

***Pseudomonas aeruginosa* airway infection
recruits and modulates myeloid-derived
suppressor cells**

Dissertation

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II. Abbreviations

APC	antigen-presenting cell
ASL	airway surface liquid
BAL	bronchoalveolar lavage
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
<i>Cftr</i> ^{-/-}	<i>Cftr</i> ^{tm1Unc*} Tg(FABPCFTR)1Jaw/J
CMP	common myeloid progenitor
ENaC	epithelial sodium channel
FABP1	fatty acid binding protein1
GM-CSF	granulocyte-macrophage colony-stimulating factor
G-MDSC	granulocytic/ neutrophilic myeloid-derived suppressor cell
GR-1	myeloid differentiation antigen GR-1
HLA-DR	human leukocyte antigen – antigen D Related
HSC	heamatopoietic stem cell
IMC	immature myeloid cell
IL	Interleukin
iNOS	inducible nitric oxide synthase
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
M-CSF	mayrophage colony-stimulating factor
M-MDSC	monocytic myeloid-derived suppressor cell
NADPH	nicotine adenine dinucleotide phosphate

NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononucleated cell
PMN	neutrophil granulocyte
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
ROS	reactive oxygen species
SCF	stem-cell factor
TCR	T-cell receptor
Th	T helper cell
TLR	toll-like receptor
Treg	Regulatory T cell

III. Summary

Tumor associated myeloid suppressive cells have been described in the scientific field about 30 years ago. In the year 2007 these cells were given the name myeloid-derived suppressor cells (MDSCs) due to their myeloid origin and their immunosuppressive potential. MDSCs are commonly found in cancer patients, where they are known to impair the patients' immune response leading to severe tumor progression. Besides cancer, MDSCs are likewise found to be recruited in inflammatory and infectious diseases, where their role is often discussed. According to the disease type and/ or the pathogen involved they can be either beneficial or detrimental for patients.

Cystic Fibrosis (CF) is a monogenetic disease caused by mutations in the gene encoding for the chloride channel Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Morbidity and mortality of CF patients is mainly due to a steady decline in the lung function, caused by infections and hyperinflammatory immune responses leading to lung injury. *Pseudomonas (P) aeruginosa*, a gram-negative flagellated opportunistic pathogen, is the most common pathogen causing chronic infections and leading to severe problems in CF patients. Previous studies from our group revealed that MDSC numbers are increased in CF patients, especially in patients with chronic *P. aeruginosa* infections. Among patients with chronic *P. aeruginosa* infections, the amount of MDSCs positively correlated with better lung function.

In this study we investigated the interaction of *P. aeruginosa* and MDSCs as well as assessed the impact of CFTR deficiency. Therefore we performed *in vivo* acute pulmonary infections in wildtype as well as in *Cftr*^{-/-} mice followed by flow cytometry (FACS) analysis to quantify MDSCs in different tissues. We could demonstrate that acute pulmonary *P. aeruginosa* infections potently induce CD11b⁺Ly6G⁺Ly6C^{low} cells. These markers describe the profile of granulocytic/ neutrophilic MDSCs (G-MDSCs). *In vitro* expansion of wildtype or *Cftr*^{-/-} splenocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) also resulted in the induction of CD11b⁺Ly6G⁺Ly6C^{low} cells. To check for their suppressive nature and thereby distinguish the induced CD11b⁺Ly6G⁺Ly6C^{low} cells from neutrophil granulocytes, we isolated the cells and assessed their suppressive capability using CFSE based T cell suppression assays. The isolated CD11b⁺Ly6G⁺Ly6C^{low} cells

from lungs, spleens, bone marrow and bronchoalveolar lavage (BAL) of infected mice potently suppressed T cell proliferation *ex vivo* in a dose-dependent manner, which provided evidence that they are G-MDSCs. Intriguingly, the suppressive capability of G-MDSCs isolated from lungs, spleens or bone marrow of *P. aeruginosa* infected mice was potently enhanced on cellular basis when compared to G-MDSCs isolated from control mice. Contrarily, G-MDSCs isolated from *in vitro* expanded *Cftr*^{-/-} splenocytes were less suppressive than G-MDSCs isolated from *in vitro* expanded wildtype splenocytes, implying a potential role of CFTR on G-MDSC suppression.

Altogether, we could demonstrate, that CFTR deficiency seems to hamper G-MDSCs' suppressive potential, whereas pulmonary *P. aeruginosa* airway infection induces G-MDSCs and enhances their suppressive capability, and thereby define a mechanism by which *P. aeruginosa* undermines host immunity *in vivo*. The pathways and mechanisms still need to be elucidated.

IV. Zusammenfassung

Tumor-assoziierte myeloische suppressive Zellen wurden vor ca. 30 Jahren beschrieben. Im Jahre 2007 wurden diese Zellen aufgrund ihrer myeloischen Herkunft und ihrem immunsuppressiven Potential myeloische Suppressorzellen (MDSC) genannt. MDSCs kommen häufig in erhöhter Anzahl in Tumorpatienten vor. Sie sind bekannt dafür, die Immunantwort des Patienten zu hemmen und ermöglichen somit verstärkte Tumorprogression. Neben Krebserkrankungen treten MDSCs auch in inflammatorischen und infektiösen Krankheiten auf, wobei ihre Rolle oft kontrovers Diskutiert wird. Abhängig von der Erkrankung und/ oder involvierten Pathogenen können MDSCs entweder gut, oder schlecht für den Patienten sein.

Zystische Fibrose (CF) ist eine monogenetische Erkrankung die durch Mutationen im Gen das für den Chloridionenkanal Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) kodiert. Die Morbidität und Mortalität der CF Patienten ist hauptsächlich auf den Funktionsverlust der Lunge zurückzuführen, die durch Infektionen und hyperinflammatorische Immunantworten beschädigt wird. *Pseudomonas aeruginosa*, ein gram-negativer flagellierter opportunistischer Erreger, sorgt bei CF Patienten häufig für Infektionen und kann dabei auch zu chronischen Infektionen führen und somit großen Schaden in der Lunge anrichten. Vorhergehende Studien unserer Arbeitsgruppe wiesen auf, dass MDSCs bei CF Patienten in erhöhtem Maße vorkommen, besonders bei CF Patienten mit chronischen *P. aeruginosa* Infektionen. Unter den Patienten mit chronischen *P. aeruginosa* Infektionen korrelierte dabei die Menge an MDSCs positiv mit der Lungenfunktion der Patienten.

In dieser Studie untersuchten wir die Interaktion von *P. aeruginosa* mit MDSCs und ermittelten den Einfluss von CFTR Defizienz. Dazu führten wir *in vivo* akute pulmonäre Infektionen in Wildtyp als auch in *Cftr*^{-/-} Mäusen durch und quantifizierten MDSCs in verschiedenen Geweben mittels Durchflusszytometrie (FACS). Dabei fanden wir heraus, dass akute pulmonäre *P. aeruginosa* Infektionen CD11b⁺Ly6G⁺Ly6C^{low} Zellen induzieren, welche zu dem Profil von granulozytären/neutrophilen MDSCs (G-MDSCs) passen. *In vitro* expansion von Wildtyp oder *Cftr*^{-/-} Splenozyten mit granulocyte-macrophage colony-stimulating factor (GM-CSF) und Interleukin-6 (IL-6) induzierte ebenfalls CD11b⁺Ly6G⁺Ly6C^{low} Zellen.

Um die suppressive Natur der induzierten CD11b⁺Ly6G⁺Ly6C^{low} Zellen zu untersuchen und sie somit von Neutrophilen Granulozyten zu unterscheiden isolierten wir sie und evaluierten ihre suppressiven Fähigkeiten mittels CFSE basierten T-Zell-Suppressionsversuchen. Die isolierten induzierten CD11b⁺Ly6G⁺Ly6C^{low} aus Lungen, Milzen, Knochenmark und bronchoalveolären Lavage (BAL) von infizierten Tieren waren in der Lage T-Zell Proliferation dosisabhängig stark zu inhibieren, womit wir aufweisen konnten, dass es sich bei den induzierten Zellen um G-MDSCs handelt. Interessanterweise war die suppressive Fähigkeit der G-MDSCs, die von Lungen, Milzen und Knochenmark von *P. aeruginosa* infizierten Mäusen isoliert wurden auf zellulärer Ebene verglichen mit G-MDSCs aus Kontrollmäusen verstärkt. Im Gegenzug dazu sind G-MDSCs, die von *in vitro* expandierten Cftr^{-/-} Splenozyten isoliert wurden weniger suppressiv als G-MDSCs die von *in vitro* expandierten Wildtyp Splenozyten isoliert wurden, was eine potentielle Rolle von CFTR auf die Suppressivität von G-MDSCs vermuten lässt.

Insgesamt konnten wir zeigen, dass pulmonäre *P. aeruginosa* Infektionen G-MDSCs induzieren und sie in ihrer suppressiven Fähigkeit verstärken und somit konnten wir einen Mechanismus aufweisen, mit welchem *P. aeruginosa in vivo* die Immunantwort untergräbt, während CFTR Defizienz die G-MDSC suppressive Fähigkeit *in vitro* abschwächt. Die involvierten Signalwege und Mechanismen, die hierbei involviert sind müssen, noch bestimmt werden.

V. Publications

Original Publications

Hasan H. Öz, Benyuan Zhou, Pina Voss, Melanie Carevic, Carolin Schroth, Nina Frey, Nikolaus Rieber, Andreas Hector, and Dominik Hartl. Submitted August 2016. *Pseudomonas aeruginosa* airway infection recruits and modulates neutrophilic myeloid-derived suppressor cells. Submitted to *Frontiers in Cellular and Infection Microbiology*

Öz HH, Hartl D. 2016. Innate Immunity in Cystic Fibrosis: Novel Pieces of the Puzzle. *J Innate Immun.*

Carevic, M., **H. Oz**, K. Fuchs, J. Laval, C. Schroth, N. Frey, A. Hector, T. Bilich, M. Haug, A. Schmidt, S. E. Autenrieth, K. Bucher, S. Beer-Hammer, A. Gaggar, M. Kneilling, C. Benarafa, J. L. Gao, P. M. Murphy, S. Schwarz, B. Moepps, and D. Hartl. 2016. CXCR1 Regulates Pulmonary Anti-Pseudomonas Host Defense. *J Innate Immun.*

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Hector, A., H. Schafer, S. Poschel, A. Fischer, B. Fritzsching, A. Ralhan, M. Carevic, **H. Oz**, S. Zundel, M. Hogardt, M. Bakele, N. Rieber, J. Riethmueller, U. Graepler-Mainka, M. Stahl, A. Bender, J. S. Frick, M. Mall, and D. Hartl. 2015. Regulatory T-cell impairment in cystic fibrosis patients with chronic pseudomonas infection. *American journal of respiratory and critical care medicine* 191: 914-923.

Rieber, N., A. Gavrilov, L. Hofer, A. Singh, **H. Oz**, T. Endres, I. Schafer, R. Handgretinger, D. Hartl, and J. Kuemmerle-Deschner. 2015. A functional inflammasome activation assay differentiates patients with pathogenic NLRP3

mutations and symptomatic patients with low penetrance variants. *Clin Immunol* 157: 56-64.



Name: Hasan Halit Öz

List of Publications in the thesis

Paper 1: Hasan H. Öz., Benyuan Zhou, Pina Voss, Melanie Carevic, Carolin Schroth, Nina Frey, Nikolaus Rieber, Andreas Hector, and Dominik Hartl. Submitted August 2016. *Pseudomonas aeruginosa* airway infection recruits and modulates neutrophilic myeloid-derived suppressor cells. Submitted to *Frontiers in Cellular and Infection Microbiology*

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Paper 3: Kolahian, S., **H. H. Oz**, B. Zhou, C. M. Griessinger, N. Rieber, and D. Hartl. 2016. The emerging role of myeloid-derived suppressor cells in lung diseases. *Eur Respir J* 47: 967-977.

Contribution to the publications

Paper 1

Pseudomonas aeruginosa airway infection recruits and modulates neutrophilic myeloid-derived suppressor cells

I performed and/ or supervised all experiments with assistance from Pina Voss, Benyuan Zhou, Melanie Carevic and Carolin Schroth, analyzed the data, made figures and contributed in writing and proof-reading of the manuscript. Nina Frey performed immune fluorescence stainings and microscopy. Andreas Hector, Nikolaus Rieber and Dominik Hartl designed this study and supervised experiments, D. Hartl wrote the manuscript.

Paper 2

Pathogenic fungi regulate immunity by inducing neutrophilic myeloid-derived suppressor cells

I performed all *in vivo* experiments for this paper together with A. Singh and M. Carevic, analyzed data, made graphs and contributed to writing the manuscript. A. Singh, I. Schäfer and M. Ballbach performed *in vitro* experiments. Other authors provided important materials and contributed to the manuscript. N. Rieber and D. Hartl designed and supervised this study and wrote the manuscript.

Paper 3

The emerging role of myeloid-derived suppressor cells in lung diseases

I contributed in writing and proof reading of the manuscript with other authors. S. Kolahian wrote the manuscript, D. Hartl supervised this review.

1. Introduction

1.1 The immune system

The immune system is a complex system consisting of physical and chemical barriers, various specific cells and a plethora of soluble components. Altogether, it is the defense mechanism of the body to fight against pathogens and malignant tumor cells and to maintain cellular homeostasis. It splits into two main branches, the innate and the adaptive immune system. The innate immune system consists of antimicrobial agents like the complement system or defensins, and innate immune cells. Among these are monocytes, neutrophil granulocytes (PMN) and dendritic cells, which are also called phagocytes, because of their ability to phagocytose pathogens. They recognize conserved molecular structures on pathogens via toll-like receptors. After recognition of the so called pathogen-associated molecular patterns like lipopolysaccharide or flagellin, the innate immune cells get activated and are able to eliminate pathogens and produce a specific set of cytokines and chemokines. Cytokines are soluble mediators between cells that are recognized and induce specific intracellular mechanisms further activating the immune response; chemokines are soluble mediators that lead other immune cells to the site of inflammation (1-6). Phagocytes have furthermore the ability to activate the adaptive immune response. They process the phagocytosed pathogens into small parts, called antigens, and present them on their surface via major histocompatibility complex (MHC) molecules to the main initiators of the adaptive immunity, the T cells. CD8⁺ T cells, also called cytotoxic T cells, recognize endogenous antigens on MHC class I (MHC-I) molecules while CD4⁺ T cells, also called T helper cells (Ths), recognize exogenous antigens on MHC class II (MHC-II) molecules via their T-cell receptor (TCR). T cells have very specialized TCRs that only bind specific antigens. Upon recognition of the MHC-II molecule with the presented antigen and receiving a secondary costimulatory signal from APCs, naïve Ths are activated, produce high amounts of cytokines and start clonal proliferation and differentiation into effector T-lymphocytes (1).

1.1.1 T cell subtypes

Depending on the cytokine milieu, naïve Ths are differentiating into different subtypes of T-helper cells as the main ones being Th1, Th2, Th17 and T cell suppressive regulatory T cells (Tregs).

Th1 cells activate cellular immune responses by activating macrophages and cytotoxic T cells. Th2 cells activate humoral immune responses by activating B cells that turn into plasma cells and produce high amounts of antibodies. These bind specifically to foreign structures like recognized antigens on pathogens, neutralizing them and simplifying phagocytosis of these pathogens (1, 7-11). Th17 cells are contributing to pathogen elimination at mucosal surfaces, but are also associated with autoimmunity and autoinflammation (12). Tregs are the main suppressive cells counteracting pro-inflammatory T cell activation and act as an intrinsic way to stop overshooting inflammatory T-cell responses (13).

As T cells are important to mediate and enhance immune responses, unbalanced activation of T cells can cause severe damage to the host leading to autoimmune diseases and hyper-inflammation (14-18).

1.2 MDSCs

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of myeloid cells that are potently able to suppress T cells. Initially MDSCs have been described in cancer patients (19-22) and tumor-bearing mice. More recently MDSCs were also found in infectious and autoinflammatory disease conditions like inflammatory bowel disease (23), rheumatoid arthritis (24, 25), polymicrobial sepsis (26) and cystic fibrosis (27). In cancer, MDSCs are viewed as pathologically induced cells that help the tumor cells to evade immune responses by suppressing T-cell responses. Increased amounts of MDSCs in cancer patients correlate with a worse survival and higher metastatic burden (22). In infectious and autoinflammatory diseases it is speculated that MDSCs are induced as physiological immune regulators, but their role is controversially discussed and needs further understanding.

1.2.1 MDSC origin and expansion

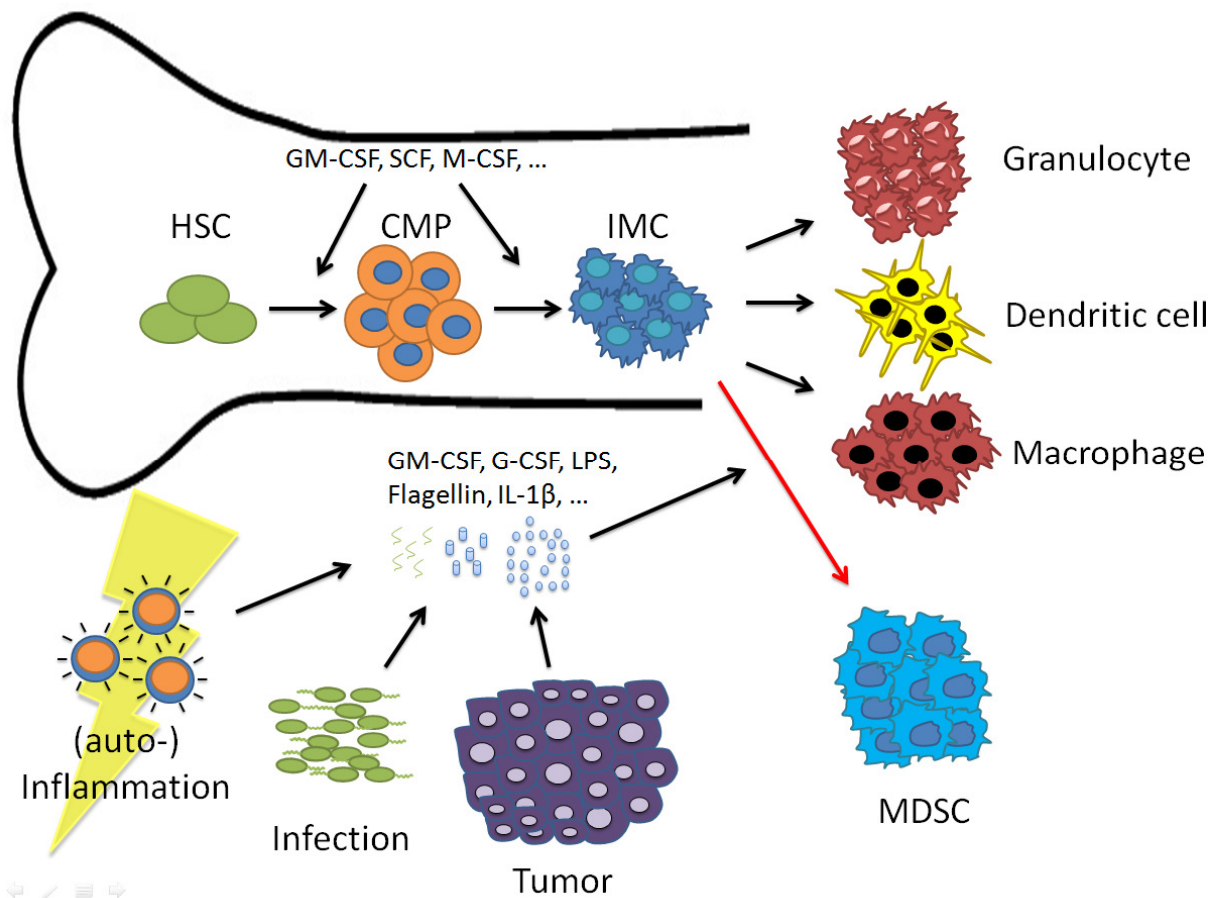


Figure 1 MDSC homeostasis

Adapted from Gabrilovich *et al.* 2009 © Nature publishing group

Immature myeloid cells (IMCs) are the basis of myeloid innate immune cells like granulocytes, dendritic cells and macrophages. They differentiate from common myeloid progenitor (CMP) cells, which are derived from haematopoietic stem cells (HSCs). This process is controlled by soluble factors like granulocyte/macrophage colony-stimulating factor (GM-CSF), stem-cell factor (SCF), macrophage colony-stimulating factor (M-CSF) and many others as well as cell-bound molecules (not shown). Under normal conditions, immature myeloid cells migrate to peripheral organs, where they differentiate into granulocytes, dendritic cells and macrophages. Under pathologic conditions however, soluble factors produced by tumors, pathogens or (auto-) inflammatory responses recruit immature myeloid cells, leading to an accumulation and activation of the cells and inhibit their further differentiation. These cells have the ability to potently suppress immune responses, especially T cells and are therefore named myeloid-derived suppressor cells (MDSCs) (28).

In the bone marrow of healthy individuals, hematopoietic stem cells differentiate into common myeloid progenitor cells, which in turn differentiate into immature myeloid cells (IMCs). These migrate out of the bone marrow and further differentiate into mature myeloid cells like macrophages, dendritic cells and granulocytes. This differentiation and recruitment is directed by a series of soluble factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), stem-cell factor (SCF), macrophage colony-stimulating factor (M-CSF) and others. In pathological conditions IMCs are recruited to sites of infection, trauma or tumor, where their expansion is

promoted whereas their differentiation into mature myeloid cells is blocked and their suppressive phenotype is activated by soluble factors like GM-CSF, other cytokines (IL-1 β , IL-6, IL-10) or bacterial PAMPs (LPS and flagellin) produced by tumor cells, bacteria or cells mediating inflammatory responses (28).

1.2.2 MDSC function

MDSCs are most commonly known for their ability to suppress T-cell responses via direct suppression or the induction of Tregs (29). The suppression of T cells requires direct cell to cell contact, thus indicating that MDSCs act through receptors on the cell surface or through soluble short-lived molecules (28). Several mechanisms have been described to be involved in the suppressive activity of MDSCs.

Inducing and releasing high amounts of reactive oxygen species (ROS) like superoxide anions and hydrogen peroxide is one of the main characteristics related to MDSC suppression by causing oxidative stress. In T cells ROS suppress various signaling pathways and IL-2 production (30) negatively affecting T-cell signaling, activation and proliferation (31). Another mechanism is the metabolism of L-arginine, a non-essential amino acid, by inducing the enzymes arginase1 (Arg1) and inducible nitric oxide synthase (iNOS) leading to a shortage of L-arginine in the microenvironment. T cells are unable to synthesize L-arginine and thereby have a diminished proliferation (19, 32). iNOS also produces nitric oxide (NO), that is involved in T cell suppression via inhibition of transcription factors (33), MHC-II expression (34) as well as induction of apoptosis, leading to cell death (35). NO also reacts with superoxide anions to form peroxynitrite, a very powerful oxidant inducing nitration and nitrosylation of sulfur-containing amino acids (36). High amounts of peroxynitrite have been described to correlate with T-cell unresponsiveness (37) and direct contact of MDSCs with T cells lead to nitration of the T-cell receptor and CD8 molecules, disrupting antigen-specific stimulation of T cells (38). MDSCs are also known to modulate the activity and function of other myeloid cells (39) for example by releasing IL-10 MDSCs suppress pro-inflammatory IL-12 responses by macrophages and DCs decreasing their potential to activate T cells (40).

The induction of regulatory T cells (Tregs) by MDSCs has also been described (41, 42), but is controversially discussed (43) and requires further investigation.

1.2.3 MDSC subtypes

MDSCs are a phenotypically heterogeneous group of myeloid cells, which can be grouped into two subtypes, granulocytic MDSCs (G-MDSCs), which resemble granulocytes, and monocytic MDSCs (M-MDSCs), which resemble monocytes. G-MDSCs are the predominant subset in most diseases (44, 45) and produce high amounts of ROS but little amounts of NO, while M-MDSCs produce high amounts of NO and low amounts of ROS. Both subsets have elevated Arg1 activity (43, 44).

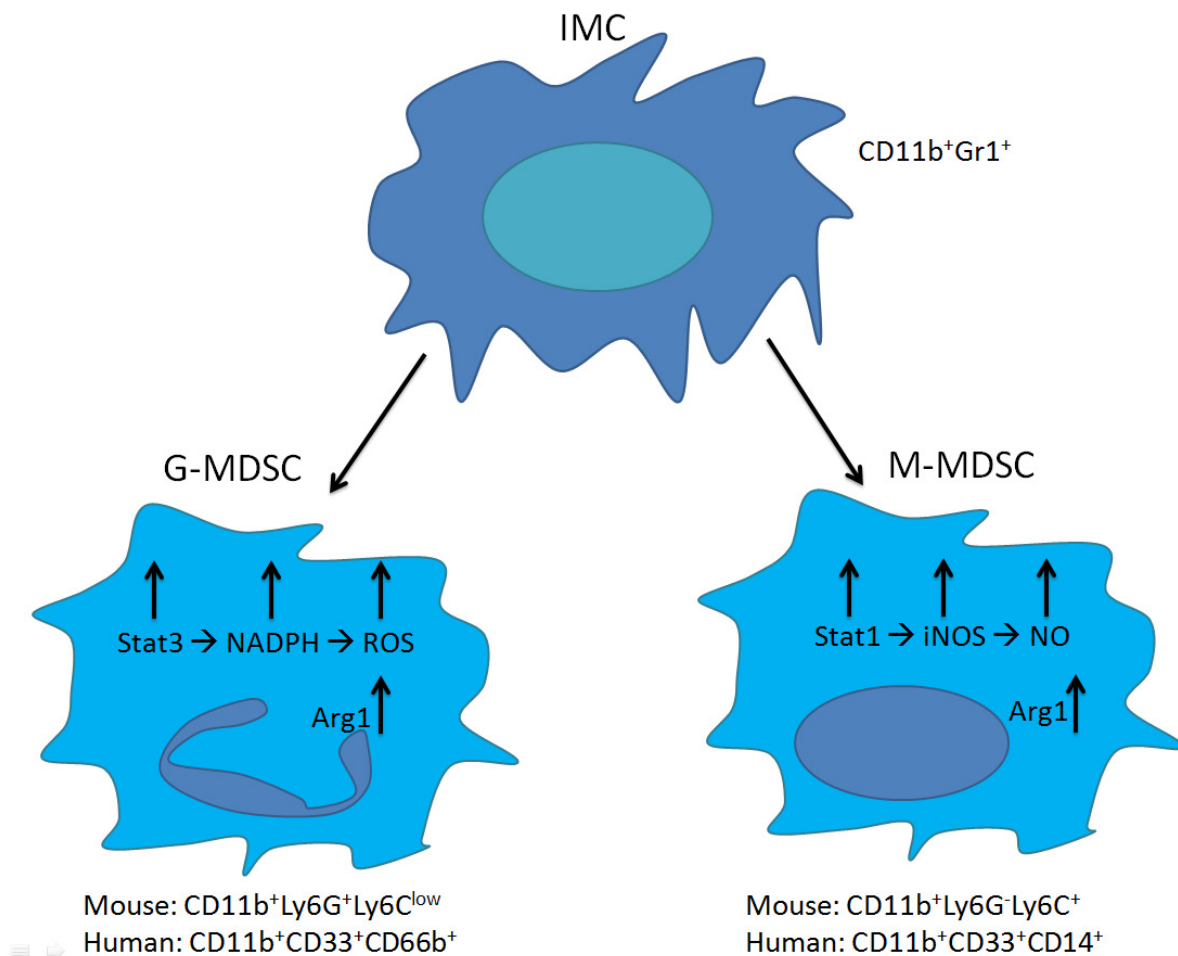


Figure 2 MDSC function and subtypes

Adapted from Gabrilovich *et al.* 2009 © Nature publishing group

MDSCs divide into two subtypes, granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). While both groups potently suppress T cells, they use different mechanisms. While Stat3 activation in G-MDSCs leads to high levels of NADPH and therefore inducing high amounts of reactive oxygen species (ROS), Stat1 activation in M-MDSCs leads to high levels of inducible nitric oxide synthase (iNOS) and nitric oxide (NO). Both populations share high arginase1 (Arg1) activity which further increases their suppressive potential. Commonly used markers are for murine G-MDSCs CD11b⁺Ly6G⁺Ly6C^{low} and for human G-MDSCs CD11b⁺CD33⁺CD66b⁺. For murine M-MDSCs the markers CD11b⁺Ly6G⁻Ly6C⁺ are commonly used and for human M-MDSCs CD11b⁺CD33⁺CD14⁺ respectively (28).

Due to their heterogeneity there is no definite way of labeling MDSCs, but commonly used markers in human studies are CD33 and CD11b as myeloid cell markers and in particular CD66b or CD15 as marker for G-MDSCs whereas CD14 as marker for M-MDSCs. Additionally missing or less expressed markers HLA-DR⁻ or F4/80⁻ might be used to be able to distinguish MDSCs from granulocytes or monocytes respectively (43). For mouse studies the myeloid marker CD11b is used, as well as the myeloid differentiation antigen GR-1 (GR-1), that is including the granulocyte marker Ly6G and the macrophage marker Ly6C. G-MDSCs are specified as CD11b⁺Ly6G⁺Ly6C^{low} and for M-MDSCs as CD11b⁺Ly6G⁻Ly6C⁺ (44). These markers are shared with granulocytes and macrophages, making it difficult to clearly distinguish MDSCs from their mature myeloid “siblings”. Therefore to safely identify MDSC populations, their suppressive potential has to be evaluated.

1.3 Cystic fibrosis

Cystic fibrosis (CF) has first been characterized in 1938 and is the most common lethal inherited disease in the Caucasian population. While in the first years of discovery life expectancy was only a few months due to meconium ileus and pancreatic malabsorption (46) nowadays life expectancy has increased to more than 40 years (47, 48) by enzyme replacement therapy, correcting nutritional defects, airway clearance therapies and aggressive treatment against pathogens (49-51) with progressive airway disease being the main cause of mortality (52).

1.3.1 CF pathogenesis

CF is caused by gene defects in the *cystic fibrosis transmembrane conductance regulator (CFTR)* gene, which is encoding for a cAMP-dependent chloride ion channel (53, 54). More than 2000 mutations have been found to cause CF, with the $\Delta F508$ mutation being the most common one. Dysfunctional or lacking CFTR protein leads to deregulated ion fluxes, especially leading to hyper-reabsorption of Na^+ by the epithelial sodium channel (ENaC), causing dehydration of the airway surface liquid (50, 55). This in turn hampers ciliary movement and causes thick mucus stasis in CF airways that reduces the ability to clear pathogens from the airways (56, 57). Besides these physiochemical disturbances in the airways it is also speculated that CFTR deficiency intrinsically leads to a hyper-inflammatory condition, for example via accumulation of ceramides, which further promotes lung injury (50, 58-60).

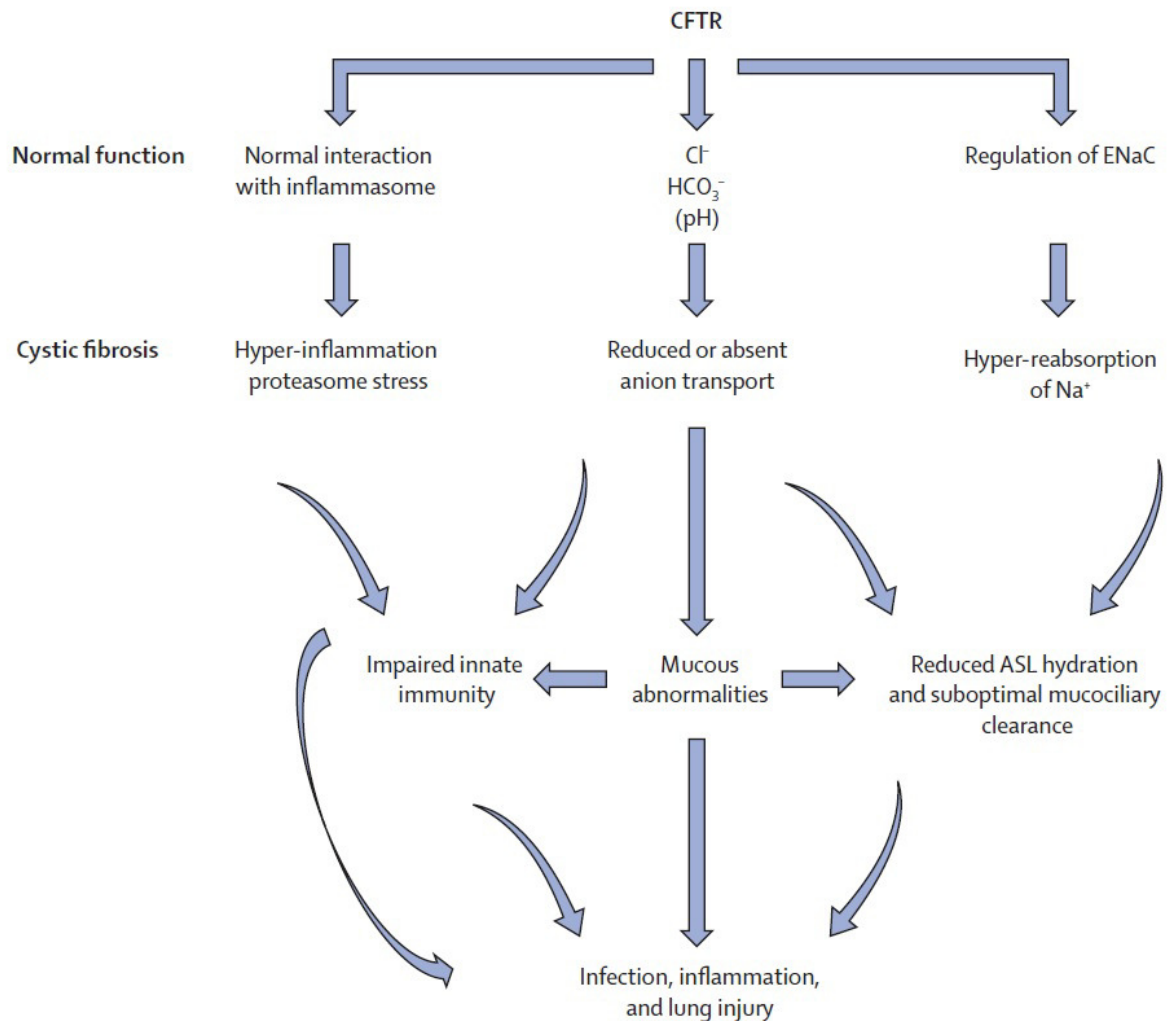


Figure 3 Effects of CFTR dysfunction:

Adapted from J Stuart Elborn 2016 © The Lancet

Disrupted anion transport and absent regulation of the epithelial sodium channel (ENaC) leads to mucus abnormalities and reduced airway surface liquid (ASL) hydration, hampering the mucociliary clearance. In combination with disrupted interactions with the inflammasome and impaired innate immunity this leads to poor pathogen clearance allowing chronic infections, a hyper-inflammatory state and therefore causing severe lung injury (52).

The abundance of pathogens and the general inflammatory condition leads to severe tissue damage and bronchiectasis via secretion of elastase, cathepsin S and matrix metalloproteinases by neutrophils (61-64). While in the early years of disease progression pathogens like *Haemophilus influenzae* and *Staphylococcus aureus* are mainly responsible for infections, in the later years, as the epithelial cell integrity is disrupted, opportunistic gram-negative bacteria like *Pseudomonas aeruginosa* and fungi like *Aspergillus fumigatus* take over (58, 65-67). Recent publications point out that CF patients suffer from unopposed Th2/Th17 driven T-cell responses, which further fuel the hyper-inflammatory state (68-70) and precede *P. aeruginosa*

infections (69). Fitting to these findings previous work from our group also demonstrated that Tregs, normally able to counteract the Th2/Th17 driven inflammation are diminished in CF patients, especially in patients with chronic *P. aeruginosa* infections (71).

1.3.2 Treatment of CF

Treatment of CF includes pancreatic enzyme replacement and nutritional monitoring (72-74), prevention and treatment of infections using oral, inhaled and intravenous applied antibiotics (75, 76) as well as clearance of airways (75, 77).

More innovative therapies target the reconstitution of the CFTR protein either by usage of small molecules or gene therapy. While for some mutations the small molecule ivacaftor has shown to improve CF patients' constitution, however the effect on the most common F508del mutation was rather minor (78-80). Restoration of *CFTR* on a genetic base has recently been reported to have beneficial effects (81). In general, these studies suggest that CFTR function can be restored, but further studies are needed to increase the efficiency (82).

All of these therapies are delaying disease progression and respiratory failure, but cannot fully prevent it, and therefore in the long run lung transplantation will be needed, alluding the need for new therapeutic approaches.

1.3.3 CF mouse model

Ever since the murine *Cftr* gene has been described 1991 different mouse models were generated with targeted mutagenesis (83). The biggest problem of most of these models was that the mice had severe pathological complications in the intestines, often leading to death prior to lung disease. The transgenic *Cftr*^{tm1Unc} Tg(FABPCFTR)1Jaw/J (*Cftr*^{-/-}) mouse counteracts this problem by expressing human CFTR in the gut under the control of the FABP1 (fatty acid binding protein1) promoter, rescuing the mice from intestinal obstruction (84). These mice show inflammatory lung conditions, presumably due to ceramide accumulation in the respiratory epithelium (60), leading to an increased susceptibility to infections in aged mice (>16 weeks old) (85). Another study showed no correlation between age and susceptibility towards intratracheally applied *P. aeruginosa* infections, but implied that CF mice are a suitable model for studying early host defense mechanisms in CF (86).

1.4 *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic, gram-negative, flagellated, rodshaped, facultative anaerob bacterium. It has a highly regulated big genome ranging from 5.5 – 7 Mbp (87) with more than 500 regulatory genes found in the PAO1 strain (88) enabling the survival in various environmental habitats (89, 90). *P. aeruginosa* is under normal conditions cleared by the immune system, but in immunocompromised hosts, or in inflammatory conditions like CF, where the host epithelium is disrupted, *P. aeruginosa* can cause severe infections. Once *P. aeruginosa* gets a hold in the host, it has several mechanisms to escape immune responses as being formation of biofilms (91), high intrinsic resistance to antibiotics (92) and release of virulence factors (93-95). It is the most predominant pathogen in CF lung disease (52).

2. Aim of study

Excessive inflammation due to unbalanced T-cell responses is a major problem in autoinflammatory (like inflammatory bowel disease or rheumatoid arthritis) and infectious diseases, causing collateral damage to the host. In CF, unbalanced Th2/Th17 responses seem to play a major role in the persistence of the inflammatory state next to a high number of recruited neutrophils. Our group revealed, that CF patients have increased numbers of granulocytic myeloid-derived suppressor cells (G-MDSCs) in bronchoalveolar lavage and in peripheral blood compared to healthy donors, especially CF patients with chronic *P. aeruginosa* infections. Furthermore percentages of G-MDSCs positively correlated with lung function in chronic *P. aeruginosa* infected CF patients.

The aim of this work was to further investigate how *P. aeruginosa* interacts with MDSCs in acute pulmonary infections and what impact CFTR deficiency has. Using *in vivo* infection models and *Cftr*^{-/-} mice we mimicked the MDSC induction of *P. aeruginosa* infected CF patients. With further *ex vivo* suppression assays we analyzed the capability of induced MDSCs to suppress polyclonal T cell expansion. To assess the impact of CFTR deficiency on G-MDSC suppression of T cells, we compared the suppressive capability of *in vitro* expanded G-MDSCs from CFTR deficient to wildtype mice. In order to clarify if G-MDSCs are beneficial or detrimental in acute pulmonary *P. aeruginosa* infections, we adoptively transferred G-MDSCs into wildtype mice prior to intranasal infection and monitored the survival of the mice. With this study, we want to gain further insights into the role of MDSCs in *P. aeruginosa* infections in the context of CF using *in vivo* models in order to elaborate the modulation of MDSCs as a therapeutic approach in CF.

3. Discussion

The aim of this study was to investigate the interaction of myeloid-derived suppressor cells (MDSCs) and the opportunistic pathogen *P. aeruginosa* in the context of cystic fibrosis (CF). While MDSCs have been widely studied in various cancers, where they are associated with tumor immune evasion and therefore negatively correlate with patient survival, MDSCs have also been shown to be involved in autoinflammatory and infectious diseases. Previous studies from our group revealed, that G-MDSCs, but not M-MDSCs are increased in peripheral blood of patients with CF compared to healthy donors. The highest increase was found in patients with chronic *P. aeruginosa* infections and correlated positively with clinical pulmonary parameters. Further *in vitro* assays, as well as *in vivo* pulmonary infection models in wildtype (WT) and *Cftr*^{-/-} mice we gained more insights into the role of MDSCs in infective inflammatory diseases.

3.1 *P. aeruginosa* infections induce MDSCs

To investigate the role of G- and M-MDSCs upon bacterial infections, we used an acute pulmonary infection model in WT and *Cftr*^{-/-} mice with the *P. aeruginosa* lab strain PAO1. We saw a local (lung and bronchoalveolar lavage (BAL)) and systemic (spleen) induction of G-MDSCs in mice after acute pulmonary *P. aeruginosa* infections. G-MDSCs seem to play the major role, as M-MDSCs were only slightly induced in lungs and spleens, what resembles the situation in CF patients and *in vitro* experiments with human peripheral blood mononucleated cells (PBMCs) (27). G-MDSCs were decreased in bone marrow of infected animals and since it is a fairly short time of infection duration before the organs were harvested (12 – 16 h), we assume that G-MDSCs are mainly recruited from the bone marrow to the airways and spleens. Gender of the mice had no influence on basal MDSC amounts or induction through infection (data not shown).

Our *in vivo* studies with 8 – 12 weeks old *Cftr*^{tm1Unc-Tg(FABPCFTR)1Jaw/J (*Cftr*^{-/-}) mice revealed that CFTR deficiency has no influence on the basal amount of MDSCs (G- and M-MDSCs) as well as on the recruitment of MDSCs during acute pulmonary *P. aeruginosa* infections when comparing *Cftr*^{-/-} to WT littermates. Further studies on older *Cftr*^{-/-} mice might give further insights, as older *Cftr*^{-/-} mice develop inflammatory responses due to CFTR deficiency (86), reflecting the human CF condition better. Another discrepancy is that CF patients often suffer from persisting bacterial}

infections, whereas in the acute infection model in *Cftr*^{-/-} mice *P. aeruginosa* is cleared efficiently within a few days (86). Therefore we will further try an artificial bead model with encapsulated *P. aeruginosa* that will mimic the bacterial persistence in the lungs (96).

3.2 *P. aeruginosa* enhances G-MDSC activity

Since in our recent and previous studies G-MDSCs seem to be key players in *P. aeruginosa* infections (27), our further studies were focused on G-MDSCs only. As a next step, we analyzed the suppressive capability of basal G-MDSCs as well as acute pulmonary *P. aeruginosa* infection induced G-MDSCs isolated from lungs, BAL and bone marrow of C57Bl6/J mice. In all three compartments we could demonstrate a dose dependent suppression of proliferation of freshly isolated splenic CD4⁺ T cells at ratios 1:1, 1:2, 1:4 and 1:8 with the highest suppression at the 1:1 G-MDSC to T-cell ratio. G-MDSCs isolated from infected mice had an enhanced suppressive capability at a single cell level, indicating that *P. aeruginosa* infections enhance G-MDSC activity. Intriguingly G-MDSCs had not only enhanced suppressive capabilities at the site of inflammation (lung), but also a systemic activation of G-MDSCs could be observed in G-MDSCs isolated from splenocytes and bone marrow cells.

The cellular mechanisms that are activated in G-MDSCs following infection remain to be elucidated, although ROS and IL-10 production of G-MDSCs seem not to be involved (data not shown/ unpublished observation). Further interesting targets to investigate could be arginase-1 production, as it is a major suppressive mechanism of MDSCs (19, 32) or transforming growth factor-beta (TGF- β) secretion (41, 42). For the mechanism triggering G-MDSC activation via *P. aeruginosa*, we suspect that soluble mediators that are spread throughout the mice are responsible for the enhanced suppressive activity, as we could not detect any bacteria in spleens and bone marrow of infected animals. Flagellin could be the most prevalent target, as it is known to be shed by *P. aeruginosa* and has been shown to potently induce G-MDSCs in human *in vitro* approaches (27), but other soluble mediators should also be taken into account. Another factor driving G-MDSC activation could also be endogenous inflammatory signals triggered the infection.

CFTR deficient G-MDSCs on the other hand were less suppressive. While G-MDSCs isolated from *Cftr*^{-/-} mice were still able to suppress T-cell proliferation, the suppressive effect on a single cell level was diminished when compared to G-MDSCs

isolated from wild type littermates. As *Cftr*^{-/-} mice only slowly start to develop inflammatory symptoms with higher age (>16 weeks) (86) and in our experiments cells isolated from 8 – 12 week old mice were used, this effect can most likely be directly linked directly to CFTR deficiency. Via this system we therefore have the ability to investigate cellular mechanisms linking CFTR to MDSC suppression. While this finding is interesting, it is yet too preliminary to draw solid conclusions for CF patients. If MDSCs from CF patients are also less suppressive, this could at least partially explain the overshooting Th2/Th17 responses of CF patients, as T-cell responses would be less suppressed by MDSCs. Investigation of the effect of *P. aeruginosa* infections on G-MDSCs from *Cftr*^{-/-} mice could give further insights.

3.3 G-MDSCs effect *in vivo*

Recent findings from our group revealed, that adoptive transfer of G-MDSCs prior to systemic *Candida (C) albicans* (systemic candidiasis) infection improves the survival of infected Balb/c mice, probably by diminishing pathogen beneficial Th17 responses and by generally decreasing overshooting inflammatory responses (97, 98). In CF patients with chronic *P. aeruginosa* infections MDSCs positively correlated with clinical lung function parameters (27). Therefore we wanted to determine the potential protective effect of G-MDSCs by adoptively transferring G-MDSCs prior to pulmonary acute *P. aeruginosa* infection into mice. Similar to the findings in pulmonary *Aspergillus fumigatus* infections (98) adoptive transfer of G-MDSCs had no effect on the severity of infection as well as on the weight recovery. Higher concentrations of *P. aeruginosa* lead to death via acute sepsis (99-101) within two to three days, irrespective of adoptive G-MDSC transfer. Based on these findings, we speculate that G-MDSCs have a higher impact on systemic fungal infections (like systemic candidiasis) compared to acute local bacterial airway infections *in vivo*. Another explanation could be the methodological discrepancy, as both, G-MDSCs and *C. albicans* were administered intravenously, whereas *P. aeruginosa* was given intranasally. The close proximity in the systemic *C. albicans* infection might give the transferred G-MDSCs an edge, whereas in the pulmonary infections the G-MDSCs actively have to migrate to the site of infection from the circulation. To clarify the effect of local proximity, future studies have to be conducted transferring G-MDSCs into the airways prior to intranasal PAO1 infection. Furthermore, the beneficial effect of G-MDSCs on the clinical lung function parameters of chronically infected CF patients with chronic *P. aeruginosa* infections seems to be a long-term effect rather

than, as simulated in our model, an immediate immune response (27). Therefore it would be relevant to perform our *in vivo* studies with a chronic infection model of *P. aeruginosa* (102) combined with adoptive transfer of G-MDSCs i) systemically and ii) locally (intratracheal application) of bone marrow situated G-MDSCs from control mice, *P. aeruginosa* infection induced G-MDSCs and G-MDSCs from *Cftr*^{-/-} mice.

3.4 Concluding remarks

Emerging evidence suggests that MDSCs are essential not only in malignant diseases, but also infectious and inflammatory diseases, for example inflammatory bowel disease (23), rheumatoid arthritis (24, 25), chronic obstructive pulmonary disease (103, 104), tuberculosis (105), sepsis (106, 107), cystic fibrosis (27). This study demonstrates that acute pulmonary *P. aeruginosa* infection recruits and functionally modulates G-MDSCs and thereby undermines host immunity *in vivo*. Further studies need be performed to understand the cellular mechanisms, as it might give insights into general MDSC recruitment and activation in infectious and inflammatory diseases.

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Appendix: Publications in the thesis

Paper 1

Hasan H. Öz., Benyuan Zhou, Pina Voss, Melanie Carevic, Carolin Schroth, Nina Frey, Nikolaus Rieber, Andreas Hector, and Dominik Hartl. Submitted August 2016.
Pseudomonas aeruginosa airway infection recruits and modulates neutrophilic myeloid-derived suppressor cells. Submitted to *Frontiers in Cellular and Infection Microbiology*

1 ***Pseudomonas aeruginosa* airway infection recruits and modulates**
2 **neutrophilic myeloid-derived suppressor cells**

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5 **Running title:**

6 *P. aeruginosa* modulates MDSCs

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20

21

22 **Abstract**

23 *Pseudomonas aeruginosa* is an opportunistic pathogen that causes infections mainly in patients
24 with cystic fibrosis (CF) lung disease. Despite innate and adaptive immune responses upon
25 infection, *P. aeruginosa* is capable of efficiently escaping host defenses, but the underlying
26 immune mechanisms remain poorly understood. Myeloid-derived suppressor cells (MDSCs) are
27 innate immune cells that are functionally characterized by their potential to suppress T- and
28 natural killer (NK)-cell responses. Here we demonstrate, using an airway *in vivo* infection model,
29 that *P. aeruginosa* recruits and activates neutrophilic MDSCs, which functionally suppress T-cell
30 responses. We further show that the CF gene defect (cystic fibrosis transmembrane conductance
31 regulator, CFTR) modulates the functionality, but not the recruitment or generation of
32 neutrophilic MDSCs. Collectively, we define a mechanism by which *P. aeruginosa* airway
33 infection undermines host immunity by modulating neutrophilic MDSCs *in vivo*.

35 **Author Summary**

36 Infections with *Pseudomonas aeruginosa*, an opportunistic gram-negative bacterium, represent a
37 major cause of morbidity and mortality in patients with cystic fibrosis (CF) lung disease. Upon
38 airway infection, *P. aeruginosa* activates the innate immune system. However, in spite of a
39 substantial and sustained presence of phagocytic and lymphocytic immune cells at the infected
40 airway compartment, the host is not able to efficiently eliminate *P. aeruginosa* in CF. The
41 underlying immune mechanisms remained poorly understood. Myeloid-derived suppressor cells
42 (MDSCs) are anti-inflammatory innate immune cells that potently suppress T cellular immune
43 responses. We showed in a previous study that neutrophilic MDSCs accumulate in patients with
44 CF infected with *P. aeruginosa* and correlate with lung function in those patients. Here, we
45 demonstrate that *P. aeruginosa* airway infection recruits and functionally activates neutrophilic
46 MDSCs and thereby define a mechanism by which *P. aeruginosa* airway infection undermines
47 host immunity by modulating neutrophilic MDSCs *in vivo*.

49 Key words: myeloid-derived suppressor cells, MDSCs, bacteria, infection, *Pseudomonas*, CF,

50 cystic fibrosis, CFTR, lung, T cells

51

52

53 Introduction

54 Infections with *Pseudomonas aeruginosa*, an opportunistic gram-negative bacterium, represent a
55 major cause of morbidity and mortality in patients with cystic fibrosis (CF) lung disease, chronic
56 obstructive pulmonary disease (COPD), ventilated patients and patients undergoing
57 immunosuppression (1, 2). Upon infection, *P. aeruginosa* activates the innate immune system
58 and induces a rapid recruitment of neutrophilic cells to the site of inflammation, followed by
59 activation of the adaptive response characterized by T-cell infiltration (1). Despite a substantial
60 and sustained presence of phagocytic and lymphocytic immune cells at the infected
61 compartment, the host is not able to efficiently eliminate *P. aeruginosa*, particularly in
62 pulmonary disease conditions, such as CF (caused by mutations in the cystic fibrosis
63 transmembrane conductance regulator, CFTR, gene) or chronic obstructive pulmonary disease
64 (COPD, caused by cigarette smoke) (1, 3, 4). The underlying immune mechanisms remained
65 poorly understood.

66 Myeloid-derived suppressor cells (MDSCs) are innate immune cells that are functionally
67 characterized by their potential to suppress T- and natural killer (NK)-cell responses (5, 6).
68 MDSCs can be sub-divided into neutrophilic and monocytic MDSCs as defined by surface
69 marker profiles. Rieber *et al.* demonstrated in a previous study that neutrophilic MDSCs
70 accumulate in patients with CF infected with *P. aeruginosa* (7). We further showed that
71 neutrophilic MDSCs are clinically relevant in *P. aeruginosa*-infected CF patients, because
72 percentages of neutrophilic MDSCs correlated with lung function in those patients. However, the
73 mechanisms by which *P. aeruginosa* airway infection regulates neutrophilic MDSCs *in vivo*
74 remained elusive.

75 Here we used a well-established *P. aeruginosa* airway infection model (8, 9) to
76 investigate the mechanisms by which *P. aeruginosa* skews host immunity *in vivo*. Our studies
77 demonstrate that (i) *P. aeruginosa* airway infection triggers the recruitment of neutrophilic, but
78 not monocytic MDSCs, (ii) *P. aeruginosa* infection enhances the suppressive capacity of
79 neutrophilic MDSCs and (iii) CFTR partially overlaps with *P. aeruginosa* in modulating
80 neutrophilic MDSCs.

81 **Materials and Methods**

82 *Ethics statement*

83 All animal studies were reviewed and approved by the Regierungspräsidium Tübingen,
84 Tübingen, Germany (approval ID: K4/12), and were carried out according to the guidelines of
85 the German law of protection of animal life.

86

87 *Bacteria*

88 *P. aeruginosa* wild type strains (PAO1) were used as published previously by our group (9).
89 Strains of the culture collection were streaked on agar plates and incubated at 37°C overnight.
90 Colonies were then inoculated into lysogeny broth overnight. The next day, a 1:10 dilution in
91 lysogeny broth was performed and bacteria were cultured at 37°C for 4 hours. The optical
92 density was measured at 600 nm.

93

94 *Mouse models*

95 Mice were bred at the animal facility of the Institute of Pharmacology (Tübingen). C57Bl/6J or
96 *Cftr*^{-/-} (*Cftr*^{tm1Unc}-Tg(*FABPCFTR*))*Jaw/J* mice were used. *Cftr*^{-/-} mice were compared with age-
97 and background strain-matched *Cftr*^{+/+} littermates. The mouse model of acute pulmonary *P.*
98 *aeruginosa* infection was performed as published by our group (9). Mice were infected with
99 2x10⁶ or 4x10⁶ CFU of *P. aeruginosa* (PAO1) utilizing previously established procedures (9).
100 Intranasal applications were carried out under antagonizable anesthesia. Briefly, an inoculum of
101 2x10⁶ and 4x10⁶ CFU were administered intranasally (50 µl/nostril). After infection, body weight
102 was monitored once a day over one week. For FACS analysis mice were sacrificed 16 hours after
103 infection.

104

105 *MDSC in vitro generation*

106 Bone marrow cells and splenocytes were isolated from *Cftr*^{+/+} and *Cftr*^{-/-} mice and cultured in
107 RPMI1640 (Merck Millipore) with supplements, in detail 10 % fetal bovine serum (Sigma), 100
108 U/ml Penicillin-Streptomycin (Merck Millipore) each, 2 mM L-Glutamine (Merck Millipore), 10
109 mM HEPES (Merck Millipore). MDSC generation was induced by addition of 40 ng/ml
110 recombinant mouse GM-CSF (Biolegend) and 40 ng/ml recombinant mouse IL-6 (Biolegend) as
111 published by Marigo *et al.* (10). Cells were either fed with fresh media and cytokines on d3 and
112 d6 or collected and analyzed by flow cytometry at d3, d6 and d10. For suppression assays cells
113 were collected on d6.

114

115 *MDSC characterization, isolation and adoptive transfer*

116 Murine MDSCs were phenotypically characterized as described previously (11) by using CD11b,
117 Ly6G (neutrophilic MDSCs) and Ly6C (monocytic MDSCs) markers followed by T-cell
118 suppression assays to distinguish them from non-suppressive neutrophilic or monocytic effector
119 cells respectively. Flow cytometry was performed on a FACS Calibur (BD). Murine MDSCs
120 were isolated from different organs/tissues/fluids as described previously (11) using MACS
121 (MDSC isolation kit, Miltenyi). For adoptive transfer experiments, CD11b⁺Ly6G⁺ neutrophilic
122 MDSCs were isolated from the bone marrow of healthy female C57Bl6/J wildtype mice by
123 MACS (MDSC isolation kit, Miltenyi Biotec, Germany). Transfer was performed by transferring
124 8-10x10⁶ neutrophilic MDSCs per animal into eight to twelve weeks old female C57Bl6/J

125 wildtype mice via lateral tail vein injection. 24 hours after the neutrophilic MDSC transfer, mice
126 were infected with *P. aeruginosa* (PAO1) as described above.

127

128 *T-cell suppression assays*

129 T-cell suppression assays were performed as described previously (11) using the CFSE method
130 according to the manufacturer's protocol (Invitrogen). In brief, CD11b⁺Ly6G⁺ neutrophilic
131 MDSCs were isolated from different organs/tissues/fluids by using MACS (MDSC isolation kit,
132 Miltenyi Biotec, Germany) and were co-cultured for three days (37°C, 5% CO₂) with MACS
133 sorted CFSE stained CD4⁺ T cells from splenocytes at MDSC : T cell ratios 1:1, 1:2, 1:4 and 1:8
134 in RPMI1640 with supplements as mentioned before. The number of T cells per well was kept at
135 10⁵ and MDSCs were added accordingly. T-cell proliferation was stimulated with CD3/CD28-
136 beads (mouse T-cell activation kit, Miltenyi Biotec, Germany) and recombinant mouse IL-2 (50
137 U/ml, Biolegend). CFSE-fluorescence intensity was analyzed by flow cytometry to determine the
138 percentage of polyclonally proliferated T- cells. For the graphs the data was normalized to the
139 proliferation of the stimulated control T-cell proliferation (without addition of MDSCs).

140

141 *BALF*

142 Bronchoalveolar lavage was extracted through the trachea with 2 ml PBS. Living BAL cells
143 were counted using trypan blue dye exclusion. After erythrocyte lysis with ACK lysis solution,
144 cells were incubated with an Fc receptor block (1 µg/1 × 10⁶ cells; BD Bioscience) to reduce
145 nonspecific antibody binding.

146

147 *Flow Cytometry*

148 The panel of antibodies used to stain BALF, lung, spleen or bone marrow cells included CD11b
149 (clone M1/70), Ly6G (clone 1A8) and Ly6C (clone AL-21) (all from BD Biosciences). Flow
150 cytometry was performed using BD FACS Calibur (BD Bioscience), and data were analyzed
151 with FlowJo software.

152

153 *Statistics*

154 Statistical analysis was done using GraphPad Prism 6.0 (Graph Pad Software, La Jolla, CA,
155 USA). Differences between the groups were determined by Students' t test. A *P* value of <0.05
156 was considered to be significant.

157 **Results**158 *Pseudomonas* infection triggers neutrophilic MDSC recruitment

159 We characterized the recruitment of neutrophilic and monocytic MDSCs in response to acute *P.*
160 *aeruginosa* airway infection in different pulmonary (BALF, lung) and extra-pulmonary (spleen,
161 bone marrow) compartments *in vivo* (Figure 1A, Figure 1D, Figure 1E). To this end, we
162 analyzed MDSCs based on their (i) phenotypic characteristics (Figure 1A) and their (ii)
163 functional capacity to dose-dependently suppress CD4⁺ T-cell proliferation (Figure 1B, Figure
164 1C). Utilizing these approaches we could demonstrate that acute *P. aeruginosa* airway infection
165 lead to an increase of neutrophilic MDSCs in BALF and lung tissue by both percentages as well
166 as total cell numbers and lead to an increase of MDSC percentages in the spleen (Figure 1A,
167 Figure 1D), whereas the percentages or total cell numbers of neutrophilic MDSCs decreased in
168 the bone marrow compartment (Figure 1D). Percentages of monocytic MDSCs remained
169 unchanged in BALF and bone marrow but increased in lung tissue had a tendential increase in
170 spleen, however to a far lesser extent than neutrophilic MDSCs upon *P. aeruginosa* airway
171 infection (Figure 1E). Total monocytic MDSC numbers also increased in lungs upon *P.*
172 *aeruginosa* airway infection but remained unchanged in spleen. Collectively, these studies
173 indicate that *P. aeruginosa* airway infection has a substantial effect on MDSCs *in vivo* by
174 triggering the recruitment of neutrophilic MDSCs into the pulmonary compartment and
175 increasing the percentage of neutrophilic MDSCs in the spleen.

176

177 *Pseudomonas* infection enhances the suppressive capacity of neutrophilic MDSCs

178 Next, we sought to dissect whether acute *P. aeruginosa* airway infection not only recruits
179 neutrophilic MDSCs, but also shapes their functional characteristics in terms of suppressing T-
180 cell proliferation. For this purpose, we isolated neutrophilic MDSCs from lungs, bone marrow
181 and spleens using MACS technology and tested their capacity to suppress polyclonal T-cell
182 proliferation (Figure 2A). These studies demonstrated that spleen-isolated neutrophilic MDSCs
183 suppressed polyclonal T-cell proliferation in a dose-dependent manner (Figure 2B). This effect
184 that was enhanced upon acute *P. aeruginosa* airway infection, but depended on its magnitude
185 and significance on the origin of isolated neutrophilic MDSCs (bone marrow, spleens or lungs)
186 and the MDSC-to-T-cell ratios applied (Figure 2B, Figure 2C). In general, neutrophilic MDSCs
187 isolated from the lung had the strongest suppressive capability, followed by neutrophilic MDSCs
188 isolated from bone marrow, while splenic neutrophilic MDSCs showed the weakest suppression
189 of polyclonal T-cell proliferation (Figure 2C). When viewed in combination, these experiments
190 provide evidence that (i) neutrophilic MDSCs in *P. aeruginosa* airway infection functionally
191 dampen T-cell proliferation and that (ii) *P. aeruginosa* airway infection topically (pulmonary)
192 and systemically enhances the suppressive capacity of neutrophilic MDSCs.

193

194 *Adoptive transfer of neutrophilic MDSCs has no impact on weight loss and weight recovery in*
195 *acute Pseudomonas* infection

196 To assess whether neutrophilic MDSCs bear therapeutic potential in *P. aeruginosa* airway
197 infection, we isolated neutrophilic MDSCs from bone marrow by MACS technology, checked
198 their functionality *ex vivo* in T-cell suppression, adoptively transferred the cells i.v. prior to acute
199 *P. aeruginosa* airway infection and monitored the impact of the adoptively transferred MDSCs
200 on weight loss and weight recovery after acute *P. aeruginosa* airway infection (Figure 3). Two
201 different infection doses were assessed, 2x10⁶ (Figure 3A) or 4x10⁶ (Figure 3B) CFU. These

202 investigations demonstrated that the adoptive transfer of neutrophilic MDSCs had no significant
203 impact on weight loss or weight recovery upon acute *P. aeruginosa* airway infection at 2×10^6 or
204 4×10^6 CFU (Figure 3). Both experimental groups successfully cleared the pathogen, as we could
205 not find any residual CFU of *P. aeruginosa* at d5 (2×10^6 CFU) or d6 (4×10^6 CFU) p.i. (data not
206 shown).

207

208 *CFTR modulates function, but not recruitment or generation of neutrophilic MDSCs*

209 Since *P. aeruginosa* airway infections play a predominant role in patients with CF, we sought to
210 dissect the role of CFTR in regulating the recruitment and functionality of neutrophilic MDSCs
211 in our experimental systems. For this purpose, we first analyzed whether the absence of
212 functional CFTR has an impact on the recruitment of neutrophilic or monocytic MDSCs into the
213 airways or into non-pulmonary compartments *in vivo* (Supplementary Figure 1, Supplementary
214 Figure 2). Our data suggest that *Cftr* deficiency has no significant effect on the recruitment of
215 neutrophilic MDSCs (Supplementary Figure 1) in any body compartment *in vivo*, when
216 analyzing *Cftr*^{-/-} mice compared to *Cftr*^{+/+} mice. *Cftr* deficiency also had no significant effect on
217 the recruitment of monocytic MDSCs (Supplementary Figure 2), although the total numbers of
218 monocytic MDSCs were decreased in the bone marrow of *Cftr*^{-/-} mice compared to *Cftr*^{+/+}. Next,
219 we assessed whether the lack of CFTR has an effect on the *in vitro* generation of neutrophilic
220 MDSCs (Supplementary Figure 3). Consistent with the *in vivo* recruitment studies, these
221 analyses showed that the lack of CFTR had no effect on the generation of neutrophilic MDSCs *in*
222 *vitro* (Supplementary Figure 3). Finally, we sought to determine whether the absence of CFTR
223 has a functional consequence for neutrophilic MDSCs in MDSC - T cell interaction assays. In
224 contrast to the recruitment and generation studies, these functional assays provided evidence that
225 *in vitro* generated *Cftr*^{-/-} neutrophilic MDSCs were impaired in suppressing T-cell proliferation
226 compared to their *Cftr*^{+/+} counterpart cells, most strongly at a 1:4 and 1:8 MDSC : T-cell ratios
227 (Figure 4). In summary, these investigations demonstrated that CFTR has no effect on the
228 recruitment or generation of MDSCs, but regulates, at least partially, MDSC-mediated T-cell
229 suppression.

230 **Discussion**

231 MDSC generation and functionalities have been studied thoroughly in several types of cancer
232 (12-18), yet their regulation and functional role in infectious disease conditions remained poorly
233 defined. Here we studied the role of MDSCs in the setting of airway *P. aeruginosa* infection,
234 which is highly relevant for several human diseases, particularly CF, COPD, ventilation-
235 associated pneumonia and burn-related infections (2, 19-21).

236 These present studies build on previous findings from our group demonstrate that (i)
237 *P. aeruginosa* airway infections in patients with CF were associated with increased percentages
238 of neutrophilic MDSCs in the peripheral blood and that (ii) percentages of circulating
239 neutrophilic MDSCs in *P. aeruginosa* infected CF patients were positively correlated with the
240 lung function outcome (7). Despite these intriguing results, we so far do not have any
241 mechanistic explanation(s) how *P. aeruginosa* infection modulates MDSC recruitment and
242 function and why, unexpectedly, percentages of circulating neutrophilic MDSCs were associated
243 with a beneficial disease outcome in patients with CF lung disease. To dissect this host-pathogen
244 interaction *in vivo*, we systematically investigated the MDSCs in different immune and airway
245 compartments in a well-established model of acute *P. aeruginosa* infection (8). Our results here
246 confirmed and extended previous findings obtained from human CF patients that *P. aeruginosa*
247 infections induced MDSC subsets, mainly being neutrophilic MDSCs. *In vivo*, acute *P.*
248 *aeruginosa* airway infections triggered neutrophilic MDSC recruitment from the bone marrow
249 into bronchoalveolar and pulmonary compartments, where neutrophilic MDSCs were
250 functionally active in suppressing polyclonal effector T-cell responses.

251 Intriguingly, acute bacterial *P. aeruginosa* airway infections not only recruited
252 neutrophilic MDSCs to the infected airway compartment, but also enhanced, at least partially,
253 their T-cell suppressive potential at a single cell level, surprisingly even at sites distant from the
254 primary site of airway infection, such as the bone marrow. The underlying mechanisms for this
255 phenomenon remain to be solved in the future, but based on our previous finding that
256 neutrophilic MDSCs express Toll-like receptor 5 (TLR5) (7) and its ligand flagellin is shed from
257 *P. aeruginosa* bacteria and can be found in the circulation after *P. aeruginosa* airway infections
258 (Hartl et. al., unpublished observation), we tempt to speculate that *P. aeruginosa*-derived
259 pathogen-associated molecular patterns (PAMPs), prototypically flagellin, skew neutrophilic
260 MDSCs systemically, including the bone marrow compartment, to boost their
261 immunosuppressive functionalities, a hypothesis remaining to be tested in future investigations.
262 Overall, the precise *in vivo* kinetics, dynamics and compartmentalizations in the complex setting
263 of *P. aeruginosa* infection remain to be deciphered in future *in vivo* studies.

264 Besides *P. aeruginosa* infection, we assessed the effect of *Cftr* deficiency on the
265 induction and function of neutrophilic MDSCs, as it is pathophysiological the case for CF
266 patients *in vivo* (7). Our *in vivo* model, however, failed to identify a significant role for *Cftr* in
267 MDSCs homeostasis, at least in our experimental settings, since *Cftr* deficient mice displayed the
268 same amounts of neutrophilic MDSCs as their wildtype littermates in BALF, lungs, spleens and
269 bone marrow, precluding a major effect of *Cftr*. We also observed no difference on the
270 recruitment and accumulation of neutrophilic MDSCs in BALF and lungs of *Cftr* deficient mice
271 compared to wildtype littermates upon acute pulmonary *P. aeruginosa* infection *in vivo* as well
272 as on neutrophilic MDSC generation *in vitro*.

273 In contrast to the neutrophilic MDSC-enhancing effect of *P. aeruginosa* infections, *Cftr*
274 deficiency, however, differentially modulated the suppressive capability of *in vitro*-expanded

275 neutrophilic MDSCs. The underlying mechanism requires further investigation, particularly
276 given the complex role of *Cftr* deficiency in driving a hyper-inflammatory milieu (22, 23)
277 associated with MDSC induction (7, 24). A possible explanation for the diminished suppressive
278 activity of *Cftr*^{-/-} neutrophilic MDSCs could be a scenario where MDSCs may lose their
279 suppressive function via inflammasome activity, as discussed by Koehn et al. (25).

280 Monocytic MDSC recruitment was also not affected by *Cftr* deficiency, yet the total
281 amount of cells in bone marrow of *Cftr*^{-/-} mice was diminished compared to *Cftr*^{+/+} littermates,
282 leading to a decreased net total amount of monocytic MDSCs in bone marrow. In-depth
283 characterization of this phenomenon was, however, beyond the scope of this study, as the focus
284 lied mainly on neutrophilic MDSCs, the major CF-relevant MDSC population (7).

285 Inspired by previous findings from our group that adoptive transfer of neutrophilic
286 MDSCs was protective in an invasive/systemic *in vivo* fungal infection model (11), we tested the
287 potential of adoptively transferred neutrophilic MDSCs to affect the outcome of *P. aeruginosa*
288 infection *in vivo*. However, in our bacterial infection model, the adoptive transfer of neutrophilic
289 MDSCs did not affect the weight loss and/or weight recovery of *P. aeruginosa* infected mice and
290 the bacterial clearance at d5 or d6 p.i.. Based on these findings, we speculate that neutrophilic
291 MDSCs have a more substantial impact on invasive/systemic fungal infections compared to
292 compartmentalized *P. aeruginosa* airway infections *in vivo*. The precise mechanisms underlying
293 this difference remain to be defined, but may relate to the fact that in invasive/systemic fungal
294 infections the protective effect of MDSCs was conferred by MDSCs adoptively transferred i.v.,
295 the same route as fungal pathogens were inoculated, whereas in contrast in the case of *P.*
296 *aeruginosa* airway infections, bacteria were inoculated topically into the airways (intranasally)
297 while MDSCs were adoptively transferred i.v.. Consequently, in systemic fungal infections both
298 fungal pathogens and MDSCs were in close proximities, whereas in the case of *P. aeruginosa*
299 airway infections, MDSCs would have to actively enter the airway compartment from the
300 circulation.

301 Our study has several limitations. (i) We were using an acute *P. aeruginosa* infection model for
302 our proof-of-concept MDSC studies; yet a chronic infection/ colonization model, which is much
303 harder to standardize and read-out, would resemble the situation of CF patients more closely; a
304 task for future investigations; (ii) our studies were unable to identify the molecular mechanisms
305 by which *P. aeruginosa*/*P. aeruginosa*-derived factors enhance neutrophilic MDSC activity.
306 Preliminary studies failed to demonstrate significant involvements of ROS and IL-10 (data not
307 shown), issues requiring further immunological and biochemical studies; (iii) lymphocytes
308 express *Cftr*, suggesting that *Cftr* in T cells could affect the functional outcome of MDSC-Tcell
309 interactions in CF lung disease, though preliminary data from our group showed no significant
310 impact of T cellular *Cftr* on MDSC-T cell studies (data not shown); (iv) we observed in our
311 suppression assays that beyond CD4⁺ cells, also non-CD4⁺ cells in the fraction of lymphocytes
312 were proliferating. While we have no clear explanation for this observation and its
313 immunological relevance, we speculate that these non-CD4⁺ T cells may arise due to the high
314 stimulation trigger during our culture conditions. As the population consistently appeared
315 throughout the experiments, we expect no substantial influence on the outcome of our respective
316 read-out studies; (v) data and knowledge on the dynamics and kinetics of adoptively transferred
317 MDSCs *in vivo* are limited. Preliminary investigations showed that neutrophilic MDSCs reach
318 lungs of mice within 24 h after lateral tail vein injection (data not shown). It is further known
319 from previous studies that adoptively transferred MDSCs can be recovered from splenocytes 5
320 days post injection (25), yet studies focusing on the kinetics and dynamics of adoptively

321 transferred MDSCs in the pulmonary compartment are lacking to the best of our current
322 knowledge; (vi) Our adoptive transfer study lacks further in-depth readouts, such as lung
323 inflammation parameters, amounts and characteristics of parenchymal T cells or kinetics on
324 pathogen clearance. Since our MDSC adoptive transfer studies did not reveal any impact of
325 MDSCs on morbidity, we did restrict our read-outs to the minimum. Further studies are required
326 to dissect the temporal and spatial *in vivo* kinetics and dynamics of MDSCs and T cells in the
327 infected pulmonary compartment.

328

329 Collectively, when viewed in combination, this study demonstrates that acute *P. aeruginosa*
330 airway infection recruits and functionally modulates neutrophilic myeloid-derived suppressor
331 cells and thereby defines a mechanism by which *P. aeruginosa* undermines host immunity *in*
332 *vivo* by modulating neutrophilic MDSCs.

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335

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421

422

423 **Author contributions**

424 H.Ö., B.Z., C.S. and P.V. performed *in vitro* and *in vivo* experiments. C.S. contributed to
425 reagents and analysis tools. M.C. and N.F. performed *in vivo* experiments. D.H., A.H., H.Ö., and
426 N.R. designed the study, supervised experiments and wrote the manuscript. H.Ö. and D.H.
427 analyzed the data.

428

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Competing Interests

434 The authors have declared that no competing interests exist.

435

436

437 **Fig 1 *Pseudomonas aeruginosa* airway infection recruits MDSCs**

438 (A) Neutrophilic (PMN-) MDSCs and monocytic (M-) MDSCs in BALF, lung, bone marrow and
439 spleen were sequentially gated based on FSC/SSC, CD11b, Ly6G and Ly6C. PMN-MDSCs were
440 assessed as CD11b⁺Ly6G⁺Ly6C^{intermediate}, M-MDSCs as CD11b⁺Ly6C⁺Ly6G^{low}.

441 (B) Left panel: Gating strategy to assess CD4⁺ T cell proliferation using CFSE staining and flow
442 cytometry. CD4⁺ cells were gated from the lymphocyte population in FSC/SSC and further gated
443 for CD4⁺. T-cell proliferation was assessed based on the CFSE fluorescence of CD4⁺ T cells.

444 Right panel: Representative histograms of co-cultured PMN-MDSCs isolated from BALF of
445 PAO-1 infected mice and T cells at ratios 1:1, 1:2, 1:4 and 1:8 as indicated.

446 (C) Ratio-dependent (suppressed) proliferation of CD4⁺ T cells by co-culturing with
447 neutrophilic MDSCs isolated from BALF of *P. aeruginosa*-infected mice is shown. The values
448 are normalized to the proliferation of activated T cells without addition of MDSCs (T cells only)
449 as indicated as proliferation index.

450 (D) Percentages (top row) and total amounts (bottom row) of neutrophilic MDSCs in BALF,
451 lung, bone marrow and spleen in acute *P. aeruginosa* infection compared to non-infected control
452 animals. Percentages of neutrophilic MDSCs (PMN-MDSCs) were acquired as % of
453 CD11b⁺Ly6G⁺Ly6C^{intermediate} cells of viable cells (see Fig 1A). Total cell amounts were
454 calculated from cell counts of single cell suspensions from isolated organs/tissues/fluids prior to
455 FACS staining. ** $p < 0.01$

456 (E) Percentages (top row) and total amounts (bottom row) of monocytic MDSCs in BALF, lung,
457 bone marrow and spleen in acute *P. aeruginosa* infection compared to non-infected control
458 animals. Percentages of monocytic MDSCs (M-MDSCs) were acquired as % of
459 CD11b⁺Ly6C⁺Ly6G^{low} cells of viable cells (see Fig 1A). Total cell amounts were calculated

460 from cell counts of single cell suspensions from isolated organs/tissues/fluids prior to FACS
461 staining. ** $p < 0.01$

462 **Fig 2 *Pseudomonas aeruginosa* airway infection functionally modulates MDSCs**

463 After *P. aeruginosa* airway infection CD11b⁺Ly6G⁺ neutrophilic MDSCs were isolated from the
464 lung, the bone marrow or the spleens and tested for their potential to suppress polyclonal T cell
465 proliferation. T-cell suppression assays were performed as described previously (11) using the
466 CFSE method. In brief, isolated CD11b⁺Ly6G⁺ neutrophilic MDSCs were co-cultured for three
467 days (37°C, 5% CO₂) with T cells (CD4⁺ splenocytes) at MDSC : T cell ratios 1:1, 1:2, 1:4 and
468 1:8. T cells were activated with CD3/CD28-beads and recombinant mouse IL-2. CFSE-
469 fluorescence intensity was analyzed by flow cytometry to determine polyclonal T-cell
470 proliferation.

471 (A) Strategy to assess the effect of acute *P. aeruginosa* infection on MDSC-mediated T-cell
472 suppression *ex vivo*. CD4⁺ T cells were isolated by MACS from splenocytes of C57Bl6/J mice
473 and stained with CFSE. Ly6G⁺ cells were isolated from lungs, bone marrow and spleens of
474 infected and control C57Bl6/J mice. The isolated cells were co-cultured under T-cell
475 proliferative conditions (with CD3-CD28-coupled beads and rIL-2), collected after 3 days
476 incubation at 37°C 5% CO₂, stained for CD4 and analyzed by flow cytometry.

477 (B) Representative histograms showing CFSE stained control CD4⁺ T-cell proliferation (top) and
478 the suppressed proliferation caused by co-culture with isolated neutrophilic MDSCs (see Fig 2A)
479 from spleens of PBS treated (left) and *P. aeruginosa* infected (right) C57Bl6/J mice with
480 decreasing PMN-MDSC : T-cell ratios (1:1, 1:2, 1:4 and 1:8). Each peak represents one cycle of
481 cell division.

482 (C) Graph showing the proliferation of T cells under T-cell proliferative conditions when co-
483 cultured with suppressive neutrophilic MDSCs isolated from bone marrow, spleens and lungs of
484 PBS treated or PAO1 infected C57BL6/J mice (see Fig 2A). T-cell proliferation was assessed
485 based on CFSE fluorescence of CD4⁺ T cells. (see Fig1B). Shown are MDSC : T-cell ratios of
486 1:1 and 1:2. The values are normalized to the proliferation of activated T cells without addition
487 of MDSCs (T cells only), indicated as proliferation index. * $p < 0.05$

488 **Fig 3 Adoptive transfer of neutrophilic MDSCs has no impact on weight loss and weight**
489 **recovery in acute *Pseudomonas* infection**

490 For adoptive transfer experiments, CD11b⁺Ly6G⁺ neutrophilic MDSCs were isolated from the
491 bone marrow of wildtype mice by MACS. Transfer was performed by transferring 8-10x10⁶
492 neutrophilic MDSCs per animal into wildtype mice via lateral tail vein injection. 24 hours after
493 the neutrophilic MDSC transfer, mice were infected with doses of 2x10⁶ (A) or 4x10⁶ (B) CFU *P.*
494 *aeruginosa* (PAO1) as described above in detail and weight loss was monitored.

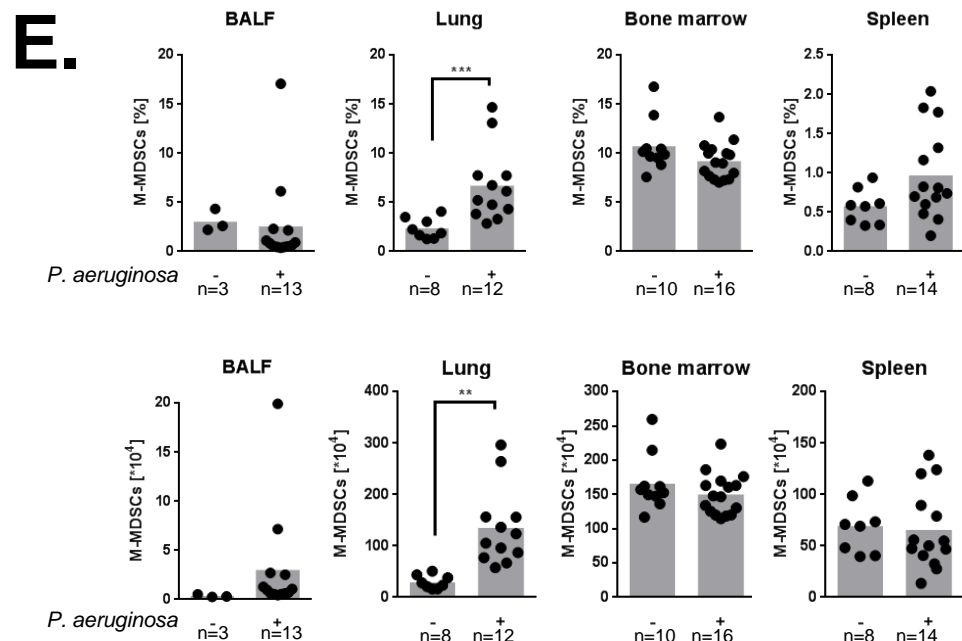
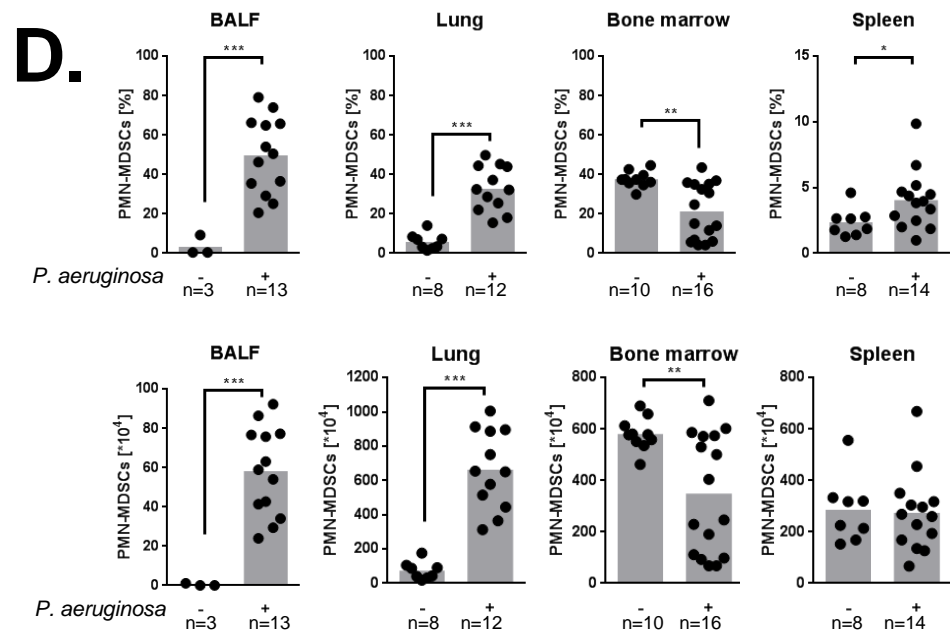
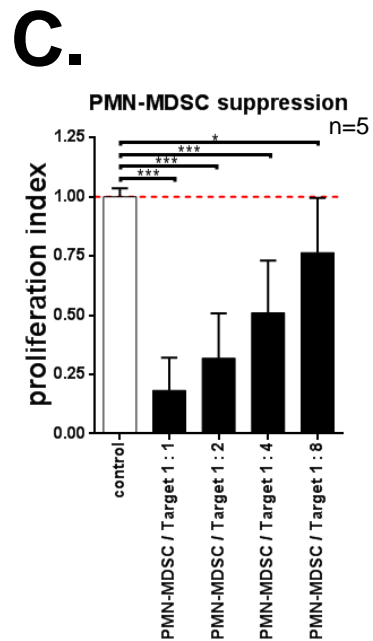
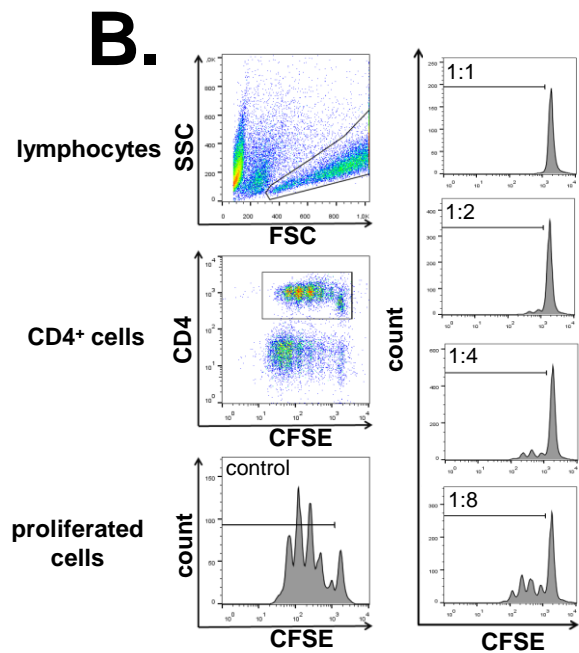
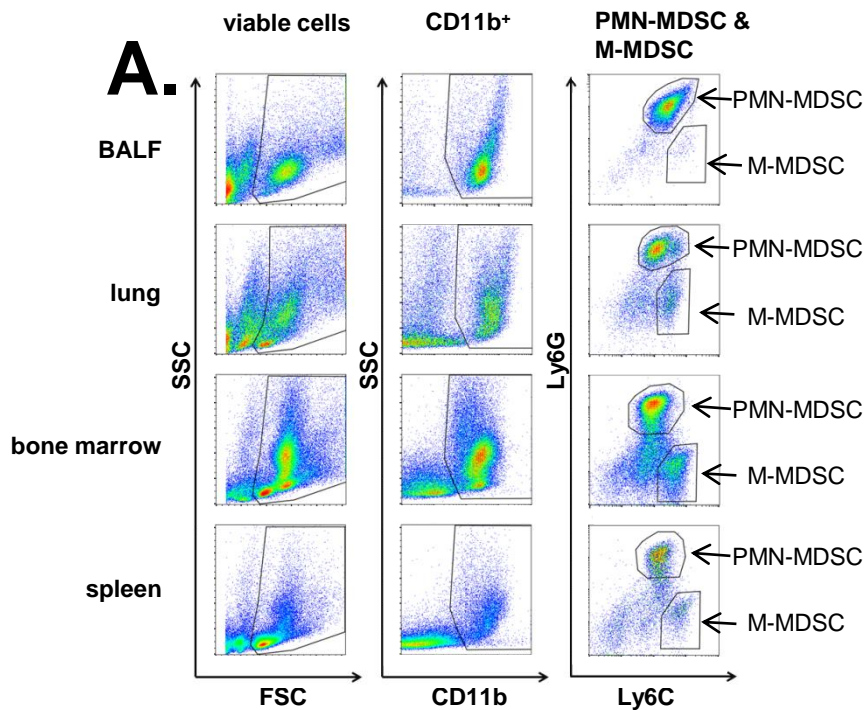
495 **Fig 4 Role of CFTR in MDSC functionality**

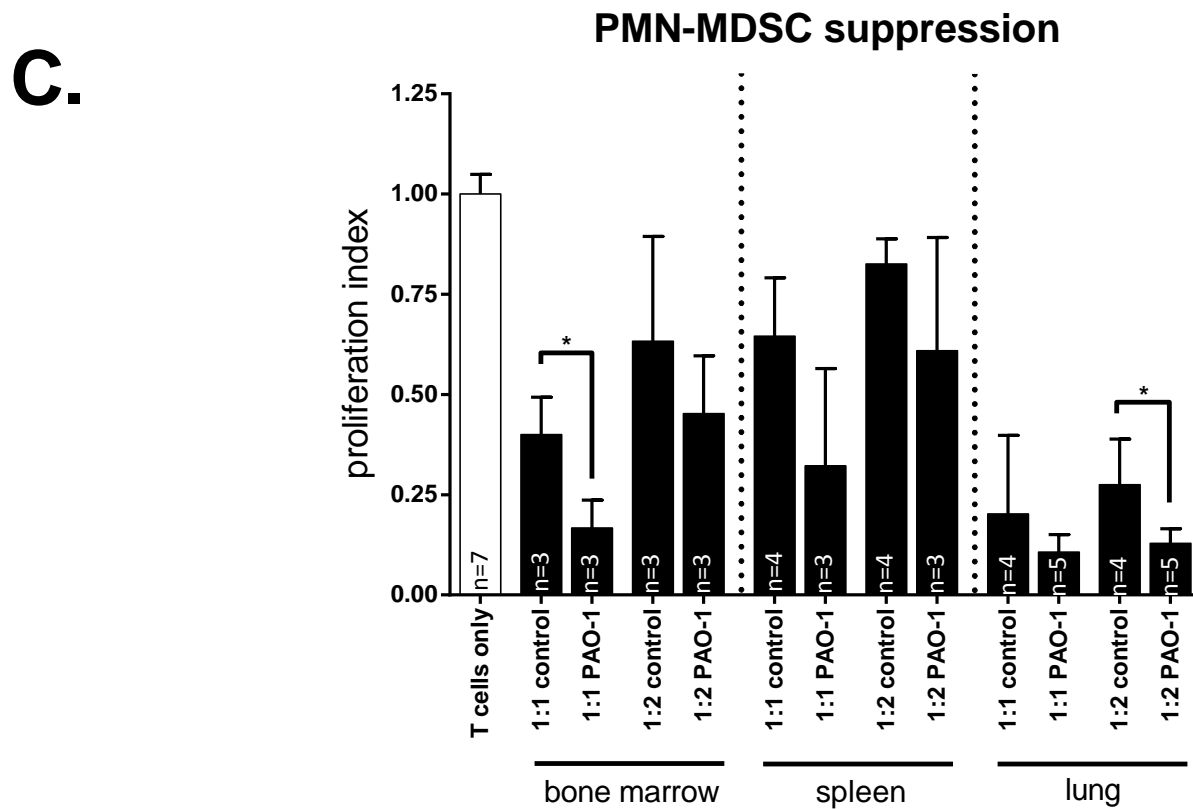
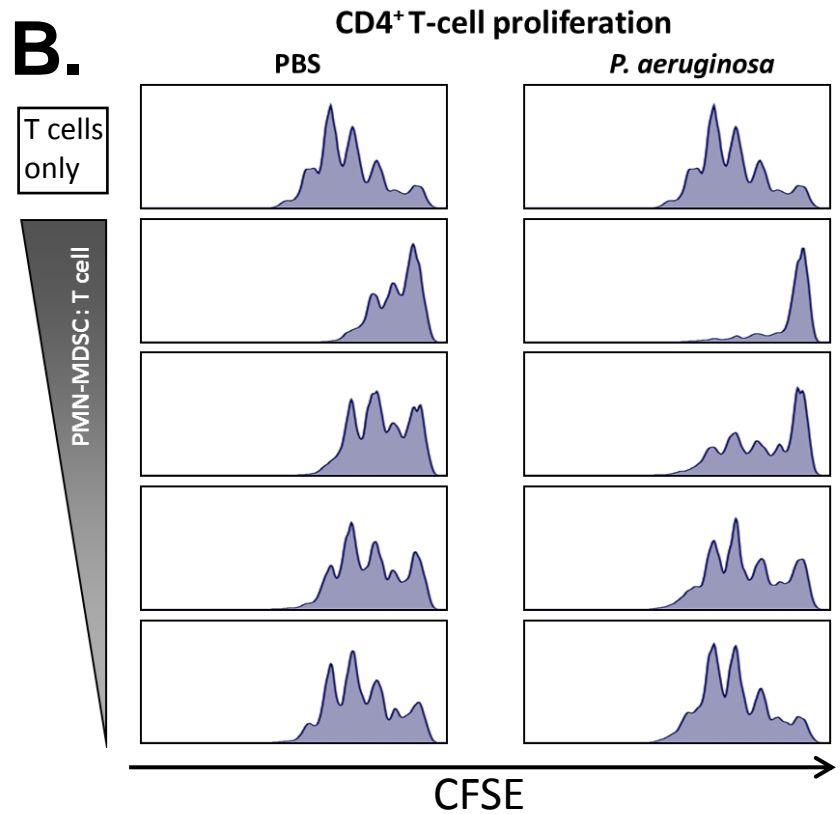
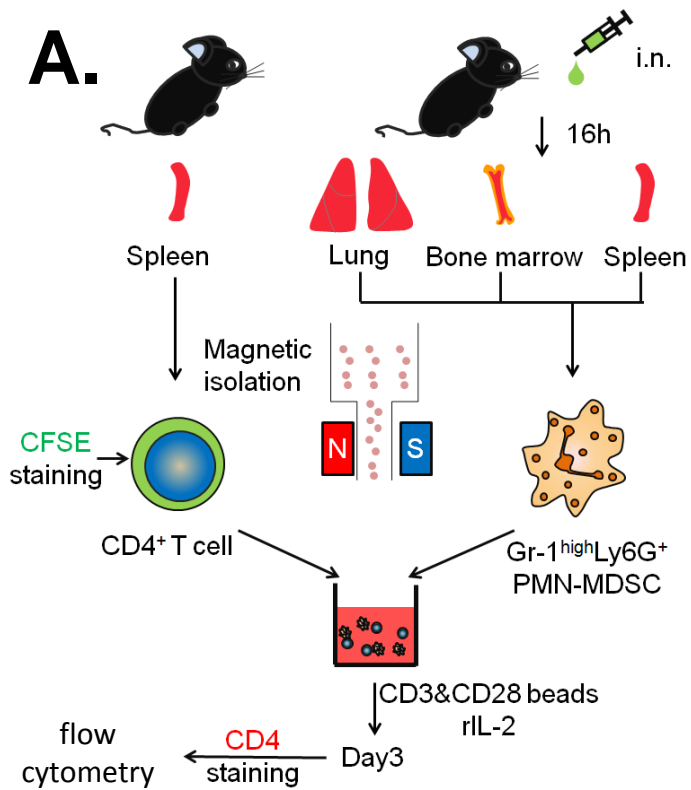
496 Neutrophilic MDSCs were isolated from 6 days *in vitro* expanded splenocytes in supplemented
497 RPMI and GM-CSF and IL-6 of *Cftr*^{+/+} and *Cftr*^{-/-} mice and tested for their potential to suppress
498 polyclonal T cell proliferation. T-cell suppression assays were performed as described previously
499 (11) using the CFSE method. In brief, bone marrow isolated *Cftr*^{+/+} or *Cftr*^{-/-} CD11b⁺Ly6G⁺
500 neutrophilic MDSCs were co-cultured for three days (37°C, 5% CO₂) with T cells (CD4⁺
501 splenocytes) at MDSC : T cell ratios 1:1, 1:2, 1:4 and 1:8. T cells were activated with
502 CD3/CD28-beads and recombinant mouse IL-2. CFSE-fluorescence of CD4⁺ T cells was
503 analyzed by flow cytometry to determine polyclonal T- cell proliferation. The values are

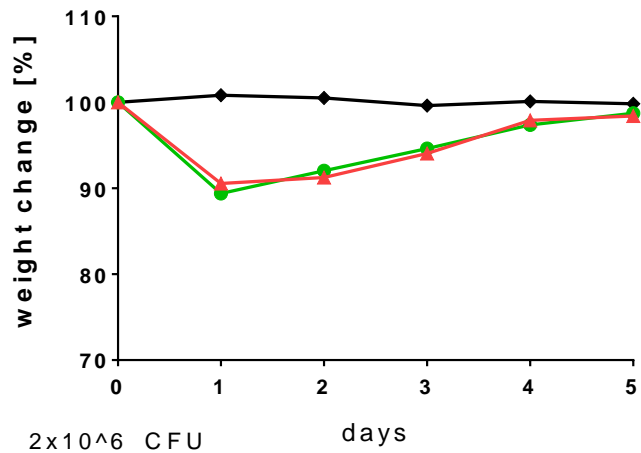
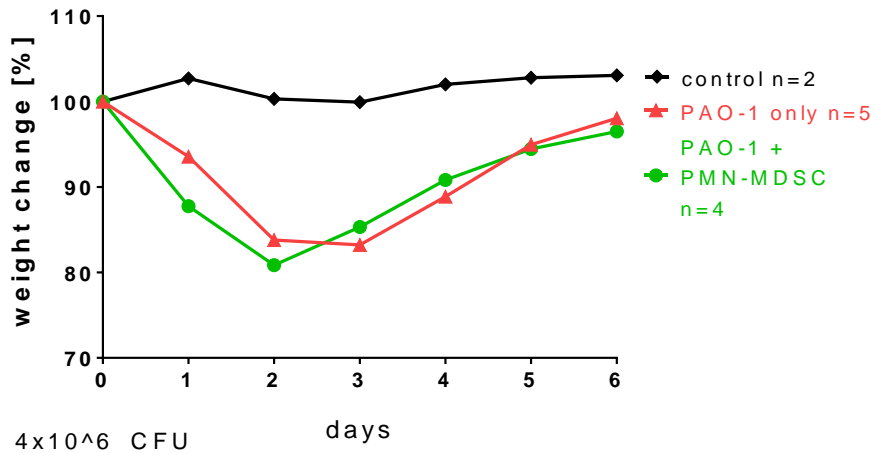
504 normalized to the proliferation of activated T cells without addition of MDSCs (T cells only),

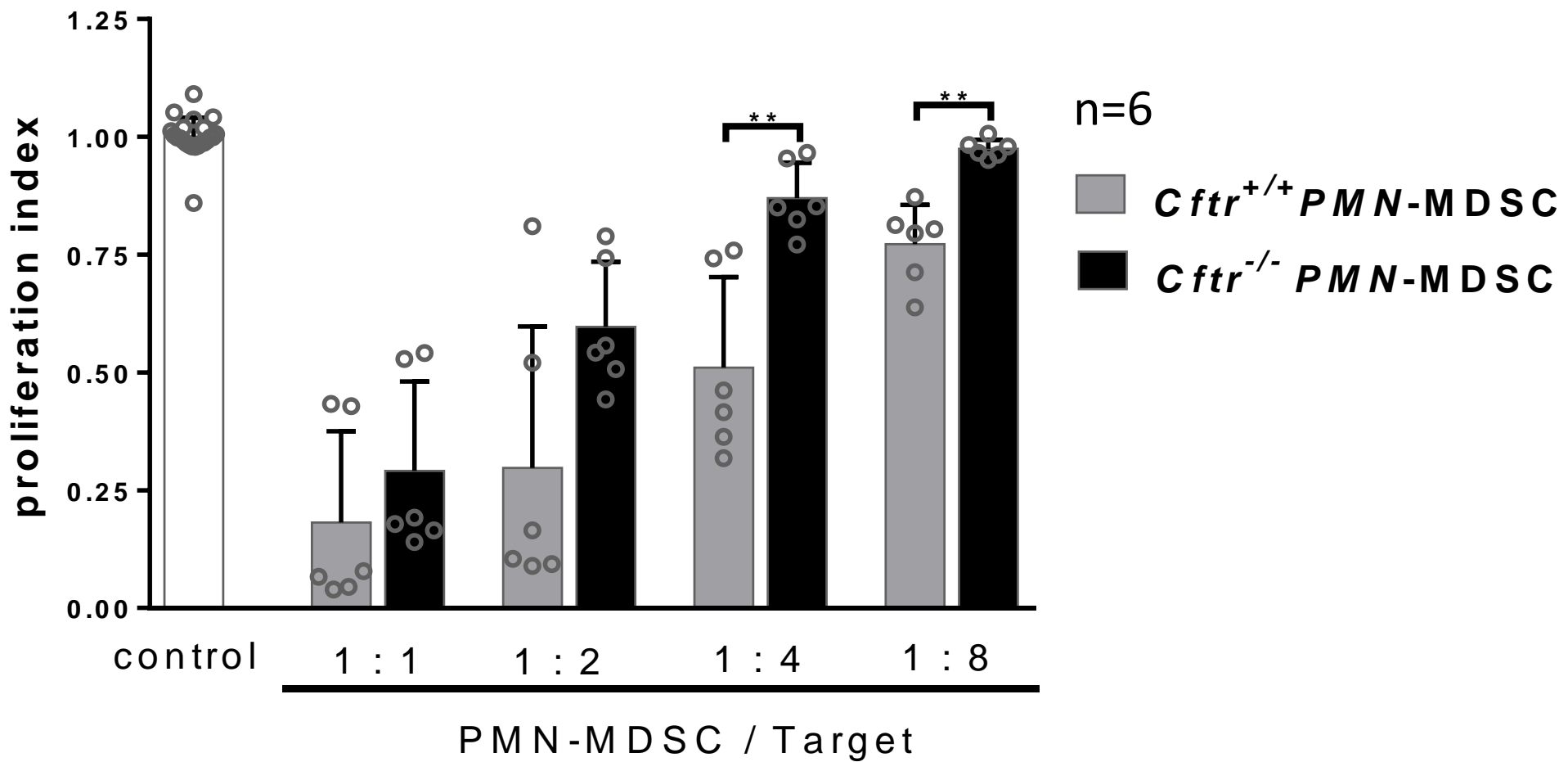
505 indicated as proliferation index. ** $p < 0.01$

506





A.**B.**



Supp Figure 1

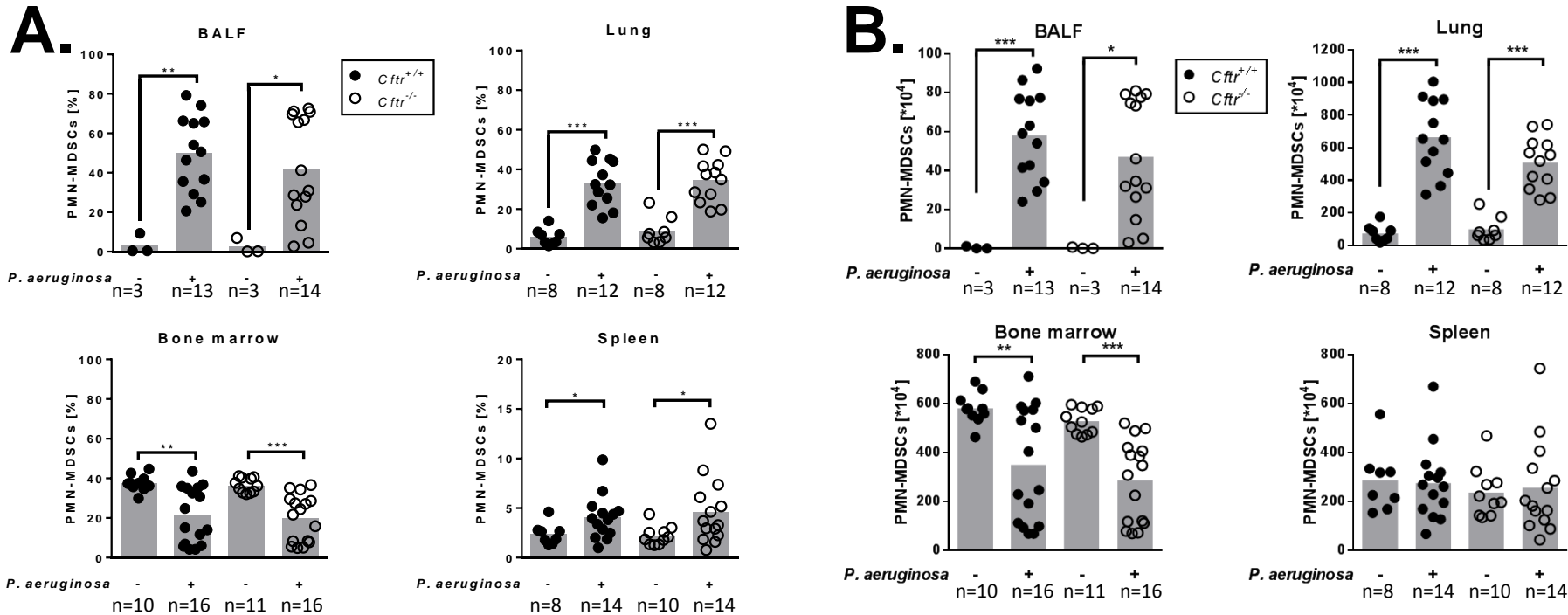


Fig S1 Role of CFTR in PMN-MDSC recruitment

Percentages (A) and total cell amounts (B) of PMN-MDSCs in BALF, lung, bone marrow and spleen 16h after acute *P. aeruginosa* infection in *Cfr*^{+/+} and *Cfr*^{-/-} mice compared to PBS treated mice. Percentages were acquired as % of Ly6G⁺CD11b⁺Ly6C^{intermediate} cells of viable cells (see Fig 1A). Total cell amounts were calculated from cell counts of single cell suspensions from isolated organs/tissues/fluids prior to FACS staining. Filled circles represent *Cfr*^{+/+} while clear circles represent *Cfr*^{-/-} mice. Each circle represents a biological replicate, n numbers are indicated under each bar. For details see methods. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Supp Figure 2

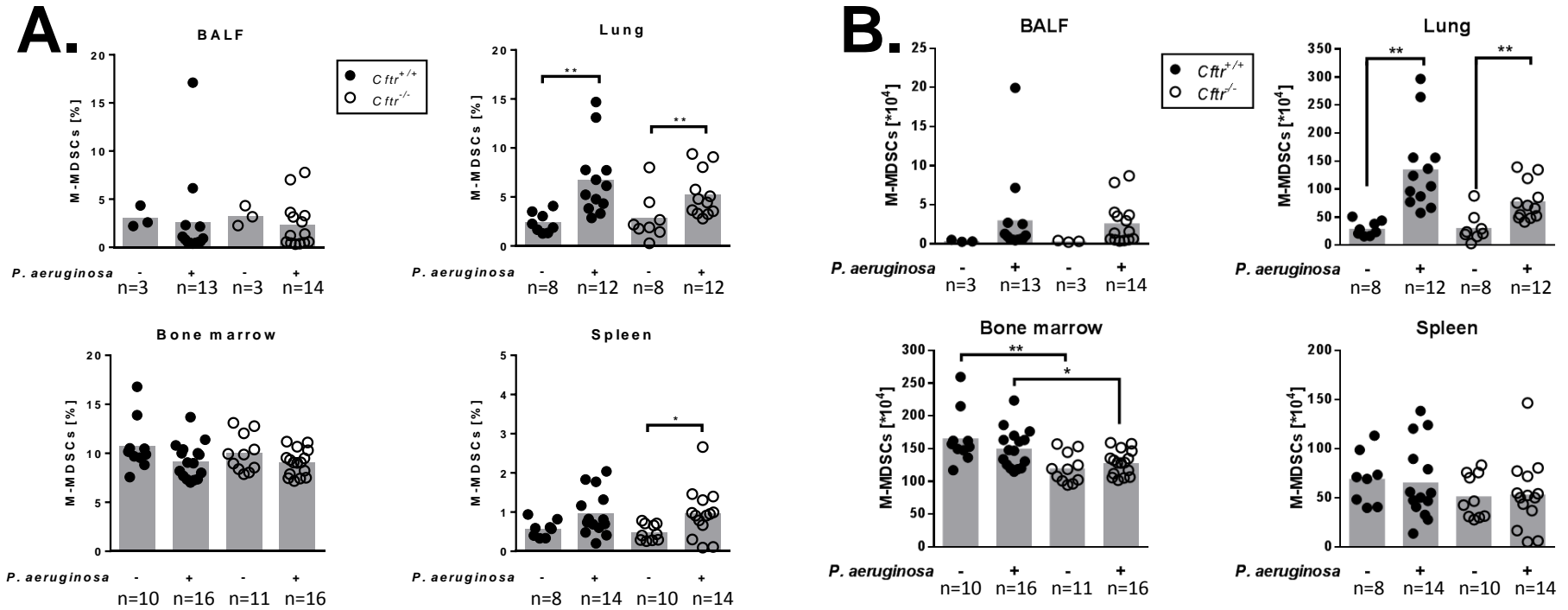
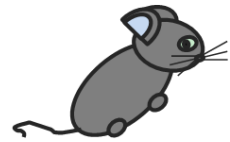


Fig S1 Role of CFTR in M-MDSC recruitment

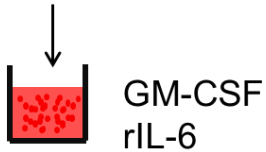
Percentages (A) and total cell amounts (B) of M-MDSCs in BALF, lung, bone marrow and spleen 16h after acute *P. aeruginosa* infection in *Cfrt*^{+/+} and *Cfrt*^{-/-} mice compared to PBS treated mice. Percentages were acquired as % of Ly6C⁺CD11b⁺Ly6G^{low} cells of viable cells (see Fig 1A). Total cell amounts were calculated from cell counts of single cell suspensions from isolated organs/tissues/fluids prior to FACS staining. Filled circles represent *Cfrt*^{+/+} while clear circles represent *Cfrt*^{-/-} mice. Each circle represents a biological replicate, n numbers are indicated under each bar. For details see methods. * $p < 0.05$; ** $p < 0.01$

Supp Figure 3

A.



Bone marrow Spleen



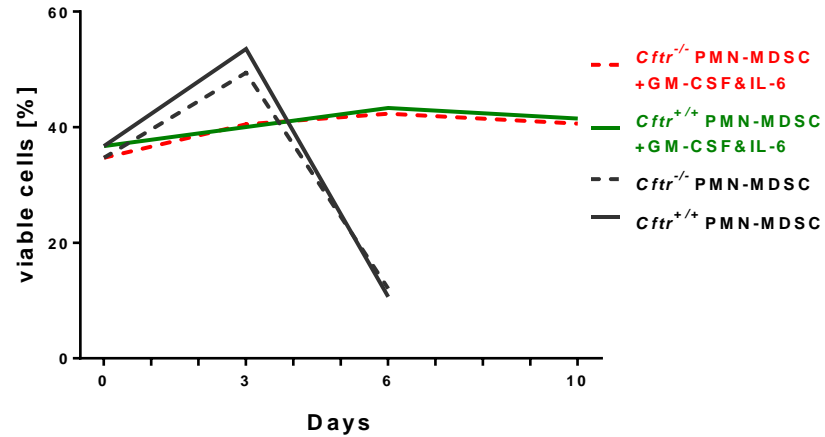
Day 0 Day 3 Day 6 Day 10

CD11b, Ly6G, Ly6C staining

FACS

B.

Bone marrow



Spleen

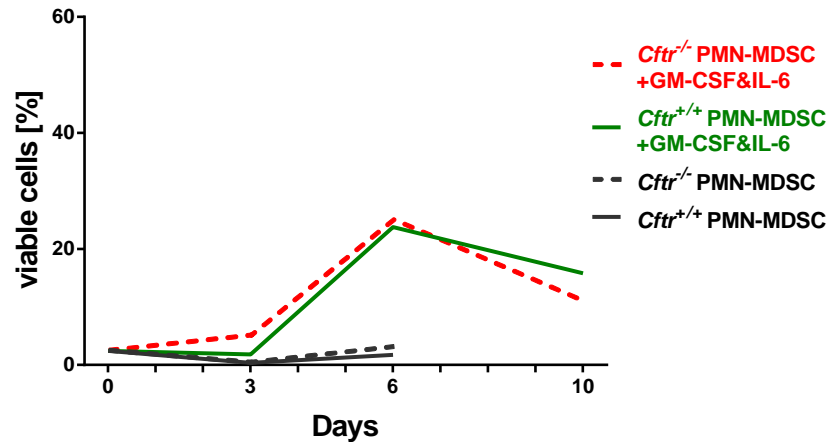


Fig S3 Role of CFTR in MDSC generation

(A) MDSC generation approach. Bone marrow cells and splenocytes were isolated from *Cftr*^{+/+} and *Cftr*^{-/-} mice and cultured in RPMI1640 (Merck Millipore) with supplements (for details see methods section). MDSC generation was induced by addition of 40 ng/ml rmGM-CSF and 40 ng/ml rmIL-6. Cells were either fed with fresh media and cytokines on d3 and d6 or collected and analyzed by flow cytometry at d3, d6 and d10.

(B) Percentages of Ly6G⁺CD11b⁺Ly6C^{im} cells at d0 (n=6), d3 (n=1), d6 (n=6) and d10 (n=3) generated from *in vitro* expanded *Cftr*^{+/+} and *Cftr*^{-/-} bone marrow cells (top) and splenocytes (bottom).

Paper 2

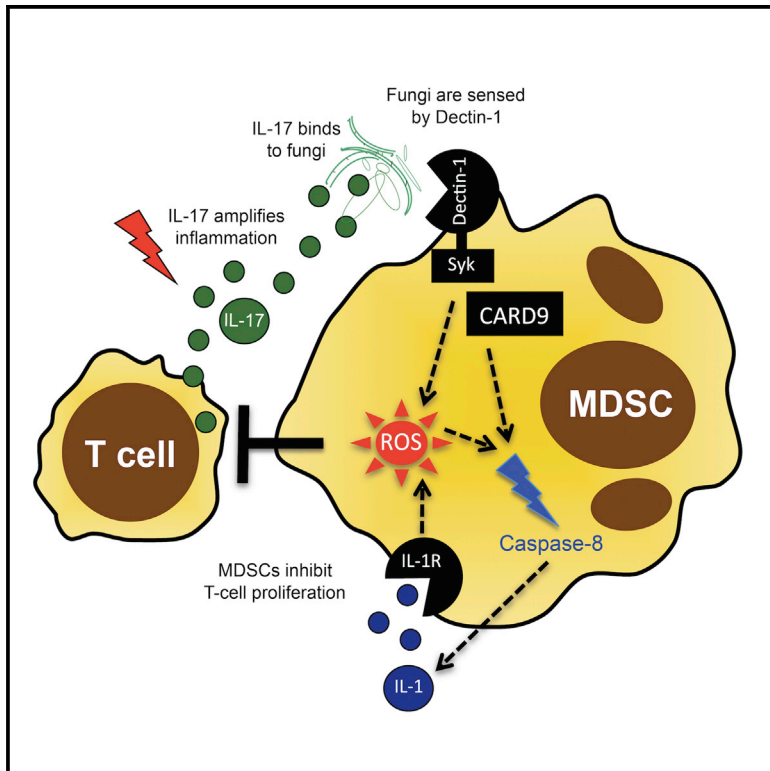
Rieber N, **Singh A**, Öz H, Carevic M, Bouzani M, Amich J, Ost M, Ye Z, Ballbach M, Schäfer I, Mezger M, Klimosch SN, Weber A, Handgretinger R, Krappmann S, Liese J, Engeholm M, Schüle R, Salih HR, Marodi L, Speckmann C, Grimbacher B, Ruland J, Brown GD, Beilhack A, Loeffler J and Hartl D (2015).

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Cell Host & Microbe

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

Graphical Abstract



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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

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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

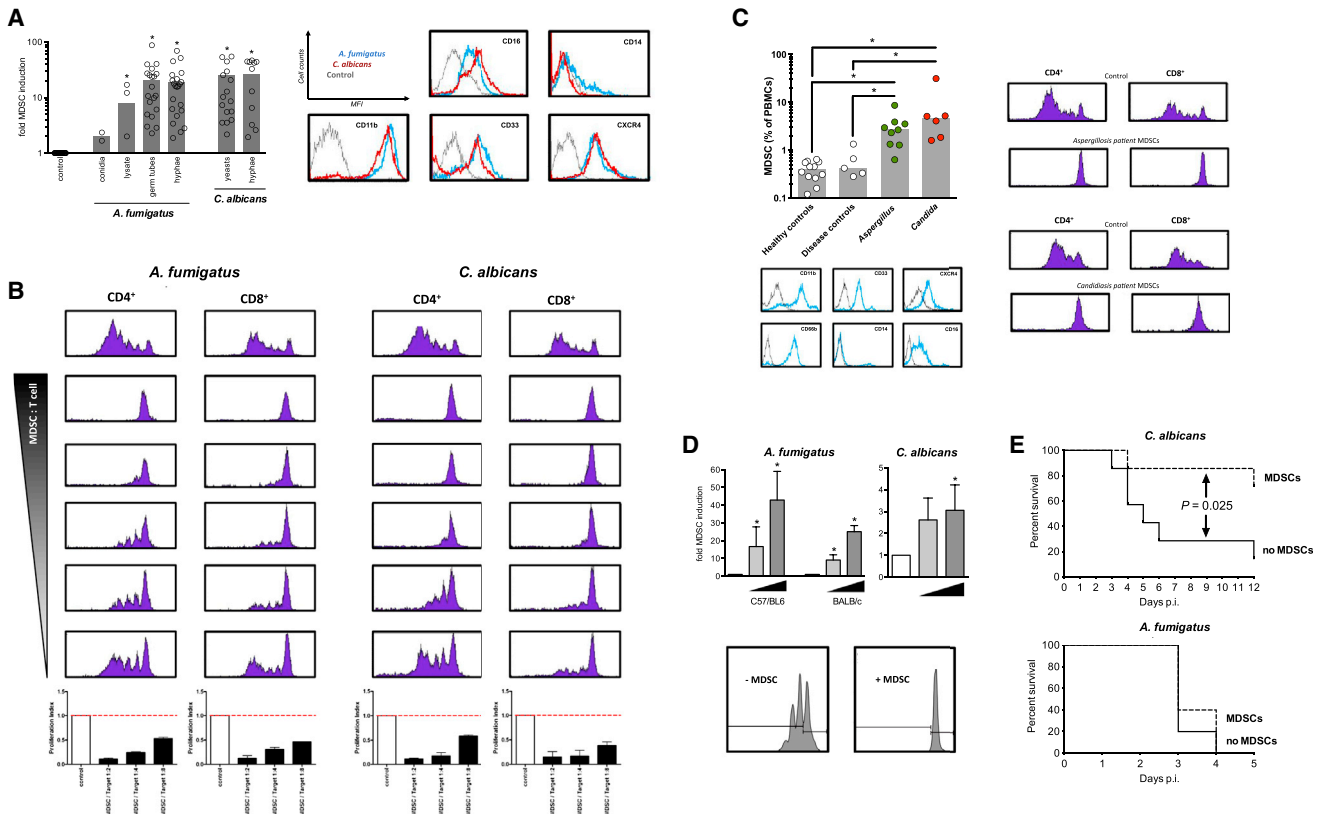


Figure 1. Fungi Induce Functional MDSCs In Vitro and In Vivo

(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.

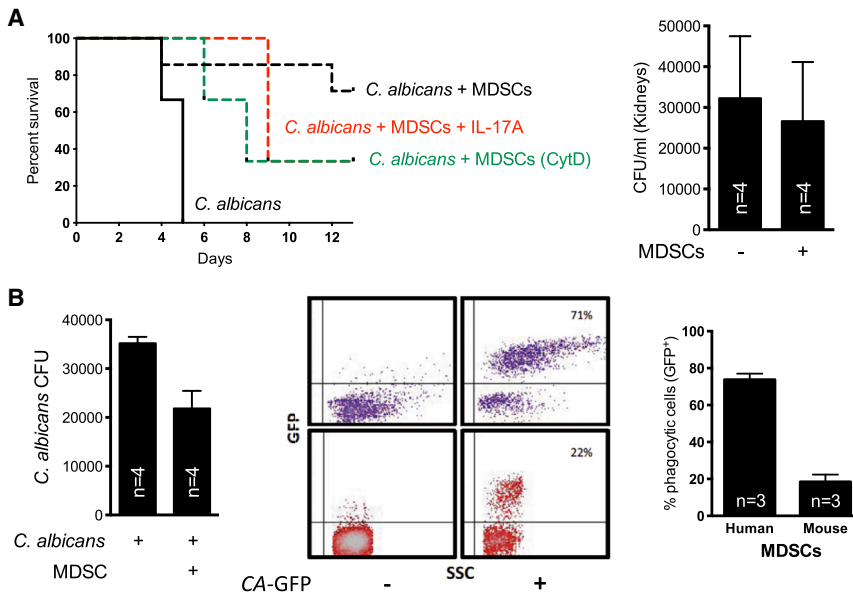


Figure 2. Antifungal Functions

(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 down-regulated 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

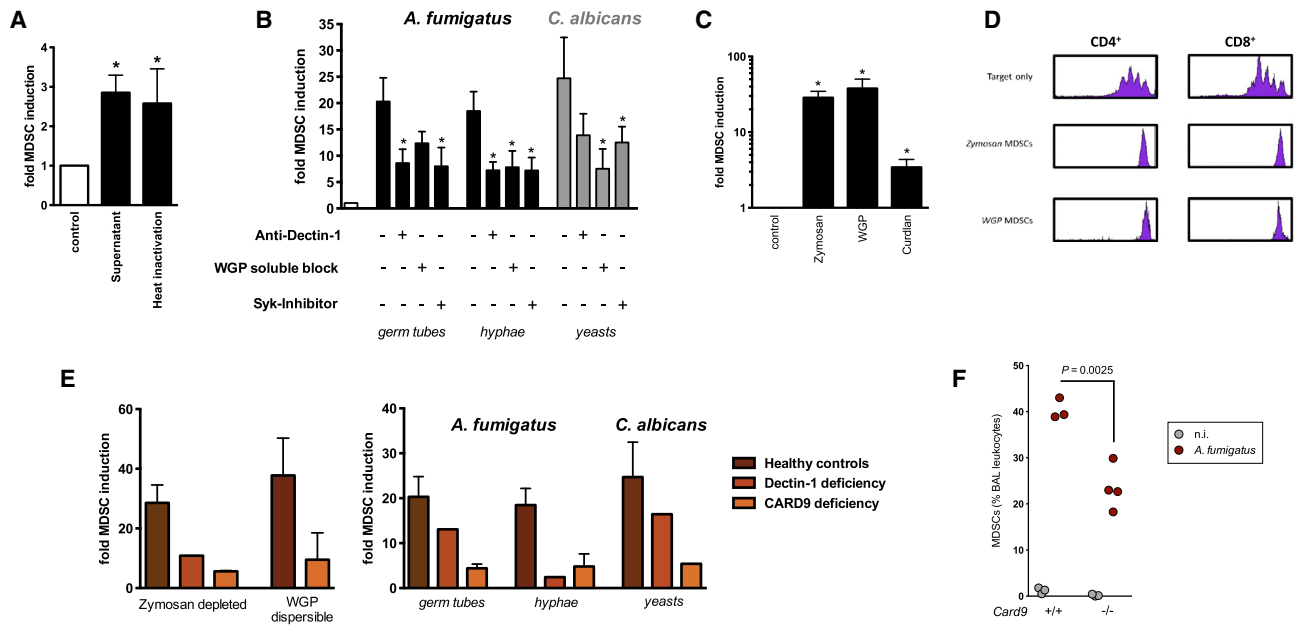


Figure 3. Fungi Induce MDSCs through a Dectin-1-, Syk-, and CARD9-Mediated Mechanism

(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.

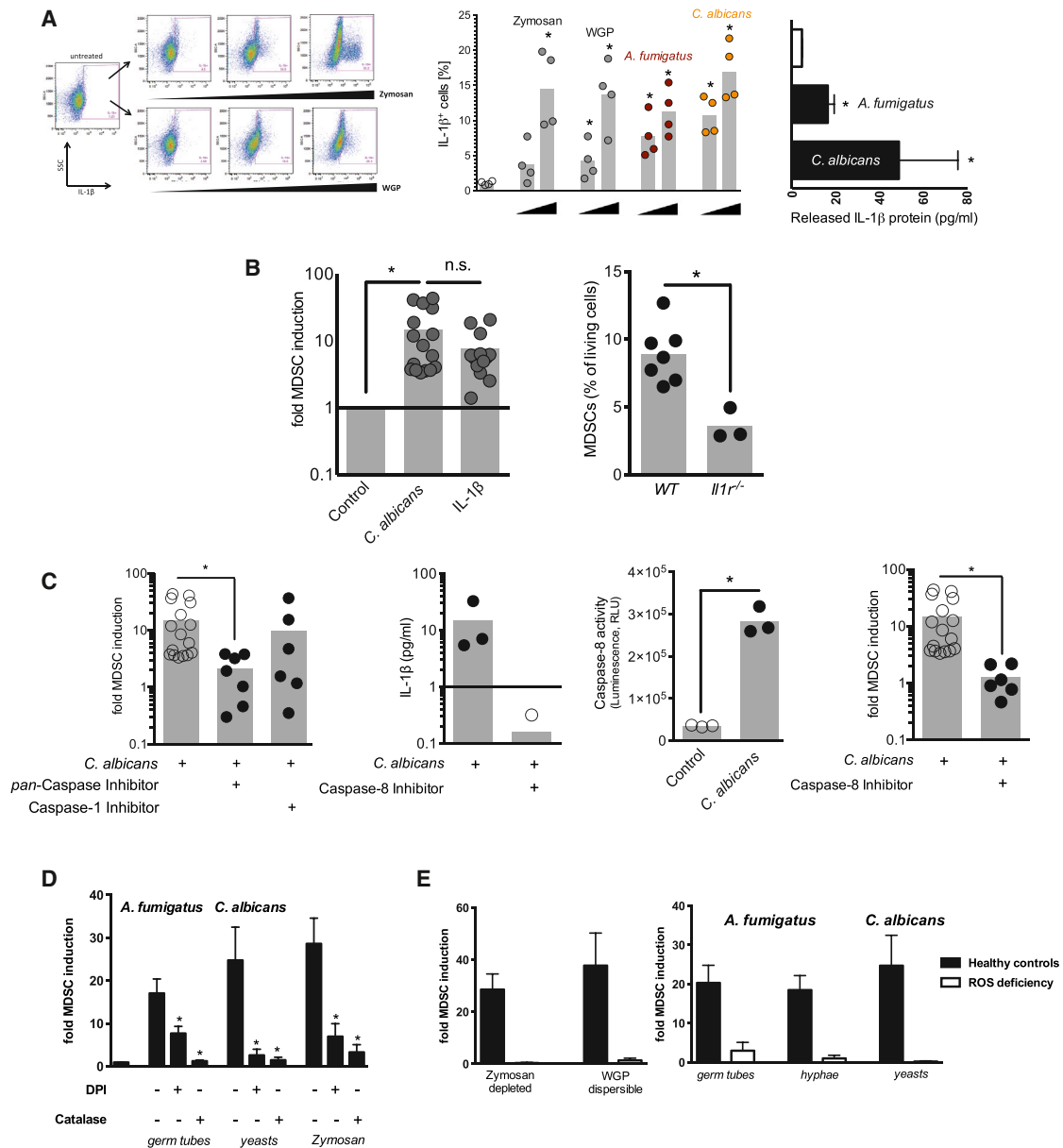


Figure 4. Fungal MDSC Induction Involves IL-1 β , Caspase-8, and ROS

(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 2×10^5 /ml and 1×10^6 /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 *Il1r1*^{-/-} 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.

(legend continued on next page)

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 μ g/ml) for 6 days. PBMCs were pretreated where indicated with the NADPH oxidase inhibitor DPI (0.1 μ M) or the H₂O₂ 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 μ g/ml) or dispersible WGP (20 μ 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

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Supplemental Data

Figure S1, related to Figure 1

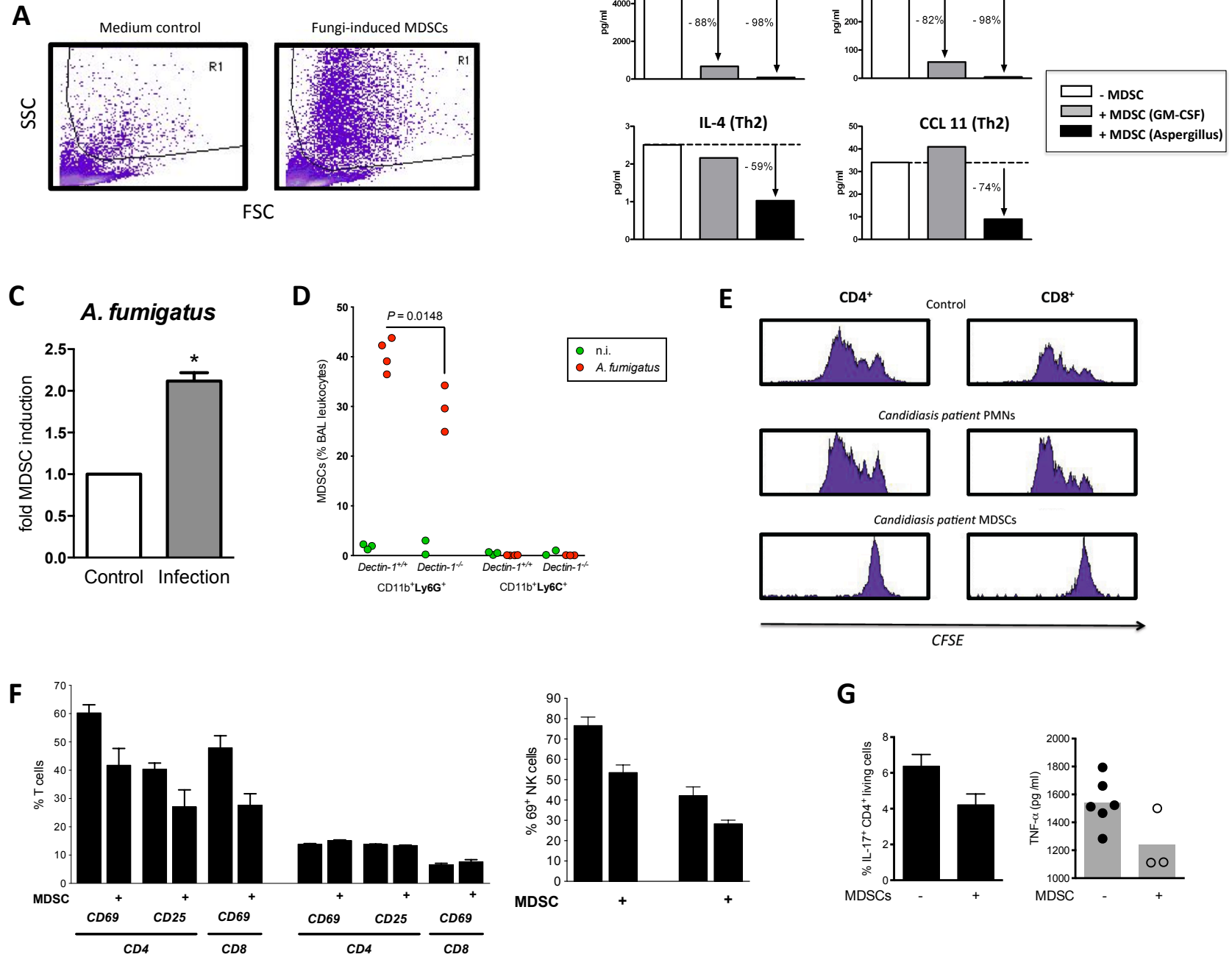


Figure S1. Phenotypic and functional MDSC characteristics

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 gatings 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

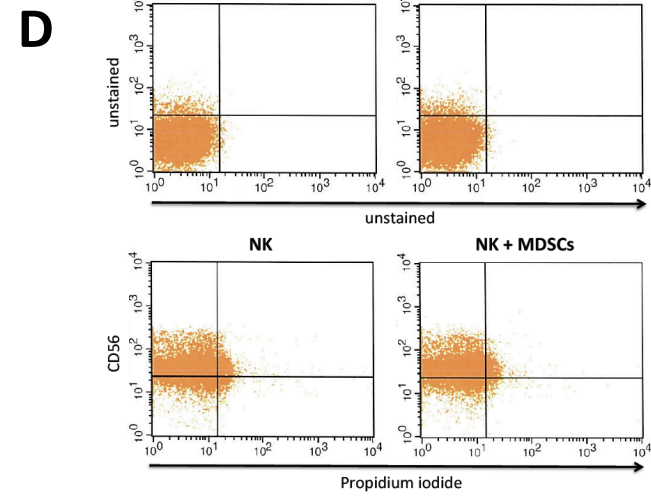
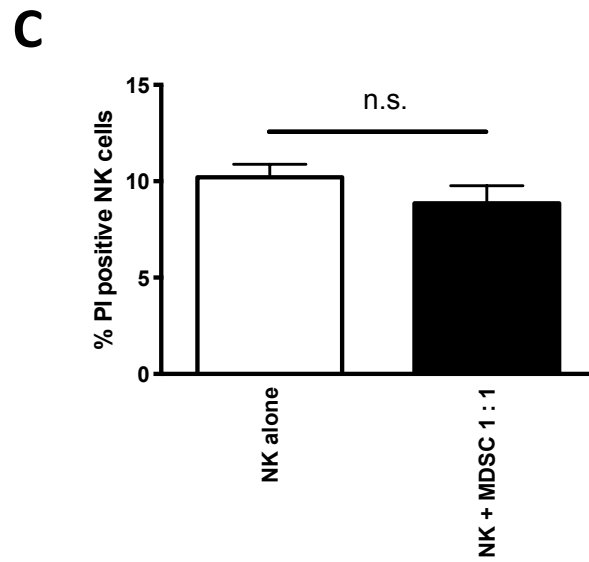
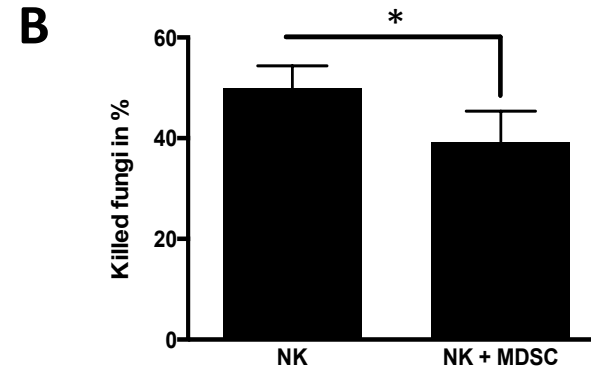
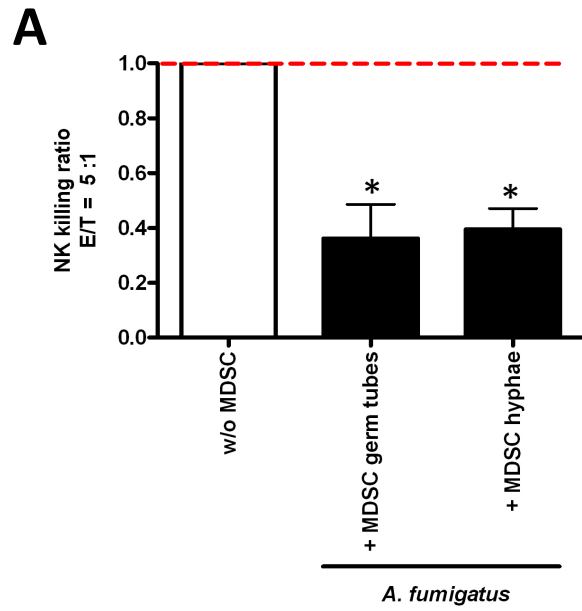


Figure S2: *Aspergillus*-induced MDSCs decrease antifungal NK killing activity

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

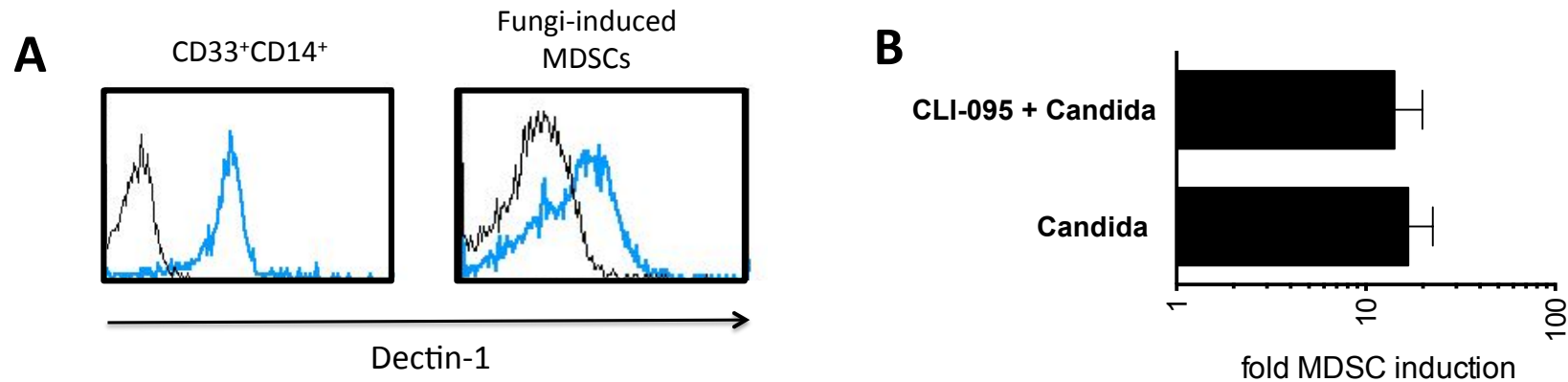


Figure S3: Dectin-1 and TLR4

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

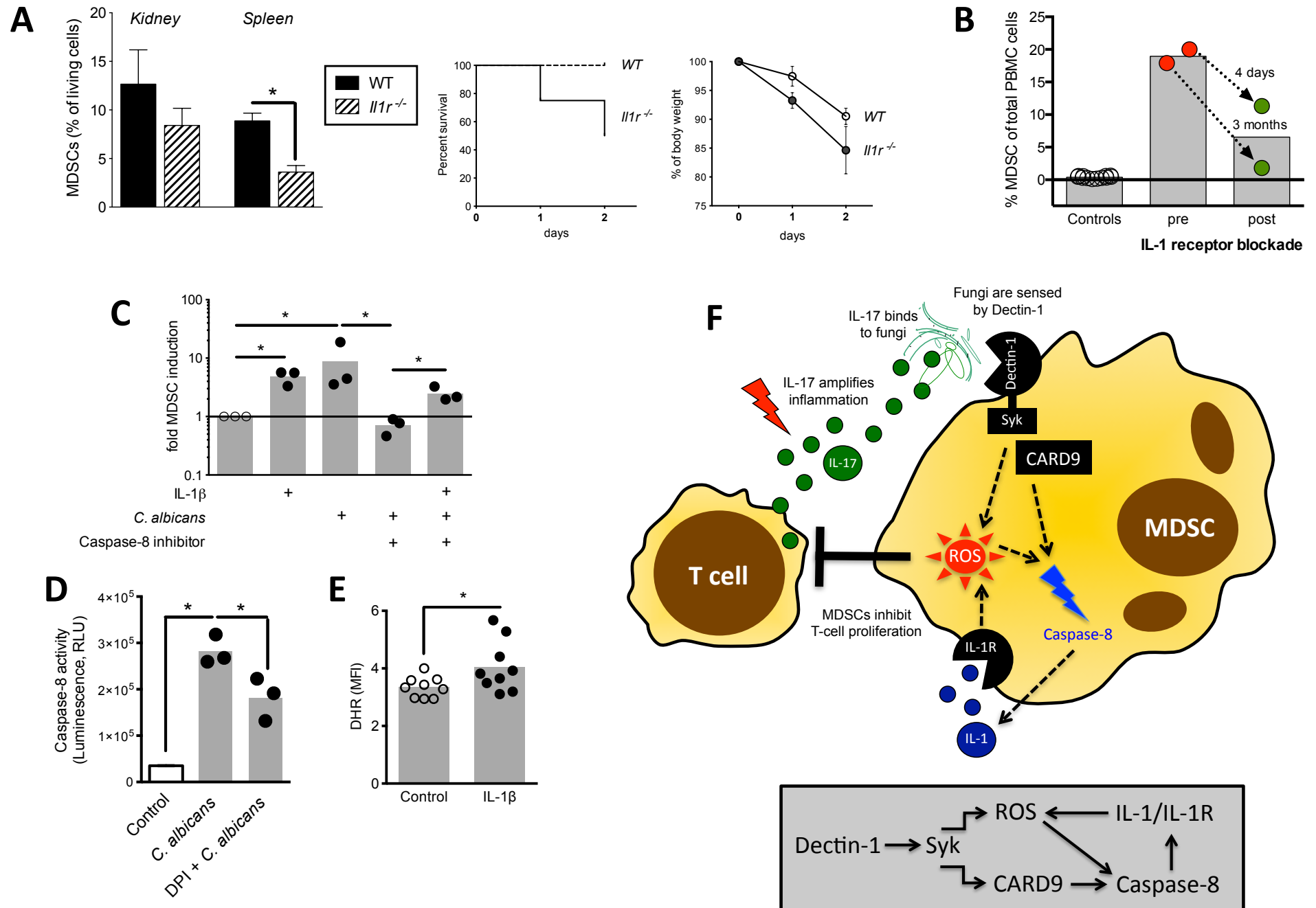


Figure S4: IL-1, Caspase-8 and ROS

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 IL1-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 Cytofix/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 1x10⁴ or 1x10⁶ *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 2.5x10⁵ or 5x10⁵ blastospores per animal in 200 µl 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 4-5x10⁶ 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 1x10⁵ blastospores of *C. albicans* (SC5314 in 100 µl 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 (1µg/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 (5µg 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 1x10³ or 2x10⁵ *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 4x10⁶ 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 3

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The emerging role of myeloid-derived suppressor cells in lung diseases

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ABSTRACT Myeloid-derived suppressor cells (MDSCs) are innate immune cells characterised by their potential to control T-cell responses and to dampen inflammation. While the role of MDSCs in cancer has been studied in depth, our understanding of their relevance for infectious and inflammatory disease conditions has just begun to evolve. Recent studies highlight an emerging and complex role for MDSCs in pulmonary diseases. In this review, we discuss the potential contribution of MDSCs as biomarkers and therapeutic targets in lung diseases, particularly lung cancer, tuberculosis, chronic obstructive pulmonary disease, asthma and cystic fibrosis.



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Myeloid-derived suppressor cells are involved in various lung diseases and represent promising therapeutic targets <http://ow.ly/WKZKh>

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Myeloid-derived suppressor cells

Definition

Suppressive myeloid cells were initially identified more than three decades ago in patients with cancer [1–3]. Later on, these cells were uniformly termed as myeloid-derived suppressor cells (MDSCs) [4] and defined by 1) their myeloid origin and 2) their ability to suppress T-cell responses. Despite a plethora of studies on MDSCs in mice and men, the precise haematopoietic origin and lineage-association still remain a matter of debate [5, 6]. Broadly accepted is the notion that MDSCs stem from immature myeloid cells (IMCs) and can be subdivided into granulocytic/neutrophilic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). Current MDSC concepts suggest that differentiation of IMCs into mature granulocytes, macrophages or dendritic cells (DCs) in bone marrow is skewed towards MDSCs in cancers [7]. While tumour-derived factors, such as granulocyte/macrophage colony-stimulating factor (GM-CSF), have been proposed to induce MDSCs in malignancies, the signals that drive MDSC generation in non-malignant infectious and inflammatory conditions remain incompletely understood [7, 8]. MDSCs are not defined as a single subset of cells, but rather represent a group of phenotypically heterogeneous myeloid cells that share a common biological activity. Human MDSCs have been described to commonly express the myeloid markers CD11b and CD33 as well as CD66b/CD15 for G-MDSCs and CD14 for M-MDSCs [9]. Murine MDSCs express the surface markers CD11b and Gr1 and lack the expression of cell-surface markers that are specific for macrophages or DCs [10]. Sub-phenotyping divides murine MDSCs into monocytic (Ly-6G^{low}CD11b⁺Ly-6C^{high}SSC^{low}) and granulocytic/neutrophilic (Ly-6G^{high}CD11b⁺Ly-6C^{intermed}) subsets [7, 11].

Expansion

MDSCs have been reported to expand in malignant, infectious and autoimmune conditions [7, 8]. The factors driving MDSC expansion comprise a broad variety of pro-inflammatory factors, including interferon (IFN) γ [12–14], cyclooxygenase (COX) 2, stem-cell factor and prostaglandin E (PGE) [15, 16], GM-CSF [17], transforming growth factor (TGF) β [18–21], and interleukin (IL) 1 β [22]. Signal transducer and activator of transcription 3 (STAT3) is regarded as the main transcription factor that regulates the expansion of MDSCs. STAT3 activation increases survival and proliferation of myeloid progenitor cells and regulates MDSC expansion through inducing the expression of S100A8 and S100A9 proteins [23, 24]. The combination of IL-6 and GM-CSF has been identified to generate MDSCs from both human and murine immune cells [25, 26]. Beyond GM-CSF and IL-6, microbial factors have been described to induce MDSCs. Particularly, the opportunistic bacterium *Pseudomonas aeruginosa* was found to potently induce MDSC generation through flagellin [27]. Moreover, fungal infections with *Aspergillus fumigatus* and *Candida albicans* induced a distinct subset of MDSCs through the pattern recognition receptor Dectin-1 and its downstream adaptor protein caspase recruitment domain-containing protein 9, which further involves the generation of reactive oxygen species (ROS) as well as caspase-8 activity and IL-1 production [28]. The retinoblastoma gene was found to regulate M-MDSC differentiation towards G-MDSCs in tumour-bearing mice [29]. Under hypoxic conditions in the tumour microenvironment, MDSCs suppress both antigen-specific and non-specific T-cell activity *via* hypoxia-inducible factor (HIF) 1 α [30]. HIF-1 α also redirects MDSCs differentiation toward tumour-associated macrophages, which further supports the immune-suppressive network in the tumour microenvironment [30]. MDSCs have been reported to have a shorter lifespan in comparison to their counterparts, granulocytes and monocytes, in tumour-free mice mediated by tumour necrosis factor (TNF)-related apoptosis-induced ligand receptors and endoplasmic reticulum stress [31]. LPS and IFN- γ treatment in combination has been shown to trigger the expansion of splenic myeloid precursors into functionally suppressive MDSCs, blocking their development into DCs [32]. In addition, it was reported that G-MDSCs are expanded in neonatal cord blood and efficiently modulate innate and adaptive immune responses by suppressing T and natural killer (NK) cell responses [33]. These neonatal cord blood MDSCs may weaken cellular anti-microbial host defence responses and may contribute to the increased lung infection susceptibility in neonates.

Function

The mechanisms listed below have been implicated in MDSC-mediated suppression of T-cell function:

Reactive oxygen species

ROS have been implicated in MDSC-derived T-cell suppression as common mechanism in neoplastic conditions, inflammation and microbial infections [34]. MDSCs in both tumour-bearing mice and patients with cancer produce ROS and inhibition of ROS production diminished the suppressive effect of MDSCs [35–38]. It has been further shown that ligation of integrins, expressed on MDSCs, contribute to increased ROS production following the interaction of MDSCs with T-cells [39]. Several cytokines, such as TGF- β , IL-6, and GM-CSF, have been described to induce the production of ROS by MDSCs [40].

Arginase, nitric oxide synthase and nitric Oxide

Arginase (ARG) 1 and inducible nitric oxide synthase (iNOS) are involved in L-arginine metabolism. iNOS generates nitric oxide (NO) from L-arginine, and arginase converts L-arginine into urea and L-ornithine. MDSCs express high levels of arginase and iNOS, and utilise these enzymes to deprive arginine and, thereby, inhibit T-cell function [41–43]. The shortage of L-arginine inhibits T-cell proliferation by decreasing T-cellular CD3 ζ expression [44] and inhibiting the upregulation of the cell-cycle regulators cyclin D3 and cyclin-dependent kinase 4 [45]. On the other hand, NO suppresses T-cell function through inhibition of Janus kinase 3 and STAT5 in T-cells [46], inhibition of MHC class II expression [47] and the induction of T-cell apoptosis [48].

Peroxynitrite

Peroxynitrite is one of the most powerful oxidants, and is a product of a chemical reaction between NO and superoxide anion (O₂⁻). Peroxynitrite induces the nitration and nitrosylation of the amino acids cysteine, methionine, tryptophan and tyrosine. Increased levels of peroxynitrite are associated with tumour progression in many types of cancer [49–54], which has been linked with T-cell unresponsiveness.

Induction of regulatory T-cells

MDSCs were found to promote the development of CD4⁺CD25⁺FOXP3⁺ regulatory T-cells (Tregs), an effect that required the activation of tumour-specific T-cells and the presence of IFN- γ and IL-10 [55, 56].

Subset-specific mechanisms:

The two main subsets of MDSC employ different mechanisms to suppress T-cell proliferation. The G-MDSC expresses high levels of ROS and low levels of NO, whereas the M-MDSC conversely expresses low levels of ROS and high levels of NO, while both subsets express arginase [57]. The suppressive activity of the G-MDSCs was shown to be ARG1-dependent, in contrast to the STAT1- and iNOS-dependent mechanism of M-MDSCs [58].

Non-T-cell related mechanisms of suppression

While initially described as merely T-cell suppressive, emerging evidence suggests that MDSCs also interact with and modulate the function of other immune cells, particularly including macrophages [59], NK cells [60, 61], and Tregs [61]. Moreover, MDSCs, tumour-associated macrophages (TAMs) and DCs have been reported to interact and to cross-promote their immunosuppressive activities within the tumour microenvironment [62]. MDSCs in the tumour microenvironment were described to rapidly differentiate into TAMs through a HIF-1 α mediated mechanism [31]. MDSCs, in turn, producing high levels of IL-10, downregulate macrophage IL-12 production, promote TAMs and macrophage M2 polarisation and facilitate the development of Tregs [59, 63]. Furthermore, regulatory DCs (regDCs) have been described in cancer contexts as distinct DC subpopulation, which directly inhibit effector T-cells and indirectly induce or activate Treg cells and MDSCs [64]. Mechanistically, the immunosuppressive effects of regDCs were found to be mediated through IL-10, TGF- β , COX-2, iNOS, arginase and indoleamine 2,3-dioxygenase (IDO) [65–67].

MDSC plasticity/fibrocytic MDSCs

Recent studies suggest that Gr1⁺ myeloid-derived monocytic cells and MDSCs can transdifferentiate into extracellular matrix (collagen type I)-producing fibrocytes, a mechanism involving CD4⁺ T-cells, IL-2, IL-4, IFN- γ and TNF, GM-CSF/G-CSF, Kruppel-like factor 4 and fibroblast-specific protein 1 [68–70]. Fibrocytic MDSCs were found to interact with activated T-cells in a cell contact dependent manner, resulting in the production of IDO and leading to Treg expansion [69]. Fibrocytes can migrate into the tumour stroma microenvironment and further differentiate into myofibroblasts and promote tumorigenesis [71, 72] as well as metastasis [73]. Targeting fibrocytic MDSCs could represent a strategy to prevent the formation of the pre-metastatic niches and subsequently suppress metastasis formation.

Other mechanisms

Less established mechanisms used by MDSCs to suppress immune responses include: 1) upregulation of cyclooxygenase 2 and PGE₂ [16]; 2) secretion of TGF- β [22]; and 3) sequestering cysteine as well as limiting the availability of cysteine, which is an essential amino acid for T-cell activation and proliferation [74]. Several studies demonstrate that the immunosuppressive functions of MDSCs require cell–cell contact, suggesting that MDSCs act through cell-surface receptors and/or the release of short-lived paracrine mediators [7]. MDSCs produce the anti-inflammatory cytokine IL-10 and dampen both CD4⁺ T-cells and NK cell responses [7, 75, 76], while promoting the expansion of Treg [63] and M2-like macrophages [59].

MDSCs in lung diseases

Some of the different lung diseases in which MDCs play a role and the mechanisms that are used are shown if figure 1.

Lung cancer and lung metastasis

T and NK cells are essential for tumour elimination in the lung [77]. Accordingly, factors that regulate their activity are of high interest for lung cancer treatment strategies. Accumulating evidence suggest that MDSCs are responsible for inhibiting host T-cell activity against tumour-associated antigens and consequently impair the effectiveness of anti-cancer immunotherapeutic approaches [78]. MDSCs numbers were found to be associated inversely with responsiveness to chemotherapy and positively with shorter survival in patients with lung cancer [79, 80]. Several studies support the concept that MDSCs dampen T-cells in lung cancer through direct contact and through mechanisms involving a plethora of mediators and mechanisms, such as iNOS, ARG1, TGF- β , IL-10 and the induction of Tregs [55, 75, 81–86]. MDSCs are recruited to the tumour site by the CC chemokine ligand (CCL) 2, CXC chemokine ligand (CXCL) 12, and CXCL5 [87]. The tumour microenvironment stimulates MDSCs to acquire immunosuppressive properties, which are mediated through STAT1, STAT3, STAT6 and nuclear factor κ B transcription factors [7]. Activated MDSCs, in turn, produce ARG1, iNOS2, IDO, NADPH oxidase and immunosuppressive cytokines that have the potential to inhibit cytotoxic T lymphocytes, DC, and NK cells as well as expand CD4⁺CD25⁺FoxP3⁺ Tregs [88, 89].

Many tumour types show an organ tropism of metastatic outgrowth, which was first proposed by Stephen Paget's seed and soil theory in 1889 [90]. Colon cancer, as an example, induces predominantly metastasis in the lung. A decade ago KAPLAN *et al.* [91] first described the concept of the premetastatic niches appearing in the lung as target organ. Signalling factors and cytokines of the primary tumour, *e.g.* vascular endothelial growth factor, placental growth factor, lysyl oxidases, and TNF or TGF- β , lead to the recruitment of immature bone marrow derived cells, which mainly consist of G-MDSCs, and form pre-metastatic niches in organs distinct from the location of the primary tumour. Within the pre-metastatic niche the main drivers for the infiltration of circulating CXC receptor (CXCR) 4⁺ tumour cells are the remodelling of the extracellular matrix by matrix metalloproteinase 9 (MMP9), expression of the adhesion molecule fibronectin, pro-inflammatory S100A8/9 signalling and finally the release of the chemokine stromal cell-derived factor 1, the agonist for CXCR4 [91]. Also hypoxia of the primary breast tumour accompanied by angiogenesis signalling promotes the infiltration of G-MDSCs with potent immunosuppression of NK cells [92].

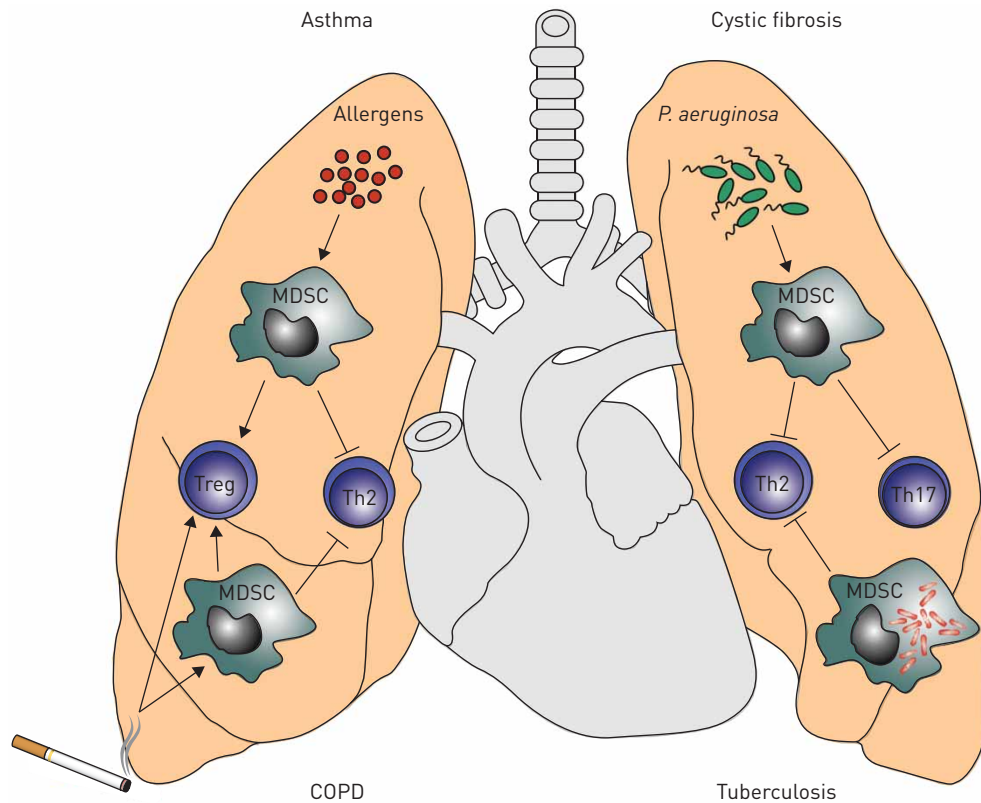


FIGURE 1 The role of myeloid-derived suppressor cells in lung diseases. In response to allergens (asthma), cigarette smoke (chronic obstructive pulmonary disease (COPD)), *P. aeruginosa* bacteria (cystic fibrosis) or *Mycobacterium tuberculosis* bacteria (tuberculosis), myeloid-derived suppressor cells (MDSCs) accumulate in the lungs and cooperate with regulatory T-cells (Tregs) to suppress T-helper type 2 (Th2) cells or Th17-driven inflammation. In tuberculosis, MDSCs phagocytose the mycobacteria and dampen surrounding T-cell responses, thereby supporting immune-evasion.

Data from animal lung cancer models showed that COX-2 is involved in MDSC regulation through the production of PGE₂ and ARG1 [43]. SRIVASTAVA *et al.* [93] demonstrated that Snail, an activating transcription factor in epithelial-mesenchymal transition, induces tumour growth and metastasis by increasing MDSCs *via* increasing intracellular expression of ARG1 in murine lung tumour microenvironment. In other murine models of lung cancer, targeting of MDSCs using antibodies improved antitumor activity *via* enhancing effector and memory T-cell responses, as well as NK cell and antigen-presenting cell activities [94–96]. Peripheral blood MDSCs levels were shown to correlate with a higher tumour burden and a worse prognosis [97–99]. Several approaches have been pursued to eliminate MDSCs in cancer, particularly abrogation of MDSCs using all-trans retinoic acid [100, 101], chemotherapy (gemcitabine, 5-fluoro-uracil) [102, 103], MMP9 inhibition (amino-biphosphonates) [104], MDSC proliferation inhibitors such as tyrosine kinase inhibitors (sunitinib and sorafenib) [97, 105, 106], MDSC recruitment inhibitors (CXCR2 antagonists) [109], MDSC function/activation inhibitors such as COX-2 inhibitors [16, 43, 108] and phosphodiesterase-5 inhibitors (sildenafil) [109]. Another novel approach to abrogate MDSCs, so called “peptibody” treatment showed complete depletion of blood, splenic, and intratumoural G- and M-MDSCs in tumour bearing mice, without affecting pro-inflammatory immune cell types [110]. Based on these studies, the translational and therapeutic potential of targeting MDSCs, in combination with conventional therapies, could be a promising approach for future anti-cancer therapy in human patients. Overall, the development of novel therapeutic agents that eliminate the activity of MDSCs in human lung cancer should accelerate our understanding of their biological role within the tumour microenvironment.

Asthma

CD11b⁺Gr1^{int}F4/80⁺MDSC-like cells were found to accumulate in allergic asthma and to suppress lung DC-mediated reactivation of primed Th2 cells in a toll-like receptor (TLR) 4- and MyD88-dependent fashion, mediated by IL-10 and ARG1 [75, 111]. It has been further shown that tumour-derived MDSCs suppress Th2-dominant inflammation in asthmatic mice, reduce recruitment of inflammatory cells and suppress production of IgE and Th2 cytokines in a TGF-β1 dependent manner [112]. The chemokine CCL2 was found to recruit MDSCs into lung tissues in airway inflammation [112]. Aspirin treatment dampened the accumulation of G-MDSCs in the inflamed lung accompanied by increased Th2 airway responses [113]. It has further been demonstrated that COX and its product, PGE₂, play an important role in the regulation of activation and accumulation of MDSCs through PGE₂ and PGE₄ receptors [113, 114]. MDSCs and IL-10 levels significantly increased and negatively correlated with IL-12 levels during the onset of asthma in both human and mice. IL-10 not only suppressed the production of pro-inflammatory factors by macrophages, but also reduced cytotoxic effects and decreased NO production. Therefore, it is proposed that MDSCs play a dual role in asthma by upregulating anti-inflammatory IL-10 and downregulating pro-inflammatory IL-12 [76]. In a different study, MDSCs enhanced mast cell-mediated secretion of several inflammatory cytokines, including TNF, IL-6, IL-13, macrophage inflammatory protein-1α and monocyte chemoattractant protein-1. The mutual interaction of MDSCs and mast cells enhanced the activities of each cell type, resulting in exacerbated inflammation and airway hyperresponsiveness [115]. Three different populations of CD11b⁺Ly-6G⁺ myeloid cells infiltrated the lung in a mouse model of allergic airway inflammation where they differentially generate the reactive free radicals NO and O₂⁻. The Ly-6C⁺Ly-6G⁻ subset (predominant NO producer) and the Ly-6C⁺Ly-6G⁺ subset were found to suppress T-cell proliferation. In contrast, the O₂⁻ that generates the Ly-6C⁻Ly-6G⁺ subset enhanced T-cell responses [116]. Superoxide-producing myeloid-derived regulatory cells (MDRSCs), present in high numbers in the airways of patients with mild asthma or chronic obstructive pulmonary disease (COPD), but not in healthy controls, were, on the other hand, found to enhance proliferation of CD4⁺ T-cells [117].

COPD

NO producing MDRSCs were found in the airways of patients with mild asthma, but not in COPD patients or healthy control individuals and, were found to suppress activated CD4⁺ T-cells [117]. Smoking upregulated and activated circulating MDSCs in COPD patients, but not in smokers with normal lung function [118]. In COPD patients the MDSC activation was accompanied by down-regulation of the T-cell receptor ζ chain expression in T-cells [118]. In addition, it has been shown that MDSCs were elevated in the bone marrow, spleens, and lungs after 4 months of cigarette smoke exposure, while this was paralleled by decreased pulmonary DCs [119]. However, these phenotypic MDSCs lacked immune suppressive activity, and thus were not *bona fide* MDSCs [119]. In a further study, MDSCs were also increased in patients with COPD and correlated with elevated levels of Tregs, which is in agreement with studies that suggest reciprocal control of these two cell types [120]. In summary, these studies suggest that the accumulation of MDSCs in COPD may underlie the blunted immune response observed in COPD.

Tuberculosis

Development of active tuberculosis (TB) is known to correlate with impaired T-cell responses, but the underlying immune mechanisms remained incompletely understood [121–125]. Both patients with acute (household exposure within 3 months) and chronic TB were recently described to show significantly higher frequencies of MDSCs that inhibited functions of CD4⁺ and CD8⁺ T-cells, including T-cell proliferation, altered T-cell trafficking as well as production of IL-2, IFN- γ and TNF [126]. The frequency of CD3⁻CD244^{high} cells with MDSC phenotypes were significantly higher in active TB patients and were inversely associated with the activation and functionality of CD4⁺ and CD8⁺ T-cells [127]. In murine experimental pulmonary TB, MDSCs readily phagocytosed *Mycobacterium tuberculosis*, and released both pro-inflammatory (IL-6, IL-1 α) and immunomodulatory (IL-10) cytokines, while retaining their suppressive capacity [128]. Excessive MDSC accumulation in lungs correlated with elevated surface expression of IL-4R α and increased TB lethality, whereas targeted depletion of MDSCs ameliorated disease in this animal TB model [128]. In summary, these findings indicate that MDSCs accumulate in lungs during pulmonary TB and play a dual role in host-pathogen interaction: MDSCs phagocytose and harbour *M. tuberculosis* bacteria intracellularly, providing a cellular shelter, while simultaneously dampening surrounding hostile T-cell responses.

Pulmonary hypertension

Pulmonary hypertension is a progressive syndrome with dysregulated inflammatory processes [129]. Immunohistochemical analysis of lung sections from patients with pulmonary hypertension indicated that immature DCs are present in peribronchovascular regions of vascular remodelling [130]. In a rat model of monocrotaline-induced pulmonary hypertension, DCs with immature myeloid phenotype were recruited to remodelled vessels [130]. In addition, monocyte-derived DCs from patients with pulmonary hypertension were defective in their ability to stimulate T-cells in an allostimulatory mixed-leukocyte reaction assay. In this respect, abnormalities of T lymphocyte subsets have been documented in patients with pulmonary hypertension [131, 132]. In pulmonary hypertension patients, circulating activated MDSC numbers were significantly increased in comparison to control subjects and correlated with increasing mean pulmonary artery pressure [133]. A direct mechanistic role for MDSCs in pulmonary hypertension and inflammation-associated vascular remodelling has not yet been defined.

Cystic fibrosis

Cystic fibrosis (CF) patients are impaired in eradicating *P. aeruginosa* infections and show skewed T-cell proliferation and immune responses, but the underlying reasons remained poorly understood [134–136]. Recently, it has been shown that G-MDSCs accumulate in CF patients, particularly in patients chronically infected with *P. aeruginosa* and correlate with CF lung disease activity [28]. Flagellated *P. aeruginosa* induced MDSC generation, corresponding to TLR5 surface expression on G-MDSCs. Moreover, G-MDSCs in CF patients were further characterised by an upregulation of the chemokine receptor and HIV-coreceptor CXCR4 on the surface of MDSCs. Functionally, both CF patient-isolated and flagellin-induced MDSCs suppressed T-cell proliferation and modulated Th17 cells, as key antibacterial T-cell populations in CF. Percentages of circulating G-MDSCs correlated with pulmonary function in CF patients chronically infected with *P. aeruginosa*. MDSCs could, therefore, represent a novel therapeutic target in CF patients, particularly in patients chronically infected with *P. aeruginosa* [26].

Pulmonary infection

Efficient innate host defence is crucial for the elimination of invading pulmonary pathogens [137, 138], but uncontrolled immune activation leads to collateral tissue damage. Neutrophilic cells that are rapidly recruited to the site of infection produce ROS and proteases to clear infection. However, due to a relatively short life span, neutrophils rapidly undergo apoptosis, secondary necrosis or neutrophil extracellular trap formation at the pulmonary site of infection. MDSCs have been described to efficiently efferocytose apoptotic neutrophils, mediated by IL-10 [139]. Clearance of dead neutrophils by MDSCs may, therefore help to resolve lung inflammation, preventing lung injury and ultimately restore tissue homeostasis. Studies in *Stat1*^{-/-} mice showed that bacterial infection significantly increased pulmonary MDSCs, while decreasing neutrophils [139]. These observations suggest that increasing MDSCs *via* STAT1 inhibition in combination with effective antibiotic therapy may be beneficial in the context of non-resolving bacterial pneumonia. On the other hand, it has been shown that expansion of MDSCs and absence of invariant NK T-cells in influenza A infection suppresses influenza-specific immune responses [140]. These paradoxical effects in viral and bacterial infection may be due to different MDSC kinetics and/or downstream responses [139, 140]. MDSCs accumulate in the lungs during pneumocystis pneumonia (PCP) [141]. At the pulmonary site, MDSCs interact with alveolar macrophages through programmed cell death protein 1 and programmed death-ligand 1, leading to macrophage suppression through histone modification and DNA methylation of the PU.1 gene, finally resulting in PU.1 downregulation. MDSCs employ the same

mechanism to interact with monocytes, leading to PU.1 downregulation and inhibition of their differentiation into alveolar macrophages, resulting in decreased numbers and activity of alveolar macrophages during PCP [142].

Pulmonary inflammation

Exposure of mice to lipopolysaccharide (LPS) triggers the recruitment of a MDSC-like phenotype (CD11b⁺Ly6G^{int}Ly6C^{low/-}F4/80⁺CD80⁺) into the lung [139], where they reside, in contrast to DCs, which traffic readily to the lymph nodes [75]. LPS-induced lung MDSCs were further shown to blunt the ability of pulmonary DCs to promote Th2 responses [75, 143–145]. It is speculated that lung MDSCs compromise Th2 cell survival, thereby reducing the size of the memory T-cell pool [75, 146, 147]. Thus, collectively, it appears that an important effector function of TLR-induced MDSCs is not directed to the lymph node to influence the development of adaptive immune functions, but rather to control local pulmonary immune responses. Recent studies further suggest that TLR4 activation by LPS induces GM-CSF and IL-6 production leading to STAT5 and STAT3 activation, which in turn drives MDSC generation [82, 139, 148, 149]. At the cellular level, MDSCs induce Tregs [150] by increasing Foxp3 expression through an IL-10-, TGF- β - and ARG1-dependent mechanism [150]. It has been further shown that glucocorticoids induce a distinct anti-inflammatory phenotype in mouse monocytes, which phenotypically resemble MDSCs with respect to the expression of CD11b, Ly-6G and IL-4R α chain [151].

Conclusions and outlook

Emerging evidence suggests that MDSCs, as immunosuppressive myeloid cells, play a critical role in malignant, infectious and inflammatory lung diseases, particularly lung cancer, TB, COPD, pulmonary hypertension, asthma and CF. Amongst the MDSC subsets, granulocytic MDSCs appear to represent the major population accumulating in pulmonary diseases. Pathways orchestrating MDSC generation, recruitment, activation and suppressive functions are diverse and future studies are required to narrow down the most relevant ones for therapeutic targeting approaches. Mechanistically, a more precise understanding of how host- or pathogen-derived cues modulate MDSC generation and function will help to develop tailored MDSC inhibitors for conditions where MDSC cause harm to the host, such as lung cancer and pulmonary infection (for instance TB), where pulmonary MDSCs favour the survival of malignant cells or pathogens. Conversely, adoptive cellular transfer or specific activation of MDSCs may represent an attractive therapeutic strategy to dampen immune responses in the setting of immune over activation, as found in allergic, autoimmune and auto-inflammatory pulmonary disease conditions. Findings from both mouse models and human patients indicate a potential therapeutic role for vitamin A and D, tyrosine kinase inhibitors, chemokine receptor antagonists, COX inhibitors and phosphodiesterase-5 inhibitors in regulating MDSCs. Clinical interventional studies are the next consequent step to systematically assess the safety and efficacy of these MDSC-interfering approaches in pulmonary diseases.

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