4 Discussion

Despite numerous approaches to find a cure for multiple myeloma (MM), to date this haematological malignancy remains irredeemable. In MM mechanisms develop, which mediate myeloma cell homing to the bone marrow. As a consequence, interactions of MM cells with the surrounding bone marrow stromal cells (BMSCs) trigger pathways causing the release of cytokines, which promote MM cell growth, survival, drug resistance, angiogenesis, or migration. Oncogenic mutations (translocations) enable the cells to survive and proliferate, resulting in immortalization of the malignant cell clone, and in later disease stages lead to stroma-independent growth, selection of a single clone for malignant expansion and escape from the BM microenvironment [3, 5]. Over the past years, many new targets have been proposed, based upon the advanced understanding of genetics, biology, and the signalling pathways underlying disease initiation and progression, and the components involved in MM pathology. Combination treatment and novel drugs such as thalidomide and its derivates (immunomodulatory agents), bortezomib (proteasome inhibitor), and others (recently reviewed by Barlogie *et al.* [115], Richardson [46], Morgan & Davies [116], or Bruno *et al.* [20]), have improved the clinical situation of MM patients, especially in combination with haematopoietic stem cell transplantation (HSCT).

One of the main reasons for recurrent onset of the active disease is the persistence of minimal residual disease (MRD) after chemotherapy and HSCT. Major hope is placed upon the use of immunotherapeutic strategies (i.e., adoptive immunotherapy) in patients with MRD to intervene exactly at this stage of disease progression. It is assumed that the amount of enduring malignant cells in patients with MRD is low enough to be efficiently targeted by specific antibodies [14, 51]. The use of tumour directed monoclonal antibodies (mABs) and derivates thereof for the treatment of haematopoietic diseases has already proven applicable (e.g. anti-CD20 (Rituximab), anti-CD52 (Campath-1H), anti-CD33 (Myelotarg)), and by now several novel constructs have entered clinical trials (recently reviewed by Wu & Senter [68] and Adams & Weiner [55]). The development of effective clinical therapeutics based on mABs bears various impediments: murine-derived mABs may be highly affine and specific, but in the human host, they are also immunogenic, have short serum half-lives, and lack efficient interaction with some human immune effector cells. With the creation of recombinant bispecific single-chain (bssc) ABs, most of the problems associated with conventional mABs have been overcome: bssc-ABs are compact molecules (approximately 50kDa), which

comprise on a single polypeptide chain the Fv-fragments (variable region) from two antibodies, connected by a short linker. One arm of the bssc-antibody is specific for a tumour-associated antigen (TAA), the other one is designed to target the effector cell. Thus, bssc-ABs stand out due to high specificity, efficient induction of cytotoxic response, good bioavailability and retention time, enhanced tumour infiltration capability, and economic production with high yields of clinical grade material.

Recent focus has been set on the use of a bssc-anti-CD19xCD3 antibody (MT103) for treatment of B-cell malignancies [76-81]. In *in vitro* cytotoxicity assays with lymphoma cell lines MT103 displayed significantly higher and more rapid lytic activity than the conventional bispecific anti-CD19xCD3 mAB. Induction of specific, B-cell directed lysis by MT103 takes place at low antibody concentrations and does not require (co-) stimulation of the effector T-cells. However, MT103 is not applicable for the treatment of multiple myeloma, since many of the specific markers on B-cells, such as CD19 and CD20, are strongly downreglated or entirely absent on plasma cells. In consequence, the situation for MM patients is less promising with regard to immunotherapy and this fact has prompted the search for suitable surface antigens on plasma cells.

4.1 Investigating plasma cell-specific antibodies and antigens

4.1.1 Screening of hybridoma supernatants

The group of Dr. Axel Greiner (Institute of Pathology, University of Würzburg) has generated stable, mucosa-associated lymphoid tissue (MALT) lymphoma derived immunoglobulin secreting hybridoma cell lines. Two of the antibodies, which were identified after screening the immunoglobulins for plasma cell specificity, namely anti-Wue-1 and anti-Mue-1, were selected for this study. Some knowledge about specificity and characteristics of the anti-Wue-1 antibody and its antigen had already been gained [41], but anti-Mue-1 (an immunoglobulin of the IgM-κ subtype) was an entirely novel antibody, which had not yet been investigated.

One of the basic requirements for the direct approach of identifying specific antibodies from hybridoma supernatants, and consequently of gaining rapid and explicit information about the associated antigens, is the applicability of methods like immunohistochemistry, flow cytometry (FACS), immunoprecipitation (IP) and/or Western blotting (WB). Analysis of anti-Mue-1 using those methods revealed that the antibody recognized a protein of approximately

25kDa. With two-dimensional WB followed by MALDI/MS analysis the antigen was identified as human λ-light chain. Although in this case the identified antigen was not a novel plasma cell-specific protein the experiment gives solid proof of principle.

On the other hand, the attempted identification of the WUE-1 antigen demonstrated the pitfalls of this approach. Here, the basic requirement of an antibody or antigen that is suitable for analysis by IP and/or WB was not met. Therefore, methods had to be refined and alternative approaches had to be considered, which made the whole identification process time consuming and laborious.

4.1.2 Anti-Wue-1 is highly specific for human plasma cells

The justification as to why such numerous attempts were undertaken to identify the WUE-1 antigen lie within its prospective suitability as target for immunotherapeutic approaches in the treatment of MM. Preliminary experiments with anti-Wue-1, an antibody of the IgG_1 subtype, demonstrated extraordinary specificity for plasma cells and mucosa-associated lymphoid tissue (MALT) lymphoma with plasma cell differentiation. Thus, it was postulated that anti-Wue-1 specifically recognizes a novel, hitherto unknown membrane associated structure on normal and malignant human plasma cells [41]. Moreover, the recombinant MM directed bssc-anti-Wue-1xCD3 AB (MT105) demonstrated specificity for primary myeloma cells and MM cell lines, when used in cytotoxicity assays. In 9/11 cases efficient T-cell mediated lysis of NCI-H929 and primary myeloma cells was induced in the presence of 2-10µg/ml bssc-anti-Wue-1xCD3 and at a ratio of 1:2 effector to target cells. It was shown that both, allogeneic and autologous effector cells were able to induce specific lysis, where normally an allogeneic response is MHC restricted. This demonstrates the capability of MT105 to induce MHC independent cytotoxicity, an important feature for possible clinical application. In accordance with the results obtained with MT103 [76-81], additional stimulation of PBMCs was not required to induce a cytotoxic response, addition of non-specific bssc-anti-EpCAMxCD3 or bssc-anti-CD19xCD3 did not trigger the effector cells, and target cell lysis was prevented by the parental anti-Wue-1 mAB in a competitive manner. Moreover, a WUE-1 negative control cell line was not affected by MT105 and in the absence of T-cells no cytotoxic effects were observed [82]. These results clearly show that bssc-anti-Wue-1xCD3 initiates a specific and efficient immunoresponse and is therefore a highly interesting molecule for antibody-based treatment of MM.

4.1.3 ARH77 and NCI-H929 cell lines are used for studies of WUE-1

Knowledge of the molecular structure of the molecule that is recognized by anti-Wue-1 is not only a prerequisite for further characterization of the molecule, but also for the application of the bssc-antibody as a novel agent in MM immunotherapy. Moreover, insight into the nature of the WUE-1 antigen can help to generate antibodies for immunotherapy, which display higher specificity, better bioavailability, and probably less side effects.

Primary cells from the bone marrow of MM patients would be the ideal research material for studies of WUE-1. However, the amounts of cells required for such extensive analysis cannot be granted by biopsies from patients. Thus, it was imperative to identify a suitable cell line, which stably expressed the WUE-1 antigen, and therefore could provide unlimited supply of antigen-bearing cells for thorough investigation. The human plasma cell leukaemia cell line ARH77 and the human multiple myeloma cell line NCI-H929 met the requirements. FACS analysis of ARH77 cells with anti-Wue-1 confirmed stable, reproducible antigen expression, whereas on NCI-H929 WUE-1 expression was more variable and less strong. On the other hand, the slow proliferation rate of the NCI-H929 cells rendered this cell line particularly suitable for the cytotoxicity assays with bssc-anti-CD19xCD3 and bssc-anti-Wue-1xCD3 (when ARH77 cells were used in cytotoxicity assays the decrease of target cells, due to T-cell mediated lysis, was blotted out by their high proliferation rate). Both cell lines were used for applications such as FACS analysis, immunoprecipitation, Western blot, and for functional assays.

4.1.4 Western blot or immunoprecipitation failed to identify WUE-1

The main difficulty encountered in the attempt to identify and isolate WUE-1 was that the anti-Wue-1 antibody does not function in conventional biochemical applications, such as Western blotting (WB) and immunoprecipitation (IP). Controversially, the antibody is applicable for immunostaining (immunohistochemistry) and flow cytometry, i.e. when the cells are still intact. This indicates that preparation of cell lysates, isolation of (membrane) proteins, and denaturing gelelectrophoresis are likely to destroy the recognition site for the antibody, thus resulting in failure of WB or IP. In the attempt to identify a setting of gentle conditions suitable for WB or IP analysis of ARH77 protein preparations was carried out with

various different methods. The main issues addressed were (A) lysis conditions, (B) protein preparation, (C) electrophoresis, (D) blotting conditions, (E) labelling and detection, and (F) crosslinking and immunoprecipitation (detailed information is provided in the chapter 2). Moreover, it was attempted to achieve renaturation of the proteins either directly after SDS-PAGE by washing the acrylamid gels free from SDS, followed by an "in gel" WB, or by renaturing the proteins directly on the Western blot membrane (data not shown).

Despite all efforts to optimize the assay conditions, it was neither possible to immunoprecipitate WUE-1 nor to identify a specific band for the antigen on a Western blot. The circumstance, that even after crosslinking of the anti-Wue-1 mAB to the cell surface precipitation of the antigen failed remains obscure. Identification of a specific band on Coomassie or silver nitrate stained gels was attempted by comparison of protein patterns of immunoprecipitates obtained from WUE-1 positive and WUE-1 negative cell lines, respectively. There may be the probability that the IP was actually successful, but if the specific WUE-1 band is weak, it could be overlaid by other proteins of similar size. Moreover, the amount of protein recovered by IP may be insufficient to be detected by Coomassie or silver nitrate staining. In this case, it may be an option to perform the IP, recover the proteins, and subject the entire sample to analysis by MALDI/MS.

Taken together, the results gave rise to the assumption that WUE-1 is probably a conformational epitope, or a structure comprised of more than one distinct moiety. On intact cells (such as used for FACS analysis), the epitope is displayed in its native state and is recognized by the anti-Wue-1 AB. Upon cell disruption or lysis, the spatial conformation of the epitope is altered or the single moieties are disrupted, so that the specific antibody-binding site is destroyed. A further explanation of the failure to detect a specific band for WUE-1 on a WB could be that the antibody recognizes an abundant carbohydrate structure. A variety of glycoproteins have been reported to be tumour-associated antigens, in fact heavy glycosylation appears to be a common feature on malignant cells [117]. This issue will be discussed in more detail below (section 4.1.6)

4.1.5 Is the anti-Wue-1 binding affinity sufficient for analytical assays?

Due to the difficulties, which were encountered with immunoprecipitation and Western blotting it was postulated that anti-Wue-1 exhibits only low binding affinity to its antigen.

This issue was investigated by performing FACS analysis with ARH77 cells, which were incubated with either PE-conjugated anti-Wue-1 (red fluorescent) or Alexa Fluor® 488-conjugated anti-Wue-1 (green fluorescent). Then both cell populations were mixed and incubated. One would assume that after some time an exchange of labelled antibody would occur between the two cell populations if the binding affinity of anti-Wue-1 was low, resulting in a double-stained cell population. However, this was not observed, indicating that once the antibody has bound to its antigen it remains stably bound.

In a recent publication addressing the issue of binding affinities, it was proposed that antibodies which bind to multivalent cell surface antigens can be considered to bind with infinite affinity, i.e. the dissociation rate is zero, thus bivalent antibody binding can be considered predominantly irreversible [118]. This proposition is based on a mathematical model, which takes into account that bivalent binding on the cell surface occurs in two dimensions and depends on the mobility of reactants on the cell surface. The author states that in cases where antibodies bind bivalently approximately 80-90% of AB binds irreversibly and only a small fraction of 10-20% of the AB dissociates rapidly. It is concluded, that bivalent antibody binding is much stronger than monovalent binding, but that binding strength depends on the nature of the antigen and on whether the structure allows for bivalent binding [119]. This model would be in accordance with the results obtained with the PE-, and Alexa Fluor 488-labelled anti-Wue-1 antibodies.

Moreover, the author states that weak monovalent antibody binding, i.e. an increased dissociation rate, may be caused by incubation at low temperatures, addition of soluble, competing AG, or excess of AB. Low temperatures may result in "freezing" of the membrane, i.e., reduced mobility of membrane components, and thus inhibition of bivalent binding. Excess AB or competing antigen is thought to interfere with antibody binding to the second binding site, consequently causing "wobbling" and detachment of second arm of the bivalently bound AB. This hypothesis may explain the low binding affinity of anti-Wue-1 in IP reactions (especially when the antibodies were added prior to cell lysis and in the presence of crosslinkers), since the reactions are performed at low temperature and with an excess of antibody.

4.1.6 Is WUE-1 a carbohydrate antigen?

Carbohydrate molecules cover the cell surface of mammalian cells, mainly in shape of glycolipids or glycoproteins, which are related to certain cell types or differentiation stages.

Various tumour-associated antigens comprise cell surface carbohydrates, thus representing valuable markers for the identification of human cancer. Analysis of tumour specific glycoproteins has therefore been subject of many investigations.

Real *et al*. [120] for example describe a class I melanoma antigen (i.e. an antigen exclusively present on tumour cells), which was characterized to be a glycoprotein due to its binding affinity to lentil lectin, but the researchers did not succeed in their efforts to immunoprecipitate the molecule. Interestingly, some of the features, which were associated with glycoprotein antigens apply also to WUE-1. For example instable expression (i.e. unpredictable cyclic variation) of class I antigens was described for *in vitro* cultured cells, as was noted for WUE-1 on NCI-H929 cells. Moreover, poor immunogenicity and the impossibility to isolate the protein by immunoprecipitation was reported [120-122]. The latter was mainly attributed to insufficient antibody levels, inefficient methodology, poor labelling of the AGs, the composition carbohydrate or aminoacid composition of the antigen, loss of antigen expression or the slow rate of antigen synthesis.

The probability that anti-Wue-1 recognizes a carbohydrate antigen was investigated by performing a carbohydrate specific ELISA. This method was successfully used to identify antibodies specific for blood group-related carbohydrate antigens [100, 123-125]. The carbohydrate antigens are mimicked by polyacrylamid (PAA) conjugated saccharides [126] which are coated into the microtiter plates. Specificity of the antibody binding to carbohydrate antigens can be analyzed by periodate oxidation of the carbohydrates prior to antibody incubation. Periodate oxidation is a common tool for structural analysis of glycoproteins. By means of treatment with periodic acid it is possible to identify mABs, which are directed against carbohydrate-containing epitopes associated with glycoproteins or glycolipids. Anti-carbohydrate antibodies can be roughly divided into ABs with a groove-type binding site, which bind at non-terminal locations along linear chains and antibodies with a pocket-type binding site that reacts at the non-reducing terminus of carbohydrate chains. Mild periodate oxidation at acid pH cleaves carbohydrate vicinal hydroxyl-groups, for example on cell surface glycoconjugates, thus altering the antigen structure and preventing antibody binding, but without altering the structure of the polypeptide chains. Especially glucose and other saccharides are cleaved by periodic acid, due to abundance of vicinal diol moieties. More drastic conditions (longer periods of periodate oxidation followed by borohydride reduction) on the other hand may be destructive to the peptide moiety of the glycoproteins [127, 128]. Most anti-carbohydrate ABs are directed against non-reducing terminal carbohydrate structures

(partial review by Hakomori & Kannagi [129]), which are generally susceptible to cleavage by periodic acid. Electrophoretic separation of membrane proteins prior to periodate oxidation permits direct identification of glycoproteins possessing periodate sensitive epitopes on a Western blot. However, not all carbohydrate antigens are sensitive to treatment with periodic acid, e.g. determinants consisting of linear sugar chains with linkages on carbon C3, determinants located at branch points and those containing partially *O*-acetylated sialic acids [101]. Generally, the possibility exists that periodate oxidizes certain amino acids (cysteine, methionine, tryptophane, tyrosine, and histidine) even when they are substituted, as it is the case in a polypeptide chain [128].

By means of the carbohydrate ELISA, anti-Wue-1 was found to be reactive with the monosaccharides β-D-glucose and β-D-galactose, but not with any of the blood group-specific polysaccharide antigens. After treatment of the PAA-conjugates with periodic acid binding of a blood group specific (anti-Thomsen-Friedenreich) antibody to its antigen was abolished (positive control), whereas binding of anti-Wue-1 to β-D-glucose and β-D-galactose was not altered. As mentioned above, not all carbohydrates are sensitive to periodate oxidation, but glucose is one of the saccharides that contains vicinal hydroxyl groups susceptible to cleavage. Therefore, if anti-Wue-1 was specific for β-D-glucose, an effect should have been noted after periodate oxidation.

The question as to whether WUE-1 is a carbohydrate antigen was further addressed by trying to block the anti-Wue-1 binding site with lectins. Lectins are carbohydrate-binding molecules, containing at least two identical carbohydrate-binding sites, via which they can bind cells and/or carbohydrate-conjugates (polysaccharides, glycoproteins, glycolipids). The specificity of a lectin is determined by the saccharide, which competitively inhibits agglutination and by the sterical location of the carbohydrate on the cell surface, which has to be fully accessible. Here, ARH77 cells were preincubated with a panel of biotinylated lectins with various specificities. Then FACS analysis with anti-Wue-1 antibody was performed. However, binding of anti-Wue-1 to ARH77 cells was not altered.

The question whether WUE-1 may possibly be a carbohydrate antigen or at least contains a carbohydrate moiety remains controversial. On the one hand, it was shown that anti-Wue-1 does bind β-D-glucose and β-D-galactose monosaccharides in the carbohydrate ELISA and the reactivity is not abolished by periodate oxidation. On the other hand, anti-Wue-1 shows no reactivity with the more complex blood group specific polysaccharides, which are more likely

to be present on the cell surface. This may indicate that anti-Wue-1 reactivity with β-D-glucose and β-D-galactose is a non-specific artefact. This assumption is strengthened by the fact, that incubation with lectins does not block anti-Wue-1 binding to ARH77 cells. On the other hand, carbohydrate epitopes are generally present in many copies on the cell surface and are spaced irregularly. With regard to antibody binding this may suggest that a mixture of bi- and monovalently bound AB exists on the cells in varying amounts, depending on the antigen preparation. The variation of the amounts of bi- and monovalently bound AB is particularly relevant if antigens are immobilized, as for example fixation to ELISA plates. This may result in a changed conformation and antigen density in solid phase binding assays, and therefore different results as obtained with intact cells [119].

Probably the best way to investigate involvement of carbohydrate structures in anti-Wue-1 binding to target cells would be enzymatic digestion of the carbohydrate determinant. Alternatively, impact of glycosylation inhibitors such as tunicamycin on anti-Wue-1 binding to surface glycoproteins could be examined.

4.1.7 Is WUE-1 a protein?

The failure of WUE-1 immunoprecipitation and Western blot analysis gave rise to the question whether WUE-1 is a protein at all. Bearing in mind the results from the carbohydrate ELISA and the lectin FACS analysis (section 4.6.1), it is unlikely that the antibody binds to a molecule that does not comprise a protein epitope. Nonetheless, in order to provide evidence that WUE-1 is at least in part a protein enzymatic digestion of cell surface proteins with Pronase or phospholipase C (PLC) was performed on ARH77 cells. Pronase is a broad specificity protease mixture, which contains various types of endo- and exopeptidases (e.g. serine and metalloproteases; carboxypeptidases and aminopeptidases), therefore cleaving virtually all types of proteins. PLC is specific to glycosylphosphatidylinositol (GPI) anchored proteins.

Normal transmembrane proteins are restricted in their free movement within the plasma membrane. They tend to cluster, and are often immobilized by interaction with cytoskeletal proteins. On the other hand, cell surface proteins, which are attached within the membrane by GPI-phospholipid anchors, maintain a high mobility. Usually the lack of a transmembrane domain prevents interactions of GPI-anchored proteins with the cytoskeleton, thus, their

mobility is not restricted by cytoskeletal structures. Many GPI-anchored proteins are co-receptors or adhesion molecules, and freedom of movement in the membrane may be advantageous for their interactions with ligands. Functions suggested for GPI anchors include (1) allowing proteins an increased lateral mobility, (2) mediation of the release or secretion of proteins by activation of a lipase, (3) involvement in signal transduction of receptor-mediated events, (4) regulation of endocytosis or protein turnover, and (5) targeting protein to apical surfaces [130]. The signal transduction mechanisms involving GPI-anchored proteins that lack intracellular domains are not fully understood. Some studies suggest that protein tyrosine kinases are part of the signal transduction mechanism by which GPI-anchored proteins mediate T-cell activation, but very little is known about the mechanisms involved in GPI-mediated signal transduction or indeed even how such signals are transduced across the plasma membrane. It was proposed, that proteins involved in signal transduction might be confined to different lipid microdomains (rafts) to form clusters. Clustering of GPI-anchored proteins could activate signalling pathways leading either to signal transduction mediated by enzymes which get activated in the rafts, or might lead to interaction with signal-transducing proteins that become activated in consequence [131].

Concerning WUE-1, it was clearly demonstrated by FACS analysis that the molecule is sensitive to Pronase treatment. The serine protease inhibitor PMSF greatly prevented digestion of WUE-1 by Pronase, leading to the conclusion that the WUE-1 antigen contains a protein epitope, which, is sensitive to digestion by serine proteases. FACS analysis of PLC treated cells showed that binding of anti-Wue-1 antibody was not affected by this enzyme, indicating that WUE-1 most likely does not belong to the family of GPI-anchored proteins.

4.2 Eukaryotic screening of expression libraries

4.2.1 Expression cloning and immunoselection failed to identify WUE-1

In addition to the standard biochemical procedures to identify the WUE-1 antigen, cDNA libraries were screened for WUE-1 expressing clones. One library was generated from the WUE-1^+ cell line ARH77. Additionally, a MM library generated from polyA^+ mRNA from four MM patient bone marrow samples, was kindly provided by Dr. Sun-Jin Choi, University of Pittsburgh Medical Centre, USA. The libraries were transiently transfected into COS7 cells and screened by means of immunoselection with anti-Wue-1 antibody ("panning"). Selection

of positive clones occurs when transfectants expressing the target protein bind to a solid-phase matrix via protein-protein (antibody/antigen) interaction. Cells, which do not express the target protein will not adhere and will be washed away. Plasmid DNA contained within the adhered tissue culture cells is isolated and transformed into *E. coli* cells for amplification and large-scale plasmid DNA preparations. After several rounds of transfection and immunoselection the fraction of cells which carrying the plasmid with the sequence of interest are enriched.

The pitfall of screening the cDNA libraries by means of anti-Wue-1 mAB is that stable antibody-antigen interaction was once more crucial. Depending on the structure of the WUE-1 antigen screening by expression cloning may prove problematic. If WUE-1 consists of just one protein, probably with more than one conformational epitope immunoselection should be applicable. A possible explanation for the failure of anti-Wue-1 binding in that case would be that in COS7 cells glycosylation, protein folding, or presentation at the cell surface differ from the conditions found in tumour cells (e.g. MM or MALT lymphoma). On the other hand, if WUE-1 consists of more than one distinct protein domains panning with anti-Wue-1 is bound to fail, since the probability that exactly those two WUE-1 epitopes are expressed within the same COS7 cell is highly unlikely. In fact, the results obtained from the screen support this concern. The clones, which were identified after 3-4 rounds of panning were all non-specific. Furthermore, the majority of the identified clones coded for proteins which are not cell surface associated (e.g. GAPDH or transcription factors). It is possible that the selected clones had a growth advantage resulting from the protein they were expressing.

4.2.2 Functional screening with a WUE-1 specific chimeric T-cell receptor lacks sufficient sensitivity

Alongside the immunoselection approach a functional assay screen was developed for the cDNA expression libraries. A functional screen can be performed either with transiently or stably transfected eukaryotic cells. If analysis of a library is performed by means of transient expression, it is required to subdivide the library into smaller pools of clones prior to library amplification, in order to prevent the dilution of a positive cell signal with an excess of negative clones. The initial pool size as well as the number of pools required in the screen is determined by the sensitivity of the available assay. It has to be ensured that a single clone within a pool is still theoretically detectable within the population of transfected cells. Each

clone pool is then amplified separately and transfected into the eukaryotic cells. The transfected cells are subsequently tested for the expression of the desired clone by means of a bioassay. Once a pool is identified as containing the clone of interest, it is subdivided into smaller pools for a second round of prokaryotic amplification, eukaryotic transfection, and screening. After several rounds of enriching for the desired clone, a single clone can be isolated and further analyzed as required.

In this project, a novel functional assay was developed for screening of the expression libraries on basis of chimeric T-cell receptors (TCRs). The molecules combine antibody like specificity with the capability to trigger efficient MHC-independent immune response in grafted cytotoxic T-lymphocytes (CTLs). Compared to strategies relying either only on CTLs, or on antibodies, a major advantage of using CTLs transfected with a chimeric TCR is that virtually any antigen for which a mAB exists can be targeted. Moreover, the requirements of MHC restriction in the interaction of effector cells with target cells are bypassed, the tumour cell binding of CTLs grafted with chimeric receptors is not affected by downregulation of HLA class I antigens or by defects in the antigen-processing machinery. Furthermore, CTLs grafted with a chimeric receptor can be targeted not only to protein antigens, but also to structures such as gangliosides and carbohydrates, which are not recognized by normal TCRs [132]. An advantage of chimeric TCRs over the use of bispecific antibodies (which also rely on a combination of T-cell and AB-based mechanisms to destroy tumour cells) is that by stable transfection of T-cells with a chimeric TCR (i.e. viral transduction) CTLs can be generated which will constitutively express the chimeric receptor. In contrast, bispecific antibodies remain bound to target and effector cells only for a limited amount of time until they are proteolytically degraded [84]. Upon activation by antigen, T-cells transfected with a chimeric receptor will secrete cytokines that recruit or modulate other components of the immune system at the tumour site, thus amplifying the immune response to tumour cells. Secretion of cytokines can be used as readout for a functional screen. To date several chimeric TCR-constructs with various specificities have successfully been tested *in vitro* and *in vivo*. The constructs are aimed for immunotherapy of B-cell lymphoma (anti-CD20), Hodgkin's lymphoma (anti-CD30), gastrointestinal tumours (CEA), Neuroblastoma (NCAM), and gp120 (HIV) [86, 87, 93, 94]. Here, the fact, that WUE-1 expression was shown exclusively on normal and malignant plasma cells [41], and specific lysis of WUE-1 positive target cells was efficiently induced by the use of bssc-anti-Wue-1xCD3 antibody [82] has prompted the development of a WUE-1 specific chimeric TCR for potential immunotherapeutic use in MM. Two

recombinant chimeric T-cell receptors were generated for this study, comprising the anti-Wue-1 single-chain variable (Fv) domain attached to a human IgG Fc sequence, the transmembrane moiety of CD28, and an intracellular CD3ζ signalling domain. For the functional screen with the chimeric scWue1 TCRs an interferon gamma (IFN-γ) ELISA was chosen as readout. Expression of the chimeric TCR on the cell surface of grafted Jurkat cells was successfully demonstrated by triggering the IFN-γ release through addition of Fc specific anti-human IgG to the cell-cultures. This proves that (at least) the extracellular Fc moiety of the chimeric WUE-1-specific TCR is presented on the cell surface. However, when anti-Wue-1 TCR-grafted Jurkat cells were co-cultured with WUE-1^+ cell lines (ARH77, NCI-H929) IFN-γ was not detected in the cell culture supernatants.

A possible explanation for this phenomenon may be that due to steric inhibition antigen/antibody (AG/AB) binding did not occur when whole cells were used. The small anti-Fc antibody molecule on the other hand can easily access its binding site. Moreover, transfection efficiency and the number of chimeric TCR molecules on the cell-surface of the grafted Jurkat cells may strongly influence AG/AB linking. Low transfection efficiency or low TCR expression levels may result in insufficient triggering of the immunoresponse. On the other hand, too many TCR molecules per cell may result in steric hindering of antigen binding. Conversely, the group of Dr. Max Topp (University of Würzburg) was able to show that T-cells grafted with the anti-Wue-1 chimeric TCR were able to specifically kill primary multiple myeloma cells in a cytotoxicity assay (personal communication). This result indicates that the anti-Wue-1 TCR specifically recognizes the antigen on primary target cells and a cytotoxic response is initiated upon AB/AG binding. As to whether this positive result was due to the use of different effector T-cells, other effector:target (E:T) cell ratios, different transfection methods, or the fact that primary MM cells were used instead of cell lines needs to be further investigated.

Although the methods for generation of chimeric TCRs are well established little is known about the impact of the recombinant receptors on antigen recognition and subsequent effector cell activation. Additional co-stimulatory molecules on the target cells may further influence recombinant receptor mediated T cell activation, especially when recognition of heterologous target antigens (e.g. carbohydrate antigens) and subsequent activation of different T cell effector populations (e. g. $CD4^+$ or $CD8^+$ T-cells) is concerned [85]. In the context of the effector:target (E:T) cell ratios which are required for efficient target cell lysis ratios of as low

as 1:20 have been reported [92]. Other constructs require an E:T ratio within a range of 10:1 [93] up to 90:1 [133], depending very much on the target antigen.

It can be concluded that prior to performing the library screen further optimization experiments are necessary to ensure (1) high transfection rates for COS7 and Jurkat cells, (2) sufficient amounts of cells/well for efficient recovery and amplification of plasmid DNA, (3) sensitivity of detection of the IFN-γ ELISA. Particular focus has to be shed on the format of the screen. A 96-well format, as it was used in this study, may not be sufficient for use with cellular co-cultures, although it is suited for IFN-γ ELISA after stimulation with anti-Fc mAB. It seems more advisable to start with a large format and to determine the ideal E:T cell ratios for successful IFN-γ ELISA first and continue with the scale-down from there. Limiting to the scale-down is not only the sensitivity of IFN-γ detection, but also the minimum number of cells/well, which are needed to be able to still recover and amplify plasmid DNA for use in further rounds of screening. Finally, as discussed in section 4.2.1, the positive outcome of the library screen is very much dependent on the structure of the antigen, i.e. whether it is a conformational epitope that needs to be glycosylated, folded, and presented properly in COS7 cells, or whether it is comprised of different proteins.

4.3 Conclusion: What we have learned about the WUE-1 antigen

The antibody anti-Wue-1 and its associated antigen WUE-1 are certainly very controversial molecules. Up to now, the antigen is only scarcely characterized, mainly because immunoprecipitation and Western blotting keep failing. It is conceivable that WUE-1 comprises either a conformational epitope, or the epitope consists of more than one distinct proteins, probably also bearing a carbohydrate moiety.

In applications such as immunohistochemistry anti-Wue-1 monoclonal antibody specifically recognizes normal and malignant plasma cells (i.e. MM cells and MALT lymphoma cells with plasma cell differentiation). The anti-Wue-1 monoclonal antibody binds with high affinity to intact cells in applications such as flow cytometry. The antibody seems to be only weakly affine when used for immunoprecipitation or immunopanning and Western blot analysis with anti-Wue-1 mAB does not work. Binding of the mAB to its antigen cannot be blocked by incubation of the cells with lectins and was not abolished after treatment of WUE-1^+ cells with phospholipase C, which cleaves glycosyl-phosphatidylinositol (GPI) protein

anchors. However the WUE-1 molecule was found to be sensitive to digestion by serine proteases.

In cytotoxicity assays with bispecific single-chain anti-Wue-1xCD3 antibody T-cell mediated lysis of primary MM cells or WUE-1 positive MM cell lines is specifically induced. Immunoresponse (i.e. IFN-γ release) of T-cells grafted with the recombinant chimeric anti-Wue-1 TCR can be triggered through addition of Fc-specific mAB, but IFN-γ was not detected in co-cultures of WUE-1^+ cell lines and effector T-cells grafted with the WUE-1-specific TCR. On the other hand specific target cell lysis was demonstrated in cytotoxicity assays with anti-Wue-1 TCR-grafted effector T-cells and primary myeloma target cells. It has to be kept in mind that the use of cell lines can only be a model to mimic "natural" conditions. During enhanced periods of subculturing, freezing and thawing, cell lines are likely to undergo mutations, which may slowly change their phenotype. Thus, results obtained with primary cells should be considered more trustworthy. Thus, results obtained with primary cells cannot be directly compared to those obtained with cell lines.