Immunohistochemical Expression of CD23 and CD40 May Identify Prognostically Favorable Subgroups of Diffuse Large B-cell Lymphoma: A Nordic Lymphoma Group Study[1]

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ABSTRACT

Purpose: **In search for subgroups of diffuse large B-cell lymphoma (DLBCL) with different histogenetic origin and prognosis, as has been described by gene expression profiling, we examined tumor specimens from 125 patients with DLBCL, uniformly treated by either cyclophosphamide-Adriamycin-vincristine-prednisone or methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin in a multicenter trial set by the Nordic Lymphoma Group 1989–1994.**

***Experimental Design: Bcl*-6, CD10, and CD40 were chosen as markers for a germinal center phenotype, CD23 as a marker of pre/early germinal center origin, and CD138 as a marker for postgerminal center origin. In addition, expression of the apoptotic regulators *bcl*-2 and bax was analyzed. Immunohistochemical analysis was performed using the EnVision method.**

Results: **CD10 was positive in 51%, *bcl*-6 in 97%, and CD138 only in 2% of the cases. No prognostic conclusions could be drawn from analysis of these factors. CD40 was positive in 76% of the cases. This group was associated with superior time to treatment failure ($P = 0.027$) and overall survival (OS; $P = 0.0068$). By Cox regression analysis, positivity for CD40 was shown to be a prognostic factor for OS, independent of International Prognostic Index. CD23 was positive in 16% of the cases (all CD5 negative and all CD40 positive). This group showed a strong tendency for better OS ($P = 0.033$). CD40 expression correlated with bax but not with *bcl*-2 expression.**

Conclusions: **CD23 and CD40 expression seems to be prognostically favorable in DLBCL. This may be secondary to a germinal center origin or attributable to increased apoptosis via induction of bax and/or enhanced T-cell interaction, resulting in improved autologous tumor response. Confirmatory studies are necessary.**

INTRODUCTION

DLBCL[3] is the most frequent lymphoma subtype and encompasses the majority, ~60–70%, of the aggressive lymphomas. Biologically and clinically, it shows considerable heterogeneity. Eventually, 30–40% of the patients with advanced stage DLBCL who are treated with current therapies will be long-time survivors, whereas the rest will succumb to the disease. There is an obvious need for prognostic information to design a more risk-adapted primary therapy. The IPI is one available, validated tool, based on clinical features (age, stage, level of lactate dehydrogenase, performance status, and number of extranodal sites; Ref. 1). However, the IPI is not an ultimate instrument because a considerable proportion of patients classified as "low" or "low-intermediate risk" eventually shows therapeutic failure. Consequently, attempts have been made to identify other biological prognostic factors to be used separately or combined with the IPI. According to the recent WHO classification, there are several morphological variants of DLBCL: (*a*) centroblastic; (*b*) immunoblastic; (*c*) T-cell/histiocyte-rich; (*d*) anaplastic; (*e*) plasmablastic; (*f*) lymphomatoid granulomatosis type; and (*g*) a variant expressing full-length anaplastic large cell lymphoma (2). The prognostic importance of morphological subclassification remains a disputable issue.

By DNA microarray analysis, Alizadeh *et al.* (3, 4) identified two main groups of DLBCL with different prognosis: (*a*) "germinal center DLBCL," expressing genes characteristic of germinal center B cells (with ongoing somatic mutations); and (*b*) "activated B-like type DLBCL," expressing genes normally induced during *in vitro* activation of peripheral blood B cells.

The result of this study brought about a new challenge: a search for a protein expression pattern which would best distinguish between the two groups. Although immunohistochemical characteristics of the non-neoplastic germinal center are known, a normal counterpart of "activated B cell" is not easily defined.

Trying to identify subgroups of DLBCL with different histogenetic origin and prognosis, the following immunohistochemical markers were used: (*a*) *bcl*-6; and (*b*) CD10, as well as established markers of the germinal center (5–8) together with CD40, essential in the germinal center reaction. CD23 was chosen as a possible pre/early germinal center marker. CD138/

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[3] The abbreviations used are: DLBCL, diffuse large B-cell lymphoma; IPI, International Prognostic Index; RR, relative risk; CI, confidence interval; TTF, time to treatment failure; OS, overall survival.

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Syndecan-1 is a plasma cell-associated antigen also present on immunoblasts. When studied in HIV lymphomas, positivity for this antigen, in combination with negative *bcl*-6 expression, is thought to represent a postgerminal center origin (9). Analysis of CD5 expression was included because it has been associated with poor prognosis in several studies (10–12).

Analysis of *bcl*-2 protein expression was already performed on this patient material confirming that *bcl*-2 expression is associated with increased risk of therapeutic failure (13). In the present study, analysis of the proapoptotic *bcl*-2 analogue, bax, was included.

Table 1 List of antibodies

Antibody (clone)	Dilution	Source
CD5 (4C7)	1:40	NovoCastra Laboratories (Newcastle upon Tyne, UK)
CD10 (56C6)	1:5	NovoCastra Laboratories
CD23 (1B12)	1:50	NovoCastra Laboratories
CD40 (11E-9)	1:40	NovoCastra Laboratories
CD138 (B-B4)	1:20	Serotec Ltd. (Oxford, UK)
Bcl-6 (PG-Bbp)	1:5	DAKO
Bax (B-9)	1:120	Santa Cruz Biotechnology (Santa Cruz, CA)

PATIENTS AND METHODS

Patients and Follow-Up. Tumor tissue was selected from a group of 405 patients with aggressive lymphomas, originally included in a trial set by the Nordic Lymphoma Group. Patients were included in this trial between 1989 and 1994 and randomized to treatment either with cyclophosphamide, doxorubicin, vincristine, and prednisone or with methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin. No superiority of methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin compared with cyclophosphamide, doxorubicin, vincristine, and prednisone could be demonstrated in this study (14).

Inclusion criteria for the Nordic Lymphoma Group trial were aged between 18 and 67 years, high-grade lymphoma according to the updated Kiel classification, and stage II-IV disease. Patients with central nervous system involvement, transformed low-grade, discordant, lymphoblastic, or Burkitt lymphoma were excluded. OS was defined as time from randomization to death. TTF was defined as time from randomization to progression or relapse (15). The last clinical observations were made during spring 1998 when the original study was analyzed. After this period, follow-up data concerning treatment failure are incomplete. Because median follow-up already was 67.5 months, this was not prolonged in the current study. Of originally 267 patients with diagnosis of DLBCL, paraffin blocks from 150 patients were available. All cases included were reevaluated according to the Revised European-American Classification of Lymphoid Neoplasms classification by a hematopathologist. Cases (14) of cyclin D1-positive large cell/blastic variants of mantle cell lymphoma, 1 case of prolymphocytic variant of small lymphocytic lymphoma, 2 cases of small lymphocytic lymphoma with large proliferation centers, 2 cases of Burkitt-like lymphoma, and 6 cases with incomplete follow-up data were excluded.

The remaining cases were morphologically subtyped as: (*a*) 107 of 125 (86%) centroblastic (large and noncleaved); (*b*) 14 of 125 (11%) were immunoblastic; and (*c*) 4 of 125 (3%) were T cell/histiocyte-rich, B-cell lymphoma. There were no differences between the original cohort of 267 patients and our remaining material of 125 patients concerning bulky disease and IPI. At the time of diagnosis, 47% scored low risk, 34% scored low-intermediate risk, 12% scored high-intermediate risk, and 7% scored high risk ($n = 122$). A portion (74%) of the patients was <60 years of age, and the rest, 26%, thus between 60 and 67 years of age.

Immunohistochemistry. The paraffin blocks were cut at 4–6 μm, dried overnight at 60°C, and deparaffinised in xylene. Subsequently, sections were rehydrated through graded alcohol in water. Heat-induced epitope retrieval was achieved by boiling sections in EDTA buffer at pH 8.9 in a microwave oven at 1000 W for 20 min (4 × 5 min). After boiling, sections were allowed to cool at room temperature for 20 min, rinsed thoroughly with water, and placed in a Tris-buffered saline for 5 min. Endogenous peroxidase was blocked with peroxidase block solution provided in the EnVision kit for 5 min, and slides were rinsed/washed with Tris-buffered saline. The primary antibodies were incubated for 30 min in room temperature. The list of the antibodies and their sources and dilutions are shown in Table 1. The immunostaining was performed using the EnVision method (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. Scoring of intensity of the immunostaining was done semiquantitatively (0 = no staining, 1+ = weak staining, 2+ = moderate staining, and 3+ = strong staining of the tumor cells). The cases were interpreted as negative if <10% of tumor cells were positive.

Multitissue blocks were prepared using 4-mm diameter modified, punch-biopsy needle. Generally, two different representative areas were cut out from the paraffin blocks. In total, this provided 25.5 mm^2 for evaluation.

Statistical Analysis. Survival analyses by different levels of expression were computed using Kaplan-Meier method and were compared using the Log-rank test. $P < 0.01$ was required for statistical significance (16). Cox regression analysis was used for multivariate analysis. For analysis of correlation between immunohistochemical markers, Spearman's test was used.

RESULTS

The results of immunostainings are summarized in Table 2. A vast majority, 97%, of the tumors was positive for *bcl*-6. Three of 4 patients with *bcl*-6-negative tumors died in <20 months of follow-up. However, the number of *bcl*-6-negative cases was too small for statistical analysis. Clinically, the *bcl*-6-negative group did not show any common features.

A portion (51%) of the tumors was positive for CD10. No correlation to prognosis was found. There were no major differences in clinical presentation between the CD10-positive and CD10-negative groups. A composite phenotype consisting of *bcl*-6 and CD10-positive cases did not show any prognostic advantages.

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Table 2 Results of immunostainings

Epitope	*n*	Intensity of staining (%)			
		Negative	1+	2+	3+
Bcl-6	124	4 (3)	30 (24)	49 (40)	41 (33)
CD10	124	61 (49)	10 (8)	24 (19)	29 (23)
CD23	124	104 (84)	13 (10)	7 (6)	
CD40	121	29 (24)	47 (39)	28 (23)	17 (14)
CD138	124	122 (98)	2 (2)		
CD5	125	116 (93)	3 (2)	6 (5)	
Bax	115	0	36 (31)	39 (34)	40 (35)

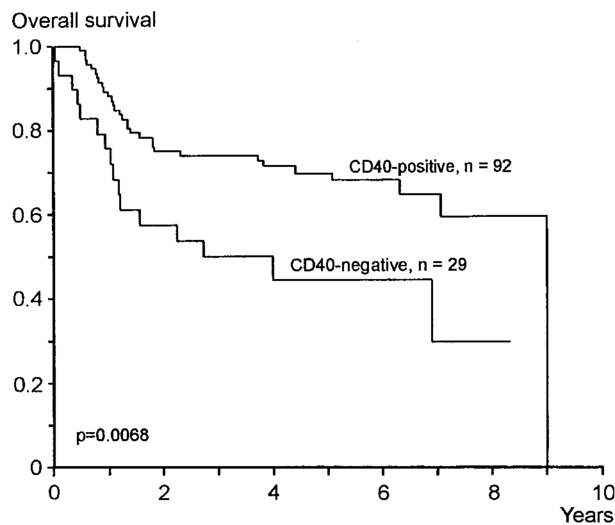

Fig. 1 OS in 121 patients with DLBCL in relation to CD40 expression.

Only 2 of 124 tumor specimens were positive for CD138. No statistical analysis could thus be performed.

A portion (76%) of the tumors was CD40 positive. There was a strong association between the expression of CD40 and improved OS ($P = 0.0068$), with a median survival of 33 months for negative cases and 108 months for positive cases (Fig. 1). By Cox regression analysis, positivity for CD40 was shown to be a prognostic factor for OS, independent of IPI (RR = 0.42, 95% CI 0.22–0.82). The positive group also displayed a tendency for longer TTF ($P = 0.027$; Fig. 2). There was no significant difference to be found between the CD40-negative and CD40-positive cases regarding the individual parameters of the IPI. CD40 expression did not show any correlation with nodal *versus* extra nodal presentation. When the *bcl*-6-positive group was analyzed separately regarding expression of CD40, there was still a trend for superior OS ($P = 0.0178$) but not TTF ($P = 0.0645$). CD40 expression did not correlate with CD10 expression. A portion (16%) of the tumors was CD23 positive (all CD5 negative). This group showed a tendency for better OS ($P = 0.033$), with a median survival of 85 months for negative cases. For CD23-positive cases, median survival was not reached (Fig. 3). The difference in TTF was not significant. There was a strong association between CD23 and CD40 expression ($r_S = 0.25$, $P = 0.005$). All CD23-positive lymphomas coexpressed CD40. There was no significant difference to be found between the CD23-negative and CD23-positive groups regarding the individual parameters of the IPI. When CD23-positive cases were excluded from the CD40-positive population, there was still a difference in OS between CD40-positive and CD40-negative cases ($P = 0.032$; Fig. 4).

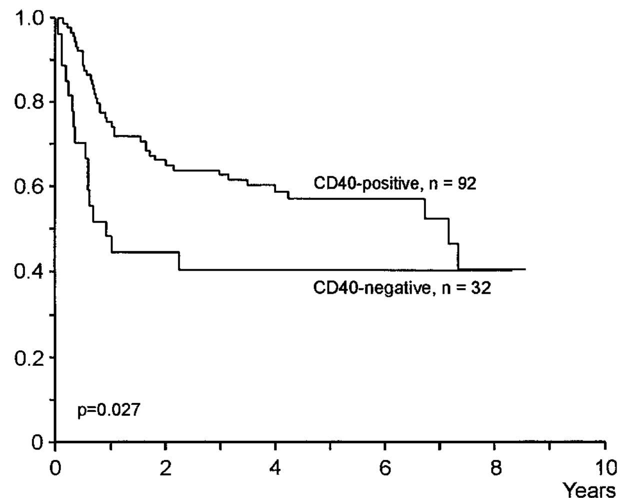

Fig. 2 Failure-free survival in 124 patients with DLBCL in relation to CD40 expression.

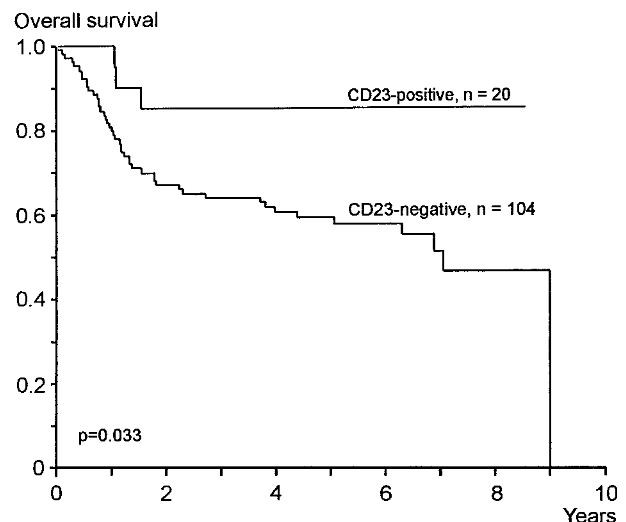

Fig. 3 OS in 124 patients with DLBCL in relation to CD23 expression.

Only 9 of 125 (7%) lymphomas were CD5 positive. All were negative for cyclin D1 and CD23. CD5 expression was associated with an unfavorable prognosis both regarding TTF and OS (Figs. 5 and 6). CD5-positive cases had a median TTF of 7 months, compared with 86 months for the CD5-negative group ($P = 0.0002$). CD5-positive cases were also found to have a median OS of 15 months, compared with 108 months for the negative cases ($P = 0.0008$). By Cox regression analysis, positivity for CD5 was shown to be a negative

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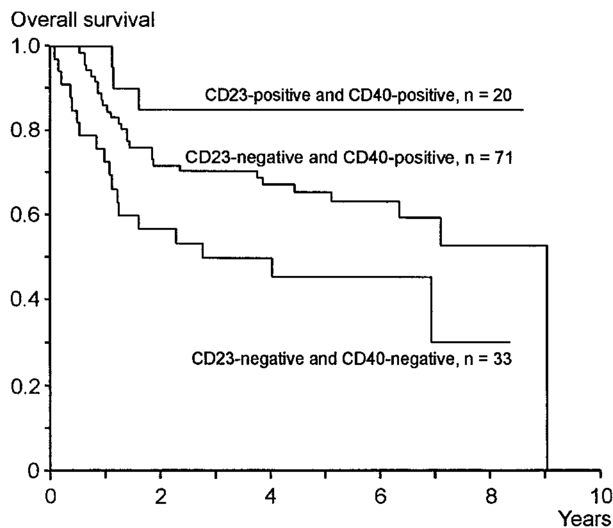

Fig. 4 OS in 124 patients with DLBCL in relation to three expression patterns: (*a*) tumors coexpressing CD23 and CD40; (*b*) tumors expressing CD40 but not CD23; and (*c*) tumors without CD40 and CD23 expression. There was no difference in OS between groups a and b (P = 0.09). There was a trend for difference in OS between groups b and c (P = 0.032).

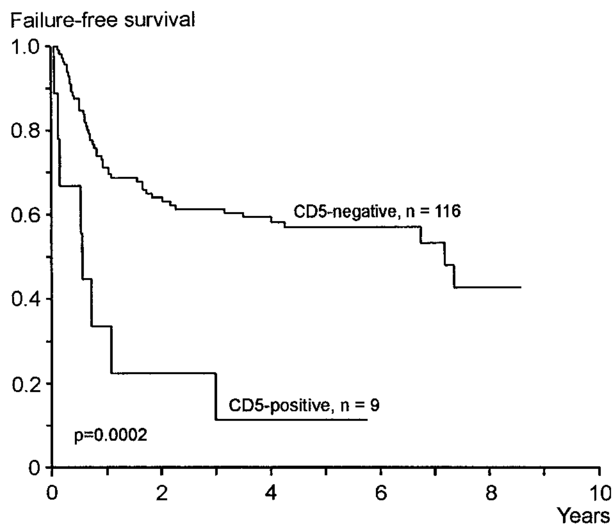

Fig. 5 Failure-free survival in 125 patients with DLBCL in relation to CD5 expression.

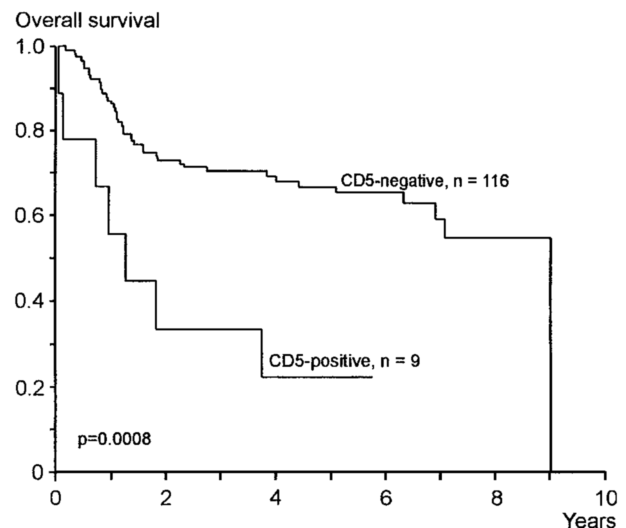

Fig. 6 OS in 125 patients with DLBCL in relation to CD5 expression.

prognostic factor for TTF (RR = 3.6, 95% CI 1.6–8.1) and OS (RR = 3.6, 95% CI 1.5–8.5), independent of IPI. Clinically, there was no difference between the groups at the time of diagnosis.

In an earlier study performed on the same patient material, we were able to confirm that *bcl*-2-positive tumors were associated with significantly shortened TTF (13). In the present study, we could not find any statistical correlation between the expression of *bcl*-2 and CD5, CD23, or CD40, respectively. There was a positive association between the expression of CD40 and bax ($r_S = 0.23$, $P = 0.012$). Because all tumors to a variable degree were positive for bax (Table 2), no survival analysis was performed.

DISCUSSION

In this study of DLBCL, we have examined the expression of markers associated with different stages of B-cell development. Several immunohistochemical groups could be defined which also appeared to correlate with clinical outcome. No correlation with prognosis was found concerning expression of CD10 and *bcl*-6, alone or in combination. Regarding CD10 expression and prognosis, this contributes to earlier, somewhat conflicting results (17–19). The majority (97%) of the tumors was *bcl*-6 positive in our study, prohibiting a meaningful survival analysis. In previous studies, *bcl*-6 protein was detected in 55–97% of DLBCL (20–23). Skinnider *et al.* (7) found that the expression of *bcl*-6 was seen more frequently in DLBCL with large noncleaved (centroblastic) morphology compared with immunoblastic morphology. The number of cases in our study with immunoblastic morphology was small (11%), which might be in agreement with the low number of cases with CD138 expression (2%; Ref. 9). Thus, a selection bias might contribute to the high frequency of *bcl*-6-positive tumors. The usage of a potentially more sensitive staining technique, the EnVision method, may also contribute to these results.

With this study, we could confirm earlier reports that the minority of DLBCL expressing CD5 does poorly. Somewhat unexpected were the findings on CD40 and CD23 and their strong relation to prognosis.

CD40 is a cell surface molecule, member of the tumor necrosis factor-receptor family, and expressed on all stages of B-cell development but also on monocytes, dendritic cells, endothelial cells, fibroblasts, and some epithelial cells (24). It is expressed in the majority of B-cell malignancies (non-Hodgkin lymphoma, acute lymphoblastic lymphoma, chronic lymphocytic leukemia, and myeloma) and in 35–100% of solid tumors (25). The natural ligand of CD40, CD40L, is expressed mainly on activated T-lymphocytes. Cross-linking with CD40L in the

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presence of interleukin-4 promotes immunoglobulin production and isotype switching to IgG4 and IgE (24).

We found CD40 positivity in 76% (92 of 121) of our cases, which is roughly similar to the results of Vyth-Dreese *et al.* (26), who demonstrated CD40 expression in 67% (8 of 12) of the DLBCL cases. Even though CD40 is thought to be constitutively expressed by B cells, it was recently demonstrated that it is highly expressed by B cells predominately in germinal centers, where it is necessary for their maturation (27). CD40 as well as CD40 L is regulated by the AT-hook transcription factor AKNA (27), which is mainly expressed by germinal center B-lymphocytes. These data suggest that CD40 expression is associated with the germinal center reaction. However, the specificity of CD40 as a marker of germinal center origin in lymphoma has to be further evaluated. In our study, expression of CD40 and the more established germinal center marker, CD10, was not correlated. DLBCL that expresses CD40 could be potentially sensitive to CD40 signaling caused by their interaction with cells that express CD40L. *In vitro* studies have shown CD40/CD40L interaction to provide a critical survival signal for B-cell lymphoma cell lines, in some cases reducing chemotherapy sensitivity (28–33). The same CD40/CD40L interaction, however, has also been shown to have a negative effect on tumor growth. Immune stimulation has been observed after CD40 cross-linking, including enhanced antigen presentation and the triggering of antigen-specific T-cell responses. This effect has been studied and used therapeutically in Phase I trials, including not only B-cell malignancies but also solid tumors. French *et al.* (34–36) report that CD40 cross-linking (after treatment with monoclonal antibodies against CD40) in CD40-positive, B-cell lymphoma lines may stimulate the neoplastic B cells to become more effective as antigen-presenting cells and thereby more prone to present their own tumor antigens to autologous killer T cells.

Another possible mechanism may be attributable to differences in apoptosis modulation. Data indicate that although CD40 activation up-regulates the expression of CD95 (Fas) on B-cell neoplasms (37), tumor B cells are resistant to this pathway (38). In a recent study by Szocinski *et al.* (39), CD40 stimulation of Burkitt lymphoma cell lines results in apoptosis via bax induction, in a pathway independent of CD95. Data also indicate that this bax induction is independent of p53 and not compensated by *bcl*-2. In our study, we could not find any correlation between CD40 and *bcl*-2 expression. However, the expression of CD40 and bax correlated significantly, which indicates that CD40 may be a true positive prognosticator in DLBCL.

CD23 is involved in the regulation of IgE synthesis and has a proinflammatory function. As well as being a low affinity receptor for IgE, CD23 works as an adhesion molecule interacting with CD11b, CD11c, and CD21, the latter a molecule potentially involved both in T cell-dependent and -independent responses. CD23 is expressed on naïve B cells, monocytes, and follicular dendritic cells and commonly on B-chronic lymphocytic leukemia cells (40).[4] In human tonsillar tissue, CD23 is a precentroblast marker, expressed on naïve (nonswitched) B cells both in the mantle zone (41) and early germinal center phase (19, 42–44). In B-CLL, serum soluble CD23 is a well-known prognostic factor in early stages (45), but the prognostic value of determining membrane-bound CD23 expression remains unclear (46).

CD23 is not a well-studied prognostic factor in DLBCL, although it has been associated with prolonged disease-free survival (47). In our small but distinct subgroup of CD23-positive DLBCL, the malignant cells were clearly positive, without dendritic cells in the background. All CD23-positive tumor specimens coexpressed CD40. *In vitro* studies have shown that CD40 activation can stimulate the expression of CD23 expression (24, 48).

It is thus possible that CD23 is a marker of a specific histogenetic stage, representing an early germinal center origin corresponding to an early, not yet isotype-switched centroblast. However, the expression of CD23 might also be representing functional properties of the tumor, as discussed above.

In conclusion, CD40 expression in DLBCL appears to be associated with favorable prognosis. A possible explanation might be germinal center origin, in line with the DNA microarray findings of Alizadeh *et al.* and the more recent findings of Rosenwald *et al.* (49). More likely, however, is that CD40 positivity in DLBCL represents other biological properties of the tumor. Enhanced T-cell interaction with improved autologous tumor response and/or increased apoptosis via bax induction seems to be the most probable explanations. CD23-positive DLBCL may constitute an even more favorable subpopulation of CD40-positive DLBCL. Bias cannot be excluded, because not all of the cases in the randomized study could be included in the survival analyses. Furthermore, this is a retrospective study, and data with regard to prognosis must be interpreted with caution.

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