The differential expression of LCK and BAFF-receptor and their role in apoptosis in human lymphomas

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Background and Objectives. We explored the expression of LCK and BAFF-R (B-cell activating factor receptor) both of which are known to play a role in signaling and apoptosis, in routine tissue biopsies. It was hypothesized that their expression patterns might yield information on apoptosis as it occurs in normal and reactive lymphoid cells, and also be of value for the detection of lymphoma subtypes.

Design and Methods. Both molecules were studied in paraffin-embedded tissue sections and cell lines by immunoperoxidase staining, and were also studied by western blotting. Human tonsillar B-cell subsets were analyzed by flow cytometry for LCK expression.

Results. LCK was detected for the first time in germinal centers and, at lower levels, in mantle zone B cells. The presence of LCK in B cells was confirmed by western blotting. Cross-linking surface IgM reduced LCK expression whereas cross-linking surface CD40 appeared to have the opposite effect. BAFF-R was present on mantle zone B cells but absent or weakly expressed in germinal center cells. Most lymphomas of germinal center origin (e.g. follicular lymphoma) and also many mantle cell lymphomas, chronic lymphocytic leukemia (CLL) and most T-cell neoplasms expressed LCK. In contrast, BAFF-R was expressed in a variety of B-cell lymphomas, but often absent in grade 3 follicular lymphomas and diffuse large B-cell lymphomas (DLBCL). Both LCK-positive and BAFF-R-positive DLBCL tended to be of *germinal-center* phenotype.

Interpretation and Conclusions. The reciprocal expression pattern of LCK and BAFF-R in germinal center and mantle zone B cells may reflect their opposing roles in apoptosis. Their detection in lymphoma tissue biopsies may therefore be of clinical relevance in predicting response to treatment.

Key words: LCK, BAFF-R, lymphoma. western blotting, immunohistochemistry.

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There is evidence from many studies that one contributing factor to lymphoid neoplasia is increased resistance to cellular apoptosis. The first well-documented example (aberrant expression of the anti-apoptotic protein BCL2 in lymphoma), was followed by the identification of many other protein abnormalities, comprising both the acquisition of anti-apoptotic molecules and the loss of proteins that promote this type of cell death.[1-3] Furthermore, defective apoptosis may be relevant not only to the initial transforming step but also to the subsequent response to chemotherapy. This is reflected by the fact that the expression of anti- or pro-apoptotic proteins has been correlated with clinical progress in many lymphoid neoplasms, including diffuse large B-cell lymphoma (DLBCL),[4,5] mantle cell lymphoma,[6,7] lymphoma,[8,9] mucosal associated lymphoid tissue (MALT) lymphoma,[10,11] myeloma[12,13] and Hodgkin's disease.[14]

In this study, we analyzed the expression in routine lymphoma biopsies of two other lymphoid-associated molecules that are known to play a role in signaling and apoptosis. Our underlying hypothesis was that their expression might throw new light on the susceptibility to apoptosis of normal and reactive lymphoid cells, and possibly aid the detection of lymphoma subtypes and the identification of signaling pathways relevant to pathogenesis and therapy. One of these molecules is the tyrosine kinase LCK, a protein that has been extensively studied over many years because of its role in signal transduction after antigen binding but which has only recently been shown to play an important role in promoting apoptosis.[15-19] In the present study, we report for the first time that LCK is present at high levels in normal germinal center cells, a site at which many B cells undergo apoptosis, and we also document its expression in lymphoid neoplasms. The second apoptosis-related marker that we have been able to detect in routine biopsy samples is the receptor for B cell-activating factor (BAFF-R), a member of the tumor necrosis factor (TNF) family-receptors. This molecule was first identified as one of three receptors that bind the B-cell survival factor of the TNF-family or BAFF (also known as BLyS, TALL-1, THANK and zTNF4). BAFF-R regulates the survival of resting B cells,[20-22] in part through protecting cells from apoptosis.[23] BAFF-R has been detected by flow cytometry in non-Hodgkin's lymphoma[24,25] and myeloma,[26] and more recently two studies demonstrated its

expression by immunohistochemistry using two different anti-BAFF-R antibodies.[25,27] We report here that BAFF-R shows a reciprocal expression pattern in normal B cells to LCK, in keeping with their opposing roles in B-cell apoptosis. We also documented its expression in neoplastic B cells. Since these two molecules can be detected in paraffin-embedded tissue, we suggest that it would be of interest to investigate retrospectively their relationship to clinical behavior in uniformly treated B-cell lymphomas.

Design and Methods

Tissue samples

Paraffin-embedded sections of normal lymphoid tissues (tonsil, thymus, spleen, *Toxoplasma* lymphadenitis and bone marrow) were obtained from the routine diagnostic service of the authors' institutions. Cryostat sections of reactive tonsil (obtained from the routine diagnostic service) were also used for studying the two antibodies. The lymphoma samples were studied as sections of tissue arrays containing 0.6-1 mm cores[28] or as sections of routinely processed biopsies (i.e. seven cases of T-cell-rich B-cell lymphoma, five cases of lymphoma, four cases of MALT lymphoma and four cases of hairy cell leukemia). A total of 417 lymphomas were studied for LCK and 394 for BAFF-R. They covered a broad spectrum of subcategories and had been diagnosed on the basis of conventional histologic and immunohistologic criteria according to the WHO classification.[29] Biopsies were also studied from cases of small lymphocytic lymphoma/chronic lymphocytic leukemia (CLL) that had been investigated previously for *IgH* mutation (*Campo E, unpublished*).

Antibodies

Two antibodies against LCK were evaluated: a monoclonal antibody (Cat. No. 05-435) from Upstate (Dundee, UK) and a polyclonal rabbit affinity purified reagent (Cat. No. 2752) from Cell Signaling Technology (Boston, MA, USA). Both antibodies gave very similar results, but the latter antibody, raised against a peptide sequence in the carboxyl-terminal region of the molecule, was chosen for this study since it worked at a higher dilution. Antibody to BAFF-R (Cat. No. ab5965), a rabbit polyclonal reagent raised against a peptide sequence, was obtained from Abcam (Cambridge, UK). Two antibodies against Zap-70 were used: a polyclonal rabbit (Cat. No. 2705) from Cell Signaling Technology and a monoclonal antibody from Upstate (Cat. No. 05-253). Other antibodies used in this study were monoclonal anti-BCL6 (Dr. G. Roncador, CNIO, Madrid, Spain), anti-MUM-1/IRF-4 (Prof. B Falini, Istituto di Ematologia, Policlinico Monteluce, Perugia, Italy), anti-CD10 (NCL-CD10-270, Novocastra, UK), anti-CD40 (clone G28-5),[30] anti-IgD and anti-human CD38 (both from BD Pharmingen, San Jose, CA, USA), anti-nucleophosmin (J. Cordell, NDCLS, Oxford, UK) and anti-BCL2.[31] The following polyclonal antibodies were used: goat anti-actin (sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-human IgM F(ab')$_2$ fragments (anti-IgM) (Jackson Immunoresearch, West Grove, PA, USA) and goat anti-rabbit IgG (Cat. Nr. 4050-095, Southern Biotech, Birmingham, AL, USA).

Immunohistochemistry

The antibodies were tested at a range of dilutions in PBS or TBS containing 10% human serum (to minimize possible non-specific binding) and then used at a concentration that gave background-free selective cellular labeling. The protocols for antigen retrieval and staining have been described previously.[32] A peroxidase-based Envision™ method[33] was used to stain the cell pellets and cytospin preparations of cell lines. Each staining was scored as negative, positive, weakly positive or not evaluable. The analysis and scoring of tissue arrays was aided by microscope-associated SpotBrowser®V2 software (Alphelys, Plaisir, France) allowing immediate recording of data and images in electronic format. Output data were compatible with Cluster and TreeView programs,[34] which enabled hierarchical cluster analysis to be performed in order to illustrate the relationship between a number of molecules in lymphoma subtypes.

Cell lines

The source of two T-cell lymphoma-derived cell lines (CCRF-CEM and Jurkat) and techniques for cell culture have been described previously.[32] B-cell lymphoma-derived cell lines were obtained from the Sir William Dunn School of Pathology, Oxford, UK (lines FL-18 and Raji) and from Dr. R.E. Davis, Center for Cancer Research, NCI, Bethesda, USA (lines SU-DHL-4, SU-DHL-6, SU-DHL-10, OCI-Ly3 and OCI-Ly10). Cells were cultured as previously described.[32] Cytospin preparations of cell lines were prepared and fixed in acetone for 10 min before air-drying overnight at room temperature and storage at -20°C.

Functional studies of LCK in isolated human tonsillar B cells

Dense B lymphocytes were isolated from human tonsils from different individual donors by sheep erythrocyte rosetting to deplete T cells followed by Percoll density gradient centrifugation, as previously described.[35] These purified dense human tonsillar B cells were then treated for 0 to 24 hr with medium, goat anti-human IgM F(ab')$_2$ fragments (anti-IgM) or anti-CD40 antibodies. Dense human tonsillar B cells (1×10^6 per sample) were suspended in PBS/2% BSA and incubated for 30 min on ice in a total volume of 200 μL containing 20 μL FITC-conjugated anti-human IgD and 0.25 μL PerCP-Cy5.5-conjugated anti-human CD38. Cell surface-stained cells were fixed in 200 μL PBS/1% paraformaldehyde for 60 min with shaking at room temperature. After washing, cells were permeabilized with freshly prepared 0.1% saponin in PBS/2% fetal calf serum for 30 min on ice. Finally, cells were incubated with 150 ng rabbit anti-LCK polyclonal antiserum or control rabbit antiserum for 30 min on ice, washed and incubated with 0.5 μg RPE-conjugated goat anti-rabbit IgG. Cells were washed and fixed with PBS/1% paraformaldehyde prior to analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Data were analyzed using Cellquest software.

Western blotting

Protein extracts from the above-mentioned cell lines were analyzed by western blotting for LCK and BAFF-R, using a protocol described elsewhere.[32] Cell line lysates were blot-

ted for nucleophosmin as a control for equal protein loading. Whole cell lysates from approximately 10×10^6 dense human tonsillar B cells, as described elsewhere,[36] were blotted with the rabbit anti-LCK serum, or anti-actin as a control for equal protein loading.

Results

Immunostaining of normal tissue biopsies for LCK and BAFF-R

The results of immunostaining routine paraffin-embedded samples of normal tissues are illustrated in Figure 1. T-cell areas in peripheral lymphoid tissue along with both cortical and medullary thymocytes were strongly positive for LCK. In reactive lymphoid tissue, LCK was also found in germinal center B cells; in contrast, B cells in mantle zones were LCK-negative or only weakly positive. However, in cryostat sections (in which molecules may be better conserved) of reactive tonsil the mantle zone B cells seemed more clearly positive than in paraffin sections although the staining was weaker than in T cells. Other B-cell populations (splenic marginal zone cells in the spleen, *monocytoid* B cells characteristic of toxoplasmosis, and plasma cells) were consistently LCK-negative. Hematopoietic cells in the bone marrow were negative for LCK and a screen of normal non-lymphohemopoietic tissue also showed no reactivity with cells other than leukocytes.

BAFF-R was found in peripheral lymphoid tissue in small B cells in the mantle zone of reactive lymphoid follicles and also marginal zone B cells in the spleen. In contrast, *monocytoid* B cells were negative. Germinal center cells were negative to weakly positive (similar results were also observed using cryostat sections of human tonsil). In the thymus, only scattered cells in the cortex and in the medulla were BAFF-R-positive.

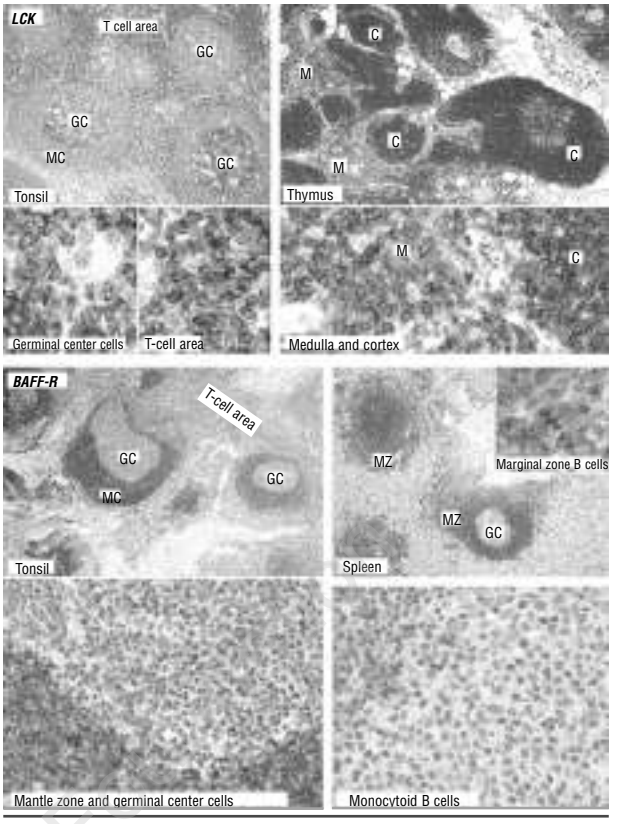

Figure 1. Immunostaining of normal hematopoietic tissue for LCK kinase and BAFF-R. LCK is expressed by T cells in tonsil in interfollicular areas and by germinal center (GC) cells. LCK is also strongly expressed by thymocytes in the cortex (C) and medulla (M). Mantle zone (MC) cells in secondary B-cell follicles in spleen and tonsil are positive for BAFF-R, whereas germinal centers (GC) are not stained, or only weakly so. Marginal zone (MZ) cells in the spleen also express BAFF-R. Monocytoid B cells in a lymph node affected by toxoplasmosis are BAFF-R-negative. All stains for LCK and BAFF-R performed, unless otherwise stated, on paraffin sections by the immunoperoxidase technique.

Immunostaining of lymphoma biopsies for LCK and BAFF-R

The expression patterns of LCK and BAFF-R in paraffin-embedded neoplastic lymphoid biopsies are summarized in Table I and illustrated in Figures 2 and 3. No correlation was noted between the expression of these two markers.

LCK

Expression of LCK among B-cell neoplasms was frequent in CLL/small lymphocytic lymphoma (10/15 cases) and mantle cell lymphoma (14/17 cases) (Figure 2). Fourteen cases of CLL were stained for Zap-70, and no clear correlation was found with LCK (since five cases co-expressed LCK and Zap-70, five lacked both markers and four expressed only LCK). However no cases were LCK-negative and Zap-70-positive. LCK was also found, but less commonly, in nodal and splenic marginal zone lymphomas and in follicular center lymphoma (Figure 2). In contrast, MALT lymphomas and hairy cell leukemia were negative for LCK (Figure 2). Among high grade B-cell lymphomas, LCK was absent in the small numbers of mediastinal B-cell lymphomas and T-cell-rich B-cell lymphomas tested, but it was detectable in a minority of DLBCL (18/86 cases). When DLBCL cases were assigned to *germinal center* and *non-germinal center* categories, using the algorithm proposed by Hans *et al.*,[37] it was evident that LCK was expressed in almost half of the germinal center group (15/31), but in only a minority (2/28) of the non-germinal center type (Figure 4). Lymphomas representing the earliest and latest stages of B cell maturation (i.e. lymphoblastic and plasmacytic neoplasms, respectively) were also negative for LCK (Figure 2), as were cases of classical and lymphocyte-predominant Hodgkin's disease (Figure 2). T-cell neoplasms, including lymphoblastic lymphoma, were frequently positive for LCK (Figure 2), in keeping with the expression of this molecule from early stages of T-cell maturation, although it is of interest that ALK-positive lymphomas (13 cases) were consistently negative (Figure 2).

BAFF-R

The majority of B-cell lymphomas showed reactivity for BAFF-R. Among small cell lymphomas, BAFF-R was present in many cases of CLL (9/15), in almost all mantle cell lymphomas (14/17), in nodal and splenic marginal zone lymphomas (26/39) and also in some cases of MALT lymphomas (5/7). In marginal zone lymphomas (nodal and extranodal) the positivity of BAFF-R was variable, being weak in a group of cases (Table 1). Among follicular center lymphomas, BAFF-R expression was inversely related to

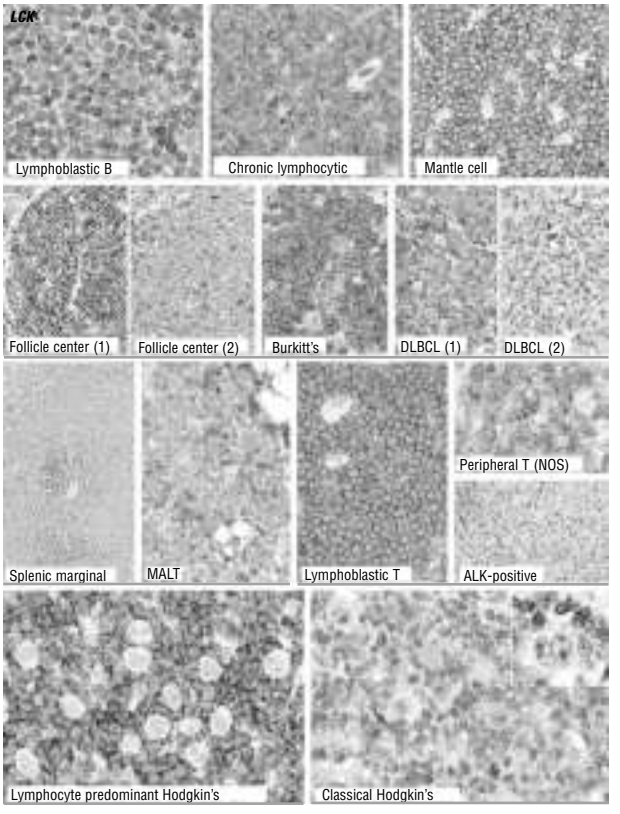

Figure 2. **Representative examples of lymphoma subtypes immunostained for LCK kinase. All performed, unless otherwise stated, on paraffin sections by the immunoperoxidase technique.**

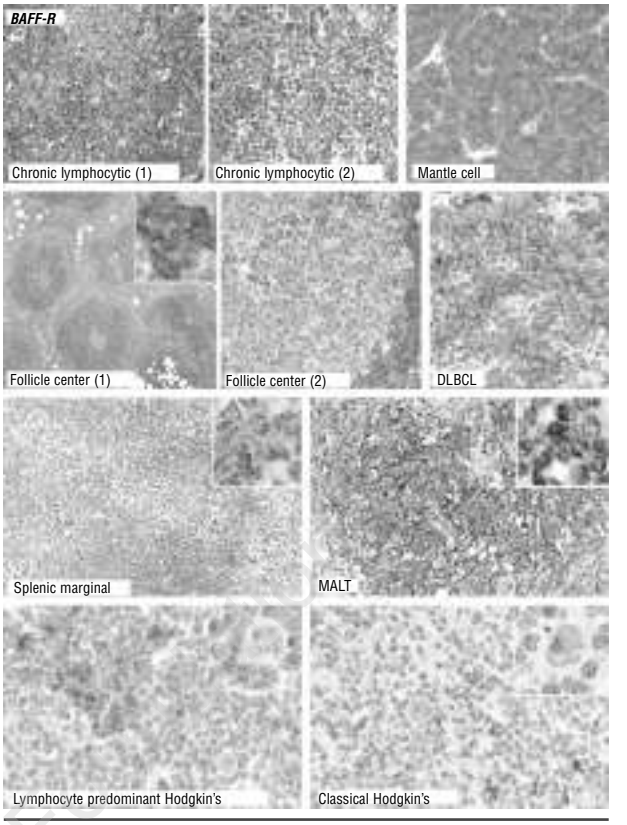

Figure 3. **Representative examples of lymphoma subtypes immunostained for BAFF-R. All stains performed, unless otherwise stated, on paraffin sections by the immunoperoxidase technique.**

histological grade, grades 1 and 2 being positive in 82% and 69% of cases, respectively, whereas only 30% of grade 3 cases expressed BAFF-R. Only a small proportion of DLBCL (all showing a germinal-center phenotype) were BAFF-R-positive (12/92). Interestingly, all Burkitt's lymphomas were BAFF-R-negative. BAFF-R was absent in T/NK-cell lymphomas and also in classical and lymphocyte-predominant Hodgkin's disease (Figure 3).

Western blotting analysis and immunostaining of cell lines

In order to investigate the specificity of the antibodies used, to provide confirmatory evidence for the results obtained from the lymphoma cases, and to identify sources of cells of potential value for future biochemical/functional studies, we analyzed the following lymphoma-derived cell lines by western blotting: FL-18 (follicular lymphoma), Raji (Burkitt's lymphoma), SU-DHL-4, -6, -10, OCI-Ly3 and OCI-Ly10 (DLBCL). A strong band of the expected size for LCK (between 50 and 60 kDa) was found in lysates of T-cell lines (Jurkat and CCRF-CEM) (Figure 5A). A band of moderate intensity was seen in the FL-18 line and of weak intensity in lysates of the SU-DHL-4 line (Figure 5A). Immunostaining of cytospin preparations for LCK showed results corresponding to the western blotting data: an example of SU-DHL-4 labeling is illustrated in Figure 5A.

BAFF-R was also investigated using the same cell lines (Figure 5B) by blotting and immunohistochemistry. A prominent band (approximately 37 kDa) (along with others) was found in the cell lysates of SU-DHL-4, SU-DHL-6 and FL-18 (Figure 5B). A weaker band of the same size (Figure 5B) was seen in the OCI-Ly10 and Raji cell lines, but this was not seen in the OCI-Ly3 line. In the lines containing the 37 kDa band, weaker bands of higher molecular

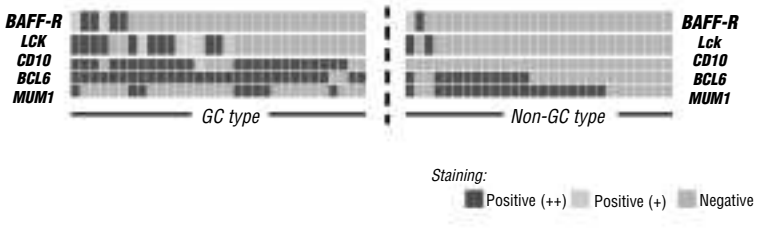

Figure 4. **Expression of LCK and BAFF-R in 59 cases of diffuse large B-cell lymphoma. These had all also been immunostained for BCL6, CD10 and MUM1 in order to assign them to germinal center (GC) and or non-GC categories, following the scheme proposed by Hans *et al*.[37] Fifteen of the 31 cases in the GC group expressed LCK. In contrast, only two of the 28 cases in the non-GC category were positive and one of these also expressed the GC-associated marker BCL6.**

Table 1. **Immunostaining of lymphoma samples for LCK and BAFF-R (positive cases/total cases).**

Neoplasm	*LCK*	*BAFF-R*
B cell non-Hodgkin's		
Lymphoblastic lymphoma/leukemia	0/5	0/6
Chronic lymphocytic leukemia/lymphoma	10/15	9/15
Mantle cell lymphoma	14/17	14/17
Follicular center lymphoma		
Grade 1	9/27	23/28
Grade 2	6/21	16/23
Grade 3	18*/56	18/60
Burkitt's lymphoma	7/20	0/12
Diffuse large B-cell lymphoma	18/86	12/92
Primary mediastinal B-cell lymphoma	0/5	1/5
T cell-rich B-cell lymphoma	0/7	0/7
Marginal zone lymphoma		
Nodal	4/14	9†/15
Splenic	4/30	17‡/24
MALT lymphoma	0/9	5§/7
Lymphoplasmacytic lymphoma	0/3	0/3
Hairy cell leukemia	0/4	0/4
Myeloma/plasmacytoma	0/5	0/5
Post-transplant lymphoproliferative disorder	0/3	0/3
T/NK cell non-Hodgkin's		
Lymphoblastic lymphoma/leukemia	10/11	0/12
Peripheral T-cell lymphoma (NOS)	11/13	0/13
Lennert's lymphoma	1/1	0/1
Angioimmunoblastic lymphoma	1/1	0/1
Natural-killer (NK) cell lymphoma	1/4	0/4
ALCL, ALK-negative lymphoma	2/5	0/5
ALK-positive lymphoma	0/13	0/10
Hodgkin's disease		
Classical	0/25	0/11
Lymphocyte-predominant	0/17¶	0/11

**In one case, approximately 50% of the tumor cells were positive. †Four out of nine cases were weakly positive. ‡Six out of seventeen cases were weakly positive. §In four out of five cases, the tumor cells were weakly positive. ¶In two cases, single cells were LCK-positive.*

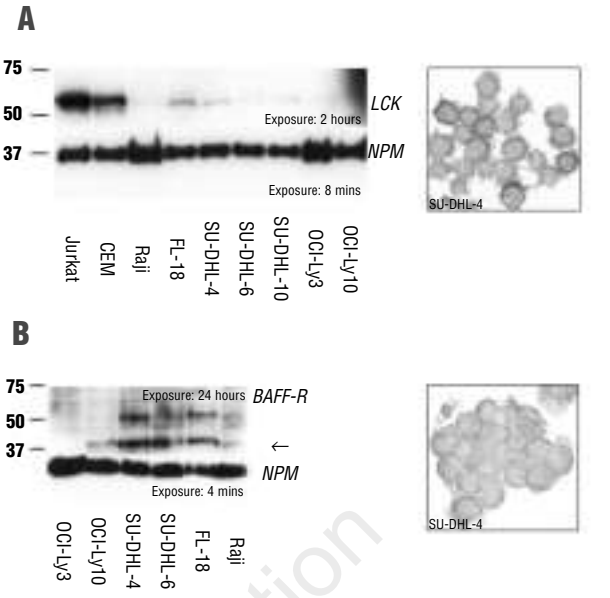

Figure 5. Western blotting and immunocytochemical labeling for LCK and BAFF-R in human cell lines. A. LCK: A strong band of the expected molecular weight was found in two T-cell-derived lymphoma lines (Jurkat and CCRF-CEM) and a band was seen in a follicular lymphoma line (FL-18) and a DLBCL line (SU-DHL-4). Much weaker bands were seen in two other DLBCL lines, SU-DHL-6 and SU-DHL-10. An example of LCK detection in a cell line by immunocytochemistry is shown on the right (immunoperoxidase, 40X). B. BAFF-R: The anti-BAFF-R antibody detected a band of approximately 37 kDa (arrow) (in keeping with the published mouse anti-BAFF-R)[27] in two germinal-center type DLBCL-derived cell lines (SU-DHL-4 and -6) and in the follicular lymphoma-derived cell line FL-18. A weaker band was found in the activated-type DLBCL-derived cell line (OCI-Ly10) and in Raji (Burkitt's-derived lymphoma cell line). On the right is an example of the SU-DHL-4 line immunostained for BAFF-R (immunoperoxidase, 40X). In both experiments blotting for the nuclear protein nucleophosmin (NPM) was used as loading control. Times of exposure of the X-ray films to the chemiluminescent detection reagent are indicated.

weight were seen (particularly of approximately 50 kDa). BAFF-R was also investigated using the same cell lines by immunohistochemistry. Strong labeling was found in the SU-DHL-4 and -6 lines, whereas the FL-18 line was weakly positive. OCI-Ly3, OCI-Ly10 and the two T-cell lymphoma-derived lines (Jurkat and CCRF-CEM) were BAFF-R-negative.

Functional studies of LCK in isolated human tonsillar B cells

In order to complement our immunohistochemical analysis of tissues (Figure 1), immunoblotting of lysates of highly purified human tonsillar B cells with anti-LCK was performed, revealing a band of the expected size for LCK (Figure 6A). To determine the relative levels of LCK in distinct dense human tonsillar B-cell subsets, B cells were surface stained with anti-IgD and anti-CD38, and LCK levels quantified by intracellular staining. Each B-cell subset expressed readily detectable levels of LCK, with $CD38^{+}IgD^{-}$ germinal center B cells expressing the highest LCK levels (Figure 6B), consistent with the immunostaining data (Figure 1). Moreover, LCK expression increased in cells after CD40-ligation whereas B-cell antigen receptor (BCR) cross-linking (cells stimulated with anti-IgM $F(ab')_2$ fragments) consistently lowered LCK expression (Figure 6A). An apparent reduction of LCK expression was seen in the absence of any stimuli at 24 hours, compared to 0 hr, but this may reflect higher rates of spontaneous loss through apoptosis in germinal center B cells compared to either naïve or memory B-cell populations.

Discussion

LCK: its role and expression in lymphoid cells

LCK was first documented in the 1980s as a member of the Src family of non-receptor tyrosine kinases.[38,39] Early studies revealed that it associates with the cytoplasmic domains of CD4 and CD8,[40] and subsequent investigations showed that it is inactive in resting cells (because of phosphorylation of a C terminal tyrosine), but that it can be activated by CD45-mediated dephosphorylation.[41,42] Studies of LCK in lymphoid cells have focused largely on T cells, but there have been a few studies indicating that LCK can also be found in B cells. For example, there are independent reports that LCK can physically associate in B cells

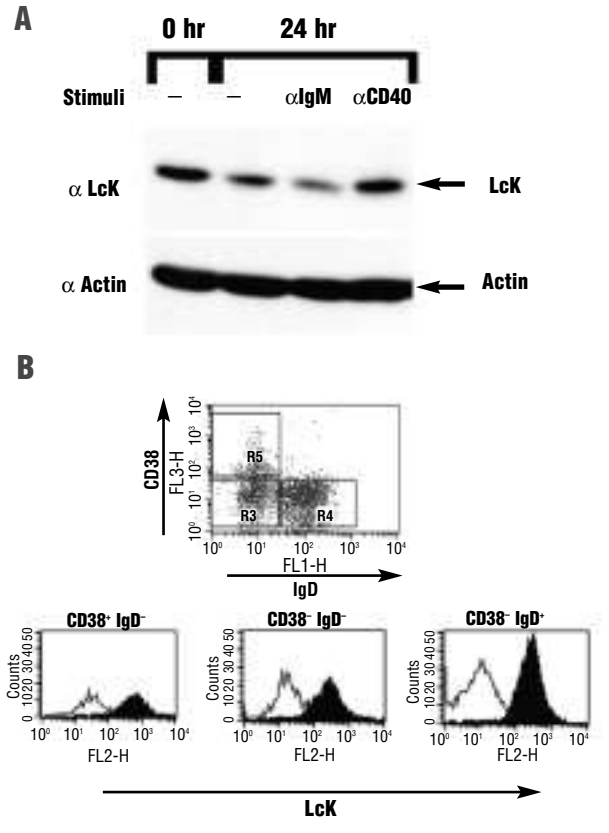

Figure 6. A. BCR and CD40 engagement regulates LCK expression in human tonsillar B cells. Highly purified dense human tonsillar B cells were treated for 0 or 24 hr with medium (–), anti-IgM ($F(ab')_2$) or anti-CD40 antibodies. RIPA cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose. Immunoblots were probed with either anti-LCK or anti-actin as a control for equal protein loading. B. LCK expression in human tonsillar B cell subsets. Dense human tonsillar B cells were surface-stained with anti-IgD-FITC and anti-CD38-PerCPCy5.5, permeabilized with 0.1% saponin and stained for intracellular LCK with rabbit anti-LCK (or rabbit control serum) in combination with goat anti-rabbit IgG-RPE. The dot plot shows the surface expression levels of IgD (horizontal axis) and CD38 (vertical axis), and the histograms depict either LCK expression (black bars) or staining with the control serum (white bars) on $CD38^+IgD^-$, $CD38^-IgD^-$, and $CD38^-IgD^+$ cell populations.

with membrane Ig, with CD19 and with CD20 (although the latter association may be indirect).[16,43,44] However the subtypes of B cell that may express LCK are not clearly defined. It has been reported that LCK is detectable by proteomic analysis of nuclear proteins at a pro-B cell stage (but not in later cells).[45] While some evidence suggests LCK is selectively expressed by B cells of the B1 subset,[46,47] a recent study found that mouse B2 B cells express more LCK than do B1 B cells.[48] Furthermore, there are also reports that LCK expression can be induced when B cells are activated/stimulated, e.g. by infection with Epstein-Barr virus or BCR cross-linking.[46,47,49-51] Our experiments showed that expression of LCK increases in human tonsillar B cells after CD40-ligation and decreases in BCR-stimulated B cells (Figure 6A). Furthermore, FACS analysis of intracellular/cell surface staining of different human tonsillar B-cell subsets confirmed that germinal center B cells express the highest levels of LCK although detectable levels of LCK were also found in IgD^+CD38^-(naïve) and in IgD^-CD38^+ (memory) B cells (Figure 6B). These results are consistent with those of Frances *et al.*,[48] who detected LCK in splenic B2 follicular B cells.

Although initial studies focused on the role of LCK in lymphoid cell activation, more recent investigations have uncovered an important role for this kinase in apoptosis initiated by various stimuli.[15,16,18,19] From a clinical point of view it is of particular interest that apoptosis, in response to ionizing radiation and anti-cancer drugs, is LCK-dependent.[17,52] Furthermore, it has recently been reported that LCK-deficient lymphoid cells are resistant to apoptosis induced by a number of unrelated chemotherapeutic agents, and that this defect is reversed when LCK is re-expressed.[53] Our own observations of LCK expression in normal lymphoid tissues show for the first time that this molecule is strongly expressed by B cells in germinal centers (whereas it is weakly expressed by resting small B cells). A report that B cells require LCK to enter the S phase may be of relevance[54] since many germinal center B cells are proliferating. Furthermore, expression of the *LCK* gene is induced by the transcriptional co-activator OCA-B (BOB.1), a factor needed to generate germinal centers.[55] However, it is also possible that our findings indicate a role for LCK in the high level of apoptosis that occurs in germinal center B cells.

We also detected expression of LCK in a spectrum of B-cell neoplasms. Our observation that it is found in many cases of CLL correlates with two previous reports of its presence in this neoplasm.[46,56] It is of interest that LCK and CD5 are both expressed in this disorder but found at lower levels in its putative non-neoplastic counterpart, the mantle zone B cell. It is therefore possible that a shared mechanism accounts for this co-ordinated up-regulation of both molecules. It may be added that our analysis of a small number (no=14) of CLL cases for Zap-70 (a marker for poor prognosis cases with unmutated IgH genes) revealed no correlation with LCK expression.

There have been no previous studies of protein expression in lymphoid neoplasms other than CLL, although LCK protein has been detected in Burkitt's-derived and lymphoblastoid cell lines,[56] and three microarray studies have shown expression of mRNA encoding LCK in a number of fresh samples of B-cell lymphomas of different subtypes.[57-59] A finding of major interest in the present study is that LCK was often absent in lymphomas arising from germinal centers, despite its strong expression in the normal counterpart of these tumors. Thus, LCK was expressed in no cases of lymphocyte-predominant Hodgkin's disease (0/17) and in only 32% of follicular lymphomas (33/104), 35% of Burkitt's lymphomas (7/20), and 48% of DLBCL of putative germinal center subtype (15/31). It is therefore possible that the loss of LCK expression in neoplastic germinal center cells represents one process by which these cells escape elimination by apoptosis.

BAFF-R: its role and expression in lymphoid cells

The TNF family ligand BAFF (BLys, TALL-1, THANK and zTNF4) is a type II transmembrane molecule, expressed either as a cell surface protein or in soluble form.[60-62] Three receptors for BAFF have now been identified, BAFF-R, TACI and BCMA, each apparently serving a different function in B cells.[63,64] BAFF-R stands out in that it plays a major role in B-cell survival, and mice defective for BAFF-R do not devel-

op mature B cells.[63,64] Thus, it was of particular interest to determine how BAFF-R is expressed in B-cell neoplasms.

Investigations in BAFF and BAFF-R knock-out mice have shown that this ligand receptor is not needed for the formation of germinal centers but that these structures are rapidly attenuated (and follicular dendritic cell development and immune complex trapping are abnormal)[65] and that antibody responses are reduced.[21] Other studies have demonstrated that BAFF enhances the survival of B cells,[20-22] and this appears to depend on anti-apoptotic signals via BAFF-R (in concert with signaling from the BCR)[23] including down-regulation of the pro-apoptotic protein Bim.[66] Of more importance in a clinical context are observations that B-cell neoplasms, including B-CLL and myeloma, are resistant to apoptosis by virtue of the binding of BAFF to its receptor.[26,67,68]

Our immunohistological findings of strong staining of small B cells in tissue sections (Figure 3) are in keeping with recent flow cytometry experiments showing that BAFF-R is present on human peripheral B cells,[25,69] and with recent immunohistological studies using different monoclonal antibodies.[25,27] Its absence (or only weak expression) on germinal center B cells has not been previously reported (with the exception of the recent studies from Nakamura *et al.*[27] and Rodig *et al.*[25]), but is in keeping with the concept that this receptor's major role is in the survival of peripheral B cells (i.e. corresponding to mantle zone cells) and that its down-regulation on germinal center cells facilitates the process of apoptosis at this site. It is also of interest that normal splenic marginal zone cells were strongly positive for BAFF-R (Figure 1), since there is experimental evidence from murine studies that BAFF-R engagement is needed to generate this B cell population.[21,23]

Flow cytometric studies of all histological subtypes of non-Hodgkin's lymphomas have shown a variable degree of BAFF-R expression,[24] and it has also been reported that BAFF-R is present on myeloma and CLL cells.[24-26,68,70] There is evidence that in CLL and myeloma, the neoplastic cells can also secrete BAFF, thereby promoting neoplastic plasma cell growth through an autocrine anti-apoptotic mechanism.[26,68,70] Although BAFF-R expression in neoplastic cells has not previously been explored by immunohistological techniques in lymphoid neoplasms, we and two other groups recently investigated BAFF-R expression by immunohistochemistry using different antibodies;[25,27] the data obtained in the three studies were similar. In the present study, BAFF-R was investigated in 394 cases of lymphoid neoplasia and we showed that BAFF-R is expressed in small cell lymphomas. Its strong expression in CLL and mantle cell lymphoma may reflect the fact that BAFF-R is strongly expressed in the putative normal cell counterpart of those lymphomas, namely mantle zone B cells. This is also true for marginal zone lymphomas whose positivity for BAFF-R is in keeping with this receptor's expression in normal splenic marginal zone cells. A different scenario is observed among follicular center lymphomas. BAFF-R was expressed in almost all cases of grade 1 follicular center lymphoma (in contrast to its absence or weak expression on normal germinal center cells) but it tended to be absent in many cases (70%) of grade 3 follicular center lymphoma and in DLBCL (six of the eight BAFF-R-positive cases showed a germinal center phenotype).

This tendency to lose BAFF-R is compatible with a recent report that BAFF-R was expressed in only two of six cases of grade 3 follicular lymphoma,[27] contrasting with very frequent expression in grade 1 and 2 cases.[27] One explanation is that grade 3 follicular lymphoma is a different entity in terms of molecular pathogenesis from DLBCL of germinal center origin, one of its characteristics being a tendency to lose BAFF-R. It may be relevant that the meshwork of follicular dendritic cells, a cell type known to produce BAFF (the ligand for BAFF-R),[71] is prominent in grade 1 and 2 follicular lymphoma but tends to be lost in grade 3 disease.

Potential clinical relevance of LCK and BAFF-R expression

The presence of LCK in many CLL cases contrasted with its observed absence in the small number of MALT lymphomas studied. If this is a consistent finding, it might be exploited in the diagnosis of small B-cell neoplasms. Furthermore, its differential expression in these two neoplasms may contribute to their contrasting clinical and biological features. In addition, the fact that both LCK and BAFF-R are variably expressed in different categories of B-cell lymphoma may prove of value for a better understanding of signaling pathways (e.g. activated following ligand binding) in tumoral and normal B-cells and might be relevant to the response of these tumors to chemotherapy, given the known involvement of these molecules in B-cell apoptosis. For example, our observation that a number of B-cell neoplasms arising from germinal centers lose LCK expression suggests that it would be of interest to see whether this loss correlates with resistance to chemotherapy, for example, to establish whether LCK expression in DLBCL of germinal center subtype is associated with poorer response to treatment or overall survival. Since the antibodies used in this study work reliably on routine biopsies, the correlation between LCK and/or BAFF-R expression and clinical behavior could easily be investigated in a retrospective study of archival material. Furthermore, if treatment strategies that specifically target such pathways are devised in the future, the ability to detect components such as those documented in this paper may prove of direct clinical relevance.

JCP and ST contributed equally to the work reported in this paper. TM and DYM conceived and designed the study, interpreted the data and, together with M-LH and EAC, wrote the manuscript; EC and SP provided samples relevant to this study; JCP, ST and MJ performed the immunostaining; JCP, HR and GC performed the western blotting of cell lines; TM and ML-H reviewed the immunostaining; AC and EAC were involved in the isolation of human B cells and FACS analysis. The final version of this manuscript was approved by all the authors.

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