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2023

Document Version:
Publisher's PDF, also known as Version of record

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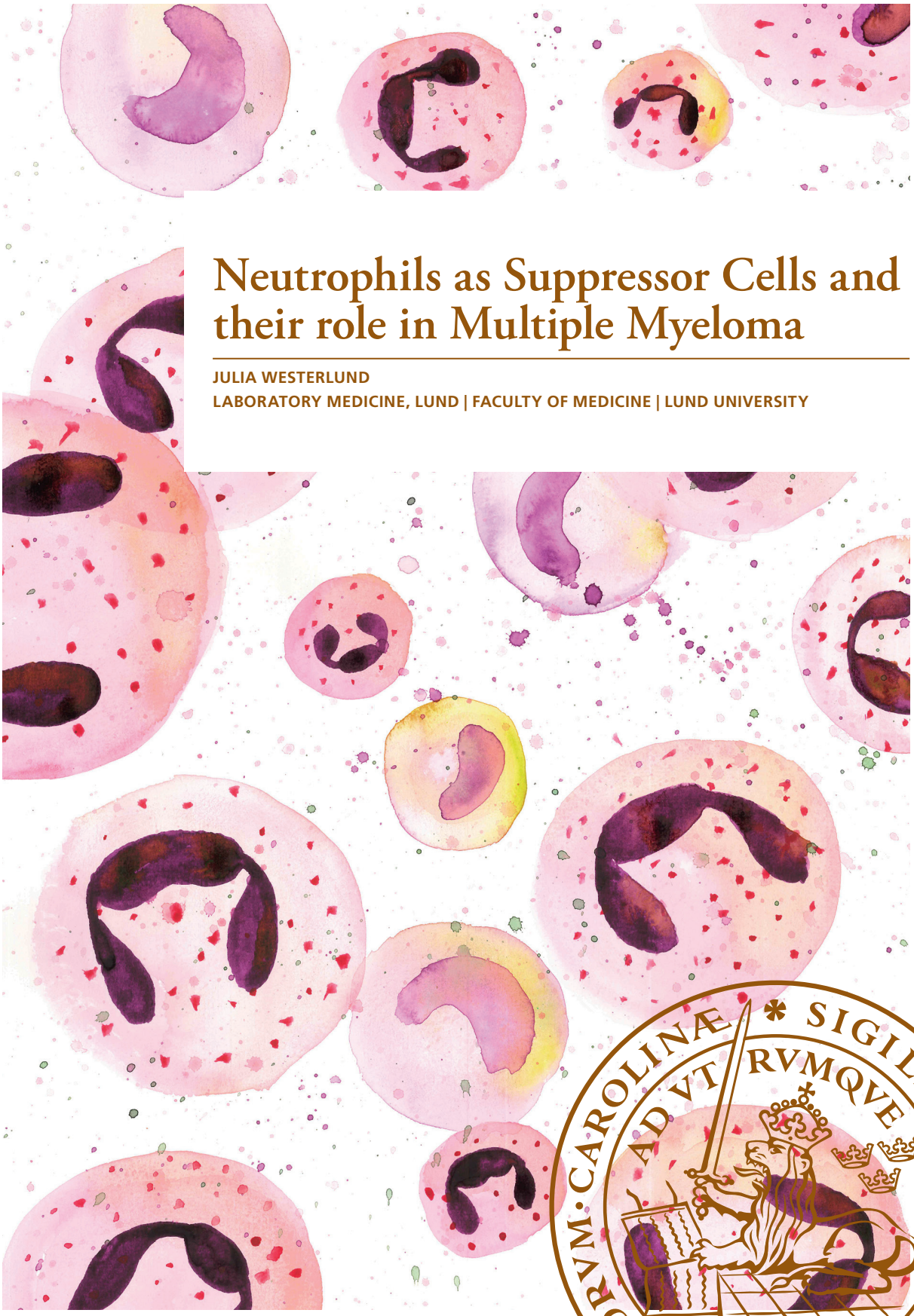

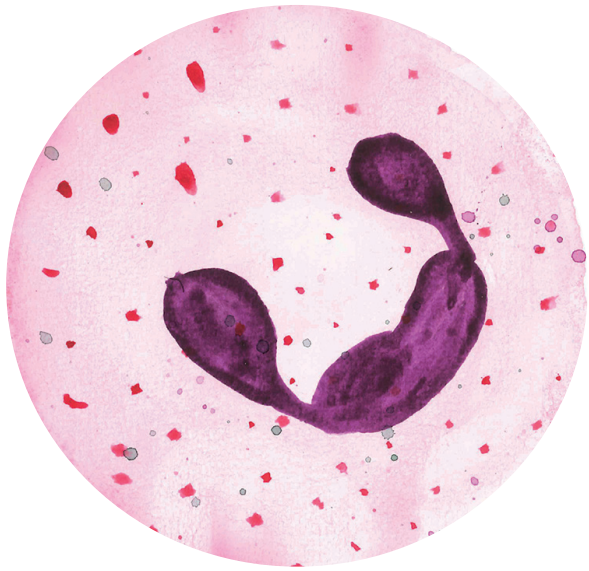

Printed by Media-Tryck, Lund 2023 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Laboratory Medicine, Lund

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2023:84
ISBN 978-91-8021-424-7
ISSN 1652-8220

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Julia Westerlund

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on June 9, 2023, at 09.00 in Segerfalksalen, BMC A10, Sölvegatan 19, Lund.

Faculty opponent
Professor Johan Bylund, Gothenburg University, Sweden

Organization: LUND UNIVERSITY

Document name: Doctoral dissertation **Date of issue** 2023-06-09

Author: Julia Westerlund **Sponsoring organization:**

Title and subtitle: Neutrophils as suppressor cells and their role in Multiple Myeloma

Abstract:

Multiple Myeloma (MM) is an incurable plasma cell malignancy of the bone marrow (BM), where monoclonal plasma cells proliferate uncontrollably and produce monoclonal antibodies. MM patients have an increased risk of severe and recurrent infections, and since neutrophils are one of the most important cells to fight infection, MM patients seem to have neutrophils with impaired functions.

Myeloid-derived suppressor cells (MDSC) are characterized as immature myeloid cells that inhibit T cell responses. T cells are important for tumour clearance, and it is common that cancer patients have exhausted T cells. MDSC are often increased in the blood of cancer patients and correlate with disease severity. The neutrophil-like MDSC subset called polymorphonuclear (PMN)-MDSC has been described as a prominent T cell inhibitor. However, in recent years it has become evident that neutrophils share this suppressive ability.

This thesis evaluates aspects of neutrophil and MDSC immune regulatory functions in the setting of MM. We further evaluate if MM patients have neutrophils with impaired anti-microbial functions.

We discovered that newly diagnosed MM patients do not have increased levels of MDSC and that these subsets lack significant inhibitory capacity. However, we found that neutrophils have a superior suppressive ability compared to PMN-MDSC, in both health and disease. The inhibitory effect of healthy donor neutrophils requires cell-cell contact and is mediated by reactive oxygen species (ROS). The inhibition occurs within hours, and leads to reduced proliferation and T helper cell cytokine production.

BM neutrophils from MM patients have strong suppressive abilities, suggesting that these cells contribute to the T cell exhaustion in the BM of MM patients. We also found that neutrophils from MM patients have a reduced phagocytic and ROS producing ability, indicating impaired anti-microbial effector functions. When investigating neutrophil function in Lenalidomide treated patients, we found that Lenalidomide restores the anti-microbial effector functions. Taken together, neutrophils from the bone marrow of MM patients have altered abilities and their role in both health and disease needs further investigation.

Key words: Neutrophils, MDSC, Multiple Myeloma, Immunology, Hematology, T-cells.

Classification system and/or index terms (if any) Supplementary bibliographical information

Language English **ISSN and key title**: 1652-8220

ISBN: 978-91-8021-424-7

Recipient's notes **Number of pages**: 75

Price Security classification

Signature Date 2023-04-25

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Coverphoto by Irma Sjölin

Faculty of Medicine
Department of Laboratory Medicine, Lund

ISBN 978-91-8021-424-7
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2023

Till mormor

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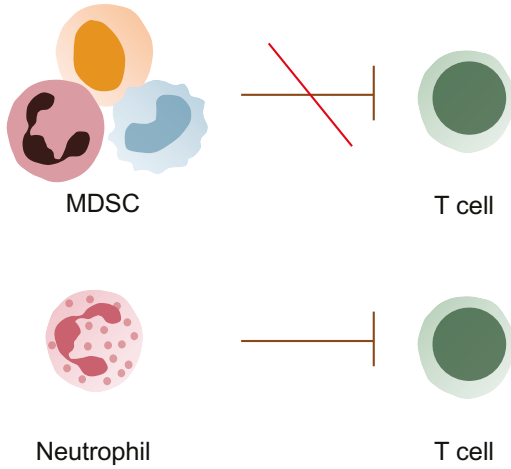
Populärvetenskaplig sammanfattning

Rökning, rattfylla, mobbning eller farliga dyk på grunt vatten. Grupptryck har i alla tider fått människor till att göra dumma saker som de kanske egentligen inte vill. När cancerceller börjar växa i kroppen kan de påverka celler i sin omgivning till det negativa, som ett slags grupptryck som får närliggande celler att gör saker de normalt sätt inte gör i en frisk kropp. I den här avhandlingen undersöker jag hur det här grupptrycket påverkar olika celler vid cancerformen myelom.

Myelom är en typ av benmärgscancer som drabbar ca 600 personer i Sverige varje år. Sjukdomen uppstår i benmärgen, som är kroppens blodkroppsfabrik. Där bildas bland annat plasmaceller, en slags vit blodkropp, vars jobb är att skapa antikroppar. Antikroppar är viktiga vid kroppens normala försvar mot bakterier och virus. Vid myelom har en plasmacell omvandlats till en cancercell som ohämmat delar sig och bildar allt fler myelomceller. Ganska snabbt tar de över benmärgen och tränger undan andra celler som ska bilda andra livsviktiga blodkroppar. Myelom är en obotlig sjukdom, men tack vare nya läkemedel har livslängden ökat under de senaste åren och sjukdomen går idag att leva med under en längre tid.

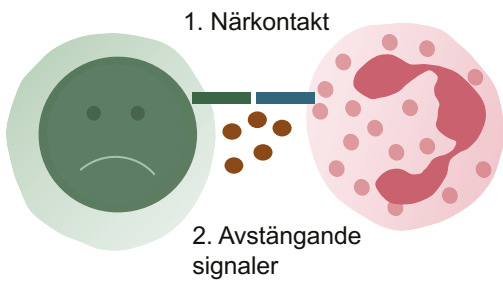
När myelomcellerna tar över benmärgen släpper de ifrån sig signalmolekyler som påverkar omgivande celler. Bland annat påverkas cellerna i immunförsvaret, kroppens skydd mot infektioner och cancer, som blir försvagade och mindre effektiva. Än vet man vet inte exakt hur myelomcellernas negativa grupptryck påverkar cellerna i sin omgivning, även om det är en viktig pusselbit i jakten på ett botemedel mot sjukdomen.

I det första projektet i den här avhandlingen undersökte vi om de så kallade MDSC-cellerna har påverkats av grupptrycket från myelomcellerna. MDSC-celler inaktiverar T-celler, kroppens mördarceller som dödar både bakterier och cancerceller. Om T-cellerna är avstängda tillåts myelomcellerna härja fritt. Medan andra forskare tidigare har sett att MDSC-celler verkar öka i blodet när en person får cancer. Men i vår studie, såg vi inte någon skillnad i antalet MDSC-celler hos myelompatienter jämfört med hos friska personer. MDSC-cellerna hade inte heller förmågan att inaktivera T-cellerna. Med andra ord verkar MDSC-celler inte ha så stor betydelse för utvecklingen av myelom. Istället hittade vi att en annan typ av vita blodkroppar, neutrofiler, hade förmågan att inaktivera T-celler. Neutrofiler är kroppens bästa försvar emot infektioner, och dessa verkar kunna stänga av T-cellerna i benmärgen hos patienter med myelom.



MDSC-cellerna har beskrivits i flertalet olika cancerformer, där de beskrivs öka i mängd och ha förmågan att stänga av T-celler – våra mördarceller. I första studien i den här avhandlingen undersöker vi MDSC-cellerna och om de påverkats av myelomcellernas grupptryck, något de inte verkar ha gjort. MDSC-cellerna hade inte ökat i mängd, och kunde inte heller stänga av T-cellerna. Istället såg vi att neutrofiler, kroppens bästa försvar mot bakterier, hade påverkats av myelomcellerna i benmärgen och stängde av T-cellerna.

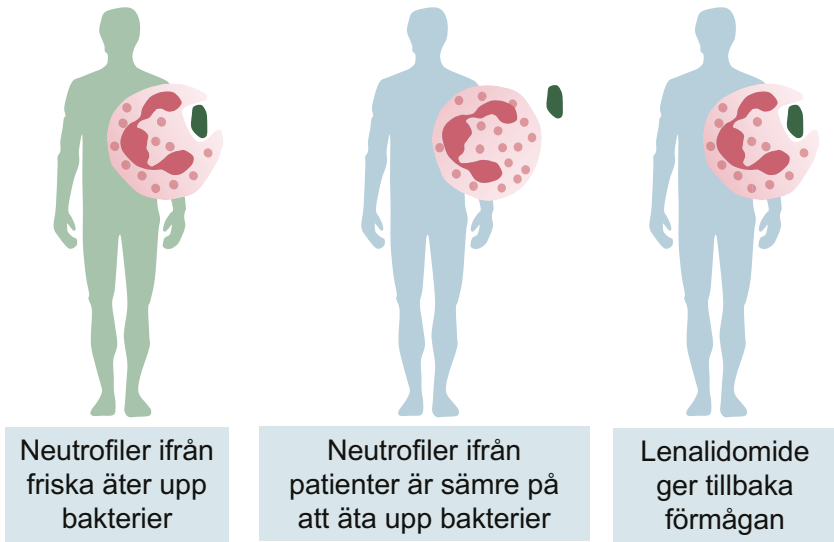
I det andra och tredje arbetet i den här avhandlingen undersöker vi neutrofilernas förmåga att stänga av T-cellerna. Neutrofiler som finns i blod kan inaktivera T-celler. Detta gäller både neutrofiler som renats fram från friska och sjuka, men av neutrofilerna som är upprenade ifrån benmärgen så är det bara patienternas neutrofiler som har denna förmåga. Vi har även studerat hur friska neutrofiler från blodet stänger av T-celler och upptäckte att det krävs både närkontakt och avstängande signaler.



Neutrofiler (rosa) stänger av T-cellerna (gröna) genom att ha närkontakt och genom att släppa ifrån sig avstängande signaler.

I det fjärde och sista arbetet undersöker vi ifall myelomcellernas närvaro kan ha påverkat neutrofilernas förmåga att försvara kroppen mot bakteriella infektioner.

Myelompatienter har nämligen en ökad risk för att få infektioner och eftersom neutrofilerna är kroppens främsta försvar emot bakterier så tror man att dessa kan vara försvagade hos patienterna. Ett bra sätt för neutrofilerna att försvara kroppen mot bakterier är genom att äta upp dom. När vi testade neutrofilernas förmåga att äta upp bakterier såg vi att de inte alls var lika bra på det som neutrofiler ifrån friska personer. Vi såg också att neutrofiler från patienter som hade behandlats med läkemedlet lenalidomide hade återfått sin förmåga att äta upp bakterier.



Myelompatienternas neutrofiler är sämre på att äta upp bakterier än vad neutrofilerna hos friska är. Lenalidomide-behandling kan dock ge tillbaka neutrofilerna deras förmåga att äta bakterier.

I den här avhandlingen har vi undersökt hur myelomcellernas grupptryck påverkar MDSC-celler och neutrofiler. Myelomcellerna verkar inte ha ha så stor påverkan på MDSC-celler, men desto större påverkan på neutrofiler. Neutrofiler i benmärgen förändras av grupptrycket så att de inaktiverar T-celler, vilket leder till att de inte kan stoppa myelomcellernas framfart. Dessutom tappar neutrofilerna sin förmåga att äta upp bakterier vilket kan förklara varför myelompatienter lättare blir sjuka. Detta är en pusselbit i jakten på att ta reda på hur myelomcellerna påverkar andra celler, vilket i framtiden kan hjälpa till att utveckla nya läkemedel som minskar sjukdomens dödlighet.

List of Publications and Manuscripts

Paper I

Westerlund, J., Askman, S., Pettersson, Å., Wichert, S., Hellmark, T., Johansson, Å., Hansson, M. Myeloid-derived suppressor cells and their role in Multiple Myeloma. *Manuscript.*

Paper II

Petersson, J., Askman, S., Pettersson, Å., Wichert, S., Hellmark, T., Johansson, Å., Hansson, M. (2021). Bone marrow neutrophils of multiple myeloma patients exhibit myeloid-derived suppressor cell activity. *Journal of Immunology Research.*

Paper III

Westerlund, J., Askman, S., Pettersson, Å., Hellmark, T., Johansson, Å., Hansson, M. (2022). Suppression of T cell proliferation by normal density granulocytes led to CD183 downregulation and cytokine inhibition in T cells. *Journal of Immunology Research.*

Paper IV

Askman, S., **Westerlund, J**., Pettersson, Å., Hellmark, T., Johansson, Å., Wichert, S., Hansson, M. Newly diagnosed multiple myeloma patients have decreased neutrophil functions in blood and bone marrow that is restored with lenalidomide therapy. *Manuscript.*

Abbreviations

Arg-1	Arginase 1
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor
BCR	B cell receptor
BM	Bone marrow
CCL	C-C chemokine ligand
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CGD	Chronic granulomatous disease
CSR	Class switch recombination
CTL	Cytotoxic T lymphocyte
CXCL	CXC ligand
CXCR	CXC chemokine receptor
DC	Dendritic cell
ECM	Extracellular matrix
eMDSC	Early-state Myeloid-Derived Suppressor Cell
EMM	Extramedullary multiple myeloma
FACS	Fluorescence activated cell sorting
fMLF	N-Formylmethionine-leucyl-phenylalanine
GC	Germinal center
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
G-MDSC	Granulocytic MDSC, same as PMN-MDSC

HLA-DR	Major histocompatibility complex class II
IFN	Interferon
Ig	Immunoglobulin
IKZF1	IKAROS Zinc finger 1, Ikaros
IKZF3	IKAROS Zinc finger 3, Aiolos
IL	Interleukin
LDG	Low Density Granulocyte
LDN	Low Density Neutrophil
Lin	Lineage
Lox-1	Lectin-type oxidized LDL receptor-1
Mac-1	Macrophage-1 antigen
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-Derived Suppressor Cell
MGUS	Monoclonal Gammopathy of Undetermined Significance
MHC	Major histocompatibility complex
MHC-II	Major histocompatibility complex class II
MM	Multiple Myeloma
M-MDSC	Monocytic Myeloid-Derived Suppressor Cell
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
M protein	Myeloma protein, Myeloma Immunoglobulin
NADPH	Nicotinamide adenine dinucleotide phosphate
NDG	Normal Density Granulocytes
NDN	Normal Density Neutrophil
NETs	Neutrophil extracellular traps
NK cell	Natural Killer cell
NO	Nitric oxide
NOX2	NADPH oxidase II complex
PD-1	Programmed cell death protein 1

PD-L1	Programmed death-ligand 1
PMN-MDSC	Polymorphonuclear Myeloid-Derived Suppressor Cell
RBC	Red blood cell
rTEM	Reversed endothelial cell migration
ROS	Reactive oxygen species
SHM	Somatic hypermutation
SMM	Smoldering Multiple Myeloma
TAM	Tumour associated macrophage
TAN	Tumour associated neutrophil
TCR	T cell receptor
TEM	Transendothelial cell migration
Tfh	T follicular helper cell
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumour necrosis factor
Treg	Regulatory T cell

Introduction

The immune system

The immune system is what is keeping us alive. It protects us against foreign pathogens, as well as against cancer cells. The immune system is a complex network of cells, tissues, organs, and substances, that communicate with one another and help the body fight infections and diseases. It can be divided into the innate immune system and the adaptive immune system.

The innate immune system is the first line of defence against foreign substances. It acts fast, within minutes, and has no immunological memory. The innate immune system is ancient and found in all multicellular organisms. It consists of physical barriers, such as the skin and the mucosa, but also of cellular and humoral components, such as antibodies and the complement system. The cellular components consist of cells of the myeloid lineage, including neutrophils, monocytes, mast cells, basophils, dendritic cells (DCs), and eosinophils. (1, 2)

The adaptive immune system is activated by the components of the innate immune system. In contrast to the innate immune system, which acts fast, the adaptive immune system is much slower and takes time to evolve. It generates an antigen-specific immune response, with an immunological memory. The adaptive immune system consists of two different kinds of lymphocytes, called T cells and B cells. These cells can be further divided into different subsets with specific functions. (1)

The cells of the immune system are formed from a pluripotent hematopoietic stem cell in the bone marrow (BM), that differentiates into either a myeloid progenitor cell or a lymphoid progenitor cell (fig. 1). The myeloid progenitor cells give rise to cells of the myeloid lineage, including red blood cells, monocytes, neutrophils, eosinophils, basophils, megakaryocytes and mast cells. Monocytes can further differentiate into DCs and macrophages. The lymphoid progenitor cells gives rise to T cells, B cells and NK cells. B cells give rise to plasma cells, which are the antibody producing cells. (3)

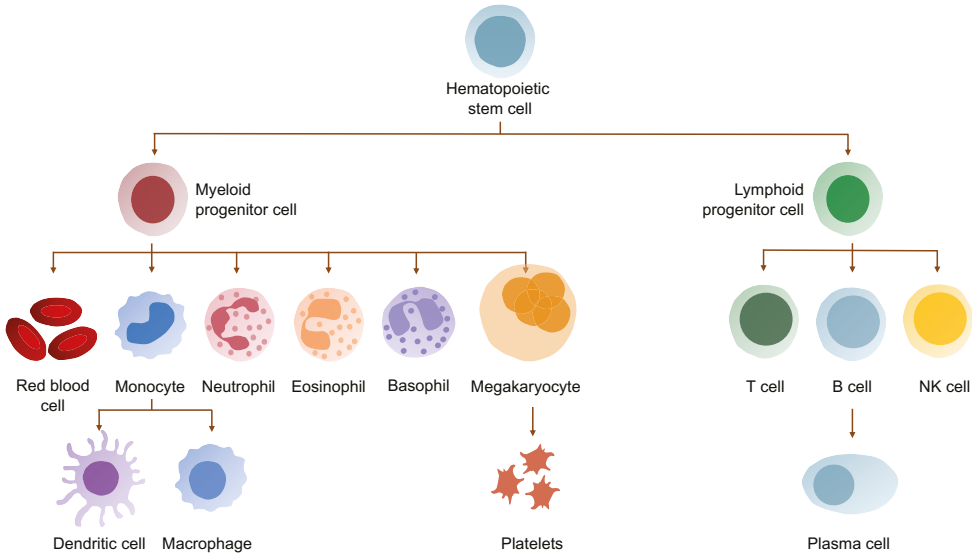


Figure 1. Hematopoietic stem cell differentiation into immune cells in the BM.
Hematopoietic stem cells differentiatie into myeloid and lymphoid progenitor cells, that in turn differentiatite into several different immune cells. The myeloid progenitor cell can differentiate into red blood cells, monocytes, neutrophils, eosinophils, basophils, megakaryocytes that form platelets, DCs, and macrophages. The lymphoid progenitor cell can differentiate into T cells, B cells, and NK cells. The B cell can further differentiate into plasma cells. In this thesis we mainly focus on neutrophils and T cells, in the plasma cell malignancy Multiple Myeloma.

T cells

T cells are part of our adaptive immune system, which generates antigen-specific immune responses. T cells have T cell receptors (TCRs) on their cell surface, which are used to recognise antigens presented on major histocompatibility complex class II (MHC-II) by antigen presenting cells (APC), such as DCs. The cells of the innate immune system can attack any antigen they encounter, but T cells circulate until they encounter their specific antigen. All T cell clones are specific for one antigen, so no T cell clone is like the other. T cells are highly important for our immune defence, both in fighting infections and fighting cancer.

T cells are $CD3^+$ cells that can be divided into multiple different subsets, which all have important functions within the immune system. The two major subsets are the $CD4^+$ cells and the $CD8^+$ cells (4). The $CD4^+$ cells are T helper (Th) cells and can be further divided into several different subsets, including Th1, Th2, Th9, Th22, regulatory T cells (Tregs), and follicular helper cells (Tfh) (4, 5). The different subsets develop from naïve T cells under the influence of specific cytokines, and are characterized based on their unique cytokine profiles (fig. 2) (5).

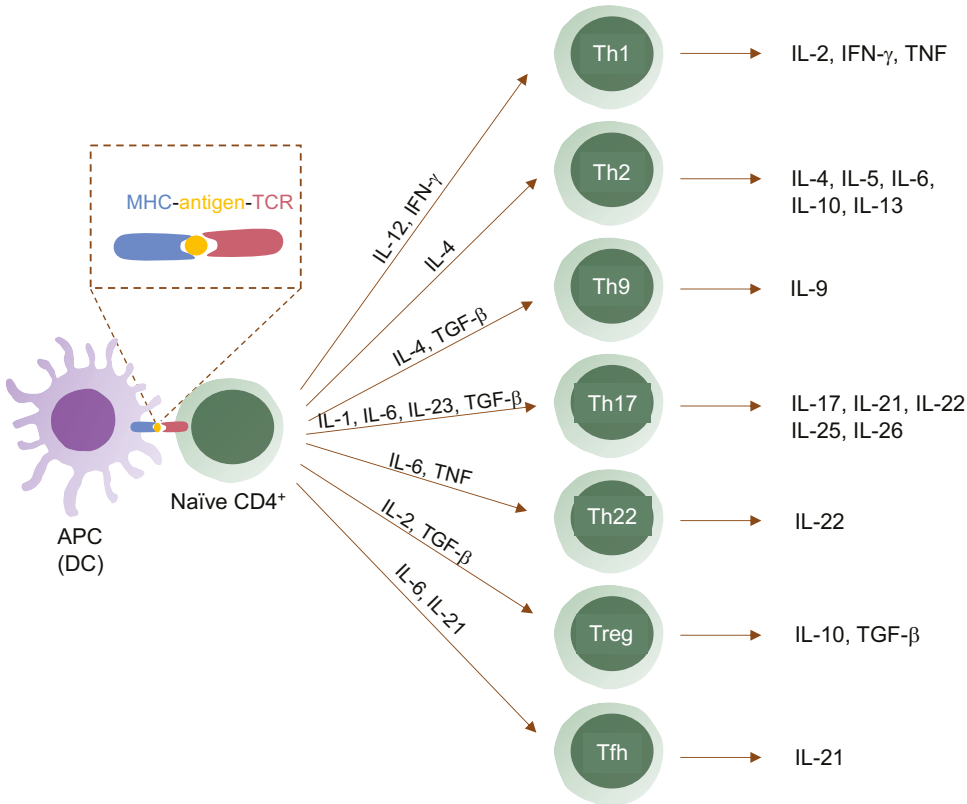


Figure 2. T helper (Th) subsets.
Naïve T cells are activated by antigen presenting cells (APCs), as represented by a dendritic cell (DC) in this figure. The APC present the antigen on a MHC, and the T cell recognises its cognate antigen using its TCR. The different Th subsets develop under the influence of specific cytokines, as implicated in the figure. The different Th subsets have unique cytokine profiles.

Th1

Th1 cells are most often defined by their ability to produce IL-2, IFN-γ and TNF, as well as their expression of the transcription factor T-bet (4). These cells are important for immunity against intracellular pathogens as well as the immune response against cancer cells (6). Th1 cells also contribute to the development of autoimmune diseases (6).

Th2

Th2 cells are characterized based on their expression of the transcription factor GATA3, and their production of IL-4, IL-5, and IL-13 (6). They also produce IL-6 and IL-10. Th2 cells are important in the immune defences against large parasites, such as helminths (6). They are also of major importance in allergy and atopic illness (4). Th2 cells are mostly present in the intestinal tract and the lungs, where they

carry out their functions (4). Th2 cells are described as anti-inflammatory in autoimmune diseases, as they suppress Th1 responses (4).

Th17

Th17 cells express the transcription factor RORγt and produce IL-17 (6). Th17 cells are important in the defence against extracellular pathogens, including bacteria and fungi (4). IL-17 secretion leads to recruitment of neutrophils, activation of innate immune cells, enhanced B cell function, and increased release of pro-inflammatory mediators (4).

Tregs

Tregs are a subpopulation of T cells with the ability to modulate and suppress the immune system, maintain tolerance to self-antigens and prevent autoimmune diseases. Tregs are defined by the expression of the transcription factor FoxP3, and the production of IL-10 and TGFβ (6). Tregs were described for the first time in 2001 (7-10) and are important for the regulation of the immune system, as patient who lack Tregs develop systemic autoimmunity. This is observed in patients who suffer from the rare disease IPEX, a genetic disorder with a mutation in the FoxP3 gene (11-13). Tregs can be induced by the tumour microenvironment, and their suppressive abilities leads to an immunosuppressed state with a reduced anti-tumour response that allows for tumour progression (6, 14).

$CD8^+$ T cells

After activation by APCs, $CD8^+$ T cells undergo massive expansion and differentiates into cytotoxic T-lymphocytes (CTLs) that can migrate to sites of infection and efficiently kill pathogens (15). The CTL mainly use two different cytotoxic pathways for killing: perforin/Granzyme and Fas/FasL (6, 16). Perforin/granzyme is fast acting. Perforin forms pores in the cell membrane that destroys the integrity of the cell (17) while the co-secreted granzymes enter the cell to induce apoptosis (16, 18). Fas/FasL is much slower, and involves the binding of the cell surface death receptor Fas on the targeted cell to FasL expressed on the cell surface of CTLs, which leads to a death-inducing signal in the target cell (16). CTLs do not only bind to and kill pathogens, they are also prominent tumour cell killers (18, 19).

The T cell response against tumour cells

T cells play a central role in the elimination of tumour cells, as well as in the development and progression of tumours (20, 21). During the early stages of tumour initiation, immunogenic antigens are produced and presented on the tumour cells, which can activate the immune system (19). APCs will take up the antigen and present it to the naïve T cells in the lymph nodes (19). There, naïve T cells will become primed and activated, and then migrate to the tumour site where they

activate a prominent effector immune response that eliminates the tumour cells (19). The presence of T cells within a tumour is associated with an improved prognosis in several different cancer types (22). Both $CD8^+$ CTLs and Th1 cells are important for tumour clearance. When the CTL have recognized a tumour cell, it binds to it and creates an immunological synapse into which granule proteins, such as perforin and granzyme B, are released (18, 19). This interaction will kill the cancer cell. Th1 cells are also part of the anti-tumour response and through the release of the pro-inflammatory cytokines IL-2, TNF-α, and IFN-γ, they promote the priming and activation of CTLs, induce anti-tumoral activity in macrophages and NK cells, and increases overall antigen presentation (19).

Even if T cells are incredibly good at destroying tumour cells, some tumours still manage to escape the immunosurveillance and form tumours by altering the immune defence (23). Tumour cells evade the immune system through two different strategies – avoiding immune recognition and promoting an anti-tumour microenvironment (19). The tumour cells can avoid immune recognition by losing their tumour antigens, and by secreting suppressive molecules and upregulating inhibitory checkpoint molecules, such as PD-L1, they promote a pro-tumour environment with reduced T cell function (19). Tumour cells also release factors that recruit immune cells that act in a pro-tumour fashion, such as M2 macrophages, myeloid-derived suppressor cells (MDSC) and Tregs (19). Tregs suppress the priming, activation and cytotoxicity of several immune cells, such as CTLs, Th1 cells, macrophages, neutrophils and NK cells (24).

The formation of a pro-tumour microenvironment is complex and requires evolution of the tumour cells, so that they can promote the changes necessary for immune evasion and for promoting a suppressive environment. It also requires crosstalk between several different immune cells. How the different immune cells interact within the tumour microenvironment is not well investigated, and the complexity is not well understood.

Myeloid-derived Suppressor Cells

MDSC seem to play an important role in the immune suppression often observed in cancer patients, as they inhibit T cell responses. MDSC levels increase during cancer progression and correlate with disease severity (25, 26). They also seem to decrease as a response to tumour restriction (25). In some cancers, for example advanced breast cancer, MDSC levels could be used as a prognostic marker as patients with low levels have a better overall survival than patients with high levels (27).

MDSC are defined as a heterogenous group of different immature cellular subsets with myeloid origin that exhibit immune regulatory functions. MDSCs can be divided into three different subsets based on their phenotypic features. The two main

groups are the polymorphonuclear (PMN)-MDSCs and the monocytic (M)-MDSCs, but a third subset called early (e)-MDSCs has also been described in humans (26, 28, 29).

Identification of MDSC subsets

In the very beginning of MDSC research, there was no consensus on how to define the MDSC subsets. Different groups used different markers for identification, and it was difficult to determine if people were researching the same subset or not. In 2016, Bronte et. al. published a paper containing recommendations for how to study human and mouse MDSC (26). It contained guidelines for which markers to use, how to gate the cells, as well as which functional tests to use. According to their definition, human PMN-MDSC are characterized as $CD11b^{+}CD14^{-}CD15^{+}$ or $CD11b^{+}CD14^{-}CD66^{+}$ cells, while M-MDSC are categorized as $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}CD15^{-}$ cells (fig. 3) (26). These markers are also present on neutrophils and monocytes, respectively, making it difficult to distinguish them from one another. The eMDSC subset is characterised as $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$, where Lin^{-} (Lineage-) includes CD3, CD14, CD15, CD19, CD20 and CD56 (fig. 3) (26). All MDSC are found in the low-density fraction after a density gradient centrifugation. The criteria for defining MDSC does not just include phenotypic markers. The functional characteristics, such as the ability to inhibit T cell proliferation, needs to be evaluated as well (26).

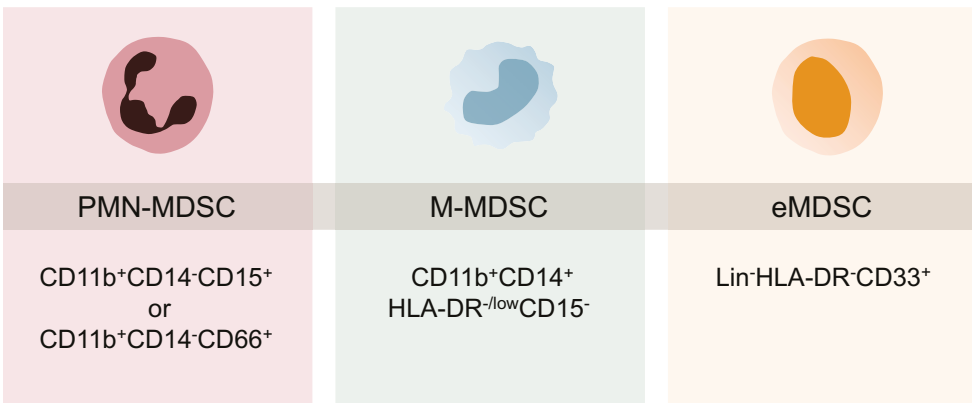


Figure 3. The different MDSC subsets and their cell surface markers.
There are three different MDSC subsets defined in humans. PMN-MDSC are defined as $CD11b^{+}CD14^{-}CD15^{+}$ or $CD11b^{+}CD14^{-}CD66b^{+}$ cells that are found in the mononuclear cell layer after a density gradient centrifugation. M-MDSC are defined as $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}CD15^{-}$ cells and eMDSC as $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$ cells.

The definition of PMN-MDSC as a unique subset has met some controversy, as they are so similar to neutrophils (30). The only way to distinguish PMN-MDSC from neutrophils is to perform a density gradient centrifugation (26). PMN-MDSC ends

up in the low-density layer together with the mononuclear cells. The granulocytes that are found in this layer are either immature neutrophils that have a lower density due to lower levels of granules, and a smaller cells size (31, 32), or NDG that have been activated and degranulated (33). PMN-MDSC are sometimes referred to as low density granulocytes (LDG), while neutrophils that end up in the high-density layer sometimes are referred to as Normal Density Granulocytes (NDG). In some studies, PMN-MDSC are referred to as granulocytic (G)-MDSC. Throughout this thesis, the low-density cells that express neutrophil markers will be referred to as PMN-MDSC, as the term LDG usually is used in autoimmune diseases.

M-MDSC are monocyte-like cells, and difficult to distinguish from normal monocytes. Mature monocytes are HLA-$DR^{+}CD11b^{+}$ cells and can be further divided into three distinct subsets, including the classical monocytes ($CD14^{++}CD16^{-}$), the non-classical monocytes ($D14^{lo}CD16^{+}$), and the intermediate monocytes ($CD14^{+}CD16^{+}$) (34-36). M-MDSC are distinguished from ordinary monocytes based on HLA-DR expression, where M-MDSC are HLA-$DR^{-/low}$ (26).

Mice are commonly used as an animal model when studying MDSC. In mice, MDSC have specific markers, making it possible to distinguish them from neutrophils and monocytes. Murine PMN-MDSCs are defined as $CD11b^{+}Ly6G^{+/low}Ly6C^{low}$ cells, while M-MDSCs are defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells (28). No equivalent of eMDSCs have been identified (28).

PMN-MDSC are often defined as immature cells, and a good way to distinguish mature and immature neutrophils from one another is by looking at nuclear segmentation. However, science has not yet found a way to isolate cells based on their nuclear segmentation. We are therefore stuck with cell surface markers. There are a few differences between mature and immature neutrophils when it comes to cell surface markers, for example CXCR4, CXCR2, CD11b, CD18, CD62L, but these markers changes with activation (CD11b, CD18), migration(CD62L) and ageing (CXCR4) (37). Several attempts have been made to find unique markers for human MDSC. Lectin-type oxidized LDL receptor-1 (Lox-1) has been suggested to be a PMN-MDSC specific marker (38-40) and S100A9 have been suggested to be a M-MDSC specific maker (41, 42).

According to the literature, a cell can only be called a true MDSC if they are found in the low density layer after a density gradient centrifugation, has the correct phenotypic markers, and are immune suppressive (26). An algoritm for MDSC identification has been suggested (fig. 4) (26).

If the proposed MDSC cell lacks inhibitory effect against T cells, it should not be called an MDSC. However, biochemical and molecular traits could be investigated and if they have MDSC properties the cell could be called an MDSC like cell (26). Non-suppressive MDSC (MDSC like cells) may regulate other aspects of tumour onset and progression (26). If the investigated cell is not an MDSC like cell, it is probably an ordinary neutrophil, eosinophil or monocyte (26).

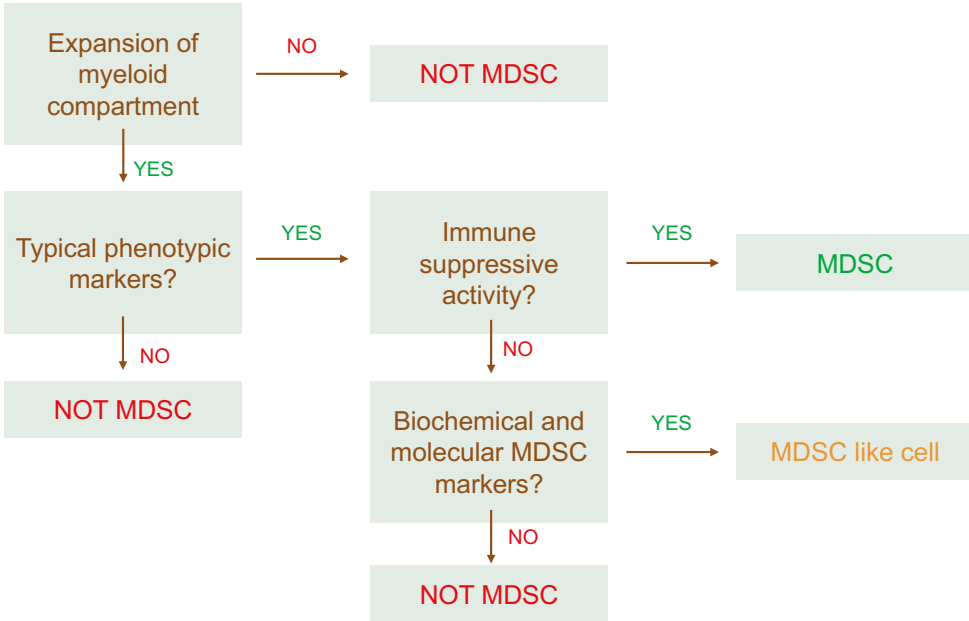


Figure 4. Algoritm for MDSC identification, suggested by Bronte et. al (26).
Step-by-step approach for MDSC identification. The first step is to investigate if there is an increase of MDSC in the patient setting compared to a healthy setting. If no, the cells are not MDSC. If yes, continue to investigate phenotypic markers. If they are present, continue with immune suppressive ability. The figure has been modified and can be found in its whole in the original article (26).

Inhibitory mechanisms

The main feature of MDSC, that has allowed them to be distinguished from monocytes and neutrophils, is their characteristic immune suppressive effects (26, 28). The main mechanisms of action that has been described are the production of reactive oxygen species (ROS), Arginase-1 (Arg-1), and nitric oxide (NO), as well as the induction of Tregs (43, 44). The expression of PD-L1 may also be important for the MDSC ability to inhibit T cell responses (45). It is not certain if all these are important for the inhibitory effect, since results vary between groups (46). It is also possible that the mechanisms of action differ depending on type of disease, as well as the severity of the disease. In any case, the proposed mechanisms are described below.

ROS interacts with macromolecules, such as lipids and DNA, and can regulate both protein structure and function leading to changes in several biological processes important for a normal immune function. ROS inhibits T cells through several mechanisms, including inhibition of DNA synthesis and by alteration of T cell receptor signalling (47). ROS downregulates expression of the CD3ζ-chain, making the T cell unable to respond to stimuli. ROS also leads to reduced cytokine secretion from T cells. Studies have shown that MDSCs with inhibited ROS production no

longer have T cell inhibitory properties. Several known tumour-derived factors, such as TGF-β, IL-10, IL-3, IL-6, platelet-derived growth factors, and granulocyte macrophage colony-stimulating factor (GM-CSF) can promote MDSC ROS production. (48, 49).

Production of Arg-1 leads to depletion of L-arginine in the microenvironment (48). Shortage of L-arginine affects the T cell by reduced expression of the CD3ζ-chain, making the T cells unable to respond to stimuli, and prevents upregulation of cell-cycle regulators, preventing the T cells from dividing (48). L-arginine levels directly impact the metabolic fitness and the survival of T cells, which is essential for their ability to perform anti-tumour responses (50).

NO suppresses T cells through many different mechanisms, leading to for example induction of apoptosis, and inhibition of intracellular signalling (48). This method for inhibition is mainly used by M-MDSC (34).

Several studies have also suggested that MDSC can induce Tregs, leading to further T cell inhibition (48). However, results vary, and more research is needed in order to unravel the impact MDSC have on other cellular subsets.

PMN-MDSC

PMN-MDSC are present already in the early stages of cancer (51), and their suppressive abilities seem to increase during cancer progression (52). Compared to neutrophils, PMN-MDSC have been described to have less granules, reduced levels of CD16 and CD62L, and an increase in arginase 1, peroxynitrite, CD11b and CD66b (26).

The MDSC subsets are derived from myeloid progenitor cells and are hypothesised to arise during pathological conditions (53). Normal neutrophils are produced in a process called myelopoiesis, which will be described in more detail later. In short, neutrophils develop from a myelocyte and gain maturity in a step-by-step process, as described in fig. 5. The process occurs in the BM, and mature neutrophils are released into the blood. During acute infections, such as viral- or bacterial infections, myelopoiesis is triggered by strong activation signals that provide an increased number of myeloid cells ready to eliminate the threat (28).When the threat has been eliminated, the number of myeloid cells is restored to normal (28). During several pathological conditions, for example cancer, the activation signals for myelopoiesis may be weak but prolonged, leading to aberrant myelopoiesis (28). MDSC are thought to be produced during this pathological activation (28, 54). The aberrant myelopoiesis leads to a “left shift” in myelopoiesis, defined as an increased ratio of immature myeloid cells in the blood – such as metamyelocytes and band cells (fig. 5) (54).

MDSC formation have been suggested to be a two-step process (48). The first step is the mobilization of immature myeloid cells from the BM, followed by an activation signal that mediates the suppressive ability of immature myeloid cells – turning them into MDSC (48). Normal granulopoiesis is regulated by GM-CSF, granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF). These growth factors also seem to be important in the formation of MDSC (53). There are, however, contradictory results regarding the immaturity of MDSC as mature phenotypes also have been shown to inhibit T cells, and maybe be even better at it (55).

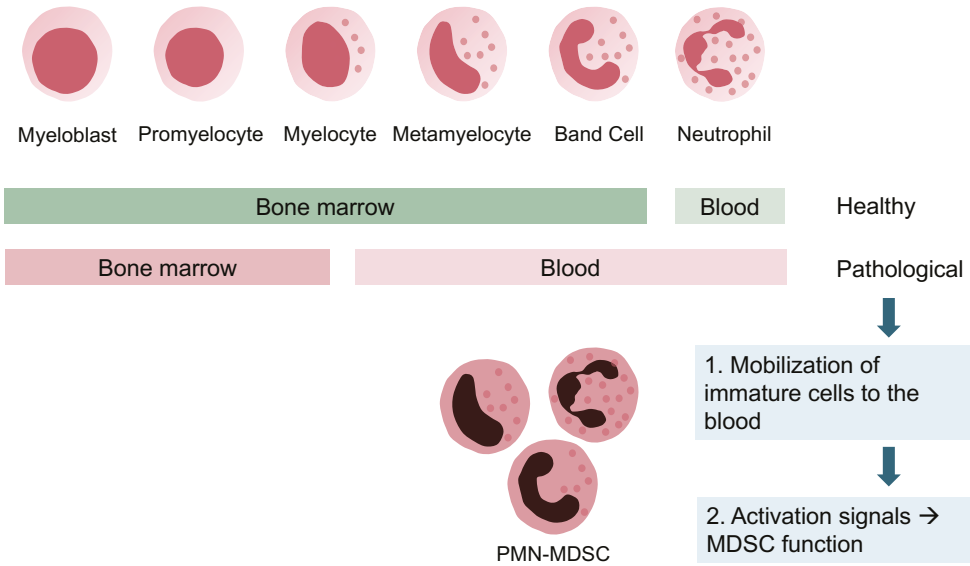


Figure 5. A hypothesis behind the formation of PMN-MDSC.
In a healthy setting, only the mature neutrophils reach the blood. Under pathological conditions, immature cells are thought to mobilize into the blood. Activation signals then lead to the induction of MDSC (inhibitory) functions.

In autoimmune diseases, the PMN-MDSC subset is referred to as low-density granulocytes (LDG) or low-density neutrophils (LDN). These cells have a different role in an autoimmune setting than in cancer. PMN-MDSC are suppressive cells, while LDG are pro-inflammatory and activates T cells (54, 56). As in cancer, LDG are found in increased numbers in several different autoimmune diseases and increased levels correlate with disease severity (57). There are also contradictory results regarding weather or not these cells are of immature or mature origin, or a mixed population that comprise of an activated mature neutrophil subset and an immature subset (57). LDG are prone to release neutrophil extracellular traps (NETs), and increase the proliferation and cytokine production in T cells (57).

M-MDSC

It is not certain if M-MDSC are immature cells, as immature monocytic cells are very similar to mature monocytes by both morphology and cell surface markers, so the suppressive M-MDSC may be of mature origin (54). It is likely that that is the case as monocytes have a high plasticity and can change phenotype and function depending on the local microenvironment (34, 58). To date, M-MDSC have been suggested to originate from a monocyte through pathological myelopoiesis, activation, or a combination of both (59).

M-MDSC are enriched in cancer patients and the levels correlate with disease severity (54). They are also increased in patients with severe infections, and patients who suffer from inflammatory conditions (54).

When cells enter the tumour microenvironment, they encounter a hostile hypoxic environment filled with oxidative agents, pro-inflammatory cytokines and low levels of nutrients (44). M-MDSC, and monocytes, are attracted to the tumour site by different cytokines (CCL2, CCL5, CSF1) (60). At the site of the tumour, at least in mice, they seem to develop into tumour associated macrophages (TAMs) (61, 62). In humans it is difficult to determine specific myeloid subsets in tumour tissues, and further validate their suppressive abilities *in vivo* (54). It is the signals in the tumour microenvironment that drives the differentiation of monocytes/M-MDSC into TAM (44). There are two kinds of TAM, the anti-tumour M1 TAM, and the pro-tumour M2 TAM. M2 TAM can directly inhibit CTL through PD-L1, increase the recruitment of Tregs, inhibit DCs, and remodel the extracellular matrix (63). M1 TAM, on the other hand, can induce a robust anti-tumour response by enhancing antigen presentation and activate the adaptive immune response (63). Besides the M-MDSC ability to develop into TAM, they seem to be able to differentiate into DCs or fibrocytes during cancer progression (64). There have also been suggestions of PMN-MDSC as tumour associated neutrophils (TAN) (40).

eMDSC

Little is known about eMDSC, as they are sparsely investigated. The eMDSC subset has only been observed in humans, which may be the reason why they are not as thoroughly investigated as the other MDSC subsets. Their origin is not known, but they are more immature compared to PMN-MDSC (26). The levels of eMDSC are not always increased in cancer patients, but their suppressive abilities seem to be present despite this (65, 66).

Obstacles in MDSC research

One of the biggest obstacles of MDSC research is the fact that they are only present in low numbers – both in healthy donors and patients who have increased levels (26). This makes it difficult to use them in functional assays, and consequently

many researchers skip them. By skipping the functional assay, it is impossible to determine if the investigated subset have a true MDSC phenotype. Another obstacle is the lack of specific phenotypic markers, making it difficult to isolate the subsets and secure the purity of the cells. It is also difficult to determine the origin of the cells when they cannot be properly distinguished.

A lot of research and effort has been put into defining the role of MDSC in cancer, and they seem to have a tremendous impact in tumour formation and progression. By their ability to inhibit T cell responses, they may promote a pro-tumour environment favourable for tumour cell survival and progression. However, the origin of the MDSC cell types and their similarity to neutrophils and monocytes has gained some interest the past years – are they unique cellular subsets or not?

Neutrophils

Neutrophils are the most abundant white blood cell in the peripheral blood (60-70 %) and distinct themselves morphologically from other cell types with their multi-lobular nuclei and their high content of intracellular granules (67, 68).

Neutrophils are most known for their strong antimicrobial properties. They are the first cells to arrive at the site of infection and have the ability to seek and kill invading pathogens (68).

Neutrophil development

Neutrophils are produced in the BM and are derived from pluripotent hematopoietic cells, in a process called myelopoiesis. Myelopoiesis is a process that give rise to not only neutrophils, but also monocytes and DCs. Under the influence of different transcription factors and the growth factor G-CSF, pluripotent hematopoietic cells start to develop into myeloblasts and commit to the neutrophil lineage (69). Myeloblasts then develop into promyelocytes and further into myelocytes (fig. 6) (69). During these stages, the nucleus is still round, but when they progress into metamyelocytes the nucleus turns into a kidney shape (69). The next developmental stage is to turn into a band cell, and then into a mature neutrophil with the classic segmented nucleus (69). The developmental process from myeloblast into mature neutrophil takes around 14 days (70).

As the neutrophils mature in the BM, they synthesise proteins that are sorted into different granules (fig. 6) (71). The content of the granules depend on which transcriptional program that is active at the time of creation (68).The first granules to be produced are the primary (azurophilic) granules, and their major protein content is myeloperoxidase (MPO) (71). They also contain different proteases, defensins and other antimicrobial proteins (71). Then the secondary (specific) granules are formed, filled with for example lactoferrin and antimicrobial proteins,

followed by the formation of tertiary (gelatinase) granules (69). Tertiary granules contain Arg-1 and matrix-degrading enzymes such as matrix metalloproteinase-9 (MMP9), among others (69, 72). Neutrophils also contain secretory vesicles that contain a reservoir of membrane proteins, such as adhesion molecules and receptors that are important for adhesion to the vascular endothelium (73). The secretory vesicles and the tertiary granules are most readily released to the cell surface, followed by the secondary granules (73). Primary granules are not often released but play an important role in the degradation of phagocytosed material (73, 74). As the neutrophil mature they increase in density due to increased granular content as well as changes in cell size (31).

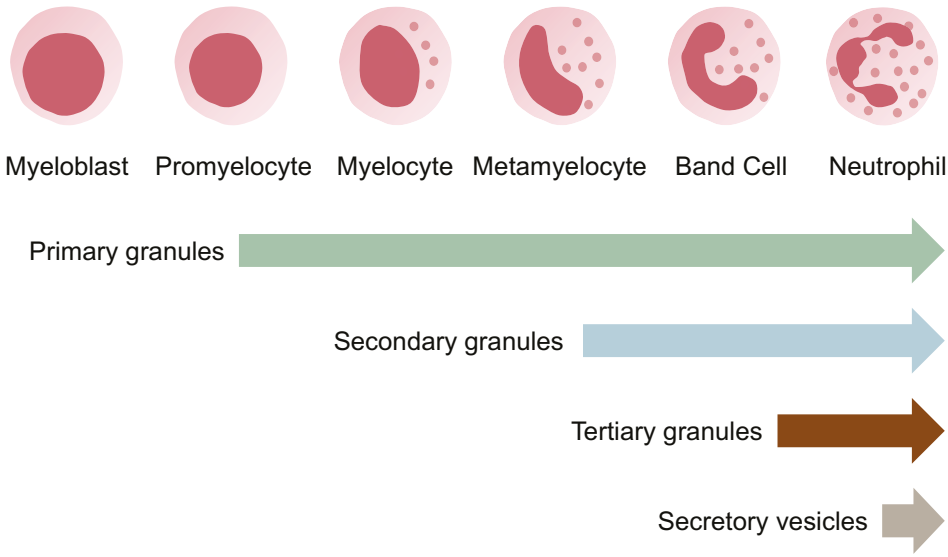


Figure 6. Neutrophil development and granule formation.
Neutrophils are produced in the BM, from a pluripotent hematopoietic stem cells into a myeloblast, promyelocyte, myelocyte, metamyelocye, band cell and lastly into a neutrophil. Granules are formed during these process, starting with primary granules. The secondary and tertiary granules are formed after that, and lastly the secretory vesicles. (69)

When the neutrophils have matured, they are released into the circulation. Circulating neutrophils are considered to be short-lived, with a lifespan of mere hours (75). This has, however, been challenged by observations of neutrophils living for several (5.4) days (76). The lifespan is dependent on several factors and can be altered as a response to different signals in the microenvironment, coming from for example ongoing infections and inflammations (77). Apoptotic neutrophils remain intact for at least 24 h, with no release of granules (77).

Neutrophils and their antimicrobial properties

Neutrophils circulate the blood in a dormant state and will not start their activation process until the vascular endothelium signals that an infection has occurred (78). The circulating neutrophils will roll, adhere, and then cross the endothelium in a process called transendothelial cell migration (TEM) (78, 79). Once on the other side, the neutrophil will follow a gradient of chemo-attractants and be the first cell type to reach the site of infection, where it will (hopefully) kill the pathogen (78).

Neutrophils have an arsenal of different effector functions that can be used to kill pathogens, and clear away infections. The main ones are phagocytosis, degranulation, oxidative burst, and the release of NETs (68) (fig. 7).

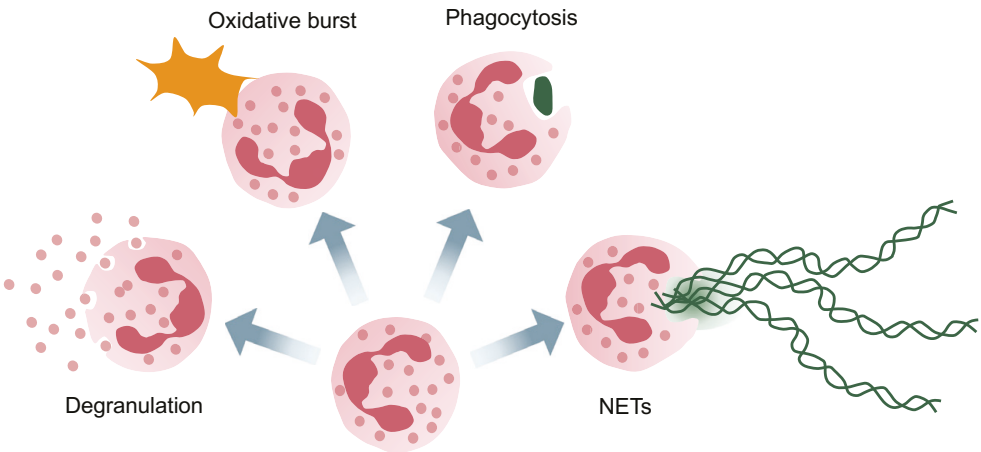


Figure 7. Overview of neutrophil effector functions.
The most common effector functions are degranulation, oxidative burst, phagocytosis and NET formation.

Phagocytosis is a receptor-mediated process in which a pathogen is engulfed and killed within the cell. The pathogen is internalized into the neutrophil in a plasma membrane surrounded vacuole, called the phagosome. The phagosome will then fuse with neutrophilic granules so that antimicrobial molecules and proteolytic enzymes come in contact with the engulfed particle (73, 74). At the same time, the NADPH oxidase will assemble on the phagosomal membrane, which allows the production of ROS. Together, these create a toxic environment that degrades the pathogen within the phagosome. (68, 74)

Oxidative burst is the rapid release of ROS (68). ROS are produced by the NADPH oxidase II (NOX2) complex, present on the cell membrane, when the neutrophil become activated (68). ROS production is important for an efficient immune system, as patients who suffer from chronic granulomatous disease (CGD) suffer from frequent and severe infections (80). CGD is a rare genetic syndrome where the

NOX2 complex is non-functional, making it impossible for phagocytes to produce ROS and clear away certain bacteria and fungi (80).

The neutrophilic granules, as described above, contain several different antimicrobial peptides and proteolytic enzymes, important for the killing and digestion of pathogens (71).

Another microbial-killing mechanism that neutrophils are capable of, is the ability to undergo NETosis, which is the release of NETs into the extracellular space. NETs consists of decondensed chromatin in combination with antimicrobial proteins from either granules or the cytosol (81). NETs are thought to trap the pathogen and kill it by exposing it to high concentrations of the antimicrobial proteins (68). NET formation can be induced when a neutrophil encounter certain pathogens and the process is dependent on ROS (81).

Neutrophils as Myeloid-Derived Suppressor Cells

In recent years, it has become evident that neutrophils have a far more complex role in the immune system than originally thought. Besides their antimicrobial properties, they have the ability to modulate the adaptive immune system (82). It is not only PMN-MDSC that have suppressive abilities, NDG (also known as ordinary neutrophils) can inhibit T cell responses under certain conditions (55, 83-86). Studies suggest that mature NDG from healthy donors have to be pre-activated in order to gain inhibitory functions (55).

As for PMN-MDSC, both ROS and Arg-1has been suggested to be the main effectors of NDG mediated T cell inhibition (83, 85, 87-90). Other mechanisms have also been proposed. For example, peripheral blood NDG from patients with colon cancer inhibit T cell proliferation through NDG-secreted MMP9 (91). MMP9 activates TGF-β in the microenvironment and promotes the inhibition of T cells (91). There are also studies that suggest the necessity of cell-cell contact (92, 93) and others who claim that soluble factors are responsible for the inhibition (56, 91). For cell-cell contact, the integrin Mac-1 (CD11b/CD18) complex seem to be important, as blocking of CD11b prevent activated NDG from inhibiting T cells (83, 93). Mac-1 binds to ICAM-1 on T cells and forms an immunological synapse, in which ROS can be released (93, 94). Others do, however, claim that an upregulation of PD-L1 is more important for the inhibitory effect (92). Binding of PD-L1 to PD-1, which is present on T cells, leads to reduced activation and cytokine production in T cells. As for PMN-MDSC, it is possible that different diseases and the disease severity, yields NDG with different inhibitory mechanisms and different levels of inhibitory effect.

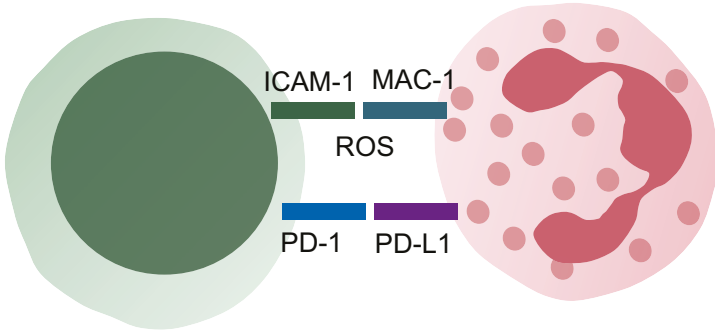


Figure 8. Two proposed mechanisms of T cell inhibition that require cell-cell contact.
One proposed mechanism is the formation of an immunological synapse. The Mac-1 complex, which consists of CD11b and CD18, on neutrophils binds to ICAM-1 on T cells and forms an immunological synapse in which ROS can be released. The second proposed mechanism that requires cell-cell contact is the binding of PD-1 to PD-L1.

Researchers argue about whether PMN-MDSCs are a unique granulocytic subset or a type of neutrophil. Both mature and immature neutrophils can have suppressive abilities (93, 95, 96), and many researchers believe that suppressive neutrophils are a pathological response to tumorigenesis and not separate neutrophil subsets (37, 97-99). Both immature and mature neutrophils are present in the blood of cancer patients (32), as well as in solid human tumours (40, 100, 101). The number of immature and mature neutrophils may vary depending on the type of cancer, as well as the severity of the disease (32, 40, 100, 101). Immature granulocytes are, as mentioned above, different from mature granulocytes based on their segmented nucleus. Several studies have shown that PMN-MDSC from cancer patients have a segmented nucleus just like mature neutrophils, and that neutrophils with hypersegemented nuclei sometimes are the best immune inhibitors (82, 93). These studies suggest that PMN-MDSC are not always immature, but mature cells that have gained inhibitory functions.

The suppressive effect observed in both MDSC and NDG studies seem to be influenced by the disease they are studied in, which neutrophil isolation method that is used, and which T cell assay that is used. More research is needed to unravel the inhibitory function of neutrophils, both in health and disease.

Plasticity

Neutrophils have high plasticity and the ability to adapt to its surrounding microenvironment. Change in function seem to be induced by several factors, one of them being migration. Under normal conditions, when neutrophils have fulfilled their antimicrobial role, they will be phagocytosed by macrophages and removed from the tissue (79). However, studies have shown that some neutrophils have the ability to undergo reversed transendothelial cell migration (rTEM), meaning that

they can go back into the circulation after they have encounted inflammation in a tissue (79). The neutrophils that undergo rTEM have an altered phenotype (ICAM-1^{high}CXCR1^{low}) and are more rigid, less prone to transmigrate again, better at producing ROS and less prone to undergo apoptosis (102, 103). The ICAM-1^{high}CXCR1^{low} neutrophil subset has been found in patients with rheumatoid arthritis and atherosclerosis, suggesting a pathogenic role for these cells (102). Studies have also shown that neutrophils lose their capacity to suppress T cell proliferation after they have migrated towards inflamed joints in JIA (104).

Another example of neutrophils that have adapted to a certain microenvironment are TAN – neutrophils that have entered the tumour site. Neutrophils, and also PMN-MDSC, are attracted to the tumour site by different cytokines (CXCL1, CXCL5, CXCL6, CXCL8, CXCL12) (60). Increased levels of TAN are one of the most powerful prognostic markers for poor survival in cancer (105). There are two different kinds of TAN, the antitumour N1 TAN and the protumour N2 TAN (106). The protumour functions of N2 TAN include degradation of the extracellular matrix, stimulation of tumour cell proliferation, promotion of metastasis formation and increase angiogenesis (107). N2 neutrophils have been reported to be very similar to PMN-MDSC, both phenotypically and functionally (26, 108). PMN-MDSC, in this case as TAN, colocalize with T cells in head and neck cancer tumours (40). T cells that are near PMN-MDSC have reduced levels of granzyme B, while PMN-MDSC in these areas have a higher expression of LOX-1 and Arginase-1, suggesting a T cell inhibitory function of these cells (40). Strong down-regulation of T cell function by PMN-MDSC correlates with impaired survival (40). The antitumour functions of N1 TAN include promoting tumour cell death by releasing their antimicrobial killing machinery, and releasing factors that recruit other immune cells to help with the tumour destruction (109, 110).

Neutrophils have also been shown to have antigen presenting capacities by acquiring surface expression of MHC-II (111, 112), as well as the ability to transdifferentiate into monocytes *in vitro* (113). Neutrophils truly exhibit high levels of plasticity, as they seem to gain new abilities during different conditions. The role of neutrophils during different diseases, such as in cancer and autoimmunity, has just started to unravel and they seem capable to both supressing and activating T cell responses – either as they are with a high density or as low-density cells. The field of neutrophils, PMN-MDSC and LDG is to date confusing, and more research is needed in order to determine their true origins and functions in different disease settings.

Monocytes

Monocytes are cells of the innate immune system, which protects us against foreign pathogens (114). They can present antigens to other immune cells and have the ability to phagocytose. Mature monocytes are HLA-DR^{+}CD11b^{+} cells and are divided into three distinct subsets, which includes the classical monocytes (CD14^{++}CD16^{-}), the non-classical monocytes (D14^{lo}CD16^{+}), and the intermediate monocytes (CD14^{+}CD16^{+}) (34-36). When a monocyte migrate into a tissue, it becomes either a macrophage or a DC (114). Macrophages are often divided into the pro-inflammatory M1 subset and the anti-inflammatory M2-subset (115). Monocytes/macrophages have high plasticity, and it is surrounding signals that determine their phenotype and function (115). As mentioned above, macrophages and M-MDSC that have entered a tumour site become TAM, that can be of either M1 or M2 phenotype.

B cells

B cells are lymphocytes, a part of our adaptive immune system together with T cells. B cells are our antibody producing cells and are therefore a major part of the humoral immunity as well as the cellular immunity. Besides their ability to produce antibodies and thereby playing a role in the immune system against pathogens, B cells can present antigens on their MHC-II.

B cell differentiation occurs in two steps – an early antigen independent step and a late antigen dependent step. In the first step, precursor B cells undergo VDJ-recombination and mature into naïve B cells that circulates the blood and lymph nodes in search for their cognate antigen. Upon antigen encounter, the B cell will be activated and move into the dark zone of the germinal center (GC) where it will undergo clonal expansion as well somatic hyper mutations (SHM). The clonal expansion and the SHMs will create several centroblasts out of one B cell, with either increased or decreased affinity for its specific antigen. The centroblasts will then move into the light zone of the GC where they will become centrocytes. The centrocytes ability to bind to its cognate antigen will then be tested. If the SHM have led to decreased affinity of the immunoglobulin it will proceed into apoptosis, but if it the SHM has led to increased affinity the centrocyte will undergo class switch recombination (CSR) and produce high affinity IgG or IgA antibodies. The B cell will also differentiate into either a memory B cell or an antibody producing plasma cell. (116)

Antibodies

Antibodies, also known as immunoglobulins, are either present on the cell surface as B cell receptors (BCRs) or secreted into the extracellular space as antibodies where they bind to their cognate antigen to neutralize it (117). Just like the TCR, BCRs/antibodies are specific for one certain antigen and all B cell clones only produce one kind of antibody (117).

There are five different antibody isotypes – IgM, IgD, IgG, IgA and IgE (117). Besides these, there are also 4 subclasses of IgG, named IgG1, IgG2, IgG3 and IgG4 (117). An antibody consists of two heavy chains and two light chains, where the N-terminus regions of the chains make up the antigen-binding site. The constant portion of the antibody, the Fc region, mediates the effector functions of the antibody (117).

There are three ways for antibodies to exert their function. The first one is through neutralization, they bind to the target, for example a virus, a prevents it from entering a host cell (117). The second mechanism is binding of the Fc part to a FcR on macrophages and other immune cells, and thereby activating it (117). This mechanism can induce phagocytosis. The third way is to activate the classic pathway of the complement system by binding to C1q (117). Which effector mechanism that occurs is decided by the antibody isotype. For example, IgM and IgG3 activate the complement system, IgG1activate macrophages, and IgE activates mast cells.

Multiple Myeloma

Multiple Myeloma (MM) is an incurable plasma cell malignancy of the BM (BM), characterised by clonal expansion of malignant plasma cells. MM is clinically characterized by the CRAB criteria – increased calcium levels, renal failure, anaemia and osteolytic bone lesions (118).

Epidemiology

MM is the most common haematological malignancy and accounts for 1 % of all cancer cases (118). In the western world, the age-standardized incidence is 5/100 000 people (118). This is in line with the incidence in Sweden, which lies around 4.7/100 000 people (119). MM is more common in older people, and the incidence is thought to increase as a result of an ageing population (119). The median age of diagnosis lies around 66-70 years of age, but 37 % of all patients are 65 years of age or younger (118). The incidence of developing MM below the age of 30 is 0.02-0.3 %, which is extremely rare (118). MM is more common in males compared to

females, and the disease is twice as common in African Americans compared to European Americans (118, 120).

The Multiple Myeloma plasma cell is a malignant post-germinal center plasma cell

The MM plasma cell is a long-lived plasma cells, meaning it has undergone the B cell maturation process and has encountered antigen, as described above. MM plasma cells produce high affinity antibodies with high levels of SHM on their heavy chains, indicating that the MM plasma cells are derived from a post-GC B cell (116) (fig. 9). MM plasma cells produce abnormal amounts of monoclonal immunoglobulins and light chain proteins (kappa or lambda) (121). IgG, IgM and IgA are the predominant immunoglobulins produced by MM plasma cells, but rare cases of IgE and IgD MM patients have occurred (121). The immunoglobulins produced by MM plasma cells are usually referred to as Myeloma proteins (M proteins). The malignant plasma cell is derived from one B cell clone, so all M proteins that are produced are against the same antigen.

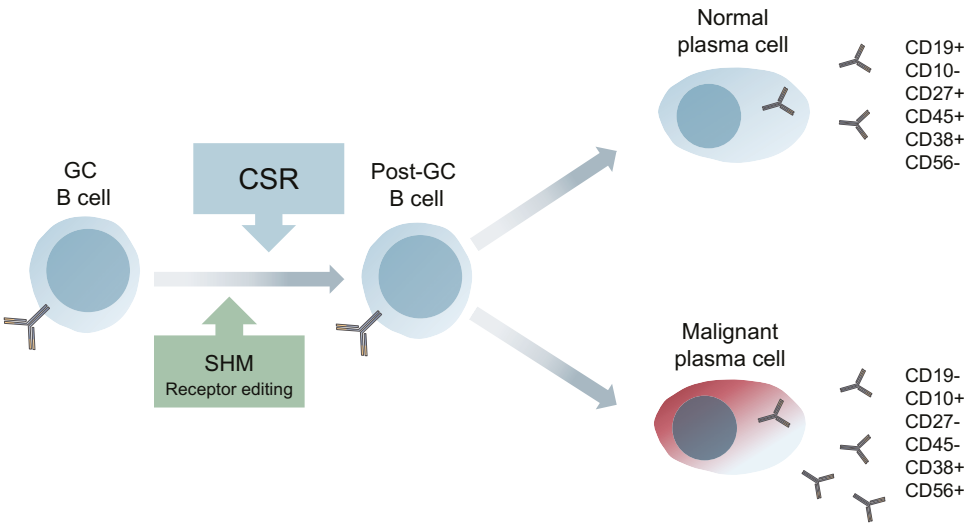


Figure 9. Both normal plasma cells and malignant plasma cells are derived from a post-germinal center (GC) B cell.
In the GC, the B cell undergo somatic hyper mutations (SHM) as well as the class switch recombination (CSR) process, enabling the cell to produce high affinity antibodies. The post-GC plasma cell will later develop into either a memory B cell or a plasma cell. The cell surface markers CD19, CD10, CD27, CD38, CD45 and CD56 can be used in order to distinguish between normal and malignant plasma cells. The picture is modified from an original picture by Furukawa et al. (122)

Malignant plasma cells express unique immunophenotypes, that differ from person to person. Healthy plasma cells and MM plasma cells are usually distinguished by their expression of cell surface markers. Healthy BM plasma cells are $CD38^{+}CD138^{+}CD19^{+}CD45^{+}CD56^{-}CD10^{-}CD27^{+}$ cells, and while MGUS, SMM and MM plasma cells also are $CD38^{+}CD138^{+}$ only 10% of the patients have plasma cells that are $CD19^{+}$, 1 % $CD45^{+}$ and 30 % $CD56^{-}$ (122-124) (fig. 9).

Multiple myeloma is preceded by a premalignant disorder

MM is preceded by a premalignant condition called MGUS (125, 126). MGUS is associated with a life-long risk of developing MM and each year 1 % of the patients with MGUS will develop MM (116, 125, 126). MGUS is present in around 2 % of the general population aged 50-70 years old, and in 5 % of the population aged 70 years or older (127). Smoldering multiple myeloma (SMM) is also preceding MM but is a part of the MM diagnosis. Just like MGUS, SMM patients do not have any clinical symptoms but have an overproduction of immunoglobulins from monoclonal plasma cells (128). Each year, 10 % of SMM patients progress into MM (116, 125, 126).

MGUS, SMM and MM are all diagnosed based on the levels of M proteins, BM plasma cells and the presence of end organ damages (fig. 10) (128). A patient is diagnosed with MGUS if the levels of M proteins in serum are < 3 g/dl and the BM plasma cells are < 10 % (128). If the M protein level is > 3 g/dl and the BM plasma cell level is > 10 %, the patient is diagnosed with SMM (128). If a patient has increased levels of M protein and increased BM plasma cell levels in combination with end organ damages, the patient is diagnosed with MM and treatment will start (128).

MM can evolve into extramedullary MM (EMM), which is a more aggressive form om MM (123). Almost all human MM cell lines have been generated from EMM tumors (123).

M – protein < 3 g/dl	M – protein > 3 g/dl	M – protein Present in blood or urine
BM clonal plasma cells <10 %	BM clonal plasma cells >10 %	BM clonal plasma cells >10 %
No end organ damages	No end organ damages	Presence of end organ damages

Figure 10. Diagnostic criteria for MGUS, SMM and MM.
MGUS, SMM and MM are different spectrums of the same disease, where SMM is the stage between MGUS and MM. MGUS has the lowest tumor burden of the three, while MM has the highest. They are all diagnosed based on the level of M protein, BM plasma cells and the presence of end organ damages.

The BM microenvironment in Multiple Myeloma

The BM microenvironment is essential for the survival of both healthy and malignant plasma cells. Malignant plasma cells rarely metastasize outside of the BM to form EMM, indicating the importance of the BM niche for its survival (129, 130). The BM microenvironment consists of two compartments – the cellular compartment and the non-cellular compartment (129). The cellular compartment is composed of several different cell types, for example BM stromal cells (BMSC), DCs, macrophages, monocytes, osteoclasts and osteoblasts (129). The non-cellular compartment consists of the extracellular matrix (ECM), the oxygen concentration, as well as cytokines, growth factors and chemokines (129). All compartments of the BM microenvironment interact with each other and promotes several different biological processes throughout the BM (129).

Plasma cells are attracted to its niche by a gradient of CXCL12, produced by for example the BMSCs, which binds to CXCR4 on the surface of the plasma cells (131). The BMSC and the ECM anchor the plasma cells to the niche (132) (131). Many immune cells, such as megakaryocytes, eosinophils, macrophages, and DCs, and neutrophils, express CXCR4 and can therefore co-localize with the plasma cells in the BM niche (131). The cells of the cellular compartment produce different

factors that are essential for the survival of plasma cells, such as APRIL, BAFF and IL-6 (131). APRIL and BAFF protect myeloma cells from apoptosis (133), while IL-6 stimulate plasma cell proliferation (134).

Tumour cells are capable of escaping immune surveillance by altering the immune defence (23). An example of this is that T cells from MM patients show signs of exhaustion and senescence, and T cells from the tumour microenvironment of MM patients lacks reactivity towards autologous tumour cells (135, 136). Interestingly, MGUS patients have T cells with prominent responses against their own tumour cells (137), indicating that the T cells from the microenvironment in MGUS patients has not been fully affected by the presence of the tumour cells. The exact mechanisms behind the reduced capacity of T cells remains unknown and is probably multifactorial. One mechanism could be through PD-L1 and PD-1 interactions. MM plasma cells express PD-L1, something healthy donor plasma cells do not (138). In combination with this is PD-1 upregulated on T cells from MM patients (139). This upregulation of PD-L1 on MM T cells could be one of several mechanisms behind the lowered anti-tumour response in MM. Another mechanism that potentially play a part in T cell inhibition, is inhibition by MDSC. MDSC in MM have high levels of PD-L1, that potentially binds to PD-1 on T cells (140).

MDSC levels have been reported to be increased in the BM of MM patients and MM bearing animals (88, 89, 141-143) and there they seem to inhibit T cell responses (142, 144, 145). BM PMN-MDSC seem to inhibit by producing ROS as well as Arg-1 (87, 88). Contradictory results regarding MDSC impact on MM tumour growth have been reported (88, 142, 144). Studies have, however, indicated that removal of $CD11b^+$ cells from the BM of tumour bearing mice significantly reduced MM tumour growth and improved the response to chemotherapy, while increased levels of $CD11b^+$ cells due to GM-CSF administration led to decreased survival and decreased sensitivity to chemotherapy (141, 146). The tumour microenvironment is important for the survival of plasma cells even during treatment. Both PMN-MDSC and neutrophils can protect MM plasma cells from chemotherapy, and the chemoprotective effect does not require cell-cell contact (141). Besides their role in the microenvironment, a high neutrophil-lymphocyte ratio is associated with a poor prognosis (147).

Besides their role in the BM microenvironment, an increase of PMN-MDSC in peripheral blood have been reported to correlate with disease progression and promote resistance to therapy (87, 143, 144). Peripheral blood NDG from MM patients have an altered gene expression profile compared to healthy NDG and seem to inhibit T cell proliferation through Arg-1 (85).

The tumour microenvironment is full of interactions between the cellular and non-cellular compartments. Once a tumour has been established, they work together in

a pro-tumour fashion. The role of neutrophils and MDSC within this environment has just started to be investigated and remains to be fully discovered.

Treatments

The median overall survival of MM patients has increased during the past decades, from 1-2 years to 7-8 years, due to the development of new pharmaceuticals (121). When deciding on a treatment, several different parameters are considered, including stage of the disease, genetic profile, age, and overall health of the patient (121). There are five main classes of MM treatments, which often are used in combination with one another (121). These are corticosteroids, chemotherapy, proteasome inhibitors, immunomodulatory drugs (IMiDs), and monoclonal antibodies (121). Younger patients (below 70 years of age) with no severe co-morbidity are eligible for an autologous stem cell transplantation (148). Furthermore, the most recently emerging therapy is a T-cell dependent treatment where chimeric antigen receptor (CAR) T cells or bi-specific antibodies (binding both T cells and tumor cells) are used with promising results (149).

Corticosteroids

Corticosteroids are often used in combination with other treatments to treat MM (121). Corticosteroids, such as dexamethasone and prednisone, are thought to induce apoptosis of the malignant plasma cells through several different mechanisms (150).

Chemotherapy

Melphalan and cyclophosphamide are two examples of chemotherapy that are used to treat MM. Melphalan in combination with the corticosteroid prednisone has been used as a standard therapy since the 60s but has recently been changed to more effective therapies (151). It is still in use before autologous stem cell transplantation.

Proteasome inhibitors

Proteasome inhibitors, such as bortezomib, are used as first-line treatments in combinations with IMiDs, corticosteroids and therapeutic antibodies. The proteasome is a multienzyme complex which provides a pathway for degradation of intracellular proteins that has been targeted for destruction through ubiquitination. The proteasome maintains the balance of protein homeostasis within the cell and degrades misfolded or cytotoxic proteins. Inhibition of the proteasome leads to the accumulation of misfolded or unfolded proteins within the cell, and this cause the activation of the protective unfolded protein response that leads to growth arrest and apoptosis (121, 132).

Immunomodulatory drugs

Lenalidomide and thalidomide are two examples of IMiDs (152). IMiDs are all small molecules with similar chemical structures but they differ in pharmacological properties, such as half-life, metabolism, clearance, and side-effect profiles (152). IMiDs affect the very complex relationship between the MM plasma cells and its microenvironment and can turn the cellular compartment from pro-tumour into anti-tumour. They can, for example, inhibit angiogenesis (153, 154) and inhibit the adhesion of BMSC to malignant plasma cells, which leads to decreased IL-6 production in BMSC (155). Treatment with IMiDs also lead to increased apoptosis of MM plasma cells, and surrounding cells produce less APRIL, BAFF and IL-6 (152).

One known mechanism for the anti-myeloma effect of lenalidomide, is the selective ubiquitination and subsequent degradation of Ikaros (IKZF1) and Aiolos (IKZF3), two essential transcription factors in MM (156). The degradation is performed by the CRBN-CRL4 ubiquitin ligase (156). Lenalidomide treatment stimulates T-cell proliferation through TCR signalling and increase the production of IL-2 and IFN-γ production (140). It is the depletion of IKZF1 and IKZF3 that lead to lenalidomide-induced IL-2 production in T-cells (156). IL-2 is important for T-cell responses as it stimulates proliferation and Th differentiation (157). Interestingly, Lenalidomide treatment increases myeloma-specific T-cell responses as well as overall T-cell function (158). A single amino acid substitution in IKZF3 conferred resistance to lenalidomide-induced degradation and rescued Lenalidomide-induced inhibition of cell growth (156).

Lenalidomide is a treatment with many immunomodulatory functions, and it is still uncertain how and in which ways IMiDs affect all immune cells. More research is needed to unravel all the possible mechanisms that are behind the good effect of this treatment.

Monoclonal antibodies

Today, monoclonal antibodies are part of the initial treatment of MM. The first monoclonal antibody for MM treatment was daratumumab, a monoclonal antibody that target CD38 (159). CD38 is highly expressed on MM plasma cells, as well as on normal plasma cells, but it is not highly expressed on other cell types making it an appealing target (160, 161). The monoclonal antibodies bind to CD38 and induce killing of the cells through several different mechanisms (162). The killing-mechanisms include complement-dependent cytotoxicity by binding of the antibody to C1q, antibody-dependent phagocytosis, antibody-dependent cellular toxicity, tumour cell apoptosis and modulation of enzymatic activity (162). It also decreases the immunosuppressive activity of Tregs (161). Isatuximab is another antibody against CD38 that is commonly used when treating MM (163). The two antibodies target different epitopes of CD38 and their tumour-killing mechanisms differ slightly (161). Unlike daratumumab, isatuximab can induce apoptosis in MM

plasma cells directly, without having to cross-link to other cells or the complement system (161).

Bispecific antibodies

Bispecific antibodies, which are antibodies with two binding sites directed at two different antigens or epitopes, are one of the recently emerging therapies in MM (149, 164). Teclistamab is a bispecific antibody that targets CD3 on T cells and B cell maturation antigen (BCMA), which is overexpressed on MM plasma cells (165). It redirects the $CD3^+$ T cells to the BCMA expressing plasma cells to induce cytotoxicity and tumour cell death, mediated by the release of perforin and granzyme from CTLs (165). Teclistamab was recently granted conditional approval in EU for the treatment of adult patient with relapsed and refractory MM who have received three or more prior therapies but still have disease progression (165).

CAR T cells

Another therapy that also induces killing of malignant cells by T-cells, are the CAR T cells (166). CAR T cells have the cell killing capacity of CTLs, but have been engineered so that they possess highly specific receptors for their target antigen (166). CAR T cell production can take up to 6 weeks and require isolation of T cells from patient blood. The isolated T cells are then activated and transduced with CAR via viral vectors (166). The CAR transduced T cells are then expanded, after which they are infused into the patient (166). CAR T cells have recently been approved for the treatment of MM (166).

Aims

This thesis evaluates aspects of neutrophil and MDSC immune regulatory functions in the setting of the hematological malignancy MM. We further evaluate if MM patients, who have a 7-fold increased risk for infections, have neutrophils with impaired anti-microbial effector functions. The aims of this thesis were to:

- Investigate the levels and function of MDSC in MM and the premalignant condition MGUS
- Investigate the neutrophil induced T cell inhibition in the blood and BM of healthy donors and MM patients
- Further investigate the mechanism behind the neutrophil suppressive ability and how their presence affects T cells, beyond inhibiting proliferation
- Investigate if neutrophils in MM patients have an impaired phagocytic ability as well as an impaired ability to perform oxidative burst, and what effect lenalidomide treatment has on these neutrophil effector functions

Methods

In paper I, II and III, we use a T cell proliferation assay, in order to evaluate the suppressive effect of MDSC and neutrophils on T cell proliferation. An overview of this method can be viewed in Figure 11 on the next page.

Isolation of T cells

The first step of the T cell proliferation assay is to isolate T cells. The T cells are isolated from healthy donor blood, whereas the MDSC or neutrophils are isolated from another individual. We have performed this assay comparing autologous and allogenic settings of the co-culture, using healthy donors, and have not observed any differences on the outcome of the assay using allogenic co-cultures. T cells derived from MM patients already show signs of exhaustion (135), which could affect the outcome of the proliferation assay if patient samples were used.

To isolate the T-cells, we performed a density gradient centrifugation (Lymphoprep) to isolate the mononuclear cells, followed by magnetic isolation that uses negative selection (Stemcell technologies). An antibody cocktail, with antibodies against all cell types except T-cells, are added to the sample and then incubated with magnetic beads that bind to the antibodies. The sample is then put in a magnet, where all antibody bound cells will be attached to the magnet. The T-cells are not bound and can be poured of, yielding a very pure sample (>97 % purity). We have also tried other T-cell isolation kits, using positive selection, but the result was not as pure (~95 %). Magnetic isolation using positive selection means that the isolated cells have been isolated by antibody-binding to their surface, while negative selection leaves the cells untouched. Antibody-binding to the cell surface could possibly affect the function of the cell and influence downstream assays. The method we use today is trustworthy since it constantly yields a pure result and provides untouched T cells.

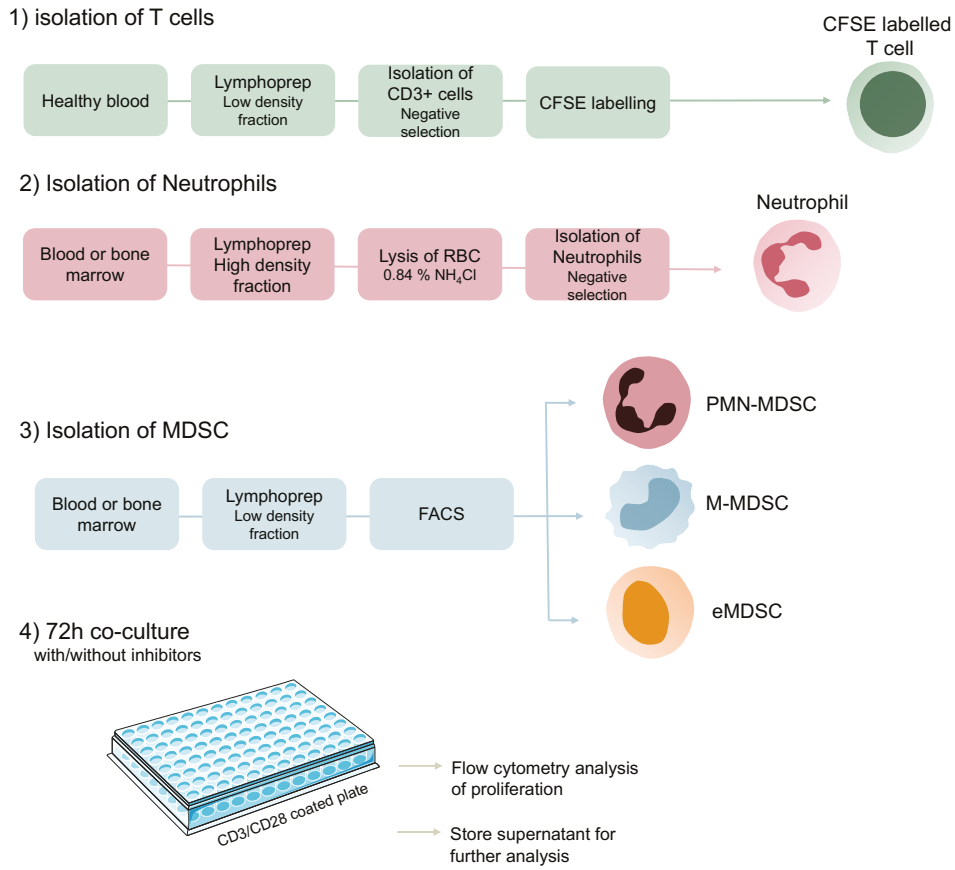


Figure 11. Overview of the T cell proliferation assay

T cells were isolated from healthy donors by a density gradient centrifugation (Lymphoprep), followed my magnetic isolation of CD3+ cells using negative selection. T cells were then stained with the cell staining dye CFSE to track proliferation. Neutrophils were isolated from either blood or bone marrow by first a density gradient centrifugation, to get rid of any possibly contaminating MDSC. The red blood cells were then lysed using NH_4Cl, whereupon neutrophils were isolated by magnetic isolation using a Neutrophil kit (StemCell technologies). MDSC were isolated from the mononuclear cell layer through fluorescence activated cell sorting (FACS) after density gradient centrifugation. T-cells (100 000 cells) were cultured with either MDSC or neutrophils (50 000) for 3 days after which proliferation was measured using flow cytometry. Supernatant was stored for further analysis.

The isolated cells were then stained with carboxyfluorescein succinimidyl ester (CFSE), which is a cell staining dye that binds to intracellular molecules, making it possible to track proliferation. There are other methods besides CFSE staining that could be used to track proliferation, for example ^{3}H-thymidine incorporation or Ki-67 expression, or the use of other analogues of CFSE. ^{3}H-thymidine incorporation is a strategy where radioactive ^{3}H-thymidine is incorporated into new strands of chromosomal DNA during cell division. Ki-67 is a proliferation marker that is active

during the active phases of the cell cycle but absent during the resting phase. We have used CFSE since it is a cost-effective and simple method. Ki-67 is more expensive but provides increased sensitivity (167) and ^{3}H-thymidine incorporation does not seem to bring any advantages compared to the other two (167). However, CFSE is toxic in high concentrations, and we learned the importance of titrating CFSE the hard way. Apparently, different batches differ in their potency, and when changing to a new batch with a slightly higher potency than the first batch – the T cells died from the CFSE staining and the proliferation assay did not work properly. According to manufacturer's instructions, the CFSE concentration for staining should be between 1-10 μM. We titrated the concentration, investigated the T-cells ability to proliferate after three days and investigated their morphology by light microscopy. We found that high concentrations (>5 μM) killed some of the cells, while the lowest concentration (1 μM) did not have an effect on T-cell proliferation or morphology. Since CFSE staining causes spill-over into many channels, it is not possible to do a viability assay using flow cytometry.

Isolation of neutrophils and MDSC subsets

The most common isolation method in MDSC research is FACS. Two advantages of FACS sorting are the ability to isolate high purity populations and the capability of measuring several parameters at once. In the beginning of MDSC research, there was no consensus on how to gate and isolate the different MDSC subsets, making it impossible to compare findings between groups. In 2016, Bronte et. al. published a paper providing recommendations for how to study human and mice MDSC (26). It contained guidelines for which markers to use, how to gate the cells, as well as which functional tests to use. To build on this, their research group has, together with 12 other groups, formed a network called Mye-EUNITER (www.mye-euniter.eu) which performed an extensive study with the goal to standardize and facilitate the comparative analysis of human circulating MDSC in cancer, inflammation, and infection (65). The old gating strategy from 2016 was changed for a new one, published in 2020 (65). When starting this project, we tried to make a protocol that followed the pre-existing guidelines. Several different antibody panels have been used, with several different markers. We have changed fluorochromes and antibodies, leading to the 11-color panel we are using today (described in paper I). There are several markers that are necessary to have in the panel in order to isolate all three MDSC subsets at once. We had to use the same fluorochrome for the Lineage markers (CD3, CD19, CD56) if we wanted to be able to investigate all three subsets simultaneously, while investigating if LOX-1 is a specific PMN-MDSC marker. The gating strategy used in paper I is a combination of the guidelines published by Bronte et. al. 2016 (26) and knowledge gained during a lecture by Sven Brandau (Action chair at Mye-EUNITER) at the 1st European Symposium on Myeloid Regulatory Cells in Health and Disease (ESMRC) held in

Essen, Germany 2018. When analysing and overlapping the PMN-MDSCs found by gating according to the 2016 paper, the 2020 paper and the PMN-MDSCs in paper I, it was found to be the same cellular subset. This makes it possible to compare our data with other published results, where a similar gating strategy has been used.

Another method that can be used, instead of FACS, is magnetic bead isolation. This has been used to isolate LDG in systemic lupus erythematosus (SLE) (56). After the density gradient centrifugation, to gain the low-density cells, the cells are isolated with a neutrophil kit that uses negative selection (Stemcell technologies). Three advantages of this method compared to FACS is that the method is fast and easy, and the cells will be untouched by antibodies. FACS sorted cells are not untouched, as several antibodies have been bound to their cell surface. This could affect the function of the cells and how they respond to other stimuli, possibly affecting the T-cell proliferation assay. There have been reports suggesting that neutrophils lose their ability to produce ROS and inhibit T-cell proliferation after FACS sorting (55). However, our comparison between FACS sorted neutrophils and magnetically isolated neutrophils did not show any difference in T-cell inhibitory effect, suggesting that FACS sorting (in our hands) do not affect neutrophil function. Since PMN-MDSC and neutrophils are similar, we concluded that PMN-MDSC are not affected by the sorting. We have also tried to isolate PMN-MDSC by magnetic isolation, but this approach did not yield enough cells to perform any downstream experiments. A disadvantage with FACS sorting is that rare populations are difficult to sort, as the sorting efficacy becomes low, leading to cell loss (168). This could possibly affect the sorting of MDSC, as they are rare. However, we have not managed to isolate the rare subsets using magnetic isolation either, suggesting that FACS has been the best option for our experiments. Another disadvantage of magnetic isolation is that it cannot be used for the isolation of M-MDSC and eMDSC.

Since magnetic isolation is faster, cheaper, and easier, we chose to isolate our neutrophils using this method. Prior to magnetic isolation, we performed both Lymphoprep and RBC lysis. Many researchers do not perform the magnetic isolation of neutrophils after the RBC lysis (104), as the high-density layer mostly contain neutrophils. There are, however, other granulocytes in this layer as well, including eosinophils and basophils. The levels of these, compared to neutrophils, are relatively low and may not affect the outcome of the study. The choice of RBC lysis method can affect the viability and integrity of neutrophils, and the two gentlest methods seem to be hypotonic shock or NH_4Cl (169).

Co-culture

The methods used for investigating the suppressive abilities of neutrophils, MDSC and monocytes affect the outcome of the study. To stimulate the T-cells, several

different methods can be used. The two most common ones in MDSC research are dynabeads coated with CD3/CD28 or plate-bound CD3/CD28. The beads can be phagocytosed by cells that have phagocytic ability and neutrophils can cleave antibodies on their surface, leading to a false inhibitory effect as the T cell stimuli is lost (99, 170). Plate bound CD3/CD28 removes this artefact and is the preferred method for these kinds of assays (99, 170). Dynabeads could be used when studying soluble mediators instead of cell-cell interactions (56).

There are several aspects of a method that can influence the outcome. All methods discussed have both pros and cons. Since neutrophils are sensitive cells, we have tried to be as gentle with them as possible.

Results

Study I: Myeloid-derived suppressor cells and their role in Multiple Myeloma

Background and aim

MDSC are known for their suppressive abilities against T cells. They are often increased in the blood of cancer patients and increased levels have been shown to correlate with disease severity in MM, as well as in other cancer types. There are three different MDSC subsets, M-MDSC, PMN-MDSC and eMDSC.

The neutrophil-like PMN-MDSC subset is suggested to be different from ordinary neutrophils by their ability to inhibit T cell responses, and by their low density. PMN-MDSC are found in the low-density fraction after a density gradient centrifugation, while neutrophils have a normal density. Neutrophils are therefore referred to as NDG.

In this study, we aimed to investigate if MDSC levels were increased in the blood and BM of newly diagnosed MGUS and MM patients, compared to healthy donors. We also aimed to investigate the immunosuppressive function of the MDSC subsets by performing a T cell proliferation assay. The inhibitory ability of PMN-MDSC was compared to the inhibitory ability of NDG.

Method

We obtained blood and BM from 7 patients diagnosed with MGUS, 11 patients diagnosed with MM, and from 12 healthy controls. The samples were collected from the patients at the time of diagnosis. Therefore, they had no ongoing treatments for their disease.

The three different MDSC subsets were isolated from blood and BM using FACS. NDG were isolated using magnetic separation. PMN-MDSC, M-MDSC, eMDSC or NDG were cultured together with T cells in an allogenic co-culture. In some experiments, the neutrophil activator fMLF or the ROS inhibitor catalase were added. Proliferation was measured after 3 days. An overview of the method can be viewed in Figure 11.

Key findings

PMN-MDSC, M-MDSC and eMDSC levels were not increased in the BM of MGUS or MM patients, nor increased in the peripheral blood. However, eMDSC levels were decreased in the peripheral blood of MM patients compared to healthy donors.

The MDSC subsets were not very strong inhibitors. In fact, M-MDSC increased the proliferation of T cells. PMN-MDSC had a minor inhibitory effect and were interestingly not as suppressive as NDG. The neutrophil activator fMLF increased the inhibitory effect of blood PMN-MDSC and the overall suppressive effect of PMN-MDSC may be ROS mediated.

Conclusion

This study showed that MDSC levels were not increased in the peripheral blood or BM of newly diagnosed MGUS and MM patients, compared to healthy donors. According to the algorithm for MDSC research (fig. 4), this would indicate that the isolated cells cannot be categorized as MDSC. M-MDSC did not suppress T cell proliferation, instead the proliferation increased. It is possible that the M-MDSC has not yet been formed during the early stages of MM, and that we are observing the activating effect of monocytes on T cell proliferation. Suppressive abilities have been suggested to correlate with disease severity, which could explain the low inhibitory effects of eMDSC and PMN-MDSC in this study. Interestingly, both blood and BM PMN-MDSC were not as suppressive as the NDGs from the same donor.

Study II: BM neutrophils of multiple myeloma patients exhibit myeloid-derived suppressor cell activity

Background and aim

This study was born from the findings in paper I, where we found that NDG were more inhibitory than PMN-MDSC, which is the proposed inhibitory subset. Indeed, research from the past years have indicated that NDG also have an inhibitory capacity, but only under certain conditions.

MM patients have a 7-fold increased risk of infections, which suggests an impaired neutrophil function. In this study, we investigated, for the first time, the T cell suppressive effect of MM NDG from the BM. We also investigated blood NDG and aimed to unravel the mechanism used for suppression.

Method

In this study, NDG from 21 MM patients were investigated. 16 of the patients were newly diagnosed and untreated, whereas 5 patients were previously diagnosed and treated for the disease.

NDG were isolated from the blood and BM by a density gradient centrifugation followed by magnetic isolation. The NDG were then used in a T cell proliferation assay together with healthy donor T cells. For a more detailed overview of the T cell proliferation assay, see fig. 11. IFN-γ levels in the supernatant were measured through an enzyme-linked immunosorbent assay (ELISA).

Besides the proliferation assay, the levels of mature, immature, and immature $CD11b^-$ neutrophils were evaluated. Blood and BM were stained with a neutrophil antibody panel and evaluated using flow cytometry.

Results

NDG from the peripheral blood of MM patients have the ability to inhibit both T cell proliferation and T cell IFN-γ production. NDG from healthy donors shared these abilities and the inhibition was dose-dependent. The inhibitory effect was mediated through the production of ROS, and not through the production of Arg-1.

NDG from the BM of MM patients also inhibited both T cell proliferation and T cell IFN-γ production. However, healthy donor NDG did not inhibit T cell proliferation, only the production of IFN-γ. Next, we wanted to investigate if an increased level of immature neutrophils were responsible for the observed suppression. However, we did not find any differences in the levels of mature, immature, or immature $CD11b^-$ cells in the blood or BM of healthy donors compared to MM patients. These data indicate that the suppressive ability of MM BM neutrophils is not due to a skewed ratio of mature/immature cells.

The NDG did not have to be pre-activated to inhibit proliferation. Instead, they seemed to become activated within the first few hours of co-culture as the neutrophil activation markers CD11b and CD66b increased during this period.

Conclusion

This study showed that blood NDG inhibit T cell proliferation and IFN-γ production, and thereby exhibit MDSC abilities. The NDG did not have to be pre-activated to inhibit the T cells, but activation with fMLF increased the suppression. The inhibition was mediated by the production of ROS, and Arg-1 was not important for the inhibitory effect. There was no difference in suppressive abilities between healthy donor and MM blood NDG.

BM NDG from healthy donors, on the other hand, did not suppress T cell proliferation. They did, however, suppress IFN-γ production. BM NDG from MM patients suppressed both, indicating a more suppressive phenotype compared to healthy donor BM NDG.

Taken together, these findings indicate that healthy donor NDG have immunoregulatory abilities that needs to be further investigated. We also show that BM neutrophils from MM patients are more suppressive than BM NDG from healthy donors.

Study III: Suppression of T cell proliferation by normal density granulocytes led to CD183 downregulation and cytokine inhibition in T cells

Background and aim

This article builds on the findings from paper II where we show that blood neutrophils can inhibit T cell proliferation, as well as IFN-γ production, and that the suppressive effect is mediated by ROS. Besides not being able to proliferate, little is known about what happens to the T cells when they encounter neutrophils. In this study, we investigated the effect NDG have on different T cell subsets, mainly the T helper subsets Th1, Th2 and Th17, by investigating cell surface markers and cytokine production. We also further evaluated the mechanisms behind the suppression.

Method

In this study, blood from 19 healthy donors were collected. NDG and T cells were isolated from the blood, as described in fig. 11, and used in allogenic co-cultures measuring T cell proliferation. To further evaluate the mechanisms behind suppression, ROS, Arg-1, TGF-β and CD11b was inhibited. In some experiments, NDG were activated with fMLF.

To evaluate how rapid NDG inhibit T cell proliferation occur, as well as how NDG effect different T cell subsets, time experiments were performed. T cell phenotype and viability was measured at several time points between 0.5-72h.

On the supernatant from the 3-day cultures, the levels of different Th1, Th2 and Th17 cytokines were measured by performing a cytometric bead array (CBA).

Key findings

As shown in paper I, blood neutrophils from healthy donors inhibit T cell proliferation by producing ROS. The inhibition is contact dependent, as blocking of CD11b restored the T cells ability to proliferate. T cell inhibition occurs within the first few hours of co-culture, and Arg-1 and TGF-β were not important for the inhibition.

Since we previously have seen a decrease of IFN-γ in co-cultures, and IFN-γ mainly is produced by CTLs and Th1 cells, we hypothesised that NDG have different effects on different T cell subsets. Firstly, we evaluated the levels of $CD4^+$ T cells, $CD8^+$ T cells, Th1 cells, Th2 cells and Th17 cells within the first 5 hours of co-culture. We did not see any difference in the levels of $CD4^+$ T cells or $CD8^+$ T cells between cultures of only T cells, and cultures with T cells in combination with NDG. However, we did observe a decrease of Th1 cells and an increase of Th2 and Th17 cells. The expression of CD183, a Th1 cell surface marker, was completely lost on T cells that had been in contact with NDG. In order to evaluate if the presence of NDG promotes the formation of Th2 and Th17 subsets and inhibits the Th1 subset, we measured seven different Th1, Th2 and Th17 specific cytokines. We found that all seven cytokines were inhibited in the presence of NDG. Taken together, these data indicate that the presence of NDG leads to downregulation of CD183 on T cells, and that they inhibit cytokine production in all Th subsets investigated.

CD183, also known as CXCR3, has 4 known ligands – CXCL4 (PF4), CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC). When a ligand binds to CD183, it is removed from the cell surface. We wanted to investigate if the observed loss of CD183 was dependent on ligand binding. However, blocking the ligands did not protect CD183 expression on the T cells, indicating that the NDG-induced downregulation of CD183 is not mediated by ligand binding.

Conclusion

Blood neutrophils inhibit T cell proliferation by producing ROS and the inhibition is cell-cell contact dependent. The inhibition of the T cells is rapid and occurs within the first few hours of interaction.

NDG downregulate CD183 expression on T cells, which could potentially lead to the misinterpretation that NDG inhibits Th1 cells while promoting the formation of Th2 and Th17 cells. However, it is important to note that cytokine production is also inhibited in all Th subsets, indicating a more general effect on T cell activation and function rather than a specific effect on Th1 cells. The CD183 loss is not mediated by ligand-binding.

This study provides new insights into how healthy donor NDG inhibit T cells. Their immune suppressive role seems to be more diverse than just having an inhibitory effect on T cell proliferation.

Study IV: Newly diagnosed multiple myeloma patients have decreased neutrophil functions in blood and BM that is restored with lenalidomide therapy

Background and aim

Immune dysfunction and reduced levels of polyclonal antibodies are a characteristic feature of MM, leading to an increased risk of recurrent infections that are a major cause of morbidity and mortality. MM patients have a 7-fold risk of developing bacterial infections, and since neutrophils are the most important cell type for fighting bacteria, we hypothesised that neutrophils have impaired effector functions. The processes of phagocytosis and oxidative burst are two of the effector functions that are important for clearance of bacteria.

Lenalidomide has a well-established immunomodulatory effect when treating MM. However, its impact on neutrophil function and specifically phagocytosis is not yet fully established.

In this study, we investigated the phagocytic ability as well as the PMNs ability to perform oxidative burst, in both newly diagnosed patients who suffer from MGUS, SMM and MM, as well as in lenalidomide treated patients.

Method

In this study, blood and BM from 21 MGUS patients, 8 SMM patients, 19 newly diagnosed and untreated MM patients, 12 MM patients with lenalidomide treatment, and 28 healthy donors, was collected.

The neutrophil effector functions of phagocytosis and oxidative burst was evaluated, in both blood and BM. To measure phagocytosis, the neutrophils (PMN) were allowed to phagocytose fluorescently labelled opsonized *E. coli*. The level of phagocytosis was quantified with flow cytometry. For oxidative burst, the neutrophils (PMN) were stimulated with either opsonized *E. coli*, or PMA. Oxidative burst was measured using flow cytometry and quantified by the conversion of DHR-123 into R-123. DHR-123 is non-fluorescent until oxidised into R-123 by ROS.

The levels of mature neutrophils, immature neutrophils, and immature $CD11b^-$ neutrophils were evaluated in the blood and BM using flow cytometry.

Key findings

Newly diagnosed patients with either MGUS, SMM or MM, have PMNs with a reduced ability to perform phagocytosis and produce ROS. However, patients with MM that are in remission and treated with lenalidomide have PMN with restored phagocytic function.

Similar findings were observed when measuring oxidative burst. The newly diagnosed patient group exhibited a lower potential of producing ROS. So did the PMN from lenalidomide treated patients, except for BM PMN stimulated with PMA, which also had a lower potential of producing ROS.

Conclusion

These findings demonstrate that PMN in newly diagnosed patients with MGUS, SMM and MM have a decreased phagocytic ability as well as a decreased ability to produce ROS. Patients treated with lenalidomide have PMN with restored phagocytic function and an increased ability to perform oxidative burst.

Lenalidomide is known for its immunomodulatory effects, but its impact on neutrophil function has been unknown. In this study, we show that lenalidomide restores PMN function in MM patients, measured by their ability to perform phagocytosis and oxidative burst.

Discussion

Neutrophils have, for a long time, been viewed as a cell with only one function – to seek and kill invading pathogens. In recent years, it has become evident that their abilities reach further than that – they have immune regulatory functions as well. The immune regulatory ability of neutrophils was first described in PMN-MDSC, a neutrophil-like subset that differ from ordinary neutrophils by its low density and its ability to inhibit T cell responses. However, in recent years, the ability of ordinary neutrophils to act as T cell suppressors has started to gain interest. This thesis evaluates aspects of neutrophil immune regulatory functions as well as their antimicrobial effector functions in the setting of the hematological malignancy MM.

According to the MDSC literature, when comparing blood PMN-MDSC and neutrophils from healthy donors with the same cells from cancer patients, the PMN-MDSC from the cancer patient should have the strongest inhibitory capacity (26). Interestingly, in paper I, we show that blood neutrophils, regardless of the disease status of the donor, are more suppressive than PMN-MDSC. PMN-MDSC from cancer patients should also be more suppressive than the same subset in healthy donors (26), something we did not observe in paper I. One explanation for this could be that the cells defined as PMN-MDSC are mature neutrophils that have started to degranulate, as degranulated neutrophils seem to lack T cell inhibitory capacity (171). Granule release, more importantly the release of MPO, seem to be important for the suppressive effect of neutrophils (83). MPO converts ROS into the more stable form of hypochlorous acid, instead of the less stable H_2O_2, and MPO-deficient patients seem to lack neutrophils with T cell inhibitory functions (83).

The suppressive effect of neutrophils was further investigated in paper II and III. Several different mechanisms have been proposed for neutrophil-induced T cell inhibition. In paper II and III, we conclude that neutrophils from healthy donors suppress T cell proliferation via ROS production and that the inhibition is contact dependent, as in line with data from other groups (83). T cells and neutrophils seem to form an immunological synapse through the Mac-1 complex, in which ROS can be produced (93, 94). As shown in paper III, both ROS and cell-cell contact is necessary for the inhibition of the Th cytokines as well. These data do, however, only reflect the inhibitory mechanism used by neutrophils that are derived from healthy donors. It is possible that the microenvironment in different disease conditions alter the neutrophil function, making it possible to inhibit the T cells by other mechanisms. For example, in colon cancer, where neutrophils seem to release

MMP9 that in turn activates TGF-β that inhibit T cells (91) or in MM, where Arg-1 seem to play an important role (85).

Previous reports regarding neutrophils suppressive ability have been contradictory, either showing that circulating neutrophils cannot suppress T cells, or are suppressive but only after being pre-activated (55, 83-86), or suppressive even when not pre-activated (92, 171). Indeed, pre-activated neutrophils have an increased ability to suppress T cells. In our hands, the neutrophils seem to become activated in the co-culture when they encounter the T cells, indicated by increased levels of CD11b and CD66b on the cell surface. The findings in paper III suggest that the inhibition of T cells occurs within the first few hours of contact, as indicated by decreased levels of the T cell activation markers CD25 and CD69. To our knowledge, this aspect of neutrophil-T cell interaction has not been investigated previously. It is a rapid response that happens before any of the two cell types have had the time to die, which minimizes the concern that the observed inhibition is just an artefact of neutrophil cell death (171).

Minns et. al. have investigated if different *in vitro* methods model different *in vivo* situations (171). The method used in paper I, II and III, where activated T cells encounter neutrophils, would according to them model the interaction in lymph nodes were recently activated T cells encounter neutrophils. This type of interaction leads to T cell suppression (171), as also seen in our studies. To investigate the interaction between T cells and neutrophils in the tissue, the T cells should be pre-activated for 24h before encountering the neutrophil. This type of interaction does not lead to T cell inhibition, but instead enhances the proliferation (171). These findings further support the complexity of neutrophil-T cell interaction, suggesting that it is context dependent. Neutrophils have been shown to be present in lymph nodes, in the interfollicular zone were naïve T cells become activated (172). However, these neutrophils were distinct from circulating neutrophils as they expressed MHC-II, which suggest a T cell activation potential (172). There are two different routes that a neutrophil can take to the lymph nodes. The first one is through afferent lymphatics from the site of infection, and the second is from the circulation (173). The neutrophils that arrive from a site of inflammation are likely to be highly activated, and perhaps antigen presenting (173).

MM is associated with a 7-fold risk of bacterial infections and a 10-fold risk for viral infections (174). Within two months after diagnosis, 10 % has died from their disease and 22 % of those has died from infections (174). Due to several different factors, the immune system of MM patients has lost its proper function. Studies have shown impaired functions of several different immune cells, including for example macrophages, dendritic cells, and T cells (135, 175, 176). In paper I, II and IV, we show that neutrophils from MM patients act differently compared to healthy donor neutrophils.

T cells from MM patients display features of exhaustion and senescence, especially the T cells derived from the tumour site in the BM (135). In paper II, we show that BM neutrophils from MM patients can suppress T cell proliferation, while healthy donor BM neutrophils lack this ability. Taken together, these data may indicate that the neutrophils present in the BM inhibit T cells *in vivo*. The tumour environment found within the MM BM may promote a more suppressive neutrophil phenotype. As mentioned above, the suppressive effect of MM neutrophils from the circulation may be at least partly due to increased expression of ARG1 (85). More research is needed to truly unravel which mechanisms that are specific for BM derived neutrophils in MM, as they have the biggest capacity for T cell inhibition.

Many studies have suggested that PMN-MDSC levels are increased in MM patients (88, 141, 143, 144, 177, 178). The increased levels of immature neutrophils, that often are associated with cancer, do not have to indicate an MDSC phenotype and many of the studies that have described increased levels of MDSC have not investigated their inhibitory potential. We and others have shown that mature neutrophils have suppressive abilities. The increased levels of immature neutrophils in cancer patients could function as a source for mature neutrophils as it has been shown that immature neutrophils can mature within the circulation (179). It is also possible for immature neutrophils to mature within tumours, as PMN-MDSC that have been transferred to tumour sites have gained a more mature phenotype (95). However, the results in paper I, II, and IV do not indicate increased levels of either immature neutrophils or PMN-MDSC in MM. However, it is possible that both levels and inhibitory function increase with disease progression, as PMN-MDSC from newly diagnosed patients are not as suppressive as PMN-MDSC from patients with a progressed disease (88).

The neutrophil dysfunction observed in MM patients seem to start at an early stage, as MGUS and MM neutrophils have unique gene expression profiles compared to healthy donor neutrophils (85). For example, Arg-1 production is increased, as well as the expression of FcγRI/CD64, which suggests an activated phenotype (85). Besides that, the FcγRIIIa/CD16 is downregulated, in combination with a lowered ability to perform phagocytosis and oxidative burst (85). This lowered ability to perform phagocytosis and oxidative burst is also shown in paper IV.

An interesting finding in paper IV, is that neutrophils from patients treated with lenalidomide have an increased ability to perform both phagocytosis and oxidative burst, compared to neutrophils from newly diagnosed patients. It is well known that lenalidomide has immunomodulatory effects. For example, lenalidomide treatment seem to skew BM macrophages, which have a pro-tumour M2 phenotype, towards anti-tumour M1 macrophages (180). However, its impact on neutrophil function is not well established. The increased ability of lenalidomide treated patients to phagocytose and perform oxidative burst have been observed in other studies as well (181). However, the mechanism behind these increased abilities remains to be

unravelled. IMiDs have been suggested to play a role in cytoskeleton reorganization (182), which together with lipid remodulation of the plasma membrane, is essential for extending the cell membrane around the target during phagocytosis (183). If lenalidomide improves this function, it might explain the improved phagocytic capacity. Interestingly, both FcγRI/CD64 and FcγRIIIa/CD16 can mediate phagocytosis (184). However, newly diagnosed MGUS and MM patients have reduced phagocytic abilities, despite having an increase of FcγRI/CD64 (85). Lenalidomide treatment increases the already high levels of FcγRI/CD64 on neutrophils, and restores their phagocytic capacity (181). It is possible that FcγRI/CD64 play a role in this restored function, and so might other FcR such as FcγRIIIa/CD16.

The MM plasma cells are highly dependent on the BM microenvironment for survival and proliferation. Monocytes and macrophages produce different factors that can promote the growth and survival of the malignant plasma cells (185, 186). Studies have shown that the levels of non-classical monocytes increase with tumour load in MM patients (187) and similar findings have been seen for M-MDSC (188, 189). However, in paper I, we show that newly diagnosed MM patients do not have increased levels of M-MDSC, and the cells isolated as M-MDSC did not inhibit T cell proliferation. Instead, the proliferation increased in the presence of the isolated M-MDSC subset. The cells in paper I may be ordinary monocytes, with a slightly lower HLA-DR expression, as monocytes have been shown to stimulate T cell proliferation (190). As the disease progresses, it is possible that the levels of M-MDSC increase and that they gain suppressive abilities, but newly diagnosed patients seem to lack this MDSC subset.

The eMDSC subset is rarely investigated and its role in MM, as well as in other diseases, remains to be unravelled. In paper I we show that MM patients have decreased levels of eMDSC. A similar finding has been seen for patients with glioma as well (65). The few samples examined in paper I did not show a major decrease of T cell proliferation, and it seem like the eMDSC subset is the least inhibitory MDSC subset (65). To our knowledge, the levels of eMDSC in MM have not been investigated previously, and not its inhibitory function either. However, there is a study that suggests that eMDSC from MM patients in remission have increased levels of Arg-1, which suggests an inhibitory phenotype (191).

A loved child seems to have many names – PMN-MDSC, G-MDSC, LDG, NDG, immature neutrophil, mature neutrophil, NDN, neutrophil, TAN, N1, and N2. Are they specific subsets or just neutrophils during different conditions? When reading the current literature, recent studies imply the latter. It has been suggested that immune cells develop from hematopoietic stem cells under a phenotypic continuum, and not through a series of distinct and stable progenitor cells that have committed to a certain lineage (192), as proposed in fig. 6. Further, it has been shown that terminally differentiated neutrophils can polarize towards other cell phenotypes,

such as dendritic cells and monocytes (111-113). If a neutrophil can turn into a monocyte, it is not unbelievable that they can have different functions during different disease conditions. It is evident that neutrophils exhibit high plasticity and the ability to adapt to its surrounding signals.

Neutrophils are an important key player in our immune system. Besides its ability to kill invading pathogens, its immune regulatory role has just started to unravel. In healthy individuals, this immune regulatory role may be a break to stop the immune system from spiralling during infections and inflammations.

In cancer, with its ability to inhibit T cell responses, neutrophils may promote a pro-tumour environment, as immune evasion is an important step in tumour progression (22, 193). T cells from the BM of MM patients lack an anti-tumour response, and display features of exhaustion and senescence (135, 136). By inhibiting T cells in the BM, neutrophils may act in a pro-tumour way. T-cell inhibition is important for tumour progression, and all the new treatments of MM have a focus on improving T-cell abilities to clear away the tumour cells. It is important to know the mechanisms that are behind tumour evasion, as it could be a target for treatments in the future.

Besides having a suppressive role, neutrophils from MM patients seem to have reduced ability to perform phagocytosis and oxidative burst – two important anti-microbial mechanisms. Indeed, MM patients are at an increased risk of bacterial infections. Lenalidomide might be a good treatment option for patients who suffer from recurrent infections, as it seems to increase their antimicrobial effector functions.

The mechanisms behind neutrophil-induced T cell inhibition in the BM of MM patients' needs to be further investigated, and so does the immune regulatory role of neutrophils in both health and disease.

Acknowledgements

I want to deeply thank everyone who has helped me with this thesis and supported me throughout my PhD, without you this work would not have been possible.

First, I would like to thank **Markus,** my supervisor. Thank you for letting me into your research group and believing in me. You have given me responsibility and room to explore my own ideas, yet you have guided me when I have needed it. Your passion for helping your patients have broadened my view from cells and immunology, to see the patient's perspective.

Thomas, my co-supervisor, my deepest thanks to you for all the times you have helped me – with everything from small computer-stuff to scientific reasoning. Your great scientific knowledge has helped me to become a better scientist in all aspects. Thank you for letting me be a part of the vasculitis group as well.

Åsa J, my co-supervisor, thank you for guiding me and helping me throughout my thesis. Your knowledge of immunology, neutrophils and flow cytometry has been tremendously helpful.

Åsa P, I don't even know how to thank you properly for these years. It has been a joy working with you. Thank you for all the help with experiments and calculations (det är alltid så många nollor), and all the support. I will look back on all our late evenings with MDSC-sorting with fondness. Your help in this thesis is invaluable.

Thank you to all the past and present members of the myeloma research group. **Lena**, I miss having you around. Thank you for all the help in the lab. **Sandra**, my PhD colleague, thank you for teaching me more about Multiple Myeloma and helping me with patient data. I also want to thank you for all the cookies and great discussions.

I also want to thank all the past and present members of **the vasculitis group**, for letting me be a part of your group. Your input on my work and all the great discussions during the lab meetings has helped a lot.

A big thanks to my old office-buddies, **Sabine** and **Tobbe**. I know we're not supposed to live in the past, but sharing an office with the both of you was the most fun period of my PhD. It was, however, not just fun because of you guys – but also because of **Daniel** and his daily visits. Thank you all for the help, support, and laughs. You are all amazing friends (and scientists) and I miss having you around daily.

Since we used to share our laboratory space with the rheumatology group, I also want to thank them for their participation in my PhD. Thank you **Anki**, **Gittan**, **Robin**, **Andreas**, **Lina** (snäckan <3), and all other past and present members. Your support and presence have meant a lot to me.

Also, thanks to all my past and present **colleagues at B13** for creating a lovely working environment. An extra thanks to the **hematogenomics group** for the Tuesday seminars, the fika, and all the help in the cell culture room.

A big thank you to the students I have supervised, you have taught me a lot. **Becky**, thank you for dancing to *Think About Things* with me and for becoming a true friend.

My dear friends at BMC: **Kristus, Aalex, Vendela, Emil** and **Alexandra**. Thank you for corridor conversations, lunches, and fika-breaks. A special thanks to **Aalex** for proofreading this thesis.

Helen at the Hematology department, thank you for providing me with both patient samples and healthy donor samples. I am truly grateful for your helpful work, without you I would have had nothing to do my experiments on.

When I am at it, I also want to thank my old supervisor **Mo**. Thank you for introducing me to the lab life when I was only nineteen. You gave me all the support I needed to eventually try my own wings.

Tack till **alla mina kompisar** utanför denna vetenskapliga värld, för att ni får mig att må bra. Tack för att ni lyssnat på alla gånger jag berättat om att jag minsann har tagit ut äggstockarna på en bananfluga (coolt ju!!!!), och för att ni funnits där, stöttat och lyssnat när jag klagat på att livet som doktorand inte alltid är så lätt. Extra stort tack till **Irma** som tog sig tid till att måla den fantastiska framsidan.

Jag vill också tacka min kära familj. **Pappa**, **mamma** och **Lasse,** tack för att ni har ställt upp och hejat på mig under alla lägen. Tack för all er kärlek. Tack till mina syskon, **Sabina** och **Fabian**, för att ni alltid får mig att skratta. Tack till **mormor**, utan dig hade jag aldrig kommit in på den naturvetenskapliga banan – jag hade väl följt dina fotspår och jobbat med mode. Vem hade jag ens varit då? Tack **morfar**, **Gunilla**, **Carro**, **Jennie**, **Lisbet** och **Leif**.

Johan, min kära man, tack för att du alltid är min stöttepelare.

Vidar, du är ljuset. Att få komma hem till dig och din pappa efter en lång dag av skrivande på denna avhandling är det bästa som finns.

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