Modulation of Neutrophil Extracellular Trap Formation in Health and Disease

Ava Hosseinzadeh
To my family
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Manuscript, 2016

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Infection and Immunity, 2013
Abstract

The critical prompt innate immune response is highly built upon the influx of neutrophils from the blood stream to the site of infection. In the battlefield, neutrophils sense pathogen-associated molecular patterns (PAMPs) through their pattern-recognition receptors (PRRs) to launch a number of responses with the goal to defeat the invading pathogen. Neutrophils’ wide spectrum of responses ranges from reactive oxygen species production (ROS), phagocytosis, cytokine and chemokine secretion, and neutrophil extracellular trap (NET) formation. The NET scaffold is composed of nuclear chromatin which is armed with antimicrobial proteins. DNA traps are able to ensnare and kill microbes in the extracellular space and NET release concurs with cell death of the neutrophil. An increasing body of literature describes that NETs impose deleterious effects on the host itself in addition to their antimicrobial activity. These hazardous effects mainly stem from pro-inflammatory and tissue-destructive activity of NETs. These two diverse outcomes of NETs result in a series of effects on both host and pathogen. Therefore, it seems rational that NET formation is tightly regulated and not happening spontaneously. The opportunistic fungal pathogen *Candida albicans* captured and killed by NETs. This fungus has the remarkable ability to grow as budding yeast or as filamentous hyphae, and reversibly alternate between these morphotypes. Hyphae are the tissue-destructive, invasive and pro-inflammatory form of *C. albicans*, whereas yeast is the proliferative, non-invasive form. Hence, it is important to find out how neutrophils discriminate between distinct growth forms of *C. albicans* and how NET release is regulated in this regard.

To assess neutrophils responses towards each growth form of *C. albicans*, the mere ratio of each fungal morphotypes is an insufficient measure to describe comparable amounts used in infection experiments; we therefore used dry mass of fungal cells to serve as a common denominator for amounts of fungal cells with different morphotypes. As assessment of dry mass is laborious, we developed a quick correlative method, which quantified fungal metabolic activity corresponding to the actual dry mass. We applied this
method in consecutive studies investigating the neutrophil responses specific to different morphotypes of *C. albicans*.

Positive and negative regulators of NET formation were investigated for this thesis in a mechanistic fashion. To identify how NET release is negatively regulated during *C. albicans* infection we focused on anti-inflammatory receptors on neutrophils. We observed that adenosine signals via adenosine receptor reduces the amount of NETs exclusively in response to *C. albicans* hyphae, the invasive, pro-inflammatory form. We identified adenosine receptor A3 as the responsible receptor suggesting that targeting of adenosine A3 would be a promising approach to control invasive fungal infection, since particularly during immune reconstitution invasive mycoses are frequently accompanied by hyperinflammation which additionally worsens the patient’s state.

As unbalanced inflammation is harmful to the host, a situation reflected in autoimmune diseases, such as systemic lupus erythematosus, we aimed to find molecules, which are able to inhibit NET formation. Thus, we introduced the non-toxic agent tempol”. During ROS-depended stimulation of NET formation via *C. albicans* and phorbol esters, the stable redox-cycling nitroxide tempol efficiently blocked NET induction. We therefore proposed tempol as a potential treatment during inflammatory disorders where NET formation is out of balance. In quest for positive regulators of NET formation we found the major addictive component of tobacco and electronic cigarettes, nicotine, as compelling direct inducer of NET release. Interestingly, nicotine is associated with exacerbated inflammatory diseases exerting its pro-inflammatory activity via acetylcholine receptor by targeting protein kinase B (known as Akt) activation with no effect on NADPH oxidase complex in a ROS independent fashion. In consideration of neutrophils role in smoking-related diseases we propose targeting Akt could lower the undesirable effect of NET.

In conclusion, this thesis identified new modulators of NET formation in response to fungal infection and more broadly to other NET-inducing stimuli, which might have implications in forthcoming therapies.
Abbreviations

AChR acetylcholine receptor
AIDs acquired immune deficiency syndrome
Akt protein kinase B
ALS agglutinin-like sequence
AMP antimicrobial peptide
ATP adenosine triphosphate
BCY1 bypass of cyclic-AMP requirement 1
BPI bactericidal permeability-increasing protein
cAMP cyclic adenosine monophosphate
CFU colony forming unit
CGD chronic granulomatous disease
CLR C-type lectin receptors
COPD chronic obstructive pulmonary disease
CPH1 Candida pseudohyphal regulator 1
CR complement receptor
CST20 C. albicans STE20
CTA1 Catalase A
CWPs Cell wall proteins
CXCR chemokine receptor
DAMPs damage-associated molecular patterns
DPI diphenylene iodonium
EFG1 enhanced filamentous growth
ERK extracellular signal-regulated kinases or classical MAPK
ET extracellular trap
FcR Fc receptor
GAS group A streptococcus
G-CSF granulocyte-colony-stimulating factor
GI gastrointestinal tract
GM-CSF granulocyte-macrophage colony-stimulating factor
GPCRs G-protein coupled receptors
GPI glycosyl-phosphatidylinositol-anchored protein
GPX glutathione peroxidases
H2O2 hydrogen peroxide
HOCl hypochlorous acid
HST7 homologue to STE7
HWP1 hyphal cell wall protein 1
ICAM intracellular adhesion molecule
ICs immune complexes
IFF11 secreted protein required for cell wall structure and virulence
IFNα interferon-alpha
IL-8 interleukin-8
ITAM immunoreceptor Tyr-based activation-like motif
IRIS immune reconstitution inflammatory syndrome
LPS lipopolysaccharide
MAPK mitogen activated protein kinase
<table>
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<tr>
<td>MIG1</td>
<td>multiple inhibitor of galactose 1 gene</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<td>MPO</td>
<td>myeloperoxidase</td>
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<td>MSU</td>
<td>monosodium urate</td>
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<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NE</td>
<td>neutrophil elastase</td>
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<tr>
<td>NET</td>
<td>neutrophil extracellular trap</td>
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<tr>
<td>NLRs</td>
<td>nucleotide-binding oligomerization domain-like receptors</td>
</tr>
<tr>
<td>Nox2</td>
<td>gp91phox</td>
</tr>
<tr>
<td>NRG1</td>
<td>negative regulator of glucose-repressed genes</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>superoxide</td>
</tr>
<tr>
<td>PAD</td>
<td>peptidylarginine deiminases</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PGRPs</td>
<td>peptidoglycan recognition proteins</td>
</tr>
<tr>
<td>PI3Ks</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>Pir</td>
<td>proteins with internal repeats</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
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<tr>
<td>PMN</td>
<td>polymorphonuclear cell</td>
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<tr>
<td>PRRs</td>
<td>pattern-recognition receptors</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>RAF</td>
<td>serine/threonine-specific protein kinases</td>
</tr>
<tr>
<td>RAS1</td>
<td>homologous Ras-like proto-oncogene 1</td>
</tr>
<tr>
<td>RFG1</td>
<td>repressor of filamentous growth</td>
</tr>
<tr>
<td>RIGI</td>
<td>retinoic-acid-inducible gene I</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SAP</td>
<td>secreted aspartyl proteinase</td>
</tr>
<tr>
<td>SK3</td>
<td>small conductance calcium-activated potassium channel 3</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SMR</td>
<td>suspended microchannel resonator</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TIR</td>
<td>TLR-interleukin 1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor-alpha</td>
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<tr>
<td>TPK</td>
<td>Takashi’s protein kinase</td>
</tr>
<tr>
<td>TUP1</td>
<td>thymidine uptake 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>XTT</td>
<td>2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide</td>
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</table>
Introduction

Fungal pathogens

The kingdom of fungi include about 1.5 million species [1] and within this immense number only a few pathogenic species exist that may cause superficial to systemic infections in humans.

Most fungi are opportunistic, not primary pathogens, and thus cause infections (mycoses) predominantly in immunocompromised individuals. Typical opportunistic mycoses are aspergillosis and candidiasis.

These mycoses also represent the main route of infections. Either the fungi are environmental or ubiquitous, such as Aspergillus spp. Spores of the opportunistic mould are commonly present in the air both indoor and outdoor and inhaled on a regular basis. Breathing in spores usually is harmless unless the immune system is weakened as in immunocompromised patients. Then, spores may cause infections in lungs or sinuses and may disseminate to other parts of the body [2]. The other type of fungi are commensals such as Candida spp. These colonize skin and mucosal tissues of humans asymptotically and may use the opportunity of a weakened immune system to spread to deeper body niches and to cause disease (Fig. 1) [3].

The enormous increase in frequency and severity of fungal infections in recent years are mainly due to an increase number of immunocompromised patients such as organ transplant recipients (bone marrow or solid organs), acquired immune deficiency syndrome (AIDS) or cancer. Advances in medical treatment such as chemotherapy and immunosuppressive drugs as well lead to increased numbers of opportunistic infections [4, 5]. Other factors such as high antifungal therapy cost, lack of sensitivity or specificity in current medical diagnosis as well as delayed diagnosis result in emerging fungal infections [6].
**Candida albicans**

*Candida albicans* is a universal fungal organism. Fredrik Berg, David Gruby and John Bennett between 1839 and 1844 reported about oral thrush in infants caused by a yeast. The names for the causative agent changed over time until Christine Berkhout in 1923 termed it *C. albicans* [7]. *C. albicans* causes a wide range of infections from benign colonization on epithelial surfaces to systemic candidiasis in immunocompromised patients. In hosts with normal immune defence the fungus often colonizes the mucosal surfaces of the oral, gastrointestinal (GI) and urogenital tract as part of the common microbiota without affecting the host [5, 8, 9]. Overgrow of *C. albicans* can lead to hazardous colonization which establishes as superficial, mucosal infection in form of oral thrush [10], vaginal yeast infections [11, 12] and diaper rash or disseminates to invasive oropharyngeal and esophageal candidiasis [13, 14]. *C. albicans* may moreover disseminate even further to cause systemic infection by invasion into deep organs and blood [15]. Within a dozen of fungal genera, *Candida* genus plays a major role. Candidiasis are different types of infections caused by species of the genus *Candida*. Among the *Candida* species the opportunist *C. albicans* is the most common cause. Roughly 92% of *Candida*-derived blood stream infections (candidaemia) are caused by the species *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* [16].

Not surprisingly, also within this group *C. albicans* is the most common cause of blood stream infections accounting for 66% of the cases worldwide [17]. In susceptible patients with compromised immunity, candidaemia results in high mortality rates of 30-50% [18]. This percentage is unacceptably high, even in comparison to the most aggressive types of bacterial and viral sepsis [19, 20]. In order to parasitize humans four general criteria must be fulfilled by a fungal pathogen: (1) It must grow at high temperature at or above 37 °C, (2) it must penetrate host tissue barriers that they will parasitize, (3) it must be able to acquire food within components of
host tissue, and (4) finally it must tolerate and survive attacks by human immune defence [21].

Fig. 1: Model for commensal and pathogenic status of *Candida albicans*
In immunocompetent hosts, the *C. albicans* is in the commensal state. The situation changes in immunocompromised hosts in which the defence system is unable to block virulence factors and the opportunistic fungi may become pathogenic. Severity of disease reciprocally correlates to the host immune status [Adapted from Brown and Ernst et al., 2003].

*Candida albicans* biology
A number of dimorphic fungal pathogens are capable to reversibly transit between yeast and hyphal growth forms. Morphogenic transition between these forms is frequently stimulated by growth in a host and related to the capability to invade host tissue [22]. *C. albicans* is a trimorphic fungus with remarkable power to grow as unicellular budding yeast and as filamentous hyphae or pseudohyphae, depending on the environment (Fig. 2). These filamentous forms are morphologically distinct from each other. True hyphae form long parallel germ tubes, whereas pseudohyphae are constricted at the septation sites, are wider than hyphae and usually
branched to a higher extent [23]. *C. albicans* undergoes reversible transitions between morphotypes under certain conditions. Hyphal transition can be readily induced by neutral to alkaline pH, temperature above 37 °C, hypoxia or by presence of serum [24].

*C. albicans* morphotypes vary widely in diameter and length depending on the growth condition. Measurements of yeast cells grown resulted in an average calculated volume of 88 μm³ [25]. Hyphal width and length of *C. albicans* vary considerably. Under optimal growth conditions, a diameter of 2.6 μm for a germ tube and 3.4 μm for a mature hypha has been observed [26, 27]. The length of cultured *C. albicans* hyphae was found to be saturated at a value of about 70 μm [28].

![Fig. 2: Candida albicans morphotypes](image)

*C. albicans* can reversibly switch between budding yeast and filamentous forms. Filamentous forms are pseudohyphae and true hyphae. Pseudohyphae consist of a chain of un-separated fungal cells that have constrictions at the sites of separation. True hyphae are characterised by formation of long parallel germ tube [23].

*C. albicans* morphogenesis is often induced by extracellular factors such as mentioned alteration in microenvironments. Morphologic transition is regulated in *C. albicans* in a complex network involving many different genes to steer multiple signal transduction pathways. Two well-characterized
pathways involved in yeast-to-hypha transition are *Candida* pseudohyphal regulator 1 protein-mitogen activated protein kinases (Cph1p-MAPK) and enhanced filamentous growth 1 protein-cyclic adenosine monophosphate (Efg1p-cAMP) mediated pathway (Fig. 3). Homologous to Ras-like proto-oncogene 1 protein (Ras1p) functions upstream of both of these pathways as an important regulator of hyphal induction [29]. There are several catalytic subunits in each pathway such as Takashi’s Protein Kinase 1 and 2 proteins (Tpk1p or Tpk2p), bypass of cyclic-AMP requirement 1 protein (Bcy1p) in the cAMP-mediated pathway and *C. albicans* STE20 (Cst20p), the homologue to Ste7p (Hst7p) in MAPK-mediated pathway as well as downstream and parallel transcription regulators that play a role in morphogenesis regulation which will not be further discussed here. To down-regulate or reverse filamentous growth *C. albicans* uses transcriptional repressor thymidine uptake 1 (Tup1p) which negatively regulates transcription of hypha-specific genes. Tup1p is recruited by DNA-binding proteins, such as repressor of filamentous growth (Rfg1p), negative regulator of glucose-repressed genes (Nrg1p) or multiple inhibitor of galactose 1 gene (Mig1p) that subsequently in combination with Tup1 act as repressor of hyphal morphogenesis (Fig. 3) [29].

An enhanced filamentous growth protein mutant strain (∆efg1) has a strong but not complete defect in filamentous growth [30, 31]. Overexpression of Efg1p induces pseudohyphae in certain environmental conditions, such as a microaerophilic milieu (typically 2-10% O₂) [30]. Interestingly, the double mutant strain ∆efg1 ∆cph1 has an extreme filamentous growth defect, with no detectable filament formation under any tested conditions. Thus, Cph1p and Efg1p define elements of two separate pathways that together are essential for filamentation in *C. albicans* [32].
**Fig. 3: Morphogenesis signalling pathways**

Selective signal transduction pathways and regulators of morphogenesis in *C. albicans*. Cph1-MAPK and Efg1-cAMP pathway regulate yeast-hyphal transition [Adapted from Biswas, Van Dijck et al., 2007].

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**Candida albicans cell wall composition**

The fungal cell wall is the outer-most layer of *C. albicans* cells that surrounds the cell membrane. It is an essential organelle to maintain structural integrity, to protect from mechanical stress, to limit entry of large toxic molecules, to provide a stable osmotic environment, and to serve as a physical contact interface with the environment. To understand how fungal cells are recognized by the host immune system, it is necessary to know the components and the dynamics of the cell wall (Fig. 4). The three main components are glucan, chitin and glycosylated proteins [33, 34] composing a three dimensional network. In fungal cell walls β-glucans are the most abundant polysaccharides (50-60% of the cell wall biomass). They are polymers of glucose joined by β-(1,3) or β-(1,6)-glycosidic linkages. Glucans remain protected from the host receptors by other cell wall components [35, 36]. Another carbohydrate polymer is chitin composed of more than units of
N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose, GlcNAc) joined by β-1,4-linkages in a linear and antiparallel fashion [37]. Lipids are minor components of the cell wall and have some relevance in adhesion, protection and signalling. Phospholipomannan is made of linear chains of β-(1,2)-joined mannose residues that polymerize to chains of 8 to 18 monomers linked to phosphoinositol ceramide [37].

Most models suggest that the scaffolding components of the cell wall are mainly found close to the cell membrane although they can be identified throughout the thickness of the cell wall. The cell wall composition is constantly remodelled during growth and in particular during morphotype transition [34]. For instance during the budding process of yeasts, a scar is formed on the mother cell after separation due to the exposure of chitin and glucan throughout the entire layer of the cell wall. At this site the components of the inner layers of cell wall, such as chitin and β-(1,3)-glucan, can become exposed at the surface. In hyphae which lack a budding mechanism glucans are hidden by the outermost situated phosphomannoprotein complex, a layer of glycosylated cell wall proteins covalently attached to the carbohydrate backbone via β-(1,6)-glucan bridges. Continuous masking of β-glucan enables the hyphae to escape from host receptors recognizing β-glucan (Dectin-1) [33, 38].

The cell wall additionally serves as the first barrier of defence against external insults. Fungi encounter diverse environmental stresses including nutritional shortages, pH changes, altering osmotic conditions, high or low oxygen levels, antifungal drugs, and host immune attacks [39]. To overcome environmental stresses, such as osmotic pressure, the cell wall architecture is remodelled by enzymatic activity of chitinases or glucanases to control cell wall elasticity during hyper osmotic stress by modulating chitin- β-glucan linkages [40]. For instance, at high NaCl concentrations the cell volume decreases and as a result the cell wall thickness increases rapidly. Cell wall architecture changes by compression of β-glucan coils and sliding chitin and β-glucan polymers over one another [34].
Candida albicans cell wall major components

β-(1,3)-glucans and chitins are the main structural components located closely towards the cell membrane and form the skeleton of the cell wall. The outer layer is attached to the inner layer mainly by β-(1,6)-glucans. The outer layer is mainly composed of mannoproteins and mannan [Adapted from Netea, Brown et al., 2008].

**Candida albicans cell wall proteins are virulence factors**

Cell wall proteins (CWPs) are crucial for *C. albicans* and have different roles such as cell wall remodelling, adhesion to host interaction, cellular structure, and morphogenesis [37].

CWPs can be clustered into two main groups. The first group comprises CWPs which are covalently-linked to the polysaccharide backbone, the so-called Pir proteins, originally named after a group of genes coding for putative proteins with internal repeats, and glycosyl-phosphatidylinositol-(GPI)-anchored proteins. The GPI anchor supports targeting of proteins to the membrane or the cell wall and often GPI-anchored proteins are found at both locations such as for instance secreted aspartyl proteinase (Sap9p) and Sap10p [41]. The second group comprises non-covalently linked and secreted proteins. An example for this group is the secreted virulence factor Iff11p which is essential for invasion and adherence to host tissue [42]. Adherence
of *C. albicans* to host tissue is mediated by a range of GPI-anchored CWPs [43].

In immunocompromised hosts *C. albicans* in the GI tract is allowed to form hyphae and to express CWPs such as agglutinin-like sequence 3 (Als3p) or hyphal wall protein 1 (Hwp1p) [44]. *C. albicans* can invade epithelial cells by either active penetration, endocytosis [45] or proteolytic digestion of tight junctions [46-48]. Although both growth forms can induce endocytosis, *C. albicans* hyphae are thought to be more efficient due to the observation that the yeast-locked mutant strain Δefg1 is less capable to induce endocytosis than wild-type cells [49]. Als3p can additionally bind to host receptors E- and N-cadherin that induces endocytosis and further mediates invasion to endothelial cells [50]. By combining these different invasion mechanisms the fungus is ultimately able to escape the commensal niche and to disseminate into deeper tissue and to the blood stream [51, 52]

**Cell wall components are potential targets for antifungal therapy**

The cell wall of fungi is the contact surface to host tissue, harbours numerous virulence factors and is unique to the fungal kingdom. The latter is important, since the fungal kingdom is the most closely related kingdom to the animal kingdom, a fact which renders design of antifungal drugs targeting conserved pathways particularly problematic. These features make the fungal cell wall a very suitable target for antifungal compounds. Two distinct approaches can be potentially employed to target cell walls: (i) by targeting cell wall integrity to impair cell wall biogenesis and eventually cell viability, and (ii) by targeting putative cell wall virulence factors to disarm the pathogen [53]. The relatively newly developed echinocandins for instance target the glucan synthase complex Fks1p responsible for the synthesis of β-(1,3)-glucan[54]. Inhibition of Fks1p causes reduced β-(1,3)-glucan and thus lyses fungal cells [54, 55]. Unfortunately, emergence of echinocandin-resistant strains is already a clinical concern.

Despite echinocandins the majority of available antifungal drugs used against *C. albicans* do not target the cell wall, but the cell membrane. There
are for instance azoles, amphotericin B, and polyene compounds. They target ergosterol synthesis (azoles) or bind ergosterol to induce pores (amphotericin and polyenes) [56]. Ergosterol is the functional equivalent to cholesterol in mammalian cell membranes [57]. In general, available antifungal treatment is not sufficiently efficient, mainly due to lack of specificity and sensitivity as well as due to toxic side effects [17].

Defence mechanism of Candida albicans

To facilitate pathogenicity, many fungal pathogens have developed strategies to resist attack from phagocytes such as macrophages and neutrophils [58]. They are able to survive and replicate in phagocytes [59] by cell surface modification strategies such as glucans rearrangements to avoid pathogen recognition [60] and ultimately phagocytosis and ROS resistance. Upon infection, phagocytes launch a quick response by releasing reactive oxygen species (ROS) to destroy microbes. ROS diffuses through the fungal cell wall. To evade the harmful effect of ROS from phagocytes, C. albicans uses superoxide dismutase (SOD) proteins, which are able to detoxify superoxide, the first component of the ROS cascade, into hydrogen peroxide (H₂O₂) [61]. H₂O₂ is converted to water and oxygen to reduce antimicrobial activity by catalase enzyme (Cta1p) [3, 61, 62] or detoxified by glutathione peroxidases (Gpxps) via oxidation of thiol groups into glutathione molecules [63]. SODs and Gpxps are however not restricted to host responses, but are essential for C. albicans, as high levels of ROS are produced intrinsically during phases of rapid growth [64]. SODs in C. albicans comprise a family of six members Sod1p to Sod6p which are localized intracellularly or at the cell surface. They are classified according to the metal cofactor(s) bound. Sod1p and Sod3p (CuZn SODs) are cytoplasmic, Sod2p is mitochondrial (Mn SOD), and Sod4p to Sod6p are cell surface localized (CuZn SODs) [65]. Sod5p plays a major role in the superoxide removal to evade host immune surveillance [66].
Cell number versus cell size: the difficulties to determine initial amounts of different *Candida albicans* morphotypes

Many human fungal pathogens such as *C. albicans*, *Histoplasma capsulatum* and *Blastomyces dermatitidis* are capable of growing as budding yeast or filamentous form a trait termed fungal polymorphism [67]. The possible individual contribution of *C. albicans* yeast and filamentous forms during colonization on skin and mucosae, invasion to deep tissues and to the bloodstream have been under debate for a long time [22, 68]. To understand the contribution of *C. albicans* morphotypes to pathogenicity, the role of the two best characterized growth forms, yeast and hyphae, need to be investigated meticulously during the interaction with different immune cells.

The mere number of yeast cells in multiplicity of infection (MOI), commonly reflects the ratio of fungi interacting with a defined number of host cells. In case of dimorphic fungi, MOI does not indicate the exact amount of fungi used to set infection. *C. albicans* hyphae grow as long filaments without separation of individual cell, whereas budding yeasts separate from mother cells. Thus, upon growth sizes and ergo interaction surfaces of one hypha and one yeast can differ considerably.

Other methods such as counting colony forming units (CFU), spectrophotometric analysis and accessing metabolic activity by reduction of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) in colorimetric assays also have been introduced. Additionally, more sophisticated methods, such as suspended micro-channel resonator (SMR) measurements in combination with coulter counter was applied to analyse single cell mass, volume and density [69] which could monitor density changes during growth of a yeast. Most of these laborious methods are limited due to the long time needed to measure, which makes them inappropriate for fast use prior to infection experiments. Hence, in respect to multimorphic fungal pathogens, there is a demand for rapid methods to precisely determine biomass of all types of morphotypes for improved investigations in host-fungal interactions.
Immune system overview

The immune system serves to protect the host from pathogens. Microbes that pass through the first physical or chemical defence barriers such as skin, mucosae (gastrointestinal or respiratory tract) and body fluids (tears, saliva, digestion enzymes) encounter cells from the innate and adaptive immune system (white blood cells). Pathogens are recognized by a set of inherited receptors on innate immune cells and by a large reservoir of genetically recombined receptors on adaptive immune cells to disarm and neutralize invading microorganisms. Cells of the adaptive immune system are lymphocytes, mainly B and T cells that are capable of specific recognition, high adaptation and long term memory. Despite these excellent features, the adaptive immune response takes about 3-4 days. In contrast to slow adaptive immune responses the innate immune system, launches immediate defence against invading microorganisms. Innate immune cells consist of natural killer cells, mast cells, eosinophils, basophils, macrophages, dendritic cells and abundant number of neutrophils, constituting around 70% of all circulating white blood cells [70].
Neutrophils

Polymorphonuclear leukocytes (shortly neutrophils or PMNs) were first discovered by Paul Ehrlich who identified lobulated nuclei (2-5) and the granular, cytoplasmic vesicles of granulocytes. Ehrlich classified these granulocytes as eosinophils, basophils, and neutrophils [71]. Neutrophils, also known as neutrophilic granulocytes, are professional phagocytes which have critical roles in host defence against microbial infections and are among the first cells to be recruited to a site of infection. In addition to the powerful antimicrobial activity, neutrophils can contribute to significant adverse effects in pathogenesis of numerous inflammatory diseases. For instance improper activation of neutrophils can lead to tissue damage [72]. Neutrophils are cells from the haematopoietic system maturing in the bone marrow in a process termed granulopoiesis. Once matured, neutrophils leave the bone marrow and enter circulation where they are the most abundant white blood cells. Mature neutrophils are equipped already with most of the antimicrobial weaponry to be able to react fast upon invasion of pathogens. Therefore, the life span of these cells is controlled tightly. Neutrophils are terminally differentiated, unable to proliferate and short-lived. Per day, an astonishing number of approximately 1-2×10^{11} neutrophils are produced in the bone marrow of the whole body [73]. Their release from bone marrow generally regulated by chemokines with a CXC motif and their specific receptors expressed on myeloid cells. CXC chemokine receptor 4 (CXCR4) expression is important to maintain neutrophils in bone marrow [74]. Expression of other receptors later during neutrophil maturation in bone marrow such as CXCR2 mediates neutrophil release from bone marrow into circulation [74]. Neutrophils are directed from blood to the site of infection in a controlled way. Initial attachments of circulating neutrophils to endothelial cells is mediated by endothelial cells which react to cytokines like TNFα, IL-1β and IL-17 [75]. Recognition of these cytokines by endothelial cells results in expression of P-selectin, E-selectin, and integrin superfamily receptors on luminal surfaces [76]. On the other hand, neutrophils express
ligands like P-selectin glycoprotein ligand 1 (PSGL1) and L-selectin for expressed selectins to mediate first adherence on endothelial cells and slower rolling in circulation [77]. After slowing down, neutrophils start expression of β2 integrins and Mac-1 for immunoglobulin superfamily, intracellular adhesion molecule-1 and 2 (ICAM-1 and ICAM2) to mediate firm adhesion [78]. Neutrophils take two roads to migrate from endothelial barriers into afflicted tissues: Paracellular migration to squeeze between endothelial cells and transcellular migration to penetrate directly through an endothelial cell [79]. Once neutrophils pass from circulation to the tissue they encounter chemotactic factors such as interleukin-8 (IL-8) by endothelial cells or macrophages which are sensed by specific receptors on neutrophil, CXCR1 and CXCR2, to mediate exact migration towards the site of infection [75, 80].

Mature neutrophils are present in bone marrow, circulation as well as organs such as spleen, liver and lung [81]. These so called organ-marginated granulocytes could serve as an additional reservoir of mature neutrophils to be quickly deployed to the site of infection or inflammation [82]. Additionally, presence of neutrophils in theses organs could constantly protect them from microbial invasion. Residing in tissue, neutrophils may function for additionally 1-2 days before they will become apoptotic there (discussed below) and be cleared by macrophages [75, 83]. The exact lifespan of neutrophils in circulation is still under debate, ranging between 16 h and about 5.4 days [84]. Nevertheless, upon priming neutrophil longevity increases several fold during inflammation and ensures their presence at the inflammatory site [81].

**Apoptosis**

Neutrophils are short-lived and usually released into circulation 10-24 h before migrating into tissues upon recruitment. Unused, aging neutrophils in absence of a stimulus start a spontaneous apoptotic program. Apoptosis describes the death program our cells commence in order to be silently removed and recycled. It is an essential process that occurs throughout life in
all tissues. After having fulfilled crucial functions, such as phagocytosis and ROS production (see related parts), neutrophils also eventually start apoptosis by the cleavage and activation of caspases-3 and-8 [85]. During the process, small membrane-bound vesicles containing strongly condensed chromatin called apoptotic bodies are formed. Apoptotic bodies are a characteristic morphological hallmark of apoptosis and promote macrophages to clear apoptotic cell debris [86, 87]. The larger and long-lived phagocytic macrophages recognize and engulf apoptotic neutrophils and transport them to the liver for final removal. The process of uptake of apoptotic cells by macrophages is called efferocytosis [75, 83, 88].

**Neutrophil antimicrobial effectors**

As first line of defence against microbes neutrophils launch several antimicrobial activities including (i) the production of ROS, (ii) phagocytosis and intracellular killing, (iii) release of cytokines, such as IL-8 or tumour necrosis factor α (TNF-α) to recruit additional cells and to promote inflammatory responses [89, 90], (iv) release of extracellular enzymes and antimicrobial granule proteins, such as myeloperoxidase (MPO), neutrophil elastase (NE) and gelatinase in a process named degranulation, and (v) release of neutrophil extracellular traps (NETs). In addition to the well-known antimicrobial activity of neutrophils, they are increasingly recognized for their role in inflammation-mediated tissue damage and in modulation of immune-related functions.

**Neutrophil oxidative burst and NADPH oxidase complex**

In neutrophils the major source of reactive oxygen species (ROS) during the oxidative burst is the NADPH oxidase complex. NADPH oxidase is a multi-subunit complex (Fig. 5). The complex comprises two membrane-bound subunits, gp91phox (Nox2) and p22phox, collectively known as cytochrome b558 [91]. Nox2 subunits require p22phox for functionality [92]. The membrane-bound proteins are activated by translocation of the cytosolic subunits p40phox, p47phox, p67phox, and Rac to cytochrome b558. The
complex catalyzes electron reduction of oxygen using NADPH as an electron donor [93]. Oxidation of cytosolic NADPH creates electrons, which are transported from the cytosol across membranes to the phagosome or to the extracellular milieu. This culminates in transfer of electrons to molecular oxygen to produce superoxide \( \text{O}_2^- \). Superoxide is a highly-reactive, free radical that can both spontaneously or enzymatically dismutates to generate \( \text{H}_2\text{O}_2 \). Subsequently, either spontaneously or by reaction with other molecules ROS, such as singlet oxygen, ozone, chloramine, or hydroxyl radical are formed. \( \text{H}_2\text{O}_2 \) furthermore serves as specific substrate for the neutrophil enzyme MPO to generate an additional, very potent ROS product hypochlorous acid (HOCl) [94-96]. ROS kill microorganisms by oxidizing proteins and lipids. In addition, acidification of the phagosome enhances the effectiveness of pH-sensitive antimicrobial compounds. Acidification in neutrophils in contrast to macrophages is modest and neutrophil phagosomes are initially alkaline. The pH change is depending on proton consumption for example during the process of dismutation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) [97, 98].

As these ROS products mediate oxidative damage against invading pathogens they impose at the same time a large potential damage to host tissues. Therefore, neutrophil oxidative bursts need to be tightly regulated and are only transiently active. In recent years an additional role of ROS production has increasingly been recognized. It has been shown that ROS may play a critical role as signalling molecules that regulate diverse physiological signalling pathways. ROS additionally regulate cellular functions by reacting with macromolecules such as DNA, proteins, and lipids. The beneficiary effects of ROS involve for instance in cell proliferation, migration, and cell differentiation. On the other hand, excess of ROS may cause malignant signalling and contribute to disease amplification in cardiovascular complications or hypertension [99].
Inhibition of reactive oxygen species (ROS)

Diphenylene iodonium (DPI) is a commonly used potent inhibitor of NADPH oxidase activity. NADPH oxidase inhibition by DPI is mediated by targeting the flavin-containing subunit, withdrawing an electron from the oxidase and subsequently inhibiting superoxide formation (Fig.5) [94, 100]. It has been shown that DPI has a toxic effect by inhibiting pentose phosphate pathway and tricarboxylic acid cycle on glial cells [101] which makes it undesirable for usage in ROS-driven diseases. Unlike DPI, antioxidants do not act by inhibiting NADPH oxidase activity. They scavenge and neutralize the products of the complex along with SODs. The SOD mimetic tempol (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) is currently applied in clinical studies to assess therapeutic usage, such as for improving blood pressure [102] and also in brain tumour patients receiving radiotherapy, a topical application of tempol is suggested to protect normal cells from radiotherapy-induced alopecia and damage without protecting radiotherapy-targeted cancerous cells [103, 104]. Such data suggests that ROS are critically important and cause damages in several diseases [105].

Fig. 5: NADPH oxidase complex
NADPH oxidase is a multi-subunit protein complex producing superoxide upon activation and assembly. The DPI targets the flavin containing subunit of the complex. Superoxide is generated by transfer of electrons to molecular oxygen (O₂). The product is further dismutated by the SOD enzyme into hydrogen peroxide H₂O₂ [ Adapted from Williams and Griendling, 2007].
**Phagocytosis**

Another antimicrobial feature of neutrophil is phagocytosis. To facilitate phagocytosis microbes are opsonized, in other words immune-marked, by molecules called opsonins. Important opsonins are antibodies, immunoglobulins with a variable region that harbours microbe-specific entities and a constant region which can be recognized by receptors on immune cells. These antibodies coat microbial surfaces by binding to their specific counterpart, the so called antigen. Antibody binding enhances and expedites the recognition of the constant part of the antibody by host receptors. Another example of opsonins are complement proteins found in serum. Once activated, complement proteins can bind and mark pathogens to either induce a membrane attack complex for lysis of microbial cells or to bind specific complement receptors on immune cells to induce subsequent cellular immune functions.

Two pathways mediate phagocytosis. The first is antibody-mediated by Fc (fragment crystallisable)-receptor that binds to the constant Fc region of antibodies attached to the invaded pathogen. Upon detection of the opsonized microbe by phagocyte receptors, the antibody–mediated engulfment appears rapidly by forming lamellipodia via rearranging cytoskeleton proteins. This type of engulfment is a rapid process happening in 30 seconds for neutrophils for instance [106]. The second pathway is complement and complement receptor-mediated in which the pathogenic microbe appears to sink into phagocytes [107].

The neutrophil phagosome, the membrane-enclosed vesicle forming upon engulfment, is a hostile environment for microbes. The assembly of NADPH oxidase to the phagosome results in the generation of large amounts of ROS molecules in the phagosome. Additionally fusion of specific granules and release of antimicrobial properties such as MPO contribute to the potent antimicrobial milieu to form mature phagosomes with almost neutral pH values [106].
Neutrophil cytokines

Granulocytes are generally believed to be poor producers of secretory effector proteins, referred to as cytokines. However, activated neutrophils do release numerous cytokine hat have specific effects on communication and interactions between cells. Specialized cytokines, so called chemokines, have the main function to recruit immune cells towards higher concentrations of the respective chemokine, such as interleukins made by leukocytes in infected or damaged areas. Cytokines may interact and communicate directly on cells that produce them (autocrine action), on nearby cells (paracrine action), or on distant cells (endocrine action) [108]. Neutrophils express a number of different groups of cytokines to modulate immune responses, such as chemokine receptors CC (CCL2) and CXC (CXCL8), granulocytes-colony stimulating factors (G-CSF), pro-inflammatory cytokines (IL-1α), immunoregulatory cytokines (IFNα), anti-inflammatory cytokines (transforming growth factor beta, TGFβ1), angiogenic and fibrogenic factors (vascular endothelial growth factor, VEGF), TNF-superfamily members (TNFα) amongst others [109]. G-CSF is one of the cytokines that increases the production of neutrophils as a potent hematopoietic regulator of the bone marrow to produce granulocytes and to release matured cells into blood. For instance, chemotherapy related-neutropenia (low level of neutrophils) in cancer patients makes them susceptible for infections and sepsis. Therefore, G-CSF treatment is used to accelerate recovery after chemotherapy. It is also used to mobilize pluripotent hematopoietic cells into the blood of donors. These enriched hematopoietic stem cells are then used for transplantation to cure blood cancers. The discovery of this transplantation technique granted the Nobel prize in 1990 [110, 111].

Neutrophil granules

Ehrlich described neutrophils first due to their ability to take up neutral to basic dyes, particularly the dye azure. The fact that azure was incorporated by a specific set of granules resulted in the name azurophilic granules and
separated these vesicles form specific granules, which did not take up azure. Neutrophils tertiary granules were identified later by electron microscopy [71]. Granules of neutrophils contain antimicrobial molecules and enzymes that target microbes and degrade extracellular matrix components [112]. Primary or azurophilic granules contain large amounts of cationic proteins. The cationic nature is shared by almost all antimicrobial peptides (AMPs) and proteins. Azurophilic granule contents have different components, such as MPO (discussed above), defensins and bactericidal permeability-increasing protein (BPI), the latter to directly targeting microbial membranes [113]. Cathepsin G, proteinase 3 and NE [114] are serine proteases with the purpose to degrade engulfed microbes by proteolytic activity.

Secondary or specific granules contain components, such as peptidoglycan recognition proteins (PGRPs) [115] that bind cell wall peptidoglycans to inhibit growth and lysozyme that degrades peptidoglycan to lyse bacterial cells [112, 115, 116]. Additionally, lactoferrin sequesters iron and copper to remove these essential elements required for bacterial growth [113].

Besides antimicrobial activities, secondary granules have other functions such as the chemotactic ability of matrix metalloproteinase-8 (MMP-8) to recruit neutrophils to the site of inflammation [116]. The tertiary (gelatinase) granules contain gelatinase a matrix metalloproteinase-2 (MMP-2) which breaks down extracellular matrix proteins to facilitate neutrophil migration through the extracellular matrix to the site of infection. In addition, the proteolytic activity of MMP-2 can also cause damage to bacterial cell walls [113]. There are also other enzymes synthesized by neutrophil that are not specific to one particular granule type, such as LL37 a member of the cathelicidin AMP family [117].
Neutrophil pattern recognition receptors (PRRs) recognize fungal pathogen associated molecular patterns (PAMPs)

Cells of the immune system evolved to express different classes of membrane-bound pattern recognition receptors (PRRs) to identify and bind conserved molecular motifs associated with pathogens called pathogen-associated molecular patterns (PAMPs) as well as ligands from damaged or dead cells called damage-associated molecular patterns (DAMPs). PRRs are sentinels of the immune defence which trigger cell signalling for cytokine or chemokine production, phagocytosis, degranulation or other functions in order to launch a pathogen-tailored response.

Diverse pathogens, such as viruses, bacteria, protozoa or fungi with different biochemical compositions and entry sites may be recognized by host PRRs with very similar, often overlapping mechanisms [118].

Examples discussed below illustrate clearance of *C. albicans* by the innate immune system as mediated by PRRs-mediated sampling of PAMPs (Fig. 6). For fungal recognition, over the past 50 years the dogma was that major players of innate immunity, for instance neutrophils and macrophages, unselectively recognize and destroy fungi [119]. Within the past decade this concept has changed and it has been appreciated that innate immune cells indeed specifically recognize microbes by dedicated PRRs [33].

PPRs are classified into four major classes. To the first class belong Toll-like receptors (TLRs). So far, thirteen TLR have been described whereof TLR1 to TLR10 are represented in humans. TLRs comprise cell membrane-associated (TLR1, TLR2, TLR4, TLR5 and TLR6 and TLR10) and intracellular (TLR3, TLR7, TLR8 and TLR9) receptors [120]. *C. albicans* and other fungi for instance are recognized by TLR4 via O-linked mannan from CWPs. A further example is TLR6 which was shown to recognize zymosan particles as homodimers or as heterodimers TLR2/TLR6, [33]. Zymosan is a crude preparation of *Saccharomyces cerevisiae* cell walls. The Second class is composed of C-type lectin receptors (CLRs), such as Dectin-1 and
complement receptor 3 (CR3), which are membrane-bound receptors. Dectin-1 recognize β-(1-3) glucan and initiates phagocytosis of *C. albicans* [121]. The importance of these receptors for antifungal immunity is demonstrated by the fact that for instance Dectin-1 deficiency in humans leads to a highly increased risk to acquire mucosal candidiasis [122]. CR3 recognizes and binds both to mannann and glucan components of the fungal cell wall. Additionally, collaboration of dectin-1 with CR3 is important during phagocytosis [123, 124]. After interactions with PAMPs, PRRs further converge signals to often common adopter molecules, such as myeloid differentiation primary response gene 88 (MyD88), and mediate downstream intracellular signalling via kinases to activate transcription factors [125]. Upon phagocytosis most of the internalized receptors are degraded or recycled [126].

The third class comprises the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic-acid-inducible gene I (RIGI) receptors for recognition of bacterial peptidoglycans or viral nucleic acids. These intracellular classes of receptors have not been described for fungal recognition yet [33].

The forth intracellular class is composed of signalling domains of receptors that are responsible for the functional activity of respective receptors, such as the TLR-interleukin 1 receptor (TIR) domain for TLRs or the immunoreceptor Tyr-based activation-like motif (ITAM) domain for some CLR [33].
Fig. 6: PRRs and fungal PAMPs interaction
Certain C. albicans PAMPs are recognized by PRRs on immune cells. Dectin-1 recognizes β-(1-3) glucan, TLR2 recognizes phospholipomannan, TLR4 recognize O-linked mannan, TLR6 recognizes zymosan and intracellular TLR9 recognizes fungal DNA. The PRRs signal to adaptor molecules, intracellular molecules and transcription factors [Adapted from Netea, Brown et al., 2008].

Additional receptor types expressed on neutrophil surfaces are immunoglobulin superfamily receptors, such as Fc receptors which recognize constant regions of antibodies [127]. Another large family of receptors are G-protein coupled receptors (GPCRs). Adenosine and acetylcholine receptors (AChR) belong to this family. Nicotine acetylcholine receptor (nAChR) recognizes nicotine. Adenosine receptors specifically bind adenosine and mediate signals into the cell upon stimulation by extracellular nucleoside.

These receptors could exert inflammatory or anti-inflammatory responses in neutrophils depending on the stimulus. Extracellular adenosine may inhibit
TNF-α release [128], whereas nicotine modulates proinflammatory response in neutrophils via nAChRs by inducing IL-8 release [129].

Nicotine, the major toxic component of cigarettes, is associated with numerous diseases that may have deadly outcomes. Smoking results in the absorption of considerable amounts of nicotine into the blood from where it reaches different organs. Nicotine can induce infiltration of leukocytes, such as neutrophils, and the infiltration in turn may lead to damage of surrounding tissues. Such damage can aggravate inflammatory disorders, such as chronic obstructive pulmonary disease (COPD) [130]. The effect of nicotine is mediated through various nAChRs, which have been initially described as ligand-gated cation channel superfamily consisting of (α2-10) and (β2-4) subunits [131]. According to previous reports, nicotine can delay spontaneous apoptosis of neutrophils and suppress phagocytic activity in neutrophils. In contrast, the oxidative burst is unaffected and degranulation of neutrophil elastase is rather enhanced [130, 132].

Examples for GPCRs on neutrophils mediating anti-inflammatory signalling are adenosine receptors. Adenosine is an endogenous purine nucleoside and a breakdown product of adenosine triphosphate (ATP) with a short half-life in tissue. Upon adenosine generation, the enzyme adenosine deaminase can rapidly degrade adenosine into inosine. Alternatively, adenosine is phosphorylated by adenosine kinase to form adenosine monophosphate (AMP). Notably, despite the short tissue half-life, adenosine has potent signalling functions [133]. Adenosine can be released constitutively by neutrophils among many other cell types. Under baseline conditions the extracellular concentrations of adenosine is approximately 300 nM in circulation [134-136]. In inflamed and injured tissue, adenosine generation and release is greatly amplified and levels can rise up to 100-fold above the average concentrations reaching 10 μM in tissues [134]. Inactivation of extracellular adenosine deaminase can further increase adenosine levels in the extracellular milieu [137]. Additionally, the expression of nucleoside transporters, responsible for clearance of adenosine can be inhibited
resulting in extracellular adenosine accumulation [138]. Adenosine acquires various physiological roles in different organs. In the brain, adenosine is an inhibitory neurotransmitter. It promotes sleep and suppresses arousal. In the heart, it improves blood circulation by dilating coronary blood vessels, preventing coagulation, and decreasing heart rate [128, 139]. Adenosine signals via four widely expressed GPCRs encoded by the genes ADORA1, ADORA2A, ADORA2B, and ADORA3. The respective receptors are termed A₁, A₂A, A₂B, and A₃. Neutrophils respond to adenosine via expression of all four adenosine receptors. At low concentrations, adenosine can act via A₁ and A₃ receptor subtypes, which have high-affinity for adenosine and promote neutrophil chemotaxis and phagocytosis [140, 141]. At higher concentrations, adenosine acts on the low-affinity A₂A and A₂B receptors to inhibit neutrophil trafficking. Signalling via A₂A and A₂B additionally leads to inhibition of oxidative burst, inflammatory mediator production, and granule release [128, 141-143]. Modulation of neutrophil function by adenosine is relevant in a broad array of diseases, including ischemia reperfusion injury, sepsis, and sterile acute lung injury [128]. For example in neutrophil-mediated reperfusion injury, the tissue damage stems from increased neutrophil infiltration mediating oxidative damage to endothelial cells. Administration of adenosine agonist targeting A₂A and A₃ receptor has shown to improved symptoms of reperfusion injury [144, 145].

**Neutrophil extracellular trap (NET) formation**

Besides apoptosis or necrosis, another distinct form of cell death has been described in neutrophils, which was named neutrophil extracellular trap (NET) formation. A decade passed since NETs were discovered [146]. NETs are fibres of extracellular chromatin which are heavily laden with antimicrobial proteins and enzymes. NETs are released into the extracellular space to trap and kill microbes [146]. Different pathogens interact with NETs [83]. Gram-positive and Gram-negative bacteria, protozoa [147] and virus particles have been described to be ensnared by NETs [148-150]. Moreover, NETs are critical for antifungal defence. They ensnare and kill fungi, for
instance *C. albicans* [151], *Aspergillus spp.* [152] or *Cryptococcus neoformans* [153]. Indeed, NETs inhibit growth and germ tube formation in *C. albicans* [151] along with other potent antimicrobial functions of neutrophils, such as phagocytosis, ROS production, and degranulation of granular proteins.

Neutrophils form NETs in a ROS dependent manner upon stimulation with yeast or hyphae of *C. albicans*. In 2009 Ermert et al. demonstrated that large filaments of *C. albicans* more potently induced NETs than smaller yeasts from the same pathogens. Similarly, hyphae induced more ROS in neutrophils as similar amounts for yeasts did [154, 155]. As for microbial-induced NET formation ROS production were essential [156] (this will be discussed in more detail in following paragraphs) the observations were in good agreement with each other. A more recent study suggested that neutrophils sense microbe size and exclusively launch a NET program when stimulated with large filamentous hyphae that cannot be easily phagocytosed [157, 158]. Neutrophil undergo a series of morphologic changes starting from the nucleus and ending with the cell membrane. After the nucleus has expanded, the nuclear membrane disassembles and nuclear and cytoplasmic contents can mix [146, 156]. Finally, the plasma membrane integrity is lost and mixed DNA-protein content is expelled to the outside to form NETs (Fig. 7). Antimicrobial NET components have diverse functions to combat microbes. Massive release of histones to the extracellular space are extremely effective antibacterial components by promoting bacterial lysis [159]. Highly positively charged peptides and proteins such as defensins, LL-37, MPO, BPI and cationic proteases such as NE, cathepsin G, proteinase-3 are able to disrupt microbial cell walls and membranes or inhibit microbial growth. Neutrophils also have factors that restrict nutrient supply to microbes; for instance granular protein lactoferrin that chelates iron and cytoplasmic protein calprotectin that sequesters zinc ions to prevent access of microbes to these essential micro nutrients [160, 161].
Fig. 7: Mechanism of NET induction in response to *Candida albicans*

Neutrophil starts to phagocyte *C. albicans*. In addition, neutrophils release NETs and subsequently trap and inhibit the growth of *C. albicans*. In response to *C. albicans* NET induction started by losing the characteristic lobes of nucleus and become delobulated. Upon nucleus expansion, the nuclear membrane integrity is lost and the nucleus chromatin content is released into cytosol, mixed with granule contents and cytosolic proteins. Eventually, the cell membrane ruptures. At this final stage the mixture of chromatin and proteins is released into the extracellular milieu to form NET, which now traps and kills *C. albicans*. 


Other NET-forming cells

The phenomenon of extracellular trap (ET) formation was first described in human neutrophils. This observation was soon followed by findings on other immune cells that were able to release similar ETs, such as eosinophils [162], mast cells [163], basophils [164], and macrophages [165]. Contribution of other cells to promote NETosis has been shown in platelets-neutrophils interactions which may result in NET release in circulation in organs like liver sinusoids and pulmonary capillaries during sepsis to ensnare and kill bacteria to eventually decrease further dissemination [166]. However, the phenomenon of ET formation is not restricted to humans, but seems to be a conserved mechanism among many different species. ETs have been detected in plants [167], and various animal groups, such as insects [168], fish [169], birds [170], and rodents [154].

NET-evasion mechanisms

Most microbes trapped in NETs are killed by an arsenal of NET-associated antimicrobial weapons. The antimicrobial mechanism could be due to the charge-mediated mechanism whereby negatively charged microbes are trapped in AMPs in NETs. Some microbes, however, evolved strategies to escape from the DNA backbone of NETs by nuclease activity. *Staphylococcus aureus* [171] and *Vibrio cholerae* [172] for instance, are able to apply this escape mechanisms by induction and secretion of extracellular nucleases.

The inhibition of AMPs is another mechanism described in Group A streptococcus (GAS). This Gram-positive microbe can sequester NET-bound cathelicidin via M1 protein together with DNase expression, which allows the survival in NETs and increases virulence [173]. In addition, microbial surface structures for instance capsules of *Streptococcus pneumonia* and GAS prevent trapping and consequently killing in NETs [174, 175]. Similarly, extracellular fibrils of the emerging animal and human fungal pathogen *Cryptococcus gattii* renders it less-susceptible to neutrophil killing and enhances its resistance to NETs [176].
**Different mechanisms proposed for extracellular trap formation**

NETosis is a unique form of cell death that is morphologically distinct from other forms of programmed-cell death. Similarly, as neutrophils do not randomly engulf microbes, they neither release NETs in an uncontrolled or spontaneous fashion. The signalling and subcellular events that lead to NET formation and regulatory mechanisms have not yet been fully understood. A few cellular events were proposed to be involved in NET formation, however mechanisms may diverge depending on the type of triggering stimuli.

Different stimuli have been described to induce NETs such as lipopolysaccharide (LPS), phorbol myristate acetate (PMA), IL-8, interferons, as well as complement proteins [177] or activated platelets [166]. In the presence of foreign antigens, antibodies bind to them and form immune complexes (ICs) which act as specific epitopes. ICs cause a number of responses in neutrophils including complement deposition, opsonisation, phagocytosis, and upon endocytosis of soluble ICs release NETs [178].

The first mechanism to describe NET formation was proposed by Fuchs et al. This process involves ROS production, nuclear envelope and granule membranes dissolve allowing mixing of the cytoplasmic components [156]. Chromatin degradation is mediated by azurophilic granule components which are translocated to the nucleus. Particularly NE and MPO promote histone degradation and chromatin decondensation upon nuclear translocation [156, 179]. In addition, deimination of histone-associated amino acid arginine to form citrulline, termed as citrullination, is one of the important steps characterized in chromatin decondensation during NET release. This mechanism mediated by peptidylarginine deiminases (PADs), a family of calcium-dependent enzymes that catalyze the process of citrullination of proteins. A PAD4-null mouse strain failed to release NETs [180, 181].

Generation of ROS at the phagosomal membrane is a critical early step in NET formation. Neutrophil activation and release of harmful cellular contents and granular proteins are strongly regulated by production of ROS.
There are several studies to support NET induction in a ROS-dependent manner. Pretreatment of neutrophils with a potent NADPH oxidase inhibitor, DPI, abrogates NET formation in human and murine neutrophils upon stimulation with protein kinase C (PKC) activator PMA [156, 182].

Importance of ROS for NET induction is highlighted in certain situations such as chronic granulomatous disease (CGD). These individuals have inherited mutations in NADPH oxidase complex subunits rendering the complex non-functional and resulting in lack of the oxidative burst in phagocytes. Consequently, CGD phagocytes are impaired in antimicrobial activity. In addition, as ROS are important signalling molecules, CGD patients show sterile inflammation and hyper-inflammatory states during infections [183]. As mentioned above, microbial-induced NET formation requires ROS production and therefore neutrophils from CGD patients additionally are unable to form NETs [152]. Thus, impaired NET release among other reduced functions renders CGD patients more susceptible towards microbial infections. Particularly invasive pulmonary *aspergillosis* is a very frequent and refractory infection in CGD patients. Notably, gene therapy to complement NADPH oxidase function could also restore NET formation by neutrophils and subsequently killing of large filamentous *Aspergillus* hyphae [152, 184].

However, other pathways of NETosis which are NADPH oxidase-independent have been described. One report indicates that NET-like structures are composed of mitochondrial DNA which were released upon priming cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) and stimulation by a complement component. Mitochondrial DNA release was measured after 15 minutes and neutrophils remained viable even after the release [185]. However, there was no kinetic of NET formation indicated maybe due to the fact that neutrophils have limited number of mitochondria and their DNA contents are relatively lower compared to nuclear DNA. A few other studies suggested rapid NET release mechanisms via small conductance calcium-activated potassium channel 3 (SK3) or
mitochondrial ROS [186] whereas a consecutive study rejects the involvement of mitochondrial ROS in NET formation [187].

As NET formation is accompanied by plasma membrane rupture and consequently cell death, the silent cellular death named apoptosis which is initiated for unhazardous removal of neutrophils is presumably regulated in a distinct manner. Indeed, neutrophils may block apoptosis to allow NETosis. Not caspase-activation, but the serine/threonine-specific protein kinases, mitogen-activated protein kinases, and extracellular signal-regulated kinases (Raf-MEK-ERK) pathway was shown to be crucial for induction of NET formation [188]. In line with this, protein kinase B (known as Akt) was shown to act as a molecular switch between NETosis and apoptosis. When Akt is activated, cells undergo NETosis. The inhibition of Akt commits the cell towards apoptosis [189]. Additionally, stimulation of neutrophils with PMA, form large autophagosome-like vacuoles. During autophagy, a regulated cellular process to recycle the cell’s own resources, inhibition of phosphoinositide 3-kinase (PI3Ks) could block vacuolization and subsequently chromatin decondensation and thereby preventing NET formation which promoted apoptosis instead. Thus, PMA-induced NET formation requires both autophagy and ROS production [190].

Several studies have suggested that NET release may not always be associated with cell death, but could also be performed by cells which remained alive after NET expulsion. During this version of NETosis, DNA release from the nucleus occurs by packing of DNA in budding vesicles derived from the nuclear membrane, which subsequently are released into the extracellular space [191, 192]. Once the vesicles reached outside of the cells, they ruptured to release their packed chromatin. This process was described to be oxidant-independent. In doing so, NETing neutrophils induced by Gram-positive bacteria could retain other defence functions, such as migration and phagocytosis to prevent further bacterial dissemination. This mechanism of vital NET release stimulated during S. aureus infection, is a rapid process occurring within 1 h [81, 193]. In contrast, lytic NET
release begins after 2 h and is usually ongoing to 4 – 6 h, depending on the stimulus [194].

**NET-associated diseases**

NETs are efficient antimicrobial entities that entangle and kill microbes. However, their potent antimicrobial activities harbour an intrinsic harmful potential for the host by directly attacking surrounding tissue or by over-activation of inflammation. For instance, it has been described that NET-associated protein components, in particular histones, can mediate host cell toxicity on epithelial and endothelial cells in lung tissue [195]. Therefore, the different contribution of NETs to human disease will be discussed in the following paragraphs.

As indicated above, NETs are involved in various inflammatory disorders and can act as a double-edged sword, promoting pathology. In malignancies, such as cancer, systemic effects are frequently induced by increased release of pro-inflammatory immune effector molecules. G-CSF is associated with neutrophilia and produced by various tumors [196]. Neutrophils from cancer patients have been shown to be more prone to release NETs and increased NETs were associated to induction of thrombosis, the second most common cause of death in cancer patients [197]. Moreover, postsurgical infection in cancer patients was linked to adverse oncologic outcomes [198]. Unwanted infections led to microvascular NET deposition. The NETs trapped microbes, but along with the primary target additionally circulating lung carcinoma cells and thereby promoted metastasis.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease causing damage to many different body sites from the skin on the outside to deep-seated organs inside. The immune system mistakenly reacts against the individual’s own proteins and DNA. Resulting self-antibodies form complexes with the host molecules which bind to cell surfaces and blood vessels in vital organs such as kidneys leading to hazardous inflammation [199]. SLE patients have high titers of auto-antibodies directed against neutrophil components, such as MPO, which are additionally abundant on
NETs [200, 201]. During disease progression, the number of neutrophils undergoing NET formation is considerably increased leading to the described effects [202-204]. In addition, some SLE patients show an impaired ability to remove NETs from the system, as auto-antibodies tightly binding NET components block access of DNase1 and thereby prevent dismantling of NET [202]. The increased NET phenotype could be successfully diminished in mice by application of a PAD4 inhibitor, indicating that NET formation was involved [202-205]. An additional complication in SLE is a resulting amplification loop leading to organ damage. NETs stimulate inflammasomes in macrophages which subsequently release IL-1β and IL-18 cytokines. These in turn promote additional NET release. More NET release causes additional inflammasome activation and so forth [165, 206].

For atherosclerosis, a strongly inflammatory disease in which life-threatening conditions are not uncommon, NET involvement has been suggested as well. The disease is associated with chronic inflammation of the vessel wall in large arteries, often manifesting as myocardial infarction or stroke. The inhibition of PAD4 prevented NET formation in a murine model of atherosclerosis. Blocking of NET formation decreased atherosclerotic lesion size, and delayed carotid artery thrombosis [205].

However, NET release is not exclusively harmful during inflammation. Accumulation of neutrophils in gout disease in response to monosodium urate (MSU) crystals resulted in an acute inflammatory reaction [207]. Under high density of neutrophils, NET aggregates are formed in a ROS-dependent manner. These MSU-induced NET aggregates could efficiently resolve neutrophil-derived cytokines and chemokines by degradation via released serine proteases, which disrupted additional neutrophil recruitment and aggregate formation.
Until now, NETs have been associated in prevention and progression of many diseases. Ongoing research on NETs will generate novel insights to elucidate different mechanisms regulating NET formation. A better understanding of this form of neutrophil cell death may likely lead to more effective and safer therapeutic approaches to master NET-associated diseases.
Methodology

In this section, main methods used in papers I, II, III and IV are briefly discussed.

Fungal strains and cultivation

The clinical isolate *C. albicans* strain SC5314, Δefg1, Δtup1, Δsod5, Δsod4Δsod5Δsod6 [208], and *Saccharomyces cerevisiae* wild-type strain UMY2067 were used. The strain SC5314 was chosen as a wild-type strain [209]. To grow *C. albicans* in yeast growth forms, strains were cultured in 20 ml YPD (1% yeast extract, 2% bacto peptone, 2% glucose) at 30 ºC, at 300 rpm on an orbital shaker. To induce hyphae, the cells were inoculated in RPMI medium (RPMI 1640, Lonza) and incubated at 37 ºC with the starting 0.1 OD600 nm. To inoculate Δtup1 50 mg wet pellet in 20 ml YPD after centrifugation was measured to obtain approximately similar starting amounts as other inoculated strains.

Dry mass quantification

The dry mass of *C. albicans* morphotypes was used instead of cell number. First, the mass for each strain was obtained by weighting dried cell pellet at 80 ºC on top of pre-weighted 2.5 cm circular glass microfiber filters (Whatman, 1.2 μm pore size). Then the metabolic activity was measured by XTT method. These values were then applied to correlative formula to obtain a correlation factor [155]. The correlation factor was generated for different strains at different time points.

Mouse strains

C57BL/6 and/or male gp91phox− (B6.129S-Cybbtm1Din/J) mice were purchased from Jackson Laboratory and were kept under pathogen-free condition in the Umeå Center for Comparative Biology (UCCB) animal facility.
Neutrophil isolation

Neutrophils were isolated from fresh human blood of healthy volunteers according to the principles expressed in the Declaration of Helsinki. Mice neutrophils from bone marrow were also purified according to local Ethical Committee (Umeå djurförsöksöketiska nämnd) with the permit number A79-14. Neutrophils were isolated as described previously [155, 210] by a combination of differential equilibrium density centrifugation on Histopaque and discontinuous Percoll gradients. The viability of isolated cells was assessed using a cell counter by trypan-blue staining.

ROS measurement

ROS were measured with a luminol-based assay. Neutrophils incubated with 50 µM luminol (Sigma-Aldrich) and 1.2 u/ml horseradish peroxidise (Sigma-Aldrich) for 15 min at cell culture condition (37 °C, 5% CO₂) into white 96-well plates (Nunc) in RPMI. The production of ROS were measured in triplicate for a total of 3 h in a luminometer (Infinite 200, TECAN). ROS quantification is presented either as relative light units (RLU) over time or as total ROS by measuring the integral of the curve.

ROS scavenging

Removal of ROS were measured for different masses, morphotypes and strains of C. albicans. Cells were added into white 96-well plates (Nunc) containing hydrogen peroxide (0.5 mM), 50 µM luminol and 1.2 u/ml horseradish peroxidise (all from Sigma-Aldrich) in RPMI, and incubated in cell culture condition. RLU was measured in triplicate for a total of 20 min in a luminometer (Infinite 200, TECAN).

Immunostaining of neutrophils

Neutrophils were seeded on cover slips coated with 0.01% poly-L-lysine (Sigma-Aldrich) with a concentration of 1 × 10⁵ cells in 24-well plate. Cells were fixed using 2% final concentration paraformaldehyde (Sigma-Aldrich).
For immune staining, antibodies directed against histone H1 (Acris) and NE (Calbiochem) or polyclonal anti-\textit{Candida} antibody (Acris) were applied. Secondary antibodies conjugated to Cyanine dyes (Cy 2 and Cy 3, Jackson ImmunoResearch) or DAPI were used to stain DNA. Cells were washed and mounted in Mowiol 4-88 (Calbiochem) and images were captured using (Nikon 90i fluorescence microscope) and analyzed with NIS-Elements software ver. 3.20.

**NET quantification**

NETs were quantified either microscopically or by fluorescence assay.

**Microscopic quantifications**

NETs were stained by staining released DNA with DAPI or other markers for NETs such as NE and histone antibodies. In average, 6-10 images of the same sample at different fields were captured randomly. Image J software (version 1.44p) was used for quantification and analysis. In general, more than 250 cells for each condition were counted. Carefully the area of each cell was marked using the software. Pixels in marked area from every image were converted to μm². Finally, NET forming cells presented in percentage.

Notably, human neutrophils have an average diameter of 10 μm in intact stage and the surface area of approximately 80 μm². Cells that exceeded area of 100 μm² considered as cells undergoing NET formation.

**DNA fluorescence (cell death) assay**

This assay was carried out using sytox green dye (2.5 μM, Invitrogen). The dye is impermeable in live cells and stains only dead cells. Sytox green intercalate with DNA to produce fluorescence. The relative fluorescence directly correlates to the amount of DNA released by dead cells, which can be expressed as percentage of dead cells in comparison to 100% lysis controls. DNA fluorescence assay was used in combination with microscopy to quantify NETs.
Animal experiment

Mice were kept in the animal facility Umeå Centre for Comparative Biology (UCCB). Animal experiments were conducted in accordance with recommendations from Swedish animal protection laws and applicable guidelines (djurskyddslagen 1988:534; djurskyddsförordningen 1988:539; djurskyddsmyndigheten DFS 2004:4), with ethical committee permit number as stated above. Mice were infected with $5 \times 10^5$ fresh cultured \textit{C. albicans} yeast cells. The cells were washed and resuspended in 1X PBS prior to intravenous injection. Seven male mice per group were injected peritoneal one hour after infection with Cl-IB-MECA (100 $\mu$g/Kg), MRS1191 A$_3$ antagonist (50 $\mu$g/Kg) dissolved in 100 $\mu$l PBS or with 100 $\mu$l 1X PBS only as control. After 3 days treatment as stated, mice were euthanized and kidneys were harvested. Each kidney was stored in a tube containing 5 ml ice cold 1X PBS plus kanamycin (45 $\mu$g/ml), ampicillin (100 $\mu$g/ml) to prevent bacterial contamination. Isolated kidneys were homogenized prior to colony forming units (CFU) measurement (refer paper II).

Colony forming units

Human or murine neutrophils stimulated with \textit{C. albicans} or isolated kidney of mice were homogenized. Serial dilutions were made prior to plating on YPD agar plates. Plates were incubated over night at 30 $^\circ$C to determine CFU the day after.

Cytokine measurement

Briefly, human neutrophils infected with different masses of \textit{C. albicans} yeast and hyphae; after 6 h incubation supernatants were collected. The IL-8 concentration per $10^5$ neutrophils was determined using human IL-8 ELISA kit (ELISA MAX Deluxe, Biolegend) according to manufacturer’s instruction.
Chemotaxis assay

Neutrophil migration was measured in a transwell system (BD Falcon, HTS FluoroBlok Insert, 3 μM pore size). Neutrophils were labelled with BCECF-AM (Sigma-Aldrich), a cytoplasmic fluorescent dye, at a final concentration of 3.3 μM. For base line migration the RPMI with 0.05% HSA and as 100% control only labelled neutrophils were used. The samples were monitored for fluorescence under cell culture condition in a fluorescence spectrophotometer (FLUOstar Omega, BMG). Chemotaxis was plotted as percentage of total signal.
Aims

NETs released by neutrophils serve to trap and kill microorganisms and are essential to control the opportunistic fungal pathogen *C. albicans*. However, at the same time NETs may have deleterious effects on the host, stemming from pro-inflammatory and tissue-destructive activity of NET and exposed neutrophil components. These diverse effects of NETs suggest that NET formation is not a spontaneous, but rather a tightly regulated phenomenon.

**Paper I**

How do neutrophils differentially respond towards different morphologies of *C. albicans*?

**Paper II**

How is NET release negatively regulated during *C. albicans* infection in response to adenosine as an anti-inflammatory agent?

**Paper III**

How does antioxidant tempol affect neutrophil ROS and ROS-dependent NET formation?

**Paper IV**

How does nicotine, the major toxic agent of tobacco, trigger the release of NETs?
Results and Discussion

Paper I

Neutrophils ROS response pattern is depended on Candida albicans amounts and morphotypes

To investigate host-microbe interactions involving different growth forms of polymorphic C. albicans and particularly to render these studies comparable, an exact defined ratio of these growth forms needs to be obtained. Due to the different nature of ellipsoid yeasts and filamentous hyphae, MOIs to determine pathogen to immune cell ratios is less useful for polymorphic fungi. As yeast growth results in a rise in cell number, whereas filamentous growth in elongation of cells we used a dry mass approach for the accurate determination of amounts of different C. albicans morphotypes in infection experiments.

We showed that dry mass unlike MOI directly correlates to the cell surface area. The surface area of the pathogenic, microbial cell is considered the host-interacting interface contributing to a variety of virulence traits. We demonstrate that the ratio of cell surface area per µg yeast versus hyphae remained constant around the value 1, whereas the ratio of cell numbers in µg yeast to hyphae increased over time. After 3 h the mass of a hypha is almost equal to the mass of 3 yeast cells. With the established tools in our hands, we set the stage to study human neutrophil responses specifically towards different morphologies of C. albicans using the same mass of yeasts and hyphae.

We studied neutrophil ROS responses with different amounts of C. albicans yeasts and hyphae. Surprisingly, we observed that at low infection doses under 5 µg of live C. albicans yeasts and hyphae, the yeasts induced more ROS than the hyphae. In contrast, when using higher amounts of yeasts and hyphae (above 10 µg), the hyphae induced higher neutrophil
ROS responses than the equivalent amount of yeasts. As we observed similar neutrophil responses depended on amounts of *C. albicans* morphotypes using mutant strains arrested in either growth form, we could conclude that the initial form of infection determined the outcome of neutrophil ROS responses rather than morphotype changes occurring during the infection experiment (Fig. 8).

**Fig. 8:** Amounts and morphotypes of *Candida albicans* shape neutrophil ROS responses
Neutrophil ROS generation were measured in a luminol-based assay. Different DM amounts of *C. albicans* were used to infect $10^5$ neutrophils. Each data point represents AUC corresponding to the total ROS in the course of 3 h. Neutrophils were infected with *C. albicans* yeasts (closed circles) or hyphae (open squares). The dashed line represents the best-fit curve from 3 different donors. Masses to MOI conversion for $10^5$ neutrophils: (yeasts) 1 µg$\pm$0.4, 5 µg$\pm$2.1, 10 µg$\pm$4.1, 20 µg$\pm$8.2, 30 µg$\pm$12.3; (hyphae) 1 µg$\pm$0.2, 5 µg$\pm$0.9, 10 µg$\pm$1.7, 20 µg$\pm$3.4, 30 µg$\pm$5.1 [Re-produced from Hosseinzadeh and Urban 2013].
**Candida albicans** yeasts are efficient in detoxification of neutrophils ROS

We aimed to understand whether lower levels of neutrophil ROS stimulated with high amounts of *C. albicans* yeasts were a result of an efficient detoxification of ROS or derived from active suppression of ROS production. For this purpose, we infected neutrophils with two different loads of *C. albicans* at two different time points. The first infection induced a characteristic ROS peak. The secondary (spiked) infection with larger amounts of *C. albicans* immediately resulted in rapid reduction of neutrophil ROS, but subsequently a second ROS peak arose indicating that ROS production could still occur and was not actively suppressed. In line with this, we also performed the spiking experiment with a mutant strain deficient in surface-localized SodP 4-6. This strain has previously been shown to be impaired in detoxification of phagocyte-induced ROS [208]. When used in a spiking experiment the mutant strain was unable to decrease neutrophil ROS production and a secondary ROS peak was absent. Thus, the declined ROS pattern in neutrophils in dependence of higher amounts of yeasts used to infect neutrophils is not due to active suppression of ROS by *C. albicans*, but rather to efficient ROS detoxification (Fig. 9).
Fig. 9: Detoxification of neutrophil ROS by Candida albicans

Spiking ROS assay: ROS production over time is shown for total of 3 h as relative light units (RLUs). For each sample, 10⁵ neutrophils were infected with C. albicans yeasts (closed circles) and after 30 min re-infected with 15 µg C. albicans hyphae (open circles, A) or C. albicans hyphae (closed squares) re-infected with 15 µg C. albicans yeasts (open squares, B). The first infection dose was 3 µg of wild-type C. albicans yeasts (black circles, C) or 3 µg of wild-type C. albicans hyphae (black squares, D); 30 min thereafter, the neutrophils were re-infected with 3 µg (gray symbols) or 10 µg (white symbols) Δsod4/5/6 mutant strain in hyphae (C) or yeasts (D) growth form. A representative experiment from 3 different donors is shown. Data are presented as means of 3 technical replicates ±SD. Masses to MOI conversion for 10⁵ neutrophils: (yeasts) 2 µg ≈ 0.8, 3 µg ≈ 1.2, 4 µg ≈ 1.6, 10 µg ≈ 4.1, 15 µg ≈ 6.2; (hyphae) 2 µg ≈ 0.3, 3 µg ≈ 0.5, 4 µg ≈ 0.7 10 µg ≈ 1.7, 15 µg ≈ 2.6. [Re-produced from Hosseinzadeh and Urban 2013].
Candida albicans morphotypes define IL-8 secretion pattern in neutrophils

Interestingly, the distinct pattern of neutrophil responses to different amounts and morphotypes of C. albicans was not restricted to ROS production, but similarly reflected by a second important activity of neutrophils: the release of IL-8. At lower doses, C. albicans yeasts triggered more IL-8 secretion than hyphae, whereas at higher doses hyphae stimulated higher amounts of released IL-8 (Fig. 10). Again, this pattern was rather dependent on the initial morphotype used to infect neutrophils as shown by morphotype-locked mutant strains.

Fig. 10: Neutrophil IL-8 response triggered with different amounts and morphotypes of wild-type C. albicans

Neutrophils IL-8 secretion was measured after 6 h by ELISA. Neutrophils were infected with C. albicans yeasts (closed circles) or hyphae (open squares). The dashed line represents the best-fit curve of 3 individual donors. Masses to MOI conversion for $10^5$ neutrophils: (yeasts) 1 µg = 0.4, 5 µg = 2.1, 10 µg = 4.1, 20 µg = 8.2, 30 µg = 12.3; (hyphae) 1 µg = 0.2, 5 µg = 0.9, 10 µg = 1.7, 20 µg = 3.4, 30 µg = 5.1 [Reproduced from Hosseinzadeh and Urban 2013].

To summarize, dry mass application revealed new insight into neutrophil ROS and IL-8 response patterns against C. albicans in respect of amounts as well as growth morphology. The response largely depended on the initial morphotype used for infection. The method is versatile and applicable to study host-pathogen interactions using polymorphic microbes.
**Paper II**

**NET formation induced by Candida albicans hyphae is modulated by adenosine**

NETs have strong antifungal activity against invasive *C. albicans* hyphae [151], however can additionally promote excessive inflammation in infected tissues [197]. Therefore, we investigated the immune-regulatory activity of adenosine in response to *C. albicans*. Neutrophils release NETs in response to *C. albicans* yeast and pre-grown hyphae under cell culture conditions which are hypha-inducing (Fig. 11 A). Addition of adenosine blocked NET release, however exclusively in cells infected with hyphae (Fig. 11 A, second and third row). In contrast, the level of NET release in cells infected with pre-grown yeast remained unchanged upon adenosine treatment (Fig. 11 A, fourth and fifth row).

More precise quantification of NET formation was used, either directly by microscopic analysis or indirectly by DNA fluorescence, to investigate the extent of NET inhibition by adenosine in response to PMA, *C. albicans* pre-grown hyphae or yeast. Both methods led to similar observations. Adenosine reduced NET induction only in response to *C. albicans* hyphae to approximately 50% without affecting the level of NET release upon PMA or infection with yeast 10 h after inoculation (Fig. 11B and C).

Adenosine levels can rise a 100-fold in damaged tissue reaching up to 10 μM to protect the host from further self-inflicted damage and to keep inflammation in balance [134]. Thus, we speculate that adenosine could dampen NET formation during candidiasis to allow optimal host response by controlling further inflammation induced by the fungus.
Fig. 11: NET induced by Candida albicans hyphae is blocked by adenosine

Immunofluorescence staining of neutrophils in presence or absence of 10 μM adenosine, 10 h post infection with C. albicans. Human neutrophils were stained with DAPI (blue channel), antibody against histone H1 (red channel) and C. albicans with an antibody directed against C. albicans cell wall (green channel, A). NET formation was quantified in microscopic and DNA fluorescence assay (B and C) 10 h post infection stimulated with C. albicans yeast, hyphae or PMA. Data are shown as means ±SD from 6 independent donors, in 3 technical replicates. Significance was analyzed by one-way ANOVA, P < 0.05.
Adenosine regulates NET formation via A<sub>3</sub> receptor in response to Candida albicans hyphae

Four expressed adenosine receptors on neutrophil were investigated to understand how NET inhibition by adenosine is mediated only towards C. albicans hyphae. Specific agonists targeting each four different subtypes of adenosine receptor A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> were applied to explore the responsible receptor agonist(s) that mediates NET down regulation when neutrophil triggered with C. albicans hyphae. Our results indicate that specific agonist for A<sub>3</sub> receptor, IB-MECA, resulted in 50% NET release reduction in response to C. albicans hyphae (Fig. 12).

To confirm this observation specific antagonist targeting A<sub>3</sub> receptor was applied to prevent A<sub>3</sub> selective agonist or adenosine interaction with the receptor. Treated neutrophils with A<sub>3</sub> receptor antagonist following by adding specific A<sub>3</sub> agonist or adenosine does not result in NET release reduction (Fig. 12). In conclusion, adenosine mediates NET reduction in response to hyphae via receptor A<sub>3</sub>.

![Graph showing DNA fluorescence](image)

**Fig. 12: Adenosine signals via A<sub>3</sub> to inhibit NET release induced by Candida albicans hyphae**

NET formation was quantified by DNA fluorescence assay, 10 h post infection with C. albicans hyphae. Data are shown as means ±SD from 6 independent donors, in 3 technical replicates. Significance was analyzed by one-way ANOVA, P < 0.05.
A3 modulates fungal infection in mouse kidneys

To study the potential relevance of A3 receptor in systemic candidiasis, wild-type mice were tail-vain infected with C. albicans. Post infection, groups of mice were intraperitoneally administered either with A3 agonist, antagonist or PBS with one dose per day for 3 days. Thereafter, kidneys were harvested and analysed for fungal burden. A3 antagonist-treated mice showed a significantly decreased C. albicans burden in their kidneys, in contrast to A3 agonist-treated mice showing a higher fungal load compared to PBS-administered control mice (Fig. 13A). These results indicate that A3 antagonist treatment supported the clearance of infection in kidneys and suggests the potential therapeutic application of A3 antagonist as adjunct therapy during systemic candidiasis. Additionally, MPO levels were measured in homogenized kidneys to determine neutrophil infiltration. Neither treatment with A3 agonist nor with A3 antagonist resulted in significant changes of MPO levels, indicating that A3 does not contribute to regulation of neutrophil infiltration into kidneys during candidiasis (Fig. 13B).

Fig. 13: A3 regulates antifungal activity
Mice were infected with C. albicans yeasts by tail vein injection. Mice were subsequently administered with PBS, A3 agonist or antagonist with one dose per day. After 3 days kidneys were harvested and CFU counts were performed (A). MPO activity was determined from harvested mouse kidney lysates (B). Data are shown as means ±SD from 7 mice per treated group, in three technical replicates. Significance was analyzed by one-way ANOVA, P < 0.05.
$A_3$ signals via PI3K to regulate NET release in response to *Candida albicans* hyphae

We demonstrated that adenosine-mediated NET reduction of hypha-induced NETs were dependent on $A_3$. To elucidate downstream signalling from $A_3$ we investigated the potential involvement of PI3K. This kinase has been shown to stimulate the initiation of autophagy, a process required for NET formation [190, 211]. Inhibition of PI3K resulted in a reduction of PMA-induced NET formation. Similarly, we showed here that blockage of PI3K resulted in a 50% reduction of hypha-induced NET release (Fig. 14). Combination of adenosine or $A_3$ receptor agonist to PI3K inhibitor resulted in a slightly stronger, but similar reduction of NETs to background levels (Fig. 14). This indicates that reduction of hypha-induced NETs by adenosine mediated by $A_3$ targets PI3K for further downstream signalling.

![Figure 14](image)

**Fig. 14: A3 signals via Pi3K to regulate NETs in response to *Candida albicans* hyphae**

NET formation was quantified in DNA fluorescence assay, 10 h post infection with *C. albicans* hyphae. Data are shown as means ±SD from 6 independent donors, in three technical replicates. Significance was analyzed by one-way ANOVA, $P < 0.05$. 

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Adenosine reduces ERK1/2 activity in infected neutrophils with Candida albicans hyphae

Human neutrophils via activation of ERK mediate C. albicans killing [188]. We therefore tested the involvement of ERK1/2 in adenosine signalling upon hypha-induced NET formation. Our results indicate ERK1/2 is efficiently phosphorylated in neutrophils triggered with C. albicans. Upon treatment with different concentration of adenosine in response to C. albicans hyphae ERK1/2 activation in neutrophils reduced from 60% to 40% in a dose-dependent manner (Fig 15A). Additionally, NET quantification demonstrated reduction in NET release upon inhibition of ERK1/2. Combination of adenosine and ERK1/2 inhibitor resulted in similar NET reduction indicating that adenosine targets ERK1/2 for downstream signalling in neutrophils triggered with C. albicans hyphae (Fig. 15B).

Fig. 15: ERK1/2 activity is reduced in neutrophils in response to Candida albicans hyphae
Human neutrophils were incubated with 10 μM adenosine, or ERK inhibitor prior to infection with C. albicans hyphae. Total and phosphorylated ERK1/2 protein levels were detected by immunoblotting (A). NET formation was quantified in DNA fluorescence assay, 10 h post infection with C. albicans hyphae (B). Data are shown as means ±SD from 6 independent donors, each in three technical replicates. Significance was analyzed by one-way ANOVA, P < 0.05.
In conclusion, this study demonstrates that hypha-induced NET formation is modulated by adenosine via A$_3$. This adenosine receptor further signals to PI3K/Akt and ERK 1/2 to regulate NET release. The host might use this mechanism to reduce the amount of NETs released in response to invasive fungi to prevent overwhelming tissue damage caused by excessive amounts of NETs. Targeting anti-inflammatory receptors such as A$_3$ could be used as therapeutic approach during invasive mycosis to prevent these potentially harmful responses and associated tissue damage.
**Paper III**

**ROS scavenger tempol blocks NET formation**

ROS are essential for NET formation stimulated by PMA and the majority of microbial inducers, including *C. albicans*. We therefore reasoned that molecules that scavenge ROS are potential NET inhibitors. To test this hypothesis, we analyzed neutrophils ROS and NET release in the presence of tempol. Tempol is a non-toxic, stable nitroxide which scavenges radicals mimicking SOD proteins [212].

Our data shows that tempol removed ROS from neutrophils in a dose-dependent manner upon stimulation with potent NET inducer PMA and both growth forms of *C. albicans* (Fig. 16). NET release was determined microscopically and in time-lapse DNA fluorescence assays. We demonstrate that tempol was able to significantly reduce the amount of NET release upon PMA and *C. albicans* stimulation (Fig. 17).
Fig. 16: Tempol scavenges neutrophil ROS triggered by different growth forms of Candida albicans
The total amount of ROS generated by neutrophils is plotted against increasing concentrations of tempol. Neutrophils were infected with C. albicans (MOI 3). The total amount of ROS produced over 3 h were calculated as area under the curve (AUC). Neutrophils infected with (MOI 3) of C. albicans yeast (A) hyphae (B) and PMA (C). One representative experiment from 3 different donors is shown. Data are presented as means of 3 technical replicates ±SD. Significance was analysed by Tukey one-way ANOVA (P < 0.05) [Re-produced from Hosseinzadeh, Messer et al. 2012].
Fig. 17: Tempol prevents NET formation induced by different growth forms of Candida albicans and PMA

Neutrophils stimulated with 100 nM PMA (A,B and C) or C. albicans morphotypes (MOI 3, D) incubated with or without tempol. Microscopic analysis of percentage of NET formation (A and B) and percentage of DNA release was measured in fluorescent assay by using sytox green dye (C). Data are presented after 6 h as means ±SD of 3 independent experiments from 3 different donors. Significance was analysed by one-way ANOVA P < 0.05) [Re-produced from Hosseinzadeh, Messer et al. 2012].
Besides their beneficial role to trap and kill microbes, NETs have hazardous effects in other diseases affecting the immune system and promoting inflammation. Since the ROS-related microbicidal arsenal of phagocytes could be negatively affected by tempol, it might not be suggested to use tempol during acute infections. Otherwise, in diseases such as SLE, it is important to control undesired NET formation. Therefore, the proposed nontoxic ROS scavenger, tempol, could provide a promising approach to inhibit ROS-dependent NET formation in such diseases.
Paper IV

Nicotine induces NET formation

Nicotine is the major addictive component of tobacco and is a considerable cause of inflammatory disease, disability and death. Nevertheless, it has been reported that apoptosis can be inhibited by nicotine [213]. Since Akt was shown to be a molecular switch for neutrophils to either commit for NET formation or apoptosis induction [189] and nicotine-mediated apoptosis inhibition was Akt-dependent as well [130], we reasoned that nicotine possibly could also modulate NET formation. Excessive NET release induced by nicotine provides a possible explanation for the inflammatory potential of this molecule.

Therefore, we investigated, how neutrophils respond in direct contact to nicotine. Immunofluorescence microscopy quantification revealed that 1 mM nicotine induces NET formation with similar efficiency as PMA (Fig. 18).

**Fig. 18: Nicotine triggers NETs**

NET release was measured microscopically from $10^5$ neutrophils after 10 h incubation with 100 nM PMA or 1 mM nicotine. Data are shown as means ± SD from 6 independent donors. Significance was analysed by one-way ANOVA, $P < 0.05$. 

- Nicotine
- + Nicotine
- PMA
**Nicotine-mediated NET induction is independent of NADPH oxidase**

To understand whether nicotine-induced NET generation is dependent on Nox2, we used gp91\textsuperscript{phox−} mice that are defective in their Nox2 activity. Therefore, phagocytes from these mice cannot produce an oxidative burst nor release NETs upon microbial or PMA stimulation. PAD4 enzyme was previously shown to be essential for decondensation of chromatin and NET release \cite{180}. PAD4 catalyses the modification of histone H3 to citrullinated H3. To demonstrate that histone H3 modification is also required for nicotine-mediated NET formation antibody directed against citrullinated histone H3 (Cit-H3) was used. Cit-H3 staining significantly increased in stimulated neutrophils compared to unstimulated controls and was abundantly present on nicotine-induced NETs. This indicates that alike other NET stimuli nicotine activates a PAD4-dependent NET release mechanism. More importantly, nicotine induced NETs in both wild-type and gp91\textsuperscript{phox−} neutrophils in a comparable manner (Fig. 19, second row). As expected, PMA stimulation of neutrophils from gp91\textsuperscript{phox−} mice failed to form NETs in contrast to WT mice (Fig. 19, third row).
**Fig. 19: Nicotine induces NET independent of NADPH oxidase activity**

NET release from $10^5$ WT and gp91phox− was measured after 10 h incubation with 1 mM nicotine or 100 nM PMA. NET formation is visualized by using DAPI (blue channel) and primary antibody directed against Cit-H3 (green channel). Scale bar represents 10 μm.

Taken together, our results demonstrate that nicotine-induced NET release is a conserved feature in human and mouse neutrophils. Nicotinic acetylcholine receptors (nAChRs) are responsible for mediating the signalling effect by nicotine (data not shown). Further downstream, this pathway is mediated via Akt activation. Blockage of nAChR or Akt with specific Akt inhibitor (10 μM XI inhibitor) abrogates NETs induced by nicotine (Fig. 20). Moreover, nicotine-induced NET formation involves histone H3 citrullination, but is ROS-independent (Fig. 19).
**Fig. 20: Inhibition of Akt blocks NET formation**

NET release was measured by DNA fluorescence assay after 10 h incubation with 100 nM PMA or 1 mM nicotine in presence or absence of Akt inhibitor XI (10 µM, Merck Millipore). Data are shown as means of 3 technical replicates ± SD from 6 independent donors. Significance was analysed by one-way ANOVA, P < 0.05.

Our data suggests that nicotine usage due to increased NET formation may lead to unwanted inflammation and tissue injury. Thereby, inhibition of Akt could provide a therapeutic strategy to control inflammatory nicotine-related diseases. On the other hand, deeper knowledge of the nicotine-induced pathway of NET formation is likely to support development of therapies for diseases in which NET formation is impaired.
Conclusions and Outlook

Multimorphic *C. albicans* has the remarkable ability to transiently switch between different growth morphologies, such as yeast and filamentous hyphae. The hyphal form is considered the invasive, tissue destructive form. To delineate immune responses directed against individual morphotypes we have developed a dry mass approach which allowed precise determination of amounts of yeast and hyphae. This enabled us to meticulously investigate responses of neutrophils mediated to different forms of *C. albicans*. Neutrophils are crucial in antifungal immunity. They have different defence mechanisms to target microbes and to destroy them, such as for instance NET release. Invasive fungal infections are often accompanied by immune reconstitution inflammatory syndrome (IRIS), an overwhelming and hazardous inflammatory response of the host. Uncontrolled NET release, is also associated with tissue damage. Agents that downregulate NET formation could therefore be used as adjunct therapies during invasive mycosis. This thesis demonstrates that during *Candida* infection the host effector molecule adenosine downregulates NET release mounted specifically towards *C. albicans* hyphae, which might contribute to downregulation of inflammation and self-inflicted damage. On the search for additional compounds with the ability to block ROS-dependent NET formation and thereby potentially reducing IRIS, we identified the nitrooxide tempol.

To understand the whole picture we also investigated the pro-inflammatory side. As a proinflammatory mediator, nicotine was identified here as an efficient stimulus of NET formation, thereby revealing a previously unknown explanation for a strong contribution of the alkaloid to inflammatory effects in tobacco consumers. We found that nicotine-induced NET formation is dependent on the kinase Akt, but independent of the production of ROS. Downstream signalling pathways of positive NET regulators, such as nicotine, could thus provide valuable targets for therapies of NET-related diseases. In summary, our findings are likely to have implications in
forthcoming therapies involving modulation of NET-mediated effects during infections and other inflammatory disorders.

**Paper I**

- The mass assessed from dry mass correlation to metabolic activity is a better way to study immune response towards dimorphic fungi.
- Neutrophil response pattern depends on amount and morphotypes of *C. albicans*.

\[
\text{correlative formula} \quad \begin{array}{c}
C. albicans \text{ metabolic activity} \\
\text{if: } C. albicans \text{ amounts (μg)}
\end{array} \rightarrow C. albicans \text{ mass (μg)} \begin{cases} 
\text{yeast > hyphae} & \rightarrow \text{ROS ↓} \\
\text{yeast < hyphae} & \rightarrow \text{ROS ↑}
\end{cases}
\]

**Paper II**

- Adenosine, the endogenous purine nucleoside, downregulates NET formation only in response to *C. albicans* hyphae.
- Adenosine inhibits hypha-associated NET release via A₃R.
- Adenosine inhibits NET via A₃R by targeting downstream PI3K/Akt and ERK1/2 signalling molecules.

\[
\begin{array}{c}
? \\
C. albicans \text{ hyphae} \rightarrow \text{Receptor(s)} \rightarrow \text{PI3K} \rightarrow \text{Akt} \rightarrow \text{ERK1/2} \rightarrow \text{NET}↓ \\
\text{Adenosine} \rightarrow \text{A₃R} \rightarrow \text{ROS} \rightarrow \text{Inflammation}↓
\end{array}
\]
Paper III

- The antioxidant ROS scavenger tempol detoxify neutrophils ROS and subsequently suppress NET formation. Thereby tempol could be a promising approach to control unwanted NET in associated diseases.

\[ C. \textit{albicans} \text{ yeast/hyphae/PMA} \rightarrow \text{ROS} \rightarrow \text{NET} \downarrow \]

\[ \text{Tempol} \quad \text{inflammation} \downarrow \]

Paper IV

- Nicotine induces NET in NADPH oxidase-independent mechanism.
- Nicotine triggers NET through nAChR via activation of Akt.
- Nicotine induces undesired NET release, which could contribute in nicotine related inflammatory diseases.

\[ \text{Nicotine} \rightarrow \text{nAChR} \rightarrow \text{Akt} \rightarrow \text{NET} \uparrow \rightarrow \text{inflammation} \uparrow \]
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