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Characterization of Wue-1, a novel monoclonal antibody that stimulates the growth of plasmacytoma cell lines

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Abstract A new monoclonal antibody, Wue-1, which specifically recognizes normal and malignant plasma cells, is characterized. Biochemical studies showed that monoclonal antibodies (mAbs) recognize a protein of 94 kDa. Using triple-staining flow cytometry and double-labeling immunohistochemical techniques, two populations of plasma cells, i.e. lymphoplasmocytoid plasma cells located in the germinal center of lymphoid organs and reticular plasma cells at the paracortex or medullary cords of secondary lymphoid tissues, were distinguished. Wue-1 is expressed when B-cell markers become lost and secretory activity with plasma cell morphology appears. Cell surface markers were identified on normal plasma cells and compared with their malignant counterpart in vivo. Terminal plasma–cellular differentiation of malignant low- and high-grade B-cell lymphoma and anaplastic plasmacytoma, otherwise difficult to identify with conventional B-cell markers on tissue sections or fluorescence-activated cell sorter analyses, were detectable by Wue-1. In cell culture, Wue-1 enhanced the proliferation of myeloma cell lines but not normal plasma cells in a dose-dependent manner. Since Wue-1-induced proliferation was increased by interleukin (IL)-6, Wue-1 recognizes a so far unidentified antigen with functional properties. Therefore, Wue-1 represents a useful new tool for therapy and for the in vivo and in vitro studying of B-cell lymphomas and the mechanisms of B-cell differentiation.

Keywords Monoclonal antibody · Lymphoma · Myeloma · Therapy

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Introduction

The difficulties in identifying plasma cell subtypes compared with B-lymphocyte subsets result from the low frequency of plasma cells in bone marrow and lymphoid organs and from the loss of typical B-cell surface antigens at the plasma cell level. Therefore, most information regarding normal plasma cell differentiation has been deduced from the extrapolation of findings in malignant myelomas.

Several monoclonal antibodies (mAbs) have been raised to detect plasma cell-associated antigens, i.e. PC-1 [3], PCA-1 [2], MM4 [32], or VS38 [6, 28, 30, 33], but they do cross-react with a broad panel of other cell types or are not suitable for fluorescence-activated cell sorter (FACS) analyses or cell sorting due to their lack of membrane-bound antigen expression. Likewise, antibodies against CD38 are still used for immunophenotyping and multiparameter flow cytometric analyses [17, 20, 25, 31] of plasma cells and malignant myeloma, even though CD38 is an activation-associated [10, 19] rather than a differentiation-associated antigen, lacking lineage restriction [1]. At present, major interest in the analysis of mature plasma cell neoplasias has focused on the identification of precursor cells that give rise to mature malignant plasmacytoma. However, the definition of terminal differentiation steps in normal B cells and malignant B-cell lymphoma has been hampered by the lack of useful markers.

Additionally, it is not known whether plasma cell-related antigens in myeloma cells are residual constituents of normal developmental regulation pathways or play a role in the pathogenesis of the malignant transformation as exemplified by plasmacytoma cell lines [16].

We describe here a new mAb, designated Wue-1, which recognizes an intracellular and membrane-bound protein in normal and malignant human plasma cells with functional effects on malignant but not on normal plasma cells.

Materials and methods

Generation of mAb Wue-1

Briefly, the mAb Wue-1 was produced by means of immunizing BALB/c mice with a cell line derived from a pulmonary low-grade mucosa-associated lymphoid tissue (MALT)-type B-cell lymphoma H3302, which was generated in the Institute of Pathology in Würzburg. Mouse spleen cells were obtained and fused with the NSO cell line according to standard techniques [12]. After repeated subcloning, a stable hybridoma cell line producing a mouse immunoglobulin IgG2α was identified. This was determined using an enzyme-linked immunosorbent assay (ELISA).

Immunoglobulin-containing supernatant was purified by means of ammonium sulfate precipitation, followed by protein A affinity chromatography, and labeled with fluorescein isothiocyanate (FITC) or Biotin using *N*-hydroxy-succimid ester (Sigma, Germany) according to standardized procedures [13]. As a control, purified Igs of the same species and isotype (Dianova, Germany) were used.

Specificity tests of Wue-1

Immunohistochemical stainings were performed on 4 µm cryostat sections of fresh-frozen surgical specimen. Special attention was given to those organs known to be populated by plasma cells, i.e., bone marrow, lymph nodes, thymuses, and tonsils. Furthermore, cytospin preparations of normal peripheral blood lymphocytes, secondary lymphoid tissues, and lymphoma cell suspensions separated as described elsewhere [18], and myeloma/plasmocytoma cell lines were subjected to immunocytochemical testing. For double staining on cryostat sections, bound biotin-labeled antibodies were detected using Cy3-streptavidin (Dianova, Germany) as described [13]. The immunoperoxidase method was applied using a three-step incubation procedure with diluted purified mAb Wue-1 and the isotype control as described in detail elsewhere [22]. Biopsy tissues were kept at –70°C as snap-frozen blocks until fresh sections were prepared at the time of experiments. The following mAbs were used for indirect staining: CD22, CD23, Ki67, CD10, bcl-2 (all Dako), 4D12 (Spencer, London [29]), and VS38 (Gatter, [33]).

Flow cytometric analysis was performed using a FACScan (Becton Dickinson) with an argon ion laser tuned at 488 nm using LYSIS II for data acquisition and analysis using triple immunostaining with directly conjugated mAbs ($CD19_{HD\ 37}$, Sigma; $CD3_{UCHT-1}$, Sigma; $CD14_{Leu-M3}$, Becton Dickson; $CD54_{84M10}$, Immunotech, Krammer, Heidelberg [8]; $CD66L_{DREG\ 56}$, Dianova; $CD40_{MAb89}$ Banchereau; APO-1, Dardilly [4]; $CD38_{AT\ 13/5}$, Serotec). SerotecWue-1 was used either directly conjugated with FITC or as a biotinylated antibody detected with streptavidin-phycoerythrin (PE, Sigma). Instrument set-up samples included an unstained sample and samples stained with CD19-FITC, CD19-PE, and CD19-QR. The instrument set-up was standardized using CD19+ B lymphocytes from normal tonsils, referenced by means of gating on the fluorescence intensity of CD19 lymphocytes followed by adjustment of the light-scattering detectors to locate the B cells in a standard position in the correlative display of forward and sideward light scattering. The fluorescence detectors were adjusted using a tight light scattering gate obtained from the light scattering of the CD19+ lymphocytes, followed by adjustment of the three fluorescence detectors of an unstained sample. Each measurement contained 20,000 cells. Dead-cell discrimination was performed with 7-amino-actinomycin D (7-AAD, Calbiochem, Germany) in combination with dual color immunofluorescence, as described elsewhere [27].

Biochemical characterization

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% acrylamide Laemmli slab gel with a 3% stacking gel. Sample buffer contained 20% glycine, 0.125 mol/l Tris-HCl (pH 6.8), and 6% SDS with or without 10% 2-mercaptoethanol. Before electrophoresis, samples were heated to 56°C for 30 min. Molecular-weight markers were obtained from Pharmacia, LKB (Freiburg, Germany). After SDS-PAGE, the proteins were transferred to 0.45-µm nitrocellulose membranes by means of Western blot semi-dry transfer for 1 h at 4 C. To block free binding sides, filters were incubated for 30 min in phosphate-buffered saline (PBS) buffer (pH 7.4; 50 mmol/l) containing 5% dried skimmed milk. After three washes with PBS buffer, the membranes were incubated with the primary antibody overnight at 4 C. Bound antibody was targeted with peroxidase-conjugated rabbit anti-mouse Ig and visualized with a freshly prepared mixture of 20 ml Tris-buffered saline, 4 ml 4-chloro-1-naphthol (3 mg/ml in methanol), and 5 µl hydrogen peroxide. Another murine mAb with the same isotype served as a control and did not show any reactivity.

For immunoprecipitation, 2×10^7 cells were extracted at 4°C for 90 min with 200 µl lysis buffer under rotation. The composition of the lysis buffer was as previously described [24] with the exception of the fivefold higher Triton X-100 concentration (0.5%). Cell debris was spun down for 5 min, and the supernatants were transferred to fresh Eppendorf tubes. The supernatants were diluted five times with detergent-free lysis buffer in order to achieve a Triton X-100 concentration of 0.1%. The ensuing antibody incubation was for 4 h on ice. The antibody dilutions were 1:20 for the antibody designated Max1 (0.5 mg/ml) and 1:100 for the antibody designated Max2 (2.5 mg/ml). The solutions containing the immune complexes were transferred to a tube containing 40 ml slurry of protein A agarose beads (Pierce) previously washed with lysis buffer. Incubation was for 45 min at 4°C under rotation. The beads were pelleted and washed three times with 400 µl lysis buffer, while the supernatants were discarded. The immune complexes were recovered by means of boiling the agarose beads for 10 min in 25 µl Laemmli sample buffer. After filtration of the protein solution through 45-µm ultrafree-MC filter units (Millipore), the proteins were resolved on an 8% SDS-polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane. In contrast to the previously described immunoblots, immunodetection was performed using the ECL system (Amersham, UK) according to the manufacturer's instructions. The antibody dilution used for immunodetection was 1:100.

Functional analyses

Plasmacytoma cell lines L-363 and NCI-H929 were obtained from the German department of human and animal cell cultures (DSM No ACC49 and ACC163). Normal plasma cells were obtained after negative depletion of normal tonsil lymphocytes with immunobeads (Dynal, Germany) coupled to anti-CD2 (for T-cell-), anti-CD19 (for B-cell-), and anti-CD14 (for myeloid/macrophage depletion). A ficoll-paque (Pharmacia LKB, Freiburg, Germany) density gradient centrifugation was followed by with FACS analyses to guarantee more than 95% Wue-1+ plasma cells. Cells were maintained in culture containing RPMI1640 medium supplemented with 10% fetal calf serum and 0.03% gentamycin. Cells (5×10^4) were seeded in a round-bottom 96-well plate and grown in 200 µl medium supplemented with and without soluble antibodies at different concentrations in the absence or presence of 100 IU recombinant human IL-6 (a kind gift from Prof. W. Sebald, Institute of Biochemistry, Würzburg). DNA synthesis was determined by means of [3H] TdR incorporation for 16 h after 5 days of culture.

Results

Wue-1 recognizes two different human plasma cell populations in vivo

Using immunohistochemistry, Wue-1-positive cells were identified inside germinal centers, within marginal

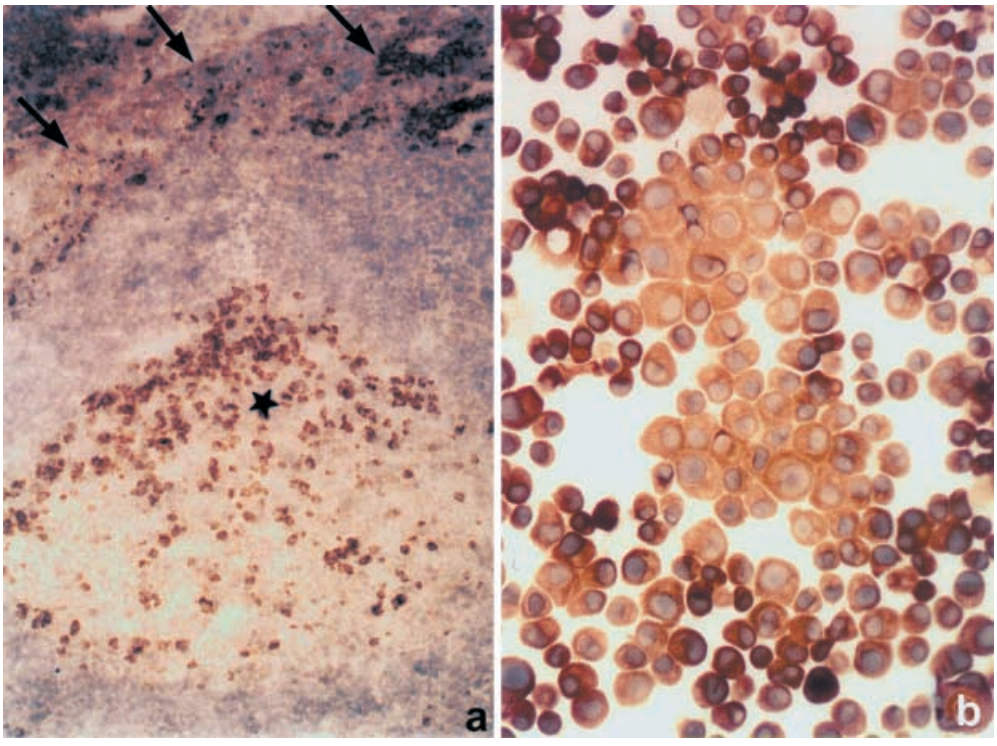

Fig. 1 Wue-1 immunoperoxidase stain of normal tonsil cryostat section (a) showing plasma cells in the light zone of a germinal center (*star*), while the mantle and T-cell zone outside are negative. Nearby and in the adjacent epithelium, another zone of plasma cells is present (*arrow*). (×200). **b** Plasma cell line NCI with strong reactivity with Wue-1

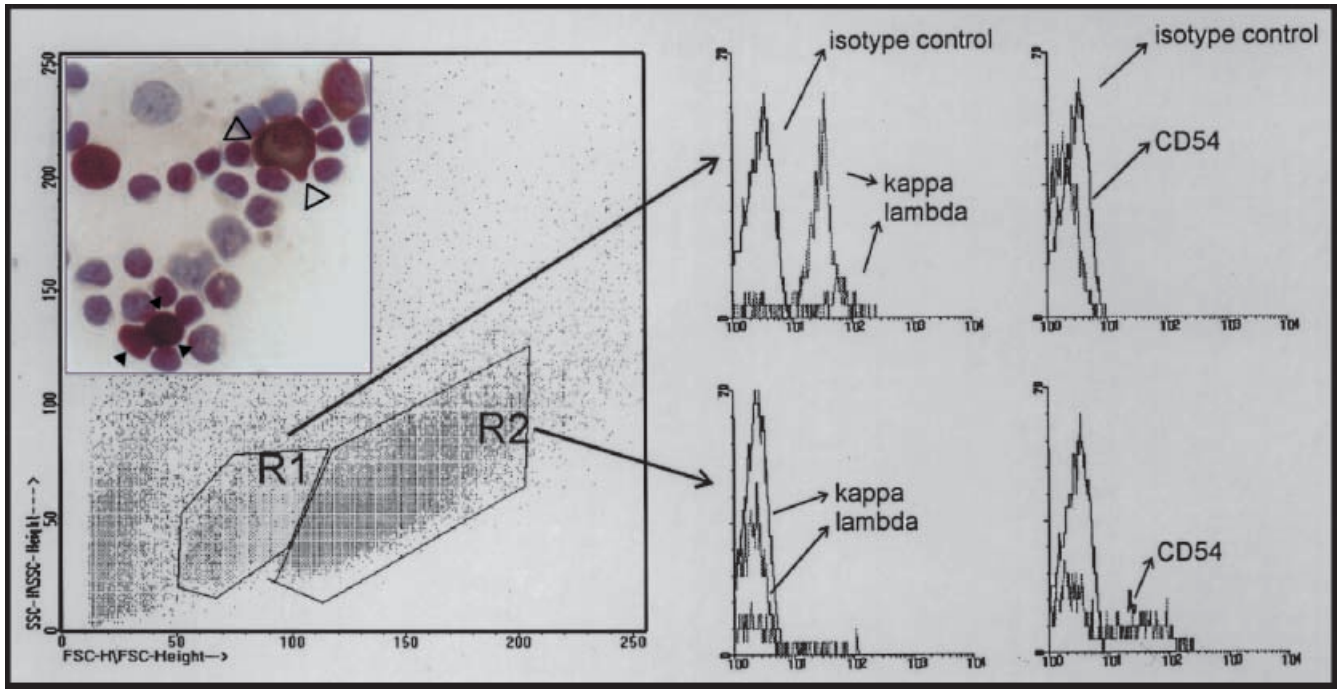

Fig. 2 Fluorescence-activated cell sorter (FACS) analysis of a normal tonsil cell suspension. Based solely on size (*x-axis*) and granularity (*y-axis*), two populations of plasma cells (R1 and R2) were identified. The R1-Wue-1$^+$ population was small, with low granularity, and expressed surface immunoglobulin but no CD54 (*right histogram, top*). In contrast, the R2-Wue-1$^+$ population was larger, with more granularity, and expressed no surface immunoglobulin except for CD54 (*right histogram, bottom*). The inset on the left shows a cytospin preparation and immunostaining with Wue-1 of the same cell suspension with mature plasma cells (*open triangle*) and lymphoplasmacytoid plasma cells (*filled triangle*)

zones, and adjacent to or within the epithelium of normal tonsils (Fig. 1a). Microscopic examination showed the typical morphological features of mature plasma cells known as the Marschalko type and a second type of plasma cell, the lymphoplasmacytoid type (Fig. 2, inset). The light microscopic difference between the two plasma cell types was mirrored by differences in the expression of cell surface markers as determined using FACS analysis. Forward and sideward light scattering of a Wue-1+

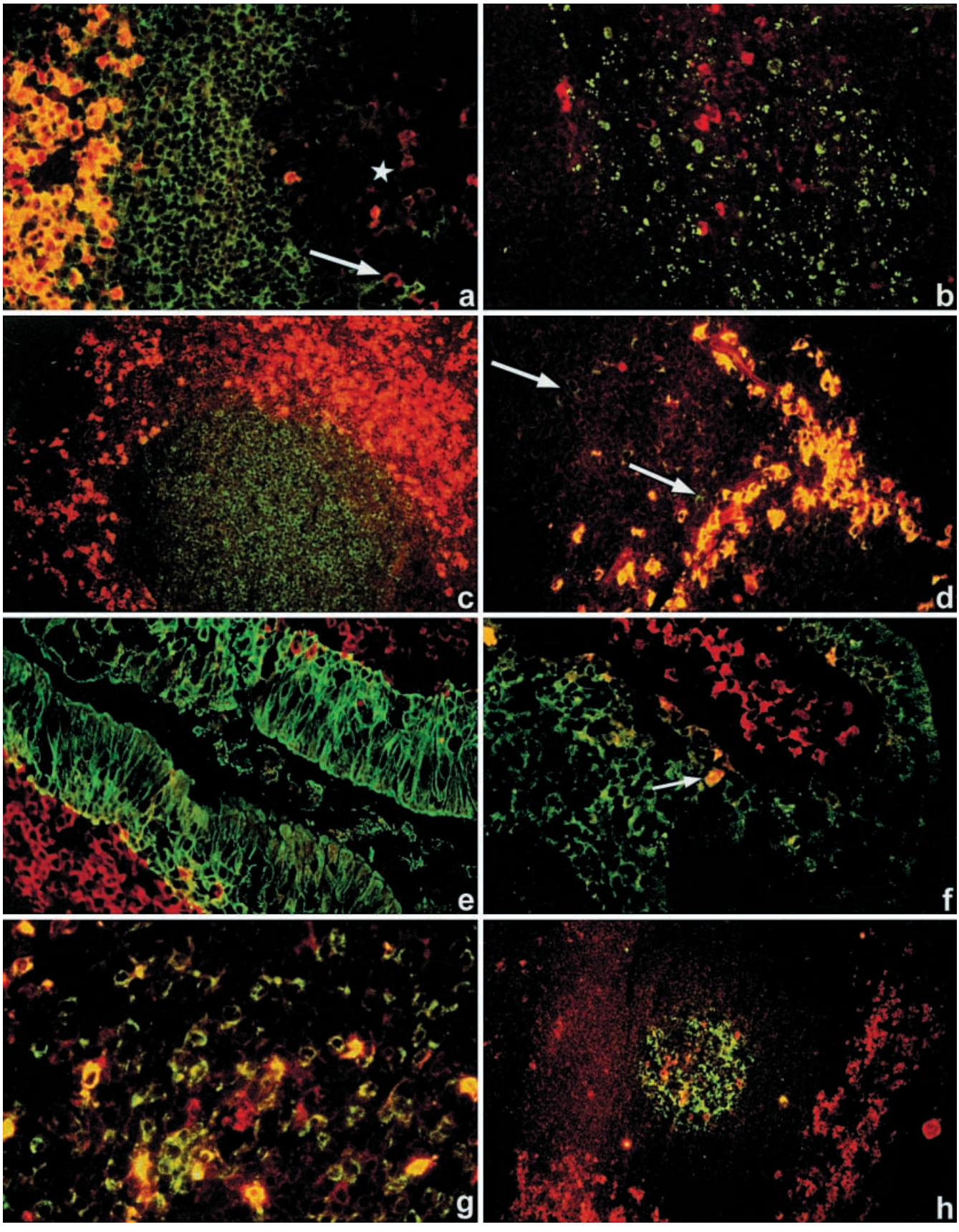

Fig. 3

plasma cell population of small cells expressing surface Ig was distinguished from a large cell population devoid of Ig. This distinction was confirmed through the combination of Wue-1 with other differentiation- and lineage-restricted markers and through comparison of FACS data with the anatomical location of plasma cells in normal lymphoid tissues, as outlined in Table 1 and Fig. 3a–h. The small cell population equivalent to the lymphoplasmocytoid cells did not express CD23 or CD44. However, B-cell markers (CD19, CD22, CD40) and APO-1 and human leukocyte antigen (HLA)-DR in germinal centers of lymph follicles were expressed. Additionally, 4D12, a specific marker for marginal zone B cells [29], was co-expressed with Wue-1 in all germinal center lymphoplasmoid cells but only in 10% of lymphoplasmoid cells in the paracortex (Fig. 3g). The large cell population equivalent to mature plasma cells occurs only outside germinal centers and co-expressed bcl-2 and CD54 weakly but expressed CD44 strongly (Fig. 3a). The B-cell markers (CD19, CD22, or CD23; Fig. 3h) were not expressed in these mature plasma cells. 4D12, APO-1 (Fig. 3f), and HLA-DR were also not expressed. Non-malignant lymphoplasmocytoid and plasma cells were found to co-express Wue-1 with CD38 and VS38 (Fig. 3d). Both subpopulations were negative with Ki-67, indicating their transition into the G0 phase (Fig. 3b). No staining of plasma cells could be detected using antibodies specific for macrophage- (CD14, CD68) and T-cell markers (CD3; Fig. 3c) or CD10, CD66L, or cytokeratin 8 (Fig. 3e). Wue-1 mAb was found to be unreactive with most normal tissues, including peripheral blood, liver, kidney, heart, muscle, skin, bladder, ovary, and testis but stained weakly secretory epithelia in gastrointestinal tissues.

Wue-1 recognizes terminal differentiated malignant lymphoma

As shown in Table 2, the reactivity of Wue-1 was demonstrable in all multiple myelomas and plasmacytomas tested, irrespective of their site of derivation. Furthermore, lymphoma B cells that were recently found to exemplify the terminal phase of B-cell differentiation, like low-grade MALT-type lymphomas with plasma cell differentiation and immunocytomas, were stained (Fig. 4a, b; Table 2) [26]. In MALT-type lymphoma, a clear cut distinction could be made between those cases with plasma cell differentiation (Wue-1+) and those without Wue-1. Those negative for Wue-1 stained all malignant B-cell lymphomas of early or mid-stage B-cell differentiation like mantle cell lymphomas, Burkitt's lymphoma, or B-chronic lymphocytic leukemia (CLL) and all T-cell lymphomas and Hodgkin's lymphomas. Strong staining with Wue-1 was also found with all plasma cell lines investigated

Table 1 Double immunofluorescence with Wue-1 and normal tonsil tissue. – No staining; (+) weak staining; + strong staining as qualified by two independent observers; *Ig* immunoglobulin; *ICAM* intercellular adhesion molecule

Antigen	Germinal center	Paracortex
Cytoplasmic Ig	+	+
Surface Ig	+	–
Ig isotype	IgM>>IgG>IgA	IgG>IgM>IgA
CD19	(+)	–
CD22	(+)	–
CD23	–	–
CD40	(+)	–
4D12	(+)	(+)
CD38	+	+
VS38	+	+
CD136	(+)	+
CD44	–	+
CD3	–	–
CD14	–	–
Ki67	–	–
HLA-DR	+	–
CD66L	–	–
CD54 (ICAM-1)	–	+
CD10	–	–
CD95 (APO-1)	(+)	–
bcl-2	–	(+)
bcl-6	–	–
Cytokeratin 8	–	–
CD68 (KiM-1)	–	–

Table 2 Reactivity of different lymphoma subtypes with Wue-1 according to the revised European–American (REAL) classification [15]. *MALT* mucosa-associated lymphoid tissue

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

◀ **Fig. 3** Double-immunofluorescence staining with different monoclonal antibodies (mAbs; *green*) and Wue-1 (*red*) on normal tonsil cryostat sections. Double-labeled cells stained *yellow*. **a** CD44 staining mantle and marginal zone B-cells (*green*) with intense double labeling in the paracotex (*yellow*), but not in the germinal center (GC; *star*, *arrow* Wue-1+). **b** There are no proliferating Wue-1 cells in the GC when using Ki-67. **c** There is no overlap with $CD3^+$ T cells in the paracortex. **d** VS38 has an almost identical staining pattern as Wue-1, although VS38 stains additionally some non-plasma cells (*arrow*). **e** Cytokeratin 8 stains overlying tonsillar epithelium but not the scattered plasma cells between. **f** With APO-1 (FAS or CD 95), only a few intraepithelial plasma cells (*arrow*) in the basal layer of the epithelium are stained. **g** The marginal zone is stained with 4D12 and some (overall about 10%) double labeled plasma cells. **h** CD23 stains secondary lymph follicle but not plasma cells within, followed by the unstained paracortical T-cell zone (compare with Fig. 3c) and a zone with plasma cells beneath/within the epithelium (*unstained*)

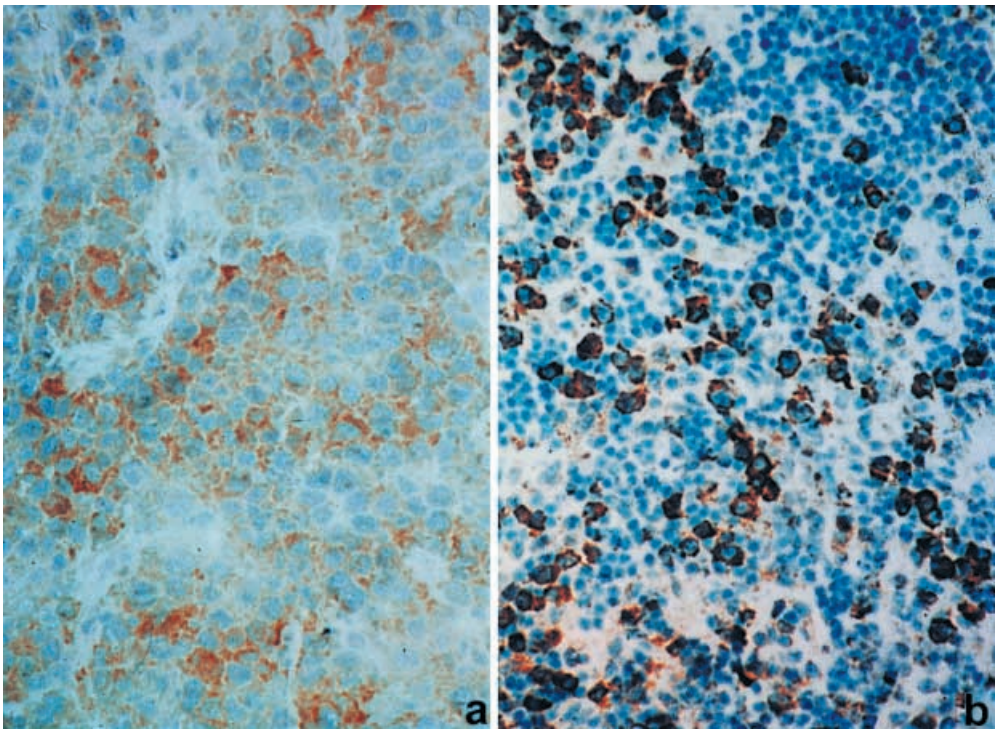

Fig. 4 **a** Immunoperoxidase with Wue-1 of a high-grade mucosa-associated lymphoid tissue (MALT)-type B-cell lymphoma with plasma cellular differentiation exhibiting weak, but visible staining of all tumor cells. **b** Plasma cellular differentiation of a low-grade MALT-type B-cell lymphoma with strong staining of some tumor cells

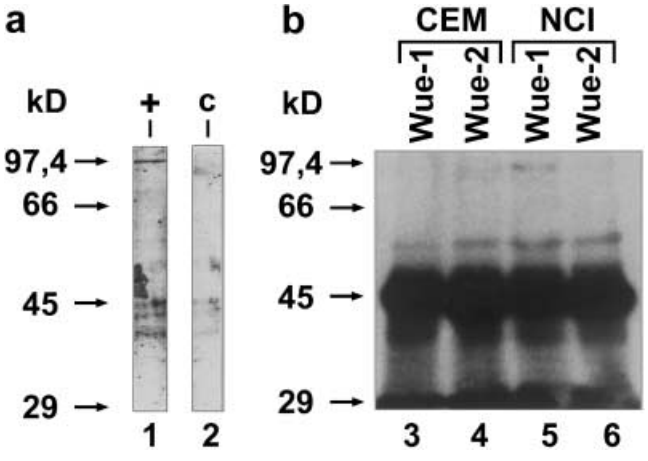

Fig. 5 **a** Western blot using plasmacytoma cell line NCI as an antigen source with immunobands at 94 kDa and 45 kDa. Molecular-weight markers are given at the *left*. Wue-1 (+) with the isotype control monoclonal antibody (mAb; *C*). **b** Immunoprecipitation using a T-cell line (CEM) as a control and another mAb, Wue-2, unrelated to plasma cell staining. The only specific band was found at 94 kDa with Wue-1 and the NCI cell line

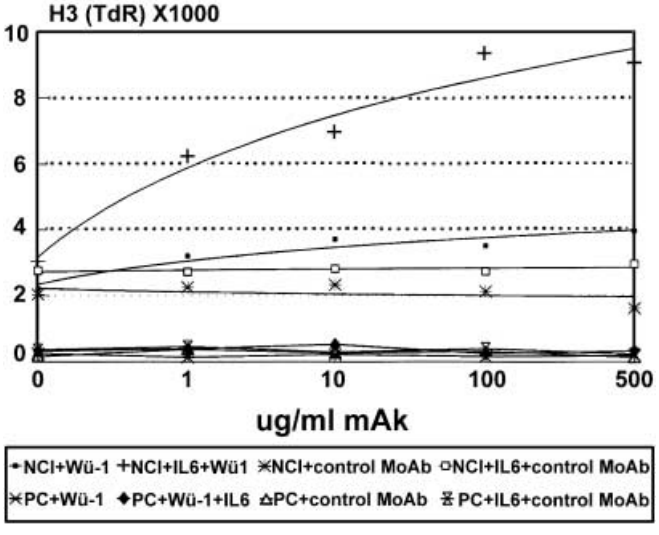

Fig. 6 Proliferation test using NCI plasmacytoma cell line and purified tonsil plasma cells (PC) with or without 100 IU interleukin (IL)-6 at different dilutions of purified monoclonal antibody (mAb; x-axis: Wue-1 and isotype control mAb). Note that non-malignant plasma cells do not proliferate. Their viability after being cultured for 6 days was below 20% in trypan blue stains. The cell line proliferation was slightly enhanced after adding IL-6 with or without control mAb, but increased up to four times after adding Wue-1 (+) and IL-6 in a dose-dependent manner

(Fig. 1b). B-cell lines (RAJI, BONNA-12, 697), monocytoid (THP-1, U936), and T-cell lines (Jurkat, CEM, C8166) stained negative (Fig. 5b and data not shown).

Wue-1 recognizes a 94 kDa molecule

Using plasma cell line NCI-H929 as a source of antigen, Wue-1 immunostained two protein bands at 94 kDa and 45 kDa under reducing conditions on a Western blot (Fig. 5a). These protein bands were also observed in tonsil or myeloma cell line extracts but with different degrees of intensity (data not shown). Through comparison of Western blot data with the immunoprecipitation data, it was found that the 94 kDa band gave the most reproducible and specific signal (Fig. 5b), while the 45-kDa

band appeared non-specific and was found in controls as well.

Wue-1 shows functional properties in plasma cell lines

To investigate the function of the Wue-1 reactive proteins, normal plasma cells were isolated from reactive tonsillitis and treated over 5 days with different dilutions of Wue-1. No proliferative response was found. This lack of effect could not be overcome by the addition of IL-6 to the culture medium. The viability of normal plasma cells decreased after 6 days in culture below 20% as determined using trypan blue stains. Normal plasma cells were not rescued from apoptosis with Wue-1 and apoptosis was not increased. Additionally, no proliferative response to Wue-1 was found for normal tonsillar B- and T cells (data not shown). In contrast, Wue-1 induced a dose-dependent proliferation in several plasma cell lines derived from multiple myeloma and plasmacytoma. This activity of Wue-1 was significantly enhanced by IL-6 (Fig. 6).

Discussion

Although normal B-cell differentiation has been studied extensively in bone marrow aspirates and lymphoid organs, little is known about the terminal differentiation of B lymphocytes into plasma cells due to the lack of specific markers. In this respect, Wue-1 is an important advance since biochemical analysis and immunophenotyping suggest that the expression pattern of the Wue-1 proteins is distinct from known CD antigens and published antibodies in the differentiation pathway of normal B- and plasma cells. In particular, using only this highly specific marker and cell size, it was possible to distinguish two developmentally distinct populations of plasma cells. By contrast, such a distinction requires multicolor FACS analysis when applying traditional and even less specific markers, such as CD38 [31]. As a consequence, we were able to perform a detailed morphologic and immunophenotypic analysis of two subpopulations in tissue sections through the use of Wue-1. The first subpopulation, comprising of lymphoplasmocytoid plasma cells, is located in germinal centers of lymphoid organs. The second subpopulation consists of mature plasma cells in the paracortex or within the marginal zone of secondary lymphoid tissues.

The mature plasma cell population was distinguished by its high forward light scattering in FACS analysis and the expression of CD44, CD54, and bcl-2 protein. Interestingly, the immature lymphoplasmocytoid subpopulation was CD44, CD54, and bcl-2 negative but CD19, 4D12, and APO-1 positive. This may indicate a close relationship of memory B cells to mature plasma cells and imply a developmental “branching” within the germinal center of late maturing B cells either into memory B cells expressing CD19, MHC class II, bcl-2, and 4D12 or into plasma cells being also bcl-2^+ but becoming CD19, MHC class II, and 4D12 negative. Outside of germinal centers, co-expression of 4D12 and Wue-1 was found in about 10% of marginal zone B cells. This may indicate a maturation of germinal center-derived memory B cells into plasma cells in the marginal zone of secondary lymphoid organs. This would explain the finding that most marginal zone plasma cells express IgG and not IgM, suggesting a maturation and immunoglobulin class switch within germinal centers as proposed recently [5, 21, 23]. However, definite proof of this idea may depend on sequencing Ig genes of memory B- and plasma cells on the single cell level to verify their relationship. So far, it cannot be excluded that lymphoplasmocytoid and mature plasma cells of Marschalko type are two unrelated subpopulations and that the former is not the precursor of the latter.

The identification of various steps in the developmental maturation of normal plasma cells by Wue-1 may be helpful to define the biological behavior of the two clinically most significant malignant counterparts in the immune system. First, MALT-type lymphomas with plasma–cellular differentiation co-expressing CD40 and 4D12 together with Wue-1 represent an example of a neoplastic process at an early stage of plasma cell development. These most frequent extranodal lymphomas were recently described to represent malignant memory B cells [11, 26], and their growth was found to depend on antigen- and T-cell mediated signals in vivo [14, 18]. Therefore, the detection of features of early plasmacytoid differentiation in vivo by Wue-1 may be of clinical relevance because antigen withdrawal can lead to MALT lymphoma regression [7] and may guide further investigations in the underlying mechanism of MALT-type lymphoma development and progression.

Second, multiple myelomas and plasmacytoma represent examples of neoplasms at a late stage of plasma cell differentiation. In these frequent hematological neoplasms in man, Wue-1 antigens will become important since they induced functional activities in malignant but not in normal plasma cells. To exploit these functional differences for diagnostic and therapeutic purposes, determination of the different signal transduction pathway after cross-linking of the antigens and assessment of cofactors that may influence the response (such as cytokines like IL-6 [9]) will be mandatory but has not yet been performed. One therapeutic application of Wue-1 is already emerging today. Due to its unique binding specificity, Wue-1 might be an ideal tool for the purging of myeloma cells for bone marrow transplantation.

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