PAPER V

Cyclin B1 is commonly expressed in the cytoplasm of primary human acute myelogenous leukemia cells and serves as a leukemia associated antigen associated with autoantibody response in a subset of patients

CYCLIN B1 IS COMMONLY EXPRESSED IN THE CYTOPLASM OF PRIMARY HUMAN ACUTE MYELOGENOUS LEUKEMIA CELLS AND SERVES AS A LEUKEMIA-ASSOCIATED ANTIGEN ASSOCIATED WITH AUTOANTIBODY RESPONSE IN A SUBSET OF PATIENTS

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Short title: Cyclin B1 in AML

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Cyclin B1 is involved in cell cycle regulation of both normal and malignant cells. Cytoplasmic expression of cyclin B1 in epithelial malignancies is associated with a specific T cell response and presumably also a humoral immune response. We therefore investigated the protein expression of cyclin B1 in native human acute myelogenous leukemia (AML) cells and the occurrence of cyclin B1 specific antibodies in AML patients. AML cell expression of cyclin B1 was detected for all 42 patients investigated by flow cytometry; but the percentage of cyclin B1 positive cells showed a wide intra patient variation. Confocal laser microscopy demonstrated that most of these patients (32/42-76%) showed abnormal cytoplasmic expression. Furthermore, both cytoplasmic and nuclear expression was maintained after 14 days of in vitro culture and differentiation of the AML cells in the direction of a dendritic cell phenotype. AML engraftment in NOD/LtSz-*Prkdc*^{scid}/*B2m*$^{-/-}$ mice showed no clear correlation with cyclin B1 expression. Cyclin B1 specific serum antibodies could be detected for 8 out of 66 consecutive patients with untreated AML. Fourteen of these patients were in addition tested later following intensive chemotherapy; three of them had detectable antibody levels that decreased following treatment. Our studies demonstrate that primary human AML cells show aberrant cytoplasmic expression of cyclin B1 for a majority of patients; this expression pattern has been associated with induction of specific immune responses in patients with epithelial malignancies and a specific humoral immune response was also detected for a subset of patients with untreated AML.

Key Words: Acute myeloid leukemia – Cyclin B1 – Leukemia associated antigens

INTRODUCTION

There is an expanding list of autoantibodies to tumor-associated antigens (TAAs), and the identification of cancer-associated autoantibody signatures may therefore become useful as an early diagnostic or prognostic specimen (1-3). However the number of autoantibodies that have been detected in acute myelogenous leukemia (AML) is relatively few compared to many other malignancies. Among these leukemia associated antigens (LAAs) that induce a humoral immune response in AML patients are Wilms tumor gene product (WT1) (4, 5), the M-phase phosphoprotein 11 (MPP11) (6) and receptor for hyaluronan acid-mediated motility (RHAMM) (7), whereas T cell recognized LAAs include WT-1, Proteinase3, PRAME (8, 9), RHAMM (10), BCL-2 (11) and OFA-iLRP (12). Vaccination with LAA peptides are now tried in the treatment of AML (13, 14).

Cyclins and the cyclin-dependent protein kinases (CDKs) are major regulators of cell cycle progression (15, 16). Cyclin B1 associates with it's catalytic partner P34/CDK1 during interphase (17), and this complex phosphorylates their substrates to drive cells through the G_2-M phase complex (17, 18). The cyclin B1/CDK1 complex thereby controls chromosomal condensation, reorganization of microtubules, destruction of the nuclear membrane, and Golgi apparatus fragmentation (19). Accumulation of cyclin B1 usually begins late in S-phase, reaches maximal level at G_2/M transition when it is rapidly degraded before onset of anaphase (15, 17, 20). However, detection of cyclin B1 in the G_1 phase has also been reported (21-26).

Cyclin B1 is believed to shuttle between the nucleus and the cytoplasm in interphase, but is mainly localized in the cytoplasm (27, 28), whereas upon inception of prophase cyclin B1 rapidly accumulates in the nucleus (20, 29, 30). The mechanism of nuclear import of cyclin B1 is unclear. The G2/M regulator 14-3-3sigma, the tumor suppressor protein p53, phosphorylation of cyclin B1 within the cytoplasmic retention signal (CRS) region, and the nuclear localization signal (NLS) regions of cyclin F all seem to be important for the cytoplasmic localization (27, 31-37). Furthermore, previous experimental observations suggest that p53 inactivation is essential for the aberrant cytoplasmic expression (overexpression and in wrong location) in malignant cells (38).

Several studies have described cytoplasmic accumulation of cyclin B1 in epithelial malignancies

(39-45). These studies identified cyclin B1 as a shared human epithelial tumor-associated antigen (TAA) that could be recognized by naive T cells from healthy donors primed against in vitro generated dendritic cells loaded with peptides eluted from HLA-class I molecules of the breast cancer cell line MS-A2(45). This study determined that (i) cyclin-B1-derived peptides could be presented by HLA-A*0201 and possibly HLA-A*0206; (ii) several cyclin-B1-derived peptides could be recognized by HLA-A2-restricted T cells; and (iii) cyclin-B1 specific and HLA-A2-restricted T cells could be detected in the T cell repertoire of healthy individuals, breast cancer patients as well as patients with squamous cell carcinoma of the head and neck (45). In contrast, no cytoplasmic cyclin B1 staining was observed for corresponding normal cells, i.e. human airway bronchoepithelial cells and mucosal cells surrounding head and neck carcinomas. Thus, recognition of cyclin-B1 as a TAA in epithelial cancers by in vitro and in vivo generation of specific T cell responses was associated with predominant cytoplasmic cyclin-B1 expression in the malignant cells. The aim of the present study was therefore to investigate whether a similar cytoplasmic pattern and cyclin B1 specific immune responses could be detected in human acute myelogenous leukemia (AML); a hematological malignancy where antileukemic T cell reactivity is important both for the graft-versus-leukemia effect after allogeneic stem cell transplantation and possibly also as a target for vaccination therapy after conventional chemotherapy (46).

MATERIALS AND METHODS

Acute leukemia patients

The study was approved by the local Ethics Committee and all samples were collected after informed consent. *AML cells* derived from 43 consecutive patients with high peripheral blood blast counts were included in the major part of this study. The clinical and biological data for these patients are presented in Table 1. *Serum samples* were derived from another group of 66 consecutive AML patients (median age 58 years, range 23-83 years), and the results for these samples were compared with a group of 13 healthy individuals (median age 44 years, range 33-69 years).

Preparation of human AML blasts

Leukemic peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Ficoll-Hypaque; NyCoMed, Oslo, Norway; specific density 1.077) from the peripheral blood of patients with a high percentage of AML blasts among blood leukocytes (see Table 1). Cells were cryopreserved in 20% fetal calf serum (FCS) plus 10% dimethylsulphoxide (DMSO) in liquid nitrogen before use. The percentage of blasts among leukemic PBMC generally exceeded 95% (47-49), the contaminating cells being small lymphocytes.

***In vitro* differentiation of human AML blasts in dendritic cell direction**

Unless otherwise stated RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 100 µg/mL of garamycin was used for culture of AML cells. Recombinant human cytokines were used at the following concentrations in AML blast cultures: 50 ng/ml IL-4, 100 ng/ml GM-SCF, and 50 ng/ml TNF-α (Peprotech, Rocky Hill, NJ). For differentiation in dendritic cell direction AML cells were seeded in 24-well plates (1 x 10^6 cells/ml, 2 ml per well) and cultured for 6 days in culture medium containing GM-CSF and IL-4. On day 6 TNF-α was added together with GM-CSF and IL-4 by exchanging 1 ml of the medium per well, and the cells were cultured for additional 8 days. Fresh medium with cytokines was also added on day 3 and day 9.

Membrane molecule expression was analyzed by flow cytometry (FCM) using either PE, FITC or APC-conjugated HLA-DR, CD40, CD80 and CD83 monoclonal antibodies (Becton Dickinson, San Jose, USA). The cut-off for positive cells was defined as a fluorescence corresponding to 1% positive cells when using an isotypic control antibody

Analysis of Cyclin B1 expression and localization

AML blasts were thawed and directly fixed in 2 % (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes, thereafter permeabilized by incubation in 0.2 % Saponin (Sigma-Aldrich Co., St.Lois, MO) in PBS for 8 minutes, followed by blocking overnight at 4°C in 2 % bovine serum albumin (BSA)(Roche, Mannheim, Germany) and antibody staining. Primary antibody (anti-cyclin

B1; Biosciences PharMingen, San Diego) and isotypic control (mouse IgG_1; BD Biosciences PharMingen) was diluted 1:50 in 2 % BSA, 0.2 % Saponin in PBS. Secondary conjugated antibody (Alexa Fluor 488 anti-mouse IgG (H+L); Molecular Probes, Eugene, Oregon) was diluted 1:500 in 2 % BSA, 0.2 % Saponin in PBS. Each step was followed by three rinses in 0.05 % BSA, 0.2 % Saponin in PBS for 10 minutes each. Cells were then either postfixed in 2 % (w/v) paraformaldehyde in PBS, containing 10 µg/ml of the DNA-specific dye Hoechst 33342, before mounting on glass slides with SlowFade Light antifade reagent (Molecular Probes), or diluted in PBS for analysis by Flow cytometry.

Confocal microscopy was performed with a Leica TCS SP2 AOBS (Acousto-Optical Beam Splitter) (Leica Microsystems, Germany) at Molecular Imaging Centre (Bergen, Norway).

Flow cytometry was performed using a FACS Calibur System (Becton-Dickinson, Mountain View, CA). The gates were set according to FSC and SSC which were acquired in linear mode. 10 000 events were collected. The data was analyzed on CellQuest software (Becton-Dickinson) or FlowJo software (Tree Star, Inc., OR, USA). The cutoff for positive cells was defined as a fluorescence corresponding to 1% positive cells when using an isotypic control antibody.

Gel electrophoresis (SDS-PAGE) and western blot were performed using a modification of a previously described method (50). AML cells were lysed in the lysisbuffer. Supernatant aliquots of 50 µg were added conventional sodium dodecyl sulphate (SDS) loading buffer before separation in Novex NuPAGE Bis-Tris/MOPS, 4-12 % pre-cast gels (Invitrogen Corporation, Ca, USA). As a control lane, crude extract from the cell line HL60(51) was included in each gel. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) by electroblotting. Equal protein loading was confirmed by staining the gels with Bio-Safe coomassie (BioRad Laboratories, Oslo, Norway). The membranes were probed with primary mouse anti-human Cyclin B1 (NeoMarkers, Labvision Corp., Fremont, CA, USA) or rabbit anti-human Cyclin B1 (Santa Cruz Biotechnologies, CA, USA) diluted 1:300. Thereafter they were probed with corresponding horse radish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Inc., PA, USA), and finally incubated with substrate SuperSignal West Pico (Pierce Biotechnology Inc., IL, USA), all

by conventional western blot method. The Membranes were captured for imaging of chemiluminescence by Kodak Image Station 2000R, and band intensity was analyzed by Kodak 1D Image Analysis Software (Eastman Kodak Company, CT, USA).

Two-dimensional electrophoresis

Two-dimensional electrophoresis and immunoblotting was performed as previously described(52). Briefly, cells were washed in NaCl (9 mg/ml) and then lysed in 7% trichloroacetic acid (TCA). Protein precipitate was washed in 5% TCA and water saturated ether to remove salts. The protein pellet was suspended in sample buffer for two-dimensional gel electrophoresis (7 M urea, 2 M thiourea, 100 mM dithiothreitol, 1.5% Ampholyte 3 – 10, 0.5% Ampholyte 5 – 6, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate). Two-dimensional gel electrophoresis (2DE) was performed using the ZOOM® IPGRunner™ System from Invitrogen (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturers' instructions. After the second dimension, the proteins were transferred to polyvinylidene fluoride membrane by standard electroblotting. The p53 protein was detected using Bp53-12 monoclonal antibody (Santa Cruz Biotechnology, CA, USA) and secondary horse radish peroxidase conjugated mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) visualized using the Supersignal West Pico Chemoluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA).

Xenograft models

NOD/LtSz-*Prkdc*^{scid}/*B2m*^{null} (53) mice (abbreviated as NOD/SCID/β2m^{null}, originally obtained from Dr. Leonard Schultz, Jackson Laboratories, Bar Harbor, Maine, USA) were expanded and maintained under defined flora conditions in individually ventilated (HEPA filtered air) sterile microisolator cages (Techniplast, Buguggiate, Italy) at the universities animal facility. All experiments were approved by The Norwegian Animal Research Authority and conducted according to The European Convention for the Protection of Vertebrates Used for Scientific Purposes. NOD/SCID/β2m^{null} mice 6-8 weeks old were irradiated from a photon radiation source (BCC Dynaray CH4, 4 megavolt photon irradiation source, with a sub-lethal dose of 3 Gy (100 cGy/min) 4-6 h prior to transplantation. Frozen cells were

thawed quickly at 37^{o}C and diluted with RPMI 1640 medium (Gibco, Grand Island, NY, USA). Cells were then washed twice, counted and the cell viability was determined by the trypan blue exclusion test. The AML cells (approximately 5x10^{6}), were suspended in 300µl of RPMI before injection via the dorsal tail vein. Recipient mice were monitored and sacrificed when they became moribund or after 12 weeks, following institutional guidelines. At sacrifice, blood was collected via cardiac puncture, while the animal was maintained under anesthesia (1.5% Isoflurane). Following death, femurs, tibia and spleens were recovered, cell suspensions prepared in Stemspan and the relative percentages of human cells determined by flow cytometric analysis of human CD45 expression on leukocytes. Mice where engraftment was <1 % was assumed unengrafted.

Analysis of cyclin B1 antibodies

Serum levels of cyclin B1-specific antibodies were analyzed as described in detail previously(2). Briefly, microtiter plates were coated with 200 µl of 0.5 µg/ml purified recombinant cyclin B1 proteins in PBS. Human sera were diluted 1:200 and incubated with antigen-coated wells for 90 minutes at room temperature followed by washing in PBS, and developed with horseradish peroxidase-conjugated goat antihuman IgG (Caltag Laboratories, San Francisco, CA) using the substrate 2,2'-azinobis (Boehringer Mannheim GmbH, Mannheim, Germany). Each sample was tested in duplicate and the average $A_{490\ nm}$ was used for data analysis.

Analysis of the data

For statistical analysis of the data the Wilcoxon signed rank test was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 4.00 (GraphPad Software, San Diego, California USA). Differences were regarded as significant when $P < 0.05$.

RESULTS

Flow-cytometric analysis of cyclin B1 levels in native AML blasts

The expression of cyclin B1 in native human AML cells was examined for 42 patients (Table 1,

patients 1-42; cells from patient 43 were not available for this part of the study). A detailed presentation of the overall results is included in Table 2 (left columns), and the results are summarized in Fig. 1. Cyclin B1 expression was detected for most patients, but the levels showed a wide variation both when comparing the percentage of positive cells and the mean channel fluorescence intensity (Table 2). The median percentage of positive cells was 28.5% (variation range 1-91%); more than 50% cyclin $B1^+$ positive cells were observed for 9 patients and more than 25% for 22 patients. The expression levels showed no correlation with morphology (FAB classification), genetic abnormalities or response to induction chemotherapy.

Nuclear and cytoplasmic distribution of cyclin B1 in native human AML cells

Cyclin B1 expression was investigated by confocal laser microscopy for 42 patients (Table 1, patients 1-42) and detectable levels observed for 41 patients (Table 2). Most patients showed combined nuclear and cytoplasmic expression (31/42), a minority showed only nuclear expression (9/42) whereas cytoplasmic expression alone or undetectable cyclin B1 expression was rare (1 patient each).

The cyclin B1 expression patterns varied. The most common pattern was several individual/single spots either in the nucleus or in the cytoplasm, this is referred to as individual spots. In contrast, for several patients we also detected cells with positive staining of most of the nucleus or cytoplasm; the pattern was consistent with confluence of multiple small spots and is referred to as confluent staining. These two distinct patterns can seen in Fig. 2. An individual spot distribution was most common and observed for all 9 patients with nuclear expression alone and for the majority of patients with combined nuclear and cytoplasmic expression (22/31). A mixture of cells with individual spots and confluent staining was observed for a minority of the patients (8 out of the 31 patients with combined nuclear and cytoplasmic expression), and for all these patients confluent staining was detectable only for a minority of the positive cells. In contrast, confluent expression alone was rare and observed only for 2 patients.

Taken together our results demonstrate that cyclin B1 expression in native human AML cells varies between patients and shows two major patterns: (i) combined nuclear and cytoplasmic expression with

individual spot (eventually in combination with confluent) distribution (approximately 70% of patients); or (ii) nuclear expression alone with individual spots (approximately 20% of patients). The expression patterns showed no correlation with morphology (FAB classification), genetic abnormalities or response to induction chemotherapy.

Molecular analysis of cyclin B1 in native human AML cells

The protein level of cyclin B1 in native AML blasts was also examined for 36 patients by western blotting. These results indicate that (i) detectable cyclin B1 levels were observed for all except 4 patients; (ii) the levels varied between patients (median relative expression 0.13, variation range undetectable-42.9), this is illustrated by the results presented in Fig. 3; and (iii) the 62 kDa band was detected for all cyclin $B1^+$ patients (Fig. 3). As for the flow cytometric analysis the expression levels showed no correlation with morphology (FAB classification), genetic abnormalities or response to induction therapy (data not shown).

Expression of cyclin B1 in AML blasts with dendritic cell characteristics

AML blasts derived from 11 patients were cultured for 14 days in medium with and without GM-CSF+IL4 (days 1-14) + TNFα (days 7-14). Before culture ten of the patients showed either nuclear or nuclear and cytoplasmic cyclin B1 expression in their primary AML cells (Tables 1, 2; patients 1, 3-5, 10, 14-16 and 36), whereas the last patient (Table 1, patient 43) showed high cyclin B1 expression in Western blotting (see Fig. 3). Viable cells were observed after 14 days of culture for all except one patient (Table 1, patient 22), and the results for these 10 patients are presented in Table 3. Firstly, AML cells showed no or only low expression of the dendritic cell-associated CD40, CD80 and CD83 molecules when tested before culture (Table 3) and after culture in medium alone (data not shown). Increased AML cell expression of CD40 (4 out of 10 patients), CD80 (4/10), CD83 (9/10) and also HLA-DR (5/10) was observed for most patients after culture with GM-CSF+IL4+TNFα (Table 3). Secondly, primary AML cells showed relatively high frequencies of cyclin $B1^+$ cells (median 44.5%, range 17-92%) before culture, and cyclin $B1^+$ cells were detected for all patients also after 14 days of culture with IL4+GM-CSF+TNFα. These frequencies of cyclin $B1^+$ cells after culture with the

exogenous cytokines were equal to or higher than for cells cultured in medium alone but lower than for the primary AML cells. Thirdly, cells cultured in the presence of GM-CSF+IL4+TNFα showed higher viability than cells cultured in medium alone, and abnormal cytoplasmic expression of cyclin B1 usually persisted after culture with these exogenous cytokines (patient 5 being an exception).

p53 expression in patients with and without abnormal cytoplasmic cyclin B1 expression

Previous experimental studies suggest that p53 is important for localization of cyclin B1 to the cytoplasm through its regulation of cyclin B1 shuttling between nucleus and cytoplasm, and a recent study described that p53 inactivation was essential for the cytoplasmic localization in malignant cells(32, 33, 38). We therefore compared the p53 molecular profile for AML cells with high and absent cytoplasmic cyclin B1 expression (Fig. 4). These results demonstrated that the expression of various p53 isoforms showed a considerable variation between patients and was independent of the cyclin B1 expression.

Cyclin B1 expression and engraftment of human AML cell in NOD/SCID/β2m^{null} mice.

We selected 6 patients for these studies: 3 patients with high nuclear and cytoplasmatic expression of cyclin B1 and 3 with low expression. All patients were Flt3-ITD^{+} and 5 of the patients showed a normal karyotype (Table 4). While 9 out of 13 human AML samples with varying degree of cyclin B1 expression engrafted in NOD/SCID/β2m^{null} mice, no correlation could be made between survival of recipient mice and cyclin B1 expression. While engraftment and subsequent survival differed between samples it appeared independent of cyclin B1 expression. For example, mice engrafted with material from two separate FAB M4 patients, both exhibiting less than 40 % cyclin B1^{+} cells (Table 1, patients 16 and 31) had drastically different disease latencies (Table 4). Conversely, patient samples demonstrating relatively equivalent levels of Cyclin B1 and different FAB classification (patients 15 and 16) were similar in disease latency while one sample (patient 3) with high expresser of cyclin B1 engrafted in only one mouse.

Cyclin B1 reactive antibodies can be detected for a subset of AML patients

Serum antibody levels were compared for 66 patients with untreated AML and 13 healthy controls (Fig. 5, left part). A detectable level was defined as an absorbance reading exceeding mean +2SD for 13 control samples. The serum level exceeded this limit for 8 patients. Furthermore, 14 of these patients were tested later before and following intensive chemotherapy, three patients then showed detectable levels prior to therapy and these levels decreased following treatment (Fig. 5, right part). Patients with detectable antibody responses were heterogeneous with regard to AML cell morphology (FAB classification), expression of the CD34 stem cell marker, cytogenetic and genetic Flt3 abnormalities; and their AML blasts showed a wide variation in HLA-DR expression (31-99% HLA-DR^+ cells).

Twenty patients were tested for cyclin B1 expression by flow cytometry; cyclin B1 localization by confocal microscopy and antibodies against cyclin B1. Only 2 of these patients had detectable cyclin B1 antibodies in their sera, their median frequencies of cyclin $B1^+$ cells determined by flow cytometry were 46 and 91% respectively and both showed cytoplasmic cyclin B1 staining. Both the cyclin B1 expression and the serum patient groups included unselected patients, and the group of patient investigated for autoantibodies will therefore include a high percentage of patients with cytoplasmatic cyclin B1 expression. It is therefore justified to conclude that only a subset of patients with cytoplasmatic cyclin B1 expression will have detectable cyclin B1 antibodies.

DISCUSSION

T cell mediated antileukemic reactivity is important after allogeneic stem cell transplantation in AML (54). The leukemia-reactive T cells recognize various antigens, including peptides derived from normal proteins with aberrant expression or overexpression by the AML cells (46). In the present study we describe that primary human AML cells usually show an abnormal cytoplasmic cyclin B1 expression that has been associated with induction of cyclin B1-specific immune responses (45, 55). This is true both for T cells primed against the human MS-A2 breast cancer cell line as well as lung adenocarcinoma cells, and for in vivo primed cyclin-specific T cells detected in patients with squamos

cell carcinomas of the head and neck. The humoral immune responses against cyclin B1 in cancer patients are also regarded as T cell dependent .

We included large groups of consecutive AML patients in our study. For the antibody investigations we included unselected patients, whereas for the AML cell studies we selected patients with relatively high peripheral blood blast counts so that enriched AML cell population could be prepared by density gradient separation alone. By using this simple preparation procedure the risk of separation-induced phenotypic alterations could be minimized (49).

Cyclin B1 expression was investigated by three different methodological approaches, and all methods demonstrated that most patients showed detectable expression even though the levels showed a wide variation between patients. Less than 10% cyclin $B1^+$ cells were only detected for a minority of patients (6 out of our 42 patients), and our western blot analysis demonstrated that a 62 kDa isoform was detected for all patients tested as well as for the HL60 AML cell line.

AML cell populations are heterogeneous and have a hierarchical organization with only a small cell subset (usually <0.5%) being capable of clonogenic long-term in vitro proliferation (48, 49). Furthermore, the majority of AML cells will undergo spontaneous apoptosis during in vitro incubation, and the percentage of viable cells will therefore decrease gradually during incubation. After 3 days of culture usually >50% of the cells will be apoptotic/necrotic and for certain patients this number will be >95% (56). We detected cytoplasmic as well as nuclear cyclin $B1^+$ cells for a large fraction of AML cells from most patients, and abnormal cytoplasmic expression persisted after 14 days of in vitro culture (Table 3). Taken together these results indicate that abnormal cytoplasmic cyclin B1 expression is seen both for the more mature majority and for the minority of more immature long-term surviving/proliferating cells within the hierarchically organized AML clones.

A dendritic AML cell phenotype can be induced by in vitro culture of native AML cells in the presence of exogenous cytokine combinations (57, 58). We selected an in vitro model that has been shown in previous studies to induce this phenotype for native human AML cells (59). Our results demonstrated that phenotypic characteristics common with normal dendritic cells could be induced in several patients as would be expected. Furthermore, cytoplasmic cyclin B1 expression was maintained in AML cell populations that showed expression of dendritic cell-associated membrane molecules.

Thus, cytoplasmic cyclin B1 expression is maintained among long-term proliferating AML cells induced to differentiate in dendritic cell direction. Cyclin B1 specific immune responses will therefore be expected to be enhanced when using AML-dendritic cell vaccines.

We investigated xenografting for a selected subset of patients. All except one patient had normal karyotype, and all 6 patients had Flt3-ITD and would therefore be expected to show a similar degree of high engraftment in immunocompromized mice (60). However, leukemia-free survival after xenografting showed no association with cyclin B1 level or expression pattern. A recent study described that leukemia-free survival of xenografted AML cells (i.e. AML-free survival of the xenotransplanted animals) is significantly correlated with the patients' AML-free survival after intensive chemotherapy, and the ability of the AML cells to repopulate immunocompromized mice was an independent prognostic parameter for these patients (61). Our results suggest that cyclin B1 expression is not a part of a biological phenotype with rapid engraftment and early death of xenotransplanted animals due to the development of AML. Our results from the xenograft model thereby indirectly suggest that cyclin B1 expression does not have a major prognostic impact with regard to relapse risk after intensive chemotherapy. This is also supported by a recent clinical study (43) and our own observation of no significant correlation between cyclin B1 expression and response to induction chemotherapy. This is different from other malignant disorders where cyclin B1 expression correlates with staging of head and neck cancer (39, 62, 63) and prognosis in patients with lung, tongue and esophageal cancer (64).

Several previous studies suggest that p53 is important for the regulation of cyclin B1 shuttling between nucleus and cytoplasm and thereby possibly also for the abnormal localization of cyclin B1 in primary AML cells (32, 33, 38). This hypothesis is also supported by a previous study describing that p53 inactivation was essential for the abnormal cytoplasmic cyclin B1 localization in malignant cells (38). Furthermore, the molecular variation of the p53 protein in human AML cells has recently been characterized (52), and we therefore compared the p53 molecular forms for AML cells with high and absent cytoplasmic cyclin B1 expression. Although our results document a considerable p53 variation between patients, there was no association between p53 pattern and cyclin B1 expression. The most

likely explanation for this is that p53 is one out of several regulators that determine the cytoplasmic expression.

Cytoplasmic cyclin B1 expression was common in AML. Several previous studies have demonstrated that such an expression in malignant cells is associated with induction of a cyclin B1-specific immune response. This can be both a cellular and humoral immune response, and previous studies strongly suggest that the humoral immune responses in cancer patients are T cell dependent (2). Our present results demonstrate that a cyclin B1-specific antibody response could be detected for a subset of AML patients (13%); this is lower than for patients with prostate cancer, similar to patients with lung cancer, gastric cancer, and higher than for patients with hepatocellular carcinoma and colorectal cancer. The frequency is also higher than for healthy individuals and patients with systemic lupus erythematosus and systemic sclerosis (<2%).

This is one of the very few studies of autoantibody responses to TAAs in non-solid tumors, since most of the studies reported in the literature include patients with solid tumors. These data show that autoantibody responses in AML patients to cyclin B1 are about 13%, a number which falls in the range of autoantibody responses which have been reported for solid tumors (65, 66). In our studies (66), antibodies to seven selected TAAs varied from 9% to 15.6% for a variety of solid tumors including breast, lung, colorectal, gastric, prostate and liver cancers. In a compilation of antibodies to p53 in patients with various types of cancer, the frequency of antibodies rarely exceeded this range for any specific tumor. At the present time, there are no good insights into this phenomenon, but the study cited above (66), show that every category of solid tumor is associated with antibody responses to multiple TAAs and the further interesting finding was that for some solid tumors, there was a specific profile of autoantibody responses which overlapped with other tumors but were clearly not identical. It would be of interest to determine whether in non-solid tumors such as AML, autoantibodies to multiple TAAs are also detectable and whether a specific profile of antigen-antibody responses might also be present.

Previous studies have demonstrated that HLA-A2 restricted cyclin B1-specific T cells can be detected both for healthy individuals as well as patients with epithelial malignancies (61). Furthermore, HLA-A*0201 is a very common tissue type, and cyclin B1 specific T cells would

therefore be expected to be included in the T cell repertoire for a large proportion of individuals, including healthy stem cell donors. These observations suggest that cyclin B1 specific T cell reactivity may contribute to post transplant antileukemic T cell reactivity in AML, and cytoplasmic cyclin B1 expression in residual AML cells may enhance this reactivity. This cyclin B1 specific reactivity will possibly be AML specific because cytoplasmic cyclin B1 is not detected in normal $CD34^+$ hematopoietic stem cells (49).

To conclude, abnormal cytoplasmic cyclin B1 expression is frequently observed in primary human AML cells. This expression is associated with cyclin B1-specific immune responses in several malignancies, and cyclin B1 specific antibodies could be detected for 10-15% of AML patients. However, our observations suggest that the cytoplasmic expression persist during differentiation, cyclin B1 specific immune reactivity may therefore be enhanced and become important during immunotherapy (e.g. AML dendritic cell vaccines).

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LEGENDS TO FIGURES

Figure 1.

Expression of cyclin B1 by native AML cells examined by flow cytometry. Leukemia cells were derived from 42 consecutive patients and the results are expressed as the percentage of positive cells compared with an isotypic control. The median value (dotted line) is denoted in the figure.

Figure 2.

Cyclin B1 expression patterns in native AML cells analyzed by confocal laser microscopy. The figure shows results for 7 representative patients. Leukemia cells were fixed and stained with a cyclin B1 specific antibody (green). Hoechst staining reveals the nucleus (blue). The semi quantitative estimation of cyclin B1 expression was decided visually by comparison with isotypic controls and is indicated in the lower right corner for each picture: -, ≤ isotypic control; +, <10% cyclin $B1^+$ cells ; ++, 10-50% positive cells; +++, >50% positive cells. This classification was based on the analysis of at least 50-100 cells. The patient numbers (see Tables 1 and 2) are indicated in the upper left corner of each picture. (LEFT) One representative isotypic control is presented (isotype), and the lower picture shows one cell with staining equal to isotypic control (patient 37, overall staining -). (MIDDLE LEFT) The 3 patients 6, 21, 25 all showed stronger nuclear and cytoplasmic staining (+ and ++). (MIDDLE RIGHT AND RIGHT) Patients 11, 14 and 15 were all classified as +++ and a majority of the cells showed nuclear and cytoplasmic staining. The figure presents representative cells for these three patients. Most of the cyclin $B1^+$ cells in this figure showed single spot staining, but for patients 15 and 21 examples of typical confluent staining are shown.

Figure 3.

Analysis of cyclin B1 expression in native AML cells by Western blot. The figure shows the molecular expression of 62 KDa cyclin B1 for 6 AML cell samples and the HL60 AML cell line. The patient numbers refer to Tables 1 and 2. The band intensity of each AML sample was standardized to the cyclin B1 band for the HL60 AML cell line that was included in each gel (right part, equal

intensity defined as 1.0). Equal protein loading was confirmed by staining the gels with Coomassie blue (see lower part of the figure).

Figure 4.

p53 protein pattern for AML cells with and without cytoplasmic cyclin B1 expression. Mapping of the p53 protein was performed by two-dimensional gel electrophoresis and immunoblot (2DI) of AML blasts, identifying the putative p53 cleavage product or short form (Δp53, lower dotted circle) and spots corresponding to different isoforms of full length p53 (αp53, upper dotted circle). The molecular weight (kDa) and isoelectric point (pI) are denoted in the figure. The left panel show p53 pattern for AML cells with cytoplasmic cyclin B1 expression (Tables 1 and 2, patients 3, 14-16, 19, 38), while right panel show p53 pattern for AML cells without cytoplasmic cyclin B1 expression (patients 4-6, 18, 31, 35).

Figure 5.

Serum levels of cyclin B1 specific antibodies in AML patients and healthy controls. (LEFT) Serum antibody levels were examined for 9 healthy controls and for 68 AML patients at the time of diagnosis. We calculated the mean optical density of the controls, and the mean + 2 standard deviations is indicated in the figure. The observations represent the mean of duplicate determinations. (RIGHT) Antibody levels were determined before and following intensive chemotherapy for 12 patients, and for 3 patients detectable cyclin B1 antibody levels (filled squares) were observed prior to therapy. The results for these three patients are presented in the figure (dotted line indicate mean + 2SD for the healthy controls).

Table 1. Clinical and biological characteristics of acute myeloid leukemia patients[1]

Patient	Sex	Age (years)	Previous chemotherapy or preleukemic disease	FAB classification	Membrane molecule expression[2]					Cytogenetic abnormalities	Flt3 abnormality[3]	Response to first induction Chemotherapy[4]
					CD13	CD14	CD15	CD33	CD34			
1	F	75		AML-M2	-	-	+	+	+	nt	nt	nt
2	M	74		AML-M0	-	-	+	+	+	90-94,XXYY	wt	nt
3	M	72		AML-M5	+	+	+	+	-	Normal	wt	nt
4	F	58		AML-M2	+	-	+	+	-	Normal	ITD,wt-	-
5	M	65		AML-M2	+	-	-	-	+	Multiple	wt	+
6	F	64	MDS	AML-M1	+	-	-	+	+	del(11)(q14), del(20)(q11),+21	wt	-
7	F	70	Relapse	AML-M1	+	-	+	-	+	nt	ITD	nt
8	M	69	MDS	AML-M1	+	nt	+	+	+	nt	-	-
9	M	79		AML-M1	+	-	-	-	+	Normal	ITD	nt
10	M	74	MDS	AML-M4	+	-	+	+	-	Multiple	-	nt
11	F	60		AML-M1	+	-	-	+	+	Normal	wt	+
12	F	63		AML-M4	+	-	-	+	+	Normal	ITD	-
13	M	74	MDS	AML-M5	+	-	-	+	+	nt	ITD	nt
14	M			AML-M4	+	-	+	+	+	Inv(16)	-	+
15	F	51		AML-M2	+	-	+	+	+	Normal	ITD,wt-	+
16	M	29		AML-M4	+	-	+	+	+	Normal	ITD, D835	-
17	F	44		AML-M1	+	-	-	+	+	del 7 (q22)	ITD	-
18	F	59		AML-M2	+	-	-	+	+	-7	-	-
19	F	56		AML-M1	+	-	-	+	+	Normal	wt	+
20	M	43		AML-M5	+	-	+	+	+	inv(16)(p13q22),	D835	+
21	F	79		AML-M5	+	-	+	+	nt	del(12)(p11) add(11)(p15)	D835	Nt
22	F	40		AML-M5	-	-	+	+	-	t(9,11) (p22;q23) +8	-	+
23	M	40	MDS	AML-M6	+	-	+	+	+	Normal	ITD	-
24	F	45	Chemotherapy	AML-M4	+	-	+	+	-	Normal	-	+
25	F	81		AML-M2	+	-	-	+	-	nt	-	Nt
26	M	79		AML-M4	-	-	+	+	-	nt	ITD	nt
27	M	82		AML-M1	-	-	nt	+	+	nt	-	nt
28	M	56		AML-M2	+	-	+	+	+	Normal	ITD	nt
29	F	45		AML-M2	+	-	-	+	-	Normal	ITD, D835	+
30	F	75		AML-M5	+	-	+	+	+	Normal	ITD	nt

31	M	61		AML-M4	-	-	+	+	-	Normal	ITD	+
32	F	74		AML-M2	nt	nt	nt	nt	nt	nt	-	nt
33	F	80		AML-M2	+	-	-	+	+	Multiple	-	nt
34	M	30		AML-M3	+	-	-	+	-	t(15;17)	ITD	nt
35	M	33		AML-M1	+	-	-	+	+	+4	ITD	-
36	F	61		AML-M4	+	+	+	+	-	Normal	-	nt
37	F	78		AML-M4	+	+	+	+	-	-4,-5,+der(8)t(8;?)(q21;?), inv(16),+20,+21	-	nt
38	M	43		AML-M2	+	-	+	+	-	Normal	-	nt
39	M	82		AML-M5	+	+	+	+	-	45X	-	nt
40	M	73		AML-M1	+	-	+	+	-	+8	-	nt
41	F	46		AML-M4	+	+	+	-	-	Normal	D835	+
42	M	60		AML-M1	+	-	+	+	+	Normal	ITD	-
43	M	36	CML	AML-M2	+	nt	nt	+	+	t(9;12)	-	nt

1. Abbreviations; female (F), male (M), acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), chronic myelogenous leukemia (CML).
2. Patients were regarded as positive when more than 20% of the blasts cells stained positive judged by flow cytometric analysis.
3. Flt3 abnormalities were internal tandem duplications (ITD), Asp(D) 835 mutations (D835), and loss of wild type (wt-) (nt, not tested).
4. Response (+) was defined as complete remission after one induction cycle with cytarabine 200 mg/m^2 days 1-7 together with an anthracycline (daunorubicine 45 mg/m^2 or idarubicin 12 mg/m^2 days 1-3). Patients who did not receive chemotherapy or had AML relapse (39 patients) were classified as not treated/tested (nt).

Table 2. Cyclin B1 expression by native human AML blasts: ranking of patients is based on percentage of cyclin $B1^+$ cells in the flow cytometric analysis.

	Flow cytometry analysis of cyclin B1		Confocal microscopy of cyclin B1 localization and morphology		
Patient	**Frequency of positive cells**	**MCFI**	**Nuclear expression**	**Cytoplasmic expression**	**Distribution**
1	91	80	+++	+++	Individual
2	91	96	-	++	Confluent
3	67	48	+++	+++	Individual /confluent
4	59	35	+	-	Individual
5	57	46	+	-	Individual
6	55	35	+	-	Individual
7	53	35	+++	+++	Individual
8	53	35	+	-	Individual
9	51	39	++	++	Individual /confluent
10	48	39	+++	+++	Individual /confluent
11	46	35	+++	+++	Individual
12	43	43	++	++	Individual
13	42	29	+	+	Confluent
14	41	42	+++	+++	Individual
15	36	47	+++	+++	Individual /confluent
16	35	38	+++	+++	Individual
17	33	34	+	-	Individual
18	31	31	+	-	Individual
19	30	37	+++	+++	Individual /confluent
20	29	31	+++	+++	Individual
21	29	24	++	++	Individual /confluent
22	28	44	+++	+++	Individual
23	24	28	++	++	Individual
24	23	18	++	++	Confluent
25	22	26	++	++	Individual
26	22	21	++	++	Individual
27	21	19	++	++	Individual
28	19	17	++	++	Individual
29	17	18	+	+	Individual
30	16	25	++	++	Individual /confluent
31	16	15	+	-	Individual
32	15	7	++	-	Individual
33	14	15	+	+	Individual
34	12	5	+	+	Individual
35	11	15	+	-	Individual
36	10	18	-	++	Confluent
37	9	8	-	-	-
38	7	20	+++	+++	Individual
39	6	12	+	+	Individual /confluent
40	4	19	++	++	Individual
41	4	15	+	+	Individual
42	1	1	++	++	Individual

Flow cytometric results are presented as the frequency of positive cells and the mean channel fluorescence intensity (MCFI) within the gated blast population. Confocal microscopy results are ranked as undetectable (-), <10% positive cells (+), intermediate 10-50% positivity (++) and >50% positive cells (+++) when examining at least 50-100 cells.

Table 3. Cyclin B1 expression by AML blasts cultured for 14 days either in medium alone without exogenous cytokines or in medium containing IL4 + GM-CSF + TNFα.[1]

	Expression of DC-associated membrane molecules[2] (GM-CSF + IL4 + TNFα - expanded cells/native AML cells)				Cyclin B1 localization (confocal laser microscopy)[3]				Cyclin B1 expression (flow cytometric analysis)[4]			
					Without exogenous cytokines		GM-CSF + IL4 + TNFα		Without exogenous cytokines		GM-CSF + IL4 + TNFα	
Patient	CD40	CD80	CD83	HLA-DR	Nucl	Cyto	Nucl	Cyto	Percent positive cells (percent viable cells)		Percent positive cells (percent viable cells)	
1	0/0	↑ 2/0	↑ 8/1	↑ 36/0	+	-	+	-	1	(2)	8	(21.1)
3	0/0	0/0	↑ 10/6	↑ 68/22	ne	ne	+	+	0	(5)	19	(3)
4	↑ 7/0	0/0	↑ 16/0	↓ 23/59	-	-	-	+	2	(1.2)	10	(9.5)
5	0/0	0/0	0/0	↑ 2/7	-	-	-	-	0	(67)	0	(67)
10	↑ 8/0	0/0	↑ 3/0	↑ 73/14	ne	ne	+	+	ne	(0)	19	(4.6)
14	↑ 54/0	↑ 22/0	↑ 29/2	↑ 60/31	+	-	-	-	10	(24.5)	8	(21.2)
15	0/0	↑ 4/0	↑ 11/5	↑ 44/20	+	-	+	+	4	(4.5)	2	(19.2)
16	0/0	0/0	↑ 43/1	↓ 38/53	+	-	+	+	2	(5.6)	8	(10)
31	↑ 33/2	↑ 28/0	↑ 21/0	↑ 60/31	-	-	+	+	0	(1.1)	11	(27)
43	↓ 0/33	0/0	↑ 40/0	↓ 47/76	+	+	+	+	0	(5.5)	3	(31.5)

1. Eleven patients were examined; for one exceptional patient (Table 1, patient 22) no viable cells were detected after in vitro culture and this patient is left out from the table. Abbreviations; not tested (nt), nuclear expression (Nucl), cytoplasmic expression (Cyto), dendritic cells (DC), not evaluable because of no viable cells (ne).
2. The results are presented as the percentage of positive cells by flow cytometric analysis of cultured/native AML cells. Arrows illustrate increased (↑) or decreased (↓) membrane molecule expression. Cells cultured in medium alone showed low expression of all molecules that did not differ from the primary/native cells (data not shown).
3. Confocal microscopy analysis: Expression was ranked as undetectable (-) or detectable (+) for cells cultured with and without exogenous IL4+GM-CSF+TNFα.
4. Flow cytometric analysis: percentage of positive cells after culture with and without exogenous IL4+GM-CSF+TNFα. The percentages of viable cells after in vitro culture are presented in brackets.

Table 4. Engraftment of AML cell populations with different cyclin B1 expression in NOD/SCID/β2m^{null} mice.

	Cyclin B1 expression and distribution							Survival (Days)	
Patient	**Nuclear expression**	**Cytoplasmic expression**	**Distribution**	**Frequency of Cyclin B1^{+} cells**	**FAB classification**	**Cytogenetic examination**	**Flt3 abnormality**	**Mouse id.**	**Mouse Survival**
3	+++	+++	spotted / even	67	M5	Normal	ITD	2.1	36
								2.2	>84
								2.3	>84
15	+++	+++	spotted / even	36	M2	Normal	ITD	3.1	37
								3.2	43
16	+++	+++	spotted	35	M4	Normal	ITD	7.1	34
								7.2	36
13	+	+	spotted	42	M5	Normal	ITD	27.1	46
								27.2	>84
17	+	-	spotted	33	M1	del7	ITD	37.1	>84
								37.2	39
31	+	-	spotted	16	M4	Normal	ITD	39.1	13
								39.2	16

The mice were sacrificed when they became moribund. Mice that did not show signs of illness were sacrificed after 12 weeks, these mice are classified as no engraftment (engraftment > 84 days).

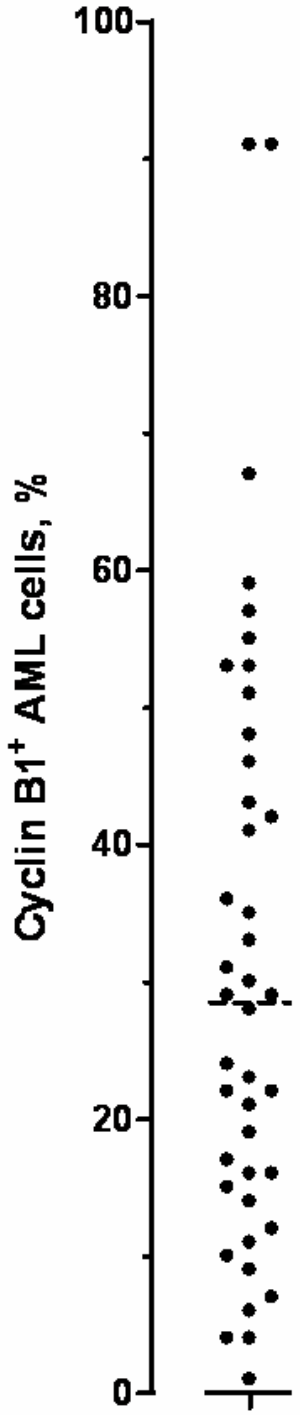


Figure 1

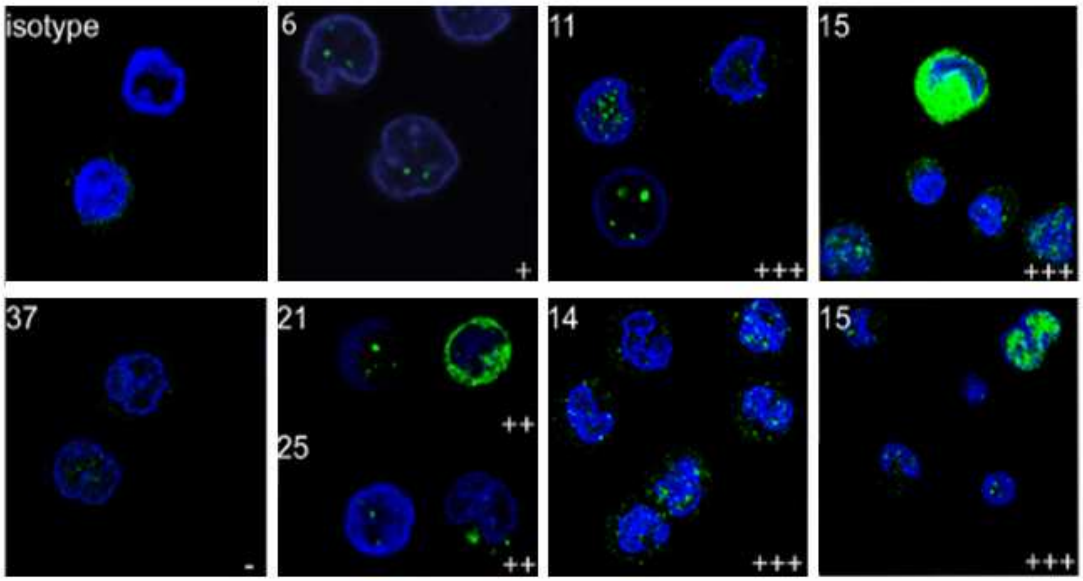


Figure 2

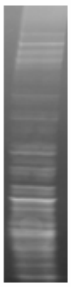
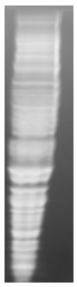
Patients	1	15	24	25	34	43	HL60
Expression	1.1	0.2	0.5	0.6	0.6	1.6	1.0
Cyclin B1							
Coomassie gel							

Figure 3

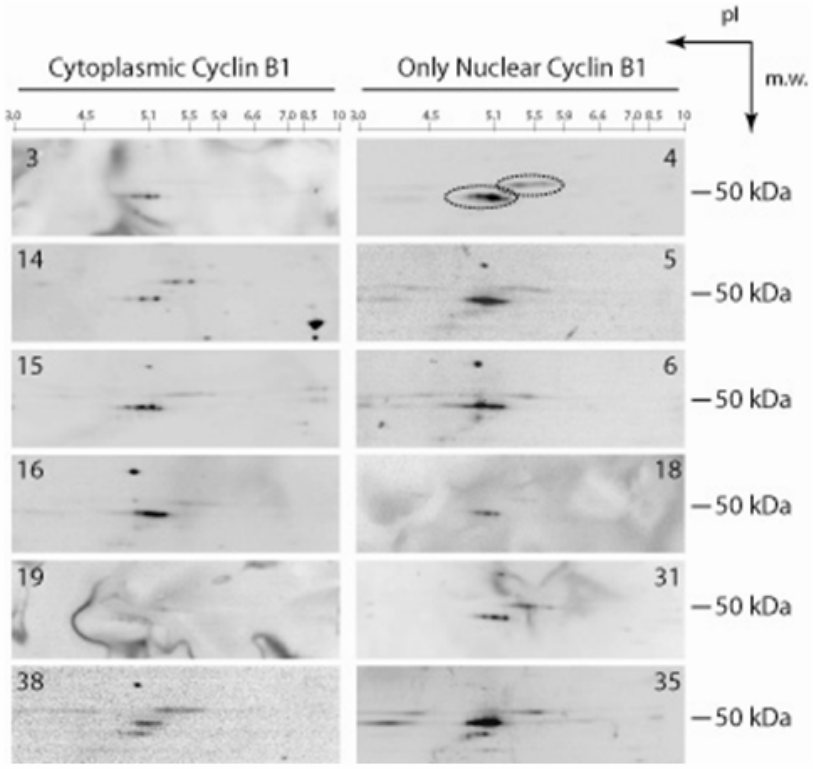

pI
m.w.
Cytoplasmic Cyclin B1
Only Nuclear Cyclin B1
3.0 4.5 5.1 5.5 5.9 6.6 7.0 8.5 10
3.0 4.5 5.1 5.5 5.9 6.6 7.0 8.5 10
3
4
50 kDa
14
5
50 kDa
15
6
50 kDa
16
18
50 kDa
19
31
50 kDa
38
35
50 kDa

Figure 4

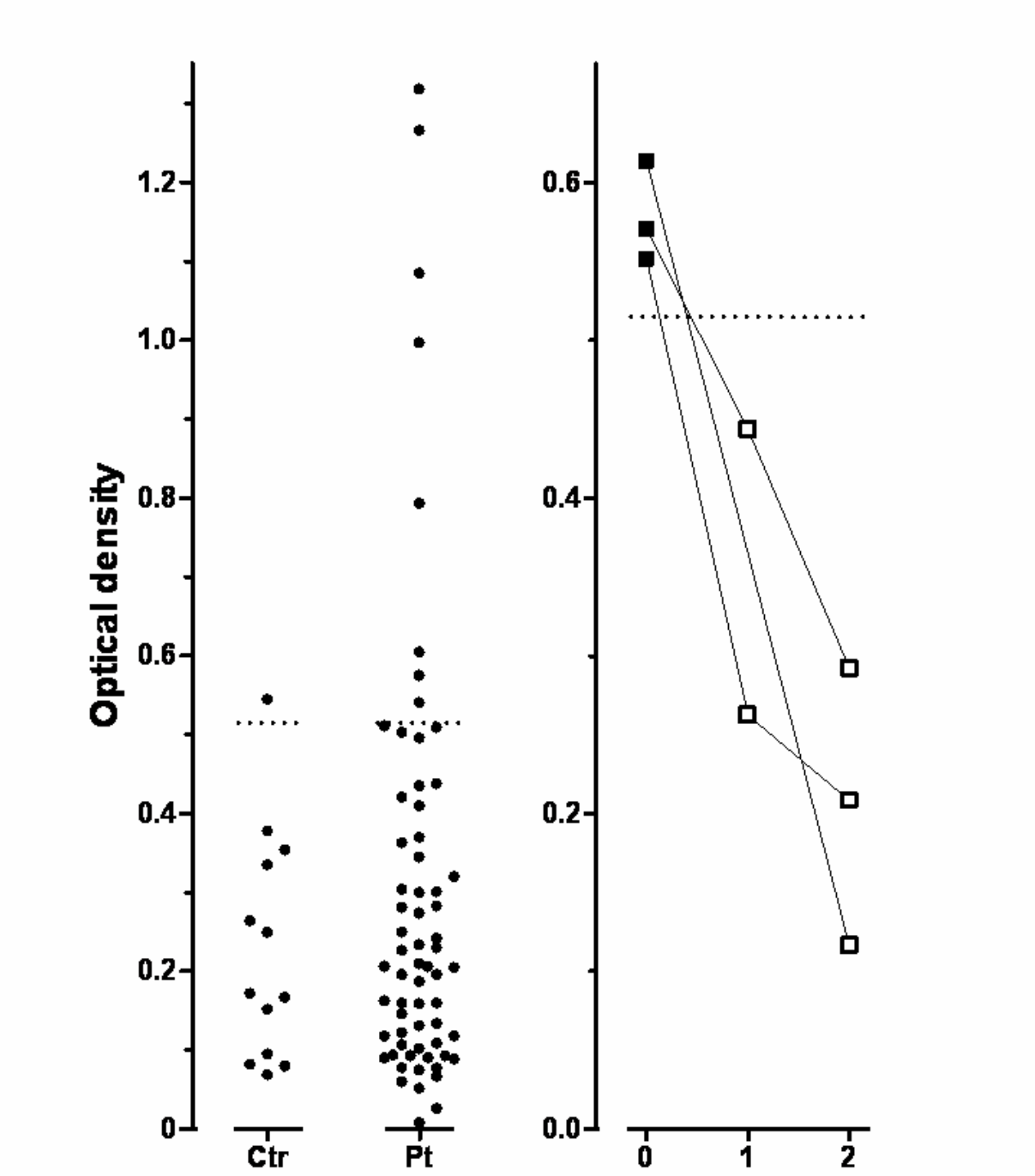


Figure 5