Loss of B7.2 (CD86) and Intracellular Adhesion Molecule 1 (CD54) Expression Is Associated with Decreased Tumor-infiltrating T Lymphocytes in Diffuse B-cell Large-cell Lymphoma[1]

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ABSTRACT

Tumor-infiltrating CD8+ T-lymphocytes (T-TILs) are thought to be relevant to immunosurveillance of several tumor types including B-cell non-Hodgkin's lymphoma. B- and T-lymphocyte interactions via cellular adhesion molecules (CAMs), recognition molecules (HLAs), and costimulatory molecules (CSMs) are necessary for optimal antigen-specific T-cell activation to occur and may be important in generating effective host T-TIL responses. We previously found that low T-TIL response (CD8+ T cells < 6%) correlates with statistically shorter relapse-free survival in patients with diffuse large-cell lymphoma (DLCL). We now extend our observations in 71 DLCL patients by analyzing malignant B-cell expression of the following molecules important in T-cell activation: (*a*) recognition molecules [MHC I (MAS and MCA) and MHC II (HLA-DR, -DP, -DQ)]; (*b*) CAMs [leukocyte function antigen 1 (CD11a and CD18) and intracellular adhesion molecule 1 (CD54)]; and (*c*) CSMs [B7.1 (CD80) and B7.2 (CD86)]. Eighteen patients (25%) had low a T-TIL response, and 53 patients (75%) had a high T-TIL response. Overall, expression of the MHC class II molecules HLA-DR and HLA-DQ was most conserved. The loss of B7.2 ($P = 0.04$), intracellular adhesion molecule 1 ($P = 0.0004$), MAS ($P = 0.02$), and HLA-DR ($P = 0.0004$) expression was significantly associated with decreased T-TIL response. In 100% of patients with low T-TIL responses, at least one HLA, CAM, or CSM was undetectable on the malignant B cells by immunohistochemical staining (mean number of molecules lost = 2.67). In contrast, 49% of patients with high T-TIL responses had no losses in HLA, CAM, or CSM expression (mean number of molecules lost = 0.89). The mean number of absent molecules (HLA, CAM, or CSM) was significantly associated with T-TIL response ($P = 0.0001$). We conclude that loss of HLA, CAM, or CSM expression on malignant B cells is associated with a poor host T-cell immune response. In addition, because patients with low T-TIL response had lost expression of multiple cellular adhesion, recognition, and costimulatory molecules, our results suggest that a combination of immunorestorative therapies may be required to generate effective antitumor T-cell responses in B-cell DLCL.

INTRODUCTION

DLCL[3] is the most common subtype of potentially curable NHLs. A variety of clinical characteristics including age, stage, extranodal site of involvement, LDH, and performance status are useful in predicting clinical outcome but provide little understanding of the biology of the disease. Infiltrating T lymphocytes isolated from B-cell lymphomas recognize specific epitopes of the malignant clone (1–3). Previously, we reported that the number of T-TILs in the initial lymphoma biopsy from patients with B-cell DLCL was predictive of relapse-free survival (4). A host T-TIL response of <6% (low T-TIL response) correlated with statistically shorter relapse-free survival in a series of 82 consecutively treated patients (4). In this well-characterized series of patients, loss of MHC class I and class II (HLA-DP and HLA-DR) expression correlated with shortened relapse-free survival and/or overall survival and decreased host T-TIL response (5–7).

CAM, HLA, and CSMs are important determinants of B-cell stimulation and activation of T cells. Whereas the MHC class I and II antigens are necessary for presentation of specific antigenic epitopes, T-cell recognition is insufficient for effective CTL responses. ICAM-1/LFA-1 interactions permit the leukocyte adhesion required for CTL generation (8, 9). B7.1 and B7.2 are CSMs that provide the second signal required for stimulated T cells to become activated. Without costimulation, T cells will undergo apoptosis, and an anergic immune response will prevail, even in the presence of optimal recognition and presentation of antigens via the MHC-T-cell receptor interaction (10–12). Thus, multiple interactions between the T cell and antigen-presenting cell are necessary for optimal and effective T-cell responses.

Because HLA, CAM, and CSMs play an important role in generating cytotoxic T-cell responses, we hypothesized that

Received 4/25/00; revised 8/1/00; accepted 8/2/00.
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[1] Supported in part by gifts to the Arizona Cancer Center from Anne Mangelsdorf and Mary Dabney.
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[3] The abbreviations used are: DLCL, diffuse large-cell lymphoma; T-TIL, tumor-infiltrating T-lymphocyte; CAM, cellular adhesion molecule; CSM, costimulatory molecule; NHL, non-Hodgkin's lymphoma; ICAM-1, intracellular adhesion molecule 1; LFA, leukocyte function antigen; IFN-α, interferon α.

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their expression on malignant B cells may determine the host T-TIL response. In this study, we examine the role of B7.1, B7.2, ICAM-1, and LFA-1 expression in host T-TIL responses. Our results significantly expand our previous data, suggest a hypothesis by which malignant B cells may evade host immune surveillance, and provide a rationale for immunotherapy strategies for treating B-cell lymphoma.

MATERIALS AND METHODS

Clinical and Demographic Data. From 1978 to 1987, 115 consecutively accrued patients with a diagnosis of DLCL having fresh tissue available for analysis were evaluated at the Arizona Cancer Center. Tumors were classified as B-cell lymphoma according to previously defined criteria that required restricted immunoglobulin light or heavy chain expression and/or reactivity with pan-B-cell antigens without T-cell antigen expression (13). Patients were excluded due to inadequate tissue (10 patients), T-cell phenotype (23 patients), indeterminate phenotype (2 patients), prior history of follicular lymphoma (8 patients), and AIDS-related lymphoma (1 patient). Information on cell adhesion, recognition, and costimulatory molecules, survival, stage, B symptoms, T-TILs, and patient characteristics was available for the remaining 71 cases. The clinical details including demographics, prognostic factors, and treatment for this group have been described previously in detail (4).

Tissue Immunohistochemistry. B- and T-cell antigens, percentage of T-TILs, and immunoregulatory molecules [recognition molecules, MHC I (MAS and MCA) and MHC II (HLA-DR, DP, and DQ); cellular adhesion molecules, LFA-1α (CD11a), LFA-1β (CD18), and ICAM-1 (CD54); and costimulatory molecules, B7.1 (CD80) and B7.2 (CD86)] were analyzed using a three-stage avidin-biotin horseradish peroxidase 3,3′-diaminobenzidine immunoperoxidase technique on snap-frozen tissue sections as described previously (5, 7). Negative controls excluded the primary antibody and substituted an irrelevant isotype-matched monoclonal antibody. Positive control specimens were reactive tonsil tissue sections placed at the bottom of each glass slide assay ("same slide control").

Positive expression of a specific HLA, CAM, or CSM was determined as surface staining in any of the tumor cells. Loss of expression (negative expression) constituted an absolute absence of staining in the malignant B cells of the tumor specimen, whereas the "same slide control" was positive. The immunoregulatory molecules were also grouped into families. Loss of expression of any of the tested antigens included in the family classified the family as negative (lost expression). All cases were reviewed independently and then reviewed jointly by two of the authors (A. G. and T. M. G.), who were blinded to the clinical staging and percentage of T-TILs of the specimens. The tabulated results are the consensus findings of the two authors.

The number of T-TILs was determined as described previously (4). Briefly, host T-TILs were quantitated by counting the number of CD8+ cells among the total number of lymphoid cells counted as indicated by methylene blue counterstaining. A cut point of 6% T-TILs was established previously to distinguish tumor specimens with low (<6%) and adequate (>6%) T-TIL expression as determined by predictive power for relapse-free survival in the lymphoma patients (4).

Antibodies used in detecting B cells, cytotoxic (CD8+) T cells, HLA-DP, HLA-DR, HLA-DQ, and class I HLA-A, -B, and -C have been described previously (4–6). LFA-1α, LFA-1β, and ICAM-1 (CD54) antibodies were purchased from Immunotech (Marseilles, France). B7.1 (CD80) antibody was purchased from Becton Dickinson (Mountain View, CA), and B7.2 (CD86) antibody was generously provided by Repligen Corp. (Cambridge, MA).

Statistical Methods. Two-sample t tests were used to test the association between individual molecules or molecular families and T-TILs as a continuous measurement. $P < 0.05$ was considered statistically significant. Several regression models were fit with all HLAs, CAMs, and CSMs entered as independent variables to examine the correlation between T-TILs, specific molecules, and molecular families. Logistic regression models were also fit with the outcome variable being T-TIL response (<6% *versus* >6%). Statistical significance was determined as $P < 0.05$.

RESULTS

Seventy-one patients with B-cell DLCL were reviewed, and their tumors were divided into high T-TIL expression [53 (75%) of patients] and low T-TIL expression [18 (25%) of patients]. In a previously published study of these lymphoma patients, the cut point of 6% T-TILs predicted progression-free survival (4). This provided the rationale for our choice of the 6% cutoff in dividing our patients into high or low host T-TILs. In the high T-TIL expression group, T-cell infiltration in the lymphoma specimens ranged from 6.23–37.9% (number of CD8+ T cells/total number of cells), with a mean infiltration of 18.2%. In the low T-TIL expression group, T-cell infiltration ranged from 0.07–5.46% with a mean of 2.6%.

Table 1 shows the expression of the 10 HLA, CAM, and CSMs analyzed in the lymphoma specimens. B cells function as effective antigen-presenting cells *in vivo* and thus normally express all examined molecules. This finding was confirmed in the "same slide control" tonsil sections. Overall, MHC class II expression was most conserved because 94% of the malignant B cells expressed HLA-DR and HLA-DP. The CSM B7.2 was also highly conserved, being present in 90% of the cases. In nine patients, the malignant B cells expressed only one of the CSMs, B7.1 or B7.2, and in five patients, expression of both B7.1 and B7.2 was lost.

The expression of each HLA, CAM, and CSM was also related to host T-TIL response in Table 1. Loss of B7.2 ($P = 0.04$), MAS ($P = 0.02$), HLA-DR ($P = 0.0004$), and ICAM-1 ($P = 0.0004$) expression was significantly associated with low T-TIL (Figs. 1 and 2). The number of absent HLA, CAM, and CSM on the surface of the malignant B cells was highly associated with host T-TIL response ($P = 0.0001$). In the 18 patients with a low host T-TIL response, a mean number of 2.67 (range, 1- 7) HLA, CAM, or CSM were lost as compared with a mean number of 0.89 (range, 0–4) molecules lost in the lymphomas characterized by a high host T-TIL response. In addition, every lymphoma specimen with a low host T-TIL response had loss of expression of at least one HLA, CAM, or CSM on the malignant B cells. In contrast, 26 (49%) of the B-cell lymphomas with high host T-TIL response had not lost expression of any of the 10 HLA, CAM, and CSMs analyzed. Expression of each HLA, CAM, and CSM was highly correlated

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Table 1 Expression and association of host T-TIL response with cellular recognition, adhesion, and costimulatory molecules expressed by B-cell DLCLs

HLA, CAM, and CSM	No. of tumors (%) positive for molecule	No. of tumors (%) negative for molecule	Mean T-TIL if molecule positive	Mean T-TIL if molecule negative	T-TIL significance[a]
B7.1	56 (81%)	13 (19%)	15.2%	10.7%	$P = 0.15$
B7.2	62 (90%)	7 (10%)	15.3%	6.1%	$P = 0.04$[b]
MAS	59 (84%)	11 (15.7%)	15.5%	7.8%	$P = 0.02$[b]
MCA	61 (87%)	9 (12.9%)	14.9%	10.1%	$P = 0.20$
HLA-DR	66 (94%)	4 (5.7%)	14.9%	5.7%	$P = 0.0004$[b]
HLA-DP	65 (94%)	4 (5.8%)	14.5%	8.7%	$P = 0.17$
HLA-DQ	55 (80%)	14 (20.3%)	15.6%	9.8%	$P = 0.07$
ICAM-1	55 (93%)	4 (6.8%)	15.6%	1.6%	$P = 0.0004$[b]
LFA-1a	61 (88%)	8 (11.6%)	15.0%	10.0%	$P = 0.22$
LFA-1b	44 (68%)	21 (32%)	14.2%	14.2%	$P = 0.99$

[a] Specific HLA, CAM, or CSM association with T-TIL (two-sample *t* test).
[b] Significant at the $P < 0.05$ level.

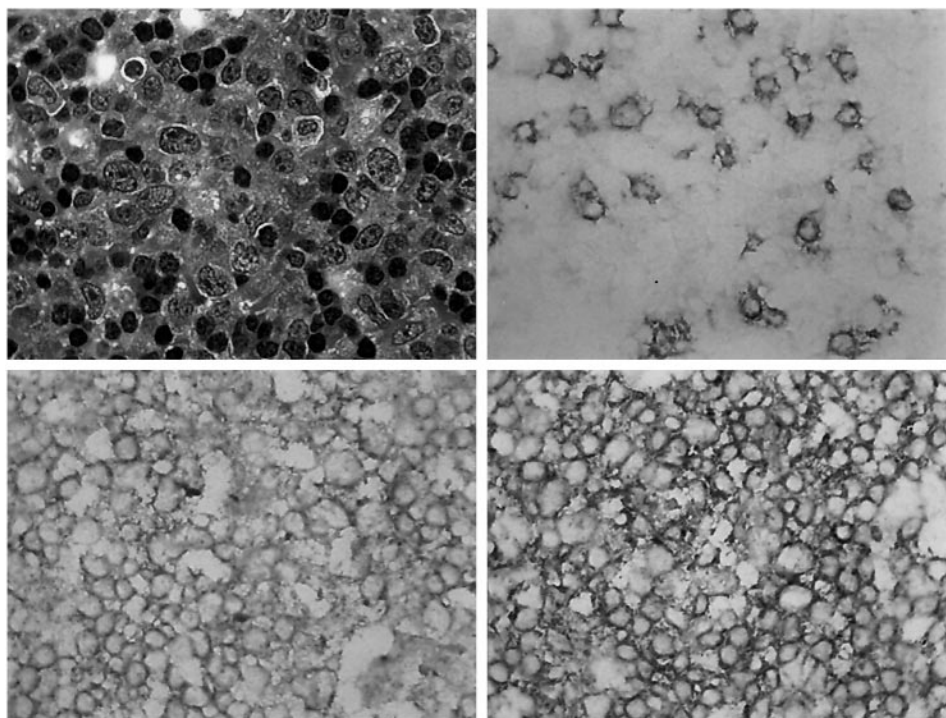

Fig. 1 Large-cell lymphoma with high T-TIL response (CD8 = 28%) and expression of B7.1 (CD80) and B7.2 (CD86). *Top left*, specimen stained with H&E. Immunohistochemical staining with antibodies against CD8 (*top right*) B7.1 (*bottom left*), and B7.2 (*bottom right*). ×1000.

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with expression of the other molecules, thus only B7.2, HLA-DR, and ICAM-1 expression independently correlated with host T-TIL response at a statistically significant level. The total number of tumor specimens examined for each molecule (MAS, MCA, and HLA-DR, $n = 70$; B7.1, B7.2, HLA-DP, HLA-DQ, and LFA-1a, $n = 69$; LFA-1b, $n = 65$; and ICAM-1, $n = 59$) is less than 71 because not all patients were evaluable secondary to inadequate tissue or indeterminate results.

In Table 2, the data are consolidated into HLA, CAM, and CSM families. Tumors were considered negative or to have lost family expression if any of the antigens tested in that family were absent on the malignant B cells (see "Materials and Methods"). Expression in the LFA family was lost most frequently (32%) but had no effect on host T-TIL responses. Host T-TIL responses were statistically correlated to expression of the B7, ICAM-1, and MHC (class I) families.

Because our results suggested that several cellular molecules predicted T-TIL response, we then examined whether analysis of each HLA, CAM, and CSM was necessary for predicting T-TIL. Using a logistic regression model, a forward selection procedure was used to enter variables into a model one by one, until no more variables added statistically relevant information to predicting T-TIL response at the $P = 0.05$ level. When considering each individual variable, the final model included only B7.2, HLA-DQ, MAS, and ICAM-1 expression. When the molecular families, instead of the individual molecules, were entered separately into the regression model, the final model included only MHC class I, MHC class II, and

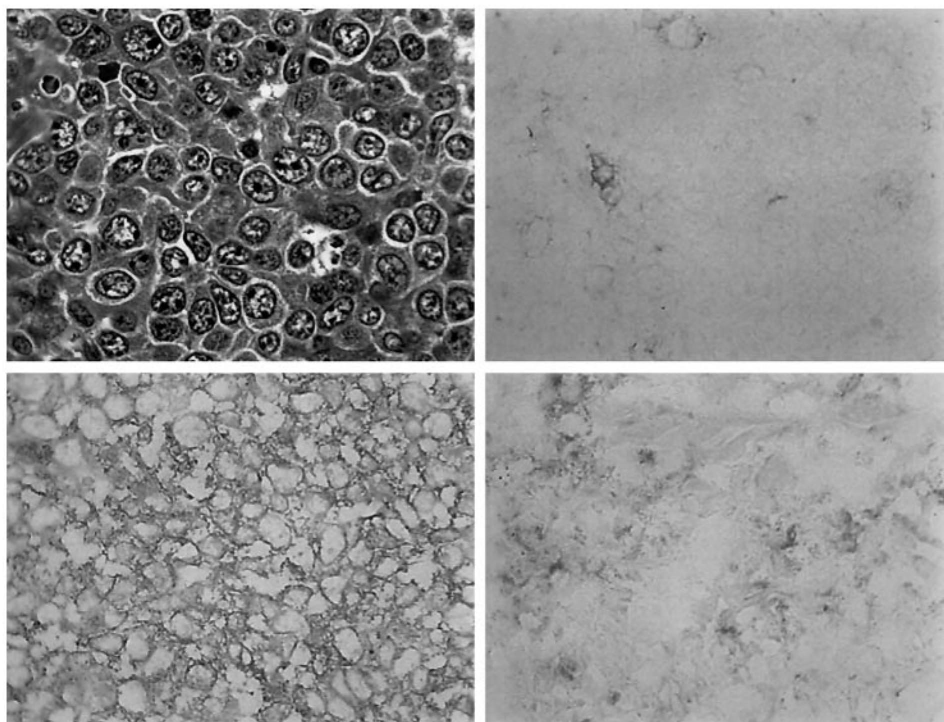

Fig. 2 Large-cell lymphoma with low T-TIL response (CD8 = 2.45%) and expression of B7.1 (CD80) and absence of B7.2 (CD86). *Top left*, specimen stained with H&E. Immunohistochemical staining with antibodies against CD8 (*top right*) B7.1 (*bottom left*), and B7.2 (*bottom right*). ×1000.

Table 2 Expression and association of host T-TIL response with molecular families

Molecular family	No. of tumors (%) positive	No. of tumors (%) negative	Mean T-TIL (family positive)	Mean T-TIL (family negative)	T-TIL significance[a]
B7	54 (78%)	15 (22%)	15.7%	9.6%	$P = 0.04$[b]
MHC I	56 (80%)	14 (20%)	15.7%	8.8%	$P = 0.03$[b]
MHC II	54 (76%)	17 (24%)	15.6%	10.0%	$P = 0.06$
LFA	44 (68%)	21 (32%)	14.2%	14.2%	$P = 0.99$
ICAM-1	55 (93%)	4 (7%)	15.6%	1.6%	$P = 0.0004$[b]

[a] Association of the molecular family with T-TIL (two-sample *t* test).
[b] Significant at the $P < 0.05$ level.

ICAM-1 families. Thus, by logistic analysis, multiple variables are significant, and additive information is gained by examining the expression of several HLA, CAM, and CSM.

DISCUSSION

In this study, we find that loss of the CSM, B7.2 (CD86) and loss of the CAM, ICAM-1 (CD54) are statistically associated with decreased T-cell infiltration in tumors from patients with B-cell DLCL. These studies significantly add to our prior findings in which we established an association between recognition molecule (MHC class I and II) expression, host T-TIL response, and relapse-free survival in this same group of lymphoma patients (4–7). The results from these prior studies, combined with our new findings, suggest the hypothesis that a low host T-TIL response in B-cell DLCL is a multifactorial event characterized by cumulative loss of surface expression of HLA, CAM, and CSM necessary for optimal T-cell activation by B cells. Furthermore, these results suggest that loss of these immunoregulatory molecules on malignant B cells may favor tumor progression and growth, although prospective studies would be needed to confirm this hypothesis. These results have potential implications for immunotherapy approaches for lymphoma patients and suggest combination immunorestorative agents and strategies may be necessary to sufficiently restore malignant B-cell immune function to generate effective and specific antitumor T-cell responses.

Tumor cells use multiple strategies in obtaining a growth advantage over normal cells, including overexpression of growth factors or their receptors, loss of apoptotic controls, and increased cell cycling. Despite these genetic mutations that increase the cell growth of tumor cells, survival also requires tumor cells to evade immune recognition. Because tumor cells are potentially susceptible to T-cell-mediated cytotoxicity, tumor cells have also developed mechanisms to escape immune recognition. Examples of these strategies include the release of immunosuppressive cytokines such as interleukin-10 and transforming growth factor β as well as the loss of surface molecules crucial to T-cell recognition and stimulation (14–16). B cells normally express or can be stim-

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ulated to express the molecules necessary for optimal T-cell recognition and stimulation (17). Thus, malignant B cells, by retaining expression of HLA, CAM, and CSMs, would be able to initiate and stimulate T-cell cytotoxic and helper responses. Prominent infiltrates of normal and proliferating T cells can often be observed in lymphoma specimens (7). In addition, the ability of malignant B cells to stimulate a strong host T-cell response has been associated with longer disease-free survival and improved prognosis in patients with B-cell malignancies (4, 18). This suggests that cytotoxic (CD8+) T cells can inhibit the growth and progression of B-cell malignancies in patients.

A variety of surface molecules are necessary for optimal T-cell-B-cell interactions (10, 11). T-cell activation is thought to require two distinct signals. The first signal (recognition) requires interaction of the T cell receptor with a MHC-peptide complex. The second signal is provided by the CSMs, B7.1 and B7.2, on the antigen-presenting cell and CD28 and CTLA-4 on the reacting T cell. If a costimulatory signal is not received, the T cell undergoes apoptosis instead of activation (12). Adhesion molecules, including LFA-1 and ICAM-1, further promote immune recognition and T-cell stimulation by docking B and T cells such that effective antigen presentation and T-cell stimulation occur (9, 19, 20). Antibodies directed against LFA-1 or ICAM-1 block lysis of target cells by CTLs and abrogate lymphokine-activated killer cell and natural killer-mediated cell killing *in vitro* (8, 9, 21). Thus, each of these surface molecules, MHC class I, MHC class II, B7.1, B7.2, LFA-1, and ICAM-1, plays a major role in T-cell activation and the generation of cell-mediated immunity.

Our results suggest that loss of immune recognition, adhesion, or costimulatory molecules on tumor cells is a mechanism by which B-cell lymphomas inhibit host cytotoxic T-cell responses. Every tumor with a low host T-TIL response also had loss of expression of at least one HLA, CAM, or CSM important for B-cell-T-cell interaction and cytotoxic T-cell generation. In fact, in the majority of patients with low T-TIL responses, not just one but several HLA, CAM, or CSM were undetectable (a mean of 2.67 molecules lost in each low T-TIL tumor). This suggests that evading host T-TIL responses is a multifactorial process involving the progressive loss of several surface molecules necessary for T-cell recognition and activation.

Others have also found that loss of immunoregulatory molecules effects lymphoma biology and prognosis (22, 23). Terol *et al.*(22) found that absent or weak ICAM-1 expression predicted disseminated (stage IV) disease and poor survival in several subtypes of NHL, including 61 patients with DLCL. They noted that ICAM-1 maintained prognostic importance within the subset of patients with stage IV disease and thus suggested that ICAM may play other important biological roles. Our results are consistent with this hypothesis as we found ICAM expression to play an important role in generating host T-TIL responses.

Terol *et al.* (23) also published results on LFA-1 expression in 64 DLCL specimens. Their results of 41% LFA loss are in excellent agreement with our own results, in which 32% of DLCL specimens lost LFA expression (23). Interestingly, they did not find that LFA expression predicted overall or relapse-free survival. In our cases, LFA expression was the most common defect observed, yet it was not associated with low T-TIL response and thus may explain the failure of LFA to predict prognosis.

Dorfman *et al.* (24) analyzed CSM expression on NHL specimens and found low levels of expression on all germinal center-derived lymphomas examined using a highly sensitive immunohistochemical technique. The low level of B7 expressed on the surface of malignant B cells was nonetheless functional and able to prevent T-cell anergy but was insufficient to initiate T-cell proliferation *in vitro* (24). Only 7 specimens with DLCL were analyzed in their study, as compared with 70 patients in our series. Thus, the increased number of specimens with undetectable B7.1 and B7.2 expression found in our series may be related to the increased number of patients analyzed, the selection of all stages of NHL for our study, and antibody clone selection. For each specimen examined, we similarly analyzed B cells from tonsillar tissue and confirmed positive CSM expression in germinal center B cells, thus verifying the sensitivity of our assay method. The association we found between undetectable B7.2 expression and T-TIL response may reflect ineffective B7 function including loss of T-cell proliferation as suggested by Dorfman *et al.* (24) or an inability to recruit T cells.

In the literature, there are also reports in which lymphoma ICAM or LFA-1 expression does not predict prognosis (25, 26). These results may be due to imbalances in important clinical prognostic features such that clinical outcome relative to the biological variable could not be accurately tested. In our series, we have chosen a biological end point (T-TIL response) to avoid the limitations that confounding prognostic variables might have on a relatively small group of patients ($n = 71$) analyzed retrospectively. Differences in immunohistochemical technique including paraffin or fresh tissue and choice of antibody clone might also account for the variability in results, as suggested by Medeiros *et al.* (26). The importance of our findings to the clinical outcome of patients with B-cell NHL awaits the results from prospective clinical trials involving uniformly staged and treated patients.

The mechanism for progressive loss of immune molecules in DLCL is not known but is important for determining optimal immunorestorative therapies. Because the genes for these molecules are located on different chromosomes, allelic loss is an unlikely mechanism to explain our results. Others have found that loss of HLA-DR expression in lymphomas is not associated with gene deletions but rather with transcriptional inhibition (27). Transcriptional or translational inhibition is an attractive hypothesis for explaining aberrant expression because altered expression or binding of a single regulatory factor that targets a common enhancer or promoter sequence can inhibit the transcription and expression of several proteins. Whereas allelic loss may be best corrected by gene therapy strategies, transcriptional and posttranscriptional events may be corrected with cytokine therapies (28). We are currently pursuing additional studies to determine the mechanism by which malignant B cells alter surface molecular expression.

Our results predict that therapies designed to increase expression of a single immunoregulatory molecule will be successful in only a minority of cases. Interferon-α (IFN-α) 32 has been linked to restoration of LFA-1α (CD11a) and ICAM-1 (CD54) expression on malignant B cells (29, 30) and enhanced susceptibility to CTL killing in hairy cell leukemia (29). From our results, 17 of 71 (24%) patients with DLCL have altered expression of MHC class I, ICAM-1, and/or LFA-1α, and, of these, 59% have additional HLA, CAM, or CSM losses as well. Thus, the multitude and heterogeneity of altered HLA, CAM, and CSM expression in DLCL may be one reason for the disappointing and conflicting results of IFN-α

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treatment in B-cell NHL (31–33). More effective antitumor responses may be obtainable by designing therapies that target more immunoregulatory molecules.

To summarize, the results presented in this study suggest that in B-cell DLCL, a deficient host T-TIL response is a multifactorial event characterized by combined loss of cellular adhesion, recognition, and costimulation molecules. Our results suggest that a combination of immunorestorative cytokine or gene therapies may be required to generate effective antitumor responses in B-cell lymphoma.

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