Origin and pathogenesis of nodular lymphocyte–predominant Hodgkin lymphoma as revealed by global gene expression analysis

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The pathogenesis of nodular lymphocyte–predominant Hodgkin lymphoma (NLPHL) and its relationship to other lymphomas are largely unknown. This is partly because of the technical challenge of analyzing its rare neoplastic lymphocytic and histiocytic (L&H) cells, which are dispersed in an abundant nonneoplastic cellular microenvironment. We performed a genome-wide expression study of microdissected L&H lymphoma cells in comparison to normal and other malignant B cells that indicated a relationship of L&H cells to and/or that they originate from germinal center B cells at the transition to memory B cells. L&H cells show a surprisingly high similarity to the tumor cells of T cell–rich B cell lymphoma and classical Hodgkin lymphoma, a partial loss of their B cell phenotype, and deregulation of many apoptosis regulators and putative oncogenes. Importantly, L&H cells are characterized by constitutive nuclear factor κB activity and aberrant extracellular signal-regulated kinase signaling. Thus, these findings shed new light on the nature of L&H cells, reveal several novel pathogenetic mechanisms in NLPHL, and may help in differential diagnosis and lead to novel therapeutic strategies.

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Abbreviations used: BL, Burkitt lymphoma; B-NHL, B cell non-HL; cHL, classical HL; DLBCL, diffuse large B cell lymphoma; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FC, fold change; FDR, false discovery rate; FL, follicular lymphoma; GC, germinal center; HL, Hodgkin lymphoma; HRS, Hodgkin and Reed/Sternberg; L&H, lymphocytic and histiocytic; NLPHL, nodular lymphocyte–predominant HL; PCA, principal component analysis; TCRBL, T cell–rich B cell lymphoma; UBD, ubiquitin D.

Hodgkin lymphoma (HL), one of the most common malignant lymphomas, comprises two entities, classical HL (cHL) and nodular lymphocyte–predominant HL (NLPHL). The tumor cells—Hodgkin and Reed/Sternberg (HRS) cells in cHL and lymphocytic and histiocytic (L&H) cells in NLPHL—usually represent <1% of cells in the tumor, being dispersed in a prominent inflammatory cellular background. Tumor cells of both entities are derived from germinal center (GC) B cells, but they are genetically, morphologically, and phenotypically different. HRS cells often carry "crippling" mutations in originally functional IgV gene rearrangements and, thus, likely derive from preapoptotic GC B cells (1). The process of somatic hypermutation is silenced in these cells. HRS cells lack expression of most B cell molecules (2) and express markers typical for other hematopoietic lineages (1). L&H cells, on the other hand, express functional IgV genes and often show intraclonal V gene diversification (1), a feature they share with normal GC B cells. Also immunohistochemically,

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The online version of this article contains supplemental material.

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 205 No. 10 2251-2268
www.jem.org/cgi/doi/10.1084/jem.20080809

The Journal of Experimental Medicine

L&H cells resemble GC B cells, as they express BCL6 (a key transcription factor for the GC B cell program) (3), activation-induced cytidine deaminase (the enzyme critical for somatic hypermutation and class switching in GC B cells) (4), and the GC B cell markers human GC-associated lymphoma (5) and centerin (6). However, L&H cells frequently lack expression of some pan–B lineage markers, e.g., CD19, and the GC markers CD10 and PAG (7–9). GC and NLPHL also resemble each other in their follicular growth pattern and in their association with follicular dendritic cells and $CD57^+$ T helper cells. Many of these features of NLPHL are observed in follicular lymphoma (FL), too, suggesting a close relationship between these lymphomas (10).

Only very little is known about the pathogenetic mechanisms in NLPHL. Comparative genomic hybridization analysis revealed the occurrence of recurrent but nonspecific chromosomal abnormalities in NLPHL (11). Approximately 50% of NLPHLs harbor BCL6 oncogene rearrangements (12, 13). Moreover, various protooncogenes are targeted by aberrant somatic hypermutation in L&H cells, which may alter the function of these genes and thereby contribute to NLPHL pathogenesis (14).

Because of the extraordinary technical difficulties in analyzing tumor cells present in tissues in such a small amount, no systematic large-scale gene expression study of L&H cells has been performed so far. In this study, we succeeded in establishing a reliable method to microdissect primary L&H cells from frozen tissue sections, isolating RNA from the cells and analyzing their global gene expression after a two-rounded in vitro transcription. This allowed us to clarify the following questions: (a) does the gene expression profile of L&H cells define NLPHL as a distinct entity; (b) what is the histogenetic origin of L&H cells; (c) to which other malignant B cells are L&H cells most closely related in terms of gene expression; (d) can we identify L&H cell–specific genes that might be involved in the pathogenesis of NLPHL and represent potential new diagnostic markers or therapeutic targets; and (e) which signaling pathways are active in L&H cells that could promote their growth and survival?

RESULTS

Gene expression profiling of isolated L&H cells defines NLPHL as a distinct entity closely related to T cell–rich B cell lymphoma (TCRBL), a subset of diffuse large B cell lymphoma (DLBCL), and cHL

Using Affymetrix U133 Plus 2.0 microarrays (representing ~47,000 transcripts), gene expression profiles were generated from microdissected L&H cells of five cases of NLPHL and compared with the main subsets of normal B cells ($CD77^+$ and $CD77^-$ GC B cells and plasma cells isolated from tonsils, and naive and memory B cells isolated from peripheral blood; 5 donors each), with tumor cells of B cell non-HLs (B-NHLs; 5 FLs, 5 Burkitt lymphomas [BLs], 11 DLBCLs, and 4 TCRBLs, a rare variant of DLBCL), and with HRS cells of 12 cHLs. Unsupervised hierarchical clustering divided the 67 profiles into two main branches comprising the malignant lymphomas and the normal B cell subsets, respectively. (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080809/DC1). Among the latter, three main subbranches separate plasma cells and resting peripheral blood cells (naive and memory B cells) from GC B cells. To better determine the relatedness of L&H cells to the other lymphomas, unsupervised clustering was restricted to the tumor samples (Fig. 1). FLs and BLs clustered separately from each other in one branch of the dendrogram, which also comprised the majority of DLBCLs (6 out of 11) and 1 TCRBL. NLPHLs clustered in the other branch, close to most TCRBLs (3 out of 4) and some DLBCLs (3 out of 11) and cHLs. The L&H profiles were more similar to each other than to any other B cell lymphoma, implying that NLPHL represents a distinct entity among the B cell lymphomas analyzed. In contrast, DLBCLs, which are known to be composed of more than one subentity (15, 16), did not form a separate branch but were intermingled with other lymphomas.

We next examined the relationship of L&H cells with other lymphomas by principal component analysis (PCA) of the mean expression vectors of each entity (Fig. 2 A). NLPHL showed a closer relatedness to TCRBL and cHL than to DLBCL, BL, or FL, which was in line with the results of the unsupervised hierarchical clustering (Fig. 1).

Close relatedness of L&H and HRS cells is confirmed by supervised analysis

In a supervised comparison between the profiles of L&H and HRS cells, only 43 genes were differentially expressed (≥1.8 fold change [FC]) in a statistically significant manner (false discovery rate [FDR] < 0.05 after the *t* test; unpublished data). Notably, applying the same filter criteria, approximately sevenfold more (295) genes turned out to distinguish NLPHL from FL (unpublished data), which resembles NLPHL in various aspects. Because cHLs are often heterogeneous in their expression of immunohistological markers (17), we relaxed the FDR filter to <0.1 and revealed 129 genes differentially expressed between NLPHL and cHL, of which 6 were expressed at higher levels in HRS cells and 123 were expressed at higher levels in L&H cells.

Three of the six genes up-regulated in cHL, namely the chemokines *CCL22* (*MDC*) and *CCL17* (*TARC*), and *IL6*, are known to be expressed in HRS cells more frequently and/or at higher levels than in L&H cells (18–20), thus validating our analysis. High expression of the Jun dimerization protein p21SNFT is also known for HRS cells (21).

Other genes known to be differentially expressed between HRS and L&H cells, e.g., *CD30* and *GATA3*, were not found. This is because their mRNA expression levels in the cHL cases were too heterogeneous to fulfill the filter criteria.

Most of the 123 genes up-regulated in L&H cells are expressed at similar levels in GC B cells, indicating that these are (GC) B cell genes down-regulated in HRS cells. Thus, only 27 genes remain that are up-regulated in L&H cells compared with HRS and GC B cells (Table I).

Up-regulation of these genes in L&H as compared with HRS cells is mostly moderate (two- to threefold). Among them, we identified KISS1 receptor (*KISS1R*), SLAM family member 7 (*SLAMF7*), protein kinase C ζ, and serum/glucocorticoid-regulated kinase (*SGK*). Collectively, we identified surprisingly few genes that showed consistent differences in expression between HRS and L&H cells.

Only a few genes distinguish L&H cells from lymphoma cells of TCRBL

The supervised comparison of L&H cells and the lymphoma cells of TCRBL, a highly heterogenous entity (22), revealed 42 genes consistently differentially expressed (FC ≥ 1.8; $P < 0.05$ using the *t* test; FDR < 0.2; Fig. S2 and Table S1, available at http://www.jem.org/cgi/content/full/jem.20080809/DC1). All 42 genes showed enhanced expression in L&H cells compared with TCRBL and include autotaxin (*ENPP2*), the protooncogene cortactin (*CTTN*), and α kinase 2 (*ALPK2*).

Identification of "L&H cell–specific" genes

To identify genes specifically up- or down-regulated in L&H cells, a supervised comparison of the NLPHL profiles with those of the normal B cells and B-NHL was performed. The 12 cHL profiles were not included because the high similarity between NLPHL and cHL in terms of gene expression would bias the analysis, as they represent one third of all lymphoma samples. 49 genes are consistently up-regulated in the L&H cells, and 5 genes are down-regulated (Table II; and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20080809/DC1). The expression pattern of several of the 54 genes in NLPHL is relatively similar to that observed in the same TCRBLs and DLBCLs (TCRBLs 1, 2, and 4, and DLBCL 2, 3, and 11) that cluster near the L&H cell profiles in the unsupervised hierarchical clustering (Fig. 1), consistent with the reported biological and phenotypical relatedness of these lymphomas.

Notably, the genes up-regulated in L&H cells have not been previously described to be expressed in these cells. They include the transcription factors *EOMES*, the cathepsins *CTSB* and *CTSK*, matrix metalloprotease 12 (*MMP12*), a member of the tumor protein family D52 (*TPD52L1*), a member of the Ras oncogene family (*RAS42*), the protooncogene *CTTN*, the serine/threonine kinase *PRKCZ*, vascular cell adhesion molecule 1 (*VCAM1*), *KISS1R*, and insulin-like growth factor 2 receptor (*IGF2R*). The five genes down-regulated in L&H cells represent B lineage–specific genes (Table II).

L&H cells show a similar relatedness to GC and memory B cells

To identify the closest normal counterpart of L&H cells, PCA was performed using genes differentiating GC B cells from naive B cells, memory B cells, or plasma cells, and memory B cells from naive B cells or plasma cells (Fig. 2 B). In the comparisons of GC B cells to naive B cells and plasma cells, L&H cells were more similar to GC B cells. L&H cells were also more similar to memory B cells than to plasma cells. However, when compared with GC B cells and memory B cells, L&H cells were equidistant from both. This, together with the much higher similarity of L&H cells to memory than to naive B cells (Fig. 2 B), suggests that L&H cells resemble an intermediate developmental stage in the transition between GC B cells and memory B cells.

Comparison of L&H cells to GC B cells

To investigate whether there are deregulated signaling pathways in L&H cells that promote their growth and survival, we compared the L&H cell profiles with those of their nonmalignant B cell counterparts. We selected GC B cells for this analysis, although the PCA also indicated some similarity of L&H cells to memory B cells, because the overall features of L&H cells (a GC-like growth pattern, the expression of key GC B cell factors, and ongoing hypermutation) strongly support a GC B cell origin of L&H cells. We identified 963 differentially expressed genes (558 up-regulated and 405 down-regulated in L&H cells; FDR ≤ 0.05 after the *t* test; FC ≥ 1.8; unpublished data). Several of the up-regulated genes are known for their

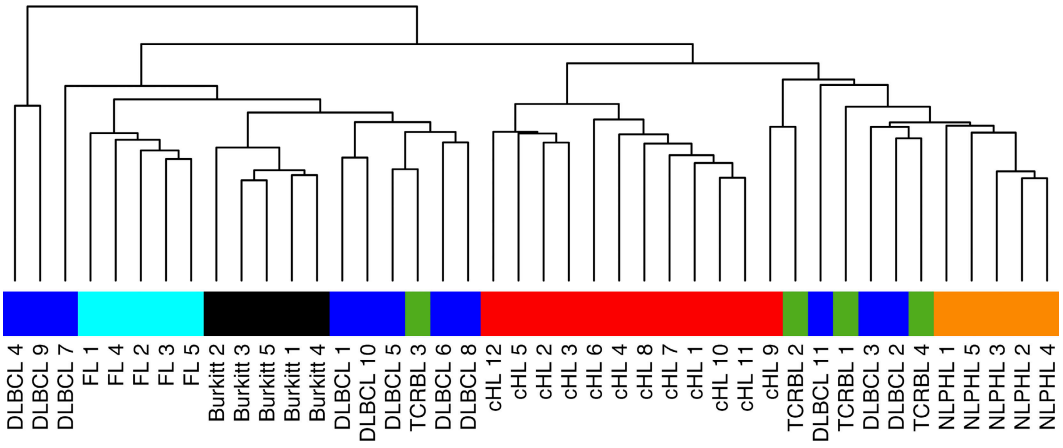

Figure 1. Unsupervised hierarchical clustering of lymphoma samples. The dendrogram is based on the expression data of the 582 most variable genes (BL, black; cHL, red; DLBCL, dark blue; FL, light blue; NLPHL, orange; TCRBL, green).

expression in L&H cells, i.e., RAS family member *RAB13* (23), EBV-induced gene 3 (*EBI3*) (24), TNF-α–induced protein 3 (*TNFAIP3*; *A20*) (25), *FYN* (26), *CFLAR* (*c-FLIP*) (27), the chemokine ligands *CXCL9* (*MIG*) and *CXCL10* (*IP10*) (18), and transferrin receptor (*TFRC; CD71*) (28) (see Fig. 4; Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20080809/DC1; and not depicted).

The other up-regulated genes, previously not reported to be expressed in L&H cells, include the B cell survival factors *BAFF* (*TNFSF13B*; 10.5-fold up-regulated) and *APRIL* (*TNFSF13*; 4.5-fold up-regulated), and the transcription factors *STAT1* and *STAT2* (17.7-/8.7-/6.4-fold and 1.8-fold up-regulated, respectively) and *EOMES* (4.1-fold up-regulated). A direct target of EOMES, IL2RB (CD122) (29), is also 7.4-fold up-regulated. β-2 microglobulin (*B2M*), which is up-regulated by STAT1 and is part of MHC I, is fivefold up-regulated in L&H cells. STAT1 can promote leukemia development by maintaining high MHC I expression (30).

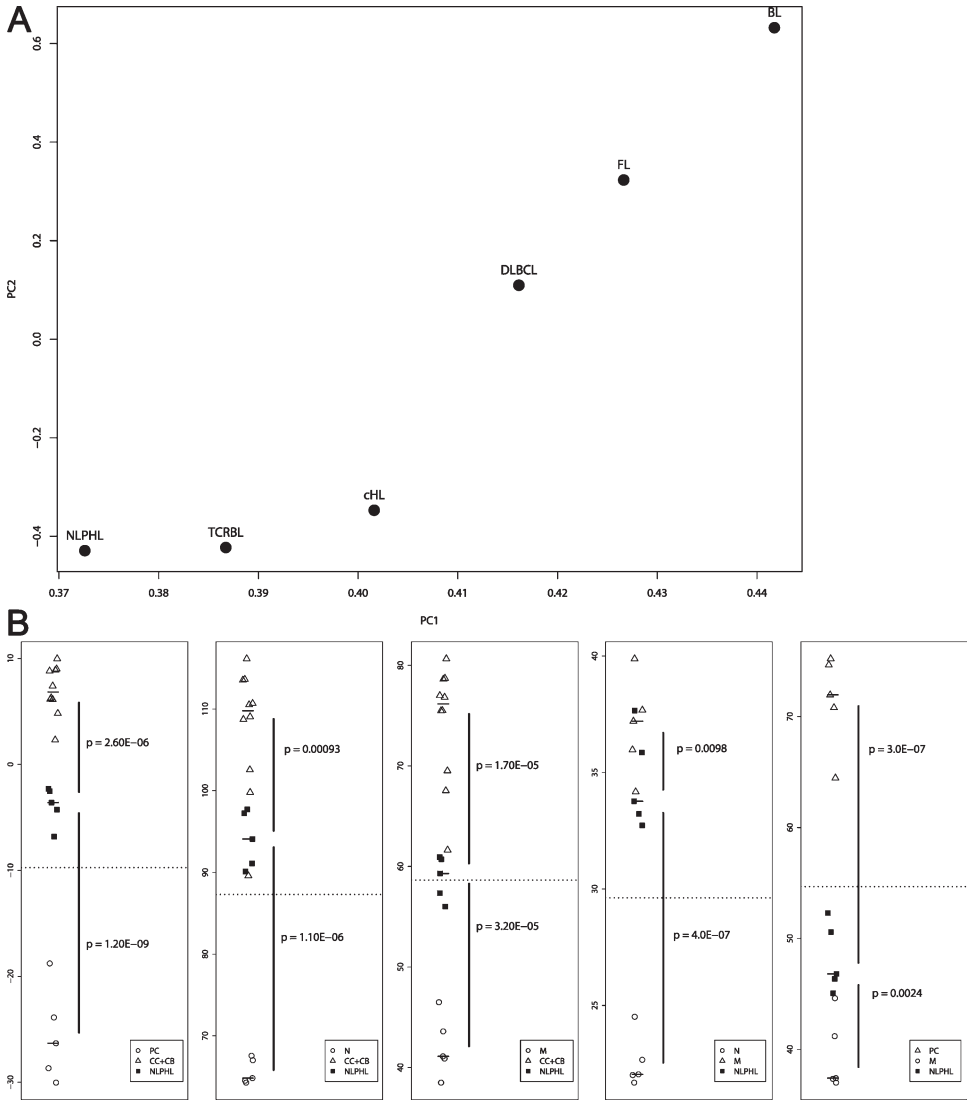

Figure 2. Relatedness of L&H cells with other lymphomas and with normal B cells by PCA. (A) 3,364 genes, whose median expression was above background in at least one lymphoma entity, were used by PCA to assess the relatedness of L&H cells (mean of 5 samples) to the malignant B cells of other lymphomas (mean of 4–12 samples). The first two principal components are shown (accounting for 88.1% and 5.5% of the total variance, respectively). (B) Genes discriminating between GC B cells and naive B (N), memory B (M), or plasma cells (PC; FC ≥ 4; FDR < 0.05 after *t* test; 229, 128, and 130 probe sets), and between naive and memory B cells (FC ≥ 2.5; FDR < 0.05 after *t* test; 47 probe sets) or between memory B cells and plasma cells (FC ≥ 4; FDR < 0.05 after *t* test; 135 probe sets) were used by PCA to identify the closest normal counterpart of L&H cells. In each of the five graphs, the y axis indicates the first principal component score, the horizontal solid lines show the median score of each cell subset, and the horizontal dotted line represents the mean of the median scores of the two normal subsets being compared. Also shown are the p-values (obtained by *t* test) of the pairwise comparisons (vertical lines) between L&H cells and normal B cells. The percentage of total variance accounted for by the first principal component is as follows: GC B versus plasma cells, 89%; GC B versus naïve B cells, 93.9%; GC B versus memory B cells, 92.5%; memory versus naïve B cells, 86.6%; and memory B versus plasma cells, 92.8%.

Among the genes differentially expressed between L&H and GC B cells, several up-regulated genes have known oncogenic potential, namely *FYN*, *SGK*, and *CTTN* (each about threefold up-regulated). The protein-tyrosine kinase FYN is implicated in the control of cell growth. SGK, a serine/threonine protein kinase, acts as an oncogene in breast cancer cells through activation of NF-κB (31). Cortactin is overexpressed in several cancers and contributes to tumor cell invasiveness and metastasis (32). Additionally, several members of the Ras oncogene family, i.e., RAB13, RAB20, RAB27A, RAB42,

Table I. Genes differentially expressed between NLPHL and cHL

	FC	Gene symbol	Affymetrix probe set ID	Gene name
Genes up-regulated in cHL compared with NLPHL				
	33.6	*CCL22*	207861_at	chemokine (c-c motif) ligand 22; MDC
	15.6	*CCL17*	207900_at	chemokine (c-c motif) ligand 17; TARC
	7.2	*SNFT*	220358_at	Jun dimerization protein p21SNFT
	4	*ODZ2*	231867_at	odz, odd Oz/ten-m homolog 2 (*Drosophila*); teneurin-2
	3.1	*IL6*	205207_at	IL-6 (IFN, β 2)
	2.3	*MGST3*	201403_s_at	microsomal glutathione S-transferase 3
Genes up-regulated in NLPHL compared with cHL (and GC B cells)				
	4.9	*KISS1R*	242517_at	KISS1 receptor; GPR54
	3.9	*CHIT1*	208168_s_at	chitinase 1 (chitotriosidase)
	3.9/2.9	*GBP1*	231577_s_at 202269_x_at	guanylate binding protein 1, IFN-inducible, 67 kD
	3.8	*SGK*	201739_at	serum/glucocorticoid-regulated kinase
	3.2	*CHI3L1*	209396_s_at	chitinase 3-like 1 (cartilage glycoprotein-39)
	2.9	*SLAMF7*	222838_at	SLAM family member 7
	2.9	*GLA*	214430_at	galactosidase, α
	2.9	*TSC22D1*	215111_s_at	TSC22 domain family, member 1
	2.8	*STAT1*	AFFX-HUMISGF3A/ M97935_3_at	signal transducer and activator of transcription 1, 91 kD
	2.8	*EOMES*	231776_at	eomesodermin homolog (*Xenopus laevis*)
	2.8	*ITM2A*	202747_s_at	integral membrane protein 2A
	2.6	*TMEM163*	223503_at	transmembrane protein 163
	2.6	*ATP6V1A*	201972_at	ATPase, H^+ transporting, lysosomal 70 kD, V1 subunit A
	2.5	*PLXNA1*	221538_s_at	plexin A1
	2.4	*DRAM*	218627_at	damage-regulated autophagy modulator
	2.4	*DUSP2*	204794_at	Dual-specific phosphatase 2; PAC1
	2.4	*PLEK*	203471_s_at	pleckstrin
	2.3	*CKS2*	204170_s_at	CDC28 protein kinase regulatory subunit 2
	2.3	*PRKCZ*	202178_at	protein kinase C, ζ
	2.3	*LIMS1*	212687_at	LIM and senescent cell antigen-like domains 1
	2.2	*F11R*	223000_s_at	F11 receptor
	2.2	*SLC31A1*	203971_at	solute carrier family 31 (copper transporters), member 1
	2.1	*CTSH*	202295_s_at	cathepsin H
	2.1	*BID*	211725_s_at	BH3 interacting domain death agonist
	2.1	*NOD27*	226474_at	nucleotide-binding oligomerization domains 27
	1.8	*RAB27A*	210951_x_at	RAB27A, member RAS oncogene family
	1.8	*PSMB8*	209040_s_at	proteasome (prosome, macropain) subunit, β type, 8 (large multifunctional peptidase 7)

129 genes are differentially expressed between L&H and HRS using ≥1.8 as the FC cut-off, and a *t* test p-value of <0.05/FDR of <0.1 as the statistical cut-off. Out of the 123 genes expressed at higher levels in L&H than in HRS cells, only 27 are also up-regulated in comparison to GC B cells, and they are shown in this table. The remaining 96 genes are down-regulated in HRS cells compared with GC B cells (unpublished data).

Table II. L&H cell–specific genes

	FC	Gene symbol	Affymetrix probe set ID	Gene function
Genes up-regulated in NLPHL compared with normal B cells and B-NHL				
	13	*MMP12*	204580_at	matrix metalloprotease; involved in ECM degradation, metastasis and angiogenesis
	9.8	*CHIT1*	208168_s_at	
	8.5	*CTSK*	202450_s_at	involved in ECM degradation and tumor invasion
	7.6	*CYP27B1*	205676_at	
	7.1	*ZBED2*	219836_at	
	6.7	*ALPK2*	228367_at	
	6.3		230741_at	
	6.2	*KISS1R*	242517_at	involved in ERK activation; overexpressed in several cancers
	5.5	*RGS4*	204337_at	regulator of G protein signaling
	5	*TPD52L1*	203786_s_at	member of the tumor protein D52 family; involved in cell proliferation and calcium signaling
	4.6	*PLA2G7*	206214_at	
	4.6	*NPL*	221210_s_at	
	2.9		240449_at	
	4.5	*UBD*	205890_s_at	may modulate tumorigenesis; high levels of *UBD* in cells lead to increased mitotic nondisjunction and chromosome instability
	4.5	*SLC1A3*	202800_at	
	4.3	*VCAM1*	203868_s_at	mediates leukocyte–endothelial cell adhesion
	4.2	*MAF*	206363_at	oncogene; transcription factor; overexpressed in multiple myelomas
	4	*CD2*	205831_at	
	4	*DRAM*	218627_at	
	3.8		230391_at	
	3.8	*TMEM163*	223503_at	
	3.7	*CTSB*	227961_at	involved in ECM degradation and apoptosis; overexpressed in several cancers
	3.5	*SCARB2*	224983_at	
	3.4	*PTGDS*	211663_x_at	
	3.4	*SERPING1*	200986_at	complement component C1 inhibitor
	3.4	*IGF2R*	201392_s_at	involved in tumor cell surveillance
	3.4	*ALAS1*	205633_s_at	
	3.4	*SLC31A1*	203971_at	
	3.3	*RASGEF1A*	230563_at	
	3.3	*SLC26A11*	226679_at	
	3.2	*CTTN*	201059_at	oncogene; involved in tumor cell invasion and metastasis; overexpressed in several cancers
	3.1	*SCPEP1*	218217_at	
	3	*EOMES*	231776_at	transcription factor highly homologous to T-bet
	3	*RAB42*	1552846_s_at	member of the RAS oncogene family
	3	*C1orf54*	219506_at	
	2.9	*PLA2G2D*	220423_at	
	2.9	*LOC642705*	228066_at	
	2.9	*NPC1*	202679_at	
	2.9	*LMNA*	203411_s_at	lamin proteins are thought to be involved in nuclear stability and chromatin structure
	2.9	*EGR2*	205249_at	transcription factor
	2.9	*FPR1*	205119_s_at	appears to mediate motility, growth and angiogenesis of human glioblastoma

and RAB7L1, are expressed at increased levels in L&H cells as compared with GC B cells. Besides the genes mentioned in this section, the analysis revealed several signaling pathways, survival mechanisms, and distortions of differentiation programs that likely contribute to the pathogenesis of NLPHL, and they are presented in the following paragraphs.

L&H cells show a partial loss of their B cell phenotype. To obtain a comprehensive overview about the expression of B cell markers in L&H cells, we selected informative probe sets for B cell markers known from the literature, as well as for genes we found in our profiles to be up-regulated in normal B cells compared with $CD4^+$ and $CD8^+$ T cells. We thus generated a list of 61 B cell markers (94 informative probe sets). 60 out of the 61 genes showed statistically significantly reduced expression in L&H cells as compared with GC B cells, and only 1 gene, *IL21R*, was 2.2-fold up-regulated (Fig. 3 A). The down-regulated B lineage–specific genes included B cell receptor signaling molecules like *CD79B*, *CD22*, *LYN*, *SYK*, and *BLNK*; the transcriptional coactivator for Ig gene expression *POU2AF1* (*BOB1*); the Fc receptor–like molecules *FCRLA*, *FCRL2* (*IRTA4*), and *FCRL3* (*IRTA3*); and the GC B cell–specific transcription factors basic leucine zipper transcription factor 2 (*BACH2*), IFN regulatory factor 8 (*IRF8*), *BCL6*, and *MYBL1* (*a-myb*). Furthermore, transcription factors/modulators important for B cell development, lineage commitment, and maintenance, i.e., EBF, E2A, PAX5, Ikaros, BCL11A, and deltex 1 (DTX1), showed reduced expression in L&H cells. To quantify the B cell gene expression in each entity, we computed the first principal component of the expression matrix of the 61 B cell genes in GC B cells (representing high expression of B cell genes) and in $CD4^+$ and $CD8^+$ T cells (representing low expression of B cell genes). We then used the score displayed by each entity along this component as a measure of B cell gene expression and found it to be highly different across entities (10^{-9} using the Kruskal-Wallis test; Fig. 3 B). L&H cells showed significantly reduced B cell gene expression compared with GC, naïve, and memory B cells. FL, BL, and DLBCL also showed significant B cell gene down-regulation compared with GC B cells, suggesting that this is a common feature among all of these GC B cell–derived lymphomas. Nevertheless, the extent of B cell gene down-regulation in L&H cells is higher than in FL ($P = 0.016$) and in DLBCL and BL (although not reaching statistical significance). However, down-regulation of B cell markers in NLPHL is not as extensive as in cHL.

L&H cells show an antiapoptotic phenotype and create an immunosuppressive environment. Comparing the genes differentially expressed between L&H and GC B cells, 38 genes associated with apoptosis were identified (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20080809/DC1), of which 28 were expressed at increased and 10 were expressed at decreased levels in L&H cells. All 21 genes with antiapoptotic functions in the extrinsic and/or intrinsic apoptosis pathway were expressed at increased levels in L&H cells, including

Table II. L&H cell–specific genes *(Continued)*

	FC	Gene symbol	Affymetrix probe set ID	Gene function
	2.7	*PRKCZ*	202178_at	serine/threonine kinase; involved in proliferation and differentiation; activates NF-κB
	2.7	*TPCN2*	229250_at	
	2.6	*ADFP*	209122_at	
	2.5	*SQSTM1*	201471_s_at	
	2.4	*VPS37C*	219053_s_at	
	2.3	*PNKD*	233177_s_at	
	2.3	*RTN4*	214629_x_at	
	2.1	*SLIC1*	228869_at	
	2	*ABCC1*	202804_at	involved in multidrug resistance
Genes down-regulated in NLPHL compared with normal B cells and B-NHL				
	−2.3	*CD79B*	205297_s_at	B lineage–specific gene
	−3.8	*BCNP1*	230983_at	B lineage–specific gene
	−4.3	*IGHM*	209374_s_at	B lineage–specific gene
	−4/−4	*TCL1A*	39318_at	B lineage–specific gene
			209995_s_at	
	−2.8	*FCRL2*	1563674_at	B lineage–specific gene

49 genes specifically up-regulated by L&H cells compared with normal and other malignant B cells were identified according to the following criteria: (a) expression above background level in ≥4 out of 5 L&H cell profiles and in ≤6 out of the 50 normal and other malignant B cell profiles; and (b) greater than or equal to twofold up-regulation in L&H cells and with a *t* test p-value of ≤0.05. Five genes specifically down-regulated in L&H cells were identified according to the following criteria: (a) expression above background level in ≤1 out of 5 L&H cell profiles and in ≥33 out of the 50 normal and other malignant B cell profiles; and (b) greater than or equal to twofold down-regulation in L&H cells and with a *t* test p-value ≤0.05. The 12 cHL profiles were excluded from the comparison (see Results). For genes with known gene functions, these are indicated.

cathepsin B, cystatin A (*CSTA*), autotaxin, CD59, defender against cell death 1 (*DAD1*), BAX inhibitor 1 (*TEGT*), galectin 3 (*LGALS3*), peroxiredoxin 1 (*PRDX1*), and *c-FLIP*.

10 out of the 15 proapoptotic genes were down-regulated in L&H cells, including caspase 2 (*CASP2*), Ataxia telangiectasia mutated (*ATM*), serine/threonine kinases 17a and 17b (*STK17A* and *STK17B*), and TNF receptor–associated factor 5 (*TRAF5*; Fig. S4). The function of the remaining five proapoptotic genes (Fig. S4), expressed at increased levels in L&H cells, may be inhibited by the increased expression of cystatin A and c-FLIP and the reduced expression of caspase 2 (33–35).

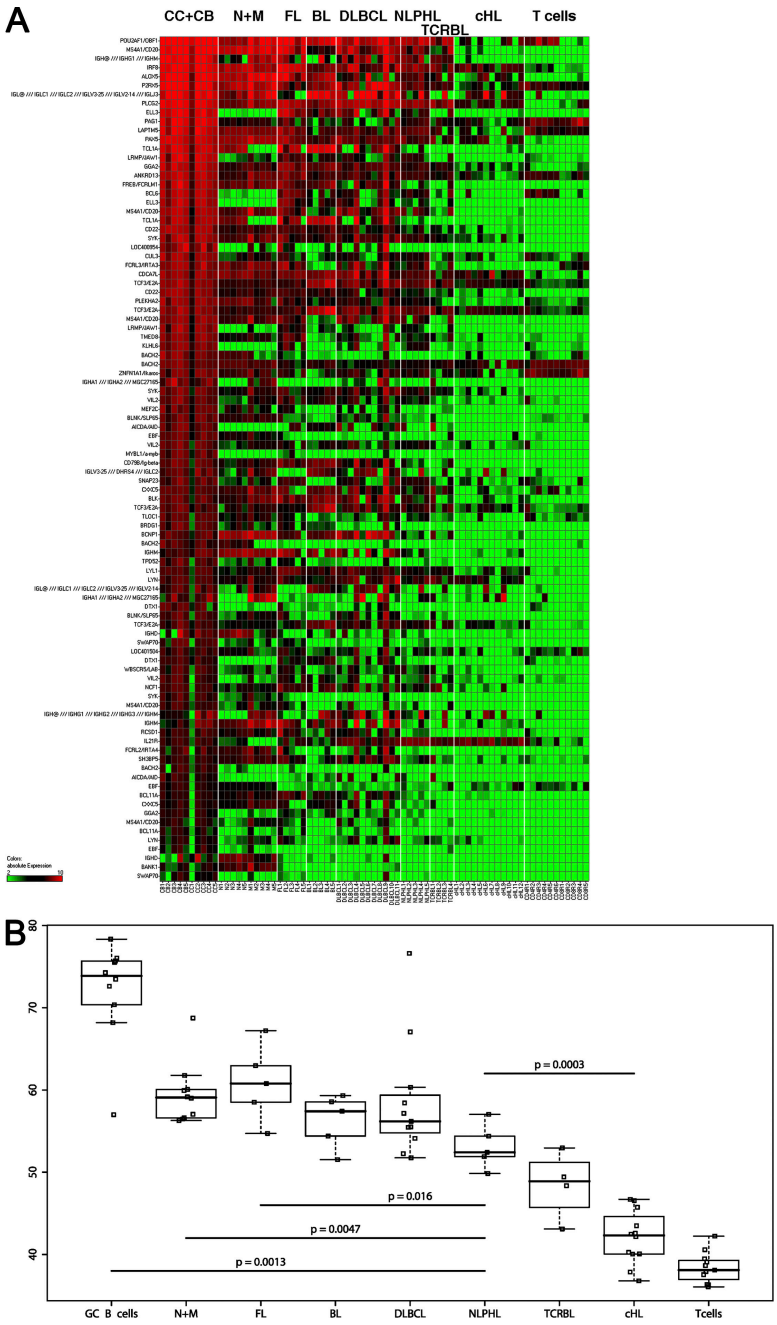

Figure 3. Partial loss of B cell markers in NLPHL. (A) Heat map of the absolute expression values of B cell markers, which are encoded by the color bar (base 2 logarithmic scale). (B) Individual samples (squares) are placed along the first principal component (which accounts for 92.2% of the total variance) of the expression matrix of 61 selected B cell markers. Box plots summarize the score of each group in this component. The vertical lines ending in horizontal lines (whiskers) extend to the most extreme data points if they extend not more than 1.5 times the box width (the interquartile range). Otherwise, the whiskers end at an observed value that is not more than 1.5 times the box width away from the box. p-values of the pairwise comparisons using a Wilcoxon test are indicated.

In addition, the L&H cells have acquired several mechanisms to escape from immune surveillance by T and NK cells. The galectins 1, 2, and 3, which are all up-regulated in L&H cells (Fig. S4), can mediate proapoptotic signals to neighboring T cells and could thereby counteract the elimination of L&H cells by T cells (36). Cathepsin B cleaves perforin (37), and secreted IGF2R/M6PR acts as a sink for granzyme B secreted by $CD8^+$ cytotoxic T cells (38). MMP9, which is strongly up-regulated in L&H cells (Table III), participates in resistance to NK cell–mediated cytotoxicity (39). Different MHC I molecules are up-regulated between two- and fivefold in L&H cells (unpublished data), which may prevent the elimination of these cells by NK cells. CD59 (3.6-fold up-regulated) inhibits the formation of the membrane attack complex. Additionally, soluble annexin A2 (sixfold up-regulated) has immunosuppressive properties (40). Collectively, survival of the L&H cells seems to be promoted by the up-regulation of multiple antiapoptotic genes, the down-regulation of numerous proapoptotic genes, and the creation of an immunosuppressive environment.

L&H cells are characterized by a strong constitutive NF-κB activity. Among the genes differentially expressed between L&H and GC B cells, numerous NF-κB target genes were observed. To study NF-κB activity in L&H cells in more detail, we generated a comprehensive list of 62 informative probe sets for 50 NF-κB target genes (Fig. 4). GC B cells showed no significant expression of the NF-κB target genes, and HRS cells had strong expression, as expected (41, 42). Thus, this list of NF-κB target genes is well suited to discriminate cases with and without NF-κB activity. Strikingly, 48 out of 50 genes showed a strong expression by L&H cells, at levels comparable to HRS cells, and only 2 genes (*CCL22* and *IL6*) showed signal intensities below background (Fig. 4 A). In line with published data, NF-κB activity is low in FL and BL and highly diverse in DLBCL (43, 44). Approximately half of the DLBCLs (including DLBCLs 2, 3, and 11, which show some similarity with NLPHL; Fig. 1 and Fig. S3) show expression of most NF-κB target genes. TCRBLs also seem to have strong NF-κB activity in most cases. To quantify NF-κB activity across different entities, we computed the first principal component of the expression matrix of NF-κB target genes in HRS cells (representing high NF-κB activity) and GC B cells (representing low NF-κB activity). We then used the score displayed by each entity along this component as a measure for NF-κB activity and found it to be highly different across entities (3×10^{-9} using the Kruskal-Wallis test; Fig. 4 B). NLPHL shows the strongest NF-κB activity in this PCA, with high statistical significance compared with GC B cells, FL, BL, and DLBCL, and with a trend also compared with cHL and TCRBL (Fig. 4 B).

To confirm NF-κB expression and activity in L&H cells, we performed immunohistochemistry with an antibody recognizing an epitope that is only accessible when NF-κB heterodimers contain active p65 (45). In 19 out of the 19 cases of NLPHL analyzed, strong p65 staining was observed in L&H cells (Fig. 5 A). Collectively, L&H cells show a strong constitutive NF-κB activity and increased expression of the vast majority of NF-κB target genes in our study.

Extracellular signal-regulated kinase (ERK) activation plays a role in at least a proportion of NLPHL cases. We identified several genes up-regulated in L&H cells compared with GC B cells that indicate activation of the ERK pathway. This pathway is implicated in malignant transformation and the regulation of cellular growth and proliferation. KISS1 receptor (10.4-fold up-regulated), galectin 1 (4.5-fold up-regulated), chitinase 3–like 1 (*CHI3L1*; 36.3-/20.4-fold up-regulated),

Table III. Immunohistochemical expression of genes with higher transcript levels in L&H than in GC B cells

Gene name	Affymetrix probe set ID	FC up-regulation in L&H cells versus GC B cells	Positivity in primary NLPHL cases (%)[a]	Positivity in GC B cells
CTSB	200839_s_at	44.7	19/19 (100)	faint background staining
	200838_at	14.6		
	213274_s_at	14.3		
	213275_x_at	14.1		
	227961_at	6.2		
MMP9	203936_s_at	60.2	15/16 (94)	faint background staining
MMP12	204580_at	25.4	15/17 (88)	faint background staining
LGALS3	208949_s_at	25.8	15/15 (100)	negative
IL21R	221658_s_at	2.2	7/7 (100)	positive
EOMES	231776_at	4.1	10/15 (67)	negative
p-STAT1	209969_s_at	17.7 (STAT1β)	11/19 (58)	negative
	200887_s_at	8.7 (STAT1α)		
	AFFX-HUMISGF3A/M97935_3_at	6.4 (STAT1α)		

[a]A case was scored as positive if ≥20% of L&H cells showed expression of the respective marker. For CTSB, MMP9, MMP12, LGALS3, and IL21R, the vast majority of L&H cells were positive. Positivity for the expression of EOMES and p-STAT1 in L&H cells ranged from 20–80%. p-STAT1 was detected in a fraction of L&H cells in 11 out of 19 cases of NLPHL, which might underestimate the frequency of positive cases for reasons discussed regarding p-ERK staining.

SLAM family member 7 (13.9-fold up-regulated), and syndecan binding protein (*SDCBP*; 2.6-fold up-regulated) activate ERK (46–50). RAS homologue gene family member H (*RHOH*), which is 2.3-fold down-regulated, was previously described to suppress ERK activation (51). *PLAU* (9.1-fold up-regulated) is an ERK target gene as well as a major determinant

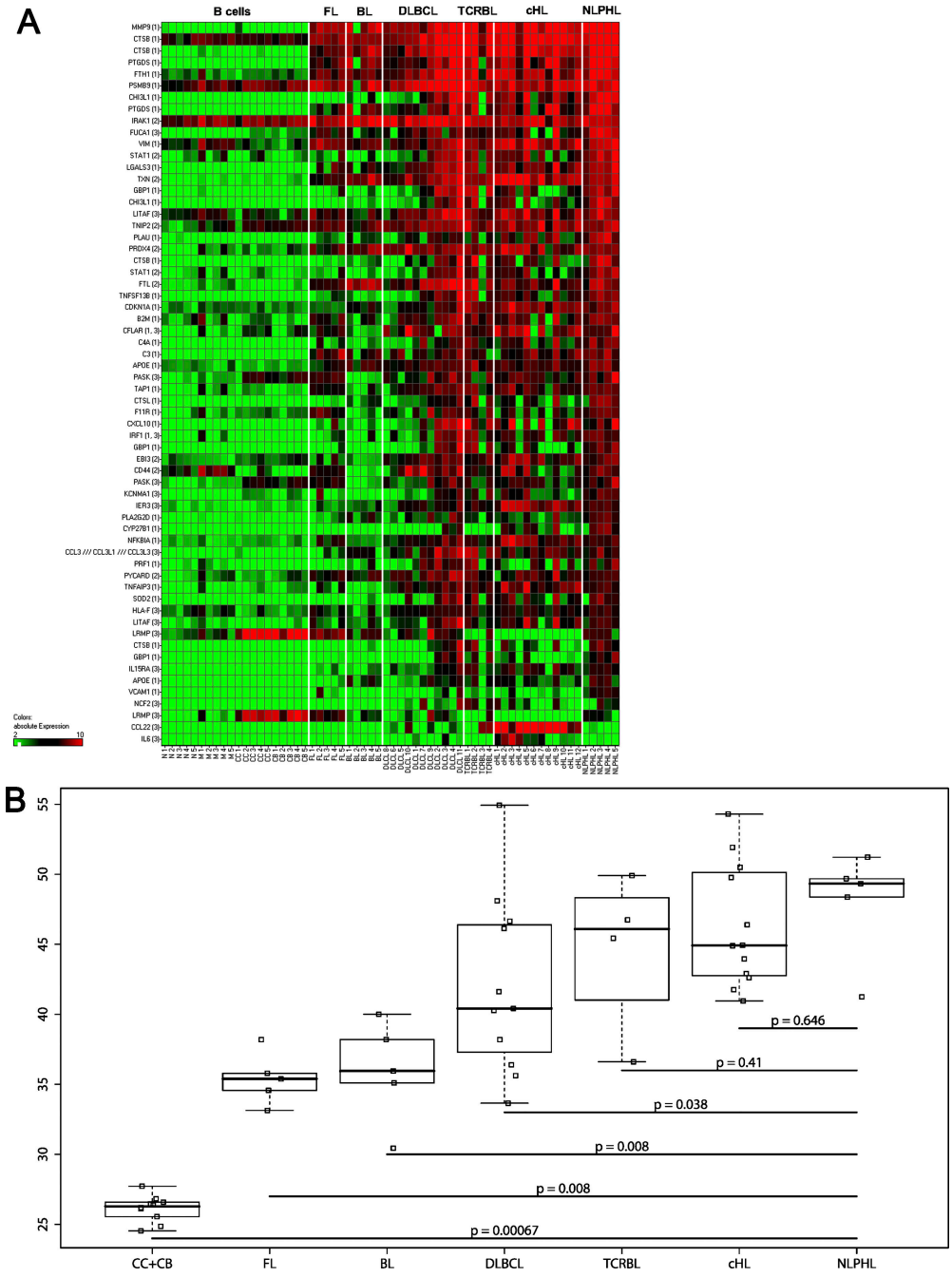

Figure 4. NF-κB activity in NLPHL. The list of NF-κB target genes experimentally validated in cHL cell lines (reference 89) was expanded by including other NF-κB target genes described in the literature or other sources (available at http://www.nf-kb.org). After excluding all noninformative probe sets (signals below background in all samples), we obtained a list of 62 probe sets, corresponding to 50 NF-κB target genes, of which 48 turned out to be consistently up-regulated in L&H versus GC B cells (FDR < 0.05 after *t* test; FC ≥ 1.8). (A) Heat map of NF-κB target genes. The sources of the NF-κB target genes are (1) http://www.nf-kb.org, (2) the literature, and (3) Hinz et al. (reference 89), as indicated in parentheses after the gene names. The absolute expression values on a logarithmic scale (base 2) are encoded by the color bar. (B) Individual samples (squares) are placed along the first principal component (which accounts for 80.1% of the total variance) of the expression matrix of 50 NF-κB target genes. Box plots summarize the scores displayed by each group in this component. The vertical lines ending in horizontal lines (whiskers) extend to the most extreme data points if they extend not more than 1.5 times the box width (the interquartile range). Otherwise, the whiskers end at an observed value that is not more than 1.5 times the box width away from the box. p-values of the pairwise comparisons using a Wilcoxon test are indicated.

of ERK activation (52). Furthermore, several additional transcriptional targets of the ERK pathway, namely *DR5*, *STAT3*, *MMP9*, *CDKN1A* (*p21*$^{CIP1/WAF1}$), and cathepsin B (53–57), are up-regulated in L&H cells. Four out of the six are also NF-κB target genes (Fig. 4 A).

To determine whether L&H cells aberrantly express activated ERK, we performed immunohistochemical staining of paraffin sections of NLPHL and normal tonsils using antibodies against total ERK and phosphorylated ERK (p-ERK). As previously reported (58), normal GC B cells were ERK positive, but p-ERK was not detectable (unpublished data). Among NLPHL, 10 out of 14 cases expressed ERK, and 4 out of 12 cases were positive for p-ERK in a fraction of L&H cells (Fig. 5, B and C). The true fraction of cases with activated p-ERK is likely higher because of a loss of phosphogroups caused by insufficient preservation of material (59). Because *STAT1* can be phosphorylated by ERK and thereby activated (60), we stained NLPHL for p-STAT1 and revealed nuclear localization of p-STAT1 in a fraction of L&H cells in more than half of the cases (Fig. 5 J). Thus, the frequent detection of p-ERK, together with the increased expression of numerous ERK activating and target genes, indicates a deregulated activation of this signaling pathway in L&H cells.

Extracellular matrix (ECM) degradation and tissue remodeling. L&H cells turned out to express a large number of ECM-degrading molecules, including MMP9, MMP12, and several cathepsins, at higher levels than GC B cells and at similar or higher levels than the more aggressive DLBCL and BL (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20080809/DC1). This was unexpected given the indolent behavior of NLPHL, and the correlation in B-NHL

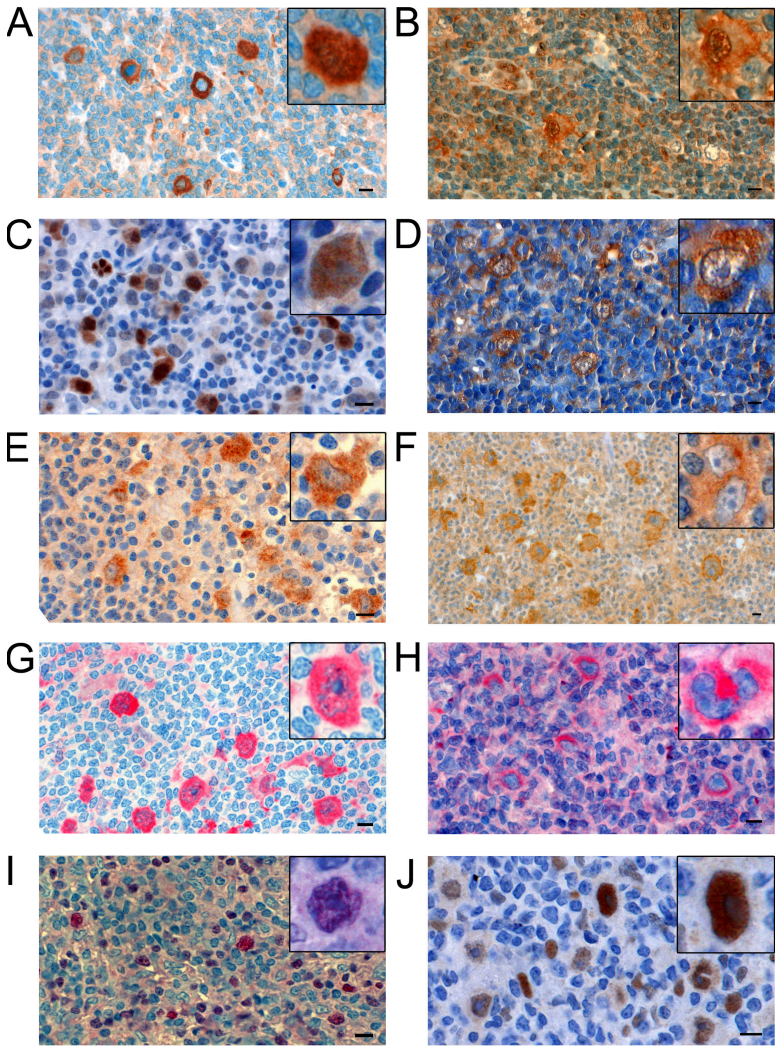

Figure 5. Protein expression in NLPHL cases. (A–J) Immunohistological stainings of paraffin sections of lymph nodes from patients with NLPHL for active NF-κB p65 subunit (A), total ERK (B), active p-ERK (C), CTSB (D), MMP9 (E), MMP12 (F), LGALS3 (G), IL21R (H), EOMES (I), and p-STAT1 (J). The anti–NF-κB p65 antibody recognizes only p65 that is no longer bound by IκB factors, and thus identifies active NF-κB factors, although the staining appears often predominantly cytoplasmatic (reference 45). Insets show L&H cells at higher magnification. Some insets (A, B, D, E, G, and J) are magnified using Photoshop CS software (Adobe). Bars, 10 µm.

between expression levels of ECM-degrading molecules and clinical aggressiveness (61). Besides a role for tissue remodeling in NLPHL, up-regulation of these genes may have other functions in L&H cells, such as inactivation of apoptosis-inducing perforin by cathepsin B (37).

Validation of protein expression in primary NLPHL cases

To further validate the reliability of our analysis and to verify whether the up-regulated mRNA levels in L&H cells correspond to elevated protein levels, we investigated NLPHL and reactive tonsils for protein expression of cathepsin B, MMP9, MMP12, galectin 3, p-STAT1, EOMES, and IL21R by immunohistochemistry, as suitable antibodies were available and as these molecules may be of pathogenetic relevance (Fig. 5, D–J). With the exception of IL21R (see Discussion), normal tonsillar GC B cells showed no or only weak background staining for these markers, as expected (Table III). All of the markers tested showed consistent and often strong expression in L&H cells in most or even all NLPHLs analyzed (Table III). Although the Affymetrix data indicated only a twofold up-regulation of IL21R transcripts in L&H cells versus GC B cells, in some NLPHL cases we detected stronger staining in L&H cells than in the infiltrating lymphocytes. Overall, the results of the immunohistochemical stainings confirmed the up-regulated expression of these genes in L&H cells compared with GC B cells in the great majority of cases.

DISCUSSION

Gene expression profiling of microdissected lymphoma cells

As the present work represents one of the first global transcriptome studies with microdissected lymphoma cells, the reliability of the results obtained is a critical issue. Certainly, when microdissecting L&H cells from tissue sections, a low level contamination with RNA from rosseting $CD4^+$ T cells cannot be excluded. However, for several reasons it is evident that the results obtained in the present analysis are not compromised by such a contamination. First, the immunohistochemical study of genes identified in the comparison of microdissected L&H cells and cytometrically isolated GC B cells revealed that all markers were indeed consistently expressed in L&H cells (Table III and Fig. 5). Second, several typical markers of activated $CD4^+$ T cells (e.g., GATA3, CTLA4, and IL7-R), which were clearly detected in tonsillar-activated $CD4^+$ T cells, gave signals only in the range of the background noise in L&H cells (unpublished data). Third, $CD4^+$ T cells do not show an NF-κB signature (unpublished data), ruling out that the strong NF-κB signature detected in L&H cells could derive from contaminating T cells. Finally, a low level contamination with RNA from T cells should furthermore largely be averaged out in the differential gene expression studies, as T cells are also surrounding the other lymphoma cells microdissected as single cells (lymphoma cells from cHL and TCRBL), and T cells are also present at low frequency in the lymphoma microenvironment of the lymphomas in which lymphoma cell–rich areas were microdissected (DLBCL, FL, and BL).

L&H and HRS cells are very similar in their gene expression patterns

NLPHL and cHL are classified as two subtypes of HL, and several genes were already identified as being aberrantly expressed by both L&H and HRS cells (18, 23, 24). However, several studies stressed many clinical, phenotypic and genetic differences between L&H and HRS cells and also pointed to distinct histogenetic origins of the tumor cells (1, 3, 4, 62). On this background, it was unexpected to find only a few genes differentially expressed between L&H and HRS cells. Our approach is, however, partly underestimating the differences between L&H and HRS cells, as HRS cells may be quite heterogeneous in gene expression, and we focused on genes with consistent differential expression. Many of the phenotypic differences between L&H and HRS cells seen in our analysis and also described in the literature (4, 26, 63, 64) are related to the strong down-regulation of B cell markers in HRS cells, which is not as extensive in L&H cells (discussed in the third following section). Nevertheless, we identified a few additional differences in the gene expression of L&H and HRS cells (Table I). Future studies should reveal which of the differences reflect or are responsible for distinct pathogenetic mechanisms in NLPHL and cHL.

Based on many similarities in their histological picture (a nodular growth pattern, and an association with $CD57^+CD4^+$ GC T helper cells and follicular dendritic cells), the phenotype of the lymphoma cells ($CD20^+$, $BCL6^+$, activation-induced cytidine deaminase$^+$, centerin$^+$, human GC-associated lymphoma$^+$), and genetic features (selection for functional IgV genes and ongoing somatic hypermutation), one might have expected that L&H cells are actually more similar to FL cells than to HRS cells. However, our study shows that at the level of global gene expression, L&H cells are much more closely related to HRS than to FL cells.

Only a few genes distinguish L&H cells and the lymphoma cells of TCRBL

L&H cells turned out to be most closely related to the neoplastic cells of TCRBL and a subgroup of DLBCL, thus expanding on a genome-wide view of the reported biological, phenotypical, and clinical relatedness of these lymphomas. Despite their morphological and phenotypical similarities, the clinical presentation and treatment strategies of TCRBL and NLPHL are different. Therefore, the differential diagnosis between NLPHL and TCRBL has important therapeutic implications. So far, only the transcription factor PU.1 has been reported to be expressed more frequently in L&H cells than in the lymphoma cells of TCRBL. We identified 42 differentially expressed genes (Fig. S2 and Table S1) that might be suitable for establishing new immunohistochemical markers for differential diagnosis.

L&H cells resemble GC B cells at the transition to memory B cells

Although L&H cells clearly resemble GC B cells in many phenotypic and genetic aspects, their gene expression profile

(Fig. 2 B) suggests that they might originate from transformed late GC B cells in the process of differentiation toward memory B cells. This hypothesis would be consistent with our finding of constitutive NF-κB activity in L&H cells, as a subset of centrocytes in the GC light zone presumably developing toward memory B cells shows active NF-κB, whereas the majority of GC B cells lacks NF-κB activity (41). However, it cannot be excluded that NF-κB activation is acquired after neoplastic transformation of a GC B cell into a L&H cell, thus being unrelated to the cell of origin of NLPHL.

L&H cells show a partial loss of their B cell phenotype

Previous immunohistochemical studies reported that L&H cells usually express general B cell markers and GC B cell–specific molecules (3–6, 26, 62–64). However, at the level of sensitivity of immunohistochemistry, L&H cells also lack expression of some B lineage markers (65). These data are, however, not quantitative and are restricted to a relatively small number of proteins. Analyzing a comprehensive set of 61 B lineage–specific genes, we identified 60 genes that were not expressed or transcribed at reduced levels in L&H cells in comparison to normal GC B cells, including many already described.

As plasma cells down-regulate many B cell genes, it has to be considered whether the low expression of multiple B cell genes reflects a (partial) differentiation of L&H cells or their precursors toward a plasma cell phenotype. However, the PCA suggested that L&H cells are more similar to GC and memory B cells than to plasma cells. Moreover, for key plasma cell markers (i.e., BLIMP1, XBP1, and CD38), the expression levels of L&H cells were similar to the ones of GC B cells and lower than in plasma cells (unpublished data). Thus, the down-regulated B cell phenotype of L&H cells cannot be explained by a plasma cell differentiation.

The diminished expression of the B cell–specific transcription factors PAX5, E2A, EBF, Ikaros, and BCL11A, and of the Notch1 inhibitor deltex 1, is likely to result in a reduced expression of multiple B cell genes and, hence, is presumably a major cause of the partial loss of the B cell phenotype in L&H cells. Furthermore, the aberrant expression of ID2, a negative regulator of E2A, in L&H cells (66) may also contribute to the diminished expression of several B lineage–specific genes in these cells. This could also be caused by epigenetic silencing, but promoter methylation does not explain the loss of expression of CD10, CD19, or LCK in L&H cells (65).

Only a single B cell–associated gene, *IL21R*, was up-regulated in L&H cells, and it also showed a tendency for increased expression at the protein level. As follicular T helper cells are the main producers of IL21 and as IL21R triggering improves the survival of normal GC B cells, sustained IL21R expression may be important for the survival of L&H cells and their interaction with surrounding T helper cells (67).

In cHL, the global loss of the B cell phenotype may be of selective advantage for the HRS (precursor) cells to escape the apoptotic program in GC B cells with destructive IgV mutations (2). However, L&H cells express functional B cell receptors, rendering this concept unlikely for NLPHL. Moreover, L&H cells still express many genes at immunohistochemically detectable levels, and they are closely associated with normal constituents of GC, indicating microenvironmental interactions like those of normal GC B cells. Therefore, it appears that (some) B cell functions are still essential for the survival and proliferation of those cells. Perhaps the diminished expression and/or function of B cell genes in L&H cells is a side effect of the so far largely unknown transforming event in these cells. Indeed, for *PAX5*, mutations were recently described in L&H cells, although their functional consequences are still unclear (14).

Identification of L&H cell–specific genes

We identified 49 genes as being significantly up-regulated in L&H cells in comparison to normal B cells and B-NHL. By immunohistochemistry, we showed protein expression of MMP12, CTSB, and EOMES (Fig. 4) in L&H cells of primary NLPHL, thereby confirming our microarray data. Indeed, the genes showing deregulated expression in L&H cells may represent interesting new diagnostic markers and might be of special interest for the pathogenesis of NLPHL. Protein kinase C ζ is involved in cell growth, proliferation, and differentiation, as well as in the activation of NF-κB (68). KISS1 receptor activates ERK signaling (46). High levels of ubiquitin D (UBD) were shown to increase mitotic nondisjunction and chromosome instability promoting tumorigenesis (69). Up-regulated UBD in L&H cells might therefore contribute to chromosomal abnormalities in NLPHL.

L&H cells show deregulation of apoptosis regulators

L&H cells show up-regulated expression of genes encoding antiapoptotic molecules and down-regulation of most pro-apoptotic genes. The pattern is not only a characteristic feature of L&H cells in comparison to the apoptosis-prone GC B cells, but also in comparison to the other normal B cell subsets, indicating a tumor-specific deregulation of molecules regulating apoptosis (Fig. S4). Notably, HRS cells and most TCRBLs and DLBCLs show a similar expression pattern of pro- and antiapoptotic genes as L&H cells, whereas FLs and BLs behave differently in this regard. This indicates similar pathogenic mechanisms in NLPHLs, TCRBLs, and some DLBCLs to escape apoptosis, further supporting the similarity of these malignancies.

L&H cells are characterized by a strong constitutive NF-κB activity

Constitutive NF-κB activity is involved in deregulated proliferation, malignant transformation, and resistance to apoptosis in a variety of tumors, including cHL (70). In previous studies, active RelA was detected in L&H cells in a small collection of 6 NLPHL cases (71), and cREL was found in only 3 out of 15 NLPHLs (72). These studies did not address whether these NF-κB factors indeed cause expression of NF-κB target genes. To clarify this, we analyzed the expression of a large set of NF-κB target genes in L&H cells and found it to be at least as high

as in HRS cells (Fig. 4). Strong immunostaining for the active form of p65 in L&H cells in 19 out of 19 NLPHLs (Fig. 5 A) further confirms constitutive NF-κB activity in L&H cells.

The mechanisms underlying the strong NF-κB activity in L&H cells are still unclear. Genomic gains of the *REL* gene, as frequently seen in cHL, are presumably not involved, because L&H cells rarely show active c-REL (72). A contribution by EBV, as in cHL, does not play a role in NLPHL, as L&H cells are virtually never EBV infected. Remarkably, whereas T cells rosetting around HRS cells in cHL frequently express CD40L and may thus cause NF-κB activation by triggering CD40 on the HRS cells, rosetting T cells surrounding L&H cells rarely express CD40L, rendering a contribution of this signaling pathway in L&H cells also unlikely (73). Whether inactivating mutations of the IκBα or the IκBε genes as in cHL are involved in the pathogenesis of NLPHL remains to be clarified.

Implications for NLPHL pathogenesis, treatment, and diagnosis

The present work revealed important novel insights into the nature and pathogenesis of the L&H cells in NLPHL. Although L&H cells clearly resemble GC B cells in many phenotypic and genetic aspects, their gene expression profile seems to place them at the transition of GC B cells to memory B cells. We identified several pathogenetic mechanisms in NLPHL, including strong constitutive activity of NF-κB and activation of the ERK signaling pathway. Activation of the ERK pathway is critical for a large number of Ras-induced cellular responses and has been implicated in the pathogenesis of several cancers. Its aberrant activation in L&H cells was previously unknown and might be of pathogenic relevance. Several pharmacological agents that inhibit NF-κB or ERK are currently under clinical investigations (74, 75) and may become suitable for therapy in NLPHL. Further pathogenetic mechanisms in NLPHL involve the suppression of apoptosis and the creation of an immunosuppressive environment to escape from immune surveillance. The role of other genes that emerged to be specifically deregulated in L&H cells requires further investigation.

Because HRS and L&H cells show marked differences in immunohistochemical analysis (1, 62), their close relatedness in terms of gene expression profile appears surprising. Similarities among these tumor cells include constitutive NF-κB activity and ERK signaling, which suggest that NLPHL and cHL may share similar pathogenetic mechanisms. The gene expression profile of HRS and L&H cells also resemble each another because of down-regulation of B lineage–specific genes, although this event is not as severe in L&H cells as in HRS cells. L&H cells turned out to be even more similar to TCRBL (and a subset of DLBCL) in terms of gene expression. Because the distinction between NLPHL and TCRBL is, however, clinically important (76), the genes discriminating between L&H cells and TCRBL identified in this paper may become valuable markers for the differential diagnosis of NLPHL and TCRBL.

MATERIALS AND METHODS

Purification of normal B cell subsets and selection of lymph node biopsies of lymphoma patients

Naive and memory B cells were isolated from the peripheral blood of five healthy adult donors. Tonsils for the isolation of centrocytes, centroblasts, and plasma cells were obtained from patients undergoing routine tonsillectomy. Approval by the Internal Review Board in Essen for these studies was obtained. Tonsils and blood samples were immediately cooled at 0–4°C, and cells were further processed and immediately sorted after staining at this low temperature to avoid changes in gene expression caused by RNA turnover or signaling effects of the antibodies used for staining. 2,000 naive B cells were isolated by magnetic cell separation, depleting $CD27^+$ (memory B cells, T cell subpopulation) and $CD11b^+$ (monocytes, macrophages, granulocytes, NK cells) cells, using the MACS system (Miltenyi Biotec), followed by FACS sorting of IgD^+ $CD27^-$ cells. 2,000 memory B cells were isolated by MACS depletion of $CD11b^+$ and $CD3^+$ cells, followed by FACS sorting of $CD20^+$ $CD27^+$ cells. 2,000 tonsillar GC B cells and plasma cells were isolated by FACS sorting according to the following marker expression: $CD20^{high}$ $CD38^+$ $CD77^+$ (centroblasts), $CD20^{high}$ $CD38^+$ $CD77^-$ (centrocytes), and $CD20^{low}$ $CD38^{high}$ (plasma cells). We used CD77 to separate centroblasts and centrocytes. However, it has meanwhile become clear that this marker is unsuitable to clearly separate the two GC B cell subsets for gene expression studies (77, 78).

As human transitional B cells have recently been identified as $CD20^+$ $CD38^+$ IgD^+ peripheral blood B cells (79), the isolated GC B cells might potentially be contaminated by transitional B cells. However, as only 5–15% of GC B cells express IgD and at least half of these are GC founder cells with mutated V genes (80), transitional B cells could account at most for ~5% of sorted GC B cells. Importantly, transitional B cells also express $CD5^+$, and tonsillar $CD20^+$ $CD38^+$ IgD^+ $CD5^+$ B cells were found to lack CD77 expression (79, 81). Thus, our sorted $CD77^+$ GC centroblast population is devoid of transitional B cells, and as there are no significant differences in gene expression between $CD77^+$ and $CD77^-$ tonsillar GC B cells (77, 78), we also conclude that the gene expression profile of $CD77^-$ centrocytes is not significantly contaminated by RNA from transitional B cells.

The total RNA of all lymphoma cases was isolated from frozen tissue sections with TRIzol (Invitrogen), followed by RNeasy Mini Kit column purification (QIAGEN) to check RNA quality with a 2100 Bioanalyzer Nano Assay (Agilent Technologies). Among the cases with good RNA quality (28S/18S ratio > 1.5; no signs of degradation in the electropherogram), 5 cases of NLPHL, 12 cases of cHL, 4 cases of TCRBL, 11 cases of DLBCL, 5 cases of FL, and 5 cases of BL, diagnosed according to the World Health Organization classification, were selected for gene expression profiling. One out of the five NLPHLs (NLPHL3) was microdissected from a lymph node taken at relapse; in another area of the lymph node, a transformation to DLBCL was observed, which was not present in the lymph node taken at disease onset and which was also subjected to microdissection and gene expression profiling (representing 1 out of the 11 DLBCLs mentioned, DLBCL3). For the analysis of B cell gene expression, we used an additional 11 profiles from human tonsillar $CD4^+$ and $CD8^+$ T cells that will be published as part of a future study.

Microdissection of lymphoma cells

5-μm-thick frozen sections of lymph nodes from lymphoma patients were mounted on membrane-covered slides (PALM), incubated with hematoxylin containing 200 U/ml RNase inhibitor (Roche) for 4 min, washed in molecular biology grade water for 2 min, incubated in 2% eosin for 15 s, washed again, and dried at 37°C for 3 h. Microdissection was performed using the laser microdissection and pressure catapulting (LMPC) technique with a UV laser beam (PALM). cHL, NLPHL, and TCRBL tumor cells were microdissected as single cells according to their characteristic morphological criteria concerning nuclear shape and diameter, chromatin distribution, nucleoli, and the amount and density of cytoplasm. In DLBCL, FL, and BL cases, areas with at least 95% tumor cells were selected so that these tumor cells could be microdissected in areas. Cells were directly catapulted into lysis buffer (Purescript; Gentra) and pooled in groups of 1,000–2,000 tumor cells.

RNA isolation and generation of cRNA and microarray hybridization

The Purescript RNA isolation kit (Gentra) was applied using 80 μg glycogen (Roche) as a carrier and reducing all reagents to one tenth of the amounts given in the standard protocol. The T7 RNA polymerase-based RiboAmp RNA amplification kit (version C; Arcturus) was used to amplify the RNA by in vitro transcription. In the first cDNA synthesis, 1.5 μg of T4 gene 32 protein (Ambion) was added to the reaction to improve the specificity and yield of the reaction. The first cDNA synthesis was followed by an in vitro transcription (IVT) and a second cDNA synthesis using random hexamers. 100 ng of the second cDNA synthesis product were used in the labeling reaction with the BioArray High Yield Transcription Labeling Kit (ENZO Life Science) with the following modifications: the incubation time was prolonged to 8 h and 150 U of the ENZO T7 RNA polymerase was substituted with 300 U of Stratagene T7 RNA polymerase (Stratagene). cRNA yield and electropherogram profiles were controlled after the first and second IVT on a 2100 Bioanalyzer RNA 6000 Pico or Nano Assay (Agilent Technologies), respectively. Fragmentation of cRNA, microarray hybridization to GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix), washing steps, and scanning of the microarrays was performed according to the Affymetrix protocol. The complete gene expression dataset has been deposited in the Gene Expression Omnibus under accession no. GSE 12453.

Immunohistochemistry

Immunohistochemical stainings were performed on paraffin sections of tonsils obtained by routine tonsillectomy and of lymph node biopsies from patients with NLPHL. Antibodies and staining procedures are summarized in Table S2 (available at http://www.jem.org/cgi/content/full/jem.20080809/DC1).

Statistical analysis of the microarray data

Microarray quality. Data analysis for quality of the technical performance of the microarrays was performed using the GCOS 1.4 software (Affymetrix). Default algorithm parameters were applied, and measured signal intensities of each probe set were scaled to a target value of 2,000 for the 100 control transcripts, as provided by Affymetrix (available at http://www.affymetrix.com/Auth/support/downloads/mask_files/hgu133_plus_2norm.zip). Additionally, the ".DAT" files were visually examined for possible artifacts. Scaling factors (SF), percentages of present calls (%P), and signal ratios of probe sets interrogating different segments (3′/M) of transcripts were largely comparable across the samples, with similar variations among samples of the various groups (SF mean = 7 [0.5–21.1]; %P mean = 24.6 [14.9–41.8]; 3′/M mean = 7.6 [1.8–13.1]), thus indicating that different isolation methods (LMPC or FACS) or slight differences in the RNA quality of live sorted cells (normal B cell subsets) and microdissected lymphoma cases did not have a measurable impact on the expression profiling analysis. Further statistical analysis was done with the statistical computing environment R (82). Additional software packages (affy, geneplotter, multtest, and vsn) were taken from the Bioconductor project, as previously described (83).

Microarray preprocessing. Probe level normalization was conducted using the variance stabilization method of Huber et al. (84). This method renders the variance of probe intensities approximately independent of their expected expression levels. Parameters (offset and a scaling factor) are estimated for each microarray, assuming that the majority of genes are not differentially expressed across the samples. In view of the computational complexity of the algorithm, parameters are estimated on a random subset of probes and are then used to transform the complete arrays. To compute probe set summaries, for each probe set an additive model on the logarithmic scale (base 2) was fitted to the normalized data of all arrays with the robust median polish method, considering differences in probe affinities via the probe effect (85, 86).

Unsupervised hierarchical clustering. Unsupervised hierarchical clustering was performed for the genes with a standard deviation ≥ 1 across all samples using the Manhattan distance and the mean linkage method. To prove the stability of the resulting dendrogram, we used Pvclust (87), an R software package for assessing the uncertainty in hierarchical cluster analysis.

Heat map. A heat map is a false color display of a matrix of numerical values. Heat maps were generated with Spotfire software (Spotfire DecisionSite 9.1, 1996–2007).

Differential gene expression. Many of the genes on the microarray are not expressed in most of the samples or have only a small variability across the samples. For each pairwise comparison, we first used a global filter to reduce the dimension of the microarray data. We applied an intensity filter (the signal intensity of a probe set should be >100 in at least 25% of the samples if the group sizes are equal) and a variance filter (the interquartile range of log2 intensities should be at least 0.5 if the group sizes are equal). If the group sizes are not equal, the signal intensity of a probe set should be >100 in at least a fraction α of the samples, where α is the smaller group size minus one, divided by the total sample size of the two groups.

The interquartile range of log2 intensities should be at least 0.1 if the group sizes are not equal. After the global filtering, we applied the two-sample *t* test (assuming equal variance in both groups) to identify genes that are differentially expressed between the two groups. To account for the multiple testing, we used the FDR, as described by Benjamini and Hochberg (88). In addition, FC values between the two groups were calculated for each gene. Differentially expressed genes were determined with FDR and FC criteria.

PCA. For preselected gene sets and predecided groups of samples, we used PCA analysis. The first principal component is used as an expression signature for the given gene set, which is then applied to the samples of all groups. A Wilcoxon test was performed to detect significant differences between the groups after PCA.

Online supplemental material

Fig. S1 shows an unsupervised hierarchical clustering of all normal and malignant samples analyzed. Fig. S2 shows a heat map of 42 genes differentially expressed between L&H cells and TCRBL. Fig. S3 shows a heat map of genes differentially expressed between L&H cells and all other normal and neoplastic samples. Fig. S4 shows a heat map of apoptosis-associated genes that show a significantly different expression in L&H cells compared with GC B cells. Fig. S5 shows a heat map for the expression of genes associated with ECM degradation and tissue remodeling in normal and neoplastic B cells. Table S1 lists the genes differentially expressed between L&H cells and TCRBL. Table S2 provides the conditions for immunohistochemistry. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080809/DC1.

We are grateful to Ralf Lieberz, Nicole Dieckert, Yvonne Michel, Ingrid Kremser, and Kerstin Heise for excellent technical assistance, and especially to Ralf Lieberz for excellent support in establishing immunohistochemical stainings. We are indebted to Roland Schmitz and Ewerton Marques-Maggio for stimulating discussions, and to Ulf Klein and Andreas Rosenwald for critically reading the manuscript. We thank Dr. Detlef Güssow and Melanie Kühnl for the opportunity to scan the Affymetrix arrays in their laboratory, and Prof. Ahmet Dogan for reviewing the diagnosis of two NLPHL cases.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (KU 1315/2-1, 2-2, and 5-2; and BR1238/6-1, 6-2, and 6-3), the German José Carreras Leukemia Foundation (SP 03/11), the Deutsche Krebshilfe e.V. (107230), and the Associazione Italiana per Ricerca sul Cancro (to B. Falini). E. Tiacci was the recipient of a fellowship (F 05/01) granted by the German José Carreras Leukemia Foundation.

The authors have no conflicting financial interests.

Submitted: 15 April 2008
Accepted: 27 August 2008

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