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Lymphoblastoid cell line with B1 cell characteristics established from a chronic lymphocytic leukemia clone by in vitro EBV infection

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Abbreviations: Ab, antibody; CDR, complementarity determining region; CLL, chronic lymphocytic leukemia; EBV, Epstein-Barr virus; FISH, fluorescence in situ hybridization; *IG*, immunoglobulin; *IGHV*, *IG* heavy chain variable; *IGL/KV*, *IG* lambda/kappa light chain variable; LCL, lymphoblastoid cell line; LMP-1, EBV-encoded latent membrane protein 1; MDA-LDL, oxidized low density lipoprotein of the malonedialdehyde type; miR, micro-RNA; nLDL, native low density lipoprotein; oxLDL, oxidized low density lipoprotein; SNP, single nucleotide polymorphism; RQ-PCR, real-time quantitative polymerase chain reaction

Chronic lymphocytic leukemia (CLL) cells express the receptor for Epstein-Barr virus (EBV) and can be infected in vitro. Infected cells do not express the growth-promoting set of EBV-encoded genes and therefore they do not yield LCLs, in most experiments. With exceptional clones, lines were obtained however. We describe a new line, HG3, established by in vitro EBV-infection from an *IGHV1–2* unmutated CLL patient clone. All cells expressed EBNA-2 and LMP-1, the EBV-encoded genes pivotal for transformation. The karyotype, FISH cytogenetics and SNP-array profile of the line and the patient's ex vivo clone showed biallelic 13q14 deletions with genomic loss of *DLEU7*, miR15a/miR16–1, the two micro-RNAs that are deleted in 50% of CLL cases. Further features of CLL cells were: expression of CD5/CD20/CD27/CD43 and release of IgM natural antibodies reacting with oxLDL-like epitopes on apoptotic cells (*cf*. stereotyped subset-1). Comparison with two LCLs established from normal B cells showed 32 genes expressed at higher levels (> 2-fold). Among these were *LHX2* and *LILRA*. These genes may play a role in the development of the disease. *LHX2* expression was shown in self-renewing multipotent hematopoietic stem cells, and *LILRA4* codes for a receptor for bone marrow stromal cell antigen-2 that contributes to B cell development. Twenty-four genes were expressed at lower levels, among these *PARD3* that is essential for asymmetric cell division. These genes may contribute to establish precursors of CLL clones by regulation of cellular phenotype in the hematopoietic compartment. Expression of CD5/CD20/CD27/CD43 and spontaneous production of natural antibodies may identify the CLL cell as a self-renewing B1 lymphocyte.

Introduction

Chronic lymphocytic leukemia (CLL) develops as an expansion of clonal CD5[+] B cell with mature phenotype. The proliferating compartment is in the bone marrow and in the lymph nodes, while the cells in the blood are arrested in G0/G1 phase. The gene expression profile of CLL cells is similar to that of memory B cells[1,2] and their phenotype is reminiscent of antigen experienced B cells with CD23, CD27, IgM, IgD expression.[3] The precise cell(s)-of-origin has not yet been established. Based on mouse B1 cell studies, a human B cell population (CD20[+], CD27[+], CD43[+], CD5[+]/CD5[-]) characterized by spontaneous release of natural IgM antibodies (Abs), efficient T cell stimulation and tonic intracellular signaling was recently identified as human B1 cells.[4] It was

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pointed out that these cells share most properties with malignant CLL cells at least of the poor prognosis type,[4] and may constitute the normal counterpart of CLL. Evidence was also recently presented that CLL pathogenesis involves self-renewing hematopoietic stem cells. From its multi-linear/polyclonal differentiation products, mono/oligo-clonal cell population(s) with highly biased *IGHV*-gene rearrangements arise,[5] most likely as a result of (auto)antigen driven selection in a unique compartment (*i.e.* pleural cavity or peritoneum) with special function for innate B cells producing natural Abs.

CLL cells express the CD5 and CD6 scavenger receptors.[6,7] The majority of cases produce natural Abs.[8-10] These Abs are produced both by restricted immunoglobulin heavy chain variable (*IGHV*) gene "stereotyped" CLL cases (~30% of all CLL) and by non-stereotyped cases. The Abs recognize epitopes on apoptotic cells and on certain microbial structures.[8,11] CD5 expression and this functional specificity defines the B1 "innate" B lymphocyte subset.[8,9] While $CD5^+$ B cells represent a high proportion of the B cells in cord blood (> 80%), primary follicles of fetal lymph nodes, and spleen, they represent only 5–20% of the B cells in adult blood and in adult lymphoid tissues.

Epstein-Barr virus (EBV) is not involved in the etiology of CLL. The relationship of CLL B cells and EBV is however unique. CLL cells separated from blood, express the EBV-receptor CD21 and can be infected by the virus in vitro. It is therefore remarkable that CLL cells generally do not carry EBV genome. In the in vitro infected CLL cells, the expression of EBV-encoded proteins is restricted and they do not exhibit the complete growth program. The infected cells do not express the EBV encoded cell membrane localized protein LMP1 that is essential for the transformation. Consequently they do not yield lymphoblastoid lines.[12-14] In rare cases, proliferating lines were obtained by infection of the cells sometimes with simultaneous application of an activating stimulus.[12,15] In these cases, the encoded EBV genes required for transformation are expressed and the derived LCLs resemble morphologically those derived from normal B cells. Similarly to LCLs derived from cord blood B lymphocytes, the CD5 marker initially present disappeared during prolonged cultivation, in most cases.[16]

We describe here the characteristics of an LCL established by in vitro EBV infection of CLL cells. We emphasize the authenticity of the cell line as demonstrated by the karyotype with biallelic chromosome 13q14 deletions and the unmutated *IGHV* genes being identical with the ex vivo patient CLL cells. Its phenotype, CD5/CD19/ CD20/ CD27/CD43-positive, and its Ab production are features of innate B1-like B cells. We attempted to trace characteristics that may have been retained from the cell(s)-of-origin of the CLL cell and therefore we compared expression of a panel of genes with that of normal B cell derived LCLs. We found higher expression of *LHX2* a gene, which was shown to promote self-renewal of a multi-potent hematopoietic progenitor cell. This gene may contribute to the establishment of the malignancy and may have been maintained in the LCL.

Results

Establishment of the lymphoblastoid cell line(s) by EBV infection. The mononuclear cell population derived from the patient's blood was incubated with the B95–8 EBV virus for 1 h, washed and cultured with or without CD40L-expressing L-cell fibroblasts.[17] Lines were established from the freshly collected samples and thereafter on four occasions from aliquots of the sample that was stored frozen. The latency period for the establishment of growing cell population was longer than it is usually in experiments when normal B lymphocytes are infected in vitro. For all parameters tested, the independently obtained lines were identical. One of the cell lines designated HG3 was selected and unless indicated these results are presented.

Cell surface phenotype with innate B1-like features. All established cell lines strongly expressed CD5. **Figure 1A** shows the expression of a CD5, CD20, CD27, CD43 markers on the HG3 cells. The cell line showed a weak expression of the monocyte/ macrophage marker CD14, and expression of the activation markers CD38 and CD70 (**Fig. 1A**). The line was also positive for CD19, CD21, CD31, CD69 (not shown). Upon prolonged

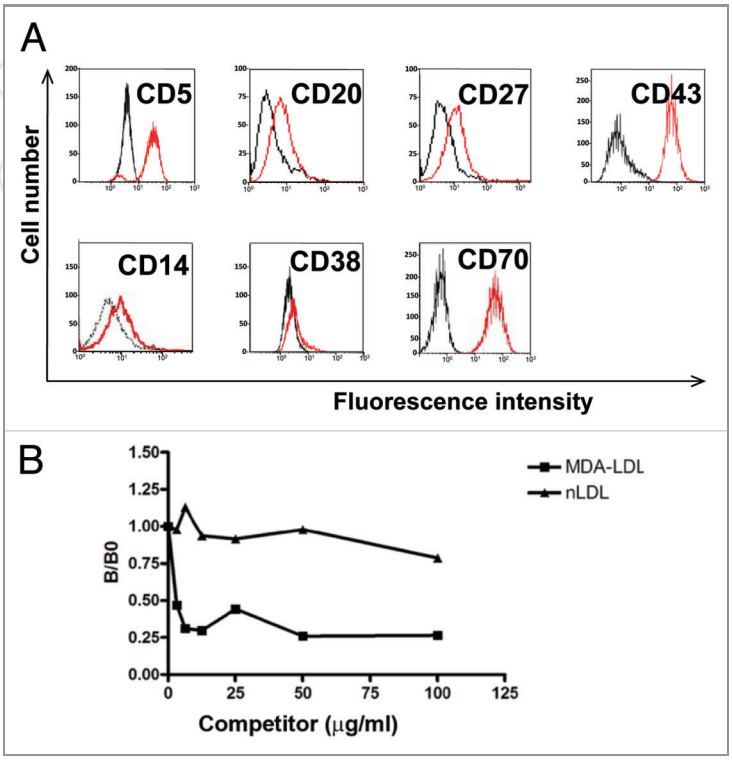

Figure 1. (A) Human B1 phenotypic markers expressed on HG3. Indirect immunofluorescence analysis with appropriate mAbs followed by FITC-of Cy-ChromeTM-conjugated anti-mouse Ig. Ten thousands events were counted in a FACScan flow cytometer. (B) Competition chemiluminescent ELISA for oxLDL. MDA-LDL competition ELISA was performed for HG3 CLL IgM mAb isolated from cell supernatant and purified by affinity chromatography. Plates were coated with MDA-LDL, then IgM was allowed to react with increasing amounts of soluble competitor MDA-LDL or native LDL (nLDL). B/B_0 indicates the ratio between bound (cpm) and bound (cpm) without competitor.

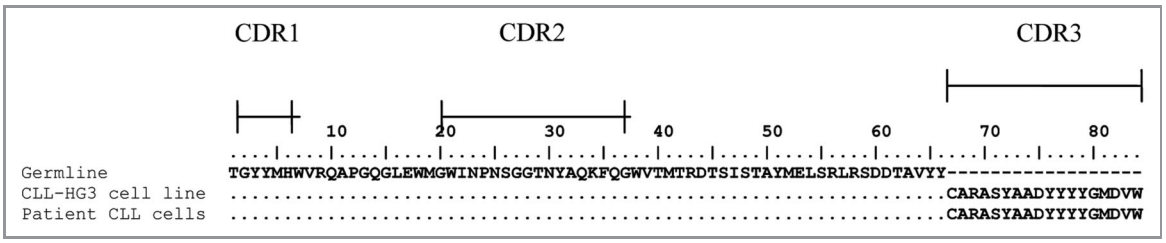

Figure 2. Identical *IGHV1–2* gene rearrangements in HG3 and the patient's CLL cells.

cultivation, CD5-expression was slightly decreased, whereas the CD14, CD19, CD20, CD21, CD23, CD27, CD31, CD38, CD43, CD69, CD70, IgM and λ−light chain expression were retained.

***IG* gene rearrangements and antigen-binding specificity.** The *IG* gene sequence of the cells in a frozen stored ex vivo patient sample and in the HG3 cell line were identical with unmutated *IGHV1–2* rearrangements (**Fig. 2**). The unmutated *IGLV2–14* light chain gene rearrangements were identical as well (not shown). It did not belong to any known stereotyped subset.[18] The HG3 cells spontaneously released IgM mAb that bound to oxidized low density lipoprotein (oxLDL) epitopes of the malonedialdehyde type (MDA-LDL), but not to native LDL (**Fig. 1B**). This specificity was previously shown for CLL Abs belonging to the stereotyped subset-1.[8,11] However, ex vivo CLL cells did not release IgM unless they were activated by CpG .

Karyotype, FISH, SNP-array and RQ-PCR analysis. The karyotypes of the ex vivo cells and the cell line showed biallelic 13q14 deletions, 46,XY,del(13)(q14)x2 (**Fig. 3**). It was confirmed in fluorescence in situ hybridization (FISH) and single nucleotide polymorphism (SNP) array analysis. The biallelic deletions included genomic loss of *DLEU7*, miR15a and miR16–1 (**Fig. 4**). Real-time quantitative polymerase chain reaction (RQ-PCR) confirmed loss of miR15a expression, however, miR16–1 RQ-PCR was positive. In addition, insertions were detected on chromosomes 11 and 14 in the cell line (**Fig. 3A and B**) but not in the ex vivo CLL cells.

Gene expression profiles. In comparative analysis of the expression of 21,632 genes in the HG3 line vs. two LCLs derived from normal B cells, IARC-171 and LCL-3M (**Table S1A and S1B**) 32 genes were found to be expressed relatively higher and 24 genes were expressed relatively lower. For a few selected genes the results were validated by quantitative PCR.

Genes expressed with a 2-fold (or over) difference in the HG3 cells included *LHX,* and *LILRA4.* Among the relatively low expressed genes were *PARD3, FCRLA* and *TCL1*. The *LHX2* (LIM homeobox 2) gene was expressed 20-fold higher in HG3 compared with LCL-3M (**Table 1**). In three of four additional CLL-LCLs (232B4, I83-E95, CI, MEC1), high expression of *LHX2* was detected (**Table 1**). The *LILRA4* [leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 4] gene also showed higher expression (**Fig. 5**). Reduced expression was found for *FCRLA* (cytochrome p450, family 1, subfamily B, polypeptide 1) (**Fig. 5 and Table 1**), *CYP1B1* (**Fig. 5**), the polarity complex gene *PARD3* [par-3 partitioning defective 3 homolog (*C. elegans*)] (**Table 1**) and *TCL1A*.

Expression of EBV encoded proteins EBNA-2 and LMP-1 in HG3. The EBV encoded nuclear antigen EBNA-2 and the latent membrane protein LMP-1 were expressed in all cells as seen by immunofluorescence. This characterizes the growth program denoted as type III EBV expression. As in LCLs generated from normal B lymphocyte population, the levels of both proteins were variable and they were not related. **Figure 6** shows the simultaneous staining with both antibodies. Similar results were detected by single staining for each antigen (not shown).

Discussion

Despite recent advancements in the CLL field, the cell(s)-of-origin and the transforming event(s) are largely unknown. The expression of CD5 on CLL cells is an obligatory marker for classification of the disease. CD5 appears early in the differentiation of T lymphocytes and it is abundantly expressed on the mature T cells. In addition, CD5 can be detected in a subset of B lymphocytes with frequencies varying in different developmental stages, anatomical localization and function. The $CD5^+$ B cells, referred to as B1 cells in mice, are self-renewing and produce natural Abs. A small fraction of human B cells with a $CD20^+CD27^+CD43^+CD70^-$ phenotype (partially overlapping with $CD5^+$ B cells), showing natural IgM secretion and a capacity to stimulate T cells, was recently detected in umbilical cord and in adult peripheral blood.[4] These cell are candidates for the B1 subset in humans,[4] and may be one of the proposed CLL precursor(s).[19] The CLL-derived permanent cell line described in this study resembles these human B1 cells both with regard to its surface marker profile and to its function as a natural Ab secreting cell. An exception is CD70, which is expressed in HG3, but low or absent in the putative human B1 population. This is explained, however, by findings in previous reports showing that ex vivo CLL cells are CD70-negative[4,20] or weakly positive,[21] whereas all LCLs are CD70-positive.[22] The B1 cell capacity to stimulate T cells, which is one of the functional markers, appear not to be shared by resting ex vivo blood CLL cells, whereas activated CLL cells do stimulate.[23] The B1 function of tonic intracellular signaling is found in CLL cells isolated from lymph nodes, which display gene expression profiles that indicate BCR activation.[24,25] However, in LCLs, the BCR signaling is hi-jacked/blocked by the virally encoded protein LMP2A.[26] With these notions, the HG3 line recapitulates a B1 cell.

The human $CD5^+$ B cells represent a minority in the adult B cell population, while in the cord blood > 80% of the cells are

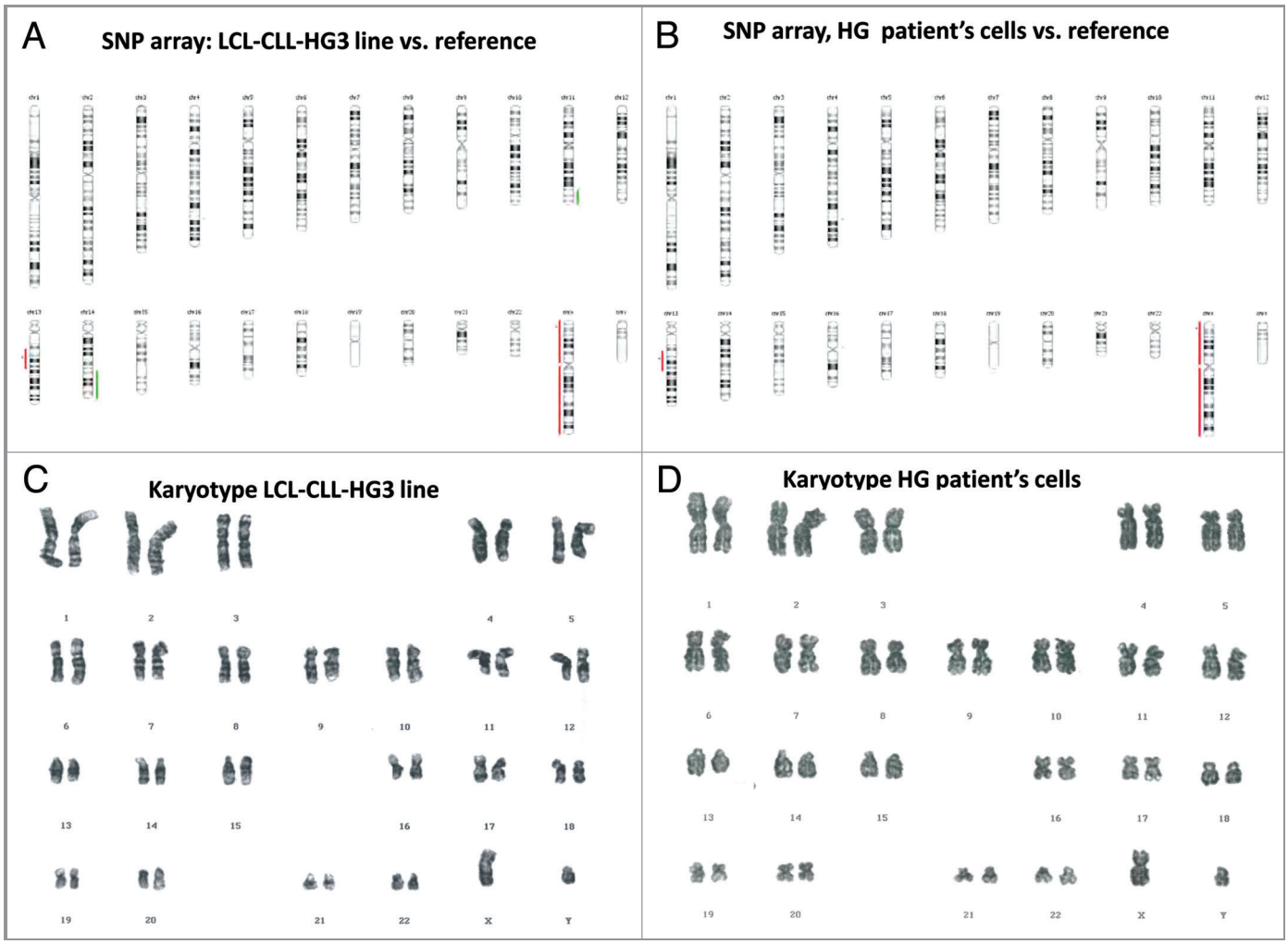

Figure 3. SNP array analysis and karyotype reveal biallelic chromosome 13 deletions. (A and B) show results from SNP array analysis in HG3 cell line and HG patient. Red marking show deletions and green marks show amplifications. (C and D) G-banding are showing identical biallelic deletions of chromosome 13 in HG3 cell line and in HG patient's leukemic clone.

positive. Ab production of the $CD5^+$ B subset is "innate," prompt and T cell independent. It contributes, however, to the development of T cell-dependent, adaptive Ab response.[27] The $CD5^+$ B subset is considered to play a role in the pathogenesis of autoimmune diseases.[28] The production of 'natural' IgM Abs by CLL clones suggests that they are derived from B cells with

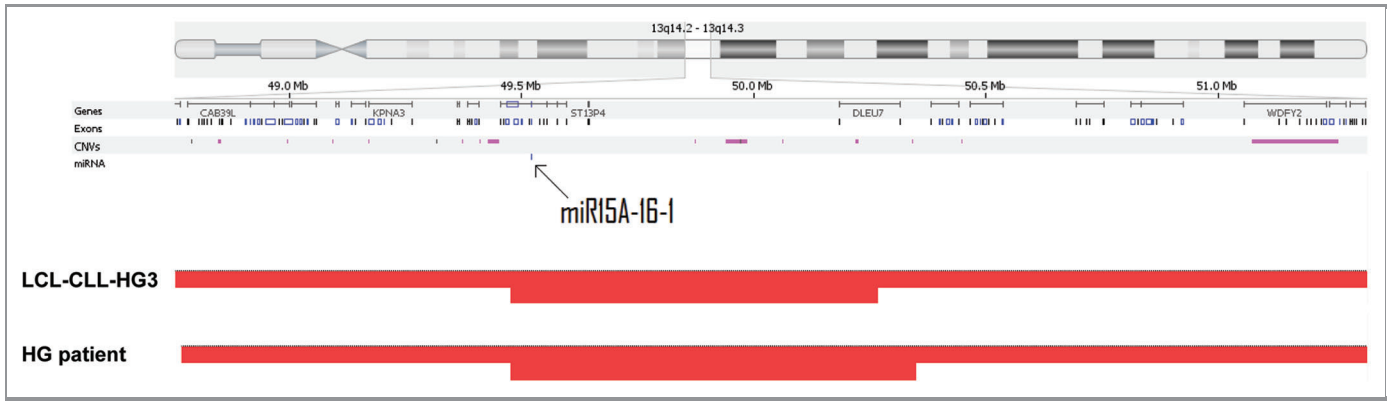

Figure 4. Zoom in picture of the homozygous 13q4 deletion. The array revealed that *DLEU-7*, miR16–1 and miR15a were deleted.

Table 1. *LHX2, PARD3 and FCRLA* mRNA levels in CLL-derived LCL lines related to its expression in LCL-3M derived from normal B cells

Cell line	Derivation	*LHX2* (fold higher expression)	*PARD3* (fold lower expression	FCRLA (fold lower expression)
LCL-3M	normal B cells	(1)	(1)	(1)
HG3	CLL	19.7	3.8	6.0
CI (Corina I)	CLL	30.9	-	-
MEC1	CLL/PLL	30.0	-	-
I83-E95	CLL	12.6	-	-
232B4	CLL	0.9	-	-

RQ-PCR analysis. The values represent means of triplicate measurement obtained in 2 independent experiments. GAPDH was used as reference.

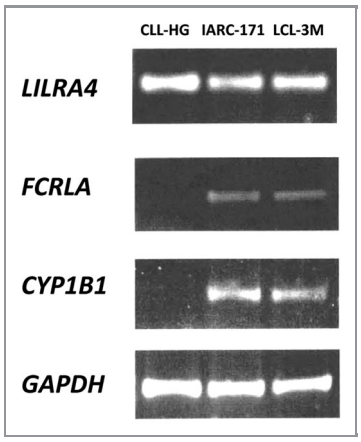

Figure 5. Gene expression difference observed by RT-PCR. Comparison between gene expression of *LILRA4, FCRLA* and *CYP1B1* using RT-PCR in the CLL-HG cell line and 2 LCL lines IARC-171 and LCL-3M derived from normal B cells.

reactivity against self and neo-self epitopes and by that the proliferation of cells may be induced by (auto)antigenic pressure. One of the most frequent epitopes recognized by natural Abs present on apoptotic cell membranes/blebs and on the surface of multiple bacterial species is the oxLDL-like epitope.[8,11] This was also recognized by the HG3 IgM Abs.

The expression of the CD5 marker in the context of the EBV-induced transformation of B cells deserves closer investigation. In contrast to normal B lymphocytes, EBV infected CLL cells do not develop to LCLs.[17,29] The virally encoded protein expression is restricted, the cells express EBNAs, but not LMP-1 that is also required for transformation.

The HG3 cell line and other CLL derived LCLs initially expressed CD5, but it diminished (in some cases) after prolonged cultivation. In cord blood B cells CD5-expression disappeared already 72 h after infection.[30] The HG lines are unique as they maintained CD5 expression. The transformed cells that yielded the HG3 cell line appeared with a delay in the in vitro infected CLL cultures. This suggests that the phenotype of a few CLL cells changed in the culture, including probably the appearance of cellular factors, which were required for expression of the transforming EBV genes. The recent report that CLL cells share characteristics with spontaneous IgM secreting B1 cells[4] may explain why mature CD5$^+$ B cells that harbor IgM and IgD secretory granulae, cannot be immortalized by EBV. We previously showed that secretory differentiation in the LCLs accompanied downregulation of EBNA,[31] and slowdown of proliferation. 10–20% of the natural Ab producing CD5$^+$ B cells have the secretory machinery, and this differentiated phenotype may be incompatible with expression of the EBV transforming genes. We suggest, based on the presence of early pro-B cell markers in the HG3 cells, that these cells are less mature and thus transformable by EBV. More mature lymphoblasts/plasmablasts cannot be transformed by EBV.

In comparing proliferating CLL cell with normal proliferating B cells it can be assumed that influences on the gene expression arising from tissue culture microenvironment or presence of EBV would be the same for all LCLs, whether CLL-derived (HG3) or derived from normal B cells (LCL-3M and IARC-171). Our gene profile comparison with conventional

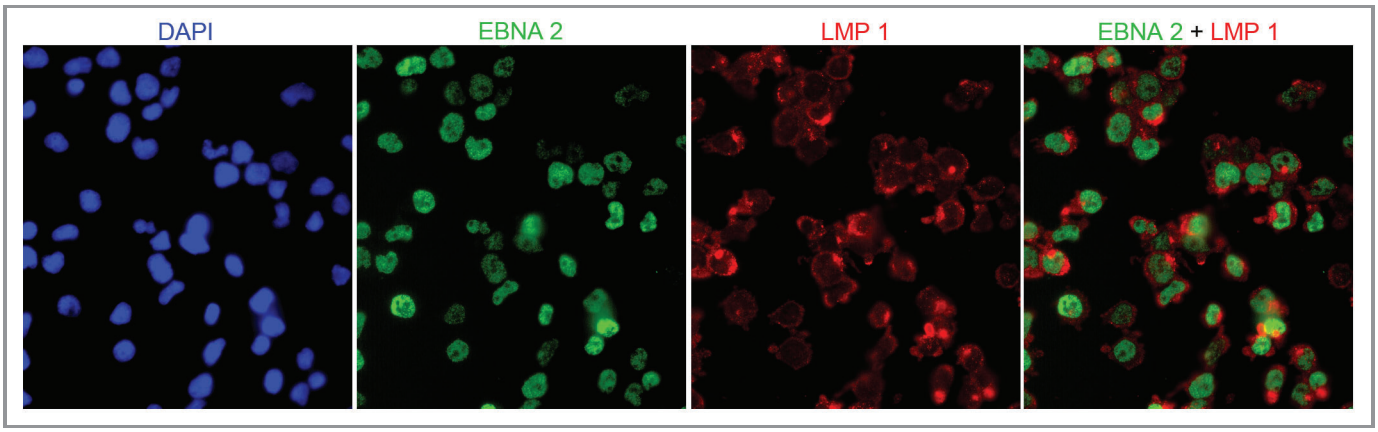

Figure 6. Multicolor immunofluorescence for EBNA-2 and LMP-1. From left to right: DAPI staining (Vectashield) for nuclear DNA; EBNA-2 staining with mouse anti-EBNA-2 mAb clone PE2, followed by goat anti-mouse IgG_1-Alexa488; LMP-1 staining with anti-LMP-1 mAb clone S-12, followed by anti-mouse IgG-Alexa594; Merged image of EBNA-2 and LMP-1.

normal B cell derived LCLs showed elevated expression of some genes involved in early B cell development, among these, the higher expression of the *LHX2,* which was shown to promote self-renewal of a multi-potent hematopoietic progenitor cell, and *LILRA4* may be of particular significance in view of the recent findings of Kikushige et al.[5] showing that self-renewing hematopoietic stem cells are the primary targets in pathogenesis of human CLL.

LHX2 codes for a regulatory protein involved in the differentiation of lymphoid multipotient progenitor cells with self-renewal properties.[32,33] It is also expressed in chronic myelogeneous leukemia (CML).[34] In embryonic mouse and rat liver tissue, *LHX2* was found to be expressed early during B cell differentiation in a subepithelial lymphoid compartment. Recent studies in CLL and in normal blood B cells[35] showed that *LHX2* was lower (or absent) in the CLL cells.[1,2,35] However, our comparison of CLL-LCL and conventional LCLs showed the opposite relationship. It is possible that the different results are explained by the fact that we analyzed proliferating cells, whereas blood CLL cells as analyzed previously by others, represent resting G_0/G_1 cells. The *LHX2* expressing embryonic lymphoid cells may resemble a unique human $CD5^+$ B cell population, detected in the marginal zone equivalent in tonsil subepithelial areas.[36] These B cells were shown to produce Abs to T-independent antigens (natural Abs), and it was characterized by a memory B1-like profile with $CD5^+CD20^+CD27^+$ phenotype, thus resembling CLL B cells and the B1 cells.

The *LILRA4* gene, also known as ILT7 (immunoglobulin-like transcript 7) and CD85 g, is a member of a large family of leukocyte-Ig-like receptors,[37] which is mostly expressed on plasmacytoid dendritic cells. It was recently identified as receptor for bone marrow stromal cell antigen-2 (BST2; CD317)[38] that is important for pre-B cell development. Thus, the expression of the gene in HG3 may reflect the developmental stage of the CLL cell precursor. It was also found that a genetic variant of *LILRA4* (LILRA4 P27L) was associated with progression free survival in a cohort of 977 CLL patients.[39]

The majority (89%) of CLL cases express the macrophage marker CD14,[40] also found in HG3, whereas in a panel of 37 conventional LCLs, all were negative for CD14.[41] CD14 functions as a receptor for LPS, as well as a receptor for apoptotic cells,[42] and it is a co-receptor for Toll-like receptors (TLR) 7 and 9, which are expressed in CLL cells.[43,44] Interestingly, soluble CD14 derived from monocytes was recently shown to be a growth factor for CLL.[45]

Based on CLL mAb specificity with binding to epitopes on apoptotic cells (oxLDL-like epitopes and non-muscle myosin)[8,46] and certain microbes, we assumed that the CLL precursor B cells belong to a B1 cell subset endowed with specialized innate B cell scavenging functions for prompt removal of apoptotic cells. In addition, several reports describe co-receptors for apoptotic cells, including CD36 (receptor for oxLDL-like epitopes) and CD31 that is involved in tethering apoptotic cells.[47] CD5 and CD6 are scavenger receptors for certain microbes i.e., β-glycan of fungal cell wall components.[48,49] For this function, CLL and $CD5^+$ cells share many properties with macrophages. Whether the CLL cell (s)-of-origin belong to a bipotential B-macrophage progenitor, which has also been identified in bone marrow of adult mice,[50] requires further studies. CD31 was found to be relatively higher expressed in HG3 cell line. One of the receptors for CD31 is CD38, which is found on all proliferative i.e., Ki67-positive CLL cells in the proliferative compartment of pseudofollicles.[51] CD31 have important functions for removal of apoptotic cell,[47] and the CD31/CD38 interaction may induce self-renewal CLL proliferation. CD38 was also expressed on the HG3 cells.

One of the genes that were expressed at a low level comparing HG3 vs. LCLs from normal B cells was *PARD3*, first identified in *C. elegans*. It is essential for asymmetric cell division and polarized growth. *PARD3* is thought to be a master regulator of apical-basal cell polarity, a process that has been indirectly implicated in tumorigenesis. Loss of *PARD3* leads to abnormal cell contacts, and it was recently shown that disruption of polarity complex genes (*PARD3*) is frequent in human cancers.[52] We speculate that during the leukemogenesis of CLL, disrupted *PARD3* could lead to loss of cell-contacts in the normal (subepithelial) compartment, inducing dissemination of the cells.

The karyotype of HG3 line and the patient's CLL clone showed biallelic 13q14 deletions, with genomic loss of *DLEU7*, miR15a and miR16–1. These are two micro-RNAs that are reduced in most CLL cases, with or without 13q14 deletion.[53] The complex regulation of miR15a/16–1 is not fully understood, but they are suggested to be involved in regulating proliferation and apoptosis.[54-56] They are expressed as a cluster within intron 4 of *DLEU2* and are regulated by the *DLEU2* promoter.[54] Thus, any genetic alteration of *DLEU2* can also affect miR-15a/16–1 expression. In our analysis of miR15a and miR16–1 in HG3, we found that the two miRs were deleted, whereas this genomic loss did not conform with the RQ-PCR that revealed loss of mR15a and relatively high expression of miR16–1. The reason for detecting miR-16–1 in RQ-PCR, although it is deleted, is because miR-16–2 of the miR-15b/16–2 cluster (chromosome 3) has exactly the same sequence (kindly pointed out by Drs. C. Croce and U. Klein). This may indicate that miR-15a is probably more important than miR-16 during CLL leukemogenesis. It is important to note that the chromosome 13q14 deletion does not counteract the EBV induced transformation event.

In summary, the LCL derived from an IgM *IGHV* unmutated CLL case is authentic and maintained characteristics of the CLL cell, such as $CD5^+$, $CD20^+$, $CD27^+$, CD43, natural IgM, λ Ab secretion with restricted target structure recognition. The biallelic chromosome 13q14 deletion was identical in the patient and cell line, including the loss of *DLEU7* and miR15a/16–1. When comparing the gene expression pattern of the cell line with LCLs derived from normal B lymphocytes, we could single out two genes, *LHX2* and *LILRA4* which could have significance for the development of the CLL condition. *LHX2* is expressed in self-replenishing hematopoietic stem cells, and *LILRA4* is involved in early B cell development. *LHX2* expression was not detected in ex vivo blood CLL cells, however it could be turned on in dividing CLL precursors and silenced in resting cells. The CLL derived HG3 LCL is the first cell line that resembles human

B1 cells both with regard to its surface marker profile ($CD5^+CD20^+CD27^+CD43^+$) and its function *i.e.* spontaneous natural Ab-secretion.

Materials and Methods

Cell culture. Peripheral blood mononuclear cells (PBMC) were collected from venous blood of a 70 y old male CLL patient (Rai stage II) upon informed consent according to the Ethical committee of Karolinska University Hospital guidelines. The white blood cell count was 184.1 × 10^9/l. The lymphocytes were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden). Sixty-four percent of the cells were B lymphocytes, all were monoclonal for IgM, λ-light chains, $CD5^+$, $CD23^+$, $FMC7^+$, $CD22^+$, $CD52^+$, $CD38^+$. Twenty percent of the PBMCs were T cells, 3% were NK cells and the CD4/CD8 ratio was 0.3. T cells and macrophages were removed as described.[17] CLL cell ($CD5^+/CD19^+$) purity in the CD3-depleted populations was more than 98% and the cells expressed the EBV receptor CD21. The CLL cells were exposed to the B95–8 EBV strain for 1 h, washed and then cultured with CD40L human L-cell fibroblasts in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.[17] Cells were frozen and stored in liquid nitrogen. EBV-infected cell lines were initiated on several occasions. The efficiency of the infection was indicated by the expression of the EBV encoded nuclear protein 2 (EBNA-2) in 20–30% of the cells in 4 d old cultures, as detected by immunofluorescence staining. The LCLs derived from normal B cells (LCL-3M, IARC-171), or from CLL cells: CI,[57-59] MEC1,[60] I83-E95[15] and 232B4[15] were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Phenotype analysis of the CLL LCL. Flow cytometry analysis was performed with mAbs against CD5, CD14, CD19, CD20, CD23 (Dako); CD27, CD43 (BioLegend); CD31, CD38 (AbD Serotec); CD69, CD70 (BD Biosciences PharMingen). These were detected with FITC-, or Cy-ChromeTM -conjugated anti-mouse IgG (Dako). The cells were washed once with PBS/1% FCS, incubated for 30 min at 4°C with indicated Abs, washed once with PBS/1% FCS and resuspended in 500 μl PBS/1% formaldehyde. Ten thousand events were collected in a FACScan flow cytometer, and the results were analyzed using CellQuest software (BD Biosciences).

Immunofluorescence staining. The cells were deposited on glass slides in a cytospin centrifuge and fixed in acetone-methanol (2:1). For single staining, following rehydration in balanced salt solution (BSS), the slides were incubated with primary Ab at room temperature for 60 min. For EBNA-2, mouse mAb PE2 (IgG_1, Leica Biosystem NovoCastra Ltd., dilution 1:50) and for LMP-1, S-12 (IgG_{2a}, culture supernatant, dilution 1:50) were used. Following washing in BSS, fluorochrome labeled anti-mouse Ab was added and the slides were incubated for 30 min at room temperature for visualization. EBNA-2 was visualized with goat anti-mouse IgG_1-Alexa Fluor 488 (Invitrogen, dilution 1:200) and LMP-1 was detected with goat anti-mouse IgG_{2a}-Alexa Fluor 594 (Invitrogen, dilution 1:200). The slides were mounted with Vectashield containing DAPI (Vector Laboratories, Inc.). For double staining, first EBNA-2 was stained as described above. Thereafter the slides were washed and the reagents for detection of LMP-1 were applied. Images were generated with a Leitz DM RB microscope (Leica Microsystems) using a 100x/1.32 N.A. oil immersion objective lens. Images were captured with a Hamamatsu dual-mode cooled charged-coupled-device camera (C48880) and Hipic 6.4.0 software (Hamamatsu Photonics) as 8-bit uncompressed TIFF files. Pictures are edited for optimal color contrast by using Photoshop CS3 (Adobe systems).

G-banding and FISH analysis. G-banding of chromosomes and FISH was performed by standardized routine methods as described elsewhere.[61] The probes for FISH analysis were the following: p53(17p), ATM(11q), D13S1319(13q14.3), LAMP1 (13q34) and CEP12(trisomy 12).

Microarray experiments and data analysis. Microarray production was performed as described by Takahashi et al.[62] with slight modifications. Briefly, 21,632 cDNA clones were PCR amplified directly from bacterial stocks purchased from Research Genetics. After ethanol precipitation and transfer to 384-well plates, clones were printed onto poly-L-lysine coated glass slides. Two micrograms of $poly(A)^+$ RNA from HG3 cells and 2 μg of $poly(A)^+$ RNA from each of the two LCLs derived from normal B cells, IARC-171 and LCL-3M, were reverse transcribed with oligo(dT) primer and Superscript II (Life Technologies) in the presence of Cy5-dCTP and Cy3-dCTP (Amersham Pharmacia Biotech), respectively. The Cy3- and Cy5-labeled cDNA probes were hybridized to prewarmed (42°C) slides for 20 h at 42°C. After hybridization, slides were washed, dried and scanned immediately in a confocal fluorescent scanner. Images were analyzed by using the software Genepix Pro 3.0 (Axon Instruments). The ratios of net fluorescence from the Cy5-specific channel to the net fluorescence from the Cy3-specific channel were calculated for each spot, representing CLL-LCL mRNA expression relative to the LCLs derived from normal B cells. Ratios were log-transformed (base 2) and normalized so that the average log-transformed ratio equaled zero.

SNP-array. High density SNP array on high quality DNA prepared from HG3 and peripheral blood sample from the HG patient was performed according to standard protocols for Affymetrix GeneChip® 250 K arrays (Affymetrix, Inc.). Briefly, total genomic DNA was digested with a restriction enzyme (*Nsp1*), ligated to an appropriate adaptor for the enzyme, and subjected to PCR amplification using a single primer. After digestion with DNase I, the PCR products were labeled with a biotinylated nucleotide analog using terminal deoxynucleotidyl transferase and hybridized to the microarray. Hybridized probes were captured by streptavidin-phycoerythrin conjugates. Copy number normalization, to produce log(2) ratios, was performed using the Copy Number Analysis Tool (CNAT) 4.0.1. The reference set was 82 normal samples analyzed at the Uppsala Array Platform. Subsequent copy number analysis and group comparisons were performed using the software BioDiscovery Nexus Copy Number 3.0 and its built in Rank Segmentation algorithm, segmenting each interval and estimating copy numbers.

PCR amplification of *IGHV* and *IGLV/KV* rearrangements. DNA was isolated as described above. *IGHV* and *IGLV/KV* subgroup-specific PCR amplification was performed using consensus primers, as previously described.[64] The sequence reactions were performed using either the BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems) or the DYEnamic ET Dye Terminator Kit (Amersham Biosciences) and analyzed with an automated DNA sequencer (ABI 377 or ABI3700, Applied Biosystems; and MegaBACE 500 DNA Analysis System, Amersham Biosciences). The sequences were aligned to *IG* sequences in the IMGT database. *IGHV* sequences with less than 98% identity to germline were defined as mutated.

Reverse transcription-PCR and RQ-PCR. The cells were thawed, washed in PBS and total RNA extracted utilizing the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed with 1–2 μg of total RNA in a 20 μl reaction mixture with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). RT-PCR: Separation and visualization of the PCR products was performed according to standard protocols. RQ-PCR was performed using the TaqMan Fast Universal PCR Master Mix and the TaqMan Gene expression assay (Applied Biosystems): The primers and probes for *LHX2, LILRA4, PARD3, FCRLA* and *GAPDH* (Hs99999905_m1) used as an internal control, were designed and quality controlled by Applied Biosystems. All reactions were performed in triplicates twice on a 7500 Fast Real-Time PCR system (Applied Biosystems). miR RQ-PCR: RNA was extracted using Trizol solution (Invitrogen) and 10 ng of total RNA was used for the expression analysis of miR-15a (assay ID 00389) and miR16–1 (assay ID 00391) according to manufacturer's protocol (Applied Biosystems). RNU6B (assay ID 001093) was used as an internal control.

Chemiluminescent ELISA for oxLDL Abs. CLL mAb binding to oxLDL was determined in chemiluminescent ELISA according to previously described methods.[65] The most frequently used model of oxLDL, malonedialdehyde LDL (MDA-LDL) was used. Abs against native LDL (nLDL), was also tested. Competition ELISA was performed for the oxLDL-reactive CLL mAb clones. B/B_0 indicates the ratio between bound (cpm) and bound (cpm) without competitor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplementary Material can be found at:
www.landesbioscience.com/journals/oncoimmunology/article18400

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