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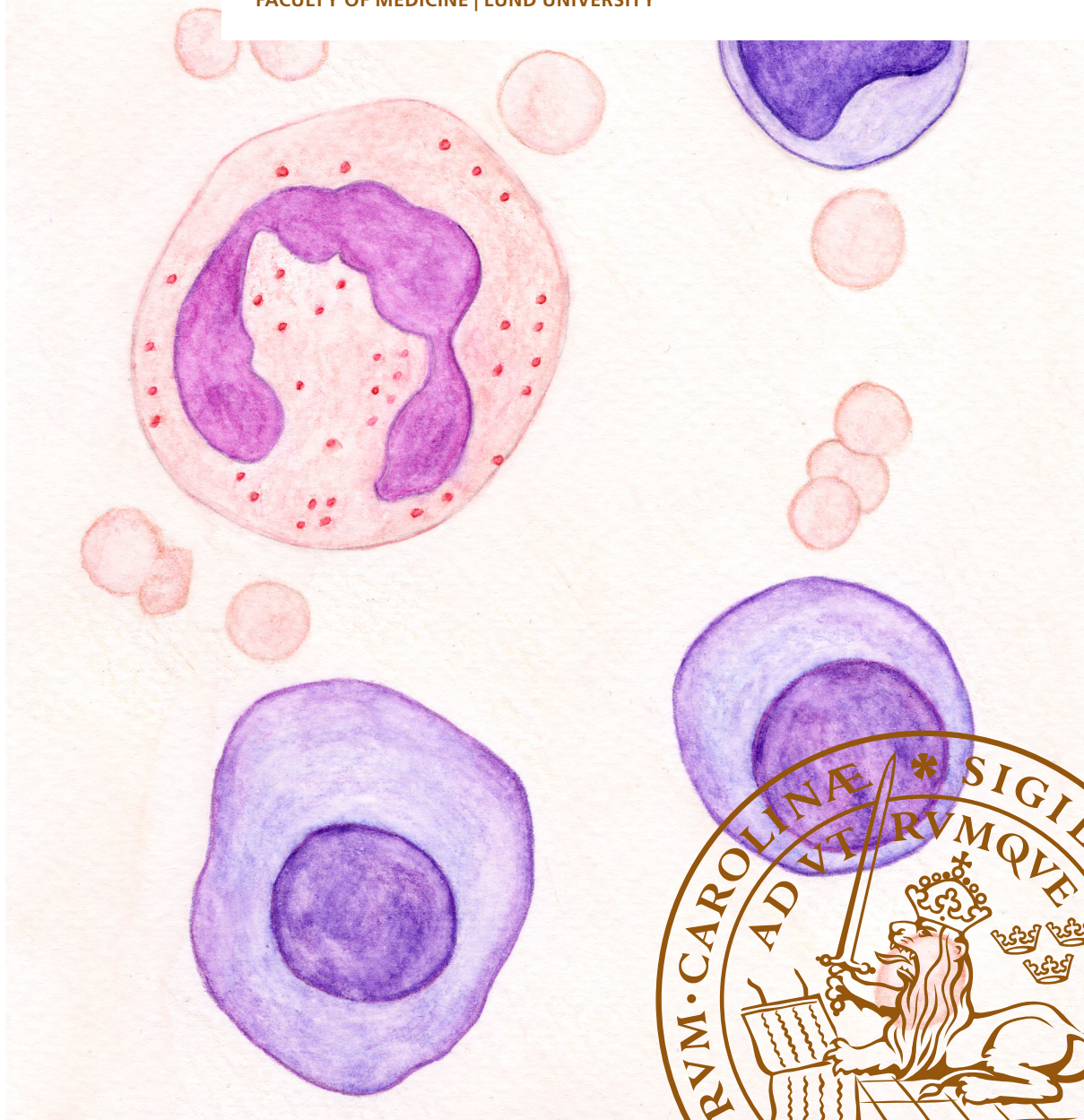
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Multiple Immunological Aspects in Myeloma

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Multiple Immunological Aspects in Myeloma

Multiple Immunological Aspects in Myeloma

Sandra Askman



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine, Lund University.

To be publicly defended on January 16th at 13.00 BMC I, Falck-Hillarp Auditorium, Sölvegatan 17, Lund.

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Professor Johan Bylund, University of Gothenburg

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Abstract:

Multiple myeloma (MM) is the second most common hematological malignancy, characterized by clonal proliferation of neoplastic plasma cells in the bone marrow, causing hematopoietic suppression and impaired antibody production. Bacterial infections remains a major cause of mortality in MM. As in most malignancies, myeloma cells modify their microenvironment to promote tumor growth, including suppression of the immune system. This thesis investigated different aspects of neutrophil function, including the immunoregulatory role of normal density granulocytes (NDGs) and myeloid-derived suppressor cells (MDSCs).

In paper I, neutrophils from patients with monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), and newly diagnosed MM exhibited reduced phagocytic ability and capacity of oxidative burst compared with healthy controls. The impairment was most pronounced in bone marrow (BM) samples and might contribute to the increased susceptibility to bacterial infections in MM. Lenalidomide treatment was associated with restored neutrophil function.

In paper II, levels and suppressive abilities of MDSCs as well as NDGs, were investigated. No increase in MDSC levels was detected in peripheral blood (PB) or BM, and they exhibited limited suppressive ability toward T cells, suggesting that MDSCs are not major contributors to MM pathogenesis. NDGs, however, displayed immunoregulatory activity, potentially contributing to MM development.

In paper III, NDGs induced a contact-dependent suppression of T cell proliferation, mediated by CD11b and reactive oxygen species (ROS) produced by NDGs. NDGs also induced a loss of the T helper (Th)1 surface marker CD183, which was unrelated to ligand binding. Moreover, incubation with NDGs suppressed cytokine production by Th1, Th2 and Th17 cells.

In paper IV, we tested whether NDGs from PB or BM of MM patients exerted suppressive effects on T-cells, similar to MDSCs. NDGs from the BM of MM patients, but not healthy donors, inhibited T cell proliferation. This effect could not be explained by alterations in maturity levels of NDGs in the bone marrow. NDGs in PB from both MM patients and controls inhibited T cell proliferation and IFN- γ production. PB NDGs did not need to be preactivated to mediate suppressive effects. Instead, they became activated during coculture, indicating that contact with activated T cells is important for their regulatory function. The inhibitory effect was ROS dependent and reversed with the inhibitor catalase. This suggests that NDGs possesses MDSC-like properties that might support myeloma cell growth.

Key words: Multiple myeloma, phagocytosis, phagoburst, MDSC, NDG, T cell proliferation

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Populärvetenskaplig sammanfattning

Multipelt Myelom utgör den näst vanligaste typen av blodcancer. Liksom andra typer av blodcancer utgår myelom från kroppens blodbildande organ – benmärgen – som finns inuti skelettets hålrum. Även om mekanismerna bakom sjukdomens uppkomst ännu inte är fullständigt klarlagda, är det fastställt att myelom innebär en klonal tillväxt av elakartade plasmaceller.

Plasmaceller är en typ av vita blodkroppar som spelar en viktig roll i kroppens immunförsvar. De producerar ett mångfacetterat spektrum av antikroppar vars uppgift är att känna igen specifika smittämnen, till exempel vissa bakterier eller virus. När kroppen infekteras av ett sådant smittämne binder de plasmacellsproducerade antikropparna till smittämnet. Det leder i sin tur till att andra delar av immunförsvaret aktiveras varpå smittämnet kan elimineras.

Vid myelom finns en onormalt stor klon av en viss sorts plasmaceller (så kallade myelomceller). Detta medför en överdriven och ensidig produktion av en enda sorts (monoklonal) antikropp. Följden blir en rad fysiologiska rubbningar: De sjuka cellerna tränger undan den normala blodbildningen i benmärgen, vilket kan orsaka blodbrist (anemi), brist på vita blodkroppar (leukopeni) – inklusive andra typer av plasmaceller och brist på blodplättar (trombocytopeni).

Sammantaget innebär detta en ökad risk för symtom som trötthet, blekhet, andfåddhet samt en större blödnings- och infektionsbenägenhet. Myelomcellerna bidrar också till en ökad nedbrytning av skelettet, vilket leder till en ökad risk för benbrott (fraktur), skelettsmärta och förhöjda kalciumnivåer i blodet. Dessutom kan den stora mängden monoklonala antikroppar täppa till njurarnas filter (glomeruli), vilket ofta medför försämrad njurfunktion.

I denna avhandling har olika aspekter av immunförsvaret vid myelom studerats.

I det första arbetet har vi tittat på hur funktionen av neutrofila granulocyter påverkas. Neutrofila granulocyter är en typ av vita blodkroppar som är viktiga för att bekämpa framför allt bakteriella infektioner. En av studierna visade att dessa celler har en nedsatt förmåga att äta upp (fagocytos) och förgöra (oxidativ burst) bakterier. Vi såg även att den nedsatta förmågan förbättrades i samband med behandling med ett vanligt myelomläkemedel (lenalidomid). Sammantaget bedöms den nedsatta förmågan att äta upp och förgöra bakterier kunna bidra till den ökade risken för bakteriella infektioner hos myelompatienter.

I det andra arbetet har vi studerat den immunhämmande förmågan hos så kallade myeloderiverade suppressorceller (MDSC) och neutrofila granulocyter. Myeloderiverade suppressorceller är en typ av immunceller som bromsar immunförsvaret, bland annat genom sin hämmande effekt på T celler, vilket gör att kroppens försvar mot infektioner försvagas. MDSC cellerna har även visat sig kunna bidra till utvecklingen av vissa cancerformer. Vi kunde inte se att MDSC cellerna hade någon hämmande effekt på T cellerna vid myelom. Däremot kunde vi observera att neutrofilerna hade en dämpande effekt på T cellerna, vilket potentiellt kan bidra till myelomutvecklingen.

I det tredje arbetet har vi undersökt hur neutrofila granulocyter påverkar T cellers förmåga att föröka sig. T celler anses spela en viktig roll vid bekämpandet av olika typer av tumorsjukdomar. Vi såg dels att T cellers förmåga att föröka sig (proliferera) hämmades av de neutrofila granulocyterna, dels att neutrofilerna minskade produktionen av vissa av deras signalmolekyler (cytokiner). Detta är i linje med resultaten från det andra arbetet.

I det fjärde arbetet såg vi att de neutrofila granulocyterna inte behövde aktiveras för att ha en dämpande effekt på T cellerna. Det var tillräckligt att de odlades tillsammans (co-cultivated) med T cellerna för att uppnå sin hämmande förmåga. Neutrofilernas hämmande effekt på T cellerna visade sig vara beroende av produktionen av fria syreradikaler (reactive oxygen species).

Sammantaget pekar resultaten på att funktionen hos de neutrofila granulocyterna är nedsatt vid myelom, vilket kan öka känsligheten för bakteriella infektioner. Vi har även sett att neutrofila granulocyter har en hämmande effekt på T celler, som spelar en viktig roll i att bekämpa tumorsjukdomar. Det är därför möjligt att neutrofila granulocyter, via sin T cellsdämpande effekt, bidrar till utvecklingen av myelom.

List of Papers

Paper I

Askman, S., Westerlund, J., Pettersson, Å., Hellmark, T., Johansson, Å., Wichert, S., Hansson, M. (2024) Decreased neutrophil function in newly diagnosed multiple myeloma patients is restored with lenalidomide therapy. *European Journal of Haematology*

Paper II

Westerlund, J., **Askman, S.**, Pettersson, Å., Wichert, S., Hellmark, T., Johansson, Å., Hansson, M. Myeloid-derived Suppressor Cells (MDSC) suppress T-cell proliferation less than mature neutrophils in Blood and Bone Marrow from Multiple Myeloma patients. *Submitted*

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

Petersson J., **Askman, S.**, Petterson Å., Wichert S., Hellmark T, Johansson Å CM (2021) Bone Marrow Neutrophils of Multiple Myeloma Patients Exhibit Myeloid-Derived Suppressor Cell Activity *Journal of Immunology Research*

Abbreviations

ADC Analog-to-digital converter
ADCC Antibody dependent cellular cytotoxicity
ADCP Antibody dependent cellular phagocytosis
AE Adverse effect
Akt Protein kinase B
Amp Amplifier
APCs Antigen presenting cells
APRIL A proliferation inducing ligand
BAFF B cell activating factor
BCMA B cell maturation antigen
BMSC Bone marrow stroma cell
BP Bandpass
BsAbs Bispecific monoclonal antibodies
CAR T chimeric antigen receptor T cell
CBA Cytometric bead array
CFSE Carboxyfluorescein succinimidyl ester
CLL Chronic lymphocytic leukemia
CM Costimulatory molecule
CR Complement receptor
CRAB Hypercalcemia, renal failure, anemia, lytic bone lesions
CRBN Cereblon
CRS Cytokine release syndrome
CRL4 E3 Cullin4A-RING E3
CSR Class-switch recombination
CTLA-4-Ig Cytotoxic T lymphocyte antigen 4–immunoglobulin
CTL Cytotoxic T Lymphocyte
CUL4A Cullin-4A
DC Dendritic cell
DDB1 DNA damage binding protein 1
DHR 123 Dihydrorhodamine 123
E. Coli Escherichia coli
e-MDSC Early myeloid-derived suppressor cell
ER Endoplasmic reticulum
FACS Fluorescence-Activated Cell Sorter
FCAP Flow cytometric analysis program

FCM Flow cytometry
 FcγR Fcγ receptor
 fMLF Formylmethionyl-leucyl-phenylalanine
 FLC Free light chain
 FSC Forward scatter
 GAL Galactose oxidase
 G-CSF Granulocyte colony-stimulating factor
 (G)-MDSC Granulocytic myeloderived suppressor cell
 GMP Granulocyte-monocyte progenitor
 GPRC5D G protein coupled receptor family C group 5 member D
 HIF-1 Hypoxia-Inducible Factor 1
 ICAM-1 Intercellular Adhesion Molecule 1
 ICANS Immune effector cell-associated neurotoxicity syndrome
 ICOS Inducible costimulator
 IMC Immature myeloid cell
 IMiD Immunomodulatory drug
 iNOS Inducible nitric oxide synthase
 IRF4 Interferon regulatory factor 4
 IS Immunological synapse
 ITAM Immunoreceptor tyrosine-based activation motif
 IVD In vitro diagnostic
 LDG Low density granulocyte
 LDN Low density neutrophil
 LFA-1 Lymphocyte Function Associated Antigen 1
 LMPP Lymphoid-primed multipotent progenitor
 LP Longpass
 LOX-1 Lectin-type oxidized LDL receptor 1
 mAbs Monoclonal antibodies
 Mac-1 Macrophage-1 antigen
 MHC Major histocompatibility complex
 MICA and MICB Major histocompatibility complex (MHC)-class I related chain A and B
 MIP-2 Macrophage Inflammatory Protein-2
 M-MDSC Monocytic myeloderived suppressor cells
 MDSC Myeloderived suppressor cell
 MDE Myeloma defining events
 MGUS Monoclonal gammopathy of undetermined significance
 MHC II Major histocompatibility complex class II
 MIP-1α Macrophage inflammatory protein-1
 MM Multiple myeloma
 MPP Multipotent progenitor cell
 MMP9 Matrix metalloprotease 9
 mTOR Mammalian target of rapamycin

NADPH Nicotinamide adenine dinucleotide phosphate
 NDG Normal density granulocyte
 NFAT2 Nuclear factor of activated T cells 2
 NK cells Natural killer cells
 NKG2D NK receptor group 2 member D
 NO Nitric oxide
 nor-NOHA N ω -hydroxy-nor-L-arginine
 OS Overall survival PI Proteasome inhibitors
 P-Alb Plasma albumin
 PBMC Peripheral blood mononuclear cells
 PBS Phosphate-buffered saline
 P-Ca Plasma calcium
 PCD Plasma cell dyscrasias
 PD-1 Programmed cell death protein 1
 PDMM Previously diagnosed multiple myeloma
 PHA Phytohemagglutinin
 PI3K Phosphoinositide 3-Kinase
 PHA Phytohemagglutinin
 PKC ζ Protein kinase C zeta
 PMA Phorbol-12-myristate-13-acetate
 PN Peripheral neuropathy
 PMN-MDSC Polymorphonuclear myeloderived suppressor cells
 PRR Pattern recognition receptor
 PSGL1 P-selectin glycoprotein ligand 1
 OBL Osteoblasts
 OCL Osteoclast
 OPG Osteoprotegerin
 RANKL Receptor Activator of Nuclear Factor κ B Ligand
 RBX1 RING-box protein 1
 SH2 Src Homology 2
 SINE Selective inhibitor of nuclear export
 SLAMF7 Function of signaling lymphocytic activation molecule
 SLO Secondary lymphoid organ
 SHM Somatic hypermutation
 SMM Smouldering myeloma
 SP Shortpass
 SSC Side scatter
 XPO1 Exportin 1
 TD T cell dependent
 Tfh cells T follicular helper cells
 TNF- α Tumor necrosis factor-alpha
 Tregs T regulatory cells
 TSP Tumor suppressor proteins

Theoretical framework

Multiple Myeloma (MM)

Multiple myeloma is the second most common hematological malignancy. Each year 600 patients in Sweden are diagnosed with the disease [1]. The prevalence is rising due to increasingly number of elderly and improved treatment possibilities [2]. The malignancy is caused by clonal proliferation of neoplastic plasma cells/myeloma cells infiltrating the bone marrow causing hematopoietic suppression and inhibition of normal antibody production. The myeloma cells produce monoclonal antibodies, detectable in serum as M protein [1].

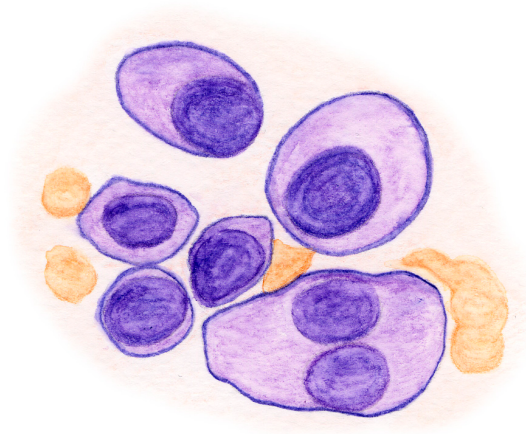


Figure 1 Illustration of malignant plasma (myeloma) cells.

Monoclonal gammopathy of undetermined significance (MGUS), smouldering multiple myeloma (SMM) and multiple myeloma (MM) all represents a spectrum of clonal plasma cell proliferative disorders [3]. More than 3% of the population over 50 years of age presents with the early, premalignant, asymptomatic stage of MGUS, with a 1% annual risk of progression to myeloma or other lymphoproliferative disorders [1, 4].

MGUS is characterized by the presence of serum M-protein levels below 3 g/dL, fewer than 10% clonal plasma cells in bone marrow, no evidence of end-organ damage, B-cell lymphoma or other diseases known to produce M-protein [5].

SMM, represents a heterogeneous intermediate stage, prevalent in approximately 0.5% of the general population over 40 years of age. It carries a 10% risk of progression to MM within the first 5 years following diagnosis, a 3% annual risk over the next 5 years, and a 1.5% annual risk thereafter [3, 6]. SMM is defined by monoclonal protein (IgG or IgA) ≥ 3 g/dL or urinary monoclonal protein ≥ 500 mg/24 h and/or 10%-60% clonal bone marrow plasma cells in the absence of myeloma defining events or amyloidosis [6].

The rate of progression to multiple myeloma is influenced by disease burden and the underlying cytogenetic abnormalities, where patients harboring t(4;14) translocation, del(17p) or gain(1q) being at higher risk of progression from MGUS or SMM [3, 6]. The diagnosis of multiple myeloma requires the presence of one or more myeloma defining events (MDE) in addition to either $\geq 10\%$ clonal plasma cells on bone marrow examination or a biopsy-proven plasmacytoma [6].

MDE include the established CRAB criteria (hypercalcemia, renal failure, anemia, or lytic bone lesions) as well as three new specific biomarkers: clonal bone marrow plasma cells $\geq 60\%$, a serum free light chain (FLC) ratio ≥ 100 (provided the involved FLC level is ≥ 100 mg/L and urinary monoclonal protein excretion is ≥ 200 mg/24 h), and more than one focal lesion on magnetic resonance imaging (MRI). Each of these new biomarkers is associated with an 80% risk of progression to symptomatic end-organ damage, and the updated criteria enable diagnosis and treatment initiation prior to the onset of organ injury [6].

The most common symptom of myeloma is skeletal pain due to osteolytic bone lesions. Other manifestations include anemia, fatigue and recurrent infections [1].

Rapid evolution of myeloma treatment in the last two decades has led to significantly improved outcome. Initially *immunomodulatory drugs (IMiDs)* such as thalidomide and lenalidomide and the *proteasome inhibitor (PI)* bortezomib were introduced [6, 7]. Later another IMiD (pomalidomide), additional PIs (carfilzomib, ixazomib) together with *monoclonal antibodies* (elotuzumab, daratumumab, isatuximab, belantamab mafodotin), *bispecific antibodies* (teclistamab, talquetamab, elranatamab), and *chimeric antigen receptor T cell* were approved [6].

Immunomodulatory Drugs (IMiDs)

Thalidomide was first introduced in 1957 as an antiemetic for pregnant woman. Unfortunately, it proved to be highly teratogenic and was withdrawn from market only a few years later [8, 9]. Later thalidomide proved to be effective in treatment of advanced stages of myeloma and was reintroduced as the first IMiD in 2006 [10, 11]. Chemical structure modification of thalidomide resulted in production of the analogs lenalidomide and pomalidomide with more significant immunomodulatory effects and better safety profile [7].

IMiDs have pleiotropic effects including immunomodulation, with alterations in cytokine production, T cell activation, augmentation of NK cell function, anti-angiogenesis activity, and effect on the microenvironment in myeloma [12].

Molecular target of IMiDs

Cereblon (CRBN), an important protein of the E3 ubiquitin ligase complex, regulate protein degradation, thereby controlling multiple cell functions [13]. By binding to CRBN, IMiDs alter its substrate specificity, resulting in the ubiquitination and proteasomal degradation of IKZF1 and IKZF3, which are essential for myeloma cell survival [9, 11, 14], leading to myeloma cell death. The depletion of IKZF1 and IKZF3 also known as Ikaros and Aiolos, results in downregulation of the oncogenes interferon regulatory factor 4 (IRF4) and c-Myc [14], two key drivers of multiple myeloma pathogenesis. IRF4 is highly expressed in plasma and B cells, where it regulates plasma cell differentiation and immunoglobulin class switching. In MM, IRF4 is frequently overexpressed due to activating mutations or translocations and has emerged as a master regulator of the malignancy-specific aberrant gene expression program [15]. Similarly, c-Myc is overexpressed in approximately 40% of NDMM cases, supporting the notion that Myc activation is an early event in MM pathogenesis. Its overexpression correlates with clinical features such as hypercalcemia and extramedullary myeloma, and c-Myc upregulation is associated with poorer overall survival (OS) [16].

T cell activation by IMiDs – multiple targets

IMiDs, has been shown to activate T-cells via different mechanisms. T cell proliferation and differentiation are regulated by numerous cytokines, most importantly interleukin-2 (IL-2). High IL-2 levels in PB correlate with favorable prognostic factors and improved survival in MM [17]. Ikaros and Aiolos function as repressors of the IL-2 promoter.

Lenalidomide and pomalidomide enhance the ability of the E3 ubiquitin ligase complex to induce ubiquitination and subsequent degradation of Ikaros and Aiolos.

This results in de-repression of the IL-2 promoter, ultimately increasing IL-2 expression [18].

IMiDs also interact with Zeta-chain-associated protein kinase 70 (ZAP-70), a tyrosine kinase mainly expressed in T cells, natural killer (NK) cells and a subset of B cells [19]. After TCR engagement, CD4-associated Lck is positioned close to the CD3 complex, where it phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs). The doubly phosphorylated ITAMs then interact with the Src Homology 2 (SH2) domains of ZAP-70. Upon ITAM binding, ZAP-70 is phosphorylated by Lck resulting in activation of ZAP-70 autophosphorylation and catalytic activity. Activated ZAP-70 subsequently phosphorylates LAT and SLP-76, which function as scaffolds to recruit additional signaling molecules involved in T cell activation, proliferation, and differentiation [20]. IMiDs binds directly to ZAP-70 thereby triggering its activation [21].

CD28 is a critical costimulatory molecule expressed on T cells. IMiD-induce CD28 phosphorylation and activation. The interaction between CD28 and its ligands B7-1 and B7-2 on antigen presenting cells (APCs) results in activation of protein kinases such as Lck and Fyn that phosphorylates CD28. This entails recruitment and activation of downstream targets such as phosphatidylinositol 3-kinase (*PI3K*), Growth factor receptor-bound protein 2 (GRB-2-SOS) and NF- κ B. Cytotoxic T lymphocyte antigen 4-immunoglobulin (CTLA-4-Ig) inhibits T-cell proliferation by blocking co-stimulation through the B7-CD28 pathway. CTLA-4-Ig is inappropriately expressed in MM patients, however this co-stimulatory blockade is partially overcome by IMiD-induced CD28 phosphorylation, leading to activation of NF- κ B [22]. NF- κ B has a role in regulation of T cell differentiation and effector function. Upon activation CD4⁺ T cells differentiate into different subsets including Th1, Th2, Th17 and T follicular (Tfh) cells. NF- κ B promotes Th1 differentiation by regulating TCR signaling. It also functions in innate immune cells to mediate the secretion of cytokines such as IL-2, further enhancing Th1 differentiation [23]. Recent investigations have demonstrated elevated levels of the Th1 cytokine IFN- γ during lenalidomide treatment, and that this elevation is abolished upon disease progression [24].

Suppressor of cytokine signaling (SOCS1) functions as a negative regulator of IL-6 signaling. In MM, SOCS1 is silenced by hypermethylation resulting in uncontrolled IL-6 signaling, enabling MM cell growth and immune suppression.

Both Lenalidomide and pomalidomide has the capacity to demethylate and induce SOCS1 gene transcription in myeloma cells. The re-expression of SOCS1 in addition to suppression of myeloma cell IL-6 signaling enhances the susceptibility of MM cells to Cytotoxic T Lymphocyte (CTL) mediated cytotoxicity [25].

Augmentation of NK cell function

Natural killer (NK) cells exert cytotoxic and suppressor activity against virus infected cells and cancer cells by lysis and secretion of inflammatory cytokines such as IFN- γ [26, 27]. The capability of NK surveillance and cytotoxicity decreases with progressive MM disease [27]. NK cells eliminate tumor cells through natural cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC). IMiDs activate phosphoinositide 3-kinase (PI3K) and its downstream targets, protein kinase C ζ (PKC ζ) and protein kinase B (Akt). This leads to nuclear translocation of nuclear factor of activated T cells 2 (NFAT2), which triggers IL-2 production enhancing both natural and ADCC-mediated NK cell cytotoxicity [28].

Receptors such as NK receptor group 2 member D (NKG2D) and CD16/Fc γ RIII represents activating receptors expressed on NK cells [29]. After activation, efficient lysis requires the formation of a tight adherent contact, known as the immunological synapse (IS), between NK cells and target cells. The formation of a mature cytolytic synapse occurs in several stages, ultimately resulting in the secretion of lytic granules at the IS [30]. Lenalidomide lowers the threshold for NK cell activation through CD16 and NKG2D, indicating a reduced need for activating ligands. In addition, lenalidomide augments actin remodeling at the NK immune synapse [27].

T regulatory cells

T regulatory cells (Tregs) are elevated in several malignancies, including multiple myeloma. Tregs play an important role as regulators of the immune response, functioning as controllers of autoimmune diseases and suppressors of anti-tumor immunity [31]. Lenalidomide and pomalidomide exert their inhibitory effects by acting on the forkhead box transcription factor (FOXP3), a specific marker of Tregs, necessary for its suppressive activity [31, 32].

Apoptosis in MM cells by IMiDs

The anti-inflammatory phenotype of several cytokines in MM supports immunosurveillance escape and tumor growth [24]. TNF- α induces adhesion molecules between myeloma cells and bone marrow stroma cells (BMSCs).

This results in activation of NF- κ B and subsequent IL-6 secretion [33]. IL-6 promotes proliferation and inhibits apoptosis of malignant plasma cells [12]. Lenalidomide induces apoptosis in myeloma cells by directly inhibiting pro-inflammatory cytokines such as IL-6 and TNF- α , and indirectly by disrupting interactions between multiple MM cells and BMSCs [12].

Anti-angiogenesis activity of IMiDs

The growth of blood vessels, known as angiogenesis, is essential for physical development and repair. However, an imbalance in angiogenesis contributes to the pathogenesis of numerous disorders, and bone marrow angiogenesis progressively increases along the spectrum from MGUS to advanced MM [34-36]. Several aspects of angiogenesis are affected in MM. Below is a description of known angiogenic disturbances, followed by an overview of the effects of lenalidomide on these abnormalities.

Vascular endothelial growth factor (VEGF) is a pro-angiogenic molecule that is important for endothelial cell survival and vascular development. Myeloma cells produce VEGF, which stimulates bone marrow stromal cells to secrete IL-6 and additional VEGF. In turn, stromal cells promote VEGF production by myeloma cells in a paracrine loop, thereby supporting tumor growth [35, 37]. Hypoxia-Inducible Factor (HIF)-1 is a heterodimeric transcription factor essential for the ability of cells to sense and adapt to changes in oxygen levels [38, 39]. It consists of a constitutively expressed β -subunit and an oxygen-regulated α -subunit [38]. Under normal oxygen conditions, HIF-1 α is transcribed in the nucleus. Once translated, HIF-1 α is degraded by the proteasome. During hypoxic conditions, HIF-1 α re-enters the nucleus, forming a transcription complex with the HIF-1 β subunit. This complex ultimately functions as a regulator of target genes, such as vascular endothelial growth factor (VEGF) and cathepsin D. HIF-1 plays a critical role in tumor proliferation due to its upregulation under hypoxic conditions, such as in the bone marrow microenvironment in MM [38, 40]. Lenalidomide acts as a potent inhibitor of HIF-1 α , thereby suppressing an aggressive tumor phenotype and its accompanying ability for tissue invasion [41].

The close association of MM cells with stromal and endothelial cells in the microenvironment contributes to elevated cytokine stimulation of pathways such as the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, which is vital for the growth and survival of MM cells [42]. Activation of the PI3K/AKT/mTOR pathway increases secretion of VEGF through both HIF-1-dependent and HIF-1-independent mechanisms [43].

In addition to its activation in tumor cells, the PI3K/AKT/mTOR pathway is also essential for endothelial cell migration. Lenalidomide inhibits VEGF-induced PI3K/AKT/mTOR pathway activity, consequently impairing blood vessel development [41]. Capillary tube formation is a specialized endothelial cell function essential for establishing a continuous vessel lumen. The molecules cadherin-5, CD31, β -catenin, and F-actin form a functional complex that regulates this process. [44]. Lenalidomide inhibits the VEGF-induced association between these molecules, thereby preventing the formation of the vessel lumen [41].

Inhibition of Osteoclastogenesis

Normal bone remodeling is a tightly regulated process characterized by a balance between the activity of osteoblasts for bone formation and osteoclasts for bone resorption [45]. MM results in severe dysfunction of both bone formation and resorption, associated with painful osteolytic bone lesions, pathological fractures, hypercalcemia, and spinal cord compression [46].

The abnormal bone remodeling process is induced by interactions between bone marrow stromal cells (BMSCs) and myeloma cells in the bone marrow microenvironment [46, 47]. Myeloma cells drive bone destruction by suppressing osteoblast (OBL) formation and by directly and indirectly stimulating osteoclast (OCL) formation [46]. Receptor Activator of Nuclear Factor κ B Ligand (RANKL) and Osteoprotegerin (OPG) constitutes indicators of bone remodeling. OPG suppresses osteoclastogenesis by its inhibition of RANKL and stimulation of OBLs [48].

In MM, the RANKL/OPG ratio is elevated due to increased RANKL production and decreased OPG production, triggered by the adhesion of myeloma cells to BMSCs. Elevated levels of soluble RANKL/OPG correlate with both the extent of bone pathology and patient survival [48]. Lenalidomide influences several aspects of osteoclastogenesis in myeloma. Lenalidomide decreases RANKL secretion from BMSCs in myeloma patients. [48]. In addition, lenalidomide suppresses secretion of the OCL-derived growth and survival factors MIP-1 α , BAFF and APRIL [49]. Macrophage inflammatory protein-1 (MIP-1 α) is chemokine secreted by macrophages that performs various functions, including activation of bone resorption cells and induction of bone destruction [49]. A proliferation inducing ligand (APRIL) and B cell activating factor (BAFF) are both TNF superfamily molecules associated with pathological bone resorption in myeloma [50].

Moreover, lenalidomide inhibits OCL formation and activation by targeting PU.1 and Phosphorylated ERK (pERK). PU.1 is a transcription factor involved in the early stages of osteoclastogenesis, promoting the commitment of hematopoietic cells to macrophage lineage [48]. Endoplasmic reticulum (ER) stress can disrupt intracellular homeostasis in osteoclasts (OCLs) and osteoblasts (OBLs) [51]. pERK functions as an ER stress sensing protein essential for osteoclast differentiation [52].

Proteasome inhibitors (PI)

Proteasomes are large complexes that degrade proteins into oligopeptides, enabling the recycling of essential intracellular proteins. Bortezomib acts by reversibly inhibiting the proteasome. Although many intracellular processes are affected, the reduction in nuclear signaling by nuclear factor kappa B (NF- κ B) is considered a central mechanism for inducing apoptosis in myeloma cells [53]. Because of their

high production and secretion of proteins, myeloma cells are especially sensitive to proteasome inhibitors. Carfilzomib is a second-generation PI that binds irreversibly to the proteasome. Like bortezomib, it is characterized by rapid onset and can be safely administered to patients with renal impairment [54]. Ixazomib is the first oral PI that presents with a lower incidence of peripheral neuropathy (PN) compared to bortezomib [55].

Monoclonal antibodies (mAbs)

The cell surface protein CD38 is highly expressed on myeloma cells. The binding of daratumumab to CD38 activates several mechanisms including initiation of complement cascade, activation of cytotoxic processes, induction of phagocytosis and direct cellular apoptosis [56]. Isatuximab is another immunoglobulin G1 (IgG1) mAbs targeting CD38. Binding to CD38 induces several Fc-dependent mechanisms such as antibody dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement dependent cytotoxicity (CDC). ADCC mediated by the activation of Fcγ receptors on the NK cells is suggested to be the dominant effector mechanism [57]. Elotuzumab targets SLAMF7 which is highly expressed on MM cells. This stimulates robust ADCC through engagement of FcγRIIIA on NK cells and ADCP by macrophages [58].

Bispecific monoclonal antibodies (BsAbs)

B cell maturation antigen (BCMA) is highly expressed in MM. Elevated BCMA levels correlates with plasma cell burden in bone marrow, and high levels indicates poor clinical outcome [59, 60]. Teclistimab is a bispecific antibody with dual binding sites directed at CD3⁺ on T cells and BCMA⁺ on myeloma cells. This interaction results in T-cell activation and subsequent lysis of BCMA⁺-expressing MM cells [61]. Elranatamab targets the same antigens as Teclistimab and demonstrates similar efficacy [62]. GPRC5D (G protein–coupled receptor, family C, group 5) is an orphan receptor that is primarily expressed on plasma cells and hard keratinized tissues. GPRC5D expression is associated with high-risk myeloma disease [63]. By simultaneously binding GPRC5D and CD3⁺, talquetamab recruits and activates T cells, thereby inducing the killing of GPRC5D-expressing malignant plasma cells [63].

Chimeric antigen receptor T (CAR T)

Chimeric antigen receptor T (CAR T) are defined as fusion proteins with an antigen recognition domain and T cell signaling domains [64]. CAR T consists of an extracellular, a transmembrane and an intracellular domain [65]. Single chain variable fragment (scFv), is the most commonly used ligand binding domain of the extracellular region [66]. scFV are fusion proteins composed of the immunoglobulin heavy and light chain variable regions connected by a peptide binding [67]. Binding of surface cancer antigens to scFV typically results in a MHC independent T cell activation [68]. In the last thirty years CAR T cell therapies has evolved into five generations. This is achieved by modifications of the endodomain structure, addition of costimulatory molecules (CMs) and impact on cytokine activation [69].

The principles of CAR T therapy can be divided into several steps. Initially blood is harvested from patient followed by leukapheresis, T cell enrichment and activation [65]. Next T cells undergo genetic transduction typically using a viral vector inserting the CAR gene into the T cell genome. T cells are then expanded. After lymphodepleting chemotherapy, CAR T cells are infused to patient. Finally CAR T cells proliferate and targets cancer cells [70]. Several anti-BCMA CAR T cell therapies have shown impressive clinical results in treatment of multiple myeloma [71].

Although CAR T cells have demonstrated clinical benefit to many patients, they are associated with significant adverse effects (AEs) such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) [72].

CRS can present with a wide variety of symptoms, from mild to life-threatening. Mild symptoms include fatigue, arthralgia, myalgia and headache. Severe manifestations include high fever and hypotension, which may progress to an uncontrolled systemic inflammatory response syndrome requiring vasopressors for circulatory support, vascular leakage, disseminated intravascular coagulation and multi-organ failure [73]. Since CRS often precedes the development of ICANS it could be considered an initiating event of ICANS [74]. ICANS is typically characterized as a toxic encephalopathy that begins with symptoms such as word-finding difficulties, impaired fine motor skills, dysphasia, and confusion. In more advanced stages seizures, motor weakness and coma might occur [74].

The pathophysiology of CRS (Cytokine Release Syndrome) can be divided into five phases. The first phase involves the trafficking of CAR T cells to the tumor site following infusion. The second phase is characterized by CAR T-cell proliferation and cytokine production by both the tumor microenvironment's cellular components and the CAR T cells themselves. The third phase is marked by elevated cytokine levels and expansion of CAR T cells in the peripheral blood, accompanied by a systemic inflammatory response that results in endothelial injury and vascular leakage in several organs. The fourth phase involves the diffusion of cytokines and

transmigration of CAR T cells into the cerebrospinal fluid (CSF), along with breakdown of the blood-brain barrier (BBB). This phase correlates with the onset of ICANS. Finally, the fifth phase is defined by a decline in cytokine levels and resolution of the systemic inflammatory response. Treatment of short-term CAR T cell associated toxicities starts with supportive care. Management of prolonged or severe CRS should be treated with the IL-6 inhibitor tocilizumab and corticosteroids [75]. In most cases both CRS and ICANS are reversible and without permanent neurological deficits [74].

Neutrophils

Neutrophils, also designated polymorphonuclear leucocytes (PMNs) or normal density granulocytes (NDGs), are the most common leucocytes in peripheral blood [76, 77]. Pathogen recognition and recruitment of neutrophils constitute key elements in host-defense particularly against bacteria and fungi [78, 79].

Activation of neutrophils involves a multistep process initiated by the neutrophil recruitment cascade, followed by phagocytosis and intracellular destruction mediated by reactive oxygen species (ROS) and granular components [79, 80]. Every day, bone marrow granulopoiesis originating from hematopoietic stem cells (HSCs) produces approximately 10^{11} PMNs [76, 81].

Hematopoiesis

The hematopoietic process begins with multipotent hematopoietic stem cells (HSCs), capable of dividing into either self-renewal or multipotent progenitor cells (MPPs). The MPPs are capable of further differentiation into increasingly specialized cells, ultimately resulting in the production of either leucocytes, erythrocytes or thrombocytes [82].

Differentiation

In neutrophil differentiation, MPPs are transformed into lymphoid-primed multipotent progenitors (LMPPs). LMPPs then develop into the granulocyte-monocyte progenitors (GMPs). Under the influence of granulocyte colony-stimulating factor (G-CSF), GMPs differentiate into myeloblasts. Myeloblasts proceed through a multistep process, including the stages of promyelocyte, myelocyte, metamyelocyte and band cells stages ultimately resulting in mature neutrophils [76].

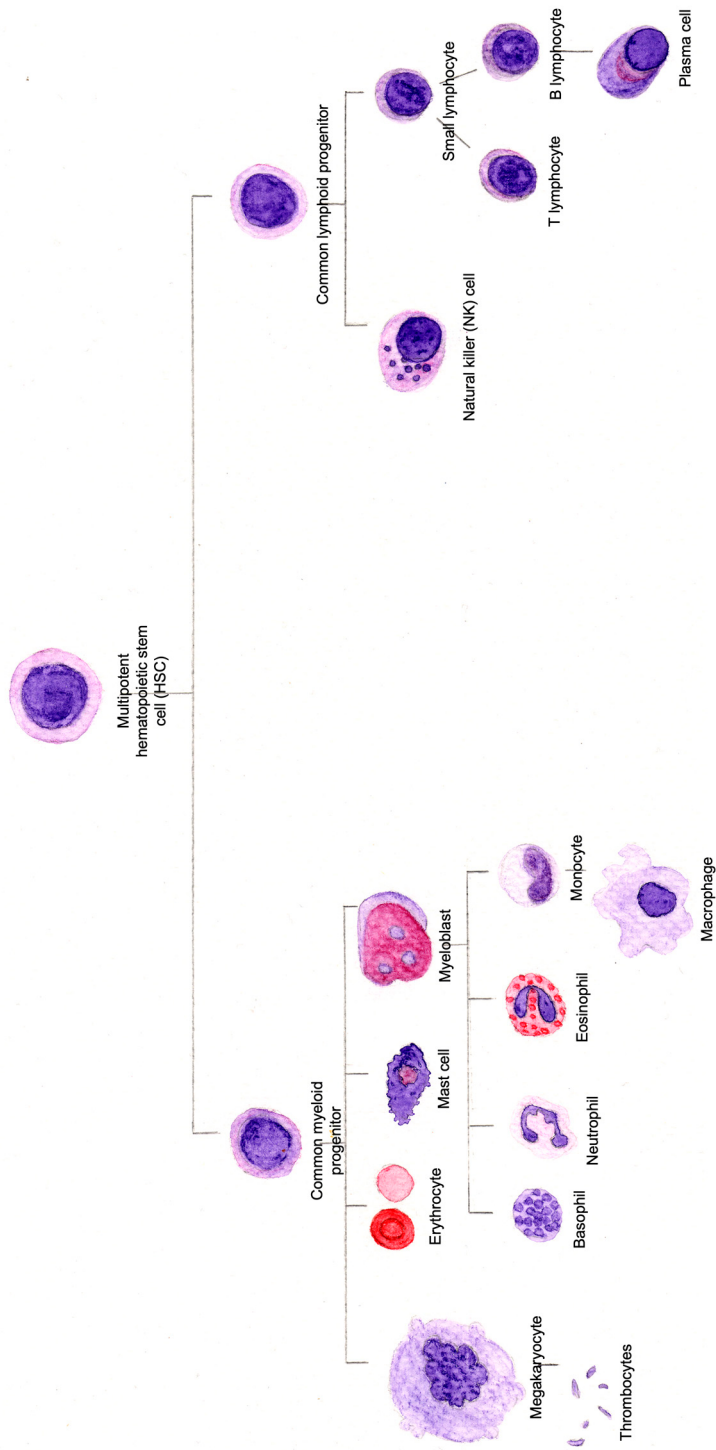


Figure 2 Diagram illustrating the differentiation of haematopoietic stem cells into fully mature blood cells.

Release from bone marrow to blood stream

After maturation, neutrophils exit the hematopoietic compartment by transcellular migration through the bone marrow endothelium into the bloodstream. The ability of neutrophils to pass through the endothelium is regulated by vascular endothelial cadherin, affecting the endothelial permeability [81]. Granulocyte colony-stimulating factor (G-CSF) is the principal regulator of physiological granulopoiesis, exerting its effects by binding to the G-CSF receptor, a member of the class I cytokine receptor family. In addition to committing progenitor cells to the myeloid lineage and inducing proliferation of granulocytic precursors, G-CSF also promotes the release of mature neutrophils from the bone marrow [83]. Neutrophil release from the hematopoietic compartment is further stimulated by the CXC chemokine, macrophage inflammatory protein-2 (MIP-2) [84].

Neutrophil recruitment to tissues

Neutrophil recruitment to tissues involves a multistep process, including tethering, rolling, adhesion and trans-endothelial migration [80]. Neutrophil sequestration is initiated by changes in the vascular endothelium. These changes are induced either by inflammatory mediators released from tissue-resident sentinel cells in response to microbial encounter or by direct activation through pattern recognition receptor (PRR)-mediated detection of the pathogen [85].

PRR detection results in increased expression of adhesion molecules such as P- and E-selectin capturing neutrophils through binding of glycosylated ligands such as P-selectin glycoprotein ligand 1 (PSGL1) [85]. In order to resist the shear forces of blood flow and induce the process of rolling, stronger interactions are needed. This is initiated when chemokines such as CXCL-8 binds to its receptor on the neutrophil, triggering the activation of the integrins Lymphocyte Function Associated Antigen 1 (LFA-1) and Macrophage-1 antigen (Mac-1), ultimately resulting in firm adhesion [81].

Inflammatory cytokines like tumor necrosis factor-alpha (TNF- α) are necessary for the endothelial expression of adhesion molecules such as Intercellular Adhesion Molecule 1 (ICAM-1) binding to LFA-1 receptor on neutrophils. Robust interaction between ICAMs and integrins terminates rolling and results in arrest. This allows neutrophils to transmigrate across the endothelium through either trans- or paracellular migration. The passage through the basement membrane is mediated by matrix metalloproteinases expressed on the neutrophil surface. Ultimately, extravasated neutrophils migrate towards a concentration gradient of chemokines secreted by cells at the infection site [81].

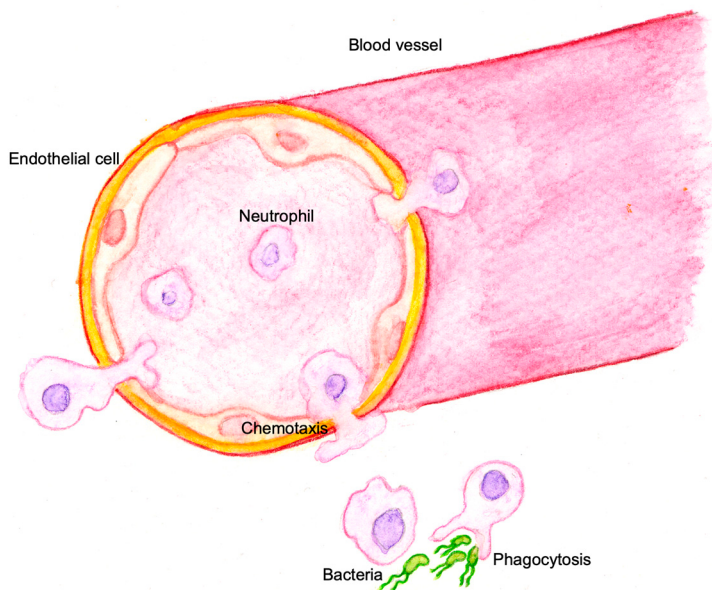


Figure 3 Diagram illustrating diapedesis, showing neutrophil migration through the endothelial wall of a blood vessel in response to chemotactic signals, and subsequent phagocytosis of bacteria.

Phagocytosis

Phagocytosis is defined as the process for cellular uptake and elimination of microbial pathogens and apoptotic cells $> 0,5 \mu\text{m}$ [86-88]. Professional phagocytic cells includes neutrophils, eosinophils, macrophages, monocytes, dendritic cells (DCs) and osteoclasts [88]. The phagocytic process involves several phases: detection, activation, phagosome formation and transformation into the phagolysosome [86].

Detection

Detection of target particles is mediated by phagocytic receptors, which are further divided into opsonic and non-opsonic receptors. Opsonic receptors are subdivided into Fcγ receptors (FcγRs) and complement receptors (CRs). FcγRs are glycoproteins that recognize the Fc region of IgG molecules, whereas CRs bind complement components deposited on microorganisms or cells. Non-opsonic receptors include receptors that recognize microorganisms and apoptotic cells [86].

Activation and phagosome formation

Interaction with the phagocytic receptor induces a series of signaling events that trigger phagocytosis. The internalization of IgG-opsonized particles by FcγRs is the most well-understood model of phagocytosis [89]. After recognition via FcγR, a phagocytic cup forms as pseudopods extend around the particle. Membrane fusion at the distal end seals the vacuole, which eventually separates into an intracellular phagosome [90].

Maturation into phagolysosome

During maturation, the phagosome transitions through early, intermediate, and late stages, becoming progressively acidic while gaining degradative capacity [91, 92]. Ultimately, the phagosome fuses with lysosomes to form the highly acidic phagolysosome [86]. A characteristic feature of neutrophils is their ability to induce an oxidative burst through the enzyme complex NADPH oxidase, which is located on the phagolysosomal membrane [86, 91]. NADPH oxidase catalyzes the production of superoxide from NADPH and oxygen [92]. The superoxide then dismutates to hydrogen peroxide (H_2O_2), which reacts with chloride ions (Cl^-) to form the highly potent microbicidal substance hypochlorous acid. Furthermore, the phagolysosome contains several other hydrolytic enzymes that contribute to the degradation of ingested microorganisms [86].

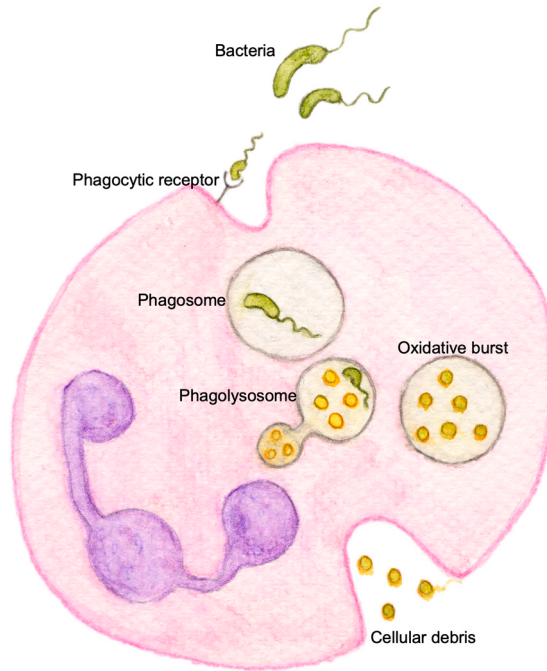


Figure 4 Stepwise illustration of phagocytosis, showing bacteria engulfed by a neutrophil into a phagosome that fuses with a lysosome to form a phagolysosome, leading to bacterial degradation via oxidative burst and release of cellular debris.

Myeloderived suppressor cells (MDSCs)

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of activated immature myeloid cells (IMCs) that expand in malignant diseases, primarily suppressing T cells [93-96]. Circulating levels of MDSCs correlates to tumor stage and shorter survival in both solid and non-solid tumors [95]. MDSCs consist of two major subsets: polymorphonuclear (PMN)-MDSCs, which are phenotypically and morphologically similar to neutrophils, and monocytic (M)-MDSCs, which resemble monocytes [96]. A third, more immature subpopulation referred to as early-stage MDSC (e-MDSC), represents an additional subtype that lacks macrophage and granulocyte markers [97, 98]. Although PMN-MDSCs phenotypically resemble neutrophils, they can be separated by density gradient centrifugation: PMN-MDSCs are recovered at a lower density (1.077 g/mL), whereas neutrophils are found at a higher density (1.1–1.2 g/mL) [99].

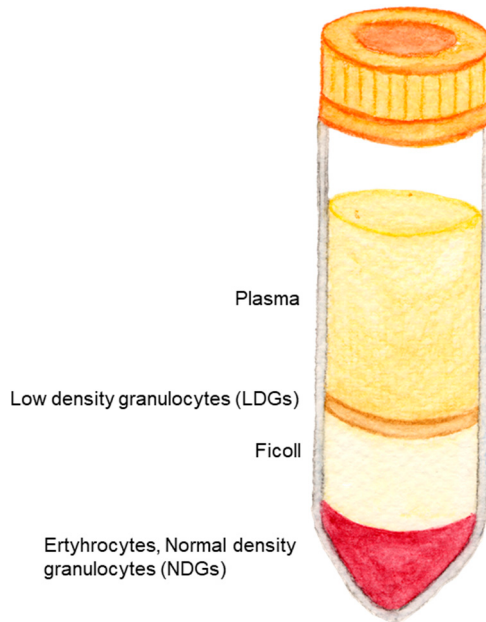


Figure 5 Separation of normal-density and low-density granulocytes by density gradient centrifugation. Ficoll forms a barrier that separates blood components according to their density: Plasma remains at the top (least dense). Peripheral blood mononuclear cells (PBMCs) including lymphocytes, monocytes, and LDGs, which are enriched for PMN-MDSCs, accumulate at the plasma–Ficoll interface. NDGs and erythrocytes, having a greater density, pass through the Ficoll layer and form a pellet at the bottom.

The role of MDSCs could be considered to protect the host from extensive tissue damage caused by an uncontrolled immune response during unresolved inflammation. However, malignant cells appear to exploit this immunosuppressive effect to evade elimination by the immune system. [100]. The expansion and activation of MDSCs are influenced by several factors that can be divided into two principal groups. The first group consists of factors produced by tumor cells that promote MDSC expansion by stimulating myelopoiesis and inhibiting maturation into fully differentiated myeloid cells. The second group includes factors primarily produced by stromal tumor cells and activated T cells, which lead to the direct activation of MDSCs. The immunosuppressive activity of MDSCs requires direct cell–cell contact, suggesting that they function either through binding to cell surface receptors or through the release of short-lived soluble mediators. The mechanisms involved in MDSC mediated T cell suppression includes release of arginase 1, inducible nitric oxide synthase (iNOS), ROS and peroxynitrite [94].

It has been questioned whether cells with similar morphology and phenotype to neutrophils and monocytes should have their own designation [100].

Several lines of evidence suggest that MDSCs possess their own specific nature, representing a relatively stable and distinct state of functionally active neutrophils

and monocytes. These characteristic features include an intrinsic suppressive activity, a unique genomic profile, a specific phenotypic expression and biochemical features distinguishing them from neutrophils and monocytes [96]. The distinct biochemical features that distinguish MDSCs from neutrophils and monocytes are described in more detail below.

Classical neutrophil and monocyte activation has evolved to protect the host from bacterial and viral infections and to provide support for remodeling after resolved inflammation. This activation is characterized by efficient phagocytosis, respiratory burst, and the release of pro-inflammatory cytokines. Myeloid activation is relatively short-lived, and the response terminates when the pathogen is eliminated. Pathological activation results from persistent, low-level stimulation in response to malignancies and chronic inflammation. Myeloid cells generated under these conditions are poorly phagocytic, secrete large amounts of reactive oxygen and nitrogen species, and predominantly produce anti-inflammatory cytokines. As a consequence, these cells are unable to perform the normal myeloid functions. Instead, they acquire the potent immunosuppressive properties that are characteristic of MDSCs [100].

B cell development and plasma cell differentiation

Immunological function

B lymphocytes are fundamental components of the adaptive immune response, performing critical functions such as antibody production, antigen presentation and cytokine secretion.

Development

B cell development proceeds through a tightly regulated series of stages, from hematopoietic stem cells to differentiated plasma and memory cells. This process is directed by transcription factors such as PU.1, Ikaros, E2A, Pax-5 and BCL6. These transcriptional regulators govern the gene expression necessary for processes such as V(D)J recombination, somatic hypermutation and immunoglobulin class switching, all of which are essential for proper B cell lineage commitment and the maintenance of immunological tolerance [101].

Dysregulation of these pathways – whether through genetic or epigenetic alterations or as a consequence of chronic inflammation – can impair B cell function and contribute to the development of B cell malignancies. B cell development and maturation involve two distinct, consecutive processes; antigen-independent

precursor development in the bone marrow and antigen-dependent B cell activation in secondary lymphoid organs (SLOs) [101].

Bone marrow

The process of V (D) J recombination occurs in the bone marrow and involves the rearrangement of the heavy-chain (V_H , D_H , and J_H) and light chain (V_L - J_L) immunoglobulin gene segments. This process generates a B cell antibody repertoire capable of recognizing more than 5×10^{13} different antigens. After successful recombination and selection, naïve B cells migrate to SLOs for further activation [102].

T cell dependence

Depending on the nature of the activation, B cell responses can be T cell-independent or T cell-dependent. T cell-independent activation, which occurs without cytokine help from T cells, typically produces short-lived plasmablast and low-affinity antibodies. In contrast, T cell-dependent, cytokine-mediated activation elicits a more complex B cell response, generating plasma and memory B cells with high-affinity receptors and long-lasting immunity. However, this process is susceptible to genomic errors that can contribute to oncogenesis, and evidence suggests that the majority of myeloma cases originates from mutations acquired during T cell-dependent immune responses [103].

T cell dependent activation requires B cell receptor-mediated endocytosis of protein antigens. These antigens are subsequently degraded and presented on major histocompatibility complex class II (MHC II) molecules. Recognition of the presented peptide by a cognate T cell receptor induces an immunological synapse that triggers T cell activation [103].

Activation

T cell activation leads to the expression of CD40 ligand on T cells, which in turn induces CD40 signalling in B cells and the release of T cell-derived cytokines IL-4, IL-6 and IL-21 – essential for potent B cell activation [103].

However, IL-6 not only promotes B cell activation but also functions as a potent growth factor for plasma cells and myeloma cells [103]. The stimulation induced by T cells ultimately triggers B cell proliferation and germinal center formation, where B cells undergo affinity maturation through somatic hypermutation (SHM) and immunoglobulin class switching resulting in the generation of either plasma cells or memory B cells [103, 104].

Plasma cells

The newly generated long-lived plasma cells, capable of sustaining high levels of antibody secretion, migrate into specialized niches – particularly in the bone marrow – where they complete their maturation and persist for long period of times [105, 106].

The pathogenesis of multiple myeloma remains incompletely understood. However, the rapid proliferation and somatic mutation of the B cell receptor predispose B cells to oncogenic mutations that cause B cell malignancies, including plasma cell neoplasms such as myeloma. Nevertheless, the genetic alterations that occur in the germinal center are not sufficient to cause myeloma; rather, they impart the proliferative potential to plasm cells, which are normally non-dividing [103].

T helper (Th) cells

Immunological function

CD4⁺ together with CD8⁺ cells, constitute the majority of T lymphocytes. CD4⁺ cells are involved in multiple functions, including the activation of innate immune cells, B lymphocytes, cytotoxic T cells, and non-immune cells. In addition, CD4⁺ cells play an important role in the suppression of immune reactions [107].

Role of CD4⁺ T cells in multiple myeloma

Low CD3⁺CD4⁺ T-cell counts have been observed in myeloma patients. Those with reduced counts showed decreased progression free survival (PFS) and overall survival (OS) compared to patients with higher counts [108].

Development

Different signal transducer and activator of transcription (STAT) proteins drives the differentiation of naïve CD4⁺ T cells into various T helper (Th) cell subsets, depending on the cytokine environment [109]. Activated STAT induces the transcription of lineage-specific transcription factors—T-bet, generally associated with Th1; GATA3, typically linked to Th2; and ROR γ t, often correlated with Th17—although co-expression can occur, reflecting the plasticity between subsets [110].

Th1

Th1 cells are particularly important for the defense against viral and bacterial infections [109]. They produce interleukin-2 (IL-2), interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α), eliciting macrophage activation and inducing phagocyte-dependent responses and cell-mediated immunity [111].

Th2

Th2 cells are critical for the defense against helminth infections, through their signature cytokines IL-4, IL-5, 10 and IL-13 [109, 111]. These cytokines are responsible for inducing B-cell immunoglobulin switching to IgG1 and IgE, activating eosinophils, and inhibiting several macrophage functions [110, 111].

Th17

Th17 cells, vital for host defense against intra- and extracellular bacterial infections, fungal infections, and for regulating the gut microbiota, act through their typical cytokines; IL-17A, IL-17F, and IL-22 [109].

T follicular helper (Tfh) cells represent a distinct subset of CD4⁺ T helper cells whose primary role is to support germinal center reactions.

Tfh

Tfh cells are critical providers of T cell help to B cells, essential for germinal center formation, affinity maturation and development of most high affinity antibodies and memory B cells [112]. Tfh differentiation involves the transcription factor B cell lymphoma 6 (Bcl6) where IL-21 serves as a characteristic cytokine [112, 113].

MDSCs and Th cells

T cells are the main targets of myeloid-derived suppressor cells (MDSCs), and both PMN-MDSCs and M-MDSCs can inhibit T-cell proliferation and modulate T-cell differentiation into functional Th subsets [114]. The interaction between MDSCs, tumors, and tumor-derived factors contributes to T-cell dysfunction in malignancies. There is bidirectional crosstalk between T cells and MDSCs: while MDSCs suppress T-cell function, CD4⁺ T cells interacting with antigens presented by MDSCs can induce their conversion into non-specific suppressors [115].

Flow cytometry

In this thesis, flow cytometry has been one of the principal methods employed. Below is a description of the main principles underlying this technique.

Due to its high sensitivity, specificity and rapid analysis, multiparametric flow cytometry (FCM) is an important tool in a wide variety of disciplines, including immunology, tumor research, and in the diagnosis and monitoring of hematological malignancies [116, 117]. The characteristics that can be analyzed include cell size, membrane-bound and intracellular proteins, cytoplasmic complexity, as well as DNA and RNA content [117]. In MM, flow cytometry is useful for differentiating plasma cell dyscrasias (PCDs) from reactive plasmocytosis and other B cell neoplasms with extensive plasmocytic differentiation [116].

Based on different surface antigen expression and clonality analysis, FCM is useful for distinguishing abnormal plasma cells (APCs) from normal plasma cells (NPCs). Generally, plasma cells express a CD38+++ CD138+ CD45⁻ /low immunophenotype [118]. APCs typically show overexpression of CD56, CD117, CD28, CD33, and CD200 and low expression of CD19, CD45, CD27, CD81, CD38 [116].

Below is an overview of the principles of flow cytometric analysis.

Principles

FCM analyses single cells or particles suspended in a buffered salt-based solution as they pass through single or multiple lasers. Each particle is analyzed for visible light scatter and one or more fluorescence parameters [119].

The samples are prepared for fluorescence measurement through staining with different fluorescently conjugated antibodies, fluorescent dyes or transfection and expression of fluorescent proteins. A conventional flow cytometer consists of three systems: fluidics, optics and electronics [119].

Fluidics

In the fluidics system, sample cells are injected into the flow chamber together with a faster moving surrounding sheath fluid. As the two fluids pass through the narrow part of the flow chamber, the sample cells are compressed into a narrow stream by the process of hydrodynamic focusing. This results in single cell alignment, enabling individual cell analysis as they pass through the laser interrogation point [120].

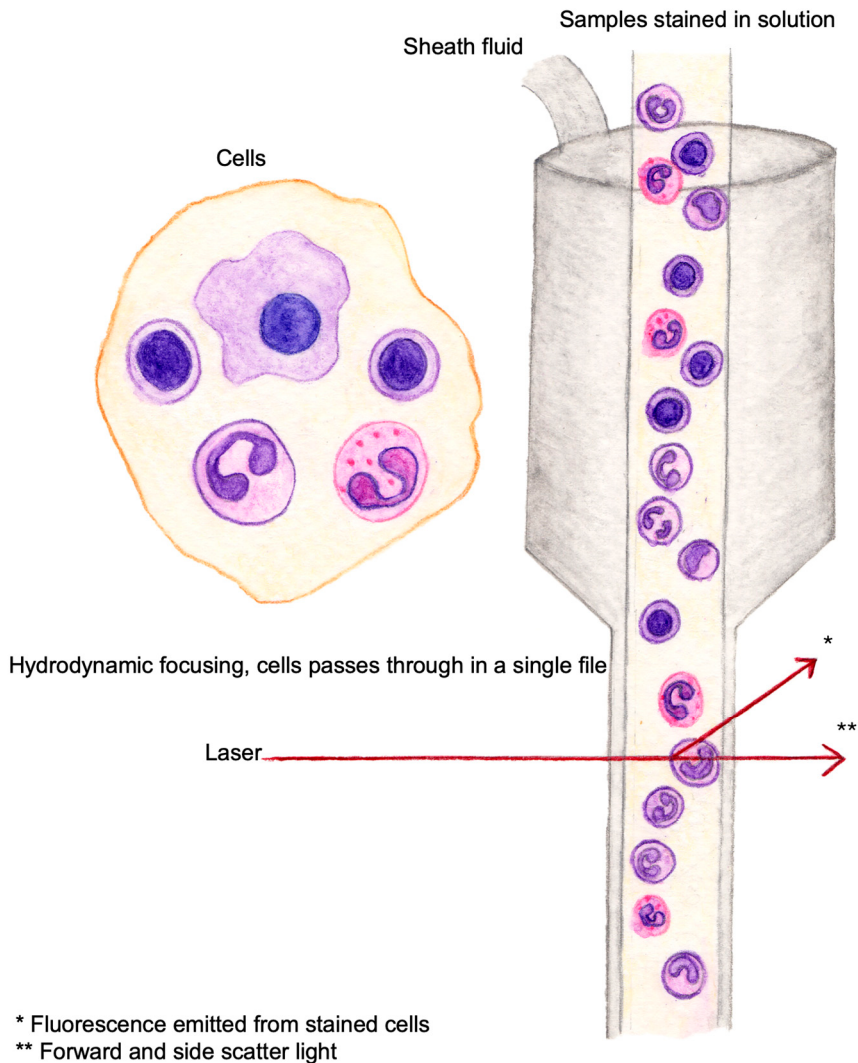


Figure 6 Cells suspended in sheath fluid are hydrodynamically focused to pass through the flow cell in a single file. Each stained cell is intercepted by a laser beam, generating forward and side scatter light as well as fluorescence signals. These emitted signals are detected and analyzed to determine properties such as the physical characteristics of cells (including size and internal complexity), chemical characteristics (encompassing intracellular components or metabolic markers), and immunophenotypic characteristics (based on the expression of specific surface or intracellular antigens).

Optics

The optical system comprises excitation optics and collection optics. The excitation optics, which consist of lasers that illuminate the sample, excite the fluorochromes attached to cells or particles. When the fluorochromes return to their ground state, the excess energy is released as photons, resulting in fluorescence [119].

The wavelength range at which a fluorochrome absorbs light and becomes excited is referred to as the excitation or absorption wavelength, while the wavelength range of the emitted light is termed the emission wavelength. The emitted wavelength is longer than the absorption wavelength; this difference is known as the Stokes shift [121]. The flow cytometer measures the intensity of the emitted light at specific wavelengths, allowing quantification of fluorescence signals from labeled cells or particles [119].

Commonly used lasers include violet (405 nm), blue (488 nm), green (532 nm), green (552), yellow-green (561 nm), red (640 nm) and ultraviolet (355 nm). A series of dichroic filters directs the fluorescent light according to predefined wavelengths, allowing light of a specific wavelength to pass through one detector while reflecting other wavelengths to different detectors. Bandpass filters define the range of the detected wavelengths, enabling the measurement of individual fluorochromes [119].

Visible light scatter is measured in two directions: forward scatter (FSC) and side scatter (SSC). FSC correlates with cell size, while SSC reflects the internal complexity or granularity of the cell [119]. Photomultiplier tubes (PMTs) and photodiodes (PDs) serve as optical detectors, which convert visible and fluorescent light signals into photoelectrons, subsequently transformed into voltage signals [122].

Electronics

The electronic system includes the hardware of the flow cytometer, a computer and a software that mediates communication between the machine and the computer for sample acquisition and analysis. The electronics primarily assists in signal generation by creating voltage pulses, where the intensity of light from each individual cell is measured by each photodetector as peak height intensity, area intensity and width [117].

The signal value detected by each photodetector for every cell are recorded digitally and may be amplified or reduced, either linearly or logarithmically. The electronic system can also apply a threshold to exclude signals generated by debris or unwanted particles during data acquisition. Additionally, the electronics can compensate for spectral overlap when fluorescence emissions from different dyes are detected simultaneously by multiple photodetectors. Data for each parameter from a specified number of cells is generated and stored in a flow cytometry (fcs) format [117].

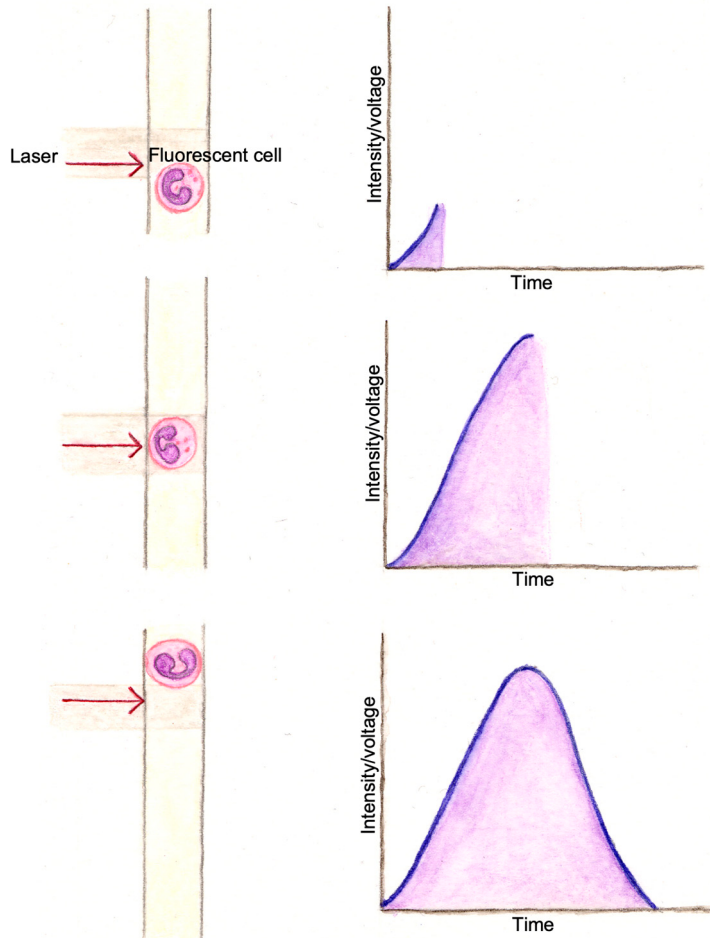


Figure 7 Illustration of a voltage pulse where a stained cell passes through the laser beam, scatters light and emits fluorescence that are captured by optical detectors. The resulting optical signals are converted into electrical voltage pulses, where the pulse height, width, and area correspond to the intensity, duration, and total emitted signal from the cell. Analysis of these pulse parameters enables the discrimination of individual cells based on their optical and fluorescence properties.

Data analysis

Software analysis provides several methods for visualizing and analyzing data, including dot plots, density plots, contour plots and histograms. Among these, dot plots and histograms are the most commonly used. A dot plot is a type of graph used during sample acquisition to analyze specific cell events. The signals detected by FSC, SSC, and fluorescence detectors are converted into values that appear on the dot plot. Each dot represents an event. A cluster of dots can be marked, being

referred to as a gate. Gating enables the selection of specific cell populations, allowing further analysis of their cell characteristics. A histogram illustrates the distribution of signal intensities emitted by the cells and shows how gates can be applied to distinguish different intensity ranges [117].

Fluorescence-Activated Cell Sorter (FACS)

Cell sorting is an important extension of conventional flow cytometry. Specific flow cytometers that not only analyze the properties of the cells but also enable the precise separation of cells into distinct subgroups based on critical phenotypic data are known as fluorescence-activated cell sorters (FACS). The principle of FACS lies in its ability to label cells with fluorescent dyes and then sort them according to their fluorescence intensity [117].

The two major types of cell sorters are mechanical and electrostatic sorters, with the latter being the most used. In mechanical sorting, only one cell type is sorted, while the remaining cells are discarded as waste. In contrast, the widely used electrostatic sorter allows for the simultaneous sorting of multiple cell types. In electrostatic cell sorting, hydrodynamic focusing directs individual cells past lasers. The fluid stream becomes electrically charged when a cell meets the sorting criteria. The charged droplets are then rapidly deflected through an electrical field into designated collection tubes [117].

In Paper I of this thesis, the ability of PMNs to perform phagocytosis and oxidative burst was evaluated using flow cytometry.

Phagocytosis

Labeling of bioparticles or bacteria with fluorescent dyes enables the detection of phagocytosis by flow cytometry. This is accomplished by using a pH sensitive dye that fluoresces upon exposure to the acidic environment of the phagosome, indicating that the bacteria has been phagocytosed [119].

Phagoburst

The capacity of PMNs to perform oxidative burst can be assessed by measuring the intracellular production of ROS. This is achieved using fluorescent probes such as dihydrorhodamine 123 (DHR 123) [123]. DHR123 is a non-fluorescent derivative of rhodamine 123 that is converted into a green fluorescent compound upon reaction with reactive oxygen intermediates, enabling detection by flow cytometry [124].

Aims and rationale

The overall aim of this thesis

The overall aim of this thesis was to explore how altered functions of neutrophils, myeloid-derived suppressor cells (MDSCs), and various T cell subsets contribute to immune dysregulation in multiple myeloma and its premalignant stages.

Paper I

Aim

The aim was to investigate the capacity of polymorphonuclear leukocytes (PMNs) to perform phagocytosis and oxidative burst in patients with MGUS, SMM, multiple myeloma and healthy controls and to assess the impact of lenalidomide treatment on PMN function.

Rationale

Multiple myeloma patients have a substantially elevated risk of developing bacterial infection, and infections constitute a major cause of morbidity and mortality. Neutrophilic granulocytes, also referred to as polymorphonuclear leucocytes (PMNs), are essential for immunological defense against bacterial infections. In paper I, we examined the ability of PMNs to perform phagocytosis and oxidative burst in patients with MGUS, SMM and NDMM, compared to healthy controls and patients receiving lenalidomide treatment. Peripheral blood (PB) and bone marrow (BM) samples were analyzed by flow cytometry using the commercially available PhagoTest and PhagoBurst kits. Patients with MGUS, SMM and NDMM showed reduced neutrophil function compared to healthy controls. The immune dysfunction was most pronounced in the bone marrow.

Interestingly, patients on lenalidomide treatment had restored neutrophilic function, both in terms of phagocytosis and oxidative burst, potentially reducing the risk of infection in MM patients.

Paper II

Aim

The intention was to examine whether MDSCs play an immunosuppressive role in the tumor microenvironment of MGUS and MM, and if their presence and function are linked to disease progression.

Rationale

The tumor microenvironment in the bone marrow might influence the progression from the premalignant condition MGUS to MM. MDSCs are thought to play an important regulatory role in various diseases and seem to correlate with poor prognosis in several types of malignancies. MDSCs represent a heterogenous group of myeloid-derived subsets characterized by their ability to inhibit T-cell responses. MDSCs can be further divided into the neutrophil like polymorphonuclear (PMN)-MDSC, the monocyte like (M)-MDSC and the immature early (e)MDSC. We investigated the levels and functions of all MDSC subsets in the BM of patients with MGUS and MM and compared them with the corresponding MDSC subsets in PB.

We found no elevated levels of MDSCs in either bone marrow or peripheral blood in MGUS or MM patients. PMN-MDSCs appeared to have only a minor inhibitory effect, whereas normal density granulocytes (NDGs) were more suppressive. Notably, eMDSC levels were decreased in the PB of MM patients. Our data indicate that MDSCs do not appear to play a key role in the pathogenesis of MM. Instead, NDGs may play a more significant role due to their stronger immunoregulatory effects.

Paper III

Aim

The aim was to investigate whether normal density granulocytes (NDGs) from healthy donors preferentially inhibited Th1 cells and to evaluate the myeloid-derived suppressive effect on different T-cell populations.

Rationale

NDGs can suppress T-cell responses in a manner similar to MDSCs. We investigated whether NDGs from healthy donors preferentially inhibited T helper 1 (Th1) cells and examined their myeloid-derived suppressive ability on different T-cell populations.

We found that NDG-induced suppression of T-cell proliferation was contact-dependent, mediated by integrin CD11b, and dependent on the production of reactive oxygen species (ROS) by NDGs. The inhibitory effect did not affect the CD8⁺/CD4⁺ ratio, indicating equal sensitivity in these populations. Further analysis of the CD4⁺ T helper subsets revealed that NDGs induced a loss of the Th1 surface marker CD183, independent of ligand binding to CD183. Additionally, incubation with NDGs led to reduced Th1, Th2 and Th17 cytokine production.

We therefore conclude that NDGs do not preferentially inhibit Th1 cells. Instead NDGs generally suppresses both Th cells and cytotoxic T-cells, while specifically downregulating the Th1 marker CD183.

Paper IV

Aim

The objective was to investigate whether normal NDGs in PB and BM could suppress T-cells in a manner similar to MDSCs.

Rationale

Neutrophils are the most abundant leukocytes in peripheral blood, with a well-established role in the innate immune system as the first line of defense against invading pathogens. However, in recent years, they have also been shown to play a broader role in regulating the adaptive immune system, including suppression of T-cell proliferation. We investigated whether NDGs in BM and PB of MM patients could suppress T-cells in a manner similar to MDSCs. We found that bone marrow NDGs from MM patients suppressed T-cell proliferation, in contrast to those from healthy donors. This inhibitory effect could not be explained by differences in the proportion of mature or immature NDGs in the bone marrow.

Moreover, NDGs isolated from the blood of both myeloma patients and healthy individuals inhibited T-cell proliferation and IFN- γ production. Unlike previous studies, NDGs did not need to be preactivated to exert its suppressive effects. Instead, they became activated during coculture, indicating that contact with activated T-cells is critical for their regulatory function. The inhibitory effect was dependent on the production of reactive oxygen species and could be reverted by the addition of its inhibitor, catalase. The MDSC-like suppression mediated by bone marrow NDGs may play an important role in supporting the growth of malignant plasma cells in MM.

Methodology

Paper I

Study population

Peripheral blood (PB) and bone marrow (BM) aspirates were collected at the Department of Hematology, Skåne University Hospital, Lund, between February 2017 and March 2020, from patients with MGUS, SMM, or newly diagnosed myeloma, as well as from healthy controls and patients in remission receiving lenalidomide treatment. None of the participants exhibited ongoing symptoms of infection.

Sample collection

PB was collected in vacutainer tubes containing sodium heparin, and BM aspirates were drawn into tubes with the culture medium RPMI 1640. Samples were stored in room temperature, protected from light and analyzed within 24 hours.

Phagocytosis assay

The ability of phagocytosis was investigated using the in vitro diagnostic (IVD) certified PhagoTest assay (Glycotop Biotechnology, GmbH, Germany). This flow cytometry-based method measures the percentage of PMN having ingested fluorescein-labeled opsonized *Escherichia coli* (*E. coli*). The geometric mean fluorescein intensity (MFI) corresponded to the number of ingested bacteria per cell.

Oxidative burst assay

The production of reactive oxygen species (ROS) was investigated using the IVD-certified PhagoBurst assay (Glycotop Biotechnology, GmbH, Germany) according to the manufacturer's protocol and gating strategy, after ex vivo activation with phorbol-12-myristate-13-acetate (PMA) or opsonized *E. coli*. The magnitude of ROS production was measured as geometric MFI in ROS-producing cells. At least 15,000 PMNs were collected based on forward and side scatter properties, after excluding dead cells and debris according to leucocytic DNA content.

Instrumentation and calibration

Fluorescence-activated cell sorting (FACS) Fortessa was used for the first 3.5 months. Thereafter, FACS Canto II was used. Both instruments employed FACS Diva software for data collection. The gain on the FACS Canto II was adjusted using several repeated samples to achieve MFI values comparable to those obtained with the FACS Fortessa.

Data analysis software and gating strategy

Kaluza software (Beckman Coulter, Brea, CA) was used for analysis, and the gating strategy was performed according to the manufacturer's instructions. The PhagoBurst assay failed in five cases, and in four cases only BM samples were collected. The PhagoTest analysis failed in seven cases, and in five cases only BM samples were collected. Failures were mainly due to FACS instrument issues or nonfunctioning positive controls.

Immunophenotyping

PB samples were collected in heparin tubes, and BM aspirates were collected in tubes containing the culture medium RPMI 1640. Specimens were stored overnight at room temperature. Within 24 hours of sampling, PB and BM erythrocytes were lysed with 0.84% NH_4Cl . The remaining cells were stained with a neutrophil antibody panel. Samples were processed on FACS Aria Fusion (BD) and analyzed using Kaluza software. Single cells were gated based on forward scatter height and area properties. PMNs were gated based on CD45 expression and side scatter properties. From this gate, neutrophils were defined as $\text{CD45}^+\text{CD14}^-\text{CD193}^-$ cells. Neutrophils were further subdivided into mature, immature, and CD11b^- immature populations based on their expression of CD11b and CD62L.

Statistical analysis

The Kruskal-Wallis test was used for comparisons involving more than two groups, followed by Dunn's multiple comparison test. Correlations were assessed using Spearman's rank correlation test. Statistical analyses were performed using GraphPad Prism v9.1.0. P-values <0.05 were considered statistically significant.

Paper II

Study population

Peripheral blood (PB) and bone marrow (BM) aspirates were collected at the Department of Hematology, Skåne University Hospital, Lund between 2018 and 2021 from patients with recently diagnosed MGUS or MM without treatment. None of the participants exhibited ongoing symptoms of infection.

Isolation of MDSC subsets

PB and BM mononuclear cells were isolated by Lymphoprep (Stemcell Technologies, Cambridge, UK) density gradient centrifugation and stained with an MDSC antibody panel. Samples were kept on ice and sorted on a FACS Aria Fusion (BD, Franklin Lakes, NJ, USA) into PMN-MDSCs (CD45⁺Lin⁻CD33⁺/dimHLA-DR⁻CD66b⁺CD11b⁺), M-MDSCs (CD14⁺HLA-DR⁻/low), and e-MDSCs (CD45⁺Lin⁻CD33⁺/dimHLA-DR⁻CD66b⁻CD11b⁺). Cells were sorted into RPMI-1640 medium without L-glutamine (Sigma, Malmö, Sweden) supplemented with 10% fetal calf serum, 10⁴ U/ml penicillin, 10 ng/ml streptomycin, and 2 mM L-glutamine (all from Gibco™, Thermo Fisher, Waltham, MA, USA). The MDSCs were used in the T-cell proliferation assay immediately after sorting.

Isolation of neutrophils

Neutrophils were isolated from BM or PB by density gradient centrifugation (Lymphoprep) followed by erythrocyte lysis with 0.84% NH₄Cl. Cells were further purified using the EasySep™ Human Neutrophil Isolation Kit (Stemcell Technologies, Cambridge, UK). Purity, assessed by flow cytometry, was >95%. Impurities did not form any clear cell populations and was considered debris mixed with an occasional lymphocyte or monocyte. In a control experiment, neutrophils were isolated from the lysed RBC layer by FACS.

T-cell proliferation assay

CFSE-labeled healthy donor T cells (1×10⁵) were co-cultured with FACS-sorted PMN-MDSCs, M-MDSCs, or eMDSCs (5×10⁴). T-cell proliferation was assessed after 3 days using a CytoFLEX flow cytometer (Beckman Coulter). In some experiments, PMN-MDSCs and eMDSCs were preactivated with 1 mM of the neutrophil activator N-formylmethionyl-leucyl-phenylalanine (fMLF). The ROS inhibitor catalase (Sigma, Malmö, Sweden) was added in certain experiments to assess the role of ROS.

Statistical analysis

Due to a low sample size, the data was assumed to be non-normally distributed and nonparametric tests were used. For more than two groups, the Kruskal Wallis test was used to compare groups. Dunns's multiple comparison test was used for multiple comparisons. When comparing two groups, the Mann Whitney test was used. We assumed the difference in proliferation to be normally distributed. In groups with more than 4 experiments, paired t-test was used to evaluate significance. All analyses were conducted in GraphPad Prism v9.

Paper III

Study population

The study included 19 healthy donors without any known diseases or ongoing infections, consisting of 12 females and 7 males. The median age of the overall population was 44.5 years, with 47.9 years for the women and 38.7 years for the men.

Isolation of T-cells

PB was collected in 5 mL heparin tubes, and the mononuclear cells (PBMCs) were isolated by Lymphoprep™ (Axis-Shield) separation. T cells were then purified using the EasySep™ human T cell isolation kit (Stemcell technologies) with >97% purity; remaining cells (1-3%) were mainly debris or occasional monocytes. For proliferation assays, cells were stained with 1 μ M carboxyfluorescein succinimidyl ester (CFSE) (BD Horizon) for 10 min at 37°C.

Isolation of neutrophils

Normal density granulocytes (NDGs) were isolated from PB collected in heparin tubes. PBMCs were separated by Lymphoprep™, followed by RBC lysis with 0.84% NH₄Cl. NDGs were then purified using the EasySep™ Human Neutrophil Isolation Kit (Stemcell technologies). Flow cytometry confirmed >95% purity; remaining cells were considered debris with occasional lymphocytes or monocytes.

T-cell proliferation assay with NDGs

CFSE-stained T-cells (100,000/well) were cocultured with NDGs (5,000–50,000/well) for 3 days in flat-bottom 96-well plates (Eppendorf, Hamburg, Germany) coated with anti-CD3 (1 μ g/ml, clone OKT-3, Invitrogen) and anti-CD28

(2 µg/ml, clone CD28.2, Invitrogen) antibodies. Control wells were pretreated with phosphate-buffered saline (PBS). Cultures contained RPMI-1640 without L-glutamine (Sigma) supplemented with 10% fetal calf serum (Gibco™), penicillin (104 U/ml), streptomycin (10 ng/ml), and L-glutamine (2 mM) in a final volume of 200 µl. To assess NDG-mediated inhibition of T-cell proliferation, cocultures were treated with the ROS inhibitor catalase (Sigma), the arginase inhibitor nor-NOHA (300 µM, AH diagnostics), the TGF- β inhibitor Galunisertib (0.1 µg/ml, Cayman), or anti-CD11b antibody (1 µg/ml, clone ICRF44, eBioscience). All substances were used in concentrations that preserved T-cell viability. In some assays, neutrophils were activated with fMLF (1 µM). After 3 days, supernatants were stored at -80 °C and T-cell proliferation was analyzed using a CytoFLEX (Beckman Coulter).

Cytometric Bead Array (CBA)

The supernatant from the 3-day long T-cell and NDGs cocultures were thawed, diluted 1:10, and used in a human Th1/Th2/Th17 cytometric bead array (CBA). The presence of interleukin-(IL-) 2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF), interferon (IFN)- γ , and IL-17A was measured. The data was analyzed using Flow Cytometric Analysis Program (FCAP) array multiplex assay analysis software.

Time experiment

Healthy donor T-cells (100,000 cells/well) were cocultured with NDGs (50,000 cells/well) in a final volume of 200 µl per well. At multiple time points between 0.5 and 72 h, cells were collected and analyzed. Viability was assessed using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Pharmingen), and T-cell phenotype was evaluated on a CytoFLEX flow cytometer using a T-cell antibody panel. Flow cytometry data were analyzed with Kaluza software (Beckman Coulter).

Statistical analysis

Because the data were not normally distributed, nonparametric tests were applied. The Wilcoxon matched-pairs signed-rank test was used to compare groups. All analyses were conducted in GraphPad Prism v9.

Paper IV

Study population

Peripheral blood (PB) and bone marrow (BM) aspirates were collected at the Department of Hematology, Skåne University Hospital, Lund, between 2018 and 2020 from 16 patients with newly diagnosed multiple myeloma (MM). In addition, 5 patients with previously diagnosed myeloma who had already received treatment for the disease, as well as 31 healthy controls, were included. None of the participants had ongoing symptoms of infection.

Normal Density Neutrophil Isolation

NDGs were isolated from the RBC layer after density gradient centrifugation using Lymphoprep (Stemcell Technologies, UK). The RBC pellet was lysed with 0.84% NH₄Cl for 15 min, and NDGs were further purified using the EasySep™ Human Neutrophil Isolation Kit (Stemcell Technologies). Blood NDGs with >95% purity and bone marrow NDGs with 90% purity were used for analysis. The remaining cells consisted mostly of debris with occasional lymphocytes or monocytes and did not form distinct populations.

T-cell proliferation assay

Mononuclear cells were isolated from PB of healthy donors using Sodium Heparin Vacutainer™ CPT tubes (BD). T-cells were purified from the mononuclear cell layer with the EasySep™ Human T-cell Isolation Kit (Stemcell Technologies) at >97%; the remaining 1-3% consisted mostly of debris or occasional monocytes. Purified T-cells were labelled with 1 µM carboxyfluorescein succinimidyl ester (CFSE) (BD Horizon) in PBS and washed. CFSE-labelled T-cells (10⁵ cells) were then added into a flat-bottom 96-well plate (Eppendorf) coated with anti-CD3 (1 µg/ml, clone OKT-3, Invitrogen) and anti-CD28 (2 µg/ml, clone CD28.2, Invitrogen) antibodies. Cells were cultured in RPMI-1640 medium (without L-glutamine, Sigma) supplemented with 10% fetal calf serum (Gibco™, Thermo Fisher), 10⁴ U/ml penicillin (Gibco™, Thermo Fisher), 10 ng/ml streptomycin (Gibco™, Thermo Fisher), and 2 mM L-glutamine (Gibco™, Thermo Fisher). NDGs (5 × 10⁴ cells) were added to the healthy T-cells (10⁵ cells) at a 1:2 ratio, with additional ratios (1:1, 2:1, and 3:1) tested in selected experiments. The effects of the ROS inhibitor catalase (Sigma) and the arginase inhibitor nor-NOHA (300 µM, AH diagnostics) were also evaluated. To activate neutrophils, N-formylmethionyl-leucyl-phenylalanine (fMLF) was included in a subset of experiments. After 3 days, supernatants were collected and stored at -80°C, and proliferation of CFSE-labeled T-cells was measured by flow cytometry (Accuri C6+, BD).

Cytokine production measurement

Interferon gamma (IFN- γ) production was measured in the supernatants using a Quantikine ELISA (R&D system). All samples were diluted 1:50.

Flow cytometry

PB and BM samples were lysed using 0.84% NH₄Cl and stained with the following antibodies: CD14—PerCP Cy5.5 (clone: M5E2, BD), CD80—FITC (Santa Cruz Biotechnology), Siglec8—PE (clone: 7C9, BioLegend), CD16—APC H7 (clone: 3G8, BD), CD66b—Alexa flour 700 (clone: G10F5, BioLegend), CD184—APC (clone: 12G5, eBioscience), CD11b—BV785 (clone: ICRF44, BioLegend), CD62L—BV650 (clone: DREG-56, BioLegend), CD193—V510 (clone: 5 E8, BD), CD45—V450 (clone: 2D1, BD), HLA-DR—PE Cy7 (clone: L243, BD), and CD69—PE/- DAZZLE (clone: FN50, BioLegend). The samples were then analyzed on FACS Aria Fusion (BD).

Single cells were gated, and neutrophils identified as CD45⁺CD14⁻CD193⁻. They were subdivided into mature, immature, and CD11b⁻ immature subsets based on CD11b and CD62L expression. The same antibody panel was used to assess activation pre- and post-isolation and to analyze purity, with acquisition on a Cytotflex (Beckman Coulter, Brea, CA, USA). Data were analyzed using Kaluza software (BD).

Statistical analysis

The data is not normally distributed; therefore, nonparametric tests were used. Wilcoxon matched pairs signed rank test or Mann-Whitney was used to compare groups. Spearman correlation test was used to analyze the correlation between age and suppressive ability. All analyses were conducted in GraphPad Prism v8.

Results

Paper I

Phagocytosis is essential for neutrophils to clear infections. Patients with NDMM, SMM, and MGUS had circulating PMNs with a significantly reduced capacity for phagocytosis, measured as the percentage of PMNs that had ingested opsonized *E. coli*, compared with healthy controls ($p=0.031$, Figure 8A).

In addition, the number of ingested bacteria per cell in peripheral blood was also reduced in NDMM patients compared with patients receiving lenalidomide treatment ($p=0.045$, Figure 8B). The reduced phagocytic function was similarly observed in bone marrow (Figure 8C-E), where patients with NDMM, SMM and MGUS had a reduced percentage of PMNs capable of phagocytosing *E. coli* compared with healthy controls ($p=0.039$) and patients receiving lenalidomide treatment ($p=0.0243$, Figure 8C).

The number of phagocytized bacteria per cell in BM was reduced in NDMM patients, in contrast to the lenalidomide cohort ($p=0.033$, Figure 8D). After subdividing patients into NDMM, SMM and MGUS groups, the percentage of PMNs that had ingested *E. coli* was reduced in the SMM group compared with healthy controls ($p=0.043$) and patients in lenalidomide treatment ($p=0.021$, Figure 8E). No significant differences were observed in the NDMM and MGUS groups.

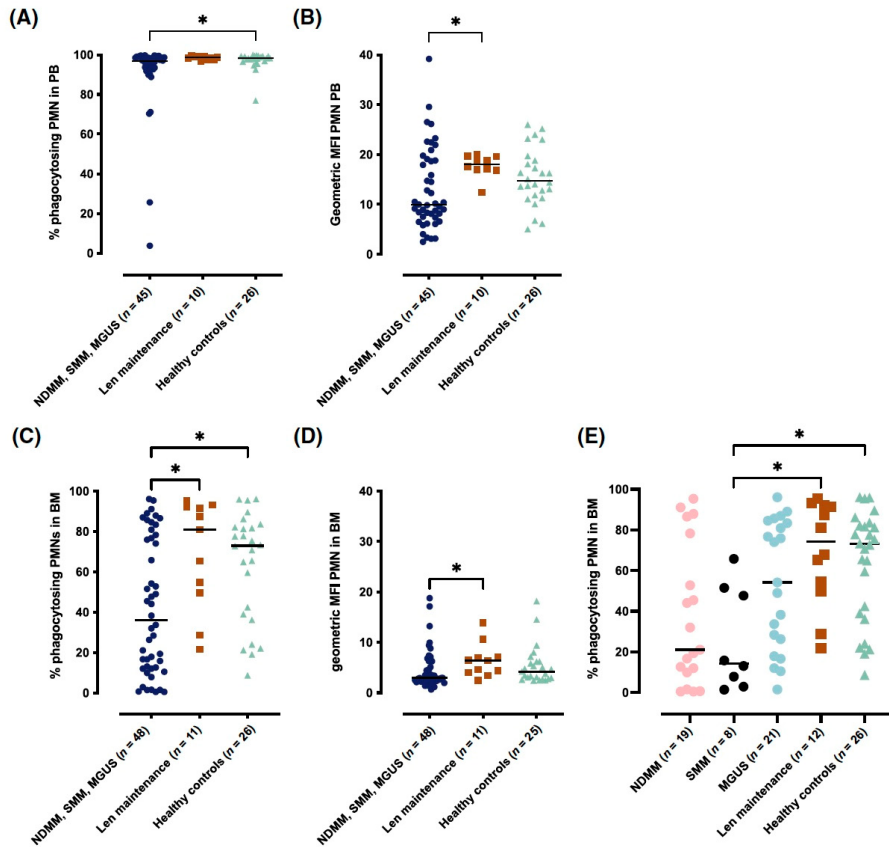


Figure 8 Blood and bone marrow of polymorphonuclear leukocyte (PMN), and their ability to phagocytose in newly diagnosed patients with newly diagnosed multiple myeloma (NDMM), smoldering MM (SMM), and monoclonal gammopathy of undetermined significance (MGUS), Lenalidomide-treated MM patients, and healthy controls. (A) Phagocytosis is measured as the percentage of blood PMNs that have ingested fluorescein-labeled opsonized *E. coli*. (B) Phagocytosis is measured as the number of ingested bacteria per cell (geometric MFI) in the blood. (C) Phagocytosis measured as the percentage of bone marrow PMNs that have ingested fluorescein-labeled opsonized *E. coli*. (D) Phagocytosis measured as the number of ingested bacteria per cell (geometric MFI) in the bone marrow. (E) Phagocytosis measured as the percentage of bone marrow PMNs that have ingested fluorescein-labelled opsonized *E. coli*, where the newly diagnosed patient group has been divided into separate groups depending on disease. Significance was tested using the Kruskal–Wallis test and Dunn's multiple comparison test was used to test the difference between groups (* $p < 0.05$). Horizontal lines indicate median

Of note, several patients in the newly diagnosed, untreated group had bone marrow neutrophils with markedly reduced phagocytic capacity. Approximately 50% of NDMM and SMM patients and 25% of MGUS patients had fewer than 20% phagocytosing PMNs, compared with only a few percent among healthy controls.

Altogether, our findings indicate that neutrophils from patients with newly diagnosed plasma cell disorders exhibit impaired phagocytic function, which is restored by lenalidomide treatment.

The capacity of oxidative burst is impaired in circulating neutrophils of patients with plasma cell disorders

Oxidative burst in neutrophils is the rapid release of ROS in order to eliminate invading pathogens. The group with plasma cell disorders exhibited a reduced proportion of ROS-producing neutrophils in blood upon stimulation with physiological stimuli, with *E. coli*, compared to patients receiving lenalidomide treatment ($p=0.0020$). Similarly, the plasma cell disorder group demonstrated a lower percentage of ROS-producing neutrophils than healthy controls after strong stimulation with PMA ($p=0.029$). When analyzing only the ROS-producing cells, the magnitude of ROS production was comparable between groups following stimulation with *E. coli*. In contrast, the plasma cell disorder group exhibited reduced ROS production compared to healthy donors after stimulation with PMA ($p=0.041$).

After stimulation of bone marrow PMNs with *E. coli*, the newly diagnosed group exhibited a lower percentage of PMNs that produced ROS in relation to the lenalidomide-treated group ($p=0.031$) and the healthy controls ($p=0.028$, Figure 9A). Upon stimulation of BM PMN with PMA, the patients with plasma cell disorders demonstrated a diminished percentage of PMN that produced ROS compared to healthy controls ($p=0.00020$), whereas no difference was seen compared with the lenalidomide-treated group (Figure 9B). In fact, the lenalidomide-treated group also had a reduced percentage of PMN that produced ROS compared with the healthy controls ($p=0.0040$; Figure 9B).

The degree of ROS production was higher in the lenalidomide-treated group compared with the patient group upon stimulation with *E. coli* ($p=0.0053$; Figure 9D). However, no difference was observed between the groups when ROS production was stimulated with PMA (Figure 9E). When subdividing the patient group into MGUS, SMM, and NDMM, we observed a lower percentage of BM PMN producing ROS in the SMM group compared with healthy controls ($p=0.022$) and with patients in the lenalidomide-treated group ($p=0.017$; Figure 9C). The MGUS and NDMM groups did not show a significantly reduced percentage of ROS-producing BM PMN. Nevertheless, these groups contained a higher proportion of individuals with $<20\%$ ROS production relative to the lenalidomide-treated group and the healthy controls.

Overall, these data indicate that PMN from newly diagnosed patients have impaired ROS production. Patients treated with lenalidomide did not exhibit this impaired function, except upon BM PMN stimulation with PMA.

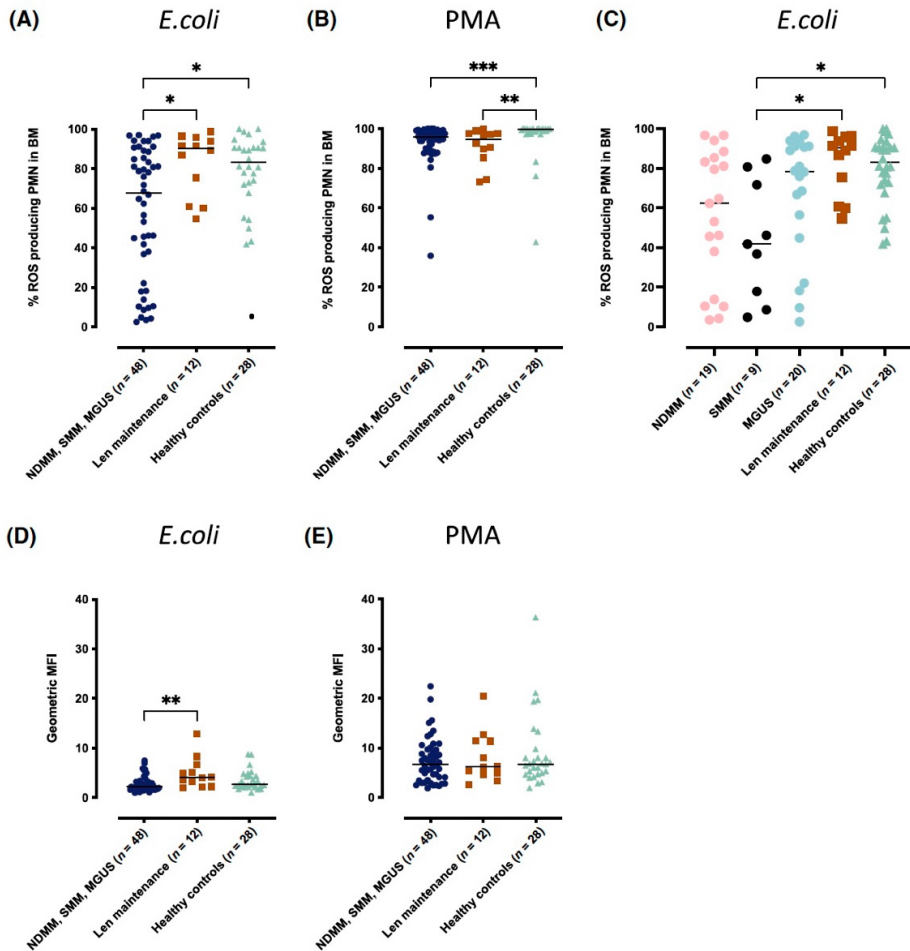


Figure 9 Bone marrow polymorphonuclear leukocyte (PMN) and their ability to perform oxidative burst when stimulated with opsonized *E. coli* or phorbol-12-myristate-13-acetate (PMA), in patients with newly diagnosed multiple myeloma (NDMM), smoldering MM (SMM), and monoclonal gammopathy of undetermined significance (MGUS), lenalidomide treated MM patients, and healthy controls. (A) Percentage of PMNs, which have produced reactive oxygen species (ROS) after incubation with opsonized *E. coli*. (B) Percentage of PMNs, which have produced ROS after stimulation with PMA. (C) Percentage of PMN that have produced ROS after incubation with *E. coli*, where the patient group has been divided into separate groups depending on disease. (D) Geometric MFI correspond to degree of ROS production after stimulation with *E. coli*. (E) Geometric MFI correspond to degree of ROS production after stimulation with PMA. Significance was tested using the Kruskal–Wallis test and Dunn's multiple comparison test was used to test difference between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Horizontal lines indicate median.

Impaired neutrophil function could not be explained by hypercalcemia, increased inflammatory activity, or degree of tumor cell infiltration

One hypothesis was that untreated NDMM patients might have an increased tendency for hypercalcemia due to enhanced osteoclast activity and renal impairment, possibly affecting intracellular signaling in phagocytosis. In this cohort, three NDMM patients and one MGUS patient had a calculated corrected calcium concentration in plasma (P-Ca) slightly above the normal range; however, there was no significant difference in P-Ca, plasma albumin (P-Alb), or calculated corrected P-Ca levels among the different patient cohorts.

Furthermore, no correlation was observed between these parameters and BM PMN oxidative burst or phagocytosis. Moreover, neutrophil dysfunction could not be explained by increased inflammatory activity, as measured by serum C-reactive protein, plasma haptoglobin, or plasma orosmucoid. In untreated patients (NDMM, SMM and MGUS), there was also no correlation between the percentage of plasma cell infiltration and phagocytic capacity.

Neutrophil maturation and activation

To investigate whether neutrophil maturation and activation status affected phagocytic activity and oxidative burst, BM and PB samples were stained with an antibody panel targeting neutrophil and activation markers, including CD11b, CD62L, CD66b, and CD69. Consistent with our previous findings [77], no differences in neutrophil expression of maturation and activation markers were observed.

Paper II

Myeloid-derived suppressor cells (MDSCs) are a heterogenous group of myeloid-origin cells that exhibit immunoregulatory functions, characterized by their inhibitory effect on T cells. Together with PMN-MDSCs and M-MDSCs, early MDSC (eMDSC) constitute the different MDSC subsets.

Circulating eMDSCs are decreased in MM patients

As MM originates in the bone marrow, we investigated the levels of all BM MDSC subsets. Because blood is more accessible and more commonly analyzed, we compared BM levels with those in PB. No statistically significant differences in the levels of PMN-MDSCs, M-MDSCs or eMDSC in BM were observed among MGUS, MM and healthy donors.

Similarly, no significant differences in PMN-MDSCs or M-MDSCs in PB were detected between cohorts. However, eMDSCs were significantly decreased in the PB of MM patients compared with healthy controls ($p=0.041$).

LOX-1 is not a specific marker for patient PMN-MDSC

Lectin-type oxidized LDL receptor 1 (LOX-1) has been suggested as a specific PMN-MDSC marker, reported to be highly expressed on PMN-MDSC from cancer patients. In MM, increased LOX-1-expression in BM compared to PB has also been described [125]. Therefore, we investigated the levels of LOX-1 to determine whether it could serve as a specific PMN-MDSC marker in MM.

BM PMN-MDSCs from MM patients have a higher expression of LOX-1, compared to blood PMN-MDSCs from the same patient ($p=0.0004$). However, PMN-MDSCs from MGUS patients and healthy donors also express LOX-1 and in similar levels as PMN-MDSCs from MM patients. These findings indicate that LOX-1 is not a specific PMN-MDSC marker, only restricted to cancer patients. In addition, LOX-1 expression levels seem not to be increased in the BM of MM patients and are present also in a healthy setting.

The isolated MDSC subsets are not strong inhibitors of T-cell proliferation

The main characteristic of all MDSC subtypes is their ability to suppress T-cell responses, including T-cell proliferation. There was considerable variability in the ability of BM PMN-MDSCs to inhibit T-cell proliferation between individuals. No trend toward differences in inhibitory effect was observed among the different groups. Nevertheless, PMN-MDSCs from all cohorts showed a small inhibitory effect, although the number of successful experiments and the level of inhibition was too low to reach statistical significance.

M-MDSCs in BM had no inhibitory effect on T-cell proliferation, instead a minor increase of proliferation with only a few percent was seen in most experiments reaching significance for the healthy controls ($p=0.02$). A similar trend was observed for PB M-MDSCs. T cell proliferation was neither affected in BM nor PB after coculturing with eMDSCs.

In summary, none of the MDSCs populations had a strong inhibitory effect on T cell proliferation.

PMN-MDSC are not as suppressive as NDG in T-cell proliferations assays

As PMN-MDSCs and NDGs are considered very similar, we compared their ability to suppress T-cell proliferation. Previous published data from our group showed that NDGs suppress T-cell responses in a manner similar to MDSCs [77]. We found PMN-MDSC to be less suppressive than NDGs. PB NDGs from both healthy donors ($p<0.0001$) and MM patients ($p<0.001$) had a strong inhibitory effect on T-cell proliferation, whereas PB PMN-MDSC exhibited only a marginal suppressive effect.

When comparing the suppressive effect of PB PMN-MDSCs with NDGs from the same MM patients ($n=4$), NDGs were significantly more suppressive than PMN-MDSCs ($p=0.038$). In the BM, only MM NDGs had a major suppressive effect ($p=0.0006$). MM PMN-MDSCs, healthy donor NDGs and healthy donor PMN-MDSCs were less suppressive. In all but one case, PMN-MDSCs were less suppressive than NDGs from the same donor ($n=5$). Altogether, these data indicate that PMN-MDSCs are less suppressive than NDGs.

Activation of circulating PMN-MDSC increases suppression of T-cell proliferation

Our group [77] and others [126] have previously shown that the neutrophil activator N-Formylmethionyl-leucyl-phenylalanine (fMLF) can enhance NDG suppression of T-cell proliferation. Therefore, we investigated whether fMLF activation of PMN-MDSCs could increase their suppressive ability. fMLF had only a minor effect on BM PMN-MDSCs but exerted a more pronounced effect on PB PMN-MDSCs, indicating that the suppressive effect of PB PMN-MDSC can be enhanced by fMLF activation.

ROS mediates the suppressive effect of PMN-MDSCs on T-cell proliferation

We have previously demonstrated that ROS are important for NDG-mediated T-cell inhibition [77, 127]. To investigate whether PMN-MDSC mediated suppression is dependent on ROS, the inhibitor catalase was added to the co-cultures. Catalase abrogated the suppression of T-cell proliferation in both BM and PB from MGUS and MM patients as well as healthy donors, indicating that the suppressive effect might be mediated by ROS.

Paper III

We have previously demonstrated that NDGs has an inhibitory effect on T-cell proliferation through the production of ROS [77]. In this study, we further evaluated the potential inhibitory mechanisms of neutrophils in the suppression of T-cell proliferation. T cells were co-cultured with NDGs alone or with NDGs in combination with an inhibitor. After 3 days, T-cell proliferation was measured, and the effects of catalase, N-hydroxy-nor-L-arginine (nor-NOHA), Galactose oxidase (GAL), and anti-CD11b were determined. As control, T cells and activated T cells were cultured alone with the same inhibitors without any signs of toxicity or affection on proliferation.

Consistent with our previously published data, non-activated NDGs inhibited T cell proliferation ($p < 0.0001$), with an inhibition range of 33% to 78% (Figure 10 (a)). The ROS inhibitor catalase partially preserved T-cell proliferation ($p = 0.048$). Blocking CD11b with an anti-CD11b antibody partially restored T-cell proliferation by interfering with cell-cell contact between NDGs and T cells (Figure 10 (a)). In contrast, addition of the arginase inhibitor nor-NOHA had no effect on T-cell proliferation. Transfer of supernatant from cultured NDGs to stimulated T-cells had no effect on proliferation. The TGF- β inhibitor GAL was added to investigate whether the inhibition of T-cell proliferation was mediated by TGF- β activated matrix metalloprotease. GAL did not restore proliferation.

NDGs activated with fMLF demonstrated similar patterns of inhibition and restoration (Figure 10 (b)). fMLF activated NDGs inhibited T-cell proliferation ($p=0.0039$) with an inhibition range of 53% to 93%. In all cases, fMLF-activated NDGs were more inhibitory than non-activated NDGs from the same donors. Catalase ($p=0.016$) and anti-Cd11b ($p=0.031$) both restored proliferations, whereas nor-NOHA and GAL did not. These data indicate that both nonactivated and activated NDGs are potent T-cell inhibitors, and that the inhibition is mediated by ROS production and cell-cell contact, but not through arginase or TGF- β activation.

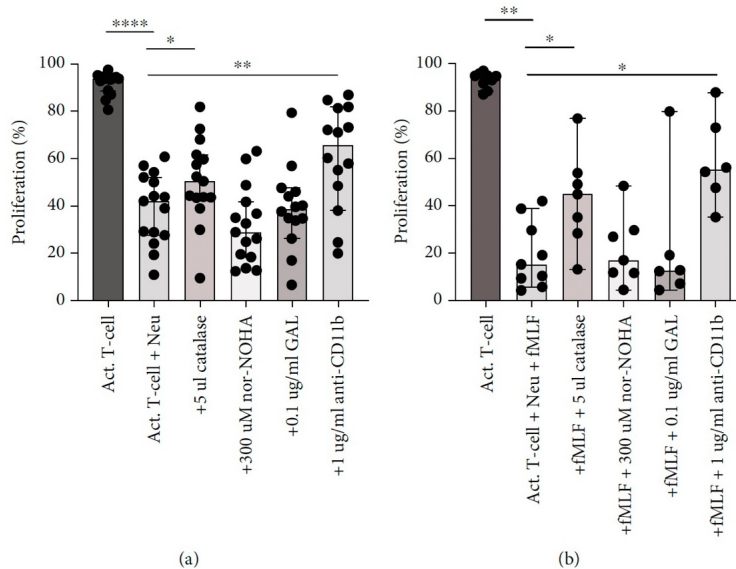


Figure 10 Inhibition of T-cell proliferation by NDGs and the effect of catalase, nor-NOHA, GAL, and CD11b on proliferation restoration. (a) Inhibition of T-cell proliferation by nonactivated NDGs (n = 15). Neutrophils suppress T-cell proliferation (p < 0.001), and addition of ROS inhibitor catalase protects T-cell proliferation (p = 0.048). The arginase inhibitor nor-NOHA did not have a protective effect and neither did TGF- β inhibitor GAL. Addition of anti-CD11b antibody protected T-cell proliferation (p = 0.0040). (b) Inhibition of T-cell proliferation by fMLF activated NDGs (n = 9). fMLF activated NDGs inhibit T-cell proliferation (p = 0.0039), and the proliferation was improved by catalase (p = 0.016) and anti-CD11b (p = 0.031). nor-NOHA and GAL did not improve proliferation. Graphs indicate median with 95% CI, and statistical significance was tested using Wilcoxon matched pairs signed rank test.

NDG-induced T-cell suppression emerges rapidly, beginning within the first few hours of coculture

CD25 (IL-2 receptor α -chain) and CD69 (transmembrane C-type lectin) are common T-cell markers used to monitor T-cell activation. CD69 is an early activation marker involved in T-cell proliferation, whereas CD25 represents a later activation marker. To investigate the rate of T-cell response to NDGs, the expression of CD25 and CD69 was measured on nonactivated T cells, activated T cells, and activated T cells in coculture with NDGs. Measurements were performed at six time points between 0.5 and 5 hours. T-cells and NDGs remained viable throughout the experiment. During the first 5 hours of coculture, both CD25 and CD69 expression increased on activated T-cells (Figure 11) CD69 increased more than CD25, as expected, since it is a marker of early T-cell activation. NDGs inhibited the accumulation of CD25 and CD69 on the T-cell surface, indicating that NDG-mediated inhibition is rapid and occurs within the first few hours of coculture.

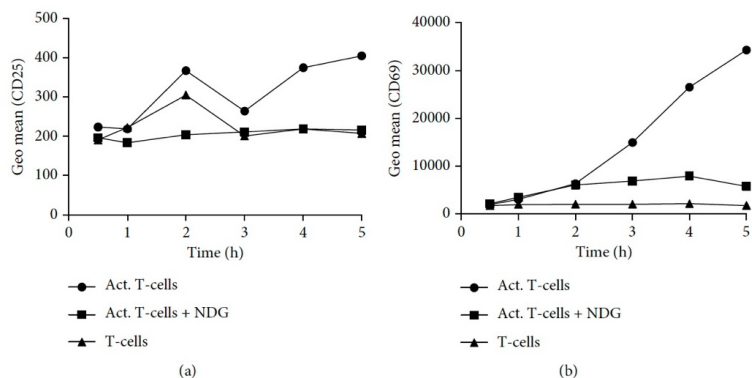


Figure 11 CD25 and CD69 expression on CD3⁺ T-cells during 0.5–5 hours coculture with NDG. (a) The geometric mean of CD25 on T-cells, activated T-cells, and activated T-cells cocultured with NDGs for 0.5–5 h. (b) The geometric mean of CD69 on T-cells, activated T-cells, and activated T-cells cocultured with NDGs for 0.5–5 h. Graphs show the median of 5 experiments.

NDGs Downregulate the Th1 Cell Surface Marker CD183 on T-Cells

Reduced IFN- γ is commonly used to represent MDSC-induced inhibition of cytokine production in T cells. Since IFN- γ is mainly produced by CD8⁺ cells and Th1 cells, we hypothesized that NDGs might have a differential inhibitory effect on the Th subset. To investigate this, T cells were cocultured with NDGs at a 1:2 ratio for 0.5–72 hours to track the levels of CD4⁺ and CD8⁺ T cells over time. The CD4⁺ cells were gated into Th1, Th2, and Th17 cells based on their expression of CD183 (CXCR3) and CD196 (CCR6). Th1 were defined as CD3⁺CD4⁺CD183⁺CD196⁻ cells, whereas Th2 were characterized as CD3⁺CD4⁺CD183⁻CD196⁻ and Th17 cells as CD3⁺CD4⁺CD183⁻CD196⁺.

We found no change in the ratio of CD4⁺ to CD8⁺ T cell subsets. However, Th1 decreased already after 30 minutes after coculture and did not recover during the 5-hour period (Figure 12 (a)–(c)). In contrast, both Th2 and Th17 subsets increased (Figure 12 (d)–(e)). In the coculture samples, CD183 expression of Th1 cells declined at 30 minutes, and was completely lost after 120 minutes (Figure 12 (f)). No decline of CD183 was observed in controls incubated without NDGs (Figure 12 (f)). Activated T-cells increase in size and autofluorescence, making it impossible to accurately track the different T-helper subsets after 5 hours. In contrast, this does not occur when T-cells are cultured without stimulation, as they do not proliferate, which makes it possible to observe the different subsets even after 5 hours of culture. When comparing nonactivated T cells cultured with and without NDGs, the decline in CD183 was less rapid than in activated T cells but became evident after 24 hours. This indicates that NDGs suppress CD183 expression on T cells overall, not only on activated T-cells. The observed decline in CD183 may not reflect a true Th1 decline but rather a reduction in overall CD183 expression.

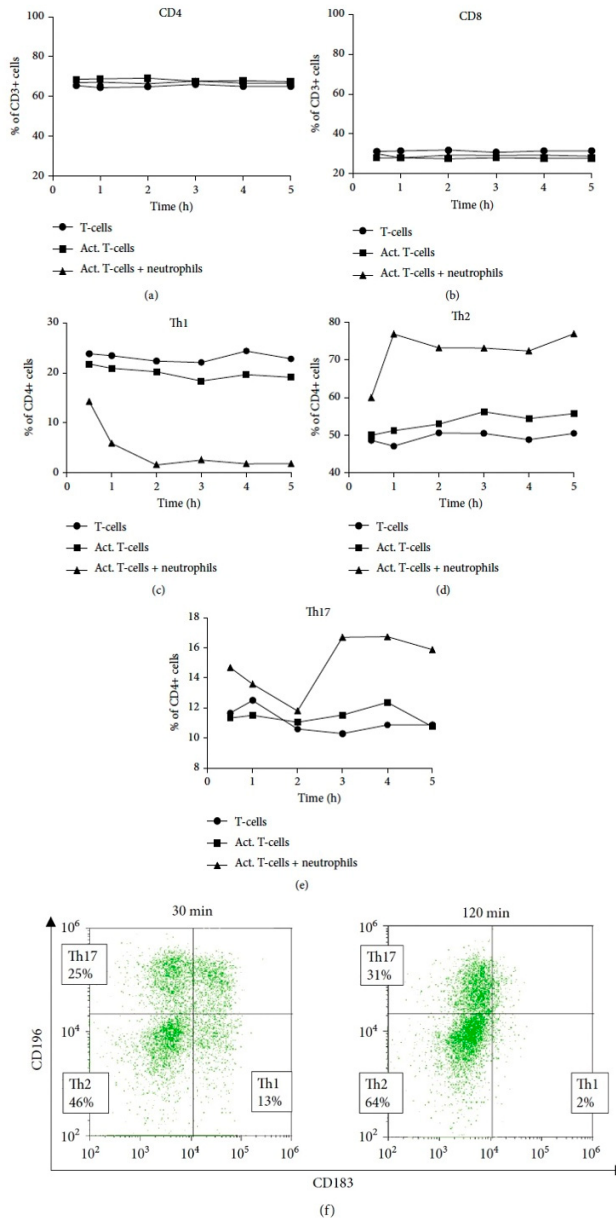


Figure 12 NDGs downregulate the Th1 marker CD183 on T-cells. T-cells were cultured together with NDGs for 0.5–5 hours, and T-cell subsets were evaluated at different time-points. NDGs did not change the percentage of (a) CD4+ cells and (b) CD8+ cells. However, the percentage of (c) Th1 cells decreased, and the percentage of (d) Th2 and (e) Th17 cells increased. (f) Subsets of T helper cells were distinguished based on their expression of CD196 and CD183. Graphs (a)–(e) show the median of 5 experiments.

NDGs Suppress Cytokine Production in T Helper Subsets.

To evaluate whether NDGs promote the formation of Th2 and Th17 subsets, we performed a cytometric bead array (CBA). The CBA measured seven Th1-, Th2-, and Th17-specific cytokines, including IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ , and TNF, in the supernatant from 3-day cocultures. IL-2, TNF and IFN- γ are Th1 cytokines, whereas IL-4, IL-6 and IL-10 are Th2 cytokines, and IL-17A is a Th17 cytokine. We found that the presence of NDGs inhibited the production of all the measured T cell cytokines ($p = 0.0001$) (Figure 13) in a dose-dependent manner.

The addition of the ROS inhibitor catalase to cocultures partly rescued the production of all cytokines, including IL-2 ($p = 0.020$), IL-4 ($p = 0.0059$), IL-6 ($p = 0.0002$), IL-10 ($p = 0.0024$), TNF ($p = 0.0002$), IFN- γ ($p = 0.0007$), and IL-17A ($p = 0.0137$). The arginase inhibitor nor-NOHA did not preserve cytokine production. The presence of anti-Cd11b increased the production of IL-2 ($p = 0.0010$), IL-4 ($p = 0.020$), IL-6 ($p = 0.023$), IL-10 ($p = 0.0024$), TNF ($p = 0.0005$), and IFN- γ ($p = 0.0068$), but not IL-17A.

These results indicate that both ROS and Cd11b-mediated cell-cell interactions inhibit the production of all measured cytokines, except IL-17A, where cell-cell contact does not appear to play a role. Altogether, these data further suggest that NDGs do not promote the loss of Th1 cells or the formation of Th2 and Th17 subsets. Instead, they point to the downregulation of CD183 being due to cleavage or internalization of the receptor.

CD183, also known as CXCR3, has four known ligands, CXCL4 (PF4), CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) [128, 129]. Upon ligand binding to CD183, the receptor is rapidly removed from the surface of T cells in a dose-dependent manner. Internalized CD183 is degraded, and receptor replenishment requires de novo synthesis, which occurs over several hours [130]. To investigate whether the loss of surface CD183 depended on binding of CXCL4, CXCL9, CXCL10, or CXCL11, antibodies against these ligands were added to the culture. After 2 hours, CD183 expression on T cells was evaluated by flow cytometry. As previously indicated, NDGs suppressed CD183 surface expression on T cells. Blocking CXCL4, CXCL9, CXCL10, or CXCL11 did not preserve CD183 expression on T cells. Several concentrations (0.5-50 $\mu\text{g/ml}$) were tested, but none restored CD183 levels on the T cell surface. Our data indicate that NDG-induced suppression of CD183 is not mediated by ligand binding.

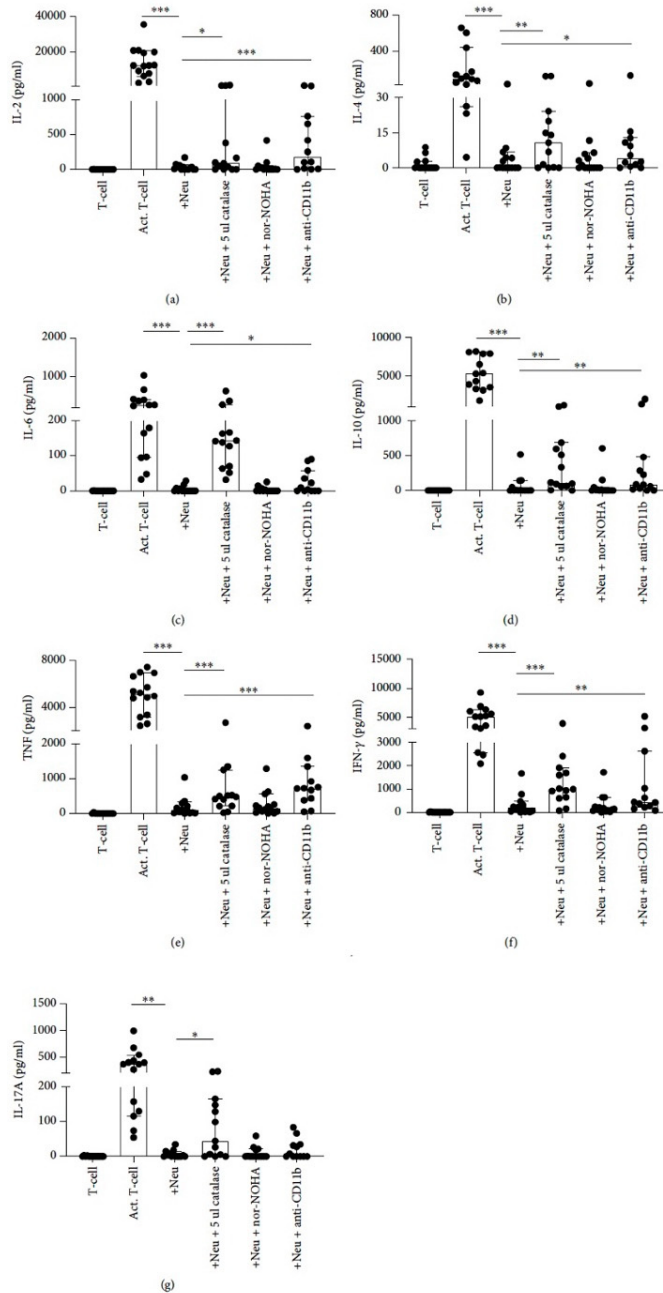


Figure 13 NDGs inhibit the cytokine production of Th cells. After 3 days of coculture together with the inhibitors catalase, nor-NOHA, and anti-CD11b, the supernatant was saved and used in CBA to measure the presence of (a) IL-2, (b) IL-4, (c) IL-6, (d) IL-10, (e) TNF, (f) IFN-g, and (f) IL-17A. Graphs show median with 95% CI (n = 14).

Paper IV

NDGs were isolated and cocultured with healthy donor T cells to investigate their suppressive effect. After 3 days of coculture, T cell proliferation and IFN- γ production were measured. IFN- γ is mainly produced by CD8⁺ T-cells and Th1 cells, and its concentration in the supernatant is commonly used to assess the suppressive activity of MDSCs [100]. NDGs in PB of healthy donors, without preactivation with a neutrophil activator, inhibited proliferation T cell proliferation ($p < 0.0001$, inhibition range 5-84%) as well as IFN- γ production ($p = 0.0078$). NDGs in the PB of MM patients inhibited both proliferation ($p = 0.002$, inhibition range 13-75%) and IFN- γ production ($p = 0.0312$) (Figure 14 (a)-(d)).

Because of age differences between healthy donors and patients, we assessed whether age correlated with NDGs' suppressive ability. No correlation was observed, indicating that the inhibitory capacity of NDGs in PB is independent of age. However, the inhibitory capacity of NDGs was dose dependent (Figure 14 (e)). Since inhibition was already observed at a 1:2 ratio, this ratio was used for all experiments unless otherwise indicated. Mixing NDGs from one donor with T cells from another did not induce alloreactivity.

These data demonstrate that unstimulated peripheral blood NDGs have an inhibitory effect on T cells. Several groups have shown that blood NDGs become suppressive after activation. Therefore, we investigated whether activation could further potentiate the inhibitory activity of NDGs. After activation with fMLF we observed increased inhibition compared to nonactivated NDGs ($p = 0.0078$) (Figure 14 (f)).

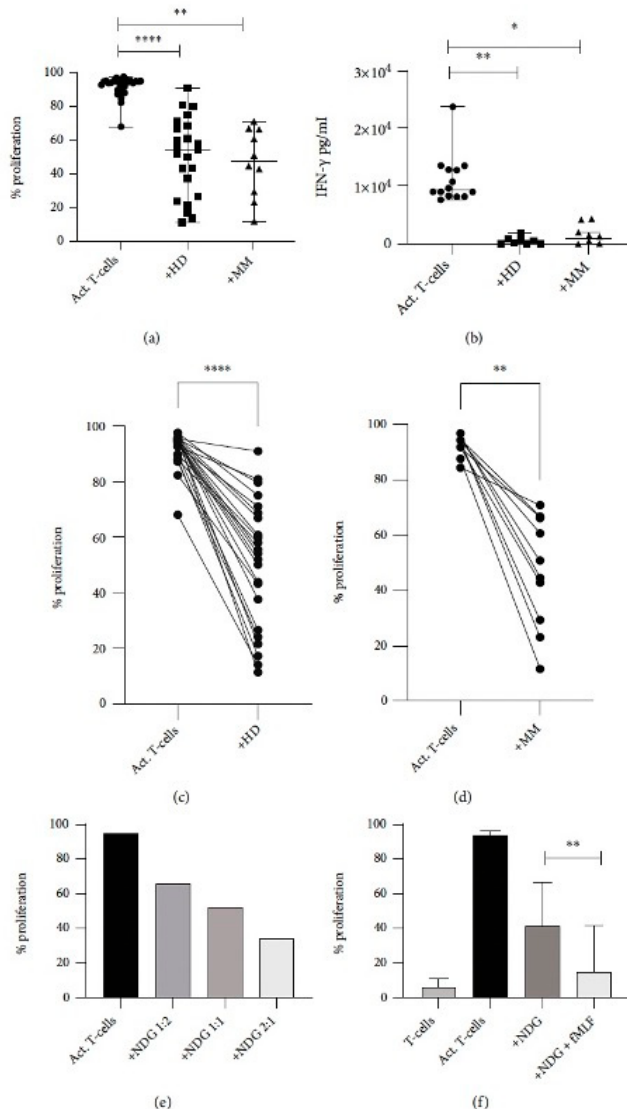


Figure 14 Inhibition of T-cell proliferation and IFN- γ production by peripheral blood normal density granulocytes. (a) Inhibition of proliferation with healthy donor (HD) and MM NDG. HD NDG inhibits T-cell proliferation ($p < 0.0001$), and the median proliferation is 54% (95% CI: 38–67). MM NDG also inhibits T-cell proliferation ($p = 0.002$), and the median proliferation is 48% (95% CI: 23–67). (b) The median of IFN- γ production for each group is 9400 pg/ml (95% CI: 820–13500), 460 pg/ml (95% CI: 0–1900), and 900 pg/ml (95% CI: 0–2000), respectively. Both HD NDG ($p = 0.0078$) and MM NDG ($p = 0.0312$) inhibit the production of IFN- γ . (c) The inhibitory effect of HD NDG ($p < 0.0001$) differs between 5 and 84% and (d) between 13 and 75% for MM NDG ($p = 0.0156$). (e) The inhibition of T-cell proliferation by NDG is dose dependent (data from representative experiment). (f) NDGs become more inhibitory in the presence of the activator fMLF ($n = 9$) ($p = 0.0078$). Error bars indicate median with range. Statistical significance was tested with Wilcoxon matched pairs signed rank test.

Bone Marrow NDGs from Myeloma Patients Effectively Suppress T Cells

How different immune cells within the bone marrow are affected by the presence of malignant plasma cells remains largely unknown. Therefore, we investigated whether NDGs from the malignant bone marrow exert a different effect on T cell proliferation compared to those from healthy donors. BM NDGs were isolated by magnetic separation and cocultured with T cells. The isolated bone marrow NDGs contained cells from different stages of maturation. BM NDGs from MM patients inhibited T cell proliferation ($p = 0.002$, inhibition range 1.7-60%). Interestingly, NDGs from healthy donors did not show an inhibitory effect ($p = 0.1016$, inhibition range +2–26%) (Figure 15).

Because of age differences between the healthy donors and the patient group, a correlation between age and suppressive ability was assessed. No correlation was found between age and the suppressive ability of bone marrow NDGs, indicating that the suppressive capacity of BM NDGs is independent of donor age. The production of IFN- γ was inhibited by both MM NDGs ($p = 0.0312$) and healthy donor NDGs ($p = 0.0078$). These data indicate that BM NDGs from MM patients suppress T cell proliferation, whereas BM NDGs from healthy donors exert only a minor effect.

Next, we investigated whether the suppressive effect observed in PB was due to activation of NDGs during the isolation process. Whole blood, as well as freshly isolated NDGs from the same healthy participants, was stained with an antibody panel including the neutrophil activation markers CD11b and CD66b. No differences in expression were observed indicating that the cells were not activated during the isolation process.

Since a T cell inhibitory effect was still observed, we hypothesized that NDGs were activated during coculture and that this late activation caused the inhibition. Therefore, the expression of CD11b and CD66b on NDGs was measured at several time points. After a few hours in culture, both alone and in the presence of activated T cells, the number of viable NDGs began to decline. However, before the decline in viable NDGs, we observed an increase in both CD11b and CD66b expression (Figure 16).

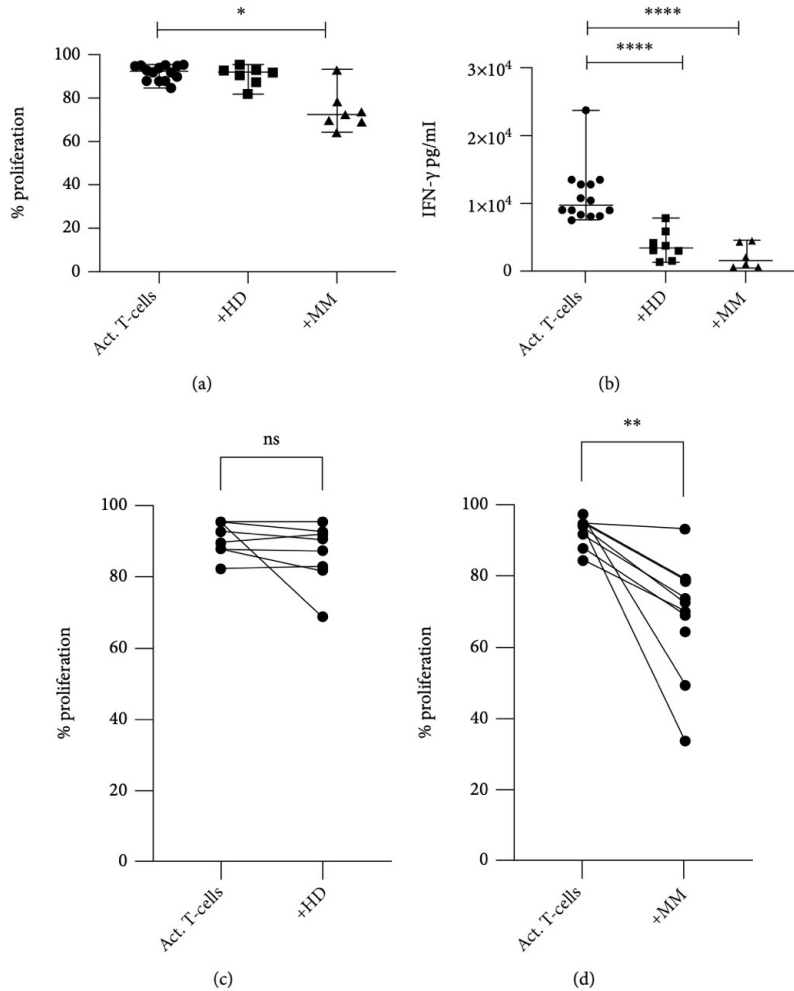


Figure 15 Inhibition of T-cell proliferation and IFN- γ production by bone marrow NDG. (a) Median proliferation for each group is 94% (95% CI: 88–95), 91% (95% CI: 82–93), and 71 (95% CI: 50–79), respectively. The proliferation of T-cells was not inhibited by HD NDG ($p = 0.1016$) but by NDG from MM patients ($p = 0.002$). (b) The median IFN- γ production for each group is 9700 pg/ml (95% CI: 8200–13500), 3500 pg/ml (95% CI: 1450–7800), and 1600 (95% CI: 600–4500), respectively. Both HD NDG ($p = 0.0078$) and MM NDG ($p = 0.0312$) have the ability to inhibit IFN- γ production. (c) The effect of HD NDG on T-cell proliferation ($p = 0.1016$) differed between +2% and -26% proliferation. (d) Inhibition by NDGs from MM patients ($p = 0.002$) differed between 1.7 and 60%. Error bars indicate median with range. Statistical significance was tested with Wilcoxon matched pairs signed rank test. Normal density neutrophils become activated by T-cells over time.

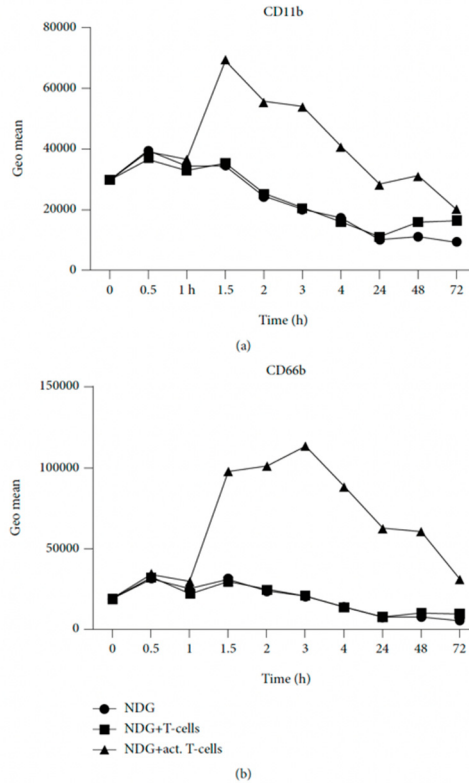


Figure 16

Neutrophil activation markers CD11b and CD66b increase during coculture with CD3/CD28 activated T-cells. (a) CD11b expression over time (72 h) on NDG alone in culture (ring), on NDG in coculture with CD3/CD28 activated T-cells (square), and on NDG in coculture with nonactivated T-cells (rectangle). (b) CD66b expression over time. These are representative plots from two experiments.

The peak in CD11b occurred at an early stage, approximately 1.5-2 hours after the addition of NDGs to the culture, whereas CD66b peaked at 2-3 hours. The expression of both markers gradually declined over time, in parallel with decreasing cell viability. NDGs cultured alone, or cocultured with nonactivated T cells, showed no signs of activation. These data suggest that NDGs become activated during coculture with activated T cells.

Catalase Can Restore T Cell Proliferation

Next, we investigated whether the inhibitory effect of NDGs was dependent on ROS and/or Arginase-1. Healthy T cells were cocultured with healthy donor NDGs in the presence of either the ROS inhibitor catalase or the arginase inhibitor nor-NOHA. Catalase converts extracellular hydrogen peroxide (H_2O_2) into water and oxygen, while nor-NOHA blocks the active site of arginase-1. Catalase and nor-NOHA alone had no effect on T cell proliferation. Addition of catalase restored the proliferative capacity of T cells ($p = 0.0312$) with 7-54 percentage units (Figure 17(a)). This indicates that the inhibitory effect of blood NDGs on T cells is at least partly mediated by ROS.

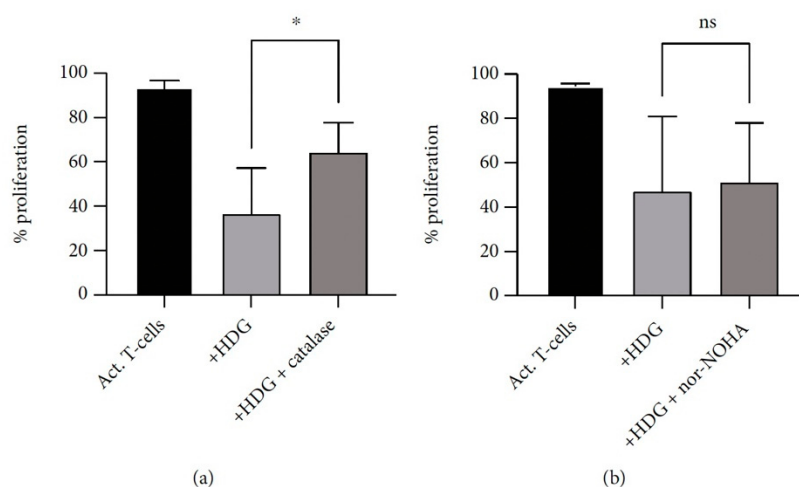


Figure 17

Catalase partially recovers the proliferation of T-cells. (a) Addition of the ROS inhibitor catalase to the coculture reverted the inhibitory effect of healthy blood NDG ($n = 6$), and T-cell proliferation was partially recovered ($p = 0.0312$). (b) When adding the arginase inhibitor nor-NOHA, the proliferation of T-cells was not recovered ($p = 0.780$) ($n = 10$).

The addition of nor-NOHA did not restore proliferation in the majority of the experiments ($p = 0.780$) (Figure 17(b)). In two out of ten experiments, the proliferation was restored with $> 20\%$, while nor-NOHA further inhibited or had no major effect on the proliferation in the other experiments. These data suggest that arginase-1 is not the main actor responsible for the T cell inhibition induced by healthy donor NDGs, while ROS play a more important role.

Levels of Immature and Mature Neutrophils in the Blood and Bone marrow

Aarts et al. demonstrated that bone marrow-derived NDGs with a mature phenotype have a superior inhibitory capacity compared to those with an immature phenotype [131]. Therefore, we investigated if the observed inhibitory effect when using bone marrow NDGs from MM patients - but not from healthy controls – could be dependent on an increased frequency of mature NDGs. To test this, both bone marrow and whole blood were evaluated with a granulocyte antibody panel and analyzed by flow cytometry.

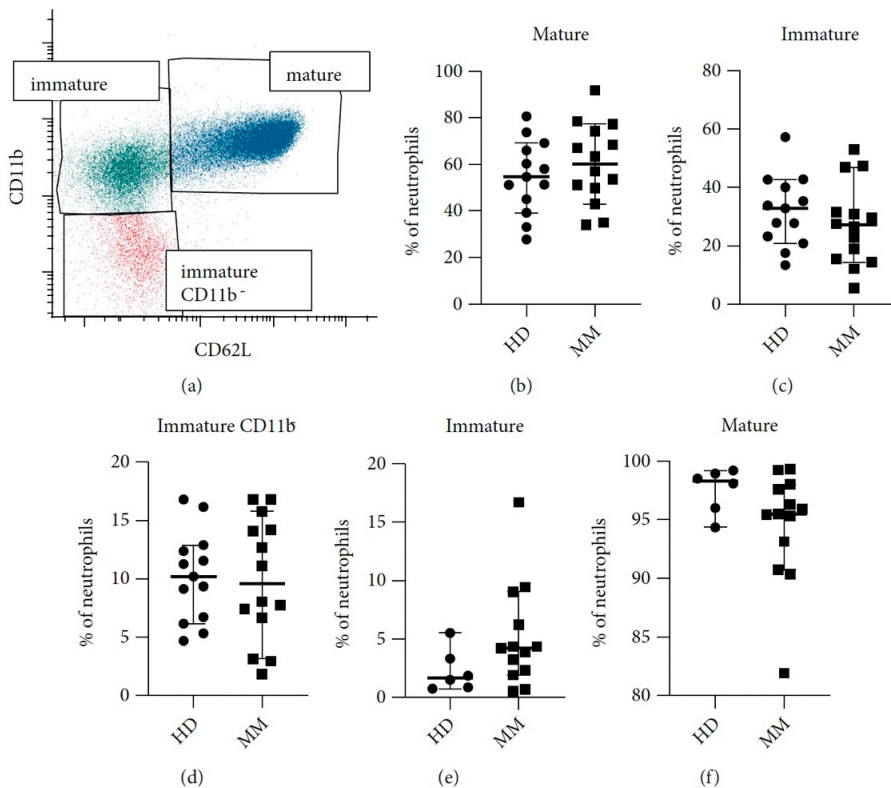


Figure 18

Percentage of mature, immature, and immature CD11b⁻ neutrophils in blood and bone marrow. (a) Gating strategy for the division of bone marrow neutrophils into mature, immature, and immature CD11b⁻ cells. The percentage of (b) mature, (c) immature, and (d) immature CD11b⁻ cells in the bone marrow of healthy donors (HDs) and MM patients. (e) The percentage of immature and (f) mature neutrophils in the blood of healthy donors and multiple myeloma patients. Statistical significance was tested with Mann–Whitney, but none was found.

Neutrophils were divided into mature, immature, and CD11b⁻ immature subsets based on their expression of CD11b and CD62L (Figure 18(a)). Among the immature cells, the CD11b⁻ subset is more immature than those expressing CD11b.

In the bone marrow, there was no significant difference in the frequency of mature, immature and immature CD11b⁻ cells between healthy donors and patients (Figure 18(b)-13(d)). These data indicate that the increased inhibitory effect of bone marrow NDGs from MM patients is not due to a skewed ratio of immature and mature neutrophils in MM patients. In peripheral blood, there was a tendency towards an increase of immature cells in MM patients (Figure 18(e)-(f)).

Discussion

Paper I

MM patients have an increased risk of developing severe bacterial infections, particularly during the early course of the disease, when infections constitute a major cause of morbidity and mortality [132, 133]. The risk is most pronounced during the first 3 months and is often caused by encapsulated bacteria [134]. Reduced levels of polyclonal immunoglobulins are a consistent feature of active disease, but numerous other immune system defects have also been described, including B- and T-cell dysfunction, impaired NK-cell function, and neutropenia [134, 135].

Phagocytosis and oxidative burst are complex, multistep processes that play a crucial role in eliminating bacteria [136, 137]. We hypothesized that a reduction in PMN phagocytic function might contribute to the increased risk of high mortality bacterial infections observed in newly diagnosed MM patients. Although the study population was limited, our data indicate that PMN dysfunction is common in patients with NDMM and may already occur at the premalignant stage of MGUS. As expected, BM phagocytes were the most affected. These findings are consistent with previously published data by Romano et al. [138, 139]. The compromised neutrophil function in our assays could not be explained by hypogammaglobulinemia (lack of immunoglobulins) since opsonized *E. coli*, with immunoglobulin and complement from pooled sera, was used as stimuli.

The activation and regulation of NADPH oxidase in granulocytes remains incompletely understood. However, different agonists appear to engage various combinations of kinases, thereby influencing both the activity of the NADPH complex and the amount of ROS produced [140]. PMA is known to for its potent ability to fully activate the NADPH complex, whereas stimulation with *E. coli* induces a lower degree of phosphorylation of its regulatory subunits, leading to reduced ROS production. These differences might partly explain the varying results observed between the two stimuli.

Due to its broad antimyeloma activity, the immunomodulatory drug (IMiD) lenalidomide has contributed to significantly improved outcomes in MM [141, 142]. Although much is understood about its wide-ranging effects, little is known regarding the impact of lenalidomide on phagocytic function. Interestingly, we

found that MM that patients in remission and ongoing lenalidomide treatment had neutrophils with restored, normal capacity for phagocytosis and ROS production. IMiDs have been suggested to play a role in cytoskeletal reorganization [143], which, together with lipid remodeling of the plasma membrane, is essential for extending the cell membrane around the target particle [90]. If lenalidomide enhances actin filament formation, this might explain the improved phagocytic capacity observed in the lenalidomide treated cohort.

The CD16 Fc receptor, important for the initiation of phagocytosis, has previously been found to be downregulated in high density neutrophils from MGUS and MM patients, together with reduced phagocytic activity and capacity of oxidative burst [138]. A hypothesis is that lenalidomide could upregulate CD16 and thereby restore phagocytosis. The FcγRI/CD64 is, in contrast to CD16, upregulated in NDMM patients, suggesting that the neutrophils from these patients are chronically activated. Lenalidomide treatment further increases both the CD64 expression, as well as the neutrophils ability to perform oxidative burst and phagocytose. [139].

In summary, our results suggest PMN dysfunction might be an additional factor contributing to the increased susceptibility to bacterial infections in patients with newly diagnosed plasma cell disorders, and that treatment with lenalidomide can partially restore PMN function. Further studies of the effects of other IMiDs are required to clarify the underlying mechanisms.

Paper II

MDSCs are considered to play an important role in the tumor microenvironment, promoting tumor immune evasion through several mechanisms involving various molecules and cytokines. In this study, we provide insight into how different MDSC subsets act in the peripheral blood and bone marrow of MGUS and MM patients.

We found no increase in the numbers of PMN-MDSCs in either the peripheral blood or bone marrow of MGUS and MM patients. Other groups have observed increased levels of PMN-MDSCs in the bone marrow [144, 145] and peripheral blood of MM patients [144, 146-149].

We have only investigated samples from newly diagnosed, untreated patients. Other studies have focused on different MM patient groups, such as those with relapsed disease. In MM patients, PMN-MDSCs have been shown to increase with disease progression [147] and to correlate with poor prognosis [145]. Similar to our results, newly diagnosed patients did not show an increase of PMN-MDSCs in the blood [147]. Blood-derived PMN-MDSCs exhibit modest suppression of T-cell proliferation, though their inhibitory effect is weaker than that of NDGs.

Notably, this suppressive activity is consistent across healthy donors, MGUS patients, and MM patients, suggesting that disease status has no significant influence on PMN-MDSC function. This observation is consistent with data from Favaloro et. al., which demonstrated that peripheral blood PMN-MDSCs are as suppressive as those from healthy donors [146]. However, other studies have reported contradictory results, showing that MDSCs from MM patients are highly suppressive, whereas MDSCs from healthy donors lacks this ability [144]. Suppression of T cell proliferation can be enhanced by activating PMN-MDSCs with the neutrophil activator fMLF, and this effect appears to be mediated by reactive oxygen species, as the radical scavenger catalase abrogates the inhibitory effect.

In 2010, M-MDSCs were described to be increased in the peripheral blood of newly diagnosed MM patients [150]. This is inconsistent with the results of our study. Similarly, Ramchandran et. al. reported that M-MDSC levels are not elevated in MM patients [144]. However, their data indicate that M-MDSCs from MM patients can inhibit T cell responses, a finding that does not agree with our results. Studies have demonstrated that normal monocytes can increase T cell proliferation [151], and in our experiments, M-MDSCs behaved in a similar manner. The gating of HLA-DR^{-low} cells is complex, as there is no clear boundary between HLA-DR⁺ cells, and HLA-DR^{-low} populations. It is possible that different gating strategies yield different results and that our population might have included monocytes, as M-MDSCs appear to be absent. It is also possible that patients in other studies had more severe disease than those in our study, as M-MDSC levels have been reported to be higher in patients with progressive disease and to correlate with poor prognosis and reduced overall survival [152].

To our knowledge, no other researchers have evaluated the levels and function of eMDSCs in peripheral blood and bone marrow of MGUS and MM patients compared to healthy donors. Interestingly, we found a decrease in eMDSCs in the blood of MM patients, compared to healthy donors.

Casetta et. al. investigated eMDSC levels in 6 different solid cancers and observed that in 5/6 cancer types, blood eMDSC levels were comparable to those in healthy donors. However, similar to our findings, in 1/6 cancer types the eMDSC level was decreased in cancer patients. Unlike Casetta et al., we did not observe a T cell suppressive response [97]. In recent years, it has become evident that neutrophils also exhibit immune regulatory functions, in the same manner as PMN-MDSCs [77, 126, 127, 131, 138, 153-156].

Previous data from our group indicate that blood NDGs, from both MM patients and healthy individuals, can suppress T cell proliferation and IFN- γ production [77]. In this study, we compared the suppressive ability of PMN-MDSCs and NDGs and found that NDGs are stronger suppressors of T cell responses than PMN-MDSCs. Others have conducted similar experiments and found only limited differences

[156]. The major distinction between PMN-MDSCs and NDGs appears to be their density, suggesting that PMN-MDSCs might represent ordinary neutrophils with fewer granules, as granularity is directly proportional to density. PMN-MDSCs are thought to be more immature than NDGs, and during maturation neutrophils increase in both size and granularity [157].

An important limitation in MDSC research is the lack of consensus on markers for their identification, and the absence of specific markers for the different subsets. Two papers have been published that propose guidelines for the identification of PMN-MDSC, M-MDSC and e MDSC in peripheral blood [97, 100]. Without these guidelines, it is difficult to compare the results from different groups, as it is not certain that the same cells are being investigated. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a class E scavenger receptor, has been suggested as a specific PMN-MDSC marker [125]. However, attempts to reproduce these findings have failed [97, 126]. LOX-1 appear to be expressed not only on PMN-MDSCs but also on neutrophils, making it a poor marker for PMN-MDSCs [126]. It has also been suggested that PMN-MDSCs from the bone marrow of MM patients exhibit higher LOX-1 expression compared to peripheral blood from the same patients [125]. We and others have shown that LOX-1 is present on PMN-MDSCs, but the expression levels do not differ from those on PMN-MDSCs found in healthy donors [97].

Several different methods are used to functionally assess suppression of T cell proliferation, which complicates comparisons between studies. Examples include using lipopolysaccharide-matured dendritic cells to activate T cells [144], CD3/CD28-coated beads [146], CD3/CD28 coated plates [97] or the use of peripheral blood mononuclear cells (PBMCs) instead of purified T cells.

Another limitation in MDSC research is their low abundance. It is often difficult to obtain sufficient cells for experiments from all patients, which might introduce bias, as individuals with higher amounts of MDSC levels are more likely to be evaluated. Over the past decades, suppressive neutrophils have been described under various names—PMN-MDSCs, granulocytic (G)-MDSCs, low-density neutrophils (LDNs), low-density granulocytes (LDGs), and immature myeloid cells (IMCs). These terms all refer to neutrophil-like cells isolated from the low-density fraction after density gradient centrifugation, although they are associated with different disease contexts. While these cells share a common isolation profile, their functional roles appear to vary across pathological conditions.

This raises a critical question: Do these designations represent distinct cell populations, or are they simply reflections of neutrophil plasticity in response to diverse microenvironments?

Paper III

The regulation of the immune system and the interaction between myeloid cells and T cells in health and disease is complex. In this study, we demonstrate that NDGs could exert MDSC activity and inhibit T cell proliferation by cell-cell contact and ROS production. This inhibitory effect is rapid and can be observed within the first few hours of contact. In addition, we demonstrate that NDGs suppress CD183 expression on the surface of T cells, resulting in an almost complete loss of the receptor. Furthermore, NDGs generally suppress the production of Th-signature cytokines.

Several different mechanisms have been proposed for myeloid-derived suppression of T cells. Our findings of suppression mediated by contact dependence through CD11b and by ROS production are consistent with previous data from Aarts et. al [126]. They also investigated cell-cell contact using coculture with the Transwell technique and found that direct contact was necessary for NGD-induced suppression of T cells. However, other mechanisms might play important roles in different disease contexts. In this study, we could not observe a suppressive role for MMP9-activated TGF- β , a mechanism described by Germann et. al. in neutrophils from patients with colon cancer [155].

CD11b, together with CD18, forms the integrin Mac-1 complex [126, 158]. Blocking CD11b prevents NDGs from inhibiting T cell proliferation, indicating that cell-cell contact through the Mac-1 complex is necessary for the inhibitory effect. However, another study reported that CD11b plays only a minor role, and that upregulation of programmed death ligand 1 (PD-L1) is more important for the inhibitory function of neutrophils [159]. This mechanism could not be evaluated, as the antibodies we used to block PD-L1 resulted in T cell death. Nevertheless, our findings, together with those of the aforementioned groups, demonstrate that CD11b contributes to the observed effect and that cell-cell contact is important for the inhibitory effect.

Neutrophils are highly sensitive cells that readily become activated, and the purification process as well as handling prior to culture could potentially influence experimental outcomes. Several studies have reported that NDGs in peripheral blood require activation to exhibit MDSC function [126, 131, 138, 153, 154]. However, we and others have observed inhibitory capacities even in the absence of neutrophil activators [77, 159, 160].

In our previous study, we demonstrated that NDGs are not activated during the isolation process but become activated approximately one hour after coincubation with activated T cells, indicated by increased surface expression of the neutrophil activation markers CD11b and CD66b [77]. Interestingly, the loss of CD183 from the T cell surface occurred more rapidly than neutrophil activation. In the present study, we observed that the decrease in CD183 expression began within the first 30

minutes of coculture. The decline in T cell activation markers occurred after 2 hours [77]. Here, we show that NDGs do not exert an inhibitory effect specifically on Th1 cells but rather display a broader inhibitory capacity, as they downregulate the production of all measured Th cytokines in a dose-dependent manner. Neutrophils therefore do not appear to drive Th responses in a specific direction but instead broadly suppress T cell cytokine production across all three Th subsets.

CD183 (CXCR3) is the receptor for four different chemokines, including CXCL4 (PF4), CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) [128, 129]. There are two isoforms of CD183, CXCR3-A and CXCR3-B. CXCL9, CXCL10, and CXCL11 bind to both isoforms, whereas CXCL4 binds only to CXCR3-B [128]. CXCL9, CXCL10, and CXCL11 are all induced by IFN- γ and promote Th1 immune responses and Th1 migration [130, 161]. A study by Romagnani et al. demonstrated that CXCL10 promotes Th1 responses and enhances IFN- γ production, while CXCL4 promotes Th2 responses by inducing the Th2-type cytokines IL-4, IL-5, and IL-13 in naïve CD4⁺ T-cells [129].

Furthermore, downregulation of CD183 has been associated with reduced migration of CD8⁺ T-cells in tumor patients [162]. Since CXCR3 binding leads to receptor internalization and thereby removal from the T cell surface [130], we hypothesized that these chemokines might be responsible for the loss of CD183 on T cells and perhaps played a role in promoting Th1 or Th2 responses. However, when adding blocking antibodies towards the four chemokines, either alone or in combination, we could not revert the loss of the receptor. The loss of CD183 is not due to binding of its chemokine.

NDGs produce CXCL9, CXCL10, and CXCL11 only after incubation with IFN- γ and an activator [163, 164]. Upon activation, NDGs release extracellular contents, including proteases, which can cleave surface receptors and potentially terminate cytokine responses [165]. If neutrophils release a protease that cleaves CD183 from the surface, it could possibly lead to the shutdown of at least the Th1 cytokine response. However, all Th cytokines were inhibited by neutrophils, indicating that removal of CD183 cannot be the only mechanism for cytokine inhibition. NDGs suppressed all measured Th-type cytokines, including IL-2, IL-4, IL-6, IL-10, IL-17A, TNF and IFN- γ . These cytokines modulate the function of both the adaptive and innate immune system [107]. Th cells are critical for initiating anti-tumor responses [166], and impaired Th cell function has been associated with the development of autoimmune diseases [107].

The ROS inhibitor catalase restored the cytokine production, indicating that ROS is part of the mechanism responsible for the suppression of these cytokines. The arginase inhibitor nor-NOHA did not preserve cytokine production or proliferative capacity. Therefore, arginase and nitric oxide do not seem to be important in our experimental set-up. When the close contact between neutrophils and T cells was

disrupted by blocking CD11b, the production of all cytokines, except IL-17, was partially restored.

This suggests that cell-cell contact is important for the suppression of most cytokines, except for IL-17. Multiple pathways likely act in concert to mediate the inhibitory effects of neutrophils, and these mechanisms need to be further unraveled. In this study, we showed that NDGs can interact with T cells, leading to inhibition of both proliferation and cytokine production. NDGs suppress T cells by ROS production, whereas arginase and TGF- β do not appear to be part of these inhibitory mechanisms. The Th1 marker CD183 was rapidly downregulated from the T cell surface following cocubation with NDGs, independent of chemokine binding. These data indicate that NDGs can regulate the adaptive immune response by inhibiting T cell proliferation and cytokine production.

Paper IV

In the past decade, a regulatory function of neutrophils has been recognized in both health and disease. LDGs and PMN-MDSCs were the first neutrophil subsets described as immunoregulatory, and recently, a similar role has been demonstrated for activated NDGs. In this study, we show that bone marrow NDGs from MM patients can suppress T-cell proliferation, whereas bone marrow NDGs from healthy individuals do not. Furthermore, NDGs do not need to be preactivated to exert their inhibitory effect on T cells; rather, they become activated during coculture with activated T cells. Previous reports have shown that peripheral blood NDGs activated by a neutrophil activator can exhibit MDSC functions, such as suppressing T cell proliferation [126, 131, 138, 153, 154]. However, our data differ from these studies, as we observed an inhibitory effect of NDGs even in the absence of neutrophil activators, using a similar method. Consistent with our findings, de Kleijn et al. reported a minor inhibitory capacity of nonactivated blood neutrophils when cocultured with phytohemagglutinin (PHA)-activated CD3⁺ lymphocytes [159].

There are several different methods that can be used for neutrophil isolation, each of which may potentially affect neutrophil function. In this study, we found no evidence of NDG activation resulting from the isolation process, as assessed by surface expression of CD11b and CD66b. Instead, our data suggest that NDGs become activated by coculture with activated T cells. No activation was observed when NDGs were cocultured with non-activated T cells, indicating that T cell activation might be an important factor for neutrophil activation and a prerequisite for neutrophil-mediated suppression.

An interesting finding in this study was that bone marrow NDGs from healthy donors did not affect T cell proliferation but did reduce IFN- γ production. In contrast, bone marrow NDGs from MM patients were able to inhibit both

proliferation and IFN- γ production. The reason for this partial inhibition observed when using bone marrow NDGs from healthy donors remains unknown. In the MDSC literature, the absence of IFN- γ in the supernatant after coculture with T cells is considered a marker of inhibition [100]. IFN- γ is mainly produced by activated CD8⁺ T cells and activated CD4⁺ Th1 cells, and our findings may suggest selective inhibition of these subsets, while other T cell subsets retain their ability to proliferate in the presence of healthy donor bone marrow NDGs.

ROS have been suggested to inhibit T cells through several mechanisms, including suppression of DNA synthesis and alterations in T cell receptor signaling [167]. In this study, we observed that the inhibitory effect of NDGs could be partially reversed by the addition of catalase, suggesting that NDGs inhibit T cell proliferation, at least in part, through ROS production. This finding is consistent with results from other groups who have studied NDGs from healthy donors [131, 154, 168, 169].

A limitation in our study is that the age of the control group differs from that of the patient group, with the healthy donors being younger. It is well established that neutrophil function changes with age, generally showing reduced function in the elderly [170]. For example, neutrophils from older individuals have a diminished ability to produce ROS upon stimulation and a reduced capacity for phagocytosis [170, 171]. However, the spontaneous release of ROS appears to be higher in elderly individuals than in young adults [172]. As the function of neutrophils from elderly is supposed to be less potent, and the ROS production after stimulation is decreased, we should observe a decreased inhibitory effect when using neutrophils from older patients. In this study, however, we observed a similar inhibitory effect in peripheral blood, as well as an increased inhibitory effect in the bone marrow. This suggests that the inhibitory effect in the bone marrow is not dependent on donor age. Instead, it is more likely related to the patient's underlying disease. An earlier report showed that NDGs from the peripheral blood of MM patients are chronically activated and inhibit PHA-induced T-cell proliferation [138]. In contrast, our study did not support these findings, as we observe no increased inhibitory effect of MM NDGs isolated from blood compared to NDGs from healthy donors. We only observed an increased inhibitory effect of NDGs from MM patients when using bone marrow NDGs.

In systemic lupus erythematosus, NDGs have been suggested to restrict T cell proliferation in an arginase-dependent manner [173]. A similar effect has been proposed for blood NDGs from both chronic myeloid leukemia patients [174] and MM patients [138]. Our data do not support arginase-dependent inhibition. However, it is possible that both ROS and arginase-1 play important roles, with their contributions varying depending on disease phenotype and experimental settings. Germann et al. reported that peripheral blood NDGs from patients with colon cancer inhibit T cell proliferation, and that this inhibitory effect can be reversed by the addition of a transforming growth factor beta (TGF- β) inhibitor. They suggested

that NDG-secreted matrix metalloprotease 9 (MMP9) activates TGF- β in the microenvironment, thereby promoting T cell inhibition [155]. Moreover, other studies have suggested that the inhibitory effect is either contact dependent [158, 159] or mediated through soluble factors [173].

The integrin Mac-1 (CD11b/CD18) has also been suggested to play an important role in the inhibitory effect of NDGs [126, 158]. When CD11b is blocked on activated NDGs, they are prevented from exhibiting MDSC functions [126]. However, another study reported that CD11b plays only a minor role, and that the upregulation of programmed death-ligand 1 (PD-L1) is more important for the inhibitory effect of NDGs [159]. Further studies are needed, investigating several inhibitory factors, in order to unravel how NDGs inhibits T cell responses in different diseases. Bone marrow NDGs from MM patients show an increased T cell suppressive effect compared to bone marrow NDG from healthy donors.

It is well known that tumors can affect surrounding cells and promote a protumor microenvironment, and it is possible that the tumor milieu in the bone marrow preactivates neutrophils, making them more prone to inhibit T cell proliferation. For example, the presence of TGF- β at a tumor site can mediate an “immunogenic switch” from proinflammatory, antitumor N1 neutrophils to anti-inflammatory, protumor N2 neutrophils [175]. The presence of IFN- γ has also been suggested to promote a T-cell suppressive phenotype in NDGs [159]. In MM, bone marrow PMN-MDSCs and NDGs produce soluble factors that protect human MM cell lines from the cytotoxicity of the MM treatments doxorubicin and melphalan [149].

The observed inhibitory effect of bone marrow NDGs might contribute to the growth of malignant plasma cells in MM patients. By inhibiting T cells, MM plasma cells get a greater chance to survive. This hypothesis is supported by findings showing that T cells from the bone marrow of MM patients exhibit characteristics of both exhaustion and senescence [135]. The frequencies of mature, immature, and CD11b- immature NDGs did not differ between healthy controls and MM patients in our study. Therefore, the observed difference in inhibition in the bone marrow cannot be explained by the frequency of mature and immature cells. Aarts et al. reported that only mature bone marrow NDGs exhibit MDSC abilities when activated with fMLF [131]. Whether the degree of maturity is important for the suppressive capacity of bone marrow NDGs in MM patients remains to be determined.

It is evident that neutrophils from MM patients have altered function in both blood and bone marrow. In this study, we provide new evidence showing that NDGs isolated from the bone marrow of MM patients are more immunosuppressive than bone marrow NDGs from healthy donors. A deeper knowledge regarding the neutrophil-induced T-cell suppression could form the basis for a new therapeutic target in MM as bi-specific T-cell engagers and chimeric-antigen-receptor T-cells are new emerging therapies in MM.

General discussion

Although significant progress has been made in both survival and treatment options for patients with myeloma, the underlying pathogenesis, including disease related disturbances of the immune system, remains incompletely understood. In particular, during the first year after diagnosis, myeloma patients are at high risk of life-threatening, primarily bacterial infections [132]. A well-established explanation for this elevated susceptibility is immunoparesis [176], resulting from the displacement of the normal, diverse antibody repertoire by clonal plasma cells.

In paper I we demonstrated that neutrophils in patients with newly diagnosed myeloma, exhibited reduced phagocytic capacity and oxidative burst compared to healthy controls. Given that neutrophilic granulocytes play a crucial role in defense against bacterial infections, our findings provide a new perspective that complements the established model of immunoparesis. Interestingly, the commonly used immunomodulatory drug lenalidomide restored both phagocytic function and respiratory burst activity. Lenalidomide exerts pleiotropic effects, as described in detail in the theoretical framework. In addition to these known effects, our results add further complexity to the functional understanding of lenalidomide, suggesting that it might directly or indirectly influence neutrophilic granulocytes. This also raises the question of whether lenalidomide could function by bridging the innate and adaptive immune system.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that exert immunosuppressive effects, enabling tumor immune escape [177]. In paper II we investigated the levels and suppressive effect of MDSCs and normal density granulocytes (NDGs) in MM and found that NDGs had a stronger immunoregulatory activity toward T cells than MDSCs, suggesting that NDGs potentially contribute to disease development. Our findings also highlight the ongoing challenge posed by the lack of a consensus definition for MDSCs. It is possible that MDSCs and neutrophils represent a continuum within the same myeloid lineage, differentiated by the context of their activation. A potential explanation has been proposed by others [100].

In this model, neutrophils activated in response to acute infections acquire a classical neutrophil effector phenotype, characterized by efficient phagocytosis, release of an appropriate amount of reactive oxygen species (ROS), and secretion of proinflammatory cytokines. In contrast, chronic or low-level stimulation, such as that occurring in the tumor microenvironment or during persistent inflammation, drives the emergence of MDSCs. These cells exhibit impaired phagocytic capacity

and secrete high levels of ROS, nitrogen species, and immunosuppressive cytokines, contributing to immune evasion and pathological inflammation [100].

Traditionally, NDGs have been regarded as short-lived, purely innate effector cells, specialized in phagocytosis and oxidative burst. However, in paper III we identified a contact-dependent suppression of T cell proliferation by NDGs that was mediated by CD11b and ROS. This finding supports the idea that neutrophils might act as a functional bridge between the innate and adaptive immune systems, actively modulating adaptive immunity through interactions with T cells and potentially other lymphocytes.

In paper IV we observed that bone marrow NDGs from myeloma patients, but not from healthy controls, exerted suppressive effects on T cells, similar to MDSCs. This effect could not be explained by alterations in NDG maturity in the bone marrow. Our results suggest that BM NDGs possess MDSC-like properties that might support myeloma cell growth. In addition, NDGs in peripheral blood from both MM patients and healthy controls inhibited T cell proliferation and IFN- γ production. Peripheral blood NDGs did not need to be preactivated to mediate suppressive effects; instead, they became activated during coculture. The inhibitory effect was ROS-dependent and was reversed by the addition of the inhibitor catalase.

The fact that NDGs became activated during coculture highlights the importance of direct contact with activated T cells for the acquisition of suppressive capacity. The observation that the effect is ROS dependent supports the concept that oxidative stress constitutes a central mechanism of neutrophil mediated immunoregulation. Altogether, these data indicate that neutrophilic granulocytes function as dynamic regulators at the interface between innate and adaptive immunity.

Future perspectives

In order to bring clarity to the field of MDSCs and NDGs and enable comparisons between studies, it will be important to establish a consensus on the classification of the various myeloid cell types.

As a follow-up to our findings in paper IV, it would be of interest to investigate whether neutrophils also influence other components of the immune system beyond T cells. In particular, it would be valuable to investigate whether neutrophils can modulate the activation, differentiation, and effector functions of other lymphocyte subsets, such as B cells, thereby providing further insight into the immunoregulatory roles of neutrophils and their contribution to the modulation of the adaptive immune response.

Since the use of T cell-dependent therapies, including bispecific antibodies and CAR T cells, is expected to increase in the future, it would also be highly relevant to further elucidate T cell interactions with other cell types, including neutrophils.

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