Monozytäre Zellantwort gegen *Aspergillus fumigatus*:
Untersuchung der Phagozytose, Genexpression und Peptidpräsentation

Monocytic cell responses to *Aspergillus fumigatus*:
Investigation of phagocytosis, gene expression and peptide presentation

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for my parents, my brother and my sister

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1. Introduction

Living organisms on earth are separated into five different categories called kingdoms. These kingdoms include the *Monera* (prokaryotes), the *Protista* (eukaryotes), the *Fungi*, the *Plantae*, and the *Animalia*. Being one of these five major kingdoms, fungi play a vital role in life on earth. They exist in single cells or chains of cells together, and are largely hidden from man's view. We only see the "fruit" of a fungus. They feed by absorbing nutrients from the organic material that they live in. They digest their food before they absorb it by secreting acids and hydrolytic enzymes.

Like all other organisms, there are good fungi and there are bad fungi, when considered from man's view of life. Good fungi include the ones man loves to eat, the mushrooms or yeast in bread; the ones man loves to drink, the yeast making alcohol in beer; and those who are useful to man's life, like those composting waste into their gardens. A bad fungus doesn't actually exist; it's a good fungus living its own way, which is sometimes different. Unfortunately, good fungi become bad when they're useless or harmful to man, like toxic mushrooms. But man can decide by himself to eat the toxic mushroom or to leave it. On the other hand, other fungi don't need man's decision to harm him; they just take unintentionally advantage of his weakness or illness, settle down and spread in his body. This leads mostly to infections which become severe or even lethal in some cases.

As some fungi defend themselves by being toxic to other organisms, humans have their robust immune system which stands in the way and eliminates almost every foreign organism or particle that represents a risk or danger to the human body. This immune system works usually faultless; nevertheless, in some cases it mistakes self tissues for non-self and mounts an inappropriate attack causing autoimmune diseases. In other cases, the immune system is weak due to illness or other factors, making it easier for pathogens to enter the body. After recuperation, it is able to eliminate these pathogens again. Yet in other few cases, the immune system is completely turned down, for example during transplantation surgeries, in order to prevent repulsion of the new organ or cells. This short period is used by pathogens like spores of some filamentous fungi to enter the host lungs and spread out into other organs causing infections which lead to the death of the patient. The main fungus causing these infections is called *Aspergillus fumigatus*.

1.1 Overview of the immune system

The immune system consists of a variety of specialized cells, enzymes, and other serum proteins which are spread throughout the blood and tissues like the spleen, the thymus, the lymph nodes, the bone marrow and other glands. Through evolution, the system developed complexity and specificity. It enables organisms to survive the large number of pathogens in the environment and inside the organism. The immune system is divided into two major components - the innate (or native) defense system and the acquired (or adaptive) immune system.

The innate immune system is the first line of defense, which responds against foreign and infectious agents as soon as they enter the body. It consists generally of phagocytic cells, like monocytes/macrophages and neutrophils, and the complement system. The phagocytic cells are attracted to the infected site via chemotactic substances released by the infectious organism or other activated immune cells. Upon arrival and contact, they engulf, kill and digest the pathogen (bacteria, virus or fungus). So called antigen processing and presentation takes place and the antigenic peptides are presented on their cell surface in order to be recognized by T lymphocytes. The complement system consists of at least 20 serum proteins which, when stimulated, respond with a series of chain reactions. The primary effect of this chain reaction is to increase the blood flow towards the infection area, so that the phagocytes can reach this area more easily.

The acquired immune system is adaptive and specific. It learns to recognize and respond to previously unseen molecules or foreign bodies. This immunity is acquired by the development of antibodies after an infection occurs, after immune cells have sometime already made contact with the disease or after vaccination. It is divided into humoral and cellular immunity. Humoral immunity is represented by the antibodies released by B lymphocytes upon stimulation by T lymphocytes, which recognize MHC-presented peptides on the surface of B cells. These antibodies are then found in body fluids, e.g. in plasma and mucus. They have many functions; among others opsonization and immobilization of bacteria, neutralization of toxins, complement activation and mucosal protection. On the other hand, cellular immunity involves the activation of monocytes/macrophages and natural killer cells (NK cells), the production of antigen-specific cytotoxic T lymphocytes (CTL), and the release of various cytokines in response to an antigen. Unlike B cells, T cells receptors (TCR) don't recognize antigens, they only recognize peptides bound on MHC molecules. $CD8^+$ T cells recognize endogenous peptides presented on MHC-I molecules present on most

nucleated cells. CTLs recognize peptides on MHC-I molecules on infected and tumor cells and destroy them. $CD4^+$ T cells recognize exogenous peptides on MHC-II molecules present on antigen presenting cells (APC) in lymphoid tissues initiating the immune response and releasing cytokines. T cells can be further subdivided by a distinct cytokine production, into so called helper T cells. T_h1 cells predominantly produce cytokines like interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which stimulate a cellular immune response by activating monocytes and macrophages. In contrast, T_h2 cells predominantly produce interleukin (IL) -4, IL-5 and IL-10, which stimulate B cells to boost an IgE-mediated allergic reaction.

Thus, the human immune system is an amazing constellation of responses to attacks from outside the body. It provides the body with a defense against infection, afforded by the presence of circulating antibodies and white blood cells. The system is remarkably effective, most of the time.

1.2 Antigen presenting cells

Antigen presenting cells are immune system cells which digest antigens from foreign cells or pathogens and present them, complexed with MHC, to T cells. This alerts the immune system to prepare to fight the invader. That is, when APCs present cancer cell proteins to T cells, the T cells are primed to attack the cancer. There are three main types of professional antigen-presenting cells: Dendritic cells, macrophages and B cells. These APCs are very efficient at phagocytosis, which allows them to present exogenous as well as internal antigens. They possess co-stimulatory molecules: cell-surface molecules that deliver essential signals to T cells, allowing the T cells to become activated and mature into fully functional forms.

The primary function of dendritic cells is to capture and present protein antigens to naive T lymphocytes. Most dendritic cells are derived from monocytes and are referred to as myeloid dendritic cells. They are located throughout the epithelium of the skin, the respiratory tract, and the gastrointestinal tract where, in their immature form, they are attached by long cytoplasmic processes. Upon capturing antigens and becoming activated by proinflammatory cytokines, the dendritic cells detach from the epithelium, enter lymph vessels, and are carried to regional lymph nodes. By the time they enter the lymph nodes, they have matured and are now able to present antigen to the populations of naive T lymphocytes located in the cortex of the lymph nodes. Macrophages are also derived from monocytes. Their primary function is to capture and present protein antigens to effector T lymphocytes. This interaction results in the

activation of that macrophage. B lymphocytes capture and present protein antigens to effector T4 lymphocytes. This interaction eventually triggers the T4 cell to produce and secrete various cytokines that enable that B lymphocyte to proliferate and differentiate into antibody-secreting plasma cells.

1.2.1 Monocytes

Monocytes are classified as cells of the mononuclear phagocytic system. They are the biggest phagocytic cells (18-25 μm) and make approximately 5-8 % of the total leukocytes and have bean-shaped nuclei. They are produced by the bone marrow from haematopoietic stem cell precursors, and are able to migrate and circulate in the blood for about one to three days before they move into different tissues throughout the body.

In the tissues, monocytes mature into different types of macrophages, like kupffer cells, alveolar macrophages and histiocytes as well as into dendritic cells (DC) (Zhou and Tedder, 1996). Effete monocytes are destroyed in the spleen. Monocytes also consist of different subsets, which are morphologically and phenotypically different and therefore also have different roles (Muller, 2001; Geissmann *et al.*, 2003).

Monocytes can recognize pathogens via pathogen-recognition receptors (PRR), e.g. Toll-like receptors (TLR). They phagocytose these foreign bodies and digest them. Monocytes are also capable of killing infected host cells via antibody-mediated cellular toxicity. Crucial for the immune response is the secretion of inflammatory cytokines by the monocytes, especially TNF-α (Frankenberger *et al.*, 1996), IL-1β (Stordeur *et al.*, 2003), IL-6 (Navarro *et al.*, 1989) and IL-8 (Chaly *et al.*, 2000). These and other cytokines bind to cytokine receptors on target cells initiating inflammation and activating both the complement pathways and the coagulation pathway. Chemokines, like the monocytes chemotactic protein-1 (MCP-1) and MCP-3, as well as the macrophage inflammatory protein-1 (MIP-1) and MIP-3α play also a significant role in chemotaxis (Loetscher *et al.*, 1994; Allavena *et al.*, 1994), activating and attracting T lymphocytes and NK cells to sites of infection.

Being part of the APC lineage as a precursor cell of DCs and macrophages, monocytes are able to process endogenous or exogenous antigens and present the peptides on their MHC surface molecules in order to get recognized by the different T cells subsets. The T cells then get activated which play a crucial role in defeating the pathogen. Primary monocytes are not efficient stimulators of T cells. This is in part due to low cell surface expression of MHC molecules and co-stimulatory molecules as CD40, CD80 and CD86 (Fleischer *et al.*, 1996;

Laupeze *et al.*, 1999). Critical for the development of mature monocytes capability is the expression of surface class II MHC molecules stably loaded with peptide. This expression is also dependent on the expression of the invariant chain (Ii) (Laupeze *et al.*, 1999), cathepsins and, in particular, HLA-DM. DM acts intracellularly on class II molecules loaded with class II-associated Ii-derived peptides (CLIP) to catalyze peptide exchange and stabilize empty class II molecules. Many of these molecules, MHC-II, DM, and Ii are regulated coordinately, in part through dependence on expression of class II transactivator (CIITA) (Reith and Mach, 2001). Furthermore, GM-CSF was shown to play a big role in inducing the expression of these molecules (Hornell *et al.*, 2003).

1.2.1.1 Phagocytosis by monocytes

Phagocytosis or "cell eating" is defined as a form of endocytosis. In the process of phagocytosis, the cell changes shape by sending out projections which help engulf an endo- or exogenous particle. Monocytes can phagocyte particles with a diameter ≥ 2 µm. They phagocyte foreign particles and pathogens like bacteria, viruses and fungi as well as apoptotic cells, mainly red blood cells and platelets.

Monocytes, like all professional phagocytes, recognize opsonized particles as well as specific molecules called pathogen-associated molecular patterns (PAMP) via PRR and phagocyte them (Janeway, 1992). Opsonization means the coating of pathogens with molecules, especially antibodies (mainly IgG) and C3b, which are recognized by specific phagocytes receptors and so makes them more susceptible to phagocytosis. PAMPs are structures on pathogens that are not shared with the host cells, e.g. lipopolysaccharide (LPS, also called endotoxin) of gram-negative bacteria and peptidoglycan of gram-positive bacteria. Considering fungi, monocytes have a PRR called mannose receptor (MR) that recognizes PAMPS containing mannose and fucose on the surfaces of the pathogen (Stahl and Ezekowitz, 1998).

Phagocytosis, unlike pinocytosis and endocytosis (uptake of smaller molecules, < 0.5 µm), is actin-dependent and leads to the polymerization of actin at the site of ingestion, and the internalization of the particle via an actin-based mechanism. After internalization actin is shed from the phagosome, and the phagosome matures by a series of fusion and fission events with components of the endocytic pathway, culminating in the formation of the mature phagolysosome (Aderem and Underhill, 1999). In the Phagolysosomes, both oxygen-dependent and independent processes lead to the degradation of the internalized pathogen.

Superoxide (O^{2-}), H_2O_2 and other active radicals are responsible for killing bacteria and fungi in the oxygen-dependent way (Philippe *et al.*, 2003). The oxygen-independent way is characterized by a drop in the pH-value which activates lysosomal enzymes responsible for degradation.

Monocytes were also shown to phagocytose *A. fumigatus* conidia and a genome-wide expression analysis was done to investigate the genes regulated during phagocytosis (Cortez *et al.*, 2006).

1.3 Major histocompatibility complex

Major histocompatibility complex (MHC) molecules are glycoproteins expressed at the surface of almost all vertebrate cells. They are highly polymorphic genes found in humans on chromosome number 6. Each person has their own collection of MHC molecules, but monozygotic human twins have the same histocompatibility molecules on their cells. The term "histocompatibility" refers to the difficulty of finding compatible grafts between a donor and a patient. MHC in man is called "Human Leukocyte Antigen" (HLA). MHC was first identified by Peter Gorer in 1937 as a blood group locus that controlled the presence of antigens on the surface of mouse erythrocytes. In 1980 George Snell, Jean Dausset and Baruj Benacerraf received the Nobel Prize in Physiology or Medicine for their contributions to the discovery and understanding of the MHC (Snell, 1980; Marx, 1980). There are two major types of MHC protein molecules - class I and class II - that span the membrane of almost every cell in an organism and present antigens, respectively, to CD8 and CD4 T cells.

Table 1.3: Expression of MHC molecules I and II in different cell types. (-) stands for no expression, (+) to (+++) stands for normal to high expression. DCs express MHC in very high levels.
(www-immuno.path.cam.ac.uk/~immuno/part1/lec07/lec7_97.html)

Cell Type	**MHC I**	**MHC II**
T cells	+++	Varies, inducible in some species
B cells	+++	++
Macrophages	+++	+
Dendritic cells	+++ x10	+++ x10
Granulocytes	++	-
Endothelium	++	- (inducible)
Hepatocytes	+	-
Neurons	-	-

A third type of MHC protein molecules, MHC class III proteins, include complement proteins which are involved in the antibody response, the inflammatory cytokines, TNF-α and -β and two heat shock proteins which help cells deal with heat, stress and viral infection.

MHC class I and class II molecules are characterized by distinctive α and β polypeptide subunits that combine to form αβ heterodimers characteristic of mature MHC molecules. MHC Class I molecules consist of two proteins: the soluble β2m (not encoded in MHC locus) and the α chain consisting of the α1, α2, α3, and transmembrane/cytoplasmic tail domains. The peptide (typically 8-11 amino acids long) is held in the peptide-binding groove between the α1 and α2 subunits. The peptide's ends are tethered into two pockets located on the MHC. These MHC pockets interact with the side chains of the terminal amino acids.

MHC Class II is a symmetrical molecule made of two transmembrane proteins, the α chain (α1-α2 globular domains) and the β chain (β1-β2 domains). The peptide cleft is between subunits α1 and β1, and it holds peptides differently than MHC Class I (12-16 amino acids). Instead of tethering peptide at its ends, MHC Class II works more as a clamp, tethering the peptide in the center through multiple H-bonds, van der Waals, and electrostatic interactions; the ends of the MHC Class II "clamp" are open. This allows more flexibility in the length and types of peptide that can bind.

1.3.1 Antigen processing and presentation

MHC-I molecules can bind peptide epitopes from endogenous proteins found within the cytosol like viral proteins produced during viral replication, tumor antigens produced by cancer cells and self peptides from human cell proteins. These proteins are degraded by the proteasomes, where proteases and peptidases are active. A transporter protein called TAP located in the membrane of the cell's endoplasmic reticulum then transports these peptide epitopes into the endoplasmic reticulum (ER) where they bind to newly made MHC-I molecules. The MHC-I molecules with bound peptides are then transported to the Golgi complex and placed in exocytic vesicles. These vesicles carry the MHC-I/peptide complexes to the cytoplasmic membrane of the cell where they become anchored to its surface. On APCs, TCRs and CD8 molecules on the surface of naive CD8 T cells recognize then these peptide epitopes bound to the MHC-I molecules, whereas those on infected and tumor cells are recognized by TCRs and CD8 on the surface of CTLs.

MHC-II molecules are made primarily by APCs. MHC-II molecules have a deep groove that can bind peptide epitopes from exogenous proteins which come from phagocytosed microbes and pathogens. After degradation in phagolysosomes takes place, these proteins are degraded by proteases into a series of peptides. While MHC-II is being assembled within the endoplasmic reticulum, a protein called the invariant chain (Ii) attaches to the peptide-binding groove of the MHC-II molecules preventing peptides designated for binding to MHC-I from binding to MHC-II. The MHC-II molecules with bound Ii chain are transported to the Golgi complex, and placed in vesicles. The vesicles then fuse with the peptide-containing phagolysosomes. The Ii chain is removed and the peptides are now free to bind to the grooves of the MHC-II molecules. The MHC-II/peptide complexes are then transported to the cytoplasmic membrane of the APC where they become anchored to its surface. Now they can be recognized by complementary-shaped TCRs and CD4 molecules on the surface of naive CD4 T cells.

1.4 *Aspergillus* species

In 1729, *Aspergillus* was first described by an Italian priest and biologist named P. Micheli. In studying the shape of the fungus, he was reminded of an aspergillum, an instrument used for the dispersion of holy water, thus the name. *Aspergillus* is a member of the *deuteromycetes*, or Fungi Imperfecti, which is a group reserved for fungi for which there is no known sexual state. However, the sexual state (teleomorph) for many of the species of *Aspergillus* is known, and most of these are in the *Ascomycota* genus *Emericella*, which forms cleistothecia (closed ascocarps) under certain conditions. *Ascomycota* are 'spore shooters'. They are fungi which produce microscopic spores. It is likely that all members of the genus *Aspergillus* are closely related and should be considered members of the *Ascomycota*. There are around 185 species under the genus *Aspergillus*. Around twenty species have been reported so far as causative agents of opportunistic infections in humans. Among these, *Aspergillus fumigatus* is the most frequently isolated species, followed by *Aspergillus flavus* and *Aspergillus niger*. Among the other species not often isolated as opportunistic pathogens are *Aspergillus clavatus*, *Aspergillus glaucus* group, *Aspergillus nidulans*, *Aspergillus oryzae*, *Aspergillus terreus*, *Aspergillus ustus* and *Aspergillus versicolor*.

1.4.1 *Aspergillus fumigatus*

Aspergillus fumigatus was first well-described and illustrated in the year 1863 by J. B. Georg W. Fresenius. Its natural ecological niche is the soil, wherein it survives and grows on organic debris (Latgé, 1999).

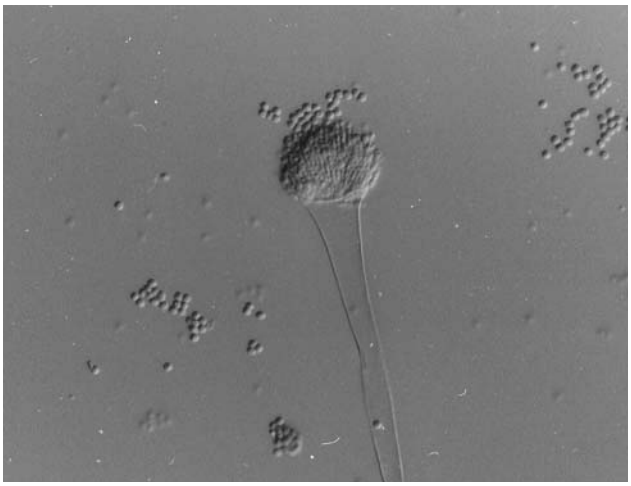


Figure 1.4.1: Light microscopy of typical *A. fumigatus* sporulating structures (from Latge, 1999).

This thermophilic filamentous fungus grows and sporulates mainly in humid environments, and it is one of the most ubiquitous of those with airborne conidia (Mullins *et al.*, 1976). It sporulates abundantly, with every conidial head producing thousands of green conidia into the atmosphere with a diameter of 2.5 – 3 μm. The small size of the conidia keeps them airborne indoors and outdoors. Some mutant strains had also been identified which have pigmentless (white) conidia (Jahn *et al.*, 1997).

The conidiophores originate from the basal foot cell located on the supporting hyphae and terminate in a vesicle at the apex (figure 1.4.1). The chains of conidia are borne directly on the vesicles (20 – 30 μm in diameter). No sexual stage is yet known for this species. However, accumulating evidence suggest that a sexual stage for *A. fumigatus* may yet be identified (Dyer and Paoletti, 2005). *A. fumigatus* is a fast grower: the colony size can reach about 4 cm within a week. It is a thermophilic species, with growth occurring at temperatures as high as 55^{o}C and survival maintained at temperatures up to 70^{o}C (Leslie *et al.*, 1988).

The cell wall of *A. fumigatus* is a complex structure mainly composed of polysaccharides and where the majority of the antigens secreted by the fungus during its *in vitro* and *in vivo* growth are located (Latge *et al.*, 1993; Bernard and Latge, 2001). The main polysaccharides are β(1-3)-glucan, galactomannan and chitin. Along with the polysaccharides and other

molecules and enzymes, toxins like gliotoxin and fumagillin and helvolic acid are also located in the cell wall of conidia, but also in hyphae (Rementeria *et al.*, 2005).
Just recently, Nierman *et al.* (2005) published the complete genome sequence of *A. fumigatus*. The report details the complete 29.4 million base-pair genome sequence of *A. fumigatus* clinical isolate Af293, which consists of eight chromosomes containing 9926 predicted genes.

1.4.2 *Aspergillus fumigatus* and the immune system

All humans inhale several hundred conidia per day (Chazalet *et al.*, 1998; Hospenthal *et al.*, 1998). In healthy individuals, inhaled conidia cause rarely allergic diseases or pulmonary infections. They are usually antagonized efficiently by the innate immune system; thus eliminated primarily by pulmonary alveolar macrophages before they are able to germinate and invade tissue organs. In immunosuppressed persons, conidia are able to reach the alveoli of the lungs and germinate into hyphae, which in term invade the neighboring blood vessels. Over the last years, *A. fumigatus* has become the most prevalent fungal pathogen, causing severe and mostly fatal invasive infections in immunocompromised patients, like those with acute leukemia or after solid-organ or bone marrow transplantation (Andriole, 1993; Denning, 1998). The mortality rate is 50% to 80% in these patients (Denning, 1996; Wald *et al.*, 1997). Invasive aspergillosis (IA) occurs in 10 to 25 % of all leukemia patients, in whom the mortality rate is 80 to 90 %, even when treated (Denning, 1995; Groll *et al.*, 1996). Invasive pulmonary aspergillosis is characterized by hyphal invasion and destruction of pulmonary tissue. The fungus has a tendency to invade blood vessels, and the clinical syndrome is often termed “angioinvasive pulmonary aspergillosis” (Oren and Goldstein, 2002). The invasive infection can affect any organ of the body, but especially the heart, lungs, brain, and kidneys. In addition, other diseases like bronchopulmonary aspergillosis and aspergilloma are caused by the fungus. Bronchopulmonary aspergillosis is characterized by fungus colonization of the mucus within the bronchi, evoking a severe allergic reaction. In aspergilloma, the fungus forms a mycelial ball in a lung cavity produced by an earlier attack of tuberculosis. The wall of the cavity may erode, causing the patient to spit blood, and necessitating surgical intervention.
Innate and adaptive immune effector mechanisms have been reported to be important in the successful control of IA. *A. fumigatus* activates primarily immune effector cells such as monocytes/macrophages and DCs. Several studies already described the role of these cells in defending the host organism against this pathogen. Monocytes and macrophages are able to

phagocytose conidia of *A. fumigatus* by the help of many receptors and surface molecules, such as TLRs or Pentraxin-3 (PTX3) (Wang *et al.*, 2001; Ibrahim-Granet *et al.*, 2003; Garlanda *et al.*, 2002). The phagocytosed spores are taken down in the phagolysosome, and protein antigens are degraded into a series of peptides, which are presented to $CD4^+$ T cells by MHC-II molecules. It has also been shown that human monocytes bind to and destroy *A. fumigatus* hyphae with either oxygen-dependent or -independent mechanisms (Diamond *et al.*, 1983). Yet, there are not enough data till now concerning the interaction and killing of *A. fumigatus* by human monocytes. Most studies focused on murine models or human alveolar macrophages.

DCs were also shown to play a vital role in host defense against *A. fumigatus*. They were shown to bind and phagocytose *A. fumigatus* conidia, and initiate a T cell response in the lymph nodes (Grazziutti *et al.*, 2001; Bozza *et al.*, 2002; Serrano-Gomez *et al.*, 2004). TLR2 and TLR4 were shown to play a major role in the activation of DCs and also granulocytes by *A. fumigatus* (Braedel *et al.*, 2004).

1.4.3 *Aspergillus fumigatus* antigens

Many *A. fumigatus* allergens or antigens that primarily induce immune responses have been identified and characterized. They can be classified according to their production and localization region as well as their structure and function and can be further subclassified into proteins, polysaccharides, and glycoproteins (Kurup and Kumar, 1991).

A large number of enzymes such as chymotrypsin, proteinases, elastases, ribonucleases, catalases, and superoxide dismutases are included into the antigens' list. An immunodominant *A. fumigatus* antigen was reported belonging to a catalase subclass which recognized over 90% of serum samples from patients with aspergilloma (Lopez-Medrano *et al.*, 1995). Superoxide dismutase (SOD) was isolated from *A. fumigatus* and specifically reacted with the sera from patients with confirmed aspergillosis on Western blots (Hamilton *et al.*, 1995). Also proteases purified from membrane vesicles of *A. fumigatus* were reported to be involved in the pathogenesis of aspergillosis (Piechura *et al.*, 1990). Also among the common protein antigens or allergens produced by both *A. fumigatus* conidia and hyphae, Asp f1 was detected in aspergillosis patients, which is sometimes called mitogillin, and was shown to have cytotoxic activity. A synthetic peptide epitope of Asp f1 (LNPKTNKWEDK) was used for improved immunodiagnosis of allergic bronchopulmonary aspergillosis and showed high diagnostic efficiency (Madan *et al.*, 2004). Another allergen, Asp f16, was shown to activate a

T_h1 response and an HLA class II retricted epitope (HLA-DRB1-0301, TWSIDGAVVRT) was identified in a murine model, as well as CTL responses directed to class I (HLA-B*3501) restricted epitopes, in vitro (Ramadan *et al.*, 2005; Ramadan *et al.*, 2005).

β-1,3-glucan and chitin are the main components of the *A. fumigatus* cell wall, along with galactomannan and a linear β-1,3/1,4-glucan. Galactomannan was detected in serum of patients with invasive aspergillosis (Dupont *et al.*, 1987). Today, the detection of galactomannan in blood of patients is used to diagnose invasive aspergillosis in humans (Hope *et al.*, 2005). Along with these polysaccharides and glycoproteins, *A. fumigatus* is known to produce various immunosuppressive mycotoxins. Recent studies have made progress in the determination of mycotoxins as virulence factors. Gliotoxin was also found to be detectable in the sera of aspergillosis mice and of aspergillosis patients. Gliotoxin is an inducer of apoptotic cell death in a number of cell types, including monocytes, macrophages and other immune cells, by the inhibition of transcription factor NF-κB (Stanzani *et al.*, 2005; Suen *et al.*, 2001; Pahl *et al.*, 1996). Fumagillin, another *A. fumigatus* toxin, was shown to play a significant role in damaging epithelial cells in the respiratory tract and influencing colonization of the airways (Amitani *et al.*, 1995).

1.5 Cytokines and chemokines in fungal infections

Cytokines are secreted glycoproteins (most between 15 and 40 kDa) that provide a means for intracellular signaling. They act via cell receptors and mediate profound effects on cell proliferation, differentiation, and activation. They regulate the immune system, specifically the interleukins (IL, now numbering 18), the interferons (IFN-α, -β, and -γ), the tumor necrosis factors (TNF-α) and lymphotoxins, and the hemopoietic colony stimulating factors (granulocyte CSF, macrophage CSF, and granulocyte–macrophage CSF) (Kelso, 2000). Chemokines are small chemotactic cytokines (6-14 kDa) that attract leucocytes to sites of infection and inflammation. They are key regulators of migration in lymphoid tissues. To date, at least 40 distinct chemokines have been well characterized and are produced by a variety of cell types (Baggiolini *et al.*, 1997; Rollins, 1997).

Cytokines and chemokines play an important role in host defense against fungal infections. Inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8 have been shown to be expressed by immune cells during fungal infections (Frankenberger *et al.*, 1996; Roilides *et al.*, 1998; Stordeur *et al.*, 2003), as well as many chemokines which attract fellow immune cells to the

infection and inflammation site, such as the chemokines of the MCP or the MIP family (Loetscher *et al.*, 1994; Allavena *et al.*, 1994; Yang *et al.*, 2003).

TNF-α (17 kDa) is a member of a superfamily of cytokines produced primarily by monocytes and macrophages which induce necrosis of tumor cells and possess a wide range of pro-inflammatory actions. It was shown to be over-expressed by monocytes, macrophages and other leucocytes upon contact or interaction with fungal pathogens, mainly in the lung (Liebhart *et al.*, 2002; Pylkkanen *et al.*, 2004). It has shown to be a critical proximal signal in the initiation and maintenance of innate pulmonary immunity in animal models of pneumonia and human pneumonia (Moussa *et al.*, 1994; Gosselin *et al.*, 1995).

Interleukins are the cytokines that act specifically as mediators between leucocytes. IL-1, originally known as Lymphocyte Activating Factor (LAF), activates T lymphocytes, which then proliferate and secrete IL-2. IL-6 appears to be directly involved in the responses that occur after infection and injury and may prove to be as important as IL-1 and TNF-α in regulating the acute phase response. IL-8 acts chemoattractant on T cells and neutrophils and helps to bring them to the site of an inflammation. It was demonstrated that these interleukins are secreted among others, upon stimulation of monocytes with fungal pathogens in vitro (Castro *et al.*, 1996), but also from cells of patients with airway infections (Cembrzynska-Nowak *et al.*, 1998; Mazzarella *et al.*, 2000).

MCP and MIP belong to the family of C-C chemokines. It has been reported that MCP chemokines are major attractants of human $CD4^+$ and $CD8^+$ T lymphocytes (Loetscher at al., 1994) but also MIP chemokines have differential effects on lymphocytes (Schall *et al.*, 1993). Critical for the host defense against fungal pathogens and their infections is MCP-1 (CCL2) and its receptor CCR2. Recent studies showed the role of CCL2 and CCR2 in response and clearance of *A. fumigatus* conidia. $CCR2^{-/-}$ mice showed less neutrophil recruitment into the airways as well as an increase in inflammation and subepithelial fibrosis (Blease *et al.*, 2000). Also MIPs, mainly MIP-1α, have been reported to play a role in chemotaxis during fungal infections. They were shown to be up-regulated in cells of patients with fungal infections (Shahan *et al.*, 1998; Mehrad *et al.*, 2000).

1.6 Clinical approaches to *Aspergillus fumigatus* infections

Invasive Aspergillosis comprises a spectrum of diseases caused by *Aspergillus* species. These include allergy as well as invasive, inflammatory, granulomatous, narcotising diseases of lungs, and other organs. The three principal entities are: allergic bronchopulmonary

aspergillosis, pulmonary aspergilloma and invasive aspergillosis. The type of disease and severity depends upon the physiologic state of the host.

Allergic aspergillosis has been successfully treated with corticosteroids, and intraconazole (Stevens *et al.*, 2000). Aspergillomas may be treated by surgical resection; however, this approach may cause morbidity and mortality, therefore it should be reserved for patients at high risk. Invasive aspergillosis may be treated with voriconazole and amphotericin B (Herbrecht *et al.*, 2002) as well as itraconazole (Denning and Stevens, 1990). In addition to those, a new promising group of antifungals called "echinocandins" is nowadays being used in treatment of the rapid progressive invasive aspergillosis. They are less toxic to the host than previous drugs and inhibit the β-1,3-glucan synthase. The most prominent of them is caspofungin which showed already positive outcomes in aspergillosis therapy (Kartsonis *et al.*, 2005), along with micafungin and anidulafungin. These antifungals may be used in combination, in order to get maximum effects.

A new promising tool that may in the near future play a significant role in therapy of aspergillus infections is specific gene silencing of redundant genes with RNA interference (RNAi). RNAi is a method based on the application of a short double-stranded RNA into the cell, which results in down-regulation of the mRNA with the complementary sequence. Some studies with promising results have been made in this field, silencing specific aspergillus genes of interest (Mouyna *et al.*, 2004).

1.7 Aims of the study

Aspergillus fumigatus is one of the most widely distributed microorganisms on earth. It is the leading cause of infection-related death in leukemia and bone-marrow transplant patients. These are two crucial arguments for researchers to work in this field. This study was initiated to investigate a still little-researched field in immune response against this pathogenic fungus: the role of monocytes.

Our first aim was to investigate phagocytosis of *A. fumigatus* conidia by human monocytes. Monocytes are the biggest phagocytes in blood; therefore having a crucial role in phagocytosis of foreign pathogens escaping the lungs and entering the bloodstream. So do peripheral blood monocytes phagocytose conidia in vitro? At what time and what about the phagocytosis rate? For this purpose, phagocytosis was microscopically analyzed using fluorescent dyes after 3 and 6 hours of incubation.

Our second aim was to study the regulation of gene expression in monocytes after incubation with *A. fumigatus*. How do *A. fumigatus* conidia and hyphae affect gene epression in monocytes? Are any fungal-specific genes regulated in monocytes after interaction? Do conidia and hyphae regulate different genes? Which ones? For this purpose, we analyzed gene expression after 3, 6 and 9 hours of incubation, using real-time RT-PCR, microarray gene chip analysis and RNA interference.

Our third aim was to investigate peptides presented on different HLA molecules of monocytes, after incubation with *A. fumigatus*. Are *A. fumigatus* peptides detectable on HLA class II molecules on monocytes? Or maybe on class I? Are different peptides presented by stimulated cells than by non-stimulated ones? We tried to answer these questions by extracting HLA presented peptides after stimulation with *A. fumigatus*. Analyses followed by ESI/Q-Tof mass spectrometry in combination with HPLC (LC-MS).

2. Material

2.1 Cell cultures

Different cultures and subsets of monocytes were used in the following experiments, according to the aim and the magnitude of the methods used, in order to get the best possible basis for each different procedure.

2.1.1 Peripheral blood monocytes

For pre-experiments, monocytes were won from buffy coats of healthy donors, supplied by the local blood bank. For more decisive and essential experiments, monocytes were won from fresh blood, isolated from healthy donors.

2.1.2 Cell lines

THP-1 cells were purchased form the "German National Resource Centre for Biological Material" (DSMZ): Accession number ACC 16. THP-1 is a human acute monocytic cell line ($CD14^+$), established from the peripheral blood of a 1-year-old boy with acute monocytic leukemia (AML) at relapse in 1978. HLA haplotypes of THP-1 are HLA-A2, -A9, -B5, -DRW1 and -DRW2. The cells were described to be phagocytic (Tsuchiya *et al.*, 1980).
JY cells, a human lymphoblastoid cell line (LCL), were purchased from "The European Collection of Cell Cultures" (ECACC): Accession number ECACC-94022533. HLA haplotypes of JY are HLA-A2, -B7, -Cw7 and -DR4.

2.2 *Aspergillus fumigatus* cultures

The used *Aspergillus fumigatus* cultures stemmed from a well characterized clinical isolate from the sputum of a patient with cystic fibrosis (isolate number: VA15701.2), supplied by the institute for medical microbiology in Tuebingen.

2.3 Chemicals, solutions and kits

Solutions for cell culture:

Phosphate Buffered Saline: PBS-DULBECCO (1x)	Biochrom KG, Germany
RPMI 1640 Medium with 25 Mm HEPES & Glutamax-I	GIBCO, Invitrogen, UK
HBSS Hanks' Balanced Salt (1x)	GIBCO, Invitrogen, UK
FICOLL separating solution	Biochrom KG, Germany
EDTA 0.5 M, pH 8	BIO Whittaker, USA
FCS	SIGMA Cell Culture, US
Monocyte Isolation Kit, human	Miltenyi Biotec, Germany
CD14-Microbeads	Miltenyi Biotec, Germany
Trypan blue 0.4%	GIBCO, Invitrogen, UK
Anaerocult C	MERCK, Germany

Reagents for fungal cultures:

Yeast Nitrogen Base	GIFCO Laboratories, US
Sabouraud-2%-glucose agar	MERCK, Germany
Ethanol (99%)	MERCK, Germany
Tween 20	MERCK, Germany

Reagents for FACS:

BSA	Sigma-Aldrich, USA
Formaldehyde	Sigma-Aldrich, USA
Fluorescence tagged antibodies:	
Anti-IgG (FITC) and anti-CD14 (FITC)	Becton Dickinson, USA
FACS-Flow	Becton Dickinson, USA
FACS-Safe	Becton Dickinson, USA
FACS-Rinse	Becton Dickinson, USA

Reagents for RNA work:

RNeasy Mini Kit and QIAshredder	Qiagen, Germany
ß-Mercaptoethanol	ICN Biomedicals Inc, US
AMV 1st strand cDNA synthesis kit	Roche GmbH, Germany
Affymetrix gene chip array: HG-U133A	Affymetrix, USA

Kits, primer and probes for Real-Time-PCR:

LightCycler FastStart DNA Master Hybridization Probes	Roche GmbH, Germany
LightCycler-h-ALAS Housekeeping Gene Set	Roche GmbH, Germany

Primer:		TIB MOLBIOL, Germany
TNF-α as	5' –ggCgTTTgggAAggTTggAT	5 µM
TNF-α se	5' – CTCTggCCCAggCAgTCAgA	5 µM
hu IL-6 S	5' – CTTTTggAgTTTgAggTATACCTAg	5 µM
hu IL-6 A	5' – CgCAgAATgAgATgAgTTgTC	5 µM
IL-1β S	5' – CAgggACAggATATggAgCAA	5 µM
IL-1β A	5' – gCAgACTCAAATTCCAgCTTgTTA	5 µM

Probes:		TIB MOLBIOL, Germany
TNF-α LC	5' – Red640-CCACTggAgCTgCCCCTCAgCT p	0.3 µM
TNF-α FL	5' – gCATTggCCCggCggTTC X	0.3 µM
hu IL-6 LC	5' – Red640-CCACAAATgCCAgCCTgCTgAC p	0.3 µM
hu IL-6 FL	5' – TAgATgCAATAACCACCCCTgACCCA X	0.3 µM
IL-1β LC	5' – Red640-gTACAgATTCTTTTCCTTgAggCCCA p	0.3 µM
IL-1β FL	5' – gCTTATCATCTTTCAACACgCAggACA X	0.3 µM

Solutions for agarose gels:

Agarose		Sigma-Aldrich, USA
TAE-Puffer	(10x) 0.4 M Tris-acetate 0.01 M EDTA pH 8.3	Life Tech., Scotland
Gelstar	GmbH, 99.9% DMSO 0.01% dye	Biozym Diagnostik Germany
Gel loading buffer	65% (w/v) sucrose 10 mM Tris-HCl pH 7.5	Invitrogen, Germany

10 mM EDTA	
0.3% (w/v) Bromphenol blue	
100 bp DNA-Ladder	Invitrogen, Germany

Reagents for microscopy:

Fluoprep	BioMerieux, France
DePeX	SERVA, Germany
Antibodies:	
Mouse monoclonal anti-CD14	Dako, Denmark
Goat anti-mouse IgG (conjugate: Cy3)	Dako, Denmark
Fungi-Fluor Kit	Polysciences Europe
Sol. A: 0.05% Cellufluor:	GmbH, Germany
Sol. B: Evans blue	

Reagents and kits for work with RNAi

siRNAs:	
non-specific control-siRNA (20 µM)	Qiagen, Germany
GAPDH-siRNA (20 µM)	Ambion, Texas, USA
TLR2, TLR4 & TLR8 (2-For-Silencing siRNA duplexes)	Qiagen, Germany
RNAi Starter kit (RNAiFect)	Qiagen, Germany
Oligofectamine™	Invitrogen, Germany
Silencer siRNA Transfection kit	Ambion, Texas, USA

Reagents for isolating HLA-peptides

"Complete" protease inhibitor tablets	Roche, Germany
CHAPS	Roche, Germany
CNBr-activated Sepharose 4B	Amersham Phar., Sweden
Protein A-Sepharose, CL-4B	Amersham Phar., Sweden
TFA	Applied Biosystems, USA
Bio Brene Plus	Applied Biosystems, USA
O-methylisourea hemisulfate 94%	ACROS organics, USA
$^{1}H_4$- and $^{2}D_4$-NicNHS ester	Weik, Uni. of Tuebingen

Reagents for western blots

40% Acrylamide, N,N'-Methylenebisacrylamide (29:1)	Roth, Germany
Ammoniumpersulfate, 10% (APS)	Sigma, Germany
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma, Germany
Polyclonal rabbit anti-human-ß2 microglobulin antibody	Anogen, Canada
anti-rabbit horseradish-peroxidase antibody	Amersham Bio., Germany
monomer HLA-B*1501	Walter, Uni. of Tuebingen

Reagents for ELISA

Quantikine Immunoassay kits	R&D Systems, USA

2.4 Instruments

Real-time RT-PCR:	
LightCycler™-Instrument	Roche, Germany
LC Carousel Centrifuge	Roche, Germany
Computer	Windows NT 95
Software	LC Software Version 3.5
Incubators (35°C, 37°C)	Heraeus 6220, Germany
MACS Separation Columns	Miltenyi Biotec, Germany
Centrifuges:	
Multifuge 3 S-R & 1.0R	Heraeus, Germany
Biofuge 13	Heraeus, Germany
Centrifuge 5415R & 5417R	Eppendorf, Germany
Centrifuge RC 5C Plus, Rotor GS-3	Sorvall, Kendro, Germany
Ultracentrifuge L-80, Rotor Ti45	Beckman Coult., Germany
Sterile bench	Heraeus, Germany
Water baths	Memmert, Germany
Heating block Thermomixer 5437	Eppendorf, Germany
Pipettes (10 µl, 100 µl, 1000 µl)	Eppendorf, Germany
Microscopes:	
Laborlux S	Leitz, Portugal
Diavert	Leitz Wetzlar, Germany
Axiolab	Zeiss, Germany

Fluorescence Microscopy:	
Microscope	Leica DMRE, Germany
Camera	Diagnostic Instr. Inc, USA
Object slides 76x26 mm	Langenbrick, Germany
Cover slips 24x60 mm	Langenbrick, Germany
FACS-Instrument:	
FACS Calibur	Becton Dickinson, USA
Tubes (5 ml)	Becton Dickinson, USA
Analysis software	Cell Quest
Photometers:	
GeneQuant II	Pharmacia Biot., Germany
UV-Spectrometer Ultrospec 3000	Pharmacia Biot., Germany
Quartz cuvettes	Hellma, Germany
Agarose gel:	
Gel scanner: ImageMaster	Pharmacia Biot., Germany
Gel chamber: HORIZON 11·14	BRL, Life Technol., USA
siRNA electroporation:	
Electroporator instrument: EPI 2500	Fischer, Germany
Electroporation cuvettes	Biorad, Germany
MHC-peptide-Isolation:	
Table pump P-1	Pharmacia LKB, Sweden
Magnet rotor: RCT basic	IKA Labortech., Germany
Branson Sonifier 250	Branson Ultrasonics, USA
Pressure filtration unit: Sartolab P Plus	Sartorius AG, Germany
Microliter syringes	Hamilton, Switzerland
Centrifugal filter devices: Centricons	Millipore corp., USA
Cryo Freezing Container	Nalgene, H. le Spring, UK
SDS gels and Western Blots:	
Voltage source Power Pac 200	Bio-Rad, Germany
Mini-Protean II electrophoresis cell	Bio-Rad, Germany
Semi-Dry electroblotunit transfer-blot	CTI, Idstein, Germany
Nitrocellulose membrane Hybond ECL	Pharmacia Biot., Germany
Filter paper: Whatman 3MM	Whatman, Maidstone, UK

Image-Station LAS-1000	FujiFilm, Germany
Edman and Mass-spectrometry:	
Lyophilisation instr.: Vaco5	ZIRBUS, Germany
SpeedVac vacuum concentrator	Bachhofer, Germany
Pre-column pump ABI 140D Delivery System	Perkin Elmer, Boston, US
μCapillary-LC-System Ultimate	Dionex, Germany
Fused-silica-μCapillary-column (5 µm C18-Material)	LC Packings, Germering
Pre-column C18-Material, 300 µm, 10 mm	Dionex, Germering
online-ESI capillary, 360/20 µm AD / ID capillary	New Objective, USA
offline-ESI capillary, Medium Capillaries Q-TOF ES387	Proxeon, Odense, DK
Hybrid-Q-Tof-Mass-spectrometer	Micromass, UK
Edman ABI "Procise" 494A Protein-Sequencer	Applied Biosystems, USA
C18-columns-5 PTH 5 µm	Perkin Elmer, Boston, US
Peptide cleanup C-18 spin tubes	Agilent, Germany

3. Methods

3.1 Preparation of cell cultures

Blood from buffy coats (normally 50 ml of 500 ml originally donated) was always diluted (1:1) with Hanks buffer before use. Fresh blood from healthy donors was usually also diluted 1:1 with Hanks buffer before further use.

3.1.1 Isolation of PBMC from human blood

Peripheral blood mononuclear cells (PBMCs), which contain the monocytes, the lymphocytes, the thrombocytes and the NK cells, were isolated with a FICOLL-gradient. Through centrifuging, erythrocytes, granulocytes and dead cells are pelleted and the PBMCs stay in the supernatant, because their density is smaller than that of the FICOLL (1.07 g/l), in contrast to the erythrocytes and the dead cell fragments. Briefly, the diluted blood was carefully layered on the FICOLL and 20 min at 2000 rpm brakeless centrifuged. The white layer originated above the FICOLL contains the mononuclear white blood cells. This interphase was carefully separated and the cells washed with Hanks buffer (Centrifugation: 10 min, 1300 rpm). The cell pellet was resuspended in hanks, cells were counted with a Neubauer cell chamber and put on ice till the next separation steps.

3.1.2 Cell-counting with the Neubauer chamber

20 µl from the cell suspension were added to 180 µl Hanks buffer (1:10 dilution). 20 µl from this dilution were added to 20 µl Trypan blue (1:2 dilution) and a chamber was filled with this dilution. Living cells could be distinguished from dead cells (blue) and counted in 16 of the small squares, which build one of 4 big squares, each with 1 mm side length (Figure 3.1.2). The cell number was then calculated using the following formula:

No. of counted cells x 10^4 x 2 x 10

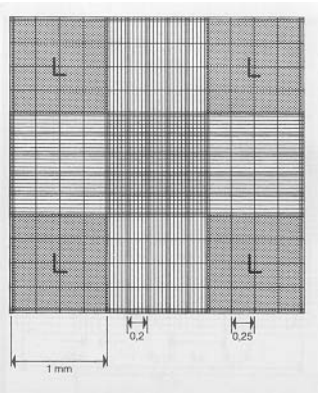


Figure 3.1.2: Neubauer chamber (www.ub.es/biocel/wbc/tecnicas/contajecelular.htm)

3.1.3 Extraction of monocytes

Monocytes were extracted from the PBMCs with the help of magnetic cell separation (Miltenyi *et al.*, 1990). We used two different methods, a positive selection with CD-14 microbeads and a negative selection with the "Monocyte isolation kit".

Being conjugated to a monoclonal mouse anti-human CD14 antibody, the microbeads bind specifically to the monocytes surface-receptor CD14. After application to a magnetic column, the monocytes-microbeads-complexes remain in the column, whereas the other cells pass through. The monocytes can be eluted afterwards, when taking the column off the magnet. The monocytic culture obtained revealed a high purity (> 95%), but a slight activation through the beads was taken in consideration. Briefly, 10^8 PBMCs were resuspended in 800 µl Hanks/2 mM EDTA/1% FCS buffer and incubated with 200 µl CD14-Microbeads for 15 min at 4°C. The 20-fold volume of Hanks/2 mM EDTA/1% FCS was then added and 10 min at 1300 rpm and 4°C centrifuged. A MACS LS-column was fixed to a special magnet and washed with 3 ml Hanks/2 mM EDTA/1% FCS. The cells were resuspended in 500 µl of the same buffer, applied to the column and washed 3 times with the buffer. After taking the column off the magnet, it was washed 2 times with the buffer and the monocytes were depleted through pressing them out with a special plunger. The monocytes were counted, resuspended in RPMI/20% FCS and incubated on ice till further use.

With the "Monocyte Isolation Kit", the other cells were bound to microbeads (CD3 for T cells, CD7 for hematopoietic stem cells, CD19 for B cells, CD45RA for naive T cells, CD56 for NK cells and IgE for mast cells), whereas the monocytes remained untouched. Unfortunately, we couldn't achieve a monocytic culture purity as with the positive selection (>75%). Nevertheless, a possible activation through the microbeads could be ruled out, which is essential for gene expression analysis. Briefly, 10^8 PBMCs were resuspended in 600 µl Hanks/2 mM EDTA/1% FCS buffer and 200 µl FCR blocking reagent in addition to 200 µl hapten-antibody-cocktail were added, then 5 min at 4°C incubated. Washing followed with 10-fold volume with Hanks/2 mM EDTA/1% FCS followed and centrifuged at 1300 rpm, 10 min and 4°C. The Pellet was resuspended in 600 µl of the same buffer and 200 µl FCR blocking reagent as well as 200 µl anti-hapten microbeads, and incubated 15 min at 4°C. Washing followed again with 10-fold volume with Hanks/2 mM EDTA/1% FCS. The MACS LS-column was treated as done with the positive selection method. The Pellet was resuspended in 500 µl of the same buffer and applied to the column, washed 3 times with the

buffer. The fractions obtained (containing the monocytes) were collected, the cells counted and resuspended in RPMI/20% FCS as well, till further use.

3.1.4 Generation of dendritic cells

Immature dendritic cells (iDCs) were generated from the $CD14^+$-monocytes through addition of cytokines. In brief, 1000 ng/ml IL-4 and 100 ng/ml GM-CSF were added every 2 days to the monocytes (10^6 cells/ 1 ml RPMI/10% FCS), in addition to fresh medium. After one week, the iDCs were counted and directly used. The generation of iDCs was tested with FACS by analyzing the surface expression of CD14, which was down-regulated. The iDCs were used in RNA interference experiments.

3.1.5 Culturing THP-1 and JY cells

The THP-1 cell line was cultured as well in RPMI/10% FCS at 37°C and 5-10% CO_2. These cells have a doubling time of about 2 days, so they were splitted 1:2 every two days and fresh medium was added. They could be kept under these conditions for a long time in culture and grew continuously.
The JY human lymphoblastoid cell line was cultured under the same conditions, but splitted 1:3-1:5 every three days.

3.1.5.1 Freezing and thawing of cells

In order to have always THP-1 cells in case of contaminations or other conditions, aliquots of $5x10^6$ cells were frozen from time to time and stored at -80°C. In detail, $5x10^6$ cells were centrifuged (1500 rpm, 5 min) and resuspended in 1 ml cold FCS/10% DMSO and directly frozen in special reservoirs surrounded with isopropanol, at -80°C.
Thawing was done by carefully incubating the frozen aliquot in warm water followed by direct washing with PBS (centrifugation at 1500 rpm, 5 min), resuspending in RPMI/10% FCS and culturing at 37°C.

3.2 Preparation of *Aspergillus fumigatus* cultures

An *A. fumigatus* sample collected from the sputum of a well characterized cystic fibrosis patient (isolate number VA15701.2, Institute for Medical Microbiology, Tübingen) was crossed out on a sabouraud-2%-glucose-agar culture plate and incubated 5-7 days at 37°C. The culture was ready to use for experiments first after the fungus was dense and with a greenish color.

3.2.1 Isolation of fungal conidia

The dense fungal colonies were washed with PBS/0.01% Tween using a syringe and a canula in order to repeat the washing process and so that a contamination with the bigger hyphal fragments could be excluded. Tween was used in order to reduce the surface hydophobicity of the conidia (Tronchin *et al.*, 1995). The conidial suspension was washed once with the same buffer and the conidia counted with the Neubauer chamber. After centrifugation (9000 rpm, 5 min), the pellet was resuspended in RPMI/10% FCS to the proper concentration. The conidia were then instantly used in experiments, either for incubation with cells or for generation of hyphae.

3.2.2 Generation and killing of hyphae

In order to obtain a purposely wanted number of hyphal cells, hyphae were generated from a well defined number of conidia. For this purpose, $5x10^7$ conidia were incubated in 50 ml "Yeast Nitrogen Base – medium" (10^6/ml) at 37°C for 18 h. The originated mycelial hyphae were washed twice with hanks buffer (5000 rpm, 5 min). Because only inactivated hyphae were to be used in our experiments in order to avoid overgrowth of cell cultures, we incubated the hyphal fragments directly in 70% ethanol-PBS for 24 h at 4°C. The hyphal suspension was then washed twice with hanks, resuspended in RPMI/10% FCS and had a final concentration of 10^6 in 1 ml (Wang *et al.*, 2001). Aliquots were frozen at -80°C until further use. The inactivation efficacy was controlled by cultivating the hyphae on Sabouraud-2%-Glucose agar plates. After few days, no fungal cultures could be detected. However, it cannot be ruled out that ethanol-inactivation may lead to slightly different monocytic responses if compared to live hyphae.

3.3 Incubation experiments

For time-dependent gene expression analysis, monocytes were incubated with two states of *A. fumigatus*, viable conidia and inactivated hyphae. For phagocytosis experiments, monocytes and dendritic cells were incubated with conidia, and for analyzing the effects of immunosuppressive drugs on expression of surface markers, dendritic cells were incubated with these drugs along with hyphae. Furthermore, THP-1 cells were incubated with hyphae too, for identification of possible *A. fumigatus* peptides on MHC class II molecules.

3.3.1 Incubation of monocytes with *A. fumigatus* conidia and hyphae

Human monocytes were isolated with the "Monocyte Isolation Kit" from three healthy donors and incubated with two states of *A. fumigatus*, hyphae and conidia, in addition to the negative control (medium), each for 3, 6 and 9 hours at 37°C, separately. Thus, 27 incubation experiments were performed in total.
Briefly, $5x10^6$ freshly isolated monocytes were incubated with 10^7 viable conidia (1:2 ratio) and/or 10^6 hyphae (5:1 ratio) in 3 ml RPMI/10% FCS total volume, at 37°C and 5-10% CO_2 for 3, 6 and 9 hours. The same no. of cells were incubated only in RPMI/10% FCS (medium control). RNA was directly isolated, its concentration was measured with standard UV-photometry and the RNA immediately frozen at -80°C until further use.

3.3.2 Incubation of THP-1 cells with *A. fumigatus* hyphae

In order to get enough material for peptide analysis on HLA molecules, a very large number of cells was necessary. For this purpose, cells were grown in culture flasks at 37°C in RPMI/10% FCS, then in bottles until they reached a certain concentration. At this point, they were stimulated for 6 h at 37°C with *A. fumigatus* hyphae. Briefly, the cells were grown in a bottle until their count reached about $5x10^8$ in total. These were then stimulated with 10^8 hyphae (5:1 ratio), in correlation with the gene expression assays. After 6 h, the cells were collected by centrifugation for 10 min at 1600 rpm and 4°C, followed by once washing with PBS (1600 rpm, 10 min). The pellets were immediately frozen at -80°C till further use. This procedure was repeated until enough cells were available (about $4x10^9$). At the same time, THP-1 cells were grown and harvested without being stimulated with hyphae, to have a non-stimulated comparison sample.

3.4 Fluorescence microscopy

Fluorescence microscopy is a widely used technique to detect cellular structures, or molecules inside or outside the cells of interest. Fluorescence is the emission of light that occurs within nanoseconds after the absorption of light that is typically of shorter wavelength. When a cell or cellular compartment is stained with a fluorescent dye and the dye is excited with light, the light emitted then from the dye is viewed through a filter that allows only the emitted wavelength to be seen, against a dark background (figure 3.4). The large spectral range of nowadays available fluorophores allows simultaneous imaging of different cellular, subcellular or molecular components (Lichtman and Conchello, 2005).

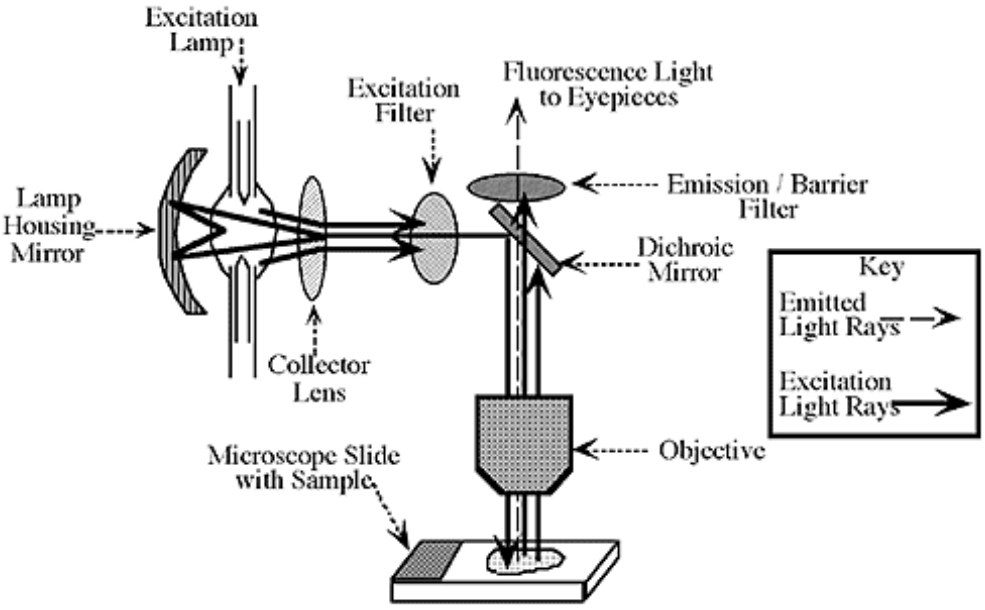


Figure 3.4: Components of the fluorescence microscope optical train.
(www.vysis.com/images/Content/FluorescenceMicroscopeDiag.gif)

For the microscopic analysis, monocytes were isolated from 10 ml of fresh blood samples from healthy donors using the positive selection method, where CD14-microbeads bind specifically to the monocytes in order to get a highly pure culture, which is essential for optimal microscopic images. A potential activation of the monocytes by the beads could be taken into account, since no gene expression analysis was performed.

3.4.1 Fluorescent labeling of *A. fumigatus* conidia and monocytes

Conidia were labeled with the Fungi-Fluor™ kit, according to the manufacturer's protocol. Fungi-Fluor contains a 0.05% solution of cellufluor, which binds nonspecifically to beta-linked polysaccharides found in cell walls of various organisms, such as chitin and cellulose. In our case, it will bind to the chitin which is a main component of the cell wall of *A. fumigatus*.

Monocytes were labeled with a monoclonal mouse anti-CD14-antibody followed by a goat anti-mouse Cy3-labeled antibody (both kindly provided by C. Sinzger, Virology institute, University of Tuebingen).

3.4.2 Incubation of monocytes with *A. fumigatus* conidia

For the incubation experiments with the monocytes, two strategies were followed; conidia were stained either before (a), or after incubation with the monocytes (b). This enabled us to differentiate between phagocytosed and unphagocytosed conidia, and study the correlation in both experiments.

(a) After staining the conidia, they were directly incubated with monocytes at a 1:2 ratio, first at 4°C for 30 min, then at 37°C for 6 hours. The suspension was then put on slides, left to dry and the cells fixed with 99% ethanol. A few drops of Fluoprep were added to fasten the cover slip on the slide. The slides were incubated at 4°C in the dark until the Fluoprep got hard. Then microscopy was performed.

(b) The same procedure as in (a), only that the conidia staining was done after 6 hours of phagocytosis, followed by a washing step with PBS and then fixing with ethanol.

3.5 Fluorescence Activated Cell Sorting (FACS)

The term "FACS" is Becton-Dickinson's registered trademark and is an acronym for Fluorescence-Activated Cell Sorter, a main technique in flow cytometry. Flow cytometry measures simultaneously size, granularity and fluorescent intensity of single cells in a suspension. It also facilitates quantitative analysis of the expression of cell surface and/or intracellular molecules, by measuring the relative fluorescent intensity of a molecule stained before.

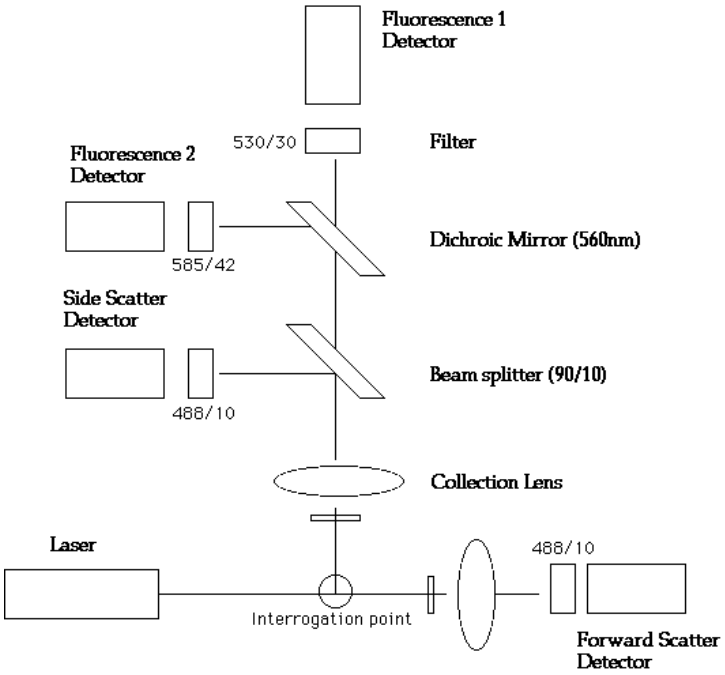


Figure 3.5: A simplified digram of a typical analytical flow cytometer.
(www.pathology.wustl.edu/html/facilitiesfacsfc.html)

The modern flow cytometer consists of a light source, collection optics, electronics and a computer to translate signals to data. The light source of choice is a laser which emits coherent light at a specified wavelength. Scattered and emitted fluorescent light is collected by two lenses (one set in front of the light source and one set at right angles) and by a series of optics, beam splitters and filters, specific bands of fluorescence can be measured (figure 3.5).

3.5.1 Staining of monocytes

Monocytes were analyzed using FACS in order to specify their purity in a suspension after separation. This was achieved by staining the suspension cells with a FITC-conjugated anti-CD14 antibody. The CD14 antigen is part of the functional heteromeric LPS receptor complex which is strongly expressed on most human monocytes and macrophages in peripheral blood.

The staining took place by incubating the monocytes with the antibody for 30 min at 4°C in the dark. After washing twice with hanks/10% FCS, the cells were fixed with 1-2% formaldehyde, and stored at 4°C in the dark till analysis. FACS analysis was done using standard software and hardware, i.e. the “Cell Quest” software and the FACS-Calibur from “Becton Dickinson”.

3.5.2 Staining of THP-1 and JY cells

Relative MHC surface expression was analyzed on THP-1 cells, before and after incubation with *A. fumigatus*. Staining was performed with specific monoclonal antibodies previously purified from culturing hybridoma cells supernatant (W6/32 for HLA-A*B*C*, L243 for HLA-DR, BB7.2 for HLA-A*02, B123.2 for HLA-B* and GAP-A3 for HLA-A*03), which were again stained with a secondary FITC-conjugated goat anti-mouse IgG, F(ab')2 antibody. The JY cell line was used as a positive control. Briefly, 500 µl (5 µg/ml) from each antibody were prepared and the antibodies were diluted 1:5 (1 µg/ml) and 1:10 (0.2 µg/ml). Cells were diluted to 2 x 10^5 cells/well in 100 µl FACS buffer (PBS + 2.5% FCS), washed 3x with that same buffer (centrifuge: 1800 rpm, 2 min, 4°C), pelleted at last and then the antibodies were added. Incubation followed for 30 min at 4°C in the dark, then 4x washing with FACS buffer. 100 µl of a 1:200 dilution of the secondary FITC-conjugated antibody were added to the cells. Again incubation followed for 30 min at 4°C in the dark. Washing followed (4x) with FACS buffer and direct fixing of the cells was performed, using 200 µl FACS buffer with 1% paraformaldehyde. FACS analysis was afterwards performed (see above).

3.6 Methods in molecular biology

For studies of gene expression analysis, molecular biology methods played a major role in our research group and were performed at a high grade of accuracy and purity, which are two major requirements in working with nucleic acids.

3.6.1 RNA isolation

RNA was isolated with the RNeasy Minikit and the QiaShredder spin columns (QIAGEN) according to the manufacturer's protocol. Briefly, after incubation, the adherent monocytes were directly treated with RLT lysis buffer in the incubation wells, in order to get as much as possible cells for a sufficient RNA yield. After a few washing steps, 45 µl total RNA were eluted. The RNA concentration and purity (260nm/280nm ratio) were then measured with standard UV-photometry, using 6 µl RNA. An 8 µl aliquot was taken for cDNA synthesis and the remaining RNA (31 µl) immediately frozen at -80°C until further use, i.e. for microarray chip analysis.

3.6.2 cDNA synthesis

cDNA synthesis was performed using the AMV 1st strand cDNA synthesis kit (Roche), following the manufacturer's protocol. Briefly, 8 µl RNA were first incubated for 10 min at 65°C with 2.2 µl oligo-p(dT)-primer [0.8 µg/µl] and then for 5 min on ice. The following 9.8 µl mix was added:

4 µl $MgCl_2$ [25 mM], 2 µl 10x reaction-buffer, 2 µl dNTPs [10 mM], 1 µl RNase-inhibitor [50 units/µl] and 0.8 µl reverse transcriptase [≥ 20 units]

The 20 µl mixture was incubated for 1 h at 42°C and the reaction then stopped in a 95°C water bath for 5 min. cDNA concentration was then measured using standard UV-photometry and the remaining cDNA frozen at -20°C until further use, i.e. for gene expression analysis with real-time PCR.

3.6.3 Real-Time PCR using the LightCycler instrument

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (Livak *et al.*, 1995). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. However, real-time PCR does not detect the size of the amplicon and thus does not allow the differentiation between DNA and cDNA amplification. There are three main fluorescence-monitoring systems for DNA amplification: (1) SYBR Green, (2) hybridization probes and (3) TaqMan probes.

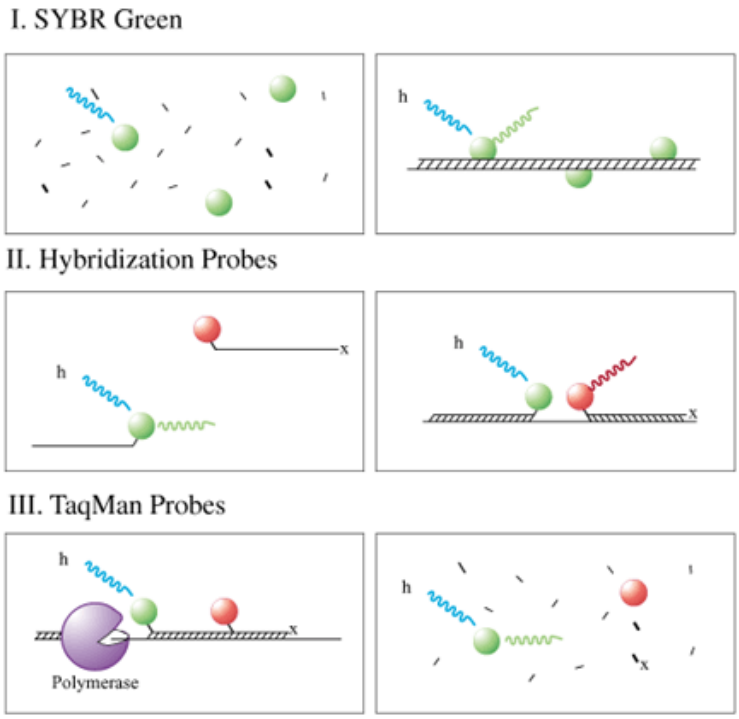


Figure 3.6.3a: The three main fluorescence-monitoring systems compatible with the LightCylcer™. (www.idahotech.com/lightcycler_u/lectures/quantification_on_lc.htm)

We used for some RNAi assays SYBR Green, but usually followed in our study the method using the hybridization probes (in addition to standard primers) with the LightCylcer™ (LC) instrument. The detection principle is based on the so called Fluorescence Resonance Energy Transfer (FRET), the phenomenon of energy transfer from a donor to an acceptor fluorophor. Hybridization probes are designed as a pair of oligonucleotides, of which one probe is labeled with the donor (3´Fluo) and one with the acceptor (5´ LCRed 640 or LCRed 705) dye. As FRET decreases with the sixth power of distance, the probes have to be designed to hybridize to adjacent regions of the template DNA (separated by 1-5 nucleotides). If both probes hybridize, the two dyes are brought close together and FRET to the acceptor dye results in a signal measurable by the built-in fluorimeter of the LightCycler™ (figure 3.6.3a).

The LightCyler uses air for heating and cooling. Ambient air is drawn into the machine by a small fan and warmed with a heating coil. Since air has a very low thermal capacity, the instrument can attain a thermal ramping rate of about 20°C per second. Thus, heating and cooling occur about ten times faster than in a conventional thermal cycler. PCR occurs in specially designed glass capillaries (20 – 100 µl volume). A typical amplification cycle requires only 30 to 60 seconds; an amplification reaction with 30 cycles is usually complete in about 30 minutes. For fluorescence excitation, the instrument uses a blue LED, which emits

light at 470 nm. This LED is an integral part of the fluorimeter (figure 3.6.3b). The donor dye (fluorescein) is excited by the blue LED and the energy emitted excites the acceptor dye, which then emits fluorescent light at a different wavelength. This fluorescence is directly proportional to the amount of target DNA generated during PCR.

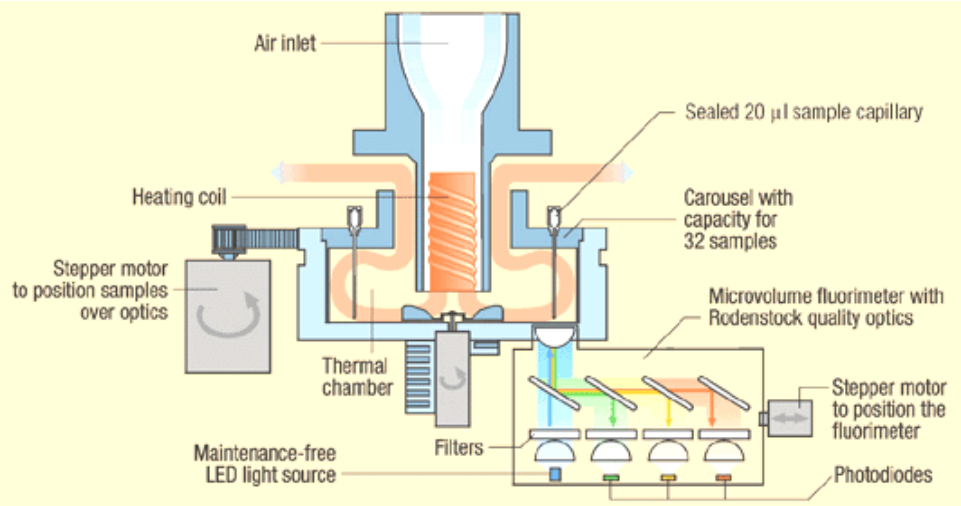


Figure 3.6.3b: Cross section of the LightCycler instrument. (www.hisco.co.jp/prodhs/lightcycler/)

3.6.3.1 Performing the PCR

To obtain precise quantitative results, cDNA standards were used along with our samples in the PCR run, forming a standard curve according to the cycle number of each standard sample, in order to be able to evaluate and calculate the copies of the sample-DNA with the help of that standard curve (Kuhne and Oschmann, 2002).
10 µl of each standard (10^6 copies – 10^3 copies), 4 µg of each cDNA sample and 10 µl H_2O (negative control) were pipetted into LC capillaries. H_2O was added to the cDNA to obtain 10µl final volume. Then 10 µl of the master mix was added to all capillaries. This mix was composed of:

2.6 µl H_2O
2.4 µl $MgCl_2$
1 µl of each probe
0.5 µl of each Primer
2 µl Taq-polymerase.

After application of the capillaries in the LC carousel and centrifuging them shortly, the carousel was placed in the LC and the run started. 45 cycles were performed using the following parameters:

1st Denaturation	95°C	9 min
Amplification	95°C	9 sec (Denaturation)
	54°C	15 sec (Annealing)
	72°C	25 sec (Elongation)
Melting	95°C	20 sec (Heating: 0.2°C/sec till 95°C)
	50°C	20 sec
Cooling	40°C	3 min

The samples were collected after the run and frozen at -20°C for further analysis.
The run was now evaluated on the computer by using the special software provided by the manufacturer.

3.6.3.2 Normalization and analysis

To normalize the real-time PCR data, i.e. exclude possible errors in the reverse transcription or amplification, a PCR run was performed with the same cDNA samples, but using probes that detect a housekeeping gene. The expression of a housekeeping gene is almost equal in various cell sorts (Thellin *et al.*, 1999). We used δ-aminolevolunic acid synthase (h-ALAS) as housekeeping gene (Roche). cDNA standards were synthesized by reverse transcription of standard h-ALAS mRNA supplied in the kit, following the manufacturer's protocol. The PCR run was performed using the same parameters as for the standard runs.
Analysis was performed by comparing the number of cDNA copies of the samples to analyze with the h-ALAS copies in the same sample. The ratio was then multiplied by a specific copy number taken from the h-ALAS copies (e.g. 10^5).

$$\text{Normalized} = (\text{No. of copies of a cytokine} / \text{No. of copies of h-ALAS}) \times 10^5$$

The normalized copies were afterwards calculated relative to the non-stimulated samples (medium). All values of the non-stimulated probes were set to 1.

3.6.3.3 Agarose gels

To verify the amplification products of the real-time PCR, we analyzed them via agarose gels. 2 µl loading buffer were added to 10 µl of each sample and applied to a 2% gel (2 g agarose in 100 ml H_2O), to which 10 µl Gelstar previously were added. Gelstar stains DNA and is not toxic as ethidium bromide.
The gel was performed at 120 V for 45 min. The gels were then analyzed by a gel-scanner.

3.6.4 Microarray analysis

Microarray analysis is a tool for analyzing gene expression that consists of a small membrane or glass slide containing samples of many genes arranged in a regular pattern. Oligonucleotide microarrays are small, solid supports onto which the sequences from thousands of different genes are immobilized at fixed locations. The whole process is based on hybridization probing, a technique that uses fluorescently labeled nucleic acid molecules to identify complementary molecules, sequences that are able to base-pair with one another. The fluorescent tags are excited by the laser, and the microscope and camera work together to create a digital image of the array. This digital image is then stored, and a special program is used either to calculate the red-to-green fluorescence ratio or to subtract out background data for each microarray spot.
Samples are prepared by extracting mRNA from a cell and turning it back into DNA through reverse transcription of the mRNA into cDNA. This is followed by a labeling step during which the cDNA is then transcribed to cRNA while incorporating a label (e.g. biotin). Once labeled, the sample of cRNAs can be hybridized to the array and bound by the various oligonucleotide probes. Lastly, a staining reaction is performed in order to visualize the amount of hybridization (see figure 3.6.4).

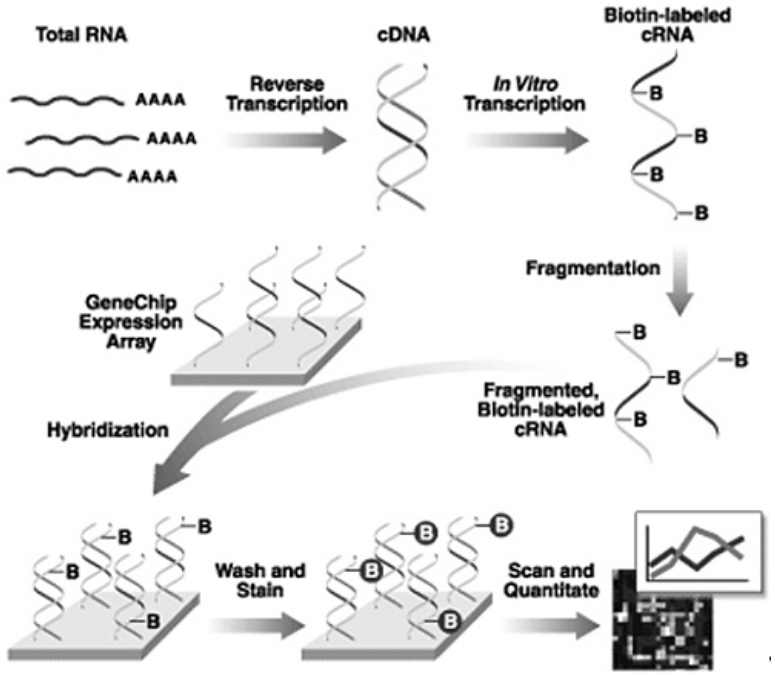


Figure 3.6.4: Standard gene expression array. Labeled cRNA targets derived from the mRNA of an experimental sample are hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it is possible to infer the abundance of each mRNA species represented. (www.affymetrix.com/en/images/expression_oveview.gif)

For genome-wide expression analysis, microarray experiments were performed for every period of incubation and donor, using the HG-U133A human genome array (Affymetrix). The previously isolated RNA (frozen at -80°C) was sent to the IZKF Microarray facility in Tübingen, were further procedures were performed for the microarray tests. Briefly, double-stranded cDNA was synthesized from 5 µg total RNA with a superscript choice kit (Invitrogen, New York, USA) with a T7-(dT)24 primer incorporating a T7 RNA polymerase promoter (Metabion, Martinsried, Germany). cRNA was prepared via in-vitro transcription (Enzo Biochemical, New York, USA) and biotin labeled. Fragmentation followed by incubation at 94°C for 35 min in the presence of 40 mM Tris-OAc (pH 8.1), 100 mM KOAc, and 30 mM MgOAc. Hybridization to the array was performed with 15 µg cRNA for 16 h at 45°C. The arrays were then automatically washed and stained with streptavidin-phycoerythrin using a fluidics station. Probe arrays were scanned thereafter at 3 µm resolution with a Genechip System confocal scanner (Agilent Technologies, Palo Alto, USA). Each cRNA generated from one incubation experiment was hybridized on one microarray separately. We ran 27 arrays in total (see 3.3.1).

3.6.4.1 Evaluation and interpretation

Affymetrix GCOS software (version 1.2) was used to scan and analyze the relative abundance of each gene based on the intensity of the signal from each probe set. Analysis parameters used by the software were set to values corresponding to moderate stringency (statistical difference threshold = 30, statistical ratio threshold = 1.5). Output from the microarray analysis was merged with the Unigene or GenBank descriptor and saved as an Excel data spreadsheet.

We analyzed the stimulated samples of a donor at a given timepoint with the non-stimulated samples of all three donors at the same timepoint, thus allowing a total of up to nine comparisons for each timepoint. For each comparison, the analysis using the Affymetrix software generates a "difference call" of no change, marginal increase/decrease, or increase/decrease, respectively. Only those genes which were found in at least 7, 8 or 9 out of 9 comparisons similarly adjusted were defined as differentially expressed genes. In some cases only 6 comparisons were possible, so genes which were found in at least 5 or 6 out of 6 comparisons were defined as differentially expressed genes. The magnitude and direction of change of a transcript were estimated as Signal Log Ratio (SLR) by comparison of two arrays, respectively. The log scale used is base 2, making it intuitive to interpret the Signal Log Ratios in terms of multiples of two. Thus, a SLR of 1.0 indicates an increase of the transcript level by 2 fold and -1.0 indicates a decrease by 2 fold. A SLR of zero would indicate no change. Categorization was based on the NetAffx™ database (www.affymetrix.com) (Liu *et al.*, 2003). Functional analysis was performed using the PathwayAssist™ software (Ariadne Genomics, Rockville, MD, USA).

3.6.5 RNA interference

1998, Fire and Mello found out that double-stranded RNA (dsRNA) down-regulates the expression of genes in *Caenorhabditis elegans* up to 10 times more than sense or anti-sense RNA (Fire *et al.*, 1998).

RNA interference (RNAi) is a conserved mechanism of the sequence-specific posttranscriptional gene silencing, at which mRNAs degrade with other homolog mRNAs to dsRNAs. dsRNA was found to induce gene silencing only at coding regions of the mRNA, not at introns and non-coding regions (Fire, 1999). RNAi has also been observed in

Drosophila. Microinjecting *Drosophila* embryos with dsRNA effected gene silencing (Guru, 2000). So the question was how is dsRNA able to trigger gene silencing?

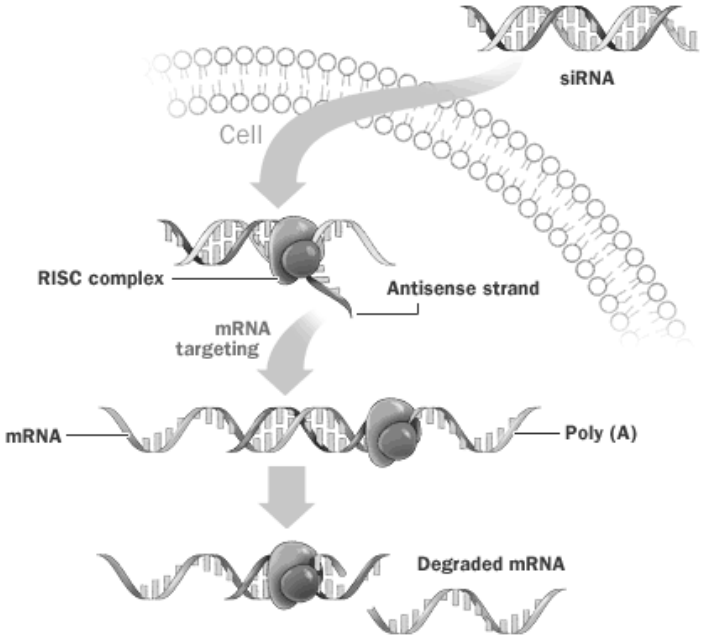


Figure 3.6.5: Simplified mechanism of RNA interference. (www.nastech.com/img/img_rna_interference.gif)

Zamore *et al.* found that dsRNA added to *Drosophila* embryo lysates was processed to 21-23 nucleotide species. They also found that the homologous endogenous mRNA was cleaved only in the region corresponding to the introduced dsRNA and that cleavage occurred at 21-23 nucleotide intervals (Zamore *et al.*, 2000). So the RNAi mechanism became clearer and clearer, leading to the following (see figure 3.6.5): input dsRNA is digested into 21-23 nucleotide small interfering RNAs (siRNAs). Evidence indicates that siRNAs are produced when the enzyme Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, processively cleaves dsRNA (introduced directly or via a transgene or virus) in an ATP-dependent, processive manner. Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNAs), each with 2-nucleotide 3' overhangs (Bernstein *et al.*, 2001). These duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, or RISC. An ATP-depending unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA (Nykanen *et al.*, 2001).

We used RNAi in order to silence specific immune-related genes, prior to incubations with *Aspergillus fumigatus*, which would give us a better understanding of the defense mechanisms

and pathways involved in the immune response to the fungus. So we tried to establish a protocol to trigger gene silencing via siRNAs in human monocytes and DCs, which was till now a difficult procedure with little success, generally in human primary cells.

3.6.5.1 Lipofection

One method, for cells friendly and gentle to transfect DNA, is lipofection. It is usually a highly efficient, lipid-mediated transfection method via liposomes, which are vesicles that can easily merge with the cell membrane since they are both made of a phospholipid bilayer.
RNAiFect™ transfection kit from Qiagen, Oligofectamine™ transfection reagent from Invitrogen and the Silencer™ siRNA Transfection kit from Ambion were used for transfection. They are all based on a lipid formulation that builds a lipid/siRNA complex which is able to merge with the cell membrane, and so the siRNA molecule can enter the cell without stressing it.
We performed various experiments, trying to transfect various siRNAs into monocytes and DCs, following the instructions of the given protocols.
Following incubation approaches were performed:

Experiments 1 & 2: IDH3 siRNA (kindly provided by Thomas Rudel, MPI, Berlin), into DCs.
(Exp. 1: RNAiFect™, Oligofectamine™ and Silencer™ kit)
(Exp. 2: RNAiFect™ and Oligofectamine™)
Experiments 3 & 4: GAPDH-siRNA, into DCs. (Silencer™ kit)
Experiment 5: GAPDH-siRNA, into monocytes. (RNAiFect™)
Experiment 6: TLR2-siRNA and TLR4-siRNA, into monocytes. (RNAiFect™)
Please note that a non-specific control-siRNA was additionally used in all 6 experiments. Incubations followed at 37°C, with 5% CO_2.

Table 3.6.5.1: Different experiments performed to transfect siRNA at different concentrations and conditions.

Exp.	No. cells	siRNA amount	Incubation times	Conditions
1	3×10^5	2.75 of 20 µM	24 h, 32 h and 48 h	in 300 µl RPMI
2	3×10^5	2.75 & 4.25 µl of 20 µM	30 h and 72 h	in 300 µl RPMI
3	5×10^5	3 µl of 20 µM	24 h and 48 h	in 1 ml RPMI/FCS
4	2×10^5	1.5 µl of 20 µM	24 h and 48 h	in 1 ml RPMI/FCS
5	4.5×10^5	3.3 µl (1 µg)	20 h and 40 h	400 µl RPMI/FCS
6	5×10^5	2.5 µl of 20 µM	9 h and 24 h	700 µl RPMI/FCS

The RNA was isolated immediately after each incubation period, frozen at -80°C, and gene expression was analyzed later with real-time RT-PCR.

3.6.5.2 Electroporation

Another method to transfect cells with siRNA is electroporation. Cells placed in a special transfection cuvette are treated with an electric pulse which disrupts temporarily the cell membrane, enabling the added oligonucleotides to enter the cell.

Encouraged by the positive results achieved in our research group with this method in DCs, we applied it also on monocytes, hoping to achieve similar results. An advantage of this method was that nothing should be added to the cells, which means less possible toxicity factors. The disadvantage is the possible stressing or even damaging of the cells through the electric pulse. Thus, proper transfection conditions had to be found out and tested, which enable the siRNA to enter the cells without stressing or damaging them.

For these experiments, we purchased TLR2-, TLR4- and TLR8-siRNA from Qiagen (Germany). So called 2-For-Silencing siRNA duplexes were purchased. So two different siRNA for each target were used in every experiment, but done in separate cell culture samples.

In brief, fresh isolated monocytes were isolated and directly used for transfections. 10^6 monocytes in 100 µl RPMI (without FCS) were taken as a standard cell number in every experiment. 1.33 µM siRNA were always used to transfect 10^6 monocytes (as prev. done in the DCs experiments). Electric voltages, pulse periods and incubation periods were only varied in order to find the most suitable conditions. Voltages were varied from 320 mV up to

360 mV; pulsing times were either 10 or 15 milliseconds (ms), and incubation periods 12 or 24 h.

For electroporation, the cells/siRNA mixture in the special cuvette was immediately electroporated with the EPI 2500, left for 15 min at room temperature to rest, then incubated by adding enough RPMI/10% FCS medium. Incubations were made at 37°C and 5% CO_2 as usual. The RNA was afterwards immediately isolated and frozen at -80°C until further analysis with real-time RT-PCR.

3.7 Immunological methods

In order to study host defense mechanisms by a special cell type against an invading organism that causes infectious diseases, immunological approaches are of high priority, due to the very large variety of antibodies and antigens present in nature. Thus, in order to understand a single specific defense response, specific and sensitive tests should be performed, and this is only possible with the help of immunological techniques.

3.7.1 Purification of monoclonal antibodies

Monoclonal antibodies (W6/32 for HLA class I and L243 for HLA class II) previously isolated from hybridoma cell supernatants were purified using protein A-sepharose, based on affinity chromatography, for further use in immunoprecipitation of MHC-peptide complexes.

1.2 g protein A-sepharose were sedimented in a column and washed with PBS for 30 min using a table pump (Flow rate: 4-5 ml/min). After measuring the concentrations of the two antibodies by measuring the absorption at 280 nm, they were applied to and let pass through the columns, two times. Antibody-binding was checked by measuring the flow-through. The columns were then washed for 3 min with H_2O in order to get rid of buffer rests. 8 ml citric acid (pH 3) were added to each column to elute the antibodies. The fractions were eluted into 18 ml of a so called coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3). The concentration was measured again. This procedure was repeated until enough of each antibody was purified (needed: 1 mg/ 1 ml cell pellet).

3.7.2 Immunoprecipitation of HLA peptides followed by acid extraction

HLA-peptide isolation was also performed via affinity chromatography. For this purpose, 37.5 mg/ mg antibody BrCN-sepharose were weighed into separate falcon tubes for W6/32 and L243 each, and activated with 40 ml 1 mM HCl for 30 min at room temperature (RT). The frozen THP-1 cell pellets (stimulated and non-stimulated) were incubated (with stirring) in a 2x concentrated lysis buffer (1.2% CHAPS in PBS, protease inhibitors) for about 1 h. After centrifugation (300 rpm, 3 min, without brake) of the activated BrCN-sepharose, the antibodies were added to couple for 2 h at RT. In the meantime, one volume of the 1x lysis buffer was additionally added to the stirring cell suspension and left to stir for additional 1 h. After coupling of the antibodies to the sepharose was over, the efficiency was checked by again measuring the antibody concentration, i.e. measuring the absorption at 280 nm. If the coupling was successful, blocking the still free sites was done by incubating the sepharose with 40 ml 0.2 M glycine for 1 h at RT. In the meantime, the lysed cells were sonified (3 x 20 sec) to make the suspension more homogeneous. Centrifugation followed at 4000 rpm for 20 min. The supernatant was collected and centrifuged in the ultracentrifuge at 40000 rpm for 1 h and 20 min, at 4°C. Meanwhile, the sepharose was centrifuged (300 rpm, 3 min, 4°C, without brake), and the pellet washed twice with PBS. Afterwards it was resuspended in 5 ml 1x lysis buffer and applied to filter columns, one for each antibody and washed 30 min with PBS (flow rate: 4-5 ml/min) with the help of a table pump. The supernatant of the centrifuged cell suspension was filtered sterile and applied to the columns, which were previously put up one above the other and linked with a flexible hose. At a flow rate of 4-5 ml/min, the lysate passed through both columns once completely, and then let to flow in a cyclic manner over night, all at 4°C. Centrifugal filter devices, so called centricons, were filled with 2.5% TFA and centrifuged at 4000 rpm over night, in order to make them polyethyleneglycole-free (PEG), which contaminates the probes and disturbs further analysis.

On the following day, the columns were washed for 30 min with PBS, then for 1 h with H_2O, to get them buffer-free. 150 µl 0.25% TFA/10 mg antibody were added to each column, then 10 µl 10% TFA, for elution. The columns were left to shake for 10 min at RT, then the eluted MHC-peptide complexes were collected in separate centricons. This procedure was repeated, but then only with 0.1% TFA; 4 fractions from each antibody were eluted from the non-simulated cells, 2 from the stimulated cells. 5% of the eluted volume was taken out for later Edman sequencing. Also small samples were collected at each step, for western blot analyses. The centricons were left to centrifuge at 4000 rpm, until the whole sample passed through.

The HLA molecules were kept therewith bound to the filter in the centricon, whereas peptides passed through. The samples were then immediately frozen at -80°C, until further use.

3.7.3 SDS-PAGE and western blots

Western blots were performed with the samples collected during the isolation procedure of HLA-peptides, in order to check the concentrations of class I HLA at each step. Samples of cell lysate before and after binding to the antibodies were used, as well as samples from the eluted peptide fractions.

After preparing the stacking (pH 6.8) and the running gels (pH 8.8) for SDS-PAGE, 10 µl of each sample were denaturated for 5 min at 95%, mixed with 3.3 µl Lämmli buffer (pH 6.8) and loaded on the stacking gel, which was already filled with running buffer (0.025 M Tris-HCl, 0.19 M Glycin, 0.1% SDS). A standard sample (monomer HLA-B*1501) was also used: 0.1 µg – 0.6 µg.

The SDS-PAGE was run at 100 V for 15 min, then at 140 V for 1 h.

Semi-dry blotting was performed by using 8 whatman filter papers and a nitrocellulose membrane, of which 4 in addition to the membrane were previously soaked in anode- (50 mM sodiumtetraborate, 20% methanol, pH 9) and the other 4 in cathode-buffer (50 mM sodiumtetraborate, 0.05% SDS, pH 9). The gel was placed on the nitrocellulose membrane, between 4 filter papers, to each side. The run was performed at 42 mA for 2 h. The membrane was then washed for 30 min with TBB buffer (3% BSA, 0.1% Nonidet P-40, 50 mM Tris-HCl pH 8, 0.15 M NaCl). The 1st antibody (anti-ß2 microglobulin 1:50000) was added and incubation followed for at least 2 h at 4°C. Washing followed 3 x for 5 min in TWB buffer (0.1% Nonidet P-40, 50 mM Tris-HCl pH 8, 0.15 M NaCl). Then the 2nd antibody (anti-rabbit horseradish peroxidase, F(ab)2 fragment, 1:5000) was added for 1 h at RT. Washing followed for 5 min with TBS (50 mM Tris-HCl pH 8, 0.15 M NaCl) and 3 x for 10 min with TWB, then 5 min with TBS again. The membrane was then developed by using ECL solution for 3 min, then photographed after different time periods in the dark room.

3.7.4 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA experiments were performed to detect the proteins of the genes over-expressed in the microarray analysis, in the supernatants of the samples, which were kept frozen at -80°C.

IL-8, CCL2 and CCL20 were analyzed using Quantikine Immunoassay kits (R&D Systems, USA), according to the manufacturer's protocol. These assays use the quantitative sandwich enzyme immunoassay technique. The developed color intensity was measured and the concentrations were calculated relatively to the standards supplied by the kit. The assays were done only once, for one single donor.

3.8 Analytical methods

3.8.1 Edman sequencing

Protein quantification of the eluted MHC-peptide fractions was performed with Edman sequencing, using the ABI "Procise" 494A Protein-Sequencer (Applied Biosystems, USA). In this process, the N-terminal amino acid of a protein reacts with phenylisothiocyanate (PITC) to form a phenylthiocarbamyl (PTC) protein. The PTC protein is then cleaved with trifluoroacetic acid, resulting in the formation of an intermediate anilinothiazolinone (ATZ). The intermediate is converted to the more stable phenylthiohydantoin (PTH) amino acid derivative and separated by HPLC, during which the detection of the PTH amino acid was performed by simultaneously measuring the absorption at 269 nm. This was compared against a standard, and identified by the sequencer software.

In brief, the protein-peptide fractions were applied to a glass fiber filter pretreated with polybrene, and dried with argon gas to prevent oxidation of the proteins. The filters were put into special cartridges and sequencing was started.

For quantitation analysis, which was done for MHCs, specific amino acids (mostly the aliphatic ones) of the α- and β-chain were taken into consideration, since the yield of their corresponding PTH-amino acid bigger is than those having functional groups; particularly methionine of the α-chain of HLA class I was mainly taken into account.

Table 3.8.1: Starting sequences of HLA class I and II α- and β-chains taken into consideration for calculating the yields of the peptide fractions.

Protein	Chain	Starting sequence
HLA class I	α	GSHSMRY
	β_2-microglobulin	IQRTPKI
HLA class II	α	LKEEHVI
	β	GDTRPRF

The resulting average amount (in pmol) was multiplied with the factor 20, since it represents only 5% of the original volume (see 3.7.2).

3.8.2 Mass spectrometry

Mass spectrometry is an analytical tool used for measuring the molecular weight (MW) of a sample. Structural information can also be generated by using certain types of mass spectrometers, usually tandem mass spectrometers, and this is achieved by fragmenting the sample and analysing the products generated. In our experiments, we used mass spectrometry in order to characterize the amino acid sequences of the HLA-presented peptides.

Sample molecules are first ionised. These ions are extracted into the analyser region of the mass spectrometer where they are separated according to their mass (m) -to-charge (z) ratios (m/z). The separated ions are detected and this signal sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of an m/z spectrum.

The ionisation method used for our analyses is electrospray ionisation (ESI, figure3.8.2). A high voltage is applied which disperses the sample into an aerosol of highly charged droplets. The charged droplets diminish in size by solvent evaporation and eventually charged sample ions, free from solvent, are released from the droplets and pass through a sampling cone or enter into an intermediate vacuum region, and from there through a small aperture into the analyser of the mass spectrometer.

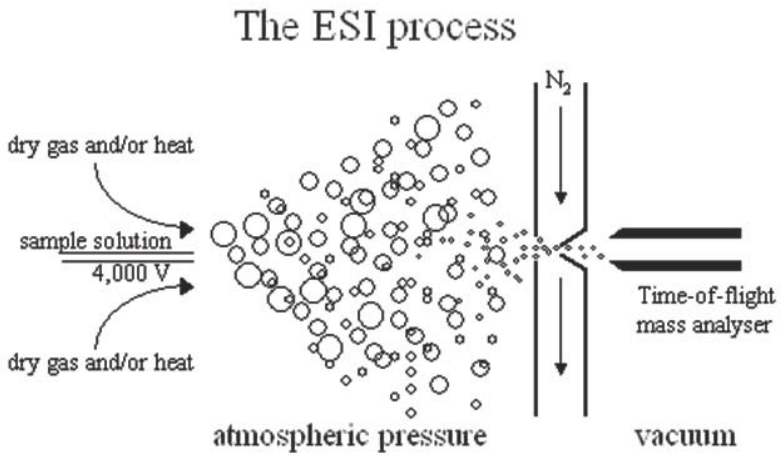


Figure 3.8.2: The ESI process (http://qbab.aber.ac.uk/roy/mss/esi.gif).

3.8.2.1 ESI/Q-Tof mass spectrometry

Nanospray ionisation, which is possible with the Q-Tof mass spectrometer (figure 3.8.2.1), is a low flow rate version of electrospray ionisation (30 – 1000 nl/min). It is a quadrupole – Time of flight tandem mass spectrometer. The quadrupole in this instance is not used as an analyser, merely as a lens to focus the ion beam into the second (time-of-flight) analyser which separates the ions according to their mass-to-charge ratio. The Q-Tof mass spectrometer is switched into "MS-MS" mode. The protonated molecular ions of each of the digest fragments can be independently selected and transmitted through the quadrupole analyser, which is now used as an analyser to transmit solely the ions of interest into the collision cell which lies in between the first and second analysers. An inert gas such as argon is introduced into the collision cell and the sample ions are bombarded by the collision gas molecules which cause them to fragment. The fragment ions are then analysed by the second (time-of-flight) analyser. In this way an MS/MS spectrum is produced showing all the fragment ions that arise directly from the chosen precursor ions for a given peptide component. Proteins and peptides are usually analysed under positive ionisation conditions, where a trace of formic acid is often added to aid protonation of the sample molecules. The m/z scale must be calibrated by analysing a standard sample of a similar type to the sample being analysed (e.g. a protein calibrant for a protein sample), and then applying a mass correction.

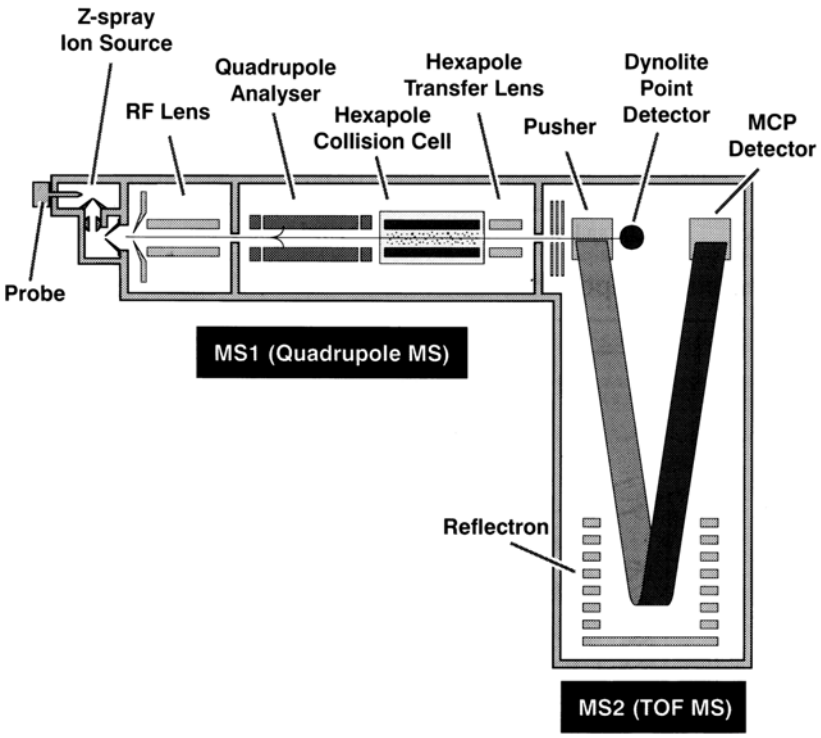


Figure 3.8.2.1: The Q-TOF mass spectrometer. It combines a quadrupole mass filter (MS1), a hexapole collision cell and TOF mass analyzer (MS2) to deliver high performance MS-MS. © Q-Tof handbook micromass.

The ESI Q-TOF mass spectrometer was controlled, the data recorded and processed via the software MassLynx 4.0 from a computer.

3.8.2.2 LC-MS

The LC-MS is a technique combining the separation power of reverse-phased HPLC (High Performance Liquid Chromatography) with the detection power of mass spectrometry in order to get optimized analytical results. In reverse-phased HPLC, compounds are separated based on their hydrophobic character. Peptides can be separated by running a linear gradient of the organic solvent. The stationary phase is generally made up of hydrophobic alkyl chains that interact with the sample. Chain lengths of C8 and C18 are generally used to capture peptides or small molecules. The separated molecules can then be ionized by the ESI spray and detected with the previously described ESI-Q-Tof method.

3.8.2.3 Experimental procedures for the class II-peptides fraction

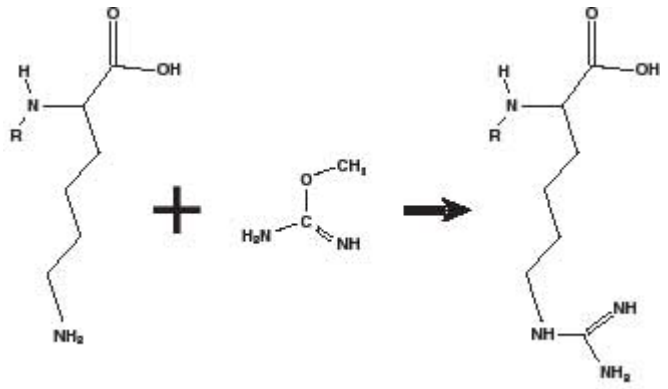
Our ESI-Q-Tof mass spectrometer is coupled to a µCapillary-HPLC system. The µCapillary column is directly connected to a gold-coated capillary, which works as the ESI source.
The sample of the class II eluted peptides of the from the stimulated cells (frozen at -80°C) was lyophilized over night (< -50°C, < 0.5 mbar) and resuspended in 450 µl solution A (4 mM ammonium acetate in H_2O, pH 3). This volume would be enough for three runs, each 150 µl.
The µCapillary-HPLC system was carried out with a flow rate of 150 – 200 µl/min, and the effective flow was reduced to 0.3 µl/min. Before each LC-MS run, the HPLC columns were equilibrated for 30 min with 15% solution B (2 mM ammonium acetate, 80% acetonitrile). Before analyzing a sample, idling was done with the *Glufib*-peptide (EGVNDNEEGFFSAR). Afterwards, 150 µl of our sample were centrifuged for 10 min at 13000 rpm to sediment dirt remains, and injected into a so called sample loop. The sample was then loaded with the help of solution A to a pre-column, where it was concentrated and desalted at 20 µl/min for 40 min. Then the pre-column was switched to the µCapillary column. For HPLC separation, a gradient varying from 15% to 45% solution B was used, over a time period of 170 min. The separated molecules were then directly ionized by the ESI spray (through a so called picotip) and detected. The complete run was followed and recorded over a time period of 200 min. The recording of the mass spectra was done with an integration time of 1 second for the Tof-analyzer and 4 seconds for the tandem-MS experiments. The interscan-delay in between was 0.1 seconds. The selection of the precursor ions $[M+H]^+$ and $[M+2H]^{2+}$ and their fragmentation was done automatically with the ESI-Q-TOF-Software MassLynx 4.0, which parameters were previously set. Two more runs were performed with the remaining 300 µl sample, following the same procedures.

3.8.2.4 Modification of HLA class I peptides

In order to analyze the isolated HLA class I peptides quantitatively and at the same time compare the ones separated from the non-stimulated THP-1 cells with those from the stimulated ones, a novel modification method was used. Briefly, the ε-aminogroup of all lysine side chains was guanidinated using O-methyl isourea hemisulfate (GUA solution), without modification of the N-terminus. This was achieved by working with pH > 10, when the amino rests of the lysines are deprotonated, and the nitrogen attacks the isourea where

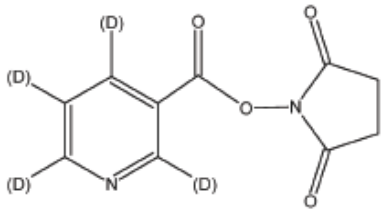
methanol is released. In a second modification step, all N-termini were nicotinylated with either normal or deuterium-enriched 1-([H_4/D_4]nicotinoyloxy) succinimide (d(NIC)-NHS). This reaction was done on a C_{18} microcolumn, which also allowed quick desalting before mass spectrometric analysis, which allowed differentiation between the compared samples. Deuterium is an isotope of hydrogen and has mass number of 2. In our case, we nicotinylated the stimulated THP-1 cells with D_4-NIC (dNIC) and the non-stimulated with H_4-NIC (NIC), which can be later detected by seeing a shift of 4 units (4 deuteriums in dNIC) in the compared LC-MS spectra.

a)



Lysine o-methyl-isourea homoarginine

b)



1-([H_4/D_4]-nicotinoyloxy) succinimide = d(NIC)-NHS

Figure 3.8.2.4: a) Modification of lysine with o-methyl-isourea. b) Structural formula of dNIC.

For this purpose, 500 µl 0.1% TFA were added to each of the previously lyophilized peptide fractions, in addition to 92 µl GUA solution (100 mg O-methyl isourea in 102 µl H_2O). The pH value was adjusted to about 10.5 by carefully adding 20-40 µl of 10 M NaOH. Incubation followed for 10 min in a 65°C-waterbath. The reaction was then stopped by adding ~ 10 µl 100% formic acid, and put on ice. Nicotinylation of the α-aminogroup followed by applying the guanidinated peptides to a C-18-reversed-phase column (Agilent) in 200 µl portions and repeated three times. The column was previously activated by washing three times with 200 µl elution buffer (50% acetonitrile, 1% formic acid) and equilibrated by washing 3 times with

200 µl H_2O with 0.1% formic acid. All centrifugation steps were done at 2000 rpm for 15 sec. 0.5 ml of the nicotinylation reagent (2.2 mg nicotinic acid-N-hydroxy-succinimide, 1ml phosphate buffer, in 500 ml H_2O) was added to the columns, which were applied to a 1 ml syringe. Every minute, ~50 µl were drawn with the syringe so that all 500 µl passed through the column. This was repeated with 0.5 ml again. Washing followed 3 times with 200 µl H_2O. In order to hydrolyze nicotinylated tyrosine rests, 0.5 ml hydroxylamine (50%) was let through the column at 50 µl/min again. After a final washing step with 200 µl H_2O, the peptides were eluted 4 times with 50 µl elution buffer, and the fractions were pooled.
For LC-MS analysis, the samples were constricted with a SpeedVac to about 10 µl, and 90 µl of solution A were added. After centrifugation for 10 min at 13 000 rpm, the samples were mixed in a 1:1 ratio, then applied into the HPLC system (see 3.8.3.2) for CID analysis. The non-mixed samples were fragmented separately afterwards.

3.8.2.5 Spectral analysis and peptide prediction

Collision Induced Decomposition (CID) was used to identify the amino acid sequence of the non-fragmented peptide ions in the general survey spectrum. Tandem-MS experiments allowed recording at the same moment 4 simultaneous fragment spectra. In this way, the 4 most detectable peptide ions were recorded at this same moment, fragmented in the collision cell and analyzed at last. The resulting MS/MS-spectra were analyzed and evaluated automatically using computer software, but the analyses were mainly done manually.
The CID fragments result from breakings in the peptide bond. The resulting charge can be either at the N-terminus (x-, y-, z-ions) or at the C-terminus (a-, b-, c-ions) (Roepstorff and Fohlmann, 1984) and are presented as peaks in the spectrum. y-, b- and a-ions are the most frequent ions detected. b-ions can further dissociate into a-ions with loss of CO (-28 Da). y-ions undergo loss of H2O (-18 Da) or NH3 (-17 Da), others gain 18 Da. Immonium ions which result from the binding of a- and y-fragments are detectable under 160 Da and are mostly a hint for special amino acids. Internal peptide fragments may also result, and make the evaluation more difficult. By calculating the interval distances from one peak to another peak, the resulting residue can be assigned to either an amino acid, or to another peptide fragment. Following are all amino acids including their residue and immonium ion masses, as well as the mass of the b1 residues of the deuterium-nicotinylated and the normal-nicotinylated ones. All masses are in dalton:

Table 3.8.2.5: Masses of natural and modified amino acid residues in MS/MS spectra.

Amino acid		Residue	Immoniumn ion	dNIC b1	NIC b1
Glycine	G	57	30	167	163
Alanine	A	71	44	181	177
Serine	S	87	60	197	193
Proline	P	97	70	207	203
Valine	V	99	72	209	205
Threonine	T	101	74	211	207
Cysteine	C	103	76	213	209
Isoleucine	I	113	86	223	219
Leucine	L	113	86	223	219
Asparagine	N	114	70	224	220
Aspartate	D	115	71	225	221
Glutamine	Q	128	101	238	234
Lysine	K	128	101	238/280	234/276
Glutamate	E	129	102	239	235
Methionine	M	131	104 (ox. 120)	241	237
Histidine	H	137	110	247	243
Phenylalanine	F	147	120	257	253
Arginine	R	156	70 / 129	266	262
Tyrosine	Y	163	136	273	269
Tryptophane	W	186	159	296	292

Mostly, not all amino acids can be detected and read out from a spectrum. Depending on the peak characters, 4 to 5 amino acids detected on the N-terminal or C-terminal side are often enough for a survey in peptide databases, which might be successful. In the case of the modified peptides, a big step is done by simply identifying the b1 residue which is nicotinylated; thus, the N-terminal amino acid is then known.

One of the online databases available is Mascot (www.matrixscience.com). If many peptide sequences are proposed, the ones which seem to mostly match with the spectrum are taken out and validated in the spectrum itself. This is carried out until the right sequence is found, which fits almost perfectly with the spectrum.

4. Results

4.1 Phagocytosis and gene expression analyses in monocytes

4.1.1 Monocytic preparations

For our essential microarray experiments, PBMCs were isolated with FICOLL centrifugation and the monocytes then with the Monocyte Isolation Kit, using 300 ml of fresh blood samples from three healthy donors. Following cell numbers were obtained:

Table 4.1.1: Number of PBMCs and monocytes extracted from the three healthy donors.

	No. of PBMCs	**No. of monocytes**
Donor 1	5×10^8	4.7×10^7
Donor 2	5×10^8	5.4×10^7
Donor 3	6×10^8	8.4×10^7

For the microscopy experiments, monocytes were isolated from PBMCs via positive labelling with CD14-microbeads in order to get pure samples for optimal microscopic images. Here for, 50 ml blood from buffy coats were taken for isolation and following cell numbers were obtained: $6{,}6 \times 10^7$ PBMCs/ml, from which $5{,}4 \times 10^6$ monocytes were isolated in total.

4.1.2 Flow cytometry of monocytes

The purity of the monocytic preparations was checked with flow cytometry; by staining the monocytes with a FITC-labelled CD14 antibody (see also 3.5.1).

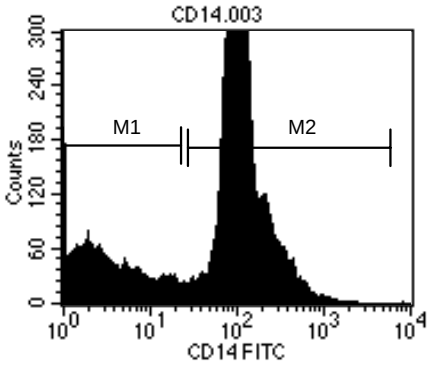


Figure 4.1.2: FACS histogram of the monocytes of one of the three donors. The CD14-positive cells represent in this example 76.16% of all cells. The remaining cells (23.29%) are most probably damaged cells, which was later assessed by cell counting.

The purity of the monocytic cultures of the other two donors was 75.5% and 63%. These purity levels were considered to be sufficient for performing microarray gene expression analysis (Szaniszlo *et al.*, 2004).

4.1.3 Phagocytosis of *A. fumigatus* conidia by monocytes

Two strategies were followed in order to analyze phagocytosis (see also 3.4.2).

(a) Conidia were fluorescently labelled with Fungi-Fluor dye prior to incubation with monocytes; after 3 hours of incubation, we prepared the samples and observed them under the microscope. Following figures were obtained:

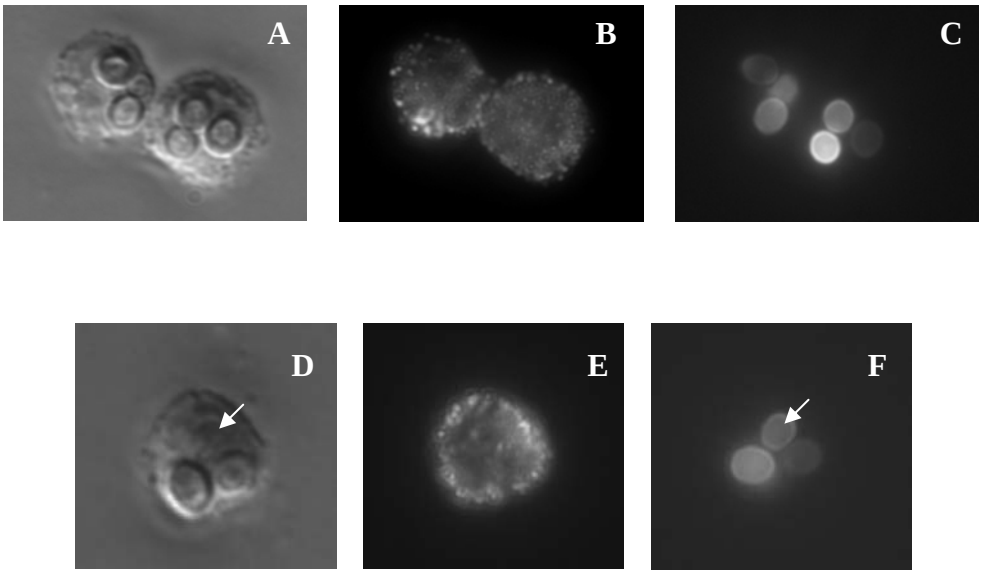


Figure 4.1a: Microscopic analyses of phagocytosis. (A, D) Light microscopic close-up of monocytes after 3 hours of phagocytosis, each phagocytosed 3 conidia. (B, C) Same pictures as A, with a fluorescent close-up of the monocytes, stained by Cy3, and the conidia phagocytosed, stained with Fungi-Fluor dye prior to phagocytosis. (E, F) Same pictures as D, showing a fluorescent close-up of the monocyte and the conidia phagocytosed which were stained with Fungi-Fluor.

We found that most of the monocytes had phagocytosed conidia. In order to quantify phagocytosis, we analyzed 100 monocytes and counted the conidia phagocytosed by them. We found that 40 monocytes phagocytosed two conidia, 23 phagocytosed three, 16 phagocytosed one, 10 phagocytosed four or more whereas only 11 phagocytosed no conidia. Thus, the majority of the monocytes phagocytosed up to three *A. fumigatus* conidia within 3

hours. The monocytes in figure 4.1a have phagocytosed each 3 conidia. The figures demonstrate that the conidia are within the cells and not on their surface. This can be clearly concluded out of figures D and F, where one conidia is almost invisible with light microscopy in D, whereas it can be well observed through fluorescent labeling in F.

(b) Conidia were fluorescently labelled with Fungi-Fluor dye after incubation with monocytes for 6 h. Microscopic observations showed the following figures:

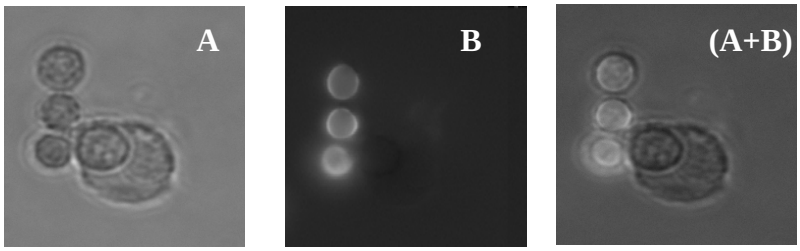


Figure 4.1b: Microscopic analyses of phagocytosis. (A) Close-up of a monocyte after 6 hours of phagocytosis with one phagocytosed swollen conidia and 3 non-phagocytosed conidia. (B) Same pictures as D, with the 3 non-phagocytosed conidia stained with the fluorescent Fungi-Fluor dye after phagocytosis.

Observing the samples after 6 hours of incubation, phagocytosed conidia were found to swell and the monocytes changed their shape accordingly, as shown in Figure 4.1b. Yet, the phagocytosis rate did not increase. Three conidia were stained whereas one was not, thus phagocytosed.
After 9 hours, the conidia had already germinated to hyphae and overgrown the monocytes, which made it difficult to make clear microscopic observations and draw any conclusions concerning phagocytosis.

4.1.4 RNA quality and quantity

In order to perform microarray gene chip experiments using the HG-U133A human genome array (Affymetrix), a minimum of about 3 µg total RNA were needed. This was achieved by isolating as much as possible RNA from the three experiments. The isolated RNA quality and quantity was measured in the 31 µl frozen at -80°C:

Table 4.1.4: RNA concentration and quality of the different incubation experiments in the three donors.

Donor 1	**Ratio 260 nm/280 nm**	**Conc. [µg/µl]**	**Total [µg] in 31 µl**
Medium 3 h	1.83	0.167	5.17
Hyphae 3 h	1.83	0.153	4.76
Conidia 3 h	2.01	0.184	5.71
Medium 6 h	1.06	0.099	3.08
Hyphae 6 h	1.77	0.161	5.00
Conidia 6 h	1.76	0.134	4.15
Medium 9 h	1.86	0.097	3.02
Hyphae 9 h	1.82	0.147	4.57
Conidia 9 h	1.82	0.138	4.30
Donor 2			
Medium 3 h	2.17	0.110	3.42
Hyphae 3 h	2.50	0.105	3.26
Conidia 3 h	1.98	0.139	4.31
Medium 6 h	2.05	0.135	4.19
Hyphae 6 h	2.06	0.122	3.80
Conidia 6 h	2.12	0.146	4.54
Medium 9 h	2.17	0.121	3.77
Hyphae 9 h	2.07	0.133	5.45
Conidia 9 h	2.14	0.138	4.30
Donor 3			
Medium 3 h	1.92	0.118	3.67
Hyphae 3 h	1.78	0.119	3.70
Conidia 3 h	1.84	0.087	2.70
Medium 6 h	1.86	0.109	3.40
Hyphae 6 h	1.79	0.118	3.68
Conidia 6 h	1.88	0.135	4.20
Medium 9 h	1.88	0.088	2.73
Hyphae 9 h	1.86	0.119	3.71
Conidia 9 h	2.09	0.101	3.14

Almost all ratios were between 1.8 and 2.1, which shows the good quality of the RNA samples. Only one exception (donor 1, medium 6 h) has a ratio of 1.06. Yet this could be taken into account.

Also all concentrations were above 3 μg, except 2 samples in donor 3, which were 2.7 μg. But these amounts are also enough for microarray assays.

4.1.5 Gene expression analysis with real-time RT-PCR

Real-time RT-PCR assays were performed for TNF-α, IL-1β and IL-6 with the cDNA synthesized from the mRNA of each of the three donors and the average expression between the three was calculated. This was done in order to check the activation of the monocytes. Three runs (one for each donor) were performed for each gene at each period with the same mRNA used later in the microarray experiments. Following quantitative results were obtained:

Table 4.1.5a: Copy numbers of TNF-α obtained from real-time PCR assays from the different experiments.

	Donor 1	**Donor 2**	**Donor 3**
	No. of copies	No. of copies	No. of copies
Medium 3 h	0.656×10^3	0.888×10^3	2.663×10^3
Hyphae 3 h	1.956×10^3	2.297×10^3	5.969×10^3
Conidia 3 h	0.547×10^3	0.155×10^3	2.438×10^3
Medium 6 h	1.549×10^3	1.695×10^3	4.782×10^3
Hyphae 6 h	9.678×10^3	11.447×10^3	60.6×10^3
Conidia 6 h	1.824×10^3	4.923×10^3	9.723×10^3
Medium 9 h	1.59×10^3	1.646×10^3	3.849×10^3
Hyphae 9 h	7.034×10^3	10.795×10^3	49.482×10^3
Conidia 9 h	3.705×10^3	6.344×10^3	9.294×10^3

Table 4.1.5b: Copy numbers of IL-1β obtained from real-time PCR assays from the different experiments.

	Donor 1	**Donor 2**	**Donor 3**
	No. of copies	No. of copies	No. of copies
Medium 3 h	7.4×10^5	4.2×10^5	27.7×10^5
Hyphae 3 h	35.3×10^5	28.1×10^5	49.8×10^5
Conidia 3 h	6.87×10^5	2.1×10^5	15.3×10^5
Medium 6 h	7×10^5	9.7×10^5	28.2×10^5
Hyphae 6 h	237×10^5	116.8×10^5	293.9×10^5
Conidia 6 h	8.3×10^5	22×10^5	63.8×10^5
Medium 9 h	3.4×10^5	4.3×10^5	11.9×10^5
Hyphae 9 h	110.4×10^5	91.2×10^5	272.1×10^5
Conidia 9 h	3.1×10^5	13.4×10^5	28.3×10^5

Table 4.1.5c: Copy numbers of IL-6 obtained from real-time PCR assays from the different experiments.

	Donor 1	**Donor 2**	**Donor 3**
	No. of copies	No. of copies	No. of copies
Medium 3 h	1.819×10^4	0.877×10^4	8.461×10^4
Hyphae 3 h	4.105×10^4	4.597×10^4	10.765×10^4
Conidia 3 h	0.412×10^4	0.405×10^4	3.533×10^4
Medium 6 h	1.003×10^4	1.403×10^4	2.465×10^4
Hyphae 6 h	9.190×10^4	19.157×10^4	55.035×10^4
Conidia 6 h	0.934×10^4	2.361×10^4	7.551×10^4
Medium 9 h	0.421×10^4	1.299×10^4	2.339×10^4
Hyphae 9 h	5.875×10^4	13.455×10^4	12.434×10^4
Conidia 9 h	0.703×10^4	2.506×10^4	2.943×10^4

These data represent the normalized results (see also 3.6.3.2).

The average expression level of each cytokine was then calculated and presented in the following diagrams:

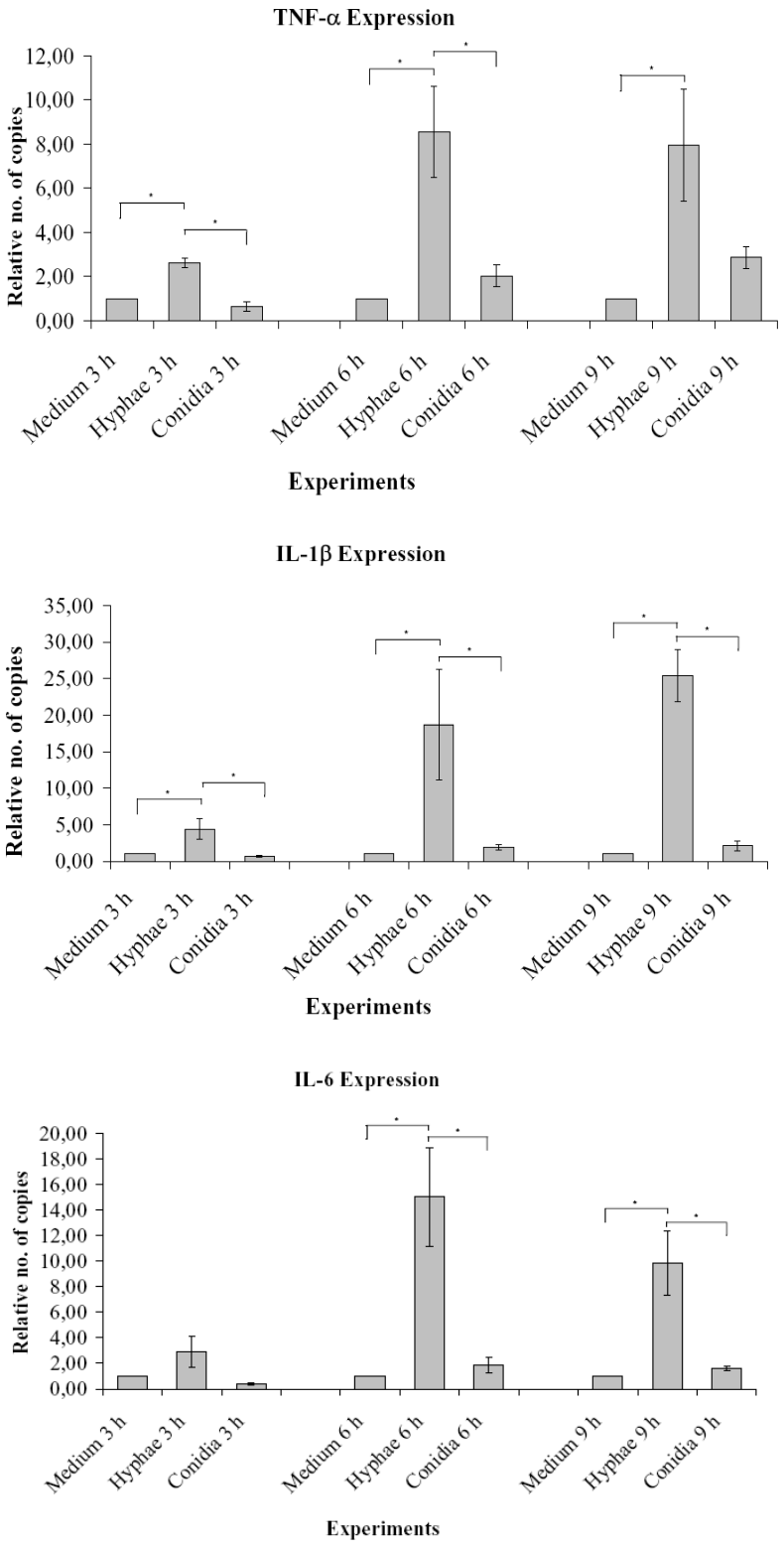


Figure 4.1.5: Expression analysis of the cytokines TNF-α, IL-1β and IL-6 by real-time PCR. The data shown represent the mean ± SEM of three donors for each cytokine. ∗, p values < 0.05 were considered statistically significant.

The diagrams show conidia to stimulate cytokine expression only after 6 and 9 hours, but not after 3 hours. Yet the expression levels are as not as high as stimulations with inactivated hyphae. They stimulate cytokine expression already after 3 hours, and the expression levels get much higher after 6 and 9 hours. The standard deviations of the expression levels between the 3 donors seem to be in some cases relatively high, meaning that the different monocytic samples reacted differently with the same *A. fumigatus* culture, thus donor-dependent.

4.1.6 Agarose gel electrophoresis

The cDNA samples were collected after the amplification procedure and analyzed with agarose gel electrophoresis. The same 10^6 standards were used as for the RT-PCR.

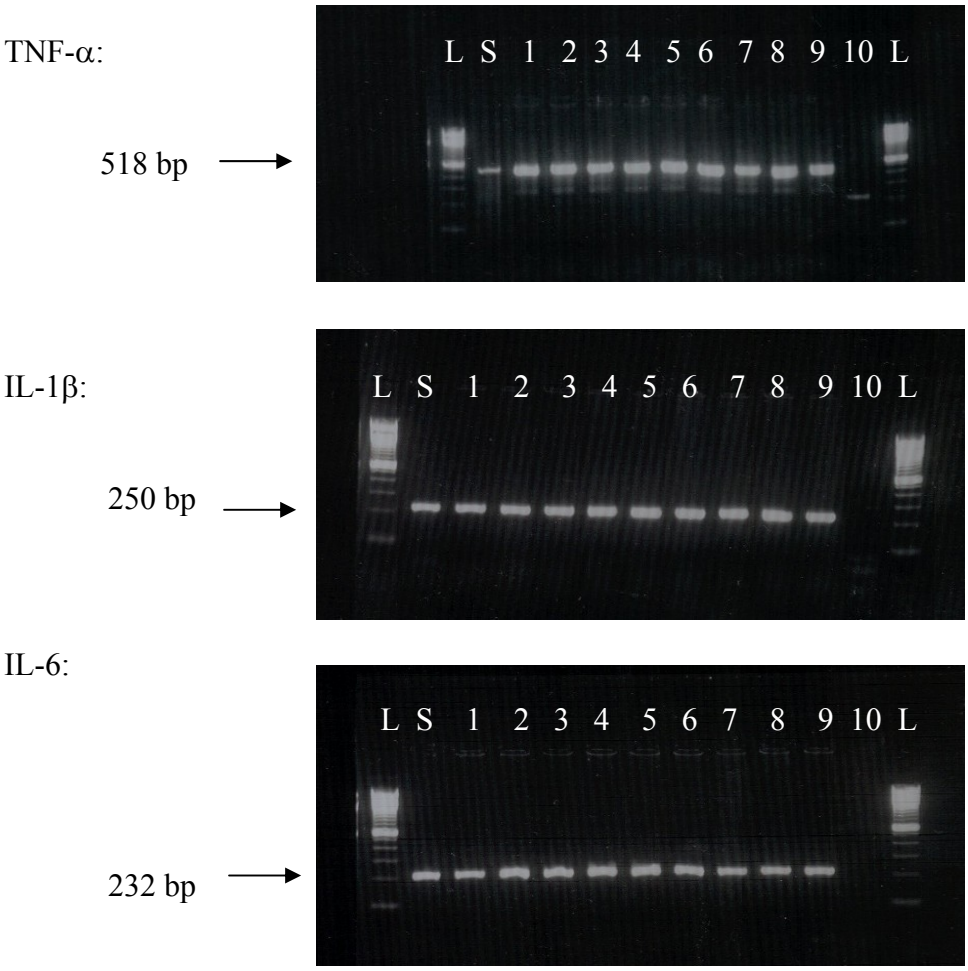


Figure 4.1.6: Agarose gel electrophoresis of TNF-a, IL-1b and IL-6 with the cDNA of the real-time PCR assays.

Samples analyzed:

L: Ladder	5: Hyphae 6 h
S: 10^6 Standard	6: Conidia 6 h
1: Medium 3 h	7: Medium 9 h
2: Hyphae 3 h	8: Hyphae 9 h
3: Conidia 3 h	9: Conidia 9 h
4: Medium 6 h	10: Negative control

The samples were shown to have the same size as the standards at each amplification. TNF-α has 518 bp, IL-1β has 250 bp and IL-6 has 232 bp.

4.1.7 Microarray analyses

In order to have a broader understanding of genes involved in *Aspergillus*-directed monocytic defense strategies, gene regulation after incubation was studied by microarray chip analyses using the human HG-U133A Affymetrix-array.

Analysis revealed a wide range of differentially regulated genes, of which we were interested in genes involved in the cellular immune response. Genes were regulated in different scales among the three donors, certainly depending on the donor. Among these genes, we analyzed the ones commonly regulated between the three, and found out that 602 genes were differentially regulated after 3 hours of incubation with inactivated hyphae and 206 after incubation with conidia. At 6 and 9 hours of incubation, the number of genes showed only slight differences between conidia and inactivated hyphae; 418 and 399 genes were differentially regulated after incubation with conidia, 375 and 456 genes after incubation with hyphae, respectively. Table 4.1.7 summarizes the number of the commonly up- and down-regulated genes after every incubation period. These genes varied in their expression level depending on the incubation time and the morphological state of the fungus, with signal log ratios varying between -3.7 and +4.7 for conidia, and between -4.1 and +3.5 for hyphae.

Table 4.1.7: Number of genes differentially regulated by viable conidia and inactivated hyphae.

Number of genes	by viable conidia			by inactivated hyphae		
	3 h	**6 h**	**9 h**	**3 h**	**6 h**	**9 h**
up-regulated	99	166	231	287	147	214
down-regulated	107	252	168	315	228	242
Total	**206**	**418**	**399**	**602**	**375**	**456**

4.1.7.1 Interpretation

Hyphae and conidia have different surface structures and molecules and therefore interact differently with immune cells (Rohde *et al.*, 2002). This was clearly observed after analyzing the microarray data. Yet, similarities in gene expression profiles and levels can also be found.
During the interpretation of our data, we set two main questions to be answered:

(1) What is the difference between the genes regulated after incubation with viable conidia, and those regulated after incubation with inactivated hyphae? What are the similarities?

(2) How does the expression level vary between the different incubation periods?

In order to answer these questions, the analysis of the expression profiles was done manually. We searched for and picked out the immune relevant genes, which were found among the commonly regulated genes mentioned above (see 4.1.7). These included cytokines, chemokines, their receptors, TLRs, as well as genes coding for signal-transduction and adhesion molecules.
We generally found hyphae, although inactivated, to stimulate more immune relevant genes than viable conidia. In addition, hyphae stimulated monocytes to express these genes already after 3 hours and the expression level increased after 6 and 9 hours for most of these genes. On the contrary, conidia stimulated monocytes to express such genes only after 6 and 9 hours, despite phagocytosis during the first 3 hours. This was also observed by real-time RT-PCR analyses of TNF-α, IL-1β and IL-6 (see 4.1.5)
In the following two tables, we present all immune related genes which were differentially regulated after incubation with conidia and hyphae, and which were more than about 2 fold up-or down-regulated, in comparison to the non-stimulated samples.

Tables 4.1.7.1a & b: Immune related genes differently regulated by *A. fumigatus* conidia and hyphae. Values in brackets represent the signal log ratio and the standard deviation between the 3 donors (SLR, SD).

a	Cytokines	Cytokine Receptors	Chemokines	Chemokine Receptors	TLRs	Adhesion molecules	Other
Conidia 3 h		*IL-7R* (-0.93, 0.76) *CSF1R* (-0.73, 0.36)				*ITGAE* (-0.73, 0.32) *TFPI* (-0.73, 0.25) LGALS2 (2.05, 1.69) THBD (1.18, 0.63) SERPIN B9 (0.74, 0.44)	*CD86* (-0.95, 0.57) *STAT1* (-0.67, 0.29) PTX3 (1.87, 1.16)
Conidia 6 h	IL-8 (2.19, 0.87) TNFSF3 (1.11, 0.36)	*TRAIL-R2* (-1.05, 0.5) *IFNγR1* (-0.83, 0.28) IL-1RN (2.58, 1.72) IL-2Rγ (1.03, 0.38) IL-3Rα (1, 0.46)	CXCL5 (2.23, 1.3) CXCL3 (1.74, 0.84) CCL2 (1.48, 0.97)	*CCR2* (-2.19, 1.51)	*TLR1* (-1.22, 0.62)	*F8A* (-0.85, 0.39) *PECAM1* (-2.09, 0.9) F3 (3.95, 2.22) SERPIN B2 (1.16, 0.7) ALCAM (1.02, 0.47)	*NCF4* (-1.01, 0.77) *CASP8* (-1.43, 0.65) *MHCIIβ* (-1.34, 0.66) MET (2.35, 0.78) STAT3 (0.79, 0.31)
Conidia 9 h	IL-8 (4.05, 0.56) IL-1α (3.53, 1.18) CSF1 (2.42, 2.33) TGFβ1 (1.18, 0.81) TNF-α (1.62, 1.87) TNFSF3 (1.18, 0.56)	*CSF3R* (-1.1, 0.26) *TRAIL-R2* (-0.76, 0.28) IL-3Rα (1.94, 0.43) IL-2Rγ (1.33, 0.33) CSF2Rα (0.8, 0.27) TRAF1 (0.71, 0.46)	CCL20 (4.3, 4.13) CXCL5 (4.29, 2.31) CCL2 (2.33, 0.82) CCL7 (1.39, 0.74)	*CCR2* (-2.03, 2.27)		*CLECSF14* (-1.64, 1.21) *LGALS2* (-2.17, 1.54) *CD33* (-2.51, 0.36) SERPIN E1 (3.29, 1.95) uPA (1.62, 1.04) ICAM1 (1.59, 1.26) SERPIN B2 (1.33, 0.65)	SOCS3 (2.34, 1.54) MET (2.21, 1.72) MMP9 (1.99, 0.6) C3 (1.39, 0.41)

b	Cytokines	Cytokine Receptors	Chemokines	Chemokine Receptors	TLRs	Adhesion molecules	Other
Hyphae 3 h	IL-6 (1.77, 1.47) IL-1α (1.43, 1.48) IL-10 (1.32, 0.82) IL-1β (1.05, 0.7)	*CSF2Rα* (-0.94, 0.36) TNFRSF6 (1.41, 1.01)	CCL7 (1, 1.21) CCL5 (0.82, 1.03)	*CCR5* (-1.07, 0.94)		*ALCAM* (-1.34, 0.92) LGALS2 (2.45, 1.4) ADAM19 (2.04, 1.59) ITGA2B (1.15, 0.99) uPA (0.78, 1.03) F13A1 (0.88, 1.01)	PTX3 (2.27, 0.93)
Hyphae 6 h	TNFSF3 (1.95, 0.68)	CRLF2 (1.62, 1.48) TRAF1 (1.34, 0.98) IL-1R2 (0.78, 0.59)	CXCL3 (1.73, 1.05) CXCL1 (1.66, 1.09)		*TLR7* (-2.71, 1.33) *TLR8* (-3.47, 1.44)	*PECAM1* (-0.82, 0.83) *CLECSF2* (-1.32, 0.96) SERPIN B2 (1.37, 0.72)	C3 (1.04, 0.67) MMP9 (1.26, 1.14)
Hyphae 9 h	IL-8 (3.51, 1.27) IL-1α (2.7, 2.96) TGF-α (1.88, 1.24)	*CSF3R* (-1.21, 0.66) *TRAIL-R2* (-0.84, 0.32) IL-1RAP (2.04, 0.8) IL-7R (1.57, 0.64) IL-3Rα (1.41, 0.63) IL-2Rγ (1.15, 0.6)	CCL2 (2.63, 0.64) CXCL6 (2.56, 0.98) CCL7 (2.12, 0.56)			*CLECSF2* (-0.56, 0.1) *CLECSF14* (-2.21, 1.32) SERPIN E1 (2.4, 2.03) THBD (2.29, 0.7) uPA (1.81, 0.88) ICAM1 (1.22, 1.77)	*CD244* (-1.53, 0.28) *CD163* (-2.3, 0.98) MMP9 (2.35, 0.62) MET (1.68, 1.97) LAT (1.26, 0.64) MAPK8 (1.21, 0.91) C3 (0.96, 0.48)

After 3 hours, conidia did not stimulate the expression of cytokines or chemokines. Only the receptors of IL-7 and CSF1 were down-regulated (about 2 fold). However, genes encoding for adhesion and surface molecules were differentially regulated. On the contrary, hyphae stimulated monocytes to express cytokines and chemokines already after 3 hours, in analogy to the RT-PCR experiments. IL-1α, IL-1β, IL-6, IL-10, and CCL7 were consistently up-regulated (SLRs between 1 and 1.8). We also observed the over-expression of the soluble pattern recognition receptor (PRR) pentraxin-3 by both stimuli, which is involved in innate immunity and plays a role in pathogen recognition. It was shown to bind to *A. fumigatus* conidia and facilitate the interaction with mononuclear phagocytic cells (Garlanda *et al.*, 2002). PTX3 was between 3.6 (conidia) and 4.8-fold (hyphae) up-regulated after 3 hours, which indicates its role in pathogen recognition and binding. No over-expression was detected after 6 and 9 hours. Interestingly, no significant changes in the expression levels of TLRs were detected at this timepoint.

After 6 and 9 hours, the conidia germinated to hyphae, which is reflected in the gene expression profile of the monocytes at these timepoints. Similarities in the expression patterns were observed between conidia and hyphae. The expression of IL-8, IL-10, IL-6, TNF and IL-1 was up-regulated to varying degrees with signal log ratios from 1 to 4 by both stimuli. Transforming growth factor-α (TGF-α) and -β1 (TGF-β1) were also up-regulated after incubation with inactivated hyphae and conidia, respectively. In addition, cytokine receptors were up- or down-regulated, for example TRAIL-R2, CSF receptors, TNF receptors and some interleukin receptors such as IL-7R, IL-2Rγ and IL-3Rα. Different chemokines and their receptors, such as CCL2, CCL7, CCL20 and members of the GRO-family, were also found to be up-regulated after incubation with both stimuli. The urokinase-type plasminogen activator (uPA) was found to be up-regulated (about 3 to 4-fold) by both stimuli at these timepoints. Nevertheless, differences in the expression patterns were also observed. We found the expression levels of TLR7 (6.5-fold decrease) and TLR8 (11.3-fold decrease) to be strongly down-regulated after 6 hours of incubation with inactivated hyphae, but remained unchanged after incubation with conidia. TLR1 was down-regulated (2.3-fold) after 6 hours incubation with conidia. Beside the TLRs, chemokine receptor CCR2 was found to be down-regulated (>4-fold) after incubation with conidia, but no change after incubation with inactivated hyphae.

4.1.7.2 Analysis with PathwayAssist™

PathwayAssist™ searched several databases for interactions between the genes previously detected by the Affymetrix-array and subsequently constructed so-called biological association networks. Upon loading the gene ID numbers into this software, it displayed pathways for each incubation period. After the 9-hour incubation period, two pathways were generated by this software for both stimuli, which were for the most part similar (Figure 4.1.7.2). They show CCL2 (SLR 2.4) inducing uPA expression (SLR 1.6) along with other genes such as IL-8, IL-1α, TGF and intracellular adhesion molecule 1 (ICAM1). In addition, uPA may directly induce CCL2 expression. Serine protease inhibitors (SERPINs) that are known to inhibit uPA expression were also up-regulated (SLR 1.3 to 3.3). Thus, two feed-back pathways were proposed which may reflect possible monocytic defense strategies in the case of an *A. fumigatus* infection. Yet, a slight difference can be noted: CCR2 (receptor for CCL2) is strongly down-regulated (SLR -2) after the 9-hour incubation period, but only after incubation with the viable conidia.

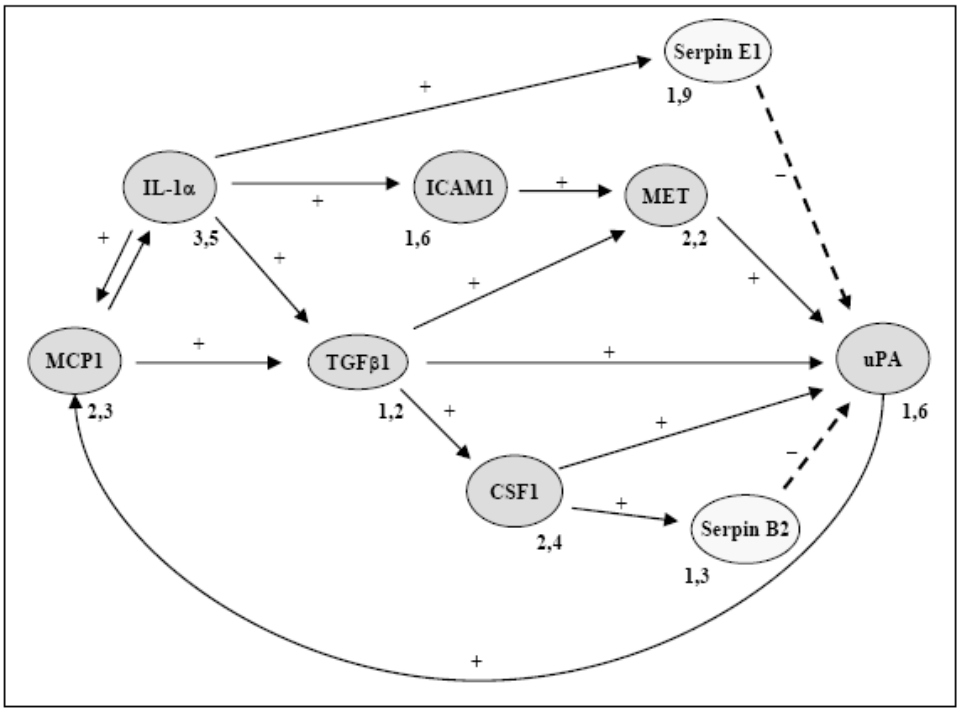


Figure 4.1.7.2: Pathway proposed by PathwayAssist, showing the interaction of MCP1 (CCL2) and uPA, after the 9h-stimulation of monocytes with viable conidia. The numbers below the genes represent the SLR.

4.1.8 ELISA

To confirm over-expressed genes on a protein level, quantitative determination of the concentrations of IL-8, CCL2 and CCL20 in the supernatants of each incubation experiment were performed.

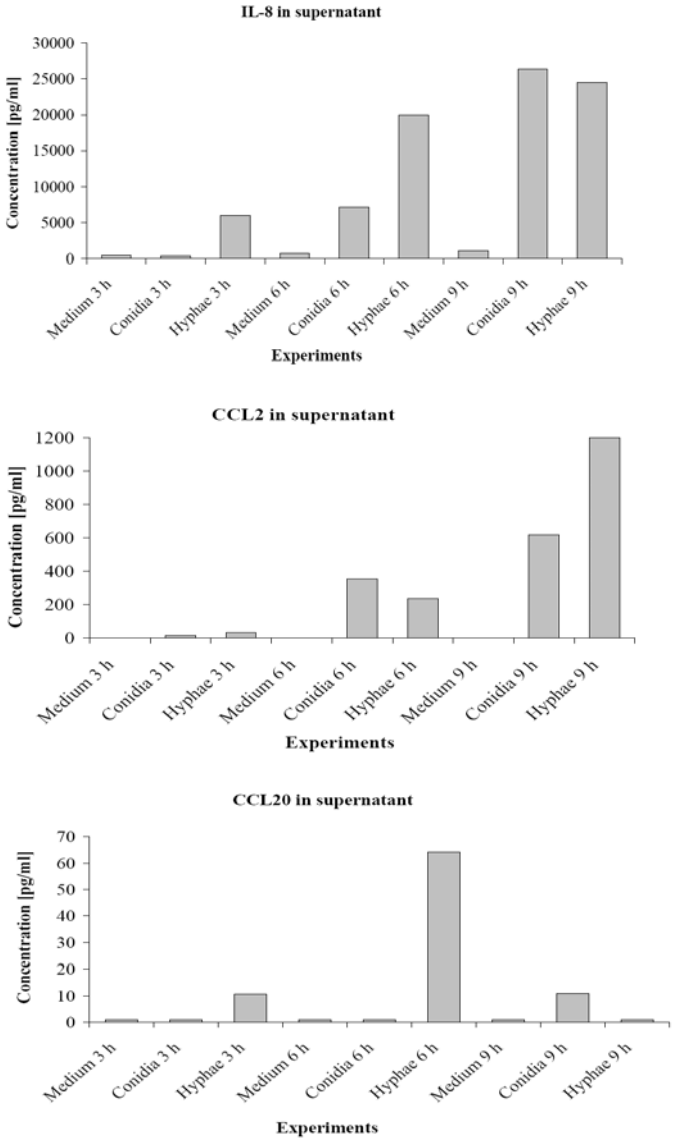


Figure 4.1.8: ELISA assays showing the concentrations [pg/ml] of IL-8, CCL2 and CCL20 in the supernatants. The assays were done only once, for one single monocyte preparation.

A steady increase of their concentrations in culture supernatants after 6 and 9 hours was observed.

4.1.9 RNA interference

To analyze a potential role of TLRs in monocyte activation, we used RNAi in order to silence specific genes, prior to incubations with *Aspergillus fumigatus*. We tried to establish a protocol to transfect the primary monocytes with siRNA. We tried lipofection i.e. lipid-mediated transfection, and electroporation.

4.1.9.1 Lipofection

RNAiFect™ transfection kit from Qiagen, Oligofectamine™ transfection reagent from Invitrogen and the Silencer™ siRNA Transfection kit from Ambion were used for transfection. For detailed procedures see also paragraph 3.6.5.1.
In the first two experiments, using isocitrate dehydrogenase 3 (IDH3) siRNA, SYBR Green real-time RT-PCR showed following results:

Table 4.1.9.1a: Experiment 1, using 3 different transfection kits to transfect IDH3 siRNA into monocytes. mRNA was isolated and real-time PCR performed after 24, 32 and 48 h.

	IDH3 mRNA level change after		
	24 h	**32 h**	**48 h**
Oligofectamine™	50% down	25% down	50% down
RNAiFect™	75% down	66% down	66% down
Silencer™	50% down	25% down	50% down

Table 4.1.9.1b: Experiment 2, using 2 different transfection kits to transfect IDH3 siRNA into monocytes. mRNA was isolated and real-time PCR performed after 30 and 72 h.

	IDH3 mRNA level change after			
	30 h		**72 h**	
	2.75 µl siRNA	**4.25 µl siRNA**	**2.75 µl siRNA**	**4.25 µl siRNA**
Oligofectamine™	25% up	No change	No change	50% down
RNAiFect™	25% up	25% down	25% up	25% down

Silencing IDH3 in monocytes using three different siRNA transfection kits and different time periods showed big differences throughout the experiments. As in exp. 1 the tendency is a

clear decrease with all three kits, in exp.2 there is no consens. The siRNA induced even an increase in IDH3 expression.

In the experiments 3, 4 and 5, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA and analyzed it with SYBR Green RT-PCR too:

Table 4.1.9.1c: Experiments 3 & 4, using Silencer™ transfection kit to transfect GAPDH siRNA into DCs. mRNA was isolated and real-time PCR performed after 24 and 48 h.

	GAPDH mRNA level change after			
	24 h		**48 h**	
	3 µl	**1.5 µl**	**3 µl**	**1.5 µl**
Silencer™	50% down	No change	75% down	No change

The GAPDH expression was down-regulated with GAPDH siRNA in DCs when using 3 µl of the 20 µM siRNA solution, yet not enough. The 1.5 µl didn't show any effects on expression level.

Table 4.1.9.1d: Experiment 5, using RNAiFect™ transfection kit to transfect GAPDH siRNA into monocytes. mRNA was isolated and real-time PCR performed after 20 and 40 h.

	GAPDH mRNA level change after	
	20 h	**40 h**
RNAiFect™	50% down	No change

The GAPDH expression was reduced after 20 hours, although not enough. An unspecific siRNA showed the same effects (data not shown). After 40 h, there was no change detected.

In the next step (Exp. 6), we used TLR2 and TLR4 siRNA on monocytes with RNAiFect™. Evaluation was done by hybridization probes real-time RT-PCR assays:

Table 4.1.9.1e: Experiment 6, using RNAiFect™ transfection kit to transfect TLR2 and TLR4 siRNA into monocytes. mRNA was isolated and real-time PCR performed after 9 and 24 h.

siRNA	**TLR4 mRNA level change after**	
	9 h	**24 h**
TLR2	No change	No change
TLR4	No change	25% down

Unfortunately, TLR2 and TLR4 siRNAs didn't produce any silencing in monocytes with the transfection reagent.
In general, silencing effects up to 90% or above must be achieved in order to successfully turn down a specific gene function. Thus, lipofection with 3 different reagents did not result in satisfactory gene silencing.

4.1.9.2 Electroporation

Due to the variable and non-consensus results obtained by transfecting the siRNA with lipid mediators, we performed electroporation experiments to get the siRNA into the cells. So called 2-For-Silencing siRNA duplexes were purchased, specific for TLR2, TLR4 and TLR8. Transfection experiments were varied in time, voltage and siRNA concentrations (see also paragraph 3.6.5.2). DCs were also transfected in some cases in order to compare the silencing effects with the monocytes.

Experiment 1:

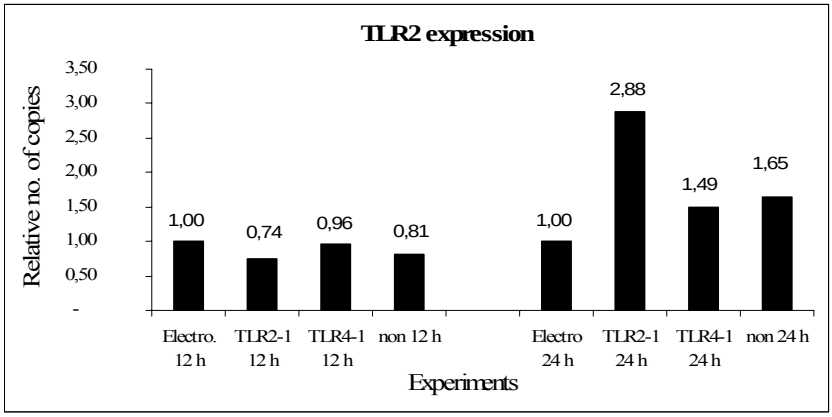


Figure 4.1.9.2a: Experiment 1; 10^6 monocytes were electroporated with 3 µM TLR2- and TLR4-siRNA for 10 ms at 330 mV. mRNA was isolated and real-time PCR performed after 12 and 24 h for TLR2.

After 12 h, no significant decrease could be observed in TLR2 expression. Surprisingly, its expression increases quite strong after 24 h. A non-specific effect could also be observed by TLR4-siRNA and the non-treated sample.

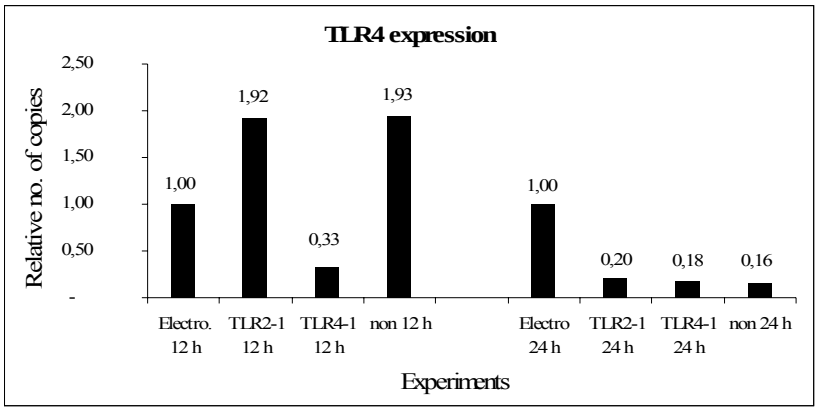


Figure 4.1.9.2b: Experiment 1 as well, performing real-time PCR for TLR4.

The same samples as before show a quite strong TLR4 decrease after 12 h, but TLR2-siRNA and the non-treated show non-specific effects. These effects are unfortunately also seen after 24 h, where all three samples show a strong down-regulation in TLR4 expression.

Experiment 2:

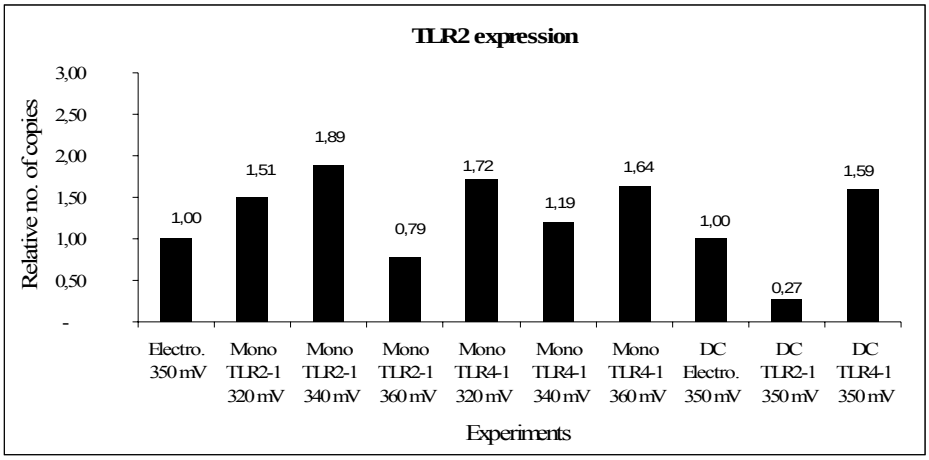


Figure 4.1.9.2c: Experiment 2; 10^6 monocytes were electroporated with 3 µM TLR2- and TLR4-siRNA for 15 ms at 320, 340, and 360 mV. mRNA was isolated and real-time PCR performed after 12h for TLR2. DCs were also transfected, at 350 mV.

TLR2 is strongly down-regulated in DCs, whereas monocytes did not show differences in expression levels depending on the voltage used.

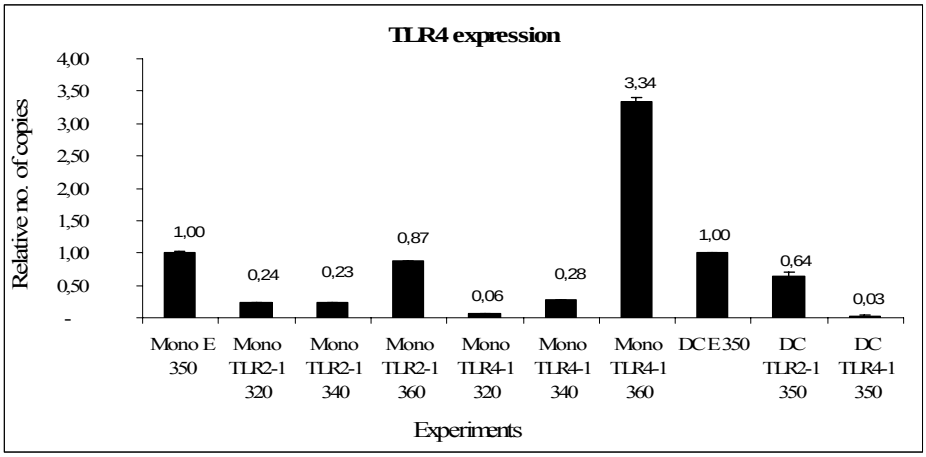


Figure 4.1.9.2d: Experiment 2 as well, performing real-time PCR for TLR4.

TLR4 expression was in one sample strongly reduced (0.06, at 320 mV), as in the DCs. But TLR2-siRNA showed non-specific effects and also caused reduction of TLR4 expression.

Experiment 3:

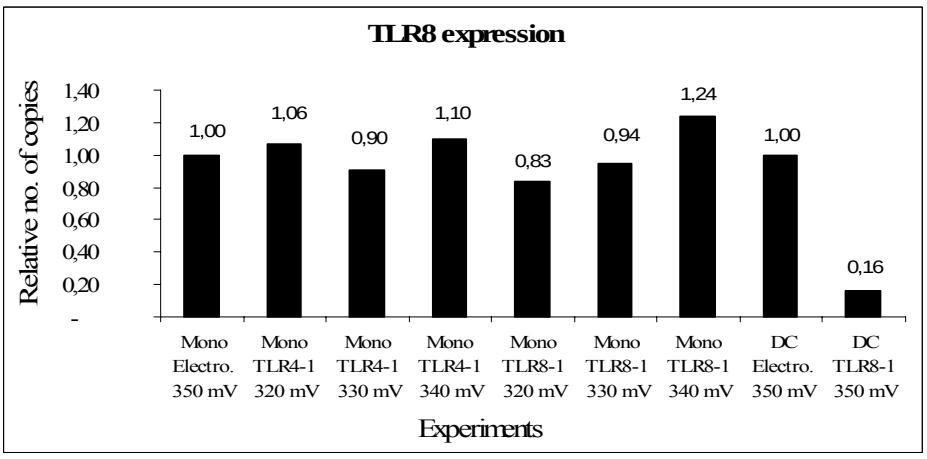


Figure 4.1.9.2e: Experiment 3; 10^6 monocytes were electroporated with 3 µM TLR4- and TLR8-siRNA for 15 ms at 320, 330 and 340 mV. mRNA was isolated and real-time PCR performed after 24 h for TLR8. DCs were also transfected, only with TLR8-siRNA at 350 mV.

Analyzing the TLR8 expression, as usual only experiments with DCs were successful, showing a strong down-regulation of the targeted gene. Monocytes were not affected in any case.

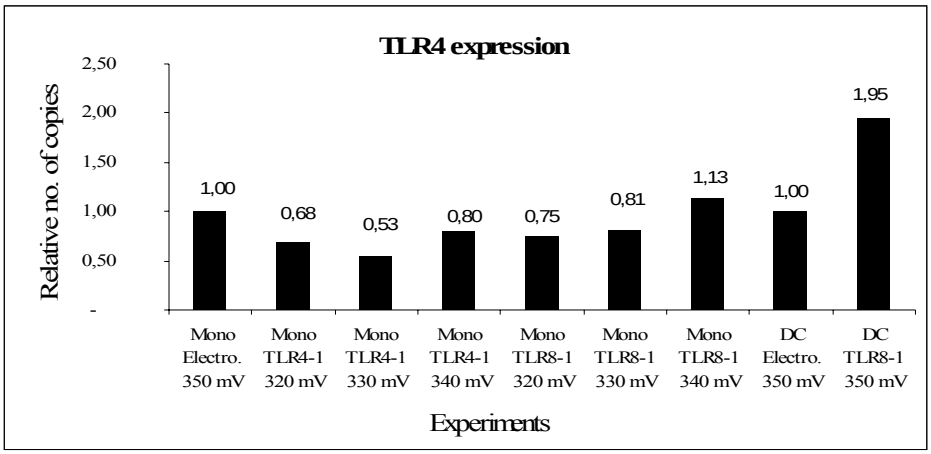


Figure 4.1.9.2f: Experiment 3 as well, performing real-time PCR for TLR4.

The same samples show a slight decrease in TLR4 expression in monocytes (0.5-0.8), but yet not enough to turn down the function of the targeted gene.

Overall, no standard protocol could be established to use RNAi transfections in monocytes. Silencing achieved was too variable to apply this method for further evaluations. In contrast, DCs showed stable results when transfected with siRNA.

4.2 Analysis of HLA-presented peptides in the THP-1 cell line

Having the ability to phagocytose like normal peripheral blood monocytes (Tsuchiya *et al.*, 1980), monocytic THP-1 cells were incubated with *A. fumigatus* hyphae for 6 h in order to find specific *A. fumigatus* peptides and other peptides presented on the HLA molecules.

4.2.1 Cell numbers and yield of harvested THP-1 cells

THP-1 cells were grown in roller bottles in RPMI/10% FCS and harvested at different time periods, depending on their growth and density. Fractions of these cells were incubated with *A. fumigatus* hyphae for 6 hours, then harvested by centrifugation. The pellets were frozen immediately at -80°C in 3-5 ml volume batches (see 3.3.2).
Overall, the following cell yields were achieved:

Table 4.2.1: Number of THP-1 cells and total pellet volume harvested with and without stimulation.

Cell line	Experiment	Yield	Cell no.
THP-1	non-stimulated	33 ml pellet	~ 6.5 x 10^9
THP-1	+ *A. fumigatus* hyphae	20 ml pellet	~ 4 x 10^9

4.2.2 Flow cytometric analysis of THP-1

To analyze the relative surface expression of different HLA molecules on THP-1 cells, we performed flow cytometric analyses non- as well as with stimulated cells, and compared them. Class I (W6/32), class II (L243), HLA-A*02 (BB7.2), HLA-A*03 (GAP-A3) and HLA-B* (B123.2) molecules were analyzed with specific monoclonal antibodies (see also 3.5.2). Cells of the JY cell line were analyzed too, used as a positive control.
The following figures show the expression levels of the different HLA molecules, when stained with 1 µg/ml antibody:

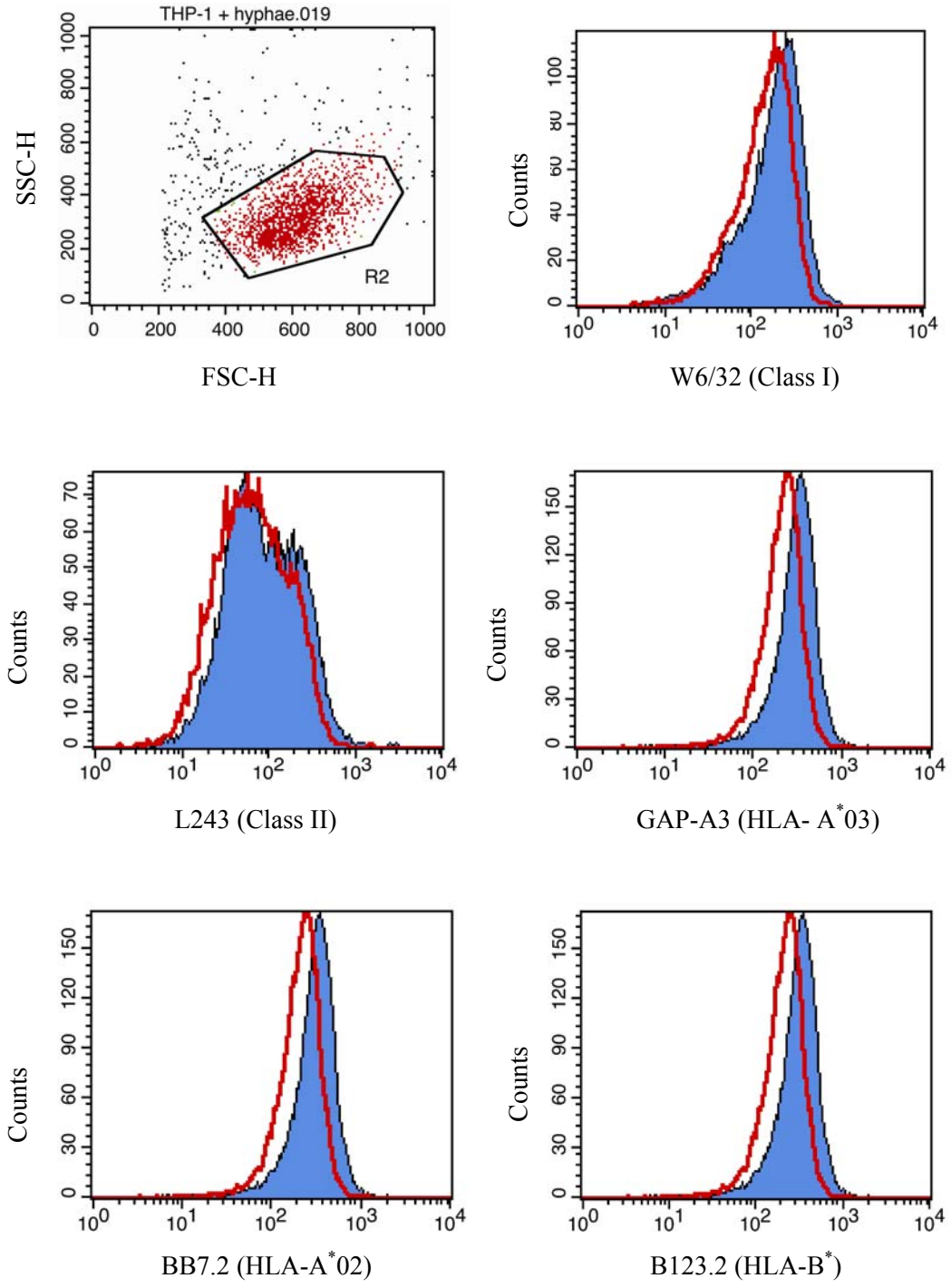


Figure 4.2.2: Surface expression of different HLA molecules on THP-1. The first figure shows the gated cells after stimulation with the hyphae. The blue area shows the expression level in the non-stimulated cells and the red line shows the expression level of the stimulated cells.

The figures show enough expression of all these HLA molecules in the non-stimulated sample. Interestingly, their expression goes down a little after stimulation with *A. fumigatus*

hyphae (red line, figure 4.2.2), but it is still high enough to perform peptide analysis. Comparison was also made with the JY cell line, which shows positive expression levels for these molecules (data not shown).

4.2.3 Monoclonal antibodies and immunoprecipitation

Monoclonal antibodies W6/32 and L243 were purified for use in immunoprecipitation experiments to isolate HLA-presented peptides. For the experiment with the non-stimulated cells, we used 34.7 mg W6/32 and 33 mg L243. For those stimulated with hyphae for 6 hours, we used 20 mg W6/32 and 19.3 mg L243.

4.2.4 Edman sequencing of the isolated peptide samples

The eluted samples from the centricons were analyzed with Edman-sequencing. Analysis of the stimulated samples showed following results:

Table 4.2.4: Results of the Edman-sequencing of HLA class I and HLA class II sample fractions

Protein	Chain	Starting sequence	Yield [pmol]
HLA class I	α	GSHSMRY	> 50
	β_2-microglobulin	IQRTPKI	> 40
HLA class II	α	LKEEHVI	30-50
	β	GDTRPRF	< 10

So the average of 50 pmol was used for both fractions and calculated to 100% volume:
→ 50 x 20 = 1000 pmol = 1 nmol

Unfortunately, the analysis of the non-stimulated samples showed a far less yield of class II peptides. Class I peptide fractions couldn't be analyzed, for technical reasons. Nevertheless, peptide analyses with mass spectrometry were later performed.

4.2.5 Western blot analysis of the isolated peptide samples

Western blot analysis was done with different samples taken during the immunoprecipitation and with the eluted peptide fractions to analyze the class I molecules, using an anti-ß2 microglobulin antibody. The monomer HLA-B*1501 was used as a standard.

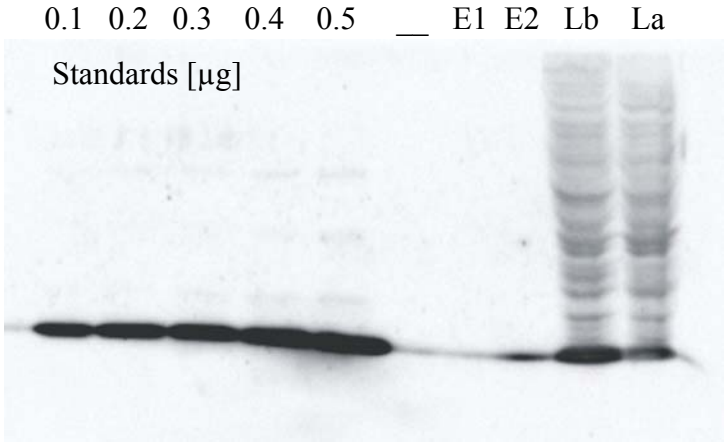


Figure 4.2.5a: Western blot analysis of lysate fractions and eluted peptide samples of the non-stimulated THP1 cells. E1 represents the eluted fractions 1 and 2 of the W6/32-specific class I peptides; E2 represents the fractions 3 and 4. Lb is a cell lysate sample before antibody precipitation; La after antibody precipitation.

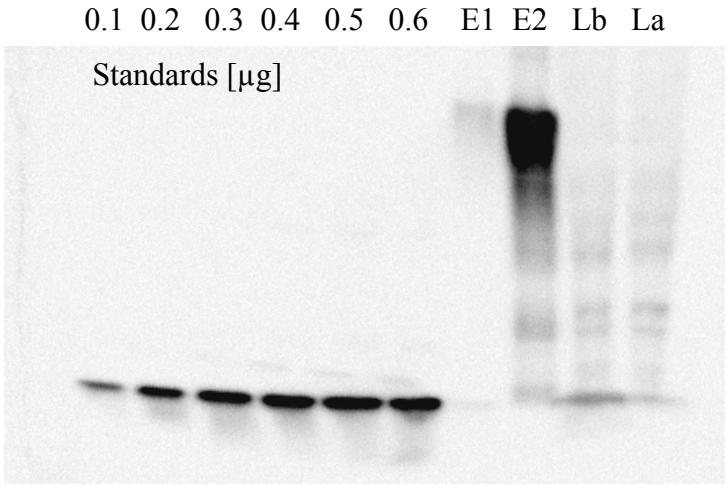


Figure 4.2.5b: Western blot analysis of lysate fractions and eluted peptide samples of the stimulated THP-1 cells. E1 represents the eluted fractions 1 and 2 of the W6/32-specific class I peptides; E2 represents the fractions 1 and 2 of the L243-specific class II peptides. Lb is a cell lysate sample before antibody precipitation; La after antibody precipitation.

The eluted class I fractions of the non-stimulated cells (figure 4.2.5a) show a variation in peptide amount. Fractions 1 and 2 together (E1) obviously don't contain much peptide whereas the fractions 3 and 4 (E2) do. This was taken into consideration, because Edman analysis couldn't be done for these fractions. The lysate fractions show also clearly the decrease in peptide amounts after antibody precipitation; thus the peptides were bound successfully to the specific antibodies (W6/32).
The W6/32-specific fractions 1 and 2 (E1) from the stimulated THP-1 cells (figure 4.2.5b) unfortunately don't show any peptides, yet the Edman sequencing revealed enough yield for mass spectrometry. The E2 fraction of the class II peptides was only used to see if unspecific bindings take place, for the antibody used is class I specific. As in figure 4.2.5a, the lysate fractions show also a decrease in La due to peptide binding to the antibodies.

4.2.6 LC-MS and tandem-MS runs

Three LC-MS/MS runs were made with the L243-specific class II eluted peptide fractions from the stimulated THP-1 cells (see also 3.8.2.3). The W6/32-specific class I eluted peptides from both stimulated and non-stimulated cells were first modified (Guanidination and nicotinylation). LC-MS (survey scan) was done after mixing both samples. Afterwards, the modified samples were fragmented separately (LC-MS/MS) (see also 3.8.2.4).

4.2.6.1 LC-MS/MS runs of class II eluted peptides from stimulated cells

After idling the spectrometer with the *Glufib*-peptide (EGVNDNEEGFFSAR), the L243-specific class II eluted peptide samples of the stimulated THP-1 cells were analyzed in three separate runs (each 150 µl).
Survey scan analyses were first performed (LC-MS), afterwards peptide fragmentation (MS/MS), in order to identify possible *A. fumigatus* peptides presented on the cells. The following chromatograms show the survey scan analyses of the three fractions. Every run was recorded for about 200 minutes.

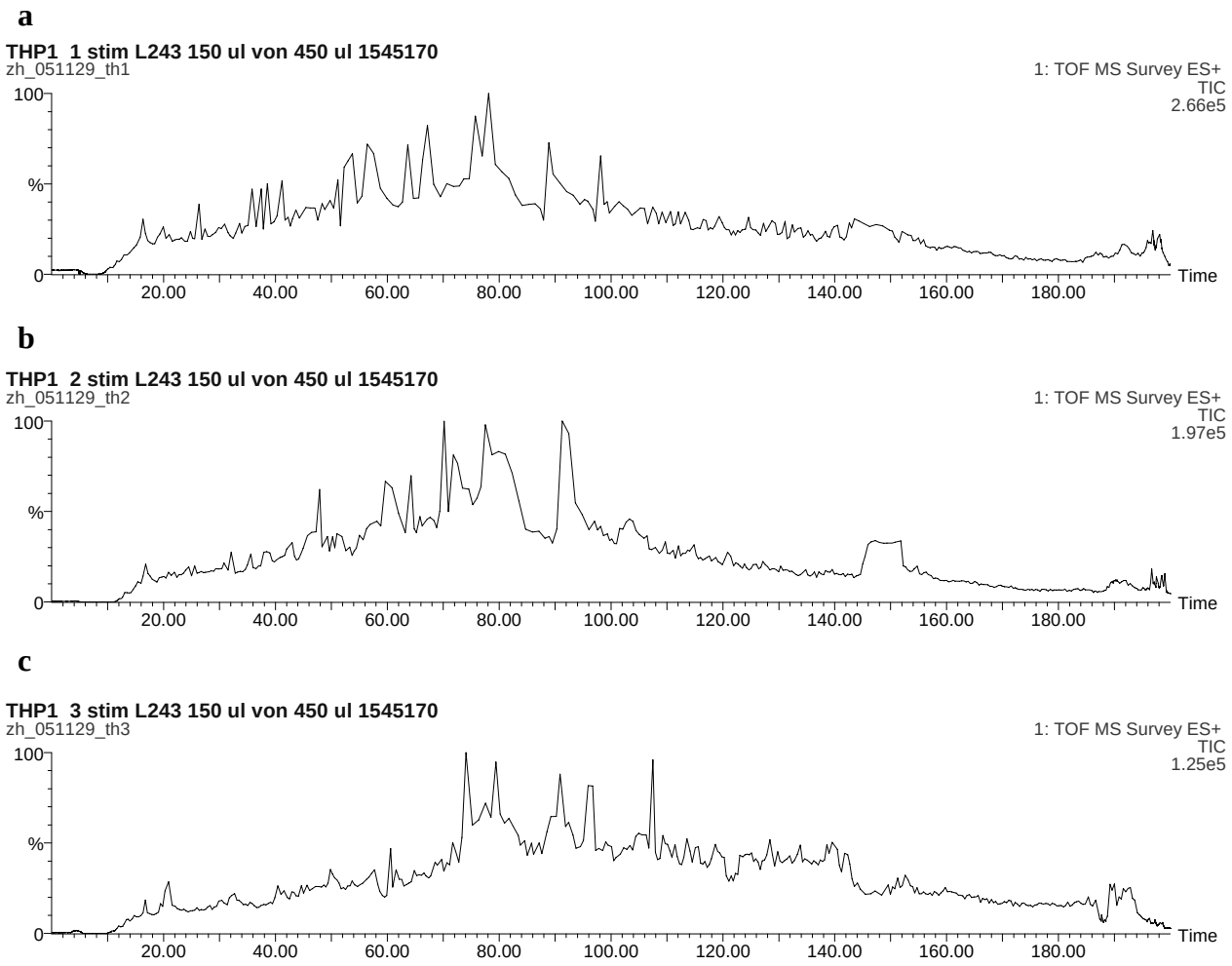


Figure 4.2.6.1a: TOF MS survey scan showing the total ion current (TIC) of all three runs performed with the class II eluted fractions. The relative ion intensity is plotted against the retention time (RT) [min].

Similarities can be observed between run a and b, concerning the retention time where the highest intensity peaks are seen. Run c is a little bit different, but has also high peak intensities. The TIC in run a (~ 3 x 10^5) is higher than in b and c (2 x 10^5 and 1.25 x 10^5), which leads to more and better fragmented peptides. This is due to the wearing out of the picotip used for the three runs, which leads to alteration of the total ion current; thus less ions reaching the detector.

Fragmentation (TOF MS/MS) of the peptide ions followed automatically, and the resulting peptide peaks were analyzed first with automated software, then evaluated manually. The following spectrum represents one of the peptides found by manual analysis, shown as an example.

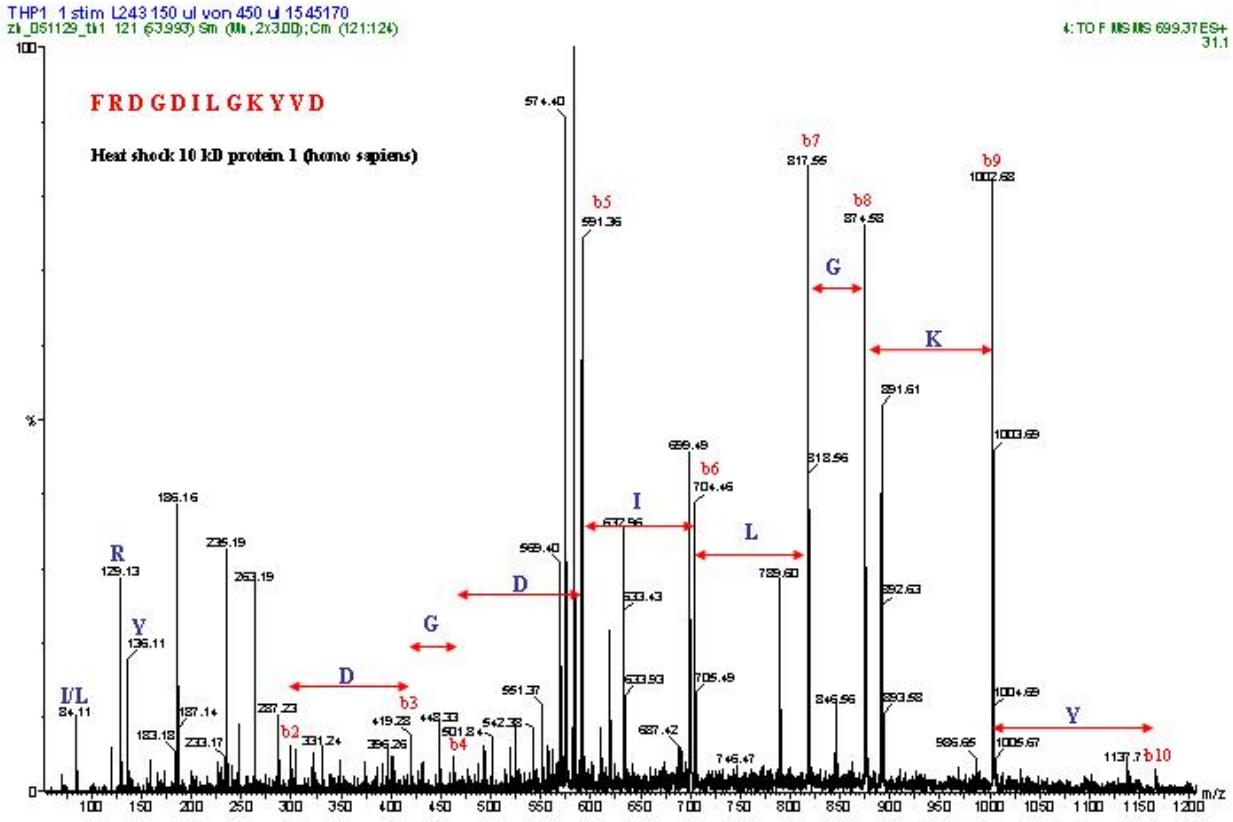


Figure 4.2.6.1b: MALDI Q-Tof MS/MS spectrum of FRDGDILGKYVD, a class II peptide identified from the first fragmentation run. It represents the last 12 amino acids of the human heat shock 10kD protein 1. The spectrum shows the identified amino acids and their associated b-series ions. The b-ions represent the peptide fragments with charges on the N-terminal ends.

The spectrum shows the amino acids detected between the b-ions series. The intensities of b5, b6, b7, b8 and b9 were high enough, so that the amino acids K, G, L and I could be easily detected. We could also find b10, yet not as easily as the others. The peaks at m/z 84, 129 and 136 represent the immonium ions of I/L, R and Y, respectively. The peptide spectrum showed a mass of 699.37 Da (see fig. 4.261b), but had a doubly-charged parent ion $[M+2H]^{2+}$, thus weighing 1397.74 Da. With its actual mass, we were able to find the remaining amino acids by searching online databases like mascot (www.matrixscience.com) and fitting the results to the spectrum until the most suitable peptide is found; in this case FRDGDILGKYVD, a heat shock protein.

The same procedures were done for many other spectra, also in runs b and c, in order to hopefully find *A. fumigatus* specific peptides. Unfortunately, we only found human peptides presented on class II HLA molecules, as shown in the following list:

Table 4.2.6.1: Class II peptides found by analyzing spectrums of three MALDI Q-Tof MS/MS runs performed from stimulated THP-1 cells. All these peptides belong to the human species, thus originate from the monocytes. APLP2, amyloid beta (A4) precursor-like protein 2; DNPEP, aspartyl aminopeptidase; SNAG1, sorting nexin associated golgi protein 1.

Run	RT [min]	Mass [Da]	Peptide sequence	Amino acids	Related protein
a	20.11	1372.84	GGALQPSPQQLYGG	14	SNAG1 protein
a	36.25	1701.91	FSETGAGKHVPRAVFV	16	Tubulin alpha 6
a	43.14	2410.15	WISKQEYDESGPSIVHRKCF	20	Actin
a	46.66	1094.62	RHLDNLLLT	9	Phosphoinositide-3-kinase
a	53.99	1397.74	FRDGDILGKYVD	12	Heat shock 10 kD protein 1
a	103.1	1742.86	IPPFHPFHPFPALPE	15	APLP2 protein
b	48.68	1094.78	PSLSHNLLVD	10	DNPEP protein
b	74.75	1360.76	DVELDDLGKDEL	12	Disulfide isomerase-rel. prot. 5

The three runs produced many MS/MS spectra, of which we could only evaluate a few (Table 4.2.6.1). Two peptides (RHLDNLLLT and PSLSHNLLVD) are, according to their length, characteristically class I peptides, yet presented on Class II. Alpha-tubulin is a component of microtubules and actin a structure protein, and both belong to the cellular cytoskeleton. APLP2 plays a role in the regulation of hemostasis and DNPEP is an aminopeptidase. So most of these peptides do not play a vital role in immune-related pathways, except the heat shock 10kD protein 1, which was shown to exert anti-inflammatory activity by inhibiting toll-like receptor signaling in murine macrophages and human monocytes (Johnson *et al.*, 2005). Unfortunately, no *A. fumigatus*-specific peptides could be detected. Only the 20-amino-acid-long actin-related peptide WISKQEYDESGPSIVHRKCF is in addition to humans also *A. fumigatus*-specific; thus we can take a small possibility into consideration that this peptide originated from the *A. fumigatus* hyphae.

4.2.6.2 LC-MS of the mixed class I modified fractions

Class I isolated peptides of both stimulated and non-stimulated cells were modified (Guanidination and nicotinylation) prior to LC-MS and LC-MS/MS analysis (see also 3.8.2.4). The with D_4-NIC (dNIC, stimulated cells) and H_4-NIC (NIC, non-stimulated cells) modified peptides were mixed in a 1:1 ratio and analyzed with LC-MS, resulting in the following survey scan:

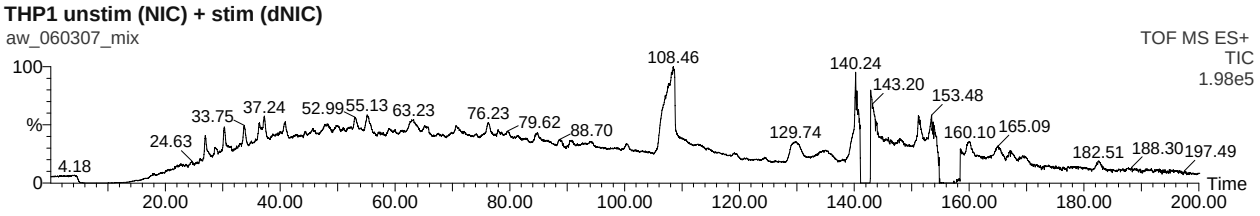


Figure 4.2.6.2a: LC-MS survey scan showing the total ion current (TIC) of the mixed class I fractions, previously modified by guanidination and nicotinylation. The relative ion intensity is plotted against the retention time [min].

By analyzing each peak of this survey scan (figure 4.2.6.2a), we could detect a series of peak pairs (dNIC and NIC) present, with a difference of 4 units (4 deuteriums in dNIC; deuterium has mass number of 2). Thus these peaks represent the guanidinated N-termini of the modified peptides and show us the number of peptides presented by stimulated (dNIC) and non-stimulated (NIC) cells. This number depends on many factors, above all the mixing and the modification process itself. More than 100 peak pairs were detected, some with low, others with high intensities, revealing a successful modification.

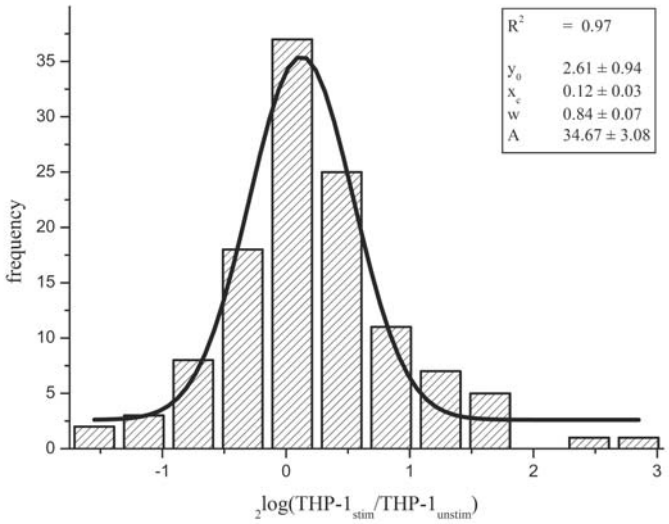


Figure 4.2.6.2b: Frequency distribution of the binary-logarithmic ratios of stimulated THP-1 to non-stimulated THP-1 from the identified dNIC/NIC-modified pairs and proximity of these ratios through a Gaussian curve.

The normal distribution of the peptide ratios of dNIC to NIC is given in figure 4.2.6.2b. The R^2 value of the gauss curve is 0.97 (close to 1) and the median value X_c is 0.12 (close to 0), meaning the mixing of dNIC and NIC peptides was generally done well, which enables almost quantitative conclusions of the peptide amounts. These values were obtained by calculating the ratios of the intensities of all dNIC/NIC peak pairs and building the binary

logarithm of these ratios. In brief, all peptides were modified and almost all mixed successfully in a 1:1 ratio (dNIC/NIC).

4.2.6.3 LC-MS/MS run of the modified samples separately

After analyzing the modified peptides as a mixture, we analyzed them separately with LC-MS/MS in order to identify them and to differentiate between peptides presented on simulated and non-stimulated cells. Following survey scans were obtained:

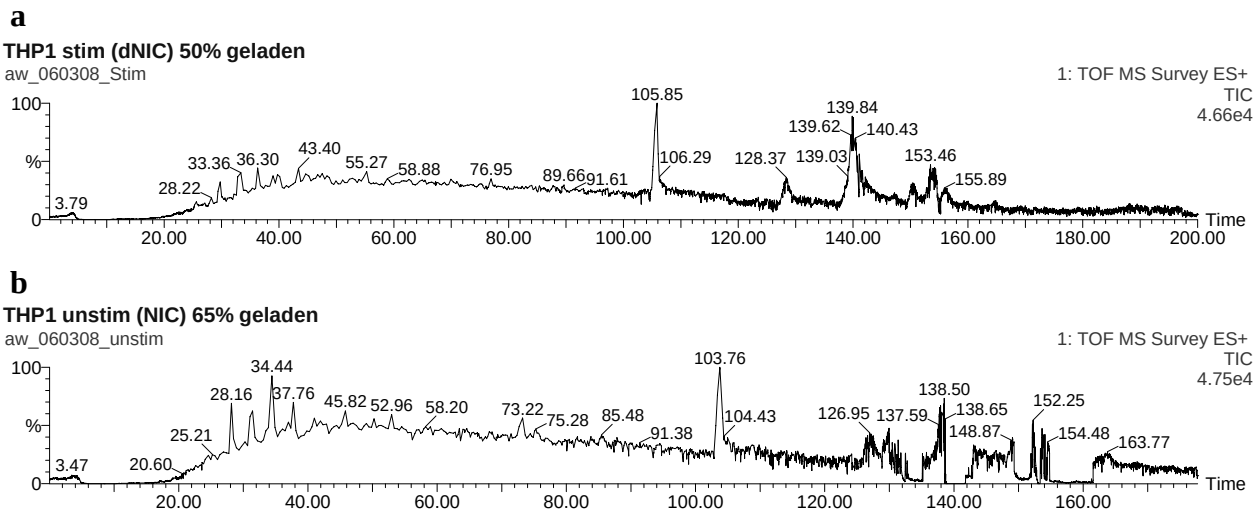


Figure 4.2.6.3a: LC-MS survey scans showing the TIC of the modified class I peptides analyzed separately. (a) TIC of the class I peptides isolated from stimulated cells and modified with dNIC. (b) TIC of the class I peptides isolated from the non-stimulated cells and modified with NIC.

The scans show almost the same TIC intensities (~ 4.7 x 10^4) and the retention times of the main peaks are also very close, differing in ~ 2 min from one another. We analyzed most spectra of the stimulated peptides, after fragmentation in MS/MS mode, and obtained peptides present in both fractions as well as peptides only present in the stimulated fraction.

Following peptides were found in both fractions:

Table 4.2.6.3a: Peptides identified as pairs in both stimulated and non-stimulated fractions.

RT [min]	Mass [Da]	Peptide sequence	AA	Related protein
36.4	1140.75	RPAPVEVTY	9	PTP4 A1 (Protein tyrosine phosphatase)
52.34	1159.8	NVIRDAVTY	9	HIST2H4 (histone 2, H4)
54.8	1078.8	AIVDKVPSV	9	coatomer protein complex, subunit γ 1
55.75	1085.67	LIDDVHRL	8	SRPR (signal recognition particle receptor)
70.43	1109.72	FIDTTSKF	8	RPL3 (ribosomal protein L3)

Table 4.2.6.3a shows peptides identified in both fractions. They represent relatively unspectacular proteins except maybe PTP4A1 (also called PRL1), a tyrosine phosphatase believed to regulate cell proliferation and to have a role in tumorgenesis (Werner *et al.*, 2003).

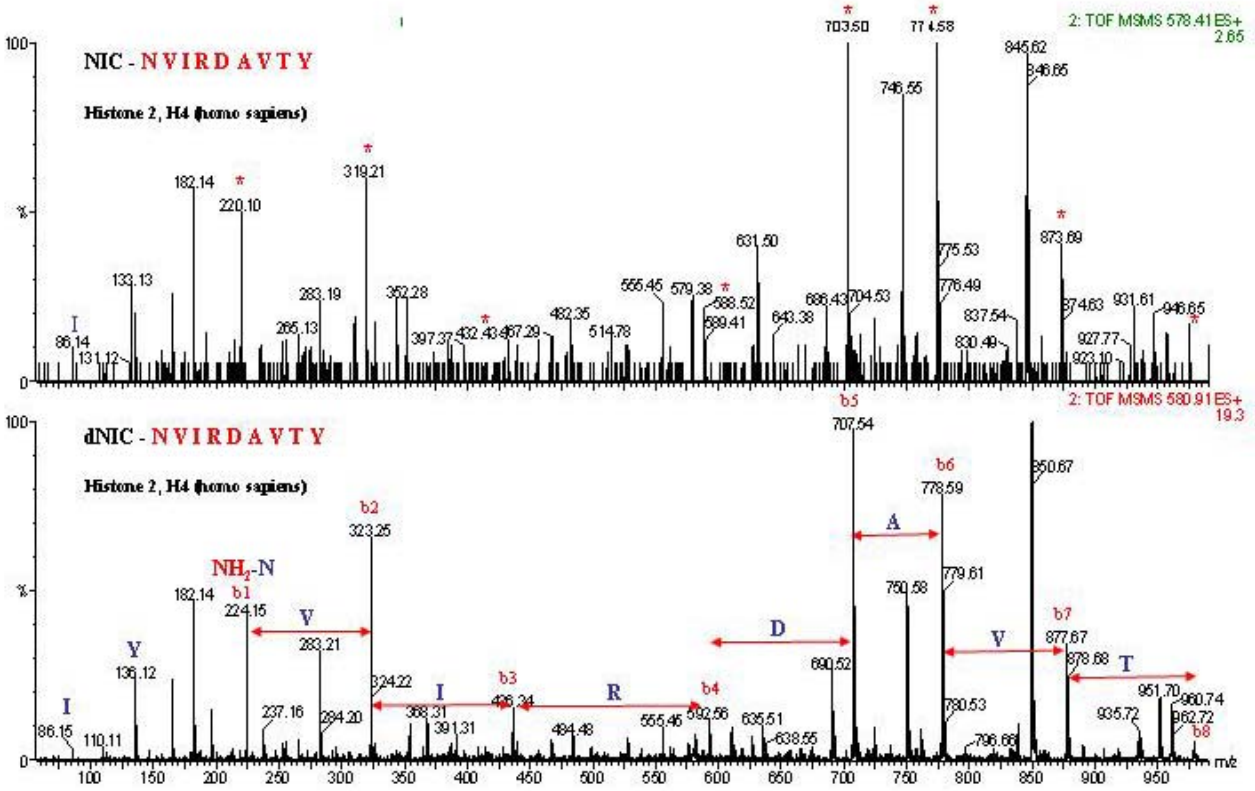


Figure 4.2.6.3b: MALDI Q-Tof MS/MS spectra of NIC- and dNIC-modified NVIRDAVTY, a class I peptide identified in both stimulated and non-stimulated cells. It is a 9 amino acid long peptide. The figure shows the dNIC-peptide in the lower half, compared to the NIC-peptide in the upper half. The easily to identify b-ion series in the dNIC-spectrum is shown. They differ by 4 Da to the corresponding peaks in the NIC-spectrum (*). By finding b1, the N-terminal amino acid is found, in this case N.

The peptides in table 4.2.6.3a were identified by analyzing and simultaneously comparing the NIC- and the dNIC modified peptide spectra, as shown in figure 4.2.6.3b.

The peptide shown in this figure was easily identified, starting by b1 which gives the N-terminal amino acid, in this case N (see table 3.8.2.5). The other b-ions could be identified by comparing the masses of the similar peaks; they must have a difference of 4 Da. The amino acids I/L and Y could also be assumed, by finding their immonium ions at 86 and 136 m/z. The rest is done by mascot software, which gives back the whole peptide that fits in this spectrum.

In addition to these, we were also able to identify peptides only presented by stimulating cells, thus dNIC-modified, shown in the following table:

Table 4.2.6.3b: Peptides identified only in the fraction of the stimulated cells.

RT [min]	Mass [Da]	Peptide sequence	AA	Related protein
26.9	1162.81	VAEPNRRVL	9	LRRC41 (leucine rich repeat containing 41)
27.9	961.7	KAHLGTAL	8	RPS23 (ribosomal protein S23)
32	1183.82	TIIDSKSKSV	10	HMGCS1 (3-hydroxy-3-methylglutaryl-CoA synth. 1)
33.45	1000.5	FIDTTSKF	8	RPL3 (ribosomal protein L3)
38.8	1051.37	ALADGVQK'V	9	APOL1 (apolipoprotein 1)
39	1070.73	SLIDKTTAA	9	SNRPC (small nucl. Ribonucl. polypep. C)
44.47	962.64	IDTTSKF	7	RPL3 (ribosomal protein L3)
52.87	1279.82	KFIDTTSKF	9	RPL3 (ribosomal protein L3)

The peptides found (table 4.2.6.3b) represent mostly a peptide of the ribosomal protein L3 (K-F-IDTTSKF). Another ribosomal protein peptide is also shown, that of RPS23. APOL1 is the major plasma apoprotein of HDL and HMGCS1 catalyzes the conversion of (S)-3-hydroxy-3-methylglutaryl-CoA and CoA to acetyl-CoA, acetoacetyl-CoA, and H2O.

So as in class II, we couldn't find any *A. fumigatus*-specific peptides presented on HLA class I molecules.

5. Discussion

Aspergillus fumigatus is a fungal pathogen which can cause allergic but also lethal infections in immunosuppressed patients, like patients undergoing solid organ or stem cell transplantations or AIDS patients. It is exceptional among microorganisms in being both a primary and opportunistic pathogen as well as a major allergen. Most *Aspergillus* species don't grow at 37°C, but *A. fumigatus* does, which may distinguish pathogenic species from non-pathogenic ones (Pitt, 1994). *Aspergillus* species may cause serious clinical syndromes, of which the invasive pulmonary aspergillosis is the most dangerous. Invasive aspergillosis has emerged as a major cause of infection-related mortality in immunocompromised patients. Spores or conidia of *A. fumigatus* enter the lungs by inhalation. Since they don't face there any interception by cells of the immune system, they are able to settle down in the alveoli. Immune cells have been previuosly totally eliminated or are only present in very few numbers. After a short while they germinate to hyphae, which are capable of tissue invasion. *Aspergillus* hyphae typically invade pulmonary parenchyma and blood vessels causing pulmonary hemorrhage, arterial thrombosis and infarction. Invasive aspergillosis (IA) occurs in 10 to 25 % of all leukemia patients, in whom the mortality rate is 80 to 90 %, even after treatment (Denning, 1995; Groll *et al.*, 1996).

Non-specific immunity plays a major role in host defense against *A. fumigatus*. It includes three defense strategies: anatomical barriers (through the ciliary action of the mucous epithelium), humoral factors such as complement, and phagocytic cells and their related antimicrobial products.

Having a major role in protection against the fungus, phagocytic cells have been studied by several groups (Sturtevant and Latgé, 1991; Roilides *et al.*, 1998; Ibrahim-Granet *et al.*, 2003). We contributed to these studies by analyzing phagocytosis of *A. fumigatus* conidia by human monocytes. We also analyzed gene expression of monocytes after incubation with conidia and hyphae and at last we analyzed HLA peptide-presentation by the monocytic cell line THP-1 after incubation with *A. fumigatus* hyphae.

5.1 Phagocytosis and gene expression analyses in monocytes

Monocytes play a major part in innate immunity. They initiate immune responses by phagocytosis, killing of pathogens and production of a wide range of co-stimulatory

molecules, inflammatory cytokines and chemokines (Janeway and Medzhitov, 2002). Considering *A. fumigatus*, alveolar macrophages represent the first line of defense against its spores. Monocytes attack and damage escaping conidia and hyphae in the blood stream by phagocytosis and secretion of oxidative metabolites and non-oxidative compounds, thereby preventing establishment of invasive infections (Schaffner *et al.*, 1982; Levitz *et al.*, 1986). For a broader understanding of defense strategies used by monocytes, genome-wide expression profiling was performed and gene expression patterns were correlated with phagocytosis of conidia in a time-dependent manner, after incubation with *A. fumigatus* live conidia and inactivated hyphae.

We found that in the majority, monocytes phagocytosed two to three conidia, some more than three, few phagocytosed one and very few phagocytosed no conidia. Thus the majority of the monocytes were found to phagocytose up to 3 conidia within the first 3 hours (figure 4.1a). This is in line with other studies on phagocytosis of *A. fumigatus* conidia (Cortez *et al.*, 2006). The number of phagocytosed conidia remained unaltered after 6 hours, but the conidia were swollen and the monocytes changed their shape accordingly (figure 4.1b). This behavior is not always similar, depending on the phagocytosed particle (Aderem and Underhill, 1999). Pathogens are able to swell and after a certain period of time destroy the phagocyte. In our experiments, non-phagocytosed conidia germinated to hyphae and overgrew the monocytes. The phagocytosis rate of monocytes of various *Aspergillus* species was shown to differ according to the pathogenicity of the conidia (Henwick *et al.*, 1993; Akpogheneta *et al.*, 2003).

Until now, most researchers studied cytokine expression either in murine models or in human macrophages (Schelenz *et al.*, 1999; Pylkkanen *et al.*, 2004), but only very few studies were performed on human monocytes and only a limited number of cytokines was investigated (Roilides *et al.*, 1998; Wang *et al.*, 2001; Cortez *et al.*, 2006). After analyzing the real-time PCR data, we could observe increased cytokine expression in monocytes only after 6 and 9 hours, when incubated with conidia. Thus phagocytosis didn't stimulate any up-regulation of the three inflammatory cytokines tested (TNF-α, IL-1β and IL-6). After 6 and 9 hours, a little up-regulation was observed, probably due to the swelling of the phagocytosed conidia (see also fig 4.1b) and the beginning of generation to hyphae of the non-phagocytosed ones. Such activities stimulate many immune-related pathways in the cell, such as cytokines responsible for host defense and phagocyte activation. Similar results were shown previously for the interaction of endothelial cells with *Pseudomonas aeruginosa*. An inflammatory response was only detected at the end of phagocytosis (Grassme *et al.*, 2003). Also the results obtained by

Cortez *et al.* (2006) are comparable to ours. On the contrary to conidia, inactivated hyphae stimulated an early and high up-regulation of all three cytokines. This is due to the different toxins, surface antigens and cell wall components on hyphae (Bernard and Latge, 2001; Rementeria *et al.*, 2005). Hyphae represent the invasive form of the fungus, leading to the logical conclusion that they have far stronger effects on host cells than conidia, not to forget they are also bigger. They are too big for phagocytosis, stimulating cells to produce instantly inflammatory cytokines and chemokines to attract fellow immune cells for help.

Genome-wide expression profiling demonstrated a differential regulation of many genes, mostly related to the inflammatory response such as cytokines and chemokines and their receptors, as well as genes coding for signal-transduction, adhesion molecules and many others. In comparison to conidia, inactivated hyphae induced a strong differential gene expression after 3 hours of incubation already, but the total number of differentially regulated genes was similar after 6 and 9 hours, regardless of the stimulus. Conidia started to germinate to hyphae after 3 hours. Therefore, as mentioned before, after 6 and 9 hours of incubation, hyphal elements are present in all experiments and may account for similarities in gene expression profiles and numbers of differentially regulated genes at 6 and 9 hours. Yet standard deviations of several SLR values are rather high, most probably due to individual differences between the monocytic preparations. Yet a clear trend concerning the regulation tendency of the genes was noticed.

Activation of immune defense responses during *Aspergillus fumigatus* infection is, among other things, dependent on so-called pattern recognition receptors (PRRs), such as the mannose receptor, and some integrins, as well as TLRs and PTX3. Some *A. fumigatus* cell-wall molecules act as PAMPs which activate PRRs, like mannans, for example. Foreign particles and pathogens are recognized by more than one receptor on the cell surface. Due to the many different surface antigens of a pathogen, the response triggered by more or less specific receptors may differ, releasing cellular responses dependent on the downstream genes activated by these receptors (Rohde *et al.*, 2002). PTX3 is a lectin-like molecule which plays an essential part in antigen recognition and defense against foreign agents and pathogens, among others conidia of *A. fumigatus* (Garlanda *et al.*, 2005). PTX3 production is induced by primary inflammatory signals, such as agonists for different members of the TLR family. Our data demonstrated that PTX3 expression was up-regulated after 3 hours of incubation by both conidia and inactivated hyphae. Thus PTX3 may have promoted phagocytosis of the conidia during the first 3 hours. These findings are in line with recent reports that describe the role of PTX3 in promoting phagocytosis of *A. fumigatus* conidia and in activating the anti-fungal

cellular response (Garlanda *et al.*, 2002; Gaziano *et al.*, 2004). Interestingly, also hyphae induced PTX3 expression. However, we presume that in parallel to LPS, hyphae can induce PTX3 production but cannot be bound by PTX3 (Garlanda *et al.*, 2005).

Toll-like receptors (TLRs) belong to the family of pathogen recognition receptors which recognize pathogen-associated molecular patterns (PAMPs). TLR2 and TLR4 revealed no changes in their expression profiles in our experiments. These results were confirmed with real-time PCR (Data not shown). TLR2 and TLR4 were shown to be essential for *A. fumigatus* recognition, activating the cellular immune response by inducing genes such as MyD88, IRAK and the transcription factor NF-κB, which in turn induces transcription of cytokines and chemokines as well as other inflammatory genes (Meier *et al.*, 2003; Braedel *et al.*, 2004). Our data demonstrate the expression of a broad range of cytokines and chemokines, downstream of the TLR signaling pathway. Most likely this reflects the incubation periods we used, starting at 3 hours, which is longer compared to earlier studies (Meier *et al.*, 2003; Braedel *et al.*, 2004). Nevertheless, similar results were observed by Cortez *et al.* (2006). Yet more interesting is the strong down-regulation of TLR7 and TLR8, which are responsible for single-stranded RNA (ssRNA) recognition (Heil *et al.*, 2004). TLR7 and TLR8 were strongly down-regulated only after incubation with inactivated hyphae, but not conidia. This coincides with the results of an earlier study, in which neutrophil activity against *Aspergillus fumigatus* was assessed. Bellocchio *et al*. (2004) could show that an increased expression of TLR7 and TLR8 on neutrophils inhibits fungal damage, but does not affect their phagocytosis activity. This may explain why monocytes down-regulate the expression of these TLRs when stimulated by hyphae, but not when phagocytosing conidia.

As predicted, inflammatory genes such as cytokines and chemokines (IL-8, IL-1 and CCL2) were for the most part similarly expressed after incubation with both stimuli, despite slight differences depending on the maturation state of the fungus and the incubation time. This was also confirmed by ELISA assays (figure 4.1.8). Interestingly, some genes, mostly receptors, showed large differences in their regulation. CCR2, the receptor for CCL2 (MCP1), which is an important chemokine that induces the recruitment of monocytes and neutrophils to infection sites, was strongly down-regulated after incubation with conidia, but was found unchanged after incubation with hyphae. Recent studies described the significant role of CCR2 in clearance of *A. fumigatus* conidia from the airways of mice. $CCR2^{-/-}$ mice showed less neutrophil recruitment into the airways as well as an increase in inflammation and sub epithelial fibrosis (Blease *et al.*, 2000). Lundien *et al*. (2002) demonstrated that CCR2 expression is inhibited in bronchial epithelial cells when incubated with LPS, a method which

may be used by bacteria to promote cell infection. These findings may resemble *A. fumigatus* infection of monocytes. *A. fumigatus* conidia were able to inhibit CCR2 expression in monocytes, which may play a role in facilitating the infection process. In contrast to viable conidia, inactivated hyphae obviously lose their ability to inhibit CCR2 expression.

Our microarray data demonstrate an increase in CCL2 and urokinase-type plasminogen activator (uPA) expression. These results were similar for both stimuli, conidia and inactivated hyphae. uPA binds to its receptor (uPAR) resulting in the conversion of plasminogen to plasmin, which activates pro-collagenase that degrades the extra cellular protein matrix and interstitial tissue and facilitates migration and invasion of tumor as well as immune cells. Plasmin acts as a chemoattractant for monocytes (Syrovets *et al.*, 1997) and induces expression of inflammatory cytokines and chemokines at sites of inflammation (Weide *et al.*, 1996; Syrovets *et al.*, 2001; Burysek *et al.*, 2002). Higher levels of uPA were also detected at injury and inflammation sites in immunocompromised patients, such as AIDS patients (Angelici *et al.*, 1996; Angelici *et al.*, 2001; Kusch *et al.*, 2002; Kunigal *et al.*, 2003), for example. A feedback pathway generated by PathwayAssist™ revealed the interaction of CCL2 and uPA, which is largely similar for conidia and hyphae (figure 4.1.7.2). This pathway gives an overview of a possible monocytic defense strategy which involves IL-1, TGF and CCL2, along with uPA, in causing lung injury. Recently, in a murine model, Maus *et al*. (2005) described the effect of CCR2 expression levels in recruited monocytes on CCL2 concentrations in inflamed lung tissue, limiting excess monocytic accumulation and promoting a return to homeostasis. According to our data, CCL2 may play an important part in attracting monocytes to sites of inflammation induced by *A. fumigatus*. This inflammatory process, involving the release of inflammatory cytokines (IL-1, TGFβ), ICAM1, uPA and others, might ultimately result in tissue damage. The increased cytokine and chemokine expression levels in the monocytes after incubation with *A. fumigatus* lead us to propose, in addition to the feed-back pathway, a possibly strong inflammation process that may cause local injury. Lung injuries are major complications after stem-cell transplantation (SCT). They occur in 25-55 % of transplant patients and can account for up to 50% of transplant-related mortality (Weiner *et al.*, 1986; Clark *et al.*, 1999). These recent studies report that lung injury can occur as a result of inflammation accompanied by high cytokine levels.

One limitation of the analysis of primary monocytes obtained from different persons is a possible variability between the donors which might lead to relatively high standard variations. However, gene expression analysis of different independent donors has a higher

impact compared to multiple runs from one identical donor. Furthermore, the use of adequate controls and the definition of levels of significance are essential.

To further the role of TLRs in monocytes after stimulation with *A. fumigatus*, we tried to establish a protocol to transfect the primary monocytes with siRNA. So far, no successful method was established to transfect primary monocytes and achieve a strong silencing effect (> 90%), without affecting other functions of the cell. Only one work describes a successful transfection of monocytes with CD33-siRNA using the lipofection method with Oligofectamine™ (Lajaunias *et al.*, 2005), which we also used. Other studies describe RNA interference using lentiviral vectors in monocytes (Lee *et al.*, 2004). We did not use lentiviral vectors, as this viral infection of the monocytes could potentially stimulate defense pathways and affect gene expression of immune-related genes. Our lipofection experiments revealed disappointing results, showing either weak silencing or no silencing at all. In addition, the results varied from experiment to another, showing no standardization. Although transfection with Oligofectamine™ resulted in some silencing, it was not enough to turn down the function of the target gene to an extent, where its functionality becomes non-considerable (see also 4.1.9.1). RNAiFect™ showed somehow stable results, but yet silencing was not enough as well (up to 75%, once). The weak silencing with lipofection might be due to low or even high concentrations of the reagent used. We followed the manufacturer's protocol for each reagent, and varied the concentrations of the reagent and the siRNA as well, until the levels where either very low, so that it had no effect or it was too concentrated resulting in non-specific interactions with the cell, or even cell death. Another factor we varied was incubation time. No clear tendency could be observed, where silencing was the highest. Our incubation periods varied from 9 h to 72 h, thus covering the time usually needed for siRNA to silence and for proteins to be translated.

After promising experiments were performed in our lab to transfect siRNA into DCs, we applied this method on monocytes. Several studies already showed electroporation to be an effective alternative to transfect siRNA into cells, like DCs, but also primary cells and cell lines (Gresch *et al.*, 2004; Ovcharenko *et al.*, 2005; Prechtel *et al.*, 2006). Since TLR genes were our primary targets, concerning their role in host defense against *A. fumigatus*, we used siRNAs made by Qiagen (Germany), which were previously evaluated and showed positive silencing effects. Our electroporation results were far more promising as with lipofection, yet instability in the outcome was also observed. Varying parameters like pulsing time and voltage or incubation period showed slight differences, but no constant results were obtained in monocytes. Only DCs showed stable effects. When voltage or pulsing times were raised,

monocytes couldn't withstand and perished. Unfortunately, when TLR4-siRNA silenced TLR4, TLR2 was also affected. On the otherwise, TLR2-siRNA didn't silence TLR4. (see 4.1.9.2, experiments 1 and 2). Yet, this effect wasn't seen with DCs (experiment 2). Thus, the problem lies within the monocytes themselves, not the siRNA or the transfection protocol. The same is seen when transfecting TLR8-siRNA (experiment 3). Only TLR8 in DCs gets silenced. These results lead to the fact that transfecting and successfully silencing primary cell target genes, particularly in monocytes, remain a challenge for researchers.

5.2 Analysis of HLA-presented peptides of the THP-1 cell line

The human monocytic cell line THP-1 was shown to have phagocytic abilities like normal monocytes and express monocytic surface antigens and HLA molecules (Tsuchiya *et al.*, 1980). We used this cell line to identify peptides presented on HLA class I and II molecules, after incubation with *A. fumigatus* hyphae. The advantage of using a cell line over freshly isolated cells lies mainly in the availability of large numbers of cells and the homogeneity of the cell cultures. These were two important factors which were crucial for our analyses of HLA-presented peptides. The cell line could be easily expanded and grown in roller bottles which allowed large scale stimulations with the fungus; and secondly, we could be sure to have only one cell type in the culture, excluding the possibility to have other immune cells present, as in freshly isolated blood cultures. Nevertheless, using cell lines implies also disadvantages, mainly concerning the genetic drifting which occurs in cancer cell lines through small mutations that add up over time.

We first studied cell surface expression of different HLA molecules before and after stimulation with the *A. fumigatus* hyphae and showed that THP-1 cells express high levels of class I and class II molecules. After stimulation with hyphae, the HLA class I and II expression levels were a little bit down-regulated, as shown by flow cytometry (figure 4.2.2). It is widely recognized that down-regulation of HLA class I molecules by tumor cells impairs cellular immune recognition and contributes to inefficient cytotoxic T cell-mediated tumor eradication leading to progression of disease (Morris, 1990; Seliger *et al.*, 1996). Yet down-regulation of HLA class II as well could be a mechanism triggered by the pathogen, in this case *A. fumigatus*, to cause inefficient immune recognition and to overcome the host cell. This is in line with a previous study showing THP-1 HLA class II to be strongly down-regulated during the first 2 days after phagocytosis of bacteria (De Lerma Barbaro *et al.*, 1999). On the contrary, another study showed *A. fumigatus* conidia to trigger HLA-DR expression in DCs

after 24 h of incubation (Grazziutti *et al.*, 2001), but which might be due to increased phagocytosis rates leading to higher processing and presentation levels of fungal peptides. Studies on HLA class II molecules in allergic bronchopulmonary aspergillosis patients suggest different roles for HLA-DR and HLA-DQ in response to *A. fumigatus* (Chauhan *et al.*, 2003), where some HLA-DR alleles possibly contribute to susceptibility while HLA-DQ2 contributes to resistance (Chauhan *et al.*, 2000). This reflects again a possible defense mechanism by the THP-1 cell, by down-regulating HLA-DR in response to the fungus. Yet we didn't perform further tests to investigate this more closely.

Analyzing the class II presented peptides after stimulation with hyphae, we could only identify 8 peptides, given in table 4.2.6.1. Many MS/MS spectra had low intensities; thus a high background signal which made it impossible for us to evaluate, although we had a relatively acceptable peptide yield of 1 nmol as shown in Edman-sequencing (see 4.2.4). Class II peptides are, compared to class I, much longer peptides, resulting in more complicated spectra which are more difficult to evaluate. Among the 8 identified peptides, no *A. fumigatus*-specific ones were found. This might have many reasons; first, we assume the THP-1 cells didn't phagocytose any of the hyphae due to the big volume of hyphal cells, and smaller fragments might have been present only in small amounts, which made it very difficult for us to detect them. Marr *et al.* (2001) showed that THP-1 cells do phagocytose and kill *A. fumigatus* pathogens within 6 hours, but in the case of conidia and not hyphae. Thus, stimulation of THP-1 might have happened, but not phagocytosis. Up to now, no study described phagocytosis of *A. fumigatus* hyphae. Second, we assumed that THP-1 cells might do cross-presentation, thus not present fungal peptides on class II molecules, but on class I. An earlier study showed presentation of an exogenous antigen by THP-1 on class I molecules, when targeted specifically to the receptor for IgG, FcgammaRI (Wallace *et al.*, 2001). Yet this assumption was nullified as we couldn't find fungal peptides also on class I. Third, we assume that fungal peptides were not presented, or less presented and not detected, due to less expression of class II molecules. This was also concluded by De Lerma Barbaro *et al.* (1999), where THP-1 phagocytosed bacteria, but didn't process and present specific bacterial peptides on class II molecules. So maybe mechanisms of inhibition of the transport of newly synthesized class II molecules to the cell surface were triggered by *A. fumigatus*. Similar observations were made where HLA class II molecules were internalized, but recycling was inhibited after phagocytosis of *Mycobacterium tuberculosis* by human monocytes (Clemens and Horwitz, 1995). Another study shows IL-10 to inhibit cell surface expression of class II due to block of exocytosis of mature class II molecules and inhibition of recycling

(Koppelman *et al.*, 1997). This matches with our previous findings concerning gene expression in monocytes, where we show *A. fumigatus* hyphae, but not conidia, to induce IL-10 expression after 3 hours incubation (see tables 4.1.7.1a and b). Thus IL-10 may have blocked processing and presentation of fungal peptides to a certain limit, which disabled us to detect any after 6 hours, and taken in consideration that maybe only small amounts of hyphae were phagocytosed, if ever. Stanzani *et al.* (2005) discusses that *A. fumigatus* produces many potent toxins, like gliotoxin, which inhibit antigen-presenting cell function in monocytes; but the hyphae we used were inactivated and couldn't produce toxins anymore. One can discuss and find many other reasons and mechanisms that might have lead to inhibition or down-regulation of antigen presentation, associated with no fungal peptide presentation. We assume all these factors together played a role in this. The human peptides identified belong mostly to unspectacular proteins, not involved in immune regulation pathways, except maybe FRDGDILGKYVD, the peptide shown in figure 4.2.6.1b that belongs to the heat shock 10kD protein 1. This HSP was shown to inhibit TLR signaling in macrophages and monocytes (Johnson *et al.*, 2005). This coincides surely with our gene expression data in the peripheral monocytes, showing no over-expression of TLR receptors. Yet such assumptions must be handled carefully, for THP-1 is a cell line which may differ in its immune response from fresh monocytes, as shown in a previous study on cytokine expression (Glue *et al.*, 2002). To mention is still the 20 amino acid-long actin peptide found (WISKQEYDESGPSIVHRKCF), which is human but also present in *A. fumigatus* actin. Unfortunately, one cannot figure out to which species this specific peptide really belonged to originally, so also this conclusion must be handled with care.

Identification and differentiation of HLA class I peptides from two different samples of the same cell type can be done by different modification of these peptides, like guanidination and nicotinylation. Such a method was established in our research group by C. Lemmel (Lemmel *et al.*, 2004) and further developed by A. Weinzierl in order to allow guanidination of peptides previously lyophilized in citric acid containing TFA, as well as a better nicotinylation. Through nicotinylation of the N-terminus, the b_1-ion obtains a high intensity and can be easily identified which is not the case in non-modified peptides, where usually b_2-ions are more intense and stable (Yalcin *et al.*, 1995). Through modification with NIC and dNIC (deuterium instead of hydrogen), we were able to differentiate between peptides of the stimulated cells and the non-stimulated ones. The identification was much easier, when analyzing the same peptide spectra, one with NIC and the other dNIC. Identifying the b_1 ion in dNIC was then very easy, since it differs in 4/z from the same ion in NIC. That way, we

identified 5 peptide pairs presented on HLA class I in stimulated and non-stimulated cells as well (table 4.2.6.3a). One problem that made it hard for us to detect more peptides was that we didn't obtain enough peptide yields from the immunoprecipitation, as shown with the western blot analysis, figure 4.2.5a. Unfortunately, technical reasons hindered us from quantifying the class I peptides by Edman sequencing. This was probably the main cause in not getting enough evaluable spectra. Most spectra had low intensity peaks with a high background. The five common peptides identified are, like most class II, not involved in immune regulation pathways. Only RPAPVEVTY, belonging to PTP4 A1, a tyrosine phosphatase, is believed to regulate cell proliferation and plays a role in tumorgenesis (Werner *et al.*, 2003). In addition to these commonly presented peptides, we were able to identify eight peptides only presented on the stimulated cells (table 4.2.6.3b). Four of these were peptides belonging to ribosomal proteins, three to the same one: RPL3. The other four were as well peptides which belong to irrelevant proteins, like APOL1 (Apolipoprotein 1) and HMGCS1 (3- hydroxyl-3-methylglutaryl-CoA synthase 1). Unfortunately as in class II, we were not able to detect *A. fumigatus*-specific peptides on class I, after considering THP-1 cells to perform cross-presentation (Wallace *et al.*, 2001).

5.3 General conclusion

In this investigation, monocytes are shown to play a major role in phagocytosing fungal spores. Surely they do not present the first line of defense, but yet are able to engulf escaping conidia. We could show that *A. fumigatus* induces inflammatory response in monocytes by stimulating the expression of cytokines and chemokines, which may lead ultimately to the recruitment of immune cells and possibly to lung injury, a phenomenon observed often in patients with invasive aspergillosis.

Furthermore, we analyzed peptide presentation on the monocytic cell line THP-1 after stimulation with *A. fumigatus* hyphae. Unfortunately, we couldn't find any *A. fumigatus*-specific peptides presented on either HLA class I or II molecules, most likely because THP-1 did not phagocytose any or only small, almost undetectable amounts of hyphae. We identified only human-specific peptides which belong to different proteins not involved in immune response pathways.

6. Summary

Aspergillus fumigatus is a member of a large genus of filamentous fungi found in the environment. It is pathogenic to humans, causing serious and life-threatening infections in immunocompromised patients. Many studies have been made so far investigating the interaction of *A. fumigatus* species with immune cells, most of these concentrating on macrophages, which represent the first line of defense against this fungus. Monocytes are first recruited to an infection site, as soon as conidia of the fungus escape the primary immune defense cells in the lung and enter the blood stream. This gives them a potent role in controlling the outcome of an infection.

We therefore analyzed the interaction of *A. fumigatus* live conidia and ethanol-inactivated hyphae with human monocytes on different levels. First, we investigated phagocytosis of conidia by fresh peripheral blood monocytes after 3 and 6 hours and found the majority of the monocytes to phagocytose up to 3 conidia during the first 3 hours. We further investigated gene expression in monocytes after incubation with conidia and hyphae for 3, 6 and 9 hours, using real-time RT-PCR and microarray gene chips. We found inactivated hyphae to induce strong cytokine and chemokine expression already after 3 hours and conidia after 6 and 9 hours. Genes like PTX3, which were shown to facilitate phagocytosis of conidia, were up-regulated after 3 hours. Interestingly, essential TLRs like TLR2 and TLR4 were not regulated at all. We finally found uPA and the MCP-1 (CCL2) to be potential key regulators involved in *A. fumigatus*-induced tissue damage and inflammation, as obtained by a pathway-generating software.

In a second effort to get more detailed data from the *A. fumigatus*-moncytic interaction, we analyzed class I- and class II-specific peptides presented on a monocytic cell line (THP-1) after 6-hour-incubation with inactivated hyphae. For this purpose, we isolated HLA presented peptides from stimulated and non-stimulated cells by immunoprecipitation using specific antibodies and analyzed them by ESI-Q-Tof mass spectrometry. Class I peptides were previously modified at their N-termini by different isotopes in order to differentiate the stimulated-specific from the non-stimulated-specific ones. Unfortunately, we couldn't detect any *A. fumigatus*-specific peptides on class II or I. We identified only some human peptides presented on both HLA, but these were not related to any immune relevant protein as well.

7. References

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8. Abbreviations

AIDS	Aquired immune deficiency syndrome
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	complementary DNA
Da	Dalton
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
h-ALAS	Human 5-Aminolaevulinate synthase
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IA	Invasive aspergillosis
IL	Interleukin
kDa	Kilo-dalton
LC	LightCycler™
LPS	Lipopolysaccharide
MHC	Major Histocompatibility complex
Min	Minutes
PBMCs	Peripheral blood mononuclear cells
RNAi	RNA interference
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
siRNA	small interfering RNA
Sec	Seconds
TLR	Toll-like receptor

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Aug. 2003 – Jun. 2006	PhD work and thesis in biochemistry "*Monocytic cell responses to Aspergillus fumigatus*: *Investigation of phagocytosis, gene expression and peptide presentation*" At the Department of Hematology & Oncology and the Dep. of Immunology, the university of Tübingen, Germany