Epstein-Barr Virus-Induced Gene 3 (EBI3): A Novel Diagnosis Marker in Burkitt Lymphoma and Diffuse Large B-Cell Lymphoma

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Abstract

The distinction between Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL), two types of mature aggressive B-cell lymphomas that require distinct treatments, can be difficult because of forms showing features intermediate between DLBCL and BL (here called BL/DLBCL). They can be discriminated by the presence of c-myc translocations characteristic of BL. However, these are not exclusive of BL and when present in DLBCL are associated with lower survival. In this study, we show that Epstein-Barr virus-induced gene 3 (EBI3) is differentially expressed among BL and DLBCL. Analysis of gene expression data from 502 cases of aggressive mature B-cell lymphomas available on Gene Expression Omnibus and immunohistochemical analysis of 184 cases of BL, BL/DLBCL or DLBCL, showed that EBI3 was not expressed in EBV-positive or -negative BL cases, whereas it was expressed by over 30% of tumoral cells in nearly 80% of DLBCL cases, independently of their subtypes. In addition, we show that c-myc overexpression represses EBI3 expression, and that DLBCL or BL/DLBCL cases with c-myc translocations have lower expression of EBI3. Thus, EBI3 immunohistochemistry could be useful to discriminate BL from DLBCL, and to identify cases of BL/DLBCL or DLBCL with potential c-myc translocations.

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Introduction

Burkitt’s lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL) are mature aggressive B-cell lymphomas whose distinction can be tricky. Their diagnosis relies on the patient’s clinical data and on specific features of the lymphoma including morphology, immunophenotype, and cytogenetic abnormalities. BL is a homogenous group characterized by c-myc overexpression as a result of c-myc gene translocation, and consequently increased proliferation. This translocation juxtaposes the locus of c-myc gene to one of the Ig loci (heavy chain, lambda or kappa light chains) [1]. In contrast, DLBCL encompasses a heterogeneous group of B-cell lymphomas with clinical, morphological, immunohistochemical and molecular subtypes [2,3]. Accurate diagnosis of BL and DLBCL is essential because adequate chemotherapy regimen differs between both types of lymphomas. BL is cured by high intensity chemotherapy, whereas DLBCL is usually treated by lower-dose chemotherapy regimens: cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), in association with rituximab anti-CD20 antibody (R-CHOP) [1,3–5].

Although Ig-myc translocation is the hallmark of BL, c-myc translocations are also found in other lymphomas. In particular, they are found in a subset (5 to 15%) of DLBCL and in a high proportion of lymphomas that are borderline between BL and DLBCL and were previously called « atypical BL » or « BL-like lymphoma ». These latter lymphomas are now categorized as « B-cell lymphomas, unclassifiable, with features intermediate between DLBCL and BL » [6], and will be referred to as BL/DLBCL in this study. In BL/DLBCL and DLBCL, c-myc translocations often involve non-Ig partners and are associated with a complex caryotype. Several studies have shown that these cases represent aggressive forms with poor prognosis [7–12] and the most appropriate treatments remain a matter of debate. In particular, a recent study showed that, among DLBCL patients treated with R-CHOP chemotherapy, those having c-myc gene rearrangements had an inferior prognosis compared to those without c-myc translocations, and it was suggested that treatment regimens similar to those used in BL would be more appropriate for these cases [11]. These observations highlighted the importance of identifying cases of DLBCL with c-myc translocations. However, cytogenetic studies are not systematically performed.
In previous immunohistochemical studies, we showed that Epstein-Barr virus (EBV)-induced gene 3 (EBI3), a molecule related to the p40 subunit of interleukin (IL)-12 [13], exhibited a restricted expression profile among B-cell lymphomas [14,15]. We found that EBI3, which was originally characterized as a gene induced in EBV-transformed B cells by the viral oncoprotein LMP1 [13,16], was also expressed in certain non-EBV-associated B-cell lymphomas such as DLBCL. Indeed, EBI3 was found to be expressed by tumoral cells in 18/22 cases of DLBCL [15], whereas it was not expressed in 6/6 cases of EBV-positive BL [17], consistent with the absence of LMP1 expression in EBV-associated BL. Subsequently, a study of gene profiling by Dave et al [18] showed that EBI3 was among the NF-kB regulated genes that were selectively overexpressed in DLBCL compared to BL.

These observations prompted us to further analyze the expression of EBI3 in large series of BL and DLBCL to clearly establish its differential expression profile among both types of lymphomas, and the usefulness of EBI3 immunohistochemistry for their differential diagnosis. In addition, we investigated whether EBI3 immunohistochemistry could be used as a tool to identify cases with potential c-myc gene rearrangements among BL/DLBCL and DLBCL.

Methods

Lymphomas

Formalin-fixed paraffin-embedded tumor biopsies from 184 cases of mature aggressive B-cell lymphomas diagnosed between 1987 and 2009 at Necker Hospital (Paris), Cochin Hospital (Paris) or the Henri Becquerel Cancer Center (Rouen) were included in this study. All cases were reviewed anew and classified according to the World Health Organization 2008 lymphoma classification [6]. Tumors classified as BL (n = 23) had both morphologic features and an immunophenotype typical of BL (positivity for both CD10 and Bcl6, and proliferation index measured by Ki67 positivity >90%). EBV (EBER) probe was detected in 4/18 cases, and all but one of cases were negative for Bcl2. Cases of DLBCL (n = 138) were classified into germinal center (GC) B-cell-like (GCBL) or non-GCBL types, based on the differential immunodetection of CD10, Bcl6, and MUM1, as previously described [15,19]. For these markers, status (by two pathologists (JG and FL), independently. C-myc was determined in a blinded fashion (without knowledge of cytogenetic and/or paraffin-embedded tissues (13 cases) or both techniques (12 cases). Cytogenetic data were obtained in 17/23 cases (74%) of BL, 88/138 cases (64%) of DLBCL, and in all BL/DLBCL cases. C-myc translocations were detected in 17/17 of BL cases, 12/88 (14%) of DLBCL cases and 19/23 (83%) of BL/DLBCL cases. The main characteristics of the lymphomas are summarized on Table 1.

All tissues were collected for histological examination and diagnosis purpose and were studied in accordance with the French ethical laws for studies on human tissues and with the Declaration of Helsinki. This study was approved by the institutional review board of the hospitals and cancer center that provided tissue samples and were involved in the study.

EBI3 immunohistochemistry

EBI3 immunostaining was performed on formalin-fixed paraffin-embedded tissue sections. Sections were dewaxed, rehydrated and subjected to antigen retrieval by heat pretreatment using citrate buffer. Endogeneous peroxidase activity was quenched with a peroxide-methanol buffer. EBI3 was detected using 2G4H6 mouse monoclonal antibody (IgG2a), the characterization and specificity of which had been previously reported [20], at 2-4 µg/ml. Specificity of the staining was controlled by testing in parallel an isotype-matched control mAb (RPC5, IgG2a, Cappel Durham). Binding of the primary antibody was detected by an indirect avidin-biotin peroxidase technique (BioGenex) and DAB as chromogen. Sections were counterstained with Mayer hematoxylin. Images were captured on a NanoZoomer 2.0-RS slide scanner (Hamamatsu Corporation) and processed with NDP Viewer. The percentage of EBI3-positive tumoral cells was determined in a blinded fashion (without knowledge of c-myc status) by two pathologists (JG and FL), independently.

Plasmids, cell line and transfection

An EcoRI fragment encoding an inducible c-myc-estrogen receptor (Myc-ER) fusion protein was inserted in the EcoRI site of pSG5 to construct pSG5-c-myc-ER plasmid. The green fluorescent protein (GFP) expression vector, pEGFP-C1, was obtained from Clontech.

Table 1. Characteristics of mature aggressive B-cell lymphomas analyzed by immunohistochemistry.

| Diagnosis     | No. of cases | Subtype     | c-myc translocation | No. of cases |
|---------------|--------------|-------------|---------------------|--------------|
|               |              |             | analyzed            | present      | absent      |
| BL/DLBCL      | 23           | GCB         | 23 (100%)           | 17 (100%)    | 0           |
| BL/DLBCL      | 23           | non-GCB     | 21 (91%)            | 21           | 17 (81%)    | 4 (19%)     |
|               |              | not determined | 2 (9%)               | 2           | 2           | 0           |
| DLBCL         | 138          | GCB         | 49 (35%)            | 33 (21%)     | 26 (79%)    |
| DLBCL         |              | non-GCB     | 85 (62%)            | 53 (8%)      | 49 (92%)    |
|               |              | not determined | 4 (3%)               | 2           | 1           | 1           |

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Differential Expression of EBI3 in BL and DLBCL
Karpas 1106, a DLBCL cell line derived from a patient with a mediastinal form of DLBCL, was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh, Germany. It is negative for EBV and does not carry c-myc translocation. It was cultured in RPMI 1640 medium supplemented with 20% fetal calf serum, L-glutamine and antibiotics.

For transient expression, Karpas 1106 cells (5 $\times$ 10^6 cells per cuvette) were electroporated with pSG5 control plasmid or pSG5-c-myc-ER together with GFP expression vector on a BioRad Gene Pulser Xcell electroporator at 250 V and 500 $\mu$F in 200 $\mu$l of RPMI medium containing 10% fetal calf serum. Two hours after electroporation (or at later time points in some cases), 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich) was added at a final concentration of 200 nM - unless otherwise specified - to activate c-myc-ER fusion protein. Although we did not observe any significant effect of 4-OHT on EBI3 expression in Karpas 1106 cells (not shown), 4-OHT was added to both pSG5 control and pSG5-c-myc-ER transfected cells to exclude a non-specific effect. Sixteen to 40 hours after 4-OHT addition, GFP-positive cells were isolated by electronic cell sorting on a FACSAria cytometer (IRNEM cell sorting facility). GFP-positive cells from c-myc-ER transfectants were verified to express c-myc-ER fusion protein by western blot (not shown).

**Real time quantitative PCR (RTqPCR)**

Total RNA was isolated from purified GFP-positive transfected cells or from 20 $\mu$m frozen tissues sections by TRIzol extraction followed by DNAse I digestion, or by using the RNeasy Plus Micro kit (Qiagen). RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase, and oligo(dT) or random hexamer

![Figure 1. Differential expression profile of EBI3 gene in BL and DLBCL defined by molecular profiling.](image-url)

Individual data for EBI3 gene were retrieved from series GSE4732 [18] (A, B), and pooled from series GSE4475 and GSE10172 [8,21] (C, D). Relative expression of EBI3 is shown using the authors’ scale. On (A) and (C) EBI3 expression among the different molecular types defined by gene profiling is shown. On (B) and (D), the expression of EBI3 in mBL is shown according to their original pathological diagnosis. p values are indicated. The horizontal bar indicates the mean. NS: not significant; unclass: unclassifiable.

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primers (all reagents from Invitrogen). RTqPCR for *EBI3* and *c-myc* was performed using Taqman Universal PCR Master mix and TaqMan® gene expression assays. For each sample, triplicate reactions were run for 40 cycles on Step One Plus thermal cycler (Applied Biosystems). Levels of target mRNA were normalized relative to levels of β2-microglobulin mRNA, and relative mRNA expression was calculated using the comparative cycle threshold method.

**Statistical analysis**

Statistical analysis was performed by Mann-Whitney or Student’s *t* test. Correlation was analyzed by Spearman test. A *p* value < 0.05 was considered to indicate statistical significance.

**Results**

BL and DLBCL, defined by molecular profiling, are characterized by differential expression of *EBI3* gene

First, we analyzed *EBI3* gene expression level in gene profiling studies performed in mature aggressive B-cell non-Hodgkin lymphomas that were available on Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information. Individual data for *EBI3* were retrieved from the study from Dave *et al.* [18] (GSE4732) and from two German studies (Hummel *et al.* [8], GSE4475, and Klapper *et al.* [21], GSE10172). In these studies, cases of adult or pediatric lymphomas that had originally received a pathological diagnosis of BL, DLBCL, BL/DLBCL, or high grade mature B-cell lymphomas, unclassifiable, had been analyzed for gene expression and classified into molecular BL (mBL) or non-mBL (consisting mainly of DLBCL and designed as molecular DLBCL in ref [18], using classifiers that were specific to Dave *et al.* study or to the German studies. These 3 studies totaled 111 cases of mBL and 391 cases of non-mBL. These latter were further classified into GCB (n = 148), activated B-cell like (ABC) (n = 143) and PMBL (n = 33) subtypes, or left unclassified (n = 67). Data shown on Figure 1A and 1C indicated that *EBI3* expression significantly differed between mBL and each of the non-mBL subtype (*p* < 0.0001). Thus, *EBI3* expression was low in all but one cases of mBL, whereas most cases of non-mBL, either GCB, ABC, PMBL, or unclassified, exhibited higher expression of *EBI3* gene. In Dave *et al.* study, cases of non-mBL of the GCB type were the most heterogenous for *EBI3* expression and contained the higher proportion of cases with low expression. However, this feature was not observed in the GCB non-mBL defined in the other studies, possibly due to the different ways the classification was performed in each study.

Over 50% of the cases - 28/53 (53%) (Figure 1B) and 43/57 (75%) (Figure 1D) - classified as mBL upon gene profiling by microarray analysis were initially classified as BL/DLBCL, DLBCL or aggressive NHL based on pathological diagnosis. Of note, *EBI3* level was equally low in classic BL and in cases that were re-classified as mBL upon molecular profiling (Figure 1B and 1D).

**Immunohistochemical analysis of EBI3 in BL and DLBCL**

The differential expression profile of *EBI3* observed among BL and DLBCL defined by molecular profiling was next confirmed at the protein level by analyzing by immunohistochemistry with anti-EBI3 mAb, 23 cases of classic BL and 138 cases of DLBCL (Figure 2A). No or rare EBI3-positive cells (1% positive tumoral cells at the most) were detected in BL cases including both EBV-positive or -negative cases. In contrast, numerous EBI3-positive cells, most of which were tumoral cells, were detected in a large proportion of DLBCL. These EBI3-positive tumoral cells exceeded 50% in over 60% of DLBCL cases. When a cut-off for positivity was set at ≥30% of tumoral cells (this cut-off will be used...
throughout the study), 109/138 (79%) of DLBCL cases were considered positive for EBI3, whereas all BL cases were negative (Figure 2B). When classified by immunohistochemistry in GCB and non-GCB subtypes, representing the equivalents of the GCB and ABC cell types defined by molecular profiling, 71% of the GCB cases were positive for EBI3, while this percentage reached 85% among non-GCB cases (Figure 2C), indicating a slight over-representation of EBI3-negative cases among GCB DLBCL, in line with the molecular data from Dave et al. study.

Taken together, these data obtained in a large series of BL and DLBCL indicate that positivity for EBI3 in tumoral cells strongly argues against a diagnosis of BL, while it is in favor of a DLBCL diagnosis.

Lymphomas harboring c-myc translocations are characterized by lower expression of EBI3 gene

In the studies from Hummel et al. [8] and Klapper et al. [21], cytogenetic data for the c-myc gene were available for a large number of cases (n = 245). Therefore, we investigated in patients that did not fulfill the mBL signature, i.e. cases that were classified as non-mBL or cases that could not be classified into mBL or non-mBL and were called « intermediate forms », if the presence of c-myc gene rearrangements (21% of the cases) affected EBI3 expression. Indeed, we observed that lymphomas, other than mBL, harboring myc-translocations had higher expression of c-myc, as expected, but also lower expression of EBI3 (p<0.0001) (Figure 3A). This observation was true whether cases had c-myc translocations involving Ig or non-Ig genes. In these lymphomas, an inverse correlation between EBI3 and c-myc expression was observed (Figure 3B (n = 194, p = 0.0007)). This inverse correlation was also observed when this analysis was performed by selecting cases that were originally classified as BL/DLBCL upon pathological diagnosis (Figure 3C (n = 39, p = 0.0009)).

Collectively, these data suggested that EBI3 expression level could be indicative of the level of c-myc expression and could constitute a marker to identify lymphomas with c-myc translocations, among both BL/DLBCL and DLBCL.

Overexpression of c-myc represses EBI3 gene expression

The inverse correlation observed between EBI3 and c-myc expression suggested that c-myc overexpression may repress EBI3 expression. To test this hypothesis, we used an in vitro assay by overexpressing an inducible c-myc-ER fusion protein that has
been widely used to study c-myc regulated genes [22]. This fusion protein is retained in the cytoplasm as an inactive form and translocate to the nucleus when cells are exposed to a modified estrogenic ligand, 4-OHT. The Karpas 1106 DLBCL cell line, that expresses EBI3, was transiently transfected with a GFP reporter plasmid, and either pSG5 vector control or pSG5-c-myc-ER, and cultured in the presence of 4-OHT. Twenty hours post-transfection, GFP-positive cells were isolated by cell sorting and analyzed for EBI3 expression by RTqPCR. Data shown on Figure 4A indicate that overexpression of c-myc results in statistically significant decrease of EBI3 expression (40% decrease on average, p < 0.0001). This effect was specific: it was not observed when 4-OHT was not added to the culture (Figure 4B) and was dose-dependent, as it gradually increased with 4-OHT concentration (Figure 4C). This negative effect of c-myc fusion protein overexpression on EBI3 expression was observed at all time points tested upon 4-OHT addition (Figure 4D). Treatment of transfected cells with 4-OHT for 16 or 24 hours led to a similar decrease in EBI3 expression, while treatment for 40 h resulted in a somewhat higher decrease. Altogether, these data indicate that c-myc overexpression represses EBI3 expression.

Immunohistochemical analysis of EBI3 expression in BL/ DLBCL and DLBCL characterized for c-myc gene rearrangement

Next, we investigated in DLBCL and BL/DLBCL whether their positivity or negativity for EBI3, assessed by immunohistochemistry, correlated with the absence or presence of c-myc translocation. This analysis was performed in 88 of the 138 cases of DLBCL tested for EBI3 for which cytogenetic data were available and in 23 cases of BL/DLBCL, all characterized for c-myc translocation, that we tested for EBI3 by immunohistochemistry. c-myc translocations were present in 12/88 (14%) of DLBCL cases and 19/23 (83%) of BL/ DLBCL cases.

Cases with c-myc translocation were not equally distributed among EBI3-positive or -negative cases (Figure 5A and Figure 6). Indeed, most EBI3-positive cases (67/71, 94%) did not have c-myc translocation. In contrast, while 28% of total cases had a c-myc translocation, this percentage reached 68% among EBI3-negative cases. The correlation between negativity for EBI3 and the presence of c-myc gene rearrangement was higher in BL/DLBCL than in DLBCL. Indeed, 16/17 (94%) of EBI3-negative BL/ DLBCL had a translocation for c-myc, while 12/23 (52%) of EBI3-negative DLBCL had c-myc gene rearrangement. Nevertheless, the frequency of c-myc translocation was 3.7 higher in EBI3-negative DLBCL cases compared to all DLBCL cases. Thus, consistent with the previous observations, positivity for EBI3, as determined by immunohistochemistry, argues against the presence of c-myc translocation. Conversely, negativity for EBI3 among BL/DLBCL or DLBCL indicates a higher probability for the presence of c-myc translocation, especially in BL/DLBCL cases.

Frozen tissue was available in 16 of the 88 cases with cytogenetic data. These cases included 10 cases without c-myc translocation (all DLBCL cases) that contained from 50–90% EBI3-positive

Figure 4. In vitro effect of c-myc overexpression on EBI3 expression level. Karpas 1106 cell line was transfected with a GFP reporter plasmid together with c-myc-ER expression vector or a control vector and cultured for various times in the absence or presence of 4-OHT. EBI3 expression in GFP-positive cells isolated from vector control transfected cells (Col) or c-myc-ER transfected cells (c-myc-ER) was analyzed by RTqPCR. In (A), transfected cells were cultured for 20 hours in the presence of 4-OHT (200 nM). Data are expressed as mean ± SEM from 3 independent transfections. p value is indicated. In (B), cells were cultured for 20 hours in the absence or presence of 4-OHT (200 nM). In (C), cells were cultured with increasing concentrations of 4-OHT. The expression of EBI3 in c-myc-ER transfected cells relative to that measured in control transfected cells is represented. The percentage of decrease is indicated. A higher concentration of 4-OHT (1 μM) did not result in higher repression of EBI3 expression (not shown). In (D), 4-OHT was added to the transfected cells for the last 16, 24 or 40 h of the culture. In (B)–(D), a representative experiment is shown.

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EBI3 gene expression compared to cases without c-myc translocations classified as EBI3-positive (Figure 5B, top graph, p = 0.005). On average, the EBI3/c-myc expression ratio was 27-fold higher in lymphomas without c-myc translocations than in lymphomas with c-myc translocation (Figure 5C, p < 0.005). These data indicate that EBI3 expression, analyzed at the protein level by immunohistochemistry or at the mRNA level, inversely correlates with c-myc expression.

In vivo downregulation of EBI3 expression by tumoral cells upon c-myc translocation

C-myc translocations can occur as secondary oncogenic event in follicular lymphomas that transform into DLBCL. In a previous report, we had observed that in follicular lymphomas, EBI3 was expressed by tumoral cells, mainly large cells, at a variable level that could reach up to 30% of tumoral cells [15]. To further investigate the in vivo effect of c-myc translocation on EBI3 expression, we analyzed the expression of EBI3 by immunohistochemistry in a case of follicular lymphoma that had acquired c-myc translocation during transformation (Figure 7). Whereas a substantial fraction of tumoral cells stained positive for EBI3 in the follicular lymphoma (Figure 7a), tumoral cells were all negative for EBI3 in the c-myc-positive transformed counterpart (Figure 7b). This in vivo observation further supports our in vitro data showing that c-myc overexpression downregulates EBI3 expression.

Discussion

DLBCL is a heterogeneous lymphoma with various subtypes characterized by different gene expression profiles [23–25], which hampers the identification of markers that are both specific to DLBCL and common to all subtypes. The analysis of data from gene profiling studies available on GEO, and our data obtained by immunohistochemistry, all concur to establish that DLBCL and BL are characterized by a differential expression profile of EBI3. In particular, our immunohistochemical analysis indicates that, whereas EBI3 was not expressed at significant levels in all BL cases, a large fraction of tumoral cells was positive for EBI3 in ~ 80% of DLBCL cases. In addition, we showed that an inverse correlation was observed between EBI3 expression and the presence of a c-myc translocation. Thus, 94% EBI3-negative BL/DLBCL cases exhibited c-myc translocations. In addition, while c-myc translocations were found in 14% of DLBCL cases in our series, this percentage increased to 52% among EBI3-negative cases. As mentioned earlier, the identification of c-myc translocations among BL/DLBCL and DLBCL is important, given that cases with c-myc translocations are associated with poor prognosis and decreased survival [8,9,11,12]. Despite a recent report suggesting that c-myc translocation could be identified by analyzing c-myc subcellular localization [26], the overexpression of c-myc resulting from the translocation of the gene remains difficult to assess by immunohistochemistry. Thus, EBI3 immunohistochemistry, possibly in conjunction with c-myc staining, performed routinely in all cases of BL, DL/DLBCL, and DLBCL, could not only help to discriminate BL and DLBCL, but also be useful to identify cases of BL/DLBCL and DLBCL with potential c-myc translocation and target these cases for further cytogenetic analysis by FISH. Because of practical considerations, FISH analysis is usually not routinely performed in all cases of DLBCL. Given that c-myc translocations are mostly found among EBI3-negative DLBCL cases which account for about one fifth of all DLBCL cases, targeting EBI3-negative cases for FISH analysis would allow to reduce by 80% the total number of DLBCL to test.
The factors regulating EBI3 expression in B-cell lymphomas remain to be established. In normal B cells, EBI3 is expressed at precise stages of B-cell differentiation. It is not expressed in naive B cells and in centroblasts, and is essentially expressed by a subset of germinal center B cells corresponding to activated centrocytes or cells at an early stage of plasma cell differentiation [15,27]. In activated normal B cells, EBI3 expression is positively regulated by NF-κB activation [15]. In tumoral B cell lines, including DLBCL cell lines, EBI3 expression has been shown to be dependent on NF-κB activation, and in EBV-transformed B cells to be induced by LMP1 in an NF-κB dependent manner [14,16,28]. Thus, the absence of EBI3 expression in BL may be due to its stage of differentiation (centroblast), its lack of NF-κB activation, the absence of LMP1 expression (for EBV-positive cases) or its high expression of c-myc that could repress EBI3 induction. Of note, the only mBL showing significant expression of EBI3 (Figure 1C) was a case that did not exhibit c-myc translocation.

In DLBCL, both its stage of differentiation and the activation of NF-κB, may account for EBI3 expression. Previous studies have shown that DLBCL originates from GC (GCB subtype) or post-GC (ABC subtype) normal B cells [23]. The GCB subtype was initially associated with low NF-κB activation, whereas the ABC subtype was associated with high NF-κB activation [23]. However, a more recent study has shown that not only 95% of ABC forms, but also nearly 50% of GCB forms, are characterized by substantial NF-κB activation [29]. Thus, in both GCB and ABC subtypes, the activation of NF-κB may account for EBI3 expression. The higher proportion of GCB cases among EBI3-negative cases supports this hypothesis.

Another factor regulating EBI3 expression in DLBCL is the presence of c-myc translocations resulting in c-myc overexpression that in turn can repress EBI3 expression. Indeed, c-myc translocations were present in about half EBI3-negative DLBCL

Figure 6. Immunohistochemical analysis of EBI3 expression in BL, BL/DLBCL and DLBCL. Sections from a case of BL (a), 2 cases of BL/DLBCL (b, c), and 2 cases of DLBCL (d, e) with or without c-myc translocation as indicated on each figure, were analyzed for EBI3 expression by immunohistochemistry. An inverse correlation between the presence of c-myc translocation and the positivity for EBI3 in tumoral cells was observed. Each picture is shown at the same magnification. The bar shown on (c) represents 50 μm.

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Figure 7. Immunohistochemical analysis in a case of follicular lymphoma and its transformed counterpart. Sections from a follicular lymphoma (a) that subsequently acquired a c-myc translocation and transformed into a DLBCL (b) were analyzed for EBI3 expression by immunohistochemistry. The bar represents 50 μm.

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cases. This negative effect of c-myc overexpression on EBI3 expression may be a direct effect or could result from the known negative effect of c-myc on NF-kB effectors [30,31]. The negativity for EBI3 expression among DLBCL cases may also be due to the fact that some of them may arise from transformed follicular lymphomas (our unpublished data). This hypothesis is supported by the fact that EBI3 gene expression in the German series analyzed here [8,21] was on average lower in DLBCL with t(14;18) translocation (not shown).

The biological significance of EBI3 expression in DLBCL remains to be investigated. EBI3 can associate with two different partners, p28, to form the heterodimeric cytokine IL-27, or p35, to form IL-35 [32,33]. Both cytokines have been described as important negative regulators of T-cell responses [34–36]. In a previous immunohistochemical analysis, p28 was not detected in tumor cells of DLBCL cases [15]. p28, the expression of which was analyzed only in a subset of the lymphomas of the gene profiling studies used here, did not show increased expression in DLBCL compared to BL. p35 gene was present on the gene chips used in the 3 studies, and analysis of its expression profile showed increased expression in DLBCL, especially those of the ABC subtype, compared to BL (our unpublished data). Whether IL-35 protein is produced in these lymphomas and contributes to their higher aggressiveness possibly by suppressing local anti-tumoral immune responses, remains to be established.

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Author Contributions

Conceived and designed the experiments: JG FL OD. Performed the experiments: JG FL CD OD. Analyzed the data: JG FL OD. Contributed reagents/materials/analysis tools: CB JMP JG IRW NB MCVL. Wrote the paper: OD.

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