THE DERMATOPHYTE *ARTHRODERMA BENHAMIAE* USES TWO STRATEGIES TO CONTROL COMPLEMENT IN SKIN.

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ABBREVIATIONS

ACTH adrenocorticotropic hormone

AMP antimicrobial peptides

AP alternative pathway

APC antigen presenting cell

ARPE retinal pigment epithelial cell line

ASC apoptosis associated speck-like protein containing a caspase recruitment domain

BLys B-lymphocyte stimulator

C complement

C1INH complement component 1 inhibitor

C4BP C4 bindig protein

C5aL2 C5a receptor Typ 2

C5aR C5a receptor Typ 1

CD cluster of differentiation

CFH complement Factor H

CFHR complement Factor H related protein

CSF colony stimulating factor

DAF decay accelerating factor

DMEM Dulbeccos modified Eagle`s Medium

DPBS Dulbeccos phosphate buffered saline

Dpp Dipeptidylpeptidase

DTH delayed type hypersensitivity

ECM extracellular matrix

EDTA ethylene diamine tetraacetic acid

EGTA ethylene glycol tetraacetic acid

ELISA Enzyme-linked immunosorbent assay

FBS fetal bovine serum

FHL1 Factor H like protein 1

HaCaT human keratinocyte cell line

HRP horseradish peroxidase

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IDEC Inflammatory dentritic epidermal cells

IFN-γ interferon γ

Ig immunoglobuline

IH immediate hypersensitivity

IL interleukine

iNOS inducible nitric oxide synthetase

LL-37 cathelidin

LPS Lipopolysaccharide

MAC membrane attack complex

MASP mannan binding lectin associated proteases

MBL mannan binding lectin

MCP membrane cofactor protein

MEP metalloprotease

MHC major histocompatibility complex

MMP matrixmetalloproteinase

MPO myeloperoxidase

mRNA messenger ribonucleic acid

NFκB nuclear factor kappa-light-chain-enhancer of activated B cells

NHS native human serum

NK natural killer cells

NLR nucleotide binding leucine rich repeat containing family proteins

PAF platelet activating factor

PAMPS pathogen associated molecular patterns

PMN polymorphnuclear cells

PMSF phenylmethylsulfonyl fluoride

PGE_2 prostaglandine E2

PRR pathogen recognition receptor

ROS reactive oxygen species

SALT skin associated lymphocyte tissue

SCP streptococcal cysteine protease

SCR short consensus repeat

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM scanning electron microscopy

SUB subtilisin

TCC terminal complement complex

TGF Tumor growth factor

THP-1 Human acute monocytic leukemia cell line

TLR toll like receptor

TNF tumor necrosis factor

T_H T helper cells

TRM *T. rubrum* cell wall mannans

YPD Yeast Peptone Dextrose medium

INTRODUCTION

Overview of the human immune system

The word “immune” originates from the latin “immunis” and means pure or intact. In the figurative sense it means unsusceptible to diseases. Every day humans get in contact with foreign cell, substances or microbes, like fungi, bacteria, viruses or parasites. To be protected against these foreign invaders the human body developed an elaborate and dynamic communication network of organs, tissues, cells and effector substances that specifically fend unmeant infections of the body. The immune system recognizes and attacks infective agents by using two layers of immune defense-the innate and the adaptive immunity. The innate immune system is composed of anatomic (e.g. skin, mucous membrane) and physiological (e.g. acid pH, high salt conditions, temperature, lysozyme) barriers, which build an effective mechanical and chemical defense against infections. When invaders overcome these barriers the innate immune system reacts immediately within seconds. Pathogen recognition receptors (PRR) on the surface of innate immune effector cells identify pathogen associated molecular patterns (PAMPS) and activate tissue or blood derived macrophages and dendritic cells to phagocytise foreign particles and release cytokines, which also activate inflammatory response and trigger influx of white blood cells. Humoral components of the innate immunity, like the complement system recognizes particles, gets activated, and opsonises them for phagocytes, destroy membranes by forming the membrane attack complex (MAC) and release anaphylatoxins, which are potent initiators of the inflammatory response. Furthermore attacked human cells secrete antimicrobial agents. Such are defensins and lysozymes, which destabilize membranes of invaders. Consequently the microorganism dies, because of the loss of cell homeostasis. Acute phase proteins are activated and promote inflammation, activate the complement cascade and stimulate chemotaxis of phagocytes. Cytokines and chemokines mediate the organization of the cellular part of innate immunity. The cellular components of the innate immunity, such as macrophages and neutrophils, detect and phagocytise foreign cells and release cytokines, as well as chemokines to promote inflammation. Damaged cells trigger mast cells and basophils to release histamine. Consequently blood vessels dilate and get permeable to facilitate white blood cells to move to the site of infection. In addition natural killer cells of the innate immune defense attack virus infected cells and tumor cells by releasing damaging enzymes.

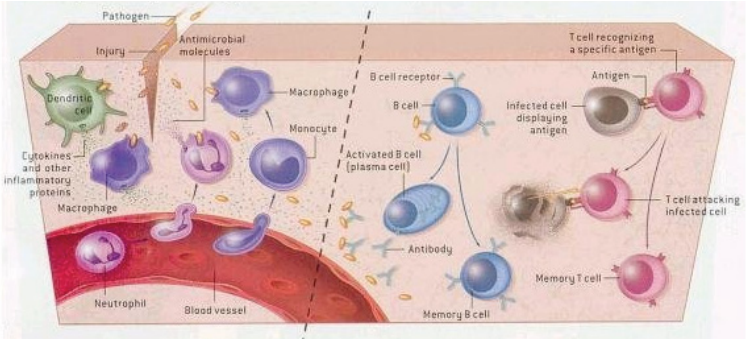


Figure 1: Overview of the immune system[1]

The immune system consists of two interactive networks- the innate and the adaptive immune system. The innate immune system includes soluble substances, like antimicrobial molecules, cytokines/chemokines and the complement system, as well as effector cells, like dendritic cells, macrophages and granulocytes. The adaptive system is composed of chemical active substances like cytokines/chemokines and antibodies. T and B lymphocytes play a major role in specific recognition and clearance of pathogens. Characteristic for the adaptive immune system is the immunological memory.

The innate and adaptive immune systems communicate at several interfaces. When microbes overcome the innate immune system, the adaptive immunity answers with a specific antigenic response and the applied immunological memory allows recognition of pathogens for a faster clearance of the infection. To initiate the adaptive immune response, phagocytes (e.g. dendritic cells) present antigens to lymphocytes. Thymus derived lymphocytes mature to effector T-lymphocytes. $CD8^+$ cytotoxic T-cells kill infected human cells. $CD4^+$-T_H1-cells stimulate macrophages to kill intracellular parasites and $CD4^+$-T_H2-cells in combination with antigens activate B-cells to antibody producing plasma cells. Antibodies bind specific to epitopes on the surface of pathogens and depict the basic part of the humoral immune answer in adaptive immunity. Phagocytes recognize the constant part of the bound antibodies with the F_c receptor. Consequently the phagocytes uptake and kill the pathogen. In addition pathogen bound antibodies activate the innate complement system. Furthermore cytotoxic γδ-T-cells and natural killer T-cells act as link between innate and adaptive immune system.

The innate immune system

The innate immune system abolishes infections of the human body with foreign substances for the most part, demonstrating a well structured and effective system of cellular and humoral components. Chemical components of the innate immune system coordinate the cellular network. Cytokines and chemokines target cells of the innate and adaptive immune system and induce inflammatory responses.

Recognition of foreign cells by the innate immune system

The central step to induce the immune system is the discrimination between self and foreign cells and particles. The innate immune system use germline encoded receptors for recognition of microbes in comparison to the adaptive immune system, which is based on receptors that are produced by somatic recombination. Innate pathogen recognition receptors (PRR) are genetically predetermined and thought to be in the hundreds.

PRRs bind pathogen-associated molecular patterns (PAMPs). These PAMPs are exclusively microbe derived. Common PAMPs are lipopolysaccharid (LPS), peptidoglycan, lipoteichoic acid, mannans, bacterial DNA, double stranded RNA and glucans. PRRs are divided into three groups of endocytic PRRs, signaling receptors and secreted proteins. Endocytic PRRs are arranged on the surface of phagocytes. Binding of endocytic PRRs to on the microbial surface expressed PAMPS results in uptake and processing of the invader. Microbe derived proteins are presented by major MHC complexes on the surface of the phagocyte. Typical endocytic PRRs are the macrophage mannose receptor or scavenger receptor. Signalling receptors recognize PAMPs and induce expression of immune response genes, like inflammatory cytokines. Typical signaling receptors are the toll like receptors (TLR). Secreted proteins, like the mannan binding lectin binds microbial carbohydrates and thus initiate the mannan binding lectin pathway of complement activation.

PRRs play a crucial role in the induction of the adaptive immune system. Failure in the innate immune recognition results in absence of an antigen specific adaptive immune response.

The humoral components of the innate immune system

Chemical components of the innate immune system coordinate the cellular network and take part in the clearance of foreign or abnormal cells. Different groups of humoral effector substances are defined. Inflammatory lipid metabolites, such as platelet activating factor (PAF) function as phospholipid activator and mediate leukocyte functions, like platelet aggregation, inflammation and anaphylaxis. Others like the derivates of the arachidonic acid, such as prostaglandins regulate physiological effects, such as smooth muscle cell contraction and relaxation. Leukotrienes mediate histamine production and chemotaxis of neutrophils, have effects in bronchoconstriction and increase vascular permeability[2]. Lipoxins inhibit

transmigration, superoxide generation and NFκB activation, but also stimulate chemotaxis of polymorphonuclear cells (PMNs) and calcium mobilization[3].

Antimicrobial peptides or substances directly kill microbes or initiate inflammatory response. Lactoferrin and transferrin limit bacterial growth by binding ion, an essential nutrient for bacteria. Enzymes like lysozyme or phospholipase found in tears, saliva or nasal secretions destruct bacterial cell walls. Epithelial cells and phagocytes contain antimicrobial peptides, such as defensins or cathelicindins (LL-37)[4]. These peptides disrupt the integrity of microbial membranes by forming pores, which leads to disruption of the osmotic stability of the cell[5]. LL-37 exhibits additional biological functions, it attracts neutrophils, monocytes, mast cells and T-cells, initiate degranulation of mast cells, regulates transcriptional response of macrophages, stimulates wound vascularization and re-epithelarization of the skin[6]. Various defensins attract monocytes, dentritic cells and T-cells or act as an antagonist to adrenocorticotropic hormone (ACTH) by binding the ACTH receptor. This inhibits the production of the immunosuppressive hormone cortisol and thus support immunological answer to infections[7].

Nitric oxide acts as a potent vasodilator and is generated by phagocytes after IFN-γ stimulation by inducible nitric oxide synthetase (iNOS). Free nitric oxide radicals act toxic on bacteria by damaging DNA and by degradation of iron sulphur centers into iron ions[8].

Three zymogen cascades generate proinflammatory peptides, the complement system, the coagulation system and the kinin system. Initiation of the intrinsic pathway of coagulation leads to the cleavage of kininogen and the release of antimicrobial acting bradykinin[9]. Platelets produce β-lysine during aggregation, which acts antimicrobial on gram positive bacteria.

In answer to inflammation liver cells release acute phase proteins, such as the C-reactive protein, the mannose binding protein, complement factors, ferritin, ceruloplasmin, serum amyloid A, haptoglobin, which stimulate inflammation or serpins, which give a negative feedback on inflammatory response.

Table 1: Role of cytokines/chemokines in actute inflammation

name	effect	source	Chromo.
cytokines			
Interleukin-1 (IL-1)	enhance PGE_2 → fever, stimulate T-cell proliferation, stimulate release of histamine from mast cells → histamine trigger vasodilation and increase vascular permeability, stimulate release of collagenase and PGE_2 by synovial cells	mononuclear phagocytes, fibroblast, keratinocytes, T- and B-cells	2
Tumor necrosis factor-α (TNF-α)	stimulate PGE_2 → fever, induce release of IL-1, stimulate release of collagenase and PGE_2 by synovial cells induce production of acute phase proteins by the liver, stimulate IL-6 synthesis	activated macrophages and monocytes, fibroblast, mast cells, some T- and NK cells	6
Tumor necrosis factor-β (TNF-β)	properties like TNF-α, induce apoptosis in transformed, virally infected and tumor cells, stimulate several PMN effector functions	activated T- and B-cells	6
Interleukin-6 (IL-6)	production of acute phase proteins by the liver, growth factor for mature B-cells, induce B-cell maturation to plasma cells, induce T-cell activation and maturation, induction of IL-2 and IL-2 receptor expression, inhibition of TNF production and limiting acute phase response	mononuclear phagocytes, fibroblasts, T-cells	7
Interleukin-11	Proliferation of plasmacytoma cell lines, production of acute phase proteins by the liver, stimulate T-cell dependent B-cell IgG secretion, increase platelet production, induce IL-6 expression by $CD4^+$ T cells	Bone marrow stromal cells, fibroblasts	19
Colony stimulating factors (CSF)-G-CSF, GM-CSF	stimulate neutrophils, GM-CSF activate eosinophils and mononuclear phagocytes	monocytes, T-cells, fibroblasts, endothelial cells	17
chemokines			
Interleukin-8 (IL-8)	endothelial adherence, diapedesis, chemotactic migration and activation of neutrophils and other cell types (e.g. monocytes, lymphocytes, basophils, eosinophils)	phagocytes, Antigen activated T-cells, endothelial and epithelial cells, neutrophils	4

prostaglandin E_2 (PGE_2), IL-1 receptor antagonist (IL-1Ra), immunglobulin G (IgG), Chromo.=Chromosome

Cytokines and chemokines play a key role in organizing the cellular component of the innate and adaptive immune system. These soluble proteins are involved in leukocyte recruitment by increasing expression of endothelial adhesion molecules and act as chemo attractants. Cytokines regulate the activation of resident cells, such as fibroblasts, endothelial cells, tissue macrophages and mast cells, as well as newly recruited cells, such as monocytes, lymphocytes, neutrophils and eosinophils. Functions of these mediators result in fever, hypotension, synthesis of acute phase proteins, leukocytosis and cachexia[10]. Important cytokines/chemokines, which are involved in acute inflammation, are listed in Table 1.

The cellular components of the innate immune system

Innate immune effector cells play important roles in the clearance of microbes, initiation of inflammation and activation of the adaptive immune response. The cellular barrier of the innate immunity is composed of neutrophils, macrophages, dentritic cells, eosinophils, basophils, mast cells and natural killer cells. As a key component in infection, neutrophils act as potent phagocytes, stimulators of innate and adaptive effector cells, undergo the respiratory burst and degranulate in response to infective agents. Neutrophils secrete TNF and other cytokines, which influence differentiation, activation and chemotaxis of macrophages and dentritic cells and recruit and activate antigen presenting cells (APC). Secreted IFN-γ helps to drive differentiation of T-cells and activation of macrophages. Otherwise neutrophils can also function as supressors of the T-cell activation. The neutrophils derived B-lymphocyte stimulator (BLys) is involved in B-cell proliferation and maturation. In addition neutrophil produce proteases, like Cathepsin G, neutrophil elastase, protease 3 or matrixmetalloproteinases (MMPs). Even neutrophil chromatin defends against microorganisms by trapping them in extracellular nets, which are decorated with proteases from azurophil granules (α-defensins and myeloperoxidase (MPO))[11]. Polymorphnuclear cells (PMN) contribute to collateral tissue damage that occurs during inflammation.

Macrophages and dentritic cells act as phagocytes and kill microbes intracellularly. The stimulation of macrophages with cytokines are followed by the production of reactive oxygen species (ROS) and microbicidal substances in response to INF-γ, as well as by increased tissue repair and suppression of inflammation in response to Interleukin (IL)-4, IL-10, IL-13 and transforming growth factor-β (TGF-β)[12]. In cause of the ability to present antigens, macrophages and dentritic cells induce the adaptive immune system. Dentritic

cells (DC) display an extraordinary capacity to stimulate native T cells and initiate the primary immune response. Furthermore DCs play a role in the induction of peripheral immunological tolerance and regulate the types of T cell immune response [13].

Eosinophils comprise granules, which efficiently kill parasites. Basophil and mast cell activation is initiated by the crosslinking of FcεRI bound IgE with multivalent antigen, which promotes the aggregation of FcεRI. Activated mast cells or basophils synthesis proinflammatory lipid mediators, secrete cytokines and chemokines or degranulate vasoactive amines, neutral proteases, proteoglycans, cytokines and growth factors[14]. Natural Killer cells (NK) distinguishes between healthy and abnormal cells and thus attack virus contaminated human cells and tumor cells. Therefore NKs use comparable killing mechanism like $CD8^+$ cytotoxic T lymphocytes (e.g. perforin and granenzymes) and secrete proinflammatory acting IFN-γ[15].

The adaptive immune system

When a pathogen evades the innate immune system and generates a threshold level of antigen, the adaptive immune system is triggered. It is composed of highly specialized cells that recognize, eliminate and remember specific microorganisms to generate immunity. The adaptive immune system is able to differentiate between specific “non-self” antigens and “self” motifs. The recognition of specific microorganisms or microorganism infected cells is tailored by V(D)J recombination and somatic hypermutation in the gene region of the antigen receptors. These mechanisms allow a small number of genes to generate a vast number of different antigen receptors on the surface of lymphocytes, which specifically binds pathogen derived antigens. Receptor specificity is stored after an accomplished infection in memory B cells and memory T cells, which mediate long-lived specific immunity.

Recognition of foreign cells by the adaptive immune system

The adaptive immune system recognizes antigens from microorganisms, parasites or infected host cells, but not antigens from the host. All nucleated host cells possess ability to present antigens by using the major histocompatibility complex I (MHC I) and activate adaptive response. Professional APCs, like dentritic cells, B-cells and macrophages express the MHC II. For example dentritic cells engulf and process microorganisms, undergo maturation and present antigens using MHC II to $CD4^+$ T helper cells passing through the

lymph node. Infected host cells processes and presents antigens of the infecting microbe by MHC I to cytotoxic $CD8^+$ T cells. The cytotoxic $CD8^+$ T cell release perforin, granzymes, and granulysin. Perforin forms pores in the membran of the cell and enables granzymes to activate the intracellular caspase system. This leads to apoptosis of the infected cell. In addition cytotoxic $CD8^+$ T cell induce apoptosis of infected cells by binding of the FAS ligand to the FAS receptor on the target cell.

The humoral components of the adaptive immune system

The humoral immune response of the adaptive immunity is mainly mediated by plasma-cell derived immunoglobulins. Immunoglobulins are also named antibodies and are classified into five groups: IgA, IgD, IgE, IgG, and IgM, which differ in its biological role and deal with different antigens. Antibodies recognise and neutralise foreign antigens of microorganisms or foreign cells. Each antibody recognizes a specific antigen unique to its target. Antibodies which bound their antigen fulfil different functions: They agglutinate the antigen, opsonise the target for removal by phagocytes and activate the classical pathway of the complement system, which induces lysis of microbial cells. Furthermore, antibodies stimulate Fc receptors on the surface of immune cells, like macrophages, dendritic cells, PMNs, mast cells and natural killer cells. Based on the type of antibodies, the different types of FcγR (IgG), FcαR (IgA) and FcεR (IgE) are defined. FcRs bind the Fc part of antibodies, which bind the antigen via the Fab portion, and thus initiate a cellular response like phagocytosis or cytokine release. FcγRIII on the surface of natural killer cells gets activated by IgG and promotes the release of proinflammatory cytokines, like IFN-γ or apoptosis inducing mediators, such as perforins and granzymes. This process is known as antibody-dependent cell-mediated cytotoxicity (ADCC). IgE binds to allergens-and interacts with FcεRI receptors on the surface of mast cells to induce a degranulation. The released granula contains histamine, proteoglycans, and serine proteases. Parasites which are not accessible for phagocytosis are coated with IgE and recognized by FcεRI on the surface of eosinophils and mast cells. Eosinophils release mediators, like the major basic protein and enzymes such as peroxidase, attack and kill helminths[16, 17]. In addition activated mast cells synthesise proinflammatory mediators, such as prostaglandins, leukotrienes, and platelet-activating factor, as well as cytokines and chemokines to mediate clearance of parasite infections[18, 19].

The cellular components of the adaptive immune system

The main cell types of the adaptive immune response are T- and B-lymphocytes. Whereas B-cells play a crucial role for the humoral immune answer; T-cells mainly act in the cell mediated response of the adaptive immune system. Different types of T-cells mediate specific functions in organisation of the cell mediated immune attack. CD 4^+ T helper cells (T_H), activate immune cells like macrophages or B-cells, CD 8^+ cytotoxic T cells (T_c or CTL) eliminate infected host cells, longliving CD 4^+ memory T cells facilitate memory and regulatory T cells maintain tolerance against self cells and molecules (suppressor T cells, T_{reg}).

B-cells are also divided into different functional groups. B2-lymphocytes are activated upon recognition of their specific antigen and mature to plasma cells, which secrete large amounts of a specific antibody. Other antibody secreting B-cell types are the B1 cells, marginal-zone B-cells and follicular B-cells. Memory B-cells exhibit function in immunological memory. Types of T- and B-lymphocytes and their functions are listed in Table 2.

Table 2: Effector cells of the adaptive immunity

name	effect	reference
T cells		
T helper cells	B cell maturation, activation of cytotixic T cells and macrophages, cytokine secretion	
T_H1	Induced by intracellular protozoa, respond with cell mediated immunity, secrete IFN-γ and TNF-β, acts on macrophages and $CD8^+$ T cells	[20, 21]
….. T_H2	Induced by intestinal helminthes, respond with induction of the humoral immunity, secrete IL-4, IL-5, IL-6, IL-10, IL-13, acts on B-cells	[20, 21]
….. T_H3 (adaptive T_{reg})	secretes transforming growth factor beta-1 (TGF-beta1), down regulates T_H1 and T_H2 response	[22]
….. T_H17	Acts in inflammation, immune defense and auto immunity, secrete IL-17, TNF-α, IL-1, IL-22	[23]
……T_{FH}	regulates B cell immunity, secrete IL-10 and IL-21 to promote B-cell survival and antibody production	[24-26]
Cytotoxic T cells	Lysis of virally infected and tumor cells, implicate transplant rejection	[27, 28]
Memory T cells	Immunological memory,	[29]
…..T_{CM} (central memory)	express L-selectin and chemokine receptor CCR7, secrete IL-2	
…..T_{EM} (effector memory)	Secrete IFN-γ and IL-4	
Regulatory T cells	Immunological tolerance, suppress T cell mediated immunity and autoreactive T cells	[25, 30]
Natural killer T cells	bridges the adaptive immune system with the innate immune system, NKT cells recognize MHC-antigen complexes and glycolipid antigen presented by CD1d, functions as discribed to both T_h and T_c cells	[31]
γδ T cells	γδ TCR, respond to small non-peptidic antigens, Vγ9/Vδ2 respond to phosphoantigens, Vγ2/Vδ2 recognize and respond to non peptidic antigens of bacteria and parasites and haematopoietic tumor cells	[32]
B cells		
Plasma cells (B2)	antibody production, APC	
B1 cells	polyspecific receptors, IgM>IgG production	
Marginal zone B cells	noncirculating, immune defense against systemic blood-borne antigens,	[33, 34]
Follicular B cells	function as a guidance system for mature resting B cells in peripheral lymph nodes	[35]
Memory B cells	immunological memory	

The complement system

The complement system is an evolutionarily ancient defense system. The complement system is next to the coagulation system, the kinin system, and the fibrinogen system as a triggered enzyme cascade. About 30 proteins in plasma, the extracellular matrix and on the cell surface are involved in complement activation, regulation and its effector functions. Since it is part of the innate immunity it can be activated within minutes after contact to foreign cells or immune complexes. Therefore mannan binding lectin recognizes surfaces of microorganism or C1q binds to antigen-antibody complexes. Products of the activated complement system are involved in opsonization of cells for phagocytosis and lysis of microorganism, foreign cells or cell waste. Thereby a central aspect of complement is the disposal of microorganism and foreign substances, but also the removal of immune complexes and apoptotic/necrotic cells to keep the body's homeostasis. Furthermore complement acts in the coordination of the cellular immune response and thereby bridges the innate and adaptive immune response.

Activation of the complement system

The activation of the complement system occurs by three different pathways- the alternative, the mannan binding lectin and the classical pathway.

The alternative pathway

The alternative pathway (AP) is activated spontaneously by conversion of soluble C3 to C3(H_2O), which results in the formation of an reactive thioester. Consequently C3(H_2O) together with the Factor B cleavage product Bb generate the initial C3 convertase (C3(H_2O)Bb), that activates C3 into C3b and the anaphylatoxin C3a. C3b bind close to the site of its generation covalently to nucleophiles at cell surfaces, immune complexes or carbohydrates. Deposited C3b forms the alternative C3 convertases (C3bBb), which induces a positive feedback loop of the cascade and thus initiates the amplification of C3b on the surface. Not deposited C3b is inactivated within a split second by water molecules.

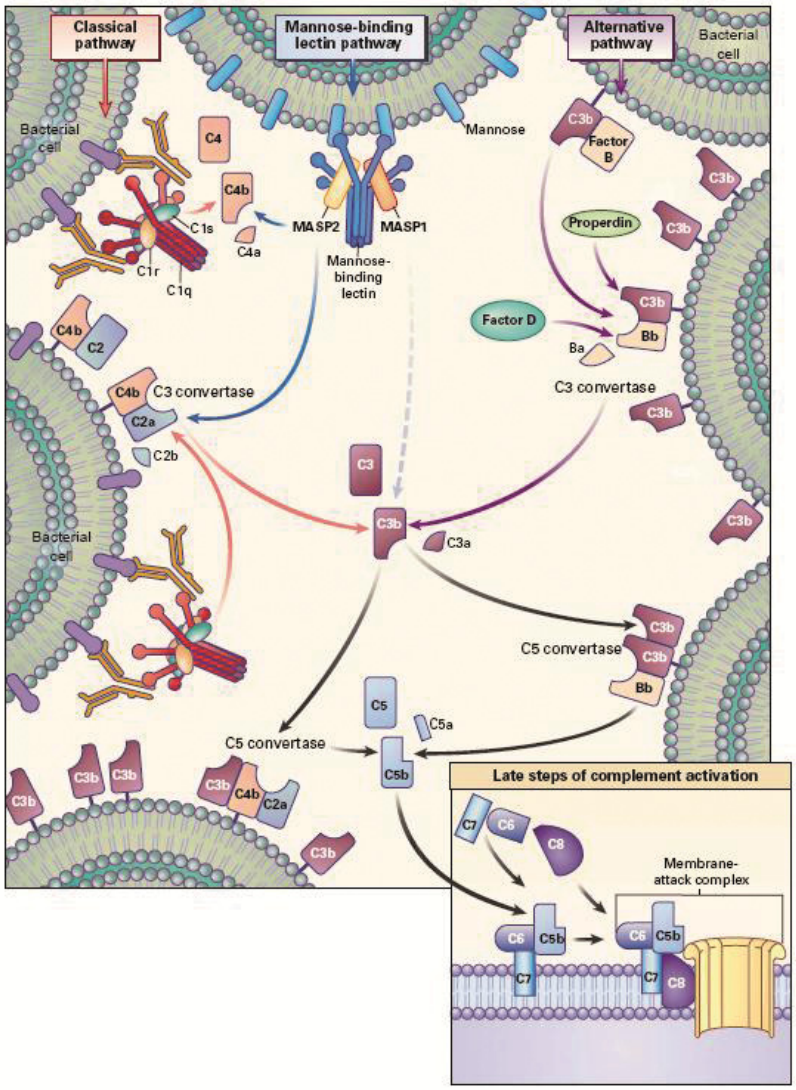


Figure 2: complement activation pathways[36]

Complement activation occurs via three pathways- the alternative, the mannan binding or lectin or the classical pathway. Activation of the alternative pathway happens spontaneously by binding of C3b to hydroxyl groups on carbohydrates on the surface. The mannan binding lectin pathway is initiated by a complex of mannan binding lectin, the mannan binding lectin associated proteases (MASP 1/2) and mannans on the surface of a bacterial cell. The classical pathway is activated by the binding of C1 to antibodies, which detects antigens on bacterial cells. All pathways converge in the generation of the C3 convertase. The C3 convertase cleaves C3 into C3b and C3a. C3b binds the C3 convertases to form the C5 convertase. The C5 convertase cleaves C5 in C5b and C5a. C5b initiates the terminal pathway. C5b binds C6, C7, C8 and several molecules of C9 to form the lytic membrane attack complex in bacterial membranes.

The classical pathway

The classical pathway (CP) is induced by binding of C1q, which is the sensor domain of C1, to immunoglobulins, pentraxins, polyanions, viruses, marker of damaged cell, prions or amyloid[37-41]. Consequently C1 conformation change and C1 component r cleave C1s. The activated C1s display an active serine protease, which cleaves C4 in C4b and the

anaphylatoxin C4a[42]. C4b binds to hydroxyl groups on surfaces and recruits C2, which is also cleaved by C1s in C2b and C2a Cleavage products C4b and C2a result in the active C3 convertase (C4bC2a) of the classical pathway.

The mannan binding or lectin pathway

The mannan binding lectin pathway (MBL) is initiated by the binding of mannan binding lectin to carbohydrates on the surface of microorganisms. This binding activates the mannan binding lectin serine proteases MASP1 and MASP2, which are homolog to C1r and C1s. MASP1 cleaves MASP2. Subsequent MASP2 cleaves C4 in C4b and the anaphylatoxin C4a. C4b recruits C2 and MASP2 cleave C2 in C2b and C2a. The major fragments C4b and C2b form the central C3 convertase C4b2b.

The terminal pathway

The C3 convertases of the alternative, the classical and the mannan binding lectin pathway cleave C3 in to C3b and C3a. Freshly generated C3b forms with the central C3 convertases the C5 convertases (C3bBbC3b, C4b2a3b) on surfaces, which cleave C5 into C5b and the anaphylatoxin C5a. C5b can initiate the terminal pathway by an aggregation between C5b, C6 and C7. The C5b-C7 complex integrates into membranes in cause of its hydrophobicity and the ability to bind phospholipids. C5b-C7 binds the eight component of complement and generate the C5b-C8 complex to penetrate into the cell membrane. Finally the C5b-C8 complex recruits up to 17 C9 molecules. Complexed C9 forms the pore of the so called membrane attack complex (MAC)[43]. MAC formation in membranes leads to a loss of cell homeostasis. The cell leaks cytoplasma and ions, which destabilize membrane potential. Moreover extracellular proteases attack the damaged cell. Consequently the cell lyses.

The complement system and it`s biological roles

(1) The induction of the terminal complement pathway results in the formation of the membrane attack complex (MAC). The MAC builds pores in membranes, such as gram $^-$ bacteria or unprotected human cells. Gram$^+$ bacteria, as well as fungi are not attacked by the membrane attack complex as the cell wall is to thick to be penetrated by complement derived pores. Pore formation in membranes destabilized the osmotic balance and cause efflux of essential ions and nutrients. The terminal complement complex (TCC) is also formed in fluids like the plasma. This sublytic complex plays a role in inflammation by cell

proliferation and by rescuing apoptotic cells[44, 45]. C5b-9 initiate Ca^{++} influx and the generation of second messenger molecules, such as cAMP, inositol phosphate mediates or arachidonate metabolites during interaction with specific cell types[46] (figure 5).

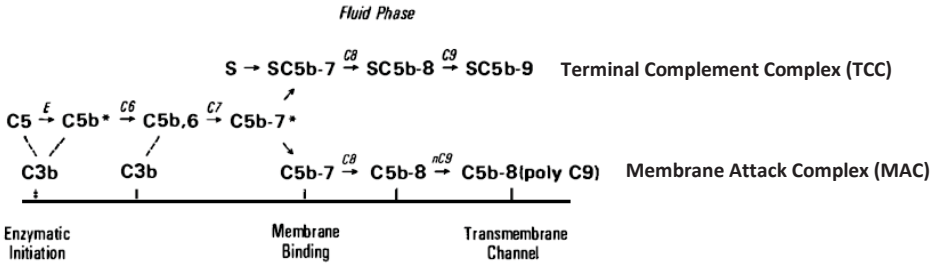


Figure 3: formation of soluble TCC and membrane bound MAC[43]

The terminal pathway of complement activation ends in the formation of either the soluble (TCC) or a membrane bound terminal complement complex (MAC). The MAC builds transmembrane channels, which results in the lysis of the cell.

(2) Furthermore activated complement system leads to the deposition of complement on surfaces and therefore to the opsonisation for phagocytes. The complement derived proteins C3b, iC3b, C4b, C1q and MBL acts as opsonisins on phagocytes. C3b and C4b bind to the complement receptor 1 (CR1) on erythrocytes, neutrophils and macrophages and induces phagocytosis[47]. The fragments iC3b and C3d of the third complement component bind to complement receptor 2 (CR2) on B cells and trigger Ig class switching and the memory function. A bound C3d molecule enhances the response to an antigen by about 20-fold. The enhancement is mediated by crosslinking of CR2 with IgM, thus CR2 amplifies signals and triggers a distinct signalling pathway[48]. The complement fragment iC3b is also recognised by complement receptor 3 and 4 (CR3/CR4) on the surface of monocytes/macrophages and neutrophils. And leads to phagocytosis of the opsonised particles either independently or in combination with the F_c receptor[47, 49,50]. Moreover complement opsonisation plays an important role in immune complex clearance from the circulation. The complement components C3b and C4b opsonise immune complexes, which results in the detection of the complexes by CR1 on the surface of erythrocytes. Erythrocytes transport the immune complexes in the liver or spleen where they give them up to phagocytes for destruction[51].

The q domain of the first component of the classical pathway binds C1q receptors on the surface of macrophages and induces phagocytosis. In addition the mannan binding lectins of the MBL pathway bind MBL receptors on phagocytes and likewise initiate phagocytosis.

(3) During complement activation the complement effector peptides C3a, C4a and C5a are formed. These complement fragments have a wide range of proinflammatory and antimicrobial activities. The complement effector peptides C3a, C4a and C5a act as potent chemoattractiv and anaphylatoxic agents. The active peptides activate endothelial cells and increases expression of adhesions, such as E-selectin, the intracellular adhesion molecule 1 or the vascular cell adhesion molecule 1 on these cells[52]. Furthermore C3a, C4a and C5a initiate vasodilation to support the migration of lymphocytes through the endothelium and have spasmogenic on smooth muscles.

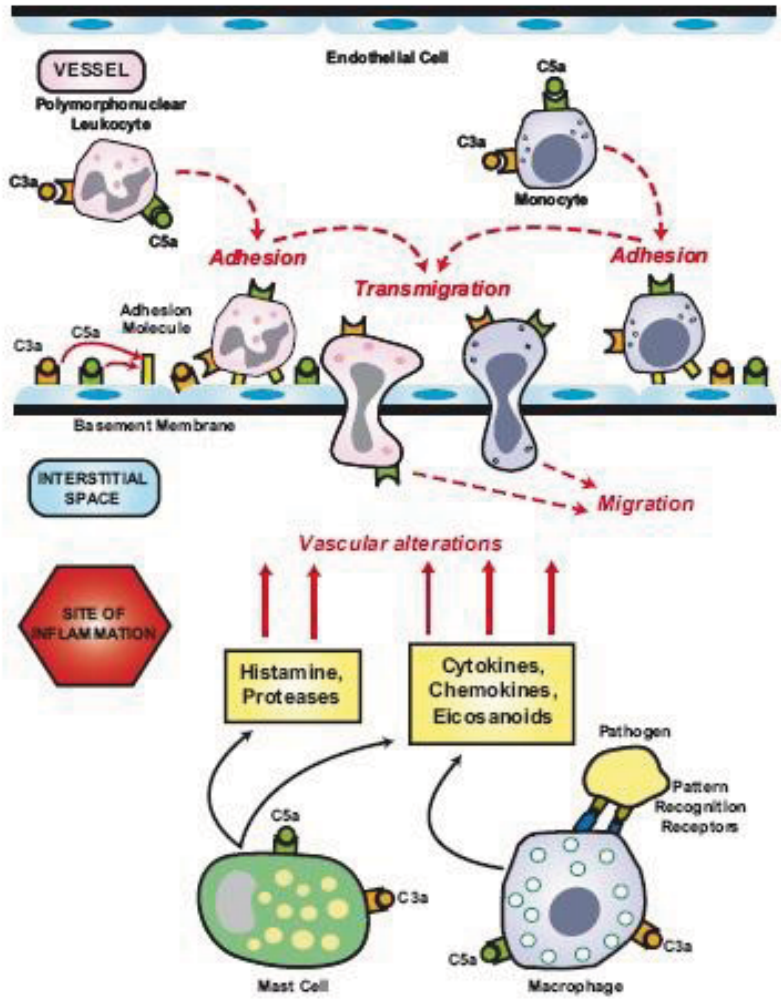


Figure 4: C3a and C5a mediated extravasion of lymphocytes[53]

The anaphylatoxins C3a and C5a increases leukocyte adhesion to the endothelium and transmigration into the interstitial tissue. In addition C3a and C5a stimulate mast cells to release histamine and proteases, which mediate vascular alterations. Macrophages recognize pathogens and induce innate immune response causing production of proinflammatory cytokines and chemokines, as well as antimicrobial eicosanoids.

C3a and C4a, but not C5a displays antimicrobial activity. Effects of C3a on gram$^-$ bacteria *E. coli* and *P. aeruginosa* were comparable to the potent keratinocyte derived peptide LL-37[54].

C3a receptors were identified on the surface of polymorphnuclear cells and monocytes[55]. C3a and C5a act on the body homeostasis. C5a mediates organ regeneration and neuroprotection, whereas C3a assists the release of progenitor haematopoietic stem cells[56-58]. The cleavage product C5a binds C5aR and C5aL2, which are expressed on phagocytes and mast cells. C5a activated mast cells release histamine and activated phagocytes to express cytokines and chemokines, like IL-1 and TNF α[59, 60]. C5a activates the arachidonic acid metabolism in neutrophils and monocytes, which lead to the production of antimicrobial acting eicosanoid. In addition C5a is a potent chemo attractant for phagocytes[36, 53] (Figure 4).

(4) Since complement acts onB cell- and T cell immunity, the linkage between innate and adaptive immunity by the complement system demonstrated[61, 62].

Regulators of the complement system

Activation of the complement system normally leads to the removal of modified host cells, microorganisms and immune complexes, whereas host cells are protected against the complement attack. To keep this in balance several complement regulators have to protect self cells and tissues by fine adjusting activation and inhibition of complement. No or defective complement regulators ends in host cell damage and the inefficient removal of apoptotic cells and immune complexes causes secondary necrosis and pathology. Thus complement has to be carefully regulated. Regulators act mainly on four central steps in the complement cascade. They interfere in complement initiation (C1INH), in C3 convertase activation and C3b amplification (Factor H, FHL1, C4BP, Properdin), C5 convertase activation (CFHR1), in TCC activation and MAC formation (clusterin and vitronectin) and control the anaphylatoxins C3a, C4a and C5a (Carboxypeptidase N)[63]. Figure 6 displays action of soluble regulators within the complement system.

Next to soluble complement regulators, surface bound regulators prevent self destruction by activated complement and control phagocytosis, leukocyte recruitment and inflammation. The complement regulators C1qR and SIGNR1 interact with C1q of the classical pathway and mediate cell adhesion, phagocytosis and inflammation[64-66]. The complement receptors (CR) CR 1-CR 4 interact with C3 fragments (C3b, C3dg, C3d and iC3b), C4b, C1q and Factor H to control C3b amplification, to enhance adhesion and phagocytosis and to regulate B cell function (CR2)[48, 63] . Cell bound complement receptor of the immunoglobulin family (CRIg) acts in phagocytosis, T cell activation and control the alternative pathway of complement

activation[67]. The membrane cofactor protein (MCP) and the decay accelerating factor (DAF) interfere in C3b inactivation and accelerate the decay of the C3 convertase[68]. Cell bound protectin inhibit the formation of the TCC. Also the anaphylatoxins C3a and C5a bind to receptors like C3aR, C5aR and C5L2 to control immune cell recruitment and inflammation[63].

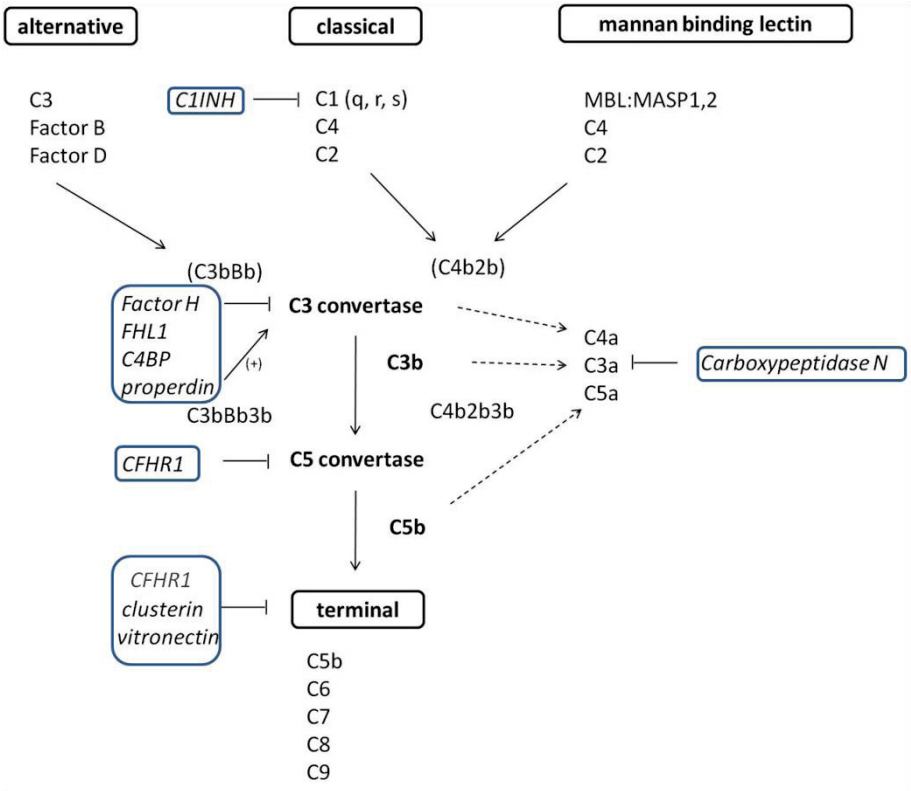


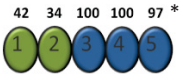
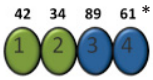
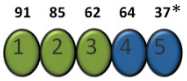
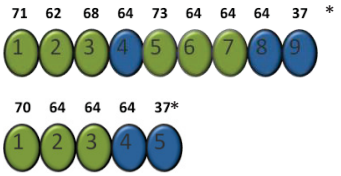
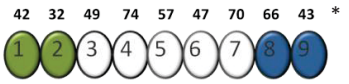
Figure 5: soluble regulators control the complement system

Soluble complement regulators act in different steps of the complement activation. C1 inhibitor (C1INH) blocks the serine protease C1s and thus controls the initiation of the classical pathway[69]. Factor H and FHL1 act as cofactor for Factor I in C3b inactivation and accelerate the decay of C3 convertase and thus prevent amplification of C3b of the alternative pathway. C4BP exhibits same function like Factor H, but controls C3 convertase of the classical and mannan binding lectin pathway. Properdin stabilizes C3 convertase of the alternative pathway and thus acts as an positive regulator of complement activation[70]. Clusterin and vitronectin interfere with components of the terminal pathway and avoid membrane integration and pore formation of the TCC[71, 72]. Carboxypeptidase N inactivates the anaphylatoxins C3a, C4a and C5a, which are generated during complement activation[73].

Factor H family

The alternative pathway of complement activation is initiated spontaneously and continuously. To avoid complement activation on self surfaces, this pathway has to be strictly controlled. Major regulators of the alternative pathway are the members of the Factor H family. Best characterized is Factor H and the Factor H like protein 1 (FHL1). Five Factor H related members are identified (CFHR 1-5).

Table 3: Factor H family: ligands, functions and structure

protein	ligands	function	structure
CFH	C3b, C3c, C3d, heparin, CRP, microbial ligands	C3b inactivation, acceleration of C3 convertase decay, cell adhesion, enhances neutrophil adhesion and antimicrobial response	
FHL1	C3b, heparin, CRP, microbial ligands	C3b inactivation, acceleration of C3 convertase decay, cell adhesion	
CFHR1	C5b, microbial ligands	inhibits C5 convertase and TCC formation, enhances neutrophil adhesion and antimicrobial response	
CFHR2			
CFHR3	C3b, C3d, heparin	enhances FH cofactor activity	
CFHR4	C3b, C3d, CRP, necrotic cells	enhances FH cofactor activity, opsonise necrotic cells by binding CRP	
CFHR5	C3b, heparin, CRP	weak C3b inactivation, acceleration of C3 convertase decay	

* Numbers represent amino acid homologies of the individual SCR domains to that of Factor H and are given in [%]

All members of the Factor H family are mainly produced in the liver and circulate in the human plasma. They are structurally composed of loop forming domains termed short consensus repeats (SCR). The *Factor H* and *CFHR* genes are located in the *Factor H* gene cluster on chromosome 1q32. Amino acid homologies of the CFHRs to Factor H are labeled within the structures in Table 3.

Complement Factor H is the best characterized member of the Factor H family. This protein is composed of 20 SCRs, in particular the SCRs 1-4 are defined as the N-Terminus and

SCRs 19-20 as the C-terminal region of the protein. Using deletion mutants of Factor H, the N-Terminus of Factor H were shown as region, which exhibits regulatory properties, such as cofactor activity for Factor I and C3 convertase decay accelerating activity. The C-Terminus binds to several ligands, like C3b, C3d, heparin or cell surface glycosaminoglycans and thus can discriminate between self and foreign surfaces[74]. Binding of Factor H to human cells regulates complement activation on self cells and thus avoids imbalance and secondary necrosis of misspent complement. The C-Terminus of Factor H is highly conserved within the members of the Factor H family, suggests similar functions of the C-Termini of CFHRs. Next to its complement regulatory function Factor H bind as an adhesion ligand to the complement receptor 3 (CD11b/CD18) on human neutrophils[75]. Increased attachment of *C. albicans* to neutrophils confirmed the binding of Factor H and also show binding of FHL1 and CFHR1 to CR3 on neutrophils. Furthermore binding of Factor H and CFHR1 enhance release of reactive oxygen species and the antimicrobial acting lactoferrin from neutrophils[76]. **FHL1** consists of the first seven SCRs of Factor H and additional four amino acids on the C-Terminus. The RGD motif in SCR 4 mediates cell adhesion and facilitates Ca^{++} dependent cell spreading of fibroblast[77]. **CFHR1** regulates complement activation on the level of C5 convertase by inhibiting C5b cell surface attachment. Subsequently MAC formation in membrans is avoided[78]. **CFHR3** enhance complement regulatory activity. **CFHR4** binds necrotic cells and the acute phase protein C-reactive protein (CRP) in its native pentameric form. CFHR4 bound CRP activates complement by the classical pathway, as determined by C3b fragment deposition, indicating a role of CFHR4 in opsonization of necrotic cells[79, 80]. **CFHR5** enhance like CFHR3 the complement regulatory activity. **CFHR2, CFHR3 and CFHR5** are poorly described. Functions in complement regulator competition on surfaces, but also independent functions of this proteins are under investigation[81].

The human skin

The human skin builds a protective shield against chemical and microbial agents, thermal and electromagnetic radiation and mechanical trauma to keep the bodies homeostasis[82]. The human skin is consists of two main compartments-the outer epidermis and the inner dermis. The epidermis contains four main strata, the stratum corneum, stratum granulosum, stratum spinosum and stratum basale. The outermost stratum corneum mainly has barrier function to resist toxic agents and prevent dehydration. It comprises corneocytes, which are

dead keratinocyte derived cells that are devoid of organelles. The stratum granulosum is characterized by dark clumps of cytoplasmatic material; the cells produce keratin and lipids. The polygonal cells of the stratum spinosum (also known as pickle cell layer) start to maturate or divide to regenerate the cells of the stratum basale. The columnar cells of the stratum basale renew the cells of the whole epidermis. They produce keratins, which are different to the keratins of the stratum spinosum. The dermis contains broader cell diversity and is anatomical divers. Next to immune cells, fibroblasts and nerve related cell types, the dermis is drained with lymphatic and vascular vessels.

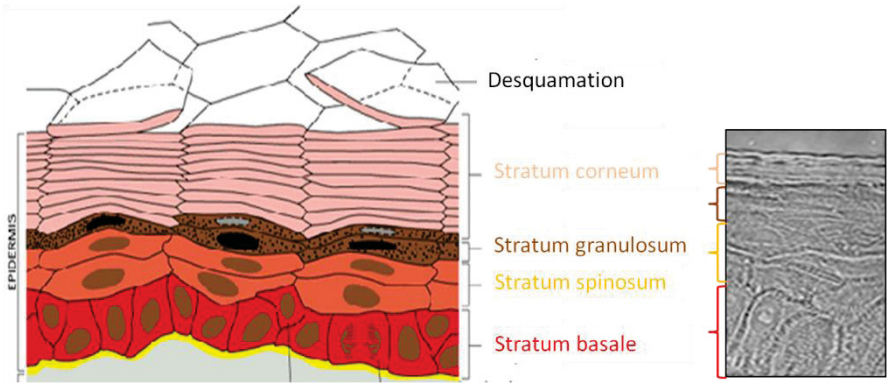


Figure 6: layers of the skin[196]

The epidermis contains four main strata-the stratum corneum, stratum granulosum, stratum spinosum and stratum basale. The stratum corneum consists of keratinized squames. The following granular cells form the stratum granulosum. The polygonal cells of the stratum spinosum border on the columnar cells of the stratum basale.

The immune system of the skin

Streitlein described first in 1983 the skin as an immunological active tissue containing individual immune cells, which continually traffic between the skin, the draining lymph nodes and the circulation. He summarized his concept as skin-associated lymphoid tissue (SALT)[83]. Later on Bos improve this concept and the term skin immune system become accepted[84]. Different resident and circuiting immune cells in the skin were identified. Specialiced cells of the epidermis, e.g. keratinocytes, malanocytes, Langerhans cells and $CD8^+$ T cells were detected in the stratum basale and the stratum spinosum. The dermis immune cells include DCs, $CD4^+$ T helper cells, γδ T cells, (i) NKT cells, as well as macrophages, mast cells, fibroblasts and nerve related cell types. Table 4 summerizes skin immune cells, their surface markers and functions.

Main cell type of the epidermis is the keratinocyte. Keratinocytes sense pathogens and danger signals by expressing pathogen recognition receptors (PRR) on their surface. Epidermal keratinocytes express toll like receptors TLR 1, TLR 2, TLR 4, TLR 5 and TLR 6 and on the endosomes TLR 3 and TLR 9[85, 86]. TLR 7 can be induced by dsRNA-TLR 3 complexes on endosomes[87]. Activation of the TLR results in a predominant T_H1-type immune response and to the release of type I interferons. Keratinocyte expressed nucleotide binding leucine rich repeat containing family proteins (NLR) recognizes pathogen and danger associated molecular patterns and results in the activation of the inflammasome (complex of NLR, an adaptor protein named ASC (apoptosis associated speck-like protein containing a caspase recruitment domain) and procaspase 1. Activated Caspase 1 cleaves pro IL-1β and pro IL-18 to generate active proinflammatory cytokines, which activate tissue resident immune cells. In addition keratinocytes potently release antimicrobial peptides (AMP), like β defensins, cathelicidins (LL-37). Under inflammatory conditions T cell derived cytokines (T_H17 response induced IL-17A, IL-22) increase the production of AMPs by keratinocytes[88]. Next to AMPs, keratinocytes produce numerous cytokines, like IL-1, IL-6, IL-10, IL-18 or TNF and chemokines, like CC-chemokine ligands 9, 10, 11 and 20 to attract effector T cells, CX-chemokine ligands 1 and 8 (IL-8) to recruit neutrophils and CCL 20 to regulate the trafficking of Langerhans cells. The linkage between innate and adaptive immune system in skin is provided by non professional antigen presentation via MHC class II molecules on keratinocytes. Thus keratinocytes provide signals for T cell proliferation, induce functional response in epitop specific $CD4^+$ and $CD\ 8^+$ memory T cells, process antigens to present it to $CD4^+$ T cells to initiate T_H1 and T_H2 response and to $CD8^+$ T cells to initiate lysis and cytokine release[89]. Keratinocytes also induce T cell anergy and tolerance[90].

The main dentritic cell (DC) types in the epidermis are Langerhans cells, which include characteristic Birbeck granule and CD 1a on the surface. Langerhans cells process lipid and microbial proteins to T-cell and induce T_H2 response and prime CD naive $CD8^+$ T cells. Inflammatory dentritic epidermal cells (IDEC) express the macrophage mannose receptor CD 206 and overexpress FcεRI[91]. Dermis derived DCs and macrophages induce T cell proliferation, and sense pathogens by the expression of TLR 2, 4, CD 206 and CD 209. Activated DC secretes cytokines and chemokines. TNF or inducible Nitric oxide synthase (iNOS) producing dentritic cells are known as TIP or IDC[92]. The early induction of plasmacytoid dentritic cells (pDC) leads to the initiation of innate immune response,

activation of myeloid DCs and consequently to an induced adaptive immune response[93]. Stressed skin cells release self DNA. Self-DNA in complex with the antimicrobial peptide LL-37 triggers TLR 9 activation in pDC, resulting in IFN-α production and in activation of the adaptive immune response[94]. Skin Macrophages, which express the scavenger receptor CD 163 and the coagulation cascade component Factor XIIIa, stay sessil, but migrate under inflammatory conditions to the lymph nodes.

Another important group of immune cells are T-cells. Next to memory T-cells and cytotoxic $CD8^+$ cells, $CD4^+$ T_H cells that produce T_H1, T_H2 and T_H17 response are identified. T_H1 response to intracellular infections leads to lymphotoxin and IFN γ production, and the activation of macrophages. T_H17 response is essential for the defense against bacterial and fungal infections[88]. T_H17 cells derived cytokines may link immune and epithelial cells to support immune response to skin infecting pathogens, e.g. IL-17 and IL-22 increase LL-37 production by keratinocytes[95]. Unconventional T cell subsets like γδ T cells and (invariant) NKT are also identified in the skin. The functional role of these subtypes is less described. Γδ T cells have been shown to produce growth factors and may contribute to the production of AMPs[96, 97]. Invariant NKT recognize bacterial glycopeptides. The presentation of self derived glycopeptides by CD1d restricted NKT cells may contribute to keratinocyte activation[98].

Table 4: skin immune sentinels

cell types	location	Main surface markers	function	literature
Keratinocytes	Epidermis	CD 1d^{+}	Immune recognition by TLRs, DC and T cell regulation and induction, neutrophils recruitment, nonprofessional APCs, antimicrobial protein (AMP) release	[56-61]
Langerhans cells	Epidermis	CD 1a, CD 207, MHC class II	APC, possible immune tolerance induction	[62]
Inflammatory dentritc epidermal cells (IDEC)	Inflamed epidermis	CD 11b, CD 23, CD 206, FcεRI, IgE, MHC class II	Antibody-allergen response	[63]
Dermal DC	Dermis	CD 1a, CD 1c, CD 206, CD 209, MHC class II	APC, cytokine and chemokine secretion	
Inflammatory DC (TIP DC)	Inflamed dermis	CD 11c	TNF, NO production	[63]
Plasmacytoid DC (pDC)	Dermis	CD 45Ra, CD123, CD 303, MHC class II	AMP, pro and anti inflammatory mediator secretion, IL-6 and TNF dependent T_H22 induction	[64,65]
macrophages	Dermis	CD 163, CD16, CD32, CD 64, factor XIIIa	IFN-α production, self DNA-LL 37 complex recognition	
CD 8^{+} T cells	Epidermis	CD 2, CD 3, CD 5,CD 8, TCR	"cytotoxic", kill antigen bearing target cells, effector cells in graft rejection	
Memory T cells	Epidermis, dermis	Cutanous lymphocyte antigen (CLA, CD 4 or CD 8	Skin-homing	[82]
CD 4^{+} T_H1 cells	Inflamed dermis	CD 2, CD 3, CD 4,CD 5, TCR	"inflammatory", IFN-γ, lymphotoxin production, macrophage induction, intracellular pathogen killing, promote skin DTH reactions	[88]
CD 4^{+} T_H2 cells	Dermis	CD 2, CD 3, CD 4, CD 5 TCR	"helper", Stimulation of B cell proliferation and differentiation, produce IL-4 and IL-5, IL-13,IL-25 clearance of helminthes	[88]
CD 4^{+} T_H17 cells	Dermis	CD 2, CD 3, CD 4, CD 5 TCR	defense against fungal and bacterial infections, IL 17, IL 22, GN-CSF, Il 6 release, AMP induction in keratinocytes	[88]
γδ T cells	Dermis	CD 3, γδ-TCR	NK like "cytotoxicity", growth factor expression, AMP production, NKG2D dependent skin cancer regulation	[99 100]

Invariant NKT cells (iNKT)	Dermis	CD 1d restricted TCR, CD 161, NK associated receptors (CD 56,NCAM-1, NKG2D)	Bacterial glycolipid recognition, clearance of infected cells by perforin pore formation and granzyme induced apoptosis, AMP production, keratinocyte induction, TNF dependent regulation of DC trafficking via α-galactosylceramide activated iNKT,	[101]
melanocyte	Epidermis		Produce melanin	
Mast cell	Dermis	CD 34, FCεRI	Pathogen defense, wound healing, anaphylaxis, allergy by release of granule molecules (histamine, serine protease, serotonin, proteoglycans), eicosanoids (prostaglandine, leukotriene, PAF), cytokines	[102, 103]
fibroblast	Dermis		Wound healing, secrete ECM precursors, growth factors, chemokines	[104]

The complement system in skin

The complement system plays a crucial role in the defence against invading microorganism, in immune recognition and in activation of innate and adaptive immune components. Althought secretion of complement components and receptors by keratinocytes and Langerhans cells is described, less is known about the function of the complement system in skin[105].

Human keratinocytes constitutively expressed C3, C5, C7, C8γ and C9 mRNA, whereas C6, C8α and C8β mRNA were not detected. They secrete C3, C7 and C9, but not C5, C6 and C8, thereby C9 production is upregulated by TNF-α [106]. In addition keratinocytes express Factor B, Factor I and the complement regulator Factor H [107,108,109]. However, Langerhans cells and macrophages are shown to express C3 receptors[110].

Activation of the complement system results in lysis of gram negative bacteria by formation of the membrane attack complex (MAC) and formation of reactive complement fragments, which exhibit function as opsonisins, anaphylatoxins and antimicrobial substances.

The complement fragments C3a and C5a were formed during complement activation of the AP/CP and act as potent chemo attractive and anaphylatoxic agents. C5a binds C5aR and C5aL2 which are expressed on phagocytes, mast cells, Langerhans cells, melanocytes and under inflammatory conditions on keratinocytes. C5a activated cells release cytokines and

chemokines, which attract phagocytes to the site of infection. In addition C5aR stimulation on DC induces T_h1 response and MHC II upregulation to enhance antigen presentation. C3a and C4a, but not C5a displays antimicrobial activity. Effects of C3a on gram$^-$ bacteria *E. coli* and *P. aeruginosa* were comparable to the potent keratinocyte derived peptide LL-37.

Overview of dermatophytes

Introduction dermatophytes

Dermatophytes are defined as fungi, which causes superficial infections in humans and animals. These fungi belong to the phylum of Ascomycota and are dedicated in the anamorph classification of *Trichophyton*, *Microsporum* and *Epidermophyton*. Teleomorph species related to the genus *Arthroderma*. Morphological, growthing of dermatophytes occur filamentous. Dermatophytes were transmitted by direct contact to infected skin of humans and animals, like rodents, dog or horses. In addition infection is transmitted indirectly by infected exfoliated skin in clothing, combs and shoes. Infections termed Tinea or ringworm. Infections of the nails are known as onychomycosis. Since dermatophytes nutrilize keratin, infections usually limited in the upper layer of the skin- the stratum corneum.

Immune response to a dermatophyte infection

The immune system reacts on a dermatophyte infection with a cell mediated response of the delayed type hypersensitivity (DTH). Main players of the DTH are macrophages and some key cytokines, like Interferon-γ (IFN-γ). CD 4^+ T cells get activated by antigen presentation via MHC class II molecules and respond with a T_H1 immune answer. Thus the proinflammatory cytokine IFN-γ is released and activate macrophages. The DTH reaction is associated with lower titers of IgG directed toward dermatophyte antigens and the absence of IgE and IgG4[111]. DTH response leads to the clearance of dermatophyte infections[112]. In contrast chronic dermatophyte infections were associated with immediate hypersensitivity (IH) reactions[113]. IH response results in less inflammation and a predominantly T_H2 response. T_H2 mediated release of IL-4 activates B-cells to switch the isotype of the antibodies to IgE and IgG4. The binding of antigen to IgE on the surface of mast cells leads to cross-linking of IgE, the degranulation of mast cells and following release of proinflammatory mediators and histamine[114].

Innate immunity response is instrumental for the cell mediated response. Phagocytes ingest fungal cells or inhibit fungal growth, trigger inflammation and present fungal antigens to T-cells. The interactions of dermatophytes and macrophages were analysed concerning morphological and immunological changes. Macrophage derived anti inflammatory cytokine IL-10 and TNF-α, which favours the growth of intracellular pathogens was upregulated under infectious conditions. Simultanous class II MHC, CD54 and CD80 costimulatory molecules, nitric oxid and IL-12 were downregulated[115]. After the intracellular differentiation of conidia into hyphae inside of macrophages, cell membrane breaking causing the death of this immune effector cells. Dense infiltration of neutrophils in infected areas of the skin were detected[116]. Consequently the respiratory burst and phagocytosis is enhanced during dermatophytosis. Even so long periods of neutrophil incubation with *T.rubrum* resulted in increased fungal viability[117]. Humoral components, like the complement system get activated during incubation with dermatophytes. Incubation of human serum with *T.rubrum* increases chemotaxis and adhesion to neutrophils. Consequently complement-opsonized spores, but not hyphae were killed by neutrophils[118]. Dentritic cells modulate and initiate immune response in skin. Immature DCs process antigen, get activated by LPS, cytokines, like IL-1β, GM-CSF and TNF-α and thus migrate to the lymph nodes and the spleen to activate naive antigen specific T cells. During maturation process DCs upregulate IL-12 release, which induce cell mediated response to intracellular pathogens by NK and T cell mediated INF-γ production[119, 120].

To understand pathophysiological mechanisms underlying an infection, interactions between fungal cells and keratinized tissues were examined. Immune recognition of fungal cells, like *C.albicans* occur via a TLR 2-dependent mechanism. It remains to be determined whether dermatophytes also activate TLR 2. Dectin-1 was shown to be expressed on DC and macrophages and mediates cellular response to conidia by inducing pro-inflammatory cytokines[121]. Sato et al showed that dectin-2 binds fungal hyphae, including *T. rubrum* hyphae, which results in upregulation of the expression of TNF-α amd IL-1 receptor antagonist[122]. Human keratinocytes react with the release of IL-8 in the presents of dermatophyte antigens like Trichophytin[123]. The cytokine IL-8 induces the accumulation of neutrophils in the stratum corneum.

Dermatophyte virulence factors

Pathogenesis of dermatophytes is poorly described. Some virulence factors contribute to the adhesion process and to immune modulation of the host cellular response during infection are defined.

Adhesion to epithelial cells is mediated by *T.rubrum* expressed carbohydrate specific adhesins on the surface of microconidia [124]. *T. mentagrophytes* adhesion may contribute to the development of long, sparse fibrilis, which connecting the fungus to the keratinocytes[125]. *Trichophyton spp*. and *M.canis* produced dipeptidylpeptidase IV (DppIV) could act like *P. gingivalis* DppIV in binding of fibronectin and thus mediate adhesion[126].

Genes for thioredoxin and and cellulose homologue in *T. mentagrophytes* were upregulated during growthing on medium supplemented with skin extracellular matrix proteins. Thioredoxin could activate fungal or host proteases or act against host derived reactive oxygen species[127].

T. rubrum cell wall mannans (TRM) inhibit lymphoproliferative response of mononuclear leukocytes in response to dermatophyte derived antigens and mitogens and bind selectively to human monocytes[128, 129]. Contrary TRM are also the major T-cell antigen[130]. In addition TRM may inhibit the turnover of the stratum corneum[131].

Dermatophyte secreted proteases

To utilize the keratin of the stratum corneum as a nutrient, dermatophtes secrete keratinolytic proteases, like multiple subtilisin- (serine) and fungalysin- (metallo) endoproteases[132]. Essential for the degradation of keratinized tissues is the reduction of the disulphide bridges of the insoluble protein network, consisting of cysteine rich loricrin and prolin containing proteins by sulfite efflux pumps encoded by the *ssu1* gene[133]. Sulfite excretion leads to sulfitolysis of the proteins and make the accessible to the activity of fungal proteases[134].

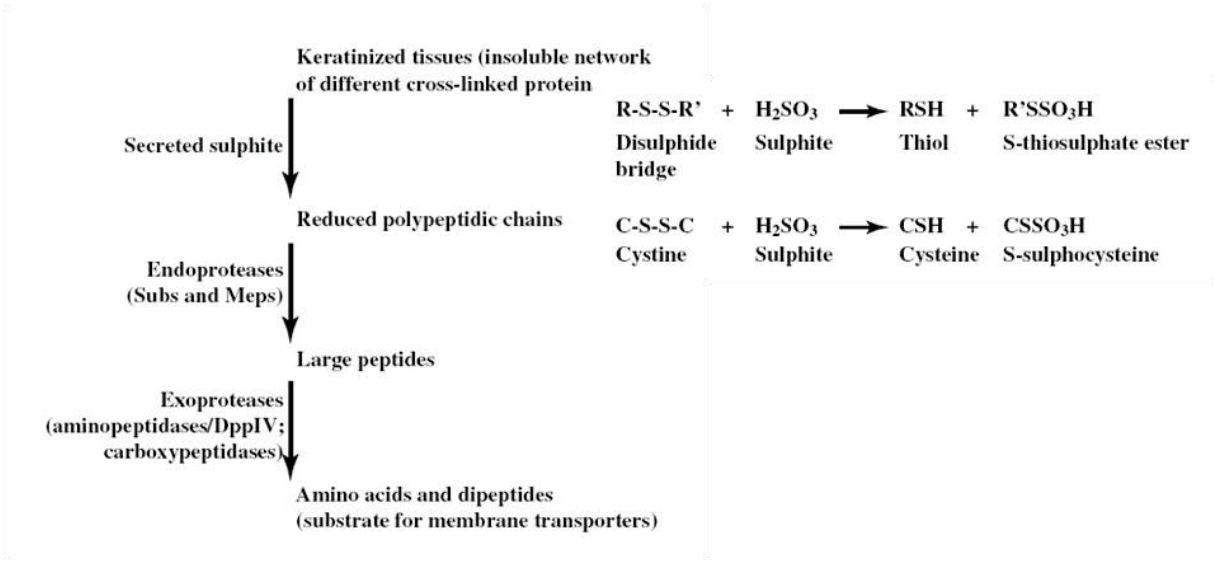


Figure 7: keratinized tissue degradation by dermatophytes[133]

Dermatophytes excrete sulphite to reduced disulphite bonds within the insoluble keratinized protein network. Reduced polypeptide chains were attacked by dermatophyte derived endo- and exopeptidases to make amino acids and dipeptides susceptible for nutrilisation.

Dermatophyte secreted endo- and exoproteases were encoded by 20 genes, not less than 15 encoded proteases are secreted in vitro during growth on protein medium. Next to endoproteolytic acting seven subtilisins (SUB) and five metalloproteases (MEP), also exoproteolytic acting two leucine aminopeptidases and two dipeptidyl-peptidases were identified as dermatophyte secreted proteases (Table 5).

Some secreted proteases of dermatophytes, like Subtilisin 3 (Sub 3) and metalloprotease 3 (Mep 3) from *M. canis* induce specific immune response[135, 136]. The protease antigens Tri r2 and Tri t4 elicit delayed-type or immediate hypersensitivity response. Tri r2 antigen is part of the SUB 6 protease of *Trichophyton* and Tri t4 was extracted from *T. tonsurans* mycelium and is the orthologue of DppV from *A. fumigatus* and *T. rubrum* [111].

Table 5: dermatophyte secreted proteases

Protease family[a]	Genes[b]	proteases	Molecular mass	substrate	inhibitor	references
M36	*Mep1-5*	Mep 1,3,4	40-48 kDa Zn dependent		Phosphoramidon, EDTA,	[137]
S8A	*Sub 1-7*	Sub 2-7	30-37 kDa, not glycosylated	N-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (synthetic)	PMSF, chymostatin	[138, 139]
S9B	*DPPIV*	DppIV	90 kDa N-linked carbohydrates	X-Pro, X-Ala	Lys-[Z(NO_2)]-pyrolidide, Lys-[Z(NO_2)]-thiazolidide	[140]
S9C	*DPPV*	DppV		X-Ala		
M28E	*LAP1*	Lap 1	33 kDa Non glycolsylated	Leucine-7-amido-4-methylcoumarin	Sensitive to different ions	[140]
M28A	*LAP2*	Lap 2	58-65 kDa			
M14	*MCPA, MCPB*	McpA, McpB				[133]
S10	*SCPA, SCPB*	ScpA, ScpB				[133]

a classification like MEROPS database b nomenclature adopted for *T.rubrum* and *M. canis*

A. benhamiae as a model organism

Most dermatophytes are difficulte to handle under culture conditions. Common dermatophytes, like *Trichophyton rubrum* or *Trichophyton mentagrophytes* are unsuitable for molecular examinations. A clinical isolate of *A. benhamiae* was shown to growth under in vitro conditions. Current works of the Leibniz-Institute for Natural Product Research and Infection Biology in Jena provide a fully sequenced genome of the dermatophytes *A. benhamiae* and *Trichophyton verrucosum.* In addition secretome analysis and transcriptome profiling of the interaction between *A. benhamiae* and human keratinocytes give molecular insights in the infection process and support work on pathogenesis of dermatophytes. Since transformation systems using *A. benhamiae* were developed, more

detailed informations about several proteins in the infection process can be obtained. Infection models in guinea pigs and reconstituted skin models are established and facilitate the research on immunological properties during dermatophyte infections. Thus *A. benhamiae* provided us the opportunity to use this fungus as a model organism to investigate dermatophyte host adaption and thus identify virulence factors of these fungi.

Infections with the dermatophyte *A. benhamiae* were initially described in the US and subsequently also in Afrika, Asia and Europe [141, 142]. This fungus causes high inflammatory infections, which causes dermatophytosis, like Tinea capitis, Tinea corporis and Tinea faciei [143]. Tinea manifests in a clinical picture by irregular alopecia, seborrheic dermatitis, rosacea, discois lupus erythematosus and contact dermatitis[141, 144]. *A. benhamiae* infects animals, like rodents, dogs or horses, but can also be transmitted to humans[145-147].

AIMS OF THIS P_HD THESIS

Dermatophytes are the major cause of superficial infections in humans and represent a prevalent worldwide health problem. Approximatly 10-20% of the world population is affected by dermatophytosis. However, fungal pathogenesis and immunological response of the human host, as well as fungal immune escape strategies are poorly understood so far. The characterization of e immune evasion of dermatophytes may help to identify new fungal targets that are helpful for therapy and will in addiotn expand our knowledge to defend such particular longlasting infections with dermatophytes.

The complement system plays a crucial role in the clearance of infections. Complement activity in the skin is poorly described. Human keratinocytes secrete several complement components. If the secreted complement components act as a complete active complement system was unknown and question of this study.

Furthermore dermatophyte interaction with the complement system is not examined in detail so far. Thus the attack of skin derived complement on *A. benhamiae* was analysed. Previous research on *T. rubrum* showed complement activation of serum during incubation with the fungus. In addition chemotactic factors are produced in serum, as shown by neutrophil attraction[118]. This indicates a role of complement in immune defense of dermatophytes. How complement affects the dermatophyte *A. benhamiae* was question of this work.

Furthermore dermatophyte infections are cleared by cell mediated immune answer. Thus dermatophytes may evade complement attack. If *A. benhamiae* inactivates complement and thus protect itself against complement attack was examined in the following assays.

Aim of this work is to get an insight in humoral immunity against dermatophytes. Moreover, immune escape strategies of *A. benhamiae* were evaluated. Therefore complement-dermatophyte interactions were examined.

MATERIAL AND METHODS

Materials

Sera, antibodies and proteins. Normal human serum (NHS) was obtained from healthy human donors or from the blood bank of the University Hospital of the Friedrich Schiller University, Jena, Germany. The study was approved by the ethical committee of the Friedrich Schiller University, Jena. Commercial goat-generated antisera against Factor H, C3, C4, C5 and C6 were used to detect Factor H and the members of the Factor H family; C3 and C3b; C4 and C4b; C5; and C6, respectively unless otherwise specified. Plasminogen antiserum obtained from goat was used to detect plasminogen and plasmin. Factor H and complement components antisera were obtained from Comp Tech, and the plasminogen antiserum was purchased from Calbiochem. Antiserum for CFHR1 and both monoclonal and polyclonal antibodies against fragments of Factor H were generated in our laboratory. Horseradish peroxidase-conjugated rabbit-anti-goat and goat-anti-rabbit sera (Dako Cytomation), and Alexa 647-conjugated rabbit-anti-goat serum (Invitrogen) were used for detection. Factor H fragments (SCR 1-4, SCR 1-7, SCR 8-11, SCR 11-15, SCR 15-18, SCR 15-19, SCR 15-20 and SCR 19-20) and CFHR1 fragments (SCR1-2, SCR 3-5) were generated in *Baculo* or *Pichia pastoris* expression system as described [148] .

***A.benhamiae* wild type and Subtilisin 3 disruption mutant.** A clinical isolate of *A. benhamiae* strain 2354 was kindly provided by Michel Monod (Centre Hospitalier Universitaire Vaudois, Lausanne)[141]. *A. benhamiae* Subtilisin 3 disruption mutant was kindly provided by Anke Burmester, department of molecular and applied microbiology, Leibniz Institut, Jena.

Cell culture

***A. benhamiae* strains and growth conditions.** *A. benhamiae* was cultivated at 28°C on Sabouraud medium supplemented with 2% glucose or medium supplemented with Keratin (20 mM potassium phosphate pH 5.5, 0.4 mM magnesium sulphate, 77 mM sodium chloride, 10g/l C/N source, 5 ml/l SL 8 micronutrient and 5 mM glucose) for 12 days. Fungal cells were extracted with isotonic water or DPBS (Lonza). Hyphae and conidia were separated by filtration using 0.2 µm filterunits (BD Bioscience). Conidia were counted using a cell counter

(Beckman, Coulter, Krefeld, Germany) or by using a Thoma chamber. Conidia were obtained after culturing *A. benhamiae* in water for over night. Culture supernatants were collected and concentrated using falcon concentrators (Amicon) with and cut off 10 kDa.

Cell culture of the keratinocyte cell line HaCaT. The human keratinocyte cell line THP-1 was grown in DMEM supplemented with 10% fetal bovine serum (FBS) and ultraglutamatine UG-1(PAA) at 5% CO_2 and 37°C. For experimental approaches HaCaTs were detached by adding trypsin/EDTA solution (PAA) and resuspended in DMEM without phenol red. Cells were counted using CASY-1 (Schärfe systems, Reutlingen).

Cell culture of the monocyte cell line THP-1. The human monocyte cell line THP-1 was grown in RPMI supplemented with 10% fetal bovine serum (FBS) and ultraglutamatine UG-1(PAA) at 5% CO_2 and 37°C. For cytokine release assays and phagocytosis approaches monocytes were centrifuged at 800 rpm for 5 minutes. Following THP-1 cells were resuspended in RPMI without phenol red and counted using CASY-1 (Schärfe systems, Reutlingen).

Isolation of blood polymorphnuclear cells. Polymorphnuclear cells (PMN) were freshly isolated from human blood by density centrifugation using polymorphPrep solution from Progen Biotechnik GmbH (Heidelberg). Afterwards PMNs were washed in 0.45% NaCl solution. Contaminating erythrocytes were lysed by washing the PMN fraction with 0.2% NaCl. The PMNs were washed with 1.2% NaCl and DPBS. Following cells were resuspended in indicator free RPMI medium.

Viability assay. Viability assays were performed by using cell titer blue reagents. Living cells are able to deoxidise resorufin and thus generate the fluorescent end product resazurin. 75.000 cells were seated in a 96 well plate and 100 µl resazurin solution from Promega (Mannheim) was added. Cells were incubated at 37°C and 5% CO_2 for 4 h and resazurin fluorescence emission signal was measured at 570 nm by using fluorescence reader safire^2.

Methods

Western Blot. Samples were separated by SDS-PAGE. Proteins were electrophoretical transferred to nitrocellulose membrane (Roth) for 1 h at 45 mA. After transfer, the membranes were blocked with 10% Roti Block in PBS II supplemented with 0.05%Tween

over night at 4°C. Specific primary antibody diluted 1:1000 in DPBS was incubated with the membrane for 2 h at RT. After washing, the membrane was incubated with an appropriated HRP-labelled secondary antiserum for 1h at RT. Following several washing steps, the blot was developed with ECL™ Western Blotting Detection Reagents (GE Healthcare). Detection carried out by 2 MF-ChemiBis 3.2 camera (Biostep).

The Secretion of Factor H, CFHR1, C3 and plasminogen by human keratinocytes were determined in the culture supernatant of the human keratinocytes cell line HaCaT. Therefore, HaCaT was grown in DMEM supplemented with 10% fetal bovine serum (FBS), gentamicin and ultraglutamatine UG-1(PAA) at 5% CO_2 and 37°C. For expression analysis keratinocytes were washed with DPBS (Lonza) and cultured in DMEM medium over night. Supernatant of keratinocytes was collected and concentrated ten times using a filter unit with a cut off 10 kDa (Millipore). Keratinocyte cultures were stimulated with 50-100 U/ml interferon-γ to detect Factor H and CFHR1 in the supernatant. Factor H was detected after Western blotting with monoclonal C18 antibody, which binds to an epitop in the C-terminus of Factor H. CFHR1, C3 and plasminogen were detected by appropriate antisera.

To determine binding of the human proteins Factor H, CFHR1 or plasminogen to *A. benhamiae*, the fungus (500 µg dry weight) was washed with washing buffer (100 mM NaCl/50 mM Tris pH 7.4) and incubated with 50% NHS/PBS II for 1 h at 37°C. Following cells were washed five times with washing buffer. Bound proteins were eluted with 3 M KSCN, separated by SDS PAGE under non-reductional conditions, transferred to a membrane and detected with appropriate antiserum.

Cofactor Assay. Purified human Factor H (1 µg) was bound to the surface of fungal cells for 15 minutes at 28°C. Conidia were washed three times to remove unbound protein and purified Factor I (0.5 µg) and C3b (1µg) were added. The mixture was centrifuged at 3500 x g at 4°C and supernatant was separated in SDS-PAGE and C3b cleavage products were detected using Western blot.

Complement cleavage assay. To characterize whether proteases secreted by *A. benhamiae* wild type or Subtilisin 3 disruption mutant cleave human complement components, purified C3a, C5a, C3b, C4b, C3, C4, C5 or C6 (1 µg) was incubated with supernatant (10 µl) obtained from *A. benhamiae* culture for 5-15 minutes at 37°C. After incubation, the mixture was

separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane and detected with appropriated antiserum using Western blotting.

Kinetics of C3b cleavage of *A. benhamiae* conidia in the presents or absence of Factor H was done. Purified human Factor H (1 µg) was bound to the surface of conidia for 15 minutes at 28°C. Cells were washed three times to remove unbound protein and purified Factor I (0.5 µg) and C3b (1µg) were added before incubation at room temperature. Additional conidia without Factor H and Factor I pretreatment were incubated with purified C3b (1µg) at room temperature. After one, five and ten minutes of incubation samples were taken from pretreated and untreated conidia. Treated and untreated conidia were centrifuged at 3500 xg at 4°C and supernatant were separated in SDS-Page. After transferring proteins to a membrane, C3b cleavage products were detected with C3 antiserum and HRP-labelled secondary goat antiserum.

ELISA. Binding of human complement proteins or plasminogen to *A. benhamiae* cells and binding domains of A. benhamiae in Factor H and CFHR1 was identified using a cell based ELISA. Fungal cells (500 µg dry weight) were bound to a filter microtiterplate (Millipore) for 1h at room temperature. Purified proteins (0.5-2.5 µg) or recombinant deletion mutants of Factor H and CFHR1 (1 µg) were added for 1 h at room temperature. After washing, bound proteins were identified with the corresponding primary antisera and appropriated HRP-linked secondary antisera. Samples were developed with Sigma Fast OPD (Sigma) and the reaction was stopped with 2M H_2SO_4. Absorption was measured at 492 nm by Multiscan Ascent microtiterplate reader (Thermo Labsystems).

To localize the *A. benhamiae* binding region in Factor H, *A. benhamiae* cells (500 µg dry weights) were immobilized on a filter microtiterplate (Millipore) for 1h at room temperature. Factor H together with domain mapped monoclonal antibody reacting with Factor H SCRs 1-4 (anti SCR 1-4); SCR 5 (B22) and SCRs 19-20 (C18) were preincubated for 1 h at 37°C. These samples were added to the immobilized fungal cells. Binding was determined by using the ELISA described above.

To measure the cytokine release of human monocytes after incubation with the supernatant derived from an *A. benhamiae* culture, fungal culture supernatant (20 %) were incubated with 1 µg C5a in a total volume of 200 µl RPMI for 10 minutes at 37 °C. Mixtures were added to human monocytes cell line THP-1 (250.000 cells/well) over night at 37 °C and 5 % CO_2.

Supernatant of treated macrophages were collected and proinflammatory cytokines TNF α, IL 6 and IL 8 were detected using cytokine ELISA kit from Immunotools (Friesoythe).

Hemolysis Assay. To analyze whether *A. benhamiae* secreted proteases affect complement attack, hemolysis assays were performed [149]. Rabbit erythrocytes were washed in 1ml HEPES buffer (20 mM HEPES, 144 mM NaCl, 7 mM Mg_2Cl, 10 mM EGTA). Supernatant derived from *A. benhamiae* wild type and Subtilisin 3 disruption mutant or from an *S. cerevisiae* culture were incubated with different concentrations of human serum for 30 minutes at 37°C. Preincubated mixtures and additional varying concentrations of human serum used as a control of complement mediated lysis were incubated with 1×10^7 rabbit erythrocytes for 30 minutes at 37°C. Treated rabbit erythrocytes were centrifuged at 2500 x g at 4°C and the absorption of the supernatant was measured at 414 nm.

To analyze whether human keratinocytes secrete functionally active complement components, culture supernatant of human keratinocytes was used in hemolysis assay. Rabbit erythrocytes were washed in 1 ml HEPES buffer and 1×10^7 cells were added to the concentrated supernatant of keratinocytes (100 µl) for 30 minutes at 37°C. Background lysis was determined by adding HEPES or DPBS buffer (100 µl) to rabbit erythrocytes (1×10^7) and incubating for 30 minutes at 37°C. Sample absorption was measured as described before.

Keratin degration assay. To determine proteolytic activity of *A. benhamiae* secreted proteases, the culture supernatant of the *A. benhamiae* wild type and Subtilisin 3 disruption mutant was collected and concentrated four times using filterunits with a cut off 5 kDa. *A. benhamiae* supernatant was incubated with chromogen charged keratin (Hide Powder Azur, Calbiochem) for 1h at 37°C. Proteolysis of keratin was followed by quantifying the released chromogen in the supernatant. (Millipore). The keratin substrate was centrifuged and the optical density of the chromgen in fungal supernatant was measured at 595 nm.

Flow cytometry. To quantify C3b deposition on *A. benhamiae* conidia, conidia ($5x10^5$) were incubated with either culture supernatant (100 µl) obtained from an *A. benhamiae* wild type or Subtilisin 3 disruption mutant or loaded with Factor H for 15 minutes at room temperature. Afterwards, purified C3b (10 µg/ml) as well as Factor I (2.5 µg/ml) were added for 15 minutes at room temperature. In addition untreated conidia were incubated with C3b. After extensive washing with DPBS, C3 antiserum diluted in DPBS was added to the conidia for 15 minutes at room temperature. After several washes with DPBS, appropriated

secondary Alexa 647-labelled antiserum was added for 15 minutes at room temperature. Additional washing steps released the unbound antiserum. Incubation of conidia with appropriated primary and secondary antiserum excluded false positive fluorescence signals. After extensive washing fluorescence intensity was measured at 647 nm by LSR II (Becton Dickinson) and evaluated in FLOW Jo 7.0 software.

Flow cytometry was also used to determine phagocytosis of prepared *A.benhamiae* conidia by human monocytes. Gfp fluorescent *A.benhamiae* mutant were kindly provided by Anke Burmester from Leibniz Institut for natural product research and infection biology, Jena. THP-1 monocytes were stimulated with 2.5 µg/ml PMA per $1x10^6$ cells over night at 37 C and 5 % CO_2. $5x10^5$ fluorescent *A. benhamiae* conidia were added to $1x10^6$ THP-1 macrophages and incubated for 30 minutes at 37°C and 5% CO_2. To separate adhered from phagocytosed cells the fungal cell wall was stained with 5 µg/ml Calcoflour. After several washings with DPBS, fluorescence signal of *A. benhamiae* conidia were measured at 355 nm and interpreted as described before.

Immunofluorescence. *A. benhamiae* (500 µg dry weights) was incubated with NHS-EDTA for 30 minutes at room temperature. Cells were washed three times with DPBS. Nonspecific binding sites were blocked with DPBS/Roti Block (Roth) for 30 minutes at room temperature. Surface bound Factor H or plasminogen was detected with polyclonal Factor H and plasminogen antiserum diluted 1:300 in DPBS/1xRoti Block for 45 minutes at room temperature. After three washes with DPBS appropriated secondary Alexa 647 conjugated antibody diluted 1:500 in DPBS/1xRoti Block was bound for 45 minutes at room temperature. After washing it three times with DPBS bound proteins were visualized by laser scanning microscopy (LSM 510 Meta, Zeiss).

Neutrophil migration assay. Polymorphnuclear cells (PMN) were freshly isolated from human blood by density centrifugation and stained with 1:10 dilution of 50 mM Calcein for 30 minutes at 37°C. 75.000 PMNs per upper well were incubated with either 8.5 µg/ml C5a diluted or a mixture of 8.5 µg/ml C5a and 20 µl culture supernatant derived from *A. benhamiae* wild type or Subtilisin 3 disruption mutant diluted in 235 µl DPBS in the lower well for 1 h at 37 °C. Medium, DPBS, gelantine, as well as activated serum and C5a des arg were added as controls. Fluorescence signal of migrated PMNs were measured using

fluorescence reader safire^2 (TecanTrading AG, Männedorf, Switzerland) at an emission wavelength of 530 nm.

Fungal killing assay. Culture supernatant derived from *A. benhamiae* wild type or Subtilisin 3 disruption mutant (20 µl), 1 µg Factor H or medium (20 µl) were incubated with 1 µg C3a for 10 minutes at 37 °C. *C. albicans* strain SC5314 were cultivated in 20 ml YPD medium over night at 30°C to get yeast cells. 5x10^4 *C. albicans* cells were diluted in 50 µl 10mM Tris buffer and incubated with C3a mixtures for 2 h at 30 °C. Afterwards cell suspension was diluted 1:100 in 10 mM Tris buffer, plated on YPD and incubated over night at 30°C. *C. albicans* colonies were counted from two replicates.

ROS release of PMN. Polymorphnuclear cells (PMN) were freshly isolated from human blood by density centrifugation using polymorphPrep solution. Afterwards PMNs were washed in 0.45% NaCl solution. Contaminating erythrocytes were lysed by washing the PMN fraction with 0.2% NaCl. The PMNs were washed with 1.2% NaCl and DPBS. To minimize preactivation of fresh isolated PMNs, a white 96 well microtiterplate (Nunc, denmark) were treated with 0.05% bovine serum albumin in DPBS (200µl/well) for 1 hour at room temperature. A 5x10^5 phagocyte suspension were placed in each well and C3a (2µg) or C3a supplemented with culture supernatant derived from *A. benhamiae* wild type or Subtilisin 3 disruption mutant was added. The samples were incubated for 3 hours at 37°C and 5 % CO_2. Detection solution (100 µl) was added to the wells for 20 minutes at room temperature to determine H_2O_2 release from human PMNs as described in the user manual of the cellestial red hydrogen peroxide assay kit (Enzo life science GmbH, Lörrach). Fluorescence was measured using fluorescence reader safire^2 at an emission wavelength of 590 nm.

RESULTS

Human keratinocytes form a functional lytic complement response.

Upon skin infection human keratinocytes represent the first cellular contact site for dermatophytes. To analyse, if keratinocytes form an intact functionally active complement system, culture supernatant derived from stimulated human keratinocytes was assayed for its complement mediated hemolysis. Supernatant derived from human keratinocytes caused lysis of rabbit erythrocytes as measured by hemolysis assays. This effect was dose dependent (Figure 8 A). The hemolytic activity of keratinocyte culture supernatant used at 60 % was comparable to that of 10% NHS. Hemolysis is specific for supernatant derived from human keratinocytes, because supernatant derived from the human epithelial cells (ARPE) lacked hemolytic activity (data not shown). The hemolytic activity of the keratinocyte supernatant was blocked by the staphylococcal complement inhibitor Sbi that inhibits complement activation at the level of C3 convertase formation (Figure 8 B). Thus, the results demonstrate that supernatant derived from human keratinocytes cause hemolysis of erythrocytes and that this lysis is complement-mediated.

To identify whether human keratinocytes release complement components, expression of C3, Factor H, CFHR1 and also plasminogen was assayed. Culture supernatant was concentrated, separated by SDS-PAGE, proteins were transferred to a nitrocellulose membrane and detected by appropriate antisera. C3 antiserum identified the 114 kDa α chain and the 75 kDa β chain of complement C3. Factor H antiserum detected a 150 kDa band, representing Factor H and two bands of 42 and 37 kDa representing the two isoforms of CFHR1 (Figure 8 C). In addition plasminogen antiserum identified a 75 kDa band as plasminogen (Figure 8 C). Thus, human keratinocytes express and secrete the complement components C3; but also the central regulators Factor H and CFHR1, as well as plasminogen.

Since complement activation results in C3b deposits on surfaces, opsonisation of keratinocyte derived complement was assayed by flow cytometry. Keratinocyte derived C3 deposited on the surface of *A. benhamiae* conidia (Figure 8 D). This confirms that human keratinocytes secrete a functionally active complement system and that dermatophytes are challenged by keratinocyte derived complement attack.

C3b deposition on *A. benhamiae* conidia leads to opsonisation and enhanced phagocytosis by human monocytes, as shown in phagocytosis assays. Both adherence and phagocytosis of *A. benhamiae* conidia by monocytes was enhanced after deposition of C3b on the surface of conidia (Figure 8 E). Adherence and phagocytosis increase dose dependent. After thirty minutes of incubation opsonisation of conidia (1.5 µg C3b) resulted in an increase of adherence (36%) to and phagocytosis (19%) by monocytes.

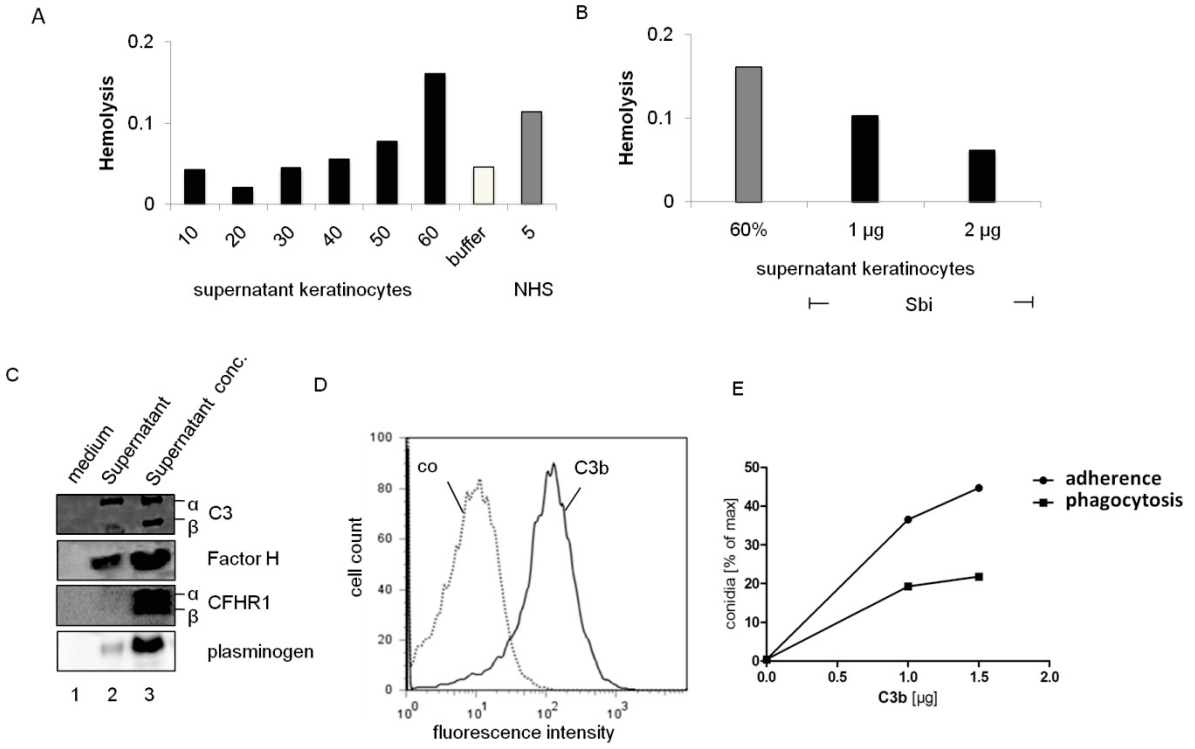


Figure 8: Keratinocytes secrete a functional active complement system

(A) Culture supernatant was collected from human keratinocytes, concentrated and assayed for hemolysis. Keratinocyte culture supernatant induced lysis of rabbit erythrocytes. This effect was dose dependent. (B) *S. aureus* derived complement inhibitor Sbi reduced hemolytic activity of keratinocyte culture supernatant, demonstrating that hemolysis is due to complement. (C) Culture supernatant was concentrated, separated by SDS-PAGE, proteins were transferred to a nitrocellulose membrane and detected by appropriate antisera. The complement protein C3; the complement regulators Factor H and CFHR1, as well as plasminogen were identified, which shows that human keratinocytes express and secrete central complement components. (D) The opsonic activity of keratinocyte derived C3 was measured by flow cytometry. C3 deposited on the surface of *A. benhamiae* conidia demonstrating that keratinocyte derived C3 is active.The abbreviation co stands for control. (E) Adherence and phagocytosis of *A. benhamiae* conidia by human monocytes was determined by flow cytometry. C3b (1.5 µg) opsonisation of fungal conidia resulted in 36% increased adherence and 19% increased phagocytosis. The effect was dose dependent.

A. benhamiae binds Factor H, CFHR1 and plasminogen.

Upon skin infection *A. benhamiae* is in contact with keratinocytes and thus exposed to complement attack. Consequently, we asked, if *A. benhamiae* binds human complement regulators Factor H, CFHR1, as well as plasminogen and uses the attached human proteins to control complement activation on the fungal surface.

Binding of Factor H, CFHR1 or plasminogen to the surface of *A. benhamiae* was assayed, using a cell based ELISA. The fungus was immobilised and purified proteins were added and after washing bound proteins were identified with appropriated antisera. *A. benhamiae* bound each of the three host proteins and binding was dose dependent (Figure 9 A-C). To confirm binding of the human proteins to the fungal surface, *A. benhamiae* was incubated in human plasma, as a source for complement proteins. After extensive washing, bound proteins were eluted, separated by SDS-PAGE, transferred to a membrane and analysed by Western blotting. In the elute fraction, Factor H was identified as a 150 kDa band and CFHR1 as 42 and 37 kDa bands (Figure 9 D, lane 2). Plasminogen was identified as a 75 kDa protein (Figure 9 E, lane 2). Thus *A. benhamiae* acquires host complement regulators Factor H and CFHR1 and also the human protease plasminogen to its surface.

In addition surface distribution of Factor H and plasminogen at the fungal surface was assayed by laser scanning microscopy. Purified proteins were attached to the fungus and then identified with the appropriate antisera. Factor H and plasminogen showed a patchy distribution, indicating clustering of the proteins on the fungal surface (Figure 9 F, G)

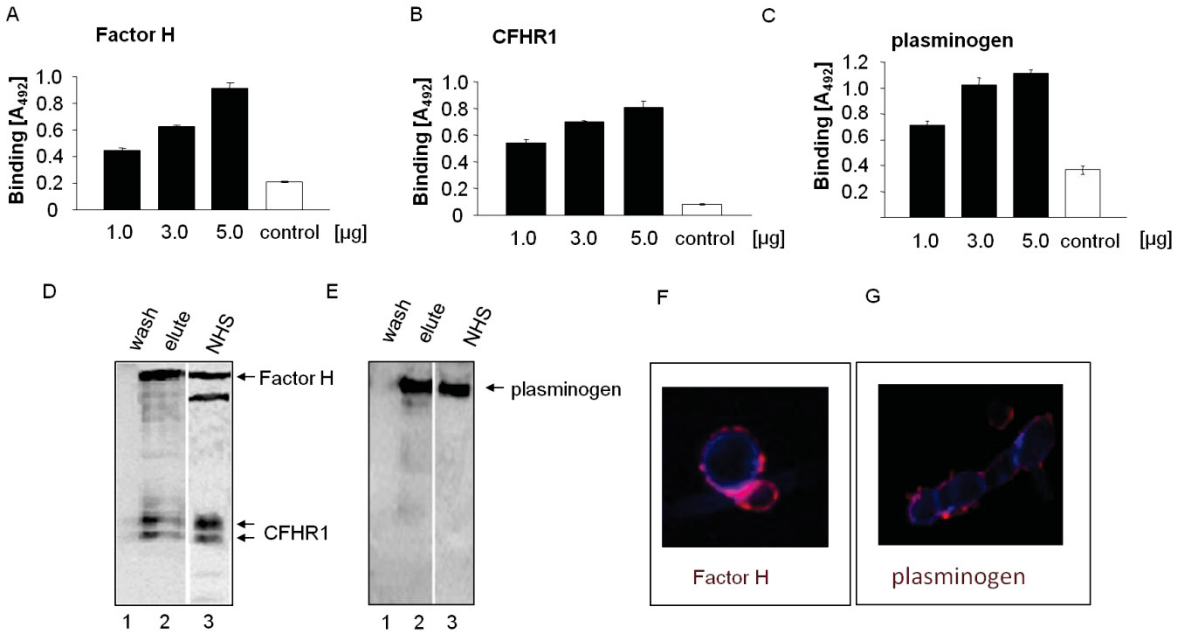


Figure 9: *A. benhamiae* binds the complement regulators Factor H and CFHR1, as well as plasminogen.

(A-C) The binding of complement components by *A. benhamiae* was assayed using a cell based ELISA. The fungus binds the complement regulators Factor H, CFHR1 and plasminogen in a dose dependent manner. (D, E) Binding of complement proteins and plasminogen to *A. benhamiae* cells was confirmed upon incubating fungal cells in human plasma. After extensive washing, bound proteins were eluted, separated by SDS-PAGE, transferred to a membrane and analysed by Western blotting. In the elute fraction Factor H, CFHR1 and plasminogen were identified as A. benhamiae binding proteins. (F, G) Fungal cells were loaded with the complement Factor H or plasminogen. Bound proteins were detected with appropriated fluorescence labelled antibodies (red). The fungal cell wall was stained with Calcoflour White in blue. Attached host proteins appear patchy on the fungal surface.

Localization of *A. benhamiae* binding sites in Factor H and CFHR1.

Factor H and also CFHR1 deletion mutants were bound to *A. benhamiae*, in order to localize the interaction domains in Factor H and CFHR1 that contact the fungus Therefore *A. benhamiae* was immobilised and various Factor H or CFHR1 deletion mutants were added. After extensive washing bound fragments were identified with appropriated antiserum. Factor H deletion mutants SCR 1-7, SCR 15-18, SCR 15-19 and SCR 15-20 bound to the fungus. Factor H deletion mutant SCR 1-4, SCR 8-11 and SCR 19-20 bound with lower intensity and SCR 11-15 did not bind to the fungus (Figure 10 A). This binding pattern shows that Factor H attaches to *A. benhamiae* via two separate regions. One binding region is located in the N-terminus, in domain SCR 1-7, likely SCR 6-7 and a second region is located in the C-terminus of Factor H within SCR 15-20 (Figure 10 D).

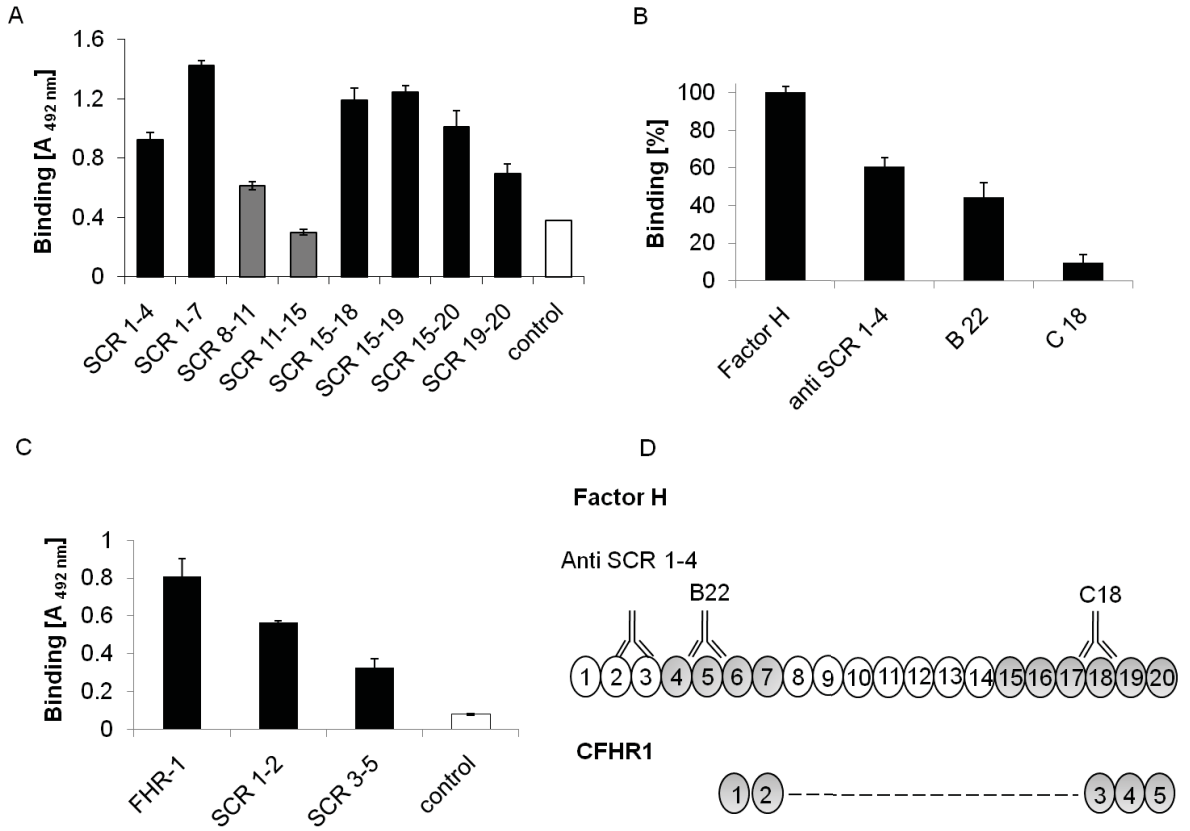


Figure 10: Localisation of *A. benhamiae* binding sites in Factor H and CFHR1.

(A) The binding of Factor H and also CFHR1 deletion mutants to *A. benhamiae* was assayed by ELISA. Factor H deletion mutants SCR 1-7, SCR 15-18, SCR 15-19 and SCR 15-20 bound to the fungus. Factor H deletion mutant SCR 1-4, SCR 8-11 and SCR 19-20 bound with lower intensity and SCR 11-15 did not bind to the fungus. (B) Specific domain mapped polyclonal antiserum, reacting with the N-terminus of Factor H (anti SCR 1-4) and domain mapped monoclonal antibodies binding to an epitop within SCR 5 (B22) or SCR 19-20 (C18) were assayed on their effect on binding of Factor H to *A. benhamiae*. Antiserum directed against the N- terminus of Factor H (anti SCR 1-4) reduced Factor H binding to *A. benhamiae* by 39%. Antibodies reacting with an epitope close to SCR 5 (B22) reduced binding by 56% and the monoclonal antibody C18, which binds to SCR 19-20, inhibited binding by 91 %. (C) Two CFHR1 deletion mutants (CFHR1 SCR 1-2, CFHR1 SCR 3-5) were analysed for binding to *A. benhamiae* cells. Both recombinant proteins bound with similar intensity to *A. benhamiae*, but each deletion mutant bound with a lower intensity as compared to intact CFHR1 to the fungus. (D)The figure shows the Factor H binding regions, which are responsible for attaching to *A. benhamiae*. One was located in the N-terminus containing SCR 1-7 and a second in the C-terminus containing SCR 15-20. CFHR1 uses two separate binding regions to contact *A. benhamiae* in the N-terminal SCR 1-2 the C-terminal SCR 3-4.

In order to verify that Factor H contacts *A. benhamiae* with two regions, the effect of a specific domain mapped polyclonal antiserum, reacting with the N-terminus of Factor H (anti SCR 1-4) and domain mapped monoclonal antibodies to SCR 5 (B22) and SCR 19-20 (C18) on binding, was assayed. Antiserum directed against the N- terminus of Factor H (anti SCR 1-4) reduced Factor H binding to *A. benhamiae* by 39%. Antibodies reacting with an epitope close to SCR 5 (B22) reduced binding by 56% and the monoclonal antibody C18, which binds to SCR 19-20, inhibited binding by 91 % (Figure 10 B). These results confirm that Factor H uses two binding regions to contact *A. benhamiae*. An N-terminal binding region

was identified in Factor H SCR 1-7. In addition the C-terminal surface attachment region of Factor H is also central for binding to the fungus. Taken together, Factor H uses two binding regions for attaching to *A. benhamiae*, an N-terminal one containing SCR 1-7 and a C-terminal region in SCR 15-20.

In addition, binding regions of CFHR1 were localized by using two CFHR1 deletion mutants (CFHR1 SCR 1-2, CFHR1 SCR 3-5). Both CFHR1 deletion mutants bound with similar intensity to *A. benhamiae*. Each deletion mutant bound with a lower intensity as compare to intact CFHR1 to the fungus (Figure 10 C). These results suggest that CFHR1 uses two separate binding regions to contact *A. benhamiae*, one is located in the N-terminus in SCR 1-2 and a second in the C-terminus in SCR 3-4 (Figure 10 D).

A. benhamiae bound Factor H displays complement regulatory activity.

As Factor H binds to *A. benhamiae*, we asked, whether the bound inhibitor displays complement regulatory function. Factor H was attached to conidia and after washing Factor I and C3b were added. After incubation, the reaction mixture was separated by SDS-PAGE and transferred to a membrane. When C3b cleavage was assayed by Western blotting, α chain fragments of 68, 43 and 41 kDa were identified (Figure 11). This shows, that Factor H bound to the surface of *A. benhamiae* maintains complement regulatory activity and acts as a cofactor for Factor I mediated C3b degradation.

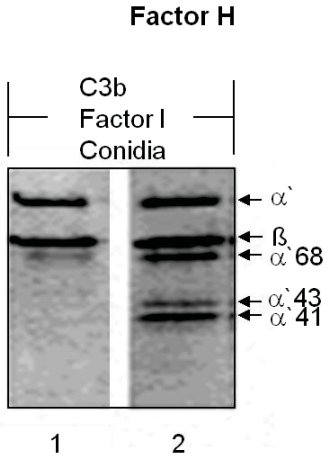


Figure 11: *A. benhamiae* bound Factor H displays complement regulatory activity.

Factor H was attached to conidia and Factor I and C3b were added. After incubation, the reaction mixture was separated by SDS-PAGE and transferred to a membrane. C3b cleavage was assayed by Western blotting. C3b α chain fragments of 68, 43 and 41 kDa were identified as typical Factor H mediated C3b cleavage products.

A. benhamiae bound complement regulator Factor H reduces C3b deposition, opsonisation and phagocytosis on conidia.

In order to analyse the influence of *A. benhamiae* bound Factor H on opsonisation, C3b deposition on conidia was measured using Factor H pretreated or untreated conidia. Factor I was added and C3b deposition on conidia was analysed by flow cytometry. C3b added to non treated conidia remained active and C3 deposited on the fungal surface (Figure 12 A black line). In comparison, when C3b was added to Factor H treated conidia supplemented with Factor I, the C3 deposition rate was reduced by 98 % (Figure 12 A red line).

A. benhamiae utilizes human complement regulator Factor H to inhibit complement activation on the fungal surface. Factor H assists C3b degradation to iC3b and thus stops assembly of the C3 convertase on the surface and amplification of C3b.To demonstrate that Factor H loaded conidia are protected against the opsonic activity of C3b, *A. benhamiae* conidia were loaded with increasing concentrations of complement regulator Factor H and Factor I as well as C3b was added. Afterwarts monocytes were added to the prepared conidia and adherence and phagocytosis were measured using flow cytometry. A decrease of adherence (36 %) and phagocytosis (25 %) was observed for conidia treated with 1 µg Factor H. Adherence of conidia to monocytes decreased dose dependent with increasing amounts of Factor H. Phagocytosis of fungal conidia by monocytes decreased, when 0.5 µg Factor H was used. Higher Factor H concentrations of 1 µg slightly increased phagocytosis (Figure 12 B). Factor I and C3b incubation with conidia resulted in no reduction of either adherence or phagocytosis (data not shown).

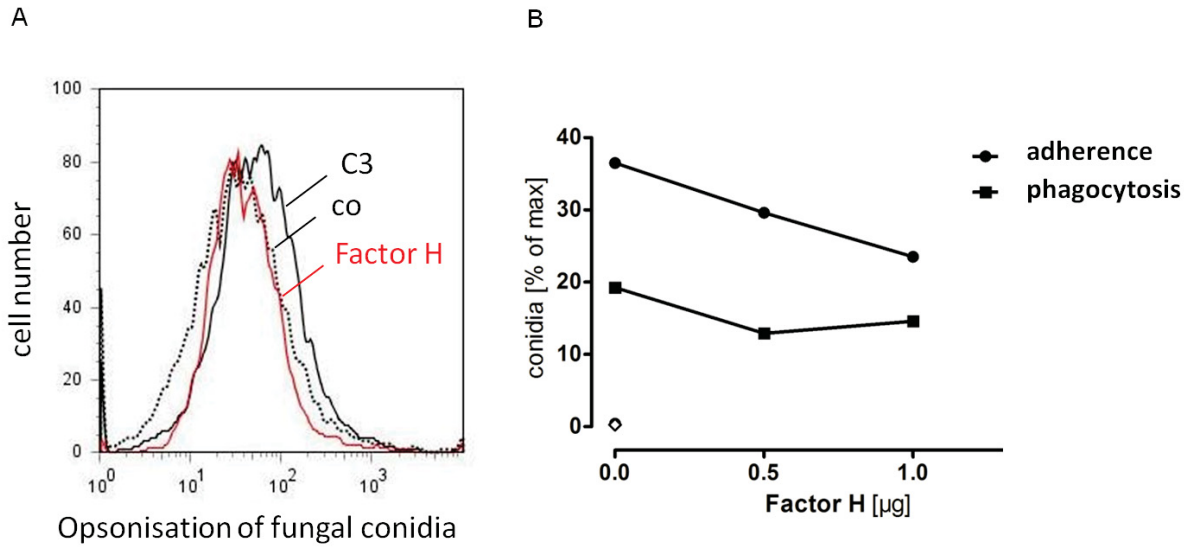


Figure 12: *A. benhamiae* bound complement regulator Factor H reduces C3b deposition, opsonisation and phagocytosis on fungal conidia.

(A) C3b deposition on conidia was measured either using Factor H pretreated conidia or using untreated conidia. Factor I was added and C3b deposition on conidia was analysed by flow cytometry. C3b added to non treated conidia remains its activity and deposited on the fungal surface (black line). In comparison, C3b added to Factor H treated conidia supplemented with Factor I showed a reduced deposition rate of 98 % (red line). (B) A. benhamiae conidia were loaded with increasing concentrations of complement regulator Factor H. Factor I as well as C3b was added. Following adherence and phagocytosis of fungal conidia by human monocytes was measured using flow cytometry. A decrease of adherence (36 %) and phagocytosis (25 %) was observed, when 1 µg Factor H was added. Adherence of fungal conidia to monocytes decrease dose dependent with increasing amounts of Factor H. Phagocytosis of fungal conidia by monocytes decrease, when 0.5 µg Factor H was used in the approach. Higher Factor H concentrations of 1 µg slightly increase phagocytosis.

A.benhamiae secretes functional active proteases

In order to assay, whether *A. benhamiae* secretes active proteases and if disruption of the Subtilisin 3 gene influences keratin degradation, culture supernatant derived from *A. benhamiae* was added to chromogen labelled keratin and proteolysis of keratin was assayed by quantifying the released chromogen in the supernatant. The chromogen content in both fungal supernatants treated samples increased. Thus, fungal supernatant has proteolytic, keratin degrading activity. Culture supernatant derived from an *A.benhamiae* Subtilisin 3 disruption mutant showed an decrease in keratin degradation as compared to culture supernatant derived from the wild type (Figure 13).

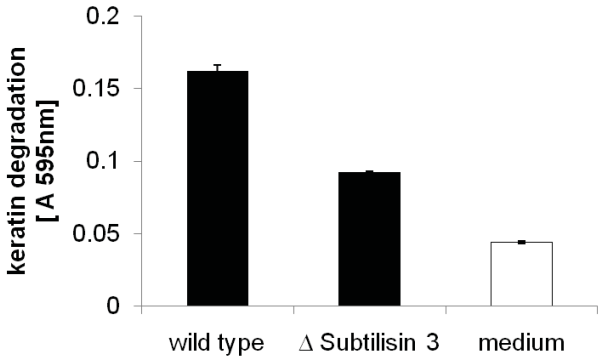


Figure 13: *A.benhamiae* secretes keratin degrading proteases

Culture supernatant derived from *A. benhamiae* wild type or Subtilisin 3 disruption mutant was added to chromogen labelled keratin and proteolysis of keratin was assayed by quantifying the released chromogen in the supernatant. The chromogen in both fungal supernatants treated samples increased. Thus fungal supernatant has proteolytic, keratin degrading activity. In comparison to the culture supernatant derived from the *A.benhamiae* wild type, the Subtilisin 3 disruption mutant shows decreased keratin degradation.

A. benhamiae derived proteases cleave complement

To assay, if secreted fungal proteases also degrade complement components, culture supernatant derived from *A. benhamiae* wild type was added to purified complement C3, C3b, C4, C4b, C5 and C6. After incubation, the reaction mixture was separated by SDS-PAGE, transferred to a membrane and cleavage was assayed by Western blotting. Fungal proteases degraded C3 and C3b, as revealed by the appearance of cleavage products with approximate mobilities of 68, 42, 40, 38, 27 and 25 kDa (Figure 14 A, B). This pattern is clearly distinct from that obtained by Factor H assisted Factor I cleavage (68, 43 and 41 kDa) (Figure 10). Incubation of fungal culture supernatant with C4 and C4b resulted in major cleavage fragments with apparent mobilities of 64, 60, 54, 50 kDa, 38 kDa and 18 kDa (Figure 14 C, D). C5 was cleaved in three major products of 60, 55, 48 and 45 kDa (Figure 14 E). In addition fungal proteases degrade C6 into four fragments with mobilities of 75, 55, 45 and 30 kDa (Figure 14 F). In contrast human IgG remained intact upon incubation with fungal supernatant (data not shown). Thus the fungal culture supernatant cleaves human complement proteins C3, C3b, C4b, C4, C5 and C6.

Complement activation results in opsonisation of foreign surfaces, assembly of the membrane attack complex and in the release of anaphylatoxins. Since opsonisation and membrane attack complex formation is inhibited by proteases in the supernatant of

A. benhamiae, degradation and inactivation of the anaphylatoxins C3a and C5a by fungal endogenous activity was analysed. C3a and C5a were incubated with the supernatant derived from an *A. benhamiae* culture. Then degradation of C3a and C5a were assayed in Western Blotting by using specific antisera. The bands of 9 kDa and 12 kDa representing C3a and C5a disappeared during incubation with fungal culture supernatant, demonstrating cleavage of the two anaphylatoxins (Figure 14 G, H).

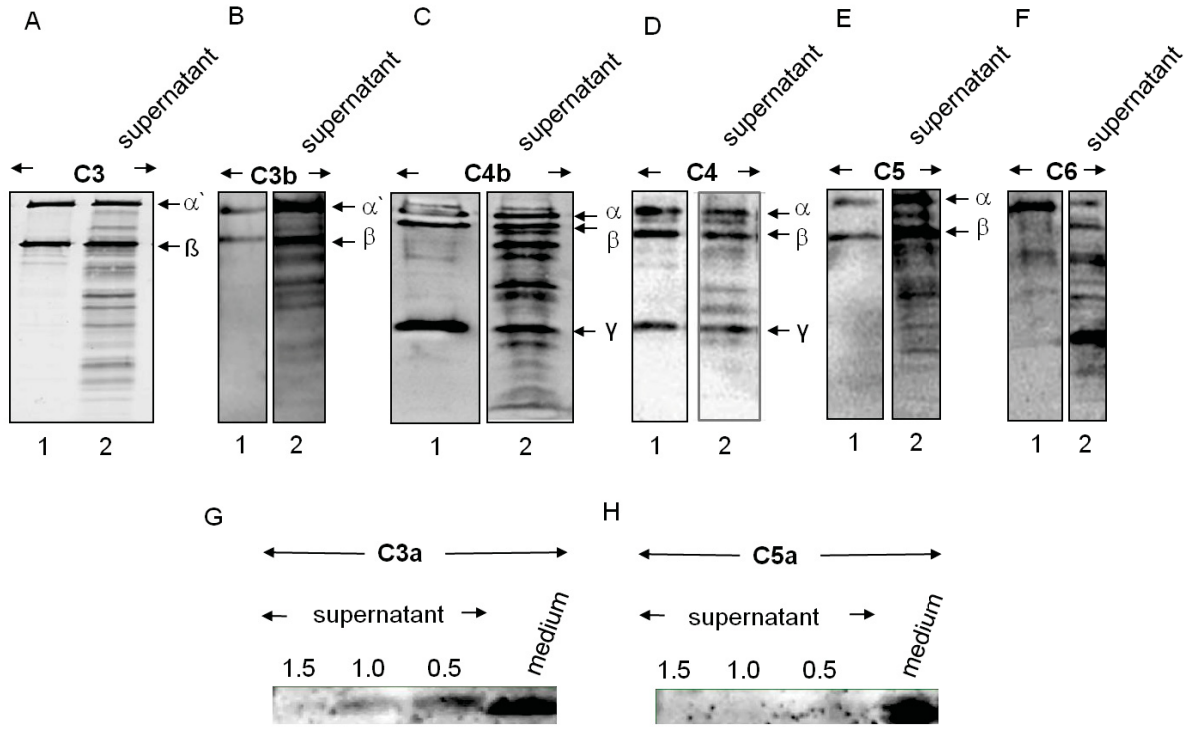


Figure 14: *A. benhamiae* derived proteases cleave complement

Culture supernatant derived from A. benhamiae wild type was added to purified C3, C3b, C4, C4b, C5, C6 and to the anaphylatoxins C3a and C5a. After incubation, the reaction mixture was separated by SDS-PAGE and transferred to a membrane and cleavage was assayed by Western blotting.(A, B) C3 and C3b was degraded by fungal proteases, as revealed by the appearance of cleavage products with approximate mobilities of 68, 42, 40, 38, 27 and 25 kDa. This pattern is distinct from that obtained by Factor H assisted Factor I cleavage (Figure 10). (C, D) C4 and C4b cleavage resulted in fragments with apparent mobilities of 60, 55, 48 and 45 kDa. (E) C5 was cleaved to products with approximate mobilities of 60, 55, 48 and 45 kDa.(F) C6 is degraded in four major products of 75, 55, 45 and 30 kDa. (G, H) The bands of 9 kDa and 12 kDa representing C3a and C5a disappeared during incubation with fungal culture supernatant, demonstrating cleavage of the anaphylatoxins.

A. benhamiae secreted protease Subtilisin 3 cleave C3a, C3b and C5b

In order to define, which of the secreted protease of *A. benhamiae* exhibits complement degrading activity; culture supernatant derived from an *A. benhamiae* Subtilisin 3 disruption mutant was collected and incubated with purified C3a, C3b and C5b. After incubation, the reaction mixtures were separated by SDS-PAGE, transferred to a membrane and cleavage

was assayed by Western blotting Complement degradation of the central components C3b and C3a by culture supernatant derived from *A. benhamiae* Subtilisin 3 disruption mutant was decreased in comparison to C3b and C3a degradation by the supernatant derived from the wild type culture. Secreted proteases in the culture supernatant derived from the Subtilisin 3 disruption mutant lacks the ability to degrade C5b (Figure 15). Complement cleavage by the fungal medium was excluded.

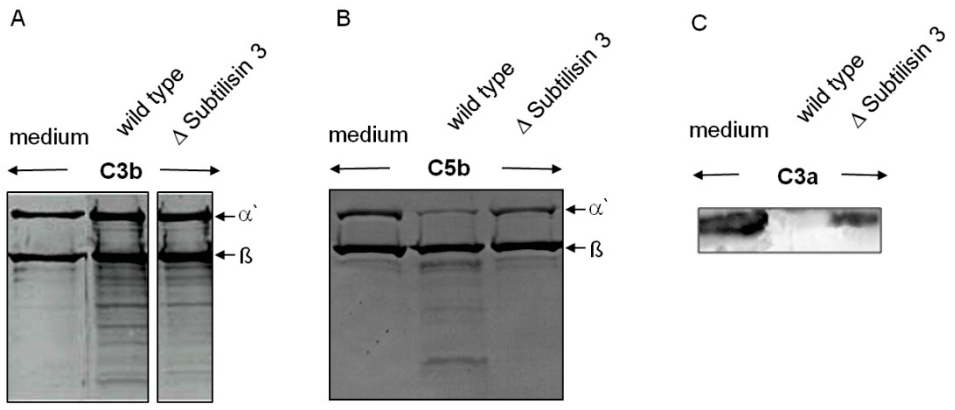


Figure 15: *A. benhamiae* secreted protease Subtilisin 3 cleave C3a, C3b and C5b

Purified C3b, C5b and C3a were incubated with culture supernatant of the *A. benhamiae* Subtilisin 3 disruption mutant. After incubation, the reaction mixtures were separated by SDS-PAGE, transferred to a membrane and cleavage was assayed by Western blotting.(A, C) Complement degradation of C3b and C3a by culture supernatant derived from *A. benhamiae* Subtilisin 3 disruption mutant was decreased in comparison to C3b and C3a degradation by the supernatant derived from the wild type culture. (B) Secreted proteases in the culture supernatant derived from the Subtilisin 3 disruption mutant lack ability to degrade C5b.

Taken together, *A. benhamiae* secretes proteases in the culture supernatant, which are functional active and degrade keratin as well as several human complement components, i.e. C3, C3b, C4, C4b, C5 and C6 and the anaphylatoxins C3a and C5a. The secreted protease Subtilisin 3 is responsible for the degradation of the central complement components C3b, C5b and the anaphylatoxin C3a.

A. benhamiae proteases inhibit complement activation on activator surfaces.

Proteases contained in the supernatant of *A. benhamiae* cleave human complement. We asked, if this cleavage affects complement activity. To this end the inhibitory effect of *A. benhamiae* culture supernatant was determined in hemolysis assays. At first fungal culture supernatant was incubated with human serum and then rabbit erythrocytes, which represent activator surfaces, were added. *A. benhamiae* culture supernatant reduced complement mediated lysis of rabbit erythrocytes. This effect was dose dependent

(Figure 16 A). Supernatant derived from *A. benhamiae* reduced lytic act ivity of serum (20%) by 77%. Culture supernatant derived from the non-pathogenic yeast *S. cerevisiae* lacked this inhibitory activity and did not affect complement mediated lysis of erythrocyte. Thus *A. benhamiae* proteases in the culture supernatant block host complement attack on surfaces. *A. benhamiae* inhibition of erythrocyte hemolysis was completely blocked by the serine protease inhibitor phenyl-methyl-sulphonyl-fluorid (PMSF). This shows that secreted serine proteases of *A. benhamiae* mediate inactivation of complement (Figure 16 B).

In order to define secreted serine proteases of *A. benhamiae*, which inactivate complement components, culture supernatant of the *A. benhamiae* Subtilisin 3 disruption mutant was incubated with NHS. Quantification of hemolysis showed a decrease of the complement inhibitor effect (21%) in comparison to culture supernatant derived from the *A. benhamiae* wild type (Figure 16 C). An effect of fungal medium on hemolysis was excluded.

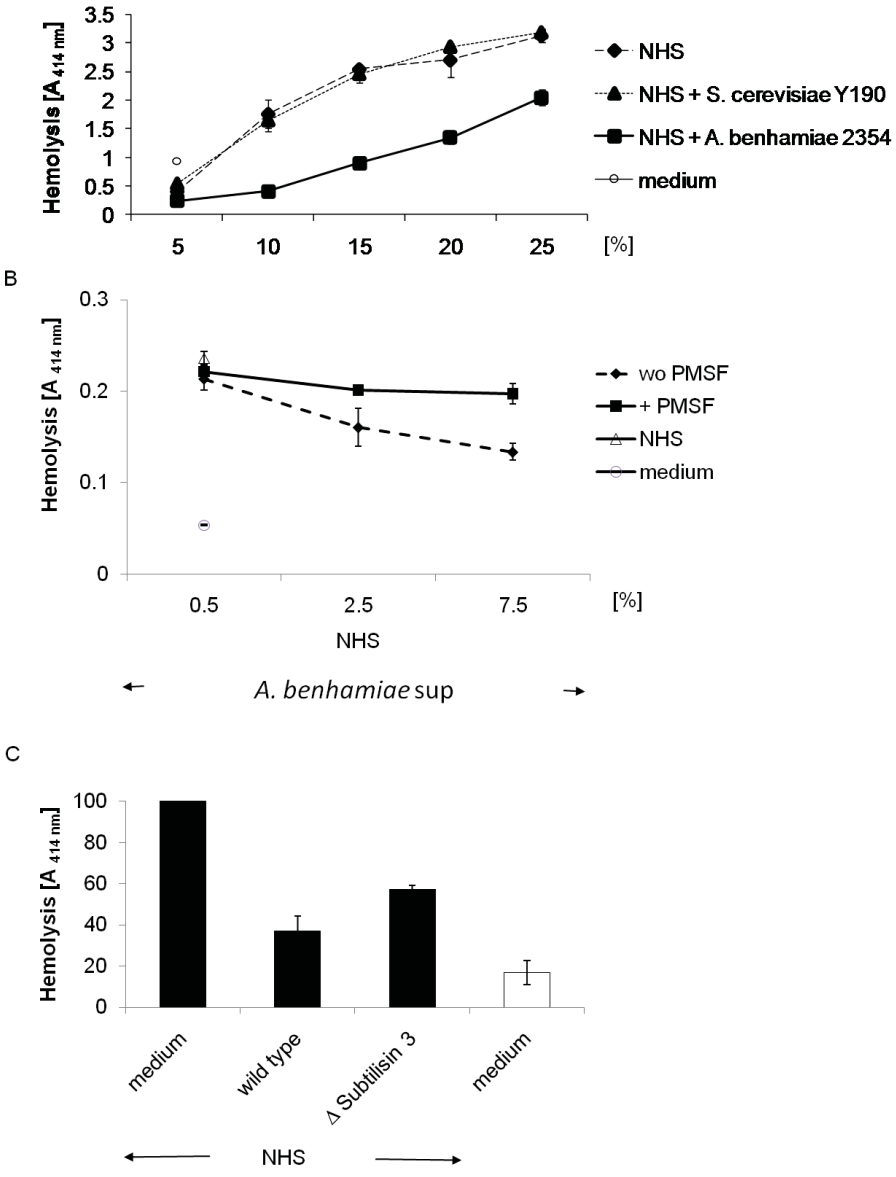


Figure 16: *A. benhamiae* proteases, particular Subtilisin 3 inhibit complement activation on activator surfaces.

Complement inhibition caused by *A. benhamiae* culture supernatant on activator surfaces was determined in hemolysis assays. Fungal culture supernatants were incubated with NHS and rabbit erythrocytes were added. The fungal medium did not cause hemolysis. (A) *A. benhamiae* wild type culture supernatant reduced complement mediated lysis of rabbit erythrocytes dose dependent. Supernatant derived from *A. benhamiae* wild type reduced lytic activity of serum by 77%. Culture supernatant derived from the non-pathogenic yeast S. cerevisiae lacked this inhibitor activity and did not affect erythrocyte lysis. (B*) A. benhamiae* inhibition of NHS (7.5%) derived hemolytic activity was completely blocked by adding 5% of PMSF. This shows that secreted serine proteases of *A. benhamiae* mediate inactivation of complement. (C) Culture supernatant derived from the Subtilisin 3 disruption mutant was incubated with NHS. Quantification of hemolysis shows a decrease of the complement inhibitor effect (21%) in comparison to *A. benhamiae* wild type culture supernatant.

Endogenous proteolytic activity of *A. benhamiae* reduces C3b deposition, opsonisation and phagocytosis on conidia.

Since *A. benhamiae* expresses and secretes proteases, which degrade the human complement component C3b, the effect of fungal culture supernatant on C3b deposition on conidia was analysed using flow cytometry. Therefore C3b was preincubated with culture supernatant derived either from the A. benhamiae wild type or from the Subtilisin 3 disruption mutant. Mixtures were added to conidia and C3b deposition was analysed on the fungal surface by flow cytometry. Not treated C3b remained its active and deposited on conidia. In comparison, fungal supernatant treated C3b showed a reduced deposition rate of 44 %. Disruption of the Subtilisin 3 gene in *A. benhamiae* resulted in the loss of complement inhibitor activity. Culture supernatant of this mutant lacks the ability to reduce C3b deposition on conidia (Figure 17 A).

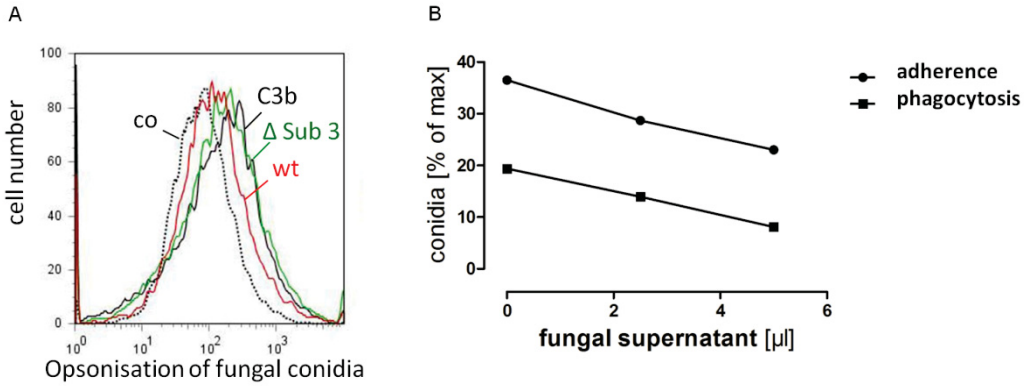


Figure 17: Endogenous proteolytic activity of *A. benhamiae*, in particular Subtilisin 3 reduces C3b deposition, opsonisation and phagocytosis of conidia.

(A) C3b was preincubated with supernatant derived from an *A. benhamiae* wild type or Subtilisin 3 disruption mutant culture. Mixtures were added to conidia and C3b deposition on the fungal surface was analysed by flow cytometry. None treated C3b remains its activity and deposited on conidia. In comparison, wild type culture supernatant treated C3b showed a reduced deposition rate of 44 %. Culture supernatant of the Subtilisin 3 disruption mutant lacks ability to reduce C3b deposition on fungal conidia. (B) Adherence and phagocytosis of treated fungal conidia by human monocytes was determined by flow cytometry. Human C3b was incubated with supernatant derived from an *A. benhamiae* wild type culture. Mixture was added to fungal conidia and incubated with human monocytes. Fungal supernatant (5%) reduces complement induced adherence at 37 % and phagocytosis at 58 %. Adherence and phagocytosis of treated fungal conidia by monocytes was dosed dependent reduced.

Endogenous proteolytic activity of *A. benhamiae* reduced deposition of complement C3b on fungal surfaces. The impact of reduced fungal opsonisation by C3b on adherence and phagocytosis by human monocytes was determined by flow cytometry. Human C3b was incubated with supernatant derived from an *A. benhamiae* wild type culture. The mixture

was added to fungal conidia and incubated with human monocytes. Fungal supernatant (5 %) reduced complement induced adherence to monocytes at 37 % and phagocytosis at 58 % (Figure 17 B). Increasing amounts of fungal supernatant caused a concentration dependent reduction of adherence and also phagocytosis of conidia by monocytes.

Culture supernatant of *A. benhamiae* reduces C3a activity.

The anaphylatoxin C3a mediates antifungal activity. In order to test, if C3a treated with culture supernatant of the *A. benhamiae* wild type or the Subtilisin 3 disruption mutant keeps its antifungal properties, killing of fungal cells was analysed. Therefore pretreated or non treated C3a was added to *C. albicans* and the fungal survivial was determined. C3a added to *C.albicans* reduced fungal growth by 78 % in comparison to the fungal growth without C3a. Pretreated C3a, which was added to the culture supernatant of *A. benhamiae* wild type resulted in increased overliving of *C. albicans* up to 194 % in comparison to nontreated C3a added to *C albicans*. C3a added to the culture supernatant of *A. benhamiae* Subtilisin 3 disruption mutant incubated with *C. albicans* leads also to significant survivial of the fungal cells, but was reduced compared to wild type culture supernatant treated C3a added to *Candida* (Figure 18 A).

In addition C3a induces ROS production in human neutrophils. Human C3a was treated with the culture supernatant of the *A. benhamiae* wild type or Subtilisin 3 disruption mutant. To determine the effect of treated and non treated C3a on the ROS production of freshly isolated PMNs, a fluorescent detection solution was used to quantify ROS release. Purified C3a increases ROS production by 87 %. Treatment of C3a with the culture supernatant of the *A. benhamiae* wild type or Subtilisin 3 disruption mutant repressed the ROS production. C3a treated with culture supernatant of the wild type leads to a reduction of ROS production by 99 %. In comparison, C3a treated with culture supernatant of the Subtilisin 3 disruption mutant showed reduction of the ROS production by 59 % (Figure 18 B). Thus C3a treated with the culture supernatant of the Subtilisin 3 disruption mutant resulted in lesser reduction of ROS by human neutrophils than C3a treatment with the culture supernatant of the wild type.

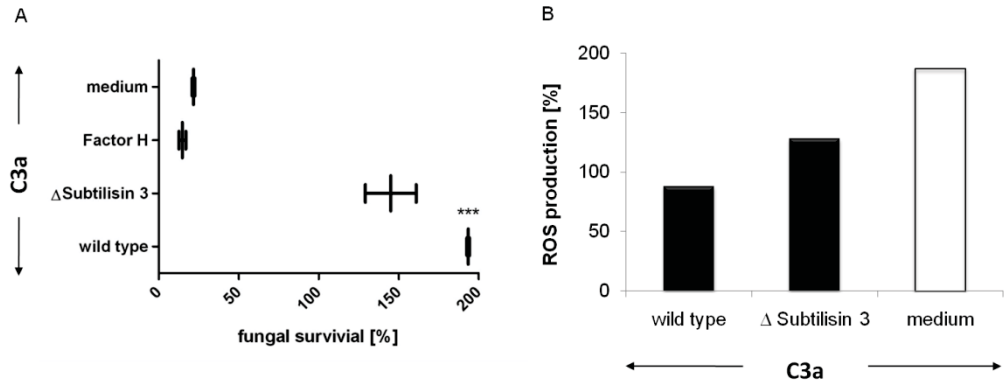


Figure 18: Culture supernatant of *A. benhamiae*, in particular Subtilisin 3 reduces C3a activity.

(A)To assay antifungal properties of C3a treated with culture supernatant of the *A. benhamiae* wild type or the Subtilisin 3 disruption mutant fungal survivial assays were performed. C3a added to *C.albicans* reduces fungal growth at 78 %. C3a added to the culture supernatant of *A. benhamiae* wild type resulted in an increased survival of *C. albicans* up to 194 %. Culture supernatant of *A. benhamiae* Subtilisin 3 disruption mutant treated C3a incubated with *C. albicans* led also to significant survival of the fungal cells, but was reduced in comparison to wild type culture supernatant treated C3a added to *Candida*. (B) To determine ROS production of freshly isolated PMNs, purified C3a was added and ROS was quantified by an flurescent detection solution. Purified C3a increases ROS release by 87 %. Treatment of C3a with the culture supernatant of the *A. benhamiae* wild type or Subtilisin 3 disruption mutant effects freshly isolated PMNs by repression of the ROS production. C3a treated with culture supernatant of the wild type leads to a reduction of ROS production by 99 % compared to non treated C3a. C3a treated with culture supernatant of the Subtilisin 3 disruption mutant resulted in a reduction of ROS production by 59 %. Thus C3a treated with the culture supernatant of the Subtilisin 3 disruption mutant resulted in lesser reduction of ROS by human neutrophils than C3a treated with the culture supernatant of the wild type.

Culture supernatant of *A. benhamiae* reduces C5a activity.

Since the proteases in the culture supernatant of *A. benhamiae* cleave C5a, the biological effects to the C5a activity were investigated. C5a induces the release of the proinflammatory cytokines IL 6, IL 8 and TNF α from human monocytes. C5a was incubated with culture supernatant of *A. benhamiae*. Afterwarts pretreated C5a or non treated C5a was added to human monocytes. To investigate secretion of proinflammatory cytokines by human monocytes after stimulation, ELISA was performed to determine cytokine and chemokine levels. Monocytes supplemented with non treated C5a produce increased IL 6 (69 %), IL 8 (88 %) and TNF α (82.5 %) levels in comparison to the control level. In comparison to the cytokine levels, which were produced by C5a treated monocytes, the preincubation of C5a with the culture supernatant of the *A. benhamiae* wild type resulted in an inhibition of IL 6 (156 %), IL 8 (154 %) and TNF α (103 %) secretion (Figure 19 A-C). The fungal medium without C5a was used to quantify C5a independent cytokine release.

To exclude cell damaging effects of the keratin medium or of fungal culture supernatants on monocytes, viability assays were done. Therefore the ability of living cells to deoxidise resazurin to the florescent end product resorufin was used. Resorufin production by human monocytes after treatment was determined by fluorescence measurement. With this assay it was excluded that different concentrations of keratin medium or culture supernatant of *A. benhamiae* had an effect on the viability of human monocytes (data not shown).

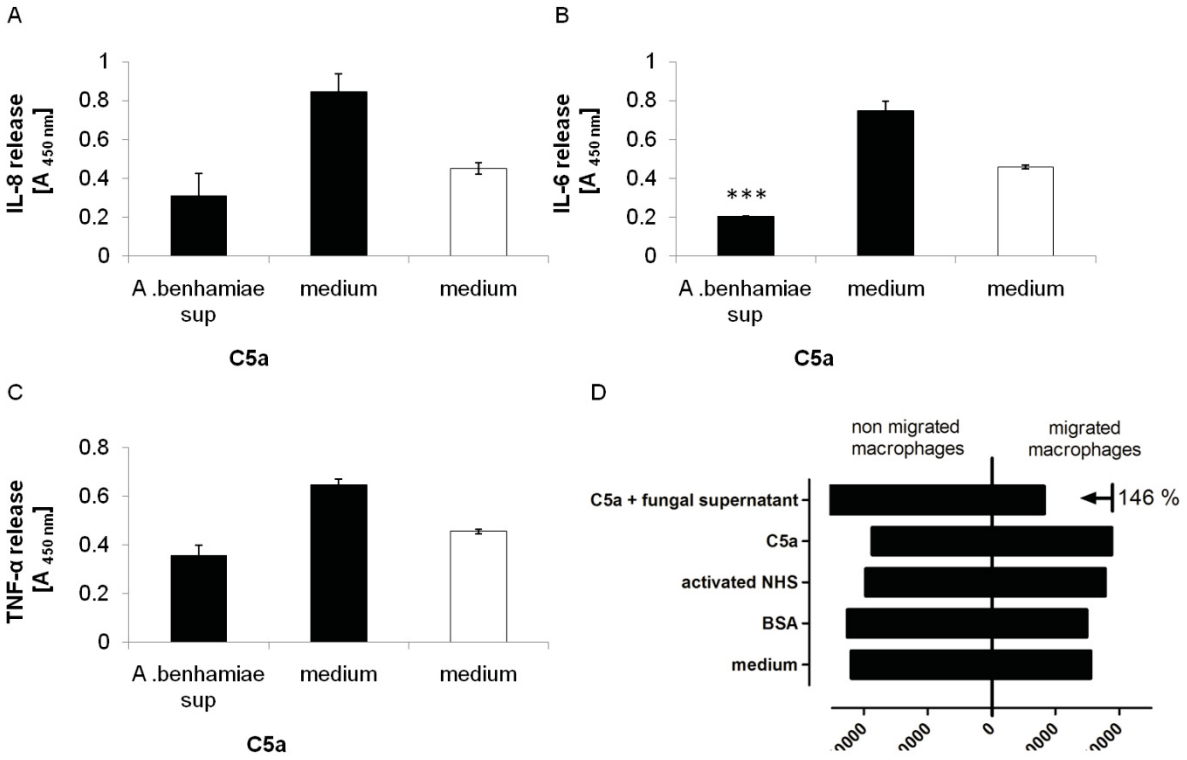


Figure 19: Culture supernatant of *A. benhamiae* reduces C5a activity.

(A-C) Secretion of proinflammatory cytokines by human monocytes after stimulation with none treated C5a or pretrated C5a was analysed using cytokines ELISA. Monocytes supplemented with C5a produce increased IL 6 (69 %), IL 8 (88 %) and TNF α (82.5 %) levels. In comparison, preincubation of C5a with the culture supernatant of the *A. benhamiae* wild type resulted in complete reduction of IL 6 (156 %), IL 8 (154 %) and TNF α (103 %) secretion. The fungal medium without C5a was used to quantify C5a independent cytokine release. (D) None treated C5a or C5a treated with supernatant of an *A. benhamiae* wild type culture was incubated with freshly isolated PMNs. Migration of PMNs was analysed using migration assay. C5a and 5 % activated serum increased neutrophil migration by 14 % and 21 % respectively. C5a incubation with culture supernatant of *A. benhamiae* resulted in complete inhibition of neutrophil migration of 146 %.

C5a is a potent chemo attractant for human neutrophils. Chemoattraction of freshly isolated PMNs by none treated C5a or C5a treated with supernatant of an *A. benhamiae* wild type culture was analysed using neutrophil migration assay. C5a and also activated serum (5 %) increased neutrophil migration by 14 % and 21 % respectively. C5a incubation with culture

supernatant of *A. benhamiae* resulted in a complete inhibition of neutrophil migration by 146 % (Figure 19 D).

Taken together, *A. benhamiae* secrete proteases, which cleave complement components. Thereby the opsonic activity of C3b, the antifungal and proinflammatory properties of C3a and the chemotattractic and proinflammatory activities of C5a were inactivated. In addition cleavage of C3, C4, C5 and C6 resulted in the inhibition of complement activation and thus the MAC is not formed on surfaces. Hence, complement degradation by fungal proteases leads to the inactivation of the complement effector functions.

A. benhamiae uses two separate mechanisms to evade host complement attack.

Here we identified two separate strategies of *A. benhamiae* to control human complement. *A. benhamiae* acquires human complement regulators and also secreted proteases to inactivate complement attack. To define, if these two complement escape mechanisms act simultaneously or in a timely separated manner, the cleavage kinetics was followed. C3b cleavage of Factor H coated and non coated conidia were examined. Therefore conidia were coated with Factor H and after washing Factor I and C3b were added. In comparison, C3b was directly added to non coated conidia. Supernatant of the samples was collected at different time points, separated by SDS PAGE, transferred to a membrane and assayed by Western blotting using C3 antiserum.

C3b cleavage was observed already after one minute of incubation with Factor H coated conidia, but not by non coated conidia (Figure 20 A, lanes 2, 5). The generated C3b products of 68, 43 and 41 kDa correspond to the pattern obtained by Factor I (Figure 20 A, lane 8). After ten minutes of incubation C3b fragments of 68, 43, 41, 35, 27 and 8 kDa appeared in samples of Factor H coated or non coated conidia (Figure 20 A, lane 4, 7). The generated cleavage fragments were comparable to that obtained by fungal secreted proteases (Figure 19 B). C3b cleavage fragments are pictured in Figure 20 B to demonstrate similarities and differences of C3b cleavage fragments derived from Factor H or from fungal secreted proteases.

The C3b cleavage kinetics indicate that *A. benhamiae* use human regulator Factor H in the initial phase in the first minutes of conidial contact with complement for complement

evasion. Later on, secreted fungal proteases efficiently cleave complement and thus inactivate complement attack. Thus, the two complement degradation strategies are independent from each other and can be separated in time.

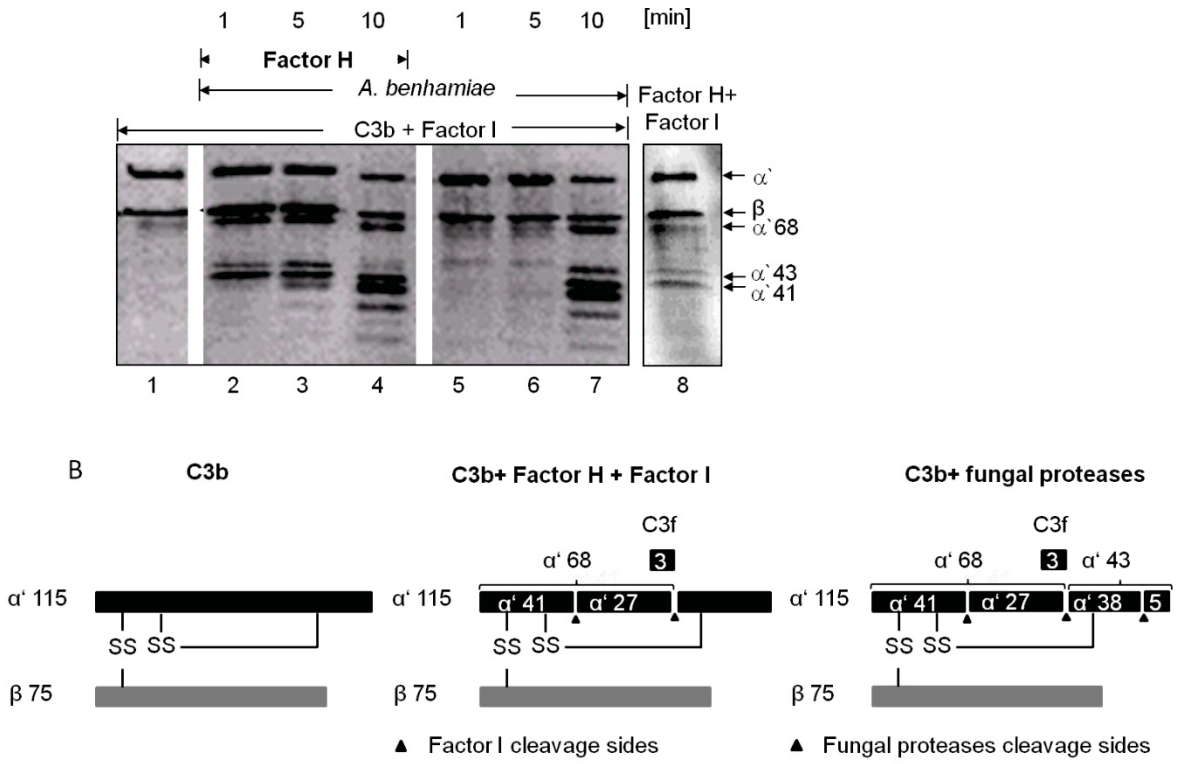


Figure 20: *A. benhamiae* uses two separate mechanisms to evade host complement attack.

(A) To separate Factor H mediated from fungal supernatant mediated C3b degradation, cleavage kinetics were done. C3b cleavage of Factor H coated and non coated conidia were examined. Therefore conidia were coated with Factor H and after washing Factor I and C3b were added. In comparison C3b was added to non coated conidia. Supernatants of the samples were collected after different time points, separated by SDS-PAGE, transferred to a membrane and assayed by Western Blotting. C3b cleavage was observed after one minute incubation by Factor H coated, but not by non coated conidia. The generated degradation products of 68 kDa, 43 kDa and 41 kDa correspond to the pattern obtained by Factor I. After ten minutes of incubation C3b fragment of 68 kDa, 43 kDa, 41 kDa, 35 kDa, 27 kDa and 8 kDa appear in samples using Factor H coated or non coated conidia. (B) The figure displays the proposed C3b cleavage pattern mediated by Factor H or fungal culture supernatant.

DISCUSSION

Scope of the study

Since prevalences of dermatophyte infections increase, much interest is given on infection and immune evasion strategies of these fungi. In comparison to systemic infecting fungi, like *C. albicans* or *A. fumigatus,* dermatophyte infections are limited on keratin-rich substrates, *e.g.* dead cell layers of the skin, hair or nails. Investigating immune evasion of dermatophytes may lead to new therapeutic targets and will expand our knowledge to defend this partial long-lasting infection with dermatophytes.

The complement system plays a crucial role in the clearance of infections. Until now, complement activity in the skin is poorly described. Human keratinocytes secrete several central complement components and regulators. The culture supernatant derived from human keratinocytes contains complement components, which are activated on surfaces and build lytic pores in membranes. Thus the secreted complement components act as a fully active complement system, which is described for the first time in this study.

It was asked whether the skin-derived complement also attacks *A. benhamiae*. The results of this work show that keratinocyte-derived C3 deposits on *A. benhamiae* conidia. Furthermore, dermatophyte opsonisation leads to increased adherence and phagocytosis by human monocytes. Thus skin cells provide active C3, which attack *A. benhamiae*.

Since other dermatophytes like *T.rubrum* was shown to exhibit resistance in serum and that dermatophyte infections are cleared by cell-mediated immune response, immune evasion of *A. benhamiae* was examined[120]. Two immune evasion strategies of *A. benhamiae* are described in this work. *A. benhamiae* immediately binds the human complement regulator Factor H on the surface after contact with complement in the skin to degrade the central complement component C3b and thus inhibits further complement activation and opsonophagocytosis. Later on, endogenous proteolytic activity of *A. benhamiae* takes over complement degradation and stops complement activation. *A. benhamiae* secreted proteases degrade also the anaphylatoxins C3a and C5a. Consequently the antifungal, chemoattractive and proinflammatory properties of these peptides are inhibited.

Taken together, this work provides an insight into the humoral immunity against dermatophytes. Moreover, complement immune escape strategies of *A. benhamiae* were identified and described to be time dependent.

Skin - a barrier with immunological properties

A. benhamiae causes superficial infection in humans and animals which leads to severe inflammation. The pathogenicity of dermatophytes is poorly understood. Moreover, nearly no attention was paid on interactions of dermatophytes with the human complement system.

To investigate the role of complement in skin, secretion and function of complement by human keratinocytes were examined. This study shows that keratinocytes secrete the central complement components, like C3 and the regulator Factor H (Figure 8). Keratinocytes continuously secrete C3 in contrast with Factor H which is generated in detectable amounts after IFN-γ stimulation.. This proves that Factor H is specifically secreted under inflammatory conditions. This finding was confirmed in several publications [107, 109, 150]. Furthermore keratinocytes secrete the complement regulator CFHR1 and the serine protease precursor plasminogen, which was shown for the first time (Figure 8). It was published that keratinocytes express and secrete additional complement components, like Factor B, Factor I and additionally, of the terminal pathway, like C5, C7, C8gamma and C9 [106-109]. Timar described in 2007, that C6, as well as C8 α and β mRNA are not constitutively expressed by human keratinocytes, but it was not excluded, that mRNA expression of these components can be induced. Thus keratinocytes secrete components to generate a fully active complement system. To prove if keratinocytes secrete complement components, which can be activated and perform effector function, keratinocyte-derived culture supernatant was analysed to form lytic pores in membranes. It was shown for the first time that keratinocyte-derived complement gets activated on surfaces. The activated complement system forms the terminal complement complex, which builds pores in unprotected membranes like rabbit erythrocytes, as shown in hemolysis assays (Figure 8 A). The staphylococci-derived complement inhibitor Sbi blocked hemolytic activity of the keratinocyte supernatant, thus demonstrating that hemolysis is caused exclusively by complement proteins (Figure 8 B). This effect is specific for keratinocytes as supernatant derived from the retinal pigment epithelial cell line ARPE lacks hemolytic activity (data not

shown). Hemolysis mediated by 60% keratinocyte supernatant was comparable to the hemolytic activity mediated by 7.5 % NHS. Since complement secretion by human keratinocytes is inducible by the proinflammatory cytokines like IFN-γ and TNF-α, complement activity may eventually increase after stimulation or infection of the cells[106, 107, 109, 151].

During the infection process, the dermatophyte *A. benhamiae* gets in contact with human keratinocytes and thus with keratinocyte-derived complement (Figure 21). If the secreted complement affects dermatophytes was a question of this dissertation.

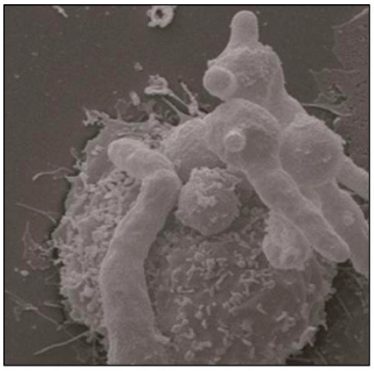


Figure 21: Dermatophyte interaction with a human keratinocyte

The picture shows a SEM micrograph of an *A. benhamiae* infection of a human keratinocyte.

Keratinocyte-derived complement attacks fungal surfaces, as shown by deposition of dermal C3 on resting *A. benhamiae* conidia (Figure 8 D). Increasing concentrations of C3 leads to better adherence and enhanced phagocytosis of *A. benhamiae* conidia by human monocytes (Figure 8 E). That C3 increases phagocytosis of microorganism via CR1 and CR3 on human monocytes was revealed by *e.g.* Schlesinger in 1990 [152, 153]. During complement activation the anaphylatoxin C5a is generated. This complement activation product C5a increases the CR3 expression on monocytes and may also increase recognition of opsonised fungal cells by monocytes[154].

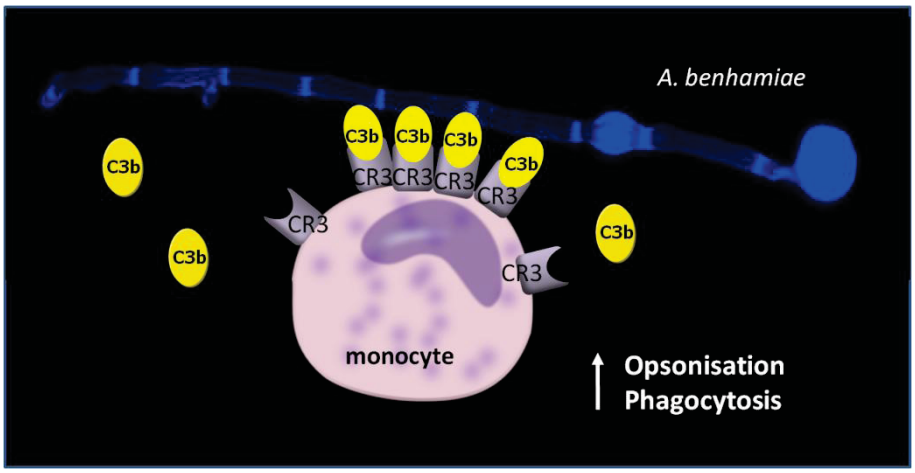


Figure 22: Keratinocyte derived C3b opsonises *A. benhamiae* conidia and increases adherence and phagocytosis by human monocytes

Keratinocyte derived C3b opsonises *A. benhamiae* cells and interacts with the CR3 on human monocytes. Following adherence and phagocytosis of *A. benhamiae* conidia by monocytes was increased.

A. benhamiae was displayed as hyphae stained in blue with Calcoflour white and visualised using laser scanning microscopy. It should be marked, that opsonisation, adherence and phagocytosis was measured using conidia. To give evidence about hyphae was not possible.

To sum up, this study demonstrates that human keratinocytes secrete central complement components and regulators. The keratinocyte derived complement is fully functionally active and attacks *A. benhamiae* conidia by opsonisation (Figure 22).

A. benhamiae binds host proteins to evade complement attack

Dermatophytes attached to human keratinocytes are exposed to a fully active host complement system. Nevertheless these pathogens still manifest in skin. How dermatophytes escape complement has not been investigated before this work.

Keratinocytes secrete the complement regulators Factor H and CFHR1 in the ECM of the skin. Thus *A. benhamiae* is exposed to these complement regulators. Interestingly, human Factor H and CFHR1 are acquired to the surface of *A. benhamiae* as shown by serum binding assays (Figure 9 D). The binding of the complement regulators was dose dependent as demonstrated in cell-based ELISA (Figure 9 A, B). The distribution of Factor H on the surfaces of *A. benhamiae* appears in clusters as illustrated by immune fluorescence (Figure 9 F).

Fungal binding regions and function of the *A. benhamiae*-bound complement regulator Factor H

The human complement regulator Factor H contacts *A. benhamiae* within two binding sites. One is located in SCR 4-7 and the second in the C-terminal SCR 15-20 of Factor H (Figure 10 A, B, D). The N-terminal SCR 1-4 of Factor H does not bind *A. benhamiae*. This is consistent with the fact that SCR 1-4, which is responsible for the cofactor activity for Factor I, is not blocked and can mediate C3b degradation [155]. *C. albicans* and *A. fumigatus* cause systemic infections in humans[156, 157]. Thus these fungi are exposed to serum complement. Both pathogens bind complement regulator Factor H and use the same contact regions, indicating common fungal binding sites of the serum- and tissue-derived Factor H [158, 159].

Factor H has complement regulatory activity as the protein acts as a cofactor for Factor I in the cleavage of C3b. The incubation of Factor H-coated *A. benhamiae* conidia with Factor I and C3b resulted in the formation of the typical Factor I C3b cleavage pattern of the α chain fragments with a mobility of 68, 43 and 41 kDa. Thus Factor H bound on the surface of *A. benhamiae* remains intact and helps to inactivate the complement component C3b (Figure 11). Following the effect of fungal-bound Factor H in inactivation of the opsonic properties of complement, adherence and phagocytosis of *A. benhamiae* conidia by innate immune cells were analysed. Factor H-coated conidia show decreased C3 deposition on the fungal surface as compared to non-coated conidia (Figure 12 A). Furthermore, the inhibition of C3b deposition can prevent C3 amplification on the fungal surface. Hence C3 convertase assembly on *A. benhamiae* is avoided and complement activation is blocked. C3b-mediated adherence and phagocytosis of Factor H-coated or non- coated *A. benhamiae* conidia to monocytes was also determined. Increasing concentrations of Factor H on the fungal surface resulted in decreasing adherence and in a lower uptake of conidia by monocytes. Higher concentration of 1.0 µg Factor H leads to a slight increase of phagocytosis (Figure 12 B). Human C3b interacts with the CR3 on human monocytes and initiate adherence and phagocytosis of C3b-opsonised cells (Figure 23). Factor H-mediated inactivation of C3b may block the interaction of C3b and CR3 on monocytes and thus decrease adherence and phagocytosis of *A. benhamiae* conidia. Since CR3 also detects the C3b degradation product iC3b, it is unclear that adherence and phagocytosis to monocytes is not increased[160].

However, it was shown that serum-opsonised zymosan down regulates the expression of CR3 on monocytes[161]. Thus keratinocyte complement proteins in combination with fungi may also decrease CR3 expression on monocytes. Furthermore, monocyte differentiation into dentritic cells results in a lack of the iC3b binding ability [162]. Since DCs act as immune cells in the skin, it should be mentioned that iC3b may not act as an opsonin for DC mediated phagocytosis.

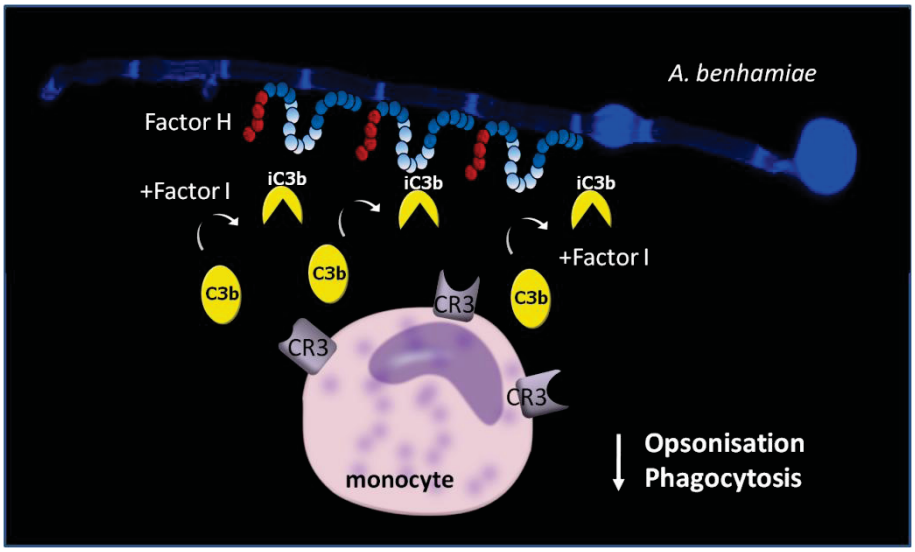


Figure 23: Factor H bound to *A. benhamiae* conidia mediate C3b degradation and consequently reduces opsonisation and phagocytosis by human monocytes

Host complement protein Factor H bind to the surface of *A. benhamiae* conidia and interacts with complement Factor I to degrade the opsonisin C3b. The interaction between C3b and the CR3 on human monocytes was inhibited and following adherence and phagocytosis of *A. benhamiae* conidia by monocytes was reduced.

A. benhamiae was displayed as hyphae stained in blue with Calcoflour white and visualised using laser scanning microscopy. Factor H mediated C3b degradation on the surface of *A. benhamiae*, opsonisation, adherence and phagocytosis studies was shown for fungal conidia, not for hyphae, which should be note during interpreting this illustration. Factor H was pictured using twenty balls standing for the 20 SCRs. Dark blue balls clarify binding regions of Factor H to *A benhamiae,* red balls mark the region SCR 1-4, which hold cofactor activity.

Fungal binding regions and function of the *A. benhamiae*-bound complement regulator CFHR1

The human CFHR1 protein is part of the Factor H family and exhibits complement regulatory properties. Using CFHR1 deletion mutants the binding region of CFHR1 to *A. benhamiae* was localized within SCR 1-2 and SCR 3-5. Both deletion mutants bind with lower intensity to the fungus than the intact protein. This shows that in intact CFHR1 both fragments contribute to binding (Figure 10 C, D). CFHR1 is a complement regulator that acts on C5 convertase and thus blocks assembly of the terminal complement complex, which forms lytic pores in membranes[78]. Since TCC is assembled, but not lytic on fungal or gram positive bacterial

surfaces, other functions of TCC are proposed[163, 164]. Consequently *A. benhamiae*-bound CFHR1 may decrease complement activation on conidial surfaces. Inhibition of terminal complement activation prevents generation of the potent chemoattractive and anaphylatoxic agent C5a. Since C5a induces phagocytes, mast cells, Langerhans cells, melanocytes and under inflammatory conditions keratinocytes and exhibits proinflammatory and chemotactic properties, C5a formation has to be avoided by *A. benhamiae* [165, 166]

Function of the *A. benhamiae*-bound human coagulation proenzyme plasminogen

A. benhamiae binds the coagulation proenzyme plasminogen on its surface. Plasminogen is converted to the active serine protease plasmin by human plasminogen activators like urokinase (uPa) or the tissue plaminogen activator (tPa), but also by pathogen-derived activators like the streptokinase (*Streptococcus spec.*) or staphylokinase (*Staphylococcus spec.*)[167-169] Pathogen-bound plasmin exhibits functions in ECM degradation and cell migration, which may assist colonisation of *A. benhamiae* in the skin [158, 170]. Furthermore plasmin affects complement degradation of C1, C2, C3 and C4[171]. Plasmin cleaves the complement C3 and thus needs no preactivation of the C3 component to C3b for degradation[172]. In comparison, Factor H exclusively assists the cleavage of the activation product C3b. Cleavage of C3 instead of C3b is beneficial as the production of the anaphylatoxin C3a during C3 activation is prevented.

This study shows that *A. benhamiae* blocks complement activation by binding the central host complement regulators Factor H, CFHR1 and plasminogen to its surface. Pathogen-attached regulators control complement and thus inhibit complement activation. The binding of human complement regulators to control complement activation was also shown for systemic infecting fungi, like *C. albicans* and *A. fumigatus* [74, 159, 173]. These fungi get in contact with serum. Tissues like the skin exhibit another biological niche and show other immunological properties. Complement regulation is shown for the first time for dermatophytes, which are exclusively in contact with the human skin.

In addition, *A. benhamiae*-bound host proteins may increase virulence of the dermatophyte by inhibiting complement activation and by degrading extracellular matrix components to facilitate tissue damage and migration. Moreover, binding of host proteins to the fungal surface may prevent immune recognition of fungal cell wall components by pattern

recognition receptors, like dectin or mannose receptors on keratinocytes and phagocytes and thus function in molecular mimicry [174, 175, 176].

A. benhamiae secretes complement-degrading proteases to evade complement attack

A. benhamiae secretes proteases

Dermatophytes secrete a high level of proteases during growth on keratin [133]. Genome analysis of *A. benhamiae* revealed 235 predicted protease-encoding genes and 87 of these sequences posses a putative secretion signal. Twenty five proteases were specific to the *Onygenales* as analysed using MEROPS, NCBI and Broad Institute databases. Nine of these twenty five proteases have a secretion signal (unpublished data). Secretome analysis of an *A. benhamiae* culture demonstrated the existence of three subtilisin-like proteases (Sub 3, Sub 4 and Sub 7), three fungalysine type metalloproteases (Mep 1, Mep 3, Mep 4), the leucine aminopeptidases Lap 1 and Lap 2, as well as the dipeptidylpeptidases DppIV and DppV.

A. benhamiae-secreted proteases, such as Subtilisin 3, degrade keratin

Secretion profiles of the dermatophyte *A. benhamiae* shows increased expression of Subtilisin 3 in cultures supplemented with keratin. To examine the role of Subtilisin 3 in pathogenesis and immune escape strategies, a Subtilisin 3 disruption mutant was generated from the *A. benhamiae* Ku 70 wild type, which was kindly provided by Anke Burmester (Department Molecular and Applied Microbiology, Leibniz-Institute for Natural Product Research and Infection Biology-HKI, Jena)

The first contact of dermatophytes with its hosts is mediated by the contact of the fungi with the cornified layers of the skin. The outer layers of the skin mainly consists of keratin, but also comprises different cross-linked proteins like elafin, filagrin, involucrin, loricrin and small proline rich proteins[177, 178]. Dermatophytes are shown to degrade the keratin-protein network of the upper layers of the skin. Specifically, *A. benhamiae* Subtilisin 3 degrades keratin[133]. Culture supernatants of the *A. benhamiae* wild type and the Subtilisin 3 disruption mutant were incubated with a chromogen-charged keratin substrate to quantify proteolytic activity. Results showed that medium supplemented with keratin induces *A. benhamiae* to

secrete proteases with keratinolytic activity (Figure 13). Culture supernatant of the Subtilisin 3 disruption mutant shows few degradation of the keratin substrate, which confirms the role of Subtilisin 3 in keratin degradation. Subtilisin 3 and Subtilisin 4 of the dermatophyte *T. rubrum* are also highly potent keratin degrading enzymes and hence suggested as virulence factors[133].

A. benhamiae secretes complement-degrading proteases

Activation of complement in the skin by *A. benhamiae* is shown above in this study (Figure 8). Since the complement system is a crucial part of the innate immune defense, *A. benhamiae* must strictly control complement activation to survive on skin. To investigate if *A. benhamiae*-secreted proteases degrade and thus inhibit complement, supernatant derived from *A. benhamiae* was incubated with the complement components C3b, C3, C4b, C4, C5 and C6. Western Blot analysis showed degradation of all the tested complement proteins (Figure 14 A-F). C3b consist of a 115 kDa α-chain and a 75 kDa β-chain.

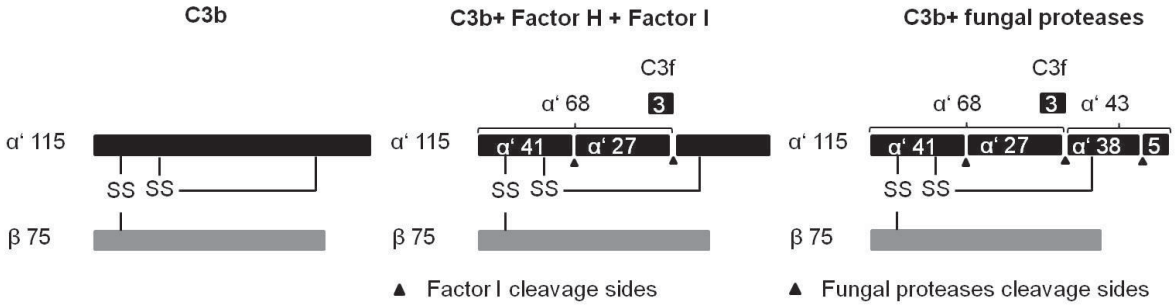


Figure 24: Cleavage of C3b by FactorH/FactorI or by *A. benhamiae*-derived proteases

C3b consist of a 115 kDa α-chain and a 75 kDa β-chain. C3b inactivation occurs by Factor H-assisted Factor I cleavage of the C3b α-chain to 68- and 43-kDa fragments by the release of a 3 kDa fragment named C3f. The 68 kDa fragment is further cleaved to 41- and 27-kDa fragments. The C3b cleavage pattern obtained from fungal proteases differs with additional cleavage products of 38 and 5 kDa. This effect may be due to an additional cleavage site within the α-chain fragment of 43 kDa by the proteases of *A. benhamiae*.

C3b cleavage pattern obtained from fungal proteases differs to those mediated by Factor I in the presence of Factor H. Although C3b α chain cleavage products of 68 kDa, 43 kDa and 41 kDa and 27 kDa occur in both cleavage patterns, additional cleavage products of 38 and 5 kDa were observed during cleavage of C3b by fungal proteases[179]. This effect may due to an additional cleavage site within the α-chain fragment of 43 kDa by the proteases of *A. benhamiae* (Figure 24). Fungal proteases also mediate C3 cleavage. The generated C3 fragments are comparable to the formed C3b fragments, indicating similar cleavage sites.

C4b and C4 are cleaved by fungal proteases. C4b consists of an 82 kDa α-chain, a 75 kDa β-chain and a 40 kDa γ-chain. Fungal proteases might have cleaved the α-chain and the γ-chain of C4b, because of their loss of intensity shown in Western Blotting.. The α-chain of C4b is cleaved to main products of 64 kDa, 60 kDa, 54 kDa, 50 kDa and 18 kDa. The γ-chain of C4b is cleaved to a 38 kDa fragment. Proposed cleavage pattern of C4b derived from *A benhamiae* secreted proteases resulted also in the generation of small 4 kDa and 6 kDa fragments (Figure 25). Host complement C4BP acts as cofactor for Factor I in C4b inactivation[180].Factor I cleaves the 82 kDa α-chain of C4b in 64 kDa (iC4b), 46 kDa (C4d) and 18 kDa (C4c) fragments. Excluding the 64 kDa fragment of the α-chain of C4b, cleavage products derived from C4BP/Factor I and fungal proteases differ, suggesting different cleavage sites within C4b. C4 cleavage by fungal proteases resulted in fewer fragments compared to the C4b cleavage of the control, indicating reduced susceptibility of C4 for *A. benhamiae* secreted proteases and the necessity for C4 precleavage to C4b for optimal fungal protease accessibility.

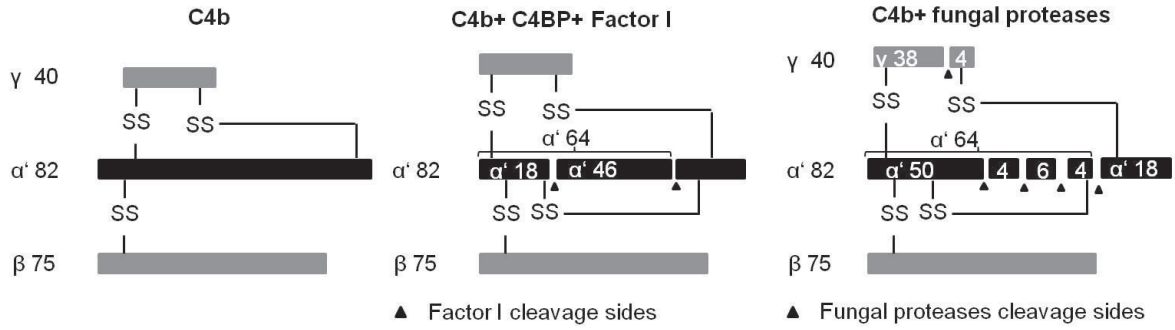


Figure 25: Cleavage of C4b by C4BP/Factor I or *A. benhamiae* derived proteases.

C4b consists of a α-, β- and a γ-chain. Host complement C4BP acts as cofactor for Factor I in C4b inactivation. Factor I cleaves the 82 kDa α-chain of C4b to 64 (iC4b), 46 (C4d) and 18 kDa (C4c) fragments. Fungal proteases attack the α-chain of C4b and products of 64, 60, 54, 50 and 18 kDa were formed. The γ-chain of C4b is cleaved to a 38 kDa fragment. Excluding the 64 kDa fragment of the α-chain of C4b, cleavage products derived from C4BP/Factor I and fungal proteases differ. C4b cleavage products derived from *A. benhamiae* secreted proteases also resulted in the generation of small 4 kDa and 6 kDa fragments.

Also, C5 and C6 are cleaved by *A. benhamiae*-derived proteases. C5 cleavage was observed by cysteine proteases of the gram negative bacterium *Porphyromona gingivalis*. The cleavage products are not described in detail[181]. Secreted aspartyl proteases of the yeast *C.albicans* degrade C5, which consequently confers escape from the complement attack[149]. The degradation and thus inactivation of C5 results to non-formation of the surface-bound membrane attack complex which consequently leads to inhibition of the terminal pathway. C6 bind C5b during formation of the lytic terminal complement complex. Degradation of C6

by *A. benhamiae*-derived proteases helps to block the terminal pathway and TCC formation. *A. benhamiae* is the first pathogen, which is described to cleave the sixth component of complement. C6 is similar in structure to complement C7. Both proteins share 33.5% identical residues including all 56 cysteine residues[182]. Since cysteine proteases of *P.gingivalis* cleave C5, cysteine-rich C6 and C7 may also be degraded by cysteine specific proteases present in the supernatant of *A. benhamiae*.

The peptides C3a and C5a are formed during complement activation and exhibit several inflammatory, chemotactic and antifungal properties. C3a and C5a were degraded by *A. benhamiae*-derived proteases (Figure 14 G, H). C3a degradation by pathogens is new and has not been described before. Human mast cell-derived chymase degrades C3a in vivo. Chymase (Merops classification: S01.140) belongs to the serine peptidase family of chymotrypsin peptidases. C5a peptidases are reported for group A and virulent group G *Streptococci*[183, 184]. Since Chymase and the C5 peptidase belong to the group of serine proteases, *A. benhamiae*-released serine proteases, like the Subtilisins, may specifically mediate C3a and C5a degradation.

Subtilisin 3 plays an important role in complement degradation

Subtilisin 3 was identified in the culture supernatant of *A. benhamiae* during growth in medium supplemented with keratin. Since serine proteases, such as Factor I, exhibit specificity for complement degradation, the function of the fungal serine protease Subtilisin 3 in complement cleavage was studied. Cleavage assays using culture supernatant of the Subtilisin 3 disruption mutant of *A. benhamiae* showed lesser degradation of C3b and nearly no degradation of C5 and the anaphylatoxin C3a (Figure 15). However, C3b degradation was not totally blocked by Subtilisin 3 disruption of *A. benhamiae* suggesting that other proteases are also involved in complement degradation. Nevertheless, these results demonstrate an important role of Subtilisin 3 in C5b and C3a inactivation.

Degradation of complement by *A. benhamiae* secreted proteases results in the loss of complement functionality

To test if the cleavage of complement proteins by *A. benhamiae* proteases leads to the inhibition of the formation of the lytic terminal complement complex on membranes, hemolysis assay using rabbit erythrocytes as a complement activator surface was done.

Culture supernatant derived from *A. benhamiae* blocked complement activation on rabbit erythrocytes. The effect was specific for *A. benhamiae,* as culture supernatant derived from the non pathogenic yeast *S. cerevisiae* lacked complement inhibitor activity (Figure 16 A). Since *A. benhamiae* secreted proteases degrade C3 and C5, the complement activation is inhibited on C3 convertase and C5 convertase level. Thus TCC formation on surfaces is prevented. The activity of secreted proteases of *A. benhamiae* was mainly inhibited by the serine protease inhibitor PMSF (Figure 16 B), which shows that serine proteases are involved in TCC inhibition. Furthermore, this indicates that the *A. benhamiae* secreted serine protease Subtilisin 3 acts in complement inactivation. To determine this, culture supernatant of the Subtilisin 3 disruption mutant was analysed in hemolysis assays[133]. A loss of Subtilisin 3 led to the reduced hemolysis inhibition (Figure 16 C). Thus, Subtilisin 3 prevents TCC formation. Since hemolysis is not totally blocked by Subtilisin 3, additional proteases in the culture supernatant of *A. benhamiae* might be involved in complement inhibition.

To examine, if secreted fungal proteases affect the opsonisic activity of C3b, culture supernatant derived from the *A. benhamiae* wild type or the Subtilisin 3 disruption mutant was incubated with C3b and added to conidia. Fungal proteases derived from the wild type inhibited deposition of C3 on conidia. In comparison, the loss of Subtilisin 3 in the fungal culture supernatant led to higher C3 deposition rates (Figure 17 A). C3b deposition on fungal surfaces opsonizes conidia for phagocytes. To measure adherence and phagocytosis of *A. benhamiae* conidia by human monocytes, C3b was treated with culture supernatant derived from the *A. benhamiae* wild type and then conidia were added before incubating the samples with monocytes. Increasing concentrations of fungal wild type culture supernatant resulted in decreasing adherence and in lower phagocytosis rates (Figure 17 B). Thus, by degrading C3b, secreted fungal proteases inhibit its opsonic activity and C3b-mediated adherence and phagocytosis by monocytes is blocked (Figure 26). Particularly, Subtilisin 3 is relevant in avoiding deposition of C3b on fungal surfaces and consequently blocking opsonophagocytosis. Consistent with these results, a recent study on a cell envelope-bound Subtilisin (SSU0757) of *Streptococcus suis* described its ability to mediate resistance of *Streptococcus* in whole blood. The study suggests that the protease degrades human serum proteins with bactericidial activity or opsonins and thus Subtilisins are involved in phagocytosis of immune cells[185]. Harris *et al*. reported about the Subtilisin CspA of *Streptococcus agalactiae*, which mediates resistance to opsonophagocytic killing by

neutrophils and mediates virulence in neonatal rat sepsis model[186]. Altogether, Subtilisins play a pivotal role in the resistance against complement attack.

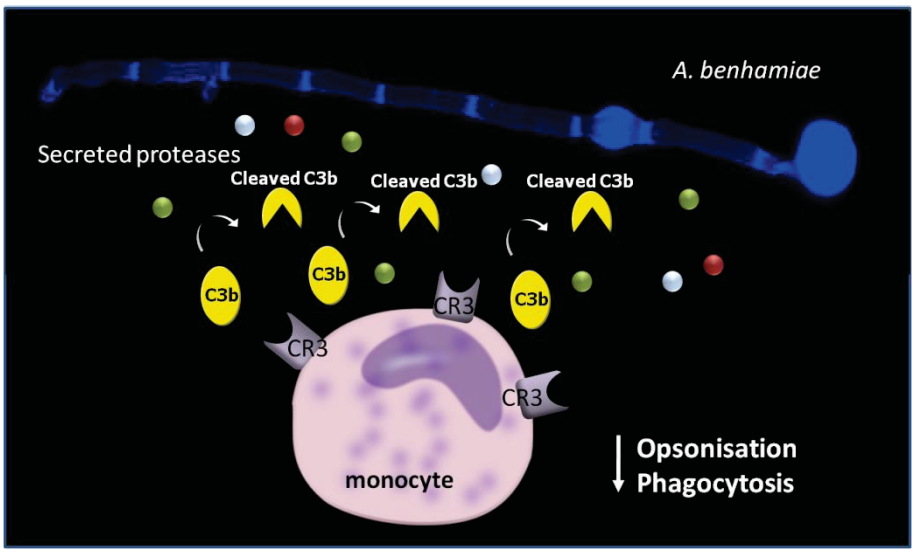


Figure 26: *A. benhamiae* secreted proteases prevent C3b-mediated opsonisation and phagocytosis by human monocytes

A. benhamiae-derived culture supernatant was incubated with C3b and added to resting fungal conidia. Analysis of the C3 deposition on the conidial surface shows reduction of the opsonin C3b in the presence of secreted fungal proteases. Thereafter, adherence and phagocytosis of *A. benhamiae* conidia by monocytes was reduced.

A. benhamiae is shown as hyphae stained in blue with Calcoflour white and visualised using laser scanning microscopy. C3b mediated opsonisation, adherence and phagocytosis studies were shown for *A. benhamiae* conidia, not for hyphae, which should be noted when interpreting this illustration. Secreted fungal proteases are illustrated using colored balls.

A. benhamiae-secreted proteases inactivate C3a and C5a

Activation of complement leads to the formation of the anaphylatoxins C3a, C4a and C5a. C3a is a potent antimicrobial peptide and its activity is comparable to the cathelicidin LL-37. Furthermore, C3a stimulates the production of reactive oxygen species by human PMNs. *A. benhamiae*-derived culture supernatant degrades the complement fragment C3a. In particular, Subtilisin 3 cleaves C3a. To analyse if the degradation of C3a by *A. benhamiae* derived proteases affects the function of C3a, the antifungal activity and the ability to stimulate ROS production of PMNs were determined.

In order to test if C3a treated with culture supernatant of the *A. benhamiae* wild type or the Subtilisin 3 disruption mutant keeps its antifungal properties, fungal killing assays were performed. C3a added to *C. albicans* reduced fungal growth. C3a-supplemented culture supernatant of *A. benhamiae* wild type resulted in increased survivial of *C. albicans*.

C3a-supplemented culture supernatant of the *A. benhamiae* Subtilisin 3 disruption mutant showed also significant survival of the fungal cells, but was lower in comparison to the activity of wild type culture supernatant (Figure 18 A). Therefore, C3a cleavage by *A. benhamiae*-derived proteases, in particular Subtilisin 3, inactivates the antifungal properties of C3a. However, the *C. albicans* growth in culture medium was increased by a combination of C3a and *A. benhamiae*-derived proteases. Investigation of stimulation of the *C. albicans* growth by *A. benhamiae*-derived culture supernatant is beyond the scope of this study

C3a induces ROS production in human neutrophils[187]. ROS production of freshly-isolated PMNs was quantified after induction with C3a or after pretreatment with C3a-supplemented fungal culture supernatant derived from the *A. benhamiae* wild type or Subtilisin 3 disruption mutant. Purified C3a increased the ROS release. In comparison, treatment of C3a with the culture supernatant of the *A. benhamiae* wild type or Subtilisin 3 disruption mutant repressed ROS production by freshly isolated blood derived PMNs. C3a treated with the culture supernatant of the Subtilisin 3 disruption mutant resulted in lesser reduction of ROS by human PMNs than C3a treated with the culture supernatant of the wild type (Figure 18 B). Evidently, C3a activity in ROS induction of PMNs is inactivated by addition of *A. benhamiae*-derived proteases. Thereby Subtilisin 3 plays an important role in the inhibition of C3a-mediated ROS stimulation of PMNs.

In conclusion, *A. benhamiae*-derived proteases block the antifungal and proinflammatory functions of C3a. Subtilisin 3 is one of the main C3adegrading proteases since it reduces C3a mediated antifungal activity by 75 % and ROS induction of PMNs by 60 %.

Since the proteases in the culture supernatant of *A. benhamiae* cleave C5a, effects on the activity of C5a were investigated. C5a induces the release of the proinflammatory cytokines IL 6, IL 8 and TNF-α from human monocytes. To investigate secretion of these cytokines by human monocytes after stimulation with C5a or C5a which was pretreated with fungal culture supernatant, cytokine assays were performed. Monocytes treated with C5a produced higher levels of IL-6, IL-8 and TNF-αin comparison to the control. Preincubation of C5a with the culture supernatant of *A. benhamiae* resulted in complete reduction of IL-6, IL-8 and TNF-α secretion by human monocytes to the level of control (Figure 19 A-C). Since the Subtilisins CspA from *S. agalactiae* and SufA isolated from *Finegoldia magna* are known

to cleave CXC chemokines, the influence of Subtilisins on chemokines were also tested. Although CspA and SufA do not cleave IL-8, the reduction of IL-8 production under the level of control could also be explained by the degradation of the chemokine through *A. benhamiae* derived Subtilisins[188, 189]. The cytokines TNF-α, IL-6 and the chemokine IL-8 act as proinflammatory mediators. The inhibition of TNF-α and IL-6 influences, amongst others, the production of acute phase proteins. IL-8 is a major attractant for neutrophils, inhibition of IL-8 production may prevent accumulation of PMN in the skin. Viability assays showed that different concentrations of keratin medium or culture supernatant of *A. benhamiae* had no effect on the viability of human monocytes which might have caused the decrease of secreted cytokines and chemokines (data not shown).

In addition, C5a is a potent chemoattractant for human neutrophils. Using neutrophil migration assays, the function of C5a or C5a treated with culture supernatant of *A. benhamiae* on freshly isolated PMNs was analysed. C5a and 5 % activated serum increased neutrophil migration. C5a incubation with culture supernatant of *A. benhamiae* resulted in complete inhibition of neutrophil migration (Figure 19 D). No effects of the fungal culture supernatant on neutrophil migration were observed (data not shown). Since neutrophil migration was blocked with the value under that of the level of the control, the influence of the C5a cleavage products in combination with the fungal culture supernatant on human neutrophils should be further analysed.

Taken together, *A. benhamiae*-derived secreted proteases degrade and inactivate C5a, which affects monocyte stimulation and neutrophil chemotaxis. Since C5a functions contribute to a proinflammatory response, *A. benhamiae* prevents the immune response by secreting C5a-degrading proteases.

Fungal secreted proteases act as potent virulence factors. *C. albicans* expresses aspartyl proteases (SAPs) during oral and cutanous candidosis *in vivo*[190]. The family of ten SAPs contributes to nutrient acquisition, facilitates adhesion and tissue invasion and degrades cells and molecules of the host immune system[191]. *A. fumigatus* expresses different classes of proteases, which are implicated with virulence, such as the alkaline serine proteases (Alp 1 and Alp 2), metalloproteases (Mep), aspartic proteases (Pep 1 and Pep 2), dipeptidyl-peptidases (Dpp IV and Dpp V), phospholipase C and phospholipase B (Plb 1 and Plb 2)[132, 192, 193]. Serine proteases, in particular, play an important role in pathogenesis as reported, for

instance, for *A.fumigatus* Alp 1, which was shown to degrade immune components[194]. Furthermore, the group of subtilisins was reported to interfere with immune components. The C5a peptidase on the surface of group A *Streptococci* degrades the anaphylatoxin C5a (S08.020, MEROPS classification) and TPP II (S08.090) which is found in mammals and has a role in the processing of antigens [195, *183*].

Taken together, *A. benhamiae*-derived proteases degrade complement and thereby inactivate complement functions by inhibiting the formation of the terminal complement complex, blocking opsonophagocytosis and inhibiting the proinflammatory, chemotactic and antifungal properties of C3a and C5a. *A. benhamiae*-secreted serine protease Subtilisin 3 is described for the first time as a main complement-degrading protease in the culture supernatant of *A. benhamiae*. Thus, *A. benhamiae* gets attacked by the skin-derived complement system and evades the attack by endogenous proteolytic activity. This study is the first description of *A. benhamiae* skin complement evasion strategies.

A. benhamiae uses two subsequent acting strategies to evade complement attack

A.benhamiae is specialized to infect human skin and thus encounters the complement system. To evade the complement system, *A. benhamiae* developed two separate mechanisms. The fungus binds the host complement regulator Factor H to mediate C3b inactivation and thus block opsonophagocytosis. In addition, *A. benhamiae* secretes potent proteases, which degrade complement components and thus inhibit complement activation. To define if this two complement escape mechanisms act simultaneously or in a timely separated manner, C3b cleavage kinetics clarified the differences between Factor H-coated and non-coated conidia in complement degradation during a time lapse study.

C3b cleavage was observed already after 1 minute incubation by Factor H-coated conidia, but not by non-coated conidia (Figure 20 A, lane 2, 5). The generated C3b products of 68 kDa, 43 kDa and 41 kDa correspond to the pattern obtained with Factor I (Figure 20 A, lane 8). The result demonstrates that Factor H-coated conidia in comparison to non-coated conidia cleave C3b and are protected against opsonophagocytosis. Furthermore, complement activation was blocked on the C3b level and formation of the anaphylatoxins C3a and C5a, as well as the formation of the TCC was inhibited within the first minute of

complement attack. After 10 minutes of incubation, C3b fragments of 68 kDa, 43 kDa, 41 kDa, 35 kDa, 27 kDa and 8 kDa appeared in samples using Factor H-coated and also non-coated conidia (Figure 20 A, lane 4, 7). The generated cleavage fragments are comparable to the pattern obtained by fungal secreted proteases (Figure 20 B). This means that proteases are secreted ten minutes later and thus mediate time-delayed complement degradation and inactivation. The C3b cleavage kinetics indicates that *A. benhamiae* uses human regulator Factor H in the first minutes of conidial contact with complement for complement evasion. Later on, secreted fungal proteases efficiently cleave complement and thus inactivate complement attack. This theory is confirmed by the fact that Factor H in fluid phase is cleaved by secreted Subtilisin 3 (data not shown). In comparison, immobilized Factor H is not attacked by Subtilisin 3. For this reason Factor H binds to the surface of *A. benhamiae* before fungal proteases are secreted. Thus, both complement degradation strategies are independent events and can be separated in time.

Conclusions

In this dissertation I show that human keratinocytes secrete a functional complement system and thus form an intact humoral innate immune defense system in skin. The complement system displays an important defense system against infecting microorganism. Activation of the complement system leads to the generation of potent and damaging activation products that ultimately result in the clearance of infectious microbes by opsonisation of microbes for phagocytosis and by the formation of the lytic terminal complement complex in membranes. Keratinocyte derived complement component C3b deposits on the conidial surface of the dermatophyte *A. benhamiae*. The opsonisation of the conidia leads to an increase of phagocytosis by human monocytes in absence of complement regulatory proteins.

Dermatophytes resist complement attack. I characterized this complement resistance and demonstrate that *A. benhamiae* uses two independent and timely separated strategies to control complement attack. Immediately upon infection the fungus acquires several host complement regulators, such as Factor H, CFHR1 and plasminogen to its surface. Fungal bound Factor H mediates cleavage of the central complement component C3b. This degradation by Factor I leads to an inactivation of C3b and thus to the inhibition of the whole complement cascade.

Furthermore I showed in this study that *A. benhamiae* secretes complement degrading proteases. Complement cleavage by these fungal proteases leads to an inactivation of the complement system on the level of the C3- and the C5 convertases.

I identified the serine proteases Subtilisin 3 as a major complement degrading protease, which is secreted by *A. benhamiae* into the culture supernatant. This protease cleaves the complement components C3b, C5b and C3a. Consequently complement activation on the fungal surfaces is blocked and in addition the antifungal and proinflammatory effector functions of the complement peptide C3a are inhibited.

Furthermore I show in this work that the immune escape strategies of *A. benhamiae* act at different times upon infection. Complement inactivation by acquiring human complement regulators occur immediately after contact with complement. Later on endogenous

proteolytic activity of *A. benhamiae* takes over the role of complement degradation and inactivation (Figure 27).

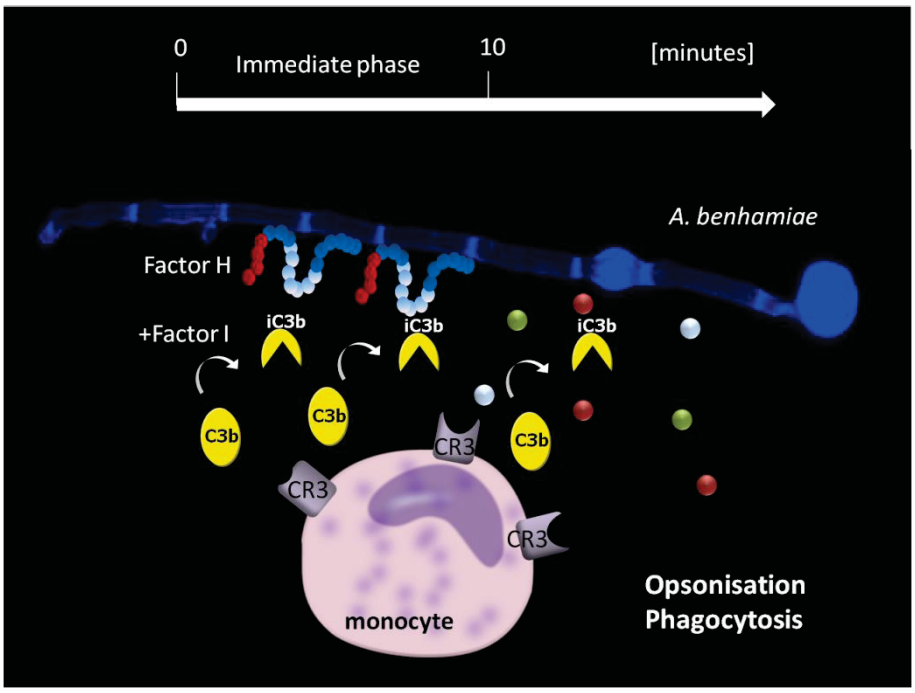


Figure 27: *A. benhamiae* uses two time dependent strategies to escape complement attack

Time lapse of the C3 cleavage by *A. benhamiae* conidia showed that the fungus immediately after contact with complement proteins binds human complement regulators to degrade C3b. Later on secreted fungal proteases take over the role in complement cleavage. *A. benhamiae* was displayed as hyphae stained in blue with Calcoflour white and visualised using laser scanning microscopy.

SUMMARY

Dermatophytes cause superficial infections of the upper layers of the skin, nails or hair. Although about 10-20% of the world population is affected the pathogenesis and the immunology against these fungi is poorly understood and requires further investigations to facilitate diagnostics and adapt therapy to dermatophytes.

Dermatophytes cause superficial infections. To colonise the skin, dermatophytes have to evade the local immune system. The skin is mainly composed of keratinocytes, which are immunocompetent cells. This work shows that keratinocytes secrete a functional complement system in skin. The complement system displays an important defense mechanism against infecting microorganism. Complement activation generates C3b that opsonises surfaces in order to allow phagocytosis. Keratinocyte derived C3b deposits on the conidial surface of the dermatophyte *A. benhamiae*. The opsonisation of the conidia leads to an increase of phagocytosis by human monocytes.

Former studies showed that dermatophytes exhibit resistence against serum derived complement. Hence dermatophytes developed strategies to evade complement attack. This work identified two separate acting complement escape strategies of the dermatophyte *A. benhamiae*. Initially the fungus aquires keratinocyte derived complement regulatory proteins, such as Factor H and CFHR1 on its surface. Fungal bound Factor H mediate cleavage of the central complement component C3b. The degradation leads to an inactivation of C3b and thus to the decrease of opsonophagocytosis of the conidia. Furthermore C3b cleavage results in the inhibition of the following complement cascade enzymes and avoids the formation of the anaphylatoxins C3a, C4a and C5a, as well as the formation of the lytic terminal complement complex.

The hyphal form of *A. benhamiae* secretes after in vitro stimulation with keratin a multiplicity of proteases in the culture supernatant. These proteases are associated with the virulence of pathogenic dermatophytes. This study shows that *A. benhamiae* secreted proteases cleave and inactivate several complement components. The *A. benhamiae* mediated cleavage of the complement proteins C3, C3b, C4, C4b, C5, C6 and of the anaphylatoxins C3a and C5a leads to the inactivation of the respective complement effector function. The proteolytic activity of *A. benhamiae* inactivates the opsonic properties of C3b.

Thus the phagocytosis of conidia is reduced. The cleavage of C3, C4, C5 and C6 blocks the formation of the terminal complement complex on surfaces and consequently the lysis of complement sensitive cells. Moreover *A. benhamiae* derived proteases inhibit the antifungal properties of C3a, as well as the C3a mediated release of reactive oxygen species by human granulocytes. The endogenous proteolytic activity of *A. benhamiae* inhibits the C5a mediated chemoattractive effect on granulocytes, as well as the C5a induced release of cytokines by human monocytes.

The serine proteases Subtilisin 3 was identified as major complement degrading protease in the supernatant of an *A. benhamiae* culture. This protease cleaves the complement components C3b, C5b and C3a. Thereby Subtilisin 3 inactivates C3a and C5a properties in complement activation, opsonisation and inflammation, as well as the antifungal properties of C3a.

Complement inactivation by acquiring human complement regulators or endogenous proteolytic activity of *A. benhamiae* are timely separated mechanisms. In the first minutes of the contact with keratinocyte derived complement, human derived complement regulators on the surface of *A. benhamiae* mediate complement inactivation. When synthesis of fungal proteases is induced, the endogenous proteolytic activity of *A. benhamiae* takes over the role of complement degradation and inactivation.

ZUSAMMENFASSUNG

Dermatophyten verursachen oberflächliche Infektionen des stratum corneum der Haut, der Nägel oder Haare. Etwa 10-20% der Weltbevölkerung sind von Dermatomykosen betroffen. Trotzdem sind die Pathogenese und die immunologische Antwort des Menschen auf Dermatophyteninfektionen nur ansatzweise beschrieben und bedürfen weiterer Forschung, um die Diagnostik der Hautpilzerkrankungen zu erleichtern und die Therapien der Erkrankungen auf den Pilz abzustimmen.

Dermatophyten manifestieren sich in den keratinreichen Schichten der Hornhaut und bilden normalerweise keine systemischen Infektionen aus. Um die Haut zu besiedeln müssen Dermatophyten das lokale Immunsystem umgehen. Die Haut wird hauptsächlich durch verschiedene Differenzierungsstadien der Keratinozyten aufgebaut. Keratinozyten sind immunologisch potente Zellen. Unter anderem wurde in dieser Arbeit gezeigt, dass diese Zellen diverse Komplementkomponenten in die Haut sezernieren. Das durch Keratinozyten sezernierte Komplement agiert in einem System und wird auf ungeschützten Oberflächen aktiviert. Die Komplementkomponente C3b opsonisiert Oberflächen für die Phagozytose. Mit dieser Arbeit konnte gezeigt werden, dass sich C3b auf Konidien des Dermatophyten *A. benhamiae* ablagert. Folglich wird die Phagozytose jener opsonisierten Konidien durch humane Monozyten erhöht.

Frühere Studien zeigten, dass Dermatophyten resistent gegen Serumkomplement sind. Demzufolge bilden Dermatophyen Strategien aus, um dem Angriff durch Komplement zu entgehen. In dieser Arbeit wurden zwei unabhängige Komplementevasionsstrategien des Dermatophyten *A. benhamiae* identifiziert. Zunächst bindet der Pilz Komplementregulatoren, wie Faktor H oder CFHR1, die durch Keratinozyten sekretiert werden. Ist Faktor H an die Oberfläche von Konidien gebunden, so spaltet es mittels Faktor I das Opsonisin C3b. Die Spaltung bewirkt eine Inaktivierung des C3b und damit eine Reduzierung der Opsonophagozytose der Konidien von *A. benhamiae*. Des Weiteren wird durch die C3b Spaltung die Aktivierung der folgenden Kaskadenenzyme unterbunden und damit die Freisetzung der Anaphylatoxine C3a, C4a und C5a, sowie der Aufbau des Membranangriffskomplexes verhindert. Im Gegensatz zur konidialen Form von *A. benhamiae* sekretiert die hyphale Form des Pilzes nach in vitro Stimulation mit Keratin eine Vielzahl an Proteasen in den Kulturüberstand. Diese Proteasen werden vielfach mit der Virulenz der

pathogenen Dermatophyten in Zusammenhang gebracht. Diese Studie zeigt, dass sekretierte Proteasen des Dermatophyten *A. benhamiae* humane Komplementproteine spalten und inaktivieren. Die Spaltung der Komplementproteine C3, C3b, C4, C4b, C5, C6 und der Anaphylatoxine C3a und C5a führt zur Inaktivierung der jeweiligen Komplementeffektorfunktionen. Die opsonisierenden Eigenschaften der Komplementkomponente C3b wird proteolytisch inaktiviert und damit die Phagocytoserate der Konidien verringert. Die Spaltung der Komponenten C3, C4, C5 und C6 verhindert den Aufbau des Membranangriffskomplexes auf Oberflächen und die damit verbundene Lyse von Zellen. Des Weiteren blockieren die sekretierten Proteasen von *A. benhamiae* die antifungalen Eigenschaften von C3a, sowie die C3a induzierte Ausschüttung von reaktiven, oxygenen Spezies durch Granulozyten. Die C5a vermittelte chemoattraktive Wirkung auf Granulozyten, sowie die C5a induzierte Ausschüttung von Zytokinen durch Monozyten wird ebenfalls durch endogene proteolytische Aktivität von *A. benhamiae* inhibiert.

Diese Studie identifiziert die Protease Subtilisin 3 als wichtige, Komplement spaltende Protease in vitro. Die sekretierte fungale Protease Subtilisin 3 spaltet die Komplementkomponenten C3b, C5b und C3a und inaktiviert dabei deren Komplement aktivierenden, opsonisierenden und antifungalen Eigenschaften und proinflammatorischen Effekte. Somit ist Subtilisin 3 hauptsächlich an der Komplementspaltung und Inaktivierung beteiligt.

Die Inaktivierungsprozesse von Komplement durch die wirtseigenen Komplementregulatoren oder sekretierten fungalen Proteasen sind zeitlich getrennte Ereignisse. Die Inaktivierung des Komplements durch wirtseigene Regulatoren an der Oberfläche von Konidien ist in den ersten Minuten des Kontaktes mit Komplement essentiell. Fungale Proteasen werden erst nach ihrer induzierten Synthese sekretiert und vermitteln so in den ersten Minuten keinen Schutz vor Komplementangriffen. Im weiteren Verlauf übernimmt die endogene proteolytische Aktivität von *A. benhamiae* die Inaktivierung des Komplementsystems.

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EIGENSTÄNDIGKEITSERKLÄRUNG

- Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt.

- Die vorliegende Dissertation wurde von mir selbst angefertigt und alle benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen sind in dieser Arbeit angegeben.

- Alle Personen, die mich bei der Auswahl und Auswertung des Materials, sowie bei der Herstellung des Manuskripts unterstützt haben, habe ich benannt.

- Die Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen.

- Dritte Personen haben weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegtenDissertation stehen.

- Diese Arbeit wurde bisher weder an einer anderen Hochschule als Dissertation noch als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.

Jena, 09.Dezember 2010 _____

Susann Schindler

PUBLICATION LIST, OVERVIEW OF ORAL AND POSTER PRESENTATIONS AND GRANTS

PUBLICATIONS

Gropp K, Schild L, Hube B, Schindler S, Zipfel PF, Skerka C (2009) The yeast *Candida albicans* evades the human complement system by secretion of aspartic proteinases. Mol Immunol 47(2-3):465 – 475

Behnsen J, Lessing F, Schindler S, Wartenberg D, Jacobsen ID, Thoen M, Zipfel PF, Brakhage AA (2010) Secreted *Aspergillus fumigatus* Protease Alp 1 degrades Human Complement Proteins C3, C4 and C5. Infection and Immunity 78 (8):3585-3594

Zipfel PF, Schindler S, Reuter M, Gropp K, Skerka C *Candida* and Complement, in: The Mycota. AA Brakhage, PF Zipfel, edts, Springer 2008

Schindler S, Brakhage AA, Zipfel PF The dermatophyte *A. benhamiae* evades complement attack in skin by two timely separated strategies. (in preparation)

Schindler S, Brakhage AA, Zipfel PF Endogenous proteolytic activity of the dermatophyte *A. benhamiae* degrades and inactivates the complement effector peptides C3a and C5a. (in preparation)

Schindler S, Brakhage AA, Zipfel PF The *A. benhamiae* secreted protease Subtilisin 3 mediate complement resistence in skin. (in preparation)

RESEARCH PRESENTATIONS

Susann Schindler, Axel Brakhage, Peter F. Zipfel (2010) Innate Innate immune escape strategies of the dermatophyte *Arthroderma benhamiae.* **Interdisciplinary Forum on Superficial Fungal Infections**, Jena, Germany (oral presentation)

Susann Schindler, Axel Brakhage, Peter F. Zipfel (2009) *Arthroderma benhamiae* binds host proteins and evades complement attack. **Statusworkshop DGHM-eukaryotic pathogens** 2009, Würzburg, Germany (oral presentation)

Susann Schindler, Axel Brakhage, Peter F. Zipfel (2008) *Arthroderma benhamiae* uses a dual strategy to evade host complement attack. **DMykG Meeting** 2008, Jena, Germany (oral presentation)

Susann Schindler, Axel Brakhage, Peter F. Zipfel (2008) Immune evasion of the human dermatophyte *Arthroderma benhamiae*. **Workshop Infectivity and Host Cells**, Wittenberg, Germany (oral presentation)

Susann Schindler, Axel Brakhage, Peter F. Zipfel (2009) *Arthroderma benhamiae* evades complement attack by binding host proteins. **Third FEBS Advanced Lecture Course: Human Fungal Pathogens**, La Colle sur Loup, France (poster)

Susann Schindler, Axel Brakhage, Peter F. Zipfel (2007) *Arthroderma benhamiae* evades complement attack by binding host proteins. **Annual Meeting of DGHM**, Göttingen, Germany (poster)

Susann Schindler, Axel Brakhage, Peter F. Zipfel (2008) *Arthroderma benhamiae* uses two strategies to evade host complement attack. **ILRS Symposium**, Dornburg, Germany (poster)

Susann Schindler, Axel Brakhage, Peter F. Zipfel (2008) *Arthroderma benhamiae* uses a dual strategy to evade host complement attack. **Annual meeting of DGHM**, Dresden, Germany (poster)

SCHOLARSHIPS RECEIVED

Travel Fellow Ship for statusworkshop eukaryotic pathogens, Würzburg, Germany

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RESEARCH INTERESTS

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  Immune evasion of human dermatophytes

EDUCATION

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RESEARCHES PERFORMED

- **Interactions of complement proteins with the human serine protease plasminogen**
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  (diploma thesis)

- **Compstatin- Factor H constructs, complement inhibitors as therapeutical agent**
  Department of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology, Jena

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- **Identification of heavy metal resistant microorganism in the Wismut area**
  Microbial phytopathology, Friedrich Schiller university, Jena, Germany

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