Myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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aus Schillingsfürst

Tübingen

2016

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	22.07.2016
Dekan:	Prof. Dr. Wolfgang Rosenstiel
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Abbreviations

ADAM	A disintegrin and metallopeptidase
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BM	Bone marrow
CAPS	Cryopyrin-associated periodic syndromes
CARD	Caspase recruitment domain
CCL	C-C motif ligand
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CFSE	Carboxyfluorescein succinimidyl ester
CINCA	Chronic, infantile, neurological, cutaneous and articular
CMP	Common myeloid progenitor
CNS	Central nervous system
CRP	C-reactive protein
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	C-X-C motif ligand
CXCR	C-X-C chemokine receptor type 4
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
ECP	Extracorporeal photopheresis
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ESR	Erythrocyte sedimentation rate
FCAS	Familial cold autoinflammatory syndrome
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FMF	Familial Mediterranean fever

FOXP3	Forkhead box P3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-MDSC	Granulocytic myeloid-derived suppressor cells
GvHD	Graft-versus-host disease
HLA	Human leukocyte antigen
H_2O_2	Hydrogen peroxide
HSC	Hematopoietic stem cell
HSP	Heat shock protein
IDO	Indoleamine 2,3-dyoxygenase
Ig	Immunoglobulin
IL	Interleukin
ILT2	Immunoglobulin-like transcript 2
IL-1RA	Interleukin-1 receptor agonist
IL4Rα	Interleukin-4 receptor alpha
IMC	Immature myeloid cell
IFN	Interferon
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
LAG3	Lymphocyte-activation gene 3
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
M-MDSC	Monocytic myeloid-derived suppressor cell
MPO	Myeloperoxidase
mRNA	Messenger ribonucleicacid
MSC	Mesenchymal stem cells
MWS	Muckle-wells syndrome
NACHT	Nucleotide-binding and oligomerization domain
NADPH	Nicotinamide adenine dinucleotide phosphate

NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B cells
NK	Natural killer
NKG2D	Natural-killer group 2, member D
NLR	Nucleotide-binding oligomerization domain like receptor
NLRP	NOD-like receptor family, pyrin domain containing
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOMID	Neonatal-onset multisystem inflammatory disease
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PFAPA	Periodic fever, aphthous stomatitis, pharyngitis and adenitis
PYD	Pyrin domain
ROS	Reactive oxygen species
SAA	Serum amyloid A
SCF	Stem cell factor
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
TGF	Tumor growth factor
Th	T helper
TNF	Tumor necrosis factor
Tr1	Type 1 regulatory
TRAPS	Tumor necrosis factor receptor-associated periodic syndrome
T_{reg}	Regulatory T cell
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Summary

Recurrent episodes of systemic inflammation are the hallmarks of cryopyrin-associated periodic syndromes (CAPS), leading to symptoms like fever, urtricaria and sensorineural hearing loss. Due to mutations in the *NLRP3* gene, encoding for cryopoyrin, one of the key components of the NLRP3 inflammasome, patients show elevated levels of IL-1β. Previous studies demonstrated that the overactivation of the inflammasome resulting in high levels of IL-1β leads to elevated numbers of myeloid-derived suppressor cells (MDSC). This innate immune cell population is heterogeneous and able to suppress T-cell responses, which renders MDSCs key players in the balance between innate and adaptive immunity. This led us to the hypothesis that MDSCs might be increased in CAPS patients, due to induction of NLRP3 inflammasome-dependent factors, being able to influence the disease outcome by regulating the autoinflammation.

Therefore, we investigated the potential role of MDSCs in CAPS by combining *ex vivo* and *in vitro* studies. We could show that granulocytic MDSCs (G-MDSCs), defined as SSC^{high}, $CD66b^{+}$, $CD33^{+}$, $CXCR4^{+}$, $CD16^{+}$, $CD14^{-}$ and $HLA\text{-}DR^{low}$, were generally elevated in CAPS patients compared to healthy age-matched controls, even under effective anti-IL-1 therapy. Patient's G-MDSCs were shown to be functionally competent as they suppressed polyclonal $CD4^{+}$ and $CD8^{+}$ T-cell proliferation. Also, cytokine secretion of IFNγ and IL-17 by T cells was decreased through G-MDSCs. In addition, we could show that G-MDSCs had a direct anti-inflammatory effect by suppressing monocytic IL-1β secretion. Furthermore, several cytokines and growth factors elevated in CAPS patients could be identified, which were already associated with G-MDSC-induction, like GM-CSF and VEGF. These findings were corroborated by *in vitro* studies showing that several IL-1 family cytokines and autoinflammation-associated alarmins were able to induce functional G-MDSCs, meaning that these identified factors might account for induction of G-MDSCs in patients besides IL-1β.

Taken together, an increased G-MDSC population in CAPS might represent a so far underappreciated autologous anti-inflammatory mechanism in autoinflammatory conditions and might be used for therapeutic approaches in the future.

Zusammenfassung

Die Cryopyrin-assoziierten periodischen Syndrome (CAPS) sind definiert durch wiederkehrende Episoden systemischer Entzündungsreaktionen, unter anderem gekennzeichnet durch Fieber, Hautauschläge und sensorineurale Schwerhörigkeit. Die Syndrome haben ihre genetischen Ursachen in Mutationen im Gen für *NLRP3*, welches als Cryopyrin exprimiert wird. Cryopyrin ist einer der Hauptbestandteile des NLRP3 Inflammasoms, wodurch die Folge dieser Mutation ein erhöhter Level an IL-1β ist. Verschiedene Studien konnten zeigen, dass eine Überaktivierung des Inflammasoms und somit auch erhöhte IL-1β Sekretion zu einer Induktion von myeloiden Suppressorzellen (MDSCs) führt. MDSCs sind eine heterogene Zellpopulation des angeborenen Immunsystems und in der Lage T-Zellantworten zu supprimieren. Dadurch nehmen MDSCs eine wichtige Rolle im Gleichgewicht zwischen angeborener und erworbener Immunität ein. Diese Beobachtungen führten uns zu der Hypothese, dass MDSCs in Patienten mit CAPS erhöht sein könnten, induziert durch Faktoren, die durch das NLRP3 Inflammasom sezerniert werden. Dadurch ist es möglich, dass MDSCs regulierend auf den Krankheitsverlauf von CAPS Patienten Einfluss nehmen.

Hierfür studierten wir die mögliche Funktion von MDSCs in CAPS durch eine Kombination von *ex vivo* und *in vitro* Experimenten. Wir konnten zeigen, dass granulozytäre MDSCs (G-MDSCs), per Definition SSC^{high}, $CD66b^{+}$, $CD33^{+}$, $CXCR4^{+}$, $CD16^{+}$, $CD14^{-}$ und $HLA\text{-}DR^{low}$, in CAPS Patienten erhöht sind im Vergleich zu gesunden Spendern gleichen Alters. Dies konnte auch für Patienten mit effektiver anti-IL-1 Therapie gezeigt werden. Die G-MDSCs der Patienten waren funktionell, indem sie die Proliferation von $CD4^{+}$ und $CD8^{+}$ T Zellen Dosis-abhängig supprimierten. Zusätzlich konnte eine verminderte Zytokinsekretion von IFNγ und IL-17 gezeigt werden. Darüber hinaus konnten wir eine verminderte Ausschüttung von IL-1β durch Monozyten beobachten, was auf eine direkt anti-inflammatorisch Wirkung von MDSCs in CAPS Patienten hinweist. In weiteren Experimenten wurden verschiedene Zytokine und Wachstumsfaktoren identifiziert, die im Serum der Patienten erhöht sind. Hierzu gehören zum Beispiel GM-CSF und VEGF, von denen bekannt ist, dass sie G-MDSCs induzieren. Diese Entdeckung wurde in *in vitro* Experimenten vertieft, in denen gezeigt werden konnte, dass Mitglieder der IL-1 Familie, sowie verschiedene bei Autoinflammation erhöhte Alarmine, in der Lage

sind, funktionelle G-MDSCs zu induzieren. Dies bedeutet wiederum, dass neben IL-1β noch weitere Faktoren für die erhöhte Anzahl an G-MDSCs bei CAPS Patienten verantwortlich seien dürften.

Zusammenfassend konnten wir zeigen, dass CAPS Patienten mehr G-MDSCs besitzen als gesunde Kontrollen und dass G-MDSCs somit einen autologen Mechanismus der Entzündungsbegrenzung darstellen. Diese Ergebnisse könnten in Zukunft für zusätzliche Therapieoptionen autoinflammatorischer Krankheiten von Nutzen sein.

List of publications

Original publications

Ballbach M, Hall T, Brand A, Neri D, Singh A, Schäfer I, Herrmann E, Hansmann S, Handgretinger R, Kümmerle-Deschner J, Hartl D, Rieber N.
Induction of myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes. J Innate Immun 06/2016.

Ralhan A, Laval J, Lelis F, **Ballbach M**, Grund C, Hector A, Hartl D.
Current concepts and controversies in innate immunity in cystic fibrosis lung disease. J Innate Immun (accepted 05/2016)

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, Hartl D.
Pathogenic fungi regulate immunity by inducing neutrophilic myeloid-derived suppressor cells. Cell Host Microbe 03/2015.

Mack I, Hector A, **Ballbach M**, Kohlhäufl J, Fuchs KJ, Weber A, Mall MA, Hartl D.
The role of chitin, chitinases, and chitinase-like proteins in pediatric lung diseases. Molecular and Cellular Pediatrics 02/2015.

Conference abstracts

Oral presentations

Ballbach M.
Myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes
Conference on Molecular Mechanisms of Inflammation
Trondheim, Norway, May 30th – June 2nd 2016

Ballbach M.

Myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes

11th Annual Meeting – Network Meeting of the DFG training and graduate programs

Obertrubach, Germany, July 4th – 6th 2016

Ballbach M.

Role of the inflammasome for MDSC homeostasis

9th Annual Meeting - Network Meeting of the DFG training and graduate programs

Kloster Schöntal, Germany, June 23rd – 25th 2014

Poster presentations

Ballbach M, Hall T, Brand A, Neri D, Singh A, Schäfer I, Hansmann S, Handgretinger R, Kümmerle-Deschner J, Hartl D, Rieber N.

Induction of myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes.

Novel Concepts in Innate Immunity

Tübingen, Germany, September 23rd – 25th 2015

Ballbach M, Hall T, Brand A, Neri D, Singh A, Schäfer I, Hansmann S, Handgretinger R, Kümmerle-Deschner J, Hartl D, Rieber N.

Induction of myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes.

4th European Congress on Immunology (ECI)

Vienna, Austria, September 6th – 9th 2015

Ballbach M, Hall T, Brand A, Neri D, Singh A, Schäfer I, Hansmann S, Handgretinger R, Kümmerle-Deschner J, Hartl D, Rieber N.

Induction of myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes.

10th Annual Meeting – Network Meeting of the DFG training and graduate programs

Blaubeuren, Germany, June 6th – 8th 2015

Ballbach M, Hall T, Schäfer I, Mezger M, Rieber N, Hartl D.
Role of the inflammasome in MDSC homeostasis
44th annual meeting of the german society for immunology
Bonn, Germany, September 17th – 20th 2014

ERKLÄRUNG NACH § 5 ABS. 2 NR. 7 DER PROMOTIONSORDNUNG DER MATH.-NAT. FAKULTÄT

-ANTEIL AN GEMEINSCHAFTLICHEN VERÖFFENTLICHUNGEN-
NUR BEI KUMULATIVER DISSERTATION ERFORDERLICH!

DECLARATION ACCORDING TO § 5 ABS. 2 NO. 7 OF THE PROMO OF THE FACULTY OF SCIENCE

-SHARE IN PUBLICATIONS DONE IN TEAM WORK-

Name: Marlene Ballbach

List of Publications:

1. **Ballbach M**, Hall T, Brand A, Neri D, Singh A, Schäfer I, Herrmann E, Hansmann S, Handgretinger R, Kümmerle-Deschner J, Hartl D, Rieber N.
   Induction of myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes. J Innate Immun 06/2016.

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, Hartl D.
   Pathogenic fungi regulate immunity by inducing neutrophilic myeloid-derived suppressor cells. Cell Host Microbe 03/2015.

3. Mack I, Hector A, **Ballbach M**, Kohlhäufl J, Fuchs KJ, Weber A, Mall MA, Hartl D.
   The role of chitin, chitinases, and chitinas-like proteins in pediatric lung diseases. Molecular and Cellular Pediatrics 02/2015.

Nr.	Accepted for publication yes/no	Number of all authors	Position of the candidate in list of authors	Scientific ideas of candidate (%)	Data generation by candidate (%)	Analysis and Interpretation by candidate (%)	Paper writing by candidate (%)
			Optional, the declaration of the own share can also be done in words, please add an extra sheet.				
1	**Yes**	**12**	**1**				
2	**Yes**	**27**	**9**				
3	**Yes**	**8**	**3**				

I certify that the above statement is correct.

Date, Signature of the candidate

I/We certify that the above statement is correct.

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

Contribution to the publications in the thesis

Paper 1:

Induction of myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes

I performed MDSC *in vitro* assays, analysed patient samples with help of I. Schäfer, as well as various cytokine assays in this study. Furthermore I performed data analysis, made figures, contributed in writing and proof reading of the manuscript. N. Rieber and D. Hartl designed the study, supervised the project and wrote the manuscript. Other authors contributed materials, experiments, ideas and contributed to the manuscript.

Paper 2:

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

I helped with MDSC *in vitro* assays, discussed data and contributed to writing and proof-reading of the manuscript. N. Rieber and D. Hartl designed and supervised the study and wrote the manuscript.

Paper 3:

The role of chitin, chitinases, and chitinase-like proteins in pediatric lung diseases

Together with the co-authors I contributed to writing and proof-reading of the article. The manuscript was written by I. Mack and D. Hartl.

1. Introduction

1.1 Cryopyrin-associated periodic syndromes (CAPS)

Cryopyrin-associated periodic syndromes (CAPS) are a group of inherited autoinflammatory diseases with varying degrees of severity, including familiar cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disorder (NOMID).

CAPS is a very rare disease with an estimated prevalence of 1 to 10 cases in one million (Farasat, Aksentijevich, and Toro 2008), although the assumed prevalence is probably higher due to unawareness of physicians and mis- or late diagnosis of the disease (Stych and Dobrovolny 2008; Giat and Lidar 2014). Caucasians seem to be affected more frequently, but there are no gender preferences (Kummerle-Deschner 2012).

CAPS is caused by mutations in the nucleotide-binding oligomerization domain (*NOD)-like receptor family, pyrin domain containing 3* (*NLRP3*) gene coding for cryopyrin, which leads to overactivation of the NLRP3 inflammasome, resulting in systemic inflammation driven by excessive secretion of interleukin (IL)-1β.

1.1.1 The NLRP3 inflammasome

Inflammasomes are part of the innate immune defence against pathogens or cellular stress and present molecular platforms for the maturation of pro-inflammatory cytokines (Martinon, Burns, and Tschopp 2002). The most extensively studied inflammasome is the NLRP3 inflammasome. As a multiprotein, intracellular complex it leads to the secretion of the proinflammatory cytokines IL-1β and IL-18 in a caspase-1 dependent manner. This process is tightly regulated by two steps, namely a priming and an activation step, to avoid abnormal stimulation of the inflammasome and consequent tissue damage.

Different ligands, like pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs) or pathogens bind to receptors on the cell surface and lead to a signal cascade, resulting in activation of nuclear factor ‘kappa-light-chain-enhancer’ of activated B cells (NFκB) (Bauernfeind et al. 2009), followed by the transcriptional upregulation of the *IL1B* transcript (Hiscott et al. 1993). The transcript of IL-18 however is constitutively expressed in most cells (Marshall et al.

1999). Besides priming with a Toll-like receptor (TLR) agonist, also proinflammatory cytokines like tumor necrosis factor (TNF) are able to induce activation of NFκB (Franchi, Eigenbrod, and Nunez 2009).

After priming, a second signal is required to activate caspase-1, which is able to translate the cytokine proforms into their mature forms. This activation step is conducted by a large number of different agents, like potassium efflux (Petrilli et al. 2007; Kahlenberg and Dubyak 2004), reactive oxygen species (ROS) (Cassel et al. 2008; Cruz et al. 2007; Dostert et al. 2008), crystals (Martinon et al. 2006; Hornung et al. 2008) or adenosine triphosphate (ATP) (Mariathasan et al. 2006), whereby the whole mechanism has not been solved so far and is still highly debated. Upon activation, the NLRP3 complex, consisting of C-terminal leucine rich repeats (LRRs), a central nucleotide-binding and oligomerization domain (NACHT) and an N-terminal pyrin domain (PYD), oligomerizes. Due to conformational changes, the NLRP3 can now recruit the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (ASC) via its PYD domain. ASC also contains a CARD domain, with which it can in turn bind the CARD of procaspase-1. After autocleavage, caspase-1, now in its active form, is able to mature IL-1β and IL-18, followed by their secretion into the extracellular space (see figure 1) (Martinon, Burns, and Tschopp 2002)

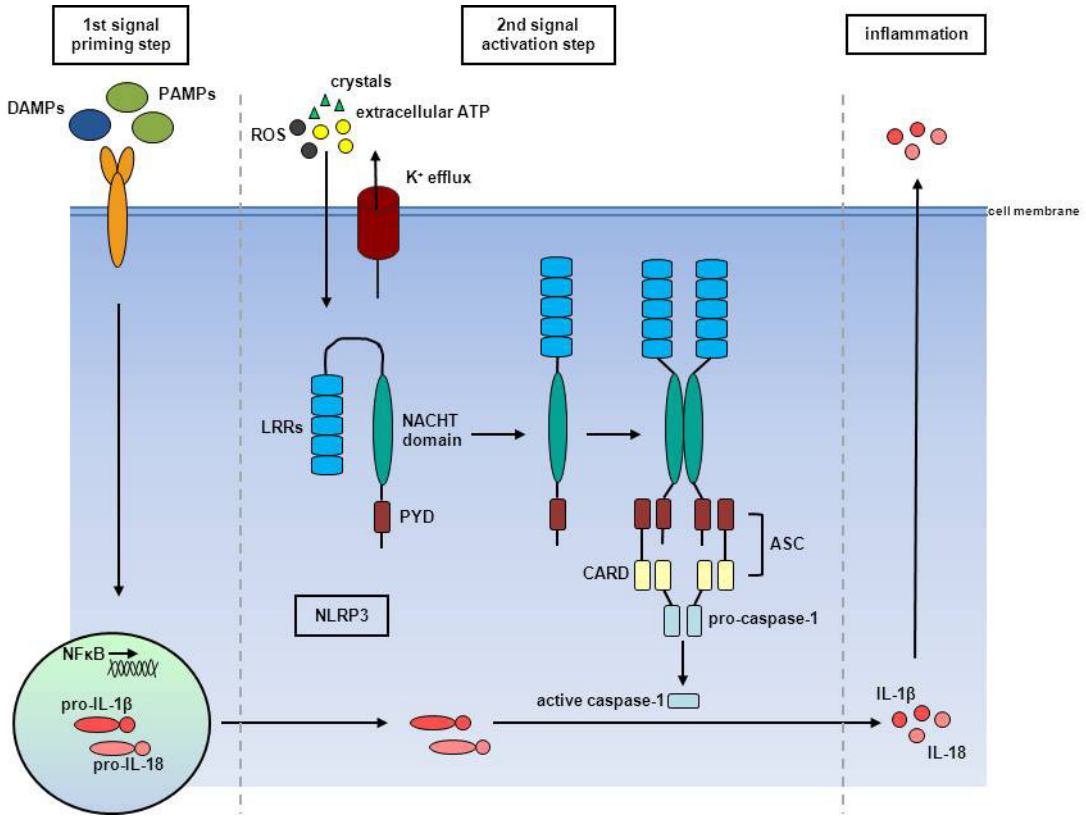

Figure 1: The NLRP3 inflammasome
The NLRP3 inflammasome is a multiprotein, intracellular complex, which leads upon activation, to the maturation and secretion of IL-1β and IL-18 in a caspase-1 dependent mechanism. It is activated in a two-step model, including priming via e.g. DAMPs or PAMPs, leading to transcriptional upregulation of IL-1β, and activation, via ROS, ATP, crystals and K^+-efflux. Upon oligomerization of the NLRP3 and recruitment of ASC, the active form of caspase-1 matures the proinflammatory cytokines, which get secreted in the extracellular space (adapted from Tschopp and Schroder 2010).

1.1.2 Genetics and pathogenesis of CAPS

The underlying cause of CAPS is mutations in the NACHT domain of the *NLRP3* gene coding for cryopyrin, located on chromosome 1q44. These mutations are either inherited autosomal-dominantly or *de novo* gain-of-function mutations, latter ones especially seen in NOMID (Aksentijevich et al. 2002). Up to date there are about 177 different sequence variants of the *NLRP3* gene known (http://fmf.igh.cnrs.fr/ISSAID/infevers/search.php, May 2016), as well as more than 90 heterozygous mutations which have been associated with CAPS and there is a strong correlation between phenotype and genotype. Regardless, carriers of the identical mutation sometimes show a heterogeneous phenotype (Levy et al. 2015). Around 40% of NOMID patients cannot be associated with a genetic mutation, though in most of these patients somatic mosaicism could be identified (Tanaka et al.

2011), implicating the involvement of genetic mosaicism in the pathogenesis of CAPS.

Cryopyrin is a member of the nucleotide-binding oligomerization domain-like receptor (NLR) family and is crucial for the formation of the NLRP3 inflammasome. As the mutation leads to gain-of-function, patients possess an overactivated NLRP3 inflammasome, leading to high levels of IL-1β, most importantly. As IL-1β is a potent, proinflammatory cytokine, its excessive secretion leads to various inflammatory symptoms.

1.1.3 Clinical manifestation of CAPS patients

CAPS patients suffer from chronic or recurrent episodes of systemic inflammation, involving skin, joints, muscles, eyes and the central nervous system (CNS) and are commonly associated with progressive sensorineural hearing loss.

FCAS represents the mildest form of CAPS and is induced upon exposure to cold, resulting in a systemic inflammatory response. Besides fever accompanied by shivering and urtricaria, patients also develop conjunctival infections as well as arthralgia. Patients additionally suffer from daily rashes, fatigue, headache, myalgia and conjunctivitis even without a clear trigger. The episodes vary in length and severity depending on the length of cold exposure, but normally resolve within 24 hours (Stych and Dobrovolny 2008).

The intermediate phenotype of MWS is more chronic, resulting in episodes of fever, headache, urtricaria, arthralgia and arthritis (Muckle and Wellsm 1962). In contrast to FCAS there is no specific trigger inducing the attacks. The onset of the disease is already in the early childhood and is accompanied by progressive sensorineural hearing loss (Kuemmerle-Deschner et al. 2013; Biswas and Stafford 2010) as well as secondary amyloidosis as a late complication. Latter one can result in proteinuria and renal failure (Aganna et al. 2002).

NOMID, also known as chronic, infantile, neurological, cutaneous and articular (CINCA) syndrome, is the most severe form of CAPS and already starts in the neonatal phase. It is associated with chronic urtricaria-like rashes, joint-involvement like arthropathy (Hill et al. 2007) and arthritis as well as CNS symptoms. Latter ones are shown through chronic aseptic meningitis, intracranial pressure, headaches,

seizures and goes as far as cognitive and mental deficits (Goldbach-Mansky 2011). Patients also evolve a progressive sensorineural hearing loss (Ahmadi et al. 2011) and additionally suffer from uveitis, conjunctivitis, optic neuritis and papillary involvement (Dollfus et al. 2000). The disease is also present morphologically; patients are of short stature, have an enlarged head and a characteristic saddle-back nose (Goldbach-Mansky 2011). Additionally, extremities are rather short and thick. As NOMID is defined by a neonatal onset accompanied by end-organ damage, there is a high mortality among patients, often leading to death before adulthood, especially when left untreated (Prieur et al. 1987).

As symptoms in all CAPS diseases are chronic and often occur unpredictable and even daily, life quality of patients is largely impaired, especially due to the chronic fatigue and pain patients suffer from. Patients commonly live to avoid exposure to cold temperature and are thereby readily compromised in their professional and social life (Stych and Dobrovolny 2008).

1.1.4 Diagnosis and current treatment options

As CAPS are very rare diseases and symptoms are unspecific and overlap with other conditions, diagnosis occurs rather late, also because physicians are frequently unaware of the disease. Often, a variety of diagnostic tools is necessary, as there is a strong variability of disease severity, making it hard to point out a specific diagnosis.

Commonly inflammatory markers are assessed (e.g. erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and serum amyloid A (SAA)), accompanied by skin biopsies and genetic testing for *NLRP3* mutations. As the latter one sometimes already correlates with the phenotype of the disease, a prediction of the outcome might be possible. Additionally, as CAPS is an autosomal-dominant disease, family history is taken into account as well as physical examination. Early diagnosis is crucial to be able to prevent end-organ damage and reverse symptoms, although not all symptoms are reversible, especially bone deformations in NOMID patients.

Before the discovery of the disease mechanism of CAPS, patients could only be treated to ameliorate symptoms with anti-inflammatory drugs. But since the mutation and the pathogenesis based on the NLRP3 inflammasome and IL-1β has been

revealed, several drugs are on the market, which target IL-1β to supress the excessive inflammatory state in patients and avoid organ damage.

Canakinumab is the most commonly used drug for CAPS patients and shows a very high remission rate. It is a human anti-IL-1β monoclonal antibody which is specific for IL-1β without cross-reactivity to IL-1α and IL-1 receptor agonist (IL-1RA) (Alten et al. 2008). Its high half-life of 21 to 28 days makes it possible that injections only have to be given every 8 weeks and it leads to a rapid remission of symptoms (Lachmann et al. 2009). The European Medicines Agency (EMA) approved Canakinumab for all CAPS patients older than 2 years; the Food and Drug Agency (FDA) gave approval for the antibody in patients with FCAS or MWS that are older than 4 years.

Anakinra, a recombinant IL-1RA, was approved in July 2014 by the EMA for all CAPS patients older than 8 months. The FDA approved Anakinra for NOMID patients that are 4 years or older. This agonist has a half-life of about 4 to 6 hours and has thus to be given daily through subcutaneous injections. It blocks the activity of both IL-1α and IL-1β and has been shown to be effective in the reduction of daily symptoms and proteinuria, as well as in the prevention of cold-induced attacks (Hawkins, Lachmann, and McDermott 2003; Hoffman et al. 2004).

Rilonacept is a fusion protein made up of the extracellular domains of a humanized IL-1 receptor, type 1 (IL-1R1) and an IL-1 receptor accessory protein (IL-1RAcP), which is fused to the Fc protein of immunoglobulin G_1 (IgG_1) and functions as an IL-1 trap, targeting both IL-1α and IL-1β (Stahl, Radin, and Mellis 2009). With a half-life of about 6.3 to 8.6 days, it has to be injected subcutaneously once a week, which is an advantage over the daily injections of Anakinra. However, the monthly costs of Rilonacept are still higher. Rilonacept has been shown to be effective in ameliorating symptoms which occur on a daily basis and is well tolerated (Hoffman et al. 2008). It is FDA approved for patients with FCAS and MWS older than 12 years.

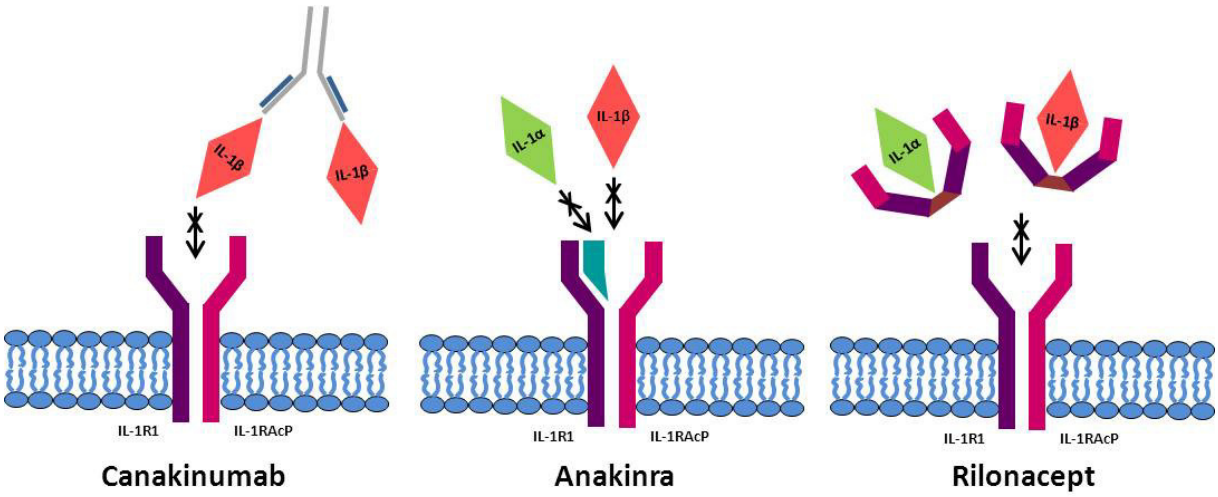

Figure 2: Current treatment options for CAPS patients.

Treatment for CAPS patients is based on targeting IL-1β to suppress the excessive inflammation in the patients. Canakinumab is a fully human monoclonal antibody binding IL-1β specifically. Anakinra acts as a recombinant IL-1 receptor agonist, preventing IL-1β and IL-1α to bind to the IL-1 receptor. Rilonacept functions as an IL-1 trap. It is a fusion protein made up of the extracellular domains of a humanized IL-1 receptor, type 1 (IL-1R1) and an IL-1 receptor accessory protein (IL-1RAcP) fused to the Fc protein of IgG1, preventing IL-1β and IL-1α to bind to the IL-receptor (adapted from (Federici, Martini, and Gattorno 2013).

1.2 Myeloid-derived suppressor cells (MDSCs)

1.2.1 Origin and expansion of MDSCs

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells (IMCs). They derive from cells in the bone marrow, which – under healthy conditions – migrate into the periphery and mature into granulocytes, dendritic cells and macrophages. Under certain circumstances though, like cancer, infections, inflammation or trauma, this differentiation is blocked, resulting in the expansion of MDSCs (see figure 3).

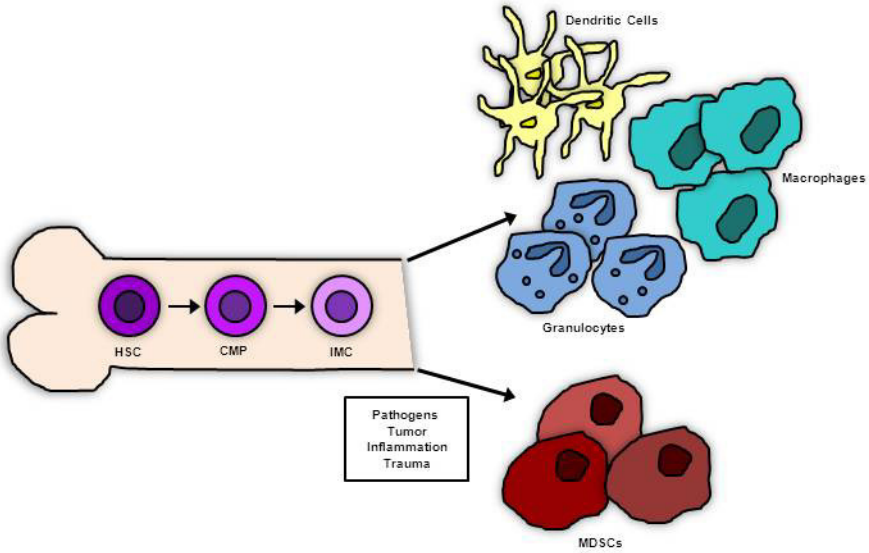

Figure 3: Origin and expansion of MDSCs

Under pathological conditions, MDSCs expand from IMCs in the bone marrow, which normally differentiate into granulocytes, dendritic cells or macrophages. This expansion is driven by pathogens, cancer, inflammation or trauma. HSC: hematopoietic stem cell: common myeloid progenitor, IMC: immature myeloid cell (adapted from (Gabrilovich and Nagaraj 2009).

Expansion and activation of MDSCs is regulated by factors, which can be divided into two different groups. First, tumor-derived factors are able to induce expansion of MDSCs by stimulating the myelopoiesis and concurrently inhibiting the differentiation into mature myeloid cells. These factors include cyclooxygenase-2, prostaglandins (Sinha, Clements, Fulton, et al. 2007; Obermajer et al. 2011), growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) (Serafini et al. 2004), granulocyte colony-stimulating factor (G-CSF) (Sawanobori et al. 2008), macrophage colony-stimulating factor (M-CSF) (Menetrier-Caux et al. 1998), vascular endothelial growth factor (VEGF) (Gabrilovich et al. 1998), stem-cell factor (SCF) (Pan et al. 2008) and IL-6 (Bunt et al. 2007). Also the heat shock protein (Hsp) 72 (Chalmin et al. 2010) and the complement factor C5a (Markiewski et al. 2008) have been shown to be involved in MDSC expansion. The main signalling cascade activated by these factors is the janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3) pathway (Gabrilovich and Nagaraj 2009).

The second group is responsible for activation of MDSCs and include factors deriving from activated T cells or tumor stromal cells. Examples are proinflammatory cytokines, such as interferon (IFN)-γ (Gallina et al. 2006), tumor growth factor (TGF)-β (Yang et al. 2008), TNF-α (Hu et al. 2014; Sade-Feldman et al. 2013), IL-1β (Song

et al. 2005), IL-4 (Bronte et al. 2003), IL-12 (Heim et al. 2015) and IL-13 (Highfill et al. 2010), as well as chemokines, like C-C motif ligand 2 (CCL2) (Huang et al. 2007), C-X-C motif ligand (CXCL) 5 and CXCL12 (Yang et al. 2008). Also TLR agonists (Delano et al. 2007) and proinflammatory molecules like S100A8 or S100A9 (Sinha et al. 2008) belong to this group, which conducts its signals via STAT1, STAT6 or NFκB (Gabrilovich and Nagaraj 2009).
In addition to soluble factors, MDSCs are controlled by the membrane-bound death-receptor Fas (CD95), resulting in apoptosis when bound by Fas-ligand on activated T cells (Sinha et al. 2011).

1.2.2 Subsets of MDSCs

As MDSCs are a very heterogeneous cell population of immature myeloid cells, no specific cell marker is known so far. Nevertheless it is possible to distinguish between granulocytic (G-MDSCs) and monocytic MDSCs (M-MDSCs).
MDSCs are commonly defined as expressing the common myeloid marker cluster of differentiation (CD) 33, a sialic acid-binding lectin, and the integrin CD11b and being negative for myeloid or lymphoid lineage specific markers (CD3, CD19, CD56). Also MDSCs show a low expression of the major histocompatibility complex (MHC) class II surface receptor human leukocyte antigen (HLA) DR (Almand et al. 2001), but are positive for MHC class I. G-MDSCs have additionally been described as $CD15^+$ and $CD66b^+$ (Rodriguez et al. 2009), whereby M-MDSCs are positive for CD14 as a monocytic marker. Recently, more markers have been assessed for identification of MDSCs, including VEGF receptor 1 (VEGFR1) and the α-chain of the IL-4 receptor (IL4Rα/CD124) (Mandruzzato et al. 2009), as well as intracellular markers like S100A9 (Zhao et al. 2012) and indoleamine 2,3-dyoxygenase (IDO) (Jitschin et al. 2014; Mougiakakos et al. 2013) for M-MDSCs. Potential other markers for M-MDSCs are CD80, CD83 and dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Poschke et al. 2010).
Although a lot of markers already have been assessed in the phenotypic identification of MDSCs, no consensus was made so far, which still renders identification of MDSCs a challenge and makes it necessary to include functional tests (Mandruzzato et al. 2016).

1.2.3 Effector functions of MDSCs

The main function of MDSCs is suppression of T-cell proliferation, which is pleiotropic as there are many different ways of inhibition (see figure 4). One way of MDSC-mediated T-cell suppression is depletion of L-arginine from the microenvironment. This conditionally essential amino acid serves as a substrate for two different enzymes: inducible nitric oxide synthase (iNOS) and arginase. iNOS converts L-arginine into nitric oxide (NO), while arginase turns L-arginine into urea and L-ornithine. The depletion of L-arginine leads to a dysregulation of T-cell proliferation, as it is needed for expression of CD3ζ (Rodriguez et al. 2002) and upregulation of cell cycle regulators, like cyclin D3 or cyclin-dependent kinase 4 (CDK4) (Rodriguez, Quiceno, and Ochoa 2007). NO additionally inhibits the expression of MHC class II (Harari and Liao 2004), induces T-cell apoptosis (Rivoltini et al. 2002) and blocks signalling via JAK3 and STAT5 in T cells (Bingisser et al. 1998).

Other important factors involved in suppression of T cells mediated by MDSCs are ROS (Kusmartsev et al. 2004; Schmielau and Finn 2001). Effects of ROS like hydrogen peroxide (H_2O_2) range from impairment of CD3ζ expression (Otsuji et al. 1996) to T-cell death (Hildeman, Mitchell, Aronow, et al. 2003; Hildeman, Mitchell, Kappler, et al. 2003) and are the main effector functions of G-MDSCs, while M-MDSCs rather suppress via NO (Movahedi et al. 2008; Youn et al. 2008). Alternatively, MDSCs are able to down-regulate L-Selectin (CD62L) on T cells by cleavage through expression of a disintegrin and metallopeptidase domain 17 (ADAM17), weakening the homing of naïve T cells to lymph nodes (Hanson et al. 2009). In 2010, Srivastava et al. showed that MDSCs also inhibit T cells by depriving cysteine from the microenvironment, which is an essential amino acid for T-cell activation and function (Srivastava et al. 2010).

Besides T-cell suppression, MDSCs additionally have the ability to impair the development of natural killer (NK) cells and were shown to affect their production of IFNγ and cytotoxicity towards tumor cells. This NK-cell inhibition is mainly mediated by membrane bound TGF-β1 and the downregulation of natural-killer group 2, member D (NKG2D), which is the main activating receptor on NK cells (Elkabets et al. 2010; Li et al. 2009; Liu et al. 2007).

Moreover, as an indirect way of their suppressive action, MDSCs can induce the *de novo* development of regulatory T cells (T_{regs}), which are forkhead box P3 (Foxp3)

positive. Different studies have been published on this topic and show that the induction requires cytokines like IFNγ or IL-10 or direct cell-to-cell contact (Huang et al. 2006; Yang et al. 2006). This topic is still controversial though, as no clear mechanism has been shown so far (Gabrilovich and Nagaraj 2009).

Lastly, it has also been demonstrated that MDSCs disrupt innate immune responses by cross-talk with macrophages, which is cell contact dependent. This results in an elevated production of IL-10 by MDSCs and thereby to a decrease of the IL-12 production by macrophages, which skews immunity towards a type 2 response (Sinha, Clements, Bunt, et al. 2007).

Taken together, MDSCs have various mechanisms to affect immune responses and are thereby considered key players in balancing innate and adaptive immunity.

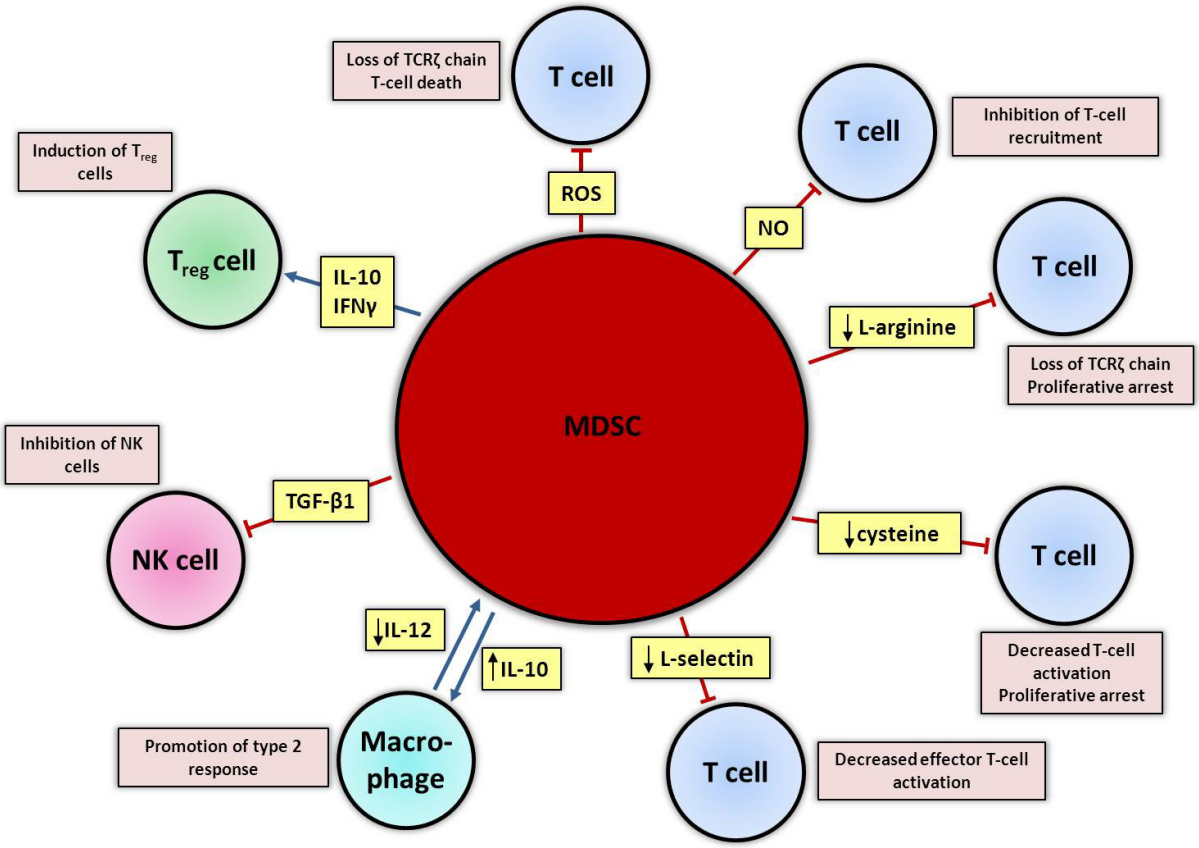

Figure 4: Pleiotropic functions of MDSCs

MDSCs are able to suppress T-cells through various effector mechanisms, like ROS, NO, depletion of L-arginine and cysteine as well as down regulation of L-selectin on T cells. Additionally, MDSCs can inhibit NK cells via membrane-bound TGF-β1. Besides suppression, MDSCs can induce regulatory T cells via IL-10 or IFNγ. They also interact with macrophages by increasing their secretion of IL-10, leading to a decreased IL-12 production of macrophages, resulting in a promoted Th2 response (adapted from (Gabrilovich, Ostrand-Rosenberg, and Bronte 2012).

1.2.4 MDSCs in pathological conditions

MDSCs have first been described in 1964 in tumor-bearing mice (Lappat and Cawein 1964), further described as natural suppressor cells in the 1980's (Maier, Holda, and Claman 1989; Strober 1984) until they were named MDSCs in 2007 (Gabrilovich et al. 2007). Most studies on MDSCs have been published in correlation to cancer, showing an expansion of this suppressive cell population and opening perspectives for new cancer therapies. But recently, MDSCs have also been associated to bacterial (Tebartz et al. 2015), fungal (Rieber et al. 2015) and parasitic (Goni, Alcaide, and Fresno 2002; Voisin et al. 2004) infections as well as to acute and chronic inflammation and autoimmune diseases (Haile et al. 2008; Marhaba et al. 2007; Zhu et al. 2007). Additionally, MDSCs are known to be elevated in patients with traumatic stress (Makarenkova et al. 2006), sepsis (Delano et al. 2007) or after transplantation (Dugast et al. 2008). This variety shows that MDSCs expand commonly in different pathological conditions and are important in many distinct settings, making it even more important to study this cell population, not only because they show such pleiotropic and diverse effector functions. Depending on the pathological environment, MDSCs can have a beneficial or harmful influence. Numerous studies show that MDSCs have a negative influence on antitumor immunity, as they inhibit immunosurveillance and contribute to tumor progression (Almand et al. 2001; Bunt et al. 2006; Nagaraj and Gabrilovich 2008). Nevertheless, as they act anti-inflammatory, they are considered beneficial when it comes to autoinflammatory diseases or infections.

2. Aim of the study

In recent years, MDSCs have gathered a substantial amount of attention in terms of one of the immunotherapeutic strategies for cancer treatment, as well as in settings of inflammatory diseases. Several publications have shown that MDSCs get induced upon inflammation. Importantly, IL-1β has been associated with MDSC induction.

As IL-1β is the driving proinflammatory factor in CAPS patients, we aimed to address the question if MDSCs are induced in CAPS patients. Moreover, we were interested if MDSCs also affect other immune cell types important in these autoinflammatory diseases besides $CD4^+$ and $CD8^+$ T cells. Therefore we evaluated the influence of MDSCs on Th17 cells, which have been associated to autoinflammation previously. Also, to assess the influence of MDSCs on the main cell type responsible for the excessive inflammation in CAPS patients, monocytes and their secretion of IL-1β were investigated. Furthermore, we tried to elicit other inflammation-associated factors that might be involved in MDSC induction not only in CAPS patients but also in healthy individuals.

Our findings provide new insights into the autologous anti-inflammatory response mechanism in CAPS and might be beneficial in developing new treatment options in addition to the currently available standard therapies.

3. Discussion

The central aim of this study was to investigate the presence and role of MDSCs in an autoinflammatory setting, particularly in CAPS. Until now, MDSCs have been thoroughly investigated and characterised in different forms of cancer, both in mice and human. Also knowledge about MDSCs in other pathologies, such as bacterial, parasitic and fungal infections or autoimmune diseases was broadened in the last years. Nevertheless, there is still scarce information when it comes to autoinflammatory diseases, especially in the human system. As it is known that inflammation and notably IL-1β lead to induction of G-MDSCs, we hypothesized that G-MDSCs are induced in the autoinflammatory setting of CAPS. To further investigate this, we combined patient data and *in vitro* studies to analyse the role of G-MDSCs and their influence on different cell types, including T-cell subsets and monocytes. Thereby we could show that G-MDSCs present a direct anti-inflammatory mechanism, which might be useful for the development of new therapies. In this part the difference of the identified cells compared to neutrophils will be discussed as well as how these cells are induced in the setting of CAPS. Furthermore, their influence on different T-cell subsets and on monocytes will be elucidated as well as their possible influence on other cell types. Lastly, the options of a therapeutic use of MDSCs in CAPS patients will be evaluated.

3.1 Definition of MDSCs – comparison to neutrophils

MDSCs are characterized to be lineage negative and as this cell population is so heterogeneous, there is no exclusive marker to phenotype MDSCs further, especially human ones. Therefore it is important to include functional assays, like T-cell proliferation assays, to assess the identity of MDSCs.

This becomes critical as G-MDSCs are very similar to neutrophils in their morphology and phenotype, as for example both are positive for CD66b, CD33, CD16 or CXCR4. Until now there is no reliable marker to distinguish neutrophils from G-MDSCs, as there is no consensus in the literature for the use of markers and some findings are even contradictory (Mandruzzato et al. 2016; Pillay et al. 2013). So an exclusive marker would be ideal in order to distinguish both cell types as well as a definition of

the differences between these two cell types on a molecular and functional level (Dumitru et al. 2012). Morphologically, the gold standard of neutrophil identification is done via May-Grünwald-Giemsa staining. Upon staining, neutrophils are seen under the microscope as cells with a nucleus, which is either formed like a band or (hyper-) segmented. Additionally, the cytoplasm is of light pink or purple with visible granules. In studies where the morphology of neutrophils and G-MDSCs was compared, G-MDSCs are mostly shown as young and immature cells, which are suppressive in contrast to the mature neutrophils (Greifenberg et al. 2009). Nevertheless there are also publications presenting data where G-MDSCs with a more mature morphology similar to neutrophils are suppressive (Dumitru et al. 2012). Taken together, morphology alone is not suitable for identification of immunosuppressive G-MDSCs and distinction from neutrophils. In this study we saw a similar morphology of G-MDSCs and neutrophils, following the concept of G-MDSCs being a rather heterogeneous cell population without a clear demarcation between immature and mature cells.

To find differences between the two very close cell types it is worthy to look into their gene expression profile. One study from Fridlender et al. found that G-MDSCs have a lower messenger ribonucleic acid (mRNA) expression of granule proteins, nicotinamide adenine dinucleotide phosphate (NADPH) complex subunits and peroxidases and a higher expression of arginase 1 (Fridlender et al. 2012). Moreover, Youn et al. revealed an upregulated expression of myeloperoxidase (MPO) and proteins involved in the cell-cycle of G-MDSCs, whereby neutrophils showed an upregulation of different cytokines, chemokines and proteases (Youn et al. 2012). Nevertheless differences in gene expression profiles cannot be used for isolation of these cells.

As G-MDSCs are of low density, they are found in the mononuclear layer when blood is separated by density centrifugation, whereby neutrophils have higher density and can be found in the pellet, on top of the erythrocytes. Still, neutrophils can degranulate and thereby loose some density ending up in the fraction of peripheral blood mononuclear cells (PBMCs), mixing themselves with G-MDSCs (Bryk et al. 2010). Furthermore, activation of neutrophils can lead to an acquired immune suppression, for example through release of intracellular arginase 1 resulting from induction of exocytosis or neutrophil death (Rotondo et al. 2009). This results in an inhibition of T-cell activity, e.g. through a decreased expression of the CD3ζ chain of

the T-cell receptor (Munder et al. 2006). As activation of neutrophils can lead to suppression, G-MDSCs are in some publications defined as a subtype of neutrophils (Rodriguez et al. 2009; Pillay et al. 2013).

In this study we identified G-MDSCs as SSC^{high}, $CD66b^{+}$, $CD33^{+}$, $CXCR4^{+}$, $CD16^{+}$, $CD14^{-}$ and $HLA\text{-}DR^{low}$, with a mature morphology and additionally showing their suppressive capacity compared to conventional neutrophils. These functional tests include T-cell proliferation tests with carboxyfluorescein succinimidyl ester (CFSE)-stained T cells, cytokine release by T cells as well as assays to assess the effector molecules released by G-MDSCs.

The comparison of G-MDSCs to neutrophils can in the same way be applied to M-MDSCs and monocytes, which are also very similar as they both express, besides others, the monocytic marker CD14 and are morphologically very similar. But as in autoinflammation G-MDSCs are the more relevant subtype of MDSCs, the focus is laid on them instead of on M-MDSCs, which might be of higher relevance in cancer settings (Montero et al. 2012).

3.2 Induction of MDSCs

CAPS patients have an underlying mutation leading to elevated inflammasome activity. The high levels of IL-1β secreted mainly by monocytes lead to an excessive autoinflammation in these patients. We could show that IL-1β also induces the generation of G-MDSCs *in vitro* from immature myeloid cells by binding to the IL-1 receptor. This leads to the activation of the JAK-STAT pathway resulting in upregulation of ROS and arginase 1, which get released and in turn are able to supress T-cell proliferation. Also G-MDSCs can have direct cell-to-cell contact with T cells, thereby adding to the suppression. Which receptors are relevant for this has not been addressed so far in the human setting, but inhibitory receptors, like programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA4) seem likely (Ioannou et al. 2012). Especially PD-1 seems to be involved in T-cell impairment by MDSCs, which could be seen in mice (Liu et al. 2008) as well as in colorectal cancer patients (Zhang et al. 2013). Also lymphocyte-activation gene 3 (LAG3) has been discussed for its involvement in MDSC-mediated T-cell suppression

as it is up-regulated, together with PD-1, on T cells after co-culture with MDSCs (Pinton et al. 2015).

Through this inhibition, T cells, as important effector cells in this autoinflammatory setting, are restricted in some way, but as IL-1β levels are very high and the inflammation in the patients is very severe, the little amount of G-MDSCs alone is not sufficient to fight the disease.

Noteworthy is that the patients in this study are under the treatment of Canakinumab, meaning that they already show less IL-1β compared to the naïve state. As there are still more G-MDSCs compared to healthy age-matched controls seen in the flow cytometry analysis, other factors must additionally account for G-MDSC induction in these patients. As seen by multiplex analysis and enzyme-linked immunosorbent assay (ELISA), GM-CSF is elevated in the patients. GM-CSF as an already established G-MDSC-inducing factor is used commonly as a positive control in MDSC *in vitro* studies (Lechner, Liebertz, and Epstein 2010; Rieber et al. 2013). Besides GM-CSF, VEGF is another candidate elevated in the serum of patients and known to be a G-MDSC-inducing factor, as it blocks the differentiation of dendritic cells while driving the accumulation of MDSCs (Gabrilovich et al. 1998). Kujawski et al. also showed that MDSCs themselves secrete VEGF, leading to an autocrine feedback loop which sustains the accumulation of MDSCs (Kujawski et al. 2008). As both growth factors could be shown to be independent of IL-1β by ELISA of sera of patients before and after therapy with Canakinumab, they are possible candidates for inducing G-MDSCs in the absence of IL-1β. Fibroblast growth factor (FGF) is elevated as well, even under Canakinumab treatment, but when tested *in vitro* for G-MDSC stimulation no effect could be seen, meaning that FGF alone is not responsible for MDSC induction. This has already been published in a broader context by Keller et al. in 2008 showing that FGF is secreted in an NLRP3-dependent manner, which is however independent from catalytic caspase-1 activity and IL-1β (Keller et al. 2008). Also the chemokines monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α (CCL2 and CCL3) were shown to be elevated in the serum of patients, but were unable to induce suppressive G-MDSCs *in vitro.* In the case of IL-8, which has also been shown to be elevated in patient's sera, its concentration goes down concurrently with IL-1β under Canakinumab treatment, making it not a relevant candidate for G-MDSC induction, which also could be shown through *in vitro* stimulation assays. Furthermore, other IL-1 family

members could be shown to be able to induce G-MDSCs *in vitro*, including IL-18 and IL-1α. Especially IL-18, which is also cleaved by caspase-1 upon NLRP3 inflammasome activation, seems a legit candidate. Additionally Lim et al. showed in 2014 that IL-18 is able to induce MDSCs in mice (Lim et al. 2014). Both cytokines were validated in functional assays *in vitro* and proved to induce suppressive G-MDSCs to at least the same extent as IL-1β.

Besides the discussed and in this study further investigated factors, other inflammation-associated factors can be considered to be responsible for G-MDSC induction. This includes several pro-inflammatory cytokines like IFNγ (Gallina et al. 2006) or TNF-α (Hu et al. 2014; Sade-Feldman et al. 2013). Not published data from our laboratory shows that both cytokines are able to induce suppressive G-MDSCs which is concordant with the literature. Nevertheless, so far it has not been shown if these cytokines are also elevated in CAPS patients. Further studies in our laboratory could not identify any other relevant G-MDSC-inducing factor being responsible for induction of the suppressive cells in the setting of CAPS.

Preliminary data indicate that G-MDSCs are also increased in other autoinflammatory diseases, including tumor necrosis factor receptor-associated periodic syndrome (TRAPS), familial Mediterranean fever (FMF) and periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA), which might give a hint for a common mechanism of counterregulation in autoinflammatory diseases. Additionally this could provide a rationale for the observed strong periodicity of these syndromes. Upon inflammation, G-MDSC generation would be induced from myeloid progenitor cells in the bone marrow for several days to weeks. In turn this would lead to a decreased inflammation, resulting in a disappearance of G-MDSCs over certain days due to fading inflammatory stimuli. When inflammation rises up again, a new cycle of progenitor cell differentiation can start.

These findings, especially the elevated growth factors and chemokines discussed above, might point to a subclinical inflammation in CAPS patients even under anti-IL-1 therapy. Therefore, these factors together with increased numbers of G-MDSCs may become valuable disease markers. To further assess this, larger longitudinal studies are needed for a comprehensive evaluation of elevated factors in patients and their relevance in G-MDSC induction. Nevertheless one has to keep in mind that some of these factors already have been associated with chronic disease

manifestation, for example FGF with organ fibrosis or VEGF with arteriosclerosis (Ban and Twigg 2008; Celletti et al. 2001).

3.3 Effect of MDSCs on different T-cell subsets

Through the inflammatory environment in the patients, the adaptive immune response is stimulated and contributes further to the inflammatory state. As the key cytokine IL-1β is of pleiotropic nature, its release also leads to the proliferation of T cells and their production of cytokines. As the process of inflammation is very broad, all types of T cells are stimulated. It has been shown that T cells are considerably skewed towards the highly inflammatory subtype of T helper (Th) 17 cells, a proinflammatory $CD4^+$ T-cell subset, in CAPS patients (Lasiglie et al. 2011). This is due to the high levels of IL-1β, as it is the key cytokine for Th17 differentiation in concert with IL-23, which is also elevated in the serum of patients in an IL-1β dependent manner (Kryczek et al. 2007; Sutton et al. 2006). In this study we could also see an effect of G-MDSCs on the Th17 cells as the secretion of IL-17 was significantly decreased by patients' G-MDSCs. A study from 2010 has shown that IL-17 also has an effect on MDSCs as they decrease in IL-17-deficient mice what can be reversed when IL-17 is administrated again (He et al. 2010). If this is a direct effect or an indirect, as IL-17 also triggers the production of IL-6, leading to activation of STAT3 and thereby inducing MDSCs, is not known so far (Wang et al. 2009). Nevertheless, some studies also show that MDSCs are able to induce Th17 cells by producing IL-6 and TGF-β (Chatterjee et al. 2013) or recruiting them by production of CCL4, which is a chemoattractant for Th17 cells (Ortiz et al. 2015). So apparently MDSCs and Th17 cells have a complex reciprocal relationship, which is probably influenced by several other factors as well as the microenvironment, making it necessary to perform more in depth studies to get a conclusive picture.

Besides suppression of the Th17 response we could also see an effect of G-MDSCs on the proliferation of $CD4^+$ T cells. As T-cell response in CAPS patients is, as mentioned above, skewed towards a Th17 response, one can speculate that most T cells differentiate into Th17 cells and only lower amounts into Th1 and Th2 cells. Along with the proliferation of $CD4^+$ T cells, G-MDSCs are also affecting the proliferation of $CD8^+$ T cells. These observations show that G-MDSCs have an

ubiquitary effect on T cells in CAPS patients. A more thorough investigation of the preliminary data is yet needed to clarify this.

As G-MDSCs showed to suppress proliferation of T cells as well as their secretion of IFNγ and IL-17, they might act as a physiological anti-inflammatory mechanism able to dampen the inflammation by dampening the T-cell response and also counteracting the dysregulated T-cell balance towards Th17.

In turn, recent studies reported that T cells also have an effect on MDSCs, as already discussed for Th17 cells above. Pinton et al. published data, where they show that there is an interplay between MDSCs and T cells, involving IL-10. Thereby MDSCs get active when in contact with activated T cells, while they stay harmless in the presence of resting T cells (Pinton et al. 2015). Similar results were given by another study, where Th1 cells were shown to induce MDSCs in mice (Cripps et al. 2010). Also Th2 cells have been implicated in having a direct effect on MDSCs (Nagaraj, Youn, and Gabrilovich 2013).

In summary, there is a complex relationship between MDSCs and T cells, which seems to be bidirectional. In our studies we did not investigate the influence of T cells on MDSCs, while it is still important to keep in mind for the further evaluation of the clinical use of MSDCs in CAPS patients.

3.4 Effect of MDSCs on monocytes

In the inflammatory setting of CAPS patients, monocytes are the driving cells secreting IL-1β and thereby fuelling the inflammation in these patients. In this study we asked the question whether G-MDSCs also have an effect on monocytes, as this has not been studied so far. We could show that G-MDSCs have indeed an effect on monocytes by dampening their release of IL-1β and thereby directly acting anti-inflammatory. This would mean that there is a direct negative feedback loop of MDSCs on the inflammation and not only an indirect one by inhibiting T-cell proliferation.

This observation, together with the effect of G-MDSCs on T cells leads to a complex picture of the relationship between the different cell types investigated. This is summarized in figure 5. As discussed above, inflammation fuels G-MDSC expansion mainly through IL-1β produced by monocytes. Inflammation also leads to the

engagement of T cells, leading to a reinforced inflammatory state. In turn, G-MDSCs dampen the inflammation by suppressing monocytes and T cells, thereby also reducing their own expansion as the activating stimuli of the inflammation go down.

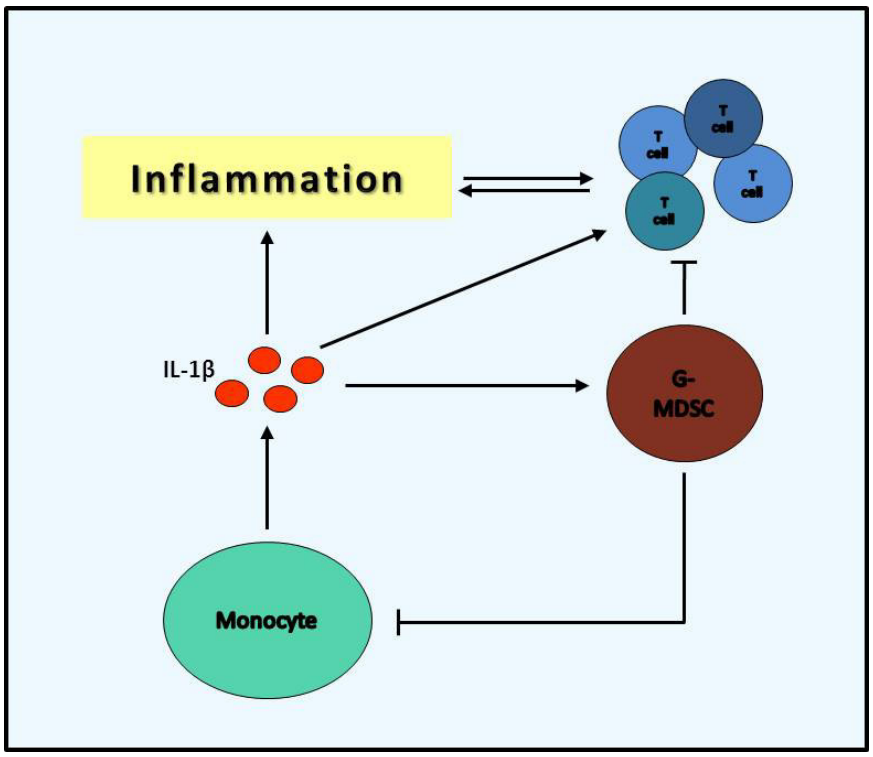

Figure 5: Complex relationship of cells in the inflammatory setting of CAPS.
Monocytes secrete IL-1β, thereby fuelling the inflammation. Additionally IL-1β, besides other factors, induces T cells, which further enhance the inflammatory state in the patients. To counteract the inflammation, G-MDSCs, which are also induced by IL-1β and other factors, act anti-inflammatory by either suppressing monocytes, thereby dampening the IL-1β secretion or by suppression of T cells.

How G-MDSCs directly affect monocytes and which other effects are involved is not known, but one can speculate, based on a paper from Sinha et al. from 2007 that there is a cross-talk between G-MDSCs and macrophages, which is cell-contact dependent (Sinha, Clements, Bunt, et al. 2007). This cell-contact results in a higher secretion of IL-10 by G-MDSCs and a dampened IL-12 secretion by macrophages, leading to a shift towards a type 2 response, thereby also working against the inflammatory Th1 and Th17 response. This could also add to the anti-inflammatory properties of the G-MDSCs in the setting of CAPS. Nevertheless, more studies have to be performed to address these questions in more depth.

3.5 Effect of MDSCs on other cell types

As the inflammasome is not only formed in monocytes, but also in other myeloid cells, it is possible that G-MDSCs also have an effect on those cells. For example, neutrophils possess an inflammasome as well (Bakele et al. 2014) and might be affected by G-MDSCs. One has to keep in mind though that G-MDSCs and neutrophils are very similar to one another, as discussed above, what makes it hard to see an effect of one cell type on the other. Some experiments regarding this question have already been performed in our lab, unfortunately without success. This was mainly due to the similarity of the cells and to their different life spans in culture, as neutrophils cannot be kept in culture that long, and G-MDSCs need some time to fulfil their suppressive function.

Also dendritic cells (DCs) might be a possible target or interaction partner for G-MDSCs. This has already been addressed in some publications, making it relevant for further studies also in correlation to CAPS patients, as DCs possess a NLRP3 inflammasome and are able to secrete IL-1β. Additionally they take up antigens for procession and present them to T cells as antigen-presenting cells (APCs). Thereby DCs might be involved in the inflammation process in the patients. In the context of cancer it has already been shown that DCs are impaired in their maturation and function. Some publications point towards the fact that this might be due to arising MDSCs, leading to the conclusion that there is an interaction between the two cell types (Greifenberg et al. 2009; Hu et al. 2011).

Another cell type, which might be involved in the inflammatory process and being influenced by MDSCs are NK cells. So far there is not a lot of data concerning NK cells and inflammation and already published data is rather contradictory, as NK cells, depending on the model system, can add to the inflammation, but also lead to an amelioration of it. Also their contribution in CAPS has not been investigated so far. Nevertheless it has been shown that MDSCs influence NK cells by reducing their cytotoxic efficacy (Liu et al. 2007). This effect is increased through inflammation (Elkabets et al. 2010). Studies giving insights into the role of NK cells in CAPS and their relationship towards MDSCs have to be performed in order to draw conclusions.

MDSCs are the regulatory cell type of the innate part of immunity, but besides MDSCs, the adaptive immunity provides T_{regs} to fulfil this job. It might be interesting to investigate this cell type in CAPS patients, as T_{regs} might help or work together with

MDSCs to dampen the excessive inflammation in these patients. Unfortunately there is no data on T_{regs} in CAPS so far. Still, it has been shown that a distinct subtype of T_{regs}, namely Foxp3$^-$ type 1 regulatory (Tr1) cells, are able to suppress the NLRP3 inflammasome by secreting IL-10 (Yao et al. 2015). Concerning the interaction of MDSCs with T_{regs} it has been shown that they might have a reciprocal relationship as they induce each other, which is regulated by IL-10 (Fujimura, Kambayashi, and Aiba 2012; Serafini et al. 2008; Huang et al. 2006). But also here, like for the NK cells, no conclusion can be drawn as to whether these cells play a major part in CAPS. Still this would be worth investigating, as T_{regs} might add to the immunosuppressive effect together with MDSCs.

Taken together, MDSCs help to dampen the inflammation in CAPS patients in various ways. Firstly, they directly act anti-inflammatory by suppressing IL-1β secretion by monocytes. Secondly, MDSCs suppress T-cell proliferation and cytokine secretion through their effector molecules. And lastly, one can speculate that they also influence the T cells indirectly via DCs by impairing their function, as the major task of DCs is to activate T cells by presenting antigens to them, which is complemented by secretion of cytokines inducing T-cell differentiation (IFNy, IL-23) (Ostrand-Rosenberg et al. 2012). An involvement of neutrophils, NK cells or T_{regs} is also possible, but at this point, due to too little or unavailable data, no certain conclusions can be drawn.

3.6 Treatment options

In addition to the different anti-IL-1 treatments, namely Canakinumab, Anakinra or Rilonacept, G-MDSCs could be used as supplementary cell-based therapy. Especially in very severe cases or in specific complications, which do not respond to conventional therapies, the addition of G-MDSCs might help to further dampen the inflammation in these patients. Thereby *in vivo* generation or *ex vivo* expansion of the cells, followed by an adoptive transfer, would be possible.

Several studies suggest the use of GM-CSF and or G-CSF for the generation of MDSCs as it has been shown that the increased production of these growth factors by tumors is linked to an expansion of MDSCs (Bronte et al. 1999; Abe et al. 2010; Shojaei et al. 2009). It already has been shown that this might be applicable for

therapeutic uses. For example Zahorchak et al. could show that the frequency of MDSCs can be increased *in vivo* in primates through sequential injection of GM-CSF and G-CSF (Zahorchak et al. 2016). Also one could use different combinations of stimuli together with GM-CSF, as shown by Lechner et al. in 2010. In this study, PBMCs are used to generate MDSCs and also different combinations of stimuli are used, which could be also applied here (Lechner, Liebertz, and Epstein 2010).

Besides from PBMCs, MDSCs can also be induced from bone marrow (BM) cells. Marigo et al. developed a method to rapidly induce MDSCs by adding GM-CSF, G-CSF an IL-6 to BM cells (Marigo et al. 2010). A similar approach was used by Highfill et al. as they use GM-CSF and G-CSF and then add IL-13 to the BM cells of mice leading to the generation of M-MDSCs with an arginase 1-dependent suppression (Highfill et al. 2010).

In a more clinical setting, different groups have established methods to generate MDSCs to treat Graft-versus-host disease (GvHD). Joo et al. showed that G-CSF leads to the induction of MDSCs and thereby to the prevention of GvHD (Joo et al. 2009). This has been deepened by another study, where it was shown that functional MDSCs expand in the peripheral blood of G-CSF-mobilized donors, with the capacity to regulate alloreactive T-cell responses *in vitro* (Luyckx et al. 2012). Additionally, Adeegbe et al. performed studies, where MDSCs were induced *in vivo* with G-CSF prolonging skin allograft survival in mice (Adeegbe et al. 2011). Another advantage of the two growth factors is that they are already available and used therapeutically. GM-CSF is used as Sargramostim, while G-CSF is given as Filgrastim to treat neutropenia after chemotherapy or BM transplantation (Mehta, Malandra, and Corey 2015).

Besides GM-CSF and G-CSF, several other stimulants were used by different groups to induce MDSCs *in vivo* or *ex vivo* for adoptive transfer. A study from 2015 showed that cannabidiol, a major component of Cannabis, is able to induce an immunosuppressive population of MDSCs in mice (Hegde et al. 2015). Morecki et al. presented data where MDSCs could be induced in mice through CpG oligodeoxynucleotide administration, leading to the suppression of alloreactivity and protection against the development of GvHD in the recipient after adoptive cell transfer (Morecki et al. 2008). In the context of transplantation, there are different reports about MDSCs, which were generated *in vivo* or *in vitro* and adoptively transferred, and which were able to inhibit allograft rejection. Zhang et al. generated

MDSCs from transgenic immunoglobulin-like transcript 2 (ILT2) mice. ILT2 is an immunosuppressive receptor, binding to HLA-G, a human immunosuppressive non-classical MHC molecule. The adoptive transfer of these MDSCs led to a longer graft survival in the recipients of an allogeneic skin transplant (Zhang et al. 2008). A study from 2009 injected mice with lipopolysaccharide (LPS) to generate MDSCs and transferred them to skin transplanted mice, resulting in delayed allograft rejection (De Wilde et al. 2009).

An already established therapy, namely extracorporal photopheresis (ECP), is used in GvHD patients, where it was shown that this treatment leads to the induction of immunosuppressive G-MDSCs (Rieber et al. 2014). ECP is a leukapheresis-based approach using psoralen and ultraviolet (UV) radiation leading to immunomodulation, which is successfully used in T cell-mediated diseases (Edelson et al. 1987). Nevertheless it is not understood how ECP exactly modulates the immune system (Knobler et al. 2009).

Taken together, there are already some possibilities for the clinical use of MDSCs, but as most studies have not been performed in a human setting and also not in an autoinflammatory setting, several points have to be discussed in order to be able to decide which option would be more feasible. Important is to choose the best course of treatment for the respective disease. *Ex vivo* versus *in vivo* generation of MDSCs for example. Which stimulant is the best to generate immunosuppressive MDSCs of the right subtype? Here the phenotype should be evaluated to secure similarity between generated and naturally occurring MDSCs. In the case of an adoptive transfer of *ex vivo* MDSCs, from which cells should MDSCs be generated from, PBMCs or BM cells? Should the transfer be autologous or allogenic? How many cells should be given to the patients, how often and by which route of administration? Latter one is especially a question concerning life span of the MDSCs in the patients, of their *in vivo* fate and their continuous functionality. Also possible side effects like the susceptibility to infections or tumors in the case of CAPS patients have to be taken into account.

As for GM-CSF one has to keep in mind that it has very broad effects and does not lead to an isolated induction of MDSCs. It has been shown that the overexpression of GM-CSF leads to a severe inflammation what would possibly add to the already existing inflammation in the patients (Shi et al. 2006). Additionally data from animal models suggest that in certain inflammatory and/or autoimmune diseases the

depletion of GM-CSF is beneficial (Hamilton 2008). GM-CSF is furthermore used as a potent adjuvant and thereby leading to the induction of other immune cells, like T cells, what would again counteract the intended immune suppression (Disis et al. 1996). To address these issues, more thorough studies have to test the usefulness of GM-CSF as treatment in CAPS patients to evaluate possible side effects, especially since in some inflammatory diseases the administration of GM-CSF even seems beneficial (Hamilton 2008).

Concerning the immunosuppressive function of MDSCs, Koehn et al. showed that it can be lost due to inflammasome activation of the MDSCs after adoptive transfer into GvHD mice opting rather for *in vivo* generation of these cells. Nevertheless this could be counteracted through genetic alteration of the MDSCs making them unable to express ASC. Until *in vivo* approaches to inhibit the inflammasome activation of MDSCs are available, multiple doses of MDSCs can be used to sustain suppression (Koehn et al. 2015).

Another problem for the *in vitro* generation might be the amount of cells one can generate or what cell number is needed, respectively. An example where adoptive transfer of cells is performed is mesenchymal stem cells (MSCs). These mesoderm-derived multipotent stem cells have immunomodulatory capacities and have shown to be effective in the treatment of GvHD (Le Blanc et al. 2004). Most clinical trials evaluating MSCs for therapeutic purposes used a dose of 0.4-10 x 10^6 cells per kilogram of body weight (Chen and Hou 2016). To achieve this many cells, it is of great need to have a protocol to generate a considerable amount of immunosuppressive MDSCs. Certainly this depends on the cells used for the generation. PBMCs are surely easier to obtain than BM cells. Another factor enabling high cell numbers is the use of allogeneic donors, as here a rapid mass production would be possible in contrast to the autologous setting. This would be accompanied by lower costs and the possibility to use young healthy donors, which might yield cells with higher efficacy. Nevertheless it has been shown, in the case of MSCs, that cells from autologous donors are more potent (Togel et al. 2009). If this is also the case for MDSCs needs to be evaluated. In summary one can conclude that a lot of different factors have to be taken into account when establishing a cell-based therapy for special indications in CAPS patients. For a conclusive decision which way would be the best, further studies and of course clinical studies are needed, to evaluate all discussed factors.

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Acknowledgment

First, I want to thank my supervisor Prof. Dr. Dominik Hartl, who gave me the opportunity to work in his lab on this interesting project. I also want to thank him for his continuous help in any matter, for always giving advice and guidance and for being a very good and sympathetic boss.

I also want to thank Junior-Prof. Dr. Alexander Weber for being my second supervisor, for his help and advice, as well as for the evaluation of my thesis.
Additionally I want to thank Prof. Dr. Hans-Georg Rammensee and Prof. Dr. Andreas Peschel, who kindly accepted and took their time to be part of my thesis committee.

Also, I want to thank Dr. Nikolaus Rieber, who was of great help in any clinical matter, for his support in finishing this thesis and for his always good mood.

My very special thanks goes to Iris Schäfer, who helped with everything around the lab, for always being there, answering every question and knowing every answer.

Furthermore I would like to thank all the members of the lab, for making the time in the lab but also outside so enjoyable. Especially Anu and Felipe, who filled these last years with so much fun every day, and also for their support and help.

I want to thank my friends for their support in good and bad times, but especially for the distraction they gave me from work.

Lastly, I want to thank my family, for everything they've done over the past years to support my studies and for just being there.

And of course I want to thank Finn, because without him on my side, his love and encouragement I would not have made it through all of this!

Appendix

Paper 1

Ballbach M, Hall T, Brand A, Neri D, Singh A, Schäfer I, Herrmann E, Hansmann S, Handgretinger R, Kümmerle-Deschner J, Hartl D, Rieber N.
Induction of myeloid-derived suppressor cells in cryopyrin-associated periodic syndromes. J Innate Immun 06/2016

(DOI:10.1159/000446615)

Research Article

Journal of Innate Immunity

J Innate Immun
DOI: 10.1159/000446615

Received: October 14, 2015
Accepted after revision: May 5, 2016
Published online: June 29, 2016

Induction of Myeloid-Derived Suppressor Cells in Cryopyrin-Associated Periodic Syndromes

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Key Words

Myeloid-derived suppressor cells · Neutrophils · Autoinflammation · NLRP3 · Cryopyrin-associated periodic syndrome · IL-1β · Chemokines · Growth factors

Abstract

Cryopyrin-associated periodic syndromes (CAPS) are caused by mutations in the *NLRP3* gene leading to overproduction of IL-1β and other NLRP3 inflammasome products. Myeloid-derived suppressor cells (MDSCs) represent a novel innate immune cell subset capable of suppressing T-cell responses. As inflammasome products were previously found to induce MDSCs, we hypothesized that NLRP3 inflammasome-dependent factors induce the generation of MDSCs in CAPS. We studied neutrophilic MDSCs, their clinical relevance, and MDSC-inducing factors in a unique cohort of CAPS patients under anti-IL-1 therapy. Despite anti-IL-1 therapy and low clinical disease activity, CAPS patients showed significantly elevated MDSCs compared to healthy controls. MDSCs were functionally competent, as they suppressed polyclonal T-cell proliferation, as well as Th1 and Th17 responses. In addition, MDSCs decreased monocytic IL-1β secretion. Multiplex assays revealed a distinct pattern of MDSC-inducing cytokines, chemokines, and growth factors. Experimental analyses demonstrated that IL-1 cytokine family members and autoinflammation-associated alarmins differentially induced human MDSCs. Increased MDSCs might represent a novel autologous anti-inflammatory mechanism in autoinflammatory conditions and may serve as a future therapeutic target.

Introduction

The cryopyrin-associated periodic syndromes (CAPS) comprise a spectrum of autoinflammatory diseases, including FCAS (familial cold-induced autoinflammatory syndrome), through MWS (Muckle-Wells syndrome), to NOMID (neonatal-onset multisystem inflammatory disease), also known as CINCA (chronic infantile neurological, cutaneous, articular) syndrome [1]. These disease conditions are characterized by recurrent episodes of systemic inflammation marked by fever, tissue inflamma-

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tion (particularly of the joints and skin), urticarial rash, and sensorineural hearing loss [2]. CAPS are caused by mutations in the *NLRP3* (Nod-like receptor family, pyrin domain containing 3) gene encoding the cryopyrin protein, a key component of the NLRP3 inflammasome that activates caspase-1, resulting in inflammation driven by excessive secretion of the cytokine IL-1β [3–6]. Disease causing mutations in the *NLRP3* gene together with the typical symptoms are an indication for a continuous anti-IL-1 therapy to prevent long-term sequelae, such as kidney failure or hearing loss [7].

The physiologically occurring IL-1 receptor antagonist (IL-1RA) is highly increased in active CAPS patients, pointing to an autologous anti-inflammatory feedback loop. Up to now there have been no reports on cellular anti-inflammatory mechanisms operative in CAPS. However, inflammatory Th17 cells have been reported to be significantly elevated in CAPS [8], providing a rationale to speculate on a counter-regulatory increase of regulatory immune cells in these patients. Myeloid-derived suppressor cells (MDSCs) represent a novel innate immune cell subset generated in tumor, infective, and proinflammatory microenvironments [9–11]. These specialized innate immune cells are characterized by their capacity to suppress T-cell responses and thereby modulate the cellular arm of the adaptive immunity [10]. Consequently, MDSCs are considered to be a key player in the balance between innate and adaptive immune responses, particularly under chronic disease conditions. In mice, where MDSCs have been studied extensively, these cells comprise both a neutrophilic and a monocytic MDSC subphenotype [10, 12–14], while studies on MDSCs in human disease conditions other than cancer are scarce. Recent evidence suggests that primarily neutrophilic MDSCs accumulate in malignancy and systemic inflammation [15–17]. The maturation stage of neutrophilic MDSCs is still a matter of debate in the field. While the suppressive phenotype has been linked to an immature state, other concepts define neutrophilic/granulocytic MDSCs as a heterogeneous group of T-cell suppressive immune cells with neutrophilic/granulocytic phenotypes, without a clear demarcation between immature and mature cells [18–21]. In this paper we favor the latter, more general approach regarding the maturity of neutrophilic MDSCs.

As murine studies have provided evidence that the inflammasome induces the generation of MDSCs as innate autologous anti-inflammatory cells [22–24], we hypothesized that MDSCs might play a role in CAPS. Our studies demonstrate for the first time that neutrophilic MDSCs are induced in CAPS patients even under effective anti-IL-1 therapy and reveal novel MDSC-inducing factors in these patients. Increased MDSCs might represent a novel autologous anti-inflammatory mechanism in autoinflammatory conditions and may serve as a future therapeutic target.

Patients and Methods

Study Population

The study was conducted at the University Children's Hospital, Tuebingen (Germany). Informed written consent was obtained from all subjects included in the study (or their legal representatives), and all study methods were approved by the local ethics committee. The study population consisted of 28 CAPS patients (16 females, 12 males) with ages ranging from 3 to 78 years (mean age 32 years). CAPS patients were diagnosed based on their characteristic clinical presentations for CAPS and *NLRP3* gene mutations [2]. Clinically, CAPS patients resembled an MWS or MWS/FCAS overlap phenotype. At the time of blood sampling for MDSC analyses, all CAPS patients received regular anti-IL-1 treatment with canakinumab and were in clinical remission. Control subjects were from 0 to 44 years of age (mean age 14 years), without signs of infections, malignancies, or inflammation at the time of blood sampling. The samples from the children in this control group were taken through a routinely inserted intravenous line before undergoing elective surgery for multiple diagnoses.

Cell Isolation and Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood samples by Ficoll density gradient sedimentation (lymphocyte separation medium; Biochrom) and washed twice in RPMI 1640 medium (Biochrom). Trypan blue staining solution (Sigma-Aldrich) at 0.5% differentiated between viable and nonviable cells and showed a viability of >90% for all cells used in this study. After Ficoll density gradient sedimentation, MDSCs were characterized as $SSC^{high}CD66b^{high}CD33^{high}CD16^{high}CXCR4^{high}HLA\text{-}DR^{low}$ cells in the PBMC fraction based on previously established human MDSC analysis methods [15, 17] (fig. 1a). PBMCs outside this neutrophilic MDSC population were CD66b negative (online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000446615). For MDSC isolation, cells were obtained from the PBMC fraction and labeled with anti-CD66b-FITC followed by two sequential anti-FITC magnetic bead separation steps (Miltenyi Biotec), according to the manufacturer's protocol. The purity of $CD66b^+$ cells after separation was >95% as assessed by flow cytometry. Conventional neutrophils (polymorphonuclear neutrophils, PMNs) were isolated from the high-density fraction of a Ficoll density gradient sedimentation (lymphocyte separation medium; Biochrom) followed by erythrocyte lysis using ammonium chloride and an additional enrichment step using magnetic bead separation for $CD66b^+$, as described above. The morphology of the cells (MDSCs and conventional neutrophils isolated from CAPS patients) was analyzed by May-Grünwald-Giemsa staining of cytospins (fig. 1b). Isolated MDSCs featured typical morphological characteristics of neutrophils, identifying them as neutrophilic MDSCs [25]. Antibodies against CD4, CD8, CD16, and CCR2 were purchased from BioLegend, anti-human CD33 from Miltenyi Biotec, and antibodies

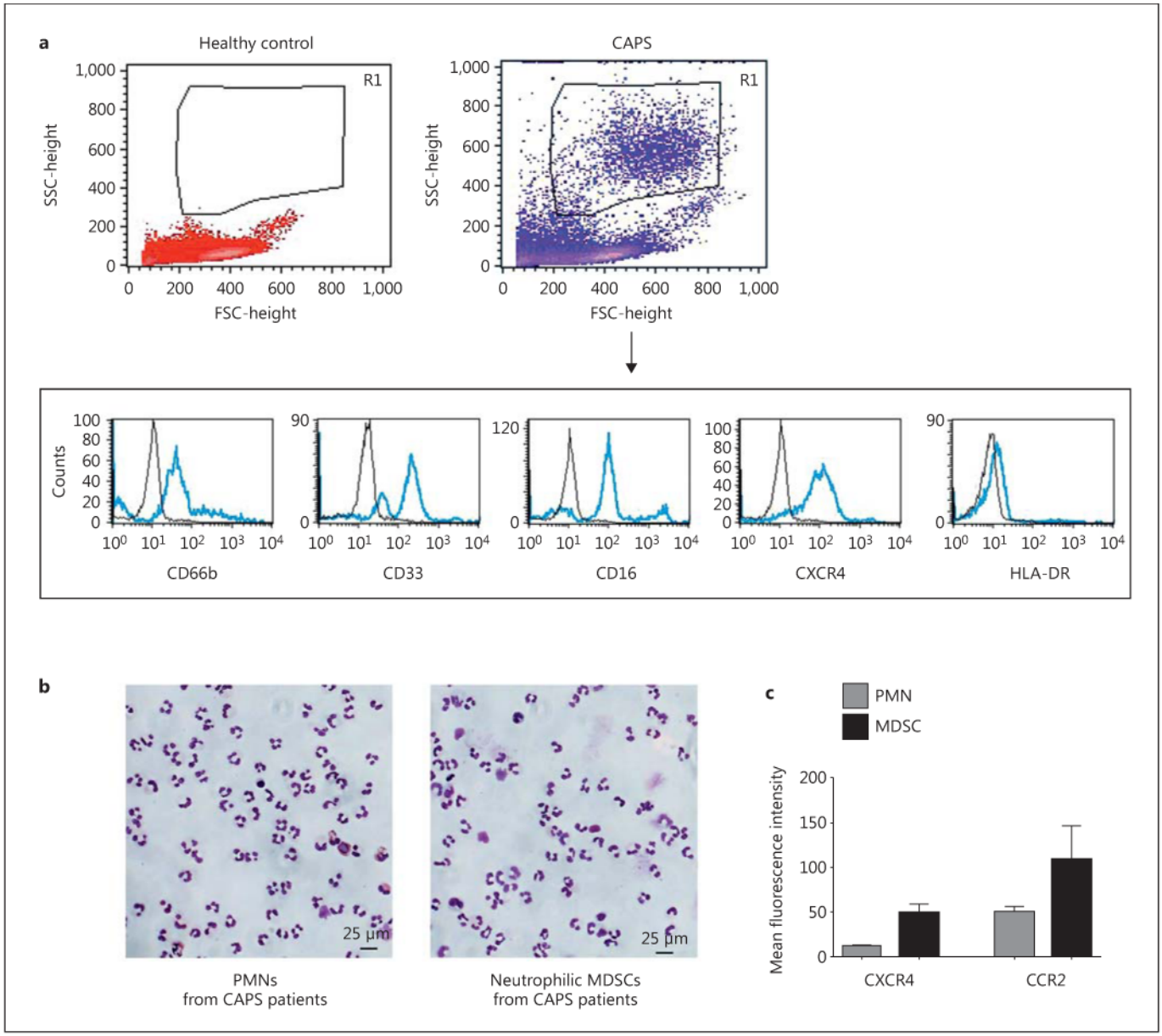


Fig. 1. Phenotypic characterization of MDSCs. PBMCs were isolated from heparinized blood of CAPS patients and healthy age-matched controls using Ficoll density gradient sedimentation. **a** MDSCs were identified by flow cytometry as neutrophilic cells which are CD66b^{high}, CD33^{high}, CD16^{high}, CXCR4^{high}, and HLA-DR^{low}, as shown representatively in the FSC-SSC (forward scatter-side scatter) dot blots and histograms. **b** Representative May-Grünwald-Giemsa-stained cytospins of conventional neutrophils (PMNs) and neutrophilic MDSCs are shown. **c** Mean fluorescence intensities of chemokine receptors CXCR4 and CCR2 in PMNs and neutrophilic MDSCs were determined by flow cytometry.

against CD66b and HLA-DR from BD Pharmingen. Anti-human CXCR4 (clone 12G5) was obtained from eBioscience. Mouse IgG1-FITC, mouse IgM-FITC, mouse IgG1-PE, and mouse IgG1-APC (BD Pharmingen) were used as isotype controls. Results were expressed as percentage of positive cells and mean fluorescence intensity. Quantifications were performed with BD CellQuest and FlowJo (FlowJo, LLC) analysis software. For all in vitro flow cytometry assays at least 3 independent experiments were performed.

In vitro Characterization and Isolation of Human MDSCs

Human MDSCs were characterized in vitro according to a previously published protocol [26]. Isolated human PBMCs from

healthy controls were cultured in 12-well flat bottom plates (Corning) or 25-cm^2 flasks (Greiner Bio-One) at 5×10^5 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated FCS (PAA Laboratories), 2 mM glutamine (Sigma-Aldrich), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Biochrom) (subsequently referred to as 'complete medium') for 6 days, and granulocyte-macrophage colony-stimulating factor (GM-CSF, 10 ng/ml; Genzyme), IL-1β, IL-1α (both PeproTech), IL-18 (MBL International), IL-33 (GenWay Biotech), S100 proteins (ProSpec), calprotectin (Hycult Biotech), and hybrid serum amyloid A (SAA; PeproTech) were added at different concentrations as indicated in the respective figures. For all assays, at least 3 independent experiments were performed. PBMCs cultured in complete medium alone were run in parallel as a control for each donor. Medium and supplements were refreshed after 3 days. After 6 days, all cells were collected from PBMC cultures. Adherent cells were removed using nonprotease cell detachment solution Detachin (Genlantis). MDSCs were determined as $SSC^{high}CD33^+CD14^-$ cells. The number of MDSCs as a percentage of all cells in medium-only cultures was set to 1-fold for every single experiment. The MDSC induction due to the specific stimuli is presented as x-fold compared to medium control. For functional assays $CD33^+$ cells were isolated from each culture using anti-CD33 magnetic microbeads and LS column separation (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated cell populations was >90%, as assessed by flow cytometry. A May-Grünwald-Giemsa-stained cytospin of these isolated $CD33^+$ cells is given in online supplementary figure S3. The complete flowcytometric characterization of surface markers of the in vitro MDSC population is shown in online supplementary figure S4.

T-Cell Suppression Assay and Cytokine Analysis in Supernatants

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 IL-2 (R&D Systems) and 1 μg/ml anti-CD3 antibody (OKT3; Janssen-Cilag). In a standardized way, 60,000 PBMCs per well in RPMI 1640 were seeded in a 96-well microtiter plate, and 10,000–30,000 MDSCs in RPMI 1640 or as control isolated conventional non-MDSC neutrophils in RPMI 1640 or RPMI 1640 only were added. Diphenyleneiodonium chloride (DPI, 1 μM; Sigma-Aldrich) and N^G-monomethyl-L-arginine (L-NMMA, 0.5 mM; Calbiochem) were added where indicated. The cell culture was supplemented with 10% heat-inactivated human serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. After 96 h of incubation in a humidified atmosphere at 37°C and 5% CO_2, the cells were harvested and supernatants were frozen at –20°C. $CD4^+$ or $CD8^+$ T cells, respectively, were gated and analyzed for CFSE dilution to determine polyclonal T-cell proliferation. Only propidium iodide (PI)-negative cells were considered for analysis. Supernatants of the cocultures were taken to perform an IFNγ ELISA (R&D Systems) and a multiplex cytokine array (Bio-Plex Pro human cytokine array; Bio-Rad Laboratories) according to the manufacturers' protocols.

Suppression of IL-1β Secretion

$CD66b^+$ MDSCs from heparinized blood of CAPS patients were isolated, as described above. $CD14^+$ cells were isolated from the $CD66b^-$ PBMC fraction using anti-CD14 magnetic microbeads and LS column separation (Miltenyi Biotec). $CD14^+$ cells were seeded in a 1:1 ratio with $CD66b^+$ MDSCs or with complete medium only and left unstimulated or were stimulated with 10 ng/ml LPS (Sigma-Aldrich) [27]. After 4 h of incubation in complete medium at 37°C and 5% CO_2, supernatants were taken and IL-1β secretion of $CD14^+$ monocytes was measured by an IL-1β ELISA (R&D Systems) according to the manufacturer's protocol. Conventional $CD66b^+$ neutrophils were run in parallel as control.

Cytokine Analysis in Serum

Multiplex cytokine array analysis in sera of CAPS patients before and 4 weeks after the start of anti-IL-1 therapy with canakinumab and of controls was performed using the Bio-Plex protein multiarray system (Bio-Plex Pro human cytokine array; Bio-Rad Laboratories). For the current experiments, a human 27-plex assay was used according to the recommendations of the manufacturer (Bio-Rad).

Statistical Analysis

Data are reported as means ± SEM. Differences between the control group and patients were analyzed by an unpaired t test assuming Gaussian distribution or a Mann-Whitney test assuming non-Gaussian distribution. Differences between different conditions within the patient cohort were analyzed by a paired t test assuming Gaussian distribution or a Wilcoxon test assuming non-Gaussian distribution. Differences between stimulants and medium control were analyzed by a one-sample t test. Correlations are described with Spearman's correlation coefficient r. Statistical tests were calculated using Prism 6.01 software (GraphPad Software). In all tests, differences were considered significant at $p < 0.05$.

Results

Neutrophilic MDSCs Are Increased in CAPS Patients

In a large CAPS patient cohort (n = 28), percentages of neutrophilic MDSCs were significantly increased compared to age-matched healthy control subjects (n = 36; $p < 0.0001$; fig. 2a). When compared to conventional neutrophils these neutrophilic MDSCs from the Ficoll low-density fraction (see Patients and Methods) showed the typical nuclear morphology of neutrophils and expressed the characteristic neutrophilic surface markers CD66b and CD16, but displayed increased expression of chemokine receptors CXCR4 and CCR2 (fig. 1), as reported previously [17, 28, 29]. All CAPS patients were under anti-IL-1 therapy with canakinumab and in clinical remission or had minimal residual disease activity at the time of blood sampling. We did not detect a significant correlation of neutrophilic MDSCs with common laboratory inflammation markers, namely ESR, CRP, and SAA (fig. 2b). However, with few exceptions, the values of these inflammation markers were within normal limits at the time of blood sampling.

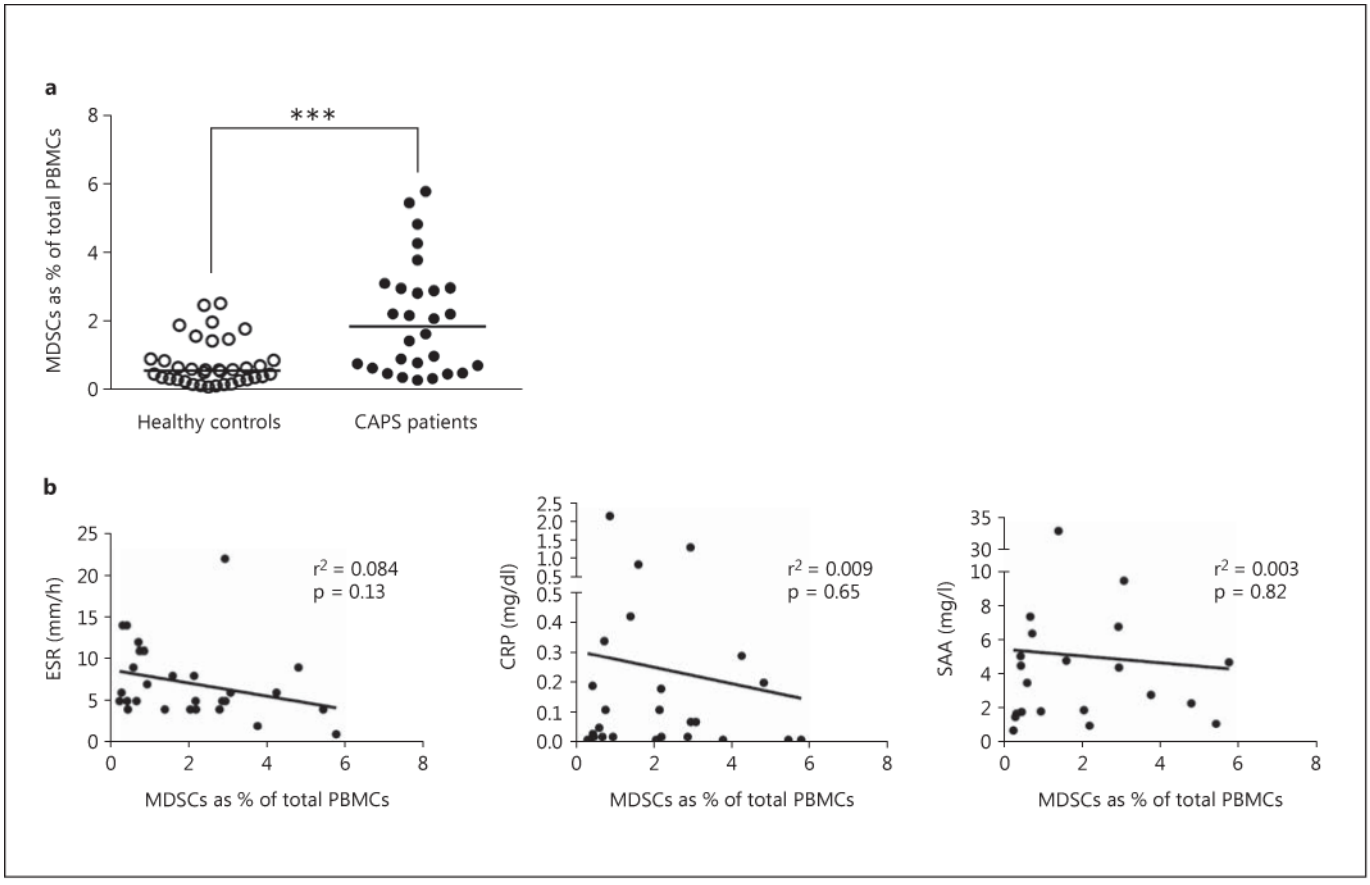
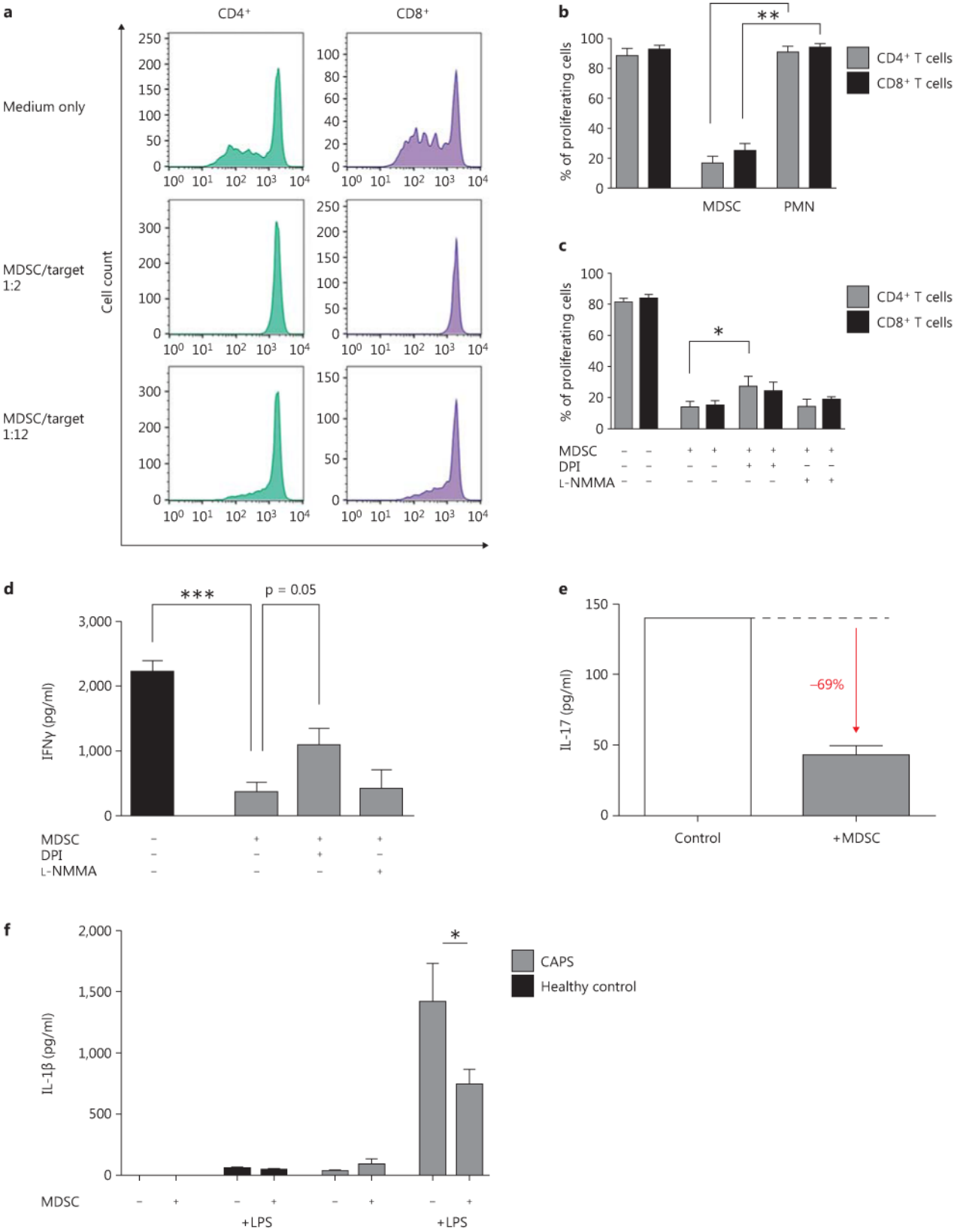


Fig. 2. MDSCs in CAPS patients. **a** Neutrophilic MDSCs are increased in CAPS patients. Percentages of MDSCs in a CAPS patient cohort (n = 28) were significantly increased compared to healthy age-matched controls (n = 36). The depicted data were assessed by flow cytometry and represent means ± SEM, whereby differences were analyzed by an unpaired t test. *** $p < 0.001$. **b** Common laboratory inflammation markers did not correlate with the number of MDSCs. Levels of ESR, CRP, and SAA as common inflammation markers were measured in clinical routine; they did not show a significant correlation with MDSCs in CAPS patients and were mostly within normal limits at the time of blood sampling. Correlation of markers with MDSCs is shown with Spearman's correlation coefficient r.

Fig. 3. MDSCs from CAPS patients are suppressive on T cells and monocytes. **a–c** MDSCs from CAPS patients are able to suppress T-cell proliferation in a dose-dependent manner involving ROS. The suppressive effect of MACS-isolated MDSCs ($CD66b^+$) from CAPS patients on T cells ($CD4^+$ and $CD8^+$ T cells) was analyzed by coculture of different MDSC-to-target (PBMC) ratios (1:2 and 1:12) and assessed by CFSE staining and flow cytometry. Shown are representative histograms (**a**) including a medium control, where PBMCs have been cultured in complete medium only, and the statistics of 3 independent experiments (**b**); conventional neutrophils (PMNs) did not have any effects on T-cell proliferation. **c** ROS were blocked by DPI and iNOS by L-NMMA. **d**, **e** IFNγ and IL-17 secretions are decreased by MDSCs. IFNγ (**d**) and IL-17 (**e**) secretions in the supernatant were measured in MDSC/PBMC coculture experiments (see **a**) by ELISA and multiplex cytokine array. **f** IL-1β secretion by monocytes is decreased by MDSCs. MDSCs ($CD66b^+$) and monocytes ($CD14^+$) were isolated by MACS from heparinized blood of CAPS patients and cocultured with or without LPS (10 ng/ml) in complete medium at 37°C and 5% CO_2. After 4 h of incubation, supernatants were analyzed for IL-1β levels with ELISA. Data are shown as means ± SEM analyzed by a paired t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(For figure see next page.)



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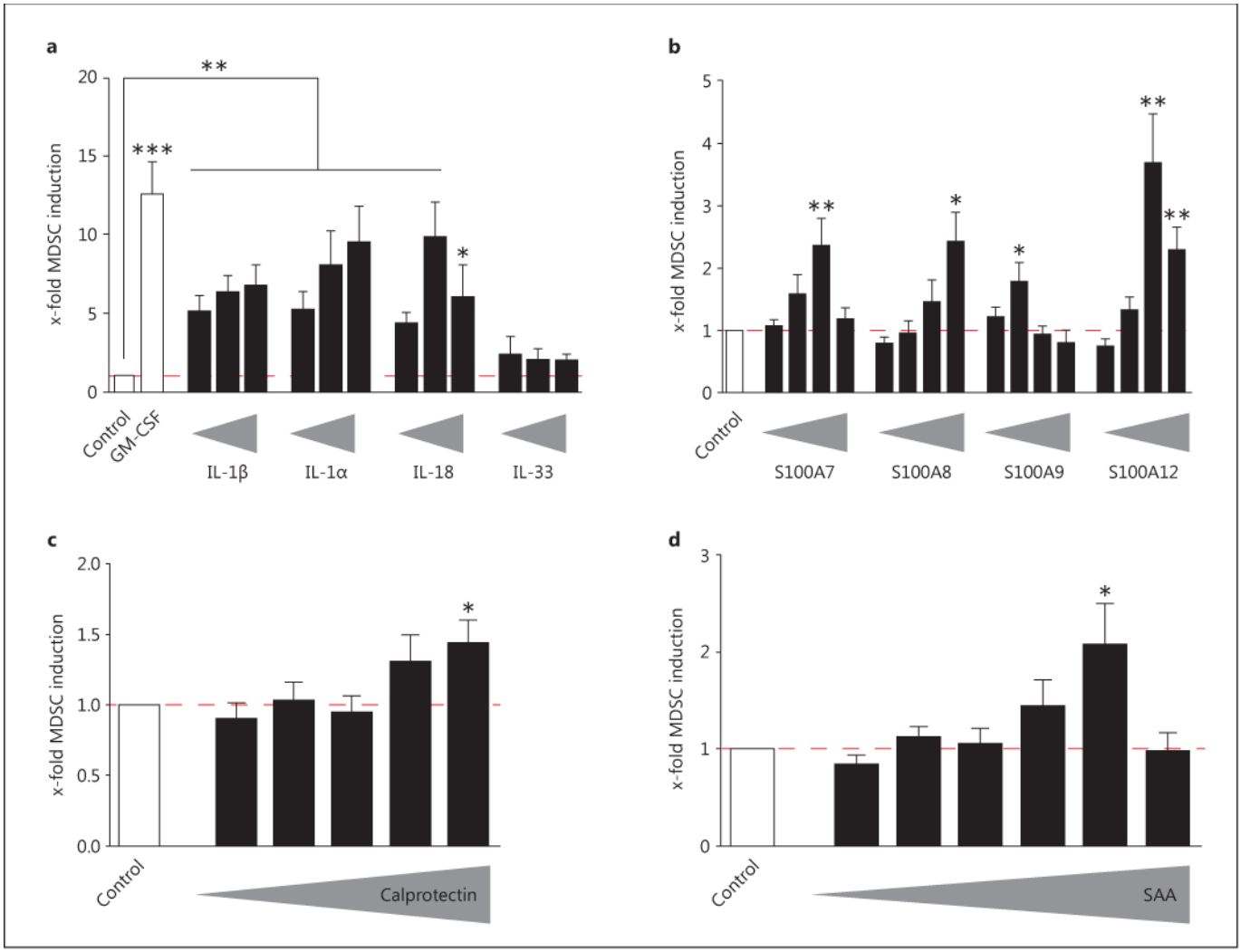

Fig. 4. Inflammasome-dependent IL-1 family cytokines and alarmins induce MDSCs in vitro. **a** IL-1 family members IL-1β, Il-1α, and IL-18 are able to induce MDSCs. PBMCs were isolated from heparinized fresh blood from healthy donors using Ficoll density gradient sedimentation. MDSCs were induced by incubating PBMCs (5×10^5/ml) with complete medium only (medium control), 10 ng/ml GM-CSF (positive control), or different concentrations (1 ng/ml to 1 μg/ml) of the respective IL-1 family cytokines. MDSCs were determined as $SSC^{high}CD33^{+}CD14^{-}$ cells. The number of MDSCs as a percentage of all cells in medium-only cultures (mean 1.69%; median 1.3%) were set to 1-fold for every single experiment. The MDSC induction due to specific stimuli is presented as x-fold compared to medium control (mean ± SEM), and differences compared to controls were analyzed by a one-sample t test. **b** S100 proteins are able to slightly induce MDSCs. General culture conditions were as described under **a**. S100 proteins S100A7, S100A8, S100A9, or S100A12 were added in different concentrations ranging from 1 ng/ml to 1 μg/ml. The x-fold induction of MDSCs compared to medium control conditions is depicted as means ± SEM, and differences were analyzed by a one-sample t test. **c** Calprotectin is able to induce MDSCs. General culture conditions were as described under **a**. Calprotectin was added in different concentrations ranging from 1 ng/ml to 2 μg/ml. The x-fold induction of MDSCs compared to medium control conditions is depicted as means ± SEM, and differences were analyzed by a one-sample t test. **d** SAA is able to induce MDSCs. General culture conditions were as described in **a**. Recombinant hybrid SAA was added in different concentrations ranging from 0.1 ng/ml to 2 μg/ml. The x-fold induction of MDSCs compared to medium control conditions is depicted as means ± SEM, and differences were analyzed by a one-sample t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

MDSCs in CAPS Patients Suppress T-Cell Proliferation, IFNγ, and IL-17 Secretion

To test the suppressive function of MDSCs in CAPS patients, we isolated neutrophilic MDSCs utilizing the magnetic bead technique and cocultured these cells together with OKT3- and IL-2-stimulated PBMCs. These experiments using T-cell CFSE labeling demonstrated that CAPS patient-derived neutrophilic MDSCs efficiently suppressed polyclonal T-cell proliferation of both $CD4^+$ and $CD8^+$ T-cell subsets in a dose-dependent manner (fig. 3a, b). Conventional neutrophils did not suppress $CD4^+$ or $CD8^+$ T-cell proliferation. To gain further insights into the mechanisms of T-cell suppression, we blocked ROS (reactive oxygen species) by DPI and NOS (nitrogen oxide synthase) by L-NMMA. These experiments showed that the production of ROS is a main mechanism, but it did not account for the entire suppressive effect (fig. 3c). In parallel with the suppression of T-cell proliferation, IFNγ secretion was diminished by MDSCs by a mechanism that also seemed to involve ROS (fig. 3d).

Since IL-17-producing T cells have been previously suggested to play a role in the pathogenesis of CAPS [8, 30], we investigated whether CAPS-isolated neutrophilic MDSCs are capable of modulating IL-17 cytokine production by $CD4^+$ T cells. These studies showed that CAPS patient-derived neutrophilic MDSCs substantially dampened released IL-17 protein (69% reduction of IL-17 protein; fig. 3e).

MDSCs in CAPS Patients Decrease IL-1β Secretion by Monocytes

Beyond the already established suppression of T-cell responses we were interested to see whether neutrophilic MDSCs would also influence the IL-1β secretion of monocytes as a cardinal pathogenetic mechanism in CAPS. Our studies demonstrate that the cocultivation of neutrophilic MDSCs with inflammasome-stimulated $CD14^+$ monocytes from CAPS patients led to a significant decrease of IL-1β in the supernatant (48% reduction of IL-1β; fig. 3f). Conventional neutrophils, however, did not show any effect on the IL-1β amount in the supernatant, which argues against an unspecific proteolytic cleavage effect by neutrophils in general (online suppl. fig. S2).

Inflammasome-Dependent IL-1 Family Cytokines and Alarmins Increase MDSCs in vitro

To analyze which factors in CAPS patients might contribute to the generation of MDSCs, we first tested IL-1 cytokine family members on their capacity to induce MDSC generation in vitro. These studies showed that the IL-1 cytokine family members IL-1β, IL-1α, and IL-18, but not IL-33, efficiently induced MDSCs to a similar extent as the well-established MDSC-inducing protein GM-CSF [26] (fig. 4a). S100 proteins, including the S100A8/A9 heterodimer calprotectin, SAA, and other increased inflammatory proteins in CAPS patients [31] with established effects on the MDSC generation in mice [32–34], elicited an overall slight impact on the MDSC amount in human in vitro cultures (fig. 4b–d).

Distinct Growth Factors and Chemokines Are Elevated in CAPS Patients

In the search for further potential MDSC-inducing factors involved in CAPS patients we applied a multiplex cytokine/growth factor array and examined frozen sera from CAPS patients before and after the initiation of an effective anti-IL-1 therapy (fig. 5; online suppl. table 1). These studies revealed that the well-established MDSC-inducing factors GM-CSF and vascular endothelial growth factor (VEGF) [10, 26, 35] were increased in CAPS sera before and still after the initiation of a clinically effective anti-IL-1 therapy (fig. 5b). Furthermore, the fibroblast growth factor (FGF), another growth factor, known to be secreted in a direct NLRP3-dependent manner [36], was increased in CAPS sera. Additionally, we found several chemokines (IL-8, MCP-1, and MIP-1α) significantly elevated in CAPS sera even under effective anti-IL-1 therapy (fig. 5c). However, a relation to MDSC induction has not yet been reported for these proteins. When viewed in combination, these studies indicate that IL-1 family cytokines on the one hand and several growth factors on the other hand seem to be involved in the generation of MDSCs in CAPS patients.

IL-1- and GM-CSF-Induced MDSCs Suppress T-Cell Proliferation and IFNγ Release

Finally, we investigated whether MDSCs induced by IL-1 family cytokines and GM-CSF exhibit a similar T-cell suppressive phenotype as ex vivo isolated neutrophilic MDSCs from CAPS patients. The proliferation assays showed that MDSCs induced in vitro with IL-1β, Il-1α, or IL-18 are able to suppress T-cell proliferation of both $CD4^+$ and $CD8^+$ T cells in a dose-dependent manner to a similar extent as GM-CSF-induced MDSCs. Results from the IFNγ secretion tests also showed a suppressive effect of in-vitro-induced MDSCs on T-cell responses, as secretion of IFNγ is markedly reduced upon cocultivation of the two cell types. Collectively, in these experiments we showed that in-vitro-induced MDSCs recapitulated the

T-cell suppressive characteristics of ex vivo isolated neutrophilic MDSCs from CAPS patients, robustly suppressing both $CD4^+$ and $CD8^+$ T-cell activities (fig. 6).

Discussion

Our studies demonstrate for the first time that neutrophilic MDSCs accumulate in a human inflammasome-driven autoinflammatory disease condition. Neutrophilic MDSCs were induced in CAPS patients under anti-IL-1 therapy and dampened inflammatory T-cell and monocytic IL-1β responses. Neutrophilic MDSCs may represent a novel autoregulatory mechanism in inflammatory diseases, which may serve as a future therapeutic target.

Thirty years ago, IL-1RA was identified as an autologous anti-inflammatory mechanism in inflammatory conditions [37]. Later, the recombinant IL-1RA anakinra was successfully used as a targeted therapy in CAPS patients [38, 39]. In this study, we identified another autologous anti-inflammatory mechanism in CAPS patients, namely neutrophilic MDSCs. With respect to their segmented nuclear morphology, these neutrophilic cells seemed to be rather mature, which is in line with previous reports demonstrating activated mature neutrophils with a suppressive phenotype [15, 25, 32, 40]. As mentioned in the introduction, the maturation stage of neutrophilic MDSCs is still a matter of debate in the field. In this paper we follow the definition of neutrophilic/granulocytic MDSCs as a heterogeneous group of T-cell suppressive immune cells with neutrophilic/granulocytic phenotypes without a clear demarcation between immature and mature cells, as discussed in detail elsewhere [18–21]. Compared to conventional neutrophils the neutrophilic MDSCs in our population exhibited increased expression of chemokine receptors CXCR4 and CCR2, as described before [17, 28, 29]. We would like to emphasize that only quantitative differences in the expression level of chemokine receptors have been identified. To the best of our knowledge, there is still no unique marker for neutrophilic MDSCs in humans.

Neutrophilic MDSCs are known to be suppressive on T and NK cells. On the other hand, highly inflammatory Th17 cells have been reported to be increased in autoinflammatory diseases/CAPS [8, 30]. Therefore, the increased MDSCs might be regarded as an autologous feedback mechanism counteracting these enhanced T-cell activities. The immunomodulation of T-cell answers was shown in our in vitro functional studies either by means of proliferation arrest or inhibition of cytokine secretion (IFNγ and IL-17). Furthermore, this is the first study to report an effect of MDSCs on the IL-1β production of monocytes. Previously, an influence on dendritic cells has been described [41], but the IL-1β secretion has not been investigated before. The mechanisms leading to the reduction of IL-1β in MDSC/monocyte cocultures might include (1) proteolytic cleavage of IL-1β, (2) binding/uptake of IL-1β and (3) direct dampening of the inflammasome activity. We could demonstrate that conventional neutrophils did not have a similar effect on the IL-1β concentration. Dampening the amount of IL-1β directly antagonizes the effect of NLRP3 hyperactivation in CAPS. MDSCs might cooperate with other innate anti-inflammatory mechanisms like IL-1RA to limit excessive inflammation in these patients. Further studies are warranted to clarify the underlying mechanisms of this observation.

According to our in vitro data IL-1-targeted therapy in CAPS patients should decrease MDSC numbers. Unfortunately, we could not analyze fresh patient samples before and after the start of IL-1-targeted therapy, as all CAPS patients were already under anti-IL-1 therapy at study entry. Intriguingly, MDSCs were elevated even under highly effective anti-IL-1 therapy and in patients

Fig. 5. Several growth factors and chemokines are elevated in CAPS patients. **a** Multiplex cytokine array analysis. Multiplex cytokine array analysis was performed with sera of CAPS patients before (n = 22) and 4 weeks after (n = 21) the initiation of anti-IL-1 therapy with canakinumab and of healthy controls (n = 5) using the Bio-Plex protein multi-array system. The color plot represents x-fold change compared to the median value of the healthy controls (color scale bar on the right). **b** Growth factors are elevated in CAPS patients. Multiplex cytokine array analysis (see **a**) revealed the well-established MDSC-inducing factors GM-CSF, VEGF, and FGF to be elevated in CAPS patients before and after the initiation of anti-IL-1 therapy. Data sets are represented as means ± SEM, and differences were analyzed by the Mann-Whitney test for unpaired or the Wilcoxon test for paired data sets. **c** Chemokines are elevated in CAPS patients. Multiplex cytokine array analysis (see **a**) revealed the chemokines MCP-1 and MIP-1α to be elevated in CAPS patients before and after the initiation of anti-IL-1 therapy. IL-8 was significantly increased only before the initiation of anti-IL-1 therapy. Data sets are represented as means ± SEM, and differences were analyzed by the Mann-Whitney test for unpaired or the Wilcoxon test for paired data sets. * $p < 0.05$.

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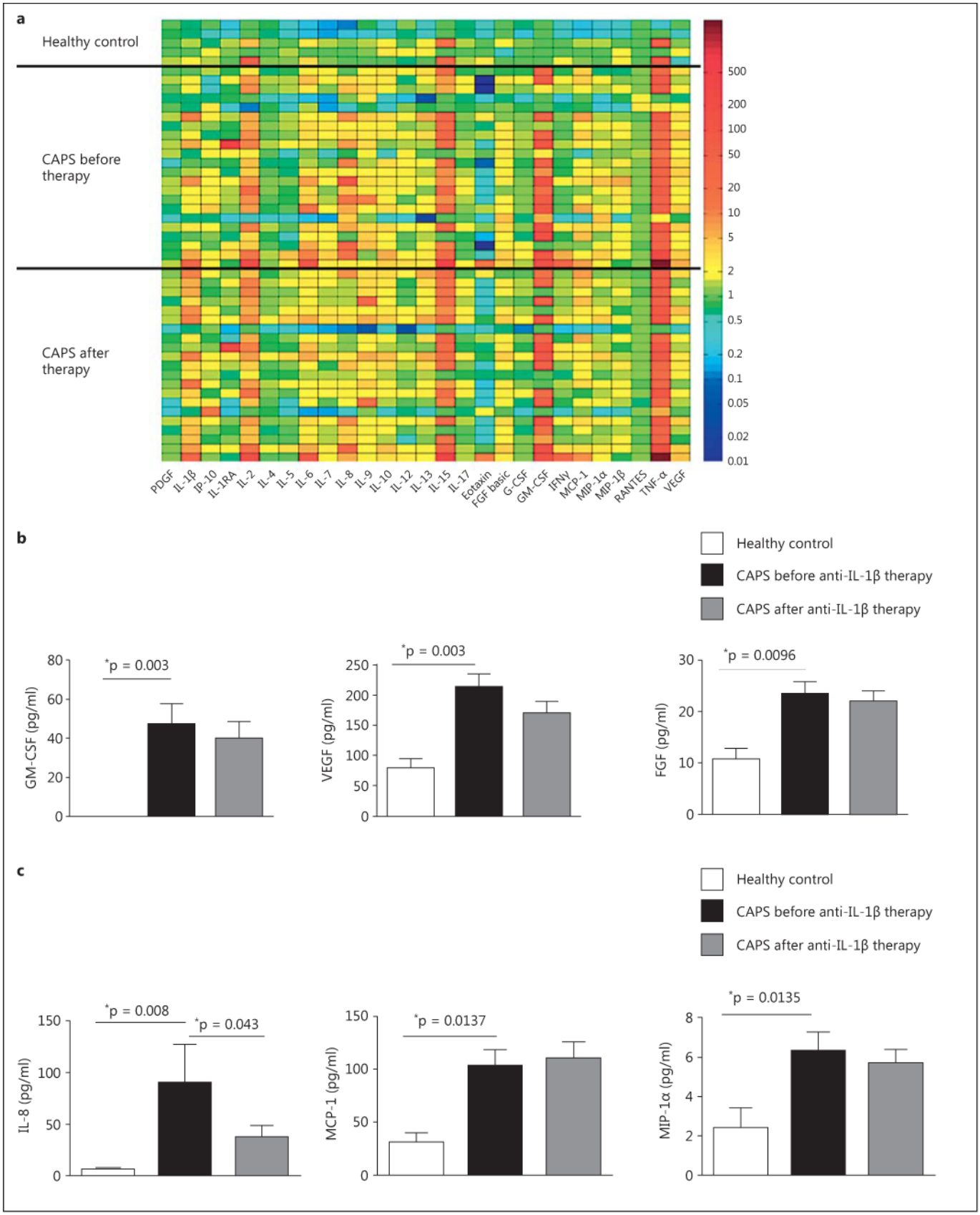

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showing clinical remission. The elevated MDSC numbers in patients with clinical remission may point to subclinical inflammatory activities possibly related to NLRP3-dependent factors other than IL-1β. It is important to note that these patients suffer from inborn defects of the NLRP3 inflammasome and have never experienced a completely normal inflammatory status. Therefore, clinical remission may be hard to define in these patients. One possible factor in the counter-regulatory feedback loop of MDSCs in autoinflammatory diseases might be SAA, which has a role in MDSC induction (e.g. in bacterial sepsis) [34]. SAA is also commonly elevated in autoinflammatory syndromes and might therefore contribute to the increase of MDSCs in these diseases. In our in vitro experiments SAA led to a slight increase in MDSCs. However, we used a recombinant hybrid SAA which elicits different inflammatory activities compared to the naturally occurring human SAA [42]. To gain further insights into which molecules might be involved in MDSC generation we applied two strategies: (1) testing of established NLRP3-dependent IL-1 family cytokines for their capacity to induce MDSCs in vitro and (2) examination of sera from CAPS patients with a multiplex cytokine/growth factor array in comparison to healthy controls. The experiments with IL-1 family members exhibited a strong inducing effect on MDSC generation for IL-1β, which has been reported previously [22, 26]. A new finding from our study was, however, that this effect is not restricted to IL-1β, but that other IL-1 family members, particularly IL-1α and IL-18, had an even higher potency in inducing MDSCs than IL-1β. A recent report showed monocytic MDSC induction by IL-18 in mice [24]. These IL-1 family members may contribute to elevated MDSCs in CAPS patients even after effective anti-IL-1β therapy. IL-33, on the other hand, had virtually no influence on MDSC generation in our studies. Analyses of sera from CAPS patients primarily exhibited elevated serum levels for growth factors and chemokines, which has not been reported before. Most of these factors did not decrease under an effective anti-IL-1β therapy, suggesting that these elevations may not depend on IL-1β secretion. For example, FGF secretion has been demonstrated to be NLRP3 dependent, but independent from catalytic caspase-1 activity and IL-1β [36]. The increased growth factors and chemokines may also point to a subclinical inflammation even under successful anti-IL-1 therapy and, together with the increased number of MDSCs, may become valuable disease biomarkers. Here, larger longitudinal studies are needed for a comprehensive evaluation. A thorough long-term observation of chronic inflammatory changes in CAPS patients is already warranted. This is underscored by the fact that several of these factors have been associated with chronic disease manifestations, like FGF with organ fibrosis [43] and VEGF with arteriosclerosis [44, 45].

Remarkably, two of the identified elevated growth factors in CAPS sera, GM-CSF and VEGF, have consistently been reported as MDSC-inducing factors [10, 26, 35]. We and others used GM-CSF as a positive control in MDSC induction assays. Therefore, our study identified several factors that may well contribute to the increased MDSC numbers even under anti-IL-1 therapy. For the moment we can only speculate on the role these cells may have in the chronic disease process. However, T cells have been shown to be considerably skewed towards highly inflammatory subtypes like Th17 cells in autoinflammatory diseases [8, 30]. MDSCs as a physiological anti-inflammatory response may counteract this dysregulated activity.

In vivo generation of MDSCs or ex vivo expansion and adoptive transfer may become a future therapeutic strategy in severe cases or in specific complications of autoinflammation not sufficiently responding to conventional therapies. The established application of mesenchymal stem cells in entities like GvHD (graft-versus-host disease) [46] may also point the way for cell-based therapies in certain autoinflammatory disease states. Possible components in this approach, already employed as medical therapies for other indications, might be GM-CSF or

Fig. 6. IL-1 family members increase T-cell suppressive MDSCs in vitro. **a** MDSCs were generated by incubating PBMCs (5×10^5/ml) from heparinized fresh blood with GM-CSF (10 ng/ml) as positive control or IL-1β (10 ng/ml), IL-1α (100 ng/ml), or IL-18 (100 ng/ml). After 6 days, MDSCs were isolated using MACS separation and cocultured with PBMCs from healthy donors stained with CFSE in different ratios (1:2 and 1:6). Proliferation was analyzed after 4 days by flow cytometry. For each cytokine at least 3 independent experiments were conducted. Respective histograms are shown. **b** The graph shows the percentage (means ± SEM) of proliferating $CD4^+$ (upper panel) and $CD8^+$ (lower panel) T cells without MDSCs (negative control) or in coculture with MDSCs, induced either with GM-CSF (positive control) or the respective IL-1 family members. Differences were analyzed by an unpaired t test. **c** IFNγ secretion by T cells was analyzed via ELISA of supernatants of coculture of MDSCs and T cells (see **a**). Data sets are represented as means ± SEM, and differences were analyzed by an unpaired t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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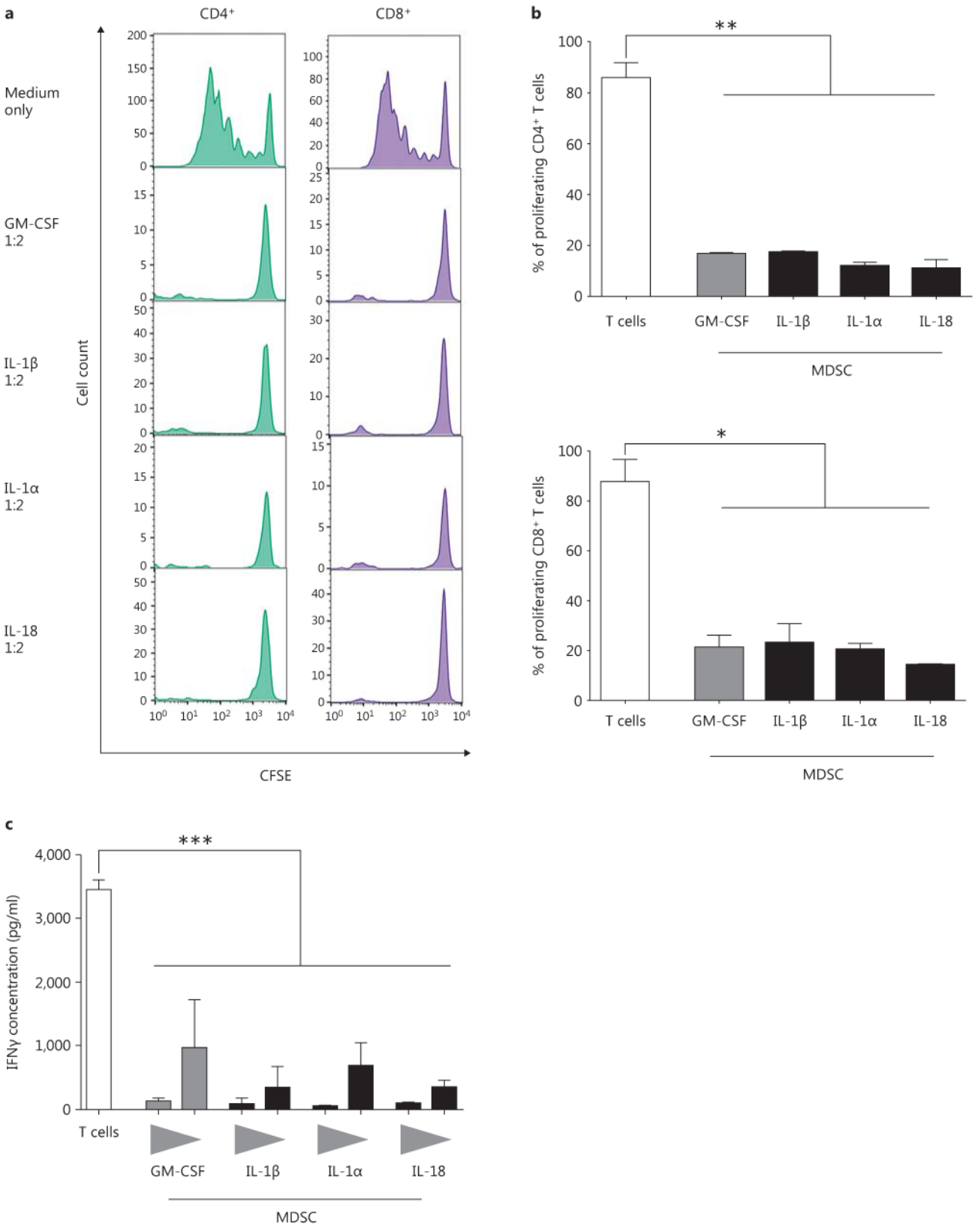

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physical therapies like extracorporal photopheresis, which has recently been associated with the induction of MDSCs in GvHD [47].

Beyond these studies, we have preliminary data that the increased percentages of MDSCs are also found in other autoinflammatory diseases, such as TRAPS (tumor necrosis factor receptor-associated periodic syndrome), FMF (familial Mediterranean fever) and PFAPA (periodic fever, aphthous stomatitis, pharyngitis and adenitis; data not shown), and might represent a common mechanism of counter-regulation in autoinflammatory diseases. This could also provide a new rationale for the observed strong periodicity of some of these syndromes, in particular PFAPA [48]. Inflammation (due to multiple reasons, e.g. chronic infections or chronic inflammasome activation) would induce the generation of MDSCs from myeloid progenitor cells in the bone marrow over several days to weeks. These MDSCs would then decrease inflammation, followed by a disappearance of MDSCs over several days due to fading inflammatory stimuli. The disappearance of MDSCs would again allow the rise of inflammation, leading to a new cycle of progenitor cell differentiation.

Taken together, our studies demonstrate that neutrophilic MDSCs are induced in CAPS patients under anti-IL-1 therapy and reveal several MDSC-inducing factors in these patients. Increased MDSCs represent a novel autologous anti-inflammatory mechanism in autoinflammatory conditions by limiting both inflammatory T-cell and monocyte responses. MDSCs may serve as a future therapeutic target in autoinflammation.

Acknowledgments

This study was supported by the German Research Foundation (DFG) Emmy Noether Programme (HA 5274/3-1 to D.H.) and an unrestricted research grant from Novartis to N.R., D.H., and J.K.-D.

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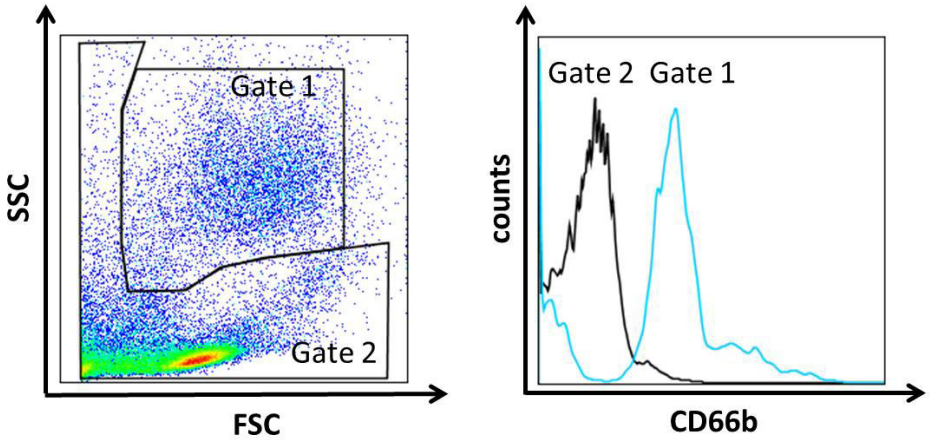
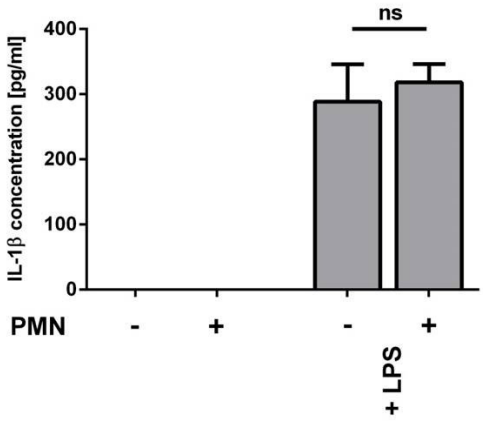
Figure S1



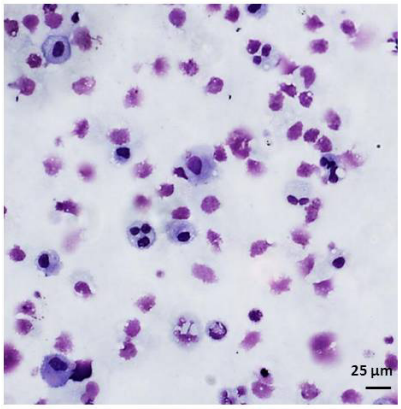
CD66b expression in cells from the PBMC fraction:
The PBMC fraction was prepared from heparinized blood samples by Ficoll density gradient sedimentation. In CAPS patients , FACS forward side scatter revealed an $FSC^{high}SSC^{high}$ "granulocytic" population (Gate 1). Only cells out of Gate 1 were CD66b positive.

Figure S2



Conventional PMNs do not decrease IL-1β secretion from CAPS monocytes:
Conventional "high-density" PMNs ($CD66b^+$) and monocytes ($CD14^+$) were MACS-isolated from heparinized blood of CAPS patients and cocultured with or without LPS (10 ng/ml) in complete medium at 37°C, 5% CO_2. After 4 hours of incubation, supernatants were analyzed for IL-1β-levels with ELISA. Data are shown as means ± SEMs analyzed by a paired t-test.

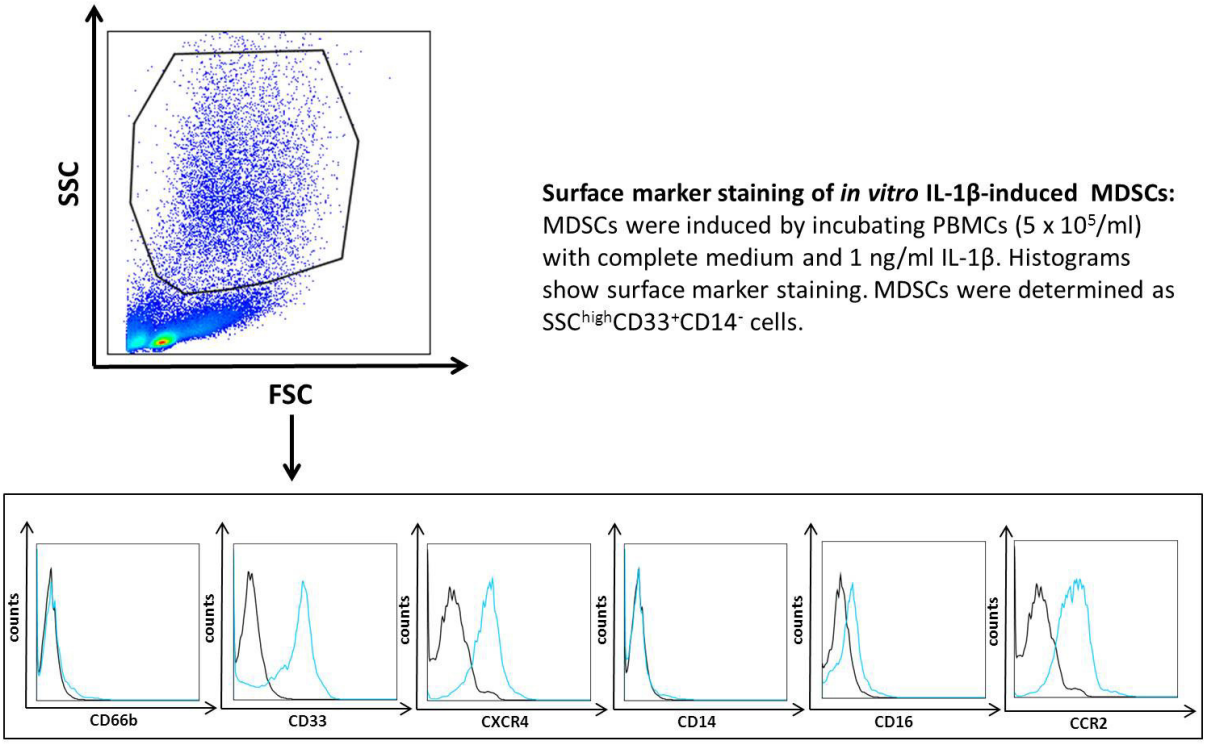
Figure S3



IL-1β MDSCs
in vitro

Cytospins of *in vitro* IL-1β-induced MDSCs:
MDSCs were induced by incubating PBMCs (5 x 10^5/ml) with complete medium and 1 ng/ml IL-1β. $CD33^+$ were MACS-isolated and cytospins were stained with May–Gruenwald–Giemsa.

Figure S4



Surface marker staining of *in vitro* IL-1β-induced MDSCs:
MDSCs were induced by incubating PBMCs (5 x 10^5/ml) with complete medium and 1 ng/ml IL-1β. Histograms show surface marker staining. MDSCs were determined as $SSC^{high}CD33^+CD14^-$ cells.

Supplemental Table S1

all: [pg/µl]

PDGF			IL-1b		
Healthy	CAPS before	CAPS after	Healthy	CAPS before	CAPS after
12316,62	11833,66	17126,18	0	1,6	14,94
10275,33	19285,2	19877,15	0	2,88	9,17
13277,79	14144,61	18939,39	2,39	2,69	8,86
13209,14	8002,62	9341,82	1,59	0	5,28
18247,06	13895,48	17366,16	2,75	0	13,4
	18794,7	18267,49		14,88	16,26
	8973,8	7053,68		2,21	0
	16943,5	11729,23		2,59	4,46
	18781,54	12951,29		8,16	2,41
	19467,32	17543,17		0	15,14
	6279,98	11525,01		1,45	4,87
	13294,69	13431,04		2,17	4,28
	19340,62	16905,79		13,99	18,62
	19663,88	20910,67		3,52	6,28
	12918,75	5349,08		2,7	4,24
	9473,19	4501,52		2,56	1,04
	7469,97	18576,76		0	5,77
	18520,67	8308,35		4,38	7,87
	8210,27	14725,17		1,63	7,51
	9950,5	16545,98		3,59	11,39
	9957,07	15802,93		8,89	52,44
	18226,67			55,26	
Median					
13209,14	13595,085	15802,93	1,59	2,64	7,51

IP-10			IL-1ra		
Healthy	CAPS before	CAPS after	Healthy	CAPS before	CAPS after
443,46	583,09	950,09	24,53	93,16	119,96
234,19	206,09	410,78	40,82	175,98	186,54
286,26	217,99	371,53	140,25	89,91	128,89
851,2	319,07	479,6	118,34	85,85	152,43
599,35	1254,24	752,39	106,97	42,68	107,78
	833,2	967,52		151,62	249,18
	576,22	514,04		78,53	17,64
	284,41	348,63		93,16	59,81
	831,01	577,22		45504,42	19242,75
	824,6	835,87		186,88	263,84
	409,19	853,29		78,53	166,24
	864,61	632,57		76,91	73,65
	990,82	991,01		250,81	111,03
	843,77	368,15		143,5	171,11
	748,64	1125,11		141,88	35,28
	1612,69	5323,36		125,64	24,52
	305,51	876,67		31,64	127,27
	896,8	522,9		109,41	88,29
	494,8	369,9		77,72	115,9
	459,23	938,34		282,6	182,48
	714,43	507,97		319,33	68,77
	721,96			94,79	
Median					
443,46	718,195	632,57	106,97	102,1	119,96

		IL-2				IL-4	
Healthy		CAPS before	CAPS after	Healthy		CAPS before	CAPS after
	0	2,65	5,03		5,87	5,58	6,1
	0	6,28	11,12		5,01	11,87	11,17
	4,35	7,83	9,99		9,54	9,46	9,92
	0	0	7,26		7,94	4,44	9,52
	46,01	0,11	5,32		9,15	6,26	10,32
		5,18	8,81			7,25	14,21
		5,87	0			8,63	3,64
		7,95	5,42			8,24	6,71
		9,74	10,66			10,99	5,91
		1,64	7,32			8,4	15,13
		4,42	4,13			5,74	8,56
		3,79	1,65			7,48	6,55
		6,77	6,67			10,22	10,74
		22,78	15,42			9,75	9,83
		7,57	4,23			8,38	7,37
		6,41	0			6,84	6,36
		0,56	7,32			3,92	9,57
		10,34	4,77			11,05	5,8
		3,3	4,35			6,15	8,23
		3,86	13,94			8,32	12,74
		12,6	155,29			10,45	7,39
		175,86				10,24	
Median							
	0	6,075	6,67		7,94	8,35	8,56

		IL-5				IL-6	
Healthy		CAPS before	CAPS after	Healthy		CAPS before	CAPS after
	0	1,8	2,76		0	7,68	8,79
	0	2,88	3,68		0	21,4	11,34
	2,1	2,98	2,35		11,52	11,24	10,89
	2,81	0	2,93		4,71	0	8,86
	2,4	0	3,75		13,91	6,69	11,1
		2,93	8,72			10,29	25,74
		2,93	0			15,81	0
		2,71	2,32			13,63	7,79
		3,47	1,7			12,85	23,1
		0	6,59			5,16	27,56
		1,33	2,74			6,99	9,88
		1,51	1,75			12,82	7,06
		2,47	3,49			30,17	10,96
		1,56	2			14,75	9,49
		1,78	0,95			18,28	9,91
		2,2	0			57,19	0,64
		0	2,64			0	14,26
		2,13	1,98			17,29	8,2
		1,13	2,15			9,67	11,48
		1,41	6,33			26,52	28,88
		9,79	2,2			51,68	360,55
		3,56				406,27	
Median							
	2,1	1,965	2,35		4,71	13,24	10,89

	IL-7			IL-8		
Healthy		CAPS before	CAPS after	Healthy		CAPS before	CAPS after
	0	10,98	12,32		0	9,46	81,42
	0	12,19	13,98		2,33	28,78	15,19
	8,98	7,53	9,83		10,48	41,56	12,84
	6,77	0	10,71		9,59	10,99	14,5
	7,67	0	16,97		9,71	4,5	16,41
		11,75	20,6			82,15	27,49
		16,45	0			19,53	0
		15,79	11,82			31,81	12,09
		9,49	9,25			66,19	10,93
		3,64	18,92			7,84	25,48
		6,35	5,89			104,02	46,21
		12,62	10,64			16,68	13,07
		14,48	11,32			828,4	17,43
		8,12	13,55			42,52	16,81
		9,59	7,29			120,36	22,38
		14,64	0			55,26	4,56
		0	13,29			33,87	74,39
		18,47	9,8			50,17	33,48
		15,6	10,07			8,65	34,61
		12,42	19,15			240,03	252,05
		23,97	12,09			152,81	49,73
		19,08				26,47	
Median							
	6,77	11,97	11,32		9,59	37,715	17,43

	IL-9			IL-10		
Healthy		CAPS before	CAPS after	Healthy		CAPS before	CAPS after
	14,13	34,14	52,46		0	6,3	7,71
	5,35	43,39	57,29		0	5,62	5,81
	25,76	52,84	54,83		3,88	3,8	4,66
	19,63	45,28	376		5,65	0	5,23
	17,17	14,45	37,21		2,34	0	5,98
		60,14	95,69			7,73	9,54
		30,79	0			4,49	0
		43,39	32,5			6,31	4,08
		52	34,81			3,05	3,18
		28,36	98,79			1,16	10,45
		25,91	46,58			7,32	6,93
		35,04	28,56			5,16	3,16
		52,69	60,37			5,98	4,1
		32,27	36,91			3,83	5,19
		177,78	278,63			4,04	3,08
		38,94	18,88			7,1	0
		18,88	35,26			0	4,72
		49,17	28,86			7,29	4,63
		64,78	35,26			4,55	3,33
		78,58	38,56			4,43	10,68
		21,51	31,23			13,56	7,16
		37,36				9,22	
Median							
	17,17	41,165	37,21		2,34	4,855	4,72

	IL-12			IL-13		
Healthy		CAPS before	CAPS after	Healthy	CAPS before	CAPS after
	27,77	38,17	45,66	1,84	11,42	20,61
	16,02	15,48	18,06	1,62	5,93	10,9
	22,6	26,77	23,37	6,41	4,68	5,57
	36,2	4,49	24,36	6,36	0,19	7,87
	15	29,58	46,29	3,49	2,67	9,71
		37,37	44,09		20,31	20,22
		26,04	0		5,43	0
		43,57	22,33		10,81	11,01
		31,45	27,18		4,92	4,32
		39,12	45,66		3,76	21,7
		44,09	31,27		6,33	11
		38,36	23,05		5,38	5,22
		40,91	25,04		6,7	6,78
		36,29	44,28		4,23	5,93
		21,08	23,32		6,94	4,32
		38,17	15,44		7,36	1,64
		6,29	47,72		0,08	8,88
		49,99	20,19		8,19	5,01
		23,5	10,88		5,04	3,8
		20,63	52,55		6,33	6,51
		52,4	53,9		8,91	7,94
		68,4			9,44	
Median						
	22,6	36,83	25,04	3,49	6,13	6,78

	IL-15			IL-17		
Healthy		CAPS before	CAPS after	Healthy	CAPS before	CAPS after
	0	18,38	20,72	24,77	26,12	35,57
	0	6,78	11,75	25,76	64,36	67,83
	11,39	5,62	13,11	51,11	57,91	58,41
	0	0	14,04	35,39	24,2	49,38
	22,11	0	8,52	49,26	25,9	56,43
		24,48	15,89		41,11	84,11
		23,62	0		36,8	16,56
		30,55	33,65		45,92	34,16
		17,88	20,5		50,74	29,55
		0	18,01		49,6	86,22
		14,77	14,08		26,79	42,84
		8,24	0		47,65	29,37
		7,96	7,53		65,1	63,86
		24,56	19,11		56,55	59,4
		8,76	9,22		49,5	35,33
		19,24	0		45,43	22,8
		0	17,79		36,27	49,13
		23,19	0		59,71	32,62
		0	13,29		26	37,42
		9,94	31,98		33,11	55,25
		20,63	325,88		31,52	49,94
		442,06			60,39	
Median						
	0	12,355	14,04	35,39	45,675	49,13

		Eotaxin				FGF	
Healthy		CAPS before	CAPS after	Healthy		CAPS before	CAPS after
	64,87	109,57	303,48		10,11	8,46	10,19
	58,94	0	60,49		9,92	27,95	27,79
	122,31	0	52,1		18,13	16,02	18,36
	120,59	33,38	40,91		4,34	6,77	11,08
	307,65	110,82	71,73		10,43	12,58	24,03
		321,24	95,66			12,84	37,85
		79,22	62,16			23,97	6,36
		173,93	165,69			26,96	17,5
		53,73	85,38			27,95	23,81
		120,96	97,23			25,44	40,21
		11,27	50,76			18,24	23,3
		51,65	75,49			17,5	8,52
		40,53	31,85			27,68	27,07
		43,67	42,75			13,01	19,26
		49,48	38,6			22,41	18,81
		167,72	256,96			22,08	9,52
		36,13	64,99			50,67	30,37
		135,4	50,08			32,98	21,57
		21,07	46,55			20,56	21,29
		0	114,19			22,08	36,55
		66,79	1124,1			40,05	26,52
		1447,39				40,53	
Median							
	120,59	52,69	64,99		10,11	22,245	21,57

		G-CSF				GM-CSF	
Healthy		CAPS before	CAPS after	Healthy		CAPS before	CAPS after
	10,52	12,71	16,12		0	29,16	16,08
	3,81	28,99	23,26		0	56,36	27,02
	20,95	18,06	22,68		0	2,71	0
	16,12	6,38	16,7		0	0	39,38
	16,22	13,74	24,69		0	0	0
		18,45	32,79			26,43	58,82
		17,86	2,04			1,32	0
		21,63	18,25			31,03	28,2
		22,11	13,98			91,63	96,9
		18,88	35,53			2,4	81,86
		12,42	16,7			43,33	45,67
		16,9	11,63			8,07	0
		19,22	19,99			98,14	0
		18,06	28,32			15,01	20,7
		19,8	11,43			60,19	47,87
		19,6	6,4			82,2	0
		10,11	14,37			5,68	88,84
		20,86	11,43			117,7	17,09
		11,43	17,86			125,49	7,56
		26,7	20,57			0	133,43
		32,41	17,57			79,53	119,84
		24,6				167,34	
Median							
	16,12	18,665	17,57		0	30,095	27,02

	IFN-g			MCP-1		
Healthy		CAPS before	CAPS after	Healthy	CAPS before	CAPS after
	60,28	88,53	134,2	7,25	128,55	249,23
	47,59	200,77	158,97	7,59	145,19	167,51
	118,31	152,8	169,37	53,31	66,95	75,5
	113,95	38,1	128,44	36,62	38,04	178,8
	235,51	74,57	176,43	46,73	28,85	91,67
		140,9	284,47		263,03	84,96
		117,83	25,48		84,13	12,16
		106,66	99,83		148,24	202,21
		163,7	287,65		91,53	83,37
		120,8	311,66		18,92	86,98
		96,4	127,47		74,32	76,31
		91,48	79,61		64,06	67,02
		193,77	166,06		112,3	115,61
		122,66	147,57		42,18	28,48
		104,23	95,42		84,59	79,86
		115,89	50,76		214,78	46,92
		53,93	223,51		15,8	113,19
		279,47	119,76		126,26	84,52
		86,55	123,14		161,24	41,42
		126,51	384,29		42,93	105,76
		587,99	2885,15		63,79	309,75
		3890,79			255,2	
Median						
	113,95	119,315	147,57	36,62	84,36	84,96

	MIP-1a			MIP-1b		
Healthy		CAPS before	CAPS after	Healthy	CAPS before	CAPS after
	0	2,37	4,25	30,82	42,83	77,58
	0	5,47	5,95	27,41	82,28	90,38
	5,12	4,6	3,84	62,86	115,86	112,6
	3,41	0	5,3	134,65	48,59	83,04
	3,47	0	6,1	102,78	27,54	55,79
		4,48	13,28		77,16	107,96
		5,62	0		104,62	44,12
		5,22	4,48		147,26	140,96
		6,49	5,3		90,23	49,03
		4,08	11,87		74,68	99,13
		5,35	5,04		152,79	160,22
		5,62	3,64		93,11	91,04
		16,24	6,99		345,43	115,42
		6,04	5,41		156,01	124,02
		5,22	6,21		153,19	85,65
		6,18	0		115,72	33,84
		17,38	8,42		28,22	119,1
		11,2	4,02		114,38	90,57
		3,93	4,55		106,27	110,52
		5,85	9,01		133,15	131,67
		11,92	5,54		127,19	54,54
		5,88			48,08	
Median						
	3,41	5,545	5,3	62,86	105,445	91,04

	RANTES			TNF-a		
Healthy		CAPS before	CAPS after	Healthy	CAPS before	CAPS after
	2610,32	2911,63	2660,65	0,37	21,08	50,08
	2426,89	2872,49	2794,62	0	68,95	58,99
	2612,28	2856,9	2699,32	41,51	54,36	61,66
	2508,67	4736,65	2868,92	0	0	44,01
	2462,17	5963,24	2785,43	64,33	2,49	68,24
		2699,46	2868,77		63,44	64,68
		2810,45	2722,64		36,5	0
		2730,06	2780,4		19,64	22,7
		2903,58	2865,22		34	64,68
		3575,08	2837,17		12,56	76,77
		2807,3	2880,41		35,07	28,8
		2813,9	2713,49		39,72	21,26
		2928,64	2594,49		39,72	29,7
		2832,16	2752,59		59,7	80,32
		2713,06	1759,26		38,65	20,9
		2614,7	3044,52		44,55	6,08
		3164,53	2686,52		2,2	57,38
		2755,2	2832,46		76,42	34
		2836,26	2577,93		37,93	34
		2615,24	2718,04		31,13	122,84
		2766,88	2803,86		181,8	2095,04
		2708,37			2144,88	
Median						
	2508,67	2823,03	2780,4	0,37	38,29	50,08

	VEGF	
Healthy	CAPS before	CAPS after
78,18	254,53	225,04
43,21	121,06	91,02
95,11	132,18	141,15
128,16	47,3	117,65
46,69	174,41	332,96
	230,69	215,43
	156,02	40
	350,46	182,47
	148,27	115,22
	178,16	190,36
	265,92	206,23
	279,48	130,78
	256,77	99,55
	147,15	154,24
	162,91	113,43
	301,76	79,4
	63,8	325,49
	304,29	115,85
	169,47	63,54
	172,28	278,92
	272,22	345,58
	485,35	
Median		
78,18	176,285	141,15

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, Hartl D.
Pathogenic fungi regulate immunity by inducing neutrophilic myeloid-derived suppressor cells. Cell Host Microbe 03/2015

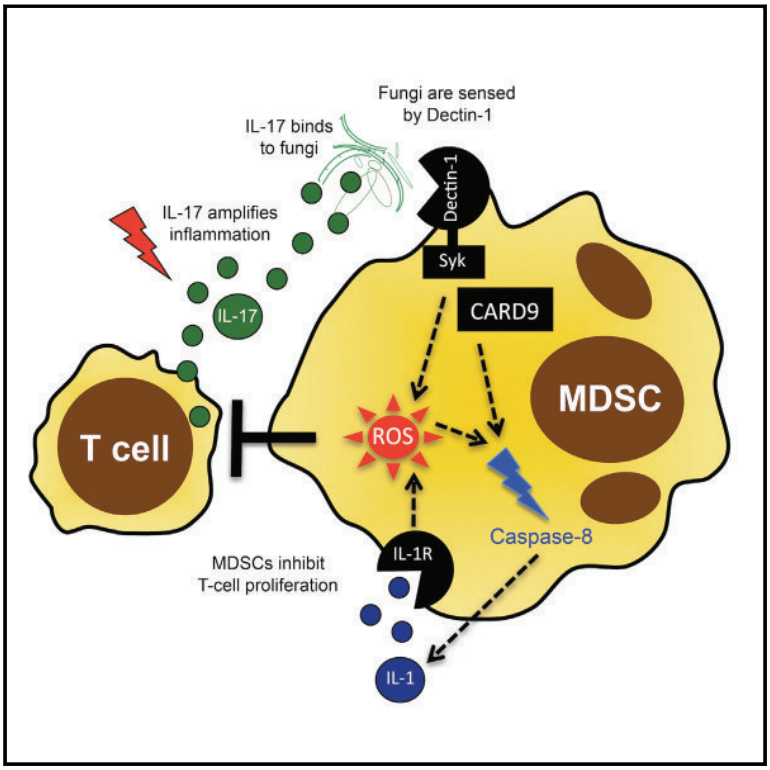
(DOI:10.1016/j.chom.2015.02.007)

Short Article

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/CARD as well as downstream ROS and IL-1β 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

Rieber et al., 2015, Cell Host & Microbe *17*, 507–514
April 8, 2015
http://dx.doi.org/10.1016/j.chom.2015.02.007

CellPress

Cell Host & Microbe
Short Article

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

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http://dx.doi.org/10.1016/j.chom.2015.02.007

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-1β 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-1β) 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-1β is involved in MDSC homeostasis (Bruchard et al., 2013). We observed an accumulation of intracellular IL-1β protein in $CD33^+$ myeloid cells followed by IL-1β release upon Dectin-1 ligand- and fungal-driven MDSC induction (Figure 4A). IL-1β 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-1β 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-1β 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-1β production (Figure 4C) and the potential of fungi to induce MDSCs (Figure 4C). Conversely, supplementing

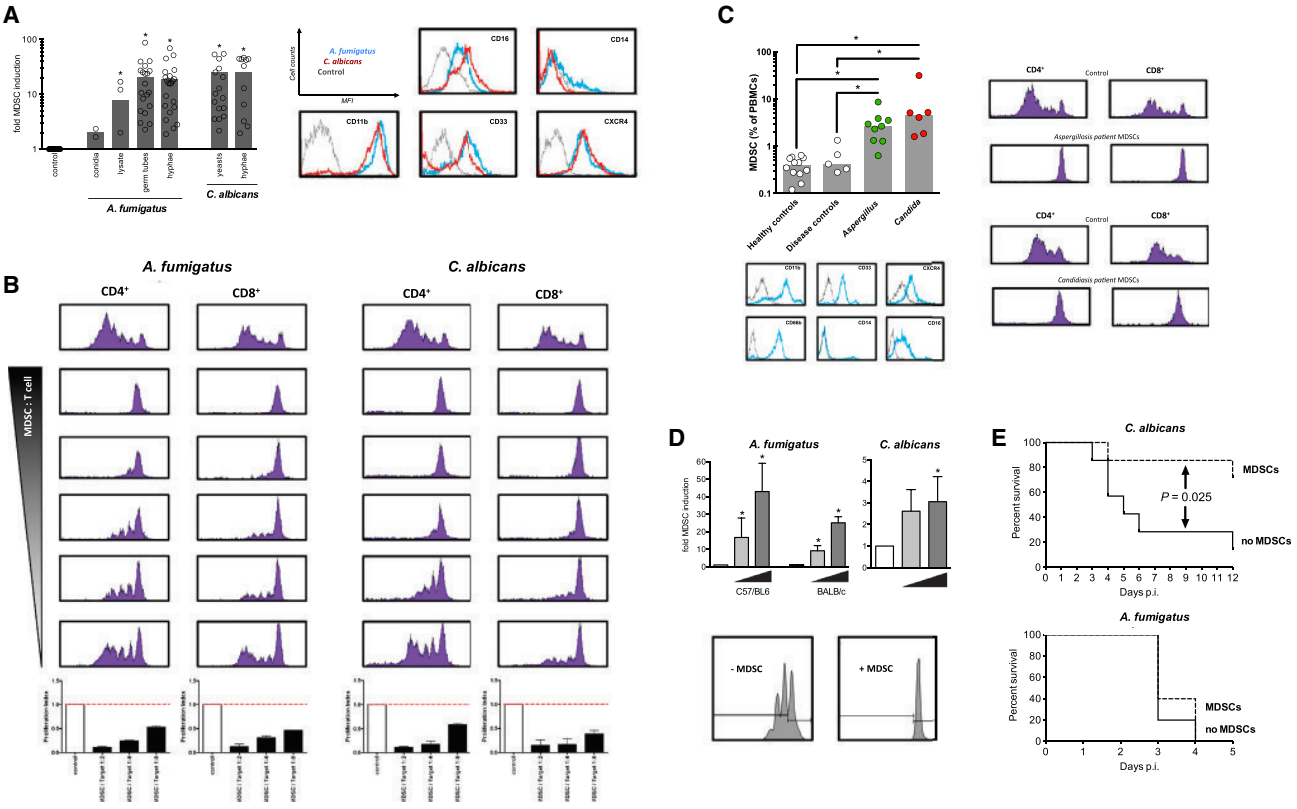

Figure 1. Fungi Induce Functional MDSCs In Vitro and In Vivo

(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 ± 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 ± 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.

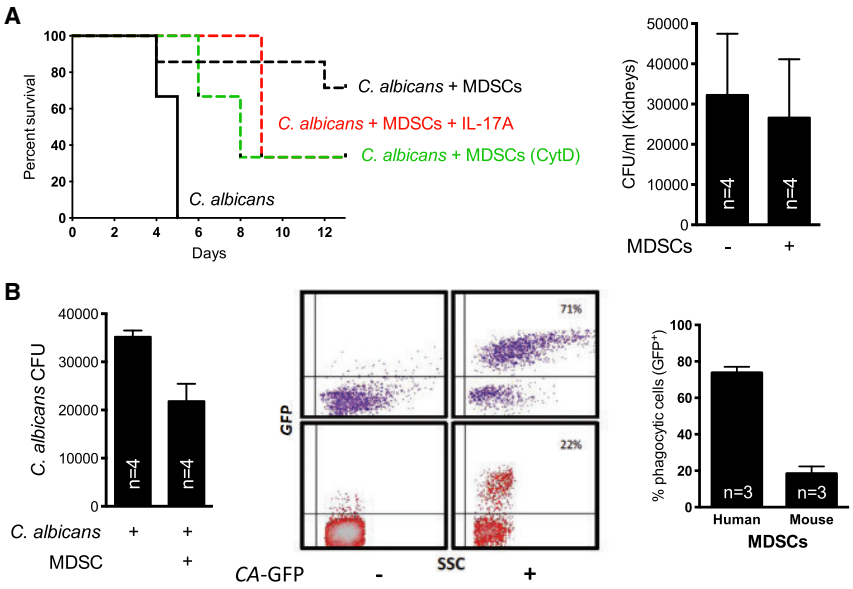

Figure 2. Antifungal Functions

(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 μg/ml, green line) or with recombinant mouse IL-17A protein (5 μg/mouse, red line).

Right panel: *C. albicans* CFUs in kidneys of BALB/c mice 5 days after adoptive transfer of MDSCs. Bars represent means ± 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 μl 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-1β 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-1β and found that ROS inhibition dampened caspase-8 activity in response to fungi (Figure S4D). IL-1β, in turn, induced ROS production during MDSC culture, suggesting a positive feedback loop between caspase-8, IL-1β, and ROS in MDSC generation (Figures S4E and S4F).

DISCUSSION

While the complete genetic deletion of pro-inflammatory cytokines, particularly TNF-α, IL-1α/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-1β. This effect seems to be specific for neutrophilic MDSCs, since monocytic MDSCs were unchanged under our experimental conditions and were previously found to be downregulated by β-glucans in tumor-bearing mice (Tian et al., 2013).

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

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

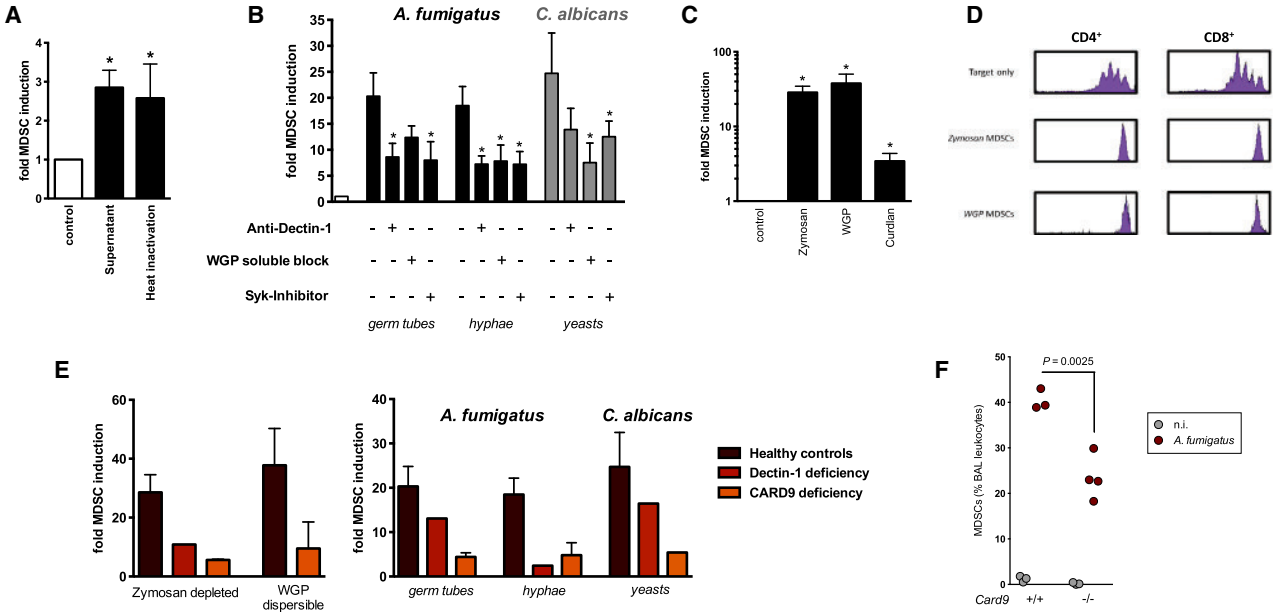

Figure 3. Fungi Induce MDSCs through a Dectin-1-, Syk-, and CARD9-Mediated Mechanism

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

(B) Dectin-1 and Syk are involved in fungal MDSC induction. MDSCs were generated in vitro by incubating isolated PBMCs (5 × 10^5 cells/ml) with *A. fumigatus* germ tubes (1 × 10^5/ml), hyphae (1 × 10^5/ml), and *C. albicans* yeasts (1 × 10^5/ml) for 6 days. Where indicated, PBMCs were pretreated for 60 min with anti-Dectin-1 blocking antibody (15 μg/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 μg/ml), dispersible WGP (20 μg/ml), or curdlan (10 μg/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 μg/ml) or dispersible WGP (20 μg/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 μg/ml) or dispersible WGP (20 μg/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 ± 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.

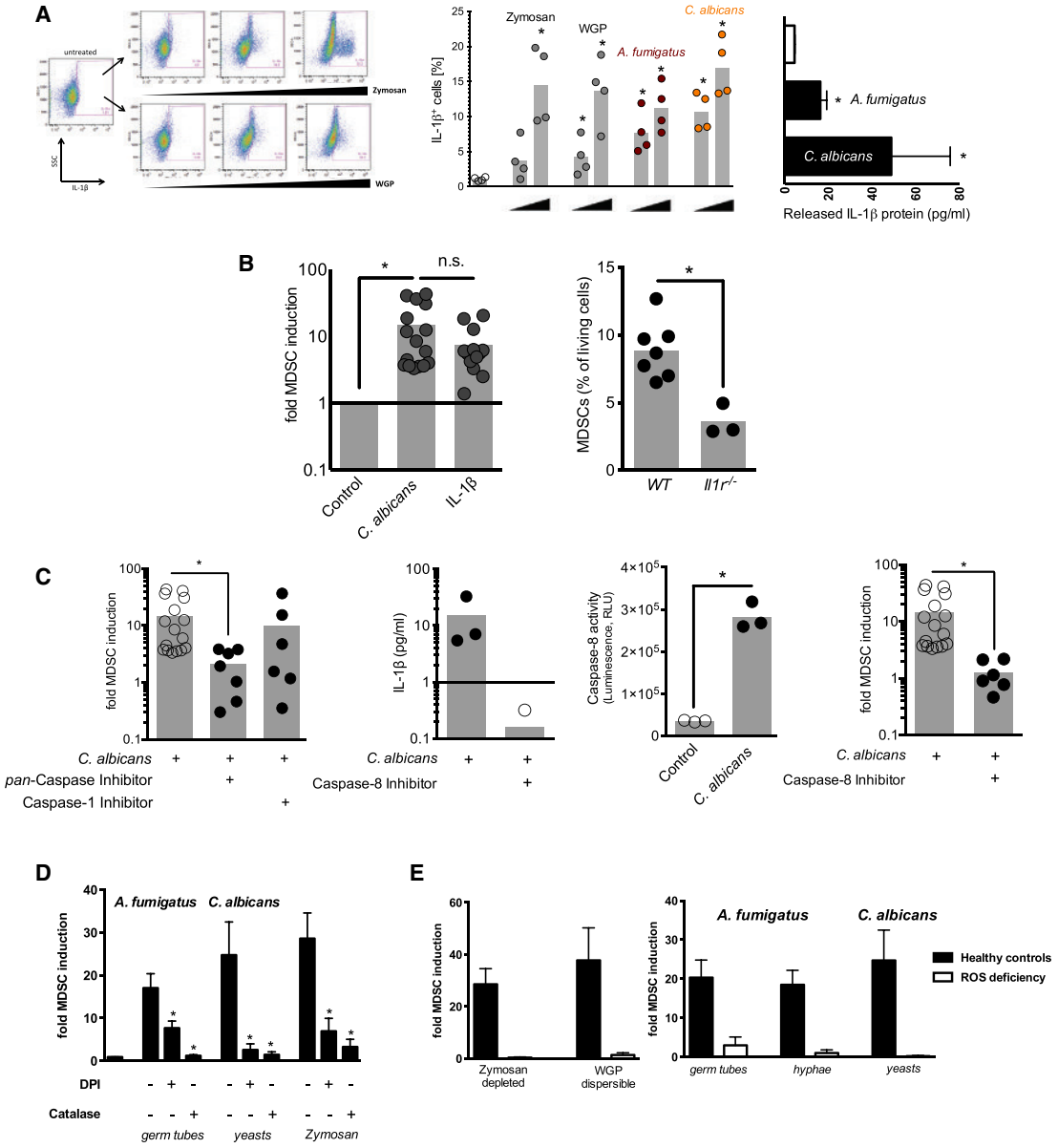

Figure 4. Fungal MDSC Induction Involves IL-1β, Caspase-8, and ROS

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

Left panel: gating strategy for intracellular cytokine staining. IL-1β was analyzed in $CD33^+$ myeloid cells using intracellular cytokine staining and flow cytometry. Zymosan depleted (20, 100, and 500 μg/ml) and WGP dispersible (20, 100, and 500 μg/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-1β 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-1β was quantified by ELISA. *$p < 0.05$ versus medium control conditions.

(B) IL-1β 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-1β protein (0.01 μg/ml) for 6 days. *$p < 0.05$.

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

(C) Fungal MDSC generation involves caspase-8. MDSCs were generated in vitro by incubating isolated PBMCs (5×10^5 cells/ml) with *C. albicans* yeasts (1×10^5/ml) for 6 days with or without pretreatment (where indicated) with the pan-caspase inhibitor Z-VAD-FMK (10 μM), the caspase-1 inhibitor Z-WEHD-FMK (50 μM), or the caspase-8 inhibitor Z-IETD-FMK (50 μM). IL-1β 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.

ACKNOWLEDGMENTS

We thank Gundula Notheis, University of Munich, and Thomas Lehrnbecher, University of Frankfurt, for patient samples. We thank Manfred Kneilling, University of Tübingen, for *Il1r*$^{-/-}$ mice. *Dectin-1*$^{-/-}$ mice were from Uwe Ritter, University of Regensburg, and originally generated by Gordon Brown, University of Aberdeen. We thank Steffen Rupp, Fraunhofer IGB Stuttgart, for the *C. albicans*-GFP strain TG6. This work was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, Emmy Noether Programme HA 5274/3-1 to D.H., the CRC/SFB685 to D.H. and A.N.R.W., and the TR/CRC124 FungiNet to A.B. and J. Loeffler), the Deutsche José Carreras Leukämie-Stiftung (DJCLS R 10/15 to A.B.), and the UK Wellcome Trust (to G.D.B.).

Received: September 30, 2014
Revised: December 17, 2014
Accepted: January 26, 2015
Published: March 12, 2015

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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 μg/ml) for 6 days. PBMCs were pretreated where indicated with the NADPH oxidase inhibitor DPI (0.1 μM) or the H_2O_2 converting enzyme catalase (100 U/l). *$p < 0.05$ blocking versus unblocked conditions.
(E) Fungal MDSC induction in patients with ROS deficiency.
Left panel: MDSCs were generated in vitro by incubating isolated PBMCs (5 × 10^5 cells/ml) from healthy controls (n = 12) or patients with CGD (n = 3) with the Dectin-1/CARD9 ligands zymosan depleted (10 μg/ml) or dispersible WGP (20 μg/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 ± 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

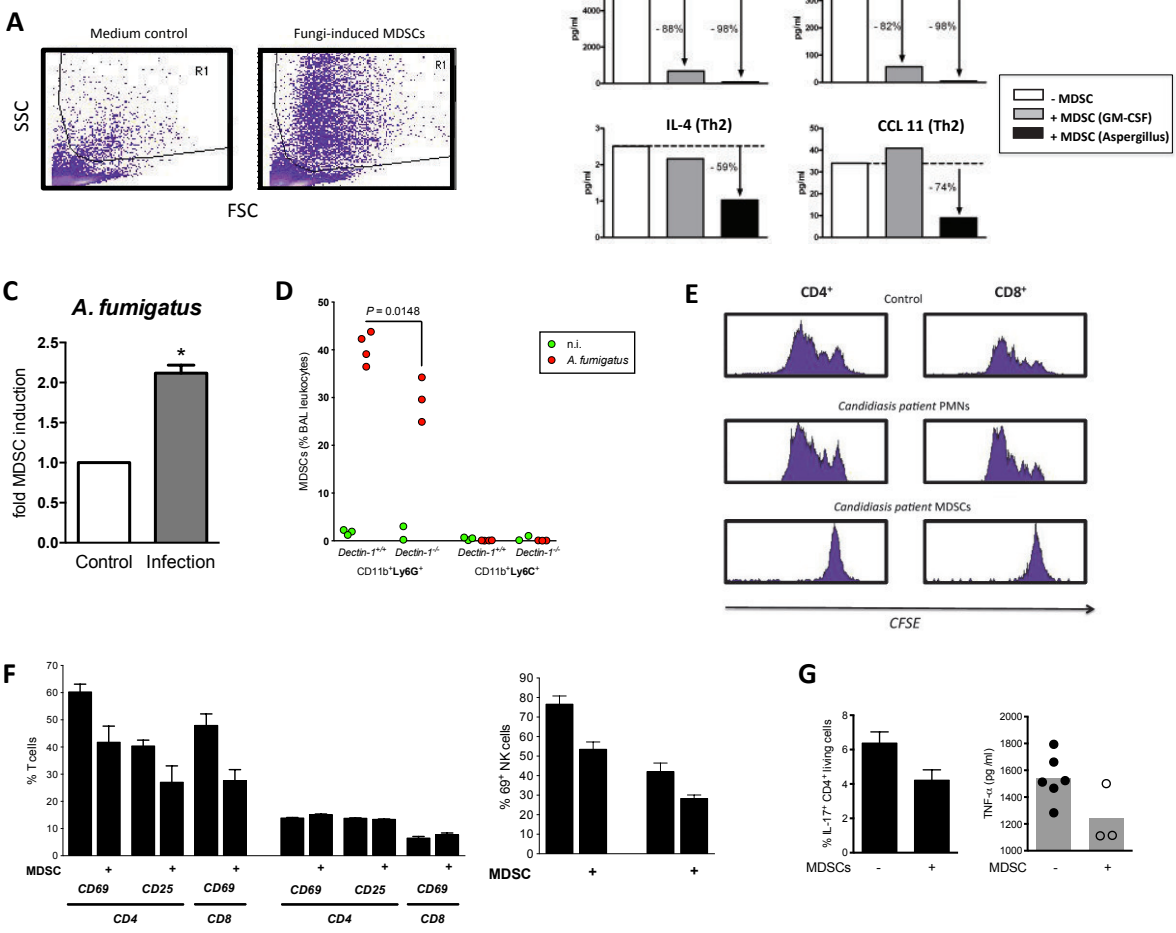

Figure S1. Phenotypic and functional MDSC characteristics

A. FSC/SSC characteristics of fungi-induced MDSCs *in vitro*
MDSCs were generated by incubating PBMCs (5 x 10^5 /ml) from healthy donors with medium only (negative control, 'Medium control') or *A. fumigatus* germ tubes (1 x 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 1x10^3 *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 ± s.e.m. **P*<0.05

D. MDSC induction in *Dectin-1*$^{-/-}$ mice
MDSC induction in *Dectin1*$^{-/-}$ mice: *Dectin-1*$^{-/-}$ mice and age-matched wildtype mice were challenged intranasally with 1x10^6 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

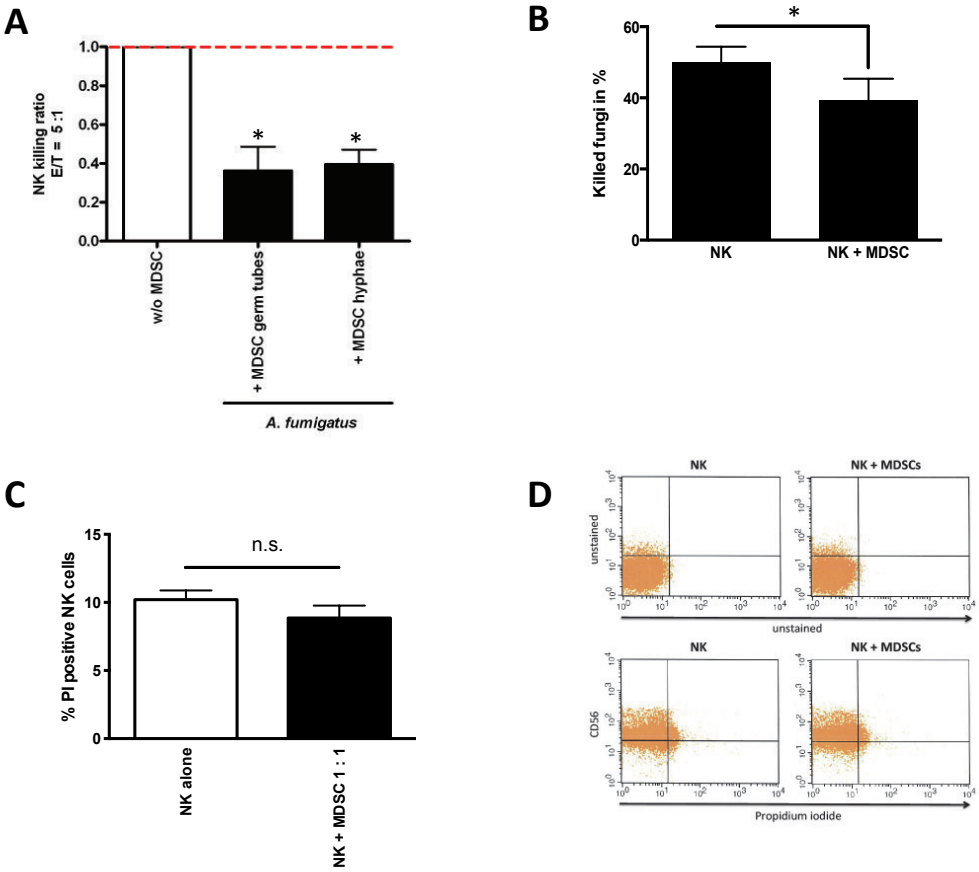

Figure S2: *Aspergillus*-induced MDSCs decrease antifungal NK killing activity

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 ($5x10^5$/ml) from healthy donors with *A. fumigatus* germ tubes ($1x10^5$/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

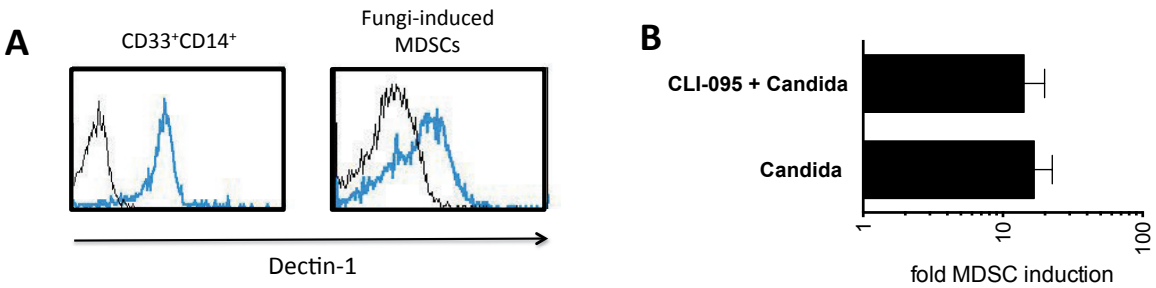

Figure S3: Dectin-1 and TLR4

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 ($5x10^5$/ml) from healthy donors with *C. albicans* (yeasts: $1x10^5$/ml) with or without 1h pretreatment with the TLR4 inhibitor CLI-095 (1µM). The x-fold induction of MDSCs compared to control conditions is depicted. Bars represent means ± s.e.m.

Figure S4, related to Figure 4

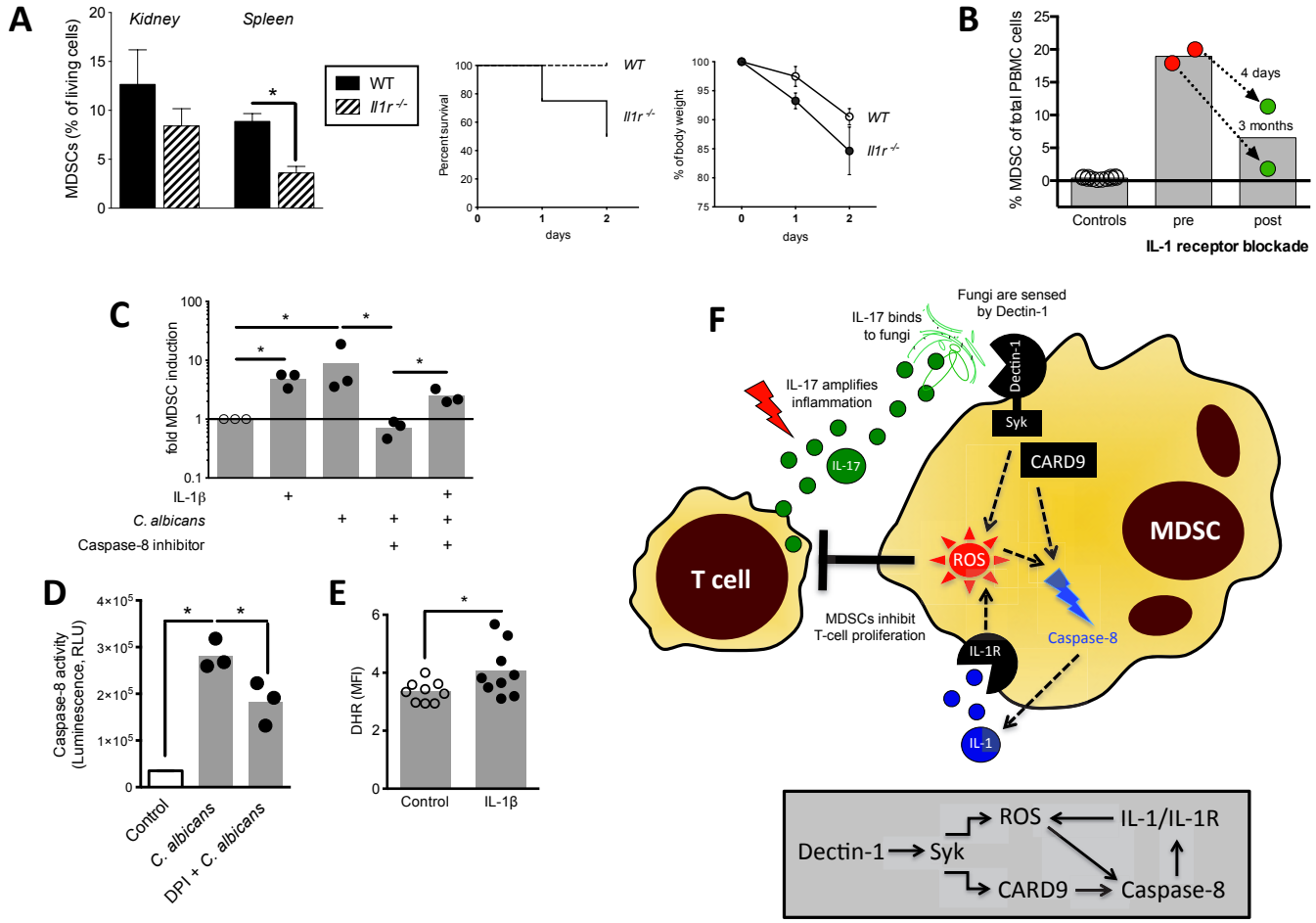

Figure S4: IL-1, Caspase-8 and ROS

A. WT and *Il1r*$^{-/-}$ mice were i.v. injected with 1×10^5 blastospores of *Candida albicans* SC5314 per animal in 100 µl 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 ± 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 (5x10^5 cells/ml) with *C. albicans* yeasts (1x10^5/ml) or recombinant human IL-1β (0.01 µg/ml) for 6 days with or without pretreatment with the caspase-8 inhibitor Z-IETD-FMK (50µM). MDSCs were quantified using flow cytometry. Bars represent means. **P*<0.05

D. MDSCs were generated *in vitro* by incubating isolated PBMCs (5x10^5 cells/ml) with *C. albicans* yeasts (1x10^5/ml) for 6 days with or without pretreatment with the NADPH oxidase inhibitor DPI (0.1 µM). 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-1β (0.1 µg/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 cm^2 flasks (Greiner Bio-One) at 5 x 10^5 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 Biotech) 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 Biotech. 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% CO_2 cells were harvested and supernatants were frozen in -20°C. For mouse T-cell suppression assays, $CD11b^+Ly6G^+$ MDSCs were isolated from bone-marrows using MACS (MDSC isolation kit, Miltenyi Biotec, Germany) and were co-cultured for three days (37°C, 5% CO_2) 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% CO_2). Brefeldin A (Sigma) was added (c_{final} = 10 µg/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 $CD33^+IL\text{-}1\beta^+$ 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-1β 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 µl 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 $1x10^4$ or $1x10^6$ *A. fumigatus* conidia for three days. At the fourth day, a bronchoalveolar lavage (BAL) was performed and $CD11b^+Ly6G^+$ and $CD11b^+Ly6C^+$ cells were quantified in BAL fluid by FACS. *C. albicans* (strain SC5314) was grown at 30°C overnight in liquid YPD (yeast extract, peptone, and dextrose) medium containing penicillin and streptomycin. Cells were collected by centrifugation, washed and resuspended in PBS. Required cell density was adjusted using a haemocytometer. For infection, female C57BL/6 mice were injected via the lateral tail vein with 2.5×10^5 or 5×10^5 blastospores per animal in 200 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 $4\text{-}5\times10^6$ MDSCs per animal into eight to twelve weeks old (18–22 g) female *BALB/c* mice via lateral tail vein. Two hours after the MDSC transfer, mice were i.v. injected with 1×10^5 blastospores of *C. albicans* (SC5314 in 100 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 where challenged intranasally with $1x10^3$ or $2x10^5$ *A. fumigatus* conidia (freshly harvested from three days old plates). For survival studies, mice were challenged once with *A. fumigatus*, for MDSC induction studies for three consecutive days, as indicated in the respective figure legends. Where indicated, MDSC transfer was performed by intravenous injection of 4×10^6 MDSCs per animal prior to infection and mice were monitored for survival as described above.

Statistical analysis

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

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Paper 3

Mack I, Hector A, **Ballbach M**, Kohlhäufl J, Fuchs KJ, Weber A, Mall MA, Hartl D.
The role of chitin, chitinases, and chitinase-like proteins in pediatric lung diseases.
Molecular and Cellular Pediatrics 02/2015

(DOI 10.1186/s40348-015-0014-6)

Mack et al. Molecular and Cellular Pediatrics (2015) 2:3
DOI 10.1186/s40348-015-0014-6

Molecular and Cellular Pediatrics
a SpringerOpen Journal

REVIEW Open Access

The role of chitin, chitinases, and chitinase-like proteins in pediatric lung diseases

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Abstract

Chitin, after cellulose, the second most abundant biopolymer on earth, is a key component of insects, fungi, and house-dust mites. Lower life forms are endowed with chitinases to defend themselves against chitin-bearing pathogens. Unexpectedly, humans were also found to express chitinases as well as chitinase-like proteins that modulate immune responses. Particularly, increased levels of the chitinase-like protein YKL-40 have been associated with severe asthma, cystic fibrosis, and other inflammatory disease conditions. Here, we summarize and discuss the potential role of chitin, chitinases, and chitinase-like proteins in pediatric lung diseases.

Keywords: Inflammation; Lung diseases; Asthma; Cystic fibrosis; Chitin; Chitinases; Chitinase-like proteins; Chitotriosidase; AMCase; CHIT1; YKL-40; BRP-39; Fungi; M2 macrophages

Introduction

The role of chitin and chitinases has been firmly established in the field of plant and microbial immunity by demonstrating that host-derived chitinases cleave chitin to protect against invading chitin-bearing pathogens, such as fungi. Although mammals lack endogenous chitin or chitin synthases, chitinases and chitinase-like proteins are endogenously expressed in their lung and other organs. Particularly, chitinase-like proteins have been described as dysregulated in a variety of diseases characterized by chronic inflammation and tissue remodeling, yet their potential role for humans has just recently begun to evolve [1,2]. Chitin is a major component of a variety of allergy-triggering environmental components, including house-dust mites or fungal spores, and fungal asthma is increasingly appreciated as an under-diagnosed disease entity [3]. Thus, an understanding of the complex immunological and pathophysiological implications of chitin-chitinase interactions in the human body is of high relevance for identifying new biomarkers and therapeutic targets for fungal diseases and other conditions, where chitin-coated microbial derivatives play a critical role. Here, we provide an overview of an emerging, yet complex field of research. Of particular interest are interspecies differences with resulting specific nomenclatures. Subsequent to an overall introduction of chitin, the role of chitinases and chitinase-like proteins in pediatric lung diseases are reviewed, leading up to a summary of ideas how these mechanisms could be exploited to improve diagnostics and therapeutics in lung diseases in childhood and beyond (Figure 1).

Review

Chitin

Chitin, a polymer of *N*-acetylglucosamine and the second most abundant polysaccharide in nature following cellulose, is an essential component of fungi, house-dust mites, exoskeletons of crabs, shrimp and insects, parasitic nematodes, and digestive tracts of many insects [1]. Chitin protects these microbes from their environment, and its turnover is regulated by biosynthesis and degradation through endogenous chitinases.

The first immune stimulatory activity of chitin and chitin derivatives in mammals was discovered and extensively explored in the middle to late 1980s, as reviewed recently [2,1]. These early studies clearly indicated that chitin has important immunologic effects *in vitro* and *in vivo*, initially highlighted by Shibata et al. who

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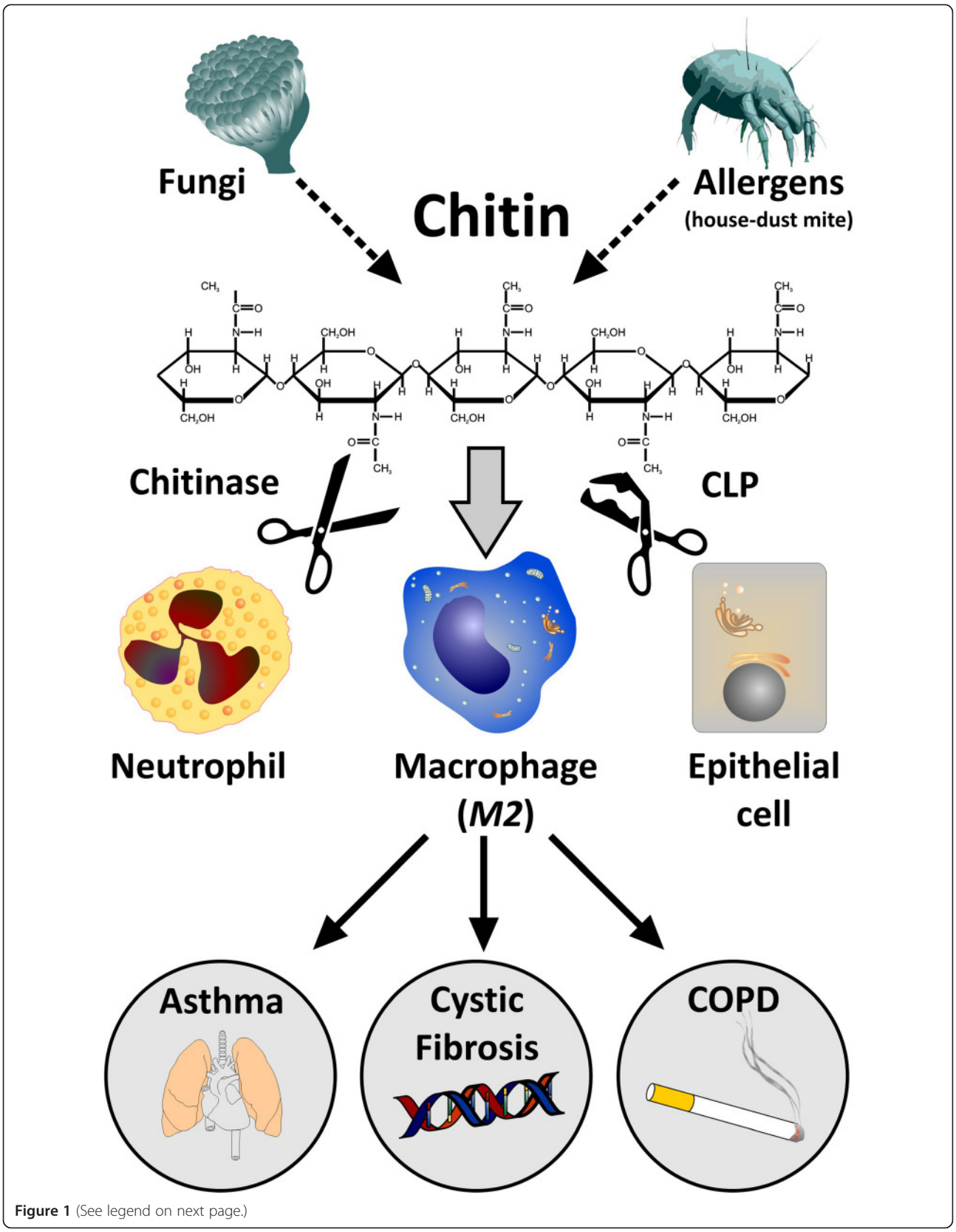

Figure 1 (See legend on next page.)

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Figure 1 The proposed role of chitin, chitinases and chitinase-like proteins (CLPs) in lung diseases. Chitin is a common component of allergy-triggering environmental components, including fungal spores and house-dust mites, which trigger an innate immune response, including chitinases (cleaving chitin; scissors) and chitinase-like proteins (binding, but not cleaving chitin; damaged scissors). Chitinases and chitinase-like proteins are mainly secreted by neutrophils, alternatively activated macrophages (M2 macrophages) and epithelial cells. The interplay of M2 macrophages, neutrophils, and epithelial cells drives inflammation and remodeling in chronic lung diseases, particularly asthma, cystic fibrosis, and COPD.

demonstrated that chitin activates peritoneal/alveolar macrophages and natural killer (NK) cells to express a number of pro-inflammatory cytokines such as interleukin-1β (IL-1β), colony-stimulating factor (CSF), and gamma interferon (IFN-γ) [4]. More recent studies by Reese et al. addressed the *in vivo* immune effects of chitin in mice [5]. They noted that after several hours of chitin exposure, innate immune cells were recruited to the lung and/or the peritoneum. These studies also demonstrated that chitin induced alternative macrophage activation and that macrophage depletion with clodronate liposome treatment prevented the recruitment of eosinophils. Van Dyken et al. further demonstrated that fungal chitin from asthma-associated home environments induced allergic eosinophilic lung inflammation [6]. Collectively, these studies strongly suggest that chitin can contribute to the development of allergic type 2 (Th2) inflammation by activating innate immune cells. Beyond these findings, chitin has also been proposed to serve as an immunoadjuvant. In murine asthma models, Shibata et al. demonstrated that orally administered chitin suppressed the production of Th2 cytokines and IgE [7] and, when used as an adjuvant, chitin caused similar effects as a Th1-promoting adjuvant [8,9]. Accordingly, one can speculate that the immune system senses chitin and that chitin can skew the Th1/Th2 immune response in a bidirectional way.

The studies noted above suggest that the size of the chitin fragment is a crucial determinant of the effector responses that it elicits. This can be seen in comparisons of large chitin polymers that are biologically inert and intermediately sized fragments, which trigger IL-17, IL-23, and TNF-α production [1], while even smaller fragments enhance the production of the anti-inflammatory cytokine IL-10. Therefore, chitin has size-dependent effects on murine immune cell function and may bind to different receptors in a size-dependent manner, similar to findings in plants [10,11]. The observation that large chitin polymers are inert, smaller fragments are pro-inflammatory, and even smaller fragments exert an anti-inflammatory effect allows for an interesting hypothesis regarding the importance of the size-dependent effects of chitin in this response. However, in all of these studies, it is difficult to rule out that chitin preparations contained mixtures of differently long chitin polymers or that 'large' chitin preparations were containing contaminating 'smaller' chitin fragments. Clearly, a more thorough study using well-defined chitin fragments is warranted. Collectively, it is tempting to speculate that chitin recognition by pattern recognition receptors triggers the induction of chitinases, leading to the generation of small-sized chitin particles that are taken up by the host cell. Recently, fungal chitin was found to dampen inflammation through IL-10 induction mediated by activation of the intracellular receptors NOD2 and TLR9 [12]. Despite these intriguing insights, the precise molecular recognition principles of chitin perception remain incompletely understood. Interestingly, this hypothesis is very similar to the established biology of another polysaccharide, hyaluronin that also serves as an alarm signal after degradation. Thus, the ability of appropriately sized polysaccharides to induce inflammation may be a more general principle of glycobiology [2,1]. When viewed in combination, chitin is a central component of potential pro-allergic microbes (e.g., *Aspergillus fumigatus* and house-dust mite) and has been shown to drive Th2-associated immune responses. Mechanisms that interfere with chitin metabolism are therefore of high relevance for allergic diseases and infections with chitin-bearing pathogens such as fungi.

Chitinases and chitinase-like proteins

Chitin-degrading enzymes, known as chitinases, are produced by humans and other mammals and are part of the 18-glycosyl-hydrolase family that encompasses both enzymatically active chitinases and chitinase-like proteins, the latter also termed chi-lectins, which lack enzymatic activity. In humans, acidic mammalian chitinase (AMCase), chitotriosidase, oviductin, and human cartilage glycoprotein (HcGP)-39/YKL-40 and YKL-39 have been described, while YM-1, YM-2, AMCase, oviductin, and breast regression protein (BRP-39) have been identified in mice [13,1,14-17]. Humans express two functional chitinases, chitotriosidase (*CHIT1*) and AMCase (*CHIA*) with an acidic pH optimum, both able to degrade chitin polymers. In mammals, chitinases [18,19] and chitinase-like proteins [20] are mainly expressed and secreted by phagocytes (mainly neutrophils and macrophages) and are induced at sites of inflammation, infection, and tissue remodeling, suggesting that these proteins play active roles in anti-infective defense and repair responses. Specifically, both chitinases and chitinase-like proteins have been linked to an alternative activation (M2) phenotype of macrophages [21,1,22], which is found in asthma and other chronic diseases, such as cystic fibrosis (CF), providing a rationale for chitinases and chitinase-like proteins to play a role in these disease conditions. While

chitotriosidase is used as disease biomarker for Gaucher disease, a disease characterized by the accumulation of lipid-laden macrophages, acidic mammalian chitinase has been linked to allergic asthma and hypersensitivities [23]. However, the major evidence for an involvement in lung diseases exists for chitinase-like proteins. Therefore, we will focus on their role in pediatric lung diseases in the chapters below.

The chitinase-like protein YKL-40 and its involvement in lung diseases

YKL-40, or also called HcGP-39 or BRP-39 in mice, lacks measurable enzymatic chitinase activity, due to mutations in its highly conserved putative active sites [17]. In mice, BRP-39 has been associated with cell growth, breast cancer, and tissue remodeling. Recent evidence from BRP-39 knock-out mice indicates that BRP-39 plays a role in T-cell, macrophage and dendritic cell responses as well as cell apoptosis and tissue repair [21,1]. In human cells, HcGP-39/YKL-40 has been shown to regulate apoptosis/proliferation/cell survival, MAPK, and cytokine pathways [24,2].

The first indication that YKL-40 could be linked to human lung diseases came from a multi-center study quantifying YKL-40 serum levels in 253 adult patients with asthma [25]. This study showed that YKL-40 serum levels were mainly increased in adult patients with severe asthma and correlated with disease severity and airway remodeling. These studies in adults were, on the one hand, confirmed for children with severe, therapy-resistant asthma [26], but, on the other hand, challenged by another study [27]. Besides increased levels of YKL-40, elevated *bona fide* chitinase activities were also found in bronchoalveolar-lavage (BAL) fluids from children with asthma [28]. YKL-40 was also found to be increased in BAL fluid after segmental allergen challenge, indicating local production of this chitinase-like protein in response to allergens [29]. Gavala et al. confirmed this finding and further demonstrated that segmental allergen challenge also increased chitinase activities [30]. Clinically, YKL-40 serum levels remained increased in patients in spite of long-term inhaled corticosteroids, which could imply that YKL-40 production is resistant to current asthma treatments and might represent an alternative therapeutic target for severe asthma. YKL-40 is mainly released by activated neutrophils [20], and neutrophilic asthma is well known to be corticosteroid-resistant [25]. Thus, increased YKL-40 levels may be a hallmark of neutrophilic asthma. A follow-up study analyzing single nucleotide polymorphisms (SNPs) in the *YKL-40/CHI3L1* gene showed a genetic association with increased susceptibility to asthma, increased bronchial hyperresponsiveness, and reduced lung function [31]. The role of *YKL-40/CHI3L1* SNPs for asthma was further confirmed in a Taiwanese population [32]. Another study found a different *YKL-40/CHI3L1* variant associated with asthma [33]. Given the enigmatic role of YKL-40, the functional importance of these variants awaits further investigation. Moreover, a Chinese study found that YKL-40 levels were increased in asthmatic patients and correlated with exacerbation, eosinophils, and immunoglobulin E [34], while a study from Poland found increased YKL-40 levels in asthma, but no correlation with disease severity or total IgE levels [35]. Besides a positive correlation between YKL-40 levels and age in subjects with asthma across all age groups, Santos et al. found no difference in circulating YKL-40 levels among asthma severities in children nor a correlation with IgE levels [27]. When viewed in combination, some but not all studies support the notion that YKL-40 is increased in severe and/or neutrophilic asthma, yet the clear relationship with age/disease progression (pediatric vs adult) and atopy remains to be defined in future studies.

Beyond asthma, other pulmonary or lung-associated disease conditions where YKL-40 levels were found to be increased were chronic obstructive pulmonary disease (COPD) [36,37], idiopathic pulmonary fibrosis [38], tuberculous pleural effusions and pneumonia [39], small-cell lung cancer [40], non-small-cell lung cancer [41], bronchiolitis obliterans syndrome [42], hyperoxic acute lung injury [43], sarcoidosis [44], allergic rhinitis [45], and CF lung disease [46]. Despite the heterogeneity of these disease conditions, alternative (M2) macrophage activation is a common feature of the majority of them, suggesting that increased YKL-40 levels reflect M2 macrophage polarization and disease conditions featuring M2 activation [24,22,2,47].

In CF, a chronic neutrophilic inflammatory disease [48,49], YKL-40 BALF levels were found to reflect airway inflammation and infection in early CF lung disease [50] and correlated inversely with lung function in adult CF patients, where YKL-40 levels were also found to be increased systemically [46]. The potential role of YKL-40 for the pathogenesis of CF lung disease is further supported by findings in *Scnn1b*-transgenic mice, a murine model of CF-like lung disease [51,52]. In this model, airway-specific overexpression of the β-subunit of the epithelial Na^+ channel ENaC mimics airway surface dehydration characteristic for CF airways and produces a CF-like lung disease with early onset of mucus obstruction, chronic airway inflammation, slowed bacterial clearance, and progressive structural lung damage [53]. Similar to patients with CF, levels of the murine homologue of YKL-40, BRP-39, were significantly increased in BALF and showed an inverse correlation with pulmonary function in *Scnn1b*-transgenic mice [46]. Collectively, these studies suggest that YKL-40/BRP-39 may be implicated in the pathogenesis of chronic airway inflammation and airflow obstruction and thus

serve as a potential biomarker of disease severity in patients with CF.

Recent studies in this mouse model of CF-like lung disease also provided first mechanistic insights on how chitinases/chitinase-like proteins (CLPs), including BRP-39, may be upregulated in CF lung disease. These studies demonstrated that CF-like airway surface dehydration causing mucociliary dysfunction and mucus obstruction [54,55] provides a robust stimulus for macrophage activation, even when *Scnn1b*-transgenic mice are kept in a germ-free environment [54, 56]. More recently, a series of gene expression studies identified signatures of alternatively activated macrophages (M2), including *Ym1, Ym2,* and *BRP-39,* in whole lungs and isolated macrophages from *Scnn1b*-transgenic mice [57, 58]. These results suggest alternative macrophage activation in mucostatic airways as a mechanism underlying elevated expression of a range of chitinases and CLPs, even in the absence of chitin-containing parasites or allergens, in the airways of patients with CF and potentially other mucoobstructive lung diseases. While the pathogenic role of chitinases/CLPs in (dys)regulation of inflammation in CF airways remains poorly understood, observations in lungs from *Scnn1b*-transgenic mice demonstrate that some proteins of the chitinase/CLP family, such as *Ym1* and *Ym2*, are expressed at such high levels that they precipitate and form sharp crystals often greater than 100 μm in size [54]. These may cause chronic mechanical irritation and injury of airway epithelial cells and phagocytes thus contributing to chronic airway inflammation. Additionally, it is intriguing to speculate that such crystals may activate the NLRP3 inflammasome, a pattern recognition receptor able to trigger inflammation in response to other crystalline or aggregated endogenous substances such as cholesterol or uric acid crystals [59,60,61].

Beyond asthma and CF, circulating YKL-40 has been further associated with decline of lung function in the general population and has been proposed as a biomarker of susceptibility to the long-term effects of cigarette smoking [62]. Studying chitinases in New York City firefighters after World Trade Center exposure revealed that increased serum chitotriosidase reduced the odds of developing pulmonary obstruction after World Trade Center-particulate matter exposure and was associated with recovery of lung function. The underlying mechanisms remain unclear [63]. As chitin is a structural component of fungi, which are well known for their role in environmental asthma, a study investigated whether the exposure to environmental fungi modulates the effect of chitinases in individuals with asthma. The study demonstrated that environmental exposure to fungi modified the effect of *CHIT1* SNPs on severe asthma exacerbations [64].

In order to dissect the cellular sources of YKL-40 in human airways and the mechanisms regulating YKL-40 expression, Park and coworkers identified human airway epithelial cells as a source of YKL-40 and demonstrated that mechanical stress potently induces CHI3L1 expression leading to increased secretion of YKL-40 protein in an EGFR and MEK1/2-dependent pathway, suggesting that mechanical stress contributes to enhanced YKL-40 levels in asthmatic lungs [65]. A further mechanistic study found that YKL-40 increased the proliferation and migration of bronchial smooth muscle (BSM) cells through PAR-2-, AKT-, ERK-, and p38-dependent mechanisms and demonstrated that YKL-40 epithelial expression was positively correlated with BSM mass in asthma [66]. Other studies showed that YKL-40 induced IL-8/CXCL8 expression from bronchial epithelium via MAPK (JNK and ERK) and NF-κB pathways [67]. Taken together, these results suggest that YKL-40-mediated IL-8 production could be related to BSM remodeling [68]. Further studies showed that the allergen ovalbumin increased YKL-40 expression in tracheal epithelial cells [69] and demonstrated that YKL-40 increased mucin5AC production in human bronchial epithelial cells [70]. Collectively, these studies extend the view that YKL-40 is mainly a marker of neutrophilic inflammation by demonstrating modulatory effects of this chitinase-like protein on airway epithelial cells. Despite these intriguing insights into the biological effects of chitinase-like proteins, their precise functional role in biological processes and disease conditions in humans still remains largely unclear.

Conclusions

Chitin, chitinases, and chitinase-like proteins remain enigmatic terms for human diseases. However, after the second look into the pathophysiology of allergic and chronic lung diseases, these ancient, insect glycoprotein-associated pathways attract high relevance as potential biomarkers and therapeutic targets. Particularly, fungal chitin-linked asthma is increasing, but treatment options and successful clinical trials are scarce, necessitating further therapeutic developments. Before those approaches can be applied clinically, several key questions remain to be answered:

- How is chitin recognized by the human immune system? Is it a novel pattern recognition receptor ligand? And if so, can this interaction be targeted and exploited therapeutically?
- What is the primary role of enzymatically active chitinase found in the human body? To defend against chitin-bearing pathogens or to skew the immune system?
- Is a dysregulation of chitin sensing or YKL-40 induction pathways associated with altered

susceptibility for diseases like fungal asthma or house-dust mite allergy?
- Is YKL-40 a potential biomarker of severe asthma, a marker of neutrophilic innate immune activation, or a marker reflecting tissue remodeling (or all of these)?
- Does YKL-40 play a causative role in these disorders? Can YKL-40 be neutralized pharmacologically? And if so, which diseases would benefit?
- Considering YKL-40 as a therapeutic target in human diseases, what is the physiologic function of this protein?

These issues remain to be solved and pave the way for an exciting novel field in pediatric immunology, bridging a gap between insects, fungi, immune cells, and the lung.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
IM and DH wrote the major parts of the manuscript and coordinated and supervised the writing process of the whole manuscript. AH, MB, JK, KF, ANW, and MM contributed to specific sections of the review. All authors read and approved the final manuscript.

Acknowledgements
We thank Peter Weber, Department of Pediatrics, Tübingen, Germany, for creating and illustrating the figure. We thank the SFB/CRC685 (D.H.), University of Tübingen, for support.

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Received: 6 November 2014 Accepted: 9 February 2015
Published online: 27 February 2015

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