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CD40 Ligand Is Constitutively Expressed in a Subset of T Cell Lymphomas and on the Microenvironmental Reactive T Cells of Follicular Lymphomas and Hodgkin's Disease

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Although CD40 has been extensively studied in B- and T-cell non-Hodgkin's lymphomas (NHLs)/leukemias, and more recently in Hodgkin's disease (HD), little is known about the expression of its ligand (CD40L) in lymphoproliferative disorders other than T-cell NHLs/leukemias. A series of 121 lymphoma/leukemia samples, including 35 cases of HD, 34 T-cell and 39 B-cell NHLs, 2 cases of adult T-cell leukemia/lymphoma, and 11 cases of T-cell acute lymphoblastic leukemia, were evaluated for CD40L expression by immunostaining of frozen tissue sections and flow cytometry with the anti-CD40L monoclonal antibody M90. CD40L was constitutively expressed by neoplastic cells in 15 of 36 (42%) T-cell NHLs/adult T-cell leukemia/lymphomas, almost invariably those displaying the $CD4^+/CD8^-$ phenotype, whereas no CD40L-expressing tumor cells could be found in B-cell NHL and HD. Among T-cell acute lymphoblastic leukemias, CD40L was detected only on 2 cases displaying a stem-cell-like phenotype. In follicular B-cell lymphomas a large number of CD40L-expressing $CD3^+/CD4^+$ T lymphocytes were found admixed with tumor cells within the neoplastic follicles and in their surrounding areas. In the nonfollicular B-cell lymphomas, CD40L-positive $CD3^+/CD4^+$ T lymphocytes were few or absent. In all HD subtypes other than the nodular lymphocytic predominance, CD40L-expressing $CD3^+/CD4^+$ T lymphocytes were numerous in the HD-involved areas and were mainly located in close proximity to the Reed-Sternberg cells. Our data indicate that in human lymphomas CD40L is preferentially expressed by a restricted subset of T-cell lymphomas, mostly with CD4 immunophenotype. Finally, we have provided morphological evidence that CD40L may play an important role in the cell contact-dependent interaction of tumor B-cells ($CD40^+$) within the neoplastic follicles or Reed-Sternberg cells ($CD40^+$) in HD-involved areas and the microenvironmental $CD3^+/CD4^+/CD40L^+$ T lymphocytes. (Am J Pathol 1995, 147:912–922)

CD40 is a cellular antigen involved in B cell growth and differentiation.[1] It is a member of the tumor necrosis factor/nerve growth factor receptor superfamily,[2,3] acting as a receptor for a specific ligand (CD40L).[1,4–6] This latter is an activation antigen of T cells with homology to ligands for other receptors of the tumor necrosis factor/nerve growth factor superfamily.[3] Whereas CD40 is constitutively expressed on the surface of normal, virally transformed, and malignant peripheral blood and tonsillar B cells,[1,2,7–9] CD40L is transiently and predominantly expressed by activated T lymphocytes of CD4 phenotype and by a small proportion of $CD8^+$ T cells.[1,4–6,10] $CD4^+$ T cells are capable of specifically activating normal and neoplastic B cells through cell

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contact-dependent interactions mediated by the binding of CD40L to the CD40 molecule.[11–13] Engagement of CD40 by CD40L triggers a number of biological events in B lymphocytes including proliferation,[1] cytokine production,[1,12–14] immunoglobulin secretion,[1,14] isotype switching,[1,4,12] homotypic adhesion,[1,12,13] and inhibition of the programmed cell death in germinal center B cells.[15] Most of these effects need the co-stimulation of additional cytokines secreted by $CD4^+$ T cells.[1,4,12–14]

In tissue sections of lymphoid organs, anti-CD40 monoclonal antibodies (MAbs) show a pan-B reactivity[7] and stain interdigitating dendritic cells[7] and tissue or isolated follicular dendritic cells.[7,16,17] Moreover, Reed-Sternberg (RS) cells of Hodgkin's disease (HD)[18,19] and virtually all B-cell non-Hodgkin's lymphomas (NHL)/leukemias,[8,9,19] but not T-cell neoplasms, express CD40.[19] Conversely, with the use of a specific MAb against CD40L (T-cell-B-cell-activating molecule),[11] it has been demonstrated that CD40L-expressing cells are $CD3^+$/$CD4^+$ T lymphocytes preferentially located in the mantle zone and germinal center light zone of secondary follicles in all peripheral lymphoid tissues.[11] $CD40L^+$ cells can also be identified within the interfollicular T-cell areas of secondary lymphoid tissues and the medulla and cortex of normal thymus.[11] Furthermore, a number (~30%) of T-cell NHLs with a relatively mature phenotype ($CD4^+$, $CD8^-$) has been shown to constitutively express CD40L.[20]

Although CD40 has been extensively studied on B- and T-cell NHLs/leukemias, myeloma plasma cells,[21] and more recently on HD,[18,19] little is known about the expression of its specific ligand in tumor cells of lymphoproliferative disorders other than T-cell NHLs/leukemias.[20] Therefore, we have investigated CD40L expression in a well characterized series of HD, follicular and diffuse B-cell NHLs in addition to T-cell NHL samples. Cases of adult T-cell leukemia/lymphoma and T-lineage acute lymphoblastic leukemias (T-ALL) were also included in the study. Expression of CD40L was assessed by staining with the recently generated anti-CD40L MAb M90, which recognizes cell surface expressed human CD40L.[4,22]

Owing to the accumulating evidence that growth of malignant lymphoid cells may be regulated by a complex network of cytokine- and cell contact-dependent interactions with non-neoplastic cells of the lymphoid microenvironment,[23,24] we were also interested in investigating the expression of CD40L on reactive T cells in tissues involved by HD and NHL. Recent data have in fact indicated that CD40 may play a role in the regulation of RS cell expansion[19] and in the contact-dependent interactions of these cells with $CD4^+$ T lymphocytes,[18,19] accumulating as reactive cells in HD tissues.[25,26] Similarly, significant functional and microenvironmental homologies of neoplastic follicles in follicular lymphomas with the reactive lymphoid follicles have been described,[27,28] and growth stimulation of follicular lymphoma cells after CD40 ligation was also reported.[29] It appeared therefore important to define the pattern of CD40L expression on T lymphocytes within the neoplastic nodules of follicular B-cell lymphomas, as compared with diffuse lymphomas. For these reasons, CD40L expression on the microenvironmental reactive lymphocytes and their nature and relationship to the RS cells of HD or tumor B cells of follicular and diffuse lymphomas were also assessed.

Materials and Methods

Tissue Samples

The study included tissue samples of 35 cases of HD covering all histological subtypes and 73 cases of NHL including 34 T-cell NHLs and 39 B-cell NHLs. Nonspecific reactive lymphadenopathies (15 cases) were also examined. The cases that formed the basis of the study were retrieved from the files of the Division of Pathology, Centro di Riferimento Oncologico, Aviano, Italy. Tissues were fixed in Bouin solution or neutral buffered formalin. In all cases a portion of unfixed tissue was snap-frozen in liquid nitrogen and stored at −80°C. Pathological specimens with B-cell NHL were classified according to the working formulation for NHL. The diagnosis of T-cell NHL was based on a correlative morphological, immunophenotypic, and when necessary genotypic analysis. They were categorized as reported elsewhere.[20] NHL cases were classified as shown in Table 1. The Rye modification of the Lukes and Butler classification was used to classify HD.[30]

Cryopreserved bone marrow and peripheral blood cells from 11 cases of T-ALL and 2 cases of acute T-cell leukemia/lymphoma in leukemic phase were also included in the study and retrieved from the files of the Leukemia Unit, Centro di Riferimento Oncologico, Aviano, Italy.

Immunohistochemistry

Deparaffinized and cryostat sections were used for immunophenotyping and lineage assignment of lymphoma cases with MAbs (βF1, CD1a, CD3, CD4, CD5, CD8, CD9, CD10, CD15, CD19, CD20, CD21, CD22, CD24, CD30, CD38, CD43, CD45, CD45RA,

Table 1. *Expression of CD40 Ligand and CD40 Receptor Protein in Hodgkin's and non-Hodgkin's Lymphomas/Leukemias*

Histological diagnosis	No. of positive/tested cases CD40L	CD40
NHLs		
B-cell type	0/39	39/39
Small lymphocytic	0/7	7/7
Follicular predominantly small cleaved cell	0/6	6/6
Follicular mixed, small cleaved cell and large cell	0/4	4/4
Follicular predominantly large cell	0/2	2/2
Diffuse small cleaved cell	0/6	6/6
Diffuse large cell	0/8	8/8
Immunoblastic	0/3	3/3
Small noncleaved cell	0/2	2/2
Monocytoid B cell	0/1	1/1
T-cell type	15/34	0/34
Small lymphocytic	0/1	0/1
Cutaneous T-cell lymphomas		
Mycosis fungoides	9/14	0/14
Non-mycosis fungoides	2/5	0/5
Lymphoblastic	1/8	0/8
Peripheral T-cell lymphomas	3/6	0/6
Adult T-cell leukemia/lymphoma	0/2	0/2
T-ALL	2/11	0/11
HD*	0/35	35/35
Lymphocyte predominance†	0/5	5/5
Nodular sclerosis	0/19	19/19
Mixed cellularity	0/10	10/10
Lymphocyte depletion	0/1	1/1

*The expression of B-cell-associated markers on RS cells was found in 11 HD cases (4 lymphocyte predominance, 6 nodular sclerosis, and 1 mixed cellularity), whereas the expression of T-cell-associated markers was found in 2 cases, all of the nodular sclerosis subtype. The RS cells in the remaining 22 HD cases were classified as of undetermined (non-B and non-T, 21 cases; T and B, 1 case) phenotype.

†The lymphocyte predominance subset included 4 cases of the nodular variant and 1 case of the diffuse variant.

CD45R0, CD68, CD74, CDw75, LN3, MB2, DBB42, DBA44, OPD4, DRC-1, Leu8, anti-k and -λ immunoglobulin (Ig) light chains, epithelial membrane antigen, vimentin, and cytokeratin (MNF116). Sources and specificities of the antibodies used in this study have been reported in detail previously.[31–33] Immunohistochemistry was performed with the avidin-biotin-peroxidase complex or alkaline phosphatase anti-alkaline phosphatase (APAAP) methods as previously described.[34,35]

Staining with CD40 and CD40L

Anti-CD40 MAb 89 (IgG1; kindly provided by Dr. J. Banchereau, Centre de Recherche Schering-Plough, Dardilly, France) and anti-CD40L MAb M90 (IgG1; kindly provided by Richard J. Armitage, Immunex Research and Development Corp., Seattle, WA) were applied to frozen sections from all cases included in the study. Immunohistochemistry was performed by the APAAP technique. Negative control experiments were performed by incubating sections with irrelevant isotype-matched mouse Ig and by omitting the primary antibody. A subclone of Jurkat T cells isolated in our laboratory[19] and showing a constitutive expression of CD40L was used as a positive control for CD40L expression (Figure 1).

Representative B-cell lymphoma cases and reactive lymph nodes were also studied either by comparing serial sections immunostained with CD40L, CD40, CD3, CD4, CD8, and CD22 or by two-color immunohistochemical staining (CD40L plus CD22 and CD22 plus CD3). Double immunohistochemistry assays were performed on cryostat tissue sections as follows: anti-CD40L or anti-CD22 MAbs were applied at working dilution for 1 hour at room temperature and sections were then immunostained by the APAAP method with naphthol AS-MX phosphate along with Fast Red TR salt for the development of alkaline phosphatase. The sections were then incubated overnight with anti-CD22 or anti-CD3 MAbs, respectively, immunostained by the avidin-biotin complex method with diaminobenzidine as substrate for the development of peroxidase, and counterstained with hematoxylin. In representative HD cases, double immunohistochemistry analysis showing CD4 plus CD15 or CD40L plus CD15 stainings were also performed.

A semiquantitative analysis was performed on frozen tissue sections for CD40L-expressing microenvironmental lymphocytes in B-cell NHL and HD. In each case the mean number of CD40L-expressing lymphocytes was derived from the number of CD40L-expressing lymphocytes counted in a total of 10 neoplastic nodules (in follicular NHL) or in a total of 20 fields at ×40 magnification (in diffuse NHL) or from the number of CD40L-expressing lymphocytes directly surrounding each RS cell on a total of 100 RS cells counted (in HD).

Flow Cytometry

MAbs used to immunophenotype NHL (see above), including anti-CD40L MAb M90 along with additional antibodies recognizing CD6, CD34, CD13, and CD33 antigens, were employed for direct and indirect immunofluorescence of isolated leukemia cells in suspension as previously described.[33] As a second step, reagents fluorescein isothiocyanate-conjugated (Technogenetics, Milan, Italy) or phycoerythrin-conjugated (Immunotech, Marseille, France) $F(ab')_2$ fragments of goat anti-mouse Ig (heavy plus light chains) were employed as appro-

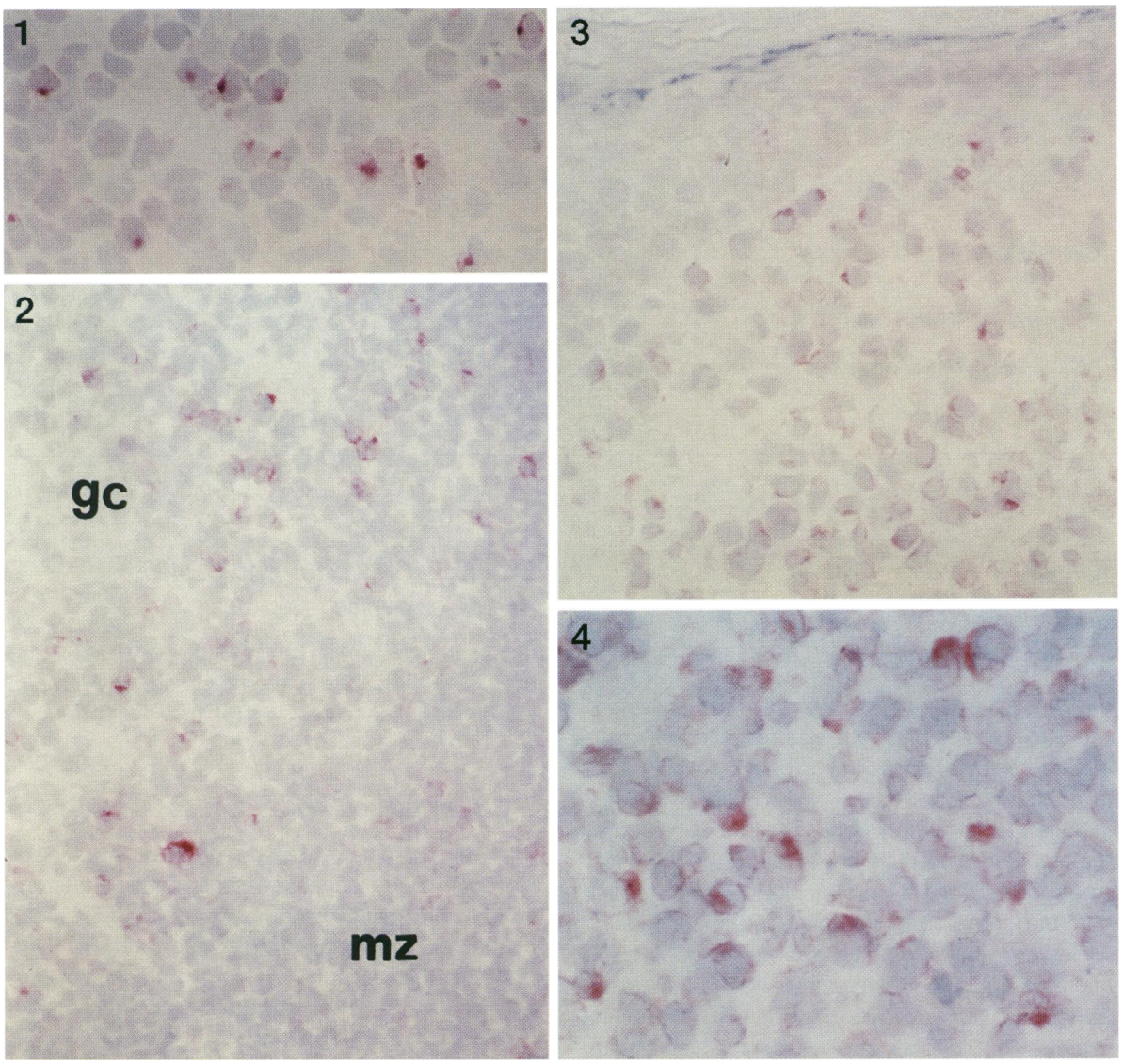

Figure 1. *Jurkat leukemic T-cell clone showing a constitutive expression of CD40L. A significant number of cells are strongly stained with anti-CD40L MAb M90 displaying a prominent dot-like pattern. Original magnification, ×250.*
Figure 2. *CD40L expression in frozen tissue section of human reactive lymph node with anti-CD40L MAb M90 and the APAAP method. CD40L positivity is manifested as dot-like or membrane staining on isolated small lymphocytes localized in the mantle zone (mz) and germinal center (gc) of a secondary follicle. Original magnification, ×250.*
Figure 3. *Immunohistochemical demonstration of CD40L expression in a non-mycosis-fungoides cutaneous T-cell lymphoma employing the APAAP method on a frozen tissue section. Tumor cells show a dot-like and membrane staining pattern with anti-CD40L MAb M90. Original magnification, ×250.*
Figure 4. *Immunohistochemical demonstration of CD40L expression in a peripheral T-cell lymphoma employing the APAAP method on a frozen tissue section. Most neoplastic cells show cytoplasmic immunoreactivity with the anti-CD40L MAb M90. Original magnification, ×400.*

priate. For two-color immunofluorescence cells were first incubated with anti-CD40L MAb M90 followed by phycoerythrin-conjugated anti-mouse Ig and subsequently with fluorescein isothiocyanate-conjugated anti-CD4, -CD7, -CD2, -CD3, and -CD5 (Becton Dickinson, Mountain View, CA). Nonspecific binding of MAbs was assessed by labeling cells with unconjugated or phycoerythrin-conjugated isotype-matched control mouse Igs (Becton Dickinson). Viable, antibody-labeled cells were identified according to their forward and right angle scattering, electronically gated, and analyzed for surface fluorescence on a FACScan flow cytometer (Becton Dickinson).

A minimum of 5000 cells were analyzed for each sample. Antibody staining was considered positive when more than 20% of tumor cells exhibited fluorescence intensity greater than that of 96% of cells stained with isotype-matched negative control antibody.

Table 2. *Expression of CD40 Ligand in T-Cell Non-Hodgkin's Lymphomas/Leukemias According to Their Immunophenotypes*

Histological diagnosis	Phenotype	Total	Positive
Small lymphocytic	$CD4^+$, $CD8^+$	1	0
Cutaneous T-cell lymphomas			
Mycosis fungoides	$CD4^+$, $CD8^-$	14	9
Non-mycosis fungoides	$CD4^+$, $CD8^+$	1	0
	$CD4^-$, $CD8^-$	2	1
	$CD4^+$, $CD8^-$	2	1
Lymphoblastic	$CD4^+$, $CD8^+$	1	0
	$CD4^-$, $CD8^-$	3	1
	$CD4^+$, $CD8^-$	4	0
Peripheral T-cell lymphomas	$CD4^+$, $CD8^+$	1	0
	$CD4^+$, $CD8^-$	5	3
Adult T-cell leukemia/lymphoma	$CD4^+$, $CD8^-$	2	0
T-ALL	$CD4^-$, $CD8^-$	5	2
	$CD4^+$, $CD8^+$	4	0
	$CD4^+$, $CD8^-$	2	0

Results

Expression of CD40L and CD40 in Non-Neoplastic Lymph Nodes

In frozen tissue sections from reactive human lymph nodes, expression of CD40L was restricted to small lymphocytes. These cells displayed a prominent dot-like pattern or punctate paranuclear staining, whereas a small number of positive cells exhibited a membrane staining pattern with the MAb M90 (Figure 2). $CD40L^+$ cells were localized in the mantle zone and germinal center light zone of secondary follicles, thus paralleling the distribution of $CD4^+$ T lymphocytes in the follicles as well as the follicular dendritic cell network stained by the DRC-1 MAb. In several of the follicles, positive cells accumulated along the boundaries between the germinal center and the mantle zone. Usually numerous CD40L-expressing cells were also immunodetected within the interfollicular and paracortical areas.

Combined analysis of serial sections and two-color immunohistochemistry indicated that CD40L expression was restricted to a fraction (no more than 20%) of $CD3^+/CD4^+$ T lymphocytes within secondary follicles. The interfollicular and paracortical areas contained a greater number of CD40L-expressing $CD3^+/CD4^+$ T lymphocytes (data not shown).

As expected, the anti-CD40 MAb showed a pan-B reactivity, staining follicular mantle and germinal center B cells, in addition to interdigitating dendritic cells. $CD40^+$ follicular dendritic cells tended to localize in the light zone of the germinal centers and progressively decreased in number toward the mantle zone (data not shown).

Expression of CD40L and CD40 in NHLs/Leukemias

Immunoreactivity with anti-CD40L MAb M90 and anti-CD40 MAb 89 in the total series of NHLs and leukemias is summarized in Table 1.

T-Cell NHLs/Leukemias

The well characterized panel of 34 different T-cell NHLs included cutaneous T-cell lymphoma (19 cases), T-cell lymphoblastic lymphoma (LBL; 8 cases), peripheral T-cell lymphoma (6 cases), and small lymphocytic T-cell neoplasm (1 case). Immunophenotypic analysis revealed different T-cell phenotypes ($CD4^+/CD8^-$, $CD4^+/CD8^+$, and $CD4^-/CD8^-$) (see Table 2). Overall, 15 of the 34 T-cell lymphomas (44%) expressed immunodetectable CD40L, whereas the CD40 antigen was not evident in any of the T-cell lymphomas investigated (Table 1). Most of the CD40L-expressing T-cell lymphomas (13/15) were $CD4^+$, $CD8^-$ neoplasms; they included 9 of 14 cases of $CD4^+/CD8^-$ mycosis fungoides (64%), 3 of 5 cases of $CD4^+/CD8^-$ peripheral T-cell lymphoma, and 1 case of non-mycosis fungoides cutaneous T-cell lymphoma. The remaining 2 $CD40L^+$ T-cell lymphomas (1 non-mycosis fungoides cutaneous T-cell lymphoma and 1 LBL) displayed the $CD4^-/CD8^-$ phenotype (Table 2).

The LBL tumor cells were characterized by a $\beta F1^+$, $CD3^+$, $CD5^+$, $CD43^+$, $CD45R0^+$, $MB2^+$, $CD4^-$, $CD8^-$, $CD1a^-$, $CD10^-$, $CD38^-$ phenotype. Seven of eight LBLs, the T-cell small lymphocytic lymphoma, and the two acute T-cell leukemia/lymphoma cases with $CD4^+$ phenotype, were $CD40L^-$

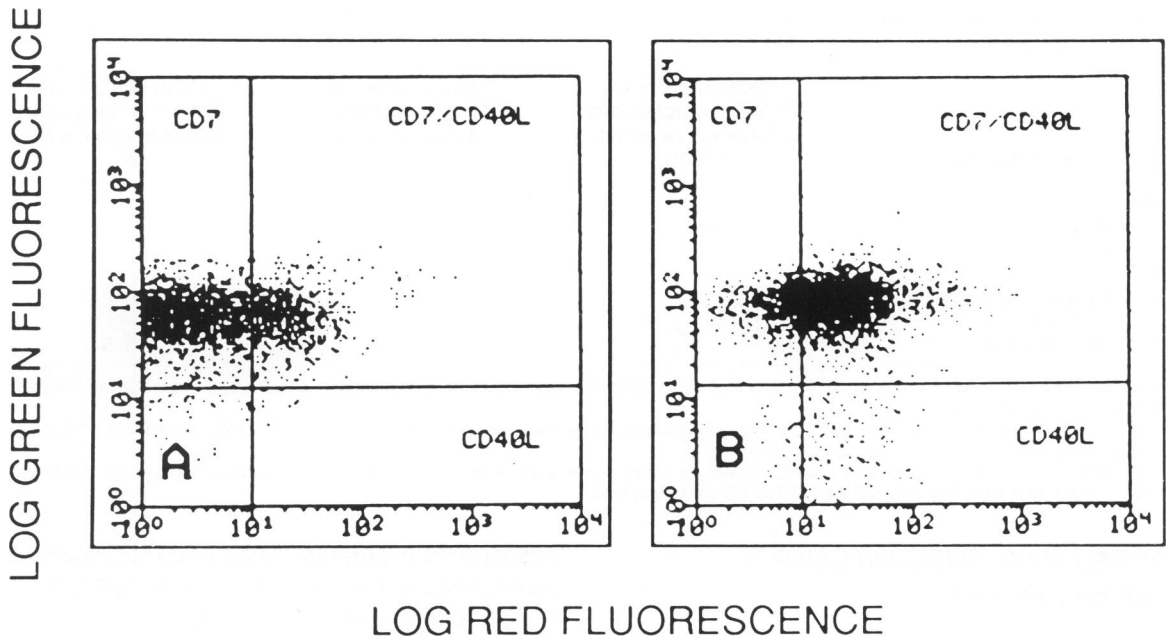

Figure 5. *Expression of CD40L by human leukemic T cells as detected by two-color immunofluorescence. Cells were incubated with anti-CD40L MAb M90 followed by phycoerythrin-conjugated anti-mouse immunoglobulins and subsequently with fluorescein isothiocyanate-conjugated anti-CD7 MAbs. Four regions are identified by setting quadrant markers at the highest levels of nonspecific fluorescence. The* x *axis represents log-green fluorescence; the* y *axis represents log-red fluorescence. Double-positive cells are identified in the upper right region.* **A:** *Bone marrow cells from a patient with pre-T-ALL with stem-cell-like phenotype (TdT$^-$, CD7$^+$, CD5$^-$, cyCD3$^+$, CD34$^+$, DR$^+$) co-express CD7 and CD40L;* **B:** *Peripheral blood cells from a patient with pre-T-ALL with a stem-cell-like phenotype (TdT$^-$, CD7$^+$, CD5$^+$, cyCD3$^+$, CD34$^+$, DR$^-$) co-express CD7 and CD40L.*

(Table 2). In all of the CD40L$^+$ lymphoma cases, a definite membrane surface and/or cytoplasmic immunoreactivity for CD40L was detected in most neoplastic cells (Figures 3 and 4). Tumor cells from most (9/11) of the T-ALL cases analyzed, encompassing pre-T (TdT$^+$, CD7$^+$, cyCD3$^+$), early T (TdT$^+$, CD7$^+$, cyCD3$^+$, CD2$^+$), and intermediate (CD1a$^+$, CD4$^+$/CD8$^+$) or mature (CD1a$^-$, CD4$^+$/CD8$^-$) thymic phenotypes, did not show surface CD40L (Tables 1 and 2). Conversely, anti-CD40L MAb M90 stained blast cells from two cases of acute lymphoblastic leukemia of T lineage (Figure 5) classified as T stem cell leukemia[36,37] on immunological grounds (TdT$^-$, CD7$^+$, CD5$^-$, cyCD3$^+$, CD34$^+$, DR$^+$ or TdT$^-$, CD7$^+$, CD5$^+$, cyCD3$^+$, CD34$^+$, DR$^-$). In both cases tumor cells lacked CD40, CD1a, CD2, CD4, and CD8 and were negative for myeloid antigens (CD13, CD33, or cytoplasmic myeloperoxidase) but displayed TCR-γ or TCR-γ and -β rearrangements (not shown), ruling out the alternative diagnosis of CD7$^+$ acute myeloid leukemia.

B-Cell NHL

The study included 39 cases of B-cell NHL covering the most frequent histological subtypes according to the working formulation. As shown in Table 1, tumor cells of all B-cell NHLs were immunostained by anti-CD40 MAbs but did not express immunodetectable CD40L. Conversely, a variable number of non-neoplastic lymphoid cells in areas involved by the lymphomas were CD40L$^+$, as shown by immunohistochemistry (Table 3). Combined serial section and two-color immunohistochemistry analysis showed that, in both follicular and diffuse NHLs, CD40L$^+$ lymphoid cells were CD3$^+$/CD4$^+$ T lymphocytes (data not shown). In follicular lymphomas a large number of CD40L-expressing T cells were immunodetected within the neoplastic follicles and in their surrounding areas (Figure 6). In particular, the mean number of T lymphocytes stained by the anti-CD40L MAb M90 in neoplastic nodules of follicular lymphoma cases ranged from 40 to 200 (Table 3). As shown by a double immunostaining of the neoplastic follicles with anti-CD22 and M90 MAbs, CD40L-expressing T lymphocytes were found to be closely admixed with CD22$^+$ tumor B cells (Figure 6). The number of CD40L$^+$ lymphoid cells was also increased in the neoplastic follicles of NHL, as compared with the amount of CD40L-expressing cells usually detected in reactive follicles (data not shown). In the nonfollicular B-cell lymphomas, CD40L$^+$ non-neoplastic lymphocytes were few or absent, ranging from 0 to 6.5 cells in a total of 20 microscopic fields examined in each single case (Table 3).

Table 3. *Semiquantitative Evaluation of CD40L-Expressing Microenvironmental Lymphocytes in B-Cell Non-Hodgkin's Lymphomas and Hodgkin's Disease*

Histological type	Mean number of $CD40L^+$ lymphocytes within each neoplastic nodule*	Mean number of $CD40L^+$ lymphocytes in diffuse areas, per field†	Mean number of $CD40L^+$ lymphocytes surrounding each RS cell‡
NHLs			
Follicular	40–200		
Diffuse		0–6.5	
HD			
Lymphocyte predominance (nodular)			0–2
Other subtypes			2.5–5

*In each case the mean number of CD40L-expressing lymphocytes was derived from the number of CD40L-expressing lymphocytes in a total of 10 neoplastic nodules.

†In each case the mean number of CD40L-expressing lymphocytes was derived from the number of CD40L-expressing lymphocytes counted in a total of 20 fields at ×40 magnification.

‡In each case the mean number of CD40L-expressing lymphocytes was derived from the number of CD40L-expressing lymphocytes directly surrounding each RS cell on a total of 100 RS cells evaluated.

Expression of CD40L and CD40 in Hodgkin's Disease

Immunoreactivity of anti-CD40L MAb M90 and anti-CD40 MAb 89 in the total series of 35 HD cases, grouped according to the histological subtypes is shown in Table 1. RS cells did not express CD40L, although they strongly stained with the anti-CD40 MAb 89 in all cases of HD examined, as expected. In all of the HD subtypes other than the lymphocytic predominance subtype (nodular variant), CD40L-expressing lymphoid cells were numerous in the HD-involved areas and were mainly located in close proximity to the RS cells (Figure 7). Two-color immunohistochemistry analysis showed that only $CD4^+$ T lymphocytes expressed CD40L (data not shown). As summarized in Table 3, a mean of 2.5 to 5 $CD40L^+$ T cells surrounded a single RS cell in the non-lymphocyte-predominance HD cases. Only scattered $CD40L^+$ lymphoid cells were conversely found within the nodules of the lymphocytic predominance subtype cases (Table 3).

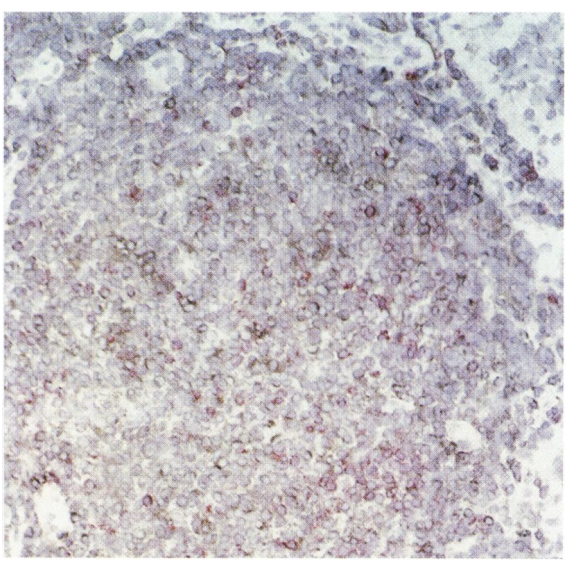

Figure 6. *Double immunostaining employing the avidin-biotin complex and APAAP methods on a frozen tissue section of a follicular B-cell lymphoma. Within a neoplastic follicle, $CD22^+$ B-cell lymphoma cells are stained brown and $CD40L^+$ T lymphocytes are stained red. Original magnification, ×250.*

Discussion

We have recently characterized by immunohistochemistry and flow cytometry the expression pattern of CD40 in human lymphomas, thus demonstrating that anti-CD40 MAbs stained consistently RS cells of HD[18,19] and neoplastic B cells but not tumor T cells from NHL.[19] Furthermore, by assessing the differential expression of CD40L in HD- and NHL-derived human cell lines we showed that only a few tumor cell lines of T-cell phenotype (HDLM2 and Karpas 299) may display CD40L mRNA by reverse transcription polymerase chain reaction.[19]

In the present report, we expand our characterization by investigating the expression and tissue distribution of CD40L in a large series of HD and B- or T-cell lymphoma/leukemia samples. In addition, we have extended our preliminary observations of CD40L expression by T lymphocytes surrounding RS cells.[18]

Anti-CD40 MAb 89 on frozen sections stained RS cells in all HD cases as well as tumor cells of all B-cell NHLs but was unreactive in all of the T-cell lymphomas (34 cases) tested. These results further corroborate the findings of our previous studies,[18,19] in which a large panel of HD and B-cell NHLs but a smaller number of T-cell lymphomas were evaluated for CD40 expression by immunostaining of paraffin-

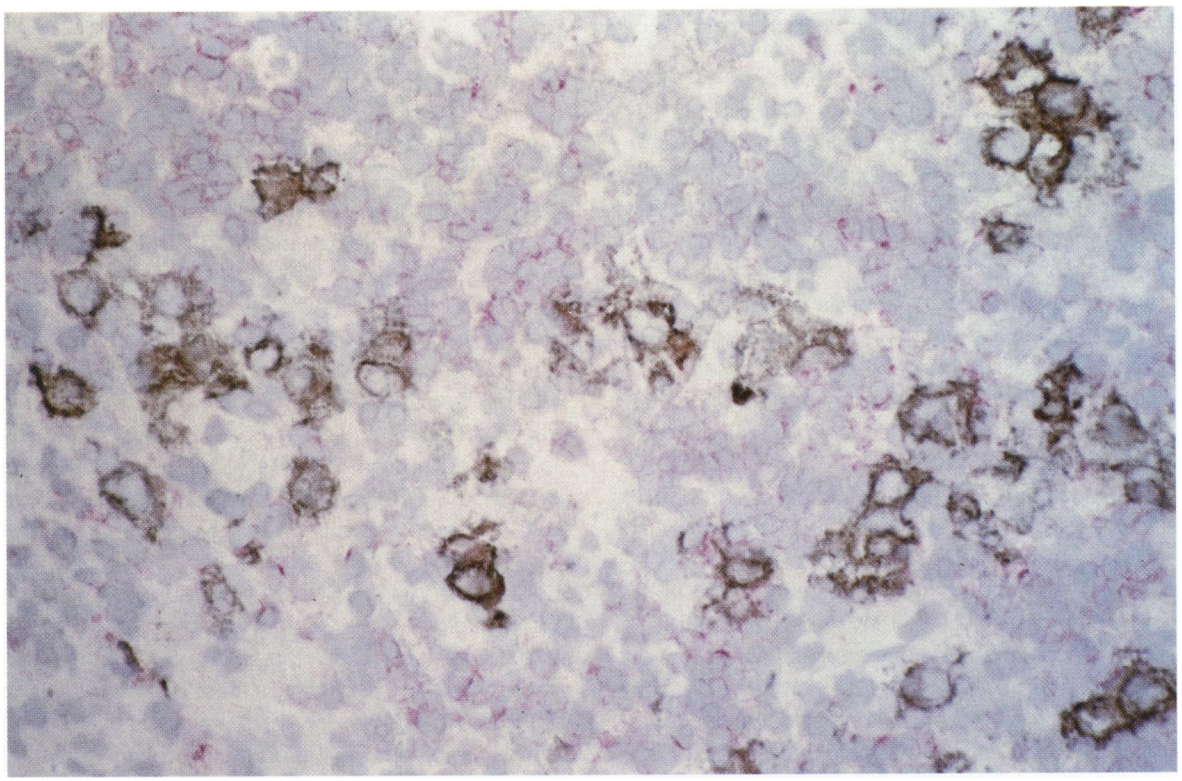

Figure 7. *Double immunostaining employing the avidin-biotin complex and APAAP methods on a frozen tissue section from a nodular sclerosing HD. CD15$^+$ RS cells are stained brown and CD40L$^+$ surrounding lymphocytes are stained red. Original magnification, ×250.*

embedded and frozen sections. On the other hand, we have shown here that CD40L, as immunodetected by the M90 MAb,[4,22] is constitutively expressed by neoplastic T cells, almost invariably displaying a CD4$^+$/CD8$^-$ phenotype. In addition, by analyzing cases of T-ALL we have shown that a restricted subset of such leukemias, previously identified as T stem cell leukemia[36,37] and characterized by a stem-cell-like phenotype (TCR-β^+ and/or -γ^+, CD34$^+$, CD7$^+$, cyCD3$^+$, CD1$^-$, CD4$^-$, CD8$^-$) and by peculiar biological and clinical features,[36,37] may express CD40L. Our data indicate therefore that, in addition to mature T cell neoplasms, specific subsets of leukemic cells of pre-T (LBL) or earlier T-cell phenotype may also display surface CD40L. Although CD40 surface expression has been detected on a subset of CD34$^+$ hemopoietic progenitors,[38] no data are so far available for the expression of CD40L by early lymphohemopoietic cells. Whether CD40L detected by us on these subsets of very immature T cell neoplasms mirrors the transformation of a normally existing progenitor or is a result of abnormal events triggered by the neoplastic change remains to be established.

In contrast with previous studies,[20] we have also investigated the expression of CD40L in frozen sections of HD and B-cell NHL, showing that no CD40L-expressing tumor cells could be found in either of these groups of neoplasms. Thus, we can conclude that in human lymphomas CD40L detected by immunohistochemistry and flow cytometry with the anti-CD40L MAb M90 is preferentially expressed by a restricted subset of mature T-cell neoplasms, most of them with a CD4$^+$ phenotype, and by rare T-cell leukemias of early stem cell phenotype. Taken together, our study largely confirms previous results obtained on a large series of T-cell NHLs with the anti-CD40L MAb 5C8.[20]

On the basis of our results it appears overall that neoplastic B cells (CD40$^+$/CD40L$^-$) and T cells (CD40$^-$/CD40L$^\pm$) mirror their putative normal cell counterparts as to the expression patterns of both CD40[1,7–9] and CD40L.[1,4,5] A significant functional difference, however, is that whereas CD40L expression in normal T lymphocytes is a transient and highly regulated process,[4,5,39] some tumor T cells may display a constitutive expression of the molecule. This finding may be particularly important in understanding lymphoid tumor growth. Although it has been shown that the CD40/CD40L co-stimulation pathway may allow for selective expansion of CD4$^+$ T cells after interactions with CD40-bearing antigen-

presenting cells[40] and that recombinant CD40L induces normal T cell proliferation,[22] the relevance of CD40L expression for cellular growth of T-cell lymphomas/leukemias still deserves additional insights.[20] Whether the constitutive expression of CD40L in some tumor T cells reflects an autocrine loop or provides a selective growth advantage by favoring cell contact-dependent interaction of tumor cells with surrounding $CD40^+$ cells and a costimulatory function remains to be elucidated. Many cellular types in the lymphoid microenvironment, including B cells,[8] monocytes,[41] and dendritic cells,[16,17] do express CD40 and are able to provide cytokines and other regulatory signals that influence tumor T cell growth.[23]

The functional significance of the expression of CD40L on reactive T lymphocytes of B-cell NHL also deserves speculation. Some of the CD40/CD40L-mediated interactions described for the normal B-cell/T-cell cross-talk[1,12,13] may in fact be operating also in lymphomas. Previous studies have also indicated that the interaction of lymphoma B cells from human follicular NHL and T helper cell clones was similar to that seen with normal B cells and T cells and that these neoplastic B cells may respond to normal activation and differentiation signals.[28] In addition to a cell contact-dependent mechanism, activated $CD4^+$ T cells may influence lymphoma growth by providing a number of cytokines involved in B cell proliferation such as interleukin (IL)-3, IL-4, IL-6, IL-9, and low or high molecular weight B cell growth factor.[23,24] Accordingly, the *in vivo* engagement of CD40 on neoplastic B cells by CD40L on activated $CD4^+$ T cells may have an important role in lymphoma cell proliferation. On the basis of our morphological findings, such a mechanism, however, appears restricted to follicular lymphomas. In this group of neoplasms a large number of CD40L-expressing $CD4^+$ T lymphocytes were found to be admixed with tumor B cells whithin the neoplastic follicles, whereas in tumor tissues of nonfollicular B-cell lymphomas, $CD40L^+$ T cells were few or absent. Our data on the distribution of $CD40L^+$ T cells further support a close functional similarity between the follicular lymphomas of supposed germinal center B cell origin and their normal counterpart. This similarity is also strengthened by the notion that these tumors tend to maintain an association with the accessory cells within the B cell areas (ie, the $DRC\text{-}1^+/CD40^+$ DRCs).[12] The combination of the functional data with our morphological findings suggests that the reactive $CD4^+/CD40L^+$ T cells in follicular lymphomas may play a role in regulating tumor growth *in vivo*. Whether the absence of $CD4^+/CD40L^+$ T cells in diffuse lymphomas indicate that tumor cells may have escaped this regulatory mechanism remains to be elucidated.

Owing to the present demonstration that also RS cells of HD express CD40 but lack CD40L, our overall results indicate that the expression of CD40 and its ligand is mutually exclusive in human lymphomas. Thus, an autocrine loop involving CD40 and CD40L appears unlikely in these disorders.

For the HD cell growth, previous data have suggested that the CD40/CD40L interaction plays a critical role in the interaction of T cells and RS cells in HD-involved tissue areas.[18,26] Furthermore, we have shown that *in vitro* rosetting of activated $CD4^+$ T cells to cultured human RS cells (L-428) is mediated in part by the CD40/CD40L adhesion pathway.[19] The data presented here show that a strong constitutive expression of CD40L can be found on reactive $CD4^+$ cells that surround RS cells *in vivo*, suggesting that in HD tissues $CD40L^+/CD4^+$ T lymphocytes might interact with tumor cells via CD40. Such interaction might result in the transmission of growth signals to RS cells. In this regard we have previously shown that recombinant CD40L promotes colony growth of HD cell lines.[19]

The almost exclusive presence of $CD40L^+$ lymphocytes surrounding RS cells in the HD subtypes other than lymphocytic predominance (nodular variant) may provide additional support for the separation of the lymphocytic predominance (nodular variant) subtype. The functional significance of the differential distribution of the $CD40L^+$ T lymphocytes among the subtypes of HD remains to be clarified.

In conclusion, we have provided morphological evidence that the CD40/CD40L pathway may play an important role in cell contact-dependent interaction of tumor B cells ($CD40^+$) within the neoplastic nodules of follicular lymphomas or RS cells ($CD40^+$) in HD-involved areas and the microenvironmental CD40L-expressing $CD3^+/CD4^+$ T lymphocytes.

References

1. Bancherau J, Bazan F, Briere F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Sealand S: The CD40 antigen and its ligand: Annu Rev Immunol 1994, 12:881–922
2. Stamenkovich I, Clark EA, Seed B: A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J 1989, 8:1403–1410
3. Smith GA, Farrah T, Goodwin RG: The tumor necrosis factor receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 1994, 76:959–962

4. Spriggs MK, Armitage RJ, Strockbine L, Clifford KN, Macduff BM, Sato TA, Maliszewski CR, Fanslow WC: Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. J Exp Med 1992, 176:1543–1550
5. Lane P, Traunecker A, Hubele S, Inui S, Lanzavecchia A, Gray D: Activated human T cells express a ligand for the human B cell-associated antigen CD40 which participates in T cell-dependent activation of B lymphocytes. Eur J Immunol 1992, 22:2573–2578
6. Hollenbaugh D, Grosmarie LS, Kullas CD, Chalupny NJ, Braesch-Andersen S, Noelle RJ, Stamenkovic I, Ledbetter JA, Aruffo A: The human T cell antigen gp39, a member of the tumor necrosis factor gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. EMBO J 1992, 11:4313–4321
7. Dörken B, Möller P, Pezzutto A, Schwartz-Albiez R, Moldenhauer G: B-cell antigens: CD40. Leucocyte Typing IV: White Cell Differentiation Antigens. Edited by W Knapp, B Dörken, WR Gilks, EP Rieber, RE Schmidt, H Stein, AEGK Von Dem Borne. Oxford, Oxford University Press, 1989, pp 90–91
8. Uckun FM, Gajl-Peczalska K, Myers DE, Jaszcz W, Haissig S, Ledbetter JA: Temporal association of CD40 antigen expression with discrete stages of human B-cell ontogeny and the efficacy of anti-CD40 immunotoxins against clonogenic B-lineage acute lymphoblastic leukemia as well as B-lineage non-Hodgkin's lymphoma cells. Blood 1990, 76:2449–2456
9. Law C-L, Wörmann B, LeBien TW: Analysis of expression and function of CD40 on normal and leukemic human B cell precursors. Leukemia 1990, 4:732–738
10. Lederman S, Yellin MJ, Krichevsky A, Belko J, Lee JJ, Chess L: Identification of a novel surface protein on activated $CD4^+$ T cells that induces contact-dependent B cell differentiation (help). J Exp Med 1992, 175:1091–1101
11. Lederman S, Yellin MJ, Inghirami G, Lee JL, Knowles DM, Chess L: Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles: roles of T cell-B cell activating molecule (5c8 antigen) and CD40 in contact-dependent help. J Immunol 1992, 149:3817–3826
12. Clark EA, Ledbetter JA: How B and T cells talk to each other. Nature 1994, 367:425–428
13. Nishioka Y, Lipsky P: The role of CD40-CD40 ligand interaction in human T cell-B cell collaboration. J Immunol 1994, 153:1027–1036
14. Armitage RJ, Macduff BM, Spriggs MK, Fanslow WC: Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. J Immunol 1993, 150:3671–3680
15. Liu Y-J, Mason DY, Johnson GD, Abbot S, Gregory CD, Hardie DL, Gordon J, MacLennan ICM: Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. Eur J Immunol 1991, 21:1905–1910
16. Schriever F, Freedman AS, Freeman G, Messner E, Lee G, Daley J, Nadler LM: Isolated human follicular dendritic cells display a unique antigenic phenotype. J Exp Med 1989, 169:2043–2058
17. Caux C, Massacrier C, Vanbervliet B, Dubois B, Van Kooten C, Durand I, Banchereau J: Activation of human dendritic cells through CD40 cross-linking. J Exp Med 1994, 180:1263–1272
18. Gruss H-J, Hirschstein D, Wright B, Ulrich D, Caligiuri MA, Strockbine L, Armitage RJ, Dower SK: Expression and function of CD40 on Hodgkin and Reed-Sternberg cells and the possible relevance for Hodgkin's disease. Blood 1994, 842305–2314
19. Carbone A, Gloghini A, Gattei V, Aldinucci D, Degan M, De Paoli P, Zagonel V, Pinto A: Expression of functional CD40 antigen on Reed-Sternberg cells and Hodgkin's disease cell lines. Blood 1995, 85:780–789
20. Inghirami G, Lederman S, Yellin MJ, Chadburn A, Chess L, Knowles DM: Phenotypic and functional characterization of T-BAM (CD40 ligand)$^+$ T-cell non-Hodgkin's lymphoma. Blood 1994, 84:866–872
21. Westendorf JJ, Ahmann GJ, Armitage RJ, Spriggs MK, Lust JA, Greipp PR, Katzmann JA, Jelinek DF: CD40 expression in malignant plasma cells: role in stimulation of autocrine IL-6 secretion by a human myeloma cell line. J Immunol 1994, 152:117–128
22. Armitage RJ, Tough TW, Macduff BM, Fanslow WC, Spriggs MK, Ramsdell F, Alderson MR: CD40 ligand is a T cell growth factor. Eur J Immunol 1993, 23:2326–2331
23. Hsu S-M, Waldron JM Jr, Hsu P-L, Hough AJ Jr: Cytokines in malignant lymphomas: review and prospective evaluation. Hum Pathol 1993, 24:1040–1047
24. Ford RJ, Tamayo A, Li DJ, Cabanillas F: Human B cell lymphomas: *in vitro* and *in vivo* studies on growth factors and cell growth. Leukemia Lymphoma 1993, 10(suppl):51–56
25. Poppema S: The nature of the lymphocytes surrounding Reed-Sternberg cells in nodular lymphocyte predominance and in other types of Hodgkin's disease. Am J Pathol 1989, 135:351–357
26. Kadin ME: Pathology of Hodgkin's disease. Curr Opin Oncol 1994, 6:456–463
27. Gloghini A, Carbone A: The non-lymphoid microenvironment of reactive follicles and lymphomas of follicular origin as defined by immunohistology on paraffin-embedded tissues. Hum Pathol 1993, 24:67–76
28. Umetsu DT, Esserman L, Donlon TA, DeKruyff RH, Levy R: Induction of proliferation of human follicular (B type) lymphoma cells by cognate interaction with $CD4^+$ T cell clone. J Immunol 1990, 144:2550–2557
29. Johnson PWM, Watt SM, Betts DR, Davies D, Jordan S, Norton AJ, Lister TA: Isolated follicular lymphoma cells are resistant to apoptosis and can be grown *in vitro* in the CD40/stromal cell system. Blood 1993, 82:1848–1857
30. Lukes RJ, Craver LF, Hall TC, Rappaport H, Rubin P:

Report of the Nomenclature Committee. Cancer Res 1966, 26:1311
31. Carbone A, Gloghini A, Volpe R: Paraffin section immunohistochemistry in the diagnosis of Hodgkin's disease and anaplastic large cell ($CD30^+$) lymphomas. Virchows Arch A Pathol Anat 1992, 420:527
32. Carbone A, Gloghini A, Volpe R: Immunohistochemistry of Hodgkin's and non-Hodgkin's lymphomas with emphasis on the diagnostic significance of the BNH9 antibody reactivity with anaplastic large cell (CD30 positive) lymphomas. Cancer 1992, 70:2691–2698
33. Pinto A, Gloghini A, Gattei V, Aldinucci D, Zagonel V, Carbone A: Expression of the c-*kit* receptor in human lymphomas is restricted to Hodgkin's disease and $CD30^+$ anaplastic large cell lymphomas. Blood 1994, 83:785–792
34. Hsu S-M, Raine L, Fanger H: A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am J Clin Pathol 1981, 75:734–738
35. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, Macdonald S, Pulford KAF, Stein H, Mason DY: Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal antialkaline phosphatase (APAAP complexes). J Histochem Cytochem 1984, 32:219–229
36. Kurtzberg J, Waldmann TA, Davey MP, Bigner SH, Moore JO, Hershfield MS, Haynes BF: $CD7^+$, $CD4^-$, $CD8^-$, acute leukemia: a syndrome of malignant pluripotent lymphohematopoietic cells. Blood 1989, 73: 381–390
37. Ferrara F, Cimino R, Antinolfi I, De Rosa C, Mele G, Mettivier V, Fusco C, Del Vecchio L: Clinical relevance of immunological dissection in T-ALL: a report on 20 cases with stem cell ($CD7^+$, $CD4^-$, $CD8^-$, $CD1^-$) phenotype. Am J Hematol 1992, 40:98–102
38. Saeland S, Duvert V, Caux C, Pandrau D, Favre C, Vallé A, Durand I, Charbord P, de Vries J, Bancherau J: Distribution of surface-membrane molecules on bone marrow and cord blood $CD34^+$ hematopoietic cells. Exp Hematol 1992, 20:24–29
39. van Kooten C, Vaillard C, Galizzi JP, Hermann P, Fossiez F, Banchereau J, Blanchard D: B cells regulate expression of CD40-ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. Eur J Immunol 1994, 24:787–792
40. Cayabyab M, Phillips JH, Lanier LL: CD40 preferentially costimulates activation of $CD4^+$ T lymphocytes. J Immunol 1994, 152:1523–1531
41. Wagner DH Jr, Stout RD, Suttles J: Role of CD40-CD40 ligand interaction in $CD4^+$ T cell contact-dependent activation of monocyte interleukin-1 synthesis. Eur J Immunol 1994, 24:3148–3154