3 Results

3.1 Functional Assays

3.1.1 Bispecific single-chain anti-Wue-1xCD3 antibody induces antigen-specific T-cell mediated lysis

Specific expression of the WUE-1 antigen on normal and malignant plasma cells has been subject of preliminary studies and has already been reported [41]. In order to provide a tool for functional analysis of WUE-1, a bispecific single-chain (bssc) anti-Wue-1xCD3 antibody has been designed, expressed and purified as described elsewhere [73, 76, 82]. Functional experiments were performed with this novel bssc-antibody using primary multiple myeloma (MM) cells and MM cell lines and the results have recently been published [82].

Here, the functionality of bssc-anti-Wue-1xCD3 as tool for T-cell mediated cytotoxicity was investigated, using the multiple myeloma cell line NCI-H929 and the lymphoma cell line Nalm6. The bssc-anti-CD19xCD3 antibody served as control. First, NCI-H929 and Nalm6 cells were analyzed with mABs for expression of WUE-1 and CD19, respectively. The histograms of the FACS analysis in figure 3.1 A show WUE-1 expression on NCI-H929 cells, whereas Nalm6 cells are WUE-1 negative. On the other hand, Nalm6 cells are strongly positive for CD19, whereas NCI-H929 cells are CD19 negative (figure 3.1 B). The results were confirmed by immunoperoxidase staining of NCI-H929 cytospins with bssc-anti-CD19xCD3 and bssc-anti-Wue-1xCD3, respectively. As the images in figure 3.2 demonstrate, NCI-H929 cells were reactive with bssc-anti-Wue-1xCD3 (fig. 3.2 B) but not with bssc-anti-CD19xCD3 (fig. 3.2 A).

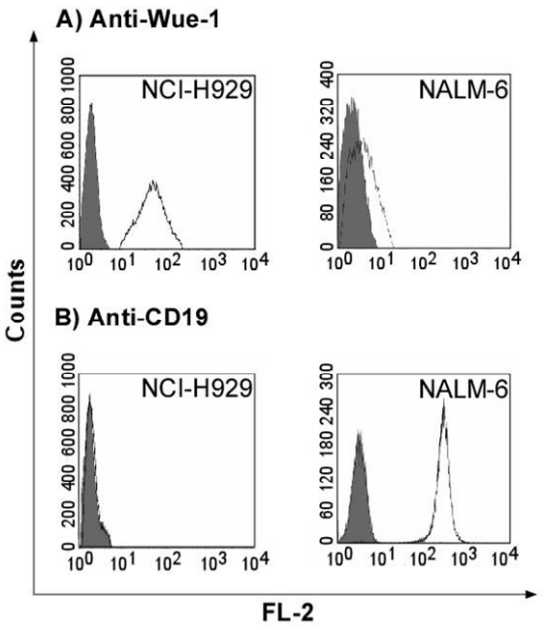

Figure 3.1: FACS analysis of NCI-H929 and Nalm6 cells. NCI-H929 multiple myeloma (MM) cells and Nalm6 lymphoma cells were analyzed for expression of WUE-1 (**A**) and CD19 (**B**). The cells were incubated with the respective primary monoclonal antibodies, followed by R-PE-conjugated anti-mouse secondary antibody.

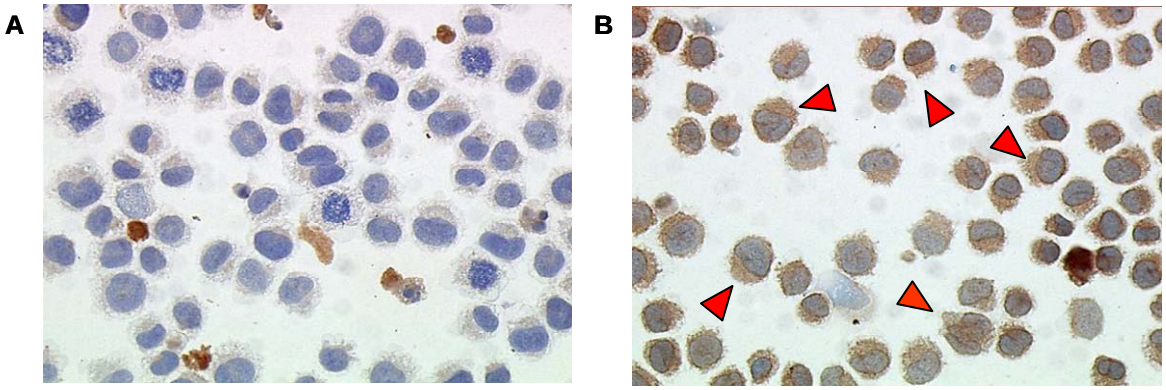

Figure 3.2: Immunohistochemical staining of NCI-H929 cells. Using the immunoperoxidase method cytospin preparations of NCI-H929 cells (WUE-1$^+$/CD19$^-$) were stained with bispecific single-chain (bssc) anti-CD19xCD3 or bssc-anti-Wue-1xCD3. **A)** The negative control antibody bssc-anti-CD19xCD3 shows no reactivity with NCI-H929 cells. **B)** Strong WUE-1 expression on the cell surface of NCI-H929 cells can be detected with bssc-anti-Wue-1xCD3 (**arrowheads**).
Images kindly provided by Dr. A. Greiner, University of Würzburg

For the cytotoxicity assay, a target cell population consisting of equal amounts of NCI-H929 and Nalm6 cells was used. This mixed population of WUE-1 positive (WUE-1$^+$) and WUE-1 negative (WUE-1$^-$) cells was used to show that even in the presence of activated T-cells cytotoxic effects of bssc-anti-Wue-1xCD3 are strictly dependent on antibody-mediated interaction of target and effector cells. Therefore, either the NCI-H929 or the Nalm6 cells in the mixed target population were labelled with the cell-membrane dye PKH-26 (Sigma), and were mixed with unlabelled effector T-cells, isolated from buffy coats of healthy donors. The cells were subsequently incubated with bssc-anti-Wue-1xCD3 (MT105), bssc-anti-CD19xCD3 (MT103), or the non-specific control antibody bssc-anti-EpCAMxCD3 (EpCAM = epithelial marker epitope 17.1a). The cells were harvested, stained with propidium iodide (PI) in order to distinguish non-viable cells, and were analyzed by FACS. The specific cytotoxicity was calculated by comparing the relative number of viable target cells (PI$^-$/PKH$^+$) in the bssc-anti-CD19xCD3 and bssc-anti-Wue-1xCD3 samples, respectively with the viable target cell fraction in the bssc-anti-EpCAMxCD3 control samples (equation see section 2.8.2). Moreover, the decrease in the viable target cell fractions was normalized against possible increase of the T-cell fraction due to proliferation, by adding a known number of CaliBRITE beads as an internal standard.

The results of the cytotoxicity assay are summarized in figure 3.3. At an effector:target (E:T) cell ratio of 10:1 and without additional stimulation of the T-cells cytotoxic effects on NCI-H929 cells were observed only in the presence of bssc-anti-Wue-1xCD3. When effector T-cells were omitted from the assay the cytotoxic effect of bssc-anti-Wue-1xCD3 was reduced

by 87% (data not shown). Incubation of NCI-H929 cells with the non-specific antibody bssc-anti-CD19xCD3 on the other hand showed no effect. *Vice versa*, specific lysis of Nalm6 B-lymphoma cells was induced with bssc-anti-CD19xCD3, but not with bssc-anti-Wue-1xCD3. The negative control antibody bssc-anti-EpCAMxCD3, did not induce specific lysis of either cell line.

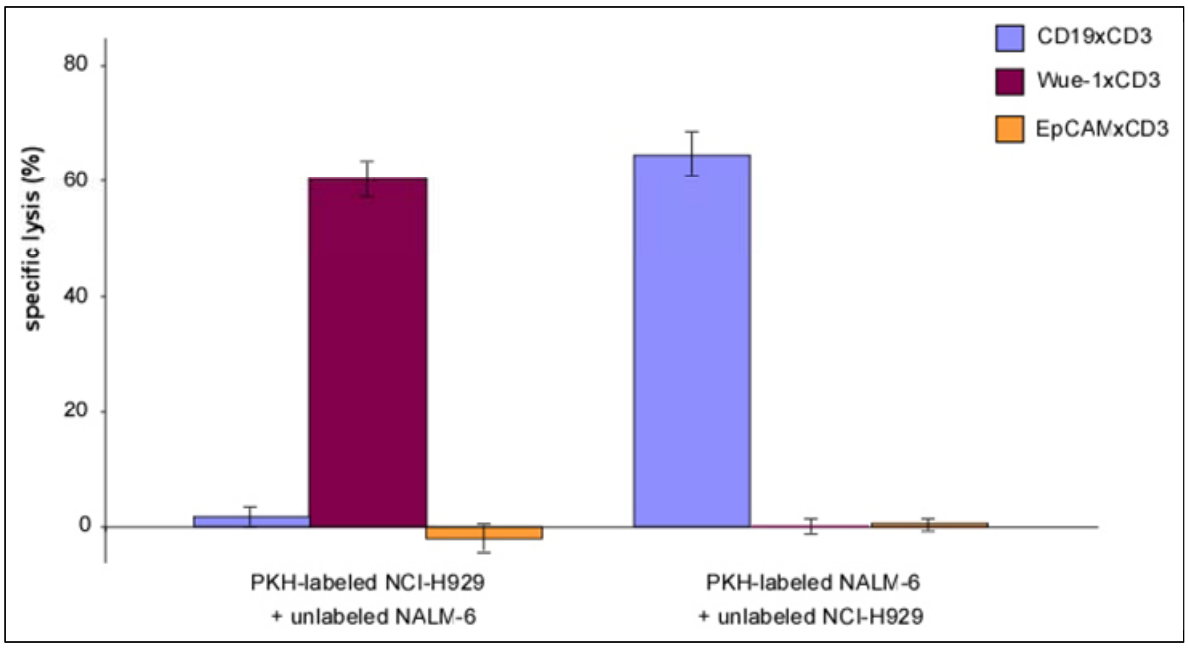

Figure 3.3: Target cell specific T-cell mediated cytotoxicity induced by bispecific single-chain (bssc) antibodies. A target cell population, consisting of equal amounts of NCI-H929 myeloma cells, labelled with the fluorescent membrane dye PKH-26 and unlabelled Nalm6 lymphoma cells (or *vice versa*), was incubated with 1µg/ml bssc-anti-CD19xCD3, 2µg/ml bssc-anti-Wue-1xCD3, or 2µg/ml bssc-anti-EpCAMxCD3 antibodies for 48h in the presence of effector T-cells, at a E:T ratio of 10:1. The percentage of specific lysis was determined by FACS analysis after staining the apoptotic cells with propidium iodide (PI).

It was also demonstrated that the cytotoxic effect of bssc-anti-Wue-1xCD3 is dose dependent. At a concentration of 2µg/ml bssc-anti-Wue-1xCD3 accounted for an average of ~20% specific lysis in three independent samples, increasing to 25-45% at a concentration of 10µg/ml (figure 3.4 A). Then, cytotoxicity assays at a constant bssc-anti-Wue-1xCD3 concentration (2µg/ml), and with varying effector:target cell ratios were performed. The results revealed that an increase in the E:T cell ratio from 1:1 to 5:1 enhances specific lysis of the target cells up to 3-fold. Thereafter the reaction seems to reach a plateau and further increase of effector cell concentration shows no additional effect (figure 3.4 B).

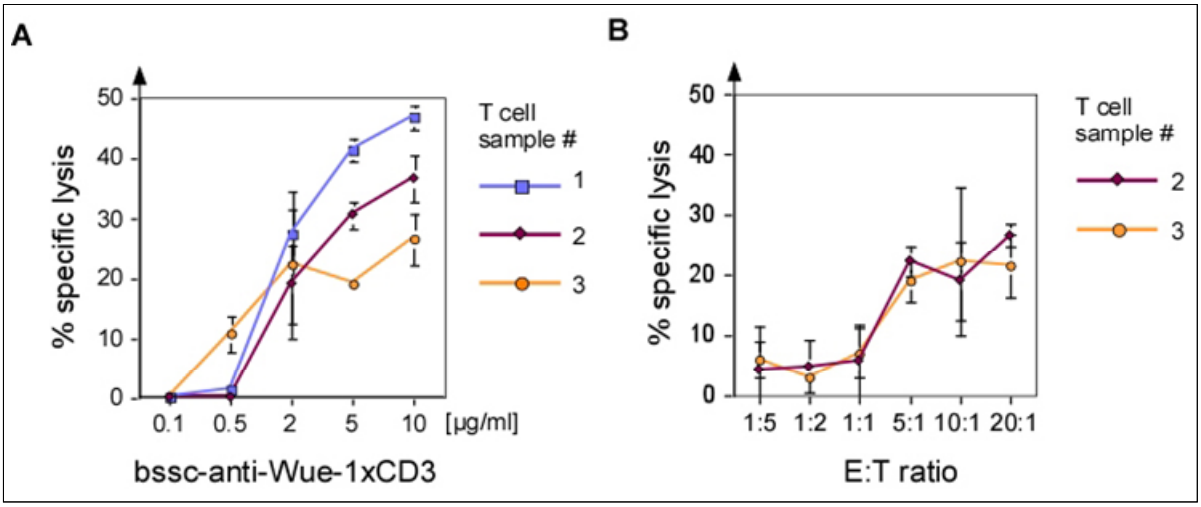

Figure 3.4: Dose dependent and effector:target (E:T) dependent specific cytotoxicity of bssc-anti-Wue-1xCD3. A) The cytotoxicity assay was performed with the MM cell line NCI-H929 at a constant E:T cell ratio of 10:1 and with increasing amounts of bssc-anti-Wue-1xCD3 antibody, ranging from 0.1µg/ml to 10µg/ml. T-cells were obtained from three independent healthy donors and were not further stimulated. **B)** The cytotoxicity assay was performed with the MM cell line NCI-H929 at a constant bssc-anti-Wue-1xCD3 concentration (2µg/ml) and with increasing E:T cell ratios, ranging from 1:5 to 20:1. T-cells were isolated from two independent donors and were not stimulated. In all assays cells and antibody were incubated for 48h prior to FACS analysis.

Finally, the antigen specificity of the bssc-anti-Wue-1xCD3 mediated lytic effect was demonstrated, using monoclonal anti-Wue-1 antibody to compete binding of the bssc-antibody. It was found, that at an eight-fold molar excess of monoclonal anti-Wue-1 antibody to bssc-anti-Wue-1xCD3 target cell lysis was reduced by up to four-fold. Addition of IgG_1 isotype control had no effect (see reference [82]). Taken together, these results give solid proof that the cytotoxic effect caused by bssc-anti-Wue-1xCD3 antibody is target cell- and antigen specific.

3.1.2 A chimeric WUE-1-specific TCR was not triggered by WUE-1 positive cells, but with Fc-specific anti-human IgG

The antigen-specific binding of bssc-anti-Wue-1xCD3 was demonstrated in cytotoxicity assays; now a novel functional screening assay was developed, using the "T-body" approach. "T-bodies" are recombinant T-cell receptors (TCRs), which comprise an antibody-derived single-chain domain to bind to a specific antigen. This scFv-fragment is attached to a human IgG Fc sequence, the transmembrane moiety of CD28, and an intracellular signalling domain e.g., the FcεRI receptor γ-chain or the CD3ζ chain [88, 111] (see also figure 2.9 A). Thus, the molecule combines antibody like specificity with the capability to trigger MHC-independent

immune response of the transfected T-cells. For the WUE-1 directed functional screen of the ARH77- or MM- expression library two recombinant chimeric single-chain (sc) anti-Wue-1-TCR constructs were created and cloned into the expression vector pBullet. The constructs were named pBullet-κ-scWue-1-Fc-CD28/CD3ζ (short: pBullet-κ-Wue-1) and pBullet-κ-HA-scWue-1-Fc-CD28/CD3ζ (short: pBullet-κ-HA-Wue-1). The latter construct bears a HA-tag at the N-terminus of the scWue-1 binding domain to facilitate detection of transfected T-cells. Moreover, constructs were designed with a κ-leader sequence to ensure surface expression of the chimeric receptor. For the functional screen with the WUE-1 specific TCRs an interferon gamma (IFN-γ) ELISA of the cell culture supernatants was chosen as readout (see also figure 2.9 B).

Prior to the actual library screen, the functionality of the assay was tested. Effector T-cells were generated by transient transfection of Jurkat cells with pBullet-κ-(HA)-Wue-1, or pBullet-607 (non-specific control). Mock transfected Jurkat cells (i.e. w/o DNA) were used as negative control. WUE-1^{+} NCI-H929 or ARH77 cells served as target cells. The co-cultures were set up in the presence of non-transfected COS7 cells to control for non-specific activation. However, IFN-γ could not be detected in the cell culture supernatants from co-cultures of ARHH77 or NCI-H929 target cells and the effector Jurkat cells. On the other hand, addition of Fc-specific anti-human IgG to transfected Jurkat effector cells was able to trigger the IFN-γ release. Addition anti-human IgG to the mock transfected Jurkat cells showed no effect (figure 3.5). This result suggests that the chimeric TCR is expressed by the transfected Jurkat cells and that it can be triggered via its extracellular Fc-domain. Moreover, 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 (unpublished data, 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. The negative results of the IFN-γ ELISA with the WUE-1^{+-} cell lines may be due to insufficient sensitivity of the assay. At this point further experiments were postponed in order to refine and improve the assay prior to its application in the library screen.

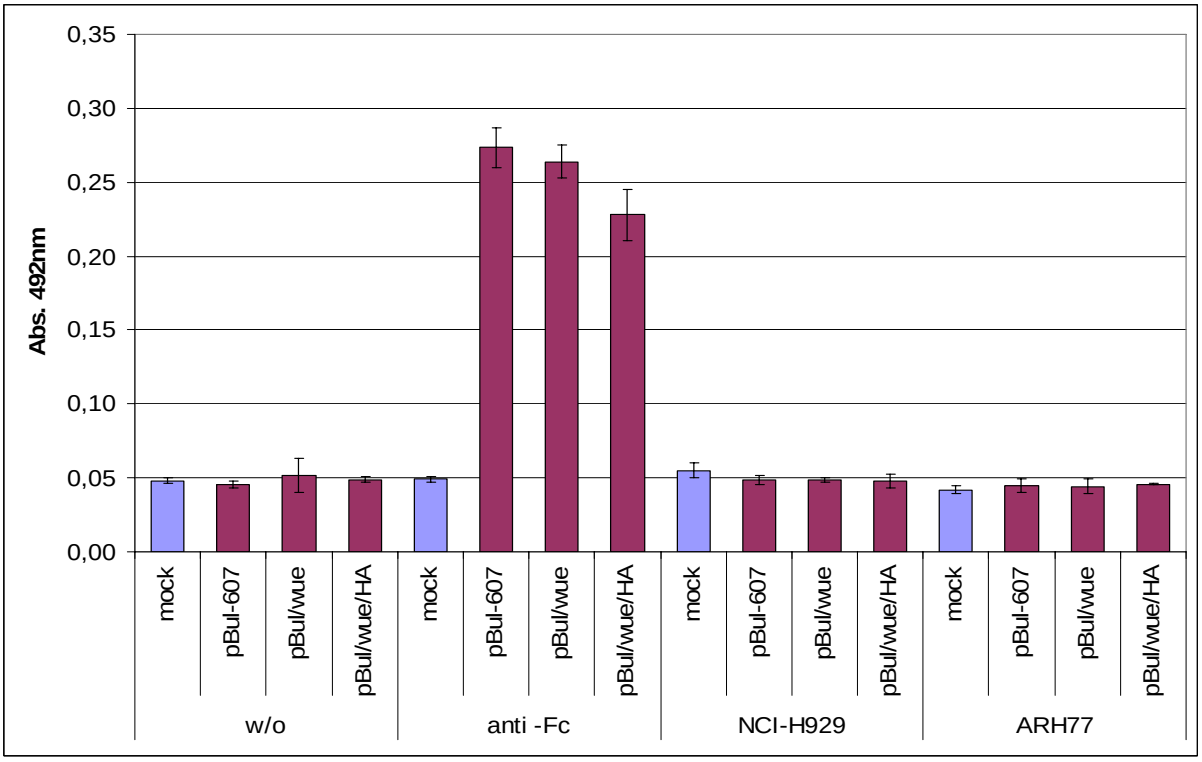

Figure 3.5: IFN-γ reporter assay with a WUE-1-specific chimeric T-cell receptor (TCR). Jurkat (T-) cells were transfected with the anti-Wue-1 chimeric T-cell receptor construct (pBullet-κ-Wue-1 or pBullet-κ-HA-Wue-1). Controls were mock transfected (w/o DNA) or transfected with parental non-specific pBullet-607. For initiation of the T-cell receptor mediated IFN-γ release the Jurkat effector cells were co-cultured with WUE-1 positive ARH77 or NCI-H929 cells 48h post-transfection. Addition of anti-human-Fc mAB served as positive control. Supernatants of the cultures were analyzed for IFN-γ by ELISA after 48h. The diagram depicts the average results of three independent experiments.

3.2 WUE-1 expression profile

In preliminary studies the expression profile of WUE-1 has been determined on a panel of primary multiple myeloma and B-cell lymphoma cells [41]. Here, FACS analysis was carried out with a panel of cell lines (table 3.1), including multiple myeloma, plasma cell leukaemia, and B-cell lymphoma. Cell lines which were found to be WUE-1 positive were ARH77 (human plasma cell leukaemia) and NCI-H929 (human MM; see also section 3.1.1). ARH77 cells show a strong and reproducible WUE-1 expression, whereas on NCI-H929 cells the expression of the antigen may vary. However, both cell lines proved suitable for FACS analysis (figure. 3.6), and the more slowly proliferating cell line NCI-H929 was successfully used for cytotoxicity experiments [82].

Table 3.1: WUE-1 expression on MM cell lines

Cell Line	Origin	WUE-1 expression
ARH77	human plasma cell leukaemia	strong expression
DOHH-2	human B-cell lymphoma	negative
Ina-6	human MM	negative
L363	human plasma cell leukaemia	negative
MM.1S	human MM	negative
NCI-H929	human MM	variable expression
OPM-2	human MM	negative
RPMI-8226	human MM	negative
U266	human MM	negative
BL60-2 (negative control line)	human Burkitt lymphoma	negative

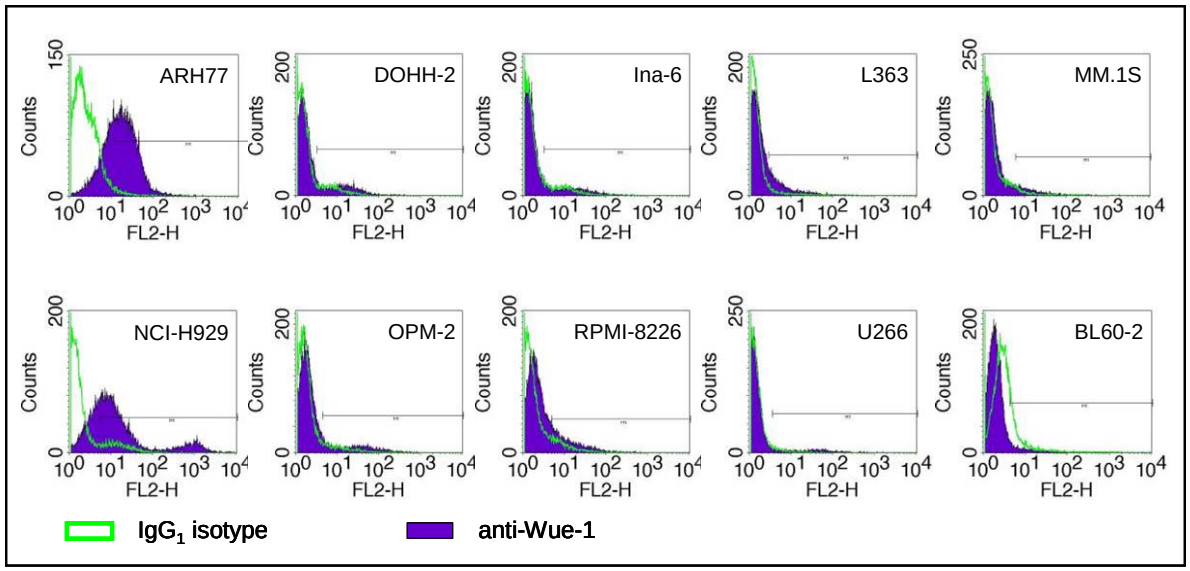

Figure 3.6: FACS analysis of WUE-1 expression on cell lines. The cells were incubated with anti-Wue-1 antibody (1:500; **filled histograms**) and IgG_1 isotype control (1:50; **open histograms**), respectively, followed by PE-conjugated anti-mouse secondary antibody (1:100). See table 3.1 for origin of the cell lines.

WUE-1 expression was also examined by means of immunohistochemistry (figures 3.2, 3.7) and cellular ELISA (figure 3.8), and was confirmed for both, ARH77 and NCI-H929 cells. No expression of WUE-1 was detected on the negative control lines Nalm6 and BL60-2. The IgG_1 isotype control samples were also negative. Thus, ARH77 and NCI-H929 were considered suitable for use in the identification process of the WUE-1 antigen and were chosen as model cell lines throughout this project.

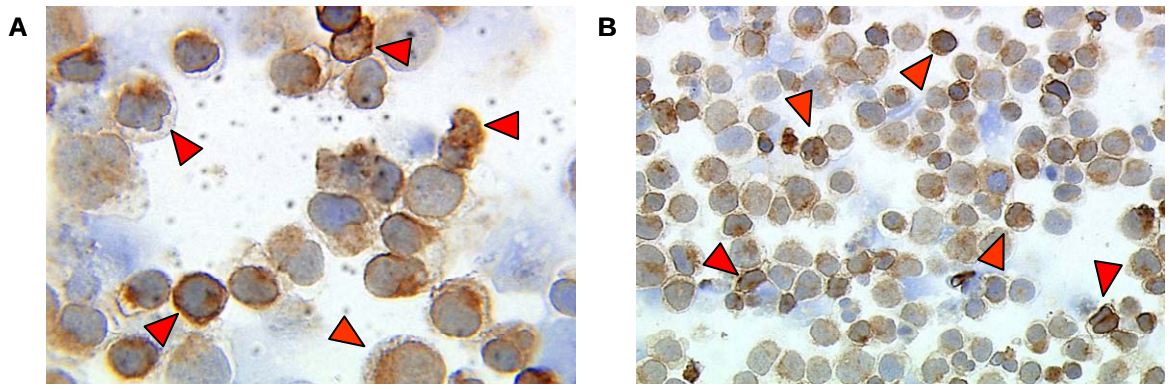

Figure 3.7: Immunohistochemical staining of ARH77 cells. Using the immunoperoxidase method cytospin preparations of ARH77 (plasma cell leukaemia) cells were stained with either with (**A**) monoclonal anti-Wue-1 or (**B**) bispecific single-chain (bssc) anti-Wue-1xCD3. With both antibodies strong WUE-1 expression is detected on the cell surface of ARH77 cells (**arrowheads**).
Images kindly provided by Dr. A. Greiner, University of Würzburg

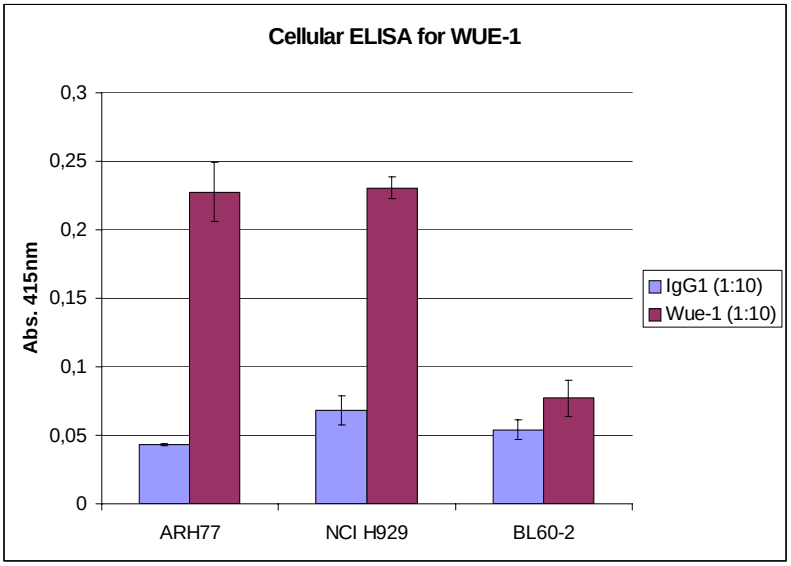

Figure 3.8: Cellular ELISA. Cells were coated into poly-L-lysine treated microtiter plates and fixed with 10% buffered formalin. The wells were incubated with diluted anti-Wue-1 antibody or with IgG_1 isotype control, followed by addition of HRP-conjugated anti-mouse secondary antibody (1:2500). The plate was measured at λ_{415nm}. The reactions were carried out in quadruples.

3.3 Immunoprecipitation (IP) and crosslinking experiments

In addition to the functional analysis with monoclonal anti-Wue-1, bispecific single-chain anti-Wue-1xCD3, or chimeric anti-Wue-1 T-cell receptors (section 3.1) a focus of this project was to identify and isolate the WUE-1 antigen. This was mainly attempted by using methods like Western blotting (WB), immunoprecipitation (IP), or expression cloning (section 3.5). Moreover, the properties of the WUE-1 antigen were to characterize, in order to gain more information about the molecule (section 3.4). A variety of different methods was performed in

the attempt to immunoprecipitate the WUE-1 antigen. The methods shared the same principle, namely to label and/or concentrate the antigen so it can be identified on a SDS PAGE gel by comparing the protein patterns of WUE-1 positive cell lines to those of WUE-1 negative control lines.

3.3.1 Immunoprecipitation with paramagnetic beads

3.3.1.1 "Protein A" Dynabeads®

In this experiment the anti-Wue-1 antibody was bound to protein A coated paramagnetic beads via its Fc region. For antibody/antigen complex formation the anti-Wue-1 coupled beads were added to ARH77 cells. In a control reaction, "Protein A" Dynabeads® were added to the cells without pre-incubation with anti-Wue-1 antibody. For negative control, BL60-2 cells per reaction were treated likewise. The cells were disrupted in a N_2-cavitation chamber, and the bead/anti-Wue-1/antigen complexes were retrieved by means of a magnet. The samples were then subjected to SDS PAGE on a 4-12% gradient gel (figure 3.9). Comparison of the band patterns from the IP reactions (lanes 2 and 4) did not reveal a specific band for WUE-1 in the ARH77 cell sample.

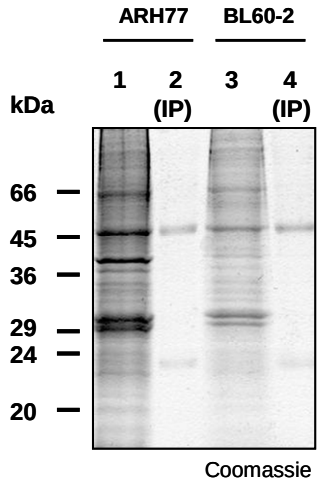

Figure 3.9: Immunoprecipitation with Protein A coated paramagnetic beads. Protein A coated paramagnetic beads were coated with anti-Wue-1 antibody. $3x10^7$ ARH77 or BL60-2 cells were incubated with the coated beads, the cells were disrupted in a N_2-cavitation chamber and the bead/anti-Wue-1/antigen complexes were retrieved. In the negative controls the anti-Wue-1 antibody was omitted. The samples were boiled in Laemmli buffer and subjected to SDS PAGE on a gradient gel. The gel was fixed and stained with colloidal coomassie. **Lane 1**: ARH77 neg. control; **lane 2**: ARH77 Wue-1 IP; **lane 3**: BL60-2 neg. control; **lane 4**: BL60 Wue-1 IP.

3.3.1.2 "Pan Mouse IgG" Dynabeads®

In contrast to the protein A coated paramagnetic beads (section 3.3.1.1) "Pan Mouse IgG" beads are coated with an antibody that recognises all mouse IgG subclasses and that is Fc specific. These beads were used in order to reduce non-specific binding of components in the

ascites fluid as may occur when protein A is used. In this experiment, immunoprecipitation was performed with WUE-1 positive ARH77 and NCI-H929 cells, and WUE-1 negative L363 and RPMI-8226 cells, respectively (see figure 3.6). The cells were pre-incubated with anti-Wue-1 ascites, followed by addition of "Pan Mouse IgG" beads. Anti-Wue-1 was omitted in the negative control reactions. The cells were disrupted in a N_2-cavitation chamber either prior to the addition of the beads or afterwards. The bound proteins were eluted from the beads with glycine buffer (pH2), and separated by SDS PAGE on a 4-12% Bis-Tris gradient gel (Novex, Invitrogen; figure 3.10). The band patterns of the WUE-1 positive ARH77 and NCI-H929 cells were compared to the patterns of the WUE-1 negative L363 and RPMI-8226 cells. Bands, which result from the paramagnetic beads (figure 3.10, lanes 4 and 5), are indicated by stars and these bands are present in all samples. Some bands, which are present in the IPs of the WUE-1 positive cells, can also be detected in the WUE-1 negative cell samples (figure 3.10, open arrowheads).

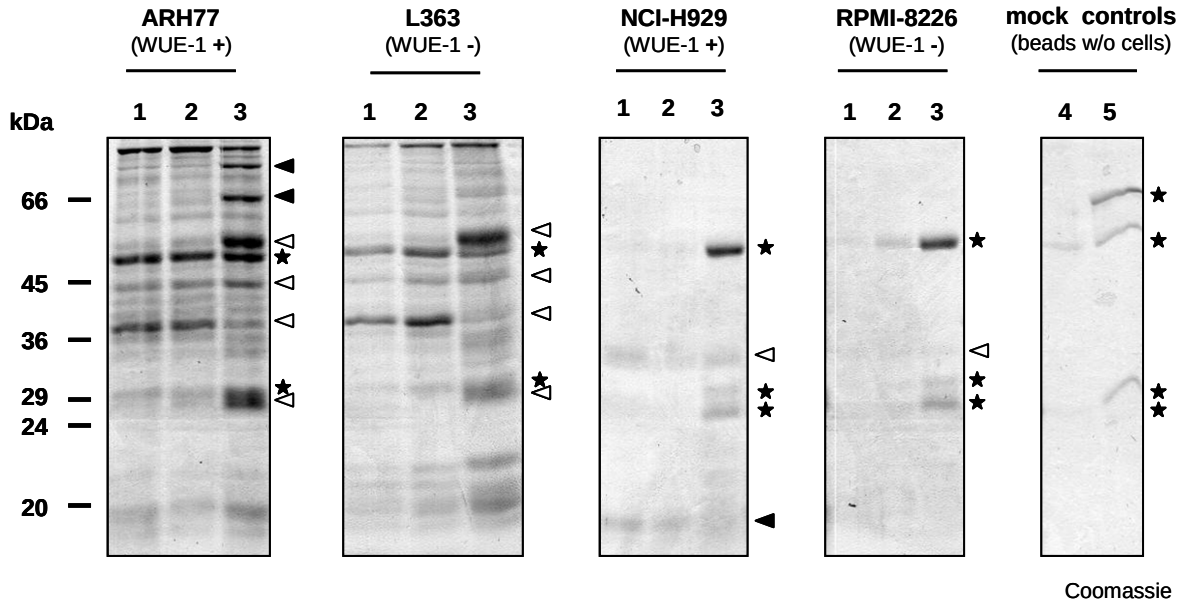

Figure 3.10: Immunoprecipitation using "Pan Mouse IgG" paramagnetic beads. 3x 10^7 WUE-1^+ ARH77 and NCI-H929 cells, and WUE-1^- L363 and RPMI-8226 cells were pre-incubated with anti-Wue-1 ascites. In the negative controls (**lane 1**) the antibody was omitted. The paramagnetic beads were added to the antibody coated cells either prior to disruption (**lane 2**), or afterwards (**lane 3**). The cells were disrupted in a N_2-cavitation chamber, the bead/antibody/membrane protein complexes were retrieved, followed by elution with glycine buffer, pH2.0. The eluted samples were subjected to SDS PAGE on a gradient gel. In a control reaction the beads were added to buffer w/o cells and processed alongside (**lane 4**). After elution the remaining beads were boiled in Laemmli buffer and the supernatant was applied onto the gel (**lane 5**). The gels were fixed, and stained with colloidal coomassie. Band patterns of the WUE-1 positive and the WUE-1 negative cell lines were compared. Bands which result from the paramagnetic beads are indicated by **stars**; strong bands which are present in WUE-1^+ as well as WUE-1^- cell lines are marked by **open arrowheads**; strong bands which are present only in the WUE-1^+ cell lines are marked by **filled arrowheads**.

In the band pattern of the ARH77 IP two strong bands of approx. 66kDa and 90kDa can be distinguished which are not present in the IP of the WUE-1$^-$ cell lines. Also, the NCI-H929 IP shows one band of approx. 20kDa which is not present in the IPs of L363 and RPMI-8226 (figure 3.10, lanes 3, filled arrowheads).Nevertheless, bands of the same sizes are also present in the negative control IPs of ARH77 and NCI-H929, where anti-Wue-1 was omitted (figure 3.10, lanes 1). Therefore, it was assumed that the bands are not WUE-1 specific. As to why the bands in lanes 3 of the IPs are stronger than in lanes 1 and 2 may be due to the sample preparation. Lane 3 contains the IP sample where the cells were first disrupted and the anti-Wue-1 antibody was added afterwards. It is possible that if the cells are first incubated with the antibody and disrupted afterwards there is less non-specific binding, thus the bands in lanes 1 and 2 are weaker.

3.3.2 Anti-Wue-1 crosslinked to metabolically [^{35}S] labelled cells

Compared to coomassie staining of protein gels the detection of radiolabelled proteins by autoradiography displays greater sensitivity. Thus metabolic protein labelling of ARH77 (WUE-1$^+$) and BL60-2 (WUE-1$^-$) cells with L-[^{35}S]-methionine was performed prior to cell lysis and IP. Moreover, the labelled cells were treated with a thiol-cleavable NHS-ester (DTSSP) after antibody incubation and prior to lysis. This was done to prevent dissociation of the antibody during the course of sample preparation and/or immunoprecipitation, thereby causing negative results in IP reactions. The crosslinker is cleaved only during sample preparation for SDS gelelectrophoresis (i.e. boiling in reducing Laemmli buffer).

For each IP reaction, 1x10^7 cells were labelled with L-[^{35}S]-methionine. The cells were then incubated with IgG_1 isotype control, anti-Wue-1, or anti-HC-10 antibodies, respectively. Anti-HC-10 is directed against human MHC class I heavy chain and served as control for successful IP. The samples were then treated with DTSSP to covalently crosslink the antibody to the antigen, lysis of the cells was performed with Triton X-100 buffer, and IP was carried out with protein A sepharose. Subsequently the immunoprecipitated proteins were separated on a 4-12% gradient gel and were analyzed by autoradiography of the dried gels (figure 3.11).

A specific band for WUE-1 was not detected in the ARH77 samples (figure 3.11, lane 2). The bands in lane 2 indicated by stars are present also in the IP of the WUE-1 negative cell line BL60-2, the three strong bands in lane 2 (indicated by open arrowheads) are also detectable in

the two control samples (IgG_1 and HC-10), thus these bands are most likely not WUE-1 specific. In the control IP with anti-HC-10 a protein of approximately 40kDa is recognized, marked with filled arrowheads (lane 3).

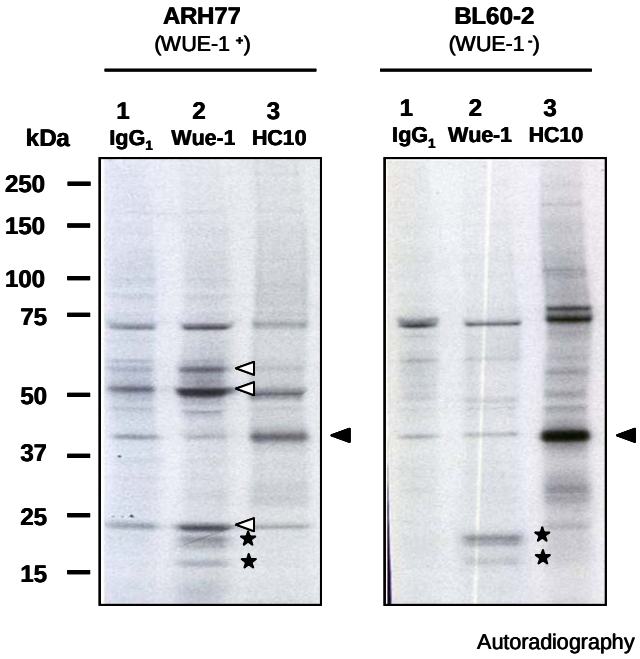

Figure 3.11: Immunoprecipitation (IP) with L-[^{35}S] methionine labelled cells. ARH77 (WUE-1$^+$) and BL60-2 (WUE-1$^-$) cells were subjected to metabolic labelling with L-[^{35}S]-methionine. 1x 10^7 cells each were incubated with IgG_1 isotype control (**lane 1**), anti-Wue-1 (**lane 2**), or anti-HC-10 (**lane 3**). The antibodies were then covalently crosslinked to the cells. The cells were lysed with Triton X-100 buffer, IP was performed with protein A sepharose. The proteins were separated on a gradient gel and were analyzed by autoradiography of the dried gel. **Open arrowheads** show bands which are present only in the IPs of WUE-1$^+$ ARH77 cells. **Stars** indicate bands which are present in the IPs of ARH77 but also BL60-2 cells. **Filled arrowheads** indicate the HC-10 positive control

3.3.3 IP with immobilized NeutrAvidin™

In this experiment, immunoprecipitation of WUE-1 was attempted by using a "sandwich" method: anti-Wue-1 antibody or IgG_1 isotype control were crosslinked to the surface of ARH77 cells, with a thiol-cleavable NHS-ester (DTSSP). The cells were disrupted by hypotonic lysis, biotin labelled anti-mouse $F(ab')_2$ secondary antibody was added to the lysates, and the [antigen/antibody/biotin anti-mouse $F(ab')_2$] complexes were captured using immobilized NeutrAvidin™. The proteins were eluted with elution buffer pH2.8 (Pierce), the eluates were neutralized, and the crosslinker was cleaved by boiling the samples in reducing Laemmli sample buffer, thus releasing the antibody bound proteins. The remaining beads were also boiled in reducing Laemmli buffer. The samples were analyzed on a 4-12% Bis-Tris gradient gel (Novex, Invitrogen) and detected by staining of the gel with silver nitrate (figure 3.12). However, a protein band specific for WUE-1 could not be detected.

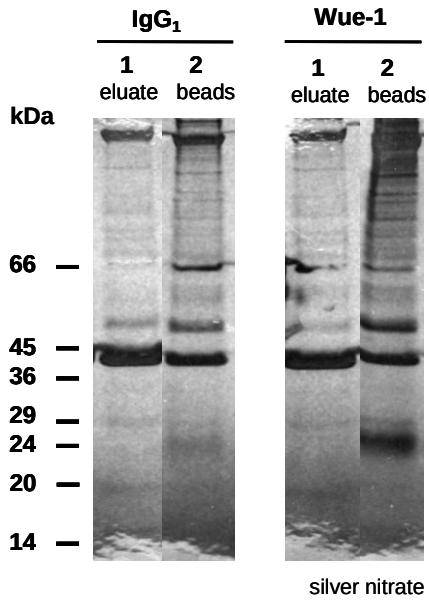

Figure 3.12: Immunoprecipitation (IP) with biotinylated anti-mouse F(ab')$_2$ and immobilized NeutrAvidin ™. Anti-Wue-1 and IgG$_1$ isotype control antibodies were crosslinked to 2x10^8 ARH77 cells each, and the cells were disrupted by hypotonic lysis. Biotinylated anti- mouse F(ab')$_2$ antibody was added at a ratio of 1:500 [antibody:total protein] and incubated o/n. NeutrAvidin™ beads were added at 1µl beads/3µg biotinylated F(ab')$_2$. The beads were collected and elution of the [antigen/antibody/biotin anti-mouse F(ab')$_2$] complexes was performed at pH2.8. The remaining beads were resuspended and boiled in Laemmli buffer. The eluates (**lanes 1**) and boiled beads (**lanes 2**) were analyzed on a gradient gel. Detection was performed by staining with silver nitrate

3.3.4 WUE-1 affinity chromatography

By means of affinity chromatography, it was attempted to isolate the WUE-1 antigen from a large volume of crude cell lysate and at the same time to obtain a concentrated eluate containing the desired protein. WUE-1 affinity chromatography was performed with lysates from biotinylated cells, thus detection of the eluted proteins could be done by Western blot analysis with streptavidin-HRP conjugate.

First, FACS analysis with anti-Wue-1 antibody and IgG$_1$ isotype control was performed using biotinylated ARH77 cells, in order to ensure that antibody binding to the antigen was not disturbed by the sulfo-NHS-biotin label. Biotinylated ARH77 cells were compared to non-labelled cells (figure 2.13). Biotinylation of the cells was monitored using a streptavidin-FITC conjugate (figure 2.13 B), and anti-Wue-1 binding was shown by incubation with R-PE conjugated anti-mouse secondary antibody (figure 2.13 C). Double staining was performed by pre-incubation of the ARH77 cells with streptavidin-FITC conjugate, followed by anti-Wue-1 incubation and R-PE conjugated anti-mouse secondary antibody (figure 2.13 D). The double stained cell population in figure 2.13 D (circle) demonstrates that cell labelling with sulfo-NHS-biotin does not disturb anti-Wue-1 antibody binding. WUE-1 affinity columns were prepared by covalently coupling anti-Wue-1 antibody to immobilized protein G, using a commercially available kit (Pierce). Cell lysates were generated from 7x10^8 sulfo-NHS labelled ARH77 and BL60-2 (negative control) cells each, by hypotonic lysis, and the lysates were cleared by centrifugation. The supernatants, containing the soluble, biotinylated proteins

were mixed to equal parts with Binding buffer pH8 (Pierce) and applied to the columns in a steady flow circuit for 5hrs. Elution of bound proteins was performed with Elution buffer pH2.8 (Pierce). The fractions from each column were pooled and protein was recovered by acetone protein precipitation. In both samples a large pellet formed, which was resuspended in 500µl H_2O. Then dialysis was performed o/n against PBS. The samples were reduced to 30µl in a Speed Vac, separated on a 4-12% Bis-Tris gradient gel (Novex, Invitrogen), and were subjected to WB analysis with streptavidin-HRP. Again, no specific bands were detected on the blot.

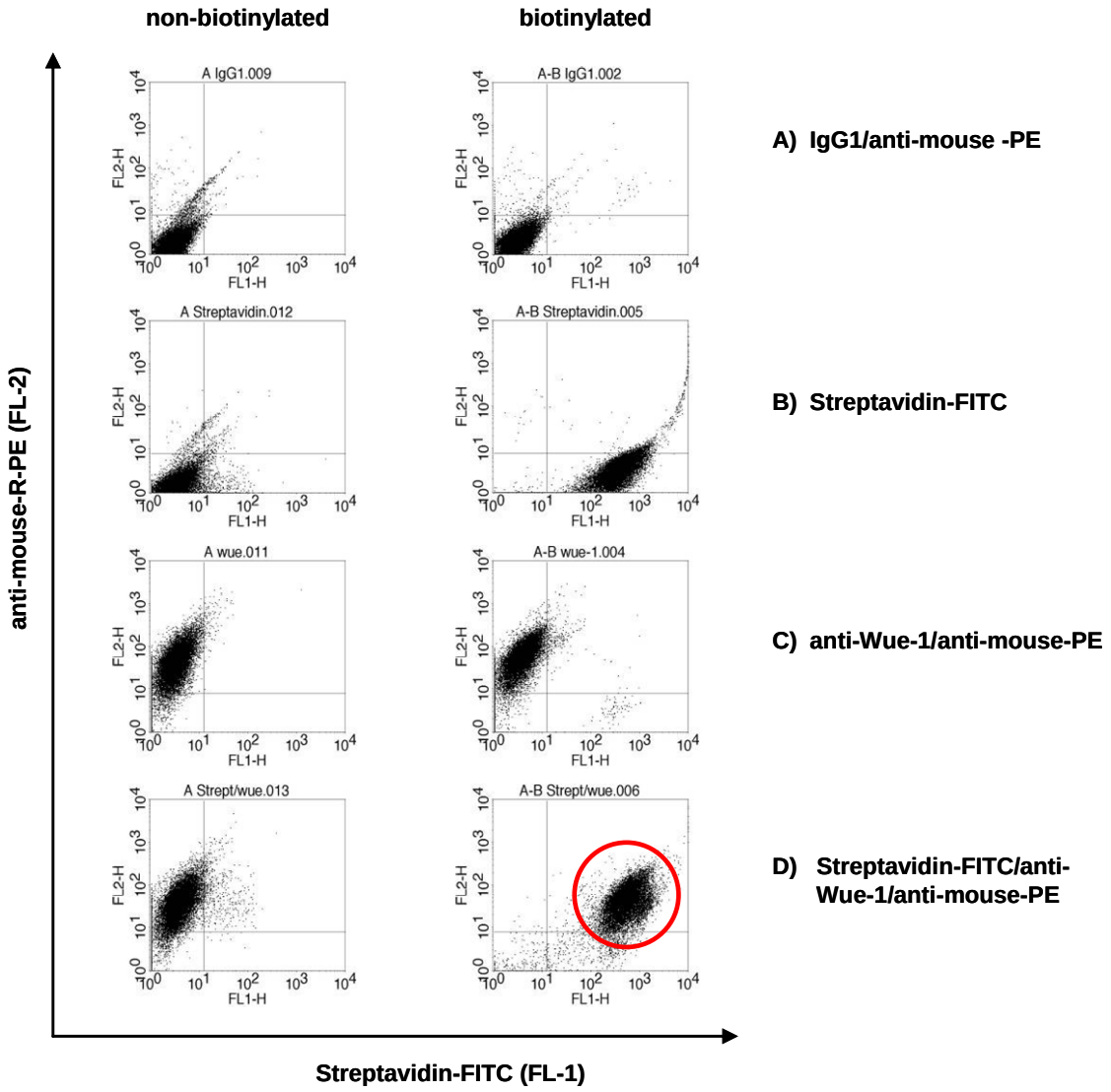

Figure 3.13: FACS analysis of biotinylated and non-biotinylated ARH77 cells. Cells were either analyzed w/o biotinylation (left row) or after treatment with sulfo-NHS-biotin (right row). Biotinylation of the cells was monitored via staining with streptavidin-FITC conjugate (**B**). IgG_1 isotype control (**A**) and anti-Wue-1 (**C**) were stained using R-PE conjugated anti-mouse secondary antibody. Double staining was performed with streptavidin-FITC conjugate followed by anti-Wue-1 and anti-mouse-PE . The double stained cell fraction is marked with a **circle** (**D**).

3.3.5 Advanced biotin label transfer (sulfo-SBED-biotin)

In a further attempt to isolate and detect the WUE-1 antigen, a photoreactive labelling method with sulfo-SBED reagent (Pierce) was used. The reagent comprises a sulfo-NHS active ester, biotin, and a photoactivatable aryl azide. Sulfo-SBED-biotin can be used to label a specific antibody. Under reducing conditions, the biotin label is transferred from the antibody to any nearby protein via the photoreactive aryl azide moiety (figure 2.5). Detection of the biotin labelled proteins can be performed by WB analysis with streptavidin-HRP conjugate. The Western blot depicted in figure 3.14 shows membrane proteins from WUE-1^+ ARH77 and NCI-H929 cells (lanes 1 and 2) compared to WUE-1^- OPM-2, U266, and BL60-2 cells (lanes 3-5) after sulfo-SBED label transfer with anti-Wue-1. It was not possible to identify a band in the samples of the WUE-1 positive cells, which may be specific for the WUE-1 antigen.

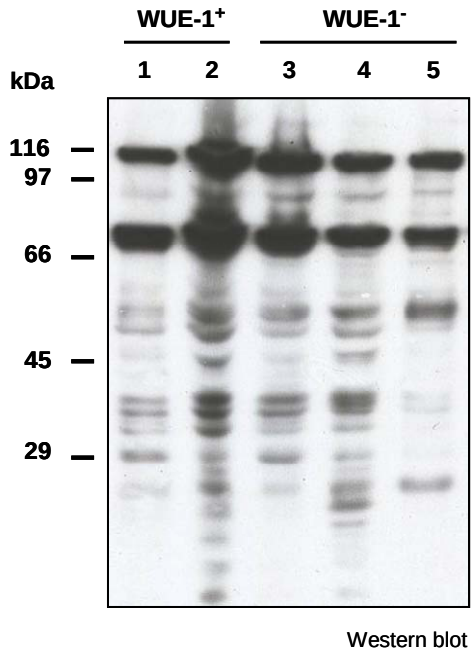

Figure 3.14: "**Sulfo-SBED-Biotin" label transfer.** Anti-Wue-1 antibody was labelled with sulfo-SBED-biotin reagent at a molar ratio of 1:8 [antibody:sulfo-SBED]. $2x10^7$ WUE-1^+ ARH77 (**lane 1**) and NCI-H929 (**lane 2**) cells, and WUE-1^- OPM-2 (**lane 3**), U266 (**lane 4**), and BL60-2 (**lane 5**) cells respectively, were incubated with 50µg sulfo-SBED-biotin labelled anti-Wue-1 antibody each. Photolysis was performed for 3min under UV light (340-380nm). The cells were disrupted in a N_2 cavitation chamber, membrane proteins were isolated and 150µg each were separated on a gradient gel under reducing conditions. Western blot analysis was performed with streptavidin-HRP conjugate (1:5000).

3.4 Biochemical analysis

Since isolation of the WUE-1 antigen with standard methods proved to be rather difficult, analytical studies were performed in order to characterize the properties of the antigen. Gaining more insight into the nature of the antigen would allow for adapting the choice of preparative methods accordingly. The following section summarizes the results of the biochemical analysis.

3.4.1 Characterization of the anti-Wue-1 antibody

3.4.1.1 Depletion of anti-Wue-1 from ascites fluid abolishes FACS signal

In order to examine whether the positive FACS signal on ARH77 cells is caused by the anti-Wue-1 antibody and not by a non-specific reaction with other factors present in the ascites fluid a depletion experiment was set up to stepwise remove the IgG from ascites fluid. Depletion was performed with protein A/G sepharose and anti-mouse antibody-coated paramagnetic beads, respectively. The Western blot in figure 3.15 shows the results: after incubation of anti-Wue-1 ascites fluid with protein A/G sepharose there is a great reduction of the signal detected with anti-mouse HRP (lane 2). Specific removal of IgG with Pan Mouse Dynabeads® further reduces the signal, which is finally eliminated by a second incubation with the Pan Mouse paramagnetic beads.

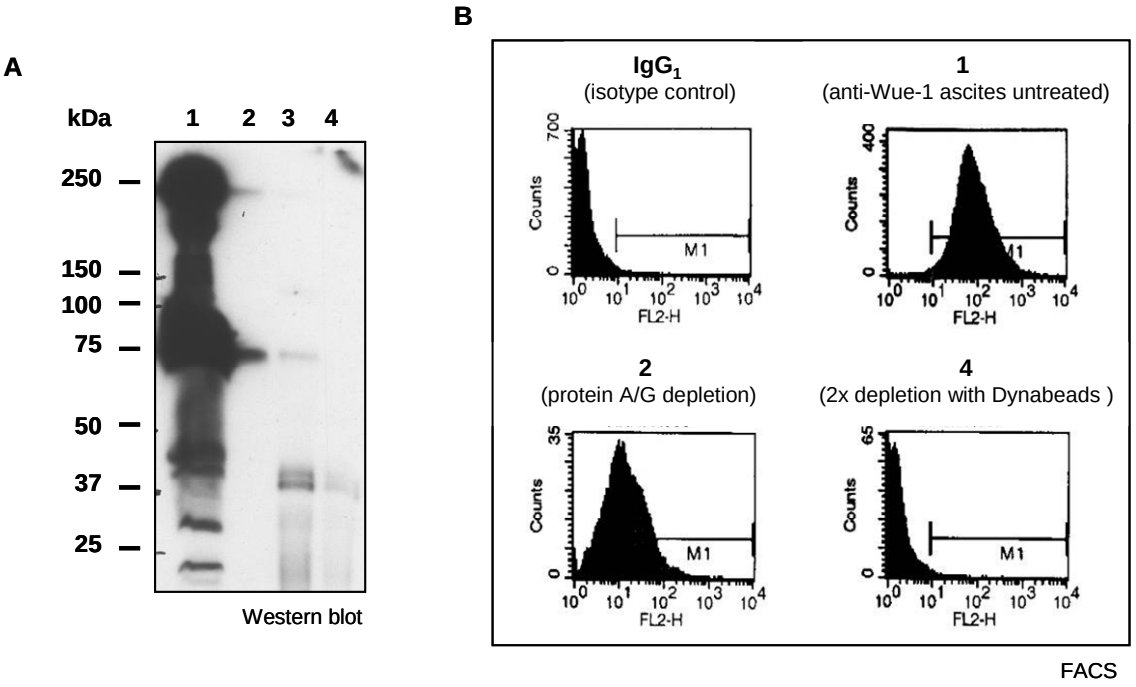

Figure 3.15: Depletion of anti-Wue-1 antibody from mouse ascites. A) Anti-Wue-1 mouse ascites (1:500 in 0.5ml PBS) was incubated with protein A/G-agarose. This was followed by two incubation steps with $4x10^7$ "Pan Mouse" Dynabeads® each. Samples of the supernatants were separated by SDS-PAGE and analyzed by WB with anti mouse-HRP (1:10.000). **Lane 1**: Wue-1 ascites (untreated); **lane 2**: Wue-1 ascites after incubation with protein A/G-agarose; **lane 3**: Wue-1 ascites after first incubation with paramagnetic beads; **lane 4**: Wue-1 ascites after second incubation with paramagnetic beads. **B)** ARH77 cells were incubated with untreated anti-Wue-1 ascites (**1** ≡ WB lane 1), protein A/G depleted anti-Wue-1 ascites (**2** ≡ WB lane 2) and anti-Wue-1 ascites depleted twice with "Pan Mouse" Dynabeads® (**4** ≡ WB lane 4). **IgG_1** ≡ IgG_1 isotype control . Secondary antibody: R-PE conjugated anti-mouse (1:100)

After incubation with protein A/G sepharose and Pan Mouse Dynabeads®, samples of the anti-Wue-1 ascites fluid were used for FACS analysis with ARH77 cells. The results are depicted in figure 3.15 B. The FACS signal on ARH77 cells was largely diminished after treatment of the ascites fluid with protein A/G sepharose (figure 3.15 B, panel 2). After further incubation of the ascites sample with Pan Mouse Dynabeads® the FACS signal on ARH77 cells was completely eliminated (figure 3.15 B, panel 4). In comparison, incubation of ARH77 cells with untreated ascites show a strong FACS signal, whereas the IgG_1 isotype control is negative (figure 3.15 B, panels 1 and IgG_1). The results indicate that the reactive substance in the mouse ascites fluid is the specific immunoglobulin, which was designated anti-Wue-1.

3.4.1.2 Biotinylation of anti-Wue-1 does not interfere with antigen binding

In order to generate a directly biotin labelled antibody for use in crosslinking experiments and for immunoprecipitation with avidin, affinity purified anti-Wue-1 antibody was labelled with NHS-biotin. The biotinylated anti-Wue-1 antibody was to be crosslinked to the cell surface of ARH77 cells prior to cell disruption and immunoprecipitation was to be carried out with immobilized NeutrAvidin™. Here it was tested, whether biotinylation of the antibody interferes with antigen binding. Biotinylation of the antibody was monitored by Western blotting (figure 3.16 A) and binding was analyzed by FACS with ARH77 cells (figure 3.16 B). Non-biotinylated IgG_1 and anti-CD54, and biotinylated lectin (ConA) served as controls. Binding of biotinylated anti-Wue-1 and ConA was visualized with streptavidin-PE conjugate, non-biotinylated antibodies were detected with R-PE labelled anti-mouse antibody. FACS analysis shows that labelling of anti-Wue-1 antibody with NHS-biotin does not inhibit binding to the WUE-1 antigen (figure 3.16 B, No. 4 and 5). However, as depicted in figure 3.16 B, panel No. 5, biotinylated anti-Wue-1 displays markedly weaker fluorescence intensity (mean fluorescence intensity = 10^1) compared to the conventional staining method with R-PE conjugated secondary antibody (mean fluorescence intensity = 10^2). By directly labelling anti-Wue-1 antibody with biotin, it was aimed to produce an efficient tool for antigen detection in immunoprecipitation experiments and/or FACS analysis. However, biotin labelling of the antibody appears to be a rather inefficient method with low sensitivity, compared to the conventional "sandwich" method with non-labelled anti-Wue-1 and R-PE-labelled anti-mouse

secondary AB. Therefore, directly biotin-labelled anti-Wue-1 was not used in further applications.

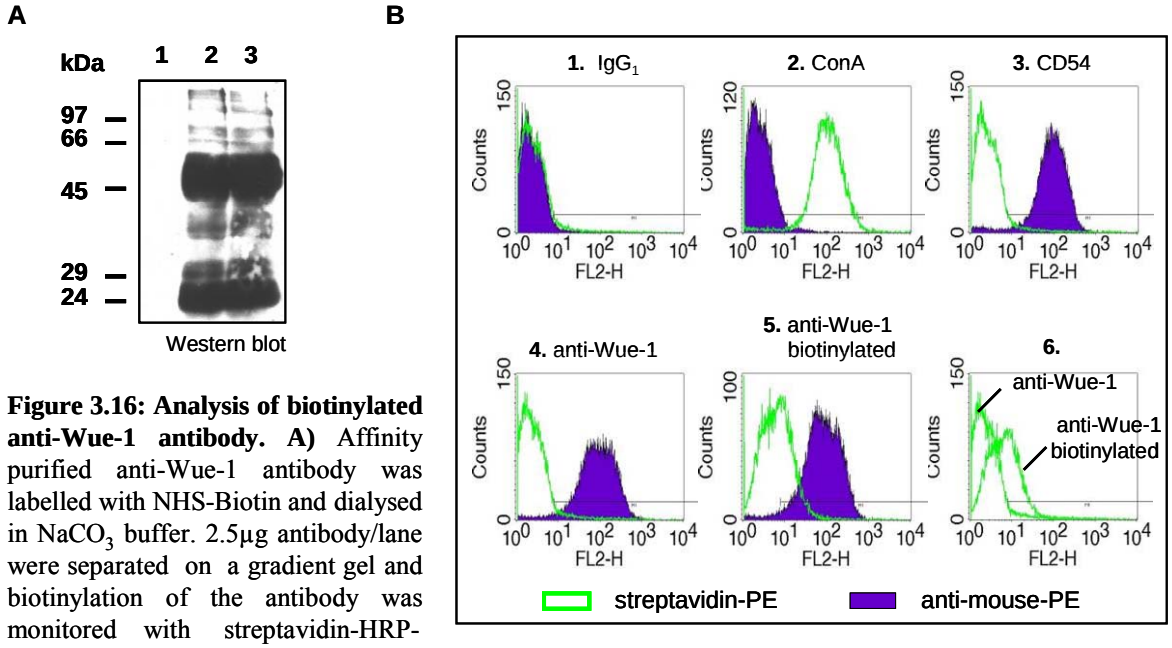

Figure 3.16: Analysis of biotinylated anti-Wue-1 antibody. A) Affinity purified anti-Wue-1 antibody was labelled with NHS-Biotin and dialysed in $NaCO_3$ buffer. 2.5µg antibody/lane were separated on a gradient gel and biotinylation of the antibody was monitored with streptavidin-HRP-conjugate (1:1000). **Lane 1**: unlabelled anti-Wue-1; **lane 2**: biotinylated anti-Wue-1 (prior to dialysis); **lane 3**: biotinylated anti-Wue-1 (after dialysis). **B)** FACS analysis of ARH77 cells with non-biotinylated and biotinylated anti-Wue-1 antibody. IgG_1, anti-CD54 and biotinylated lectin (ConA) served as controls. Binding of biotinylated antibody was visualized with streptavidin-PE conjugate (1:1000, **open histograms**) and of non-biotinylated antibody with R-PE conjugated anti-mouse secondary antibody (1:100, **filled histograms**). Panel 6 shows the comparison of untreated anti-Wue-1 with the biotinylated anti-Wue-1 (both incubated with streptavidin-PE).

3.4.1.3 Anti-Wue-1 is stably bound to the cell surface

Here, it was investigated whether anti-Wue-1 remains stably bound to the cell surface or whether a steady state of binding and dissociation of the antibody exists. In the latter case, problems may occur in applications like IP, due to insufficient stability of the antibody/antigen complex. This question was addressed by using anti-Wue-1 antibody labelled with either red fluorescent or green fluorescent Zenon™ dye (Molecular Probes). Aliquots of affinity purified anti-Wue-1 and control antibodies (IgG_1 isotype control, anti-CD54) were each labelled with Alexa Fluor® 488 (green) conjugated anti-Fc and R-PE (red) conjugated anti-Fc, respectively. ARH77 cells were incubated with either the R-PE labelled or the Alexa Fluor® 488 labelled antibodies. Excess and unbound antibody was removed by gentle wash. Both labelled cell populations were then pooled and incubated for 30min on ice.

If a steady state of antibody binding and dissociation exists, one would assume that after some time a cell population will arise which carries the red labelled as well as the green-labelled antibody. Control reactions were incubated with only R-PE labelled or Alexa Fluor® 488 labelled antibodies, respectively. The results are summarized in figure 3.17. Cell populations labelled with only one dye (figure 3.17 A and B) can be clearly determined. In the samples, where cell red fluorescent-labelled and green fluorescent-labelled cell populations were mixed double-stained cells were not detected (figure 3.17 C). The experiment shows that, at least on intact cells, anti-Wue-1 remains stably bound. Thus, if failure of the anti-Wue-1 IP is due to insufficient stability of the antibody/antigen complex this most likely occurs when the cells are lysed.

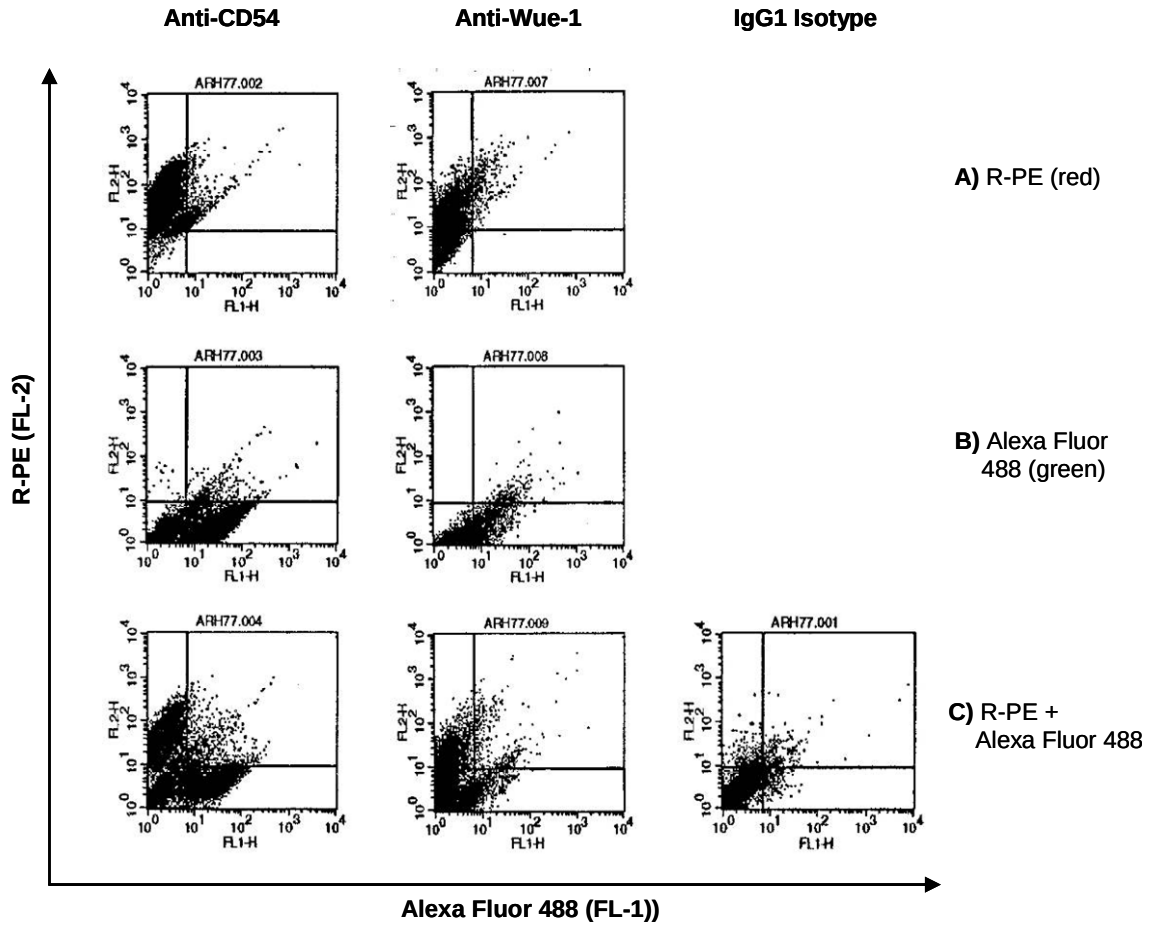

Figure 3.17: FACS analysis with Zenon™ dye labelled antibodies. ARH77 cells were analyzed for expression of CD54 or WUE-1, respectively. The cells were incubated with R-PE labelled (**A**), or Alexa Fluor 488 labelled (**B**) antibody. Both cell populations were mixed and analyzed for double stained cells (**C**). R-PE and Alexa Fluor 488 labelled IgG_1 isotype control was set up as control reaction.

3.4.2 Analysis of the WUE-1 antigen

3.4.2.1 The WUE-1 antigen is sensitive to Pronase digestion

ARH77 cells were subjected to enzymatic digestion of surface proteins in order to learn more about the nature of the WUE-1 antigen. First, the broad specificity protease mixture Pronase was tested. Pronase contains various types of endo- and exopeptidases (e.g. serine and metalloproteases; carboxypeptidases and aminopeptidases) and is therefore able to cleave virtually all proteins. ARH77 cells were treated with Pronase and subjected to FACS analysis. Alongside WUE-1, the cells were tested for the following surface antigens: CD19 (a transmembrane glycoprotein expressed during all stages of B-cell differentiation, except for plasma cells), CD29 (a heterodimeric complex of integrins, comprising the very late activation antigen (VLA) subfamily of adhesion receptors), CD48 (a GPI-anchored protein, widely distributed on leukocytes), and CD54 (or ICAM-1, a widely distributed surface protein found on lymphocytes, vascular endothelium, epithelial cells, macrophages, and DCs). The FACS results are depicted in figure 3.18 A. The histograms show that all antigens are sensitive to digestion by Pronase. The FACS signal for WUE-1 is entirely eliminated after the protease treatment, whereas the effect on the control antigens is less severe.

In a follow-up experiment, Pronase was pre-incubated with a number of different protease inhibitors (table 3.2) to narrow down the protease family, which acts upon WUE-1. ARH77 cells were then incubated with the pre-treated Pronase and analyzed by FACS (figure 3.18 B). Only PMSF yielded a notable effect, leaving the WUE-1 antigen greatly unaffected by enzymatic digestion. A slight effect was also notable after addition of EDTA or a protease inhibitor mix ("Complete mini", Roche). The results strongly indicate that WUE-1 is a molecule comprising one or more protein domains, and that is mainly sensitive to digestion by serine proteases.

Table 3.2: Specificity of protease inhibitors

Inhibitor	Inhibitor specificity
Inhibitor cocktail ("Complete mini", EDTA-free)	Broad spectrum (serine-, cysteine-, metalloproteases and calpains)
EDTA-Na_2	Metalloprotease
Pepstatin	Aspartic acid proteases (pepsin, rennin, cathepsin D, chymosin, microbial acid proteases)
PMSF	Serine proteases (chymotrypsin, trypsin, thrombin);cysteine proteases (papain)

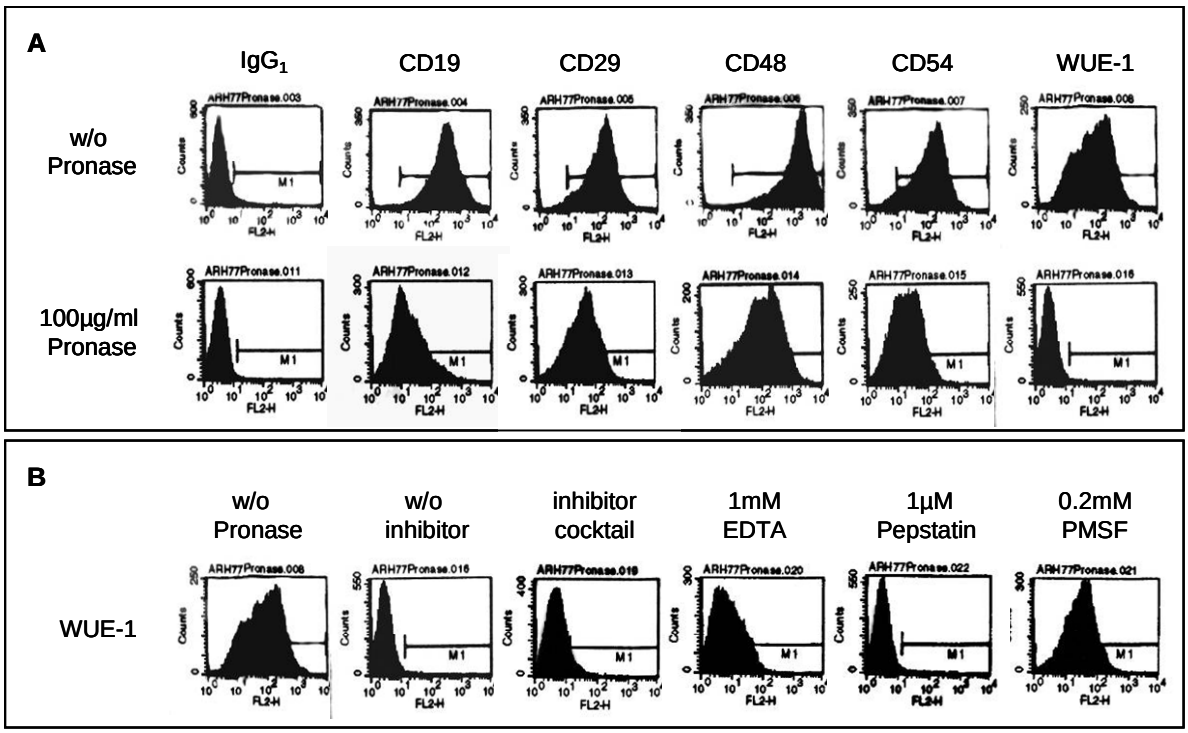

Figure 3.18: Protease (Pronase) treatment of ARH77 cells. A) Prior to FACS analysis $1x10^6$ ARH77 cells/reaction were incubated with 100µg/ml Pronase. Control reactions w/o Pronase were set up alongside. FACS analysis was performed with IgG_1 isotype control, anti-CD19, anti-CD29, anti-CD48, anti-CD54, and anti-Wue-1 primary antibodies, respectively, followed by PE-conjugated anti-mouse secondary antibody. **B)** For inhibition experiments 100µl/ml protease inhibitor cocktail (Complete mini), 1mM EDTA-Na_2, 1µM Pepstatin A, or 0.2mM PMSF were added to 100µg/ml Pronase and pre-incubated for 10min at RT. Subsequently the cells were added to the mixtures and incubated as above. The samples were then prepared for FACS analysis with anti-Wue-1 primary antibody and PE-conjugated anti-mouse secondary antibody.

3.4.2.2 The WUE-1 antigen is not affected by PI-Phospholipase C treatment

PI-phospholipase C (PI-PLC) is an enzyme that cleaves the phosphatidylinositol link by which some proteins are attached to the cell membrane. Thus, treatment with this enzyme will release glycosyl-phosphatidylinositol (GPI)-anchored proteins but will leave other proteins unaffected. By FACS analysis ARH77 cells were tested for CD19 (negative control), CD48, a GPI-anchored member of the Ig superfamily (positive control) and WUE-1. Controls were set up w/o antibody, with secondary antibody only and with IgG_1 isotype control, and samples with and w/o PI-PCL treatment were compared. As the histograms in figure 3.19 show, there is a decrease in the CD48 signal after the phosphlipase treatment (filled histogram), as was expected for this GPI anchored protein. CD19 and WUE-1 were not affected by PI-PLC treatment, suggesting that WUE-1 is a most likely not a GPI anchored protein.

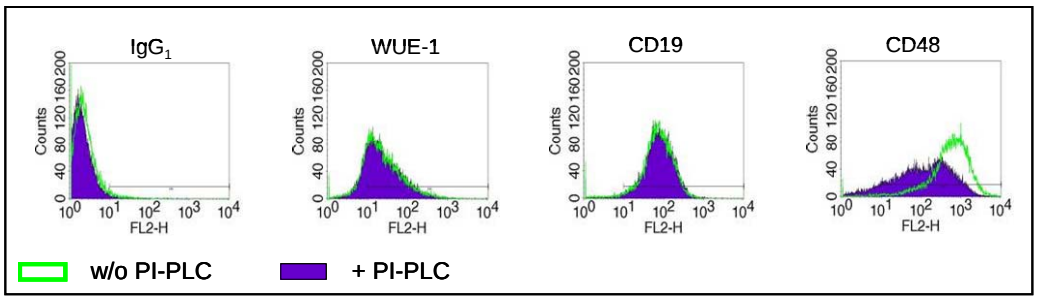

Figure 3.19: FACS analysis of ARH77 cells treated with PI-PLC. Prior to incubation with anti-Wue-1 antibody ARH77 cells were treated with PI-PLC. The overlays show FACS analysis of non-treated cells (**open histograms**) and PI-PLC treated cells (**filled histograms**), incubated with IgG_1 isotype control, anti-Wue-1, anti-CD19 or anti-CD48 (positive control) followed by PE-conjugated anti-mouse secondary antibody.

3.4.2.3 Anti-Wue-1 binds to β-D-glucose and β-D-galactose but not to blood group antigens

Here it was investigated whether anti-Wue-1 recognizes a carbohydrate (-antigen) or glycoprotein. One of the possible explanations for the failure to identify a specific band in IP reactions of WUE-1 positive cells could be that the anti-Wue-1 antibody recognizes a carbohydrate epitope that is present on multiple cell surface proteins. Consequently, applications like FACS analysis or immunohistochemistry show specific staining on the cell surface, but disruption of the cells and IP would result in a pattern of multiple bands of various sizes instead of one specific band. Moreover, by preparing cell lysates for IP or Western blotting conformational structures may be destroyed or altered, hence antibody binding is abolished.

Using the ELISA based method developed by Butschak *et al.* [100, 114] saccharide-polyacrylamid (PAA) conjugates mimicking carbohydrate antigens (table 3.3) were incubated with anti-Wue-1 antibody or IgG_1 isotype control, respectively, followed by incubation with HRP conjugated anti-mouse secondary antibody. The saccharide-polyacrylamid (PAA) conjugates were a generous gift from Dr. Uwe Karsten, MDC Berlin. The results of this carbohydrate-ELISA are summarized in figure 3.20 A. The diagram shows that anti-Wue-1 reacts with β-D-glucose and β-D-galactose, but not with any of the known blood group antigens (e.g. Lewis antigen, Thomsen Friedenreich antigen, Forssman antigen). This result does not rule out that anti-Wue-1 binds to a carbohydrate epitope on glycoproteins, but it seems unlikely that WUE-1 is a pure carbohydrate antigen.

Table 3.3: Saccharid-Polyacrylamid Conjugates

Peptide number	Structure (Short name)	Name
022	β-D-Glc (Glc)	β-D-Glucose
024	β-D-Gal (Gal)	β-D-Galactose
030	α-Gal-NAc (Tn)	T negative antigen
035	α-Neu-5-Ac	Sialic acid
043	Le^c	Lewis c antigen
048	Gal-β-1-3-Gal-NAc-α (TF_α)	Thomsen-Friedenreich α antigen
052	Gal-NAc-α-1-3-Gal NAc-α (Fs)	Forssman antigen
056	Gal-β-1-3-Gal-NAc-β (TF_β)	Thomsen-Friedenreich β antigen
086	B	Blood group B

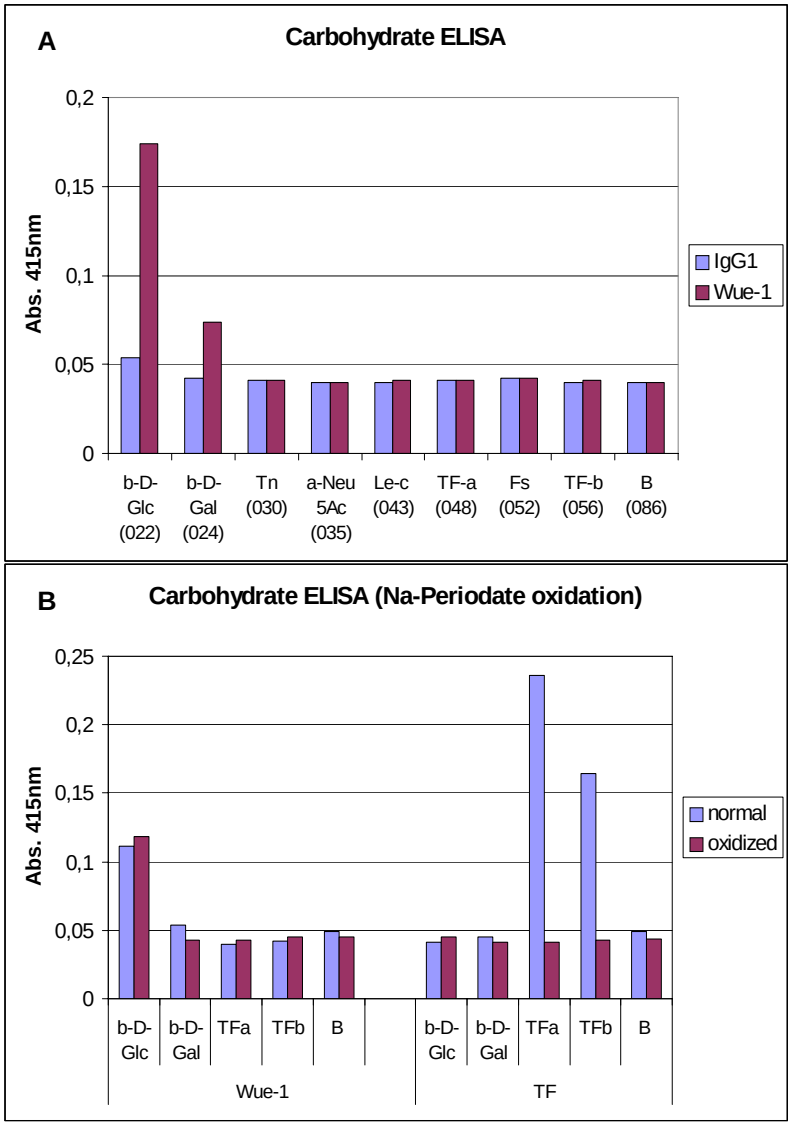

Figure 3.20: Anti-Wue-1 carbohydrate ELISA. A) PAA conjugated carbohydrates were coated into microtiter plates. The wells were incubated with diluted IgG_1 isotype control or anti-Wue-1 antibody, followed by HRP-conjugated anti-mouse-secondary antibody (1:2500). The plate was measured at λ=415nm. Numbers in brackets indicate the peptide number (as listed in table 3.3)

B) PAA conjugated carbohydrates were coated into microtiter plates. Vicinal OH-groups of the carbohydrates were oxidized with Na-periodate prior to antibody incubation. The wells were incubated with anti-Thomsen Friedenreich (positive control) or antibody, or anti-Wue-1 antibody. The ELISA was processed further as described above.

PAA: *polyacrylamid;* Glc: *glucose;* Gal: *galactose;* Tn: *T negative antigen;* α-Neu-5-Ac: *Sialic acid;* Le-c: *Lewis c antigen;* TFα/TFβ: *Thomsen Friedenreich antigen α/β;* Fs: *Forssman antigen;* B: *blood group B*

3.4.2.4 Oxidation of vicinal OH-groups does not alter anti-Wue-1 binding

Periodate oxidation is often used for structural analysis of glycoproteins. 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.

Here, the OH-groups of the saccharide-PAA conjugates (table 3.3) were oxidized with sodium periodate to investigate whether anti-Wue-1 still binds to β-D-glucose and β-D-galactose after the treatment. In a modification of the carbohydrate ELISA, the sugar conjugates were oxidized prior to incubation with anti-Wue-1. An antibody specific for Thomson Friedenreich antigens (TF) was used as control. Figure 3.20 B shows that treatment with Na-periodate abolishes specific binding of the anti-TF antibody to its target structures, i.e. TF_α (conjugate no. 048) and TF_β (conjugate no. 056). In contrast binding of anti-Wue-1 to β-D-glucose (conjugate 022) is not altered. The reaction of anti-Wue-1 with the untreated β-D-galactose (conjugate 024) conjugate is rather weak but is slightly reduced after periodate oxidation.

3.4.2.5 Binding of anti-Wue-1 to ARH77 is not blocked by lectins

A further approach to investigate whether anti-Wue-1 binds to a carbohydrate epitope was by using a panel of lectins to block the carbohydrates on the cell surface of ARH77 cells. Lectins form complexes with mono- and/or oligosaccharide structures, e.g. polysaccharides, glycoproteins, or glycolipids and several lectins display blood group specificity. The lectins used in this experiment and their specificity, are summarized in table 3.4.

WUE-1 positive ARH77 cells were incubated with biotin labelled lectins (Vector Laboratories) prior to addition of anti-Wue-1 antibody. The cells were then subjected to FACS analysis. Binding of the biotinylated lectins was detected with streptavidin-PE conjugate, and R-PE conjugated anti-mouse secondary antibody was used to monitor anti-Wue-1 binding (figure 3.21). The overlays depicted in figure 3.21 show that all lectins have bound to the cell surface of ARH77 cells (open histograms, thin outline). However, anti-Wue-1 binding was not abolished. There seems to be a slight reduction of the anti-Wue-1 FACS

signal in samples 11, 12, and 19. However, this reduction is due to toxicity of these lectins, causing destruction of cells. Although it cannot be ruled out that the WUE-1 antigen probably comprises a carbohydrate epitope, the result of this experiment strongly indicates that WUE-1 is not a pure carbohydrate antigen

Table 3.4: Lectins and their binding specificity

	Lectin	**Organism**	**Specificity**
1	Con A	Canavalia ensiformis	Mannose binding; branched α-mannosidic structures; hybrid type and biantennary complex type N-Glycans
2	SBA	Glycine max	terminal α,βGalNAc > α,βGal
3	WGA	Triticum vulgaris	Sialic acid/N-acetylglucosamine binding; GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac (sialic acid)
4	DBA	Dolichos biflorus	terminal FP > GalNAcα1-3GalNAc > GalNAcα1-3Gal; blood group A_1 (Forssman pentasaccharide: GalNAcα1-3GalNAcα1-3Galβ1-4Galβ1-4GlcNAc)
5	UEA	Ulex europaeus	Fucose binding; Fucα1-2Gal-R
6	RCA	Ricinus communis	Galactose/N-acetylgalactosamine binding; Galβ1-4GlcNAcβ1-R
7	PNA	Arachis hypogaea	Galactose/N-acetylgalactosamine binding; Galβ1-3GalNAcα1-Ser/Thr (T-antigen)
8	GSL I	Griffonia simplicifolia lectin I	α-N-acetylgalactosamine, α-galactose
9	PSA, PEA	Pisum sativum	branched α-man, complex type with N-acetylchitobiose-linked core α-fuc
10	LCA	Lens culinaris	Mannose binding; Fucosylated core region of bi- and triantennary complex type N-Glycans
11	PHA-E	Phaseolus vulgaris E	Galβ1,4GlcNAcβ1,2Manα1,6
12	PHA-L	Phaseolus vulgaris L	GlcNAcβ1,2Man, triantennary complex oligosaccharides
13	SJA	Sophora japonica terminal	Galβ1,3GalNAc>Galβ 1,3GlcNAc>αβ,GalNAc>αβ,Gal
14	WGA (succinylated)	Triticum vulgaris	Sialic acid/N-acetylglucosamine binding; GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac (sialic acid)
15	GSL II	Griffonia simplicifolia lectin II	terminal-α,β-GlcNAc; glycogen
16	DSL	Datura stramonium	$(GlcNAc\beta1\text{-}4)_3GlcNAc = (Glc\beta1\text{-}4)_2GlcNAc > Glc\beta1\text{-}4GlcNAc >> GlcNAc$
17	ECL	Erythrina cristagalli	Galactose/N-acetylgalactosamine binding; Galβ1-4GlcNAcβ1-R
18	Jacalin (AIL)	Artocarpus integrifolia	Galactose/N-acetylgalactosamine binding; (Sia)Galβ1-3GalNAcα1-Ser/Thr (T-antigen)
19	LEL	Lycopersicon esculentum	$(GlcNAc\beta\ 1\text{-}4)_3\ GlcNAc > (GlcNAc\beta1\text{-}4)_2\ GlcNAc >$ GlcNAcβ1-4GlcNAc
20	STL	Solanum tuberosum	N-acetyl-β-D-glucosamine oligomers
21	VVA	Vicia villosa	Galactose/N-acetylgalactosamine binding; GalNAcα-Ser/Thr (Tn-antigen)

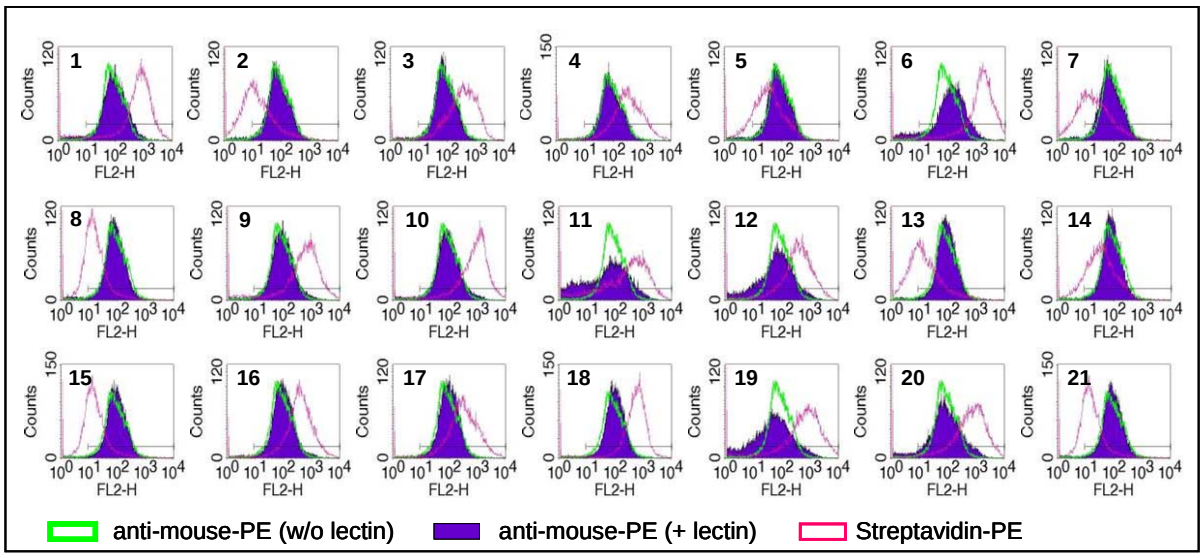

Figure 3.21: WUE-1 FACS analysis of ARH77 cells pre-incubated with lectins. Prior to incubation with anti-Wue-1 antibody ARH77 cells were pre-treated with a panel of biotin labelled lectins with different carbohydrate specificities (table 3.4). The overlays 1-21 show non-treated cells incubated with anti-Wue-1 and R-PE conjugated anti-mouse secondary antibody (**open histograms, thick outline**) compared with anti-Wue-1 FACS after lectin treatment (**filled histograms**). Binding of biotinylated lectins was monitored via staining with streptavidin-PE conjugate (**open histograms, thin outline**).

3.5 Expression libraries

cDNA expression libraries are useful tools to identify, analyze, and isolate new genes, since such a library represents the information encoded in the mRNA of a particular sample (tissue, cell, or organism). Plasmid-based functional screening is largely facilitated by cDNA libraries constructed directly into plasmid vectors. The pCMV-Script® mammalian expression vector used for creation of the ARH77 and MM libraries, respectively is designed to allow efficient protein expression in mammalian systems. The vector does not contain an ATG initiation codon, ensuring that only clones, which contain their own start codon, will be expressed. One advantage of plasmid libraries (over phage libraries) is the direct expression and efficient screening in eukaryotic cell systems. Moreover, individual colonies can easily be examined to determine the percentage of vectors containing an insert and the average insert size, either by PCR directly from the colony or by restriction analysis of individually prepared plasmid DNA.

In this project, the libraries were screened by expression cloning and immunoselection ("panning", section 2.7). Two cDNA libraries from ARH77 cells were constructed. One library was directly created as plasmid library ("pCMV-Script® XR" cDNA library), the other

one as λ-phage library ("Lambda ZAP®-CMV XR" library). The latter was converted into a plasmid-based format for use in the screen. A lambda-phage-based multiple myeloma library was kindly provided by Dr. Sun-Jin Choi, University of Pittsburgh Medical Centre, USA (see also reference [103]), which was likewise converted into a plasmid library prior to screening.

3.5.1 ARH77 pCMV® plasmid library

Size fractioning of the ARH77 cDNA yielded eight fractions. Fractions 1-6 were assumed to contain large cDNA fragments (≥ 500bp) and were pooled (referred to as "pCMV-I"). Likewise, fractions 7-8 were pooled ("pCMV-II") which were assumed to contain small (≤ 500bp) DNA fragments. The entire cDNA from both pools was ligated into the pCMV-Script® vector. Best ligation results were obtained using 10ng cDNA and 30ng vector per reaction, and a total of 11 ligations were performed and transformed individually into ultracompetent *E. coli* cells. The titer of the library (i.e. colony forming units (cfu)/µg cDNA) was determined to $6x10^5$ cfu/µg cDNA for pCMV-I and $5x10^5$ cfu/µg cDNA for pCMV-II (section 2.6.3). Size distribution of incorporated inserts was analyzed by restriction digest of 24 clones of pCMV-I and pCMV-II each, using *EcoR*I and *Xho*I (figure 3.22; note: only 13 of the 24 digests each are shown). 17 of the 24 analyzed pCMV-I clones (71%) contained inserts, with a size distribution ranging from 300 to 2000bp (figure 3.22 A). Out of the 24 analyzed pCMV-II clones only 4 (17 %) contained inserts. The size distribution varied from 300 to 3000bp (figure 3.22 B). *E. coli* cells transformed with the library were

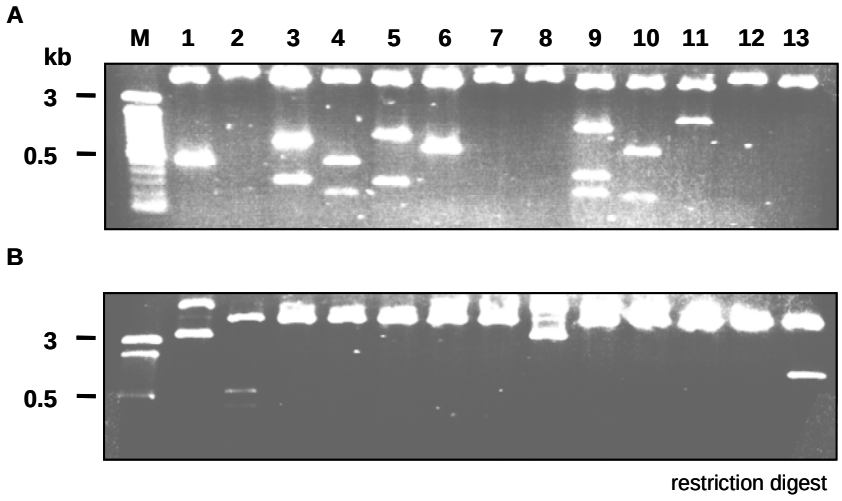

Figure 3.22: Restriction analysis of the ARH77 pCMV plasmid library (size distribution of incorporated inserts). Single colonies were picked from pCMV-I (**A**) and pCMV-II (**B**) reactions (explanation see text), plasmid DNA was prepared and analyzed by double restriction digestion, using *EcoR*I and *Xho*I. The reactions were separated on a 1% agarose/TBE/EtBr gel. **M**: 100bp+ DNA marker; **lanes 1-13**: restriction digests from single colonies (only 13 of 24 analyzed clones are shown)

plated on 530cm^2 LB-agar plates, allowing ~2x10^4 cfu/plate. The bacteria were washed off the plates and the plasmids were recovered by means of "midi preps", with each plate representing an individual plasmid pool. The recovered plasmid DNA was subsequently used for panning.

3.5.2 ARH77 "Lambda ZAP®-CMV XR" expression library

Eight ARH77 cDNA fractions were recovered by size fractioning, were pooled as described in section 3.5.1, and were named accordingly ("λ-ZAP-I" and "λ-ZAP-II"). The entire cDNA from both pools was ligated into the "Lambda ZAP®-CMV XR" vector. Best ligation results were obtained using 100ng cDNA and 1µg vector per reaction. The ligation reaction was subsequently packaged (five reactions in total) and the efficiency of packaging (i.e. plaque forming units (pfu)/µg cDNA) was determined by titration of the samples (section 2.6.2.1). For λ-ZAP-I the average titer of ~2.5x10^8 pfu/µg cDNA was determined, for λ-ZAP-II it was ~1x10^8 pfu/µg cDNA. Size distribution of incorporated inserts was analyzed by PCR of 24 λ-ZAP-I and λ-ZAP-II plaques each. 19 of the 24 analyzed λ-ZAP-I plaques (~79%) and 16 of the λ-ZAP-II plaques (~67%) contained inserts, with a size distribution ranging from 500 to 2000bp (figures 3.23 A and B; note: only 13 of each of the 24 PCR reactions are shown). Inserts ≤ 500bp were not detected.

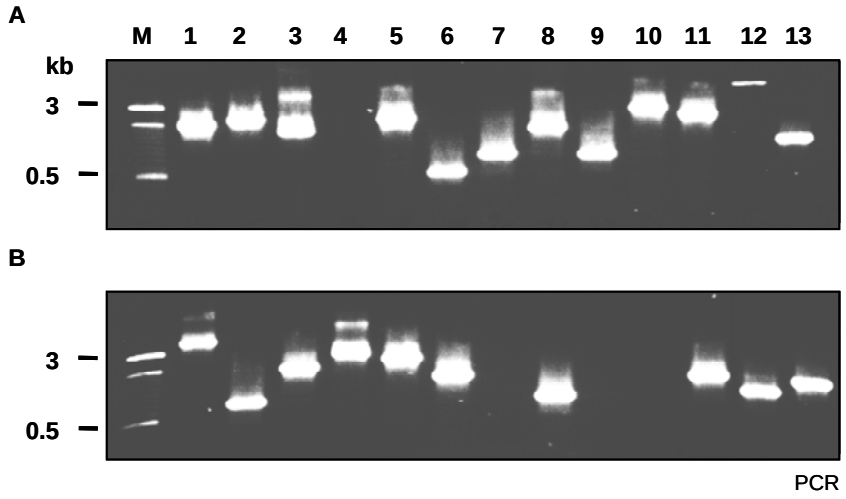

Figure 3.23: PCR analysis of ARH77 λ-ZAP library clones (size distribution of incorporated inserts). Single plaques were picked from λ ZAP-I (**A**) and λ ZAP-II (**B**) reactions (explanation see text). The inserts contained in the λ ZAP vector were amplified by PCR for 25 cycles, using $T3_{fw}$ and $T7_{rev}$ primers. The PCR products were separated on a 1% agarose/TBE/EtBr gel. **M:** 100bp+ marker; **lanes 1-13:** PCR reactions from single plaques (only 13 of 24 analyzed clones reaction are shown)

3.5.2.1 Conversion of the ARH77 λ-ZAP® primary library into a plasmid library

In vivo excision on the λ-ZAP® library was performed to isolate the "pCMV-Script® EX" phagemid vectors containing the cDNA inserts. In order to maintain the original clonal representation of the library the non-amplified, primary λ-ZAP® library (consisting of ~$1x10^7$ pfu in total) was subjected to mass excision. Titration of the mass-excised phagemid was performed and the titer was determined to $1x10^3$ cfu/µl (~$2x10^7$ pfu in total). For preparation of clonal library pools, *E. coli* cells were transformed with phagemid solution and plated on 530cm^2 plates, allowing 2.5x 10^5 cfu/plate. A total of 25 clonal pools were prepared and the plasmids were recovered from each pool by means of "maxi preps". The recovered plasmid DNA was subsequently used for panning.

3.5.3 MM expression library

The lambda-phage-based MM library was a generous gift from Dr. Sun-Jin Choi, University of Pittsburgh Medical Centre, USA. The library was constructed in the "Lambda ZAP® Express™" vector. For cDNA synthesis, mRNA from four MM patient bone marrow samples was used. The patients were phase III stage and never treated with drugs. The cDNA fragments were unidirectionally cloned into the λ-ZAP® vector (5'-*EcoR*I---*Xho*I-3'). The insert size cut off was 700bp and 11 out of 12 clones contained inserts ≥ 1.6kb. The initial titer of the primary library was $3x10^5$ pfu/ml. The primary library has been amplified once and the titer was determined to > 10^8 pfu/ml (information provided by Dr. Sun-Jin Choi).

Conversion of the amplified MM lambda phage library into a phagemid library was performed as described above (section 3.5.2). The phagemid titer was determined to $1.25x10^5$ cfu/µl. Subsequently 28 clonal pools were prepared on 530cm^2 plates from ~$2.5x10^5$ cfu each. Additionally six clonal pools were set up in shakeflasks, using $5x10^9$ cfu/200ml LB medium. The plasmids were recovered from each pool by means of "maxi preps" and the recovered plasmid DNA was subsequently used for panning.

3.5.4 Analysis of selected clones from cDNA library screens

3.5.4.1 Immunoselection with anti-Wue-1 yields non-specific results

The panning procedure was performed as described in section 2.7. Briefly, COS7 cells were transfected with plasmid DNA from the ARH77 or MM library pools. Clones were selected by incubation of the transfected COS7 cells on anti-Wue-1 coated petridishes. The plasmid DNA from attached cells was isolated, re-introduced and amplified in *E. coli*. Finally, plasmid DNA was isolated from single *E. coli* colonies either after one round, three rounds or four rounds of panning and the inserts were examined by sequence analysis and identified by database search (tables 3.5, 3.6, and 3.7).

Table 3.5: Clones isolated from the ARH77 pCMV® library by immunoselection with anti-Wue-1.
Clones were sequenced after **one round** of panning. Bold print: Clones that were analyzed by Northern blotting

Clone No.	insert size	identification
2b-1 03	900bp	HS neutrophil cytochrome b-245, light chain p22, alpha polypeptide (CYBA)
2b-1 07	**800bp**	**HS chromosome 1 specific transcript, protein KIAA0493**
2b-1 08	2500bp	HS CLL-associated antigen KW-5
2b-1 09	**900bp**	**HS GAPDH**
2b-1 11	**1500bp**	**HS hypothetical protein FLJ20203, protein KIAA1606 on chromrosome 1**
2b-1 13	**2000bp**	**HS SCG10-like protein, helicase like protein NHL, M68, and ADP-ribosylation**
2b-1 18	**1500bp**	**HS MHC class II antigen (HLA-DQA1)**
2b-1 20	**1500bp**	**HS signal sequence receptor beta, (translocon-associated protein beta), SSR2, HS TRAP beta subunit**
2b-1 27	**800bp**	**HS chromosome 16 clone RP11-5A19, CTC-277H1**
2b-1 29	700bp	HS malate dehydrogenase2, NAD (mitochondrial), MDH2, nuclear gene
2b-1 30	**1500bp**	**HS homolog of yeast MAF1**
2b-2 44	**1000bp**	**HS tumour protein, translationally-controlled 1 (TPT1)**
2b-2 46	1500bp	HS MHC, classII, DPw3-alpha-1
2b-2 47	**1250bp**	**HS arginine methyltransferase, HMT1 hnRNP methyltransferase like 1**
2b-2 48	**2000bp**	**HS hypoxia up-regulated 1 (HYOU1), oxygen regulated protein ORP 150kDa**
2b-2 55	**900bp**	**HS muscle specific gene (M9), PTD001, HSPC029, highly similar to AF077052**
3ab 57	700bp	HS small nuclear ribonucleoprotein D2 polypeptide 16.5 kDa (SNRPD2)
3ab 65	1500bp	HS triosephosphate isomerase 1 (TPI1)
3ab 67	**1200bp**	**HS hypothetical protein MGC4549**
3ab 68	2000bp	HS tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
3ab 75	2000bp	HS enolase 1 alpha (ENO1), EDAR associated death domain (EDARADD)
3ab 78	1200bp	HS MHC, classII, DR-alpha (HLA-DRA)
3ab 82	1200bp	HS guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1

Clone No.	insert size	identification
3ab 83	1200bp	HS MHC class I antigen heavy chain (HLA-CW)
3ab 84	**1500bp**	**HS CD74 antigen(invariant polypeptide of MHC classII antigen associated)**
3ab 86	**600bp**	**HS sirtuin (silent mating type information regulation 2 homolog) 2 (*S.cerev.*)**
3bb 14	**1000bp**	**HS integral membrane protein 2C (ITM2C, BRI3)**
3bb 21	**900bp**	**HS NIMA-related kinase 6 (NEK6)**
3bb 22	700bp	HS, similar to ubiquinol-cytochrome c reductase hinge protein
3bb 24	**1500bp**	**HS eukaryotic translation elongation factor 1 alpha 1 (CTCL tumor antigen)**
3bb 27	1100bp	HS chromodomain protein, Y chromosome-like
3bb 28	**1250bp**	**HS CD44 antigen**
3bb 31	900bp	HS Immunoglobulin kappa constant region
3bb 41	800bp	CDC28 protein kinase 1
3bb 44	**1100bp**	**HS hypothetical protein FLJ12287 similar to semaphorins (mRNA for SEMB)**
3bb 47	**800bp**	**HS seven transmembrane protein (NIFIE14)**

Table 3.6: Clones isolated from the ARH77 pCMV® library by immunoselection with anti-Wue-1.
Clones were sequenced after **four rounds** of panning (for comparison the numbers of the identified inserts after one round of panning are also stated)

Identification	1 round of panning	4 rounds of panning
HS GAPDH	1/78	19/42
HS MHC class II antigen (HLA-DQA1)	2/78	1/42
HS muscle specific gene (M9), PTD001, HSPC029, highly similar to AF077051 (HS eukaryotic translation initiation factor 3 subunit k (eIF3k))	3/78	16/42
HS NIMA-related kinase 6 (NEK6)	1/78	2/42
HS SCG10-like protein, helicase like protein NHL, M68, and ADP-ribosylation, factor related protein1 (ARFRP1), HS hypothetical protein FLJ14972	1/78	1/42
ribosomal protein	28/78	3/42

Table 3.7: Clones isolated from the MM phagemid library by immunoselection with anti-Wue-1.
Clones were sequenced after **three rounds** of panning

Identification	3 rounds of panning
HS eukaryotic translation initiation factor 3 subunit k (eIF3k) = HS muscle specific gene (M9)	11/27
HS GAPDH	5/27
HS immunoglobulin kappa locus, similar to anti-TNF-alpha antibody light chain F(ab') fragment,	1/27
HS MHC class II antigen (HLA-DQA1)	2/27
HS Rab9 effector p40	1/27
ribosomal protein	1/27

The results of the ARH77 pCMV® plasmid library screen are summarized in tables 3.5 and 3.6. Out of 78 clones which were isolated and sequenced after only one round of panning inserts coding for 37 different proteins were identified (table 3.5), i.e. almost every second clone carried a different insert. In contrast, after four rounds of panning only six different inserts were present within 42 sequenced clones, out of which GAPDH was identified in 45% of the clones and the eukaryotic translation initiation factor 3 subunit κ (eIF3κ) in 38% (table 3.6). This result makes clear that multiple rounds of immunoselection are required to achieve enrichment of single clones. On the other hand selection of highly abundant proteins may occur.

Table 3.7 shows the results of the multiple myeloma λ-ZAP® phagemid library screen. Here the clones were sequenced after three rounds of panning. In accordance with the ARH77 library screen, the two most frequent inserts identified in the MM library screen coded for eIF3κ (41%) and GAPDH (18%). These results indicate that immunoselection and enrichment with anti-Wue-1 mAB was rather non-specific. Interestingly, although they are the two most frequently identified inserts, neither GAPDH nor eIF3κ are cell-surface proteins. Cells, which have received plasmids that code for these proteins may experience a growth advantage, and are therefore represented in higher numbers within a pool of transfected cells.

3.5.4.2 Northern blot analysis of clones identified by panning with anti-Wue-1 does not reveal specific expression in Wue-1 positive cell lines

When database search of the sequenced clones identified inserts coding for potentially interesting proteins, e.g. transmembrane proteins, tumour associated proteins, antigens etc. (marked by bold print in table 3.5), they were further analyzed by Northern blotting. Northern blot analysis was performed with total RNA isolated from WUE-1 positive ARH77 and NCI-H929 cells. The WUE-1 negative cell lines RPMI-8226 and BL60-2, respectively served as controls. [^{32}P] labelled RNA probes from the clones of interest were generated by run off transcripts starting at the T7 promoter of the pCMV® plasmid. Specific bands were to be identified by comparing the hybridisation patterns of the WUE-1^{+} cells with those of the WUE-1^{-} cell lines. In figure, 3.24 the Northern blots are depicted. However, specific bands for the RNA probes on ARH77 or NCI-H929 samples could not be identified.

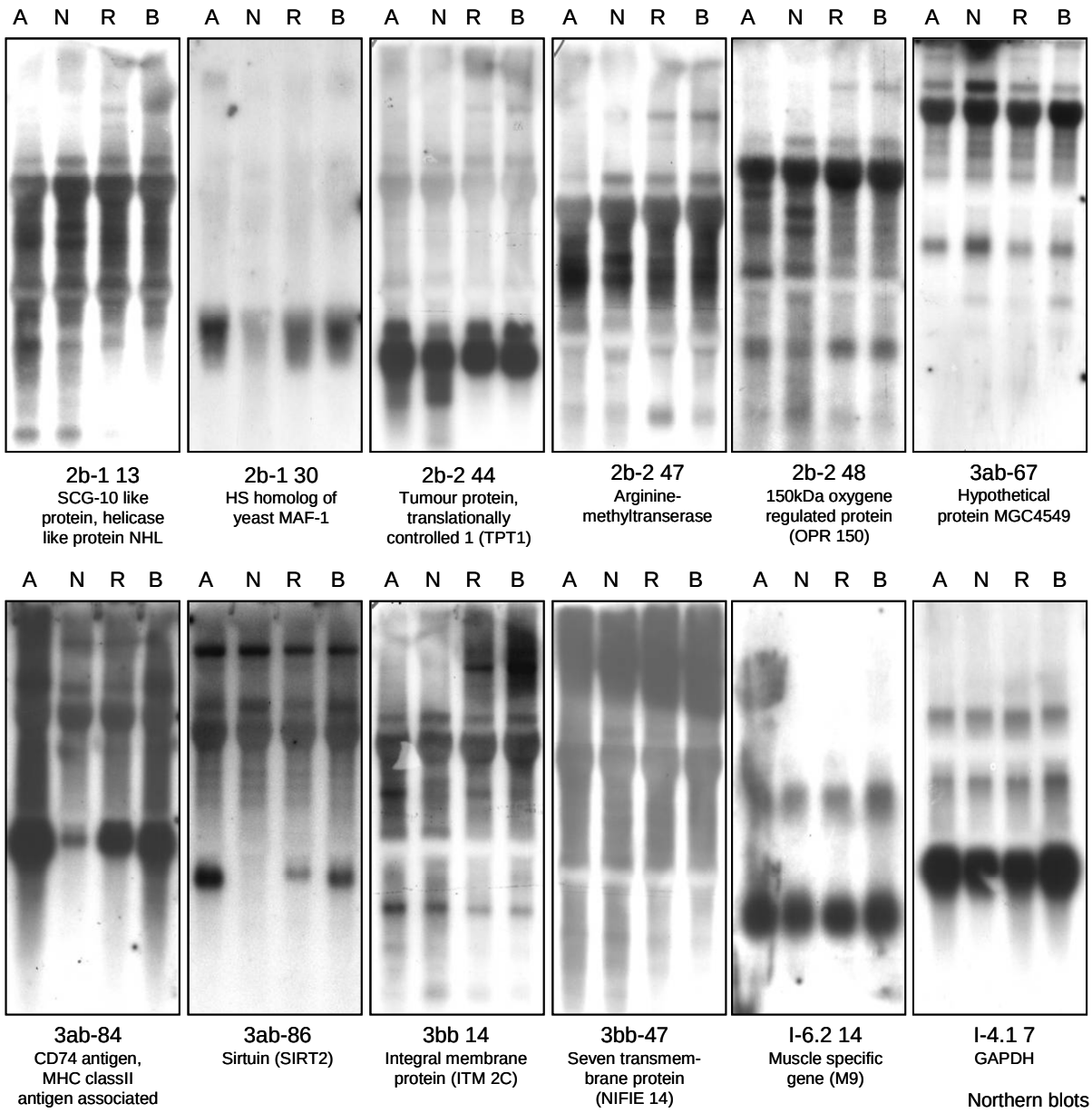

Figure 3.24: Northern blot analysis of ARH77 and MM expression library clones. Clones were isolated by expression cloning and immunoselection with anti-Wue-1 mAB. The inserts were sequenced (tables 3.5-3.7) and [^{32}P]-labelled RNA probes were generated from clones of interest. The blots depicted represent only a fraction of all Northern blot which were performed. **A**: ARH77, **N**: NCI-H929, **R**: RPMI-8226, **B**: BL60-2

3.5.4.3 FACS analysis of clones identified by panning with anti-Wue-1 does not identify a specific WUE-1 positive clone

The clones, which were subjected to Northern blot analysis, were also tested by flow cytometry. COS7 cells were transfected with the respective plasmids, 48h post transfection the cells were harvested, stained with anti-Wue-1 or IgG_1 isotype control and R-PE conjugated anti-mouse secondary antibody and were analyzed by FACS. However, expression of a protein reactive with anti-Wue-1 antibody could not be verified (data not shown).

3.6 Identification of the of MUE-1 antigen

A further goal of this project was to identify additional plasma cell-specific antigens suitable for the development of therapeutic intervention in multiple myeloma. Hybridoma cells were generated after immunization of mice with MM cell lines, and the hybridoma cell culture supernatants were subjected to FACS analysis with a panel of cell lines derived from human MM (NCI-H929, RPMI-8226), plasma cell leukaemia (L363, ARH77) and B-cell lymphoma (DOHH-2). Non-specific Burkitt lymphoma cells (BL60-2) were used as negative control. The ten hybridoma cell culture supernatants, which were tested, were kindly provided by Dr. Axel Greiner, University of Würzburg.

Out of the six cell lines, which were analyzed by FACS with the hybridoma supernatants only the human multiple myeloma cell line RPMI-8226 showed positive FACS results with three of the ten tested hybridoma supernatants. Distinct shifts of the histogram peaks were obtained with supernatants SN3, SN5 and SN9 (figure 3.25). The isotype of the antibodies, which were contained in these supernatants, was determined with a commercially available isotyping kit (Roche), and was identified to be IgM-κ subtype.

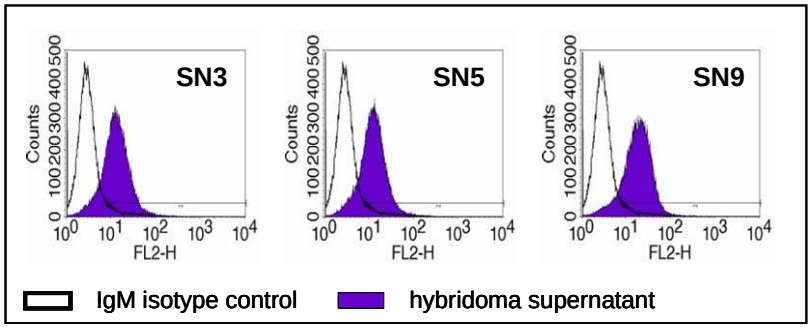

Figure 3.25: FACS analysis of hybridoma cell culture supernatants. FACS analysis was performed with RPMI-8226 cells. The cells were incubated with 500µl hybridoma supernatant (SN3, SN5, or SN9; **filled histograms**), or with IgM isotype control (1:50; **open histograms**). Secondary AB: PE conjugated anti-mouse IgM.

Supernatants SN3, SN5 and SN9 were then tested by Western blot (WB) analysis with membrane protein preparations from of the same cell panel as used for FACS analysis. The positive FACS result for the MM cell line RPMI-8226 was confirmed by WB. Supernatants SN3, SN5, and SN9 were found to be reactive with the RPMI-8226 protein samples (figure 3.26, lanes 4) and a strong band of ~25kDa was identified (figure 3.26, arrowhead) and the novel antigen was named "MUE-1". Although this band is also present in the other protein samples, the expression in RPMI-8226 is much stronger. Thus, RPMI-8226 cells were used to further investigate the unknown antigen

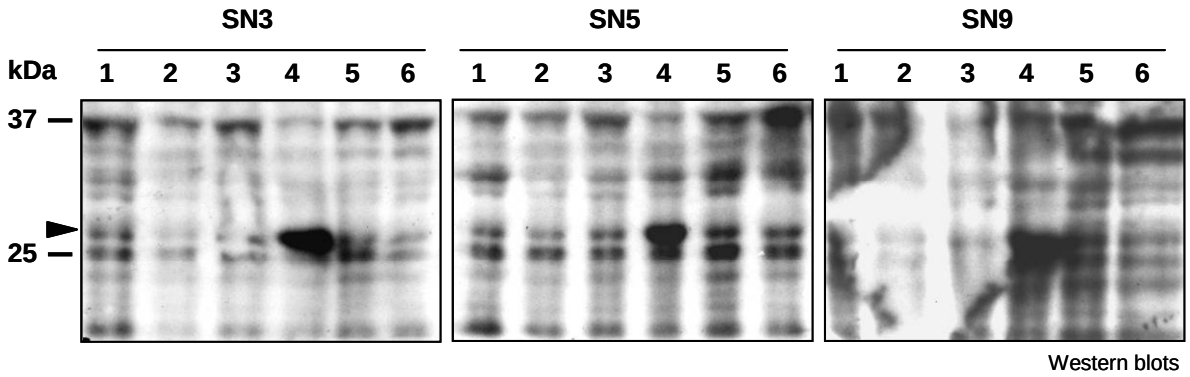

Figure 3.26: Western Blot analysis of hybridoma supernatants. 75µg/lane membrane proteins of L363 (**1**), ARH77 (**2**), NCI-H929 (**3**), **RPMI-8226** (**4**), DOHH-2 (**5**), and BL60-2 (**6**) cells were separated by SDS-PAGE on gradient gels. The blots were incubated with 5ml hybidoma SN3, SN5, or SN9. Secondary AB: anti-mouse IgM-HRP (1:10.000). **Arrowhead**: MUE-1 specific band

Subsequently, In order to identify a single, specific spot for MUE-1 2-dimentional gelelectrophoresis of RPMI-8226 cell membrane protein preparations was performed. Membrane proteins were isolated from $1x10^8$ RPMI-8226 cells and were acetone precipitated prior to electrophoresis. 50µg membrane proteins were separated in the first dimension by isoelectric focussing (IEF; pH 3-10) and then subjected to second dimension separation on a 4-12% Bis-Tris gradient gel (Novex, Invitrogen). Western blot analysis was subsequently performed with the anti-Mue-1 hybridoma supernatant SN9. With this method a single specific spot for MUE-1 was identified (figure 3.27, circle).

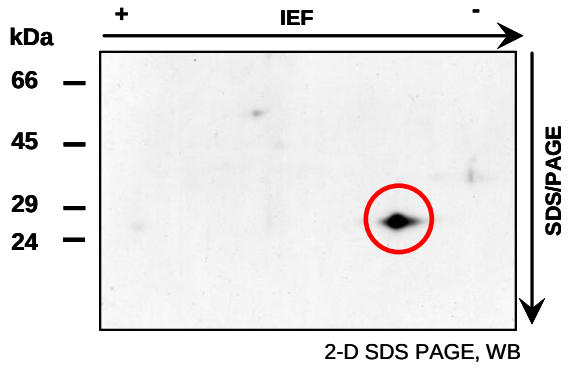

Figure 3.27: Two-dimensional Western blot analysis of MUE-1. 50µg RPMI-8226 membrane proteins (acetone precipitated) were separated in the first dimension by bioelectric focussing (IEF), pH 3-10. Second dimension separation was performed on a 4-12% gradient gel. The blot was incubated with anti-Mue-1 hybridoma SN9, diluted 1:10. Secondary AB: anti-mouse IgM-HRP (1:10.000); **Circle**: MUE-1 specific spot.

The electrophoresis was repeated and this time the gel was stained with colloidal coomassie solution. The MUE-1 spot was excised from the gel and after tryptic digestion of the protein sample, the peptides were analyzed by MALDI/MS (performed in the Department of Protein Chemistry and Proteomic Analysis from Dr. Wittmann-Liebold, MDC Berlin, Germany). The peptide sequences obtained from the spectrometry were further analyzed by database

comparison, performing a BLAST search. Database sequence comparison identified the putative plasma cell-specific antigen "MUE-1" as human λ-light chain (table 3.9). Cross-checking this result with the cytogenetic profile of RPMI-8226 cells as provided by the German Collection of Microorganisms and Cell Cultures (DSMZ) confirmed that RPMI-8226 cells express λ-light chain. The other cell lines, which were subjected to WB analysis with the hybridoma supernatants, are reported lambda-negative.

Table 3.9: Database identification of the MUE-1 peptides

Peptide sequence	Accession number	Database entry (identification)
pept 1-63 MAWALLLLTLLTQGTGSWAQSALTQPASVNGSPG QLIIISCTGPSSDIGDYQYISWYQQHPGK	gi|33700|emb|CAA40 939.1|	immunoglobulin lambda light chain [Homo sapiens]
pept 67-132 LIIYDVDKRPSGVSNRFSGSKSGNTASLTISGLQAED EADYYCSSYRGSATFEVVFGGGTKVTVLR	gi|33700|emb|CAA40 939.1|	immunoglobulin lambda light chain [Homo sapiens]
pept 132-211 AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTV AWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLT PEQWK	gi|33715|emb|CAA40 946.1|	immunoglobulin lambda light chain [Homo sapiens]
pept 215-237 SYSCQVTHEGSTVEKTVAPTECS	gi|33715|emb|CAA40 946.1|	immunoglobulin lambda light chain [Homo sapiens]

To ultimately confirm that the MUE-1 "antigen" is identical to immunoglobulin lambda light chain RPMI-8226 membrane proteins (40µg/lane) and a 0.1% solution of a human immunoglobulin preparation (Venimmun®N, Centeon) were separated by conventional SDS-PAGE on a 12% gel. The Western blots were incubated either with anti-Mue-1 (hybridoma SN9; figure 3.28 A) or anti-human λ antibody (figure 3.28 B). Moreover, in a competition experiment, anti-Mue-1 was mixed with Venimmun®N (0.1% final concentration) prior to WB analysis. Compared to the blot incubated with the original anti-Mue-1 stock the MUE-1 specific band was much weaker after incubation with the Venimmun®N spiked antibody sample (figure 3.28 A, lanes 2 and 3). This finding indicates that anti-Mue-1 is depleted through binding to a protein contained in the Venimmun®N solution and the Western blot analysis shows clearly that SN9 and anti-human λ antibody both detect the same protein.

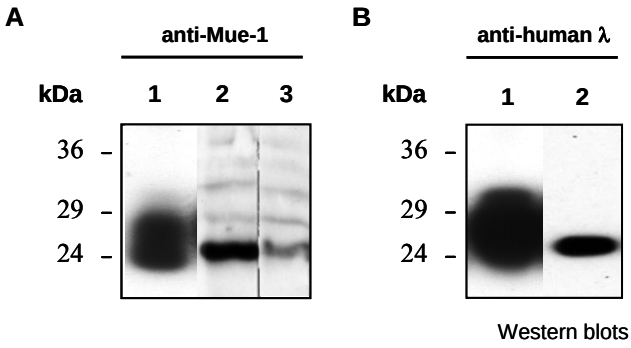

Figure 3.28: Western blot analysis of RPMI-8226 membrane proteins and Venimmun®N. A) Venimmun®N, diluted 1:1000 (**lane 1**) or 40 µg/lane RPMI-8226 membrane proteins (**lanes 2-3**) were separated on a 12% acrylamide gel and incubated with anti-Mue-1 (SN9; diluted 1:2), followed by anti-IgM-HRP secondary antibody (1:10.000). In **lane 3** anti-Mue-1 was mixed with Venimmun®N (0.1% final concentration) prior to incubation of the blot. **B)** Venimmun®N, diluted 1:1000 (**lane 1**) or 40 µg RPMI-8226 membrane protein preparation (**lane 2**) were separated on a 12% acrylamide gel and incubated with anti-human λ antibody (1:1000), followed by anti-IgM-HRP secondary AB (1:10.000).

Although in this case a novel plasma cell-specific antigen was not identified, this experiment gives proof of principle. On condition that the antigen and/or antibody are suitable for WB analysis, it was demonstrated that the strategy of screening hybridoma supernatants on cell lines, followed by protein identification with two-dimensional WB, is applicable for identification of novel antibodies, which may prove useful for development of immunotherapies.