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„Aspergillus lectin inhibition-

An alternative to the conventional treatments“

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« ***Le succès n'est pas la clé du bonheur. Le bonheur est la clé du succès. Si vous aimez ce que vous faites, vous réussirez.*** »

Albert Schweitzer

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1. ABSTRACT

As the incidence of fungal infections is steadily increasing over the past years, the ascomycete *Aspergillus fumigatus* serves as an example of a microbe with the capacity of causing life-threatening lung infections, especially in patients with compromised immune system. At the moment antifungal treatment is done by voriconazole and amphotericin B which possess a high toxicity and aim to destruct or inhibit the proliferation of the pathogen. Furthermore, voriconazole, as a representative of the azole class, shows increasing resistance rates to *A. fumigatus* mainly derived from a point mutation in the gene CYP 51A [Snelders E. et al., 2008].

Therefore, the aim of this diploma thesis is to propose an alternative to the conventional treatments of aspergillosis. Recently, it was demonstrated in collaboration with the french chemistry research group CEISAM that it is possible to inhibit the *A. fumigatus* lectin (AFL) which is expressed by *A. fumigatus* conidia and allows them to adhere to pneumocytes. This lectin recognises fucoside structures on bronchial epithelium [Houser et al., 2013; Kerr et al, 2016] and its adhesion is one of the key stages in the invasion process as well as the first step in the development of infection.

In fluorescence microscopy, an interaction model between A549 pneumocytes and the spores of *A. fumigatus* was developed and 6 saccharides that are expected to inhibit the binding of AFL were tested. Furthermore, different strains of *A. fumigatus* resistant to azoles were investigated in order to explore the effect of these strains on the interaction.

For the first time, a correlation between the inhibition of AFL by different molecules and the reduction of adherent as well as internalised *A.fumigatus* spores was observed in-vitro. Consequently, these saccharides could represent promising new drug targets for aspergillosis and the successfully standardised interaction model allows further testing which molecule finally acts best. Most interestingly, concerning testing the different strains, the mutant strain TR 46 087 showed a strong reduction in spores per cell in comparison to the other strains tested. This leads to the conclusion that certain mutations in strains have another important impact on the attachment of conidia to lung epithelial cells.

2. BACKGROUND

2.1. *Aspergillus*

Aspergillus is a saprophytic mould that can be found in the air, water, food, soil and plays an important role in the recycling environment [Sales-Campos H. et al., 2013]. The first description of *Aspergillus* was done in 1729 by the Italian priest and biologist Pietro Antonio Micheli. Under the microscope, he was reminded of the catholic aspergillum (holy water sprinkler) and therefore named it according to its morphology [Etymologia: *Aspergillus*, 2006].

Although *Aspergillus* has been long considered as a fungi in an asexual state, recently, the production of sexual means has been identified [Dyer P.S. and Paoletti M., 2005]. *Aspergillus* is highly aerobic and the genus consists of more than 300 species [Samson R.A. et al., 2014] where only a few can cause infection in human hosts. The most frequent identified pathogen is *Aspergillus fumigatus*, followed by other species such as *Aspergillus flavus* and *Aspergillus niger* [Dagenais T.R.T. and Keller N.P., 2009].

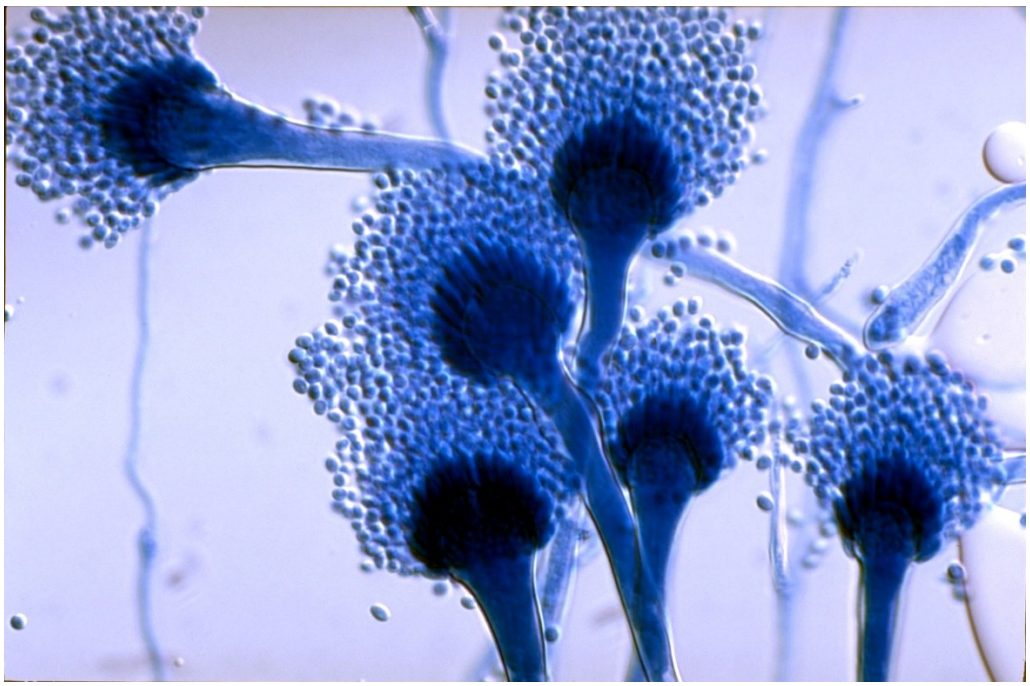


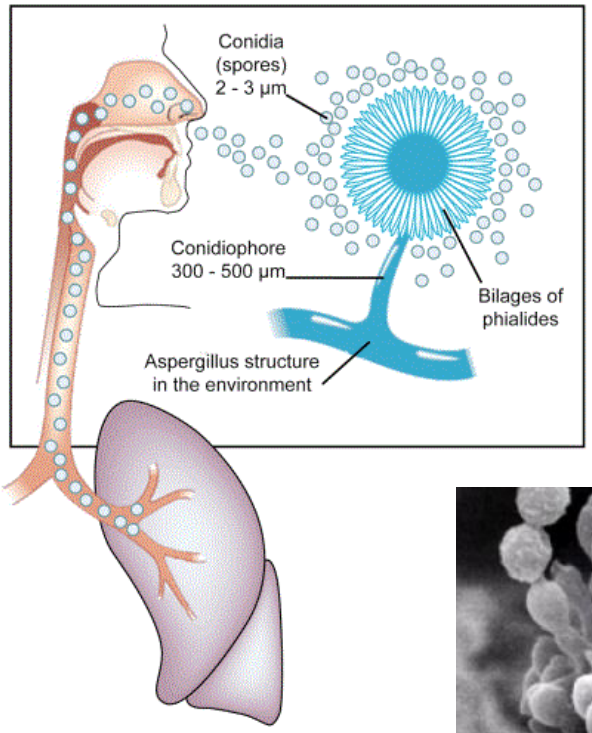
Figure 1: Morphology of *Aspergillus fumigatus* under the microscope [Alunos Online]

2.2. *A. fumigatus*- Virulence and infection

A. fumigatus posseses a multifactorial and complex virulence which results on the one hand from various virulence factors as described in part **2.7** and on the other hand from its biological characteristics. It grows between 37-50 C° which makes it better thermotolerant and more stress resistant than other *Aspergillus* species [Bhabhra R. and Askew D.S., 2005; Rhodes J.C., 2006].

Infection is usually caused by inhalation of its fungal spores, the conidia, into the lungs. Other infection sides, such as skin or cornea are also observed, but this is less frequent [Croft C.A. et al., 2016]. The conidia, which are the infectious particles of *A. fumigatus*, are very small, approximately 2-3 µm, haploid spores that are produced asexually [Croft C.A. et al., 2016] and disseminated by the air [Mullins J. et al., 1976]. Because of this small size, they can penetrate deeply into the respiratory tract where in healthy individuals, they are eliminated by mucociliary clearance and the immune system without developing disease.

Figure 2: Inhalation of conidia into the lungs [Lypro Biosciences, Inc., 2012-2018]



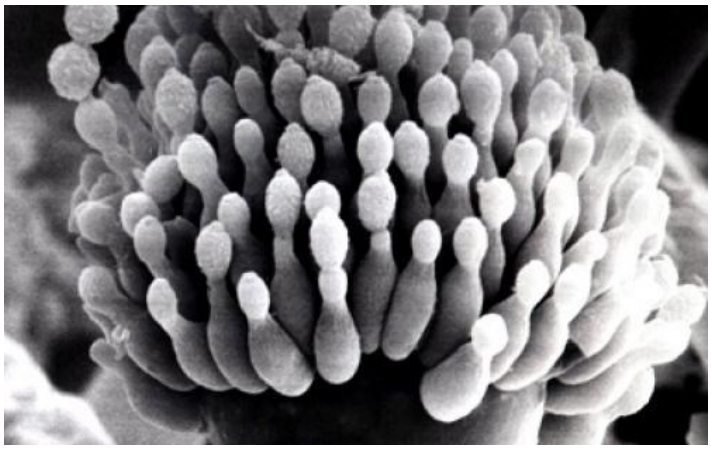


Figure 3: Conidial head of *A. fumigatus* in electron microscopy [www.aspergillus.org.uk]

2.3. *Aspergillus* disease

The group of diseases caused by any member of the genus is called aspergillosis. In-vitro studies showed that *A. fumigatus* conidia can bind [DeHart D.J. et al., 1997], be internalised [Paris S. et al., 1997] and phagocytosed [Botterel F. et al., 2008] by airway epithelium cells which may represent a source of infection.

2.3.2. Allergic bronchopulmonary aspergillosis

In the hypersensitive host, such as patients with asthma or cystic fibrosis [Knutsen A.P. and Slavin R.G., 2011], conidia are inhaled mainly into the bronchioli where they can provoke an allergic reaction which is called allergic bronchopulmonary aspergillosis [Osherov N., 2012]. ABPA is characterised by a Th2 lymphocyte response, eosinophilia and increased serum IgE levels. Its symptoms include coughing and wheezing and can lead to pulmonary fibrosis and bronchiectasis [Knutsen A.P. and Slavin R.G., 2011].

2.3.3. Invasive pulmonary aspergillosis

In the immunocompromised host, such as patients with granulocytopenia, HIV/ AIDS, cancer or patients receiving immunosuppressive therapies, as listed in **table 1**, conidia are inhaled primarily into the alveoli which leads to the most severe and sometimes life-threatening infection of *A. fumigatus* named invasive pulmonary aspergillosis [Osherov N., 2012]. IPA has a mortality rate of 25-90% [Aspergillus & Aspergillosis Website] and its typical symptoms include fever, chest pain and dyspnoea.

2.3.4. Chronic pulmonary aspergillosis and aspergilloma

Chronic pulmonary aspergillosis may be asymptomatic [Croft C.A. et al., 2016] and is defined as `fungal growth that remains localized within the lungs´ [Croft C.A. et al., 2016]. As shown in **table 1**, risc factors include mycobacterial infection as well as COPD. In patients with pre-existing lung cavities, caused by lung diseases such as tuberculosis, the growth of a fungal ball, called aspergilloma, is possible [Aspergillus & Aspergillosis Website]. Aspergilloma represents a specific pattern of CPA.

Altogether, the impact of this diseases and its symptoms depend very much upon the state of health of the host.

Disease name	**Risk factors**	**Symptoms/Manifestations**	**Reference**
Allergic broncho pulmonary aspergillosis (ABPA)	Allergic asthma	Wheezing	[Patterson K.C. and Strek M.E., **2010**]
	Cystic fibrosis	Coughing	
		Bronchiectasis	
		Generalised allergic response	
Chronic pulmonary aspergillosis (CPA)	Mycobacterial infection	Asymptomatic	[Patterson K.C. and Strek M.E., **2014**]
	COPD	Aspergilloma (Fungal ball)	
	Pre-existing lung cavity	Aspergillus nodule	
	Conditions impairing clearance	Expanding, thick walled cavities	
Invasive aspergillosis (IA)	Granulocytopenia	Fever	[Kousha M. et al., **2011**]
	HIV/AIDS	Dyspnoea	
	Immunosuppression	Chest pain	
	Cancer (particularly cancer of the blood)	Disseminated fungal growth on multiple organs	

Table 1: The primary manifestations of aspergillosis [Croft C.A. at al., 2016]

2.4. Host immune response

A successful innate immune response to *A. fumigatus* conidia starts with the recognition of pattern recognition receptors PRRs such as the C-type lectin dectin-1 [Werner et al., 2009] or toll-like receptors like TLR2 and TLR4 [Mambula S.S. et al., 2002] by pathogen-associated molecular patterns PAMPs such as fungal wall components. The C-type lectin dectin-1 represents a receptor for *β*-glucans and is responsible for protection against *A. fumigatus* infection, as described in part **2.6**.

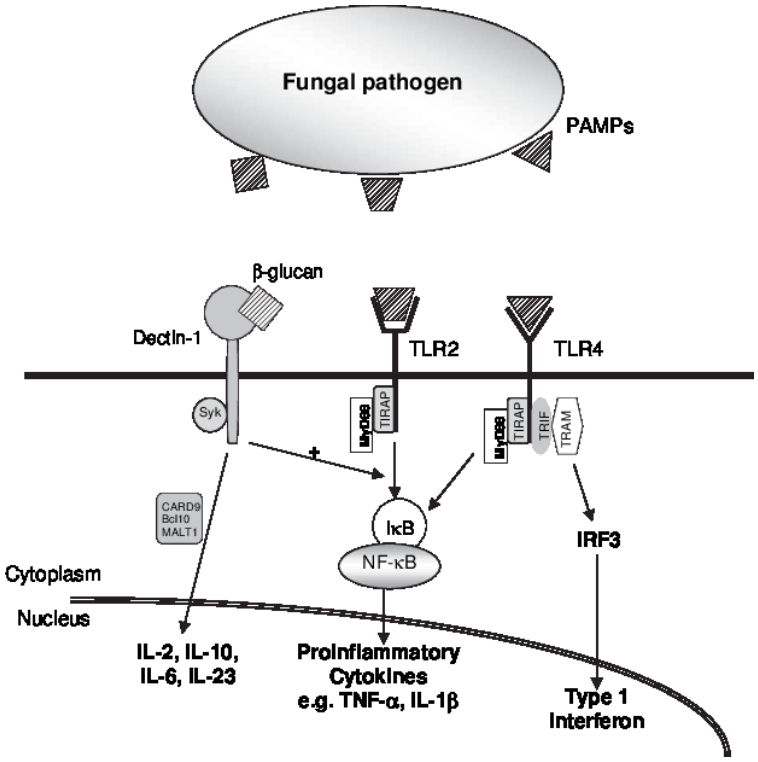


Figure 4: Pattern recognition of fungal pathogen and signaling pathways [Chai L.Y.A. et al., 2009]

This recognition activates signal transduction cascades, such as the Nf-$_K$B pathway, leading to the secretion of pro-inflammatory cytokines and chemokines by macrophages, neutrophils, monocytes and dendritic cells. First barrier against *A. fumigatus* infection are macrophages as well as neutrophils [Sales-Campos H. et al., 2013] due to their ability to form NETs [McCormick A. et al., 2010]. Monocytes react differently, either producing low or high levels of inflammatory cytokines depending on the cell phenotype [Serbina N.V. et al., 2009].

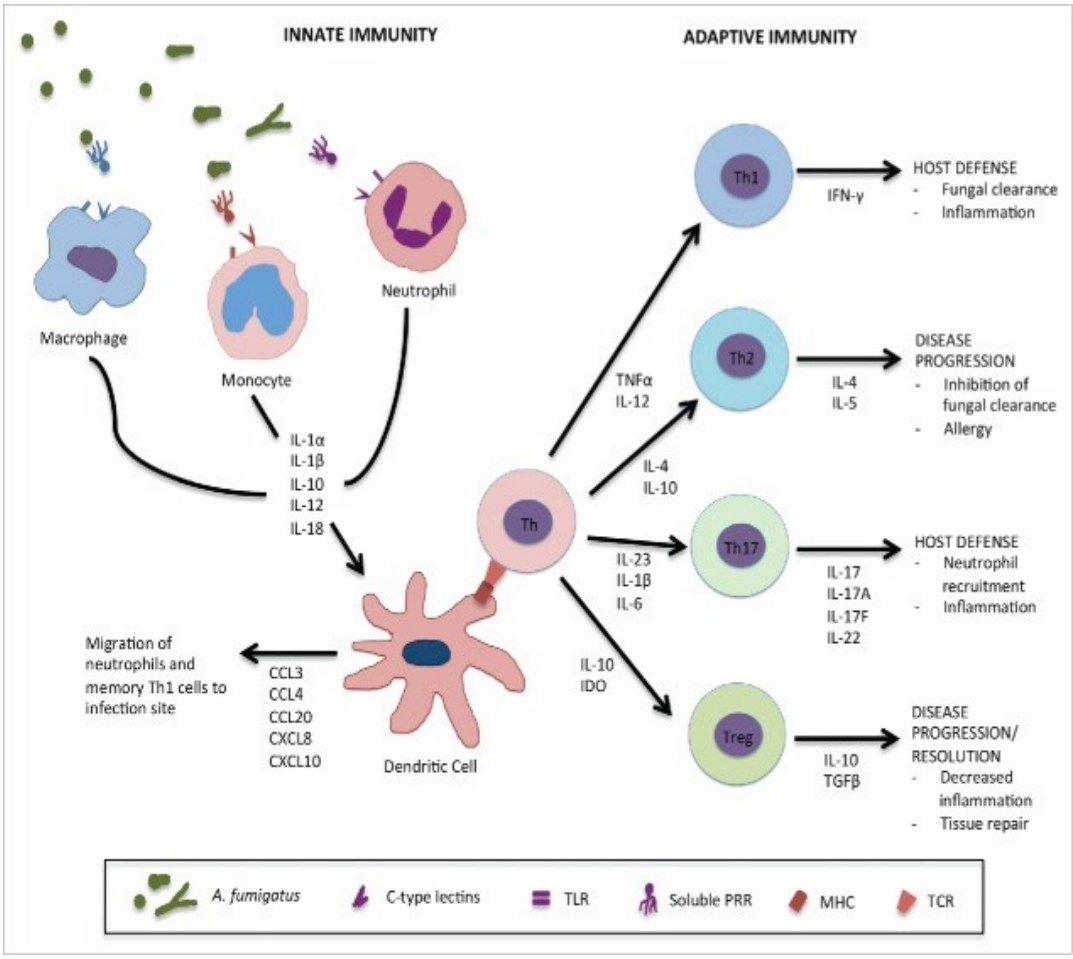


Figure 5: Host immune response against *A. fumigatus* [Al-Bader N. and Sheppard D.C., 2016]

Dendritic cells are the connection between the innate and adaptive immunity. They secrete chemokines such as CCL3, CCL4, CCL20, CXCL8 and CXCL10 resulting in the migration of neutrophils and memory Th1 cells to the infection side helping in the host defense. In addition, they interact with T-helper cells leading to the secretion of different cytokines. Th1 and Th17 cytokines play a protective role and are responsible for host defense against the fungal pathogen. Th2 and Treg cells hold a non-protective role and result in disease progression and inflammation, as shown in **figure 5**.

In general, the innate immunity is used for light infections and triggers the adaptive immunity, which is necessary for severe infections. This adaptive immune response includes CD4 (Th1, Th2, Th17 and Treg) and CD8 T-cells [Sales-Campos H., 2013].

2.5. Interaction pathogen-cell host

2.5.1. The fungal cell wall

The fungal cell wall of *A. fumigatus* plays an extremely important role in the infection process because it is the first contact of the fungus with the host [Sales-Campos et al., 2013]. It is a three dimensional construction mainly consisting of polysaccharides such as β-(1,3), β-(1,4), and β-(1,6)-glucans as well as α-1,3-glucans, chitins and galactomannans [Abad A. et al., 2010]. Its structure is not rigid, rather extremely dynamic and constantly changing, due to different environmental demands and culture conditions [Latgé J.P., 2007].

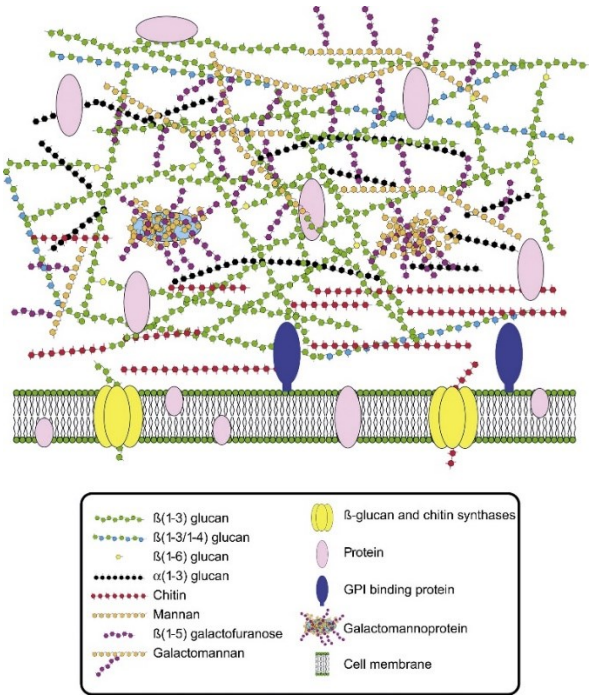


Figure 6: The fungal cell wall [Abad A. et al., 2010]

2.5.2. Lung epithelial cells

Concerning the host side, the first point of contact represents a monolayer of lung epithelial cells, either bronchial or alveolar epithelial cells [Osherov N., 2012]. Studies have shown that these lung epithelial cells are more than just a mechanical barrier against the fungal pathogen, they can better be described as an `extension of the innate immune system. They actively phagocytose a proportion of adherent conidia, and inhibit their growth. They produce cytokines and chemokines that activate both innate and acquired immunity and secrete antimicrobial peptides and lysosomal enzymes that may inhibit fungal growth´ [Osherov N., 2012].

As a result, this complex host-pathogen interaction of *A. fumigatus* cell wall components and lung epithelial cells as well as their reaction to the fungal pathogen is extremely important for the understanding of the development of aspergillosis and has to be revealed in more detail.

Therefore, in the next figures, the typical structure of a bronchiole as well as the development of ABPA, but even more important, as we worked with A549 pneumocytes which show similar characteristics to type II alveolar epithelial cells, the typical structure of an alveolus and the development of IPA, are highlighted.

2.5.3. Structure of a bronchiole and the development of ABPA

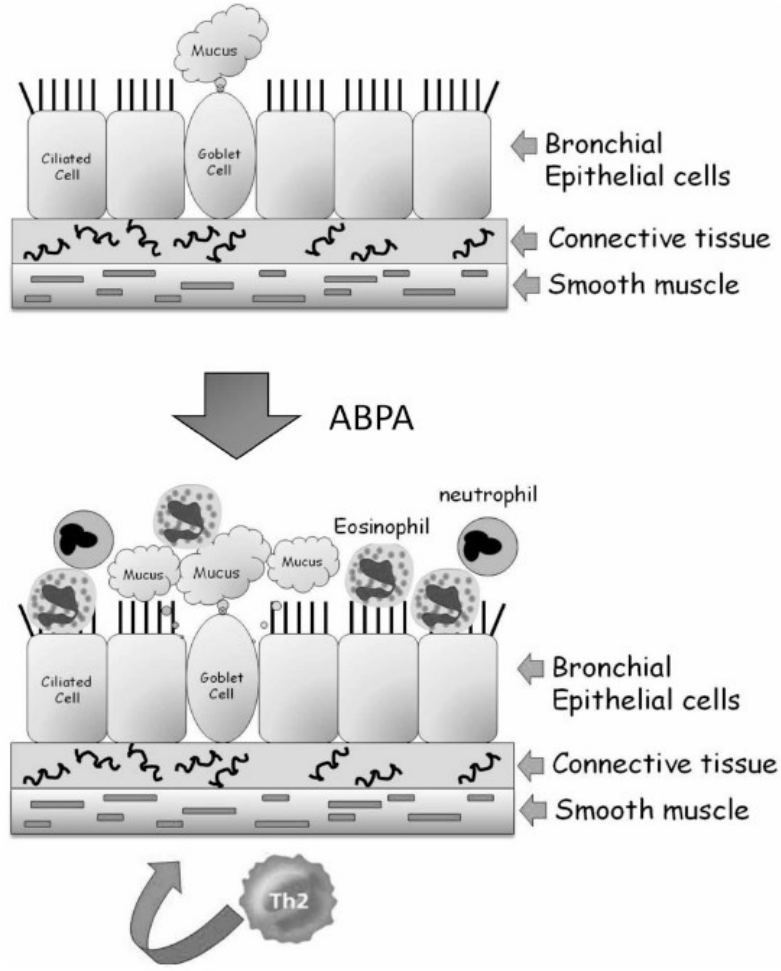


Figure 7: Structure of a bronchiole and the development of ABPA [Osherov N., 2012]

A typical bronchiole, as shown in **figure 7**, consists of an outer layer of smooth muscle cells. In the middle we can find the connective tissue and the inner layer is composed of epithelial cells. These epithelial cells are further divided into ciliated and goblet cells, which produce mucus.

By infection of ABPA, inhaled conidia trigger an enhanced Th2 lymphocyte response which leads to increased mucus production as well as the recruitment of eosinophils and neutrophils into the airway. They inflame and damage the airway which finally leads to fibrosis and bronchiectasis [Osherov N., 2012].

2.5.4. Structure of an alveolus and the development of IPA

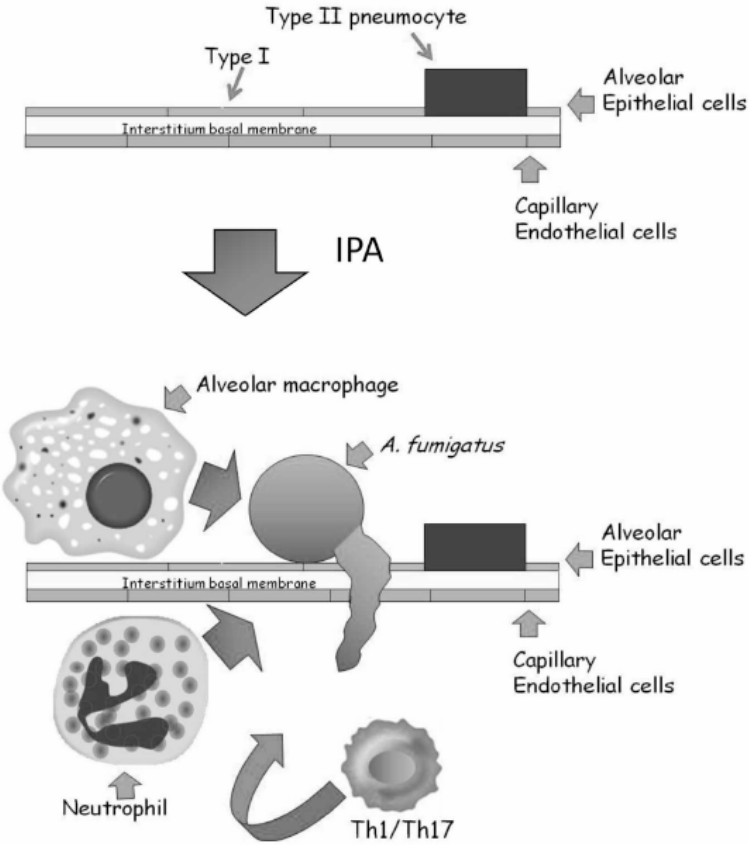


Figure 8: Structure of an alveolus and the development of IPA [Osherov N., 2012]

A typical alveolus on the other hand, as shown in **figure 8**, is composed of an outer layer of capillary endothelial cells that line the blood vessels. In the middle we can find a thin interstitium basal membrane out of collagen, laminin and fibronectin. The inner layer consists of epithelial cells, further divided into type I and type II pneumocytes.

When it comes to IPA, *A. fumigatus* conidia penetrate into the alveoli, germinate, but cannot be killed by macrophages and neutrophils because of an ineffective Th1/Th17 response in immunocompromised patients. This leads to fungal growth, entry into the blood stream and finally, tissue necrosis [Osherov N., 2012].

2.5.5. Interaction of *A. fumigatus* with a bronchial epithelial cell

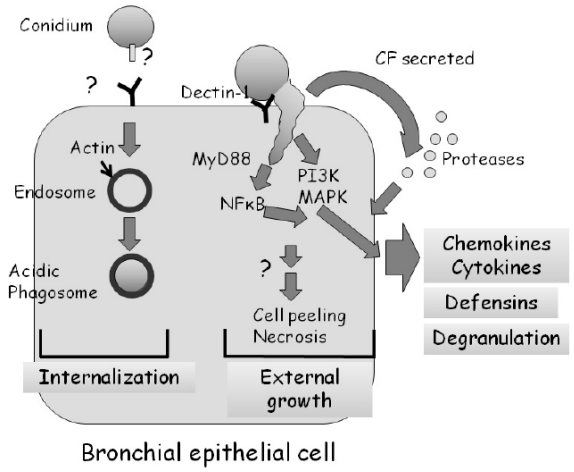


Figure 9: Interaction of *A. fumigatus* with a bronchial epithelial cell [Osherov N., 2012]

In an actin-dependant process, around 30 % of adherent conidia are internalised into the endosome and consequently, fused to an acidic phagosome. Here they remain alive for up to 20 hours which might represent a source of infection. Interestingly, 70 % of adherent conidia germinate externally, activating signal transduction cascades such as the Nf_KB or MAP-kinase pathway which leads to the production of pro-inflammatory chemokines and cytokines, dependant on dectin-1 [Osherov N., 2012].

2.5.6. Interaction of *A. fumigatus* with an alveolar epithelial cell

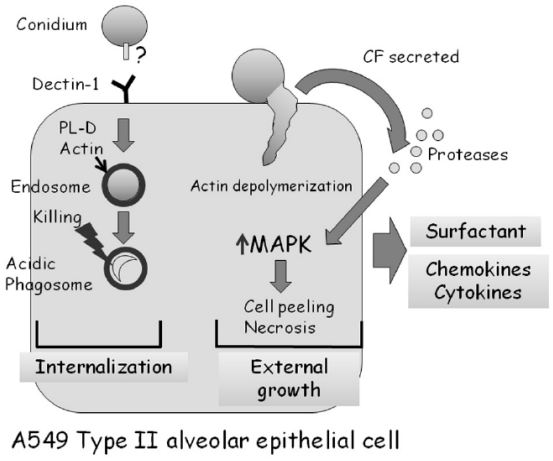


Figure 10: Interaction of *A. fumigatus* with an A549 type II alveolar epithelial cell [Osherov N., 2012]

Similar to bronchial epithelial cells, around 30 % of adherent conidia are internalised into the endosome and fused to an acidic phagosome, where dectin-1 and phospholipase-D play an important role in this process. Here they remain alive for up to 24 hours which might again represent a reservoir of infection. Likewise, 70 % of adherent conidia germinate externally, leading to actin depolymerisation, proteases secretion resulting in MAP-kinase activation, cytokine production and finally, cell death by necrosis [Osherov N., 2012].

2.6. Protective factors to *A. fumigatus* infection

- One of the most important factors for protection to *A. fumigatus* infection is represented by the C-type lectin **dectin-1**, a receptor for the cell wall component *β*-1,3-glucan. Studies have shown that mice that lack of dectin-1 are more likely to develop *A. fumigatus* lung infection than wildtype mice [Werner J.L. et al., 2009].

- Moreover, representatives of the **TLR-family** such as TLR2 and TLR4 [Al-Bader N. and Sheppard D.C., 2016], the soluble factor **PRR pentraxin-3** [Al-Bader N. and Sheppard D.C., 2016] and **vitamin D**, when converted to its active form 1,25-D_3 [Kearns M.D. et al., 2015] are suggested to play an important role in the protection to the fungal pathogen.

2.7. Virulence factors of *A. fumigatus*

As mentioned in part **2.2**, beside its biological characteristics, several factors have been demonstrated to contribute to virulence in the pathogenesis of aspergillosis.

2.7.1. Cell wall components

- **GAG-glycosaminoglycan**, a component of the hyphal cell wall which consists of N-acetyl-galactosamine and galactopyranose, `is the first polysaccharide described as a virulence factor in medical mycology´ [Briard B., 2016].

- Although a component of both cell wall of conidia and hyphae, studies have demonstrated a dangerous role of **α -1,3-glucan** in the cell wall of conidia during development of IPA [Al-Bader N. and Sheppard D.C., 2016].

- Supported by the observation that mutants that lack of an enzyme responsible for the first step of DHN-melanin production show a reduction in virulence, the specific **1, 8- dihydroxynapthalene-melanin**, which gives conidia their characteristic gray-green colour, has been demonstrated as an important factor contributing to pathogenicity as well [Heinekamp T. et al., 2012].

- Furthermore, the **hydrophobic rodlet layer**, with its main component the hydrophobic RodA protein, which is responsible for hydrophobicity on the cell wall of conidia, is suggested as a virulence factor by masking dectin-1 and dectin-2 responses and increasing fungal survival [Carrion Sde J. et al., 2013].

2.7.2. Other virulence factors

- Secondary metabolites that are produced by *A. fumigatus* and support its pathogenicity are **gliotoxin** and **LaeA** [Al-Bader N. and Sheppard D.C., 2016].

- The ability of acquisition and utilisation of **iron** from the host is another essential factor contributing to virulence in *A. fumigatus* infection [Moore M.M., 2013].

- **Adaption to hypoxia** is a critical environmental factor where the transcriptional factors SrbA and SrbB play an important role in this process [Al-Bader N. and Sheppard D.C., 2016].

- Elements of the regulatory pathway such as **MedA** [Gravelat F.N. et al., 2010], the transcription factor **Ace2** [Ejzykowicz D.E. et al., 2009] and the regulators **F1bB** and **F1bE** [Kim S.S. et al., 2013] that are responsible for spore formation have been shown to contribute to virulence in *A. fumigatus* infection as well.

- Secretion of lytic enzymes like **phospholipase** [Ghannoum M.A., 2000] and **protease** (serine protease, metalloprotease) [Farnell E., 2012] moreover, support the virulence of *A. fumigatus*.

2.8. *Aspergillus fumigatus* lectin

As many other microorganisms, *A. fumigatus* uses a lectin for the adhesion to host glycoproteins. This adhesion is described as one of the key stages in the invasion process and represents the first step in the development of infection of aspergillosis. As a result, another important factor that contributes to virulence is AFL, the *Aspergillus fumigatus* lectin. With a theoretical molecular weight of 68,9 kDa, AFL was first characterised by a homology search of *Aleuria Aurantia* lectin AAL, which shows similar sequence and structure motifs [Houser J. et al., 2013].

2.8.1. Structure

AFL is expressed by *A. fumigatus* conidia and represents a dimer with 12 binding sites. Each monomer has 6 binding sites and consists of 6 blades composed of 4 antiparallel β-sheets [Houser J. et al., 2013].

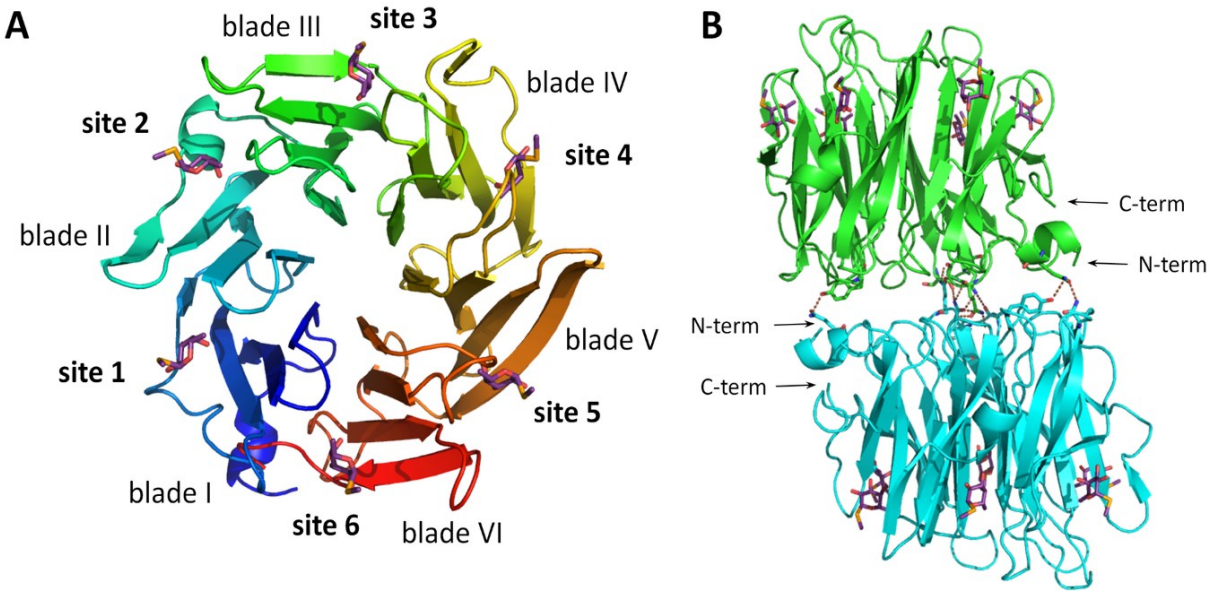


Figure 11: 3D structure of AFL [Houser J. et al., 2013]

In picture A we can see side 1-6 as well as blade 1-6 of AFL monomer. Picture B shows the AFL dimer in a side-view and the regions of contact that are located in 2 loops of blade V. Concerning its nomenclature, between blade 1 and II we can find site 1, site 2 is located between blade II and blade III, and so on [Houser J. et al., 2013].

Picture C shows the protein sequence of AFL. The colours of the secondary structure elements correspond to picture A.

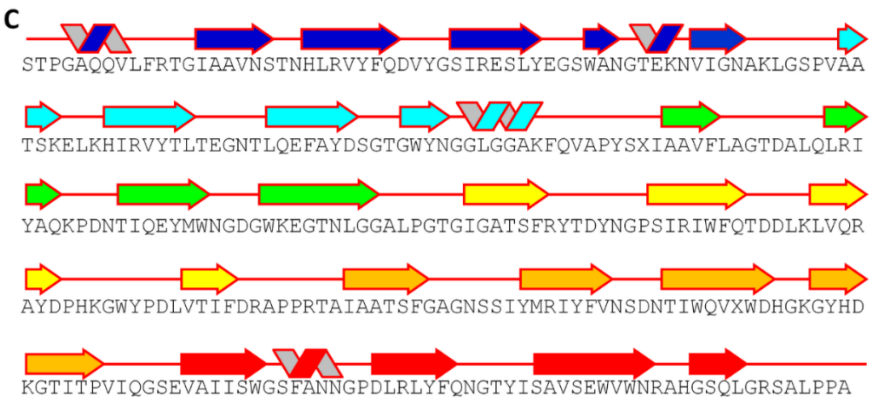


Figure 12: Protein sequence of AFL [Houser J. et al., 2013]

2.8.2. Binding sites

The binding sites are very different, each consisting of various amino acids. On the one hand we have arginine and glutamic acid/glutamine that enable polar interactions with O-3, O-4 and O-5 of fucose. The other side consists of tryptophan/tyrosine that establish hydrophobic interactions with C-3, C-4 and C-5 of fucose. Moreover, some hydrogen bonds and a water bridge are observed in the structure of AFL [Houser J. et al., 2013].

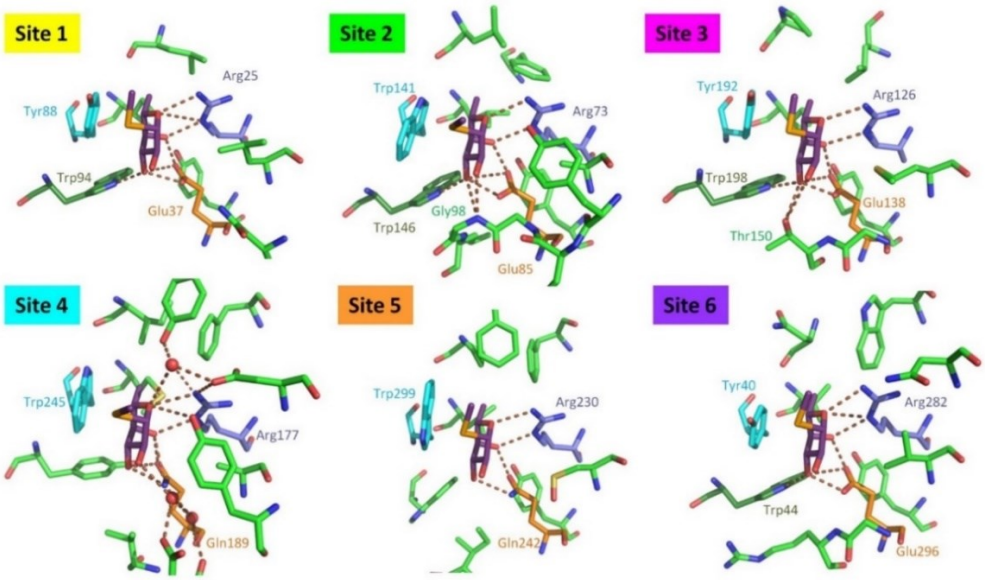


Figure 13: AFL binding sites [Houser J. et al., 2013]

2.8.3. Binding affinity

Studies have revealed that AFL strongly binds to carbohydrates such as L-fucose, but has a low affinity to other saccharides such as L-galactose, D-arabinose, D-fructose or D-mannose. This observation is proved by a hemagglutination inhibition assay where the MICs of different saccharides for AFL were tested [Houser J. et al., 2013].

As a result, with this strong ability to bind fucoside motifs, which are present on the host surface on bronchial epithelium, AFL contributes to the host-pathogen interaction and consequently, to pathogenicity and the development of infection.

2.8.4. Concept of anti-adhesive therapy

The concept of anti-adhesive therapy is to inhibit the binding of this lectin to host epithelial cells, to prevent the invasion process and as a result, the development of infection. Here we have the big advantage of a mild and safe method in comparison to the radical approach by chemotherapeutics such as voriconazole or amphotericin B. Moreover, the pathogenic cells are not affected directly by killing or arresting them, so the incidence of developing resistance mechanism is much lower [Sharon N., 2006].

2.8.5. Implication in the inflammatory response

Proved by the observation that the stimulation of bronchial epithelial cells by AFL leads to the production of IL-8, studies also suggest the contribution of AFL in the pro-inflammatory response to *A. fumigatus* [Houser J. et al., 2013]. In the experiment below, human respiratory epithelial cells were stimulated with 2 different concentrations of AFL, 0,1 µM and 0,3 µM, and the IL-8 concentration was measured. ´NS` means the negative control and ´AF` represents the positive control by stimulating with 10^6 conidia. As a result, those bronchial epithelial cells stimulated with AFL show higher IL-8 concentrations than those without. Consequently, this experiment proves the implication of AFL in the pro-inflammatory response and therefore, another way of its contribution to virulence.

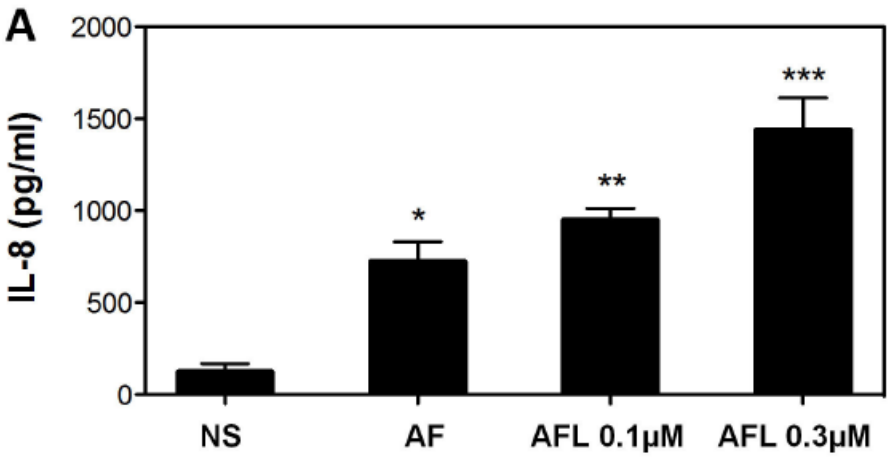


Figure 14: IL-8 concentrations after stimulation of bronchial epithelial cells with AFL [Houser J. et al., 2013]

2.8.6. Controverse studies

Another study showed conflicting results. According to Kerr S.C. et al., AFL was more described as a defense mechanism against *A. fumigatus* conidia, but not as a virulence factor. Although in vitro studies confirmed that AFL is responsible for binding of conidia to airway mucins as well as phagocytosis by lung macrophages, in vivo studies revealed that mice infected with conidia that lack of AFL are more likely to develop *Aspergillus* lung infection than with wildtype conidia [Kerr S. C. et al., 2016].

Taken together, the role of AFL is not completely understood and still remains a controverse topic. But as there is evidence for a contribution in the host-pathogen interaction, the pro-inflammatory response and consequently, the development of infection, our goal is to reveal its function and the role of possible inhibitors in more detail.

2.9. Actual treatment of aspergillosis

Released by the Infectious Diseases Society of America IDSA in 2016, first-line treatment for all invasive forms of aspergillosis is **voriconazole**. It shows best outcomes when applied to pulmonary aspergillosis, but is less applicable when used for other forms of aspergillosis.

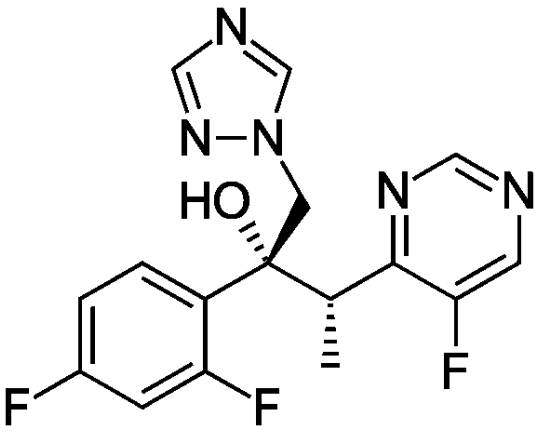


Figure 15: Voriconazole

An alternative to voriconazole for primary therapy is represented by **liposomal amphotericin B.** Furthermore, a new azole drug approved in 2015, named **isavuconazole**, represents another successful alternative to voriconazole in primary treatment. On contrary, the combination of antifungal drugs is not recommended for primary treatment. For preventative measures liposomal amphotericin B, caspofungin, micafungin or voriconazole are advised, whereas for high-risk patients in the prevention of invasive disease, posaconazole has precedence [Misch E.A. and Safdar N., 2016].

2.10. Problem of resistance to azoles

Although the number of cases is still low, azole resistance in *A. fumigatus* is increasing and evolves as a worldwide problem in public health [Verweij P.E. et al., 2016]. The mortality rate of patients with aspergillosis resistant to azoles ranges as high as 88 % [Van der Linden J.W., 2011]. On the one hand this increasing resistance results from the environment, derived from massive use of azoles in agriculture [Berger S. et al., 2017]. On the other hand, resistance emerges through long-term patient therapy with azoles [Verweij P.E. et al., 2016]. The main resistance mechanism is a point mutation in the gene CYP 51A [Snelders E. et al., 2008].

Researchers are now challenged to develop alternative treatment options, as in our case, the inhibition of the *A. fumigatus* lectin. Potential substances that inhibit this lectin could represent promising new drug targets for the treatment of *Aspergillus*-associated diseases.

3. AIM OF THIS WORK

The aim of this work is to present an alternative to the conventional treatments of aspergillosis. As at the moment, antifungal treatment is done by voriconazole and amphotericin B, which possess a high toxicity and aim to destruct or inhibit the proliferation of the pathogen, researchers are challenged to look for milder and safer methods. One approach would be to look into the corner of virulence factors. By deactivating a virulence factor which is responsible for the host-pathogen interaction, adherence of conidia to lung epithelial cells and consequently, the development of infection, it should be possible to attenuate the pathogenicity of the most frequent identified pathogen under the *Aspergillus* species, *A. fumigatus.*

In our case, the goal is to inhibit the AFL, *A. fumigatus* lectin, which is expressed by *A. fumigatus* conidia and with its 12 binding sites for fucosylated structures, it binds to bronchial epithelium. As the binding of this lectin is one of the key stages in the invasion process, implicated in the pro-inflammatory response and considered as the first step in the development of infection, the french chemistry research group CEISAM has synthesised 6 possible AFL inhibitors that will be tested. The goal is to investigate if these 6 molecules that are expected to inhibit the binding of AFL could represent promising new drugs for aspergillosis.

An interaction model of A549 pneumocytes and the spores of *A. fumigatus* in fluorescence and confocal microscopy shall be established. Therefore, A549 pneumocytes are grown on chamber slides and after confluence is reached, contaminated with the spores of *A. fumigatus*. For the better differentiation, A549 pneumocytes are stained in blue, the spores of *A. fumigatus* show autofluorescence by the attachment of GFP and the membranes of pneumocytes as well as external spores are stained in red. After standardisation of the interaction model, the impact of the molecules shall be tested. Moreover, another aim is to investigate the effect of different strains that were found to be resistant to azoles on the standardised interaction.

Future objectives of the project may be the realisation of an an ex vivo mucosal interaction model. Finally, for the best of the molecules, it is planned to carry out an in vivo model of aspergillosis after intra-tracheal infection in order to evaluate a system for the prevention of aspergillosis by nebulisation of the molecule.

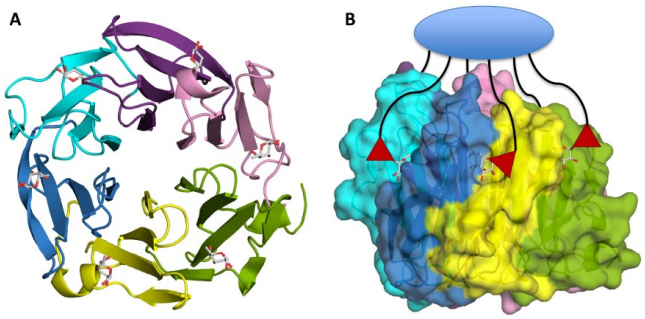


Figure 16: Binding of AFL

4. MATERIAL AND METHODS

4.1. Cell cultures

Working with cell cultures is indispensable in a microbiology research lab because it offers the opportunity to grow cells in an artificial setting, outside their natural environment, so called `in-vitro´. For a successful performance, a cell culture medium as well as a protease, e.g. trypsin, that separates cells from tissue, are necessary. The cell culture medium is essential for growing and consists of nutrients such as amino acids, vitamins, salts, glucose, but also growth factors, antibiotics or colour-indicators.

Generally, while working with cell cultures, it is distinguished between adherent cells, that need the attachment to a surface, e.g. fibroblasts, endothelial cells, epithelial cells, and suspension cultures, that are arranged detached, e.g. lymphocytes. Furthermore, the distinction between immortalised cells and primary cultures is done.

4.2. Cell culture medium

In our experiments, the RPMI 1640 with glutamine and $NaHCO_3$, catalogue number R 8758, was utilised. It had to be supplemented with ATB + 10 % FBS. For all washing steps, expired cell culture medium was used.

RPMI 1640 + ATB (100 µL: 100 mL of RPMI) + 10 % FBS
ATB = penicillin (10 000 IU/mL) + streptomycin (1mg/mL)

Inorganic Salts	g/L	**Vitamins**	g/L	**Amino Acids**	g/L
Calcium Nitrate • $4H_2O$	0,1	Riboflavin	0,0002	L-Histidine	0,015
Magnesium Sulfate (anhydrous)	0,04884	Thiamine • HCl	0,001	Hydroxy-L-Proline	0,02
Potassium Chloride	0,4	Vitamin B_{12}	0,000005	L-Isoleucine	0,05
Sodium Bicarbonate	2	**Other**		L-Leucine	0,05
Sodium Chloride	6	D-Glucose	2	L-Lysine • HCl	0,04
Sodium Phosphate Dibasic (anhydrous)	0,8	Glutathione (reduced)	0,001	L-Methionine	0,015
Vitamins		Phenol Red • Na	0,0053	L-Phenylalanine	0,015
D-Biotin	0,0002	**Amino Acids**		L-Proline	0,02
Choline Chloride	0,003	L-Arginine	0,2	L-Serine	0,03
Folic Acid	0,001	L-Asparagine (anhydrous)	0,05	L-Threonine	0,02
myo-Inositol	0,035	L-Aspartic Acid	0,02	L-Tryptophan	0,005
Niacinamide	0,001	L-Cystine • 2HCl	0,0652	L-Tyrosine • 2Na • $2H_2O$	0.02883
p-Aminobenzoic Acid	0,001	L-Glutamic Acid	0,02	L-Valine	0,02
D-Pantothenic Acid (hemicalcium)	0,00025	L-Glutamine	0,3		
Pyridoxine • HCl	0,001	Glycine	0,01		

Table 2: RPMI - 1640 media formulation

4.3. Incubator

The importance of the incubator was to provide a specific temperature of 37 °C and a gas mixture of 5 % CO_2 which represents perfect conditions for the growing of cell lines. During our experimentations, we worked with the Panasonic MCO-170AIC Co_2 incubator.

Figure 17: Incubator from outside

Figure 18: Incubator from inside

4.4. Laminar Air Flow

To avoid contamination and work under sterile conditions, the utilisation of a laminar air flow is essential. The main principle of a LAF is that air is filtered by HEPA filters where contaminations are removed and as a result, clean air reaches the working place. This air has a speed of 90 feet/min and moves in parallel patterns in the cabinet [Favero M.S. and Berquist K.R., 1968].

In general, it is distinguished between vertical and horizontal LAFs. As they also protect the user, in our experiments, a vertical LAF was used. Moreover, as we handled with the spores of *A. fumigatus*, which are highly infectious, all working steps included the utilisation of 2 different vertical LAFs at the same time, one for the preparation of the spores and another one for the growing and staining of the cell line.

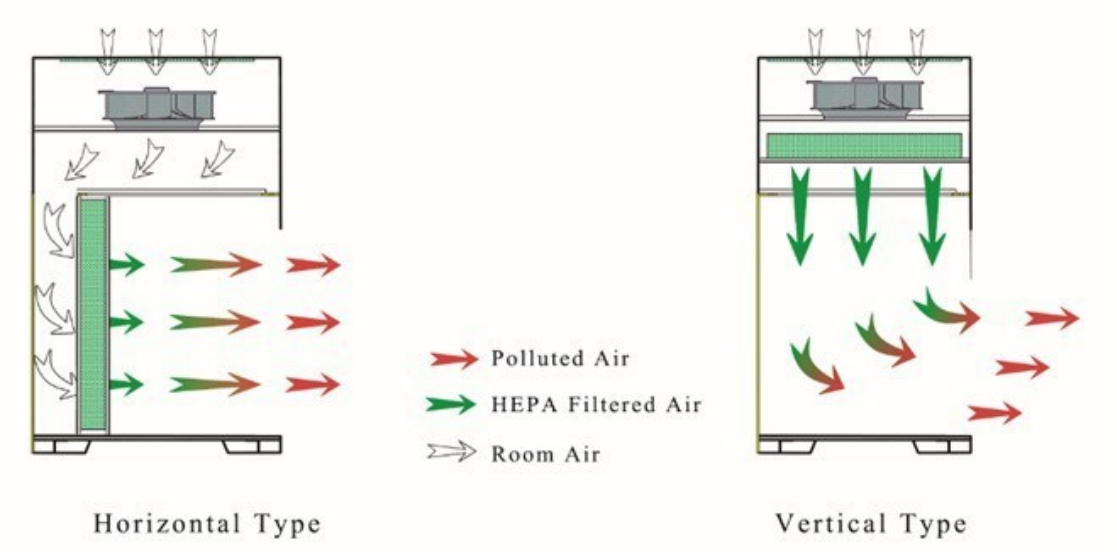


Figure 19: Horizontal vs. vertical LAF [www.Made-in-China.com]

Concerning the preparational steps, the working surface of the LAF was cleaned before and after each use with a disinfectant and the UV lamp had to be turned on around 15 min before utilisation. It was important to turn the UV lamp off during work as UV light can cause eye or skin damage such as cataract or cancer.

4.5. A549 cell line

The A549 cell line that represents a type II human alveolar epithelial cell line [Foster K.A. et al., 1998] with an adherent growth was utilised. This cell line is hypotriploid and derives from a lung carcinoma of a 58-year-old Caucasian male [A549 Cell Line Transfection Protocol Reagent Method, 2018]. It was grown in T75 cell culture flasks.

Figure 20: T75 cell culture flask with adherent A549 cell line

4.6. Preparation of the A549 cell line

4.6.1. PCR mycoplasma detection

Before starting to work, it was necessary to test if the A549 cell line was free of mycoplasma, which represents one of the main source of irritation in cell culture experiments. Therefore, the Venor®GeM OneStep mycoplasma detection kit for conventional PCR was utilised. This test uses PCR amplification for the detection of mycoplasma contamination, primarily *M. arginini, M. fermentans, M. hominis*, *M. hyorhinis, M. orale* and *M. salivarium* but also other less frequent strains. Each kit contains a OneStep mix, a rehydration buffer, a positive control DNA and a Tris/EDTA buffer for 25, 50, 100 or 250 reactions. All necessary components for PCR are included, a taq polymerase, primers and dNTPs.

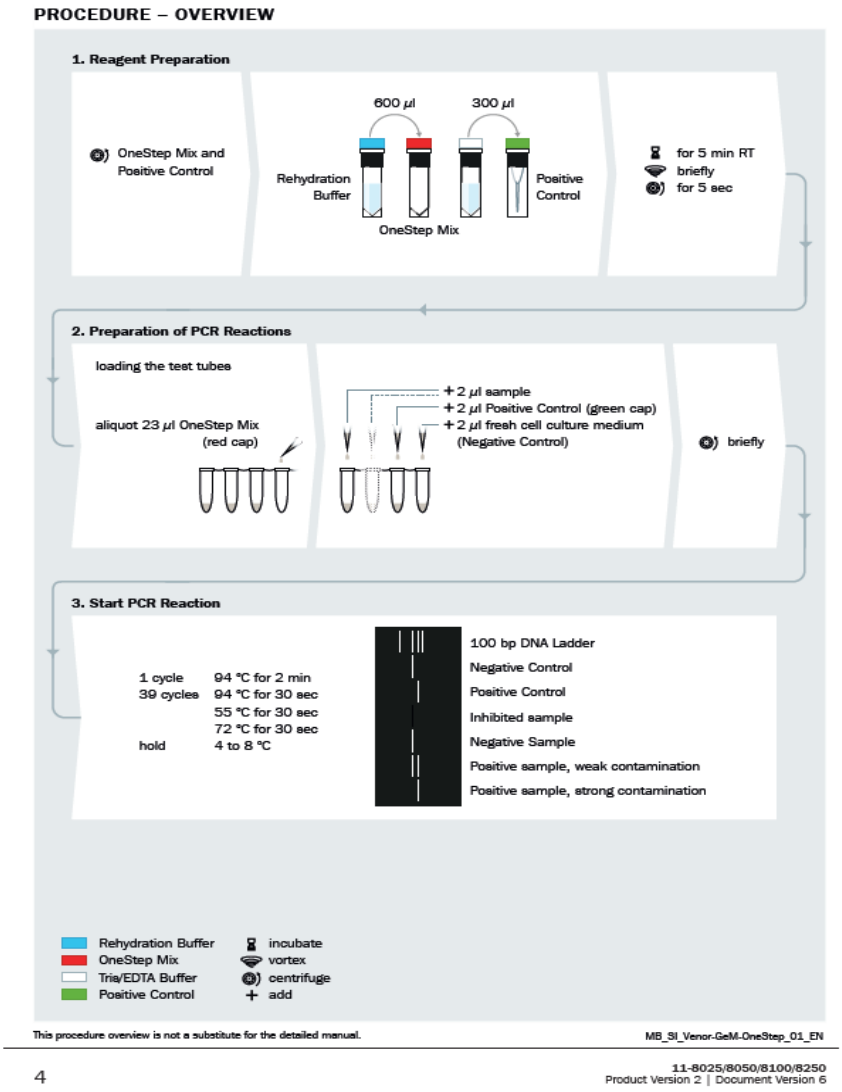


Figure 21: Procedure overview of the PCR mycoplasma detection kit [Minerva Biolabs, 2018]

4.6.2. Working steps of PCR mycoplasma detection

The first step, reagent preparation, was already done beforehand, so it was started with the second part, the preparation of the PCR reactions. 2 negative controls, 2 samples and 1 positive control, each in one PCR reaction tube, were prepared. Therefore, 23 µL OneStep mix were transferred into 5 PCR tubes, closed, vortexed and stored on ice.

Concerning the preparation of the sample, 100 µL from the supernatant of the cell culture were transferred into a reaction tube, heated for 5 min at 95 °C and centrifuged at 11 000 rpm for 15 sec. To avoid additional sample preparation, only the cell culture supernatant was finally transferred into the PCR reaction tubes of the sample.

Negative control	23 µL OneStep mix + 2 µL cell culture medium RPMI 1640 + ATB + 10 % FBS
Sample	23 µL OneStep mix + 2 µL cell culture supernatant
Positive control	23 µL OneStep mix + 2 µL positive control

Table 3: Preparation of PCR reaction tubes

After this step, the PCR reaction tubes were closed, centrifuged again and placed in the PCR cycler.

Figure 22: PCR cycler, Eppendorf Mastercycler EP Gradient

PCR Amplification:

1 cycle 94 °C for 2 min
39 cycles 94 °C for 30 sec
55 °C for 30 sec
72 °C for 30 sec
Hold at 4 °C – 8 °C

After the realised PCR amplification, an agarose gel electrophoresis followed. Therefore, a 2 % agarose gel out of 20 mL of TAE-puffer and 400 mg agarose was prepared. It was heated in the microwave until the whole powder was dissolved and the solution was clear. For the application on the agarose gel, the following solutions were used:

Marker	3 µL Buffer
	3 µL Water
	5 µL PCR marker
Sample	3 µL Buffer
	8 µL Sample
Sample ½:	3 µL Buffer
	4 µL Water
	4 µL Sample

Table 4: Solutions of the agarose gel

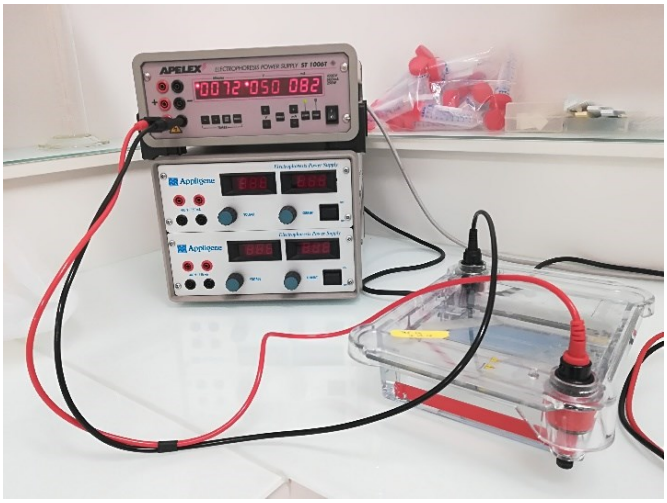


Figure 23: Electrophoresis setting: Apelex, Electrophoresis Power Supply ST 1006T & Horizontal electrophoresis apparatus, Wide Mini-Sub® Cell GT Systems

4.6.3. Interpretation of the PCR

The internal control DNA should rise at 191 bp which means that the PCR worked out successfully. The mycoplasma, depending on the species, should rise at around 270 bp. Just for explanation, in our experiment, not only the A549 cell line was tested, but also HeLa cells, that were used for another experiment.

4.6.4. Results of the PCR

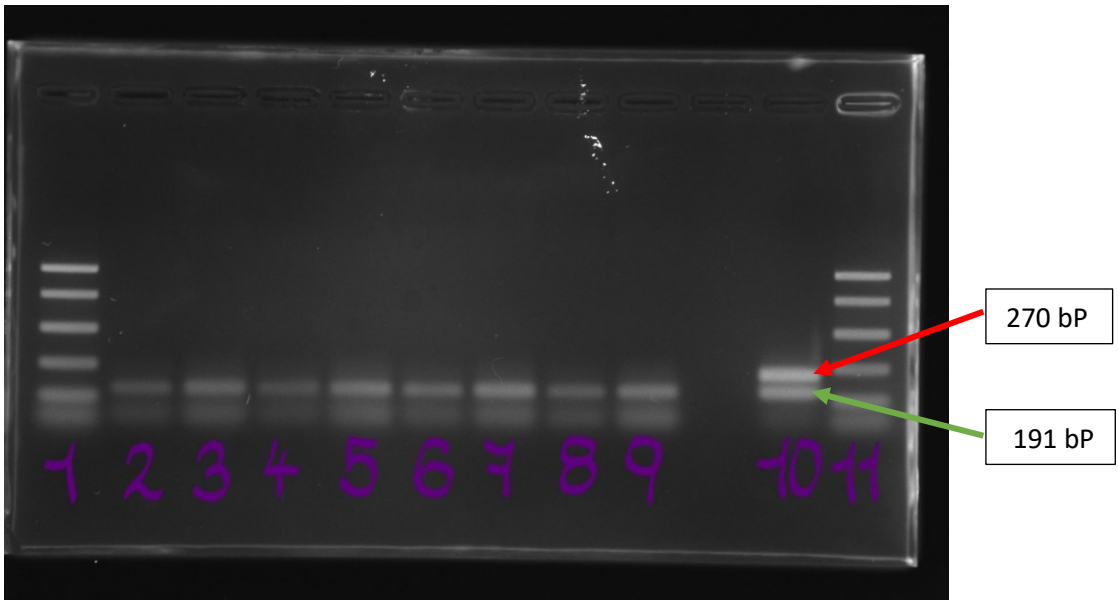


Figure 24: Results of the PCR

1	Marker: DNA ladder
2	Sample ½ negative control A549 - RPMI 1640 + ATB + 10 % FBS
3	Sample pure negative control A549 - RPMI 1640 + ATB + 10 % FBS
4	Sample ½ negative control HeLa - RPMI 1640 + ATB + 10 % FBS
5	Sample pure negative control HeLa - RPMI 1640 + ATB + 10 % FBS
6	Sample ½ A549 cells
7	Sample pure A549 cells
8	Sample ½ HeLa cells
9	Sample pure HeLa cells
10	Positive control
11	Marker: DNA ladder

Table 5: Solutions of the PCR

A separated band at 191 bp in every lane could be observed which indicates that the PCR worked out successfully. Luckily, in contrast to the positive control, no rise of a band at 270 bP was detected in the samples, which means that the A549 cell line was free of mycoplasma and we could start to work.

4.7. Maintenance of the A549 cell line

4.7.1. Changing the cell culture medium

To provide the A549 cell line constantly with fresh nutrients, the cell culture medium had to be changed every 2-3 days. Therefore, under the LAF, the old cell culture medium was replaced by new cell culture medium RPMI 1640 + ATB + 10 % FBS. To prepare this medium for use, it was taken out of the refrigerator and grown warm in the water bath until a physiological temperature of 37 °C was reached. In case too many dead cells were observed under the microscope, one washing step with expired cell culture medium was performed to remove the dead cells.

Working steps of changing the cell culture medium (every 2-3 days)

- Preparing the LAF for use
- Warming the cell culture medium RPMI 1640 + ATB + 10 % FBS and the expired cell culture medium on the water bath until physiological temperature of 37 °C was reached
- Throwing away the old cell culture medium into the trash
- (One washing step with expired cell culture medium to remove the dead cells)
- Adding 15 mL (when changed again after 2 days) or 20 mL (when changed again after 3 days) new culture medium RPMI 1640 + ATB + 10 % FBS
- Incubation at 37 °C and 5 % CO_2

Figure 25: Working place- Changing the cell culture medium

4.7.2. Trypsinisation

To maintain good growing conditions, cells had to be trypsinised once a week. Trypsinisation is essential when adherent cells reach around 70-90% confluence. Confluence means that cells cover the whole surface of the flask and cannot grow further. The trypsinisation which is called `passaging´ can only be done around 60 times at the A549 cell line and every trypsinisation counts for one `passage´.

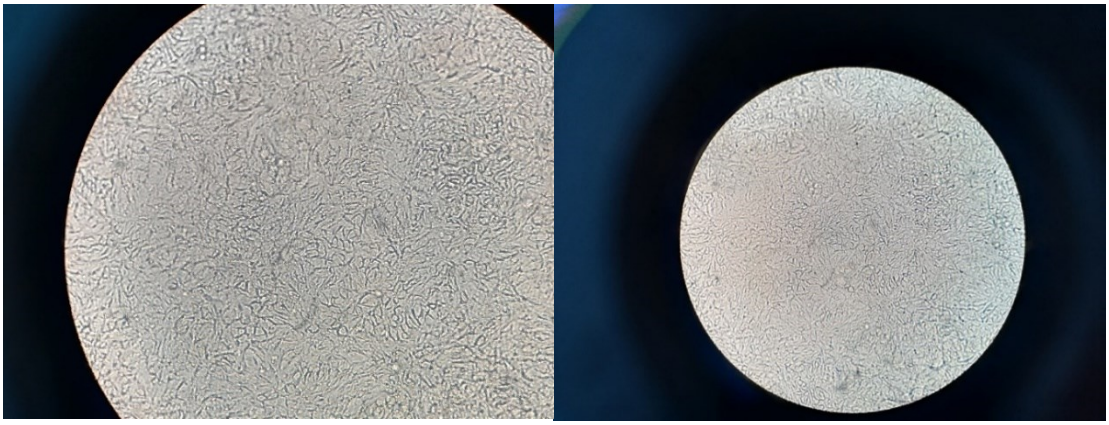


Figure 26: A549 cell line before trypsinisation, when confluence was reached

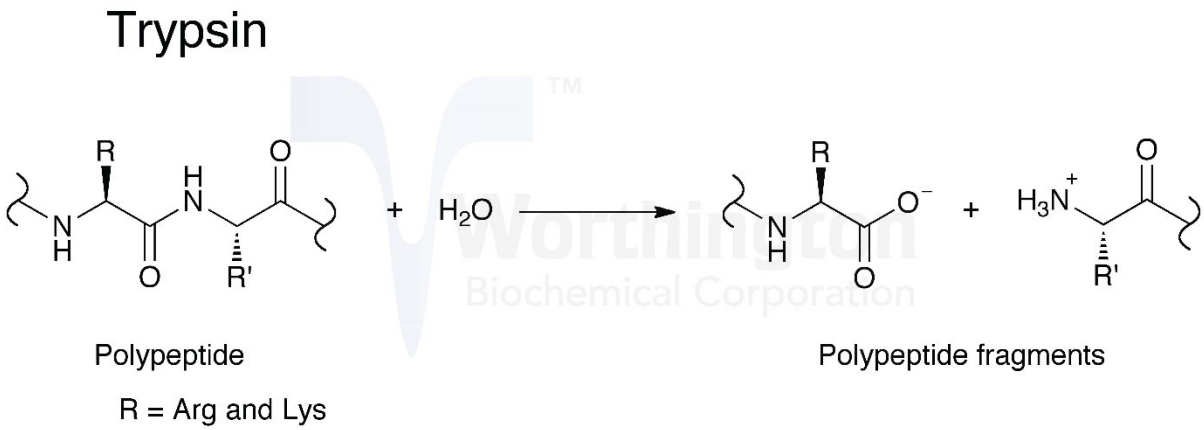


Figure 27: Reaction of trypsin [Worthington Biochemical Cooperation, 2018]

A protease, in our case trypsin, breaks down the proteins by cutting on the C-terminal side of lysine or arginine. Trypsin is mostly combined with EDTA that increases its activity by complexing calcium which is essential for cell adhesion. As a result, the complex of trypsin/EDTA detaches the cells from the surface of the flask until the cell layer is dispersed. It brings them into suspension and after dilution, they can be transferred into a new flask.

Figure 28: Working place- Trypsinisation

Working steps of trypsinisation (once a week):

- Preparing the LAF for use
- Warming the cell culture medium RPMI 1640 + ATB + 10 % FBS, the expired cell culture medium and a tube of trypsin (4 mL) on the water bath until physiological temperature of 37 °C was reached
- Throwing away the old cell culture medium into the trash
- 2 washing steps with 10 mL of expired cell culture medium to remove the dead cells
- Putting the tube of trypsin/EDTA (4 mL) into the flask, shaking gently until it reaches the whole surface of the cell layer
- Incubation at 37 °C and 5 % CO_2 until the cells are detached from the surface of the flask

Figure 29: A549 cells detached from the flask

- Adding 10 mL of new cell culture medium RPMI 1640 + ATB + 10 % FBS into the successfully trypsinised flask (FBS stopps the action of trypsin!)
- Homogenising with the detached cells by suction and discharge
- Preparation of 2 new flasks
- Distribution of the detached cells into 2 new flasks
- Incubation at 37 °C and 5 % CO_2

4.8. Lab-Tek chamber slide

Lab-Tek chamber slides from Thermo Fisher Scientific enable the cultivation of adherent cells directly on a glass slide for microscopy without transferring them before observation or coloration. In our experiments, the Nunc® Lab-Tek® Chamber Slide™ system, catalogue number 177399 was utilised. It consists of 4 removable chambers mounted on a glass slide with a cover. Concerning its geometry, each slide has a size of 75 x 25 mm and the growth area per well accounts for 1,8 cm^2 with a recommended filling volume of 500-900 µL.

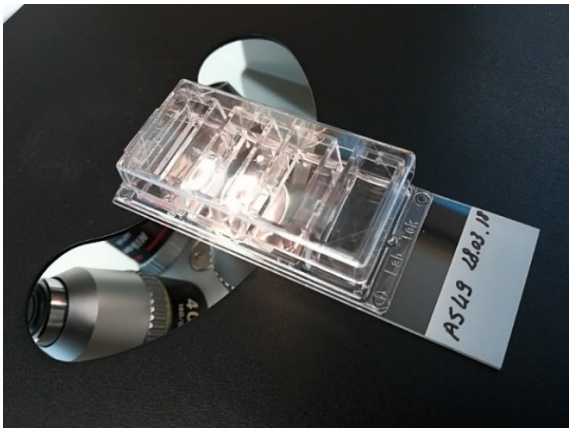


Figure 30: Lab-Tek chamber slide

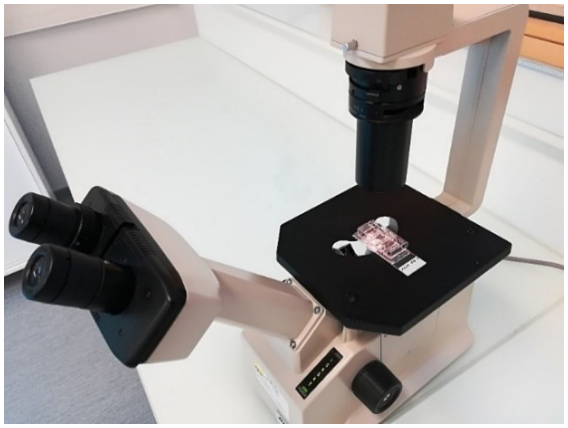


Figure 31: Lab-Tek chamber slide under the microscope

4.9. Ibidi chamber slide (8 wells)

With the similar purpose, but out of plastic and not glass, the Ibidi® cells in focus µ-slide 8 well (Biovalley S.A., 77601- Marne la Vallée Cedex 3) has a high quality similar to glass and can therefore be utilised for all kind of fluorescence experiments. In the first experiments, the Ibidi chamber slide with 8 wells, as shown in **figure 32**, was used. Concerning its geometry, the dimensions of the wells are 9,4 x 10,7 x 6,8 mm and the growth area per well accounts for 1,0 cm^2 with a recommended filling volume of 300 µL per well.

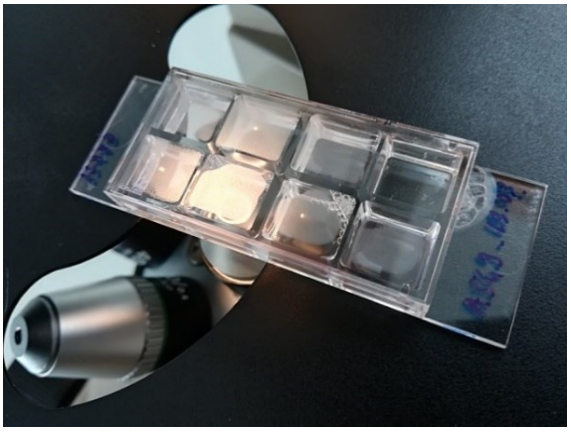


Figure 32: Ibidi chamber slide 8 wells

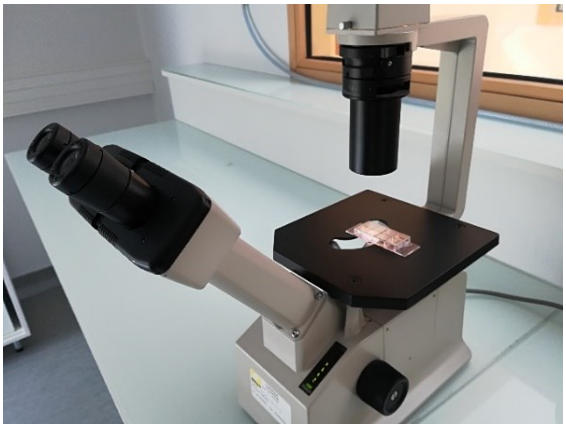


Figure 33: Ibidi chamber slide 8 wells under the microscope

4.10. Ibidi chamber slide (12 wells)

In the following experiments, the Ibidi® cells in focus µ–chamber 12 well (Biovalley S.A., 77601- Marne la Vallée Cedex 3) was utilised for the cultivation of cell cultures as well as fluorescence staining. The big advantage of this chamber slide is represented by its silicone chamber that is self-adhesive and easy to remove. It is mounted on a standard microscopy glass slide and can be used for upright microscopy, but also long-term storage of samples from cell culture microscopy. Concerning its geometry, the dimension of the 12 wells are 7,5 x 7,5 x 8 mm with a growth area per well of 0,56 cm^2 and a filling volume of 250 µL.

Figure 34: Ibidi chamber slide 12 wells

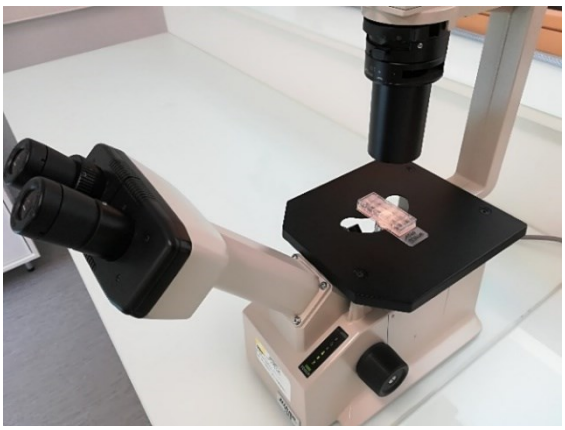


Figure 35: Ibidi chamber slide 12 wells under the microscope

4.11. Hemocytometer

The hemocytometer or `La Cellule de Malassez` (French name) allows the counting of cells in suspension in a solution. It was invented by the french doctor and scientist Louis-Charles Malassez and represents a cover glass with an engraved checked pattern of 100 small rectangles. 25 of these rectangles are further divided into 20 squares, 25 are just divided and the other 50 rectangles are plain.

The number of cells in one square accounts for 0,01 µL and the number of cells in a row (which means 10 squares) accounts for 0,1 µL. As a result, when the number of cells in a row is multiplied by 10 000 the number of cells in 1 mL is obtained.

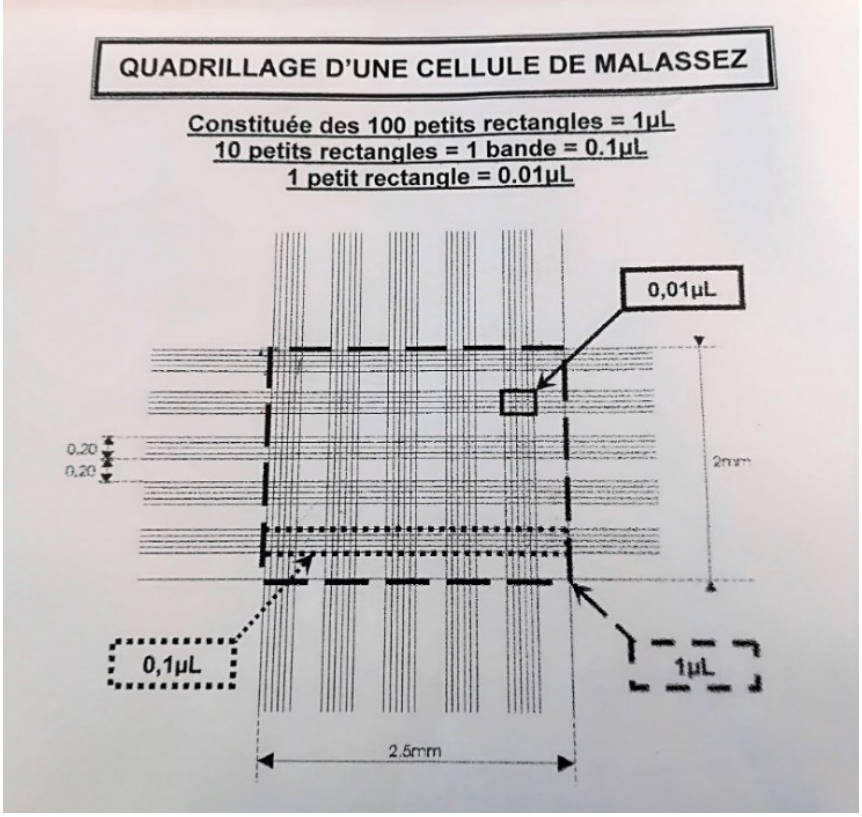


Figure 36: Counting principle of the hemocytometer or `La Cellule de Malassez`

4.11.1. Working steps of counting in the hemocytometer

Concerning the working steps of counting in the hemocytometer, after trypsinisation of a cell culture flask and homogenising the detached cells with new cell culture medium RPMI 1640 + ATB + 10 % FBS, an aliquot of this suspension was mixed with trypan blue solution at a certain proportion.

A volume of 20 µL of this mixture was put on a cover glass C-Chip™ from NanoEnTek, and the rows of the engraved pattern on the glass slide were counted under the light microscope. By considering the dilution with trypan blue solution, the number of cells in the suspension was calculated. With the rest of the suspension a centrifugation at 800 rpm and 20 °C for 5 min was performed and the obtained cell pellet was resuspended in the required volume of cell culture medium RPMI 1640 + ATB + 10 % FBS to reach the desired concentration. This required volume was easily obtained by the formula C_i x V_i = C_f x V_f.

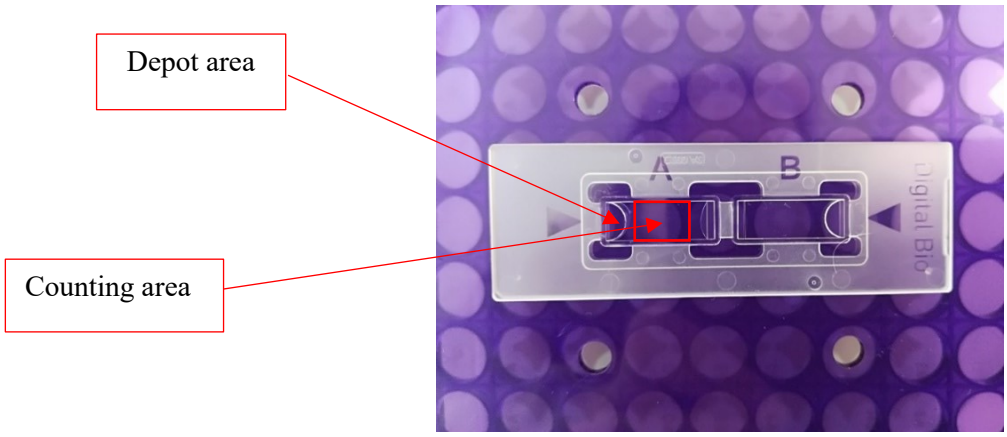


Figure 37: C-Chip™ from NanoEnTek

4.11.2. Trypan blue solution

Trypan blue solution represents an azo dye derived from toluidin. It is used in the hemocytometer to distinguish between dead and viable cells under the light microscope, as only dead cells are stained in blue. Its principle relies on a difference in cell permeability. Viable cells have an intact cell membrane and exclude the dye, in comparison to dead cells, where the cell membrane is damaged and the stain can easily penetrate [Hi Media cell culture, Enabling Breakthroughs, Product information, Trypan Blue]. As a result, colourless viable cells are counted in the hemocytometer whereas blue dead cells not.

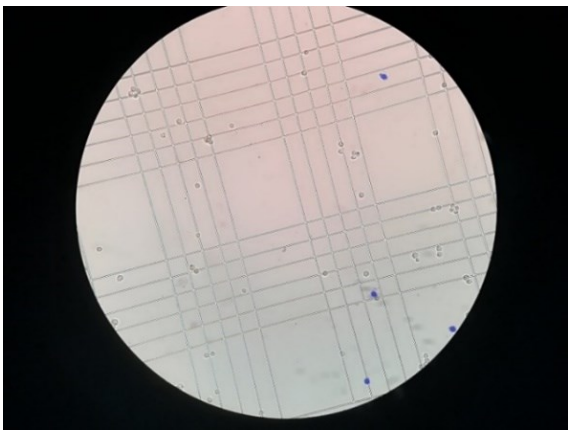


Figure 38: Viable (colourless) and dead (blue) cells in the hemocytometer

4.12. Centrifuge

The centrifuge, which works under the sedimentation principle, has the ability to separate substances such as suspensions, emulsions as well as gas mixtures by their density. By accelerating them at centrifugal force, denser substances move to the ground whereas substances with a lower density move to the top. In our experiments, the Heracleus Multifige X3 centrifuge from Thermo Fisher Scientific was utilised.

Figure 39: Centrifuge from outside

Figure 40: Inside the centrifuge

4.13. Homogeniser

During the whole experimentations, good homogenisation of samples was important, provided on the one hand by the vortex, VELP Scientifica, Zx4 Advanced IR Vortext Mixer and on the other hand, by the Gyro-rocker, Stuart, SSL3.

Figure 41: Vortex

Figure 42: Gyratory rocker

4.14. Strains of *A. fumigatus*

Aspergillus fumigatus – 90 – Green Fluorescent Protein
Aspergillus fumigatus – ATCC
Aspergillus fumigatus – TR 34 121
Aspergillus fumigatus – TR 34 089
Aspergillus fumigatus – TR 46 087
Aspergillus fumigatus – TR 46 090
Aspergillus fumigatus – WT 110
Aspergillus fumigatus – WT 08

Table 6: Different strains of *A. fumigatus*

As shown in **table 6**, we worked with 8 different strains of *A. fumigatus* during the experimentations. Mutant as well as wildtype strains were found to be resistant to azoles and further specification about *A. fumigatus*-90-GFP can be found in chapter **4.14.3**.

4.14.1. Subculturing

Subculturing a fungus means that a part of its spores are transferred from old culture to fresh medium and afterwards, incubated at a specific temperature where the fungus can grow. Therefore, the surface of the agar of a culture tube of *A.fumigatus* was scratched with a loop and the obtained spores were transferred into a new culture tube consisting of sabouraud-chloramphenicol. This culture tube was then incubated at 37 °C until the fungus grew. During our experiments, it was important to constantly subculture the strains of *A.fumigatus* as it was not possible to work with strains that were older than 1 week.

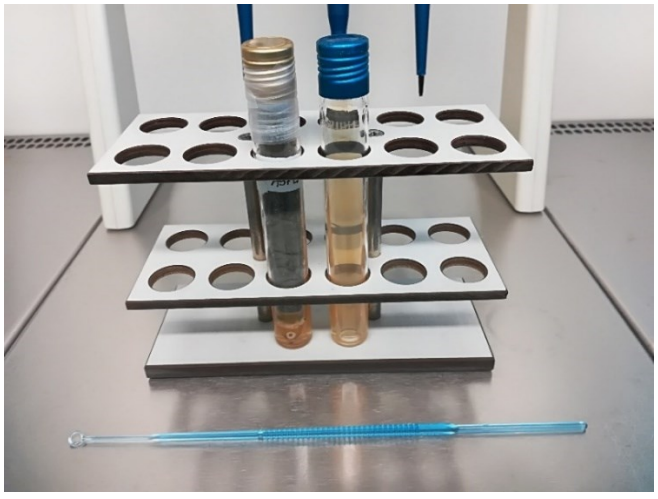


Figure 43: Working place- Subculturing

4.14.2. Filtering

All spore suspensions of *A. fumigatus* had to be filtered trough a filter whatman with the reference number 1001325 dutscher and a pore size of 11 µm in addition to a support swinnex with the reference number 044094 dutscher.

4.14.3. *Aspergillus fumigatus*-GFP

The spores of *A. fumigatus*-GFP show autofluorescence, generated by the attachment of Green fluorescent protein. GFP is isolated by the jellyfish *Aequorea victoria* and emits intense green light when rayed with blue and ultraviolet light.

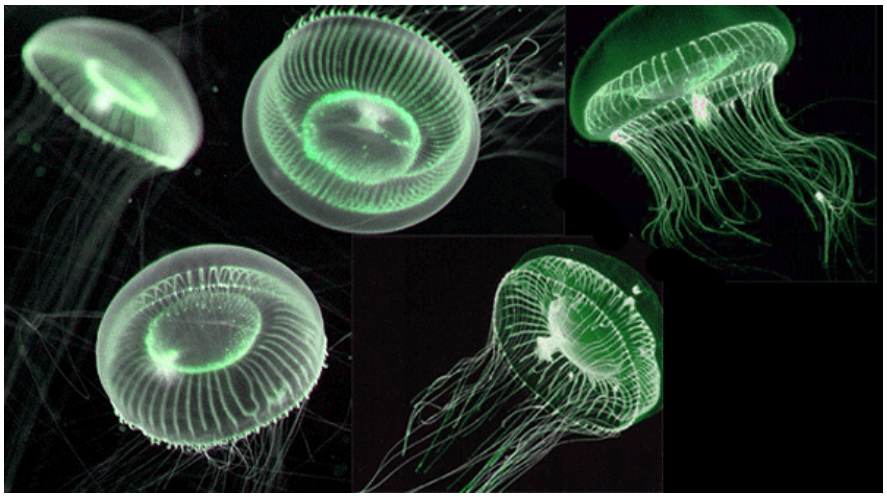


Figure 44: The jellyfish *Aequorea victoria* [Mayers K., 2016]

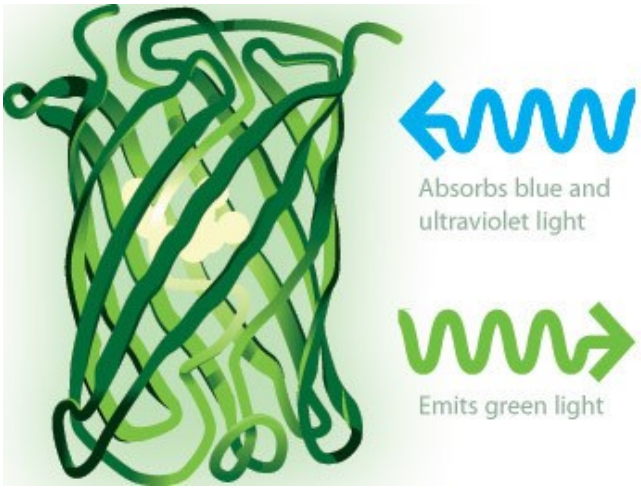


Figure 45: Absorption and emission of GFP [Jeevanshudhawan, 2015]

GFP consists of 238 amino acids where 3 of them - the residues 65-67, serine-tyrosine-glycine- form the chromophore which is responsible for emitting the green light. GFP shows maximal fluorescence when excited at 400 nm and has a minor excitation peak at 475 nm. Fluorescence emission happens at 509 nm. [John Innes Centre]. The connection between a visible protein/cell and GFP is done through DNA-technology, by positioning it behind a promotor and letting it express in another organism. This finding was revolutionary for life sciences and was awarded with the nobel price in chemistry in 2008 [Jeevanshudhawan, 2015].

4.15. Staining

4.15.1. Blue nuclei staining

NucBlue® reagent is used for staining the nuclei of cells, in our case the nuclei of the A549 pneumocytes. This blue fluorescence stain is excited by 360 nm UV light when bound to DNA with a maximum emission at 460 nm. The detection happens through a blue/cyan filter as a DAPI filter, a fluorescence stain that binds to DNA. In our experiments, the NucBlue® Live ReadyProbes® reagent, catalogue number R37605, Hoechst 33342 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazol) from Thermo Fisher Scientific was utilised.

4.15.2. Red membrane staining

As it was expected to facilitate the differentiation between internalised spores on the one hand and external spores on the other hand, membranes of pneumocytes as well as external spores were stained in red using 2 different red fluorescence stains. In the first experiments, SYTO® 17 red fluorescent nucleic acid stain - 5 mM solution in DMSO, catalogue number S-7579, was utilised. In the presence of DNA, SYTO® 17 has an absorption maxima at 621 nm and an emission maxima at 634 nm. Afterwards, wheat germ agglutinin (WGA)-Alexa was attempted.

4.16. Test substances

All test substances represent different saccharides that were already patented. As their structure was not published yet, codes for each molecule (45,46,47,72,74,75) were used concerning their identification.

4.17. Microscopy

4.17.1. Phase contrast microscopy

Phase contrast microscopy is an important technique in light microscopy, to visualise transparent and unstained objects, especially living cells and microorganisms. On contrary to stained objects, they do not absorb light. As a result, it penetrates through the object and dependant on the thickness and density, it leads to a phase shift in the light waves. Unfortunately, this phase shift is very small and invisible for human eyes, but can be enhanced by a phase-plate in the objective, resulting in a difference in brightness. Therefore, in phase contrast microscopy, tiny phase shifts are converted into changes in amplitude, which can then be observed as differences in image contrast [Murphy D.B., 2018]

During the whole experimentations, samples and cell culture flasks were observed through the Nikon TMS inverted phase contrast microscope. Especially phenomena like confluence, dead cells, which emerge as dark blue stains under the microscope, as well as the disruption of the cell layer by trypsin could be observed successfully.

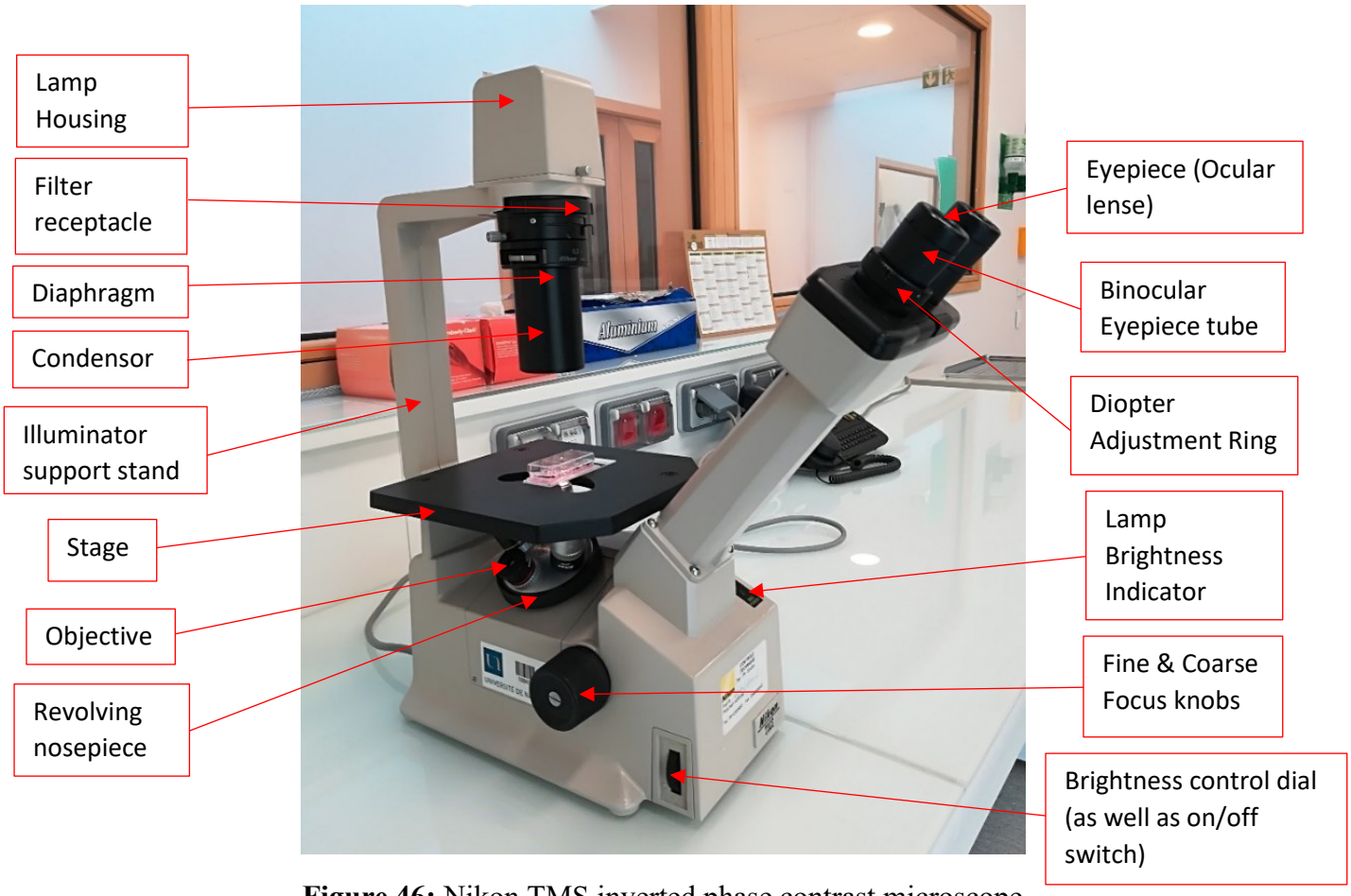


Figure 46: Nikon TMS inverted phase contrast microscope

4.17.2. Fluorescence microscopy

Fluorescence microscopy represents another unique technique in light microscopy, based on the phenomenon of fluorescence. Fluorescence happens when the excitation of a substance with short wave length leads to the emission of light with longer wave length. This phenomenon relies on the fact that an electron from the outer shell of an atom is transferred into a higher energy level. When it returns to the ground state, it sends out visible light [Powell A.L., 1947].

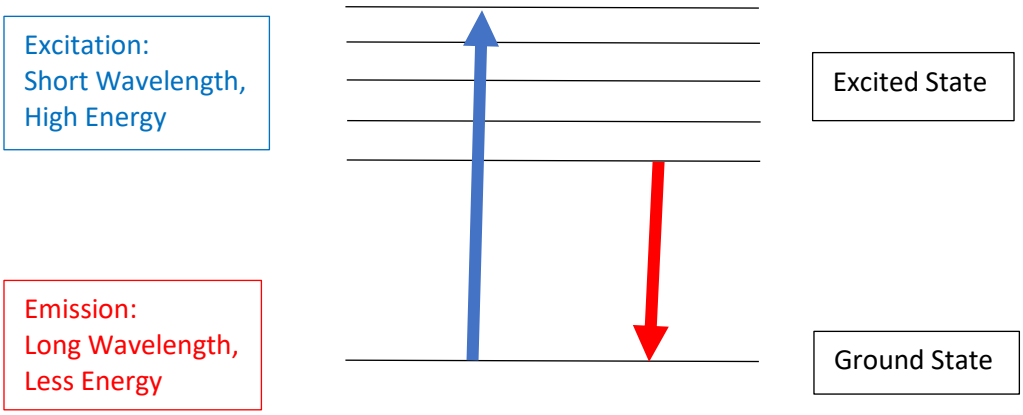


Figure 47: General principle of fluorescence

On the contrary to phosphorescence, where light emission can persist up to several days, light emission in fluorescence ceases very quickly when the energising source is removed, after around 10^{-8} seconds [Powell A.L., 1947].

Moreover, in light microscopy, the image is generated by visible light that radiates through the object, whereas in fluorescence microscopy, production of the image happens directly in the specimen, through light of much higher intensity. This specimen can either show autofluorescence, as in our experiments the spores of *A. fumigatus*-GFP, or a fluorochrome, which represents a fluorescent chemical compound, can be added.

During our experiments, the fluorescence microscope Zeiss Axioskop MC 100 was used to observe the A549 pneumocytes through the blue and the spores of *A. fumigatus*-GFP through the green filter. By changing the filters, cells as well as spores in one focus could be counted and a calculation of adherent and internalised spores/cell was performed.

Figure 48: Upright fluorescence microscope, Zeiss Axioskop MC 100

4.17.3. Confocal microscopy

Confocal microscopy represents a special technique in fluorescence microscopy where pictures in greater resolution, sensitivity and contrast are produced [Rigby P.J. and Goldie R.G., 1999]. The excitation light, which is represented by a laser, only focuses on one small area, so there is never a complete image of the whole sample. This small area can even be around 0.5 μm, which is nearly as small as the wavelength of light itself [Nwaneshiudu A., 2012]. The technique of confocal microscopy relies on the fact that optical sectioning of two dimensional images leads to the construction of three dimensional images built up by the detector. Other major advantages of this technique are the reduction of background information, which could represent a source of irritation in light microscopy, and the possibility to control depth of field [Fellers T.J. and Davidson M.W., 2012].

During our experiments, the Nikon Eclipse Ti, N-SIM confocal microscope was used to verify if all of the fluorochromes worked out properly. Moreover, three dimensional images from the interaction of spores of *A.fumigatus* and A549 pneumocytes could be achieved.

Figure 49: Room of the confocal microscope

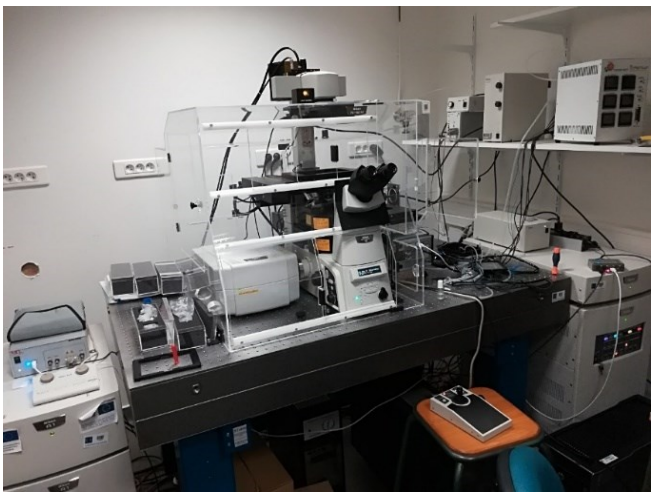


Figure 50: Confocal microscope, Nikon Eclipse Ti, N-SIM

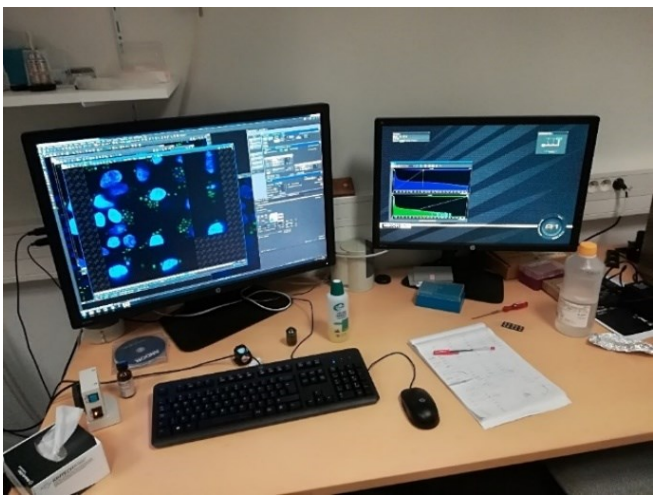


Figure 51: Screens of the confocal microscope

5. WORKING STEPS AND RESULTS

5.1. Working step 1: Nuclei staining with NucBlue®

The first working step of establishing a confluent cell culture of A549 pneumocytes which can then be stained and observed under the microscope included the application of 500 µL cell suspension into 4 wells of a Lab-Tek chamber slide. In this experiment, seeding of growth chambers of 500 000 cells/well which means 500 µL of a suspension of 10^6 cells/mL was performed.

Therefore, a trypsinisation and afterwards, the counting of the cells in the hemocytometer followed. After the already explained working steps of trypsinisation, 15 mL of new cell culture medium RPMI 1640 + ATB + 10 % FBS were added into the successfully trypsinised flask of 4 mL, which results in an initial volume of 19 mL. This volume was pipetted a couple of times to peel the cells off the wall of the flask and homogenised by suction and discharge. The 19 mL were then transferred into a recipient of 50 mL and again, homogenised, as mentioned before.

Concerning the counting in the hemocytometer, a volume of 20 µL was removed and mixed with another 20 µL of trypan blue solution, which means a dilution of ½ for the counting. Around 20 µL of this dilution were put on a glass slide C-Chip™ NanoEnTek and the rows (10 squares) were counted under the light microscope.

Figure 52: Working Place- Preparation for the counting in the hemocytometer

In one row (10 squares) the average of counted cells resulted in 45,33 cells. This means 45,33 cells/0,1 µL and consequently 4,53 x 10^5 cells/mL. As a dilution of ½ was performed, this number had to be multiplied by 2: 4,53 x 10^5 cells/mL x 2 = 9,06 x 10^5 cells/mL.

C_i = Initial concentration = 9,06 x 10^5 cells/mL
C_f = Final concentration = 10^6 cells/mL
V_i = Initial volume =19 mL
V_f = Final volume = ?

$$C_i \times V_i = C_f \times V_f$$
$$Vf = (C_i \times V_i) / C_f$$
$$(9{,}06 \times 10^5 \text{ cells/mL} \times 19 \text{ mL}) / 10^6 \text{ cells/mL} = 17{,}21 \text{ mL} \sim 17 \text{ mL}$$

With the rest of the recipient of 50 mL a centrifugation at 1200 rpm for 3 min at 20 °C was performed. The supernatant was discharged. The obtained cell pellet was resuspended in the beforehand calculated volume of new cell culture medium RPMI 1640 + ATB + 10 % FBS, which resulted in 17 mL, so that a final concentration of 10^6 cells/mL of cell culture medium RPMI 1640 + ATB + 10 % FBS was reached. In every well 500 µL at a concentration of 10^6 cells/mL, which means 500 000 cells/well were seeded in the 4 wells of the Lab-Tek chamber slide.

To maintain good growing conditions, on the next day, one washing step with expired cell culture medium followed as well as the old medium was replaced by new cell culture medium RPMI 1640 + ATB + 10 % FBS.

When confluence of the A549 pneumocytes was reached, the staining of the nuclei followed. Solutions of fluorochromes from NucBlue® reagent of 5 µL, 10 µL, 20 µL and 50 µL per 500 µL cell culture medium RPMI 1640 + ATB + 10 % FBS were prepared to see from which solution on the staining was lost. The solutions were incubated for 30 min at 37 °C and 5 % CO_2 to reach a bright nuclei staining. After one washing step with expired cell culture medium, the medium was changed to new cell culture medium RPMI 1640 + ATB + 10 % FBS. After observation under the fluorescence microscope, best outcomes were observed at the solution of 20 µL and consequently, this solution was used for the next experiments.

Figure 53: Working Material- Staining of the Lab-Tek chamber slide

500 µL of 10^6 cells/mL 5 µL fluorochrome	500 µL of 10^6 cells/mL 10 µL fluorochrome	500 µL of 10^6 cells/mL 20 µL fluorochrome	500 µL of 10^6 cells/mL 50 µL fluorochrome

Table 7: Distribution of cells and fluorochrome in the Lab-Tek chamber slide 4 wells

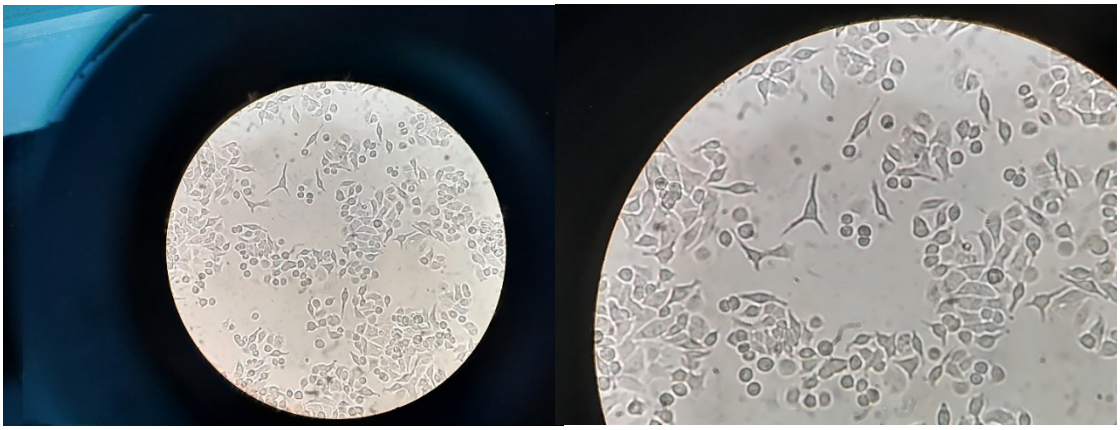


Figure 54: A549 cell line at around 50 % confluence

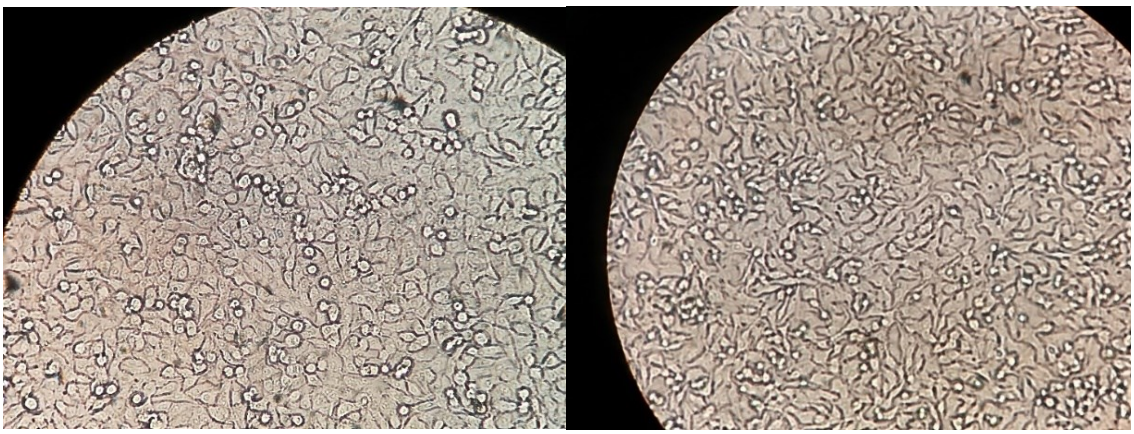


Figure 55: A549 cell line at around 70-90% confluence, shortly before staining

5.2. Working step 2: Contamination with the spores of *A. fumigatus*-GFP

Working step 2 started with the preparation of a new Lab-Tek chamber slide with 4 wells. Each well was prepared with 500 µL at a concentration of 10^6 cells/mL, which means 500 000 cells/well. After trypsinisation of a cell culture flask, 10 mL of new cell culture medium RPMI 1640 + ATB + 10 % FBS were added into the successfully trypsinised flask of 4 mL and well homogenised, as mentioned in **working step 1**. The 14 mL (10 mL cell culture medium + 4 mL trypsin) were transferred into a recipient of 50 mL and again, well homogenised.

Concerning the counting in the hemocytometer, 50 µL of this cell suspension were removed and mixed with 50 µL of trypan blue solution, which means a dilution of ½ for the counting. Around 20 µL of this dilution were put on a glass slide C-Chip™ NanoEnTek.

Under the light microscope, the average of counted cells in a row (10 squares) resulted in 108 cells, which means 108 cells/0,1µL and consequently 108 x 10^4 cells/mL. As a dilution of ½ was performed, this number had to be multiplied by 2: 108 x 10^4 cells/mL x 2 = 2,16 x 10^6 cells/mL in the suspension of 14 mL.

C_i = Initial concentration = 2,16 x 10^6 cells/mL
C_f = Final concentration = 10^6 cells/mL
V_i = Initial volume =14 mL
V_f = Final volume = ?

$$C_i \times V_i = C_f \times V_f$$
$$Vf = (C_i \times V_i) / C_f$$
$$(2{,}16 \times 10^6 \text{ cells/mL} \times 14 \text{ mL}) / 10^6 \text{ cells/mL} = 30{,}24 \text{ mL} \sim 30 \text{ mL}$$

The other part of the recipient of 50 mL was centrifuged at 800 rpm for 5 min at 20 °C. The supernatant was discharged. The obtained cell pellet was resupended in the beforehand calculated volume of 30 mL to reach a final concentration of 10^6 cells/mL. In every well 500 µL at a concentration of 10^6 cells/mL, which means 500 000 cells/well were added. The Lab-Tek chamber slide was incubated at 37 °C and 5 % CO_2.

After one day, one washing step with expired cell culture medium followed. To provide the cells with fresh nutrients, the medium was changed to new cell culture medium RPMI 1640 + ATB + 10 % FBS.

When confluence was reached, 2 suspensions of spores from *A. fumigatus*-GFP were prepared, one at a concentration of 10^6 spores per mL and the other one at a concentration of 5 x 10^5 spores per mL of cell culture medium RPMI 1640 + ATB + 10 % FBS.

The goal at this working step was to see which of the spore suspensions shows best outcomes when contaminated with the A549 pneumocytes and can be used for further experiments.

Figure 56: Culture tube of *A. fumigatus*-GFP

Therefore, 1 mL of 0,01 % Tween 20 were added into the culture tube of *A. fumigatus*-GFP and the surface of the agar was scratched with a loop. Additionally, suction and discharge with a transfer pipette was performed to remove the spores from the agar. The obtained spore suspension was aspirated and transferred into a pot of 30 mL. The procedure was repeated with another 1 mL of 0,01 % Tween 20 to obtain a final volume of 3 mL unfiltered spore suspension. With a syringe, this spore suspension was filtered into a new pot of 30 mL.

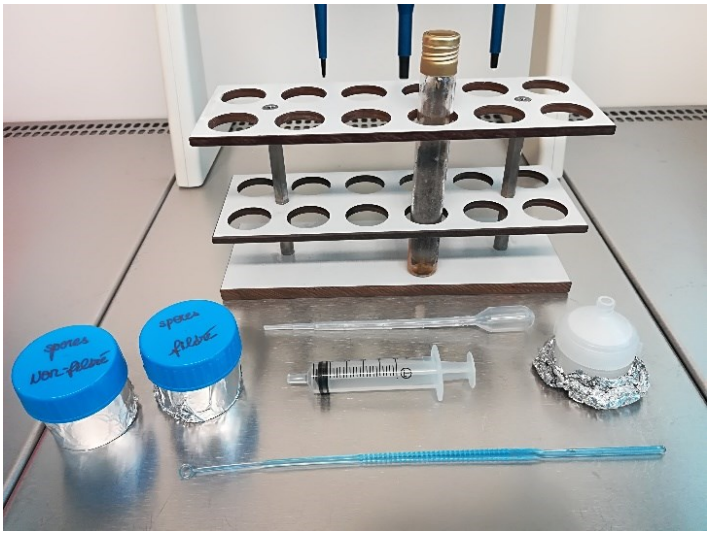


Figure 57: Working Place- Preparation of the spore suspension

Then the counting of the spores in the hemocytometer followed. Concerning the counting, a dilution of 1/100 was performed, which resulted in 5 µL of the filtered spore suspension in addition to 495 µL of 0,1 % Tween 20. From this dilution 20 µL were put on a glass slide C-Chip™ NanoEnTek.

Under the light microscope, the average of counted spores in a row (10 squares) resulted in 53 spores. This means 53 spores/0,1 µL and consequently 53 x 10^4 spores/mL in the dilution. As a dilution of 1/100 was performed, the number had to be multiplied by 100: 53 x 10^4 x 100 = 5,3 x 10^7 spores/mL in tube 1.

As a final volume 2000 µL was determined, which results in 4 wells of 500 µL (4 x 500 µL = 2000 µL) of the Lab-Tek chamber slide.

C_i = Initial concentration = 5,3 x 10^7 spores/mL
C_f = Final concentration = 10^7 spores/L
V_f = Final volume = 2000 µL
V_i = Initial concentration = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
(10^7 spores/mL x 2000 µL) / (5,3 x 10^7 spores/mL) = 377,36 µL ~ 377 µL

➔ Tube 1 (10^7 spores/mL) : 377 µL spore suspension + 1623 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (2000 µL - 377 µL = 1623 µL)

Concerning tube 2, a dilution of ½ of tube 1 was performed.

➔ Tube 2 (5 x 10^6 spores/mL) : 750 µL of tube 1 + 750 µL cell culture medium RPMI 1640 + ATB + 10 % FBS

The spore dilutions were stored under the LAF during the next step, the nuclei staining of the A549 pneumocytes in the Lab-Tek chamber slide. This staining had to be performed under another LAF, as we handled with the spores of *A. fumigatus*, which are highly infectious. After one washing step with expired cell culture medium to remove the dead cells, 500 µL of new cell culture medium RPMI 1640 + ATB + 10 % FBS in addition to 20 µL of the fluorochrome NucBlue® reagent were added into the 4 wells of the Lab-Tek chamber slide. It was incubated for 30 min at 37 °C and 5 % CO_2 until bright nuclei staining was reached. One washing step with expired cell culture medium followed and the medium was changed to new cell culture medium RPMI 1640 + ATB + 10 % FBS.

Then the Lab-Tek chamber slide was transferred under the LAF of the stored spore dilutions and after again 2 washing steps with expired cell culture medium, the A549 pneumocytes were finally contaminated with the spores of *A. fumigatus*-GFP. The first 2 wells were contaminated with 500 µL at the concentration of 10^7 spores/mL and the last 2 wells with 500 µL at the concentration of 5 x 10^6 spores/mL, as described in **table 8**. The spore dilutions with the A549 pneumocytes were incubated for 1 hour at 37 °C and 5 % CO_2.

10^6 cells/mL 20 µL fluorochrome 10^7 spores/mL	10^6 cells/mL 20 µL fluorochrome 10^7 spores/mL	10^6 cells/mL 20 µL fluorochrome 5 x 10^6 spores/mL	10^6 cells/mL 20 µL fluorochrome 5 x 10^6 spores/mL

Table 8: Distribution of cells, fluorochrome and spores in the Lab-Tek chamber slide 4 wells

After 1 hour of incubation, a fixation with methanol, which stops the development of cells and spores, was performed. Therefore, after 2 washing steps with expired cell culture medium, 500 µL of methanol were added into the 4 wells of the Lab-Tek chamber slide. The fixation with methanol was incubated for 30 min under the LAF and aspirated without washing. The Lab-Tek chamber slide was then stored in the refrigerator at 4 °C to be later observed under the microscope.

Under the fluorescence microscope, slightly better outcomes were observed at the concentration of 10^7 spores/mL, as the concentration of 5 x 10^6 spores/mL showed too little cells. However, further testings of both concentrations were necessary to standardise the Lab-Tek chamber slide to one of the attempted concentrations. Concerning the fixation of cells and spores, methanol was changed to paraformaldehyde and the culture tubes of *A. fumigatus*-GFP should be covered in aluminum foil as well as working without light should be performed in the next steps, to assure that all fluorochromes work out properly.

5.3. Working step 3: Membrane staining with SYTO® 17

Working step 3 again started with the preparation of a Lab-Tek chamber slide with 4 wells, each well with a volume of 500 µL at a concentration of 10^6 cells/mL, which means 500 000 cells/well. After trypsinisation of a cell culture flask, 10 mL of new cell culture medium RPMI 1640 + ATB + 10 % FBS were added and well homogenised with the 4 mL of trypsinised cells. This initial volume of 14 mL was transferred into a 50 mL recipient and again, well homogenised.

Then the counting in the hemocytometer followed. Therefore, 50 µL of the suspension of 14 mL were removed and mixed with 50 µL of trypan blue solution, which means a dilution of ½ for the counting in the hemocytometer. Concerning the preparation of the glass slide, around 20 µL of this dilution were put on a C-Chip™ NanoEnTek.

Under the light microscope, the average of counted cells in a row (10 squares) resulted in 100 cells, which means 100 cells/0,1 µL and consequently 100 x 10^4 cells/mL. As a dilution of ½ was performed, this number had to be multiplied by 2: 100 x 10^4 cells/mL x 2 = 2 x 10^6 cells/mL in the suspension of 14 mL.

C_i = Initial concentration = 2 x 10^6 cells/mL
C_f = Final concentration = 10^6 cells/mL
V_i = Initial volume = 14 mL
V_f = Final volume = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_f = (C_i \times V_i) / C_f$$
$$(2 \times 10^6 \text{ cells/mL} \times 14 \text{ mL}) / 10^6 \text{ cells/mL} = 28 \text{ mL}$$

The rest of the 14 mL was centrifuged at 800 rpm for 5 min at 20 °C. The supernatant was discharged and the obtained cell pellet was resuspended in the beforehand calculated volume of 28 mL to reach a final concentration of 10^6 cells/mL. 500 µL at a concentration of 10^6 cells/mL, which means 500 000 cells/well were seeded in the 4 wells of the Lab-Tek chamber slide. It was incubated at 37 °C and 5 % CO_2.

After 2 days, one washing step was performed and the medium was changed to new cell culture medium RPMI 1640 + ATB + 10 % FBS. When confluence was reached, the preparation of the spores of *A. fumigatus*-GFP followed, this time at a concentration of 10^7 spores/mL. Therefore, 2 mL of 0,01 % Tween 20 were transferred into the culture tube of *A. fumigatus*-GFP. The surface of the agar was scratched with a loop as well as suction and discharge with a transfer pipette was performed to remove the spores from the agar. The spore suspension was finally aspirated and transferred into a pot of 30 mL.

With a syringe, this suspension was filtered into another pot of 30 mL and the counting in the hemocytometer followed. Concerning the counting, a dilution of 1/100 was performed, which means 5 µL from the suspension of spores and 495 µL of 0,1 % Tween 20. 20 µL of this dilution were put on a glass slide C-Chip™ from NanoEnTek.

The average of counted spores in a row (10 squares) resulted in 45 spores. This means 45 spores/0,1 µL and consequently 45 x 10^4 spores/mL. As a dilution of 1/100 was performed, this number had to be multiplied by 100: 45 x 10^4 spores/mL x 100 = 4,5 x 10^7 spores/mL.

This time, as a final volume 2500 µL were determined, which results in 4 wells of 500 µL (4 x 500 µL = 2000 µL) of the Lab-Tek chamber slide in addition to 500 µL in case of loss of medium.

C_i = 4,5 x 10^7 spores/mL
C_f = 10^7 spores/mL
V_f = 2500 µL
V_i = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
$$V_i = (10^7 \times 2500) / 4{,}5 \times 10^7 = 555{,}56 \sim 556\ \mu L$$

➔ 556 µL spore suspension + 1944 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (2500 µL - 556 µL = 1944µL)

The prepared spore suspension was stored under the LAF during the next step, the nuclei staining of the A549 pneumocytes, which had to be performed under another LAF. After one washing step of the Lab-Tek chamber slide with expired cell culture medium, new cell culture medium RPMI 1640 + ATB + 10 % FBS as well as the staining of 20 µL of the fluorochrome NucBlue® reagent were added. It was incubated for 30 min at 37 °C and 5 % CO_2 until bright nuclei staining was reached. After these 30 min of incubation, one washing step with expired cell culture medium followed and the medium was changed to new cell culture medium RPMI 1640 + ATB + 10 % FBS.

Then the Lab-Tek chamber slide was transferred under the LAF of the spore suspensions and finally contaminated with the spores of *A. fumigatus*-GFP. All wells were contaminated with 500 µL at a concentration of 10^7 spores/mL and it was incubated for 1 h at 37 °C and 5 % CO_2.

Moreover, at this working step, a new stain for the membrane of pneumocytes as well as membranes of external spores, SYTO® 17, a red fluorescence stain, was added. With this red stain it was expected to see a better differentiation between adherent and internalised spores on the one hand, and external spores on the other hand.

Concerning the preparation of the red stain SYTO® 17, it was started from a concentration of 5 mM (=5 000 000 nM) to reach a final concentration of 50 nM. In this dilution step it was important to work with TRIS rather than PBS to avoid an interaction with the phosphates of PBS and the fluorophores of SYTO® 17.

After 2 washing steps of the Lab-Tek chamber slide with expired cell culture medium, the red fluorescence stain was finally added into 2 wells, technically speaking in the second and fourth well. The first and the third well were prepared with cell culture medium RPMI 1640 + ATB + 10 % FBS to reach two different conditions. The Lab-Tek chamber slide was then incubated for 30 min at 37 °C and 5 % CO_2.

After 30 min of incubation, SYTO® 17 was aspirated, washed 2 times with TRIS and a fixation with 4 % PFA followed. But only the first 2 wells were fixed with 4 % PFA, whereas the last two wells not, to reach altogether 4 different conditions as described in **table 9**.

Without SYTO®17	SYTO® 17	Without SYTO® 17	SYTO® 17
Fixed with PFA	Fixed with PFA	Not fixed, RPMI 1640 + ATB + 10 % FBS	Not fixed, RPMI 1640 + ATB + 10 % FBS
Washed with TRIS	Washed with TRIS	Washed with PBS	Washed with TRIS

Table 9: Distribution of SYTO®17 in the Lab-Tek chamber slide 4 wells

Therefore, after the 2 washing steps with TRIS, 500 µL of 4 % PFA were added into well 1 and well 2, incubated under the LAF for 10 min, aspirated and washed again with TRIS. The other 2 wells were also washed with TRIS, but then not fixed with PFA, but left with RPMI 1640 + ATB + 10 % FBS and afterwards washed with PBS, in case of the well without SYTO® 17 and washed with TRIS, in case of the well with SYTO® 17, to again avoid the interaction of PBS and SYTO® 17.

Observation after working step 3:
Upright fluorescence microscope, Zeiss Axioskop MC 100:

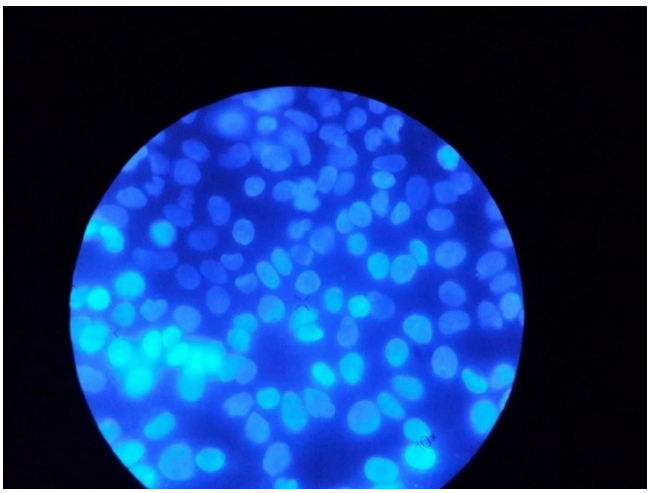


Figure 58: Nuclei of the A549 pneumocytes in blue fluorescence staining

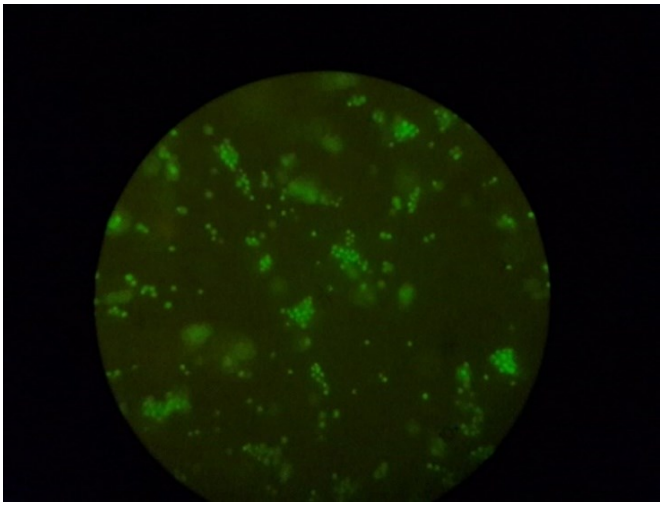


Figure 59: *A. fumigatus*-GFP spores in green fluorescence staining

After the first observation under the fluorescence microscope, the Lab-Tek chamber slide was freezed at - 20 °C to be later observed under the confocal microscope. The goal was to identify if all the fluorochromes worked out properly. Under the confocal microscope, very good outcomes with blue and green staining were observed. Unfortunately, the red stain, SYTO® 17, had to be changed to another red in the next experiments, as it did not work out properly. Moreover, it was decided to continue further working steps with Ibidi chamber slides instead of Lab-Tek chamber slides. Ibidi chamber slides have a higher quality and were expected to show better outcomes in the experiments, as they are out of plastic and not out of glass, as Lab-Tek chamber slides. Concerning the concentration of the A549 pneumocytes, a lot of aggregates were observed which were expected to be reduced by either a lower concentration or by performing the washing steps with more energy.

Confocal microscope, Nikon Eclipse Ti, N-SIM:

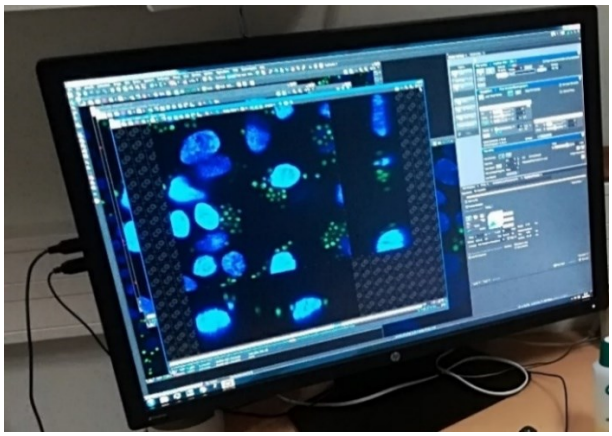


Figure 60: Nuclei and spores in the confocal microscope

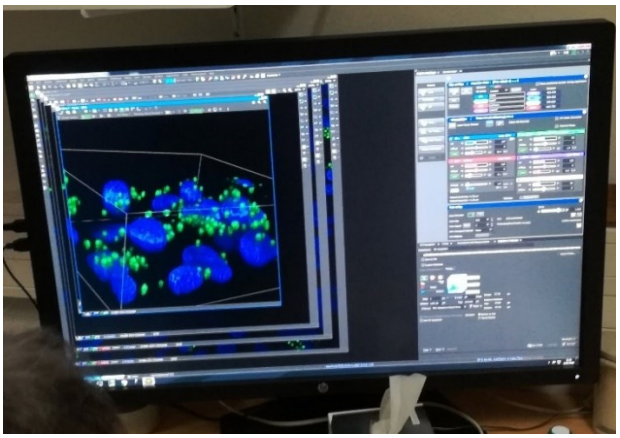


Figure 61: 3D image of nuclei and spores in the confocal microscope

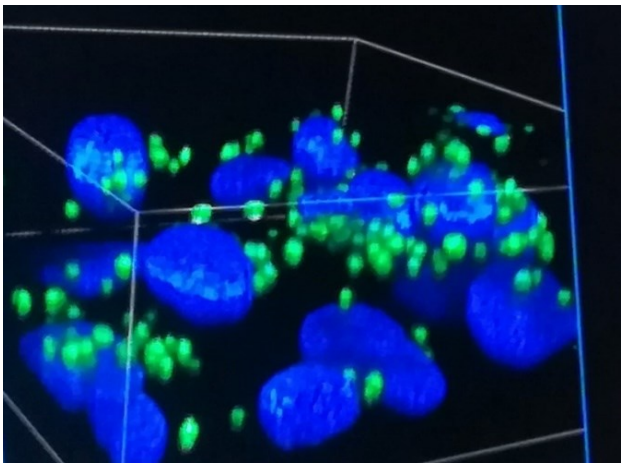


Figure 62: Zoom of 3D image of nuclei and spores in the confocal microscope

5.4. Working step 4: Washing steps with more energy

As already explained above, it was decided to continue further working steps with Ibidi chamber slides instead the material out of glass, Lab-Tek chamber slides. As a lot of cells as well as aggregates were observed in the last experiment, a dilution of ½, which results in 5 x 10^5 cells/mL was attempted. Moreover, all washing steps were performed with more energy to dissolve the aggregates.

Therefore, an Ibidi chamber slide with 8 wells was prepared, each well with a volume of 300 µL at a concentration of 5 x 10^5 cells/mL, which means 150 000 cells/well. After trypsinisation of a cell culture flask, 10 mL of new cell culture medium RPMI 1640 + ATB + 10 % FBS were added and well homogenised with the 4 mL of trypsinised cells. The resulting 14 mL were transferred into a 50 mL recipient and again, well homogenised.

Concerning the counting in the hemocytometer, 10 µL of the cell suspension of 14 mL were removed and mixed with 90 µL of trypan blue solution, which means a dilution of 1/10 for the counting. From this dilution, around 20 µL were put on a glass slide C-Chip™ NanoEnTek.

Under the light microscope, the average of counted cells in a row (10 squares) resulted in 35,67 cells. This means 35,67 cells/0,1µL and consequently 35,67 x 10^4 cells/mL. As a dilution of 1/10 was performed, this number had to be multiplied by 10: 35,67 x 10^4 cells/mL x 10 = 35,67 x 10^5 cells/mL.

C_i = 35,67 x 10^5 cells/mL
C_f = 5 x 10^5 cells/mL
V_f = 2800 µL (8 wells of 300 µL + 400 µL in case of loss)
V_i = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
$$V_i = (5 \times 10^5 \times 2800) / (35{,}67 \times 10^5) = 392{,}49\ \mu L \sim 393\ \mu L$$

➔ 393 µL spore suspension + 2407 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (2800 µL – 393 µL = 2407 µL)

The rest of the 14 mL was centrifuged at 800 rpm for 5 min at 20 °C. The cell pellet was resuspended in 10 mL of new cell culture medium RPMI 1640 + ATB + 10 % FBS and well homogenised. 393 µL of this suspension were removed and diluted in 2407 µL new cell culture medium RPMI 1640 + ATB + 10 % FBS to reach a final concentration of 5 x 10^5 cells/mL. This concentration was finally distributed into the 8 wells of the Ibidi chamber slide, 300 µL at a concentration of 5 x 10^5 cells/mL into each well. The Ibidi chamber slide was then incubated at 37 °C and 5 % CO_2.

To provide the A549 cell line constantly with fresh nutrients, after one day, the cell culture medium was changed to new cell culture medium RPMI 1640 + ATB + 10 % FBS. When confluence was reached, the contamination with the spores of *A. fumigatus*-GFP as well as the staining of the nuclei was performed.

Concerning the preparation of the spores of *A. fumigatus*-GFP, 2 mL of 0,01 % Tween 20 were added into the culture tube of *A. fumigatus*-GFP. Suction and discharge with a transfer pipette was performed to remove the spores from the agar. The supernatant was finally transferred into a recipient of 30 mL and the whole procedure was repeated with another 1 mL of 0,01 % Tween 20 to reach a final volume of 3 mL unfiltered spore suspension. This spore suspension was filtered with a syringe into another pot of 30 mL and the counting in the hemocytometer followed.

Concerning the counting, a dilution of 1/100 was performed, which means 5 µL of the filtered spore suspension in addition to 495 µL of 0,1 % Tween 20. From this dilution 20 µL were put on a glass slide C-Chip™ NanoEnTek.

Under the light microscope, the number of counted spores in a row (10 squares) resulted in 37 spores, which means 37 spores/0,1 µL and consequently 37 x 10^4 spores/mL. As a dilution of 1/100 was performed, this number had to be multiplied by 100: 37 x 10^4 spores/mL x 100 = 3,7 x 10^7 spores/mL.

C_i = 3,7 x 10^7 spores/mL
C_f = 5 x 10^6 spores/mL
V_f = 3000 µL (8 wells of 300 µL + 600 µL in case of loss)
V_i = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
$$(5 \times 10^6 \times 3000) / (3{,}7 \times 10^7) = 405{,}41\ \mu L \sim 405 \mu L$$

➔ 405 µL spore suspension + 2595 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (3000 µL - 405 µL= 2595 µL)

The spore suspension was stored under the LAF during the next step, the nuclei staining of the A549 pneumocytes. After one washing step with expired cell culture medium, new cell culture medium RPMI 1640 + ATB + 10 % FBS as well as 20 µL of the fluorochrome NucBlue® reagent were added into the 8 wells of the Ibidi chamber slide. It was incubated for 30 min at 37 °C and 5 % CO_2 to reach a brilliant nuclei staining. Then one washing step with expired cell culture medium followed and the old medium was replaced by new cell culture medium RPMI 1640 + ATB + 10 % FBS.

The Ibidi chamber slide was transferred under the LAF of the stored spore suspensions and after again 2 washing steps with expired cell culture medium, the 8

wells were contaminated with 300 µL of the spore suspensions at the concentration of 5 x 10^6 spores/mL. It was incubated for 1 hour at 37 °C and 5 % CO_2.

After 1 hour of incubation, again 2 washing steps with expired cell culture medium were performed and a fixation with 4 % PFA on the one hand, and 1% PFA on the other hand, followed. First, 300 µL of 4 % PFA were added into each of the 8 wells of the Ibidi chamber slide. It was incubated for 10 min and washed with PBS. Then another fixation with 1 % PFA was performed. PFA was removed, the Ibidi chamber slide was dried and this time, directly observed under the microscope.

Unfortunately, the observation under the microscope revealed that the cell layer was partly destroyed because the washing steps were done with too much energy. As a result, at this experiment, no further findings and insights for following working steps could be achieved.

5.5. Working step 5: Membrane staining with wheat germ agglutinin

Working step 5 started for the first time with the preparation of an Ibidi chamber slide with 12 wells, each well with a maximum volume of 250 µL. This allowed the testing of 4 more substances as in the Ibidi chamber slide with 8 wells. Moreover, because of its silicone chamber that is easy to remove, it was possible to perform all washing steps in a more convenient and gentle way. In the first 6 wells a concentration of 5 x 10^5 cells/mL and in the last 6 wells a concentration of 10^6 cells/mL was performed, as shown in **table 10**.

After trypsinisation of a cell culture flask, 10 mL of new cell culture medium RPMI 1640 + ATB + 10 % FBS were added into the successfully trypsinised flask of 4 mL and homogenised by suction and discharge. The obtained 14 mL cell suspension were transferred into a recipient of 50 mL and again, homogenised.

Concerning the counting of the cells in the hemocytometer, 10 µL of this cell suspension were removed and mixed with 90 µL of trypan blue solution, which means a dilution of 1/10 for the counting. As 10 µL represents a very small volume, it was important to add the trypan blue solution first into a small tube and then absorb the 10 µL of the cell suspension in the 90 µL of trypan blue, so that nothing remains on the wall of the tube. Then, around 20 µL of this dilution were put on a glass slide C-Chip™ NanoEnTek.

Under the light microscope, the average of counted cells in a row (10 squares) resulted in 45,17 cells. This means 45,17 cells/0,1 µL and consequently 45,17 x 10^4 cells/mL. As a dilution of 1/10 was performed, this number had to be multiplied by 10: 45,17 x 10^4 cells/mL x 10 = 45,17 x 10^5 cells/mL.

$C_i = 45{,}17 \times 10^5$ cells/mL
$C_f = 5 \times 10^5$ cells/mL
$V_f = 2000$ µL (6 wells with 250 µL + 500 µL in case of loss)
V_i = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
$$V_i = (5 \times 10^5 \times 2000) / 45{,}17 \times 10^5 = 221{,}39\ \mu L \sim 221\ \mu L$$

➔ Tube 1 (5×10^5 cells/mL) : 221 µL cell suspension + 1779 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (2000 µL - 1779 µL = 221 µL)

$C_i = 45{,}17 \times 10^5$ cells/mL
$C_f = 10^6$ cells/mL
$V_f = 2000$ µL (6 wells with 250 µL + 500 µL in case of loss)
V_i = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
$$(10^6 \times 2000) / 45{,}17 \times 10^5 = 442{,}77\ \mu L \sim 443\ \mu L$$

➔ Tube 2 (10^6 cells/mL) : 443 µL cell suspension + 1557 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (2000 µL – 443 µL = 1557 µL)

With the rest of the 14 mL a centrifugation at 800 rpm for 5 min at 20 °C was performed. The supernatant was discharged and the obtained cell pellet was resuspended in the required volume of new cell culture medium RPMI 1640 + ATB + 10 % FBS. In each of the first 6 wells of the Ibidi chamber slide 250 µL of tube 1 (221 µL cell suspension + 1779 µL cell culture medium RPMI 1640 + ATB + 10 % FBS) were distributed to reach a concentration of 5×10^5 cells/mL. In each of the last 6 wells 250 µL of tube 2 (443 µL cell suspension + 1557 µL cell culture medium RPMI 1640 + ATB + 10 % FBS) were distributed to reach a concentration of 10^6 cells/mL.

To provide the cells constantly with fresh nutrients, the medium of the Ibidi chamber slide was changed after 2 days. On day 3 confluence was reached and the staining of the nuclei as well as the contamination with the spores of *A. fumigatus*-GFP was performed. Therefore, the 12 wells of the Ibidi chamber slide were washed with expired cell culture medium and 250 µL of new cell culture medium RPMI 1640 + ATB + 10 % FBS in addition to 20 µL of the fluorochrome NucBlue® reagent were added. The fluorochrome was incubated for 30 min at 37 °C and 5 % CO_2 to reach a brilliant nuclei staining. After one washing step, the cell culture medium was changed to 250 µL new cell culture medium RPMI 1640 + ATB + 10 % FBS.

During the 30 min of incubation, the spores of *A. fumigatus*-GFP at two different concentrations, 5 x 10^6 cells/mL and 10^7 cells/mL of cell culture medium RPMI 1640 + ATB + 10 % FBS were prepared. This step had to be done under another LAF. Therefore, 2 mL of 0,01 % Tween 20 were transferred into the culture tube of *A. fumigatus*-GFP. The surface of the agar was scratched with a loop to remove the spores from the agar. Additionally, suction and discharge with a transfer pipette was performed and the supernatant was finally transferred into a pot of 30 mL. The whole procedure was repeated with another 1 mL of 0,01 % Tween 20 to obtain a final volume of 3 mL from the spore suspension of *A. fumigatus*-GFP.

With a syringe, this suspension was filtered into a new pot of 30 mL and the counting in the hemocytometer followed. A dilution of 1/100 was performed, which means 5 µL of the spore suspension + 495 µL of 0,1 % Tween 20, from which 20 µL were put on a glass slide C-Chip™ NanoEnTek to perform the counting.

In the hemocytometer, the average of counted spores in a row (10 squares) resulted in 27,3 spores. This means 27,3 spores/0,1 µL and consequently 27,3 x 10^4 spores/mL. As a dilution of 1/100 was performed, this number had to be multiplied by 100: 27,3 x 10^4 spores/mL x 100 = 2,73 x 10^7 spores/mL in tube 1.

C_i = Initial concentration = 2,73 x 10^7 spores/mL
C_f = Final concentration = 10^7 spores/mL
V_f = Final volume = 2000 µL (6 wells of 250 µL + 500 µL in case of loss)
V_i = Initial concentration = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
$$(10^7 \times 2000) / (2{,}73 \times 10^7) = 732{,}60\ \mu L \sim 733\ \mu L$$

➔ Tube 1 (10^7 spores/mL) : 733 µL spore suspension + 1267 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (2000 µL - 733 µL= 1267 µL)

C_i = Initial concentration = 2,73 x 10^7 spores/mL
C_f = Final concentration = 5 x 10^6 spores/mL
V_f = Final volume = 2000 µL (6 wells of 250 µL + 500 µL in case of loss)
V_i = Initial concentration = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
$$(5 \times 10^6 \times 2000) / (2{,}73 \times 10^7) = 366{,}30\ \mu L \sim 366\ \mu L$$

➔ Tube 2 (5 x 10^6 spores/mL): 366 µL spore suspension + 1634 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (2000 µL - 366 µL = 1634 µL)

Then the contamination with the spores of *A. fumigatus*-GFP was performed. The Ibidi chamber slide was transferred under the LAF of the spore suspensions and after 2 washing steps with expired cell culture medium, it was replaced by the different concentrations of spore suspensions. In the above row the concentration of 10^7 spores/mL and in the row beneath, the concentration of 5 x 10^6 spores/mL of cell culture medium RPMI 1640 + ATB + 10 % FBS was added. As a result, 4 different conditions were reached, as demonstrated in **table 10**. The Ibidi chamber slide was then incubated for 1 hour at 37 °C and 5 % CO_2.

10^7spores/mL 5x10^5cells/mL	10^7spores/mL 5x10^5cells/mL	10^7spores/mL 5x10^5cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL
5x10^6spores/mL 5x10^5cells/mL	5.10^6spores/mL 5x10^5cells/mL	5.10^6spores/mL 5x10^5cells/mL	5x10^6spores/mL 10^6cells/mL	5x10^6spores/mL 10^6cells/mL	5x10^6spores/mL 10^6cells/mL

Table 10: Distribution of cells and spores in the Ibidi chamber slide 12 wells

The next working step included the application of a new red fluorescent stain, as SYTO® 17 did not work out properly. Similar to SYTO® 17, WGA was used to stain the membranes of A549 pneumocytes as well as external spores in red to observe a better differentiation between internal spores on the one hand, and external spores on the other hand. Concerning the preparation of WGA, the original concentration of 1 mg/mL was diluted in cell culture medium RPMI 1640 + ATB + 10 % FBS to reach a final concentration of 5 µg/mL in 3000 µL.

C_i = Initial concentration = 1000 µg/mL
C_f = Final concentration = 5 µg/mL
V_f = Final volume = 3000 µL (12 wells of 250 µL)
V_i = Initial volume = ?

$$C_i \times V_i = C_f \times V_f$$
$$V_i = (C_f \times V_f) / C_i$$
$$(5 \times 3000) / 1000 = 15\ \mu L$$

➔ 15 µL of WGA at a concentration of 1mg/mL + 2885 µL cell culture medium RPMI 1640 + ATB + 10 % FBS (3000 µL - 15 µL = 2885 µL) was prepared to reach a final concentration of 5 µg/mL WGA

After 3 washing steps of the Ibidi chamber slide with expired cell culture medium, at the last washing step, 250 µL of 5µg/mL WGA were added into each of the 12 wells. It was incubated for 15 min under the LAF to reach a brilliant red membrane staining. WGA was aspirated and washed 3 times with expired cell culture medium. A fixation with 4 % PFA for 10 min was performed and after 2 washing steps with PBS,

PBS was finally aspirated, the Ibidi chamber slide was dried and stored in the refrigerator at 4 °C to be later observed under the microscope.

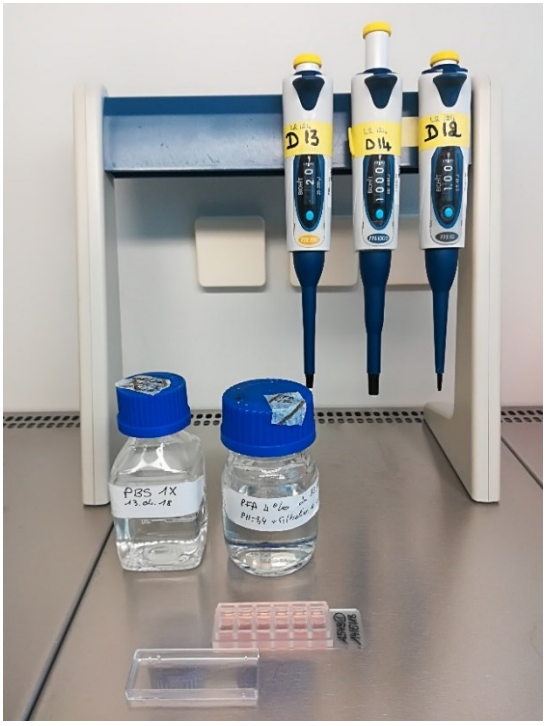


Figure 63: Working Place- Fixation of cells and spores

Under the microscope, unfortunately, the red stain WGA was too strong and overlapped to some extent with the green of the spores from *A. fumigatus*-GFP, so it was decided to leave the red stain out of the experiments. Blue and green fluorescence stains worked out very well and the Ibidi chamber slide with 12 wells was finally standardised to 10^6 cells/mL and 10^7 spores/mL of cell culture medium RPMI 1640 + ATB + 10 % FBS.

5.6. Standardisation of the Ibidi chamber slide 12 wells:

10^6 cells/mL and 10^7spores/mL

10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL
10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL	10^7spores/mL 10^6cells/mL

Table 11: Standardisation of cells and spores in the Ibidi chamber slide 12 wells

5.7. Testing the molecules

5.7.1. Working steps of testing the molecules

After the successful standardisation of an interaction model between A549 pneumocytes and the spores of *A.fumigatus*, the testing of the molecules started. Therefore, the standardised Ibidi chamber slide with 12 wells was prepared, each well consisting of 250 µL at a concentration of 10^6 cells/mL, which means 250 000 cells/well, as described above. After one day, the old cell culture medium was replaced by new cell culture medium RPMI 1640 + ATB + 10 % FBS. When confluence was reached, the preparation of the spore suspension at the standardised concentration of 10^7 spores/mL followed, as also mentioned above.

The prepared spore suspension was transferred into 6 tubes of 14 mL. In each tube 500 µL at the concentration of 10^7 spores/mL of cell culture medium RPMI 1640 + ATB + 10 % FBS was added. The 6 tubes were centrifuged at 4500 rpm for 15 min at 20 °C and the supernatant was discharged. The cell pellet was resuspended in the different concentrations of molecules at 100 µM, 10 µM or 1 µM, prior filled up with cell culture medium RPMI 1640 + ATB + 10 % FBS to 500 µL. The control only consisted of spores in addition to cell culture medium RPMI 1640 + ATB + 10 % FBS. The tubes were well homogenised and left on the gyratory rocker for 1 hour.

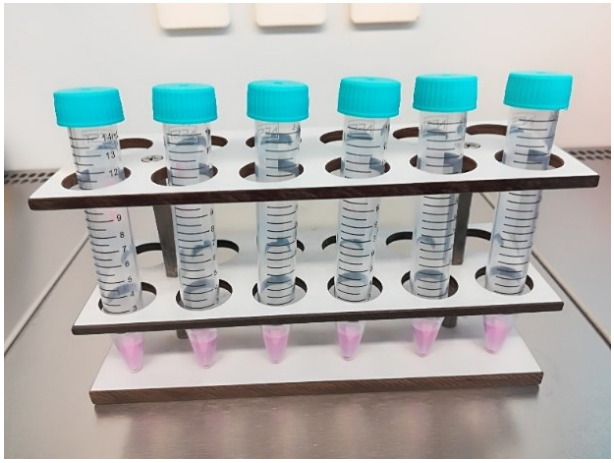


Figure 64: Tubes with spores, test substances and cell culture medium

During that hour, the already explained blue staining of the nuclei of the A549 pneumocytes in the Ibidi chamber slide was performed. After one washing step with expired cell culture medium, 250 µL new cell culture medium RPMI 1640 + ATB + 10 % FBS in addition to 20 µL of the fluorochrome NucBlue® reagent were added and incubated for 30 min at 37 °C and 5 % CO_2 to reach a brilliant nuclei staining. After one washing step with expired cell culture medium, the medium was changed to new cell culture medium RPMI 1640 + ATB + 10 % FBS. The Ibidi chamber slide was stored in the incubator at 37 °C and 5 % CO_2.

Concerning the 6 tubes on the gyratory rocker, after 1 hour, another centrifugation at 4500 rpm for 15 min at 20 °C was performed. The supernatant was discharged and the obtained cell pellet was resuspended in 550 µL new cell culture medium RPMI 1640 + ATB + 10 % FBS and well homogenised. 500 µL represents the volume of 2 wells of 250 µL of the Ibidi chamber slide. 50 µL were necessary in case of loss of medium.

Afterwards, the contamination of the Ibidi chamber slide with spores, prior incubated with the molecules and filled up with cell culture medium RPMI 1640 + ATB + 10 % FBS, followed. After 2 washing steps with expired cell culture medium, 250 µL of this mix of spores, molecules and cell culture medium RPMI 1640 + ATB + 10 % FBS were transferred into the 12 wells of the Ibidi chamber slide and incubated for 1 hour at 37 °C and 5 % CO_2.

After that hour of incubation, 2 washing steps with expired cell culture medium and a fixation with 4 % PFA was performed. One washing step with PBS followed, the cover glass was dried and stored in the refrigerator at 4 °C to be later observed under the microscope.

Under the fluorescence microscope, the blue filter was used to observe the nuclei of the A549 pneumocytes and the green filter for adherent as well as internalised spores of *A. fumigatus*-GFP. As we once worked with strain *A.fumigatus*-ATCC, which does not show green autofluorescence, normal light was used for observing the spores in that case. By switching the filters, cells as well as spores in one focus were counted and a calculation spores/cell was performed.

5.7.2. Results of testing the molecules

Testing of L-fucose, the control & molecule number 72

L-Fucose 100 µM	L-Fucose 10 µM	Control AsFu-90-GFP	72 -100µM	72-10µM	72-1µM
L-Fucose 100 µM	L-Fucose 10 µM	Control AsFu-90-GFP	72-100 µM	72-10µM	72-1µM

Table 12: Distribution of L-fucose, the control and molecule 72

Results of counting under the fluorescence microscope:

	Spores	Cells	Spores/Cell
Control AsFu-90-GFP	288,57	216,71	1.34
L-Fucose-100 µM	289,38	233,50	1,26
L-Fucose-10 µM	138,73	198,64	0,59
72-100 µM	88,30	200,20	0,45
72-10 µM	189,00	191,20	0,99(6)
72-1 µM	184,17	186,00	0,99(7)

Table 13: Results of L-fucose, the control and molecule 72

Testing of molecules number 75, 74 and 72:

75-100µM	75-10µM	74-100µM	74-10µM	72-100µM	72-10µM
75-100µM	75-10µM	74-100µM	74-10µM	72-100µM	72-10µM

Table 14: Distribution of molecule 75, 74 and 72

Results of counting under the fluorescence microscope:

	Spores	Cells	Spores/Cell
72-100 µM	327,20	284,20	1,15
72-10 µM	327,67	278,33	1,17
74-100 µM	399,25	280,00	1,43
74-10 µM	362,67	326,67	1,11
75-100 µM	370,66	276,67	1,34
75-10 µM	427,50	286,00	1,50

Table 15: Results of molecule 72, 74 and 75

Testing of molecules number 46 and 45, including the control:

46-100µM	46-10µM	45-100µM	45-10µM	Control AsFu-90-GFP	X
46-100µM	46-10µM	45-100µM	45-10µM	Control AsFu-90-GFP	X

Table 16: Distribution of molecule 46, 45 and the control

Results of counting under the fluorescence microscope:

	Spores	Cells	Spores/Cell
Control AsFu-90-GFP	322,00	201,20	1,60
45-100 µM	266,67	283,00	0,94
45-10 µM	348,00	280,00	1,24
46-100 µM	311,75	282,00	1,11
46-10 µM	326,67	272,33	1,20

Table 17: Results of the control, molecule 45 and 46

Testing of molecules number 72, 47 and the control:

72- 100 µM	72- 10 µM	72- 1 µM	47-100 µM	47-10 µM	Control AsFu-ATCC
72- 100 µM	72- 10 µM	72- 1 µM	47-100 µM	47-10 µM	Control AsFu-ATCC

Table 18: Distribution of molecule 72, 47 and the control

Results of counting under the fluorescence microscope:

	Spores	Cells	Spores/Cell
72-100 µM	286,25	272,25	1,05
72-10 µM	284,5	294,75	0,97
72-1 µM	265,75	269,25	0,99
47-100 µM	312,83	293,83	1,06
47-10 µM	356,00	306,75	1,16
Control AsFu-ATCC	310,6	295,4	1,05

Table 19: Results of molecule 72, 47 and the control

Testing of molecules number 45, 47, 46 and the control:

45- 100 µM	45- 10 µM	47- 100 µM	47-10 µM	46-100 µM	Control AsFu-90-GFP
45- 100 µM	45- 10 µM	47- 100 µM	47-10 µM	46-100 µM	Control AsFu-90-GFP

Table 20: Results of molecule 45, 47, 46 and the control

Results of counting under the fluorescence microscope:

	Spores	Cells	Spores/Cell
45-100 µM	261,14	302,29	0,86
45-10 µM	314,5	304,5	1,03
47-100 µM	267,5	304,00	0,88
47-10 µM	291,5	290,5	1,00
46-100 µM	341,00	302,00	1,13
Control AsFu-90-GFP	347,00	303,25	1,14

Table 21: Results of molecule 45, 47, 46 and the control

5.8. Testing different strains of *A. fumigatus*

5.8.1. Working steps of testing different strains

Concerning the working steps of testing different strains of *A.fumigatus*, again the standardised Ibidi chamber slide with 12 wells, each well consisting of 250 µL at a concentration of 10^6 cells/mL of cell culture medium RPMI 1640 + ATB + 10 % FBS, which means 250 000 cells/well, was prepared. After one day, the cell culture medium was changed to new cell culture medium RPMI 1640 + ATB + 10 % FBS. When confluence was reached, the different spore suspensions at the standardised concentration of 10^7 spores/mL of cell culture medium RPMI 1640 + ATB + 10 % FBS as well as the nuclei staining of the A549 pneumocytes was performed.

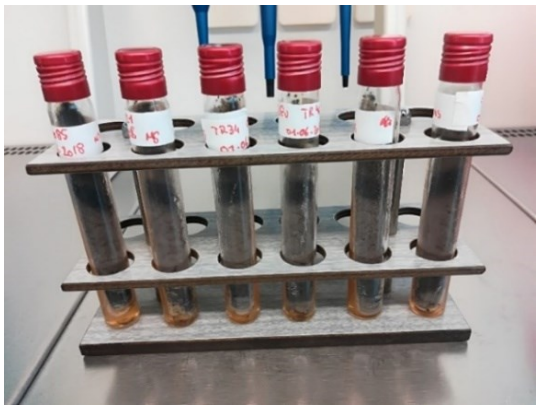


Figure 65: Different strains of *A. fumigatus*

Concerning the staining of the A549 pneumocytes in the Ibidi chamber slide, after one washing step with expired cell culture medium, new cell culture medium RPMI 1640 + ATB + 10 % FBS in addition to 20 µL of the fluorochrome NucBlue® reagent were added into the 12 wells of the Ibidi chamber slide and incubated for 30 min at 37 °C and 5 % CO_2 to reach a bright nuclei staining. After the 30 min of incubation, one washing step with expired cell culture medium followed and new cell culture medium RPMI 1640 + ATB + 10 % FBS was added.

Concerning the preparation of the different spore suspensions, each suspension was prepared at the beforehand standardised concentration of 10^7 spores/mL and stored under the LAF, as mentioned above. The Ibidi chamber slide was then transferred under this LAF and after 2 washing steps with expired cell culture medium, the 12 wells were contaminated with the different spore suspensions. The spore suspensions were incubated for 1 hour at 37 °C and 5 % CO_2 and after 2 washing steps with expired cell culture medium, a fixation with 4 % PFA for 10 min was performed. One washing step with PBS followed, PBS was aspirated, the Ibidi chamber slide was dried and stored in the refrigerator at 4 °C to be later observed under the microscope.

Under the fluorescence microscope, nuclei of the A549 pneumocytes were observed through the blue filter whereas adherent and internalised spores of different strains of *A. fumigatus*, as they do not show green autofluorescence, were observed under normal light. By switching from blue filter to normal light, cells as well as spores in one focus were counted and a calculation spores/cell was performed.

5.8.2. Results of testing different strains

WT 08	TR 34 121	TR 34 089	TR 46 090	TR 46 087	WT110
WT 08	TR 34 121	TR 34 089	TR 46 090	TR 46 087	WT 110

Table 22: Distribution of different strains

Results of the counting under the fluorescence microscope:

	Spores	Cells	Spores/Cell
WT 08	397,25	288,75	1,38
WT 110	471,50	311,25	1,52
TR 34 089	353,00	272,75	1,30
TR 34 121	378,00	287,6	1,31
TR 46 087	152,75	332,75	0,46
TR 46 090	492,00	296,25	1,66

Table 23: Results of different strains

	Spores	Cells	Spores/Cell
TR 46 087	252,00	304,33	0,83
TR 46 090	508,00	308,33	1,65

Table 24: Results of strain TR 46 087 in contrast to TR 46 090

6. DISCUSSION

As the number of fungal infections is increasing as well as their actual treatment depicts problems, the search for alternative methods is obvious. A much milder and safer method, named anti-adhesive therapy, is discussed as a promising new strategy in the treatment of severe and life-threatening lung infections. In this work the focus lies on the inhibition of AFL, the *A. fumigatus* lectin, which strongly binds to bronchial epithelium and is considered as the first step in the development of aspergillosis.

As a basis of all experiments, an interaction model between A549 pneumocytes on the one hand, and the spores of *A. fumigatus* on the other hand, was established. A549 pneumocytes were successfully standardised to 10^6 cells/mL and the spores of *A. fumigatus* to 10^7 spores/mL of cell culture medium RPMI 1640 + ATB + 10 % FBS. This interaction model allowed various advanced testings of molecules and strains in fluorescence and confocal microscopy.

For the better differentiation between cells and spores, 3 different stains were utilised. Blue staining of A549 pneumocytes worked out very well and was successfully standardised to 20 µL NucBlue® reagent of cell culture medium RPMI 1640 + ATB + 10 % FBS. Green fluorescence staining by the attachment of GFP for the spores of *A. fumigatus* resulted in satisfying outcomes as well. Although it rested unclear whether covering the culture tubes of *A. fumigatus* in aluminium foil and working without light under the LAF has a positive effect on the quality of its fluorophores or not.

Unfortunately, staining of membranes of pneumocytes and external spores in red, which should facilitate the differentiation between external spores on the one hand and adherent as well as internalised spores on the other hand, failed. Neither the first stain SYTO® 17, nor the second stain wheat germ agglutinin could reach satisfying results. Achieving this red staining rests as an perspective for the future and needs more experiments and time.

As already mentioned, the contribution of AFL in the adhesion to host glycoproteins as well as its implication in the inflammatory response, makes it to an important factor in the development of infection. Therefore, its inhibition and consequently, potential substances that inhibit the binding of this lectin, were tested.

Concerning the testing of those substances, in particular 6 different saccharides, all of them showed more or less the expected reduction in spores per cell on the interaction model. Best results were achieved with molecule 45, that reached lower adhesion and internalisation rates in 2 experiments compared to the other molecules tested on the Ibidi chamber slide. Furthermore, molecules 72 and 47 reached very good results, but have to be examined in more detail.

Unfortunately, all outcomes are still too inaccurate and unsteady to make a final decision which molecule acts best. Furthermore, as counting of cells and spores was conducted in fluorescence microscope, future efforts should be performed by taking pictures in the confocal microscope, to reach more precise results and to eliminate further dubieties.

Conflicting outcomes were obtained in the first experiment with L-fucose. As it was shown that AFL binds to fucosylated structures on bronchial epithelium [Houser J. et al., 2013], adding high concentrations of L-fucose would expect to reduce spore attachment more than with lower concentrations, according to Kerr S. C. et al. This experiment has to be repeated as the results are questionable according to the expectations.

Most interestingly, the desired inhibitions were only observed with strain *A. fumigatus*-GFP, but not with strain *A. fumigatus*-ATCC, which leads to the assumption that there is a discrepance in adhesion and internalisation in different strains.

Therefore, more different mutant as well as wildtype strains were tested on the interaction. Strains WT 08, WT 110, TR 34 089, TR 34 121 and TR 46 090 showed very high adhesion as well as internalisation levels, whereas the results of strain TR 46 087 were extremely low, although even more pneumocytes were observed. A second testing of TR 46 087 against TR 46 090 proved this first assumption and finally leads to the conclusion that indeed, different mutations in strains have an impact on the attachment of conidia to lung epithelial cells.

All in all, the idea of this project- reducing infection by inhibiting the binding of lectins- has potential for the future. If the concept works, it can be used for other diseases where lectins play an important role in the infection process. Another example would be *Escherichia coli*, where the binding of different lectins to host tissue was found to be responsible for initiating infection as well [Sharon N., 2006].

Another advantage of this method, which marks it highly valuable for the future, is the lower incidence of developing resistance mechanisms since the pathogenic cells are not affected directly by killing or arresting them, as already mentioned in chapter **2.8.4.**

To summarise, this work is the first step into a field where the highly toxic and destructive antimycotic treatment by voriconazole and amphotericin B has the chance to be replaced by a milder and safer method named anti-adhesive therapy. Due to our experiments, for the first time, a correlation between the inhibition of AFL and the reduction of adherent as well as internalised spores by different molecules was observed in-vitro. Therefore, molecules that inhibit the binding of AFL are promising substances and after further preciseness and improvement of experiments it will be possible to finally publish the best substance as a new drug target in the treatment of severe aspergillosis.

7. ABBREVIATIONS

ABPA = Allergic bronchopulmonary aspergillosis

AFL = *Aspergillus fumigatus* lectin

AIDS = Acquired immune deficiency syndrome

ATB = Antibiotics

bp = base pair

CD4 = Cluster of differentiation 4

CD8 = Cluster of differentiation 8

CPA = Chronic pulmonary aspergillosis

CCL3 = C-C chemokine ligand 3

CCL4 = C-C chemokine ligand 4

CCL20 = C-C chemokine ligand 20

CXCL8 = C-X-C chemokine ligand 8 (Synonyme for IL-8)

COPD = Chronic obstructive pulmonary disease

CO_2 = Carbon dioxide

CYP = Cytochrom P450

DAPI = 4′,6-diamidino-2-phenylindole

DHN = Dihydroxynapthtalene

DNA = Desoxyribonucleic acid

DMSO = Dimethylsulfoxide

EDTA = Ethylenediaminetetraacetic acid

dNTP = Deoxynucleotide triphosphate

FBS = Fetal bovine serum

GFP = Green fluorescent protein

HeLa = Henrietta Lacks

HEPA = High efficiency particular air filters

HIV = Human immunodeficiency virus

IgE = Immunoglobulin

IL-8 = Interleukin-8

IPA = Invasive pulmonary aspergillosis

IU = International unit

LAF = Laminar air flow

MAP = Mitogen activated protein

MIC= Minimal inhibitory concentration

min = minutes

NET = Neutrophil extracellular trap

Nf-$_K$b = Nuclear factor kappa-light-chain-enhancer of activated B-cells

PBS = Phosphate-buffered saline

PCR = Polymerase chain reaction

PFA = Paraformaldehyde

PRR = Pattern recognition receptor

rpm = rotations per minute

RPMI = Roswell park memorial institute medium

sec = seconds

TAE = TRIS-acetat-EDTA

Th1 = T-helper cell type 1

Th2 = T-helper cell type 2

Th17 = T-helper cell type 17

TLR = Toll-like receptor

TLR2 = Toll-like receptor type 2

TLR4 = Toll-like receptor type 4

Treg = Regulatory T-cell

TRIS = Tris(hydroxymethyl)aminomethane

UV = Ultraviolet

WGA= Wheat germ agglutinin

8. LIST OF FIGURES

"Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir."

9. LIST OF TABLES

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11. ABSTRACT

As the incidence of fungal infections is steadily increasing over the past years, the ascomycete *Aspergillus fumigatus* serves as an example of a microbe with the capacity of causing life-threatening lung infections, especially in patients with compromised immune system. At the moment antifungal treatment is done by voriconazole and amphotericin B which possess a high toxicity and aim to destruct or inhibit the proliferation of the pathogen. Furthermore, voriconazole, as a representative of the azole class, shows increasing resistance rates to *A. fumigatus* mainly derived from a point mutation in the gene CYP 51A [Snelders E. et al., 2008].

Therefore, the aim of this diploma thesis is to propose an alternative to the conventional treatments of aspergillosis. Recently, it was demonstrated in collaboration with the french chemistry research group CEISAM that it is possible to inhibit the *A. fumigatus* lectin (AFL) which is expressed by *A. fumigatus* conidia and allows them to adhere to pneumocytes. This lectin recognises fucoside structures on bronchial epithelium [Houser et al., 2013; Kerr et al, 2016] and its adhesion is one of the key stages in the invasion process as well as the first step in the development of infection.

In fluorescence microscopy, an interaction model between A549 pneumocytes and the spores of *A. fumigatus* was developed and 6 saccharides that are expected to inhibit the binding of AFL were tested. Furthermore, different strains of *A. fumigatus* resistant to azoles were investigated in order to explore the effect of these strains on the interaction.

For the first time, a correlation between the inhibition of AFL by different molecules and the reduction of adherent as well as internalised *A.fumigatus* spores was observed in-vitro. Consequently, these saccharides could represent promising new drug targets for aspergillosis and the successfully standardised interaction model allows further testing which molecule finally acts best. Most interestingly, concerning testing the different strains, the mutant strain TR 46 087 showed a strong reduction in spores per cell in comparison to the other strains tested. This leads to the conclusion that certain mutations in strains have another important impact on the attachment of conidia to lung epithelial cells.

12. ZUSAMMENFASSUNG

Da die Häufigkeit von Pilzinfektionen in den letzten Jahren stetig zunimmt, dient der Ascomycet *Aspergillus fumigatus* als Beispiel für eine Mikrobe mit der Fähigkeit, lebensbedrohliche Lungeninfektionen zu verursachen, insbesondere bei Patienten mit geschwächtem Immunsystem. Derzeit wird die antimykotische Behandlung mit Voriconazol und Amphotericin B durchgeführt, die eine hohe Toxizität besitzen und darauf abzielen, die Vermehrung des Pathogens zu zerstören oder zu hemmen. Darüber hinaus zeigt Voriconazol, als Vertreter der Azol-Klasse, steigende Resistenzraten gegenüber *A. fumigatus*, die hauptsächlich aus einer Punktmutation im Gen CYP 51A stammen [Snelders E. et al., 2008].

Ziel dieser Diplomarbeit ist es daher, eine Alternative zu den herkömmlichen Aspergillose-Therapien vorzuschlagen. Kürzlich konnte in Zusammenarbeit mit der französischen Chemie-Forschungsgruppe CEISAM gezeigt werden, dass es möglich ist, das von *A. fumigatus* Konidien exprimierte *A. fumigatus* Lektin (AFL) zu inhibieren und an Pneumozyten anhaften zu lassen. Dieses Lektin erkennt Fucosidstrukturen auf Bronchialepithel [Houser et al., 2013; Kerr et al., 2016] und seine Adhäsion ist eine der Schlüsselphasen im Invasionsprozess sowie der erste Schritt in der Entwicklung von Infektionen.

In der Fluoreszenzmikroskopie wurde ein Interaktionsmodell zwischen A549-Pneumozyten und den Sporen von *A. fumigatus* entwickelt und 6 Saccharide, von denen erwartet wird, dass sie die Bindung von AFL hemmen, getestet. Darüber hinaus untersuchte man verschiedene Stämme von *A. fumigatus*, die gegen Azole resistent sind, um die Wirkung dieser Stämme auf die Interaktion zu erforschen.

Zum ersten Mal wurde eine Korrelation zwischen der Hemmung von AFL durch verschiedene Moleküle und der Reduktion von adhärenten sowie internalisierten *A. fumigatus* Sporen in-vitro beobachtet. Folglich könnten diese Saccharide vielversprechende neue Arzneimittelziele für Aspergillosen darstellen, und das erfolgreich standardisierte Interaktionsmodell ermöglicht weitere Tests, welches Molekül schließlich am besten wirkt. Hinsichtlich der Testung der verschiedenen Stämme zeigte interessanterweise der Mutantenstamm TR 46 087 eine starke Reduktion von Sporen pro Zelle im Vergleich zu den anderen getesteten Stämmen. Dies führt zur Schlussfolgerung, dass bestimmte Mutationen in Stämmen einen weiteren wichtigen Einfluss auf die Anheftung von Konidien an Lungenepithelzellen aufweisen.