

Role of myeloid-derived suppressor cells in pathogenic fungal infections

Dissertation

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Anurag Singh
aus Allahabad, Indien

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Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
AMP	Antimicrobial Peptides
APC	Antigen Presenting Cells
ASC	Apoptosis Associated Speck-Like Protein
BM	Bone Marrow
CARD	Caspase-recruitment Domain
CEA	Carcinoembryonic Antigen
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CFU	Colony Forming Unit
CLR	C-type Lectin Receptors
CMC	Chronic Mucocutaneous Candidiasis
DC	Dendritic Cells
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GvHD	Graft-versus-host disease
HK	Heat-Killed
IFN	Interferon
IMCs	Immature Myeloid Cells
i.p.	Intraperitoneally
i.v.	Intravenously
IL-17RA	Interleukin-17 Receptor A
IL-1R	Interleukin-1 Receptor
MAMP	Microorganism-associated molecular patterns
MR	Mannose Receptor
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NLR	Nod-Like Receptors
OPC	Oropharyngeal Candidiasis
PAMP	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline

PMA	Phorbol Myristate Acetate
PMNs	Polymorphonuclear neutrophils
PMNL	Polymorphonuclear leucocytes
PRR	Pattern Recognition Receptors
Rag	Recombination Activating Gene 1
ROS	Reactive oxygen species
STAT3	Signal Transducer and Activator of Transcription 3
TCR	T-Cell Receptor
Th	T helper
TLR	Toll-Like Receptor
Tregs	Regulatory T Cells
WT	Wild type
YNB	Yeast Nitrogen Base
YPD	Yeast Peptone Dextrose

Summary

Myeloid-derived suppressor cells (MDSCs) have been characterized in cancer patients and tumor bearing mice based on their ability to suppress T-cell responses. Some recent studies also showed the impact of MDSC mediated immunomodulation during bacterial infections. However their role in infectious fungal diseases has not been described yet. Although, healthy individuals are also continuously exposed to fungi during their daily activities for example by inhaling fungal spores, they do not develop any pro-inflammatory anti-fungal immune responses. The underlying mechanisms for this tolerance are incompletely understood. We hypothesized that fungal infections induce MDSCs that modulate disease outcome.

We used a set of different methods that included *in vitro* studies, *in vivo* mouse models, and human patients to demonstrate that the human-pathogenic fungi *Aspergillus fumigatus* and *Candida albicans* induce a distinct subset of neutrophilic myeloid-derived suppressor cells (MDSCs), which were able to functionally suppress. We analyzed the effect of the human-pathogenic fungi *A. fumigatus* and *C. albicans* on immune cells and noticed the expansion of a distinct cell population that was based on lineage markers and side-scatter granularity. Functionally, fungi-induced myeloid cells strongly suppressed CD4⁺ and CD8⁺ T cell proliferation in a dose-dependent manner which defines MDSCs. In addition to regulating adaptive immunity, fungi-induced MDSCs also suppressed innate natural killer (NK) cell responses. To assess whether fungi induce a similar cell population *in vivo*, we quantified MDSCs in healthy controls and patients with fungal infections and challenged mice with *A. fumigatus* or *C. albicans*. Both approaches demonstrated that MDSCs accumulated in infected patients and mice. Fungi-induced MDSCs expressed neutrophilic markers in both human and mice (human: CD11b⁺CD66b⁺CD14⁻, mice: CD11b⁺Ly6G⁺) resembling neutrophilic MDSCs as described previously. Further *in vitro* studies using blocking agents, immunodeficient patient samples and knockout mouse models showed that pathogenic fungi induced neutrophilic MDSCs through the pattern recognition receptor Dectin-1 and its downstream adaptor protein CARD9. We further analyzed the mechanism underlying fungal MDSC induction, and found that it was dependent on reactive oxygen species (ROS) production and involved Caspase-8 activity and interleukin-1. Furthermore, we used a murine model to assess the impact of MDSCs during systemic candidiasis and pulmonary aspergillosis *in vivo*. After the adoptive transfer of bone marrow-

derived neutrophilic MDSCs from healthy mice to the invasive *C. albicans* model, recipient mice showed better health conditions and increased survival rate. This protective effect of MDSCs was not present in *A. fumigatus* infection model.

In summary, these studies uncover a new innate immune mechanism by which pathogenic fungi regulate host defence by inducing neutrophilic MDSCs, suggesting that this mechanism might play a broader and very important role in balancing inflammation during host-pathogen interactions. These findings also define a novel regulatory role of MDSCs during fungal infections, which might be clinically relevant in developing novel immunotherapeutic strategies for patients.

Zusammenfassung

Myeloid-derived suppressor cells (MDSC) wurden in Krebspatienten und in Tumormausmodellen durch ihre Fähigkeit T-Zellantworten zu supprimieren beschrieben. Zudem konnten einige kürzlich erschienene Studien zeigen, dass MDSCs auch während bakterieller Infektionen eine Rolle spielen. Bisher wurde allerdings der Effekt von MDSCs und ihren Rolle während infektiöser Pilzkrankheiten noch nicht untersucht. Ein Hinweis auf eine Beteiligung von MDSCs ist jedoch die Beobachtung, dass es immunkompetente Individuen gibt, die trotz ständigem Kontakt mit Pilzen keine pro-inflammatorische Immunantwort zeigen, die gegen den Pilz gerichtet ist. Dennoch sind die zugrunde liegenden Mechanismen, die zu dieser Toleranz führen, noch nicht vollständig verstanden. Daher lautet unsere Hypothese, dass MDSCs während Pilzinfektionen induziert werden und dass diese wiederum den Krankheitsverlauf beeinflussen.

Durch Kombination von *in vitro* Studien, Mausmodellen und humanen Patienten, konnten wir zeigen, dass die human-pathogenen Pilze *Aspergillus fumigatus* und *Candida albicans* einen Untertyp von MDSCs - neutrophile MDSCs - induzieren, welche in der Lage sind, T-Zellantworten zu unterdrücken. Durch die Untersuchung des Effekts der humanpathogenen Pilze *A. fumigatus* und *C. albicans* auf Immunzellen konnten wir, basierend auf deren Zelltypmarker und Granularität, die Expansion einer besonderen Zellpopulation identifizieren. Diese Pilz-induzierten MDSCs sind funktional und können daher CD4⁺ und CD8⁺ T-Zellproliferation dosisabhängig stark supprimieren, was ein Charakteristikum von MDSCs darstellt. Neben der Fähigkeit die adaptive Immunantwort zu regulieren, unterdrücken Pilz-induzierte MDSCs auch natürliche Killerzellen (NK-Zellen), die einen Teil der angeborenen Immunantwort darstellen. Um zu untersuchen, ob Pilze auch entsprechende Zellpopulation *in vivo* induzieren können, wurden MDSCs in gesunden Kontrollindividuen sowie in Patienten mit Pilzinfektionen quantifiziert. Zudem wurden Mäuse mit *Aspergillus* intranasal oder mit *C. albicans* intravenös infiziert. Beide Experimente zeigten, dass MDSCs in infizierten Patienten, wie auch in den infizierten Mäusen, erhöht sind. Die pilz-induzierten MDSCs exprimieren neutrophile Marker (human CD11b⁺CD66b⁺CD14⁻, maus: CD11b⁺Ly6G⁺), welche den neutrophilen MDSCs, wie vorher beschrieben, entspricht. Sowohl in den *in vitro* als auch den *in vivo* Experimenten werden neutrophile MDSCs durch die pathogenen

Pilze über den Pattern-recognition Rezeptor Dectin-1 und dessen nachgeschaltetes Adaptorprotein CARD9 induziert. Zudem ist die Induktion von MDSCs durch Pilze abhängig von der Produktion von ROS, wie auch der Aktivität von Caspase-8 und Interleukin-1. Des weiteren wurde ein Mausmodell genutzt, um den Einfluss von MDSCs während einer systemischen Candidose und einer pulmonaren Aspergillen Infektion *in vivo* zu untersuchen. Nach dem adoptiven Transfer von neutrophilen MDSCs, die im Knochenmark generiert wurden, von gesunden Wildtyp-Mäusen zum invasiven *C. albicans* Model, zeigten die Empfängermäuse eine bessere Fitness und eine höhere Überlebensrate. Dieser protektive Effekt der MDSCs konnte im Infektionsmodell mit *Aspergillus* nicht gezeigt werden.

Zusammenfassend wurde in dieser Studie ein neuer Mechanismus der angeborenen Immunantwort aufgedeckt, bei welchem pathogene Pilze die Immunabwehr des Wirts regulieren, indem sie neutrophile MDSCs induzieren. Dies lässt den Schluss zu, dass dieses Zusammenspiel eventuell eine sehr wichtige Rolle in der Balance der Entzündungsreaktion während der Wirt-Pathogen Interaktionen spielt. Diese Ergebnisse geben MDSCs auch eine neue regulierende Bedeutung im Verlauf von Pilzinfektionen, was von klinischer Relevanz in Hinsicht auf die Entwicklung neuer immuntherapeutischer Strategien zur Behandlung von Patienten sein könnte.

Publications

Original publications

Melanie Carevic, **Anurag Singh**, Nikolaus Rieber, Olaf Eickmeier, Matthias Griese, Andreas Hector, and Dominik Hartl.

CXCR4⁺ granulocytes reflect fungal cystic fibrosis lung disease. European respiratory Journal 2015, Accepted.

Rieber N, **Singh A**, Öz H, Carevic M, Bouzani M, Amich J, Ost M, Ye Z, Ballbach M, Schäfer I, Mezger M, Klimosch SN, Weber A, Handgretinger R, Krappmann S, Liese J, Engeholm M, Schüle R, Salih HR, Marodi L, Speckmann C, Grimbacher B, Ruland J, Brown GD, Beilhack A, Loeffler J and Hartl D.

Pathogenic Fungi Regulate Immunity by Inducing Neutrophilic Myeloid-Derived Suppressor Cells. Cell Host Microbe 03/2015.

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A screening assay based on host-pathogen interaction models identifies a set of novel antifungal benzimidazole derivatives. Antimicrobial Agents and Chemotherapy. Oct 2011

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High-throughput-screening-based identification and structure-activity relationship characterization defined (S)-2-(1-aminoisobutyl)-1-(3-chlorobenzyl) benzimidazole as a highly antimycotic agent nontoxic to cell lines. Journal of Medicinal Chemistry. Oct 2011

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Oral presentations

A. Singh, W. Abu Rayyan, L. Masri, H. Schulenburg, K. Schröppel.

Candida albicans colonizes the intestinal tract of Caenorhabditis elegans and establishes a persistent lethal infection

62. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), Hannover, 28-31 March 2010. *BIOspektrum Sonderausgabe 2010*, p. 87.

A. Singh, W. Abu Rayyan, T. Dietsche, H. Eickhoff, K.-H. Wiesmüller, C.A.

Kumamoto, K. Schröppel

Caenorhabditis elegans as a host animal model system to study morphology and virulence of major human pathogenic Candida species and to screen novel antifungal benzimidazole compounds.

64. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), Hamburg 30 Sep - 03 Oct 2012. *International Journal of Medical Microbiology*, Sept 2012, Vol. 302, Suppl. 1, p. 113.

Anurag Singh.

Regulation of MDSCs by pathogenic fungi.

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UV-activated tetracycline-induced fluorescence of Candida albicans.

61. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), Göttingen, 20-23 September 2009. International Journal of Medical Microbiology, Sept. 2009, Vol. 299, Suppl. 1, p. 14

A. Singh, W. Abu Rayyan, T. Dietsche, H. Eickhoff, K.-H. Wiesmüller, K. Schroppel. *Caenorhabditis elegans as a host model system to screen novel antifungal benzimidazole derivatives.*

63. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), Essen, 25-28 September 2011. International Journal of Medical Microbiology, Sept 2011, Vol. 301, Suppl. 1, p. 11.

Schröppel K., **Singh A.**, Dietsche T., Eickhoff H., Wiesmüller K.-H., Fischer K., and Abu Rayyan W.

A C-terminal hyphal activation motif of the transcription factor Tec1p is essential for biofilm formation and nematode infection in Candida albicans.

FINSysB Conference Candida Infection Biology– fungal armoury, battlefields and host defences. Aquafredda di Maratea, Italy, 10-14 October, 2011

N. Rieber, **A. Singh**, M. Carevic¹, H.-H. Öz, J. Ruland, G.D. Brown, J. Löffler, D. Hartl

Pilze induzieren CXCR4⁺ granulozytäre myeloide Suppressorzellen.

48. Wissenschaftlichen Tagung der Deutschsprachigen Mykologischen Gesellschaft e. V., Salzburg, Austria, 04-06. September 2014.

Poster prize

Erklärung nach § 5 Abs. 2 Nr. 7 der Promotionsordnung der Math.-Nat. Fakultät

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Declaration according to § 5 Abs. 2 No. 7 of the PromO of the Faculty of Science

-Share in publications done in team work-

Name: Anurag Singh

List of Publications:

1. Rieber N, **Singh A**, Öz H, Carevic M, Bouzani M, Amich J, Ost M, Ye Z, Ballbach M, Schäfer I, Mezger M, Klimosch SN, Weber A, Handgretinger R, Krappmann S, Liese J, Engholm M, Schüle R, Salih HR, Marodi L, Speckmann C, Grimbacher B, Ruland J, Brown GD, Beilhack A, Loeffler J and Hartl D (2015).

Pathogenic Fungi Regulate Immunity by Inducing Neutrophilic Myeloid-Derived Suppressor Cells. Cell Host Microbe 03/2015.

2. Eickmeier O, Hector A, **Singh A**, Chotirmall SH and Hartl D (2014).

Fungi in cystic fibrosis: recent findings and unresolved questions. Current Fungal Infection Reports 12/2014

3. Melanie Carevic, **Anurag Singh**, Nikolaus Rieber, Olaf Eickmeier, Matthias Griese, Andreas Hector, and Dominik Hartl (2015).

CXCR4⁺ granulocytes reflect fungal cystic fibrosis lung disease. European respiratory Journal 2015, Accepted.

Nr.	Accepted for publication yes/no	Number of all authors	Position of the candidate in list of authors	Scientific ideas of candidate (%)	Data generation by candidate (%)	Analysis and Interpretation by candidate (%)	Paper writing by candidate (%)
			<i>Optional, the declaration of the own share can also be done in words, please add an extra sheet.</i>				
1	Yes	27	2				
2	Yes	5	3				
3	Yes	7	2				



I certify that the above statement is correct.

Date, Signature of the candidate

I/We certify that the above statement is correct.

Date, Signature of the doctoral committee or at least of one of the supervisors

Contribution to the publications in thesis

Paper 1:

Pathogenic Fungi Regulate Immunity by Inducing Neutrophilic Myeloid-Derived Suppressor Cells

I performed MDSC *in vitro* assays, patient sample studies with assistance of I. Schäfer and *in vivo* murine infection studies with H. Öz., and M. Carevic. I conducted various cytokine assays and Bioplex during this study. I also performed data analyses, made figures and contributed in writing and proof reading of the manuscript. N. Rieber and D. Hartl designed this study, supervised experiments and wrote the manuscript. Other authors provided various important materials and contributed to the manuscript. D. Hartl supervised this study and wrote the manuscript.

Paper 2:

Fungi in cystic fibrosis: recent findings and unresolved questions

I contributed in writing and proof reading of this manuscript with other authors of the paper. D. Hartl supervised this study and wrote the manuscript.

Paper 3:

CXCR4⁺ granulocytes reflect fungal cystic fibrosis lung disease

Along with M. Carevic and D. Hartl, I performed experiments for this study, analyzed data, discussed the data and contributed in writing. Other authors provided related materials and contributed to the manuscript. D. Hartl supervised this study and wrote the manuscript.

1. Introduction

1.1 *Candida albicans* and *Aspergillus fumigatus*: "The Opportunistic,, fungal pathogens

Candida albicans is a commensal diploid fungus of humans as well as other mammals and a part of the normal flora of human oral, gastrointestinal, vaginal and cutaneous surfaces (1). Almost about 80% of healthy individuals are colonized with *C. albicans* with no harmful effect. However when the host encounters an immunocompromised state such as prolonged antibiotic therapy, impaired immunity due to chemotherapy, or organ transplantation, *C. albicans* may turn highly pathogenic by invading tissues causing painful cutaneous or subcutaneous infections like vaginitis, oral thrush, diaper rash, conjunctivitis, and infections of the nail and rectum, or can even spread via blood stream to various body organs causing hepatosplenic abscesses, myocarditis or pulmonary infections, resulting in a fatal systemic candidiasis infection. It is generally believed that hyphal cells expressing cell-wall proteins that facilitate adhesion to human tissues are important for tissue invasion and escape from phagocytosis mediated by neutrophils or macrophages. On the other hand, the yeast form is known to be important for dissemination of the pathogen through the blood stream (Figure 1.1). Therefore, it is highly likely that the ability to switch between the morphological forms is very important for *C. albicans* infection and virulence (3).

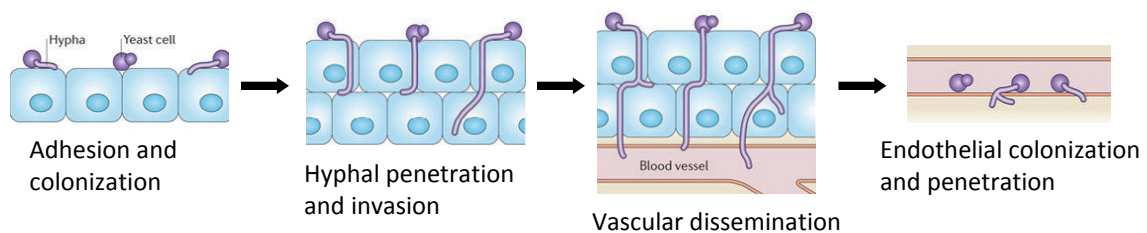


Figure 1.1 *C. albicans* morphotypes and role in tissue invasion

Adapted from Gow *et al* 2012 © Nature publishing group

The several steps in tissue invasion by *Candida albicans*, for a stylized epithelial cell surface: adhesion to the epithelium; epithelial penetration and invasion by hyphae; vascular dissemination, which involves hyphal penetration of blood vessels and seeding of yeast cells into the bloodstream; and, finally, endothelial colonization and penetration during disseminated disease.

Recently, *Aspergillus fumigatus* has been recognized as the second most common causative agent of fungal infection after *C. albicans*. In individuals with altered lung

function such as asthma and cystic fibrosis patients, *A. fumigatus* can cause allergic bronchopulmonary aspergillosis, a hypersensitive response to fungal components. Those most at risk for this life-threatening disease are individuals with hematological malignancies such as leukemia, solid-organ and hematopoietic stem cell transplant patients, patients on prolonged corticosteroid therapy, which is commonly utilized for the prevention and/or treatment of graft-versus-host disease in transplant patients, individuals with genetic immunodeficiencies such as chronic granulomatous disease (CGD) (4). Mortality rates range from 40% to 90% in high-risk populations and are dependent on factors such as host immune status, the site of infection, and the treatment (5). Being a saprophyte, *A. fumigatus* grows on soil and decaying organic debris where it produces conidia, that are disseminated by aerosolization and inhaled in large numbers by all individuals (6). For *A. fumigatus* conidia to cause invasive aspergillosis, inhaled conidia must undergo the morphogenetic change like *C. albicans*. Major steps include germination of conidia, a process that involves mitosis and emergence of the initial germ tube. In order to continue growth and consequently invade the host tissue, the germ tubes must elongate by apical extension (Figure 1.2). The result of this growth process results in long, tube-like hyphae, the characteristic morphology of pathogenic fungi that successfully invade the host tissue (7).

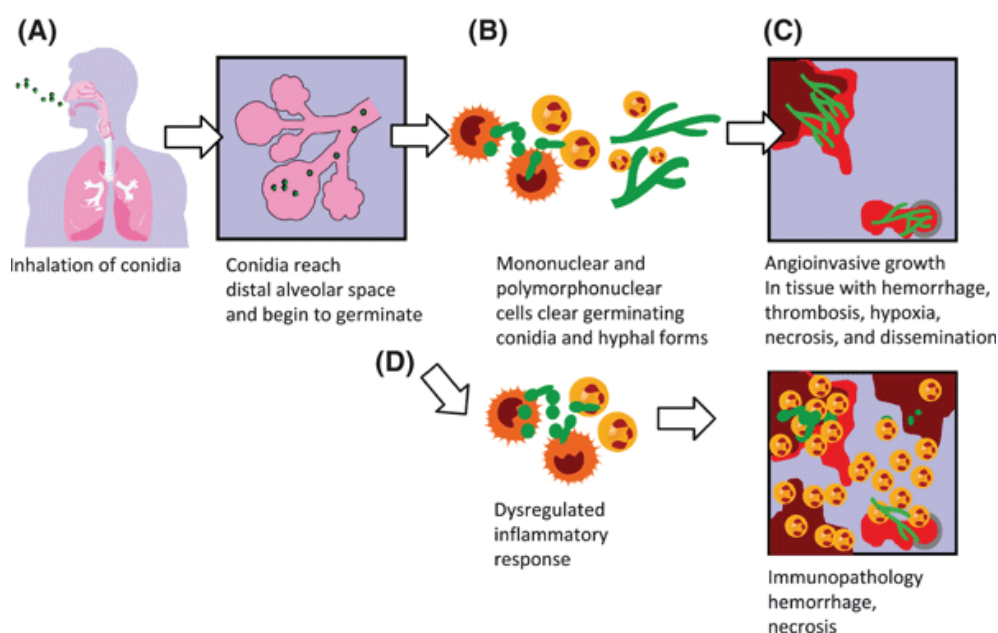


Figure 1.2 Pathogenesis of *A. fumigatus* infections

Adapted from Ben-Ami *et al* 2010 © John Wiley & Sons

Pathogenesis of invasive aspergillosis in different immunological settings. (A) *A. fumigatus* conidia are inhaled by humans, and reach the terminal airways and pulmonary alveoli. (B) In the alveoli, conidia

are destroyed by alveolar macrophages and polymorphonuclear leucocytes (PMNL). (C) In individuals with quantitative or qualitative defects in PMNLs, such as those who were exposed to cytotoxic drugs, *A. fumigatus* germination and tissue invasion proceed unabated. (D) Non-neutropenic hosts with a dysregulated immune response to *A. fumigatus*, for example patients receiving high dose corticosteroids, develop tissue damage as a result of PMNL recruitment, tissue infiltration and inflammatory necrosis.

1.2 Immunity to fungal infections

1.2.1 Recognition of fungal pathogens by pattern recognition receptors (PRRs)

During recent years, tremendous advance studies have been conducted to broaden our knowledge of how immune system of the host responds towards fungal pathogens. Especially in terms of primary interaction between the pathogen and host, involvement of several pattern recognition receptor (PRR) pathways of fungal recognition and the type of T-cell responses have been shown. Being the first line of interaction, the components of fungal cell wall are the most important factors to be recognized by PRRs (1). The fungal cell wall is a highly complex structure which maintains the integrity and viability of fungal organisms. The main structural components of the fungal cell wall are β -(1,3)-glucans, β -(1,6)-glucans and chitin (a β -(1,4)-polymer of N-acetylglucosamine (GlcNAc)) (Figure 1.3). These molecules are covalently linked to form a dense net of fibrils located in the inner layer of the cell wall (9).

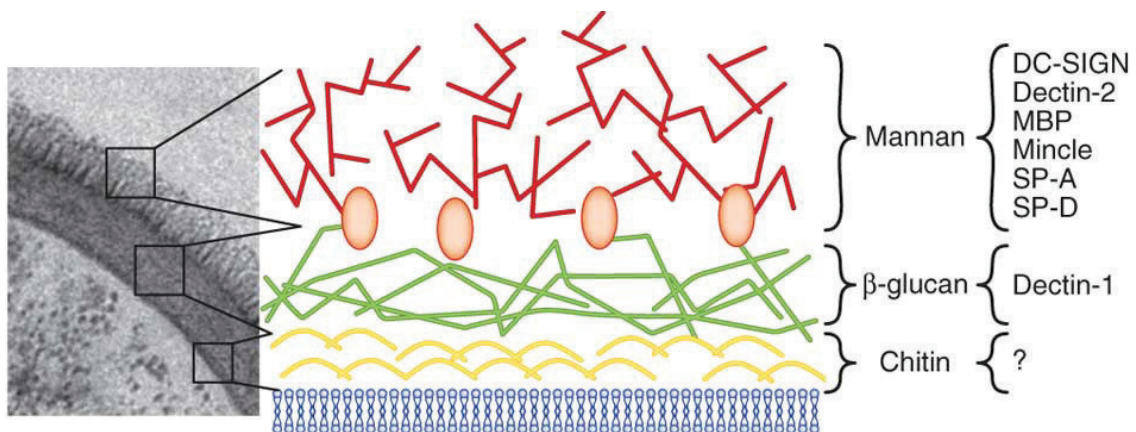


Figure 1.3 Fungal cell wall components

Adapted from Hardison and Brown 2012 © Nature publishing group

Electron micrograph of the fungal cell wall (*C. albicans*), with carbohydrate-rich layers of the fungal cell wall highlighted: mannan (mannosylated proteins), β -glucan and chitin. Although it provides a rigid framework, which gives these pathogens their shape and protection from the environment, the cell wall is a dynamic structure that changes considerably, particularly during the morphological transitions that many fungi can undergo, for example yeast to hyphae.

The outer portion of the fungal cell wall is largely composed of mannan and mannoproteins, and the inner layer is composed of β -(1,3)-glucan and chitin fragments. Expression of various cell wall proteins and carbohydrates present on the cytoskeleton is significantly changed during fungal pathogenesis and morphogenetic development. This change mostly occurs during the transition from yeast form to hyphal form, which takes place when the fungus invades target organs of the host. In response, the host immune system, mediated by several PRRs can distinguish these fungal forms, in various ways that are beginning to be unraveled.

Accumulating experimental evidence from various research group demonstrates that PRR recognition by *C. albicans* in antigen presenting cells (APCs) results in secretion of various specific cytokines including IL-1 β , IL-23 and IL-6 (1, 9, 11). Furthermore it was found that, these cytokines promote skewing of activated CD4⁺ T cells into the Th17 lineage, which expresses IL-17 (also known as IL-17A), IL-17F and IL-22. It has also been shown that IL-17 and IL-17F are closely related cytokines that operate through a common receptor (composed of the IL-17RA and IL-17RC), and IL-17R signaling is very important for an effective anti-*Candida* immune response by the host (12).

1.2.2 C-type Lectin Receptors (CLRs)

During past decade, the CLRs have been of very considerable attention in the context of antifungal immunity and appear to be comparatively more important than other PRRs in both mouse models of Candidiasis as well as in the humans (13, 14). CLRs are trans-membrane receptors which are expressed on, but not restricted to only myeloid cells including macrophages and dendritic cells. CLRs can also be found in abundance on lymphocytes, granulocytes, osteoclasts and epithelial cells of the host (15-17). The best-characterized CLRs with respect to *Candida* are Dectin-1, Dectin-2 and Mincle (Figure 1.4). Although details of their respective signaling pathways are still being elucidated, they all appear to mediate signaling through the kinase Syk1, the adaptors CARD9/Bcl-10 and the NF- κ B and Ras/Raf-1 pathways (1, 18).

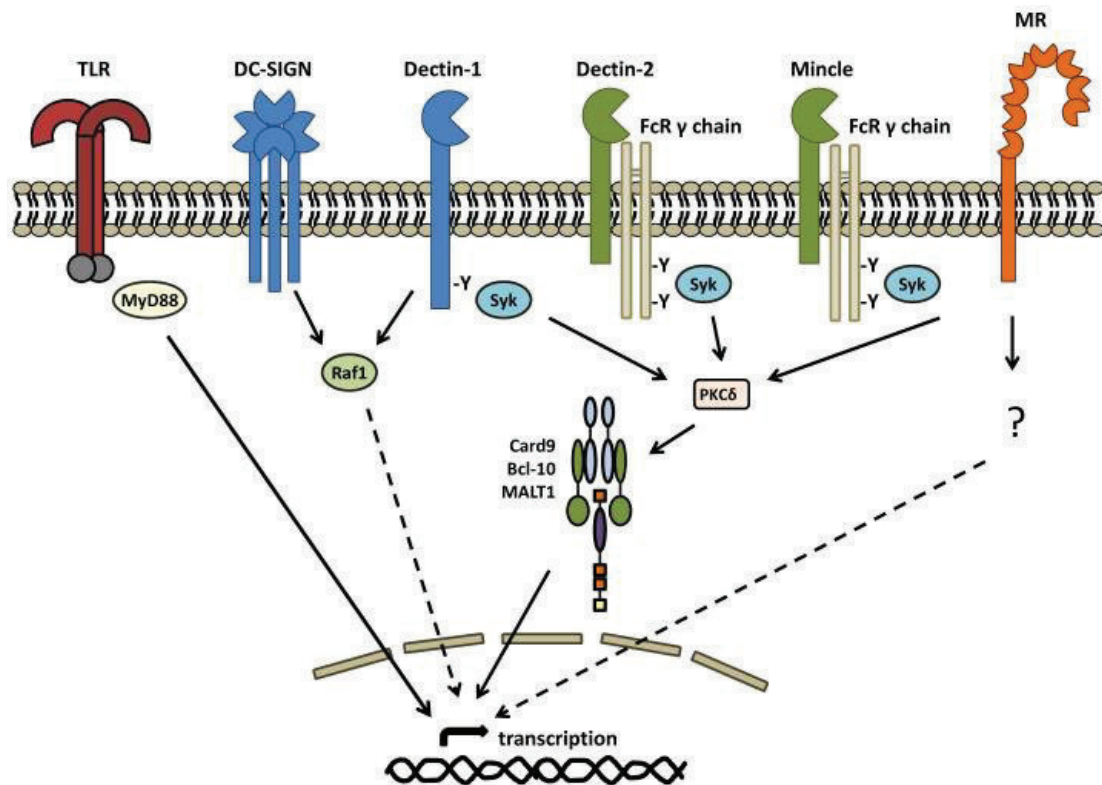


Figure 1.4 C-type Lectin Receptors in antifungal immunity and their intracellular signaling pathways

Adapted from Hardison and Brown 2012 © Nature publishing group

Dectin-1, Dectin-2 and Mincle induce intracellular signaling via immunoreceptor tyrosine (Y)-based activation motifs, which recruit and activate Syk kinase either directly or indirectly through the FcR γ adaptor chain. Signaling through protein kinase C (PKC) δ , this pathway activates the CARD9–Bcl-10–Malt1 complex, inducing gene transcription and the production of various inflammatory mediators. DC-SIGN and Dectin-1 can signal via the Raf-1 kinase pathway, which modulates (dotted line) other signaling pathways, including those induced by the Toll-like receptor (TLR) and Syk pathways. The mannose receptor (MR) can also induce intracellular signaling, but the mechanisms involved are unknown. CLR signaling can collaborate with that of the TLR to synergistically induce or repress the induction of various cytokines and chemokines.

1.2.3 Dectin-1 and CARD9

Dectin-1 is a well-defined CLR that recognises β -1,3-glucans which are usually buried underneath a layer of cell wall proteins and mannan moieties, in the cell wall of *Aspergillus*, *Candida* and many other fungal species (15, 18-20). Nevertheless, β -glucan is exposed in bud scars that are revealed during the process of hyphal transition, which facilitates *Candida* recognition and may be the essential signal that alerts the host of a transition from fungal colonization to infection (9). Upon ligand recognition, Dectin-1 signals through the Syk/CARD9 pathway via an ITAM-like motif (Figure 1.5). This motif is present in the intracellular signalling tail of Dectin-1 and uses a single phosphorylatable tyrosine residue to recruit Syk to dimerised Dectin-1 (21). Recently, the kinase PKC δ was shown to be activated by Dectin-1 and induce phosphorylation of CARD9. Both *card9*^{-/-} and *pkc δ* ^{-/-} mice are susceptible to

disseminated candidiasis (22, 23). These pathways induce cytokine and chemokine production including TNF, IL-2, IL-10, CXCL2 as well as IL-1 β , IL-6 and IL-23, key cytokines in the development of the antifungal Th17 response (14, 24). Dectin-1 also induces phagocytosis, respiratory burst and other antimicrobial effector mechanisms such as activation of the NLRP3 inflammasome via ERK-induced reactive oxygen species (ROS) production (25).

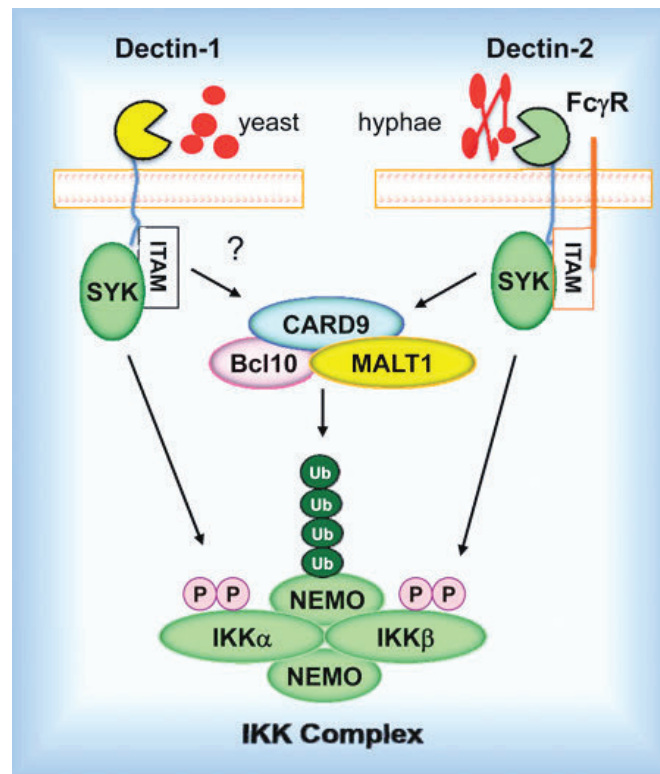


Figure 1.5 CARD9 in the C-type Lectin Receptor signaling

Adapted from Blonska *et al* 2011 © Nature publishing group

Activation of the Dectin-1 receptor by yeast-like, unicellular form of *C. albicans* or the Dectin-2 receptor by hyphal form of *C. albicans* leads to a sequential activation of tyrosine kinase Syk, the CARD9-Bcl10-MALT1 complex and IKK. In this signaling cascade Syk mediates IKK phosphorylation, whereas CARD9 controls NEMO polyubiquitination.

The importance of Dectin-1 in the control of fungal infections is highlighted by the susceptibility of Dectin-1-deficient mice to infection with *C. albicans* and *A. fumigatus* (27, 28). However, the role of Dectin-1 in fungal host defense remains a topic of debate. Whereas one study showed that *dectin-1*^{-/-} mice showed increased susceptibility to disseminated candidiasis (13, 20), another report found that *dectin-1*^{-/-} mice were resistant. Intriguingly, in the study by Saijo *et al.*, *dectin-1*^{-/-} mice were susceptible to *Pneumocystis carinii*, suggesting that the role of Dectin-1 in antifungal

immunity may be pathogen-specific. Furthermore, Dectin-1 deficiency in humans can lead to susceptibility to certain fungal infections including chronic mucocutaneous candidiasis and recurrent vulvovaginal candidiasis (29). Predisposition to fungal infections can also be seen in CARD9-deficient humans. CARD9 deficiency has been studied in a family in which eight members were affected with a mutation causing a premature stop codon (Q295X), resulting in a truncated protein (30).

In addition to promoting Th17 responses, Dectin-1 signaling appears to play a role in balancing the frequencies of Th1 and Th17 cells. Specifically, the absence of dectin-1 during lung infection with *Aspergillus fumigatus* causes reduced production of IFN γ and T-bet, a transcription factor that controls Th1 differentiation, resulting in decreased Th1 responses and correspondingly enhanced Th17 differentiation (31). Therefore, β -glucan recognition by Dectin-1 shapes the overall nature of antifungal CD4⁺ T-cell helper responses.

1.2.4 Fungal infections and the inflammasome

Inflammasome is defined as a large multiprotein complex that contains certain NOD-like receptors, RIGI-like receptors and IFI200 proteins, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD) and pro-caspase 1. Assembly of the inflammasome leads to the activation of Caspase 1, which cleaves pro-interleukin-1 β (pro-IL-1 β) and pro-IL-18 to generate the active cytokines (11). Although neither of the cytosolic receptors NOD1 nor NOD2 have been found to be required for *Candida* recognition (32), yet inflammasomes are involved. Previous studies have shown that mice deficient in the IL-1 receptor (IL-1R) or apoptosis-associated speck-like protein (ASC), an essential subunit of the inflammasome, exhibit increased oral fungal burdens in a model of oral mucosal candidiasis (33). Also, in this model, fungal dissemination was even more pronounced than mucosal infection. Consistent with these findings, *nlrp3*^{-/-} mice are more susceptible to disseminated candidiasis as well as mucosal disease (34). Furthermore, sophisticated bone marrow chimera experiments validated the role of the NLRP3 inflammasome in preventing *Candida* dissemination, and further demonstrated important involvement of the NLRC4 inflammasome compartment during oral *C. albicans* host infections (35). It was noted that NLRP3 is required in hematopoietic cells, whereas NLRC4 functions at the level of the mucosal stroma. However, both NLRP3 and NLRC4 deficiencies resulted in decreased expression of

IL-17, IL-17F and also one of the IL-17 receptor subunits (IL-17RA), showing the direct interlink of the inflammasomes and IL-17 (35). Further work showed that bone marrow derived cells (BMDCs) from *asc*^{-/-} and *caspase-1*^{-/-} mice exhibited impaired production of IL-1 β and IL-18 following *Candida* exposure, which are important for Th17 and Th1 development, respectively.

Along with these findings, splenocytes from IL-1 β - and IL-18-deficient mice showed impaired production of IL-17 and IFN γ in response to *Candida* stimulation (36). Interestingly, the development of protective Th17 responses via inflammasome activation resulted from the recognition of *C. albicans* hyphae by human macrophages. On the other hand the yeast form did not activate the inflammasome, demonstrating that this pathway is likely important for discriminating between colonizing yeast forms and invasive hyphae of *Candida* (37). Recently, another member of the NLR family, NLRP10, was demonstrated to play an important role during host defense against disseminated candidiasis (38). NLRP10-mediated anti-*Candida* immunity was shown to be dependent on induction of protective T cell responses, most likely Th1- and Th17- mediated as indicated by decreased IFN γ and IL-17 production from *nlrp10*^{-/-} splenocytes re-stimulated *in vitro* with heat killed *C. albicans* cells. However, the absence of NLRP10 did not affect the production of IL-1 β by the host, indicating that *C. albicans* recognition by NLRP10 does not affect IL-1 β production by NLRP3 and NLRC4 inflammasomes.

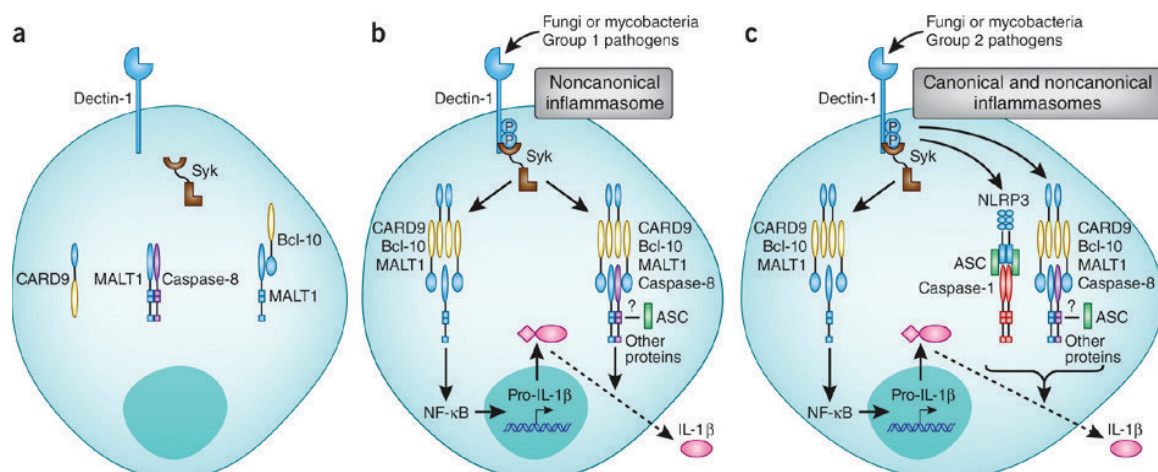


Figure 1.6 Activation of the noncanonical inflammasome
Adapted from Dapaul-Chicoine *et al* 2012 © Nature publishing group

(a) In resting dendritic cells, caspase-8 is in complex with MALT1. A second pool of MALT1 interacts with Bcl-10. After stimulation of dectin-1 with fungi or mycobacteria, engagement of Syk triggers activation of NF- κ B and transcription of the gene encoding pro-IL-1 β via the CARD9–Bcl-10–MALT1 complex. (b,c) A subset of pathogens, such as *C. albicans* (species CBS8781 and CBS8758), *Candida tropicalis*, *Candida lusitanae*, *Aspergillus fumigatus* and *Mycobacterium leprae*, stimulate the production of bioactive IL-1 β mainly via a noncanonical inflammasome composed of CARD9, Bcl-10, MALT1, caspase-8 and ASC (b). Other pathogens, such as *Mycobacterium tuberculosis* or *Mycobacterium bovis* bacillus Calmette-Guérin, engage both the classical NLRP3 inflammasome as well as the noncanonical caspase-8 inflammasome, leading to the secretion of IL-1 β (c).

In addition to the role of the NLRP3 and NLRC4 inflammasomes in processing pro-IL-1 β , a recent study exhibited a Dectin-1-dependent activation of a non-canonical Caspase-8 inflammasome (40). Dectin-1 activation resulted in the activation of Syk followed by the transcription of the IL-1 β gene through the CARD9-Bcl10-MALT1 complex. Recruitment of MALT1-caspase-8 and ASC to this scaffold resulted in processing of pro-IL-1 β to mature IL-1 β (Figure 1.6). Interestingly, the activation of this noncanonical pathway did not require *C. albicans* internalization, unlike activation of the NLRP3 inflammasome that was completely dependent on internalization. Since some *C. albicans* strains trigger IL-1 β production primarily via Caspase-8 while others also activate the NLRP3 inflammasome, it can be suggested that the ligand for NLRP3 is not present in all fungi.

Taken together, the priming of host-protective Th17 responses is likely a combined effort of multiple PRR pathways that recognize different components of pathogenic fungi, and triggers the production of cytokines that predominantly direct Th17 differentiation.

1.3 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell population of immature monocytes and granulocytes that are potent inhibitors of T cell activation. In recent years, MDSCs have been classified as the cell population responsible for modulating immune responses (41, 42). The most important function of the MDSCs has been characterized as to suppress T cells (43, 44).

1.3.1 Origin and expansion of MDSCs

In healthy individuals, immature myeloid cells (IMCs) normally reside in the bone marrow prior to their differentiation into mature granulocytes, macrophages, or dendritic cells. However, MDSCs are recruited into lymphoid and inflamed tissues during pathological and inflammatory conditions by the actions of various growth factors, such as G-CSF and GM-CSF, where alternations in cytokine homeostasis

state block their further differentiation into mature myeloid effector cells, and hence resulting in MDSC expansion (42, 45) (Figure 1.7).

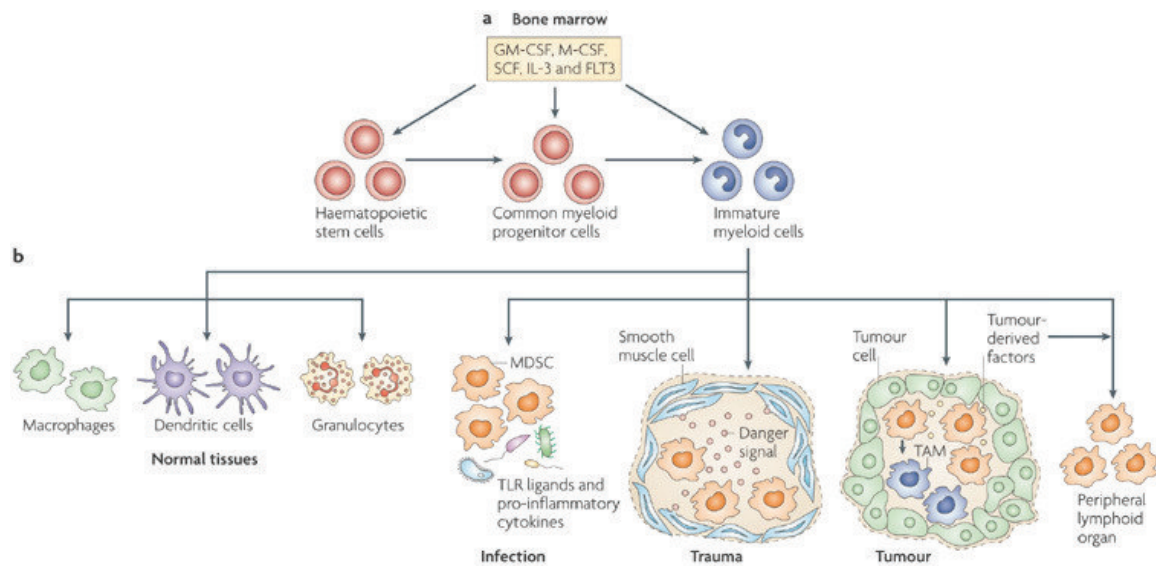


Figure 1.7 The origin of MDSCs

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Myelopoiesis of immature myeloid cells (IMCs) is controlled by soluble factors including GM-CSF, M-CSF, SCF and IL-3. Under healthy conditions, IMCs migrate to peripheral organs and differentiate into macrophages, DCs and granulocytes. Infection, trauma or tumor environment promote the accumulation of IMCs, prevent their differentiation and induce their activation to MDSC.

Importantly, the activation of IMCs in pathological conditions results in the upregulation of their expression of immune suppressive factors, such as arginase 1 (encoded by ARG1) and inducible nitric oxide synthase (iNOS; also known as NOS2), as well as an increase in their production of NO (nitric oxide) and reactive oxygen species (ROS). This results in the expansion of an IMC population that has immune suppressive activity; these cells are now collectively known as MDSCs. In terms of infection and inflammation, the precise function of MDSCs and the mechanisms of MDSC induction are not well understood and are still under investigation. However some previous work showed that in a sepsis model with Gram-negative bacteria, their induction has been shown to be dependent on TLR4-MyD88 activation (46). On the other hand in tumor models, different innate cytokines, such as IL-6, induce MDSC accumulation (47). It is also known that the suppression of IL-6 increases susceptibility to bacterial and fungal infections, indicating pleiotropic effects of IL-6 (48).

1.3.2 Mouse and Human MDSC subsets:

MDSCs are comprised of a phenotypically heterogeneous population of myeloid cells, which in mice are characterized by the co-expression of the myeloid cell differentiation antigen Gr-1 and CD11b (α M-integrin) (49, 50). In healthy mice, these cells comprise 20-30% of total bone marrow cells, 2-4% of splenocytes and are absent from the lymph nodes. Recently, two morphological and functional distinct MDSC subpopulations were described based on their expression of Gr-1. The Gr-1 antigen includes the macrophage and neutrophil markers Ly6C and Ly6G, which can be separately detected by antibodies specific for their distinct epitopes. The Ly6 family displays a group of low molecular weight molecules (12-20 kDa) that are phosphatidylinositol-anchored cell surface glycoproteins. Their triggering leads to a signal transduction resulting in T cell activation (51). Using Ly6G and Ly6C markers, two MDSC subsets were identified: CD11b⁺Ly6G⁺Ly6C^{low} MDSCs morphologically resembling polymorphonuclear granulocytes (G-MDSCs) and CD11b⁺Ly6G⁻Ly6C^{high} MDSCs with monocytic phenotype (M-MDSCs) (52). Analysis of different tumor models showed that during tumor growth both subpopulations expanded, but in most cases the accumulation of G-MDSC was more prominent (53). Novel studies defined an additional subset according to the Gr-1 staining intensities. CD11b⁺ splenocytes from different tumor models could be divided into Gr-1^{high}, Gr-1^{int} and Gr-1^{low} cells. Gr-1^{high} cells represented the G-MDSC subset, which exerted weak suppression on CD8⁺ T cells. While Gr-1^{low} cells resembled M-MDSCs with strong suppressive potential, the Gr-1^{int} subset displayed moderate suppression (54).

In humans, MDSCs are most commonly defined as CD14⁻CD11b⁺ (G-MDSCs) and CD14⁺CD11b⁺ (M-MDSCs). Even more narrowly, MDSCs are designated as cells that express the common myeloid marker CD33 but lack the expression of markers of mature myeloid and lymphoid cells, and of the MHC class II molecule human leucocyte antigen HLA-DR (2, 55). MDSCs have also been identified within a CD15⁺ population in human peripheral blood. In healthy individuals, IMCs constitute 0.5% of peripheral blood mononuclear cells (2). Rodriguez *et al.* proposed CD66b, a member of the carcinoembryonic antigen (CEA)-like glycoprotein family, as a marker for the granulocytic MDSC subpopulation in patients with renal cell carcinoma (56).

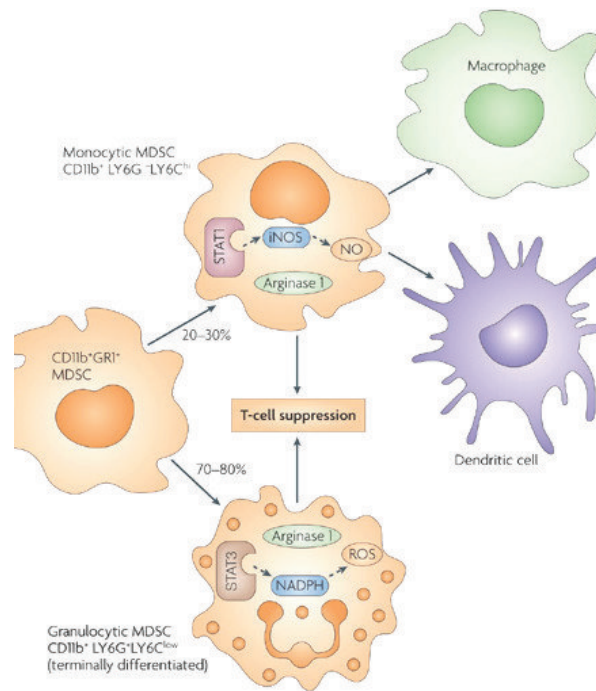


Figure 1.8 Suppressive mechanisms mediated by different subsets of MDSCs

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In most tumour models, it is predominantly (70–80%) the granulocytic subset of MDSCs that expands. G-MDSCs have increased activity of signal transducer and activator of transcription 3 (STAT3) and NADPH, which results in high levels of reactive oxygen species (ROS) but low nitric oxide (NO) production. ROS and, in particular, peroxynitrite (the product of a chemical reaction between superoxide anion and NO) induces the post-translational modification of T-cell receptors and may cause antigen-specific T-cell unresponsiveness. The M-MDSCs have upregulated expression of STAT1 and inducible nitric oxide synthase (iNOS), and increased levels of NO but low ROS production. NO, which is produced by the metabolism of L-arginine by iNOS, suppresses T-cell function through various different mechanisms that involve the inhibition of Janus kinase 3 and STAT5, the inhibition of MHC class II expression and the induction of T-cell apoptosis. Both subsets have increased levels of arginase 1, which causes T-cell suppression through depletion of L-arginine. Only monocytic MDSCs can differentiate into mature dendritic cells and macrophages *in vitro*.

1.3.3. MDSCs in pathological conditions

Previous observations and most of the recent studies on the role of MDSCs in immune responses have been attributed to the cancer research. First characterization of MDSCs was performed in tumour-bearing mice and in cancer patients. But growing evidence suggests that expansion and recruitment of MDSCs is a common feature of most pathological diseases. For instance, acute *Trypanosoma cruzi* infection, which induces T-cell activation and increases the production of interferon- γ (IFN γ), also leads to the expansion of MDSCs (57, 58). A similar expansion of MDSCs has been reported during acute toxoplasmosis, polymicrobial sepsis, acute infection with *Listeria monocytogenes*, chronic infection with *Leishmania major*, infection with helminths and very recently during *Staphylococcus*

aureus infections (46, 59-62). Expansion of MDSCs is also associated with autoimmunity and inflammation. In murine model of autoimmune encephalomyelitis, an increase in the number of MDSCs (specifically the CD11b⁺Ly6G⁻Ly6C^{hi} monocytic subset) was observed in the spleen and blood (63). An immunosuppressive MDSC population was found to be increased in normal mice following immunization with different antigens, including ovalbumin or peptide together with CFA, recombinant vaccinia virus expressing IL-2, or staphylococcal enterotoxin A (49, 64).

2. Aim of the study

Due to transplantation-associated immunosuppression and the global impact of AIDS (Acquired Immune Deficiency Syndrome), fungal diseases are on the rise. It has been shown that the anti-fungal immune response starts with an activation of the innate immune system, followed by adaptive T-cell responses but the underlying immune mechanisms are incompletely understood.

The major aim of this work was to investigate how the pathogenic fungi interact with the immune system and modulate T-cell responses by inducing myeloid-derived suppressor cells (MDSCs). Using various cellular *in vitro*, murine *in vivo* and patient studies we mimicked different immunological conditions of the host and screened how fungi induce myeloid-derived suppressor cells which functionally suppress T- and NK-cell responses. Furthermore, we analyzed the mechanism behind fungal MDSC induction focussing on Dectin-1/CARD9 signalling, reactive oxygen species (ROS), caspase-8 activity and interleukin-1. In the next step, we also performed adoptive transfer and survival experiments to check the effects of MDSCs during systemic *Candida albicans*, and nasal *Aspergillus fumigatus* infection models *in vivo*. By this study, we expect to dissect a new mechanism by which fungi regulate T-cell immunity and exploit MDSCs to escape from host defence and to establish a bistable host-pathogen interaction, suggesting modulation of MDSCs as a novel therapeutic approach in fungal diseases.

3. Discussion

The central aim of this work was to investigate the role of myeloid-derived suppressor cells (MDSCs) in pathogenic fungal infections. While MDSCs have been characterized thoroughly during various cancer related diseases, there is also a growing body of evidence that MDSCs play a key role in non-malignant conditions, particularly in chronic inflammation and infection (2, 65-71). However their phenotype and their functional relevance in infectious fungal diseases remain unknown. Our previous studies have shown that MDSCs play an important role in various pathological conditions involving *Pseudomonas aeruginosa*, cystic fibrosis and GvHD (Graft-versus-host disease) (71, 72). The present study has expanded the knowledge about immune suppressive effects of MDSCs to invasive life threatening fungal infections. Using *in vitro* assays, mouse models and patient studies, we demonstrate that a population of neutrophilic MDSCs accumulates during fungal infections and adoptive transfer of this population results in improved *C. albicans* clearance and survival of the mice. These findings suggest that MDSCs are one of the key contributors to the chronicity of *C. albicans* infection through their ability to modulate the host immune response.

3.1 Pathogenic fungi induce myeloid-derived suppressor cells (MDSCs)

In this study, we show for the first time to our knowledge, the development of functional MDSCs in response to *A. fumigatus* and *C. albicans* infection in a set of subsequent experimental models that included immunodeficient patients and *in vivo* mouse infection models. This induced/expanded function of MDSCs was characterized *ex vivo* by strong and broad suppression of both T-cell and NK-cell responses as read out by various *in vitro* proliferation and cytokine production assays. With the era of transplantation-associated immunosuppression and the global impact of AIDS, fungal infections are on the rise (8, 73-75). Fungi are sensed through the innate immune system, which triggers effector cell recruitment to protect the host. Excessive anti-fungal immune responses, however, cause tissue damage and impair fungal clearance. Protection of the host against fungi requires a fine-tuned balance between pro-inflammatory effector and counter-regulatory mechanisms. Fungal infection induces an immunosuppressive state, and in murine models CD80⁺ neutrophilic cells have been shown to be importantly involved in this process (76). In

both, patients and mice, where MDSCs have been studied extensively, these cells comprise a granulocytic/neutrophilic (G-MDSCs) and a monocytic MDSC (M-MDSCs) subtype (77, 78). Recent data suggest that the neutrophilic/G-MDSC phenotype is the predominant one in humans. Functionally, fungi-induced myeloid cells strongly suppressed CD4⁺ and CD8⁺ T-cell proliferation in a dose-dependent manner, which defines MDSCs. In addition to regulating adaptive immunity, fungi-induced G-MDSCs suppressed innate natural killer NK-cell responses.

3.2 MDSC induction involves Dectin-1/CARD9, ROS, caspase-8, and IL-1

Pathogenic fungi interact with a variety of host cells during the induction of disease. In order to cross the tissue barrier, for example during invasive aspergillosis, they normally invade nonphagocytic host cells such as epithelial and endothelial cells by triggering their own uptake. Infected epithelial cells play a key position participating in local airway inflammation via their production of cytokines and chemokines such as IL-6, IL-8, IL-1 β and others that link the innate to the adaptive immune systems. Recent studies put the gut in the centre of immunotolerance. Dectin-1 was found to control colitis and intestinal Th17 responses through sensing of the fungal mycobiome (79). The immunological events downstream of Dectin-1 and their functional impact on Th17 cells remained elusive. Fungi-induced MDSCs expressed Dectin-1 at the cell surface, and blocking Dectin-1 diminished the MDSC-inducing effect. Dectin-1 receptor activation by the particulate ligands zymosan and whole glucan particles (WGP), which operate through the phagocytic synapse, mimicked phenotypically and functionally the generation of MDSCs in vitro. Consistently, the potential of fungi or fungal patterns to induce neutrophilic MDSCs was diminished in human and murine Dectin-1 deficiency. To dissect Dectin-1 downstream signalling, we analysed the role of caspase recruitment domain 9 (CARD9), the key transducer of Dectin-1 signalling, in fungi-mediated MDSC generation in patients with genetic CARD9 deficiency and Card9 knock-out mice. These approaches demonstrated that CARD9 signalling was essential for fungal MDSC induction in humans and, to a lesser extent, in the murine system. Recent studies provided evidence that inflammasome-derived interleukin-1 beta (IL-1 β) and reactive oxygen species (ROS) are involved in MDSC and Th17 cell homeostasis. We found an accumulation of intracellular IL-1 β protein in CD33⁺ myeloid cells followed by a release of IL-1 β into the cellular microenvironment upon Dectin-1 ligand- and fungal-driven MDSC

generation. Further studies, using chemical inhibitors and human patients with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase deficiency (chronic granulomatous disease, CGD), demonstrated that ROS contributed substantially to fungal MDSC induction. Our results demonstrate that fungal Dectin-1/CARD9 signaling induces MDSCs to dampen T-cell responses and suggest that the immune homeostasis in the gut could be modulated by fungal-induced MDSCs. Beyond fungi, the Dectin-1/CARD9 pathway has been involved in bacterial and viral infections (80), suggesting that this mechanism could play a broader role in balancing inflammation at host-pathogen interfaces.

It has been previously reported that the complete genetic deletion of pro-inflammatory cytokines, in particular TNF- α , IL-1 α/β or IFN- γ , increases disease susceptibility in invasive fungal infections (1, 9, 37, 81, 82). On the other hand studies from other research groups tell that excessive inflammation causes collateral damage to the host (11, 83) indicating that efficient protection against fungi requires a fine-tuned balance between pro-inflammatory effector and counter-regulatory immune mechanisms.

3.3 Adoptive transfer of MDSCs improves survival in systemic candidiasis

One notable finding in the present study was that mice infected with lethal dose of *C. albicans* exhibited improved survival after being adoptively transferred with MDSCs. Previously it has been shown that *C. albicans* and *A. fumigatus* pathogenesis differs substantially in terms of disease progression, T-cell dependency and organ manifestation (84). Our results that neutrophilic MDSCs were protective in a murine model of systemic *C. albicans* infection, but had no effect on pulmonary *A. fumigatus* infection underlines this disparity and suggests MDSCs as potential therapeutic approach in invasive *C. albicans* rather than *A. fumigatus* infections. The MDSC-mediated effect was associated with downregulated NK- and T-cell activation and Th17 responses. It was further validated that supplementing Cytochalasin D and IL-17A *in vivo* could, at least partially, dampen the protective effect of MDSCs. Based on previous studies showing that NK cells drive hyperinflammation in candidiasis in immunocompetent mice (85) and that IL-17 promotes fungal survival (86), we speculate that MDSCs in fungal infections could act beneficial for the host by dampening pathogenic hyperinflammatory NK and Th17 responses (74, 75) Interestingly in amphibians, the fungus *Batrachochytrium dendrobatidis* has recently

been shown to paralyze lymphocyte responses, thereby dampening adaptive host defence (87), a mechanism reminiscent of MDSC activity, but the precise underlying factor mediating this effect remained elusive. Based on our findings, we speculate that therapeutic modulation of MDSCs in fungal infections has the potential to tailor the host defence towards an effective elimination of the pathogen. Accordingly, enhancing neutrophilic MDSCs may present a novel anti-inflammatory treatment strategy for fungal infections, particularly with *C. albicans*.

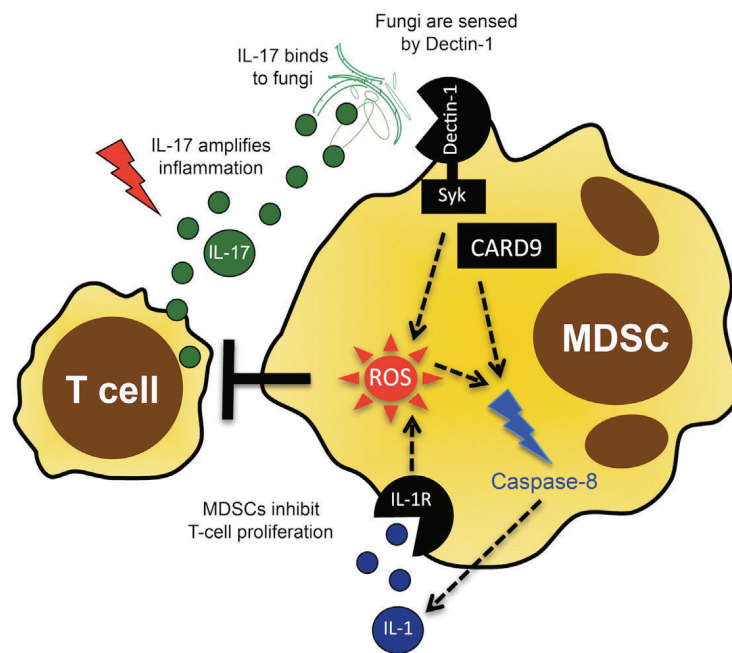


Figure 3.1 Proposed model of MDSC generation in invasive fungal infections

Adapted from Rieber *et al* 2015 © Cell Press

Fungal sensing through Dectin-1 triggers downstream signaling cascades involving Syk and CARD9, leading to caspase-8 activation. Caspase-8 drives interleukin-1 (IL-1) production. Released IL-1 binds to the IL-1 receptor (IL-1R) and enhances generation of ROS, which are essential for MDSC induction. Moreover, ROS are involved in fungal-driven Caspase-8 activation. Generated MDSCs inhibit NK and T-cell responses, such as Th17 responses that amplify inflammation and also directly affect fungal survival.

3.4 Concluding remarks

Our studies are just at the beginning to explore the role of MDSCs during pathogenic fungal infections. These data also highlight the importance of combining methods that include human and animal models, where *C. albicans* is a commensal and, as a result, the immune system is chronically exposed to the fungus. The fact that *C. albicans* is both a commensal and a pathogen (during the conditions of immunosuppression) adds an additional and intriguing level of complexity to the study of *C. albicans*-specific responses. To the best of our knowledge, this is the first study where the role of MDSCs in invasive fungal infections has been addressed. In light of these findings, we believe that induction of neutrophilic G-MDSCs and related factors are capable of regulating antifungal immune response of the host. Given the well-known key role of this cell type in immune regulation, neutrophilic G-MDSCs may modulate the immunopathogenesis of infective fungal diseases and could therefore represent a novel therapeutic target.

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Curriculum vitae

Personal details

Name: Anurag Singh
Address: Kirschenweg 3, 72076 Tuebingen
Telephone: +49-176 3041 9096
Email: anurag.singh@med.uni-tuebingen.de
Nationality: Indian
Date of Birth: 01.01.1983

Education and Qualification

2002-2006: Bachelor of Science (Hons) Microbiology, Allahabad Agricultural Institute- Deemed University, Allahabad, India.

2006-2008: Master of Science (Microbiology and Herbal Medicine) from Amity University, Noida, Uttar Pradesh, India.

2009-present: Start of PhD thesis at the Institute of Medical Microbiology and Hygiene, and continuation at University Children's Hospital, Tuebingen in Prof. Dr. Dominik Hartl's laboratory.

Appendix

Paper 1

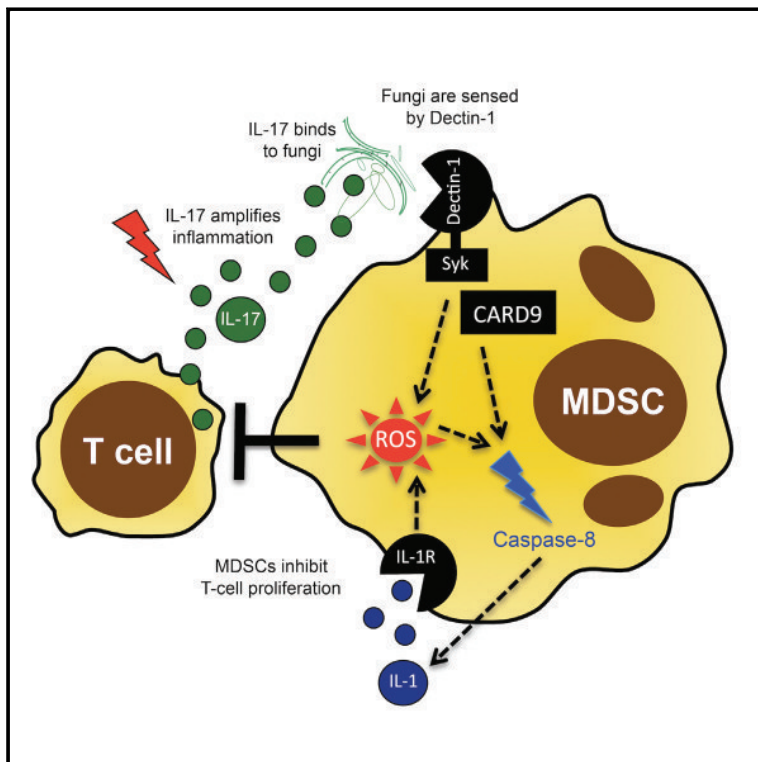
Rieber N, **Singh A**, Öz H, Carevic M, Bouzani M, Amich J, Ost M, Ye Z, Ballbach M, Schäfer I, Mezger M, Klimosch SN, Weber A, Handgretinger R, Krappmann S, Liese J, Engeholm M, Schüle R, Salih HR, Marodi L, Speckmann C, Grimbacher B, Ruland J, Brown GD, Beilhack A, Loeffler J and Hartl D (2015).

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Cell Host & Microbe

Pathogenic Fungi Regulate Immunity by Inducing Neutrophilic Myeloid-Derived Suppressor Cells

Graphical Abstract



Authors

Nikolaus Rieber, Anurag Singh, ..., Juergen Loeffler, Dominik Hartl

Correspondence

nikolaus.riever@med.uni-tuebingen.de (N.R.), dominik.hartl@med.uni-tuebingen.de (D.H.)

In Brief

Myeloid-derived suppressor cells (MDSCs) are innate immune cells that suppress T cell responses. Rieber et al. show that pathogenic fungi *Aspergillus fumigatus* and *Candida albicans* induce MDSCs through mechanisms involving Dectin-1/CARD9 as well as downstream ROS and IL-1 β production, and that transfer of MDSCs protects against invasive *Candida* infection.

Highlights

- Pathogenic fungi induce myeloid-derived suppressor cells (MDSCs)
- MDSC induction involves Dectin-1/CARD9, ROS, caspase-8, and IL-1
- MDSCs dampen T and NK cell immune responses
- Adoptive transfer of MDSCs improves survival in *Candida* infection in vivo



Pathogenic Fungi Regulate Immunity by Inducing Neutrophilic Myeloid-Derived Suppressor Cells

Nikolaus Rieber,^{1,*} Anurag Singh,¹ Hasan Öz,¹ Melanie Carevic,¹ Maria Bouzani,² Jorge Amich,³ Michael Ost,¹ Zhiyong Ye,^{1,4} Marlene Ballbach,¹ Iris Schäfer,¹ Markus Mezger,¹ Sascha N. Klimosch,⁵ Alexander N.R. Weber,⁵ Rupert Handgretinger,¹ Sven Krappmann,⁶ Johannes Liese,⁷ Maik Engeholm,⁸ Rebecca Schüle,⁸ Helmut Rainer Salih,⁹ Laszlo Marodi,¹⁰ Carsten Speckmann,¹¹ Bodo Grimbacher,¹¹ Jürgen Ruland,¹² Gordon D. Brown,¹³ Andreas Beilhack,³ Juergen Loeffler,² and Dominik Hartl^{1,*}

¹Department of Pediatrics I, University of Tübingen, 72076 Tübingen, Germany

²Department of Medicine II, University of Würzburg, 97080 Würzburg, Germany

³IZKF Research Group for Experimental Stem Cell Transplantation, Department of Medicine II, 97080 Würzburg, Germany

⁴Department of Pediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119077, Singapore

⁵Institute of Cell Biology, Department of Immunology, University of Tübingen, 72076 Tübingen, Germany

⁶Microbiology Institute – Clinical Microbiology, Immunology and Hygiene, University Hospital of Erlangen and Friedrich-Alexander University Erlangen-Nürnberg, 91054 Erlangen, Germany

⁷Department of Pediatrics, University of Würzburg, 97080 Würzburg, Germany

⁸Department of Neurology

⁹Department of Oncology

University of Tübingen, 72076 Tübingen, Germany

¹⁰Department of Infectious and Pediatric Immunology, Medical and Health Science Center, University of Debrecen, 4032 Debrecen, Hungary

¹¹Centre of Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, 79106 Freiburg, Germany

¹²Institut für Klinische Chemie und Pathobiochemie, Klinikum rechts der Isar, Technische Universität München, 81675 Munich, Germany

¹³Aberdeen Fungal Group, Section of Immunology and Infection, University of Aberdeen, AB24 3FX Aberdeen, UK

*Correspondence: nikolaus.riever@med.uni-tuebingen.de (N.R.), dominik.hartl@med.uni-tuebingen.de (D.H.)

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SUMMARY

Despite continuous contact with fungi, immunocompetent individuals rarely develop pro-inflammatory antifungal immune responses. The underlying tolerogenic mechanisms are incompletely understood. Using both mouse models and human patients, we show that infection with the human pathogenic fungi *Aspergillus fumigatus* and *Candida albicans* induces a distinct subset of neutrophilic myeloid-derived suppressor cells (MDSCs), which functionally suppress T and NK cell responses. Mechanistically, pathogenic fungi induce neutrophilic MDSCs through the pattern recognition receptor Dectin-1 and its downstream adaptor protein CARD9. Fungal MDSC induction is further dependent on pathways downstream of Dectin-1 signaling, notably reactive oxygen species (ROS) generation as well as caspase-8 activity and interleukin-1 (IL-1) production. Additionally, exogenous IL-1 β induces MDSCs to comparable levels observed during *C. albicans* infection. Adoptive transfer and survival experiments show that MDSCs are protective during invasive *C. albicans* infection, but not *A. fumigatus* infection. These studies define an innate immune mechanism by which pathogenic fungi regulate host defense.

INTRODUCTION

At mucosal sites, the human immune system is faced continuously with microbes, rendering fine-tuned immune responses essential to protect against pathogenic, while maintaining tolerance against harmless, species. This immune balance is of particular relevance for fungi, inhaled daily as spores or present in the gut microflora as commensal yeasts (Romani, 2011). While immunocompetent individuals do not develop invasive fungal infections, infections are a major problem in patients undergoing immunosuppression, for instance, at solid organ or hematopoietic stem cell transplantation (Garcia-Vidal et al., 2013).

Fungi are recognized through pattern recognition receptors, mainly C-type lectin receptors (with Dectin-1 as the prototypic one) (Steele et al., 2005), toll-like receptors (TLRs), and pentraxin 3 (PTX3) (Garlanda et al., 2002; Werner et al., 2009). A certain level of inflammation is essential to control fungal infections (Brown, 2010), but hyperinflammatory responses seem to cause more harm than good to the host. Particularly, Th17-driven hyperinflammatory responses have been shown to promote fungal growth (Zelante et al., 2012), to impair fungal clearance, and to drive tissue damage (Romani et al., 2008; Zelante et al., 2007). Generation of reactive oxygen species (ROS), indoleamine 2,3-dioxygenase (IDO) activity, and activation of the TIR domain-containing adaptor-inducing interferon- β (TRIF) pathway were found to limit hyperinflammatory responses toward *Aspergillus fumigatus* (Romani, 2011; Romani et al., 2009). Yet, the cellular mechanisms by which fungi



control T cell activation and maintain tolerogenic host-pathogen bistability remain incompletely understood.

Myeloid-derived suppressor cells (MDSCs) are innate immune cells characterized by their capacity to suppress T cell responses (Gabrilovich and Nagaraj, 2009). MDSCs comprise a neutrophilic and a monocytic subset. While the functional impact of MDSCs in cancer is established, their role in host-pathogen interactions is poorly defined. We hypothesized that fungal infections induce MDSCs that modulate disease outcome.

RESULTS

We analyzed the effect of the human-pathogenic fungi *A. fumigatus* and *C. albicans* on human immune cells and noticed the appearance of a cell population that was different from monocytes (CD14⁻), and expressed the myeloid markers CD33⁺, CD11b⁺, CD16⁺, and CXCR4 (Figures 1A and S1A). Fungi-induced myeloid cells strongly suppressed both CD4⁺ and CD8⁺ T cell proliferation in a dose-dependent manner (Figure 1B), which defines MDSCs. Fungi-induced MDSCs also suppressed innate natural killer (NK) cell responses, without affecting cell survival (Figure S2). In contrast to growth factor-induced MDSCs, fungi-induced MDSCs dampened Th2 responses, which play essential roles in fungal asthma (Kreindler et al., 2010) (Figure S1B). We quantified MDSCs in patients with invasive fungal infections and challenged mice with *A. fumigatus* or *C. albicans*. MDSCs accumulated in both *A. fumigatus*- and *C. albicans*-infected patients compared to healthy and disease control patients without fungal infections (Figure 1C). Murine studies further showed that systemic or pulmonary fungal challenge with *C. albicans* (invasive disseminated candidiasis) or *A. fumigatus* (pulmonary aspergillosis), as the clinically relevant routes of infection, dose-dependently triggered the recruitment of MDSCs in both immunocompetent and immunosuppressed conditions, with a stronger MDSC induction seen in immunocompetent animals (Figures 1D and S1C). MDSCs expressed neutrophilic markers in both man and mice, resembling the neutrophilic subtype of MDSCs (Rieber et al., 2013), while monocytic MDSC subsets were not induced (Figure S1D). Fungi-induced MDSCs functionally suppressed T cell proliferation (Figure 1C), while autologous conventional neutrophils failed to do (Figure S1E).

We adoptively transferred T cell-suppressive neutrophilic MDSCs and monitored their impact on survival in fungal infection. While a single dose of adoptively transferred MDSCs was protective in systemic *C. albicans* infection, MDSCs had no impact on *A. fumigatus* infection (Figure 1E). Septic shock determines mortality in candidiasis (Spellberg et al., 2005), and the interplay of fungal growth and renal immunopathology was shown to correlate with host survival (Lionakis et al., 2011, 2013; Lionakis and Netea, 2013; Spellberg et al., 2003). Adoptively transferred MDSCs dampened renal T and NK cell activation and systemic Th17 and TNF- α cytokine responses (Figures S1F and S1G). Conversely, supplementing IL-17A dampened the MDSC-mediated protective effect (Figure 2A). Besides these immunomodulatory effects, MDSCs might also act directly antifungal, as our in vitro studies showed that they can phagocytose and kill fungi (Figure 2B). However, direct antifungal effects could hardly explain the beneficial effect of MDSCs in candidiasis: (i)

adoptively transferred MDSCs had no effect on fungal burden in vivo (Figure 2A), (ii) inhibition of phagocytosis only partially diminished the protection conferred by MDSCs (Figure 2A), and (iii) MDSCs were exclusively protective in immunocompetent mice (*C. albicans* infection model), with no effect in immunosuppressed (neutropenic) mice (*A. fumigatus* infection model).

The potency of *A. fumigatus* to induce MDSCs was most pronounced for germ tubes and hyphae, morphotypes characteristic for invasive fungal infections (Figure 1A) (Aimanianda et al., 2009; Hohl et al., 2005; Moyes et al., 2010). The MDSC-inducing fungal factor was present in conditioned supernatants and was heat resistant (Figure 3A), pointing to β -glucans as the bioactive component. We therefore focused on Dectin-1 as β -glucan receptor and key fungal sensing system in myeloid cells. Fungi-induced MDSCs expressed Dectin-1, and blocking Dectin-1 prior to fungal exposure diminished the MDSC-inducing effect, while blocking of TLR 4 had no effect (Figures 3B and S3). Furthermore, Dectin-1 receptor activation mimicked the generation of neutrophilic MDSCs phenotypically and functionally (Figures 3C and 3D). Dectin-1 receptor signaling was confirmed by blocking of the spleen tyrosine kinase Syk, which acts downstream of Dectin-1 (Figure 3B). We further used cells from human genetic Dectin-1 deficiency and used *Dectin-1* knockout mice for fungal infection models. The potential of fungi or fungal patterns to induce neutrophilic MDSCs was diminished in human and, albeit to a lesser extent, murine Dectin-1 deficiency (Figures 3E and S1D). We analyzed the role of caspase recruitment domain 9 (CARD9), a downstream adaptor protein and key transducer of Dectin-1 signaling, in fungi-mediated MDSC generation in patients with genetic *CARD9* deficiency and *Card9* knockout mice. These approaches demonstrated that CARD9 signaling was involved in fungal MDSC induction in the human and the murine system (Figures 3E and 3F).

C. albicans induces interleukin-1 beta (IL-1 β) in vitro (van de Veerdonk et al., 2009) and in vivo (Hise et al., 2009), which is critical for antifungal immunity (Vonk et al., 2006). Recent studies further provided evidence that IL-1 β is involved in MDSC homeostasis (Bruchard et al., 2013). We observed an accumulation of intracellular IL-1 β protein in CD33⁺ myeloid cells followed by IL-1 β release upon Dectin-1 ligand- and fungal-driven MDSC induction (Figure 4A). IL-1 β protein, in turn, was sufficient to drive MDSC generation to a comparable extent as *C. albicans* did (Figure 4B). Studies in *Il1r^{-/-}* mice, characterized by an increased susceptibility to *C. albicans* infection, demonstrated that abrogation of IL-1R signaling decreased MDSC accumulation in vivo (Figures 4B and S4A), and IL-1R antagonism in patients with autoinflammatory diseases decreased MDSCs (Figure S4B). As the inflammasome is the major mechanism driving IL-1 β generation in myeloid cells through caspase activities, we blocked caspases chemically. We observed that pan-caspase inhibition largely abolished fungi-induced MDSC generation, which was not recapitulated by caspase-1 inhibition (Figure 4C). We therefore focused on caspase-8, since Dectin-1 activation was shown to trigger IL-1 β processing by a caspase-8-dependent mechanism (Ganesan et al., 2014; Gringhuis et al., 2012). Indeed, fungal MDSC induction was paralleled by a substantial increase of caspase-8 activity, and caspase-8 inhibition diminished fungal-induced IL-1 β production (Figure 4C) and the potential of fungi to induce MDSCs (Figure 4C). Conversely, supplementing

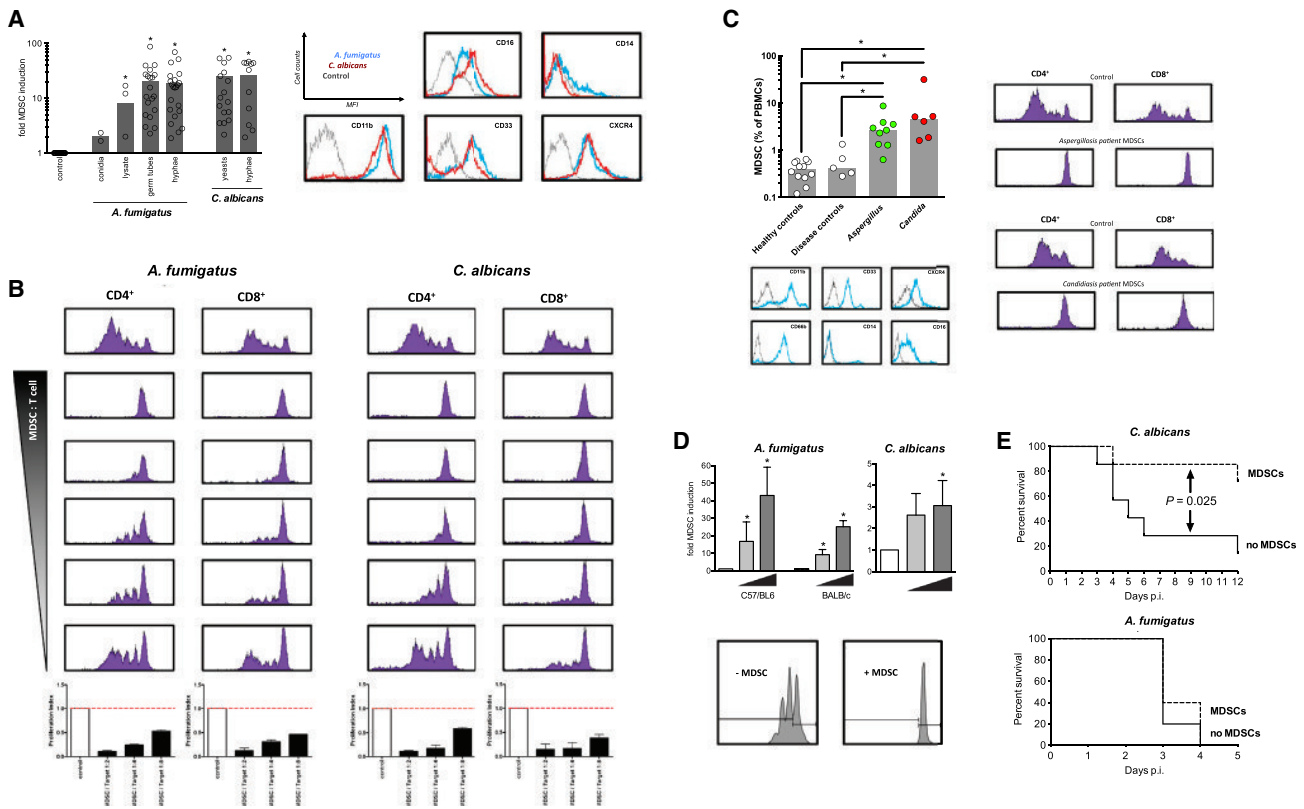


Figure 1. Fungi Induce Functional MDSCs In Vitro and In Vivo

(A) Fungal morphotypes differentially induce MDSCs.

Left panel: MDSCs were generated by incubating PBMCs (5×10^5 /ml) from healthy donors with medium only (negative control), or different morphotypes of *A. fumigatus* (conidia, 5×10^5 /ml; germ tubes, 1×10^5 /ml; hyphae, 1×10^5 /ml) or *C. albicans* (yeasts, 1×10^5 /ml; hyphae, 1×10^5 /ml). The x-fold induction of MDSCs compared to control conditions is depicted. * $p < 0.05$.

Right panel: representative histograms of fungi-induced MDSCs (CD11b⁺CD33⁺CD14⁻CD16⁺CXCR4⁺).

(B) Fungi-induced MDSCs suppress T cells. The suppressive effects of CD33⁺-MACS-isolated MDSCs were analyzed on CD4⁺ and CD8⁺ T cell proliferation. MDSCs were generated by incubating PBMCs (5×10^5 /ml) from healthy donors with *A. fumigatus* germ tubes (1×10^5 /ml) or *C. albicans* yeasts (1×10^5 /ml) for 6 days. Different MDSC-to-T cell ratios were assessed (1:2, 1:4, 1:6, 1:8, and 1:16). The lower bar graphs represent the proliferation index compared to control conditions as means \pm SEM.

(C) MDSCs in patients with fungal infections.

Left panel: MDSCs were characterized as CD14⁻ cells expressing CD33, CD66b, CD16, CD11b, and CXCR4 in the PBMC fraction. The gray line shows unstained controls. MDSCs were quantified in peripheral blood from healthy controls, immunosuppressed patients without fungal infections (disease controls, $n = 5$), or immunosuppressed patients with invasive fungal infections (invasive *A. fumigatus* infections, $n = 9$, and invasive *C. albicans* infections, $n = 6$). * $p < 0.05$.

Right panel: representative CFSE stainings, showing the effect of MDSCs isolated (MACS) from patients with invasive *A. fumigatus* infections (left) or invasive *C. albicans* infections (right) on CD4⁺ and CD8⁺ T cell proliferation.

(D) Fungi induce MDSCs in mice in vivo.

Upper left panel: C57/BL6 ($n = 3$ mice per treatment group) or BALB/c ($n = 4$ mice per treatment group) wild-type mice were not infected (white bars) or challenged intranasally with 1×10^4 (light gray bar) or 1×10^6 (dark gray bar) *A. fumigatus* conidia for 3 days. On the fourth day, a bronchoalveolar lavage (BAL) was performed, and CD11b⁺Ly6G⁺ MDSCs were quantified by FACS. The x-fold induction of CD11b⁺Ly6G⁺ MDSCs in the BAL compared to control non-infected conditions is depicted. * $p < 0.05$.

Upper right panel: C57BL/6 mice were not infected (white bars) or injected via the lateral tail vein with 2.5×10^5 (light gray bar) or 5×10^5 (dark gray bar) blastospores of *C. albicans*. On the fifth day, mice were sacrificed, and CD11b⁺Ly6G⁺ MDSCs in the spleen were quantified by FACS. The x-fold induction of CD11b⁺Ly6G⁺ MDSCs in the spleen compared to control non-infected conditions is depicted. $n = 5$ mice per treatment group. * $p < 0.05$.

Lower panel: bone marrow-isolated murine CD11b⁺Ly6G⁺ MDSCs were co-cultured for 3 days with T cells (CD4⁺ splenocytes) at a 1:2 (MDSCs:T cell) ratio. T cell proliferation was analyzed using the CFSE assay with and without MDSCs.

(E) Adoptive transfer of MDSCs modulates survival in fungal infection. For adoptive transfer experiments, CD11b⁺Ly6G⁺ MDSCs were isolated from the bone marrow of BALB/c mice by MACS and checked for T cell suppression. In (A)–(D) bars represent means \pm SEM.

Upper panel: adoptive MDSC transfer was performed by intravenous (i.v.) injection of 5×10^6 MDSCs per animal. Seven mice received MDSCs, while seven mice served as non-MDSC control animals. A total of 2 hr after the MDSC transfer, mice were i.v. injected with 1×10^5 blastospores of *C. albicans*. Mice were weighed daily and monitored for survival and signs of morbidity.

Lower panel: for invasive pulmonary *A. fumigatus* infection survival studies, mice were immunosuppressed by treatment with cyclophosphamide, and MDSC transfer was performed by i.v. injection of 4×10^6 MDSCs per animal. Five mice received MDSCs, while five mice served as non-MDSC control animals. After the MDSC transfer, mice were challenged intranasally with 2×10^5 *A. fumigatus* conidia and were monitored for survival.

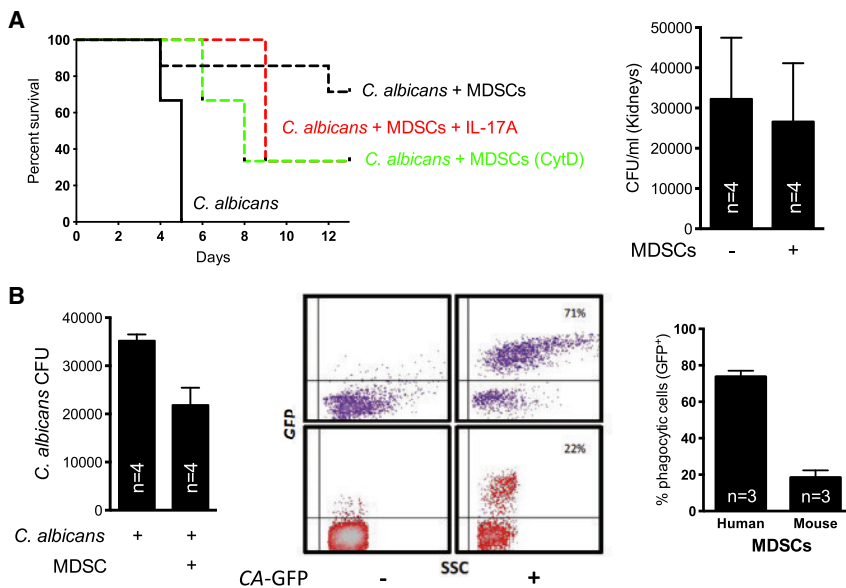


Figure 2. Antifungal Functions

(A) In vivo.

Left panel: survival in the invasive *C. albicans* infection model after adoptive MDSC transfer. Before adoptive transfer, isolated MDSCs were pretreated with cytochalasin D (CytD, 1 μ g/ml, green line) or with recombinant mouse IL-17A protein (5 μ g/mouse, red line).

Right panel: *C. albicans* CFUs in kidneys of BALB/c mice 5 days after adoptive transfer of MDSCs. Bars represent means \pm SD.

(B) In vitro.

Left panel: 1×10^6 human MDSCs were co-cultured with 1×10^5 serum opsonized *C. albicans* (10:1 ratio) for 3 hr at 37°C in RPMI. Serial dilutions were performed of the cell suspension, and 100 μ l was plated onto YPD agar plates containing penicillin and streptomycin. Plates were incubated for 24–48 hr at 37°C, and CFU were enumerated.

Middle and right panels: phagocytic capacity of human and murine MDSCs. Middle panel; upper (purple) FACS plots, isolated human granulocytic MDSCs (low-density CD66b⁺CD33⁺ cells) were co-cultured with or without GFP-labeled *C. albicans* (CA) spores (MOI = 1) in RPMI medium at 37°C for

90 min. Lower (red) FACS plots, isolated mouse granulocytic CD11b⁺Ly6G⁺ MDSCs were co-cultured with or without GFP-labeled *C. albicans* spores (MOI = 4) in RPMI medium at 37°C for 90 min. Representative dot blots are shown.

Right panel: GFP expression/fluorescence of MDSCs was analyzed by FACS and is given in the right panel as percentage of GFP⁺ MDSCs.

IL-1 β partially restored the abrogated MDSC generation upon caspase-8 inhibition (Figure S4C).

ROS are key factors in MDSC homeostasis (Gabrilovich and Nagaraj, 2009) and act downstream of Dectin-1 (Gross et al., 2009; Underhill et al., 2005). Therefore, we tested the involvement of ROS for fungal Dectin-1 ligand-induced MDSC generation using chemical inhibitors and cells from human CGD patients with ROS deficiency. These studies demonstrated that ROS contributed substantially to fungal MDSC induction (Figure 4D). Next, we investigated the interaction between ROS, caspase-8, and IL-1 β and found that ROS inhibition dampened caspase-8 activity in response to fungi (Figure S4D). IL-1 β , in turn, induced ROS production during MDSC culture, suggesting a positive feedback loop between caspase-8, IL-1 β , and ROS in MDSC generation (Figures S4E and S4F).

DISCUSSION

While the complete genetic deletion of pro-inflammatory cytokines, particularly TNF- α , IL-1 α/β , or IFN- γ , increases disease susceptibility in invasive fungal infections (Lionakis and Netea, 2013; Cheng et al., 2012; Gow et al., 2012; Netea et al., 2008, 2010), excessive inflammation causes collateral damage to the host (Carvalho et al., 2012; Romani et al., 2008), indicating that efficient protection against fungi requires a fine-tuned balance between pro-inflammatory effector and counter-regulatory immune mechanisms. Fungal infection induces an immunosuppressive state, and in murine models CD80⁺ neutrophilic cells have been shown to be importantly involved in this process (Mencacci et al., 2002; Romani, 2011; Romani et al., 1997). By combining human and murine experimental systems, we extend this concept by providing evidence for an MDSC-mediated mechanism by which fungi modulate host defense, orchestrated

by Dectin-1/CARD9, ROS, caspase-8, and IL-1 β . This effect seems to be specific for neutrophilic MDSCs, since monocytic MDSCs were unchanged under our experimental conditions and were previously found to be downregulated by β -glucans in tumor-bearing mice (Tian et al., 2013).

C. albicans and *A. fumigatus* infections differ substantially with respect to T cell dependency and organ manifestation (Garcia-Vidal et al., 2013). Our finding that neutrophilic MDSCs were protective in a murine model of systemic *C. albicans* infection, but had no effect on pulmonary *A. fumigatus* infection, underlines this disparity and suggests MDSCs as a potential therapeutic approach in invasive *C. albicans*, rather than *A. fumigatus* infections. The MDSC-mediated effect was associated with downregulated NK and T cell activation, and Th17 responses and supplementing IL-17A in vivo could, at least partially, dampen the protective effect of MDSCs. Based on previous studies showing that NK cells drive hyperinflammation in candidiasis in immunocompetent mice (Quintin et al., 2014) and that IL-17 promotes fungal survival (Zelante et al., 2012), we speculate that MDSCs in fungal infections could act beneficial for the host by dampening pathogenic hyperinflammatory NK and Th17 responses (Romani et al., 2008; Zelante et al., 2007). Accordingly, enhancing neutrophilic MDSCs may represent an anti-inflammatory treatment strategy for fungal infections, particularly with *C. albicans*.

Recent studies put the gut in the center of immunotolerance. Dectin-1 was found to control colitis and intestinal Th17 responses through sensing of the fungal mycobiome (Iliev et al., 2012). The immunological events downstream of Dectin-1 and their functional impact on Th17 cells remained elusive. Our results demonstrate that fungal Dectin-1/CARD9 signaling induces MDSCs to dampen T cell responses and suggest that the immune homeostasis in the gut could be modulated by fungal-induced

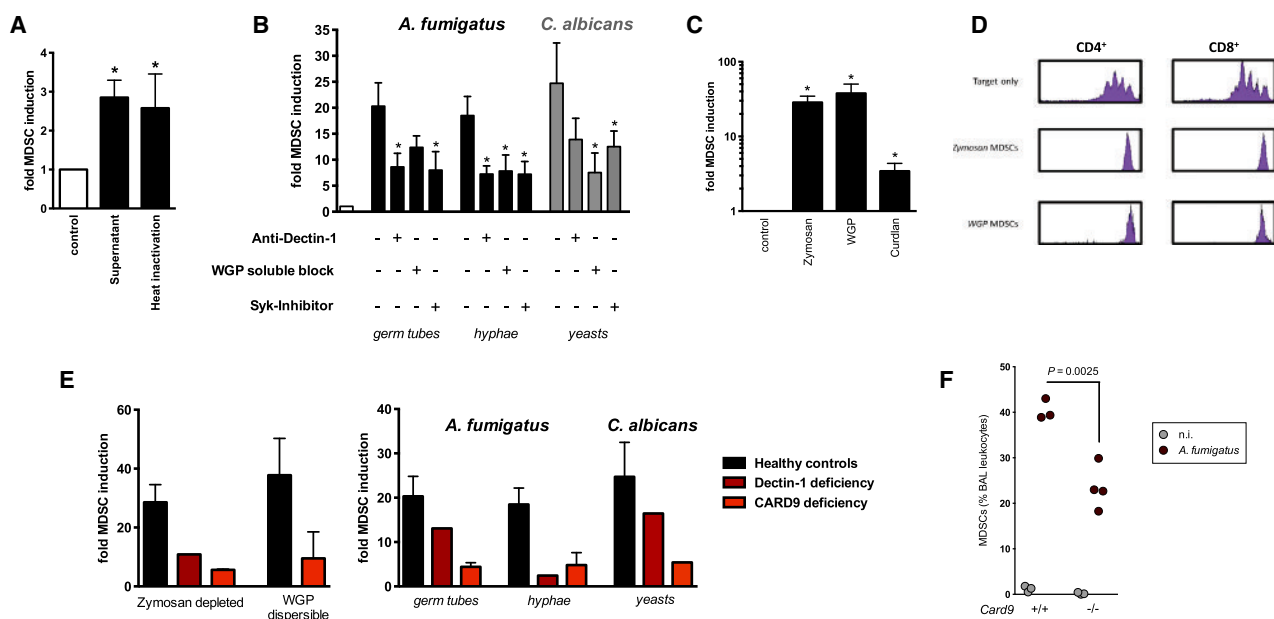


Figure 3. Fungi Induce MDSCs through a Dectin-1-, Syk-, and CARD9-Mediated Mechanism

(A) Fungal factors mediating MDSC induction are heat resistant. MDSCs were generated by incubating PBMCs (5×10^5 /ml) from healthy donors with medium only (negative control), untreated, or heat-denatured (95°C , 30 min) supernatants (SNT) of *A. fumigatus* germ tubes (4%) for 6 days. The x-fold induction of MDSCs compared to control conditions is depicted. * $p < 0.05$ versus control conditions.

(B) Dectin-1 and Syk are involved in fungal MDSC induction. MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) with *A. fumigatus* germ tubes (1×10^5 /ml), hyphae (1×10^5 /ml), and *C. albicans* yeasts (1×10^5 /ml) for 6 days. Where indicated, PBMCs were pretreated for 60 min with anti-Dectin-1 blocking antibody (15 $\mu\text{g}/\text{ml}$), soluble WGP (1 mg/ml), and a Syk inhibitor (100 nM). * $p < 0.05$ blocking versus unblocked conditions.

(C) Dectin-1/CARD9 ligands mimic fungal MDSC induction. MDSCs were generated in vitro by incubating isolated PBMCs with the Dectin-1/CARD9 ligands zymosan depleted (10 $\mu\text{g}/\text{ml}$), dispersible WGP (20 $\mu\text{g}/\text{ml}$), or curdlan (10 $\mu\text{g}/\text{ml}$). $p < 0.05$ versus control conditions.

(D) Dectin-1/CARD9 ligands induce functional MDSCs. The suppressive effects of CD33⁺-MACS-isolated MDSCs were analyzed on CD4⁺ and CD8⁺ T cell proliferation (CFSE polyclonal proliferation assay). MDSCs were generated by incubating PBMCs (5×10^5 /ml) from healthy donors with zymosan depleted (10 $\mu\text{g}/\text{ml}$) or dispersible WGP (20 $\mu\text{g}/\text{ml}$). MDSC, T cell ratio was 1:6.

(E) Fungal MDSC induction in patients with genetic Dectin-1 or CARD9 deficiency.

Left panel: MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) from healthy controls ($n = 12$), an individual with Dectin-1 deficiency, or patients with CARD9 deficiency ($n = 2$) with the Dectin-1/CARD9 ligands zymosan depleted (10 $\mu\text{g}/\text{ml}$) or dispersible WGP (20 $\mu\text{g}/\text{ml}$).

Right panel: MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) from healthy controls ($n = 12$), an individual with genetically proven Dectin-1 deficiency, or patients with CARD9 deficiency ($n = 2$) with different fungal morphotypes (1×10^5 cells/ml) for 6 days.

(F) CARD9 is involved in fungi-induced MDSC recruitment in vivo. *Card9*^{-/-} mice and age-matched wild-type mice were challenged intranasally with 1×10^6 *A. fumigatus* conidia for 3 days. On the fourth day, a BAL was performed, and CD11b⁺Ly6G⁺ MDSCs were quantified by flow cytometry. In (B), (C), and (E) bars represent means \pm SEM.

MDSCs. Beyond fungi, the Dectin-1/CARD9 pathway has been involved in bacterial and viral infections (Hsu et al., 2007), suggesting that this mechanism could play a broader role in balancing inflammation at host-pathogen interfaces.

EXPERIMENTAL PROCEDURES

Fungal Strains and Culture Conditions

A. fumigatus ATCC46645 conidia were incubated in RPMI at RT for 3 hr at 150 rpm to become swollen. Alternatively, conidia were cultured in RPMI overnight at RT, followed by germination in RPMI either at 37°C for 3 hr at 150 rpm to become germ tubes or at 37°C for 17 hr at 150 rpm to become hyphae. *C. albicans* SC5314 was grown on SAB agar plates at 25°C . One colony was inoculated and shaken at 200 rpm at 30°C in SAB broth overnight. To generate hyphae, live yeast forms of *C. albicans* were grown for 6 hr at 37°C in RPMI 1640. Killed yeasts and hyphae were prepared by heat treatment of the cell suspension at 95°C for 45 min or by fixing the cells for 1 hr with 4% paraformaldehyde followed by extensive washing with PBS to completely remove the fixing agent. The *C. albicans*-GFP strain TG6 was pre-cultured at 30°C , 200 rpm overnight in YPD medium.

Generation, Isolation, and Characterization of MDSCs

Neutrophilic MDSCs in peripheral blood were quantified based on their lower density and surface marker profiles as published previously (Rieber et al., 2013). Human MDSCs were generated in vitro according to a published protocol (Lechner et al., 2010). Murine MDSCs were characterized by CD11b, Ly6G, and Ly6C. Flow cytometry was performed on a FACS Calibur (BD Biosciences). Human and murine MDSCs were isolated using MACS (MDSC Isolation Kit; Miltenyi Biotec).

T Cell Suppression Assays

T cell suppression assays were performed as described previously (Rieber et al., 2013) using the CFSE method according to the manufacturer's protocol (Invitrogen).

Mouse Infection with *A. fumigatus* and *C. albicans*

Invasive *C. albicans* infection was established by IV injection in immunocompetent mice, whereas *A. fumigatus* infection was established by intranasal challenge in immunosuppressed mice. CD11b⁺Ly6G⁺ and CD11b⁺Ly6C⁺ cells in the spleens, BAL, and kidneys were quantified by FACS. For adoptive transfer experiments, CD11b⁺Ly6G⁺ MDSCs were isolated by MACS and transferred by IV injection of 4 or 5×10^6 MDSCs per animal.

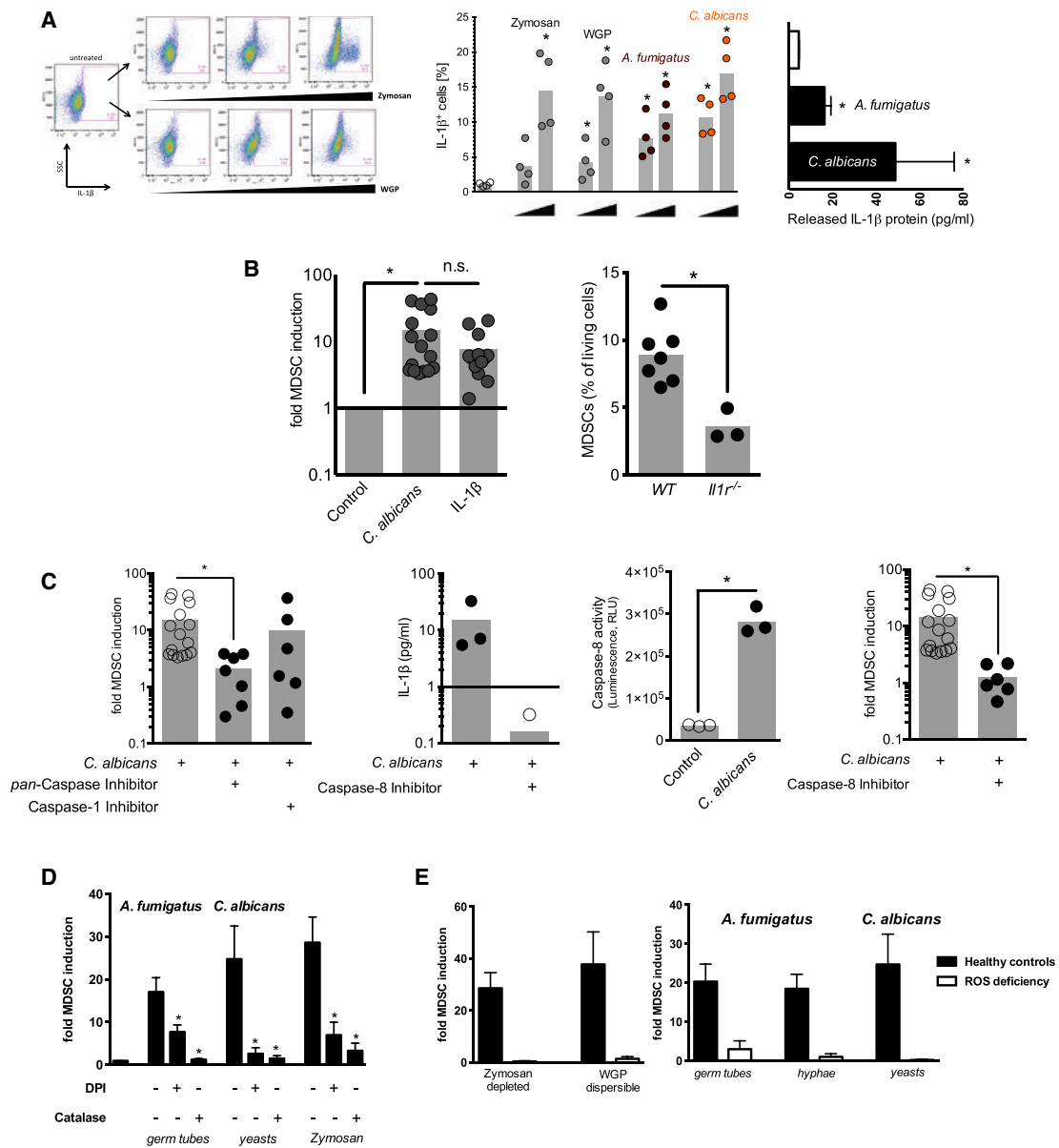


Figure 4. Fungal MDSC Induction Involves IL-1 β , Caspase-8, and ROS

(A) Intracellular accumulation and release of IL-1 β .

Left panel: gating strategy for intracellular cytokine staining. IL-1 β was analyzed in CD33⁺ myeloid cells using intracellular cytokine staining and flow cytometry. Zymosan depleted (20, 100, and 500 μ g/ml) and WGP dispersible (20, 100, and 500 μ g/ml) were used for 1 hr to stimulate cytokine production.

Middle panel: leukocytes isolated from healthy donors (n = 4) were left untreated (empty circles) or were treated for 1 hr with increasing concentrations of zymosan, WGP, *A. fumigatus* germ tubes, or *C. albicans* yeasts (each at 2×10^9 /ml and 1×10^9 /ml). IL-1 β synthesis in CD33⁺ cells was analyzed by intracellular cytokine stainings by flow cytometry. *p < 0.05 versus control/untreated conditions.

Right panel: co-culture supernatants were collected after incubating isolated PBMCs (5×10^5 cells/ml) with medium only (white bar), *A. fumigatus* germ tubes (1×10^5 cells/ml), or *C. albicans* yeasts (1×10^5 /ml) for 3 days. IL-1 β was quantified by ELISA. *p < 0.05 versus medium control conditions.

(B) IL-1 β signaling is involved in fungal-induced MDSC generation.

Left panel: MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) with *C. albicans* yeasts (1×10^5 /ml) or recombinant human IL-1 β protein (0.01 μ g/ml) for 6 days. *p < 0.05.

Right panel: MDSCs (CD11b⁺Ly6G⁺) were quantified in spleens from *Il1r*^{-/-} and age-matched WT mice 2 days after i.v. infection with 1×10^5 blastospores of *C. albicans*. *p < 0.05.

(C) Fungal MDSC generation involves caspase-8. MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) with *C. albicans* yeasts (1×10^5 /ml) for 6 days with or without pretreatment (where indicated) with the pan-caspase inhibitor Z-VAD-FMK (10 μ M), the caspase-1 inhibitor Z-WEHD-FMK (50 μ M), or the caspase-8 inhibitor Z-IETD-FMK (50 μ M). IL-1 β protein levels were quantified in cell culture supernatants by ELISA (note: two values were below detection limit). Caspase-8 activity was quantified in cell lysates using a luminescent assay. *p < 0.05.

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2015.02.007>.

AUTHOR CONTRIBUTIONS

N.R. and D.H. designed the study, supervised experiments, performed analyses, and wrote the manuscript. H.Ö., A.S., and M.C. performed murine infection studies. A.S., S.N.K., M.O., M. Ballbach, Y.Z., and I.S. performed MDSC in vitro assays. M. Bouzani and J. Loeffler performed and supervised NK cell assays. J. Loeffler and S.K. provided fungi, contributed to the design of the study, and wrote the manuscript. J.A. and A.B. performed and analyzed murine infection studies. R.H., M.M., J. Loeffler, J. Liese, A.N.R.W., M.E., R.S., H.R.S., C.S., L.M., and B.G. co-designed the study, provided patient material, and wrote the manuscript. J.R. and G.D.B. provided mice and co-designed in vivo experiments.

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(D) Fungal MDSC-inducing capacity is ROS dependent. MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) with different fungal morphotypes (1×10^5 cells/ml) or zymosan ($10 \mu\text{g/ml}$) for 6 days. PBMCs were pretreated where indicated with the NADPH oxidase inhibitor DPI ($0.1 \mu\text{M}$) or the H_2O_2 converting enzyme catalase (100 U/l). * $p < 0.05$ blocking versus unblocked conditions.

(E) Fungal MDSC induction in patients with ROS deficiency.

Left panel: MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) from healthy controls ($n = 12$) or patients with CGD ($n = 3$) with the Dectin-1/CARD9 ligands zymosan depleted ($10 \mu\text{g/ml}$) or dispersible WGP ($20 \mu\text{g/ml}$).

Right panel: MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) from healthy controls ($n = 12$) or CGD patients ($n = 3$) with different fungal morphotypes (1×10^5 cells/ml) for 6 days.

In (A)–(E) bars represent means \pm SEM.

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Cell Host & Microbe, Volume 17

Supplemental Information

Pathogenic Fungi Regulate Immunity by Inducing

Neutrophilic Myeloid-Derived Suppressor Cells

Nikolaus Rieber, Anurag Singh, Hasan Öz, Melanie Carevic, Maria Bouzani, Jorge Amich, Michael Ost, Zhiyong Ye, Marlene Ballbach, Iris Schäfer, Markus Mezger, Sascha N. Klimosch, Alexander N.R. Weber, Rupert Handgretinger, Sven Krappmann, Johannes Liese, Maik Engeholm, Rebecca Schüle, Helmut Rainer Salih, Laszlo Marodi, Carsten Speckmann, Bodo Grimbacher, Jürgen Ruland, Gordon D. Brown, Andreas Beilhack, Juergen Loeffler, and Dominik Hartl

Supplemental Data
Figure S1, related to Figure 1

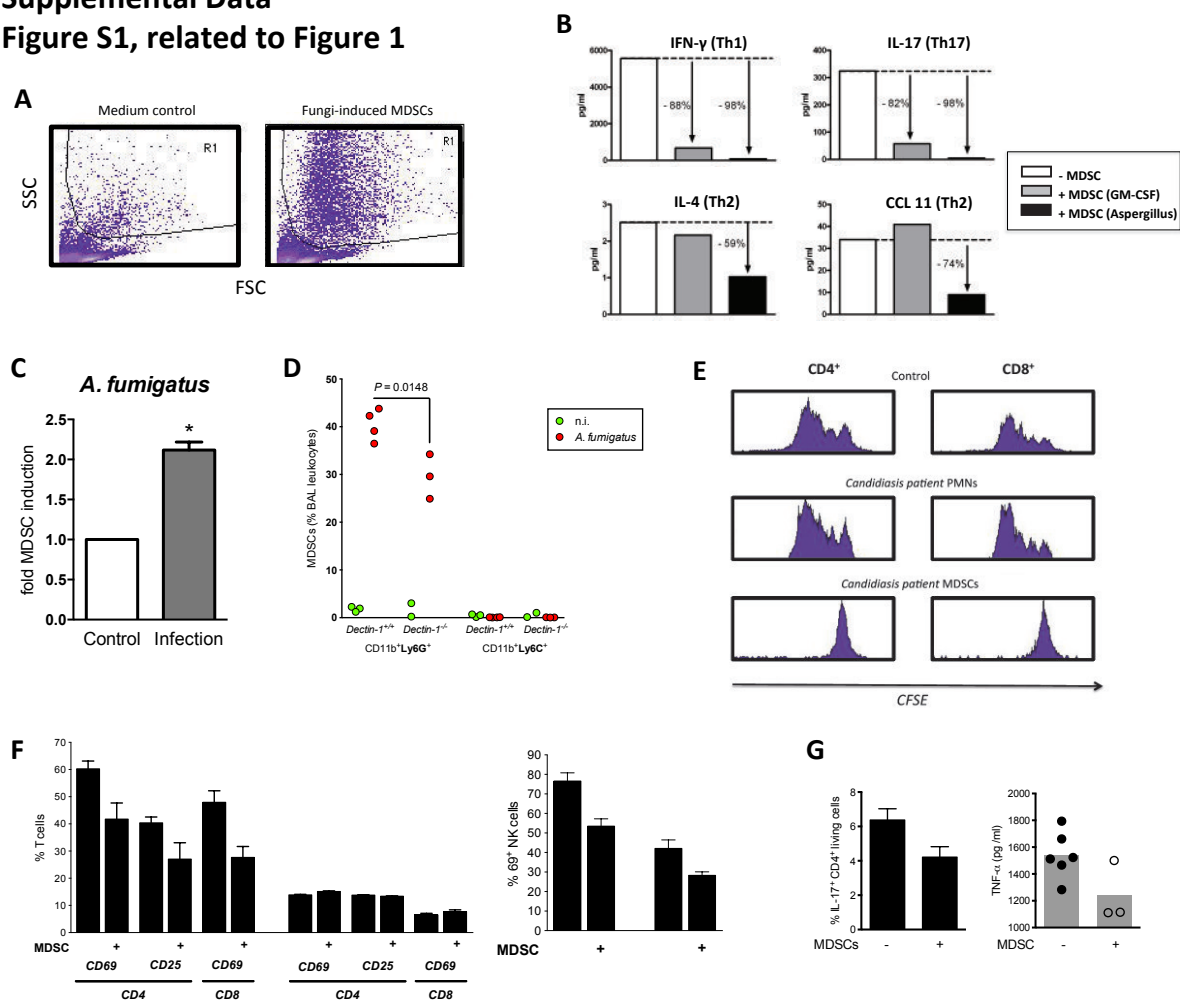


Figure S1. Phenotypic and functional MDSC characteristics

A. FSC/SSC characteristics of fungi-induced MDSCs *in vitro*

MDSCs were generated by incubating PBMCs (5×10^5 /ml) from healthy donors with medium only (negative control, 'Medium control') or *A. fumigatus* germ tubes (1×10^5 /ml, 'Fungi-induced MDSCs') for 6 days. Dot blots show representative MDSC gateings for subsequent immunophenotyping based on surface marker expression profiles as depicted in Figure 1a.

B. *Aspergillus*- and GM-CSF-induced MDSCs differentially affect Th1/Th17 and Th2 cytokine and chemokine levels

IL-2 and OKT-3 stimulated PBMCs were cultured in medium alone or together with *Aspergillus*- or GM-CSF- induced MDSCs for 96h. Cytokine and chemokine concentrations in culture supernatants were analyzed by multiplex array technology.

C. MDSC induction in immunodeficient mice

MDSC induction in immunodeficient mice: BALB/c wildtype mice were immunosuppressed with cyclophosphamide (150 mg/kg bw i.p.) and not infected (white bars) or challenged intranasally with 1×10^3 *A. fumigatus* conidia (grey bar) for three days. On the fourth day, CD11b⁺Ly6G⁺ MDSCs were quantified in lungs by FACS. The x-fold induction of CD11b⁺Ly6G⁺ MDSCs in the *A. fumigatus*-infected lung compared to control non-infected conditions is depicted. Bars represent means \pm s.e.m. * $P < 0.05$

D. MDSC induction in *Dectin-1*^{-/-} mice

MDSC induction in *Dectin1*^{-/-} mice: *Dectin1*^{-/-} mice and age-matched wildtype mice were challenged intranasally with 1×10^6 CFU *A. fumigatus* for three days. On the fourth day, a bronchoalveolar lavage was performed and granulocytic (CD11b⁺Ly6G⁺) or monocytic (CD11b⁺Ly6C⁺) MDSCs were quantified by flow cytometry.

E. *Candida*-induced neutrophilic MDSCs, but not conventional PMNs, suppress T-cell proliferation

Representative CFSE stainings, showing the effect of neutrophilic MDSCs or conventional autologous neutrophils (PMNs) isolated (MACS) from patients with invasive *C. albicans* infections on CD4⁺ and CD8⁺ T-cell proliferation.

F. Effect of MDSCs on inflammation

T- and NK-cell activation was quantified in kidneys (left bars) and spleens (right bars) (5 days p.i.) in the invasive *C. albicans* infection model with and without adoptive MDSC transfer. T and NK cell activation was measured by CD69 and CD25 surface expression on CD4⁺ T cells, CD8⁺ T cells and on CD3⁺DX5⁺NKp46⁺ NK cells. T cell graph (left): the left bars show kidney, the right bars spleen. NK cell graph (right): the left bars show kidney, the right bars spleen.

G. IL-17A was stained intracellularly in CD4⁺ splenocytes 5 days after adoptive MDSC transfer by flow cytometry. TNF- α protein levels were quantified in serum 5 days after adoptive MDSC transfer by Bioplex.

Figure S2,
related to Figure 1

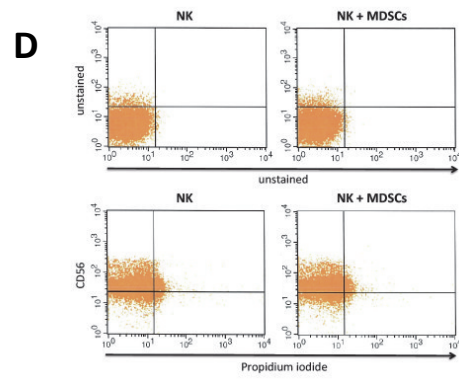
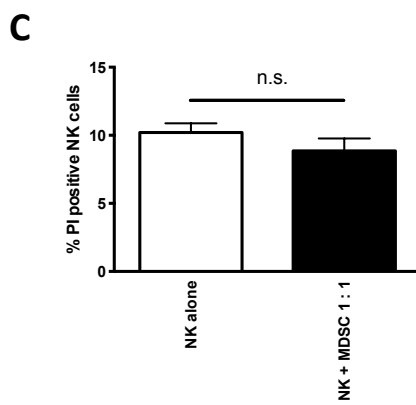
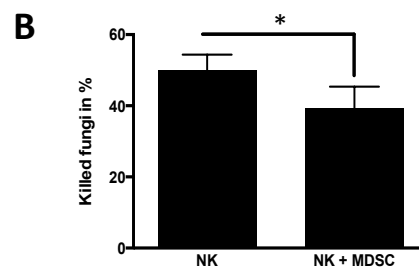
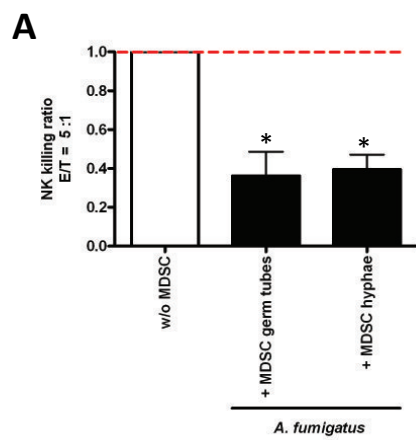


Figure S2: *Aspergillus*-induced MDSCs decrease antifungal NK killing activity

A. The suppressive effects of CD33⁺-MACS-isolated MDSCs on NK cells were analyzed by measuring the NK cell cytotoxicity against K562 tumor cell line (europium release assay). MDSCs were generated by incubating PBMCs (5x10⁵/ml) from healthy donors with *A. fumigatus* germ tubes (1x10⁵/ml). MDSC to NK cell ratio was 1:1. NK (Effector, E) to K562 (Target, T) ratio was 5:1. Bars represent means ± s.e.m. **P*<0.05;

B. Activated NK cells were co-cultured with purified MDSC at a 1:1 ratio for 16h. After co-culture, MDSC were depleted and purified NK cells were incubated with *A. fumigatus* germ tubes at a 1:1 ratio for 5h. Fungal cell viability was determined using an XTT assay. **P*<0.05

C. MDSCs and NK cells were isolated from healthy PBMCs by magnetic bead technique. NK cells were cultured in medium alone or co-cultured together with MDSCs in a ratio of 1:1 overnight analogous to the cytotoxicity assays. Dead cells were stained with propidium iodide (PI). Bar graphs show percentages of PI positive dead NK cells within all NK cells. n.s. not significant

D. NK cell viability as assessed by propidium iodide staining for NK cells (CD3⁺CD56⁺ cells) in 1:1 NK-MDSC co-culture assays. The upper panel shows unstained controls from the same cells.

Figure S3, related to Figure 3

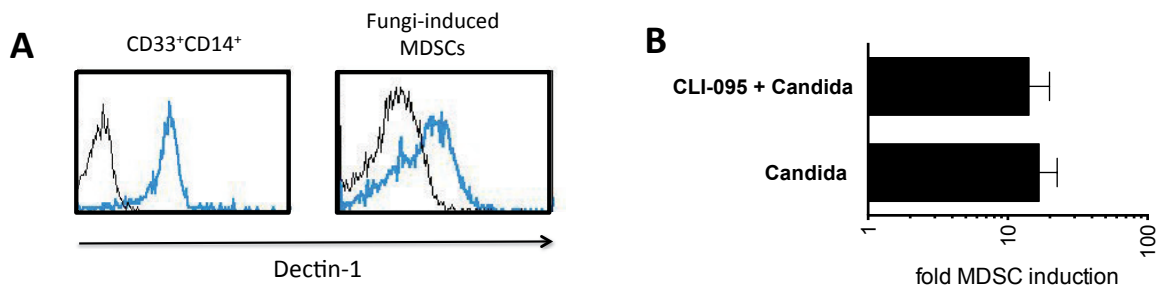


Figure S3: Dectin-1 and TLR4

A. Histograms show representative examples of Dectin-1 surface expression on fungi-induced MDSCs and CD33⁺CD14⁺ cells.

B. MDSCs were generated by incubating PBMCs (5×10^5 /ml) from healthy donors with *C. albicans* (yeasts: 1×10^5 /ml) with or without 1h pretreatment with the TLR4 inhibitor CLI-095 ($1 \mu\text{M}$). The x-fold induction of MDSCs compared to control conditions is depicted. Bars represent means \pm s.e.m.

Figure S4, related to Figure 4

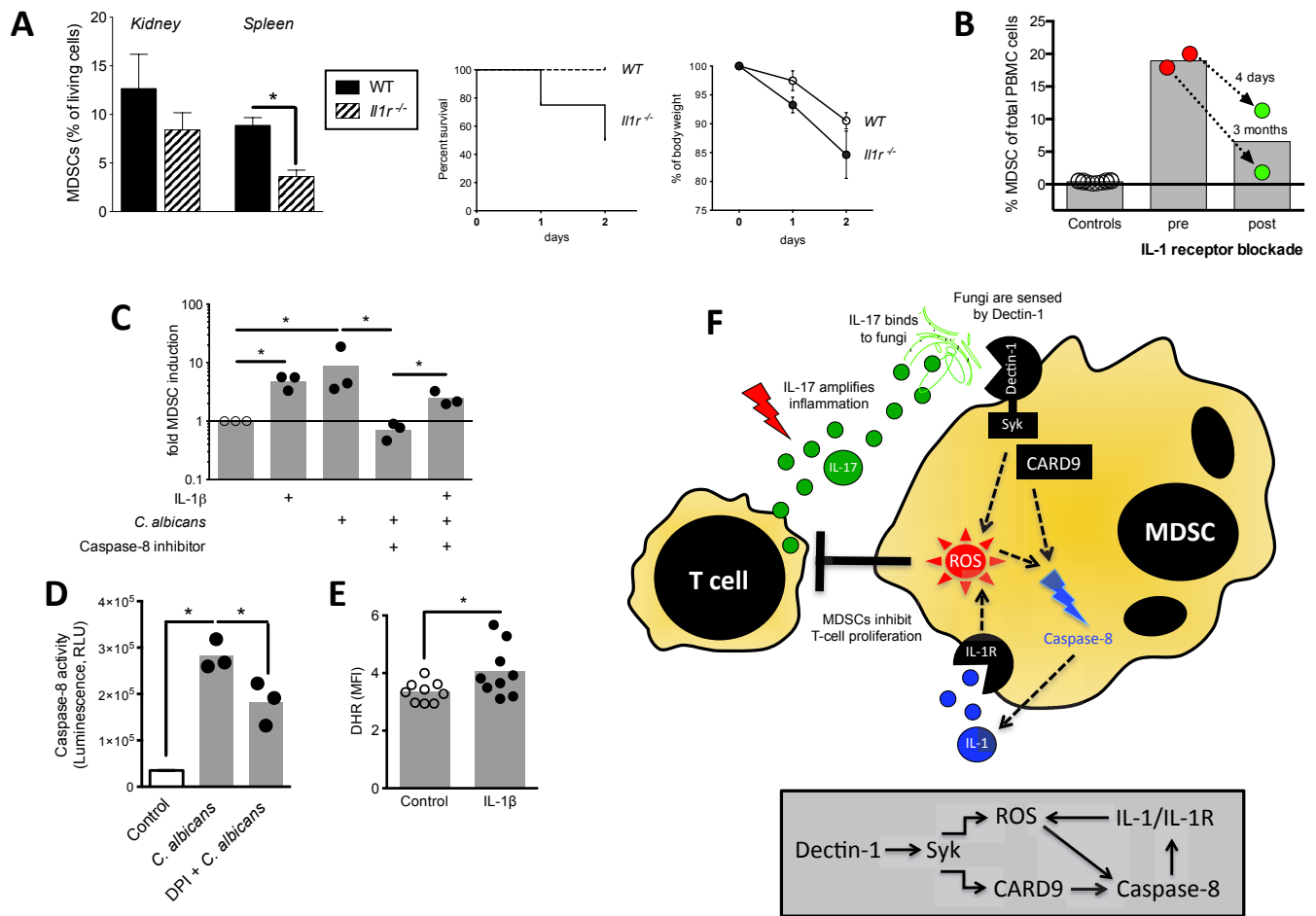


Figure S4: IL-1, Caspase-8 and ROS

A. WT and *Il1r^{-/-}* mice were i.v. injected with 1×10^5 blastospores of *Candida albicans* SC5314 per animal in 100 μ l PBS. Mice were weighed daily and monitored for survival and weight loss. Mice with a weight loss of more than 20% or with serious symptoms of illness were euthanized. For MDSC quantification, mice were sacrificed and CD11b⁺Ly6G⁺ MDSCs in the kidneys and spleens were quantified by FACS. Bars represent means \pm s.e.m. * $P < 0.05$

B. MDSCs were quantified in peripheral blood of two patients before and after systemic anti-IL-1 therapy using the IL-1-receptor-antagonist *anakinra* (3 mg/kg bw/d). Patient 1 (male, 2 years of age, 3 months on *anakinra*) had a severe chronic non-classified autoinflammatory disease and patient 2 (female, 9 years of age, 4 days on *anakinra*) suffered from systemic onset juvenile idiopathic arthritis (soJIA).

C. MDSCs were generated *in vitro* by incubating isolated PBMCs (5×10^5 cells/ml) with *C. albicans* yeasts (1×10^5 /ml) or recombinant human IL-1 β (0.01 μ g/ml) for 6 days with or without pretreatment with the caspase-8 inhibitor Z-IETD-FMK (50 μ M). MDSCs were quantified using flow cytometry. Bars represent means. * $P < 0.05$

D. MDSCs were generated *in vitro* by incubating isolated PBMCs (5×10^5 cells/ml) with *C. albicans* yeasts (1×10^5 /ml) for 6 days with or without pretreatment with the NADPH oxidase inhibitor DPI (0.1 μ M). Caspase-8 activity was measured by a luminescent assay (Caspase-Glo 8 Assay from Promega, USA). Bars represent means. * $P < 0.05$

E. PBMCs of healthy donors were treated with medium only (control) or recombinant human IL-1 β (0.1 μ g/ml) for 4 hours. After stimulation with PMA (200nM) for another 8 minutes, ROS production was measured by DHR in CD33⁺ myeloid cells. Bars represent means. * $P < 0.05$

F. Proposed model of MDSC generation in invasive fungal infections: Fungal sensing through Dectin-1 triggers downstream signaling cascades involving Syk and CARD9, leading to caspase-8 activation. Caspase-8 drives interleukin-1 (IL-1) production. Released IL-1 binds to the IL-1 receptor (IL-1R) and enhances generation of ROS, which are essential for MDSC induction. Moreover, ROS are involved in fungal-driven caspase-8 activation. Generated MDSCs inhibit NK and T-cell responses, such as Th17 responses that amplify inflammation and may also directly affect fungal survival.

Supplemental Experimental Procedures

Study subjects

The study was conducted at the University Children's Hospital Tübingen (Germany). Informed consent was obtained from all subjects included in the study and all study methods were approved by the local ethics committee. At time of blood sampling all healthy subjects were without signs of infection, inflammation, or respiratory symptoms. Nine patients with invasive aspergillosis (positive *Aspergillus* galactomannan serum test and clinical signs of invasive aspergillosis) and six patients with invasive *Candida* bloodstream infections were also included in the study after written informed consent. These patients acquired invasive fungal infection during oncologic chemotherapy, after hematopoietic stem cell transplantation or showed fungal endocarditis. Moreover, five immunosuppressed patients after hematopoietic stem cell transplantation, but without fungal infections, were included as disease control group. In two patients with autoinflammatory diseases, MDSCs were quantified in peripheral blood before and after treatment with the IL-1R antagonist anakinra (3mg/kg/d). We further obtained blood from two patients with CARD9 deficiency and a medical history of several invasive fungal infections. The CARD9 mutations were: c.883G>A(hom) and c.883C>T(hom), both resulting in a premature termination codon (Q295X) consistent with a previously defined CARD9 defect (Glocker et al., 2009). On protein level no CARD9 protein could be detected in the patient's neutrophils and monocytes. We analyzed blood from a healthy subject with a homozygous Dectin-1 stop codon mutation (Tyr238X) consistent with a previously described Dectin-1 deficiency (Ferwerda et al., 2009). This mutation was identified through a whole exome sequencing approach for his affected daughter. In addition, we obtained fresh blood samples from three patients with chronic granulomatous disease (CGD) and complete ROS deficiency.

A. fumigatus strain and culture conditions

A. fumigatus ATCC46645 conidia were frozen at -80°C in glycerol stocks. After growing on Sab agar plates at 37°C, one colony was inoculated into Sab broth and shaken at 37°C overnight. Conidia were incubated in RPMI at room temperature (RT) for 3 h at 150 rpm to become swollen. Alternatively, conidia were cultured in RPMI overnight at RT, followed by germination in RPMI either at 37°C for 3 h at 150 rpm to become germ tubes or at 37°C for 17h at 150 rpm to become hyphae. Fungi were washed twice in PBS and heat-inactivated for 30 min at 95°C. Culture supernatants from conidia, germ tubes and hyphae were centrifuged at 8000 rpm for 15 min, followed by steril filtration using a 0.2 µm filter, respectively.

C. albicans strain and culture conditions

C. albicans SC5314 was stored as frozen stocks in 35 % glycerol at -80 °C and routinely grown on Sabouraud (Sab) agar plates at 25°C. One colony was inoculated and shaken at 200 rpm at 30°C in Sab broth (1% mycological peptone and 4% glucose) overnight. Cells were harvested by centrifugation and washed twice in Dulbecco's phosphate-buffered saline (PBS). Cells were counted in a haemocytometer and density was adjusted to the desired concentration in either PBS or RPMI 1640 medium. To generate hyphae, live yeast forms of *C. albicans* were grown for 6 h at 37°C in RPMI 1640 (Gibco-BRL). Killed yeasts and hyphae were prepared by heat treatment of the cell suspension at 95°C for 45 minutes or by fixing the cells for 1 h with 4% paraformaldehyde followed by extensive washing with PBS to completely remove the fixing agent. The *C. albicans*-GFP strain TG6 (a generous gift from Dr. Steffen Rupp, Fraunhofer IGB Stuttgart) was pre-cultured at 30°C, 200 rpm overnight in YPD medium. Cells were washed twice with sterile PBS and counted using a haemocytometer prior to use.

In vitro generation and isolation of human MDSCs

Human MDSCs were generated *in vitro* according to a previously published protocol (Lechner et al., 2010). Isolated human PBMCs were cultured in 12 well flat-bottom plates (Corning) or 25 cm² flasks (Greiner Bio-One) at 5 x 10⁵ cells/ml in complete medium for 6 d, and GM-CSF (10 ng/ml, Genzyme), heat inactivated (95°C, 30min) *A. fumigatus* morphotypes (1:1 to 1:5 Aspergillus / PBMC ratio), *A. fumigatus* lysates (Miltenyi Biotec), *A. fumigatus* culture supernatants (4%), heat or formaldehyde inactivated *C. albicans* yeast and hyphae (1:5 to 1:20 Candida / PBMC ratio), curdlan (10 µg/ml, Invivogen), depleted zymosan (10 µg/ml, Invivogen) and WGP dispersible (20 µg/ml, Invivogen) were added as indicated in the respective figures. For blocking/inhibition experiments mouse anti-human Dectin-1 blocking antibody (15 µg/ml, AbD Serotec), WGP soluble (1 mg/ml, Invivogen), small molecule *syk*-inhibitor (100 nM, Calbiochem), the pan-caspase inhibitor Z-VAD-FMK (10µM, R&D Systems), the caspase-8 inhibitor Z-IETD-FMK (50µM, R&D Systems), the caspase-1 inhibitor Z-WEHD-FMK (50µM, R&D Systems), DPI (0.1µM, Sigma-Aldrich), Catalase (100 U/l, Sigma-Aldrich), the TLR4 inhibitor CLI-095 (1µM, Invivogen) and/or cytochalasin D (2µg/ml; Enzo Life Sciences) were added as indicated in the respective figures. PBMCs cultured in medium alone were run in parallel as a control for each experiment. Medium and supplements were refreshed after three days. After six days, all cells were collected from PBMC cultures. Adherent cells were removed using non-protease cell detachment solution Detachin (Genlantis). MDSCs were characterized as CD33⁺CD11b⁺CD16⁺CD14⁻ cells using recently established species-specific MDSC markers (Rieber et al., 2013a; Rieber et al., 2013b). For functional studies CD33⁺ MDSCs were isolated from each culture using anti-CD33 magnetic microbeads and LS column separation (Miltenyi Biotec) with two sequential separation steps according to manufacturer's instructions.

Flow cytometry

Neutrophilic MDSCs in peripheral blood were quantified as published previously by our group (Rieber et al., 2013a). Antibodies against human CD3, CD4, CD8, CD14, CD16, CD66b, HLA-DR and CXCR4 were purchased from BD Pharmingen. Antibodies against CD11b and CD33 were purchased from MiltenyiBiotec. Antibodies against Dectin-1 were purchased from R&D Systems. Mouse IgG1-FITC, Mouse IgM-FITC, Mouse IgG1-PE and Mouse IgG1-APC (BD Pharmingen) were used as isotype controls. Antibodies against mouse CD11b, Ly6G and Ly6C were from BD Biosciences, anti-mouse CXCR4 was from Biolegend. Anti-mouse CD4 and IL-17A were from Miltenyi Biotec. CD3, CD8, CD25, CD69, NKp46, DX5 and the corresponding isotype controls were from Biolegend. T cells were characterized by CD3, CD4, CD8, CD25 and CD69 stainings. NK were characterized by CD3⁻, NKp46, DX5 and CD69 stainings. Where indicated, T- and NK- cell activation in mice were analyzed in spleen and kidney tissues. Leukocyte enrichment/isolation from kidney tissues was performed as described previously (Lionakis et al., 2011). In brief, kidneys were aseptically removed, finely minced and digested with Liberase TL and DNase (Roche) for 30 min with intermittent shaking at 37°C. Digested tissue was passed through a 70-µm filter, washed with sterile PBS and remaining red cells were lysed with lysis buffer. Resulting suspensions were passed through a 40-µm filter and washed with PBS. Pellet was resuspended in 8 ml of 40% Percoll (GE Healthcare). Leukocyte enrichment was performed by overlaying Percoll-cell suspension on 3 ml of 70% Percoll solution, and centrifugation at 2,000 rpm without brakes for 30 min at RT. The interphase was collected carefully, washed in PBS and suspended in FACS buffer. Cells were counted using a haematocytometer. Flow cytometry was performed on a FACS Calibur (BD). Results were expressed as percent of positive cells and mean fluorescence intensity (MFI). Calculations were performed with BD CellQuestPro analysis software.

T-cell suppression assays

T-cell suppression assays were performed as described previously by us in detail (Rieber et al., 2013a). Responder-PBMCs were obtained from healthy volunteers and stained with carboxyfluoresceinsuccinimidyl ester (CFSE) according to the manufacturer's protocol (Invitrogen). PBMCs were stimulated with 100 U/ml Interleukin-2 (IL-2; R&D Systems) and 1 µg/ml OKT3 (Janssen Cilag). In a standardized way, 60,000 PBMCs per well in RPMI1640 (Biochrom) were seeded in a 96-well microtitre plate and RPMI1640 only or 3,750 (1:16) to 30,000 (1:2) MDSCs in RPMI1640 were added. The cell culture was supplemented with 10% heat-inactivated human serum, 2mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. After 96h of incubation in a humidified atmosphere at 37°C and 5% CO₂ cells were harvested and supernatants were frozen in -20°C. For mouse T-cell suppression assays, CD11b⁺Ly6G⁺ MDSCs were isolated from bone-marrow using MACS (MDSC isolation kit, Miltenyi Biotec, Germany) and were co-cultured for three days (37°C, 5% CO₂) with T cells (CD4⁺ splenocytes) at a 1:2 (MDSCs : T-cell) ratio. T cells were activated with CD3/CD28-beads (mouse T cell activation kit, Miltenyi Biotec, Germany) and recombinant mouse IL-2 (50 U/ml, Biolegend). The cell culture was supplemented with 10% fetal bovine serum and 2mM glutamine. CFSE-fluorescence intensity for human and murine assays was analyzed by flow cytometry to determine polyclonal T- cell proliferation.

Intracellular cytokine analysis

Erythrocytes were lysed with Pharm Lyse Buffer (BD Pharmingen), leukocytes were washed with cold PBS and resuspended in RPMI (3 ml) with supplements (10% human serum, 100 U/ml Penicillin, 100 U/ml Streptomycin, 2 mM L-glutamine; Gibco) with the addition of benzonase (50 U/ml; Promega). The cells were plated into a 96-well flat bottom plate (200 µl), stimulated as indicated and were cultured for one hour (37°C; 5% CO₂). Brefeldin A (Sigma) was added ($c_{\text{final}} = 10 \mu\text{g/ml}$) and cells were cultured overnight. The cells were harvested and washed with cold PBS (0.1% sodium azide). LIVE/DEAD Fixable Aqua was used to stain dead cells (Life Technologies). Fc-receptors were blocked with Flebogamma (50 µg/ml, Grifols Biologicals) and cells were stained extracellularly with anti-CD33 PerCP-Cy5.5 (BD Pharmingen). The cells were fixed and permeabilized with Cytotfix/Cytoperm (BD Pharmingen), Fc-receptors were blocked as before and IL-1β was stained intracellularly (eBioscience). Flow cytometry was performed on a FACS Canto II (BD Pharmingen). Results were expressed as percent of CD33⁺IL-1β⁺ cells. In murine infections models, IL-17A was stained intracellularly in CD4⁺ splenocytes by flow cytometry as described previously by us (Mays et al., 2013). Calculations were performed with FlowJo analysis software (Tree Star).

Cytokine and Caspase analyses

IL-1 β ELISA Kits (R&D systems) were used to quantify cytokine protein levels. Multiplex cytokine array analyses in human MDSC / PBMC co-culture supernatants and mouse serum were performed using human and mouse Bioplex protein multi-array systems (Bio-Rad). Caspase-8 activity in cell lysates was analysed using a luminescent assay (Caspase-Glo 8 Assay from Promega, USA). Assays were performed according to the manufacturer's recommendations.

NK cell cytotoxicity assay

NK cell cytotoxicity assays were performed as described previously by us (Rieber et al., 2013b). In brief, NK cells were separated by MACS and co-incubated with MDSCs for 16h in a 1:1 ratio. Afterwards cytolytic activity of NK cells against K562 tumor cell line was tested in a BATDA europium release. E:T ratio was 5:1. We used the ratios of NK cell cytotoxicity in the presence of MDSCs / NK cell cytotoxicity without MDSCs for statistical analysis. NK cell cytotoxicity without MDSCs was set to a fixed value of 1. For *A. fumigatus* killing, NK cells were pre-stimulated with 1000 IU IL-2 (MiltenyiBiotec) for 24h. Activated NK cells were co-cultured with purified MDSC at a 1:1 ratio for 16h. After co-culture, MDSC were depleted using a MACS separation column (MiltenyiBiotec) and purified NK cells were incubated with *A. fumigatus* germ tubes at a 1:1 ratio for 5h. NK cells were lysed using ddH₂O and a cell viability assay (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilid [XTT] assay) was performed to determine killing of *A. fumigatus*. Propidium iodide was used to analyse NK cell death.

Fungal phagocytosis and killing assays

MDSCs were isolated by MACS separation and the phagocyte killing assay was performed as described previously for neutrophils (Bambach et al., 2009). Briefly, 1×10^6 MDSC were cocultured with 1×10^5 serum opsonized *C. albicans* (10:1 ratio) for 3 h at 37°C in RPMI. The cells were centrifuged, and suspended in sterile water for lysis. Serial dilutions were performed of the cell suspension and 100 μ l was plated onto YPD agar plates containing penicillin and streptomycin. Plates were incubated for 24-48 h at 37°C and CFUs were enumerated. The phagocytic capacity of human and murine MDSCs was further assessed by FACS. Therefore, MACS-isolated human granulocytic MDSCs (low density CD66b⁺CD33⁺ cells) were co-cultured with GFP-labelled *C. albicans* spores (MOI=1) in RPMI medium at 37 °C for 90 min. MACS-isolated mouse granulocytic CD11b⁺Ly6G⁺ MDSCs were co-cultured with GFP-labelled *C. albicans* spores (MOI=4) in RPMI medium at 37°C for 90 minutes. GFP expression of MDSCs was analyzed by FACS.

Mouse infection with *A. fumigatus* and *C. albicans*

All animal studies were approved by the local authorities (TVA/RP IDs: AZ 35/9185.81-2 / K5/13). *A. fumigatus* conidia (strain ATCC46645) were harvested on the day of infection, submerged in 0.9% NaCl + 0.002% Tween-20, filtered, centrifuged for 10 min 3000 rpm and resuspended in 5 mL 0.9% NaCl + 0.002% Tween-20. *Card9*^{-/-} mice on a C57/BL6 background, *Dectin-1*^{-/-} mice on a BALB/c background or age-matched C57/BL6 or BALB/c WT mice, respectively, were challenged intranasally with 1×10^4 or 1×10^6 *A. fumigatus* conidia for three days. At the fourth day, a bronchoalveolar lavage (BAL) was performed and CD11b⁺Ly6G⁺ and CD11b⁺Ly6C⁺ cells were quantified in BAL fluid by FACS. *C. albicans* (strain SC5314) was grown at 30°C overnight in liquid YPD (yeast extract, peptone, and dextrose) medium containing penicillin and streptomycin. Cells were collected by centrifugation, washed and resuspended in PBS. Required cell density was adjusted using a haemocytometer. For infection, female C57BL/6 mice were injected via the lateral tail vein with 2.5×10^5 or 5×10^5 blastospores per animal in 200 μ l PBS. Control animals were given PBS only. CD11b⁺Ly6G⁺ cells in the spleens were quantified by FACS. Where indicated, *C. albicans* infection experiments (see details above) were performed in *Il1r*^{-/-} on a C57BL/6 background and matched C57BL/6 WT mice. For adoptive transfer experiments, CD11b⁺Ly6G⁺ MDSCs were isolated from the bone marrow of healthy female BALB/c mice by MACS (MDSC isolation kit, Miltenyi Biotec, Germany). Transfer was performed by injecting $4\text{--}5 \times 10^6$ MDSCs per animal into eight to twelve weeks old (18–22 g) female BALB/c mice via lateral tail vein. Two hours after the MDSC transfer, mice were i.v. injected with 1×10^5 blastospores of *C. albicans* (SC5314 in 100 μ l PBS). Mice were weighed daily and monitored for survival and signs of morbidity. Mice with a weight loss of more than 20% were euthanized. For CFU determination, mice were euthanized at day 5 post-infection. The kidneys were aseptically removed, homogenized in 1ml PBS, serially diluted, and plated in duplicate on YPD agar containing penicillin and streptomycin. CFUs were determined after 48 hrs of incubation at 37°C. To assess the impact of phagocytosis *in vivo*, MDSCs were pretreated with Cytochalasin D (1 μ g/ml, Enzo Life Sciences) prior to adoptive transfer. Where indicated, recombinant mouse IL-17A protein (Biolegend) was mixed with *C. albicans* suspension and injected via mouse tail vein (5 μ g IL-17A protein/mouse). For invasive pulmonary *A. fumigatus* infection studies, eight to twelve weeks old (18–22 g) female BALB/c mice were immunosuppressed by treatment with cyclophosphamide (150 mg/kg bw i.p., days -3 and -1). Mice were challenged intranasally with 1×10^3 or 2×10^5 *A. fumigatus* conidia (freshly harvested from three days old plates). For survival studies, mice were challenged once with *A. fumigatus*, for MDSC induction studies for three consecutive days, as indicated in the respective figure legends. Where indicated, MDSC transfer was performed by intravenous injection of 4×10^6 MDSCs per animal prior to infection and mice were monitored for survival as described above.

Statistical analysis

Statistical analysis was done using GraphPad Prism 5.0 (Graph Pad Software). Differences between the groups were determined by Students' t test. Survival was calculated using the Log-rank (Mantel-Cox) test. A *P* value of <0.05 was considered to be significant.

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Paper 2

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Fungi in cystic fibrosis: recent findings and unresolved questions. Current Fungal Infection Reports 12/2014

Fungi in Cystic Fibrosis: Recent Findings and Unresolved Questions

Olaf Eickmeier · Andreas Hector · Anurag Singh ·
Sanjay H. Chotirmall · Dominik Hartl

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Abstract Patients with cystic fibrosis (CF) suffer from chronic airway infection and inflammation. Traditionally, bacteria have been regarded the main CF pathogens while fungi have emerged and more recently warranted greater attention. Fungi are increasingly found to colonize CF airways; however, their precise clinical impact continues to spark controversy. While the clinical relevance of allergic bronchopulmonary aspergillosis (ABPA) in CF has been established, the roles of non-ABPA *Aspergillus fumigatus*, *Candida albicans*, and other more rare emerging fungi remain poorly understood. Here, we summarize and discuss recent findings in this field and refer toward unresolved questions.

Keywords Cystic fibrosis · Fungi · Pathogens

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O. Eickmeier
Department of Pediatric Pulmonology, Allergy and Cystic Fibrosis,
Children's Hospital, Christiane Herzog CF-Center,
Goethe-University, Frankfurt a.M., Germany

A. Hector · A. Singh · D. Hartl
CF Research Group, Children's Hospital, Department I, University of
Tuebingen, Tuebingen, Germany

S. H. Chotirmall
Lee Kong Chian School of Medicine, Nanyang Technological
University, Singapore, Singapore

D. Hartl (✉)
Children's Hospital and Interdisciplinary Center for Infectious
Diseases, University of Tübingen, Hoppe-Seyler-Str. 1,
72076 Tübingen, Germany
e-mail: dominik.hartl@med.uni-tuebingen.de

Fungi in Cystic Fibrosis Lung Disease

Patients with cystic fibrosis (CF) suffer from chronic progressive and infective lung disease, which determines morbidity and mortality [1]. Upon disease progression, CF airways get colonized with characteristic bacteria and fungi, mainly *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus fumigatus*, and *Candida albicans*. While the pathogenic role of bacteria, particularly *P. aeruginosa*, in CF lung disease has been established, the contribution of emerging pathogens [2], such as fungi [3–5], to the clinical course of patients with CF lung disease remains incompletely understood. Among fungi in CF, most evidence exists for *A. fumigatus*. Chronic colonization with *A. fumigatus* can lead to sensitization and, in 1–15 % of CF patients (depending on the region, the patients' age, and the diagnostic criteria), to a severe clinical phenotype termed allergic bronchopulmonary aspergillosis (ABPA). ABPA is a hypersensitivity reaction to *A. fumigatus* characterized by a strong pro-allergic T helper cell type 2 (Th2) immune response (increased total serum IgE levels—typically >1000 IU/ml, skin prick tests positive, *A. fumigatus*-specific IgE and IgG, precipitins to *A. fumigatus*, sometimes eosinophilia) with pulmonary infiltrates (typical: central bronchiectasis) and antibiotic-refractory clinical deterioration, usually treated with antifungals (mostly azols) and corticosteroids [6]. Besides ABPA, however, the significance of non-ABPA *A. fumigatus* colonization and the potential clinical impact of other fungi remain unclear [7]. Fungi are predominantly found in adult CF patients and are more prevalent in patients with preexisting severe pulmonary disease and a long-term course of inhaled antibiotics [3]. Several studies support the concept that fungi and *P. aeruginosa* frequently co-exist in CF airways, supported by a recent study showing that antibacterial treatments targeted at *P. aeruginosa* reduce the *A. fumigatus* load in CF airways [8••]. However, the interaction of fungi and bacteria is

rather complex and involves synergistic as well as antagonistic growth conditions, including biofilm formation and release of toxic metabolites [9, 10]. Despite studies, the overall clinical relevance of *C. albicans* and other more rare but emerging fungi, such as *Scedosporium spp.*, remains even more incompletely examined. Faced with the increasing prevalence of fungi in patients with CF lung disease, the challenges toward the future remain to (i) define those fungi causing harm to the host and differentiate them from innocent bystanders, (ii) develop sensitive and specific biomarkers for diagnosis and treatment monitoring, and (iii) evaluate specific antifungal and anti-inflammatory therapeutic regimens and the contexts in which these are applicable. In the chapters below, we summarize and discuss recent findings on fungi in CF, with a particular focus on studies contributing to the pathophysiological understanding and clinical treatment in CF lung disease. For more comprehensive and in-depth discussion of the field, we refer to previously published reviews [3–5, 7, 11, 12].

Recent Findings on Fungi in Cystic Fibrosis Lung Disease

Aspergillus

The airways of CF patients are frequently colonized with *Aspergillus* species, but the disease relevance of this fungal colonization pattern remains poorly understood. This is largely due to the lack of an unified and harmonized immunological classification of *Aspergillus*-associated disease phenotypes in CF lung disease. Such a classification is hampered by the fact that CF patients, beyond the severe but rare ABPA, show a wide range of immunological responses to *Aspergillus*, which require a more precise classification to define them and to assess their clinical relevance. A recent study integrated two novel methods of *Aspergillus* detection in airway fluids (sputum), namely galactomannan (GM) and reverse transcription polymerase chain reaction (RT-PCR) [13••]. Both new methods were more sensitive in detecting *Aspergillus* species in CF sputum samples compared to conventional culture-based methodology. These two new *Aspergillus* markers in combination with *Aspergillus* serology allowed the stratification / classification of CF patients into three distinct *Aspergillus* disease entities: (i) *Serologic ABPA*: patients with positive RT-PCR, positive GM, and elevated total and specific *A. fumigatus* IgE/IgG; (ii) *Aspergillus bronchitis*: patients with positive RT-PCR, positive GM, and elevated *A. fumigatus* IgG (not IgE); and (iii) *Aspergillus sensitized*: patients with or without positive RT-PCR, negative GM, and elevated *A. fumigatus* IgE (not IgG). This novel classification may help to identify and clinically follow-up different *Aspergillus* disease phenotypes in order to develop specific screening and treatment approaches. However, sub-phenotypes probably

exist within these broader classifications groups, which will require future studies to define.

Upon CF lung disease progression, both *A. fumigatus* and *P. aeruginosa* are increasingly found in CF airway fluids. However, the interaction between these two microbes in patients with CF lung disease is poorly understood, particularly the potential therapeutic implications if both pathogens are chronically detected in CF sputa. A recent study assessed the effect of short-term antipseudomonal intravenous antibiotics on the presence of *Aspergillus* in CF airways in 26 adult CF patients [8••]. This study showed that intravenous antibiotics significantly decreased the presence of *Aspergillus* in CF airways and improved lung function parameters, suggesting that the presence of *P. aeruginosa* might help *Aspergillus* to survive within the CF airway microenvironment, maybe through mixed biofilm formation, and provides a rationale for intravenous antibiotic therapy in patients with advanced CF lung disease and co-colonization with *P. aeruginosa* and *Aspergillus*.

The more CF patients that undergo lung transplantation, the more the question arises, which bacteria or fungi, found in CF airways prior to transplantation, increase the risk for invasive infections afterwards. This is of particular relevance for *Aspergillus*, as invasive aspergillosis is a critical cause of mortality in patients undergoing lung transplantation. In case of CF, it remains unclear whether the colonization with *Aspergillus* represents a risk factor for invasive aspergillosis post transplantation. A recent study addressed this question by studying 93 CF patients before and after lung transplantation with regard to their *Aspergillus* colonization status using *Aspergillus* sputum culture and bronchoalveolar lavage GM as *Aspergillus* colonization markers [14••]. The study demonstrated that 70 % of CF patients were colonized with *Aspergillus* before undergoing lung transplantation and 22.5 % developed invasive aspergillosis with a mean time of 42 days following transplantation. The related 1-year mortality was 16 %. In search of identifying *Aspergillus*-associated risk and screening factors, the authors further showed that particularly the positive intraoperative detection of *Aspergillus* resulted in a four-fold higher risk of developing invasive aspergillosis, suggesting that this method could be used for early risk assessment in CF patients undergoing lung transplantation. Moreover, the therapeutic implications of these findings for potential prophylactic approaches in CF patients scheduled for lung transplantation should be further discussed.

Traditionally, ABPA is considered rare and mainly found in older CF patients. Up to 15 % of CF patients have been reported with ABPA and an even higher

percentage with subclinical disease. These notions, however, are based on different heterogeneous reports and lack broad high-quality epidemiological evidence. Therefore, a recent study performed across countries estimated the different manifestations of aspergillosis in CF patients [15]. Incorporating various international CF registries, this study confirmed the view that ABPA prevalence substantially varies by country, probably due to the current inadequate diagnostic ABPA criteria and genetic influences. Moreover, this study implicates an underdiagnosis of ABPA that occurs in children and teenagers with an estimated rate <1 % under 4 years and increasing throughout childhood and adolescence. When reviewed in combination, this study reinforces the awareness to screen for ABPA and other *Aspergillus*-related disease phenotypes (*Aspergillus* bronchitis and *Aspergillus* sensitization, see above) as early as infancy to prevent disease progression into full-blown ABPA.

Nevertheless, it remained a matter of debate in the CF field whether *Aspergillus* colonization and/or sensitization without ABPA are associated with lung function declines. To address this question, Baxter and coworkers from the National Aspergillosis Centre, University Hospital of South Manchester, UK, performed a 2-year prospective observational cohort study including 55 adult CF patients [16]. In this cohort, 69 % showed airway colonization with *Candida* and 60 % with *Aspergillus* species. The authors did not find an association between the presence of these fungi in CF airways and lung function decline. However, patients sensitized for *Aspergillus* species showed a greater lung function decline and an increase in their need for intravenous antibiotics. Despite these intriguing findings, further studies in larger and particularly younger CF cohorts are required to dissect the relationship between fungal colonization, sensitization and longitudinal lung function declines in patients with CF.

Fungi Other Than *Aspergillus*

The clinical relevance of fungi other than *Aspergillus*, such as *Candida*, *Scedosporium*, *Pseudallescheria*, *Pneumocystis jirovecii*, and *Penicillium*, remain poorly understood, but there is an increasing number of studies analyzing these fungal species in the context of CF lung disease. Accordingly, a recent study found a high prevalence of a non-albicans *Candida* species, namely *C. dubliniensis*, which was even more prevalent than *C. albicans* in this CF study cohort [17]. *C. dubliniensis* was mainly detected in combination with *P. aeruginosa* and *S. aureus*.

Scedosporium species, mainly the *Scedosporium apiospermum* complex, are emerging and found in airway fluids from older CF patients, but the clinical relevance of this

finding is debated. A recent study did not find an association between sensitization against the *S. apiospermum* complex and poorer lung function in patients with CF [18]. Further studies are required to understand the potential role of this fungus in CF lung disease.

Similar to *Scedosporium*, *Exophiala dermatitidis* is a fungus that frequently colonizes the airways of CF patients with an unclear clinical relevance. A recent study demonstrated that *E. dermatitidis* was recovered in 17 % of CF patients, with higher levels of IgG antibodies to *E. dermatitidis* in the positive CF patients [19]. Interestingly, those patients were more often colonized with non-tuberculous mycobacteria, required more intravenous antibiotic treatment, and had a lower lung function, suggesting that this fungus could have a harmful impact on the course of CF lung disease.

A prospective multicenter study in France analyzed the prevalence of *P. jirovecii* in 104 patients with CF lung disease and found that it was present in 12.5 % of them [20]. In contrast to other fungi, the presence of *P. jirovecii* was associated with the absence of *P. aeruginosa*. Detection of *P. jirovecii* was associated with a greater lung function decline, suggesting that this fungus could play an important, but hitherto underestimated, role in patients with CF lung disease. In order to improve the methods of fungal detection, two studies were recently published. Bernhardt and colleagues used multilocus sequence typing (MLST) to characterize *S. apiospermum* and *Pseudallescheria boydii* isolates from CF patients [21]. Collectively, these studies demonstrated that MLST technology is a highly effective tool for the study of fungal colonization at an epidemiological level and to investigate whether fungal isolates are clonal or have undergone recombination. These studies also showed that CF patients are colonized by individual *S. apiospermum* and *P. boydii* strains for up to a year-long period.

Masoud-Landgraf and coworkers compared different mycological culture methods to study fungi in CF sputum [22]. These studies demonstrate that *Candida albicans*, *C. dubliniensis*, and *C. parapsilosis* were the most common yeast species and *Aspergillus fumigatus* the most common filamentous fungus, followed by *S. apiospermum/P. boydii* group and *A. terreus*. Longitudinal analyses also revealed that fungal colonization patterns in CF patients are stable and colonize the airways independent from antifungal treatments. Methodologically, this study also showed that various fungal species, particularly *E. dermatitidis*, *Rasamsonia (Geosmithia) argillacea*, were isolated only from homogenized sputum samples using mucolytics.

A significant number of CF patients suffer from ABPA or “pre-ABPA” conditions. There is no doubt on the indication to treat ABPA; however, there is still an ongoing debate what treatment regimen should be chosen. Pre-ABPA refers to non-defined clinical conditions of patients not fulfilling all ABPA criteria, but showing disease

deterioration associated with *Aspergillus* colonization and/or sensitization without any further microbial or clinical explanation. In addition, other non-IgE mediated conditions, such as *Aspergillus* bronchitis [23], have been described in CF patients. Due to the fact that *Aspergillus* is the leading pathogen and the trigger of the above-mentioned disease entities, a very interesting approach seems to be the reduction of fungal load in the airways. However, a recent placebo-controlled trial of antifungal treatment in CF patients with *A. fumigatus*-positive sputum reported a non-significant trend toward poorer lung function in the patient group receiving itraconazole compared with placebo [24]. These results were not only attributed to the small study size ($n=35$), but also due to difficulties to achieve therapeutic itraconazole levels in more than 40 % of the patients. The latter finding emphasizes the need for further clinical trials. In line with this, other administration routes, e.g., inhalative azole therapy, and alternative treatment options, such as anti-IgE (omalizumab), should be evaluated in multicenter approaches. However, due to the dynamic shift between the different disease entities, it will be difficult to define inclusion criteria for these studies, but usage of enhanced mycological techniques and modern biomarkers may help to accurate clinical stratification into new classification models.

Unresolved Questions

While major efforts have expended over the last decade in understanding the role of fungi within the CF airways, significant unresolved questions remain. Although we have improved our fungal detection and evaluation methodology, we are not able to date to conclusively comment on their clinical relevance [3]. *Aspergillus*, *Candida*, and *Scedosporium* species have been the major focus of most work to date owing to their isolation frequencies [4, 18, 25–27]. In order to determine the clinical effects of any particular fungi, investigations into their virulence, pathogenesis, and signaling pathways are imperative.

- A major challenge for CF clinicians looking toward the literature for insight on the field is the cross-sectional nature of many clinical studies making it hard to establish causality for episodes of clinical deterioration. As a consequence, a major unanswered clinical question is that of treatment: when, why, and how? A genuine need for investment in prospective placebo-controlled trials to evaluate antifungal therapies in a wide range of clinical settings is now therefore justifiable. This would begin to address
- the key and largest question in the field to date: what is the clinical significance of the fungi detected in CF samples and when and if treatment is indicated. Of course, the outcomes will vary dependent upon the fungi, CF clinical status, and the individual's response to infection.
- When and by what mechanism are fungi pathogenic? The current literature is littered with controversies and debate within this field. Do they establish infection early in infancy or later in disease? Do they cause harm initially or over time? Does synergism exist? What is the relevance of the clinical setting or fungal virulence? Should they be treated and if so what agents and for what duration? All such questions are relevant but lack data to answer effectively at present.
- Significant differences in isolation and identification techniques exist internationally between mycology laboratories resulting in some misidentification of certain fungi [3, 28]. This has been reported in the literature and impacts on our understanding of the “real” incidence of colonization and infection in CF populations. Additionally, it is likely that there is an underestimation of both importance and significance of fungi in CF. The increasing use and focus on molecular-based detection techniques and genotyping for fungi in CF represent a major step forward in standardization, and further important questions remain with regard to the diagnosis and use of biomarkers in ABPA [29]. The new immunological classifications described in this review will continue to be refined allowing the identification of sub-phenotypes of fungal “colonizers” previously unrecognized that are likely at risk of clinical deterioration [13••].
- Host-derived factors, including the state of the immune system, severity of CF disease, effect of inflammation, and the burden of treatment, all play critical roles in determining susceptibility, while fungal type, virulence, genotype, and immunoevasive capabilities all too have their place [30]. It is this combination of host and pathogen-related factors that determines if a particular fungus at a certain point in time will represent a colonizer or pathogen within the CF lung.

Recent findings provide new insights into (i) the relevance of fungal colonization and sensitization in CF beyond ABPA, (ii) the clinical impact of the interaction between fungi and bacteria, and (iii) the potential role of rare fungi for the outcome of CF lung disease. Looking into the future, our focus should remain on both developing methodology to accurately detect and attempting clinical trials to eradicate in an effort to provide an evidence base for treatment as and when it may be required.

Compliance With Ethics Guidelines

Conflict of Interest O Eickmeier, A Hector, A Singh, SH Chotirmal, and D Hartl all declare no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- Of major importance

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Paper 3

Melanie Carevic, **Anurag Singh**, Nikolaus Rieber, Olaf Eickmeier, Matthias Griesse, Andreas Hector, and Dominik Hartl (2015).

CXCR4⁺ granulocytes reflect fungal cystic fibrosis lung disease. European respiratory Journal 2015, Accepted

CXCR4⁺ granulocytes reflect fungal cystic fibrosis lung disease

Melanie Carevic¹, Anurag Singh¹, Nikolaus Rieber¹, Olaf Eickmeier², Matthias

Griese³, Andreas Hector¹, Dominik Hartl¹

¹CF Center, Department of Pediatrics I, University of Tübingen, Tübingen, Germany

²Department of Pediatric Pulmonology, Allergy and Cystic Fibrosis, Children's

Hospital, Christiane Herzog CF- Center, Goethe-University, Frankfurt a.M., Germany

³Department of Pediatrics, Ludwig-Maximilians-University, Munich, Germany

Short title: CXCR4⁺ granulocytes in fungal CF

Correspondence:

Dominik Hartl

Children's Hospital

University of Tübingen, Germany

Hoppe-Seyler-Str. 1

72076 Tübingen, Germany

Phone: +49 – 7071 – 29 – 81460

FAX: +49 – 7071 – 29 – 5482

E-mail: dominik.hartl@med.uni-tuebingen.de

ABSTRACT

Cystic fibrosis (CF) airways are frequently colonized with fungi. However, the interaction of these fungi with immune cells and the clinical relevance in CF lung disease are incompletely understood. We characterized granulocytes in airway fluids and peripheral blood from CF patients with and without fungal colonization, non-CF disease controls and healthy control subjects cross-sectionally and longitudinally and correlated these findings to lung function parameters. CF patients with chronic fungal colonization with *Aspergillus fumigatus* were characterized by an accumulation of a distinct granulocyte subset, expressing the HIV co-receptor CXCR4. Percentages of airway CXCR4⁺ granulocytes correlated with lung disease severity in patients with CF. These studies demonstrate that chronic fungal colonization with *Aspergillus fumigatus* in CF patients is associated with CXCR4⁺ airway granulocytes, which may serve as a potential biomarker and therapeutic target in fungal CF lung disease.

Word count abstract: 136

Key words: Cystic fibrosis, lung, *Aspergillus*, fungus, inflammation, biomarker, granulocytes, CXCR4

INTRODUCTION

Cystic fibrosis (CF) lung disease is characterized by chronic bacterial infection [1, 2], fungal colonization, particularly with *Aspergillus fumigatus* and *Candida albicans* [3], and airway inflammation, maintained by a continuous and non-resolving recruitment of granulocytes into the airways [4-6]. *Aspergillus fumigatus* is frequently found in respiratory tracts of CF patients, but beyond allergic bronchopulmonary aspergillosis (ABPA), its clinical relevance in CF lung disease is poorly understood [7-10]. Recent clinical observations support the notion that *Aspergillus fumigatus* airway colonization, also in the absence of ABPA, a condition that has been termed ‘*Aspergillus* bronchitis’ [11], may have a harmful effect on the outcome of CF lung disease. This relationship remains a matter of debate, but is corroborated by studies in CF and asthma patients demonstrating that *Aspergillus fumigatus* colonization [12] and/or sensitization [13, 14] affect the course of lung disease, as measured by hospitalizations and lung function parameters. Other studies found more bronchiectasis, but no decline in pulmonary function in CF patients colonized with *Aspergillus fumigatus* compared to non-colonized patients [15]. Despite these observations, the conditions and underlying mechanisms by which *Aspergillus fumigatus* modulates CF lung disease remain incompletely defined.

Granulocyte recruitment and homing are tightly regulated by chemokines, which act through seven-transmembrane domain G protein-coupled chemokine receptors [16, 17]. Granulocyte products, such as neutrophil elastase, cause pulmonary tissue remodeling and immune receptor damage and have been shown to predict the development of bronchiectasis in CF patients [18]. Consequently, therapeutic interfering with granulocyte recruitment represents a promising approach in CF lung disease [19]. We [20] and others [21, 22] have shown in previous studies

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3 that granulocytes in the airways from CF patients are unique in their phenotype as
4 they are activated and characterized by a loss of the CXCL8 receptor CXCR1 (IL-
5 8RA) [23], whereas non-canonical chemokine receptors, particularly CXCR4
6 (CD184), are upregulated on their cell surface. While CXCR1 has been involved in
7 anti-bacterial host defense in CF lung disease [23], the clinical and functional
8 relevance of CXCR4-expressing granulocytes in patients with CF lung diseases
9 remained poorly defined.
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19 We hypothesized that *Aspergillus fumigatus* colonization in CF triggers a
20 distinct innate immune response, characterized by an accumulation of distinct airway
21 granulocytes, which modulate pulmonary disease. Our studies demonstrate that a non-
22 canonical phenotype of granulocytes, expressing the HIV co-receptor CXCR4,
23 accumulates in the airways of CF patients with *Aspergillus fumigatus* colonization
24 and is correlated with pulmonary function in CF.
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MATERIAL & METHODS

Patient cohorts

The CF group included 19 male and 21 female patients with a mean age of 23 ± 14 (SD) years (see below Table 1 for patients details). Inclusion / eligibility criteria were the diagnosis of CF by clinical symptoms and positive sweat tests (sweat Cl⁻ concentration > 60 mmol/l) or disease-causing mutations in the CFTR gene, forced expiratory volume in 1 second (FEV1) > 30 % of predicted value and being clinically stable and on steady concomitant therapy at least four weeks prior to the study. Twenty CF patients were $\Delta F508$ homozygous, thirteen were $\Delta F508$ heterozygous carriers of the CFTR gene and seven had other CFTR mutations than $\Delta F508$. Bacterial and fungal species were analyzed using culture-based methods and selective media in the corresponding airway fluid samples where CXCR4-expressing granulocytes were quantified by using flow cytometry, as described below in detail, and pulmonary function testings were performed. Chronic bacterial and fungal colonization were diagnosed using the Leeds criteria [24], if the organism was present in more than 50% of patient samples in the year prior to analysis. Ten control subjects without pulmonary diseases were selected as the control group. These subjects had no suspected or proven pulmonary disease and were free of respiratory tract infections. Eight non-CF lung disease controls were included. These adult patients suffered from bronchiectasis with chronic neutrophilic bronchitis, but without CF disease characteristics or positive sweat tests. This study was conducted in accordance with the amended Declaration of Helsinki. The local independent ethics committees approved the protocol, and written informed consent was obtained from all patients. For longitudinal follow-up studies, five CF patients with *Aspergillus fumigatus* colonization and five CF patients without *Aspergillus fumigatus* colonization,

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3 matched for age and lung function prior to the longitudinal follow-up (CF patients
4 with *Aspergillus fumigatus* colonization, n=5: mean age: 22 ± 5 (SD), mean FEV₁: 73
5 ± 6 (SD) % of predicted; median FEV₁: 75 % of predicted, range: 66-82 % of
6 predicted; CF patients without *Aspergillus fumigatus* colonization, n=5: mean age: 23
7 ± 4 (SD), mean FEV₁: 73 ± 10 (SD) % of predicted; median FEV₁: 74 % of predicted,
8 range: 63-84 % of predicted) and negative for *Candida albicans*, were studied with
9 follow-up intervals / time-points between six and eight months. At each follow-up
10 time-point, percentages of CXCR4⁺ granulocytes in sputum, lung function (FEV₁ and
11 MEF₂₅) and specific IgG to *Aspergillus fumigatus* were analyzed. None of the
12 included patients had ABPA.
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28 **Sample collection and processing**

29 Induced sputum [25] and bronchoalveolar lavage (BAL) were obtained, processed and
30 stored as described previously [20, 23, 26, 27]. Cell-free sputum supernatant was
31 stored at -80°C until analysis. The obtained BALF was filtered through two layers of
32 sterile gauze. The first fraction of BALF was used because it contains higher
33 percentages of granulocytes compared to the pooled fraction. The sample processing
34 was performed immediately on ice. After centrifugation (200g, 10 min) the
35 supernatant was stored at -80°C until analysis. The cell pellet was resuspended in 5 ml
36 of PBS and used for preparation of cytopsin slides and flow cytometry.
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50 **Flow cytometry**

51 Percentages of CXCR4⁺ granulocytes were quantified by flow cytometry based on a
52 modified flow cytometric strategy described previously by us [20], as depicted in the
53 Supplementary Figure 1. Prior to staining, Fc block was used to saturate non-specific
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3 binding sites. For staining, cells were incubated with monoclonal antibodies for 40
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5 min, washed two times and analyzed by flow cytometry [20]. For analysis, cells were
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7 first gated based on their forward scatter (FSC) and side scatter (SSC) characteristics.
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9 In the next step, dead cells in sputum/airway fluid samples were excluded by using
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11 propidium iodide (PI) staining. Within viable cells, granulocytes were identified by
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13 the expression of CD15. Fluorescence-minus one (FMO) controls were used to define
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15 gating boundaries. Anti-human CXCR4 antibody (clone: 12G5) were from BD
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17 Pharmingen (Heidelberg, Germany); anti-mouse Cxcr4 antibody (clone: L276F12)
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19 were from Biolegend (San Diego, USA). All other FACS reagents were from BD
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21 Pharmingen (Heidelberg, Germany) or Immunotech (Marseille, France). Calculations
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23 were performed with Cell Quest analysis software.
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30 ELISA

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32 CXCL8 and CXCL12 protein levels were measured in triplicates by a commercially
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34 available, sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D
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36 Systems) according to the manufacturer's instructions in a subgroup of CF patients
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38 (n=30). Serum was obtained from venous blood by centrifugation at 1,000g for 10
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40 min after blood clotting. Aliquots of serum were stored at -20°C. Specific IgE and
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42 IgG antibodies to *A. fumigatus* were analyzed using a ImmunoCap system [28].
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48 Statistical analysis

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50 Differences between the patient groups were calculated using the non-parametric
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52 Kruskal-Wallis test. When a significant difference was found, the non-parametric
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54 Mann-Whitney *U* test was applied for two-group comparisons. Correlations were
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56 verified with Spearman *rho* test. A value of *P* below 0.05 was considered to be
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3 significant. Statistical analysis was performed with Prism 5.0 (Graph Pad Software,
4 San Diego, CA, USA) and STATA version 8.2 for Windows (STATA Corporation,
5 College Station, TX, USA).
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RESULTS

Accumulation of CXCR4⁺ granulocytes in CF lung disease

Inspired by our previous finding that CXCR4⁺ granulocytes accumulate in chronic lung diseases [20], we systematically quantified percentages of CXCR4⁺ granulocytes in peripheral blood (Figure 1A) and airways fluids (induced sputum, Figure 1B; and BAL fluid, Figure 1C) from CF patients, healthy controls and non-CF lung disease control subjects. These studies demonstrated that percentages of CXCR4⁺ granulocytes were increased in CF patients both in peripheral blood (Figure 1A) and, to an even greater extent, in airway fluids (Figure 1A and Figure 1B). Percentages of CXCR4⁺ granulocytes showed a positive correlation with the percentage of neutrophils in CF airway fluids ($P<0.01$ for sputum and $P<0.01$ for BALF, as assessed by cytospin staining), while no correlations were found for airway eosinophils ($P>0.05$), suggesting that CXCR4⁺ granulocytes resemble neutrophilic granulocytes in CF airways. While percentages of CXCR4⁺ granulocytes in peripheral blood from CF patients were highly overlapping with healthy and non-CF disease control subjects and only a subgroup of CF patients showed higher percentages of CXCR4⁺ granulocytes in peripheral blood compared to control subjects (Figure 1A), percentages of CXCR4⁺ granulocytes in both induced sputum (Figure 1B) and BAL fluid (Figure 1C) were not overlapping and distinctively higher in CF patients compared to healthy controls and also increased compared to non-CF disease controls (Figure 1B and Figure 1C). However, also within the CF patient cohort, percentages of CXCR4⁺ granulocytes in both peripheral blood and airway fluids showed a wide range of values, suggesting that CF disease-associated factors modulate percentages of CXCR4⁺ granulocytes in CF lung disease.

CXCR4⁺ airway granulocytes reflect fungal colonization in CF lung disease

In order to dissect the CF-related factors that modulate percentages of CXCR4⁺ granulocytes in CF airways, we performed correlation analyses, which showed that percentages of CXCR4⁺ granulocytes in CF airway fluids correlated with colonization with *Aspergillus fumigatus* ($P<0.01$), but not with other CF-related bacterial or fungal species. Stratifying the included CF patients in patients colonized with *Aspergillus fumigatus* (Figures 2A-C) or *Candida albicans* (Figures 2D-F), these analyses further demonstrated that percentages of CXCR4⁺ granulocytes in peripheral blood of CF patients did not differ significantly between *Aspergillus fumigatus* colonized or non-colonized CF patients (Figure 2A), whereas percentages of CXCR4⁺ granulocytes in induced sputum (Figure 2B) or BAL fluid (Figure 2C) were significantly increased in CF patients colonized with *Aspergillus fumigatus* compared to non-colonized patients. In contrast to *Aspergillus fumigatus*, colonization with *Candida albicans* was not associated with percentages of CXCR4⁺ granulocytes in peripheral blood (Figure 2D), induced sputum (Figure 2E) or BAL fluids (Figure 2F). Consistent with our findings in CF patients, airway fluid CXCR4⁺ granulocytes were also increased in *Aspergillus fumigatus*-colonized non-CF bronchiectasis patients compared to non-colonized patients (Figure 2B), whereas again no differences were found for *Candida albicans* (Figures 2D-F).

CXCR4⁺ airway granulocytes correlate with lung function in CF

Next, we analyzed whether percentages of CXCR4⁺ granulocytes in peripheral blood or airway fluids were correlated with clinical parameters of CF lung disease. These studies demonstrated that percentages of CXCR4⁺ granulocytes in airway fluids, but not in peripheral blood, correlated inversely with pulmonary obstruction (FEV₁) in CF

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3 patients (Figure 3A and Figure 3B). Stratifying CF patients for *Aspergillus fumigatus*
4 colonization revealed that percentages of CXCR4⁺ granulocytes in airway fluids
5 correlated inversely with lung function (FEV₁) in *Aspergillus fumigatus* colonized,
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7 but not in non-colonized CF patients (Figure 3C). Percentages of CXCR4⁺
8 granulocytes in sputum correlated with specific IgG to *Aspergillus fumigatus*
9 ($P < 0.05$), whereas no correlation with specific ($P > 0.05$) or total IgE was found
10 ($P > 0.05$). No correlations between antibiotics or other CF drugs and percentages of
11 CXCR4⁺ granulocytes were found in our study cohort. Immunological analyses in a
12 subgroup of patients further demonstrated that percentages of CXCR4⁺ granulocytes
13 in airway fluids further positively correlated with protein levels of the CXCR4 ligand
14 CXCL12 (SDF-1 alpha) (Figure 4A) in airway fluids (induced sputum supernatants),
15 while no correlation was found between percentages of CXCR4⁺ granulocytes in
16 airway fluids and CXCL8 (IL-8) (induced sputum supernatants) (Figure 4B).
17 Percentages of CXCR4⁺ granulocytes in peripheral blood did not show any correlation
18 with protein levels of these chemokines in serum of the corresponding patients
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38 To further understand the association of CXCR4⁺ airway granulocytes,
39 *Aspergillus fumigatus* colonization and lung function in the course of CF lung disease,
40 we quantified CXCR4⁺ airway granulocytes longitudinally in five CF patients with
41 *Aspergillus fumigatus* colonization (Figure 5A) and five age- and lung disease
42 severity-matched CF patients without (Figure 5B) *Aspergillus fumigatus* colonization.
43 No change in diagnosis occurred in the course of these consecutive visits that may
44 have altered patient status substantially (such as switch from *Aspergillus* colonization
45 to ABPA). Both CF follow-up groups were *Candida albicans* negative. These
46 longitudinal analyses suggested that an increase of percentages of CXCR4⁺ airway
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3 granulocytes over time was associated inversely with lung function (FEV₁) decline in
4 CF patients with *Aspergillus fumigatus* colonization (Figure 5A), whereas no
5 relationship was observed in CF patients without *Aspergillus fumigatus* colonization
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DISCUSSION

Granulocytes are major determinants of airway inflammation in CF lung disease, but their phenotype, plasticity and clinical disease relevance remain poorly understood. We and others described in previous studies a distinct neutrophil subtype present in CF airway fluids, characterized by an upregulation of the homeostatic chemokine receptor and HIV-coreceptor CXCR4 (CD184) [20, 22]. However, the pathophysiological relevance of this neutrophil phenotype remained elusive. By quantifying CXCR4⁺ granulocytes in peripheral blood and airway compartments in CF patients as well as healthy and non-CF disease control subjects, we demonstrate that CXCR4⁺ airway granulocytes were associated with chronic colonization with *Aspergillus fumigatus* in CF patients. Since CXCR4⁺ airway granulocytes also correlated with lung function, this granulocyte phenotype may represent a novel therapeutic target in fungal colonization in CF lung disease.

While the role of the CXCL8 receptors CXCR1 and CXCR2 in granulocyte recruitment and functionality has been studied in more depth in homeostasis as well as inflammatory lung diseases, such as CF, where CXCR1 is proteolytically disabled [23, 29-31], the role of CXCR4 on granulocytes in lung disease conditions remains rather enigmatic. Previous studies have shown that CXCR4 is stored intracellularly in granulocytes and is post-transcriptionally upregulated at the cell surface upon cell activation and/or ageing [20, 29, 32]. Further studies in mice indicated that the CXCL12 (SDF-1 alpha) – CXCR4 axis controls the release of granulocytes from the bone marrow and their homing following senescence [33]. Another mouse study extended this view by demonstrating that the clinically-used CXCR4 inhibitor plerixafor triggers granulocyte mobilization through a novel mechanism, involving demargination of pulmonary granulocytes and blockade of granulocyte homing to the

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3 bone marrow [34], suggesting that the pulmonary compartment represents a
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5 substantial reservoir of granulocytes, which are retained in the lung through CXCR4-
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7 mediated mechanisms. These studies therefore suggest that interfering with CXCR4
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9 signaling, at least in mice, could bear the potential to promote granulocyte homing
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11 from the airways into the bone marrow. The potential function of CXCR4 in
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13 granulocyte homeostasis is further complicated by a recent study in mice
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15 demonstrating that the rhythmic modulation of the hematopoietic niche through
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17 granulocyte clearance involves CXCR4, since aged CD62L^{low}CXCR4^{high} granulocytes
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19 were found to infiltrate the bone marrow and to regulate the hematopoietic niche [35].
20
21 However, the relevance of these murine findings for human conditions, particularly
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23 CF lung disease, remains to be established. Based on our previous experience [20]
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25 and this present study we have no evidence that CXCR4-expressing granulocytes in
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27 CF airways simply represent apoptotic or necrotic cells, but rather suggest that this
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29 granulocyte phenotype reflects microenvironmental adaption and cellular
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31 reprogramming, as proposed previously [21, 22, 36].
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38 Previous studies showed that the severity of ABPA correlated with airway
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40 neutrophilia [37], which promote lung tissue damage and bronchiectasis through the
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42 release of matrix metalloproteinases [38, 39]. Studies in patients with asthma further
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44 provided evidence that *Aspergillus fumigatus* sensitization was associated with airway
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46 granulocytes and was related to the course of disease [14]. The distinct association of
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48 CXCR4⁺ airway granulocytes with *Aspergillus fumigatus*, but not with other fungal or
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50 bacterial microbes, in our CF patient cohort, confirms and extends the importance of
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52 pulmonary granulocytes in *Aspergillus fumigatus* colonization beyond ABPA.
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54 Besides these studies, murine models of fungal asthma suggest that chemokine
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56 receptors play a critical and non-redundant role for inflammatory processes and
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3 disease outcome [40, 41], but the distinct role of CXCR4 on granulocytes in the
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5 context of fungal colonization in CF lung disease has not been defined so far.
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7 *Aspergillus fumigatus* activates the immune system through several microbial patterns
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9 and corresponding pattern recognition receptors, such as dectin-1 or TLRs [42, 43].
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11 Downstream responses include granulocyte recruitment and activation. Although the
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13 precise link between fungal patterns and CXCR4-expressing granulocytes remains to
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15 be established, a recent study in dendritic cells (DCs) demonstrated that the fungal
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17 pathogen-associated molecular pattern (PAMP) and dectin-1 ligand zymosan
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19 upregulated CXCR4 surface expression on DCs, thereby rendering DCs susceptible
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21 towards HIV infection [44]. Whether similar interactions between dectin-1 activation
22
23 and CXCR4 regulation on granulocytes are operative has to be established in future
24
25 studies, especially with regard to airway granulocytes in CF. Besides dectin-1, other
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27 pattern recognition receptors, such as specific TLRs upregulated on CF airway
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29 granulocytes, could further play a role in fungal granulocyte activation [23, 45].
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34 We found in previous *in vitro* studies that *Pseudomonas aeruginosa* induced
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36 CXCR4-expressing myeloid-derived suppressor cells through a flagellin-mediated
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38 mechanism and further showed *ex vivo* that CF patients chronically colonized with
39
40 this bacterium had increased MDSCs ('low density' granulocytes) in peripheral blood
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42 [46]. In the latter study we were, however, unable to define functional MDSCs in CF
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44 airways fluids due to limited sample material. Here, we quantified 'high
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46 density'/conventional granulocytes in blood and airway fluids and demonstrated that
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48 *Aspergillus fumigatus* colonization in CF patients was associated with increased
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50 percentages of CXCR4-expressing granulocytes in the airways, whereas no significant
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52 difference was found for peripheral blood. Though the relative contribution of
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54 MDSCs and granulocytes in different compartments in CF remains a poorly
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3 understood area of research, we speculate, based on our previous studies, that mainly
4 *Pseudomonas aeruginosa* and *Aspergillus fumigatus* modulate distinct CXCR4-
5
6 expressing myeloid cell populations systemically (*Pseudomonas aeruginosa*) or in the
7
8 airways (*Aspergillus fumigatus*). Further studies are required to dissect the differential
9
10 and compartment-specific impact of these CF-characteristic pathogens on MDSC and
11
12 granulocyte subsets. Whether CXCR4 reflects cellular plasticity and/or granulocyte
13
14 heterogeneity [47] in the CF microenvironment or even possesses a functional role
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16 remains further to be investigated.
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21 Since our studies showed that CF patients, also without *Aspergillus fumigatus*,
22
23 showed increased baseline percentages of CXCR4-expressing granulocytes compared
24
25 to healthy control subjects, we analyzed whether genetic *Cftr* deficiency in mice *per*
26
27 *se* affects CXCR4 on granulocytes. These studies demonstrated that *Cftr*^{-/-} mice
28
29 showed similar percentages of CXCR4-expressing granulocytes in the bone marrow
30
31 compared to age- and strain-matched *Cftr*^{+/+} mice (Supplemental figure 2),
32
33 suggesting, in line with our findings in non-CF bronchiectasis patients, that the
34
35 genetic CF defect alone does not provide a sufficient explanation for an increased
36
37 CXCR4 expression on granulocytes. However, besides *Aspergillus fumigatus*, our
38
39 studies failed to identify additional factors modulating CXCR4-expressing
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41 granulocytes. Consequently, *Aspergillus fumigatus*-independent microbial or non-
42
43 microbial factors affecting CXCR4-expressing granulocytes in CF remain to be
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45 further identified.
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49
50 Overall, the clinical relevance of *Aspergillus fumigatus* in patients with CF
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52 lung disease beyond ABPA remains poorly defined [8-10, 48, 49], which is, at least
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54 partially, due to the poor classification of *Aspergillus fumigatus*-associated disease
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56 phenotypes in CF. A recent study from Baxter and coworkers addressed this issue by
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3 defining a novel immunologic classification of *Aspergillus fumigatus* disease
4 phenotypes in adult CF patients [50]. Applying this classification to our CF patient
5 cohort, CXCR4⁺ airway granulocytes were increased in CF patients colonized with
6 *Aspergillus fumigatus* and with serologic signs of an anti-fungal immune response
7 (specific IgG), but without serologic signs of underlying allergy or sensitization
8 against *Aspergillus fumigatus*, a phenotype most closely resembling the class 4
9 (*Aspergillus bronchitis*) subtype as defined by Baxter et al. with a specific *Aspergillus*
10 *fumigatus* IgG cutoff of 75mg/L [50]. Further studies are required to assess whether
11 CXCR4⁺ airway granulocytes could serve as biomarker and/or potential therapeutic
12 targets in the above mentioned *Aspergillus*-associated disease phenotypes in patients
13 with CF lung disease.
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27 Regarding the generalisability and conclusions drawn from our study, the
28 following limitations have to be considered: (i) the limited size of the included CF and
29 non-CF disease control populations requires further studies with larger *Aspergillus*
30 *fumigatus*-colonized patient cohorts, particularly longitudinal studies, (ii) the lack of
31 data on CXCR4⁺ granulocytes in young CF children and (iii) the unresolved
32 mechanistic question of how *Aspergillus fumigatus* modulates CXCR4 expression in
33 CF granulocytes; issues that should be addressed in future studies. As limitation, our
34 FACS staining panel did not include specific eosinophil markers, which precludes a
35 precise flow cytometric discrimination of airway neutrophils and eosinophils.
36 However, since the majority of airway cells in our CF airway samples were
37 neutrophils (27-fold more neutrophils than eosinophils) and neutrophils strongly
38 correlated with percentages of CXCR4⁺ airway granulocytes, while we did not find an
39 association with airway eosinophils, we conclude that CXCR4⁺ airway granulocytes
40 are unlikely to represent or contain a substantial amount of eosinophils. Nevertheless,
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3 as we cannot exclude an influence of eosinophils to our findings, we termed the
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5 CXCR4-expressing cells ‘granulocytes’ instead of ‘neutrophils’ in our studies.
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8 In summary, we demonstrate that CF patients with *Aspergillus fumigatus*
9
10 colonization were characterized by an accumulation of CXCR4⁺ granulocytes within
11
12 the airways, which correlated with the severity of pulmonary obstruction. Given that
13
14 specific antagonists of CXCR4, a G-protein coupled receptor, are already in use
15
16 clinically [51], targeting CXCR4⁺ airway granulocytes may represent a potential
17
18 therapeutic approach in fungal colonization in CF. Our findings in a limited number
19
20 of patients with non-CF bronchiectasis and *Aspergillus fumigatus* colonization further
21
22 suggest, that CXCR4⁺ airway granulocytes could also play a broader role in other
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24 chronic lung disease beyond CF, such as chronic obstructive pulmonary disease
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26 (COPD) where *Aspergillus fumigatus* is also found and associated with poor lung
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28 function [52].
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COMPETING INTEREST

The authors declare that no competing interests exist.

TABLES

Table 1. Patient groups

	Cystic fibrosis	Healthy Controls	Non-CF Bronchiectasis
N	40	10	8
Age [yrs]	23 ± 14	22 ± 7	56 ± 17
Sex (m:f)	19/21	4/6	5/3
CRP, mg/dl	0.8 ± 0.5	n.d.	1.2 ± 0.8
WBC (10 ⁹ /L)	11 ± 7	7 ± 4	10 ± 6
Total IgE kUI/L	49 ± 34	n.d.	44 ± 27
FEV ₁ (% pred)	66 ± 15	n.d.	55 ± 20
MEF ₂₅ (% pred)	39 ± 27	n.d.	29 ± 16
Neutrophils in sputa (%)	82 ± 29	15 ± 9	58 ± 31
Eosinophils in sputa (%)	3 ± 2.8	0.6 ± 0.4	1.9 ± 0.7
<i>P. aeruginosa</i>	18	n.d.	2
<i>Aspergillus spp</i>	22	n.d.	3
Specific IgE to <i>Aspergillus fumigatus</i> (kU/L)	2.3 ± 1.6	n.d.	0.8 ± 0.5

Specific IgG to <i>Aspergillus fumigatus</i> (mg/L)	59 ± 35	n.d.	37 ± 19
<i>Aspergillus fumigatus</i> precipitins	2/40	n.d.	0/3
<i>Candida spp</i>	20	n.d.	2
<i>Aspergillus spp</i> and <i>Candida spp</i> co-colonization	10	n.d.	0
<i>S. aureus</i>	11	n.d.	0
<i>H. influenzae</i>	4	n.d.	0
<i>Achromobacter</i> <i>xylooxidans</i>	5	n.d.	0
<i>Stenotrophomonas</i> <i>maltophilia</i>	6	n.d.	0
Inhaled antibiotics	18	0	2
Inhaled corticosteroids	16	0	2
Dornase alpha / DNase	15	0	0
Azithromycin	11	0	1
dF508 homozygous/ heterozygous/other	20/13/7	n.d.	n.d.

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3 Results are expressed as means \pm SD; m: male, f: female; WBC: white blood count;
4
5 FEV₁: Forced expiratory volume in 1 second (% of predicted); n.d. not determined.
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8 **FIGURE LEGENDS**

9 **FIGURE 1. CXCR4⁺ granulocytes in CF patients and controls**

10
11 Percentages of CXCR4⁺ granulocytes were quantified by flow cytometry in peripheral
12
13 blood (A), sputum (B) and BAL fluid (C) from cystic fibrosis (CF), healthy control
14
15 (A, B) and non-CF bronchiectasis disease control (A, B, C) subjects. The scatter
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17 graph depicts individual patients with horizontal bars as medians.
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22 **FIGURE 2. CXCR4⁺ airway granulocytes and fungal colonization in CF patients**

23
24 Percentages of CXCR4⁺ granulocytes were quantified by flow cytometry in peripheral
25
26 blood (A, D), sputum (B, E) and BAL fluid (C, F) from cystic fibrosis (CF) patients
27
28 and non-CF bronchiectasis disease control with (+) or without (-) chronic *Aspergillus*
29
30 (A-C) or *Candida* (D-F) colonization and healthy control subjects (A, B, D, E). CF
31
32 and non-CF bronchiectasis disease control patients were stratified according to fungal
33
34 colonization independent from any other pathogens detectable in CF airway fluids.
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36 The scatter graph depicts individual patients with horizontal bars as medians.
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42 **FIGURE 3. CXCR4⁺ granulocytes and lung function**

43
44 Percentages of CXCR4⁺ granulocytes quantified by flow cytometry in peripheral
45
46 blood (A) and sputum (B) from cystic fibrosis (CF) patients in correlation to lung
47
48 function (FEV₁ in percentage of predicted). (C) CF patients with (red dots) or without
49
50 (green dots) *Aspergillus* colonization. Spearman's ρ coefficients (ρ) are depicted.
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56 **FIGURE 4. CXCR4⁺ airway granulocytes and chemokines**

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3 Percentages of CXCR4⁺ granulocytes quantified by flow cytometry in sputum from
4
5 cystic fibrosis (CF) patients in correlation to airway levels (CF sputum supernatants,
6
7 n=30) of CXCL12 (A) and CXCL8 (B). Spearman's *rho* coefficients (ρ) are depicted.
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10 11 12 **FIGURE 5. Longitudinal studies**

13
14 Percentages of CXCR4⁺ granulocytes (red lines) quantified by flow cytometry in
15
16 sputum from cystic fibrosis (CF) patients, lung function (FEV₁; full green lines; and
17
18 MEF₂₅; dashed green lines; in percentage of predicted) and specific IgG to *Aspergillus*
19
20 *fumigatus* (mg/L, blue lines) analyzed longitudinally in CF patients with (A, n=5) or
21
22 without (B, n=5) *Aspergillus* colonization. Follow-up intervals between each time-
23
24 point were six to eight months for all patients depicted. Medians and interquartile
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26 ranges are shown for each time-point.
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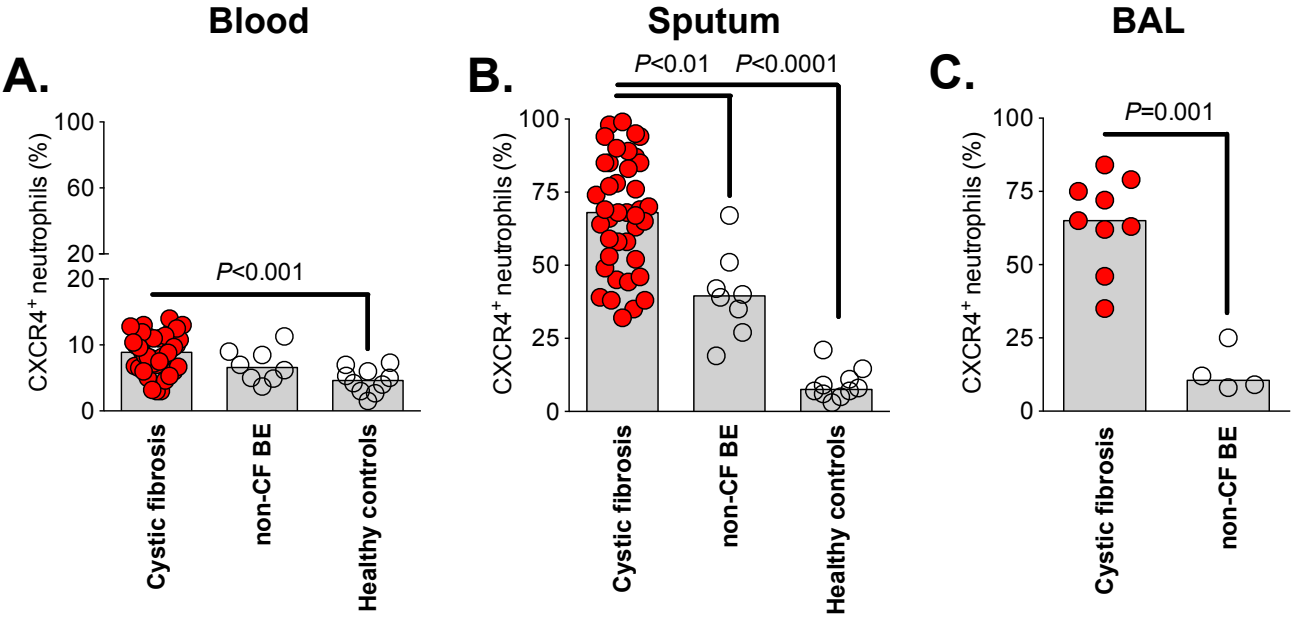
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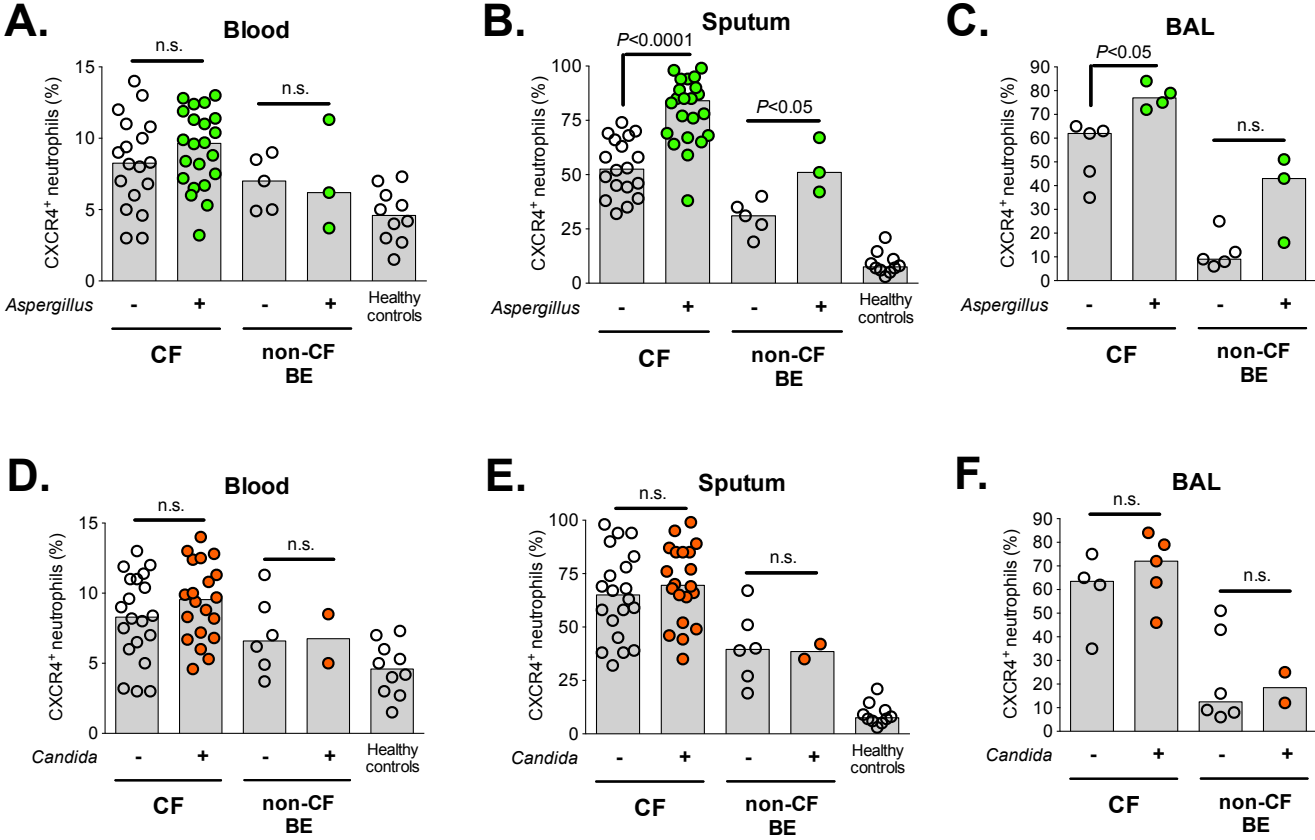
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Figure 1.



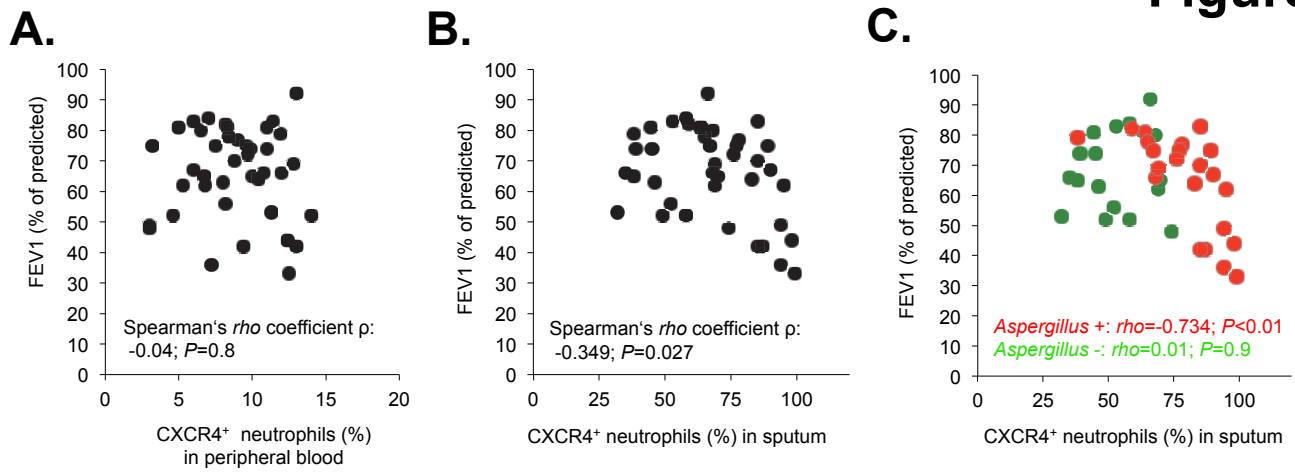
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Figure 2.



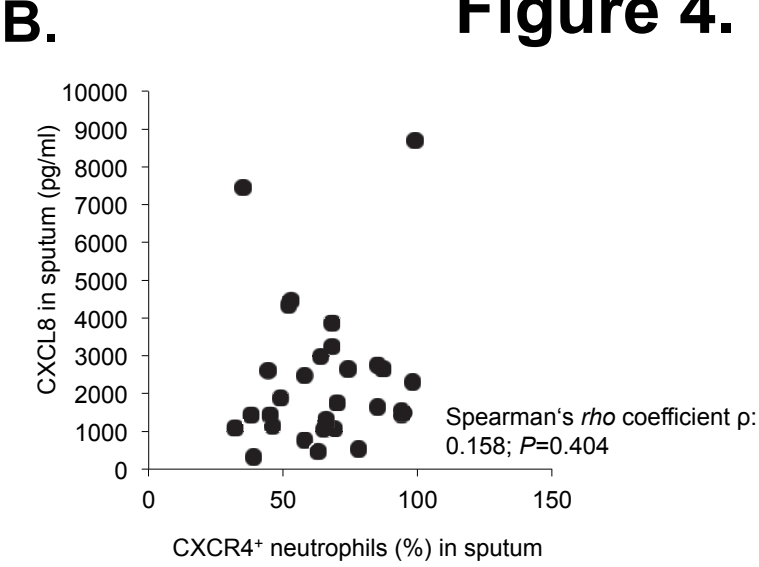
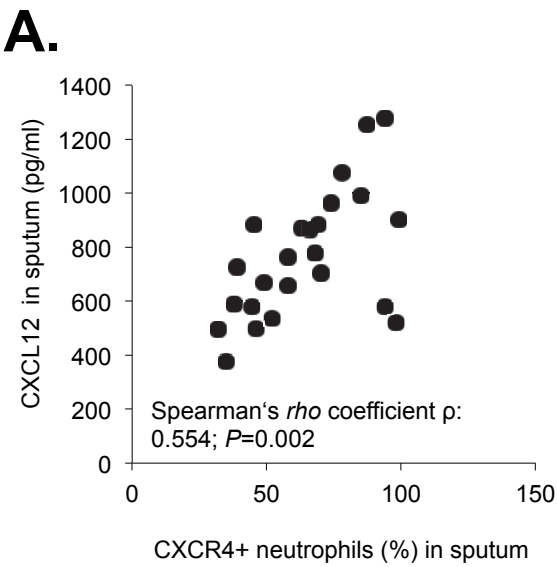
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Figure 3.



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Figure 4.

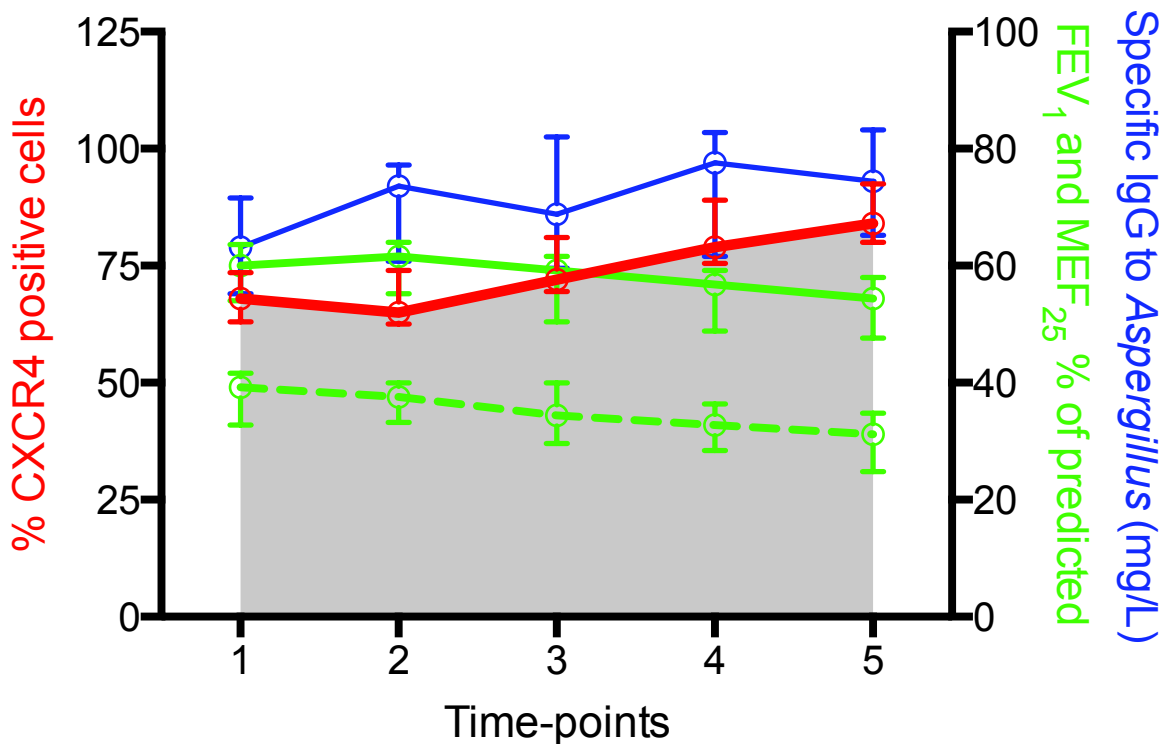


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Figure 5.

A.

Aspergillus positive



B.

Aspergillus negative

