Spatiotemporal analysis of immune cell recruitment and Neutrophil defence functions in *Aspergillus fumigatus* lung infections

Zeitliche und örtliche Analyse der Immunzellrekrutierung und der durch Neutrophile Granulozyten vermittelten Abwehr gegen *Aspergillus fumigatus* Infektionen der Lunge

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg

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Würzburg, 2016

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For my family

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List of Abbreviations

µg	Microgram
AMM	Aspergillus minimal medium
BLI	Bioluminescence Imaging
BM	Bone marrow
BM-DCs	Bone marrow-derived Dendritic cells
BSA	Bovine serum albumin
C IS	Cyclophosphamide immunosuppressed
CC IS	Corticosteroid and cyclophosphamide immunosuppressed
CCR	Chemokine receptor
CCT	Corticosteroid and cyclophosphamide treated
CD	Cluster of differentiation
CT	Corticosteroid treated
DAPI	4′,6-Diamidin-2-phenylindol
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacidic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
g	Gram
h	Hours
HPF	High Power Field

IA	Invasive aspergillosis
IC	Immunocompetent
IFM	Immunofluorescence microscopy
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
IS	Immunosuppressed
kg	Kilogram
mg	Milligram
MHC	Major histocompatibility complex
ml	Milliliter
mM	Millimolar
NETs	Neutrophil Extracellular Traps
ng	Nanogram
NRS	Normal rat serum
p.i.	Post-infection
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive Oxygen Species
SD	Standard deviation
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
WT	Wild type

Introductory statement on contributions and previous publication

This thesis was conducted in the research laboratory of Prof. Dr. Dr. Andreas Beilhack (Department of Medicine II, Würzburg University Hospital). Experimental procedures of this Ph.D project were performed by myself, with technical assistance from Dr. Jorge Amich, Mr. Berkan Arslan, Dr. Spoorthi Poreddy, Ms. Katharina Mattenheimer and Dr. Zeinab Mokhtari. Parts of this thesis were published in "*Frontiers in Microbiology*", an open access publication. This publication was written by me and all the co-authors corrected and accepted the final manuscript.

Author contributions from original publication (Kalleda et al., 2016): **NK**, JA, and AB designed the study. **NK**, JA, BA, and KM carried out experiments. NK, JA, HE, SP, MB, KH, ZM, and AB analyzed the data. **NK** wrote the manuscript. **NK**, JA, HE, SP, MB, KH, ZM, and AB revised the manuscript and all the authors approved the final manuscript.

Original publication citation: **Kalleda N**, Amich J, Arslan B, Poreddy S, Mattenheimer K, Mokhtari Z, Einsele H, Brock M, Heinze KG and Beilhack A. (2016). Dynamic immune cell recruitment after murine pulmonary *Aspergillus fumigatus* infection under different immunosuppressive regimens. *Front. Microbiol*. 7:1107.

Abstract

Humans are continuously exposed to airborne spores of the saprophytic fungus *Aspergillus fumigatus*. In healthy individuals, local pulmonary host defence mechanisms can efficiently eliminate the fungus without any overt symptoms. In contrast, *A. fumigatus* causes devastating infections in immunocompromised patients. However, local host immune responses against *A. fumigatus* lung infections in immunocompromised conditions have remained largely elusive.

Given the dynamic changes in immune cell subsets within tissues upon immunosuppressive therapy, we dissected the spatiotemporal pulmonary immune response after *A. fumigatus* infection to reveal basic immunological events that fail to effectively control the invasive fungal disease. In different immunocompromised murine models, myeloid but not lymphoid cells were strongly recruited upon infection. Notably, neutrophils and macrophages were recruited to infected lungs in different immunosuppressed regimens. Other myeloid cells, particularly dendritic cells and monocytes were only recruited in the corticosteroid model after infection. Lymphoid cells, particularly $CD4^+$ or $CD8^+$ T-cells and NK cells were highly reduced upon immunosuppression and were not recruited after *A. fumigatus* infection. Importantly, adoptive $CD11b^+$ myeloid cell transfer rescued immunosuppressed mice from lethal *A. fumigatus* infection. These findings illustrate that $CD11b^+$ myeloid cells are critical for anti-*A. fumigatus* defence under immunocompromised conditions.

Despite improved antifungal agents, invasive *A. fumigatus* lung infections cause a high rate morbidity and mortality in neutropenic patients. Granulocyte transfusions have been tested as an alternative therapy for the management of high-risk neutropenic patients with invasive *A. fumigatus* infections. To increase the granulocyte yield for transfusion, donors are treated with corticosteroids. Yet, the efficacy of granulocyte transfusion and the functional defence mechanisms of granulocytes collected from corticosteroid treated donors remain largely elusive.

We aimed to assess the efficacy of granulocyte transfusion and functional defence mechanisms of corticosteroid treated granulocytes using mouse models.

In this thesis, we show that transfusion of granulocytes from corticosteroid treated mice did not protect cyclophosphamide immunosuppressed mice against lethal *A. fumigatus* infection in contrast to granulocytes from untreated mice. Upon infection, increased levels of inflammatory cytokines helped to recruit granulocytes to the lungs without any recruitment defects in corticosteroid treated and infected mice or in cyclophosphamide immunosuppressed and infected mice that have received the granulocytes from corticosteroid treated mice. However, corticosteroid treated human or mouse neutrophils failed to form neutrophil extracellular traps (NETs) in *in vitro* and *in vivo* conditions. Further, corticosteroid treated granulocytes exhibited impaired ROS production against *A. fumigatus*. Notably, corticosteroids impaired the β-glucan receptor Dectin-1 (CLEC7A) on mouse and human granulocytes to efficiently recognize and phagocytize *A. fumigatus*, which markedly impaired fungal killing. We conclude that corticosteroid treatment of granulocyte donors for increasing neutrophil yields or patients with ongoing corticosteroid treatment could result in deleterious effects on granulocyte antifungal functions, thereby limiting the benefit of granulocyte transfusion therapies against invasive fungal infections.

Zusammenfassung

Der Mensch kommt über die Atemluft in regelmäßigem Kontakt mit Sporen des saprophyitschen Pilzes *Aspergillus fumigatus*. Glücklicherweise eliminieren die lokalen Abwehrmechanismen der Lunge den Pilz in gesunden Individuen sehr effektiv und ohne offenkundige Symptome. In immunkomprimierten Patienten hingegen verursacht *A. fumigatus* verheerende Infektionen. Allerdings ist die lokale Immunreaktion gegen *A.fumigatus*-vermittelte Infektionen der Lunge unter immunsuppressiven Bedingungen immer noch nicht ausreichend definiert.

In Anbetracht der dynamischen Veränderungen an Immunzellunterpopulationen im Gewebe nach immunsuppressiver Therapie haben wir die zeitliche und örtliche pulmonale Immunreaktion nach *A. fumigatus* infektion untersucht, um die grundlegenden immunologischen Geschehnisse aufzudecken, die in dieser Situation zur unzureichenden Kontrolle des Pilzes führen. In anderen immunsupprimierten Mausmodellen fand eine starke Rekrutierung myeloider Zellen nach Infektion statt. In besonderem Maße wurden nach der Infektion Neutrophile und Makrophagen in die Lunge immunsupprimierter Mäuse rekrutiert. Andere myeloide Zellen, insbesondere dendritische Zellen und Monozyten, wurden nur im Corticosteroid-Modell nach Infektion rekrutiert. Lymphoide Zellen, insbesondere $CD4^+$ oder $CD8^+$ Zellen und NK Zellen, waren nach Immunsuppression stark reduziert und wurden nach Infektion mit *A. fumigatus* nicht rekrutiert. Adoptiver Zelltransfer von $CD11b^+$ myeloiden Zellen stellte die Abwehr immunsupprimierter Mäuse gegen *A. fumigatus* wieder her, was die wesentliche Bedeutung dieser Zellen in der Immunabwehr unterstreicht. Diese Erkenntnisse verdeutlichen, dass $CD11b^+$ myeloide Zellen unter immunkomprimierten Bedingungen entscheidend für die Abwehr gegen *A-fumigatus* sind.

Trotz verbesserter antimykotischer Wirkstoffe verursachen Lungeninfektionen durch *A. fumigatus* eine hohe Rate an Krankheit und Sterblichkeit in neutropenischen Patienten.

Infusionen von Granulozyten wurden als Alternativtherapie für Hochrisikopatienten mit invasiver Aspergillose getestet. Um den Ertrag an Granulozyten für die Transfusion zu erhöhen, werden die Spender mit Corticosteroid-behandelt. Die Effektivität von Granulozytentransfusionen und von funktionellen Abwehrmechanismen der Granulozyten aus Corticosteroid-behandelten Spendern ist bisher unzureichend definiert. Ziel dieser Arbeit war, sich mit der Effektivität von Granulozytentransfusionen und funktionellen Abwehrmechanismen von Granulozyten aus Corticosteroid-behandelten Spendern mithilfe von Mausmodellen zu befassen.

Wir zeigen, dass die Transfusion von Granulozyten aus kortikosteroidbehandelten Mäusen keine ausreichende Kontrolle von *A. fumigatus* Infektionen in mit Cyclophosphamid supprimierten Empfängermäusen vermittelt, im Gegensatz zu Granulozyten aus unbehandelten Mäusen. Nach der Infektion halfen erhöhte Spiegel inflammatorischer Zytokine dabei, Granulozyten in die Lunge Corticosteroid-supprimierter infizierter oder mit Cyclophosphamid, supprimierter infizierter Mäuse zu rekrutieren, welche Granulozyten aus Corticosteroid-behandelten Mäusen erhalten haben. Corticosteroid-behandelte humane oder murine Neutrophile versagten *in vitro* und *in vivo* hingegen bei der Bildung neutrophiler extrazellulärer Fallen (NET, *Neutrophil Extracellular Traps*). Weiterhin zeigten Corticosteroid-behandelter Granulozyten verminderte ROS (*Reactive Oxygen Species*, reaktive Sauerstoffspezies) Produktion gegen *A. fumigatus*. Bemerkenswerterweise behinderten Corticosteroid den β-Glucanrezeptor Dectin-1 (CLEC7A) auf Maus- und menschlichen Granulozyten, was die antimykotische Abwehr merklich reduzierte. Wir schließen daraus, dass die Corticosteroid-Behandlung von Granulozytenspendern für eine erhöhte Granulozytenausbeute eine schädigende Wirkung auf die antimykotischen Funktionen der Granulozyten haben könnte, wodurch der Nutzen der Granulozytentransfusionstherapie gegen invasive Pilzinfektionen gemindert wird.

1 Introduction

1.1 *Aspergillus fumigatus* and human infections

The filamentous fungus *Aspergillus fumigatus* is a ubiquitously present environmental mold. It grows on dead-decaying organic matter and plays a crucial role in recycling environmental carbon and nitrogen (Rhodes, 2006). *A. fumigatus* mainly reproduces asexually and releases large numbers of 2-3 µM in diameter tiny thermostable airborne conidia that, due to their hydrophobic nature, propagate freely in the air. (Latge, 1999). Humans inhale hundreds to thousands of conidia daily (Chazalet et al., 1998). The small size and hydrophobic nature which is derived from the rodlet layer enables inhaled conidia to easily reach the lung alveoli by crossing innate respiratory barriers (Dagenais and Keller, 2009). Most of the healthy humans efficiently eliminate these conidia without showing any adverse symptoms by employing a combination of physiological barriers and innate immune defence mechanisms (Dagenais and Keller, 2009; Latge, 1999). However, *Aspergillus* species present several challenges to the respiratory system and are responsible for various human diseases ranging from allergic reactions to severe disseminated invasive aspergillosis, depending on the status of individual's immune system (Chabi et al., 2015; Shah and Panjabi, 2016).

1.1.1 Aspergilloma

Aspergilloma is an accumulated mass of *Aspergillus* growth typically present in the paranasal sinus or in the lung cavity and which is often formed in previously healed tuberculosis cavities, abnormal airways and sarcoid-related pulmonary cavities (Moodley et al., 2014). It is a combination of fungal hyphae, infiltrated cells, mucus and other cellular debris, without any tissue invasion. It is typically characterized by the presence of a movable round mass within a

pulmonary cavity and is usually surrounded by an airspace. A clinical symptom of advanced aspergilloma is hemoptysis, which occurs due to the disruption of blood vessels in the pulmonary cavities. In severe cases, internal bleeding also takes place, but hemoptysis frequently turns into fatal disease (Addrizzo-Harris et al., 1997; Chen et al., 1997). Aspergilloma is routinely diagnosed by identifying the presence of 'air crescent' employing computer tomography scan. Elevated levels of antibody titers are common in patients with aspergillomas (Tomee et al., 1995). Aspergillomas usually do not increase in size in the majority of the cases, but sometimes decreases or spontaneously disappears, but most patients require strong antifungal treatment to eliminate aspergillomas (Soubani and Chandrasekar, 2002).

1.1.2 Allergic bronchopulmonary aspergillosis (ABPA)

ABPA is a hypersensitive response to *Aspergillus* antigens that predominantly occurs in patients with asthma and cystic fibrosis (Shah and Panjabi, 2016). Repeated exposures to *Aspergillus* spores in susceptible patients leads to IgE-mediated type-I or IgG-mediated Type-III or cell mediated Type-IV responses, which are mainly implicated in ABPA disease manifestation (Patterson, 1998). Inhaled conidia persist and germinate in the airways leading to hyphal formation in the sputum which can interferes with mucociliary clearance. The first case of ABPA was discovered more than 60 years ago in England and, since then several cases have been reported worldwide (Hinson et al., 1952; Shah and Panjabi, 2002). The immune mechanisms involved in ABPA-induced lung damage have yet to be fully elucidated. *Aspergillus* antigens induces a polyclonal antibody, which is primarily responsible for high levels of IgE or IgG antibodies (Kurup, 2000). The chemotactic cytokines, eosinophilic infiltration and, $CD4^+$ T cell-mediated Th2 response with the subsequent production of IL-4, IL-5, and IL-13 cytokines can be attributed to ABPA (Knutsen and Slavin, 2011). ABPA is commonly treated with systemic

corticosteroids in order to reduce eosinophilic infiltrates and the associated allergic symptoms (Maturu and Agarwal, 2015).

1.1.3 Chronic pulmonary aspergillosis (CPA)

CPA is prevalent in elderly and/or weakened non-immunocompromised individuals with underlying lung disorders, such as patients suffering from chronic obstructive pulmonary disease (COPD), tuberculosis, non-tuberculous mycobacterial infection, ABPA and other lung disorders (Smith and Denning, 2011). *Aspergillus* persists in a pre-existing cavity created by an underlying lung disease. *Aspergillus* growth is limited by physiological barriers; however an uncontrolled growth of *Aspergillus* can lead to necrosis of the lung tissue, which results in chronic necrotizing pulmonary aspergillosis (Smith and Denning, 2011). Neutrophil infiltration and IFN-γ responses are important to control CPA (Kolwijck and van de Veerdonk, 2014). CPA is associated with high morbidity and mortality and requires prolonged treatment with antifungal drugs in order to eliminate the infection (Felton et al., 2010; Sales Mda, 2009).

1.1.4 Invasive pulmonary aspergillosis (IPA)

IPA results from strong immunosuppression, which allows for aggressive *A. fumigatus* growth and long hyphal formation that invades the both bronchial wall and the accompanying arterioles. IPA is the leading cause of morbidity and mortality in immunocompromised patients and IPA-associated mortality is highly prevalent, especially in haematological patients (Latge, 1999). The mortality rate of IPA is more than 50% in patients with strong neutropenia and more than 90% in haematopoietic stem-cell transplantation (HSCT) recipients (Fukuda et al., 2003; Yeghen et al., 2000). The incidence of IPA is approximately 5 to 25% in acute leukaemia patients, 5 to 10% after allogeneic HSCT, and 0.5 to 5% after treatment with strong chemotherapeutic drugs employed in blood cancers or after solid-organ transplantation (Latge, 1999). IPA also presents

a life-threatening complications in AIDS, CGD and several cancers (Kousha et al., 2011). Moreover, IPA presents complications in critically ill patients and those with COPD and other lung diseases. IPA starts with the inhalation of *A. fumigatus* spores and the entry of these spores into the lower respiratory tract. The symptoms of IPA are nonspecific and variable, including fever, cough, sputum production, pleuritic chest pain, hemoptysis, dyspnoea and unresponsiveness to antibiotics (Albelda et al., 1985; Kousha et al., 2011; Latge, 1999). IPA can also disseminate to other organs, including the brain, which can then lead to seizures, lesions, cerebral infarctions, intracranial haemorrhages, meningitis and epidural abscesses (Denning, 1998). Several methods can diagnose IPA, including computer tomography, culture and microscopy examination, identification of *Aspergillus* antigens or *Aspergillus*-specific molecules, or determination of *Aspergillus* DNA by PCR methods. To avoid IPA, high-risk patients are prophylactically treated with antifungals. The most common treatments include amphotericin B, azole derivatives and echinocandins. In addition, immunotherapies are performed depending upon the patient situation (Reichenberger et al., 2002). Nevertheless, IPA associated mortality remains very high.

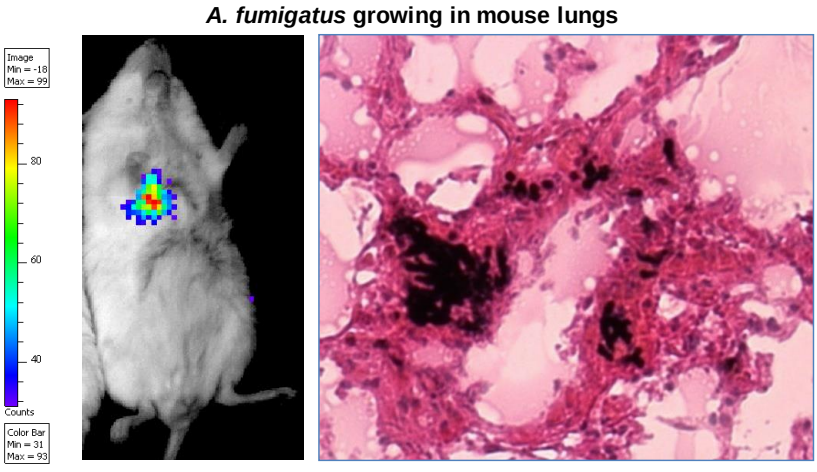

Figure. (i) *Aspergillus fumigatus* lung infection. Non-invasive in vivo bioluminescence imaging (BLI) of firefly luciferase expressing *A. fumigatus* growing in infected mouse lungs (left panel) and silver staining of a lung section shows *A. fumigatus* growth and tissue invasion (right panel).

1.2 Immune defence mechanisms against *A. fumigatus* lung infections

Inhalation of *A. fumigatus* spores is a daily occurrence for most humans due to their ubiquitous nature and surveys report that the average individual may inhale up to 200 conidia per day (Chazalet et al., 1998). These numbers are especially high in construction sites and other dirty places. However, healthy individuals can efficiently clear the infection and do not develop lung infections (Dagenais and Keller, 2009). The physical barriers of the respiratory tract, humoral immunity factors and resident and recruiting phagocytic cells act as the host's predominant defence against *A. fumigatus* lung infections (Latge, 1999). The nasal concha and the branching pattern of the bronchial tree create highly turbulent airflow that traps most of the inhaled conidia in the airway surface fluid which supports conidial removal by the ciliary action of the respiratory epithelium. This mechanism constitutes the foremost physiological antimicrobial defence in the respiratory system (Knowles and Boucher, 2002). In contrast, the tiny size of the *A. fumigatus* conidia allows them to escape from the mucociliary clearance mechanism and to enter the respiratory zone of the lung. The airway-lining mucus contains several soluble pathogen recognition receptors and microbicidal peptides. *A. fumigatus* is principally recognized by components of innate immunity, such as soluble pattern recognition molecules and cell-bound receptors. The pattern recognition receptors (PRRs), which include C-type lectin and toll-like receptor (TLR) family members, recognize pathogen-associated molecular patterns (PAMPs), such as fungal wall components (Mambula et al., 2002). The next step in the anti-*A. fumigatus* defence is the activation of the effector mechanisms of innate immunity, such as phagocytosis by resident alveolar macrophages, recruitment of other immune cells, and activation of recruited immune cells following their arrival at the site of infection. On the other hand, *A. fumigatus* conidia acquire some moisture from the surrounding environment and become swollen within 4 to 6 hours of their arrival in the lungs. If the primary innate effector mechanisms fail to clear these conidia, they will germinate and produce hyphae within 12-16 h (Kalleda et al., 2016). The

hyphal form then invades the surrounding tissues and causes respiratory difficulties and often disseminates to other organs, including the brain. Furthermore, if *A. fumigatus* is not eliminated, antigen presentation and clonal propagation of *A. fumigatus*-specific T clones lead to acquired immunity against *A. fumigatus* (Park and Mehrad, 2009).

1.2.1 Recognition of *A. fumigatus*

A. fumigatus-resting conidia, swollen conidia and hyphae present in the lung tissue are recognized by several soluble and cell-bound recognition receptors. During the conidial germination process the proteinaceous outer conidial layer disrupts and exposes predominant cell wall polysaccharides, such as β-(1,3) glucan, chitin, and galactomannan (Latge, 2007). The morphotype of the *A. fumigatus* plays an important role in the recognition of fungi by the host immune system, for instance resting conidia induce minimal inflammatory response (Gersuk et al., 2006; Hohl et al., 2005). The soluble receptors, such as lung collectins and lung surfactant proteins A and D, have been shown to bind *A. fumigatus* conidial cell wall components in a calcium-dependent manner (Allen et al., 1999; Madan et al., 2001). The components of the complement system are involved in the recognition of *A. fumigatus.* The binding of C3 to *A. fumigatus* initiates the activation of the complement alternative pathway (Kozel et al., 1989). On the other hand, mannan-binding lectin endorses the activation of the lectin complement pathway via C4bC2a (Kaur et al., 2007) and leads to a dose-dependent deposition of complement on *A. fumigatus* (Dumestre-Perard et al., 2008). Another important soluble receptor is Pentraxin-3, which belongs to the family of long pentraxins. *A. fumigatus* increases the production of pentraxin-3 in phagocytes and dendritic cells. This soluble receptor binds galactomannan on *Aspergillus* conidia and facilitates recognition by effector cells (Daigo and Hamakubo, 2012; Garlanda et al., 2002). The cell-bound receptors, such as mammalian Toll-like receptors (TLRs), recognize and mediate cellular responses to conserved PAMPs by employing the MyD88

signalling pathway which results in the production of different inflammatory cytokines and reactive oxygen species (ROS). TLR2 and TLR4 play an important role in the leukocyte-detection of *A. fumigatus* (Bellocchio et al., 2004; Dubourdeau et al., 2006). The C-type lectin-like receptor Dectin-1 is a crucial receptor for recognition of *A. fumigatus* cell wall β-glucans (Brown et al., 2003). Drectin-1 is expressed on a wide range of myeloid cells, including macrophages, neutrophils, and dendritic cells (Brown et al., 2002; Mezger et al., 2008; Taylor et al., 2002). The Dectin-1-mediated recognition of surface β-glucans on swollen conidia trigger a selective inflammatory response in order to eliminate the fungi (Gersuk et al., 2006). Dectin-1-knockout mice are highly susceptible to fungal infection mediated by an impaired production of the required cytokines and chemokines needed to eliminate the fungal infection. The reduction of inflammatory cytokines, such as IL-1β, TNF-α, CCL3, CCL4, and CXCL1, leads to a reduced pulmonary neutrophil recruitment, a reduced ROS production and an elevated pulmonary *A. fumigatus* invasion. Dectin-1 deficiency diminishes the production of pro-inflammatory mediators by alveolar macrophages and reduces lung IL-17 levels against pulmonary fungal infection (Werner et al., 2009).

1.2.2 Cytokine signalling and immune cell recruitment to infected lungs

A. fumigatus recognition by soluble or cell-bound recognition receptors is rapidly followed by the release of an initial group of cytokines, including IL-1 and TNF family members. The IL-1 gene cluster codes for IL-1α and IL-1β, both of which are important pro-inflammatory cytokines that play a key role in the recruitment of immune cells to the site of inflammation. IL-1 receptor antagonists (IL-1Ra) competitively bind to IL-1RI, thereby preventing the binding of IL-1α and lL-1β (Garlanda et al., 2013). Alveolar macrophages induce production of IL-1β in response to *A. fumigatus*, which aids in the neutrophil infiltration to the infected lungs (Nicholson et al., 1996). Recently, it was reported that 1α and IL-1β play non-redundant roles against an *A. fumigatus*

infection. It was demonstrated that IL-1α, not IL-1β, is more important for optimal immune cell recruitment and IL-1α signalling induces the production of CXCL1. On the other hand, IL-1β is essential for the activation of anti-fungal activity of macrophages (Caffrey et al., 2015). TNF is primarily secreted by myeloid cells, such as alveolar macrophages, dendritic cells, infiltrating monocytes and monocyte-derived dendritic cells, macrophages and neutrophils. TNF is highly induced in myeloid cells and in *in vivo* and *in vitro Aspergillus* antigen co-culture experiments (Brieland et al., 2001; Mehrad et al., 1999b; Schelenz et al., 1999). Neutralization of TNF results in an impaired *A. fumigatus* elimination and an elevated mortality, which is also linked with decreased levels of pulmonary chemokines, such as CXCL1, CXCL2, MCP-1, MIP-1, which leads to less neutrophil infiltration and fungal clearance (Brieland et al., 2001). Various other pro-inflammatory cytokines, such as IL-6, MCP-1 and IFNγ, have been described as vital to eliminate pulmonary *A. fumigatus* infections (Blease et al., 2001; Cenci et al., 2001). Immune cell recruitment is a complex process, which begins with the interaction of circulating immune cells and endothelial surface adhesion molecules. This is then followed by the rolling and adherence of immune cells, which leads to the extravasation of the immune cells into the extravascular space and finally to directional migration to the site of infection. *A. fumigatus* hyphae have been shown to induce the generation of E-selectin and VCAM-1in endothelial cells in both *in vitro* and in IPA murine models (Chiang et al., 2008). Many chemokine ligands and their receptors have been involved in the recruitment of innate immune cells and their anti-*A. fumigatus* defence functions: for instance, CXC chemokine ligands (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8) are critical for the recruitment of leukocytes to the infection site and, in humans, these ligands signal via two receptors, CXCR1 and CXCR2. Mouse chemokine ligands CXCL1, CXCL2, CXCL5, CXCL6 and CXCL16 signal via the CXCR2 receptor (Bonnett et al., 2006).

1.2.3 Immune cell interactions with *A. fumigatus*

The airway epithelial lining is the first contact for *A. fumigatus* following conidial inhalation and it initiates the first immune responses (Paris et al., 1997). Alveolar epithelial cells produce an array of antimicrobial peptides such as lactoferrin, chitinase, and β-defensins that have been shown to be involved in the defence against *A. fumigatus* (Alekseeva et al., 2009; Balloy and Chignard, 2009). The respiratory airway and alveolus is lined by type I and type II epithelial cells. The type-II alveolar epithelial cells and endothelial cells can internalize conidia; however, the phagocytic capacity of these cells is reduced when compared to professional phagocytes, such as macrophages and neutrophils. Moreover, epithelial cells are less efficient in eliminating *A. fumigatus* conidia (Filler and Sheppard, 2006; Wasylnka and Moore, 2003). Epithelial cells have been shown to express recognition receptors, such as C-type Lectin Receptors (CLRs) and Toll Like Receptors (TLRs), and the Dectin-1 receptor plays an important role in *A. fumigatus* recognition and the induction of inflammatory cytokines and chemokines (Sun et al., 2012). Alveolar macrophages are the key pulmonary resident leukocytes which provide the efficient first line of defence against inhaled *A. fumigatus* conidia that have entered the lung alveoli (Schaffner et al., 1982). Alveolar macrophages exhibit an impressive array of recognition receptors, phagocytic capacity and cytokine production, which helps in the elimination of resting conidia and prohibits the initial spread of fungal growth (Park and Mehrad, 2009). The recognition of *A. fumigatus* conidia by alveolar macrophages results in phagocytosis and elimination of the conidia through two mechanisms: ROS generation and phagosomal acidification (Ibrahim-Granet et al., 2003; Philippe et al., 2003). Furthermore, recognition of conidia by alveolar macrophages also induces the expression of several chemokines and cytokines which helps in the recruitment of other immune cells (Bhatia et al., 2011). Alveolar macrophages can only clear fungal conidia at lower concentrations. In order to eliminate the high fungal load, other immune cells have to be recruited to the site of infection (Balloy and

Chignard, 2009). Neutrophils are the first non-resident immune cells that are recruited to the infected lungs and they exhibit various antifungal mechanisms, such as phagocytosis, ROS production, degranulation and NET formation (Branzk et al., 2014; Park and Mehrad, 2009). Defects in neutrophil numbers or in their function serves as a major risk factor for invasive aspergillosis (Branzk et al., 2014). To escape from the neutrophil antifungal defence mechanisms, *A. fumigatus* produces toxic compounds, such as gliotoxin and fumagillin, that affects neutrophil antifungal function by prohibiting the formation of a functional NADPH oxidase, which is required for ROS production (Fallon et al., 2010; Tsunawaki et al., 2004). Circulating inflammatory monocytes ($CD11b^{+}Ly6G^{high}$), which exit the bone marrow in a CCR2-dependent manner after detecting the infection, are the precursors for the formation of monocyte-derived macrophages and dendritic cells (Geissmann et al., 2010). Inflammatory monocytes are very important in the fight against *A. fumigatus* infection as they are capable of engulfing and killing conidia and damaging the fungal hyphae. However, the role of inflammatory monocytes and their derived cells in eliminating fungal infection is still not fully defined (Espinosa and Rivera, 2016). Natural killer (NK) cells are known for their cytotoxic effects against cancer cells and virus-infected cells (Smyth et al., 2005). NK cells produce cytokines and chemokines, such as IFN-γ and TNF-α, upon infection (Lanier, 2005). They also exhibit cytolytic effects via the perforin–granzyme, Fas ligand and TRAIL pathways (Smyth et al., 2005). NK cells are important in the fight against *A. fumigatus* infections and the anti-fungal defence is mediated by IFN-γ release (Bouzani et al., 2011). Unstimulated and IL-2-stimulated NK cells kill *A. fumigatus* hyphae but not the resting conidia (Schmidt et al., 2011). Recently, it has been reported that eosinophils also play an important role in the elimination of *A. fumigatus*. Eosinophil-depleted mice exhibited deficiencies in *A. fumigatus* clearance and an increase in fungal burden mediated by the impaired production of cytokines and chemokines IL-6, IL-17A, GM-CSF, IL-1b, and CXCL1 (Lilly et al., 2014). Dendritic cells are important immune effector cells which bridge the immune responses between the innate and adaptive immune systems

and play a crucial role against *A. fumigatus* lung infections (Bhatia et al., 2011). Dendritic cells exhibit various antifungal defence functions: they can recognize *A. fumigatus* through Dectin-1, DC-SIGN, CR3 and FcgRIII recognition receptors and they can subsequently phagocytose conidia. Dendritic cells also produce cytokines (TNFα, IL-6, IL-12, IL-1α, and IL-1β) in *A. fumigatus* lung infections (Bozza et al., 2002). There are different subtypes of dendritic cells in the lung, namely conventional dendritic cells, plasmacytoid dendritic cells and monocyte-derived dendritic cells (Margalit and Kavanagh, 2015a) and each subtype of dendritic cells displays distinct interactions with *A. fumigatus* (Lother et al., 2014). Dendritic cells initiate the adaptive immune responses to *A. fumigatus* and shape the T cell-mediated immunity against *A. fumigatus* (Bozza et al., 2002). $CD4^+$ and $CD8^+$ T-cells also play essential roles in the control of fungal infections; the antifungal defence mechanisms include direct anti-fungal activity (Levitz et al., 1995), the release of antimicrobial peptides (Ma et al., 2002) and the lysis of fungus-containing phagocytes (Huffnagle et al., 1991). Cytotoxic T cells engineered to express Dectin-1 chimeric antigen receptor bind to β-glucan on *A. fumigatus* germlings and lead to the damage and the inhibition of hyphae growth *in vitro* and *in vivo* (Kumaresan et al., 2014). Increasing evidence suggests that platelets are also involved in anti-*A. fumigatus* defence. Human platelets adhere to fungal hyphae and conidia; however, they unable to phagocytose *A. fumigatus* spores (Christin et al., 1998). On the other hand, *A. fumigatus*-derived serine proteases and gliotoxin activates the platelets in a contact-independent manner (Speth et al., 2013).

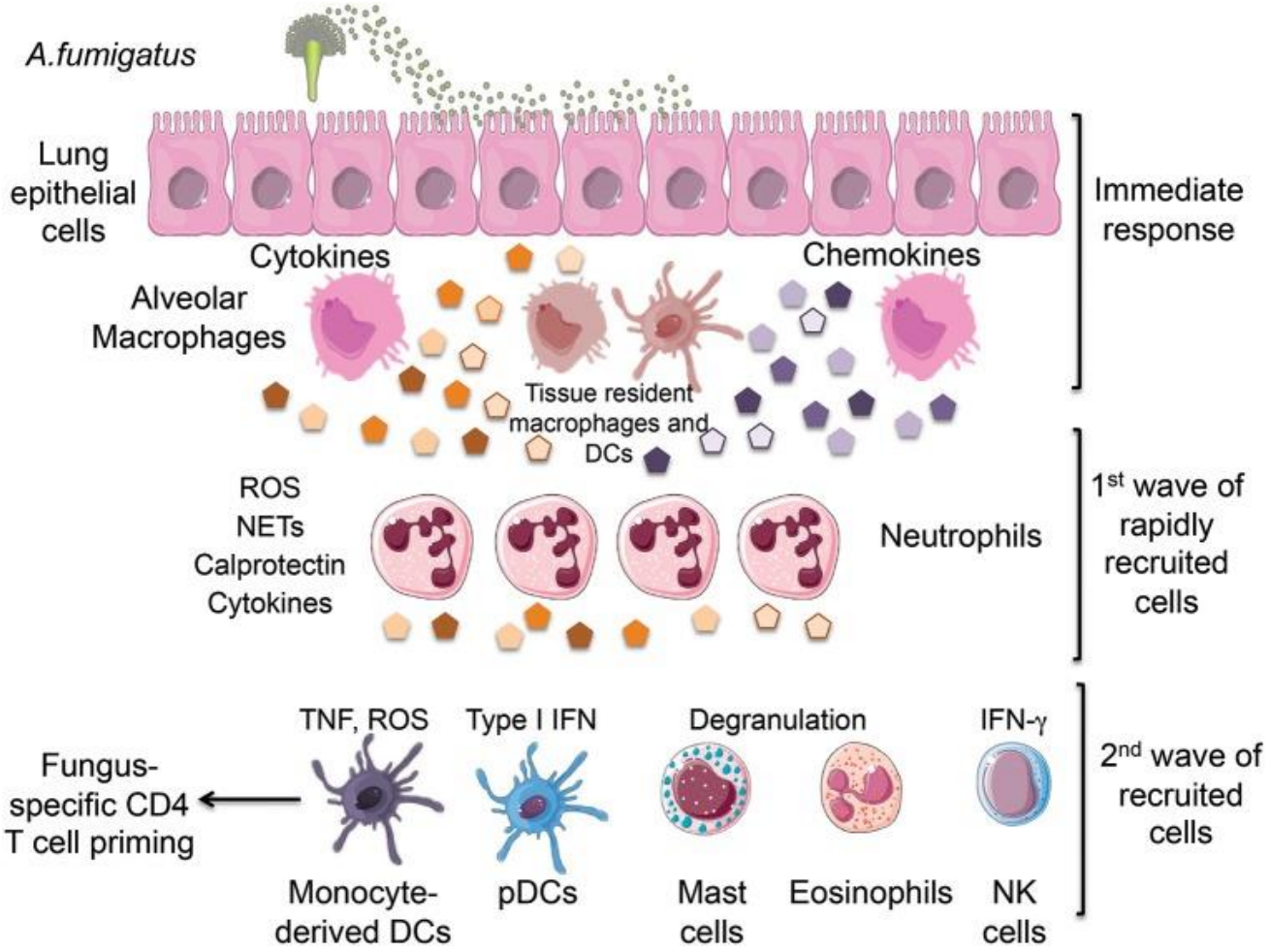

Figure. (ii) Host immune response against *A. fumigatus* lung infection. Lung epithelial cells, tissue-resident alveolar macrophages and dendritic cells initially recognise *A. fumigatus* resting conidia and initiate the production of chemokines that promote the recruitment of neutrophils. Neutrophils then also release cytokines, which support the subsequent recruitment of monocytes, pDCs, mast cells, eosinophils and NK cells. Resident and recruited immune cells use an array of immune effector mechanisms to eliminate fungal spores and provide resistance towards *A. fumigatus* lung infections. Figure adopted from Espinosa and Rivera, 2016, *Frontiers in Microbiology* (Espinosa and Rivera, 2016).

1.3 Immunocompromised patients and mouse models to study *A. fumigatus* lung infections

Healthy individuals efficiently eliminate *A. fumigatus* infection despite a continuous exposure to fungal spores (Garcia-Vidal et al., 2013) without signs of antibody- or cell-mediated adaptive immune response or symptoms attributable to *A. fumigatus* inhalation (Park and Mehrad, 2009). A steadily increasing population of immunocompromised patients is at greater risk and experiences life-threatening invasive infections by *A. fumigatus*. Although several antifungal drugs have become available to combat *A. fumigatus* infections, the mortality of this devastating disease remains as high as 90% in immunocompromised patients (Dagenais and Keller, 2009). Efforts to improve the management and the treatment of *A. fumigatus* lung infections are mostly focused on the identification of new antifungal drug targets and compounds (Segal et al., 2006). However, it is essential to develop therapies that improve the host immune defence in immunocompromised patients. To this end, an in-depth understanding of the dynamic host immune responses against *A. fumigatus* lung infections under immunocompromised conditions is a prerequisite for the successful application of novel therapeutic strategies to effectively manage and treat lung infections in high-risk immunocompromised patients. Due to various clinical therapies, patient numbers requiring the administration of immunosuppressive drugs are constantly increasing. The most commonly used immunosuppressive drugs in clinical situations with various conditions are cyclophosphamide and corticosteroids (Barnes, 2006; Emadi et al., 2009; Shaikh et al., 2012). Cyclophosphamide is a widely used antineoplastic drug and potent immunosuppressive agent used in the treatment of a wide range of diseases, such as solid tumours, hematologic malignancies, autoimmune disorders, and is used as a conditioning regimen for stem cell mobilization and hematopoietic cell transplantation (Emadi et al., 2009). Corticosteroids have been proven to be the most effective anti-inflammatory treatment for asthma and for a number of other inflammatory and immune diseases (Barnes, 2006). Some

clinical therapies also use a combination of cyclophosphamide and corticosteroids (Thone et al., 2008). The differences in *A. fumigatus* infection and inflammatory response in corticosteroid and chemotherapeutic models of invasive aspergillosis have been previously described; however, limited immune cell types and cytokines following infection have been evaluated in bronchoalveolar lavage but not in the entire lung (Balloy et al., 2005). Despite this widespread clinical use, knowledge remains limited on how these immunosuppressive treatments modulate immune cell recruitment following lethal *A. fumigatus* lung infection.

1.3 Granulocyte transfusions to treat invasive fungal infections

Despite improved antifungal therapeutics, invasive fungal infections remain a major complication in patients with prolonged neutropenia following chemotherapy for malignancies, conditioning regimens for allogeneic hematopoietic stem cell or solid organ transplantation (Dagenais and Keller, 2009; Latge, 1999; Park and Mehrad, 2009). Neutrophilic granulocytes are among the first non-resident immune cells recruited to the site of infection to eliminate the pathogens (Feldmesser, 2006). Neutrophils exhibit various anti-pathogenic mechanisms, such as phagocytosis, the release of anti-microbial compounds via degranulation and the production of cytokines or chemokines in order to recruit other immune cells (Braem et al., 2015; Bruns et al., 2010; Gazendam et al., 2016b). Importantly, neutrophils sense microbe size and selectively release Neutrophil extracellular traps (NETs) against large pathogens (Branzk et al., 2014; Bruns et al., 2010). NETs are large, extracellular web-like filaments that consist of decondensed chromatin decorated with anti-microbial factors (Zawrotniak and Rapala-Kozik, 2013). Large pathogens, such as fungal hyphae or bacterial aggregates, selectively trigger NET formation; NETs trap and kill pathogens, including filamentous fungi (Branzk et al., 2014; Brinkmann et al., 2004; Bruns et al., 2010). NET formation and NET-mediated pathogen elimination requires reactive oxygen species (ROS) production and granule proteins (myeloperoxidase and

neutrophil elastase) (Brinkmann et al., 2004; Gupta et al., 2014). Thus, patients with clinically acquired neutropenia or heritable neutrophilic granulocyte dysfunction or altered neutrophil recruitment to the site of infection or a defect in effector functions of neutrophils are at greater risk from lethal *A. fumigatus* infections.

Neutrophilic granulocyte transfusion is a logical alternate to essential therapy to treat invasive fungal infections in patients with prolonged neutropenia or aplastic anaemia or septic granulomatosis disease and visceral aspergillosis (de Talance et al., 2004; Estcourt et al., 2016). Granulocyte transfusion therapies to combat bacterial or fungal infections have been used for over half a century, since they were promoted in the 1970s (Herzig et al., 1977). However, interest in granulocyte transfusion therapy dropped quickly for multiple reasons: firstly, the introduction of improved antibiotics or antifungals, secondly, transfusion-related adverse effects and lastly, the inconsistent results obtained from granulocyte transfusion therapy related clinical trials (Cugno et al., 2015). These issues led clinicians to believe that granulocyte transfusions to control bacterial or fungal infections in neutropenic patients could provide minute benefit, though the problem of these infections in neutropenic patients is persistent (Vamvakas and Pineda, 1997). Moreover, the inconsistent results of granulocyte transfusion therapy might be, in part, explained by the administration of inadequate doses, as the common dose range from 20-30× 10^9 granulocytes/ transfusion is not sufficient (Huestis and Glasser, 1994; Safdar et al., 2014). The importance of the adequate granulocyte dose arose from early uncontrolled trails in humans, from reconsidering analysis of early controlled trials and from animal studies. Notably, normal production of neutrophils in uninfected individuals is relatively low (1x 10^9/ kg), which leads to a decreased neutrophil yield from healthy donors, and inadequate neutrophil numbers per transfusion minimizes beneficial effects (Cugno et al., 2015; Dale et al., 1998). The profound interest in granulocyte transfusion therapy was further fostered with the introduction of granulocyte colony-stimulating factor and oral corticosteroid administration to donors in order to

increase the neutrophil counts in peripheral blood (Dale and Price, 2009; Di Mario et al., 1997; Price, 2000). However, the evidence for clinical efficacy of high-dose granulocyte transfusion therapy has been elusive for a long time. Recently a multicentre clinical trial (the RING-Resolving Infection in Neutropenia with Granulocytes-study) reported that the success of granulocyte transfusion therapy depends on high doses of granulocytes and they observed no overall benefit of granulocyte transfusion on the primary outcome in patients with invasive infections (Figure. iii) (Price et al., 2015). However, the results of the RING study are not conclusive because of low patient enrolment, which limited the ability to detect a truly beneficial effect following granulocyte transfusion therapy (Drewniak and Kuijpers, 2009; Price et al., 2015).

Overall, granulocyte transfusion therapy appears to be a logical complimentary approach to treat lethal *A. fumigatus* infections, particularly for infected patients that are not responding to conventional antifungal therapies (Estcourt et al., 2016). However, the reasons behind previous inconsistent results of granulocyte transfusion therapy need to be properly investigated. In order to increase the granulocyte yield for transfusion donors are treated with corticosteroids. However, the efficacy of granulocyte transfusion and the functional defence mechanisms of granulocytes collected from corticosteroid-treated donors remain elusive.

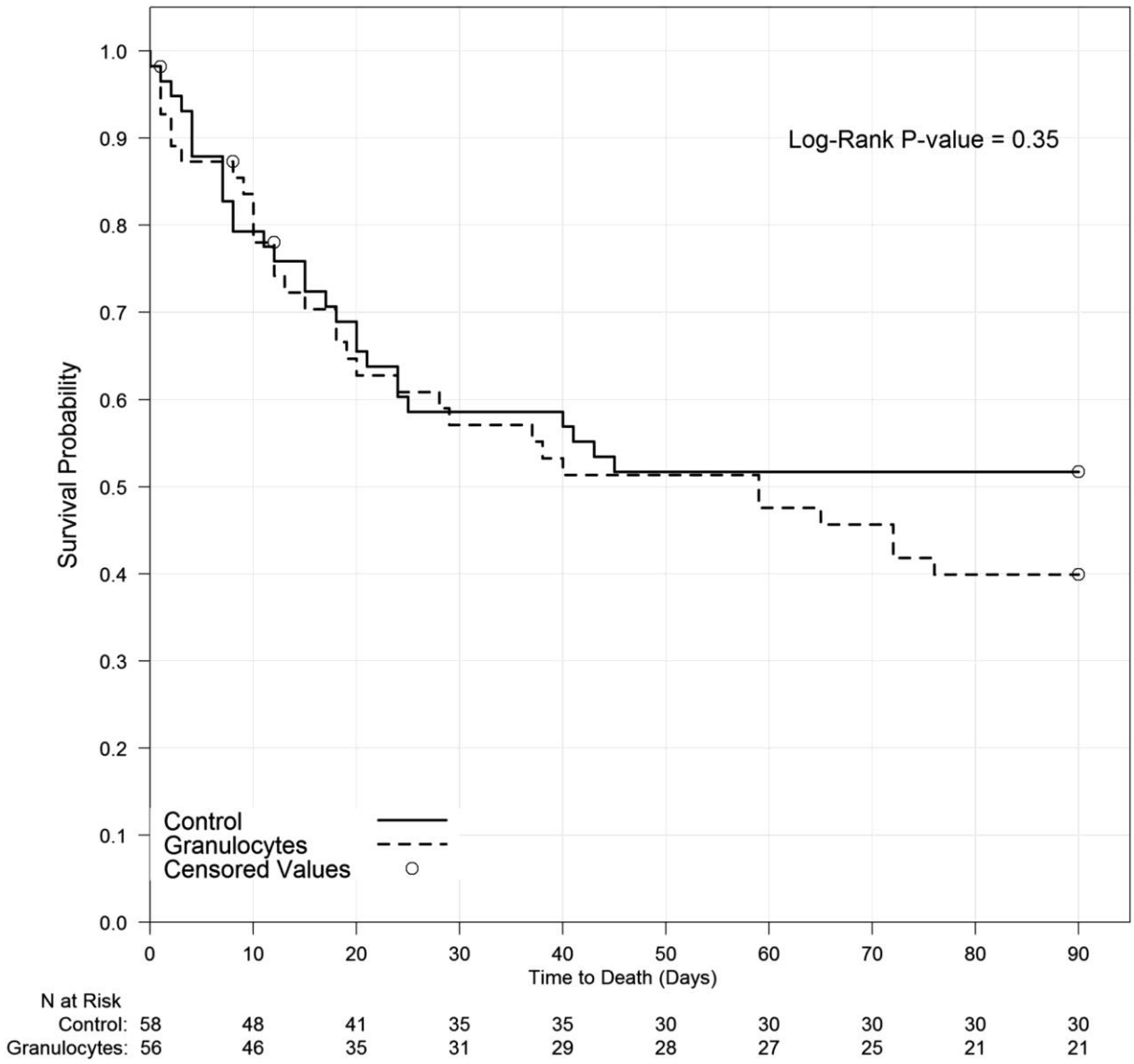

Figure. (iii) Survival in granulocyte transfusion. There is no significant difference between the survival of control group and granulocyte transfused group. Analysed using Kaplan-Meier methodology. Figure adopted from Price et al., 2015.

2 Scope and specific aims of the thesis

Invasive lung infections caused by the pathogenic mold *A. fumigatus* are life threatening complications in immunocompromised patients, for instance following hematopoietic cell- and solid organ transplantation, chemotherapy for cancer or other disease conditions leading to immune suppression. However, the timing and magnitude of host immune cell responses following *A. fumigatus* conidial inhalation, the continuous host defence throughout the different developmental stages of fungi, as well as how different immunosuppressive treatments affect the anti-*A. fumigatus* functions of immune cells remain poorly defined, especially the antifungal efficacy of neutrophilic granulocytes collected from corticosteroid-treated donors and functional anti-*A. fumigatus* defence mechanisms of these granulocytes remain elusive. It is important to address these questions for future development of novel myeloid cell-based immunotherapy in order to combat opportunistic fungal infections.

The aim of this work was to investigate immune cell responses following respiratory fungal challenge with *A. fumigatus* conidia under different immunosuppressive regimens and to study neutrophil functional defence mechanisms in the context of granulocyte transfusions in order to treat invasive fungal infections.

The specific aims of my thesis project were:

1. To determine the timing of *in vivo* host immune cell recruitment following *A. fumigatus* infection

2. To elucidate which immune cells fight against infection under immunosuppression

3.To determine the efficacy of granulocyte transfusion and functional defence mechanisms of neutrophilic granulocytes collected from corticosteroid treated donors.

3 Material and Methods

3.1 Materials

3.1.1 Chemicals

Acetone	Sigma (Deisenhofen, Germany)
D-Luciferin	Biosynth (Staad, Switzerland)
Entellan	Merck (Darmstadt, Germany)
Ethanol	Sigma (Deisenhofen, Germany)
Fetal Calf Serum (FCS)	Invitrogen (Darmstadt, Germany)
Ketamine	Pfizer (Berlin, Germany)
Normal Rat Serum (NRS)	Invitrogen (Darmstadt, Germany)
Tissue-Tek (O.C.T)	Sakura (Staufen, Germany)
Paraformaldehyde	Roth (Karlsruhe, Germany)
Trypan blue	Sigma (Deisenhofen, Germany)
Xylazine 2%	CP-Pharma (Burgdorf, Germany)
Cyclophosphamide	Sigma-Aldrich, Munich, Germany
Hydrocortisone acetate	Sigma-Aldrich, Munich, Germany
Hydrocortisone solution	Sigma-Aldrich, Munich, Germany

3.1.2 Antibodies

All the antibodies used in the study were obtained from Biolegend (Uithoorn, The Netherlands). Antibodies (clones) utilized are listed below: anti-mouse: CD90.2-PE (30-H12), CD4-APC/Cy7 (GK1.5), CD8-APC/Cy7 (53-6.7), CD11b-perCP-Cy5.5 (M1/70), CD11b-PE (M1/70) CD11c-FITC (N418), I-A/I-E-PE/Cy7 (M5/114.15.2), SiglecF-APC (E50-2440), Ly-6G-APC (1A8), FITC-

Ly-6C (HK1.4), Ly6C-PerCP-Cy5.5 (HK1.4), F4/80-APC/Cy7 (BM8), CD49b- PE/Cy7 (DX5), Dectin1-PE (RH1). Anti-human: CD45-APC (H130), CD16-PerCP/Cy5.5 (3G8), CD66b-PE (G10F5), Dectin-1-PE (15E2), anti-luciferase antibody (Abcam, USA), secondary Goat anti-Rabbit IgG, FITC conjugate antibody (Abcam, USA).

3.1.3 *A. fumigatus* strains

The clinical isolate of *A. fumigatus* ATCC46645 strain (Hearn and Mackenzie, 1980) was routinely used in all the infection experiments unless stated. Fluorescent *A. fumigatus* strains Afu-TdTomato (Lother et al., 2014) and Afu-GFP strains generated from ATCC46645 (kindly provided by Dr. Sven Krappmann) were used to determine fungal developmental stages inside lung tissue and phagocytosis assays.

3.1.4 Buffers and solutions

- FACS lysis buffer (10x): NH4Cl (89.9 g), KHCO3 (10 g), EDTA (0.37 g) in 1000 ml distilled water, sterile filtered
- PBS (10x): NaCl (80 g), Na2HPO4-2H2O (14,2 g), KCL (2 g), KH2PO4 (2 g) in 1000 ml distilled water, pH: 6,8
- Stem cell kit buffer: PBS, 0.1% Fetal bovine serum and 1mM EDTA
- PFA 4 %: 4 g PFA in 100 ml (1x) PBS, dissolved at 65 °C, pH: 7.4.
- Anesthetics: 8 ml Ketamine (25 mg/ml, Ketanest, Pfizer Pharma, Berlin, Germany), 2 ml Xylazin (2%) (Rompun, CP-Pharma, Burgdorf, Germany), 15 ml (1x) PBS
- Complete RPMI-1640: RPMI-1640 medium supplemented with 10% FCS, Penicillin (100 U/ml), Streptomycin (100 µg/ml), L-glutamine (2 mM) and β-mercaptoethanol (50 µM) (all Invitrogen, Darmstadt, Germany)
- Hutner's trace elements (1000 x): $ZnSO_4$. $7H_2O$ 2.2 g/100 ml, H_3BO_3 1.1 g/100 ml, $MnCl_2$. $4H_2O$ 0.5 g/100ml, $FeSO_4$. $7H_2O$ 0.5 g/100ml, $CoCl_2$. 6 H_2O 0.16 g/100ml, $CuSO_4$. $5H_2O$ 0.16 g/100ml, $(HH_4)6Mo_7O_{24}$. $4H_2O$ 0.11 g/100ml, Na_4EDTA. $4H_2O$ 6.0 g/100ml

- *Aspergillus* minimal medium: $NaNO_3$ 6 g/l, KH_2PO_4 1.52 g/l, KCl 0.52 g/l, $MgSO_4$ (20% [w/v]) 2.5 ml/l, Glucose (20% [w/v]) 50 ml/l, Hutner's trace elements 1 ml/l, pH 6.3 -6.5 (NaOH/ KOH) and autoclaved.

3.1.5 Commercially available kits

Cellular ROS/Superoxide Detection Assay Kit	Abcam, USA
Cytometric Bead Array	BD Bioscience (Heidelberg, Germany)
Multiplex assay kit	Biolegend, Uithoorn, The Netherlands
Mouse Myeloid cell isolation kit	STEMCELL Technologies, Cologne, Germany
Mouse Neutrophil isolation kit	STEMCELL Technologies, Cologne, Germany
Human Neutrophil isolation kit	STEMCELL Technologies, Cologne, Germany
RNeasy mini kit	Qiagen, Cologne, Germany
CDNA synthesis kit	Bio-Rad, Munich, Germany
Syber green qRT-PCR kit	Bio-Rad, Munich, Germany
Vektashield mounting medium	Vector Laboratories (Burlingame, CA)
LIVE/DEAD violet dead cell stain kit	Invitrogen, Germany
TUNEL staining kit	Roche Diagnostics, Mannheim, Germany

3.1.6 Consumables

6 well flat bottom culture plates	Sarstedt (Newton, USA)
96 well U bottom culture plates	Sarstedt (Newton, USA)
96 well V bottom culture plates	Sarstedt (Newton, USA)
10 µl tips	Sarstedt (Newton, USA)
200 µl tips	Sarstedt (Newton, USA)
1000 µl tips	Sarstedt (Newton, USA) and
15 ml and 50 ml centrifuge tube	Greiner Bio-One (Germany)
Cell strainer 70 µm	BD Biosciences (CA, USA)
Cryomolds	Sakura (Staufen, Germany)
U-100 Insulin Syringes	BD Bioscience (Heidelberg, Germany)
5, 10 and 15 ml Syringes	BD Bioscience (Heidelberg, Germany)

3.1.7 Mice

Inbred BALB/c female mice were purchased from Charles River (Sulzfeld, Germany). Firefly luciferase transgenic BALB/c.L2G85 female mice had been backcrossed from FVB/N.L2G85 mice for more than 12 generations were used in BLI experiments as donors. All the mice were maintained in the pathogen-free animal facility of the Institute for Molecular Infection Biology (IMIB), University of Würzburg, Germany. All experiments were performed with 8-12-week-old female mice. All animal experiments were carried out according to the German guidelines for animal experimentation and institutional ethical approvals. Utmost care was taken to minimize the suffering of mice by *A. fumigatus* lung infections. The responsible authority (Regierung von Unterfranken; Permit Number 55.2-2531.01-86-13) approved the study.

3.2 Methods

3.2.1 Immunosuppressive mouse models to study *A. fumigatus* lung infections

In the cyclophosphamide and corticosteroid treated (CCT) model, mice were intraperitoneally injected with 150 mg kg^{-1} cyclophosphamide (Sigma-Aldrich, Munich, Germany) and subcutaneously (s.c.) with 112 mg kg^{-1} hydrocortisone acetate (Sigma-Aldrich) on days -3 and -1 before *A. fumigatus* infection. In the corticosteroid treated (CT) model, mice were s.c. injected with 112 mg kg^{-1} hydrocortisone acetate on days -3 and -1 before infection. For adoptive cell transfer experiments mice were immunosuppressed with 150 mg kg^{-1} cyclophosphamide (Sigma-Aldrich, Munich, Germany) on days -3 and -1 before transfusion. Mice were s.c. injected with 112 mg kg^{-1} hydrocortisone acetate (Sigma-Aldrich) on days -3 and -1 to enrich corticosteroid treated neutrophils.

3.2.2 A. fumigatus culture conditions and infection strategy

All the fungal strains were cultivated on defined minimal medium (Amich et al., 2013) under standard culture conditions and handled according to German laboratory safety guidelines. Conidia were harvested from sporulating mycelium using the standard saline/ 0.01% tween solution, filtered through cell strainer and finally washed with sterile saline. Required numbers of conidia were resuspended in saline or PBS used for intra-nasal infection. Mice were anesthetized by intraperitoneal injection of ketamine (50 µg/g bodyweight) and xylacine (5 µg/g bodyweight) in 0.1 M PBS in a total volume of 10 µl/g bodyweight and intra-nasally infected with 2.5×10^5 to 1×10^6 conidia (exact dose of conidia were mentioned in each experiment at respective places) suspended in 50 µl saline/ 0.01% tween. All infected mice were monitored carefully according to the standard guidelines; briefly, mice were regularly observed twice a day and carefully monitored for weight loss and disease symptoms.

3.2.3 Preparation of lung single cell suspensions for FACS

Single cell suspensions were prepared from lungs according to the previously described protocol (Stockmann et al., 2010) with some modifications. Briefly, left and right lung lobes were dissected from euthanized mice and finely minced using surgical blades in 6 well tissue culture plates containing RPMI medium (Life Technologies, USA), and then enzymatically digested for 30 min at 37° C in the presence of 2 mg/ ml Collagenase D and 0.1 mg /ml DNase I (Roche, Mannheim, Germany), diluted with PBS + 0.5 % BSA, filtered through a 70 µm cell strainer (Greiner bio-one, Frickenhausen, Germany) and centrifuged at 1200 rpm for 5 min. The lung cell pellet was resuspended in erythrocyte lysis buffer [(168 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylene diamine tetra acetic acid (EDTA)] for 2 min, and immediately diluted with double the volume of PBS and centrifuged. Finally, single cell suspensions were diluted to desired volumes suitable for flow cytometry analyses.

3.2.4 Flow cytometry analysis (FACS analysis)

All FACS experiments were carried out using a BD FACS Canto II (BD Biosciences) and data was recorded using BD FACS Diva software and analyzed using FlowJo software version 8.0 (Tree Star, Ashland, OR, USA). For FACS analysis lung single cell suspension was transferred to a 96 well plate. To block unspecific binding to Fc receptor cells were incubated with NRS (1:20) for 5 min. Then the cells were stained with appropriate fluorochrome labeled antibodies for 30 min in at 4ºC and subsequently centrifuged for 5 min at 1500 rpm. The pellet was resuspended in PBS and analyzed on FACS. To discriminate live/dead cells, they were stained with LIVE/DEAD fixable violet dead cell stain kit (Invitrogen). A maximum of 8 colors was analyzed at a single sample. To compensate for the spillover in the emission spectrums for each fluorochrome, a control cell suspension or antibody capture beads were individually stained with single fluorochrome labeled antibody also used in the multiple staining. This compensation procedure allowed us to calculate and subtract the appropriate overlap to yield the specific signal intensity for each fluorochrome. To set gates in multicolor stained samples the fluorescence minus one (FMO) method was performed. In the FMO gating strategy samples were stained with all fluorochromes, but minus one fluorochrome at a time. All antibodies were titrated for optimal performance before their application.

3.2.5 Immunofluorescence microscopy

Tissue samples were embedded in O.C.T. within cryomolds and cryopreserved at -20°C. Cryo-embedded lung tissues were cut into 8 µm thick sections on a Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany) and mounted onto frosted slides. Slides were air-dried and fixed with acetone at room temperature for 7 min. Slides were counterstained with DAPI and mounted with mounting medium (Vector Laboratories, Peterborough, UK) or stained with hematoxylin and eosin. Luciferase expressing $CD11b^{+}$ myeloid cells were stained with anti-

luciferase antibody (Abcam, USA) and the secondary Goat anti-Rabbit IgG, FITC conjugate antibody (Abcam, USA) according to the manufacturer's instructions. To detect apoptotic cells TUNEL staining was performed using a commercial kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Images were taken using Z1 fluorescence microscope (Carl Zeiss, Gottingen, Germany) and evaluated with Zeiss AxioVision software (Carl Zeiss).

3.2.6 Cytometric Bead Array

Lungs were homogenized in 500 µl PBS using Precellys ceramic kit 1.4 mm in a Precellys 24 homogenizer. Serum was separated from cell debris by 10 min centrifugation at 13000 rpm 4ºC and immediately stored at –80ºC until further use. Cytokine/chemokine concentrations were measured using BD Cytometric Bead Array Kit (BD Pharmingen, Heidelberg, Germany) or Biolegend Multiplex assay kit (Biolegend, Uithoorn, The Netherlands) according to the manufacturer's instructions. Data were analyzed by FCAP Array v2.0 software.

3.2.7 Isolation of CD11b$^+$ myeloid cells and adoptive transfer

Mouse CD11b$^+$ myeloid cells were enriched from bone marrow (flushed from femur and tibia bones with PBS) of healthy untreated or hydrocortisone-treated BALB/c mice, using myeloid cell enrichment kit (STEMCELL Technologies, Cologne, Germany) according to the manufacturer's instructions. Cell purity was confirmed by post-enrichment FACS analysis (>90%) in all the experiments. Enriched cells were adoptively transferred via retro-orbital i.v. injection after mice were anesthetized by intraperitoneal injection of ketamine (50 µg/g bodyweight) and xylacine (5 µg/g bodyweight) in 0.1 M Phosphate-Buffered Saline (PBS) in a total volume of 10 µl/g bodyweight.

3.2.8 Bioluminescence imaging

Ex vivo lung bioluminescence imaging was performed as previously described (Chopra et al., 2015; Chopra et al., 2013). Briefly, mice were anesthetized with an intraperitoneally injected mixture of Ketamine (50 µg/g body weight) and Xylazine (5 µg/ g body weight) in 0.1 M PBS in a total volume of 10 µl/g body weight. Mice were injected with 300 mg/ kg D-luciferin and euthanized after 10 minutes to prepare lungs and immediately subjected to *ex vivo* bioluminescence imaging using IVIS Spectrum imaging system (Perkin-Elmer/Caliper Life Sciences, Mainz, Germany). Images were evaluated using Living Image 4.0 software (Caliper Life Sciences).

3.2.9 Isolation of myeloid cells and neutrophils

Mouse myeloid cells or neutrophils were enriched from bone marrow (flushed from femur and tibia bones with PBS) of healthy or hydrocortisone-treated BALB/c mice, using myeloid cell or neutrophil enrichment kits (STEMCELL Technologies, USA) according to the manufacturer's instructions. Cell purity was confirmed by post-enrichment FACS analysis (>95%) in all the experiments.

3.2.10 Phagocytosis assays

Phagocytosis capacity of neutrophils was determined by using a previously optimized FACS based assay (Lother et al., 2014). Briefly, neutrophils were incubated with conidia or germlings of a Afu-GFP strain in 5 ml round-bottom tubes (BD Falcon, Germany) with MOI = 10 and incubated at 37ºC, 5% CO2 for 3 h. GFP-fluorescence present inside the $Ly6G^+$ neutrophils was measured by flow cytometry. Dead neutrophils were excluded from analysis by light scatter properties. Background phagocytosis was normalized by subtracting GFP-fluorescence of neutrophils cultivated on ice.

3.2.11 Killing assays

Neutrophils and conidia or germlings of *A. fumigatus* ATCC46645 strain were co-cultured with MOI = 10 and incubated at 37ºC, 5% CO_2 for 3 h. Subsequently, co-cultures were treated with 2 µl of a 2.5% Triton-X solution (Merck, Germany) to lyse neutrophils and cell suspensions were plated onto solid minimal medium plates. After 24 to 48 h of incubation at 37º C, colony forming units (CFUs) were counted.

3.2.12 ROS assay

Neutrophils and conidia or germlings of *A. fumigatus* ATCC46645 strain were co-cultured with MOI = 10 and incubated at 37ºC, 5% CO_2 for 40 min. The ROS production by stimulated neutrophils was measured using Cellular ROS/Superoxide Detection Assay Kit, Abcam (cat No: ab139476) according to the manufacturer's instructions.

3.2.13 Scanning electron microscopy

Mouse neutrophils and conidia or hyphae of *A. fumigatus* ATCC46645 strain were co-cultured on cover slips (Hartenstein) with MOI = 10 and incubated at 37º C, 5% CO_2 for 3 h. Subsequently, the co-cultures were fixed in 2% glutaraldehyde in PBS and washed 3 times with PBS. The specimens were then dehydrated in a stepwise protocol by incubation in acetone followed by drying using a critical point dryer (CPD 030; BAL-TEC, Liechten-stein). Dried specimens were coated with 10 nm gold/palladium using a sputter coater (SCD 005; BAL-TEC, Liechtenstein). Images were taken using Zeiss DSM 962 scanning electron microscope (Carl Zeiss, Germany) with the software Scandium (Olympus) at the Division of Electron Microscopy, Biocenter, University of Würzburg.

3.2.14 RNA isolation and qRT-PCR analysis

Granulocytes enriched from corticosteroid treated or untreated control mice were stored in the RNA stabilization reagent (Qiagen, Germany) and were recovered and total RNA was extracted using RNeasy mini kit (Qiagen, Germany) according to manufacturer's instructions. On column DNase I treatment or isolated total RNA was subjected to RNase free-DNase I (Invitrogen, Germany) treatment to remove genomic DNA contamination. For each sample, 1 µg of total RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Biorad, Munich, Germany) according to the manufacturer's guidelines. Transcript levels of respective target genes were determined by qRT–PCR SYBR Green kit (Biorad, Munich, Germany) using CFX connect Real-time PCR system (Biorad, Munich, Germany). Relative quantification of transcripts was carried out by the comparative D cycle threshold method. Mouse GAPDH levels were used as internal control to normalize the abundance of other target gene transcripts. All the qRT-PCR assays were carried out by employing validated primers from GeneCopoeia, USA and annealing temperatures were used according to the manufacturer's instructions.

3.2.15 Statistical analyses

All the measurements were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Graph Pad Prism 6 (Groningen, The Netherlands) software. To compare cell numbers or other parameters between the two different groups the unpaired Mann-Whitney *u*-test or unpaired Student´s-t test was applied. Significant differences are marked as follows: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. To compare survival curves of infected mice, the Log-rank (Mantel-Cox) test was utilized.

4 Results

4.1 Mouse models to study spatiotemporal host immune responses against *A. fumigatus* lung infections

Every human inhale several hundreds of *A. fumigatus* conidia on daily basis, which are efficiently eliminated by innate pulmonary immune responses in healthy individuals. However, patients undergoing immunosuppressive therapy for several clinical reasons are unable to clear these conidia and susceptible to subsequent lung infections. To uncover how immunosuppressive therapy affects pulmonary control of *A. fumigatus* infection, we compared immunocompetent mice with two different immunosuppressed mouse models (**Figure 1A**). Firstly, cyclophosphamide and cortisone treated (CCT) mice and, secondly, corticosteroid treated (CT) mice to investigate pulmonary host immune responses following respiratory *A. fumigatus* infection. We examined different morphotypes of fungal developmental stages in infected lung sections at 4 h, 16 h and 40 h post-infection (p. i.) time points with immunofluorescence microscopy. We observed fungal differentiation from conidia at 4 h p. i. to germlings at 16 h p. i. and hyphae at 40 h p. i. in CT infected lung sections and CCT infected lung sections (**Fig. 1B**). However, we observed elongated filaments (hyphal growth) in CCT mice at 16 h and more clearly at 40 h (**Fig. 1B**) compared to CT infected mice. Strikingly, these results suggested that different numbers or types of immune cells might have been recruited to lungs of CT infected mice to restrict the hyphal growth. Qualitative hematoxylin & eosin staining of lung sections from immunocompetent, CT and CCT mice at 40 h p.i. exhibited different levels of infiltrated lung immune cells. Lung sections from immunocompetent mice revealed a strong pulmonary immune cell infiltration, CT mice showed less infiltration compared to immunocompetent mice, whereas CCT mice showed fewer infiltrating immune cells (**Fig. 1C**).

Next, we infected immunocompetent, CCT and CT mice with *A. fumigatus* conidia to determine their survival after *A. fumigatus* infection. Immunocompetent mice were resistant to infection, whereas CCT mice survived until 4 days p. i. and CT mice survived until 7 days p. i. (**Fig. 1D**). We hypothesized that some immune cells would have been recruited to the infected lungs to fight against infection in these immunocompromised mouse models.

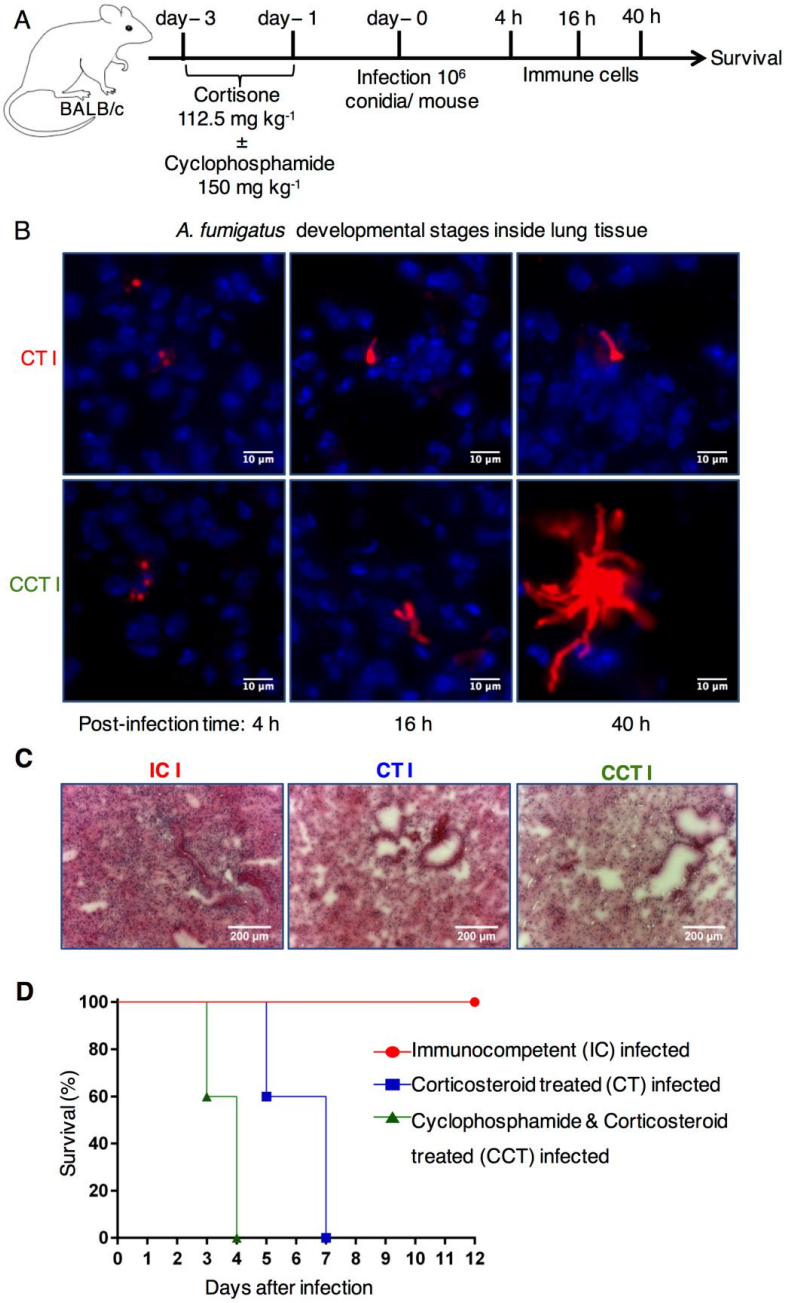

Figure 1. Immunocompromised mouse models to investigate the dynamic host immune response and survival after *A. fumigatus* infection. (A) Experimental setup. BALB/c mice were treated with hydrocortisone (112.5 mg kg^{-1}) on day -3 and day -1 (CT mice) or with cyclophosphamide (150 mg kg^{-1}) and hydrocortisone (112.5 mg kg^{-1}) on day -3 and day -1 before *A. fumigatus* infection (CCT mice). On day 0 mice were intranasally infected with 1×10^6 conidia/mouse. Pulmonary immune cell and cytokine responses were analyzed at 4 h, 16 h and 40 h post infection (p. i.). Survival was followed for 12 days p. i. **(B)** *A. fumigatus* developmental stages inside lung tissue. Immunofluorescence microscopy of lungs from immunosuppressed mice that were infected with Afu-TdTomato conidia at 4 h, 16 h and 40 h p. i. Upper panel CT mice and lower panel CCT mice, *A. fumigatus* in red color and DAPI staining for nuclei in blue color. Scale bar 10 µM. **(C)** Lung immune cell infiltration in IC, CT and CCT infected mice: Lung sections were stained with hematoxylin & eosin at 40 h p.i. and imaged in bright field microscope. Scale bar 200 µM. **(D)** Survival of mice under different immunosuppressive regimens: immunocompetent infected (IC infected), corticosteroid treated and infected (CT infected), and cyclophosphamide and corticosteroid treated and infected (CCT infected); (n=5/ group). Immunocompetent mice (IC) are resistant to infection, whereas CT (P=0.0004) and CCT (P<0.0001) mice succumb to invasive aspergillosis. However, CT mice survive infection significantly longer than CCT mice (P< 0.0001). When mice lost ≥20% weight, they reached an experimental end point and were euthanized according to animal ethics regulations. Log-rank (Mantel-Cox) test was utilized to determine differences in survival. Figure taken from my original publication (Kalleda et al., 2016).

4.2 Neutrophils and macrophages are actively recruited to infected lungs in cyclophosphamide and cortisone treated mice

To determine the timing and magnitude of immune cell recruitment at different stages of *A. fumigatus* infection in immunocompromised CCT mice, we infected them with 1×10^6 *A. fumigatus* conidia intranasally and analyzed defined immune cell populations in the lungs at 4 h, 16 h and 40 h p. i. by flow cytometry (**Fig. 2**). As determined previously, at these selected time points the fungus had evolved through different morphotypes (conidia, germlings and hyphae, respectively) that would likely trigger distinct types of immune responses. All immune populations were strongly reduced in lungs of CCT mice when compared to the immune cells in lungs of immunocompetent mice at steady-state-conditions (**Fig. 3**). Upon infection, myeloid

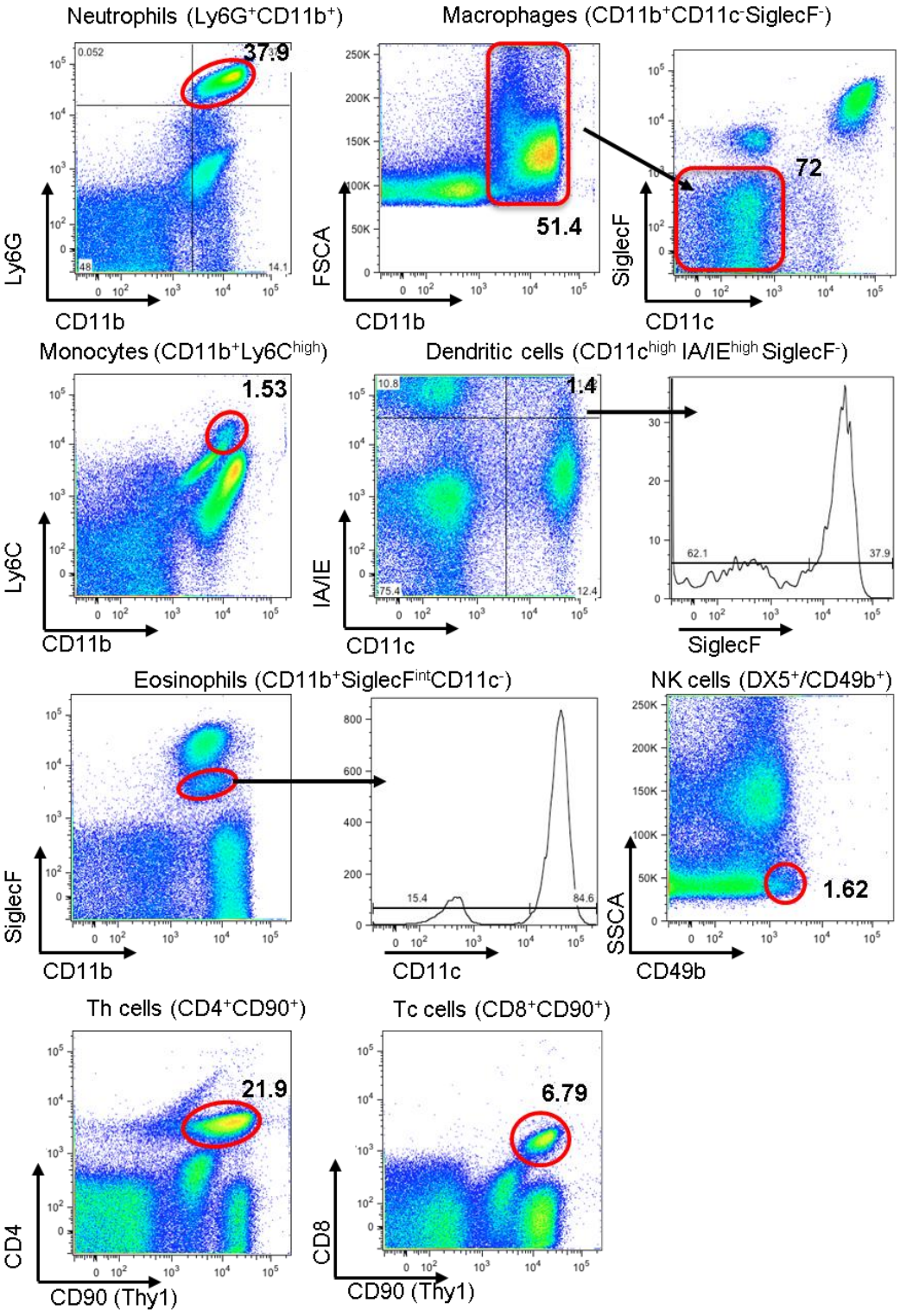
Neutrophils (Ly6G+CD11b+)
37.9
Ly6G
CD11b
Macrophages (CD11b+CD11c-SiglecF-)
51.4
FSCA
CD11b
72
SiglecF
CD11c
Monocytes (CD11b+Ly6Chigh)
1.53
Ly6C
CD11b
Dendritic cells (CD11chigh IA/IEhigh SiglecF-)
1.4
IA/IE
CD11c
62.1
37.9
SiglecF
Eosinophils (CD11b+SiglecFintCD11c-)
SiglecF
CD11b
15.4
84.6
CD11c
NK cells (DX5+/CD49b+)
1.62
SSCA
CD49b
Th cells (CD4+CD90+)
21.9
CD4
CD90 (Thy1)
Tc cells (CD8+CD90+)
6.79
CD8
CD90 (Thy1)

Figure 2. Flow cytometry gating strategy for immune cell populations in the lung. Representative dot plots show distinct immune cell phenotypes based on defined antibody stainings. Figure taken from my original publication (Kalleda et al., 2016).

cells, especially neutrophils (**Fig. 3A**) and macrophages (**Fig. 3B**) were significantly recruited to the lungs of CCT mice at 4 h p. i. However, cell numbers did not surmount numbers of non-infected immunocompetent mice under steady-state-conditions, suggesting that there were not sufficient cells to fight against infection. Despite their low number, these cells were strongly recruited at the 4 h p. i. time point; but not at 16 h and 40 h p. i. (**Fig. 3C, D**). We did not observe recruitment of other myeloid cells particularly monocytes, dendritic cells and eosinophils in CCT mice upon *A. fumigatus* infection (**Fig. 4**). Lymphoid cells, particularly NK cells, $CD4^+$ T cells and $CD8^+$ T cells were strongly reduced in the lungs of CCT mice and were not recruited upon *A. fumigatus* infection (**Fig. 3E, F, and G**), suggesting that lymphoid populations cannot play a pivotal role in the defence against *A. fumigatus* under these immunosuppressive conditions. To investigate underlying factors behind minute immune cell infiltration in CCT mice particularly at 16 h and 40 h p. i., we performed TUNEL staining to observe apoptotic cells **(Fig. 5)**. TUNEL positive cells appeared in CCT mice at 16 h and 40 h p. i., whereas in IC mice no TUNEL positive cells were observed at 40 h p. i. Despite low or reduced recruitment at 16 h and 40 h after infection, fungus growth was not controlled in CCT infected mice. Growing hyphae in the lung tissue might lead to apoptosis of some of the cells in CCT mice even at 40 h after infection, whereas in IC infected mice until 40 h fungus might have been cleared and no apoptotic cells were found in TUNEL staining.

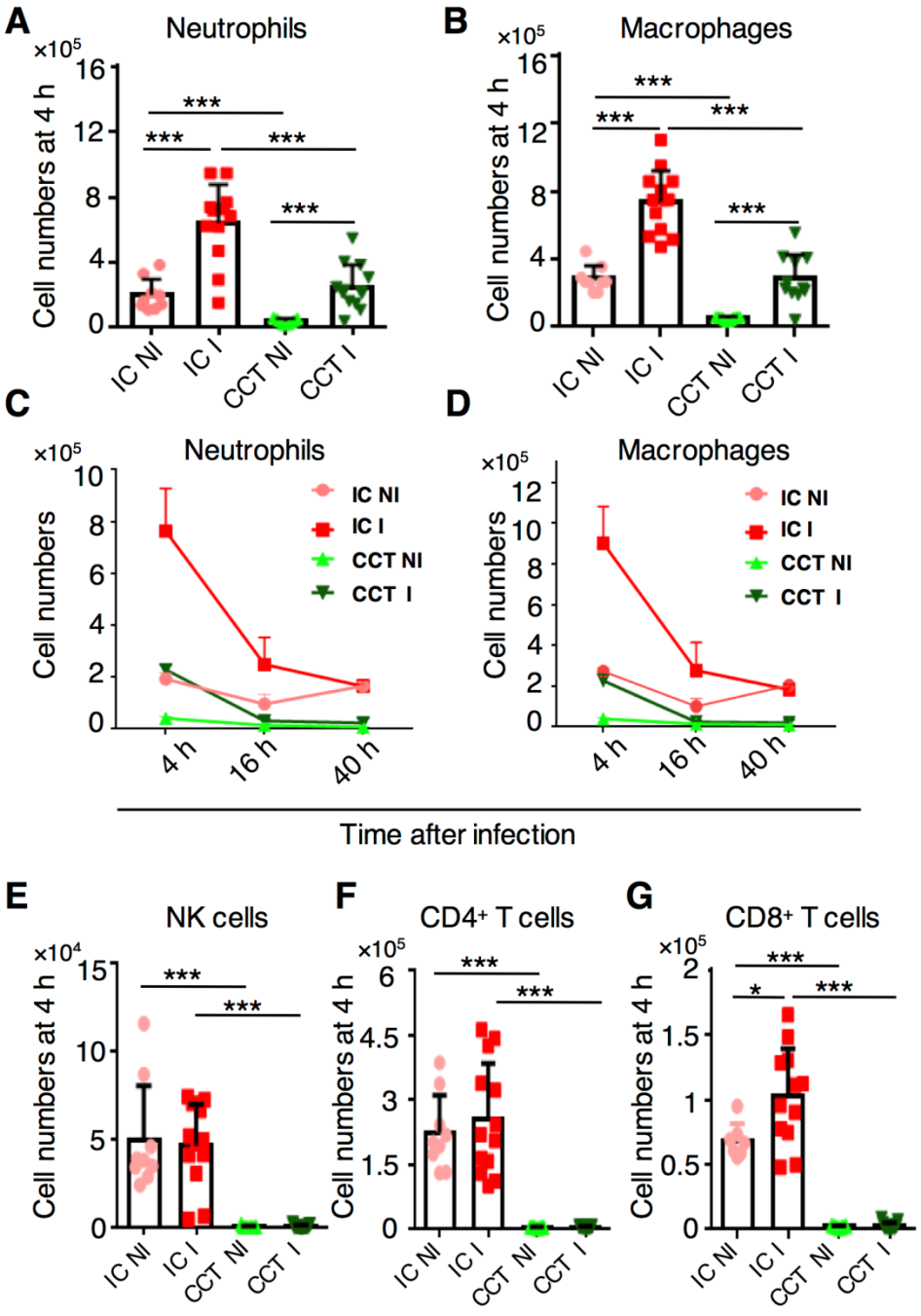

Figure 3. Immune cell response in cyclophosphamide and cortisone treated (CCT) mice after *A. fumigatus* infection. Flow cytometry of lungs from non-infected (NI) or infected (I) with 1×10^6 *A. fumigatus* conidia immunocompetent (IC) and CCT mice at indicated time points, **(A)** *In vivo* lung neutrophil and (**B**) macrophage recruitment 4 h after *A. fumigatus* infection. **(C)** *In vivo* lung neutrophil recruitment 4 h, 16 h, and 40 h after *A. fumigatus* infection. **(D)** *In vivo* lung macrophage recruitment 4 h, 16 h, and 40 h after *A. fumigatus* infection. **(E)** *In*

vivo lung NK cell, **(F)** $CD4^+$ T cell **(G)** $CD8^+$ T cell recruitment 4 h after *A. fumigatus* infection. Data are pooled from three independent experiments with at least n=3/ group of mice in each experiment. Unpaired Mann-Whitney *u*-test was utilized to determine significant differences: * *P*<0.05; ** *P*<0.01; *** *P*<0.001. Figure taken from my original publication (Kalleda et al., 2016).

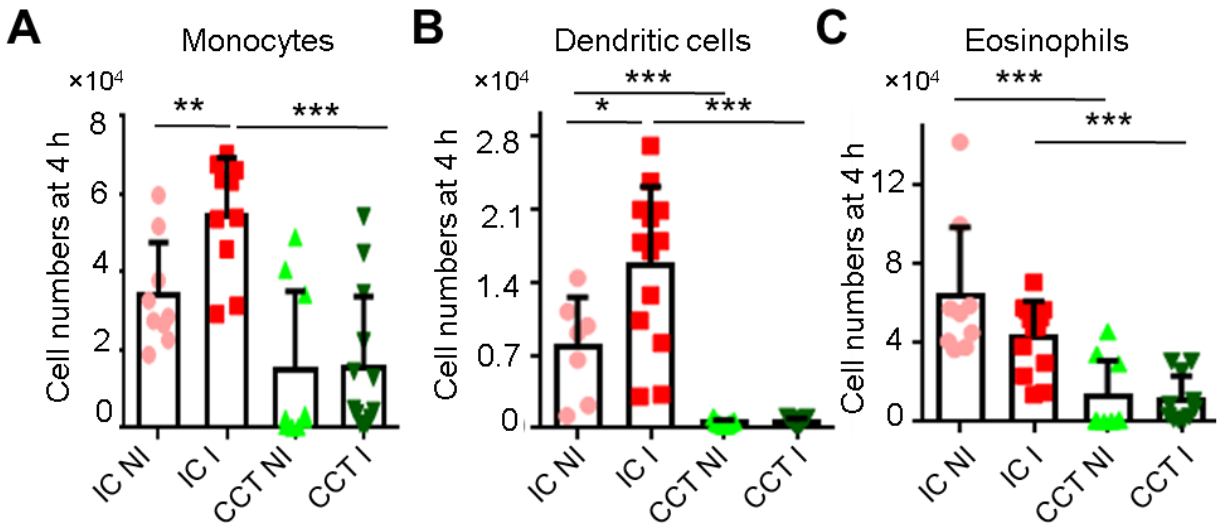

Figure 4. Immune cell response in CCT mice after *A. fumigatus* infection. Flow cytometry of lungs from non-infected (NI) or with 106 *A. fumigatus* conidia infected (I) immunocompetent (IC) and Cyclophosphamide & corticosteroid treated (CCT) mice at indicated time points, (A) In vivo lung monocyte recruitment 4 h after *A. fumigatus* infection. (B) In vivo lung dendritic cell recruitment 4 h after *A. fumigatus* infection. (C) In vivo lung eosinophil recruitment 4 h after *A. fumigatus* infection. Data are pooled from three independent experiments with at least n=3/ group of mice in each experiment. Unpaired Mann-Whitney *u*-test was utilized to determine significant differences: * P<0.05; ** P<0.01; *** P<0.001. Figure taken from my original publication (Kalleda et al., 2016).

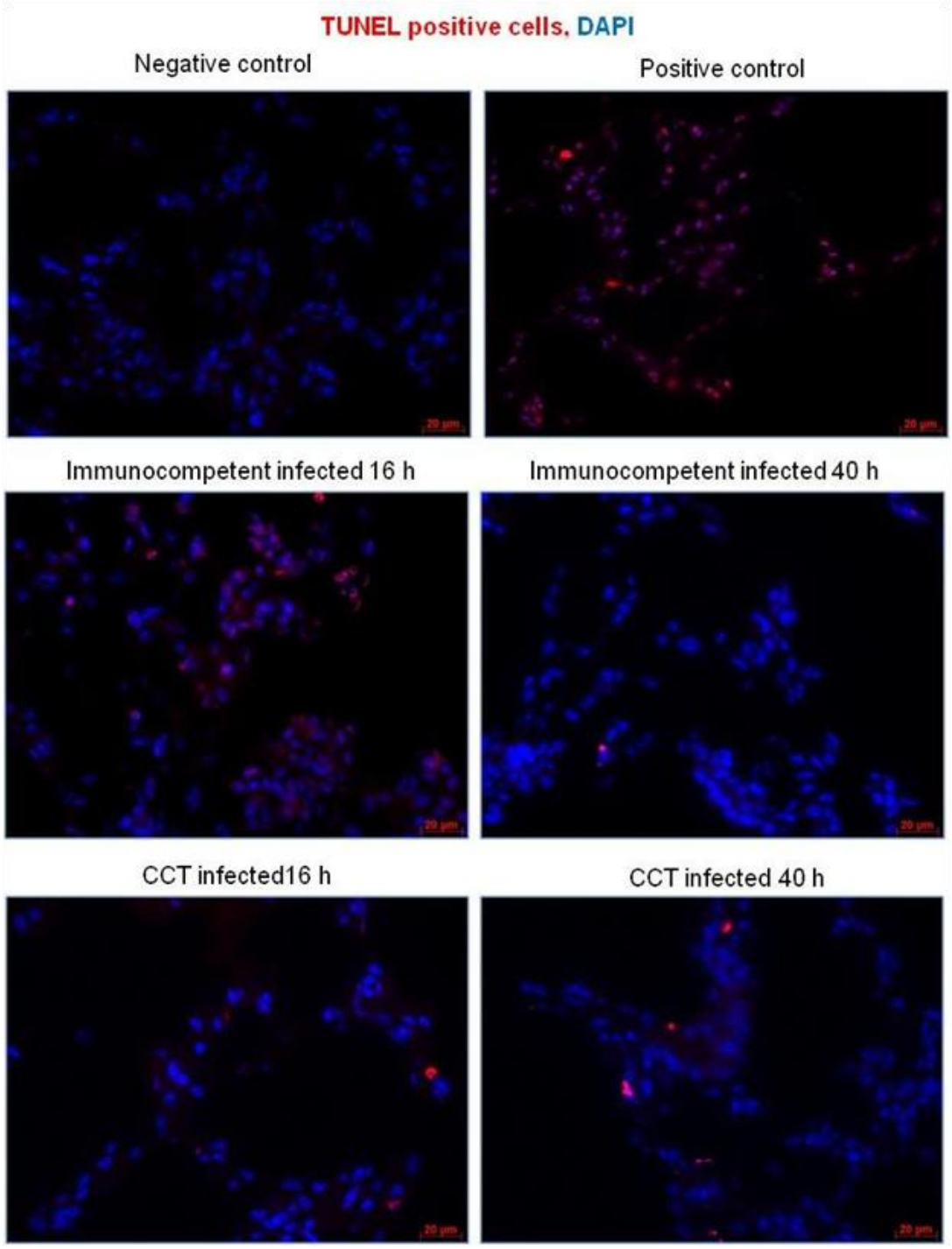

Figure 5. Detection of apoptotic cells by TUNEL staining. Lung sections of immunocompetent (IC) and corticosteroid & cyclophosphamide treated (CCT) mice at 16 h and 40 h after infection were prepared and TUNEL staining was performed using a commercial kit (TUNEL positive cells in red, DAPI staining for nuclei in blue, Scale bar 20μM). Figure taken from my original publication (Kalleda et al., 2016).

4.3 Myeloid cells are strongly recruited to the infected lungs in corticosteroid treated mice

Corticosteroids are widely used immunomodulatory drugs in patients for a variety of clinical conditions (Shaikh et al., 2012). CT mouse models are also employed to determine virulence of *A. fumigatus* mutants (Grahl et al., 2011). The phagocyte recruitment in CT mice after *A. fumigatus* infection had been previously studied (Balloy et al., 2005; Duong et al., 1998); however, the temporal kinetics of this dynamic immune cell response after *A. fumigatus* infection remains poorly defined. To determine the local host immune responses against *A. fumigatus* infection in CT mice, we infected CT mice with *A. fumigatus* conidia and analyzed immune cell recruitment at the above-specified time points of fungal development. Myeloid cells, particularly neutrophils (**Fig. 6A**), macrophages (**Fig. 6B**), dendritic cells (**Fig. 6C**) and monocytes (**Fig. 6D**) were recruited to the lungs of CT infected mice at 4 h p. i. Myeloid cell recruitment to lungs of infected mice was high at 4 h p. i. and low at 16 h and 40 h p. i. (**Fig. 6E, F, G & H**). Lymphoid cells were significantly reduced under these conditions and not recruited upon *A. fumigatus* infection (**Fig. 7**).

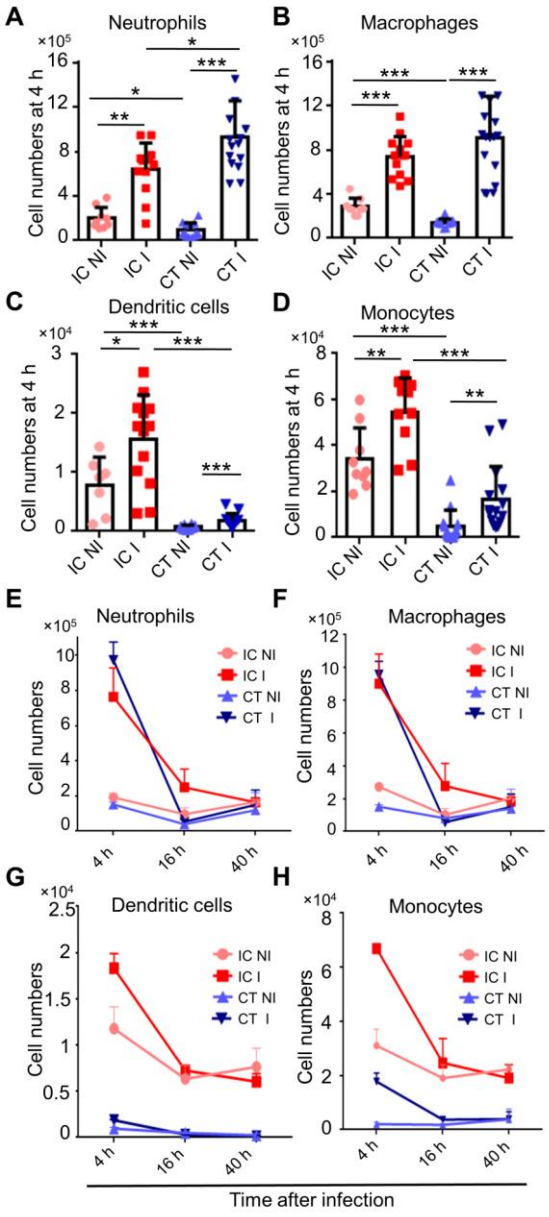

Figure 6. Host immune cell response in corticosteroid treated (CT) mice after *A. fumigatus* infection. Flow cytometric analysis of lungs from immunocompetent (IC) and CT mice non-infected (NI) or infected (I) with 1×10^6 *A. fumigatus* conidia were euthanized at indicated time points. **(A)** *In vivo* neutrophil, (**B**) macrophage, **(C)** dendritic cell and **(D)** monocyte recruitment to the lung at 4 h p. i. **(E-H)** *In vivo* recruitment of immune cells to the lung at 4 h, 16 h, and 40 h. p.i.: **(E)** neutrophils, **(F)** macrophages, **(G)** dendritic cells, and **(H)** monocytes. Data are pooled from three independent experiments with at least n=3/ group of mice in each experiment. Unpaired Mann-Whitney *u*-test was utilized to determine significant differences: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Figure taken from my original publication (Kalleda et al., 2016).

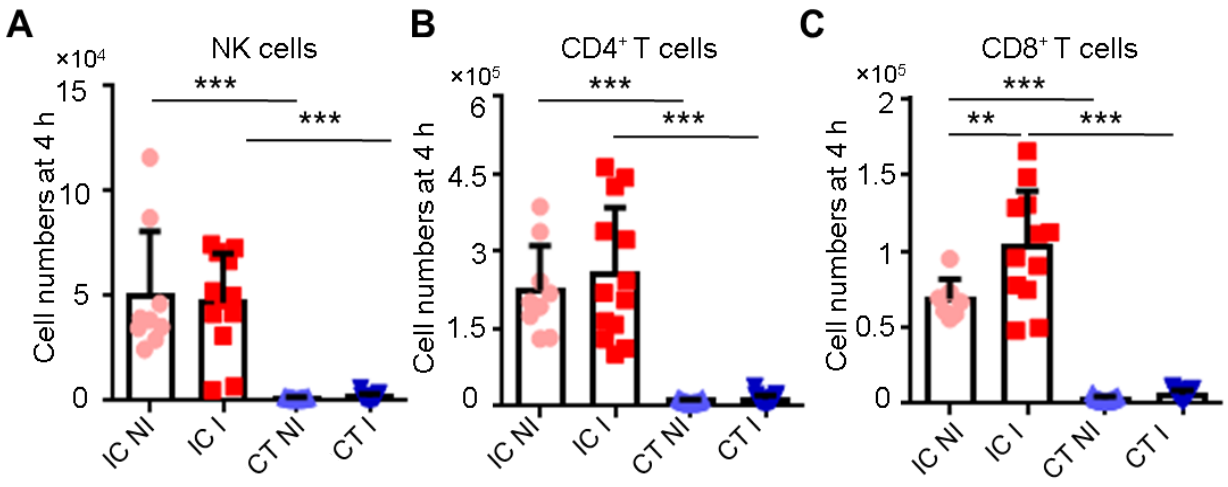

Figure 7. Immune cell response in CT mice after *A. fumigatus* infection. Flow cytometry of lungs from immunocompetent (IC) and corticosteroid treated (CT) mice, either non-infected (NI) or 4 hours after infection (I) with 106 *A. fumigatus* conidia. (A) In vivo lung NK cell (B) In vivo lung CD4+ T cell and (C) In vivo lung CD8+ T cell recruitment 4 h after *A. fumigatus* infection. Data are pooled from three independent experiments with at least n=3/ group of mice in each experiment. Unpaired Mann-Whitney *u*-test was utilized to determine significant differences: * P<0.05; ** P<0.01; *** P<0.001. Figure taken from my original publication (Kalleda et al., 2016).

4.4 Myeloid cell recruitment to infected lungs in corticosteroid treated mice correlates with increase in inflammatory lung cytokine levels

Myeloid cells were strongly recruited to the infected lungs in CT mice. To determine the lung cytokine environment at different time points after *A. fumigatus* infection in CT mice, we measured inflammatory cytokines in lung homogenates of immunocompetent, CT infected and non-infected mice. At 4 h p. i. the amount of the inflammatory cytokines MCP-1, IFN-γ, TNF-α, IL-6 and IL-12 in CT infected mice significantly exceeded cytokine levels in CT non-infected mice (**Fig. 8A**). At 40 h p. i. the levels of lung inflammatory cytokines, except IFN-γ were similar in both CT infected and non-infected mice. However, the amount of the anti-inflammatory cytokine IL-10 was significantly higher in CT infected mice compared to non-infected mice at

both 4 h and 40 h after infection (**Fig. 8B**). In contrast to CT mice, inflammatory or anti-inflammatory cytokines were below detection limits to determine in lungs of CCT mice with or without infection by the multiplex assay. Strikingly, these results suggest that increased inflammatory response in CT mice after infection is accompanied by high lung myeloid cell recruitment to the CT infected lungs.

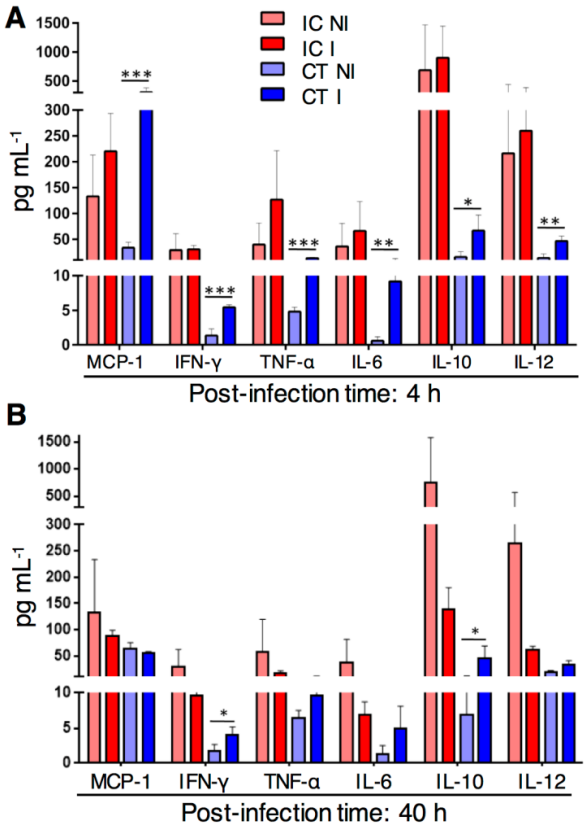

Figure 8. Inflammatory cytokine response in corticosteroid treated (CT) mice after challenge with *A. fumigatus* conidia. Cytometric Bead Array of lung homogenates from non-infected (NI) or with 1×10^6 *A. fumigatus* conidia infected (I) immunocompetent (IC) and CT mice. **(A)** *In vivo* lung cytokine environment at 4 h after *A. fumigatus* infection. **(B)** *In vivo* lung cytokine environment at 40 h p. i. Data are representative of two independent experiments with n=3 mice/group in each experiment. Unpaired Mann-Whitney *u*-test was utilized to determine significant differences: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Figure taken from my original publication (Kalleda et al., 2016).

4.5 $CD11b^+$ myeloid cells rescue cyclophosphamide immunosuppressed mice from lethal *A. fumigatus* infection

Regardless of the immune status of mice, myeloid but not lymphoid cells were recruited to the site of infection. Despite their strongly reduced immune cell numbers, this was also true for the lungs of CCT mice after *A. fumigatus* infection. To determine whether myeloid cells alone can rescue immunosuppressed mice from lethal *A. fumigatus* infection we adoptively transferred $CD11b^+$ myeloid cells into immunosuppressed mice that had been treated with cyclophosphamide (150 mg/ kg) on days -3 and -1 (**Fig. 9A**) alone, since corticosteroid models might interfere with antifungal functions of myeloid cells, as CT infected mice were not resistant to infection irrespective to strong myeloid cell recruitment to the lungs. $CD11b^+$ myeloid cells were enriched from bone marrow of BALB/c donor mice (**Fig. 9B**) and transfused intravenously to cyclophosphamide immunosuppressed (C IS) mice on day 0. This $CD11b^+$ population consisted of $CD11b^+Ly6G^{high}$ neutrophils (70±1%), $CD11b^+Ly6G^{dim}$ cells (5±0.5%), $CD11b^+Ly6G^-Ly6C^+$ monocytes (7±1%) and $CD11b^+Ly6G^-Ly6C^-$ non-differentiated neutrophilic and monocytic precursor cells (18±4%, **Fig. 10**). On day +1 we infected mice with a lethal dose of 2×10^5 *A. fumigatus* conidia and monitored their survival (**Fig. 9C**). C IS mice, which had received an adoptive $CD11b^+$ myeloid cell transfer, were resistant to a lethal infection dose, whereas, immunosuppressed and infected (control) mice were unable to clear the infection and died within 4 days after infection (**Fig. 9C**). To determine whether transfused $CD11b^+$ myeloid cells recruit to the infected lungs and directly impair *A. fumigatus* growth, we performed an adoptive cellular transfer experiment with transgenic firefly luciferase expressing $CD11b^+$ myeloid cells enriched from a BALB/c.L2G85 luciferase reporter mouse (Beilhack et al., 2005; Cao et al., 2004) and infected with TdTomato expressing *A. fumigatus* conidia with the same experimental settings as described for Fig. 9. The transfused $CD11b^+$ cells were detected in C IS infected and not infected lungs 3 days p. i. with ex vivo bioluminescence imaging (Chopra et

al., 2015). Lungs from transfused and infected C IS mice contained many $CD11b^+$ cells, whereas lungs from transfused and not infected C IS mice did not show $CD11b^+$ myeloid cells (**Fig. 9D**). To determine whether recruited $CD11b^+$ myeloid cells interacted with *A. fumigatus*, we performed fluorescence microscopy on C IS transfused and infected lung sections. Luciferase expressing $CD11b^+$ cells were detected in lung sections by staining with anti-Luciferase antibody (Rb p^{Ab} to Firefly Luciferase). Luciferase expressing $CD11b^+$ cells were found in close proximity to *A. fumigatus* and fungal hyphal formation was impaired at 3 days p. i. (**Fig. 9E**). These results indicate that adoptively transferred $CD11b^+$ cells recruit to the infected lungs and support locally the control of *A. fumigatus* fungal growth.

4.6 $CD11b^+$ myeloid cells do not rescue cortisone and cyclophosphamide immunosuppressed mice from lethal *A. fumigatus* infection

In contrast, cortisone and cyclophosphamide immunosuppressed (CC IS) mice, which had received adoptively transferred $CD11b^+$ myeloid cells, could not clear the infection and died within 5 days after infection (**Fig. 11A, 11B**). CC IS mice which had received luciferase expressing $CD11b^+$ myeloid cells showed strong influx of these cells to the lungs upon infection **(Fig. 11C)**. However, these strongly recruited cells failed to control of *A. fumigatus* growth in CC IS infected lungs **(Fig. 11D)**. These striking results indicate that corticosteroid treatment might either have caused tissue damage to recipient mice or affected the protective function of adoptively transferred $CD11b^+$ myeloid cells. However, myeloid cells significantly contributed to the host anti-*A. fumigatus* defence as adoptive transfer of $CD11b^+$ myeloid cells alone rescued cyclophosphamide immunosuppressed mice from lethal *A. fumigatus* infection.

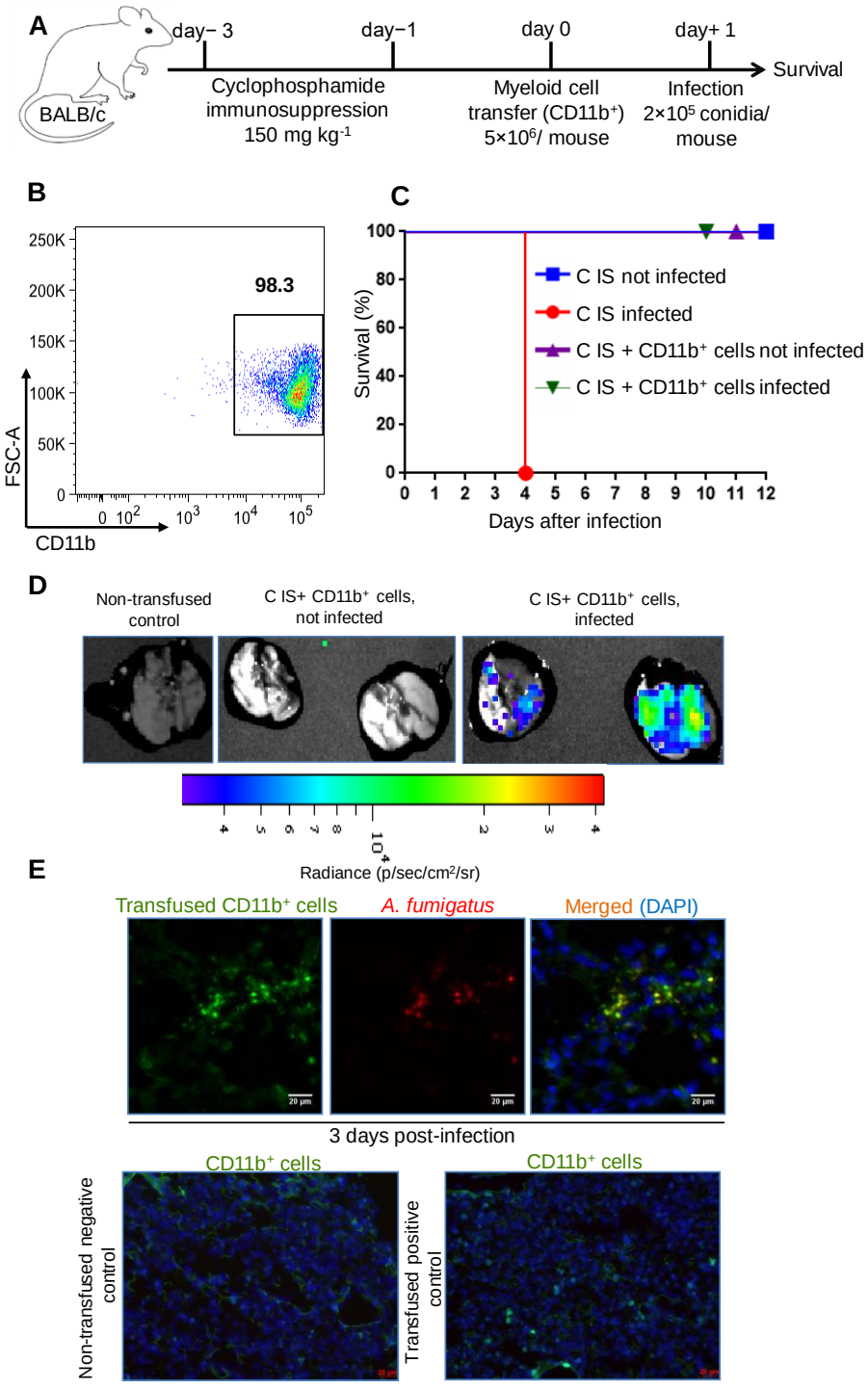

Figure 9. Adoptive CD11b⁺ myeloid cell transfer protects cyclophosphamide immunosupressed mice from lethal *A. fumigatus* infection. (A) Experimental setup for adoptive CD11b⁺ myeloid cell transfer and *A. fumigatus* infection. Mice were immunosuppressed with cyclophosphamide on day -3 and day -1. On day 0, cyclophosphamide immunosuppressed (C IS) mice were injected with 5×10⁶ cells CD11b⁺ myeloid cells/ mouse i.v. Subsequently, mice

were intranasally infected with a lethal dose of *A. fumigatus* conidia (2×10^5 conidia/ mouse). **(B)** Purity of $CD11b^+$ myeloid cells measured with flow cytometry after enrichment from bone marrow of tibia and femur bones. Cell purity always exceeded 95% . **(C)** Survival of C IS mice after *A. fumigatus* infection. C IS mice that had been transfused with $CD11b^+$ myeloid cells completely resist an otherwise lethal *A. fumigatus* infection (*P*=0.0003). All groups n=8. Data are representative of three independent experiments n=8/ group of mice in each experiment. Log-rank (Mantel-Cox) test was utilized to determine survival significance. **(D)** Bioluminescence imaging. $CD11b^+$ myeloid cells were enriched from L2G85 luciferase reporter mice and transfused to C IS mice and infected with TdTomato expressing *A. fumigatus*. *Ex vivo* bioluminescence imaging was performed 3 days p.i. n=2 mice/ group. **(E)** Immunofluorescence microscopy at 3 days p. i. of lungs from C IS mice after transfused with luciferase expressing $CD11b^+$ myeloid cells and infected with Afu-TdTomato conidia. *A. fumigatus* in red, anti-luciferase staining of transfused $CD11b^+$ cells in green and DAPI staining for nuclei in blue color. Scale bar 20 µM. Lower panel shows negative and positive controls for anti-luciferase antibody. Figure taken from my original publication (Kalleda et al., 2016).

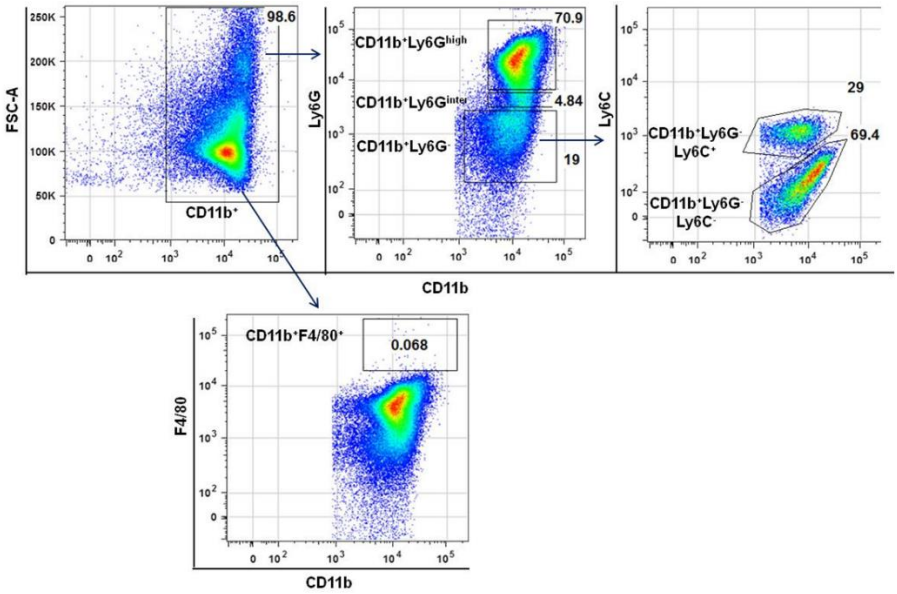

Figure 10. Flow cytometry gating strategy for CD11b+ enriched myeloid cell fraction. CD11b+ cells were enriched from mouse bone marrow using a CD11b isolation kit. Dot plots show distinct immune cell phenotypes (CD11b+Ly6Chigh: monocytes, CD11b+Ly6Ghigh: neutrophils, CD11b+F4/80: macrophages) based on defined antibody stainings. Figure taken from my original publication (Kalleda et al., 2016).

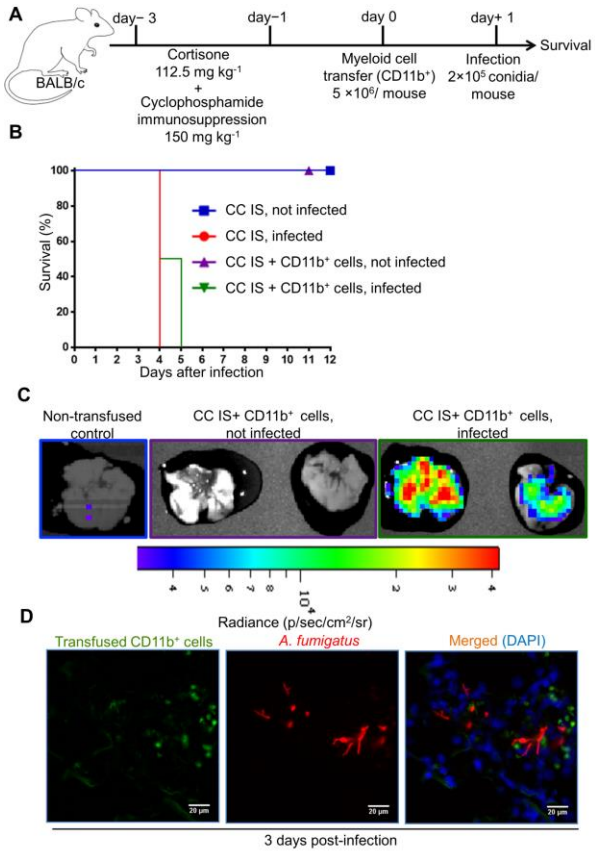

Figure 11. Adoptively transferred CD11b$^+$ myeloid cells do not protect from *A. fumigatus* infection if mice are immunosuppressed with both, cyclophosphamide and corticosteroids. (A) Experimental setup for adoptive CD11b$^+$ myeloid cell transfer and *A. fumigatus* infection. Mice were immunosuppressed with both, cyclophosphamide & cortisone on day -3 and day -1 (CC IS mice). On day 0, CC IS mice received 5×10^6 CD11b$^+$ myeloid cells i.v. and were intranasally infected with a lethal dose of 2×10^5 *A. fumigatus* conidia to determine survival. (B) Survival of mice after adoptive CD11b$^+$ myeloid cell transfer. Adoptive CD11b$^+$ myeloid cell transfer does not protect CC IS mice from lethal *A. fumigatus* infection. No differences deemed significant (Log-rank (Mantel-Cox test)) between infected CC IS mice, and infected CC IS mice that had been transfused with CD11b$^+$ myeloid cells. Data are representative of two independent experiment with n=8/ group of mice in each experiment. **(C)** Bioluminescence imaging. CD11b$^+$ myeloid cells were enriched from L2G85 luciferase reporter mice and transfused into CC IS mice and infected with TdTomato expressing *A. fumigatus*. *Ex vivo* bioluminescence imaging was performed 3 days p.i. n=2 mice/ group. **(D)** Immunofluorescence microscopy of lungs from CC IS mice that had received luciferase expressing CD11b$^+$ myeloid cells and were infected with Afu-TdTomato conidia at 3 days p. i. *A. fumigatus* in red, anti-luciferase staining for transfused CD11b$^+$ cells in green and DAPI staining for nuclei in blue color. Scale bar 20 µM. Figure taken from my original publication (Kalleda et al., 2016).

4.7 Neutrophil anti-*A. fumigatus* defence functions and granulocyte transfusions

When CT mice were infected with *A. fumigatus*, higher numbers of recruited neutrophils were observed in comparison to that of immunocompetent infected mice (**Fig. 6A**). However, CT infected mice were unable to clear the fungal infection and died from severe invasive aspergillosis (**Fig. 1D**). This prompted us to further investigate the effects of corticosteroids on neutrophil anti-*A. fumigatus* defence functions. Neutrophils exhibit various anti-fungal defence mechanisms such as phagocytosis (**Fig. 12**), release of anti-microbial compounds via degranulation and produce cytokines or chemokines to recruit other immune cells (Kolaczkowska and Kubes, 2013). Importantly, neutrophils sense microbe size and selectively release NETs (**Fig. 12**) against large fungal pathogens (Branzk et al., 2014). Patients with clinically acquired neutropenia or heritable neutrophilic granulocyte dysfunction or altered neutrophil recruitment to the site of infection or defect in effector functions of neutrophils are at greater risk by lethal *A. fumigatus* infections. Therefore, granulocyte transfusions have been tested as an alternative therapy for the management of high-risk neutropenic patients with invasive *A. fumigatus* infections. To increase the granulocyte yield for transfusion, donors are treated with oral corticosteroids (Price et al., 2015). Yet, the efficacy of granulocyte transfusion and functional defence mechanisms of granulocytes collected from corticosteroid treated donors remain largely elusive. In my thesis project, we aimed at assessing the efficacy of granulocyte transfusion and functional defence mechanisms of corticosteroid treated granulocytes using mouse models. To determine the effects of corticosteroids on granulocytes to control *A. fumigatus* infections, we performed *in vitro* human and mouse granulocyte and *A. fumigatus* co-culture experiments and granulocyte adoptive cell transfers in *in vivo* mouse models. Fluorescence and electron microscopy, flow cytometry, cytokine analysis assisted our analyses.

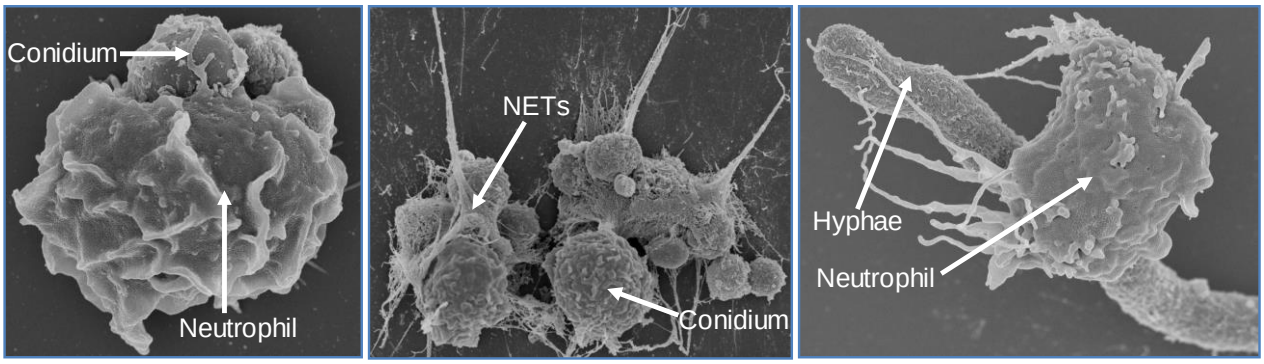

Figure. 12 Anti-*A. fumigatus* defence mechanisms of neutrophils. Scanning electron micrographs of neutrophils interacting with *A. fumigatus*. Neutrophils can phagocytose conidia, produce NETs and attack hyphae.

4.8 Granulocytes from corticosteroid treated donor do not protect cyclophosphamide immunosuppressed mice against *A. fumigatus* infection

To determine the anti-*A. fumigatus* efficacy of granulocytes collected from CT donor mice, we adoptively transfused granulocytes isolated from corticosteroid treated and untreated mice into cyclophosphamide immunosuppressed (C IS) mice (150 mg/ kg cyclophosphamide on day- 3 and day- 1). On day+ 0 we infected C IS mice with a lethal dose of 2x 10^5 *A. fumigatus* conidia (**Fig. 13A**). 15 min after infection we intravenously injected enriched granulocytes from corticosteroid treated or untreated mice (**Fig. 13B**) into C IS mice and monitored their survival (**Fig. 13C**). Transfusion of granulocytes from corticosteroid untreated mice protected most C IS mice from lethal *A. fumigatus* infection; whereas transferred corticosteroid treated granulocytes could not confer resistance (**Fig. 13C**). These results indicate that corticosteroids compromise granulocytes in fighting against lethal *A. fumigatus* infection *in vivo*.

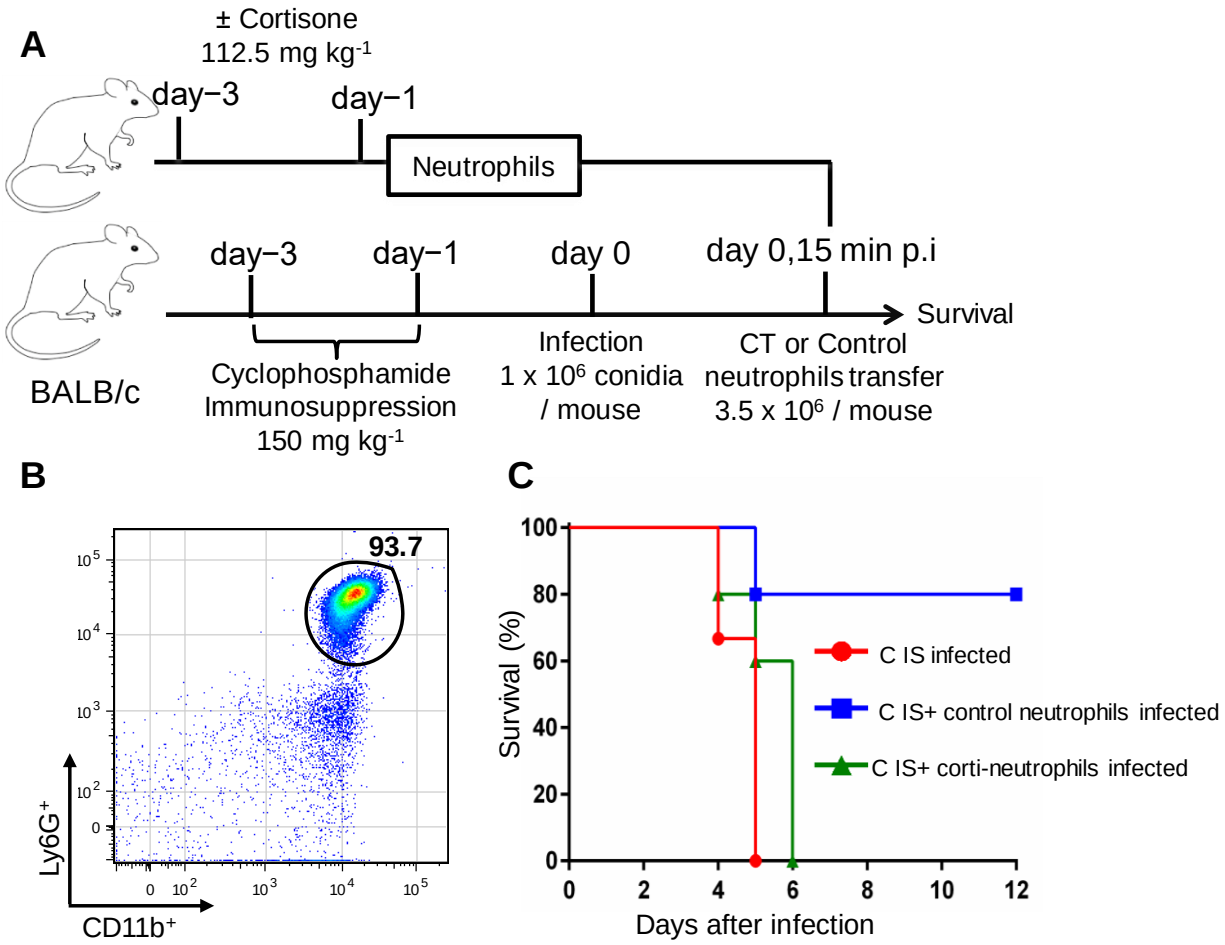

Figure 13. Granulocyte transfusions from corticosteroid treated donors do not protect cyclophosphamide immunosuppressed (C IS) mice against *A. fumigatus* infection. (A) Scheme of experimental setup for granulocyte transfusions and *A. fumigatus* infection. Mice were immunosuppressed with cyclophosphamide (C IS) on day-3 and day-1, and intranasally administered with 1x 10^6 conidia/ mouse on day-0. Granulocytes from control or corticosteroid treated mice were enriched, and 3.5x 10^6 cells/ mouse were transfused on day-0, 15 min after infection and survival was determined. **(B)** Purity of isolated granulocytes was checked with flow cytometry. For all samples, cell purity exceeded 95% **(C)** Most mice that had received granulocytes enriched from control donors recovered resistance such that 80% survived after infection. In contrast, mice that had received granulocytes enriched from corticosteroid treated donors remained as susceptible as not transferred mice and succumbed very rapidly to invasive aspergillosis, *P=0.0157*. Data are representative of two independent experiment with n=8/ group of mice in each experiment. To compare survival curves of infected mice, the Log-rank (Mantel-Cox) test was utilized.

4.9 Migration of granulocytes from corticosteroid treated donors is not impaired to the infected lungs in cyclophosphamide immunosuppressed mice

Neutrophilic granulocytes collected from CT mice did not protect C IS mice against lethal *A. fumigatus* infection. Neutrophilic granulocyte recruitment to lungs after *A. fumigatus* infection is a pivotal step in eliminating infection (Kolaczkowska and Kubes, 2013). In order to study the effect of corticosteroids on neutrophilic granulocyte recruitment to the site of infection (lungs) after *A. fumigatus* challenge we used two approaches; first we analyzed granulocyte recruitment 4 h post granulocyte transfusions. Granulocyte recruitment to the site of infection was similar in C IS infected mice which had received granulocytes collected from control or CT donors (**Fig. 14A)**. Secondly, we employed CT murine model of invasive aspergillosis. To determine the early local host neutrophilic granulocyte recruitment against *A. fumigatus* infection in CT mice, we infected CT mice with *A. fumigatus* conidia and analyzed neutrophil recruitment at 4 h post infection (p. i). by immunofluorescence staining of lung sections from corticosteroid untreated controls, CT infected and non-infected mice (**Fig. 14B**). Significantly more neutrophils were recruited to lungs in CT infected mice than in corticosteroid untreated control infected mice (**Fig. 14C**). These results indicate that corticosteroids are not interfering with the recruitment of granulocytes to the infected lungs.

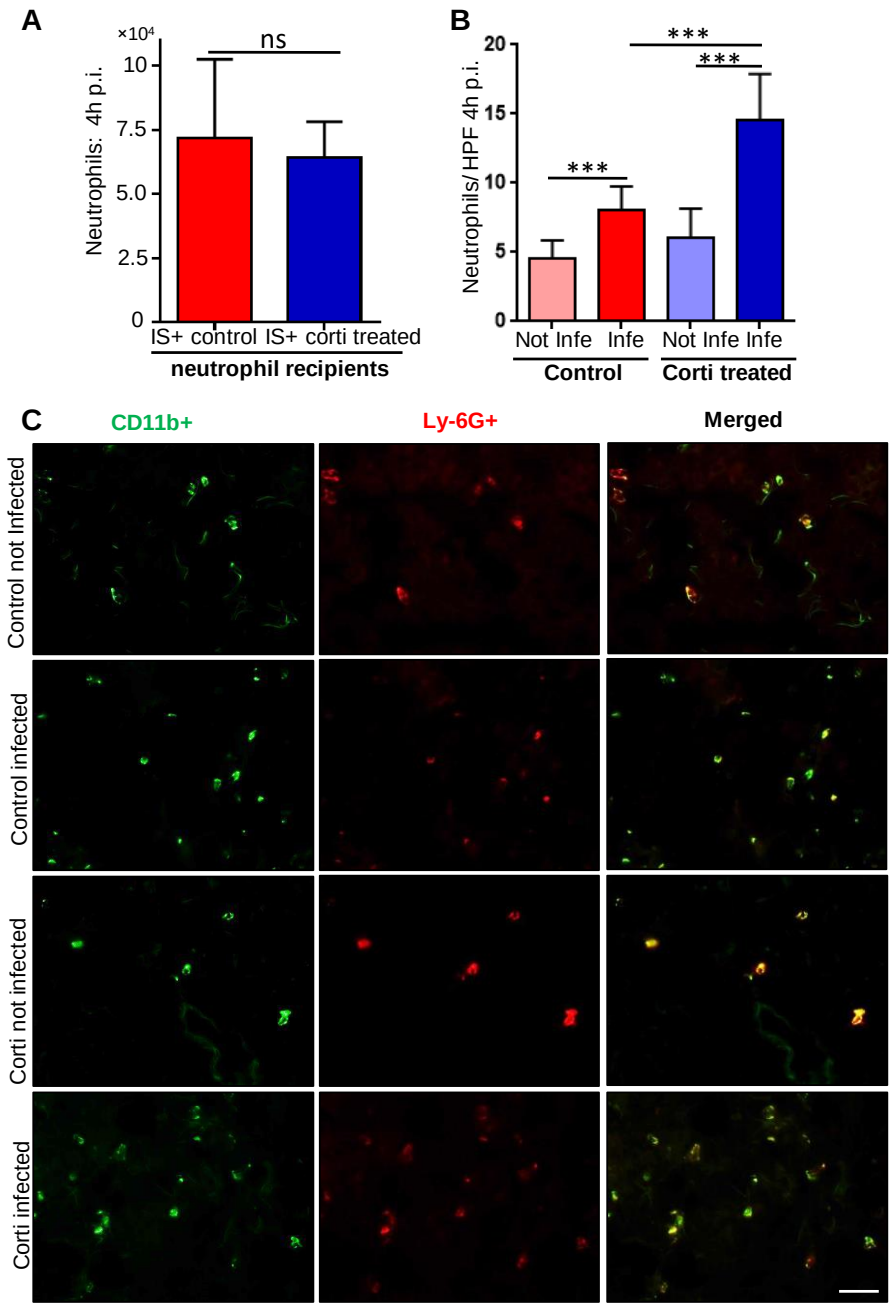

Figure 14. Granulocytes from corticosteroid treated donor are recruited to the infected lungs in cyclophosphamide immunosuppressed (C IS) mice: A. Granulocyte recruitment in transfused mice. Mice were immunosuppressed with cyclophosphamide (C IS) on day-3 and day-1, and intranasally administered with 1x 10^6 conidia/ mouse on day-0. Granulocytes from control or corticosteroid treated mice were enriched, and 3.5x 10^6 cells/ mouse were transfused on day-0, 15 min after infection and 4 h after granulocyte transfusion lung neutrophils were analyzed by FACS analysis. No significant differences were found in lung neutrophil numbers in C IS infected

mice which had received granulocytes from either untreated healthy control mice or corticosteroid treated mice. Data are representative of two independent experiments with n=4 mice/group in each experiment. **B.** Quantification of neutrophils in corticosteroid treated and infected mice. Control or corticosteroid treated mice were infected with 1×10^6 conidia/ mouse and lung sections were stained with anti-CD11b and anti-Ly6G antibodies. At least 10 immunofluorescence microscopy (IFM) images for each condition were used for quantification. **C.** Representative images of $CD11b^+Ly6G^+$ neutrophils in lung sections. Scale bar 20 µM. Unpaired Mann-Whitney *u*-test was utilized to determine significant differences: *** $P<0.001$.

4.10 Reduced proinflammatory cytokine levels after granulocyte transfusion from corticosteroid treated donors

A similar neutrophil influx was observed in the lungs of C IS-infected mice, which had received granulocytes from CT or control mice, however the C IS-infected mice were not resistant to infection if they received granulocytes from CT mice. Granulocytes, besides their primary antimicrobial defence functions are also important for their contribution to the fine tuning of host immune responses against pathogens *via* their *de novo* production and release of a wide range of cytokines and chemokines (Tecchio et al., 2014). To determine the lung cytokine and chemokine environment, we measured cytokine/ chemokines in lung homogenates of C IS-infected mice, which had received granulocytes from CT or control donors. Pulmonary IL-1α, CXCL1, CXCL5, MIP1α, CCL17 and MCP-1 levels were significantly lower in C IS-infected mice which had received granulocyte transfusion from CT donors compared to transfusion from control mice (**Fig.15**). Particularly, CXCL5 and MIP1α levels were extremely low in C IS - infected mice, which had received granulocyte transfusion from CT donors (**Fig. 15**). Since, these granulocyte derived cytokines/chemokines are important for proper functioning or regulation of immune system to efficiently clear the infection without presenting any significant damage to host tissues (Tecchio et al., 2014).

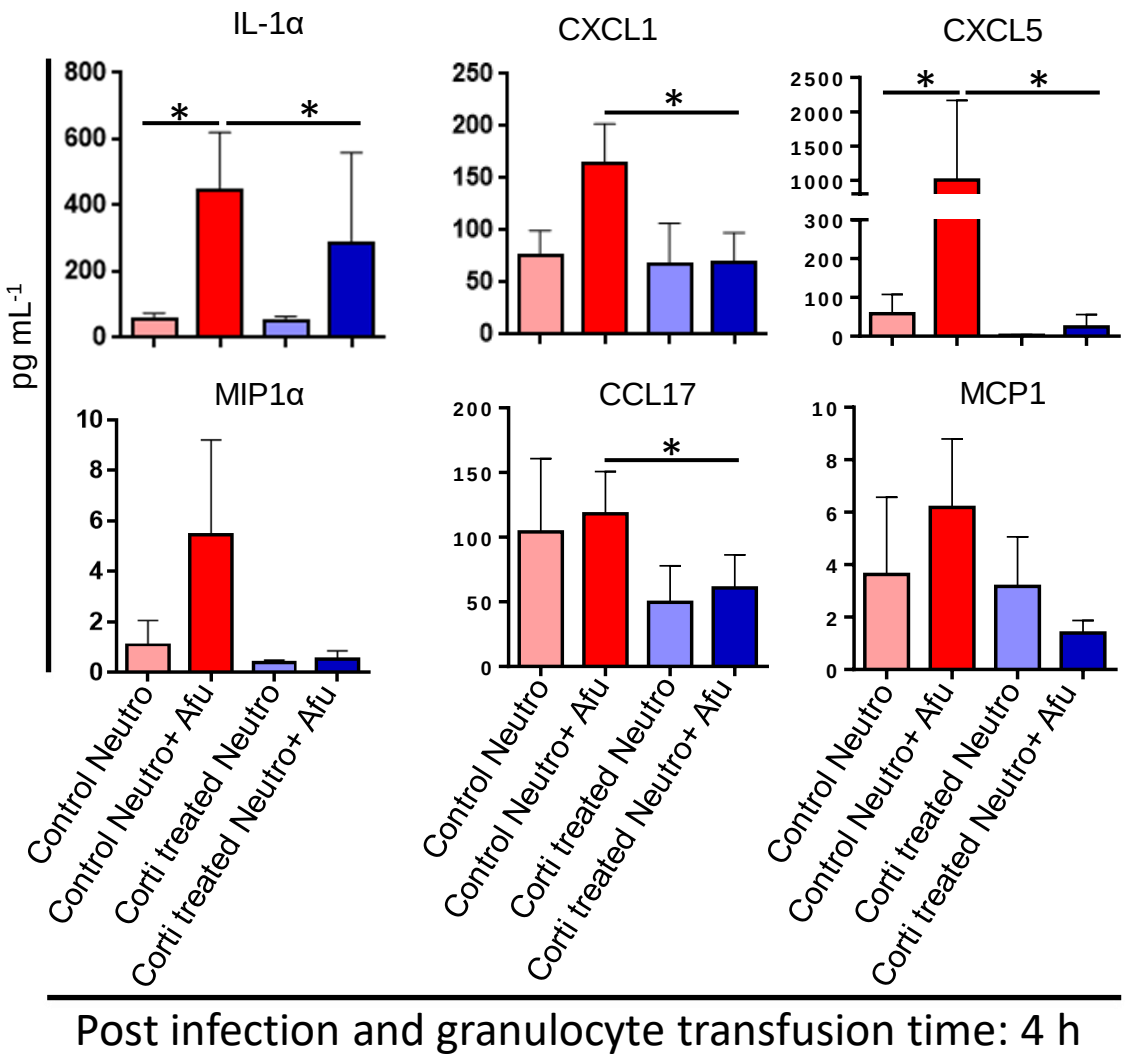

Figure 15. Reduced proinflammatory cytokine levels after granulocyte transfusion from corticosteroid treated donors: Mice were immunosuppressed with cyclophosphamide (C IS) on day-3 and day-1, and intranasally administered with 1x 10^6 conidia/ mouse on day-0. Granulocytes from control or corticosteroid treated mice were enriched, and 3.5x 10^6 cells/ mouse were transfused on day-0, 15 min after infection and 4 h after granulocyte transfusion, cytokines/ chemokines were quantified from lung homogenates using the cytometric bead array. Data are representative of two independent experiments with n=4 mice/group in each experiment. Unpaired Mann-Whitney *u*-test was utilized to determine significant differences: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

4.11 Corticosteroids impair recognition and phagocytosis of *A. fumigatus* by targeting β-glucan receptor in mouse and human neutrophils

We have observed that transfusion of neutrophilic granulocytes collected from CT donor mice could not protect from lethal *A. fumigatus* infection. On the other hand, we did not observe any impairment in granulocyte recruitment to the site of infection in C IS infected mice transfused with granulocytes collected from CT donor mice suggesting that their antifungal action might be impaired. To test this hypothesis, we directly compared the anti-*A. fumigatus* defence mechanisms of granulocytes collected from CT and untreated control mice. To this end, we isolated the neutrophils from CT mice and untreated control mice and cultured them with conidia or germlings for 3 h and measured phagocytosis of GFP-*A. fumigatus* conidia with flow cytometry. Granulocytes collected from CT donor were significantly compromised in phagocytosis of conidia or germlings when compared to granulocytes enriched from untreated control mice (**Fig. 16**). These results suggested that reduced phagocytosis of *A. fumigatus* by granulocytes collected from CT donors could result from a reduced capacity to recognize *A. fumigatus*. To test this hypothesis, we measured β-glucan receptor Dectin-1 levels in granulocytes collected from CT or untreated control mice after co-culturing with *A. fumigatus*. Granulocytes collected from CT donors expressed significantly lower Dectin-1 levels when compared to the Dectin-1 expression levels of granulocytes from control mice, which could contribute to impaired recognition of *A. fumigatus* (**Fig. 18**). Further, granulocytes enriched from CT donor mice were also significantly compromised in *A. fumigatus* killing when compared to granulocytes collected from untreated control mice (**Fig. 17**).

To determine the impact of corticosteroid treatment on human neutrophilic granulocyte defence functions against *A. fumigatus*, we treated human peripheral blood collected from healthy donors with corticosteroids for 2 h (**Fig. 19**). Neutrophilic granulocytes were enriched from

corticosteroid treated and untreated human peripheral blood and co-cultured with *A. fumigatus* conidia for 3 h and measured phagocytosis of GFP-*A. fumigatus* conidia with flow cytometry. Human granulocytes treated with corticosteroids were significantly compromised in phagocytosis of *A. fumigatus* conidia when compared to untreated human granulocytes (**Fig. 20**). Next, we determined β-glucan receptor Dectin-1expression levels in human granulocytes treated with corticosteroids or untreated control granulocytes after stimulating with *A. fumigatus*. Dectin-1 levels were upregulated in untreated control granulocytes after stimulation with *A. fumigatus* when compared to expression levels in granulocytes treated with corticosteroids and stimulated *with A. fumigatus* (**Fig. 21** upper panel). Granulocytes treated with corticosteroids were compromised in expression levels of Dectin-1 when compared to the Dectin-1 expression levels in untreated control granulocytes (**Fig.21** lower panel). Further, granulocytes treated with corticosteroids were also significantly compromised in *A. fumigatus* killing when compared to untreated control granulocytes (**Fig. 22**).

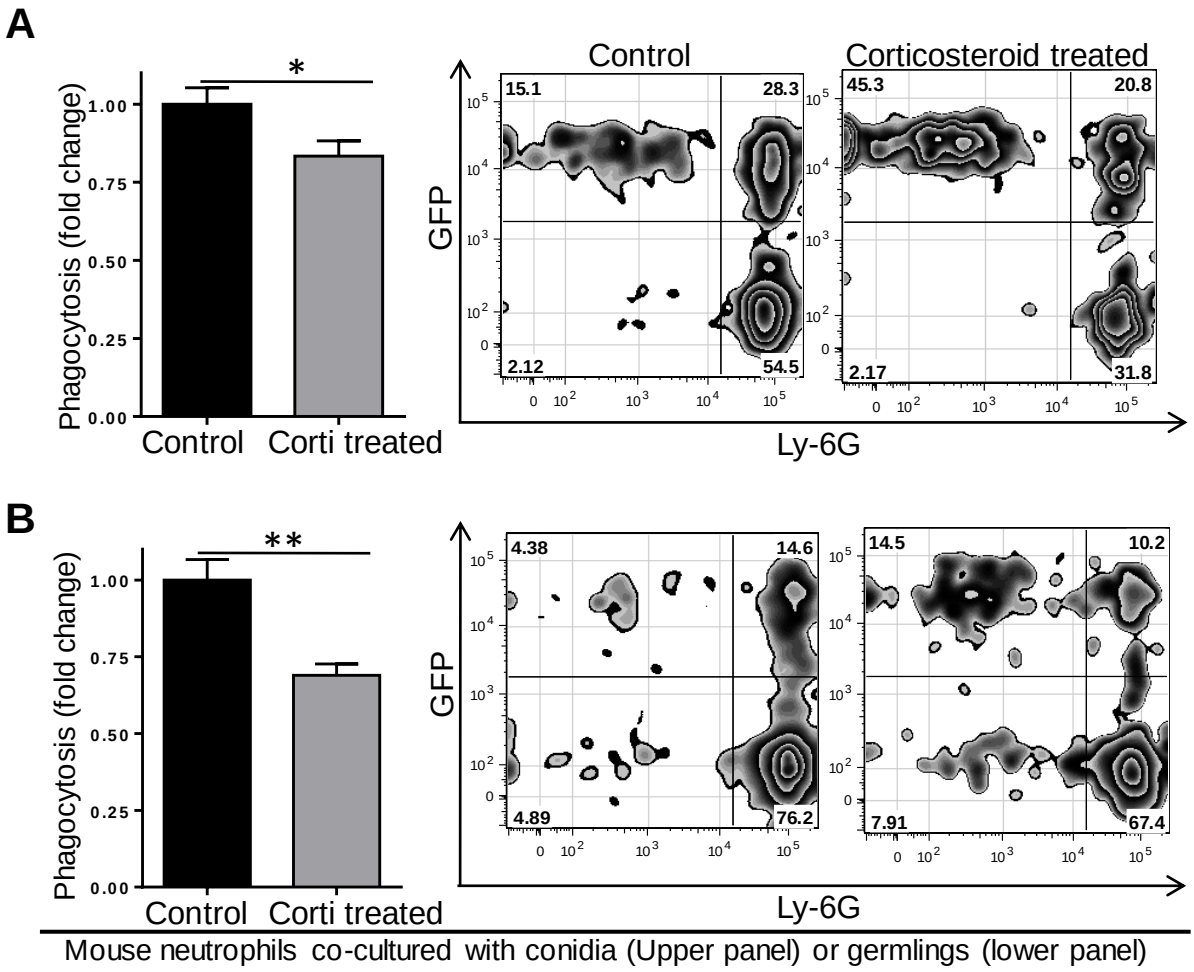

Figure 16. Corticosteroids impair phagocytosis of *A. fumigatus*: Granulocytes enriched from corticosteroid treated mice and granulocytes from untreated control mice were co-incubated with Afu-GFP conidia or germlings for 3 h and phagocytosis was measured by flow cytometry analyses. Quantification of fold change of phagocytosis demonstrates that granulocytes enriched from corticosteroid treated mice have a significantly reduced phagocytic capacity of both conidia **(A)** and germlings **(B)** when compared to phagocytic capacity of granulocytes from control mice. Data are representative of three independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant differences: * $P<0.05$; ** $P<0.01$.

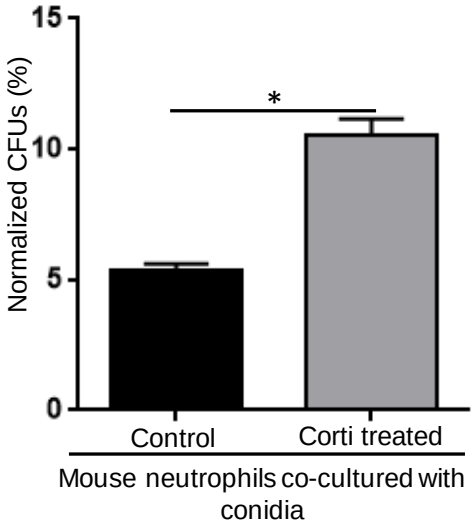

Figure 17. Corticosteroids impair fungal killing by mouse neutrophils. Granulocytes enriched from corticosteroid treated mice and granulocytes from untreated control mice were co-incubated with *A. fumigatus* conidia for 6 h and fungal killing levels were measured by CFU analyses. Data are representative of three independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant differences: * *P*<0.05.

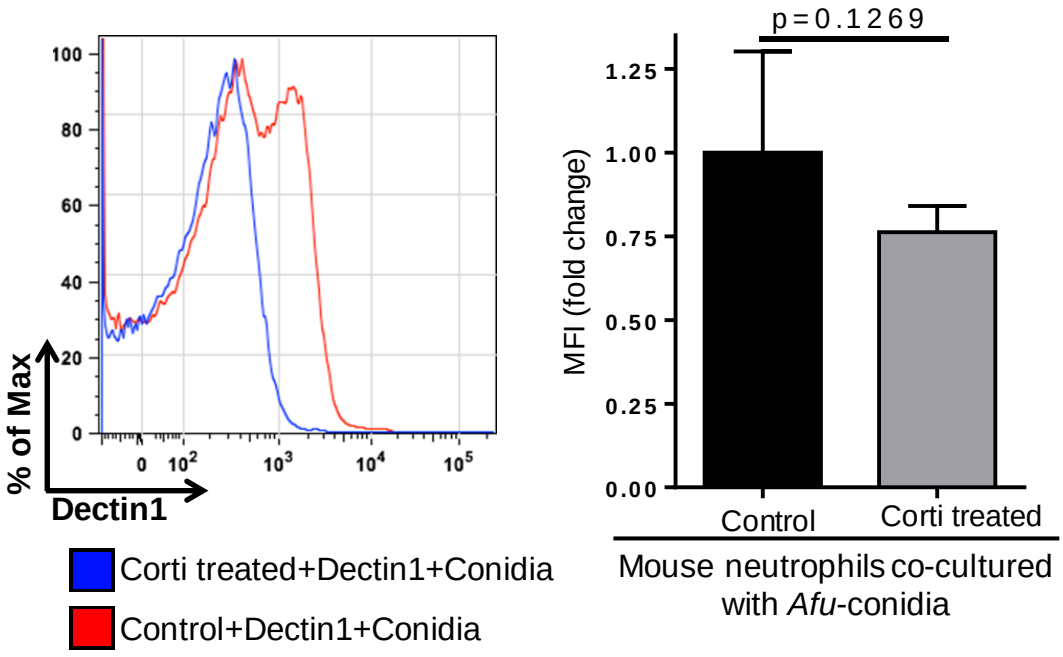

Figure 18. Corticosteroids impair Dectin-1 expression levels in mouse neutrophils after stimulation with *A. fumigatus*: Granulocytes enriched from corticosteroid treated mice and granulocytes from untreated control mice

were co-incubated with *A. fumigatus* conidia for 3 h and Dectin-1 expression levels were measured by flow cytometry analyses. Representative histograms showing Dectin-1 expression (on the left side) and quantification of Dectin-1 expression (on the right side). Data are representative of three independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant difference, *P*=0.1269.

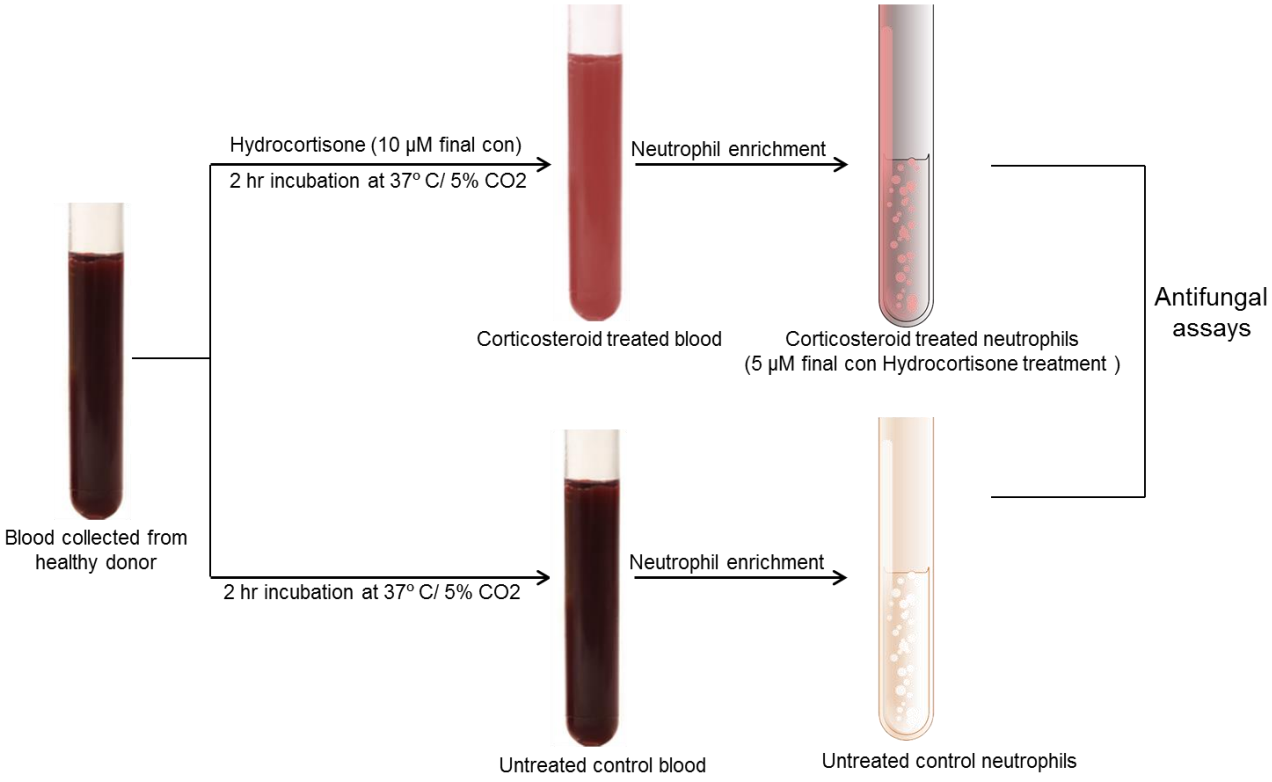

Figure 19. Corticosteroid treatment strategy for human neutrophils. Human peripheral blood was collected from healthy donors and treated with hydrocortisone or untreated blood was incubated at 37º C for 2 h and neutrophils were enriched. Further, neutrophils from corticosteroid treated blood were treated with half the initial concentration of hydrocortisone and employed these neutrophils for antifungal analyses (Test tubes pictures were adopted from shutterstock.com and modified in the figure).

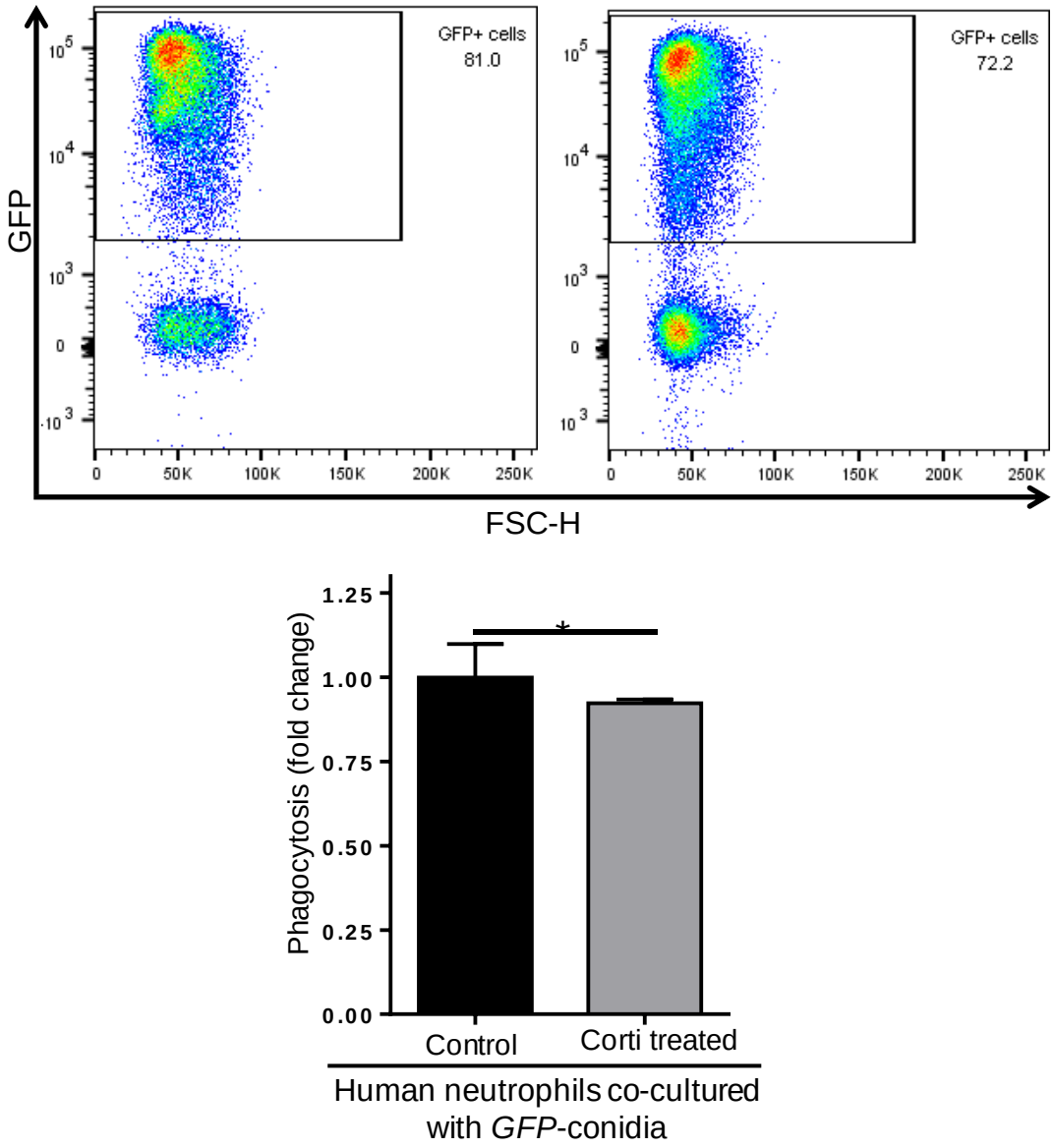

Figure 20. Corticosteroids impair phagocytosis of fungal conidia by human neutrophils. Human granulocytes enriched from corticosteroid treated blood and granulocytes from untreated control blood were co-incubated with Afu-GFP conidia for 3 h and phagocytosis was measured by flow cytometry analyses. Quantification of fold change of phagocytosis demonstrates that human granulocytes enriched from corticosteroid treated blood phagocytosed significantly fewer conidia when compared to granulocytes from control human blood. Data are representative of three independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant differences: * $P<0.05$.

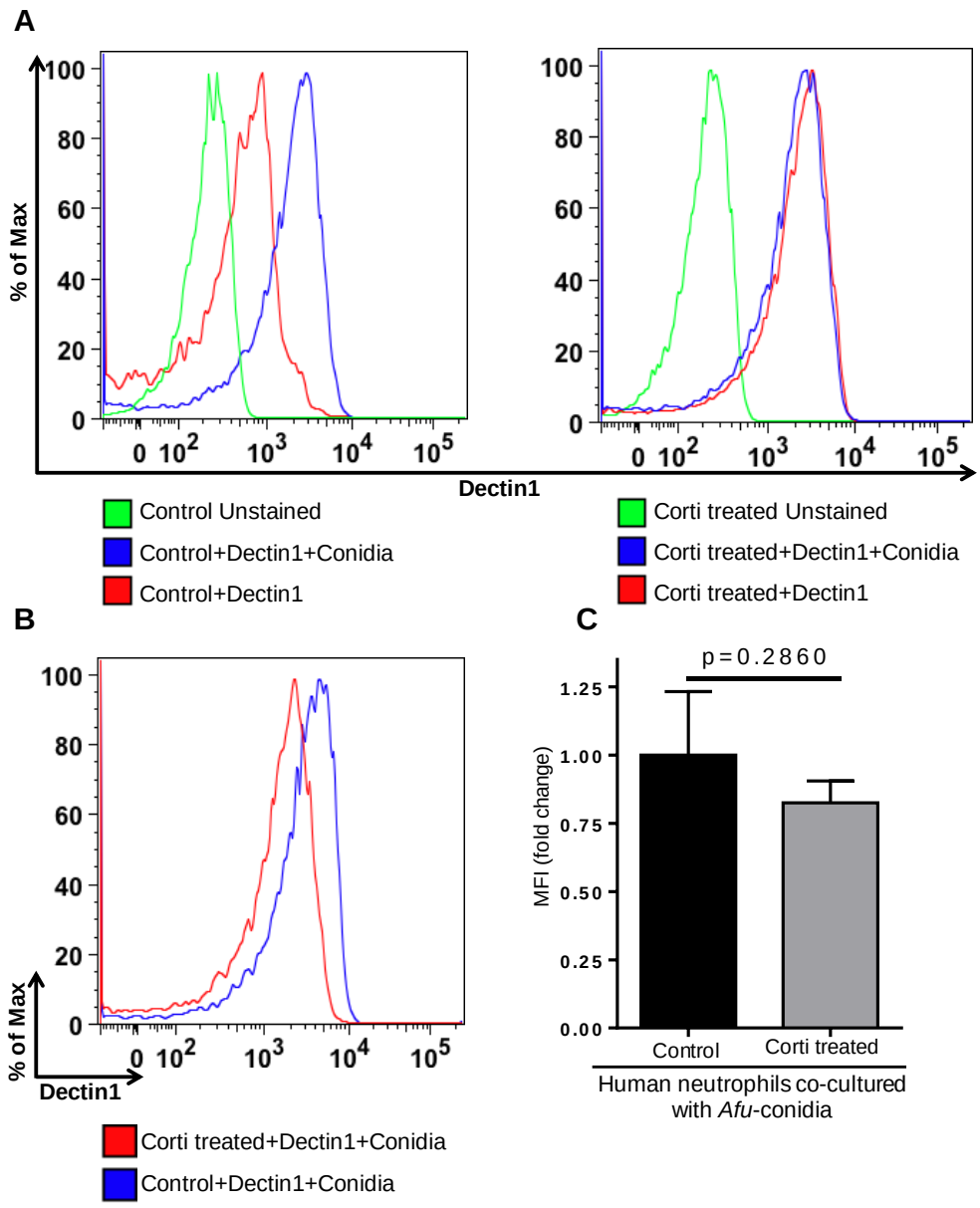

Figure 21. Corticosteroid treatment reduces Dectin-1 expression on human neutrophils after stimulation with *A. fumigatus*. Human granulocytes enriched from corticosteroid treated blood and granulocytes from untreated control blood were co-incubated with *A. fumigatus* conidia for 3 h and Dectin-1 expression levels were measured by flow cytometry analyses. Representative FACS histograms showing Dectin-1 expression in granulocytes enriched from control or corticosteroid treated human peripheral blood and quantification of Dectin-1 expression levels. Data are representative of three independent experiments with n=3 replicates/ group. Unpaired student-*t* test was utilized to determine significant differences, *P*=0.2860.

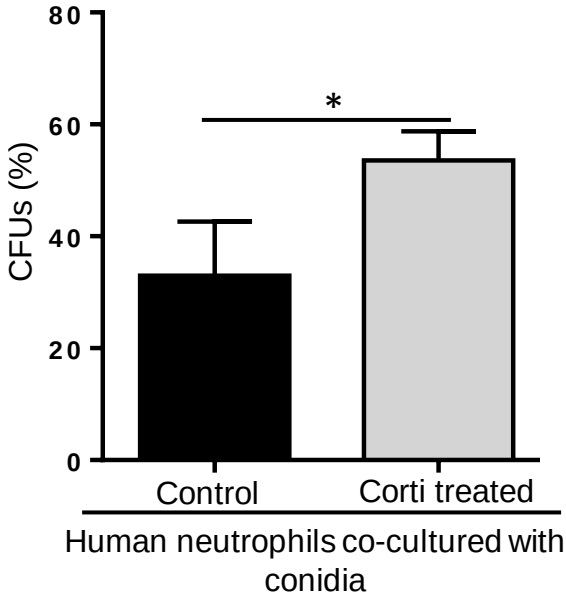

Figure 22. Corticosteroids impair fungal killing by human neutrophils. Human granulocytes enriched from corticosteroid treated human peripheral blood and granulocytes from untreated control blood were co-incubated with *A. fumigatus* conidia for 6 h and fungal killing levels were measured by CFU analyses. Data are representative of three independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant differences: * $P<0.05$.

4.12 Corticosteroids impair mouse and human neutrophils to form NETs against *A. fumigatus*

A hallmark of neutrophils is their capacity to form NETs to trap large bacterial and fungal pathogens to avoid further spread of infection (Branzk et al., 2014). Consequently, we compared the NETosis function of granulocytes collected from CT donor mice and untreated control mice after 3 h co-incubation with *A. fumigatus* conidia or hyphae in ploy-L-lysine coated plates. Granulocytes from CT mice were unable to form NETs in response to any of the *A. fumigatus* morphotypes as assessed with scanning electron microscopy (**Fig. 23A**) and

immunofluorescence microscopy (**Fig. 23B**). NET formation was significantly reduced in granulocytes from CT mice in response to *A. fumigatus* conidia (**Fig. 23C**). An important mechanism in NET formation is ROS production by neutrophils to kill or inhibit the growth of fungi (Branzk et al., 2014). Next, we assessed the production of ROS in response to *A. fumigatus* by granulocytes collected from CT or control mice. Granulocytes from CT mice produced significantly less ROS compared to granulocytes from untreated control mice when co-incubated with conidia (**Fig. 23D**), whereas ROS production did not significantly differ when co-incubated with hyphae (**Fig. 23E**). To determine NET formation under *in vivo* conditions, we infected CT and control mice with *A. fumigatus* conidia and stained for extracellular NET DNA present in infected lung sections. NETosis function was inhibited in CT and infected mice. In contrast, NETosis became clearly apparent in untreated control mice after infection with *A. fumigatus* (**Fig. 24**). NET formation is considered as a beneficial suicide program of granulocytes to minimize the spread of fungal infection. Therefore, the increase in cell survival or anti-apoptotic gene expression might impact the beneficial suicide of granulocytes and subsequent NET formation. Mcl-1 is an anti-apoptotic member of the Bcl-2 family expresses in granulocytes (Akgul et al., 2001) required for granulocyte survival. Further, it has been shown that granulocyte survival and Mcl-1 functional induction is dependent on PI3K and p38 MAPK signaling (Saffar et al., 2008). To elucidate the mechanism of corticosteroid mediated NET inhibition, we determined the transcripts of survival signaling genes, Mcl-1 and Pik3rl, in granulocytes enriched from CT mice. Expression of Mcl-1 and Pik3rl gene transcripts were significantly upregulated in granulocytes enriched from CT mice when compared to transcript levels in granulocytes enriched from untreated control mice **(Fig. 25)**.

To determine corticosteroid mediated inhibition of NETosis function against *A. fumigatus* in human neutrophilic granulocytes, we treated human peripheral blood collected from healthy donors with corticosteroids. Neutrophilic granulocytes were enriched from corticosteroid treated

and untreated human peripheral blood and co-cultured with *A. fumigatus* hyphae. NET formation was significantly reduced in neutrophils enriched from corticosteroid treated blood in response to *A. fumigatus* hyphae **(Fig. 26)**. These data indicate that cortisone treatment concomitantly impairs different antifungal defence mechanisms of neutrophils. This could explain the inability of transfused neutrophilic granulocytes collected from corticosteroid treated donor to protect immunosuppressed mice from lethal *A. fumigatus* infection.

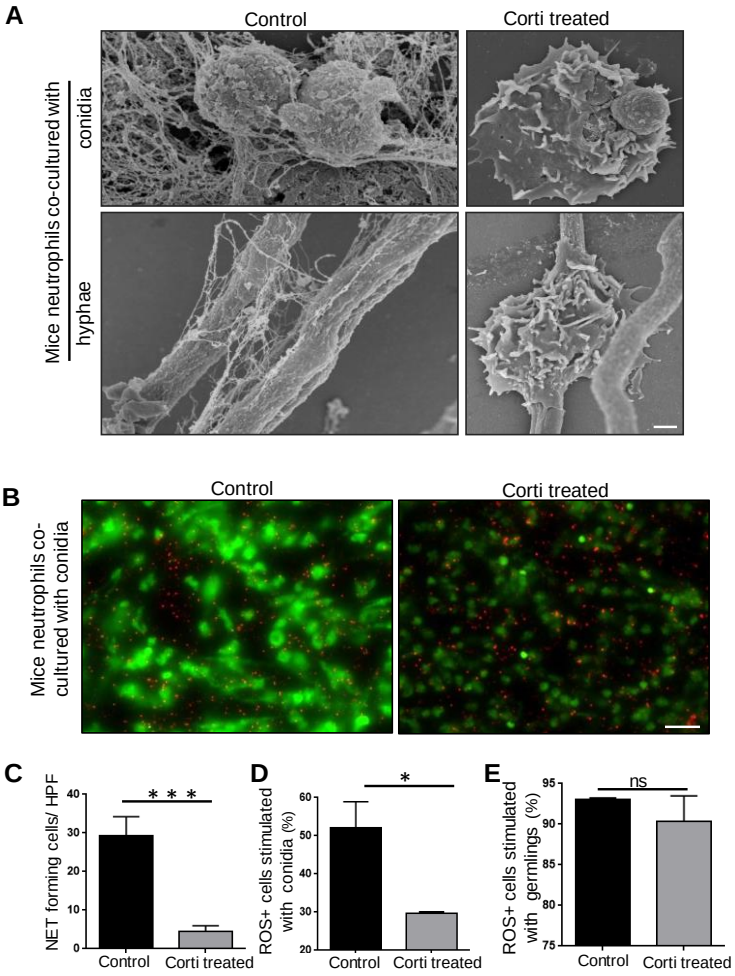

Figure 23. Corticosteroids impair NETosis function of mouse neutrophils. Granulocytes enriched from corticosteroid treated mice and granulocytes from untreated control mice were co-incubated with *A. fumigatus* conidia or hyphae for 3 h in poly-L-Lysine coated plates and NET formation was determined by scanning electron microscopy, scale bar 1 µM **(A)** or using immunofluorescence microscopy, scale bar 20 µM **(B)**. Quantification of NET

formation by determining number of NET forming cells/ HPF **(C)**. Granulocytes enriched from corticosteroid treated mice and granulocytes from untreated control mice were stimulated with *A. fumigatus* conidia or hyphae for 1.5 h in 24 well culture plates and ROS production in granulocytes were determined by FACS analyses. Quantification of ROS production by granulocytes stimulated with conidia **(D)** or hyphae **(E)**. Data are representative of three independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant differences: * $P<0.05$; *** $P<0.001$.

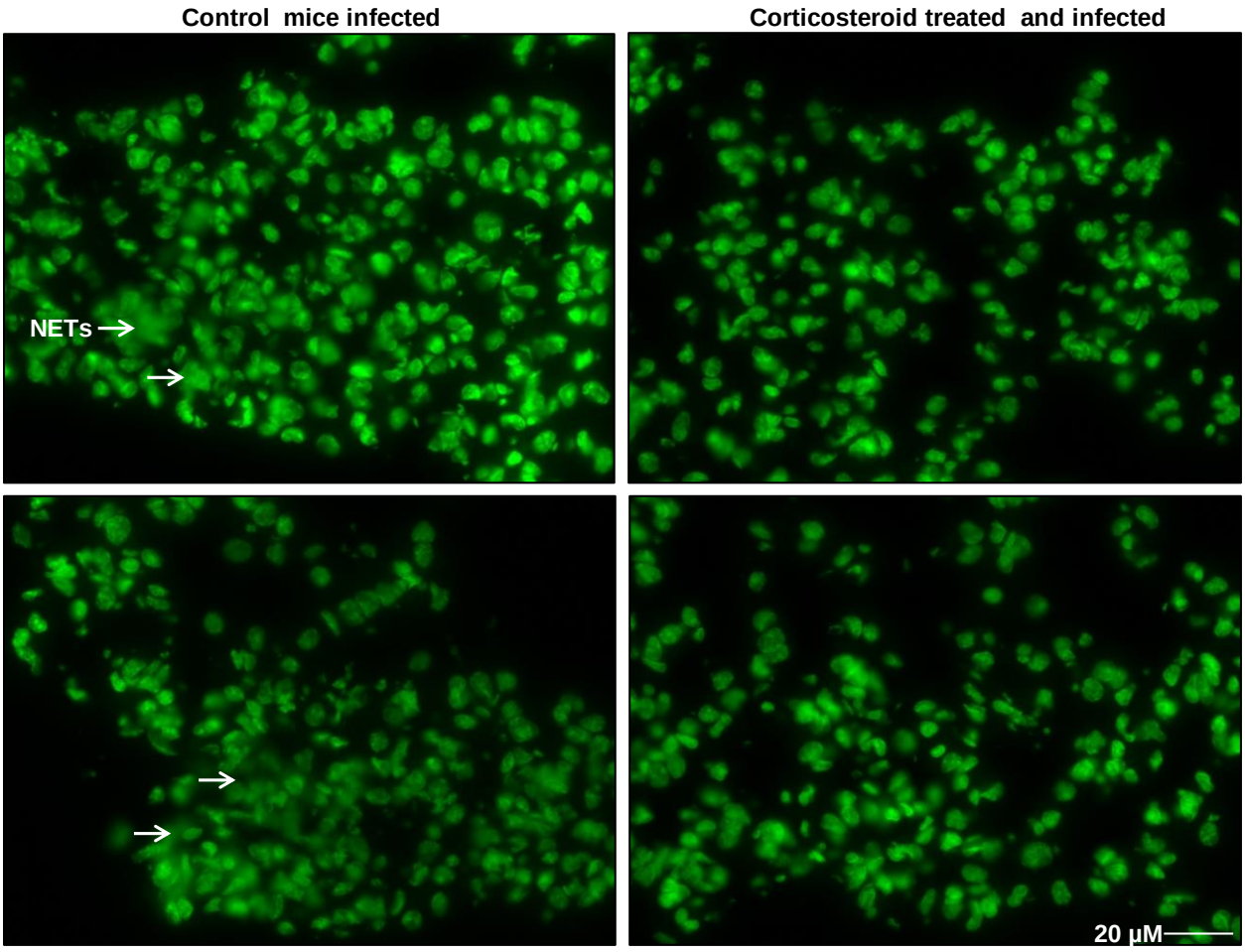

Figure 24. Corticosteroids impair NETosis in infected mouse lungs. Corticosteroid treated and control mice were infected with 1× 10^6 *A. fumigatus* conidia/ mouse. 48 h post infection mouse lung sections were prepared and Sytox staining was performed to stain extracellular NET DNA present in infected lung sections and images were taken using immunofluorescence microscopy, scale bar 20 µM. Untreated control mice showed NETosis against *A. fumigatus* (left panel) and corticosteroid treated mice did not show NETosis.

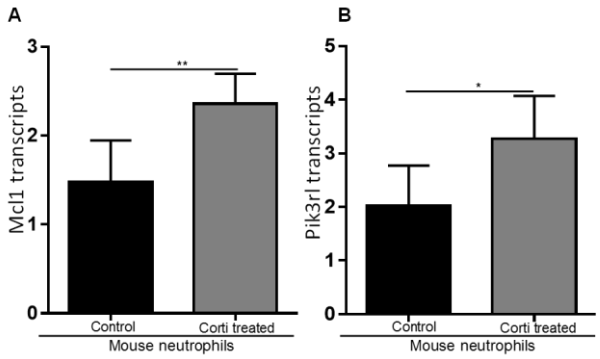

Figure 25. Corticosteroids upregulate transcripts of granulocyte survival genes. RNA was isolated from granulocytes enriched from corticosteroid treated mice and granulocytes from untreated control mice and cDNA was prepared. qRT-PCR was performed with Mcl-1 (A) or Pik3rl (B) specific primers and transcript levels were normalized with *GAPDH* transcript levels and expression levels were calculated using comparative D cycle method. Data are representative of n=4 mice/ group biological replicates. Unpaired student-*t* test was utilized to determine significant differences: * *P*<0.05; ** *P*<0.01.

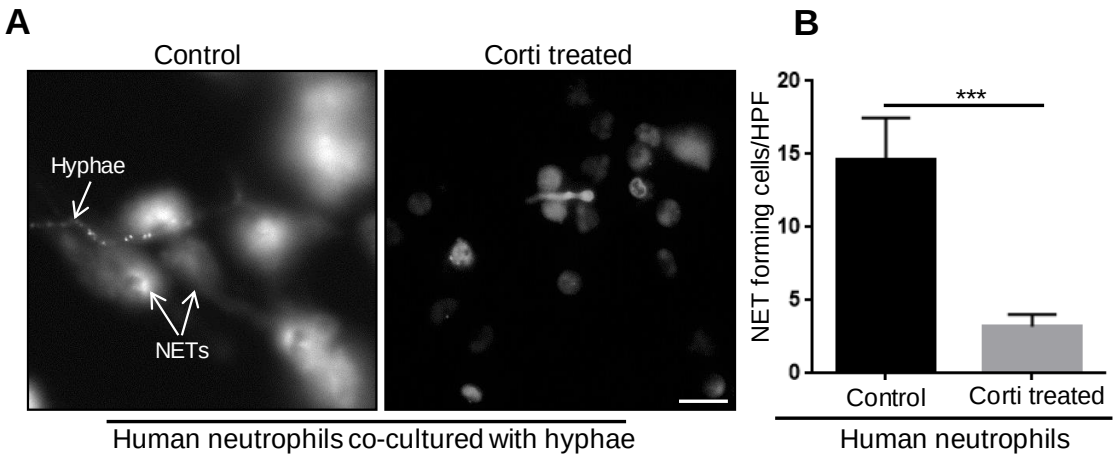

Figure 26. Corticosteroids impair NETosis function of human neutrophils. Human granulocytes enriched from corticosteroid treated human peripheral blood and granulocytes from untreated control blood were co-incubated with *A. fumigatus* germlings for 3 h in Poly-L-Lysine coated plates and NETosis was determined by Sytox staining and images were taken using immunofluorescence microscopy, scale bar 20 µM **(A)**. Quantification of NET formation by determining number of NET forming cells/ HPF **(B)**. Data are representative of three independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant differences: *** *P*<0.001.

4.13 Transfusion of CD11b$^+$ myeloid cells from corticosteroid treated mice protects cyclophosphamide immunosuppressed mice against *A. fumigatus* infection

Granulocytes from CT mice were unable to clear *A. fumigatus* infection and, similar to CT human granulocytes, exhibited several defects in terms of antifungal functions. Although neutrophils are considered as a key population in the antifungal defence repertoire (Feldmesser, 2006), other myeloid cells are also important to fight against *A. fumigatus*. Therefore, we determined the effect of corticosteroids on the complete CD11b$^+$ myeloid compartment. To this end, we adoptively transferred CD11b$^+$ myeloid cells enriched from CT and untreated control mice to C IS mice (**Fig. 27A**). On day 0 we intravenously injected enriched corticosteroid treated-CD11b$^+$ or control-CD11b$^+$ myeloid cells (**Fig. 27B**) into C IS mice that we infected with a lethal dose of 2x10^5 *A. fumigatus* conidia on day+ 1. Adoptive transfer of both, corticosteroid treated-CD11b$^+$ or control-CD11b$^+$ (5× 10^6 cells/ mouse) myeloid cells conferred resistance against *A. fumigatus* infection to the C IS mice, irrespective of prior corticosteroid exposure (**Fig. 27C**). These results suggested that the impact of corticosteroids predominantly affects the function of neutrophils. Yet, other CD11b$^+$ myeloid cell populations remained capable to provide protective defence mechanisms, since corticosteroid treated-CD11b$^+$ myeloid cells rescued C IS mice from lethal *A. fumigatus* infection. To further explore the protective defence mechanisms of corticosteroid treated CD11b$^+$ myeloid cells, we derived macrophages (**Fig. 28** upper panel) and dendritic cells (**Fig. 29** upper panel) from bone marrow collected from CT mice or untreated control mice. Fungal killing assays were performed to determine antifungal defence capability of macrophages or dendritic cells derived from bone marrow of CT or untreated healthy control mice. Macrophages or dendritic cells derived from CT or control mice bone marrow exhibited similar fungal killing capacity (**Fig. 28 and Fig. 29**) supporting the concept that corticosteroid treatment predominantly affects antifungal functions of neutrophilic granulocytes.

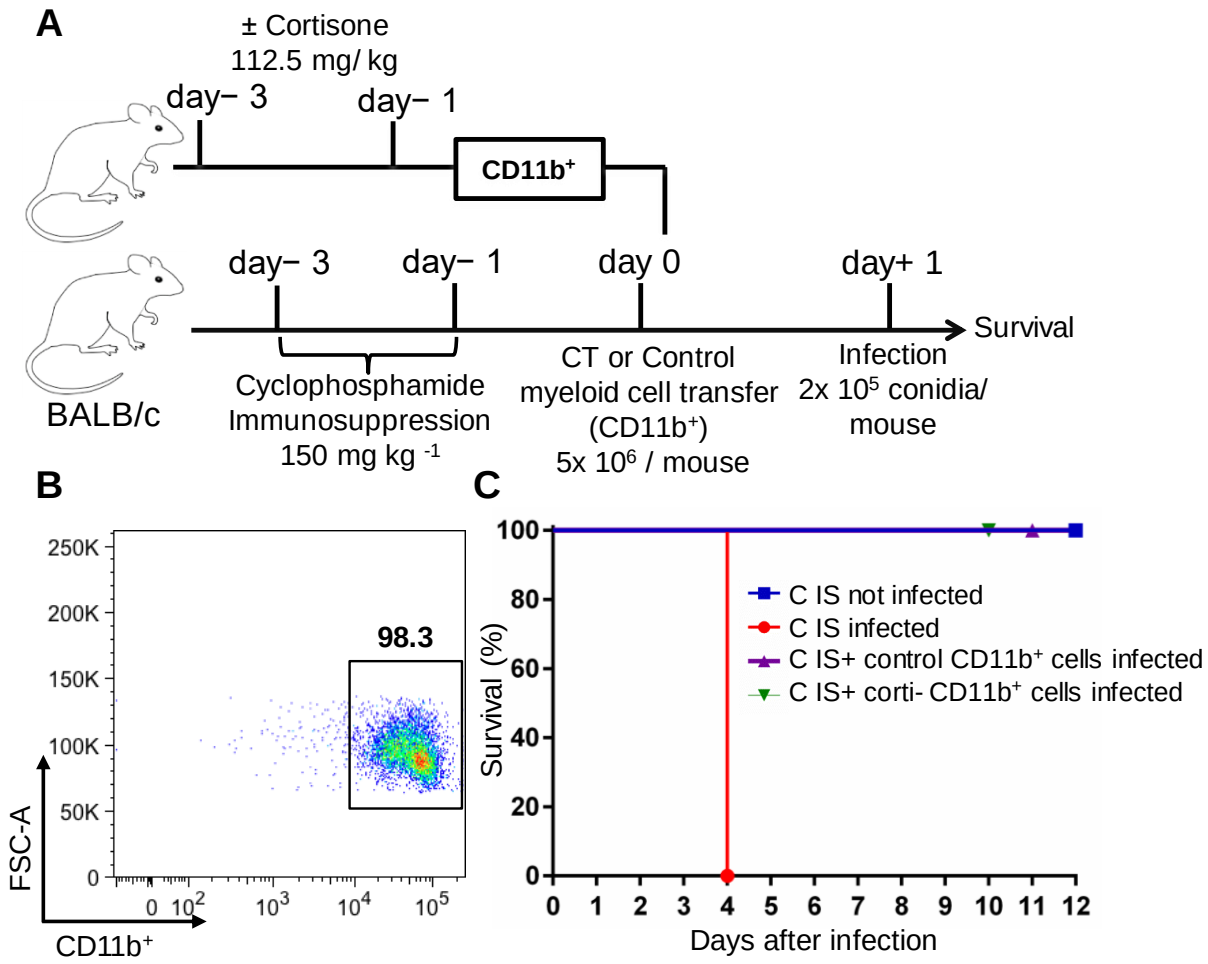

Figure 27. Adoptive transfer of corticosteroid treated-CD11b$^+$ myeloid cells protect C IS mice against *A. fumigatus* infection. (A) Scheme of experimental setup for adoptive corticosteroid treated-CD11b$^+$ myeloid cell transfer and *A. fumigatus* infection. **(B)** Purity of CD11b$^+$ myeloid cells were confirmed with flow cytometry. For all samples, purity values were above 95% **(C)** Survival of mice adoptively transferred with control or corticosteroid treated myeloid cells. Mice that had received corticosteroid treated-CD11b$^+$ myeloid cells were completely resistant to infection, in contrast to without cell transfer mice (n=8), P=0.0001. Data are representative of two independent experiment with n=8/ group of mice in each experiment. To compare survival curves of infected mice, the Log-rank (Mantel-Cox) test was utilized.

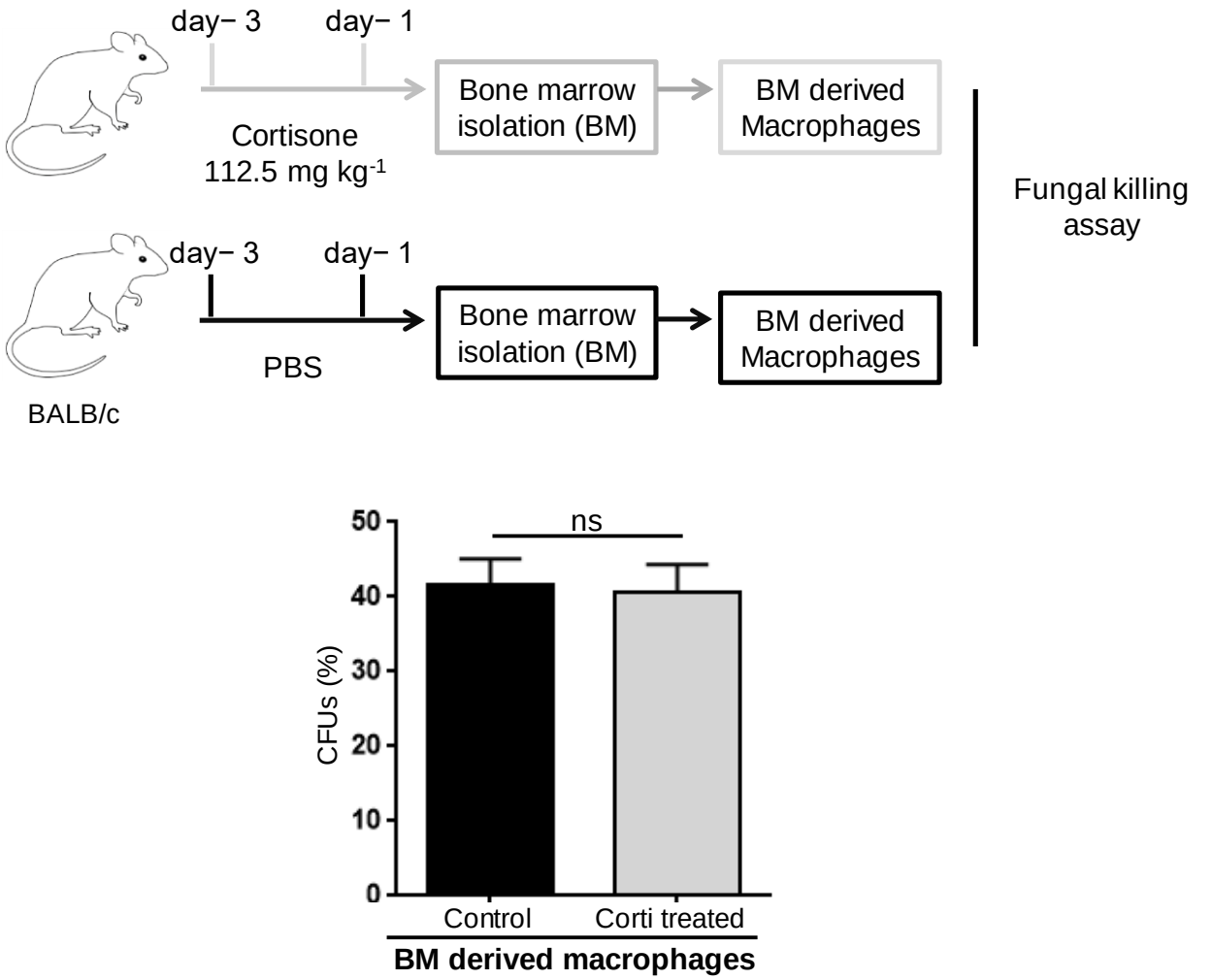

Figure 28. Killing of *A. fumigatus* by bone marrow derived macrophages. Bone marrow was isolated from corticosteroid treated or control mice and macrophages were derived. Bone marrow derived macrophages were co-cultured with *A. fumigatus* conidia and fungal survival was determined using CFU analyses (Upper panel). Quantification of fungal killing by bone marrow derived macrophages. Data are representative of two independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant differences.

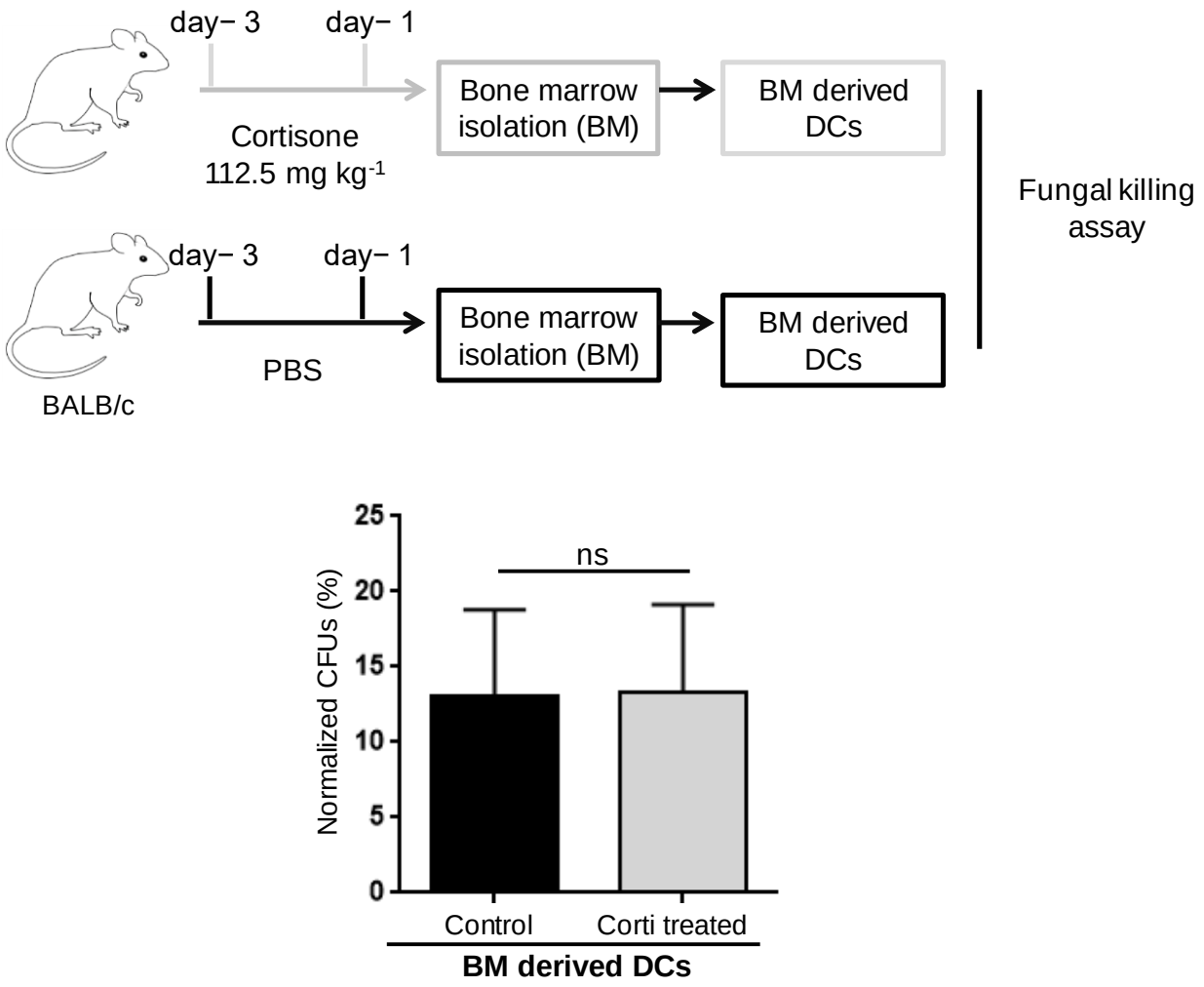

Figure 29. Killing of *A. fumigatus* by bone marrow derived DCs. Bone marrow was isolated from corticosteroid treated or control mice and DCs were derived. Bone marrow derived DCs were co-cultured with *A. fumigatus* conidia and fungal survival was determined using CFU analyses (Upper panel). Quantification of fungal killing by bone marrow derived DCs. Data are representative of two independent experiments with n=3 replicates/ group in each experiment. Unpaired student-*t* test was utilized to determine significant differences.

5 Discussion:

The timing and magnitude of host immune cell responses following *A. fumigatus* conidial inhalation, continuous host defence throughout the different developmental stages of the fungi, as well as how different immunosuppressive treatments affect the anti-*A. fumigatus* functions of immune cells remain poorly defined *in vivo*. In order to improve the management and the treatment of *A. fumigatus* lung infections in immunocompromised patients it is essential to study host pathogen interactions in preclinical *in vivo* models of aspergillosis that mimic scenarios of immunocompromised patients. In the first part of this study, we employed corticosteroid treated (CT) and corticosteroid & cyclophosphamide treated (CCT) mice models to study the host immune responses following *A. fumigatus* challenge. Given the dynamic changes in immune cell subsets within tissues upon immunosuppressive therapy, we dissected the spatiotemporal pulmonary immune response following *A. fumigatus* infection. Furthermore, we demonstrated the successful control of *A. fumigatus* infection following the adoptive transfer of $CD11b^+$ myeloid cells into cyclophosphamide immunosuppressed mice. Our results confirm that $CD11b^+$ myeloid cells are major contributors to control *A. fumigatus* lung infections under immunocompromised conditions. However, this protective effect vanished when mice were treated with cortisone and cyclophosphamide before the adoptive transfer of $CD11b^+$ myeloid cells and the infection with *A. fumigatus* conidia.

We demonstrated that more neutrophils migrated to the infected lungs of CT mice than in immunocompetent mice. However, CT infected mice were unable to clear the fungal infection and died from severe invasive aspergillosis. This prompted us to further investigate the effects of corticosteroids on neutrophil anti-*A. fumigatus* defence functions. In the past, granulocyte transfusions had been tested to treat high-risk neutropenic patients with invasive *A. fumigatus* infections. In order to increase the granulocyte yield for transfusion, donors had been treated

with oral corticosteroids (Price et al., 2015). However, the efficacy of granulocyte transfusion and the antifungal capacities of granulocytes collected from corticosteroid treated donors remained largely elusive. In order to determine the effects of corticosteroids on granulocytes to control *A. fumigatus* infections, we performed *in vitro* co-culture experiments with human or mouse granulocytes and *A. fumigatus* as well as granulocyte adoptive cell transfers in *in vivo* mouse models. Fluorescence and electron microscopy, flow cytometry and cytokine analysis assisted our analyses. The transfusion of granulocytes from corticosteroid treated mice did not protect cyclophosphamide immunosuppressed mice against lethal *A. fumigatus* infections in contrast to granulocytes from untreated mice. Corticosteroid treated human or mouse neutrophils failed to form neutrophil extracellular traps (NETs) under *in vitro* and *in vivo* conditions. Furthermore, corticosteroid treated granulocytes exhibited impaired ROS production against *A. fumigatus*. Notably, on mouse and human granulocytes corticosteroids reduced expression levels of the β-glucan receptor Dectin-1 (CLEC7A), a receptor that efficiently recognises and phagocytises *A. fumigatus*, which contributed to impaired fungal killing.

5.1 *A. fumigatus* lung infections and *in vivo* mouse models

The pivotal role of the innate immune system in eliminating *A. fumigatus* conidia in healthy individuals has long been well-recognized (Balloy and Chignard, 2009). The anatomical and physiological barriers of the respiratory tract restrict most of the airborne conidia from reaching alveolar spaces; however, the small size and hydrophobic nature of conidia allow some of them to cross alveolar epithelia and to reach alveolar spaces (Margalit and Kavanagh, 2015b). Most of the conidia in alveolar spaces are eradicated by resident phagocytes without any further development of antibody-or-cell mediated acquired immunity (Park and Mehrad, 2009). However, a compromised immune system allows for the germination of *A. fumigatus* conidia and subsequent lung infections (Margalit and Kavanagh, 2015b). In the last few decades,

several studies defined the anti-*A. fumigatus* functions of innate or adaptive immune cells (Cramer et al., 2011; Sales-Campos et al., 2013). Most of the *in vivo* studies focused on depleting a defined immune cell population from healthy murine models in order to determine the consequences of the loss of distinct cell populations on patient survival and the overall outcome of the disease. Yet, to improve management and treatment of *A. fumigatus* lung infections in immunocompromised patients it is essential to study host pathogen interactions in murine models of aspergillosis that mimic scenarios of immunocompromised patients.

We employed two clinically relevant mouse models (CT and CCT) to determine the host immune responses following a *A. fumigatus* challenge. CT and CCT mice models have often been used for virulence analysis of *A. fumigatus* mutants. CT or CCT models are selected for virulence analysis depending on the observed phenotype of the fungal mutant, for instance CT models are often used for virulence analysis of auxotroph mutants and CCT models for oxidative stress mutants (Amich et al., 2013; Chiang et al., 2008; Sheppard et al., 2005; Spikes et al., 2008; Staats et al., 2013). However, the immune status of these models under steady-state and infected conditions remained largely elusive.

5.1.1 Cyclophosphamide and corticosteroid treated mouse model

In the CCT model, mice received a combination of cyclophosphamide and corticosteroid treatment. This drug combination is widely used in treating patients with idiopathic pulmonary fibrosis (Collard et al., 2004; Kawasumi et al., 2015), acute/ subacute interstitial pneumonia (Kameda et al., 2005), refractory optic neuritis in Wegener's granulomatosis (Huchzermeyer et al., 2013) and light chain (AL) amyloidosis (Palladini et al., 2015). However, the risk of *A. fumigatus* infections associated with this treatment and immune cell responses following *A. fumigatus* infection during this treatment remained poorly defined. We confirmed that CCT mice were highly susceptible to *A. fumigatus* infection and died from infection within four days

following *A. fumigatus* challenge. Severe leukopenia permits rapid colonization of *A. fumigatus* characterized by elongated hyphae in lung tissue 40 h after infection resulting in the death of CCT-infected mice within four days following infection, which is consistent with previous findings (Amich et al., 2013). Nevertheless, despite their strongly reduced number, myeloid cells, particularly neutrophils and macrophages, were recruited to the infected lungs in CCT mice. Myeloid cell numbers in the lungs of CCT-infected mice did not exceed numbers found in the lungs of immunocompetent mice under steady-state conditions, which indicates that there were not sufficient myeloid cells recruited to the infected lungs in CCT mice to clear the infection or to prolong the life span of CCT mice. Inflammatory cytokine responses are crucial for properly resolving an *A. fumigatus* lung infection (Chotirmall et al., 2013), for instance, TNFα initially released from alveolar macrophages and later by recruited neutrophils and monocytes is important to clear a *A. fumigatus* infection (Brieland et al., 2001; Mehrad et al., 1999a; Mehrad et al., 1999b; Palladino et al., 2003). Other pro-inflammatory cytokines, such as IL-6, MCP-1 and IFNγ, have been described as vital to eliminate pulmonary *A. fumigatus* infections (Blease et al., 2001; Brieland et al., 2001; Cenci et al., 2001). All the above-mentioned cytokines were undetectable in CCT mice, both under steady-state conditions as well as following *A. fumigatus* infection. These results support the strong immunosuppressive action of high-doses of cyclophosphamide causing the high susceptibility of CCT mice to lethal *A. fumigatus* infection. The combination of cyclophosphamide and corticosteroid treatment strongly reduced lymphoid cells in CCT mice and no lymphoid cells were recruited upon infection. Infection related risk is very high with this type of treatment and clinicians might need to take special precautions to avoid infections by *A. fumigatus* throughout the treatment period.

5.1.2 Corticosteroid treated mouse model

Corticosteroids are widely prescribed in several clinical situations (Barnes, 2006; Emadi et al., 2009). We showed that corticosteroid-treated mice survived for 7 days following a *A. fumigatus* challenge, which correlates with greater myeloid cell recruitment and inflammatory lung cytokines, such as MCP-1, IFN-γ, TNFα and IL-6 levels, in the infected lungs. However, survival following infection was not greatly improved when compared to CCT infected mice, suggesting that corticosteroids may affect anti-fungal functions of immune cells rather than directly influencing myeloid cell recruitment. Therefore, further studies are warranted to completely elucidate the effects of corticosteroids on myeloid cells and their anti-*A. fumigatus* defence functions. These results prompted us to investigate the effects of corticosteroids on granulocyte transfusion therapy and the impact of corticosteroids on neutrophil antifungal defence functions. Our results were also in line with the observation that corticosteroid treatment causes strong inflammation, which might enhance tissue damage after infection (IGrahl et al., 2011a), leading to death within 7 days after infection. Overall, CCT and CT models showed lung myeloid cell but not lymphoid cell infiltration following *A. fumigatus* infection.

5.2 Adoptive transfer of $CD11b^+$ myeloid cells to treat invasive aspergillosis

The innate immune response is crucial to clear *A. fumigatus* infections (Margalit and Kavanagh, 2015b). The adoptive transfer of myeloid progenitors protects against *A. fumigatus* infections in chemically induced neutropenic mouse models (BitMansour et al., 2002; BitMansour et al., 2005) and this protection is independent to major histocompatibility complex restrictions (Arber et al., 2005). However, transfused myeloid precursors have to develop into effector cells to fight against the infection. The adoptive transfer of terminally differentiated myeloid cells might not be sustained for long enough due to the short life span of highly differentiated myeloid cells to

completely clear the infection. Thus, the transfusion of mixed myeloid population, which consists of undifferentiated precursors and terminally differentiated effector cells, might be an ideal approach to fight against *A. fumigatus* infections. This approach has not been previously investigated. In our study, we showed that $CD11b^+$ myeloid cells alone rescued cyclophosphamide immunosuppressed mice from lethal a *A. fumigatus* infection. As discussed above, the adoptive transfer of common myeloid progenitors (CMP) and granulocyte-monocyte progenitors (GMP) protected mice against disseminated *A. fumigatus* infections (BitMansour et al., 2002). This protection was only conferred when mice were infected on d+ 7 (67% survival) or d+ 11 (100% survival) following transplantation. None of the mice survived when infected on d+ 3 following transplantation (BitMansour et al., 2002). The adoptive transfer of common myeloid progenitor cells bears the benefit of providing immune-reconstitution for longer time periods, yet their requirement to firstly home to the bone marrow for further development into mature effector cells delays the host defence against *A. fumigatus* infection. The adoptive transfer of bulk $CD11b^+$ myeloid cells is advantageous as it is technically simple to achieve through enrichment with magnetic beads and it proved effective to provide early protection from an otherwise lethal *A. fumigatus* infection. The surface receptor CD11b (integrin alpha M, ITGAM) subunit forms the heterodimeric αMβ2 integrin, which is expressed on a variety of myeloid cells, including neutrophils, monocytes and macrophages. These immune populations play a pivotal role in the defence against lethal *A. fumigatus* lung infections (Balloy and Chignard, 2009). For instance, the myeloid subset of neutrophils has been shown to be critical in controlling *A. fumigatus* infection (Feldmesser, 2006). The timing of neutrophil recruitment is vital for *A. fumigatus* clearance as a small delay in neutrophils arrival leads to increased disease susceptibility (Bonnett et al., 2006; Mehrad et al., 1999a). Macrophages are effective phagocytic cells and are important for fungal pathogen clearance (Bhatia et al., 2011). Finally, circulating monocytes are major precursor cells, once they become activated with the infectious stimulus they develop into macrophages and dendritic cells (monocyte derived dendritic cells) and play

an important role in the elimination of *A. fumigatus* infections (Espinosa et al., 2014). The $CD11b^+$ myeloid cells were transfused into immunosuppressed mice and then infected with *A. fumigatus*; however, it might be interesting to perform future adoptive transfer experiments in mouse models with already established invasive aspergillosis. In real clinical scenarios, immunotherapy might be an important therapy for patients suffering from invasive *A. fumigatus* infections that have not responded to conventional antifungal drugs. Our adoptive $CD11b^+$ myeloid cell transfer experiments provided a basis for the future development of novel myeloid based immunotherapy; however, it is clear that further experiments are required to establish the right dosage of the $CD11b^+$ myeloid cells to treat already established invasive infections and to address the transfusion-related side effects. In contrast to effective infection control following adoptive $CD11b^+$ myeloid cell transfer into otherwise highly susceptible cyclophosphamide treated mice, adoptive $CD11b^+$ myeloid cell transfer did not protect corticosteroid and cyclophosphamide immunosuppressed mice from lethal infection. This clearly suggests that corticosteroid treatment enhances inflammation mediated tissue damage or impairs antifungal functions of myeloid cells. Further studies are warranted to dissect these mechanisms and to address the effects of corticosteroids on antifungal functions of immune cell subsets. Therefore, we addressed the effect of corticosteroids on granulocyte defence functions in invasive *A. fumigatus* lung infections in the second part of this study.

In the first part of this study, we provided a comprehensive analysis of immune cell responses following *A. fumigatus* infection in two clinically relevant immunocompromised mouse models. These models of invasive aspergillosis along with detailed information of the immune cell response following *A. fumigatus* infection might also help in testing the efficacy of non-conventional novel anti-fungal therapies to treat invasive *A. fumigatus* infections, for instance new small molecule inhibitors, antibodies or therapeutic RNAs (Kalleda et al., 2013). We demonstrated a successful control over *A. fumigatus* infection following an adoptive transfer of

CD11b$^+$ myeloid cells into cyclophosphamide immunosuppressed mice and our results confirmed that CD11b$^+$ myeloid cells are major contributors to the fight against *A. fumigatus* lung infections under immunocompromised conditions. These results may further support the development of novel myeloid-based immunotherapies against opportunistic fungal infections.

5.3 Neutrophilic granulocyte defence functions

Neutrophilic granulocytes are among the first non-resident immune cells recruited to the site of infection to eliminate pathogens (Mircescu et al., 2009). Neutrophils exhibit various anti-pathogenic mechanisms, such as phagocytosis, the release of anti-microbial compounds via degranulation and the production of cytokines or chemokines to recruit other immune cells (Kolaczkowska and Kubes, 2013). Importantly, neutrophils sense microbe size and selectively release NETs against large pathogens (Branzk et al., 2014).

NETs are large extracellular web-like filaments that consist of decondensed chromatin decorated with anti-microbial factors. Large pathogens, such as fungal hyphae or bacterial aggregates, selectively trigger NET formation; NETs trap and kill pathogens, including filamentous fungi (Branzk et al., 2014). NET formation and NET-mediated pathogen elimination requires ROS and the granule proteins (myeloperoxidase and neutrophil elastase) (Bell, 2004; Brinkmann et al., 2004). Thus, patients with clinically acquired neutropenia or heritable neutrophilic granulocyte dysfunction or altered neutrophil recruitment to the site of infection or defect in effector functions of neutrophils are at greater risk from lethal *A. fumigatus* infections. Neutrophils are well known for their anti-*A. fumigatus* defence functions (Segal, 2009). In patients with chronic granulomatous disease, defects in the neutrophil function led to an elevated susceptibility to *A. fumigatus* lung infections, this strongly emphasized the role of neutrophils in anti-*A. fumigatus* defence functions (Pollock et al., 1995). In the first part of this study, we showed that although more neutrophils were recruited to the lungs of CT infected

mice than in immunocompetent infected mice, these cells did not confer protection against *A. fumigatus* infections. This prompted us to further investigate the effect of corticosteroids on neutrophil antifungal defence mechanisms.

5.4 Impact of corticosteroids on granulocyte transfusion therapy

Steroid drugs are known for their immunosuppressive effects and are used in a wide range of clinical applications, such as corticosteroid replacement therapy when endogenous production is impaired, to suppress inflammation, to treat inflammatory and autoimmune diseases and mobilization of granulocytes from bone marrow (Shaikh et al., 2012; Strauss, 2015). Recent reports suggest that granulocytes collected from G-CSF/dexamethasone-treated donors are not efficient in treating infections in neutropenic patients (Price et al., 2015). Addition of recombinant G-CSF enhances granulocyte effector functions, such as phagocytosis and NADPH oxidase activation; furthermore, it has been reported that G-CSF also enhances granulocyte chemotaxis (Kitagawa et al., 1987). Treatment of granulocytes with higher doses of corticosteroids impairs *A. fumigatus* hyphae killing and re-induction of these granulocytes with IFN-γ and/ or G-CSF restores the fungal killing capacity (Roilides et al., 1993). Recently, it has been shown that human granulocytes from the G-CSF/dexamethasone-treated donors eliminated *A. fumigatus* hyphae, but not *C. albicans,* in *in vitro* conditions (Gazendam et al., 2016a). However, these studies are completely based on *in vitro* findings. Moreover, individual effects of corticosteroids (without G-CSF treatment) on granulocyte antifungal functions, particularly under *in vivo* conditions, remain poorly defined. In murine models, antibody-mediated depletion of neutrophils lead to uncontrolled *A. fumigatus* growth and subsequent infection-related mortality (Bonnett et al., 2006; Mircescu et al., 2009; Stephens-Romero et al., 2005). On the other hand, the adoptive transfer of neutrophils into immunosuppressed and infected mice might reveal more a convincing role of neutrophils against *A. fumigatus* lung infections. Here, we show that the

transfusion of granulocytes from corticosteroid treated mice did not protect cyclophosphamide immunosuppressed mice against lethal *A. fumigatus* infection in contrast to granulocytes from untreated mice. These results suggest that corticosteroids negatively regulate granulocyte antifungal defence functions.

5.5 Effect of corticosteroids on granulocyte recruitment and cytokine response

Granulocytes from corticosteroid treated mice did not protect cyclophosphamide immunosuppressed mice against lethal *A. fumigatus* infection. To further explore these deleterious effects of corticosteroids on granulocyte transfusions, we hypothesized two different scenarios: firstly, that corticosteroids might induce an impaired recruitment of granulocytes to the site of infection and, secondly, that the defence functions of corticosteroid treated granulocytes might have been impaired. Recruitment of granulocytes to the site of infection plays an important role in fungal clearance (Espinosa and Rivera, 2016). It has been previously reported in a bilayer of cultured human endothelial and bronchial epithelial cells model that higher concentrations of corticosteroids inhibit the migration of enriched neutrophils *in vitro* (van Overveld et al., 2003) or inhibit neutrophil chemotaxis (Shea and Morse, 1978). In contrast to previous *in vitro* findings, granulocytes were efficiently recruited to the site of infection in cyclophosphamide immunosuppressed and infected mice that received the granulocytes from corticosteroid treated mice. We also showed that corticosteroid treated and infected mice displayed no granulocyte recruitment defect. These results suggest that corticosteroid treatment does not play a role in granulocyte recruitment after *A. fumigatus* infection *in vivo*.

The lung cytokine/chemokines play an important role in the elimination of fungal infections by enhancing the immune cell recruitment to the site of infection or by modulating the effector functions of recruited cells (Karupiah, 2003; Schelenz et al., 1999). The IL-1 gene cluster codes

for two important pro-inflammatory cytokines such as IL-1α and IL-1β. The IL-1 family cytokines, IL-1α and IL-1β both play an important role in the recruitment of neutrophils to the site of infection. IL-1 receptor antagonists (IL-1Ra) competitively bind to IL-1RI, thereby preventing the binding of IL-1α and IL-1β (Garlanda et al., 2013). Alveolar macrophages induce production of IL-1β during invasive aspergillosis, as soon as they sense the *A. fumigatus* conidia. Increased IL-1β levels aids in the neutrophil infiltration to the infected lungs (Nicholson et al., 1996). Recently, it is shown that 1α and IL-1β play non-redundant roles against an *A. fumigatus* infection. Moreover, IL-1α, but not IL-1β, is important for optimal immune cell recruitment. On the other hand, IL-1β is essential for the activation of anti-fungal activity of macrophages (Caffrey et al., 2015). IL-1α signalling plays an important role in the elimination of *A. fumigatus* lung infections by enhancing the production of CXCL1. Il1r1-deficient mice are more susceptible to *A. fumigatus* lung infections (Caffrey et al., 2015). *A. fumigatus* lung infection activates the adaptor proteins, CARD9 and MyD88, which aids in chemokine induced granulocyte migration to the site of infection and neutrophil-mediated fungal clearance (Jhingran et al., 2015). The MyD88-deficient mice showed reduction in neutrophil chemokines such as CXCL1 and CXCL5, which leads to delayed lung neutrophil infiltration and elevated pulmonary fungal damage. Exogenous administration of CXCL1 restores the murine antifungal defences in MyD88-deficient mice, which highlight the role of CXCL1 in anti-*A. fumigatus* defence (Jhingran et al., 2015). Further, transient over expression of a CXCR2 ligand, CXCL1/KC in murine lungs displayed lower fungal burden and increased *A. fumigatus* clearance (Mehrad et al., 2002). IL-1α levels increased significantly after *A. fumigatus* infection in cyclophosphamide immunosuppressed mice that received a control neutrophil transfusion or a corticosteroid treated neutrophil transfusion. However, IL-1α and CXCL1 levels were significantly lower in cyclophosphamide immunosuppressed and infected mice that had received corticosteroid treated neutrophil transfusion when compared to IL-1α and CXCL1 levels in mice that had received control neutrophil transfusion. These results suggest that reduction in IL-1α and CXCL1 levels

contributed to impaired fungal clearance and survival defects in cyclophosphamide immunosuppressed mice that received a corticosteroid treated neutrophil transfusion. Expression levels of Dectin-1 on myeloid cells play an important role in myeloid-mediated fungal clearance (Brown and Crocker, 2016). Dectin-1-deficient mice are highly susceptible to fungal infection mediated by an impaired production of the required cytokines and chemokines to combat fungal infection. The reduction of inflammatory cytokines, such as IL-1β, TNF-α, CCL3, CCL4, and CXCL1, leads to a reduced pulmonary neutrophil recruitment, a reduced ROS production and an elevated pulmonary *A. fumigatus* invasion. Dectin-1 deficiency diminishes the production of pro-inflammatory mediators by alveolar macrophages and reduces lung IL-17 levels against pulmonary fungal infection (Werner et al., 2009). Dectin-1 is significantly reduced in granulocytes treated with corticosteroids. Dectin-1 lower expression levels on corticosteroid treated granulocytes might have contributed to impairment in production of important cytokines in cyclophosphamide immunosuppressed mice that received a corticosteroid treated neutrophil transfusion. CXCL5 is a CXC chemokine, which play an important role in neutrophil infiltration to the site of inflammation by interacting with the chemokine receptor CXCR2 (Persson et al., 2003). CXCL5 is one of the important factor in regulation of neutrophil homeostasis and play an important role in mediating neutrophil influx to the lung during the inflammatory reaction (Mei et al., 2010). CXCL5 play an important role in systemic candidiasis by interacting with CXCR1 receptor and both CXCR1 and CXCL5 are highly induced during the *Candida* infection (Swamydas et al., 2016). Recent studies show that expression levels of chemotactic cytokines including CXCL5 are highly upregulated in human DCs after exposure to *A. fumigatus* conidia (Morton et al., 2014). MIP1α is a chemokine, popularly known as CCL3 plays an important role in recruitment and activation of granulocytes (Wolpe et al., 1988). In neutropenic hosts, MIP1α is a key factor in host defence to invasive aspergillosis and plays an important role in recruitment of monocyte or macrophage populations to site of infection (Mehrad et al., 2000). MIP1α interacts with both CCR1 and CCR5 depending on route of infection of *A. fumigatus*

(Gao et al., 1997). CCL17 is reported to play an immunosuppressive role in invasive aspergillosis, systemic neutralization of CCL17 in CCR4-deficient mice increased the levels of CCL2 and improved mice survival against invasive aspergillosis (Carpenter and Hogaboam, 2005). MCP1 is highly induces in pulmonary *A. fumigatus* infection and neutralization of MCP1 resulted in elevated severity to pulmonary infection (Morrison et al., 2003). MCP1 is mainly involved in recruitment of circulating monocytes to the site of inflammation and infection (Shi and Pamer, 2011). The above mentioned important cytokines/ chemokines, such as CXCL5, MIP1α, CCL17 and MCP1 levels, were also significantly reduced in cyclophosphamide immunosuppressed and infected mice that have received a corticosteroid treated neutrophil transfusion when compared to CXCL5, MIP1α, CCL17 and MCP1 levels in mice that had received a control neutrophil transfusion. These results suggest that corticosteroids impair the antifungal functions of granulocytes by suppressing the levels of important cytokines/ chemokines which are required for the recruitment or effector functions of immune cells.

5.6 Corticosteroids and neutrophilic granulocyte antifungal functions

Corticosteroids are known for their immunosuppressive effect (Barnes, 2006) and that they inhibit neutrophil apoptosis (Liles et al., 1995). There are various mechanisms involved in the inhibition of neutrophil apoptosis by corticosteroids, such as elevation in Bcl-2 family members (Bailly-Maitre et al., 2001; Bailly-Maitre et al., 2002; Yamamoto et al., 1998); stabilization (Messmer et al., 2001) and induction (Wen et al., 1997) of inhibitors of apoptosis (IAPs); activation of NF-κB (Evans-Storms and Cidlowski, 2000; Mendoza-Milla et al., 2005); inhibition of apoptosis extrinsic pathway proteins (Baumann et al., 2005; Oh et al., 2006); and induction of serum and glucocorticoid activated kinase-1 (SGK-1) and MAPK phosphatase-1 (MKP-1) (Mikosz et al., 2001; Wu et al., 2005). Neutrophil phagocytic functions or other bactericidal properties are not significantly impaired (Schleimer et al., 1989) but phagocytic function might

be inhibited at high doses of corticosteroids (Herzer and Lemmel, 1980; Jones et al., 1983). Nevertheless, the effect of corticosteroids on neutrophil defence functions against *A. fumigatus* has largely remained elusive. Therefore, we investigated different defence functions of granulocytes from corticosteroid treated mice or corticosteroid treated human granulocytes. Based on published evidence we hypothesized that corticosteroids modulate the anti-*A. fumigatus* defence functions of neutrophils. In contrast to previous findings, we demonstrated that granulocytes collected from corticosteroid treated mice or human granulocytes treated with corticosteroids were significantly compromised in the phagocytosis of *A. fumigatus* conidia or germlings. Furthermore, we also showed that granulocytes collected from corticosteroid treated mice or human granulocytes treated with corticosteroids were significantly compromised in *A. fumigatus* elimination. These results indicate that corticosteroids reduce phagocytosis and fungal killing by mice or human granulocytes and negatively impact the granulocyte transfusions. The reduction in phagocytosis and fungal killing by corticosteroid treated mice or human granulocytes was correlated with compromised levels of β-glucan receptor Dectin-1 expression levels. Several studies have reported that Dectin-1 plays an important role in fungal recognition and the subsequent phagocytosis and killing of fungi (Dambuza and Brown, 2015; Legentil et al., 2015). Corticosteroids reduced the cell surface expression levels of Dectin-1 and subsequently resulted in the reduction of phagocytosis and killing capacity of granulocytes.

The most important anti-pathogenic function of neutrophils is to release their DNA decorated with anti-microbial proteins and form web-like networks referred to as neutrophil extracellular traps (NETs) to attack large bacterial and fungal pathogens (Brinkmann et al., 2004; Bruns et al., 2010; McCormick et al., 2010). NETs have been demonstrated to exhibit both fungicidal and fungi-static activity (Brinkmann and Zychlinsky, 2012). Recently, it has been proposed that neutrophils can discriminate between large pathogens and small pathogens and selectively form NETs (Branzk et al., 2014). Our results indicate that corticosteroid treatment can interfere with

the NETosis function of mice or human granulocytes *in vitro* and *in vivo*, which plays a pivotal role in the elimination of large bacterial and fungal pathogens. Corticosteroids are widely prescribed in patients with various conditions and a loss of NETosis function may serve as an essential risk factor for bacterial and invasive fungal infections. It has been proposed that corticosteroids prolong neutrophil survival by inhibiting the neutrophil apoptosis (Saffar et al., 2011). NETosis is considered as a form of cell death and the inhibition of neutrophil apoptosis could be the possible reason for impairment of NETosis in corticosteroid treated neutrophils. In line with previous results, we showed that corticosteroids elevate the expression levels of neutrophil survival gene transcripts, such as Mcl1 and PIK3rl, in granulocytes collected from corticosteroid treated mice. There are several studies that propose that corticosteroids inhibit neutrophil apoptosis by various mechanisms, such as through the up-regulation of anti-apoptotic Bcl-2 family members (Bailly-Maitre et al., 2001; Bailly-Maitre et al., 2002; Yamamoto et al., 1998) and the activation of NF-κB (Evans-Storms and Cidlowski, 2000; Mendoza-Milla et al., 2005). Furthermore, a growing amount of literature supports the idea that NETosis involves the production of reactive oxygen species (ROS) (Guimaraes-Costa et al., 2012; Tina Kirchner, 2012). Our results also support the idea that ROS is involved in the NETosis function of neutrophils, since granulocytes from corticosteroid treated mice were significantly compromised in ROS production.

5.7 $CD11b^+$ myeloid cells from CT mice shows protective effect in *A. fumigatus* lung infections

We showed that granulocytes from corticosteroid treated mice were impaired in several anti-*A. fumigatus* defence functions *in vitro* and *in vivo*. On the other hand, we also demonstrated that corticosteroids did not diminish defence functions of pooled $CD11b^+$ myeloid cells, since adoptive transfer of $CD11b^+$ myeloid cells enriched from corticosteroid mice also rescued the

cyclophosphamide immunosuppressed mice against *A. fumigatus* infection. In addition to neutrophils, $CD11b^+$ myeloid cells are a heterogeneous population of immune cells, including monocytes and macrophages. Monocytes are circulating precursors for dendritic cells and macrophages. Corticosteroids appeared not to impair these immune cell populations and their anti-*A. fumigatus* defence functions. Moreover, *in vitro* expanded bone marrow derived macrophages or dendritic cells showed efficient fungal killing irrespective to prior corticosteroid treatment. These results support the conclusion that besides neutrophils, monocytes and their derived populations also play a vital role in defending against lethal *A. fumigatus* lung infections.

In the second part of this study, we conclude that corticosteroid treatment of granulocyte donors for increasing neutrophil yields or patients with ongoing corticosteroid treatment could result in deleterious effects on granulocyte antifungal functions, thereby limiting the benefit of granulocyte transfusion therapies against invasive fungal infections. Importantly, corticosteroid treatment impairs the NETosis function of neutrophils and it might serve as an additional risk factor for opportunistic bacterial and fungal infections. On the other hand, corticosteroids might be useful for controlling NET-mediated tissue destruction in several clinical situations with inflammatory or autoimmune conditions. Our results may support to develop improved myeloid-based immunotherapy strategies against opportunistic fungal infection.

6 Graphical summary

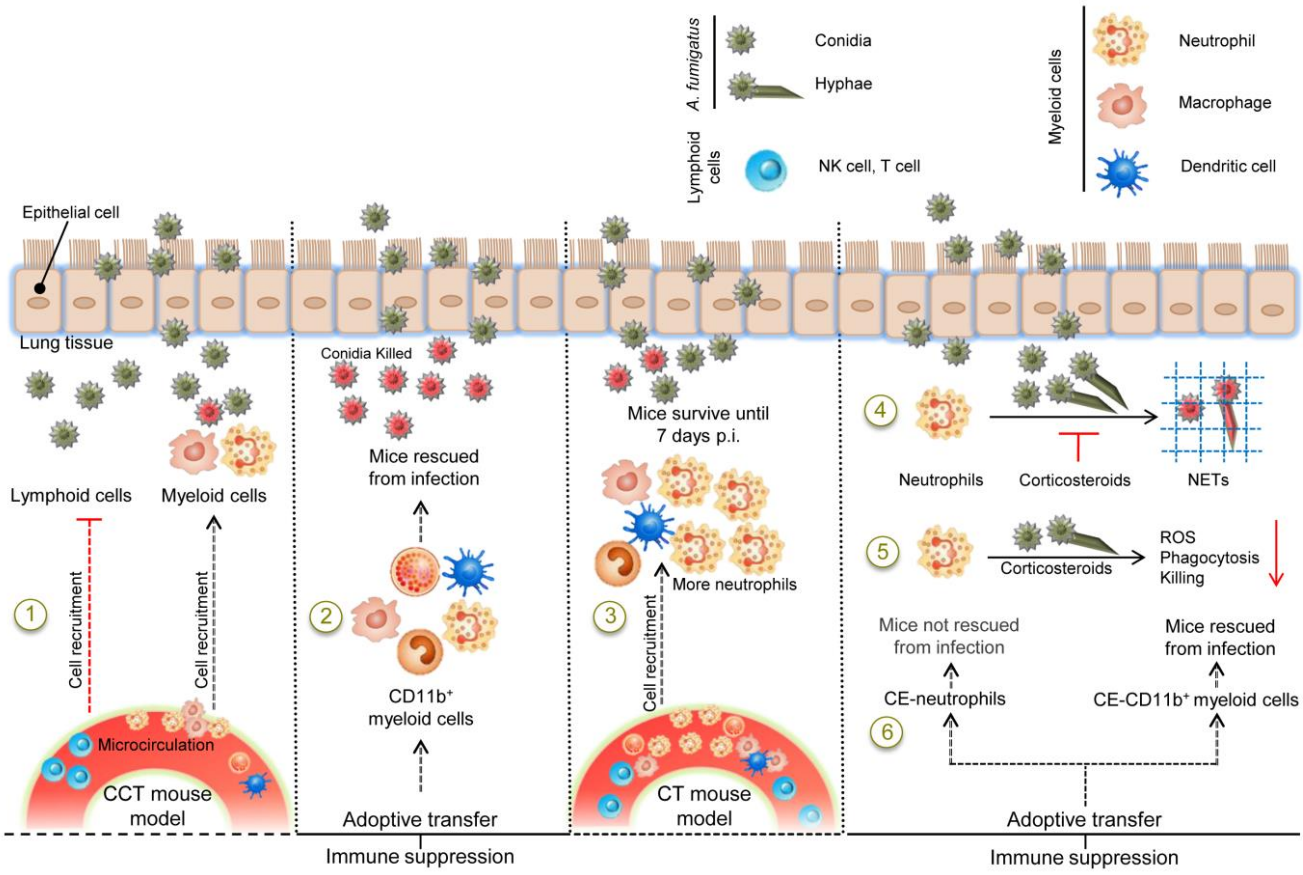

Figure 30. Visual summary. 1. *A. fumigatus* infected lungs recruit strongly myeloid but not lymphoid cells in CCT mouse model. 2. Adoptive transfer of CD11b$^+$ myeloid cells completely protected cyclophosphamide immunosuppressed mice from lethal *A. fumigatus* infection. 3. *A. fumigatus* infected lungs of corticosteroid treated (CT) mice recruited more neutrophils than untreated mice; yet, CT neutrophils could not confer protection. 4. Corticosteroid treatment impairs the vital function of neutrophils to form NETs against *A. fumigatus* conidia or hyphae. 5. Corticosteroid treatment impaired ROS production, phagocytosis and fungal killing. 6. Adoptive transfer of Corticosteroid Exposed (CE) (neutrophils from corticosteroid treated mice) neutrophils does not protect cyclophosphamide immunosuppressed mice from *A. fumigatus* infection, whereas CE-CD11b$^+$ myeloid cells (myeloid cells enriched from corticosteroid treated mice) can protect cyclophosphamide immunosuppressed mice against infection.

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Acknowledgments

First, I want to thank Prof. Dr. Dr. Andreas Beilhack for giving me the opportunity to perform my Ph.D thesis under his supervision and introducing me into the exciting world of immunology of fungal infections. I am very grateful for his support, his encouragement and freedom to develop this project. Furthermore, he gave me the opportunity to broaden my horizons by allowing me to attend several international conferences and scientific courses.

I am grateful to all the members of the Beilhack lab for providing a very nice working environment, for their help in lung single cell preparation, for helpful and constructive discussions, for excellent technical assistance, and especially for their friendliness. Special thanks to Aspergillus team, Jorge Amich, Berkan Arslan and Katharina Mattenheimer.

I am grateful to the graduate school of life sciences (GSLS, Würzburg University) for travel grants, scientific and transferable skills, for their personal support throughout the Ph.D programme. I want thank Prof. Dr. Thomas Hünig for giving me the great opportunity to be part of the Graduate College Immunomodulation (GK520).

Thanks to the DFG FungiNet Tranregio 124 research consortium for funding and excellent retreats, stimulating scientific discussions and fruitful collaborations.

I want to thank my thesis committee, Prof. Andreas Beilhack, Prof. Jürgen Löffler, Dr. Katrin Heinze and Prof. Axel Brakhage for their constant guidance and excellent support throughout my Ph.D.

Finally, I want to thank my family, especially my father Pandurangaswamy and my wife Spoorthi for their love, patience and great support. Thanks to my cute daughter Anvi for allowing me to write my thesis at home.

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Research Experience

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Publications

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- Kalleda N, Naorem A and Manchikatla RV. (2013). Targeting fungal genes by diced siRNAs: A rapid tool to decipher gene function in *Aspergillus nidulans*. *PLoS One* 8(10):e75443.
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Research awards

- Abstract achievement award, American Association of Hematology (ASH), 2016, December 3 – 6th, San Diego, California, USA.
- Full scholarship (Travel grant) for attending the 7th Advances Against Aspergillosis conference, March 3 – 5th, 2016, Manchester, UK.
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Oral Presentations

- Oral presentation at 7th Advances Against Aspergillosis, Thursday 3rd – Saturday 5th March 2016, Manchester, UK.
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- Oral presentation at 3rd Mol Micro Meeting, 7-9 May 2014, IMIB Würzburg, Germany.

- Oral presentation at International conference on Biotechnology in Human welfare, 7–9th February, 2013, Warangal, India.
- Oral presentation at International conference on Plant Biotechnology and Molecular Medicine, October 18-20, 2013, New Delhi, India.

International conferences

- 7th Advances against Aspergillosis, 3-5th March 2016, Manchester, UK. Talk: 'Corticosteroids impair neutrophils but not other CD11b+ myeloid cells to control pulmonary *Aspergillus fumigatus* infection'.
- Eureka, 10th International symposium, 14-15th October, 2015, Wuerzburg, Germany. Poster presentation: 'Myeloid cells act as major host defence against pulmonary *Aspergillus fumigatus* infections under immunocompromised conditions'.
- 49th German speaking mycological society and 1st international symposium of the CRC/Trasregio FungiNet, 16-19th September 2015, Jena, Germany. Talk: 'Corticosteroids impair 'granulocyte transfusion therapy' by targeting neutrophil antifungal functions'.
- 4th European Congress of Immunology, 6-9th Septmeber 2015, Vienna, Austria. Poster presentation: Myeloid cells act as major host defence against pulmonary *Aspergillus fumigatus* challenge under different immunosuppressive regimens'
- Gorden research conference: Immunology of of fungal infections, 17-23rd January 2015, Houston, USA. Poster presentation: 'Myeloid cells act as major host defence against pulmonary *Aspergillus fumigatus* challenge under different immunosuppressive regimens'.
- Eureka, 9th International symposium, 14-15th October, 2014, Wuerzburg, Germany. Poster presentation: 'Defining the early in vivo immune response after pulmonary *Aspergillus* challenge under different immune suppressive regimens'.
- 3rd Mol Micro Meeting, 7-9th May 2014, Wuerzburg, Germany. Talk: 'Targeting fungal genes by diced siRNA'.
- 6th Advances against Aspergillosis, 27th February to 1st March 2014, Madrid, Spain. Poster presenation: 'Silencing of fungal genes by diced siRNA'.

Affidavit (Eidesstattliche Erklärung)

I hereby declare that my thesis entitled ‘Spatiotemporal analysis of immune cell recruitment and neutrophil defence functions in *Aspergillus fumigatus* lung infections’ is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Hiermit erkläre ich an Eides statt, die Dissertation „Zeitliche und örtliche Analyse der Immunzellrekrutierung und der durch Neutrophile Granulozyten vermittelten Abwehr gegen *Aspergillus fumigatus* Infektionen der Lunge“ eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg……

Date Signature