UNIVERSITY OF CRETE

MEDICAL SCHOOL

GRADUATE PROGRAM

"MOLECULAR BASIS OF HUMAN DISEASES"

MASTER THESIS

"Exploring the mechanisms of LAP pathway activation by *Aspergillus fumigatus*"

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OCTOBER 2021

HERAKLION

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«ΜΟΡΙΑΚΗ ΒΑΣΗ ΤΩΝ ΝΟΣΗΜΑΤΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ»

ΜΕΤΑΠΤΥΧΙΑΚΗ ΕΡΓΑΣΙΑ

«Μελέτη των μηχανισμών ενεργοποίησης του LAP μονοπατιού από τον μύκητα *Aspergillus fumigatus*»

ΓΚΟΥΝΤΖΙΝΟΠΟΥΛΟΥ ΜΑΡΙΝΑ-ΕΛΛΑΔΑ

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ΟΚΤΩΒΡΙΟΣ 2021

ΗΡΑΚΛΕΙΟ

ABSTRACT

Aspergillus fumigatus is a saprophytic organism that belongs to the Ascomycetes family of filamentous fungi (molds) and contributes to the decomposition of dead organic matter for the proper recycling of carbon and nitrogen in nature. This ubiquitous filamentous fungus produces thousands of conidia (spores), which spread through the air in long distances due to their small size (2-3 um diameter) and participate in the degradation of organic material.

All humans inhale hundreds of *A. fumigatus* conidia on a daily basis, which, are successfully eliminated by lung tissue resident macrophages. However, in severely immunocompromised patients with qualitative or quantitative disorders in phagocytes, *A. fumigatus* has the ability to escape killing by phagocytes (macrophages and neutrophils), germinate into long filamentous forms (hyphae), which invade lung tissue and cause life threatening disease. Invasive fungal diseases caused by *Aspergillus* is associated with significant mortality of 40-50% despite the appropriate antifungal treatment. For this reason, understanding immunopathogenesis of fungal diseases is an unmet need for development of new therapeutic strategies aiming to restore the underlying host immune defects.

Herein, we focus on a major antifungal immune pathway termed LC3-associated phagocytosis (LAP), which is activated during phagocytosis of *Aspergillus* conidia and promotes fungal killing. More specifically, we highlight important differences in activation of LAP by live *Aspergillus* conidia as compared to different cell wall components of the fungus focusing on the role of melanin degradation and removal from the fungal cell wall during phagocytosis. In particular, previous work with melanin deficient *Aspergillus* mutants demonstrated that this molecule specifically inhibits LAP to promote virulence. In contrast to these previous findings, our preliminary work suggest an activity of melanin degradation product(s) as vita-PAMP(s) during live infection of macrophages. In addition, in order to study this phenotype in primary monocytes/macrophages of healthy individuals and patients with invasive aspergillosis, we developed and tested a cryopreservation assay in healthy human peripheral blood mononuclear cells (PBMCs), which allows evaluation of phagosome biogenesis and LAP.

ΠΕΡΙΛΗΨΗ

Ο μύκητας *Aspergillus fumigatus* είναι ένας σαπροφυτικός οργανισμός, ο οποίος ανήκει στην οικογένεια Ascomycetes των νηματοειδών μυκήτων (μούχλες) και συμβάλλει στην αποσύνθεση της νεκρής οργανικής ύλης με σκοπό τη σωστή λειτουργία των κύκλων άνθρακα και αζώτου. Το συγκεκριμένο είδος μύκητα εντοπίζεται παντού στη φύση και παράγει χιλιάδες κονίδια (σπόρια), τα οποία, λόγω του μικρού τους μεγέθους (διάμετρος 2-3 μm), εξαπλώνονται μέσω του αέρα σε μεγάλες αποστάσεις συμμετέχοντας, έτσι, στην αποικοδόμηση της οργανικής ύλης.

Όλοι οι άνθρωποι εισπνέουν, σε καθημερινή βάση, εκατοντάδες κονίδια του *A. fumigatus*, τα οποία, όμως, εξολοθρεύονται επιτυχώς από τα μακροφάγα των πνευμόνων. Ωστόσο, σε σοβαρά ανοσοκατεσταλμένους ασθενείς με ποιοτικές ή ποσοτικές διαταραχές στα φαγοκύτταρα, ο *A. fumigatus* έχει την ικανότητα να αποφεύγει τη θανάτωσή του από τα φαγοκύτταρα (μακροφάγα και ουδετερόφιλα), να εκβλαστάνει σε μακρές νηματώδεις μορφές (υφές), οι οποίες εισβάλλουν στον πνευμονικό ιστό και προκαλούν, απειλητικές για τη ζωή, ασθένειες. Οι διηθητικές μυκητιακές λοιμώξεις που προκαλούνται από τον Ασπέργιλλο έχουν συνδεθεί με μεγάλο ποσοστό θνησιμότητας (40-50%) παρά την κατάλληλη αντιμυκητιακή θεραπεία. Για τον λόγο αυτό, υπάρχει ανάγκη για κατανόηση των μηχανισμών παθογένεσης των διηθητικών μυκητιάσεων με σκοπό την ανάπτυξη νέων θεραπειών και την αποκατάσταση της υποκείμενης ανοσοανεπάρκειας.

Στη συγκεκριμένη μεταπτυχιακή εργασία, εστιάζουμε σε ένα από τα σημαντικότερα μονοπάτια που εμπλέκεται στη θανάτωση του *Aspergillus fumigatus*, τη σχετιζόμενη με την LC3 φαγοκυττάρωση (LC3-associated phagocytosis, LAP), το οποίο αποτελεί ένα ειδικό μονοπάτι αυτοφαγίας που ενεργοποιείται κατά την φαγοκυττάρωση των κονιδίων του *A. fumigatus* και στοχεύει στην εξουδετέρωσή του. Πιο συγκεκριμένα, επισημαίνουμε σημαντικές διαφορές στην ενεργοποίηση του LAP μονοπατιού από ζωντανά κονίδια του *A. fumigatus* βάσει των διαφορετικών συστατικών του κυτταρικού του τοιχώματος, εστιάζοντας στο ρόλο της αποικοδόμησης της μελανίνης και της απομάκρυνσής της από το κυτταρικό τοίχωμα του μύκητα κατά τη διάρκεια της φαγοκυττάρωσης. Συγκεκριμένα, προηγούμενες μελέτες στις οποίες χρησιμοποίηθηκαν μεταλλάξεις του *A. fumigatus* από τις οποίες η μελανίνη απουσιάζει, έδειξαν ότι το μόριο της μελανίνης έχει την ικανότητα να αναστέλλει ειδικά το LAP ενισχύοντας με αυτόν τον τρόπο την μολυσματικότητά του. Σε αντίθεση με τα ευρύματα αυτά, η εργασία αυτή προτείνει τη δράση του/των προϊόντος/προϊόντων αποικοδόμησης της μελανίνης ως vita-PAMP(s) κατά τη διάρκεια μόλυνσης των μακροφάγων με ζωντανό στέλεχος του μύκητα. Επιπλέον, προκειμένου να μελετήσουμε τον φαινότυπο αυτό σε πρωτογενή μονοκύτταρα

υγιών ατόμων και ασθενών με διηθητική ασπεργίλλωση, αναπτύξαμε και εξετάσαμε μια δοκιμασία κρυοσυντήρησης σε υγιή ανθρώπινα μονοπυρηνικά κύτταρα περιφερικού αίματος (PBMCs), η οποία επιτρέπει την αξιολόγηση της βιογένεσης των φαγοσωμάτων και του LAP μονοπατιού.

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I. INTRODUCTION

I.1. Fungal diseases and the importance of studying *Aspergillus fumigatus*

Fungal Kingdom is one of the widest and major branches of life that includes over one million species with a wide range of morphotypes, from unicellular to complex multicellular structures. Although fungi were initially considered to be plants because they grow out of the soil and have rigid cell wall, they have been classified in a separate kingdom because, as opposite to plants, they lack chlorophyll and are heterotrophic. The fungal kingdom is divided in four phyla: Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota[1]. Familiar representatives include the edible mushrooms, molds, mildews, yeasts, and the plant pathogens, smuts and rusts. The fungi reproduce by spores, both asexual and sexual, with the asexual reproduction to be more common in nature. Collectively, the term "Fungi" consists all these organisms which are eukaryotic single celled or multicellular. However, less than a hundred among the millions of all these fungal species have the ability to invade the human host and cause disease.

a. Invasive mold infections (IMIs)

Evolution has supplied humans with robust and specialized defenses against fungi. Accordingly, less than 10 fungal pathogens cause the vast majority of invasive fungal infections in humans. In particular, invasive mold infections occur exclusively in immunocompromised patients[2] due to acquired immunodeficiency as a result of immunosuppressive therapies for malignant, autoimmune, or inflammatory disorders. The most common manifestation of an IMI is pneumonia, which can disseminate to other organs.

In order for fungi to establish infection in mammalian hosts including humans, they need to meet the following criteria: (i) withstand and proliferate at or above 37°C, (ii) penetrate organism's internal tissues by avoiding host barriers, (iii) degrade and catabolize components of human tissues, and, importantly, (iv) escape surveillance of the human immune system[3]. Additionally, the establishment of an IMI requires germination of fungal conidia from the dormant, single cell stage to a multicellular vegetative stage of growth (hyphal growth).

Changes on the fungal cell wall during growth largely modulate pathogenicity and allow for establishment of invasive disease. More specifically, the fungal cell wall has considerable plasticity and changes its composition dynamically during transition from dormant conidia to germinating forms[5] expressing on its surface various unique immunostimulatory polysaccharides called "pathogen-

associated molecular patterns" or PAMPs such as chitin, mannans, and β-glucan. At this stage, the presence of PAMPs on fungi surface triggers immune responses upon activation of specific "pattern recognition receptors" (PRRs), which are expressed by innate immune cells, including C-type lectin receptors (CLRs), Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs) and others. More specifically, C-type lectin receptors, which include MICL, CLEC-2, CLEC-12B, CLEC-9A, MelLec, Dectin-1 and LOX-1 receptors, play essential roles in immunity and homeostasis. Dectin-1 (CLEC-7A) is one of the best characterized CLRs in mice and in humans and is mainly expressed on myeloid cells, including monocytes, macrophages, dendritic cells, and neutrophils and recognizes β-glucans and carbohydrates commonly found in the cell walls of plants and fungi being required to drive protective host responses to many pathogenic fungal species including *Aspergillus*. For this reason, Dectin-1 has been most studied in the context of anti-fungal immunity using mouse models[40]. However, defects in the pathogen recognition or in the pathway which is responsible for its killing allows fungi to proceed in their germination stage penetrating the colonized tissue resulting to the overlap of the organism's immune shield and leading to an invasive mold infection.

b. Aspergillosis

Continuous lung exposure to *Aspergillus* species results into the common occurrence of invasive infections in individuals with impaired immune function. Allergic reactions, lung infections, and infections in other organs are the most common health problems caused by *Aspergillus*, thus covering a wide range of clinical syndromes from aspergilloma and allergic diseases such as bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitization (SAFS) to chronic pulmonary (CP) diseases including non-invasive, chronic pulmonary aspergillosis (CPA), or invasive inflammatory disorders such as invasive pulmonary aspergillosis (IPA)[41].

Invasive pulmonary aspergillosis (IPA) is the most common invasive mold infection in solid-organ transplant recipients and it is associated with the use of corticosteroids and other immunosuppressive therapies for chronic graft-versus-host disease, metabolic abnormalities, acquired iron overload syndromes, aging, cytomegalovirus (CMV) coinfection, and other comorbidities[7, 8]. Interestingly, IPA has been described in new groups of traditionally low-risk patients[9] such as patients with malignant, autoimmune, or inflammatory diseases or patients which appear immune-metabolic abnormalities due to an underlying disease, immunosuppressive therapy or because of a previous bacterial or viral sepsis syndromes making *Aspergillus* an important model pathogen to study mechanisms of sepsis

immunosuppression (immunoparalysis). In recent years, IPA has been described increasingly in patients with respiratory viral diseases such as severe acute respiratory syndrome (SARS), the H1N1 influenza, and recently coronavirus disease 2019 (COVID-19), which are characterized by high concentrations of circulating inflammatory cytokines, acute phase reactants and ferritin, and hemophagocytosis[10]. Incompletely understood immunometabolic abnormalities in myeloid phagocytes which have been associated with different immunosuppressive conditions predisposing for development of IPA. However, the immunopathogenesis of IPA in all these patient groups remains unexplored.

For this reason, we have to take a closer look to the pathogenic mechanisms of these emerging diseases in order to identify host and pathogen molecular determinants of disease, which could represent promising future therapeutic targets.

I.2. Host defense against *Aspergillus fumigatus*

a. The innate immunity against *Aspergillus fumigatus* infections

Host defense against inhaled conidia of *Aspergillus* involves a tightly regulated response that involves multiple layers of immune system. The first important defense against *Aspergillus* is the innate immune barriers of the airway epithelium, which release various antimicrobial proteins, cytokines and chemokines. Nevertheless, some of the inhaled spores, due to the small size of *Aspergillus* resting conidia (2 to 3µm in diameter) escape this defense mechanism and reach the respiratory zone of the lung (Figure 1). In this area, fungus is recognized by pathogen recognition receptors (soluble or cell-bound). This second crucial pulmonary anti-*Aspergillus* host defense layer involves the professional phagocytes, such as alveolar macrophages and recruited

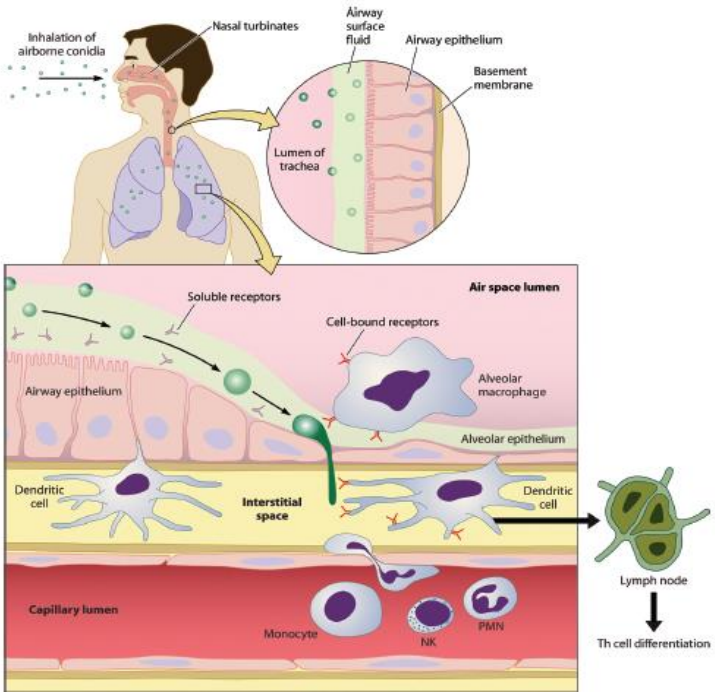

Figure 1. Schematic representation of *Aspergillus* conidia inhalation and the components of the host's defense against *A. fumigatus* (39).

monocytes/macrophages, which eliminate *Aspergillus* conidia by phagocytosis and release reactive oxygen species (ROS), cytokines and chemokines. More specifically, alveolar macrophages (AMs) are the major resident leukocytes in the lung and provide an early line of defense against inhaled conidia that reach the alveoli[11] secreting pro-inflammatory cytokines in order to restrict their spread and germination in the alveoli. On the other hand, peripheral blood monocytes are a heterogeneous population of myeloid cells that contain the precursors of tissue macrophage and dendritic cells in inflamed tissues[12]. Upon interaction with *Aspergillus* conidia, human peripheral blood monocytes proceed in important changes in the expression of hundreds of genes leading to their appearance in the lungs within hours of the onset of infection[13]. Studies in Drosophila and other insects lacking adaptive immunity show the importance of innate immune system in killing of *Aspergillus* conidia. Similarly, RAG-1-deficient mice, which lack mature T- and B- lymphocytes, can clear equally well with control mice the fungal conidia. However, if this step of defense is not successful leading to chronic inflammatory and allergic lung diseases due to *A. fumigatus*, T cell immunity proves to be extremely important.

b. The LC3-associated phagocytosis (LAP) pathway

LC3-associated phagocytosis is a specialized non-canonical autophagy pathway, implicated in the killing of extracellular pathogens, including *Aspergillus fumigatus*. However, LAP and autophagy are functionally and mechanistically distinct processes, while the LAP-engaged phagosome (LAPosome) is composed of a single membrane in contrast with the autophagy's double membrane autophagosome[14].

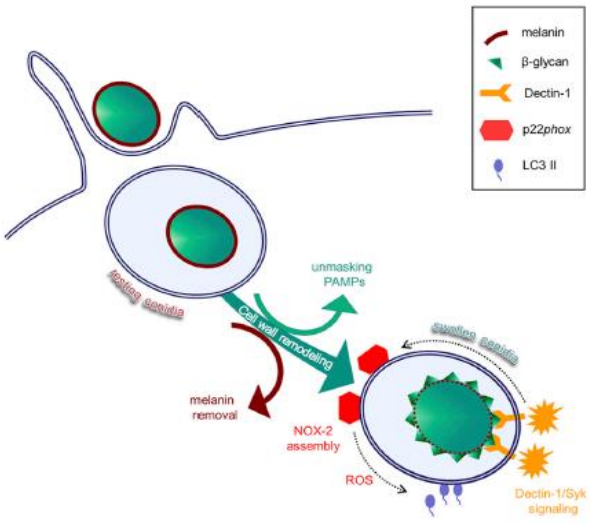

Figure 2. The LAP pathway (15).

Our group has shown that LAP constitutes one of the most important pathways which are involved in killing of *Aspergillus fumigatus*, while it seems to be a pathway regulated by Rubicon and dependent on the production of reactive oxygen species (ROS) (Figure 2)[15, 16, 22]. More specifically, the exposure of a variety of polysaccharides (Pathogen-associated Molecular Patterns, PAMPs) on the fungus cell wall surface, such as β-glucan molecules during the germination stage, makes the fungus visible to the host's immune system activating, at this way, certain receptors that recognize specific patterns-polysaccharides (Pattern

Recognition Receptors, PRRs) and are located on the surface of immune system cells. In turn, this recognition between PAMPs (pathogen) and PRRs (host) leads to the activation and production of various cytokines with the aim to kill successfully the invading fungus and avoid fungal germination and organism's conquest. In parallel, the above process induces a signaling cascade of Dectin-1/Syk kinase/NADPH oxidase[16]. As a result, reactive oxygen species (ROS) are produced activating the special pathway of LAP and the maturation of the phagosome. Therefore, the presence of NADPH oxidase plays an important role in the regulation of this pathway, the absence of which seems to lead to a predisposition to pulmonary aspergillosis. Furthermore, LAP proceeds independently of the pre-initiation complex, comprised of ULK1/2, FIP200, and ATG13, but it requires certain autophagic components, such as the Class III PI3K complex[36, 37], and elements of the ubiquitinylation-like, protein conjugation system (ATG5, ATG7, ATG16L1, ATG12)[17]. The Class III PI3K-associated protein, Rubicon, facilitates VPS34 activity and sustained PI(3)P presence on the LAPosome and stabilizes the NOX2 complex for ROS production[14], both of which are critical for the progression of LAP and the successful clearance of *Aspergillus fumigatus*.

However, exposure of PAMPs on the surface of the fungal cell wall is prevented by the presence of both a hydrophobic protein layer (Rodlet layer) and a melanin layer making *A. fumigatus* dormant conidia invisible to immune system cells. More specifically, melanin-competent conidia of *Aspergillus* appear, in contrast with the melanin-deficient conidia (albino strain, Δ*pksP*), higher ability to sequester Ca^{2+} increasing the accumulation of Ca^{2+} into the phagosomes. At this way, melanized conidia achieve to inhibit a specialized Ca^{2+} - calmodulin signaling pathway regulating LAP[22]. Thus, *A. fumigatus* follows a virulence strategy which inhibits Ca^{2+} signaling responses during phagocytosis highlighting the important role of *A. fumigatus* melanin in the development of fungal diseases[22].

I.3. *Aspergillus fumigatus* virulence strategies

In its natural environment, *A. fumigatus* represents a saprophytic organism which has to withstand environmental changes in order to survive any extreme condition. Similarly, during the human infection process, *A. fumigatus*, has to survive an aggressive environment, to fight stress-related changes in temperature, pH, water balance, oxidative damage, to find access to nutrients that are often not easily available and to overcome the host's antifungal mechanisms such as the LAP pathway that was previously referred. Important role in achieving its survival, both in natural and human environment, constitutes the *A. fumigatus* cell wall structure, which includes a variety of different important molecules.

a. *Aspergillus fumigatus* cell wall

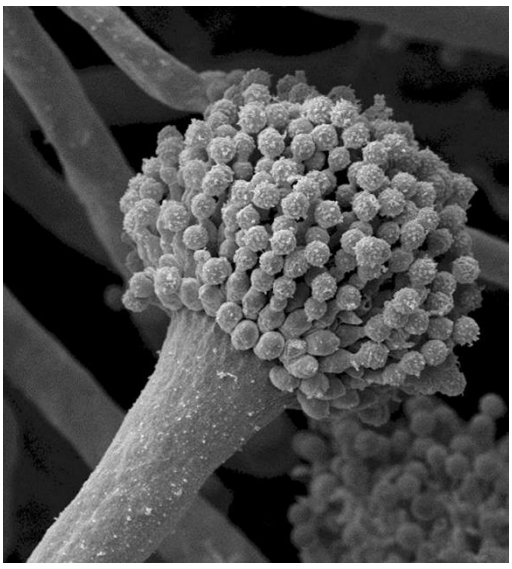

Figure 3. A conidiophore of the fungus *Aspergillus fumigatus*. (Photo: Jeannette Schmaler-Ripcke)

A. fumigatus is characterized by a dormant form, the conidium, in which germination occurs only under conditions rich in nutrients, such as water, carbon and nitrogen. More specifically, the inner cell wall of the conidia is composed of an alkali-insoluble fibrillar skeleton, which is made of a branched β-1, 3-glucan to which chitin, galactomannan and β-1, 3-glucan/β-1,4-glucan are covalently bound[19]. The outer layer of the conidia consists of the Rodlet layer (*RODA*) and a melanin layer. The Rodlet layer imparts the hydrophobic properties of the conidia and therefore its ability to spread in the air, survive environmental stress and avoid immune cell recognition[20], while the DHN (dihydroxynaphthalene) melanin molecules impart its structure and stiffness. The presence of these layers leads to the formation of a dense layer around the conidium concealing the fungus PAMPs and preventing the activation of the LAP pathway. Between these two layers, α-glucan molecules, as well as other polysaccharides, such as β-glucan, are included.

Following the process of the conidial germination in presence of carbon and nitrogen sources, water enters inside the fungus leading to the rupture of its cell wall. At this stage, termed swelling, the swollen conidia are double in size, lose the Rodlet layer and the melanin layer, which seems to shift inside the fungus in a dotted form without completely losing it. Additionally, α- (1, 3) -glucan appears on the conidia surface in order swollen conidia to form agglomerates, while β- (1, 3) -glucan molecules move from the inside to the surface of the conidia activating LAP pathway. As a result of the continuously cell wall's rearrangements during *A. fumigatus* life cycle progression, hydrophobic conidia convert to hydrophilic ones[17, 20]. When the fungus hyphal growth proceeds, a polysaccharide called galactosaminogalactan (GAG) is exposed on the surface of its cell wall, but its role in the fungus-host interactions remains unknown.

b. Melanin's intrinsic role in the fungus protection against host defense

In infectious diseases, melanins serve as virulence factors and protective molecules to microbial pathogens upon interaction with the host's immune system. More specifically, fungal melanins are negatively charged, hydrophobic pigments of high molecular weight produced by oxidative

polymerization of phenolic or indolic compounds, including catechol, 1, 8-dihydroxynaphthalene (DHN) or 3, 4-dihydroxyphenylalanine (DOPA) [21].

From the host perspective, although the mechanisms and the signaling pathways of *Aspergillus* clearance are not completely characterized, another new C-type lectin receptor, apart from Dectin-1 or Dectin-2, the MelLec receptor, which is expressed in mouse endothelial and human myeloid cells[23], seems to specifically recognize *A. fumigatus* DHN melanin and regulate inflammatory responses in human macrophages.

As far as *Aspergillus fumigatus* is concerned, dihydroxynaphthalene melanin (DHN-melanin) is an integral component of the conidial cell wall surface, which accounts for their brownish-gray color and has a central role in their pathogenicity. During *A. fumigatus* growth, melanin is removed from the surface of the fungus, but its fate remains enigmatic. Notably, Kyrmizi et al. showed that activation of physiological immune responses, which are inhibited by fungus DHN-melanin, occurs within 2–4 h following phagocytosis of *A. fumigatus* conidia by monocytes/macrophages[22], while MelLec is not able to recognize it on the fungus surface after 4h of conidial swelling[23]. Therefore, melanin is possibly present, but its removal specifically from the conidial surface appear to lead in its functional inactivation.

Additionally, Ferling I. et al. show that after 2h of infection, ROS production was increased in phagosomes containing wild type – melanized conidia of *A. fumigatus* and the pH levels displayed high values averaging 7.2 in contrast with the phagosomes containing albino conidia (pH~6.2). In these conditions, DHN-melanin was degraded *in vitro* suggesting the presence of DHN-melanin derived degradation products in phagosomes containing the melanized conidia. On the other hand, this study, also, supports that phagolysosomes containing DHN-melanin positive conidia, apart from appearing delayed phagosome maturation and intracellular transit, were leaky[24] proving a communication between phagosome lumen and the cytosol. Thus, the degradation of melanin and the leakage which is observed only for phagolysosomes containing the melanized wild type conidia lead to the hypothesis that DHN-melanin or its intracellular degradation products following phagocytosis may interfere in cell metabolism and fungal killing.

Although the molecular interactions of DHN-melanin with the immune system is incompletely understood, the biosynthetic pathway for *A. fumigatus* DHN-melanin production has been well characterized (Figure 4). More precisely, the polyketide-derived DHN-melanin is synthesized by six enzymes encoded by a gene cluster located on the second chromosome[25]. The polyketide synthase PKS or ALB1 (for “albino 1”) is responsible for the first biosynthetic step in *A. fumigatus*, resulting

in the biosynthesis of the heptaketide naphthopyrone YWA1 from the substrates acetyl-CoA and malonyl-CoA. A series of subsequent enzymatic reactions carried out by Ayg1, Arp2, Arp1, and Arp2 through hydrolysis of YWA1 to produce 1,3,6,8-tetrahydroxynapthalene (T4HN) by Ayg1, followed by serial reduction and dehydration reactions to produce scytalone, 1,3,8-trihydroxynapthalene (THN), and vermelone. Vermelone is next dehydrated to form 1, 8-DHN (DHN), which is then oxidized and polymerized by the copper oxidase Abr1 and the laccase Abr2 to form mature melanin[26]. Alb1/Ayg1/Arp1/Arp2 enzymes which function prior to the vermelone production are called early enzymes in DHN-melanin biosynthesis, while Abr1/Abr2 enzymes are the late enzymes and they are located to the cell wall surface via trafficking through the classical secretory pathway[27].

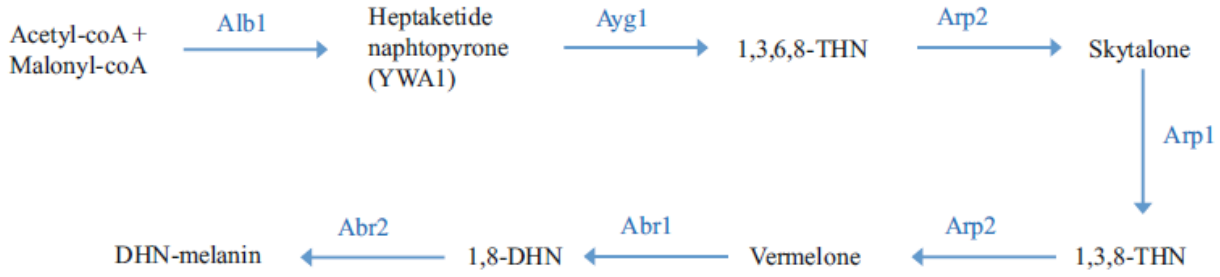

Figure 4. The biosynthetic pathway of the DHN-melanin of the *Aspergillus fumigatus* (38).

Collectively, we understand that *A. fumigatus* DHN–melanin plays an important and crucial role in fungal interaction with the host innate immune system[20] determining phagosome biogenesis and, consequently, *Aspergillus* survival. On the other hand, the innate immune system is equipped with a receptor repertoire, such as the MelLec receptor, in order to respond to the expression of virulence factors from fungi[19]. Thus, the MelLec receptor, which recognizes DHN–melanin occasionally in the cytosol and activates antifungal defenses, together with the active removal of melanin associated - germination in live fungi within the phagosome leads to the hypothesis that melanin could be considered as a PAMP[28]. Hence, dissecting the dynamics of DHN-melanin organization on the fungal cell wall and its molecular interplay with the immune system will lead to a better understanding of the melanin's role in *A. fumigatus* pathophysiology.

II. AIMS

Although LAP is activated by an increasing number of human pathogens, patients with genetic defects in LAP (e.g., CGD patients) display a unique susceptibility for invasive aspergillosis. Therefore, the dependence of anti-*Aspergillus* host defense on LAP suggests a distinct mechanism of LAP activation by this fungus, and the presence of compensatory mechanisms that counteract melanin-induced LAP blockade. Of interest, the mechanism of melanin removal from *Aspergillus* fungal cell wall occurs within hours following phagocytosis in a process that remains poorly understood.

Studying the process of LAP activation and melanin removal during live infection with *A. fumigatus* is important to understand the unique relationship of the LAP pathway with this fungal pathogen. Herein, we performed a time course of LAP activation during infection with wild type *Aspergillus fumigatus* and studied the phagosome maturation during the live infection. Furthermore, we investigated LAP activation during infection with live wild type and albino mutants and killed forms of *A. fumigatus* conidia. In order to dissect the dynamics of DHN-melanin organization on the fungal cell wall and its molecular interplay with the immune system, we performed experiments using all 7 mutants in melanin biosynthesis pathway. Finally, we established methods of cryopreservation to study this pathway in humans.

The experiments which has been conducted in this master thesis were performed at the Clinical Microbiology and Microbial Pathogenesis Laboratory of the Medical School in the University of Crete headed by the Professor George Chamilos. Following the results obtained.

III. RESULTS

III.1. Live *Aspergillus fumigatus* induces delayed and prolonged activation of LAP during infection of macrophages

Initially, we were interested in exploring LAP pathway activation over time in bone marrow-derived macrophages (BMDMs) infected with the wild type strain of *A. fumigatus*. For this purpose, we analyzed the kinetics of LC3 recruitment to the phagosome as related to the fungal killing, up to 24h post infection. In parallel, we correlated activation of LAP with phagosome maturation using established protein markers of phagosome acidification (V-ATPase) and phagolysosome fusion (Cathepsin D).

More specifically, for the assessment of LAPosome formation, we used BMDMs from GFP-LC3$^{+/+}$ transgenic mice and we infected them with the transgenic dsRed wild type *Aspergillus fumigatus* strain that allows dynamic tracking of fungal growth *ex vivo* and *in vivo*.

Firstly, we stained the infected BMDMs for the GFP which tags the LC3 protein and representative images were obtained using confocal microscopy. Of interest, we observed that LC3 accumulation in BMDMs infected with the dsRed-labeled WT *A. fumigatus* increases steadily following the first 2-4 hours of infection up to 3-fold within 6h. In contrast to previous reports of transient activation of LAP within few minutes of macrophage stimulation with bioparticles coated with microbial PAMPs (e.g., zymosan)[42], we noticed a continue increase in LC3 accumulation to the phagosome up to 12 hours of infection of macrophages with live *A. fumigatus* (Figure 5).

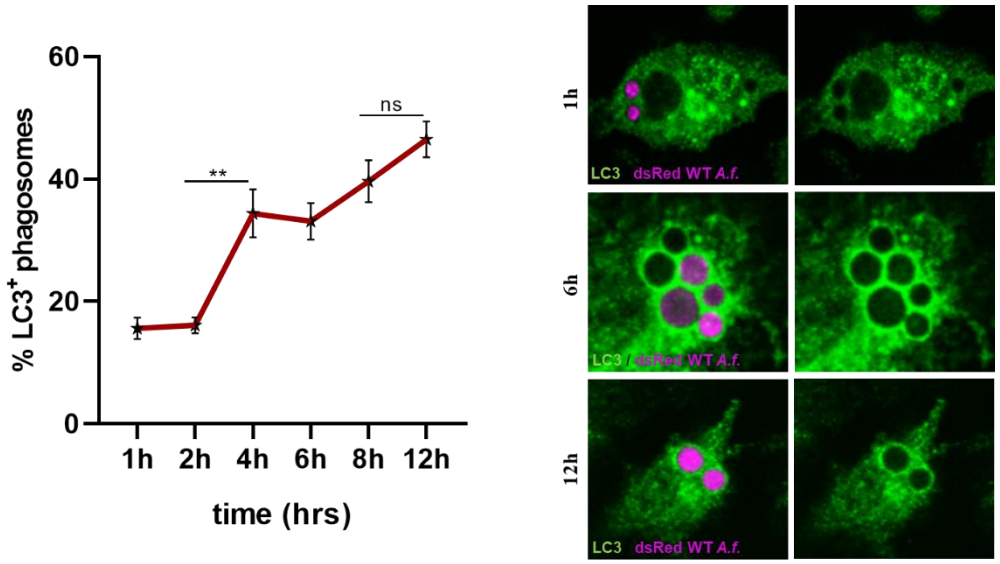

Figure 5. LC3^{+} phagosomes formation in BMDMs infected with dsRed-labeled WT *A.fumigatus* for 1, 2, 4, 6, 8 and 12 h.

We next assessed the kinetics of recruitment of other markers of phagosome biogenesis that are involved in LAPosome function. Specifically, we evaluated the localization of V-ATPase (Figure 6), which acidifies endolysosomal compartments enabling the activity of lysosomal hydrolases[33], and Cathepsin D (Figure 7), which is a lysosomal marker. We observed increased accumulation of both markers at the late time point of 6 hours after infection, a pattern that correlated with the delayed and prolonged LAPosome formation. Furthermore, we analyzed the kinetics of fungal killing by macrophages. We noticed that the prolonged and persistent activation of LAP (including LC3, V-ATPase and Cathepsin D) correlated with a delayed killing of *A. fumigatus* by the macrophages starting at 6-12h of infection (Figure 8).

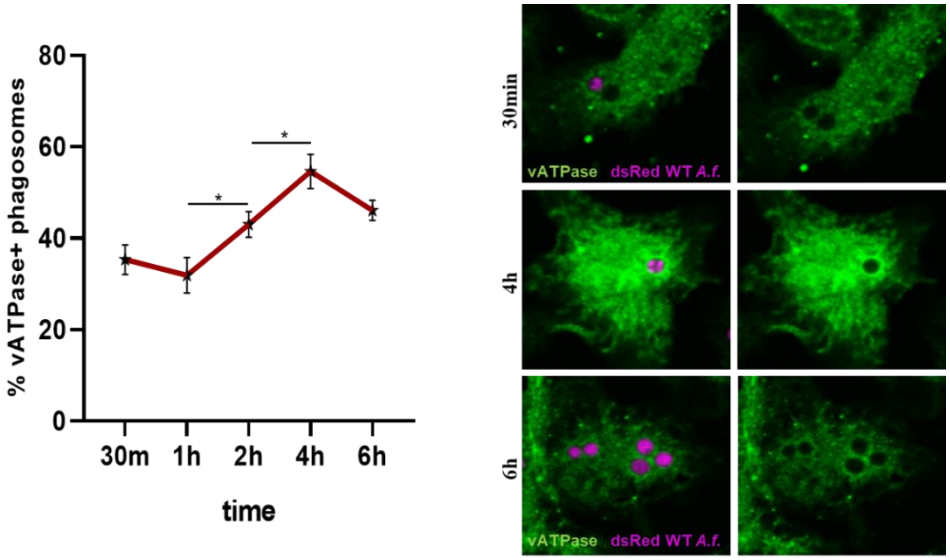

Figure 6. V-ATPase$^+$ phagosomes formation in BMDMs infected with dsRed-labeled WT *A.fumigatus* for 30minutes, 1, 2, 4 and 6 hours. .

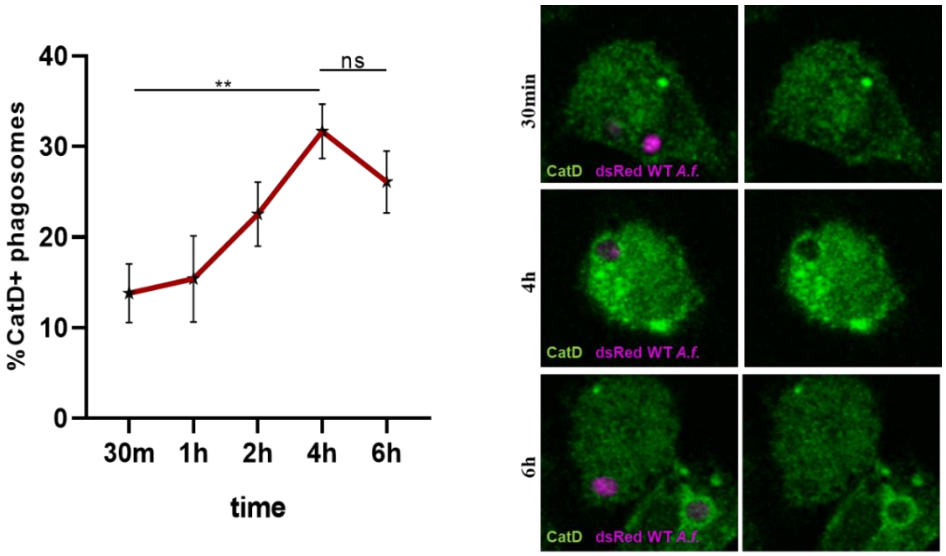

Figure 7. CatD$^+$ phagosomes formation in BMDMs infected with dsRed-labeled WT *A.fumigatus* for 30 minutes, 1, 2, 4 and 6 hours.

Another type of LAPosome formation regulates efferocytosis, a mechanism of apoptotic cell clearance by macrophages. Accordingly, mice deficient for LAP, but not canonical autophagy, accumulate apoptotic bodies in their tissues and within the cytosol of phagocytic cells[34], while deficiencies or mutations in molecules, such as ATG5 or NOX2, which constitute major components of LAP signaling

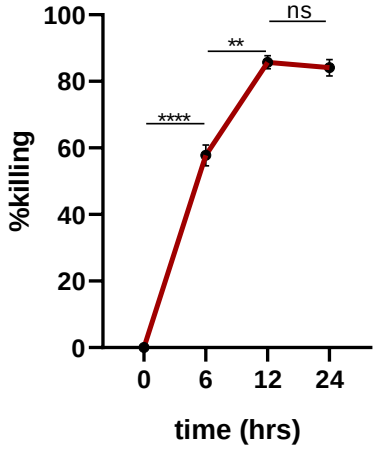

Figure 8. The percentage of conidial killing by BMDMs from B6 control mice infected with WT live dormant conidia of *A. fumigatus.*

cascade seem to lead in autoimmune disorders in which efficient engulfment and elimination of cellular debris is of particular importance. Thus, efferocytosis, can be used as a control for the phagosome maturation during live infection. For this reason, we tried to follow the LAP pathway activation evaluating over time the accumulation of the LC3 and vATPase proteins (Figure 9), infecting this time GFP-LC3$^{+/+}$ BMDMs with beads, which represent the apoptotic cells. Most specifically, the beads that we used are coated on their surface with phosphatidylcholine (PC) and phosphatidylserine (PS), which constitute the "find-me" and "eat-me" signals respectively and are released from apoptotic bodies inducing the recruitment of phagocytes and the binding of the apoptotic corpses. After the recognition of these signals, the phagocyte engulfs the apoptotic cell into an intracellular membrane-bound compartment termed the efferosome, where it is degraded.

So, we infected BMDMs with the phosphatidylcholine- and phosphatidylserine- covered beads (hereafter called efferosomes) in order to mimic efferocytosis and we performed immunofluorescence staining and confocal microscopy. We found that LC3 and vATPase recruitment in efferosomes is activated within 30 minutes to 1h (Figure 9) in contrast to infection of macrophages with live *A. fumigatus* suggesting a unique mechanism of activation of LAP by live conidia of *Aspergillus fumigatus*, which results in delayed, yet persistent LAPosome formation many hours following infection.

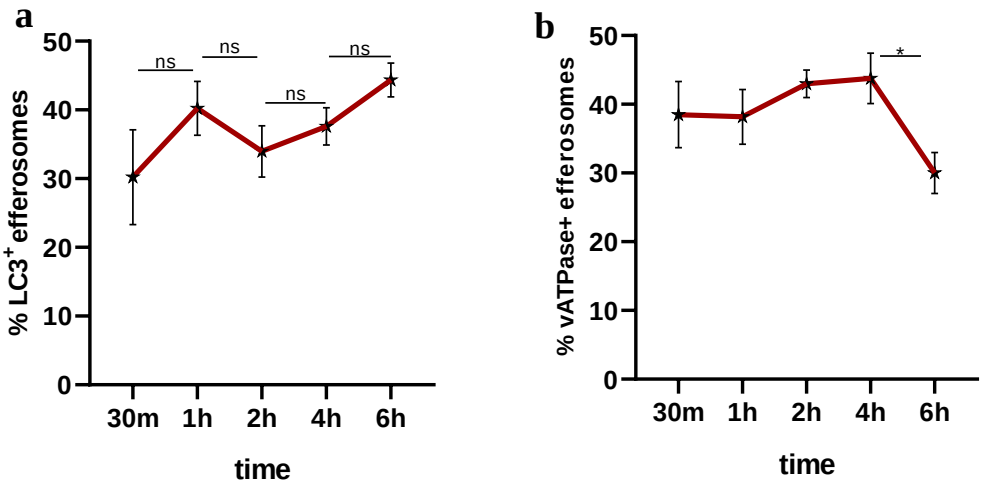

Figure 9. Percentage of **a.** LC3^{+} or **b.** vATPase^{+} efferosomes formation in BMDMs infected with phosphatidylcholine- and phosphatidylserine- covered beads for 30 minutes, 1, 2, 4 and 6 hours.

III.2. Live *Aspergillus fumigatus* conidia induce a unique mechanism of LAP pathway activation

To identify the mechanism that accounts for the unique pattern of activation of LAP following phagocytosis of live conidia of *Aspergillus*, we generated different fungal morphotypes (dormant conidia, swollen conidia), inactivated them with PFA and compared the kinetics of accumulation of LC3 in the phagosomes of BMDMs with those induced following infection with live conidia (Figure 10). In agreement with previous work, we found that dead dormant conidia do not trigger activation of LAP during infection (Figure 10a). Of interest, swollen conidia triggered early and robust LAP activation (Figure 10b). However, as opposite to the prolonged persistent LAPosome formation following infection of BMDMs with live WT conidia, we found a sharp reduction in LC3 recruitment to the phagosome of PFA-inactivated swollen conidia, within 2h of infection. Collectively, these data confirm that live conidia of *Aspergillus* induced a unique mechanism of LAPosome formation.

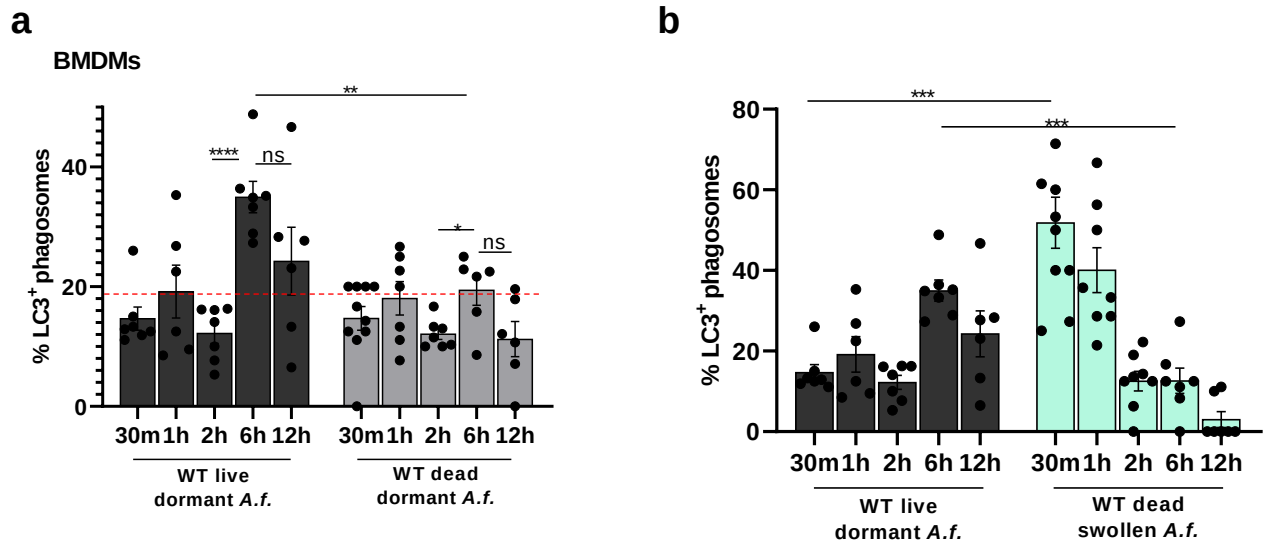

Figure 10. LC3⁺ phagosomes formation in BMDMs infected with **a.** WT live or dead dormant *A.fumigatus* or **b.** WT live dormant or WT dead swollen *A.fumigatus* conidia for 30minutes, 1h, 2h, 6h 12 h.

After this observation, we were interested in repeating the same experiment, but this time infecting primary alveolar macrophages (AMs), isolated from the lungs of healthy GFP-LC3$^{+/+}$ mice, as they constitute the early line of defense against inhaled conidia that have reached the alveoli. However, because the number of acquired cells was low after the BAL assay, we performed the experiment infecting AMs with the live wild type dormant or the dead wild type swollen conidia of *A. fumigatus* for 1 or 6 hours (Figure 11). Interestingly, the results obtained confirm the unique activation of LAP pathway in alveolar macrophages infected with the live WT conidia of *A. fumigatus*.

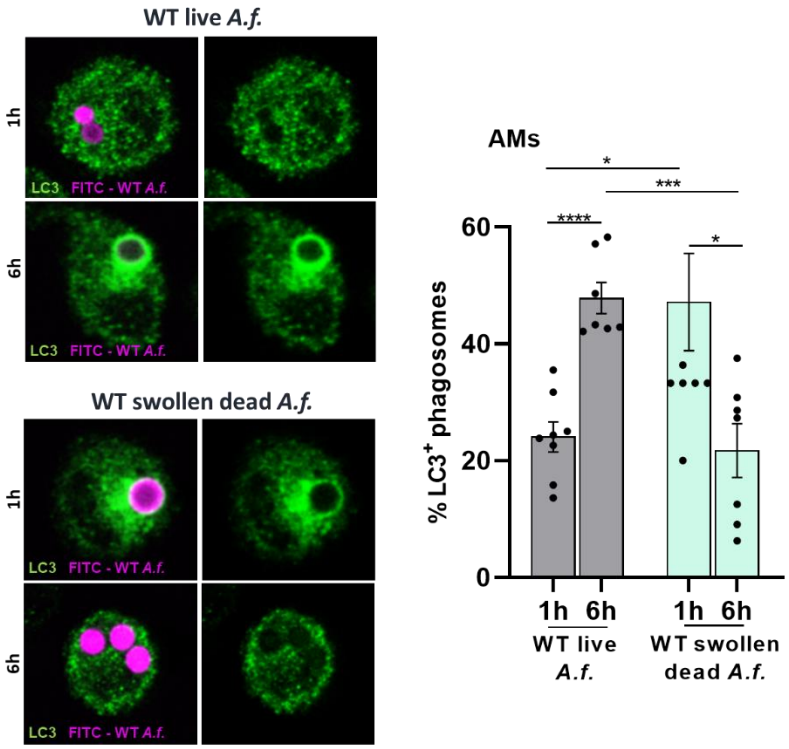

Figure 11. LC3^{+} phagosomes formation in AMs infected with WT live or swollen dead conidia of *A.fumigatus* for 1h and 6h.

III.3. The process of intracellular removal of melanin triggers persistent activation of LAP

Knowing that fungal melanin has a crucial role in fungal interaction with the host innate immune system, but without actually understanding the pathway that melanin follows during fungal swelling and germination, we proceeded to the dissection of the role and the dynamics of DHN-melanin on fungal cell wall in order to understand *A. fumigatus* pathophysiology.

For this purpose, we infected GFP-LC3$^{+/+}$ BMDMs with WT dormant live conidia and Δ*pksP* (albino) live conidia of *A. fumigatus*, whose main difference is the presence or absence of the melanin layer on their surface, respectively (Figure 12). Our results showed

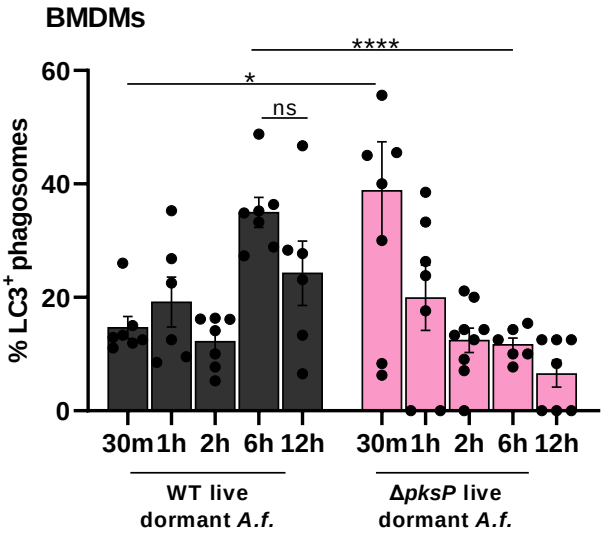

Figure 12. LC3^{+} phagosomes formation in BMDMs infected with **a.** WT live or Δ*pksP* dormant conidia of *A.fumigatus* or **b.** WT dead dormant or swollen conidia of *A. fumigatus* for 30m, 1h, 2h, 6h 12 h.

that only the melanized WT strain can cause late induction of the LAP pathway (up to 12h) in contrast with the albino strain which leads to an early induction of LC3 in the first 30 minutes after infection concluding that long-term LAP induction requires the presence of melanin.

Hypothesizing that melanin is being degraded, we were, next, interested in investigating if the production of reactive oxygen species (ROS) could be associated with the possible presence of melanin fragments in the cytosol and the induction of the unique pathway of LAP activation. For this purpose, we used B6 GFP-LC3$^{+/-}$ BMDMs or Cybb$^{-/-}$ (CGD) GFP-LC3$^{+/-}$ BMDMs as a control, because of their inability to efficiently compose the NADPH complex and produce reactive oxygen species (ROS), and we infected them with WT dormant live conidia or Δ*pksP* (albino) live conidia of *A. fumigatus* for 30minutes, 1h, 4h and 6h. In order to monitor the ROS accumulation on the phagosomes, we performed the nitro blue tetrazolium chloride (NTB) – formazan assay. Representative images from confocal microscopy show an early accumulation of ROS in the first 30 minutes of infection with the albino strain of *A. fumigatus*, which seems to be reduced over time (Figure 13). However, although we did not expect NBT accumulation in infected CGD BMDMs, we observed a small amount of ROS recruitment in their phagosomes. Therefore, this assay requires further optimization.

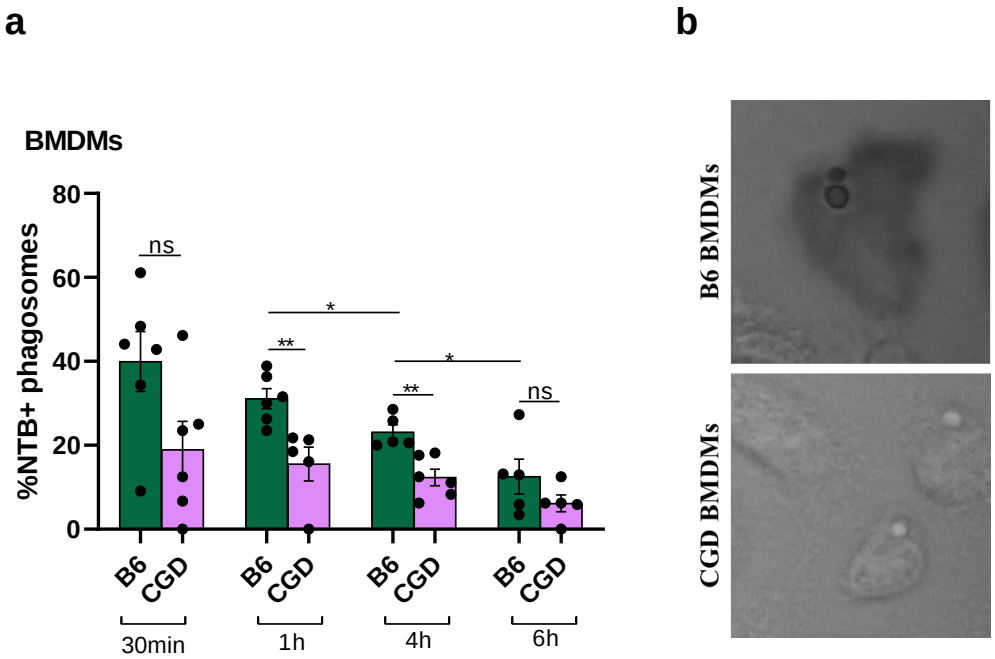

Figure 13. a. Percentage of NTB^{+} phagosomes formation in B6 or Cybb-/- (CGD) BMDMs infected with the live Δ*pksP* (albino) strain of *A.fumigatus* 30minutes, 1h, 4h and 6h. **b.** Confocal imaging of infected B6 or CGD BMDMs with the albino strain of *A. fumigatus* for 30 minutes.

III.4. Only the presence of the fully formed molecule of DHN-melanin on the *A. fumigatus* surface seems to trigger the delayed and prolonged activation of LAP in macrophages

In order to gain insight on the mechanism that melanin may activate a different LAP response, we were interested in investigating if it is the DHN-melanin molecule specifically, which is responsible for this observation. For this reason, we continued our experiments using melanin mutants, which follow the melanin's biosynthetic pathway and, more specifically, the *alb1*, *ayg1*, *arp2*, *arp1*, *arb1*, *arb2*, *B5233*, *alb*:*ALB1* mutants (Figure 14). At this way, we tried to comprehend and to get closer to the virulent mechanisms used by the fungus to invade the host's immune system. Thus, we infected GFP-LC3$^{+/+}$ BMDMs or human CD14^{+} monocytes with each of these mutants for 30min, 1h and 6h, but unfortunately the experiment did not work, technically, in terms of LAP induction of the positive control, *alb1*, in 30min of infection (data not shown here), and needs to be repeated.

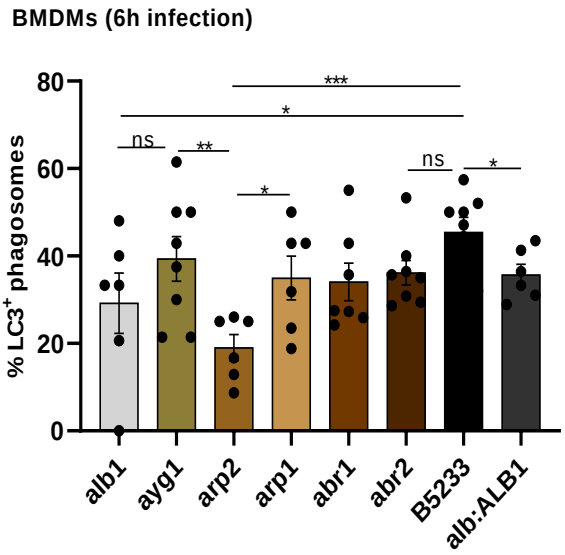

Figure 14. LC3^{+} phagosomes formation in BMDMs infected with the melanin mutants on the surface of *A.fumigatus* for 6h.

III.5. Effectiveness of LC3-associated phagocytosis (LAP) pathway in cryopreserved human primary monocytes

Cryopreservation is a process which is used in different medical areas like fertility, stem cells research, cord and peripheral blood banks and its aim is to preserve by freezing, to very low temperatures from -80°C to -200°C using liquid nitrogen organelles, cells, tissues, or any other biological constructs which are susceptible to damage. Interestingly, we proceeded in the storage of peripheral blood mononuclear cells (PBMCs) via cryopreservation, a common laboratory procedure for preserving these cells for phenotypic and functional analysis for a wide range of infectious diseases and clinical vaccine investigations[29].

At these low temperatures, any enzymatic or chemical activity which might cause damage to the biological material is effectively stopped. However, the most important factor in cryopreservation is to conserve the frozen samples intact without causing additional damage due to the formation of ice crystals during freezing. For this reason, a class of molecules termed cryoprotectants (glycerol,

DMSO) are used, whose role is to coat and protect the biological material. Additionally, another important factor which contributes in increasing the cell survival is the concentration of the FBS (Fetal Bovine Serum) that has been added to the freezing medium functioning as a natural protective component.

As far as cryoprotective agent is concerned, in our assay we used the dimethyl sulfoxide (DMSO) as it appears desirable properties such as hydrophilicity, suitable viscosity and stability and its role is to reduce the freezing point of the medium and to allow a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death[30, 43]. So, as the cell suspension is cooled below the freezing point, ice crystals are formed and the concentration of the solutes in the suspension increases. Intracellular ice can be minimized if water within the cell is allowed to escape by osmosis during the cooling process. A slow cooling rate, generally -1^{o}C per minute, facilitates this process[44]. However, as the cells lose water, they shrink in size and will quickly lose viability if they go beyond a minimum volume. Nevertheless, using the cryoprotectant DMSO these effects are minimized. Unfortunately, besides its protective role, DMSO is a toxic substance which could adversely affect the PBMCs viability while its concentration (or its time of exposure to the cells) is increased[31, 32]. Hence, optimizing cryopreservation assay we can obtain important information for the finding of promising drug targets and the development of new pharmaceutical therapies.

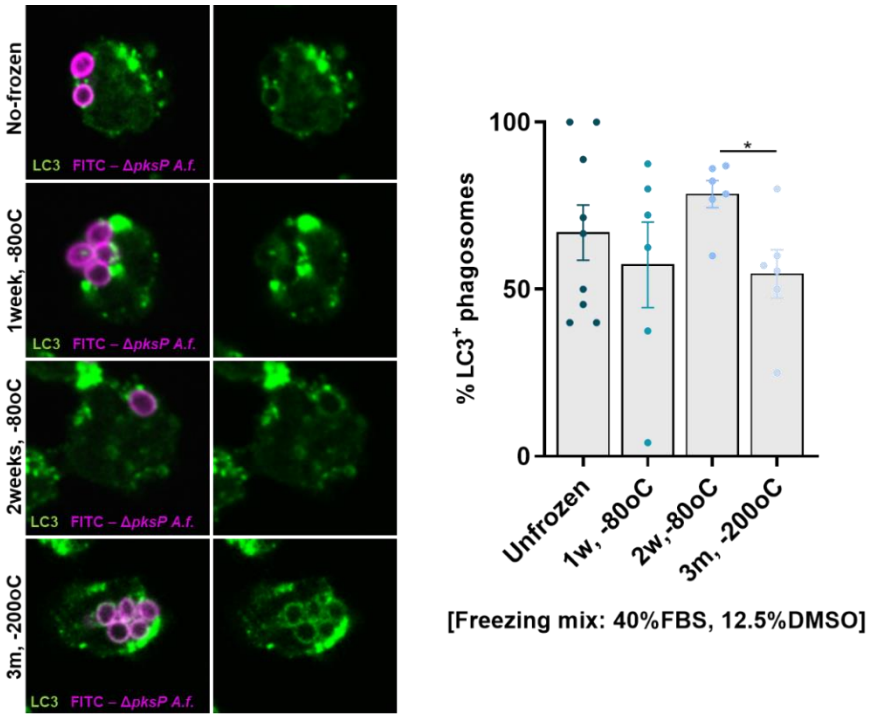

Figure 15. The effect of cryopreservation on the LC3^{+} phagosome formation in healthy human CD14^{+} monocytes frozen for 1week, 2 weeks or 3 months and infected with the FITC-labeled Δ*pksP* strain of *A. fumigatus*.

For this purpose, we tried to establish the cryopreservation method according to the pathway of interest, the LAP pathway, assessing the ability of cryopreserved human cells to induce LAP after their infection with *A. fumigatus*. Initially, we selected to freeze human healthy peripheral blood mononuclear cells (PBMCs) in a medium containing 40% FBS and 12.5% DMSO in RPMI complete. After one or two weeks in -80ºC or three months in liquid nitrogen (-200ºC) following one week in -80ºC, PBMCs were thawed and $CD14^+$ monocytes were isolated and infected for 15 minutes with the Δ*pksP* strain of *A. fumigatus*. Fixation and staining (see antibodies optimization at the Supplementary section) of the cells followed and the acquired results from the confocal microscopy showed that even after 3 months of freezing cells were able to recruit LC3 on the phagosomes (Figure 15), an observation that give us great hope to achieve studying LAP defects in various diseases.

However, we were interested in optimizing the cryopreservation assay in order to achieve better and more accurate results and cells' conservation aiming to the cryopreservation of patients PBMCs, too. For this purpose, we next used a freezing medium which contained this time 90% FBS and 10% DMSO, and after isolation of the healthy human $CD14^+$ monocytes from the thawed PBMCs and infection with the Δ*pksP* of *A. fumigatus*, we obtained better cells conservation and higher levels of $LC3^+$ phagosomes in both cases of 1week or three months after cryopreservation (Figure 16).

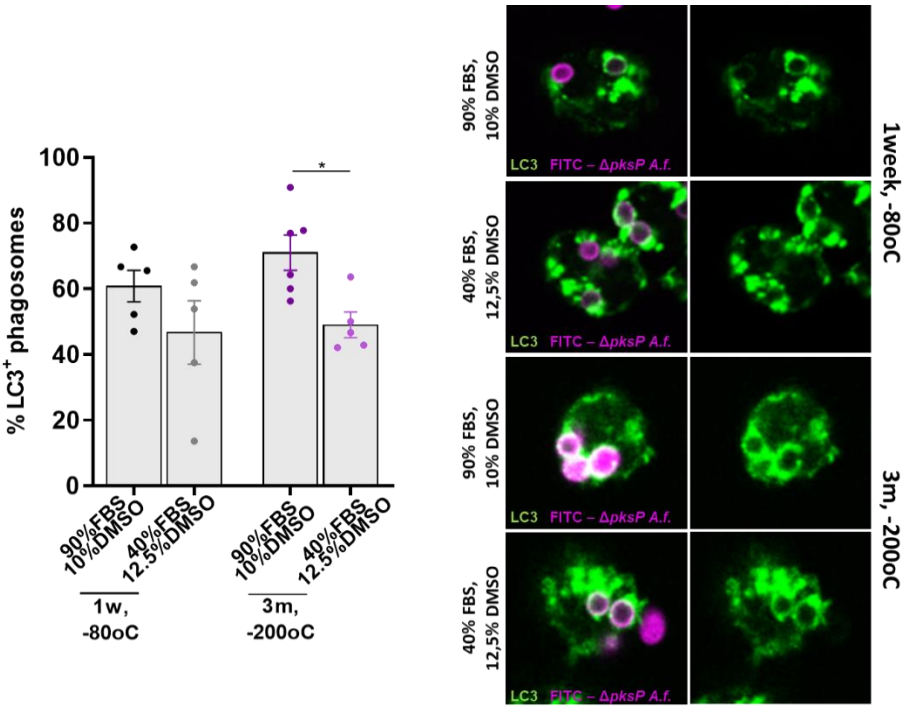

Figure 16. Comparison between the effectiveness of two different freezing mixes in the formation of $LC3^+$ phagosomes in healthy human $CD14^+$ monocytes frozen for 1week or 3 months and infected with the FITC-labeled Δ*pksP* strain of *A. fumigatus*.

Thus, our conclusions demonstrate that a 90% of FBS (in 10% DMSO) in freezing medium increases the viability of the frozen PBMCs up to 10% in contrast to using 40% of FBS (in 12.5%DMSO). As far as LAP activation is concerned, after stimulation of the healthy human $CD14^+$ monocytes with the Δ*pksP* strain of *A. fumigatus*, no important differences between the control (non-frozen cells) and the frozen cells were observed suggesting that although the frozen and thawed cells are more sensitive due to the stress[35] caused by stimulation or processing during cryopreservation, they could be used as control samples in studying LAP in many different diseases in which this pathway could possibly be defective.

IV. DISCUSSION

In the last two decades, it seems that *A. fumigatus* can cause serious diseases, such as chronic lung infections, especially in immunodeficient people. For this reason, in this master thesis, we selected to understand better the immunopathogenicity of *Aspergillus fumigatus* infections.

One of the main features of *A. fumigatus* is the presence of the melanin layer, which protects the fungus from environmental stressors including its intracellular killing by host's phagocytes. More specifically, the presence, on its cell wall surface, of the melanin and Rodlet layers results in the concealment of the immunostimulatory polysaccharides (PAMPs) making the fungus invisible by the immune system avoiding its killing. The major pathway which is responsible for the *A. fumigatus* clearance of the invaded organism is the LAP pathway, which, according to Akoumianaki et al., is blocked by the *Aspergillus* cell wall melanin in order to promote pathogenicity. However, taking into consideration the degradation of melanin inside the melanized live conidia-containing phagosomes possibly by the presence of reactive oxygen species and the phagolysosomal leakage which happens in these phagosomes, we could hypothesize that the host may counteract this inhibitory activity of melanin by the cytosolic sensing of melanin fragments may leading to metabolic rewiring and triggering of a unique pathway of activation of LAP which is, possibly, NOX-2-dependent.

Herein, we tried to understand the importance of melanin degradation and removal for the activation of a distinct pathway which leads finally to the *A. fumigatus* killing and to dissect the melanin's pathway after fungal swelling. Thus, using different strains and morphotypes of *A. fumigatus* live or killed and with quality differences in the cell wall surface composition with absence or presence of melanin, we showed the need of melanin layer to the long-term recruitment of markers of LAP activation (LC3, V-ATPase and Cathepsin D) in infected BMDMs and AMs with the live WT dormant strain of *A. fumigatus.* Furthermore, in the present work we tried to extend on our laboratory previous findings by showing that the fully formed molecule of DHN-melanin, and not the intermediate mutations of *A. fumigatus* which follow the melanin's biosynthetic pathway, are responsible for the activation of this distinct pathway which is, possibly, NADPH-oxidase-dependent and may lead to macrophage metabolic rewiring and killing of the invading *A. fumigatus* pathogen using, finally, its virulent molecule, melanin, against it.

Additionally, we tried to optimize the cryopreservation procedure of healthy human peripheral blood mononuclear cells (PBMCs) in order to test the ability of the isolated frozen-thawed $CD14^+$ monocytes to encounter *A. fumigatus* and to promote fungus killing activating LAP pathway and phagosome formation. Our results show that we achieved a ~90% viability of the thawed PBMCs and the isolated

$CD14^+$ monocytes using as a freezing medium 10% of the cryoprotectant DMSO in FBS (90%) suggesting that the success of the cryopreservation technique depends on a variety of parameters such as the PBMCs viability and functionality before freezing, freezing and thawing time, the composition of the freezing medium, the temperature of the thawing medium, and centrifugation temperature, duration and force and acquiring a promising tool for the investigation of defects of LAP or other pathways of interest, in disease.

Future perspectives

To better understand the role of melanin in host defense against *Aspergillus fumigatus*, further research is needed in order to identify the DHN-melanin fragments in the phagosome and the leakage which happens in melanized live conidia-containing phagosomes. After that, we would like to investigate if this response of degradation of melanin and the possible detection of its fragments into the cytosol is NOX-2 dependent. Thus, showing that ROS production stops in the absence of these melanin fragments, we could speculate that there is a unique signaling pathway following LC3 phagosome formation, sensing melanin in the cytosol and linking the melanin leakage with metabolic changes (e.g., glycolysis), which possibly could result in remodeling of the phagosome membrane.

Additionally, it would be important to explore whether oxidation of melanin degradation products induces activation of phagosomal or cytosolic Pattern Recognition Receptors to activate LAP. This could be an important direction to understand the immune modulatory properties of melanin pigments. Hence, understanding the mechanisms of activation of LAP by oxidized forms of melanin could be exploited as therapeutic strategy in the management of fungal diseases.

V. MATERIALS AND METHODS

V.1. Microorganisms and culture conditions

a. Culture of *Aspergillus fumigatus*

Sabouraud plates. Dissolve 22.5g Sabouraud (with chloramphenicol, Biolab) in 500ml dH_2O. Sterilize by autoclaving at 121ºC for 15min. After sterilization and the medium gets at ~55ºC, 250μl Triton 10% (0.005% final concentration) are added for unit colonies formation.

PDA plates. Dissolve 19.5g PDA (Oxoid) in 500ml dH_2O. Sterilize by autoclaving at 121ºC for 15min.

b. Strains of *Aspergillus fumigatus*

All *A. fumigatus* strains used (WT strain ATCC46645, dsRed-labeled WT, Δ*pksP*, *A. fumigatus* B-5233 (WT parental strain), B-5233/RGD12-8 (Δ*pksP*), RGD12-8/PKS33-3 (Δ*pksP*-complemented), *arp1* (RGD4-2), *arp2* (RGD10-1), *abr1* (RGD24-5), *abr2* (RGD17-2) and *ayg2* (RGD-1551)) were grown on PDA plates for 6 days at 37°C. Fungal conidia (spores) were harvested by gentle scraping in the presence of sterile PBS, washed three times with PBS, filtered through a 40-μm-pore cell strainer (Falcon) to separate conidia from contaminating mycelium, counted by a hemocytometer, and suspended at a concentration of 10^8 spores ml^{-1}. When indicated, the conidia were labelled with FITC.

FITC-labeling of *A. fumigatus* strain. Freshly harvested conidia were diluted in $NaHCO_3$ 0.1M pH 9.3 and were incubated with FITC (final concentration in DMSO, 2mg ml^{-1}) at room temperature for 2h or O/N at 4ºCand washed by centrifugation three times in PBS.

Swelling of *A. fumigatus* conidia. *Aspergillus fumigatus* dormant conidia were in the swollen state within 6 hours incubation in DMEM complete medium in 5% CO_2 and 37ºC.

***A. fumigatus* strains inactivation.** Inactivation of fungal conidia was performed by exposure to 3.7% FA (2h, room temperature) following by treatment with glycine (100 mM in PBS) and three washes in PBS and verified by c.f.u. plating. Importantly, it has been previously validated that FA inactivation of *A. fumigatus* conidia does not affect surface exposure of β-glucan, Dectin-1 signaling activation, cytokine responses and melanin inhibitory action on LAP.

V.2. Cell culture protocols

a. Generation of Bone Marrow-derived Macrophages (BMDMs)

C57BL/6 mice obtained from the IMBB Institute (Crete), and GFP-LC3 mice (obtained from RIKEN BioResource Center) were maintained in grouped cages in a high-efficiency particulate air-filtered environmentally controlled virus-free facility (24 °C, 12/12 h light/dark cycle), and fed a standard chow diet and water ad libitum. All experiments were approved by the local ethics committee of the University of Crete Medical School, Greece, in line with the corresponding National and European Union legislation.

BMDMs were generated by culturing bone marrow (BM) cells obtained from 8-week-old male or female mice in DMEM containing 20% FBS, supplemented with L929 cell-conditioned medium (30%). After 6 days, the resulting cultures consisted of macrophages (> 95% purity).

b. Murine alveolar macrophages (AMs) isolation

Alveolar macrophages (AMs) are located in the pulmonary alveoli and can be harvested by washing the lungs using the method of bronchoalveolar lavage (BAL). For alveolar macrophage isolation, mice were killed and lungs were lavaged through an intratracheal catheter with cold calcium and magnesium-free PBS supplemented with 1mM EDTA. The lavage fluids were pooled and centrifuged at 300 *g* for 10 min, and the cells were collected and measured for the culture assay.

c. Isolation and stimulation of primary human monocytes

Healthy volunteers without any known infectious or inflammatory disorders donated blood. Monocytes from healthy controls were isolated from PBMCs using magnetic bead separation with anti-CD14 coated beads (MACS Miltenyi) according to the protocol supplemented by the manufacturer. The monocytes were resuspended in RPMI culture medium supplemented with gentamicin 1%, L-glutamine 1% and pyruvate 1%. The cells were counted and their number was adjusted to $2x10^6$ ml^{-1}. A total of $2x10^5$ monocytes per condition in a final volume of 100μl were allowed to adhere to poly-L-lysine treated glass coverslips (Ψ 13 mm) for 1 h followed by stimulation with *A. fumigatus* conidia.

d. Peripheral Blood Mononuclear Cells (PBMCs) isolation

Healthy volunteers without any known infectious or inflammatory disorders donated blood. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient using Lymphocyte Separation Media (Lymphosep, Biowest). Approval for the collection of blood for functional studies on monocytes was obtained from the Ethics Committee of the University Hospital of Heraklion, Crete, Greece (5159/2014).

V.3. Cell stimulations and treatments

a. Cells infection with *A. fumigatus* strains

A total of $2x10^5$ cells (monocytes, macrophages) per condition in a final volume of 100μl were allowed to adhere to poly-L-lysine treated glass coverslips (Ψ 12 mm) for 1 h followed by stimulation with *A. fumigatus* conidia at a multiplicity of infection (MOI) of 3:1 at 37 °C for the indicated time point. After stimulation, cells were washed once with PBS to remove medium and non-phagocytosed spores and cells were fixed on the coverslips for 15 min in 4% formaldehyde without MetOH. Subsequently the coverslips were washed twice with PBS and stored in PBS at 4 °C until immunofluorescence staining.

b. Killing assay of *A. fumigatus* by the cells

Cells (1 X 10^5 per well) were plated onto 96-well round bottom plates for 1 h, and subsequently infected with the indicated *A. fumigatus* strains at a MOI of 1:10 (conidia: monocyte ratio) for 1h at 37 °C. Wells were, then, washed, and new media were added. Monocytes were then allowed to kill conidia for the indicated time points before intracellular conidia were harvested by lysis of monocytes with 0.05% Triton-X. The process of cellular lysis was confirmed by light microscopy and killing of *A. fumigatus* conidia was assessed by c.f.u. plating. Each condition was performed in triplicate.

V.4. Immunofluorescence staining protocols

a. LC3 B immunofluorescence staining

For LC3 B immunofluorescence staining, cells were seeded on coverslips pretreated with poly-L-lysine, fixed with 4% FA without MetOH for 15 min and washed twice with PBS. Then, the cells were permeabilized with ice cold methanol (-20^{o}C) for 10 min at -20^{o}C and blocked using 15 min blocking

buffer containing 2% BSA and 0.1% saponin in PBS. Cells were incubated with the primary antibody (mouse, Nanotools, 1:20 diluted in blocking buffer) for 1h at room temperature. After three washes with blocking buffer, cells were incubated with the secondary antibody (goat a-mouse-Alexa 555, 1:500 diluted in blocking buffer) for 1h at RT in dark. After three washes with blocking buffer and one with PBS, slides were placed on a drop of Mowiol and were stored in the cold room (4ºC).

b. GFP immunofluorescence staining

For GFP immunofluorescence staining, cells were seeded on coverslips pretreated with poly-L-lysine, fixed with 4% FA without MetOH for 15 min and washed twice with PBS. Then, the cells were blocked using 15 min blocking buffer containing 2% BSA and 0.1% saponin in PBS. Cells were incubated with the primary antibody (rabbit, Minothech, 1:5000 diluted in blocking buffer) for 1h at room temperature. After three washes with blocking buffer, cells were incubated with the secondary antibody **(**a-rabbit, Biotium 555 or 488, 1:500 diluted in blocking buffer) for 1h at RT in dark. After three washes with blocking buffer and one with PBS, slides were placed on a drop of Mowiol and were stored in the cold room (4ºC).

c. vATPase immunofluorescence staining

For vATPase immunofluorescence staining, cells were seeded on coverslips pretreated with poly-L-lysine, fixed with 4% FA without MetOH for 15 min and washed twice with PBS. Then, the cells were permeabilized with 0.25% Triton in PBS for 10 min and, after three washes with PBS of 5min/each, they were blocked using 30 min blocking buffer containing 3% BSA, 3%NGS in PBS-Tween (0.1%) at room temperature. Cells were incubated with the primary antibody (mouse, Nanotools, 1:100 diluted in blocking buffer) for 1h at room temperature. After three washes with PBS of 5min/each, cells were incubated with the secondary antibody (goat a-mouse-Alexa 555, 1:500 diluted in blocking buffer) for 1h at RT in dark. After three washes with PBS of 5min/each, slides were placed on a drop of Mowiol and were stored in the cold room (4ºC).

d. Cathepsin D immunofluorescence staining

For Cathepsin D immunofluorescence staining, cells were seeded on coverslips pretreated with poly-L-lysine, fixed with 4% PFA for 15 min in room temperature and washed twice with PBS. Then, the

cells were permeabilized with 0,2% Triton for 10 min in room temperature and blocked 15min using blocking buffer containing 2% BSA, 5%NGS in PBS at room temperature. Cells were incubated with the primary antibody (mouse, IgM, Santa Cruz Biotechnology, 1:50 diluted in blocking buffer) for 1h at room temperature. After three washes with blocking buffer, cells were incubated with the secondary antibody **(**goat a-mouse-Alexa 555, IgG, 1:500 diluted in blocking buffer) for 1h at RT in dark. After three washes with blocking buffer and two washes with PBS, slides were placed on a drop of Mowiol and were stored in the cold room (4^oC).

e. LAMP1 immunofluorescence staining

For LAMP1 immunofluorescence staining, cells were seeded on coverslips pretreated with poly-L-lysine, fixed with 4% PFA for 15 min at room temperature and washed twice with PBS. Then, the cells were blocked for 15min using blocking buffer containing 2% BSA and 0.1% saponin in PBS. Cells were incubated with the primary antibody (mouse, BD, 1:500 diluted in blocking buffer) for 1h at room temperature. After three washes with blocking buffer, cells were incubated with the secondary antibody (goat a-mouse-Alexa 555, 1:500 diluted in blocking buffer) for 1h at RT in dark. After two washes with blocking buffer and two washes with PBS, slides were placed on a drop of Mowiol and were stored in the cold room (4^oC).

V.5. Confocal microscopy

For immunofluorescence imaging, one day after immunofluorescence staining, images were acquired at room temperature using a laser-scanning spectral confocal microscope (TCS SP2; Leica) or they were examined under SP8 inverted confocal microscope. Phagosomes surrounded by a rim of fluorescence of the indicated protein marker were scored as positive, according to established protocols in our laboratory.

V.6. Reactive Oxygen Species (ROS) production detection

ROS measurements were performed by means of a nitro blue tetrazolium chloride (NTB) assay. Stock solution of NTB was dissolved in sterile dH_2O to a final concentration of 0.2mg/ml. NTB was added during the last 10 minutes of each time point. After the last 10 minutes, each well washed once with 1x PBS and ice-cold methanol was added for 10 minutes at -20^oC in order cells to be fixed. Followed

two washes with 1x PBS and slides were placed on a drop of Mowiol and were stored in the cold room (4^{o}C).

V.7. PBMCs cryopreservation protocol

The isolated PBMCs were washed once with PBS 1x and centrifuged at 300 x g for 10 minutes at 4^{o}C to obtain a cell pellet. Then, the pellet was resuspended in cold FBS with mild flicking (or racking). While kept on ice, the cells were counted using the Hemocytometer with viable cell exclusion dye (Trypan blue) and the concentration was adapted to 1 – 20 x 10^6 cells/mL. The proper amount of FBS was added in the cells in order 0.5ml to be shared in each of >3 vials. Then, we added dropwise 0.5ml of the cold 2x freezing mix with 20% DMSO in FBS (at a ratio of 1:1) in each vial. The final cell suspension will be in 10% DMSO and 90% FBS. The final cell concentration will be between 0.5 - 10 x 10^6 cells/mL (optimal concentration: 4 x 10^6 cells/mL). Finally we placed the cryovials in a 4^{o}C Mr. Frosty freezing container, which was placed as soon as possible into the -80°C freezer for up to 2 weeks. For the long-term storage, we transferred the vials of frozen PBMCs to liquid nitrogen (below -135°C) after 7 days of freezing in -80^{o}C.

V.8. Statistical analysis

The data were expressed as means ± standard error of the mean (SEM). Statistical significance of differences were determined by two-tailed unpaired *t*-test. A p value of < 0.05 was considered statistically significant ($* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$). Data were analyzed using GraphPad Prism software version 8.0.2.

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Additional material

43. www.invitrogen.com/cellculturebasics
44. https://www.uab.cat/doc/ATCCguide

SUPPLEMENTARY

Media

a. RPMI complete

For preparation of 250ml:

RPMI 1640	215ml
1mM Sodium pyruvate	2.5ml
10% FBS	25ml
1x Pen / Strep	2.5ml
1% Glutamine	2.5ml
10mM HEPES	2.5ml

b. DMEM complete

For preparation of 500ml:

DMEM - Glutamax	440ml
1mM Sodium pyruvate	5ml
10% FBS	50ml
1x Pen / Strep	5ml

c. DMEM conditioned

For preparation of 150ml:

DMEM - Glutamax	75ml
1mM Sodium pyruvate	1.5ml
10% FBS	30ml
1x Pen / Strep	1.5ml
L929 sup	45ml

d. MACS buffer (Miltenyi)

2mM EDTA
0.5% BSA
PBS (pH 7.2)

e. Poly-L-Lysine coverslip treatment

Sterile coverslips were treated for 1h with Poly-L-Lysine and washed twice for 10min with sterile dH_2O.

f. Freezing mix for PBMCs cryopreservation

20% DMSO (Applichem, A3672,0050)
Fetal Bovine Serum (FBS) (Gibco, Cat. Number 10270106, low endotoxin)

Optimizations

Before the cryopreservation optimization in human PBMCs, we preceded the optimization for the antibodies which target the proteins that we were interested in evaluating in thawed cells and constitute important markers of the LAP pathway, such as the LC3, the Cathepsin D and the LAMP1.

a. LC3 antibody optimization

For the LC3 antibody (mouse, Nanotools) optimization, healthy human CD14^{+} cells were infected for 2 hours with the Δ*pksP* strain of *A. fumigatus* and stained with three different concentrations of the antibody: 1:20, 1:50 and 1:100 concluding that the better concentration to use is the 1:50.

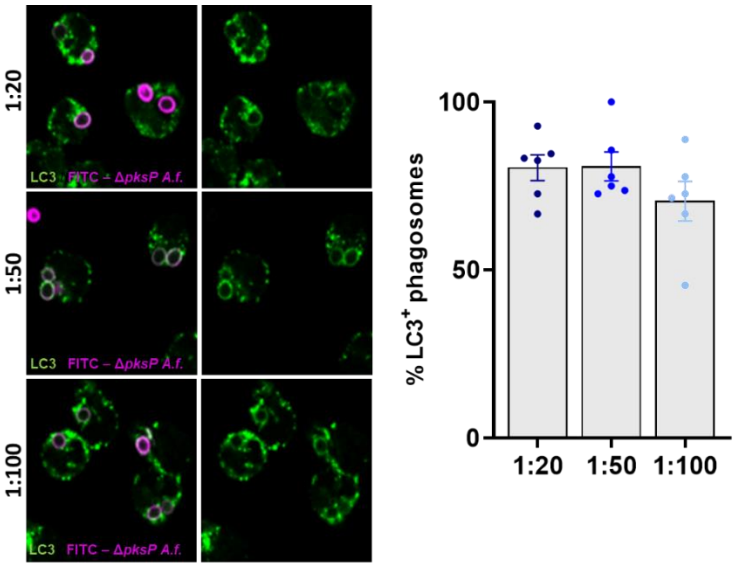

Figure 17. Human CD14^{+} monocytes infected for 15min with the FITC-labeled Δ*pksP* strain of *A.fumigatus*.The staining performed using different concentrations of the first antibody, 1:20, 1:50 and 1:100.

b. CatD antibody optimization

For the Cathepsin D antibody (mouse, Santa Cruz Biotechnology) optimization, healthy human CD14^{+} cells were infected for 15minutes and 2 hours with the Δ*pksP* strain of *A. fumigatus* and stained with two different concentrations of the antibody: 1:50 and 1:100 concluding that the better concentration to use and represents appropriately the Cathepsin D kinetics is the 1:50.

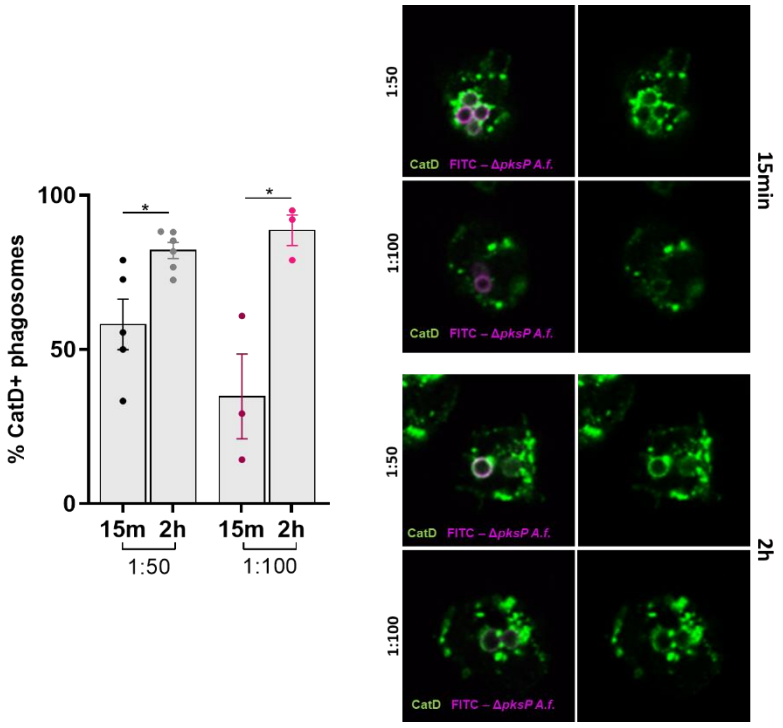

Figure 18. Human CD14^{+} monocytes infected for 15min and 2h with the FITC-labeled Δ*pksP* strain of *A.fumigatus*.The staining performed using different concentrations of the first antibody, 1:50 and 1:100.

c. LAMP1 antibody optimization

For the LAMP1 antibody (mouse, BD) optimization, healthy human CD14^{+} cells were infected for 15minutes and 2 hours with the Δ*pksP* strain of *A. fumigatus* and stained with two different concentrations of the antibody: 1:500 and 1:1000 concluding that the better concentration to use and represents appropriately the LAMP1 kinetics is the 1:500.

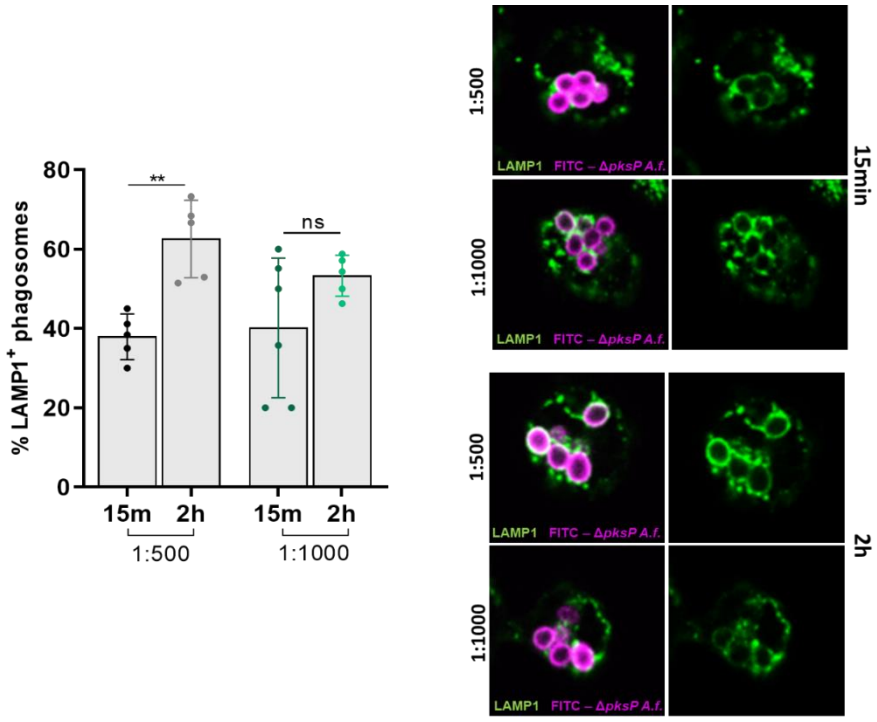

Figure 19. Human CD14^{+} monocytes infected for 15minutes and 2h with the FITC-labeled Δ*pksP* strain of *A.fumigatus*.The staining performed using different concentrations of the first antibody, 1:500 and 1:1000.