1 Introduction

1.1 Multiple myeloma (MM)

Multiple Myeloma (MM) is a clonal B-cell tumour of differentiated and usually slowly proliferating plasma cells. In the Western countries MM accounts for approximately one percent of all cancer-related deaths [1], and in the US MM constitutes approximately 20% of all haematological malignancies [2, 3]. The median age of patients with MM is 65 years but in the past 60 years, a trend towards myeloma patients under 55 years of age was noticed. However, only 3% of MM patients are younger than 40 years. Twice as many African Americans than Caucasians develop MM and its occurrence is lowest in Asian populations. The disease affects slightly more men than women (ratio 3:2) [4]. MM is yet an incurable disease, complete remission can be achieved in only 5% of the cases. The median length of survival after diagnosis is three to five years [1, 5]. The cause of MM is unknown and both, genetic and environmental factors have been implicated. Genetic predisposition is indicated by the significantly higher occurrence of multiple myeloma in African Americans and also by report of familial clusters of first-degree relatives with MM [6].

1.1.1 Course of the disease

Multiple myeloma is usually preceded by an age-dependent pre-malignant tumour called monoclonal gammopathy of undetermined significance (MGUS), which is present in 1% of adults over the age of 25 and progresses to malignant multiple myeloma at a rate of 1% per year. Although MGUS can be efficiently diagnosed by a simple blood test (it is characterized by a stable, monoclonal spike of serum IgG), it is not possible to prevent progression or even predict when progression to myeloma will occur [7]. Both MGUS and multiple myeloma secrete the same monoclonal immunoglobulin, also referred to as idiotype protein (Id), but MM is distinguished from MGUS by having a greater intramedullary tumour-cell content (>10%), the development of osteolytic bone lesions and/or an increasing tumour mass [3]. The strong link between MGUS and MM supports the two-hit hypothesis which postulates an initial oncogenic event, i.e. the immortalization of a plasma cell to form a monoclonal gammopathy and a second that leads to multiple myeloma [8].

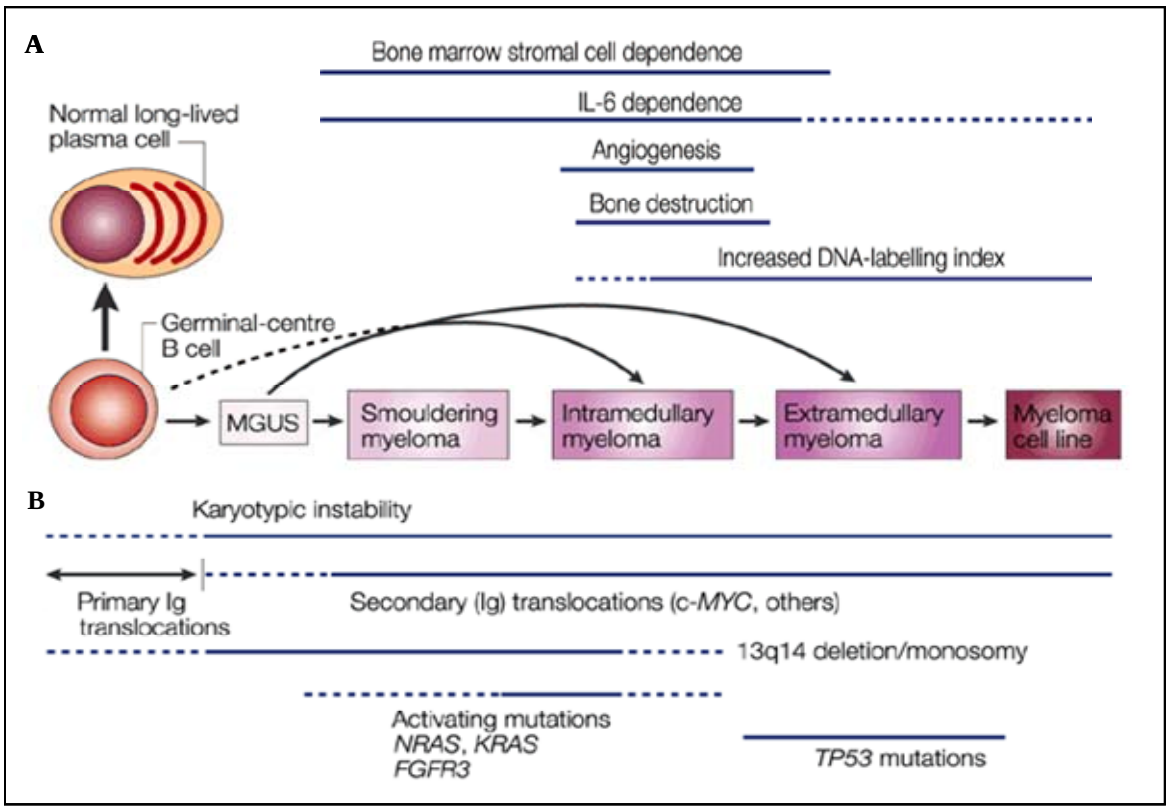

Figure 1.1: Development and molecular pathogenesis of multiple myeloma (MM). A) Developing MM occurs either from a monoclonal gammopathy of undetermined significance (MGUS), or arises directly from a normal germinal-centre B cell. Plasma cells accumulate within the bone marrow (intramedullary), leading to manifestation of clinical features. At this stage MM is usually chemosensitive and may enter a quiescent plateau phase. Localization of the tumour to blood, pleural fluid and skin (extramedullary) is characterised by resistance to chemotherapy, apoptosis, and independence from growth factors. **B)** Oncogenic events occur in MGUS and throughout the course of MM, such as karyotypic instability; primary and secondary immunoglobulin (Ig) translocations, chromosome deletion, and gene mutations (vertical lines indicate the approximate timing of events, with the dashed lines meaning some uncertainty).

Modified from Kuehl and Bergsagel, Nature Reviews Cancer, 2002 [3]

Progression of MGUS to smouldering myeloma, which has a stable intramedullary tumour-cell content of >10% but none of the other complications of malignant myeloma (e.g. osteolytic lesions) does not necessarily occur in all of the cases [3]. With progression to malignant intramedullary myeloma, complex genetic events take place in the neoplastic plasma cell. Changes also occur in the bone marrow microenvironment, i.e. induction of angiogenesis, suppression of cell-mediated immunity, and development of paracrine signalling loops (involving cytokines such as IL-6, IGF-1, and VEGF). These changes lead to interactions of myeloma cells, bone marrow stromal cells (BMSC), and microvessels which, taken together contribute to persistence of the tumour and its resistance to drugs [9]. Thus, intramedullary myeloma is associated with severe secondary features (lytic bone lesions, anaemia, immunodeficiency and renal impairment) and, in some patients with tumours

occurring in extramedullary sites (blood, pleural fluid and skin) [3]. The working multi-step model of the molecular pathogenesis of MM as proposed by Hallek *et al*. and Kuehl *et al*. [3, 5] is summarized in figure 1.1.

1.1.2 Clinical features and pathogenesis of multiple myeloma

Patients with multiple myeloma possess plasma cells with abnormal morphologic and cytogenetic features, which proliferate in the bone marrow (BM). There is also histological evidence of abnormal bone remodelling [1]. The most common clinical features of multiple myeloma depend on the progressive accumulation of MM cells within the BM and subsequently the interactions between myeloma cells and the BM microenvironment, by means of cell-cell contact, adhesion molecules, and cytokines. This leads to disruption of the normal bone marrow function (reflected by anaemia), bone marrow failure, bone destruction (diffuse osteoporosis), and damage to the surrounding bone (lytic bone lesions)[10]. In most patients, multiple discrete lytic lesions can be found at the sites where nests of myeloma cells occur. These so called "punch out lesions" can be visualized by radiography (figure 1.2) [4].

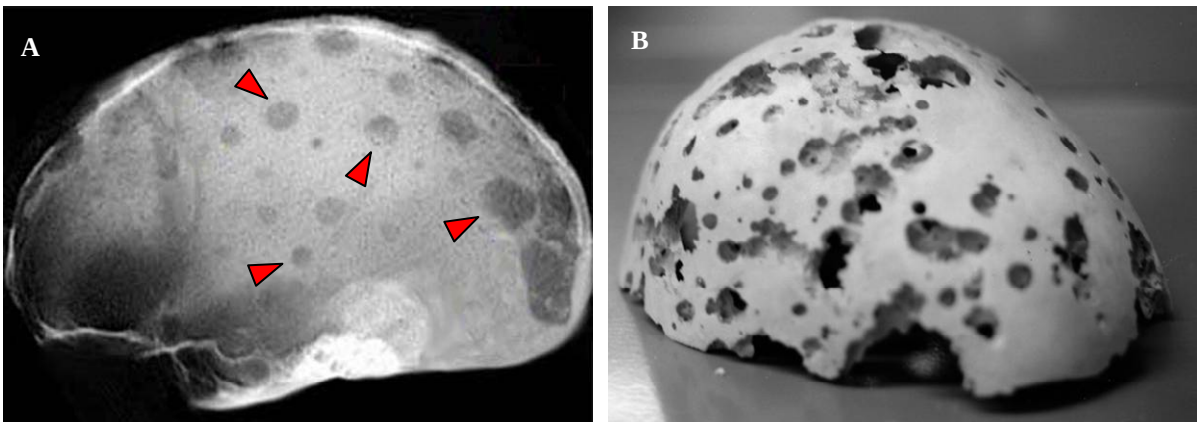

Figure 1.2: Typical bone lesions induced by multiple myeloma. A) The skull X-ray shows "punched out lesions" (arrowheads) in the skull, which are characteristic of multiple myeloma. **B)** Gross examination of the skull reveals severe bone destruction induced by MM.

Additionally, direct pathogenic effects of circulating monoclonal immunoglobulin or light chains can be observed in MM [1]. In myeloma cells, mutations have occurred in the genes responsible for IgG production. This leads to expression of proteins with abnormal aminoacid sequence and protein structure, which have lost their normal antibody function. Release of this so called "M-protein" (monoclonal protein) into the blood and thus increased plasma volume and viscosity can cause renal insufficiency. Moreover, suppression of normal immune

function, i.e. reduced levels of normal IgG and increased susceptibility to infection has been reported. [10]. Renal insufficiency and recurrent bacterial infections are also major causes of death [1].

1.1.3 Normal and malignant plasma cell development

The mature effectors of the B-cell lineage are terminally differentiated, non-dividing, antibody-secreting plasma cells (PCs). Antigen in combination with other signals (e.g. molecules of the tumour necrosis factor (TNF) family, cytokines, microbial products and two key transcriptional regulators, Blimp-1 and XBP-1), are responsible for the transition of naïve B cells in the spleenic marginal zone (MZ). Subsequently they proliferate and differentiate into mainly short-lived PCs, which remain in the local site and die within a few days. As first response to pathogens these short-lived plasma cells secrete low affinity IgM, which is not somatically hypermutated [5, 11]. Subsequently, encounter with antigen and antigen-specific T helper cells cause naïve follicular B cells to undergo proliferation, affinity maturation, and isotype switch recombination in a germinal centre (GC) reaction. In this reaction, PCs are generated that secrete high affinity antibody, mainly of switched isotype. When plasma cells receive survival signals from stromal cells, usually after migration into the BM (and possibly to the spleen) they can survive for many months [12].

Multiple myeloma results from malignant transformation of plasma cells or their precursors. The malignant plasma cells (figure 1.3) are localized to the BM in close association with stromal cells, but are rarely found in other locations. They are long-lived PCs and are still able to proliferate, although at a very low rate. The rearranged immunoglobulin genes of malignant PCs are extensively somatically hypermutated in a manner compatible with antigen selection, but in contrast to their normal counterpart the MM cells lack terminal differentiation and they produce significantly lower amounts of Ig [4]. Thus, it appears that the critical oncogenic

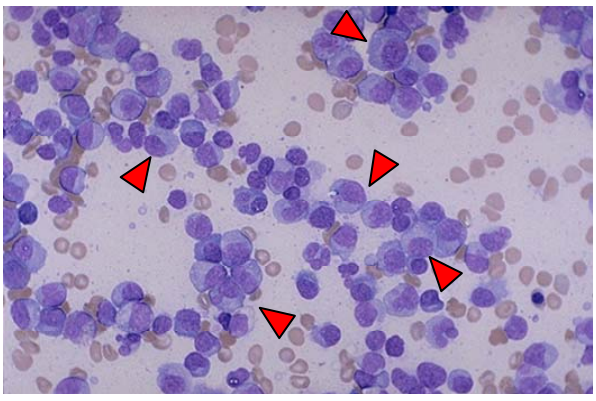

Figure 1.3: Cytomorphologic diagnosis of MM. Pappenheim stain of a bone marrow aspirate from a multiple myeloma patient. Plasma cells with eccentric nuclei and a perinuclear halo of clearer cytoplasm are clearly distinguishable (arrowheads).
Image kindly provided by Dr. A. Greiner, University of Würzburg

events in MM cells do not interfere with most of the normal differentiation process involved in generating a long-lived plasma cell [5, 12]. The origin of malignant plasma cells is not yet elucidated. Because of the presence of somatic hypermutation, the malignant clone in MM is thought to be a post-GC cell. Low-level immunoglobulin secretion and slow proliferation suggests that the malignant cell in MM could be a late cell in B-cell development (memory B-cell, plasmablast) [13, 14]. Hence, although the plasma cell is the predominant cell in MM other lymphoid cell types may also be involved in the malignant process. The shared properties concerning clonal Ig gene rearrangement, and the presence of certain specific proteins on peripheral blood lymphocytes (PBLs) and bone marrow plasma cells strongly suggests the existence of a precursor compartment [15].

1.1.4 Cytogenetic alterations and mutations

In almost all patients suffering from multiple myeloma, cytogenetically abnormal tumour cells can be detected. Like a normal long-lived plasma cell, a myeloma cell undergoes three developmentally regulated changes in the DNA structure of the immunoglobulin heavy chain and light chain (IgH and IgL) loci, including V(D)J recombination of its IgH and IgL genes, somatic hypermutation of the IgH and IgL variable regions, and productive IgH switch recombination to another IgH isotype [5]. It is speculated that errors in one or more of these processes account for genetic changes that contribute to the malignant process.

Karyotypic instability commences from the earliest stage of MM and increases with disease progression. In approximately 50% of patients with MGUS, 65% of myeloma patients, and > 90% of myeloma cell lines translocations can be observed that involve the immunoglobulin heavy-chain locus on chromosome 14q32 [16] and one of five partner chromosomes, 11q13 (cyclin D1, the most common), 4p16.3 (fibroblast growth factor receptor 3), 6p21 (cyclin D3), 16q23 (c*maf*), and 20q11 (*mafB*) [9, 17]. These translocations are markers for distinct subtypes of myeloma with important prognostic implications.

The primary translocations simultaneously dysregulate a variety of oncogenes by juxtaposing them to the strong regulatory elements of the IgH locus. This enables the cells to survive and proliferate, resulting in immortalization of the malignant cell clone [5]. As myeloma tumours become more proliferative at a later stage of disease secondary translocations occur that do not involve B-cell-specific processes. These genetic changes are characterized by dysregulation of c-*myc*, *fas*, *bcl*-2, and *PTEN*, activating mutations of *NRAS* or *KRAS2*, and also a higher incidence of monosomy 13, indicating a possible tumour-suppressor gene on

chromosome 13 [3]. Further mutations, like p53, lead to stroma-independent growth, selection of a single clone for malignant expansion and escape from the BM microenvironment [5].

Moreover, cytogenetic parameters have been found to be of high prognostic relevance: poor survival is linked to deletions on chromosome 13, hypodiploidy, and to the translocation 4;14. On the other hand prognosis is better for patients who have hyperdiploidy and for those who carry the translocation 11;14 [18].

1.1.5 Adhesion molecules, growth factors and bone marrow (BM) microenvironment

The pathogenesis of multiple myeloma depends on the presence of certain cytokines, which support the survival, proliferation, and differentiation of MM cells in the BM during the different disease stages and many of the differences in localization and growth of cancer cells compared to their normal counterparts have been associated with modified expression of specific adhesion molecules and growth factors.

After the genetic transformation events, plasmablasts start expressing various adhesion molecules, leave the germinal centre, enter the circulation and home to the BM, where they bind to bone marrow stromal cells via VLA-4/VCAM-1 (very late antigen 4/vascular cellular adhesion molecule 1) and LFA-1/ICAM-1 (lymphocyte function-associated antigen 1/intercellular adhesion molecule), which in turn induces cytokine secretion. Moreover, myeloma cells bind to each other (homotypic aggregation) to create tumour masses [19]. Myeloma cells also bind to extra cellular matrix (ECM) proteins (e.g., fibronectin) through integrins VLA-4 and VLA-5, which results in induction of drug resistance and inhibits Fas mediated apoptosis [18, 20].

Of the cytokines induced, interleukin (IL) 6 is of particular importance, since it stimulates B cells to differentiate into Ig-secreting plasma cells and acts as a growth factor for MM. Thus, IL-6 mediates the expansion of plasmablastic cells, but also of their malignant counterparts. After adhesion of MM cells to BMSC almost all IL-6 is produced by the bone marrow environment, although myeloma cells are capable of IL-6 secretion and an autocrine IL-6 loop exists in MM.[4, 5]. Subsequently, IL-6 increases cell proliferation through the JAK/STAT and the Ras/MAPK signalling pathways, prevents dexamethasone-induced apoptosis via the

PI3K/AKT signalling pathway and induces VEGF (vascular endothelial growth factor) secretion [20].

Other important molecules which were shown to stimulate growth and/or specific intracellular signalling events of MM cells include granulocyte colony-stimulating factor (G-CSF), interferon-α (IFN-α), interleukin-10 (IL-10), IL-3, granulocyte-macrophage-CSF (GM-CSF), stem cell factor (SCF), tumour necrosis factor-α (TNF-α), and hepatocyte growth factor (HGF), stromal cell derived factor 1 (SDF-1) and insulin-like growth factor 1 and 2 (IGF-1 and IGF-2) [5]. An increase in the expression of RANKL (receptor activator of nuclear factor κ-B ligand) by osteoblasts and a reduction in the level of its decoy receptor, osteoprotegerin are thought to be related to the development of bone lesions in MM. The increase in the ratio of RANKL to osteoprotegerin results in the activation of osteoclasts and bone resorption. Overexpression of RANKL is probably mediated in part by the release of macrophage inflammatory protein 1 α (MIP-1 α) by neoplastic plasma cells [9]. All these molecules are thought to be involved in signalling events related to pathogenesis of multiple myeloma and give an impression of the multitude of potential targets for compounds to overcome resistance to conventional chemotherapy (see sections 1.2.1 and 1.2.2).

1.1.6 Cell-surface antigens

Regarding the expression of specific surface antigens on normal and malignant plasma cells literature reports contradicting findings, especially concerning the expression of CD19, CD20, and CD56 [21-23], CD28 [23] and mucin 1 [24, 25]. To date it remains difficult to assign a specific phenotype for normal or malignant plasma cells, therefore impeding antibody based therapies of multiple myeloma.

During maturation into plasma cells, B-lymphocytes undergo characteristic changes in the expression of cell-surface antigens. Plasma cells exist in at least two different subpopulations i.e., early lymphoplasmacytoid plasma cells and late mature plasma cells [26]. These two populations appear phenotypically different, but both strongly express CD38 and CD138 (syndecan-1). In contrast to mature plasma cells, early lymphoplasmacytoid cells express CD22, CD35, and surface IgE receptors. Subpopulations of mature, normal plasma cells on the other hand show a very heterogeneous immunophenotype: they can express early B-cell antigens (CD19, CD20, CD10), myeloid antigens (CD13, CD33), HLA-DR (D-related human

leukocyte antigen), common haematopoietic antigens (CD45), and adhesion molecules (CD11b, CD11c) [27].

In addition to these typical plasma cell markers a number of antibodies have been described within the past years claiming to be specific for plasma cell antigens of unknown function, such as PCA-1 and PCA-2 [21], PC-1 [28], R1-3 [29], 8A, 62B1, and 8F6 [30], MM4 [31], MPC-1 [32], and HM1.24 [33-40]. However, none of the antibodies turned out to be plasma cell-specific, and often recognize cytoplasmatic antigens [1, 22].The recently discovered, plasma cell-specific antigen WUE-1 [41] is subject of this project and will be discussed in more detail below (section 1.3).

Malignant plasma cells bear similar heterogeneity in their immunophenotype as their normal counterparts according to their differentiation stage. Despite the similarity of most antigens expressed on myeloma cells and normal plasma cells, some of the antigens expressed on myeloma cells are rather unique. For example, the adhesion molecule CD56 (which seems to mediate homotypic adhesion) is highly expressed on some myeloma samples but not on normal mature plasma cells. Some MM cells or cell lines also lack expression of the B-lineage specific antigen CD19, a key member of B-cell surface signal transduction [5].

1.2 Treatment of multiple myeloma

To date multiple myeloma remains incurable. However, treatment improves the clinical situation in about 75% of patients and multiple periods of remission and relapse can occur. The occurrence of drug resistance represents the major obstacle to curing myeloma. Within the past decades understanding the factors that determine drug response and the development of drug resistance has been focus of research [42]. Insight into these crucial processes and a thorough understanding of the biology of myeloma is essential for developing effective therapeutic interventions. Ideally this will lead to "targeted therapies" (directed immunotherapy, molecularly directed therapy), which take into account the specific properties of myeloma cells, thus reducing toxicity of chemotherapy and improving the efficacy of treatment [43]. Gene array, proteomics and cell-signalling studies have contributed a great deal to identifying the *in vivo* molecular mechanisms underlying drug resistance, and were also useful in the clinical application of combination therapies [44-46].

Current approaches to improve the therapeutic outcome for myeloma patients focus on (1) enhancing the efficacy of already available chemotherapeutic drugs by identifying and

overcoming drug resistance mechanisms, (2) identifying new targets that regulate cell survival and growth of myeloma cells, and (3) developing means to enhance host immune response against myeloma cells [14].

1.2.1 Conventional therapies

The first drugs used in effective therapy of MM have been developed in the early 1960s. Since then various new compounds were introduced to fight MM. The novel agents belong to the following pharmacologic classifications: alkylating agents (melphalan, cyclophosphamide, carmustine (BCNU)), topoisomerase II inhibitors (doxorubicin and etoposide), glucocorticoids (prednisone and dexamethasone), and the anti-tubulin agent, vincristine. With the exception of the glucocorticoids, most of these agents are relatively ineffective as single agents and are best administered as a combination treatment [14].

Currently the treatment of choice for symptomatic (i.e. active) myeloma is high-dose chemotherapy with haematopoietic stem cell transplantation (HSCT). Autologous HSCT uses the patient's own stem cells, whereas allogeneic/syngeneic HSCT employs MHC (i.e. HLA) identical or twin donor bone marrow [14]. If high-dose chemotherapy with HSCT is not an option (depending on the individual situation of the patient i.e., age, state of the disease, physical fitness) conventional chemotherapy, single agent treatment (e.g., dexamethasone) or new treatments (thalidomide, bortezomib) possibly in combination with other drugs are applied [18].

1.2.2 Novel targets

The majority of novel approaches in treatment of multiple myeloma aim to overcome the mechanisms which promote MM cell growth, survival, drug resistance or migration by specifically targeting key molecules of known pathways. This can be achieved by agents such as small interfering molecules, peptides, chimeric proteins, small interfering (si) RNA, or monoclonal antibodies (mABs).

In the context of factors which are significant to intrinsic mechanisms in myeloma cells surface molecules such as CD20, CD40, CD56, CD59, CD138, caveolae, IGF-1 receptor [14, 20, 21], and cancer testis antigen [47, 48], and intercellular factors like NF-κB, telomerase catalytic

subunit [49], and p38MAPK have extensively been studied. In terms of myeloma cell/host interactions research has focussed on expression and regulation of adhesion molecules, which mediate binding to BMSC and extra cellular matrix (ECM) proteins. Induction of cytokines, and the resulting processes leading to growth, survival, and drug resistance are also issues addressed by recent research. Main factors involved were identified as interleukin (IL) 6, vascular endothelial growth factor (VEGF), stromal cell derived factor (SDF) 1, tumour necrosis factor (TNF), insulin-like growth factor (IGF) 1, transforming growth factor (TGF) β, and B cell stimulating factor (BSF) 3. Finally, the importance of patient and donor T-cells and natural killer cells in mediating anti-MM immunity in the BM microenvironment could be highlighted [44-46].

As a key molecule in MM, signalling IL-6 represents an ideal target for novel therapeutics. Thus, varieties of different approaches have been developed to prevent the action of IL-6. Among the conventional agents are all-*trans* retinoic acid (ATRA), IFN-α, and IFN-γ, which are thought to downregulate IL-6 receptor (IL-6R) and gp130. Glucocorticoids and IL-4 are reported to repress IL-6 gene transcription. Monoclonal antibodies directed against IL-6 or IL-6R are more specific antagonists of IL-6–dependent growth. Moreover, toxic fusion proteins, consisting of IL-6 fused to a toxic protein, which upon binding to the IL-6 receptor kills the MM cell, or recombinant chimeric human/murine IL-6 proteins targeting the IL-6R on MM cells were successfully used. Antisense oligonucleotides that inhibit the expression of IL-6 or IL-6R genes and the molecular modelling of IL-6 superantagonists that contain variant binding motifs with higher affinity to the IL-6R are being developed. Chemically synthesized peptides may also block the IL-6R/gp130 interaction [5].

The Ras-MAPK pathway is another, similarly important target for MM therapy, since mutations in the Ras family of genes are relatively common (with a frequency of 27% at diagnosis of MM to 46% with disease progression) in myeloma patients and cause altered signal transduction. The generation of novel inhibitors of tyrosine kinases and their substrates would be beneficial, since most tyrosine kinase inhibitors available to date (genistein, staurosporin, and erbstatin) are not specific and therefore highly cytotoxic. A key target molecule is the enzyme farnesyltransferase, which is responsible for transferring a farnesyl group to the RAS protein. Farnesylation allows RAS to attach to the inner plasma cell membrane and activate growth signals to the nucleus [5, 14]. MAPK activation is furthermore

induced by VEGF. Hence, inhibitors of the VEGF receptor may block proliferation of tumour cells and promote anti-angiogenic activity.

Proteasome inhibitors are a further group of novel drugs, which are used to inhibit for example the activation of NF-κB. They induce apoptosis of myeloma cells which are resistant to conventional therapy and importantly, inhibit the NF-κB-dependent upregulation of IL-6 in BMSCs and related paracrine growth of adherent tumour cells [14]. The molecule TRAIL (tumour necrosis factor α related apoptosis inducing ligand) can trigger apoptosis by binding to specific molecules (death receptors), which in turn activate the caspase cascade and it was found to be a potent inducer of apoptosis in primary MM cells but being non-cytotoxic to haematopoietic stem cells[50].

The interaction between cell surface integrins and ECM components, such as fibronectin, is assumed to regulate angiogenesis and stimulate myeloma growth and progression. Recent data demonstrate that adhesion to FN protects cells from DNA damage induced by DNA intercalating agents (e.g. doxorubicin), alkylating agents (e.g. melphalan), and radiation treatments [14]. Therefore, interrupting cellular adhesion may induce apoptosis and enhance the efficacy of adhesion-blocking agents. For example, bisphosphonates (pamidronate) are known to confer sensitivity to anti-MM treatment. The use bisphosphonates has also been shown to reduce skeletal complications in MM patients by blocking the development of monocytes into osteoclasts (OCs) and they are thought to promote apoptosis of OCs. Moreover the bisphosphonates prevent OCs from moving to the bone surface and these agents also seem to inhibit the production of bone-resorbing cytokines by the BMSCs (e.g. IL-6) [50].

1.2.3 Immunotherapies

Immunotherapies are being tested mainly in the setting of relapsed or resistant disease. A major drawback to successful therapy of MM are massive cytotoxicity noted after allografting, contaminating tumour cells in autografts, and most importantly, the persistence of minimal residual disease (MRD) after high-dose therapy followed by HSCT. But especially MRD offers potential for tumour cell control by adoptive transfer of immune effector cells, since at that stage of the disease they are likely to have most impact [51].

1.2.3.1 Anti-tumour vaccination

Cellular immunotherapy makes use of the unique ability of dendritic cells (DC) to sample the environment by phagocytosis, to process antigens, and to present them to naïve T-cells via major histocompatibility complex (MHC) class I and class II molecules. DC priming in turn induces clonal T-cell expansion and differentiation into effector and memory cells [51, 52]. The capability of phagocytosis can be employed to generate specific antigen (AG) presenting DC for clinical use by simply "feeding" isolated cells with tumour cells or tumour lysates. Other methods to create specific cells for anti-myeloma vaccination are fusion of DC and tumour cells by electroporation or transduction of DC with viral vectors carrying the genetic information for expression of tumour associated antigens (TAA) [14, 51].

Being a clonal malignancy of the antibody secreting plasma cell, in each multiple myeloma patient monoclonal immunoglobulin of a specific idiotype is secreted, also referred to as idiotype protein (Id). Id represents an ideal TAA for immunotherapy. It was shown that T-cells and antibodies specific for Id are present in the peripheral blood of MM patients and the presence of expanded T-cell clones is associated with prolonged survival of the patients. However the suitability of Id for immunotherapy is limited due to the fact that Id is individual to each patient, it is only weakly immunogenic, has a low level of surface expression, but is present in large amounts in the peripheral blood of patients. However, vaccination with DC pulsed with Id protein or peptide has been shown to be feasible. Moreover, it was shown that anti-Id antibody is present in the blood of MM patients and that anti-Id immunoresponse is able to kill MM cells *in vitro* and in animal models [51-53].

1.2.3.2 Specific monoclonal antibodies

Monoclonal antibodies (mABs) are a new generation of biopharmaceuticals. Currently approximately 18 mAB products are already used in disease therapy, and more than 100 are being tested in clinical trials [54]. The use of monoclonal antibodies in cancer therapy has recently been extensively reviewed by Adams & Weiner [55]. Monoclonal ABs have direct effects on tumour cells, such as blocking of growth and induction of apoptosis, but also indirect effects, which are mediated via the immune system, e.g. complement dependent cytotoxicity (CDC), or antibody dependent cellular cytotoxicity (ADCC). In order to reduce immunogenity of mouse-derived mABs in clinical applications it is favourable to chimerize (i.e. humanize) the immunoglobulin. By replacing the constant regions of a murine AB with

human IgG_1-sequences the probability of generation of human anti-mouse ABs in the patient, which may severely lessen the activity of the AB, can be significantly reduced.

A major problem of using mABs for anti-MM therapy is the so-called "antigen escape", meaning that by targeting a single antigen (AG) on the tumour cells only the AG expressing cells in the clone are deleted and the non-expressing cells will survive. Moreover, by targeting antigens on mature MM cells, the proposed "malignant stem cell" would be left unharmed. Therefore it is preferable to target multiple antigens [51].

In therapy of lymphoma and leukaemia the use of mABs directed against specific surface antigens such as CD20 (Rituximab), CD52 (Campath-1H), and CD33 (Myelotarg) has become standard practice. Unfortunately, there is only limited use for these mABs in multiple myeloma, since for example CD20 is expressed on less than 20% of MM cells freshly isolated from patients [56-58]. MM cells lack the presence of suitable surface antigens to develop mABs with satisfactory specificity and sensitivity for targeting the malignant cells. Antibodies against potential targets such as CD38 [59, 60], CD40 [61, 62], CD54 [63], CD138 [64], HM1.24 [40], NY-ESO-1 [65], VEGF [66], and Id protein [51, 67] are under current investigation.

Although unmodified mABs show some therapeutic potency, the effects are variable and not entirely curative. Therefore, current research aims to enhance the efficacy of mABs by humanization (Rituximab, Herceptin, Campath-1H) or creating immunoconjugates, i.e. mABs carrying chemotherapeutics (Mylotarg), prodrugs, inflammatory cytokines, toxins or radionucleotides (Zevalin, Bexxar) [68].

1.2.3.3 Bispecific single-chain antibodies (bssc-ABs)

Cytotoxic T-lymphocytes (CTLs) are considered the most potent effector cells of the immune system, but they cannot be engaged by ordinary monoclonal antibodies, because CTLs lack the Fcγ receptor. Therefore, recombinant antibodies and antibody fragments were engineered (by cell fusion or recombinant DNA technology) to recruit cytotoxic effector cells against a specific pathogenic target. These so called bispecific antibodies (bsABs) are designed in general with two distinct binding domains. One arm of the bsAB binds to the specific AG on the tumour cell. The other arm interacts with the activating receptor expressed on the effector cell (e.g. CD64/FcγRI or CD89 on monocytes and neutrophils, CD16 on natural killer cells, and CD3 on T-cells). CD3 is most commonly chosen as triggering molecule for bispecific

antibody approaches. It is a T-cell-specific complex comprising three different monomorphic chains (ε, γ and δ), which are associated as signal transduction units with the polymorphic T-cell receptor (TCR). Triggering the T-cells via the CD3 complex bears the advantage of being MHC independent. Thus a polyclonal CTL response against all cells bearing the target AG is initiated without the restrictions of clonotypic T-cell specificity [68]. Interaction of the bsAB with the TCR-CD3 complex brings the cytotoxic effector cells in close proximity of the target cell, simultaneously triggering the cytotoxic response, and subsequently leading to specific cell lysis. The *in vitro* effectiveness of these recombinant antibody molecules was successfully proved in the mid-1980s [69-71], but to date finding the best molecular format to produce high and affordable yields for clinical grade material and on the other hand meeting the therapeutic requirements (potency , specificity, retention time) remains challenging. Various formats of bsAB have been developed and tested since (thoroughly reviewed by Hollinger & Hudson [54] and Kufer *et al.* [72]), and the most promising molecules seem to be (tandem) diabodies and bispecific single-chain antibodies (bssc-AB) [54, 72].

Diabodies are particularly small bispecific antibodies consisting of two variable heavy chain (V_H) and two variable light chain (V_L) immunoglobulin domains from two different antibodies, connected by a short (three to twelve aminoacid) peptide linker in a fashion so that each V_H is attached to a V_L of the other antigen specificity and *vice versa* (figure 1.4 A). Diabody crystal structures revealed considerable flexibility, rendering the molecule capable of bridging surface antigens between different cells. However, the connection of the antigen-binding arms by two AA linkers is assumed to cause steric restrictions and this might become problematic in situations in which the simultaneous accessibility of two antigens on two different cells itself is restricted. The next step in the "evolution" of the conventional diabody was the generation of bssc-ABs, i.e. the arrangement of two single-chain antibody fragments (scFv) connected by a short, flexible linker on a single polypeptide chain (figure 1.4 B). In

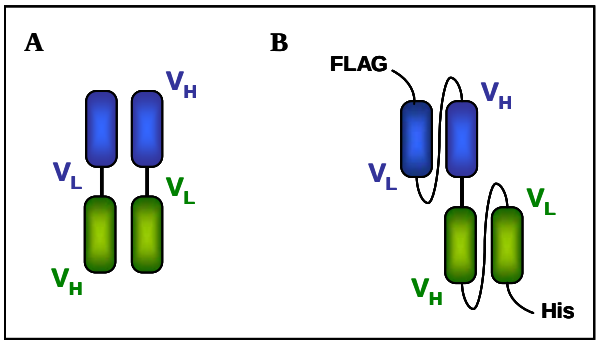

Figure 1.4: Small bispecific antibodies. Small bispecific (bs) antibodies comprise only the variable (Fv) regions of two antibodies of different specificity, connected with short peptide linkers. Lack of the Fc-domain avoid undesired biological effects and make the molecule more compact for better accessibility to the site of action. **A)** Bispecific diabody (50-55kDa): hetero-dimer of two domain-swapped Fv-moieties. **B)** Bispecific single-chain (bssc) antibody (55-60kDa): two scFv-fragments joined in tandem by a flexible peptide linker form a single molecule (optional with FLAG- or His-tags). Different colours indicate different binding specificities.

contrast to the diabody, the two binding sites in bssc-ABs can rotate freely and their axes can be bent. This ability may be advantageous when simultaneous binding of two antigen epitopes juxtaposed on two different cell surfaces is required.

1.2.3.4 Bispecific T-cell engagers (BiTEs)

BiTEs (bispecific T-cell engagers) are considered the "new generation" of bssc-ABs, which combine the proven efficacy of the CD3-triggered cytotoxic response with the advantages of the compact single-chain format. The biological activity of most therapeutic bsABs is limited due to the need for: additional signals inducing expression of the triggering receptor and/or a pre- or co-stimulation of effector cells; high concentrations of the recombinant antibody (limited drug supply, dose-limiting toxicity); and/or an excess of effector over target (E:T) cells, which is rarely encountered *in vivo*.

Studies with the novel bssc-AB constructs anti-17-1AxCD3 (anti-EpCamxCD3) and anti-CD19xCD3 (MT103) [72-81], and more recently anti-Wue-1xCD3 (MT105) [82] give proof that BiTEs indeed are capable to overcome these limiting complications. Especially use of bssc-anti-CD19xCD3 has been thoroughly studied with respect to its significance for treatment of B-cell neoplasia (non-Hodgkin lymphoma, acute/chronic lymphocytic leukaemia) and has now entered phase I clinical trials. The results so far are very promising. Sufficient amounts of MT103 for clinical application can efficiently be produced in eukaryotic cells, as properly folded, fully functional molecule and can be recovered from the cell culture supernatant without the need of renaturation steps or removing unwanted by-products [73].

In *in vitro* cytotoxicity assays with MT103, lymphoma cell lines and primary cells from lymphoma patients were co-cultured with autologous or allogeneic effector cells. These experiments have shown that, compared to the conventional bispecific anti-CD19xCD3 mAB, recombinant MT103 displays significantly higher and more rapid lytic activity selectively directed against B-cell lines and patient primary cells, but not against control cell lines. Incubation of CTLs with MT103 in the absence of B-cells did not trigger a cytotoxic response, nor did MT103 show cytotoxic effects on B-cells alone. Besides, cytotoxic effects were blocked with parental mABs anti-CD19 or anti-CD3 and controls with bssc-anti-17-1AxCD3a (directed against the epithelial marker EpCAM) showed no reactivity, proving the specificity of MT103. Importantly, MT103-mediated specific lysis was observed at extremely

low concentrations of the bssc-AB (0.2-2 pM), and with E:T ratios as low as 1:10 and pre- or co-stimulation of effector T-cells with CD28 or IL-2 was also not required [76-79]. These findings were successfully validated *in vitro* [73-77, 79, 80, 82], in mouse models [78] and most recently in chimpanzees [81].

Since CD19 is expressed almost throughout the entire B-cell lineage MT103 is potentially applicable for the treatment of the majority of B-cell neoplasia. However, one of the exceptions is multiple myeloma, since expression of CD19 on PCs is strongly downregulated or entirely absent. Therefore, a bssc-AB was generated on basis of the recently isolated plasma cell-specific antibody anti-Wue-1 [41]. This molecule was named bssc-anti-Wue-1xCD3 or MT105. Like its analogue MT103, bssc-anti-Wue-xCD3 displays extraordinary properties when tested in cytotoxicity assays with either cell lines positive for the WUE-1 antigen, or with primary cells isolated from MM patients [82]. The results are discussed in more detail in sections 3.1 and 4.3.

1.2.4 Chimeric T-cell receptors (TCRs)

As discussed in the previous sections a promising approach in adoptive immunotherapy is the initiation of MHC-independent, specific target cell lysis by means of cytotoxic T-cells. Alternatively to soluble bispecific single-chain antibodies recombinant chimeric T-cell receptors (TCRs) have been developed, which combine the antigen-specific binding properties of an antibody with the cytotoxic activity of T-cells in a single molecule [83-87]. Such a chimeric TCR (or "T-body") comprises extracellularly the single-chain Fv antibody fragment, specific for a tumour-associated antigen (TAA) and intracellularly the signalling domain from a membrane-associated receptor involved in cellular activation. T-cells can be transduced efficiently with retroviral vectors encoding the recombinant receptor. Basically each available AG-specific scFv fragment can be joined to the sequence encoding the CD3ζ-chain signalling moiety, and since it was assumed that co-stimulation, e.g. by CD28, may be beneficial for T-cell activation the latest generation of recombinant TCR-encoding vectors also comprise a CD28 sequence [88-91]. CTLs expressing a chimeric receptor engage in cytotoxic signalling cascades, leading to secretion of cytokines, recruitment of other functional components of the immune system to the site of action, and subsequently modulation and amplification of the immune response. Specific lysis of target cells by T-bodies was achieved at an effector:target cell ratio of as low as 1:20 [92]. A variety of these

chimeric TCR-constructs have already been tested successfully *in vitro* and *in vivo*, e.g. against B cell lymphoma (anti-CD20), Hodgkin's lymphoma (anti-CD30), gastrointestinal tumours (CEA) Neuroblastoma (NCAM), gp120 (HIV), and others [86, 87, 93, 94].

In this project, a recombinant chimeric TCR was created with WUE-1 antigen specificity. This construct was generated to screen a WUE-1 or MM cDNA expression library, thus bringing the T-body approach to an entirely new application.

1.3 WUE-1, a potentially plasma cell-specific antigen

As pointed out in sections 1.1 and 1.2 the main impediment of applying adoptive immunotherapy for the treatment of multiple myeloma is caused by the lack of suitable TAAs or surface-molecules on plasma cells. To date, all known markers that are expressed on plasma cells were found to be also expressed on other cell types. CD138 (syndecan-1) for example is also found on epithelial cells; CD38 is an activation AG which is also present on haematopoietic stem cells; CD56, a N-CAM splice variant, is also expressed on natural killer cells; and HM1.24 is expressed on stroma cells and other tissues. Typical pan-B-cell markers like CD19, CD20, or CD22 are downregulated or even absent on terminally differentiated B-cells. Therefore, the search for target molecules specifically expressed on (preferably malignant) plasma cells has been a major focus of all researchers working with MM.

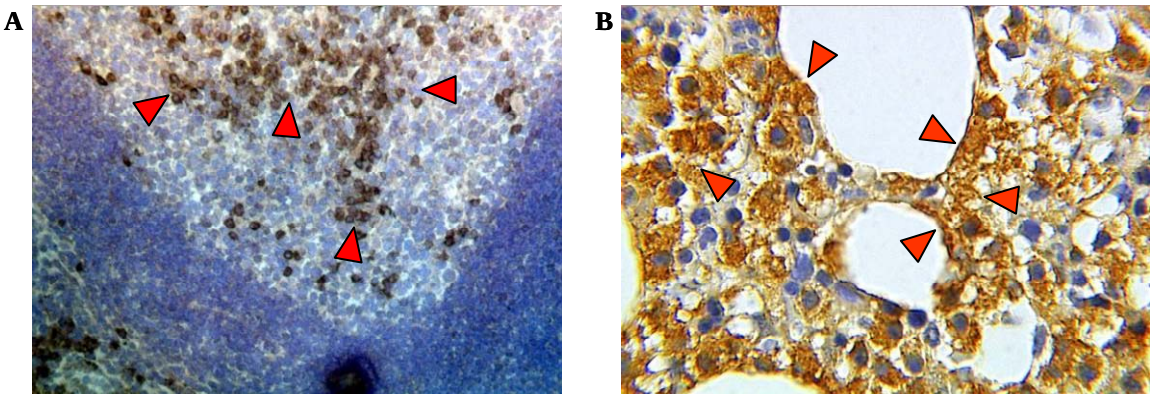

Figure 1.5: Immunohistochemical staining of WUE-1 on tonsils and primary MM cells. A) Anti-Wue-1 immunoperoxidase stain of a cryostat section of fresh-frozen normal tonsils. WUE-1 positive plasma cells in the light zone of the germinal centre are indicated by arrowheads. **B)** Anti-Wue-1 immunoperoxidase stain of a multiple myeloma biopsy. WUE-1 positive plasma cells are indicated by arrowheads. Images kindly provided by Dr. A. Greiner, University of Würzburg

In the group of Dr. Axel Greiner at the Institute of Pathology, University of Würzburg the attempt was undertaken to generate and screen antibodies, which display plasma cell specificity. Mice were immunized with the MALT-lymphoma derived cell line H3302/88 and repeated subcloning yielded stable, immunoglobulin secreting hybridoma cell lines. The secreted immunoglobulins were subsequently tested for plasma cell specificity by immunohistochemistry or FACS analysis. One antibody of IgG_1 subtype, designated anti-Wue-1, was identified, which demonstrated the desired features. It was shown that anti-Wue-1 specifically recognizes a hitherto unknown membrane associated structure on normal and malignant human plasma cells [41]. Immunohistochemical stains of normal tonsils or primary multiple myeloma cells with anti-Wue-1 monoclonal antibody have confirmed WUE-1 antigen expression on plasma cells in both samples (figure 1.5).

In table 1.1, the WUE-1 expression on various lymphoma types is summarized. The results clearly show that WUE-1 is present on all plasmacytoma and plasmacytoma-type lymphoma (plasma cell leukaemia), as well as on MALT lymphoma with plasma cell differentiation. On the other hand, samples of MALT lymphoma without plasma cell differentiation and early B-cell lymphoma are WUE-1 negative. These promising results encouraged the generation of a bispecific single-chain anti-Wue-1 x CD3 antibody for use in cytotoxicity assays to substantiate the specificity of the antibody [82] (see also sections 1.2.3.4 , 3.1.1, and 4.3).

Table 1.1: Analysis of a variety of lymphoma samples for WUE-1 expression

Lymphoma	**WUE-1 positive/total**
Plasmacytoma/myeloma	11/11
MALT-type lymphoma with plasma cell differentiation	13/13
MALT-type lymphoma without plasma cell differentiation	0/19
Immunocytoma	5/6
Diffuse large-cell lymphoma	1/13
Follicular centre lymphoma	0/23
Mantle cell lymphoma	0/10
Burkitt's lymphoma	0/5
B-cell lymphocytic lymphoma	0/5
Peripheral T-cell lymphoma	0/7
Angioimmunoblastic lymphoma	0/9
Hodgkin's disease	0/13

1.4 Objective

This work is aimed to contribute to the understanding of the biology of the terminally differentiated B-cell and its malignant counterpart, the myeloma cell. Functional characterization of the novel plasma cell-specific antigen WUE-1 could be extremely valuable for the identification of signals, which influence the differentiation and survival of human plasma cells. If WUE-1 has an impact on signalling pathways in the target cell, this may allow conclusions on growth-, survival-, and drug resistance mechanisms, thus helping to develop novel therapeutic strategies for MM (e.g., adoptive immunotherapy by means of recombinant bssc-antibody constructs, chimeric T-cell receptors).

Although the precise molecular structure of the WUE-1 antigen is still unclear, its expression profile and biochemical characteristics discriminate WUE-1 from other plasma cell-associated antigens described so far. Therefore, this PhD project is aimed at the identification, cloning and functional characterization of the novel WUE-1 antigen. Additionally, the recently generated bispecific single-chain (bssc) antibody anti-Wue-1xCD3 will be functionally characterized. Moreover, hybridoma supernatants (generated by Dr. Axel Greiner, University of Würzburg) will be screened in order to identify additional plasma cell-specific antibodies.

Preliminary *in vitro* experiments have shown expression of the WUE-1 antigen on primary MM cells, plasmacytoma and plasmacytoma-type lymphoma (plasma cell leukaemia), and on MALT lymphoma with plasma cell differentiation. In contrast, WUE-1 expression on plasma cell-lines was variable and instable. Hence, due to the need of an assured, and ideally, unlimited supply of research material further MM cell lines will be screened for stable WUE-1 expression. Because to date it was not possible to identify or isolate WUE-1 by WB or IP with standard protocols, these methods will be further optimized and refined. Moreover, a cDNA expression library from a WUE-1 positive cell line will be generated, and utilized for expression cloning and immunoselection ("panning") with monoclonal anti-Wue-1 antibody. Alongside, a recombinant chimeric T-cell receptor (TCR) will be generated, containing the single-chain anti-Wue-1 antibody domain. The anti-Wue-1 TCR-expressing effector cells will be utilized for a functional screen of a multiple myeloma cDNA library.