Master Thesis

‘Exploring molecular mechanisms of interaction of filamentous fungi with neutrophils’

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2019-2020

Heraklion

Μεταπτυχιακή Εργασία

‘Ανάλυση μοριακών μηχανισμών αλληλεπίδρασης υφομυκήτων με ουδετερόφιλα’

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2019-2020

ΗΡΑΚΛΕΙΟ

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Fungal Kingdom includes about 6 million species, however only a few hundred are associated with human diseases. Mucormycosis is a rare-yet an emerging invasive fungal disease caused by fungi of the Mucorales order, characterized with high mortality rates of almost 100% in the case of disseminated infection. Neutrophils are essential players of the host innate immunity against filamentous fungi. Although one of the major risks factors of Mucormycosis is neutropenia, the molecular mechanisms of neutrophil interaction with Mucorales remain unknown. In this project, our aim was to establish *ex vivo* imaging and killing assays to investigate the interaction between murine and human neutrophils and the main causative agent of Mucormycosis, *Rhizopus oryzae* In contrast to the other major aerial fungal pathogen *Aspergillus fumigatus,* we found that neutrophils do not phagocytose Mucorales conidia but instead selectively form clusters surrounding them, a phenomenon recently described as neutrophil swarming. Importantly, swarming was not observed against *Aspergillus* conidia. Pilot studies suggest that fungal cell wall melanin of *Rhizopus* conidia likely account for induction of swarming. Furthermore, we found that neutrophils inhibit *R. oryzae* conidia germination. Further experiments should clarify host and fungal molecular elements regulating this immune response against Mucorales and elucidate the role of swarming in disease pathogenesis.

Abstract in Greek

Το βασίλειο των μυκήτων αποτελείται από περίπου 6 εκατομμύρια είδη, εκ των οποίων μόνο μερικές εκατοντάδες σχετίζονται με ανθρώπινες ασθένειες. Η Μουκορμύκωση είναι μια σπάνια αλλά αναδυόμενη διεισδυτική μυκητίαση του αναπνευστκού που προκαλείται που υφομύκητες που ανήκουν στην τάξη Mucorales και χαρακτηρίζεται από υψηλά ποσοστά θνησιμότητας, τα οποία μπορούν να φτάσουν και το 100% σε περιπτώσεις ασθενών με γενικευμένη λοίμωξη. Τα κονίδια του γένους Mucorales εισέρχονται στον ανθρώπινο οργανισμό κυρίως μέσω της εισπνοής και ο ξενιστής αμύνεται κυρίως με μηχανισμούς της έμφυτης κυτταρικής ανοσίας. Τα κυψελιδικά μακροφάγα είναι τα κύρια κύτταρα φαγοκυττάρωσης των εισερχόμενων σπορίων. Ωστόσο, μεγάλος αριθμός εξωκυττάριων σπορίων αλληλεπιδρά με τα ουδετερόφιλα. Αν και ένας από τους κύριους παράγοντες κινδύνου ανάπτυξης Μουκορμύκωσης είναι η ουδετεροπενία, οι μοριακοί μηχανισμοί αλληλεπίδρασης των ουδετερόφιλων με τους μύκητες της τάξεως Mucorales δεν είναι γνωστοί. Σε αυτή τη μελέτη, ο στόχος μας ήταν η μελέτη των μηχανισμών αλληλεπίδρασης μεταξύ των ουδετεροφίλων και του κύριου αιτιολογικού παράγοντα της Μουκορμύκωσης, τον *Rhizopus oryzae συγκριτικά με τον μύκητα Aspergillus fumigatus.* Για αυτό το σκοπό, σχεδιάσαμε ex vivo πειράματα συνεστιακής μικροσκοπίας και δοκιμασίες θανάτωσης των μυκήτων από ουδετερόφιλα προερχόμενα από υγιείς εθελοντές και πειραματόζωα. Οι μελέτες μας αποδεικνύουν ότι τα ουδετερόφιλα κινούνται οργανωμένα προς το παθογόνο και αθροίζονται γύρω από αυτό, μια ανοσολογική απόκριση η οποία έχει πρόσφατα περιγράφει σαν φαινόμενο “σμήνους” (swarming). Το φαινόμενο αυτό είναι εκλεκτικό έναντι των μυκήτων Mucorales, καθώς δεν παρατηρήθηκε έναντι του μύκητα *Aspergillus*. Προκαταρκτικές μελέτες έδειξαν ότι η μελανίνη του τοιχώματος του μύκητα εμπλέκεται στην επαγωγή του φαινομένου swarming. Επιπλέον, τα ουδετερόφιλα δείχνουν να έχουν την ικανότητα να μπορούν να αναστείλουν και σε μικρό προσοστό να σκοτώσουν τα σπόρια (κονίδια) του μύκητα Mucorales. Μελλοντικές μελέτες θα αποδείξουν τους μοριακούς μηχανισμούς από την πλευρά του παθογόνου και του ξενιστή που επάγουν αυτή την ιδιαίτερη ανοσολογική απόκριση καθώς και το ρόλο του swarming στην παθογένεση της μουκορμύκωσης.

Introduction

Fungus [pl., fungi; Latin fungus, mushroom] is a term used to describe single celled or complex multicellular eukaryotic organisms, which compose an extremely diverse Kingdom with as many as 6 million species[1]. In nature, fungi have a wide distribution and are essential both for the environment and the humans in multiple ways. Specifically, fungi are major organic decomposers, while they are important for the production of organic acids, enzymes, vitamins and drugs. Finally, fungi are model organisms for the study of basic biology principles[2].

Invasive mold infections

Notably, only few hundreds of fungal species are associated with human diseases, mainly including superficial infections (e.g., skin and nail infections) or allergic diseases (e.g., asthma). In order to become human pathogens, fungi should be able to meet the following requirements: (a) survive and proliferate in mammalian temperatures, (b) invade surface epithelial barriers, (c) lyse human tissues and (d) survive during interaction with the human immune system. In particular, human fungal pathogens that meet these four criteria are members of the following lineages: the Zygomycota, , Ascomycota, and Basidiomycota[3,4].

It has been proposed that one of the most effective development against fungal infections is the high temperature of endothermic animals. This hypothesis was stated by Casadevall and colleagues, who conducted experiments to examine the correlation between temperature and fungal growth restriction. Results revealed that every 1^{o} increase in temperature >30^{o}C, there was a 6% decrease in the number of fungi strains that are capable of growing in such high temperatures[5,6]. Other indications that support this hypothesis are the field observations and controlled experiments displaying longer survival of fungus-infected insects that can warm themselves or find an exogenous heat source[7].

The second of the criteria of successful fungal infection is the ability to circumvent or invade human surface barriers. The most important virulence factors for this purpose is morphogenesis and adhesion molecules. Almost all human pathogenic fungi can be found in two cell shapes: (a) round or ovoid and detachable, or (b) long, filamentous, and forming multicellular mycelia. The spores can be spread in long distances and live up to 20 km in the stratosphere[8], while the filamentous cell form enables the active form of locomotion and the protection of fungi against the destruction by phagocytes[9]. Moreover, as far as the adhesion molecules are concerned, they empower fungi to stay at the sites that are in favour of their growth[4].

The lysis and the absorption of human tissues are very important for the successful fungal infection. For this reason, fungi secrete digestive enzymes that dissolve host

tissue, which will be used as a nutritious substrate. Among these enzymes are

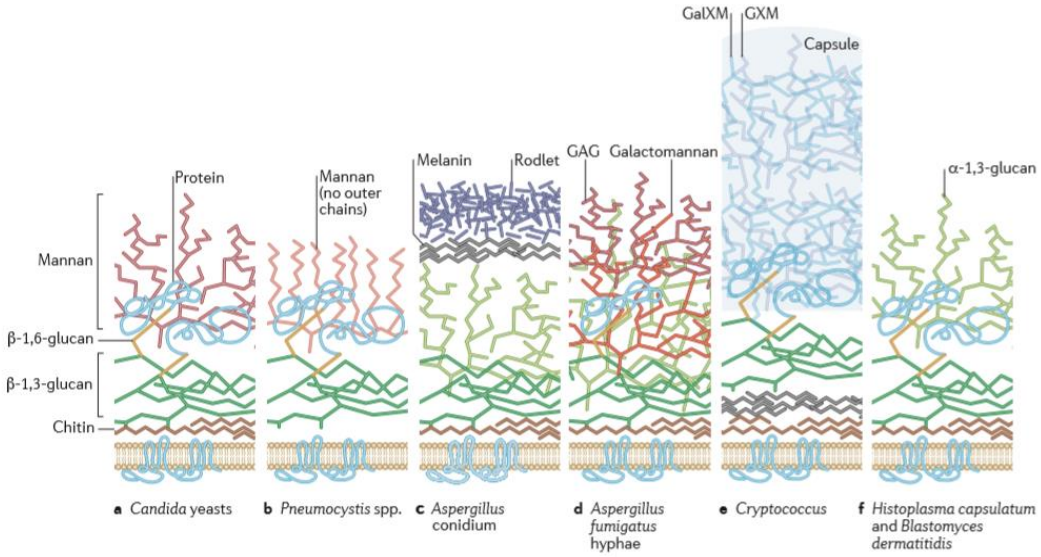


Figure 1. Cell wall of different fungi pathogens. The majority of the fungi has a core of branched β-(1,3) glucan, β-(1,6) glucan, and chitin. The difference between them are the components that are attached to this core. In the case of Candida yeasts, the outer wall is composed of highly mannosylated proteins. Aspergillus conidia have an outer layer of rodlet and a melanin layer. while A. fumigatus hyphae have have α-(1,3) glucan GM, and galactosaminoglycan (GAG) in the outer cell wall and limited glycosylated proteins. As far as *C. neoformans* is concerned, it has an outer capsule that is synthesized by glucuronoxylomannan (GXM) and lesser amounts of galactoxylomannan (GalXM) and it is attached to α-(1,3) glucan in the underlying wall. *Pneumocystis* lacks chitin and the outer chain N-mannans in its cell wall, but it has a core of N-mannan and O-mannan modified proteins. Lastly, the hyphae of *H. capsulatum* and *Blastomyces dermatitidis* have an inner core that is composed of β-(1,3) glucan and protected from being recognized by the immune system by an outer α-(1,3) glucan layer[10].

proteinases and hydrolases which will do the dissolution of host macromolecules that will be acquired by the fungi for their growth. Host withholds these elements in order to prevent fungi from growing and this has been termed as "nutritional immunity"[11]. For instance, the human host restricts the iron available for the pathogens[12–14]. On the other hand, all fungal pathogens have sophisticated mechanisms of iron assimilation that allow utilization of iron from the host[15,16].

Few fungi can evade the physiological immune response and cause invasive disease in humans. Truly, pathogenic fungi, including *Histoplasma* spp. and a handful of other dimorphic fungi (e.g., *Blastomycetes*, *Coccidioides* spp) are the ones that can downregulate the immune system or can mask their recognition and establish intracellular growth within the host cells by masking immunostimulatory molecules on the fungal cell wall surface. In contrast, the vast majority of human fungal pathogens are opportunistic (saprophytic) organisms, which are recognized and eliminated by the physiological immune system on a daily basis. Fungal cell wall polysaccharides trigger robust innate immune responses, which shape specialized antifungal adaptive immunity. Therefore, opportunistic fungal pathogens cause invasive disease exclusively in patients with severe immunodeficiency. The most important human fungal pathogens include Candida and airborne filamentous fungi, Aspergillus and the Mucorales.

Transition from a dormant, single cell stage (conidium or yeast cell) to a multicellular vegetative stage of growth (filamentous or hyphal growth) is a characteristic of invasive fungal disease caused by all opportunistic human fungal pathogens. The outer cell wall layer of fungi is a master regulator of viability, morphogenesis and virulence (Figure 1). Apart from conferring protection from environmental stressors, the fungal cell wall has a dynamic and unique composition and structure, which shape innate and adaptive antifungal immunity responses. The fungal cell wall composition is strongly affected by environmental factors, among which the interaction with the immune recognition system of humans, which allow plasticity and adaptation of fungi in different environments, including survival within the host. The vast amount of fungi species has a layered cell wall that has a more conservative inner structural skeletal layer and a more heterogeneous outer layer. The inner layer is comprised of polysaccharides, mainly b-1,3 and/or 1.6-glucan and chitin, that form bonds with proteins or other polysaccharides. Additionally, most fungi have melanin in the outer layer that protect the spores from enzymes. During germination the outer layer is removed and components of the inner layer are coming to the surface. In addition, new molecules (e.g., polysaccharides) are produced and/or released, which allows environmental adaptation during the invasive stage of growth. Mannans and β glucans are the most immunostimulatory fungal cell wall components. In the opposite, melanin, and the rodlet layer in filamentous fungi including *Aspergillus*, prevent immune recognition by masking immunostimulatory molecules. Of interest, certain cell wall molecules trigger specialized immune responses[10,17]. On the other cite, the innate immune system recognizes specialized molecular structures on the fungal cell wall. For example, DHN-melanin of *Aspergillus* and other filamentous fungi is recognized via a specific C type lectin receptor[18]. Similarly, b-1-6 glucan triggers phagocytosis of Candida yeast cells as opposite to other cell wall polysaccharides[19]. Dissecting molecular mechanisms of immune recognition of the fungal cell wall is essential for understanding pathogenesis of human fungal diseases.

Invasive Aspergillosis

Invasive Aspergillosis is the most common invasive mold infection and is caused by fungi of the genus *Aspergillus*. Aspergillus is an airborne saprophytic organism with ubiquitous distribution in nature. Physiologically, hundreds of conidia (spores) of *Aspergillus* present in the air are inhaled by humans. Inhaled conidia are constantly eliminated in the lung via a highly coordinated innate immune responses without causing inflammation or disease. The main causative agent of invasive aspergillosis is *Aspergillus fumigatus*, which is responsible for 92% of the cases, followed by *A. flavus, A. niger,* and *A. terreus*[20].

Invasive aspergillosis manifests as pneumonia. Inhaled conidia germinate to hyphae that invade epithelia, escape from the resident phagocytes and induce angioinvasion and necrotic cell death. Invasive pulmonary aspergillosis is an emerging disease that

affects mostly immunocompromised patients with high mortality rate. Specifically, a study that was conducted, analysing the underlying diseases of 960 patients with invasive aspergillosis, revealed that 48.3 % had an underlying haematological malignancy, 29.2 % were solid organ transplant recipients, 27.9 % were hematopoietic stem cell transplant (HSCT) recipients, while the rest had an associated underlying disease such as a solid tumor and inherited immunodeficiency[21].

Mucormycosis

Mucormycosis or -as previously termed, Zygomycosis, is a rare yet an emerging invasive mold infection and it is caused by opportunistic fungi belonging to order Mucorales[22]. When compared to other invasive mold infections, mucormycosis is the most devastatinged fungal disease with mortality rates of 30-50%, which approach 100% in disseminated disease[23]. This fungal disease has common epidemiological, clinical and pathogenic characteristics with other invasive mold infections, nonetheless, mucormycosis has also unique disease characteristics, which likely reflect on specialized interactions of Mucorales with the immune system. The unique biological, and pathogenetic features of Mucorales as compared to other fungi will be analysed in detail.

Biology of Mucorales

Mucorales have wide distribution and they can be found in saprobic soil organisms, o decaying organic material, plants and animals. These fungi are mainly aseptate or sparsely septate ribbon-like hyphae.

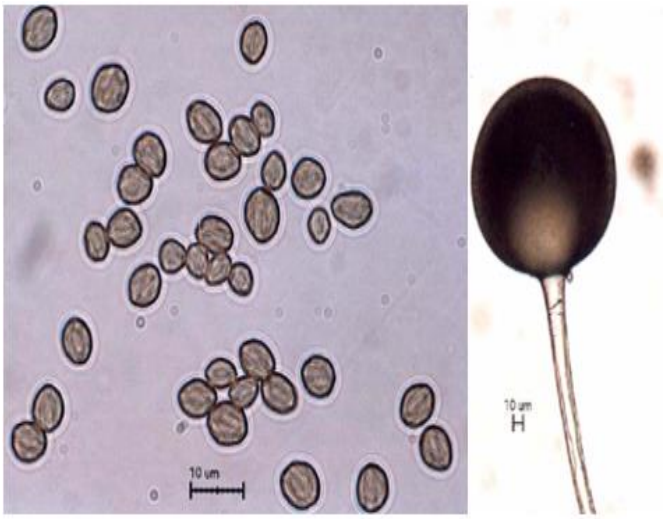


Figure 2. Rhizopus oryzae: sporangiospores (on the left) and Sporangium and sporangiophore (on the right).

Mucorales are fungi that have both sexual and asexual cycles of reproduction. In the case of asexual reproduction, fungi produce sporangiospores in a globe-like sac called sporangium in the apex of sporangiophore. These spores disperse and germinate, under favourable circumstances, creating a mycelial complex. As far as the sexual reproduction is concerned, two compatible mating type hyphae –one (-) and (+) sense each other, undergo fusion, resulting in the formation of zygospores. The dormancy of zygospores may last a considerable amount of time, ranging from a month to years, before germinating and creating a single aerial hypha that has a sporangium in the apex that will bear the meiospores. For this reason, the asexual

sporangiospores is the main source of disperse and infection[2,24]. Susceptible human patients acquire Mucormycosis by inhalation of fungal sporangiospores, and less commonly by consummation of defiled food or traumatic inoculation.

In contrast to other fungal pathogens, Mucorales have a poorly characterized cell wall. The cell wall of Mucorales conidia is mainly composed by an inner layer of polysaccharides that include chitosan, chitin, mannan and glucan and an outer layer that contains DOPA melanin and proteins. Hyphae cell wall mainly includes polysaccharides: chitosan, chitin, mucoran and mucoric acid. There are no studies on the immune recognition of Mucorales cell wall by the immune system. All available data are coming from extrapolation of studies in other filamentous fungi or indirect assessment of the immune response to the different stages of growth of Mucorales (conidia vs hyphae)[25].

The causative agents of Mucormycosis

It has been reported that eleven genus and ¬27 species under the order of Mucorales cause mucormycosis. The main causative agents of mucormycosis belong to the following Genera*: Rhizopus spp., Lichtheimia spp., Mucor spp., Cunninghamella spp., Rhizomucor spp.* and *Apophysomyces spp*. The most common agent is *Rhizopus oryzae* as it is responsible for¬70% patients of mucormycosis[26].

Shared and Unique risk factors for mucormycosis development

Similar to other invasive mycoses, mucormycosis develops in patients with defects in the numbers or function of myeloid phagocytes. Patients with hematological malignancies or transplant recipients, who develop neutropenia or receive high doses of steroid therapy, are typically at high risk of developing mucormycosis.

Diabetes mellitus is a dominant risk factor uniquely associated with mucormycosis. Studies have revealed that 17%-88% of all mucormycosis patients suffer from diabetes mellitus. Especially, in the case of diabetic acidosis (ketoacidosis), there is a marked increase in susceptibility to Mucorales infection. Other patients with metabolic abnormalities, including other types of acidosis (e.g., renal failure), malnutrition, cirrhosis, and patients with acquired iron overload syndromes are at risk for mucormycosis. Increased iron availability to Mucorales has been considered a common link in pathogenesis of mucormycosis in patients with acidosis and other metabolic diseases[26]. However, the underlying mechanism(s) is largely unknown.

Clinical forms of mucormycosis

Different forms of mucormycosis depending on the anatomical site of involvement include: rhino-orbito-cerebral (ROCM), pulmonary, gastrointestinal, cutaneous, renal, disseminated. Especially the ROCM is the most frequent form of mucormycosis while the pulmonary type is the second most common type[26].

Treatment of Mucormycosis

Mucormycosis is associated with the highest mortality rates as compared to other fungal diseases, exceeding 50% despite effective treatment. Effective therapy of mucormycosis depends on: (a) early diagnosis, (b) timely initiation of optimal antifungal therapy, (c) reversal of the underlying risk factor, and (d) radical surgery.

Early diagnosis enables the early initiation of appropriate treatment, which has been shown to improve outcome[27].

Inherent resistance of Mucorales to existing antifungal makes treatment of mucormycosis challenging. In particular, Mucorales are resistant to azole drugs that target lanosterol 14α-demethylase (LDM), preventing ergosterol biosynthesis, having as a result the synthesis of toxic sterols. This occurs due to possession of two *CYP51* paralogues and evolutionary conserved aminoacid substitution of the LDM[28].

Host defense against Mucormycosis

Innate immunity has a major role in physiological defence against Mucorales. Accordingly, mucormycosis almost exclusively occurs in patients with quantitative or qualitative defects (e.g., immunometabolic abnormalities) in professional phagocytes.

Professional phagocytes with a dominant role in antifungal immunity include neutrophils and macrophages. Neutrophils are the most abundant, short-lived cells of the immune system and comprise the first – and more efficient line of defence against bloodstream pathogens. Tissue resident macrophages in the lung comprise the first line of defense against airborne pathogens, including filamentous fungi. During fungal infection inflammatory and chemotactic signals upon sensing of filamentous fungi by epithelia and macrophages drive neutrophil recruitment at the site of infection. In addition, cross talk of monocytes/macrophages and other immune cells with neutrophils via type I and type III IFNs primes effector functions of neutrophils and results in optimal microbicidal activity[29].

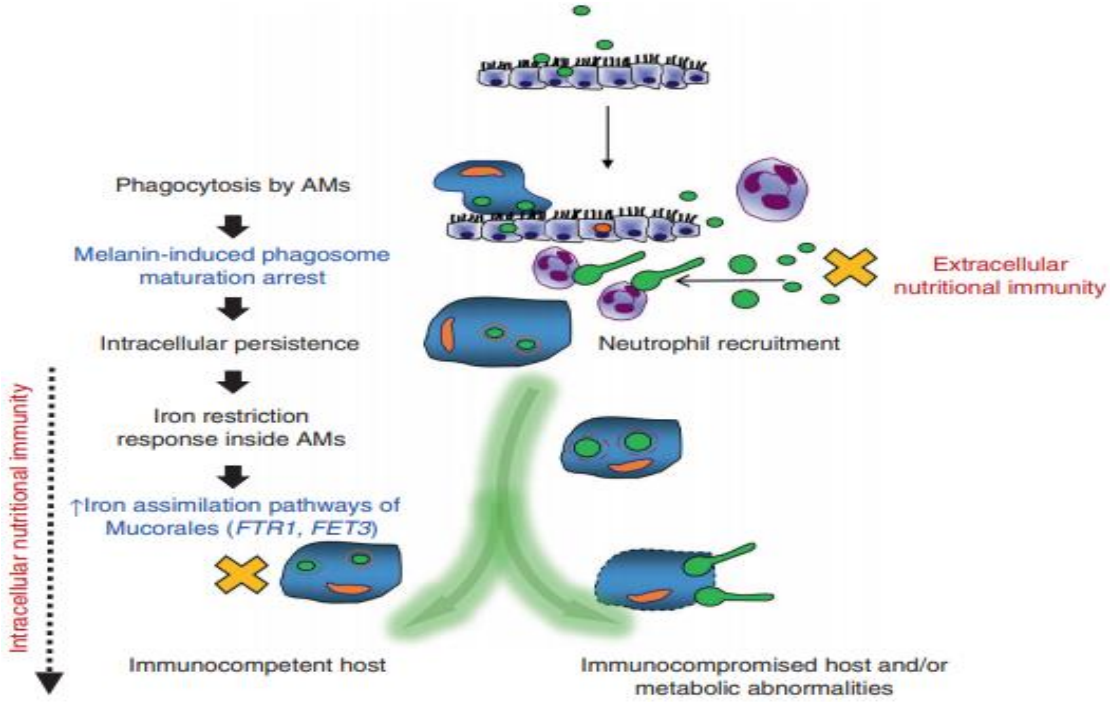


Figure 3. Macrophages engulf the majority *R. oryzae spores,* that illustrate extended persistence within phagosomes. Their ability to maintain inside the phagosomes is due to melanin-induced phagosome maturation arrest and promotion of anti-apoptotic signalling in macrophages. On the other hand, host defends itself through iron restriction competing the upregulation of fungi iron assimilation pathways[30].

This neutrophil activation typically results in the engulfment of fungal cells that escape from macrophage surveillance consequently their degradation within phagosomal compartments. Furthermore, neutrophils are the primary cells to attack germinating forms of fungi (hyphae).

Our group has recently demonstrated the major role of macrophages in immunity against Mucorales. Specifically, Mucorales conidia are predominantly phagocytosed by alveolar macrophages. Of interest, Mucorales conidia persist intracellularly for many days inside the macrophage because of their ability to induce phagosome maturation arrest. Inhibition of Mucorales growth is a major host defense mechanism and occurs via iron starvation (nutritional immunity). Accordingly, abnormalities in iron metabolism in the setting of iron overload (and possibly diabetic acidosis) result in increased iron availability to the pathogen, which leads to germination of intracellular fungal conidia and invasive disease (Fig 3)[30].

On the other site, a significant proportion of Mucorales conidia are associated extracellularly with neutrophils. Although neutropenia is a major risk factor for mucormycosis the molecular mechanisms of neutrophil interaction with Mucorales are largely unknown. Specifically, the effector mechanisms of neutrophils against Mucorales and the fate of Mucorales conidia during interaction with neutrophils are poorly understood. In addition, the mechanisms of cross talk of neutrophils with other immune cells in the lung during physiological interactions with Mucorales conidia needs to be illuminated.

Neutrophils

Polymorphonuclear (PMN) leukocytes and important cells of the innate immune system, leading the first line of host defence against invading organisms as well as injuries. In humans, they are the most abundant blood cell type, accounting for 50% to 70% of all leukocytes in the circulation.

Neutrophil production and life cycle

In adult mammals, neutrophils are generated in the bone marrow. In the case of humans, 1 to 2 x 10^{11} neutrophils are produced every day –under normal circumstances[31]. Considering this fact, it is not a surprise that bone marrow dedicates almost two thirds of its space to myelopoiesis –the term used to describe the massive generation of monocytes and granulocytes.

In bone marrow, hematopoietic stem cells are localized in niches, which can mostly be found near the endosteum or on the abluminal side of endothelial sinuses[32]. HSCs give rise to lymphoid-primed multipotent progenitors (LMPPs), from which neutrophils derive during granulopoiesis[33]. In turn, LMPPs differentiate into granulocyte–monocyte myeloid progenitors (GMPs)[34–36]. GMPS evolve in mature neutrophils with the following sequence: myeloblast, promyelocyte, myelocyte, metamyelocyte, band neutrophil and, finally, segmented neutrophil[34]. At the second step of maturation–the transition from myeloblast to promyelocyte, the first appearance of primary (azurophil) granules is noticed. The secondary (specific) granules appear during the myelocyte and metamyelocyte stage while the tertiary (gelatinase) ones are formed during the transition from band to segmented neutrophil. Finally, only mature neutrophils have secretory vesicles[34,37]. These granules are essential for the host defence as they are armed with a variety of antimicrobial effectors, among which cathelicidins, defensins, gelastase, myeloperoxidase and matrix metalloproteinases (MMPs)[37,38].

Apart from the appearance of granules, there are other changes through which neutrophil undergo during differentiation, that involve the formation of the lobulated nucleus and the expression levels of a variety of receptors.

Neutrophil release from the bone marrow

Under normal circumstances, mature neutrophils in the circulation consist only 1-2 % of the total amount of neutrophils in the body[39]. The release of neutrophils in the circulation is mainly triggered by two C-X-C chemokine receptors: CXCR4 and CXCR2. CXCR4 is responsible for the maintenance of neutrophils in the bone marrow. This neutrophil receptor binds to CLCL12, which is expressed by osteoblasts and other stromal cells, retaining the mature neutrophils in the bone marrow[40]. A considerable number of adhesion molecules contribute to the retention of neutrophils in bone marrow, among which integrin subunit α4 (ITGα4) and vascular cell adhesion molecule 1 (VCAM1)[41–43]. On the other hand, the secretion of CXCL1 and CXCL2 by endothelial cells and megakaryocytes, result in the release of the mature cells from the bone

marrow, which can also be triggered by G-CSFR and Toll-like receptor[44]. It has been established that trigger of these receptors does not result in additional cell release from the bone marrow in the case of CXCR4 absence, highlighting the importance of CXCR4 signalling in the case of homing mature and immature neutrophils in the bone marrow[40].

Neutrophils subpopulations

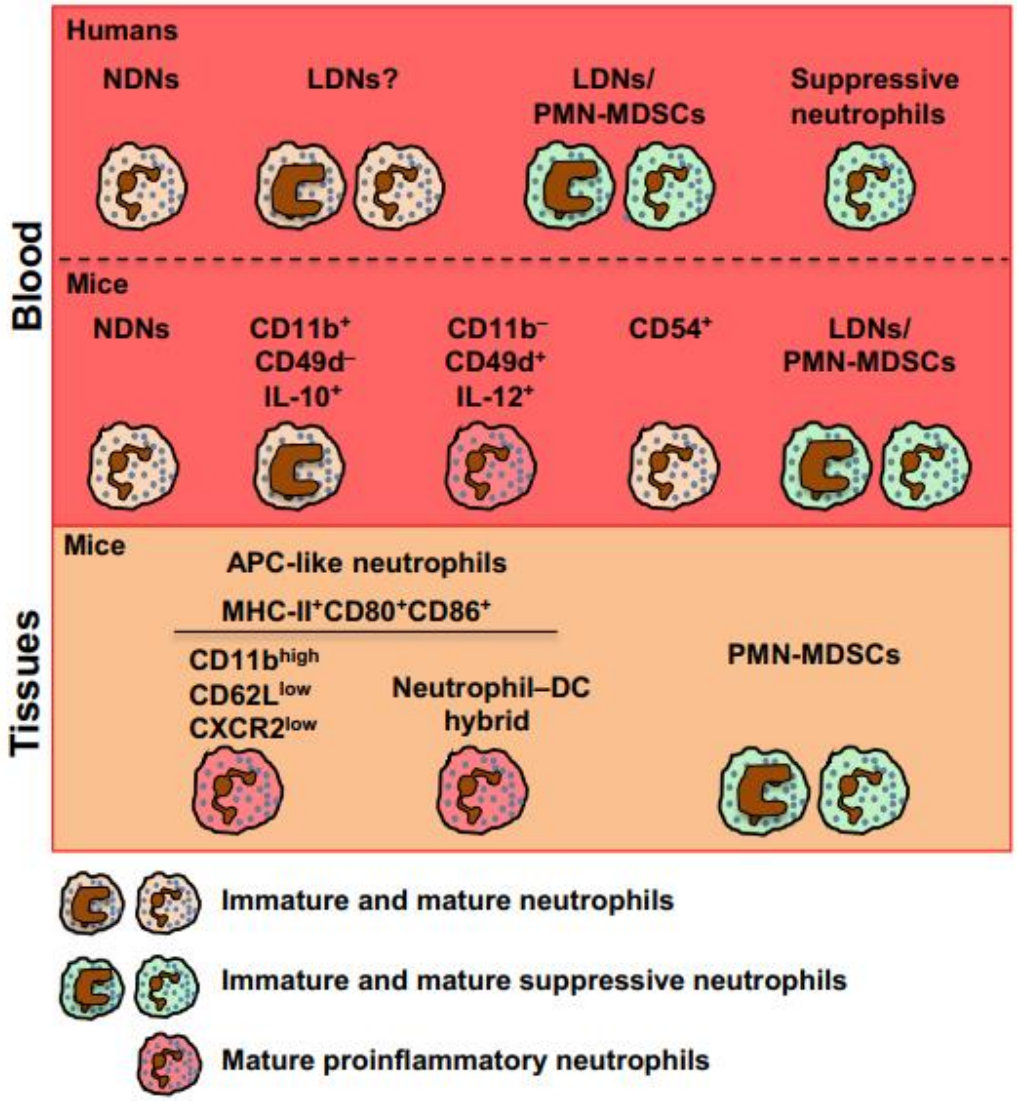


***Figure 4*.** Neutrophils population in cases of infection/inflammation. As far as humans are concerned, analysis of neutrophil subpopulations of patients with acute and chronic inflammatory conditions, among which sepsis or infection, revealed heterogeneous populations of low-density neutrophils (LDNs) within the mononuclear, in addition to normal density neutrophils (NDNs). According to studies that have been conducted, some of these heterogeneous LDNs acquire immunosuppressive properties – termed LDNs/polymorphonuclear myeloid-derived suppressor cells (PMN MDSCs, while other LDNs were proved to be proinflammatory. Regarding to mice, depending on the type of pathogen infection a different type of neutrophil subpopulations appears in blood and in tissues. Specifically, in the case of Staphylococcus aureus or Candida albicans models of infection, two subpopulations of neutrophils with opposite functions (proinflammatory, CD11b− CD49d+ IL-12+ ; anti-inflammatory, CD11b+ CD49d− IL-10+) have been identified[45].

It has been suggested that that neutrophils are not a homogenous population but instead there are different neutrophil subpopulation in circulation and in tissue (Fig 4). This diversity is due to the transcriptionally active phenotype of neutrophils, meaning that these cells have the ability to express different membrane molecules and produce cytokines[46,47]. The changes of gene transcription depend on the tissue they are found[34,48]. Specifically, it has been found that neutrophils, which are located in the vascular lumen and in the interstitial place of the lungs, are characterised by lower expression of CXCR4[49]. Furthermore, neutrophils in spleen have a $CD62L^{low}$ $CD11b^{hi}$ $ICAM\text{-}1^{hi}$ and they are more prone to netosis[50]. Another subpopulation of neutrophils includes cells that express CCR7 receptor, integrin LFA-1 and CXCR4, and they are preferentially found in lymph nodes, where they interact with T lymphocytes[51,52]. Lastly, there are neutrophils with $CD49d^{hi}$ $CXCR4^{hi}$ VEGFR1 phenotype that are recruited -in the case of hypoxia conditions, at non-vascularized tissues, in order to induce angiogenesis[53,54].

Neutrophil effector mechanisms responses

Degranulation

Neutrophil degranulation is a very essential mechanism for host protection, during which neutrophil cytoplasmic granules fuse with cell membrane and consequently exocytose soluble granule proteins or exhibit membrane granule proteins at the cell surface. The granule proteins, that are stored in a variety of granules (azurophilic, secondary, gelatinase, endocytic vesicles multivesicular bodies (MVBs) and secretory vesicles)[55–57], inside the cells, are responsible for triggering adhesion, transmigration, phagocytosis and neutrophil extracellular traps (NET) formation. Specifically, adhesion is promoted by secretory vesicles that upregulate adhesion molecules and chemotactic receptors, among which Mac-1 and CXCR2[57]. In addition, among the proteins that are contained in the granules are formyl- peptide receptor (FRP1), gelatinase B (matrix metalloproteinase-9), anti-microbial peptide cathelicidin and cytochrome b_{558}. Cytochrome b_{558} is the membrane associated subunit of the NADPH oxidase, which is an enzymatic complex whose role is to convert molecular oxygen into superoxide anion, using NADPH. The importance of this enzymatic complex is highlighted in the fact that individuals with deficiency at any of its components – such as chronic granulomatous disease (CGD) patients, are at high risk of development of life-threatening bacterial and fungal infections[58]. On the other hand, these cargoes may be toxic for the human body itself, resulting in endothelial dysfunction and systemic inflammation. For instance, myeloperoxidase (MPO) produces hypochlorite that is an oxidant able of killing microorganism and causing tissue damage. Moreover, the incontrollable release of proteolytic enzymes can lead to tissue damage and as extent to pathological conditions, in which fibrosis, sepsis and metabolic syndrome are included[59,60].

Phagocytosis

Neutrophils, together with macrophages, constitute the professional phagocytes of the cells, having the ability to engulf and eliminate pathogens. They have a considerable number of special receptors with which they can recognise their targets and initiate phagocytosis through a variety of mechanisms. After phagocytosis, cytoskeleton together with proteins and endomembranes trigger the formation of the phagosomal cup as well as its sealing. However, the formation of the phagosome is not enough for the decomposition or killing of the internalized target. In order to achieve that, a process termed 'phagosome maturation' should occur that includes fusion events of components and the removal of others via vesicular fission. Among the components that are essential for the maturation of the phagosome are microbicidal enzymes, vacuolar (V) ATPases and the NADPH oxidase complex. Defects or loss of these compartments lead to failure of phagosomal maturation and inability to defend the organism against pathogens[61].

Neutrophil extracellular traps

Apart from degranulation, neutrophils can defend human organism by using their chromatin. Precisely, neutrophils expel their chromatin outside the cell together with proteins and granules, forming structures called NETs[62]. There are different pathways that lead to the formation of NETs, with the majority of them acquiring cell death[63]. Data have shown that the induction of NETs are mainly triggered by larger microbes[64]. This indicated that NETosis is a process that be may deployed when the large size of the pathogen makes it difficult for the cells to phagocytose them. However, the exact mechanisms though which this decision is made has not been elucidated yet. Some of the physical inducers of NETosis is *Staphylococcus aureus* and hyphae from fungi. There is a variety of pathways, though which NETosis is induced. One of the pathways, that have been studied in detail, is known to require NADPH oxidase activation, which forms superoxide that through a cascade of events will lead to the release of NETs. Specifically, the main events of this cascade include the activation of proteinase 3, the dissociation of azurosome and the release of its compounds into the cytoplasm that migrate to the nucleus, where chromatin decondensation and finally nucleus swelling occur. There are also NAPDH oxidase-independent pathways, emphasizing the variety of activators that trigger NETosis. It should be mentioned that the inappropriate formation or degradation of NETs can lead to pathogenic conditions, such as Alzheimer's disease, chronic obstructive pulmonary disease, diabetes, cystic fibrosis, cancer, atherosclerosis, and various forms of arthritis[65].

Swarming

In the past few years, intravital microscopy studies have revealed a new neutrophil immune response called neutrophil swarming. Neutrophil swarming is a phenomenon of neutrophil organized chemotaxis and clustering formation and they named it after the swarming behaviour of insects because of their similarity.

Swarming is a coordinated migration mechanism that has five sequential phases: (1) initial chemotaxis of neutrophils that are close to the infected/damage site, followed by (2) communication among the swarming neutrophils and the ones from distant regions in order to accumulate, (3) swarm initiation through intercellular signal relay leading to (4) formation of neutrophil clusters, and lastly (5) resolution. Firstly, there is a small number of neutrophils that move to the infected/damaged site within the first 5-10 minutes, responding to chemotactic signals released from these sites. Secondly, more neutrophils from distant regions will follow these 'pioneers' due to signals they release. There is some data indicating that it is vital for the secondary recruitment some of the first recruited cells to die. It has been proved that even the death of one neutrophil is enough. However, the exact attractant factors released by the dying cells are responsible for the further swarming to the region. Furthermore, there are more signals released by neutrophils in order to provoke the swarming of other cells[66]. It has been proposed that one of the main regulators of this process is the lipid attractant leukotriene B4 (LTB4), which is produced by several immune cell types but neutrophils have been proved to be the main source[67,68]. LTB4 together with integrins is essential for the fourth step of swarming as well[67]. Generally, neither the mechanism of cluster resolution nor the mechanisms of the other steps have been fully elucidated yet, raising the interest for further studying. Physiologically, swarming has an important role in sterile inflammation and host defense against bacteria, fungi, and parasites.

So far, there are a few pathogens that have been described to promote this immune response, among which *Candida albicans*[68,69] and *Cryptococcus neoformans*[70].

Aims

Andrianaki et al. found that the massive influx of neutrophils in infected lung tissue with *Rhizopus oryzae*, where the cells would rather gather around the fungi than phagocytose them. Unpublished data from our group suggest that that murine neutrophils tend to form granuloma-like structures around *R. oryzae* spores. These data, together with the uncharacterized molecular mechanisms of neutrophil immune response in Mucormycosis, lead us to further investigate the interaction between these cells and Mucorales. During this Master thesis, experiments have been conducted in order to explore the immune response of neutrophils against this fungus as well as to evaluate the ability of these cells to inhibit or kill it.

Materials and Methods

Microorganisms and culture conditions

Aspergillus fumigatus ATCC46645 and *Rhizopus oryzae* ATCC557969 were cultured on Potato Dextrose Agar (PDA) plates for 4 and 6 days respectively at 37°C. Fungal conidia were harvested using spreaders and sterile PBS a via shaking the surface of the plate gently. A filtration through a 40 μm pore size cell strainer was followed and then centrifugation for 15 min at 3.000 rpm. Finally, the spores were washed 2-3 times with PBS for 5 min at 3.500 rpm and stored at 4 °C.

Swelling of conidia

Rhizopus oryzae conidia were in the swollen state within 3-4 hours incubation in RPMI medium while *Aspergillus fumigatus* spores needed approximately 5 hours.

Human neutrophil isolation

Human neutrophils were isolated using Ficoll (Histopaque) gradient density centrifugation technique. Peripheral blood was collected from healthy volunteers and diluted in 1x PBS. The blood was then carefully placed on the top of two gradients of Ficoll – Histopaque 1119 and Histopaque 1077, and centrifuged for 30 min at 600 × *g* at RT. After the centrifugation, the layer of neutrophils was collected with a clean Pasteur Pipette in a falcon tube, which was filled with PBS, and centrifuged at 300 × *g* for 10 min. Then, erythrolysis followed with resuspension of the pellet in 1 ml water for injection for 40 sec and then 1 ml of NaCl and PBS were added. The cells were centrifuged at 300 × *g* for 10 min and then the supernatant was discarded. Finally, the cells were resuspended in RPMI medium in order to be further used.

Murine neutrophil isolation

Murine neutrophils were isolated using a Percoll (Sigma) gradient density centrifugation technique. Bone marrow 2-month-old male mice was collected and flushed in room temperature in a sterile solution of PBS/EDTA. The cells were centrifuged at room temperature for 10 min at 400 × *g*, resuspended in 2 ml PBS/EDTA and then, they were carefully placed on top of 2 ml of three different Percoll concentrations (75%, 67%, and 52%) in a 15 ml Falcon tube. A centrifugation was followed for 30 min at 1100 × *g* at room temperature, resulting in three zones, peripheral blood mononuclear cells, PMNs, and red blood cells (RBCs), from the top to the bottom, respectively. PMNs were collected in PBS/EDTA/BSA medium and centrifuged in 4 °C for 10 min at 350 × *g*. The pellet was resuspended in 0.5 ml water for 20 s to lyse the remaining RBCs and then 0.5 ml of 1.8% NaCl was added. The cells were centrifuged in 4 °C for 10 min at 4000 × *g*. The pellet was resuspended in 2 ml HEPES buffer, and centrifuged again in the same conditions. Finally, the pellet was re-diluted in 1 ml RPMI w/o phenol red- 10% FBS. The mouse neutrophil survival was evaluated with trypan blue, with acceptable death rate being <4%.

Confocal microscopy

For confocal imaging, neutrophils were 2,5 µM Sytox Green and 2 µM DRAQ5. After their incubation with fungi on cover slips, they were fixed with 4% formaldehyde. The slides were examined under SP8 inverted confocal microscope.

Staining of conidia cell wall

In some experiments, fluorescent brightener 28 (Sigma-aldrich, cat#F3543) was used in order to stain the conidia cell wall. Conidia that were freshly harvested were stained with 250µg/ml for *Rhizopus* and 100µg/ml for *Aspergillus* of fluorescent brightener 28 for 1 hour at room temperature in $NaOHCO_3$. After the incubation, the conidia were washed 3-times with 1X PBS and stored at 4^oC in 1X PBS till use.

Killing assay

It was verified that neutrophils were not activated by the isolation procedure mouse Human neutrophils were isolated and placed in 96-well flat bottom plates ($2*10^5$ cells in each well) in RMPI medium w/o phenol red for 30 min at 37 °C, 5% CO2. Then, the cells were infected with fungal spores at different ratios 1:1, 1:5 and 1:10 (fungi: neutrophils). Fungal viability was tested both in the case of dormant and swollen state. Fungal spores were placed in 96-well-plate (10^4 cells in each well) for the treatment. They were sonicated at different time points (1 hour and 6 hours post infection) for 1 to 5 seconds (mention sonicator used and parameters in presence 0,05% and 0,025% Triton diluted in water. After the treatment they were in RPMI w/o phenol red for approximately 6 hours and representative photos were taken under inverted microscope, in order to evaluate fungal germination. In parallel, control fungal cells were incubated in media in the absence of neutrophils and treated with sonication, as a control to estimate the percentage of fungal cell death induced by sonication process.

Results

Imaging of neutrophil-fungal interaction ex vivo

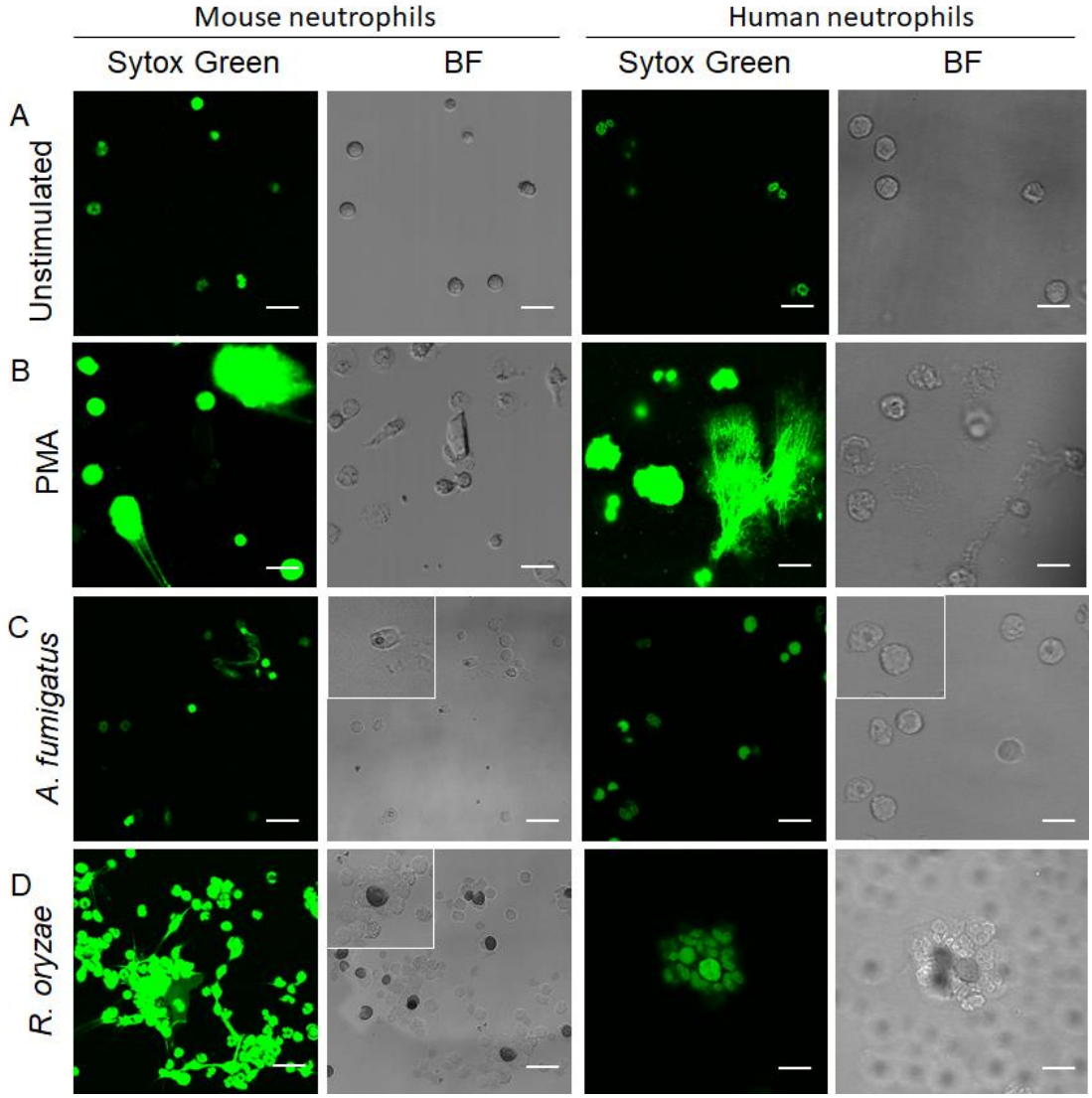


Figure 1. *Imaging of mouse neutrophils-fungi interaction ex vivo.*

Mouse neutrophils were isolated from the bone marrow of two-months-old C57/BL6 mice and were stained with Sytox green. Then, they were placed on cover slips and 6 hours after treatment, they were fixed and observed under the microscope (A) The unstimulated neutrophils are well -shaped with a distinct multi-lobulated nucleus. (B) Neutrophils exposed to 50nM PMA formed NETs, while (C) the ones that were infected with A. fumigatus, phagocytosed the pathogens. (D) In the case of R. oryzae, neutrophils formed clusters around the fungus conidia.

Previous studies suggest an important role of neutrophils (PMNs) in antifungal immune defense against Mucorales[71]. Accordingly, neutropenia is a major risk factor for mucormycosis[26]. Importantly, the molecular interactions of PMNs with Mucorales are incompletely understood. Previous studies focused on interactions of PMNs with hyphae of Mucorales. Although PMNs are able to cause damage to fungal hyphae, physiologically Mucorales conidia remain quiescent and do not germinate in vivo.

Instead, hyphal growth exclusively occurs in the neutropenic host. There is no previous model to study interactions of PMNs with Mucorales conidia. Therefore, we designed experiments both with human and mouse neutrophils in order to evaluate comparative immune response and infection outcome during infection of *R. oryzae* conidia vs *Aspergillus fumigatus* conidia. First, neutrophils were stained with Sytox Green, placed on poly-lysine treated cover slips or Ibidi slides for live imaging, left untreated (control), activated with PMA (positive control for NETosis) or infected with *R. oryzae* or *A. fumigatus* conidia and analyzed with confocal microscopy at different time points.

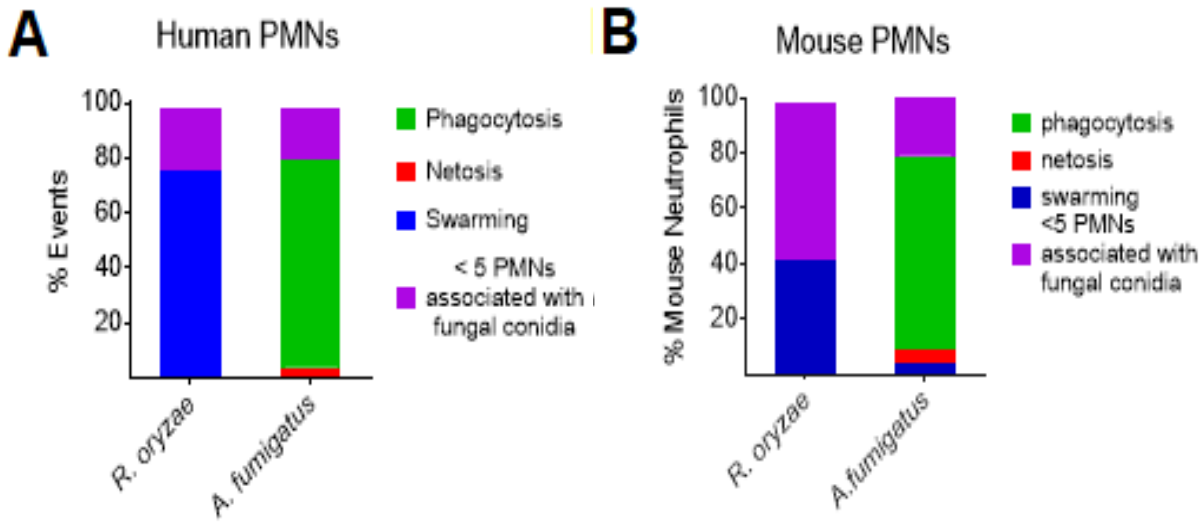
1. In order to distinguish NETosis (extracellular release of DNA filaments decorated with antimicrobial effectors), PMNs were stained with a cell permeable stain (DRAQ5) and a cell impermeable fluorescence dye (SYTOX Green). NETotic cells are shown as Sytox Green+ cells. To induce NEtosis , PMNs were incubated with PMA. In certain experiments with human PMNs, we noticed a high degree of PMN NETosis at the baselines (control unstimulated PMNs), which primed further activation of NETosis upon fungal infection. This effect was largely attributed to LPS contamination in either the FCS, Percoll or the isolation buffers. Such contaminations have not been mentioned earlier must be to be taken into account if you want to correctly evaluate NetOsis.
2. Neutrophils phagocytose conidia of *A.fumigatus* .
3. In contrast, neutrophils do not phagocytose *R. oryzae*, but instead they gather around the pathogen, forming clusters (**Fig 1**). The following responses were evaluated: (a) minimal association of PMNS with fungal cell (< 5 PMNs attached to fungal conidia), (b) NETosis, (c) phagocytosis of fungal conidia and (d) swarming. Representative images are shown in Figure 1. We used only PMNs obtained from male, non-smokers to account for increased NETosis observed in previous studies with PMNs obtained from females and smokers. To exclude the effect of circadian rhythm on PMN response all experiments were performed at the same time of the day (between 10-12 AM)[72]. Finally, we tested the effect of different media in induction of immune response. We established optimal conditions for assessment of PMNs fungal interactions by confocal imaging in RPMI w/o phenol red containing 10Mm HEPES and 0,5% human albumin in the case of humans, and 10% HI serum in the case of mice, at a MOI of 1:5 (1 fungal cell: 5 PMNs).

Selective induction of swarming during PMN interaction with Mucorales conidia.

In agreement with previous studies[30], we confirmed that *Aspergillus* conidia are effectively phagocytosed at high rates by both murine and human PMNs. In contrast

to previous report, we found minimal activation of NETosis by *Aspergillus* conidia under optimal conditions at several experiments (n = 6) (Fig.2).

In contrast to Aspergillus, Mucorales conidia were minimally phagocytosed by PMNs. Instead, PMNs formed clusters surrounding Mucorales conidia, a phenomenon that has been recently described as swarming (**Fig 2A, B**). In some experiments we noticed that swarming was associated with NETosis in some of the neutrophils in the cluster (**Fig 2 C**). Notably, PMNs swarming against Mucorales conidia also occurred under conditions of serum inactivation.



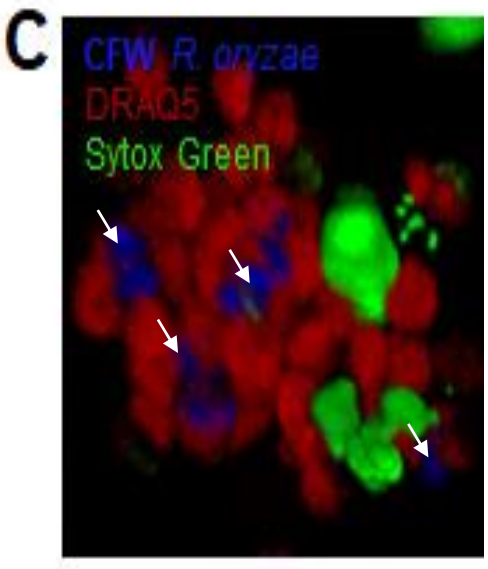


Figure 2. *R. oryzae preferentially induces both human and mouse neutrophil swarming*

(A-B) Human and mouse neutrophils do not phagocytose R. oryzae conidia, but instead they tend to swarm towards them forming clusters. Non-random association with fungi was consider to be clusters formed by at least 5 neutrophils around each R. oryzae spore. On the other hand, A. fumigatus conidia are mainly phagocytosed by both human and mouse neutrophils. **(C)** 3D projection of live mouse neutrophils stained with DRAQ5 and Sytox Green forming clusters around R. oryzae conidia, stained with Calcofluor white, after 6 hours of co-incubation.

Rhizopus melanin is involved in neutrophil swarming induction

Fungal cell wall usually triggers immune responses. Our lab had in hands the required expertise to be able to follow the role of melanin during phagocytosis of *Aspergillus*[30] We found that neutrophils tend to swarm around *R. oryzae* melanin particles **(Fig. 3)**.

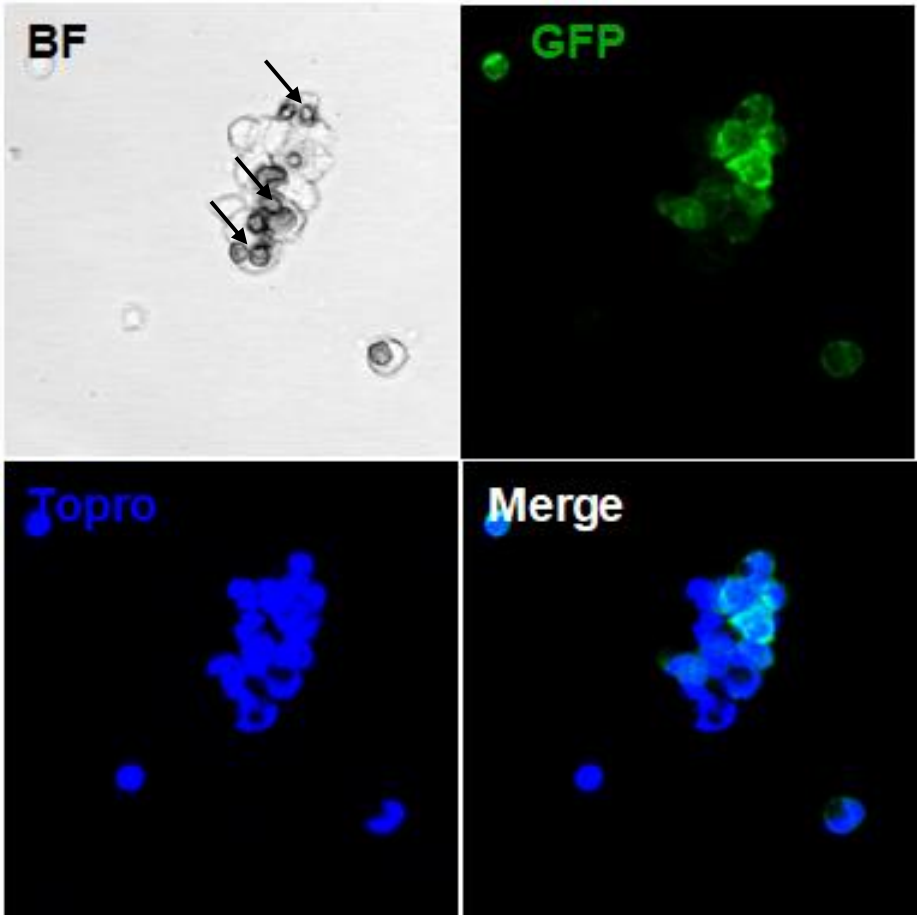


Figure 3. Mouse bone marrow isolated neutrophils from GFP/LC3 mice in interaction with melanin particles extracted from R. oryzae spores. Neutrophils form clusters around melanin particles (melanin particles).

To confirm this result, we conducted one experiment with mouse neutrophils isolated from bone marrow that were infected with regular R. oryzae spores and *R. oryzae*

spores from which melanin was chemically removed from the fungal cell wall. Neutrophils were stained with DRAQ5 and SYTOX green, while *R. oryzae* (please replace fungus name with italics throughout the text) spores were stained with Calcofluor white. We found that neutrophils do not form clusters around melanin-deficient (albino) R. oryzae spores, as opposite to stimulation with regular *R. oryzae* conidia. Collectively, this pilot studies imply that fungal cell wall melanin is involved in selective swarming of neutrophils against *Rhizopus* **(Fig. 4A, B)**. Further work is required to with purified melanin and selective removal of melanin from Mucorales cell wall is required to support thesepreliminary findings.

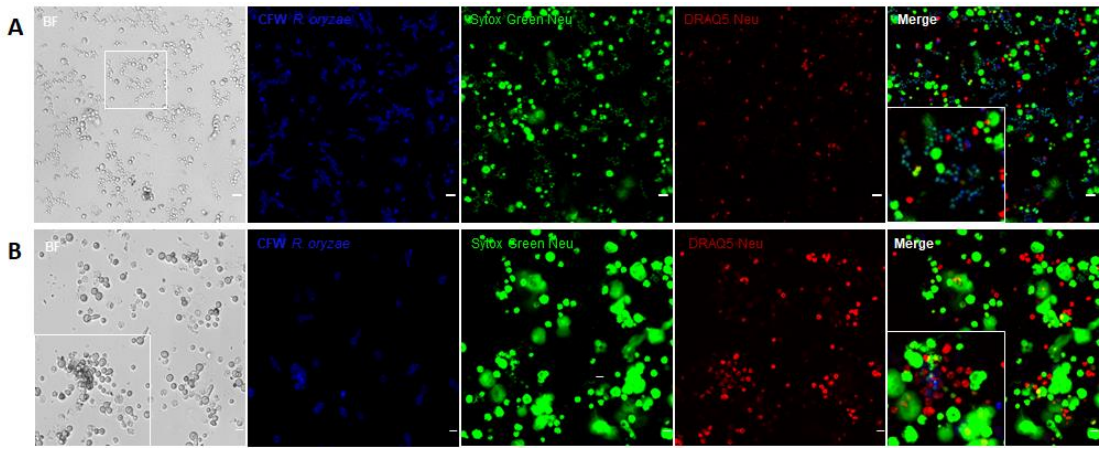


Figure 4. *Mucorales melanin is involved in neutrophil swarming induction*

Neutrophils were isolated, stained with DRAQ5 and Sytox Green and placed in ibidi plates, and were infected with R. oryzae spores with (B) and without melanin(A), all stained with Calcofluor white. The wells were observed live 6 hours post infection under SP8 reverse microscope. Neutrophils had formed clusters around R. oryzae spores with melanin, while in the case with the ones without, they were randomly dispersed in the field.

Development of an assay for evaluation of killing of fungal conidia by murine neutrophils

A

Triton (distilled water)	0,05 %	0,02 %
R. oryzae (dormant)	X	X
R. oryzae (swollen)	X	X
A. fumigatus (dormant)	X	✓
A. fumigatus (swollen)	X	X

B

Sonication (distilled water)	1 sec	2 sec	3 sec	4 sec	5 sec
R. oryzae (dormant)	✓	✓	✓	✓	✓
R. oryzae (swollen)	✓	✓	✓	✓	✓
A. fumigatus (dormant)	✓	✓	✓	✓	X
A. fumigatus (swollen)	✓	✓	✓	✓	✓

C

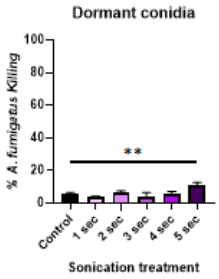


Figure 5. *Fungal killing assay in absence of active serum*

Dormant and swollen spores of both R. oryzae and A. fumigatus were treated with sonication and Triton- two possible methods of cell lysis, in order to ensure fungus survival under these conditions. (A) Triton was found to be unsuitable method as it leads to fungi death. (B) In the case of sonication treatment, only the 5 second sonication was found to provoke the death of A. fumigatus dormant conidia (C). For the evaluation of fungal survival, representative photos

*were taken and the germination of conidia was counted with ImageJ and is plotted as ± SEM and were statistically compares with one-way ANOVA, **p<0,01.*

Next, we wanted to investigate if neutrophils have the ability to kill fungal conidia. However, there was no suitable assay of neutrophil killing for filamentous fungus conidia so far. For this reason, we wanted to establish a killing protocol that will enable us to evaluate the fungal survival after co-incubation of the conidia with neutrophils for a period of time, before cell lysis. First, it was essential ensure that the cell lysis method will not affect the fungal viability. Hence, we tested possible ways of cell lysis on dormant and swollen conidia on both *R. oryzae* and *A. fumigatus.* Specifically, both 10^4 dormant and swollen spores in 96-well-plated were exposed to 0.05% and 0,02% Triton-X for 10 minutes and to 1-5 seconds of sonication, in distilled water. After the treatment, the spores were allowed to grow in RPMI medium and photos were taken under an inverted microscope in order to evaluate the germination. We found that Triton-X has a major effect on the fungal growth, therefore is an unsuitable method for cell lysis **(Fig. 5A**). On the other hand, sonication does not affect the fungal viability, except from the *A. fumigatus* dormant spores in the case of 5 seconds of sonication. We further tested sonication on neutrophils, in order to confirm that the cells are lysed efficiently **(Fig. 5B, C).** Taking all results into consideration, we decided that the best cell lysis method was the 3 seconds sonication in distilled water.

Murine neutrophils have an inhibitory effect on *Rhizopus* growth

Having established the killing assay, we further investigated if neutrophils have an effect on fungal viability. Mouse neutrophils isolated from bone marrow were placed in 19-well-plates in RPMI medium with 10% heat-inactivated serum and were infected with dormant conidia of both *R. oryzae* and *A. fumigatus,* at 3 different MOI 1:1, 1:5, 1:10 (fungal cells: neutrophils). Experiment was stopped at two different time points: 1 hour and 6 hours post infection. After the lysis treatment, the fungi were allowed to grow for 6 hours and then pictures were taken under an inverted microscope for the evaluation of germination **(Fig. 6A).** After 6 hours of incubation in our experimental conditions, there was no killing of *A.fumigatus* conidia. In contrast neutrophils were able to kill *R. oryzae* spores **(Fig. 6 B, C).** The killing increased when the

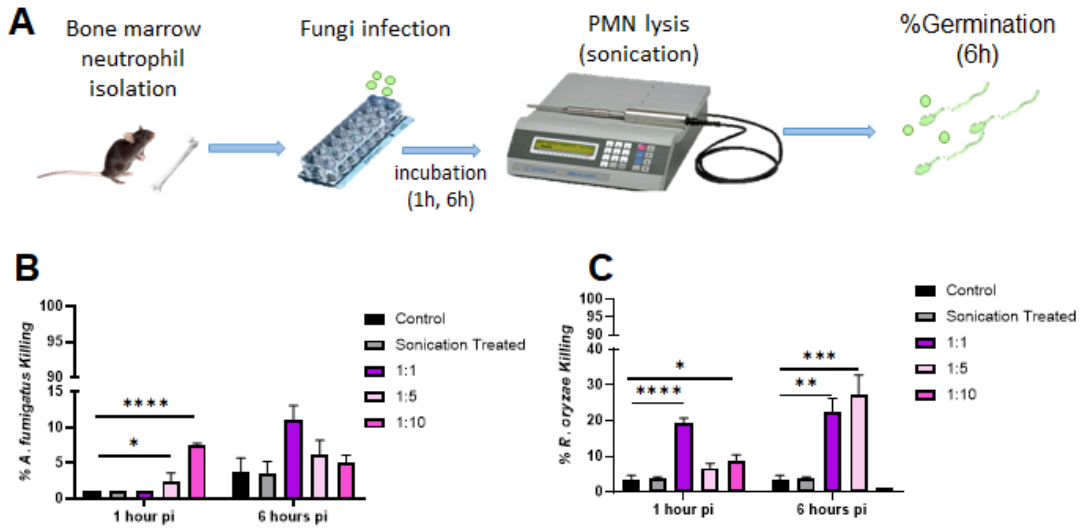


Figure 6. *Murine neutrophils have an effect on Mucorales growth*

*(A) Mouse neutrophils isolated from bone marrow were placed in 16 – plates and were infected with A. fumigatus and R. oryzae conidia at MOI 1:1,1:5and 1:10 (Fungal cells: neutrophils). The cells were lysed 1 hour and 6 hours post infection with 3 seconds sonication in distilled water, and the fungi cells were further incubated in RPMI for approximately 6 hours, in order their survival to be evaluated. Representative photos were taken under inverted microscope and the germination was evaluated with ImageJ. (B) A. fumigatus conidia are not killed by neutrophils neither in the case of 1 hour not in the case of 6 hours of co-incubation. The germination ability of A. fumigatus was plotted as ± SEM and statistically compared with one-way ANOVA, *P<0,1, ****p<0,0001. (C) This experiment indicated that neutrophils can effect the survival of R. oryzae conidi, but more experiments should be conducted in order to further confirm these results. The germination ability of R. oryzae was plotted as ± SEM and statistically compared with one-way ANOVA, *P<0,1, **p<0,01, ***p<0,001, ****p<0,0001.*

Discussion

PMNs have a pivotal role in protective immunity against fungal pathogens[73,74]. A long standing immunological concept is that PMNs are recruited at the site of infection to eliminate germinating fungal cells (hyphae), which escape from surveillance of tissue resident macrophages. Therefore, most work on fungal-PMN interactions has focused on dissecting mechanisms of PMN-induced damage to fungal hyphae. However, recent studies demonstrated a remarkable plasticity of PMN immune effector mechanisms operating against both conidia and germinating fungal hyphae. In addition, physiologically fungal cells do not germinate into the lungs. Therefore, PMNs mainly interact with conidia and not with fungal hyphae.

Specifically, it has been demonstrated that phagocytosis and ROS-mediated killing is the primary host defense mechanism of PMNs against *Candida* and *Aspergillus*[75]. In addition, NETosis and degranulation become important effector mechanisms to restrict growth against germinating forms of *Candida*[76] and *Aspergillus*[75]. Lastly, PMN swarming is new type of immune response that delays growth of large clusters of *Candida*[69] *ex vivo*, which are unable to phagocytose. However, the physiological function of NETosis and swarming in vivo has not been characterized.

Mucorales are a unique class of fungal pathogens that infect patients with a wide range of incompletely understood immunometabolic defects in phagocytes. Our group has shown that neutrophils are rapidly recruited in the lung of immunocompetent mice and form primitive granuloma like structures surrounding extracellular conidia of Mucorales. In addition, we have found that as opposite to *Aspergillus*, unopsonized *Rhizopus* conidia are minimally phagocytosed by mouse PMNs ex vivo. Finally, we found that Mucorales conidia remain dormant for many days in the lungs of mice. These studies suggest that PMNs mount a unique type of immune response against Mucorales as compared to other fungi.

Herein, we expand on previous observations and establish *ex vivo* assays to evaluate physiological mechanisms of interaction of human and mouse PMNs with Mucorales

conidia. Importantly, we found that PMNs are rapidly recruited to Mucorales conidia following infection forming “swarms” around individual fungal cells. In sharp contrast to Aspergillus, Mucorales conidia re not phagocytosed by PMNs. Furthermore, there is minimal evidence of NETosis during interaction of PMNs with both Mucorales and *Aspergillus*. Of interest, in contrast to previous studies, PMN swarming is induced by individual cells at high percentages and does not require the presence of active serum for opsonization by complement. Collectively these findings suggest a major physiological role of swarming in immunity against Mucorales. Therefore, this fungus can serve as a model pathogen to dissect molecular mechanisms of this new immunological response of PMNs and explore the physiological role during infection. Of interest, it would be essential to explore the effect of diabetic acidosis and other metabolic diseases predisposing to mucormycosis on activation of swarming. Because swarming is regulated by lipid mediators (e.g., LTB4) and complement activation (e.g., C3), it would be interesting to investigate whether defects in these effectors are implicated in disease pathogenesis.

Importantly, we established assays to evaluate killing of Mucorale conidia. These studies demonstrated that PMNs effectively inhibit Mucorales growth. The underlying mechanisms of inhibition (e.g., degranulation vs NETosis) and the effector molecules (e.g., antimicrobial peptides, lipid mediators, siderophores etc) should be characterized in future studies. In addition, how these effector mechanisms are compromised in the disease setting needs to be evaluated in future studies.

From the pathogen perspective, we found that the presence of cell wall melanin likely accounts for activation of swarming. Future studies should systematically characterize whether the type of melanin or other surface molecules induces swarming. In particular, it is intriguing that Aspergillus conidia is not inducing swarming during interaction with PMNs. One explanation could be the small size of the conidium of *Aspergillus* and the possibility for neutrophils to phagocytose their conidium while their large size prevents the phagocytosis of *Rhizopus* conidia. Another possible explanation is that Mucorales conidia contain a different type of melanin (DOPA) than the DHN melanin of *Aspergillus.* Of interest, melanin is regarded as a major virulence factor that exerts its inhibitory action on macrophages via blocking activation of Ca2+/Calmodulin signaling and downstream phagosome biogenesis during phagocytosis. In contrast, in PMNs melanin seems to act as a danger signal that triggers PMN swarming. Importantly, paracrine and autocrine Ca2+ signaling induces swarming. Therefore, it would be interesting to explore whether and how this Ca2+ response is modulated by melanin.

Importantly, the physiological role of swarming has not been convincingly demonstrated in the context of a disease. Mucormycosis is a model disease that occurs in patients with unique predisposing conditions (e.g., acidosis, iron deregulation). It is tempting to speculate that defective swarming is implicated in disease pathogenesis. Furthermore, it will be of paramount importance to identify which are the effector mechanisms (e.g., degranulation, iron starvation, NETosis) that are preferentially activated during swarming and how these are affected in the setting of mucormycosis predisposing conditions. Finally, it would be important to establish

in vivo imaging studies to investigate whether there is a differential mechanisms of induction of swarming as compared to other infectious and non-infectious stimuli.

Limitations of our work: Challenges associated with variability of human neutrophils responses, the effect of LPS contamination in activation of neutrophils, lack of a standardized inoculum of fungal conidia, susceptibility of fungal conidia to treatment during neutrophil lysis and lack of the appropriate tools to establish in vivo and live imaging assays (lack of CO2 and chamber) precluded repetition of essential experiments (e.g., effect of melanin on swarming) and capture of the dynamic nature of this immunological phenomenon of swarming. Therefore, a substantial amount of reported work has to be repeated with live imaging time lapse microscopy and in vivo imaging methods.

Collectively, our work identifies *Rhizopus* as a model pathogen for understanding PMN biology and swarming. Future studies in PMNs obtained from human patients and in the in vivo model of mucormycosis in mice will further clarify the role of swarming in physiological immunity and disease.

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