Strategies to prevent graft-versus-host disease and augment anti-fungal immunity in allogeneic hematopoietic stem cell transplant recipients

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Die vorliegende Arbeit wurde in der Zeit vom 01.07.2005 bis zum 31.10.2009 an der Medizinischen Poliklinik II in der Abteilung für Immun- und Gentherapie der Bayerischen Julius-Maximilians-Universität Würzburg unter Prof. Dr. Hermann Einsele und unter Anleitung von Dr. Max S. Topp angefertigt.

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1 Summary

Allogeneic hematopoietic stem cell transplantation (HSCT) is often the only effective treatment for patients with hematological malignancies, but its curative potential is often limited by the development of acute or chronic graft-versus-host disease (GvHD). Although extensive immunosuppressive therapy is highly efficient in the prevention or treatment of GvHD, it greatly increases the risk for life-threatening opportunistic fungal or viral infections and the recurrence of malignant disease. The possibility to selectively deplete alloreactive T cells from donor grafts prior or after transplantation would greatly diminish the need for immunosuppressive therapy in the transplant recipient and thereby greatly improve its clinical outcome.

The molecular chaperone heat shock protein of 90 kDa (Hsp90) has been previously shown to stabilize many signal transduction proteins involved in T lymphocyte activation and proliferation and is furthermore able to exert anti-apoptotic effects in different cell types. The aim of this study was therefore to investigate the possibility to selectively target activated, proliferating T cells in lymphocyte populations by inhibition of Hsp90, without compromising viability and function of non-reactive T cell populations including pathogen-specific T lymphocytes.

It could be shown in this work, that activated T cells are indeed more prone to apoptotic cell death in the presence of Hsp90 inhibitors than resting cells and that treatment of mixed lymphocyte cultures with such inhibitors eliminates the proliferation of alloreactive cells. In contrast, T cells remaining in a resting state during inhibitor treatment remain viable and also display functional virus-specific responses after inhibitor removal. These data suggest, that Hsp90 could represent a novel target for selective depletion of alloreactive T cells and that application of Hsp90 inhibitors could be a potential approach to prevent or treat GvHD without impairing pathogen-specific T cell immunity.

In the second part of this work, the immune responses to strictly defined antigens of the opportunistic pathogenic fungus *Aspergillus fumigatus* were characterized. Opportunistic fungal infections are highly prevalent in immunocompromized and immunosuppressed individuals, especially in HSCT recipients suffering from GvDH. Although antifungal treatment is permanently improved, invasive fungal infections are still often fatal. In healthy individuals clinical disease is rare, because innate and adaptive immunity act in conjunction to protect the host. Therefore one possible

strategy to prevent and treat life-threatening fungal infections in immunocompromized patients is to improve host resistance by augmenting the antifungal functions of the immune system, for example by vaccination or adoptive transfer of antigen-specific T cells.

Based on previous findings, the objective of this dissertation was to identify and characterize distinct immunogenic *A. fumigatus* antigens that could be used for clinical application like vaccination or *ex vivo* generation of antigen-specific T cells and to characterize the interaction of this antigen-specific lymphocytes with cells of the innate immune system.

First, memory T cell responses to different recombinant *A. fumigatus* proteins in healthy individuals were evaluated. The majority of tested donors displayed stable $CD4^+$ T_H1 responses to the Crf1 protein, whereas responses to the other antigens tested could only be detected in a limited number of donors, qualifying Crf1 as potential candidate antigen for clinical use. It was also possible to identify an immunodominant MHC class II DRB1*04-restricted epitope of Crf1 and to generate T cell clones specific for this epitope. This Crf1-specific T cell clones could be specifically activated by dendritic cells fed with synthetic peptide, recombinant protein or germinating *A. fumigatus* conidia or outgrown hyphae. Interestingly, these *A. fumigatus*-specific T cell clones also responded to stimulation with *Candida albicans*, which likewise causes opportunistic infections in immunocompromized patients and encodes for a glucosyl-transferase similar to *A. fumigatus* Crf1. It was also possible to show that supernatant harvested from activated Crf1-specific T cell cultures was able to significantly increase fungal killing by monocytes. These data indicate that the specified FHT epitope of the *A. fumigatus* protein Crf1 could be potentially used as antigen for vaccination protocols or for the generation of *Aspergillus*-specific effector T cells for adoptive transfer.

Zusammenfassung

Allogene, hämatopoietische Stammzelltransplantation ist häufig die einzige, effektive Behandlungsmethode für Patienten mit hämatologischen Erkrankungen, aber deren Erfolg ist häufig durch das Auftreten einer „Graft-versus-Host“-Erkrankung (GvHD) gefährdet. Obwohl durch eine weitreichende, immunsuppressive Therapie eine GvHD erfolgreich verhindert oder behandelt werden kann, erhöht diese jedoch beträchtlich das Risiko für das Auftreten lebensbedrohender opportunistischer Virus- und Pilz-Infektionen und das Wiederauftreten der malignen Erkrankung. Die Möglichkeit der selektiven Depletion alloreaktiver T-Zellen eines Transplantates bevor oder nach der Transplantation würde die Notwendigkeit immunsuppressiver Therapien des Empfängers deutlich reduzieren und dadurch den Behandlungserfolg enorm verbessern.

Für das molekulare Chaperon-Protein „Hitzeschock-Protein von 90 kDa“ (Hsp90) konnte bereits zuvor gezeigt werden, dass es viele Signal-Transduktions-Proteine, die an der Aktivierung von T-Lymphozyten beteiligt sind stabilisiert und außerdem in verschiedenen Zelltypen einen anti-apoptotischen Effekt auszuüben scheint. Deshalb war es das Ziel dieser Untersuchung festzustellen, ob es möglich ist, durch Inhibition von Hsp90 spezifisch nur die aktivierten, proliferierenden T-Zellen in einer Lymphozyten-Population zu erreichen, ohne dabei die Viabilität und Funktionalität der nicht-reaktiven T-Zell-Population, einschließlich Pathogen-spezifischen T-Lymphozyten, zu beeinträchtigen.

In dieser Arbeit konnte gezeigt werden, dass unter dem Einfluss von Hsp90-Inhibitoren in aktivierten T-Zellen tatsächlich eher Apoptose induziert wird, als in ruhenden Zellen und dass die Behandlung von gemischten Lymphozyten-Kulturen mit diesen Inhibitoren die Proliferation von alloreaktiven Zellen verhindert. Im Gegensatz dazu bleiben T-Zellen, die zum Zeitpunkt der Inhibitor-Behandlung in einem ruhenden Zustand waren überlebensfähig und zeigen zudem nach Entfernung des Inhibitors immer noch Virus-spezifische Immunantworten. Wie diese Daten zeigen, könnte Hsp90 ein neues Angriffsziel für die selektive Depletion von alloreaktiven T-Zellen sein und Hsp90-Inhibitoren deshalb möglicherweise für die Vorbeugung oder Behandlung von GvHD eingesetzt werden, ohne dabei die pathogen-spezifische Immunität zu beeinträchtigen.

Im zweiten Teil dieser Arbeit wurden die Immunantworten auf definierte Antigene das opportunistisch pathogenen Pilzes *Aspergillus fumigatus* charakterisiert.

Opportunistische Pilzinfektionen sind eine häufige Begleiterscheinung bei Personen mit beeinträchtigtem Immunsystem und immunsupprimierten Patienten, vor allem bei Patienten die sich einer allogenen Stammzell-Transplantation unterziehen müssen und unter einer GvHD leiden. Trotz ständiger Verbesserung der Behandlungsmöglichkeiten, verlaufen invasive Pilzinfektionen häufig immer noch tödlich. Gesunde Menschen erkranken nur äußerst selten symptomatisch, da sowohl die angeborene als auch die erworbene Immunabwehr dazu beitragen, eine akute Infektion effektiv zu verhindern. Eine Möglichkeit, lebensbedrohliche Pilzinfektionen bei immunsupprimierten Patienten zu verhindern könnte deshalb die Stärkung der Immunabwehr durch Verbesserung der Funktionen des Immunsystems sein, beispielsweise durch Vakzinierung oder den adoptiven Transfer antigenspezifischer T-Zellen.

Ziel dieser Arbeit war, aufbauend auf früheren Forschungsergebnissen, die Identifikation und Charakterisierung definierter immunogener *A. fumigatus* Antigene, die für die klinische Anwendung als Impfstoff oder für die *ex vivo* Generierung antigenspezifischer T-Zellen Verwendung finden könnten. Außerdem sollte die Interaktion dieser antigenspezifischen T-Lymphozyten mit den Zellen des angeborenen Immunsystems untersucht werden.

Zunächst wurden hierfür die T-Zell-Antworten gesunder Spender auf verschiedene rekombinante *A. fumigatus*-Proteine untersucht. Die große Mehrheit der getesteten Spender reagierte auf das Protein Crf1 mit einer stabilen T_H1 Antwort, während die übrigen getesteten Proteine nur in vereinzelten Spendern eine signifikante T-Zell-Antwort auslösten. Die offensichtlich weite Verbreitung von Crf1-spezifischen Gedächtnis-Zellen in der Bevölkerung lässt dieses Antigen als gut geeignet für potenzielle klinische Anwendungen erscheinen. Des weiteren wurde ein immundominantes Epitop von Crf1 identifiziert, das durch das relativ weit verbreite MHC Klasse II DRB1*04-Allel präsentiert wird.

T-Zell-Klone, die spezifisch dieses Antigen erkennen, konnten mit dendritischen Zellen, die zuvor mit synthetischem Peptid, rekombinantem Protein oder ausgekeimten *A. fumigatus* Conidien oder Hyphen inkubiert worden waren aktiviert werden. Interessanterweise reagierten diese *A. fumigatus*-spezifischen T-Zell-Klone auch auf Stimulation mit *Candida albicans*, das ebenfalls opportunistische Infektionen in immunsupprimierten Patienten auslöst und für eine ähnliche Glucosyl-Transferase wie Crf1 in *A. fumigatus* codiert. Die Kultur-Überstände von aktivierten Crf1-spezifischen T-Zell-Klonen waren außerdem in der Lage, das Abtöten des Pilzes durch Monozyten deutlich zu verbessern. Diese Daten deuten darauf hin, dass das hier beschriebene

FHT-Epitop des *A. fumigatus* Proteins Crf1 potenziell als Antigen für die Vakzinierung oder die Generierung von *Aspergillus*-spezifischen Effektor-T-Zellen für den adoptiven Transfer geeignet sein könnte.

2 Introduction

2.1 Allogeneic hematopoietic stem cell transplantation (HSCT)

Allogeneic hematopoietic stem cell transplantation (HSCT) is often the only effective treatment for patients with hematological malignancies and can also be applied for inherited disorders of blood cells (Shlomchik 2007; Auletta and Cooke 2009). HSCT recipients first receive a conditioning regimen consisting of chemotherapy and/or radiotherapy to reduce the number of malignant cells and diminish the risk of graft rejection, followed by the infusion of donor lymphocytes. The mature T cells contained in the allograft can reconstitute T cell immunity in the host, as well as eradicate remaining malignant cells to decrease the risk of disease relapse. This beneficial anti-tumor effect, also called graft-versus-leukemia (GvL) effect, is mediated by donor T cells recognizing and killing the recipient malignant cells (Bleakley and Riddell 2004). However, the allogeneic donor lymphocytes can similarly recognize healthy recipient cells as “non-self” and initiate a broad attack on different host tissues, including skin, liver and the gastrointestinal tract. This damage of host tissues is known as acute graft-versus-host disease (GvHD) and occurs in 20-60% of transplant patients. Furthermore, 60-80% of those patients develop chronic GvHD which resembles autoimmune syndromes and is a major cause of morbidity and mortality in long-term survivors of HSCT (Bacigalupo 2007; Morris and Hill 2007; Shlomchik 2007).

GvHD can occur in both, the MHC-matched or the MHC-mismatched transplantation setting (Figure 1). In both cases recipient APC, that are resistant to the conditioning regimen, seem to play an essential role in initiating the immune response against host tissues. In the matched setting donor T cells recognize peptides derived from proteins that are present only in the recipient, so-called minor histocompatibility antigens, which can be presented by recipient- as well as donor-derived antigen-presenting cells. In the mismatched setting donor T cells recognize the allogeneic MHC molecule on recipient APC itself (Shlomchik 2007). Tissue-injury can be either mediated by direct cytolytic mechanisms of the alloreactive lymphocytes or the release of soluble inflammatory mediators (Hill et al. 1997; Tsukada et al. 1999).

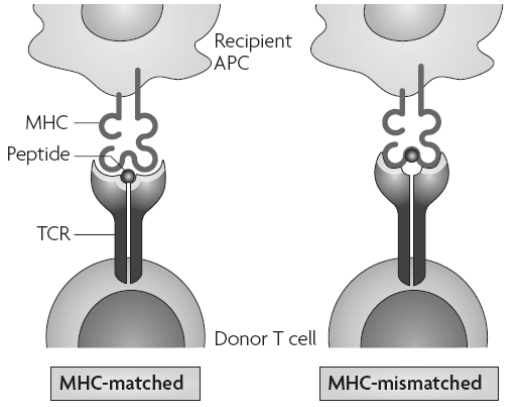


Figure 1:

T cell recognition in allogeneic stem cell transplantation. In MHC-matched transplantation donor T cells recognize peptides derived from proteins that are present in the recipient but not in the donor (so-called minor histocompatibility antigens). In the MHC-mismatched setting donor T cells recognize the recipient-specific MHC molecule itself (adapted from Shlomchik 2007).

The incidence of GvHD can be effectively reduced by extensive post-transplant immunosuppressive therapy which greatly impairs T cell functions by applying for example cyclosporine A, methotrexate or corticosteroids (Zikos et al. 1998). However, the most important complication associated with immunosuppressive treatment is severe immunodeficiency, which renders the patient susceptible to a wide range of opportunistic infections and also favours the recurrence of the malignant disease by inhibiting the GvL effect, which frequently leads to the patients death (Morris and Hill 2007). Major viral pathogens frequently causing life-threatening infections in immunocompromised patients include Cytomegalovirus (CMV), herpes simplex viruses (HSV) and adenoviruses (Lenaerts et al. 2008; Jancel and Penzak 2009). Of similar importance are invasive fungal infections caused predominately by *Aspergillus* and *Candida* species (Romani 2004; Rogers and Frost 2009). Therefore it is of considerable interest to develop therapies which are able to prevent GvHD without the need for general immunosuppression or to boost pathogen-specific immunity in HSCT recipients.

2.2 Prevention and treatment of GvHD

2.2.1 Different strategies to selectively deplete alloreactive T lymphocytes

One of the first approaches that intended to render immunosuppressive treatment after HSCT unnecessary was the application of anti-thymocyte globulin (ATG). Elimination of all T lymphocytes from the donor graft protected the patient from extensive chronic GvHD and also reduced transplant mortality (Aversa et al. 1998; Bacigalupo et al. 2006). The major drawback of this rigorous T cell depletion is however, that in addition to harmful alloreactive T cells the desirable pathogen-specific as well as tumor-specific lymphocytes are also depleted. Therefore novel transplantation protocols have the aim to selectively deplete alloreactive T cells from the lymphocyte population and at the same time spare host-compatible pathogen-specific cells and cells mediating the GvL effect. This selective T cell depletion could either be performed *ex vivo* prior to transplantation or, alternatively, in the patient after transplantation. Some of this novel approaches are based on *ex vivo* stimulation of donor lymphocytes with host-derived antigen-presenting cells (APC) followed by selective removal of the alloreactive T cells, which can be achieved by using beads or antibodies against surface antigens of recent activation, including CD25, CD69, CD71 or CD137 (Mielke et al. 2005; Wehler et al. 2007). Other possibilities are for instance photodynamic purging strategies targeting activation-based changes in p-glycoprotein (Chen et al. 2002) or the induction of anergy by B7-CD28 costimulatory blockade during T cell activation (Gribben et al. 1996). Further strategies to reduce the incidence of GvHD that are currently under investigation include the concomitant transplantation of regulatory T cells to dampen possible alloreactive immune reactions (Taylor et al. 2002; Trenado et al. 2006), administration of antibodies interfering with cytokine action or the inactivation of recipient APC, which have been shown to be predominantly responsible for inducing alloresponses in the first place (Shlomchik et al. 1999; Duffner et al. 2004; Matte et al. 2004; Chakraverty and Sykes 2007). Although all these approaches could prove their experimental feasibility, clinical data on selective allodepletion is very limited and largely based on the use of anti-CD25 immunotoxins (Solomon et al. 2005; Amrolia et al. 2006). Furthermore, instability of surface marker expression has been postulated to be in part responsible for the appearance of residual GvHD after CD25-based selective

allodepletion (Mielke et al. 2005). Therefore novel strategies are requested that can improve the selective depletion of alloreactive cells from transplants.

2.2.2 The role of heat shock protein 90 in signal transduction

Activation and proliferation of T cells are orchestrated by diverse signal transduction cascades such as the PI3K/NF-κB, Ras/MAPK or JAK/STAT pathways involving the activation of several key signaling intermediates such as ZAP-70, Raf-1, Akt, Lck, Stats and Jaks (Figure 2) (Nel 2002).

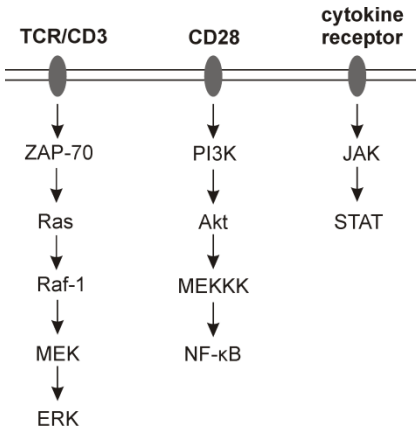


Figure 2:
Some of the signal transduction molecules involved in T lymphocyte activation.

Many of this signaling intermediates are stabilized by the molecular chaperone “heat shock protein of 90 kDa” (Hsp90) (Schulte et al. 1995; Schnaider et al. 2000; Yorgin et al. 2000; Basso et al. 2002; Xu et al. 2004; Shang and Tomasi 2006; Neckers 2007). Hsp90 is one of the most abundant cellular proteins and part of a multichaperone complex that helps in the maturation, stabilization and function of a wide variety of client proteins (Figure 3). It is constituted of two subunits, Hsp90α and Hsp90β. Furthermore, Hsp90 has also been shown to exert direct and indirect anti-apoptotic effects (Beere 2001). For example, Hsp90 stabilizes Akt, which is thought to mediate many biological actions toward anti-apoptotic responses (Sato et al. 2000). Also, the anti-apoptotic function of Bcl-2 is significantly dependent on its association with Hsp90β (Mimnaugh et al. 2004; Cohen-Saidon et al. 2006; Kuo et al. 2007).

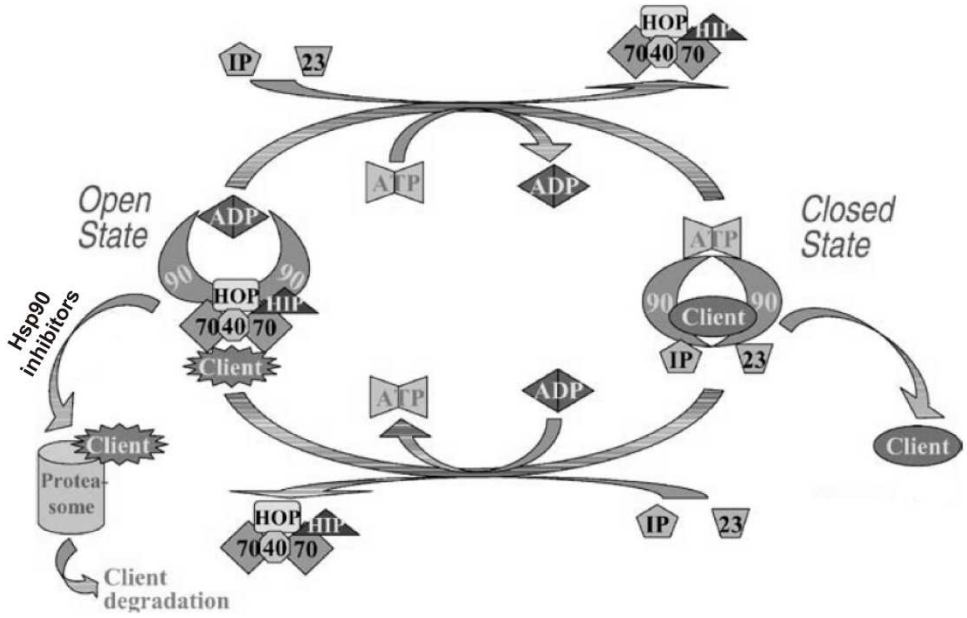


Figure 3:

The Hsp90 superchaperone complex cycle. The client protein is loaded onto Hsp90 with the help of the co-chaperones Hsp70, Hsp40, Hop and Hip in the “open” state of the complex. Upon ATP hydrolysis the co-chaperones are replaced by immunophilins (IP) and p23, the complex changes to the “closed” state and the client protein undergoes maturation. Hsp90 inhibitors lock the complex in the “open” state which results in client protein degradation (B) (adapted from Zhang and Burrows 2004).

Hsp90 also seems to play a central role in the survival and proliferation of tumor cells (Zhang and Burrows 2004; Powers and Workman 2006), which has been demonstrated with pharmacological inhibitors that block the chaperone function and are able to induce apoptosis in various cancer cell types, such as multiple myeloma (Chatterjee et al. 2007), chronic lymphocytic leukemia (Jones et al. 2004; Castro et al. 2005) or anaplastic large cell lymphoma (Schumacher et al. 2007). By inhibition of the Hsp90 chaperone its client proteins are no longer stabilized, get ubiquitinated and are rapidly degraded by proteasomes (Figure 3).

Different classes of Hsp90 inhibitors are known that all act by binding to the ATP-binding pocket of Hsp90 and thereby block the intrinsic ATPase activity of the chaperone complex. The first Hsp90 inhibitors discovered were the naturally occurring antibiotics herbimycin A and geldanamycin which were capable of inducing apoptosis in malignant cells (DeBoer et al. 1970; Whitesell et al. 1994). However, preclinical

evaluation of these substances discarded further clinical trials because of acute drug toxicity, lack of drug stability and poor solubility (Supko et al. 1995; Schulte and Neckers 1998). In contrast, semi-synthetic geldanamycin derivatives such as 17-AAG (17-allylamino-17-demethoxygeldanamycin) and 17-DMAG (17-(dimethylaminoethyl-amino)-17-demethoxygeldanamycin) showed comparable anti-tumor effects with lower toxicity and better solubility (Schulte and Neckers 1998; Zhang and Burrows 2004; Powers and Workman 2006). Recently developed novel classes of Hsp90 inhibitors are the highly potent purine-scaffold inhibitors, for example diarylpyrazoles, sheperdins or PU24FCI with markedly improved drug-like properties (Chiosis et al. 2002; Cheung et al. 2005; Plescia et al. 2005; Chiosis and Tao 2006). Some of these less toxic compounds including 17-AAG and 17-DMAG have already been tested in phase I/II clinical trials with cancer patients and have shown promising anti-tumor effects at a manageable level of toxicity (Zhang and Burrows 2004; Powers and Workman 2006).

2.2.3 Potential use of Hsp90 inhibitors for selective depletion of alloreactive T cells

As mentioned before, many Hsp90 client proteins are involved in T lymphocyte signal transduction pathways necessary for T cell activation and proliferation and can exert anti-apoptotic effects (Beere 2001; Nel 2002; Cohen-Saidon et al. 2006; Kuo et al. 2007). Some of the same signaling pathways are also active in malignant cells, which likewise have a high proliferative potential and in general show some resistance to apoptosis (Zhang and Burrows 2004; Powers and Workman 2006). Furthermore it has been shown that blockage of Hsp90 can disrupt T cell signaling events, for example by destabilizing Raf-1 (Schulte et al. 1995) or ZAP-70 (Castro et al. 2005), decreasing phosphorylation of Lck (Schnaider et al. 2000) or inhibiting phosphorylation of STAT5 (Xu et al. 2004) and is able to induce apoptosis in malignant cells (Castro et al. 2005; Chatterjee et al. 2007). Therefore it seems probable, that Hsp90 should be of greater importance for the survival of activated, proliferating cells than for resting cells and that activated T cells just as malignant cells are especially dependent on Hsp90 function. Consequently, inhibition of this chaperone should have a greater effect on activated than on resting lympohocytes in mixed cell populations. This discriminative action could potentially be exploited to selectively deplete activated, alloreactive T cells from the donor lymphocyte population after co-incubation with host-derived APC.

2.3 Augmentation of pathogen-specific immunity in HSCT recipients

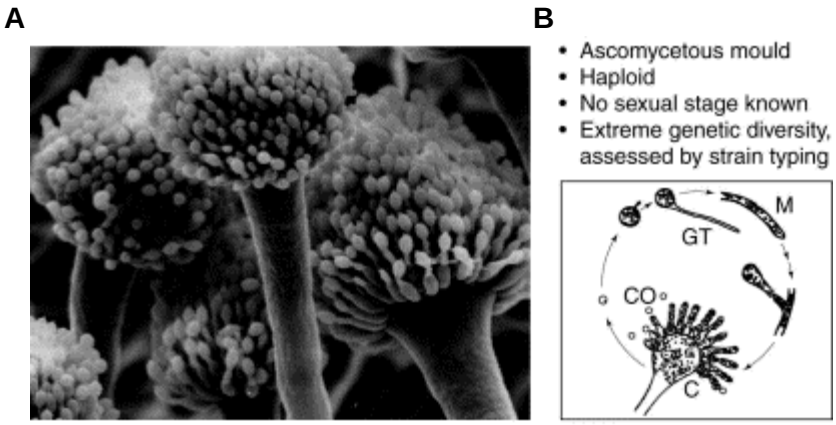
2.3.1 Fungal infections in immunocompromized individuals

The clinical relevance of systemic fungal infections has increased enormously during the last decades, mainly because of the increasing number of immunocompromized and immunosuppressed patients, new treatment modalities and the severity of immunosuppressive therapies (Latge 2001; Romani 2004; Warnock 2007). If the immune system of an individual is severely compromised as in cancer or AIDS patients and stem cell transplant recipients, fungal growth cannot be held in check and leads to the development of life-threatening, invasive disease. Especially in patients receiving allogeneic hematopoietic stem cell transplantation invasive fungal infections are leading causes of infection-related death with a mortality rate of up to 85% (Morgan et al. 2005; Nierman et al. 2005). Patients with hematological malignancies often receive cytotoxic chemotherapy or total body irradiation which causes prolonged neutropenia, virtually eliminating the most important antifungal defense mechanism (Marr et al. 2002; Fukuda et al. 2003). In addition, concomitant immunosuppressive therapy with high-dose corticosteroids which is necessary to prevent graft rejection and graft-versus-host disease (GvHD) severely impairs innate as well as adaptive immunity (Marr et al. 2002; Fukuda et al. 2003). Immunosuppressive agents like corticosteroids have profound effects on the function of innate as well as adaptive immune cells and highly predispose the patient for opportunistic fungal, bacterial and viral infections. It has been observed for example, that in the presence of glucocorticoids conidia that have been phagocytosed by macrophages are not destroyed, but germinate inside the immune cell and kill it (Schaffner et al. 1982). Corticosteroids also inhibit the maturation and differentiation of monocytes to macrophages, reduce the mobilization of PMN and suppress the respiratory burst and hyphal killing of neutrophilic granulocytes (Schaffner et al. 1982; Roilides et al. 1993; Philippe et al. 2003).

One of the most important opportunistic pathogens causing severe infections in immunocompromized patients is the fungus *Aspergillus* fumigatus.

2.3.2 The opportunistic pathogen *Aspergillus fumigatus*

The fungus *Aspergillus fumigatus* belongs to the phylum of Ascomycota and is a saprophytic, filamentous fungus predominately found in the soil, but can also be found indoors. It is nearly ubiquitously distributed in the environment and plays important roles in the recycling of environmental carbon and nitrogen. *A. fumigatus* grows on organic debris in the form of somatic hyphae and distribution occurs by thousands of small, air-borne conidia. If the disseminated conidia encounter adequate growth conditions they start germination and again grow out to form branching mycelia (Latge 1999) (Figure 4).



Latgé. TRENDS in Microbiology 2001.

Figure 4:

A. fumigatus conidiophores (A). Schematic diagram of the life cycle of *A. fumigatus*. The fungus grows in the vegetative phase in the form of somatic hyphae. Dissemination occurs by small, air-borne conidia, which can germinate to form new mycelia (B).

M, vegetative mycelium; C, conidiophore; CO, conidia; GT, germinating conidia.

Because of the small size of the conidia (2-3 µm), they can easily be inhaled and even reach the lung alveoli. It is estimated that humans inhale several hundred conidia every day (Chazalet et al. 1998). Despite this persistent contact with the fungus, healthy individuals in general do not develop symptomatic *Aspergillus* infections and clinical disease is rare. However, in immunocompromized or immunosuppressed patients infection can cause severe and often fatal disease. *Aspergillus* species, together with *Candida* and *Cryptococcus* species are therefore one of the leading causes of life-threatening, opportunistic infections in transplant recipients, cancer patients or patients suffering from AIDS (Latge 2001; Romani 2004).

About 185 *Aspergillus* species have been described so far, but only 20 of these have been associated with opportunistic infections, including *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger* (Morgan et al. 2005; Gallin and Zarember 2007). Different factors may contribute to the high pathogenicity of *A. fumigatus*, including its ability to grow at body temperature, as well as its ability to acquire nutrients or penetrate epithelial surfaces through secretion of various proteases and lipases causing tissue destruction (Hogan et al. 1996; Latge 1999). The ribotoxin restrictocin (also called AspF1) which is able to cleave the 28S rRNA of eukaryotic ribosomes also induces cell necrosis and could therefore potentially have a similar function in acquisition of nutrients (Lamy et al. 1991). Furthermore, different virulence factors including pigments, antioxidants and toxic molecules are known that allow evasion of host defense mechanisms and can even actively hamper the function of innate and adaptive immune cells, as described later.

Furthermore, *A. fumigatus* is endowed with specific virulence factors that enable the fungus to actively evade destruction by immune cells. Pigments in the conidial cell wall have been described to inhibit phagocytosis and specific scavengers of reactive oxidants like catalases and superoxide dismutases may help to protect the fungus from oxidative killing by phagocytes and granulocytes (Latge 1999). The fungal toxin gliotoxin shows a broad range of immunosuppressive properties on innate immune cells as well as cells of the adaptive immune system. It has been described for example to actively inhibit phagocytosis and the respiratory burst in granulocytes, inhibit activation of the NF-κB signal transduction pathway in T and B lymphocytes and can even induce apoptosis in monocytes, dendritic cells and thymocytes thereby suppressing *Aspergillus*-specific T cell responses (Stanzani et al. 2005; Orciuolo et al. 2007). The gliotoxin-mediated immunosuppressive effects were even observed in *in vitro* experiments with concentrations below toxin concentrations determined in the serum of patients with IA.

2.3.3 Clinical manifestations of *A. fumigatus* infections

As *A. fumigatus* generally enters its host by inhalation, infection predominantly affects the pulmonary system. The extent of fungal colonization and tissue invasion is variable, largely depending on the immunological status of the host as well as pre-existing disease (Marr et al. 2002; Romani 2004).

Patients suffering from atopic asthma or cystic fibrosis for example have a high risk of developing allergic Bronchopulmonary Aspergillosis (ABPA), a T_H2-mediated hypersensitivity lung disease which can cause symptoms ranging from airway inflammation to lung fibrosis or even fatal lung destruction (Chauhan et al. 1996; Latge 1999; Rivera et al. 2005; Romani 2008).

The most serious and often life-threatening complication caused by *A. fumigatus* is Invasive Aspergillosis (IA) which normally develops only in severely immuno-compromized patients. Especially transplant recipients are at a high risk of acquiring fungal infections during the immunosuppressive therapy accompanying the treatment, but also cancer or AIDS patients can be affected (Romani 2004). Because of the difficulty of early and correct diagnosis and the rapid progression of the disease, IA is often fatal within a time frame of 1 to 2 weeks from onset to death (Latge 1999; Safdar 2006). IA is characterized by invasive fungal growth in the lungs and accompanied by general unspecific symptoms like fever, chest pain, cough or malaise. In severe cases the fungus can enter the bloodstream and dissemination to virtually any organ, including the central nervous system, is possible (Ribaud et al. 1999; Marr et al. 2002; Fukuda et al. 2003).

2.3.4 Innate immunity to *A. fumigatus*

Healthy individuals generally do not develop symptomatic *Aspergillus* infections, because the immune system is able to deal quite efficiently with inhaled fungal conidia. The mucosal membrane of the respiratory tract serves as anatomical barrier and primary defense against fungal infections. Epithelial cells as well as immune cells secrete small cationic peptides called defensins that posses broad microbicidal activity against both bacteria and fungi (Schneider et al. 2005). Also the collectin pentraxin 3 which binds *Aspergillus* conidia and activates diverse effector pathways of immune cells seems to be an essential component of host resistance to pulmonary Aspergillosis and is able to increase resistance to infection in mice (Gaziano et al. 2004). Further anti-microbial effectors like the hydrophilic surfactant proteins A and D for example enhance phagocytosis and killing of inhaled conidia by resident alveolar macrophages in the lungs, which are the first immune cells getting in contact with the pathogen (Figure 5). Binding of conidia is probably mediated by C-type lectins recognizing structures of the fungal cell wall. The engagement of these receptors triggers signaling

pathways in the immune cell leading to phagocytosis of the conidia and the production of inflammatory cytokines and chemokines which coordinate the recruitment and activation of further innate immune cells like polymorph nuclear leukocytes (PMN) and dendritic cells (DC) at the site of infection (Dubourdeau et al. 2006; Gersuk et al. 2006). Killing of the ingested conidia by alveolar macrophages starts after a delay of several hours and only after the conidia have swollen inside the phagocytic cell with a relatively low killing rate of 90% within 24 hours (Schaffner et al. 1983). Diverse anti-microbial systems are probably involved in the killing process including non-oxidative mechanisms such as cationic proteins or degrading enzymes as well as reactive oxygen intermediates (ROI), whereas nitrogen oxide (NO) seems to be only of minor importance (Schaffner et al. 1983; Michaliszyn et al. 1995; Philippe et al. 2003).

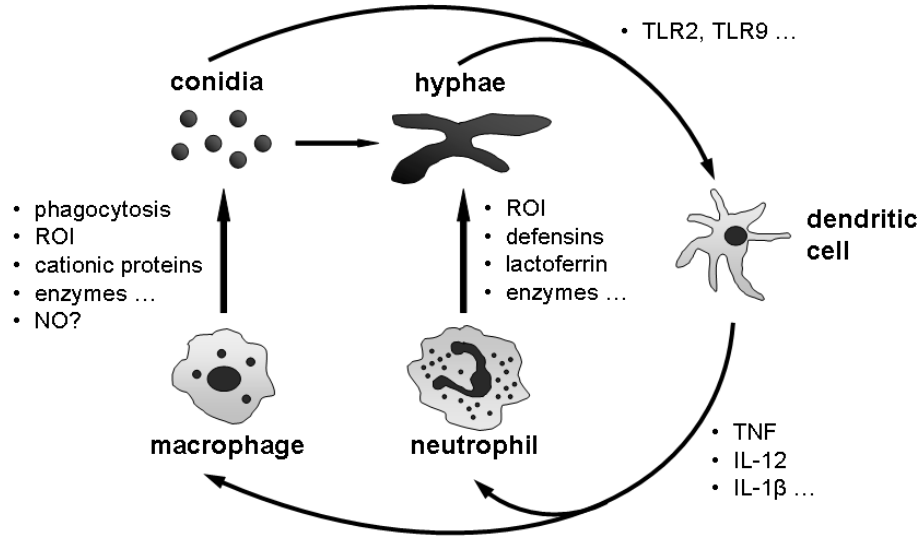


Figure 5:

Innate immunity to fungal infections. Conidia that invade the host are ingested and killed by macrophages. If some conidia escape destruction they can germinate and grow out to hyphae, which are predominantly attacked by neutrophilic granulocytes. Killing is mediated through oxidative (ROI, NO) as well as non-oxidative mechanisms (enzymes, defensins, lactoferrin …). Dendritic cells are activated by engagement of toll-like receptors (TLR's) and help to orchestrate the antifungal response e.g. by secretion of pro-inflammatory cytokines including TNF, IL-12 and IL-1β.

Eradication of conidia by resident macrophages is often not complete and the conidia that manage to escape killing can germinate and grow into hyphae, which constitute the invasive, pathogenic form of the fungus. The fungal hyphae are attacked by neutrophilic granulocytes that have been actively recruited to the site of infection by the

pro-inflammatory milieu (Bonnett et al. 2006; Gafa et al. 2007). Although neutrophils can also ingest and kill resting or swollen conidia, they are predominantly responsible for destruction of hyphae (Schaffner et al. 1982). The crucial role of neutrophils is substantiated by the fact that neutrophil recruitment is essential for survival of *Aspergillus* infections in mice (Schaffner et al. 1982) and also the dramatically increased risk of invasive fungal infections in patients with prolonged neutropenia (Marr et al. 2002). Furthermore, several types of PMN deficiencies have been identified as predisposing factors for fatal mold infections (Bonnett et al. 2006). In contrast to killing by monocytes, killing by neutrophils is rapid with a killing rate of 50% within 2 hours (Latge 2001). Killing is predominantly mediated by reactive oxygen species that are released after adherence to the hyphal wall in close proximity to the fungus resulting in irreparable damage to the fungal wall (Diamond and Clark 1982; Schaffner et al. 1982; Levitz and Farrell 1990). Killing is supported by non-oxidative antifungal agents including defensins, lactoferrin and hydrolytic enzymes like lysozyme which are released by neutrophil degranulation (Gallin and Zarember 2007). In addition to neutrophils, platelets also seem to have the ability to attach to *Aspergillus* hyphae and damage their cell wall integrity, probably facilitating fungal killing by neutrophils (Christin et al. 1998). Furthermore, recruitment of natural killer (NK) cells to the lungs seems to be a critical early host defense mechanism in fungal infections, as NK cell depletion results in impaired defense in mice (Morrison et al. 2003).

In recent years it was found that dendritic cells play an essential role in orchestrating innate immunity to fungal infections. DC recognize invariant, evolutionary conserved structures shared by large groups of pathogens, the so-called pathogen-associated molecular patterns (PAMP) on the fungal cell wall by engagement of a set of pattern recognition receptors (PRR), including for example Toll-like receptors 2 (TLR2) and 9 (TLR9) (Bellocchio et al. 2004; Romani 2008). TLR signaling in turn leads to production of chemokines that promote migration of PMN and effector memory cells to the site of infection as well as pro-inflammatory cytokines like tumor necrosis factor (TNF) and the interleukins IL-1β and IL-12 to further boost the effector functions of macrophages and neutrophils (Romani 2004; Gafa et al. 2007). DC are also critically responsible for initiating and directing adaptive immune responses as described in the next section. Because of this fundamental importance of DC in antifungal immunity, defects in Toll-like receptors or in downstream signaling pathways which abolish proper DC function can render hosts highly susceptible to Aspergillosis (Chignard et al. 2007).

2.3.5 Adaptive immunity to *A. fumigatus*

Initially it was thought, that clearance of fungal infections is predominantly mediated by innate immunity and that adaptive responses are only of marginal importance (Schaffner et al. 1982). But in recent years it has become more and more evident, that adaptive immunity is also critically involved. It was shown for example, that infection of mice with sublethal doses of *A. fumigatus* conidia or other fungal antigens can induce protective immunological memory in the animals. When the immunized mice are later rechallenged with high doses of conidia, they do not develop disease in contrast to control animals (Cenci et al. 1997; Cenci et al. 2000; Bozza et al. 2002; Ito et al. 2006). Furthermore, if T cells from immunized animals are adoptively transferred to otherwise susceptible naïve recipient mice, they are also protected, providing further evidence for the existence of *Aspergillus*-specific memory cells (Cenci et al. 2000; Bozza et al. 2003). The fact that blood lymphocytes from healthy individuals show proliferative responses after stimulation with *A. fumigatus* antigens indicates, that humans also develop adaptive immunity to this fungus and harbor long-lasting memory cells (Grazziutti et al. 1997; Hebart et al. 2002; Perruccio et al. 2005; Beck et al. 2006).

The processes of innate and adaptive immunity to fungal infections are intimately linked. Adaptive immune responses and immunological memory to *A. fumigatus* are initiated by dendritic cells that have ingested conidia and hyphae and can activate different types of T helper cells (Figure 6). Depending on the immune state of the host and the DC activation profile, the $CD4^+$ T cells can differentiate either to T_H1, T_H2 or T_H17 cells. A pro-inflammatory cytokine environment characterized by IL-12 and TNF-α generally leads to differentiation of T_H1 cells, which in turn secret cytokines including gamma interferon (IFN-γ) and colony stimulating factors (CSF) that are able to boost the effector functions of innate immune cells and thereby help to clear the infection (Romani 2008). In fact, T_H1 responses are regarded as central to protection against fungi. This is demonstrated in animal models for example by the fact that neutralization of IFN-γ leads to increased pathology, whereas administration of IL-12, IFN-γ or TNF-α or neutralization of the T_H2 cytokines IL-4 or IL-10 was able to increase resistance in infected animals (Cenci et al. 1997; Cenci et al. 1998). In contrast, immune responses of the T_H2 type are not protective and can lead to fungal allergy or even exacerbate disease (Cenci et al. 1999; Rivera et al. 2005). Susceptible mice developing predominantly T_H2 responses also showed impaired neutrophil activity and treatment with soluble IL-4 receptor cured most of the animals (Cenci et al. 1997; Rivera et al. 2005). Similarly, patients with disseminated or relapsing infection often show defective

production of IFN-γ associated with elevated levels of the type 2 cytokines IL-4 and IL-10, as well as an increase in IgE antibodies and eosinophilia (Hebart et al. 2002). The same pattern is seen in patients who are afflicted with the hypersensitivity disease ABPA (Chauhan et al. 1996).

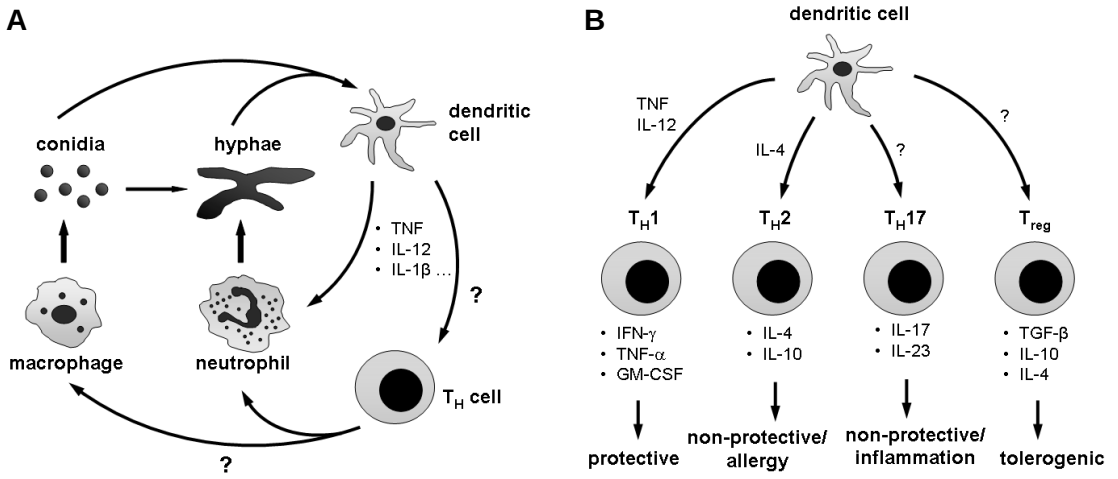


Figure 6:

Adaptive immune responses to fungal infections. Dendritic cells can directly stimulate the antifungal activity of innate immune cells by secretion of pro-inflammatory cytokines such as TNF-α, IL-12 and IL-1β. In addition they can also activate different subtypes of $CD4^+$ helper T cells with diverse effects on antifungal immunity (A). Activation of T_H1, T_H2, T_H17 and T_{reg} cells by dendritic cells. Only T_H1 responses are protective and induce immunologic memory. Differentiation of T_H2 or T_H17 cells is non-protective and associated with fungal allergy or excessive inflammation, respectively. Regulatory T cells limit inflammatory responses early in infection and counteract fungal allergy (B).

Although inflammation is a key feature of fungal immunity and serves to limit the infection, a disproportionate inflammatory response may also significantly contribute to pathogenicity and tissue destruction. The primary function of adaptive T cell responses therefore seems to be the regulation of the balance between tolerogenic and inflammatory responses (Romani and Puccetti 2007; Romani 2008). In recent years T_H17 cells have emerged as a novel, separate lineage of effector helper T cells. T_H17 responses are characterized by the signature cytokines IL-23 and IL-17 and are associated with an excessive inflammatory response and the failure to induce protective immunity (Romani and Puccetti 2007; Zelante et al. 2007; Zelante et al. 2008). In animal models the T_H17 pathway is associated with defective pathogen clearance and neutralization of IL-17 led to increased fungal control, ameliorated inflammatory pathology and restored protective T_H1 resistance (Zelante et al. 2007). In

protective immune responses excessive inflammatory responses are supposedly limited by regulatory T cells (T_{reg}) secreting immunoregulatory cytokines such as IL-10 and TGF-β to prevent tissue damage (Montagnoli et al. 2006; Romani and Puccetti 2007). Two functionally distinct types of regulatory T cells are probably involved in antifungal defense. Whereas natural T_{reg} cells limit early inflammation at the site of infection by suppression of PMN, pathogen-induced tolerogenic T_{reg} act later during adaptive immunity to inhibit T_H2 responses and fungal allergy (Montagnoli et al. 2006; Zelante et al. 2006).

The contribution of B cells and $CD8^+$ cytotoxic T cells to antifungal immunity is still unclear. Antibodies against antigens of the fungal cell wall such as mannans, capsular polysaccharides or surface proteins could for example facilitate phagocytosis by opsonization (Magliani et al. 2005), but there is yet no definitive conclusion on the role of antibodies in *A. fumigatus* immunity and the transfer of serum from immunized mice to naïve animals did not confer any protection, arguing against an essential role for B cell immunity (de Repentigny et al. 1993). The contribution of cytotoxic T lymphocytes in antifungal defense is also not clearly established to date. Cytolytic T cells are generally thought to contribute to antimicrobial activity either by releasing lymphokines which recruit and activate other cell types or by MHC-restricted lysis of infected host cells, but T cells possibly can also mediate antimicrobial activity by direct interaction with microbial targets. For instance activated $CD4^+$ and $CD8^+$ T cells as well as NK cells have demonstrated direct antifungal activity against *Cryptococcus neoformans* and *Candida albicans* depending on intimate cell to cell contact, but the receptors and cognate ligands involved in recognition and the exact mechanism of action remain largely undefined (Levitz et al. 1995). Similar direct antifungal effects of T lymphocytes have also been described for *A. fumigatus* (Levitz et al. 1995; Ramadan et al. 2005; Beck et al. 2006).

2.3.6 Prevention and treatment of *A. fumigatus* infections

The ubiquitous presence of fungal spores even in the hospital environment makes it difficult to prevent infection of immunocompromized patients. Chemoprophylaxis against fungal infections is problematic because of possible toxic side effects as well as the development of drug-resistant pathogenic strains (Cornely et al. 2007; Cornely 2008). Reduction of aerial contamination is a further efficient prophylactic method, but

although filter devices can significantly reduce the concentration of airborne conidia, it is not possible to completely eliminate the risk for infections (Latge 1999).
At present, fungal infections are mostly treated with antifungal drugs, but novel therapeutic strategies such as immune augmentation strategies, vaccination or adoptive T cell transfer are currently investigated for their clinical applicability (Table 1).

Antifungal agents	amphotericin B formulations	• broad spectrum of antifungal activity, but significant infusional toxicity
	azoles	• inhibit synthesis of ergosterol, a fungal cell membrane constituent
	echinocandins	• inhibit synthesis of glucan, a fungal cell wall constituent
Immune augmentation strategies	augmentation of neutrophil numbers	• colony-stimulating factors (G-CSF, GM-CSF) • granulocyte transfusions
	activation of neutrophils	• colony-stimulating factors (G-CSF, GM-CSF) • cytokines (IFN-γ) • chemokines • TLR activation
	activation of macrophages and dendritic cells	• colony-stimulating factors (M-CSF, GM-CSF) • cytokines (IFN-γ, TNF-α) • TLR activation
	activation or re-direction of cellular immunity	• cytokines (IFN-γ) • cytokine antagonists (IL-4, IL-10 antagonists) • TLR activation • mannan-binding lectin (MBL) • pentraxin 3 • thymosin α1
Vaccination	administration of antigenic preparations	• fungal preparations • recombinant proteins • peptides
	dendritic cell vaccination	• antigen-pulsed DC • mRNA-transfected DC
Adoptive T cell transfer	infusion of *ex vivo* generated antigen-specific T cells	

Table 1:
Current antifungal therapy and future strategies for the treatment of Invasive Aspergillosis (adapted in part from Segal and Walsh 2006).

2.3.6.1 Antifungal drugs

The most commonly used antifungal drugs include amphotericin B formulations, azoles and echinocandins. Amphotericin B was the antifungal standard therapy in the 1990's, but its application is limited by severe toxic side effects including fever, chills and

nephrotoxicity (Sau et al. 2003). The second-generation triazole voriconazole which is currently used as standard therapy is superior to conventional amphotericin B therapy because of its broad antifungal spectrum as well as its better tolerance (Odds et al. 2003; Tokimatsu and Kadota 2006). In recent studies combination of amphotericin B or azoles with echinocandins also has shown good efficiency (Segal and Walsh 2006). But the overall therapeutic efficacy of these drugs is often limited by significant toxicity and, probably even more important, the emergence of drug-resistant *Aspergillus* and other fungal strains (Howard et al. 2006; Verweij et al. 2007).

2.3.6.2 Immune augmentation strategies

Novel strategies for prevention and treatment of fungal infections investigate the possibility to improve the immune function of immunocompromized hosts. Those immunomodulatory therapies are predominantly intended to increase the number and/or fungicidal activity of innate immune cells or to stimulate antigen-specific immunity (Pirofski and Casadevall 2006; Segal et al. 2006). Administration of granulocyte infusions for instance could restore the number of phagocytic cells in neutropenic patients (Sachs et al. 2006). Preclinical studies also show therapeutic potential of a variety of cytokines, growth factors and immunomodulators in improving immune responses, influencing the T_H1/T_H2 balance or reversing the deleterious effects of immunosuppressive therapy. Augmentation of antifungal immunity could be achieved for instance by administration of IFN-γ or colony-stimulating factors (CSFs), which efficiency has been supported by extensive *in vitro* and *in vivo* preclinical data and some of which are already approved for clinical use (Nagai et al. 1995; Roilides et al. 2003; Safdar 2006). Granulocyte colony-stimulating factor (G-CSF) for instance increases granulocyte numbers, modulates their biological function and enhances oxidative mechanisms, and macrophage colony-stimulating factor (M-CSF) enhances cytotoxicity against hyphae, phagocytosis of conidia, superoxid production, chemotaxis and cytokine production in monocytes and macrophages (Roilides et al. 1995; Nemunaitis 1998). Similarly, monocytes and PMN treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) or IFN-γ exhibit enhanced conidial phagocytosis and production of reactive oxygen intermediates as well as cause increased hyphal damage (Roilides et al. 1993; Roilides et al. 1994; Gil-Lamaignere et al. 2005). Both cytokines act synergistically to substantially boost the fungicidal

functions of innate immune cells and can even reverse the suppressive effects of corticosteroids (Roilides et al. 1993; Roilides et al. 1996). TNF-α has also previously been shown to stimulate phagocytosis by monocytes and TNF-α depletion resulted in reduced recruitment of neutrophils into the lungs of infected mice as well as increased mortality (Mehrad et al. 1999). However, the toxic side-effects of this pro-inflammatory cytokine preclude its use in human therapies. A further possibility to improve antifungal immunity could be the administration of T_H1 cytokines, for example IFN-γ, or the neutralization of T_H2 cytokines such as IL-4 or IL-10 by cytokine antagonists or the targeting of distinct classes of TLRs on dendritic cells to divert the T cell response toward a favorable T_H1 response with optimal fungicidal activity in the absence of detrimental inflammatory responses (Romani 2001; Luther and Ebel 2006). Furthermore, administration of mannan-binding lectin (MBL) increases the levels of TNF-α and IL-1α in mice and can have a protective effect and even rescue corticosteroid-immunosuppressed mice (Kaur et al. 2007). Similarly, administration of pentraxin 3 or thymosin α1 has been shown to increase resistance to infection (Gaziano et al. 2004; Romani et al. 2004).
However, because of the complexity of the cytokine network as well as their diverse synergistic and antagonistic interactions, intervention is delicate and the data obtained so far have to be validated in further clinical trials.

2.3.6.3 Vaccination protocols

A further possibility to reduce the incidence of invasive fungal diseases could be the vaccination of patients who are at risk for infection or the vaccination of stem cell donors prior to transplantation. A lot of work in this area has already been done in mouse models. The initial immunization experiments were performed with viable or heat-killed *A. fumigatus* conidia or crude culture filtrates. The efficacy and type of the ensuing immune response was shown to be dependent on the immune state of the animals, as well as the type of antigen used. Vaccination with viable conidia, crude hyphal extract or culture filtrate for example was able to induce protective T_H1 responses, whereas administration of heat-killed conidia resulted in non-protective T_H2 responses (Cenci et al. 2000; Bozza et al. 2002; Ito and Lyons 2002). Remarkably, vaccinated animals were protected against infection even when they were rechallenged later during immunosuppressive corticosteroid treatment (Ito and Lyons 2002).

Protective immunity could not only be achieved with whole fungal preparations, but also with the recombinant proteins Asp f16 or Asp f3 when they were administered together with adjuvant (Bozza et al. 2002; Ito et al. 2006).
As dendritic cells show high plasticity and play a key function in initiating and directing immune responses, one way to drive immunity into the protective T_H1 direction could be vaccination with *ex vivo* generated antigen-presenting DC. Different approaches have been tested so far including transfer of DC pulsed with antigen or DC transfected with fungal mRNA. The outcome was highly dependent on the DC subset used in the experiments, and different types of DC were able to activate protective or non-protective helper T cells as well as regulatory T cells (Perruccio et al. 2004; Bellocchio et al. 2005; Montagnoli et al. 2008). Transfer of antigen-pulsed DC also accelerated the recovery of functional antifungal T_H1 responses and rescued mice after allogeneic bone marrow transplantation (Bozza et al. 2003; Perruccio et al. 2004; Bellocchio et al. 2005).
In clinical trials, vaccination of allogeneic HSCT recipients with DC loaded with peptides of cytomegalovirus (CMV) was shown to be protective against viral infection, suggesting that vaccination with fungal antigens could be similarly promising (Grigoleit et al. 2007).

2.3.6.4 Adoptive T cell transfer

Patients that are already suffering from disease could potentially profit from the adoptive transfer of pathogen-specific effector and memory T cells. Adoptive T cell therapy has already been evaluated in cancer patients or as potential anti-viral therapy (Riddell et al. 1992; Yee 2006; June 2007). That adoptive transfer of pathogen-specific T cells can indeed have curative potential was shown in studies transferring *ex vivo* generated donor-derived CMV-specific T cells into stem cell transplant recipients (Einsele et al. 2002; Perruccio et al. 2005). The viral load in the recipients dropped significantly and the infection was cleared without inducing significant alloreactivity.
Adoptive transfer of *Aspergillus*-specific T cells was performed so far mostly in mouse models, but also one human study in stem cell transplant recipients. In mice, T cell transfer from immunized animals was not only able to transfer protection to immunocompetent but also to neutropenic recipients (Cenci et al. 2000). In the human study, infusion of *Aspergillus*-specific donor T cell clones induced a stable T cell

response with a high IFN-γ/IL-10 ratio and stable T cell frequencies over time, whereas spontaneous pathogen-specific T cells in untreated patients were characterized by low frequencies and a non-protective low IFN-γ/IL-10 ratio. A single T cell infusion was sufficient to control antigenemia and infection-related mortality in the patients without eliciting harmful graft-versus-host disease (Perruccio et al. 2005).

2.4 Objectives of the project

In the first part of this work, Hsp90 inhibitors that are already successfully used in anti-cancer therapy were tested for their possible application in selective depletion of alloreactive cells from lymphocyte populations. The targeted removal of alloreactive T cells from transplants used for allogeneic hematopoietic stem cell transplantation would greatly decrease the incidence of GvHD hopefully without impairing pathogen-specific immunity and the beneficial GvL effect.
The Hsp90 inhibitor 17-DMAG was investigated for its potential to compromise the proliferation and survival of alloreactive cells in mixed lymphocyte reactions (MLRs). Furthermore, the ability of allo-depleted MLRs to respond to rechallenge with viral antigens was determined, to confirm the specificity of the depletion process.

In the second part of this work, *A. fumigatus*-specific $CD4^+$ T cell responses to strictly defined fungal antigens such as recombinant proteins or peptides were analyzed, because the development of *A. fumigatus* vaccines or the generation of pathogen-specific T cells for adoptive transfer all depend on the identification and characterization of highly immunogenic fungal antigens that can be used in a larger scale in clinical applications. It was recently shown that peripheral blood lymphocytes from healthy individuals can be induced to proliferate upon stimulation with heat-killed *A. fumigatus* conidia, cellular extract or fungal proteins (Grazziutti et al. 1997; Hebart et al. 2002; Beck et al. 2006). But one major problem regarding the use of cellular extracts is the qualitative and quantitative variability in the composition of these antigenic preparations. There has been observed a great variability between fungal extracts prepared in different laboratories and even between batches produced in the same laboratory (Latge 1999). Major factors probably responsible for antigenic differences between different preparations are for instance the incubation period, composition of the culture medium and the method of extraction. It is presumably also

of high importance whether the preparations are made from resting, swollen or germinating conidia or from mycelia. This high variability renders it difficult to use such extracts in the clinical setting and emphasizes the need for strictly defined fungal antigens such as recombinant proteins or peptides.

PBMC from a number of healthy donors were stimulated with different recombinant *A. fumigatus* proteins or peptides to determine the presence of memory cells specific for these fungal antigens. The presence of such memory cells in many different individuals would be indicative for their immunogenic potential. Antigens that showed good responses in the majority of donors were then characterized in more detail, especially for their ability to educate and stimulate cells of the innate immune system which constitute the effector arm of fungal immunity.

3 Materials

3.1 Media, chemicals and reagents

- cell culture media and supplements:

Bicoll separating solution (Biochrom)
cyclosporine A (Sigma-Aldrich)
DMEM cell culture medium (Invitrogen)
DMEM/F-12 cell culture medium (Invitrogen)
DMSO (Sigma-Aldrich)
Dulbecco's phosphate-buffered saline (PBS) (PAA)
EDTA solution 0.5 M (AppliChem)
fetal calf serum (FCS) (Biochrom)
FuGENE (Roche)
GM-CSF (PromoCell)
Holo-transferrin (Sigma-Aldrich)
human insulin (Proleukin, Chiron)
IL-1β (R&D systems)
IL-2 (Proleukin; Chiron)
IL-4 (R&D systems)
IL-6 (R&D systems)
IMDM cell culture medium (Lonza GmbH)
Lipofectamine 2000 (Invitrogen)
L-glutamine solution (Invitrogen)
neomycin (Sigma-Aldrich)
OKT-3 (Orthoclone)
Opti-MEM cell culture medium (Invitrogen)
penicillin/streptomycin (Invitrogen)
PGE_2 (Pharmacia)
puromycin (Sigma-Aldrich)
RPMI 1640 cell culture medium (Invitrogen)
sodium butyrate (Sigma-Aldrich)
TNF-α (R&D systems)
trypsin/EDTA solution (Invitrogen)

- chemicals and reagents:

ABTS (Roche)
acetic acid (AppliChem)
acrylamid (Roth)
agar (AppliChem)
agarose (Peqlab)
ampicillin (Roth)
Annexin V (Abcam)
anti-FLAG M2-agarose (Sigma-Aldrich)
APS (Sigma-Aldrich)
BCA Protein Assay Kit (Perbio)
BD OptEIA Set Human GM-CSF (BD Bioscience)
brefeldin A (BD Biosciences)
bovine serum albumin (BSA) (Sigma-Aldrich)
calcium chloride (Sigma-Aldrich)
carbonate-bicarbonate buffer pH 9.6 (Sigma-Aldrich)
CD3/CD28 Dynabeads (Invitrogen)
DNA ladder 1 kb PLUS (Invitrogen)
enhanced chemiluminescence substrate (ECL) (Perbio)
ethanol (AppliChem)
FACS permeabilizing solution 2 (BD Biosciences)
FLAG peptide (Sigma-Aldrich)
ionomycin (Sigma-Aldrich)
kanamycin (Invitrogen)
MACS cell sorting systems (Miltenyi Biotec)
MESA GREEN qPCR Kit (Eurogentec)
mMESSAGE mMACHINE T7 Ultra Kit (Ambion)
methanol (AppliChem)
milk powder (Roth)
Naïve $CD8^+$ T cell isolation kit (Miltenyi Biotec)
NBT/BCIP substrate solution (Mabtech)
Ni-NTA Purification Kit (Invitrogen)
Nonidet P40 (AppliChem)
paraformaldehyde (AppliChem)
phytohemagglutinin (PHA-M) (Sigma-Aldrich)
phosphate-buffered saline (PBS) (PAA)

peptone (AppliChem)
phorbol myristate acetate (PMA-M) (Sigma-Aldrich)
polybrene (Sigma-Aldrich)
propidium iodide (Sigma-Aldrich)
protease inhibitor cocktail (Roche)
protein marker (BioRad)
PVDF membrane (Perbio)
RDI-PHRPDIL blocker (Fitzgerald)
RDI-PHRP20 streptavidin (Fitzgerald)
RNeasy Mini Kit (Qiagen)
streptavidinALP-PQ (Mabtech)
SuperScript II Reverse Transcriptase (Invitrogen)
TEMED (Sigma-Aldrich)
Tris (Roth)
Tween 20 (Roth)
Vybrant CFDA SE Cell Tracer Kit (Invitrogen)
yeast extract (AppliChem)
17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17- DMAG) (Invivogen)

3.2 Buffers and solutions

cell lysis buffer (western blot):	50 mM Tris pH 7.5; 150 mM NaCl; 1% Nonidet P40; 0.5% sodium deoxycholate; protease inhibitor cocktail
cell lysis buffer (M2 purification):	50 mM Tris-HCl pH7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton-X-100; protease inhibitor cocktail
coomassie blue staining solution:	3 mM Coomassie Brilliant Blue R-250; 45% (v/v) methanol; 10% (v/v) acetic acid
coomassie blue destaining solution:	45% (v/v) methanol; 10% (v/v) acetic acid

ELISPOT substrate buffer:	0.1 M NaCl; 50 mM $MgCl_2$; 0.1 M Tris-HCl pH 9.5
2x HBS buffer:	50 mM HEPES; 1.5 mM Na_2HPO_4; 280 mM NaCl; pH 6.95
LB culture medium:	10 g tryptone; 5 g yeast extract; 10 g NaCl; ad 1l dH_2O; pH 7.0
LB agar:	10 g tryptone; 5 g yeast extract; 10 g NaCl; 15 g agar; ad 1l dH_2O; pH 7.0
SDS PAGE running buffer:	25 mM Tris; 192 mM glycine; 0.1% SDS
TBS:	50 mM Tris; 150 mM NaCl; pH 7.5
TBS-T:	50 mM Tris; 150 mM NaCl; 0.05% Tween-20; pH 7.5
western blot transfer buffer:	38 mM glycine; 50 mM Tris; 0.037% SDS; 20% (v/v) methanol

3.3 Antibodies, MHC class I pentamers and MHC class II tetramers

antibodies for ELISA:	anti-IFN-γ antibody (EndogenA) (Perbio)
	anti-IFN-γ antibody (EndogenB) (Perbio)
antibodies for ELISPOT:	Mab 1-D1K (Mabtech)
	Mab 7-B6-1-Biotin (Mabtech)
functional antibodies:	MHC class II blocking antibody (BD Biosciences)
	anti-CD28 antibody (BD Biosciences)
	anti-CD49d antibody (Beckman Coulter)
antibodies for western blot:	anti-FLAG antibody (Sigma-Aldrich)
	anti-Hsp90α antibody (Chemicon)

	anti-Hsp90β antibody (Chemicon)
	anti-vinculin antibody (Santa Cruz Biotechnology)
	anti-V5 antibody (Invitrogen)
	HRP-conjugated sec. antibody (Sigma-Aldrich)
antibodies for cell selection:	anti-NGFR antibody (BD Biosciences)
	anti-CD25 antibody (BD Biosciences)
antibodies for flowcytometry:	anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD25 (all BD Biosciences)
MHC class I pentamer:	A*0201 NLVPMVATV APC (Proimmune) (CMV_{pp65}-specific)
MHC class II tetramer:	DRB1*0401 FHTYTIDWTKDAVTW PE (Beckman Coulter) (*A. fumigatus* Crf1-specific)

3.4 Plasmid vectors and cDNA

For expression of recombinant proteins the eukaryotic expression vector pcDNA3.1/V5-His (Invitrogen) was used. Transgene expression is driven by a CMV promoter and transfected cells can be selected by neomycin resistance.
For production of infectious retrovirus the retroviral vector pLZNGFR-PGK (kindly provided by Stanley Riddell, Fred Hutchinson Cancer Research Center, Seattle, USA) was used. pLZNGFR-PGK allows isolation of stably transduced cells by expression of a truncated form of the nerve growth factor receptor (ΔNGFR) or puromycin resistance. The transgene is expressed via the PGK promoter, which permits relatively high expression levels in primary human B and T cells (Kondo et al. 2002; Adamopoulou et al. 2007).
For production of mRNA the vectors pGEM4Z-5'UT-3'UT-A64 and pGEM4Z-5'UT-3'UT-A64-GFP (kindly provided by Kris Thielemans, Brussels) were used.

cDNA coding for the *A. fumigatus* extracellular cell wall glucanase Crf1 (AFUA_1G16190) was kindly provided by Dr. Utz Reichard (University of Goettingen). The sequence of the Crf1 cDNA is identical to the previously published sequence AY169706.1 and differs in one nucleic acid at position 995 from the sequence

AFUA_1G16190 leading to an amino acid substitution from serine to threonine (Nierman et al. 2005).
cDNA coding for the major allergen and cytotoxin AspF1 (AFUA_5G02330) and the CipC-like antibiotic response protein CipC (AFUA_5G09330) was kindly provided by Prof. Dr. Stefan Stevanović (University of Tuebingen). The sequence of the AspF1 cDNA is identical to the previously published sequence M83781.1 and differs from the sequence AFUA_5G02330 by encoding for asparagine at position 25 instead of serine (Nierman et al. 2005).
cDNA coding for the first 80 N-terminal amino acids of the invariant chain were kindly provided by Prof. Dr. Stefan Stevanović (University of Tuebingen).

3.5 Oligonucleotides

For cloning of V5/His-tagged fusion proteins the following oligonucleotides were used (Sequence 5'-3'):

HindIII-aspf1-forw:	GGGAAGCTTGCCACCATGGCGACCTGGACATGCATC
NotI-aspf1-rev:	GGGGCGGCCGCATGAGAACACAGTCTCAAGTC
HindIII-cipC-forw:	GGGAAGCTTGCCACCATGGCTTGGGGCTGGGAG
NotI-cipC-rev:	GGGGCGGCCGCGCAACGGTCGACAGGGCG
HindIII-crf1-forw:	GGGAAGCTTGCCACCATGTATTTCAAGTACACAGCAG
NotI-crf1-rev:	GGGGCGGCCGCGAATGCCAACACGGCAGC

For cloning of FLAG-tagged fusion proteins the following oligonucleotides were used (Sequence 5'-3'):

HindIII-FLAG-aspf1-forw:
GGGAAGCTTGCCACCATGGATTACAAGGACGATGACGATAAGGCGACCTGGACATGCATC

NotI-FLAG-aspf1-rev:
GGGGCGGCCGCTTACTTATCGTCATCGTCCTTGTAATCATGAGAACACAGTCTCAAGTC

HindIII-FLAG-cipC-forw:
GGGAAGCTTGCCACCATGGATTACAAGGACGATGACGATAAGGCTTGGGGCTGGGAG

NotI-FLAG-cipC-rev:
GGGGCGGCCGCTTACTTATCGTCATCGTCCTTGTAATCGCAACGGTCGACAGGGCG

HindIII-FLAG-crf1-forw:
GGGAAGCTTGCCACCATGGATTACAAGGACGATGACGATAAGTATTTCAAGTACACAGCAG

NotI-FLAG-crf1-rev:
GGGGCGGCCGCTTACTTATCGTCATCGTCCTTGTAATCGAATGCCAACACGGCAGC

For introduction of a point mutation into the AspF1 gene the following oligonucleotides were used (Sequence 5'-3'):

HindIII-aspf1-forw:	GGGAAGCTTGCCACCATGGCGACCTGGACATGCATC
aspf1-H136L-forw:	GCGGCATTGTGGCCCTTCAGCGGGGG
aspf1-H136L-rev:	CCCCCGCTGAAGGGCCACAATGCCGC
NotI-aspf1-rev:	GGGGCGGCCGCATGAGAACACAGTCTCAAGTC

For cloning of the invariant chain-Crf1 fusion protein the following oligonucleotides were used (Sequence 5'-3'):

HindIII-li-forw:	CCCAAGCTTATGCACAGGAGGAGAAGCAG
li-crf1-forw:	CAGGGCCGGCTGGACAAAATGTATTTCAAGTACACAGCAG
li-crf1-rev:	CTGCTGTGTACTTGAAATACATTTTGTCCAGCCGGCCCTG
NotI-crf1-rev:	GGGGCGGCCGCGAATGCCAACACGGCAGC

For genetic knock-down of Hsp90β the following oligonucleotides were used (Sequence 5'-3'):

control siRNA:	OR-0030-Neg05 (Eurogentec)
Hsp90β-specific siRNA:	AUUCUUGUCGGCCUCAGCCdTdT

For real time PCR the following oligonucleotides were used (Sequence 5'-3'):

hsp90 forward:	GAGAGCCTGACAGACCC)
hsp90 reverse:	GCCCAATCATGGAGATGT)
β-microglobulin forward:	GGGTTTCATCCATCCGACAT
β-microglobulin reverse:	GATGCTGCTTACATGTCTCGA

3.6 Fungi, recombinant proteins and peptides

The fungi *A. fumigatus* strain ATCC 9197 and *C. albicans* were used. The fungi were kindly provided by Dr. Juergen Loeffler and the experiments performed at the Institute for Hygiene and Microbiology of the University of Wuerzburg with the help of Anke Hornbach.

Different *A. fumigatus* cell extracts, cell pellets and culture supernatant was kindly provided by Dr. Olaf Beck (University of Frankfurt).

The recombinant *A. fumigatus* proteins superoxide dismutase SOD (AFUA_5G09240), catalase Cat (AFUA_2G18030), aspartic endopeptidase PEP (AFUA_5G13300) and 1,3-beta-glucanosyl-transferase Gel1 (AFUA_2G01170) produced in the yeast *Pichia pastoris* were kindly provided by Prof. Jean-Paul Latgé (Institute Pasteur, Paris, France).

The following 5 synthetic peptides derived from the Crf1 sequence were designed using the SYFPEITHI algorithm (Rammensee et al. 1999) and kindly provided by Prof. Dr. Stefan Stevanović (University of Tuebingen):

designation:	**amino acid sequence:**
AST	ASTYTADFTSASALD
EVD	EVDWEVLGGDTTQVQ
FHT	FHTYTIDWTKDAVTW
GAE	GAEFTVAKQGDAPTI
VKS	VKSVRIENANPAESY

The 15mer CMV_{pp65} peptide pool (PepMix pp65) was purchased from JPT Peptide Technologies.

3.7 Cell lines

The human epithelial kidney cell line HEK 293T, the African green monkey kidney cell line COS-1, the human cervix carcinoma cell line HeLa and the human lymphoblast cell line T2 were purchased from the American Type Culture Collection (ATCC).

The HEK 293T-derivative phoenix GALV (Horn et al. 2002) and the mouse embryonic fibroblast cell line NIH/3T3 transfected with CD40 ligand (Schultze et al. 1997) were kindly provided by Stanley Riddell (Fred Hutchinson Cancer Research Centre, Seattle, USA).

4 Methods

4.1 Cloning of recombinant *A. fumigatus* proteins

4.1.1 Cloning of expression vectors

The genes encoding the *A. fumigatus* extracellular cell wall glucanase Crf1 (AFUA_1G16190), the major allergen and cytotoxin AspF1 (AFUA_5G02330) and the CipC-like antibiotic response protein CipC (AFUA_5G09330) were cloned into the eukaryotic expression vector pcDNA3.1-V5/His (Invitrogen). The cDNA was either cloned in frame resulting in fusion proteins with C-terminal V5/His-Tag or, alternatively, primers coding N- and C-terminally for FLAG-tags were used (see section 3.5).

4.1.2 Introduction of a point mutation in AspF1 by overlap PCR

To make expression of the ribotoxin AspF1 in eukaryotic cells possible, a point mutation changing the histidine residue at position 136 to leucin (AspF1-H136L) was introduced, which significantly diminished toxicity in the producer cells as previously described (Yang and Kenealy 1992; Kao et al. 1998). The point mutation was introduced by overlap PCR as shown in Figure 7 using mutated primers.

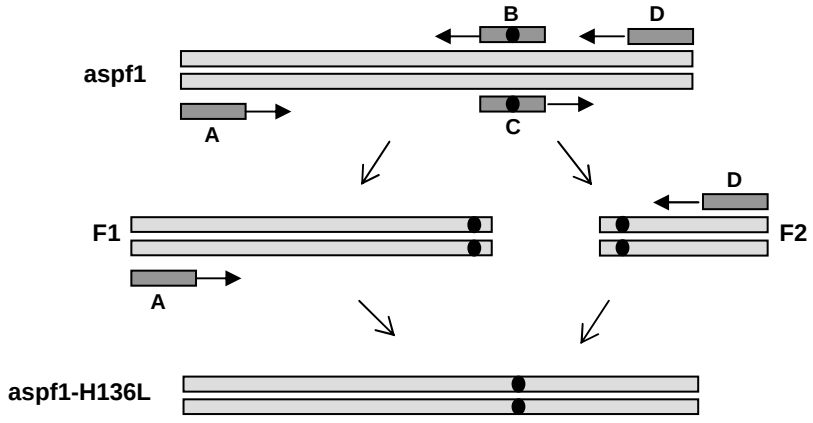


Figure 7:
Introduction of the point mutation H136L in AspF1 by overlap PCR. PCR fragment F1 was generated with the primers HindIII-aspf1-forw (A) and aspf1-H136L-rev (B) and the fragment F2 with the primers aspf1-H136L-forw (C) and NotI-aspf1-rev (D). Both fragments were used together as template in a PCR with the primers HindIII-aspf1-forw (A) and NotI-aspf1-rev (D).

4.2 *In vitro* synthesis of mRNA and cDNA

4.2.1 *In vitro* transcription

GFP-coding mRNA was *in vitro* transcribed from the vector pGEM4Z-5'UT-3'UT-A64-GFP after linearization with the restriction endonuclease SpeI using the mMESSAGE mMACHINE T7 Ultra Kit (Ambion) according to the manufacturers instructions.

4.2.2 Real time PCR

Knock-down of Hsp90β with siRNA (see section 4.6.3) was determined by real time PCR in a Bio-Rad DNA Engine Opticon TM2 device using a MESA GREEN qPCR kit according to the manufacturers instructions. RNA was purified from cells with a RNeasy Mini Kit (Qiagen). The primers used for real time PCR were hsp90 forward and hsp90 reverse and β-microglobulin was used as reference gene.

4.3 Culture and transfection of eukaryotic cell lines

4.3.1 Culture of eukaryotic cell lines

The eukaryotic cell lines HeLa and T2 were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. The cell lines HEK 293T, phoenix GALV and COS-1 were cultivated in DMEM medium supplemented with 10% heat-inactivated FCS. The cell line NIH/3T3-CD40L was cultivated in DMEM/F-12 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine. All cell culture media were supplemented with 10 µg/ml Penicillin/Streptomycin.
Adherent cells were detached by treatment with trypsin / EDTA solution.
Cells were stored frozen in culture medium containing 10% DMSO in liquid nitrogen.

4.3.2 Electroporation

HEK 293T cells were electroporated with plasmid DNA in an EasyjecT PLUS device (Equibio). $2x10^7$ cells were mixed with 30 µg plasmid DNA in 1 ml Opti-MEM medium in

4 mm electroporation cuvettes (Peqlab) and electroporated at 250 V and 1800 µF. After electroporation cells were cultivated in DMEM medium containing 10% FCS, Penicillin/Streptomycin and, if necessary, antibiotics for selection of transfected cells.

4.3.3 Calcium phosphate transfection

For transfection of HEK 293T cells, phoenix GALV cells or HeLa cells, $3x10^5$ cells were plated per well in 6-well cell culture plates. After 24h incubation when cells had grown to about 60% confluence, the medium was aspirated and replaced with 1125 µl fresh medium. For transfection of 12 wells, 24 µg plasmid DNA were mixed with 274 µl 2M $CaCl_2$ solution, 1956 µl dH_2O and 2250 µl 2xHBS buffer. The DNA was precipitated by vigorous agitation and 375 µl of the HBS/DNA solution per well spread in drops across the cell layer. After 24h the culture medium was renewed and, if necessary, supplemented with antibiotics for selection of transfected cells.

4.3.4 Lipofection

HeLa cells and COS-1 cells were transfected by lipofection with Lipofectamine 2000 (Invitrogen) or FuGENE (Roche) according to the manufacturers instructions. Best results were obtained with a cell density of $1x10^5$ cells per well in 6-well cell culture plates and a DNA to lipofectant ratio of 1:5 (1 µg DNA, 5 µl lipofectant). Cells were washed after 4h to increase viability.

4.4 Purification of recombinant proteins

4.4.1 Protein purification with a Ni-NTA Purification system

His-tagged recombinant proteins were expressed cytoplasmically in phoenix GALV cells, the cells lysed and the protein purified with a Ni-NTA Purification system (Invitrogen) according to the manufacturers instructions. Native as well as denaturing conditions and different lysis and elution buffers were tested.

4.4.2 Protein purification with anti-FLAG M2 affinity Gel

FLAG-tagged recombinant proteins were expressed cytoplasmically in phoenix GALV cells, the cells lysed with buffer without deoxycholate and the protein purified under native conditions with anti-FLAG M2 affinity Gel (Sigma Aldrich) according to the manufacturers instructions. The proteins were eluted with 100 µg/ml FLAG peptide.

4.5 Protein detection and quantification

4.5.1 Western Blot

Cells were lysed with lysing buffer containing deoxycholate, the protein concentration determined and 2-10 µg protein per lane run on SDS-polyacrylamid gels according to standard protocols. The gels were blotted onto PVDF membrane and the blots incubated first with primary antibody for 1-2h and subsequently with HRP-conjugated secondary antibody for 30 min. The blots were developed using chemiluminescent (ECL) techniques.

4.5.2 Coomassie staining

Cells were lysed with lysing buffer containing deoxycholate, the protein concentration determined and 2-10 µg protein per lane run on polyacrylamid gels according to standard protocols. The gels were incubated with coomassie staining solution for 10 min at RT and subsequently washed with destaining solution for 1-2h. For an approximate estimation of the protein concentration BSA solution of defined concentrations were used as reference.

4.5.3 Protein quantification

Protein concentration was determined using a BCA Protein Assay Kit (Perbio) according to the manufacturers instructions.

4.6 Isolation and culture of primary human blood cells

4.6.1 Isolation of PBMC from whole blood

Blood was obtained from voluntary HLA-DRB1*-typed healthy donors. Peripheral blood mononuclear cells (PBMC) were isolated via centrifugation in Biocoll Separating Solution, washed with PBS and either used directly after preparation or cryopreserved for later use.
PBMC were cultured in RPMI 1640 medium with L-glutamine, supplemented with 10% heat-inactivated, pooled human serum and 100 U/ml Penicillin/Streptomycin. To increase cell survival 5 U/ml IL-2 were added every other day and cell culture medium replenished as needed.
Cells were stored frozen in culture medium containing 10% DMSO in liquid nitrogen.

4.6.2 Isolation of T cells and T cell subsets by magnetic cell separation

$CD3^+$, $CD4^+$ or $CD8^+$ T cells or CD25-positive cells were isolated from whole PBMC by magnetic cell separation using antibody-conjugated microbeads. CD45RO-negative and -positive cells were separated using the Naïve $CD8^+$ T cell Isolation Kit according to the manufacturers instructions (all MACS cell sorting systems, Miltenyi Biotec).

4.6.3 Transfection of primary T lymphocytes with mRNA and siRNA

mRNA or siRNA were introduced into T cells by electroporation. For T cell transfection $5x10^6$ cells were pre-incubated on ice in 200 µl OPTI-MEM medium with 20µg mRNA or 40µM siRNA and electroporated using a Equibio EasyjecT plus device and 4 mm electroporation cuvettes (Peqlab) by applying 300-380 V and 300-600 µF. Following transfection the T cells were either left untreated or activated with 10 µg/ml PHA for 24h. Cell viability and transfection efficiency of T cells electroporated with mRNA was estimated flowcytometrically and genetic knock-down with siRNA was determined by western blot and real time PCR.

4.6.4 Isolation and culture of monocytes

Monocytes were isolated from whole PBMC by allowing cells to adhere to tissue culture dishes for 2h and subsequent extensive washing to remove non-adherent cells. For presentation of protein antigen, monocytes were cultured overnight in the presence of 5 µg/ml recombinant protein and harvested by mechanical abrasion the next day. For presentation of peptide antigen, monocytes were incubated overnight without antigen and mature cells harvested and pulsed with 1 µg/ml peptide antigen for 1h prior to use.

4.6.5 Generation of monocyte-derived dendritic cells

Dendritic cells were generated according to the protocol for the generation of “fast-DC” with some modifications (Dauer et al. 2003). Immature monocyte-derived dendritic cells (DC) were generated from monocytes by addition of 500 U/ml GM-CSF and 700 U/ml IL-4 to the culture medium. After 24 to 36h the dendritic cells were induced to mature by addition of 25 ng/ml TNF-α, 5 ng/ml IL-1β, 10 ng/ml IL-6 and 1 µg/ml PGE_2 for additional 24h.

4.7 Stimulation and cloning of T cells

4.7.1 Stimulation of T cells and generation of antigen-specific T cell lines

Antigen-specific T cell lines were generated by incubation of $1x10^7$ whole PBMC per well in 6-well cell culture plates with 5 µg/ml recombinant protein or 1 µg/ml peptide antigen for 7 days. Lymphocyte cultures were supplemented with 5 U/ml IL-2 every other day and culture medium replenished as needed.

4.7.2 Cloning of T cells by limiting dilution

For the generation of T cell clones specific for the *A. fumigatus* protein Crf1, PBMC were stimulated repeatedly with 1 µg/ml FHT peptide once weekly for 4 weeks or antigen-specific cells were isolated using the IFN-γ secretion assay (Miltenyi Biotec).

Subsequently T cell clones were isolated by limiting dilution in 96-well plates. For T cell cloning $3x10^2$ T cells were mixed with $7.5x10^6$ gamma-irradiated T2 cells (60 Gy) and $1x10^8$ gamma-irradiated PBMC (30 Gy) in 200 ml RPMI medium with 10% heat-inactivated, pooled human serum. The cell suspension was supplemented with 30 ng/ml OKT-3 and 50 U/ml IL-2 and allotted to 10 96-well plates. After 14 days of culture the growing clones were tested for reactivity to FHT peptide.

4.7.3 Expansion of human T cells

Primary human T cells were expanded according to the rapid expansion protocol using monoclonal antibodies to the T cell activation molecule CD3 (Riddell and Greenberg 1990; Beck et al. 2006). Briefly, $2x10^5$ PBMC or T cells were cultured on $5x10^6$ gamma-irradiated T2 cells (60 Gy) and $2.5x10^7$ gamma-irradiated PBMC (30 Gy) in 25 ml RPMI medium with 10% heat-inactivated, pooled human serum in the presence of 30 ng/ml OKT-3. The culture medium was supplemented with 50 U/ml IL-2 every 3 days. After about 14 days of culture the cells were used for experiments.

4.7.4 Stimulation of T cell clones with *A. fumigatus* antigens

Experiments with viable *A. fumigatus* conidia and hyphae and *C. albicans* were performed at the Institute for Hygiene and Microbiology (University of Wuerzburg) with the help of Anke Hornbach. For some of the experiments the fungus was killed by treatment with 70% ethanol for 15 min at RT.

4.8 Cloning of retroviral vectors and transduction of B cells

4.8.1 Cloning of retroviral vectors

The *crf1* gene and a *li-crf1* fusion gene encoding N-terminally for the first membrane-spanning 80 amino acids of the invariant chain (*li*) were cloned into the retroviral vector pLZNGFR-PGK. The invariant chain fusion protein *li-crf1* was cloned by overlap PCR as described in Figure 8.

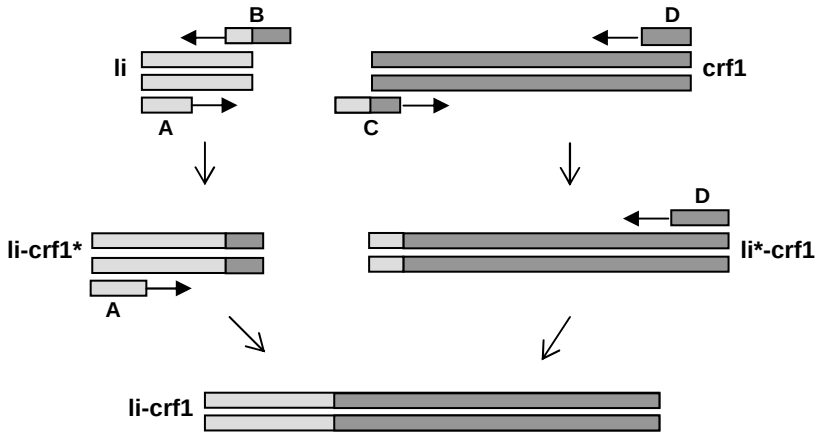


Figure 8:

Cloning of a *li-crf1* fusion gene by overlap PCR. PCR fragment li-crf1* was generated with the primers HindIII-li-forw (A) and li-crf1-rev (B) and the fragment li*-crf1 with the primers li-crf1-forw (C) and NotI-crf1-rev (D). Both fragments were used together as template in a PCR with the primers HindIII-li-forw (A) and NotI-crf1-rev (D).

4.8.2 Production of retroviral supernatant

Infectious virus used for transduction of primary B cells was produced using retroviral vectors and the retroviral packaging cell line phoenix GALV. Calcium phosphate transfection was performed as described in section 4.3.3. 24h after transfection the culture medium was exchanged and supplemented with 1 µg/ml puromycin to select stably transfected cells. For virus production $6x10^6$ stably transfected cells were plated into T-75 flasks and grown overnight without selective antibiotic. The culture medium was replaced with 7.5 ml fresh medium the next day and the cells grown overnight at 32℃. The retroviral supernatant was harvested, filtered and used for transduction of primary human B cells.

4.8.3 Generation of CD40L-activated B cells

$8x10^5$ gamma-irradiated (90 Gy) NIH/3T3 cells transfected with human CD40L were plated per well in 6-well plates. The next day $8x10^6$ PBMC in 4 ml IMDM medium supplemented with 10% heat-inactivated, pooled human serum, 4 ng/ml IL-4 and 0.7 µg/ml cyclosporine A were plated per well onto the feeder cells. The expanding B cells were transferred onto freshly prepared feeder cells every 3-4 days and transduced with retroviral vector after 7 days (see section 4.8.4) (Schultze et al. 1997).

4.8.4 Retroviral transduction of CD40L-activated B lymphocytes and NGFR selection

Retroviral transduction of B cells was performed 7 days after initiation of the B cell cultures. $6x10^6$ B cells were suspended in 3 ml of retroviral supernatant supplemented with 8 µg/ml polybrene and 4 ng/ml IL-4 per well in 6-well plates. Transduction was facilitated by spin infection (1800 rpm at 32°C for 1h). After infection 8 µg/ml polybrene and 4 ng/ml IL-4 were added per well and the cells incubated at 37°C. After 4h incubation the B cells were washed and plated onto fresh NIH/3T3-CD40L feeder cells.
Stably transduced B cells were isolated 7 days after transduction based on NGFR expression using magnetic cell separation techniques (Miltenyi Biotec). $1x10^7$ cells were incubated with 50 µl NGFR antibody in 500 µl PBS with 10% FCS for 30 min at 4°C. The labeled cells were separated by magnetic separation according to the manufacturers protocol.
Transduced CD40L-activated B cells were used in a ratio of 1:10 for restimulation of autologous T cells.

4.9 Analysis of the phenotype and effector functions of lymphocytes

4.9.1 Flowcytometry

For flowcytometric analysis $1x10^6$ cells were washed with cold PBS containing 10% FCS and the wash buffer decanted after centrifugation. Antibody (3-5 µl) was added and the samples incubated for 15 min at RT in the dark. The cells were washed with buffer, fixed with 1% paraformaldehyde and analyzed on a Becton Dickinson FACS Calibur using Cell Quest Pro software.
For assessment of apoptosis $5x10^5$ - $1x10^6$ PBMC or T cells were washed with PBS, resuspended in 100 µl Annexin V buffer (10mM Hepes/NaOH pH7.4; 140mM NaCl; 2.5mM $CaCl_2$) with 1 µl Annexin V and incubated for 10 min at RT in the dark. After addition of 300 µl Annexin V buffer cells were analyzed flowcytometrically.

4.9.2 MHC tetramer and pentamer staining

For staining with the MHC class I pentamer A*0201 NLVPMVATV $5x10^5$ cells were washed with PBS containing 10% FCS (PBS/FCS), resuspended in 100 µl PBS/FCS and incubated with 5 µl pentamer for 30 min at 37℃. Cells were washed with cold PBS/FCS, stained with anti-CD8 and anti-CD3 antibodies for 15 min at RT in the dark and analyzed flowcytometrically.
For staining with the MHC class II tetramer DRB1*0401 FHTYTIDWTKDAVTW $1x10^6$ cells were washed with PBS containing 0.1% BSA (PBS/BSA), resuspended in 50 µl PBS/BSA and incubated with 10 µl tetramer for 2h at 37℃. Cells were washed with PBS/BSA, stained with anti-CD8 and anti-CD3 antibodies for 15 min at RT in the dark and analyzed flowcytometrically.

4.9.3 Intracellular IFN-γ staining

For detection and quantification of antigen-specific T cells $1x10^6$ PBMC or T cells were stimulated with $2x10^5$ monocytes pulsed with protein or peptide antigen for 6h in the presence of 4 µg/ml anti-CD28 antibody and 4 µg/ml anti-CD49d antibody. After 1h incubation 0.5 µg/ml brefeldin A was added to prevent cytokine secretion. Control samples were activated with 0.5 µg/ml PMA and 1 µg/ml ionomycin. After 6h incubation cells were permeabilized with FACS permeabilizing solution for 12 min at RT, washed and labeled with anti-CD4, anti-CD8 and anti-IFN-γ antibody for 30 min at RT in the dark, fixed with 1% paraformaldehyde and analyzed flowcytometrically.

4.9.4 CFSE-dilution experiments

To assess T cell proliferation, PBMC were labeled with Vybrant CFDA SE Cell Tracer Kit (Invitrogen) according to the manufacturers instructions and activated with 5 µg/ml PHA, CD3/CD28 Dynabeads (Invitrogen) or allogeneic dendritic cells in a ratio of 10:1. Cultures were either left untreated or treated with 10 µM 17-DMAG 24h after culture initiation and analyzed flowcytometrically after 3-5 days.

4.9.5 Restimulation of T cells and IFN-γ and GM-CSF ELISA

To quantify secretion of IFN-γ or GM-CSF by activated T cells, $2x10^5$ T cells were plated in duplicates in 200 µl medium in 96-well plates. T cells were stimulated with $4x10^4$ monocytes per well either pulsed with 5 µg/ml protein over night or 1 µg/ml peptide for 1h. Alternatively, $5x10^6$ T cells were stimulated with $5x10^5$ dendritic cells fed overnight with protein, peptide or viable or ethanol-killed *A. fumigatus* conidia or hyphae in 3 ml medium in 12 well plates (experiments were done in the Institute for Hygiene and Microbiology with the help of Anke Hornbach). After 24h the supernatant was harvested, pooled and IFN-γ or GM-CSF ELISA performed in duplicates.

For IFN-γ ELISA, ELISA plates were coated with 100 µl/well 0.3 µg/ml Endogen M700A antibody in carbonate-bicarbonate buffer pH 9.6 overnight at 4°C. Plates were washed 3 times with wash buffer (PBS / 0.05% Tween20) and blocked with 200 µl RDI-PHRPDIL blocker for 1h at RT. After blocker removal, 50 µl of samples in different dilutions and an IFN-γ standard (0 – 200 ng/ml) were added and incubated for 1.5h with shaking. 100 µl/well 0.5 µg/ml Endogen M701B antibody in blocking buffer was added and incubated for further 1.5h. Plates were washed 5 times and incubated with 100 µl 250 ng/ml RDI-PHRP20 streptavidin in blocking buffer for 30 min. Plates were washed 6 times, incubated with 100 µl/well ABTS substrate solution for 5-15 minutes and absorption measured at 405 nm.

GM-CSF ELISA was performed with the BD OptEIA Set Human GM-CSF (BD Biosciences) according to the manufacturers instructions.

4.9.6 IFN-γ ELISPOT

IFN-γ ELISPOT was performed in duplicates either with fresh PBMC, fresh selected $CD4^+$ T cells or T cell lines pre-stimulated with antigen (5 µg/ml protein or 1 µg/ml peptide) for 1 week. ELISPOT plates (MultiScreen HTS, Millipore) were activated with 25 µl/well 35% ethanol, washed 3 times with PBS and coated with 100 µl/well 15 ng/ml Mab 1-D1K antibody overnight at 4°C. Plates were washed 6 times with PBS and blocked with 200 µl/well RPMI medium for 2h at 37°C. PBMC or T cells were plated in 3 different concentrations (between $1x10^5$ and $1x10^6$ cells per well) and stimulated with pre-loaded monocytes in a ratio of 5:1. Cells were incubated at 37°C overnight. Plates were washed 6 times with PBS and incubated with 100 µl/well 1 ng/ml Mab 7-B6-1-Biotin antibody diluted in PBS/1%BSA for 2h at RT. After 6 washes 100 µl/well streptavidinALP-PQ diluted 1:1000 in PBS/1%BSA was added and incubated for 1h at

RT. Plates were washed 6 times with PBS and once with ELISPOT substrate buffer. 100 µl/well NBT/BCIP substrate solution diluted 1:5 in substrate buffer was added and incubated in the dark for 5-30 min. Upon appearance of purple spots plates were washed repeatedly with tap water, dried and analyzed on an ELISPOT reader (CTL ImmunoSpot).

4.9.7 Plating efficiency assay

Killing of *A. fumigatus* conidia by monocytes was quantified using monocyte confrontation assays (experiments were performed in the Institute for Hygiene and Microbiology with the help of Nina Trzeciak). Monocytes were first pre-incubated with medium or supernatant from stimulated or unstimulated T cells for 30 min. Subsequently *A. fumigatus* conidia were added in a multiplicity of infection of 1. After 1, 2 and 3 hours monocytes were lysed by adding 2 ml cold dH_2O, the lysate diluted 1:5 in PBS and 90 µl of this dilution plated on Sabouraud agar. Controls were plated at 0 hours. Fungal colonies were counted after incubation at 37℃ overnight.

5 Results

5.1 Selective depletion of alloreactive T lymphocytes with Hsp90 inhibitors

5.1.1 Determination of Hsp90 protein levels in resting and activated T cells

It has been shown in our lab, that both subunits of the Hsp90 chaperone, Hsp90α and Hsp90β, are upregulated after stimulation with the mitogen PHA. To determine whether alloreactive T lymphocytes stimulated by allogeneic dendritic cells respond with similar chaperone upregulation, we established mixed lymphocyte reactions (MLRs) of PBMC of healthy donors with allogeneic dendritic cells. DC were generated using the protocol for the generation of “fast DC” described by Dauer et al (Dauer et al. 2003) with slight modifications.

To analyze if Hsp90 is selectively upregulated in alloreactive T cells after stimulation of PBMC with allogeneic DC, activated and resting T cell populations of MLRs have to be separated. To check if the T cell activation marker CD25 could be used to separate resting from activated cells, PBMC were labeled with the fluorescent dye CFSE prior to co-incubation with allogeneic DC and the surface expression of CD25 analyzed flowcytometrically 4 days after culture initiation (Figure 9A). As the great majority of highly CD25-positive cells are indeed $CFSE^{dim}$ proliferating cells, CD25 microbeads were used to separate activated alloreactive from non-reactive resting T cells of MLRs. Western blot analysis of the CD25-positive and -negative T cell populations showed only a slight upregulation of the Hsp90α subunit in activated T cells, but a more pronounced upregulation of Hsp90β (Figure 9B), which is in accordance with the data obtained by unphysiologic T cell activation with the mitogen PHA.

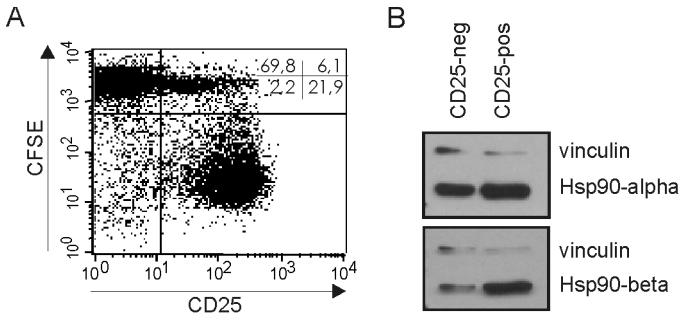


Figure 9:

PBMC were labeled with CFSE prior to stimulation with allogeneic DC and the expression of CD25 determined 4 days after culture initiation (A). A mixed lymphocyte culture was separated with CD25 microbeads 4 days after culture initiation and the expression of Hsp90 determined by western blot in both cell populations (B).

5.1.2 Inhibition of T lymphocyte proliferation in response to different antigenic stimuli by treatment with Hsp90 inhibitor

The observed upregulation of Hsp90 protein levels induced by T cell activation and the known anti-apoptotic function of Hsp90 suggested, that activated T lymphocytes should be more dependent on proper chaperone function than resting cells. Consequently, Hsp90 blockade should affect the viability of activated cells more than of resting cells. Previous experiments in our lab showed, that isolated $CD4^+$ and $CD8^+$ T lymphocytes activated with PHA showed higher apoptosis rates in the presence of 17-DMAG than resting cell populations. To verify that T cells that are activated in the presence of Hsp90 inhibitor undergo cell death and are depleted, PBMC were labeled with CFSE and activated either with the mitogen PHA, CD3/CD28 microbeads to mimic TCR stimulation or physiologically with allogeneic DC. Whereas cells readily proliferated in response to all three stimuli in untreated cultures, cultures treated with inhibitor are devoid of proliferating $CFSE^{dim}$ cell populations (Figure 10A,B). Annexin V staining showed that T cell death is induced by apoptosis, as was previously seen in mitogen activated cells (Figure 10C). To determine the minimal duration of inhibitor treatment, MLRs were analyzed at different time points after addition of 17-DMAG. As shown in Figure 10D, treatment for 24 hours is enough to deplete alloreactive cells from lymphocyte populations.

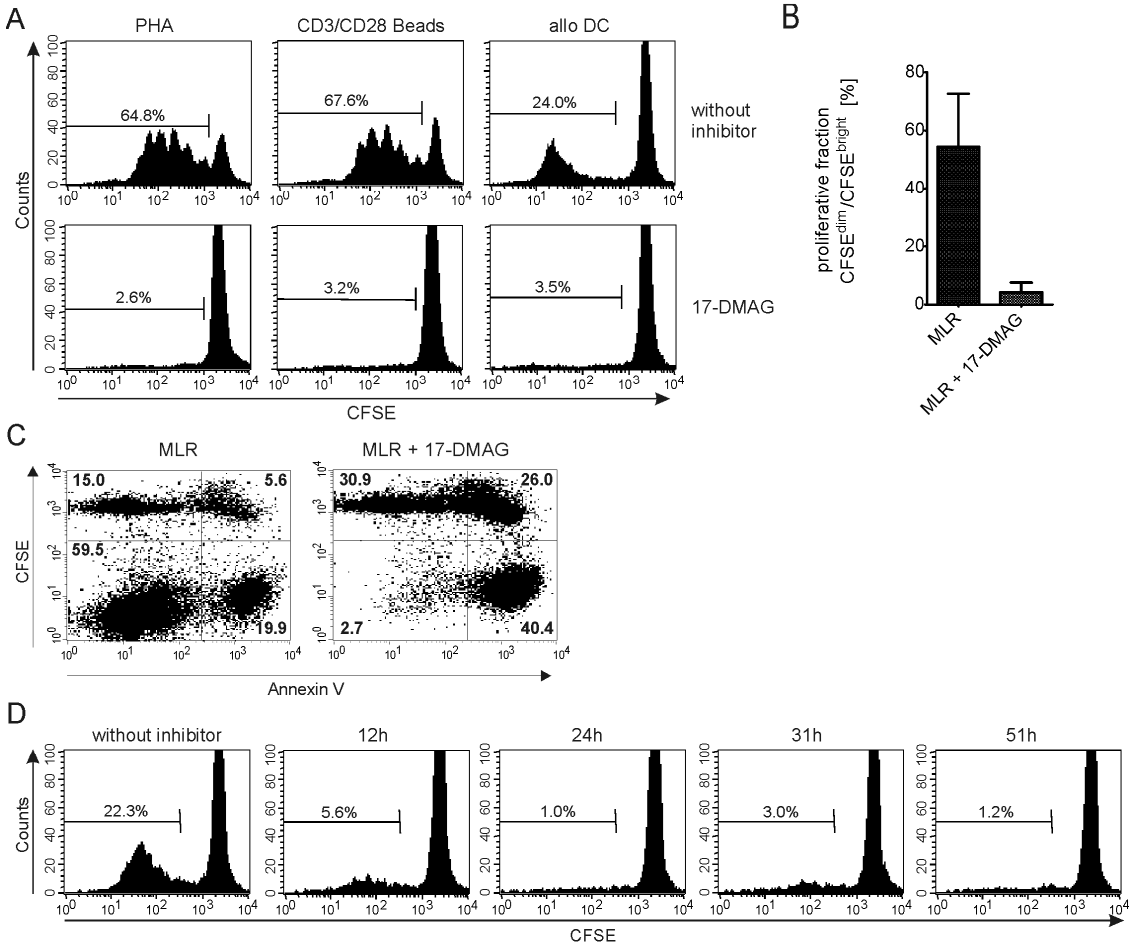


Figure 10:

PBMC were labeled with CFSE and stimulated with PHA, CD3/CD28 microbeads or allogeneic DC without inhibitor or in the presence of 17-DMAG and analyzed flowcytometrically 4 days after culture initiation (A). Reduction of the proliferative fraction in MLRs treated with 17-DMAG (n=9) (B). CFSE-labeled PBMC were stimulated with allogeneic DC, treated with 17-DMAG 4 days after culture initiation and stained with the apoptosis marker Annexin V 40 hours after inhibitor treatment (C). MLRs were treated with Hsp90 inhibitor 4 days after culture initiation and analyzed flowcytometrically 12, 24, 31 and 51 hours after inhibitor treatment (D).

Alloreactive T cells could potentially belong to different T cell compartments such as the CD45RA-positive naïve $CD4^+$ or $CD8^+$ T cell population as well as the CD45RO-positive $CD4^+$ or $CD8^+$ memory/effector population (Figure 11A). To determine if all T cell compartments can initiate an alloreaction and are equally susceptible to 17-DMAG treatment, CD45R0-positive and -negative $CD4^+$ and $CD8^+$ T cells were separately tested in MLRs (Figure 11B). As shown in Figure 11C and D all T cell compartments proliferated in response to stimulation with allogeneic DC and the alloreactive T cells in either the bulk T cell population as well as in the different T cell compartments were equally affected by 17-DMAG treatment with no significant difference between the different T cell fractions.

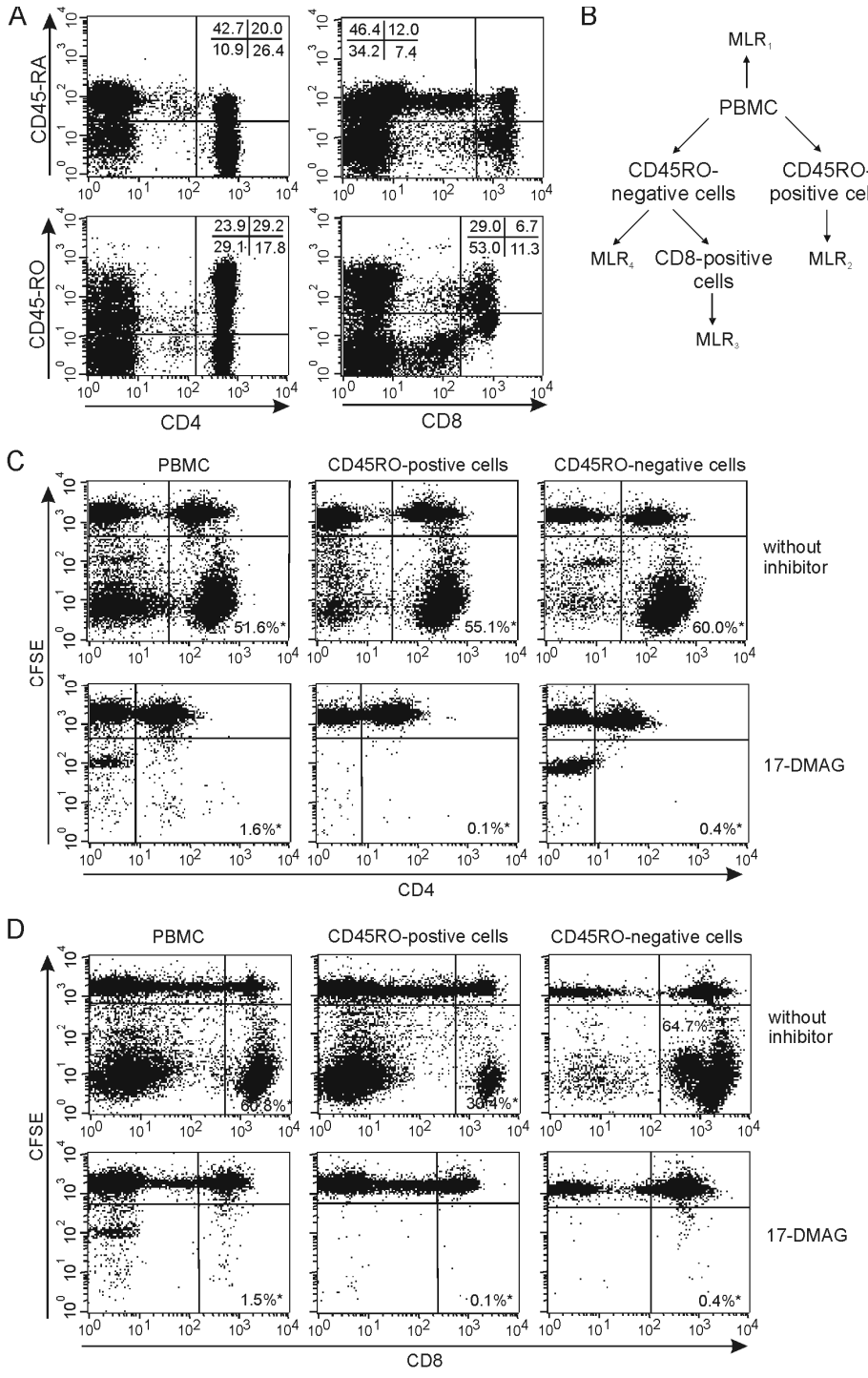


Figure 11:

PBMC were stained with antibody against CD45RA and CD45RO to determine the frequency of naïve and memory T cells in healthy donors (A). CD45RO-positive and CD45RO-negative cells were separated, labeled with CFSE, individually stimulated with allogeneic DC and analyzed flowcytometrically 4 days after culture initiation (B and C) (* indicates percentages of $CFSE^{dim}$ $CD4^+$ cells in panel C and $CD8^+$ cells in panel D).

5.1.3 Transfection of primary T lymphocytes with Hsp90-specific siRNA

Hsp90 stabilizes many key signaling intermediates and exerts anti-apoptotic effects in various cell types, including dendritic cells (Beere 2001; Cohen-Saidon et al. 2006; Kuo et al. 2007). Furthermore, inhibition of Hsp90 has recently been reported to possess the capacity to markedly affect dendritic cell maturation, antigen uptake and function (Bae et al. 2007). In mixed lymphocyte cultures treated with Hsp90 inhibitor no DC population could be detected flowcytometrically 4 days after culture initiation in contrast to untreated cultures (data not shown). It could therefore be possible, that the observed MLR inhibition is due to the dysfunction of DC entailed by Hsp90 blockage and not the selective depletion of alloreactive T lymphocytes. To address this question we selectively targeted the Hsp90 function in the T lymphocyte population with Hsp90-specific siRNA. Since the Hsp90β isoform is distinctly more upregulated than the α isoform after T lymphocyte activation (Figure 9B) and only genetic knock down of the β isoform in myeloma cells showed an significant effect (Chatterjee et al. 2007), Hsp90β-specific siRNA was used to block Hsp90 function.

Since transfection of primary human T lymphocytes is difficult, the transfection protocol was first optimized by electroporation of T cells with mRNA encoding for GFP and cell viability and transduction efficiency determined flowcytometrically. The highest transfection efficiency was obtained by electroporation with 380V and 450µF and was about 61% for $CD4^+$ cells and 65% for $CD8^+$ cells (Figure 12). These settings were therefore used for electroporation of T lymphocytes with Hsp90-specific siRNA.

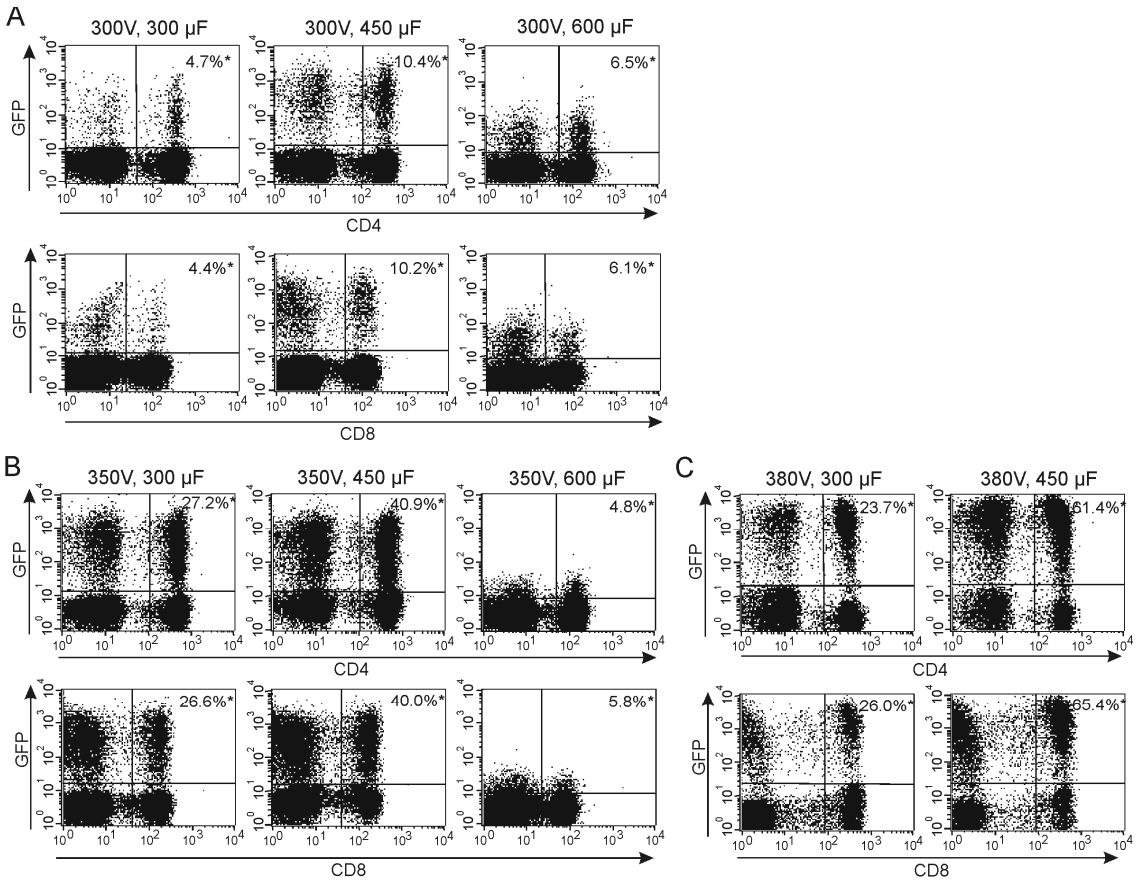


Figure 12:

PBMC were electroporated with GFP-specific mRNA using 300V (A), 350V (B) or 380V (C) and GFP fluorescence measured 2 days after transfection (* indicates percentages of $CD4^+$ or $CD8^+$ T cells).

To determine the efficiency of Hsp90 knock-down with siRNA, real time PCR was performed. T cells were transfected with control siRNA or Hsp90ß-specific siRNA and were either left untreated or activated with PHA for 24 hours. Although a distinct downregulation could be observed (Figure 13A), the efficiency varied between experiments and the knock-down was never complete. But despite the only moderate downregulation of Hsp90ß expression which is probably associated with relatively high residual activity of the Hsp90 chaperone, a significant reduction of proliferating alloreactive T cells in MLR cultures was observed in comparison to T cells transfected with negative control siRNA (Figure 13B and C). It therefore seems probable that the absence of T cell proliferation in mixed lymphocyte cultures treated with 17-DMAG is not due to inadequate stimulation of alloreactive T cells by Hsp90 inhibitor-affected DC, but rather is caused by specific interruption of Hsp90 function in the stimulated alloreactive T cell population.

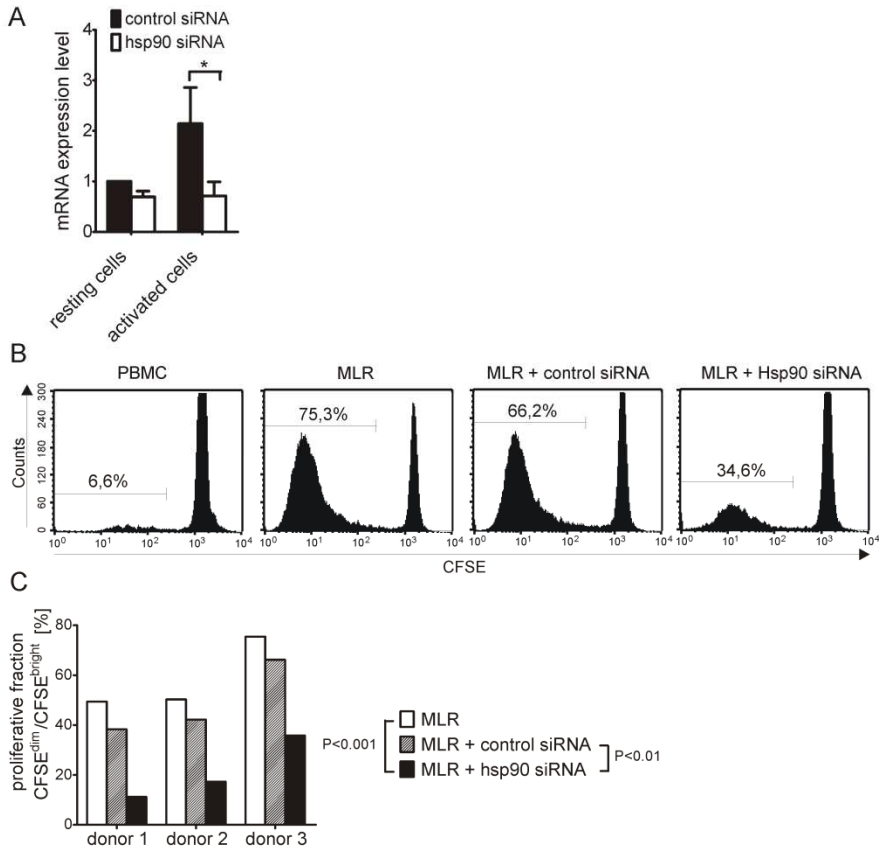


Figure 13:

Relative mRNA expression levels of PBMC transfected with control siRNA or Hsp90β-specific siRNA in resting cells and cells treated with PHA for 24h. Expression levels were determined by real time PCR (A). CFSE-dilution in mixed lymphocyte cultures of untransfected PBMC or PBMC transfected with control siRNA or Hsp90β-specific siRNA prior to culture initiation (B). Reduction of the proliferative fraction of MLRs of PBMC transfected with control siRNA or Hsp90β-specific siRNA (C).

5.1.4 Determination of T lymphocyte viability and function after treatment with Hsp90 inhibitor

Current treatment of graft-versus-host disease frequently results in a sharp increase in morbidity and mortality due to infectious complications. The applied immunosuppressive procedures such as high dose steroids generally lead to a critical

impairment of innate as well as adaptive cellular immunity. Thus novel GvHD prevention and treatment strategies should entail the capacity to spare pathogen-specific immunity and selectively eradicate alloreactive T cells. To analyze if PBMC are still able to respond to activation stimuli after inhibitor treatment, PBMC were either left untreated or treated with 17-DMAG on day 2 of the culture. From some of the cultures the inhibitor was removed by washing on day 5 and all cultures subject to stimulation with PMA/ionomycin on day 7. Whereas intracellular IFN-γ staining showed no T cell activation in the presence of the inhibitor, T cells from which the inhibitor had been removed prior to stimulation could be activated by the mitogen (Figure 14A), showing that T lymphocytes that remained in a resting state during inhibitor treatment survive and are not permanently incapacitated in their ability to react to activation stimuli. As cytomegalovirus (CMV) has been identified as one of the most prevalent pathogens causing severe infections after allogeneic transplantation, we first identified donors with CMV-specific $CD8^+$ memory T cells by staining PBMC with a MHC class I pentamer specific for the CMV protein pp65. PBMC were then stimulated for 6 days with allogeneic DC in the presence or absence of 17-DMAG. To address if viral immunity was preserved, CMV-specific $CD8^+$ T cells were again visualized with the same MHC class I pentamer after stimulation. As shown in Figures 14B and C, CMV_{pp65}-specific $CD8^+$ T cells were readily detected at similar frequencies in both the untreated as well as 17-DMAG-treated cultures. To assess the ability of CMV-specific T cells to respond to antigen after treatment with Hsp90 inhibitor, pretreated as well as untreated cultures were subject to rechallenge with autologous monocytes pulsed with a CMV_{pp65} peptide pool. To demonstrate successful depletion of alloreactive T cells, cultures were also rechallenged with the same allogeneic DC that were used to induce the alloresponse. Supernatant was harvested and the amount of secreted IFN-γ quantified by ELISA. As expected, the 17-DMAG treated cultures displayed no relevant IFN-γ production upon re-exposure to allo-DC, whereas IFN-γ was easily detected in untreated cultures (Figure 14D). However, untreated as well as inhibitor-pretreated cultures responded equally strong to rechallenge with the CMV_{pp65}-specific peptide pool, demonstrating that the resting lymphocytes surviving inhibitor treatment, including memory T cells specific for CMV_{pp65} are not compromised in their viability and function by previous treatment with Hsp90 inhibitor.

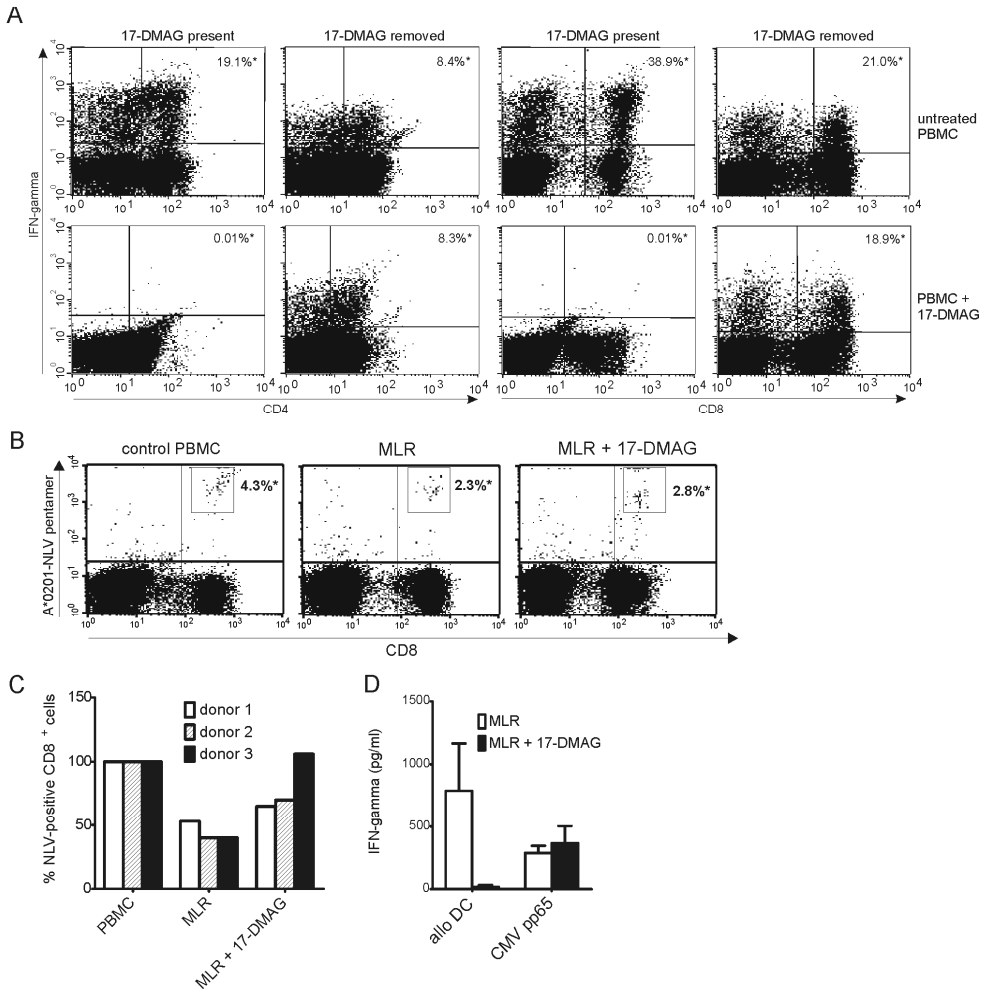


Figure 14:

PBMC were either left untreated or treated with 10µM 17-DMAG at d2. From some of the cultures the inhibitor was removed on d5 by washing the cells. All cultures were stimulated with PMA/ionomycin 7d after culture initiation and cell activation determined by intracellular IFN-γ staining (A). Quantification of CMV-specific $CD8^+$ T cells in PBMC and untreated or Hsp90 inhibitor-treated mixed lymphocyte cultures of a CMV seropositive donor using a MHC class I pentamer specific for a CMV_{pp65} epitope (A*0201-NLV) (* numbers indicate percentages of pentamer-specific $CD8^+$ cells) (B). Percentage of CMV NLV-pentamer-specific $CD8^+$ cells of PBMC and untreated or 17-DMAG-treated MLRs of 3 different donors (percentage of NLV-specific $CD8^+$ cells in untreated PBMC = 100%) (C). Quantification of supernatant IFN-γ levels after restimulation of untreated or DMAG-treated MLRs of CMV seropositive donors with the same allogeneic DC triggering the alloresponse, or autologous monocytes pulsed with a CMV_{pp65} peptide pool (D).

5.2 Characterization of *A. fumigatus* antigens

5.2.1 Expression and purification of recombinant *A. fumigatus* proteins

The *A. fumigatus* proteins major allergen and cytotoxin AspF1 (AFUA_5G02330), CipC-like antibiotic response protein CipC (AFUA_5G09330) and extracellular cell wall glucanase Crf1 (AFUA_1G16190) were cloned into the eukaryotic expression vector cDNA3.1 either with C-terminal V5/His-Tag for purification via Ni-NTA agarose or N- and C-terminal FLAG-Tags for purification via M2 agarose (Figure 15A).

Because expression of recombinant proteins in mammalian cells is in general extremely low in comparison to bacterial or yeast expression systems, the expression level of the Crf1 protein in different human (HeLa, HEK 293T, phoenix GALV) and one simian (COS-1) cell line was compared. The cell lines were transfected by electroporation, calcium phosphate transfection or two different lipofection protocols. The highest expression levels were obtained in COS-1 cells after lipofection and in phoenix GALV cells after calcium phosphate transfection (data not shown). Because calcium phosphate transfection is more economic than lipofection, phoenix GALV cells were chosen as producer cell line for the recombinant proteins.

Whereas the recombinant proteins Crf1 and CipC showed good expression, expression of AspF1 was associated with relatively high cytotoxicity for the producer cells greatly limiting the amount of recombinant protein that could be obtained (Figure 15B). This cytotoxic effect is probably due to the fact, that AspF1 is a potent ribotoxin and therefore cannot be produced efficiently cytoplasmically. Because cloning of AspF1 with N-terminal signal peptide to allow secretion of the recombinant protein did not yield satisfying results (data not shown), cytotoxicity was diminished by mutation. Yang and Kenealy and Kao et al. have previously described, that a single point mutation in the catalytic center of AspF1 and similar ribotoxins nearly abolishes catalytic activity and concomitant toxicity (Yang and Kenealy 1992; Kao et al. 1998). As expected, the substitution of the histidine residue at position 136 of AspF1 for leucine (AspF1-H136L) enables relatively high cytoplasmic expression of the recombinant protein in eukaryotic producer cells in comparison to the unmutated protein (Figure 15C).

To determine the kinetics of protein expression and the optimal harvest time point, phoenix GALV cells transfected with Crf1-FLAG were harvested 2, 4, 6, 8 and 10 days after transfection and the protein level determined by western blot, showing that expression of the recombinant protein is continuously decreasing with time, even when

the transfected cells are selected by treatment with antibiotics (Figure 15D). To improve protein expression, transgene expression from the CMV promoter of the vector was enhanced by treatment with sodium butyrate, as previously described (Palermo et al. 1991). As shown in Figure 15E, addition of 1-2 mM sodium butyrate to the culture medium significantly enhanced protein expression. For large-scale production of the recombinant proteins the producer cells were therefore treated with 2 mM sodium butyrate and harvested 2 days after transfection.

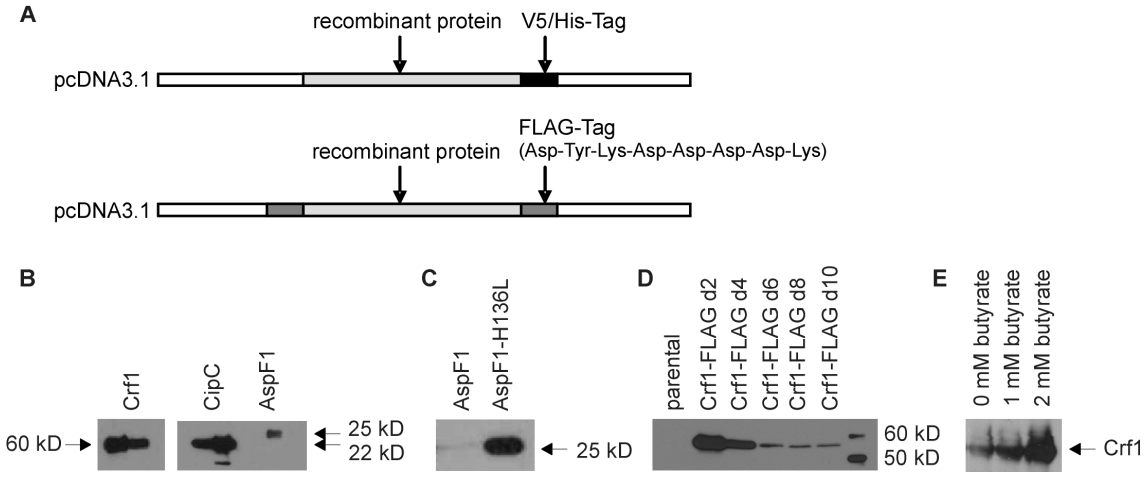


Figure 15:

Cloning of recombinant proteins with C-terminal V5/His-Tag or N- and C-terminal FLAG-Tags (A). Expression of the recombinant V5-His-tagged *A. fumigatus* proteins Crf1, CipC and AspF1 in phoenix GALV cells. Transgene expression was detected with anti-V5 antibody (B). Expression of unmutated AspF1 or AspF1 with introduced point mutation H136L in phoenix GALV cells (C). Phoenix GALV cells were transfected with pcDNA3.1-Crf1-FLAG by Ca_2PO_4 transfection and protein expression determined 2, 4, 6, 8 or 10 days after transfection with anti-FLAG antibody (D). Phoenix GALV cells transfected with pcDNA3.1-Crf1-FLAG were treated with 1 or 2 mM sodium butyrate for 2 days to enhance transgene expression from the CMV promoter (E).

Purification of the V5/His-tagged recombinant proteins was performed with Ni-NTA agarose and of the FLAG-tagged proteins with M2-agarose. Whereas enrichment of the tagged proteins from cell lysate was possible with both purification strategies (Figure 16A and C), purification with Ni-NTA agarose had the major disadvantage of massive contamination of the eluates with unspecific proteins, compared to protein purification using M2 agarose (Figure 16B and D). Therefore FLAG-tagged and M2-purified recombinant proteins were used for further experiments.

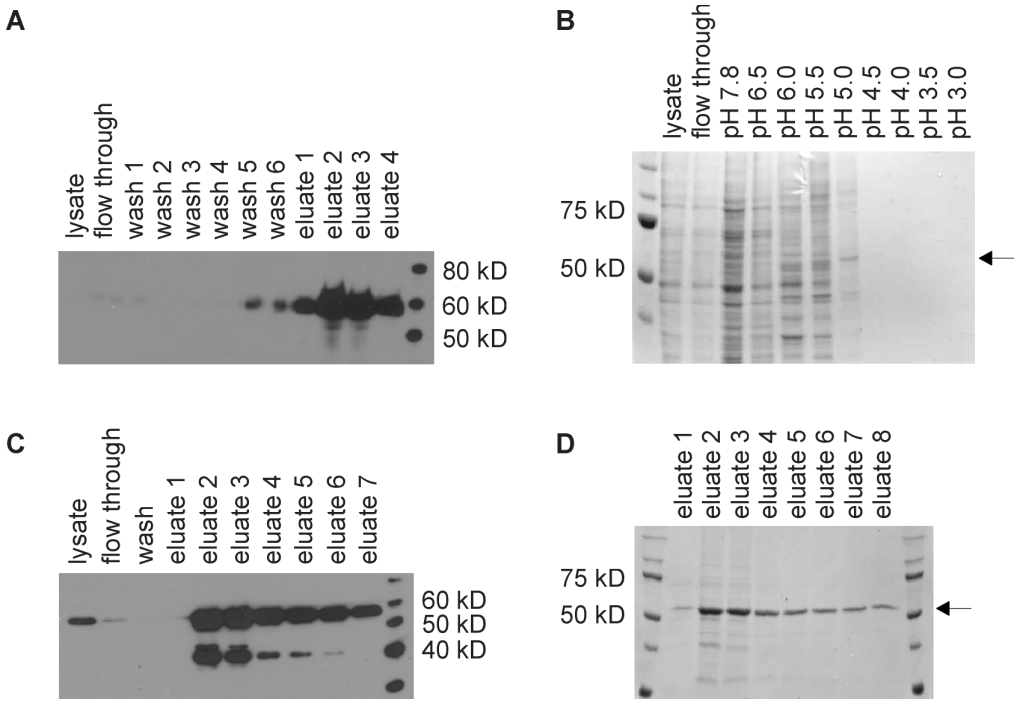


Figure 16:

Phoenix GALV cells were transfected either with pcDNA3.1-Crf1/V5-His (A, B) or pcDNA3.1-Crf1-FLAG (C, D) and the cells harvested and lysed 2 days after transfection. The His-tagged recombinant protein was purified with Ni-NTA agarose, the FLAG-tagged recombinant protein with M2 agarose and the amount of protein in the cell lysate, column flow through and successive wash fractions and eluates determined by western blot (A, C) or coomassie staining (B, D).

5.2.2 Stimulation of T cells with *Aspergillus* antigens

5.2.2.1 Stimulation with fungal cell extract

It has been shown previously by different research groups that PBMC from healthy donors respond to stimulation with *A. fumigatus* cell extract with proliferation and IFN-γ secretion (Grazziutti et al. 1997; Hebart et al. 2002; Beck et al. 2006), indicating that the permanent contact with fungus in the environment induces – in most cases asymptomatic – immune responses with concomitant differentiation of long-lived *Aspergillus*-specific memory T cells. The same result was also obtained in our lab. When PBMC of different healthy donors were stimulated with fungal cell extract for 1 week, all cultures responded with IFN-γ secretion after restimulation (Figure 17).

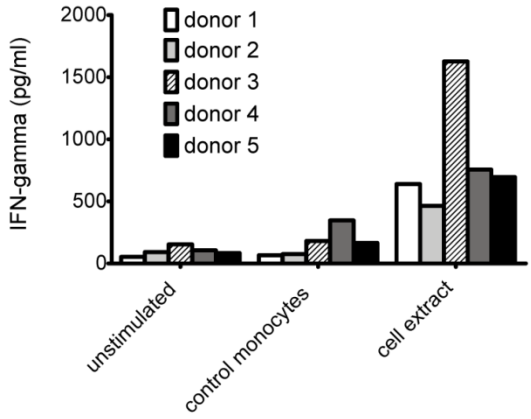


Figure 17:

PBMC of 5 different healthy donors were pre-stimulated with *A. fumigatus* cell extract for 1 week and IFN-γ secretion determined after restimulation with antigen for 24h.

5.2.2.2 Stimulation with recombinant proteins

To more specifically define immunogenic antigens that are able to induce protective immune responses, different recombinant *A. fumigatus* proteins were analyzed for their ability to stimulate T cell responses. The recombinant *A. fumigatus* proteins superoxide dismutase SOD (AFUA_5G09240), catalase Cat (AFUA_2G18030), aspartic endopeptidase PEP (AFUA_5G13300) and 1,3-beta-glucanosyl-transferase Gel1 (AFUA_2G01170) produced in the yeast *Pichia pastoris* were kindly provided by Prof. Jean-Paul Latgé (Institute Pasteur, Paris, France). PBMC of at least 7 different donors were pre-stimulated with each of the 4 proteins for 1 week and IFN-γ secretion determined after restimulation. Whereas recombinant peptidase was able to induce antigen-specific IFN-γ production in some of the donors (Figure 18A), distinct responses of PBMC to superoxide dismutase, catalse and the glucanosyl transferase Gel1 could not be discriminated, because of massive background in the control experiments (Figure 18B-D).

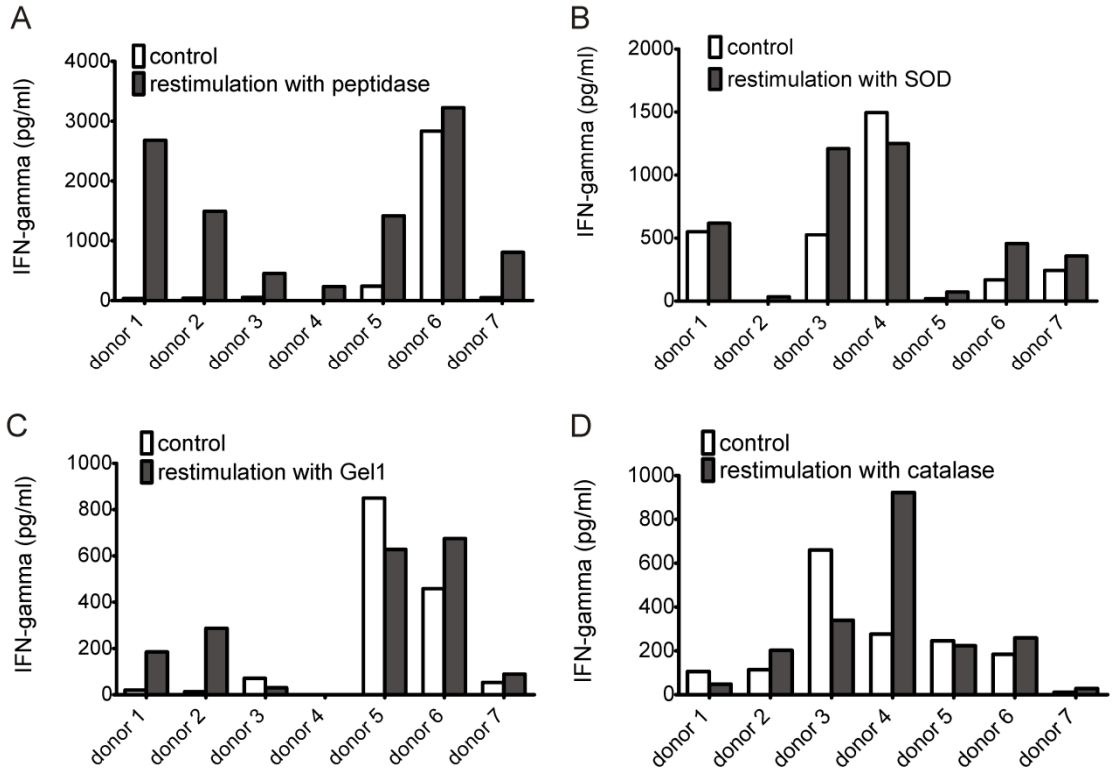


Figure 18:

IFN-γ response of different healthy donors to recombinant peptidase (A), superoxide dismutase SOD (B), Gel1 (C) and catalase (D) produced in *P. pastoris*.

To minimize the risk of unspecific T cell stimulation by contaminations in the protein preparations produced in the yeast expression system, further recombinant *Aspergillus* proteins were produced in a mammalian expression system, the human epithelial kidney cell line phoenix GALV as described in section 5.2.1.

The major allergen and cytotoxin AspF1, CipC-like antibiotic response protein CipC and extracellular cell wall glucanase Crf1 produced in the human cell line were similarly used to stimulate PBMC of healthy donors (Figure 19). T cell lines stimulated with recombinant AspF1 and CipC in most cases showed only a slight increase of cytokine production upon restimulation with the specific antigen in comparison to background levels (Figure 19A,B). The T cell response to Crf1 in contrast seemed to be more specific (Figure 19C) and therefore was analyzed in more detail.

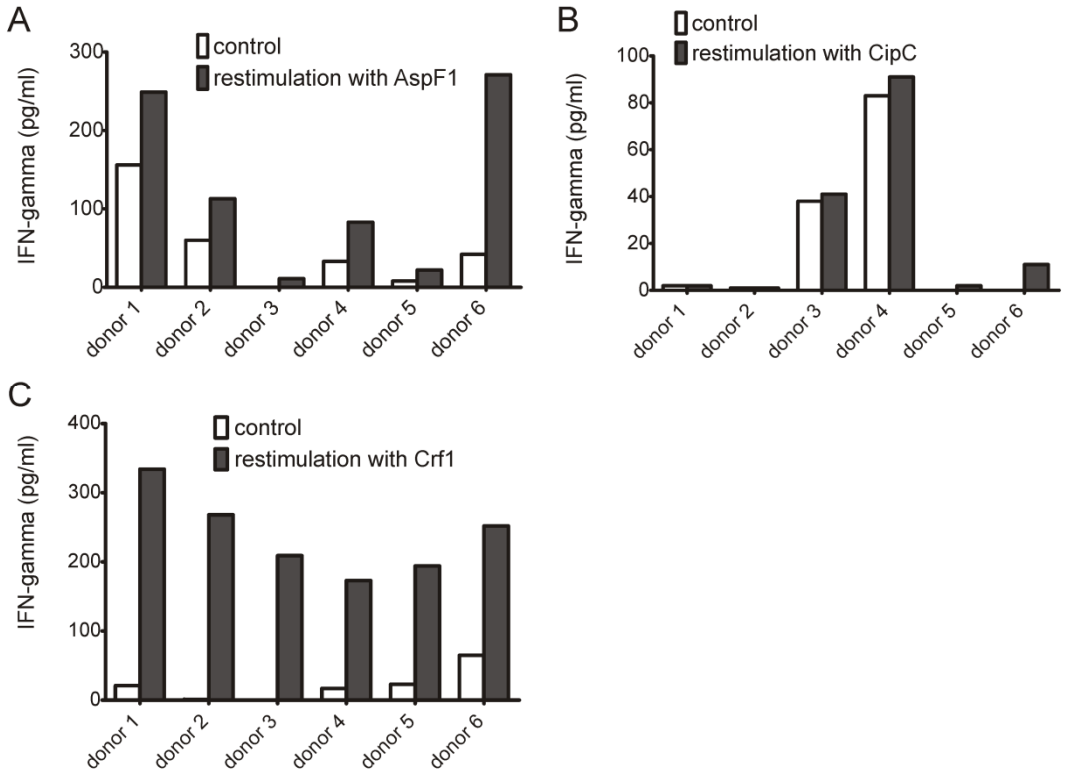


Figure 19:

IFN-γ response of different healthy donors to the recombinant proteins AspF1-H136L (A), CipC (B) and Crf1 (C) produced in the human cell line phoenix GALV.

5.2.3 Detailed characterization of the immune response to Crf1

5.2.3.1 Detection of antigen-specific responses in fresh PBMC and pre-stimulated cell lines

In the initial experiments, PBMC were pre-stimulated with antigen for 1 week before the IFN-γ response was determined after restimulation with the antigen. The pre-stimulation was done to expand the antigen-specific memory cells in the whole T cell repertoire to amplify the response and facilitate the detection of antigen-specific cells. When PBMC of the same donors that responded to Crf1 after 1 week of pre-stimulation (Figure 19C) were stimulated directly after preparation, no cytokine production could be observed (Figure 20A), indicating that the frequencies of antigen-specific T cells are extremely low in healthy donors. To exclude unspecific activation of T cells after 1

week of *in vitro* culture, the cells were also stimulated with the viral antigen CMV_{pp65} and the mitogen phytohemagglutinin PHA (Figure 20B). Whereas a significant IFN-γ response to Crf1 was only observed in the pre-stimulated cells and not fresh PBMC, neither the fresh PBMC nor the pre-stimulated cell line responded significantly to CMV_{pp65}, confirming the specificity of the Crf1 response. Fresh as well as pre-stimulated PBMC responded to the unspecific stimulus PHA to a similar degree.

To ascertain the reproducibility and determine the variability of the T cell response, one donor was challenged with the Crf1 antigen in 6 independent experiments. Although the absolute amount of IFN-γ varied noticeably between experiments, a positive response could always be detected (Figure 20C).

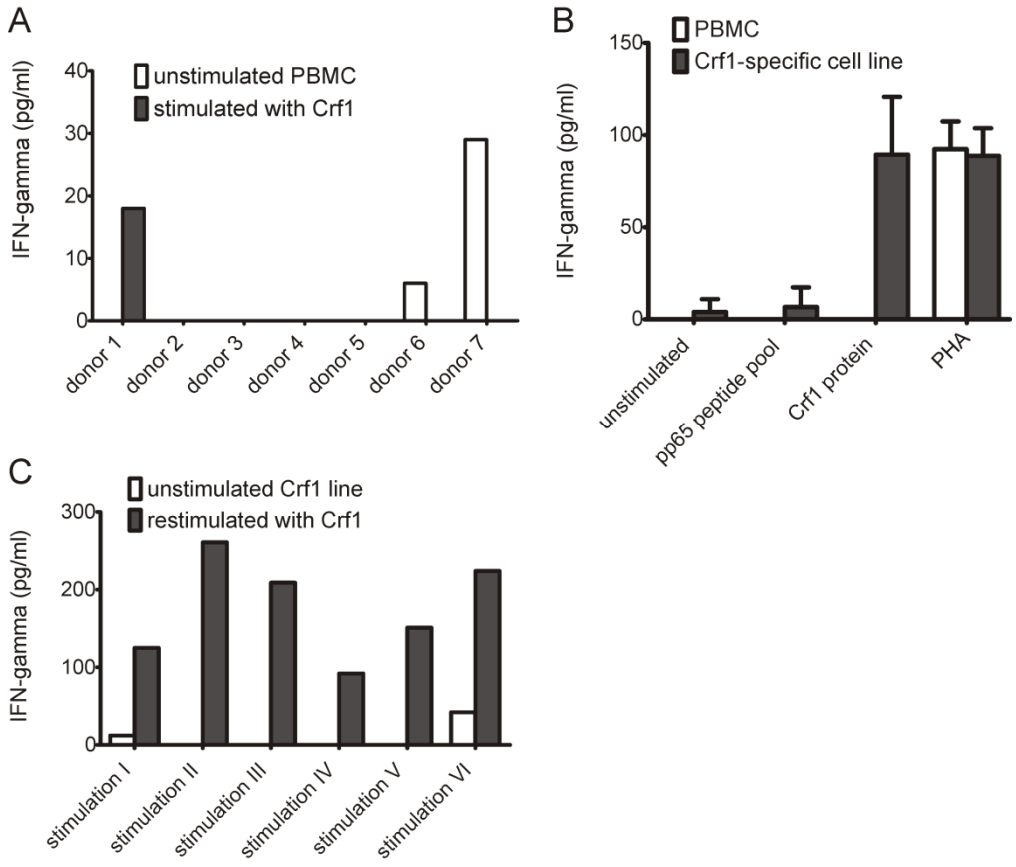


Figure 20:

Stimulation of fresh PBMC of 7 different healthy donors with recombinant Crf1 protein for 24h (A). Stimulation of either fresh PBMC or a Crf1-specific cell line pre-stimulated with protein for 7 days with control antigen (CMV_{pp65} peptide pool), recombinant Crf1 protein or PHA for 24h (representative experiment, n=3) (B). Restimulation of different Crf1-specific cell lines of the same donor to determine the variability of the antigen-specific memory response (C).

5.2.3.2 Determination of the precursor frequencies of Crf1-specific memory T cells by ELISPOT

To estimate the precursor frequencies of Crf1-specific memory T cells in the peripheral blood of healthy individuals, IFN-γ ELISPOT was performed. Although it was possible to detect Crf1-specific cells in cell lines pre-stimulated with antigen for 1 week, no reproducible response could be detected in fresh PBMC (data not shown). When up to $1x10^6$ purified $CD4^+$ T cells were analyzed per well, the response was still near background level, again suggesting extremely low precursor frequencies similar to the results obtained in IFN-γ ELISA (see section 5.2.3.1).

5.2.3.3 Verification of the specificity of the immune response to Crf1 with retrovirally transduced antigen-presenting B cells

It could not be completely ruled out that the observed response of PBMC to Crf1 is due to contamination of the recombinant protein preparation with proteins or other impurities originating from the producer cells or the purification procedure. Therefore, as independent strategy to verify that the observed T cell response is indeed Crf1-specific, autologous B cells expressing and presenting the antigen were used.

B cells that have been activated by stimulation with CD40 ligand (CD40L) expressed on feeder cells and retrovirally transduced with the antigen of interest maintain high levels of costimulatory molecules and can be used as antigen-presenting cells in an autologous system (Schultze et al. 1997; Kondo et al. 2002). The retroviral vector 95.1 (Figure 21A) also encodes for a truncated version of the nerve growth factor receptor (ΔNGFR) for detection and purification of stably transduced cells and is especially suited for transduction of human B and T cells, because the PGK promoter driving transgene expression allows comparably high expression in this primary cells.

After transduction of B cells, the endogenously synthesized antigen is predominantly presented via MHC class I and thereby potently activates $CD8^+$ T cells (Adamopoulou et al. 2007). However, $CD4^+$ cells are activated to some extent by cross presentation of the antigen. To enhance antigen presentation via MHC class II and distinctly improve the activation of $CD4^+$ T cells, the sequence coding for the membrane spanning N-terminal domain of the invariant chain (Ii) was fused to the cloned antigen (Sanderson et al. 1995; Malcherek et al. 1998). This protein domain is responsible for proper synthesis and translocation of MHC class II molecules to the MHC class II loading

compartment (Figure 21B). Every protein that is fused to this domain is translocated to the MHC class II compartment and predominantly induces $CD4^+$ T cell responses.
After retroviral transduction of CD40L-activated B cells, stably transduced cells were selected based on ΔNGFR expression (Figure 21C) and used to restimulate Crf1-specific T cell lines that had been generated by stimulation with recombinant protein for 1 week. As can be seen in Figure 21D, B cells transduced with the li-Crf1 fusion induced strong IFN-γ secretion, excluding that T cells are unspecifically activated by contaminations in the recombinant protein preparation, but are indeed Crf1-specific. Moreover, the fact that B cells transduced with Crf1 without MHC class II targeting domain show no significant cytokine production indicates that the responding cells are predominantly $CD4^+$ T helper cells and not $CD8^+$ cytotoxic T cells. Cell lines stimulated with Crf1 protein also do not respond to stimulation with B cells transduced with a li-CMV_{pp65} fusion, further excluding unspecific effects (Figure 21E).

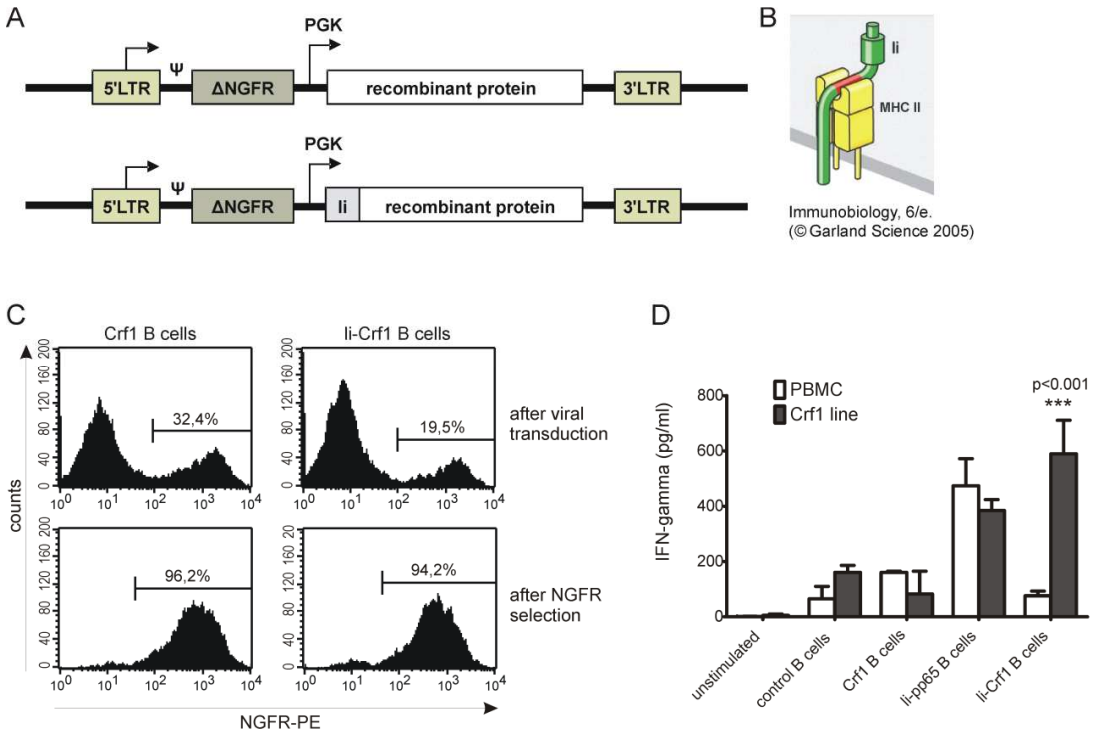


Figure 21:

Retroviral vector 95.1. Transgene expression is driven by the PGK promoter. The truncated nerve growth factor receptor (ΔNGFR) enables detection and purification of stably transduced cells. For enhanced antigen presentation via the MHC class II pathway, the cloned antigen is fused to the N-terminal part of the invariant chain li (A). Model of a MHC class II molecule with associated invariant chain li (B). B cells retrovirally transduced with the gene encoding Crf1 or a li-Crf1 fusion gene before and after selection of NGFR-positive cells (C). T cell lines generated with recombinant Crf1 protein were restimulated with untransduced control B cells or autologous B cells expressing Crf1, a li-pp65 fusion protein or a li-Crf1 fusion protein (D).

5.2.3.4 Crf1-specific memory cells are $CD4^+$ T helper cells

It has previously been shown, that successful clearance of *Aspergillus* infections is dependent on $CD4^+$ T helper responses, whereas $CD8^+$ T cell responses seem to be only of minor importance (Romani 2004). The first evidence that the Crf1-specific T cells of healthy individuals are indeed $CD4^+$ T cells was obtained with the B cell experiments described in section 5.2.3.3. Crf1-specific T cell lines did only respond to restimulation with B cells transduced with li-Crf1 which is channeled into the MHC class II presentation pathway, but did not respond to B cells transduced with Crf1 predominantly presenting the antigen on MHC class I (Figure 21D).

In further experiments the antigen-presenting monocytes were pre-treated with control antibody or MHC class II blocking antibody prior to antigen-pulsing and co-culture with Crf1-specific or CMV_{pp65}-specific T cell lines. Whereas the MHC class II blocking antibody was able to strongly diminish IFN-γ production of Crf1-specific T cell lines, the cytokine response to CMV_{pp65} was not affected, as would be expected for a MHC class I-restricted $CD8^+$-mediated memory response to a viral antigen like CMV_{pp65} (Figure 22 A,B). Similarly, T cell activation could be blocked when li-Crf1 B cells pre-treated with MHC class II blocking antibody were used as antigen-presenting cells (Figure 22C).

Exogenously administered antigen such as purified protein or peptide is predominantly processed in the MHC class II pathway and primarily activates $CD4^+$ T cell responses, possibly explaining the observed absence of $CD8^+$ memory responses. Although cross presentation of exogenous antigen on MHC class I molecules has been described for monocytes as well as dendritic cells, this process seems to be relatively inefficient (Harding and Song 1994; Maecker et al. 2001). To augment MHC class I-mediated activation of potentially present antifungal memory $CD8^+$ T cells, purified $CD4^+$ and $CD8^+$ T lymphocytes from peripheral blood were pre-stimulated with CD40L-activated B cells transduced with retrovirus coding for cytoplasmically expressed Crf1 or MHC class II-targeted li-Crf1. Despite this approach a significant $CD8^+$ recall response to Crf1 could not be detected, whereas stimulation with li-Crf1 fusion constructs was able to induce $CD4^+$ recall responses (Figure 22D). The strong response of $CD4^+$ T cells to B cells transduced with cytoplasmically expressed Crf1 is probably due to the fact, that the lymphycytes were incubated with APC for 1 week, which allows efficient activation of the $CD4^+$ memory cells by cross-presentation.

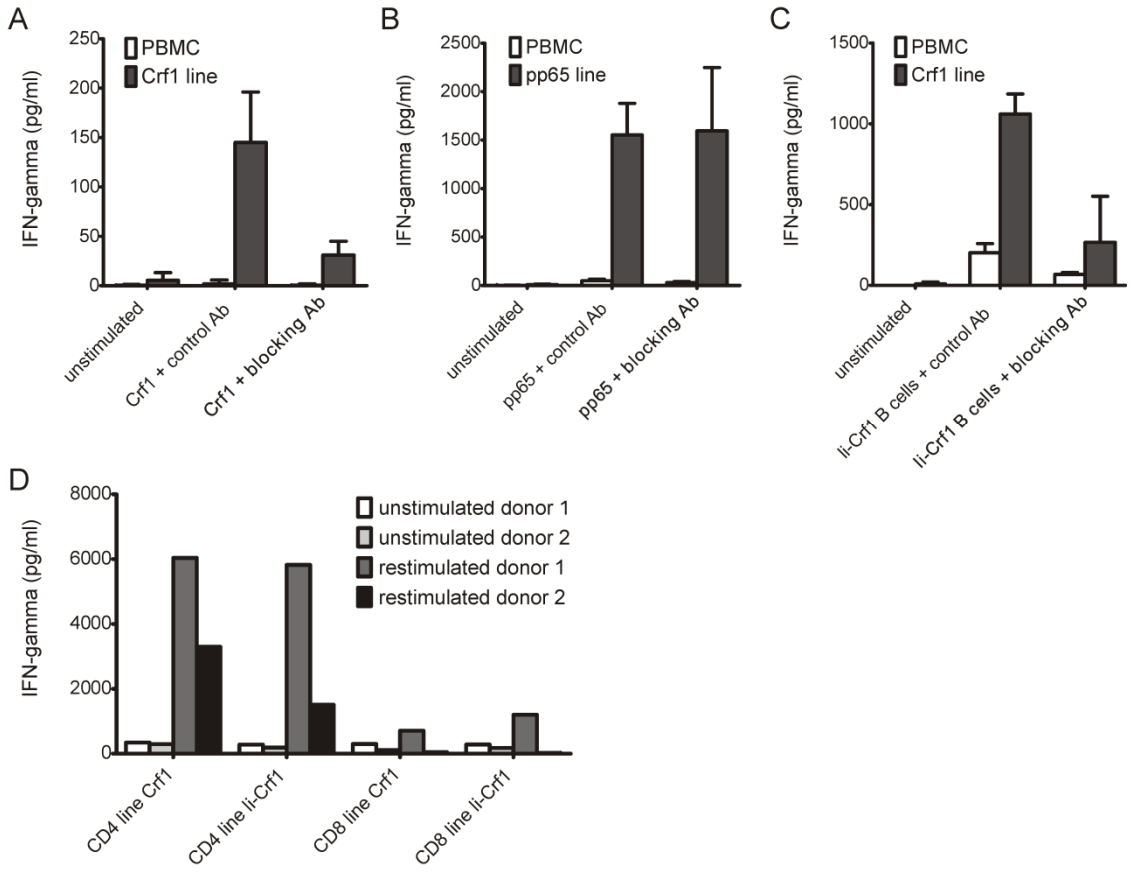


Figure 22:

A Crf1-specific cell line (A) and a CMV_{pp65}-specific cell line (B) were restimulated either with monocytes pre-treated with control antibody or with MHC class II blocking antibody for 1 hour prior to pulsing with Crf1 or CMV_{pp65}, respectively. Restimulation of a Crf1-specific cell line with li-Crf1-transduced B cells pre-treated with control antibody or MHC class II blocking antibody (C). Restimulation of $CD4^+$ and $CD8^+$ T cell lines pre-stimulated with Crf1 or li-Crf1-transfected autologous B cells for 1 week (D).

5.2.3.5 Determination of immunogenic epitopes of the Crf1 antigen

Expression and purification of whole recombinant proteins is much more laborious and expensive than synthesis of small peptides. Furthermore, elicitation of specific T cell responses is often more efficient with peptides than with proteins. For clinical application it would therefore be worthwhile to define immunogenic epitopes of an antigenic *Aspergillus* protein that could potentially be used for instance in immunization or adoptive therapy protocols. The major drawback in the use of peptides in

comparison to whole proteins lies in MHC restriction of peptide presentation. Whereas proteins are processed to many different peptide fragments by the antigen presenting cell and different fragments can be presented by different MHC class II alleles, presentation of one specific peptide is often limited to one or only few MHC alleles. Therefore one single peptide cannot be used for all patients, but only for a group of patients with respective MHC class II alleles.

Because HLA-DRB1*01 and HLA-DRB1*04 are two of the most common MHC class II alleles in the Caucasian population, 5 peptides present in the Crf1 protein sequence that are probably presented by one or both of this two alleles were tested for their immunogenicity. Sequence prediction based on the SYFPEITHI algorithm (Rammensee et al. 1999) and peptide synthesis was kindly done by Prof. Dr. Stefan Stevanović (University of Tuebingen) (Figure 23A).

To test if some of the 5 peptides are immunogenic epitopes of the Crf1 protein, PBMC of different HLA-DRB1*01- or HLA-DRB1*04-positive donors were first stimulated with recombinant protein for 1 week and then restimulated with protein or peptide and T cell activation determined by IFN-γ ELISA. When HLA-DRB1*04-positive donors were tested, it was observed, that all donors showed relatively strong reactivity to the peptide designated as FHT, whereas the other peptides induced rather weak responses only in selected donors (Figure 23B). To verify that the FHT peptide is also able to directly activate and expand Crf1-specifc memory cells, PBMC were stimulated either with the recombinant Crf1 protein or the peptide for 1 week and both cell lines restimulated with protein as well as peptide. In all cases antigen-specific IFN-γ secretion could be observed, confirming the potential use of the FHT peptide to activate and expand *Aspergillus*-specific memory cells in blood samples of healthy donors (Figure 23C). Stimulation with peptide antigen was even superior to stimulation with recombinant protein.

To facilitate detection of T cells specific for the Crf1 FHT peptide, HLA-DRB1*0401 MHC class II tetramers loaded with FHT peptide were used. Similar to the results obtained with IFN-γ ELISPOT (see section 5.2.3.2) a distinct population of FHT-specific T cells could only be detected in pre-stimulated T cell lines, but not in fresh PBMC, further suggesting rather low precursor frequencies in the peripheral blood (data not shown).

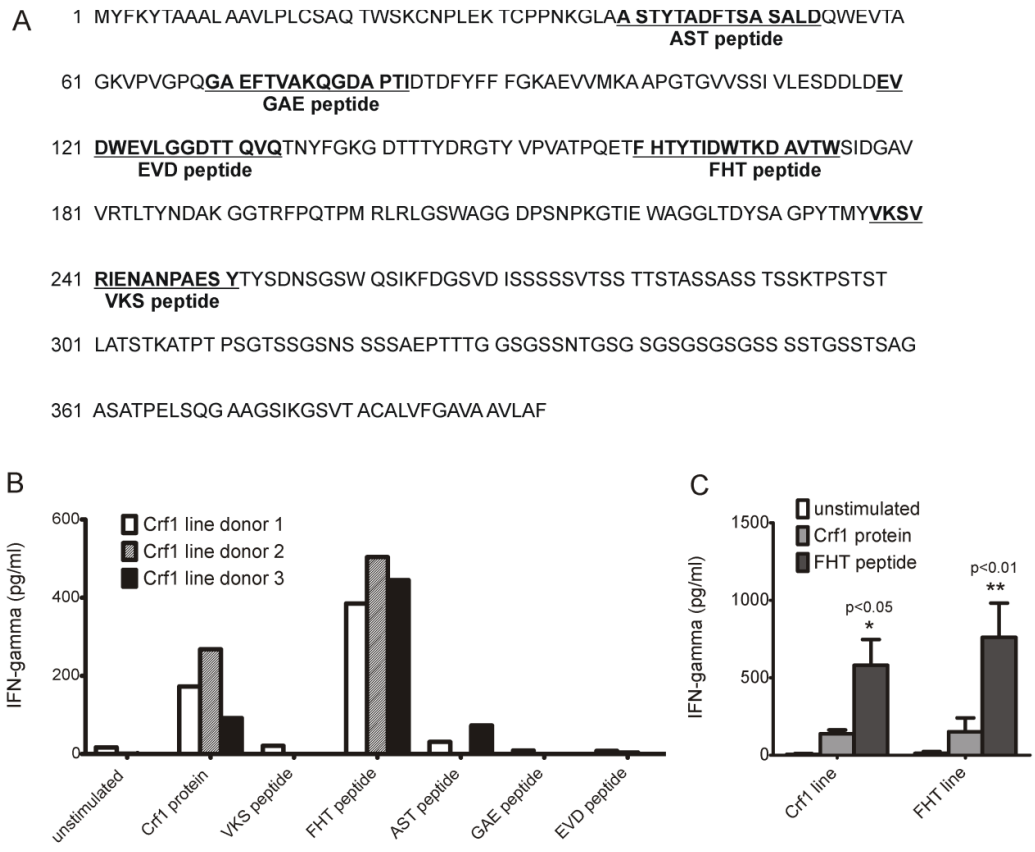


Figure 23:

Amino acid sequence of Crf1 showing 5 potential HLA-DRB1*01 or HLA-DRB1*04-restriced epitopes (A). Crf1-specific cell lines from 4 different donors were stimulated with Crf1 recombinant protein or 5 potential HLA-DRB1*01 or HLA-DRB1*04-restriced peptide antigens (B). Crf1-specific T cell lines were generated either with recombinant Crf1 protein or FHT peptide and restimulated with both recombinant protein and synthetic peptide (C).

5.2.4 Generation of Crf1-specific T cell clones

To obtain a pure population of FHT-specific T cells for further analysis, FHT-specific T cell clones were established. For efficient cloning of rare antigen-specific T cells, the specific cells have first to be enriched in the T cell population for instance by repeated stimulation with antigen for several weeks to continually increase the frequency of antigen-specific cells. Whereas activation of FHT-specific cells in an intracellular IFN-γ staining was below the detection limit in populations of fresh PBMC (data not shown), in cell populations that had been repeatedly stimulated with antigen for 3 weeks antigen-specific cells were considerably enriched (Figure 24A). The cell lines enriched for Crf1-specific cells were then used to isolate single T clones by limiting dilution.

In a further approach it was also possible to isolate Crf1-specific T cell clones by labeling pre-stimulated T cell cultures with an PE-conjugated MHC class II-FHT tetramer and subsequent isolation with anti-PE microbeads (data not shown).

To check if the isolated T cell clones are specific for the FHT peptide, they were stimulated with control antigen or the FHT peptide and IFN-γ secretion determined by ELISA and intracellular cytokine staining (Figure 24B,C). Whereas some of the clones showed no or only weak responses to restimulation with antigen, some were highly reactive to the FHT peptide. The clones that showed best responses were used for further experiments.

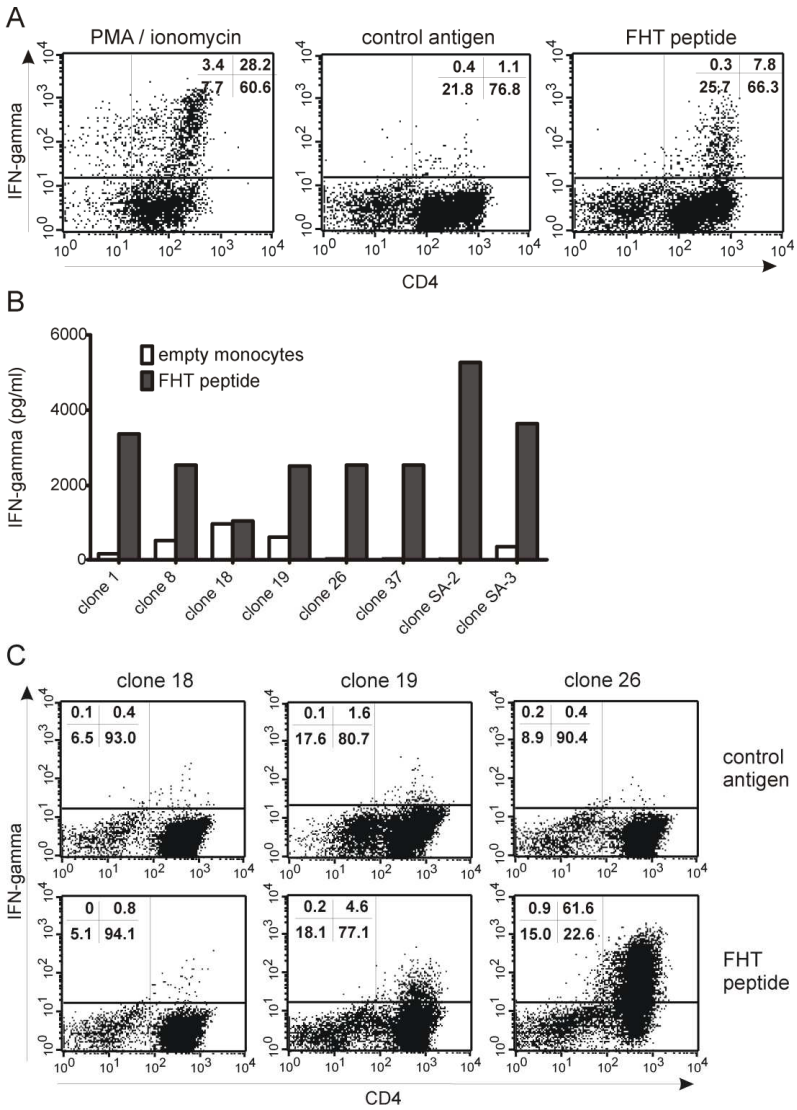


Figure 24:

Intracellular IFN-γ staining of a T cell culture enriched for FHT-specific T cells by repeated stimulation with antigen for 3 weeks (A). IFN-γ ELISA (B) and intracellular cytokine staining (C) of different FHT-specific T cell clones restimulated with monocytes pulsed with the control antigen CMV_{pp65} or monocytes presenting FHT peptide.

5.2.5 Stimulation of Crf1-specific T cell clones with different *Aspergillus* antigens

5.2.5.1 Stimulation of T cell clones with cell extract

To verify that T cell clones specific for the synthetic FHT peptide also respond to stimulation with fungal preparations which should contain the Crf1 antigen, clones were stimulated with different fractions of *A. fumigatus* cell extracts, cell pellets or culture supernatant. To some of the fungal preparations the FHT-specific clones showed good responses (Figure 25), indicating that the Crf1 protein is actually present in the growing fungus and the FHT epitope can be efficiently processed and presented by antigen presenting cells.

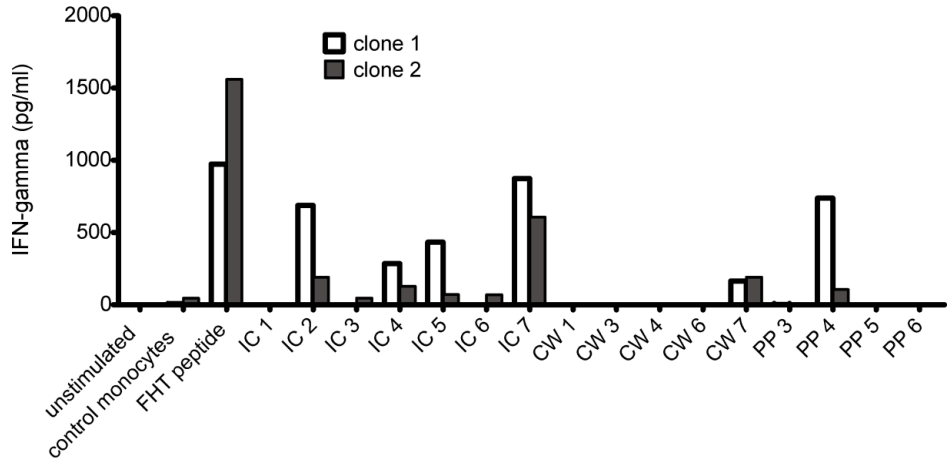


Figure 25:
Stimulation of a FHT-specific T cell clone with FHT peptide, different fractions of *A. fumigatus* cell extracts (IC 1-7), cell pellets (CW 1-7) or culture supernatant (PP 3-6) (representative experiment, n=3).

5.2.5.2 Stimulation of T cell clones with fungus

To investigate whether *A. fumigatus* FHT-specific T cells can also be specifically activated by whole fungus, FHT-specific T cell clones were restimulated with autologous dendritic cells co-incubated either with FHT peptide, ethanol-killed *C.*

albicans, *A. fumigatus*, *A. nidulans*, *A. terreus*, *A. flavus* or *A. niger*. *C. albicans* was included in the experiment, because it also causes opportunistic infections in immunocompromized individuals and encodes for a glucosyl-transferase similar to Crf1 of *A. fumigatus*. As *A. fumigatus* enters its host in the form of airborne conidia and starts invasive infection by initiation of germination and hyphal outgrowth, we investigated whether FHT-specific $CD4^+$ T cells can be activated by resting conidia as well as growing fungus. Therefore, the different fungal strains were killed with ethanol in the form of resting conidia (0h) or after germination (6h) and subsequently fed to autologous DC.

As shown in Figure 26A FHT-specific T cell clones produced high amounts of IFN-γ in response to DC fed with FHT peptide, *A. fumigatus* killed 6h after germination or C. albicans, whereas DC fed with the remaining *Aspergillus* strains as well as DC fed with ethanol-killed resting conidia (0h) failed to induce significant IFN-γ production in FHT-specific T cells. Therefore, FHT-specific $CD4^+$ T cells seem to be specifically activated by germinating *A. fumigatus* conidia and outgrown hyphae, whereas resting conidia have only limited stimulatory capacity.

Fungi and other pathogens posses distinct structures and molecular patterns that are specifically recognized by dendritic cells and other cells of the innate immune system, which respond with cytokine secretion and recruitment and activation of further immune cells (Romani 2004). To exclude the possibility that dendritic cells exposed to and activated by live *Aspergillus* fungus could potentially activate T cells unspecifically, a CMV_{pp65}-specific $CD4^+$ T cell clone was co-cultured with autologous DC fed with live *Aspergillus* conidia. As shown in Figure 26B considerably lower IFN-γ levels could be detected in cultures of T cell clones with unrelated specificity in comparison to FHT-specific $CD4^+$ T cell clones that showed robust production of cytokines. Furthermore, IFN-γ secretion by the $CD4^+$ T cell clone was greatly diminished if the antigen-presenting cells were pre-treated with MHC class II blocking antibody prior to antigen-loading (Figure 26C), showing that T cell activation is antigen-specific and MHC-restricted and not based on bystander effects.

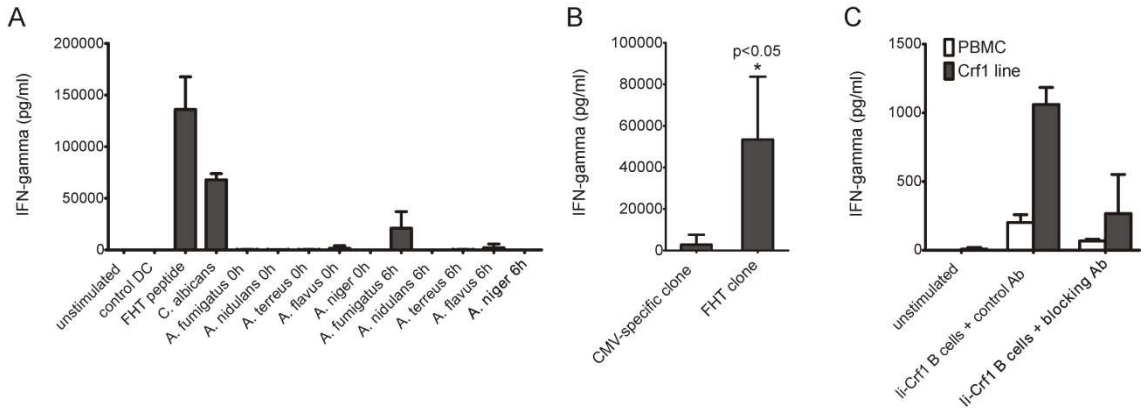


Figure 26:

Stimulation of FHT-specific T cell clones with dendritic cells fed with FHT peptide, *C. albicans*, *A. fumigatus*, *A. nidulans*, *A. terreus*, *A. flavus* or *A. niger* killed by treatment with 70% Ethanol 0h or 6h after germination (A). Stimulation of a FHT-specific T cell clone or a CMV_{pp65}-specific T cell clone with dendritic cells fed with viable *A. fumigatus* (B). Stimulation of a FHT-specific T cell clone with dendritic cells fed with viable *A. fumigatus* in the presence of control antibody or MHC class II blocking antibody (C).

5.2.6 Activation of monocytes with supernatant of activated T cell clones

To clarify if the Crf1-specific T cell clones are actually able to improve the effector function of innate effector cells, monocytes pre-incubated with supernatant taken from T cell cultures activated with FHT peptide-pulsed or fungus-pulsed dendritic cells were co-incubated with vital *A. fumigatus* conidia. After 3 hours of incubation monocytes were lysed and the lysate plated on Sabouraud agar to determine the number of viable conidia surviving inside the phagocytes (Figure 27). Whereas control supernatant taken from T cell cultures co-incubated with unpulsed DC had hardly any effect on the killing capacity of monocytes, supernatant taken from T cells activated with FHT peptide-pulsed or fungus-pulsed APC was able to significantly reduce fungal survival after phagocytosis by monocytes, indicating that factors secreted by the activated T cell clones such as IFN-γ posses the potential to enhance the antifungal mechanisms of monocytes. In addition to IFN-γ, colony-stimulating factors also have been previously described to enhance antifungal functions of innate immune cells (Gil-Lamaignere et al. 2005; Safdar 2006). GM-CSF which can be detected in culture supernatant of

Aspergillus-specific T cells clones stimulated with fungal antigens probably also contributes to enhanced fungal killing (data not shown).

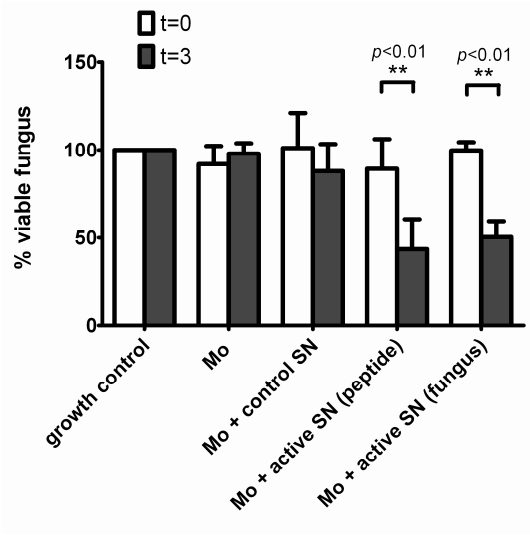


Figure 27:

Determination of fungal killing by monocytes using the plating efficiency assay. Monocytes were pre-incubated with control supernatant or supernatant of FHT-specific T cells clones activated by FHT peptide or viable fungus prior to co-incubation with *A. fumigatus* conidia. After 3 hours the monocytes were lysed and the cell lysate plated onto agar plates to determine the number of viable conidia surviving inside the phagocyte.

6 Discussion

6.1 Selective depletion of alloreactive T lymphocytes with Hsp90 inhibitors

6.1.1 Hsp90 is upregulated in alloreactive cells in mixed lymphocyte cultures

The molecular chaperone Hsp90 has been shown to stabilize many key signaling intermediates and to exert anti-apoptotic effects in various cell types, strongly suggesting a similar function in primary human T lymphocytes (Schulte et al. 1995; Yorgin et al. 2000; Beere 2001; Shang and Tomasi 2006; Neckers 2007). It was also previously shown in our lab that expression of both Hsp90 subunits α and β in T lymphocytes is activation-dependent and upregulated after stimulation of T cells with PHA. Hsp90β shows quite strong upregulation, whereas the α subunit shows only minor changes. This finding is also in good accordance with previous observations, showing induction of Hsp90 expression by mitogens and IL-2 (Ferris et al. 1988). In this work it was also confirmed, that T cells activated by physiologic stimulation with allogeneic dendritic cells similarly upregulated Hsp90 and should therefore be similarly dependent on Hsp90 function as T cell artificially activated with mitogen.

6.1.2 Inhibition of Hsp90 induces apoptosis in activated, proliferating cells

The observed upregulation of Hsp90 protein levels induced by T cell activation and the known anti-apoptotic function of Hsp90 suggested, that activated T lymphocytes should be more dependent on proper chaperone function than resting cells. Consequently, Hsp90 blockade should affect the viability of activated cells more than of resting cells. Previous experiments in our lab showed, that isolated $CD4^+$ and $CD8^+$ T lymphocytes activated with PHA showed higher apoptosis rates in the presence of the Hsp90 inhibitor 17-DMAG than resting cell populations. In this work it could be shown that proliferation of T cells that are either activated with the mitogen PHA, CD3/CD28 microbeads or physiologically with allogeneic DC is nearly abolished when the cultures

are treated with Hsp90 inhibitor. Annexin V staining showed, that alloreactive T cells that initially proliferated after contact with allogeneic DC rapidly undergo apoptosis after addition of Hsp90 inhibitor, indicating that alloreactive T cells are not only hampered in their proliferative potential by inhibitor treatment, but are actually deleted from the mixed lymphocyte culture. Non-reactive, compatible T cells on the contrary should remain resting during the co-culture and should therefore be quite unaffected by the inhibitor. Analysis of the minimal duration of inhibitor treatment necessary for abrogation of an alloresponse demonstrated, that presence of 17-DMAG for 24 hours was sufficient to block proliferation of alloreactive T cells nearly completely.

The reason for the induction of apoptosis by Hsp90 inhibition in activated cells probably lies in the corruption of signal transduction pathways necessary for T cell activation. It has been previously shown in this lab as well as by other groups that Hsp90 is necessary for the stabilization and function of important signaling intermediates and that Hsp90 blockage corrupts the signaling cascade (Schulte et al. 1995; Schnaider et al. 1998; Yorgin et al. 2000). For instance, inhibition of Hsp90 by treatment with 17-DMAG completely abrogated the phosphorylation of STAT5, STAT3 and ERK1,2, and reduced both basal and phosphorylation levels of Akt. Thus, pharmacological targeting of Hsp90 effectively abrogates phosphorylation of key signaling components leading to impairment of the respective T cell activation-induced signaling pathways (Jak/STAT5, Ras/MEK1,2/ERK1,2 and the PI3K/Akt).

Alloreactive T cells could potentially belong to different T cell compartments such as the CD45RA-positive naïve $CD4^+$ or $CD8^+$ T cell population as well as the CD45RO-positive $CD4^+$ or $CD8^+$ memory/effector population. Analysis of the different T cell subsets in separate MLRs showed, that all T cell compartments proliferated similarly in response to stimulation with allogeneic DC and that the alloreactive T cells in either the bulk T cell population as well as in the different T cell compartments were equally affected by 17-DMAG treatment with no significant difference between the different T cell subpopulations.

6.1.3 Genetic knock-down of Hsp90β also reduces proliferation of alloreactive cells

Inhibition of Hsp90 has recently been reported to possess the capacity to markedly affect dendritic cell maturation, antigen uptake and function (Bae et al. 2007). It was therefore possible, that the observed MLR inhibition was due to the dysfunction of the DC entailed by Hsp90 blockage and not the selective depletion of alloreactive T

lymphocytes. Although genetic knock-down of Hsp90β with siRNA lead only to moderate downregulation of Hsp90ß expression and was probably associated with relatively high residual activity of the Hsp90 chaperone, a significant reduction of proliferating alloreactive T cells in MLR cultures could be observed in comparison to T cells transfected with negative control siRNA. Hsp90β-specific siRNA was used to block Hsp90 function, since the Hsp90β subunit is distinctly more upregulated than the α subunit after T lymphocyte activation and only genetic knock down of Hsp90β in myeloma cells showed an significant effect (Chatterjee et al. 2007). The reduction in the proliferation of siRNA-transfected T cells suggests, that the absence of T cell proliferation in mixed lymphocyte cultures treated with 17-DMAG is not due to inadequate stimulation of alloreactive T cells by Hsp90 inhibitor-affected DC, but rather is caused by specific interruption of Hsp90 function in the stimulated alloreactive T cell population.

6.1.4 T lymphocytes depleted of alloreactive cells still exhibit functional virus-specific responses

Current treatment of graft-versus-host disease frequently results in a sharp increase in morbidity and mortality due to infectious complications. The applied immunosuppressive procedures such as high dose steroids generally lead to a critical impairment of innate as well as adaptive cellular immunity (Bacigalupo 2007; Shlomchik 2007). Thus novel GvHD prevention and treatment strategies should entail the capacity to spare pathogen-specific immunity and selectively eradicate alloreactive T cells. For instance cytomegalovirus (CMV) has been identified as one of the most prevalent pathogens causing severe infections after allogeneic transplantation. We could show, that CMV_{pp65}-specific $CD8^+$ memory T cells are not affected by Hsp90 inhibitor treatment and are readily detected at similar frequencies in both the untreated as well 17-DMAG-treated mixed lymphocyte cultures. Furthermore, untreated as well as inhibitor-pretreated cultures responded with equally strong IFN-γ production to rechallenge with a CMV_{pp65}-specific peptide pool, demonstrating that resting lymphocytes surviving inhibitor treatment, including memory T cells specific for CMV_{pp65} are not compromised in their viability and function by previous treatment with Hsp90 inhibitor. In contrast, 17-DMAG treated cultures displayed no relevant IFN-γ production upon re-exposure to the same allogeneic DC that were used to trigger the

alloresponse, whereas IFN-γ was easily detected in untreated cultures, which demonstrates that alloreactive T cells are successfully deleted from the lymphocyte pool. Potential applications of Hsp90 inhibitors may therefore be upfront depletion of alloreactive T cells by short *ex vivo* incubation of donor lymphocytes with recipient-derived antigen-presenting cells in the presence of Hsp90 inhibitor. Alternatively, Hsp90 inhibitors could also be administered to HSCT patients on the onset of GvHD.

The beneficial graft-versus-leukemia (GvL) effect of allogeneic donor lymphocyte transfusions is probably based on the same basic principles as and closely linked with the development of GvHD (Horowitz et al. 1990). Therefore the drawback of unselected T cell depletion is the increased rate of recurrence of the malignant disease (Guinan et al. 1999). However, as there is evidence that different T cell clones are capable of distinguishing between GvL and GvHD antigens (Michalek et al. 2003), approaches such as selective depletion of T cells responding to allogneic DC may overcome this limitation. Furthermore, Hsp90 blockade has been shown to have a direct anti-tumor effect on many hematological malignancies such as multiple myeloma (Chatterjee et al. 2007) and chronic lymphocytic leukemia (Jones et al. 2004; Castro et al. 2005), malignancies which are subject to allogeneic transplantation. Therefore, Hsp90 inhibitors may compensate for partial loss of GvL effects in the allodepletion procedure if administered to the transplant recipient during the onset of GvHD by not only eliminating alloreactive T cell clones but also contributing to eradication of residual malignant cells and thereby protecting from disease relapse. Direct administration of Hsp90 inhibitors to the patient could have a further important advantage, as host-DC, which seem to be primarily responsible for the induction of GvHD (Shlomchik et al. 1999; Duffner et al. 2004), are also highly susceptible to Hsp90 blockage (Bae et al. 2007) and could therefore be simultaneously deactivated in the recipient. In addition, T cells activated by antigens exclusively expressed in non-hematopoietic tissues such as skin, gut or liver could only be eliminated by direct administration of Hsp90 inhibitors *in vivo* and not in *in vitro* procedures (Wehler et al. 2007).

The results of this and previous work therefore indicate, that selective allodepletion with Hsp90 inhibitors should be a feasible approach. This novel strategy may improve the outcome after allogeneic HSCT by decreasing the incidence and severity of GvHD through selective allodepletion of donor-derived, host-reactive T cells as well as clearance of residual host DC and malignant cells. But despite the promising results obtained it should be emphasized that they cannot fully predict the clinical outcome and

it is therefore necessary to further study this novel approach for potential clinical application (Mielke et al. 2008).

6.2 Characterization of *A. fumigatus* antigens

6.2.1 Evaluation of the immunogenicity of different *A. fumigatus* proteins

It was recently shown that peripheral blood lymphocytes from healthy individuals can be induced to proliferate upon stimulation with cellular *A. fumigatus* extract. Such *ex vivo* expanded *Aspergillus*-specific $CD4^+$ T cell lines were also able to cause damage to hyphae (Hebart et al. 2002; Beck et al. 2006). Based on these findings the purpose of this thesis was the further characterization of defined fungal antigens that are able to elicit *Aspergillus*-specific immune responses and could therefore be used for potential clinical application in vaccination or adoptive transfer protocols.

Because *A. fumigatus* is nearly ubiquitously distributed in the environment, healthy individuals have permanent contact with the pathogen and should therefore possess memory T cells specific for diverse fungal antigens. To identify the specificity of those memory cells, peripheral blood mononuclear cells (PBMC) from healthy individuals were stimulated with different recombinant *A. fumigatus* proteins and the amount of IFN-γ in the culture supernatant determined. To amplify the cytokine response and thereby facilitate the identification of immunogenic proteins we first pre-stimulated fresh PBMC for 1 week with antigen and subsequently determined the cytokine amount in the supernatant after restimulation. Culture conditions biasing T_H1 $CD4^+$ T cell responses and quantification of the T_H1 cytokine IFN-γ were chosen as read-out system, because it has been repeatedly shown in the past that healthy individuals show functional T_H1 responses to *A. fumigatus*, whereas patients with fungal allergy or invasive disease show impaired T_H1 immunity and often have increased levels of T_H2 cytokines (Grazziutti et al. 1997; Hebart et al. 2002). Furthermore, only T_H1 responses are protective against *Aspergillus* infection in mice (Cenci et al. 2000; Bozza et al. 2002; Rivera et al. 2005) and adoptive transfer of functional T_H1 cells is able to control *Aspergillus*-related mortality in transplant recipients in mouse models (Bozza et al. 2003) as well as a human study (Perruccio et al. 2005), whereas T_H2 responses are often detrimental for the outcome of invasive disease (Cenci et al. 1997; Cenci et al.

1999; Cenci et al. 2000). In addition, T_H2-biased *Aspergillus*-specific T cell responses are associated with severe atopic manifestations including allergic bronchopulmonary Aspergillosis (ABPA)(Chauhan et al. 1996; Kurup et al. 2001).

PBMC of at least 6 different donors were stimulated with the recombinant *A. fumigatus* proteins superoxide dismutase, catalase, peptidase and the 1,3-beta-glucanosyl-transferase Gel1 produced in the yeast expression system *Pichia pastoris* and the major allergen and cytotoxin AspF1, CipC-like antibiotic response protein CipC and the extracellular cell wall glucanase Crf1 expressed in a human cell line. Some of the recombinant proteins were expressed in a human cell line to minimize the risk of unspecific T cell activation due to contaminating proteins or cell wall components of the producing yeast cells in the recombinant protein preparation. Although the expression levels of recombinant proteins are extremely low in eukaryotic cells compared to bacterial or yeast expression systems, optimization of the transfection and expression system in conjunction with augmentation of transgene expression by sodium butyrate yielded reasonable amounts of protein. Of the 7 antigens tested, the extracellular cell wall glucanase Crf1 induced IFN-γ production in the majority of donors and peptidase in about half the donors, whereas the remaining recombinant proteins induced distinct cytokine responses only in few donors (Table 2).

antigen	**IFN-γ response in healthy donors**
catalase	1/8 donors weakly positive
Gel1	2/10 donors positive
superoxide dismutase	1/8 donors weakly positive
peptidase	5/10 donors positive
AspF1	1/9 donors positive
CipC	1/6 donors weakly positive
Crf1	6/7 donors positive

Table 2:

IFN-γ response of healthy donors to stimulation with 7 different recombinant *A. fumigatus* proteins. Catalse, Gel1, superoxide dismutase and peptidase were expressed in the yeast *P. pastoris*. AspF1, CipC and Crf1 were expressed in the human cell line phoenix GALV.

Stimulation of PBMC with recombinant proteins expressed in the yeast expression system showed background IFN-γ levels that were about 10-fold higher than the average background levels with recombinant proteins produced in the human cell line (Figures 18/19, page 70/71), suggesting that proteins produced in the yeast expression

system could indeed induce unspecific cytokine production due to contamination of the protein preparation with yeast antigens.

As the majority of the tested donors responded to Crf1, this protein could be an interesting target for potential clinical application and was therefore analyzed in more detail. The relative sparseness of memory cells to the other proteins tested, with the exception of peptidase, could be an indication of their poor immunogenic potential and therefore probably limits their value for clinical application. The fact that rechallenge with Crf1 induced memory responses in most tested donors is somehow in accordance with previously published data. The amino acid sequence of Crf1 is to some extent identical with the proposed Asp f16 antigen, which turned out to be no genuine *Aspergillus* protein, but probably is an erroneous version of Crf1 resulting from frameshifts or other sequencing errors in the cDNA clone used for gene annotation (Bowyer and Denning 2007). Nevertheless, vaccination with the Asp f16 antigen together with CpG deoxyoligonucleotides as adjuvant was able to induce protective T_H1 immune responses in mice, most likely due to the high sequence similarity with Crf1 (Bozza et al. 2002). Also, stimulation of human peripheral blood cells with Asp f16 or an Asp f16-specific overlapping peptide pool has been shown to induce proliferation and activation of memory cells of different T lymphocyte subsets, depending on whether blood was taken from healthy individuals or patients suffering from ABPA (Banerjee et al. 2001; Ramadan et al. 2005). As the protein sequence of Asp f16 is to about 70% identical to the protein sequence of Crf1, it is most likely that the responses observed in those studies are targeted at epitopes common to both proteins. Peptidase and catalase also have been previously investigated for proliferative responses in blood of healthy donors (Hebart et al. 2002), but both proteins lead to lymphoproliferation in only some of the donors. No data were available regarding immune responses to the antigens superoxide dismutase, Gel1 and CipC. The *Aspergillus* antigen AspF1 has so far been described only in connection with T_H2-biased allergic immune responses in mice and humans (Chauhan et al. 1996; Chauhan et al. 1997; Kurup et al. 1998), possibly indicating an inherent tendency of this antigen to entail predominantly T_H2 rather than T_H1 immune responses. This could be one explanation for the relative sparseness of memory T_H1 cells in the healthy individuals tested in this study.

Crf1-specific memory responses could only be detected when PBMC were pre-stimulated with antigen for 1 week prior to restimulation, but not in PBMC directly after preparation, suggesting that the frequency of memory cells is rather low, which was

also confirmed by IFN-γ ELISPOT. However, positive responses were constant and reproducible in independent experiments. To verify the specificity of the T cell response to the Crf1 antigen and exclude unspecific T cell activation by contaminations of the recombinant protein preparation, CD40 ligand-activated B cells were transduced with retroviral constructs coding for Crf1 and used as antigen-presenting cells. To enhance presentation of the endogenously synthesized antigen via MHC class II and thereby augment $CD4^+$ T cell activation, the sequence coding for the N-terminal domain of the invariant chain (Ii) was fused to Crf1 (see section 5.2.3.3). Crf1-specific T cell lines which were generated by stimulation with the recombinant Crf1 protein did specifically respond with IFN-γ production after restimulation with Ii-Crf1-transduced B cells, demonstrating that the observed response is indeed antigen-specific and also demonstrates that the Crf1-specific memory T cells have a $CD4^+$ T_H1 phenotype. This was further confirmed by pre-treatment of APC with MHC class II blocking antibody, which strongly diminished T cell stimulation. Activation of $CD8^+$ T cells could not be detected, even when CD40L-activated B cells transduced with Crf1 where used as APC. In our experiments, the great majority of donors showed positive responses to this antigen, suggesting that Crf1-specific memory cells are relatively frequent in the population, rendering it a promising antigen for potential clinical application. Although the precursor frequency of Crf1-specific memory cells seems to be extremely low in healthy individuals as shown by IFN-γ ELISA and IFN-γ ELISPOT, it was nevertheless possible to isolate and expand functional antigen-specific T cell clones from different donors.

Ramadan et al. have already previously described the proliferation and activation of peripheral blood lymphocytes from healthy donors in response to challenge with Asp f16 protein and synthetic peptides (Ramadan et al. 2005; Ramadan et al. 2005). As would be expected, the defined epitopes of Asp f16 are located in protein regions that are also present in the Crf1 protein. The responding cells however were described as cytolytic $CD8^+$ as well as $CD4^+$ lymphocytes which could directly kill hyphae and the culture supernatant was shown to kill conidia. However, the contribution of those cytotoxic cells in host defence against *A. fumigatus* and other fungi is not clearly established to date. Although activated $CD4^+$ and $CD8^+$ T cells as well as NK cells have indeed demonstrated direct antifungal activity against *Cryptococcus neoformans*, *Candida albicans* and *A. fumigatus* depending on intimate cell to cell contact (Levitz et al. 1995; Ramadan et al. 2005), the receptors and cognate ligands involved in recognition and the exact mechanism of action remain largely undefined. In addition, infection of mice selectively depleted of neutrophilic granulocytes by antibody treatment resulted in uncontrolled fungal growth (Cenci et al. 1998), stressing the notion that the

described $CD4^+$ and $CD8^+$ cytolytic T cells supposedly do not significantly contribute to and are not sufficient for the control of *A. fumigatus in vivo*.

6.2.2 Memory T cells specific for Crf1 can be activated with synthetic peptides

Because production and purification of complete recombinant proteins is much more laborious and expensive than synthesis of small peptides, and peptides in general are more efficient in inducing specific T cell responses than proteins, it could be worthwhile to define immunogenic epitopes of an antigenic protein, especially in regard to potential clinical application such as immunization or adoptive therapy protocols. On the other hand, one major drawback in the use of peptides in comparison to entire proteins lies in the MHC restriction of peptide presentation. Whereas proteins are processed to many different peptide fragments by antigen presenting cells and different peptide fragments can be presented by different MHC class II alleles, presentation of one specific peptide is often limited to one or only few MHC alleles, implicating that one single peptide cannot be used for all patients, but only for a subgroup of patients with respective MHC class II alleles. Because HLA-DRB1*01 and HLA-DRB1*04 are two of the most common MHC class II alleles in the Caucasian population, 5 peptide fragments of the Crf1 protein sequence that are probably presented by one or both of this two alleles were tested for their immunogenic potential. One of the 5 peptides tested, the HLA-DRB1*04-restricted FHT peptide, reproducibly elicited comparatively high antigen-specific IFN-γ production in all HLA-DRB1*04-positive donors positively tested for responses to the recombinant protein and therefore seems to be a highly immunogenic epitope of Crf1. It was also possible to generate FHT-specific T cell lines and T cell clones, which responded to stimulation with FHT peptide as well as recombinant Crf1 protein. In addition, MHC class II tetramers loaded with FHT peptide could be successfully used to detect, quantify and enrich antigen-specific cells. Recent data also indicate that the FHT peptide can possibly also be presented by other MHC class II alleles, but the results have to be validated in further experiments.

6.2.3 Crf1-specific T cell clones can be specifically activated by *A. fumigatus*

Crf1-specific $CD4^+$ T_H1 T cell clones can be specifically activated by APC presenting *Aspergillus*-derived Crf1 antigen either in the form of synthetic peptide, purified protein, fungal cell extract or viable fungus. In contrast, the clones do not cross-react with APC pulsed with *C. albicans* or purified AspF1 protein. To exclude that the T cell clones are unspecifically activated independent of antigen by the pro-inflammatory milieu generated by dendritic cells after exposure to live fungus, a $CD4^+$ T_H1 T cell clone with unrelated specificity was exposed to DC fed with fungus. In contrast to the Crf1-specific clones, the CMV-specific T cell clone responded only with comparably weak cytokine secretion, indicating that T cell activation is antigen-specific. Another finding stressing the antigen-specificity of the response is that T lymphocyte activation is MHC-restricted, as pre-treatment of the antigen-presenting cells with MHC class II blocking antibody strongly impairs IFN-γ production.

One further important factor concerning immunity to *A. fumigatus* is the phenotypic switching from conidia to hyphae during germination, which may help the pathogen to evade host defence (Netea et al. 2003). The change from one phenotype to another is accompanied by loss or change of pathogen-specific signals that are important for the activation and education of antigen-presenting cells (Romani 2004) as well as determining the availability of fungal antigens during different growth phases of the pathogen. The *A. fumigatus* Crf1 FHT peptide-specific T cell clones were able to recognize their target antigen with high specificity and sensitivity on DC presenting antigen of phagocytosed ethanol-killed germinating conidia or outgrown hyphae, whereas only marginal responses were detected after phagocytosis of inactivated resting conidia. This could either suggest that the Crf1 antigen is not present in resting conidia in relevant amounts and protein expression starts only after germination, or that non-viable conidia in general induce only poor immune responses. That immune responses to *A. fumigatus* do depend on the metabolic state of the pathogen and immune cells can indeed distinguish between resting spores and germinating, potentially invasive fungus was demonstrated in vaccination experiments in mice and in *in vitro* studies with murine and human macrophages. In mice, only live conidia were able to prime robust T_H1 responses, whereas heat-inactivated spores induced T_H2 responses, indicating that fungal spores must have the potential to germinate and cause invasive infection to induce a protective immune response (Rivera et al. 2005). Also, macrophages bind and ingest resting *Aspergillus* spores efficiently, but without

initiating an inflammatory response and production of cytokines and reactive oxygen species, as is the case for germinating conidia. The differential response is probably mediated by receptors recognizing cell wall beta-glucan which is not present on the surface of dormant conidia, but only after cellular swelling and the loss of the hydrophobic proteinaceous cell wall (Gersuk et al. 2006). Similarly, degranulation of PMN is provoked only by swollen and not by resting conidia (Levitz and Farrell 1990). This confinement of the pulmonary inflammatory response toward metabolically active cells probably evolved because it has the advantage of avoiding unnecessary tissue damage (Gersuk et al. 2006).

6.2.4 Activated Crf1-specific T cell clones are able to boost the antifungal functions of innate immune cells

The protective effect of T_H1 responses in fungal infections is probably mediated by the recruitment and activation of innate immune cells by T cell-derived cytokines. The activated innate immune cells are the actual effector cells that are responsible for eradication of the pathogen. It could be shown, that culture supernatant of T cell clones activated by co-incubation with peptide-pulsed or fungus-pulsed dendritic cells was actually able to greatly enhance fungal killing by monocytes. The stimulatory effect exerted by activated antigen-specific T_H1 cells is probably based on secreted IFN-γ and colony-stimulating factors like G-CSF and GM-CSF. GM-CSF could be detected in the culture supernatant of activated T cell clones in our experiments and CSF have already proven to enhance antifungal effector mechanisms of phagocytes and granulocytes in previous studies (Roilides et al. 1993; Nemunaitis 1998; Gil-Lamaignere et al. 2005). It has also been shown that IFN-γ together with G-CSF is even able to prevent corticosteroid-induced suppression of monocyte and neutrophil function *in vitro* (Roilides et al. 1993; Roilides et al. 1996), an effect that could potentially also contribute to clearance of invasive Aspergillosis in immunosuppressed transplant recipients. This hypothesis is strengthened by mouse experiments showing that vaccinated animals are protected against subsequent lethal infection with *A. fumigatus*, even when rechallenged during corticosteroid-treatment (Ito and Lyons 2002).

Several interesting questions could be addressed in future experiments. First it would be interesting to analyse if patients that have survived an invasive *Aspergillus* infection

show enhanced numbers of Crf1-specifc memory cells to confirm their protective potential. It would also be interesting to further validate the preliminary data indicating that the FHT peptide can possibly also be presented by MHC class II alleles other than HLA-DRB1*04, which would extend its applicability in the clinical setting. Identification of additional immunogenic epitopes of Crf1 with different MHC restriction would similarly enforce its antigenic potential. As the recombinant *A. fumigatus* peptidase stimulated memory responses in about half the donors tested, it would also be worthwhile to analyze this antigen in more detail. A cocktail of several peptides corresponding to different *A. fumigatus* proteins and with different MHC restriction could be used for a greater number of patients and facilitate potential clinical application.

7 References

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8 Appendix

8.1 Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-(dimethylaminoethylamino)-17-demethoxygeldanamycin
Ab	antibody
ABPA	allergic bronchopulmonary Aspergillosis
AIDS	acquired immunodeficiency syndrome
allo-DC	allogeneic dendritic cells
APC	antigen presenting cell
APS	ammonium persulfate
ATG	anti-thymocyte globulin
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair
CD40L	CD40 ligand
cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CSF	colony-stimulating factor
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMEM	Dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence substrate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
ERK	extracellular signal-related protein kinase
F	Farad
FCS	fetal calf serum
GALV	Gibbon-Ape Leukemia Virus
G-CSF	granulocyte colony-stimulating factor

GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
GvHD	graft-versus-host disease
GvL	graft-versus-leukemia
h	hour
HBS	Hepes-buffered saline
HLA	human leukocyte antigen
HRP	horse radish peroxidase
HSCT	hematopoietic stem cell transplantation
Hsp90	heat shock protein of 90 kD
IA	invasive Aspergillosis
IFN	interferon
IL	interleukin
IMDM	Iscove's modified Dulbecco's media
JAK	janus kinase
kb	kilo base pairs
kDa	kilodalton
M	mol per liter
MAPK	mitogen-activated protein kinase
MBL	mannan-binding lectin
M-CSF	macrophage colony-stimulating factor
MEK	map-erk kinase
MHC	major histocompatibility complex
min	minute
MLR	mixed lymphocyte reaction
mRNA	messenger RNA
NF-κB	nuclear factor κB
NGFR	nerve growth factor receptor
NK cell	natural killer cell
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E2
PGK	phosphoglycerate kinase

PHA	phytohemagglutinin
PI3-K	phosphatidyl-inositol-3 kinase
PMA	phorbol myristate acetate
PMN	polymorph-nuclear neutrophilic granulocytes
PRR	pattern recognition receptor
PVDF	polyvinylidene fluoride
ROI	reactive oxygen intermediate
RT	room temperature
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SCT	stem cell transplantation
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription
TBS	Tris-buffered saline
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
T_H cell	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
V	Volt
ZAP-70	ζ-associated protein of 70 kD

8.2 Publications

Stuehler, C., S. Mielke, et al. (2009). Selective depletion of alloreactive T cells by targeted therapy of heat shock protein 90: a novel strategy for control of graft-versus-host disease. *Blood.* 114(13): 2829-36.

8.3 Curriculum Vitae

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