite the lack of the filament specific activation stimulus.

### Materials and Methods

#### Fungal strains and culture

*C. albicans* wild-type (SC5314) was used for most experiments (24). Construction of GFP-expressing *C. albicans* was described in Ref. 5. The *C. albicans* cph1Δ/Δ mutant (cph1::hisG/Δcph1, hisG::raf1/ΔhisG) and the corresponding parental strain CA4 (ura3::imm434/ura3::imm434, rps1::Clp1p10) were provided by B. Hube (HKI Jena, Jena, Germany). For infection, *C. albicans* was grown overnight in yeast extract–peptone–dextrose medium (2% D-glucose, 1% peptone, and 0.5% yeast extract in water) at 30°C, resuspended in yeast extract–peptone–dextrose medium, grown for 5 h at 30°C into the mid-log phase, and harvested in HBSS. Yeast cells were reseeded in RPMI 1640 medium and cultured at 37°C to induce filamentous growth in *C. albicans*. Both *C. albicans* yeasts and filaments were killed by incubation in 0.05% thimerosal (Sigma-Aldrich) in HBSS at 37°C for 1 h and then rinsed extensively. For subsequent differentiation between intact cells and *C. albicans* cph1Δ/Δmutant and the corresponding parental strain CA4 via FACS, Candida were labeled by incubation for 15 min with FITC (Sigma-Aldrich) dissolved in HBSS.

#### Inhibitory antibodies

The anti-C5a Ab (IFX-1) was provided by InflaRX and is a humanized mAb with mouse anti-human CD66b-V450 (clone G10F5), which was obtained (HKI Jena, Jena, Germany). For an infection, *C. albicans* was grown overnight in yeast extract–peptone–dextrose medium (2% D-glucose, 1% peptone, and 0.5% yeast extract in water) at 30°C, resuspended in yeast extract–peptone–dextrose medium, grown for 5 h at 30°C into the mid-log phase, and harvested in HBSS. Yeast cells were reseeded in RPMI 1640 medium and cultured at 37°C to induce filamentous growth in *C. albicans*. Both *C. albicans* yeasts and filaments were killed by incubation in 0.05% thimerosal (Sigma-Aldrich) in HBSS at 37°C for 1 h and then rinsed extensively. For subsequent differentiation between intact cells and *C. albicans* cph1Δ/Δmutant and the corresponding parental strain CA4 via FACS, Candida were labeled by incubation for 15 min with FITC (Sigma-Aldrich) dissolved in HBSS.

### Whole-blood model

Human peripheral blood was collected from healthy volunteers after informed consent according to guidelines from the local ethics committee. For anticoagulation, we used 50 μg/ml lepirudin (Refudlane, Cergene), a recombinant hirudin that does not influence complement activation (23). Construction of GFP-expressing *C. albicans* was described in Ref. 5. The *C. albicans* cph1Δ/Δ mutant (cph1::hisG/Δcph1, hisG::raf1/ΔhisG) and the corresponding parental strain CA4 (ura3::imm434/ura3::imm434, rps1::Clp1p10) were provided by B. Hube (HKI Jena, Jena, Germany). For infection, *C. albicans* was grown overnight in yeast extract–peptone–dextrose medium (2% D-glucose, 1% peptone, and 0.5% yeast extract in water) at 30°C, resuspended in yeast extract–peptone–dextrose medium, grown for 5 h at 30°C into the mid-log phase, and harvested in HBSS. Yeast cells were reseeded in RPMI 1640 medium and cultured at 37°C to induce filamentous growth in *C. albicans*. Both *C. albicans* yeasts and filaments were killed by incubation in 0.05% thimerosal (Sigma-Aldrich) in HBSS at 37°C for 1 h and then rinsed extensively. For subsequent differentiation between intact cells and *C. albicans* cph1Δ/Δmutant and the corresponding parental strain CA4 via FACS, Candida were labeled by incubation for 15 min with FITC (Sigma-Aldrich) dissolved in HBSS.

#### Inhibitory antibodies

The anti-C5a Ab (IFX-1) was provided by InflaRX and is a humanized mAb that blocks C5a directly and thereby neutralizes its biologic effects. Purified human IgG4.k was purchased from Sigma-Aldrich. The mouse anti-human C5aR (clone S5/1) and the corresponding purified mouse IgG2a, isotype-matched control IgG1, human IgG4, human IgG1, human IgG2, human IgG3, human IgG1k, human IgG4k, and the isotype-matched control IgG1k were purchased from Sigma-Aldrich and dissolved in HBSS.

#### Whole-blood model

Human peripheral blood was collected from healthy volunteers after informed consent according to guidelines from the local ethics committee. For anticoagulation, we used 50 μg/ml lepirudin (Refudlane, Cergene), a recombinant hirudin that does not influence complement activation (23).

In some experiments, aliquots of whole blood were preincubated with blocking Ab or the corresponding isotype control for 5 min at 37°C. HBSS or *C. albicans* in a final concentration of 1 × 10^6 yeast cells/ml whole blood was added and further incubated for various time points (as indicated) at 37°C (5). After incubation, samples were immediately subject to flow cytometry. To collect plasma samples, whole-blood aliquots were immediately placed on ice, centrifuged (10 min, 13,200 rpm, 4°C), and harvested in HBSS. Yeast cells were reseeded in RPMI 1640 medium and cultured at 37°C to induce filamentous growth in *C. albicans*. Both *C. albicans* yeasts and filaments were killed by incubation in 0.05% thimerosal (Sigma-Aldrich) in HBSS at 37°C for 1 h and then rinsed extensively. For subsequent differentiation between intact cells and *C. albicans* cph1Δ/Δmutant and the corresponding parental strain CA4 via FACS, Candida were labeled by incubation for 15 min with FITC (Sigma-Aldrich) dissolved in HBSS.

#### Isolation of human PMN

Human PMN were isolated from peripheral blood of healthy donors by density gradient centrifugation using Polymorphprep (25). Remaining RBCs were removed with ACK Lysing Buffer (Life Technologies), and purity of PMN was checked by flow cytometry to be >95%. PMN were adjusted to a concentration of 2 × 10^8 cells/ml in medium (RPMI 1640 + 5% heat-inactivated human serum).

#### Confrontation assays with human PMN

Confrontation assays with human PMN were performed as described previously (3). In selected experiments, recombinant human C5a (Hyclut Biotech) was added at different concentrations (as indicated) and PMN activation was analyzed by flow cytometry following 30 min of incubation.

#### Flow cytometry

Analyses of PMN in whole blood with regard to phagocytosis of fungal cells or their expression of cell surface activation markers were performed using differential FACS staining and subsequent measurement with the FACSCantoll. To specifically identify PMN, 100 μl whole blood was stained with mouse anti-human CD66bf-V450 (clone G10F5), which was obtained from BD Biosciences. Changes in surface expression were investigated for FCyRII (mouse anti-human CD16-allophycocyanin, clone 3G8), CD11b subunit of CR3 (mouse anti-human CD11b-allophycocyanin, clone ICRF44), C5aR (mouse anti-human C5aR-PE, clone SS/1) and L-Selectin (mouse anti-human L-selectin-allophycocyanin, clone DREG-56) using Abs obtained from BioLegend. Extracellular fungi were stained with anti-*C. albicans* Ab (Acris Abs). The stained samples were treated with BD FACS Lysing solution which lyses erythrocytes while preserving and fixing leukocytes, followed by washing and harvesting cells in BD CellWASH solution. In addition, staining of primary PMN (2 × 10^5 cells/sample) was performed as with whole blood, without lysis.

FlowJo 7.6.4 software was used for analysis. The strategy used to evaluate the association of *C. albicans* to PMN in primary PMN confrontation assay and in human blood is shown in Supplemental Fig. 1.

#### Oxidative burst

The PMN oxidative burst was measured using commercially available Burstset (Orpegen Pharma) that quantitative determine intracellular generated ROI by conversion of the fluorescent substrate dihydrothorodamine 123 to rhodamine 123. Immediately after incubation, 100 μl whole blood or 2 × 10^5 isolated PMN were treated according to the manufacturer’s instructions. Results were expressed as median fluorescence intensity of the whole PMN population.

#### Quantitative determination of C5a in human plasma

The C5a plasma concentration generated during a *C. albicans* blood infection was measured using the BD Cytometric Bead Array - Human Anaphylatoxin Kit, according to the manufacturer’s instruction.

#### Quantification of secreted proteins

The concentrations of secreted proteins within plasma samples were determined using Luminex technology (MILLIPLEX MAP Human Sepsis Magnetic Bead Panel 2 (IL-8), MILLIPLEX MAP Human Sepsis Magnetic Bead Panel 3 (lactoferrin, elastase-2, neutrophil gelatinase–associated lipocalin [NGAL]), MILLIPLEX MAP Human Cardiovascular Disease Magnetic Bead Panel 2 (myeloperoxidase [MPO]); Millipore and Procarta Immunoassay Kit - Magnetic Beads, Human (IFN-γ, TNF-α; Affymetrix). The analyses were performed according to the instructions from the manufacturer.

#### Statistics

Estimation of p values was performed with unpaired, two-sided Student t test.

### Results

**Very early PMN activation and bystander activation of cells without direct contact to *C. albicans* occurs in blood but not in isolated PMN**

Previous experiments have shown that *C. albicans* predominantly associates with PMN during ex vivo performed whole-blood infection assays and that PMN play a major role in the killing of *C. albicans* in primary confrontation assays using isolated human cells (3, 4). To gain further insight into these processes, we analyzed PMN activation in whole-blood infection in more detail. To identify the influence of blood components on PMN, activation patterns of blood PMN were compared with those of isolated PMN (Fig. 1). In contrast to isolated human PMN, PMN in whole blood were strongly activated within 10 min of infection with *C. albicans*. This activation resulted in induction of high amounts of ROI at this early time point, whereas no ROI were detected in isolated PMN after 10 min and ROI levels of isolated PMN after 60 min of coinoculation were still lower than for blood PMN after 10 min (Fig. 1). In addition, degranulation marker CD66b showed a significantly increased surface exposure on whole-blood PMN after 10 min, which was not detected for isolated PMN. Furthermore, whereas CD66b upregulation in isolated PMN at 60 min p.i. was largely restricted to those cells that were associated (phagocytosed or at least bound to) with *C. albicans*, activation of the entire PMN population occurred in blood, regardless of whether
FIGURE 2. C5a contributes to the early PMN activation during whole-blood infection with \textit{C. albicans}. Human whole blood was treated with an anti-C5a Ab (IFX-1) prior to inoculation of \textit{C. albicans} for 10 and 60 min to investigate the impact of C5a on activation of blood PMN. Samples were compared with either nontreated or IgG4-containing \textit{C. albicans}–infected blood. (A) The percentages of ROI formation in samples inoculated with \textit{C. albicans} were calculated relative to basal levels in the mock-infected sample (set to 100%). The bars show mean ± SD of at least seven independent experiments with blood from different donors; **$p < 0.01$. (B) Differential regulation of surface exposure of degranulation marker CD66b is shown in histograms. Gray filled histograms indicate basal expression on PMN in the mock-infected sample. Black open histograms indicate surface levels following \textit{C. albicans} infection and differentiate between PMN without fungal contact (dashed line) and PMN that interact with \textit{C. albicans} (dotted line). \textit{C. albicans} samples were either nontreated (a) or preincubated with IgG4 (b) or anti-C5a (c). (C) Plasma samples of whole-blood infection experiments were analyzed for secretion of antimicrobial peptides from primary (MPO, elastase-2) and secondary neutrophilic granules (NGAL, lactoferrin) and the release of IL-8. In comparison with non- (white bars) and IgG4-treated infected samples (striped bars), presence of anti-C5a Ab (black bars) significantly prevented the increase in plasma levels of lactoferrin, NGAL and IL-8 in response to \textit{C. albicans} ($t = 60$ min). Gray bars indicate basal plasma levels within mock-infected blood. Bars show means ± SD of nine independent experiments with whole blood from different donors. **$p < 0.01$. ns, nonsignificant.
the cells were in direct contact with \textit{C. albicans} or not. Analysis of the subgroup of PMN that had associated with \textit{C. albicans} after 10 min (7.3 ± 3.2% of all PMN) showed that these PMN had significantly higher CD66b levels. Interestingly, at the 10-min time point this PMN population was clearly divided into a subgroup with low increase of CD66b (similar levels to the non-\textit{C. albicans} associated PMN) and a subgroup with highly increased levels. After 60 min of coincubation, CD66b levels on blood PMN without fungal contact (86.8 ± 7.8% of all PMN) remained identical to the 10-min time point, whereas whole-blood PMN associated with \textit{C. albicans} almost exclusively showed highly increased levels. In contrast, isolated PMN showed no increase of CD66b at 10 min and a monophasic increase at 60 min. Very early activation of PMN in whole blood (10 min p.i.) occurred in advance of any signs of \textit{C. albicans} filamentation, whereas filamentation is required for activation of isolated PMN (3). Both activation within 10 min and strong participation of bystander PMN indicated a yet unknown secondary stimulus of PMN activation generated during \textit{C. albicans} blood infection, which was present in whole blood but absent in confrontation assays with isolated PMN and acted in addition to direct PMN stimulation by the fungal pathogen. The effects of the blood-specific secondary signal for PMN activation were specific because FcγRIII (CD16) showed the same pattern of surface exposure in isolated and blood PMN and was therefore not affected by the intrinsic stimulus. Decreased surface levels were exclusively observed for PMN that had engulfed \textit{C. albicans} after 60 min. PMN without contact to \textit{C. albicans} showed no change of CD16 levels both in blood and in the isolated PMN setting, indicating that the blood-specific second trigger did not act on CD16.

\textit{Complement component C5a acts as a second stimulus of PMN activation in confrontation with \textit{C. albicans}}

The proposed blood specific stimulus should 1) be able to broadly stimulate PMN activation, 2) be present in blood but not in the isolated PMN setting, and 3) act within a very short time period after inoculation with clear effects being detectable after 10 min. Because complement is among the earliest effector systems to be activated in blood and isolated PMN confrontation assays were performed in the presence of heat-inactivated human serum, we hypothesized that the unknown stimulus for the general neutrophil activation in blood might be a complement component. The most obvious candidate was the anaphylatoxin C5a, a potent chemotactic and activator of PMN. Generation of this product of complement activation in human whole blood was quantified after inoculation of \textit{C. albicans}. During a 60-min coincubation, \textit{C. albicans} rapidly induced complement activation and formation of C5a with high levels detectable at 10 min after inoculation (Supplemental Fig. 2A). In contrast, proinflammatory cytokines like IFN-γ or TNF-α, which are released by non-PMN immune cells and have previously been shown to enhance PMN activation in other settings, were only detectable starting at 60 min after inoculation (Supplemental Fig. 2B) and were therefore unlikely to account for very early PMN activation. To analyze the impact of C5a on ROI formation and activation, we treated primary human PMN with increasing concentrations of recombinant C5a (Supplemental Fig. 2C). Consistent with earlier results from other groups (21, 26), C5a resulted in elevated ROI levels and CD66b upregulation on neutrophils. In addition, L-selectin was shed from PMN surface, even at low C5a concentrations, and almost completely removed at concentrations of 50 ng/ml C5a and higher. As expected, based on results for the whole-blood infection model, only moderate but significant changes of the CD16 surface levels were detectable in response to C5a.

\textit{Blocking of C5a abrogates PMN activation in human blood at 10 min p.i.}

To determine the contribution of C5a to \textit{C. albicans}-induced PMN activation, activation of PMN in human blood after inoculation of \textit{C. albicans} was quantified in the presence of a blocking Ab. For this purpose we used a humanized anti-C5a Ab (IFX-1) with no activity against the precursor protein C5 that has been developed as a novel therapeutic agent for sepsis by InflaRx and successfully passed phase I clinical trials. Preincubation of human whole blood with the anti-C5a Ab resulted in reduced generation of ROI in PMN with levels close to background after 10 min of coincubation (Fig. 2A). However, after 1 h, presence of IFX-1 no longer had an effect on the oxidative burst. Blocking of C5a prevented CD66b upregulation in PMN without fungal contact at 10 min and 60 min after inoculation (Fig. 2B). Accordingly, this PMN subpopulation had identical CD66b surface levels as PMN from mock-infected control samples. Interestingly, we found different results for PMN with direct contact to \textit{C. albicans}. Whereas CD66b surface exposure on PMN that had engulfed \textit{C. albicans} was nearly abolished in the presence of IFX-1 at the 10-min time point, CD66b levels were only slightly reduced following 60 min of infection.

\begin{figure}[ht]
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\caption{Both C5a and \textit{C. albicans} phagocytosis trigger decrease in C5aR surface expression on PMN. Comparison of C5aR expression on primary and blood PMN after 10 min \textit{[(a), top and bottom, respectively]} and 60 min \textit{[(b), top and bottom, respectively]} confrontation with \textit{C. albicans}. Gray filled histograms show basal expression in mock-infected samples. Black open histograms indicate surface levels following \textit{C. albicans} infection and differentiate between PMN without fungal contact (dashed line) and PMN that interact with \textit{C. albicans} (dotted line). Data from one of at least three independent experiments with virtually identical results are shown.}
\end{figure}
showing that both contact to *C. albicans* and C5a act as independent signals for PMN activation. In addition, we directly analyzed the impact of C5a on degranulation by measuring secreted PMN granule contents in plasma (Fig. 2C). Plasma levels of primary granule proteins MPO and elastase-2 showed no significant differences between IFX-1-, IgG4-, and nontreated blood in response to *C. albicans*. In contrast, presence of IFX-1 partially prevented the secretion of secondary granule contents measured by lactoferrin and neutrophil gelatinase-associated lipocalin (NGAL) plasma levels. Furthermore, elevated IL-8 plasma levels in response to *C. albicans* were significantly reduced in the presence of blocking Abs to C5a, whereas IL-8 plasma concentration was not affected by the isotype-matched control Ab.

**C5a acts via C5aR**

The biological effects of C5a are largely mediated by binding to the C5aR, which is expressed on a broad range of immune cells, including PMN. Treatment of primary PMN with C5a showed a concentration-dependent decrease of surface C5aR reflecting internalization of the formed C5a–C5aR complex (Supplemental Fig. 2C). In whole blood, C5aR showed decreased surface levels on the entire PMN population after *C. albicans* inoculation. However, C5aR expression was even lower on the PMN subpopulation that engulfed *C. albicans* (Fig. 3). This phagocytosis-dependent decrease of surface C5aR could be confirmed in primary PMN confrontation assays by a specific downregulation on isolated PMN associated with *C. albicans*. PMN without fungal contact showed a C5aR surface expression comparable to cells in the mock-infected sample (Fig. 3). Using an Ab against C5aR which blocks binding of C5a, we further confirmed the contribution of C5a to activation of blood PMN (Fig. 4). The presence of the anti-C5aR Ab in whole blood inoculated with *C. albicans* led to comparable results as IFX-1-treatment, indicating that C5a acts via C5aR.

**C5a increases phagocytosis of *C. albicans* by inducing enhanced surface exposure of complement receptor 3**

Experiments so far confirmed that C5a-induced activation can directly lead to ROI formation and degranulation in human PMN. To test whether C5a also directly modulates interaction of PMN with the pathogen, we quantified phagocytosis of *C. albicans* by PMN in human blood with and without the addition of IFX-1 using a GFP-expressing mutant (Fig. 5A). Preincubation of human whole blood with the anti-C5a Ab resulted in less fungal phagocytosis (13.2 ± 6.2%; *p* < 0.001) after 10-min coinoculation when compared with *C. albicans*-infected blood that was pretreated with the corresponding isotype-matched control Ab (32.3 ± 4.5%) or nontreated (36.0 ± 8.1%). However, only moderate differences could be observed after 1 h. Identical results were obtained using anti-C5aR blocking Ab (Fig. 5B). To identify the mechanism for C5a-enhanced phagocytosis of *C. albicans*, we quantified surface exposure of CD11b as a potential uptake receptor on the surface of isolated PMN after activation by C5a and detected a concentration-dependent increase of CD11b surface expression.
expression levels after C5a stimulation (Supplemental Fig. 2C). Together with CD18, CD11b constitutes the CR3, which has already been identified as a receptor for *C. albicans* (27). To confirm C5a-induced surface recruitment of CR3, we quantified CD11b levels on blood PMN during *C. albicans* infection with and without addition of IFX-1 (Fig. 6). At 10 min p.i., we could observe a strong increase in CD11b surface exposure that was independent of whether the PMN had contact to *C. albicans* or not in nontreated samples. This increase was efficiently blocked by IFX-1. After 1 h of infection, CD11b levels on surface of PMN associated with *C. albicans* had decreased and became comparable to levels in noninfected controls. In contrast, PMN with no fungal contact still showed a higher CD11b surface expression at 60 min, which could be reduced by adding IFX-1. Because these

FIGURE 5. Blocking of C5a or C5aR decreases initial phagocytosis of *C. albicans* by PMN. Presence of the anti-C5a (A) or the anti-C5aR Ab (B) significantly reduced phagocytosis of *C. albicans* by blood PMN after 10 min of infection but not after 60 min. Non- and isotype control–treated *C. albicans* samples served as references. *C. albicans* association with blood PMN was determined by flow cytometry. Each dot represents the percentage of fungal cells phagocytosed by PMN relative to total *C. albicans* in blood (set to 100%) of independent experiments with blood from different donors. The mean ± SD is given. *p < 0.05, ***p < 0.001. ns, nonsignificant.

FIGURE 6. C5a influences CD11b expression on PMN in infected blood. CD11b expression on PMN in whole blood was analyzed 10 and 60 min after inoculation of *C. albicans* and compared with basal levels in mock-infected blood (gray filled histograms). Black open histograms indicate surface levels following *C. albicans* infection and differentiate between PMN without fungal contact (dashed line) and PMN that interact with *C. albicans* (dotted line). *C. albicans*–infected samples were either nontreated [(a), top and bottom, respectively] or preincubated with IgG4 [(b), top and bottom, respectively] or anti-C5a [(c), top and bottom, respectively]. Data from one of at least three independent experiments with virtually identical results are shown.
data indicate internalization of CD11b during uptake of *C. albicans*, we further analyzed the role of CD11b by blocking CD11b function (Fig. 7). Incubation of human blood with an anti-CD11b Ab prior to infection resulted in a significantly reduced fungal uptake during 10 min as well as one hour coincubations (Fig. 7A). Consequently, CD66b surface exposure on PMN with contact to *C. albicans* also did not reach the levels of IgG1- or nontreated controls (Fig. 7C). In contrast, whereas formation of ROI was significantly affected after 10 min, no difference to control samples could be detected after prolonged infection (Fig. 7B).

**FIGURE 7.** Phagocytosis as additional signal for early blood PMN activation during *C. albicans* infection. Blocking of CR3 by an anti-CD11b Ab showed the impact of phagocytosis on PMN activation in whole blood following 10 and 60 min of *C. albicans* infection. (A) Interactions between fungal cells and blood PMN were determined by flow cytometry. Each dot represents the percentage of *C. albicans* phagocytosed by PMN relative to total *C. albicans* in blood (set to 100%) of independent experiments with blood from different donors. The mean ± SD is given. **p < 0.01.** PMN activation is shown by ROI formation and CD66b surface exposure. (B) ROI levels in PMN in response to *C. albicans* (non-, IgG1-, or anti–CD11b-treated) were normalized against basal levels in mock-infected blood (set to 100%). The bars show mean ± SD of at least six independent experiments with blood from different donors. *p < 0.05.** (C) Changes in CD66b surface expression are shown in histograms. Gray filled histograms indicate basal expression on PMN within the mock-infected sample. Black open histograms indicate surface levels following *C. albicans* infection and differentiate between PMN without fungal contact (dashed line) and PMN that interact with *C. albicans* (dotted line). *C. albicans*-infected samples were either nontreated [(a), top and bottom, respectively] or preincubated with IgG1 [(b), top and bottom, respectively] or anti-CD11b [(c), top and bottom, respectively]. Data from one of at least three independent experiments with virtually identical results are shown. ns, nonsignificant.
Phagocytosis of *C. albicans* by blood PMN is not regulated by its morphology. Quantification of association rates of primary PMN (A) and blood PMN (B) with either *C. albicans* wild-type or the nonfilamentous mutant *cph1Δ/efg1Δ* after 60 min of confrontation was performed using flow cytometry. To discriminate between intra- and extracellular fungal cells, FITC-labeled *C. albicans* cells were used and extracellular fungi were stained with a specific anti-*C. albicans* Ab. Interestingly, anti-*C. albicans* staining to define attached *C. albicans* revealed that all fungal cells associated with blood PMN have been engulfed by the immune cells. Values correspond to the means ± SD of four independent experiments with isolated human PMN and whole blood, respectively, from different donors. PMN activation is shown by changes in surface expression levels of the activation.
C. albicans--induced phagocytosis by PMN in blood is not dependent on fungal morphology

Isolated human PMN have been shown to discriminate between yeasts and filaments of C. albicans and become specifically activated by filamentous forms of the fungus (3). These data could be confirmed and analyzed in more detail in a novel set of experiments. Both C. albicans wild-type and the nonfilamentous mutant C. albicans cph1Δ/efg1Δ showed the same association with isolated PMN (Fig. 8A). However, wild-type filaments became significantly more phagocytosed (34.1 ± 7.1%) than the mutant (12.6 ± 2.8%; Fig. 8A). As a consequence, the activation markers CD66b and CD16 showed a strongly altered surface expression on PMN with contact to wild-type filaments in contrast to only slight levels induced by C. albicans wild-type induced levels (Fig. 9A). Consequently, we hypothesized that the ability of human PMN to efficiently recognize nonfilamenting C. albicans in human blood was dependent on C5a preactivation. In line with this, IFX-1 treatment resulted in a markedly reduced PMN association for C. albicans cph1Δ/efg1Δ, whereas only minor effects could be observed for wild-type C. albicans (Fig. 9B). Comparable results could be achieved in an independent set of experiments using killed C. albicans yeast cells and filaments (Fig. 9C), indicating that C5a enables human PMN to overcome morphotype dependent recognition of C. albicans.

Discussion

In the current study, we used an ex vivo human whole-blood model to analyze stimuli required for PMN activation during systemic C. albicans infection (5). Similar models have been successfully applied to study complement function in the early immune response against bacterial pathogens and used whole blood anticoagulated with the highly specific thrombin inhibitor lepirudin (28–30). In contrast to other anticoagulants, lepirudin has been shown to preserve complement activity and thus allows physiological immune activation (23).

After inoculation with C. albicans, we found an early (10 min p.i.) and strong activation of the entire PMN population in blood that was not detectable for isolated PMN confronted with C. albicans. Blocking of either C5a or the C5a receptor during 10 min C. albicans infection reduced PMN activation, proving that C5a is the major mediator of early PMN activation in human blood. In addition to C5a, the process of C. albicans phagocytosis further contributed to activation because PMN that were associated with C. albicans showed a significantly higher CD66b surface exposure compared with PMN without fungal contact. However, inhibition of C5a–C5aR signaling also prevented the increased CD11b surface exposure in response to C. albicans and PMN phagocytosis was almost completely abolished by anti-CD11b Ab treatment under the same conditions. Taken together, these data indicate that initial phagocytosis of C. albicans strongly depends on C5a-induced upregulation of CR3. This finding, together with available data for Escherichia coli and Staphylococcus aureus, supports a predominant function of C5a in the innate immune responses toward systemic infection in general (23, 30). Following a longer coinoculation, PMN effector mechanisms (e.g., phagocytosis of C. albicans and oxidative burst) appeared to be C5a-independent and mainly influenced by other stimuli, whereas the secretion of secondary granule contents (lactoferrin, NGAL) from PMN with no fungal contact could be blocked by anti-C5a Ab treatment. The release of primary neutrophilic granules, which contain MPO and elastase-2, occurred independently of C5a, indicating different signals in the regulation of PMN degranulation. Various studies have previously demonstrated that release of each granule population from PMN is selective and depends on distinct signaling pathways (31–33). Furthermore, secretion of IL-8, a strong chemoattractant for PMN that is able to activate and degranulate PMN, was completely regulated by C5a following one hour and at least partially following longer incubation. Similarly, C5a has been shown to strongly increase IL-8 release from human whole-blood cells induced by LPS and other types of TLR agonists and to be involved in E. coli–induced IL-8 release during whole-blood infection (34), indicating the effects of C5a on IL-8 to be broad. In addition, recent data by Cheng et al. demonstrated a role for C5a in C. albicans-induced proinflammatory cytokine production by human PBMCs (22), which further supports the theory of the contribution of C5a in a broad spectrum of inflammatory responses caused by various types of pathogens. In contrast, blood PMN that had engulfed C. albicans did not further respond to C5a. Interestingly, C5aR expression on the PMN subpopulation that had phagocytosed C. albicans was lower than compared with blood PMN without fungal contact. Furthermore, we could demonstrate that physical interaction with the fungus resulted in a decrease in C5aR surface levels on primary PMN. Thus, phagocytosis may lead to downregulation of C5aR on PMN, reducing the responsiveness of this PMN subpopulation to C5a. This hypothesis is supported by recent data showing that neutrophil serine proteases, released upon PMN activation, can cleave and inactivate surface C5aR (35). In line with this, PMN associated with C. albicans showed a highly increased degranulation, reflecting the release of potential proteases. The reduced C5aR surface expression on PMN without fungal contact most likely resulted from internalization of C5a–C5aR complexes after C5a binding. Subsequent recycling of the receptor to the plasma membrane would enable C5a binding again (36). Because C. albicans uptake by PMN at 60 min p.i. was independent of C5a, we checked for other mechanisms regulating phagocytosis. Although C. albicans showed continuous filamentation in blood, phagocytosis was not strictly dependent on fungal morphology as observed for primary PMN (3, 5). In a striking difference to experiments with isolated PMN, both C. albicans wild-type and the nonfilamentous mutant cph1Δ/efg1Δ showed the same distribution in different cellular compartments of human blood and induced equal PMN activation after 60 min. Our experiments clearly suggest that this morphotype independent activation of PMN is dependent on C5a. Consequently, C5a signaling is able to override morphotype-restricted activation of human PMN in blood. In our model infection, the effects of C5a were most evi-
C5a induces PMN to phagocytose C. albicans in a morphotype-independent manner. (A) C. albicans wild-type (■) and the nonfilamentous mutant cph1Δ/efg1Δ (○) resulted in equal C5a plasma levels during whole-blood infection. Dots show means ± SD of five independent experiments with whole blood from different donors. (B and C) Samples of human whole blood were infected with either FITC-labeled C. albicans wild-type and cph1Δ/efg1Δ (B) or killed GFP-expressing C. albicans wild-type yeast cells and filaments (C). Association with blood PMN after 60 min of confrontation was determined in the presence of IFX-C. albicans* SD of three independent experiments with blood from different donors. (p 0.05.

FIGURE 9. C5a induces PMN to phagocytose C. albicans in a morphotype-independent manner. (A) C. albicans wild-type (■) and the nonfilamentous mutant cph1Δ/efg1Δ (○) resulted in equal C5a plasma levels during whole-blood infection. Dots show means ± SD of five independent experiments with whole blood from different donors. (B and C) Samples of human whole blood were infected with either FITC-labeled C. albicans wild-type and cph1Δ/efg1Δ (B) or killed GFP-expressing C. albicans wild-type yeast cells and filaments (C). Association with blood PMN after 60 min of confrontation was determined in the presence of IFX-1 and compared with either IgG4-containing or nontreated C. albicans-infected blood (set to 100%) using flow cytometry. The bars show mean ± SD of three independent experiments with blood from different donors. *p < 0.05.

dent early after inoculation (i.e., at 10 min p.i.). At later stages pathogen intrinsic stimuli related to filamentation were able to induce C5a independent PMN activation. It must, however, be noted that it is impossible to transfer these model kinetics to the in vivo situation.

To analyze this situation further, the previously established virtual infection model for the quantification of innate effector mechanisms (5) may be extended from a state-based model to a hybrid agent-based model (37). This would allow for an accurate mathematical description of the whole-blood model by explicitly accounting for mechanism of PMN activation: 1) time-dependent cleavage and diffusion of C5a and 2) morphological changes of C. albicans. The hyphal growth kinetics can be experimentally determined by an image-based systems biology approach, as was done in a previous model (38), whereas dynamic changes in C5a concentration can be fitted to whole-blood infection experiments inspected with higher temporal resolution. Such a hybrid agent-based model is extensible and resembles the whole-blood infection assay to a high degree of realism allowing for the quantification of the relative impact of complement and morphotype mechanisms.

Our data provide new insights into the regulatory role of C5a in PMN function during systemic C. albicans infection in human blood and identify C5a as an essential mediator of PMN activation in response to C. albicans. There is accumulating evidence for excessive complement activation with increased C5a levels and reduced C5aR expression on PMN in experimental sepsis and severe sepsis or septic shock, both found to be correlated with the severity of illness (21, 39–41). The present study further supports evidence that blocking of C5a may have a beneficial effect on potentially harmful systemic inflammatory responses.

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Disclosures

N.C.R. serves as CEO and R.-F.G. serves as CSO for InflaRx, which develops anti-C5a Ab (IFX-1) for clinical use.

References


