Diagnostic Usefulness and Prognostic Impact of CD200 Expression in Lymphoid Malignancies and Plasma Cell Myeloma

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Abstract

The membrane glycoprotein MRC OX-2 (CD200) is expressed in several lymphoid malignancies. However, the diagnostic usefulness and potential prognostic importance of CD200 expression have not been rigorously examined. We show that CD200 is uniformly expressed in chronic lymphocytic leukemia (CLL) and absent in mantle cell lymphoma (MCL). It is important to note that expression of CD200 is retained even in CLLs with immunophenotypic aberrancies, making CD200 a particularly useful marker for discrimination between these cases and MCL. CD200 is expressed in nearly all precursor B-lymphoblastic leukemias, with aberrant overexpression or underexpression compared with normal B-cell progenitors in 55% of cases. More than 70% of plasma cell myelomas (PCMs) expressed CD200, and loss of CD200 expression in PCM may be associated with more clinically aggressive disease. CD200 is expressed in several hematolymphoid neoplasms. Analysis of its expression has several diagnostic and potentially prognostic applications in the flow cytometric evaluation of lymphoid malignancies.

Membrane glycoprotein MRC OX-2 (also known as MOX2 and CD200; hereafter referred to as CD200) is a member of the immunoglobulin superfamily and is encoded by a gene residing at chromosome 3q12.[1] CD200 is normally expressed by endothelial cells and neurons and by B cells and a subset of T cells in the hematolymphoid system.[2] CD200 interacts with the CD200 receptor (CD200R), which has limited expression on granulocytes (neutrophils and basophils), monocytes, and cells of the reticuloendothelial system, including dendritic cells and macrophages.[3,4] Under most circumstances, CD200-mediated ligation of CD200R delivers an inhibitory signal to the target cell.[4-6] Consistent with this, macrophages from CD200-deficient mice display an activated phenotype, and the mice manifest enhanced susceptibility to experimentally induced autoimmune disease.[7]

Expression of CD200 in hematologic malignancies was first reported for chronic lymphocytic leukemia (CLL).[8] CD200 subsequently has been shown to be differentially expressed in CLL and mantle cell lymphoma (MCL); thus, evaluation of CD200 expression may have diagnostic usefulness for these CD5+ B-cell lymphomas.[9] In addition, CD200 expression, as assessed primarily by gene expression profiling, has been associated with a superior clinical outcome in acute myeloid leukemia and plasma cell myeloma (PCM).[10,11]

In this study, we report the analysis of CD200 expression by flow cytometry in several types of lymphoid malignancy. We confirm the differential expression of CD200 in CLL and MCL and, importantly, show that such differential expression is maintained in CD5+ lymphomas with an atypical or indeterminate immunophenotype. We also show that although its expression is nearly uniform in precursor B-lymphoblastic leukemia (B-ALL), there is aberrant underexpression or

overexpression of CD200 in most cases of B-ALL compared with normal bone marrow B-cell progenitors. Finally, we demonstrate that although absent in normal bone marrow PCs, CD200 is expressed in 71% (37/52) of PCMs, and negativity for this marker may be associated clinically with progression to more aggressive disease.

Materials and Methods

Routine clinical bone marrow, peripheral blood, body fluid, and lymph node samples arriving in the clinical flow cytometry laboratory at the University of Arkansas for Medical Sciences, Little Rock, were included in this study. Samples were screened morphologically and immunophenotypically to confirm the presence of neoplastic cells. Samples were then further analyzed for CD200 expression by flow cytometry as described in the following section. Lymphoid malignancies were diagnosed according to the criteria delineated in the 2008 World Health Organization classification.[12] This study was approved by the institutional review board at the University of Arkansas for Medical Sciences.

Flow Cytometry

EDTA- or heparin-anticoagulated peripheral blood or bone marrow aspirate specimens were washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 2% fetal calf serum. Cell suspensions were then incubated for 15 minutes with various cocktails of fluorochrome-conjugated antibodies. These antibodies included CD200 phycoerythrin, CD19, CD20, CD10, CD5, CD34, CD138, and CD38. All antibodies were obtained from BD Biosciences, San Jose, CA. Isotype controls were included in all analyses. RBCs were then lysed with FACS Lyse (BD Biosciences) or ammonium chloride solution, rinsed with PBS, and resuspended in PBS containing 1% formaldehyde. Analysis was performed on a FACSCanto II flow cytometer using FACSDiva software (BD Biosciences). Gating on lymphoid cells and lymphoblasts was based on CD45 vs side scatter analysis. PCs were identified based on CD138 vs side scatter analysis. CD200 expression was evaluated semiquantitatively by comparison with the isotype phycoerythrin control antibody and designated as negative or 1+ (< 1 log shift in mean fluorescence intensity [MFI] compared with isotype control), 2+ (1-2 log shift in MFI), or 3+ (>2 log shift in MFI).

Gene Expression Profiling

In PCMs in which gene expression profiling (GEP) was performed, RNA was extracted from CD138-purified PCs and analyzed using an Affymetrix-based platform with hybridization to U133 Plus2.0 microarrays (Affymetrix, Santa Clara, CA). Molecular classification and derivation of a 70-gene risk score was performed as previously described.[13,14] Molecular classification of a given case was achieved through the use of a class predictor. This predictor is composed of significantly overexpressed and underexpressed genes for each molecular subgroup and was generated using prediction analysis for microarrays (PAM) software (PAM, Stanford, CA), as described.[14] GEP-based assessment of *MOX2* expression, the gene encoding CD200, was achieved by analysis of the 2 probe sets for *MOX2* (209582_s_at; 209583_s_at), which are present on the U133 Plus2.0 microarray.

Statistical Analysis

For the analysis of PCM cases, a linear model was fit to assess differences in the mean GEP 70-gene score among the 3 CD200 groups (CD200–, CD200 1+, and CD200 2+). To compare the distribution of each of the myeloma molecular subtypes (CD-1, CD-2, HY, LB, MF, MS, and PR), a χ^2 test was performed for each molecular subtype to determine whether there was a significant difference in the distribution of the subtype within each of the CD200 expression groups.

Results

Analysis of normal peripheral blood and lymph node samples demonstrated somewhat variable CD200 expression in B and T cells. In blood, CD3+ T cells were generally CD200–, whereas CD19+ B cells were usually CD200+, with uniform positivity in some cases and more variable expression in others. In benign or reactive lymph nodes, similar patterns of CD200 expression were observed with relatively uniform expression in B cells and more heterogeneous expression in T cells **Image 1**.

CD200 Expression in Precursor Lymphoid Malignancy and Normal B-Cell Progenitors

Expression of CD200 was evaluated in several hematolymphoid malignancies by flow cytometry, the results of which are summarized in **Table 1**. While T-ALLs were uniformly negative for CD200, 19 (95%) of 20 B-ALLs analyzed were variably positive for CD200. The relative level of CD200 expression was not tightly associated with any particular cytogenetic abnormality (data not shown). CD34+ B-ALLs expressed a significantly higher level of CD200 than did CD34– cases (average MFI, 1,950 vs 686). Although brightly expressed in many B-ALLs, the level of expression of CD200 was variable, with some being only dimly CD200+ **Image 2**. These observations led us to assess the expression of CD200 in normal bone marrow B-cell progenitors or hematogones.

Stages of hematogone development are defined by the differential expression of CD10 and CD20: stage 1, CD10 bright/CD20–; stage 2, CD10+/CD20 variable; and stage 3, CD10+/CD20 bright.[15,16] As shown in **Table 2**, CD200

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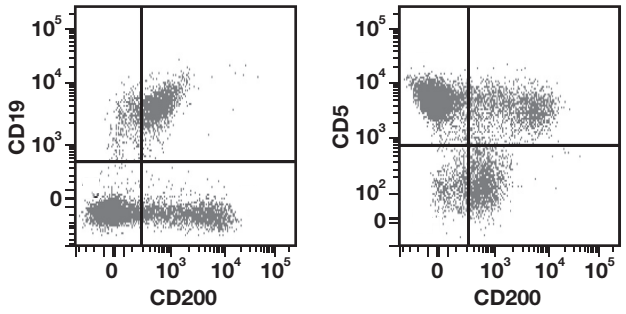

Image 1 Expression of CD200 in normal lymph node B cells and T cells. In most reactive lymph nodes, B cells manifest uniform, dim-moderate expression of CD200 (left). By contrast, T cells typically showed more variable CD200 expression, often with CD200 positivity in only a subset of cells (right).

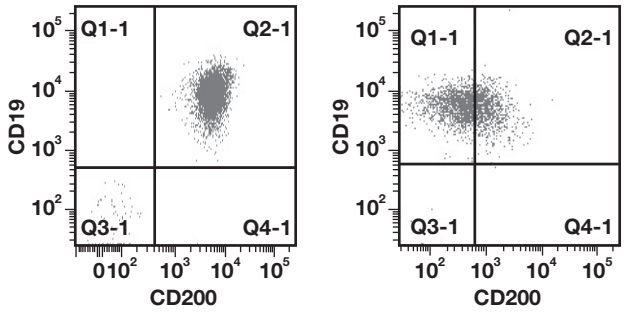

Image 2 Expression of CD200 in precursor B-lymphoblastic leukemia (B-ALL). Although positive in nearly all evaluated cases, B-ALLs nevertheless manifest variable expression of CD200. Left, A hyperdiploid B-ALL with very bright CD200 expression. Right, A B-ALL with t(4;11) with dim CD200 expression.

Table 1
Expression of CD200 by Lymphoid and Hematopoietic Neoplasms

Type	No. of Cases	No. (%) With CD200 Expression
Precursor lymphoblastic leukemia		
B lineage	20	19 (95)
T lineage	5	0 (0)
Chronic lymphocytic leukemia/small lymphocytic lymphoma	19	19 (100)
Mantle cell lymphoma	4	0 (0)
Lymphoplasmacytic lymphoma	7	3 (43)
Plasma cell myeloma	52	37 (71)

was expressed at a relatively constant level throughout all stages of hematogone development. It is interesting that the highest level of CD200 is expressed in CD34+/CD19+ B-cell progenitors, which normally comprise a minor subset of CD10 bright/CD20– cells and represent the most immature B-cell progenitors. Thus, compared with normal CD34+/CD19+ B-cell progenitors, CD200 is significantly overexpressed or underexpressed (MFI at least 2-fold greater or less than that of B-cell progenitors) in 55% (11/20) of CD34+ B-ALLs.

Differential CD200 Expression in CLL and MCL

Confirming previous reports,[9] CD200 expression was observed in 19 (100%) of 19 CLLs and was uniformly absent in the 4 MCLs (Table 1). CD200 expression was bright in all CLL cases analyzed, with at least a 1 log shift in the MFI compared with that of the isotype control antibody **Image 3A**. Although most CLLs and MCLs manifest immunophenotypes that permit their reliable distinction by flow cytometry, an aberrant immunophenotype is observed in a minority of cases, precluding a definitive diagnosis on the basis of flow cytometry alone.[17-20] Therefore, CD200 expression in such cases was evaluated to determine whether it might enhance diagnostic accuracy by flow cytometry. Two cases of CLL with aberrantly bright CD20 or surface immunoglobulin light chain expression were strongly CD200+ **Image 3B**. In addition, 1 case of MCL that aberrantly coexpressed CD23 was CD200–. Thus differential CD200 expression in CLL and MCL seems to be retained, even in cases with an otherwise indeterminate immunophenotype.

Table 2
Expression of CD200 in Bone Marrow B-Cell Progenitors

Stage	Immunophenotype*	CD200 Expression (MFI)†
—	CD19+/CD34+	1,908
1	CD10 bright/CD20–	1,068
2	CD10+/CD20 variable	851
3	CD10+/CD20 bright	1,210
Mature	CD10–/CD20 bright	1,323

* B-cell progenitors were gated on the basis of their CD45 expression and side scatter properties. The expression of CD19, CD34, CD20, and CD10 was analyzed on these gated events.
† The mean fluorescence intensity (MFI) was calculated relative to that of the isotype control for each progenitor subset.

Expression and Prognostic Impact of CD200 in PCM

To evaluate CD200 expression in normal PCs, bone marrow samples from 4 patients without a history of a PC dyscrasia were analyzed. Normal PCs were uniformly negative for CD200 **Image 4**. In contrast, CD200 was

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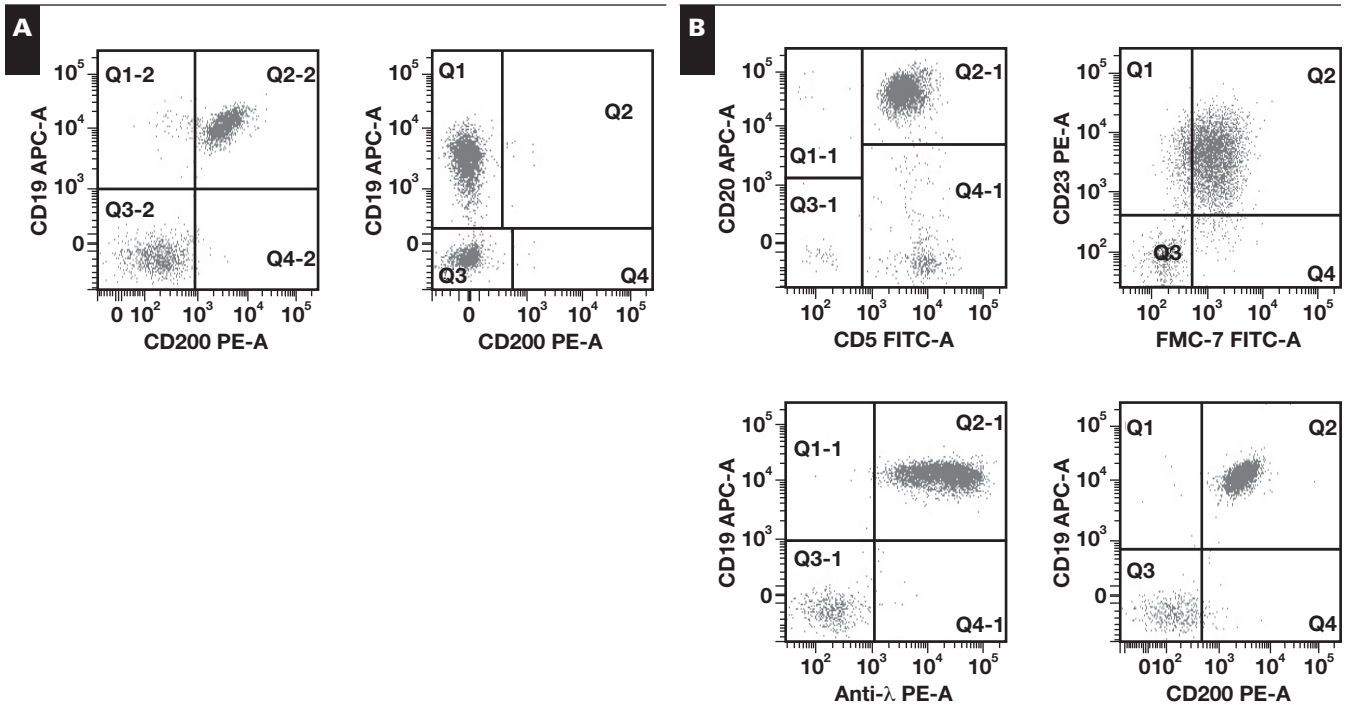

Image 3 CD200 expression in immunophenotypically atypical chronic lymphocytic leukemia (CLL). **A**, CLL with bright CD200 positivity, typical of all cases (left) and mantle cell lymphoma with no detectable CD200 expression (right); this case contained the t(11;14) by fluorescence in situ hybridization (FISH) and was cyclin D1+ by immunohistochemical studies (not shown). **B**, CD5+ B-cell lymphoma with an indeterminate immunophenotype. The patient had an absolute lymphocytosis (lymphocyte count, 10,000/μL [10×10^9/L]). Immunophenotypic analysis identified a CD5+, λ light chain–restricted neoplastic B-cell population that expressed bright CD20 (upper left), CD23 and FMC-7 (upper right), with bright surface immunoglobulin expression (lower left). These cells were also strongly CD200+ (lower right). By immunohistochemical studies, the neoplastic B cells were cyclin D1– (not shown). Although cytogenetics revealed a normal 46,XX karyotype, FISH identified +12 and del(13q14.3) and was negative for the t(11;14), cytogenetic findings typical for CLL. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

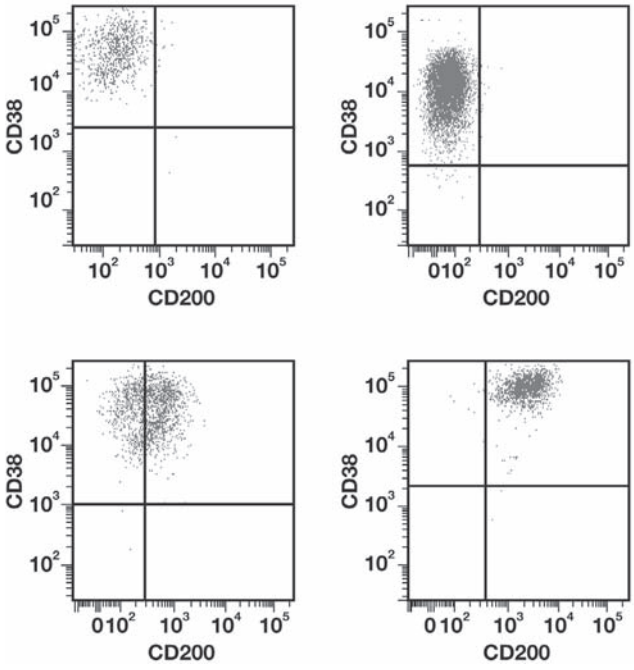

Image 4 Expression of CD200 in normal bone marrow plasma cells (PCs) and plasma cell myeloma (PCM). Benign and neoplastic PCs were identified by CD138 expression and side scatter properties. Benign PCs from 5 cases of reactive plasmacytosis were analyzed and found to be uniformly CD200– (upper left). Although most cases of PCM were CD200+, expression was variable. A CD200– case (upper right) showed extensive bone marrow involvement and had a complex karyotype by conventional cytogenetics (not shown); this case was classified as the PR subtype by gene expression profiling (GEP). A PCM with 1+ positivity for CD200 (lower left). A CD200 2+ positive PCM (lower right) that was classified as the MS subtype by GEP with a low 70-gene risk score. Fluorescence in situ hybridization confirmed the presence of the t(4;14)(p16.3;q32). Quadrants are based on negative staining obtained with isotype control antibodies.

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expressed by 37 (71%) of 52 analyzed cases of PCMs (Table 1), similar to previous reports.[10] CD200+ cases manifested variable levels of expression, with approximately half showing weak expression and the remaining cases being moderately to strongly positive (Image 3).

At our institution, GEP is routinely performed in new patients with PCM, from which a molecular subtype is assigned and a 70-gene risk score is derived, the latter being highly predictive of clinical behavior.[13,14] GEP-defined molecular subtypes include CD-1 and CD-2, in which there is dysregulated *CCND1* expression due to the t(11;14); hyperdiploid (HY), characterized by gains primarily of odd-numbered chromosomes; MF in which there is dysregulated expression of *MAF* or *MAFB*; low bone (LB), associated with low expression of *DKK1* and characterized clinically by fewer MRI-defined focal lesions; MS, characterized by dysregulated *FGFR3* and *MMSET* expression due to the t(4;14); and proliferative (PR), characterized by overexpression of several genes associated with cell cycle regulation and proliferation.

GEP analysis was also performed in 49 of the cases that had been evaluated by flow cytometry. As shown in **Table 3**, there is good correlation between the expression of *MOX2* messenger RNA, as assessed by GEP analysis, and that of CD200 protein detected by flow cytometry. Furthermore, the relative level of CD200 expression is associated with molecular subtype. For example, 90% (18/20) of LB and HY PCMs were CD200+, with uniformly high CD200 expression among LB PCMs. Somewhat surprisingly given previously published data,[10] 7 (78%) of 9 cases of PR PCM lacked CD200 expression and, as such, comprised 50% of CD200– cases. The difference in the frequency of LB PCM and PR PCM within the CD200 2+ and CD200– groups, respectively, was highly statistically significant ($P < .009$ and $P < .001$, respectively). By contrast, the frequency of other myeloma subtypes (eg, CD-2 and MS) was not statistically different among the 3 CD200 subgroups, indicating more variable CD200 expression.

Most PR PCMs harbor genetic lesions conferring aggressive clinical behavior and are associated overall with a significantly higher 70-gene risk score than other molecular subtypes.[13,21] Not surprisingly then, CD200– PCMs had a significantly higher gene risk score than PCMs with 2+ CD200 positivity ($P < .0006$) **Figure 1**. There are no reported data on the stability of CD200 expression in PCM, and we were not able to serially evaluate CD200 expression by flow cytometry. However, 27 of the patients in our series underwent 2 or more GEP analyses. With a 2-fold change in the expression for both *MOX2* probe sets as the threshold, 22 patients (81%) showed no significant variation in *MOX2* expression (data not shown). Of the 5 cases in which there was significant modulation of *MOX2* expression, 1 showed up-regulated and 4 showed down-regulated expression of *MOX2*.

Table 3
Variable Expression of CD200 in Molecular Subtypes of Plasma Cell Myeloma

	Subgroup*		
	CD200– (n = 14)	**CD200 1+ (n = 17)**	**CD200 2+ (n = 18)**
MOX2 expression†			
Set 1	911	1,427	3,768
Set 2	2,139	4,148	8,544
No. (%) of molecular subtype‡			
PR	7 (50)	2 (12)	0 (0)
CD-1	0 (0)	1 (6)	1 (6)
CD-2	4 (29)	4 (24)	2 (11)
HY	2 (14)	6 (35)	9 (50)
MS	1 (7)	2 (12)	1 (6)
MF	0 (0)	2 (12)	0 (0)
LB	0 (0)	0 (0)	5 (28)
70-Gene risk score§	0.6112	0.3558	–0.2424

* Cases were assigned to the CD200–, CD200 1+ or CD200 2+ subgroups based on independent assessment of CD200 expression by flow cytometry.
† *MOX2* is the gene encoding CD200. Its relative expression as determined by gene expression profiling (GEP) is indicated; values represent arbitrary units. Set 1 and set 2 refer to the 2 *MOX2* probe sets present on the U133 Plus2.0 microarray.
‡ The molecular subtype for each case was assigned based on GEP analysis, as described.
§ The 70-gene risk score was derived from GEP analysis, as described. A risk score greater than 0.66 is considered high-risk disease.

Discussion

CD200 is a cell surface glycoprotein expressed in normal B cells and some T-cell subsets. The expression of CD200 has been analyzed in various lymphoid malignancies. In this report, we confirm several of these previously reported findings. It is important to note that through the use of a flow cytometry–based approach, we have made several novel observations, some of which may have diagnostic and prognostic importance.

We have confirmed previous reports that CD200 is uniformly expressed in CLL, whereas its expression is not detected in MCL. While both are defined by CD5 expression, several immunophenotypic differences exist between CLL and MCL that readily allow for their distinction by flow cytometry in typical cases.[22] These include characteristic CD23 coexpression and the dim expression of CD20 and surface immunoglobulin in CLL, in contrast with the bright expression of CD20 and surface immunoglobulin and CD23 negativity typically present in MCL. However, the immunophenotype in CLL not infrequently deviates from this "classic" immunophenotype, making its distinction from MCL in such cases difficult by flow cytometry alone.[17,18,20] Common immunophenotypic variations include unusually bright expression of CD20 or surface light chain in CLL and aberrant CD23 expression in MCL. Although we were able to analyze only a limited number of cases, our findings indicate that the differential expression of CD200 is retained in immunophenotypically atypical CLL and MCL. Thus, CD200

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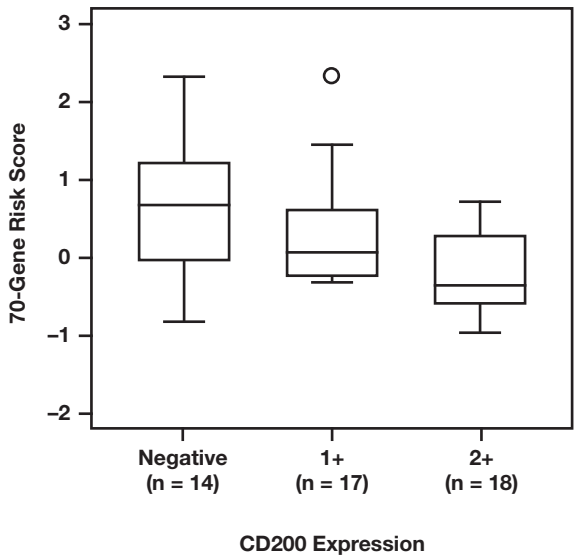

Figure 1 CD200 expression is inversely correlated with the 70-gene risk score ($P < .01$). Plasma cell myelomas were initially categorized based on their level of CD200 expression without knowledge of the 70-gene risk score. The latter was generated by gene expression profiling on CD138 immunoselected plasma cells, as described in the "Materials and Methods" section. The open circle in the CD200 1+ plot represents an outlier case.

analysis seems to have particular diagnostic usefulness in such immunophenotypically indeterminate cases. It is important to note that CD200 expression alone does not equate to malignancy because this antigen is expressed in most normal peripheral blood lymphocytes and in a subset of lymph node B cells. Therefore, the evaluation of CD200 expression must be interpreted in conjunction with that of other cell markers, including those for surface immunoglobulin light chains.

PCM is a genetically heterogeneous disease. While the investigation of underlying cytogenetic abnormalities has provided significant insight into its pathogenesis, more recent GEP-based analyses have significantly advanced our understanding of the molecular pathogenesis of PCM. Thus, 7 major molecular subtypes of PCM have been identified through GEP analysis, including CD-1, CD-2, HY, MF, LB, MS, and PR. Some of these PCM subtypes have been well characterized owing to their underlying cytogenetic abnormalities, eg, the CD-1 and CD-2 subtypes, both of which are associated in most cases with the t(11;14) resulting in aberrant cyclin D1 overexpression. It is important to note that GEP analysis has facilitated the identification of novel PCM subtypes that lack defining recurrent cytogenetic abnormalities, including the LB and PR subtypes. At our institution, GEP analysis is routinely performed on all cases of myeloma, from which molecular subtyping and a 70-gene risk score are derived.[14,21] The latter has been confirmed to be a strong predictor of poor clinical outcome.

The PR subtype comprises 18% of all newly diagnosed PCMs and is characterized by overexpression of several proliferation and cell cycle–related genes.[14] PR PCMs manifest metaphase cytogenetic abnormalities in 70% to 80% of cases, whereas such abnormalities are detected in only about 20% of other molecular subtypes.[14] Finally, several genes mapping to chromosome 1q, a region commonly amplified during disease progression and associated with poor prognosis, are frequently overexpressed in PR PCM.[23-25] Thus, PR PCM manifests several genetic features associated with poor clinical outcome and is associated with a significantly increased 70-gene risk score in most cases.

While not detectable on normal PCs, we found that CD200 is expressed in 71% of PCMs, a frequency similar to that reported by others.[10,26,27] It is interesting that the PR type is significantly overrepresented among CD200– PCMs, comprising 50% of such cases, whereas PR myelomas comprise only 6% of CD200+ cases. Not surprisingly, the CD200– group had a 70-gene risk score that was significantly higher than that of CD200+ PCMs.

These findings seem to contradict a previous report in which patients with CD200+ PCM, who comprised 78% of patients, were shown to have inferior survival compared with patients with CD200– disease.[10] However, several differences exist between our analysis and that by Moreaux et al.[10] In the latter study, CD200 expression analysis was performed only at initial diagnosis, whereas the majority of our analyses were performed on patients who had received previous antimyeloma therapy. Furthermore, in our study, flow cytometry was used to evaluate the expression level of CD200 on the cell surface of neoplastic plasma cells. By contrast, CD200 expression in the study by Moreaux et al[10] was achieved primarily through GEP-based analysis of *MOX2* expression. Although well-correlated overall, several cases in our series expressed significant levels of *MOX2* messenger RNA by GEP, yet lacked detectable protein expression as assessed by flow cytometry (data not shown). This observation may account for the somewhat lower incidence of CD200+ myelomas in the current study as compared to that of Moreaux et al.[10] Finally, although we were not able to perform serial flow cytometric analysis of CD200 expression, repeat GEP analyses suggest that *MOX2* expression is dynamically modulated during the course of disease development in a subset of patients. Furthermore, serial GEP analysis of several patients showed that the down-regulation of *MOX2* expression occurred concurrently with disease evolution to the PR subtype. The basis for this down-regulation is unknown; however, it is unlikely due to gene deletion as the gene encoding CD200, *MOX2*, is located on chromosome 3q12, a region infrequently deleted in PCM.[28] Nevertheless, these preliminary findings

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suggest that loss of CD200 expression may serve as a marker for disease progression in PCM.

We found differential expression of CD200 in precursor lymphoid malignancies, with T-lineage ALLs being uniformly CD200–, whereas B-lineage ALLs were variably positive for this marker in 95% of cases. Precursor B-lymphoblastic leukemia/lymphoma is readily distinguished from normal B-cell progenitors in most cases. However, in the setting of low-level bone marrow involvement, such distinction may be problematic. Immunophenotypic aberrancies have been detected in the majority of B-ALLs.[29] While nearly all B-ALLs were CD200+ in our analysis, more than half aberrantly overexpressed or underexpressed CD200 in comparison with normal CD34+/CD19+ B-cell progenitors. Thus, evaluation of CD200 may be useful in distinguishing between leukemic lymphoblasts and normal hematogones.

It is interesting that the 4 B-ALLs with the strongest CD200 overexpression were hyperdiploid or *TEL-AML1*+, 2 ALL subtypes that comprise approximately one half of all pediatric ALLs and that are typically associated with an excellent clinical outcome.[30-32] Although the analysis of additional cases and further confirmatory studies are needed, one implication of our findings is that CD200 may represent a potential therapeutic target in B-ALL, particularly in the subtypes manifesting high levels of CD200 expression. A humanized antihuman CD200 antibody, ALXN6000, has been developed and evaluated in a recently completed phase I/II clinical trial in patients with CLL and PCM (NCT00648739; http://ClinicalTrials.gov). An immunotherapeutic approach might obviate the significant toxicity associated with the multiagent chemotherapy presently used for ALL. Such an approach would be particularly appealing for patients with hyperdiploid or *TEL-AML1*+ B-ALL, both of which are associated with an excellent prognosis with current therapy and thus represent candidates for reduced intensity chemotherapeutic approaches.

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