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Aspects of Phagocyte Function in Multiple Myeloma

STINA WICHERT
FACULTY OF MEDICINE | LUND UNIVERSITY 2019

Aspects of Phagocyte Function in Multiple Myeloma

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Stina Wichert

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DOCTORAL DISSERTATION
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Aspects of Phagocyte Function in Multiple Myeloma

Abstract

The focus of this thesis was to investigate changes in phenotype and function of myeloid cells in multiple myeloma (MM), that could affect the course of the disease, risk of infections and response to therapy. Flow cytometry-based analysis was used to characterize the phagocyte populations and their *ex vivo* capacity for phagocytosis and oxidative burst. Samples from healthy donors were used as controls.

In the first paper we examined the effects of high-dose melphalan and autologous stem cell transplantation (ASCT) on patients´ polymorphonuclear leukocytes (PMNs) and monocytes. Patients´ PMNs, both before and after ASCT, had reduced capacity for phagocytosis. After ASCT a smaller proportion of PMNs had capacity for oxidative burst. Newly released monocytes had low surface expression of HLA-DR. Eosinophils, suggested to play a role in promoting the retention, survival and proliferation of plasma cells (PCs) in the bone marrow (BM), were markedly reduced after ASCT and slow to regenerate.

The second paper is a clinical phase 2 trial with a novel, fully human, high-affinity IgG1 monoclonal antibody against intercellular adhesion molecule-1 (ICAM-I), BI-505, in patients with smoldering MM (SMM). Previous studies reported that the BI-505 epitope of ICAM-1 was highly expressed on MM cells and the anti-myeloma activity in animal models was promising. BI-505 was well tolerated in a phase 1 study on patients with relapsed/refractory MM. Despite adequate doses, with measured concentrations sufficient to achieve complete saturation of the BI-505 epitopes, there was no efficacy on disease activity in the three evaluable patients with SMM, even if well tolerated.

In paper three, eosinophils in patients with newly diagnosed MM, SMM and monoclonal gammopathy of undetermined significance (MGUS) were analyzed. The amount of eosinophils per se did not correlate to number of PCs or disease stage. Patients´ BM eosinophils were more activated and had an increased surface expression of chemokine receptor 4 (CXCR4), which is of importance for the homing capacity to the same BM niches as PCs.

In the fourth paper we found that neutrophil dysfunction was common in patients with newly diagnosed MM and occurred already at the premalignant stage MGUS. BM phagocytes were most affected. On the contrary, MM patients in stable remission and on-going treatment with lenalidomide had neutrophils with normal, restored capacity for phagocytosis and oxidative burst.

In conclusion, our results indicate that several aspects of phagocyte functions are altered in MM and the choice of therapy might further influence these functions. The exact mechanisms remain to be clarified.

Key words: Multiple myeloma, Phagocyte function, ASCT, Eosinophils, CXCR4, anti-ICAM-1 monoclonal antibody, BI-505, phase 2 trial

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Aspects of Phagocyte Function in Multiple Myeloma

Stina Wichert

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Cover photo by Åsa Johansson and Anna Kwiecinska

The cover photo shows a plasma cell surrounded by two eosinophils. Bone marrow sample obtained from a patient with smoldering multiple myeloma and stained with May-Grünwald Giemsa.

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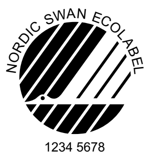

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“Rabbit’s clever,” said Pooh thoughtfully.

“Yes,” said Piglet, “Rabbit’s clever.”

“And he has Brain.”

“Yes,” said Piglet, “Rabbit has Brain.”

There was a long silence.

“I suppose,” said Pooh, “that’s why he never understands anything.”

by A. A. Milne

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Abstract

The focus of this thesis was to investigate changes in phenotype and function of myeloid cells in multiple myeloma (MM), that could affect the course of the disease, risk of infections and response to therapy.

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In the fourth paper we found that neutrophil dysfunction was common in patients with newly diagnosed MM and occurred already at the premalignant stage MGUS. BM phagocytes were most affected. On the contrary, MM patients in stable remission and

on-going treatment with lenalidomide had neutrophils with normal, restored capacity for phagocytosis and oxidative burst.

In conclusion, our results indicate that several aspects of phagocyte functions are altered in MM and the choice of therapy might further influence these functions. The exact mechanisms remain to be clarified.

List of abbreviations

AEs	adverse events
APCs	antigen-presenting cells
APRIL	a proliferation-inducing ligand
ADCC	antibody-dependent cell cytotoxicity
ADCP	antibody-dependent cell phagocytosis
ASCT	autologous stem cell transplantation
BCMA	B cell maturation antigen
BM	bone marrow
BMPCs	bone marrow plasma cells
BMSCs	bone marrow stromal cells
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocyte antigen-4
CXCR4	chemokine receptor 4
DLT	dose-limiting toxicity
FACS	fluorescence activated cell sorter
FSC	forward scatter
GrMDSCs	granulocytic myeloid-derived suppressor cells
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IFN-γ	interferon-γ
IL-6	interleukin-6

IMiDs	immunomodulatory drugs
mAbs	monoclonal antibodies
mDCs	myeloid dendritic cells
MDSCs	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
MoMDSCs	monocytic myeloid-derived suppressor cells
MTD	maximum tolerated dose
NETs	neutrophil extracellular traps
NDMM	newly diagnosed multiple myeloma
PB	peripheral blood
pDCs	plasmacytoid dendritic cells
PCs	plasma cells
PMNs	polymorphonuclear leukocytes
PMN-MDSCs	polymorphonuclear myeloid-derived suppressor cells
ROS	reactive oxygen species
SAEs	severe adverse events
SSC	side scatter
SMM	smoldering multiple myeloma
TATE	tumor-associated tissue eosinophilia
TGF-β	transforming growth factor-β
T-EMRA	T effector memory RA^+ cells
TLRs	toll-like receptors
TNF-α	tumor necrosis factor-α
Tregs	regulatory T cells

List of papers

I

Phagocyte function decreases after high-dose treatment with melphalan and autologous stem cell transplantation in patients with multiple myeloma
Wichert S, Pettersson Å, Hellmark T, Johansson Å, Hansson M. Experimental Hematology. 2016;44:342-51

II

A single-arm, open-label, phase 2 clinical trial evaluating disease response following treatment with BI-505, a human anti-intercellular adhesion molecule-1 monoclonal antibody, in patients with smoldering multiple myeloma
Wichert S, Juliusson G, Johansson Å, Sonesson E, Teige I, Wickenberg A T, Frendeus B, Korsgren M, Hansson M. PloS one. 2017;12(2):e0171205.

III

Bone marrow eosinophils in plasma cell disorders
Wichert S, Pettersson Å, Hellmark T, Johansson Å, Hansson M. Experimental Hematology. 2018;66:27-31

IV

Impaired phagocyte function in patients with newly diagnosed multiple myeloma
Wichert S, Askman S, Pettersson Å, Hellmark T, Johansson Å, Hansson M.

Introduction

Multiple myeloma (MM) is a malignant plasma cell tumor of the bone marrow (BM), clinically characterized by painful skeletal destruction, hypercalcemia, renal impairment, and anemia. It is the second most common malignancy of the blood. In most cases the malignant plasma cells produce a monoclonal immunoglobulin, "M-component". MM is preceded by a premalignant state called monoclonal gammopathy of undetermined significance (MGUS) or "asymptomatic" smoldering multiple myeloma (SMM). Immune dysfunction is a characteristic feature of MM and results in both, increased susceptibility to infections, and contributes to tumor cell survival, growth and resistance to therapy. Cells of myeloid lineage constitute a major part of the cellular component in the neoplastic BM microenvironment of MM, and recent years of research emphasize their ability to influence adaptive immune responses. This thesis focuses on phenotype and functioning of myeloid cells in MM.

Historical perspective

The first well-documented clinical case report of MM was a 39-year-old woman described by Samuel Solly in 1844. She developed severe back pain, fatigue, and died four years after the onset of symptoms. Autopsy revealed that the cancellous portion of sternum and both femurs had been replaced with a red substance and she had multiple fractures [1]. In his paper, Solly named the skeletal disease "mollities ossium". In 1845, William MacIntyre, Henry Bence Jones and John Dalrymple examined a patient with polyuria, weakness and pain in the chest. The urine specimen was opaque, acidic and of high density. They found a urine substance that precipitated by addition of nitric acid. The precipitate was soluble in boiling water but re-precipitated after the urine was cooled. Bence Jones concluded that the protein was "an oxide of albumen" [2]. When the patient died a few months later, the cause of death was "atrophy from albuminuria". Postmortem, this patient was diagnosed with "mollities ossium". MacIntyre wrote "The ribs crumbled under the heel of the scalpel" and they were soft and so brittle that they "could be easily cut by the knife" [2]. Delrymple examined two lumbar vertebrae and a rib and found the cancellous cavities filled with a red gelatiniform substance consisting of nucleated cells 1.5 to 2 times larger than average red blood cells, some with oval shapes [2]. Bence Jones emphasized in a later paper the "importance of seeking this

oxide of albumen in other cases of mollities ossium". The term "multiple myeloma" was coined by von Rustizky in 1873 when he did an autopsy of a patient and found eight separate, reddish and soft, tumors in the BM [1]. In 1889, Professor Otto Kahler from Prague described a middle-age physician with MM with all characteristic symptoms during eight years of disease progression, with skeletal pain and destructions, worsening kyphosis, pallor, anemia and proteinuria [2]. Jacobson and Walters recognized 1917 and 1921 "Bence Jones protein" in peripheral blood (PB) and hypothesized that they were derived from blood proteins through the action of abnormal cells in the BM [1]. In 1937, Tiselius manuscripts on electrophoresis and separation of serum globulins into three components, designated α, β and γ, was published. The method is based on the principle that proteins migrate at different rates according to size, shape and electrical charge. In 1939, Longworth et al. used serum protein electrophoresis in MM and demonstrated the typical tall and narrow-based "M-spike" in the gamma region, formed by the monoclonal immunoglobulin protein – the "M-component", not seen in healthy individuals [1]. Jan Waldenström from Malmö introduced in the early 60´s the concept of monoclonal versus polyclonal gammopathies. This is clinically an important distinction because patients with a narrow band of hypergammaglobulinemia have production of a monoclonal protein and have or may develop a neoplastic process, whereas patients with polyclonal hypergammaglobulinemia, with a broad band in the gamma region, have an inflammatory or infectious process [2, 3]. Patients with a monoclonal protein without evidence of malignant disease were considered to have "essential hypergammaglobulinemia" or "benign monoclonal protein", nowadays classified as "monoclonal gammopathy of undetermined significance" (MGUS) [1]. The basic chemical structure of immunoglobulins with heavy chains and light chains was determined during the 60´s, by work of Porter and Edelman, for which they shared the Nobel Prize in medicine & physiology 1972. Edelman showed that Bence Jones proteins were light chains of the myeloma protein, excreted into the urine due to the light chains´ low molecular weight [1]. In 1964, Wilson applied antisera on the surface of the agarose gel, after electrophoresis, and could thereby detect small amounts of monoclonal proteins, a method called immunofixation [2].

Tumor cell origin and disease characteristics

Plasma cells (PCs) are terminally differentiated effector cells of the B lymphocyte lineage. They produce antibodies, i.e. immunoglobulins (Igs), in response to antigenic stimulus and mediate the humoral immune response. After a primary immune response, long-lived PCs are generated in germinal centers of lymphoid tissues, by interaction with activated follicular T helper cells. They migrate as plasmablasts into the BM, where they differentiate into mature long-lived PCs. Long-lived PCs constitute

part of the immunological memory and they survive in specialized niches provided by bone marrow stromal cells (BMSCs) [4-6]. The tumor cells in MM are derived from malignantly transformed long-lived PCs or their precursors, and the first oncogenic event probably occurs in germinal centers during the process of isotype class switching and somatic hypermutation, which by nature are highly mutagenic processes [7].

The BM is the site of the disease in most cases. As the neoplastic, monoclonal PCs accumulate and gradually increase in the BM, it leads to reduced normal hematopoiesis with anemia and immune deficiencies. The normal polyclonal Ig production is replaced by monoclonal Ig produced by the malignant PC clone. This "M-component" is measurable either as a whole immunoglobulin protein in plasma or as free light chains in plasma and/or urine ("Bence Jones protein" in urine). Increasing number of malignant PCs and their interaction with BMSCs disturbs the balance between bone formation and resorption, highly promoting osteoclast activity and differentiation. This results in pathologically accelerated osteoporosis and the clinically characteristic lytic bone destructions [8]. Overproduction of monoclonal free light chains is nephrotoxic and the cause of cast nephropathy [9].

The infiltration of malignant PCs in the BM occur in three different patterns; interstitial, focal nodules or as diffuse sheets. The morphology of MM cells varies from mature types, indistinguishable from normal PCs, to immature, pleomorphic and plasmablastic. Mature PCs are usually oval in shape with an eccentric nucleus without nucleoli and "clock-face" or "cartwheel" chromatin, abundant basophilic cytoplasm and a perinuclear hof [10]. Immature forms have a higher nuclear/cytoplasmic ratio and an irregular nucleus, more prominent nucleoli and dispersed chromatin. Pleomorphic MM cells have multinucleated, polylobulated nucleus and plasmablasts are large cells resembling diffuse large B cell lymphoma with high mitotic activity. The morphological findings at diagnosis have not been included in current prognostic tools, but diffuse involvement and more immature MM cell morphology have been linked to more advanced disease and shorter survival [10-12]. The malignant clone produces one variant of immunoglobulin light chain, kappa *or* lambda, and flow cytometry analysis and/or immunohistochemical staining are used to confirm monoclonality of malignant PCs for a definitive diagnosis of MM. Flow cytometry is a valuable tool in the diagnosis, classification and monitoring of plasma cell disorders. Normal PCs have low forward/side scatter (FSC/SSC), high expression of CD38 and CD138 and are $CD19^{+}$ and $CD27^{+}$. Malignant PCs generally lack CD19 and have weaker expression of CD27 [13, 14]. Moreover, malignant PCs express a wide range of multi-lineage antigens. The aberrant surface expression of CD56, CD20, CD 117 and CD10 strengthens the diagnosis, as these antigens are generally not expressed by normal PCs [10, 13, 14]. 70-80% of MM patients have malignant PCs with aberrant CD56 expression, but the marker is usually negative in plasma cell leukemia [15-17]. CD117 expression have been linked to favorable prognosis [18]. CD20 and cyclin D1 expression correlates with the presence of translocation 11;14 [13, 19].

Despite the last 20 years of therapeutic advances, MM remains difficult to treat and is basically not curable. This has led to the concept of "MM stem cells" or "myeloma initiating cells" with tumor-initiating potential, self-renewal and pronounced chemotherapy resistance. However, MM cells exhibit high phenotypical and intra-clonal heterogeneity together with potential for plasticity, i.e. ability to modulate phenotype according to the surrounding tumor microenvironment. Different B cell precursor subsets have been proposed with conflicting experimental results. A distinct cell population responsible for tumor origin and persistence in MM have not been identified [20]. Instead, further development of diagnostic and therapeutic tools to detect and eradicate remaining subclones are needed, as well as further research to understand the intrinsic and extrinsic mechanisms responsible for disease progression. The latter is the focus of this thesis.

Diagnostic criteria and staging

Monoclonal gammopathy of undetermined significance

MGUS is present in about 2-3% of the Swedish population over the age of 50 years [21]. The prevalence increase with age to about 5% in people older than 70 years and reach 8% among those older than 80 years of age [22]. Two independent retrospective studies implies that MM is almost always preceded by a premalignant MGUS stage [23, 24]. The risk of progression of MGUS to MM is about 0.5 to 1% per year [25]. Patients with IgM immunoglobulin MGUS have a risk of progression to Waldenström´s macroglubulinemia of about 1.5% per year and only in rare cases IgM MGUS progress into MM [25]. The definition of MGUS is: serum monoclonal protein < 30 g/L, clonal BMPCs < 10% and absence of end-organ damage such as hyper**c**alcemia, **r**enal insufficiency, **a**nemia, and **b**one lesions (i.e. CRAB symptoms) or amyloidosis that can be attributed to the PC proliferative disorder [25]. Serum free light chain (FLC) assay measures unbound circulating kappa and lambda light chains. The normal ratio serum FLC kappa/lambda is 0.26-1.65. In clonal plasma cell disorders, the excessive monoclonal production of one of the light chains ("involved light chain") often results in an abnormal FLC ratio. In MGUS, about one third of the patients have an altered FLC ratio. MGUS patients without a monoclonal heavy chain expression, but a monoclonal production of kappa or lambda, are defined as "light-chain MGUS". These patients have increased levels of involved light chain in plasma, an abnormal FLC ratio, clonal BMPCs < 10%, no CRAB symptoms or amyloidosis and Bence Jones proteinuria < 500 mg/24h [25].

Smoldering multiple myeloma

In Sweden, SMM accounted for about 14% of all cases with newly diagnosed myeloma between 2008 and 2011 with an estimated incidence of 0.44 cases per 100 000 population [26]. The diagnostic criteria for SMM are: serum monoclonal protein (IgG or IgA) ≥ 30 g/L *or* urinary monoclonal protein ≥ 500 mg/24h *or* clonal BMPCs ≥ 10% and no CRAB symptoms or amyloidosis. During the first five years the risk of progression to MM is 10% per year, after that, the risk of progression decrease to 3% per year for the next five years and to 1% per year beyond 10 years of follow-up [27]. This means that 50% of patients with SMM do not progress during the first 5 years and one third of the patients are still not treatment demanding 10 years after diagnosis. Thus, in SMM there are patients with the same low risk of progression to malignant disease as MGUS, as well as "high-risk" SMM patients, who develop clinical symptoms and end-organ damage within the first two years of diagnosis. In 2014, the International Myeloma Working Group (IMWG) up-dated the criteria to include validated biomarkers associated with a risk of progression of SMM to MM of at least 80% within two years [25]. These changes are an attempt to enable earlier diagnosis of high-risk SMM patients and allow the initiation of therapy before detrimental end-organ damage occur. These new high-risk biomarkers includes ≥ 60% clonal BMPCs, a serum FLC ratio (involved/uninvolved light chain) of ≥ 100 (provided involved FLC level is > 100 mg/L) and more than one focal lesion on MRI ((magnetic resonance imaging), at least 5 mm in size) [25].

Multiple myeloma

MM is the second most common malignancy of the blood, with an incidence of about 6 to 7 per 100 000 inhabitants per year in Sweden [28]. The median age at diagnosis is 71 years [28]. Diagnostic criteria for myeloma are: ≥ 10% clonal BMPCs *or* biopsy-proven bony or extramedullary plasmacytoma with at least one more "myeloma-defining event" (MDE), i.e. any of the classical CRAB symptoms (hyper**c**alcemia, **r**enal insufficiency with creatinine clearance < 40 mL/min, **a**nemia and/or one or more osteolytic **b**one lesions on skeletal radiography, CT or CT-PET) or any of the high-risk biomarkers (≥ 60% clonal BMPCs, a serum FLC ratio (involved/uninvolved light chain) of ≥ 100 (provided involved FLC level is > 100 mg/L) and more than one focal lesion on MRI (at least 5 mm in size)) [25].

Monoclonal immunoglobulin with IgG isotype is most frequent in MM, followed by IgA [29]. Patients without a monoclonal heavy chain expression, but production of a measurable monoclonal light chain, "Bence Jones myeloma", constitutes about 14% [29]. About 90% of MM patients have an abnormal FLC ratio [25]. Patients with non-secretory MM represent 1-5%, IgD MM (2%), IgM MM (0.5%) and IgE MM is extremely rare with fewer than 50 cases reported in the literature [30, 31].

Other plasma cell dyscrasis

Include solitary plasmacytoma with or without minimal BM involvement, POEMS syndrome, systemic amyloid light-chain (AL) amyloidosis, and monoclonal gammopathy of renal significance (monoclonal immunoglobulin deposition disease, light-chain Fanconi syndrome, monoclonal gammopathy-associated membranoproliferative glomerulonephritis). Patients with these disease entities are not included in our current studies below and are described in detail elsewhere [25].

International staging system and prognosis

The latest version of staging system is based on a study of 4445 newly diagnosed MM (NDMM) patients from 11 international trials. The 5-year survival for stage I was 82% (28% of the patient cohort), for stage II 62% (62% of the patient cohort) and for stage III 40% (representing 10% of the patient cohort) [32].

Revised International Staging System(R-ISS) for multiple myeloma [32]

Stage I: serum Albumin >35 g/L, serum β-2-microglobulin <3.5, no high-risk cytogenetics* and normal LDH.

Stage II: Neither stage I or III

Stage III: serum β-2-microglubilin >5.5 <u>and</u> high-risk cytogenetics* *or* elevated LDH.

*High-risk cytogenetics include: t(4;14), t(14;16) or del(17p).

Immune dysfunction and infections

Immune dysfunction is a consistent feature of active MM and infections constitutes a major cause of morbidity and mortality [33, 34]. Yet the causative links between MM and the observed immune dysfunctions are only partly understood. Reduced levels of polyclonal immunoglobulin reflects a reduction of normal $CD19^+$ B lymphocytes and PCs with disease progression. The cytokine transforming growth factor-β (TGF-β) produced by MM cells is predominantly immunosuppressive, and inhibit normal B cell proliferation and T cell responses [33]. An up-regulated expression of interleukin (IL)-6 by BMSCs in the neoplastic BM microenvironment, stimulates MM cell growth and promotes $CD4^+$ T helper cell type 2 (Th2) differentiation. The Th2 cytokine IL-10 might in turn suppress anti-tumor Th1 differentiation [33]. Numerous other abnormalities in T-, natural killer (NK)- and NKT cell functions have been observed in MM [5, 33, 35], as well as myeloid cell dysfunctions as discussed in detail below. Neutropenia might be secondary to marrow infiltration in advanced stages, but is more often seen as a complication of chemotherapy [33].

In a large population-based study of over 9000 Swedish MM patients diagnosed between 1988 and 2004, Blimark et al. found that MM patients had a sevenfold increase in the risk for developing bacterial infections compared with age-matched controls, and during the first year following diagnosis the risk was 11-fold [34]. 9.9% of the newly diagnosed MM patients died during the first two months and 26.7% during the first year, and the cause of death was infection-related in 22% of the cases [34]. Augustson et al. showed the same trend in over 3000 UK MM patients, with a 10% mortality during the first 60 days of induction therapy, whereof bacterial infection was the direct cause of death in 45% of cases [36]. Sørrig et al. found a correlation between aggressive disease at presentation and the highest risk of developing blood stream infections and also stressed that the diagnoses of MM is often made in connection with a serious infection [37]. Patients with premalignant MGUS had a twofold increase in the risk for bacterial infections [38, 39].

Phagocytosis and Oxidative burst

In paper I and IV, neutrophil capacity for phagocytosis and oxidative burst in MM patients before and after high-dose treatment with melphalan and autologous stem cell transplantation, respectively, in newly diagnosed patients with MM, SMM and MGUS was studied. Activated phagocytes, neutrophils and monocytes-macrophages, eliminate pathogenic bacteria and fungi and remove damaged cells, by a process called phagocytosis (Fig. 1). The particles are internalized into the phagocytes´ cytoplasm in membrane-bound vesicles called phagosomes [40]. Toll-like receptors (TLRs) present on phagocytes, recognize highly conserved structures called pathogen-associated molecular patterns (PAMPs) on foreign particles. Complement and/or antibodies, (i.e. “opsonins”) attached to the surface of microbes strengthens the binding to the phagocyte, and significantly enhance the efficacy of the process [41]. Complement receptor type 1 (CR1), CR3 and CR4 and immunoglobulin G (IgG) Fc receptors are the major opsonin receptors on phagocytic cells. In paper I, we measured the surface expression of CD35 (CR1) and the high-affinity receptor FcγRI (CD64) on immature and mature neutrophils. These receptors recognize the complement proteins C3b and C4b, and the Fc portion of IgG1 and IgG3, respectively. An up-regulated surface expression of CD64 and CD35 on neutrophils have been associated with bacterial infections and may be used as early markers of septicemia [40, 42-44]. For phagocyte activation, binding and clustering of several receptors by lateral diffusion in the cell membrane is required, which means structural changes in the underlying actin cytoskeleton and loosening of its anchoring to the cell surface lipid bilayer by transmembrane proteins [41]. The plasma membrane in the region of the engaged receptors is extended and forms a cup around the particle after successive receptor binding, achieved by actin polymerization at the front and depolymerization at the base, a process dependent on activation of small GTPases of the Rho family [41]. The

phagosome closure at the top and final separation from the plasma membrane is catalyzed by the enzyme dynamin2 [41]. In the cytoplasm, phagosome vesicles fuse with lysosomes, and microbes are destroyed by reactive oxygen, nitrogen species and proteolytic enzymes inside the phagolysosomes. NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, a protein complex with five subunits, convert molecular oxygen to superoxide ions in a process where the cytosolic cofactor NADPH donates one electron [45]. Superoxide ions are used to generate reactive oxygen species (ROS) such as hydrogen peroxide, converted by myeloperoxidase to hypochlorite, that are highly toxic for bacteria and fungi [45]. ROS production is also called "respiratory burst" as it occurs during oxygen consumption by the phagocytic cell. Mutations in NADPH oxidase subunits results in defective ROS production and an inherited primary immunodeficiency disorder called chronic granulomatous disease (CGD). Children with CGD suffer from recurrent and severe infections, dysregulated inflammation and autoimmune complications [45].

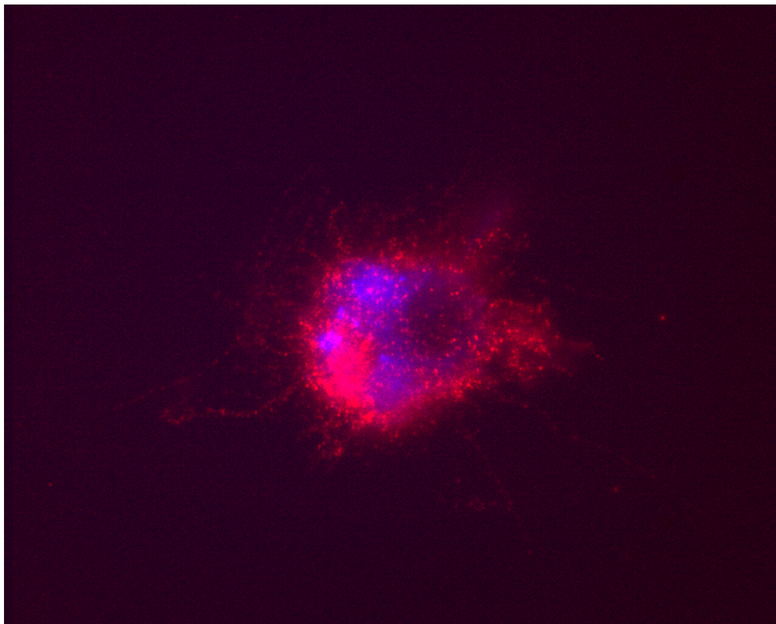

Figure 1. Macrophage phagocytosis of apoptotic neutrophils. Macrophage in red (stained with a fluorescent-labelled monoclonal antibody against CD206) and the cytoplasm of phagocytized neutrophils in blue. Photo by Thomas Hellmark.

Neutrophil extracellular traps

In 2004, the capacity of neutrophils to form so-called extracellular traps was discovered [46]. Neutrophil extracellular traps (NETs) are web-like structures of decondensed chromatin with attached antimicrobial proteins, the latter derived from neutrophil

granules including myeloperoxidase and elastase (Fig. 2). NETs trap and kill bacteria, fungi, parasites and certain viruses. They are thought to be crucial for the defense against pathogens that are too large for phagocytosis, like fungal hyphae, and to prevent the dissemination of bacteria and fungi [47]. Neutrophils can form extracellular traps via two pathways, either through a slow cell death process termed NETosis with nuclear delobulation, disassembly of the nuclear envelope, cellular depolarization, chromatin decondensation, plasma membrane rupture and release of NETs, or a *non-lytic* rapid NETosis with secretion of chromatin accompanied by the release of granule proteins through degranulation [47]. The remaining anucleated "cytoplasts" still have the capacity for phagocytosis. While the implications of NET formation in autoimmunity has been fairly well described, the role of NETs in cancer progression, metastasis and the thrombogenic property of NET formation in pathologic situations such as septicemia and malignancy only recently began to emerge [48-50]. Active MM constitutes a most thrombogenic state, with an up to 28-fold increase in the risk for venous thromboembolism compared with age-matched healthy controls, and the risk increase further on treatment with lenalidomide and corticosteroids [51-53]. The risk for venous thrombosis is highest during the first months after diagnosis [51]. It seems likely that NET formation contributes to this well-known feature of the disease, but it remains to be proven.

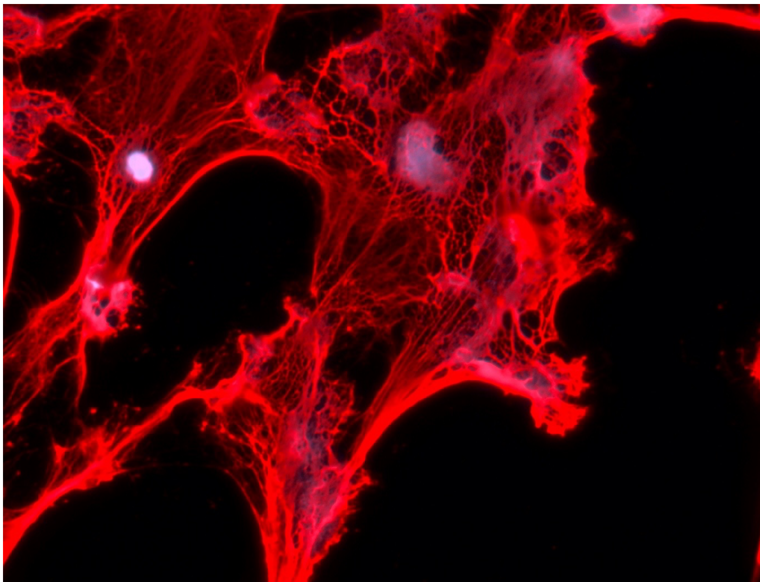

Figure 2. The figure shows neutrophil extracellular trap formation. DNA is visualised by DAPI in blue and histone proteins by Alexa Fluor 594-labelled monoclonal antibody in red. Photo by Åsa Pettersson and Thomas Hellmark.

Treatment

High-dose treatment with melphalan and autologous stem cell transplantation

The first effective treatment against MM was the alkylating agent melphalan, introduced in 1962, which combined with prednisone increased the median overall survival for MM patients from approximately six months to three-four years [1]. The dose-limiting toxicity of melphalan is primarily myelosuppression. The first trials with high-dose treatment was performed in 1983 in London by McElwain and Powles, who demonstrated that a single dose of intravenous melphalan 100-140 mg/m^2 could induce complete biochemical remission and eradication of detectable disease in the BM in three out of five previously untreated MM patients [1, 54]. Barlogie et al. was the first to introduce autologous stem cell support, i.e. a BM harvest was performed before high-dose treatment, and the frozen stored BM harvest was thawed and re-infused 24 hours after a melphalan dose of 140 mg/m^2. They could thereby achieve a faster recovery of neutrophil and platelet count and reduce the number of deaths in infectious complications [1, 55]. In the 1990s peripheral stem cell harvest was found to be superior to bone marrow transplantation regarding time to hematopoietic recovery/engraftment [1]. With modern induction therapy followed by high-dose melphalan with autologous stem cell transplantation (ASCT), about three out of four MM patients can achieve complete or very good partial remissions [56] (Abstract JCO)[1], but all patients eventually relapse with few long-term survivors (>15 years) [57]. The reconstitution of adaptive immune responses after ASCT has been extensively studied [58, 59], but the recovery of the innate immunity has received less attention. In paper I we have studied the recovery of PMNs and monocytes after ASCT.

In the late 1990s the first clinical trial with thalidomide, was conducted due to the observed anti-angiogenesis property of this agent [1, 60]. This proved to be the first new drug with single-agent anti-myeloma activity in more than three decades [60]. Subsequent studies demonstrated the potent immunomodulatory capacity of thalidomide and its newer analogs; lenalidomide and pomalidomide, as described below. Phase II trials with the first proteasome inhibitor, bortezomib, showed in 2003/2004 single-agent activity in relapsed/refractory MM patients and bortezomib became almost instantly, along with thalidomide and lenalidomide, cornerstones in MM treatment [61, 62]. Two second generation proteasome inhibitors, ixazomib and carfilzomib, are now in clinical use. The combination of a proteasome inhibitor with an immunomodulatory drug (IMiD) or alkylating agent and corticosteroids is standard of care in 1st line induction therapy for MM. High-dose melphalan 200 mg/m^2 and

[1] Abstract JCO Cavo M, J Clin Oncol. 2016;34 (suppl). Abstract 8000

ASCT are used as consolidation for patients younger than 70 years, without significant comorbidity, after 1st line induction therapy [63]. ASCT is quite often repeated as consolidation after treatment of first relapse. Median overall survival for Swedish MM patients diagnosed between 2008 and 2015 has improved after the introduction of proteasome inhibitors and IMiDs, and was 7.8 years for patients younger than 60 years, 5.4 years for patients aged 60-69, 3.3 years for patients aged 70-79 and 1.5 years for patients > 80 years of age [28].

Monoclonal antibodies

Immunotherapeutic approaches with targeted monoclonal antibodies (mAbs) against tumor-specific antigens, represents one of the major advances in modern cancer therapy. Though, the effect of mAbs in MM might be hampered by the immunosuppressive BM microenvironment [33]. A predominance of anti-inflammatory cytokines, TGF-β and IL-10, exerts a dampening effect on immune effector cells, including NK cell function and drives the polarization of macrophages towards an immunosuppressive M2 phenotype [64-66].

Most clinically available mAbs are of IgG isotype. Complement activating antibodies binds C1q-protein, resulting in complement-dependent cytotoxicity (CDC) [64]. The Fc-tail of mAbs binds to Fc-receptors on phagocytic cells. The human Fc IgG receptors includes three activating receptors; the high-affinity FcγRI (CD64), which is constitutively expressed on monocytes, macrophages and dendritic cells and expressed by neutrophils on activation, FcγRIIa (CD32a) expressed on most leukocytes and FcγRIIIa (CD16a) expressed by NK cells and certain tissue macrophages, and the inhibitory receptor FcγRIIb (CD32b) [64]. FcγRIIIb (CD16b) is expressed almost exclusively on neutrophils and primarily recognizes IgG-containing immune complexes [67]. NK cells induce apoptosis of tumor cells via antibody-dependent cell cytotoxicity (ADCC), whereas macrophages mainly eradicate opsonized tumor cells via antibody-dependent cell phagocytosis (ADCP) [64]. There is no evidence that neutrophils play any role in IgG-based anti-tumor therapies [64]. In solid malignancies, an up-regulated surface expression of CD47 on tumor cells mediates a "don´t eat me" signal, since the binding of CD47 to the inhibitory signal regulatory protein-α (SIRP-α /CD172a) on macrophages, results in reduced ADCP [64]. Clinical phase I trials with anti-CD47 mAbs are on-going and CD47 have been reported to be highly expressed on $CD38^+$ MM cells as well [68].

In 2015 two mAbs targeting MM cell antigens, daratumumab, a human CD38-specific IgG1 mAb and elotuzumab a humanized IgG1 anti-SLAMF7 (signaling lymphocytic activation family 7) mAb, were approved by the US Food and Drug Administration (FDA) for the treatment of relapsed/refractory MM patients and MM patients with high-risk cytogenetics [69]. While NK cell-mediated ADCC has been proposed the

most significant mechanism of activity in elotuzumab [70, 71], experimental studies demonstrated that daratumumab, but also elotuzumab, efficiently induced antibody-dependent macrophage-mediated phagocytosis/ADCP of tumor cells, indicating the clinical relevance of phagocytosis for the therapeutic effects [72, 73]. The combination of mAbs with lenalidomide have been described to improve NK cell-mediated ADCC and thereby the treatment response [71, 74]. In paper IV we found that untreated, newly diagnosed MM patients had BM PMNs with reduced phagocytic capacity. On the contrary, patients on treatment with lenalidomide-based therapy had PMNs with normal, restored capacity of phagocytosis. In further studies we will investigate if this also applies to monocytes-macrophages. An interesting current area of research is to what extent therapeutic mAbs can induce adaptive, long-lasting T cell immune responses, a phenomenon termed "vaccinal effect" [75]. Myeloid dendritic cells (mDCs) are important in initiation of specific immune responses, but in MM this cell population (as discussed below) was found to have reduced capacity for antigen-presentation and T cell stimulation [76, 77]. If mDCs or BM macrophages have the ability to activate self-reactive anti-tumor $CD8^+$ and $CD4^+$ T cells as a response on therapeutic mAbs in MM is not known.

Immunomodulatory drugs

Thalidomide and its synthetically modified and more potent analogs; lenalidomide and pomalidomide, are immunomodulatory drugs (IMiDs) with broad anti-myeloma activity [78]. These compounds have contributed to significantly better outcomes for patients with MM [79]. The degradation of intracellular proteins is a tightly regulated process. Incorrectly folded proteins and regulatory proteins with short half-life are marked by a polyubiquitin chain and degraded by proteolysis in cell structures called proteasomes [80]. IMiDs have been shown to exert their function by binding to cereblon (CRBN), a component of the E3 ubiquitin ligase complex. The binding of an IMiD molecule to CRBN, modulates the substrate specificity with diverse and so far not entirely understood effects [81]. Several of the positive treatment effects seen in MM could be explained by ubiquitination and thereby proteasomal degradation of the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3), both essential in B and T cell differentiation [79]. The degradation of these transcription factors has a direct anti-myeloma effect by reducing the expression of interferon regulatory factor 4 (IRF4) and the MYC oncogene in MM cells, two of the known drivers in the development and progression of plasma cell disorders [82]. In T cells, IKZF1 and IKZF3 act as transcriptional repressors of the *IL-2 gene*, and their degradation results in an enhanced expression of IL-2 by T cells, activating the T- and NK cell immune response against MM cells [83]. A paradox and unanswered question is the clear synergistic effect of IMiDs and proteasome inhibitors in the clinical setting [79].

BI-505 – a human anti-ICAM-1 antibody

The second paper in this thesis is a clinical phase 2 trial with a novel monoclonal antibody against intercellular adhesion molecule-1 (ICAM-1), BI-505, in patients with SMM [84]. BI-505 is a fully human, high-affinity IgG1 monoclonal antibody, which was isolated from a human phage-antibody library [85]. The original idea to use phage display of functional antibody fragments in the selection and development of novel therapeutic antibodies came from Sir Gregory P. Winter at the University of Cambridge and this awarded him the Nobel Prize in chemistry 2018 (shared). Recombinant single-chain variable fragment (scFv) fusion proteins were screened for selectivity for tumor B cell surface receptors and their ability to induce dose-dependent apoptosis in B lymphoma cells, but not in non-target cells [85]. Following the conversion to full IgG, BI-505 was shown specific for CD54/ICAM-1 [85]. The adhesion molecule ICAM-1 is a transmembrane glycoprotein, constitutively expressed at low levels on leukocytes and endothelial cells, and it binds to leukocyte β_2-integrins, CD11a-c/CD18 [86]. An up-regulation of ICAM-1 on endothelial cells at sites of inflammation results in increased leukocyte adhesion [86, 87].

In MM, the adhesion of malignant PCs to BMSCs triggers cytokine-mediated MM cell growth, survival and drug resistance [88]. The transcription factor "nuclear factor kappa light chain enhancer of activated B cells" (NF-κB) is a key regulator in MM cell growth and immune responses. Adhesion-mediated activation of NF-κB up-regulates the expression of adhesion molecules on both MM cells and BMSCs [88]. In the cytoplasm, NF-κB is bound to the inhibitor of κB (I-κB). Activation occurs on proteasome-mediated degradation of I-κB, which allows NF-κB to translocate to the nucleus where its binding to regulatory gene regions induce increased expression of cytokines (including TNF-α, IL-6), adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), and genes involved in anti-apoptosis, cell cycle regulation and proliferation [89, 90]. Overexpression of ICAM-1 in MM has been associated with more advanced ISS stage and multi-drug resistance [89, 91]. The increased surface expression of ICAM-1 on malignant PCs might be of importance for their co-localization and adherence to "nursery" $CD11b^+$ myeloid cells; e.g. monocytes, eosinophils and myeloid-derived suppressor cells (MDSCs) in the BM niche [92, 93], as discussed in more detail below. Proteasome inhibitors down-regulate the activation of NF-κB and related cytokine production. Moreover, MM cells, with altered and defective cell cycle proteins and high proliferation rate, accumulate damaged proteins and are therefore vulnerable for suppressed proteasomal degradation [89].

The BI-505 epitope of ICAM-1 was highly expressed on MM cells from both newly diagnosed and relapsed patients and the surface expression was 10 times higher than on normal B cells from the same patients [94]. The first experimental studies on treatment with BI-505 in SCID/xenograft mice models with four different MM cell lines showed dose-dependent tumor volume reduction. In an advanced, disseminated MM model,

BI-505 significantly enhanced animal survival compared with melphalan, bortezomib, lenalidomide and dexamethasone [94]. Moreover, a SCID/hu mice model with transplanted human bone chips and human primary MM cells demonstrated comparable BI-505 anti-myeloma activity with bortezomib [94]. In these models the anti-myeloma activity was mediated through macrophage phagocytosis (ADCP). Macrophages constituted the vast majority of FcγR-expressing cells in tumor tissue and depletion of macrophages abolished the in vivo efficacy of BI-505 [94]. On the contrary, intratumoral NK cells were scarce and their depletion had little or no effect on BI-505´s anti-myeloma activity. Pre-incubation with a recombinant soluble FcγR diminished BI-505 mediated ADCP. BI-505 did not induce CDC in tested cell lines [94].

A first-in-human trial with BI-505 in patients with relapsed/refractory MM

In 2015, the results of our first-in-human phase 1 trial with BI-505 was published [95]. The study was conducted to determine the maximum tolerated dose (MTD) of BI-505 when given as monotherapy, and to characterize its safety, tolerability, pharmacodynamics and pharmacokinetics in patients with advanced relapsed/refractory MM. Adult MM patients with at least two different previous therapies, an ECOG performance status of 2 or lower, and a life expectancy of over three months were included. All patients had a measurable M component. It was a multicenter, open-label, nonrandomized, repeat-dose, dose-escalation study. Patients in the first five dose groups (with doses of 0.0004 mg/kg, 0.001 mg/kg, 0.003 mg/kg, 0.009 mg/kg and 0.03 mg/kg) were only given two IV infusions of BI-505 on day 1, and on day 15 (one cycle). Patients in dose groups 6-11 (with doses of 0.09 mg/kg, 0.30 mg/kg, 0.90 mg/kg, 3.00 mg/kg, 10.00 mg/kg and 20.00 mg/kg) had the option to continue treatment with BI-505 (administered every two weeks). Dose group 1-5 consisted of one patient per group and the subsequent dose groups of three patients per group. A dose-limiting toxicity (DLT) was defined as any grade 3 or greater non-hematological toxicity or any grade 4 hematological toxicity, provided the toxicity was possibly related to treatment with BI-505. If a DLT was observed in any dose group, that cohort was reinforced to a total of six patients (as happened in one dose cohort (3.00 mg/kg). If a second patient in a dose group had a DLT, inclusion in that cohort was to be stopped and the dose level below identified as the MTD. Infusion-related reactions started to occur in dose group 8, at 0.9 mg/kg, and after that all patients received premedication with an antihistamine and paracetamol before each BI-505 infusion. In addition, patients in the 10.00 mg/kg and 20.00 mg/kg cohorts received corticosteroids before the first dose. The study was conducted between December 2009 and February 2013 at two sites in the United States, two sites in Sweden, two sites in Belgium and one site in Denmark. Median age of the patients was 62 years (range 46-79) and they had in median received 6 prior lines (range 2-12) of therapy. 34 patients received treatment ranging from 1-13 infusions. 29 patients completed cycle 1. The only DLT reported was a patient in the 3.00 mg/kg group who was hospitalized after

the first infusion due to a grade 3 headache, that resolved spontaneously after 48 hours. As only one DLT occurred, the MTD was not reached in this trial with doses up to 20.00 mg/kg. Adverse events (AEs) related to study treatment was reported in 26 (76%) patients. Most of these AEs (95.9%) were grade 1 or 2 in severity. Infusion-related reactions with pyrexia, chills, headache and elevated C-reactive protein, during/after the first infusion were common. To counter this, at the higher doses, all patients received premedication and the infusion started at a lower rate during the first 30 minutes. Only 6 AEs with severity grade 3 and a possibly or likely relationship to BI-505 was reported (headache, neutropenia, lymphopenia and thrombocytopenia). No grade 4 or 5 AEs with a possible or likely relationship to BI-505 was reported. A total of 26 serious AEs (grade 3-5) were reported in 14 patients, 12 of them in 10 patients occurred within 2 days of BI-505 administration. 10 of these SAEs were considered to be possibly or likely related to study treatment; headache (n=4), pyrexia (n=3), infusion-related reaction (n=1), fluid overload (n=1) and ECG T wave inversion (n=1). In this heavily pretreated patient population, no obvious treatment responses were observed. For assessment of BI-505 saturation of ICAM-1 receptors on PCs, BM samples were collected at screening and 48 hours after the end of first infusion and some samples were also collected before start of subsequent doses. Two labelled non-competing ICAM-1 antibodies were used; in addition to BI-505, enlimomab, an ICAM-1 specific mAb that recognizes a different epitope compared with BI-505, was used to estimate free ICAM-1 cell surface receptor on MM cells. Saturation of ICAM-1 BI-505 epitopes on BMPCs was achieved with doses of ≥ 3.00 mg/kg. Previous studies had shown that complete saturation of BI-505 epitopes was achieved at serum concentrations of BI-505 of about 1 µg/mL and the lowest dose at which serum concentrations were > 1 µg/mL and BI-505 epitopes were saturated throughout the entire dosing interval of 2 weeks was 10 mg/kg. In conclusion, BI-505 treatment in patients with advanced MM was generally well tolerated, with doses up to 20 mg/kg, the MTD was not reach. An optimal dose was determined to be 10 mg/kg every two weeks, which resulted in complete saturation of ICAM-1 epitopes on BMPCs during the entire dosing interval.

Novel immunotherapeutic strategies in multiple myeloma

PD-L1 (B7-H1 or CD274) is a cell surface protein that binds to the receptor programmed cell death-1 (PD-1) on T cells [96]. This interaction suppresses and terminates immune responses and is involved in peripheral tolerance and immune exhaustion. An upregulation of PD-L1 on tumor cells inhibits anti-tumor immune responses. The discovery that therapeutic blockade of “immune checkpoint inhibitors” is a potent strategy in the treatment of cancer, led to the Nobel Prize for Professor James P. Allison (CTLA-4) and Professor Tasuku Honjo (PD-1) in physiology & medicine 2018. MM cells seem to have variable expression of PD-L1 [96]. Recent studies reported that APRIL (a proliferation-inducing ligand) secreted by myeloid cells

(macrophages, osteoclasts and eosinophils) in the BM promotes MM cell growth and upregulates the expression of PD-L1 and PD-L2 on MM cells [96, 97] (Abstract ASH)[2]. In MM, the PD-1 inhibitors nivolumab and pembrolizumab, and the PD-L1 inhibitor durvalumab have been studied [96]. However, the first phase 1 studies on PD-1 inhibitors in monotherapy for patients with relapsed/refractory MM were disappointing, with no observed tumor responses. Monotherapy with these agents seemed insufficient to revert T cell exhaustion in MM, but in combination with other anti-myeloma therapies the results have been promising. Though, recently there have been concerns about the safety of PD-1/PD-L1 pathway inhibitors in combination with IMiDs in MM, and clinical trials with this combination are currently put on hold [96]. The main toxicities with these drugs are not unexpectedly different kinds of autoimmune manifestations [96].

A most interesting target in upcoming MM immunotherapies is B cell maturation antigen (BCMA) [98]. BCMA is a transmembrane protein belonging to the tumor necrosis factor receptor superfamily and its expression is restricted to PCs. Malignant PCs have increased expression of BCMA mRNA and protein compared with normal PCs [98]. The binding of the main ligand APRIL to BCMA promotes the growth and survival of PCs via activating PI3K-Akt, ERK1/2 and NF-κB signal transduction pathways and upregulation of anti-apoptotic proteins (Mcl-1, Bcl-2 and Bcl-xl) [98]. Overexpression or activation of BCMA induce increased expression of genes involved in stimulation of osteoclasts, adhesion (ICAM-1), angiogenesis (VEGF) and immunosuppression (PD-L1, IL-10, TGF-β) [97, 98]. Accessory cells of myeloid origin in the neoplastic BM microenvironment might provide the malignant PCs with APRIL [4, 97, 99]. In 2016, the first reports came on phase 1 trials with anti-BCMA chimeric antigen receptor (CAR) T-cells in patients with relapsed/refractory MM, and the results in these heavily pretreated patient cohort, were encouraging [100]. Several BCMA CAR T cells, anti-BCMA mAb with antibody-drug conjugates, bispecific T cell engagers (BiTEs) with scFv targeting BCMA and CD3ε, fully human IgG molecules with bi- and also trispecific targets including BCMA and CD3 on T cells or CD16a on NK cells, and therapeutic agents blocking the APRIL-BCMA interaction are under development or have already proceeded to clinical phase 1 or 2 trials [98].

[2] Abstract ASH Driscoll J, Blood.2017;130:4417

The bone marrow microenvironment in multiple myeloma

It is well established that interaction between tumor cells and the tumor microenvironment is of critical importance for tumor progression and response to chemotherapy [101, 102]. The BM is the primary site of growth and expansion of MM cells and both normal and malignant PCs are dependent on signals from the BM microenvironment to survive. Genetic and epigenetic aberrations in MM evolve during disease progression, but many of the aberrations are present already at the premalignant stages; MGUS and SMM, and it is believed that the neoplastic BM milieu actively contribute to the malignant transformation [5, 6, 103]. The BM microenvironment is compost of a cellular and a non-cellular compartment. The cellular compartment consists of bone marrow stromal cells (BMSCs), osteoblasts, myeloid and lymphoid cells, fibroblasts, endothelial cells and blood vessels [5, 104]. The non-cellular compartment includes the extracellular matrix, soluble factors like; cytokines, chemokines, growth factors, exosomes and microvesicles, and environmental factors such as hypoxia [6, 104]. All these factors might independently or through a complex interplay regulate MM cell growth and progression. An improved knowledge of crucial tumor-supporting mechanisms in the neoplastic BM microenvironment are important in the development of new therapeutic targets. This thesis focuses on the myeloid cells in plasma cell disorders.

Bone marrow myeloid cells in plasma cell disorders

Cells of myeloid origin constitute a major part of the cellular component in the neoplastic BM microenvironment of MM, and the majority of these cells are mature and immature neutrophils [49]. Cells belonging to myeloid lineage are granulocytes (i.e. neutrophils, basophils and eosinophils), monocytes, macrophages, dendritic cells, osteoclasts, erythrocytes and megakaryocytes [49].

Neutrophils

Neutrophils are the most abundant type of leukocyte and as the main actor in the innate immune response, they constitute the first line defense against invading pathogens. However, the classical view of granulocytes as merely short-lived, terminally differentiated phagocytes is changing. Accumulating evidence indicate the functional heterogeneity and plasticity among different granulocytic subsets and their ability to influence adaptive immune responses.

Neutrophils – "atypical" antigen-presenting cells

Professional antigen-presenting cells (APCs), i.e. dendritic cells, B-cells and monocytes-macrophages, capture and present peptides derived from extracellular proteins, including tumor antigens from ingested tumor cells, on major histocompatibility complex (MHC) class II molecules and activates $CD4^+$ T cells. Resting neutrophils have no or very low expression of MHC class II molecules, but they can be induced to express MHC class II and the co-stimulatory B7 molecules CD80 and CD86, by co-culture with antigens and memory T cells, and after exposure to specific cytokines, in particular IFN-γ [105, 106]. Cytokine-exposed neutrophils have in experimental studies been shown to gain the ability to stimulate $CD4^+$ T cells in an MHC class II restricted manner [107]. The antigen-presenting function of neutrophils seem to be induced only by antigen-specific memory T cells and not by naïve T cells. This is probably due to the more rapid and larger production of IFN-γ by the former [108]. It is thought that the acquired antigen-presenting function in granulocytes depends on the stimulation and activation of antigen-specific memory T cells by professional APCs, that in turn leads to the production of the specific cytokines required for neutrophil differentiation into "atypical" APCs in this particular microenvironment [106]. Memory T cells have higher expression of ICAM-1 than naïve T cells, and ICAM-1 binds to CD11b/CD18 on neutrophils [109]. CD66b is a unique marker for neutrophils, and this molecule can function as a receptor for galectin-3, expressed by human $CD4^+$ memory T cells [110, 111]. These receptor-ligand interactions between neutrophils and memory T cells might both initiate and amplify the antigen-presenting capacity by neutrophils. Although, the capacity of neutrophils to present antigens under certain conditions is well documented in these experimental settings, their actual contribution compared to professional APCs in physiological and pathological conditions including MM remains unclear.

Neutrophils – capacity of cross-presentation

After the initial interaction of the T cell receptor (TCR) on T cells with the antigen-MHC complex on APCs, the additional binding of CD28 on T cells to the B7 molecules; CD80 (B7-1) and CD86 (B7-2) on APCs, amplifies the TCR signal and is crucial for an adequate T cell response. Cytotoxic T lymphocyte antigen-4 (CTLA-4), one of the key immune checkpoint molecules, is a counteracting inhibitory regulator of this interaction. CTLA-4 is expressed on activated T cells and Tregs, binds to CD80/86 with higher affinity than CD28, and attenuates the T cell activation [112, 113]. In adaptive tumor immune responses, tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) are required for efficient tumor cell eradication. All nucleated cells have the capacity to present peptides from cytosolic proteins on MHC class I molecules and initiate $CD8^+$ T cells responses. Tumor cells might escape immune evasion by downregulation of MHC class I molecules. To enhance the differentiation of $CD8^+$ T cells into tumor-specific CTLs, professional APCs, particularly dendritic cells, might

present peptide tumor antigens on both MHC class I and class II molecules together with co-stimulatory CD80/86, a process called "cross-presentation" [114]. Tumor-infiltrating neutrophils can be induced to cross-present antigens of tumor cell origin on MHC class I and thereby promote tumor-specific CTL responses [106, 115]. In a BM microenvironment dominated by T cell dysfunction, with increased expression of inhibitory receptors (such as CTLA-4) on T cells, as described in MM, tumor-infiltrating neutrophils and eosinophils with acquired CD80/CD86 expression and/or APC function, might have the opposite effect as proposed in paper III [35].

Neutrophils – extracellular traps (NETs)

NETs have in an autoimmune setting been shown to promote malignant transformation of B cells to lymphoma via stimulation of NF-κB-signaling [116]. If NET formation could increase the risk of progression from MGUS and SMM to MM is not known.

Myeloid-derived suppressor cells

Activation of normal phagocytes as a response on infection or tissue remodelling after injury results in phagocytosis, oxidative burst and the release of pro-inflammatory cytokines. This reaction is short-lived and terminated on cessation of the stimuli. Chronic inflammatory conditions, autoimmune diseases and cancer is accompanied by a sustained myelopoiesis and the accumulation of immature myeloid cells. These cells deviate from the normal myeloid differentiation pathway, are pathologically activated and have the ability to suppress immune responses. The immunosuppressive function of these cells might be aimed to limit the extensive tissue damage that could be caused by prolonged antigen stimulation and uncontrolled immune responses. The phenotypical, morphological and functional heterogeneity of myeloid-derived suppressor cells (MDSCs) has made them difficult to define and classify, especially as there is no specific marker *or* combination of markers unique to human MDSCs. Recently a standardized nomenclature and characterization of MDSCs have been proposed, including minimal phenotypical and functional characteristics for the three subsets of MDSCs defined so far [93]. The phenotypical markers for polymorphonuclear MDSCs (PMN-MDSCs) are $CD11b^{+}/CD14^{neg}/CD15^{+}$ (or $CD66b^{+}$). Previously these cells were designated "granulocytic" MDSCs, and that is the term we have used in paper I. Surface markers for Monocytic MDSCs (M-MDSCs) are $CD11b^{+}/CD14^{+}/HLA\text{-}DR^{neg/low}/CD15^{neg}$. CD11b can be replaced by CD33 as very few $CD15^{+}$ cells are $CD11b^{neg}$. PMN-MDSCs are $CD33^{dim}$ and M-MDSCs $CD33^{++}$. The proposed markers for early-stage MDSCs (eMDSCs) comprising immature progenitors are Lin^{neg}(CD3/14/15/19/56)/$HLA\text{-}DR^{neg}/CD33^{+}$ [93]. The proposed gating strategy by flow cytometry cannot discriminate neutrophils from PMN-MDSCs and monocytes from M-MDSCs. The only method available to separate MDSCs from

mature normal neutrophils and monocytes is by Ficoll gradient centrifugation. MDSCs enrich in the mononuclear low-density fraction, but the separation is not perfect and the low-density fraction will contain both mature and immature cells. In the absence of unique MDSCs markers, single-cell functional analysis and detection of MDSCs in unseparated peripheral blood (PB) samples is as yet not possible. To define cells as MDSCs they have to fulfil the phenotypic criteria in combination with a proved ability to suppress T-cell activity, such as inhibition of T cell proliferation or INF-γ production [93].

MDSCs – promote disease progression and chemoresistance in MM

A characteristic feature of MDSCs is their potent ability to suppress T cell-mediated anti-tumor immune responses [117, 118]. The release of reactive oxygen species (ROS) by PMN-MDSCs and peroxynitrite, derived from the up-regulation of inducible nitric oxide synthase (iNOS) by M-MDSCs, inhibit T cell receptor signal transduction [118]. M-MDSCs have an up-regulated expression of arginase-1, which depletes the extracellular matrix of the essential amino acid arginine, required for T-cell proliferation [119]. MDSCs also have an indirect immunosuppressive effect by the induction of regulatory T cells (Tregs) [120-122]. Recent years of research have demonstrated that MDSCs, mainly PMN-MDSCs, accumulate in the BM of patients and animals with MM [102, 123-126]. MDSCs might support MM disease progression by direct stimulation of MM cell proliferation [124], inhibition of tumor-specific CTLs [123], induction of Tregs [125] and increased angiogenesis [127]. It is well known that the tumor microenvironment affects the tumor cell chemosensitivity, but the cellular mechanisms and the relative contribution of each cell population is not entirely understood. The chemoprotective effect of BMSCs, macrophages, dendritic cells and osteoclasts seem to be dependent on a direct MM cell contact through adhesion molecules, e.g. CD28, ICAM-1 or P-selectin glycoprotein ligand-1 (PSGL-1) [128-130]. Ramachandran et al. showed in MM mouse models that elimination of BM $CD11b^+$ cells reduced MM cell growth and resulted in improved chemosensitivity [102]. Interestingly, both PMN-MDSCs and mature neutrophils from BM of MM patients could reduce the cytotoxic effect of doxorubicin and melphalan on different human MM cell lines [102]. BM immature myeloid cells (with the same phenotype as PMN-MDSCs) from healthy donors had a similar effect. Moreover, the protective effect of neutrophils did not require cell-cell contact, as the addition of supernatant from these myeloid cells were sufficient to increase MM cell chemoresistance [102]. The soluble factor/s/ responsible for the neutrophil-induced MM cell chemoresistance remains to be defined.

Eosinophils

Eosinophils, the end effector cells in parasitic infections, have been associated with several pathological conditions like asthma, hypereosinophilic syndrome and graft-versus-host disease (Fig. 3) [131]. As a source of over 35 cytokines, chemokines and growth factors, they are suggested to have immunomodulatory properties, which also could affect tumor growth [132, 133]. In similarity with neutrophils, eosinophils have been shown to express MHC class II and co-stimulatory molecules, process antigens and stimulate T cells to proliferate and produce cytokines in an antigen-specific manner [106, 134]. In mice, purified and in vitro activated eosinophils induced the recruitment of tumor specific CTLs [135]. Tumor-associated tissue eosinophilia (TATE), have been observed in several types of solid cancers and has generally been linked to favorable prognosis [131, 133]. In contrast, TATE in the setting of Hodgkin lymphoma nodular sclerosis constitutes a poor prognostic factor [136]. Recently, Lingblom et al. could demonstrate that a subset of "regulatory eosinophils" have direct T cell suppressive effects. [137]. This regulatory subset of eosinophils had gained surface expression of CD16. The protein galectin-10, abundantly stored in human eosinophils, was crucial for the T cell suppressive effect and could be detected in immune synapses between lymphocytes and eosinophils [137].

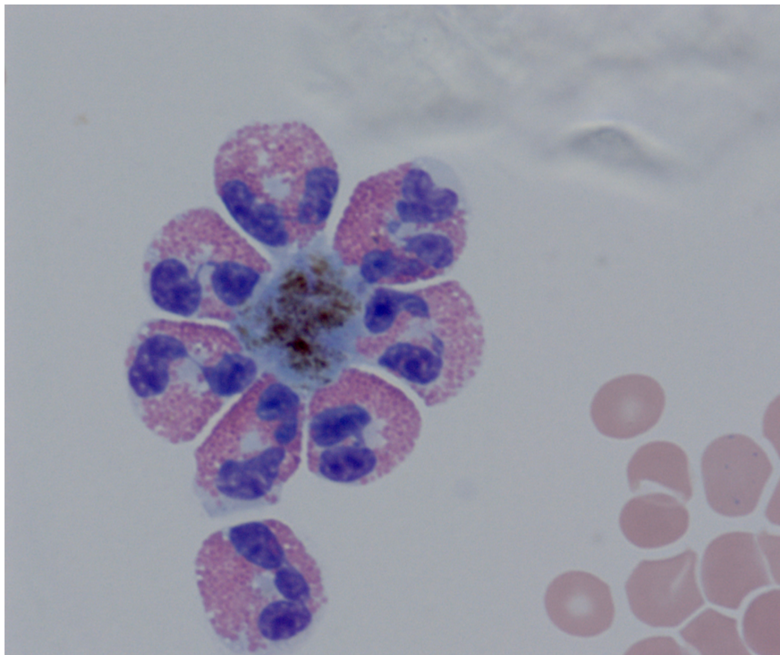

Figure 3. Purified eosinophils stained with May-Grünwald Giemsa. Photo by Åsa Pettersson and Thomas Hellmark.

Eosinophils – of importance for the survival, proliferation and retention of plasma cells in the bone marrow

Presence of eosinophils were in experiments on mice shown to be required for the retention of normal PCs in the BM and for the survival of long-lived PCs [4]. Mice deficient in eosinophils had significantly fewer BMPCs and depletion of eosinophils with an anti-siglec-F antibody resulted in decreased frequency of PCs in the BM [4]. Depletion of eosinophils by IL-5 blockade in a mouse MOPC315 BM model of MM reduced the early stages of MM growth, but more advanced stages were unaffected [99]. Wong et al. have with BM biopsies from 10 patients with MGUS, SMM and MM shown that eosinophils are in close proximity to malignant PCs in the BM and moreover, the frequency of eosinophils increased with disease progression [138]. Expression of chemokine receptor 4 (CXCR4)/CD184 on plasma cells and eosinophils might explain their co-localization to the same BM niches as a response on chemokine ligand 12 (CXCL12), also called stromal cell-derived factor-1 (SDF-1) secreted by BMSCs [4, 139-141]. The cytokines IL-6 and APRIL have been shown to promote survival and proliferation of normal and malignant PCs in mice and are secreted by eosinophils, BMSCs and megakaryocytes [4, 99]. In *in vitro* experiments on human MM cell lines, the presence of eosinophils promoted plasma cell proliferation in half of the cases and cell-free culture supernatant from BM or PB eosinophils was sufficient to induce MM cell proliferation [138]. The tumor-supporting effect of eosinophils might therefore be mediated by a soluble factor. Blocking the activity of APRIL and IL-6 in these experiments on human MM cells lines did not remove the eosinophil-promoting effect, indicating that there might be other/additional soluble factors, which remains to be identified [138]. Eosinophils have also been suggested to play a role in modulating B cells numbers in mice and humans [142]. Despite these experimental data indicating the importance of eosinophils for PCs in the BM, very little is actually known about their clinical relevance in MM. In paper III we have analyzed frequency and phenotype of eosinophils in PB and BM samples from 23 patients with newly diagnosed MM and MGUS.

Monocytes and macrophages

Macrophages, which usually constitutes a small part of BM cells, expand in malignant plasma cell disorders [130, 143]. Zheng et al. found that macrophages represented about 11% of the cells in BM samples from MM patients, compared with less than 1% in control samples [130]. The chemokine (C-C motif) ligands (CCL) 2, CCL3 and CCL14 are highly expressed by MM cells and BMSCs, and these cytokines stimulated the recruitment of monocytes to the BM microenvironment [144]. The level of CCL 3 and CCL14 correlated to number of BM macrophages [144].

Macrophage - MM cell interactions promote chemoresistance in MM

In vitro co-culture of human MM cells with macrophages were shown to protect the malignant cells from dexamethasone- and melphalan-induced apoptosis [130]. Macrophages that were pre-exposed to tumor-culturing conditioning medium for 72 hours were designated "tumor-associated" and had a more protective effect than normal macrophages. The chemotherapy-protective effect was abolished if macrophages and MM cells were separated by Transwell inserts, indicating that the protective effect was dependent on a direct cell-cell contact. Addition of an antibody specific for the adhesion molecule ICAM-1 (other than BI-505) was also shown to block the protective effect of tumor-associated macrophages against melphalan-induced MM cell apoptosis, confirming the importance of a direct cell-cell contact [130]. Subsequent studies demonstrated that two paired cell surface protein interactions were critical for the macrophage - MM cell interactions conferring drug resistance. MM cells have surface expression of PSGL-1 and ICAM-1, which binds to selectins, respectively CD11/CD18, on macrophages, and antibodies against these four molecules and genetic knockdown abrogated the protective effect of macrophages [129]. Moreover, the interaction of macrophages and MM cells via these adhesion molecules activated MM cells pro-survival signaling by downstream activation of Src and ERK 1/2 kinases and c-MYC accumulation [129]. The presence of macrophages mediated multidrug resistance of MM cells, both against conventional chemotherapy, corticosteroids and bortezomib [129]. We have performed a first-in-human phase I study of a human, monoclonal ICAM-1 antibody, BI-505, in patients with advanced relapsed/refractory MM. Even if well tolerated, in single therapy, none of these heavily pretreated patients showed any obvious treatment response [95]. In paper II we performed a subsequent phase 2 study with BI-505 for patient with SMM as discuss below [84]. If the addition of BI-505 to other anti-myeloma therapies could sensitize or revert the increasing drug-resistance, characterizing more advanced disease stages in MM, is so far not tested in a clinical setting.

Increased amount of BM macrophages with M2 phenotype associated with unfavorable prognosis

Tumor-associated macrophages arise from *in situ* maturation of monocytes in a neoplastic microenvironment. Activated macrophages exist in different functional subtypes classified as M1 (pro-inflammatory) and M2 (immunosuppressive) [49, 145]. M2 have low expression of TNF-α and IL-12 and increased IL-10 production. Macrophages predominantly display the M2 phenotype in myeloma BM microenvironment and an increased number of BM $CD68^{+}/CD163^{+}$ M2 macrophages was associated with a poor prognosis [143, 145]. IL-10 is a MM growth factor and might enhance angiogenesis through VEGF secretion and as discussed in paper IV affect neutrophil migration [145-147].

Dendritic cells

The two major subsets of dendritic cells (DCs); myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) normally represent less than 1% of hematopoietic cells in the BM [49]. Peripheral blood DCs from MM patients were functionally defective in antigen-presentation and T cell stimulation [76, 77]. mDCs are professional APCs, critical in the initiation of specific immune responses. mDCs have as professional APCs surface expression of the B7-1 molecules CD80/86. MM cells have high expression of CD28 and co-culture with CD80/CD86 expressing mDCs protected the malignant PCs from dexamethasone induced apoptosis [148]. Blocking this surface molecule interaction abrogated mDCs´ ability to protect MM cells from melphalan-induced apoptosis *in vitro* and sensitized MM cells to melphalan and reduced tumor burden in a mouse MM model [149]. pDCs are also known as "interferon-producing cells". They possess TLR-9 and are involved in mediating antiviral immune responses. pDCs have been associated with B cell development into plasmablasts and differentiation to antibody-secreting PCs. In BM samples from MM patients, the percentage of pDCs was increased to 4.7 +/- 0.8% compared with 0.4 +/- 0.2% in control samples [150]. Presence of pDCs enhanced growth and survival of malignant MM cells from human MM cell lines and primary tumor cells from MM patients, but not normal PCs [150]. pDCs were also shown to enhance proteasome activity in MM cells and thereby increase their resistance against the proteasome inhibitor bortezomib [150]. The TLR-9 agonist C792 restored BM-derived pDCs immune function and blocked pDC-induced MM cell growth and drug resistance in experimental studies [151].

Megakaryocytes

Megakaryocytes, constitutes less than 0.1% of the hematopoietic cells in the BM. In a mouse MOPC315 BM model of MM, megakaryocytes were found to be in close contact with malignant plasma cells [99]. Megakaryocytes secrete APRIL and IL-6 and co-localized with MM cells constitutes a local source of these MM promoting growth factors [99].

Osteoclasts

In healthy subjects, there is a fine-tuned balance between bone formation by osteoblasts and bone resorption by osteoclasts. At some stages of the disease, 80-90% of MM patients are affected by skeletal manifestations including bone pain, lytic bone destructions, pathological fractures and hypercalcemia [8]. This is caused by an overactivation of osteoclasts in a process driven by MM cells and their influence on the surrounding cells in the BM microenvironment. One of the most well-studied drivers of osteoclastogenesis is RANKL (receptor activator of nuclear factor-κB ligand).

Malignant PCs express and promote the expression of RANKL by BMSCs, activated T-cells and osteoblasts, which binds to the receptor RANK on osteoclast progenitors and promote the maturation and differentiation of osteoclasts [8]. Osteoprotegerin is a soluble receptor secreted by osteoblasts and stromal cells. Osteoprotegerin also binds to RANK on osteoclasts and inhibit osteoclast differentiation [8]. Syndecan-1 (CD138) on normal and malignant PCs cells degrades osteoprotegerin, and in MM with increased PCs infiltration this interaction further amplifies the osteoclast activation and bone resorption [152]. RANKL signaling in malignant PCs enhanced the release of TNF-α, IL-6 and IL-8, promoting growth and survival of the malignant PCs [153]. Direct cell-cell contact between MM cells and osteoclasts mediated by integrins supported MM cell growth and therapy resistance [154]. Lawson et al. developed a method to track myeloma cell appearance over time in the tibia of living mice by intravital two-photon microscopy [155]. Murine MM cells were double-labelled with both a fixed genetic reporter and a membrane bound dye, retained by non-dividing dormant MM cells. The injected MM cells entered the BM and directly migrated to an endosteal niche adjacent to osteoblasts. Co-culture of MM cells with osteoblasts maintained the cells in a dormant, non-proliferating state [155]. Dormant, non-dividing MM cells were resistant to treatment with chemotherapy [155]. Stimulation of bone resorption and osteoclast activity with soluble RANKL resulted in a reduction of dormant MM cells and increased tumor burden [155]. The authors of this paper raised the question if inhibitors of bone resorption, such as bisphosphonates, could prevent activation of dormant MM cells and sustain remission, *or* if a short-term treatment with an osteoclast activating agent could facilitate the eradication of remaining minimal residual disease (MRD) [155]?

In summary, the growth and survival of MM cells, and their sensitivity to treatment, are strongly dependent on the BM microenvironment and the accessory cells. Further research is needed to clarify these interactions. This might open up for new treatment approaches, which could work synergistically with more direct MM cell-targeting therapies.

Aims of the studies

Paper I

The aim of the first study was to investigate the innate immunity of patients with MM after treatment with melphalan and autologous hematopoietic stem cell transplantation (ASCT), with a focus on subpopulations and functioning of polymorphonuclear leukocytes (PMNs) and monocytes during the early phase of reconstitution of the immune system, and the putative presence of myeloid-derived suppressor cells (MDSCs).

Paper II

BI-505 is a fully human, high-affinity IgG1 monoclonal antibody directed against ICAM-1. The BI-505 epitope was strongly expressed on myeloma cells from both newly diagnosed and relapsed patients. The anti-myeloma activity of BI-505 has been evaluated in animal models and it was well tolerated in our earlier phase 1 trial. In this phase 2 trial the effects of BI-505 in patients with SMM were studied.

Paper III

Experimental data indicate that eosinophils are important for the survival, proliferation and retention of PCs in the BM. The clinical significance of eosinophils in plasma cell disorders in humans is largely unknown. The aim of this study was to examine the frequency and phenotype of mature siglec-8 positive eosinophils in BM samples from patients with newly diagnosed multiple myeloma (NDMM), SMM and MGUS compared with healthy controls. We wanted to investigate if there was a correlation between percentage of eosinophils and the degree of plasma cell infiltration and/or disease stage. Moreover, we wanted to investigate if differences in BM eosinophil count could be linked to variations in T cell subsets.

Paper IV

The aim of this study was to examine neutrophil function in PB and BM samples from patients with untreated, newly diagnosed MM (NDMM), SMM and MGUS, compared with healthy controls and compared with treated MM patients in stable remission.

Methods

Paper I

Study population

After written informed consent, PB samples collected in heparin tubes were obtained from 14 patients with MM before and after treatment with high-dose melphalan and autologous stem cell support. Samples were collected the day before administration of melphalan, on day +11 to +14 (at the time point of expected first sign of engraftment) after transplantation, and one sample was collected from 10 of the patients on day +15-20 at the time point of discharge from inpatient care. As controls, we used PB samples from 40 healthy blood donors. When we began to analyze these samples, we found interesting differences compared with the control samples and we managed to obtain at least one additional follow-up PB sample from each patient, but the time from autologous stem cell transplant varied greatly, from day +47 and up to day +637 (Supplementary Table E1). All patients were still in stable remission, without signs of disease progression, when the PB sample for follow-up/steady state was collected.

PMNs and monocyte phenotypes

The expression profile of different surface markers by PMNs and monocytes was analyzed using flow cytometry. To lyse erythrocytes, 0.84% ammonium chloride was used. The remaining leukocytes were washed with phosphate buffered saline (PBS), resuspended in PBS with 0.5% bovine serum albumin, and stained with the following three panels of monoclonal fluorescent-labelled antibodies (BD Bioscience California, USA):

(1) CD10, CD16, CD14, CD35, CD49d, CD64, siglec-8, and CD193

(2) CD10, CD16, CD14, CD11b, CD11c, and CD62L

(3) CD10, CD16, CD14, CD11b, HLA-DR, CD33, and CD66b [156].

A flow cytometer, FACS Canto II, with the DIVA software (Becton Dickinson, BD, New York, USA) was used for data collection and Kaluza software (Beckman Coulter, Brea, CA) for analysis. The final elaborated gating strategies are illustrated and described in (Supplementary Fig. E1 (for panel 1 and 2), Fig. E2 (for panel 3) and supplementary methods, Paper I). To set up/compose the different panels of mAbs, we

reviewed the literature for PMN surface markers that changes during maturation and activation [157] and a work by Dumitru et al. published in June 2012, proposed a gating strategy for MDSCs with the mixture of mAbs against the surface antigens in the 3rd panel [156]. An anti-Siglec-8 mAb was added to panel 1 from the inclusion of the seventh patient in July 2013, as we learned to gate eosinophils. Two different gating strategies were used to sort eosinophils, panel 1 and panel 3, and we could conclude that they showed comparable results with good correlation, in control samples (rs: 0.94, $p < 0.0001$) and patient samples (rs: 0.98, p: 0.0004) [158, 159]. In the final manuscript the eosinophil counts achieved from the 3rd panel were presented (Table 2, Paper I).

Phagocytosis and oxidative burst

PMNs and monocytes capacity for phagocytosis and production of ROS were investigated within 24 hours of sampling using the PhagoTest respectively PhagoBurst assays (Glycotope Biotechnology, GmBH, Germany), according to the manufacturer's protocols. These flow cytometry-based methods measure the percentage of PMNs and monocytes having ingested fluorescein-labelled opsonized *Escherichia coli* (*E. coli*) and number of cells that have produced ROS after *ex vivo* activation with phorbole-12-myristate-13-acetate (PMA) or opsonized *E. coli*. Mean fluorescence intensity (MFI) corresponds in the PhagoTest assay to number of ingested bacteria per cell and for the PhagoBurst assay to degree of ROS production in response on the different stimuli.

Statistical analysis

GraphPad PRISM version 6.0a software was used for statistical calculations in Paper I, III and IV. The Friedman test was used for repeated comparisons of patient samples, collected at different times before and after ASCT. The Mann-Whitney U test was used for two group comparisons between patients and healthy controls. The Spearman´s rank correlation coefficient was used to quantify covariation. All p-values were considered significant at $p < 0.05$.

Paper II

Study design

A single-arm, open-label, phase 2 study to assess the efficacy, safety and pharmacodynamics of BI-505 in patients with SMM. Planned sample size was four to ten patients. This was thought to be a first pilot study to assess if single therapy with BI-505 had any effect on disease activity, measured as decrease in M protein level in plasma and urine, and/or decrease in percentage of BMPCs, before planning a larger randomized trial. Following a screening period of up to 14 days, eligible patients were to receive five intravenous infusions of BI-505 over a seven-week period; 3.00 mg/kg

on treatment day 1 and 10.00 mg/kg on treatment day 8, 22, 36 and 50. This was considered one dosing cycle (cycle 1). M protein level in plasma and urine was determined throughout the study. Bone marrow sampling was performed at screening and on treatment day 50 or at the "end of study visit" 28 days after the last dose of BI-505. Patients with at least a minimal response (MR), based on M protein level day 50, were allowed to continue to cycle 2, which was to comprise three bi-weekly BI-505 infusions of 10 mg/kg. MR was defined as a 25-49% reduction in plasma M protein level [160]. Patients who achieved at least a partial remission (PR, ≥ 50% reduction in M protein level) on treatment day 92, were allowed to continue to cycle 3 with three additional bi-weekly doses of BI-505. The dosing regimen was based on data from the phase I study [95]. To reduce the risk of infusion-related reactions observed in the phase 1 study, the starting dose was lowered to 3.00 mg/kg, which was estimated to lead to complete saturation of all ICAM-1 epitopes on patients BMPCs during the first week. Subsequent doses of 10.00 mg/kg would result in complete epitope saturation during the subsequent two-week dosing intervals, with trough plasma concentrations above 1 µg/L, needed for full receptor saturation in the BM. Furthermore, the first and second doses were administered over four hours, with a lower initial infusion rate, compared to two hours infusions in the previous study protocol. As an additional precaution, all patients received paracetamol and an antihistamine (cetirizine) before each infusion.

The study protocol, patient information and consent form were reviewed and approved by an Independent Ethics Committee, The Regional Ethical Review Board in Lund, Sweden (www.epn.se), prior to inclusion of patients. The study was conducted in compliance with the protocol, regulatory requirements, good clinical practice (GCP) and the ethical principles of the latest revision of the Declaration of Helsinki as adopted by the World Medical Association. Written informed consent was obtained from all patients before enrollment. EudraCT number: 2012-004884-29. ClinicalTrials.gov Identifier: NCT01838369.

Study Population

Adult patients ≥ 18 years old with a diagnosis of SMM based on the 2009 IMWG criteria [161], i.e. a serum monoclonal (M) protein (IgG or IgA) ≥ 30 g/L and/or clonal BMPCs ≥ 10% <u>and</u> no CRAB symptoms or amyloidosis. Patients should have an Eastern Cooperative Oncology Group (ECOG) performance status of 0-1. Patients with significant co-morbidity were excluded and systemic corticosteroid use was not allowed within four weeks prior to screening. Patients were also excluded if they have had any prior or current treatment with proven or potential impact on PCs survival and proliferation. For details see Appendix paper II.

Safety and efficacy assessments

The primary objective was to assess the tumor response rate, defined according to the EBMT response criteria [160], including measurement of plasma and urine M protein,

serum free light chains and percentage of BMPCs. The secondary objectives were to further assess the clinical safety of BI-505 and the pharmacodynamics. AEs were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0.

Paper III

Study population

Patients referred to the Department of Hematology, Skåne University Hospital in Lund, Sweden, due to a newly discovered M component, or other symptoms consistent with MM, were consecutively included during one year, from the 1st February 2017 to the 31st January 2018. After written informed consent, PB and BM aspirates were collected. The patients were subsequently diagnosed with MGUS, SMM and MM. For comparison, PB and BM samples were also obtained from healthy controls.

Eosinophil phenotype and T-cell characterization

PB samples collected in heparin tubes, and BM aspirates collected in tubes with the culture medium RPMI 1640, were analyzed by flow cytometry. The following two panels of monoclonal fluorescent-labelled antibodies (BD Bioscience California, USA, for details on fluorochromes, see Supplementary Table E1) were used;

(1) PMNs and monocytes: CD45, CD14, Siglec-8, CD16, CD193, CD184, CD80, HLA-DR, CD62L, CD66b, CD11b and CD69.

(2) T-cells: CD3, CD4, CD8, CD45RA, CD197, CD196, CD183, CD25, CD127, CD194, CD45RO and HLA-DR.

The flow cytometer, FACS Fortessa, was used for the first 3.5 months and thereafter we used FACS Aria Fusion. DIVA software was used for data collection and Kaluza software (Beckman Coulter, Brea, CA) for analysis.

Eosinophils were gated on CD16 negative PMNs with strong expression of siglec-8 and their surface expression of CXCR4/CD184 (ability for homing to BM), CD80 (B7-1), HLA-DR (antigen-presenting capacity), CD193/CCR3 (the receptor for eotaxin), CD69 (marker of activation) and CD62L/L-selectin (adhesion to activated endothelial cells) were measured by MFI. Samples with < 100 cells gated were excluded. Monocytes were gated on typical FSC/SSC, CD45 and CD14 positive cells. The T cell subsets were gated according to standard gating strategy [162].

Statistical analysis

The Mann-Whitney U test was used for two group comparisons between patients and healthy controls and Wilcoxon matched-pairs signed rank test for comparisons between samples from PB respectively BM from each individual. The Kruskal-Wallis test was used for > two group comparisons. The Spearman´s rank correlation coefficient was used to quantify covariation. All *p*-values were considered significant at $p < 0.05$.

Paper IV

Study population

Patients referred to the Department of Hematology, Skåne University Hospital in Lund, Sweden, due to a newly discovered M component, or other symptoms consistent with MM, were consecutively included from February 2017 to June 2018 (The same patient cohort as the previous study on BM eosinophils, but extended inclusion period with nine additional patients). After written informed consent, PB and BM aspirates were collected. The patients were subsequently diagnosed with MGUS, SMM and MM. These samples were compared with BM samples from MM patients in stable remission on treatment with lenalidomide, and with BM samples from healthy controls.

Phagocytosis and oxidative burst

PB samples collected in heparin tubes, and BM aspirates collected in tubes with the culture medium RPMI 1640 were stored overnight. PMNs capacity for phagocytosis and production of ROS was investigated within 24 hours of sampling using the PhagoTest respectively PhagoBurst assays (Glycotope Biotechnology, GmBH, Germany), according to the manufacturer's protocols and as described in methods for paper I. For the first 3.5 months we used the flow cytometer FACS Fortessa, after that we switched to use the FACS Canto II. DIVA software was used for data collection and Kaluza software (Beckman Coulter, Brea, CA) for analysis.

Statistical analysis

The Kruskal-Wallis test was used for > two group comparisons, followed by Dunn´s multiple comparisons test. The Mann-Whitney U test was used for two-group comparisons. Correlation was assessed by Spearman´s test. $p < 0.05$ was considered significant.

Results and discussion

Paper I

Fourteen patients with MM from Malmö/Lund receiving high-dose melphalan with autologous stem cell transplantation at the Department of Hematology, Skåne University Hospital in Lund, were consecutively included from January 2013 until May 2014. They had all achieved at least partial remission on induction therapy before inclusion. All patients experienced neutropenic fever and were treated with broad-spectrum antibiotics. Median time point for neutrophil engraftment according to EBMT criteria with absolute neutrophil count ≥ 0.5 x 10^9/L was 12.5 days (range 11-18). It was not until day +13 that all patients had sufficient PMNs for flow cytometry-based analysis of neutrophil functioning, with a white blood cell (WBC) count of ≥ 0.3 x 10^9/L (median 1.7, range: 0.3-7.4). Day +13 was thereby selected as the first time point for testing after ASCT in this study. We compared the expression of selected surface markers on circulating PMNs and monocytes and their functioning on day +13 with samples collected before high-dose treatment, and at the time point of discharge from inpatient care on day +15-20, and also with a later follow-up sample ≥ 1.5 months after ASCT. As controls we used PB samples collected from 40 healthy blood donors.

The low-affinity receptor for IgG, CD16, first appears at the metamyelocytes stage [157], and has the highest expression on mature segmented neutrophils; whereas the zinc-binding metalloprotease CD10 is limited to the late segmentation stage [157]. First of all we found that PMNs defined by typical forward and side scatter properties, could be divided into three populations based on degree of CD16 expression: mature $CD16^{high}$ neutrophils ($CD16^{+++}$ /$CD10^{+}$), immature $CD16^{weak}$ neutrophils at the band and metamyelocytic stages ($CD16^{+}$/$CD10^{negative}$), and $CD16^{negative}$ PMNs ($CD16^{negative}$/$CD10^{negative}$) [163] (Supplementary Fig. E1, Paper I).

On day +13 and day +15-20, there was an expanded pool of immature $CD16^{weak}$ PMNs and a corresponding drop in more mature $CD16^{high}$ PMNs (Table 1, Paper 1). CD62L (L-selectin) has been suggested as a marker for recently released neutrophils and we observed that both mature and immature PMNs had elevated expression of CD62L after ASCT, consistent with a rapid transfer from bone marrow to peripheral blood (Fig. 1 C+G, Paper I) [164]. Expression of CD10 was significantly reduced on $CD16^{high}$ PMNs, not only on day +13, but also on day +15-20, consistent with a lack of

segmented neutrophils (Fig. 1A, Paper I). CD49d, a VCAM-1 and fibronectin antigen, is mainly expressed on myeloid precursors and mature eosinophils [157]. Expression of CD49d was increased in the expanded population of CD16^{weak} PMNs after ASCT (Fig. 1E, Paper I), indicating their more immature nature.

Mature CD16^{high} PMNs showed increased surface levels of CD35 (CR1) and the high-affinity FcγRI, CD64, indicating increased activation [42, 43, 157, 165] (Fig. 1 B+D, Paper I). CD35 expression on PMNs was further accentuated in patients who had received treatment with G-CSF, a phenomenon previously observed in healthy volunteers [166]. PMNs surface expression of CD11b and CD11c did not change during the treatment course. The surface expression of CD35 and CD64 was also increased on the more immature subset of neutrophils, the CD16^{weak} population. As CD64 is normally expressed on immature myeloid cells until the metamyelocytic stage, after which the surface expression for mature resting neutrophils usually drops to low levels [157]. The increased surface level of CD64 in this cell population might therefore not only be a sign of activation, but also reflect increased immaturity (Fig. 1H, Paper I).

The proportion of CD16^{negative} PMNs did not vary during the treatment course and was similar to that in healthy controls (Table I, Paper I). In healthy controls and in patients′ samples collected before treatment and at follow-up more than 1.5 months after ASCT, circulating CD16^{negative} PMNs consisted mainly of eosinophils. For recently transplanted patients the CD16^{negative} PMN population consisted of promyelocytes and myelocytes. An unexpected finding in this study was the almost complete absence of eosinophils on day +13 and on day +15-20 after ASCT. From one of the last included patients, we obtained a control sample on day +47 and there were still no detectable eosinophils. Siglec-8 is expressed late in eosinophil differentiation [159], and to be sure that we had not excluded immature eosinophils, we also used an alternative gating strategy to identify eosinophils based on CD16^{negative}/CD33^{dim}/SSC$^{moderate-high}$ PMNs [158] (panel 3 of mAbs, Table 2, Supplementary methods and Supplementary Fig. E2, Paper I). However, there was a strong correlation between the two gating protocols. This also means that the PMN pool on day +13 in median consisted of 53.8% (10th-90th percentile: 3.4-99.4, n=14) immature CD16^{negative} + CD16^{weak} PMNs, and 44.9% on day +15-20 (30.9-70.7, n=10).

We also found that a reduced proportion of PMNs in MM patients were able to phagocytose bacteria both before and up to at least day +18-20 post ASCT (Fig. 3, Paper I). Moreover, a smaller proportion of patients′ PMNs had capacity for oxidative burst after ASCT (Fig. 4, Paper I). 12 out of 14 patients had restored PMN phagocytic capacity in follow-up samples ≥ 1.5 months after ASCT.

Monocytes rapidly reappeared in PB and these newly released monocytes, in samples from day +13 after ASCT, had reduced antigen-presenting function with low expression of HLA-DR (Fig. 2B, Paper I). Absence of HLA-DR expression is otherwise

a characteristic attributed to M-MDSCs, but the percentage of HLA-DR^{negative} monocytes was similar to that in healthy controls in pretreatment and follow-up samples (Fig. 2A, Table 2, Paper I). Loss of HLA-DR expression on circulating monocytes has been observed in critically ill patients, especially in sepsis, and the ability to regain the antigen-presenting function was correlated to clinical outcome [167].

MDSCs are described to enrich in the mononuclear low-density fraction after Ficoll gradient centrifugation. In this trial we analyzed the whole leukocyte population, without previous density gradient separation. Since most mature neutrophils are CD33^{dim}/CD11b^{+}/CD66b^{+}/CD14^{neg}/HLA-DR^{neg}, we were only able to identify a small subpopulation of putative GrMDSCs in the CD16^{negative} PMN population representing immature myeloid cells and eosinophils. The PMN population with the phenotype: CD16^{neg}/CD33^{dim}/CD11b^{+}/CD66b^{+}/CD14^{neg}/HLA-DR^{neg}, representing potential immature granulocytic MDSCs (GrMDSCs) or just HLA-DR negative eosinophils (!), was small in both patients and controls and remained unchanged for the patients after recovery of the ASCT treatment (Table 2, Paper I). Instead, the 3rd panel of mAbs was used for the identification of eosinophils, myelocytes, promyelocytes and promonocytes and to define HLA-DR^{negative} monocytes (as outlined in Supplementary Methods and Supplementary Fig. E2, Paper I).

Our results indicate that several aspects of phagocytic functions are altered after high-dose melphalan and ASCT. The more immature PMN pool after ASCT showed reduced capacity for phagocytosis and oxidative burst. HLA-DR expression by monocytes was significantly depressed. These alterations might contribute to a weakened immune response and increased susceptibility to bacterial infections. An unexpected finding was the almost complete absence of eosinophils and their delayed regeneration after high-dose treatment. We found this particular interesting while, as discussed in paper III, experimental studies had shown that eosinophils promote the survival, proliferation and retention of PCs in the BM [4, 99, 138, 142]. A speculation and unanswered question is if the prolonged elimination of eosinophils after ASCT could augment the effect of high-dose melphalan in MM? This finding led to the subsequent study on eosinophils in newly diagnosed MM and MGUS (Paper III).

Paper II

BI-505 is a fully human, high-affinity IgG1 monoclonal antibody directed against ICAM-1/CD54. The BI-505 epitope was strongly expressed on MM cells from both newly diagnosed and relapsed patients [94]. BI-505 showed promising anti-myeloma activity in animal models [94] and it was well tolerated in our earlier phase 1 trial [95]. In this phase 2 trial the effects of BI-505 in patients with SMM were studied. The primary purpose of this pilot study was to evaluate anti-tumor effect before promoting a large randomized trial. Few treatment studies, aiming to prevent or delay the development of manifest disease, have been conducted in SMM [168-178], and standard of care is close observation without treatment until progression [179]. To keep in mind is that with the used IMWG diagnostic criteria from 2009 [161], before the addition of "high-risk" biochemical markers, 50% of SMM patients will not progress during the first 5 years and one third of the patients will still not be treatment demanding 10 years after diagnosis [27]. Consequently, only therapies with minimal side effects is to be considered. Our hypothesis was that BI-505 would be a possible treatment approach for SMM patients with potential to eliminate tumor cells with minimal long-term side effects. Moreover, we hypothesized that untreated SMM patients at an early disease stage, would be particularly suitable for treatment with monoclonal antibodies, since the therapeutic effects are dependent on a functional immune system, including competent effector cells, such as NK cells and macrophages.

The study was conducted between April 2013 and December 2014 at the Department of Hematology, Skåne University Hospital in Lund. Four SMM patients were enrolled, all in good clinical shape (ECOG 0), three males and one female. The age of the patients was 50, 64, 66 and 67 years. One patient discontinued due to migraine and onset of symptoms before start of premedication and first dose of BI-505 infusion. This patient received a dose of 0.27 mg/kg on treatment day 1 and no further doses. Three of the patients completed the first cycle of treatment defined as five doses of BI-505, a total of 43 mg/kg, over a seven-week period. In the three evaluable patients, BI-505 showed a benign safety profile. Of reported 22 AEs, 10 were judged to have a probable relationship to the study drug, nine infusion-related reactions grade 1 to 2 and one case of mild C-reactive protein increase. One other AE was judged as possibly related to study treatment, prolonged symptoms of bronchitis after a common cold. One SAE grade 3, acute coronary syndrome, occurred one month after the last dose of BI-505. The patient was treated by percutaneous transluminal coronary angioplasty and the relationship to the study drug was judged unlikely. He had well-known risk factors for cardiovascular complications (long term smoking, treatment for hypertension and hyperlipidemia), and it turned out that he had triple vessel coronary artery disease. BI-505 concentrations in BM aspirates were at the end of study treatment; 12.9 µg/mL (on day 50), 21.6 µg/mL (on day 69) and 66.1 µg/mL (on day 50), indicating complete saturation of BI-505 epitopes on ICAM-1, as saturation were known to occur at a BI-

505 concentration of 1 to 3 µg/L [95]. None of the patients achieved a response as defined per protocol (Table 1, Paper III). After inclusion of the fourth patient, the sponsor decided to terminate the study.

Previous studies with BI-505 in animal models showed that the anti-myeloma activity mainly was mediated through macrophage phagocytosis (ADCP) [94]. In paper IV (unpublished data) we demonstrate that the phagocytic capacity of BM PMNs is reduced, not only in patients with manifest MM but also in patients with MGUS and SMM. Four patients out of seven with SMM had less than 20% of BM PMNs with capacity for phagocytosis (Fig. 3, paper IV). As a next step we will investigate if this also applies to monocytes-macrophages? BM macrophages in MM predominantly display the M2 phenotype [143, 145], but less is known about their phagocytic capacity. One study of tumor-associated macrophages in the setting of breast cancer showed preserved capacity for phagocytosis [180]. The combination of mAbs with lenalidomide have been described to improve NK cell-mediated ADCC and our data (paper IV) shows that the phagocytic capacity of PMNs are restored in MM patients on lenalidomide-based treatment [71, 74]. If concomitant therapy with lenalidomide induce more efficient antibody-dependent macrophage-mediated phagocytosis/ADCP of tumor cells remains to be proven. A retrospective speculation is that the binding of ICAM-1 on MM cells to CD11a/CD18 (lymphocyte function-associated antigen-1, LFA-1) on NK cells might be involved in mediating a first cell-cell contact and adhesion, and if BI-505 interferes with this interaction, it could affect NK cell-mediated tumor cell cytotoxicity? If BI-505 has this property is however not known. An experimental study published in 2014 showed that the addition of a mAb against CD18 significantly decreased NK cell activity and MM cell killing in elotuzumab treated MM cell line cultures [71]. Addition of an anti-ICAM-1/CD54 mAb did also significantly reduce MM cell killing by elotuzumab, but to a lesser extent than the anti-CD18 mAb [71]. On the other hand, in more advanced disease stages, the increased surface expression of ICAM-1 on malignant PCs might be of importance for their co-localization and adherence to "nursery" $CD11b^{+}$/CD18 (macrophage-1 antigen (MAC-1), complement receptor 3) myeloid cells; e.g. eosinophils, MDSCs and monocytes-macrophages, in the BM niche [4, 92, 93, 99, 129, 130]. Theoretically, the addition of an anti-ICAM-1 mAb to other anti-myeloma therapies in this setting could possibly sensitize or revert the increasing drug-resistance in advanced disease stages, but this is so far not shown in any clinical trial.

The number of included patients in this phase 2 clinical trial with BI-505 was too few to draw conclusions about efficacy on disease activity. A total of 22 MM patients, 19 with advanced refractory disease in the phase 1 trial and three with SMM in this phase 2 study, received repeated doses of BI-505 of ≥ 3.00 mg/kg (enough for complete saturation of the BI-505 epitopes on ICAM-1 on BMPCs for one week) and none of them achieved any disease response.

Paper III

This far, the knowledge about eosinophils and their effect on MM are mainly based on murine models and *in vitro* experiments using MM cell lines. The clinical relevance of eosinophils in plasma cell disorders is unknown. In this study we investigated the frequency and phenotype of eosinophils in PB and BM from patients with newly diagnosed MM (NDMM), SMM and MGUS, and whether the eosinophil population in BM might be correlated to degree of PCs infiltration or T cell subsets. A rationale for increased differentiation, accumulation and activation of eosinophils in MM, would be an up-regulated expression of IL-6 by BMSCs in the neoplastic BM microenvironment, that not only stimulates MM cell growth, but also promotes $CD4^+$ Th2 differentiation and induction of Th2 cytokines including IL-5 [33]. IL-5, fundamental for the differentiation and maturation of eosinophils, could be targeted with neutralizing mAbs (mepolizumab and reslizumab), or a blocking mAb against the IL-5 receptor, IL-5Rα (benralizumab) [181]. These agents are in clinical use for patients with severe asthma with minimal adverse effects reported [182].

A total of 23 patients were included. Nine patients were subsequently diagnosed with NDMM, five patients with SMM and nine patients with MGUS. Four additional patients (one only PB, three BM + PB) with NDMM had started treatment with high dose corticosteroids before sampling, which could affect the surface antigen expression on monocytes and eosinophils, as well as the T cells, and were therefore excluded from those analyses. None of the patients had any sign of on-going infection at the time-point of sampling. BM and PB samples from 19 healthy controls were also collected during this year. For measurement of degree of antigen expression with MFI, three NDMM patients, two MGUS patients and six healthy controls were excluded due to the use of another flow cytometer (FACS Fortessa) during the first 3.5 months.

There was no significant difference between the percentages of BM eosinophils for patients with NDMM, SMM and MGUS (n=23, in median 3.1% eosinophils of PMNs (range: 0.9-7.5%)) compared with healthy controls (n=19, in median 2.5% eosinophils of PMNs (range: 0.1-7.3%), (Fig. 1A, Paper III). Three out of nine patients with NDMM, one out of five patients with SMM, three out of nine MGUS patients, and five out of 19 healthy controls had > 4.0% eosinophils of PMNs in BM (Supplementary Table E2). Hence, there was no correlation with disease stage or between the percentage of BM eosinophils and the degree of PCs infiltration (Supplementary Table E2).

Four patients with NDMM had at the time-point of sampling already started treatment with corticosteroids and had significantly reduced percentage of eosinophils in PB (Fig. 1B, Paper III). This difference could not be explained by a corresponding increase in absolute neutrophil count (ANC) as the median ANC for patients was 3.7 x 10^9/L (range: 1.5-7.8) and for patients on corticosteroids, only moderately higher 4.9 x 10^9/L

(range: 4.8-5.5). Compared to other leukocyte subsets, eosinophils have higher expression of glucocorticoid-receptors [183]. There are two isoforms of the glucocorticoid-receptor, but the most expressed form is the "α-splice variant", which on activation is pro-apoptotic for eosinophils and makes them susceptible to treatment with corticosteroids [184, 185]. Nevertheless, BM samples from three of these patients were collected (n=3, 40 mg prednisolone daily for 2 days, 24 mg betamethasone for 4 days, and 20 mg dexamethasone for 4 days), and still showed 3 to 4% eosinophils of PMNs, which was on the same level as the median values for the patient cohort as a whole and not obviously reduced (Fig. 1A, Supplementary Table E2, Paper III). This raises the question of whether BM eosinophils are less sensitive to corticosteroids than their circulating counterpart. Do they have higher expression of the β-isoform glucocorticoid-receptor, which has been associated with glucocorticoid resistance in other diseases [184]? *In vitro*, addition of dexamethasone during eosinophil differentiation of $CD34^+$ human cord blood stem cells, resulted in enhanced eosinophil proliferation and differentiation [186]. While inhibiting apoptosis during early eosinophil differentiation, dexamethasone increased apoptosis of mature eosinophils [186].

BM eosinophils from patients with NDMM, SMM and MGUS had an increased surface expression of CXCR4/CD184 compared with BM eosinophils from healthy controls (Fig. 2, Paper III). CXCL12/SDF-1 secreted by BMSCs, binds to and activates CXCR4 on hematopoietic cells and this interaction is known to be important for the homing capacity to the BM [4, 139-141]. An increased expression of the receptor CXCR4 on patients′ eosinophils, might enhance their capacity for homing to the BM niches of hematopoietic stem cells and long-lived PCs, the origin for most plasma cell diseases.

As a sign of increased activation of BM eosinophils, CD69 expression was elevated compared with samples from healthy controls ($p < 0.05$, Fig. 3A, Paper III), more apparent in BM samples from patients with manifest disease, NDMM (p: 0.02 compared to healthy controls, data not shown) [187]. The expression of CD62L/L-selectin, the adhesion receptor for activated endothelial cells, tended to be lower, albeit not significant (p: 0.07, Fig. 3B, Paper III). The expression of CD193/CCR3, the receptor for eotaxin, on eosinophils from PB and BM, did not differ between patients and controls, nor did the expression of CD69 and CD62L on eosinophils in PB (data not shown).

Eosinophils were here shown to have surface expression of CD80 (B7-1) (Supplementary Fig. E1, Paper III). Eosinophils increase the expression of CD80 further upon activation and CD80 expression on BM eosinophils was correlated to CD69 expression (n: 17, rs: 0.56, p: 0.02, data not shown) in patient samples.

CTLA-4 binds to CD80 with higher affinity than the co-stimulatory CD28, and attenuates T cell activation [112, 113]. Both $CD4^+$ and $CD8^+$ BM T cells in patients

with MM are recently shown to have enhanced expression of the inhibitory receptor CTLA-4 [35]. Two out of 10 patients with MM in this study had BM eosinophils with a low, but measurable surface expression of HLA-DR (Fig. 3C, Paper III) and it has been reported that only few MHC class II molecules are needed for a cell to achieve APC function and interact with CD4⁺ T cells [188]. Together with the expression of CD80, this might be sufficient to engage the high affinity co-inhibitory receptor CTLA-4 on BM CD4⁺ T cells in MM patients, which would contribute to immunological tolerance in the tumor microenvironment. CD80 (B7-1) has also been found to interact with the programmed death-ligand-1 (PD-L1), with an affinity between CD28 and CTLA-4 [189]. PD-L1 is constitutively expressed on hematopoietic cells, and *in vitro* trials showed that T cell binding of PD-L1 to B7-1 resulted in down-regulation of T cell proliferation and cytokine production [189].

A previous study by Zelle-Rieser et al., with BM samples from patients with NDMM and age-matched controls, have shown that myeloma patients had decreased naïve CD8⁺ T cells and an expanded pool of CD8⁺ T effector memory RA⁺ cells (T-EMRA) [35]. Moreover, CD8⁺ CD45 RA⁺ T cells from patients′ BM, were on *in vitro* activation shown to actually reduce their capacity for proliferation and cytotoxic activity, consistent with T cell exhaustion [35]. We could in this study confirm that patients with NDMM had decreased naïve CD8⁺ T cells and a markedly increased proportion of CD8⁺ T-EMRA (Supplementary Fig. E2, Paper III). We also showed that patients with MGUS had a similar pattern of CD8⁺ T cell distribution (Supplementary Fig. E2, Paper III). An increase in exhausted memory T cells is described in settings with continuous antigen exposure like chronic infections and cancer [190]. The effector functions of exhausted memory T cells are impaired and a hallmark of these cells is a higher and sustained expression of inhibitory receptors like PD-1 and CTLA-4 [190].

Tumor-infiltrating neutrophils can be induced to cross-present antigens of tumor cell origin on MHC class I and thereby promote CD8⁺ T cell responses [106, 115]. If eosinophils, here shown to express CD80, also could be shown to have the capacity of cross-presentation, it would partly explain that their presence in solid tumors usually implicates an improved prognosis by activating tumor-specific CD8⁺ CTLs through TCR-peptide/MHC-binding followed by CD28-CD80 co-stimulation. In a microenvironment dominated by T cell dysfunction and increased expression of inhibitory receptors on T cells, as described in MM [35] and Hodgkin lymphoma [191], tumor-infiltrating eosinophils might have the opposite effect.

MM cells have high expression of CD28 and increased CD28-expression correlated clinically with disease progression [192]. MM cell co-culture with CD80/CD86 positive mDCs was shown to protect MM cells from drug-induced apoptosis [148, 149]. CD80-expressing BM eosinophils might theoretically have the same protective effect.

The distribution of $CD4^+$ T cell subsets, including regulatory T cells (Tregs) (Supplementary Fig. E3, Paper III), did not differ between patients and healthy controls. Increased eosinophils in the BM could not be correlated to Th2 or Th17 polarization of $CD4^+$ T central memory (T-CM) or T effector memory (T-EM) cells (data not shown). When analysing patients with MM, i.e. NDMM and SMM separately, a correlation between proportion of BM eosinophils and BM $CD8^+$ T-EM and T-CM cells was noted (n: 13, rs: 0.58, *p*: 0.04 and rs: 0.64, *p*: 0.02, data not shown), but each of these cell populations and variations were small, especially for T-CMs, while these data should be interpreted with caution, and there was no correlation between percentage of eosinophils and $CD8^+$ T-EM and T-CM cells when including MGUS patients and healthy controls (*p*: 0.50 and *p*: 0.24, data not shown).

Previous experimental data have shown that eosinophils directly benefits the retention, survival and proliferation of normal and malignant PCs in the BM [4, 99, 138]. Lately, the role of eosinophils as a major source of BMPCs survival factors has been challenged, whereas other myeloid cells have been reported to replace these function in eosinophil-deficient mice [193]. In this study we found no evidence for a direct correlation between amount of BM eosinophils and degree of BMPCs infiltration or disease stage in patients with newly diagnosed MM, SMM and MGUS. This does not exclude that eosinophils are one of the myeloid cell populations in the BM microenvironment that can support MM cell growth, either directly through cell-cell interactions e.g. via the binding of CD11b/CD18 and CD80 on eosinophils, to ICAM-1, respectively CD28 on MM cells, or indirectly through secretion of tumor-promoting soluble factors [138], or through T-cell suppressive effects, as suggested by us and others [137].

Paper IV

During 17 months, 11 patients with NDMM, seven patients with SMM and 14 patients with MGUS were included. During the same period, four MM patients in complete remission (CR) on lenalidomide maintenance therapy (10 mg daily), were included, as well as five patients in stable remission (three in stringent CR and two patients in good stable partial remission (PR) on lenalidomide-based treatment, and below we have named this group of nine patients "Len maintenance" (Table 1, Paper IV). For comparison, BM samples from 20 healthy controls were collected. In two cases, the PhagoBurst assay failed for control samples and in two other cases for the PhagoTest assay. None of the patients or healthy controls had any sign of on-going infection at the time-point of sampling. For the first 3.5 months we used the flow cytometer FACS Fortessa, after that we switched to use a FACS Canto II. Since this potentially could affect the MFI values, the first three patients with NDMM, two MGUS patients and four (PhagoTest) respectively five (PhagoBurst) healthy controls, were excluded from that part of the analysis (Table 1, Paper IV).

Several of the patients with NDMM, SMM and MGUS, had circulating PMNs with reduced *ex vivo* capacity for phagocytosis, measured as geometric MFI, that corresponds to number of phagocytized opsonized *E. coli* bacteria per cell, especially compared with the "Len maintenance" cohort (Fig. 1B, Paper IV). However, the most evident differences were observed for BM PMNs (Fig. 2 and 3, Paper IV). Patients with NDMM, SMM and MGUS had reduced percentage of BM PMNs with capacity to phagocytose opsonized *E. coli* bacteria and with the capacity to produce ROS on stimulation with *E. coli*, compared with healthy controls and compared with the "Len maintenance" cohort (Fig. 1, 2 and 3, Paper IV). These data indicate that neutrophil dysfunction is common in patients with NDMM and may occur already at the premalignant stage MGUS (Fig. 3, Paper IV). The compromised neutrophil function in these assays could not be explained by lack of immunoglobulins (hypogammaglobulinemia), since opsonized *E. coli*, with immunoglobulin and complement from pooled sera, was used as stimuli.

On the contrary, patients in stable remission and on-going treatment with lenalidomide, were shown to have PMNs with normal, restored capacity for phagocytosis and ROS production (Fig. 1, 2 and 3, Paper IV). Previous population-based data have shown a rate of early toxic deaths on conventional chemotherapy of about 10%, whereof infections was the cause in 22 to 45% of the cases [34, 36]. In an analysis of data from two multicenter, randomized clinical trials on first-line treatment with thalidomide, lenalidomide *or* bortezomib for patients not eligible for high-dose melphalan with autologous stem cell transplantation, including together over 1100 NDMM patients from May 2006 to Sept 2016, the rate of early deaths during the first two months was down to 1% and a two-year cumulative incidence of 4.1%. Causes of

toxic deaths were cardiac events in 28%, infections in 26%, and vascular complications in 16%. The majority of included patients were fit elderly patients < 80 years, without significant comorbidities. Although not directly comparable with older population-based data, this might indicate that induction treatment with novel agents reduce the risk of serious infections at the expense of newer toxicity [194].

Cytoskeletal remodelling is an essential part of the phagocytic process, and there is experimental evidence that IMiDs can improve RhoA GTPase activity and enhance actin filament formation [195-197]. This might be one explanation for the improved phagocytic capacity observed in the lenalidomide-treated patient cohort.

One hypothesis was that untreated NDMM patients might have an increased tendency for hypercalcemia that could affect phagocyte function. The lenalidomide treated patient cohort, with restored phagocyte function, did all receive regular bisphosphonate infusions and were therefore expected to have lower plasma calcium (P-Ca) levels. However, in this material, the decreased phagocyte function at diagnosis could not be correlated to variation in P-Ca levels, inflammatory activity *or* degree of BM plasma cell infiltration (Fig. 4 and Table 1, Paper IV).

Normal and malignant PCs produce IL-10, and this cytokine was detectable in sera from patients with advanced MM [147]. BM macrophages with M2 phenotype, the predominant variant in MM, also contributes to a locally increased IL-10 level [145]. In *in vitro* experiments, IL-10 has been shown to attenuate the ability of PMNs to phagocytose *E. coli* [198]. In a murine model of autoimmune disease, IL-10 directly inhibited neutrophil migration toward the anaphylatoxin C5a and IL-10 blockade could reduce the incidence of bacterial airway infections in a murine model of advanced MM [147]. Overexpression of IL-10 by MM cells and M2 macrophages might therefore be one of the factors leading to dysfunction of BM PMNs, at least in more advanced stages of the disease.

If the observed fully restored phagocytic capacity in the lenalidomide treated patient cohort merely is a result of effective plasma cell eradication, or if the treatment itself further improves these cellular functions remains to be elucidated, as well as if the phagocytic improvement also applies to monocytes-macrophages.

In this study we demonstrate that neutrophil dysfunction is an additional factor that might contribute to the increased susceptibility to bacterial infections in patients with newly diagnosed MM. Further studies are required to clarify the causes, which are likely to be multifactorial.

Conclusions

- Newly regenerated PMNs after high-dose melphalan with autologous stem cell transplantation (ASCT) had reduced capacity for phagocytosis and oxidative burst.
- Eosinophils, suggested to play a role in promoting the retention, survival and proliferation of plasma cells in the bone marrow (BM), were markedly reduced after ASCT and slow to regenerate.
- We found no evidence for a direct correlation between amount of BM eosinophils and degree of BM plasma cell infiltration, or disease stage in patients with newly diagnosed MM, SMM and MGUS.
- BM eosinophils from patients were more activated and had an increased homing capacity to the same BM niches as plasma cells.
- Patients on treatment with corticosteroids had low levels of circulating eosinophils, but preserved levels of eosinophils in the BM.
- Neutrophil dysfunction was common in patients with newly diagnosed MM and occurred already at the premalignant stage MGUS. BM phagocytes were most affected. The decreased phagocyte function at diagnosis could not be explained by blood calcium levels, inflammatory activity *or* degree of BM plasma cell infiltration.
- MM patients in stable remission and on-going treatment with lenalidomide had neutrophils with normal, restored capacity for phagocytosis and oxidative burst.

Limitations

MM is a rather uncommon disease and therefore it takes time to recruit and limits the number of patients. Moreover, in the third and fourth studies we wanted to identify and include patients at diagnosis, prior to initiation of treatment. Flow cytometry-based analysis of freshly collected PB and BM samples from MM patients implicated logistical requirements, from sampling to conducted analysis within 24 hours, and access to the same flow cytometry instrument (shared by all others in the laboratory). In the third and fourth trial we chose after the first 3.5 months to switch from one flow cytometer to another. The use of another FACS instrument could potentially affect the MFI-values, and therefore, we had to exclude some patient and control samples. In flow cytometry-based experiments, predefined panels of mAbs are used. Analysis of granulocytes with short life span, sensitive for any kind of manipulation, also implies that the samples could not be re-analyzed later on with additional mAbs, as could be done in experiments using frozen material or cell lines.

Planning the BI-505 phase 2 study, the prerequisite set by the sponsor, was a limited amount of remaining, available study drug. The calculated upper limit for number of patients, who could receive treatment and be included was a maximum of 10 patients. The clearly defined stop criteria in the protocol is also an expression for the limited supply of mAb. After the inclusion of the fourth patient, the company decided to terminate the study. They chose to prioritize another clinical trial with BI-505 as maintenance treatment after high-dose melphalan and ASCT.

Future considerations

In the fourth paper, we could show that BM PMNs had reduced capacity for phagocytosis. Does this also apply to monocytes-macrophages? If so, it might have implications for the treatment response on targeted mAbs. One hypothesis is that increased levels of IL-10 in MM, produced by PCs and M2 macrophages (and to a lesser extent $CD4^+$ Th2 differentiated T cells and Tregs?) in the BM [145, 147, 199], may suppress phagocyte functions. Do MM patients have increased levels of IL-10 in serum and/or BM aspirates, and if so, could the IL-10 level be correlated to PMNs′ and monocytes-macrophages′ capacity for phagocytosis? That would at least partly explain why BM PMNs were more affected than PB PMNs in our previous experiment (Paper IV).

An interesting observation was the exceptionally good PMN phagocyte function in our patients on lenalidomide maintenance therapy. There is some experimental evidence that IMiDs can improve RhoA GTPase activity and enhance actin filament formation [195-197], which is an essential part of the phagocytic process. Therefore, we want to test if patient PMNs with poor ability for *ex vivo* phagocytosis in the PhagoTest assay, could be directly improved by the addition of lenalidomide in the test tube. Another explanation might be that the treatment with lenalidomide, relatively fast could revert the immunosuppressive state in the BM microenvironment of MM patients, thus reducing the production of IL-10 and thereby restore neutrophil function? Later on, the treatment will in responding patients also lead to a reduction of MM cells. Paiva et al. reported increased T helper cell Th1 differentiation, T- and NK cell activation in patients with SMM during treatment with lenalidomide and dexamethasone [200]. Can a short-term treatment (a few days?) with lenalidomide improve phagocyte function in patients with SMM (– for those with less than 20% BM PMNs with capacity for phagocytosis)? Long-term treatment with this agent increase the risk of secondary malignancies [201], but if short-term treatment directly could restore neutrophil dysfunction in case of serious infections in MM patients, it would be of clinical interest. Likewise, it would mean that MM patients on treatment with lenalidomide, during infectious episodes, not necessarily have to stop the treatment, if neutrophil count is normal? The combination of mAbs with lenalidomide have been described to improve NK cell-mediated ADCC [71, 74]. Could treatment with lenalidomide also improve macrophage-mediated ADCP?

We have during the last year set up a method to sort MDSCs after Ficoll gradient centrifugation and to measure their T cell suppressive capacity. One thought is that these cells might have a reduced capacity for phagocytosis of microbes, but a preserved or increased capacity for phagocytosis of damaged cells?

A novel area of research is the role of NETs formation in MM. Kim et al. reported elevated indirect markers for NETs formation in different hematological malignancies [202]. A dysregulation of NETosis might contribute to the increased tendency for thrombosis in septicemia and malignancies [50]. If myeloma patients have an increased, pathologically disturbed propensity for NETs formation, is this at the expense of a decreased capacity for phagocytosis and a reason for the increased susceptibility to infections? NETs have been shown to entrap, but not always kill microbes [203, 204]? What mechanisms regulate these processes? As a first step it would be interesting to investigate if MM patients have PMNs with increased degree of extracellular traps formation compared with healthy controls (can be studied with live imaging techniques using fluorescence microscopy [205, 206]), and if so, does an increased tendency for NETs formation correlate to an increased frequency of thrombosis or bacterial infectious episodes? Patients on first line treatment with lenalidomide and dexamethasone have an even higher risk of thrombosis and if increased NETs formation is part of the pathogenesis, this would be reflected in repeated samples, collected at diagnosis prior to treatment, and during the first treatment cycles.

This was just some suggestions and thoughts how to proceed with on-going projects… Much remains to be elucidated regarding the immune dysfunction in MM and phagocyte function in health and disease.

Stina Wichert

November 2018

Populärvetenskaplig sammanfattning på svenska

Bakgrund

Multipelt myelom (MM) är den näst vanligaste formen av blodcancer. Cancercellerna vid MM härstammar från så kallade ”plasmaceller” i benmärgen. Benmärgen är det organ som står för nybildningen av blodceller och hos vuxna finns benmärg framförallt inuti kotkroppar, bäckenben, lårben och överarmsben, men även i platta ben, som skallben, bröstben och skulderblad. Plasmaceller är ”utbildade” celler i immunförsvaret, som bildar specifika antikroppar mot olika främmande ämnen, inklusive virus och bakterier och de utgör en del av kroppens immunologiska minne. Vid MM bildar de sjuka plasmacellerna vanligen en enda unik typ av antikropp och i takt med att de sjuka cellerna förökar sig allt mer ohämmat, så blir mängden av den bildade antikroppen allt högre och kan mätas i blodet, som en ”M-komponent”. En M-komponent är ett protein i blodet, som man normalt sätt inte ska ha. Det är dock inte så ovanligt att man med åren utvecklar en liten M-komponent i blodet, vilket innebär att man har en liten klon av plasmaceller i benmärgen. Såvida denna M-komponent/plasmacellsklon är liten och inte tillväxer ohämmat, så har patienten inte några symtom eller obehag av detta och tillståndet kallas för ”monoklonal gammopati av oklar signifikans” (MGUS). Man kan uppskatta att ca 2-3% av den svenska befolkningen över 50 år, har en sådan liten M-komponent. Numera vet vi att sjukdomen MM nästan alltid föregås av MGUS och risken för att utveckla en behandlingskrävande myelomsjukdom är ca 1% per år för patienter med MGUS. Om M-komponenten i blodet är mer än 30 g/L eller om andelen plasmaceller i ett benmärgsprov överstiger 10% av andelen vita blodkroppar, så är risken för att utveckla MM betydligt högre och tillståndet kallas då för ”smoldering” MM (SMM). För att ställa diagnosen MM, så ska sjukdomen vara behandlingskrävande, vilket i princip innebär påvisad organpåverkan, som gör att sjukdomen måste bromsas. När de sjuka plasmacellerna ökar i antal, så leder det till mindre plats i benmärgen för den normala blodbildningen, vilket i första hand drabbar bildningen av röda blodkroppar och leder till ”anemi”, i dagligt tal ”blodbrist” och trötthet. De sjuka plasmacellerna stimulerar nedbrytning av skelettet, som får urspaningar, så kallade lytiska destruktioner, det blir skört och ett vanligt första symtom på sjukdomen är smärtsamma frakturer redan efter en mindre belastning. Ökad skelettnedbrytning leder till högt kalciumvärde i blodet, som i sin tur ger symtom i

form av trötthet, förstoppning och njurpåverkan. Delar av M-komponenten, de lätta kedjorna, fälls ofta ut i och skadar njurarna. Patienter med MM har ett sämre immunförsvar och risken för bakteriella infektioner är 7 gånger högre jämfört med åldersmatchade kontroller. Även patienter med MGUS hade i en studie en dubbelt så hög risk för bakteriella infektioner. Orsakerna till att immunförsvaret är försvagat vid MM är inte helt klarlagda, men i takt med att de sjuka plasmacellerna tar över i benmärgen, så minskar andelen normala så kallade B-lymfocyter och plasmaceller, och den normala uppsättningen med antikroppar, som är riktade mot många olika främmande agens, minskar. Det försvagade immunförsvaret gör också att kroppen får allt svårare att hålla tumörsjukdomen ”i schack”. Samtidigt så är plasmaceller, både normala och sjuka, beroende av den omgivande benmärgsmiljön för att överleva och såväl andra typer av vita blodkroppar, som bindvävens celler, skyddar dem och förser dem med tillväxtfaktorer. De senaste 15 åren har flera nya effektiva läkemedel utvecklats, utöver äldre former av cellgifter, som påtagligt bidragit till förlängd överlevnad för flertalet patienter, men fortfarande finns det inte någon egentlig botande behandling mot MM och med tiden blir sjukdomen alltmer svårbehandlad.

Särskilt framställda antikroppar riktade mot proteiner, som överuttrycks på tumörcellers yta, används idag ofta som läkemedel, vilket då underlättar för immunförsvaret att känna igen dessa celler som sjuka. Vid MM har det varit svårt att ta fram antikroppsbehandlingar som är verksamma och det beror sannolikt på att immunförsvaret är så pass hämmat, att det inte räcker med att ge ett läkemedel som märker in tumörcellerna, utan man måste samtidigt ge andra läkemedel som aktiverar de celler som verkligen avdödar tumörcellerna. I ett arbete som föregick denna avhandlingen så genomfördes en så kallad klinisk fas 1 prövning med en ny antikroppsbehandling mot MM. Denna antikropp fick namnet BI-505 och riktade sig mot ett protein, ICAM-1, som oftast är överuttryckt på sjuka plasmaceller. När vi gav BI-505 till patienter med långt gången avancerad myelomsjukdom, så var behandlingen väl tolererad, men den gav inte någon mätbar effekt på sjukdomen.

Frågeställning och syfte

I den här avhandlingen har jag främst studerat vita blodkroppar av den typ som kallas granulocyter och monocyter och hur deras antal, funktion och cellyteproteiner påverkas vid plasmacellssjukdom i olika stadier och av den behandling som ges. Det är celler, som känner igen skadade celler och främmande partiklar. Efter att de bundit partiklarna till sin cellyta, så tas de upp och bryts ned/oskadliggörs inne i de vita blodkropparnas cytoplasma. Processen att ta upp t ex bakterier och antikroppsinmärkta partiklar kallas ”fagocytos” och celler med denna förmåga kallas därför ”fagocyter”. Den här typen av celler kan inne i blåsor i cytoplasman, där partiklarna bryts ned, bilda reaktiva syreradikaler, vilket är viktigt för att kunna avdöda bakterier. Om dessa cellers funktion försämras, så ökar känsligheten för infektioner. Nedsatt funktion hos monocytära celler

leder till sämre respons på de antikroppsbehandlingar, där förmågan att destruera de antikroppsinmärkta tumörcellerna genom fagocytos, är viktig.

Metod

Den här avhandlingen bygger på fyra delarbeten. I delarbete I, III och IV har blod- och (i de två senare även) benmärgsprover från patienter med plasmacellssjukdom analyserats med hjälp av flödescytometri (se nedan) och korrelerats med kliniska data. Vi har undersökt vilka och hur mycket av utvalda cellyteproteiner som granulocyter och monocyter uppvisar, vilket avspeglar deras mognadsgrad och aktivering. Ändrade ytproteinmönster kan tyda på ändrad funktion. Vi har också mätt cellernas förmåga till fagocytos av bakterier, samt förmåga till bildning av reaktiva syreradikaler. För jämförelse har vi erhållit prover från friska försökspersoner. Delarbete II är en klinisk fas 2 prövning med en ny terapeutisk antikropp (BI-505) riktad mot ICAM-I vid SMM.

Flödescytometri

För flödescytometrisk analys används instrument med avancerad optik, lasrar och elektroniska detektorer, där celler i vätska får passerar genom en smal kanal där de enskilda cellerna en efter en belyses med laserljus. Förenklat så mäts ljusets spridning av två detektorer. Ljuset som mäts i laserstrålens längsriktning kallas ”forward scatter” (FSC) och motsvarar cellernas storlek. Intensiteten av FSC motsvarar cellernas diameter och detekteras med en fotomultiplikator. Den andra detektorn mäter spridning av ljuset vid 90 graders vinkel mot laserkällan och hur mycket ljus som reflekteras av cellen beror på cellens komplexitet, bl a hur kärnan ser ut och mängden granula i cytoplasman, och mäts i hur mycket ”side scatter” (SSC) den ger. Genom att använda ett set av lasrar, som emitterar ljus med olika våglängder, så kan man detekterar olika fluorokromer som absorberar och sedan fluorescerar då de belyses av ljus med ett specifikt excitationsspektrum. Genom att märka in riktade antikroppar mot specifika proteiner på cellernas yta med fluorescerande ämnen, så kan man, dels identifiera och karakterisera olika celltyper, men uppmätt intensitet av den specifika fluorescens-signalen (MFI) för olika markörer ger också ett relativt mått på hur mycket ytprotein som uttrycks av en cellpopulation. För att märka in cellyteproteiner, som uttrycks i hög grad av ett stort antal celler i provet, så får man använda fluorokromer med ”svagt ljus” och för mer ovanliga, sällsynta ytproteiner får man använda fluorokromer som ger en ”stark” signal för att kunna detektera dem. För att mäta granulocyters och monocyters förmåga till produktion av reaktiva syreradikaler använde vi ett testkit med en substans, dihydrorhodamine 123, som emitterar ljus då den vid närvaro av reaktiva syreradikaler omvandlas till rhodamine 123 och därmed avger en mätbar fluorescerande signal [207]. För att mäta cellernas förmåga till fagocytos användes *E. coli*- bakterier, märkta med en fluorescerande substans.

Resultat och diskussion

I det första arbetet studerade vi granulocyter och monocyter hos MM patienter före och efter så kallad ”högdosbehandling med autolog stamcellstransplantation”. Behandlingen innebär att man ger en hög dos av ett cytostatikum, melfalan, vilket i den givna dosen slår ut inte bara de sjuka plasmacellerna, utan även den egna blodbildningen, som ”räddas” genom återgivning av i förväg insamlade och frysförvarade egna blodstamceller. Den här behandlingen har god förutsättning att ge långvarig symtomfrihet, men risken för infektioner under de första veckorna efter behandlingen är mycket hög. Den vanligaste typen av vit blodkropp i både blod och benmärg är neutrofila granulocyter. De utgör första linjens försvar mot främmande agens. Trots att antalet neutrofila granulocyter hade börjat återhämta sig dag +13 efter stamcellstillförseln, så hade de en profil av cellyteproteiner, som tydde på hög grad av omognad och aktivering och de hade nedsatt förmåga att oskadliggöra bakterier när vi testade dem med flödescytometriska analyser och jämförde med prover från friska kontroller. Graden av omognad och den nedsatta funktionen hos de neutrofila granulocyter kvarstod i regel vid provtagning några dagar senare när patienterna bedömdes vara i så pass gott skick att de kunde skrivas ut från sjukhuset. Patienternas monocyter hade mindre av ett protein på sin yta, HLA-DR, som behövs för att bygga upp en riktad immunrespons mot främmande och skadliga partiklar. En annan celltyp, eosinofila granulocyter, saknades nästan helt efter den här typen av behandling och återhämtade sig långsamt. Eosinofila har rapporterats vara viktiga för plasmacellers överlevnad i benmärgen och vi spekulerade därför över om avsaknaden av dessa celler under första tiden efter högdosbehandling, kunde bidra till den många gånger goda och långvariga behandlingseffekten.

Det andra arbetet är en klinisk fas 2 prövning med BI-505 till patienter med SMM. Läkemedlet hade ingen effekt när det gavs till patienter med avancerad myelomsjukdom. Tanken med att ge läkemedlet till patienter i ett tidigt, ännu icke behandlingskrävande skede, var att fördröja ett eventuellt insjuknande i fullt utvecklad myelomsjukdom, genom att minska tumörklonen och att de här patienterna förmodades ha ett mer aktivt och friskt immunförsvar, som kunde känna igen och avdöda de av BI-505 inmärkta tumörcellerna. Samtidigt är det långt ifrån alla patienter med SMM, som någonsin blir behandlingskrävande och den här behandlingen bedömdes därför lämplig eftersom den inte förväntades ge någon form av bestående biverkningar, vilket de flesta andra typer av anti-myelom läkemedel kan göra. Tanken med den här fas 2 prövning var att inkludera 4-10 patienter med SMM, för att utröna om läkemedlet hade någon effekt på sjukdomsbördan mätt som M-komponent eller andel plasmaceller i benmärgen, innan man tog nästa steg och planerade för en större studie med fler patienter. Efter den 4:e inkluderade patienten avslutades studien. Det var ingen av de 3 patienter som fullföljde hela den 7 veckor långa planerade behandling med BI-505, som uppvisade några tecken på behandlingssvar.

I det tredje och fjärde delarbetena, så samlade vi in blod- och benmärgsprover från patienter, som kom för utredning på grund av en nyupptäckt M-komponent eller misstanke om MM, och som därefter diagnosticerades med plasmacellssjukdom i olika stadier, MGUS, SMM och manifest MM. Experimentella studier främst på möss tyder på att eosinofila granulocyter har betydelse för både normala och sjuka plasmacellers överlevnad i benmärgen, men den kliniska betydelsen av dessa celler vid plasmacellssjukdom hos människor är inte känd. Vid flera andra typer av cancer så verkar närvaron av eosinofila i tumörvävnaden medföra en gynnsammare prognos, medan ökad mängd eosinofila vid en viss typ av lymfom (Hodgkinlymfom av typen nodulär skleros) innebär mycket dålig prognos. Andelen eosinofila i benmärgsprover från våra patienter kunde inte korreleras till vare sig sjukdomsstadium eller andel plasmaceller i benmärgen, och skiljde sig inte signifikant från andelen eosinofila i benmärgsprover från friska kontroller. Däremot var eosinofila från patienternas benmärgsprover mer aktiverade och hade högre uttryck av en receptor (CXCR4), som är av betydelse för inbindningen av dessa celler till samma områden i benmärgen där plasmaceller är lokaliserade.

Det fjärde arbetet visar att det är vanligt att neutrofila granulocyter har nedsatt funktion vid diagnos av MM och detta kan ses redan i tidiga stadier av plasmacellssjukdom, som MGUS. Funktionsnedsättningen var mest uttalad för neutrofila i benmärgen. Nedsatt neutrofil funktion är ytterligare en faktor, som skulle kunna bidra till den ökade risken för bakteriella infektioner vid MM. Ett intressant fynd var att neutrofila granulocyters förmåga till fagocytos var fullt återställd hos patienter som uppnått god sjukdomsrespons och som stod på längre tids behandling med läkemedlet lenalidomid.

Slutsatser

Resultaten visar att celler med förmåga till fagocytos är påverkade vid plasmacellssjukdomar och kan påverkas ytterligare av de olika behandlingar som ges, vilket i sin tur får betydelse inte bara för risken för infektioner, utan sannolikt även inverkar på hur väl sjukdomen svarar på behandling.

Populärwissenschaftliche Zusammenfassung auf Deutsch

Hintergrund

Das Multiple Myelom (MM) ist die zweithäufigste Form von Blutkrebs. Bei dieser Bluterkrankung entarten sogenannte ”Plasmazellen” im Knochenmark zu Krebszellen. Als blutbildendes Organ befindet sich das Knochenmark in den großen Knochen des Skelettes, d. h. in der Wirbelsäule, den Oberschenkel- und Oberarmknochen, sowie den Schädelknochen und dem Brustbein. Plasmazellen zählen zu den ”ausgebildeten” Zellen des Immunsystems, die spezifische Antikörper gegen unterschiedliche Bakterien und Viren bilden und außerdem als ein Teil des immunologischen Gedächtnisses betrachtet werden können. Beim MM bilden die entarteten Plasmazellen nur einen einzigen Antikörpertyp, der bei ungehemmter Vermehrung kranker Plasmazellen im Blut als ”M-Komponente” messbar wird. Auch wenn dieses Protein an sich als krankhaft betrachtet wird, sind geringe M-Komponenten bei gesunden Menschen nicht ungewöhnlich und Ausdruck für kleinere Ansammlung von gleichartigen, entarteten Plasmazellen im Knochenmark. So lange diese Plasmazellen keine Beschwerden verursachen und sich nicht ungehemmt vermehren, wird dieser Zustand als ”Monoklonale Gammopati Unklarer Signifikanz” (MGUS) bezeichnet. Man geht davon aus, dass bei 2-3% der schwedischen Bevölkerung im Alter von über 50 Jahren eine solche niedrige M-Komponente zu finden ist. Es ist außerdem bekannt, dass einem MM in fast allen Fällen ein MGUS vorausgeht. Patienten mit MGUS haben ein jährliches Risiko von ca. 1%, ein behandlungsbedürftiges MM zu entwickeln. In Fällen, in denen die M-Komponente im Blut 30g/l überschreitet oder sich mehr als 10% entartete Plasmazellen im Knochenmark nachweisen lassen, spricht man von einem ”smoldering” MM (SMM). Um die Diagnose MM stellen zu können, muss die Erkrankung behandlungsbedürftig sein, d. h. es treten Organschäden oder Beschwerden auf, die Behandlung erforderlich machen. Die ungehemmte Vermehrung von Plasmazellen kann z. B. zu einer Verdrängung der normalen Blutbildung im Knochenmark und dadurch zu Blutarmut, sog. Anämie, führen. Aufgrund von knochenabbauenden Eigenschaften der bösartigen Plasmazellen kommt es nicht selten zu größeren Defekten im Skelett, sog. ”Aufklarungen” oder ”lytischen Destruktionen”. Diese können zu Frakturen oder Skelettschmerzen führen und sind häufig das erste Symptom des MM. Außerdem kann aus dem Skelett freigesetztes Kalzium selber zu

Beschwerden führen. Teile der M-Komponente, die sog. ”leichten Ketten” können bei der Filtration in der Niere ausfallen und schwere Nierenschäden verursachen. Patienten mit sowohl MM als auch mit MGUS haben ein geschwächtes Immunsystem und ein deutlich erhöhtes Risiko für bakterielle Infektionen verglichen mit gesunden Kontrollpersonen. Die Ursache für diesen Immundefekt ist nicht eindeutig bekannt. Man weiß aber, dass mit steigender Anzahl bösartiger Plasmazellen die Anzahl gesunder antikörperproduzierender B-Lymphozyten und Plasmazellen weniger wird und dadurch das Spektrum unterschiedlicher Antikörper abnimmt. Außerdem hat es das geschwächte Immunsystem schwerer, die entarteten Zellen unter Kontrolle zu halten. Zugleich sind sowohl gesunde als auch entartete Plasmazellen in ihrem Überleben abhängig von den umgebenen Zellen im Knochenmark, wie z. B. andere weiße Blutkörperchen oder Bindegewebszellen, die sie schützen und mit Wachstumsfaktoren versehen. Die Entwicklung neuer Medikamente hat in den letzten 15 Jahren wesentlich zur Verlängerung des Überlebens von Myelomapatienten beigetragen. Bisher gibt es jedoch keine Behandlung die zur Heilung von MM führt und die Erkrankung ist mit zunehmender Dauer und Anzahl von Behandlungslinien immer schwerer zu kontrollieren.

Speziell hergestellte Antikörper, die gegen bestimmte Proteine auf der Oberfläche von Tumorzellen exponiert werden, werden heute häufig in der Behandlung von Krebserkrankungen angewendet, um dem Immunsystem zu erleichtern, diese als entartet zu erkennen. Beim MM ist es bisher schwerer gewesen, eine solche Antikörperbehandlung zu entwickeln. Wahrscheinlich beruht dies auf der Tatsache, dass einzig die Markierung der Tumorzellen für das kräftig gehemmte Immunsystem beim MM nicht ausreicht, sondern dass die attackierenden Immunzellen selber stimuliert werden müssen. In einer Arbeit, die dieser Abhandlung vorrausging, wurde eine sogenannte ”Phase 1” Studie mit einer neuen Antikörperbehandlung für MM durchgeführt. Dieser Antikörper mit Namen BI-505 war gegen das Oberflächenprotein ICAM-1 gerichtet, dass von entarteten Plasmazellen produziert wird. Diese Behandlung wurde von Patienten mit Myelom gut vertragen, hatte aber nicht den gewünschten Effekt auf die Myelomerkrankung.

Fragestellung und Ziel

Die Forschung in dieser Abhandlung konzentriert sich auf Granulozyten och Monozyten, zwei Typen von weißen Blutkörperchen. Untersucht wurde, wie Zellanzahl, Funktion und Oberflächenproteine, sowohl von der Myelomerkrankung selber, wie auch durch die Behandlung beeinflusst werden. Diese Zellen erkennen fremde Partikel, Bakterien und antikörpermarkierte Zellen, können diese in das Zellinnere, das sogenannte Zytoplasma aufnehmen und zerstören. Dieser Prozess wird als ”Phagozytose” bezeichnet, weshalb Immunzellen mit dieser Eigenschaft auch ”Phagozyten” genannt werden. Die Zerstörung im Zellinneren geschieht mit Hilfe von Sauerstoffradikalen, die von der phagozytierenden Zelle gebildet werden. Eine

beeinträchtigte Phagocytosefunktion kann daher, sowohl zu einer erhöhten Infektionsneigung, als auch zu einem eingeschränkten Effekt von Behandlung mit Antikörpern führen.

Methoden

Diese Abhandlung besteht aus vier Teilarbeiten. In Arbeit I, III und IV wurde Blut- und Knochenmarkproben von Patienten mit MM analysiert und die Ergebnisse mit klinischen Daten korreliert. Mit Hilfe von Durchflusszytometrie wurden Granulozyten und Monozyten in bezog auf den Reifegrad und die Aktivierung der Zellen untersucht und die Fähigkeit zur Phagozytose und Bildung von Sauerstoffradikalen im Vergleich zu Zellen gesunder Versuchspersonen gemessen. Arbeit II beinhaltet eine Phase 2 Studie, in der ein neuer, gegen ICAM-1 gerichteter Antikörper (BI-505) in der Behandlung von SMM getestet wurde.

Durchflusszytometrie

Bei der Durchflusszytometrie kann mit Hilfe von komplizierter Optik, Laserstrahlen und elektronischen Detektoren charakteristische Merkmale einzelner Zellen wie z. B. Größe, Form und Struktur, sowie Oberflächenproteine bestimmt werden. Dazu passieren die Zellen in einer Lösung mit hoher Geschwindigkeit eine elektrische Spannung oder/und einen Lichtstrahl. Die erzeugten Effekte werden von elektronischen Detektoren gemessen und ergeben ein Bild über die Eigenschaften der Zelle. Durch Markierung von Oberflächenproteinen mit fluoreszierenden Antikörpern können durch Verwendung mehrerer Laser verschiedene Oberflächenproteine gleichzeitig erkannt und ihre Menge bestimmt werden. Um die Fähigkeit zur Produktion von Sauerstoffradikalen in Granulozyten und Monozyten zu messen, wurde Dihydrorhodamine 123 benutzt, dass nur im Kontakt mit reaktiven Sauerstoffradikalen zur Substanz Rhodamine 123 umgewandelt wird, und dabei ein fluoreszierendes Signal abgibt. Um die Fähigkeit zur Phagocytose zu bestimmen, wurden *E. coli*–Bakterien benutzt, die mit einer fluoreszierenden Substanz markiert wurden.

Ergebnisse und Diskussion

In der ersten Arbeit wurden Granulozyten und Monozyten von Patienten mit MM vor und nach einer sogenannten "Hochdosisbehandlung mit autologer Stamzelltransplantation" analysiert. Bei dieser Behandlung wird eine hohe Dosis des Zytostatikums Melphalan verabreicht, dass in dieser Dosis nicht nur die entarteten Plasmazellen, sondern auch die gesunde Blutbildung eliminiert. Diese wird durch die Wiedergabe von im voraus gesammelten und eingefrorenen Blutstammzellen "gerettet". Durch diese Behandlung kann langanhaltende Symptomfreiheit erreicht werden. Allerdings ist das Risiko für Infektionen innerhalb der ersten Wochen hoch. Neutrophile Granulozyten machen den Großteil der weißen Blutkörperchen aus und

stellen die erste Abwehr gegenüber fremden Erregern dar. Obwohl diese neutrophilen Granulozyten am Tag +13 nach Hochdosisbehandlung angefangen hatten, sich zu vermehren, ließ das Profil von Oberflächenproteinen auf einen hohen Grad von Unreife und Aktivierung schließen, und die Fähigkeit, Bakterien zu phagozytieren war deutlich beeinträchtigt. Diese Funktionseinschränkung bestand auch noch Tage später bei Entlassung der Patienten. Weitere durchflusszytometrische Analysen zeigten, dass die Monozyten der Patienten eine deutlich verminderte Menge von HLA-DR besaßen, ein Oberflächenprotein, dass gebraucht wird um eine zielgerichtete Immunabwehr gegen fremde Zellen und Partikel aufzubauen. Ein anderer Zelltyp, eosinophile Granulozyten waren praktisch nicht messbar und erholten sich nur langsam nach dieser Behandlungsform. Diese Zellen sind möglicherweise von Bedeutung für das Überleben von Plasmazellen im Knochenmark. Spekulativ kann man daher annehmen, dass dies einer der Gründe für den langanhaltenden Behandlungseffekt sein kann.

Die zweite Arbeit ist eine Phase 2 Studie zur Behandlung von Patienten mit SMM mit BI-505. Dieser Antikörper hatte keinen Effekt in der Behandlung von Patienten mit fortgeschrittenen MM. Der Gedanke war nun, Patienten früher im Erkrankungsverlauf zu behandeln und mit Hilfe eines noch intakten Immunsystems die Entwicklung einer manifesten Myelomerkrankung aufhalten zu können. Da nicht alle Patienten mit SMM im Laufe ihres Lebens ein MM entwickeln, schien es außerdem verlockend, eine Behandlung geben zu können, die im Gegensatz zu vielen anderen Myelombehandlungen bisher keine bestehenden Nebenwirkungen gezeigt hat. Diese Phase 2 Studie sollte 4-10 Patienten umfassen, um vor Planung von weiteren größeren Studien herauszufinden, ob BI-505 einen Effekt auf die SMM Erkrankung hat. Die Studie wurde abgebrochen, nachdem für keinen der drei Patienten, die die vollständige, 7 Wochen lange Behandlung mit BI-505 erhalten hatten, ein Therapieeffekt gezeigt werden konnte.

Im dritten und vierten Teil von Arbeit wurden Blut- und Knochenmarkproben von Patienten gesammelt, die aufgrund einer neuaufgetretenen M-Komponente oder Verdacht auf MM untersucht und später mit MGUS, SMM oder manifest MM diagnostiziert wurden. In experimentellen Studien wurde gezeigt, dass eosinophile Granulozyten für das Überleben von normalen und entarteten Plasmazellen im Knochenmark von Bedeutung sind. Die klinische Bedeutung dieser Zellen in Plasmazellerkrankung im Menschen ist jedoch bisher nicht bekannt. Bei vielen anderen Krebsformen scheint das Vorkommen dieser Zellen im Tumorgewebe mit einer besseren Prognose assoziiert zu sein; bei einem bestimmten Subtyp von Hodgkinlymphom ist eine erhöhte Anzahl von eosinophilen Granulozyen dagegen mit einer sehr schlechten Prognose verknüpft. Der Anteil von eosinophilen Granulozyten im Knochenmark der Patienten in diesen Studien konnte nicht mit dem Erkrankungsstadium oder der Anzahl Plasmazellen im Knochenmark korreliert werden, und unterschied sich nicht signifikant von gesunden Kontrollen. Allerdings waren die eosinophilen Granulozyten in den Patientenproben in einem mehr

aktivierten Zustand und zeigten einen erhöhten Gehalt des Rezeptors CXCR4 auf ihrer Oberfläche, der für die Bindung von Zellen im Knochenmark eine Rolle spielt, und zwar in gleicher Lokalisation wie die der Plasmazellen.

In der vierten Arbeit wird gezeigt, dass eine eingeschränkte Funktion der neutrophilen Granulozyten bei der Diagnose von MM häufig ist und dass dies schon in früheren Stadien von Plasmazellerkrankungen wie MGUS zu beobachten ist. Die Beeinträchtigung war vor allem bei neutrophilen Granulozyten im Knochenmark zu finden und könnte zum erhöhten Risiko für bakterielle Infektionen bei Patienten mit MM beitragen. Eine interessante Beobachtung war, dass sich bei Patienten die eine gute Therapieeffekt erreicht hatten, und die über lange Zeit mit dem Medikament Lenalidomid behandelt wurden, die Funktion der Phagozytose vollständig erholt hatte.

Schlussfolgerung

Unsere Resultate zeigen, dass Zellen mit der Eigenschaft zur Phagozytose bei Plasmazellerkrankungen in ihrer Funktion beeinträchtigt sind und dass diese außerdem durch die gegebenen Behandlungen beeinflusst werden. Dies ist sowohl für das Infektionsrisiko, sowie wahrscheinlich auch für das Ansprechen auf Behandlung von Bedeutung.

Translated from Swedish to German by my dear friend and colleague:

Anna Lübking, M.D. Specialist in Hematology.

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Paper I

Experimental Hematology

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Phagocyte function decreases after high-dose treatment with melphalan and autologous stem cell transplantation in patients with multiple myeloma

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High-dose melphalan with autologous hematopoietic stem cell transplantation (ASCT) is the standard of care for younger patients with newly diagnosed multiple myeloma and is aimed at achieving as deep and complete a response as possible after various combinations of induction therapy. However, it is frequently associated with infectious complications. This study investigated the effects of high-dose treatment with autologous stem cell support on patients' innate immunity, with a focus on subpopulations and functioning of recently released polymorphonuclear leukocytes (PMNs) and monocytes in peripheral blood. Flow cytometry-based analysis was used to measure the degree of PMN maturation and activation, before and after ASCT and compared with healthy controls. After high-dose treatment and ASCT, a smaller proportion of patients' PMNs had the capacity for oxidative burst. Moreover, patients' PMNs, both before and after ASCT, had a reduced capacity for phagocytosis. Eosinophils, which recently have been suggested to play a role in promoting malignant plasma cell proliferation, were markedly reduced after ASCT, with slow regeneration. HLA-DR expression by monocytes was significantly depressed after ASCT, a characteristic often attributed to monocytic myeloid-derived suppressor cells. Our results suggest that several aspects of phagocytic function are impaired for at least 20 days after ASCT. **Copyright © 2016 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.**

Multiple myeloma (MM) is a malignant plasma cell tumor of the bone marrow clinically characterized by painful skeletal destruction, renal impairment, and anemia. Survival can be extended with several new treatment options, but MM remains incurable and fatal [1,2]. Current therapies include novel agents such as proteasome inhibitors and immunomodulatory drugs (IMiDs) in combination with chemotherapy and corticosteroids. High-dose melphalan with autologous hematopoietic stem cell transplantation (ASCT) has been part of first-line treatment for younger patients with MM for more than two decades [1].

The reconstitution of the adaptive immune system after ASCT has been studied extensively [3,4], but the recovery of innate immunity has received less attention. Polymorphonuclear leukocytes (PMNs), primarily neutrophils, are the main effector cells in the defense against acute bacterial infections [5]. In MM [6], and especially after ASCT, infections are a major clinical problem, and impaired neutrophil function may contribute to this susceptibility. A few small studies have suggested that the capacity of phagocytes for oxidative burst and phagocytosis is altered in lymphoproliferative and plasma cell disorders [7,8] because of the disease or treatment. Corticosteroids and proteasome inhibitors may interfere with the nuclear factor κB (NF-κB) pathway, central to the activation of the innate immune response by Toll-like receptors [9–11].

In MM, different lines of evidence have indicated that the bone marrow microenvironment is important either in promoting or in preventing disease progression, and that the surrounding stromal cells, osteoclasts, osteoblasts, and myeloid and lymphoid cells are all involved in tuning the tumor immune response [12]. Furthermore, the therapeutic effects of treatments, such as those using IMiDs [13] and monoclonal antibodies [14], are partly mediated via the microenvironment.

AJ and MH shared last authorship.

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells such as myeloid progenitors of monocytes, granulocytes, and dendritic cells [15]. In healthy individuals, MDSCs are present in low numbers and are thought to be involved in regulation of immune responses and tissue repair [16]. MDSCs expand during inflammatory conditions and cancer and have the ability to suppress several T-cell functions [17] and promote tumor progression [18]. In recent years, MDSCs have been considered a potential target in hematologic malignancies, to enhance the effect of immune modulating agents [15].

The aim of this study was to investigate the innate immunity of patients with MM after treatment with melphalan and ASCT, with a focus on subpopulations and functioning of PMNs and monocytes during the early phase of reconstitution of the immune system and the putative presence of MDSCs. Our results indicate that alterations in phagocyte phenotype and function may contribute to the suppressed immune response and increased susceptibility to infections in MM patients after ASCT.

Methods

Patients and controls

After informed consent, heparinized venous blood samples were obtained from 14 patients with MM before and after treatment with high-dose melphalan and ASCT (Supplementary Table E1, online only, available at www.exphem.org). Samples were collected the day before administration of high-dose melphalan, at the time of expected first sign of engraftment on days +11–14, and (one additional sample) on days +15–20 after ASCT, and were compared with samples from 40 healthy blood donors. Samples from each patient were also collected at least 1.5 months after ASCT.

PMNs and monocyte phenotypes

Expression of selected surface markers was analyzed using flow cytometry. Briefly, peripheral blood was lysed, using 0.84% ammonium chloride. The remaining leukocytes were stained using three different panels of monoclonal fluorescent-labeled antibodies (BD Biosciences, San Jose, CA): (1) CD10, CD16, CD14, CD35, CD49d, CD64, siglec-8, and CD193; (2) CD10, CD16, CD14, CD11b, CD11c, and CD62L; (3) CD10, CD16, CD14, CD11b, HLA-DR, CD33, and CD66b [18]. A FACSCanto II was used with DIVA software (Becton Dickinson, Franklin Lakes, NJ) for data collection and with Kaluza software (Beckman Coulter, Brea, CA) for analysis. Additional details regarding gating strategies are provided under Supplementary Methods and in Supplementary Figures E1 and E2 (online only, available at www.exphem.org).

Phagocytosis and oxidative burst

Phagocytosis was investigated using the PhagoTest assay (Glycotope Biotechnology, Heidelberg, Germany), according to the manufacturer's protocol. This flow cytometry-based method measures the percentages of PMNs and monocytes that ingested fluorescein-labeled opsonized *Escherichia coli* and the mean fluorescence intensity (MFI), which corresponds to the number of ingested bacteria per cell. Production of reactive oxygen species (ROS) in peripheral blood PMNs and monocytes was investigated using the PhagoBurst assay, (Glycotope Biotechnology), according to the manufacturer's protocol, after ex vivo activation with phorbol-12-myristrate-13-acetate (PMA) or opsonized *E. coli*.

Statistical analysis

GraphPad PRISM (San Diego, CA) Version 6.0a software was used for statistical calculations. The Friedman test was used for repeated comparisons of patient samples, collected at different times before and after ASCT. The Mann–Whitney U test was used for two-group comparisons between patients and healthy controls. Spearman's rank correlation coefficient was used to quantify covariation. All p values were considered significant at $p < 0.05$.

Results

Patient characteristics

Patients with multiple myeloma from Malmö/Lund, receiving high-dose treatment at the Hematological Clinic, Skåne University Hospital, Sweden, were consecutively included from January 2013 to May 2014. Of the 14 patients, 10 were under first-line treatment. The other 4 patients had had one or two prior therapies: 3 had undergone ASCT and 1 had early progress/failure on first-line treatment with bortezomib in combination with alkylating agents. This last patient had received lenalidomide-based induction therapy, and the other 13 had received bortezomib-based induction therapy, before the current high-dose treatment. They had all achieved at least partial remission on induction therapy before inclusion in this trial: 6 partial remission (PR), 7 very good partial remission (VGPR), and 1 complete remission (CR) (response criteria according to the International Myeloma Working Group [19]). All patients were stem cell harvested after standard cyclophosphamide with granulocyte colony-stimulating factor (G-CSF) mobilization. Blood samples were obtained from patients before melphalan infusion on day −1 and at the time of expected first sign of engraftment on days +11–14. All patients were treated for neutropenic fever with broad-spectrum antibiotics; however, none showed any clinical signs of engraftment syndrome [20]. On day +11, 12 of 14 patients had a WBC count $\leq 0.3 \times 10^9$/L (data not shown). We found that it was not until day +13, that all patients had a sufficient amount of PMNs for fluorescence-activated cell sorting (FACS)-based analysis of neutrophil functioning, with a WBC count $\geq 0.3 \times 10^9$/L (range: 0.3–7.4). Day +13 was thereby selected as the first time point for testing after ASCT in this study. The time point of neutrophil engraftment according to EBMT criteria with ANC (absolute neutrophil count) $\geq 0.5 \times 10^9$/L was a median of 12.5 days post-ASCT (Supplementary Table E2, online only, available at www.exphem.org). An additional sample was collected from 10 patients on days +15–20 post-ASCT,

which, with one exception, was the time point of discharge from inpatient care. One patient developed a severe infection and was still on treatment with intravenous antibiotics on day +18 post-ASCT. We also obtained at least one blood sample from every patient at follow-up ≥1.5 months after ASCT (Supplementary Table E1). During the same period, 40 healthy blood donors were evaluated and used as controls.

Proportion of mature neutrophils decreases, but neutrophils are more activated, after ASCT

To characterize the regeneration of patients' innate immunity after ASCT, the maturation and activation of PMNs were investigated using flow cytometry. The low-affinity receptor for IgG, CD16, first appears at the metamyelocyte stage [21] and has the highest expression on mature segmented neutrophils, whereas the zinc-binding metalloprotease CD10 is limited to the late segmentation stage [21] of neutrophils. PMNs defined by typical forward-scatter (FSC) and side-scatter (SSC) properties were divided into three populations: $CD16^{high}$ ($CD16^{+++}$/$CD10^{+}$), $CD16^{weak}$ ($CD16^{+}$/$CD10^{negative}$), and $CD16^{negative}$ ($CD16^{negative}$/$CD10^{negative}$) [22]. Monocytes were excluded based on CD14 gating.

Mature neutrophils ($CD16^{high}$) were similarly distributed in healthy controls and MM patients before treatment and in the samples collected more than 1.5 months after ASCT (median = 82.4%, 84.9%, and 84.0% of PMNs, respectively) (Table 1). On day +13 post-ASCT, blood leukocyte counts had started to regenerate (median = 1.7×10^9/L). However, the proportion of mature neutrophils in these leukocytes was down to a median of 46.2% of PMNs. This reduced level was also found on days +15–20. In this study population, there was no difference in median values for proportion of mature neutrophils between patients that had received two or more doses of G-CSF and those that had fewer than two doses of G-CSF on day +13 (37.7% [range: 0.24–95.5, n = 6] vs. 46.2% [range: 28.3–97.6, n = 8], p = 0.719), respectively on days +15–20 (57.1% [range: 50.1–67.2, n = 5] vs. 51.5% [range: 29.2–63.4, n = 5], p = 0.310). Furthermore, expression of CD10 was significantly reduced on $CD16^{high}$ PMNs on day +13 and days +15–20 after ASCT (Fig. 1), suggesting a lack of segmented neutrophils.

Complement receptor type 1 (CD35) and high-affinity FCγ-receptor I (CD64) have been described as increasing after activation [21,23,24] and as clinically useful early markers of sepsis [25]. High CD62L (L-selectin) expression, in contrast, is characteristic of recently released neutrophils [26]. Surface expression of CD35, CD62L, and CD64 was markedly increased on $CD16^{high}$ PMNs on day +13, and increased expression of CD62L and CD64 was still observed on days +15–20 post-ASCT (Fig. 1). The surface level of CD11b and CD11c (complement receptors 3 and 4, respectively) did not change during the course of treatment (data not shown).

All patients had neutropenic fever after ASCT, and for those patients (n = 7) who had received at least one dose of G-CSF until day +13 (Supplementary Table E2), the CD35 expression by $CD16^{high}$ PMNs was significantly increased (p = 0.018). The patient who received nine doses of G-CSF before day +13 had by far the highest expression of CD35. This is consistent with earlier observations indicating increased CD35 and CD64 expression after G-CSF treatment [27] (Fig. 1).

Table 1. WBC counts and proportions of lymphocytes, monocytes, and PMNs in relation to total number of leukocytes and proportions of $CD16^{high}$, $CD16^{weak}$, and $CD16^{negative}$ PMNs in relation to total number of PMNs, before and after high-dose treatment with ASCT and compared with healthy controls

	Healthy controls (n = 40)	Pretreatment (n = 14)	Day +13 post-ASCT (n = 14)	Days +15–20 post-ASCT (n = 10)	>1.5 mo post-ASCT (n = 16)	p value Friedman[a] (n = 10)
WBC count ($\times 10^9$/L)		4.9 (2.2–7.7)[b]	1.7 (0.3–7.2)	3.4 (1.3–5.8)	5.4 (2.3–8.1)	
Lymphocytes (% of total number of leukocytes)	43.6 (27.5–65.4)	47.6 (10.3–67.1)	14.5 (1.4–54.5)	22.8 (5.5–38.2)	39.5 (13.7–54.6)	0.0223
Monocytes (% of total number of leukocytes)	6.7 (3.9–11.5)	5.6 (1.6–11.0)	13.8 (7.7–39.3)	12.1 (5.4–38.1)	3.8 (1.6–11.4)	0.0012
PMNs (% of total number of leukocytes)	48.5 (27.0–66.8)	45.6 (29.6–87.0)	72.8 (7.7–90.8)	62.4 (37.7–75.2)	52.5 (42.2–83.9)	NS
$CD16^{high}$ (% of total number of PMNs)	82.4 (59.9–93.6)	84.9 (62.2–97.3)	46.2 (0.6–96.5)	55.1 (29.3–67.0)	84.0 (66.6–94.3)	0.0047
$CD16^{weak}$ (% of total number of PMNs)	5.4 (0.9–17.4)	3.6 (0.9–15.9)	16.6 (1.7–94.9)	28.5 (6.8–65.6)	2.6 (1.5–7.8)	0.0008
$CD16^{negative}$ (% of total number of PMNs)	9.1 (2.4–19.5)	8.1 (0.8–31.6)	13.1 (1.7–53.0)	11.3 (4.7–40.6)	11.5 (4.2–31.3)	NS

[a]Friedman test calculated for the patients for whom blood samples were available at all four time points.
[b]Median (10th–90th percentiles).

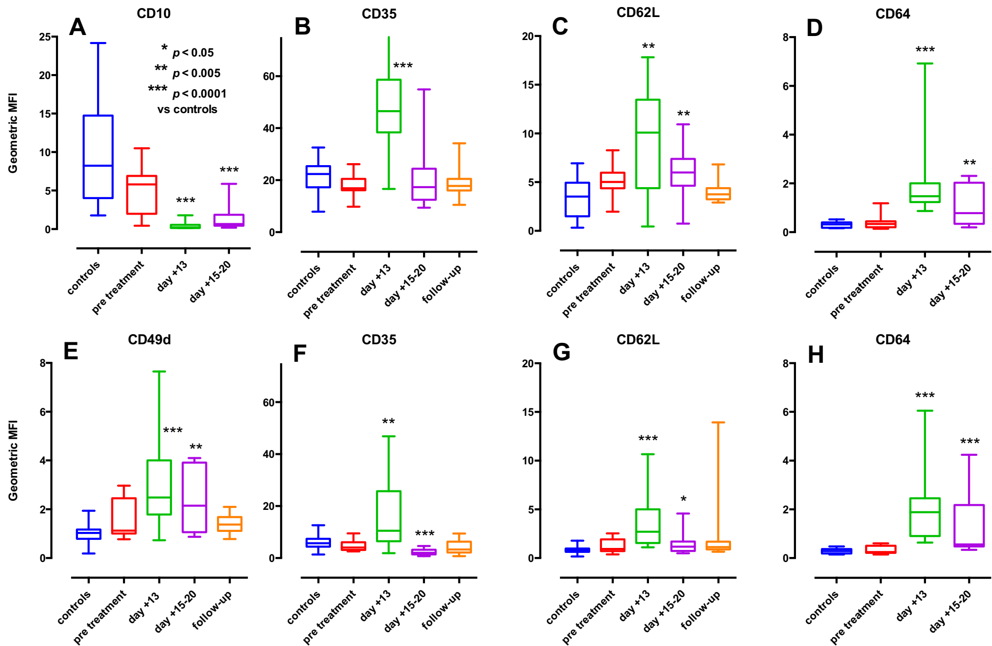

Figure 1. Expression of surface antigens by PMNs, measured as geometric MFI, on CD16^{high} PMNs (**A–D**) and CD16^{weak} PMNs (**E–H**), before and after high-dose treatment with ASCT and compared with healthy controls.

In summary, mature neutrophils decreased, but were more activated after ASCT, and the increased expression of CD62L is likely consistent with a high bone marrow release.

Expanded population of immature CD16^{weak} neutrophils after ASCT

The proportion of CD16^{weak} PMNs, representing immature neutrophils [28] at the band and metamyelocytic stages [21], was elevated in early post-ASCT samples. Although in pretreatment samples a median of 3.6% of PMNs were CD16^{weak}, the median value had risen to 16.6% on day +13 and reached 28.5% on days +15–20 post-ASCT (Table 1). By >1.5 months after ASCT, the median had fallen to 2.6%. In healthy controls, CD16^{weak} PMNs constituted a median of 5.4% of PMNs.

CD49d, a VCAM-1 and fibronectin antigen, is expressed mainly on myeloid precursors [21], although mature eosinophils also have high expression. Expression of CD49d was slightly, but significantly increased in the expanded population of CD16^{weak} PMNs after ASCT (Fig. 1), indicating a more immature nature.

Similar to CD16^{high}, increased expression of CD35, CD62L, and CD64 was observed on CD16^{weak} PMNs on day +13 post-ASCT (Fig. 1).

CD64 is normally expressed on immature myeloid cells until the metamyelocytic stage, after which surface expression drops to very low levels on mature resting, nonactivated neutrophils [21,23]. The increased expression of CD64 in this population after ASCT can therefore be interpreted not only as a sign of activation, but also, and perhaps rather, as a sign of increased immaturity in comparison to the corresponding PMN population in healthy controls.

Absence of eosinophil granulocytes after ASCT

The proportion of CD16^{negative} PMNs (Table 1) did not vary during the course of treatment and was similar to that in healthy controls. However, this gate includes a variety of PMN populations whose relative proportions changed markedly (Table 2, eosinophils, myelocytes [MCs], and promyelocytes [PMCs]). In healthy controls, CD16^{negative} PMNs represented a median of 9.1% of PMNs and were mainly eosinophils.

Eosinophils are CD16^{negative}/CD10^{negative} granulocytes with FSC/SSC partially overlapping with neutrophils, but partly with a markedly lower FSC and higher SSC [29,30]. With this in mind, our gate for PMNs was set to include the whole population of eosinophils. Eosinophils gated on CD16^{negative} PMNs, with strong expression of

Table 2. Proportions of eosinophils, myeloid precursors, and MDSCs, in relation to total number of leukocytes, before and after high-dose treatment with ASCT and compared with healthy controls

	Healthy controls (n = 31)	Pretreatment (n = 14)	Day +13 post-ASCT (n = 14)	Days +15–20 post-ASCT (n = 10)	>1.5 mo post-ASCT (n = 8)[a]	p value Friedman[b] (n = 7)[c]	p value (days +13 and +15–20 vs. controls)
Eosinophils (% of total number of leukocytes)	3.12 (1.18–6.65)[d]	1.15 (0.02–7.60)	0.08 (0.03–0.73)	0.13 (0.01–1.53)	4.16 (0.03–12.4)	0.0265	<0.0001 <0.0001
GrMDSCs (% of total number of leukocytes)	0.05 (0.01–0.17)	0.05 (0.01–0.15)	0.10 (0.0–0.79)	0.11 (0.0–0.32)	0.06 (0.02–0.22)	NS	NS
MCs (% of total number of leukocytes)	0.07 (0.02–0.13)	0.18 (0.03–0.76)	2.27 (0.05–11.1)	1.02 (0.15–12.4)	0.21 (0.05–3.64)	NS	<0.0001 <0.0001
PMCs (% of total number of leukocytes)	0.02 (0.0–0.05)	0.06 (0.01–0.43)	0.55 (0.00–5.42)	1.57 (0.16–6.28)	0.02 (0–1.01)	0.0075	<0.0001 <0.0001
Promonocytes (% of total number of leukocytes)	0.32 (0.0–0.74)	0.34 (0.01–1.02)	0.81 (0.07–4.90)	0.30 (0.0–2.35)	0.51 (0.26–0.77)	NS	0.0033 NS
MoMDSCs (% of total number of leukocytes)	0.46 (0.14–1.52)	0.60 (0.12–3.92)	4.15 (0.83–17.5)	1.80 (0.25–6.49)	0.51 (0.17–4.25)	0.0019	<0.0001 0.0018
% MoMDSCs (% of monocytes)	10.9 (2.8–27.9)	14.8 (3.2–56.2)	54.3 (8.4–82.4)	14.4 (2.2–50.2)	14.4 (6.3–55.6)	NS	<0.0001 NS

[a] n = 7 for promonocytes and n = 14 for MoMDSCs.
[b] Friedman test calculated for the patients for whom blood samples were available at all four time points.
[c] n = 6 for promonocytes and n = 10 for MoMDSCs.
[d] Median (10th–90th percentiles).

siglec-8, were CD49d^{+++} (panel 1 of mAbs) and represented a median of 7.6% of PMNs and 4.3% of leukocytes in healthy controls (n = 19). Siglec-8 is expressed late in eosinophil differentiation [31], and mature eosinophils were almost undetectable on day +13 and days +15–20 post-ASCT. To be sure that we had not excluded immature eosinophils, an alternative gating based on CD16^{negative}/CD33^{dim}/SSC$^{moderate\text{-}high}$ PMNs was also used to detect eosinophils [30] (Table 2, panel 3 of mAbs). However, there was a strong correlation between the two gating protocols. One patient (No. 13, Supplementary Table E1) still had no detectable eosinophils on day +47 post-ASCT. This patient was not being treated with corticosteroids (No. 13, Supplementary Table E3, online only, available at www.exphem.org). In later follow-up samples (n = 15, Supplementary Table E1), eosinophils constituted a median of 5.9% of leukocytes, with a range (10th and 90th percentiles) of 0.5–17.2, if gated on siglec-8.

In this trial, the presence of eosinophils was markedly reduced after ASCT and these were slow to regenerate.

Myeloid precursors

The presence of myeloid precursors in the CD16^{negative} population was evaluated on the basis of CD33, CD66b, and CD11b expression. As expected, the proportion of CD16^{negative} myeloid precursors—myelocytes (MCs) and promyelocytes (PMCs)—was significantly elevated on day +13 and days +15–20 post-ASCT. Although MCs accounted for a median of 0.18% of leukocytes before treatment, this rose to 2.27% on Day +13 and 1.02% on days +15–20 post-ASCT. Similarly, PMCs accounted for a median of 0.06% of leukocytes in pretreatment samples, rising to 0.55% on day +13 and 1.57% on days +15–20 post-ASCT (Table 2).

Granulocytic MDSCs

We were able to define a small subpopulation of CD16^{negative}/CD14$^{negative/weak}$/CD33^{dim}/HLA-DR^{negative}/CD66b^{+}/CD11b^{+} PMNs, representing potential granulocytic MDSCs (GrMDSCs). This putative GrMDSC population was small in both patients and controls, with a median value of 0.04% to 0.11% of leukocytes; a slight, but not significant, increase could perhaps be discerned after ASCT (Table 2).

Monocytes have reduced expression of HLA-DR after ASCT

Monocytes are another important phagocyte in peripheral blood, and like PMNs, they rapidly reappeared in peripheral blood after ASCT (Table 1). Promonocytes were significantly increased on day +13 post-ASCT (Table 2), indicating a shift toward more immature cells. In addition, the newly released monocytes had reduced expression of HLA-DR compared with those of healthy controls on day +13 post-ASCT (Fig. 2).

Monocytic MDSCs (MoMDSCs) are described as CD14^{high}/CD33^{high}/HLA-DR$^{negative/weak}$ monocytes [18,32–34]. The proportion of HLA-DR^{negative} monocytes of leukocytes was higher in MM patients than in the healthy controls on day +13 and days +15–20 post-ASCT (Table 2). The proportion of HLA-DR^{negative} monocytes tended to covariate with generally low HLA-DR expression (sr: −0.8275, $p < 0.0001$) on monocytes. There was no significant difference in HLA-DR expression by monocytes

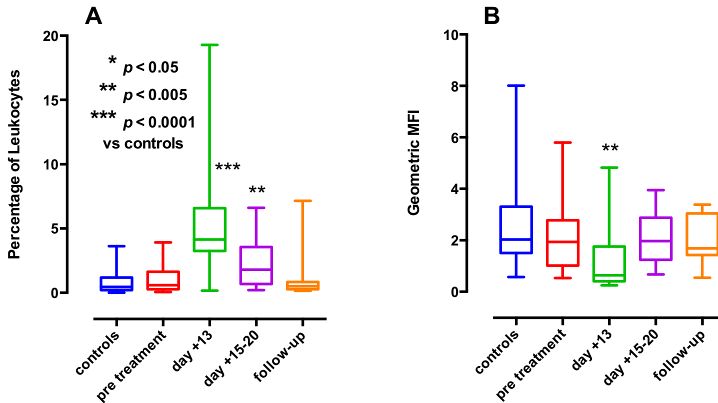

Figure 2. HLA-DR^{negative} monocytes and total degree of HLA-DR expression by monocytes. Proportion of HLA-DR^{negative} monocytes in relation to total number of leukocytes (**A**), and degree of HLA-DR expression by monocytes, measured as geometric MFI (**B**), before and after high-dose treatment with ASCT and compared with healthy controls.

between patients that had received two or more doses of G-CSF and those that had fewer than two doses of G-CSF (Supplementary Figure E3, online only, available at www.exphem.org). The presence of HLA-DR^{negative} monocytes/MoMDSCs in pretreatment samples and samples taken >1.5 months post-ASCT was similar to that in healthy controls (Table 2).

Compromised phagocytosis and oxidative burst after ASCT

Phagocytosis and oxidative burst are important effector mechanisms in the defense against invading bacteria. To evaluate the function of the newly regenerated PMNs and monocytes after ASCT, the capacity for oxidative burst and phagocytosis were investigated after ex vivo stimulation.

The proportion of PMNs able to phagocytose opsonized *E. coli*, before treatment and on day +13 and days +18–20 post-ASCT, was smaller in MM patients than in the controls (Fig. 3A). At follow-up, >1.5 months post-ACST, PMNs from 12 of 14 MM patients exhibited normal phagocytosis patterns (Fig. 3A). In MM patients, a tendency toward a decreased amount of ingested bacteria per phagocytosing cell was observed (Fig. 3B). The monocyte population was small and further decreased after ASCT. No influence on monocyte phagocytosis was observed (data not shown).

After phagocytosis, PMNs and monocytes produce ROS into the phagosome to kill bacteria. Here, we investigated oxidative burst after activation with PMA or *E. coli*. After ASCT, reduced proportions of PMNs producing ROS were observed on day +13 and days +15–20 (Fig. 4). However, the proportion of PMNs that produced ROS was as efficient in ROS formation as PMNs from healthy controls (data not shown). Monocytes exhibited a pattern similar to that of the PMNs from MM patients, with a decreased percentage of cells producing ROS after PMA activation on day +13 compared with controls (data not shown).

These results suggest that PMNs have a compromised capacity for phagocytosis and oxidative burst after ASCT, which might increase patients' susceptibility to bacterial infections.

Discussion

In this study, we found that newly regenerated innate immunity effector cells are either compromised (neutrophils and monocytes) or absent (eosinophils) after high-dose treatment with melphalan and ASCT. This might lead to a weakened immune response, have implications for defense against infections, and might raise the question of prolonged antibiotic prophylaxis.

At high doses, melphalan effectively eradicates bone marrow cells, and it normally takes 10–14 days before the autologous graft starts to release detectable numbers of leukocytes into peripheral blood. In this study, all patients had measurable leukocyte counts on day +13, and we observed a general tendency toward more immature cells and signs of high bone marrow release. There was an expanded population of immature CD16^{weak} cells in the neutrophil pool and a corresponding drop in the proportion of mature CD16^{high} cells. The high surface levels of CD35 and CD64 found on mature CD16^{high} cells were consistent with increased PMN activation after ASCT [21,23,24]. CD35 expression on PMNs was further accentuated in patients who had received G-CSF treatment, a phenomenon previously described in healthy volunteers [27].

CD62L has been suggested as a marker for recently released neutrophils [26,35]. In line with this, we observed

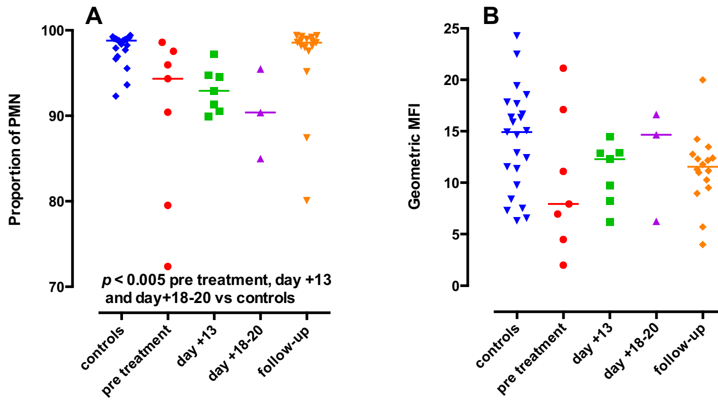

Figure 3. Capacity of PMNs for phagocytosis, measured as the percentage of PMNs that ingested fluorescein-labeled opsonized *Escherichia coli* (**A**), and geometric MFI corresponding to number of ingested bacteria per cell (**B**), before and after high-dose treatment with ASCT and compared with healthy controls.

that both mature (CD16^{high}) and immature (CD16^{weak}) neutrophils had elevated expression of CD62L after ASCT, indicating a rapid transfer from bone marrow to peripheral blood.

Monocytes rapidly reappeared in peripheral blood, and these newly released monocytes had reduced HLA-DR expression. This is in line with a previous study by Krause et al. [36] and a recent observation in pediatric patients after hematologic stem cell therapy [37]. Both of these studies included patients with more diverse hematologic and oncologic disorders than ours. Loss of HLA-DR expression on circulating monocytes is a hallmark of immune dysfunction in critically ill patients, especially in sepsis, where the ability to regain the antigen-presenting function by increasing HLA-DR expression has been found to correlate with clinical outcome [38]. Treatment with glucocorticoids may decrease surface levels of HLA-DR by acting at a transcriptional level [39]. All patients in this study received one dose of hydrocortisone as premedication before grafting of thawed autologous peripheral stem cells on day 0, and most of them received betamethasone as an antiemetic from the start of melphalan infusion on days −1 to +1 with respect to ASCT. Only three patients were still on treatment with steroids at the start of regeneration

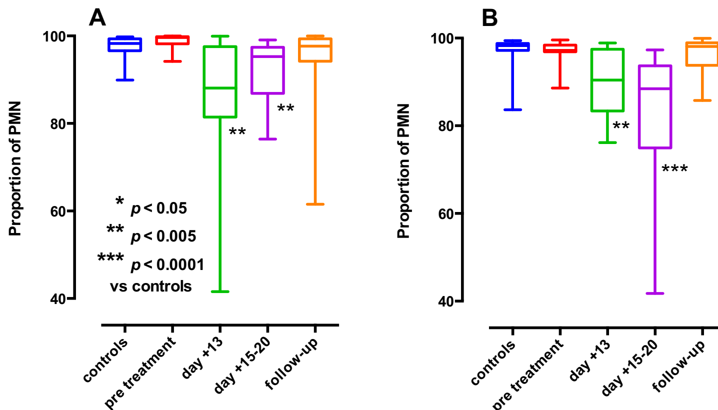

Figure 4. Capacity of PMNs for oxidative burst, measured as percentage of PMNs that produced reactive oxidants on stimulation with PMA (**A**) and *Escherichia coli* (**B**), before and after high-dose treatment with ASCT and compared with healthy controls. Controls: $n = 38$; pretreatment, $n = 13$; day +, $n = 13$; days +15–20, $n = 9$; follow-up, $n = 16$. Friedman test: $p < 0.05$; $n = 8$ (**A** and **B**).

(Supplementary Table E3). Endogenous increased cortisol level, in the stressful condition early after ASCT, is thus a more plausible contributor to changed HLA-DR expression than treatment with glucocorticoids.

Eosinophils, another subset of PMNs sensitive to treatment with corticosteroids [40], were absent up to 20 days after ASCT. This is a particularly interesting finding, as there is evidence that eosinophils play an important role in promoting the survival of B cells and enhancement of malignant plasma cell proliferation in the bone marrow [41,42]. Hence, the elimination of eosinophils may augment the effect of high-dose treatment with melphalan and autologous stem cell support. On the other hand, Carretero et al. reported that activated eosinophils are critical accessory cells for the recruitment of tumor-specific CD8+ T cells in mouse models [43]. Therefore, the role of eosinophils in MM and other tumors is likely complex.

Phagocytosis and oxidative burst are important effector mechanisms in bacterial defense, and changes in phagocytic activity have been associated with sepsis and post-transplant complications in the allogeneic setting [44]. Here we reported that a reduced proportion of PMNs in MM patients were able to phagocytose bacteria both before treatment and up to at least days +18–20 post-ASCT. The decreased phagocytosis before ASCT indicates a possible association with MM or bortezomib pretreatment. ASCT seems to have restored the phagocytosis capacity of PMNs in MM patients, as 12 of 14 patients exhibited normal phagocytosis at follow-up $>$1.5 months post-ASCT. Furthermore, we found a reduced percentage of PMNs and monocytes with capacity to produce ROS after ASCT. The decreased proportion of ROS-producing cells might have a negative impact on the defense against certain kinds of bacteria [45]. Stuehler et al. recently reported impaired ROS production and impaired PMN-mediated killing capacity in response to *Aspergillus fumigatus* for up to 1 year in patients after allogeneic stem cell transplantation [46]. Furthermore, ROS produced by phagocytes could also control anti-tumor defense by regulating survival and activation of natural killer and T cells [47,48].

During the past few years, the role of MDSCs in regulating immune responses in malignant tumor diseases has received attention [17,18]. In human solid cancers, an increase in circulating MDSCs has been found to correlate with advanced clinical cancer stage and metastatic tumor burden [49]. Choi et al. found that the number of $CD15^{+}$/$CD16^{low}$ PMNs in blood samples from patients with terminal cancer was elevated and that a corresponding in vitro activated PMN population from healthy donors, with phenotype $CD16^{low}$/$CD66b^{++}$/$CD15^{+}$, suppressed both proliferation and cytotoxic activity in normal T cells [50]. GrMDSCs have been described as enriching among mononuclear cells on density gradient separation, although at subsequent sorting on myeloid markers such as $CD66b^{+}$ and $CD11b^{+}$/$CD14^{negative}$, they were not morphologically distinguishable from normal granulocytes [51]. A few reports have described increased proportions of GrMDSCs in MM patients: Görgün et al. found an expanded GrMDSC population in patients with relapsed/refractory disease [52], and Ramachandran et al. reported an increase in GrMDSCs in untreated MM patients [53], with the capacity to depress interferon-γ secretion and proliferation of T cells in response to allogeneic dendritic cells in vitro. We identified a small population of putative immature GrMDSCs in treated MM patients before high-dose treatment with melphalan and ASCT that remained after recovery from the ASCT treatment.

Brimnes et al. previously reported an expanded population of monocytes with low expression of HLA-DR, a phenotype described for MoMDSCs in eight patients with newly diagnosed MM [32]. We found an increased proportion of MoMDSCs on day +13 and days +15–20 after ASCT in MM patients. There was no increase in MoMDSCs before high-dose treatment or $>$1.5 months after ASCT compared with healthy controls. It should be pointed out that all patients in this study had achieved disease control, at least partial remission, on induction treatment before collection of the first samples, and this might have affected the number of MDSCs, as they seem to correlate with tumor progression and outcome [54].

Conclusions

We report that the newly regenerated PMN pool in patients with MM had an altered phenotype—more immature and activated—and had a compromised capacity for oxidative burst and phagocytosis, at least up to 20 days, after high-dose treatment with melphalan and autologous stem cell support. In addition, the decreased HLA-DR expression on monocytes might interfere with their ability to present foreign antigens to the adaptive immune system. These results indicate that several phagocyte functions are affected after high-dose melphalan and ASCT, which might contribute to a weakened immune response. Furthermore, we were surprised by the almost complete absence of eosinophils and their delayed regeneration after high-dose treatment, as eosinophils may promote malignant plasma cell proliferation.

Acknowledgments

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Author contributions

Laboratory work was conducted by ÅP and ÅJ. SW and MH recruited the patients. SW carried out the FACS analysis, compiled the data, and drafted the article. ÅJ, MH, TH, and SW contributed to the design of the study and writing. All authors approved the final article.

Conflict of interest disclosure

The authors report no potential conflicts of interest.

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Supplementary methods

Supplementary Figure E1 illustrates the gating strategy for (panel 1 and 2) lymphocytes, monocytes, PMNs ($CD16^{high}$, $CD16^{weak}$, $CD16^{negative}$), and eosinophils. Supplementary Figure E2 illustrates the gating strategy for (panel 3) eosinophils, GrMDSCs, myelocytes, promyelocytes, promonocytes, and MoMDSCs. Panel 3 (CD10, CD16, CD14, CD11b, HLA-DR, CD33 and CD66b) was used to further analyze $CD16^{negative}$ PMNs, myeloid precursors, and eosinophils and to identify putative MDSCs [18].

The majority of lymphocytes and natural killer cells were excluded by CD33 negativity, and mature monocytes/MoMDSCs were selected based on $CD14^{++}$ expression. The remaining cells were gated on $CD16^{negative}$/$CD10^{negative}$ and further selected on SSC and CD33 expression.

Eosinophils were sorted on $CD33^{dim}$/$SSC^{moderate\text{-}high}$ [29,30]. Eosinophils are $CD66b^{+}$ and weakly express HLA-DR [55], more than PMN in general, and therefore “HLA-DR negativity” for MDSCs was in this analysis defined as the MFI limit at which eosinophils were just above the threshold for positive.

Immature GrMDSCs were identified as $CD16^{negative}$/$CD14^{neg/weak}$/$CD33^{dim}$/HLA-$DR^{negative}$/$CD66b^{+}$. As we used whole blood samples, it was not possible to discriminate mature GrMDSCs, expressing CD16, whereas most PMNs are $CD33^{dim}$/$CD66b^{+}$/$CD11b^{+}$/$CD14^{neg/weak}$/HLA-$DR^{neg/weak}$.

Myeloid precursors are $CD33^{+}$, with stronger expression of CD33 than their more mature counterparts [29]. PMCs were gated on SSC/$CD33^{+}$/$CD66^{+}$/$CD11b^{negative}$, and MCs were gated on SSC/$CD33^{+}$/$CD66^{+}$/$CD11b^{+}$. The more differentiated MCs do express CD11b, complement receptor 3, expression of which is maintained during further neutrophilic maturation [21].

Promonocytes (included in the $CD33^{+}CD14^{neg}CD16^{neg}$ $CD10^{neg}$ gate) have, together with monocytes, the most pronounced expression of CD33, but are $CD14^{negative}$ [29].

MoMDSCs were defined as $CD14^{high}$/$CD33^{high}$/HLA-$DR^{neg/weak}$ [18,32–34].

Supplementary Table E1. Patient characteristics

Patient	Sex	Age	M-Comp Class	ISS	FISH	Prior therapy	Induction	Cell dose	NF	Response[a]	Day >1.5 mo
1	F	66	G	I	Negative	None	RT 30 Gy VCD × 3	5.3	Y	PR	+509
2	M	46	Lambda		Not done	2 (1 prior ASCT)	VCD × 3	4.2	Y	VGPR	+637
3	M	51	G	II	Negative	None	VCD × 3	4.1	Y	CR	+489
4	M	38	G	II	Negative	None	VCD × 3	6.3	Y	CR	+475
5	F	57	A	II	Negative	RT for breast cancer	VCD × 3	3.2	Y	CR	+553
6	F	47	G	II	Negative	None	VCD × 3	3.4	Y	VGPR	+285 +425
7	M	57	G	II	del(17)p	None	VCD × 3	3.1	Y	VGPR	+200
8	M	66	G	II	Negative	1	RD × 2	3.3	Y	PR	+301
9	M	61	G	III	Negative	1 (prior ASCT)	VD × 3	2	Y	VGPR	+267
10	F	67	G	II	Negative	None	VCD × 3	5.6	Y	VGPR	+122
11	F	64	G	I	Not done	2 (1 prior ASCT)	VCD × 3	4.8	Y	CR	+96
12	M	65	G	II	Negative	None	VCD × 3	4.5	Y	VGPR	+250
13	M	64	Kappa	III	del(17)p	None	VCD × 3	3.5	Y	CR	+47 +194
14	F	46	A + G	II	Negative	None	VCD × 4	3.2	Y	PR	+162

C = cyclophosphamide; Cell Dose (CD34 + cells × 10^6/kg in autograft); D = dexamethasone; Day >1.5 mo (days post-ASCT, as the last blood sample for follow-up was obtained); FISH = fluorescence in situ hybridization, presence of del(17)p, t(4:14), or t(14:16); Induction = therapy before current high-dose treatment; ISS = International Staging System stage at diagnosis); NF = presence of neutropenic fever post-ASCT; R = lenalidomide; RT = radiotherapy; V = bortezomib.
[a]Criteria of International Myeloma Working Group.

Supplementary Table E2. Time to engraftment and G-CSF treatment[a]

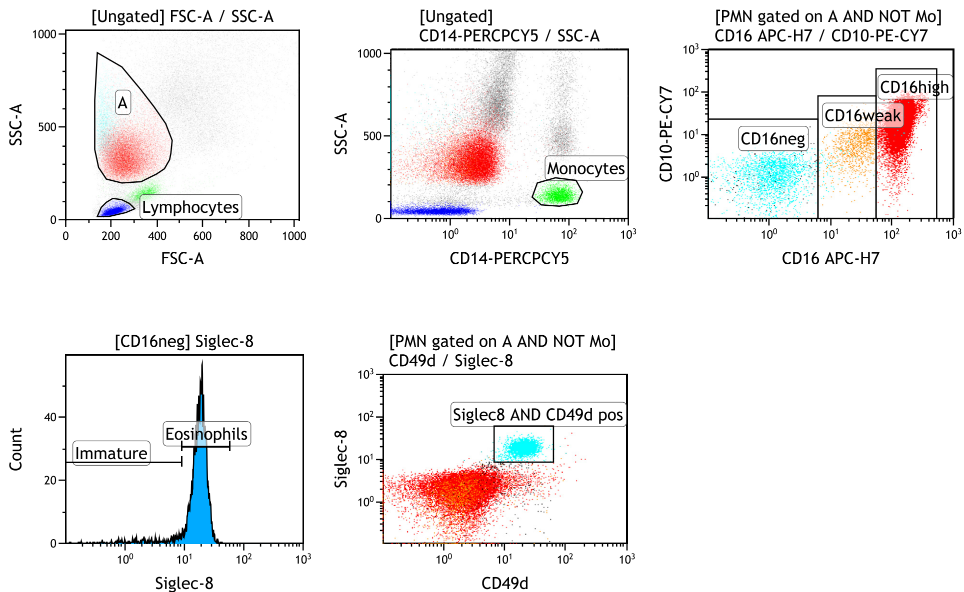
	Days posttherapy				
	Plt ≥20	Plt ≥50	ANC ≥0.5	ANC ≥1.0	G-CSF
1	+10	+11	+12	+12	+7
2	+11	+12	+12		None
3	+17	+19	+18	+25	+21–22
4	+13	+18	+18	+21	None
5	+16	+33	+14	+14	+19
6	+14	+16	+16	+16	+14–15
7	+13	+15	+13	+13	+11–12
8	+13	+19	+15	+16	None
9	+25	Not achieved	+13	+15	+4–15
10	+14	+17	+12	+12	+9–12
11	+12	+15	+11	+12	None
12	+14		+12	+13	+9–12
13	+12	+19	+12	+12	+8–12
14	+11	+14	+12	+13	+9–12

ANC = absolute neutrophil count (×10^9/L); Plt = platelet count (×10^9/L).
[a]Engraftment is defined according to EBMT criteria: the first of three consecutive days with neutrophil count ≥0.5 × 10^9/L and platelet count ≥20 × 10^9/L respectively ≥50 × 10^9/L (without transfusion). Median value for ANC ≥0.5 × 10^9/L was 12.5 days post-ASCT.

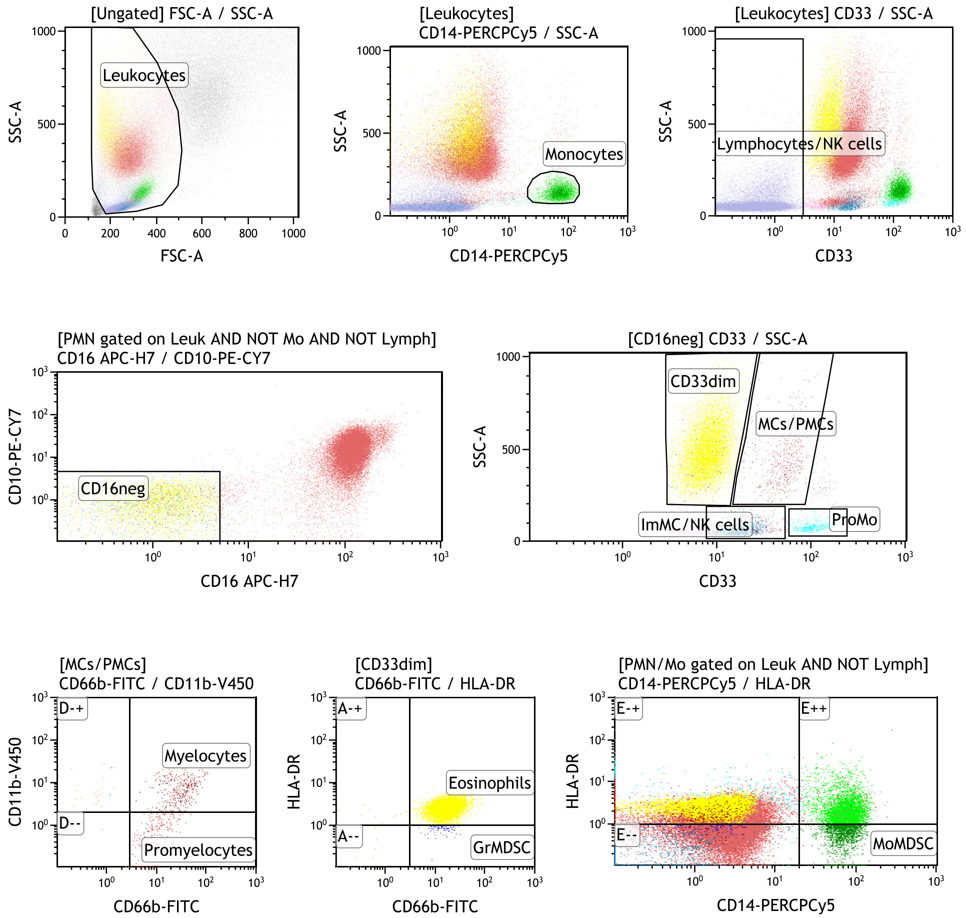
Supplementary Table E3. Treatment with betamethasone (mg)[a]

Patient	−1	0	+1	+2	+3	+4	+5	+6	+7	+8	+9	10	11	12	13	14	15	16	17	18	19	20
1	8	8	8	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2																						
3	4	4	4	4	4	4																
4	8	8	8																			
5	8	8	8						4	4	4	4	4	4	4	4	4	1	1	1	1	1
6																						
7		8	8	8																		
8	8	8	8								4											
9	8	8	8																			
10	8	8	8	8	8	4	8		4	8	8	8	8	8								
11	8	8	8					4														
12	8	8	8	8																		
13	8	8	8																			
14	8	8	4			4	4	8														

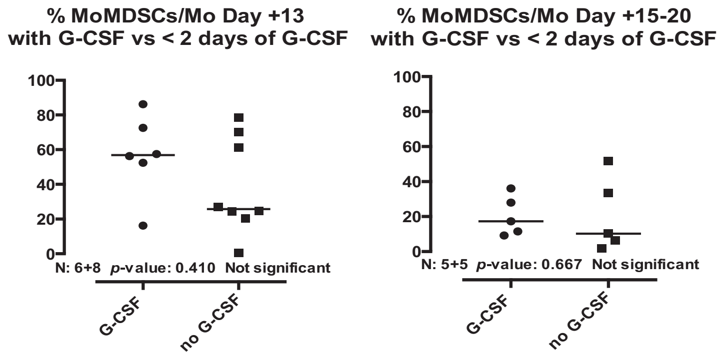
[a]Doses of betamethasone in milligrams (mg) given during the treatment, from start of melphalan infusion on day −1 to day +20 post-ASCT. The day on which thawed autologous peripheral stem cells were grafted is day 0. In addition, all patients received premedication with one dose of 100 mg hydrocortisone intravenously 30 min before transplantation on day 0.



Supplementary Figure E1. Gating strategy for (panel 1 and 2) lymphocytes, monocytes, PMNs (CD16^{high}, CD16^{weak}, CD16^{negative}), and eosinophils.

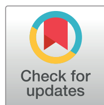


Supplementary Figure E2. Gating strategy for (panel 3) eosinophils, GrMDSCs, myelocytes, promyelocytes, promonocytes, and MoMDSCs.



Supplementary Figure E3. Effect of G-CSF on HLA-DR expression by monocytes, measured as the proportion of HLA-DR^{negative} monocytes in relation to total number of monocytes in patients who had received two or more doses of G-CSF compared with those who had received fewer than two doses of G-CSF on days +13 (left) and +15–20 (right).

Paper II



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RESEARCH ARTICLE

A single-arm, open-label, phase 2 clinical trial evaluating disease response following treatment with BI-505, a human anti-intercellular adhesion molecule-1 monoclonal antibody, in patients with smoldering multiple myeloma

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Abstract

Background

Smoldering multiple myeloma (SMM) is an indolent disease stage, considered to represent the transition phase from the premalignant MGUS (Monoclonal Gammopathy of Undetermined Significance) state towards symptomatic multiple myeloma (MM). Even though this diagnosis provides an opportunity for early intervention, few treatment studies have been done and the current standard of care is observation until progression. BI-505, a monoclonal antibody directed against intercellular adhesion molecule 1 (ICAM-1) with promising anti-myeloma activity in preclinical trials, is a possible treatment approach for this patient category with potential to eliminate tumor cells with minimal long-term side effects. BI-505 was well tolerated in an earlier phase 1 trial.

Methods and findings

In this phase 2 trial the effects of BI-505 in patients with SMM were studied. Four patients were enrolled and three of them completed the first cycle of treatment defined as 5 doses of BI-505, a total of 43 mg/kg BW, over a 7-week period. In the three evaluable patients, BI-505 showed a benign safety profile. None of the patients achieved a response as defined per protocol. EudraCT number: 2012-004884-29.

AB provided support in the form of salaries for these authors but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Competing Interests: We have the following interests: ES, IT, ATW, BF, MK are, or were, full-time employees of BioInvent International AB at the time of this study. IT, ATW and BF are currently employed and own stock (>50,000) in the company. BF and MH are inventors on patents relating to BI-505 and anti-ICAM antibody therapy. These declarations do not alter our adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

Conclusions

The study was conducted to assess the efficacy, safety and pharmacodynamics of BI-505 in patients with SMM. BI-505 showed no clinically relevant efficacy on disease activity in these patients with SMM, even if well tolerated.

Trial registration

ClinicalTrials.gov Identifier: NCT01838369.

Introduction

Smoldering multiple myeloma (SMM), first described as a clinical entity by Kyle and Greipp [1] in 1980, accounts for 10 to 15% of all myeloma diagnoses [2]. It is an asymptomatic plasma cell dyscrasia, with the risk of progression to symptomatic multiple myeloma or systemic amyloidosis of 10% per year during the first 5 years, after that, the risk of progression decrease to 3% per year for the next 5 years and to 1% per year beyond 10 years of follow-up [3]. The International Myeloma Working Group (IMWG) has defined smoldering multiple myeloma as a disorder where the patient has a serum monoclonal (M) protein (IgG or IgA) $\geq$3 g/100 ml and/or clonal bone marrow plasma cells (BMPCs) of $\geq$10%, but no CRAB symptoms (increased **c**alcium level, **r**enal failure, **a**nemia or destructive **b**one lesions) [4]. The current standard of care is observation without treatment until progression to symptomatic multiple myeloma [5]. Only one clinical trial so far has shown a benefit in overall survival for high-risk SMM patients on treatment with lenalidomide/dexamethasone compared to observation [6]. The IMWG criteria has recently been updated [7], allowing, in addition to the classical CRAB symptoms, presence of one of the following three "myeloma defining events (MDEs)": BMPCs $\geq$ 60%, serum involved/uninvolved free light chain ratio $\geq$ 100 or $>$ 1 focal lesions on MRI, to be sufficient for a diagnosis of symptomatic multiple myeloma, implying the possibility of early treatment, before irreversible end-organ damage has occurred.

The cell-surface receptor, intercellular adhesion molecule 1 (ICAM-1), is a transmembrane glycoprotein, which is constitutively expressed at low levels on many cell types including different leukocytes subsets and endothelial cells [8]. Up-regulation of ICAM-1 on endothelial cells at sites of inflammation cause increased leukocyte adhesion with subsequent extravasation and tissue infiltration [9]. In multiple myeloma, the bone marrow microenvironment and adhesion of multiple myeloma cells to bone marrow stroma cells (BMSCs) is essential for tumor cells growth and survival [10]. Adhesion molecules like ICAM-1 on multiple myeloma cells increase the binding capacity to BMSCs and overexpression of ICAM-1 have been associated with more advanced disease and drug resistance [11–13]. Since several independent observations indicate that ICAM-1 is highly expressed and involved in the pathogenesis of myeloma, it constitutes an attractive novel target for immunotherapy of multiple myeloma [14–16].

BI-505 is a fully human, high-affinity IgG1 monoclonal antibody directed against ICAM-1. The BI-505 epitope was strongly expressed on myeloma cells from both newly diagnosed and relapsed patients [16] and the anti-myeloma activity of BI-505 has been evaluated in animal models representing early as well as late-stage disease [16]. In vitro and in vivo mode-of-action studies provide strong evidence for Fc:FcgR-dependent antitumor mechanisms, e.g.,

macrophage-mediated antibody-dependent cellular phagocytosis and FcγR cross-linking-induced antibody tumor programed cell death, underlying BI-505's therapeutic activity [16].

A phase 1, multicenter, open-label, nonrandomized, repeat-dose, dose-escalation study of BI-505 in patients with relapsed/refractory multiple myeloma has been conducted [17]. The study was a first-in-human trial to evaluate the tolerability, safety, pharmacodynamics (PD) and pharmacokinetics (PK) of BI-505 following intravenous administration at doses of 0.0004–20.0 mg/kg once every second week. Thirty-four patients were treated with BI-505. The drug was generally well tolerated. Only 3 patients withdrew because of treatment-emergent adverse events (AEs).

In this phase 2 trial the effects of BI-505 in patients with SMM were studied. The primary purpose of this pilot study was to evaluate anti-tumor effect before promoting a large randomized trial. This patient category, untreated and at an early stage of the disease, may be particularly suitable for treatment with monoclonal antibodies, since the therapeutic effects are dependent on a functional immune system, including competent effector cells, such as natural killer cells and macrophages. More advanced disease is characterized by profound immune dysfunction and effective therapies at these stages is likely to require a potential to restore the immune response against myeloma [18, 19]. Moreover, monoclonal antibodies, such as BI-505, offers the possibility of targeted therapy to selectively eliminate tumor cells with minimal side effects, which is preferable for patients with SMM, in whom severe side effects are not acceptable from a risk-benefit perspective.

Methods

Study design

This was a single-arm, open-label, phase 2 study designed to assess the disease response following treatment with the monoclonal human antibody BI-505, when administered to patients with SMM. The sample size, 4 to 10 patients, was estimated to minimize the number of patients exposed to BI-505 while obtaining sufficient information to assess effects on disease activity, measured as M protein levels in plasma and urine, and change in percentage of BMPCs.

Following a screening period of up to 14 days, eligible patients were to receive 5 intravenous infusions of BI-505 over a 7-week period: 3 mg/kg body weight (BW) BI-505 on treatment day 1, 10 mg/kg BW BI-505 on treatment day 8 and then 3 infusions of 10 mg/kg BW BI-505 every second week. This was considered 1 dosing cycle of 50 days (Cycle 1).

Patients were evaluated for disease activity based on data collected on treatment day 50. Patients with at least a minimal response (MR) based on M protein levels were allowed to continue to cycle 2, which was to comprise 3 bi-weekly intravenous doses of BI-505 (10 mg/kg BW). Patients who completed Cycle 2 and had at least a partial response (PR) on treatment day 92 were allowed to continue to cycle 3, which was to comprise 3 additional bi-weekly intravenous doses of BI-505 (10 mg/kg BW).

Disease activity, measured as M protein levels in serum/urine, was to be determined throughout the study and was evaluated according to the EBMT criteria [20]. Minor response (MR) was defined as a 25 to 49% reduction in serum M protein level.

The dosing regimen selected for this study was supported by safety, PK and receptor saturation data from the phase 1 study in patients with relapsed/refractory multiple myeloma [17]. In order to reduce the risk of infusion-related reactions observed in the phase 1 trial, the initial dose (3 mg/kg) was lower than subsequent doses (10 mg/kg). Furthermore, the first dose and second doses were administered over a longer time period compared to subsequent doses (4 hours vs. 2 hours). All patients remained at the clinical unit for 10 hours after the start of the

first and second infusions and were closely monitored for any untoward reactions. For subsequent infusions, patients were observed for at least 3 hours after the start of the infusion. All patients received paracetamol and an antihistamine (cetirizine) before each infusion.

Based on data from the phase 1 study, where the full PK profile of BI-505 was assessed, dosing with 3 mg/kg BW BI-505 would lead to complete saturation of all ICAM-1 epitopes on the patient's bone marrow myeloma cells during the first dosing interval (1 week) [17]. Similarly, dosing with 10 mg/kg BW BI-505 would lead to complete saturation during the subsequent 2-week dosing intervals, with trough plasma concentrations above 1 μg/ml, which is needed for full receptor saturation in the bone marrow.

The study protocol, patient information and consent form were reviewed and approved by an Independent Ethics Committee, The Regional Ethical Review Board in Lund, Sweden (www.epn.se), prior to inclusion of patients. The study was conducted in compliance with the protocol, regulatory requirements, good clinical practice (GCP) and the ethical principles of the latest revision of the Declaration of Helsinki as adopted by the World Medical Association. Written informed consent was obtained from all patients before enrollment. EudraCT number: 2012-004884-29. ClinicalTrials.gov Identifier: NCT01838369.

Patient population

The study included patients $\geq$ 18 years old with diagnosis of SMM based on the 2009 IMWG criteria [4], i.e.; serum M protein level $\geq$3 g/100 ml and/or BMPCs of $\geq$10% and absence of end-organ damage such as lytic bone lesions, anemia, hypercalcemia and renal failure that could be attributed to a plasma cell disorder. All patients had measurable disease, defined by a serum M protein $\geq$1 g/100 ml, Eastern Cooperative Oncology Group (ECOG) performance status 0–1, adequate renal and hepatic function. Systemic corticosteroid use was not allowed within 4 weeks prior to screening. Patients were excluded if they had clinical suspicion of progression to symptomatic multiple myeloma, if they had any prior or current treatment with proven or potential impact on plasma cell proliferation and survival, or had used any investigational agent within the last 3 months. They were also excluded if clinical findings indicating cardiac or renal amyloid light-chain (AL) amyloidosis (Additional details are provided in S1 Appendix).

Safety and efficacy assessments

Adverse events (AEs) were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. Other safety evaluations were physical examinations including Eastern Cooperative Oncology Group (ECOG) performance status, vital signs, electrocardiograms and laboratory tests (including complete blood count, clinical chemistry, coagulation, and urinalysis).

The primary objective was to assess the tumor response rate, defined according to the EBMT response criteria [20], which included measurements of serum and urine M-protein, serum free light chains, and plasma cell levels in bone marrow. The secondary objectives were to further assess the clinical safety of BI-505 and the pharmacodynamics (PD).

Pharmacodynamic assessment in blood

The proportion of monocytes, NK and NKT cells in blood samples, collected immediately before and after each BI-505 infusion, was analysed using flow cytometry with monoclonal fluorescent-labelled antibodies with panels including CD14, CD3 and CD56.

Statistical analysis

There was no statistical analysis. All patients and results are listed.

Results

Patients' characteristics

The study was conducted between April 2013 and December 2014 at the Department of Hematology, Skåne University Hospital in Lund, Sweden. Four patients were screened. Bone marrow aspirate smear from the first patient showed at screening 8% BMPCs. The actual sample was assessed to be somewhat diluted by peripheral blood. However, a previous investigation at diagnosis had shown 20% BMPCs and the SMM diagnosis considered confirmed. All four patients enrolled in the study fulfilled the inclusion and exclusion criteria. All patients had an ECOG 0. The ages of the patients were 50, 64, 66 and 67 years (Table 1). Three were male and one was female. In three patients the immunoglobulin was IgG; in one patient it was IgA. The light chain was kappa in three patients and lambda in one patient. A CONSORT flowchart is provided in (Fig 1).

The three patients who completed the first dosing cycle received a dose of 2.25 to 2.98 mg/kg BW on treatment day 1 and a dose of 10.00 mg/kg BW on treatment day 8, 22, 36 and 50.

One patient discontinued for safety reasons as judged by the investigator, after suffering from headache, hypertension, vomiting and fatigue on treatment day 1. This was a patient with a medical history of migraine, and onset of symptoms before start of the first BI-505 infusion. These 4 AEs were all judged to be unrelated to the study drug. This patient received a dose of 0.27 mg/kg BW on treatment day 1 and no further doses. The infusion was stopped after 1 hour because of hypertension. The headache and vomiting were resolved on the same day they developed; the fatigue was resolved the day after it developed; and the hypertension was resolved after 136 days.

The Sponsor terminated the trial due to re-assessment of the developmental path for BI-505, focusing on maintenance after high dose melphalan and autologous stem cell transplantation.

Efficacy assessments

Three patients were assessed for tumor response based on change in serum M protein level on treatment day 50 (Table 1). None of these patients achieved MR, a requirement for continuation to Cycle 2. Between treatment day 1 and 50 (after 4 doses of BI-505), there were no clinically significant changes in serum M protein level; 1.8 to 1.9 g/100 ml, 3.0 to 3.6 g/100 ml and 0.9 to 0.9g/100 ml.

Table 1. Patients' characteristics and efficacy assessments.

Pat No	Sex	Age	M- Comp Class	Diagnosis SMM yr. before inclusion	Serum M protein at diagnosis g/100 ml	Serum M protein Day 1 g/100 ml	Serum M protein Day 50 g/100 ml	Urinary M protein at screening mg/24 h	Urinary M protein Day 50 mg/24 h	BMPCs (%) at screening	BMPCs (%) Day 50 resp. Day 69
1	M	66	A kappa	2.8	1.9	1.8	1.9	78	62	8	12
2	M	64	G kappa	2.3	0.7	0.9	0.9	497	664	17	9
3	M	67	G lambda	2.5	2.8	3.0	3.6	18	0	11	
4	F	50	G kappa	8.4	2.0	2.2				11	

doi:10.1371/journal.pone.0171205.t001

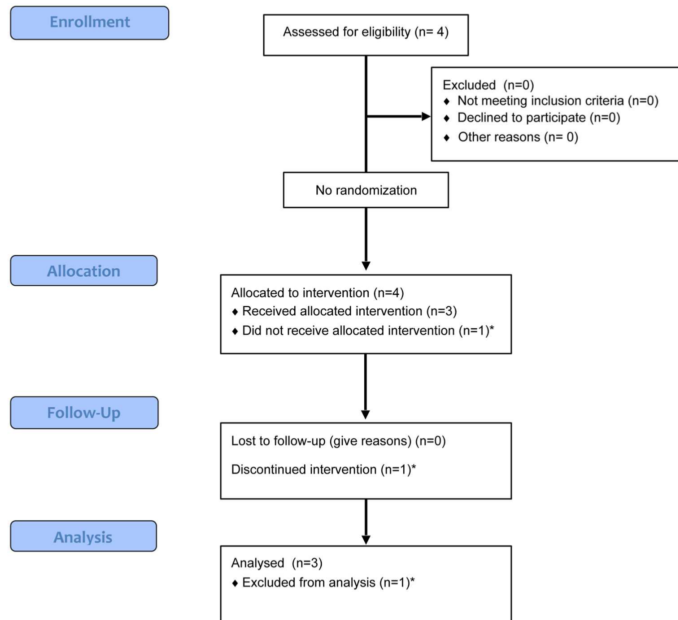

Fig 1. CONSORT flowchart for A single-arm, open-label, phase 2 clinical trial with BI-505, a human anti-intercellular adhesion molecule-1 monoclonal antibody, in patients with smoldering multiple myeloma.

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There were no clinically significant changes in twenty-four hour urinary M protein production between screening and treatment day 50 (Table 1); 78 mg to 62 mg, 497 mg to 664 mg and 18 mg to 0 mg.

Change in percentage of BMPCs was assessed in two patients (Table 1). The percentage of BMPCs was 8% at screening and 12% on treatment day 50 for the first patient and 17% at screening and 9% at the End of Study Visit on day 69 for the second patient. For the other two patients, the percentage of BMPCs was only assessed at screening.

Bone marrow concentrations of BI-505

BI-505 concentration in bone marrow was assessed as previously described [17]. For the three patients assessed, BI-505 concentrations of 12.9 μg/ml (day 50), 21.6 μg/ml (day 69) and 66.1 μg/ml (day 50) were detected in bone marrow aspirates. This indicated complete saturation of ICAM-1, as saturation occurs at a BI-505 concentration of 1 to 3 μg/ml [17].

Table 2. Adverse events.

Adverse Events	n	Severity	Relationship to Study Treatment
Overall, treatment-related AEs	11	Grade 1: (9) Grade 2: (2)	Probable/Certain (10) Possible (1)
Infusion-related reactions; pyrexia, chills, body ache	9	Grade 1: (7) Grade 2: (2)	Probable/Certain
C-reactive protein increase	1	Grade 1: Mild	Probable/Certain
Bronchitis	1	Grade 1: Mild	Possible
Nasopharyngitis/viral infection	1	Grade 2	Unlikely
Headache	2	Grade 1–2	Unrelated
Hypertension	2	Grade 1–2	Unrelated
Fatigue	1	Grade 1: Mild	Unrelated
Vomiting	1	Grade 1: Mild	Unrelated
Insomnia	1	Grade 1: Mild	Unrelated
Nicotine dependence	1	Grade 2: Moderate	Unrelated
Blood cholesterol increase	1	Grade 1: Mild	Unrelated
Acute coronary syndrome	1	Grade 3: Severe	Unrelated

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Adverse events

A total of 22 AEs were reported in the four patients and 10 of them were judged to have a "Probable/Certain" relationship to the study drug (Table 2); 9 infusion-related reactions with pyrexia, chills and body ache and one case of C-reactive protein increase. One other AE, prolonged symptoms of bronchitis after a common cold, was judged to have a "Possible" relationship to the study drug. Most of the reported AEs were mild. One patient was discontinued after developing AEs (judged to be unrelated to the study drug) on treatment day 1 as described above.

One AE, acute coronary syndrome that occurred one month after the last dose of BI-505, was judged to be serious. This serious adverse event (SAE, Grade 3) was treated by percutaneous transluminal coronary angioplasty. The relationship to the study drug was judged as "Unlikely."

Pharmacodynamic assessment in blood

For two patients, blood samples for FACS-analysis were obtained immediately before and after each infusion for exploratory correlative analysis. The proportions of monocytes, NK and NKT cells in peripheral blood after completion of the first infusion (Fig 2), were shown to be reduced. This could be an indirect sign of activated immune response against target cells for BI-505. However, the levels recovered before the second infusion and further decrease was not seen in subsequent infusions (data not shown).

Discussion

Few treatment studies, aiming to prevent or delay the development of manifest disease in SMM, have been conducted [6, 21–31]. Up to the present, standard of care is close observation without treatment until progression [5]. Theoretically, targeted therapy with monoclonal antibodies, without expected long-term side effects, would be an attractive treatment strategy for patients with SMM. BI-505 is a human monoclonal IgG1 antibody with high specificity for ICAM-1, selected by functional screening for its ability to induce dose-dependent programmed cell death in B lymphoma cell lines [32] and with promising anti-myeloma activity in several murine myeloma models including hu-SCID mice with human fetal bone chip and human primary myeloma cells [15, 16].

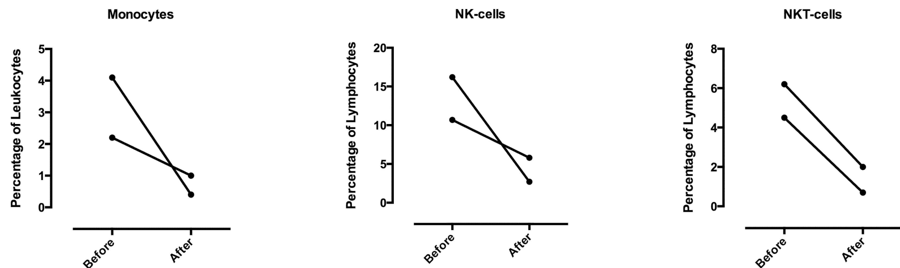

Fig 2. Reduced proportions of monocytes, NK and NKT cells after the first dose of BI-505. Percentage of monocytes, NK and NKT cells in blood samples collected before and immediately after completion of the first BI-505 infusion in patients No 2 and No 3, as analyzed by flow cytometry.

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Previous clinical trials, with a murine IgG2a anti-ICAM-1 antibody, targeting another ICAM-1 epitope than BI-505, enlimomab, have been performed, with the aim of reducing detrimental inflammatory activity in ischemic stroke, rheumatoid arthritis and burn injuries [33–36]. Altogether, more than 400 patients have received treatment with the murine anti-ICAM-1 antibody in these trials. The randomized stroke trial did not show any benefit for treatment with enlimomab and treated patients experienced more adverse events, mainly fever and pneumonia. The results of the other trials were cautiously positive and the treatment well tolerated. A follow-up study with a second treatment course of enlimomab in patients with rheumatoid arthritis was associated with cases of serum sickness-like reactions [37], probably due to complement-activating properties of murine monoclonal antibodies of IgG2a isotype and the murine nature of the antibody [38]. In contrary, BI-505 is a fully human antibody of IgG1 isotype with less complement activating properties. In pre-clinical studies, BI-505 did not show any induction of apoptosis in resting or activated normal ICAM-1 expressing peripheral blood B cells or human microvascular endothelial cells [16]. Moreover, BI-505 added in solution did not induce cytokine release from peripheral blood mononuclear cells or T cell proliferation, which otherwise might contribute to adverse events to antibody therapy [16].

A first-in-human, phase 1, study of BI-505 in patients with advanced relapsed/refractory multiple myeloma has shown good tolerability, with doses up to 20 mg/kg, the maximum tolerated dose was not reached [17]. An optimal dose, used in the current study, was determined to be 10 mg/kg every two weeks, which resulted in complete saturation of ICAM-1 epitopes on BMPCs during the entire dosing interval [17]. In the heavily pretreated patient cohort in the phase 1 study, no objective responses were observed, but 24% (7/29) achieved stable disease for more than 8 weeks [17].

This study was conducted to assess the efficacy, safety and pharmacodynamics of BI-505 in patients with SMM. The aim was to enroll four to ten patients. Four patients were enrolled and three of them completed the first cycle of treatment, defined as 5 doses of BI-505 (a total of 43 mg/kg BW) over a 7-week period. All three patients who received treatment doses of BI-505 experienced, despite pre-medication, infusion-related adverse events with fever, chills and body pain during the 1st, the 1st and 2nd, respectively the 1st to 4th infusion, causing transient discomfort, although classified no worse than mild to moderate. One of the patients had after the 2nd doses of BI-505 a sustained period of low-grade fever (19 days) and moderate, spontaneously resolving, C-reactive protein increase (15 days), without evidence of infection. CRP elevation was observed in 15% of patients (5/34) in the previous phase 1 study and might be a

sign of secondary cytokine release [17]. Overall, BI-505 showed no clinically relevant efficacy on disease activity in the three evaluable patients with SMM, even if well tolerated.

The four patients in this study had a stable M component for over 2 years before enrollment (Table 1) and none of them have so far (November 2016; 6.4, 4.9, 5.0 resp. 10.4 years after diagnosis of SMM), progressed to symptomatic multiple myeloma. Several new prognostic variables are emerging making it possible to identify SMM patients at high risk for disease progression [39–41].

BI-505´s in vivo anti-myeloma activity was shown to be macrophage-dependent and was associated with significant recruitment of monocyte/macrophages to tumor lesions and myeloma-infiltrated bone marrow [16] (and unpublished data). BI-505´s anti-myeloma activity was found in early and disseminated experimental models of MM, or when combined with novel drugs e.g. the proteasome inhibitor bortezomib or the immune modulatory drug lenalidomide [16]. However, addition of lenalidomide or bortezomib to the treatment with BI-505 for patients with SMM, would imply a higher degree of expected toxicity, such as pancytopenia, susceptibility to infections and increased risk of secondary malignancies (lenalidomide) and peripheral neuropathy (bortezomib), counteracting the idea of a new treatment choice, without severe side effects for this patient category [41]. Recently published data on a clinical trial with carfilzomib/lenalidomide/dexamethasone to 12 patients with SMM reported that two patients developed secondary malignant neoplasms and one patient discontinued treatment due to a serious adverse event with grade 3 congestive heart failure [31].

Coming clinical trials will focus on exploring BI-505 activity in settings of lower tumor burden. A clinical phase II study exploring the addition of BI-505 to high dose melphalan and autologous stem cell transplantation has started recruiting patients (www.clinicaltrials.gov Identifier: NCT02756728).

Supporting information

S1 Appendix. Inclusion and exclusion criteria.
(DOCX)

S2 Appendix. TREND statement checklist.
(PDF)

S3 Appendix. Clinical study protocol.
(PDF)

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Supervision: BF MK MH.

Visualization: SW.

Writing – original draft: SW.

Writing – review & editing: SW GJ ÅJ ES IT ATW BF MK MH.

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S1 Appendix Paper II, Inclusion and Exclusion Criteria

Inclusion Criteria

- Diagnosis of smoldering multiple myeloma based on the IMWG criteria: Serum M protein level ≥3 g/100 ml and/or ≥10% bone marrow plasma cells AND absence of end-organ damage such as lytic bone lesions, anemia, hypercalcemia or renal failure that could be attributed to a plasma cell proliferative disorder
- Male or female, 18 years or older
- Ability to understand and willingness to sign an informed consent form
- Measurable disease, defined by a serum M protein of ≥1.0 g/100 ml
- Eastern Cooperative Oncology Group (ECOG) performance status 0 to 1
- Adequate hepatic function (aspartate transaminase [AST] and alanine transaminase [ALT] ≤2.5 times the upper limit of normal [ULN]; bilirubin ≤1.5 times the ULN)
- Adequate renal function (calculated serum creatinine clearance ≥ 50mL/min)
- Females of childbearing potential and males (and their partners): Agreement to use adequate contraception during the study and for at least 12 weeks after discontinuation. Adequate contraception: oral/systemic contraception, intrauterine device, last natural menstruation at least 24 months prior to baseline, surgical sterilization before baseline or hysterectomy prior to baseline
- No systemic corticosteroid use within 4 weeks prior to screening

Exclusion Criteria

- Diagnosis of symptomatic multiple myeloma or clinical suspicion of ongoing progression to symptomatic multiple myeloma
- Clinical findings indicating cardiac or renal amyloid light-chain (AL) amyloidosis
- Prior or current treatment with a proven or potential impact on myeloma cell proliferation or survival (including conventional chemotherapies, biological therapies, immunomodulatory drugs and proteasome inhibitors), as judged by the Investigator

- Use of any investigational agent within the last 3 months
- History of allogeneic stem cell or solid organ transplantation
- Prior malignancy in the last 2 years, with the exception of SMM, adequately treated basal cell or squamous cell carcinoma, cervical carcinoma in situ, prostate cancer Gleason < 6 and prostate-specific antigen (PSA) < 10 ng/mL, radically excised lobular or ductal carcinoma in situ (LCIS/DCIS) ≤ 15 mm breast cancer in women over 40 years old, or any malignancy for which the subject had undergone potentially curative therapy with no evidence of the disease for three years
- Evidence of significant active infection, requiring intravenous antibiotics, within 14 days before enrollment
- Current active infectious disease or positive serology for human immunodeficiency virus (HIV), hepatitis C virus (HCV) or hepatitis B surface antigen
- Patients with a history of cerebrovascular disease or atrial fibrillation were excluded (unless the event occurred more than two years previously and was adequately treated; and atrial fibrillation that was well controlled with medication)
- Patients with other severe conditions requiring treatment and close monitoring, e.g. cardiac failure of New York Heart Association (NYHA) grade > 3, unstable coronary disease or oxygen-dependent chronic obstructive pulmonary disease (CORP)
- Significant autoimmune disease requiring systemic treatment with corticosteroids or other immunosuppressive drugs during the previous 24 months (including rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, psoriasis, multiple sclerosis, hemolytic anemia and glomerulonephritis, and other conditions that required such therapy (mild autoimmune phenomena and inactive disease were not exclusion criteria)
- Breast-feeding or positive pregnancy test
- Substance abuse or other concurrent medical conditions that could confound study interpretation or affect the patient´s ability to tolerate treatment or complete the study

Paper III

Experimental Hematology 2018;66:27−31

Experimental Hematology

BRIEF COMMUNICATION

Bone marrow eosinophils in plasma cell disorders

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In experimental studies, eosinophils have been shown to promote the survival, proliferation, and retention of plasma cells in the bone marrow (BM). The clinical significance of eosinophils in plasma cell disorders (PCDs) in humans is largely unknown. This study focuses on the frequency and phenotype of eosinophils in the BM and peripheral blood (PB) in patients with untreated PCD compared with healthy controls. The number of eosinophils per se did not correlate with the number of BM plasma cells or disease stage. The expression of chemokine receptor 4, which is important in the homing capacity to bone marrow stromal cells, was significantly higher in patient eosinophils and increased with disease stage. BM eosinophils from patients, especially from those with manifest disease, were more activated. Another finding in this study was that eosinophils in PB and BM from both patients and healthy controls expressed CD80 (B7-1). We discuss probable immunomodulatory consequences of surface expression of CD80 by eosinophils in conditions with marked T-cell exhaustion (e.g., multiple myeloma). Finally, we found that patients treated with corticosteroids had low levels of circulating eosinophils but preserved levels of eosinophils in the BM. **© 2018 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.**

Experimental data indicate that eosinophils are important for the survival, proliferation, and retention of plasma cells (PCs) in the bone marrow (BM) and they tend to accumulate in the same BM niches [1−3]. Eosinophils might function as antigen-presenting cells (APCs) [4,5] and have been shown to have immunomodulatory properties that could affect tumor growth [6−8]. Recently, Lingblom et al. found a subset of "regulatory eosinophils" with a T-cell-suppressive effect mediated by the protein galectin-10 [9]. Tumor-associated tissue eosinophilia (TATE) has been observed in several types of solid cancers and has generally been linked to favorable prognosis [7,10]. In contrast, TATE in the setting of Hodgkin lymphoma nodular sclerosis constitutes a poor prognostic factor [11].

The clinical relevance of eosinophils in plasma cell disorders (PCDs) is unknown. The aim of this study was to investigate the frequency and phenotype of eosinophils in peripheral blood (PB) and BM from patients with newly diagnosed PCD in different stages, premalignant monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), and manifest multiple myeloma (MM) and whether the eosinophil population in BM might be correlated to the degree of PC infiltration or T-cell subsets.

Methods

Patients and controls

The study was approved by an independent ethics committee (www.epn.se; reference number 2016/768). After obtaining written informed consent, PB and BM aspirates were collected from patients with MGUS, SMM, and MM at diagnosis and compared with samples from healthy controls.

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Eosinophil phenotype and T-cell characterization

PB and BM samples were analyzed by flow cytometry (for details, see Supplementary Table E1, online only, available at www.exphem.org). Eosinophils were gated on CD16-negative polymorphonuclear neutrophils (PMNs) with strong expression of siglec-8. The T-cell subsets were gated according to standard gating strategy [12].

Statistical analysis

GraphPad PRISM version 6.0a software was used for statistical calculations. The Mann−Whitney U test was used for two-group comparisons and the Kruskal−Wallis test was used for comparisons of more than two groups. Spearman's rank correlation coefficient was used to quantify covariation. $p < 0.05$ was considered significant.

Results and discussion

Patient characteristics

Patients referred to the Department of Hematology, Skåne University Hospital, in Lund, Sweden, due to a newly discovered M component or other symptoms consistent with a PCD were consecutively included from February 1, 2017 to the January 31, 2018 (Supplementary Table E2, online only, available at www.exphem.org). None of the patients had any history of allergic asthma or atopic dermatitis and no ongoing symptoms of infection. A total of 23 patients were included. Nine patients were subsequently diagnosed with newly diagnosed MM (NDMM), five patients with SMM, and nine patients with MGUS. Four additional patients (one only PB, three BM + PB) with NDMM had started treatment with high-dose corticosteroids before sampling, which might affect T-cell subsets and antigen expression, so only the results for proportion of eosinophils are included and reported separately. BM and PB samples from 19 healthy controls were also collected (Supplementary (ii) Data on healthy controls). For measurement of degree of antigen expression with mean fluorescence intensity, three NDMM patients, two MGUS patients, and six healthy controls were excluded due to the use of another fluorescence-activated cell sorting instrument during the first 3.5 months (Supplementary Tables E1 and E2, online only, available at www.exphem.org).

Eosinophils with altered phenotype in PCDs

The median percentage of eosinophils out of PMNs in BM samples was 3.1% (range 0.9−7.5%) for patients with PCD compared with 2.5% (range 0.1−7.3%) for healthy controls (not significant; Figure 1A). Three out of 9 patients with NDMM, 1/5 patients with SMM, 3/9 patients with MGUS, and 5/19 healthy controls had >4.0% eosinophils of PMNs in BM (Supplementary Table E2, online only, available at www.exphem.org).

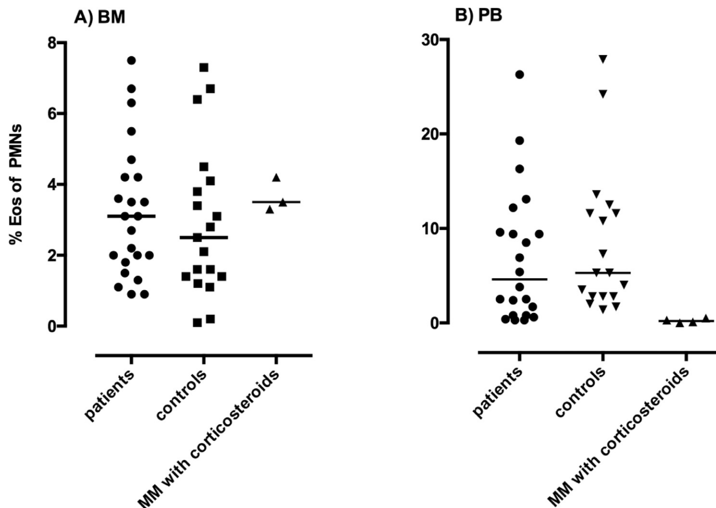

Figure 1. Percentage of eosinophils of PMNs in BM and PB. **(A)** There was no significant difference between the percentages of BM eosinophils for patients with PCD ($n = 23$) compared with healthy controls ($n = 19$) and no reduction of BM eosinophils for NDMM patients after 2−4 days of treatment with high-dose corticosteroids ($n = 3$, 40 mg prednisolone daily for 2 days, 24 mg betamethasone for 4 days, and 20 mg dexamethasone for 4 days). **(B)** NDMM patients treated with corticosteroids ($n = 4$) had a significantly reduced percentage of eosinophils in PB both compared with healthy controls ($n = 18$) ($p = 0.0003$) and with untreated patients with PCD ($n = 22$) ($p = 0.002$).

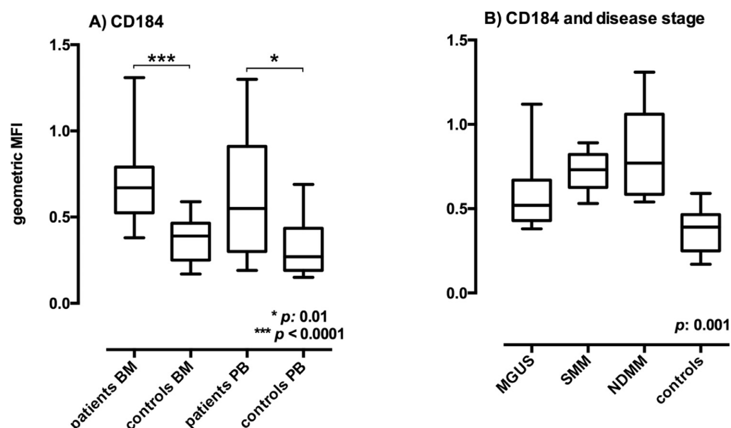

Figure 2. Expression of surface antigen CXCR4/CD184 by eosinophils measured as geometric mean fluorescence intensity and compared with healthy controls. **(A)** CD184 expression on eosinophils in BM and PB for patients with PCD (BM, $n = 17$; PB, $n = 15$) and healthy controls (BM, $n = 13$; PB, $n = 12$). For each individual, there was no significant difference between surface expression of CD184 on eosinophils from BM-samples compared with those collected in PB for either patients or healthy controls (Wilcoxon matched-pairs signed rank test). **(B)** Expression of CD184 on BM eosinophils according to disease stage (NDMM, $n = 5$; SMM, $n = 5$; MGUS, $n = 7$; healthy controls, $n = 13$).

Therefore, there was no correlation with disease stage or between the percentage of BM eosinophils and the degree of PC infiltration (Supplementary Table E2, online only, available at www.exphem.org).

The four patients with NDMM who had started corticosteroid therapy had significantly lower levels of eosinophils in PB (0.0%, 0.1%, 0.3%, and 0.5%) compared with the other patients (median 4.6%, range: 0.3–26.3%) and controls (median 5.3%, range: 1.4–27.9%) ($p = 0.005$; Figure 1B). This difference could not be explained by a corresponding increase in absolute neutrophil count (ANC) because the median ANC for patients was 3.7×10^9/L (range: 1.5–7.8) and, for patients on corticosteroids, it was only moderately higher at 4.9×10^9/L (range: 4.8–5.5). Eosinophils have a high expression of glucocorticoid receptors [13] and the most expressed "α-splice variant" is pro-apoptotic for eosinophils [14,15]. Nevertheless, the proportions of BM eosinophils from the three patients examined after 2–4 days of treatment with corticosteroids were not reduced compared with the median values for the patient group as a whole or healthy controls (3.5%, 4.2%, and 3.3%, respectively) (Figure 1A and Supplementary Table E2, online only, available at www.exphem.org). This raises the question of whether BM eosinophils are less sensitive to corticosteroids than their circulating counterparts. In vitro, the addition of dexamethasone during eosinophil differentiation of human cord blood stem cells resulted in enhanced early eosinophil differentiation and proliferation, but increased apoptosis of mature eosinophils [16].

CXCL12/SDF-1 secreted by BM stromal cells binds to and activates chemokine receptor 4 (CXCR4/CD184) on hematopoietic cells and this interaction is known to be important for the homing capacity to the BM [1,17–19]. BM eosinophils from patients with PCD had an increased surface expression of CD184 compared with healthy controls ($p < 0.0001$). Furthermore, the expression was correlated with disease stage ($p = 0.001$; Figure 2). Also, patient eosinophils from PB had an increased expression of CD184 ($p = 0.01$; Figure 2). This implies that eosinophils are important players in the BM microenvironment that could contribute to PCD development in humans.

CD69 expression, a marker for activation, was elevated in BM eosinophils compared with healthy controls ($p < 0.05$; Figure 3A) and this was more apparent in BM samples from patients with NDMM ($p = 0.02$ vs. healthy controls; data not shown) [20]. The expression of CD62L/L-selectin, the adhesion receptor for activated endothelial cells, tended to be lower in patient BM eosinophils, but this was not significant ($p = 0.07$; Figure 3B).

Proposed immunomodulatory properties for eosinophils in the setting of MM

Eosinophils in BM and PB from patients and from healthy controls were found to have surface expression of CD80 (Supplementary Figure E1, online only, available at www.exphem.org). Eosinophils increase CD80 expression further

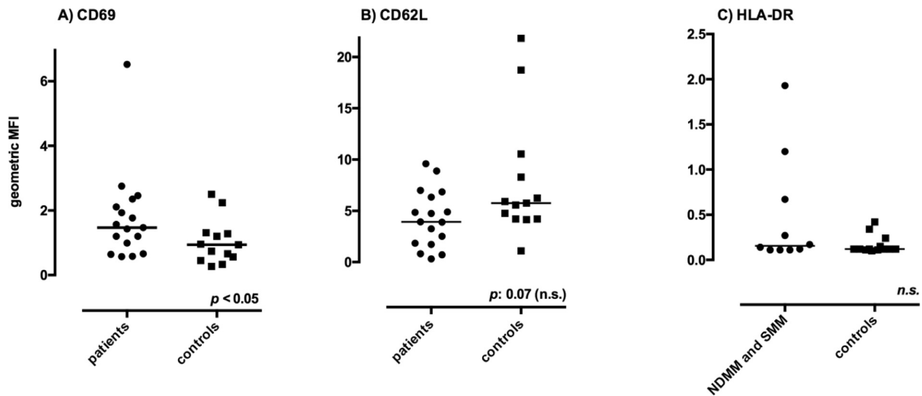

Figure 3. Expression of the surface antigens CD69, CD62L, and HLA-DR by BM eosinophils measured as geometric mean fluorescence intensity and compared with healthy controls. **(A)** CD69 in patients with PCD ($n = 17$) and healthy controls ($n = 13$). **(B)** CD62L in patients with PCD ($n = 17$) and healthy controls ($n = 13$). **(C)** HLA-DR data for myeloma patients (n=10), NDMM and SMM, and healthy controls ($n = 13$).

upon activation and CD80 expression on BM eosinophils was correlated to CD69 expression ($n = 17$, $r = 0.56$, $p = 0.02$; data not shown) in patient samples. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) binds to CD80 with higher affinity than the co-stimulatory CD28 and attenuates T-cell activation [21,22]. Two out of 10 patients with MM in this study had BM eosinophils with a low but measurable surface expression of HLA-DR (Figure 3C). It has been reported that only few major histocompatibility complex (MHC) class II molecules are needed for a cell to achieve APC function and interact with CD4$^+$ T cells [23]. Together with the expression of CD80, this might be sufficient to engage the high-affinity receptor CTLA-4, the expression of which is elevated in BM T cells in MM patients [24]. Eosinophils would thereby contribute to immunological tolerance in the tumor microenvironment. CD80 has also been found to interact with the programmed death ligand-1 (PD-L1) with an affinity between CD28 and CTLA-4 [25]. PD-L1 is constitutively expressed on hematopoietic cells and in vitro trials showed that T-cell binding of PD-L1 to CD80 resulted in downregulation of T-cell proliferation [25].

A previous study with BM samples from patients with NDMM and age-matched controls showed that MM patients had decreased naive CD8$^+$ T cells and an expanded pool of CD8$^+$ T effector memory RA$^+$ cells (T-EMRA) [24]. Moreover, CD8$^+$ CD45 RA$^+$ T cells from patient BM were shown by in vitro activation to have reduced capacity for proliferation and cytotoxic activity, which is consistent with T-cell exhaustion [24]. We can confirm here that patients with NDMM had decreased naive CD8$^+$ T cells and a markedly increased proportion of CD8$^+$ T-EMRA (Supplementary Figure E2, online only, available at www.exphem.org). We also show that patients with the premalignant state MGUS (Supplementary Figure E2, online only, available at www.exphem.org) have a similar pattern of CD8$^+$ T-cell distribution. An increase in exhausted memory T cells is described in settings with continuous antigen exposure such as chronic infections and cancer [26]. The effector functions of exhausted memory T cells are impaired and a hallmark of these cells is a higher and sustained expression of inhibitory receptors [26]. (For further results regarding T-cell subsets see, Supplementary Figures E2 and E3, online only, available at www.exphem.org.)

Tumor-infiltrating neutrophils can be induced to cross-present antigens of tumor cell origin and thereby promote CD8$^+$ T-cell responses [5,27]. They activate tumor-specific cytotoxic T lymphocytes through TCR-peptide/MHC class I binding followed by CD28–CD80 co-stimulation. If eosinophils, shown here to express CD80 (Supplementary Figure E1, online only, available at www.exphem.org), also have the capacity of cross-presentation, it would partly explain that their presence in solid tumors usually implicates an improved prognosis. In a microenvironment dominated by T-cell dysfunction and increased expression of inhibitory receptors on T cells, as described in MM [24] and Hodgkin lymphoma [28], tumor-infiltrating eosinophils might have the opposite effect.

To conclude, the number of BM eosinophils did not correlate with disease stage, but they were more activated in patients with PCD and had increased surface expression of the BM homing receptor CXCR4.

Eosinophils express CD80 and, in MM with increased T-cell expression of CTLA-4, might contribute to a dampened antitumor immune response.

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Laboratory work was conducted by ÅP and ÅJ. SW and MH recruited the patients. SW carried out the FACS analysis, compiled the data, and drafted the manuscript. ÅJ, MH, TH, and SW contributed to the design of the study and writing the manuscript. All authors approved the final manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

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Supplementary materials

Supplementary methods (i)

Supplementary Table E1. – Antibody panels for characterization of eosinophils and T cells

Panels of monoclonal fluorescent-labelled antibodies (BD Bioscience California, USA):						
(1) PMNs, eosinophils, monocytes	CD45 BV421	CD14 PerCP	Siglec-8 PE	CD16 APC-Cy7	CD193 BV510	CD184 APC
	CD80 FITC	HLA-DR PECy7	CD62L BV650	CD66b ALEXA700	CD11b BV786	CD69 PE-CF594
(2) T cell subsets	CD3 ALEXA700	CD4 BV421	CD8 BV510	CD45RA PE-Cy7	CD197 APC	CD196 PE
	CD183 FITC	CD25 PerCP-Cy5-5	CD127 PE-CF594	CD194 BV786	CD45RO BV650	HLA-DR APC-H7

Peripheral blood (PB) samples collected in heparin tubes, and bone marrow (BM) aspirates collected in tubes with the culture medium RPMI 1640, were analyzed by flow cytometry. To lyse erythrocytes, 0.84% ammonium chloride was used. The remaining leukocytes were washed with phosphate buffered saline (PBS), resuspended in PBS with 0.5% bovine serum albumin, and stained with these two panels of monoclonal fluorescent-labelled antibodies.

A FACS (fluorescence-activated cell sorting) instrument Fortessa was used for the first 3.5 months and thereafter we used FACS Aria Fusion with FACS Diva software for data collection. Kaluza software (Beckman Coulter, Brea, CA) was used for analysis.

Eosinophils were gated on CD16 negative PMNs (Polymorphonuclear leukocytes) with strong expression of siglec-8 and their surface expression of CXCR4/CD184 (ability for homing to BM), CD80 (B7-1), HLA-DR (antigen-presenting capacity), CD193/CCR3 (the receptor for eotaxin), CD69 (marker of activation) and CD62L/L-selectin (adhesion to activated endothelial cells) were measured by mean fluorescence intensity (MFI) and compared to healthy controls. Samples with < 100 cells gated were excluded. Monocytes were gated on typical FSC/SSC, CD45 and CD14 positive cells. The T cell subsets were gated according to standard gating strategy [1].

Supplementary, patients and healthy controls (ii)

Supplementary Table E2. - Patients' characteristics

Pat No	FACS	Sex	Age	Diagnosis	ISS	FISH	M component class	M protein g/L	% PCs BM	% Eos BM	WBC count	ALC
1	**F**	**M**	**80**	**NDMM**	**I**	**del(17)p**	**IgG lambda**	**5**	**1**	**3.1**	**8.5**	**2.4**
2	**F**	**M**	**79**	**NDMM**	**II**	**Negative**	**IgA kappa**	**27**	**10**	**5.5**	**9.8**	**2.0**
3	**F**	**F**	**72**	**NDMM**	**III**	**Negative**	**FLC lambda**	**4**	**4**	**1.5**	**6.8**	**1.5**
4	**A**	**F**	**72**	**NDMM**	**II**	**Negative**	**IgG kappa**	**63**	**52**	**7.5**	**Not done**	**Not done**
5	**A**	**F**	**61**	**NDMM**	**II**	**Negative**	**IgG kappa**	**87**	**43**	**0.9**	**6.1**	**2.6**
6	**A**	**F**	**56**	**NDMM**	**I**	**Negative**	**FLC lambda**	**2.5**	**9.5**	**1.1**	**8.9**	**3.9**
7	**A**	**M**	**74**	**NDMM**	**I**	**Negative**	**IgA lambda**	**2.5**	**7**	**4.2**	**5.3**	**1.5**
8	**A**	**F**	**70**	**NDMM**	**III**	**Negative**	**FLC kappa**	**0.7**	**12**	**3.5**	**10.1**	**1.6**
9	**A**	**M**	**62**	**NDMM**	**II**	**Negative**	**IgG kappa**	**36**	**13**	**1.3**	**5.7**	**2.9**
10	**F (only PB)**	**M**	**65**	**MM with steroids**	**II**	**Negative**	**IgG lambda**	**26**	**15**		**Not done**	**Not done**
11	**F**	**F**	**62**	**MM with steroids**	**III**	**del(17)p**	**FLC kappa**	**14**	**23**	**3.5**	**8.1**	**2.6**
12	**F**	**F**	**68**	**MM with steroids**	**≥ or II**	**Negative**	**IgG kappa**	**29**	**10**	**4.2**	**5.6**	**0.6**
13	**A**	**F**	**57**	**MM with steroids**	**I**	**Negative**	**IgG kappa**	**18**	**46**	**3.3**	**8.0**	**Not done**
14	**A**	**M**	**73**	**SMM**	**N/A**	**Negative**	**IgA kappa**	**5**	**11**	**2.0**	**4.6**	**1.2**
15	**A**	**M**	**80**	**SMM**	**N/A**	**Negative**	**IgG kappa**	**29**	**27**	**2.0**	**7.5**	**2.2**
16	**A**	**F**	**81**	**SMM**	**N/A**	**Negative**	**IgG kappa**	**29**	**12**	**6.7**	**5.5**	**0.6**
17	**A**	**M**	**79**	**SMM**	**N/A**	**Negative**	**FLC kappa**	**2.8**	**3**	**3.5**	**2.8**	**0.9**
18	**A**	**M**	**74**	**SMM**	**N/A**	**Negative**	**IgA kappa**	**11**	**17**	**1.8**	**Not done**	**Not done**
19	**F**	**M**	**66**	**MGUS**	**N/A**	**Negative**	**IgA kappa**	**14**	**2.5**	**4.7**	**8.6**	**5.2**
20	**F**	**M**	**80**	**MGUS**	**N/A**	**Not done**	**IgA kappa**	**4.8**	**3**	**2.0**	**6.8**	**0.5**
21	**A**	**M**	**73**	**MGUS**	**N/A**	**Negative**	**IgG kappa**	**15**	**8**	**3.1**	**5.4**	**1.1**
22	**A**	**M**	**73**	**MGUS**	**N/A**	**Negative**	**IgG lambda**	**12**	**5**	**0.9**	**10.5**	**3.5**
23	**A**	**F**	**51**	**MGUS**	**N/A**	**Negative**	**IgG lambda**	**12**	**6**	**6.3**	**4.3**	**1.9**
24	**A**	**F**	**71**	**MGUS**	**N/A**	**Negative**	**IgG lambda**	**10**	**4**	**2.2**	**3.5**	**1.5**
25	**A (only BM) only**	**M**	**50**	**MGUS**	**N/A**	**Negative**	**IgG kappa**	**9**	**1**	**4.2**	**Not done**	**Not done**
26	**A**	**F**	**76**	**MGUS**	**N/A**	**Negative**	**IgG kappa**	**14**	**5**	**2.7**	**5.6**	**1.7**
27	**A**	**M**	**79**	**MGUS**	**N/A**	**Negative**	**IgG lambda**	**22**	**2**	**3.6**	**7.3**	**2.5**

Abbreviations: FACS - Flow cytometry instrument used, F: Fortessa, A: Aria Fusion. Diagnosis: NDMM (Newly Diagnosed Multiple Myeloma), MM with steroids (NDMM with initiated treatment with corticosteroids), SMM (Smoldering Multiple Myeloma) and MGUS (Monoclonal Gammopathy of Unknown Significance). ISS (International Staging System) at diagnosis. FISH (Fluorescence In Situ Hybridization) - presence of t (4;14) or t(14;16) translocation, or 17p deletion (del(17) p). %PCs (Plasma Cells) in bone marrow. WBC (white blood cell) count and ALC (Absolute Lymphocyte Count) in 10^9/L.

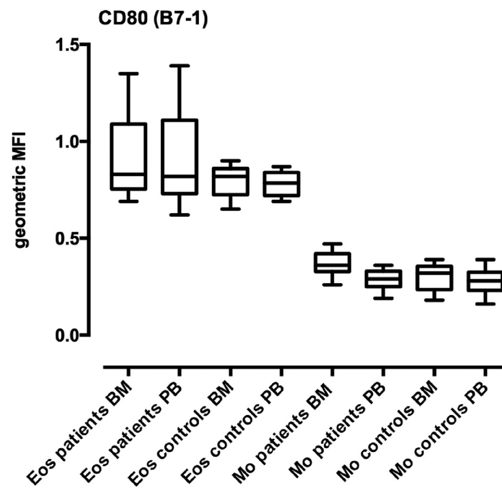
Data on healthy controls

Median age: 22.5 (range: 19–64) years

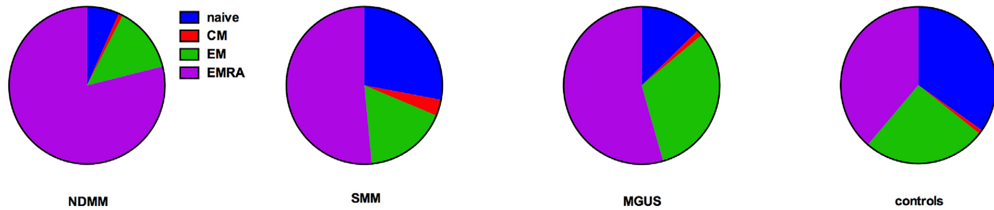
Gender: Male n=7, Female n=12

All control persons reported that they were in good health without any on-going medical treatment. None of them had any obvious symptoms of infection, allergic asthma or atopic dermatitis.

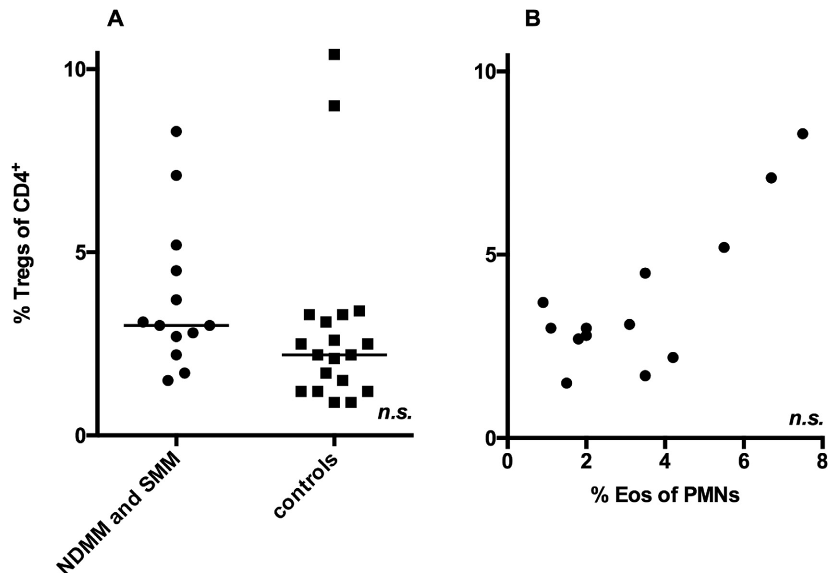
Supplementary results (III)



Supplementary Figure E1. Expression of the surface antigen CD80 (B7-1) on eosinophils (Eos) in comparison with monocytes (Mo) in BM and PB, measured as geometric MFI. Eosinophils in patients with PCD, BM (n=17) and PB (n=15), eosinophils in healthy controls, BM (n=13) and PB (n=12). Monocytes in patients with PCD, BM (n=18) and PB (n=17), monocytes in healthy controls, BM and PB (n=13). Activated monocytes have surface expression of CD80, required for adequate co-stimulatory signals in T cell interactions. Eosinophils in BM and PB from patients, as well as healthy controls, were found to have surface expression of CD80 above average for the monocyte populations in BM and PB.



Supplementary Figure E2. Distribution of CD8$^+$ T cell subsets in BM − median values for naïve/central memory (CM)/effector memory (EM) and effector memory RA+ (EMRA) CD8$^+$ T cells for patients with NDMM (n=8), SMM (n=5), MGUS (n=9) and healthy controls (n=19). Patients with NDMM had significantly lower proportion of naïve CD8$^+$ T cells ($p < 0.0001$) and increased proportion of CD8$^+$ T effector memory RA$^+$ cells (T-EMRA) in BM (*p*: 0.0009), compared to healthy controls. (One patient with total lack of CD4$^+$ T cells was excluded, however, this patient had also a reduced amount of naïve CD8$^+$ T cells). In median, the NDMM patients′ naïve T cells accounted for 6.1% (range: 1.6-18.7) of CD8$^+$ T cells compared to 26.8% (range: 5.8-72.8) in BM-samples from healthy controls, and CD8$^+$ T-EMRA accounted for 71.7% (range: 38.0-86.6) compared to 36.1% (range: 9.8-75.3). This was in accordance with a previous study by Zelle-Rieser on BM samples from NDMM patients [2]. In this material, we analysed BM samples from patients with MGUS as well. They were also shown to have significantly lower levels of naïve CD8$^+$ T cells, median 10.6% (range: 0.3-34.2, *p*: 0.0008), and increased proportion of CD8$^+$ T-EMRA, median 45.2% (range: 30.2-87.9, *p*: 0.01), compared to BM samples from healthy controls. Four group comparisons (NDMM, SMM, MGUS, controls) with Kruskal-Wallis test was significant for difference in proportion of naïve CD8$^+$ T cells (*p*: 0.0001) and CD8$^+$ T-EMRA (*p*: 0.003), but not for CD8$^+$ T central memory (T-CM) and T effector memory (T-EM) cells. The proportion of eosinophils in BM samples from patients with NDMM (n: 8) and SMM (n: 5) correlated with CD8$^+$ T-EM cells and CD8$^+$ T-CM cells (rs: 0.58, *p*: 0.04 and rs: 0.64, *p*: 0.02, data not shown), but each of these cell populations and variations were small, especially for T-CMs, while these data should be interpreted with caution, and there was no correlation between percentage of eosinophils and CD8$^+$ T-EM and T-CM cells when including MGUS patients and healthy controls (*p*: 0.50 and *p*: 0.24, data not shown).



Supplementary Figure E3. Percentage of Tregs in BM and correlation between percentage of Tregs and percentage of eosinophils (Eos) in BM – (A) Percentage of Tregs of $CD4^+$ T cells in BM for patients with multiple myeloma, i.e. NDMM (n=8) and SMM (n=5), compared to healthy controls (n=19). (B) Correlation between percentage of Tregs of $CD4^+$ T cells in BM with percentage of BM eosinophils of PMNs for patients with NDMM (n=8) and SMM (n=5). We could not show any significant increase in percentage of regulatory T cells (Tregs) in BM samples (A) and no significant correlation between percentage of BM eosinophils and percentage of Tregs in patients with NDMM and SMM (B) or healthy controls (data not shown). Two controls were found to have an increased amount of Tregs, 9.0 and 10.4% respectively of $CD4^+$ T cells in BM, and both had very low proportions of BM eosinophils, 0.1 and 0.2%. The distribution of $CD4^+$ T cell subsets; naïve/CM/EM/EMRA, and further subdivision of T-CMs and T-EMs in Th1/Th2/Th17, did not differ between patients and controls (data not shown). Increased eosinophils in the BM could not be correlated to Th2 or Th17 polarization of central memory or effector memory T helper cells (data not shown).

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Paper IV

Impaired phagocyte function in patients with newly diagnosed multiple myeloma

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CONFLICTS OF INTERESTS: None

Abstract

In multiple myeloma (MM), a characteristic feature is suppression of adaptive immune responses and most distinctive, reduced levels of normal polyclonal immunoglobulins. Recurrent and severe bacterial infections are common and a major cause of mortality. In this study we show that neutrophil dysfunction might be an additional factor contributing to the increased susceptibility to bacterial infections in patients with plasma cell disorders, and this may occur in early disease stages. Polymorphonuclear leukocytes (PMNs) from newly diagnosed patients with MM, smoldering MM and monoclonal gammopathy of undetermined significance (MGUS) had reduced phagocytic capacity. The dysfunction was most prominent for PMNs in bone marrow samples. On the contrary, myeloma patients in stable remission and on-going treatment with lenalidomide were found to have neutrophils with normal, restored capacity for phagocytosis and oxidative burst. The compromised neutrophil function in these *ex vivo* assays could not be explained by hypogammaglobulinemia, since opsonized *E. coli* was used as stimuli. In this material, decreased phagocyte function could not be correlated to plasma calcium levels, inflammatory activity or degree of BM plasma cell infiltration.

Introduction

Multiple myeloma (MM) is the second most common malignancy of the blood, with an incidence of about 6 to 7 per 100 000 per year in Sweden [1]. The disease is characterized by an uncontrolled growth of an abnormal malignant plasma cell clone in the bone marrow (BM), producing monoclonal immunoglobulins that can be detected as a paraprotein in serum ("M component"). MM arise from a premalignant disease, monoclonal gammopathy of undetermined significance (MGUS), which is a common condition with a prevalence of 2 to 3% in the Swedish population over the age of 50 years [2]. In contrast to MGUS, which is without symptoms, MM is clinically characterized by anemia, lytic bone destructions and renal failure [3]. Patients that fulfil the diagnostic criteria for MM, but are without symptoms and not treatment demanding, are defined as smoldering MM (SMM) [3].

Immune dysfunction, with reduced levels of polyclonal immunoglobulin is a characteristic feature of active MM, and infections constitutes a major cause of morbidity and mortality [4]. In a large population-based study of over 9000 Swedish MM patients, Blimark et al. found that MM patients had a sevenfold increase in the risk for developing bacterial infections compared with age-matched controls [5]. The risk for bacterial infections was even more pronounced during the first year following diagnosis, reaching 11-fold, and was the cause of death in 22% of the cases [5].

Augustson et al. showed the same trend in UK MM patients, with a 10% mortality during the first 60 days of induction therapy, whereof bacterial infection was the direct cause of death in 45% of cases and contributed to death in 50% [6]. Only 8% of patients dying of infections had neutrophils less than 2.0 x 10^9/L [6]. Sørrig et al. found a correlation between aggressive disease at presentation and the highest risk of developing blood stream infections [7]. Authors also implied that MM is diagnosed in relation to a serious infection for a large number of patients [7]. Patients with MGUS had a twofold increase in the risk for bacterial infections [8, 9].

Activated phagocytes, neutrophils and macrophages, are essential in first line defence against microbes. They recognize pathogen-associated molecular patterns (PAMPs) on foreign particles and eliminate pathogenic bacteria and fungi by the process of phagocytosis. Antibodies or complement (i.e. "opsonins") attached to the surface of microbes strengthens the binding to the phagocyte, and increase the efficacy of the process [10]. Bound microbes are internalized into the phagocytes′ cytoplasm in membrane-bound vesicles, phagosomes [11]. In the cytoplasm, phagosome vesicles fuse with lysosomes and the microbes are destroyed by reactive oxygen species (ROS), nitrogen species, and proteolytic enzymes inside the phagolysosomes [12].

We have previously shown that neutrophils from MM patients, in at least partial remission on induction therapy, had reduced capacity for phagocytosis [13]. The phagocytic functions was further affected in newly regenerated neutrophils after autologous stem cell transplantation [13].

The aim of this study was to examine neutrophil function in peripheral blood (PB) and BM samples from patients with untreated, newly diagnosed MM (NDMM), SMM and MGUS, compared with healthy controls and compared with treated MM patients in stable remission.

Patients and Methods

Patients and controls

The study protocol, patient information and consent form were reviewed and approved by an Independent Ethics Committee, The Regional Ethical Review Board in Lund, Sweden (www.epn.se), Ref No 2016/768. PB and BM aspirates were collected from patients with MGUS, SMM and MM at diagnosis and compared with samples from MM patients in stable remission on treatment with lenalidomide, and with samples from healthy controls.

Phagocytosis and oxidative burst

Peripheral blood (PB) samples collected in heparin tubes, and bone marrow (BM) aspirates collected in tubes with the culture medium RPMI 1640 were stored overnight. Polymorphonuclear leukocytes (PMNs) capacity for phagocytosis and production of ROS was investigated within 24 hours of sampling using the PhagoTest respectively PhagoBurst assays (Glycotope Biotechnology, GmBH, Germany), according to the manufacturer's protocols. These flow cytometry-based methods measure the percentage of PMNs having ingested fluorescein-labelled opsonized *Escherichia coli* (*E. coli*) and number of cells that have produced ROS after *ex vivo* activation with phorbole-12-myristate-13-acetate (PMA) or opsonized *E. coli*. Mean fluorescence intensity (MFI) correlates in the PhagoTest assay to number of ingested bacteria per cell and for the PhagoBurst assay to degree of ROS production in response on the different stimuli. The flow cytometer FACS Fortessa was used for the first 3.5 months and thereafter we used FACS Canto II. DIVA software was used for data collection and Kaluza software (Beckman Coulter, Brea, CA) for analysis.

Statistical analysis

The Kruskal-Wallis test was used for > two group comparisons, followed by Dunn´s multiple comparisons test. The Mann-Whitney U test was used for two-group comparisons. Correlation was assessed by Spearman´s rank correlation test. Statistical calculations were performed using GraphPad PRISM version 6.0a software. $p < 0.05$ was considered significant.

Results

Patients characteristics

From February 2017 until June 2018, 11 patients with NDMM, seven patients with SMM and 14 patients with MGUS were included at the Department of Hematology, Skåne University Hospital in Lund, Sweden (Table 1). During the same period, four MM patients in complete remission (CR) on lenalidomide maintenance therapy (10 mg daily), were included, as well as five patients in stable remission (three in stringent CR and two patients in good stable partial remission (PR) on lenalidomide-based treatment, and below we have named this group of nine patients "Len maintenance" (Table 1). For comparison, BM samples from 20 healthy controls were collected. In two cases, the PhagoBurst assay failed for control samples and in two other cases for the PhagoTest assay. None of the patients or healthy controls had any sign of on-going infection at the time-point of sampling. For the first 3.5 months we used the flow

cytometry instrument FACS Fortessa, after that we switched to use a FACS Canto II. Since this potentially could affect the MFI values, the first three patients with NDMM, two MGUS patients and four (PhagoTest) respectively five (PhagoBurst) healthy controls, were excluded from that part of the analysis (Table 1).

Compromised neutrophil function in patients with newly diagnosed plasma cell disorders

Several of the patients with NDMM, SMM and MGUS, had circulating PMNs with reduced capacity for phagocytosis, measured as geometric MFI corresponding to number of phagocytized bacteria per cell, especially compared with the "Len maintenance" cohort (Dunn´s multiple comparisons test ($p < 0.01$) Fig. 1B). Using the Mann-Whitney U test, there was a reduced phagocytic capacity also compared with healthy controls (Mann-Whitney U test ($p < 0.05$), Fig. 1B). A reduced proportion of PB PMNs from newly diagnosed patients produced ROS on activation with opsonized *E. coli*, compared with patients on lenalidomide therapy (Dunn´s test ($p < 0.01$), Fig. 1A).

The reduced phagocytic functioning in newly diagnosed patients was evident in the comparison of BM PMNs (Fig. 2). Patients with NDMM, SMM and MGUS had reduced percentage of BM PMNs with capacity to phagocytose opsonized *E. coli* bacteria compared with healthy controls (Dunn´s test ($p < 0.05$), Fig. 2A) and compared with the "Len maintenance" cohort (Dunn´s test ($p < 0.001$), Fig. 2A). A reduced percentage of BM PMNs from newly diagnosed patients produced ROS on stimulation with *E. coli*, compared with healthy controls (Dunn´s test ($p < 0.01$), Fig. 2A) and compared with the "Len maintenance" cohort (Dunn´s test ($p < 0.01$), Fig. 2A). Number of phagocytized bacteria per cell was reduced for BM PMNs from newly diagnosed patients compared with the "Len maintenance" patient cohort (Dunn´s test ($p < 0.01$), Fig. 2B) and degree of ROS production on stimulation with *E. Coli* was also reduced for BM PMNs from the newly diagnosed patients compared with healthy controls (Dunn´s test ($p < 0.05$), Fig. 2B) and the "Len maintenance" cohort (Dunn´s test ($p < 0.01$), Fig. 2B).

Analysis with further subdivision of patients according to disease stage at diagnosis showed that both patients with NDMM, and patients with SMM, had significantly reduced percentage of BM PMNs with capacity for phagocytosis and oxidative burst on stimulation with *E. Coli*, compared with the "Len maintenance" cohort (Dunn´s test ($p < 0.01$ and $p < 0.05$, respectively), Fig. 3). Using the Mann-Whitney U test, both patients with NDMM, and patients with SMM, had reduced percentage of BM PMNs with capacity for phagocytosis and ROS production, compared with healthy controls (p: 0.005 and p: 0.008, respectively for phagocytosis, and p: 0.005 and p: 0.006, respectively for PhagoBurst, Fig. 3). Seven out of 11 patients with NDMM,

four out of seven with SMM and five out of 14 with MGUS had less than 20% of BM PMNs with capacity for phagocytosis (Fig. 3). MM patients in stable remission and on-going treatment with lenalidomide, were shown to have PMNs with normal, restored capacity for phagocytosis and ROS production (Fig. 1, 2 and 3).

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 that could affect phagocyte function. On the other hand, the lenalidomide treated patient cohort, with restored phagocyte function, did all receive regular bisphosphonate infusions and were supposed to have lower plasma calcium (P-Ca) levels. In this material, three NDMM patients had a calculated corrected P-Ca level slightly above the normal range, but there was no significant difference in P-Ca, plasma albumin (P-Alb), or calculated corrected P-Ca levels for the different patient cohorts (Table 1, Fig. 4A-C) and no correlation could be shown for these parameters and BM PMNs capacity for phagocytosis or oxidative burst (n= 41, data not shown). In this study, neutrophil dysfunction could not be explained by increased inflammatory activity. For acute phase reactants, we could only detect a small, but significant differences in P-Orosomucoid levels between patients with NDMM and SMM (Dunn´s test ($p < 0.05$), Mann-Whitney U test (p: 0.02), Fig. 4F). However, none of the patients had any apparent inflammatory activity as measured by conventional parameters (Table 1, Fig. 4), and for each parameter; CRP, P-α-1-antitrypsin, P-Orosomucoid and P-Haptoglobin, there was no significant correlation to BM PMNs capacity for phagocytosis or oxidative burst (n=41, data not shown).

For untreated patients; NDMM, SMM and MGUS, there was no correlation between percentage of plasma cell infiltration and capacity for phagocytosis (n=32, data not shown).

Discussion

These data indicate that neutrophil dysfunction is common in patients with NDMM and may occur already at the premalignant stage MGUS. Phagocytosis and oxidative burst are complex, multistep procedures and the mechanisms that directly and/or indirectly inhibit the phagocyte function in MM are incompletely understood and with certainty multifactorial [10-12]. BM phagocytes were most affected. One can speculate if this reflects an increased proportion of PMN myeloid-derived suppressor cells (PMN-MDSCs) included in these unmanipulated BM samples. Flow cytometry cannot discriminate neutrophils from PMN-MDSCs. The only method available to separate

MDSCs from mature normal neutrophils is by Ficoll gradient centrifugation, where PMN-MDSCs enrich in the mononuclear low-density fraction [14]. MDSCs, mainly PMN-MDSCs, are described to accumulate in the BM of patients and animals with MM [15-19]. These functionally altered immature myeloid cells have direct and indirect immunosuppressive properties and might have reduced phagocytic capacity [20, 21]. Overexpression of IL-10 by malignant plasma cells and M2 macrophages might also be a contributing factor to the observed dysfunction of BM PMNs in untreated MM [22]. Normal and malignant PCs produce IL-10, and this cytokine was detectable in sera from patients with advanced MM [23]. The predominant variant of BM macrophages in MM are of immunosuppressive M2 phenotype and secrete IL-10 [24]. In a murine model of autoimmune disease, IL-10 directly inhibited neutrophil migration toward the anaphylatoxin C5a and IL-10 blockade could reduce the incidence of bacterial airway infections in a murine model of advanced MM [23].

The compromised neutrophil function in these assays could not be explained by lack of immunoglobulins (hypogammaglobulinemia), since opsonized *E. coli*, with immunoglobulin and complement from pooled sera, was used as stimuli. Moreover, in this material, the decreased phagocyte function at diagnosis could not be correlated to variation in P-Ca levels, inflammatory activity *or* degree of BM plasma cell infiltration.

Interestingly, we also found that MM patients in stable remission and on-going treatment with lenalidomide-based therapy had neutrophils with normal, fully restored capacity for phagocytosis and ROS production. If this merely is a result of effective plasma cell eradication or if the treatment itself could further improve these cellular functions remains to be elucidated, as well as if the phagocytic improvement also applies to monocytes-macrophages. Thalidomide, and its synthetically modified and more potent analogs; lenalidomide and pomalidomide, are immunomodulatory drugs (IMiDs) with broad anti-myeloma activity [25]. These compounds have contributed to significantly better outcomes for patients with MM [26]. In a previous experimental study on human MM cells co-cultured with bone marrow stromal cells and MDSCs, treatment with lenalidomide did not alter MDSCs frequency or suppressive activity [17]. In an *ex-vivo* study on human monocytes and T cells, pomalidomide was found to enhance the formation of actin filaments, induce changes in actin and microtubule cytoskeleton and to stimulate monocyte migration by activation of the Rho GTPase RhoA [27]. This effect was achieved in minutes after drug exposure, without any co-stimulation. Lenalidomide have been shown to restore defect T cell GTPase activation induced by chronic lymphocytic leukemia (CLL) cells [28] and to enhance actin remodelling at natural killer (NK) cell immune synapses [29]. Cytoskeletal remodelling is an essential part of the phagocytic process, and if lenalidomide enhance the formation of actin filaments, this might be one explanation for the improved phagocytic capacity observed in lenalidomide treated patients.

There is an increased risk of severe bacterial infections in MM [5, 6]. Reduced levels of polyclonal immunoglobulins are a consistent feature of active disease, but numerous other defects of the immune system have been described including B- and T-cell dysfunctions and defective NK cell function [4, 30]. In this study we show that neutrophil dysfunction might be an additional factor contributing to the increased susceptibility to bacterial infections in patients with newly diagnosed plasma cell disorders. Further studies are needed to clarify the underlying causes.

Acknowledgments

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Authorship and Disclosures

Laboratory work was conducted by ÅP and ÅJ. SW, SA and MH recruited the patients. SW carried out the FACS analysis, compiled the data and drafted the manuscript. ÅJ, MH, TH, SA and SW contributed to the design of the study and writing up. All authors approved the final manuscript. The authors report no potential conflicts of interest.

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Table 1 - Patient characteristics

No.	Diagnosis	F/C	Gender	Age	M component class	M protein g/L	% BMPCs	WBC count	CRP	Albumin	Ca^{2+}	Ca^{corr}	α-1AT	Orosomucoid	Haptoglobin
1	NDMM	F	M	80	IgG lambda	5	1	8.5	6.2	34	2.3	2.4	1.4	1.2	2.8
2	NDMM	F	M	79	IgA kappa	27	10	9.8	2.4	38	2.5	2.5	1.7	1.2	3.3
3	NDMM	F	F	72	FLC lambda	4	4	6.8	2.3	40	2.4	2.4	1.5	1.2	1.9
4	NDMM	C	F	72	IgG kappa	63	52	-	0.6	31	2.4	2.6	0.9	0.9	1.5
5	NDMM	C	F	61	IgG kappa	87	43	6.1	0.6	41	2.4	2.4	1.1	0.3	<0.1
6	NDMM	C	F	56	FLC lambda	2.5	9.5	5.3	4.6	46	2.6	2.5	1.5	1.1	1.3
7	NDMM	C	M	74	IgA lambda	2.5	7	5.3	0.7	44	2.7	2.6	1.3	0.8	1.9
8	NDMM	C	F	70	FLC kappa	0.7	12	10.1	1.0	42	2.3	2.3	1.2	1.4	2.5
9	NDMM	C	M	62	IgG kappa	36	13	5.7	0.6	34	2.2	2.3	1.1	0.6	1.1
10	NDMM	C	F	85	IgG lambda	28	20	7.7	5.4	31	2.3	2.5	1.6	1.2	1.4
11	NDMM	C	M	70	IgG lambda	59	50	8.7	8.0	32	2.5	2.7	1.3	1.1	1.9
12	SMM	C	M	73	IgA kappa	5	11	4.6	1.3	42	2.4	2.4	1.1	0.6	0.8
13	SMM	C	M	80	IgG kappa	29	27	7.5	1.6	41	2.3	2.3	1.7	0.5	1.8
14	SMM	C	F	81	IgG kappa	29	12	5.5	1.6	36	2.3	2.4	1.4	0.6	1.4
15	SMM	C	M	79	FLC kappa	2.8	3	2.8	0.9	40	2.3	2.3	1.2	0.4	0.1
16	SMM	C	M	74	IgA kappa	11	17	-	0.6	40	2.4	2.4	1.0	0.9	1.7
17	SMM	C	M	77	FLC kappa	0.6	13	4.2	1.6	42	2.5	2.5	1.3	0.6	1.0
18	SMM	C	F	77	IgG lambda	41	23	7.4	3.6	33	2.4	2.5	1.1	0.8	1.5
19	MGUS	F	M	66	IgA kappa	14	2.5	8.6	4.5	36	2.4	2.5	1.5	1.0	0.8
20	MGUS	F	M	80	IgA kappa	4.8	3	6.8	12	34	1.2	1.3	1.7	1.0	<0.1
21	MGUS	C	M	73	IgG kappa	15	8	5.4	3.0	40	2.3	2.3	1.4	1.0	1.6
22	MGUS	C	M	73	IgG lambda	12	5	10.5	1.8	35	2.3	2.4	1.1	0.6	1.0

23	MGUS	C	F	51	IgG lambda	12	6	4.3	4.9	40	2.3	2.3	1.3	0.9	1.6
24	MGUS	C	F	71	IgG lambda	10	4	3.5	2.0	39	2.4	2.4	1.8	0.5	0.6
25	MGUS (only BM)	C	M	50	IgG kappa	9	1	-	2.2	41	2.2	2.2	1.1	0.7	1.8
26	MGUS	C	F	76	IgG kappa	14	5	5.6	0.8	42	2.3	2.3	1.3	0.7	0.8
27	MGUS	C	M	79	IgG lambda	22	2	7.3	2.3	44	2.4	2.3	1.5	0.5	1.1
28	MGUS	C	M	75	IgG lambda	15	2	5.4	2.6	34	2.2	2.3	1.2	1.0	1.3
29	MGUS (only BM)	C	M	73	IgG lambda	8	9	7.3	1.1	45	2.6	2.5	1.3	0.7	2.0
30	MGUS	C	M	72	IgA lambda	11	7	9.0	9.6	36	2.3	2.4	2.0	0.9	0.5
31	MGUS	C	M	69	IgA lambda	6.1	3	-	2.4	23	2.2	2.5	1.5	0.6	4.0
32	MGUS	C	F	78	IgG kappa	13	7	4.2	0.9	38	2.3	2.3	1.2	0.5	0.2
33	Len maintenance	C	M	70	IgG kappa	CR1	sCR	3.5	0.7	36	2.3	2.4	1.2	0.6	1.1
34	Len maintenance	C	F	69	IgG kappa	CR1	sCR	3.6	0.6	41	2.5	2.5	1.6	0.9	1.1
35	Len maintenance	C	F	50	FLC kappa	CR1	sCR	-	0.6	41	2.5	2.5	1.0	0.8	0.8
36	Len maintenance	C	M	42	IgG kappa	CR1	sCR	-	0.6	42	2.4	2.4	1.3	0.7	0.1
37	Dara/Len	C	F	75	IgG lambda	CR1	sCR	4.6	11	32	2.2	2.4	1.4	1.2	2.3
38	Len/Dex +/- Ixa #52	C	M	70	IgG kappa	PR2	0.5	2.1	1.3	36	2.1	2.2	1.1	0.5	1.0
39	Len/Dex#19	C	M	74	FLC lambda	PR1	2	4.7	11	32	2.2	2.4	1.5	1.0	2.6
40	Len/Dex +/- Ixa #63	C	F	75	IgG kappa	CR2	sCR	2.4	1.9	34	2.2	2.3	1.4	0.7	0.5
41	Len/Dex +/- Ixa #69	C	F	64	IgG kappa	CR2	sCR	-	1.3	35	2.2	2.3	1.1	0.6	1.5

Abbreviations: F/C- Flow cytometry instrument used, F: Fortessa, C: Canto II. Diagnosis: NDMM (Newly Diagnosed Multiple Myeloma), SMM (Smoldering Multiple Myeloma) and MGUS (Monoclonal Gammopathy of Undetermined Significance). Pat no 33 to 36 (Len maintenance) were all in first stringent complete remission (sCR) and on treatment with Lenalidomide 10 mg daily. Pat no 37 to 41 were all on treatment with Lenalidomide (Len) 25 mg day 1-21 (28 day cycles) in combination with Daratumumab (n=1), Dexamethasone (Dex) (n=1), or Dexamethasone + Ixazomib (Ixa) or placebo (n=3). % BMPCs (% Plasma Cells in Bone Marrow), WBC count (White Blood Cell count, normal range (3.5-8.8)) in 10^9/L, CRP (C-reactive protein, (<3)) in mg/L, Albumin (Albumin concentration in plasma, (34-45)) in g/L, Ca (Calcium concentration in plasma, (2.2-2.5)) in mmol/L. Formula used to calculate a "corrected" P-Ca^{corr} in mmol/L: P-Ca + 0.02 x (40 – P-Alb). α-1AT (α-1-antitrypsin in plasma, (0.9-1.8)) in g/L. Orosomucoid (0.5-1.2) and Haptoglobin (0.2-1.9) in plasma in g/L. CR (Complete Remission) and PR (Partial Remission).

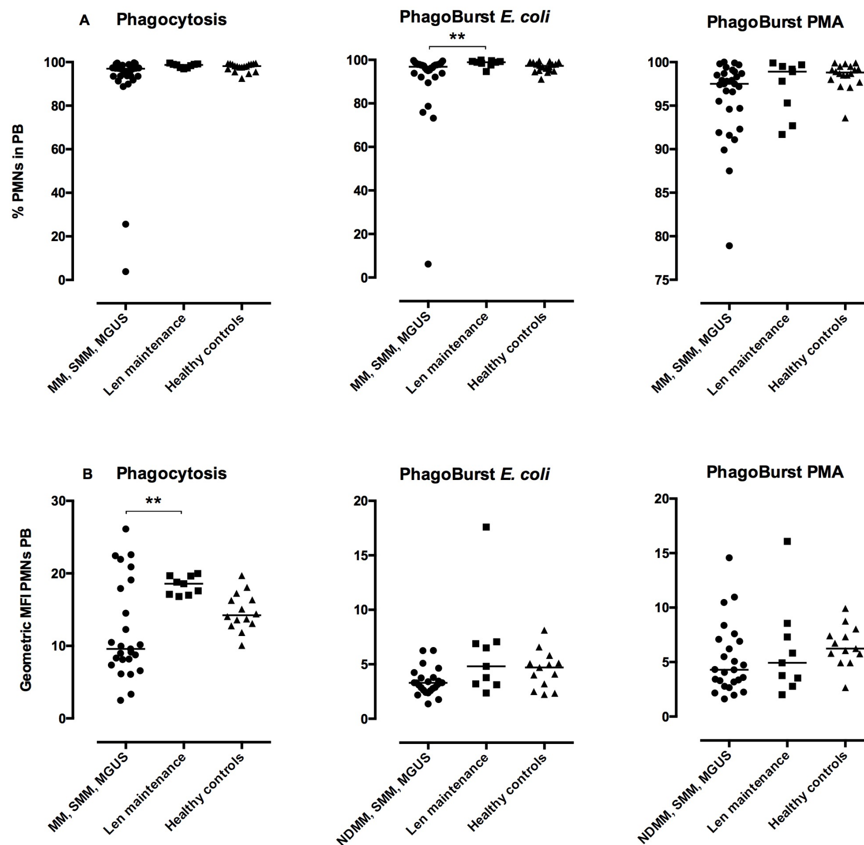

Figure 1 – Capacity of PB PMNs for phagocytosis and oxidative burst. In (A), neutrophil functions were measured as the percentage of PMNs having ingested fluorescein-labelled opsonized *E. coli*, and percentage of PMNs, which have produced reactive oxidants on stimulation with opsonized *E. coli* and PMA. NDMM, SMM, MGUS (n=30), "Len maintenance" (n=9), and healthy controls (n=18). For Phagocytosis, Kruskal-Wallis test showed significant difference (*p*: 0.03). The Mann-Whitney U test was significant for difference between the untreated patient cohort and the "Len maintenance" cohort (*p*: 0.02), but not for the untreated patients compared with healthy controls (*p*: 0.0526, *N.S.*). For PhagoBurst on stimulation with *E.Coli*, Kruskal-Wallis test showed significant difference (*p*: 0.008) and Dunn´s test was significant for difference between the untreated patient cohort compared with the "Len maintenance" cohort ($p < 0.01$)**. Kruskal-Wallis test showed no significant difference between the cohorts for PhagoBurst on stimulation with PMA (*p*: 0.0511, *N.S.*). In (B), geometric MFI corresponded to number of ingested bacteria per cell for phagocytosis and degree of ROS production on stimulation with *E. coli* and PMA. NDMM, SMM, MGUS (n=25), "Len maintenance" (n=9), and healthy controls (n=14 for Phagocytosis and n=13 for the PhagoBurst assays). For Phagocytosis, Kruskal-Wallis test showed significant difference (*p*: 0.08) and Dunn´s test was significant for difference between the untreated patient cohort compared with the "Len maintenance" cohort ($p < 0.01$)**. The Mann-Whitney U test was significant for difference between the untreated patient cohort and the "Len maintenance" cohort (*p*: 0.02) and for untreated patients compared with healthy controls (*p*: 0.047). For PhagoBurst on stimulation with *E.Coli*, Kruskal-Wallis test showed significant difference (*p*: 0.04). Mann-Whitney U test was significant for difference between the untreated patient cohort and the "Len maintenance" cohort (*p*: 0.03), but not for the untreated patients compared with healthy controls (*p*: 0.055, *N.S.*). Kruskal-Wallis test showed no significant difference between the cohorts for PhagoBurst on stimulation with PMA. Horizontal lines represents the median values.

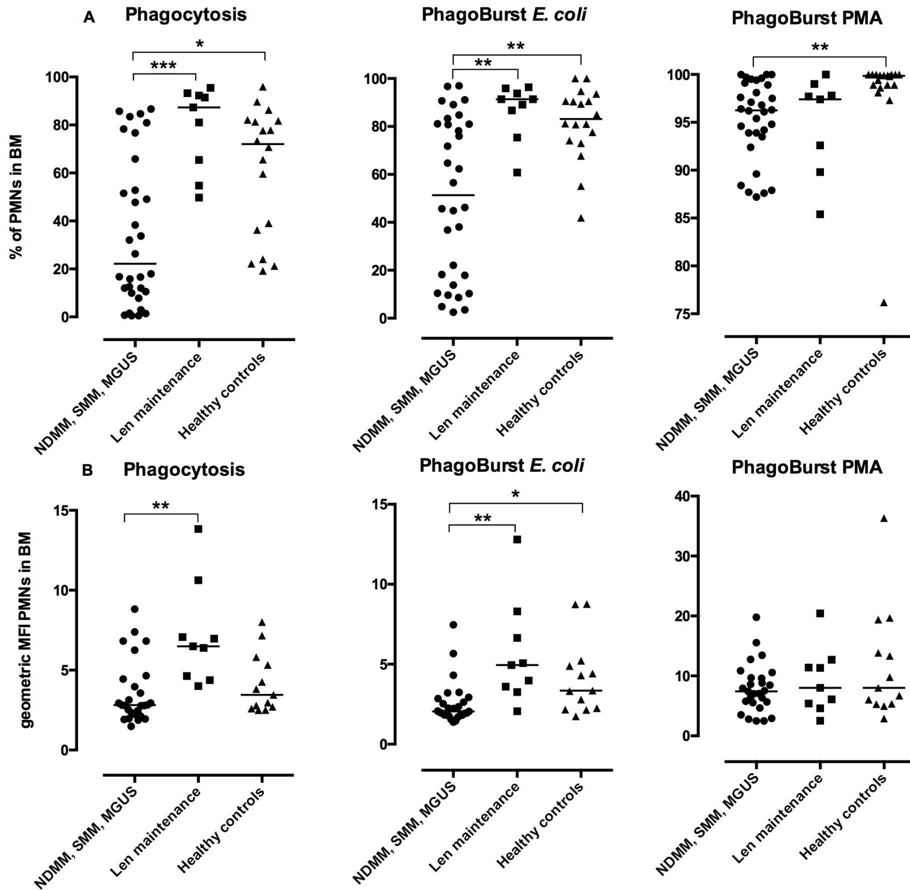

Figure 2 – Capacity of BM PMNs for phagocytosis and oxidative burst. In (A), neutrophil functions were measured as the percentage of PMNs having ingested fluorescein-labelled opsonized *E. coli*, and percentage of PMNs, which have produced reactive oxidants on stimulation with opsonized *E. coli* and PMA. NDMM, SMM, MGUS (n=32), "Len maintenance" (n=9), and healthy controls (n=18). For Phagocytosis, Kruskal-Wallis test showed significant difference (*p*: 0.0002) and Dunn´s multiple comparisons test was significant for difference between the untreated patient cohort compared with both healthy controls ($p < 0.05$)* and the "Len maintenance" cohort ($p < 0.001$)***. For PhagoBurst on stimulation with *E.Coli*, Kruskal-Wallis test showed significant difference (*p*: 0.0004) and Dunn´s test was significant for difference between the untreated patient cohort, compared with both healthy controls ($p < 0.01$)**and the "Len maintenance" cohort ($p < 0.01$)**. For PhagoBurst on stimulation with PMA, Kruskal-Wallis test showed significant difference (*p*: 0.001) and Dunn´s test was significant for difference between the untreated patient cohort and healthy controls ($p < 0.01$)**. In (B), geometric MFI corresponded to number of ingested bacteria per cell for phagocytosis and degree of ROS production on stimulation with *E. coli* and PMA. NDMM, SMM, MGUS (n=27), "Len maintenance" (n=9), and healthy controls (n=13). For Phagocytosis, Kruskal-Wallis test showed significant difference (*p*: 0.025) and Dunn´s test was significant for difference between the untreated patient cohort compared with the "Len maintenance" cohort ($p < 0.01$)**. For PhagoBurst on stimulation with *E.Coli*, Kruskal-Wallis test showed significant difference (*p*: 0.0004) and Dunn´s test was significant for difference between the untreated patient cohort, compared with both healthy controls ($p < 0.05$)* and the "Len maintenance" cohort ($p < 0.01$)**. Kruskal-Wallis test showed no significant difference between the cohorts for PhagoBurst on stimulation with PMA. Horizontal lines represents the median values.

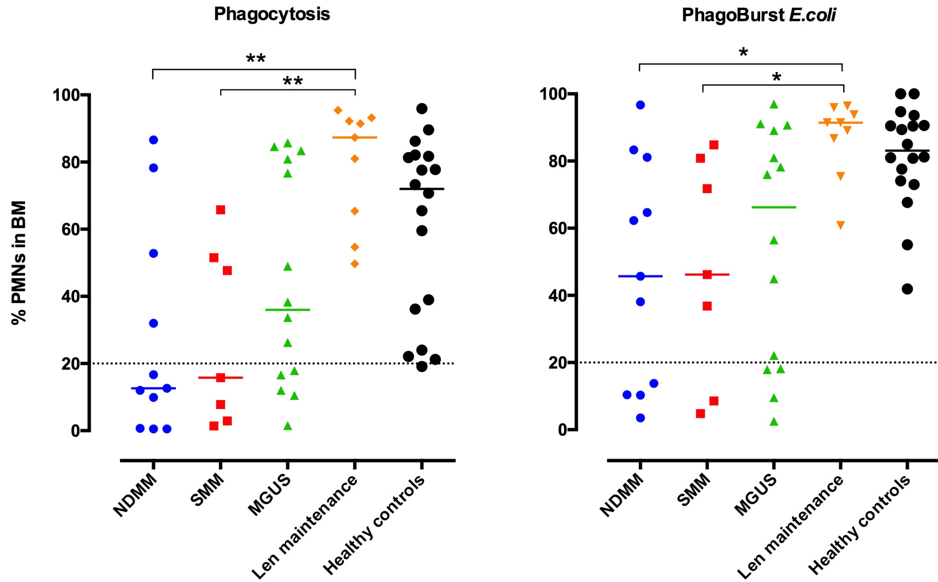

Figure 3 – Capacity of BM PMNs for phagocytosis and oxidative burst, according to disease stage and treatment, and compared with healthy controls. Phagocytosis was measured as the percentage of PMNs having ingested fluorescein-labelled opsonized *E. coli* and oxidative burst as percentage of PMNs, which have produced reactive oxidants on stimulation with opsonized *E. coli*. NDMM (n=11), SMM (n=7), MGUS (n=14), "Len maintenance" (n=9), and healthy controls (n=18). For Phagocytosis, Kruskal-Wallis test showed significant difference (*p*: 0.0004) and Dunn´s test was significant for difference between NDMM and the "Len maintenance" cohort (*p* <0.01)** and for SMM patients compared with the "Len maintenance" cohort (*p* <0.01)**. The Mann-Whitney U test was significant for difference between NDMM patients and healthy controls (*p*: 0.005), and SMM patients and healthy controls as well (*p*: 0.008). The Mann-Whitney U test was significant for difference between MGUS patients compared with the "Len maintenance" cohort (*p*: 0.003), but not compared with healthy controls. For PhagoBurst, Kruskal-Wallis test showed significant difference (*p*: 0.0003) and Dunn´s test was significant for difference between NDMM and the "Len maintenance" cohort (*p* <0.05)* and for SMM patients compared with the "Len maintenance" cohort (*p* <0.05)*. The Mann-Whitney U test was significant for difference between NDMM patients and healthy controls (*p*: 0.005), and SMM patients and healthy controls as well (*p*: 0.006). The Mann-Whitney U test was significant for difference between MGUS patients compared with the "Len maintenance" cohort (*p*: 0.02), but not compared with healthy controls (*p*: 0.055, *N.S.*). BM PMNs from patients in stable remission on long-term lenalidomide treatment were here shown to have normal, restored capacity for phagocytosis and oxidative burst. Horizontal solid lines represents the median values.

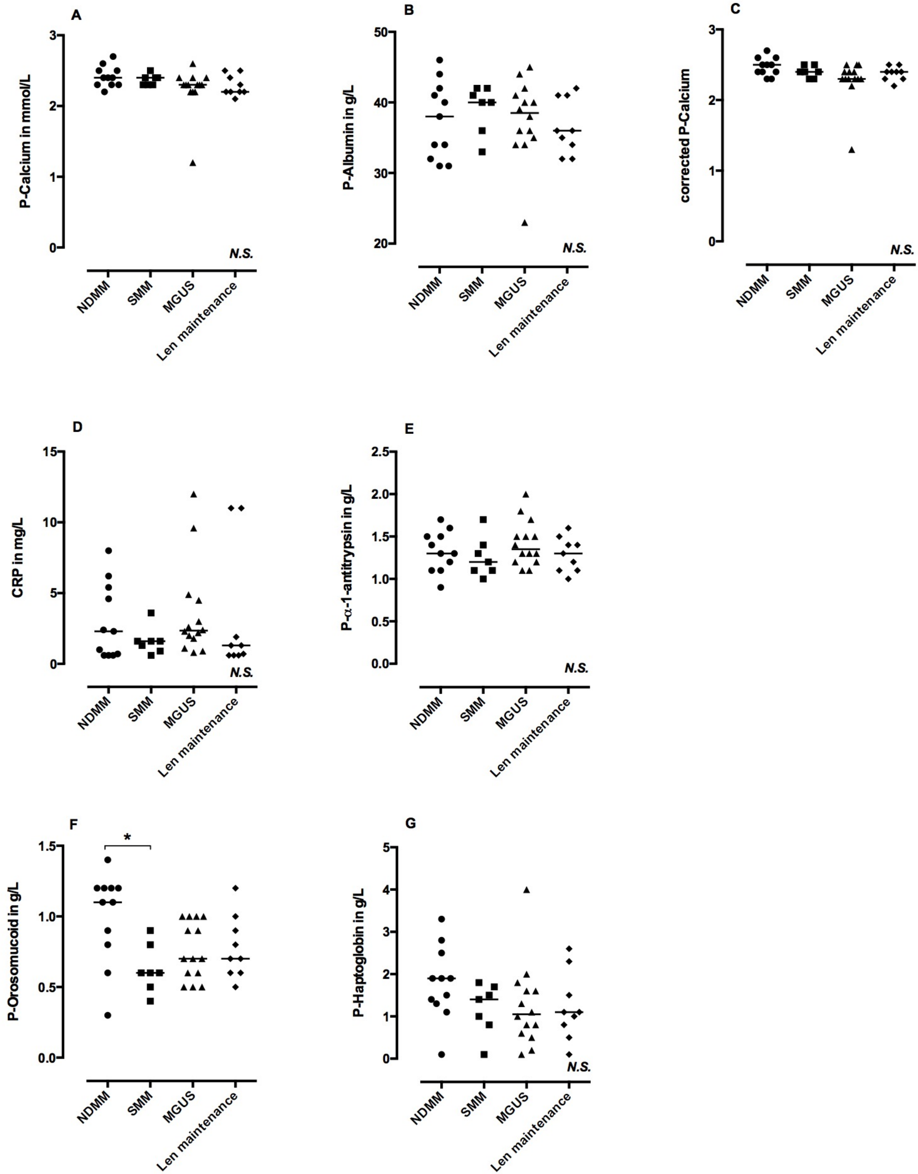

Figure 4 – Plasma levels of calcium, albumin and acute phase reactant proteins for the different patient cohorts. NDMM (n=11), SMM (n=7), MGUS (n=14) and “Len maintenance” (n=9). *p*-values for Kruskal-Wallis test was not significant for most parameters, with the exception of P-Orosomucoid (*p*: 0.0276) and subsequent Dunn´s multiple comparisons test was significant for difference in P-Orosomucoid levels between NDMM and SMM patients ($p < 0.05$)*. Horizontal lines represents the median values.

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