Aus der medizinischen Klinik mit Schwerpunkt Rheumatologie und klinische Immunologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Influence of megakaryocytes and megakaryocytopoiesis on plasma cell survival

Zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Nam Matthias Ly

aus *Herten*

Datum der Promotion: 02.03.2018

For Margo and Michelle

Living is easy with eyes closed
Misunderstanding all you see
It's getting hard to be someone
But it all works out
It doesn't matter much to me

John Lennon – Strawberry Fields Forever

Abstract

Introduction: Autoimmune diseases like SLE are associated with pathogenic antibodies and malignant neoplasms such as multiple myeloma with abnormal PCs. In both cases, pathogenesis can be related to LLPCs, which require a specific microenvironment composed of stromal cells, haematopoietic stem cells and cytokines. MKs constitute an important niche component as major sources of APRIL and IL-6. TPO is the crucial growth factor for MKs and c-mpl, its receptor. Thus, alterations in megakaryopoiesis can change survival conditions for LLPCs in the niche. Our group found an SNP in the crucial TPO binding domain of c-mpl in SLE-prone mice, and the first part of this thesis aimed to find evidence for a similar mutation in SLE patients. The second part evaluated the effect of selected HNCs in co-culture with murine MM cells.

Methods: Genomic DNA from 40 SLE patients and 15 healthy donors was submitted for gene sequencing after target DNA was amplified. Cell lysates of megakaryocytes from SLE-prone mice were examined with ELISA for increased Akt phosphorylation downstream of c-mpl. The second part involved co-culture of a murine MM cell line with selected primary HNCs. After determining optimal cell confluence at which MOPCs depend on external growth factors, the number of MKs available for seeding was improved by combination of BM culture, BSA density gradient, staining of surface markers and manual cell separation using magnetic immunobeads.

Results: There was no evidence for a corresponding SNP in samples extracted from SLE patients, not sufficient proof for increased activation of Akt downstream signalling. Co-culture of megakaryocytes demonstrated that MKs support growth and survival of MOPC during nutrient deprivation. The same effect was observed for granulocytes, however not to the same extent. It was also confirmed that the expression profile of CD138 is a potential marker of very early apoptosis.

Discussion: Results from the co-culture experiments confirm that MKs have a pivotal influence on PC survival. Extraction of sufficient numbers of MKs proved to be the most challenging aspect of these series of experiments and future studies should aim to optimize efficiency of cell recovery further. Granulocytes also showed a positive effect on PC survival; subsequent research should focus on the examination of distinct subpopulations, including eosinophils and basophils. As several factors are involved in the multi-component survival niche, there is a plethora of prospective therapeutic strategies for the treatment of autoimmune or malignant diseases linked with LLPCs.

Abstrakt

Einleitung: Autoimmunerkrankungen wie SLE sind durch pathogene Antikörper und bösartige Neubildungen wie das multiple Myelom durch abnormale PCs charakterisiert, deren Persistenz auf langlebige Plasmazellen zurückgeführt werden kann. Letztere benötigen ein spezifisches Milieu aus Stromazellen, hämatopoetischen Stammzellen und Zytokinen. MKs stellen eine wichtige Komponente dieser Überlebensnische dar. TPO ist der essenzielle Wachstumsfaktor für MKs und c-mpl sein Rezeptor. Veränderungen in der Megakaryopoese können die Überlebensbedingungen für LLPCs in der Nische beeinflussen. Unsere Arbeitsgruppe hat einen SNP in der essenziellen Bindungsdomäne von c-mpl in einem SLE Mausmodell gefunden und der erste Teil dieser Arbeit beabsichtigt, eine ähnliche Mutation in SLE Patienten zu finden. Der zweite Teil beurteilt den Effekt von HNCs in Co-Kultur mit murinen Plasmozytomzellen.
Methodik: Genomische DNA von 40 SLE Patienten wurde isoliert, sequenziert und mit DNA von 15 gesunden Probanden verglichen. MKs aus einem SLE Mausmodell wurden anhand von ELISA auf erhöhte Akt Phosphorylierung downstream von c-mpl untersucht. Im Folgenden wurden murine MM Zellen mit ausgewählten HNCs kultiviert. Nach Bestimmung der optimalen Zelldichte wurde die Anzahl verfügbarer MKs durch Knochenmarkskultur, BSA Dichte Gradienten, Färbung von Zelltypmarkern und magnetischer Zelltrennung optimiert.
Ergebnisse: Es gab keinen Anhalt für einen entsprechenden SNP in den gewonnenen Proben, eine vermehrte Aktivierung der Akt Signalkaskade konnte nicht eindeutig belegt werden. Co-Kultur von MKs zeigte, dass diese Wachstum und insbesondere Überleben von MOPCs unter suboptimalen Bedingungen unterstützen. Ein ähnlicher Effekt wurde auch für Granulozyten beobachtet. Des Weiteren bestätigte sich auch die Rolle von CD138 als möglichen Indikator für sehr frühe Apoptose.
Diskussion: Die Ergebnisse der Co-Kulturen bestätigten, dass MKs einen bedeutenden Einfluss auf das Überleben von Plasmazellen haben. Die Isolation von MKs in ausreichenden Zahlen war eines der anspruchsvollsten Aspekte dieser Arbeit und sollte in zukünftigen Studien weiter optimiert werden. Granulozyten zeigten ebenso einen positiven Effekt, nachfolgende Arbeiten sollten sich auch auf Unterpopulationen wie Eosinophile oder Basophile konzentrieren. Da mehrere Faktoren die Überlebensnische beeinflussen, gibt es eine Fülle an möglichen neuen Optionen für die Behandlung von LLPC-assoziierten autoimmunen oder neoplastischen Erkrankungen.

Table of Contents

1. Introduction

1.1 The Immune System and Humoral Immunity

The immune system plays a crucial role in defending its host organism against pathogenic microorganisms and can be divided into two main components: the innate and the adaptive immune system. Working in synergy, the innate immune system confers an immediate and unspecific first-line defence against many common bacterial pathogens, while the adaptive immune system offers a highly specific yet time-delayed response to a wide variety of infectious agents. This second reaction is mediated by B and T lymphocytes and depends on the presentation of antigens by appropriate effector cells. Humoral immunity is irrefutably linked to the activity of antibodies which are produced by plasma cells, the latter representing the final developmental step in B cell maturation (1).

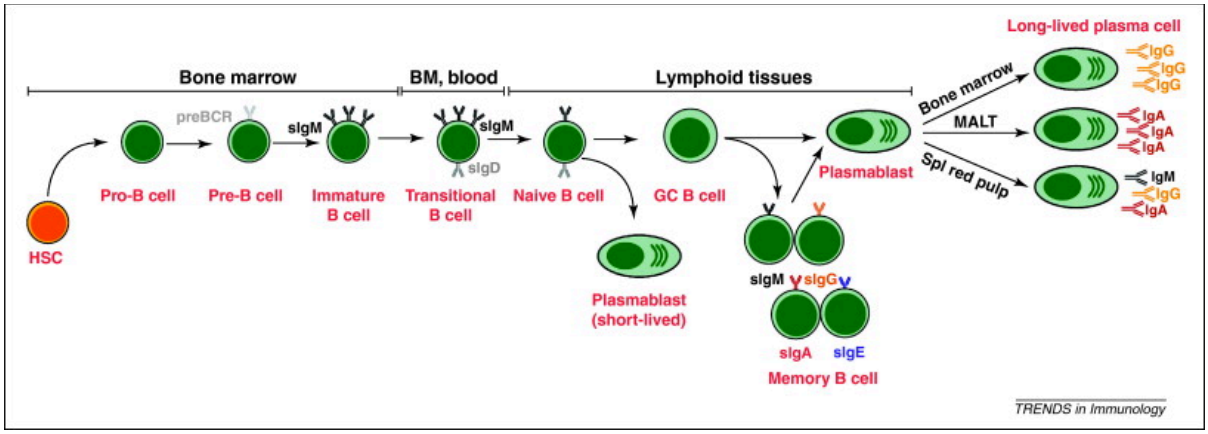

Figure 1: Stages in B Cell Development (2)

In adults, B cells originate from haematopoietic stem cells in the bone marrow. Somatic recombination of light and heavy immunoglobulin (Ig) chains eventually gives rise to an immature B cell, which expresses IgM on its cell surface. Further development continues with a transitional B cell that carries both IgM and IgD on its membrane. Subsequently, naïve but immunocompetent B cells can be stimulated by contact with antigen within the periphery, which differentiate into memory B cells or plasma cells. The latter are terminally differentiated, can no longer undergo cell division and are functionally distinct as antibody-producing cells. During their maturation, the levels of

several cell surface molecules are modified, allowing the identification of distinct subsets of B cell development based on the customary pattern of their respective membrane markers. By means of flow cytometry, plasma cells can be selected due to an increased expression of CD138 (Syndecan-1), CD9, CXCR4 and BLIMP-1, whereas surface molecules such as MHC II, B220, membrane-bound IgM and IgD, CD19, CD20 and CD40 that are typical for their precursor B cells are down-regulated (3).

Plasma cells and plasma blasts are functionally distinct from other cells in the B cell lineage due to their ability to secrete antibodies in the form of soluble immunoglobulin proteins. Certain antibody responses, e.g. levels of protective antibodies after vaccination, can be sustained for years and even decades after antigen stimulation (4) despite the short circulatory half-life of immunoglobulin of less than one week (5). It was thus assumed that stable antibody titres were dependent on continuous production and secretion by plasma cells. However, since the latter were also thought to be intrinsically short-lived with a projected survival span between days (6) and weeks (7), this in turn implied a continuous replenishment of plasma cells from a pool of memory B cells.

Evidence pointing towards a population of long-lived plasma cells (LLPCs), which were first mentioned by Manz (8) and Slifka (9) in the early 1990s, provided a new conceptual approach to explaining the persistence of stable antibody titres and it was even suggested that these could have a lifespan that could potentially extend to the natural lifetime of the individual host (9, 10). However, LLPCs are not intrinsically long-lived but require a specific microenvironment with anti-apoptotic signals to maintain their longevity. These so-called survival niches consist of multiple components, with non-haematopoietic stromal cells and haematopoietic stem cells providing a basic framework as well as producing and secreting cytokines that promote migration and support the survival of LLPCs within their niche (2).

For this reason, any changes regarding the factors governing the survival of plasma cells, in particular long-lived ones, can have a crucial impact on the pathogenesis of diseases that are defined by the pathological production of antibodies, e.g. autoreactive antibodies in systemic lupus erythematosus (SLE) or excessive, malignant plasma cells as in multiple myeloma (MM), both of which will be further discussed in the following sections.

1.2 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that commonly affects multiple organs and tissues such as the kidneys, heart, skin, joints and the nervous system and is characterized by an inappropriate response of the adaptive immune system to autologous antigens. Given the wide range of associated symptoms, the American College of Rheumatology established several criteria that should help make or exclude the diagnosis of SLE, including clinical symptoms such as discoid rashes, photosensitivity, arthritis and nephritis, but also including laboratory evidence in the form of lupus-specific antibodies such as anti-double-stranded DNA (anti-dsDNA), anti-phospholipids or anti-nucleosomes (11). Approximately 35 genes have been associated with SLE, however, alterations in multiple genes are needed to significantly increase the risk of developing the disease (12) and genes that are most notably affected have an immunoregulatory function (13). Other possible pathogenic influences include epigenetic modulation, viral infections and environmental factors such as exposure to tobacco smoke during pregnancy (14). Gender also plays a major part in determining the risk of developing SLE with the female-male ratio for SLE prevalence as high as 10:1 between the ages of 20-30. There is also evidence for epidemiological variations between ethnic groups, such as African-Americans, Asians and Hispanics who are more commonly affected than others (15).

Circulating immune complexes and antibody-antigen complexes are thought to be responsible for most clinical manifestations of systemic lupus erythematosus. One proposed mechanism for the production of auto-reactive antibodies in SLE is that defective apoptosis and impaired clearing of cellular fragments causes a wide variety of auto-antigens to be exposed and become available as a target for auto-reactive lymphocytes (16). While over 100 different autoantibodies have been associated with SLE (17), certain anti-nuclear antibodies such as anti-dsDNA and anti-Smith antibodies correlate with progression and clinical activity of the disease (18) and are widely used as standard diagnostic markers. As plasma cells exclusively produce and secrete antibodies, they are naturally implicated in the pathogenesis of SLE and represent suitable targets for immunosuppressive treatment. High doses of cyclophosphamide or steroids are usually able to successfully manage a flare-up of disease activity by

purging plasmablasts and plasma cells and thereby decreasing the number of circulating anti-dsDNA antibodies for a number of patients.

1.2.1 Long-Lived Plasma Cells in SLE

Nonetheless, certain autoantibodies persist in SLE despite aggressive immunosuppressive treatment, suggesting the existence of a population of non-proliferating, long-lived plasma cells that are resistant to treatment and can continue to maintain stable antibody titres.

Experiments with a murine model of SLE (NZB/W) by Hoyer and colleagues (19) demonstrated that both short- and long-lived plasma cells contribute to the maintenance of autoimmunity in SLE: NZB/W mice were fed with Bromodeoxyuridine (BrdU), a synthetic nucleoside, which is incorporated into the DNA of proliferating cells, for 14 to 21 days. Throughout the whole feeding time, 40% of all $CD138^+$ plasma cells did not take up any BrdU, indicating that these were non-proliferating, long-lived plasma cells. After immunosuppressive treatment with cyclophosphamide, short-lived $BrdU^+$ cells were depleted, while long-lived $BrdU^-$ cells resisted treatment and continued to secrete auto reactive antibodies, in particular anti-dsDNA antibodies. For human SLE, however, the majority of anti-dsDNA antibodies seem to originate from short-lived plasma cells, whereas other antibodies, such as anti-RNA and anti-cardiolipin antibodies, are unaffected by immunosuppressive treatment and could be generated by long-lived plasma cells (20).

Rituximab, a monoclonal antibody against CD20, which is found on the surface of B cells, but not on mature, antibody-secreting plasma cells, has been suggested as a potential therapeutic option for SLE. Studies in lupus-prone mice revealed that long-term treatment with rituximab for 12 weeks was able to significantly reduce the production of auto-reactive antibodies (21). Despite this, research by Anolik and colleagues (22) showed that rituximab therapy depleted B cells (as precursors to short-lived plasma cells) in SLE patients, but did not arrest the production of autoantibodies, whose titres could be maintained well by LLPCs as mentioned above (19). Additionally, two large-scale, randomized controlled studies aiming to evaluate the safety and efficacy of rituximab treatment failed to attain their primary end points (23, 24), with

recent research now focussing on targeting B-cell activating factors such as a proliferation-inducing ligand (APRIL), B-Cell Activating Factor (BAFF). In 2009, Voll and Hiepe described two potential therapeutic approaches that could target LLPCs, but are restricted to severe cases, refractory to other therapeutic options: Firstly, immunoablation with anti-thymocyte globulin followed by autologous stem cell transplantation in human SLE patients and secondly, treatment with the proteasome inhibitor bortezomib in the murine model (25).

1.3 The Multi-Component Plasma Cell Niche

Plasma cells themselves are not inherently long-lived and depend on a suitable microenvironment, namely the so-called plasma cell niche, for their survival. While these survival niches are located mainly in the bone marrow (26), they can also be found in various other locations such as the spleen (19) or in chronically inflamed tissue (27). Their actual number is assumed to be limited, implying constant competition between newly formed plasma blasts and older, terminally differentiated plasma cells for residency in the survival niche (10).

The current model proposes a multi-component plasma cell niche that is composed of cells of haematopoietic and mesenchymal origin that promote plasma cell migration and viability in conjunction with cytokines and membrane-bound factors. Tokoyoda et al. (28) noticed that the majority of plasma cells within the bone marrow survival niche are in contact with a subset of stromal cells expressing the chemokine CXCL12, so-called CXCL12-abundant reticular (CAR) cells. CXCL12 is also known as stromal-derived factor SDF-1. These CAR cells serve as a basic framework for cells within the niche and mediate migration of plasma cells via CXCR4 (the receptor for CXCL12). Additionally, CAR cells also impart homing and adhesion through the VCAM-1 receptor and its ligand VLA4 that is expressed on the surface of plasma cells. Even though all CAR cells are VCAM-1^+, they only represent 17% of all VCAM-1^+ cells in the bone marrow (28).

Other $CXCR4^+$ cells that are recruited to the bone marrow niche compartment via CXCL12 comprise a multitude of cells including monocytes and dendritic cells (29), eosinophils (30), basophils (31, 32), neutrophils (33) and megakaryocytes (34). It should also be noted that CXCL12, next to its role in delivering haematopoietic cells to the niche, has also been shown to support plasma cell survival in vitro (27).

The aforementioned haematopoietic niche components (HNC) promote plasma cell longevity by producing essential survival factors such as APRIL, BAFF and IL-6. APRIL and BAFF are members of the TNF superfamily with shared homology, and Benson and colleagues demonstrated that the survival of LLPCs is dependent on the presence of either BAFF or APRIL alone (35). There are three receptors for BAFF and APRIL: Firstly, the BAFF-receptor which, as its name suggests, interacts specifically with BAFF. TACI on the other hand is able to bind both ligands, whereas BCMA shows a significantly higher affinity for APRIL than BAFF. Previous research has shown that BCMA knockout mice have a limited capacity to accommodate LLPCs when compared to wild-type mice (36). IL-6 has also been shown to be crucial for supporting plasma cell survival in vitro, either by itself for murine LLPCs (37) or when combined with APRIL/BAFF or CXCL12 respectively in human LLPCs (38). The most important sources of IL-6 and APRIL within the bone marrow plasma cell niche are dendritic cells, macrophages, eosinophils and megakaryocytes. Furthermore, it should be noted that stromal CAR cells are also able to produce IL-6.

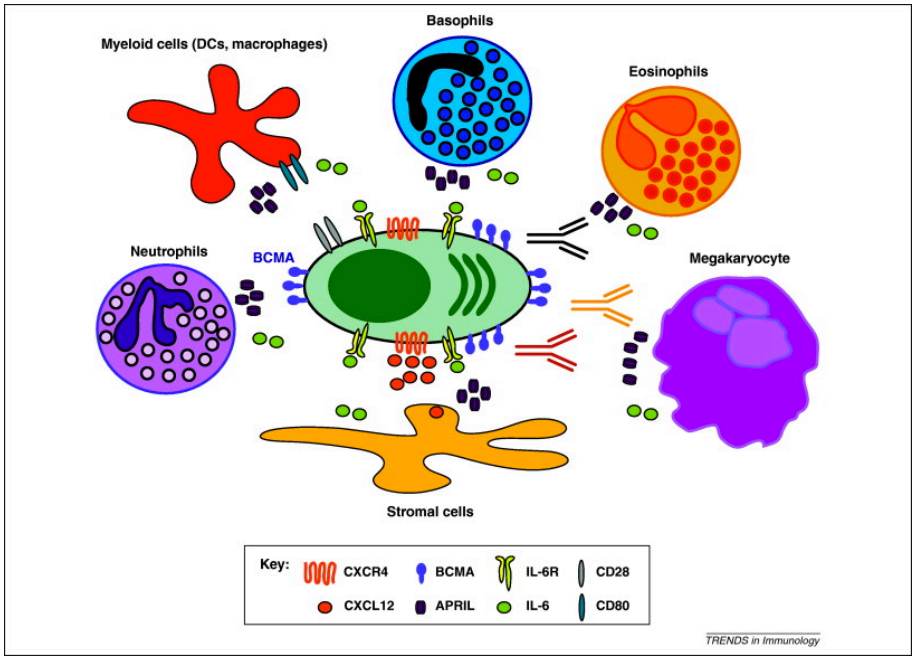

Figure 2: Model of the multi-component plasma cell niche, based on research by Winter et al. (39) : Haematopoietic niche components migrate to the bone marrow microenvironment via the CXCL12-CXCR4 axis and contribute to a suitable microenvironment by producing pro-survival cytokines such as IL-6 and APRIL. (2)

Megakaryocytes are attracted to the niche as they express CXCR4 and it has been demonstrated by Winter et al. (34) that they are localized in close proximity to LLPCs in the bone marrow. Furthermore, they promote plasma cell survival by being the main

source of APRIL and IL-6. For this reason, any alterations in megakaryocytopoiesis and associated subsequent changes in the numbers of megakaryocytes will change the survival conditions for LLPCs within the bone marrow microenvironment with a direct effect on overall viability and survival. Correspondingly, mice that are deficient for the c-mpl gene display a reduced number of plasma cells in the bone marrow compartment. c-mpl is the receptor for thrombopoietin (TPO), which in turn is the crucial growth factor for megakaryocytopoiesis. In the following sections, the role of megakaryocytes as a significant constituent of the multi-component plasma cell niche will be further evaluated.

1.4 Megakaryocytopoiesis

The c-mpl receptor belongs to the superfamily of haematopoietic receptors (41, 42) and is highly conserved among species (43). It is expressed on the cell surface of megakaryocytes, $CD34^{+}/CD38^{-}$ stem cells and platelets. The mature gene product for human mpl (CD110) has a molecular weight of approximately 85 kDa (44) and is composed of twelve exons corresponding to distinct functional domains (45): Exon 1 encodes a putative signal peptide that is removed in the mature protein, while Exons 2 to 9 are assigned to two duplicate extracellular cytokine receptor domains, each exhibiting structural features such as specific cysteine residues and the WSXWS motif that are typically conserved within the cytokine receptor superfamily (46). Of these two functional domains the cytokine receptor homology (CRH) distal to the cell membrane is essential for ligand binding as well as maintaining inhibition of the preformed receptor in the absence of its ligand (47). Exon 10 corresponds to the transmembrane domain, while Exons 11 and 12 encode for its intracellular counterpart. This cytoplasmic domain includes two regions called BOX1 and BOX2 that are also highly conserved within the cytokine receptor superfamily (48). Together, these regions mediate the receptor function through mutual phosphorylation

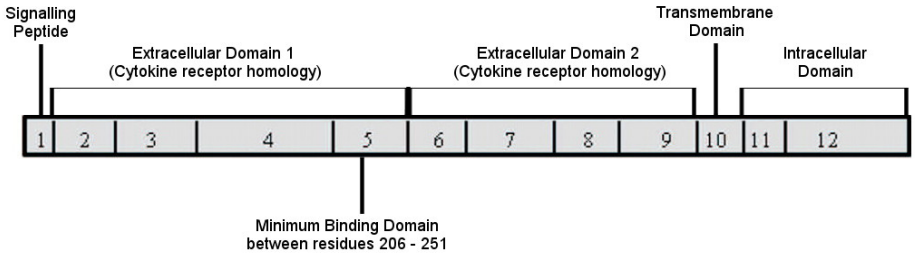

Figure 3: Exon structure of c-mpl. Exon 5 of the extracellular domain 1 contains the minimum-binding domain for TPO between residues 206-251. Modified from Walne (40)

of tyrosine residues and are pivotal for the subsequent activation of specific signalling cascades such as JAK2 or STAT (49), which will be discussed in more detail further below.

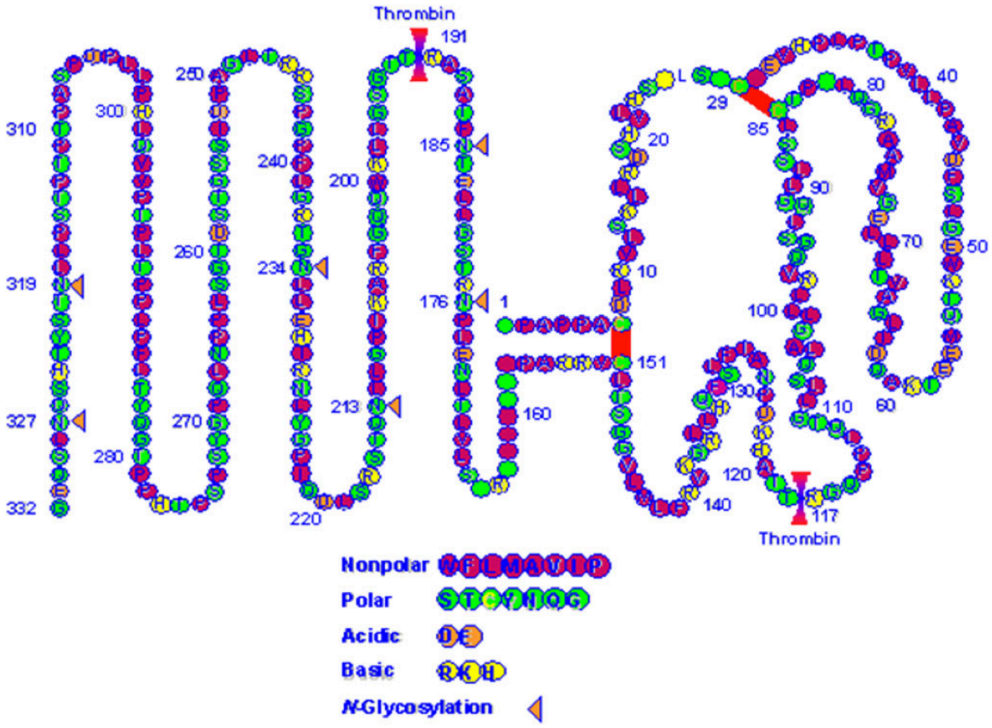

Figure 4: Thrombopoietin Structure (50). Amino acids 1 -153 designate the amino terminal domain that shares sequence homology with EPO and is comprised of four alpha helices. Amino acids 154-332 represent the carboxy terminal region, which undergoes glycosylation and mediates TPO stability.

TPO itself is the essential and critical factor for megakaryocytopoiesis (51), it supports the proliferation of precursor cells, stimulates polyploidy (52) and enhances endomitosis (53). The human TPO gene is situated on chromosome 3q26.3-27 (54) and encodes a protein with a predicted molecular weight of 36 kDa (55), consisting of two terminal domains that have separate structural and functional properties: The NH2 terminal domain is crucial for receptor binding to c-mpl, its secondary protein structure consisting of the typical four alpha helix structure common to other haematopoietic cytokines such as EPO (56). The carboxy terminal domain consists of 178 amino acids and has no importance for receptor binding (50, 57). Experiments with truncated forms of TPO lacking the carboxy terminus showed that the amino terminal region itself is sufficient to fully activate c-mpl (58).

TPO is produced primarily in the liver (11) and released immediately into circulation without being stored (50). As mentioned previously, the c-mpl receptor is also present on platelets (59), where it primarily mediates TPO breakdown. In fact, serum concentrations of TPO are inversely proportional to platelet counts, comparable to the relationship between erythrocyte counts and EPO. Li and colleagues (60) demonstrated the mechanism of TPO clearance using radioactively labelled recombinant TPO: After binding to c-mpl, the ligand-receptor complex undergoes internalization followed by degradation with 80% of all platelet-bound TPO removed within the first 60 minutes. The c-mpl receptor itself is not available for subsequent ligand binding after undergoing internalization and ubiquitin-mediated degradation (61).

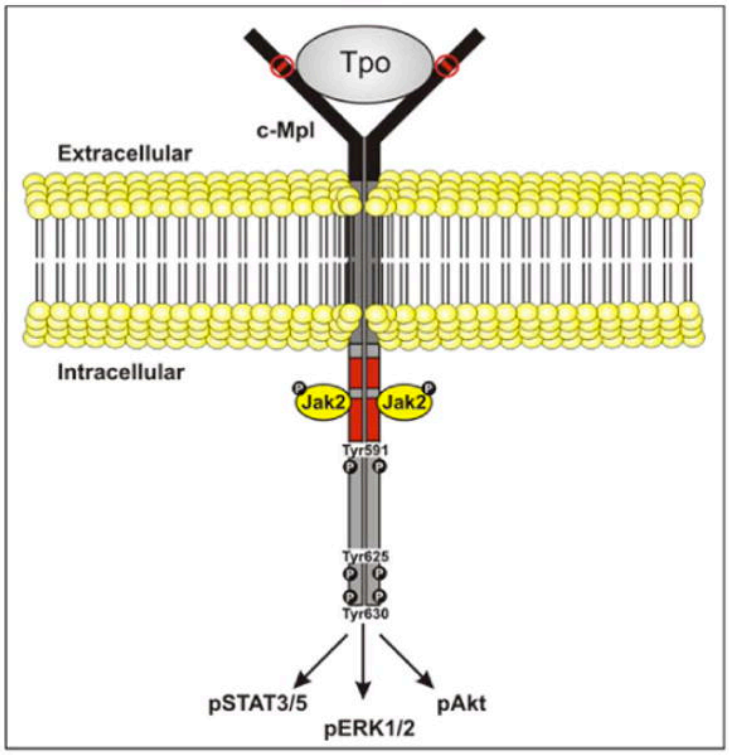

Figure 5: Downstream signaling mediated by TPO binding to its cytokine receptor, c-mpl. Autophosphorylation of JAK activates other pathways involved in regulation of cell cycle and survival such as STAT3/5, MAPK/ERK, PI3K/Akt. (62)

The active c-mpl receptor requires dimerization of two homologous subunits: TPO binding to the distal CRH induces a conformational change in the c-mpl receptor, initiating several signal cascades that activate each other through reciprocal phosphorylation (63, 64). Downstream signalling is mediated primarily via Janus Kinase 2 (JAK2), which also promotes localization of the c-mpl receptor to the cell surface as well as protecting it from degradation (65). Other signalling cascades that are activated through phosphorylation by JAK2 include: The MAPK/ERK pathway affecting endomitosis (66), STAT signalling involved in both early differentiation and proliferation (STAT3) as well as late maturation (STAT5) (67) and the PI3K/Akt pathway supporting cell survival (68).

Research by Chen et al. in 2010 regarding the putative binding domain of the TPO receptor discovered that residues 206 to 251 of the extracellular domain 1 of c-mpl, corresponding to exon 5 of the c-mpl gene, are required as minimum binding domains for TPO (42). Data from our research group (not published) found evidence of a single nucleotide polymorphism (SNP) in this exon in DNA obtained from SLE-prone NZB and NZB/W mice. This SNP has not been previously described in human mpl, and thus the primary working hypothesis was to find evidence for a similar mutation in a cohort of SLE patients. Proof of a mutation could implicate transformed kinetics of TPO receptor binding and an altered activation of signalling pathways. Additionally, the first part of this thesis focused on finding evidence for increased Akt signalling. Akt is a protein kinase involved in downstream signalling of c-mpl and is regulated by phosphorylation of its residues Threonine 308 (Thr308) by PI3K (69) and Serine 473 (Ser473) by mTORC2 (70). Upon activation of these, Akt is then able to target several downstream substrates such as forkhead and glycogen synthase kinase beta 3 (GSK-3β) that are both able to promote cell proliferation and survival in megakaryocytes (62). Variations in the number of MKs available for the PC niche on the other hand can ultimately have a significant effect on PC survival as mentioned above.

1.5 Multiple Myeloma

Multiple myeloma (MM) is characterized by the unchecked proliferation of monoclonal, malignant plasma cells within the bone marrow and is accompanied by a series of distinctive symptoms that can be associated with the production of irregular antibodies by abnormal plasma cells. Plasma cells in MM are characteristically located in the bone marrow, where their survival depends on cytokines produced and secreted by cells within the microenvironment, as well as on direct cell-cell contact similar to the factors that govern the long-term survival of long-lived plasma cells in protective immunity or autoimmune disease, as described above.

MM is the second most common haematological malignant neoplasm, frequently diagnosed in men, with a median peak onset at approximately 65 years of age. It is considered incurable, yet recent therapeutic advances have increased median survival to 5-7 years (71). Typical MM associated symptoms can be explained by the

unregulated proliferation of plasma cells: Multifocal deposition of antibodies can lead to renal failure and polyneuropathy, while expansion within the bone marrow suppresses other haematopoietic lineages, leading to anaemia, thrombocytopenia and leukopenia. Osteolytic destruction of bone marrow is furthermore associated with pathological bone fractures and increased serum calcium. The current view suggests a model in which asymptomatic precursor stages such as monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM) progress to MM with a risk of about 30% over the course of 25 years (72).

Our current understanding of MM pathogenesis is constantly evolving. While previous research aimed at identifying the cell of origin in multiple myeloma, e.g. $CD138^-$ B Cells (73), recent investigations have shifted their focus to examine the complex role of the microenvironment for plasma cell survival in MM, a strategy that has also permitted the development of new therapeutic strategies.

1.5.1 The Bone Marrow Niche in Multiple Myeloma

Pro-Survival Growth Factors

A multitude of myeloma growth factors has been extensively described in the literature among which IL-6, BAFF, APRIL, Insulin-like growth factor 1 (IGF-1) and CXCL12/SDF-1 confer the most significant pro-survival and anti-apoptotic stimuli. Bone marrow stromal cells (BMSC) are able to produce IL-6, even though there have been reports of autocrine production of IL-6 by myeloma cells (74). Other local sources of IL-6 within the BM microenvironment include osteoclasts and bone marrow endothelial cells (BMEC) (75). IGF-1 is the second essential growth factor for myeloma cells (76) and is expressed mainly by osteoclasts, even though there are also indicators for autocrine production by MM cells (77). When compared to IL-6, it shows a stronger anti-apoptotic effect through sustained activation of the transcription factor NF-κB, the PI3K/Akt pathway and by inducing the phosphorylation of the forkhead transcription factor (78). All receptors for APRIL and BAFF exhibit heterogeneous expression profiles in different cell lines of MM and respond differently when stimulated by their respective ligands. As is the case for IGF-1, osteoclasts produce the majority of APRIL and BAFF.

Furthermore, there is additional evidence of autocrine APRIL and BAFF production by a subtype of myeloma cells suggestive of a potential autonomous survival loop (79).

Neovascularisation

Another important factor to be considered for the establishment of the BM survival niche is neovascularisation. The formation of new blood vessels represents an important hallmark of tumour growth in MM (80), parallels disease progression and depends on stimulation by pro-angiogenic factors. The cytokine VEGF-1 has a critical role in myeloma-associated angiogenesis and is expressed mainly by stromal and endothelial cells in the bone marrow. However, myeloma cells themselves are also able to produce VEGF-1, which can create a paracrine loop with an additional distinct pro-survival effect due to an up-regulation of IL-6 secretion from bone marrow stromal cells (81).

Homing and Adhesion

SDF-1 or CXCL12 is secreted by BMEC and mediates early homing of myeloma cells to the BM niche via its cell surface receptor, CXCR4. MM cells can then closely interact with other components within this microenvironment, such as BMSCs, HNCs, fibroblasts, adipocytes, various number of extracellular matrix proteins, as well as osteoblasts and osteoclasts (75). The adhesion of myeloma cells to the niche is mediated by several molecules including CD44, VLA-4 and CD138 (82). These factors have the potential to induce the activation of several intracellular signalling pathways that can augment the secretion of pro-survival cytokines within myeloma cells and other cells in the niche, thus creating a circulus vitiosus which is continuously perpetuated by autocrine and paracrine activity. Other signalling cascades involved in survival, proliferation and migration of myeloma cells within the bone marrow niche include the PI3K/Akt and JAK/STAT pathways that are also implicated in megakaryocytopoiesis, as mentioned above. Syndecan-1 or CD138 is found on the surface of most MM cells and mediates adhesion to extracellular matrix proteins by binding to type 1 collagen (75). It is typically associated with a poor clinical outcome, as higher serum levels are correlated with elevated tumour mass (83).

The Role of Osteoclasts and Osteoblasts

The two cellular factors that modulate the development of osteolytic bone lesions in MM are osteoclasts and osteoblasts: Firstly, stimulation of osteoclastogenesis is influenced by the expression of receptor activator of NF-κB (RANK) and its ligand (RANKL). Osteoblasts and BMSC both produce RANKL and osteoprotegerin (OPG), the former inducing osteoclast differentiation and maturation after binding to RANK on osteoclast precursor cells, while the latter acts as a decoy receptor for RANKL, thus preventing excessive overstimulation of osteoclastogenesis (84). This homeostatic balance is disturbed in MM, as cell adhesion via VCAM-1 to BMSC decreases the secretion of OPG and increases the production of RANKL (85), with additional evidence for an increased inactivation of OPG by CD138 (86). Osteoclasts are an important source of factors that stimulate myeloma cell growth such as IL-6, IGF-1 and APRIL/BAFF, as mentioned above. However, a multitude of these survival factors also serve as osteoclast-activating factors, thereby creating a self-perpetuating circle of bone destruction promoting increased tumour growth.

Consequences for therapeutic options

This complex network of cell-to-cell interactions that is interwoven with the release of cytokines promoting growth and survival of myeloma cells has permitted an expansion towards new potential therapeutic approaches. These could involve the role of the bone marrow microenvironment in myeloma pathogenesis and could focus on migration, adhesion, angiogenesis, soluble cytokine survival factors and haematopoietic niche components, all of which could complement current therapeutic options and improve overall survival. Despite this, MM is still considered incurable and treatment currently focuses on inducing complete remission or terminating disease progression, thereby reducing the risk of associated end organ damage. The advent of novel agents such as the proteasome inhibitor bortezomib or the immune modulatory drugs thalidomide and its derivative lenalidomide, have greatly improved the median survival of MM patients (87).

Based on recent findings regarding the role of the bone marrow microenvironment in myeloma, additional treatment approaches can now not only target the tumour cell

alone but also its interactions with the survival niche, thereby increasing the efficacy of current treatments, reducing adverse effects and potentially overcoming drug resistance. Due to the multitude of growth factors involved in myeloma survival, targeting these cytokines appears to be a sensible approach. The inhibition of IL-6 for instance, enhanced the cytotoxicity of chemotherapeutic agents and has even achieved a complete and sustained remission (88). Furthermore, BAFF and APRIL depletion by a TACI fusion protein augmented the apoptotic effect of treatment with anti-IL6 antibodies (79). Targeting of cell attachment represents another potential strategy: Syndecan-1 is not only a primary diagnostic marker in multiple myeloma, but also mediates cell adhesion to extracellular matrix proteins as described above. Research by Ikeda and colleagues demonstrated that the chimeric antibody nBT062 managed to inhibit the adhesion of myeloma cells to BMSC (89). A similar effect was observed with Natalizumab, a monoclonal antibody against the cell attachment molecule integrin-α4, which also interrupted VEGF-1-mediated neovascularization, IGF-1-associated cell migration as well as sensitizing myeloma cells for treatment with bortezomib (90). The last in the long line of potential promising targets is the CXCR4-CXCL12 axis, which mediates initial homing and migration of myeloma cells to the bone marrow niche. The CXCR4 inhibitor AMD3100 effectively disrupted the adhesion of myeloma cells to BMSC, reflected by an increased level of dislocated MM cells found in the circulation and augmented sensitivity to treatment with bortezomib (91). A further study also indicated that AMD3100 could mobilize $CD34^+$ haematopoietic progenitor cells, thereby targeting homing and mooring of HNC within the survival niche (92).

1.6 Aims and Goals

SLE is a prototypical autoimmune disease that is characterized by the production of auto-reactive antibodies both by short- and long-lived plasma cells. LLPCs are able to escape conventional immunosuppressive treatment within special niches typically found in the bone marrow. These survival niches confer an ideal microenvironment for long-term residency of LLPCs and consist of several components, with stromal cells providing basic scaffolding for homing and adhesion of plasma cells, while haematopoietic niche cells promote survival by producing growth factors. Megakaryocytes are localized in close proximity to plasma cells as well and are an

important source of the pro-survival cytokines IL-6 and APRIL/BAFF. Their development depends on the c-mpl receptor and its ligand, TPO. Recent research has identified the minimum-binding domain within the extracellular domain 1 of the c-mpl gene. A single nucleotide polymorphism in this gene locus has been identified in our group for SLE-prone mice, and the first part of this thesis aims to discover and identify a corresponding mutation in the human c-mpl gene in a cohort of patients with systemic lupus erythematosus. Evidence for such a mutation, in turn, could be linked with a significant activation of signalling pathways such as PI3K/Akt that promote megakaryocytopoiesis, increase the number of available megakaryocytes and subsequently modulate the plasma cell niche through augmented levels of pro-survival cytokines. However, increased downstream signalling could also occur independently of a mutation within the c-mpl gene. The second part of this thesis will focus on the effect of selected HNCs such as granulocytes and megakaryocytes on the viability and growth of murine myeloma cells, a disease that is also influenced by plasma cell survival within a protective microenvironment.

2. Materials and Methods

2.1 Materials

2.1.1 Sequencing of c-mpl Exon 5

2.1.1.1 Materials Used

The following represents a summary of all materials and their respective compositions, if applicable, for the c-mpl exon 5 sequencing experiments that were carried out:

Name of reagent	Composition	Manufacturer
Erythrocyte Lysis Buffer	NH_4Cl [80.2 g/L] $KHCO_3$ [10.01 g/L] EDTA [3.72 g/L]	DRFZ
QIAGEN Protease		Qiagen
Buffer AL		Qiagen
Ethanol 96%		DRFZ
PBS	NaCl [8 g/L] Na_2HPO_4 [1.44 g/L] KCl [0.2 g/L]	Roth Sigma-Aldrich Sigma-Aldrich
EDTA		DRFZ
Buffer AW1		Qiagen
Buffer AW2		Qiagen
Buffer AE		Qiagen
TopTaq Master Mix	1.25 units TopTaq DNA Polymerase 1 x PCR Buffer (1.5 mM $MgCl_2$) 200 µM of each dNTP	Qiagen
CoralLoad		Qiagen
Forward primer	TCAGGCCTCCAAATTAATGG	
Reverse primer	GTTCCTGTCCAGTGGCAAGT	
Agarose		DRFZ

Tris-acetate-EDTA	Trishydroxymethylaminomethane 40mM Acetic Acid for pH titration EDTA 1mM	DRFZ
Ethidiumbromide	0.7 mg/mL	DRFZ
DNA Binding Buffer		GeneOn
Wash Buffer		GeneOn
Elution Buffer		GeneOn
RPMI medium with 1% FCS	RPMI 1640 1% FCS L-glutamate [10 mM/L] Penicillin [100 U/mL] Streptomycin [100 µg/mL]	Life Technologies Invitrogen Invitrogen Invitrogen Invitrogen
IMDM	GlutaMAX HEPES [25 mM/L] Penicillin [100 U/mL] Streptomycin [100 µg/mL] 5% FCS	Life Technologies Life Technologies Invitrogen Invitrogen Invitrogen
Thrombopoietin (TPO)	10 µg recombinant murine TPO from E.coli	Peprotech Inc
CD41-FITC	Clone: MWReg30	AbD Serotec
DAPI-PB		Southern Biotech
RPMI medium with 10% FCS	RPMI 1640 10% FCS L-glutamate [10mM] Penicillin [100 U/mL] Streptomycin [100 µg/mL]	Life Technologies Invitrogen Invitrogen Invitrogen Invitrogen
BSA		DRFZ
Cytofix		BD Pharmingen

Perm Wash 10x		BD Pharmingen
Wash Buffer		Abcam
Lysis Buffer		Abcam
Enhancer Solution		Abcam
ADHP		Abcam
ADHP Dilution Buffer		Abcam
Assay Control Lysate		Abcam
Stop Solution		Abcam
Capture Antibody	pT308, pS473, total Akt1	Abcam
Detection Antibody	pT308, pS473, total Akt1	Abcam

Table 1: Reagents used in the c-mpl exon 5 sequencing experiments

Name	Specifics	Manufacturer
QIAamp Mini Spin Columns		Qiagen
Eppendorf tube	0.5, 1.5, 2.0 mL	Eppendorf
Collection tubes	2 mL	Qiagen
Microcentrifuge tubes		GeneOn
GF-1 columns		GeneOn
Cell Culture Flask	25, 50 cm^2	Corning Costar
Needle	26 gauge	Braun
Syringes	6, 10 mL	Braun
Pipette tips	10, 200, 500 µL	Sarstedt
Scissors		DRFZ
Scalpel		DRFZ
6 well plate		Sigma Aldrich
Falcon tubes	15, 50 mL	Fisher Scientific
96-well PhosphoTracer assay plate		Abcam

Table 2: Materials used in the c-mpl exon 5 sequencing experiments

2.1.1.2 SLE Patients

40 SLE patients currently receiving treatment at the Charité Clinic for Rheumatology and Clinical Immunology were selected for further examination and 10 mL to 20 mL whole blood samples recovered after informed consent was obtained. Patients were selected according to current disease status, disease activity index (SLEDAI), chronic disease progression, last known medication, autoimmune diseases in family history and known allergies. The control group consisted of 15 male and female donors who were not ill and had not taken any relevant concomitant medication immediately prior to or at the time of sample recovery.

2.1.2 Co-Culture of MOPC315.BM and HNCs

2.1.2.1 Materials Used

The following represents a summary of all materials and their respective composition used for the co-culture experiments that were carried out:

Name of reagent	Composition	Manufacturer
PBS	NaCl [8 g/L] Na_2HPO_4 [1.44 g/L] KCl [0.2 g/L]	Roth Sigma-Aldrich Sigma-Aldrich
PBS with BSA	PBS as above with the addition of 0.5 % BSA	Biomol
Thrombopoietin (TPO)	10 µg recombinant murine TPO from E.coli	Peprotech Inc.
RPMI medium with 1% FCS	RPMI 1640 1% FCS L-Glutamate [10 mM/L] Penicillin [100 U/mL] Streptomycin [100 µg/mL]	Life Technologies Invitrogen Invitrogen Invitrogen Invitrogen

RPMI medium with 10% FCS	RPMI 1640 10% FCS L-glutamate [10mM] Penicillin [100 U/mL] Streptomycin [100 µg/mL]	Life Technologies Invitrogen Invitrogen Invitrogen Invitrogen
IMDM	GlutaMAX HEPES [25 mM/L] Penicillin [100 U/mL] Streptomycin [100 µg/mL] 5% FCS	Life Technologies Life Technologies Invitrogen Invitrogen Invitrogen
Cytofix		BD Pharmingen
Perm Wash 10x		BD Pharmingen
EDTA		DRFZ
Ethanol 70%		DRFZ
BSA		DRFZ
Annexin V Binding Buffer		eBioScience
Korsoline		DRFZ
Aqua dest		DRFZ

Table 3: Solutions, culture media, buffers and reagents used in the MOPC315.BM co-cell culture experiments

Name	Specifics	Manufacturer
96 Flat bottom Microplate		Sigma Aldrich
6 well plate		Sigma Aldrich
Falcon tubes	15, 50 mL	Fisher Scientific
Glass pipettes	5, 10, 20 mL	DRFZ
Cell Culture Flask	25, 50 cm^2	Corning Costar
Cell strainer	20 µL	BD Biosciences
Syringes	6, 10 mL	Braun
Needles	26 gauge	Braun
Pipette tips	10, 200, 500 µL	Corning Costar
Eppendorf tube	0.5, 1.5, 2.0 mL	Eppendorf
Scissors		DRFZ

Scalpel		DRFZ
Tweezers		DRFZ

Table 4: Materials used for MOPC315.BM co-cell culture experiments

Antigen-conjugate	Clone	Source
DAPI-PB		Southern Biotech
CD138-PE	281-2	BD-Pharmingen
Gran-FITC	RB6-8C5	DRFZ
Gran-BIO	RB6-8C5	DRFZ
CD4-PE	GK1.5	DRFZ
IgD-PE	11.26c	DRFZ
CD41-FITC	MWReg30	AbD Serotec
CD41-PE	MWReg30	AbD Serotec
Streptavidin-PE		BD-Pharmingen
Annexin V-APC		eBioScience
Propidium Iodide		Sigma Aldrich

Table 5: Antibodies used for cell surface staining for flow cytometry

Name	Manufacturer
MidiMACS™ Separator	Miltenyi Biotec GmbH, Germany
MidiMACS™ Multistand	Miltenyi Biotec GmbH, Germany
LS & LD Columns	Miltenyi Biotec GmbH, Germany
Anti-PE	Miltenyi Biotec GmbH, Germany
Anti-BIO	Miltenyi Biotec GmbH, Germany

Table 6: Material used for manual cell separation (MACS)

2.1.2.2 Mice

All co-culture experiments in this doctoral thesis were performed with primary cells, from 12 to 20 week-old BALB/c mice, as the MOPC315.BM cell line is derived from that strain. The experiments that examined Akt phosphorylation in this thesis were

completed using NZB and NZW mice, a mouse model that develops a lupus-like phenotype, with each strain featuring limited characteristics of autoimmune disease.

All mice were born and raised in the breeding unit of the federal institute for risk assessment (Bundesinstitut fuer Risikobewertung) in Marienfelde, Germany under specific pathogen-free conditions and transferred to the animal (mouse) maintenance facility of the DRFZ, where they were kept during the course of the experiments. All animal experiments were conducted by trained and certified personnel after approval by the responsible local ethics committee at the “Landesamt fur Gesundheit und Soziales” (Berlin, Germany) had been obtained.

2.1.2.3 MOPC Cells

Multiple myeloma in mice can be induced by intraperitoneal injection of oil, creating so-called mineral oil induced plasmocytoma (MOPC) cells that can serve as a murine model to study the pathophysiology of multiple myeloma. These cells can be translocated by subcutaneous or intraperitoneal injection and induce local, extramedullary tumour growth. The MOPC315.BM cell line used in these series of experiments was a kind gift from Dr. Rudolf Manz (Universitätsklinikum Schleswig-Holstein, Lübeck, Germany). It was first described by Hofgaard et al. (93) in 2012 and can be grown in vivo and in vitro. It represents a cell line that has the ability to migrate to, and to expand within, the bone marrow following intravenous injection, thereby creating a suitable model to study the bone marrow microenvironment in the context of myeloma disease. The cells were previously labelled with GFP by retroviral transfection and cultured in RPMI 1640 medium supplemented with 10% FCS before co-culture with haematopoietic niche component cells.

2.1.2.4 Instruments and Devices Used

Evaluation of flow cytometry was performed on a MACSQuant® Analyzer (Miltenyi Biotec), generating flow cytometry standard (*.fcs) files that were further examined using FlowJo Version 7.6.5. All graphs and statistical analyses were prepared with GraphPad Prism Version 5.0.

2.2 Methods

2.2.1 Sequencing of c-mpl Exon 5

2.2.1.1 DNA Extraction and Gene Sequence Analysis

Genomic DNA was recovered from peripheral blood samples from patients with SLE who were recruited from the Charité – University Medicine Berlin Clinic for Rheumatology and Clinical Immunology. Whole blood was initially centrifuged at 300 g for 10 minutes, its serum removed for storage and erythrocyte lysis buffer added at a ratio of 5:1. After 20 minutes of incubation at ambient temperature, the sample was again centrifuged at 300 g for 10 minutes and its supernatant discarded. The pellet was then recovered and genomic DNA extracted using the QIAamp DNA Mini Kit standard protocol (Qiagen). Concentration and purity of isolated DNA was measured by a NanoDrop™ (Thermo Scientific) spectrophotometer before a polymerase chain reaction was conducted according to the standard protocol of a TopTaq Master Mix Kit (Qiagen). Subsequently, amplified DNA was separated by gel electrophoresis at 100 Volts for 30-45 minutes. The gel was composed of 1% agarose with tris-acetate-EDTA used as an electrophoresis buffer and 5 µL of ethidium bromide added as a nucleic acid dye. The separated DNA target fragment was recovered from the gel following the standard procedures of an AmbiClean PCR/Gel DNA extraction kit (GeneOn) and sent to GATC Biotech for sequencing. The results were then screened for the SNP described above using the BioEdit sequence alignment software.

2.2.1.2 Analysis of Akt Signalling

Bone marrow recovered from femora and tibiae of NZW and NZB mice was cultured separately in IMDM medium with TPO added at a concentration of 10 ng/mL every 36 hours; all subsequent resuspension steps were performed with the distal end of the pipette tip cut off as described in section 2.2.2.2. After three days in culture, the medium was substituted with RPMI medium containing 1% FCS so as to deprive the cells of TPO for 12 hours. Following this, half of the bone marrow culture was washed with

sterile PBS at 300 g for 8 minutes, resuspended in RPMI medium with 10% FCS and transferred to a 6 well plate to be incubated for 30 minutes with additional TPO at the same concentration as above, while the other half remained unstimulated. After a second wash step with PBS with EDTA, cells of both stimulated and unstimulated bone marrow cultures were resuspended in 100 µL of BD Cytofix, kept on ice and washed again with BD Perm/Wash Buffer before they were stained for 20 minutes with CD41-FITC and DAPI-PB at a concentration of 1:400 and 1:50, respectively. Afterwards, cells were resuspended in 500 µL PBS/BSA with EDTA and added to tubes coated with 3% BSA in PBS before undergoing fluorescence-activated cell sorting, the aim being to select $CD41^+$ megakaryocytes according to polyploidy. Finally, partial and total Akt phosphorylation of extracted cells was examined with an enzyme-linked immunosorbent assay kit (abcam Phosphotracer Akt pT308/pS473/total Akt1) following the instructions of the standard PhosphoTracer assay protocol.

2.2.2 Co-Culture of MOPC315.BM and HNCs

2.2.2.1 MOPC Cell Confluence

In cell biology, confluence refers to the amount of surface area in a culture vessel that is covered with cells. As cells increase their level of confluence in a culture, growth slows down or can cease entirely due to contact inhibition. However, some transformed cells can continue to proliferate after confluence has been reached. The first set of experiments aimed to find the optimal cell density for MOPC cells, at which autocrine production of survival factors becomes insufficient and the cells would become dependent on external growth factors produced by HNCs. MOPC cells were cultured for 2 days in RPMI 1640 medium by adding either 1% or 10% FCS. Cell confluence was calculated using the following values: Within a 96-well plate used for cell cultures, each well provided a space of 32 mm^2. MOPC cells have a diameter of 0.014 mm (94) and take up a cell surface area of 1.54×10^{-4} mm^2, indicating that a cell confluence of 100% is attained when approximately 200,000 cells are present per well. Absolute cell numbers for initial seeding on day 0 were determined via the automated CASY® Cell Counter (Roche Life Sciences) while overall cell viability on day 2 was based on the uptake of the nuclear dye DAPI. Due to ease of use and logistical as well as practical

considerations, any subsequent cell counts were henceforth performed on the MACSQuant® Analyzer (Miltenyi Biotec).

2.2.2.2 Day -3: Bone Marrow Culture

In order to promote the differentiation of haematopoietic stem cells to megakaryocytes through the action of TPO, and thus increase the numbers of megakaryocytes harvested for the upcoming co-culture experiments, murine bone marrow was isolated and pre-cultured. Two BALB/c mice were sacrificed by cervical dislocation and both femora and tibiae were recovered by separating muscles and tendons from the bones using a scalpel. Each bone was then cut at its proximal as well as its distal end, allowing marrow cells to be obtained by carefully flushing all bone cavities with Iscove's modified Dulbecco's medium (IMDM) using a 26 gauge needle attached to a 6 mL syringe. To prevent the destruction of megakaryocytes by shearing forces, approximately 5-10 mm of a 500 µL pipette tip were cut off with a scalpel whenever a single cell suspension was created as was previously described (95). The use of a cell strainer was omitted for the same reason. These isolated bone marrow cells were then cultured in IMDM supplemented with Glutamax™, 5% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), HEPES (25 mMol/L) and recombinant mouse TPO at a concentration of 10 ng/mL. The extracted cells were then incubated at 37° Celsius and 5% CO_2 for 3 days. Additional TPO was added to the culture medium when its half-life of 19-25 hours approached (96).

2.2.2.3 Day 0: BSA Density Gradient

On the day of the co-culture experiment, the flask containing the bone marrow cell culture was cautiously removed from the incubator, without stirring its contents. A supernatant volume of approximately 25-30 mL was removed by vacuum aspiration via a Pasteur pipette. The remaining volume of BM culture was then resuspended with a 10 mL glass pipette and transferred to a 50 mL Falcon tube. Any remaining cellular content within the flask was rinsed with 20 mL of PBS with EDTA and added to the aforementioned falcon tube. Following a washing step of 300g for 8 minutes, the pellet was resuspended in 1 mL PBS after the supernatant had been discarded. In analogy to

the bone marrow culture, the distal end of the 500 µL pipette tip was cut off each time before resuspending the solution. This single-cell suspension was slowly added to a BSA density gradient that had been prepared simultaneously by the addition of the following layers within a 15 mL falcon tube: 1.5 mL of 3% BSA, 1.5 mL of 1.5% BSA and 1.5 mL of PBS only. After 30 minutes at ambient temperature, the topmost layer was removed and fractions II and III transferred to a second 15 mL Falcon tube. After another washing step with PBS with EDTA at 300g for 8 minutes, the pellet was resuspended in 500 µL PBS/BSA with EDTA and the sample kept on ice.

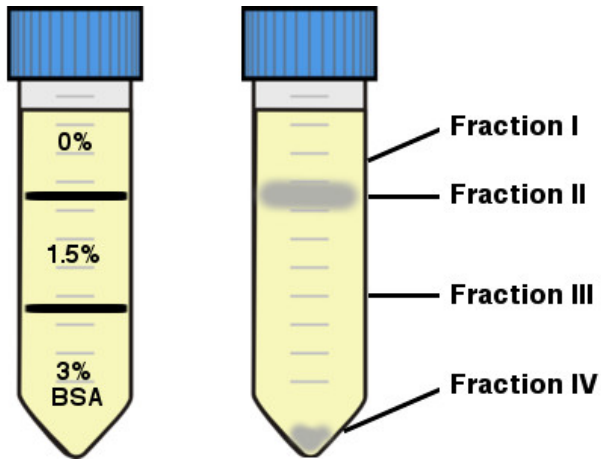

Figure 6: Schematic representation of purification of megakaryocytes by means of BSA density gradient, before the application of sample specimen (left) and after 30 minutes at ambient temperature (right). Fraction II represents the buffy coat with leukocytes and platelets, while fraction IV exemplifies the pellet

2.2.2.4 Day 0: Cell Extraction

During the separation of megakaryocytes in the BSA density gradient, one BALB/c mouse was sacrificed by cervical dislocation and its spleen and femora recovered. The spleen was kept in a small petri dish filled with PBS, gently pushed through a cell strainer using a syringe stamp. The single cell suspension was then transferred to a 50 mL falcon tube. Any remaining cell debris was collected by rinsing the petri dish with PBS containing EDTA and added to the aforementioned falcon tube. Following a wash step at 300g for 8 minutes, the pellet was then resuspended in 1 mL PBS/BSA with EDTA and kept on ice. At the same time, murine bone marrow was flushed using the

same methods as described above in section 2.2.2.2, washed with PBS containing EDTA for 8 minutes at 300g and its pellet resuspended in 500 µL PBS/BSA with EDTA.

2.2.2.5 Primary Immunostaining

With regard to the isolation of HNCs, the initial steps involved primary staining of each cell population using appropriate cell surface markers conjugated with fluorochromes, allowing the identification of specific groups of cells during subsequent flow cytometry analyses and simultaneously serving as a prerequisite for further purification of HNCs via secondary staining with magnetic immunobeads.

B and T lymphocytes were isolated from murine splenic cells and labelled with IgD-PE and CD4-PE, respectively. Granulocytes were obtained from murine bone marrow on the day of seeding and stained with Gran1-BIO. An earlier experiment attempting to grow these for 3 days simultaneously with megakaryocytes failed, as the majority of granulocytes did not survive (Data not shown). Megakaryocytes, having been cultured in IMDM medium for three days and purified with a BSA density gradient, were marked with CD41-PE. All stains were performed at a concentration of 1:250 using PBS/BSA with EDTA for 20 minutes, while all samples were kept on ice. All samples were then washed with PBS with EDTA and centrifuged at 300g for 8 minutes, followed by the removal of supernatant and resuspension of the samples in 300 µL PBS/BSA containing EDTA for further staining. As mentioned above, the distal end of the pipette tip was cut off each time megakaryocytes were resuspended.

2.2.2.6 Manual Cell Separation

Subsequently, HNCs that had been previously marked by surface staining were isolated and purified by a second staining step using the MACS microbead technology (Miltenyi Biotec). This technique involves targeting particular cells of interest by specific antibodies that are conjugated to very small magnetic particles approximately 50 nm in size. The actual cell separation is based on the action of several MACSMidi™

separators, inducing a strong magnetic field within different MACS cell separation columns, allowing the retention of cells that have been previously magnetically labelled.

In order to isolate the HNCs that were required for the co-culture experiments, we performed a positive selection using LD columns for megakaryocytes and LS columns for all other HNCs. B and T lymphocytes, as well as megakaryocytes were labelled with Anti-PE (1:20), while granulocytes were stained with Anti-BIO (1:20) and Streptavidin-PE (1:400). All stains were performed using PBS/BSA with EDTA for 20 minutes, while being kept on ice. All samples were then washed with PBS with EDTA, centrifuged at 300g for 8 minutes, their supernatants removed and then resuspended in 1 mL of PBS/BSA with EDTA. As mentioned above, the distal end of the pipette tip was cut off for all suspension steps that involved megakaryocytes. Each cell separation column was calibrated with 1 ml of PBS/BSA with EDTA before the application of 1 ml of sample volume. Unlabelled cells passed through the column and constituted the negative or depleted fraction of the sample volume. After the column was washed three times by adding 1 ml PBS containing EDTA, respectively, it was removed from the cell separator and the retained, magnetically labelled cells were then carefully eluted using a plunger and 3 ml of PBS/BSA with EDTA. This sample volume was consecutively split into two falcon tubes containing 1.5 ml of separated cells each, filled up with PBS with EDTA and washed at 300 g for 8 minutes, its supernatant discarded and the pellet resuspended in 500 µL of RPMI medium supplemented with either 1% or 10% FCS. MOPCs taken directly from their cell culture flask underwent the same separation into two fractions. After careful resuspension of all respective sample populations, a volume of 50 µL each was taken, diluted with 150 µL of RPMI medium containing the appropriate amount of FCS and DAPI-PB was added at a concentration of 1:200 in order to analyse the purity of extraction on MACSQuant for the following cell culture experiments, while all remaining sample volumes were kept on ice.

2.2.2.7 Cell Purity and Cell Counts

The absolute cell counts of isolated cells available for the co-culture experiments and purity of extraction were assessed in the following steps via the MACSQuant Analyzer.

Purity was based on the proportion of PE^+ cells of all selected cells after the removal of debris and doublets by electronic gating via the FlowJo flow cytometry analysis software. MACSQuant analysis (including cell counts) was based on an uptake volume of 50 µL, representing a quarter of the sample volume that was set aside for measurement. All $DAPI^-$ cells were then multiplied by 4/50 in order to obtain the concentration of viable cells within this small portion of purified cells. The absolute number of available cells that remained for seeding could then be found by multiplying this value by 450. In order to determine the required cell sample volumes, the following calculations had to be considered: MOPC cells were required to be seeded in triplicates at a concentration of 1,000 cells per well and co-cultured with HNCs at a ratio of 1:10, with each well holding a volume of 200 µL. Additionally, seeding was performed twice so as to permit for a day 0 analysis. For the HNCs, this necessitated a minimum of 70,000 (surplus buffer) available cells resuspended in RPMI medium containing 1% or 10% FCS respectively, which when divided by the concentration of available cells, provided the total required volume. HNCs and MOPCs were added to the co-culture within a sample volume of 100 µL each. For this reason, the total required volume of HNCs was filled up with RPMI medium containing either 1% or 10% FCS until a total volume of 700 µL was attained. Similar considerations were taken into account when sample volumes for MOPCs were determined and 36,000 cells including surplus buffer were set aside for seeding in the co-culture experiments.

2.2.2.8 Co-Cell culture

As mentioned above, MOPC and HNC sample volumes were adjusted to be 100 µL respectively and to contain 1,000 or 10,000 cells each, translating to a ratio of 1:10 for myeloma to feeder cells. Individual cell populations were seeded in triplicates in RPMI medium containing either 1% FCS (suboptimal conditions) or 10% FCS and incubated at 37° Celsius and 5% CO_2 for two days. Isolated megakaryocytes were supplemented with TPO at a concentration of 20 ng / mL on day 0 and after 36 hours had passed as described under 2.2.2.2. As mentioned earlier, TPO administration alone did not have a direct effect on plasma cell numbers (34). This observation was also evaluated in the experiments with the MOPC315.BM cell line. Both HNCs and MOPCs were resuspended before seeding in order to guarantee a uniform distribution of cells. The

96-well plate used for cell cultivation was wrapped in transparent film to prevent condensation forming within the culture plate. After seeding was completed a second, identical duplicate cell culture was performed, allowing an analysis of day 0 samples with the MACSQuant Analyzer.

	1	2	3	4	5	6	7	8	9	10	11
FCS	1%	1%	1%	1%	1%		10%	10%	10%	10%	10%
A	BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only		BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only
B	BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only		BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only
C	BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only		BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only
Day 0	BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only		BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only
Day 0	BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only		BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only
Day 0	BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only		BCells + MOPC	TCells + MOPC	Gran + MOPC	MK + MOPC + TPO	MOPC only

Table 7: Schematic representation of seeding performed for co-culture experiments with MOPC and HNCs. All cell populations were seeded in triplicates, including an identical, duplicate co-culture to represent values for day 0.

2.2.2.9 Cell Viability

The primary end point for the co-culture experiments was to measure the survival of murine MM cells when grown together with haematopoietic niche components. Viability of MOPC cells was measured on day 0 and on day 2 using Annexin V-APC as a marker

for early apoptosis and DAPI-PB as a marker for late, terminal apoptosis.

Phosphatidylserine (PS) is a component of the phospholipid cell membrane and is found on the cytoplasmic side in normal viable cells. It is translocated to the external surface of the membrane during early to intermediate stages of apoptosis. This externalisation precedes other apoptotic processes such as cytoplasmic condensation or loss of membrane integrity and attachment (97). Annexin V is a protein with high affinity for negatively charged phospholipids like PS and can be used as a probe to detect early apoptotic cells. DAPI, on the other hand is a molecule that has a strong affinity for regions of double-stranded DNA that is rich in Adenine-Thymine and is a commonly used viability stain (98). While early apoptotic cells have not lost the integrity of their nuclear membrane and are thus not permeable to nuclear dyes, dead cells can be discriminated by their uptake of DAPI. However, it should be noted that DAPI is able to permeate live cells when applied at very high concentrations (99). Additionally, the expression of the plasma cell marker CD138 or Syndecan-1 was also analysed in order to evaluate its potential as a supplementary marker for very early apoptosis.

The following experimental procedure was applied to samples on day 0 (immediately after seeding) and on day 2, following the incubation period. All staining and washing steps were performed within the 96-well cell culture plate. Initially, two wash steps were implemented by spinning the culture plate at 300 g for 8 min, discarding 150 µL of supernatant and filling up with the same volume of PBS in the first wash and Annexin V binding buffer in the second wash. Cells were then stained using Annexin V binding buffer and DAPI-PB at a concentration of 1:50, Annexin V-APC at 1:100 and CD138-PE at 1:400 for 15 minutes at 4° Celsius, washed and resuspended in binding buffer once more, resulting in a total volume of 200 µL per well. An uptake volume of 100 µL was used for analysis with MACSQuant, generating flow cytometry standard (fcs) files, which were examined using the flow cytometry software FlowJo. Depending on their pattern of fluorescence, cells were then determined to be viable ($DAPI^-$, Annexin V^-), early apoptotic ($DAPI^-$, Annexin V^+) or late apoptotic / dead ($DAPI^+$, Annexin V^+).

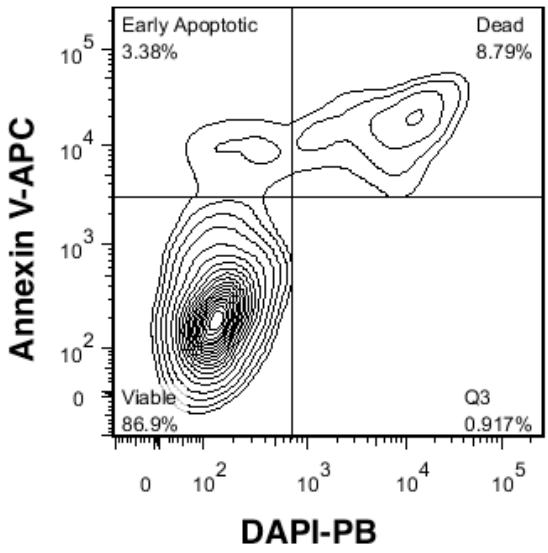

Figure 7: Exemplary flow cytometry data for electronic gating performed to evaluate viability on day 0 and on day 2.

3. Results

3.1 Sequencing of c-mpl Exon 5

In the first part of this thesis, blood samples obtained from 40 selected SLE patients currently receiving treatment in the Charité Clinic for Rheumatology and Clinical Immunology were evaluated for SNP within the minimum-binding domain of extracellular domain 1. For this purpose, genomic DNA was extracted from peripheral blood samples and exon 5 of c-mpl amplified using polymerase chain reaction. This was followed by a separation of DNA fragments by gel electrophoresis and recovery of target DNA for sequence analysis. Previously, unpublished data from our group described an SNP in SLE-prone NZB and NZW mice within the extracellular binding domain of TPO, and the primary aim of the experimental part of this thesis was to find evidence for a similar mutation in SLE patients. After all samples were extracted and assessed using DNA sequence alignment software, there was no indication of such a mutation in the c-mpl gene of those patients.

A further set of experiments focused on the examination of downstream signalling of c-mpl, in particular activation of the Akt signalling cascade, as one of the major pathways affecting megakaryocyte proliferation and differentiation. This included an analysis of total and partial Akt phosphorylation of megakaryocytes pre-cultured for three days and originating from either NZB or NZW mice with or without stimulation by TPO prior to ELISA examination. Even though different patterns of Ser473 or Thr308 phosphorylation were observed, there was no conclusive evidence for an increased activation of the Akt pathway after the analysis of all cell lysates. However, since the experimental data was based on one single study, this could justify further potential follow-up examinations.

3.2 Co-Culture of MOPC315.BM and HNCs

These series of experiments aimed to assess the influence of known haematopoietic niche cell components such as granulocytes and megakaryocytes as well as B and T

lymphocytes on the growth and viability of murine plasmocytoma cells using the example of the MOPC315.BM line. Before the actual co-culture experiments were performed, several aspects of the experimental setup had to be considered and which shall be presented accordingly and chronologically in this thesis:

First, murine myeloma cells were cultured at different cell densities in order to determine the optimal seeding conditions at which the former would be dependent on external cues such as pro-survival growth factors secreted by HNCs. Secondly, the preparation of HNCs from BALB/c mice underwent several modifications and adjustments before a suitable experimental approach could be determined that permitted successful co-culture with MOPCs. The results for the latter constitute the principal part of this thesis and will be presented with a focus on growth and viability of MOPCs after two-day culture as well as providing an additional outlook on the expression profile of the plasma cell surface marker CD138 and its potential role as a very early marker of apoptosis.

3.2.1 Dependence on Confluence

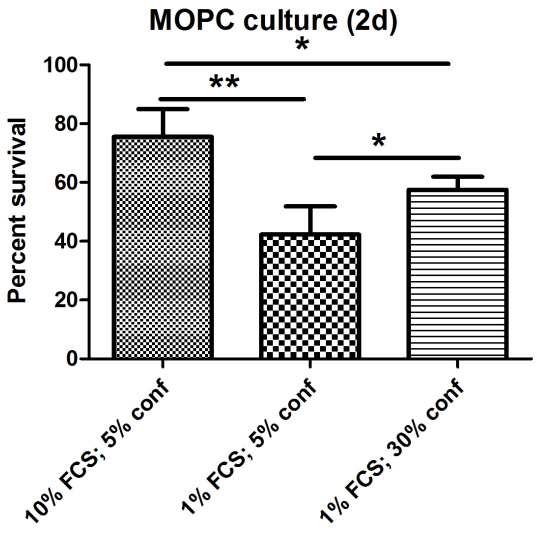

Figure 8: Initial two day MOPC Culture. The bars indicate the mean and SD for the overall survival of MOPCs. The asterisks represent values that are significantly lower/higher when compared to each other (: p <0.005; *: p <0.05)**

In the first preliminary experiment, MOPCs were incubated for 2 days in RPMI 1640 medium containing 1% and 10% FCS respectively, and seeded at different levels of cell confluence. MOPC survival was then evaluated by flow cytometry analysis of viable $DAPI^-$ cells. Earlier reports have indicated that MOPC viability can be dependent on external factors such as IL-6, which can be produced by some MM cell lines themselves, thus creating an autocrine survival loop (74, 100, 101). The experimental evidence from our initial study confirmed this relationship: MOPC cells grown under optimal conditions in medium with the addition of 10% FCS demonstrated a survival rate of approximately 80%, while cells grown in a medium supplemented with only 1% FCS revealed significant differences in survival rates in

dependence of their cell density. Based on this data, a second experiment followed including multiple, incrementing levels of cell density for MOPC cells grown under suboptimal conditions in RPMI medium which was only supplied with 1% FCS.

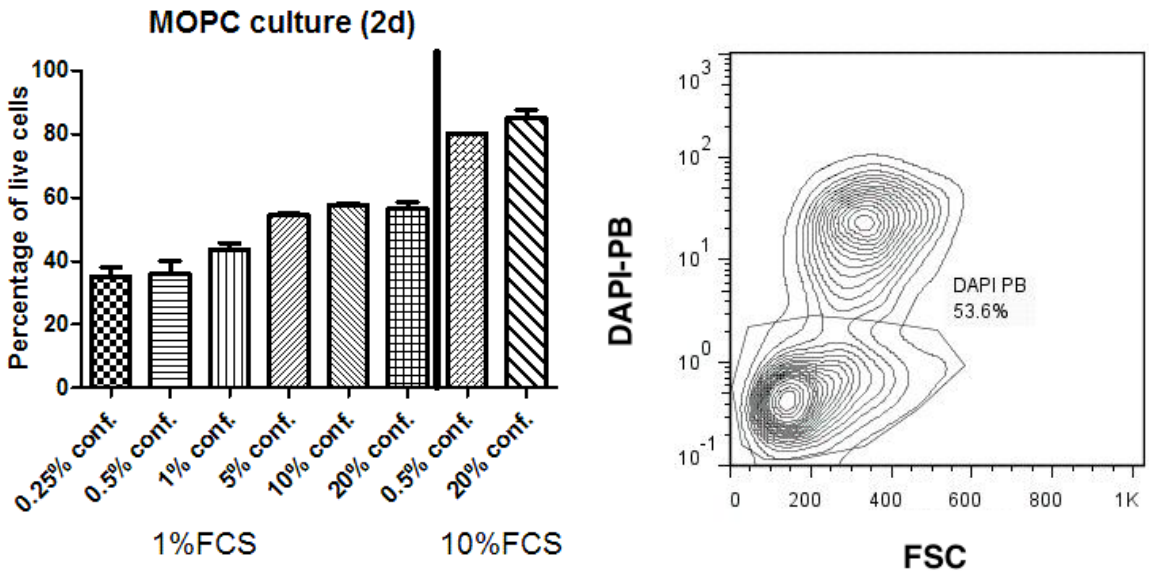

Figure 9: Left: Follow-Up MOPC Cultures to test for dependence on confluence. The bars indicate the mean and SD for the percentage of live, DAPI negative cells after the removal of cell debris and doublets. Right: Exemplary flow cytometry data showing gating on viable, DAPI negative MOPCs after the removal of cell debris, grown in 1% FCS and seeded at 5% confluence.

Once again, MOPC cells that were grown in a medium supplemented with 10% FCS showed little difference in overall survival even within a wide range of confluence (80.0% for 0.5% confluence and 85.0% for 20% confluence). On the other hand, the frequency of viable cells cultured in a medium with 1% FCS revealed an increase proportional to the confluence, reaching a plateau between 5% and 10% confluence with a survival rate of 54.5% to 57.5%. Any increases in cell density thereafter had no significant effect on overall viability. Correspondingly, MOPC survival began to decrease for confluences lower than 5%.

As mentioned above, some myeloma cells lines can support their own survival through the autocrine production of growth factors such as IL-6. At higher cell densities, this could have a major impact on growth due to MOPCs potentially stimulating themselves,

whereas on the other hand, their dependence on external growth factors would be much more pronounced at lower cell confluences. Based on the promising results of these preliminary experiments and due to the technical limitations involved in cell detection at remarkably low cell counts, the optimal confluence for the forthcoming co-culture experiments was thus determined to be 0.5% or 1000 cells per well (96-well plate).

Another value that was analysed in these precursory experiments was the fraction of $CD138^-$ cells among all GFP^+ cells. Jourdan and colleagues (102) reported in 1998 that the plasma cell surface marker Syndecan-1 was present only on viable myeloma cells and quickly lost during early or intermediate stages of apoptosis. The results of our investigation provided further evidence for this observation and revealed an inverse relationship between Syndecan-1 and cell density: Decreasing levels of cell confluence were associated with a higher percentage of $CD138^-$ among all $GFP^+/DAPI^-$ cells. Based on the assumption that MOPCs would lose cell surface markers like CD138 more readily and earlier than they would lose a protein located in the intracellular fluid itself such as GFP, this could indicate that Syndecan-1 loss has the potential to represent earlier stages of apoptosis. For this reason, the expression profile of CD138 on MOPCs was included in the planning of future co-culture experiments, and will be analysed and evaluated in the subsequent sections.

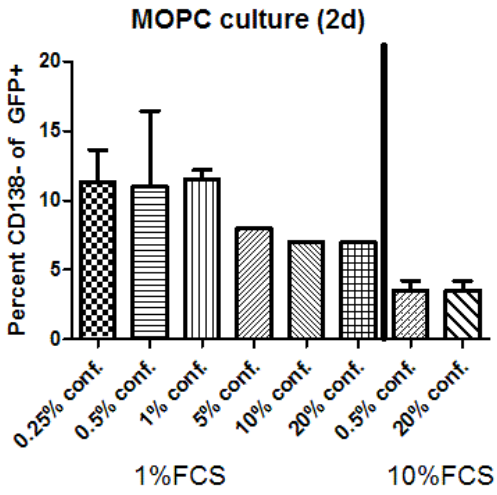

Figure 10: Left: Expression profile of CD138 in GFP^+ cells in the follow-up MOPC cultures tested for dependence on confluence. The bars indicate the mean and SD for the percentage of $CD138^-$ cells among all GFP^+ cells.

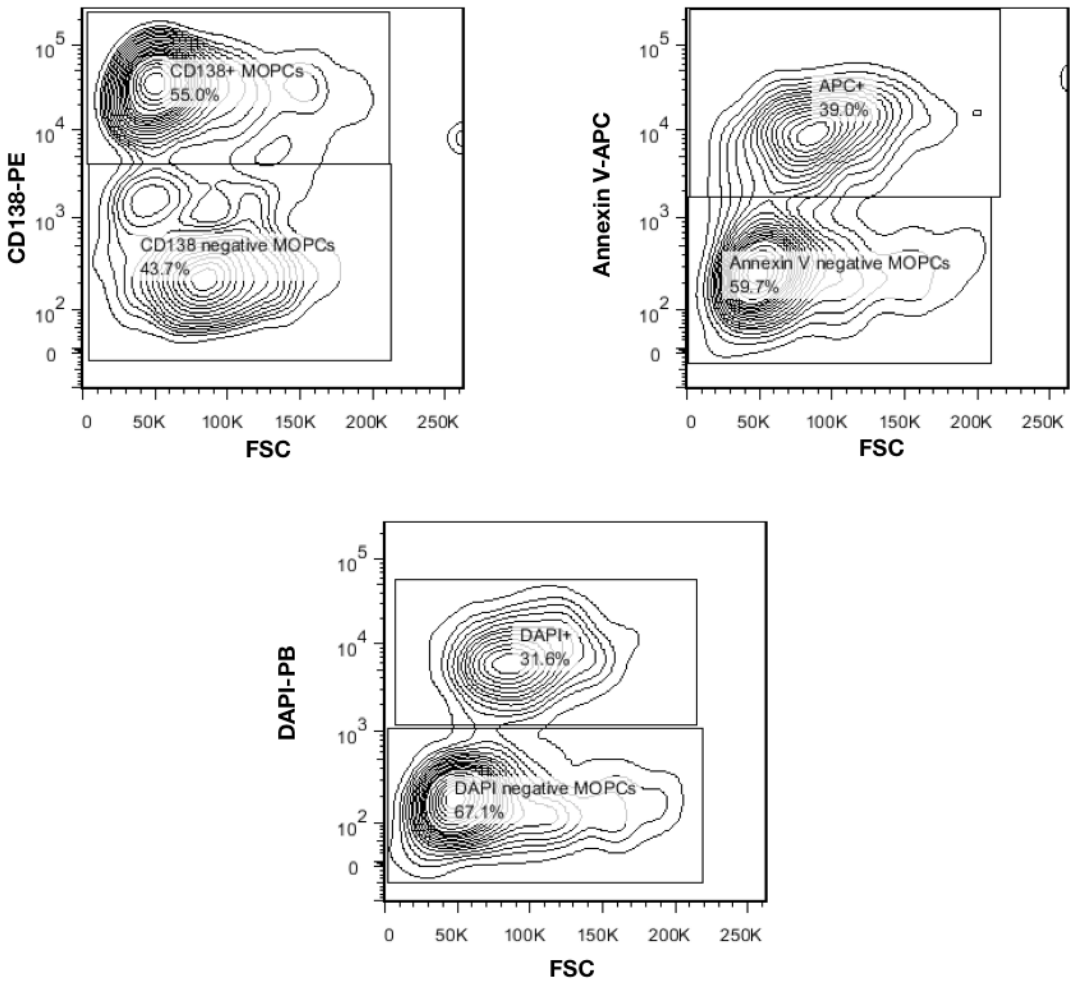

Figure 11: Exemplary flow cytometry data comparing the expression of CD138, Annexin V and DAPI of MOPCs grown over two days in medium supplemented with 1% FCS and seeded at 0.5% confluence. While 43.7% of MOPCs were negative for CD138 only 39.0% or 31.6% were positive for Annexin V or DAPI respectively.

3.2.2 Enrichment of Megakaryocytes

After determining the optimal seeding confluence of MOPCs, HNCs had to be isolated in sufficient numbers of at least 10,000 cells per well to obtain a ratio of 10:1 for HNC feeder cells to myeloma cells, while at the same time accomplishing satisfactory purity and viability.

B and T lymphocytes, as well as granulocytes were successfully and consistently isolated with a high purity and viability of over 85%. Cell viability was based on the uptake of the nuclear dye DAPI, while purity was evaluated as the fraction of PE^+ cells

of all gated cells after the removal of doublets and cell debris. After recovery from spleen or bone marrow by primary staining of their respective cell surface markers, they were marked with immunomagnetic beads and underwent manual cell separation (MACS, Miltenyi Biotec). A detailed description of methods and materials employed can be found in sections 2.2.2.4 to 2.2.2.6. Figure 12 shows exemplary flow cytometry data that has been collected for these three cell types.

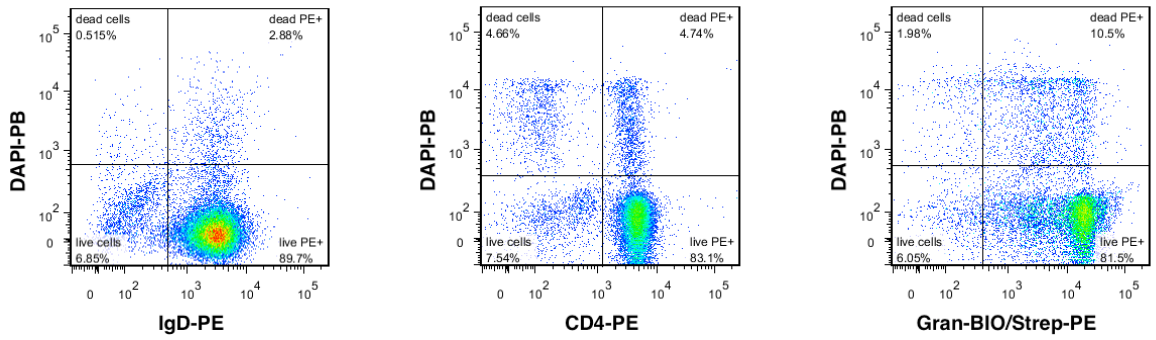

Figure 12: Flow Cytometry Data regarding purity of extraction for B cells (left), T cells (centre) and granulocytes (right) after primary immunostaining and manual cell separation. All aforementioned cell populations were consistently isolated at high purity (>85%) and viability (>85%).

Nonetheless, the isolation of viable megakaryocytes from bone marrow in sufficient quantities and purity turned out to be more challenging due to their low frequency within the bone marrow and technical difficulties based on their morphological properties and required several modifications of experimental steps involved before adequate results were achieved. As for the other cell types to be co-cultured, viability and purity of cells extracted was analysed using flow cytometry. The first co-culture experiments involved direct preparation of MKs from murine bone marrow via surface staining with CD41-FITC and was followed by manual cell separation after magnetic labelling using anti-FITC microbeads (MACS, Miltenyi Biotec). In this first attempt, absolute frequency and purity of isolated megakaryocytes were unsatisfactory (6.72% of all gated cells) even though the majority of isolated megakaryocytes was viable (87.47%).

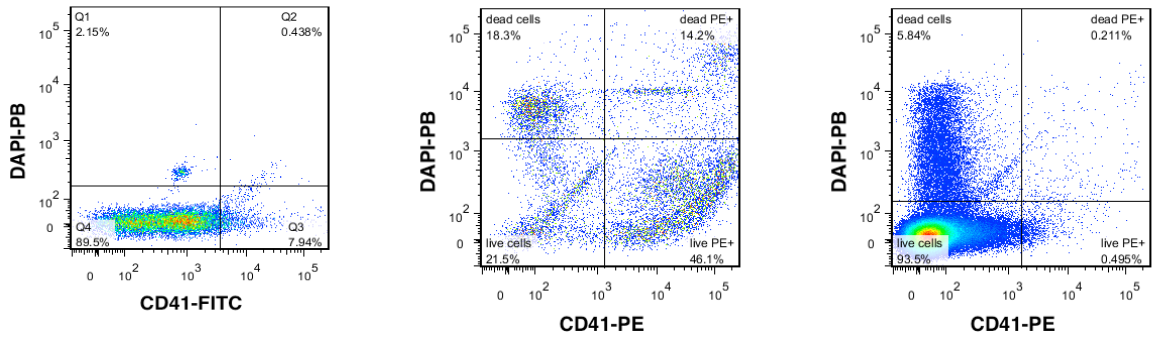

Figure 13: Left: Exemplary flow cytometry data for the first MK extraction experiment. The majority of cells isolated were viable (>95%), but only a small fraction was made up of CD41-FITC$^+$ megakaryocytes. Centre: Exemplary flow cytometry data for late MK extraction protocol, involving three-day culture with IMDM medium and twofold manual cell separation using LD cell separation columns. After the removal of cell debris, 60% of all gated cells were PE$^+$, of which 75% were viable. Right: Exemplary flow cytometry data for late MK extraction protocol, showing the MK negative fraction after manual cell separation.

The following experiment was aimed to increase the absolute number of available MKs by pre-culturing bone marrow from one BALB/c mouse in RPMI 1640 medium containing 10% FCS for 3 days with additional stimulation by TPO at a concentration of 20 ng/mL. This experiment also evaluated the effects of two different cell separation columns used during the manual cell separation steps, with the LD depletion column showing a slight enrichment of MKs at 15.12% of all selected cells, of which 72.75% were viable. The LS column, a positive selection column that had previously been only used for granulocytes as well as B and T lymphocytes, produced an inferior result for megakaryocytes, with a purity of only 3.84% at a 39.32% survival rate. For these reasons, all future manual cell separations involving MKs were henceforth performed with an LD column.

In order to further augment the absolute number of MK and MK progenitor cells, bone marrow gathered from freshly harvested tibiae and femora of two BALB/c mice were cultured for three days in IMDM medium as it has previously been described (103). A more detailed description of any other supplements that were added to this medium can be found under 2.2.2.2. In previous experiments, all isolated HNCs were kept in PBS while their purity was analysed with MACSQuant. As the enhancement of MK purification became more complex and time consuming, this proved to be rather detrimental to the survival of HNCs. With all staining and washing steps considered, the

total time between the primary extraction of HNCs and co-culture with MOPCs could range between 5 and 6 hours. For this reason, HNCs were from this point on resuspended in RPMI 1640 medium containing the respective concentrations of FCS at which they would be later co-cultured in order to maintain their viability during the course of the cell culture preparation. Other modifications included changing the primary surface marker from CD41-FITC to CD41-PE e.g., which improved the accuracy of discrimination between MKs and GFP/FITC-labelled MOPCs during flow cytometry analysis. Additionally, a second sequential cell separation step using an LD column was implemented to enhance MK separation. All of these factors combined generated a purity of 60.30%, with 76.45% of all selected cells being viable.

Despite these promising results, the generation of appropriate numbers of MKs had to undergo further calibration and enhancement, as total seeding time had meanwhile increased to a maximum of 5.5 hours and some HNCs were already found to be in early apoptosis at the time of seeding, as measured by their binding of Annexin V. To effectively address this, a different separation technique, i.e. concentration of megakaryocytes through the use of a bovine serum albumin (BSA) density gradient instead of twofold manual cell separation using depleting LD columns, was considered.

An auxiliary experiment followed aiming to evaluate the efficacy of a BSA density gradient in purifying MKs: Spare humeri from a previous co-culture experiment were used to extract bone marrow and then added to a falcon tube containing BSA which had previously been layered at different concentrations. A detailed description of materials, methods and procedures involved in preparing this BSA density gradient can be found under 2.2.2.3. After 30 minutes at ambient temperature, each fraction (Figure 6) was recovered and stained with CD41-FITC and propidium iodide. The latter serves as a DNA probe and allows the selection and separation of $FITC^+$ megakaryocytes and platelets based on their PI uptake, which ultimately is an indicator for DNA content and ploidy. The measurement of megakaryocyte frequency and ploidy distribution for each fraction was based on a modified technique of the methods described by Tomer et al. (104): After the removal of cell debris, $CD41^+$ cells were electronically gated. In the second step, PI uptake was evaluated and plotted against the number of cells using a histogram. Using this gating strategy, it was possible to determine the relative frequency

of cells with variable DNA content for each fraction, thus helping to identify the fractions that should be selected after density gradient separation in order to improve the numbers of available megakaryocytes for the co-culture experiments. In this case, Fraction 2 showed the greatest absolute number of $CD41^+$ cells, while Fraction 3 showed progressive increases in cellular DNA content, representing different stages of MK maturation. Selection of these two fractions instead of all four during MK extraction allowed the removal of 38% of bone marrow cells and an increase in the frequency of available MKs to 2.17%, this is then followed by the staining of cell surface markers and manual cell separation using immunomagnetic beads.

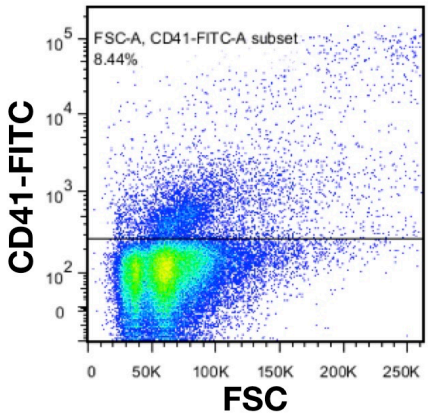

Figure 14: Pseudocolor plot showing cells labelled with CD41 vs. forward scatter (FSC), approximating cell size. In this BSA gradient experiment, $CD41^+$ cells were selected by electronically gating for the population shown above the horizontal line in this figure. The selected population was then evaluated for PI uptake as shown in the next figure.

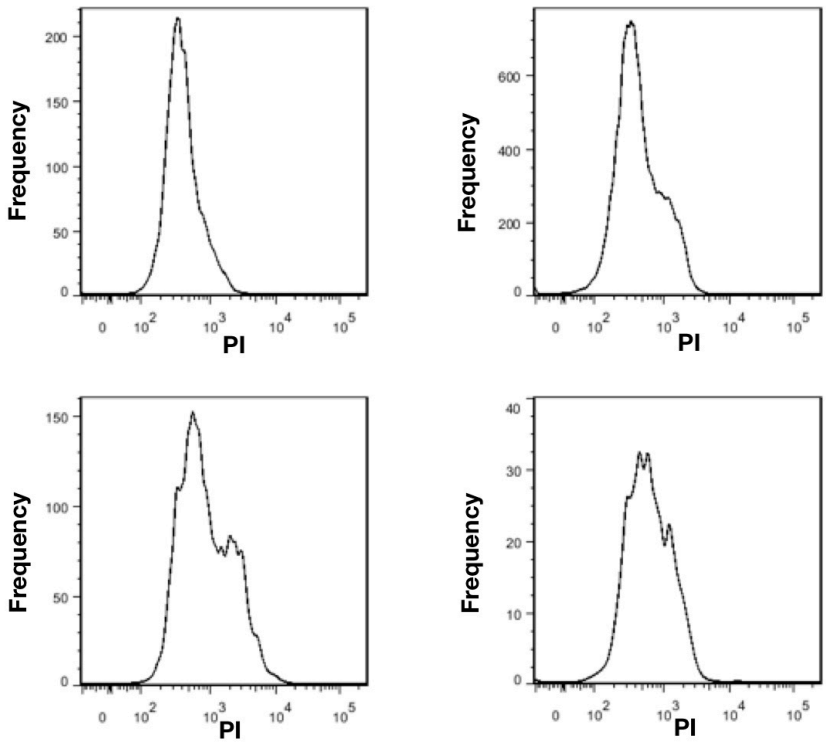

Figure 15: Propidium Iodide histogram for CD41^{+} population as gated in Figure 14, e.g. Top Left: Fraction 1 contains a single peak with regard to PI uptake and most likely does not contain MKs of different maturational stages; Top right: Fraction 2 had the largest absolute frequency of cells positive for CD41 and with variable PI uptake; Bottom left: Fraction 3 shows a great variety in DNA content reflecting different stages of MK maturation and ploidy. Bottom right: Fraction 4 also shows a variety in PI uptake, however it is also the fraction that contains the lowest number of cells.

Finally, cultivation of bone marrow from two BALB/c mice for 3 days in IMDM medium containing TPO, the additional application of a BSA density gradient before surface staining with CD41-PE, manual cell separation with one LD column, and optimizing the preparation of bone marrow suspensions by cutting off the distal end of all pipettes as described in 2.2.2.2 to account for the fragility of MKs, successfully produced satisfactory results when MKs could be isolated at a purity of 61.23% with 74.58% viability. Experimental procedures were arranged so as to minimize time lost between each step, e.g. murine spleen and bone marrow were recovered during BSA density gradient isolation or staining solutions were prepared during wash steps. All logistical considerations and practical modifications considered, total seeding time was optimized to a total of 4 hours and 20 minutes.

HNC	FCS1%		FCS10%	
	PURITY	VIABLE	PURITY	VIABLE
B Cells	85.81%	87.08%	86.62%	90.05%
T Cells	71.50%	82.80%	70.40%	84.80%
Granulocytes	90.72%	87.33%	88.61%	87.97%
Megakaryocytes	61.23%	74.58%	56.41%	82.43%

Table 8: HNC Purity and viability according to final extraction protocol.

3.2.3 Co-Culture Experiments

The primary aim of these co-culture experiments was to evaluate the influence of different HNC populations in supporting the in vitro growth of murine malignant plasma cells, using the example of the MOPC315.BM model. Employing the methods and techniques described in the above sections, HNCs were isolated from BALB/c mice and grown in co-culture with MOPC at a ratio of 10:1 (HNC to MOPC) for a total of 2 days. Altogether, eleven experiments were required before all HNCs were successfully and simultaneously isolated with sufficient purity and viability. MOPCs were seeded in triplicates at low cell density (0.5% confluence or 1000 cells per well) in RPMI medium containing either 1% or 10% FCS. MOPC growth and survival was assessed on day 0 and day 2 by means of flow cytometry using a combination of the DNA viability dyes, DAPI and Annexin V as described in 2.2.2.9. Based on the results of the preliminary experiments regarding cell confluence, the fraction of $CD138^-$ cells among all GFP^+ MOPCs was also evaluated. MOPCs that were grown alone were considered the control group (CTRL) and values for all other co-cultures were evaluated in reference to this.

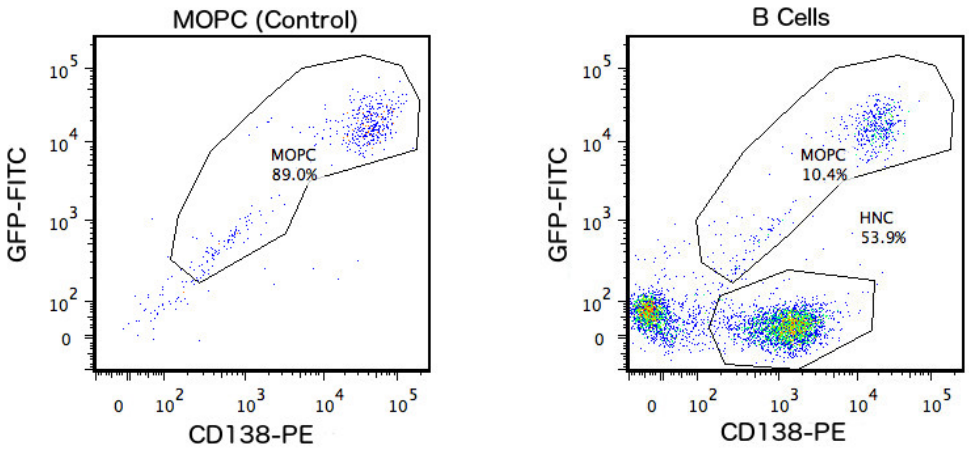

Figure 16: Exemplary flow cytometry data for MOPC grown alone and in co-culture with HNCs on day 0 in RPMI medium containing 1% FCS.

Megakaryocytes support murine myeloma cell growth under suboptimal conditions

After two days in culture, total MOPC cells were quantified by flow cytometry (MACSQuant, Miltenyi BioTec) using the same electronic gating that had been in place during day 0 analysis and which was performed immediately after seeding had occurred (ref. Fig 16). With regard to the absolute cell number of MOPCs grown in medium supplemented with 1% FCS, the control group exhibited a mean growth of 164.85% after two days. Megakaryocytes and granulocytes both represent haematopoietic niche components and have been described earlier. Co-Culture of MOPCs under suboptimal conditions with these cell types resulted in a significant increase of total MOPCs. In comparison to granulocytes, megakaryocytes especially supported MOPC growth and demonstrated a manifest two-fold increase in MOPC numbers ($p < 0.05$) corresponding to a growth rate of approximately 270%. B and T lymphocytes had no effect on MOPC growth and their cell numbers closely matched the ones for the control group.

When compared to MOPC cells grown in medium containing 10% FCS, megakaryocytes had no distinctive supportive effect on cell counts and growth. Co-cultivation of MOPCs with all other cell types under these conditions resulted in higher plasma cell numbers that were statistically significant ($p < 0.05$); this increase however was not correlated with significantly different growth rates (refer Table 9).

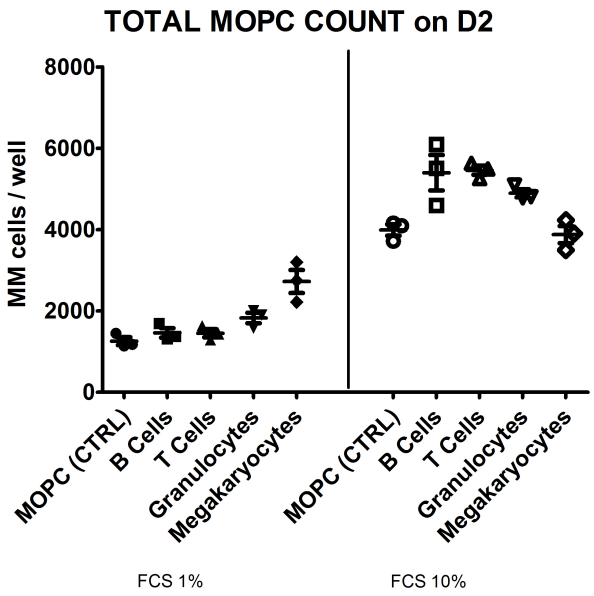

Figure 17: Total MOPC cell counts after (co-)cultivation in RPMI 1640 medium for two days with either 1% FCS or 10% FCS. Data points represent triplicate values from final experiment, mean and SD are shown.

	HNC	MEAN	STD	MEDIAN	p <0.05
FCS1%	MOPC	164.85%	19.82%		
	B Cells	166.52%	30.24%		
	T Cells	183.40%	26.40%		
	Granulocytes	227.75%	37.33%		
	Megakaryocytes	267.53%	47.13%		✔
FCS10%	MOPC	649.43%	159.57%	680.73%	
	B Cells	696.68%	76.47%		
	T Cells	881.72%	80.26%		
	Granulocytes	732.88%	165.20%	728.96%	
	Megakaryocytes	461.40%	17.78%		

Table 9: Total MOPC cell growth after culture in RPMI supplemented with 1% FCS or 10% FCS for two days. Average mean based on triplicate values from final experiment with standard deviation, median and significantly different means (p<0.05) when compared to control group

Megakaryocytes support the survival of myeloma cells under suboptimal conditions

After evaluating the changes in total MOPC cell numbers depending on co-cultivation with different HNC populations, flow cytometry analysis now focused on the overall survival of MOPCs, employing the viability dyes DAPI and Annexin V to establish

subgroups of live, early-apoptotic and late-apoptotic MOPCs as described above in 2.2.2.9.

The first parameter to be analysed was the ratio of viable, i.e. DAPI and Annexin V negative myeloma cells on day 2 in comparison to the day of seeding. When grown under suboptimal conditions, there were approximately 1.5 times as many viable cells on day 2 than day 0 for the control group. While this increase in live cells was consistent for all other cell types, co-cultivation with megakaryocytes specifically substantiated the strongest supportive effect on survival and was associated with a three-fold increase in viable cells ($p<0.05$) over two days, complementing the changes in absolute MOPC numbers and growth associated with co-cultivation with megakaryocytes as observed above. When grown in medium supplemented with 10% FCS, there was no significant effect of any HNC or B cells and T cells on the ratio of viable cells after two-day cultivation.

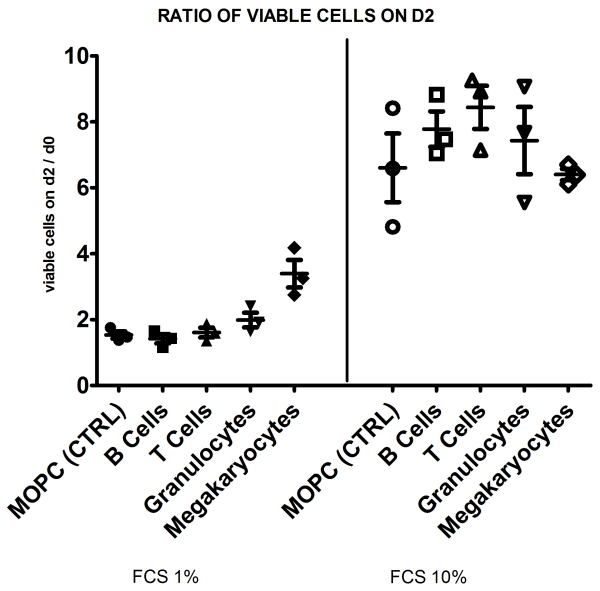

Figure 18: Ratio of viable MOPCs on day 2 compared to day 0. Data points represent triplicate values from final experiment, mean and SD are shown.

In comparison to the ratio of viable cells on day 2 and day 0, the relative fraction of viable (DAPI and Annexin V negative) and early apoptotic (DAPI negative, Annexin V positive) MOPC cells after two-day cultivation provided additional insights with regard to the influence of the different cell types that were examined on overall plasma cell

survival. On average, a monoculture of MOPCs resulted in approximately 85% viable and 10% early apoptotic cells. MOPCs grown in conjunction with megakaryocytes in RPMI medium containing 1% FCS yielded the highest relative frequency of live cells with a mean of 93.07% ($p<0.05$), while simultaneously demonstrating a four- to five-fold decrease in the overall fraction of early apoptotic cells (median 2.11%, $p<0.005$) when compared to control. Co-cultivation with granulocytes on the other hand was correlated with a slight, statistically not significant increase in MOPC viability (mean 88.13%) and approximately half as many early apoptotic cells (mean 5.78%, $p>0.05$). It should also be noted that MOPCs grown with B lymphocytes were associated with a statistically significant lower proportion of viable cells (mean 82.22%, $p <0.05$) and a higher proportion of early apoptotic cells (mean 12.02%, $p <0.05$).

Under optimal conditions, i.e. in medium supplemented with 10% FCS, co-cultivation with T lymphocytes was linked to a significantly lower percentage of viable cells (mean 84.37%, $p <0.05$) in comparison to the control group (mean 93.10%). There was no significant effect of any HNC or B and T cells on the relative frequency of early apoptotic cells.

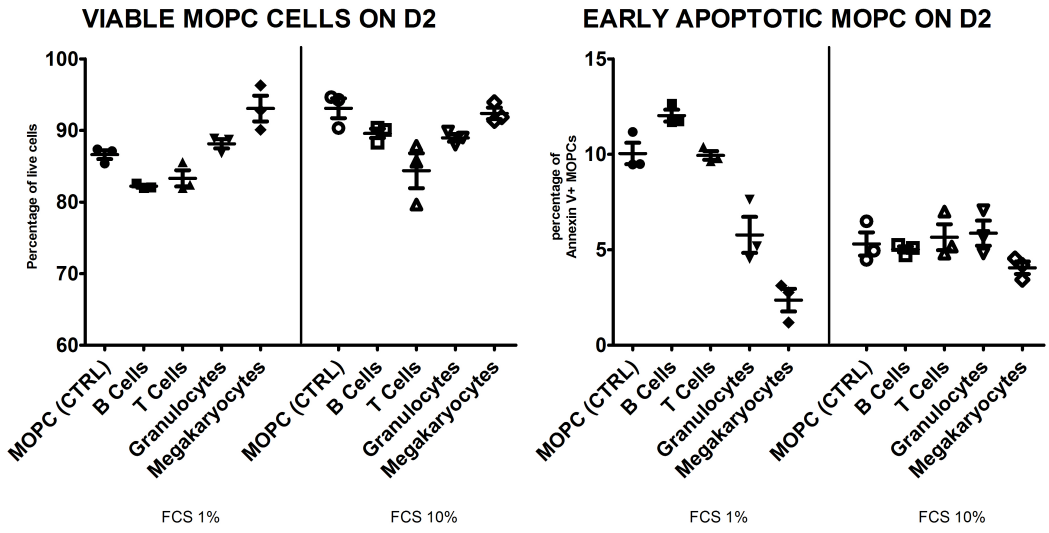

Figure 19: Relative frequency of live (DAPI and Annexin V negative) and early apoptotic (DAPI negative and Annexin V positive) MOPCs after culture in RPMI 1640 medium with either 1% FCS or 10% FCS for two days. Data points represent triplicate values from final experiment, mean and SD are shown.

To summarize, this data confirms that megakaryocytes support MOPC growth and survival, with an emphasis on the latter as evidenced by overall higher frequencies of viable cells and a lower percentage of early apoptotic cells when compared to the control group. This supportive effect of megakaryocytes can be seen in particular under suboptimal conditions and is in agreement with the notion that megakaryocytes constitute an important component of the haematopoietic survival niche through the production of pro-survival cytokines and thus promote the survival of plasma cells.

CD138 is a potential marker of early apoptosis

Based on the promising results during the incipient confluence experiments, the last parameter to be evaluated was the expression profile of the plasma cell surface marker CD138 or Syndecan-1 as a potential marker of early apoptosis for MOPCs. Firstly, all GFP^+ MOPCs were selected by electronic gating and subdivided into a Syndecan-1 positive and negative population. The latter was then further analysed for expression of Annexin V. Under suboptimal conditions, i.e. in the medium supplemented with 1% FCS, MOPCs co-cultured with MKs possessed the lowest fraction of $CD138^-$ cells (mean 9.04%, $p < 0.005$) in comparison to the MOPC control group (mean 13.67%), which is in accordance with the earlier observation that megakaryocytes promote the survival of murine multiple myeloma cells. When grown in the medium with 10% FCS, MOPCs co-cultured with T lymphocytes (mean 5.28%) and granulocytes (mean 5.50%) exhibited a slight, but statistically significant (both $p<0.05$) higher percentage of $CD138^-$ MOPCs than the control group (mean 3.86%).

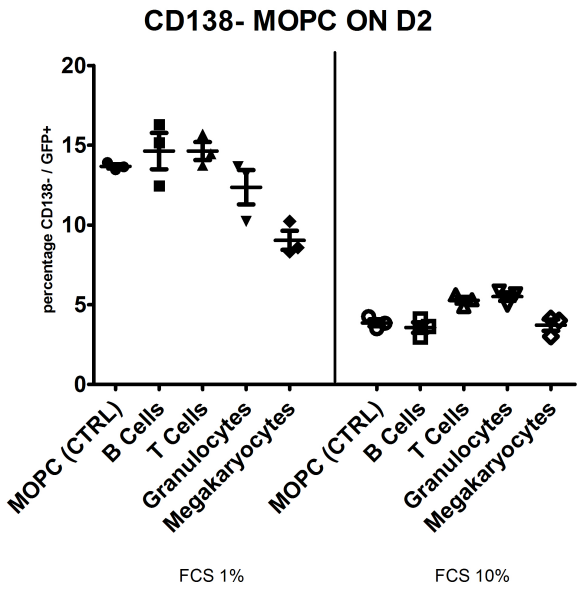

Figure 20: Percentage of CD138$^-$/GFP$^+$ MOPCs after (co-) cultivation in RPMI 1640 medium with either 1% FCS or 10% FCS for two days. Data points represent triplicate values from final experiment, mean and SD are shown.

		MEAN	STD	MEDIAN	p <0.05
FCS1%	MOPC	53.70%	5.05%		
	B Cells	54.53%	8.41%		
	T Cells	47.87%	7.51%		
	Granulocytes	53.00%	6.36%		
	Megakaryocytes	29.77%	25.31%	41.70%	
FCS10%	MOPC	45.00%	1.13%		
	B Cells	58.63%	6.65%		
	T Cells	56.03%	3.16%		
	Granulocytes	43.73%	3.35%		
	Megakaryocytes	40.43%	4.22%		

Table 10: Percentage of Annexin V$^+$ of all CD138$^-$ MOPCs after culture in RPMI with 1% FCS or 10% FCS for two days. Average mean based on triplicate values from final experiment with standard deviation, median and relative change when compared to control group.

Based on the assumption that the shedding of CD138 could represent an earlier stage of apoptosis, the following examination compared the overall percentage of early apoptotic MOPCs to that of Syndecan-1 negative MOPCs after two days growth in co-culture. Under suboptimal conditions, this was associated with a significantly higher fraction of CD138$^-$ than Annexin V positive cells for the control group ($p < 0.005$) as well as for T cells ($p < 0.005$), granulocytes ($p < 0.05$) and megakaryocytes ($p < 0.005$). When grown under optimal conditions, differences between Annexin V positive and CD138

negative MOPCs were only significant for B Cells ($p < 0.05$) however, these changes were associated with a decrease in the percentage of $CD138^-$ cells for all cell types that were analysed.

		Annexin V+	CD138-	p <0.05
FCS1%	MOPC	10.05%	13.67%	✔
	B Cells	12.02%	14.63%	
	T Cells	9.94%	14.63%	✔
	Granulocytes	5.78%	12.36%	✔
	Megakaryocytes	2.11%	9.04%	✔
FCS10%	MOPC	5.30%	3.86%	
	B Cells	4.74%	3.57%	✔
	T Cells	5.66%	5.28%	
	Granulocytes	5.87%	5.50%	
	Megakaryocytes	4.06%	3.72%	

Table 11: Comparison of relative frequency of Annexin V^+ and $CD138^-$ MOPCs on day 2. Data based on average mean values from figures 19 and 20.

The final analysis juxtaposed the expression patterns of Annexin V and CD138 in MOPCs and demonstrated a clear dichotomy between MOPCs that are either positive for Syndecan-1 or positive for Annexin V, in concordance with previous research by Jourdan and colleagues (102), who showed that Syndecan-1 was present on viable, human myeloma cells and was rapidly lost upon induction of apoptosis. This observation was consistent for all cell types and indicates that the loss or shedding of Syndecan-1 could be a promising marker for very early, intermitting apoptosis that would precede externalization of phosphatidylserine as measured by Annexin V uptake.

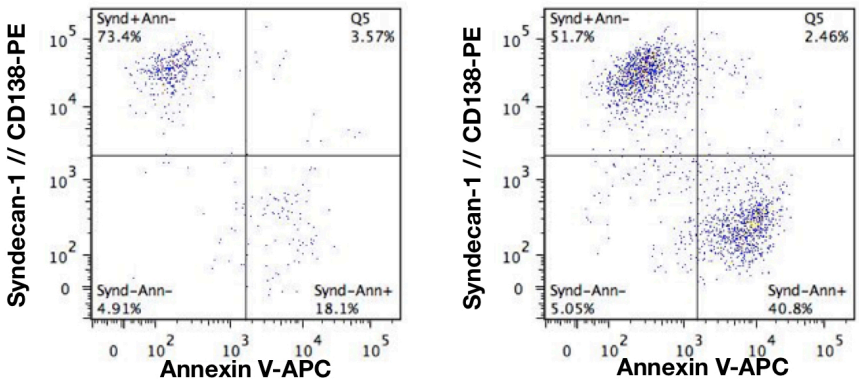

Figure 21: Exemplary flow cytometry Data juxtaposing the expression of CD138 vs. Annexin V for gated MOPCs on day 0 (left) vs. day 2 (right).

4. Discussion

4.1 Discussion of Main Results

4.1.1 Megakaryocytopoiesis and Plasma Cell Survival

Long-lived plasma cells in the bone marrow possess a crucial role in the pathogenesis of diseases associated with the production of autoreactive or malignant antibodies. Their prolonged survival depends on anti-apoptotic stimuli generated inside a suitable microenvironment, the so-called multi-component plasma cell survival niche composed of cellular and humoral components (2); among the former, cells of haematopoietic origin including megakaryocytes (34), eosinophils (30), basophils (31, 32), monocytes and other myeloid cells like dendritic cells and macrophages (29) have been implicated to promoting plasma cell survival by producing and providing essential pro-survival cytokines such as IL-6, APRIL or BAFF (35).

Among these, megakaryocytes represent one particularly important haematopoietic niche component, as evidenced by c-mpl knockout mice that presented with lower numbers of plasma cells in accordance with lower megakaryocyte counts (34). Similarly, eosinophils (30) have also been shown to co-localize with long-lived plasma cells in their bone marrow survival niches, and eosinophil depletion has also been correlated with the loss of plasma cells. The principal objective of this thesis was to investigate the influence of megakaryocytes and megakaryocytopoiesis on the survival of plasma cells within their niches in autoimmune disease using the example of SLE as well as in malignant neoplastic disease using the example of multiple myeloma.

4.1.2 Gene Sequence Analysis of c-mpl Exon 5

In the first part of this thesis, genomic DNA from a cohort of 40 SLE patients was amplified and screened for a mutation in exon 5 of the c-mpl gene, the receptor for TPO and a key regulator of megakaryocytopoiesis. Mutations in this gene can be either loss-of-function mutations as it has been previously described for congenital amegakaryocytic thrombocytopenia (105) or gain-of-function mutations as seen in

familial essential thrombocythaemia (106). The analysis performed in this study was based on previous research by Chen and colleagues (42) who identified the presumed minimum-binding domain for TPO within the c-mpl cytokine receptor of which amino acid residues Aspartate^{235} and Leucine^{239} in exon 5 have been outlined as particular crucial binding sites for TPO interaction. In addition to this, unpublished data from our research group described a single nucleotide polymorphism in proximity of this gene location in SLE-prone mice. In this context, the primary working hypothesis was that evidence for a similar mutation in the human thrombopoietin receptor gene of SLE patients could be associated with an increased activation of downstream signalling pathways that are involved in megakaryocytopoiesis. In turn, an increase in the number of available megakaryocytes should be accompanied by more favourable survival conditions for autoreactive plasma cells due to an augmented production of pro-survival cytokines like IL-6 and APRIL. However, there have only been rare reports of thrombocytosis in SLE while on the other hand lupus-associated thrombocytopenia has previously been linked to the presence of anti-c-mpl antibodies (107).

This study could not draw a proper conclusion with regard to this issue: No mutation within exon 5 of the human c-mpl gene was found by me, and a subsequent examination of downstream signalling using an enzyme-linked immunosorbent assay (ELISA) showed a different pattern of phosphorylation of Akt in both NZB and NZW mice. There was however, no conclusive evidence for an increased phosphorylation of Akt in these mice. Nonetheless, this does not exclude the existence of a potential SNP in another gene locus with the ability to modify c-mpl function or that other signalling pathways other than PI3K/Akt could be affected in SLE.

It should be noted that this analysis was performed in a small cohort of only 40 SLE patients and future studies could deliver more insight when a larger population is recruited and examined. Initially, these studies could investigate whether SLE patients have a higher megakaryocyte count than healthy subjects. Research could also focus on potential mutations within the other exons that encode for the extracellular cytokine receptor domain, namely exons 2 to 9. In addition to this, there have been reports of point mutations in other regions of the c-mpl gene like the transmembrane or juxtamembrane region correlating with an activation of c-mpl signalling pathways despite the absence of TPO and presenting with clinical signs typical of

myeloproliferative disorders (108). Likewise, an examination of other downstream signalling cascades such as MAPK/ERK (66) or JAK/STAT (67) as well as non-TPO-dependent signalling pathways such as notch and integrin signalling or the CXCL12/CXCR4 axis (109) could be considered for future research in order to elucidate potential alterations in megakaryocytopoiesis and their resulting influence on the longevity of autoreactive long-lived plasma cells within the bone marrow survival niche.

4.1.3 Co-Culture Experiments with MOPC315.BM

4.1.3.1 MOPC Growth in Dependence of Confluence

The second, subsequent part of this thesis similarly aimed to investigate the influence of megakaryocytes on the survival of (malignant) plasma cells by co-culturing selected haematopoietic niche components in conjunction with the murine MOPC315.BM cell line. MOPC315.BM myeloma cells can be used to study the microenvironment of long-lived plasma cells as they express CXCR4, which mediates homing and adhesion of cells to the bone marrow survival niche by acting as a receptor for CXCL12 produced by CAR cells. Recent research by Wong et al. (110) demonstrated that MOPC315.BM growth could be stimulated by the external growth factors IL-6 and APRIL which to a large part are produced by megakaryocytes as mentioned above. Earlier studies have shown that IL-6 is an essential tumour growth factor in multiple myeloma (111) as well as being involved in myeloma-associated drug-resistance (112). In their investigation, MOPCs were seeded at low or high cell density and cultivated in medium that contained either 1% or 5% FCS. When seeded at low cell confluence, the addition of the pro-survival cytokines IL-6 and/or APRIL led to an increased growth of MOPCs irrespective of the concentration of FCS that had been added to the medium. At higher cell confluences however, this effect was not as prominent, suggesting that under these given conditions the need for external growth factors is diminished. This can be explained by the fact that myeloma cells possess the ability to stimulate themselves through autocrine production of IL-6 as previously reported by Frassanito and colleagues (74) or also by supplying additional cell-to-cell contacts as proposed by the authors themselves (110).

Our incipient experiments confirmed this dependence on confluence for MOPCs grown in vitro: Cell density had virtually no impact on the frequency of MOPC survival when cultured in medium containing 10% FCS, whereas there were vast differences when these cells were cultured in medium containing only 1% FCS: Any MOPCs that were grown at cell densities higher than 1-5% confluence were not linked to a higher rate of surviving cells, suggesting that a seeding confluence of less than 1% will ensure that MOPCs are dependent on external survival factors and are at the same time not capable of auto-stimulation. Cell confluences lower than 0.25% (500 cells) did not yield a utilisable result, possibly due to cells being lost during the process of staining. However this did not have an impact on the seeding confluence of 0.5% (1000 cells) that was ultimately chosen for the co-culture experiments.

4.1.3.2 Megakaryocyte Extraction

Regarding the subsequent experimental course of this thesis, the separation of megakaryocytes from bone marrow proved to be one of the more challenging parts of the co-culture experiments, due to their morphological properties, their tendency to aggregate, and their relative distribution in the bone marrow. They are more fragile due to their larger cell size and therefore more susceptible to destruction by shearing forces and they only constitute approximately 0.1% of all nucleated cells within the bone marrow (113). Over the last few decades, a variety of techniques have been proposed to increase the number of purified megakaryocytes for in vitro use: Velocity sedimentation (114), density gradient centrifugation (115), counterflow centrifugal elutriation (116), fluorescence-activated cell sorting (117) and manual cell separation using immunomagnetic beads (118) to name but a few. After various attempts, we successfully isolated megakaryocytes in sufficient numbers for co-cultivation by means of a combination and adaptation of several techniques: Firstly, a 3-day bone marrow culture that was stimulated with TPO in order to increase the total number of available megakaryocytes (103), enhancing cell purification through a BSA density gradient centrifugation (modified from Nakeff et al. (115)) and primary surface staining with CD41-PE followed by magnetic activated cell sorting (118). All suspension steps were performed with the distal end of the pipette tip cut off as previously described and suggested by Levine et al. (95).

The removal of the distal pipette tip proved to be one of many crucial features that enabled the successful isolation of megakaryocytes for the purpose of cultivation. Damage to the cell membrane when single cells suspensions were created could have been responsible for a higher fraction of dead or apoptotic cells during earlier cell preparations. Attempts to improve the purity of megakaryocyte extraction also included modification of manual cell separation techniques e.g. by depletion of unwanted cells followed by positive selection of $CD41^+$ cells. However, it should be taken into account that with regard to the experiments performed in this thesis the total extraction time of HNCs turned out to be a decisive limiting factor: For this reason, the introduction of any additional purification step would have to be carefully considered as it could potentially have a tremendous impact on the overall survival of the isolated cells. A potential strategy to address this is to increase the total number of available megakaryocytes that can be harvested and to further evaluate and optimize the conditions in which purified megakaryocytes can survive as long as possible before being seeded in co-culture with MOPCs: Panuganti et al. e.g. recently described a method that promoted in vitro expansion and maturation of megakaryocytes by optimizing environmental conditions in terms of providing optimal pH and partial oxygen pressure in conjunction with a mix of several cytokines including TPO, SCF in high doses, as well as IL-3, IL-6 and IL-11 (119).

4.1.3.3 Megakaryocytes Support MOPC Growth & Survival

Megakaryocytes have been shown to co-localize with long-lived plasma cells (34) and MOPCs (110), they home in to the plasma cell niche via the CXCL12/CXCR4 axis and presumably contribute to the multi-component plasma cell niche in autoimmune disease and multiple myeloma by being a major source of the aforementioned pro-survival cytokines IL-6 and APRIL, which support both long-lived plasma cells and myeloma cells (32, 34, 69).

Complementing these previous observations, it is demonstrated here that MKs are capable of supporting MOPC growth in vitro: Co-cultivation with megakaryocytes under suboptimal conditions in medium with added 1% FCS was associated with the highest increase of total MOPC cell numbers and was also accompanied by the largest growth

rate over two days. This effect did not persist when MOPCs were cultivated in medium containing 10% FCS. Notably, co-culture with MKs also revealed an even more pronounced effect on the survival of myeloma cells: MOPCs grown together with megakaryocytes did not only represent the population with the highest number of viable cells after two days, but co-cultivation was also associated with the highest percentage of total viable cells and the lowest fraction of early apoptotic and $CD138^-$ cells. Once again, this result was only observed under suboptimal conditions, for MOPCs grown in medium supplemented with 1% FCS, indicating that these supportive properties of megakaryocytes might only be relevant under suboptimal conditions at which MOPCs are most dependent on the external supply of local growth factors by appropriate HNCs.

4.1.3.4 Influence of Granulocytes on MOPC Growth & Survival

Granulocytes have also been shown to contribute to the plasma cell niche: Previous research by Chu and colleagues demonstrated that eosinophils, which also express CXCR4 and hence can be recruited to the survival niche via CXCL12, are localized in close proximity to long-lived plasma cells and contribute to plasma cell survival by producing the survival factors APRIL and IL-6 as well as supporting the differentiation of plasma blasts to plasma cells (30). Denzel et al. (32) described a similar role for basophils: IL-6 and IL-4 derived from activated basophils was associated with B cell proliferation and supported antibody production in the presence of activated T cells. As shown here, granulocytes supported both the growth and survival of the MOPC315.BM cell line, but not to the same extent as co-cultivation with megakaryocytes did.

Nonetheless, it should be taken into account that granulocytes were not separated into their subtypes for the purpose of co-cultivation with MOPCs. A preliminary experiment attempting to sort eosinophils, neutrophils and basophils by means of flow cytometry was not successful, as the isolated cells were not viable and therefore not suitable for co-cultivation. Since the primary focus of this thesis was to elucidate the impact of megakaryocytes and megakaryocytopoiesis on plasma cell survival, and since performing an additional sorting step would have further increased the total recovery time for all isolated cells, granulocytes were purified as a whole and co-cultured as such

with MOPCs. As a result, the supportive effect of granulocytes on MOPC growth and survival observed in these co-culture experiments cannot recognizably be attributed to the influence of a single granulocyte population like eosinophils or basophils alone, as previous research would indeed suggest. Moreover, neutrophils constitute the vast majority of all granulocytes and it cannot be ruled out with certainty that they represent one of the cell populations responsible for supporting MOPCs in co-culture, as they also co-localize with plasma cells and have recently been implicated as a key component of the BM microenvironment in human SLE and murine models as evidenced by local production of IFN-a, APRIL and BAFF (33). Based on these facts, a prospective study could, e.g. focus exclusively on the examination of granulocyte subpopulations and their supportive effect on MOPC growth and survival, in conjunction with a study of cytokine production for each cell population.

4.1.3.5 CD138 is a Suitable Marker for Very early Apoptosis

Syndecan-1 or CD138 is a heparan sulfate proteoglycan that functions as an integral membrane protein and mediates various tasks, including cell signalling, proliferation and binding to extracellular matrix proteins. Its expression is subject to up- and down-regulation during B Cell differentiation (120), it serves as an immunohistochemical marker for both normal and malignant plasma cells (121) and high serum levels are correlated with a poor clinical outcome in patients with multiple myeloma (83). Several findings indicate that Syndecan-1 plays a key role in the pathogenesis of MM: Yang et al. (122) found that CD138 was able to promote tumour growth and metastasis in an in vivo model of multiple myeloma, while other research revealed its role as an important regulator of the bone marrow microenvironment, e.g. by mediating intercellular adhesion of myeloma cells (123) or to type 1 collagen (124).

Jourdan et al. have demonstrated that Syndecan-1 is only found on viable multiple myeloma cells and promptly released during early apoptosis (102). The experimental data put forward in this thesis is in agreement with this and revealed a distinct dichotomy of MOPCs that are positive for either CD138 or Annexin V, independently of the cell population that they were co-cultured with. In combination with an intermediary

population of cells that had already lost CD138, but was still negative for Annexin V, this observation could be possibly explained by an apoptosis sequence in which MOPCs lose the plasma cell surface marker CD138 first before externalizing phosphatidylserine from the cytoplasmic side to the outer cell membrane which then can be detected and measured by Annexin V. Together, these results indicate that the loss or shedding of Syndecan-1 is a promising marker for very early, intermitting apoptosis preceding Annexin V uptake.

Staining for CD138 could be used in combination with other viability dyes in subsequent studies to evaluate plasma cell survival, while selective gating strategies could allow the identification of distinct subpopulations each representing a separate stage of apoptosis. Nevertheless the dichotomy between CD138 expression and Annexin V uptake was observed in experiments with malignant plasma cells and will require further analyses with normal plasma cells in order to ascertain whether the shedding of CD138 is indeed an appropriate marker for very early apoptosis. At the same time, the immediate loss of CD138 upon the initiation of programmed cell death also implies that cell samples have to be handled with great care and that environmental conditions, including the kind of medium used, temperature, pH etc. can have a great impact on cell survival and consequently CD138 expression.

4.2 Outlook and Concluding Remarks

Long-lived plasma cells require a special microenvironment with specific conditions guaranteeing continuous survival. The multi-component plasma cell niche has been at the centre of ongoing research regarding the pathogenesis of diseases involving autoreactive or malignant plasma cells and the vast multitude of elements that are implicated permits an extensive selection of promising targets for further examination. Insights gained from these studies can potentially open up new treatment options – in particular to provide alternatives for patients that are refractory to conventional therapy. In autoimmune disease such as SLE, an effective intervention could combine depletion of short-lived plasma cells (e.g. with rituximab) with specifically targeting one or several of the many mechanisms involved in the maintenance of LLPCs within their survival niche. It should however be noted that protective antibodies could also be affected by

such a treatment, possibly explaining some adverse events observed in randomized clinical trials associated with B cell targeted therapy.

Megakaryocytes have been previously described as a key haematopoietic niche component, and the primary focus of this thesis was to confirm their influence on plasma cell survival. The significance of the experimental evidence put forward here merits further investigation in greater depth, as megakaryocytes were shown to promote the survival and growth of murine malignant plasma cells in co-culture. These *in vitro* observations also complement the findings by Wong et al. who demonstrated *in vivo* that MOPC.315BM cells co-localize with megakaryocytes (110). Furthermore, Winter et al. previously showed that manipulation of megakaryocyte counts had a noticeable impact on the survival of plasma cells: Stimulation with TPO was correlated with higher plasma cell numbers during a specific immune response, whereas c-mpl-deficient mice showed an absolute reduction of plasma cells in the bone marrow (34).

Aside from megakaryocytes, there are other HNCs implicated in the multi-component plasma cell survival niche such as eosinophils, basophils and neutrophils, and detailed studies of distinct granulocyte subpopulations could confirm a stimulatory effect on plasma cell survival as suggested earlier. Likewise, this can be applied to other cells with potential involvement including monocytes (125), dendritic cells (126) and macrophages (127) or osteoclasts for the survival niche in multiple myeloma (75). The stromal cells that constitute the scaffolding framework within the bone marrow microenvironment also represent a prospective target for future research: Patterns of recruitment, homing and adhesion to the niche, mainly mediated by the CXCR4-CXCL12 and to a smaller extent by the VCAM-1-VLA4 axis, have a profound effect on the relative distribution of niche components and soluble cytokines and can influence long-term plasma cell survival. Manipulation on a ligand (CXCL12 or VLA4) or receptor (CXCR4 or VCAM-1) level could thus impede cell adhesion and attachment or even induce dislocation of resident plasma cells, as already indicated by continuing research with CXCR4 inhibitors such as AMD3100 (91). CD28 is a co-stimulatory molecule found on T cells and its signalling has also been linked with the long-term survival of bone marrow plasma cells in murine experimental studies (128) as well as human myeloma cells (129) and might potentially open up additional treatment options.

HNCs as well as stromal CAR cells contribute to the multi-component plasma cell niche by producing cytokines that confer resistance to apoptosis. Of these, APRIL, BAFF and IL-6 have been extensively studied and specified as essential survival factors for LLPCs, yet there is no scarcity of other growth-promoting cytokines that might also be implicated, including IGF-1 (130), TNF-α, TGF-β, VEGF, IL-4 and IL-5 (37, 131). Experimental evidence by Cassese and colleagues showed that long-lived plasma cells require stimulation by not one but multiple growth factors acting together in synergy (37). Megakaryocytes and eosinophils are found in close proximity to LLPCs and both have been put forward as major local sources of either IL-6 or APRIL (30, 34) in the bone marrow survival niche, a role that is also assumed by osteoclasts in the MM microenvironment. Belimumab, a monoclonal antibody against BAFF, has recently been approved for the treatment of SLE. Large-scale clinical trials have confirmed a mild beneficial effect that allowed for lower prednisone doses and was correlated with reduced disease activity (132). Atacicept, on the other hand, is a recombinant fusion protein between human IgG and the TACI receptor, which can inhibit both APRIL and BAFF. Despite encouraging results owing to reduced titres of anti-dsDNA antibodies and a decrease in the overall B cell count, there have been safety concerns due to two fatal infections which were however not associated with hypergammaglobulinaemia (133, 134). Tocilizumab is a monoclonal antibody against the IL-6 receptor and is mainly used for the treatment of rheumatoid arthritis. One open-label phase I study in patients with SLE showed an improvement in disease activity scores and lower levels of antibodies against double-stranded DNA (135). In summary, targeting individual survival factors on a receptor level (IL-6 receptor or TACI receptor antibody) has the potential to complement already existing therapeutic regimens: Thalidomide e.g. is an immunomodulatory drug that down-regulates the production of IL-6 within the bone marrow survival niche in MM (136), while the proteasome inhibitor Bortezomib down-regulates the transcription of VEGF, IGF-1 and IL-6 (137) and suppresses signalling cascades mediated by IL-6 (138).

To conclude, understanding megakaryocytopoiesis, including the detailed mechanisms that control cell survival and proliferation has the potential to deliver new insights into the pathogenesis of diseases defined by an aberrant production of autoimmune or malignant antibodies by long-lived plasma cells. The mechanisms that define the maintenance of LLPCs in their bone marrow niche are being progressively researched

and new discoveries permit the development of alternative therapeutic strategies, which can modify specific components of the bone marrow survival niche. One promising novel strategy that could influence plasma cell survival may be to target a combination of several niche components: Depleting cellular components such as megakaryocytes and eosinophils simultaneously, in conjunction with blocking the function of essential pro-survival cytokines, has the potential to alter the composition of the microenvironment and deprive plasma cells of vital anti-apoptotic cues. Combined therapeutic strategies such as these can minimize the toxicity of conventional immunosuppressive treatment and improve overall clinical benefit for patients by reducing treatment-associated side effects and can manage disease flare-ups and progression by preventing the regeneration of pathogenic antibodies.

5. References

1. Janeway CA Jr TP, Walport M, Travers P, Shlomchik MJ. Immunobiology: The Immune System in Health and Disease. 5th Edition. New York: Garland Science; 2001.
2. Tangye SG. Staying alive: regulation of plasma cell survival. Trends in immunology. 2011;32(12):595-602.
3. Fairfax KA, Kallies A, Nutt SL, Tarlinton DM. Plasma cell development: from B-cell subsets to long-term survival niches. Seminars in immunology. 2008;20(1):49-58.
4. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. The New England journal of medicine. 2007;357(19):1903-15.
5. Vieira P, Rajewsky K. The half-lives of serum immunoglobulins in adult mice. European journal of immunology. 1988;18(2):313-6.
6. Cooper EH. Production of lymphocytes and plasma cells in the rat following immunization with human serum albumin. Immunology. 1961;4:219-31.
7. Ho F, Lortan JE, MacLennan IC, Khan M. Distinct short-lived and long-lived antibody-producing cell populations. European journal of immunology. 1986;16(10):1297-301.
8. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. Nature. 1997;388(6638):133-4.
9. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. Immunity. 1998;8(3):363-72.
10. Manz RA, Arce S, Cassese G, Hauser AE, Hiepe F, Radbruch A. Humoral immunity and long-lived plasma cells. Current opinion in immunology. 2002;14(4):517-21.
11. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Schaller JG, Talal N, Winchester RJ. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis and rheumatism. 1982;25(11):1271-7.
12. Sestak AL, Furnrohr BG, Harley JB, Merrill JT, Namjou B. The genetics of systemic lupus erythematosus and implications for targeted therapy. Annals of the rheumatic diseases. 2011;70 Suppl 1:i37-43.
13. Harley JB, Moser KL, Gaffney PM, Behrens TW. The genetics of human systemic lupus erythematosus. Current opinion in immunology. 1998;10(6):690-6.
14. Simard JF, Costenbader KH, Liang MH, Karlson EW, Mittleman MA. Exposure to maternal smoking and incident SLE in a prospective cohort study. Lupus. 2009;18(5):431-5.
15. Manzi S. Epidemiology of systemic lupus erythematosus. The American journal of managed care. 2001;7(16 Suppl):S474-9.
16. Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. Nature reviews Rheumatology. 2010;6(5):280-9.
17. Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. Seminars in arthritis and rheumatism. 2004;34(2):501-37.
18. Hiepe F, Dorner T. [Autoantibodies and antibody-secreting cells]. Zeitschrift fur Rheumatologie. 2005;64(6):389-95.
19. Hoyer BF, Moser K, Hauser AE, Peddinghaus A, Voigt C, Eilat D, Radbruch A, Hiepe F, Manz RA. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. The Journal of experimental medicine. 2004;199(11):1577-84.

20. Liu Z, Zou Y, Davidson A. Plasma cells in systemic lupus erythematosus: the long and short of it all. European journal of immunology. 2011;41(3):588-91.
21. Wang W, Rangel-Moreno J, Owen T, Barnard J, Nevarez S, Ichikawa HT, Anolik JH. Long-term B cell depletion in murine lupus eliminates autoantibody-secreting cells and is associated with alterations in the kidney plasma cell niche. Journal of immunology. 2014;192(7):3011-20.
22. Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ, Sanz I. Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. Arthritis and rheumatism. 2004;50(11):3580-90.
23. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC, Utset TO, Gordon C, Isenberg DA, Hsieh HJ, Zhang D, Brunetta PG. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. Arthritis and rheumatism. 2010;62(1):222-33.
24. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, Maciuca R, Zhang D, Garg JP, Brunetta P, Appel G; LUNAR Investigator Group. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis and rheumatism. 2012;64(4):1215-26.
25. Voll R, Hiepe F. [Depletion of plasma cells - a novel strategy in the therapy of systemic lupus erythematosus in mice and man]. Zeitschrift fur Rheumatologie. 2009;68(2):150-3.
26. Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KG, Dorner T, Hiepe F. Competence and competition: the challenge of becoming a long-lived plasma cell. Nature reviews Immunology. 2006;6(10):741-50.
27. Cassese G, Lindenau S, de Boer B, Arce S, Hauser A, Riemekasten G, Berek C, Hiepe F, Krenn V, Radbruch A, Manz RA. Inflamed kidneys of NZB / W mice are a major site for the homeostasis of plasma cells. European journal of immunology. 2001;31(9):2726-32.
28. Tokoyoda K, Egawa T, Sugiyama T, Choi BI, Nagasawa T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. Immunity. 2004;20(6):707-18.
29. Mohr E, Serre K, Manz RA, Cunningham AF, Khan M, Hardie DL, Bird R, MacLennan IC. Dendritic cells and monocyte/macrophages that create the IL-6/APRIL-rich lymph node microenvironments where plasmablasts mature. Journal of immunology. 2009;182(4):2113-23.
30. Chu VT, Frohlich A, Steinhauser G, Scheel T, Roch T, Fillatreau S, Lee JJ, Lohning M, Berek C. Eosinophils are required for the maintenance of plasma cells in the bone marrow. Nature immunology. 2011;12(2):151-9.
31. Rodriguez Gomez M, Talke Y, Goebel N, Hermann F, Reich B, Mack M. Basophils support the survival of plasma cells in mice. Journal of immunology. 2010;185(12):7180-5.
32. Denzel A, Maus UA, Rodriguez Gomez M, Moll C, Niedermeier M, Winter C, Maus R, Hollingshead S, Briles DE, Kunz-Shughart LA, Talke Y, Mack M. Basophils enhance immunological memory responses. Nature immunology. 2008;9(7):733-42.
33. Palanichamy A, Bauer JW, Yalavarthi S, Meednu N, Barnard J, Owen T, Cistrone C, Bird A, Rabinovich A, Nevarez S, Knight JS, Dedrick R, Rosenberg A, Wei C, Rangel-Moreno J, Liesveld J, Sanz I, Baechler E, Kaplan MJ, Anolik JH. Neutrophil-mediated IFN activation in the bone marrow alters B cell development in human and murine systemic lupus erythematosus. Journal of immunology. 2014;192(3):906-18.

34. Winter O, Moser K, Mohr E, Zotos D, Kaminski H, Szyska M, Roth K, Wong DM, Dame C, Tarlinton DM, Schulze H, MacLennan IC, Manz RA. Megakaryocytes constitute a functional component of a plasma cell niche in the bone marrow. Blood. 2010;116(11):1867-75.
35. Benson MJ, Dillon SR, Castigli E, Geha RS, Xu S, Lam KP, Noelle RJ. Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. Journal of immunology. 2008;180(6):3655-9.
36. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, Lin LL, Mantchev GT, Bram RJ, Noelle RJ. BCMA is essential for the survival of long-lived bone marrow plasma cells. The Journal of experimental medicine. 2004;199(1):91-8.
37. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, Muehlinghaus G, Szyska M, Radbruch A, Manz RA. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. Journal of immunology. 2003;171(4):1684-90.
38. Jourdan M, Cren M, Robert N, Bollore K, Fest T, Duperray C, Guilloton F, Hose D, Tarte K, Klein B. IL-6 supports the generation of human long-lived plasma cells in combination with either APRIL or stromal cell-soluble factors. Leukemia. 2014;28(8):1647-56.
39. Winter O, Mohr E, Manz RA. Alternative cell types form a Multi-Component-Plasma-Cell-Niche. Immunology letters. 2011;141(1):145-6.
40. Walne AJ, Dokal A, Plagnol V, Beswick R, Kirwan M, de la Fuente J, Vulliamy T, Dokal I. Exome sequencing identifies MPL as a causative gene in familial aplastic anemia. Haematologica. 2012;97(4):524-8.
41. Vigon I, Mornon JP, Cocault L, Mitjavila MT, Tambourin P, Gisselbrecht S, Souyri M. Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(12):5640-4.
42. Chen WM, Yu B, Zhang Q, Xu P. Identification of the residues in the extracellular domain of thrombopoietin receptor involved in the binding of thrombopoietin and a nuclear distribution protein (human NUDC). The Journal of biological chemistry. 2010;285(34):26697-709.
43. Vigon I, Florindo C, Fichelson S, Guenet JL, Mattei MG, Souyri M, Cosman D, Gisselbrecht S. Characterization of the murine Mpl proto-oncogene, a member of the hematopoietic cytokine receptor family: molecular cloning, chromosomal location and evidence for a function in cell growth. Oncogene. 1993;8(10):2607-15.
44. Hitchcock IS, Chen MM, King JR, Kaushansky K. YRRL motifs in the cytoplasmic domain of the thrombopoietin receptor regulate receptor internalization and degradation. Blood. 2008;112(6):2222-31.
45. Mignotte V, Vigon I, Boucher de Crevecoeur E, Romeo PH, Lemarchandel V, Chretien S. Structure and transcription of the human c-mpl gene (MPL). Genomics. 1994;20(1):5-12.
46. Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. Proceedings of the National Academy of Sciences of the United States of America. 1990;87(18):6934-8.
47. Sabath DF, Kaushansky K, Broudy VC. Deletion of the extracellular membrane-distal cytokine receptor homology module of Mpl results in constitutive cell growth and loss of thrombopoietin binding. Blood. 1999;94(1):365-7.

48. Benit L, Courtois G, Charon M, Varlet P, Dusanter-Fourt I, Gisselbrecht S. Characterization of mpl cytoplasmic domain sequences required for myeloproliferative leukemia virus pathogenicity. Journal of virology. 1994;68(8):5270-4.
49. Drachman JG, Kaushansky K. Dissecting the thrombopoietin receptor: functional elements of the Mpl cytoplasmic domain. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(6):2350-5.
50. Kuter DJ. The biology of thrombopoietin and thrombopoietin receptor agonists. International journal of hematology. 2013;98(1):10-23.
51. Kaushansky K, Broudy VC, Lin N, Jorgensen MJ, McCarty J, Fox N, Zucker-Franklin D, Lofton-Day C. Thrombopoietin, the Mp1 ligand, is essential for full megakaryocyte development. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(8):3234-8.
52. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. Blood. 1995;85(7):1719-26.
53. Kuter DJ, Beeler DL, Rosenberg RD. The purification of megapoietin: a physiological regulator of megakaryocyte growth and platelet production. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(23):11104-8.
54. Suzukawa K, Satoh H, Taniwaki M, Yokota J, Morishita K. The human thrombopoietin gene is located on chromosome 3q26.33-q27, but is not transcriptionally activated in leukemia cells with 3q21 and 3q26 abnormalities (3q21q26 syndrome). Leukemia. 1995;9(8):1328-31.
55. Foster DC, Sprecher CA, Grant FJ, Kramer JM, Kuijper JL, Holly RD, Whitmore TE, Heipel MD, Bell LA, Ching AF. Human thrombopoietin: gene structure, cDNA sequence, expression, and chromosomal localization. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(26):13023-7.
56. Park H, Park SS, Jin EH, Song JS, Ryu SE, Yu MH, Hong HJ. Identification of functionally important residues of human thrombopoietin. The Journal of biological chemistry. 1998;273(1):256-61.
57. Gurney AL, Kuang WJ, Xie MH, Malloy BE, Eaton DL, de Sauvage FJ. Genomic structure, chromosomal localization, and conserved alternative splice forms of thrombopoietin. Blood. 1995;85(4):981-8.
58. Foster D, Lok S. Biological roles for the second domain of thrombopoietin. Stem cells. 1996;14 Suppl 1:102-7.
59. Methia N, Louache F, Vainchenker W, Wendling F. Oligodeoxynucleotides antisense to the proto-oncogene c-mpl specifically inhibit in vitro megakaryocytopoiesis. Blood. 1993;82(5):1395-401.
60. Li J, Xia Y, Kuter DJ. Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: binding, internalization, stability and pharmacokinetics. British journal of haematology. 1999;106(2):345-56.
61. Saur SJ, Sangkhae V, Geddis AE, Kaushansky K, Hitchcock IS. Ubiquitination and degradation of the thrombopoietin receptor c-Mpl. Blood. 2010;115(6):1254-63.
62. Geddis AE. Megakaryopoiesis. Seminars in hematology. 2010;47(3):212-9.
63. Kaushansky K. Lineage-specific hematopoietic growth factors. The New England journal of medicine. 2006;354(19):2034-45.
64. Alexander WS, Metcalf D, Dunn AR. Point mutations within a dimer interface homology domain of c-Mpl induce constitutive receptor activity and tumorigenicity. The EMBO journal. 1995;14(22):5569-78.

65. Royer Y, Staerk J, Costuleanu M, Courtoy PJ, Constantinescu SN. Janus kinases affect thrombopoietin receptor cell surface localization and stability. The Journal of biological chemistry. 2005;280(29):27251-61.
66. Rojnuckarin P, Drachman JG, Kaushansky K. Thrombopoietin-induced activation of the mitogen-activated protein kinase (MAPK) pathway in normal megakaryocytes: role in endomitosis. Blood. 1999;94(4):1273-82.
67. Drachman JG, Sabath DF, Fox NE, Kaushansky K. Thrombopoietin signal transduction in purified murine megakaryocytes. Blood. 1997;89(2):483-92.
68. Miyakawa Y, Rojnuckarin P, Habib T, Kaushansky K. Thrombopoietin induces phosphoinositol 3-kinase activation through SHP2, Gab, and insulin receptor substrate proteins in BAF3 cells and primary murine megakaryocytes. The Journal of biological chemistry. 2001;276(4):2494-502.
69. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Current biology : CB. 1997;7(4):261-9.
70. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005;307(5712):1098-101.
71. Raab MS, Podar K, Breitkreutz I, Richardson PG, Anderson KC. Multiple myeloma. Lancet. 2009;374(9686):324-39.
72. Blade J, Rosinol L, Cibeira MT, de Larrea CF. Pathogenesis and progression of monoclonal gammopathy of undetermined significance. Leukemia. 2008;22(9):1651-7.
73. Matsui W, Wang Q, Barber JP, Brennan S, Smith BD, Borrello I, McNiece I, Lin L, Ambinder RF, Peacock C, Watkins DN, Huff CA, Jones RJ. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. Cancer research. 2008;68(1):190-7.
74. Frassanito MA, Cusmai A, Iodice G, Dammacco F. Autocrine interleukin-6 production and highly malignant multiple myeloma: relation with resistance to drug-induced apoptosis. Blood. 2001;97(2):483-9.
75. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nature reviews Cancer. 2007;7(8):585-98.
76. Georgii-Hemming P, Wiklund HJ, Ljunggren O, Nilsson K. Insulin-like growth factor I is a growth and survival factor in human multiple myeloma cell lines. Blood. 1996;88(6):2250-8.
77. Mahtouk K, Moreaux J, Hose D, Reme T, Meissner T, Jourdan M, Rossi JF, Pals ST, Goldschmidt H, Klein B. Growth factors in multiple myeloma: a comprehensive analysis of their expression in tumor cells and bone marrow environment using Affymetrix microarrays. BMC cancer. 2010;10:198.
78. Mitsiades CS, Mitsiades N, Poulaki V, Schlossman R, Akiyama M, Chauhan D, Hideshima T, Treon SP, Munshi NC, Richardson PG, Anderson KC. Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene. 2002;21(37):5673-83.
79. Moreaux J, Legouffe E, Jourdan E, Quittet P, Reme T, Lugagne C, Moine P, Rossi JF, Klein B, Tarte K. BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. Blood. 2004;103(8):3148-57.
80. Ribatti D, Vacca A. The role of microenvironment in tumor angiogenesis. Genes & nutrition. 2008;3(1):29-34.

81. Dankbar B, Padro T, Leo R, Feldmann B, Kropff M, Mesters RM, Serve H, Berdel WE, Kienast J. Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood. 2000;95(8):2630-6.
82. Van Riet I, Van Camp B. The involvement of adhesion molecules in the biology of multiple myeloma. Leukemia & lymphoma. 1993;9(6):441-52.
83. Seidel C, Sundan A, Hjorth M, Turesson I, Dahl IM, Abildgaard N, Waage A, Borset M. Serum syndecan-1: a new independent prognostic marker in multiple myeloma. Blood. 2000;95(2):388-92.
84. Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. Jama. 2004;292(4):490-5.
85. Pearse RN, Sordillo EM, Yaccoby S, Wong BR, Liau DF, Colman N, Michaeli J, Epstein J, Choi Y. Multiple myeloma disrupts the TRANCE/ osteoprotegerin cytokine axis to trigger bone destruction and promote tumor progression. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(20):11581-6.
86. Tricot G. New insights into role of microenvironment in multiple myeloma. Lancet. 2000;355(9200):248-50.
87. Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Zeldenrust SR, Dingli D, Russell SJ, Lust JA, Greipp PR, Kyle RA, Gertz MA. Improved survival in multiple myeloma and the impact of novel therapies. Blood. 2008;111(5):2516-20.
88. Chari A, Pri-Chen H, Jagannath S. Complete remission achieved with single agent CNTO 328, an anti-IL-6 monoclonal antibody, in relapsed and refractory myeloma. Clinical lymphoma, myeloma & leukemia. 2013;13(3):333-7.
89. Ikeda H, Hideshima T, Fulciniti M, Lutz RJ, Yasui H, Okawa Y, Kiziltepe T, Vallet S, Pozzi S, Santo L, Perrone G, Tai YT, Cirstea D, Raje NS, Uherek C, Dalken B, Aigner S, Osterroth F, Munshi N, Richardson P, Anderson KC. The monoclonal antibody nBT062 conjugated to cytotoxic Maytansinoids has selective cytotoxicity against CD138-positive multiple myeloma cells in vitro and in vivo. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(12):4028-37.
90. Podar K, Zimmerhackl A, Fulciniti M, Tonon G, Hainz U, Tai YT, Vallet S, Halama N, Jager D, Olson DL, Sattler M, Chauhan D, Anderson KC. The selective adhesion molecule inhibitor Natalizumab decreases multiple myeloma cell growth in the bone marrow microenvironment: therapeutic implications. British journal of haematology. 2011;155(4):438-48.
91. Azab AK, Runnels JM, Pitsillides C, Moreau AS, Azab F, Leleu X, Jia X, Wright R, Ospina B, Carlson AL, Alt C, Burwick N, Roccaro AM, Ngo HT, Farag M, Melhem MR, Sacco A, Munshi NC, Hideshima T, Rollins BJ, Anderson KC, Kung AL, Lin CP, Ghobrial IM. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. Blood. 2009;113(18):4341-51.
92. Hubel K, Liles WC, Broxmeyer HE, Rodger E, Wood B, Cooper S, Hangoc G, Macfarland R, Bridger GJ, Henson GW, Calandra G, Dale DC. Leukocytosis and Mobilization of CD34+ Hematopoietic Progenitor Cells by AMD3100, a CXCR4 Antagonist. Supportive cancer therapy. 2004;1(3):165-72.
93. Hofgaard PO, Jodal HC, Bommert K, Huard B, Caers J, Carlsen H, Schwarzer R, Schunemann N, Jundt F, Lindeberg MM, Bogen B. A novel mouse model for multiple myeloma (MOPC315.BM) that allows noninvasive spatiotemporal detection of osteolytic disease. PloS one. 2012;7(12):e51892.

94. Bloom W FD. Textbook of Histology. 9th ed. Philadelphia, USA: W.B. Saunders; 1968.
95. Levine RF, Fedorko ME. Isolation of intact megakaryocytes from guinea pig femoral marrow. Successful harvest made possible with inhibitions of platelet aggregation; enrichment achieved with a two-step separation technique. The Journal of cell biology. 1976;69(1):159-72.
96. Thomas GR, Thibodeaux H, Errett CJ, Mathias J, Marian M, Meng G, Vandlen RL, Eaton DL. In vivo biological effects of various forms of thrombopoietin in a murine model of transient pancytopenia. Stem cells. 1996;14 Suppl 1:246-55.
97. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood. 1994;84(5):1415-20.
98. Kapuscinski J. DAPI: a DNA-specific fluorescent probe. Biotechnic & histochemistry : official publication of the Biological Stain Commission. 1995;70(5):220-33.
99. Zink D, Sadoni N, Stelzer E. Visualizing chromatin and chromosomes in living cells. Methods. 2003;29(1):42-50.
100. Gerlo S, Haegeman G, Vanden Berghe W. Transcriptional regulation of autocrine IL-6 expression in multiple myeloma cells. Cellular signalling. 2008;20(8):1489-96.
101. Chauhan D, Uchiyama H, Urashima M, Yamamoto K, Anderson KC. Regulation of interleukin 6 in multiple myeloma and bone marrow stromal cells. Stem cells. 1995;13 Suppl 2:35-9.
102. Jourdan M, Ferlin M, Legouffe E, Horvathova M, Liautard J, Rossi JF, Wijdenes J, Brochier J, Klein B. The myeloma cell antigen syndecan-1 is lost by apoptotic myeloma cells. British journal of haematology. 1998;100(4):637-46.
103. Murphy GJ, Leavitt AD. A model for studying megakaryocyte development and biology. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(6):3065-70.
104. Tomer A, Harker LA, Burstein SA. Flow cytometric analysis of normal human megakaryocytes. Blood. 1988;71(5):1244-52.
105. Ballmaier M, Germeshausen M, Schulze H, Cherkaoui K, Lang S, Gaudig A, Krukemeier S, Eilers M, Strauss G, Welte K. c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. Blood. 2001;97(1):139-46.
106. Kondo T, Okabe M, Sanada M, Kurosawa M, Suzuki S, Kobayashi M, Hosokawa M, Asaka M. Familial essential thrombocythemia associated with one-base deletion in the 5'-untranslated region of the thrombopoietin gene. Blood. 1998;92(4):1091-6.
107. Kuwana M, Okazaki Y, Kajihara M, Kaburaki J, Miyazaki H, Kawakami Y, Ikeda Y. Autoantibody to c-Mpl (thrombopoietin receptor) in systemic lupus erythematosus: relationship to thrombocytopenia with megakaryocytic hypoplasia. Arthritis and rheumatism. 2002;46(8):2148-59.
108. Staerk J, Defour JP, Pecquet C, Leroy E, Antoine-Poirel H, Brett I, Itaya M, Smith SO, Vainchenker W, Constantinescu SN. Orientation-specific signalling by thrombopoietin receptor dimers. The EMBO journal. 2011;30(21):4398-413.
109. Yu M, Cantor AB. Megakaryopoiesis and thrombopoiesis: an update on cytokines and lineage surface markers. Methods Mol Biol. 2012;788:291-303.
110. Wong D, Winter O, Hartig C, Siebels S, Szyska M, Tiburzy B, Meng L, Kulkarni U, Fahnrich A, Bommert K, Bargou R, Berek C, Chu VT, Bogen B, Jundt F, Manz RA. Eosinophils and megakaryocytes support the early growth of murine MOPC315 myeloma cells in their bone marrow niches. PloS one. 2014;9(10):e109018.

111. Klein B, Zhang XG, Jourdan M, Boiron JM, Portier M, Lu ZY, Wijdenes J, Brochier J, Bataille R. Interleukin-6 is the central tumor growth factor in vitro and in vivo in multiple myeloma. European cytokine network. 1990;1(4):193-201.
112. Honemann D, Chatterjee M, Savino R, Bommert K, Burger R, Gramatzki M, Dorken B, Bargou RC. The IL-6 receptor antagonist SANT-7 overcomes bone marrow stromal cell-mediated drug resistance of multiple myeloma cells. International journal of cancer Journal international du cancer. 2001;93(5):674-80.
113. Corash L, Levin J, Mok Y, Baker G, McDowell J. Measurement of megakaryocyte frequency and ploidy distribution in unfractionated murine bone marrow. Experimental hematology. 1989;17(3):278-86.
114. Nakeff A, Maat B. Separation of megakaryocytes from mouse bone marrow by velocity sedimentation. Blood. 1974;43(4):591-5.
115. Nakeff A, Floeh DP. Separation of megakaryocytes from mouse bone marrow by density gradient centrifugation. Blood. 1976;48(1):133-8.
116. Shoff PK, Levine RF. Elutriation for isolation of megakaryocytes. Blood cells. 1989;15(1):285-305.
117. Tomer A, Harker LA, Burstein SA. Purification of human megakaryocytes by fluorescence-activated cell sorting. Blood. 1987;70(6):1735-42.
118. Tanaka H, Ishida Y, Kaneko T, Matsumoto N. Isolation of human megakaryocytes by immunomagnetic beads. British journal of haematology. 1989;73(1):18-22.
119. Panuganti S, Schlinker AC, Lindholm PF, Papoutsakis ET, Miller WM. Three-stage ex vivo expansion of high-ploidy megakaryocytic cells: toward large-scale platelet production. Tissue Eng Part A. 2013;19(7-8):998-1014.
120. Sanderson RD, Lalor P, Bernfield M. B lymphocytes express and lose syndecan at specific stages of differentiation. Cell Regul. 1989;1(1):27-35.
121. Chilosi M, Adami F, Lestani M, Montagna L, Cimarosto L, Semenzato G, Pizzolo G, Menestrina F. CD138/syndecan-1: a useful immunohistochemical marker of normal and neoplastic plasma cells on routine trephine bone marrow biopsies. Mod Pathol. 1999;12(12):1101-6.
122. Yang Y, Yaccoby S, Liu W, Langford JK, Pumphrey CY, Theus A, Epstein J, Sanderson RD. Soluble syndecan-1 promotes growth of myeloma tumors in vivo. Blood. 2002;100(2):610-7.
123. Stanley MJ, Liebersbach BF, Liu W, Anhalt DJ, Sanderson RD. Heparan sulfate-mediated cell aggregation. Syndecans-1 and -4 mediate intercellular adhesion following their transfection into human B lymphoid cells. The Journal of biological chemistry. 1995;270(10):5077-83.
124. Ridley RC, Xiao H, Hata H, Woodliff J, Epstein J, Sanderson RD. Expression of syndecan regulates human myeloma plasma cell adhesion to type I collagen. Blood. 1993;81(3):767-74.
125. Belnoue E, Tougne C, Rochat AF, Lambert PH, Pinschewer DD, Siegrist CA. Homing and adhesion patterns determine the cellular composition of the bone marrow plasma cell niche. Journal of immunology. 2012;188(3):1283-91.
126. Garcia De Vinuesa C, Gulbranson-Judge A, Khan M, O'Leary P, Cascalho M, Wabl M, Klaus GG, Owen MJ, MacLennan IC. Dendritic cells associated with plasmablast survival. European journal of immunology. 1999;29(11):3712-21.
127. Kim J, Denu RA, Dollar BA, Escalante LE, Kuether JP, Callander NS, Asimakopoulos F, Hematti P. Macrophages and mesenchymal stromal cells support survival and proliferation of multiple myeloma cells. British journal of haematology. 2012;158(3):336-46.

128. Rozanski CH, Arens R, Carlson LM, Nair J, Boise LH, Chanan-Khan AA, Schoenberger SP, Lee KP. Sustained antibody responses depend on CD28 function in bone marrow-resident plasma cells. The Journal of experimental medicine. 2011;208(7):1435-46.
129. Nair JR, Carlson LM, Koorella C, Rozanski CH, Byrne GE, Bergsagel PL, Shaughnessy JP Jr, Chanan-Khan A, Lee KP. CD28 expressed on malignant plasma cells induces a prosurvival and immunosuppressive microenvironment. Journal of immunology. 2011;187(3):1243-53.
130. Sprynski AC, Hose D, Caillot L, Reme T, Shaughnessy JD, Jr., Barlogie B, Seckinger A, Moreaux J, Hundemer M, Jourdan M, Meissner T, Jauch A, Mahtouk K, Kassambara A, Bertsch U, Rossi JF, Goldschmidt H, Klein B. The role of IGF-1 as a major growth factor for myeloma cell lines and the prognostic relevance of the expression of its receptor. Blood. 2009;113(19):4614-26.
131. Ribatti D, Moschetta M, Vacca A. Microenvironment and multiple myeloma spread. Thromb Res. 2014;133 Suppl 2:S102-6.
132. Hui-Yuen JS, Li XQ, Askanase AD. Belimumab in systemic lupus erythematosus: a perspective review. Ther Adv Musculoskelet Dis. 2015;7(4):115-21.
133. Cogollo E, Silva MA, Isenberg D. Profile of atacicept and its potential in the treatment of systemic lupus erythematosus. Drug Des Devel Ther. 2015;9:1331-9.
134. Isenberg D, Gordon C, Licu D, Copt S, Rossi CP, Wofsy D. Efficacy and safety of atacicept for prevention of flares in patients with moderate-to-severe systemic lupus erythematosus (SLE): 52-week data (APRIL-SLE randomised trial). Annals of the rheumatic diseases. 2014.
135. Illei GG, Shirota Y, Yarboro CH, Daruwalla J, Tackey E, Takada K, Fleisher T, Balow JE, Lipsky PE. Tocilizumab in systemic lupus erythematosus: data on safety, preliminary efficacy, and impact on circulating plasma cells from an open-label phase I dosage-escalation study. Arthritis and rheumatism. 2010;62(2):542-52.
136. Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Richardson PG, Hideshima T, Munshi NC, Treon SP, Anderson KC. Apoptotic signaling induced by immunomodulatory thalidomide analogs in human multiple myeloma cells: therapeutic implications. Blood. 2002;99(12):4525-30.
137. Roccaro AM, Hideshima T, Raje N, Kumar S, Ishitsuka K, Yasui H, Shiraishi N, Ribatti D, Nico B, Dammacco F, Richardson PG, Anderson KC. Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. Cancer research. 2006;66(1):184-91.
138. Hideshima T, Chauhan D, Hayashi T, Akiyama M, Mitsiades N, Mitsiades C, Podar K, Munshi NC, Richardson PG, Anderson KC. Proteasome inhibitor PS-341 abrogates IL-6 triggered signaling cascades via caspase-dependent downregulation of gp130 in multiple myeloma. Oncogene. 2003;22(52):8386-93.

6. Abbreviations

ADHP	Acetyl-3, 7-dihydroxyphenoxazine
APC	Allophycocyanin
APRIL	A Proliferation-Inducing Ligand
BAFF	B Cell Activation Factor
BCMA	B Cell Maturation Antigen
BM	Bone Marrow
BMEC	Bone Marrow Endothelial Cells
BMSC	Bone Marrow Stromal Cells
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CAR	CXCL12-abundant reticular cell
CD	Cluster Of Differentiation
cDNA	Complimentary DNA
CFU	Colony-Forming Unit
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DAPI	4’6-Diamidino-2-Phenylindole
DNA	Deoxyribonucleic acid
dsDNA	double-stranded DNA
DRFZ	Deutsches Rheumaforschungszentrum
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence-activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescin Isothyocynate
GFP	Green Fluorescent Protein
GSK-3β	Glycogen Synthase Kinase 3 Beta
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNC	Haematopoietic niche cells
Ig	Immunoglobulin
IGF-1	Insulin-Like Growth Factor 1

IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
JAK2	Janus Kinase2
kDa	kilodalton
kb	kilobase
LLPC	Long-Lived Plasma Cell
MALT	Mucosa-Associated Lymphoid Tissue
MAPK/ERK	Mitogen-activated Kinase Extracellular Signal-Regulated Kinase
MGUS	Monoclonal Gammopathy Of Undetermined Significance
MHC	Major Histocompatibility Complex
MK	Megakaryocyte
MM	Multiple Myeloma
MOPC	Mineral Oil Induced Plasmocytoma
MPL	Myeloproliferative Leucaemia Virus
mTORC2	Mammalian target of rapamycin complex 2
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of activated B Cells
NZB/W	New Zealand Black/White
OPG	Osteoprotegerin
PI3K	Phosphatidylinositol 3-Kinase
PB	Pacific Blue
PBS	Phosphate Buffered Saline
PC	Plasma Cell
PE	Phycoerythrin
PEG-rh MGDF	PEGylated Recombinant Human Megakaryocyte Growth And Development Factor
PS	Phosphatidylserine
RANK	receptor activator of NF-κB
RANKL	RANK ligand
rhTPO	Recombinant Human Thrombopoietin
RPMI	Rosewell Park Memorial Institute
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency
SDF-1	Stromal Cell-Derived Factor 1
SD	Standard Deviation

SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SMM	Smouldering Multiple Myeloma
SNP	Single Nucleotide Polymorphism
STAT	Signal Transducer and Activator of Transcription
STD	Standard Deviation
TACI	Transmembrane Activator And Calcium-Modulator And Cyclophilin Ligand Interactor
TNF-α	Tumour Necrosis Factor Alpha
TGF-β	Transforming Growth Factor Beta
TPO	Thrombopoietin
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VLA-4	Very-Late Antigen-4

7. Statutory Declaration – Eidesstattliche Versicherung

„Ich, Nam Matthias Ly, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Influence of megakaryocytes and megakaryocytopoiesis on plasma cell survival“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Betreuer angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

8. Acknowledgement – Danksagung

I would like to thank everyone who has supported me in the last few years while I have been working on this thesis. Firstly, I would like to thank my supervisor Oliver Winter for his advice during all parts of the thesis, especially his patience during the experimental part and also for the ongoing support and assistance during data analysis and writing. I would also like to thank Professor Falk Hiepe for the overall supervision of this doctoral thesis.

Next I would like to thank my wife Michelle: For all your patience and understanding, for coping with the hours I spent working and studying. I am sure I would not have been able to do it all without your love and support. You have always been there for me and believed in me even when I did not. Thank you.

Up next, Margo: Maybe one day when you are old enough you will end up reading this. (I sure do hope so.) You are the best thing I have done in my life and being able to watch you grow up is something that fills me with joy every day. Remember that your Mama and Papa love you dearly and are immensely proud of you.

Last but not least I would like to thank my parents for their love and care, for all their sacrifices, I would not be where I am without them.

9. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

For privacy reasons, my resume will not be published and included in the electronic version of my thesis.

This page has been left intentionally blank.