Myeloid-derived suppressor cells regulate B-cell responses

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“Knowledge we learn with the masters and books.
Wisdom we learn with life and the lowly.”
Cora Coralina

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III. Abbreviations

AIDS	Acquired immunodeficiency syndrome
APRIL	A proliferation-inducing ligand
ASC	Antibody secreting cell
BAFF	B-cell activating factor
BCG	Bacillus Calmette-Guérin
BCR	B-cell receptor
BM	Bone marrow
Bregs	Regulatory B cells
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Common lymphoid progenitor
CLRs	C-type lectin receptors
CpG ODN	Cytosine-phosphate-Guanine oligodeoxynucleotide
CSF	Colony-stimulating factor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
eMDSCs	Early-stage myeloid-derived suppressor cells
F (ab)	Fragment antigen-binding
FOXP3:	Forkhead Box P3

GC	Germinal center
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-MDSCs	Ganulocytic-myelod-derived suppressor cells
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen - antigen D related
HSC	Hematopoietic stem cell
IDO	Indoleamine-pyrrole 2,3-dioxygenase
Ig	Immunoglobulin
IL	Interleukin
IMC	Immature myeloid cell
INF	Interferon
iNOS	Inducible nitric oxygen synthase
ION	Ionomycin
L-NMMA	L-NG-monomethyl Arginine
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Monocyte colony-stimulating factor
MDSCs	Myeloid-derived suppressor cells
MHC-II	Major histocompatibility II
M-MDSC	Monocytic myeloid-derived suppressor cell
MZ	Marginal zone

NADPH	Nicotinamide adenine dinucleotide phosphate
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NLRs	NOD-like receptor
NO	Nitric oxide
Nor-NOHA	Nω-hydroxy-nor-Arginine
NOS2	Nitric Oxide Synthase 2
PAMPs	Pathogen-associated molecular patterns
PGE_2	Prostaglandin E2
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cell
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
SCF	Stem cell factor
STAT	Signal transducer and activator of transcription
TAMs	Tumor associated macrophages
TCR	T cell receptor
TD	Thymus-dependent
Tfh	T follicular helper cell
TGF	Transforming growth factor
TI	Thymus-independent

TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	Regulatory T cell
VEGF	Vascular endothelial growth factor

IV. Summary

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell group, which share close phenotypical similarities with conventional myeloid cells. In contrast to conventional myeloid cells, MDSCs have the ability to suppress other immune cells. MDSCs have been reported to suppress dendritic cells (DCs), natural killer (NK) and natural killer T cells (NKT) cells. However, the “gold standard” to characterize and discriminate them from other myeloid cells is the ability to suppress T-cell function. MDSCs apply multiple mechanisms of suppression, including production of reactive oxygen and nitrogen species, arginase-1 and production of several immunomodulatory cytokines. MDSCs have been described to arise in several forms of cancer, where they correlate with poor prognosis. Beyond cancer, MDSCs have been involved in chronic inflammatory and autoimmune diseases. However, in these situations the effects of MDSCs are still controversial and need further investigation. B cells are the key players of the humoral adaptive immune response. Their main function is to produce antibodies. B cells are involved in eliminating mainly extracellular invasive pathogens. Moreover, they are important modulators of several diseases, such as systemic lupus erythematous and rheumatoid arthritis, where autoantibodies lead to the development of chronic inflammation and loss of tissue function. MDSCs suppress T cells and other immune cells functions, but their ability to modulate B-cell responses is still poorly understood. The aim of this study is to study interactions between human polymorphonuclear-MDSCs (PMN-MDSCs) and B cells. For that purpose, we performed B-cell proliferation assays by co-culturing activated B-cell with PMN-MDSCs. The data was then assessed by flow cytometry, image stream, and ELISA. Our studies demonstrate that human PMN-MDCs differentially modulate B-cell function by suppressing B-cell proliferation and antibody production in a stimulus- and dose-dependent fashion. We further demonstrate that this MDSC-mediated effect is cell-contact dependent and involves established mediators such as arginase-1, nitric oxide (NO), reactive oxygen species (ROS) as well as B-cell death. Collectively, our studies provide novel evidence that human MDSCs modulate B cells, which could have future implications for immunotherapy approaches.

V. Zusammenfassung

Myeloische Suppressorzellen (MDSCs) sind eine heterogene Zellpopulation, die konventionellen myeloischen Zellen phenotypisch ähnlich sind. Im Vergleich zu konventionellen myeloischen Zellen haben sie die Fähigkeit andere Immunzellen zu supprimieren, insbesondere T-Zellen. MDSCs haben mehrere Suppressionsmechanismen zu denen unter anderem die Produktion von reaktiven Sauerstoff- und Stickstoffspezies, Arginase 1 sowie diverse immunmodulatorische Zytokine zählen. MDSCs akkumulieren in Tumorpatienten, bei denen sie mit einer schlechteren Prognose im Zusammenhang stehen. Sie spielen nicht nur bei Tumorerkrankungen, sondern auch bei chronisch inflammatorischen Erkrankungen und Autoimmunerkrankungen eine Rolle. Die pathophysiologische Relevanz von MDSCs bei diesen Erkrankungen ist bislang allerdings noch unzureichend verstanden. B-Zellen sind wichtige Immunzellen der humoral adaptiven Immunantwort, da ihre Hauptaufgabe darin besteht, Antikörper zu produzieren. Sie sind hauptsächlich für die Eliminierung von extrazellulären Pathogenen zuständig. B-Zellen sind zudem Hauptmodulatoren von diversen Krankheiten, wie systemischer Lupus Erythematodes und rheumatoider Arthritis, bei denen Autoantikörper zur Entwicklung von chronischen Entzündungsreaktionen und Gewebeschäden führen. MDSCs supprimieren T-Zellen und modulieren weitere Immunzellen. Eine mögliche Interaktion mit B-Zellen ist jedoch bislang kaum untersucht. Das Ziel dieser Studie war es, mögliche Interaktionen zwischen polymorphonukleären MDSCs (PMN-MDSCs) und B-Zellen zu untersuchen. Dazu führten wir B-Zell Proliferationsversuche durch, in denen wir aktivierte B-Zellen mit PMN-MDSCs ko-kultivierten. Die Daten wurden mittels Durchflusszytometrie, ImageStream und ELISA analysiert. Wir konnten zeigen, dass humane PMN-MDSCs B-Zellen differentiell modulieren, indem sie dosisabhängig die B-Zell Proliferation und die Antikörperproduktion supprimieren. Wir konnten zudem demonstrieren, dass dieser Effekt abhängig von Zellkontakt ist und für PMN-MDSCs typische Mechanismen wie die Expression von Arginase-1, Stickstoffoxid und reaktive Sauerstoffspezies sowie auch B-Zelltod involviert sind. Zusammenfassend konnten wir neue Erkenntnisse gewinnen, dass humane MDSCs B-Zellen modulieren und somit neue potentielle Targets für Immuntherapien darstellen.

VI. List of publications

In peer-reviewed journals

Felipe Lelis, Anurag Singh, Katja Fromm, Jennifer Jaufmann, Annkathrin-Chiara Teschner, Simone Pöschel, Iris Schäfer, Sandra Beer-Hammer, Nikolaus Rieber, Dominik Hartl. Submitted in November, 2016. Myeloid-derived suppressor cells modulate B-cell responses. Submitted to *Immunology letters.*

Singh A, **Lelis F**, Braig S, Schäfer I, Hartl D, Rieber N. Differential Regulation of Myeloid-Derived Suppressor Cells by *Candida* Species. *Frontiers in Microbiology*. 2016; 7,1624.

Ralhan A, Laval J, **Lelis F**, Ballbach M, Grund C, Hector A, Hartl D. Current Concepts and Controversies in Innate Immunity of Cystic Fibrosis Lung Disease. *J of Innate Immun.* 2016; 8, 531-540.

Gustavo E.A. Brito-Melo, Rodrigo Nicolato, Antonio Carlos P. de Oliveira, Gustavo B. Menezes, **Felipe J.N. Lélis**, Renato S. Avelar, Juliana Sá, Moisés Evandro Bauer, Bruno R. Souza, Antonio L. Teixeira, Helton José Reis Increase in dopaminergic, but not serotoninergic, receptors in T-cells as a marker for schizophrenia severity. *Journal of Psychiatric Research*. 2012; 46(6), 738-42.

Bethania A Avelar; **Felipe JN Lelis**; Renato S Avelar; Mathias Weber; Elaine M. Souza-Fagundes; Olindo A Martins-Filho; Mirian TP Lopes; Gustavo EA Brito-Melo. The crude latex of *Euphorbia tirucalli* L. (Euphorbiaceae) modulates the cytokine response of leukocytes, especially CD4+ T lymphocytes. *Brazilian Journal of Pharmacognosy*. 2011; 21(4), 662-67.

Posters in events (selected)

F Lelis *et al.* (2016) Myeloid-derived suppressor cells modulate B-cell responses**.** Immunology Training Network of Tübingen, Erlangen and Würzburg. 11th Annual Meeting: Tagungszentrum Blaubeuren, Germany.

F Lelis and D Hartl (2016) Myeloid-derived suppressor cells modulate B-cell responses. B-cells at the intercept of innate and adaptive immunity. Stockholm, Sweden.

Lelis *et al.* (2015) The interaction between B lymphocytes and myeloid-derived suppressor cells. Novel Concepts in Innate Immunity. Tuebingen, Germany.

Lelis *et al.* (2015) The interaction between B lymphocytes and Myeloid-derived suppressor cells. Autumn School of Immunology. Currents concepts in Immunology. Mersemburg, Germany.

Lélis, Felipe José Nobre; ***et al.*** (2005) Analysis of chemokines receptors on the surface of peripheral blood leukocytes in individuals infected with *Micobacterium leprae*. In: VII Academic and Scientific and Technologic Initiation Journey of the Federal University of the Vales of Jequitinhonha and Mucuri. Diamantina, Brazil.

Lélis, Felipe José Nobre; ***et al.*** (2005) Analysis of the increased expression of dopamine and serotonin in lymphocytes' surface of schizophrenic patients In: VII Academic and Scientific and Technologic Initiation Journey of the Federal University of the Vales of Jequitinhonha and Mucuri. Diamantina, Brazil.

Erklärung nach § 5 Abs. 2 Nr. 8 der Promotionsordnung der Math.-Nat. Fakultät

-Anteil an gemeinschaftlichen Veröffentlichungen-

Nur bei kumulativer Dissertation erforderlich!

Declaration according to § 5 Abs. 2 No. 8 of the PromO of the Faculty of Science

-Share in publications done in team work-

Name: Felipe Jose Nobre Lelis

VII. List of Publications in the thesis

Paper 1: **Felipe Lelis**, Anurag Singh, Katja Fromm, Jennifer Jaufmann, Annkathrin-Chiara Teschner, Simone Pöschel, Iris Schäfer, Sandra Beer-Hammer, Nikolaus Rieber, Dominik Hartl. Submitted November, 2016. Myeloid-derived suppressor cells modulate B-cell responses. Submitted to *Immunology letters.*

Paper 2: Singh A, **Lelis F**, Braig S, Schäfer I, Hartl D, Rieber N. Differential Regulation of Myeloid-Derived Suppressor Cells by *Candida* Species. *Frontiers in Microbiology*. 2016; 7:1624.

Paper 3: Ralhan A, Laval J, **Lelis F**, Ballbach M, Grund C, Hector A, Hartl D. Current Concepts and Controversies in Innate Immunity of Cystic Fibrosis Lung Disease. *J of Innate Immun.* 2016; 8: 531-540.

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 (%)
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I certify that the above statement is correct.

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Date, Signature of the candidate

I/We certify that the above statement is correct.

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

Contribution

Paper 1

Myeloid-derived suppressor cells modulate B cell responses

I performed and/ or supervised all experiments with assistance from Katja Fromm, Jennifer Jaufmann, Annkatrin-Chiara Teschner and Iris Schäfer. I also designed the experiments, analyzed the data, made figures and contributed to writing and proof-reading of the manuscript. Simone Pöschel performed the Image stream measurements and analyzed the data. Anurag Singh, Nikolaus Rieber and Dominik Hartl designed this study and supervised experiments Sandra Beer-Hammer and Dominik Hartl wrote the manuscript.

Paper 2

Differential Regulation of Myeloid-Derived Suppressor Cells by Candida Species

I performed part of the experiments for this paper together with A. Singh, S. Braig and I. Schaefer. I also helped A. Singh analyzing data, making graphs and contributed to the manuscript preparation. A. Singh, D. Hartl and N. Rieber designed and supervised this study and wrote the manuscript.

Paper 3

Current Concepts and Controversies in Innate Immunity of Cystic Fibrosis Lung Disease

I contributed in writing and proof reading of the manuscript with other authors. J. Laval, M. Ballbach, C. Grund, A. and Hector. A. Ralhan wrote the manuscript, D. Hartl.

1. Introduction

1.1The innate immune system

The immune system defends the human body against several microorganisms such as, viruses, bacteria, fungi and parasites, which are commonly known as pathogens. It is also involved in the recognition and elimination of non-self-substances (antigens), which we are regularly exposed [1, 2]. By using a complex network of physical barriers (skin and mucosa), chemical compounds (defensins and complement proteins), tissues (bone marrow and blood), organs (thymus and spleen) and cells (leukocytes or white blood cells), the immune system constantly avoids the establishment of many infective microbes in our body [3, 4]. However, these invaders may cause infections and can be lethal when the immune system fails in eliminating them [5, 6].

The immune responses are divided into two main categories, innate and adaptive immune responses [7]. The innate immune response is the first line defense of our body, and its effects are immediately observed after the invasion of the human body by an antigen or pathogen. The innate immune system comprises the physical, chemical and cellular barriers of our body [8-10]. The skin and mucosa prevent the invasion of pathogens or allergens (antigens) physically [11, 12], accompanied by chemical components of the mucous, skin and tissue, such as defensins and proteins of the complement system. The former act as an antimicrobial and play important role to eliminate the unwanted invader. The latter is a complex system of proteins that bind to pathogen`s cell wall and either lyse the pathogen or attract immune cells to eliminate it [13, 14].

Microorganisms which pass the physical and chemical barriers of the immune system will then face the cellular defense mechanisms. Langerhans's cells (Figure 1) are the macrophages that reside in the skin and the first cells to encounter a pathogen [15, 16]. They recognize and phagocyte the invaders by using pattern recognition receptors (PRRs), which are involved in several signaling pathways, such as host cell activation and cytokines production. PRRs identify the pathogen-associated molecular patterns (PAMPs) present on microbes [10, 17, 18].

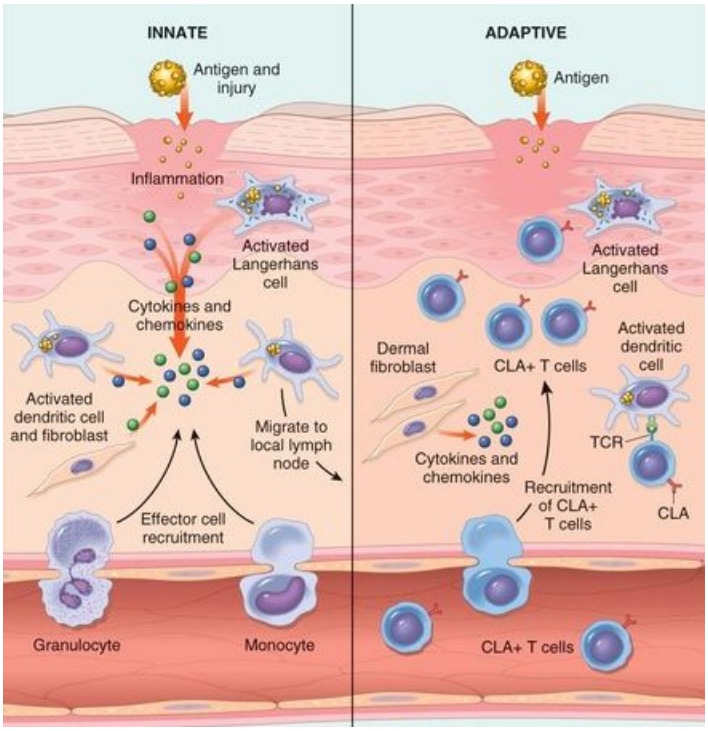

Figure 1: Comparison between innate and adaptive immune system. In the left panel is depicted the nonspecific defense mechanisms of the innate immune response that come into play immediately after a pathogen invasion in the body. These mechanisms include physical barriers such as skin, chemicals with antimicrobial activities and cells such as macrophages, neutrophils, monocytes, mast cells, monocytes and NK cells. In the right panel is shown the mechanisms that comprise the adaptive immune system composed of highly specialized, systemic cell, such as lymphocytes T and B. They act in a very specific manner in order to eliminate pathogens and prevent the establishment of infections. Modified from Kumar et al. (2014) [9].

Furthermore, macrophages can phagocyte the invader microbe forming the phagosome. The phagosome merges with cytoplasmic granules that contain digestives enzymes (lysozymes) and form the phagolysosome, which kills the pathogen. Macrophages are also involved in the production and release of reactive oxygen species (ROS) and nitrogen species, such as nitric oxide (NO), collectively responsible for the respiratory burst. These anionic chemical species are involved in the elimination of the pathogen, they also enhance the acidity intracellularly thus induce cell death [19-22]. In parallel, macrophages secrete pro-inflammatory cytokines, such as interferons, as well as chemokines contributing to the development of a complex network of cells and proteins with vasoactive and cell activation capacities, called inflammation [23]. The inflammatory process happens in order to

eliminate the invader and heal the injury. The physical signs of inflammation are redness, heat, pain and tumor [24].

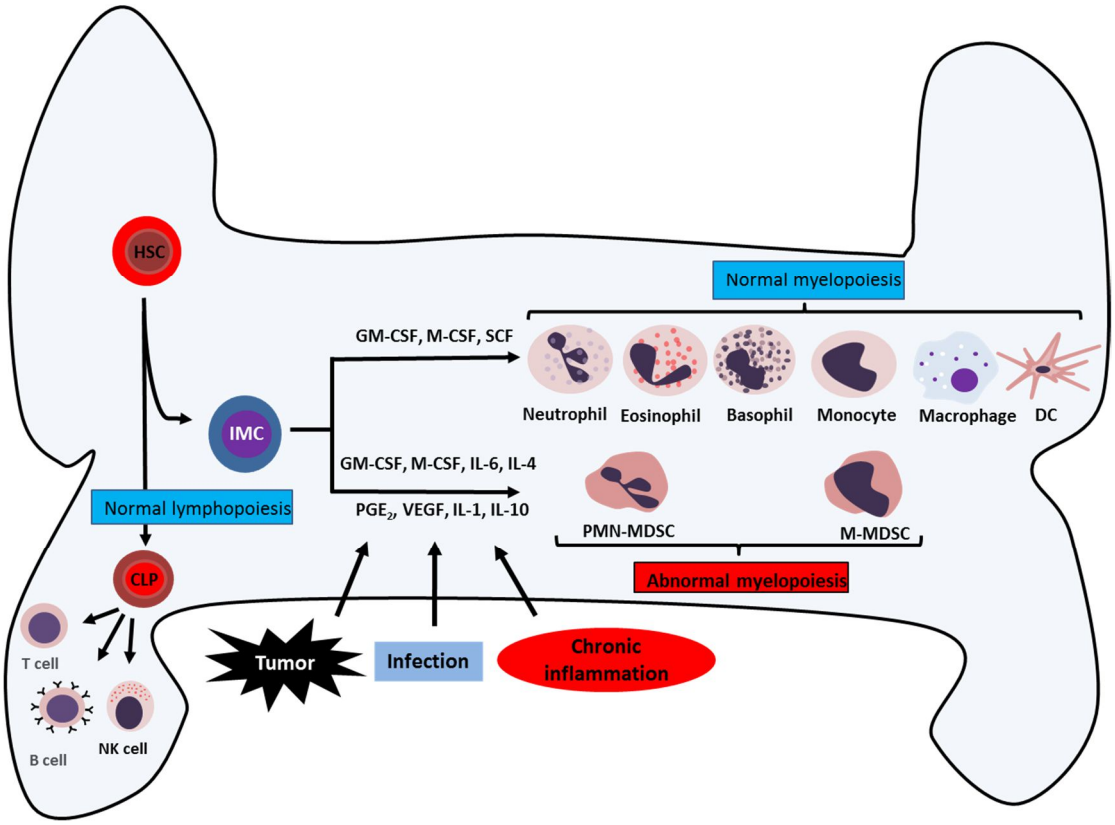

Figure 2: **Development of myeloid and lymphoid cells in the bone marrow.** Homeostatic myelopoiesis and lymphopoiesis both originated from the hematopoietic stem cell (HSC) in the bone marrow. In parallel, it is depicted the changes caused by excessive amount of soluble and non-soluble factors in the tumor and inflammed microenvironment and in infections, contributing to the abnormal differentiation of immature myeloid cells (IMCs) leading to the appearance, expansion and accumulation of MDSCs (abnormal myelopoiesis). Adapted from Gabrilovich and Nagaraj (2009) [25].

After macrophages, neutrophils [26, 27] and monocytes [28] enter the tissues (Figure 1). They leave the peripheral blood attracted by chemokines, such as CC motif ligand 2 (CCL2), interleukin 8 (IL-8) released by the cells in the inflamed environment as well as due to the enhanced permeability of the blood vessels due to cytokine activity. The neutrophils act similarly to macrophages, they phagocyte and kill invading microbes also by means of digestive enzymes from their different types of granules, they also are involved in the respiratory burst and production of cytokines [29, 30].

Natural killer cells (NK cells) and mast cells are also innate cells. The former play important roles in the immune responses, for instance, eliminating intracellular pathogens and producing INF-γ and the latter are key effector in allergic reactions [31-34].

Dendritic cells (DCs) are the link between the innate and adaptive responses, they sense, uptake, process and present antigens to T cells in secondary lymphoid organs (spleen and lymph nodes) thereby initializing the adaptive immune response [35].

T cells and B cells are the effector cell types of the adaptive immune response. While T cells act as helper cells by activating other immune cells (T $CD4^+$) or mediating direct cytotoxicity to infected cells (T $CD8^+$), B cells produce highly specific antibodies. The adaptive immune response may take hours or days after the initial pathogen invasion to begin its defensive mechanisms [36]. It is highly specific against recognized antigens such as proteins, lipids, carbohydrates or nucleic acids of pathogens [37, 38]. After eliminating the harmful agent, the adaptive immune response develops memory cells, which remembers encountered pathogens and upon a re-encounter, they respond in a faster and stronger way [39-41].

1.2 Myeloid-derived suppressor cells

1.2.1 Definition, origin, generation and expansion

The immunosuppressive activities of myeloid cells were firstly appreciated, almost forty years ago, when Bennet et al. (1978) [42] observed that Bacillus Calmette-Guérin (BCG) enhanced the suppressive activity of bone marrow cells, by inhibiting the development of cell-mediated immunity. Nowadays it is known that these cells arise and accumulate from an abnormal hematopoiesis, and due to changes in the cellular microenvironment caused by the presence of tumors and excessive colony-stimulating activity. [43, 44]. Consistent investigations have been performed in order to compile evidences about the generation of these immune-suppressive cells, as well as to characterize them, phenotypically, morphologically and functionally. It is now known that some cells subsets from lymphoid and myeloid origin can suppress the immune system, under both pathological and physiological conditions. Regulatory T and B cells

(Tregs and Bregs), tumor-associated macrophages (TAMs), dendritic cells (DCs), mesenchymal stromal cells and myeloid-derived suppressor cells (MDSCs), comprise the main cell types involved in this phenomenon [45-50].

Myeloid-derived suppressor cells (MDSCs) are described as an immature and heterogeneous cell population, arising from an abnormal differentiation of the immature myeloid cells (IMCs) in the bone marrow (Figure 2). In cancer, MDSCs have been described to positively correlate with bad prognosis of patients, induction of angiogenesis, tumor progression and metastasis [25, 50].

Several tumor and inflammatory cell-derived factors induce the generation and accumulation of MDSCs [51]. The altered cellular microenvironment, with a persistent production of cytokines and chemokines lead to the expansion and activation of MDSCs. Cytokines such as stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interleukin (IL)-13, IL-10, vascular endothelial growth factor (VEGF) and chemokines such as, CCL2 or monocyte chemoattractant protein-1 (MCP-1), C-X-C motif chemokine 12 (CXCL12) and CXCL18 (IL-8) and other factors, such as prostaglandin E_2 (PGE_2) are also important players in the induction of MDSCs (Figure 2) [25, 50-56].

1.2.2 Characterization of human myeloid-derived suppressor cells

1.2.2.1 Phenotypically

MDSCs comprise a heterogeneous group of myeloid cells and that makes it difficult to phenotypically identify them. In humans, two subtypes of MDSCs have been described, granulocytic or polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). Human PMN-MDSCs are characterized according their surface markers as $CD11b^+CD14^-CD15^+CD66b^+$ and M-MDSCs as $CD11b^+CD14^+HLA\text{-}DR^{-/low}CD15^-$. The general phenotypic features of MDSCs can be discriminate in the early stage of these cells (eMDSC), as lineage negative or Lin^- (CD3, CD14, CD56, CD15, and CD19), $HLD\text{-}DR^-$ and $CD33^+$ (Table 1). Between CD33 and CD11b either one or both can be used as a myeloid marker [55, 57].

Table 1: **Human MDSC subtypes and surface markers for phenotyping identification**

MDSC subtype	Surface markers
PMN-MDSC	$CD11b^{+}CD14^{-}CD15^{+}$ (or $CD66b^{+}$)
M-MDSC	$CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}CD15^{-}$.
eMDSC	Lin^{-} (CD3, 14, 56, 15 and 19), $HLD\text{-}DR^{-}$ $CD33^{+}$

eMDSC: early-stage myeloid-derived suppressor cell; M-MDSC: monocytic myeloid-derived suppressor cell; PMN-MDSC: polymorphonuclear myeloid-derived suppressor cell.

Due to the phonotypical similarities among MDSCs, neutrophils or monocytes, only the identification by receptor markers is not enough to distinguish them. It is necessary additional functional or suppressive assays. In addition, PMN-MDSCs can be separated from neutrophils by their gradient of centrifugation behavior. PMN-MDSCs after Ficoll density centrifugation dislocate to the low-density or peripheral blood mononuclear cell (PBMC) fraction, whereas the neutrophils move to the high-density fraction [25, 51, 55, 58, 59].

1.2.2.2 Functional and biochemical identification

According to Bronte et al. (2016) [55], the “gold” standard to identify MDSCs is by verifying their suppressive activity towards T cells [54, 60-62]. Therefore, the myeloid phenotypic description (Table 1) plus proliferative and functional assays involving co-culture of T cells and MDSCs are sufficient information to characterize MDSCs. [55, 63, 64].

Moreover, MDSCs can also be discriminated from other myeloid cells by biochemical and genetic markers. In this regard, it is important to observe that MDSCs compared to conventional myeloid cells have enhanced expression of NADPH oxidase (Nox2) thus they produce large amount of ROS [65]. In addition, they also show an increased expression of arginase-1 (*arg1*) [66]. High expression of nitric oxide synthase 2 (*nos2*) or inducible nitric oxide synthase (iNOS) has also been described in MDSCs. iNOS leads to increased production of nitric oxide (NO) [25, 67]. Furthermore, it is also observed higher activity of the transcription factors signal transducer and activator of transcription 1 and 3 (STAT1 and STAT3) as shown in Figure 3 [25, 55, 68]. All the above-mentioned factors are involved in the suppressive mechanisms of MDSCs [51]. The biochemical peculiarities maybe are the bottom line in distinguishing MDSCs from neutrophils and monocytes [55].

1.2.2.3 Suppressive functions of myeloid-derived suppressor cells

MDSCs use of multiple mechanisms to suppress the anti-tumor activity of other immune cells, and it may be due to their heterogeneity [25, 54, 69, 70] (Figure 3). According to Kumar et al. (2016) [51], these mechanisms are localization dependent (tumor or peripheral lymphoid organs) and include ROS, NO, arginase-1, indoleamine 2,3-dioxygenase (IDO), suppressive cytokines (IL-10, Tumor growth factor-beta (TGF-β)), tumor necrosis factor-alpha (TNF-α) and induction of immune-regulatory cells. Taking together the suppressive activities of MDSCs, they can be specific and non-specific. [71-79].

Increased production of ROS (Figure 3) is a key characteristic of MDSCs, especially PMN-MDSCs. This mechanism is mediated by antigen-specific interaction with the target cell thus require cell-to-cell contact and can be induced by tumor microenvironment factors, such as IL-10, TGF-β, and GM-CSF. [25, 80-82]. ROS are strongly oxidative compounds that inhibit T-cell proliferation and function by inducing loss of CD3 ζ-chain [67, 69, 83]. The formation of ROS in myeloid cells is mediated by NADPH oxidase, which induces superoxide anion (O_2^-) formation [65, 69]. O_2^- strongly reacts with NO leading to the formation of peroxynitrite ($ONOO^-$), capable of nitrating amino acids from the T-cell receptor (TCR), inducing T-cell anergy. In addition, ROS is also involved in T-cell apoptosis, inhibition of proliferation and nitration of many other T cell-related proteins [59, 80, 84].

Another important suppressive mechanism used by MDSCs is the deprivation of L-arginine (Figure 3), which is substrate for arginase-1 and inducible nitric oxide synthase (iNOS). These two enzymes play important roles in the contact-independent suppression of T-cell function and proliferation [66]. Arginase-1 catalyzes L-arginine into urea and L-ornithine and iNOS originates nitric oxide (NO) [25, 85, 86]. Excessive activity of arginase-1 deprives L-arginine in the tumor environment abrogating T-cell proliferation, also via decreasing CD3 ζ-chain and inducing cell cycle arrest [87]. NO induces T-cell apoptosis and it is involved in suppression mediated by inhibition of kinases and STATs [88]. NO is more involved in M-MDSCs modulatory actions and arginine-1 is used by both MDSCs subtypes [82] (Figure 3).

MDSCs can promote the *de novo* development of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) (Figure 3), as an indirect mechanism of suppression. Many mechanisms are suggested for MDSCs generation of Tregs such as presence of IL-10, and INF-γ in the cell environment and activation of tumor-specific T cells [89]; via expression of cytotoxic lymphocyte antigen 4 (CTLA-4), arginase-1 and antigen tumor presentation by MDSCs [78, 90]. Despite there are indications of the MDSCs involvement in Tregs generation, it is rather controversial once there are scientific finds showing the opposite or limited collaboration of MDSCs in this phenomenon, but these two immunoregulatory cell type might cross-talk in the cellular network of the immune response [91].

ROS, iNOs, arginase-1 inhibitors, such as diphenyleneiodonium (DPI), L-NG-monomethyl Arginine citrate (L-NMMA), and Nω-Hydroxy-nor-L-arginine (nor-NOHA) respectively, are used to address the mechanisms applied by MDSCs to suppress immune functions [92].

Many other biological mechanisms seem to play roles in the MDSCs functions, such as indoleamine-pyrrole 2,3-dioxygenase (IDO) [93-95], suppressive cytokines, such as IL-10 and TGF-β [55, 96, 97], depletion of essential amino acids such as cysteine [51, 98] and the S100 proteins, S100A8/9 [99]. All these mechanisms are involved in abrogating or decreasing T-cell immune functions and/or proliferation. It is important to notice that even though MDSCs apparently use of multiple mechanisms to exert their suppressive functions, not all of them happen simultaneously, but rather according to the different states of differentiation of these cells [51].

1.2.3 Myeloid-derived suppressor cells and diseases other than cancers

Despite most of the investigations and the acquired knowledge regarding MDSCs come from studies in tumors [54, 98, 100], it is important to consider, that the appearance and accumulation of this immunosuppressive cell population is not restricted to tumors. Likewise, they have also been described in several other pathological conditions such as chronic inflammation, autoimmune diseases, infections, trauma, transplantation, sepsis, as well as in the steady-state. MDSCs play different roles in different pathologies, and their suppressive activities can be either

beneficial or detrimental. In addition, MDSCs can modulate the immune response to Th1 or Th2, generate Tregs and induce apoptosis in immune cells [67, 92, 101-109].

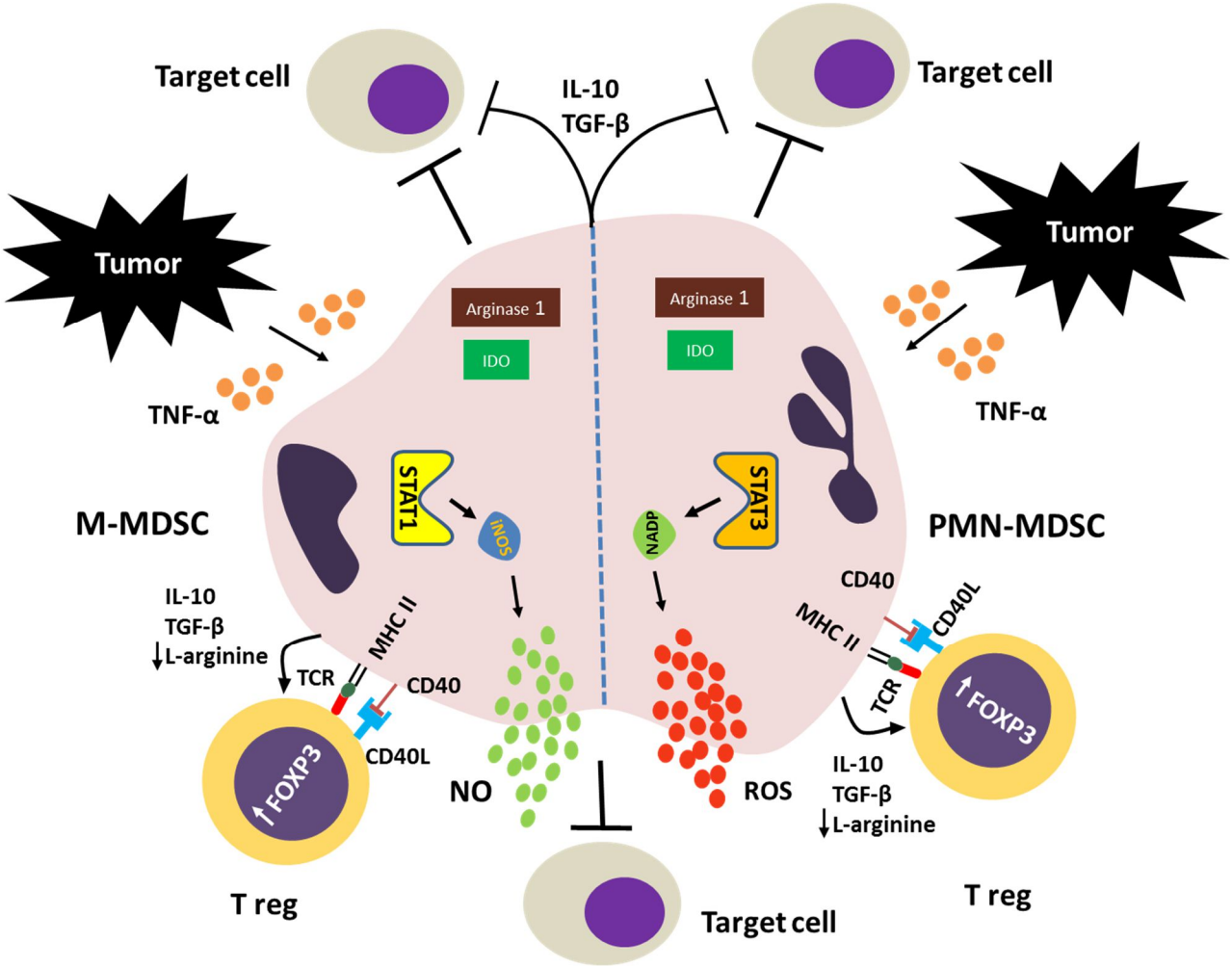

Figure 3: **Main mechanisms of suppression applied by MDSCs**. Both PMN-MDSC and M-MDSCs are shown and their main mechanisms of immunosuppression are depicted, such as production of ROS, upregulation of arginase-1, production of NO and induction of Tregs. The main cytokines and transcription factors involved are also shown. These mechanisms activate MDSCs and are involved in the induction and accumulation of these suppressive cells in tumor and inflammatory microenvironments. Adapted from Gabrilovich and Nagaraj (2009) [25], Bodogai et al. (2015) [110], Yu et al. (2013) [93], Kumar et al. (2016) [51] and Srivastava et al. (2010) [111].

1.2.4 Clinical implications of myeloid-derived suppressor cells

A vast number of studies reveal that MDSCs can exert their suppressive activities on T, DCs, NK, NKT cells and monocytes [80, 112, 113]. B cells have been described to cross-talk with MDSCs and experience their suppressive effects in several contexts such as HIV infection [114, 115], autoimmune disease [116] , tumor [110] and in the induction of regulatory B cells (Bregs) [117, 118].

MDSCs are known to inhibit T cells anti-tumor microenvironment immune responses, and to communicate in several ways with other immune cells, making then a complex web of cells cross-talking. This elevates the importance in targeting MDSCs and other myeloid suppressive cells in the clinical or therapeutic implications of tumor treatment. Table 2 shows a summary of some compounds which have been described for potential use to decrease MDSCs in the tumor microenvironment as well as their main mechanisms [98].

1.3 B cells

1.3.1 Definition and development

B cells were discovered about fifty years ago by Cooper and colleagues (1965) [119], by doing experiments with the “Bursa of Fabricius” from chickens, leading to the name B cells. They are the main immune cells involved in the humoral adaptive immune response, which is mediated by the secretion of antibodies. Antibodies are glycoproteins belonging to the immunoglobulin’s superfamily, they confer the first line protection mainly against extracellular pathogens, such as bacteria and microbial products [120-122]. B cells are involved in both, hemostasis [123] and pathological conditions such as autoimmune diseases, tumors, infections and hypersensitivity [124-129].

B cells have their primordial importance for reasons that involve the elimination of a body´s invaders by mounting a polyclonal antibody response [130], as well as providing one of the most powerful tools of the contemporary immunological research, the technology of monoclonal antibodies [131]. Recently they are being used with

enormous success in therapies for cancer and autoimmune diseases [132-134]. Antibody-secreting cells (ASC), plasmablasts and plasma cells produce antibodies in secondary lymphoid organs, from where they are distributed via circulatory and lymphatic systems throughout the body [135, 136]. Moreover, B cells are key elements in developing vaccines because of their remarkable high-specificity and -affinity antibody production against a particular antigen, and the capacity to confer immunological memory and mount a greater immune response after re-encountering with the same antigen [137, 138].

The development of B cells takes place in the bone marrow (BM) from the common lymphoid progenitor (CLP) (Figure 2). In contrast to T cells, they leave the BM fully developed but in immature state, expressing IgM as B-cell receptor (BCR) [139]. T cells on the other hand, are originated in the BM, but the final stage of development takes place in the thymus [140]. Immature B cells drive through the peripheral blood [141] and other tissues towards the secondary lymphoid organs, by means of chemokines, for example, CXCL13, CCL12, [142], where they finish their maturation process, start co-express IgM and IgD and are ready to encounter their specific antigen to become activated [143]. Nevertheless, it is fundamental that before leaving the BM they undergo the central tolerance test (self-antigen autoreactivity test), and in the process of maturation in the peripheral lymphoid organs, pass through the peripheral tolerance and are checked a second time. B cells which fail autoreactivity tests are deleted by the programmed cell death, apoptosis or become anergic [144-146].

1.3.2 Human B-cell phenotyping

After leaving the BM B cells can assume several phenotypes, which characterize specific cell stages and fate. For instance, a mature-naïve B cell express $CD19^{+}$ (pan B-cell marker) $IgM^{+}IgG^{+}CD24^{low}$ as surface markers and it is ready to recognize its cognate antigen via the BCR (Figure 4 A and B). The encounter of a mature B cell with an antigen pre-activates it, leading to the upregulation of co-stimulatory molecules, such as CD80, CD86, CD40, CD69 and MHC II as well as the common B-cell markers CD19, IgM, IgD, CD24, and others. Following the activation path, B cell may present the antigen it captured via MHC II to its cognate follicular T helper cell (Tfh), and thus

becoming fully activated [147, 148]. The full activation confers to B cells the ability to form germinal centers (GCs) and become antibody secreting cells (ASC) by undergoing substantial changes on the cell surface, genetically (class switching, somatic hypermutation), morphologically and functionally (antibody secretion) [136], turning them into plasmablasts, with phenotype $CD19^{+}CD27^{high}$ $CD38^{high}$ and $CD138^{-}$ and finally into long-lived plasma cells which are $CD19^{low\text{-}med}IgD^{-}$ $CD27^{high}CD38^{high}CD138^{high}$ [149]. Alternatively, they can differentiate into memory B cells, $CD19^{+}IgD^{-}CD27^{+}$. Memory B-cell markers vary according to their different stage of differentiation and way of activation, for instance, there are switched, non-switched and others stages of memory B cells. [149-151].

Table 2: **Therapeutic approaches and pharmacological regulation of myeloid cells in cancer (MDSCs).**

Therapeutic treatment	Molecular events	Effect on myeloid cells
Nitrosapin, Triterpenoids	Downregulation of ARG1, iNOS, and $ONOO^{-}$	Inhibition of MDSCs suppressive effects
Sunitinib, Gemcitabine	STAT3 inhibition and decreasing effects of GM-CSF	Inhibition of MDSCs expansion
Celecoxib	Downregulation of PGE_2, CCL2 and increase expression of CXCL10	Inhibition of MDSCs suppressive effects
CCL2-specific monoclonal antibody	Act on CCL2 binding to CCR2 and with VEGFA upregulation	Inhibition of metastatic spread
5-fluorouracil, docetaxel, vitamin D3	MDSCs apoptosis	Inhibition of MDSCs expansion
All-*trans* retinoic acid	Differentiation of immature myeloid cells to mature leukocytes	Inhibition of MDSCs accumulation

ARG1: arginase-1; iNOS: Inducible nitric oxide synthase; $ONOO^{-}$: peroxynitrite; MDSCs: Myeloid-derived suppressor cells; STAT3: Signal transducer and activator of transcription 3; GM-CSF: Granulocyte-macrophage colony-stimulating factor; CCL, CC-chemokine ligand 2; CCR2, CC-chemokine receptor 2; PGE2: prostaglandin E2; VEGFA: Vascular endothelial growth factor A. Adapted from Gabrilovich, Ostrand-Rosenberg [98]

Many other combinations of markers are also used to phenotype human B cells and they are also used as biomarkers for various diseases involving B cells, such as systemic erythematous lupus [152], Sjögrens syndrome [153], and rheumatoid arthritis [133, 149]. In addition, B10 or Bregs cells exhibit the intrinsic B-cell activation markers plus the capacity to produce and release high amounts of IL-10 cytokine and thereby induce Th2 immune responses [48, 154] (Figure 4 A).

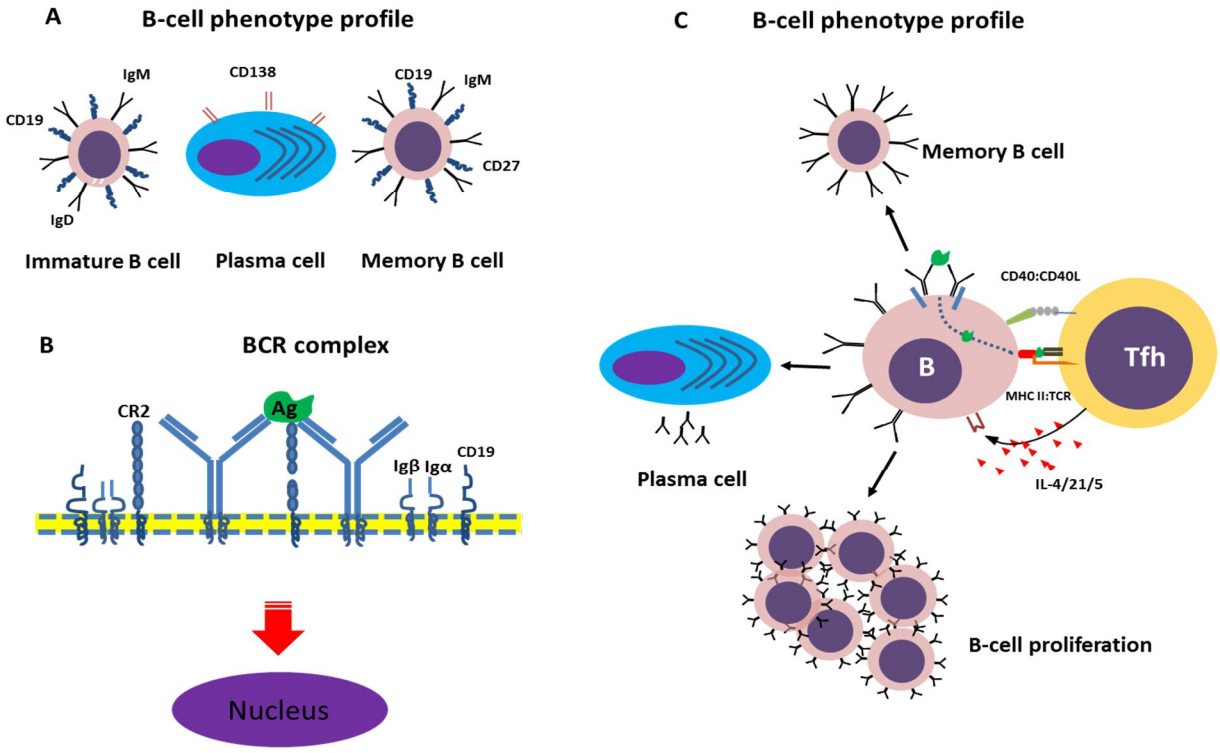

Figure 4: **Characteristics and interactions of B cells** (A) B-cell phenotypic profiles for immature, memory B cell and plasma cell. (B) B-cell receptor (BCR) and the linkage of an antigen and co-receptors are also represented, for example, CD19 (pan marker for B cells). (C) A B-cell up-taking, processing and present an antigen to a cognate Tfh cell, forming a linked recognition followed of the formation of germinal centers (GCs). Adapted from Kaminski et al. (2012) [149], Shlomchik and Weisel (2012) [122] and Yuseff et al. (2013) [120].

1.2.3 B-cell activation

A naïve mature B cell in a secondary lymphoid organ is subject to encounter its cognate antigen and begin its activation process [151, 155, 156]. The first step is the recognition of a particular epitope of the antigen via BCR [157] It may happen via cross-link of the receptor by multivalent antigens (Figure 4B), such as lipopolysaccharide (LPS) [158] or by mitogens, for instance, poke-weed [159, 160]. The outcome of this type of activation, called thymus-independent (TI), is proliferation and differentiation of the TI activated B cells into short-lived plasma cells, which can mainly produce IgM as antibody, with this type of activation B cells do not develop the ability of somatic hypermutation or antibody class-switching. What's more, the antibodies produced have low affinity for the antigen and no immunological memory is developed, however, it is controversial [161, 162]. It may be better understood as an B-cell innate response to a microbe, because it is fast and intense, delaying then the establishment of an infection [163].

Another way to induce TI B-cell activation is via toll-like receptors (TLRs) agonists, for instance, human B cells express in a great deal intracytoplasmic TLR-9 so that CpG ODN DNAs can induce B-cell activation. It has been used for the *in vitro* activation of B cells, a combination of CpG ODN and BCRs agonists such as IgM $(Fab`)_2$ portion with pronounced success in research [128].

The thymus-dependent (TD) activation of B cells is a more complex process, and is described as the process after a B cell meeting a microbial antigen, via BCR. It then internalizes, processes and presents the antigen via MHC II to a cognate follicular T helper cells (Tfh). This process happens in a secondary lymphoid organ, for instance, spleen and is a reciprocal communicative interaction between B and T cells [135, 164].

By presenting antigen to a T cell (the B-cell APC function), the T cell also becomes activated and upregulates molecules such as CD40L (CD154), CD28 which link to their respective receptors on the B-cell surface, CD40 and CD80 (B7-1) or CD86 (B7-2) respectively [151, 165]. In addition, the Tfh also produce cytokines, such as IL-4, IL-21, IL-2, IL-6, IL-5 and others, which function as growth factor and induce differentiation, antibody class-switching and secretion and activation of genes involved

in B-cell survival (anti-apoptotic genes), up-regulation of MHC II, and costimulatory molecules (B7-1 and B7-2) and formation of germinal centers (GC) [151, 163, 166-169]. Other proteins involved in the B-cell activation or fate are the cytokines A proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF), they are both tumor necrosis factor superfamily ligands (TNFSF ligand) and are remarkably important for B-cell activation and fate in homeostasis and diseases such as autoimmune diseases and cancer [170, 171]. The former is linked to development and survival, and the latter is involved in activation, proliferation, and differentiation [172, 173]

2. Aim of study

MDSCs are key components of the immunosuppressive tumor microenvironment, and they have been described as unpleasant components due to their contribution to tumor progress, positively correlation with patient tumor burden, and development of metastasis. They play also a role in inflammatory, infective diseases and trauma. MDSCs are known to suppress the immune function of T cells in several pathological conditions and homeostasis. The effects of MDSCs on B cells remain elusive, and the pieces of information provided so far, is mainly obtained from studies using murine systems, which are sub-optimal of normal human B-cell function or genuine diseases. B cells play important roles in several diseases, such as lymphomas, autoimmune diseases and infections, they play also a pivotal role in the maintenance of homeostasis. Due to that, it is important to reveal the possible interactions between MDSCs-B cells and then make more robust assumptions towards therapeutically performances and treatment of pathologies involving these both cells types.

In light of the foregoing, the aim of this project was to depict the interactions between polymorphonuclear-MDSCs and B cells. Using *in vitro* assays, we performed cell co-cultures using isolated MDSCs and B cells in different ratios. We applied different ways of B-cell activation, one is an unspecific way by using phorbol 12-myristate 13-acetate (PMA) plus ionomycin (ION) and the other using specific stimulus, CpG oligodeoxynucleotides (ODN CpG) plus IgM $(Fab`)_2$. From the co-cultures, we performed B-cell proliferation tests, by following carboxyfluorescein succinimidyl ester (CFSE) dilution of the CD19 positive cells by flow cytometry, and collection of supernatant to observe the titer of immunoglobulin M (IgM) by ELISA. In addition, we applied live-dead tests by using annexin V and propidium iodide (PI). In order to depict the mechanism involved in the interactions between PMN-MDSCs and B cells, we applied transwell systems to check on cell-contact dependence and tests of inhibitors for arginase-1 (Nor-NOHA), iNOs (L-NMMA), ROS (DPI) and IDO (1-MT). To confirm physical contact between PMN-MDSC: B cell we used microscopy flow cytometry technology (image stream).

3. Discussion

The aim and focus here is the investigation of the interactions between human MDSCs and B cells in homeostasis. MDSCs exert their suppressive mechanisms in pathological conditions, such as chronic inflammation, autoimmune diseases, infections and various types of cancers as well as in homeostasis. The tumor microenvironment milieu is highly immunosuppressive and a great deal of this phenomenon is due to the accumulation and expansion of suppressive myeloid cells, mainly MDSCs. These cells are closely related to the patient poor prognosis as well as the development of metastasis. They are involved in a complex cell web cross-talk and they have been described to regulate T-, DCs, NK-, NKT-cells and neutrophils responses [59, 69, 174]. A few studies have been carried out regarding the effects of MDSCs on B cells, most of them were conducted using mouse models, which are only sub-optimal systems to reflect human B-cell function or genuine diseases [149]. Our experimental results in this study reveal that human PMN-MDSCs in a dose-dependent fashion and by means of different suppressive mechanisms dampen B-cell function at several levels, by suppressing B-cell proliferation and antibody production, depending on the B-cell stimulation type. In order to depict the mechanisms involved in these interactions we demonstrate that the MDSC-mediated effects are cell contact-dependent, involve arginase-1, NO, ROS and cell death. All in all, our studies established a novel function of PMN-MDSCs by regulating B-cell homeostasis, which could have future implications for the immunotherapy approaches.

3.1 PMN-MDSCs suppress B-cell proliferation and antibody production in a dose- and stimulus-dependent manner

To investigate the effects of human MDSCs on B-cell proliferation and antibody production we isolated PMN-MDSCs, PMNs (used as control) and B cells and co-cultured them upon non-specific and specific B-cell stimulation. Our data demonstrate that PMN-MDSCs but not PMNs strongly inhibited specific activated (with CpG plus IgM) B-cell proliferation, in a dose-dependent fashion. Next, we evaluated the IgM titer, and found that MDSCs also significantly decreased IgM secretion also in a dose-depended manner. Control using conventional human neutrophils had no significant

effect on proliferation or IgM secretion. As PMN-MDSCs share common phenotypic characteristics with conventional neutrophils, it is important to notice that in a study using mice systems it was found that neutrophils interacted and stimulate B cells in the marginal zone (MZ B cells) of the spleen ("B cell-helper neutrophils"). MZ B cells recognize PAMPs due to poor diversification of BCR and they are considered innate ASCs, which produce IgM mainly. Particularly, splenic neutrophils triggered antibody production by MZ B cells (IgM) and immunoglobulin class switching to IgG and IgA isotypes [175]. This B-cell thymus dependent activation side of the neutrophils, is probably due to their capacity to produce BAFF and APRIL cytokines, which are CD40L-ralated molecules. Interestingly, neutropenic patients exhibited lower MZ B cells, but it is controversially discussed [176, 177]. In respect to MDSCs, recent finds based on murine studies point towards an immunosuppression of MDSCs on B-cell immune functions in infective diseases, such as acquired immune deficiency syndrome (AIDS) and BM5 retrovirus infection [114, 178]. Moreover, autoimmune disease/autoimmune arthritis was also target of instigation for MDSC-B-cell interactions. The study showed in a mouse model hat MDSCs suppressed autologous B-cell proliferation and antibody production [116]. Unspecific stimulated B-cells with PMA and ionomycin was not affected by PMN-MDSCs suppressive mechanisms, as the direct and simultaneous stimulation of protein kinase C (PKC) and Ca^{2+} opening channels is probably too strong to be affected.

3.2 PMN-MDSC-mediated B-cell suppression is cell-contact dependent and involves arginase-1, nitric oxide, reactive oxygen species, cell death.

MDSCs use a variety of molecular mechanisms to suppress T cells, some of the mechanisms are cell-contact dependent and involve ROS, iNOS, arginase-1, cytokines and other factors. [51, 98, 179]. An interesting and novel aspect is that MDSCs are also involved in the induction of Bregs (IL-10 producing B cells) by means of arginase-1 and iNOS production [117]. Here we also demonstrate that PMN-MDSCs require cell-to-cell contact to suppress B-cell proliferation by using transwell inserts systems. Transwell separation of PMN-MDSCs from CpG/IgM stimulated B cells, restored B-cell proliferation compared to the cell-to-cell contact cultures. This phenomenon indicates contact dependence, and was confirmed by proliferation assays (CFSE and flow

cytometry). Microscopic flow cytometry (Image Stream) confirmed the cell contact between B cells and PMN-MDSCs. Investigations using mouse systems further support to our findings. To give illustration of that, in the rheumatoid arthritis studies performed by Crook et al. (2015) [116], MDSCs required cell contact in order to inhibit B-cell proliferation. In contrast, it has been published that B-cell lymphopoiesis can be inhibited by MDSCs contact independently by the MDSCs production of IL-1 [180].

A key approach to depict MDSCs suppressive functions, is by inhibiting their mechanisms of suppression [92, 102, 181, 182]. In this regard, we applied inhibitors for arginase-1 (Nor-NOHA), iNOS (L-NMMA), NADPH/ROS (DPI) and IDO (1-methyl-D-tryptophan (1-MT)) in the cell culture with B cells and PMN-MDSCs in different ratios. Inhibition of arginase-1 and iNOS restored B-cell proliferation to almost 100% of the baseline B-cell proliferation. DPI effects were less pronounced; however, we could still observe a significant recovery in B-cell proliferation. Inhibition of IDO with 1-MT did not result in recovery of B-cell proliferation, as it seems to be toxic to B cells, as it is supported by Pigott and Mandik-Nayak (2012) [183]. Studies using murine models support the involvement of MDSCs` arginase-1, NO and ROS in B-cell suppressive functions by means of inhibitors usage as well as the involvement of the negative-checkpoint regulator V-domain Ig suppressor of T-cell activation (VISTA). In addition, by using L-NMMA, it was also described the contribution of iNOS/NO, arginase-1 (Nor-NOHA) and IL-10 in inhibition of B-cell proliferation and responsiveness [114, 115, 178, 180]. To our knowledge, there is no indication that ROS inhibition has been directly tested on MDSCs-B cells analysis, but in previous studies of our lab involving MDSCs-T cells, DPI has successfully been applied to inhibit ROS production of MDSCs [102, 184].

Besides the proliferative and IgM evaluations, we also demonstrate here that human PMN-MDSCs regulate B-cell death of CpG/IgM activated. We observed a decrease in B-cell apoptosis in presence of MDSCs. Moreover, we also show a significant increase on B-cell necrosis. No differences were found in co-culture with conventional PMNs. Information that MDSCs induce B-cell death is scarce. It is described that MDSCs promote T-cell apoptosis, nonetheless. It is also proclaimed that MDSCs promote T-cell death via a mechanism involving IDO in a breast cancer study [93]. In a review

article, Rodriguez and Ochoa (2008) [66] suggested that the peroxynitrites originated from MDSCs as product of ROS and NO reactions due to the deprivation of L-arginine, lead to T-cell death. Our finds showed that the B-cell death profile is skewed to necrosis rather than apoptosis. It may be explained due to the fact that MDSCs` oxygen and nitrogen species additionally to low levels of L-arginine, accelerate the physiological cell-death fate or directly induce necrosis. The B-cell death was decreased by using transwell inserts as well as in the presence of the inhibitors such as L-NMMA, Nor-NOHA and DPI.

4. Conclusion

In conclusion, our study demonstrates that the suppressive potential of human MDSCs is not restricted to T-, DC, NK-, or NKT-cell responses, but also regulates B cells. PMN-MDSCs were found to regulate B-cell proliferation and antibody production, depending on the B-cell stimulus. Mechanistically, these effects involved cell-to-cell contact, cell death, arginase-1, NO and ROS indicating that the interactions between PMN-MDSCs and B cells involve multiple mechanisms. Taken together, our finds help to shed light on future investigations to reveal in deep the MDSCs-B-cell interactions, and in this way to bring novel and significant immunotherapeutic insights, for instance, in autoimmune diseases to dampen exacerbate B-cell responses and autoantibody production. Future studies in preclinical disease models and patients are warranted to address this potential role of MDSCs as regulators of B-cell activities.

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INTERNSHIP EXPERIENCE

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FOREIGN LANGUAGE SKILLS

English: Advanced level
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COMPUTING SKILLS

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Appendix

Paper 1

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Myeloid-derived suppressor cells modulate B-cell responses

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Short title: MDSCs modulate B cells

Abstract

Myeloid-derived suppressor cells (MDSCs) are key regulators of adaptive immunity by suppressing T-cell functions. However, their potential action on or interaction with B cells remained poorly understood. Here we demonstrate that human polymorphonuclear MDSCs differentially modulate B-cell function by suppressing B-cell proliferation and antibody production. We further demonstrate that this MDSC-mediated effect is cell contact dependent and involves established mediators such as arginase-1, nitric oxide (NO), reactive oxygen species (ROS) as well as B-cell death. Collectively, our studies provide novel evidence that human MDSCs modulate B cells, which could have future implications for immunotherapy approaches.

Key words: myeloid-derived suppressor cells, MDSCs, B cells

Abbreviations

CFSE:	Carboxyfluoresceinsuccinimidyl ester
DAPI:	4',6-diamidino-2-phenylindole
DC:	Dendritic cell
DPI:	Diphenyleneiodonium
FACS:	Fluorescence-activated cell sorting
PMN-MDSCs:	Polymorphonuclear Myeloid-derived suppressor cells
IDO:	Indoleamine 2,3-dioxygenase
iNOS:	Inducible nitric oxide synthase
IL-:	Interleukin-
L-NMMA:	L-N^G-monomethyl Arginine citrate
MACS:	Magnetic-activated cell sorting
MDSCs:	Myeloid-derived suppressor cells
1-MT:	1-methyl-tryptophan
NK cells:	Natural killer cells
NKT cells:	Natural killer T cells
NO:	Nitric oxide
Nor-NOHA:	Nω-hydroxy-nor-Arginine
PBMCs:	Peripheral blood mononuclear cells
PMNs:	Polymorphonuclear leukocytes
ROS:	Reactive oxygen species
TNF-α:	Tumor necrosis factor alpha
IgM:	Immunoglobulin M

1. Introduction

Myeloid-derived suppressor cells (MDSCs) are innate immune cells that are functionally characterized by their capacity to dampen T-cell responses [1,2]. Phenotypically, MDSCs are a heterogeneous population and comprise a neutrophilic/polymorphonuclear (PMN-MDSC) and a monocytic (M-MDSC) subset as defined by their respective surface marker profiles [3]. In humans, polymorphonuclear MDSCs represent the predominant subtype [4,5] and have been studied in several forms of cancer as well as in infections, auto-immune and auto-inflammatory disease conditions [1,6].

While initially MDSCs were found to suppress $CD4^+$ and $CD8^+$ T-cell responses, subsequent investigations expanded their role by demonstrating that MDSCs were also involved in the regulation of and interaction with natural killer (NK) cells, dendritic cells (DC), neutrophils, macrophages and natural killer T (NKT) cells [7-9]. The molecular and cellular mechanisms involved in MDSC generation and function seem to be diverse and complex, but major mediators described to be involved so far include arginase 1 activity (arginine deprivation), reactive oxygen species (ROS), nitric oxide synthase (NO), indoleamine 2,3-dioxygenase (IDO) and tumor necrosis factor alpha (TNF-α) [1,6,8,9]. Recently, the transcriptional regulation and the proteome landscape of MDSCs have further deepened our insights into their generation, homeostasis and regulation [10,11].

In spite of multiple MDSCs-immune cell interactions described [7-9], the potential role of MDSCs in regulating B cells, as key adaptive immune cell population, remained poorly understood. Several recent observations already suggested that MDSCs and B cells functionally interact in mice [12-16], but the characteristics of human MDSCs-B-cell interactions and the underlying mechanisms have not been defined yet.

Here we demonstrate that human PMN-MDSCs differentially regulate key B-cell functions, particularly B-cell proliferation and antibody production, depending on the B-cell stimulus. We further demonstrate that this MDSC-mediated effect is cell contact dependent and involves arginase-1, NO, ROS and cell death.

2. Material and Methods

2.1. Cell isolation

The study was conducted at the University Children's Hospital Tuebingen (Germany). All study methods were approved by the local ethics committee. Buffy coats were provided by *DRK-Blutspendedienst* Baden-Württemberg-Hessen Institute, Ulm, Germany. Peripheral blood mononuclear cells (PBMCs) and (high-density) polymorphonuclear leukocytes (PMNs) were prepared from blood samples by Ficoll density gradient sedimentation (Biochrome). After one washing step in RPMI 1640 medium (Biochrome), erythrocytes were lysed in Lysis buffer (0.829% ammonium chloride, 0.1% potassium hydrogen carbonate, 0.00372% Ethylenediaminetetraacetic acid disodium salt dihydrate in water). PMN-MDSCs were isolated and characterized as described in our previous studies [17-19]. In brief, PMN-MDSCs in the Ficoll PBMC interphase fraction were labelled with anti-CD66b-fluorescein isothiocyanate (FITC) and isolated by an anti-FITC sequential magnetic bead separation step using the autoMACS®Pro Separator (Miltenyi Biotec), according to the manufacturer's protocol. The B-cell isolation strategy was based on previous protocols [20] using negative selection from the PBMCs fraction. Therefore, cells were labelled with anti-CD43-FITC followed by anti-FITC sequential magnetic bead separation using autoMACS®Pro Separator. One further labelling step with a combination of anti-CD3 magnetic beads and anti-CD14 magnetic beads was performed, followed by a second magnetic bead separation step using autoMACS®Pro Separator. Microbeads against CD3, CD14 or FITC were purchased from Miltenyi Biotec.

2.2. Cell culture, CFSE labeling and B-cell activation

Isolated B cells were stained with carboxyfluorescein-succinimidyl ester (CFSE, Life Technologies) at day 0, according to the manufacturer's protocol. For the CD86 and HLA-DR/MHC-II receptor analysis, no CFSE-staining was performed. For activation, isolated B cells were stimulated with PMA (1 µM, Sigma) plus ionomycin (0.5 µg/mL, Sigma) or 5 µg/mL IgM (Jackson ImmunoResearch) plus 2.5 µg/mL CpG ODN (InvivoGen). 5 x 10^5 B cells per well were seeded in RPMI-1640, in a 48-well microtitre plate (Life Sciences) and PMN-MDSCs or PMNs as a control in RPMI-1640 were co-cultured at the ratios 1:5, 1:2 or 1:1 (MDSC:B cell). The transwell assay was performed using a transwell insert system (6.5 mm diameter inserts with 0.4 µm pores, Greiner BIO-ONE). Where indicated, inhibitors of Arginase-1, iNOS, ROS and IDO production, namely Nω-hydroxy-nor-Arginine 300 µM (Nor-NOHA/Enzo Life Sciences), L-N^G-monomethyl arginine citrate 300µM (L-NMMA/Calbiochem) or Methyl-Tryptophan 500 µM (1-MT/Sigma Aldrich) were added to the cell culture (for details see Table 1 and [21]). Where indicated, diphenyleneiodonium chloride 1 µM (DPI/Sigma Aldrich) was incubated for 1 h at 37°C with PMN-MDSCs before these cells were washed once to get rid of excessive DPI as described in Table 1 and previously [22,23]. The cell culture was supplemented with 10 % human serum, 2 mM L-glutamine (Millipore) and 100 U/mL penicillin/streptomycin (Millipore). After 120 h of incubation at 37°C and 5 % CO_2, cells were harvested and supernatants were frozen at -20°C.

2.3. Immunoglobulin M analysis

Immunoglobulin M (IgM) analysis in collected supernatants from the CFSE-culture was performed using human IgM Ready-SET-GO from eBioscience, according to the manufacturer's protocol with the exception that supernatants were used in a 1:100 dilution.

2.4. Flow cytometry

For B-cell stainings, harvested cells were stained with a PE-conjugated (Biolegend) or FITC-conjugated (BD Biosciences) anti-CD19-antibody for 10 min. To assess B-cell proliferation, the CFSE fluorescence intensity was analyzed. For necrosis and apoptosis studies, propidium iodide (PI, BD Bioscience) and Annexin V (BD Bioscience) stainings were performed as to the protocols of the manufacturer, respectively. For the analysis of cell surface receptors, CD86-receptor was stained using anti-CD86-APC antibody (Biolegend) and MHCII-receptors were stained with anti-HLA-DR-PerCP (Biolegend). 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. Supplementary Figure 1 shows the flow cytometric gating strategy.

2.5. ImageStream

The Image Stream^{x} mk II (Merck Millipore) system with the INSPIRE instrument software was used for acquisition and the IDEAS® data analysis software for image analysis. In brief, human B cells and PMN-MDSCs were isolated from human peripheral blood. Following incubation, cells were stained for CD66b-PE (Biolegend) for PMN-MDSCs, CD19-FITC (BD) for B cells and DAPI for nuclear stain (ThermoFisher). Cell doublets were identified, a valley mask on the DAPI image was created in order to define the contact region between conjugates and an “interface” mask was applied in order to define the overlap area between MDSCs and B cells. MDSC-B-cell interactions were visualized.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0. Differences between the groups were determined by ANOVA when comparing 5 groups and un-paired, two-tailed Student's t-test for comparing 2 groups. All results are presented as mean + SEM. A *P* value < 0.05 was considered significant.

3. Results

3.1. PMN-MDSCs suppress B-cell proliferation and antibody production in a dose- and stimulus-dependent manner

We systematically assessed the potential of isolated human PMN-MDSCs to suppress B-cell responses. PMN-MDSCs were defined by their low-density, respective surface markers and by their characteristic to suppress T-cell responses as published previously by our group [17-19]. Conventional (high-density) non-MDSC human PMNs cells were used as control cell population. Initially, we tested the effect of MDSCs on non-specific B-cell activation, using PMA and ionomycin. These results demonstrated that neither PMN-MDSCs nor control neutrophils/PMNs had an effect on PMA/ionomycin-induced B-cell proliferation (Figure 1A). Next, we analyzed the effect of MDSCs on specific B-cell activation, elicited by IgM $F(ab`)_2$ plus ODN CpG, as described previously [24,25]. These studies, in contrast to our findings on PMA/ionomycin-activated B-cell proliferation, demonstrated that PMN-MDSCs dose-dependently suppressed B-cell proliferation, with the most potent suppression observed at 1:1 ratio, whereas control PMNs did not (Figure 1B). Furthermore, we investigated whether MDSCs modulate the expression of co-stimulatory molecules and activation markers on the surface of B cells. These studies demonstrated that PMN-MDSCs decreased the surface expression of IgM F(ab`)2/CpG-induced CD86 expression on human B cells in a dose dependent fashion, but had no significant effect on HLA-DR/MHC-II expression (Figure 2A). Finally, we assessed whether MDSCs affect antibody production by B cells. These data demonstrated that PMN-MDSCs significantly suppressed IgM $F(ab`)_2$/CpG-induced-IgM production. Conventional PMNs did not show any significant effect on IgM suppression (Figure 2B). Taken together, these data demonstrated that human PMN-MDSCs modulate B-cell responses in a dose- and B-cell stimulus-dependent manner.

3.2. PMN-MDSC-mediated B-cell suppression is cell contact dependent and involves arginase-1, nitric oxide, reactive oxygen species and cell death

Next, we sought to dissect the mechanisms involved in MDSC-mediated B-cell suppression. Since MDSCs have been described to mediate target cell suppression through cell contact as well as paracrine soluble mediator mechanisms, we analyzed whether MDSC-mediated B-cell suppression required cell-to-cell contact by using a Transwell co-culture system as applied previously by our group [17]. These investigations demonstrated that MDSC-mediated suppression of B-cell proliferation required cell-to-cell contact, since Transwell-separated B cells were not affected by increasing numbers of PMN-MDSCs (Figure 3A). Imagestream analysis confirmed the physical interaction of PMN-MDSCs and B cells in our assays (Supplementary Figure 2). In a subsequent step, we reasoned which MDSC-related factors are involved in MDSC-mediated cell contact dependent B-cell proliferation. Based on previous studies [21,23] we focused on arginase-1, NO, ROS and IDO as factors potentially involved in T-cell suppression. For this purpose, we pretreated PMN-MDSCs by using specific inhibitors for these mediators such as Nor-NOHA, L-NMMA, DPI and 1-MT, respectively, in MDSC-B-cell co-culture assays as indicated in Table 1. These investigations demonstrated that these inhibitors, with exception of 1-MT, dampened the effect of MDSCs on B-cell proliferation (Figure 3B). In addition, we investigated whether the effects of MDSCs are related to induction of B-cell apoptosis and/or necrosis by flow cytometry using Annexin V and propidium iodide (PI) staining (Figure 4). These analyses demonstrated that human PMN-MDSCs, in high concentrations, prevented B-cell apoptosis (Figure 4A), but instead B-cell necrosis was induced when co-cultured at 1:2 and 1:1 ratios (Figure 4B). No pronounced effect was observed in late apoptosis when assessed by the double positive B cells for Annexin V and PI (Figure 4C). Again, conventional PMNs had no significant effect on B-cell apoptosis or necrosis

(see right panels of Figure 4A-C). In line with the effect on proliferation and antibody production the PMN-MDSC-induced cell death of the B cells was also cell contact dependent (Figure 5A). Consistently, inhibitors of the MDSC effector pathways also interfered with the MDSC-mediated effect on B-cell death (Figure 5B). Collectively, these studies demonstrate that human PMN-MDSCs dampen B-cell proliferation through a mechanism involving cell-to-cell contact, B cell death, arginase-1, NO and ROS.

4. Discussion

MDSCs have been described to regulate T-, DC, NK-, NKT-cell and neutrophil responses [7-9]. However, their potential effect on human B-cell homeostasis and function remained largely elusive. Here we demonstrate that human PMN-MDSCs dose-dependently and differentially regulate B-cell function at several levels, by suppressing B-cell proliferation and antibody production, depending on the B-cell stimulus. Mechanistically, we also demonstrate that the MDSC-mediated effects are cell contact-dependent and involve arginase-1, NO, ROS and cell death. Collectively, our studies established a novel function of PMN-MDSCs by regulating B-cell homeostasis, which could have future implications for immunotherapy approaches.

Evidence on MDSC-B-cell interactions is scarce. Polymorphonuclear MDSCs share common phenotypic characteristics with conventional neutrophils. In mice, neutrophils were found to interact and stimulate B cells in the marginal zone of the spleen („B cell-helper neutrophils“) [26]. Particularly, splenic neutrophils triggered immunoglobulin class switching and antibody production by activating B cells. Interestingly, neutropenic patients exhibited lower marginal zone B cells. While these intriguing findings were challenged by another study which failed to detect functional “B cell-helper neutrophils” in the human spleen, the potential interaction between neutrophils and B cells remains an active and controversial field [27].

Regarding MDSCs, recent murine studies point towards an interaction of MDSCs and B cells in the contexts of murine acquired immune deficiency syndrome (AIDS) and BM5 retrovirus infection [13,14,28] and autoimmune disease / autoimmune arthritis [12]. In the latter study, MDSCs in a mouse model of autoimmune arthritis suppressed autologous B-cell proliferation and antibody production via NO and prostaglandin E2 (PGE2) and in a cell contact dependent manner. Moreover, IL-1-producing murine MDSCs were found to inhibit B-cell lymphopoiesis [15]. In

addition, tumor-evoked B regulatory cells (tBregs) were found to activate the function of MDSCs through a TGF-beta receptor type-1/2 -mediated mechanism [29]. In murine models, MDSCs co-cultured with splenocytes triggered the expansion of IL-10-producing B cells, which was blocked upon inhibition [30]. In patients with non-small cell lung carcinoma, interleukin-10 (IL-10) producing Breg cells correlated positively with MDSCs [31].

Despite these intriguing insights into MDSC-B-cell interactions, these concepts were mainly based on findings in the murine system, while human data on the reciprocal MDSC-B- cell relationship remained elusive. Here we comprehensively dissected the effect of human PMN-MDSCs on B-cell homeostasis and function. Our studies demonstrate that human MDSCs supress B-cell proliferation and antibody production in a dose- and B cell stimulus-dependent manner. Furthermore, our investigations showed that, in line with studies on MDSC-T-cell interactions, MDSC-mediated B-cell suppression was cell-cell contact dependent and involved soluble mediators such as arginase-1, NO and ROS as well as cell death. Control conventional human neutrophils had no significant effect on proliferation, B-cell death or effect on IgM production. The cellular mechanism(s) underlying this functional difference between human PMN-MDSCs and conventional human neutrophils in regulating B-cell functions remain to be dissected in future investigations. However, based on previous studies demonstrating that both cell-to-cell contact / cellular proximity and the soluble mediators above mentioned were involved in T-cell suppressive activities of a distinct PMN-MDSC-like neutrophil subset induced during acute systemic inflammation in humans [32], we tempt to speculate that arginase-1, iNOS and ROS activities may represent common key mechanisms involved in PMN-MDSC-mediated suppression of both T- and B-cell responses.

5. Conclusion

In summary, our studies demonstrate that the suppressive potential of human MDSCs is not restricted to T-, DC, NK-, or NKT-cell responses, but also regulates B cells at several levels. PMN-MDSCs were found to regulate B-cell proliferation and antibody production, depending on the B-cell stimulus. Mechanistically, these effects involved cell-to-cell contact, cell death, arginase-1, NO and ROS. Based on these findings, the *in vivo* induction / expansion or the adoptive transfer of sorted MDSCs may represent a novel immunotherapeutic tool in autoimmune diseases to dampen B-cell responses and autoantibody production. Future studies in preclinical disease models and patients are warranted to address this potential role of MDSCs as regulators of B-cell activities.

Authorship

F.L. performed the experiments, analyzed the data and co-wrote the manuscript. A.S. co-supervised the study, co-wrote the manuscript. K.F. performed the experiments, co-wrote the manuscript and analyzed the data. J.J. and A.T. performed the experiments, analyzed the data. I.S. performed the experiments. S.P. performed the imagestream analyses. S. B-H.co-supervised the study and co-wrote the manuscript. N.R. co-supervised the study and analyzed data. D.H. supervised the study and co-wrote the manuscript.

Conflict of Interest Disclosure

All authors declare that no conflicts of interest exist.

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Figure legends

Fig. 1. MDSCs suppress B-cell proliferation in a dose- and stimulus-dependent manner

Human polymorphonuclear MDSCs (PMN-MDSCs), conventional neutrophils (PMNs) and B cells were isolated from buffy coats and co-cultured at 1:5, 1:2 and 1:1 ratios (MDSCs:B cells). Isolated B cells cultured in medium only served as negative controls and when cultured in presence of PMA (1 µM) plus ionomycin (0.5 µg/mL) or IgM F(ab`)$_2$ (5 µg/mL) plus CpG (2.5 µg/mL) served as positive controls. (A) Effect of MDSCs/PMNs on non-specific PMA/ionomycin-induced B-cell proliferation (CFSE assay, n=13). (B) Effect of MDSCs/PMNs on specific IgM F(ab`)$_2$/CpG-induced B-cell proliferation (CFSE assay, n=9). Bars represent means +SEMs; *P< 0.05.

Fig. 2. MDSCs supress B-cell antibody production in a dose- and stimulus-dependent manner

Human polymorphonuclear MDSCs (PMN-MDSCs), conventional neutrophils (PMNs) and B cells were isolated from buffy coats and co-cultured at 1:5, 1:2 and 1:1 ratios (MDSCs : B cells). Isolated B cells cultured in medium only served as negative controls and when cultured in presence of IgM F(ab`)$_2$ (5 µg/mL) plus CpG (2.5 µg/mL) served as positive controls. (A) Effect of MDSCs on IgM F(ab`)2/CpG-induced surface expression of CD86 and MHC-II (HLA-DR) (FACS, n=6). (B) Effect of MDSCs/PMNs on IgM F(ab`)$_2$/CpG-induced IgM levels (ELISA, n=8). Bars represent means +SEMs; *P< 0.05

Fig. 3. MDSC-mediated B-cell suppression is cell contact-dependent and involves soluble mediators such as arginase-1, nitric oxide (NO) and reactive oxygen species (ROS)

Human polymorphonuclear MDSCs (PMN-MDSCs) and B cells were isolated from buffy coats and co-cultured at 1:5, 1:2 and 1:1 ratios (MDSCs:B cells). Isolated B cells cultured in medium only served as negative controls and when cultured in presence of IgM F(ab`)$_2$ (5 µg/mL) plus CpG (2.5 µg/mL) served as positive controls (white bars). (A) The effect of transwell filters (0.4 µm pore size, n=8, light-gray bars) was compared to the cell to cell contact tests (n=9, dark-gray bars). In (B) the effect of arginase-1, iNOS, ROS and IDO inhibition (using Nor-NOHA, L-NMMA, DPI and 1-MT, respectively; n=8; dark-gray to light-gray bars gradient) on MDSCs is depicted. Except for 1-MT, all inhibitors mediated suppression of IgM F(ab`)$_2$/CpG-induced B-cell proliferation (CFSE assay) when compared to the positive control (CFSE assay, n=9,white bar). Bars represent means +SEMs; *P< 0.05.

Fig. 4. MDSC-mediated B-cell suppression involves cell death

Human B cells and polymorphonuclear MDSCs (PMN-MDSCs) or conventional neutrophils (PMNs) were isolated from buffy coats and co-cultured at 1:5, 1:2 and 1:1 ratios (MDSCs:B cells). Isolated B cells cultured in medium only served as negative controls and when cultured in presence of IgM F(ab`)$_2$ (5 µg/mL) plus CpG (2.5 µg/mL) served as positive controls. The effect of MDSCs/PMNs on specific IgM F(ab`)$_2$/CpG-induced B-cell (A) apoptosis (Annexin V staining, CFSE assays, n=9), (B) necrosis (PI staining, CFSE assays, n=9) and (C) late apoptosis (Annexin V, PI staining, CFSE assays, n=9). Bars represent means +SEMs; *P< 0.05.

Fig. 5. MDSC-mediated induction of B-cell death is cell contact-dependent and involves arginase-1, nitric oxide (NO) and reactive oxygen species (ROS)

Human polymorphonuclear MDSCs (PMN-MDSCs) and B cells were isolated from buffy coats and co-cultured at 1:5, 1:2 and 1:1 ratios (MDSC:B cells). Isolated B cells cultured in medium only served as negative controls and when cultured in presence of IgM F(ab`)$_2$ (5 µg/mL) plus CpG (2.5 µg/mL) served as positive controls. (A) The effect of transwell filters (0.4 µm pore size, n=8, light-gray bars) was compared to the cell to cell contact tests (n=9, dark-gray bars). In (B) the effect of arginase-1, iNOS, ROS and IDO inhibition (using Nor-NOHA, L-NMMA, DPI and 1-MT respectively, n=8 dark gray to light gray bars gradient) on MDSCs is depicted. All inhibitors mediated suppression of IgM/CpG-induced B-cell death as determined by PI staining compared to the positive control (CFSE assays, n=9, white bar). Bars represent means +SEMs; *P< 0.05).

Table 1: Description of the inhibitors used with respective concentrations, supplying companies, targets and references

Inhibitor	Concentration (µM)	Company	Target
Nor-NOHA*	300	Enzo Life Sciences	arginase-1
L-NMMA*	300	Calbiochem	iNOS
DPI*	1	Sigma Aldrich	ROS
1-MT*	500	Sigma Aldrich	IDO

*Added to the cell culture and incubated at 37°C, 5%CO_2 for 120 hours.

** DPI was incubated for 1 h at 37°C with PMN-MDSCs before cells were washed once to get rid of excessive DPI as reported previously [22].

Figure 1.

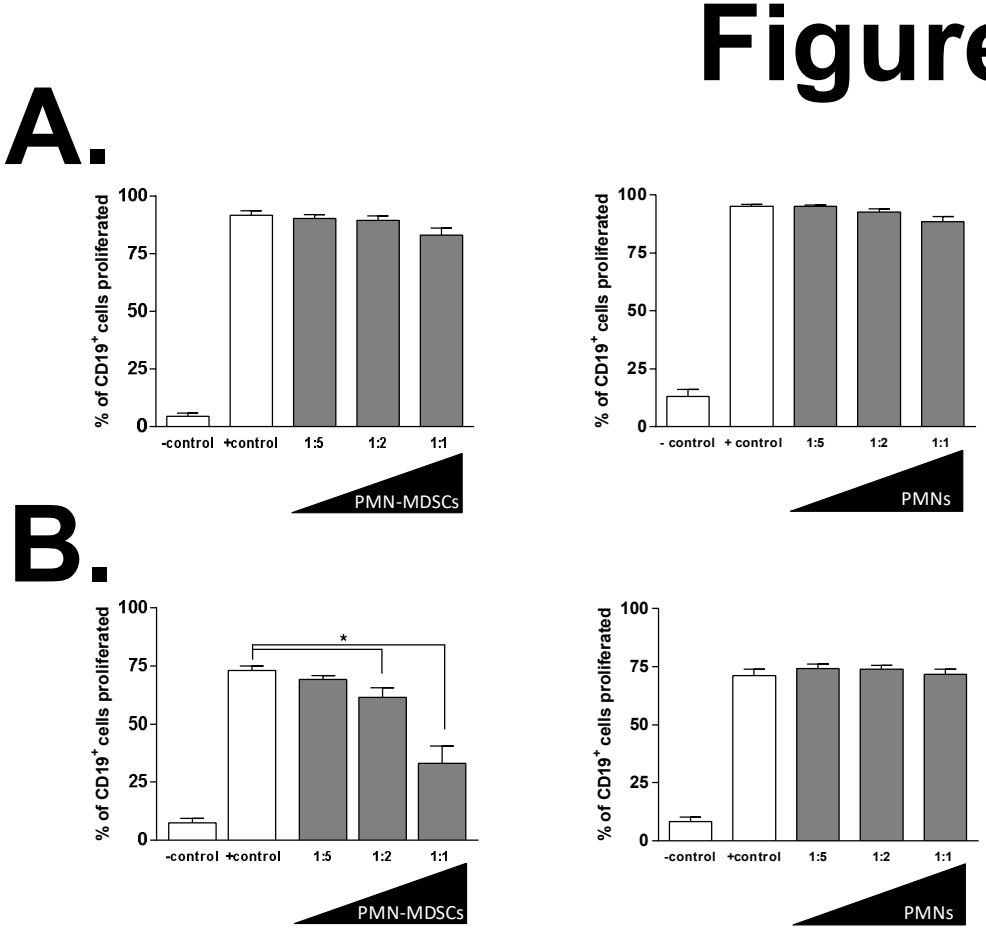
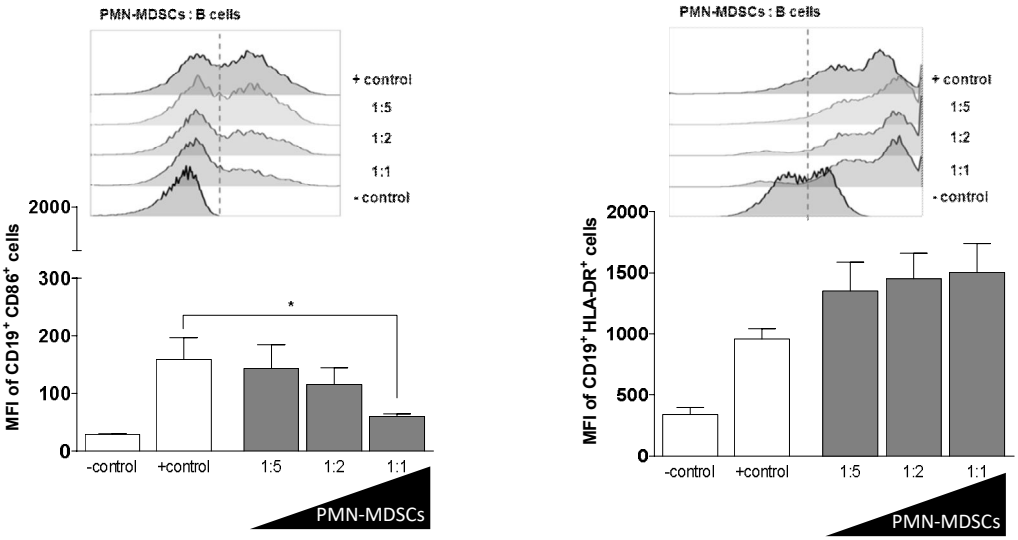

A.
B.
% of CD19+ cells proliferated
100
75
50
25
0
-control
+control
1:5
1:2
1:1
PMN-MDSCs
- control
+ control
PMNs
*

Figure 2.

A.


PMN-MDSCs : B cells
+ control
1:5
1:2
1:1
- control
2000
300
200
100
0
MFI of CD19+ CD86+ cells
*
-control
+control
1:5
1:2
1:1
PMN-MDSCs
PMN-MDSCs : B cells
+ control
1:5
1:2
1:1
- control
2000
1500
1000
500
0
MFI of CD19+ HLA-DR+ cells
-control
+control
1:5
1:2
1:1
PMN-MDSCs

B.

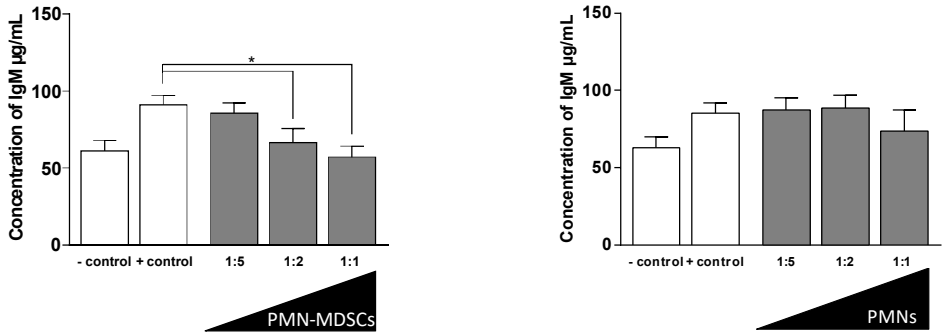
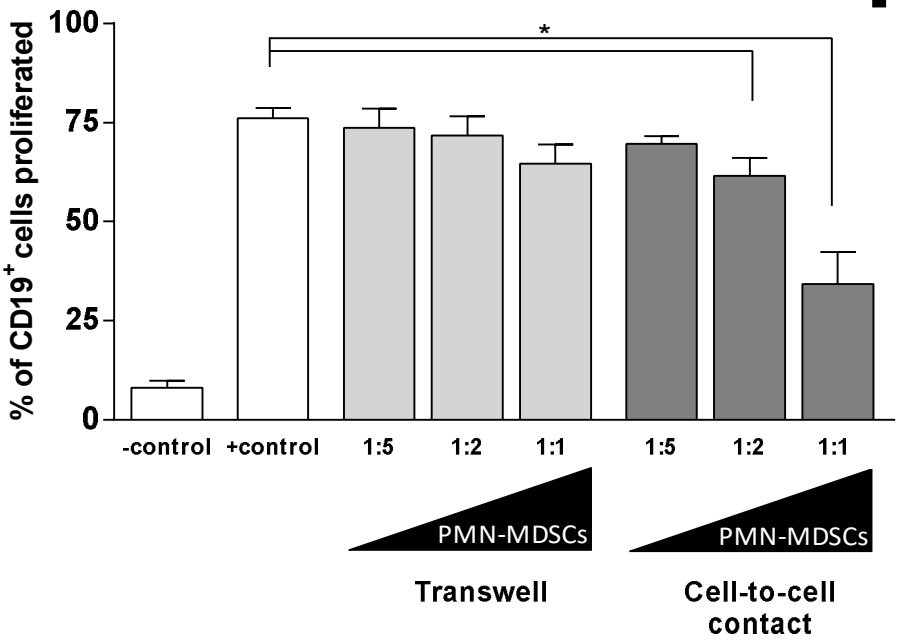

150
100
50
0
Concentration of IgM µg/mL
*
- control
+ control
1:5
1:2
1:1
PMN-MDSCs
150
100
50
0
Concentration of IgM µg/mL
- control
+ control
1:5
1:2
1:1
PMNs

Figure 3.

A.


% of CD19+ cells proliferated
100
75
50
25
0
*
-control
+control
1:5
1:2
1:1
1:5
1:2
1:1
PMN-MDSCs
PMN-MDSCs
Transwell
Cell-to-cell contact

B.

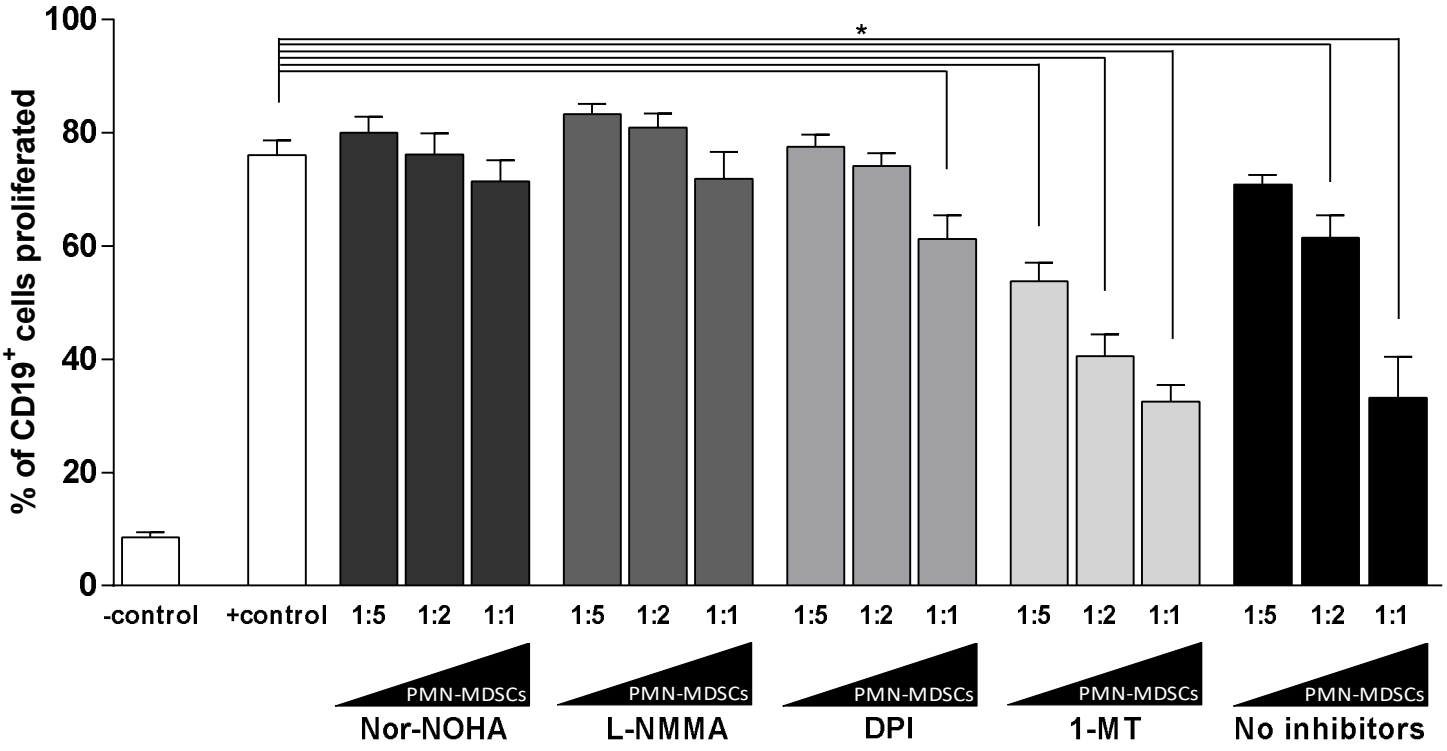
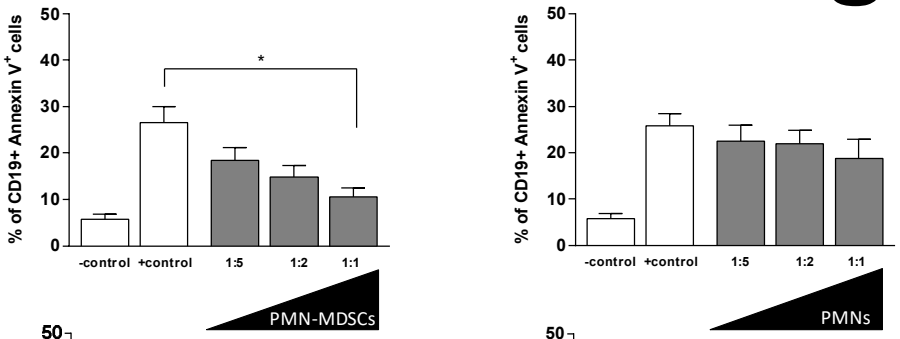

% of CD19+ cells proliferated
100
80
60
40
20
0
*
-control
+control
1:5
1:2
1:1
PMN-MDSCs
Nor-NOHA
L-NMMA
DPI
1-MT
No inhibitors

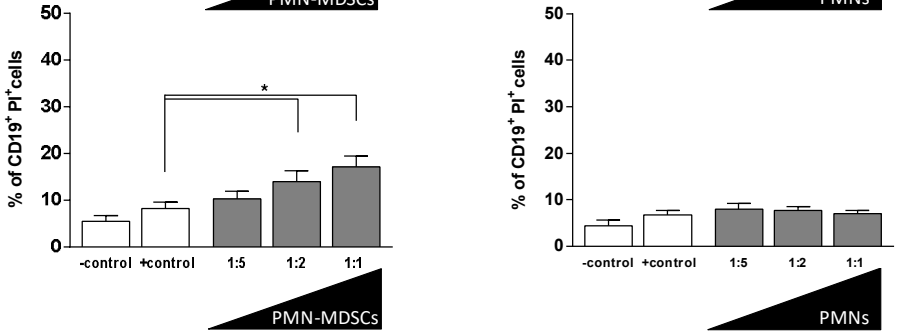
Figure 4.

A.


% of CD19+ Annexin V+ cells
50
40
30
20
10
0
*
-control
+control
1:5
1:2
1:1
PMN-MDSCs

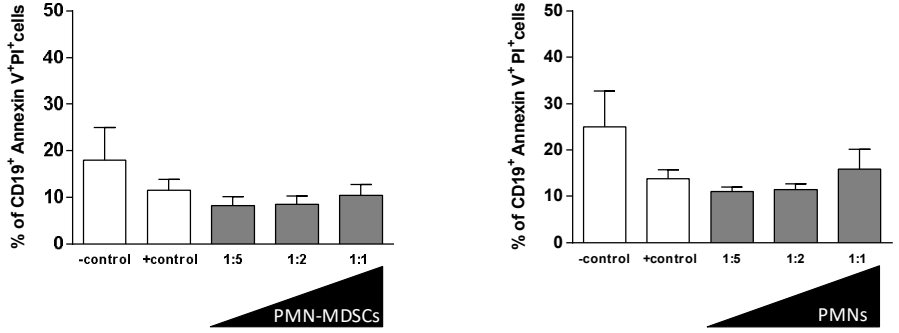
% of CD19+ Annexin V+ cells
50
40
30
20
10
0
-control
+control
1:5
1:2
1:1
PMNs

B.


% of CD19+ PI+cells
50
40
30
20
10
0
*
-control
+control
1:5
1:2
1:1
PMN-MDSCs

% of CD19+ PI+ cells
50
40
30
20
10
0
-control
+control
1:5
1:2
1:1
PMNs

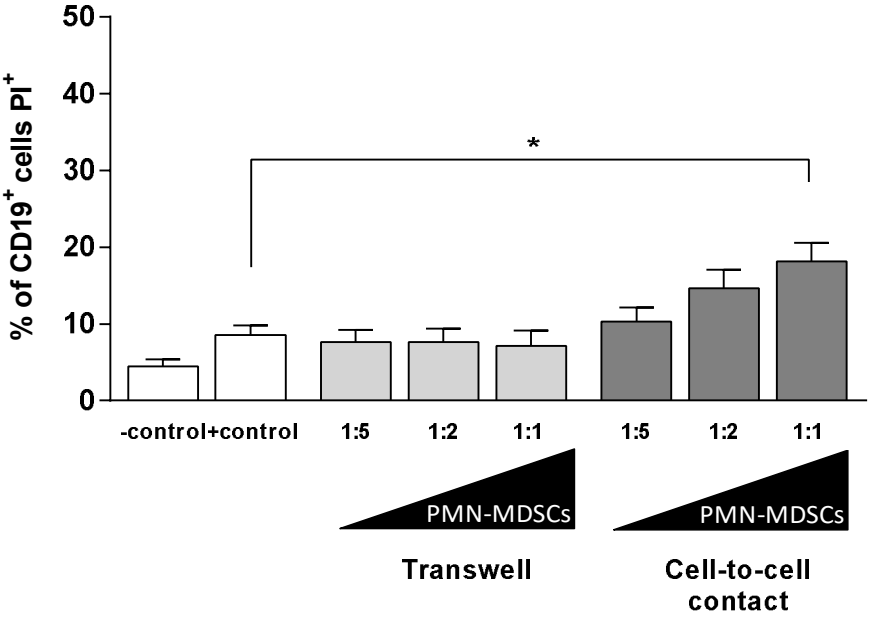
C.


% of CD19+ Annexin V+PI+cells
50
40
30
20
10
0
-control
+control
1:5
1:2
1:1
PMN-MDSCs

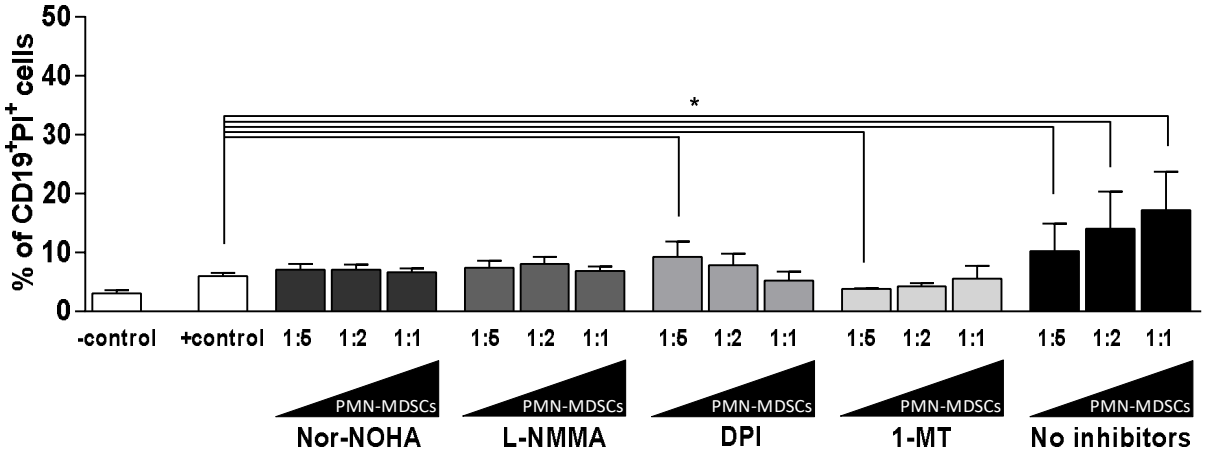
% of CD19+ Annexin V+ PI+cells
50
40
30
20
10
0
-control
+control
1:5
1:2
1:1
PMNs

Figure 5.

A.


50
40
30
20
10
0
% of CD19+ cells PI+
*
-control
+control
1:5
1:2
1:1
1:5
1:2
1:1
PMN-MDSCs
PMN-MDSCs
Transwell
Cell-to-cell contact

B.


50
40
30
20
10
0
% of CD19+PI+ cells
*
-control
+control
1:5
1:2
1:1
PMN-MDSCs
Nor-NOHA
L-NMMA
DPI
1-MT
No inhibitors

Highlights

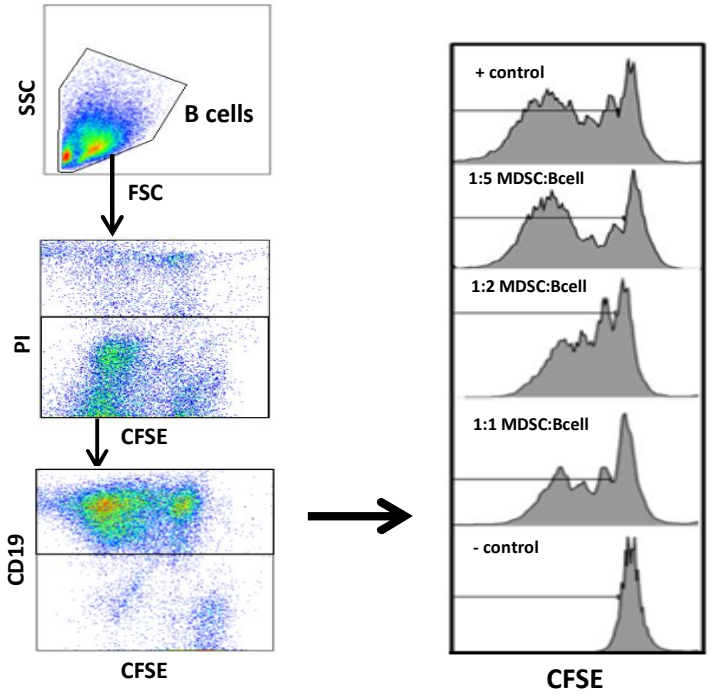
● Myeloid-derived suppressor cells (MDSCs) are important mediators of the immune cells.

● MDSCs impair B-cell proliferation and induce B-cell death

● MDSCs decrease B-cell IgM responses and down regulate the expression of important activation surface markers

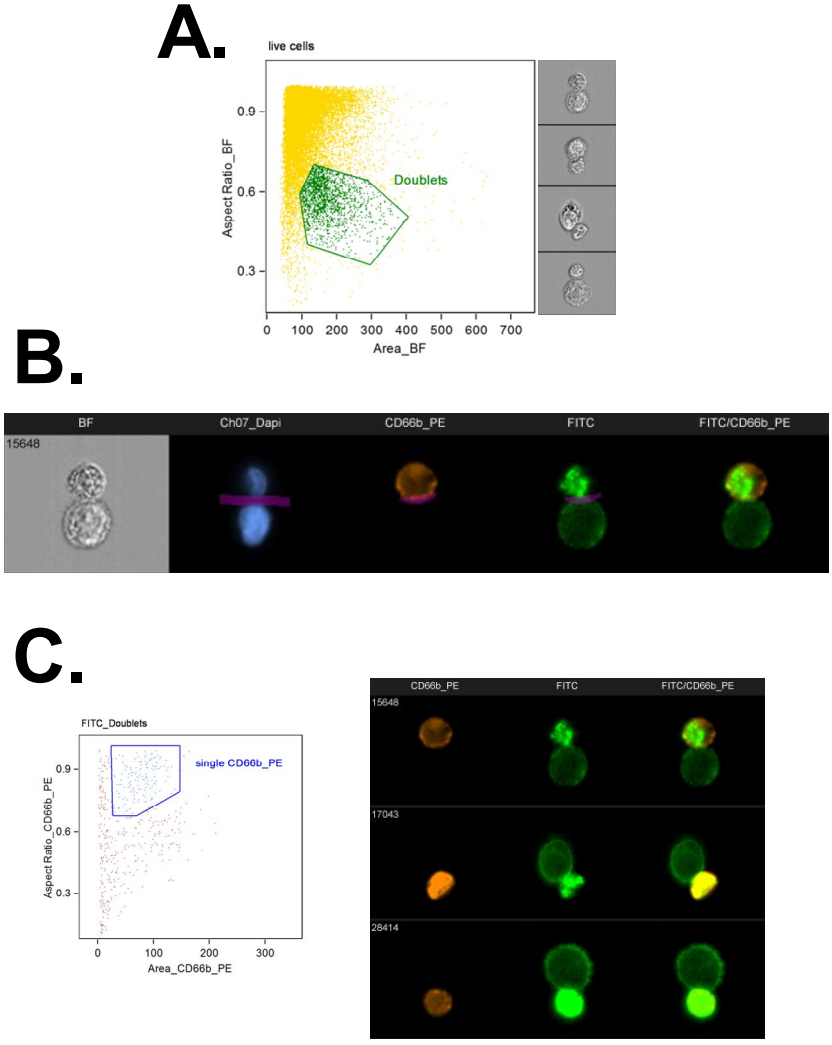
● MDSCs use of reactive oxygen species (ROS), arginase-1, and nitric oxide (NO) to modulate B-cell immune responses

Supplementary Figure 1



Supplementary Figure 1. Flow cytometry strategy: Human polymorphonuclear MDSCs (PMN-MDSCs) and B cells were isolated from buffy coats and co-cultured at 1:5, 1:2 and 1:1 ratios (MDSC:B cells). Isolated B cells cultured in medium only served as negative controls and when cultured in presence of IgM $F(ab`)_2$ (5 µg/mL) plus CpG (2.5 µg/mL) served as positive controls. The plots on the left side depict the Side scatter (SSC) vs Forward scatter (FSC), followed by the exclusion of the dead or PI positive cells then the CD19, CFSE positive cells. On the right side, histograms are depicted showing the inhibition of PMN-MDSC on the B-cell proliferation in a dose-dependent manner, comparing to the positive control.

Supplementary Figure 2.



Supplementary Figure 2. MDSC – B cell interactions
Physical MDSC-B cell interactions were visualized by using Image Stream technology. In brief, human B cells and PMN-MDSCs were isolated from human peripheral blood and stained for CD66b (MDSCs), CD19 (B cells) and DAPI (nucleus). (A) Doublets were identified by plotting Aspect ratio vs Area of the Brightfield image. (B) A valley mask on the DAPI image and an interface mask (shown in purple) on $CD66b^+$ PE cells were created to define contact area between cell conjugates (C) Upper panel: From all doublets the single $CD66b^+$ cell population was isolated by plotting Area vs Aspect ratio of CD66b signal. Lower panel: Representative images of MDSC – B cell contacts / immune synapses are shown.

Paper 2

Singh A, **Lelis F**, Braig S, Schäfer I, Hartl D, Rieber N. Differential Regulation of Myeloid-Derived Suppressor Cells by *Candida* Species. *Frontiers in Microbiology*. 2016;7:1624.

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Differential Regulation of Myeloid-Derived Suppressor Cells by *Candida* Species

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Myeloid-derived suppressor cells (MDSCs) are innate immune cells characterized by their ability to suppress T-cell responses. Recently, we demonstrated that the human-pathogenic fungi *Candida albicans* and *Aspergillus fumigatus* induced a distinct subset of neutrophilic MDSCs. To dissect *Candida*-mediated MDSC induction in more depth, we studied the relative efficacy of different pathogenic non-*albicans Candida* species to induce and functionally modulate neutrophilic MDSCs, including *C. glabrata, C. parapsilosis, C. dubliniensis*, and *C. krusei*. Our data demonstrate that the extent of MDSC generation is largely dependent on the *Candida* species with MDSCs induced by *C. krusei* and *C. glabrata* showing a higher suppressive activity compared to MDSCs induced by *C. albicans.* In summary, these studies show that fungal MDSC induction is differentially regulated at the species level and differentially affects effector T-cell responses.

Keywords: *Candida*, anti-fungal immunity, myeloid-derived suppressor cells, MDSCs, T-cell suppression, Dectin-1, Dectin-2

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INTRODUCTION

Candida species cause one of the most prevalent fungal infections worldwide (Pfaller and Diekema, 2007; Brown et al., 2012). Among various *Candida* species, *Candida albicans* has been the model organism for the most research studies focused on immunity against *Candida* infections (Papon et al., 2013). However, the genus *Candida* consists of multiple species that show a considerable variation in terms of their virulence and phenotype and recent studies showed that particularly diseases caused by NAC species are on the rise (Merseguel et al., 2015).

While *C. albicans* is well characterized in terms of recognition through PRRs mainly CLRs like Dectin-1, Dectin-2, mannose receptor (MR) and Mincle (Brown, 2010; Plato et al., 2015), recognition of NAC species is less precisely defined. In contrast to *C. albicans*, phagocytosis of *C. parapsilosis* by neutrophils was not impaired following Dectin-1 blockade *in vitro* (Linden et al., 2010) and, *dectin-1*$^{-/-}$ bone marrow macrophages showed no defect in binding to *C. glabrata* (Kuhn and Vyas, 2012). Interestingly, studies indicated that Dectin-2 also played a more important role in *C. glabrata* infection than Dectin-1 (Ifrim et al., 2014).

Abbreviations: CFSE, Carboxyfluoresceinsuccinimidyl ester; DPI, Diphenyleneiodonium chloride; ELISA, Enzyme-linked immunosorbent assay; FACS, Fluorescence-activated cell sorting; GM-CSF, Granulocyte-macrophage colony-stimulating factor; G-MDSCs, Granulocytic myeloid-derived suppressor cells; IL-, Interleukin; MACS, Magnetic-activated cell sorting; MDSCs, Myeloid-derived suppressor cells; M-MDSCs, Monocytic myeloid-derived suppressor cells; NAC, Non-*albicans Candida*; PBMCs, Peripheral blood mononuclear cells; PRR, Pattern recognition receptor; ROS, Reactive oxygen species.

There is also some evidence that T-cell responses are differentially involved in immunity to NAC species. For example, *C. albicans* and *C. parapsilosis* were shown to induce different T-cell responses (Tóth et al., 2013), but underlying mechanisms by which different *Candida* species exert a differential immune response remained elusive.

Myeloid-derived suppressor cells are characterized by their ability to suppress T-cell responses and have mainly been studied in cancer (Bronte, 2009; Gabrilovich and Nagaraj, 2009). However, expansion and involvement of MDSCs has also been reported during various infectious disease conditions, such as polymicrobial sepsis, tuberculosis, and *Staphylococcus aureus* infections (Delano et al., 2007; Du Plessis et al., 2013; Tebartz et al., 2014). Recently, we showed that *C. albicans* induces a distinct subset of neutrophilic myeloid-derived suppressor cells (G-MDSCs) which is mediated by a Dectin-1/CARD9 signaling pathway, leading to dampening of T-cell and NK-cell responses (Rieber et al., 2015).

To further broaden our understanding of how MDSCs play a role in modulating the host immune response to *Candida* infections, we studied the relative efficacy of different pathogenic NAC species to induce neutrophilic MDSCs, including *C. glabrata, C. krusei*, *C. parapsilosis,* and *C. dubliniensis*.

Our data demonstrate that the generation of MDSCs is largely dependent on the *Candida* species and morphotype. Further results also show, that Dectin-1 but not Dectin-2 has an important role during NAC induced MDSC generation.

MATERIALS AND METHODS

Study Subjects

The study was conducted at the University Children's Hospital Tübingen (Germany). MDSCs were analyzed in primary cell cultures from peripheral blood obtained from healthy subjects. Informed consent was obtained from all subjects included in the study and the local ethics committee approved all study methods. At the time of blood sampling, all healthy subjects were without any signs of infection, inflammation, or respiratory symptoms.

Candida Species and Culture Conditions

Candida albicans, *C. krusei*, *C. glabrata*, *C. dubliniensis*, and *C. parapsilosis* strains were stored as frozen stocks in 35% glycerol at −80°C and routinely grown on Sabouraud (Sab) agar (1% mycological peptone, 4% glucose, and 1.5% agar) and YPD agar (1% yeast extract, 2% bacteriological peptone, 2% glucose, and 1.5% agar) plates at 25°C. One colony was inoculated and shaken at 150 rpm at 30°C in YPD broth (1% yeast extract, 2% bacteriological peptone, and 2% glucose) overnight. Cells were harvested by centrifugation and washed twice in sterile 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 medium (Gibco-BRL). Heat-inactivated *Candida* cells were prepared by heat treatment of the cell suspension at 90°C for 30 min.

In vitro MDSC Generation and Flow Cytometry

Human MDSCs were generated *in vitro* as described previously (Lechner et al., 2010; Rieber et al., 2015). In brief, isolated human PBMCs were cultured in 24 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; referred to as "complete medium") for 6 days, and GM-CSF (10 ng/ml, Genzyme), heat-inactivated *C. albicans*, *C. glabrata*, *C. krusei*, C. *dubliniensis*, and *C. parapsilosis* were added at a ratio of 1:5 (Fungi:PBMC) as indicated in figures. Dectin-1 antagonist Laminarin obtained from *Laminaria digitata* (100 μg/ml, Sigma) and Dectin-2 antagonist whole mannan particle preparation isolated *from Saccharomyces cerevisiae* (100 μg/ml, Sigma) were added in cell culture where indicated. For ROS inhibition assays, PBMCs were incubated with NADPH oxidase inhibitor DPI (DPI, 0.1 μM; Sigma-Aldrich) for 1 h prior to adding the stimulants.

The number of MDSCs in % 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. Medium and supplements were refreshed on day 4 and supernatants were frozen for ELISA. After 6 days, all cells were collected from PBMC cultures using non-protease cell detachment solution Detachin (Genlantis). G-MDSCs were characterized as $CD33^+CD11b^+$ $CD14^-$ cells as described before (Rieber et al., 2013, 2015).

Cell Isolation and T-Cell Suppression Assays

For functional assays, $CD33^+$ MDSCs were isolated from *in vitro* cultures using anti-CD33 magnetic microbeads and autoMACS®Pro Separator (Miltenyi Biotec) according to manufacturer's instructions. Morphology of the MDSCs was analyzed by cytospin staining. For cytospin stainings 5 x 10^4 $CD33^+$ cells were centrifuged in a Cytospin three centrifuge (Shandon) at 800 rpm for 15 min followed by staining with May-Grunwald-Giemsa method (Supplementary Figure S1). T-cell suppression assays were performed as described previously (Rieber et al., 2015). PBMCs were obtained from healthy volunteers and stained with CFSE according to the manufacturer's protocol (Invitrogen). PBMCs were stimulated with 100 U/ml IL-2 (R&D Systems) and 1 μg/ml OKT3 (Janssen Cilag). Cell number was adjusted to 5×10^5 cells per ml and a total of 60,000 PBMCs per well were seeded in RPMI1640 (Biochrom) medium, in a 96-well microtitre plate and different numbers of MDSCs in RPMI1640 were added to get an MDSC:T-cell ratio 1:2, 1;4, 1:8, 1:16, and 1:32. 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% CO2, cells were harvested and supernatants were frozen in −20°C. CFSE-fluorescence intensity was analyzed by flow cytometry to determine T-cell proliferation.

Flow Cytometry

Antibodies against human CD4, CD8, and CD14 were purchased from BD Pharmingen. Antibodies against CD11b and CD33 were purchased from Miltenyi Biotec. Flow cytometry was performed using a FACSCalibur (BD). Results were expressed as percent of positive cells and mean fluorescence intensity (MFI). Calculations were performed with BD CellQuest Pro analysis software and FlowJo.

Cytokine Analysis in Culture Supernatants

IL-1β (R&D systems) and GM-CSF (Biolegend) ELISA Kits were used to quantify cytokine protein levels in cell culture supernatants. Released IFN-γ protein was quantified by using the Human IFN-γ DuoSet (R&D Systems). All assays were performed according to the manufacturer's recommendations.

Statistical Analysis

Statistical analysis was performed in GraphPad Prism version 6.0 using a one-sample *t*-test. In all tests, differences were considered significant at $P < 0.05$ ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

RESULTS

Different *Candida* Species Induce Functional G-MDSCs

First, we assessed the ability of NAC species to induce human G-MDSCs and to control their function. G-MDSCs were defined by their surface markers ($CD11b^{+}CD33^{+}CD14^{-}$) and by their characteristic to suppress T-cell responses. By comparing *Candida* species, we found a differential pattern of MDSC induction among all *Candida* species. While *C. albicans* (9.1-fold) was the strongest inducer of G-MDSCs, *C. krusei*, and *C. glabrata* (5.5- and 6.1-fold, respectively) also induced high amounts of MDSCs, followed by *C. parapsilosis* (3.5-fold) and *C. dubliniensis* (2.1-fold), which was least potent in comparison to others (**Figure 1A**). G-MDSC induction by *C. albicans* was observed for different fungal morphotypes and even occurred using filter sterilized *C. albicans* yeast supernatants (**Figure 1B**). M-MDSCs ($CD11b^{+}CD33^{+}CD14^{+}$) were not induced during these culture conditions. (Supplementary Figure S2).

MDSCs Induced by Non-*albicans* *Candida* Species Are More Suppressive than MDSCs Induced by *C. albicans*

The key function attributed to MDSCs is to suppress T-cell responses. (Bronte et al., 2016). Therefore, we performed functional assays to screen for T-cell suppression capability of *Candida*-induced MDSCs. CFSE assays showed that NAS-induced myeloid cells strongly suppressed both $CD4^{+}$ and $CD8^{+}$ T cell proliferation in a dose-dependent manner. Interestingly, MDSCs induced by *C. krusei* and *C. glabrata* exhibited an even higher suppressive activity than MDSCs induced by *C. albicans*, an effect which was significant at MDSC:T cell ratios of 1:8 and 1:16. (**Figures 2A,B**). Apart from T-cell proliferation assays, we also investigated the impact of fungi-derived MDSCs on IL-2 and OKT3-induced T cell cytokine production. These studies demonstrated that MDSCs efficiently suppressed IFN-γ secretion (**Figure 2C**).

Dectin-1, but not Dectin-2, Is Involved in MDSC Induction by Non-*albicans* *Candida* Species

In our previous work we showed that Dectin-1 plays a key role in *C. albicans*-induced MDSC generation. Several studies also reveal the role of Dectin-1 and also Dectin-2 (Saijo and Iwakura, 2011) in immune mechanisms against NAC species. We therefore focussed on Dectin-1 and Dectin-2 as β-glucan and mannan receptors, essentially involved in recognition of fungi. As shown for *C. albicans,* blocking of Dectin-1 prior to co-culture with fungal cells diminished the MDSC-inducing effect significantly in *C. glabrata*. For *C. krusei*-induced MDSCs we observed a similar, however, not significant effect. On the other hand, blocking of Dectin-2 had no effect (**Figure 3A**) suggesting that Dectin-2 is dispensable for *Candida*-mediated MDSC generation. Since fundamental differences have been reported between host recognition of *C. albicans* morphotypes (Lowman et al., 2014), we next examined the impact of Dectin-1 blockage on MDSC generation. In case of *C. albicans* yeast cells and hyphae, Dectin-1 blockage significantly inhibited the MDSCs. Dectin-1 blockage also led to a similar trend for filter sterilized *C. albicans* yeast cell supernatant, however, it was not significant (**Figure 3B**).

Candida-Mediated MDSC Generation Is Associated with GM-CSF, IL-1β, and ROS Production

The cytokine GM-CSF has been involved in MDSC generation (Gabrilovich and Nagaraj, 2009; Dolcetti et al., 2010) and previous studies showed that GM-CSF is secreted upon stimulation with fungal pathogens. (Li and Dongari-Bagtzoglou, 2009; Svobodová et al., 2012). Therefore we hypothesized that GM-CSF might play a role in *Candida*-mediated MDSC generation and analyzed the amount of GM-CSF in conditioned medium obtained from PBMC-*Candida* co-culture. Our results demonstrate that *C. albicans* stimulation leads to a high amount of GM-CSF release in comparison to *C. glabrata* and *C. krusei* (**Figure 4A**). In addition to GM-CSF, the inflammasome product IL-1β has been previously involved in MDSC induction (Elkabets et al., 2010; Lechner et al., 2011; Ballbach et al., 2016). Hence, we quantified IL-1β protein in our assays and found that *C. albicans, C. glabrata*, *and C. krusei*, all three major pathogenic *Candida* species lead to high amounts of IL-1β secretion upon PBMC stimulation (**Figure 4B**). These results indicate that the two MDSC-related cytokines GM-CSF and IL-1β seem to be associated with fungal MDSC induction. ROS have been consistently involved in MDSC generation and function (Gabrilovich and Nagaraj, 2009). To check the role of ROS, MDSCs were generated *in vitro* by incubating isolated PBMCs (5×10^{5} cells/ml) with different *Candida*

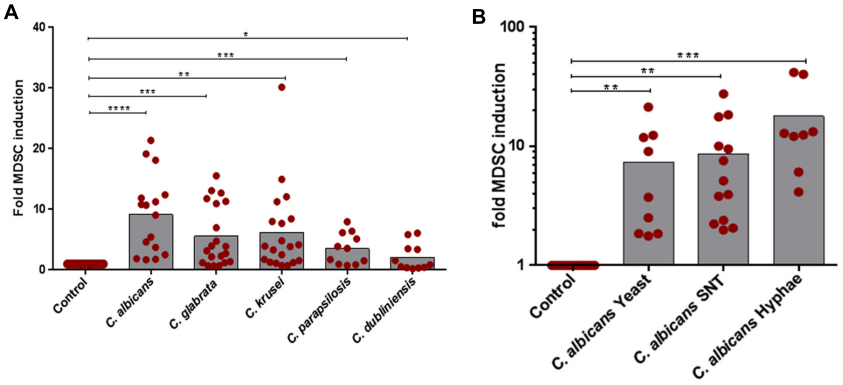

FIGURE 1 | *In vitro* MDSC generation by different *Candida* non-*albicans* species and *C. albicans* morphotypes. MDSCs were generated by incubating freshly isolated PBMCs (5×10^5/ml) from healthy donors with medium only (negative control) or indicated stimulants. **(A)** PBMCs were cultured with heat killed yeast cells of *C. albicans, C. glabrata, C. krusei, C. parapsilosis*, and *C. dubliniensis* (1×10^5/ml) for 6 days ($n = 11$–20) or **(B)** with heat killed *C. albicans* yeast cells (1×10^5/ml), filter sterilized *C. albicans* yeast supernatant (5% SNT), or *C. albicans* hyphae (1×10^5/ml) for 6 days ($n = 8$–13). Granulocytic MDSCs ($CD11b^+CD33^+CD14^-$) were quantified by using Flow Cytometry. The number of MDSCs in % of all cells in medium only cultures was 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. Significant differences between control and G-MDSCs induction by stimulants are indicated by an asterisk (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

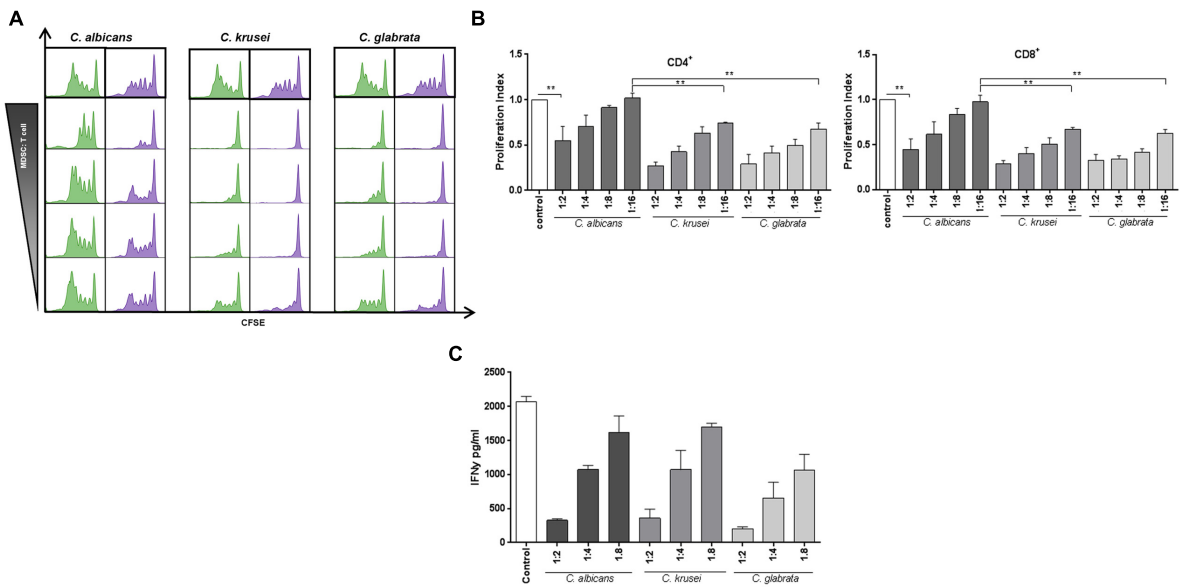

FIGURE 2 | *Candida*-induced MDSCs suppress T cell responses. MDSCs generated by *Candida* species are able to suppress T-cell proliferation and function in a dose dependent manner. The suppressive effects of $CD33^+$-MACS-isolated MDSCs on $CD4^+$ (green) and $CD8^+$ (lilac) were assessed by T-cell proliferation (CFSE polyclonal proliferation) assay. MDSCs were generated by incubating PBMCs (5×10^5/ml) from healthy donors with heat killed yeast cells of various *Candida* species (1×10^5/ml) or *C. albicans* yeasts for 6 days. **(A)** Representative CFSE stainings, showing the effect of *in vitro C. albicans, C. krusei*, and *C. glabrata* induced MDSCs on $CD4^+$ and $CD8^+$ T-cell proliferation. Different MDSC to T cell ratios were assessed by using a wide range of MDSC:Target ratio (1:2, 1:4, 1:6, 1:8, and 1:16). **(B)** The bar graphs represent the proliferation index compared to control conditions. Even at a higher MDSC:target ratio of 1:16, MDSCs induced by *C. krusei*, and *C. glabrata* show higher suppressive activity in comparison to *C. albicans*. Data is shown as mean ± SEM ($n = 4$) **$P < 0.01$. **(C)** IFNγ secretion of T cells is decreased by MDSCs. IFNγ secretion in the supernatant was measured on day 4 of MDSC/T cell co-culture experiments by ELISA. The concentration is given in pg/ml ($n = 3$).

stimulants (1×10^5 cells/ml) and pretreatment for 1 h with the NADPH oxidase inhibitor DPI (0.1 μM) where indicated. These experiments showed that ROS contributed substantially to fungi-mediated MDSC induction *in vitro* (**Figure 4C**).

DISCUSSION

Previous studies from our group demonstrated that pathogenic fungi *A. fumigatus* and *C. albicans* induce MDSCs, which

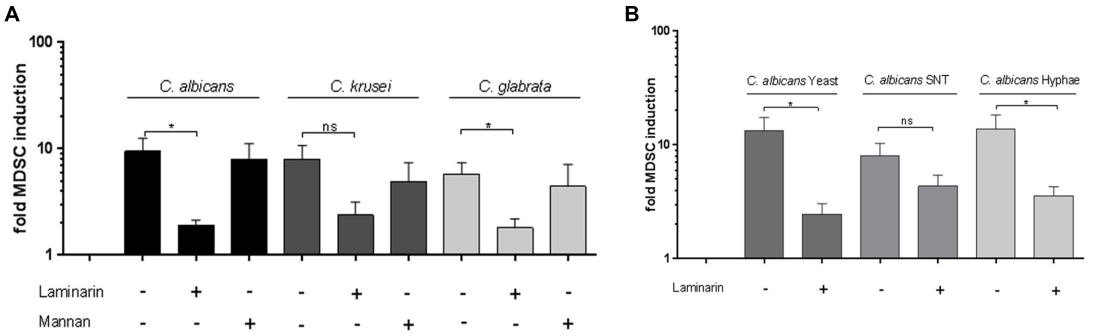

FIGURE 3 | Dectin-1 is involved in *Candida*-mediated MDSC induction *in vitro*. MDSCs were generated *in vitro* by incubating isolated PBMCs (5×10^5 cells/ml) with stimulants and inhibitors. **(A)** with heat killed yeast cells of *C. albicans*, *C. krusei*, and *C. glabrata* (all 1×10^5/ml), ($n = 8$–11) or **(B)** with heat killed *C. albicans* yeast cells (1×10^5/ml), filter sterilized *C. albicans* yeast cell supernatant (5% SNT) or *C. albicans* hyphae for 6 days ($n = 8$–13). Where indicated, prior to stimulation, PBMCs were pretreated for 60 min with Dectin-1 inhibitor Laminarin (100 μg/ml) or Mannan (100 μg/ml) from *Saccharomyces cerevisea* to mimic Dectin-2 binding without receptor activating capacity. (*$P < 0.05$, Bars represent SEM).

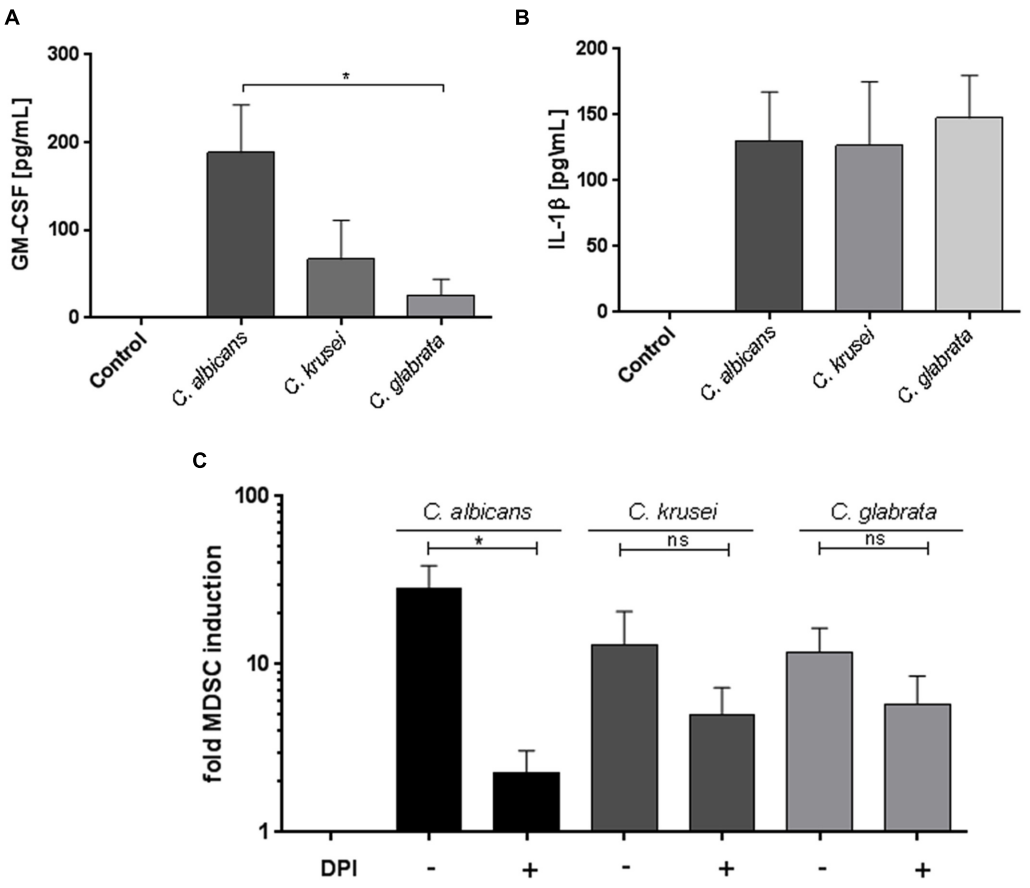

FIGURE 4 | *Candida*-mediated MDSC generation involves GM-CSF, IL-1β, and ROS. GM-CSF, IL-1β, and ROS are involved in *Candida*-mediated MDSC generation. Freshly isolated PBMCs (5×10^5 cells/ml) were cultured in medium only, or with heat killed yeast cells of *C. albicans* (1×10^5/ml), *C. krusei* (1×10^5 cells/ml), and *C. glabrata* (1×10^5 cells/ml) for 4 days. For quantification of cytokines, co-culture supernatants were collected on day 4. **(A)** GM-CSF ($n = 8$) and **(B)** IL-1β ($n = 6$) levels were quantified by ELISA. **(C)** MDSCs were generated *in vitro* by incubating isolated PBMCs (5×10^5 cells/ml) with with heat killed yeast cells of *C. albicans* (1×10^5/ml), *C. krusei* (1×10^5 cells/ml), and *C. glabrata* (1×10^5 cells/ml) for 6 days. Prior to stimulation, PBMCs were pretreated for 60 min where indicated with the NADPH oxidase inhibitor DPI (0.1 μM; $n = 6$) (*$P < 0.05$, Bars represent SEM).

suppress T cell responses (Rieber et al., 2015). In this study, we compared the capacity of *C. albicans*, *C. glabrata*, *C. krusei*, and *C. parapsilosis* to induce G-MDSCs and the relative strength of *Candida*-induced G-MDSCs to suppress T-cell proliferation and cytokine production.

Candida species are found as commensal organisms at mucosal surfaces in the human body. Since *C. albicans* is the most prominent fungus isolated from clinical samples, research related to anti-fungal immune response is largely centered on it. However, recent clinical studies have reported a rise in the NAC species isolated from clinical samples of fungal infections. NAC species associated with disease mainly include *C. glabrata*, *C. krusei*, *C. dubliniensis*, and *C. parapsilosis* (Butler et al., 2009). Here we extend our previous findings by showing that the strength of *Candida*-mediated MDSC induction substantially depends on the *Candida* species. While *C. albicans* was the strongest inducer of MDSCs, *C. dubliniensis* showed the lowest capacity. Importantly, our studies further show that not only the extent, but also the functionality of MDSCs is regulated by distinct *Candida* species. Collectively, these studies add to our understanding of how different *Candida* species differentially modulate host immunity.

Candida species consist of a diverse range of virulence factors and morphotypes. Although limited in number, studies using *in vitro* methods and *in vivo* infection strategies demonstrate that host innate immune responses to *Candida* challenge including activation and function of neutrophils (Dementhon et al., 2012; Svobodová et al., 2012; Duggan et al., 2015), dendritic cells (Bourgeois et al., 2011), and macrophages (Seider et al., 2011) differ depending on the *Candida* species. In addition to different species, we also used *C. albicans* yeast and hyphal forms and filter sterilized supernatant from yeast cultures to study the impact of different fungal morphotypes and soluble products during fungi-mediated MDSC generation. *C. albicans* yeast to hyphae morphogenesis has been attributed as a crucial virulence factor during fungal pathogenesis. Various studies demonstrate that immune cell recognition and subsequent immune response toward different morphotypes of *C. albicans* differs (Lewis et al., 2012; Lowman et al., 2014) due to differential exposure of cell wall components, e.g., β-glucans (Wheeler et al., 2008; Gow et al., 2011). However, in our studies, we did not find a difference in MDSC induction after stimulation with *C. albicans* yeast and hyphae (Rieber et al., 2015) or supernatants. Further studies involving various morphotypes of different NAC species and secreted fungal virulence factors will help to dissect the mechanism underlying *Candida*-mediated MDSC generation and function. T cells are pivotal immune cells during *C. albicans* infection and patients with decreased CD4+ T cells were found to be highly susceptible to mucocutaneous and invasive Candidiasis (Fidel, 2011; Lionakis and Netea, 2013). Interestingly, *C. glabrata* and *C. krusei*-generated MDSCs were more suppressive on T cell proliferation than *C. albicans*-generated MDSCs and this phenomenon was recapitulated in the suppression of IFNγ release. There is some evidence suggesting differential T-cell responses depending on the *Candida* species. *C. albicans* and *C. parapsilosis* were found to induce different T-cell responses and cytokines. Human PBMCs stimulated with heat killed *C. parapsilosis* yeast cells showed higher production of IL-10 but lower amounts of IL-1β, IFNγ, IL-17, and IL-22, when compared to cells stimulated with *C. albicans* (Tóth et al., 2013). Another study reported distinct T-cell generation in response to *C. albicans* and NAC species and T cells generated after stimulation with *C. albicans* displayed cross-reactivity only with *C. tropicalis* but not *C. glabrata* (Tramsen et al., 2007). Our findings now also hint toward a species-dependent innate immune response against different *Candida* species. The induction of MDSCs might contribute to a fine-tuned balance between pro-inflammatory effector and counter-regulatory immune mechanisms, which has been demonstrated to be crucial for an effective anti-fungal immune response (Zelante et al., 2011, 2012; Rieber et al., 2015).

Candida albicans is recognized by different classes of PRRs among which, the CLRs including Dectin-1and Dectin-2 are the most important ones described so far. In our previous work, we showed that dectin-1 mediated signaling was prominent in fungi-induced MDSC generation. While Dectin-1 has been shown to be the key PRR for *C. albicans* (Taylor et al., 2007; Marakalala et al., 2013), Dectin-2 has emerged as a leading PRR to recognize both *C. albicans* and *C. glabrata* (Saijo et al., 2010; Ifrim et al., 2014). Therefore we focussed on these two PRRs to clarify their role in *Candida*-mediated MDSC generation. In consistence with our previous findings for *C. albicans* (Rieber et al., 2015), we found that blockage of Dectin-1 but not Dectin-2 led to diminished MDSC generation by *C. albicans*, *C. glabrata*, and *C. krusei*. Our results demonstrate that *Candida*-mediated MDSC induction is dependent on the type of *Candida* species, which is in line with the notion that anti-fungal immune responses are species- and strain-specific and vary in terms of recognition by the host immune system (Netea et al., 2010; Marakalala et al., 2013). Future studies will be essential to expand the understanding how differential adaptation of *Candida* strains plays a role in MDSC generation. Different morphotypes of *C. albicans* induce an altered immune response. It has been reported that *C. albicans* yeast cells and hyphae are differentially recognized by Dectin-1 and Dectin-2 during host-pathogen-interaction (Saijo et al., 2010; Saijo and Iwakura, 2011). We observed a similar MDSC induction independent of the *C. albicans* morphotype. Dectin-1 blockage significantly inhibited the MDSC generation by *C. albicans* yeast cells and hyphae, and led to a similar trend for *C. albicans* supernatant. This hints toward the presence of a soluble Dectin-1 ligand in *C. albicans* supernatant that contributes to MDSC generation. Interestingly, while yeast mannan particles have been described to impact not only Dectin-2, but also other PRRs like MR, DC-SIGN, and Mincle (Netea et al., 2015), we did not observe any effect of mannan treatment on *Candida*-mediated MDSC generation in our studies.

To elucidate the mechanism of *Candida*-mediated MDSC induction, we further focused on two key cytokines, GM-CSF and IL-1β, both reported to play an important role in MDSC generation and homeostasis (Elkabets et al., 2010; Lechner et al., 2011; Gabrilovich et al., 2012; Bayne et al., 2016), as well as during fungal pathogenesis (Svobodová et al., 2012; Netea et al., 2015). Stimulation of PBMCs with *C. albicans* and NAC species led to release of GM-CSF and IL-1β. *C. albicans*-mediated release

of GM-CSF was significantly higher than that of *C. glabrata,* possibly explaining the stronger induction of MDSCs upon *C. albicans* stimulation. All three species *C. albicans*, *C. glabrata*, and *C. krusei* released similar amounts of IL-1β upon PBMC stimulation. Since Dectin-1 was found to be the key receptor for *Candida*-mediated MDSC generation, and previous studies demonstrated that ROS act downstream of Dectin-1 (Branzk et al., 2014), and ROS have been shown to be involved in MDSC homeostasis (Corzo et al., 2010; Gabrilovich et al., 2012), we further examined the role of ROS for *Candida*-mediated MDSC induction. These studies demonstrated that ROS contributed substantially to NAC-mediated MDSC induction *in vitro*.

CONCLUSION

Our results demonstrate that *Candida*-mediated MDSC induction is differentially regulated at the species level and differentially affects effector T-cell responses. In our previous study using a systemic infection mouse model for *C. albicans*, we showed that adaptive transfer with MDSCs leads to a protective effect against invasive Candidiasis. While the classical MDSC inducing factor GM-CSF has already been proposed as one of the leading candidates for anti-fungal adjunctive therapy (Vazquez et al., 1998; van de Veerdonk et al., 2012), *in vivo* generation of MDSCs or *ex vivo* expansion and adoptive transfer might become an interesting approach for future therapeutic strategies against infections caused by *Candida* species.

AUTHOR CONTRIBUTIONS

AS designed the study, performed the experiments, analyzed the data, and wrote the manuscript. FL, SB, and IS performed the experiments. DH and NR co-designed the study, supervised experiments, discussed data, and co-wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01624

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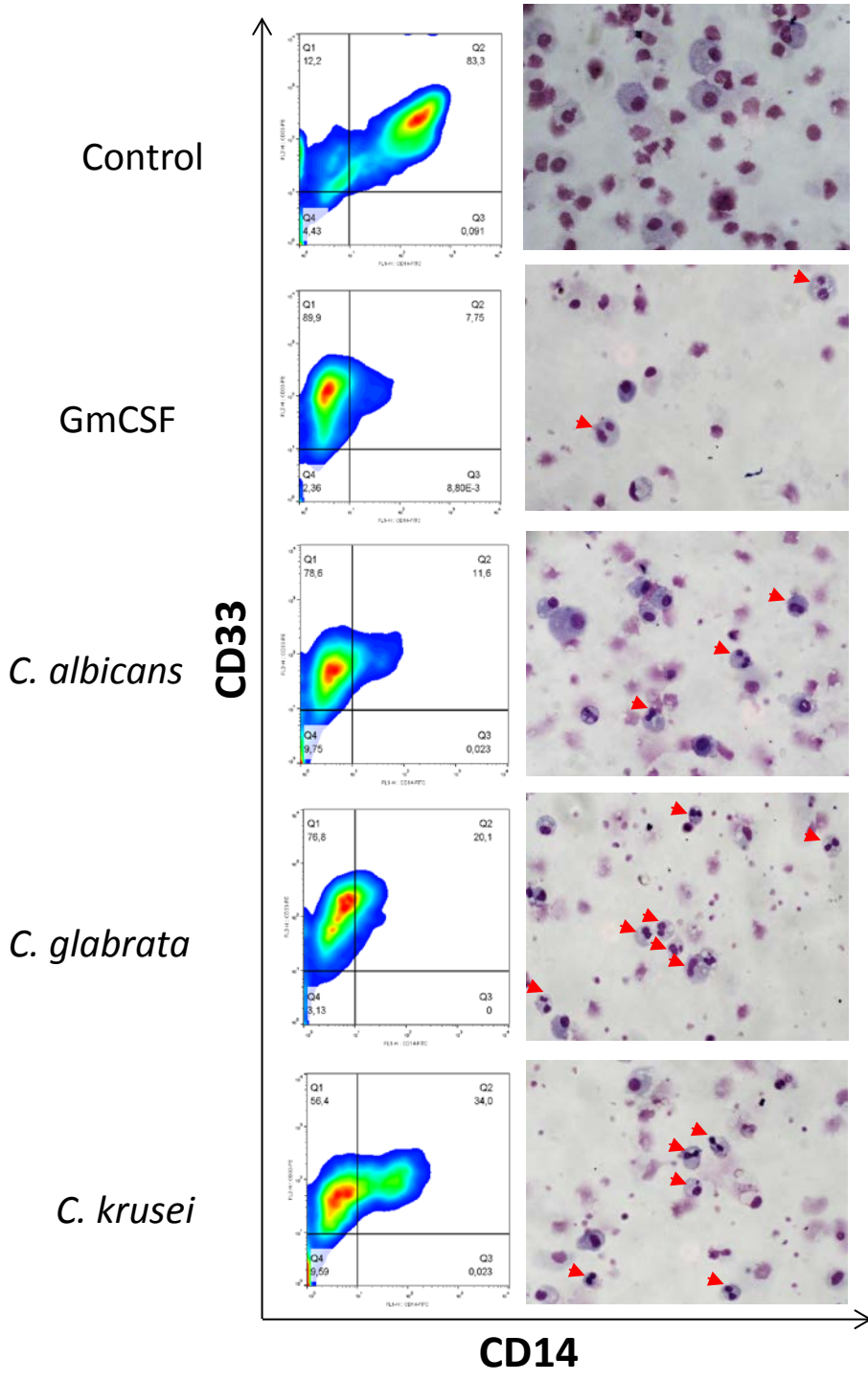
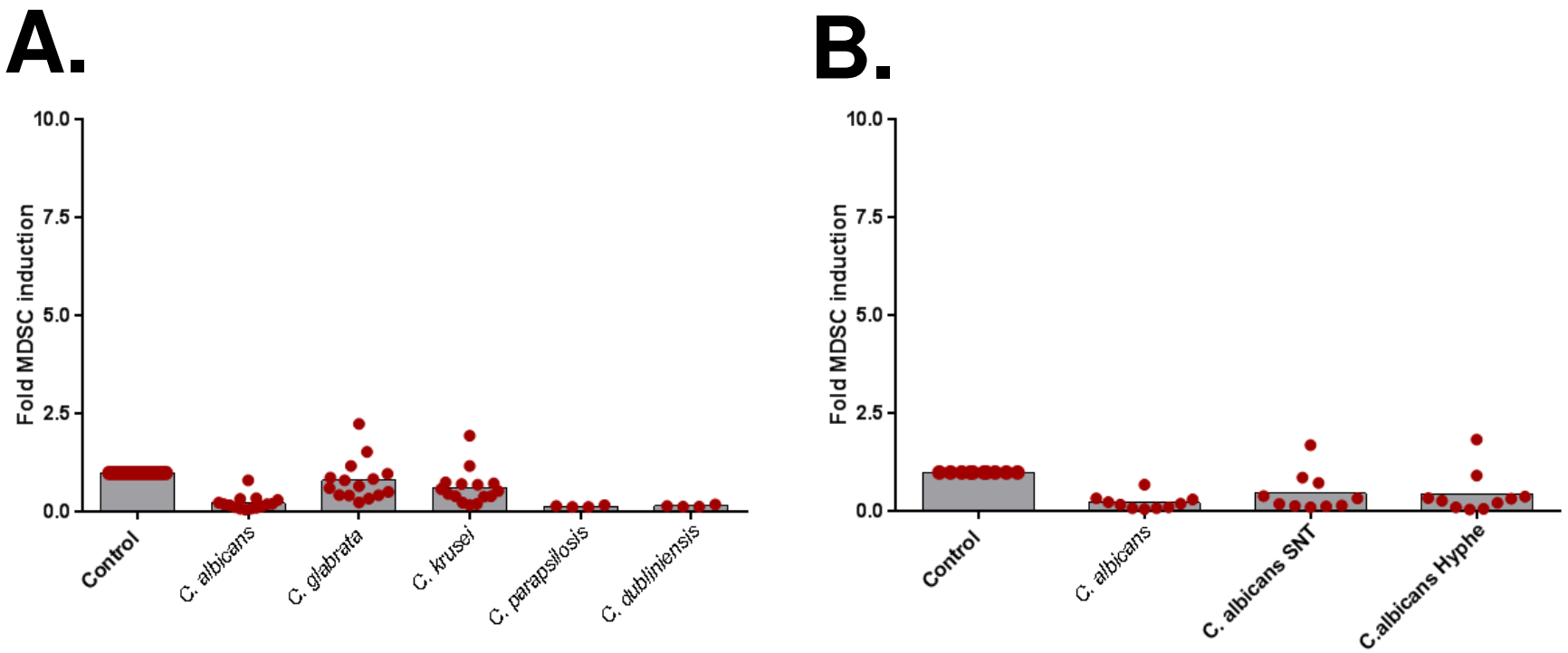
Supplementary Figure S1



FACS gating and photomicrograph showing granulocytic morphology of *in vitro Candida*-induced MDSCs:
MDSCs were generated *in vitro* by incubating isolated PBMCs ($5x10^5$ cells/ml) with GM-CSF, heat killed yeast cells of *C. albicans*, *C. krusei* and *C. glabrata* (all $1x10^5$/ml) for 6 days. Phenotyping was done by selecting $CD33^+CD14^-$ cells.
For microscopy, $CD33^+$ MDSCs were MACS-isolated after 6 days culture and cytospins were stained with May–Gruenwald–Giemsa. Pictures were obtained by using a reverted Zeiss Axiovision Microscope mounted with a Canon 550D camera. Cells with a granulocytic-MDSC morphology are marked with red arrow.

Supplementary Figure S2



M-MDSCs are not induced after fungal stimulation of human PBMCs:
MDSCs were generated by incubating freshly isolated PBMCs (5 x 10^5 /ml) from healthy donors with medium only (negative control) or indicated stimulants. (A) PBMCs were cultured with heat killed yeast cells of *C. albicans, C. glabrata, C. krusei, C. parapsilosis and C. dubliniensis* (1 x 10^5 /ml) for 6 days (n=4-15) or (B) with heat killed *C. albicans* yeast cells (1 x 10^5 /ml), filter sterilized *C. albicans* yeast supernatant (5% SNT) or *C. albicans* hyphae (1 x 10^5 /ml) for 6 days (n=10). M-MDSCs ($CD11b^+CD33^+CD14^+$) were quantified by using Flow Cytometry. The number of MDSCs in % of all cells in medium only cultures was 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). No M-MDSCs were found in the cell population.

Paper 3

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Current Concepts and Controversies in Innate Immunity of Cystic Fibrosis Lung Disease

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Key Words
Cystic fibrosis · Lung disease · Host defense · Immune response · Neutrophils · Pattern recognition receptors · Toll-like receptor

Abstract
Cystic fibrosis (CF) lung disease is characterized by chronic infection and inflammation. The inflammatory response in CF is dominated by the activation of the innate immune system. Bacteria and fungi represent the key pathogens chronically colonizing the CF airways. In response, innate immune pattern recognition receptors, expressed by airway epithelial and myeloid cells, sense the microbial threat and release chemoattractants to recruit large numbers of neutrophils into CF airways. However, neutrophils fail to efficiently clear the invading pathogens, but instead release harmful proteases and oxidants and finally cause tissue injury. Here, we summarize and discuss current concepts and controversies in the field of innate immunity in CF lung disease, facing the ongoing questions of whether inflammation is good or bad in CF and how innate immune mechanisms could be harnessed therapeutically.

CF Lung Disease

Cystic fibrosis (CF) lung disease, the most common inherited lethal disease in Caucasians [1], is characterized by an early [2], nonresolving [3] and harmful [3, 4] activation of the innate immune system. CF is caused by mutations in the CF transmembrane conductance regulator *(CFTR)* gene, mainly expressed at the apical membrane of epithelial cells [5]. However, besides *CFTR*, other genes ('modifier genes') also appear to play a significant role in modulating lung disease severity and immune response [6–9], particularly genetic variants of transforming growth factor β_1 (TGF-β_1) [10–12], mannose-binding lectin (MBL2) [13] and interferon-related developmental regulator 1 (IFRD1) [14, 15]. Exome sequencing has revealed that the variants in dynactin protein, DCTN4, are linked with the chronic infections in CF [16]. A more recent meta-analysis [17] has identified 5 loci: *MUC4/MUC20*, *SLC9A3*, *HLA Class II* and *AGTR2/SLC6A14* to be associated with the lung function in CF. Labenski et al. [18] have reported 2 cytokine receptor genes, *INFGR1* and *IL1B*, and a transcription factor, STAT3, which is associated with the basic *CFTR* defect as candidate modi-

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fier genes in a study comparing F508del homozygous CF patient subsets. Some lesser-known genetic variations linked to CF lung disease are *EDNRA* [19], *IL-8* [20] and *SERPINA1* [9].

Studies from regions with CF newborn screening indicate that the innate immune system, as reflected prototypically by neutrophil products present in CF airway fluids, is operative in infants with CF and predicts the later outcome of irreversible pulmonary disease [2]. Based on these and other studies, innate immune cells have come into the focus of understanding and treating CF lung disease [3]. Whilst there are several studies supporting the notion that unopposed neutrophil products, such as extracellular elastase, are detrimental for tissue integrity and innate immune cell receptors [3, 21] and can be used as noninvasive biomarkers for CF airway inflammation [22, 23], therapeutic approaches to dampen excessive neutrophilic inflammation in CF lung disease have remained largely unsuccessful [24]. Neutralizing neutrophil elastase (NE) by using antiproteases showed some effects in preclinical and clinical studies; however, the benefits for lung function are so far not convincing [25]. Interfering with neutrophil recruitment through CXCR2 antagonists was safe and showed anti-inflammatory potential, yet no beneficial effects on lung function were found [26]. As CF airways are chronically colonized with bacteria and fungi [27], completely abrogating neutrophil recruitment into the lung bears the inherent risk of unleashing bacterial and fungal infections. Collectively, innate immune pathways are activated early in CF and seem to cause more harm than good within the pulmonary microenvironment; however, the therapeutic implications of these insights remain a matter of debate. To dissect the innate immune response in CF and develop future pharmacotherapeutic strategies, we have composed this review, embedded in a thematic CF series in the *Journal of Innate Immunity*.

Current Controversies in Innate Immunity of CF Lung Disease

Innate immunity comprises both cellular and humoral factors. Here, we focus on the cellular components of innate immunity and their pathogenic, diagnostic and/or potentially therapeutic role in CF lung disease. However, before considering innate immune cells as pharmacotherapeutic targets, one must understand their activation and effector functionalities. Therefore, we start with summarizing and discussing the mechanisms by which innate immune cells sense and are activated by CF pathogens. Based on this, we focus on the role of neutrophils, probably the key type of innate immune cell in CF lung disease, including their distinct innate immune receptor profiles and phenotypes in the proinflammatory CF airway microenvironment. Overall, our review should stir a discussion of the following controversies in the field:

- Is inflammation good or bad in CF lung disease? The correlation between neutrophil activation and irreversible lung tissue remodeling (bronchiectasis) [2] suggests a harmful role, but without functional neutrophils (as exemplified in patients with the primary immunodeficiency chronic granulomatous disease), we cannot efficiently defend against bacteria and fungi. Consequently, dampening neutrophil activation would be reasonable, while completely abrogating neutrophil influx or function might be dangerous.
- How does harmful proinflammatory neutrophil activation in CF get dampened? Harmful unopposed neutrophil functions, such as unopposed protease release and neutrophil extracellular trap (NET) formation should be controlled, but how? Antiproteases show limited success so far, but studies are ongoing. NET formation still represents a controversial area [28]. On the one hand, NETs can entrap pathogens and may therefore act beneficially. On the other hand, abundant NETs, as found in CF airways, can obstruct the airway lumen and correlate with decreased lung function in CF patients [29]. Recombinant DNase (Dornase alfa) is clinically effective in CF patients by cleaving DNA strands and facilitating airway mucus clearance [30]. A recent study suggested that the majority of extracellular DNA in CF airways is derived from NETs [31]. Thus, the clinical effectiveness of recombinant DNase might support the concept that the prevalence of NETs causes more harm than good in CF lung disease. However, DNases cleave extracellular DNA and do not prevent de novo NET generation or release. Approaches how to target NET generation may involve interfering with reactive oxygen species (ROS) or MAPK, which have been found important for NET formation [32, 33]. Studies comparing the effect of inhibiting intracellular NET generation versus cleaving free extracellular DNA strands would shed more light on the kinetics and dynamics of NET-pathogen interactions in lung disease and beyond. Alternatively, specific neutrophil phenotypes, such as olfactomedin-4- or CD177-expressing neutrophil subsets, could be targeted [28]. Their functional role and CF disease relevance remains to be defined.

- When should inflammation be targeted? At first glance, the earlier, the better, in order to prevent inflammation-associated tissue damage and avoid irreversible pulmonary tissue remodeling as soon as possible in the course of disease. On the other hand, neutrophils could be essential in early host-pathogen interactions by restricting airway pathogen colonization in the first years of life, when the airways are intensively exposed to environmental microbes and vaccinations are performed. Further investigations into CF lung disease are required to define the time windows when inflammation could be targeted safely without significantly impairing the protective innate immune defenses.

Innate Immune Activation in CF Lung Disease

Sensitive microbial detection mechanisms as well as tailored immune responses are required to efficiently protect the host from pathogens. Simultaneously, inflammation has to be tightly controlled and limited to avoid overshooting immune responses and collateral tissue injury. In 1989, Janeway [34] proposed the pattern recognition theory, stating that the microbial presence is sensed by the host innate immune system through the detection of distinct molecular structures called pathogen-associated molecular patterns (PAMPs) that are expressed by the pathogen but are absent in the host. To sense the presence of microorganism, the cells of the immune system possess germline-encoded pattern recognition receptors (PRRs) with 4 different families having been currently identified. These families include transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) as well as cytoplasmic proteins such as the retinoic acid-inducible gene *(RIG)*-I-like receptors (RLRs) and NOD-like receptors (NLRs). Apart from PAMPs, PRRs also recognize host-derived patterns/molecules, termed damage- or danger-associated molecular patterns (DAMPs).

CF lung disease is mainly characterized by bacterial and fungal colonization and infection. Therefore, in the sections below, we will focus on these 2 microbial entities and the corresponding innate immune responses.

Bacterial Recognition: TLRs

The main bacteria commonly identified in CF lungs in early disease/infancy are *Staphylococcus aureus* and *Haemophilus influenzae*, followed in adolescence and adulthood by the major CF pathogen *Pseudomonas aeruginosa*. However, beyond these 'classical' CF bacteria, microbiome studies indicate that a much broader variety of bacterial species, including anaerobes, colonize CF airways [35–37]. TLRs are the main innate immune receptors (PRRs) to sense bacteria. Ten and 12 TLRs have been identified in humans and mice, respectively, and TLR1–9 are conserved in both species [38]. The PRRs responsible for the recognition of *P. aeruginosa* in CF lung disease are TLRs, Asialo-GM1 receptors [39] and the NLRC4/IPAF inflammasome [40]. TLR2, TLR4, TLR5 and/or TLR9 have been reported to sense *P. aeruginosa* [41]. The bacteria-derived ligands known to bind TLR2 are lipoproteins, components of the extracellular capsule and secreted toxin, ExoS, with C-terminal-specific interaction [42–44]. Reports have shown a role for TLR2 in the recognition of mannuronic acid polymer, a major component of the alginate capsule and slime GLP, produced by mucoid and nonmucoid strains of *P. aeruginosa* [45, 46]. Lipopolysaccharide (LPS) is mainly sensed through TLR4 and, after recognition, the TLR4/LPS complex is rapidly endocytosed and trafficked for lysosomal degradation in order to terminate further inflammatory cascades [47]. The lipid A component of LPS ligates TLR4, inducing a potent immune response [48], with the hexacyclated form being a strong activator of TLR4-mediated signaling in humans [49]. Hexacylated lipid A is often produced by bacterial strains adapted to the chronic CF microenvironment [50, 51], leading to escape from the host antimicrobial peptides and increased recognition by human TLR4. In contrast to this structural peculiarity, a recent study by Di Lorenzo et al. [52] sheds new light on the activation mechanism of TLR4/MD2 complex by penta-acylated lipid A produced by the CF isolates of *Burkholderia cenocepacia.* TLR5 specifically binds to flagellin, a primary constituent of flagella important for microbial motility [53]. However, the correlation between bacterial motility and immune evasion by *P. aeruginosa* remains controversial [54]. An in vivo study highlighted the proinflammatory role of flagellin-mediated TLR5 activation [55]. Descamps et al. [56] reported that TLR5, rather than TLR4, is essential for bacterial phagocytosis and killing by murine alveolar macrophages (AMs) in vitro and in vivo. The authors also demonstrated that nonflagellated *P. aeruginosa* or mutants defective in TLR5 activation are resistant to AM clearing, which is dependent on TLR5 signaling and IL-1β production. The intracellular function of TLR9 is characterized by detection of unmethylated CpG motifs in bacterial DNA [57, 58]. Synergistic effects of TLR2, TLR6 and TLR9 have been reported using in vivo studies

[59]. Further studies report a resistant phenotype of TLR9$^{-/-}$ mice to *P. aeruginosa* infection compared to wild-type mice [60]. These unexpected findings are attributed to increased airways cytokine production leading to effective bacterial clearance in the lungs of the TLR9$^{-/-}$ mice.

The NLRC4 and NLRP3 Inflammasomes

NLRs are cytosolic proteins that respond to a variety of ligands, from bacterial and viral components to particulate matter and crystals. The mammalian NLR family comprises >20 members, containing a C-terminal leucine-rich repeat domain, a central nucleotide-binding NACHT domain and an N-terminal protein-protein interaction domain composed of a caspase activation and recruitment domain (CARD) or Pyrin domain [61–63]. The transmembrane secretion systems of intracellular pathogens or bacteria serve as cytosolic microbe-associated molecular patterns (MAMPs) that may interact with NLRs [64–66]. Regarding human pulmonary pathogens, NLRC4 and NLRP3 are the 2 most widely studied NLRs that orchestrate immune responses [67–69]. In addition to TLR5, bacterial flagellin is sensed by NLRC4 [70, 71]. Sutterwala et al. [40] have further described that NLRC4 triggers the activation of the inflammasome upon infection with *P. aeruginosa*, resulting in macrophage cell death and the secretion of the proinflammatory cytokines, IL-1β and IL-18. This activation cascade was shown to be IPAF-dependent, but flagellin-independent. Moreover, in vivo studies revealed an increased susceptibility of NLRC4-deficient mice against *P. aeruginosa* infection [72]. In addition to *Pseudomonas*, other Gram-negative bacteria, such as *Salmonella*, *Legionella* and *Shigella*, have also been found to activate the NLRC4 inflammasome [73–75]. In a recent study, the role of NLRP3 inflammasome activation in the CF lung has been described in association with elevated levels of ceramide [76]. The authors demonstrated an upregulation and recruitment of the adapter protein apoptosis-associated speck-like protein (ASC) and caspase-1 in the lungs of CF mice. The activation of NLRP3 is characterized by a canonical two-step deubiquitination mechanism that is initiated by priming through TLR signaling (e.g. TLR4), inducing NF-κB-dependent NLRP3 protein synthesis, followed by a second signal leading to full NLRP3 inflammasome assembly [77]. In CF airway epithelial cells, *P. aeruginosa* infection has been shown to trigger mitochondrial dysfunction and enhance mitochondrial Ca^{2+} uptake, leading to NLRP3 inflammasome activation [78, 79].

Fungal Recognition

With constant inhalation of fungal spores, the human airway immune system has evolved a plethora of fine-tuned defense mechanisms for effective fungal clearance, involving, mainly, AM, neutrophils and antimicrobial peptides [80–85]. With ageing and more intensified antibiotic treatments, prevalence rates of fungal colonization increase in CF lung disease, traditionally known to be mainly colonized by a bacterial community [86–88]. The reported emerging rate of filamentous fungal species, such as *Aspergillus fumigatus*, in CF, is found to be most frequent; however, other important filamentous fungi including *Scedosporium* sp. and *Exophiala dermatitidis* have also been identified [89, 90]. The sensitization of CF patients to the airway microenvironment presents a wide range of unresolved questions. However, previous reports have proposed a crucial role for dendritic cells and Th2-associated chemokines, like CCL17 [91]. Phagocytic cells play an essential role in protection against the fungal infections, and abrogation of these cells leads to increase susceptibility towards pathogens [92]. The receptors involved in these processes include secreted factors such as pentraxin-3 (PTX3), C-type lectins, complement system and membrane PRRs such as TLRs [93]. Previous reports have shown that *A. fumigatus* conidia are recognized by TLRs [94, 95] and β-glucan receptor dectin-1 on dendritic cells, AM and lung epithelial cells [96, 97]. TLRs, in particular TLR2 [98, 99], TLR4 [100, 101] or an interplay between TLR2, 4 and 9 via an MyD88-dependent pathway [96], are described as playing an important role in the host immune response to *A. fumigatus*. The endocytic PRR dectin-1 is crucial in the recognition and internalization of specific morphotypes of *A. fumigatus* in AM [102, 103], and a novel mechanism of dectin-1 induction in human bronchial epithelial cells and its consequences for innate immune responses against *A. fumigatus* have been described by Sun et al. [97]. Secreted receptor pentraxin PTX3 also plays an important role in the clearance of fungal burden in vivo after *A. fumigatus* pulmonary infection. PTX3 levels in a CF patient's respiratory secretions and sputum samples were found to have decreased [104]; this could be one of the explanations for recurrent lung infections in CF lungs. Another study showed that a serum opsonin, H-ficolin, modulates host immune response by binding to *A. fumigatus* [105]. The authors further showed that following pathogen recognition, there is an enhanced activation of the lectin complement pathway and fungal association with lung epithelial cells.

Innate Immune Cells

Airway epithelial cells form the first line of defense against microbial infections and serve as a central player in the mucociliary clearance of the lung. The key innate immune functions of the epithelium include (1) secretion of a variety of antimicrobial substances, (2) release of chemokines, cytokines and growth factors that mediate leukocyte recruitment, (3) modulation of adaptive immunity and (4) tissue repair and remodeling [3, 106, 107]. Direct interaction between the CFTR protein and pathogens has been previously suggested, where CFTR serves as a receptor for *Salmonella typhi* [108] and *P. aeruginosa* [109, 110] when expressed on intestinal or airway epithelial cells, respectively. Moreover, *A. fumigatus* spores are readily ingested by airway epithelial cells and the uptake and killing of conidia are both impaired in epithelial cells lacking CFTR [111]. The bronchial epithelium has been previously shown to modulate its sensitivity towards microbial recognition by regulating receptor expression levels [112]. Upon pathogen recognition by specific PRRs, the activation of intracellular signaling cascades initiates proinflammatory and antimicrobial responses. Bacterial infection in CF can exacerbate lung inflammation by exaggerating proinflammatory gene expression via TLR activation in airway epithelial cells [43]. In vitro as well as in vivo studies have shown that excessive cytokine release upon *P. aeruginosa* exposure to CF airway epithelial cells is mainly mediated by TLR5/flagellin or TLR4/LPS interactions [113, 114]. In particular, intracellular TLR4 trafficking seems to be dysregulated and attenuated in human CF airway epithelial cells compared to non-CF cells [115–117]. Hyperresponsiveness of primary airway epithelial cells to LPS, despite expressing normal levels of TLR4, has been attributed to the reduced surface expression of coreceptor CD14 and lower levels of the costimulatory molecule MD2 [118]. Conflicting studies have been reported regarding the localization of TLR5 on airway epithelial cells, with apical dominance on human and murine cells [119–122] and basolateral expression on polarized human nasal and bronchial epithelium [123–125]. Specific cell source, modulation of culture conditions and/or specific stimuli might explain these discrepancies. A strong synergism between TLR2/PGN- and TLR4/LPS-mediated IL-8 production and IL17A was found in human bronchial epithelial cell lines [126]. Recently, genotyping of TLR polymorphisms revealed that CF airway epithelial cells are homozygous for TLR1 SNP 1602S and possess a diminished innate immune response towards *Mycobacterium abscessus* infection. [127]. In a separate study, TLR SNPs were associated with CF lung function decline [128]. A recent study [129] demonstrated that *S. aureus* filtrates inhibit *P. aeruginosa* filtrate-mediated IL-8 production.

The CF airways are characterized by a neutrophil-rich environment. Neutrophils have been mainly implicated in controlling bacterial and fungal infections, but can also lead to airway damage upon activation through the release of enzymes (proteases) and oxidants [28]. Neutrophils are the first cell type recruited to the CF airway compartment. The recruitment of blood neutrophils into the airway compartment is mainly regulated through chemokines, such as IL-8, and lipid-mediators, such as LTB_4. The efficient antibacterial function of neutrophils in the CF airway micromilieu is impeded due to several mechanisms, such as proteolytic damage of airway neutrophils, neutrophil cell death and bacterial/fungal biofilm formation that prevents phagocytosis [3]. At the site of infection, neutrophils sense PAMPs or DAMPs via PRRs. Expression and functionality of TLRs in neutrophils have been studied in the context of CF lung disease. Collectively, TLR2, TLR4 and TLR5 are suggested to be most essential for neutrophil-*P. aeruginosa* interactions. CF airway neutrophils express remarkably high levels of TLR5, which correlates with lung function in CF patients [130, 131]. In a separate study, TLR surface expression was investigated on circulating and induced sputum neutrophils in CF patients. Compared to healthy controls, decreased expression of TLR2 was detected on circulating neutrophils in CF patients [132]. Furthermore, an inverse relationship between TNF-α serum levels and TLR2 surface expression on circulating neutrophils has been described [130]. DAMPs such as proline-glycine-proline and high-mobility group box protein-1 (HMGB1) have been implicated in CF lung disease. A high concentration of these mediators is found in CF airways and they serve as neutrophil chemoattractants to drive lung inflammation [133]. S100A12, a member of the S100/calgranulin family and a neutrophil-derived DAMP, was found in abundance in CF airway fluids leading to activation of downstream metabolic and stress pathways following neutrophil entry into CF airways [134].

Novel Therapeutic Concepts

Despite a plethora of proinflammatory innate immune pathways having been studied and determined as playing a significant role in CF lung disease, therapeutic exploitation of these pathomechanisms remains scarce. For a broader and more in-depth discussion of this aspect, we

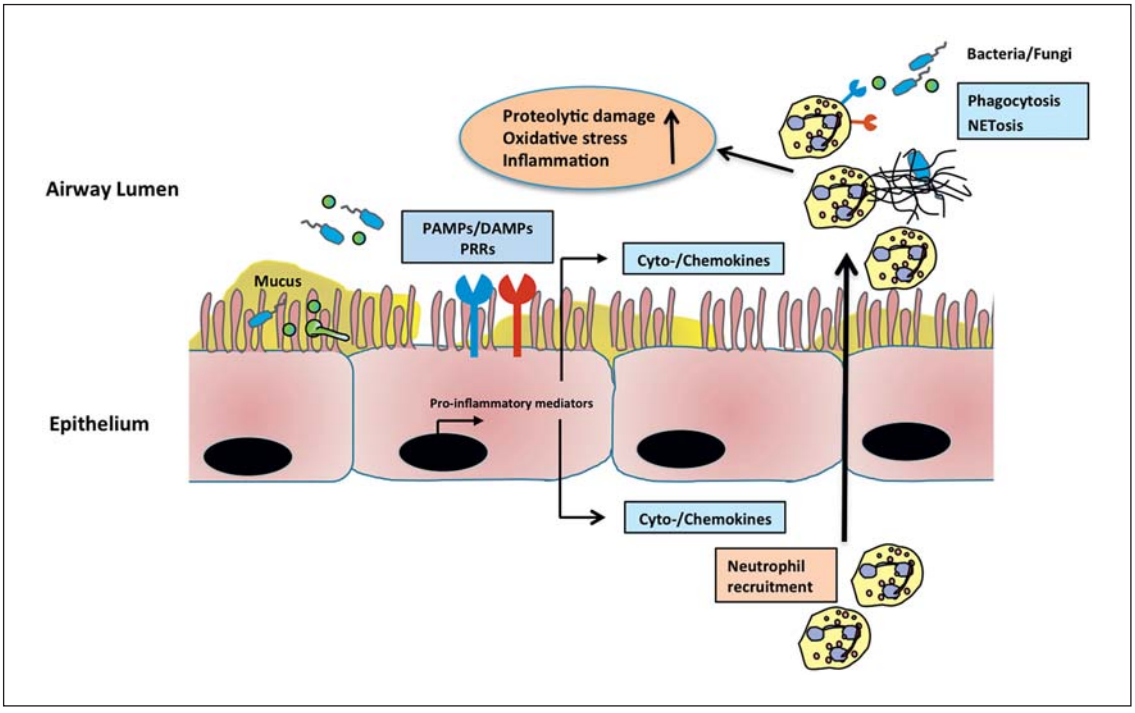

Fig. 1. Innate immune activation in CF airways. Due to continuous production of cytokines and chemokines, especially IL-8, neutrophils are recruited into the CF airways. Bacterial and fungal PAMPs and host-derived DAMPs further activate downstream signaling pathways through the activation of PRRs, and lead to enhanced cytokine and chemokine production. Infiltrated neutrophils release proteases and oxidants, resulting in perpetuated inflammation and tissue injury.

refer to thematic review articles [24, 135]. Ibuprofen represents a clinically available anti-inflammatory drug that has been shown to slow lung function decline in pediatric/adolescent CF lung disease [136–139], but its broad clinical usage outside the USA is restricted by drug-monitoring requirements. Correlations between lung function and inflammatory markers in CF airway fluids (neutrophil counts, IL-8 and NE) have been demonstrated in multicenter CF patient cohorts [22], suggesting that targeting neutrophil-related products may be beneficial in CF lung disease. However, clinical studies aiming to neutralize free NE activity in CF airways by the delivery of antiproteases, such as α-1 antitrypsin, showed modulated airway inflammation but failed to show convincing effects on lung function [25]. In contrast, the use of the oral antioxidant *N*-acetylcysteine, as a strategy to rebalance antioxidant deficiencies in CF, shows beneficial effects on lung function, but has no effect on neutrophilic inflammation [140]. Future studies are required to reconcile these findings and to further assess the therapeutic potential of antiprotease or antioxidant approaches in CF lung disease [24, 141]. The antibiotic azithromycin is known to have anti-inflammatory effects. A clinical trial [142] showed that azithromycin treatment reduced circulating neutrophil counts and systemic blood biomarkers, including C-reactive protein, serum amyloid A and calprotectin, and was correlated with the improvement in lung function and weight gain. Other anti-inflammatory therapeutic approaches include sildenafil (phosphodiesterase inhibitor) [143], CXCR2 inhibition [26] and others less-advanced ones that are not discussed here. Collectively, therapeutic interventions to dampen inflammation in CF remain an appealing yet challenging approach.

Conclusions and Outlook

There is broad consensus about the concept that the innate immune system is activated early and strongly in CF lung disease, leading to the continuous recruitment of neutrophils into CF airways [3]. These neutrophils release proteases that cause harm to the host by degradation

of the pulmonary tissue and the immune receptors (fig. 1). However, controversy exists as to whether the targeting of innate immune pathways, by neutrophil recruitment and/or activation, represents a promising strategy in CF lung disease. On the one hand, there are clear relationships between neutrophil products, prototypical NE and decreased lung function [22] as well as bronchiectasis [2]. On the other hand, targeting excessive proteolytic activities in CF has clinically not been successful so far. Interfering with neutrophil recruitment through CXCR2 inhibition represents a causative anti-inflammatory approach [26], but has also not shown any clinical benefits for lung function. Novel strategies to dampen innate immunity in CF in the future could involve anti-inflammatory pro-resolution lipid mediator pathways, such as resolvins [144], and the endocannabinoid system [145]. However, most of these pathways have mainly been assessed in acute lung inflammation models and not in chronic CF lung disease. Both preclinical and clinical studies are warranted to evaluate these and other anti-inflammatory mechanisms in the context of CF lung disease.

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Disclosure Statement

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