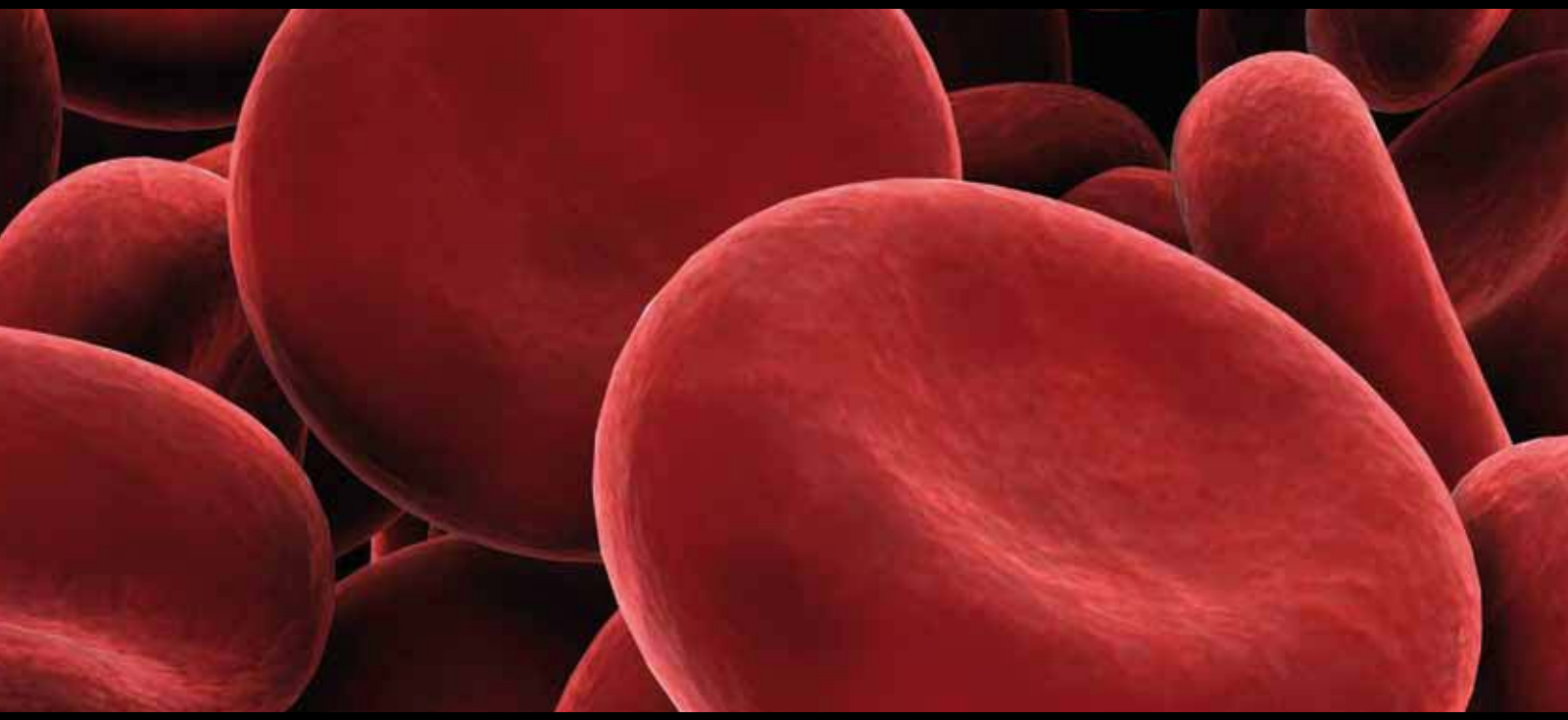
Advances in Hematology

Aggressive B-Cell Lymphomas

Guest Editors: Kikkeri N. Naresh, Ian Magrath, Martine Raphael, and Lorenzo Leoncini



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Editorial Board

Contents

Aggressive B-Cell Lymphomas, Kikkeri N. Naresh, Ian Magrath, Martine Raphael, and Lorenzo Leoncini
Volume 2012, Article ID 604603, 1 page

EBV Reactivation and Chromosomal Polysomies: *Euphorbia tirucalli* as a Possible Cofactor in Endemic Burkitt Lymphoma, Susanna Mannucci, Anna Luzzi, Alessandro Carugi, Alessandro Gozzetti, Stefano Lazzi, Valeria Malagnino, Monique Simmonds, Maria Grazia Cusi, Lorenzo Leoncini, Cornelia A. van den Bosch, and Giulia De Falco
Volume 2012, Article ID 149780, 11 pages

Posttransplant Lymphoproliferative Disorders, Hazem A. H. Ibrahim and Kikkeri N. Naresh
Volume 2012, Article ID 230173, 11 pages

A Role for RNA Viruses in the Pathogenesis of Burkitt's Lymphoma: The Need for Reappraisal, Corry van den Bosch
Volume 2012, Article ID 494758, 16 pages

Grey Zone Lymphomas: Lymphomas with Intermediate Features, Sylvia Hoeller and Christiane Copie-Bergman
Volume 2012, Article ID 460801, 7 pages

Novel Therapies for Aggressive B-Cell Lymphoma, Kenneth A. Foon, Kenichi Takeshita, and Pier L. Zinzani
Volume 2012, Article ID 302570, 22 pages

Anaplastic Lymphoma Kinase-Positive Large B-Cell Lymphoma: An Underrecognized Aggressive Lymphoma, Elizabeth A. Morgan and Alessandra F. Nascimento
Volume 2012, Article ID 529572, 6 pages

Microenvironment-Centred Dynamics in Aggressive B-Cell Lymphomas, Matilde Cacciatore, Carla Guarnotta, Marco Calvaruso, Sabina Sangaletti, Ada Maria Florena, Vito Franco, Mario Paolo Colombo, and Claudio Tripodo
Volume 2012, Article ID 138079, 12 pages

Murine Models of B-Cell Lymphomas: Promising Tools for Designing Cancer Therapies, Sabrina Donnou, Claire Galand, Valérie Touitou, Catherine Sautès-Fridman, Zsuzsanna Fabry, and Sylvain Fisson
Volume 2012, Article ID 701704, 13 pages

AIDS-Related Non-Hodgkin's Lymphoma in the Era of Highly Active Antiretroviral Therapy, Prakash Vishnu and David M. Aboulafia
Volume 2012, Article ID 485943, 9 pages

The Role of Autologous Stem Cell Transplantation in the Treatment of Diffuse Large B-Cell Lymphoma, Marco Gunnellini, Rita Emili, Stefano Coaccioli, and Anna Marina Liberati
Volume 2012, Article ID 195484, 10 pages

Towards Curative Therapy in Burkitt Lymphoma: The Role of Early African Studies in Demonstrating the Value of Combination Therapy and CNS Prophylaxis, Ian Magrath
Volume 2012, Article ID 130680, 7 pages

Molecular Profiling of Aggressive Lymphomas, Maura Rossi, Maria Antonella Laginestra, Anna Gazzola, Maria Rosaria Sapienza, Stefano A. Pileri, and Pier Paolo Piccaluga
Volume 2012, Article ID 464680, 9 pages

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Editorial
Aggressive B-Cell Lymphomas

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Aggressive B-cell lymphomas (ABLs) are a group of B-cell malignancies, that is, a relatively common form of cancer across the world. This issue on aggressive B-cell lymphomas compiles 12 exciting manuscripts, most of which are very meticulously performed reviews of the available current literature.

Three areas of lymphoma pathology and biology that are leading to a greater understanding of relatively recently defined entities are discussed. These include anaplastic lymphoma kinase (ALK)-positive large B-cell lymphoma (ALK+ LBCL), and the so-called grey zone lymphomas or lymphomas with intermediate features and the post-transplant lymphoproliferative disorders. ALK+ LBCL are characterised by gene rearrangement involving clathrin and *ALK* genes (t(2;17)(p23;q23)). Grey zone lymphomas include two unclassifiable B-cell lymphomas, one with features intermediate between diffuse large B-cell lymphoma (DLBCL) and classical Hodgkin lymphoma (cHL) and the second with features intermediate between DLBCL and Burkitt lymphoma (BL).

The biology of common lymphomas is addressed in two manuscripts. M. Rossi et al. review the current literature on molecular subsets of DLBCL, the distinction between DLBCL and BL, and gene expression characteristics of the grey zone lymphomas. This paper is accompanied by M. Cacciatore et al.'s manuscript that explores the importance of the microenvironment in lymphoma biology.

Two of the manuscripts deal with those aspects of endemic BL pathogenesis that have hitherto not been explored in great depth. The paper by S. Mannucci et al. explore the possibility of *Euphorbia tirucalli* acting as a cofactor in BL. They show that, in lymphoblastoid cell lines, *E. tirucalli* can modulate the EBV latency genes, result in polysomy for chromosome 8, and cause upregulation of c-MYC. Furthermore, Dr. C. van den Bosch raises question about a possible role for RNA viruses in the pathogenesis of BL.

Dr. I. Magrath reviews the evolution of BL treatment in Africa—from cyclophosphamide monotherapy to multiagent combination chemotherapy including central nervous system prophylaxis using combinations of cyclophosphamide, vincristine, prednisone, and methotrexate. He also highlights the benefits of collaboration between resource-rich and resource-poor settings. P. Vishnu and D. M. Aboulafia review how the treatment of HIV-associated lymphomas has evolved since the introduction of highly active antiretroviral therapy (HAART). The use of rituximab, in conjunction with multiagent chemotherapy, and where required high-dose chemotherapy (HDCT) and autologous stem cell rescue (ASCT) have all contributed to the success of treating HIV lymphomas. Treatment of ABL is further reviewed in two paper—one on ASCT in DLBCL and the other on novel therapies by K. Foon et al. To complete the issue, S. Donnou reviews the currently available murine models useful for studying lymphomagenesis, the lymphoma microenvironment, and the efficacy of new therapies.

By compiling these paper, we hope to enrich our readers and researchers with respect to these particularly common, yet usually highly treatable aggressive B-cell malignancies.

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Research Article

EBV Reactivation and Chromosomal Polysomies: *Euphorbia tirucalli* as a Possible Cofactor in Endemic Burkitt Lymphoma

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Burkitt lymphoma is endemic in the Equatorial Belt of Africa, its molecular hallmark is an activated, *MYC* gene mostly due to a chromosomal translocation. Especially in its endemic clinical variant, Burkitt lymphoma is associated with the oncogenic Epstein-Barr virus (EBV), and holoendemic malaria acts as an amplifier. Environmental factors may also cooperate in Burkitt lymphomagenesis in the endemic regions, such as plants used as traditional herbal remedies. *Euphorbia tirucalli*, a plant known to possess EBV-activating substances, has a similar geographical distribution to endemic Burkitt's Lymphoma and is used as a hedge, herbal remedy and toy in the Lymphoma BeltI. In this study we aimed at determining if exposure to *Euphorbia tirucalli* could contribute to lymphomagenesis, and at which extent. Lymphoblastoid and cord blood-derived cell lines were treated with plant extracts, and the expression of EBV-coded proteins was checked, to assess EBV reactivation. The occurrence of chromosomal translocations was then investigated by FISH. Our preliminary results suggest that *E. tirucalli* is able to reactivate EBV and determine chromosomal alterations, which leads to c-MYC altered expression. The existence of genomic alterations might determine the accumulation of further genetic alteration, which could eventually lead to a transformed phenotype.

1. Introduction

Burkitt's Lymphoma (BL), a high-grade Non-Hodgkin's lymphoma, is endemic in the Lymphoma Belt of Africa, which lies between 10°N and 10°S of the Equator [1, 2]. Within these geographical boundaries, BL accounts for up to 70% of children cancer with rates up to 10 cases of Burkitt's Lymphoma per 100,000 children under the age of 14 years [3]. Burkitt's Lymphoma characteristically has a translocation involving a deregulated, activated, *MYC* gene on chromosome 8 and immunoglobulin genes on chromosome 14, or, more rarely, chromosomes 2 or 22 [1], though alternative pathogenetic mechanisms leading to *MYC* activation have also been described [4, 5]. Burkitt's Lymphoma is associated with the oncogenic Epstein-Barr virus [6], in particular, 98% of Burkitt's Lymphoma cases in the Lymphoma belt show positivity to EBV [7]. EBV is recognised as a Class 1 human carcinogen and is thought to play a pivotal role in lymphomagenesis in endemic Burkitt's Lymphoma [8]. Holoendemic malaria acts as an amplifier and has been shown to be able to activate the latent EBV in B-lymphocytes in children in the Equatorial Belt [9, 10]. The combination of malaria and early infection with the Epstein-Barr virus is thought to be responsible for boosting the incidence of Burkitt's lymphoma a hundred-fold in Africa, compared with rates in the France, and the USA [11, 12]. Children who subsequently develop the endemic Burkitt's Lymphoma have raised antibody levels to the EBV Viral Capsid Antigen (VCA) of EBV several years before they actually develop the tumour [13]. Raised levels of this antibody are also found in the relatives of children

with Burkitt's Lymphoma [14] and in those who have used traditional herbal remedies [15], which have been shown to be capable of activating the EBV [16].

Euphorbia tirucalli, a plant known to possess EBV-activating substances, has a similar geographical distribution to endemic Burkitt's Lymphoma [17] and is used as a hedge, herbal remedy, and toy in the Lymphoma Belt. This plant is found significantly more often at the homes of Burkitt's Lymphoma patients [17, 18] and the incidence of Burkitt's Lymphoma has fallen in Northern Zambia following the eradication of thickets of *E. tirucalli* [19].

Euphorbia tirucalli possesses a milky, rubbery sap which contains a 4-deoxyphorbol ester [20] closely related to the tumour-promoter substance TPA (12-O-tetra-decanoy-phorbol-13-acetate), which is derived from another Euphorbia, *Croton tiglium*. These TPA-related extracts of *Euphorbia tirucalli* present in the plant are secreted into the soil around the plant in active form [21] and can activate the latent EBV within the cell [16, 22], enhance EBV-mediated cell transformation [21], and modulate EBV-specific T-cell activity [21], myelopoiesis, and cellular immunity [23]. EBV and TPA can work synergistically in nude mice to produce both T- and B-cell lymphomas [24]. TPA's activity is also potentiated by the association with nucleic acids. Other plants commonly found in the Lymphoma belt and elsewhere in the tropics, typically those belonging to the Euphorbiaceae, and Thymelaeaceae families, are also known to induce the EBV lytic phase [16]. Some, but not all of these Euphorbiae, contain identical or similar phorbol esters to those found in *E. tirucalli* [25]. EBV-activation has been shown to be dependent on cellular protein kinase C (PKC), irrespective of the extracts' tumour-promotion abilities [26]. The TPA-related substances present in *E. tirucalli* could be expected to exhibit similar properties to those of TPA.

A research paper of the early nineties reported that *E. tirucalli* extracts can induce chromosomal abnormalities when added to EBV-infected cord blood B-lymphocytes [27]. Aya et al. showed that the cells multiplied rapidly following exposure to the extracts and, after one year of culture, ten percent of the chromosomal abnormalities induced by these plant extracts affected chromosome 8 with activation of the oncogene *MYC*, thus reproducing the crucial translocation characterizing Burkitt's Lymphoma [27], and that cells treated with the Euphorbia extracts produced lymphomas when injected into nude mice [27].

These observations suggest that *Euphorbia tirucalli* extracts and, possibly, extracts of other plants, which are known to have similar EBV-activating properties, in conjunction with other environmental factors, could play an important role in lymphomagenesis in endemic African Burkitt's Lymphoma. Despite the interesting observation reported by Aya in the early nineties, no further study since then has elucidated the molecular mechanisms by which some plant extracts may act as a cofactor in lymphomagenesis. Therefore, it is also interesting trying to explain the close link between the geographic distribution of *E. tirucalli* and the incidence of BL in the endemic areas. The aim of our study was to determine if exposure to *E. tirucalli* extracts could result in EBV reactivation and induction of genomic alteration, which might contribute to transformation. Therefore, we treated both a lymphoblastoid cell line (LCL) and a cord-blood- (CB-) derived cell line newly infected with EBV with this plant extracts. It was considered that the LCLs are likely to have accumulated preexisting genetic abnormalities [28] having been cultured for a number of years, whereas the cord-blood cell line should be a better *in vitro* model to mimic the effects of the plant extracts *in vivo* on EBV-infected cells. Cells were treated with different concentrations of *E. tirucalli*, and we monitored its effect on cell proliferation, EBV antigen expression, and induction of genomic alterations, such as chromosomal translocations.

Our results indicate that exposure to the plant extracts is able to reactivate EBV from its latent phase, as indicated by the expression of the EBV Zebra antigen following the treatment. In addition, the expression of EBV early antigens was also observed, along with a marked up regulation of LMP1, EBNA1, and EBNA2. In addition, though the specific chromosomal translocations present in Burkitt's lymphoma were not detected after *E. tirucalli* exposure, we observed the occurrence of polysomies involving chromosome 8, as demonstrated by the existence of multiple signals for *MYC* by FISH, which may result in overexpression of c-MYC, both at the mRNA and the protein level. In addition, increased expression for BCL2 was also observed, even in the absence of any genetic translocations involving this gene.

All together, our results suggest that *E. tirucalli*, through EBV reactivation and induction of genetic alterations leading to *MYC* overexpression, could contribute to the malignant transformation process.

2. Materials and Methods

2.1. Cell Lines and Cell Culture. The human lymphoblastoid cell line (LCL) was a kind gift of Prof. A. Lanzavecchia (IRB, Bellinzona, Switzerland). The human cord-blood- (CB-) derived cell line was obtained from cells newly infected and immortalized by EBV, following the protocol described by Pelloquin et al. [29]. Mononucleated cells were isolated from cord blood by Ficoll fractionation. After isolation, purified EBV obtained from B95.8 cell line (kindly provided by dr. M. Kleines, Austria) was added to the cell culture, in a 1 : 1 ratio. Cells were cultured in the presence of Cyclosporin A at a final concentration 2 μg/mL. The efficiency of infection was demonstrated by cluster formation in the cell culture after an overnight incubation with the virus. The establishment of the cell line was achieved after one month. Immunophenotype of the established CB cell line ($CD79^+$, $CD34^-$, $CD138^-$, $IRF4^-$, $BCL2^+$, and $BCL6^-$) was assessed as described below. Before treatment, cell caryotype was also assessed to confirm the absence of chromosomal translocations and aneuploidies. An EBV-negative Burkitt lymphoma-derived cell line (Ramos) was also used and treated as follows. For daily experiments and treatment with *E. tirucalli*, cells were cultured in RPMI supplemented with 10% FBS, 1% l-glutamine, penicillin/streptomycin, with 5% CO_2, at 37°C.

2.2. Immunocytochemisty. Immunocytochemical studies (ICC) were performed on representative cell smears of both

treated and untreated cells using microwave pretreatment or proteolytic digestion of slides for antigen retrieval. A large panel of antibodies (Table 1) recognizing the various EBV antigens was applied, in conjunction with the streptavidine-peroxidase method (Ultravision Detection System Anti-Polyvalent, HRP by Lab Vision Corporation, and Liquid DAB Substrate Chromogen System by DAKO), to visualize antibody binding. Protein expression was then quantified by counting the percentage of positive cells per HPF in 10 randomly chosen HPFs.

2.3. E. tirucalli Treatment and Cell Proliferation. *E. tirucalli* plant extracts were prepared as described by Ito et al. [30]. Briefly, *E. tirucalli* extracts were obtained using 200 mL ether under reflux for 72 hours. The ethereal solution was then evaporated down and the resultant oily extracts were then dissolved in methanol and served as the test substance for EBV antigen activation. Cells were treated with different concentration of *E. tirucalli* (0.1, 0.5, 1, and 10 μg/mL), resolved in methanol. As a normal control, untreated cells were cultured with the same amount of methanol. For the proliferation assay, cells were counted each day for 4 days. Statistical significance was assessed by the analysis of variance (ANOVA) test. For detection of EBV-specific responses, Ramos cells, an EBV-negative Burkitt lymphoma-derived cell line, were used as negative control and were cultured as previously described.

2.4. Cell Death Analysis. Cell death was evaluated by several approaches. Cell viability was checked by Trypan Blue exclusion test. Cell cycle analysis was performed by flow cytometry on a FACStar (BD Bioscience, CA). Forward Scatter (FSC) and Side Scatter (SSC) signals were recorded in linear mode. Dead cells and debris were gated out using scatter properties of the cells and additionally using propidium iodide (PI) at a concentration of 1 μg/mL. Data was analyzed using CellQuest software (BD Bioscience, CA). Apoptosis was detected by DNA laddering on a 1% agarose gel. Caspase staining for caspase 3 and 8 was detected by immunocytochemistry, as previously described.

2.5. Fluorescence In Situ Hybridization (FISH). Briefly, *MYC* and *BCL2* rearrangements were sought using the *MYC* FISH DNA Probe-Split Signal using standard procedures (*BCL2*, IgH, IgL, *BCL6*, break-apart probes, and *MYC* dual color probe). Briefly, smeared cells were air-dried, immersed in a jar filled with pretreatment solution, and warmed at 98°C for 10 min by means of a Whirlpool JT 356 microwave. Subsequently, the slides were cooled for 15 min at RT. After two passages of 3 min each in Wash Buffer, excess buffer was tapped off and the slides digested with cold Pepsin for 20 min in a Dako Cytomation Hybridizer (Dako, Denmark). The slides were then washed twice in Wash Buffer for 3 min, dehydrated using increasing graded ethanol series, air-dried, and finally 10 μL of probe mix were applied to each tissue section. The slides, covered with coverslip and sealed with rubber cement, were then incubated in the DakoCytomation Hybridizer (Dako, Denmark) according to the manufacturer's recommendations. The next day, slides were treated with stringency buffer at 65°C for 2 min then placed twice in Wash Buffer for 3 min, dehydrated using increasing graded ethanol series, air-dried, and counterstained applying 15 μL of Fluorescence Mounting Medium. Hybridization signals were visualized using a Leica microscope equipped with a triple-band filter for detecting green fluorescent protein (GFP)/spectrum green, Texas red/spectrum orange, and DAPI/spectrum blue. Images were captured and archived using Leica FW4000 software. One hundred nonoverlapping interphase nuclei were scored for each tumor specimen. In normal nuclei, two yellow fusion signals (2F) are detected, whereas in nuclei with translocations, a yellow (or red-green juxtaposed) signal is obtained from one red and one green segregated signal (1F1R1G). The results were further confirmed by additional FISH analysis using split-signal probes for IgH and IgL loci as well as an LSI *IGH*/*MYC* CEP 8 Tri-color dual-fusion probe (Vysis, Abbott Molecular IL, USA) specific for the detection of the translocation t[8; 14]. All reagents, instruments, and split-signal probes were kindly provided by DakoCytomation (Glostrup, Denmark). To specifically detect chromosome 8, the centromeric probe Zyto Light SPEC CMYC/CEN8 Dual Color Probe (ZytoVision, Germany) has been used. To establish the *MYC*:chromosome 8 ratio, 100 nuclei were randomly chosen and signals for *MYC* and chromosome 8 were counted.

2.6. qRT-PCR. Real-time PCR for *MYC* and *BCL2* was performed using FluoCycle SYBR green (Euroclone, Celbio, Italy) according to the manufacturer's instructions and *HPRT* as an internal control.

Primer sequences for *MYC* amplified a region of 129 bp: LEFT: AGCGACTCTGAGGAGGAAC; RIGHT: TGTGAGGAGGTTTGCTGTG. Primer sequences for *BCL2* amplified

Table 1: List of antibodies and their respective concentrations.

Primary antibody	Dilution	Company
EBNA-1	1 : 50	Novus Biologicals
EBNA-2	1 : 50	Dako
ZEBRA	1 : 50	Santacruz
LMP1	1 : 50	Dako
EA	1 : 50	Santacruz
Gp350	1 : 100	Santacruz
BCL6	1 : 30	Dako
BCL2	1 : 150	Dako
CD20	1 : 150	NeoMarkers
CD27	1 : 50	NeoMarkers
IgM	1 : 10000	Dako
CD30	1 : 50	NeoMarkers
CD10	1 : 20	NeoMarkers
CD79	1 : 50	NeoMarkers
IgD	1 : 50	NeoMarkers
Irf-4	1 : 50	Dako
CD138	1 : 100	Dako
Caspase 3	1 : 50	Abcam

a region of 258 bp: LEFT: 5′-TTGCCACGGTGGTGGAGG-A-3′; RIGHT: 5′-ACAGCCAGGAGAAATCAAACAG-3′. Primer sequences for *HPRT* amplified a region of 191 bp: LEFT: AGCCAGACTTTGTTGGATTTG; RIGHT: TTT-ACTGGCGATGTCAATAAG. Differences in gene expression were calculated using the ΔΔCt method [31].

2.7. Indirect Immunofluorescence. For c-MYC detection, cells were smeared on positively charged slides after *E. tirucalli* treatment (Day 5) and fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Permeabilization was achieved by washing cells in PBS, 0.2% Triton X-100, and 1% BSA. Saturation was performed for 1 hour at room temperature in goat serum (Zymed laboratories, Milan, Italy). All of the antibodies were diluted in goat serum. Primary antibody incubation was carried out at room temperature for 1 hour, using anti-c-MYC (9E10 sc-40: Santa Cruz Biotechnology, Santa Cruz, CA) 1 : 50. Secondary goat anti-mouse antibody, conjugated with Alexafluor568 (Molecular Probes, Invitrogen, Milan, Italy), was diluted 1 : 100 in goat serum and incubated at room temperature for 45 minutes. The slides were examined on an Axiovert 200 microscope (Carl Zeiss, Germany) and processed with Zeiss software (Carl Zeiss, Germany). Nuclei were counterstained by DAPI.

Table 2: Immunocytochemistry (ICC) of EBV-encoded proteins in cells treated with *E. tirucalli* at the concentration of 10 μg/mL versus untreated cells, expressed as percentage of positive cells out of total cells. Comparable results were obtained in LCL and cord blood-derived cells.

	Control (%)	*E. tirucalli* 10 μg/mL (%)
Zebra	0	6
Ea-d	40	50
LMP1	50	90
Gp350	0	0
EBNA1	5	30
EBNA2	10	80

Table 3: FISH analysis on *E. tirucalli* treated versus untreated cells.

	Control	*E. tirucalli* 10 μg/mL
BCL2	No translocation	No translocation
BCL6	No translocation	No translocation
IgH (chromosome 14)	No translocation	No translocation
IgL (chromosome 22)	No translocation	No translocation
c-MYC (chromosome 8)	No translocation	Polysomies, no translocation

3. Results

3.1. E. tirucalli Treatment Affects Cell Proliferation. We treated LCLs and the cord-blood-derived cell line with increasing concentrations of *E. tirucalli*, as reported in Section 2, using methanol-treated cells as a control. The effects of the treatment on cell proliferation and cell death were monitored. A dose-dependent reduction in cell proliferation was observed after *E. tirucalli* treatment (Figure 1(a)), accompanied by high rates of cell deaths (Figure 1(b)).

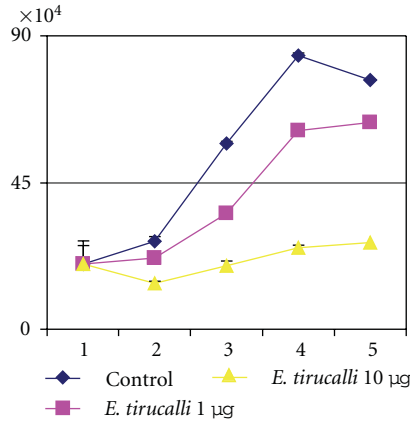
Cell death seemed to be due to necrosis rather than to apoptosis, as no DNA laddering nor caspase activation was observed following *E. tirucalli* treatment (Figures 1(c) and 1(d)). To assess whether cell death was due to the toxicity effects of the plant extracts or to reactivation of EBV, we treated an EBV-negative cell line using the same experimental conditions, and we observed a similar reduction in cell proliferation accompanied by an increase in cell death suggesting that cell death was due to plant toxicity (Figure 2).

3.2. E. tirucalli Modulates the Expression of EBV-Antigens. There are three different latency programs of EBV, characterized by the differential expression of its coded genes [32]. In additions, the expression of some EBV-genes, such as Zebra, indicates the shift from the latent to the lytic phase of the virus [33]. Reactivation of EBV as a consequence of TPA-analogous treatment had been reported by literature [24]. We, therefore, treated cells with different concentrations of *E. tirucalli* and monitored the expression of EBV-coded genes by ICC. After *E. tirucalli* treatment, we observed the expression of Zebra, which was not expressed by untreated cells. In addition, enhanced expression of the EBV early antigen (EA), LMP1 and EBNA2 expression was also observed, whereas no significant variation of the EBV early antigen gp350 was detected following *E. tirucalli* treatment. Higher concentrations of plant extracts (10 μg/mL) resulted in a more marked EBV protein expression. Comparable results were obtained in LCL and cord-blood cell lines. Table 2 summarizes immunocytochemical results obtained in treated versus untreated cells. Figure 3 shows ICC results.

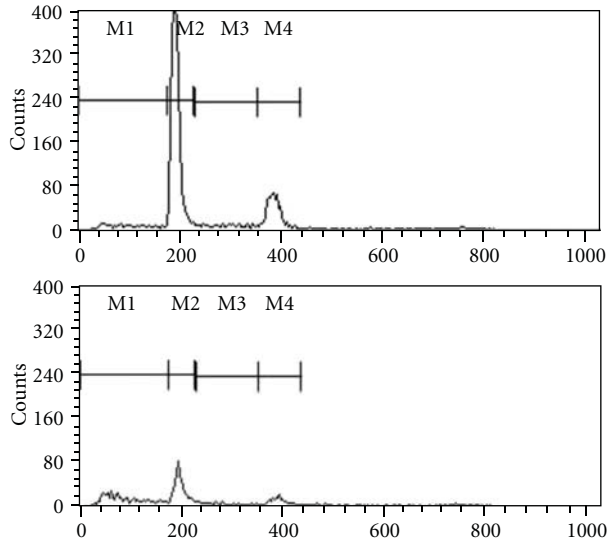
3.3. E. tirucalli Induces Chromosome 8 Polysomy. E. tirucalli has been shown to induce genetic alterations, particularly those involving the oncogene *MYC* [27]. To detect the onset of specific chromosomal translocations after *E. tirucalli* treatment, we performed FISH analysis to identify the most frequently described chromosomal translocations occurring in aggressive B-cell lymphomas, using probes designed to detect *MYC*, *BCL-2, BCL6,* and their respective Ig partners. Using this approach, no balanced translocations involving *BCL2, BCL6,* IgH, and IgL were detected. Figure 4 summarizes FISH results for *BCL2* and *MYC*.

Multiple signals for *MYC* were detected in about 17% of cells, ranging between 3 and more copies, in contrast to the normal 2 copies, though no specific chromosomal translocation could be identified. To assess whether these signals were dependent on gene amplification or to a chromosome 8 polysomy, we used a centromeric probe to detect both *MYC* and centromere signals. Our results indicated a chromosome 8 polysomy, as more than 2 signals for the centromeres were detected, together with multiple signals for *MYC* (Figure 5). Table 3 summarizes FISH results.

3.4. c-MYC Expression Is Upregulated following E. tirucalli Treatment. The presence of multiple copies of *MYC* is compatible with an overexpression of the c-MYC protein, as



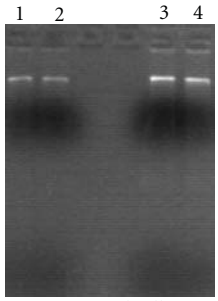
(a)



Untreated cells	%
M1	6.65
M2	66.82
M3	6.24
M4	17.41

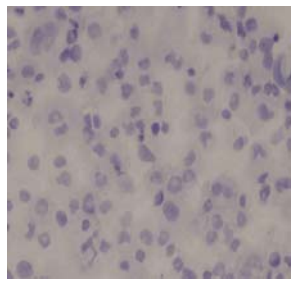
Treated cells (10 μg/mL)	%
M1	37.78
M2	37.12
M3	8.8
M4	12.19

(b)



1-2: Untreated cells
3-4: Treated cells

(c)



(d)

Figure 1: (a) Cord-blood-derived cells were treated with different concentration of *E. tirucalli* for five days, and proliferation has been monitored. Methanol-treated cells, with no *E. tirucalli* extract, have been used as a control. A dose-dependent decrease of proliferation rate is observed in *E. tirucalli*-treated cells ($P < 0.05$). The graph is representative of three different experiments. Error bars represent standard deviation between duplicates. (b) Cell cycle analysis by FACS on untreated (upper part) and *E. tirucalli*-treated cells (lower part). Tables indicate the percentage of cells in each cell cycle stages, where M1 indicates total number of dead cells (apoptotic and necrotic cells), M2 indicates G0/G1, M3 cells in S phase, and M4 the G2/M phase. Treated cells show a higher number of the M1 fraction. (c) Electrophoresis on agarose gel of untreated (lanes 1-2) and *E. tirucalli*-treated cells (lanes 3-4). No DNA laddering indicative of cell death by apoptosis is visible. (d) ICC for caspase 3. No caspase activation is detected following *E. tirucalli* treatment.

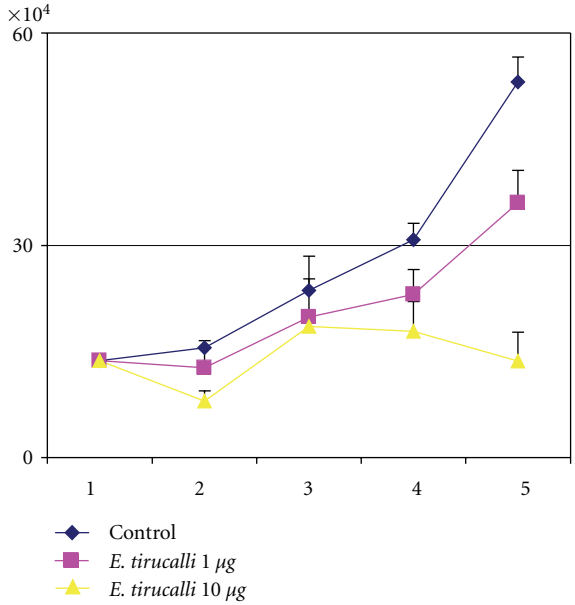


Figure 2: To rule out the possibility that the decrease of cell proliferation and the concomitant increase in cell death observed in *E. tirucalli*-treated cells was due to induction of the lytic pathway of EBV, an EBV-negative cell line (Ramos) was cultured in the same experimental conditions as cord blood derived cells. *E. tirucalli* treatment had the same effects on cell proliferation and cell death, independently of EBV status. Treated cells showed a decreased cell proliferation ($P < 0.05$). The graph is representative of three different experiments. Error bars represent standard deviation between duplicates.

occurs in Burkitt lymphoma. Therefore, we checked the expression level of c-MYC by qRT-PRC and immunofluorescence. As a consequence of chromosome 8 polysomy, up-regulation of c-MYC was observed, as expected, at both levels (Figures 6(a) and 6(b)).

3.5. BCL2 Is Overexpressed following E. tirucalli Treatment. *BCL2* deregulation is often observed in lymphomas, as in the case of follicular lymphoma. Though FISH revealed the absence of any chromosomal translocations involving *BCL2*, which maps on chromosome 18, we detected up-regulation of its expression level in treated cells, both at the mRNA (Figure 6(a)) and protein levels (Figure 7), which may be compatible with the acquirement of an anti-apoptotic capability by treated cells.

4. Discussion

A potential transforming capability by *E. tirucalli* extracts has been suggested by a single publication in the last twenty years [27]. No further studies have been performed since then to highlight through which molecular mechanisms it occurred. In this study, we report the results on cell proliferation and cell death, expression of EBV-antigens, and induction of chromosomal abnormalities in LCL and cord-blood-derived cell lines after treatment with *E. tirucalli* plant extracts. Results have been almost completely matching between the two cell lines, though the freshly established cord-blood-derived cell line is more likely to represent the *in vivo* situation in respect with an LCL, as a prolonged *in vitro* culture in the latter could determine the accumulation of preexisting genetic abnormalities.

E. tirucalli treatment determined a reduction of cell proliferation and a concomitant increase of cell death. This result is in contrast to what reported by literature for PBMCs treated with *E. tirucalli* [34], which seems to result in an increased proliferation rate following treatment. Quite interestingly, we were not able to reproduce these results neither in LCLs or cord-blood-derived cells. One possible explanation could be that the increase of cell proliferation previously reported [34] has been observed mostly on the $CD3^+$ subpopulation within PBMC, which represents lymphoid T-cells, whereas our results are referred to EBV-infected B-cells.

Cell death was quite high in all the treated cells, being highest with the highest concentrations and seemed to depend on necrosis due to the plant toxicity, rather than to apoptosis, as no DNA laddering nor caspase activation was detected in cells treated with *E. tirucalli* (Figures 1(c) and 1(d)). The possibility that cell death was due to activation of the lytic cycle of EBV, as possibly suggested by Zebra expression, was ruled out by treating an EBV-negative cell line using the same experimental conditions. Cell death levels in this cell line were comparable to those observed in EBV-positive cells, thus suggesting that cell death was due to plant toxicity.

On the contrary, treatment with *E. tirucalli* extracts modulated the expression of EBV-antigens. In particular, the expression of early antigens and a marked upregulation of LMP1 were observed after treatment. LMP1 expression may be relevant for NF-κB activation [35] and apoptosis [36]. A similar pattern of expression of EBV antigens expression was observed in LCL and cord blood cells, with the exception of gp350, which was not expressed in cord-blood after treatment, whereas a weak expression was observed in LCL.

Although it has been previously reported that cells treated with *E. tirucalli* and cultured for one year accumulated genetic abnormalities, here we report for the first time that as less as a five-day treatment of *E. tirucalli* was able to determine genomic abnormalities both in LCL and CB. In particular, in both cases, polysomies were observed, being more evident the higher the concentration of *E. tirucalli* was. The possibility that the observed polysomies observed in LCL could be due to the accumulation of several genetic alterations, which may happen to cells cultured for many years, was ruled out by the observation that polysomies were induced by *E. tirucalli* treatment also in newly established CB cells, with a normal caryotype. In particular, polysomy of chromosome 8 was detected by FISH through specific centromeric probes, resulting in an increased number of copies of the *MYC* oncogene, as detected by FISH. Though Aya et al. reported the occurrence of specific chromosomal translocation in about 10% of treated cells after one year of culture, we did not detect any specific translocations. One possible explanation could be that our analyses have been performed after only five days of treatment, which were

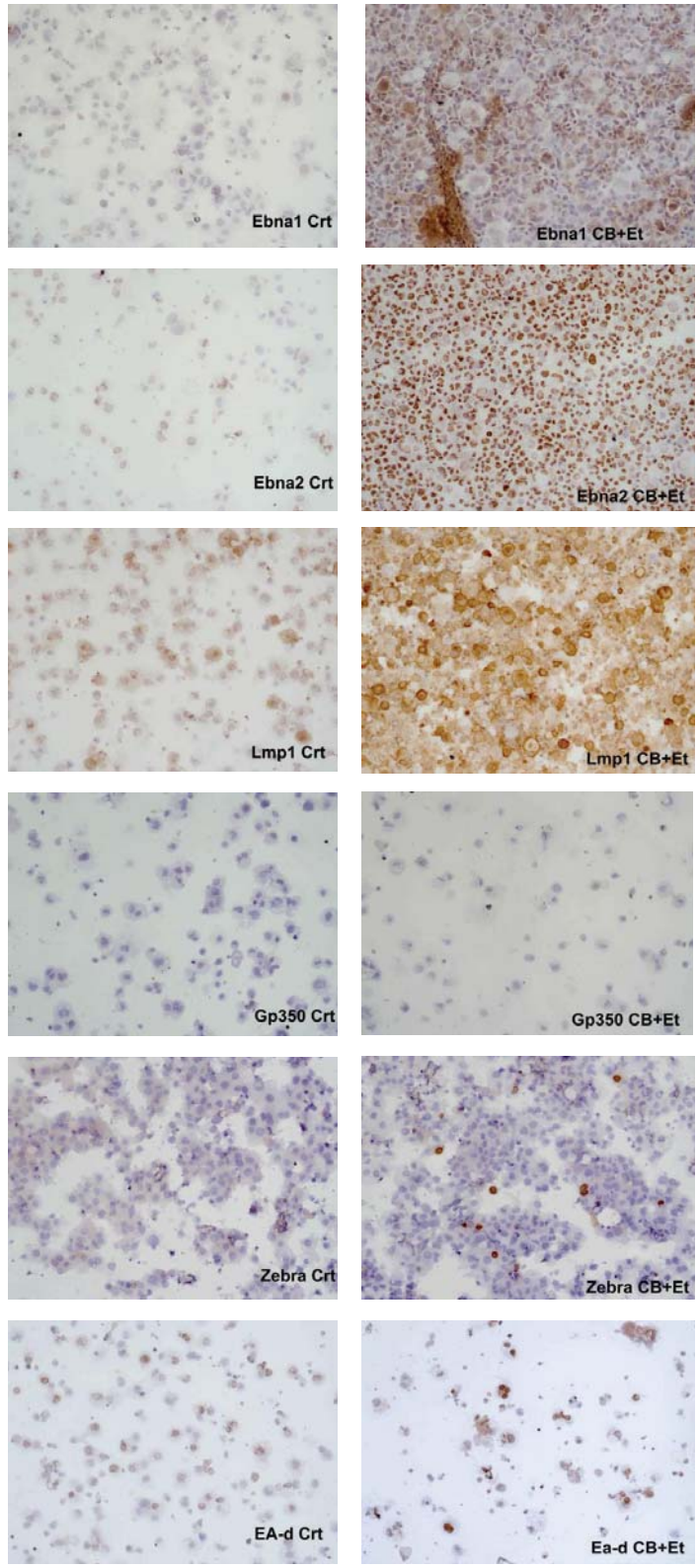


Figure 3: Immunocytochemistry of untreated (a) and *E. tirucalli*-treated cells (b). The expression of EBV-coded products was monitored.

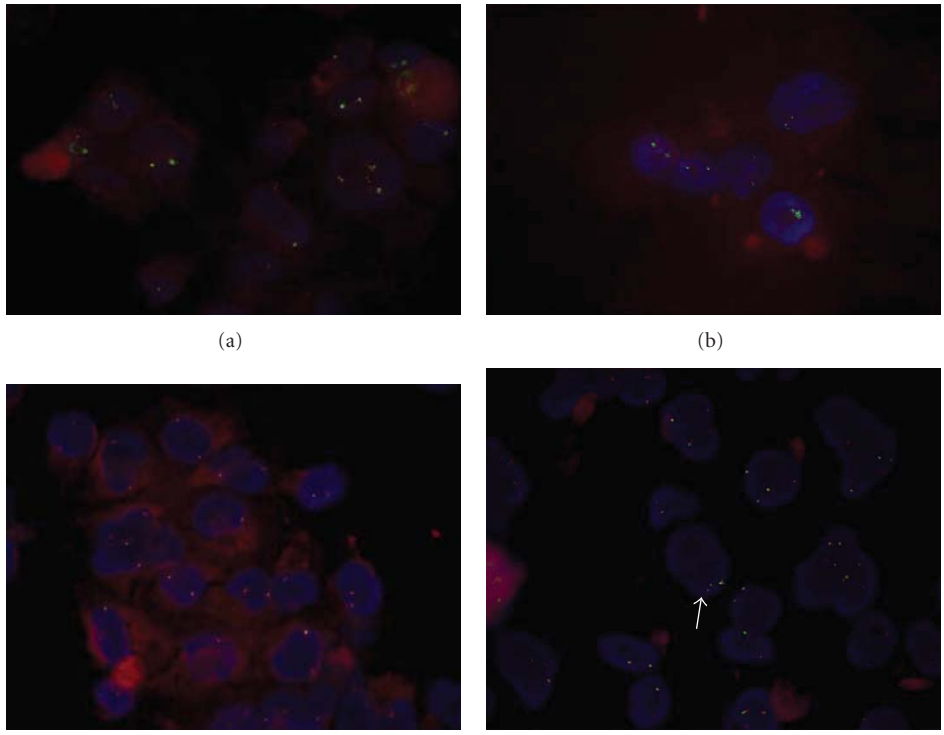


Figure 4: FISH analysis for *BCL2* (a, b) and *MYC* (c, d) for untreated (a, c) and *E. tirucalli*-treated cells (b, d). No balanced translocations have been detected, though multiple signals for *MYC* have been identified. Arrows indicate cells with multiple *MYC* signals.

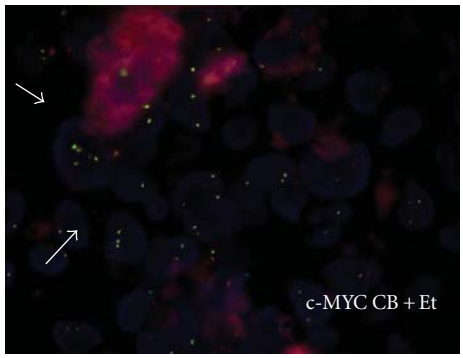
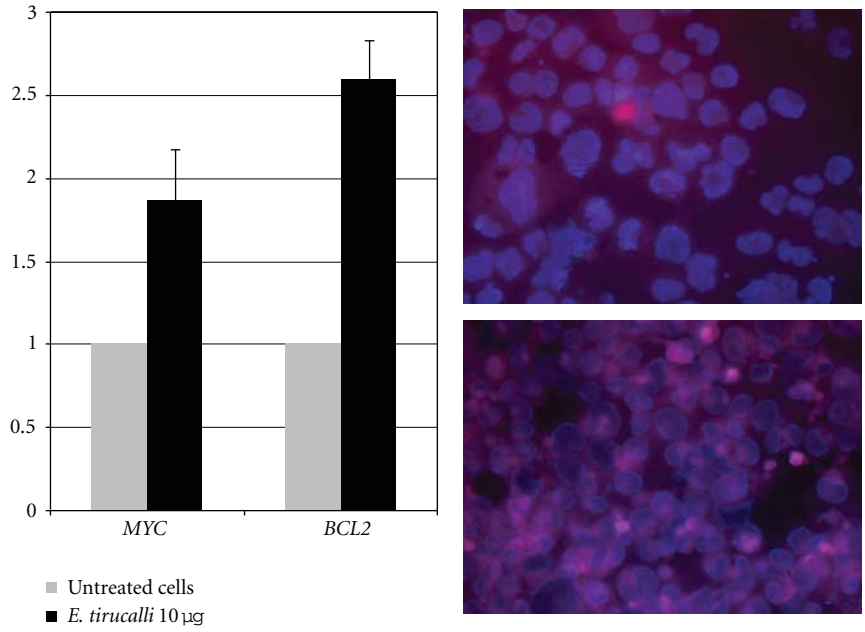


Figure 5: To assess whether multiple *MYC* signals relied on *MYC* gene amplification or on chromosomal 8 polyploidy, a centromeric probe for chromosome 8 was used. Our results indicated the presence of multiple signals for chromosome 8 in cells treated with *E. tirucalli*, consistent with a polysomy of chromosome 8. Arrows indicate cells with multiple chromosome 8 and *MYC* signals.

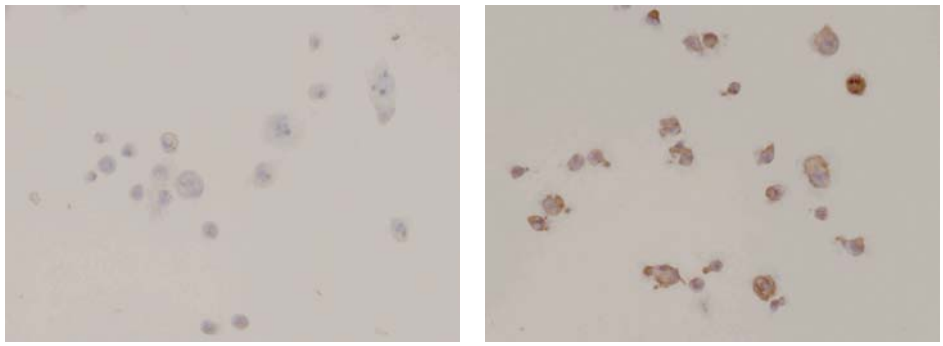
enough to induce polysomies, but may be not sufficient to let the chromosomal translocation occur. It is presumable that keeping these already genetically altered cells growing for a longer period of time might determine the onset and the accumulation of further genetic alterations, as translocations. In any case, the increased number of *MYC* copies could mimic the effects of the *MYC* activation due to the translocation, as observed in most BL. It is worth noting, nevertheless, that BL cases lacking *MYC* translocation do exist, in which the *MYC* expression level is increased due to different mechanisms [4, 5, 37]. Of interest, polysomies were detected only for chromosome 8, suggesting a predilection for a specific genetic locus alteration after *E. tirucalli* treatment.

Interestingly, EBV reactivation may be crucial as its proteins may induce the expression of cellular genes. In particular, EBNA2 may induce *MYC* expression [38], whereas LMP1 may induce *BCL2* expression [39]. This may be of help to explain *BCL2* hyperexpression following *E. tirucalli* treatment, as no chromosomal translocations, neither genetic alterations involving *BCL2* have been detected. It is reasonable to hypothesize *BCL2* upregulation may be due to LMP1, whose expression is induced by *E. tirucalli*. In addition, EBNA2 overexpression, which is itself induced by *E. tirucalli,* may also contribute to hyperexpression of *MYC*. The observation of multiple signals for chromosome 8 indicates an additional mechanism explaining *MYC* upregulation, which can synergistically act with EBNA2-induced *MYC* expression, in determining higher expression levels of c-MYC. The overexpression of c-MYC should lead to both an increase of cell proliferation and cell death, as *MYC* has proliferative and proapoptotic effects, thus keeping balanced cell number. *E. tirucalli*-treated cells showed a marked upregulation of the antiapoptotic gene *BCL2*, though no genetic alterations for this gene had been detected by FISH. The upregulation of *BCL2* may counteract the proapoptotic effect due to c-MYC overexpression and could give the treated cells a growth



(a) (b)

Figure 6: (a) qRT-PCR for *BCL2* and *MYC* in untreated and *E. tirucalli*-treated cells. A marked up-regulation of both genes is observed following treatment. The graph is representative of three different qRT-PCR experiments. Error bars represent standard deviation between duplicates. (b) Immunofluorescence of untreated (upper panel) or *E. tirucalli*-treated cells (lower panel). c-MYC expression increases following treatment. Magnification 40x.



(a) (b)

Figure 7: ICC for *BCL2* in untreated (a) and *E. tirucalli*-treated cells (b). Magnification 40x.

advantage, which may contribute to malignant transformation.

Collectively, our preliminary data suggest that *E. tirucalli* may cooperate in inducing malignant transformation, due to its modulation of the expression of the latency genes of EBV, and the upregulation of two key factors as *BCL-2* and *MYC*. In particular, the overexpression of c-MYC seems to rely on the induction of polysomies after treatment, rather than chromosomal translocations.

These observations suggest that *Euphorbia tirucalli* extracts and, possibly, extracts of other plants, which are known to have similar EBV-activating properties, could act as cofactors for lymphomagenesis in endemic African Burkitt's lymphoma.

Acknowledgment

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Review Article

Posttransplant Lymphoproliferative Disorders

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Posttransplant lymphoproliferative disorders (PTLDs) are a group of diseases that range from benign polyclonal to malignant monoclonal lymphoid proliferations. They arise secondary to treatment with immunosuppressive drugs given to prevent transplant rejection. Three main pathologic subsets/stages of evolution are recognised: early, polymorphic, and monomorphic lesions. The pathogenesis of PTLDs seems to be multifactorial. Among possible infective aetiologies, the role of EBV has been studied in depth, and the virus is thought to play a central role in driving the proliferation of EBV-infected B cells that leads to subsequent development of the lymphoproliferative disorder. It is apparent, however, that EBV is not solely responsible for the "neoplastic" state. Accumulated genetic alterations of oncogenes and tumour suppressor genes (deletions, mutations, rearrangements, and amplifications) and epigenetic changes (aberrant hypermethylation) that involve tumour suppressor genes are integral to the pathogenesis. Antigenic stimulation also plays an evident role in the pathogenesis of PTLDs. Plasmacytoid dendritic cells (PDCs) that are critical to fight viral infections have been thought to play a pathogenetically relevant role in PTLDs. Furthermore, regulatory T cells (Treg cells), which are modulators of immune reactions once incited, seem to have an important role in PTLDs where antigenic stimulation is key for the pathogenesis.

1. Introduction

Post-transplant lymphoproliferative disorders (PTLDs) are a group of diseases that range from benign polyclonal to malignant monoclonal lymphoid proliferations. They develop as a consequence of immunosuppression. PTLDs are characterised by the following: they are usually derived from B cells with preferential presentation as non-Hodgkin's lymphoma (as against Hodgkin's lymphoma), usually originate in extranodal sites, rarely affect skin, behave aggressively, and frequently harbour the Epstein-Barr virus (EBV) genome. Whilst most are high-grade B-cell non-Hodgkin's lymphoma (NHLs), a few are classical Hodgkin's lymphomas. Rare cases have also been shown to be either of T-cell or NK-cell lineages [1, 2].

T-cell neoplasms constitute 10% to 15% of all PTLDs, and about 75% of T-cell PTLDs, have been shown to be negative for EBV and to behave more aggressively. T-PTLDs usually develop later than B-PTLDs and patients are less likely to respond to reduction in immunosuppression [3, 4].

The abnormal B cells in solid organ transplant recipients originate usually from those of the recipient, while in recipients of bone marrow transplant they are of donor origin [5, 6].

2. Onset, Frequency of Occurrence, and Risk Factors of PTLD

PTLDs are classified as either early onset lesions which develop within one year, or late onset lesions, which develop more than one year after transplantation [7, 8].

The occurrence of PTLD varies between different studies, but the overall frequency is less than 2% in transplant recipients [9]. It differs according to many factors such as the age of the patient, the organ transplanted, type and dosage

regimen of immunosuppressive drugs, and the pretransplant EBV serostatus [10].

(1) The age of the patient. children are more prone to developing PTLDs as they are usually naïve for Epstein-Barr virus (EBV) infection [10].

(2) The organ transplanted. the frequency of PTLD differs according to the type of organ transplanted. (Table 1) summarizes the frequency of PTLDs in transplant recipients [6, 9].

(3) Type and dosage regimen of immunosuppressive drugs. It has been reported that the risk of developing PTLD increases with the use of certain drugs such as tacrolimus and OKT3, especially when they are combined [11]. Despite the fact that immunosuppressive drugs are an established risk factor, it is still not well-understood whether the risk is due to the cumulative dose or peak levels of immunosuppressive drugs. The cumulative dose, however, is more likely to be the incriminating factor [12].

(4) The pretransplant EBV sero-status. EBV-naive recipients, being incapable of initiating an EBV-specific cytotoxic T-lymphocyte (CTC) response, are more liable to develop PTLD [12, 13]. Nonexposure to EBV before transplantation remains the most important predisposing factor [13].

3. Clinical Presentation

The clinical manifestations vary from nonspecific symptoms in the form of fever, sweats, malaise, weight loss, and features of primary EBV infection in some patients, to sudden enlargement of tonsils, lymph nodes, or other extranodal lymphoid organs. Other organs such as the central nervous system, bone marrow, spleen, lung, small intestine, liver, and kidney may also be affected [10].

4. Pathological Features and Classification of PTLDs

Clinicopathologic features of major types of posttransplant lymphoproliferative disorders are summarised in Table 2. The classification of PTLDs is currently based on the WHO classification of lymphoid neoplasms (Table 3). Three main pathologic subsets/stages of evolution are recognised: early, polymorphic, and monomorphic lesions [3].

4.1. Early Lesions. Early lesions form one end of the spectrum of PTLD and mostly develop within one year after transplantation. These include two morphological types: plasma cell hyperplasia and infectious mononucleosis-like lesions. Early lesions more frequently involve tonsils, adenoids or lymph nodes than true extranodal sites. They do not invade or disturb the architecture of the affected tissue [3].

Plasmacytic hyperplasia shows numerous polytypic plasma cells and occasional immunoblasts. Infectious mononucleosis-like lesions show marked paracortical expansion by a cellular infiltrate composed of numerous immunoblasts and a mixed population of T cells and plasma cells. These lesions often show spontaneous regression or regress following reduction in immunosuppression [3]. Immunoblasts in infectious mononucleosis-like lesions frequently harbour EBV and express EBV-encoded RNA (EBER) or EBV-LMP-1. Early lesions rarely harbour clonal cytogenetic changes [14].

Table 1: Frequency of PTLD in different types of transplants.

Organ transplanted	Reported risk of developing PTLD % and references
Kidney	1%
Liver	2–5%
Heart	2–5%
Lung	1.8–7.9%
Heart-lung	9.4%
Small bowel	up to 30%
Pancreas	2.1%
Bone marrow	<1%

4.2. Polymorphic PTLDs. Polymorphic PTLDs affect nodal and extranodal tissues and show loss of tissue architecture and necrosis. Polymorphic PTLDs are composed of a mixed population of immunoblasts, plasma cells, intermediate-sized lymphoid cells (incorporating a full range of B-cell morphology and differentiation), as well as occasional Hodgkin Reed Sternberg-like cells [15] (Figure 1). The B-cells are usually monotypic but may be polytypic. Nonetheless, a clonal pattern of *IgH* or episomal EBV genome is observed [16, 17]. The majority of the lesions exhibit EBV latency type II or III (expressing EBER and EBV-LMP-1 with variable expression of EBV-EBNA2 and other viral antigens). A variable proportion of cases show regression in response to reduction in immune suppression while other cases may progress and require chemotherapy [3].

4.3. Monomorphic PTLDs. Monomorphic PTLDs (mPTLDs) can be either of B cell or T-cell lineage and resemble the typical types of non-Hodgkin lymphomas (NHLs) seen in immunocompetent patients, and they are usually monoclonal. They disturb the tissue architecture and spread to other organs. They are classified according to the WHO classification of lymphomas in immunocompetent patients. Monomorphic B-PTLDs show features of different morphologic variants of diffuse large B-cell lymphoma (DLBCL) in immunocompetent patients (iDLBCL) (immunoblastic, centroblastic, or anaplastic), Burkitt's lymphoma (BL), or plasmablastic lymphoma (PL). Almost all cases display a clonal pattern of *IGH* rearrangement, and EBV-positive cases show episomal EBV genome. mPTLDs can be EBV-negative, tend to be more aggressive, and only rarely respond to a reduction in immune suppression [3, 18]. In addition, genetic alterations of 3q27, 8q24.1, and 14q32 have been described in monomorphic B-PTLDs [19]. The identification of similar cytogenetic alterations and clonal relationship

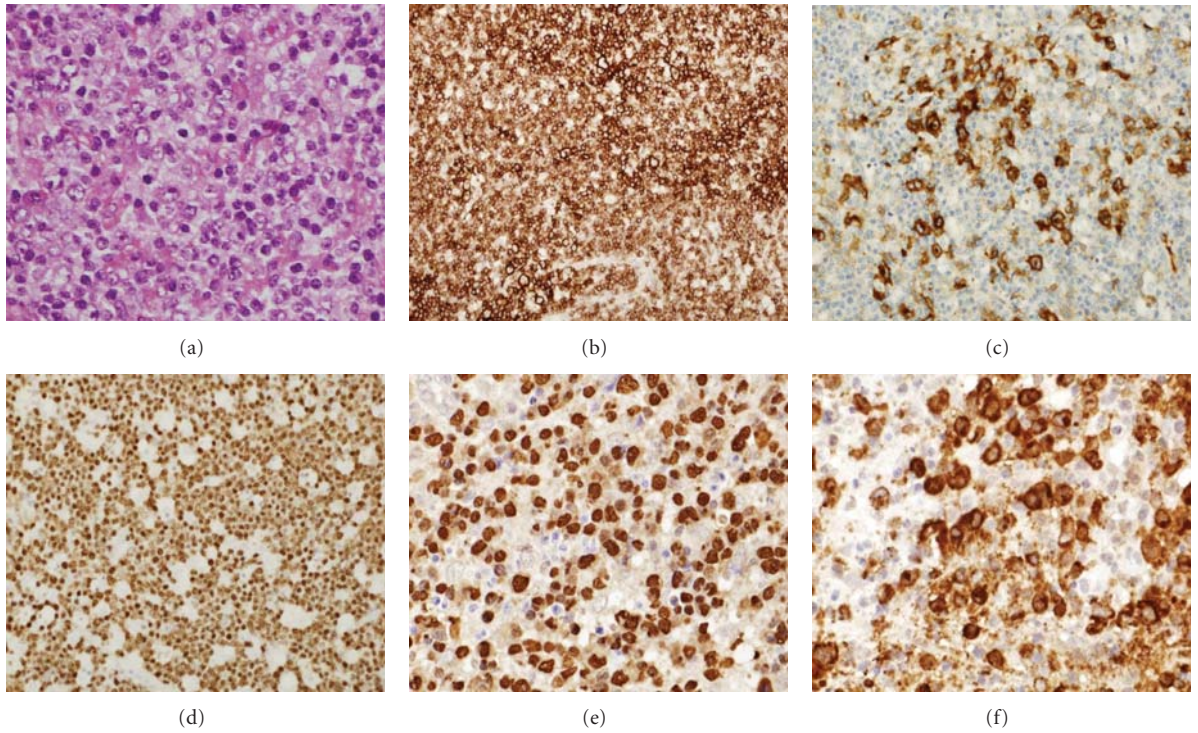

Figure 1: A typical case of polymorphic PTLD. (a) Infiltrate is a mix of plasma cells, small lymphoid cells and larger cells with nucleoli. The cells are positive for CD20 (b), CD30 (c), MUM1 (d), EBER (e), and EBV-LMP-1. Magnification: (b,d): ×100; (a,e,f): ×200.

Table 2: Clinicopathologic features of major types of posttransplant lymphoproliferative disorders.

	Early lesions (plasmacytic hyperplasia and infectious mononucleosis-like)	Polymorphic PTLD	Monomorphic PTLD
(i) Clinical features			
(a) Age	Children and young adults	All age groups	All age groups
(b) Organ involved	Tonsils or lymph nodes	Lymph nodes, GIT, lung or allograft	Lymph node, any extranodal site, including bone marrow
(c) Regression	Usually regress either with minimal reduction of immunosuppressive drugs or spontaneously	Some cases regress, others progress	Very rare. Most cases progress rapidly
(ii) Histopathological features			
(a) Tissue architecture	No or partial effacement	Nearly complete effacement	Complete effacement
(b) Nature of infiltrate	Comprised mainly of plasma cells and lymphoplasmacytoid cells in plasmacytic hyperplasia, and immunoblasts and plasmablasts in infectious mononucleosis-like lesion	Composed of a mixture of plasma cells, small lymphocytes, and large activated cells	Monotonous, similar to that of usual type B-cell NHL
(c) Atypia	Absent	Present/absent in large cells	Present
(d) Necrosis	Absent	Variable	Present (geographic)
(iii) Molecular features			
(a) *Ig* gene	Polyclonal in most cases	Usually monoclonal; may be oligo or polyclonal	Monoclonal
(b) EBV	Usually nonclonal	Clonal	Clonal
(c) Structural alterations of oncogenes and TSG	Usually absent	Usually absent	Usually present

Ig: immunoglobulin, EBV: Epstein-Barr virus, PTLD: posttransplant lymphoproliferative disorder, NHL: non-Hodgkin's lymphoma, TSG: tumour suppressor gene.

Table 3: Categories of posttransplant lymphoproliferative disorders.

(1) Early lesions
(a) Reactive plasmacytic hyperplasia
(b) Infectious mononucleosis-like lesions
(2) Polymorphic PTLD
(3) Monomorphic PTLD (classified according to lymphoma they resemble)
B-cell neoplasms
(a) Diffuse large B-cell lymphoma (DLBCL)
(b) Burkitt's lymphoma
(c) Plasma cell myeloma
(d) Plasmacytoma-like lesions
(e) Others*
T-cell neoplasms
(a) Peripheral T-cell lymphoma not otherwise specified
(b) Hepatosplenic T-cell lymphoma
(c) Others
(4) Classical Hodgkin's lymphoma-type (HL-PTLD) and HL-like PTLD**

* Indolent small B-cell lymphomas developing in transplant recipient are not included among the PTLD.
**HL-like PTLDs are better categorized either as a polymorphic or monomorphic PTLD based on the overall morphology.

between polymorphic PTLDs and mPTLD supports the hypothesis that PTLDs progress along a continuum from polyclonal early lesions to monoclonal mPTLD [3, 20].

Plasmablastic lymphomas (PBL), which were originally described in HIV-infected people affecting the oral cavity, may occur as a PTLD. Nearly 60–75% of cases of PBL are EBV associated [21–23].

There are only a few cases of PTLDs reported in the literature that demonstrate both B- and T-cell clones. In a recently published study, however, monoclonal expansion of T-cell population which seems to arise from $CD8^+$ T cells has been found to occur frequently in B-PTLDs, and these clonal T-cell populations coexist with monoclonal B-cell population in B-PTLDs. However, these clonal T-cell expansions do not constitute a clinical T-cell lymphoma [24–26].

4.4. Hodgkin's Lymphoma-PTLD and HL-Like PTLD. The histological features of HL-type PTLD are similar to mixed cellularity or lymphocyte-depleted subtypes. The infiltrate is composed of scattered large pleomorphic mono- and binucleated Hodgkin/Reed-Sternberg giant cells in a background of small lymphocytes, B-immunoblasts intermingled with histiocytes, plasma cells, a few eosinophils, and neutrophils. The neoplastic cells are usually $CD30^+$, and $CD15^+$, $EBER^+$, $CD45^-$, OCT-2^-/BOB1 [3]. In HL-like PTLD, the EBV^+ cells are $CD45^+$, $CD20^+$, and $CD15^+$ and EBV^+ small and medium-sized lymphoid cells may be present. Distinguishing HL-PTLD from HL-like lesions is sometimes difficult, and it has been suggested that the latter are better diagnosed as either a polymorphic or monomorphic PTLD based on the overall morphological features [3].

5. Aetiology and Pathogenesis

The pathogenesis of PTLD is multifactorial. EBV plays an important role in driving the proliferation of EBV-infected B cells. It is widely perceived, however, that it is not solely responsible for the "neoplastic" state, and that accumulation of different aberrations in protooncogenes and suppressor genes, and hypermethylation of suppressor genes are integral parts of the pathogenesis [27] (Figure 2).

5.1. Viruses

5.1.1. EBV. EBV is an oncogenic double-stranded DNA virus that infects and persists in memory B cells. Two phases of EBV infection have been recognized. The lytic phase is characterized by the expression of all EBV proteins and active viral replication, leading eventually to cell death and the release of virions. The latency phase involves infection of lymphoid B cells via their CD21 receptors, resulting in the formation of EBV episomes and the expression of a limited number of viral proteins [28].

This results in persistence of the virus in the lymphoid cells and their progeny without destruction of the infected cell. LMP-1 and LMP-2 viral proteins are believed to act as oncogenes, allowing B cells to escape cell death and proliferate autonomously [28]. There are three different latency patterns that correspond to the differentiation stages of B cells. These patterns are thought to play a major role in protecting EBV-infected cells from immunosurveillance [29–31]. EBV-infected naive B cells expressing all latent antigens are said to have "type III latency." Infected naive B cells enter the germinal centre where they multiply and form clones. They express EBNA1, LMP-1, and LMP-2, and this is known as "type II latency" [32]. However, some only express EBNA-1 and 2 as well as small noncoding Epstein-Barr RNAs (EBERS) and are said to have "type I latency" as seen in Burkitt's lymphoma [33, 34].

Most PTLDs are associated with EBV, but nonetheless a proportion (42% reported in one study) is EBV-negative, including 53% of the mPTLD cases [35, 36]. There is a debate as to whether EBV-negative PTLDs are in fact incidental lymphomas developing in transplant patients, or true PTLDs that can regress following reduction of the immunosuppression [37]. It has to be noted that the lack of identifiable EBV, based on EBER or LMP-1 staining, does not necessarily imply that EBV-DNA is absent in all of these cases, or that EBV did not play a role in the pathogenesis of the EBV-negative lymphoid proliferations [38]. It has been suggested that EBV-negative PTLD may develop as a result of "hit and run" oncogenesis as does EBV-negative classical Hodgkin lymphoma (cHL) [38, 39]. Chronic antigenic stimulation on the background of immune suppression is thought to play an essential role in the pathogenesis of EBV-negative PTLD [40].

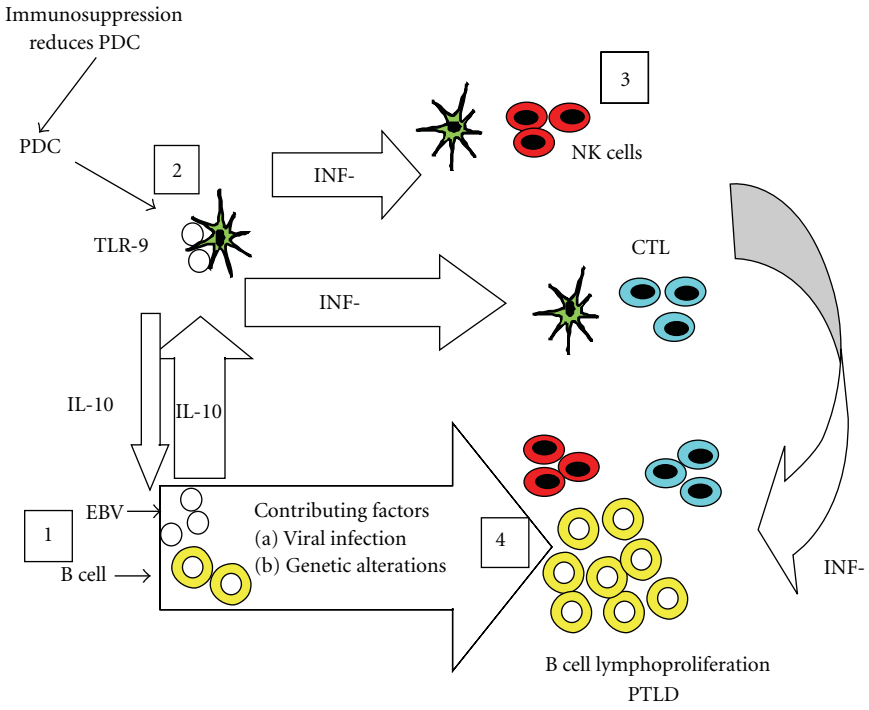

Figure 2: A proposed model of pathogenesis of EBV infection in the development of PTLDs in sold organ transplant recipients. CTL: cytotoxic T lymphocytes, IL-10: interleukin-10, INF-α: Interferon-α, NK cells: natural killer cells, PDC: plasmacytoid dendritic cells, TLR-9: toll-like receptor-9.

B-PTLDs have been shown to be more frequently associated with type-A EBV genotype than type-B EBV [41, 42].

5.1.2. HHV-8. HHV-8 is a gamma-herpes virus that, like EBV, infects B cells and acquires an episomal configuration in the nucleus and results in a state of latency. In the posttransplant setting, it has only been detected in cases of primary effusion lymphoma [43, 44]. In a recently published study, KSHV/HHV-8 was found to be consistently absent in PTLD [45].

5.2. Molecular Alteration of Cellular Genes. Different genetic alterations among PTLDs are summarised in Table 4.

5.2.1. Microsatellite Instability. Lymphomas developing in immunocompetent patients are often characterized by relative genomic stability. In contrast, a small subset of PTLD is associated with microsatellite instability, which results from defects in DNA mismatch repair mechanisms [46]. These cases show mutations involving multiple genes, including *BAX* and *CASPASE5* (proapoptotic factors) and *RAD50* (a DNA repair gene) [27].

5.2.2. Aberrant Somatic Hypermutation (ASHM). B cells in the germinal centre (GC) are subjected to a physiological phenomenon known as "somatic hypermutation" (SHM), which involves the introduction of single nucleotide substitution into their *IgV* genes [47]. It involves not only the *IgH* gene but also nonimmunoglobulin genes such as *BCL6* and *Fas/CD95*. In more than 50% of DLBCLs, SHMs can also affect some proto-oncogenes such as *PAX5*, *PIM-1*, *RhoH/TTF*, and *c-MYC* gene, which are involved in the pathogenesis of lymphoid neoplasms including some cases of PTLD [47].

5.2.3. Other Genetic Alterations

BCL6 Gene. The *BCL6* gene is located on chromosome 3q27 and encodes a transcriptional repressor [48]. *BCL6* rearrangement is very rarely seen in PTLDs, although it is the target of SHM in approximately 50% of PTLDs [14, 19].

c-MYC Gene. *c-MYC* gene is located on chromosome 8q24 and is the target of chromosomal breaks in most posttransplant Burkitt's lymphomas (PT-BL) [14, 49].

BCL2 Gene. *BCL2* gene, an antiapoptotic gene, is located on chromosome 18q21. Although the *BCL2* is amplified in a proportion of PTLDs, its rearrangement is a very rare event in PTLDs [14, 50].

TP53 Gene. The *TP53* gene is a tumour suppressor gene located on 17p13.1 and is mutated or deleted in a small proportion of mPTLDs (DLBCL) [16].

IGH Gene. *IGH* gene is located on 14q32 and breakpoints involving the gene are detected in a small proportion of

Table 4: Summary of different genetic alterations among PTLDs.

Genetic alteration	Frequency
BCL6 gene	
(1) Rearrangement	Rare in PTLD
(2) SHM	50% of PTLD
c-Myc gene rearrangement	100% PT-BL
BCL2 gene	
(1) Rearrangement	Very rare in PTLD
(2) Amplification	A proportion of PTLD
P53 gene mutation/deletion	Small proportion of mPTLD
Translocations involving IG genes	A small proportion of PTLD. Rarely in florid follicular hyperplasia in posttransplant setting
PAX5 gene	
(1) Rearrangement	Very rare in PT-DLBCL
(2) SHM	Very rare in PT-DLBCL
(3) Amplification	A proportion of PTLD
Chromosomal gains	
(1) 3q27, 7q, 8q24, 12q, 12p, 18q21, 21q	
(2) 5p and 11p	PT-DLBCL = iDLBCL
(3) 6q25.3	Recurrent in PT-BL
(4) 1q, 11q, and of chromosome 7	PT-DLBCL
Chromosomal loss	
(1) 1p, 6q, 9p, and 17p13	Common to PTLD and lymphomas immune competent patients
(2) 4q, 17q, and Xp	In PTLD but not common in other lymphomas
(3) 12p, 4p, 4q, 12q, 17p, and 18q	Frequent in PT-DLBCL
(4) 11q25	Recurrent in PT-BL
(5) 2p16.1 (FRA2E)	30% of PT-DLBCL (both in EBV positive and negative cases)
(6) 17p	PT-DLBCL
Aberrant hypermethylation of	
(1) *MGMT*	75% pPTLD and 93% mPTLD.
(2) *DAP-kinase*	75% mPTLD
(3) *TP73*	20% mPTLD
(4) *SHP1*	~77% PT-DLBCLs, 75% pPTLDs, 66% PT-BLs
(5) *CDKN2A*	A small proportion of mPTLD

iDLBCL: immunocompetent diffuse large B cell lymphoma, mPTLD: monomorphic posttransplant lymphoproliferative disorders, pPTLD: polymorphic posttransplant lymphoproliferative disorders, PT-BL: posttransplant Burkitt lymphoma, PT-DLBCL: posttransplant diffuse large B cell lymphoma, SHM: Somatic hypermutation.

PTLD and rarely in florid follicular hyperplasia in post-transplant setting [14].

PAX5 Gene. *PAX5* is the target of t(9;14)(p13;q32) as well as ASHM in a very small proportion of mPTLD (DLBCL) [51, 52]. A proportion of PTLDs has also been reported to have *PAX5* gene amplification [53].

Other Chromosomal Changes. Comparative genomic hybridisation (CGH) analysis of PTLDs highlights some genetic changes similar to those occurring in the lymphoma of immunocompetent patients; for example, gains of 3q27, 7q, 8q24, 12q, 12p, 18q21, and 21q and losses of 1p, 6q, 9p, and 17p13. In addition, PTLDs show losses of 4q, 17q and Xp that are not common in other lymphomas [50, 53]. It has been demonstrated that posttransplant-DLBCLs (PT-DLBCLs), with a frequency similar to iDLBCLs, show gains of chromosomes 5p and 11p. Moreover, deletions of 12p, 4p, 4q, 12q, 17p, and 18q are frequently seen in PT-DLBCLs [53]. The finding that iDLBCLs and a proportion of PTLDs (especially PT-DLBCLs) share some histogenetic and pathogenetic pathways is reinforced by the presence of recurrent chromosomal aberrations common to both PTLDs and iDLBCLs [54]. In addition, recurrent deletions on 11q25 and gains on 6q25.3 were observed in PT-BLs [53]. Rinaldi et al. [55] using high-density genome-wide SNP-based arrays, reported similar genomic complexity among PT-DLBCLs, HIV-DLBCLs, and iDLBCLs. Nonetheless, PT-DLBCLs displayed a genomic profile with distinctive features. It has been reported that the del(13q14.3) targets the locus coding for different noncoding RNAs [56]. The absence of del(13q14.3) in PT-DLBCLs is the most significant difference between PT-DLBCLs and iDLBCLs [55, 57]. Del(13q14.3) is thought to be involved in immunosurveillance escape in the view that immunodeficiency-related lymphomas including PTLDs lack del(13q14.3) [55].

PT-DLBCLs have *IgV* mutational status and gene expression profiles similar to post-GC B cells [3, 20]. Nonetheless, iDLBCLs of post-GC phenotype display genetic lesions that are different from PT-DLBCLs [55, 58, 59]. PT-DLBCLs have been reported to have gains of 1q, 11q, and of chromosome 7, in addition to losses at 17p (*TP53*) [55]. Compared with PT-DLBCLs, iDLBCLs were found to be more frequently associated with gains of 18q (BCL2 and NFATC1), and LOH at 6q21-q22 (approximately 7 Mb telomeric from PRDM1(BLIMP1)) and at 6p21.32-p21.33 (HLA-DR locus) [55].

Craig et al. [60] used Affymetrix HU133A GeneChips to show that EBV-positive mPTLDs overexpress several interferon-induced genes as compared to EBV-negative mPTLDs. Furthermore, EBV-negative PTLDs overexpress genes corresponding to the B-cell receptor signalling pathways and a group of proliferation-related genes. These suggest that EBV-negative PTLDs are biologically distinct from EBV-positive PTLDs and are more similar to iDLBCL [60].

When compared with EBV-negative PT-DLBCLs, EBV-positive PT-DLBCLs have been described as having less recurrent lesions. However, del(2p16.1) is common in both EBV-negative and positive PT-DLBCLs [60].

"Fragile sites" are regions with marked genomic instability, present throughout the genome, that are often the sites of DNA breakage in malignant tumours and in cells exposed to specific chemical agents [61]. PT-DLBCLs have been described to have frequent interstitial deletions at 1p32.2, 2p16.1, 3p14.2, 4p14, 14q13.2, 20p12.3, and 20q13.32. Some of these deletions involve fragile sites such as *FRA1B*, *FRA2E*, and *FRA3B*. Del(2p16.1) (*FRA2E*) is the most common aberration in PT-DLBCLs, and the involvement is significantly higher than in iDLBCLs [55]. Some viruses including EBV and HHV-8 have been shown to incorporate themselves into the host genome, mainly at fragile sites, resulting in local genomic instability at the insertion sites [55]. Iatrogenically immunosuppressed posttransplant patients are more susceptible to a wide range of viruses which could integrate into the genome, particularly at these fragile sites [55]. The dissimilar pattern of breakage at fragile sites reported in PT-DLBCLs and HIV-DLBCLs might be due to differences in the integration sites for various viruses [55].

5.2.4. Epigenetic Alteration (DNA Hypermethylation). Hypermethylation is an epigenetic phenomenon that alters the gene activity without changing its base sequences and is accomplished through DNA methyl transferase enzyme [62]. Aberrant hypermethylation (AH), which is a mechanism for tumour suppressor gene silencing alternative to deletion and/or mutation, has been implicated in the pathogenesis of lymphoproliferative disorders in the posttransplant setting [63].

Hypermethylation of Death-Associated Protein Kinase (DAP-k). *DAP-k* is a serine-threonine kinase, which plays an important role in apoptosis triggered by TNFα, INFγ, and the FAS ligand. About 75% of mPTLDs display *DAP-k* hypermethylation [64].

Hypermethylation of O6-Methylguanine-DNA Methyl-Transferase (MGMT). MGMT is one of the DNA repair genes that serves to protect against DNA damage. MGMT is methylated in nearly 75% and 93% polymorphic PTLDs, and mPTLDs respectively [65].

Hypermethylation of P73. *P73* is a tumour suppressor gene that bears some functional and structural resemblance to *TP53*. It plays a role in cell cycle regulation and apoptosis and is hypermethylated in about 20% mPTLDs [63].

Hypermethylation of P16. *P16* is a tumour suppressor gene located on chromosome 9p21. It hinders the G1-S cell cycle transition by inhibiting the phosphorylation of Retinoblastoma protein. Martin et al. [66] described downregulation of P16/INK4a in subsets of mPTLDs (DLBCLs) that had an aggressive course but were not associated with EBV. There is a rare case report of an EBV-positive mPTLD (plasmablastic type) that showed *P16* hypermethylation [67].

Hypermethylation of SHP1 Gene. The *SHP1* gene is located on chromosome 12p13 and encodes the SHP1 protein. The protein is expressed in hematopoietic cells and potentiates its negative effect on cell cycle regulation by inhibiting the JAKs/STATs pathway. In B-lymphocytes, therefore, it inhibits proliferation, and its deficiency through AH results in overgrowth [68]. Cerri et al. [69] reported *SHP1* methylation in 76.5% of the PT-DLBCLs, 75% of the polymorphic PTLDs, 66% of the PT-BLs, and in a case of PT-myeloma.

5.3. Antigen Stimulation. Antigenic stimulation plays an important role in the pathogenesis of immunodeficiency-associated lymphomas. Under normal circumstances B cells express the B-cell receptor (BCR), and the loss of a functional receptor through the acquisition of mutations results in apoptosis [47]. It has been demonstrated that EBV, through expression of LMP2A which simulates a BCR, protects BCR-lacking GC B cells from death, leading to lymphoma development [47]. There are a few reports of the existence of EBV-negative PTLDs that lack expression of sIg, pointing to the possibility of as yet unidentified genetic mechanisms that may rescue EBV-negative, BCR-lacking lymphocytes [20]. Molecular signs of antigen stimulation are evident in a fraction of PTLDs that exhibit a functional BCR [47].

5.4. Role of Microenvironment

5.4.1. Role of Plasmacytoid Dendritic Cells (PDCs). PDCs are potent antigen-presenting cells that originate from the hematopoietic stem cells in the bone marrow under the effect of some cytokines, principally Flt3L [70]. In the posttransplant scenario, EBV-stimulated PDCs produce insufficient concentrations of IFN-α. Furthermore, the numbers of circulating blood PDC precursors are reduced in renal and cardiac transplant recipients. These are thought to play a significant role in the development of lymphoproliferative disorders [71, 72]. In addition, EBV-stimulated PDCs produce the immunosuppressive cytokine IL-10, thereby allowing the

virus-infected B cells to escape immunorecognition [73]. IL-10 inhibits expression of costimulatory molecules, which in turn results in inability of monocytes and macrophages to activate T cells [74]. In addition, IL-10 suppresses the production of IFN-α and IFN-γ by PDCs, T cells, and NK cells. It also has an inhibitory effect on antigen-specific activation and proliferation [75]. PDCs numbers are increased in some malignant neoplasms including cutaneous T-cell lymphoma [76]. PDCs are markedly decreased in number and are qualitatively altered in non-Hodgkin lymphoma, compared with reactive lymph nodes [76]. However, in some cases of classical Hodgkin lymphoma (cHL), there are increased numbers of PDCs present which may be attributed to the cytokines released in the microenvironment of cHL [76]. The observation of PDC clusters in tumour samples suggests that PDCs may also play an important role in the pathogenesis of cutaneous marginal zone B-cell lymphoma [77]. Based on the finding of significantly higher numbers of PDCs in the tumour microenvironment of early lesions of B-PTLDs compared to polymorphic and monomorphic lesion, and in PT-DLBCL compared to iDLBCL, PDCs have been suggested to play a pathogenetically relevant role in PTLDs [78].

5.4.2. Role of Treg Cells. Treg cells are $CD4^+$ and $CD25^+$ T lymphocytes that are a subset of immunoregulatory cells, and have the ability to suppress immune responses. There is a subpopulation of Treg cells which express CD8 and not CD4 [79]. When Treg cells undergo activation via their TCR, they inhibit the proliferation of $CD4^+$ and $CD8^+$ T lymphocytes, through the release of cytokines such as IL-10 and TGF-β [80, 81]. The intratumoural Treg cells have been shown to have an inhibitory effect on the production and release of perforin and granzyme B, which is necessary for the effector functions of $CD8^+$ cells and cytotoxic T-cell-mediated lysis of tumour cells [82]. Treg cells are also known to have a direct effect on B lymphocytes and inhibit the production of immunoglobulins [83]. Treg cells can suppress the growth of some tumours in addition to their role in suppressing the antitumour immune response [84]. Higher numbers of Treg cells have been described as predictors of both improved survival in follicular lymphoma and therapeutic response [85]. Treg cells are found in higher numbers in tissue samples of B-cell lymphomas as compared to reactive lymph nodes or tonsils. This is thought to be due to the attraction of Treg cells to the tumour microenvironment through CCL22 secreted by the lymphoma cells [85, 86]. It has been previously shown that in recipients of solid organ transplants who are on multiple immunosuppressive drugs, the levels $FOXP3^+$ Treg cells reduce in the peripheral blood, possibly due to redistribution into tissues and lymphoid organs [87]. The numbers of Treg cells in the tumour microenvironment of PTLDs have been shown to have no impact on patient survival [88].

6. Conclusion

PTLDs are group of diseases that range from benign polyclonal to malignant monoclonal lymphoid proliferations. Genetic and epigenetic alterations as well as viruses, notably EBV, contribute towards the development of PTLDs. Common genetic rearrangements which are frequent in immune competent lymphoma are rare in PTLDs. Microenvironment-resident PDCs and Treg cells are likely to play a critical role in the pathogenesis of PTLDs. Therefore, further studies investigating the cytokines secreted by PDCs and Teg cells are required to substantiate and further clarify their precise role in the pathogenesis of PTLD.

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Review Article

A Role for RNA Viruses in the Pathogenesis of Burkitt's Lymphoma: The Need for Reappraisal

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Certain infectious agents are associated with lymphomas, but the strength of the association varies geographically, suggesting that local environmental factors make important contributions to lymphomagenesis. Endemic Burkitt's Lymphoma has well-defined environmental requirements making it particularly suitable for research into local environmental factors. The Epstein-Barr virus and holoendemic Malaria are recognized as important cofactors in endemic Burkitt's Lymphoma and their contributions are discussed. Additionally, infection with Chikungunya Fever, a potentially oncogenic arbovirus, was associated with the onset of endemic Burkitt's Lymphoma in one study and also with space-time case clusters of the lymphoma. Chikungunya Virus has several characteristics typical of oncogenic viruses. The Flavivirus, Hepatitis C, a Class 1 Human Carcinogen, closely related to the arboviruses, Yellow Fever, and Dengue, is also more distantly related to Chikungunya Virus. The mechanisms of oncogenesis believed to operate in Hepatitis C lymphomagenesis are discussed, as is their potential applicability to Chikungunya Virus.

1. Introduction

It has been estimated that approximately 20% of all cancers, worldwide, are attributable to infectious agents [1]. This is likely to be an underestimate because of under-reporting and under-ascertainment, particularly in resource-poor countries, where the burden of infection-related cancers is almost four times that of the more prosperous countries [1]. A number of infectious agents, comprised of a variety of different types of organisms, have been shown to be associated with lymphomas. It is highly probable that this number will continue to expand as diagnostic methods improve, new organisms emerge and general advances in knowledge are made.

Some of the organisms which have been linked with different types of lymphomas have already been designated Class 1 Human Carcinogens by the World Health Organisation. They are the DNA Herpes viruses, the *Epstein-Barr virus* [2] and *Kaposi Sarcoma Herpesvirus* [2, 3], the retroviruses *Human Immunodeficiency Virus type 1* and *Human T Cell Lymphotropic Virus Type 1* [4], the Hepatitis viruses, *Hepatitis B*, a DNA virus, and *Hepatitis C*, an RNA virus [5], and the bacterium, *Helicobacter pylori* [6]. In addition, the bacteria *Campylobacter jejuni* [7], *Chlamydia psittaci* and *pneumoniae* [8, 9], *Borrelia burgdorferi* [10, 11] and the RNA Alphavirus *Chikungunya virus* [12], an arbovirus, have been found to be associated with various different forms of lymphoma. The *Epstein-Barr virus* (EBV) [2], the protozoon, *Malaria* [13], and the vector-borne Alphavirus, *Chikungunya virus* (CHIKV), have been linked specifically with endemic Burkitt's Lymphoma (eBL), perhaps the best studied of all lymphomas. Studies of associations between lymphomas and different infectious organisms often show considerable geographic differences in the strength of the association, suggesting that local environmental factors, including lifestyle-related ones, as yet unidentified, may play important roles in lymphomagenesis [9, 14, 15].

The infectious agents linked with lymphomas are thought to promote lymphomagenesis by processes linked with chronic antigenic stimulation. They establish persistent infections, accompanied by overt or silent chronic inflammation, leading to cytokine activity, the activation of cyto-oncogenes, with or without chromosomal abnormalities, and the inactivation of tumour-suppressor genes [16–18]. Some viruses, including EBV and *Hepatitis C* (HCV) [19], can cause a polyclonal B cell proliferation, a risk factor

for Non-Hodgkins Lymphomas. Immunosuppression may be important, as with HIV-associated Lymphomas [18]. Oncogenic viruses may or may not appear to co-operate: in HIV infection, the incidence of EBV-positive Burkitt's Lymphoma is increased [18], whereas that of HCV-associated lymphomas is reduced [19, 20].

As we learn more, our understanding of the process of oncogenesis is changing from the view that it is confined to a series of irreversible genetic changes in the cell, culminating in full-blown malignancy, to an appreciation of the important contribution made by epigenetic changes and the balance of forces promoting or opposing apoptosis, many driven by infectious agents. Some of these changes are reversible, and, in a few cases, and under certain conditions, the process of oncogenesis can be reversed, as will be discussed later.

This paper will concentrate on those aspects of lymphomagenesis, particularly apparent co-operation between cofactors, which are best exemplified in endemic Burkitt's Lymphoma (eBL), often described as the "Rosetta Stone" of cancer [21]. It will discuss, drawing on research into lymphomagenesis in HCV infection, how the arbovirus, CHIKV, shown to be associated with the onset of eBL [12, 22], might contribute to lymphomagenesis.

2. Burkitt's Lymphoma

Burkitt's Lymphoma (BL), an aggressive non-Hodgkins Lymphoma (NHL), has an extremely rapid doubling time of 24–48 hours as almost all the cells are cycling at one time [23]. It has been calculated, based on the phenomena of seasonality and time-space case clusters sometimes observed in the endemic form of Burkitt's Lymphoma (eBL), that the latent period for this lymphoma is likely to be as short as one year [24]. The rapid growth, coupled with a short induction period could, theoretically, make the train of events involved in lymphomagenesis easier to unravel.

There are three types of BL: endemic or "African," sporadic and HIV-associated. BL can also arise in association with severe immunosuppression as in organ transplants [25, 26]. All BLs have one of three translocations, of which *t(8:14)* is by far the commonest. They involve the *C-MYC* oncogene on the long arm of chromosome 8, and an immunoglobulin chain gene [27]. The *C-MYC* gene, which plays an important role in cellular proliferation, becomes deregulated and activated as a consequence of the BL translocation. This occurs due to proximity to Ig transcriptional enhancers [28] or to structural alterations within *C-MYC* [29]. The linking of *C-MYC* to immunoglobulin sequences leads to constitutive *MYC* expression and the cell is unable to leave the cycling phase [30]. Although a deregulated *C-MYC* plays an important role in lymphomagenesis in eBL [31], working in conjunction with the EBV [32], it cannot institute tumorigenesis unaided [33]. The *TP53* mutations, commonly found in BL, may be accompanied with a gain in transforming ability and loss of growth suppression [34, 35], but are thought to contribute to tumour progression rather than lymphomagenesis.

Endemic and sporadic forms of BL have different breakpoints within both the *MYC* locus on chromosome 8 and the Ig heavy-chain locus on chromosome 14. There are also clinical, molecular, and cytological differences and varying degrees of EBV positivity, which exhibit a geographic gradient [14]. These differences, together with the well-defined climatic requirements for the endemic form, highlight the probable importance of as yet unrecognised lymphomagenic environmental factors, which may differ throughout the world.

BL cells are B lymphocytes with rearranged immunoglobulin genes, secreting immunoglobulin chains which correlate with the site involved in the translocation, suggesting that an active immunoglobulin locus is directly involved [36]. Cloning of translocation breakpoints from endemic cases has revealed evidence of V-D-J (variable diversity joining) recombinase involvement in the genesis of the translocations [37] strongly suggesting that eBL arises while the cell is actively arranging its IgG genes. It appears that the deletions and insertions seen in Ig V_HDJ_H mutations occur as the result of an antigen-driven selection process and that the *C-MYC*/Ig translocation happens due to hypermutation in B-cells entering or transiting Germinal Centres [35, 38, 39].

2.1. Lymphomagenesis of Endemic Burkitt's Lymphoma. It is thought that lymphomagenesis in eBL begins in infancy. Heavy primary EBV infection results in a degree of immune tolerance. EBV-infected cells proliferate and some are immortalised and transformed [40]. Heavy malarial infection further stimulates expansion of the B-cell pool and suppresses T cells involved in EBV control. The final stage of lymphomagenesis is the development of the characteristic translocation, leading to deregulation of *C-MYC* and the development of a malignant clone.

2.1.1. EBV and Lymphomagenesis. Much has been learned about the mode of action of EBV in oncogenesis, but much remains to be learned [41]. EBV is associated with various forms of Non-Hodgkins Lymphoma (NHL), including Post-transplant lymphomas, some AIDS-related large-cell lymphomas, BL, and also Hodgkins Lymphoma. The extent of the association varies geographically by type of lymphoma and location. The evidence for a causal relationship is the strongest with eBL where the EBV genome is incorporated into 90% or more of cases in the African Lymphoma Belt [42]. No preferential integration site in the human chromosome [43] has been shown, the virus being integrated into the genome at a number of different sites in cell lines, but human-mouse hybrid cell studies suggest that the EBV genome is consistently associated with Chromosome 14 [44].

2.1.2. EBV-Antibody Studies. EBV seems to be actively involved in all stages of eBL development, as judged by EBV-antibody responses. The association between eBL and raised levels of EBV-VCA (Viral Capsid Antigen) and EBV-EA (Early Antigen) antibodies, both associated with actively replicating virus, is one factor implicating EBV as an active

participant in lymphomagenesis. In a Ugandan prospective study, children who subsequently developed BL had significantly higher titres of EBV-VCA IgG antibodies up to 6 years before the onset of the lymphoma [45, 46], and these were the only antibodies showing a significant increase. There was no further rise after the onset of BL. Chronic rather than acute EBV infection appeared to be relevant to lymphomagenesis. EBV-EA antibody levels were shown to increase as the tumour grew, and to decline after treatment [47], again implicating an active rather than a latent phase of EBV infection. Additionally, EBV-specific antibody-mediated cellular cytotoxicity appeared to have a prognostic significance for BL patients [48].

Raised levels of antibodies to EBV-VCA and EBV-EA antigens were also found in the relatives of BL patients [49], those exposed to chronic malaria [50, 51] and users of herbal remedies [52], which included plants producing tumour-promoter substances with EBV-potentiating activity. *Euphorbia tirucalli* is one of a number of such plants growing commonly in Africa and, more notably, around the homes of BL patients [53, 54]. It is possible that exposure to these plants, which secrete their active principles into the soil [55, 56], thus potentially contaminating environmental air and water, could account for some of the rises in EBV-antibodies seen in eBL. It has been suggested that the relatives of BL patients have raised EBV-antibody titres [49] because they share a similar immune dysfunction, due to similar exposures to environmental factors.

2.1.3. Potential Contributions of EBV to Lymphomagenesis. The EBV could potentiate lymphomagenesis in several ways. EBV is able to stimulate and maintain B-cell proliferation because of CD40 and B Cell Receptor (BCR) mimicry, increasing the size of the B cell pool, and, thereby, the chances of translocations and other cytogenetic changes occurring [57]. EBV can immortalize and transform lymphocytes and may also collaborate, in as yet unidentified ways, with the changes induced by the *C-MYC* translocation [32, 58]. EBV proteins such as EBNA1 may induce epigenetic changes, with subsequent cellular dysregulation [59, 60]. EBV also encodes products which can interact with, or mimic, a variety of cellular molecules, signals, and cytokines, many of which have antiapoptotic actions, and which promote lymphomagenesis [18, 30, 57, 61–63]. EBV infection also protects cells damaged by mutations from destruction by apoptosis, thus allowing them to replicate [62] and this function may be extremely important in lymphomagenesis [63, 64].

2.1.4. EBV Infection and Immunological Control. Over 90% of the world's population is infected with EBV. Once infected, people become lifelong carriers of the virus which persists in two main forms: circulating latently infected cells and a localized lytic infection in epithelial cells in the mouth and pharynx, possibly also the urogenital tract and salivary glands [65]. In generalized immunodeficiency states such as HIV infection and transplant patients, or the more specifically EBV-linked Duncan's Syndrome, proliferation of B-cells can proceed unchecked [42, 66] and may evolve from a polyclonal reactive process to a monoclonal malignant lymphoma [67]. The polyclonal activation and proliferation subsequent upon primary EBV infection is normally controlled by inhibitory immunological mechanisms, as it is usually followed by the development of cellular immunity and antibodies to the various EBV antigens. Killer cells and EBV-specific cytotoxic lymphocytes are also generated, the latter playing a crucial role in controlling circulating EBV-infected cells [68]. It has also been suggested that, because BL cells have a "resting" rather than B-blast phenotype, together with the accompanying changes in expression of certain EBV, HLA and adhesion molecules, the BL cell is not rejected by the EBV-specific immune response [30].

2.1.5. EBV Latency. After the acute infection has subsided, a type of EBV latency is found where most latency transcripts are undetectable [65]. However, BL cell lines display a unique Type I latency where the EBV nuclear protein, EBNA1, and the EBV RNA transcripts, the EBERs, and BART (BamA rightward transcripts) are expressed [69, 70]. However, some authorities believe that the concept of BL cells predominantly exhibiting type I latency is an oversimplification [71–73].

EBNA1 is responsible for maintaining the EBV episome in latently infected cells [74]. There are EBNA1 binding sites in the human genome and, as EBNA1 can bind both RNA and DNA, it could influence the expression of viral or cellular genes [75], possibly by eliciting demethylation and subsequent activation or dysfunction of cellular functions [59]. EBNA1 can up regulate the recombinase-activating genes which mediate V-D-J combination and are usually only expressed in immature lymphoid cells [76]. EBNA1 is indispensable for B cell transformation and can enhance B cell immortalization several thousandfold [77]. Although EBNA1 does not appear to be oncogenic on its own, as it is consistently expressed in EBV-infected cells, including latently infected cells, without oncogenic sequelae, EBNA1 transgenic mice can develop monoclonal B-cell lymphomas similar to those induced by transgenic *C-MYC* expression [78]. EBNA 1 and *MYC*, the murine analogue of the human oncogene *C-MYC*, seemed to cooperate in lymphomagenesis in a transgenic mouse model, suggesting the possibility of a similar action in man [32].

The RNA transcripts, EBERs 1 and 2, appear to produce resistance to apoptosis, conferring a malignant phenotype [79, 80]. They can modulate expression of LMP1 considered to be the EBV oncogene [81], upregulate *BCL-2*, inhibit apoptosis by binding protein kinase, block apoptosis due to Interferon-α signalling, stimulate production of Interleukin (IL)-10, an autocrine growth factor for BL cells, induce colony growth of cells in agar, and are tumorigenic in immunodeficient mice [58, 82, 83]. In addition, a binding site for *C-MYC*, found in the promoter for EBER 1, permits cooperation with *C-MYC* and a role in lymphomagenesis [58]. LMP1, only found in a small minority of BLs, but uniformly present in Naso-Pharyngeal Carcinoma, has transforming ability, is tumorigenic in nude mice [84], inhibits apoptosis in B lymphocytes, and induces expression of the antiapoptotic *BCL2* oncogene [85].

Table 1: Reports of Case clustering in endemic Burkitt's Lymphoma.

Location	Dates	Space only	Space and time	Author
West Nile,	1961–65		+	Williams et al. [97, 98], Pike 1972,
Uganda	1972-73			Siemiatycki et al. [104]
Mengo District and Bwamba County, Uganda	1966–68		+	Morrow et al. [103, 106], 1974
Aliba, Uganda	1962-63		+	Pike et al. [101]
Malawi	1987–90		+	van den Bosch [22, 109]
West Kenya	1999–04	+	Not tested	Rainey et al. [99]
Cameroon	2003–2006	+	Not tested	Wright et al. [100]

2.2. Role of Malaria. In sub-Saharan Africa, 90% of children are infected with EBV by the age of 2 years and have a degree of immune tolerance to it [86] which is exacerbated by the immunomodulatory effects of chronic malaria. Holoendemic malaria undoubtedly contributes to the greatly increased numbers of BL cases, nearly all EBV-positive, seen in the Lymphoma Belt. Malaria produces polyclonal B cell activation [87], a five-fold increase in EBV-positive cells during acute malarial infection [88], inhibition of EBV-specific cytotoxic T cells [89], an increase in EBV-transformed B cells [89], and higher circulating levels of EBV-positive cells in children [51]. The combination of EBV and holoendemic malaria has been credited with amplifying the incidence of BL in African children approximately a hundred-fold. Rates of BL are 0.04–0.08/100,000 in Western Europe, increasing to 1-2/100,000 in countries of intermediate prevalence such as Algeria, and up to 10/100,000 in the African Lymphoma Belt [86]. Similarly, BL EBV-positivity ranges from 10–15% in France, up to 85% in Algeria and over 90% in the Lymphoma Belt of Africa [86].

2.3. Arboviruses and the Epidemiology of Endemic Burkitt's Lymphoma. While it is recognized that EBV and malaria make important contributions to BL endemicity, yet the sporadic form of BL can occur in the absence of both of these infections and, if early EBV infection and Holoendemic malaria are the only prerequisites for eBL, then the tumour should be much commoner than it is within the African Lymphoma Belt, where malarial transmission is intense. The Belt lies between the latitudes 10° north and south of the equator with an extension along the eastern coastal margin of Mozambique. BL is endemic within the Lymphoma Belt wherever mean minimum temperatures exceed 15.5°C and annual rainfall is above 50 mls [90, 91]. The lymphoma appears to be associated with water and is absent from arid areas [92]. The climatically defined Lymphoma Belt coincides with the geographic distribution of holoendemic malaria, vectors of certain arboviruses such as Chikungunya Virus (CHIKV) [93], and EBV-activating plants such as *Euphorbia tirucalli* [54], all of which conform with one of Chapin's zones of flora and fauna [90, 91, 94].

Endemic BL exhibits unusual features such as seasonality [95, 96], shifting foci, or lymphoma "hot-spots" which change location from year to year [97, 98] and both spatial [99, 100] and space-time case clusters [95, 97, 98, 101–104]. The clustering was very striking when it occured; in the Aliba outbreak, four of the five cases from this small village presented within one year [101] and unrelated cases in clusters in Malawi were often very close in space and time, with one unrelated case-pair living in neighbouring huts. Statistically significant clustering at intervals of less than 2.5 kms and less than 60 days was seen in Malawi [105]. Clustering was more pronounced in older children in both Uganda and Malawi [98, 104]. The case clusters are summarized in Table 1.

Although the phenomena of seasonality, shifting foci, and clustering have been observed and are well documented, they are not always found, even when sought [103, 105]. Clustering can best be explained by an environmental cofactor which moves around and is variable from year to year, such as an infectious disease, especially one like measles which can cause epidemics and clusters [101]. Neither malaria, nor infections with other common parasites such as Schistosoma, a Class 1 human carcinogen, can provide a convincing explanation for the phenomena. Heavy Schistosomal infection exerts a considerable effect upon the immune system and could potentially contribute to lymphomagenesis by inducing a skewing of the immune response away from the TH1 cell-mediated immunosurveillance towards a B-lymphocyte dominated TH2 response [106]; indeed Schistosomal lesions adjacent to BL lesions have been noted [107]. Foci of Schistosomal infections could explain spatial clustering, but not space-time clustering or shifting foci of BL cases. However, insect-borne viruses, known as arboviruses, are particularly well suited to explain the occurrence of space-time case clusters. The epidemiology of eBL mimics that of certain arboviruses, including their temperature requirements, age, and geographic distributions more closely than that of malaria [22, 91, 108], as shown in Table 2. An arbovirus, which is endemic, but causes periodic epidemics, could explain the existence of the time-space case clusters during an epidemic, and their absence, in the intervening periods, when it is endemic [22, 56]. Morrow et al. [105] observed that the incidence of the tumour was inversely related to age, suggesting that intense malarial transmission was associated with earlier age of onset. This observation could also apply to arboviral infection as both infections are

Table 2: Arboviruses and Malaria—a comparison.

Characteristic	Arboviruses	Malaria
Epidemiology in Lymphoma Belt	Endemic and occasionally epidemic	Holoendemic + 3 types less intense
Age acquisition immunity	Mimics age distribution of eBL	By age of 5 yrs if holoendemic
Altitude Barrier	5000 ft at Equator, 3000 ft in Zambia—same as BL	Up to 8,000 ft at Equator
Geographic Distribution	Dependent on vectors—usually mosquitoes	Dependent on anopheline mosquitoes
Replication Temperature Requirements	Yellow fever stops <15.5–18°C Same as BL	Malaria stops <20°C (PF > 18°C, PV > 17°C, PM > 16°C)
Effect malaria suppression	None	Reduced
Effect malaria eradication	Eradicated	Eradicated

Table 3: Characteristic arboviral signs and symptoms seen in eBL patients immediately preceding development of lymphoma.

Sign or symptom	Total number (%)	Time before BL in days (range)	CHIK IgG/M+ On admission	CHIK IgG/M+ after 14/7
Rash	9 (8)	8 (2–14)	0	5
Sore eyes	16 (18)	19 (7–28)	1	10
Joint pains	32 (37)	14 (2–28)	9	16
Mouth ulcers	14 (16)	13 (3–21)	2	10
Fever	27 (31)	16 (1–56)	10	19
Bleeding	14 (16)	19 (14–28)	3	6

increased where mosquitoes thrive. Both malaria and most arboviruses are vectored by mosquitoes.

2.4. Space-Time Case Clusters of Endemic Burkitt's Lymphoma. A statistically significant association between infection with the arbovirus, CHIKV and the onset of eBL was observed in Malaŵi, at the time of a CHIKV epidemic, when space-time case clusters were also being observed [12]. BL patients were significantly more likely to be CHIKV seropositive on first admission or to have seroconverted three weeks afterwards than either hospital or local controls ($P = 0.002$ and 0.009, resp.) [12]. A majority of BL patients, irrespective of CHIKV seropositivity, gave a history of signs and symptoms typical of arboviral infection, such as rashes, oral lesions, and bleeding tendencies, occurring shortly before the appearance of BL, as summarized in Table 3 [12, 22, 56]. Rashes and oral lesions preceding BL onset had been seen previously in Uganda and attributed to Herpes or Measles infection [110]. However, most unimmunized children in tropical Africa acquire measles by 1–4 years, whereas BL is not seen before the age of 2-3 years, peaks at 5–8 years, depending on the degree of endemicity and is rarely seen after the age of 18 years [110].

Ugandan BL serological studies showed that antibodies to various arboviruses, which included CHIKV, were significantly more likely to be found in BL patients, and to a lesser extent, their families, than controls, but no one arbovirus predominated [109, 111]. This would be consistent with more than one arbovirus being associated with BL. This possibility is also suggested by the observation that three patients, seronegative for CHIKV, seroconverted for Yellow Fever during the course of their first admission, and other cases, seronegative for both viruses, had high titres of antibody to Sandfly Fever, denoting recent infection [56]. Additionally, some Ugandan space-time case clusters [101] occurred during or following an epidemic of O'nyong-nyong, an arbovirus closely related to CHIKV [112, 113] and others [95, 97, 98, 101–103] also occurred during periods when CHIKV activity was recorded in East and Central Africa, viz. 1958, 1960-61, 1963-65, 1967, 1971, 1973 [114].

3. Arboviruses

Arboviruses occur world-wide, particularly in the tropics and where vector-control is poor. They are an important group of diseases, with considerable economic consequences for the livestock industry [115], and a considerable burden of morbidity and mortality in humans [115, 116] although human disease is too often unrecognized or misdiagnosed [116, 117], except when large-scale epidemics occur as with the frequent outbreaks of Dengue in South-East Asia [118], or the recent CHIKV epidemic in the Indian Ocean [93]. Climatic conditions are important in determining arboviral outbreaks, with rainfall pattern, temperature, and humidity all playing a role [118].

There are many arboviruses, but only a minority are of medical importance. Arboviruses are RNA viruses, dependent on arthropod hosts for their transmission. They are classified on the basis of antigenic relationships, structure and manner of replication, into five main groups shown in Table 4 [119]. There is considerable cross-reactivity among different, but related, arboviruses. Viral reassortment is thought to occur in nature and, possibly, to explain the origin of some of these viruses [120].

Table 4: Classification of arboviruses.

Family	Genus	Disease	Vector
Flaviviridae	Flavivirus Formerly Casal's Gp B	Yellow Fever	Aedes Mosquitoes
		Dengue	Aedes Mosquitoes
		Japanese Encephalitis	Culicine Mosquitoes
		Saint-Louis Encephalitis	Culicine Mosquitoes
		West Nile Fever	Culicine Mosquitoes
		Tick-borne Encephalitis	Ticks
Togaviridae	Alphaviruses Formerly Casal's Gp. A	Chikungunya, O'Nyong-Nyong, Sindbis Ross River Fever, Barmah Forest	Aedes and Culicine Mosquitoes
		Mayaro	Culicine Mosquitoes
		Equine Encephalitis	
Bunyaviridae	Bunyavirus Nairovirus Phlebovirus	Bunyamwera Virus	Aedes Mosquitoes
		Crimea-Congo Haemorrhagic Fever	Ticks
		Rift Valley Fever	Mosquitoes and Ticks
		Sand-fly Fever	Sandflies

3.1. Characteristics of Arboviral Infection. Arboviruses are best known for causing acute febile illnesses and only recently has the magnitude of long-term arthritic, ocular, and central nervous system sequelae, as seen in the recent Indian Ocean CHIKV epidemic [121–123], been fully appreciated [124, 125]. Subclinical infection occurs frequently and persistent infection is extremely common [121, 126, 127]. Disease is most severe in the very young and the elderly. Arboviruses can produce immunosuppression which is dependent on the age of the patient and the degree of leukopenia induced by the virus [128]. In the presence of mosquito saliva, the natural route of infection, CHIKV can skew the immune response towards the TH2 type postulated to be a risk factor for BL [106, 129]. Arboviruses can also produce the phenomenon of immune enhancement whereby pre-existing, nonneutralising, viral antibodies, due to prior infection with a different, but related serotype, enhance viral replication [130, 131], facilitating viral entry into cells and promoting the release of cytokines [132], thus increasing severity of disease. Certain strains and genotypes may be more virulent, or replicate at a higher rate, and thereby exacerbate disease severity [133]. Both the Flavivirus Dengue, and the Alphavirus CHIKV, can cause a severe form of the disease known as "Shock Syndrome" [130, 131]. Dengue serotype-crossreactive CTL clones showing high avidity for antigen produce higher levels of inflammatory cytokines than serotype-specific clones [133]. *In vitro* experiments show that Alphavirus infection inhibits host protein synthesis drastically, whilst virally encoded genes are expressed liberally [134]. Alphaviruses, Flaviviruses, and Reoviruses are particularly well suited to be vectors for heterologous genes. They are being investigated as vectors for miscellaneous treatments and vaccines and show considerable promise. However, caution needs to be exercised in view of their propensity for mutation, reassortment and establishing persistent infections [134–136].

3.2. Oncogenic Potential of Arboviruses. Arboviruses have the potential to be oncogenic since they exhibit persistence *in vivo* and [121] and *in vitro* [125, 137]. Persistence is enhanced, *in vitro*, if arboviruses are cultured in EBV-infected cell lines as EBV opposes the arboviral tendency to apoptosis [138]. Mice brain cells infected with CHIKV showed loss of contact inhibition and morphological alterations suggesting they had been transformed [137]. Viral isolates related to CHIKV and Bunyamwera induced tumours when injected into Swiss albino mice which could be transmitted to other animals [139]. In a series of early experiments inspired by the arboviral cofactor hypothesis, Reoviruses, which are classified as arboviruses [119], were detected in ten BL biopsies. Antibodies to Reovirus type 3 were commoner in BL cases than in controls [140–142], but no clear-cut relationship between high levels of Reovirus childhood infection and BL incidence could be established [143]. Reoviruses were reported as inducing a lymphoma in a rabbit [144, 145] and BL-like lesions in mice [146–148], but it was finally decided the tumours were induced by a Murine Leukaemia virus, the Reoviruses having been commensals [149].

Acute infection with the arbovirus, West Nile virus, can potentiate the actions of the tumour promoter, TPA, 12-o-tetradecanoylphorbol-13-acetate, when applied to the skin of nude mice, producing an increase in the number and size of papillomata [150]. TPA is derived from a Euphorbia, one of the EBV-activating plants considered potential cofactors in eBL lymphomagenesis [22, 151, 152].

4. Hepatitis C

It has already been mentioned that the Flavivirus, HCV, a Class 1 Human Carcinogen [5] is most closely related not only to Hepatitis G, another apparently oncogenic Flavivirus, but also to the Arboviruses, Yellow Fever, and Dengue [153–156]. Hepatitis G accounts for up to 9% of all NHLs in some studies, showing a stronger association with lymphomas than HCV in several studies [157, 158].

HCV, a Hepacivirus [155], belonging to the Flaviviridae family, produces a chronic infection, often relatively silent in

the majority of cases, which persists despite the production of antibody. Important manifestations of the disease are cirrhosis, autoimmune disease, lymphoproliferative conditions such as Mixed Cryoglobulinaemia, and a well-documented association with both low- and high-grade NHLs [154]. HCL accounts for 7.4–37% of NHLs overall [159], the strength of the association varying geographically [160], being particularly high in Italy [161, 162] but absent in some countries, including those of Northern Europe [163], indicating the existence of important environmental cofactors. Populations with high HCV prevalence have a greater propensity to develop HCV-associated NHL [162].

It is of particular interest and relevance to this paper that rare instances of sporadic Burkitt's lymphoma arising in connection with chronic HCV infection have been recorded [164–166]. They included a number of primary hepatic BLs [164], a cardiac lymphoma with variant BL translocation and a gingival BL arising in a renal transplant patient with chronic HCV infection [165, 166]. Under-ascertainment of HCV-associated BL is likely, unless there is a high index of diagnostic suspicion.

4.1. Possible Oncogenic Mechanisms of HCV. HCV replicates by way of an RNA-dependent polymerase which lacks a proof-reading function [154]. High rates of genetic variations during replication result in the production of mutant viruses capable of escaping the immune attack and establishing persistent infection. Chronic antigenic stimulation occurs during a lengthy induction period. HCV induces Toll-Like Receptor 4 and consequent enhanced production of Beta-Interferon and Interleukin-6 [167]. HCV directly stimulates B cell expansion, causing a clonal or polyclonal B cell expansion by producing a variety of cytokines and chemokines [167, 168], which may result in mixed cryoglobulinemia, the development of the antiapoptotic *t(14;18)* translocation in some patients, and, in a few cases, NHL [162, 168, 169].

The virus can greatly enhance mutations of both immunoglobulins and proto-oncogenes by inducing error-prone polymerases and acting on cellular enzymes to enhance production of Nitrous Oxide leading to DNA double-strand breaks, hypermutation of immunoglobulin, proto-oncogene, and tumor suppressor genes, with amplification of the mutated proto-oncogenes [170, 171]. HCV infection inhibits multiple DNA repair processes [172]. Chromosomal abnormalities and polyploidy are frequently found in HCV-infected peripheral blood cells and HCV is thought to inhibit the mitotic checkpoint [173]. The HCV Core and NS3 proteins are responsible for the inhibition of DNA repair, mediated by nitric oxide and reactive oxygen species and both have oncogenic potential, since they can transform certain cell lines [174–176]. The Core Protein can impair cell cycle regulation *in vivo*, affecting the function of human pRb/p105 and other cell growth regulatory proteins, thus uncoupling cell cycle progression from mitotic control and permitting random mutations and rearrangements of the genome [175, 176]. Part of the HCV genome encoding the nonstructural protein NS3 is involved in cell transformation as cells expressing this sequence proliferated rapidly, displayed characteristics associated with malignancy, and were tumorigenic in nude mice [174, 176]. The HCV NS5A protein is also thought to have oncogenic potential, by opposing *TP53* and acting as a *BCL2* homologue [177, 178]. The HCV protein E2 enhanced the expression of antiapoptotic *BCL2* family proteins and increased the expression of costimulatory molecules CD80, CD86, and CD81, both of which mechanisms are likely to contribute to HCV-associated B cell lymphoproliferative disorders [162]. Thus, HCV chronic infection acts in a number of different ways, resulting in B cell activation and a subset of cells which are more likely to express *BCL2* and to be intrinsically resistant to apoptosis [162, 177–180].

4.2. Pathogenesis. The frequency of HCV-associated NHL is much lower than that of HCV infection, suggesting that additional factors are required for lymphomagenesis, which are likely to include cellular interactions with the virus and its products. HCV-associated Cryoglobulinaemia seems to precede the development of both high- and low-grade NHLs [181, 182] and it has been suggested that particular HCV genotypes may be more prone to develop NHLs [169].

HCV directly stimulates B cell expansion, causing a clonal or polyclonal B cell expansion [183]. Serum levels of Rheumatoid Factor were found to be increased in patients with a clonal expansion, suggesting that the expanded B-cell clones belong to the Rheumatoid Factor producing B-cell subset [183, 184] and that, in some cases at least, they can evolve into NHL [185].

Up to half of all HCV carriers have mixed cryoglobulinaemia, composed of HCV antigen and antibody. Cryoglobinaemia, and the severity of disease, appears to be linked to the wide range of antibodies produced in HCV infection, consequent, to some extent upon the frequent genetic mutations that the virus produces in the course of the disease. HCV is also associated with monoclonal gammopathies, particularly when infection is due to Genotype 2a/c [186]. Cryoglobulinaemia is associated with the development of the *t(14;18)* translocation which consists of the rearrangement and activation of *BCL2*, the antiapoptotic B-cell lymphoma/leukaemia gene and its juxtaposition with the Immunoglobulin heavy chain gene IgH on chromosome 14 [182]. Development of the *t(14;18)* translocation, the commonest form of translocation found in lymphomas, is thought to be favoured by chronic antigenic stimulation [182, 187]. This translocation can occur in normal people without malignancy, suggesting that, on its own, it is insufficient to induce a malignant outcome [182]. Chronic antigenic stimulation is considered to be a factor in the clonal evolution of HCV-associated immunocytomas [187]. Both premalignant and malignant lymphoproliferations in an HCV-infected type II Mixed Cryoglobulinemic patient appear to be sequential phases of an antigen-driven pathological process [188]. Effective antiviral treatment leads to the disappearance of the translocation [189, 190] and in some cases, resolution of the lymphoma [162, 191] highlighting the importance of both translocation and virus in the

process of lymphomagenesis and the potential reversibility of the process.

EBV coinfection seems to increase the oncogenicity of HCV, at least as regarding its contribution to the incidence of Hepatocellular carcinoma [192]. HCV replication is enhanced in the presence of EBV [193], due to an interaction with EBNA1, thus increasing the effect of antigen-driven oncogenic processes. EBV could also potentiate the effects of HCV's mutator actions because EBV can rescue error-bearing cells from apoptosis [63]. In addition, EBV-infected B cells tend to accumulate more somatic hypermutations, to have more replacement mutations and to occupy a skewed niche within the memory compartment, due to their exclusion from the CD27(+)IgD(+)IgM(+) subset, which protects them from the immune system, since they cannot be distinguished from uninfected cells [194].

5. Similarities between Hepatitis C and Arboviruses

HCV is part of the Family Flaviviridae to which those Arborviruses which are Flaviviruses belong. The arboviruses most closely related to HCV are the Flaviviruses Yellow Fever and Dengue. CHIKV is an Alphavirus, belonging to the Togaviridae Family. Flaviviruses are closely related to Alphaviruses, being previously classified as an Alphavirus subgroup, and were only allocated their own family when sufficient differences were noted [153]. It is conceivable that CHIKV, already known to be potentially oncogenic [137, 139] and additionally those arboviruses, closely related to HCV such as Yellow Fever, might deploy oncogenic mechanisms similar to those of HCV because of their shared characteristics, and that some, or all, related Flaviviruses and Alphaviruses could share such potential.

It has been shown that Arboviruses, as a group, can exhibit persistence and initiate autoimmune disease [119, 126, 127]. CHIKV, as demonstrated during the recent epidemic [121–123], can persist and give rise to chronic infection. Not only autoimmune disease, but also cryoglobulinaemia, has been found to be common in chronic forms of this infection [195]. CHIKV infection, like Dengue, has the ability to induce Haemorrhagic and Shock Syndrome forms of disease [131, 132] which both unleash a huge release of cytokines [132, 196, 197]. Both are thought to be related to crossreactivity with antibodies produced as a result of previous exposure to closely related serotypes of the virus [131–133, 198–201]. Antibody-dependent immune enhancement can also occur during infection, resulting in high levels of replicating virus. Arboviruses have a rapid replication cycle of four hours and, as with HCV, they generate a high rate of genetic variations during viral replication resulting in the production of mutants capable of escaping attack by the immune system. This process is also likely to generate faulty cells requiring either DNA repair or apoptosis, particularly as arboviral RNA polymerases do not have proof-reading ability. If antibody-dependent immune enhancement occurred, it could produce a rapid increase in viral replication and infected cells, unleashing prodigious amounts of cytokines [132], which could exert effects such as those seen in chronic antigenic stimulation. This could challenge the capacity of cellular DNA repair mechanisms at the very least.

In the Lymphoma Belt setting, chronic EBV infection would provide an expanded pool of B-lymphocytes, thought to be a key factor in lymphomagenesis, because of the enhanced potential for the development of chromosomal abnormalities. This effect would have been amplified still further by the mitogenic effect of holoendemic malaria. CHIKV infection has been shown to be associated with the onset of eBL [12, 22] and an acute arboviral infection could be the reason that the BL cell that is actively rearranging its IgG genes [37]. It is conceivable that acute CHIKV infection in such a setting, particularly if the infection had been preceded by infection with a closely related arbovirus, could initiate a release of cytokines which could have an effect analogous to the antigenic stimulation seen in HCV infection. CHIKV has a very short replication time and also readily produces mutations as its polymerase lacks a proof-reading function. In addition, EBV could cooperate with the arbovirus, by helping error-bearing cells to survive and might also assist the arbovirus to establish persistent infection, as seen *in vitro* [138]. EBV is known to increase the rate of HCV replication [193] but it is unknown whether it exerts a similar effect on arboviruses, though conceivable that this might be the case with those arboviruses closely related to HCV.

Arboviruses readily act as vectors for heterologous genes [134], suggesting the possibility that they could act as vectors within the cell, possibly in conjunction with EBV. Their association with autoimmune disease raises the possibility that they could interact with cellular mechanisms though molecular mimicry, thought to be a factor in autoimmune disease [202].

6. Conclusion

A role in lymphomagenesis has been confirmed for HCV [5] and is probable for Hepatitis G [158], suggesting that closely related flaviviruses, such as Yellow fever, and other related groups of arboviruses, could also have lymphomagenic potential. CHIKV is already known to have oncogenic potential [137, 139]. High levels of CHIKV activity were documented around the time when space-time case clusters of eBL were occurring in Malawi [105], and there was a statistically significant association between recent infection with that virus and the onset of eBL [12, 22]. High levels of CHIKV activity were also recorded in NW Cameroon [203] around the time when extremely high rates of eBL were recorded, up to 20/100,000, and spatial clusters were observed [100]. Although no analysis for space-time clustering was performed in Cameroon, it is likely that this was occurring, particularly in one area where the spatial clustering was very pronounced. In addition, the early space-time case clusters recorded in Uganda occurred at a time when epidemic CHIKV [114] and O'nyong-Nyong Virus activity was observed [200, 202]. O'nyong-nyong, like

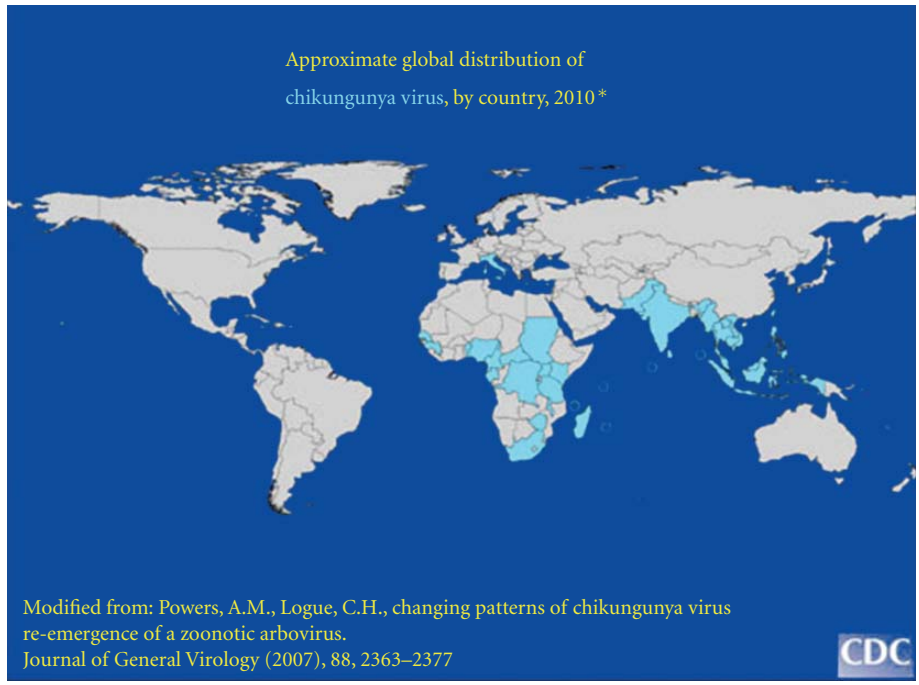

Figure 1

CHIKV, is an Alphavirus and is antigenically extremely closely related to CHIKV [112]. It could appear that not only CHIKV, but possibly both viruses, could be linked with the eBL case clusters seen contemporaneously with their epidemics. It is also possible that CHIKV could have acted as a cofactor for late-stage eBL pathogenesis, in view of the link between recent CHIKV infection and the onset of eBL recorded in Malaŵi [12, 22].

As mentioned previously, CHIKV has the potential to be oncogenic since it can transform mouse brain cells [135] and is tumorigenic in nude mice [138]. CHIKV has also recently been shown to give rise to cryoglobulinaemia [195], a lymphoproliferative state analogous to the Mixed Cryoglobulinaemia seen in chronic HCV infection. HCV-associated Mixed Cryoglobulinaemia is thought to be associated with a 35-fold risk of lymphoma development and to evolve into HCV-associated NHL in 8–10% of cases [162]. In eBL lymphomagenesis, oncogenic arboviruses might interact synergistically with EBV, possibly aided by exposure to tumour-promoting, EBV-activating plant extracts [22, 54]. Further research needs to be done to investigate the association between CHIKV, possibly other arboviruses, and eBL. The requisite research is difficult to carry out in the absence of epidemics, which only occur at lengthy intervals, but is long overdue. Such work is likely to elucidate the mechanisms of lymphomagenesis, not only in eBL, but also in sporadic BL. HCV has been shown to be associated with a few cases of sporadic BL and other RNA viruses, apart from HIV, might also contribute to the small number of sporadic BL cases.

Alphaviruses causing disease similar to CHIKV, are not confined to the Lymphoma Belt, but, as shown in Figure 1, are found in Asia, Australia, the Americas, and Europe. CHIKV vectors are currently extending their range considerably and it would be of interest to see how much an updated map of the geographic distribution of eBL differed from the Lymphoma Belt of Africa as originally defined by Burkitt in the 1950s [204]. A CHIKV outbreak recently occurred in Northern Italy [205], where the vector, *Aedes albopictus*, is now endemic. Local transmission in Italy and new areas invaded by the virus, may offer the dubious advantage of providing arboviral research material where pre-existing expertise and research facilities are readily available. Our knowledge about chronic CHIKV disease has already advanced due to the recent epidemic in the Indian Ocean and India [195], and it is to be hoped that progress will also be made with the assessment of the oncogenic potential of this hitherto underestimated virus.

Global warming and other factors contingent on the emergence of infectious organisms, and viruses in particular, will almost certainly contribute to an increased disease burden [206] in future, due not only to acute infection, but also the more challenging, often initially inapparent, sequelae of chronic infections. It is likely that more infectious agents, particularly viruses, including some yet to be identified, will be implicated in lymphomagenesis and oncogenesis and their study will continue to illuminate oncogenic processes, aided by advances in molecular biology and improved diagnostic methods.

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Review Article

Grey Zone Lymphomas: Lymphomas with Intermediate Features

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The current classification of lymphoid neoplasms is based on clinical information, morphology, immunophenotype, and molecular genetic characteristics. Despite technical and scientific progress, some aggressive B-cell lymphomas with features overlapping between two different types of lymphomas remain difficult to classify. The updated 2008 World Health Organization (WHO) classification of Tumours of the Hematopoietic and Lymphoid Tissues has addressed this problem by creation of two new provisional categories of B-cell lymphomas, unclassifiable; one with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma and the second with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma. We review here the diagnostic criteria of these two provisional entities and discuss new scientific findings in light of the 2008 WHO classification.

1. Introduction

The current classification of lymphoid neoplasms is based on clinical information morphology, immunophenotype, and molecular genetic characteristics. Most lymphomas can be accurately classified. However, some lymphomas present with features transitional between diffuse large B-cell lymphomas (DLBCLs) and classical Hodgkin lymphoma (cHL) or DLBCL and Burkitt lymphoma (BL), and these are difficult to classify [1]. These lymphomas have been reported in the literature using different terms, such as borderline lymphomas, B-cell lymphomas unclassifiable, atypical Burkitt lymphoma, Burkitt-like lymphomas, or gray zone lymphomas. The term "Gray Zone Lymphoma" was firstly used in 1998 at the "Workshop on Hodgkin's disease and related diseases" to designate lymphomas at the border of cHL and other entities [2]. This term was then further extended to lymphomas with overlapping features between BL and DLBCL. The 2008 updated WHO classification of Tumours of the Hematopoietic and Lymphoid Tissues proposed to assign these gray zone lymphomas to provisional categories called B-cell lymphomas unclassifiable with features intermediate between DLBCL and cHL (BCLu-DLBCL/cHL) and B-cell lymphomas unclassifiable with features intermediate between DLBCL and BL (BCLu-DLBCL/BL) [3]. The reason to create these provisional categories is to enable to collect for further studies and to maintain the "purity" of well-defined categories. This would be particularly relevant for conducting clinical studies. This paper focuses on these two provisional entities introduced in the 2008 WHO classification of Tumours of the Hematopoietic and Lymphoid Tissues.

2. B-Cell Lymphoma, Unclassifiable, with Features Intermediate between Diffuse Large B-Cell Lymphoma and Classical Hodgkin Lymphoma (BCLu-DLBCL/cHL)

Primary mediastinal diffuse large B-cell lymphoma (PMBCL) and classical Hodgkin lymphoma of nodular sclerosing subtype (cHL-NS) have clinical, histopathological,

and molecular similarities (Table 1). Both lymphomas present as an anterior mediastinal mass with involvement of the thymus and/or supraclavicular lymph nodes and affect preferentially young women. Median age of presentation is slightly older in PMBCL (35 years) than in cHL-NS (30 years) [4]. The histopathological features of PMBCL include a diffuse proliferation of large cells with clear abundant cytoplasm and fine compartmentalizing sclerosis. Reed-Sternberg-like cells may be present [5, 6], and distinction from cHL-NS can sometimes be difficult. The neoplastic cells in PMBCL express B-cell markers (CD20, CD79a, CD19, PAX5) and lack expression of HLA class I antigens and surface immunoglobulin (Ig). However, expression of Ig-associated transcription factors BOB1, OCT2, and PU1 is preserved in contrast to cHL [7, 8]. CD30 is expressed in 70% of cases and tumour cells are typically CD23 positive. Seventy per cent of PMBCL and 10% of cHL express the MAL protein linking them histogenetically to the thymic asteroid medullary B cells [9, 10]. EBV is absent in PMBCL.

On gene expression profiling studies, the PMBCL gene signature differs from that of germinal centre B-cell-like and activated B-cell-like DLBCL. Highly expressed genes include *MAL*, *Interleukine 4 induced gene 1 (IL4I1)*, *TARC*, *NFkB2,* and *PDL1/L2* [11, 12]. Interestingly, the PMBCL gene signature appeared to be more related to cHL gene signature as both represent downregulation BCR pathway signalling, constitutive NF-kappa B activation, activation of the cytokine-JAK-STAT pathway, high expression of extracellular matrix elements, overexpression of the TNF family members, and aberrant activation of the P13K/AKT pathway [11–17]. Recent studies have highlighted many genetic similarities as well. Both entities show gains at 2p15 (*REL* locus) and 9p24 (*JAK2* locus) and breaks at *CIITA* (38% of PMBCL and 15% of cHL) [18–20]. The presence of *CIITA* rearrangement significantly correlates with shorter disease-specific survival for patients with PMBCL [20]. Altogether, these features point to a similar histogenesis.

In recent years, cases with morphological and immunophenotypic features transitional between PMBCL and the nodular sclerosis subtype of cHL have been reported. These cases, which were initially referred to as "gray zone lymphomas," were assigned in the 2008 WHO classification to a provisional category designated B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and cHL (BCLu-DLBCL/cHL). These lymphomas with intermediate features have been reported mostly from Western countries, and they seem to be less frequent in sub-Saharan Africa and Asia [3]. BCLu-DLBCL/cHL usually presents with mediastinal manifestations, but also include occasional cases involving nonmediastinal lymph node sites. Involvement of lung (by direct extension), liver, spleen, and bone marrow are documented. In contrast to PMBCL, nonlymphoid organs are rarely infiltrated [3]. Interestingly, these lymphomas are probably more frequent in young men and have a more aggressive clinical course and poorer outcome than either cHL or PMBCL [21, 22]. From the morphological point of view BCLu-DLBCL/cHL shows typically sheet-like, confluent growth of pleomorphic tumour cells embedded in a diffusely fibrotic stroma. The majority of tumour cells classically resemble lacunar cells and Hodgkin cells. However, the tumour shows marked variation of morphological aspects ranging from cHL to DLBCL/PMBCL in the same tumour. There is usually a sparse inflammatory infiltrate present with only scattered eosinophils, lymphocytes, and histiocytes. Typically necrotic areas do not include neutrophilic infiltrates. Immunohistochemically, B-cell program is usually preserved in the tumour cells with expression of the transcription factors PAX5, OCT-2, and BOB.1, but this profile is accompanied by expression of typical "cHL markers" like CD15 and CD30. Surface Ig expression is absent. MAL, a typical marker for PMBCL [10], is expressed in at least a proportion of cases [3]. Diagnostic criteria include, for example, cases morphologically resembling PMBCL but with strong expression of CD15, absence of CD20 or presence of EBV [3]. Cases rich in tumour cells resembling cHL, which are strongly positive for CD20 and/or other B-cell markers, are also included in this category [23].

The existence of composite (cHL and PMBCL at the time of diagnosis) or sequential/metachronous lymphomas (cHL following a diagnosis of PMBCL or vice versa) suggests that some lymphomas in the mediastinum show lineage plasticity with a shift over time toward the one or the other entity [21] which may be due to epigenetic and not genetic mechanisms. Based on this consumption Eberle et al. studied the DNA methylation status which is the best established epigenetic marker so far from 10 mediastinal gray zone lymphomas (MGZLs) compared to 10 cHL-NS, 10 PMBCL, and 10 nodal DLBCL cases [24]. MGZL cases had epigenetic profiles intermediate to cHL and PMBCL but clearly distinct from DLBCL. PMBCL and cHL-NS presented with distinct methylation signatures. cHL-NS showed presence of de novo hypermethylation and absence of de novo hypomethylation within CpG islands and in a fraction of promoters outside CpG islands. These results are in line with other studies suggesting that the development of Reed-Sternberg cells may be due to gene silencing by DNA methylation [25, 26]. In contrast, PMBCL showed both de novo hypermethylation and hypomethylation. Interestingly, *HOXA5* hypomethylation was exclusively found in MGZL, and the biological relevance of this finding remains to be explored.

Eberle and colleagues studied the genetic features of 27 MGZL and 6 mediastinal composite or synchronous/metachronous lymphomas by fluorescence in situ hybridization. They demonstrated amplification in 2p16.1 (*REL/BCL11A* locus) and alterations in 9p24.1 (*JAK2/PD2* locus) in 33% and 55% of the patients, respectively. In addition, rearrangement of the *CIITA* locus at 16p13.13 and gains of 8p24 (*MYC*) were both observed in 27% of the cases each [27]. These very recent findings underline the plasticity of mediastinal BCLu-DLBCL/cHL not only on morphologic and immunophenotypic but also on molecular grounds.

Identification of BCLu-DLBCL/cHL will hopefully enable to better characterize them and above all to develop an optimal therapeutic approach. The clinical management of these patients is actually a challenge for clinicians as cHL and PMBCL require different therapies. Due to the rarity of the disease and the complexity of diagnostic criteria, the

Table 1: Common and distinguishing features of PMBCL, cHL-Nodular sclerosis (NS), and B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and cHL (BCLu-DLBCL/cHL). Modified after Hasserjian et al. [23].

	PMBCL	cHL-NS	BCLu-DLBCL/cHL
Common features			
Age	Young patients	Young patients	Young patients
Gender	Female predominance	Female predominance	Male predominance
Localization	Mediastinal mass eventually supraclavicular lymph nodes	Mediastinal mass eventually supraclavicular lymph nodes	Mediastinal mass eventually supraclavicular lymph nodes or more rarely other lymph nodes
Morphology	Compartmentalizing fibrosis	Fibrosis in thick bands	Confluent, sheet like growth of pleomorphic tumor cells with diffuse fibrotic stroma Variability from area to area
Therapy response	Radiotherapy sensitive	Radiotherapy sensitive	
Immunophenotype	Lack of Ig-Expression Lack of HLA I expression Frequent CD30 expression Expression of MAL and CD23	Lack of Ig-expression Lack of HLA I expression CD30 expression	Transitional features between PMBCL and cHL B-cell program often preserved
Genetic and molcular features	Expression of HLA-I REL (2p15) and JAK2 gains (9p24) *CIITA* breaks Activation: NF-kappaB, JAK-STAT (incl. STAT6), and P13K/AKT pathway High expression of extracellular matrix elements, overexpression of TNF family members	Expression of HLA-I REL (2p15) and JAK2 gains (9p24) *CIITA* breaks Activation: NF-kappaB, JAK-STAT (incl. STAT6), and P13K/AKT pathway High expression of extracellular matrix elements, overexpression of TNF family members	REL (2p15) and JAK2 gains (9p24) *CIITA* breaks
Distinguishing features			
Morphology	Clear cells often homogenous (but Reed Sternberg cells may occur) Little or no inflammatory background	Hodgkin cells and Reed Sternberg cells Typical inflammatory background	
Immunophenotype	B-cell markers preserved (CD20, CD79a, PAX5) B-cell transcription factors present (BOB.1 and OCT-2) CD15 absent Absence of EBV	B-cell markers lacking or only weakly or heterogeneously expressed (especially PAX5) B-cell transcription factors usually negative CD15 may be present EBV may be present	

optimal therapy for these patients remains unclear. In a small series of patients with mediastinal BCLu-DLBCL/cHL, Traverse-Glehen et al. suggested that patients might benefit better from therapy designed for aggressive B-cell non-Hodgkin lymphoma than those applied to patients with cHL [21]. However, these results need validation on a larger series of patients.

3. B-Cell Lymphoma, Unclassifiable, with Features Intermediate between Diffuse Large B-Cell Lymphoma and Burkitt Lymphoma (BCLu-DLBCL/BL)

In order to understand the concept of this new category of lymphoma, we will briefly review the diagnostic criteria for Burkitt lymphoma (BL) according to the updated WHO classification of lymphoid neoplasms.

BL includes three epidemiologic variants, the endemic (so-called African type with 100% EBV association), the sporadic, and the immunodeficiency-associated (mostly HIV infected patients). Additionally, cases presenting with a leukemic picture (formerly known as L3 ALL) and without significant lymphadenopathy are also included. The atypical/Burkitt-like and the plasmacytoid variants are no longer mentioned [23].

The diagnostic criteria for BL are quite strict. Morphologically, the tumour cells are medium sized, with monotonous cytology presenting with round nuclei with finely clumped and dispersed chromatin and multiple basophilic paracentrally situated nucleoli. The cytoplasm is deeply basophilic and usually contains lipid vacuoles (which may better be seen in imprints). The growth pattern is diffuse, and the tumour cells often seem to grow in a cohesive way. The proliferation fraction is extremely high (>90%) with many mitotic figures accompanied by

Table 2: Common and distinguishing features of BL, DLBCL, and B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (BCLu-DLBCL/BL).

	BL	DLBCL	BCLu-DLBCL/BL
Common features			
Age	Young children and less frequent young adults	Less frequent in children but frequent in adults of all age groups	Mainly diagnosed in adults
Gender	Male predominance	No real predominance	
Localization	Often extranodal (jaw and iliac region)	Nodal and extranodal	Often extranodal (no predominant region) often widespread disease leukemic presentation is possible
Morphology	Frequent mitotic figures and apoptosis often with starry sky pattern	Frequent mitotic figures and apoptosis may be present	Frequent mitotic figures and apoptosis often with starry sky pattern resembling BL
Immunophenotype	CD10, BCL-6 positive, BCL-2 negative	"BL immunophenotype" (CD10, BCL-6 positive, BCL-2 negative) may be present	Variable depending on morphologic features (see text)
Genetic and molecular features	Typical *IG-MYC* fusion, simple karyotype	Typical *IG-MYC* fusion may be present	Often non-*IG-MYC* fusion complex karyotype
Distinguishing features			
Morphology	Medium-sized blastic cells with basophilic cytoplasm, no inflammatory background, sometimes cohesive growth pattern Small nucleoli at the periphery, mitotic rate always very high (Ki67 > 90%)	Pleomorphic large blastic tumor cells, often inflammatory infiltrate, mitotic rate variable	
Genetical and molecular features	Typical *cMYC* fusion with IG light or heavy chain locus, simple karyotype More complex karyotypes possible (sign of progression)	Other types of *cMYC* fusions can be present (other than IG as a partner), complex karyotype possible	Combination of *BCL2* and/or *BCL6* breaks possible (so-called "double or triple hit lymphomas")

a high fraction of apoptosis and often a starry sky pattern due to the background occurrence of tingible body macrophages [3]. In contrast with previous classification, increased nuclear irregularity, slight nuclear pleomorphism, and/or more prominent, single nucleoli are allowed if the immunophenotype and the molecular characteristics fit with the diagnosis of BL. These lymphomas previously classified as "Burkitt-like" or "atypical Burkitt" lymphoma are now included in the "Burkitt lymphoma" category, and hence the terms "Burkitt-like" or "atypical Burkitt" lymphoma should not be used any longer. This approach is supported by molecular studies, which have revealed that the cases classified morphologically as "atypical" BL have a molecular signature similar to classical BL [23, 28].

The immunohistophenotype required for the diagnosis of BL is strong CD10 and BCL6 positivity, negativity for BCL2, and a Ki67 index of near 100% (at least 90%) [3]. Weak positivity for BCL2 is accepted, but strong expression for BCL2 and a proliferation fraction below 90% are strong contraindications for a diagnosis of BL [23].

On genetics, most cases show rearrangement of *MYC* at 8q24 to the *IG heavy* chain (14q32) or less frequent to the *kappa* (22q11) or *lambda* (2p12) light chain loci. The breakpoints are different in endemic and sporadic BL. Endemic BLs present with breakpoints occurring within the VJ region of the *IGH* locus, while sporadic BL mainly present with breakpoints occurring within IGH switch regions of the *IGH* locus, which may point to the differing maturation status of the two types [29, 30]. Importantly, up to 10% of BL may lack a *MYC* gene translocation by FISH. To date it is not clear if this is due to a failure of detection or if such lymphomas really exist. However, *MYC*-negative BL in adults is characterised by downregulation of microRNA hsa-mir-34b and recurrent duplications of chromosome 11 [28, 31, 32]. However, these *MYC*-negative BLs have to be completely typical on morphological and immunophenotypic grounds to be classified as BL although this opinion is not shared by all authors [1].

However, in view of the strict diagnostic criteria, there remain cases, which are not completely typical, either on morphological and/or immunohistochemical/genetic grounds and therefore are difficult to assign to either BL or DLBCL category (classical common and distinguishing features between these two entities are listed in Table 2). Gene expression profiling (GEP) studies reveal that though the GEP signature of BL and DLBCL are distinct, in a proportion

of B-cell lymphomas the GEP signature is intermediate between BL and DLBCL [28]. Such cases were diagnosed either as BL or DLBCL on morphologic and immunohistochemical grounds by expert hematopathologists, and possibly inappropriate therapy was rendered for some patients. Based on these observations, the recent WHO classification created a provisional entity, B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (BCLu-DLBCL/BL). The reasons for the creation of this new entity are mostly similar to those of BCLu-DLBCL/cHL. Firstly, it intends to collect cases with intermediate features under the same name, and secondly it segregates "clean" BL and DLBCL, which is extremely helpful for clinical trials. On the other hand, it also creates difficulties to clinicians, as the therapeutic strategies differ greatly in adults between BL and DLBCL, and there has been no consensus, on how to treat patients with BCLu-DLBCL/BL.

BCLu-DLBCL/BL are relatively rare and mainly diagnosed in adults [3]. They represent up to 5% of adult aggressive B-cell lymphomas and usually occur in extranodal sites sometimes associated with leukemic involvement [33]. By definition, BCLu-DLBCL/BL harbour intermediate morphological and immunohistochemical features between BL and DLBCL [3]. They may be medium or large cells, usually with a high proliferation fraction and starry sky pattern, with an atypical immunophenotype (lack of CD10 and/or strong BCL2 expression) that precludes the diagnosis of BL. Most of them are of germinal centre subtype with expression of CD10, BCL6 and lack of MUM1 [33].

Cytogenetic characterization of these BCLu has shown that a proportion of them harbours a complex karyotype with two main genetic events—usually c*MYC* alterations together with *BCL2* and/or *BCL6,* less commonly *CCND1* rearrangements, designated the so-called "double hit" lymphomas (DHL). Some patients may have a previous history of low-grade lymphoma such as follicular lymphoma, CLL, or mantle cell lymphoma, and the acquisition of *cMYC* alteration may represent a secondary genetic event [33].

Importantly, lymphomas with a typical DLBCL morphology that have a *MYC* breakpoint are excluded from the category of BCLu-DLBCL/BL. Up to 15% of DLBCL have *MYC* translocations [23, 34], and they are generally associated with an inferior outcome [35, 36].

The clinical evolution of patients with double-hit lymphomas is dramatic with a median survival of 4.5 months, and they are usually resistant to either conventional CHOP-like regimens or to intensive therapy used to treat BL. However, factors associated with a better survival have been identified, which include non-*IGH MYC* partner, BCL2 protein expression, and rituximab inclusive chemotherapy [37].

Altogether, this category of lymphoma appears heterogeneous and remains difficult to diagnose in day-to-day practice based on morphological and immunohistochemical grounds. Interphase FISH with BCL2, BCL6, and *cMYC* DNA probes provides a useful diagnostic tool to identify these DHL. Adult cases in which BL or DLBCL/BL is a diagnostic consideration should be tested for *MYC*, *BCL2,* and *BCL6* rearrangements, and if *MYC* break is associated with *BCL2* and/or *BCL6* rearrangements, the case should be classified as DLBCL/BL irrespective of other features [23].

A very recent paper from the group of Reiner Siebert [38] reviewed the "grey zone" between BL and DLBCL from a genetic perspective. This paper aims to clarify the different definitions of intermediate lymphomas and to propose a subclassification based on genetic aberrations. The "intermediate lymphoma" group from GEP studies and the BCLu-DLBCL/BL from the WHO classification are by far not identical. The intermediate group of GEP is defined of a group of lymphomas not meeting the profiling of either molecular BL or for molecular DLBCL. Therefore, this category represents a wastebasket for all lymphomas, which do not fit into the two molecularly defined entities. On the other hand, the BCLu-DLBCL/BL entity defined by the WHO contains all lymphomas, which does not meet the criteria of either BL or DLBCL on morphologic, immunohistochemical, and classical genetic grounds and represents a heterogeneous group of diseases. However, the following aggressive B-cell lymphomas are excluded from BCLu-DLBCL/BL: cases with typical DLBCL morphology with a very high proliferation index, typical DLBCL with a *MYC* translocation, and typical BL in which a *MYC* rearrangement cannot be demonstrated and those with *IG-MYC* rearrangement as the only abnormality, since they probably correspond to real BL with atypical morphology. Salaverria and Siebert [38] proposed a simple approach based predominantly on age and genetic aberrations to classify these aggressive B-cell lymphomas into biologically meaningful and clinically recognizable subgroups.

In children, the classification into BCLu-DLBCL/BL has currently no influence on therapy or outcome and either intermediate lymphomas according to both GEP and WHO classification are infrequent in patients under the age of 18 years [3, 39] and do not seem to have an adverse prognosis. Almost all childhood "intermediate lymphoma" present with *IG-MYC* fusion, and *BCL2* breaks are almost always absent. Therefore, it seems that in children "intermediate lymphomas" represent rather true biologic BL, which were classified as "intermediate" in the GEP due to secondary aberrations [38]. In contrast some morphological DLBCLs in children show a GEP signature of molecular BL with more than half of them being *IG-MYC* positive, suggesting that the presence of the *MYC* fusion is mostly responsible for its given molecular signature. Since the *MYC* fusion is very likely to be the first event in lymphoma development, complex karyotypes are indicators of disease progression and inferior outcome and do not indicate an *IG-MYC* fusion as a secondary event in children.

In adult patients the situation is rather different and the subclassification of aggressive B-cell lymphomas has a true impact on treatment decisions and prognosis. Salaverria and Siebert [38] suggest that adult aggressive B-cell lymphomas lacking typical BL morphology and phenotype can be classified thereafter into four different genetic subcategories according to their *MYC* status as follows.

(1) *IG-MYC-Positive Mature Aggressive Lymphomas with Simple Karyotype Lacking Typical BL Morphology and/or*

Phenotype. These cases represent a spectrum ranging from cases that would be classified as BL to up to DLBCL based on the WHO classification, since the WHO considers morphology, immunophenotype, and genetic characteristics as being equally relevant [3]. Since the molecular BL signature can also be found in classical DLBCL cases [28], this points to the fact that such cases might be candidates for this molecular BL group.

(2) *IG-MYC-Positive Mature Aggressive B-Cell Lymphomas with Complex Karyotype, Lacking Typical BL Morphology, and/or Phenotype Carrying a High Genetic Complexy*. These cases can correspond to BL with progression or DLBCL with secondary *MYC* break. However, like in the first group it is not easy to set a cutoff between complex and simple karyotype, since there a standard reference method is not defined.

(3) *Non-IG-MYC-Positive Mature Aggressive B-Cell Lymphomas*. *MYC* translocations can involve partners other than the IG heavy or light chain loci. Those translocations are almost exclusively considered as secondary events. These translocations are exceedingly rare in BL but represent up to half of *MYC* translocations in BCLu-DLBCL/BL [3, 28]. Those cases are probably cases with a different primary genetic event (*BCL2*, *BCL6* break, or others) and acquire the *MYC*-break secondarily and may develop very complex karyotypes.

(4) *Double Hit-Positive Mature Aggressive B-Cell Lymphomas*. These lymphomas carry either *IG-MYC* fusion or non-*IG-MYC* fusion in combination with either *BCL2* and/or *BCL6* breaks. By definition these lymphomas would also conform to the previously defined group. But this specific group comprises of >30% of *MYC*-translocation-positive lymphomas in elderly patients and has an aggressive clinical course. However, Salaverria and Siebert [38] pointed out that further studies are needed to clarify: if double hit lymphomas are different from other *MYC*-positive non-BL cases and if the different types of combinations (DLBCL/BL or DLBCL with *BCL2* and/or *BCL6* break and *IG-MYC* or non-*IG-MYC* fusion) influence the outcome and behavior of these lymphomas.

In addition, there are also *MYC*-translocation-negative aggressive B-cell lymphomas with features intermediate between BL and DLBCL. Here the "intermediate" status is defined according to the WHO criteria by histology and immunophenotype. This group probably represents a heterogeneous group of mainly DLBCL, but little is known about this category to date. Salaverria and Siebert [38] suggest that this genetic classification should be tested for reproducibility and clinical impact in future clinical trials.

In conclusion, since the publication of the 2008 WHO classification, several groups have tried to better characterize these two new provisional entities of B-cell lymphomas, unclassifiable with intermediate features. Eberle and colleagues [24, 27] have shed new insights on the epigenetic and cytogenetic characteristics of BCLu-DLBCL/cHL which highlight the plasticity of the tumour cells and the molecular continuum between PMBCL and cHL. Regarding the second category of B-cell lymphoma, BCLu-DLBCL/BL, the situation is more complex. This category appears extremely heterogeneous and remains difficult to diagnose in day-to-day practice based on morphological and immunohistochemical features. The genetic approach proposed by Salaverria is interesting and supports the fact that the detection of chromosomal abnormalities in the diagnostic workup of aggressive B-cell lymphomas is becoming increasingly important.

Further studies are needed to better define the diagnostic criteria of BCLu-DLBCL/BL and allow clinicians to conduct clinical trials to define the optimal therapy which remains unclear to date.

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Review Article

Novel Therapies for Aggressive B-Cell Lymphoma

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Aggressive B-cell lymphoma (BCL) comprises a heterogeneous group of malignancies, including diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma, and mantle cell lymphoma (MCL). DLBCL, with its 3 subtypes, is the most common type of lymphoma. Advances in chemoimmunotherapy have substantially improved disease control. However, depending on the subtype, patients with DLBCL still exhibit substantially different survival rates. In MCL, a mature B-cell lymphoma, the addition of rituximab to conventional chemotherapy regimens has increased response rates, but not survival. Burkitt lymphoma, the most aggressive BCL, is characterized by a high proliferative index and requires more intensive chemotherapy regimens than DLBCL. Hence, there is a need for more effective therapies for all three diseases. Increased understanding of the molecular features of aggressive BCL has led to the development of a range of novel therapies, many of which target the tumor in a tailored manner and are summarized in this paper.

1. Introduction

Many variations of aggressive B-cell lymphoma (BCL) exist, each with distinct molecular, biological, and cytogenetic characteristics [1]. Examples include diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma, and mantle cell lymphoma (MCL). Malignant lymphomas can arise at multiple stages of normal B-cell development, with the germinal center serving as the probable origin of many types of lymphoma [2]. In the germinal-center reaction, mature B cells are activated by antigen, in conjunction with signals from T cells. During this process, B-cell DNA is modified, which results in an altered B-cell receptor. These genetic modifications are prerequisite to a normal immune response but are also the source of genetic defects that result in accumulated molecular alterations during the lymphomagenesis process [3–5].

DLBCL is the most common lymphoid malignancy, accounting for approximately 25 to 30% of all adult lymphomas in the western world [6]. Chemoimmunotherapy with rituximab plus anthracycline-based combination regimens has substantially improved long-term disease control, with more than 50% of patients still in remission 5 years after treatment [7–10]. There are 3 histologically indistinguishable molecular subtypes of DLBCL: the activated B-cell-like (ABC) subtype, the germinal-center B-cell-like (GCB) subtype, and primary mediastinal BCL (PMBL) [11–13]. These subtypes differ in terms of gene expression [13, 14] and are believed to originate in B cells at different stages of differentiation [15]. In addition, the process of malignant transformation differs for each subtype, resulting in distinctive patterns of genetic abnormality [11, 15]. Clinical presentation and responsiveness to targeted therapies also vary across the subtypes.

Gene expression in GCB lymphomas is characteristic for germinal-center B cells [11, 15, 16], with, for example, deletion of the tumor suppressor gene *PTEN* [17], and *p53* mutations [18] being specific to GCB lymphomas. Genetic abnormalities that are characteristic for ABC DLBCL include, for example, deletion of the *INK4α/ARF* tumor suppressor locus on chromosome 9 and amplification of a 9-Mb region on chromosome 19 [19]. Loss of these tumor suppressors impedes the action of chemotherapy and may contribute to the poor prognosis associated with this subtype. PMBL,

although not easily differentiated clinically from other lymphoma subtypes, is readily distinguishable by gene-expression profiling [12, 13] such as deletion of *SOCS1*, a suppressor of JAK signaling [20–22].

Burkitt lymphoma, an aggressive BCL characterized by a high degree of proliferation of the malignant cells and deregulation of the *MYC* gene, relies on morphologic findings, immunophenotyping results, and cytogenetic features for diagnosis [2]. However, DLBCL and Burkitt lymphoma can have overlapping morphologic and immunophenotypic features, and the characteristic t(8;14) translocation found in Burkitt lymphoma also occurs in ≤15% of DLBCL cases [23]. While the regimen of rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone (R-CHOP) is typically used as a first-line treatment for DLBCL, Burkitt lymphoma requires more intensive chemotherapy regimens [24].

MCL, a mature B-cell lymphoma, is almost invariably associated with the t(11;14) translocation with overexpression of cyclin D1 [25]. Several morphologic variants exist, some of which are predictive of a poorer prognosis [26]. Deletions of the *INK4α/ARF* locus on chromosome 9p21 [27] and mutations of *p53* in 17p13, for instance, are also associated with a more aggressive histology [27–29].

Significant progress has been made in the management of patients with aggressive DLBCL. Addition of rituximab to the CHOP regimen (R-CHOP) [30] has resulted in fewer patients with disease progression. However, recent trial results have provided no evidence to indicate that rituximab combined with CHOP given every 14 days (R-CHOP14) improves overall survival (OS) or progression-free survival (PFS) compared with the standard regimen of R-CHOP given every 21 days (R-CHOP21) in newly diagnosed DLBCL [31].

Consequently, a substantial unmet need still exists. Depending on the DLBCL subtype, patients experience significantly different survival rates following chemotherapy, with the ABC subtype in particular being associated with a poorer outcome [11, 19, 32]. Recurrent disease, especially after rituximab exposure, is also a concern, and patients with early relapse after rituximab-containing first-line therapy have been shown to have a poor prognosis [33]. In MCL, the addition of rituximab to conventional chemotherapy regimens has increased overall response rates (ORRs), but not OS compared with chemotherapy alone [34].

As we further our understanding of the molecular characteristics of aggressive BCL, we hope it will lead to the design of therapies that target the tumor and its microenvironment more directly and more effectively.

2. Cytotoxic Therapies

Several new cytotoxic agents are being investigated for the treatment of aggressive lymphomas (Table 1). Bendamustine has shown single-agent and combination activity in indolent lymphomas [35–37]. Although approved for this indication in some countries, evidence supporting its use in treating aggressive lymphomas has been limited. Recently, a feasibility and pharmacokinetic study of bendamustine in combination with rituximab in relapsed or refractory (R/R) aggressive B-cell non-Hodgkin lymphoma (NHL) confirmed that bendamustine 120 mg/m^2 plus rituximab 375 mg/m^2 was feasible and well tolerated and showed promising efficacy [38]. A subsequent phase II study of bendamustine as monotherapy showed a 100% ORR and a 73% complete response (CR) in R/R MCL patients [39]. Preliminary data of another study of bendamustine in combination with rituximab in elderly patients with R/R DLBCL demonstrated an ORR of 52% [40]. A phase III study of this combination showed better efficacy than a fludarabine-rituximab combination in patients with relapsed follicular, other indolent NHLs and MCL [41]. In another phase III study in previously untreated indolent BCL and MCL patients, the bendamustine-rituximab regimen was superior to R-CHOP in terms of CR and PFS [42]. Retrospective analyses of clinical use in Italy [43] and Spain [44] have indicated that treatment with bendamustine alone, or in combination with rituximab, is efficacious and has an acceptable safety profile in heavily pretreated NHL and chronic lymphocytic leukemia (CLL) patients. The most common adverse events associated with bendamustine were hematologic or gastrointestinal in nature and mild to moderate in intensity.

The activity profile of the gemcitabine-oxaliplatin (GEMOX) combination makes it an attractive regimen for use as salvage therapy for several types of lymphoma. Phase II studies have demonstrated significant activity of GEMOX in combination with rituximab (GEMOX-R) in R/R DLBCL [45] and MCL [46]. The major toxicities observed with this regimen were grade 3 or 4 neutropenia and thrombocytopenia. Promising activity with acceptable toxicity has been shown for GEMOX-R in patients with R/R B-cell NHL who are ineligible for high-dose therapy [47] or subsequent transplant [48]. A phase III trial of the novel aza-anthracenedione pixantrone dimaleate [49] was prompted by the absence of reliable durable efficacy in patients with aggressive NHL who have relapsed following multiple lines of therapy. This trial showed superior efficacy compared with a number of alternative third-line single-agent therapies. Neutropenia and leukopenia were the most common grade 3 or 4 adverse events. A second phase III trial, comparing pixantrone-rituximab with gemcitabine-rituximab in patients with R/R DLBCL that are not eligible for stem cell transplantation (SCT), is currently recruiting (NCT01321541). A liposomal formulation of vincristine has also shown activity in patients with aggressive NHL that have relapsed after second-line therapy [50]; grade 3 or 4 neurotoxicity occurred in 32% of patients.

Other novel agents target mitotic spindle proteins; Eg5, for example, has emerged as a unique mitotic spindle target [51]. SB-743921 is a novel kinesin spindle protein inhibitor that has shown significant activity in both *in vivo* and *in vitro* models of aggressive DLBCL. In a phase I/II dose-finding study, activity was observed in heavily pretreated NHL and Hodgkin lymphoma (HL) patients, with neutropenia (47%) reported as the most frequent grade 3 or 4 toxicity [52].

Clofarabine is a second-generation purine analog approved by the United States Food and Drug Administration

Table 1: Cytotoxic therapies in clinical development for the treatment of aggressive NHL. [B: bendamustine; CLL: chronic lymphocytic leukemia; CR: complete response; CRu: unconfirmed CR; DLBCL: diffuse large B-cell lymphoma; EFS: event-free survival; F: fludarabine; HL: Hodgkin lymphoma; MCL: mantle cell lymphoma; MOA: mechanism of action; mOS: median overall survival; mPFS: median progression-free survival; NHL: non-Hodgkin lymphoma; ORR: overall response rate; OS: overall survival; P: pixantrone; PFS: progression-free survival; PR: partial response; R: rituximab; R-CHOP: cyclophosphamide, doxorubicin, vincristine, prednisolone plus rituximab; R/R: relapsed or refractory; SD: stable disease.]

Drug	MOA (target)	Eligibility (and design)	Phase	Randomized	Results
Bendamustine [46]	Alkylating agent	R/R NHL/CLL	Registry	No	ORR: 84.6% in MCL
Bendamustine [45]	Alkylating agent	R/R Lymphoma (±R)	Retrospective analysis	No	MCL: ORR: 67%; 1-year OS: 68%; 1-year PFS: 15% DLBCL: ORR: 31%; 1-year OS: 27%; 1-year PFS: 10%
Bendamustine [42]	Alkylating agent	R/R DLBCL (+R)		No	ORR: 51.6%
Bendamustine [40]	Alkylating agent	R/R aggressive NHL	I	No	B 90 mg/m^2: ORR: 33%; B 120 mg/m^2: ORR: 100%
Bendamustine [41]	Alkylating agent	R/R MCL/NHL	II	No	ORR: 100% in MCL
Bendamustine [44]	Alkylating agent	Previously untreated follicular + indolent + MCL (with R versus R-CHOP)	III	Yes	BR versus R-CHOP: CR: 40.1% versus 30.8% (P = .0323); PFS: 54.8 months versus 34.8 months (P = .0002); OS: no difference
Bendamustine [43]	Alkylating agent	Follicular + indolent + MCL with R versus FR	III	Yes	BR versus FR: CR: 83.5% versus 52.5% (P < .0001); PFS: 30 months versus 11 months (P < .0001); OS: no difference
Pixantrone [49]	Aza-anthracenedione	R/R aggressive NHL (versus other single agents)	III	Yes	P versus comparator: ORR: 37% versus 14%; CR/CRu: 20% versus 6%; PFS: 4.7 versus 2.6 months; mPFS (P = .007); mOS: 8.1 versus 6.9 months (HR = 0.88, P = .554)
SB-743921 [51]	Kinesin spindle protein inhibitor	R/R HL or NHL	I/II dose-finding study	No	4 PRs: 3 in HL, 1 in marginal-zone NHL; 1 durable SD (>17 months) in DLBCL
Gemcitabine/oxaliplatin [45]	Chemotherapy	R/R DLBCL with rituximab	II	No	ORR: 43%; CR: 34%; 12-month PFS rate: 29%; 12-month OS rate: 41%
Gemcitabine/oxaliplatin [46]	Chemotherapy	R/R MCL with rituximab		No	CR/CRu: 77%; 2-year PFS rate: 41%; 2-year OS rate: 58%
Gemcitabine/oxaliplatin [47]	Chemotherapy	R/R B-cell NHL with rituximab	II	No	ORR: 83%; CR/CRu: 50%; 2-year EFS: 42%; 2-year OS: 66%
Gemcitabine/oxaliplatin [48]	Chemotherapy	R/R B-cell NHL (transplant ineligible) with R		No	GemOx: ORR: 57%; CR: 30% R-GemOx: ORR: 78%; CR: 50%
Liposomal vincristine [50]	Chemotherapy	R/R NHL	II	No	ORR: 25%; CR/CRu: 5%

(FDA) for intravenous use in R/R pediatric acute lymphoblastic leukemia (ALL). Purine analogs demonstrate significant clinical activity in NHL, with a phase I preliminary evaluation of an oral formulation of clofarabine in relapsed or refractory NHL reporting an ORR of 35%, with no grade 3 or 4 nonhematologic toxicities [53].

3. Antibodies

3.1. Anti-CD20 Monoclonal Antibodies (mAbs) (Table 2). The chimeric anti-CD20 mAb rituximab improved therapeutic outcomes considerably for patients with B-cell malignancies, particularly when combined with chemotherapy [54].

Table 2: Therapeutic antibodies in clinical development for the treatment of aggressive NHL. [CR: complete response; CRu: unconfirmed CR; DLBCL: diffuse large B-cell lymphoma; EFS: event-free survival; EOTR: extent of tumor resection; FL: follicular lymphoma; HL: Hodgkin lymphoma; mAb: monoclonal antibody; MCL: mantle cell lymphoma; mDR: median duration of response; MOA: mechanism of action; mPFS: median progression-free survival; MZL: marginal zone B-cell lymphoma; NHL: non-Hodgkin lymphoma; ORR: overall response rate; OS: overall survival; PFS: progression-free survival; PR: partial response; R-CHOP: cyclophosphamide, doxorubicin, vincristine, prednisolone plus rituximab; R: rituximab; R/R: relapsed or refractory; RR: response rate.]

Drug	MOA (target)	Eligibility (and design)	Phase	Randomized	Results
Ofatumumab [60]	Anti-CD20 mAb	R/R DLBCL	II	No	ORR: 11%; CR: 4%; mDR: 6.9 months; mPFS: 2.5 months
GA101 [67]	Anti-CD20 mAb	R/R DLBCL and MCL	II	Yes	EOTR: All: 28%; DLBCL: 29%; MCL: 27%
Veltuzumab [56]	Anti-CD20 mAb	R/R NHL	I/II	No	ORR: DLBCL: 43%; MZL: 83%, including CR/CRu: 33% ORR: FL: 44%, including CR/CRu: 27%
Epratuzumab [69]	Anti-CD22 mAb	R/R NHL (with rituximab)	II	No	ORR: 47%; DLBCL: CR: 33%
Epratuzumab [70]	Anti-CD22 mAb	Previously untreated DLBCL (with R-CHOP)	II	No	ORR: 95%; CR/CRu: 73%; 1-year EFS rate: 80%; 1-year PFS rate: 82%; 1-year OS rate: 88%
Milatuzumab [57]	Anti-CD74 mAb	R/R NHL (with veltuzumab)	I/II	Dose-finding	PR: 1/3 in Cohort 1 (8 mg/kg); 2/3 in Cohort 2 (16 mg/kg)
Dacetuzumab [77]	Anti-CD40 mAb	R/R DLBCL (with rituximab and gemcitabine)	Ib	Dose-finding	ORR: 54%
Lucatumumab [74]	Anti-CD40 mAb	R/R HL or NHL	Ia/II	Dose-finding	RR: R refractory: 40%; ORR: DLBCL: 11% (phase Ia); ORR: DLBCL: 15% (phase II)
Blinatumomab (MT103) [83]	Single-chain bispecific anti-CD19 and CD3 mAb construct	R/R NHL	I	Dose-finding	FL: 11/21 responses; MCL: 3/21 responses

However, resistance and reduced response to retreatment led to the development of second-generation humanized (or primarily human) mAbs, which have greater cytotoxicity and stronger direct effects on B cells.

Veltuzumab is a humanized CD20 mAb with complementarity-determining regions differing from rituximab by only 1 amino acid, a characteristic believed to account for the markedly reduced off-rates demonstrated by veltuzumab compared with rituximab [55]. A major response was demonstrated in a phase I/II dose-escalation trial in patients with R/R NHL, with no evidence of immunogenicity [56]. B-cell depletion was observed from first infusion, even at the lowest dose of 80 mg/m^2. Adverse events were transient, mild to moderate, and occurred mostly at first infusion, a notable finding given the short infusion times. A phase I study with veltuzumab in combination with the anti-CD74 antibody milatuzumab in patients with R/R NHL is ongoing [57].

The fully human CD20 mAb, ofatumumab, has been FDA-approved for the treatment of fludarabine- and alemtuzumab-refractory CLL [58] and is currently being evaluated in NHL. Ofatumumab induces B-cell depletion via mechanisms similar to rituximab, but with substantially more complement-dependent cytotoxicity. Recent *in vivo* data suggest ofatumumab may be more potent than rituximab in both rituximab-sensitive and rituximab-resistant models and may potentiate the antitumor activity of chemotherapy agents commonly used in the treatment of B-cell NHL [59]. Initial results from a phase II study in relapsed or progressive DLBCL showed that single-agent ofatumumab is well-tolerated with evidence of efficacy [60]. In this patient population, response to the last systemic treatment appeared to influence response to ofatumumab; a subsequent study of ofatumumab in combination with ifosfamide, carboplatin, etoposide (ICE) or dexamethasone, Ara-C, and cisplatin (DHAP) chemotherapy regimens (NCT00823719) is ongoing.

GA101 is a novel humanized CD20 mAb that binds CD20 in a manner completely different to that of rituximab and ofatumumab [61]. In preclinical studies it has demonstrated superior efficacy compared with both agents [62, 63], and an initial phase I trial with dosing every three weeks demonstrated promising activity with no dose-limiting toxicity (DLT) [64]. A second dose-finding study in patients with R/R NHL ($n = 22$; ORR: 25% at 3 months) [65] has been followed by a phase II study in heavily pretreated patients with R/R DLBCL and MCL. Treatment was well tolerated,

and promising evidence of efficacy was shown [66]. Recent *in vivo* studies have shown enhanced inhibition of tumor growth for GA101 in combination with bendamustine, fludarabine, and the B-cell lymphoma 2 (Bcl-2) family inhibitors ABT-737 and ABT-263 [67].

3.2. Novel Targeted mAbs (Table 2). The humanized mAb, epratuzumab, targets CD22 which is a B-cell marker thought to play a role in B-cell activation, cell-surface receptor circulation, and modulation of antigen-receptor signaling [68]. In a phase II trial in patients with R/R NHL, the combination of epratuzumab and rituximab resulted in considerable ORRs in both follicular lymphoma and DLBCL [69]. In a subsequent phase II study, in which epratuzumab was added to R-CHOP as first-line therapy for DLBCL, an ORR of 95% was reported. Substantial responses were documented even when patients were separated into low- and high-risk international prognostic index (IPI) groups [70]. Positron emission tomography (PET) scan data confirmed a functional CR rate of 87% in this study, with attainment of PET negativity by completion of therapy being associated with a good outcome [71].

Milatuzumab is a humanized anti-CD74 mAb in clinical evaluation for the treatment of multiple myeloma (MM), CLL, and NHL. In preclinical trials, milatuzumab monotherapy has demonstrated therapeutic activity against various B-cell malignancies, while the addition of milatuzumab to numerous agents including rituximab and fludarabine enhanced the therapeutic efficacy in a variety of B-cell malignancy cell lines [72]. As milatuzumab combined with rituximab was shown to cause MCL cell death [73], further evaluation of this combination in MCL is warranted. A dose-escalation study of a milatuzumab-veltuzumab regimen in R/R NHL is ongoing [57].

Lucatumumab (HCD122), a mAb that is a pure antagonist of the CD40 transmembrane receptor, has been evaluated clinically in CLL and MM and is currently under evaluation in a variety of lymphomas, including DLBCL and MCL [74]. Initial efficacy has been shown in an ongoing phase Ia/II trial in patients who had progressed after multiple prior therapies, with DLTs limited to clinically asymptomatic and reversible grade 3 or 4 elevations of amylase and/or lipase and grade 3 or 4 elevations of alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST).

The humanized anti-CD40 mAb, dacetuzumab (SGN-40), has demonstrated antiproliferative and apoptotic activity against a panel of high-grade BCL cell lines [75]. Dacetuzumab was shown to enhance the antitumor activity of rituximab in NHL cell lines and xenograft models, suggesting that antibody-mediated signaling through both CD20 and CD40 may be an effective strategy in the treatment of NHL [76]. Dacetuzumab in combination with rituximab and gemcitabine for the treatment of NHL is currently being evaluated in a phase Ib study [77].

Small modular immunopharmaceuticals (SMIPs) are single-polypeptide chains consisting of a single-chain Fv linked to human IgG hinge, CH2, and CH3 domains [78]. TRU-016, a novel humanized anti-CD37 SMIP protein, has demonstrated single-agent activity as well as synergy with bendamustine, rituximab, rapamycin, and temsirolimus and an additive benefit with doxorubicin [79–81]. TRU-016 is currently being evaluated in a phase I study in relapsed NHL and CLL (NCT00614042).

3.3. Bispecific Antibodies (BsAbs). New mAbs are being tested in combination with rituximab, including BsAbs that target CD20 and CD22 simultaneously [82]. HB22.7 is an anti-CD22 mAb that specifically blocks the interaction of CD22 with its ligand, has direct cytotoxic effects, and initiates CD22-mediated signal transduction. The cell binding, signaling patterns, and lymphomacidal activity of a BsAb (Bs20x22) combining rituximab and HB22.7 have been evaluated using a xenograft model of human NHL. Efficacy was demonstrated by *in vitro* cytotoxicity and apoptosis assays, p38 activation, and xenograft models. Bs20x22 appeared to be more efficacious than the combination of rituximab and HB22.7 and eliminated the need for sequential administration of 2 separate mAbs.

The recent creation of an anti-CD20/human leukocyte antigen (HLA)-DR-interferon- (IFN-) $\alpha 2\beta$ BsAb immunocytokine (designated 20-C2-2β) is expected to have greater *in vivo* potency than IFN-α due to improved pharmacokinetics and targeting specificity and may potentially be useful in a variety of hematopoietic tumors that express either CD20 or HLA-DR [83].

Bispecific T-cell engager molecules (BiTEs) are antibodies that target both an antigen on malignant cells and CD3 on the surface of T cells [84]. In a phase I trial in relapsed NHL, the anti-CD19/CD3 BiTE antibody, blinatumomab, produced multiple responses in 52 patients. Implementation of a double-step dose-escalation procedure avoided treatment discontinuations due to CNS events [85].

Recently, preclinical data have been presented for a number of other agents, including anti-HLA-DR humanized mAb IMMU-114 [86], anti-CD47 antibody [87], anti-CD137 antibody [88], and the anti-CD19 mAb XmAb5574 [89].

3.4. Antibody-Drug Conjugates (ADCs) (Table 3). ADCs are mAbs attached to cytotoxic drugs via chemical linkers [90]. Inotuzumab ozogamicin (CMC544) is composed of the anti-CD22 antibody inotuzumab and calicheamicin, a cytotoxic agent derived from the bacteria *Micromonospora echinospora*, which acts by cleaving DNA [91]. A phase I trial with 48 patients with R/R lymphoma showed ORRs of 69% and 33% for follicular lymphoma and DLBCL, respectively [92]. Inotuzumab ozogamicin was well tolerated; the most frequent adverse event was thrombocytopenia, which occurred at grade 3 or 4 in 57% of patients. In a phase I/II trial where inotuzumab was combined with rituximab in patients with relapsed follicular lymphoma or DLBCL, the response rates and 6-month PFS were 88% and 100% for follicular lymphoma and 71% and 66% for DLBCL, respectively [93]. Recently, preliminary results from a trial of inotuzumab plus rituximab in relapsed DLBCL patients followed by SCT were reported [94]. A best ORR of 21% was observed, with no new safety concerns. The inotuzumab-rituximab combination was also used in a study in Japanese patients with R/R B-cell NHL, resulting in an ORR of 80%; adverse

Table 3: Antibody-drug conjugates and radiolabeled antibodies in clinical development for the treatment of aggressive NHL. [CR: complete response; CRu: unconfirmed CR; DLBCL: diffuse large B-cell lymphoma; HDT-ASCT: high-dose therapy/autologous stem cell transplantation; mAb: monoclonal antibody; MOA: mechanism of action; mPFS: median progression-free survival; NHL: non-Hodgkin lymphoma; ORR: overall response rate; OS: overall survival; PFS: progression-free survival; R: rituximab; R-CHOP: cyclophosphamide, doxorubicin, vincristine, prednisolone plus rituximab; RIT: radioimmunotherapy; R/R: relapsed or refractory.]

Drug	MOA (target)	Eligibility (and design)	Phase	Randomized	Results
I-131 tositumomab [96]	Anti-CD20 radioimmunotherapy	Previously untreated DLBCL (with R-CHOP)		No	1-year PFS rate: 75%; 1-year OS rate: 83%
Inotuzumab ozogamicin (CMC-544) [93]	CD22 targeted cytotoxic immunoconjugate	R/R $CD22^+$ and $CD20^+$ NHL (with R)	I	No	ORR: 80%; 1-year PFS rate: 89%
Inotuzumab ozogamicin (CMC-544) [94]	CD22 targeted cytotoxic immunoconjugate	R/R $CD22^+$ and $CD20^+$ DLBCL prior to HDT-ASCT (with R)		No	ORR: 21%
^{90}Y-epratuzumab tetraxetan [92]	Radiolabeled humanized anti-CD22 mAb	R/R NHL	I/II	Dose-finding	ORR: 62%; CR/CRu: 48%; mPFS: 9.5 months
^{90}Y-epratuzumab tetraxetan [97]	Radiolabeled humanized anti-CD22 mAb	Consolidation after first-line R-CHOP in DLBCL	II	No	Improved remission status 6 weeks after RIT: 30.7%
Brentuximab vedotin (SGN-35) [104]	Antitubulin monomethyl auristatin E (MMAE) anti-CD30 mAb conjugate	R/R lymphoma	I		ORR: 46%; CR: 29%

events leading to discontinuation included neutropenia and hyperbilirubinemia [95]. Further studies of this combination in NHL are ongoing (NCT00299494; NCT01232556).

^{90}Y-epratuzumab-tetraxetan is a radiolabeled, humanized anti-CD22 antibody that has been used for fractionated radioimmunotherapy (RIT) and has shown high rates of durable CRs with manageable hematologic toxicity in previously treated patients with indolent and aggressive NHL [96]. A phase II study, currently underway, is assessing ^{90}Y-epratuzumab-tetraxetan as consolidation therapy after first-line chemotherapy in disseminated DLBCL patients over 60 years of age [97]. 31% of patients in whom a CR, unconfirmed CR, or worse, was reported with R-CHOP improved their remission status 6 weeks after RIT. The common grade 3 or 4 toxicities reported were neutropenia (78%) and thrombocytopenia (74%). A phase I/II study of ^{90}Y-epratuzumab-tetraxetan combined with veltuzumab in patients with R/R aggressive NHL is currently recruiting (NCT01101581). Preclinical data indicate that the efficacy of epratuzumab conjugated with SN-38 (the active component of the topoisomerase I inhibitor, irinotecan) may potentially be enhanced when combined with the CD20 immunotherapeutic, veltuzumab [98].

^{90}Y-ibritumomab tiuxetan (^{90}Y-IT), an anti-CD20 murine antibody linked to a beta-emitting isotope, is approved for use in indolent lymphoma [99]. In a phase II trial, ^{90}Y-IT induction followed by rituximab maintenance in patients with R/R DLBCL had an acceptable toxicity profile and the 2-week outpatient ^{90}Y-IT infusion produced response rates and durations similar to those of more prolonged cytotoxic chemotherapy regimens. Another phase II trial showed 6 cycles of fludarabine and mitoxantrone (FM) followed by ^{90}Y-IT in previously untreated, indolent, nonfollicular NHL to be tolerable and effective, with a CR rate of 50% after FM chemotherapy increasing to 100% at the end of the treatment regimen [100]. The Eastern Cooperative Oncology Group (ECOG) carried out a phase II study of R-CHOP followed by ^{90}Y-IT in previously untreated MCL. This trial showed that failure-free survival appeared prolonged over that expected with R-CHOP alone and the regimen was considered to be safe, with neutropenia and thrombocytopenia being the most frequent adverse events [101].

Consolidative RIT with iodine-131 tositumomab was administered in a phase II trial in 86 patients with previously untreated DLBCL [102]. In this trial, 5 patients died of toxicities possibly related to therapy, including 1 case of febrile neutropenia, 1 case of acute myeloid leukemia (AML), and one case of renal failure; 2 deaths were caused by cardiac ischemia, 1 of which occurred after a gastrointestinal bleed in a patient that became thrombocytopenic after iodine-131 tositumomab. The 1-year PFS and OS estimates were 75% and 83%, respectively; given that the estimated historical 1-year PFS rate with R-CHOP alone in this population is 74%, a consolidation strategy utilizing iodine-131 tositumomab after 8 cycles of CHOP (6 with rituximab) for DLBCL does not appear to be promising in regard to 1-year PFS or

OS. The authors concluded that in this population of DLBCL, early progressions, deaths, and declining performance status during CHOP limit the number of patients who can ultimately benefit from a planned consolidation approach. The use of novel agents earlier in therapy may have a greater impact in DLBCL than consolidation or maintenance approaches [102]. A phase II study of iodine-131 tositumomab for 1st- or 2nd-relapse indolent BCLs, or BCLs that have transformed to a more aggressive histology, has been completed recently (NCT00950755).

The binding properties, internalization kinetics, and clinicopathological activity of the ADC, brentuximab vedotin (SGN-35), were described recently [103]. In a phase 1, weekly dosing study, brentuximab induced multiple objective responses in patients with R/R CD30-positive lymphomas [104]. DLTs included diarrhea, vomiting, and hyperglycemia.

A novel ribonuclease-based immunotoxin comprising quadruple ranpirnase (Rap) site specifically conjugated to an anti-CD22 IgG has shown potent antilymphoma activity in *in vivo* and *in vitro* assays [105].

4. Additional Novel Strategies

Adoptive transfer of autologous T-cells expressing anti-CD19 chimeric antigen receptors (CARs) is a potential new approach for treating B-cell malignancies [106]. A phase I clinical trial of B-cell malignancies treated with autologous anti-CD19-CAR-transduced T cells is ongoing, with data published on five patients, having received two doses of cyclophosphamide 60 mg/kg and five doses of fludarabine 25 mg/m^2 followed by infusions of anti-CD19-CAR-transduced T cells and administration of high-dose interleukin-2 (IL-2). Initial results appear promising.

Therapeutic vaccination holds enormous potential as a complementary treatment for NHL, and IL-2 has a wide range of immunologic effects and is able to induce regression of metastatic human tumors [107]. In a preclinical study, a therapeutic vaccine using tumor cells activated by *Salmonella* infection and IL-2 has been shown to induce antitumor immunity in BCL. This approach may have therapeutic value in promoting systemic immunity against human NHL.

To circumvent cytotoxic T-lymphocyte (CTL) tolerance of tumor-associated antigens, noncognate cytotoxic T cells have been retargeted against CD20$^+$ tumor cells using [Fab' x MHC class I/peptide] conjugates. The ability of [Fab' x MHC class I/peptide] constructs to cause proliferation of OT-1 cells (a transgenic T-cell receptor model in which the CD8+ T-cells express a T-cell receptor specific for the SIINFEKL peptide of ovalbumin) *in vitro* suggests that it may be possible to use a single molecule to generate a secondary cytotoxic T-cell response and, subsequently, to retarget it, thus increasing the feasibility of the approach if adopted in the clinical setting [108].

5. Other Targeted Therapies

5.1. Immunomodulating Agents (Table 4). Thalidomide and its newer derivative, lenalidomide, have multifaceted antitumor effects that include immunomodulatory effects via natural-killer-cell recruitment and cytokine modulation, antiangiogenesis, and the ability to alter tumor and stromal-cell interactions [109]. An early study of thalidomide plus rituximab found responses in 13/16 patients with relapsed MCL, although follow-up was limited [110]. More recently, data from 58 patients in a French compassionate-use study provided good response data with limited toxicity [111]. Lenalidomide monotherapy was evaluated in a phase II study of 49 patients with R/R aggressive NHL, including 15 with MCL [112, 113], and demonstrated an ORR of 35% with a median duration of response (DR) of 6.2 months. Cytopenias, fatigue, constipation or diarrhea, rash, and fever were common adverse events. A larger, international, confirmatory phase II study in patients with R/R DLBCL or MCL showed an ORR of 35%. Adverse events included grade 3 or 4 neutropenia (41%) and thrombocytopenia (19%) [114]. Pooled data of patients who had received prior SCT from these 2 studies suggest lenalidomide to be efficacious, with an ORR of 39%, and well tolerated [115].

Preclinical evidence for synergistic activity of the lenalidomide-rituximab combination in MCL [116, 117] is supported by results of a phase I/II study, which has shown a 53% ORR in patients with R/R MCL. Grade 3 or 4 toxicities included neutropenia (37 events in 45 patients) [118]. The evolving role of lenalidomide in relapsed MCL is further strengthened by data from a phase II trial of lenalidomide in combination with dexamethasone (ORR: 55%; grade 3 or 4 neutropenia in 48% of patients) [119], and with rituximab and dexamethasone (ORR: 57%) [120]. Lenalidomide is also being evaluated in combination with R-CHOP (R2-CHOP) in a phase I/II trial in patients with aggressive BCLs [121]. A second phase I study is ongoing [122]. Interim analysis of a phase I/II trial of lenalidomide plus R-CHOP21 showed multiple CRs and moderate hematologic toxicity (grade 3 or 4 neutropenia: 28%) [123]. Recruitment is ongoing for a phase I/II study of lenalidomide, rituximab, and bendamustine in aggressive BCL (NCT00987493).

5.2. Proteosome Inhibitors (Table 5). Bortezomib, a reversible inhibitor of the chymotrypsin-like activity of the 26S proteasome, disrupts normal homeostatic mechanisms in cells [124]. This agent is used widely to treat MM and is now also approved for use in MCL. Its activity in combination with other agents has been investigated in several recent studies. R-CHOP plus bortezomib produced an ORR of 91% in previously untreated MCL patients, with neutropenia (23%) and thrombocytopenia (14%) among the grade 3 or 4 cytopenias that were reported [125]. A phase II study of bortezomib in combination with bendamustine and rituximab in patients with R/R indolent and MCL produced an ORR of 84%, although the triple regimen appeared to be more toxic than the bendamustine-rituximab regimen alone [126]. Interim data from a phase II study suggested promising results for a regimen of bortezomib plus dose-dense CHOP every 2 weeks as first-line treatment in disseminated DLBCL [127]. A recent study by Dunleavy and colleagues [128] showed that although bortezomib alone had no activity in DLBCL, when combined with chemotherapy it demonstrated a significantly higher response in ABC

Table 4: Immunomodulatory agents in clinical development for the treatment of aggressive NHL. [B: bortezomib; CR: complete response; CRR: complete response rate; CRu: unconfirmed CR; Dex: dexamethasone; MCL: mantle cell lymphoma; mDR: median duration of response; mEFS: median event-free survival; MOA: mechanism of action; mPFS: median progression-free survival; NHL: non-Hodgkin lymphoma; ORR: overall response rate; OS: overall survival; PR: partial response; R: rituximab; R-CHOP21: cyclophosphamide, doxorubicin, vincristine, prednisolone plus rituximab, every 21 days; R/R: relapsed or refractory; TTF: time to treatment failure.]

Drug	MOA (target)	Eligibility	Phase	Randomized	Results
Thalidomide [111]	Immunomodulator	R/R MCL (alone, with R, with B, with RB, with other agents)	NA	No	ORR: 50%; CR: 20.7; 2-year TTF rate: 10.9%; 2-year OS rate: 49.6%
Lenalidomide [113]	Immunomodulator	R/R aggressive NHL	II	No	ORR: 35%; CR/CRu: 12%; mDR: 6.2 months; mPFS: 4.0 months
Lenalidomide [114]	Immunomodulator	R/R aggressive NHL	II	No	ORR: 35%; CR/CRu: 13%; mPFS: 3.5 months
Lenalidomide [116]	Immunomodulator	R/R MCL	II	No	ORR: 53%; CR: 20%; mPFS: 5.6 months
Lenalidomide [117]	Immunomodulator	R/R MCL (with Dex)	II	No	ORR: 55%; CR: 24%
Lenalidomide [118]	Immunomodulator	R/R MCL (with R)	I/II	Dose-finding	ORR: 53%; CRR: 31%; mPFS: 14.0 months
Lenalidomide [120]	Immunomodulator	R/R Indolent or MCL (with R/Dex)	II	No	ORR: 57%; mEFS: 12.0 months; 78%
Lenalidomide [121]	Immunomodulator	Previously untreated DLBCL (with R-CHOP21)	I/II	Dose-finding	CR: 15/21; PR: 1/21

compared with GCB DLBCL. These results indicate that bortezomib specifically benefits non-GCB DLBCL patients, who normally exhibit inferior outcomes relative to GCB subtype patients after therapy with CHOP or R-CHOP. An ongoing phase II study of R-CHOP with or without bortezomib is prospectively enrolling only those patients with the non-GCB subtype DLBCL [129].

The combination of bortezomib and rituximab in a weekly schedule has been shown to be effective with little hematologic toxicity in a phase II study in R/R indolent BCL and MCL [130]. In another phase II study, a combination of bortezomib plus rituximab, doxorubicin, dexamethasone, and chlorambucil (RiPAD+C) was shown to be feasible and well tolerated as a first-line therapy in elderly MCL patients [131]. Bortezomib was used in place of vincristine in the standard rituximab, cyclophosphamide, vincristine, and prednisone (R-CVP) regimen in a phase I trial in R/R indolent DLBCL and MCL [132]. The R-CBorP regimen appeared to be well tolerated and the efficacy data looked promising. Several other phase I studies are further exploring potential uses of bortezomib, with positive data reported for its use in combination with conatumumab [133], gemcitabine [134], and ^{90}Y-IT [135].

Numerous trials that are ongoing or recruiting, are investigating the combination of bortezomib with rituximab-ICE (RICE) (NCT01226849; NCT00515138), tositumomab (NCT00398762), and vorinostat (NCT00837174). Preclinical data support further combination regimens, including romidepsin [136], autophagy inhibitors [137], the murine double minute (MDM2) inhibitor, nutlin-3 [138], and the BH3 mimetic, obatoclax [139].

NPI-0052 is a proteasome inhibitor with a novel bicyclic structure [140]. In a phase I study, NPI-0052 produced dose-dependent pharmacologic effects, with less peripheral neuropathy, neutropenia, and thrombocytopenia than was typically noted with other proteasome inhibitors. MLN9708 has shown activity in preclinical models of lymphoma [141, 142]. Further, the novel proteasome inhibitor carfilzomib has been shown to interact synergistically with histone deacetylase inhibitors (HDACIs) [143].

5.3. Phosphatidylinositol 3-Kinase (PI3K) Pathway (Table 5). The PI3K-signaling pathway plays a major role in regulating cell growth and survival and is often deregulated as a result of the mutation or amplification of Akt [144, 145]. The mammalian target of rapamycin (mTOR) kinase is an essential mediator of growth signaling that originates from PI3K. mTOR activation by Akt leads to cell proliferation and survival by modulating critical molecules such as cyclin D1.

The rapamycin analogs, everolimus (RAD001) and temsirolimus, are approved by the FDA for renal cell carcinoma and have demonstrated activity against lymphoma cells both *in vitro* and *in vivo* [146, 147]. Everolimus was evaluated in a single-agent phase II study in patients with relapsed aggressive NHL in whom standard therapy failed [148]. Significant responses were noted; grade 3 or 4 events included anemia (14%), neutropenia (18%), and thrombocytopenia (38%). In another single-agent phase II study, everolimus showed moderate activity in patients with R/R MCL; grade 3 or 4 anemia and thrombocytopenia were reported in 11% of patients [149]. A phase II study of the combination of everolimus and rituximab in R/R DLBCL has just been

Table 5: Targeted therapies in clinical development for the treatment of aggressive NHL. [ABC: activated B-cell-like DLBCL; CR: complete response; CRu: unconfirmed CR; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; GCB: germinal-center B-cell-like DLBCL; HDACI: histone deacetylase inhibitor; HL: Hodgkin lymphoma; Hsp: heat shock protein; mAB: monoclonal antibody; MCL: mantle cell lymphoma; mDR: median duration of response; MOA: mechanism of action; mOS: median overall survival; mPFS: median progression-free survival; mTOR: mammalian target of rapamycin; MZL: marginal zone B-cell lymphoma; NHL: non-Hodgkin lymphoma; ORR: overall response rate; OS: overall survival; PI3K: phosphatidylinositol 3-kinase; PFS: progression-free survival; PR: partial response; R: rituximab; R-CHOP: cyclophosphamide, doxorubicin, vincristine, prednisolone plus rituximab; R-ICE: rituximab, ifosfamide, carboplatin, and etoposide; RiPAD+C: bortezomib plus rituximab, doxorubicin, dexamethasone, chlorambucil; R/R: relapsed or refractory; TLR: Toll-like receptor; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.]

Drug	MOA (target)	Eligibility	Phase	Randomized	Results
Bortezomib [127]	Proteasome inhibitor	Previously untreated DLBCL (with R-CHOP)	I/II	Dose-finding	CR/CRu: 92%
Bortezomib [128]	Proteasome inhibitor	R/R DLBCL (with chemotherapy)	II	No	ABC versus GCB: ORR: 83% versus 13%; mOS: 10.8 versus 3.4 months
Bortezomib [130]	Proteasome inhibitor	R/R indolent nonfollicular + MCL (+R)	II	No	ORR: 53%; CR: 26.5%; PR: 26.5% 2-year OS: 80%; 2-year PFS: 25%
Bortezomib [126]	Proteasome inhibitor	R/R indolent + MCL with R/bendamustine	II	No	ORR: 84%; CR/CRu: 52%
Bortezomib [131]	Proteasome inhibitor	Previously untreated MCL (RiPAD + C)	II	No	ORR: 80%; CR: 51% after 4 cycles
NPI-0052 [141]	Proteasome inhibitor	Multiple tumor types	I/II	Dose-finding	Clinical benefit observed in multiple tumor types including MCL, HL, cutaneous MZL, and FL
Everolimus [150]	mTOR inhibitor	R/R MCL	II	No	ORR: 12%
Everolimus [151]	mTOR inhibitor	R/R NHL	II	No	ORR: 30%; mDR: 5.7 months
Everolimus [152]	mTOR inhibitor	R/R NHL	I	2 dose cohorts	2 responses in DLBCL and 2 responses in FL, in 13 patients
Everolimus [149]	mTOR inhibitor	R/R MCL	II	No	ORR: 20%; mDR: 5.45 months
Temsirolimus [153]	mTOR inhibitor	R/R MCL (2 doses, compared with investigators' choice therapy)	III	Yes	ORR: 22% (temsirolimus 175/75 mg) versus 2% (investigators' choice); mPFS: 4.8 months (temsirolimus 175/75 mg) versus 3.4 months (temsirolimus 175/25 mg) versus 1.9 months (investigators' choice); OS: 12.8 months (temsirolimus 175/75 mg) versus 9.7 months (investigator's choice)
Temsirolimus [154]	mTOR inhibitor	R/R MCL (with rituximab)	II	No	ORR: 59%; CR: 19%; PR: 40%
Vorinostat [167]	Deacetylase inhibitor	R/R lymphoma (with R-ICE)	I	No	19/27 responses
Vorinostat [168]	Deacetylase inhibitor	R/R lymphoma (with DOXIL)	I	Dose-finding	4/14 disease control
Oblimersen sodium [183]	Bcl-2 antisense oligonucleotide	R/R B-cell NHL (with R)	II	No	ORR: 42%; ORR in FL: 60%
PF-3512676 [209]	TLR9-antagonist	R/R NHL (with R)	I	Dose-finding	ORR: 24%; ORR in extended treatment cohort: 50%
17-AAG [216]	HSP90 inhibitor	R/R MCL or HL	II	No	ORR: 11% (all PR)
Bevacizumab [221]	Anti-VEGF mAb	Previously untreated DLBCL (with R-CHOP)	II	No	1-year PFS rate: 77%; 2-year PFS rate: 69%; 1-year OS rate: 86%; 2-year OS rate: 79%
Aflibercept [220]	VEGF fusion protein	Previously untreated B-cell lymphoma (with R-CHOP)	I	Dose-finding	ORR: 100%; CR: 80%
CAL-101 [157]	PI3K inhibitor	R/R NHL	I	No	RR: relapsed MCL: 73%; RR: refractory MCL: 40%
Valproic acid [171]	HDACI	R/R NHL	II	No	ORR: 29% (all PR)

completed (NCT00869999). Preliminary results from a phase II study in MCL patients refractory to bortezomib reported promising single-agent activity and good tolerability [150]. A Japanese phase I study in patients with R/R NHL has also shown preliminary evidence of activity of everolimus in NHL [151]. Phase I/II studies exploring the novel combinations of everolimus and panobinostat (LBH589) [152] or bortezomib (NCT00671112) are ongoing.

A phase III study of R/R MCL comparing temsirolimus with physician's choice demonstrated an ORR of 22% and 2%, respectively [153]. A phase II study of temsirolimus plus rituximab produced a 59% ORR; the most common grade 3 or 4 adverse event in rituximab-sensitive and -refractory patients was thrombocytopenia (17% and 38%, resp.) [154]. Temsirolimus also shows some activity in DLBCL with an ORR of 28%, a CR of 12%, and a median PFS of 2.6 months [155].

The PI3K p110δ isoform is preferentially expressed in cells of hematologic origin and in a variety of malignant cells [156]. CAL-101 is a potent p110δ inhibitor and has shown acceptable safety and promising pharmacodynamic and clinical activity in a variety of hematologic malignancies, as a single agent [157–159] and in combination with rituximab or bendamustine [160].

SF1126 is a dual PI3K/mTOR inhibitor and is currently in phase I development in B-cell malignancies [161]. Other novel approaches under investigation in preclinical trials include combining mTOR inhibitors with rapamycin-resistant T cells [162], targeting the PI3K/Akt/survivin pathway with the protease inhibitor, ritonavir [163], dual mTORC1/mTORC2 inhibition [164], and use of immunosuppressive agents (e.g., fingolimod; FTY720) to downregulate cyclin D1 and pAkt [165].

5.4. DACs/HDACIs (Table 5). Several groups of HDACIs have been developed, and they all show activity in lymphoma, mostly cutaneous [166]. HDACIs have been shown to promote apoptosis and to reduce angiogenesis. Vorinostat, registered for R/R cutaneous T-cell lymphoma (CTCL), works synergistically with other drugs, but its role in the treatment of DLBCL is not clear yet. A number of phase I studies of vorinostat-combination regimens in relapsed lymphoma are either ongoing or have been completed recently. These studies have incorporated R-ICE/ICE [167], pegylated liposomal doxorubicin [168], and conatumumab [133]. Preclinical evidence supporting the clinical development of vorinostat plus the novel Aurora kinase inhibitor, MK-5108, has also been presented [169]. A recent safety and tolerability analysis of prior phase I and II trials of vorinostat-based therapy in CTCL, other hematologic malignancies, and solid tumors, highlighted fatigue (62%) and nausea (56%) as the most common drug-associated adverse events, with fatigue (12%) and thrombocytopenia (11%) the most common grade 3 or 4 adverse events [170].

Valproic acid functions as a HDACI, although data on its activity are limited [171]. A recent phase II trial in refractory lymphoma produced 4/14 responses (all partial responses (PRs)). An earlier phase I study with decitabine showed dose-limiting myelosuppression and infectious complications which precluded dose escalation to a minimum effective dose [172].

Panobinostat is an oral pan-DACI that has shown activity in a variety of cancers. Responses have been documented in a phase II study in relapsed HL [173] and in combination with everolimus in a phase I/II study in R/R HL and NHL [152]. It is also being investigated in DLBCL, where preclinical activity has been observed in combination with decitabine [174].

The HDACI, belinostat, has broad preclinical activity [175]. Interim results from a phase I study in patients with lymphoid malignancies provided evidence of tumor shrinkage, and a phase II, Southwest Oncology Group (SWOG) study in patients with R/R aggressive B-cell NHL is ongoing (NCT00303953).

PCI-24781 is a broad-spectrum HDACI, which has shown activity in lymphoma cell lines and models [176]. It has also demonstrated safety and initial clinical benefit in a phase I study in R/R lymphoma.

Entinostat (SNDX-275) is an oral, class I isoform-selective HDACI [177]. A number of responses have been observed in an ongoing phase II study in R/R NHL, and synergistic preclinical activity has been reported in combination with bortezomib [178].

Preclinical activity has also been observed with panobinostat [179, 180] and the oral heat-shock-protein- (Hsp-) 90 inhibitor, SNX-2112 (in combination with bortezomib and rituximab) [181].

5.5. Cell Death (Bcl Family) (Table 5). The intrinsic cell-death pathway is triggered at the mitochondria by a range of signals, with the most important regulators residing in the Bcl-2 family [182]. The Bcl-2 antisense nucleotide, oblimersen, was evaluated in a phase II study in combination with rituximab in patients with recurrent B-cell NHL. An ORR of 42% was found and most toxicity was low in grade and was reversible [183].

ABT-263 (navitoclax) is currently being investigated in clinical trials of lymphoma, as monotherapy [184] and in combination with rituximab [185]. The experimental Bcl-2 inhibitor, ABT-737, is in preclinical development for MCL [186] and DLBCL [67]. Other agents in preclinical development include obatoclax (in combination with bortezomib) [140, 187] and YM155 [188, 189].

5.6. Kinase Inhibitors (Table 6). Aurora kinases A and B (AAK and ABK) are oncogenic serine/threonine (S/T) kinases that play central roles in the mitotic phase of the eukaryotic cell cycle [190]. Overexpression of Aurora kinases during the cell cycle can override mitotic and spindle checkpoints leading to aneuploidy in many human cancers. Gene expression profiling in aggressive B- and T-cell NHL has shown the Aurora kinases to be overexpressed suggesting that they may be key component genes of the "proliferative" signature.

MLN8237 is a selective AAK inhibitor, which showed synergy with docetaxel in preclinical models of MCL [191]. In a phase I study in patients with advanced hematologic malignancies, durable responses were observed, with neutropenia (46%) and thrombocytopenia (36%) being the

Table 6: Kinase inhibitors currently in clinical development for the treatment of aggressive NHL. [CDK: cyclin-dependant kinase; CLL: chronic lymphocytic leukemia; CR: complete response; DLBCL: diffuse large B-cell lymphoma; ERK: extracellular signal regulated kinase; FFP: freedom from progression; FL: follicular lymphoma; HL: Hodgkin lymphoma; JAK: Janus kinase; MCL: mantle cell lymphoma; mPFS: median progression-free survival; NHL: non-Hodgkin lymphoma; MEK: mitogen-activated protein kinase kinase; MM: multiple myeloma; mTOR: mammalian target of rapamycin; ORR: overall response rate; OS: overall survival; PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth factor receptor; PFS: progression-free survival; PR: partial response; R/R: relapsed or refractory; RTK: receptor tyrosine kinase; SD: stable disease; SLL: small lymphocytic lymphoma; TKI: tyrosine kinase inhibitor; VEGFR: vascular endothelial growth factor receptor.]

Drug	MOA (target)	Eligibility	Phase	Randomized	Results
Dinaciclib [183]	CDK1, 2, 5, 9 inhibitor	R/R in low-grade lymphoma and DLBCL	I	No	PR: DLBCL: 1/7; SD: FL: 2/7; MCL: 1/1
Fostamatinib [198]	Syk inhibitor	R/R B-cell NHL and CLL	I/II	No	ORR: DLBCL: 22%; FL: 10% SLL/CLL: 55% in SLL/CLL: MCL: 11%; overall mPFS: 4.2 months
Dasatinib [202]	RTK inhibitor of BCR-ABL, SRC, c-Kit, PDGF and ephrin receptor kinases	R/R NHL	I/II	No	ORR: 32%; 2-year PFS: 13%; 2-year OS: 50%
Enzastaurin [201]	Protein kinase beta inhibitor	R/R DLBCL	II	No	FFP: 22%
PCI-32765 [204]	Bruton's tyrosine kinase inhibitor	R/R B-cell NHL	I	Dose-finding	ORR: 43%
SB1518 [208]	JAK2 inhibitor	R/R lymphoma	I	Dose-finding	PR: 3/26 (2 in MCL)
Sorafenib [221]	TKI inhibitor of RAF/MEK/ERK/c-kit/Flt3, VEGFRs, PDGFRs, RET	R/R NHL	II	No	ORR: 10%; CR: 5%
Sorafenib [222]	TKI inhibitor of RAF/MEK/ERK/c-kit/Flt3, VEGFRs, PDGFRs, RET	R/R lymphoma (with Akt inhibitor perifosine)	II	No	ORR: 23% (all PR; all in HL)
Sorafenib [223]	TKI inhibitor of RAF/MEK/ERK/c-kit/Flt3, VEGFRs, PDGFRs, RET	R/R MM or lymphoma (with mTOR inhibitor everolimus)	I/II	Dose-finding	ORR: 33%

most common treatment-related adverse events [192]. A subsequent phase II study in patients with aggressive NHL is ongoing (NCT00807495).

The selective ABK inhibitor, AZD1152, potently inhibited a range of tumor xenografts in immunodeficient mice [193] and is currently in phase I/II development for DLBCL (NCT01354392). Aurora kinases in preclinical development include the novel pan-Aurora/JAK-2 kinase inhibitor AT9283 [194].

A number of cyclin modulators are currently in development, including the cyclin-dependant kinase (CDK) inhibitors flavopiridol, which is in a phase I/II study in relapsed MCL/DLBCL (NCT00445341), and dinaciclib (SCH 727965), which has shown clinical responses in a phase I study in heavily pretreated diffuse large cell lymphoma [195]. A phase I dose-escalation study of the cyclin D modulator ON 013105 in patients with R/R lymphoma is ongoing (NCT01049113) after showing promising *in vitro* and *in vivo* data in MCL [196].

Fostamatinib is a spleen tyrosine kinase (Syk) inhibitor which has shown synergistic activity with a number of agents in *in vivo* models of DLBCL [197]. In a recent phase I/II study in NHL and CLL, substantial responses were observed in a number of tumor types. Common toxicities included diarrhea, fatigue, cytopenias, and hypertension [198].

Activation of protein kinase C (PKC) and its overexpression have been associated with a less favorable outcome in DLBCL [199]. Enzastaurin is an inhibitor of PKC-β [200]. In a phase II study in R/R DLBCL, prolonged freedom from progression (FFP) was observed with little grade 3 toxicity. Preliminary results from a subsequent study in aggressive NHL also indicate single-agent activity [201]. A phase III study with daily enzastaurin to prevent relapse in DLBCL patients in remission after R-CHOP treatment is currently ongoing (NCT00332202).

Dasatinib has shown single-agent activity in a phase I/II study in R/R NHL [202]. Pleural effusions and cytopenias were the main grade 3 or 4 toxicities. A phase II study in R/R DLBCL (NCT00918463) is currently recruiting.

Bruton's tyrosine kinase (Btk) is a mediator of B-cell signaling, and PCI-32765 is a selective, irreversible inhibitor of Btk [203]. In a phase I study in patients with R/R B-cell malignancies, PCI-32765 induced durable responses with minimal toxicity [204]. Encouraging initial clinical results with the anaplastic lymphoma kinase (ALK) inhibitor crizotinib in advanced chemoresistant ALK^+ lymphoma patients have also been observed [205].

The benzimidazole AZD6244 (ARRY-142886) is a novel, 2nd-generation mitogen-activated protein kinase (MEK) inhibitor [206]. Considerable cell death was shown in DLBCL cell lines, primary cells, and in an *in vivo* xenograft model, at clinically achievable concentrations.

5.7. JAK/STAT Pathway. The Janus kinase 2 (JAK-2)/signal transducers and activators of transcription (STAT) pathway play a key role in the proliferation and pathogenesis of hematologic malignancies [207]. A phase I study of the novel JAK-2 inhibitor, SB1518, has provided evidence of activity in patients with relapsed lymphoma. Degrasyn, a novel, small-molecule inhibitor of the JAK/STAT pathway, has been shown to interact synergistically with bortezomib *in vivo* to prevent tumor development and to prolong survival time in a xenotransplant severe combined immunodeficient (SCID) mouse model of MCL [208].

5.8. Toll-Like Receptor (TLR) Agonist (Table 5). PF-3512676 is a novel TLR9-activating oligonucleotide with single-agent antitumor activity that augments preclinical rituximab efficacy [209]. Preliminary antitumor activity for the combination was found by a phase I study in patients with recurrent, indolent, and aggressive NHL, while grade 3 or 4 neutropenia occurred in 4/50 patients.

Evaluation of a combination regimen involving a TLR7/8 dual agonist (IMO-4200) with rituximab, bortezomib, or cyclophosphamide, in human xenograft and murine syngeneic lymphoma models suggests that the antitumor activity of these agents in the treatment of NHL and other hematologic malignancies could be enhanced using this strategy [210]. The transforming-growth-factor- (TGF-) β-activated kinase 1 (TAK-1) inhibitor, AZ-Tak1, has been shown to inhibit X-linked inhibitor of apoptosis protein (XIAP), activate caspase-9, and induce apoptosis in MCL cell lines [211].

Immunostimulatory CpG oligodeoxynucleotides (ODNs) are potent activators of T-cell immunity and antibody-dependent cellular cytotoxicity (ADCC) and are under investigation as immunotherapeutic agents for a variety of malignancies, including BCL [212]. Anti-CD20 antibody-CpG conjugates have been shown to eradicate rituximab-resistant BCL in a syngeneic murine lymphoma model. A recent demonstration of the divergent effects of CpG ODNs on normal versus malignant B cells may suggest a novel mechanism of action for CpG ODNs as therapeutic agents for BCL [213].

5.9. Heat Shock Proteins (Hsps) (Table 5). Hsps are chaperones needed for the correct functioning of proteins involved in cell growth and survival [214]. Inhibition of these proteins results in increased degradation of key proteins such as kinases, signal transducer proteins, and mutated oncogenic proteins. GUT-70, a tricyclic coumarin derived from *Calophyllum brasiliense*, has shown pronounced antiproliferative effects in MCL with mutant-type *p53* (*mt-p53*), a known negative prognostic factor for MCL, through Hsp90 inhibition. These findings suggest that GUT-70 could be potentially useful for the treatment of MCL [215].

The small-molecule 17-AAG (17-allylamino-17-demethoxygeldanamycin) can induce cell death in a dose- and time-dependent manner by reducing the cellular contents of critical survival proteins, including Akt and cyclin D1 in a range of lymphoma cell lines [216]. Several clinical responses were observed in a phase II study of 17-AAG in patients with R/R MCL or HL. SNX-2112 was found to exert effects in combination with bortezomib and rituximab in rituximab-resistant NHL cell lines [181]. SNX-2112 is currently in phase I clinical trials.

5.10. Angiogenesis (Table 5). Tumor angiogenesis is important in a variety of hematologic malignancies [217]. Bevacizumab, already widely studied in solid tumors, has also been evaluated in lymphoma. In a phase II SWOG study of R-CHOP plus bevacizumab in patients with advanced DLBCL, the observed 1-year PFS estimate trended higher than the historical estimate. However, as significant toxicities were associated with the addition of bevacizumab the regimen was not recommended for further evaluation [218]. In a phase II study of single-agent sunitinib in R/R DLBCL, no evidence of activity was recorded and hematologic toxicities were greater than anticipated [219]. The vascular-endothelial-growth-factor- (VEGF-) 1/2 fusion protein, aflibercept, has been evaluated in a phase I study in combination with R-CHOP in untreated patients with BCLs [220]. The 6 mg/kg dose of aflibercept is used in all ongoing phase III trials in other indications, and the combination with R-CHOP resulted in high response rates in this study. The main grade 3 or 4 adverse events included hypertension (8/25), febrile neutropenia (4/25), and asthenia (4/25).

Preliminary results are available from 2 recent phase II trials with sorafenib. In a single-agent study in heavily pretreated patients with R/R NHL, a number of responses were noted and therapy was overall well tolerated [221]. In a phase II study in combination with the Akt inhibitor perifosine in R/R lymphomas, a number of PRs were observed, with thrombocytopenia (18%) the most common drug-related hematological toxicity [222]. A phase II study in recurrent DLBCL is currently ongoing (NCT00131937). The combination of sorafenib and everolimus was shown to be well tolerated, with activity observed, especially in HL, in a phase I trial in patients with lymphoma or MM [223].

5.11. Additional Targeted Agents and Novel Therapeutics. Farnesyltransferases are key cellular enzymes involved in the prenylation of proteins [224]. Prenylated proteins are important for malignant cell growth. The oral farnesyltransferase inhibitor, tipifarnib, has been assessed in a phase II study in patients with relapsed, aggressive, indolent, or uncommon lymphoma. Tipifarnib had a good tolerability profile and demonstrated activity in lymphoma, with responses in

Table 7: Overview of ongoing or recently completed phase III studies mentioned in this paper, with agents in clinical development for the treatment of aggressive NHL. [B: bendamustine; CR: complete response; DLBCL: diffuse large B-cell lymphoma; F: fludarabine; MCL: mantle cell lymphoma; NA: not applicable; NHL: non-Hodgkin lymphoma; PFS: progression-free survival; R-CHOP: cyclophosphamide, doxorubicin, vincristine, prednisolone plus rituximab; R: rituximab; R/R: relapsed or refractory; SCT: stem cell transplantation.]

Drug	Indication	Study identifier	Study status	Results
Enzastaurin	DLBCL in remission after R-CHOP treatment	NCT00332202 PRELUDE	Ongoing; not recruiting	NA
Inotuzumab ozogamicin + R versus investigator's choice of gemcitabine + R or B + R	R/R aggressive NHL	NCT01232556	Recruiting	NA
B + R versus F + R [41]	R/R follicular, indolent, and MCL	NCT01456351	Completed; final results presented at ASH 10	B + R had better efficacy than F + R
B + R versus R-CHOP [42]	Previously untreated follicular, indolent, and MCL	NCT00991211	Completed; final results presented at ASH 09	B + R superior to R-CHOP for CR and PFS
Single-agent pixantrone dimaleate versus investigator's choice therapy [49]	Third-line treatment of R/R aggressive NHL	NCT00088530 EXTEND (PIX301)	Completed; final results presented at ASH 10	Pixantrone superior to other single-agent therapies
Pixantrone + R versus gemcitabine + R	R/R DLBCL patients not eligible for SCT	NCT01321541 PIX-R (PIX306)	Recruiting	NA

patients with heavily pretreated DLBCL, HL, and T-cell types, although little activity was observed in follicular NHL.

MLN4924 is an investigational inhibitor of Nedd8-activating enzyme (NAE), which plays a crucial role in regulating the activity of the cullin-RING E3 ligases (CRLs) [225]. Preclinical activity has been demonstrated in a novel primary human DLBCL xenograft model [226] and a phase 1 dose-escalation study of multiple dosing schedules is currently underway in patients with R/R MM or lymphoma [225].

Potential molecular targets for novel therapeutics (or "theranostics") are beginning to be identified through an emerging area in lymphoma biology involving energy metabolism. Personalized medicine approaches using bifunctional imaging and therapeutic agents are based on the premise that glucose metabolism rates are high in aggressive B-cell lymphomas [227]. Use of this bifunctional pathway as a targeted therapy has been explored recently with ^{187}rhenium-ethylenedicysteine-N-acetylglucosamine, a synthetic glucose analog, which accumulates in cancer cell nuclei and in various tumors in animal models. Biodistribution data revealed that radioactivity was retained in tumor tissue 2 hours after injection with little uptake in the plasma when compared with tumor tissue. The compound was excreted over a longer incubation period, and the retention time in lymphoma tissue was longer than that of other tissues. The results suggest that the metallic pharmaceutical agent 187Re-ECG may be a potential candidate for targeted therapy in aggressive R/R lymphomas.

The recently developed, small-molecule MDM2 antagonist, nutlin-3, inhibits the MDM2-p53 interaction, resulting in stimulation of p53 activity and apoptosis [228]. The cytotoxic effects of nutlin-3 on ALL cells suggest that the agent may be a novel therapeutic for refractory ALL [138, 228].

Stromal-cell-derived-factor-1 (SDF-1) is a chemokine that binds to the CXCR4 chemokine receptor and stimulates B-cell growth [229]. CXCR4 is frequently overexpressed on tumor cells, and the SDF-1/CXCR4 axis is thought to play a role in promoting survival, angiogenesis, and metastasis. Treatment with the CXCR4 antagonist, AMD3100, has been shown to enhance antibody-mediated cell death in disseminated lymphoma models, suggesting a potential role for CXCR4 antagonists in combination with a B-cell targeted therapy in the treatment of B-cell malignancies in the clinical setting.

MCL is characterized by the translocation t(11; 14)(q13; q32) [230]. All-trans retinoic acid (ATRA) is a key retinoid that acts through nuclear receptors that function as ligand-inducible transcription factors [231]. MCL cells express retinoid receptors; therefore ATRA may exert antiproliferative effects and, thus, may have a role in treatment. In a recent study, a novel approach to deliver ATRA to MCL cells in culture involved stably incorporating the water-insoluble bioactive lipid into nanoscale lipid particles, termed nanodisks (ND), comprised of disk-shaped phospholipid bilayers stabilized by amphipathic apolipoproteins. ATRA-ND was shown to enhance apoptosis and cell-cycle arrest in MCL

cell lines, resulting in increased p21, p27, and p53 expression and decreased cyclin D1 expression; these results suggest that ATRA-ND may represent a potentially effective approach to the treatment of MCL.

Hypoxia-inducible-factor-1 (HIF-1) is a transcription factor that serves as a master regulator of cellular responses to hypoxia and regulates genes required for adaptation to hypoxic conditions [232]. HIF-1a is commonly activated in cancer cells, including under normoxic circumstances, by oncogene products or by impaired activity of tumor suppressor genes. PX-478, the novel, small-molecule HIF-1a inhibitor, has been shown to downregulate HIF-1a protein at low concentrations effectively and to induce cell death in DLBCL cells.

6. Conclusion

In addition to the numerous cytotoxic combination regimens already available, a myriad of new agents are in development, targeting key molecular pathways critical to aggressive B-cell growth (Table 7). As monotherapy, or in combination with chemotherapy or other targeted agents, these new pharmacotherapies are likely to provide additional clinical benefit to patients with aggressive B-cell NHL and represent continued progress in the search for individualized treatments. As individualized therapy will depend on the identification of predictive markers, future clinical trials should incorporate the identification of molecular markers in their "smart" trial design. How the search for individualized treatment will affect drug development and improve clinical trial design remains to be seen.

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Review Article

Anaplastic Lymphoma Kinase-Positive Large B-Cell Lymphoma: An Underrecognized Aggressive Lymphoma

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Anaplastic lymphoma kinase-(ALK-) positive large B-cell lymphoma (ALK+ LBCL) is a rare, aggressive tumor characterized by an immunoblastic or plasmablastic morphologic appearance, expression of ALK, CD138, CD45, EMA, and often IgA by immunohistochemistry, and characteristic chromosomal translocations or rearrangements involving the *ALK* locus. The morphologic and immunophenotypic overlap of this tumor with other hematologic and nonhematologic malignancies may result in misdiagnosis. The tumor has been identified in both pediatric and adult populations and demonstrates a male predominance. Presentation is most often nodal, particularly cervical. No association with immunocompromise or geographic location has been recognized. The most common gene rearrangement is between *clathrin* and *ALK* (t(2;17)(p23;q23)), resulting in the CLTC-ALK chimeric protein, although other fusions have been described. Response to conventional chemotherapy is poor. The recent introduction of the small molecule ALK inhibitor, crizotinib, may provide a potential new therapeutic option for patients with this disease.

1. Introduction

Anaplastic lymphoma kinase-(ALK-) positive large B-cell lymphoma (ALK+ LBCL) was first described by Delsol and colleagues in 1997 [1] and is now listed as a distinct entity in the 2008 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues [2]. ALK+ LBCL is an aggressive tumor with a poor response to conventional therapies. Although it appears to be very rare, it may in fact be underrecognized due to its morphologic and immunophenotypic overlap with other hematologic and nonhematologic entities Awareness of this diagnosis, particularly in a new era of ALK inhibitor therapies, is necessary for hematopathologists as well as general surgical pathologists.

2. Clinical Features and Epidemiology

Fewer than 100 cases of ALK+ LBCL have been described in the literature (reviewed in [3–6], subsequent cases reported in [7–13]). The neoplasm has been diagnosed in both pediatric and adult age groups, ranging in age from 9 to 85 years old, with a median age of 37 to 44.5 years, and with a male predominance of 3–5 : 1. The proportion of patients under age 18 has been found to comprise approximately 15–20% of the total. One confirmed case occurring in a patient with the human immunodeficiency virus (HIV) has been reported [12]. In general, patients do not appear to be immunocompromised. Furthermore, patients with this tumor do not appear to be restricted geographically; cases have been reported from Europe, the United States, and Asia. Our institution recently diagnosed ALK+ LBCL on a biopsy sample from a 29-year-old man living in Rwanda [S. Rodig, personal communication].

ALK+ LBCL most commonly presents in lymph nodes, particularly cervical, although extranodal involvement has been reported in a wide variety of sites, including the GI tract, epidural space, ovaries, skeleton, nasopharyngeal or nasal area, tongue, brain, and liver. Bone marrow and splenic involvement have also been described. The majority of patients present with advanced stage disease. No tumors have been positive for the Epstein-Barr virus (EBV) by in situ hybridization or for human herpes virus-8 (HHV8) by immunohistochemistry [5, 6].

3. Diagnosis

The diagnosis of ALK+ LBCL requires synthesis of the morphologic features and immunohistochemical findings and is aided by cytogenetic data when available. The neoplastic cells are intermediate to large sized, and immunoblastic- or plasmablastic-appearing with round nuclei, dispersed chromatin, a single, prominent, central nucleolus and moderate amounts of eosinophilic to amphophilic cytoplasm (Figure 1). The tumor grows in sheets in nodal and extranodal sites, and, within the lymph node, often demonstrates sinusoidal invasion. The tumor cells are almost always negative for CD20 and CD79a but are always positive for CD138 and CD38, consistent with a postgerminal center phenotype (Figure 2). ALK stains 100% of tumors, and its staining pattern may be suggestive of the underlying cytogenetic abnormality (see Genetics and Pathogenesis). CD45, EMA, and MUM-1 are also frequently positive, and CD4 staining may be seen. The tumors often express IgA with monotypic light chain restriction. Other markers, including keratins, have infrequently been reported.

Due to the morphologic and immunophenotypic overlap with other hematologic and nonhematologic malignancies, this neoplasm may be misdiagnosed. Indeed, several cases in the literature were initially classified as poorly differentiated or anaplastic carcinoma [6], ALK-positive anaplastic large cell lymphoma (ALK+ ALCL) [14], or extramedullary plasmacytoma [5, 10]. Further, the plasmablastic morphologic features overlap with other aggressive large B-cell lymphomas with plasmablastic differentiation. It is possible that the small number of cases in the literature may represent underreporting due to failure of recognition of the entity. Immunohistochemical studies can greatly assist in the interpretation of these tumors (Table 1).

4. Genetics and Pathogenesis

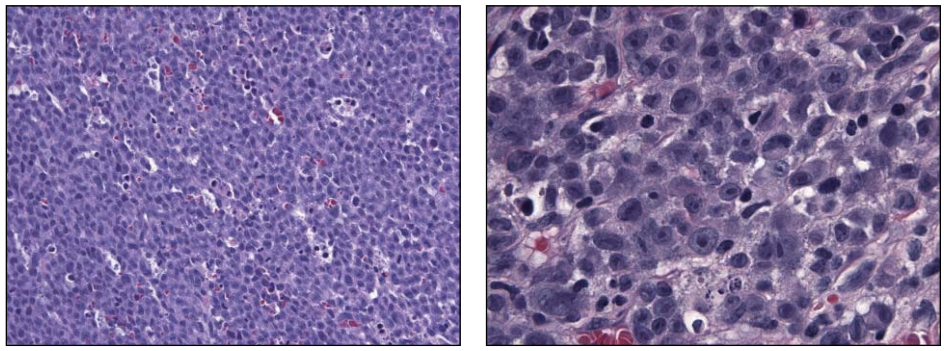
Anaplastic lymphoma kinase (ALK) is a tyrosine kinase of the insulin receptor superfamily. In all cases of ALK+ LBCL in which cytogenetic or molecular attempts were successful, chromosomal translocation or rearrangement involving the *ALK* locus has been identified by karyotype, FISH, and/or RT-PCR. Although it was initially suggested that a full-length ALK protein was expressed by these tumors, it is now clear that these tumors commonly express the product of a fusion gene that incorporates the 3′ portion of *ALK* encoding the tyrosine kinase domain.

Several genetic partners fused to *ALK* have been described. The most common is *clathrin (CLTC-ALK)*, the product of t(2;17)(p23;q23) (first described in [15–17]). Interestingly, this translocation has been described in less than 1% of cases of ALK+ ALCL [2] and has also been identified in inflammatory myofibroblastic tumors (IMT) [18]. In both ALK+ LBCL and ALK+ ALCL, immunohistochemical staining with anti-ALK antibodies gives a granular cytoplasmic pattern in neoplastic cells because clathrin encodes for a coated vesicle protein involved in intracellular transport. Less commonly described in ALK+ LBCL is the nucleophosmin-ALK (NPM-ALK) fusion protein, resulting from t(2;5)(p23;q35) [9, 19, 20]. In contrast, NPM-ALK is the most common chimeric protein in ALK+ ALCL [2]. In both ALK+ LBCL and ALK+ ALCL bearing this chimeric protein, immunohistochemical staining with ALK gives a nuclear and cytoplasmic staining pattern. As in ALK+ ALCL, the distinct ALK staining patterns may act as a surrogate for the underlying gene rearrangement in ALK+ LBCL.

As awareness of this rare lymphoma increases, investigators are using more sensitive and directed techniques to confirm the presence of ALK rearrangements in tumors that immunophenotypically are compatible with the diagnosis. Van Roosbroeck and colleagues utilized sophisticated technique including metaphase FISH, RT-PCR, and 5′ RACE PCR to identify a cryptic *SEC31A-ALK* fusion generated by insertion of the 5′ end of *SEC31A* (4q21) upstream of the 3′ end of *ALK* [9]. This had previously only been described in one case of IMT [21] and was subsequently confirmed in a second case of ALK+ LBCL by Bedwell and colleagues [8]. In all three cases, the *SEC31A-ALK* fusion was the result of complex rearrangements rather than a single reciprocal translocation event (which would not be possible due to the opposite transcriptional orientation of *ALK* and *SEC31A*); this likely accounts for the rarity of both its occurrence and its detection. As pointed out by Van Roosbroeck et al., Stachurski and colleagues' report of an ALK+ LBCL with complex karyotype and cryptic insertion of 3′-*ALK* gene sequences into chromosome 4q22-24 may, potentially, represent a *SEC31A-ALK* fusion [22]. In all cases, ALK immunohistochemical staining was granular cytoplasmic.

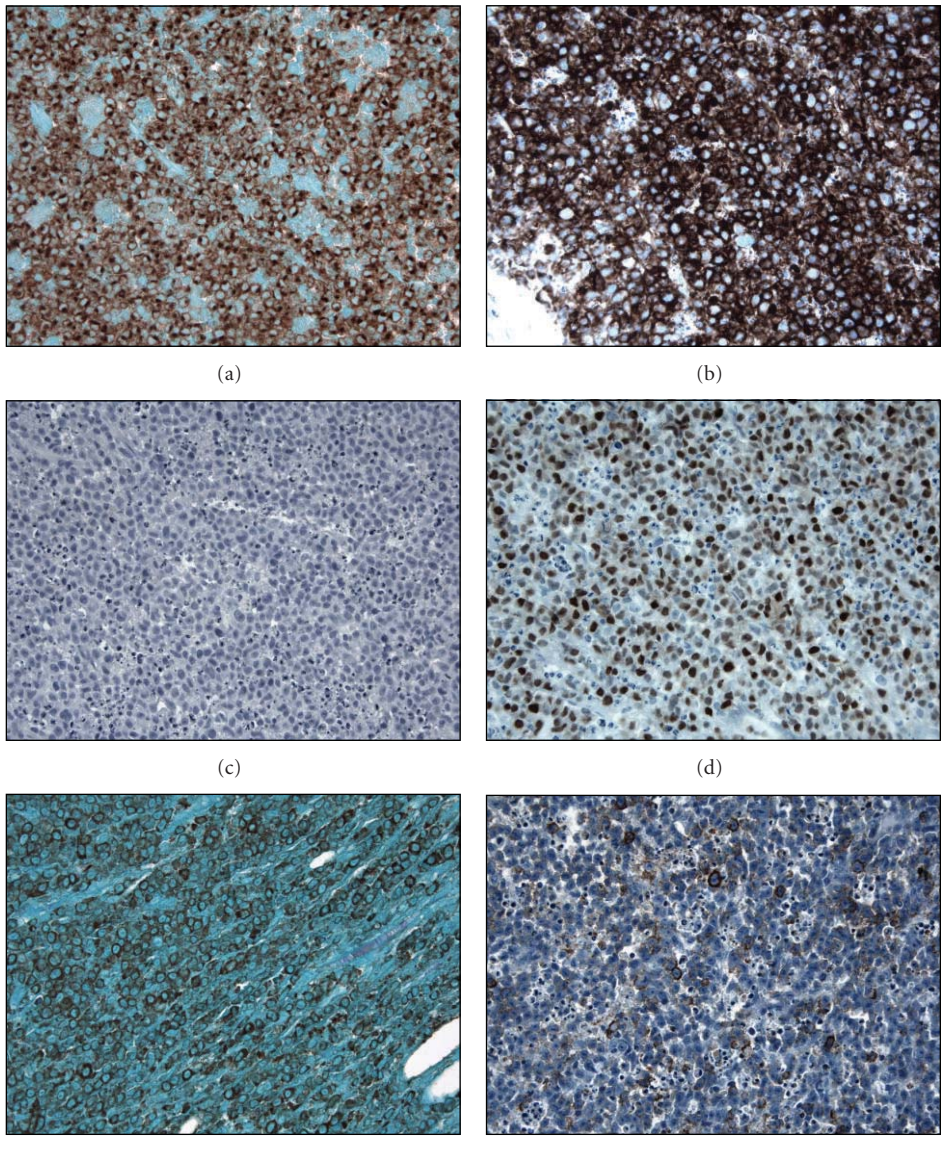
In addition to these reproducible genetic events, additional case reports have demonstrated 5′*ALK* gene deletion [23]; duplication of the *ALK* gene region/additional copy of chromosome 2 [24]; complex karyotype with two independent *ALK* translocations: t(X;2)(q21;p23) and t(2;12)(p23;q24.1) showing granular cytoplasmic ALK staining [12]; *SQSTM1-ALK*, generated from t(2;5)(p23.1;q35.3), showing diffuse cytoplasmic ALK staining with ill-demarcated spots [13].

Several experimental models support the role of *ALK* fusion genes in disease pathogenesis. Expression of a *SEC31A-ALK* construct in the interleukin-3-(IL3-) dependent cell line Ba/F3 permitted growth factor-independent growth upon cessation of IL3 administration [9]. Addition of an ALK inhibitor resulted in decreased ALK tyrosine phosphorylation and decreased phosphorylation of downstream effectors ERK1/2 and STAT3, and, less robustly, AKT and STAT5 [9]. STAT3, but not STAT5, has been found to be highly hyperphosphorylated on tyrosine 705 in two cases of ALK+ LBCL with the *CLTC-ALK* fusion protein [7]. Injection of 3T3 cells expressing *SQSTM1-ALK* into nude mice produced subcutaneous tumors [13]. A *CLTC-ALK*-positive DLBCL cell line, generated from a patient with systemic relapsed disease, formed subcutaneous tumors in NOD-SCID mice, which morphologically and immunophenotypically recapitulated the ALK+ LBCL phenotype, including the granular cytoplasmic ALK staining pattern [14]. As in ALK+ ALCL, one could hypothesize that ALK chimeric proteins allow for constitutive activation of the ALK tyrosine



(a) (b)

Figure 1: ALK+ LBCL morphology. (a) The tumor grows diffusely with a sheet-like architecture (400x, H&E). (b) The tumor cells are intermediate to large sized with round nuclei, dispersed chromatin, centrally placed nucleoli and moderate amounts of amphophilic cytoplasm (1000x, H&E).



(a) (b)

(c) (d)

(e) (f)

Figure 2: ALK+ LBCL immunohistochemical profile. All tumors are positive for ALK (a) and CD138 (b). CD20 (c) is almost always negative, but MUM-1 (d) is frequently positive. IgA is often expressed (e). EMA can show focal to diffuse positivity (f). All microphotographs are 400x.

Table 1: Differential diagnosis of ALK+ LBCL and predominant staining patterns with several antibodies. This table summarizes the predominant staining patterns for these entities, with the understanding that exceptional cases have been reported. Typically, ALK+ LBCL demonstrates a postgerminal center phenotype and is negative for CD30. Rare cases expressing CD20, CD79a, or CD30 have been reported [5].

	ALK+ LBCL	ALK+ ALCL	DLBCL, NOS	Poorly differentiated carcinoma	Extramedullary plasmacytoma
CD45	+	+	+	−	
CD20	−	−	+		−
CD79a	−		+		+
MUM-1	+		+/−		
CD138	+	−	−	+/−	+
ALK	+	+	−	−	−
CD30	−	+	+/−		
EMA	+	+		+/−	
Keratin	+/−			+/−	
EBER	−	−			

kinase independent of ligand binding, resulting in unchecked activation of downstream effectors.

5. Outcome and Treatment Strategies

As opposed to ALK+ ALCL, which has an overall 5-year survival rate approaching 80% [2], ALK+ LBCL has a dismal prognosis and poor response to conventional therapy regimens. Laurent and colleagues retrospectively analyzed clinical outcome data in 31 patients diagnosed with ALK+ LBCL [6]. All patients with documented therapy ($n = 30$) received cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) or CHOP-derived chemotherapy; 11 additionally received radiation therapy, and 3 underwent subsequent autologous stem cell transplantation. The 5-year survival rate was 25% with a median survival of 12 months in advanced stage disease. Overall survival was significantly shorter for patients with stage III or IV disease as defined by the Ann Arbor staging system compared to those who presented with stage I or II disease.

Another review of 46 published cases and 4 new cases of ALK+ LBCL analyzed outcomes in 41 cases with available treatment data [5]. Twelve of 32 patients treated with chemotherapy received CHOP; of these, 6 patients relapsed and 4 patients died of progressive lymphoma. Of all treated patients, eighteen (44%) had relapsed/refractory disease, and 7 of 8 patients who underwent salvage hematopoietic stem cell transplantation (4 autologous, 1 allogeneic, 3 unspecified) died between 3 and 44 months after diagnosis. Notably, a subsequently published case report described relapse and death within 100 days of front-line autologous transplantation after treatment with CHOP and radiation therapy [10]. Overall, Beltran and colleagues documented that 56% of reported patients died, most commonly (90% of cases) due to progressive lymphoma, with an overall survival time of 24 months. Similarly to Laurent and colleagues, they found that the strongest correlative factor to survival was clinical stage at presentation, calculating an 18-month median survival in patients with advanced disease versus not reached in early-stage presentations [5].

While a systematic, prospective study of treatment regimens would be difficult in this rare disease, it is apparent from the reported literature that ALK+ LBCL is an aggressive disease with poor response to conventional therapies. More recently, interest has turned to the potential use of a new class of drugs, namely, ALK inhibitors. In addition to ALK+ LBCL, mutations or gene rearrangements leading to growth-factor-independent ALK activation have been implicated in the oncogenesis of several neoplasms including ALK+ ALCL [25], 50% of IMTs [26, 27], a subset of sporadic and familial neuroblastoma [28, 29], and 5% of nonsmall cell lung cancer (NSCLC) [30, 31]. The use of a small molecular ALK inhibitor is an attractive possibility in these diseases, and the potential is garnering excitement in the oncology field.

Recently, and in a remarkably short time since the first description of the ALK rearrangement in NSCLC, the Food and Drug Administration approved the use of crizotinib (Xalkori Capsules, Pfizer, Inc.), the small-molecule dual inhibitor of the c-Met and ALK receptor tyrosine kinases, for the treatment of patients with locally advanced or metastatic NSCLC with an ALK rearrangement [30]. Crizotinib has also been tried on a case-by-case basis in other tumors. Butrynski and colleagues recently reported a sustained partial response in a patient with an ALK-translocated IMT, while no response was observed in a patient with an ALK-non-rearranged IMT [32]. Gambacorti-Passerini and colleagues administered crizotinib to two patients with relapsed ALK+ ALCL; clinical improvement was seen within one week for both patients, and complete response was sustained at six months and five months, respectively [33].

There is some experimental evidence to suggest that ALK inhibitors may be efficacious in the treatment of ALK+ LBCL. Upon identifying a *SEC31A-ALK* fusion in a patient with ALK+ LBCL, Van Roosbroeck and colleagues demonstrated that the selective ALK inhibitor NVP-TAE684 (TAE-684) could inhibit the growth of a Ba/F3 cell line expressing the *SEC31A-ALK* construct in a dose-dependent

manner [9, 34]. Cerchietti and colleagues established a cell line expressing CLTC-ALK, the most common fusion protein identified in ALK+ LBCL, and demonstrated that TAE-684 could inhibit cell growth *in vitro* and could regress murine tumor xenografts *in vivo* [14]. To date, no reports of the use of crizotinib in patients with ALK+ LBCL have appeared in the literature. However, at least one Phase 1B clinical trial, studying the safety and clinical activity of crizotinib in tumors with genetic events involving the *ALK* gene locus, is currently recruiting patients (NCT01121588), and reference was made to a patient enrolling in an inhibitor trial in a recent case report [12].

6. Conclusion

ALK+ LBCL is a rare, aggressive B-cell lymphoma with characteristic morphologic, immunophenotypic and cytogenetic/molecular findings. Awareness of this entity is important in both the hematopathology and general surgical pathology fields. Although response to conventional therapy has been poor, the possibility of a targeted therapy provides an intriguing option for patients with this disease.

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Review Article

Microenvironment-Centred Dynamics in Aggressive B-Cell Lymphomas

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Aggressive B-cell lymphomas share high proliferative and invasive attitudes and dismal prognosis despite heterogeneous biological features. In the interchained sequence of events leading to cancer progression, neoplastic clone-intrinsic molecular events play a major role. Nevertheless, microenvironment-related cues have progressively come into focus as true determinants for this process. The cancer-associated microenvironment is a complex network of nonneoplastic immune and stromal cells embedded in extracellular components, giving rise to a multifarious crosstalk with neoplastic cells towards the induction of a supportive milieu. The immunological and stromal microenvironments have been classically regarded as essential partners of indolent lymphomas, while considered mainly negligible in the setting of aggressive B-cell lymphomas that, by their nature, are less reliant on external stimuli. By this paper we try to delineate the cardinal microenvironment-centred dynamics exerting an influence over lymphoid clone progression in aggressive B-cell lymphomas.

1. Introduction

B-cell malignancies represent a heterogeneous group of diseases characterized by different biological features and clinical behaviour, the latter ranging from indolent to highly aggressive. As for most neoplasms, the natural course of B-cell malignancies is characterized by tumour progression, featured by a flow of events leading to the enhancement of proliferative and invasive capabilities, towards the establishment of a more aggressive phenotype. Even if most of the processes involved in cancer progression are inherent to the neoplastic clone, this event is, actually, the result of an articulated mechanism, which seems to require the constant crosstalk between neoplastic cells and the faulty surrounding microenvironment. An ever-increasing amount of evidences suggest that this bijective relationship is a prime determinant of cancer natural history and evolution. Much has been so far discovered about the role of tumour intrinsic mechanisms of neoplastic progression, and the focus of research has been progressively shifting toward the study of microenvironment-centred dynamics. Cancer-associated microenvironment represents a multifaceted entity, which not only provides structural support to neoplastic cells (proper stroma) but also acts as a "fertile soil" that, through humoral factors (bioactive molecules such as cytokines, chemokines, and adhesion molecules), nonmalignant cellular elements of the stroma (fibroblasts and endothelial cells) and the immune system (macrophages, mast cells, B and T lymphocytes) fosters tumour clone survival and expansion, local invasion/spreading, and escape from the immunological response.

The relative contribution of these branches of the tumour microenvironment may vary in the diverse tissues and organs in which lymphomas arise as well as in different lymphoma

histotypes, yet, their relevance is proved by their influence over the disease clinical outcome.

The contribution of microenvironment to lymphoma progression has been deeply investigated in indolent lymphomas (ILs), comprising chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL), lymphoplasmacytic lymphoma (LPL), Marginal zone lymphoma (MZL), and follicular lymphoma (FL), all sharing common features such as low proliferative rate of neoplastic cells and long time to disease progression and/or treatment.

ILs are indeed characterized by a constant crosstalk with the surrounding microenvironment, which plays a role in their pathogenesis and that eventually affects several aspects of their natural history.

A prototypical example of the influence of tumour microenvironment over lymphoma progression is provided by CLL. It has been shown that CLL clones characterized by CD38 and CD49d expression, harbouring an unfavourable prognosis, are able to attract CD68+ monocytes (macrophages) at site of infiltration, by CCL3 and CCL4 chemokines synthesis.

Macrophages (Ms), recruited by the neoplastic cells, in turn, release proinflammatory mediators such as TNF, inducing upregulation of the vascular cell adhesion molecule VCAM-1 on the surrounding stroma; the following VCAM-1/CD49d binding significantly increases neoplastic cell proliferation and survival [1]. Such an interchained sequence of events involving the CLL stroma thus represents a direct link with the acquisition of a clinically appreciable aggressive pace of the disease, providing a precious insight into the potential influence of microenvironment-centred dynamics over disease course.

The pressure of immune-cell-engendered stromal changes over lymphoid clone progression can be identified in ILs other than CLL and also involving, besides Ms, other cells of the innate and adaptive immune system. Indeed, bone marrow (BM) mast cells (MCs) are commonly found in association with neoplastic BM infiltrates in patients with LPL, supporting tumour expansion through vigorous CD154-CD40 stimulation [2].

In FL, neoplastic cells benefit of the association with follicular helper T cells (Th), follicular dendritic cells (FDCs), Ms and FOXP3-expressing T regulatory cells (Tregs), for the shaping of an aberrant stromal microenvironment permissive for germinal centre (GC), neoplastic B cells [3, 4], also T helper 17 (Th17) and other IL-17-producing cells are likely to play a role in this setting.

In line with their strong reliance from lymphomatous/leukemic microenvironment, ILs show a diversified degree of tropism for stromal niches, which they colonize and subdue. Relevant examples are provided by FL cells tropism for osteoblastic/paratrabecular BM niches, rich in cellular and extracellular components (Jagged-1 Notch ligand, β-1integrins, type I collagen, osteopontin, and SPARC matricellular proteins) shared by the GC environment itself [5], and by splenic MZL cell homing to sinusoidal vascular niches of the BM and spleen sharing chemoattractive and adhesive signals (CXCL-12, hyaluronan, and ICAM-1) [6, 7].

The tight relation and mutual influence between neoplastic cells and their stromal microenvironment, which we have outlined for ILs, may appear less germane to aggressive lymphomas that, by their nature, show a stronger proliferative attitude and a high invasive behaviour. In these malignancies, the microenvironment role has been marginally considered in tumour progression and, therefore, poorly studied.

The aim of this paper is to highlight the actual relevance of the stromal microenvironment in the natural history of B-cell aggressive lymphomas, trying to provide a detailed perspective of the relevant interactions involving bystander immune and mesenchymal cells and extracellular components of the stroma.

2. Bystander Immune Cells

The tumour microenvironment is populated by cells of the adaptive and innate immune system that, interacting with cancer cells, may contravene to their primary "guardian" function by actively contributing to tumour onset and progression (Figure 1).

The amount, composition, and location of both adaptive and innate immune system components vary greatly between the different types of malignant lymphoma and exert a diverse influence on the prognosis [8].

T-cell-/histiocyte-rich large B-cell lymphoma (THRLBCL) represents a paradigmatic variant in which the neoplastic large B cells constitute a minority of the tumour burden in the context of a dense microenvironment rich in T cells, with or without histiocytes [9]. Such a picture displays a high degree of homology with that of classical Hodgkin's lymphoma, in which neoplastic cells induce the recruitment of a constellation of immune cells (lymphocytes, granulocytes, Ms, DCs) from the peripheral circulation that, in turn, induce a favourable environment to the neoplasm maintenance and progression [10–12].

Tumour associated macrophages (TAMs) of THRLBCL are recruited within neoplastic infiltrates mainly by clone-derived macrophage chemotactic proteins (MCPs) and represent a major component of the infiltrate. Within the cancer-associated microenvironment, TAMs display a peculiar dual-faceted attitude; they can kill tumour cells but at the same time favour their growth by inducing immunosuppression and producing angiogenic factors and metalloproteases [13]. In most aggressive B-cell lymphomas, TAM protumoral function neatly prevails over their participation to antitumour immunity. Specifically, in THRLBCL IFN-γ-induced TAM activation determines the synthesis of the chemoattractant protein CCL-8 (MCP-2) and of the immunomodulatory molecule indoleamine 2,3-dioxygenase (IDO), that give rise to a self-feeding immunosuppressive loop [14].

A significant M infiltration is observed in other aggressive B-cell lymphomas such as in Burkitt's lymphoma (BL), in which Ms are functionally involved in neoplastic apoptotic cell engulfment and are stimulated by IL-10 to the synthesis

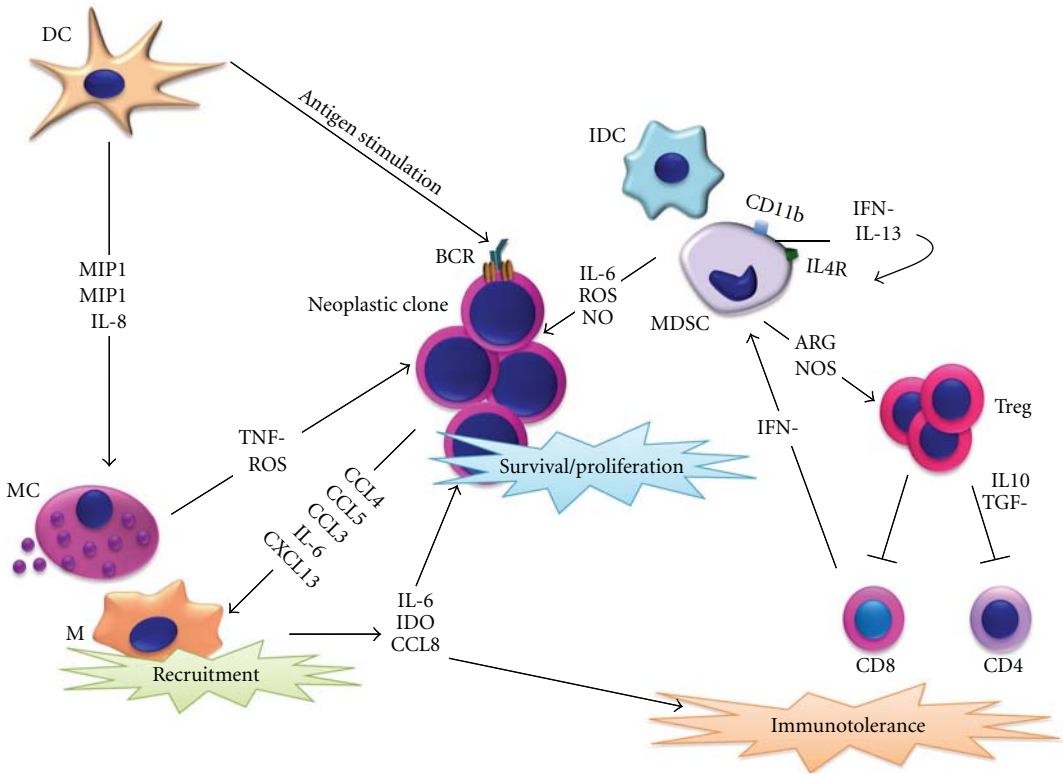

Figure 1: Immune system cells, both adaptive and innate, engender a fertile microenvironment through direct interaction and release of factors in the milieu. (M: monocyte/macrophage; MC: mast cell; DC: dendritic cell; IDC: immature dentritic cell; MDSC: myeloid derived suppressor cell; T reg: T regulatory lymphocyte. Black arrows indicate activation pathways. Red lines indicate inhibitory pathways).

and release of B-cell trophic factors such as BAFF/BLyS [15]. In addition to directly stimulate neoplastic B cells, CD68-expressing Ms induce activation of the neighbouring mesenchymal cells as demonstrated by VCAM-1 upmodulation in areas of prominent M infiltration (Figures 2(a) and 2(b)).

Similarly, in diffuse large B-cell lymphoma (DLBCL), neoplastic cells recruit T cells and CD14+ monocytes by CCL-5 release, also engendering a histiocyte-enriched microenvironment [16]. Besides local synthesis of soluble mediators active on neoplastic B cells, infiltrating Ms and other professional antigen-presenting cells (APCs) can support neoplastic B-cell proliferation and rescue from apoptosis by sustained B-cell-receptor (BCR) stimulation [17].

BCR signalling pathway can be triggered in neoplastic cells by canonical antigen ligation or by antigen-independent adhesive signals modifying the actin cytoskeleton and in both cases involves the activation of the Syk kinase [18]. BCR stimulation by environment-generated signals can be relevant for the fitness of neoplastic B cells, yet, in several aggressive B-cell lymphomas, as in DLBCL, constitutive activation of the BCR pathway (i.e., tonic signalling) can be observed, which is not dependent on external stimuli [19, 20]. On these bases, inhibition by Syk targeting, irrespectively of the neoplastic cell-intrinsic or cell-extrinsic source of BCR stimulation, could be envisaged as an appealing therapeutic prospect [21].

Among monocytic/macrophagic CD14-expressing cells recruited by DLBCL clones, intratumoral precursors of dendritic cells (DCs) have been identified basing on their expression of the DC marker DC-SIGN and on the acquisition of DC morphology. DCs found within tumour infiltrates are commonly "frozen" in an immature status (iDCs) by soluble factors of the tumour milieu such as IL-4, IL-6, GM-, and M-CSF. iDCs are also recruited from the BM myeloid cell reservoir through CCL-3 and CCL-4 chemokine interaction with CCR-1/-2/-5 receptors and on their turn participate to the recruitment of other myeloid cells at sites of infiltration (e.g., by IL-8, RANTES, TARC, and MDC) [22, 23]. Among BM-derived myeloid cells that might be coopted by neoplastic B cells or bystander cells in the lymphoma-associated microenvironment, a relevant population is represented by myeloid-derived suppressor cells (MDSCs). With iDCs, MDSCs share an immature myeloid phenotype and are characterized by the expression of the CD11b, CD33, and IL4r. Both iDCs and MDSCs empower the regulatory milieu associated with the expanding clone by the inhibition of T-cell responses through nitric oxide (NO) and reactive oxigen species (ROS) release and induction of Treg skewing. It is conceivable that the restoration of the physiologic crosstalk between DCs (or other APCs) and T cells, or the functional inactivation of such myeloid regulatory cells [24, 25], might induce effective Th-1-oriented cytotoxic responses against the B-lymphoid clone. Indeed, a dense

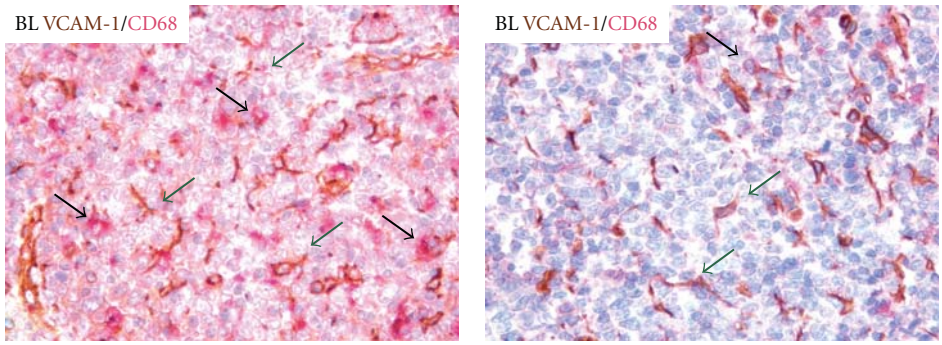

Figure 2: Adhesion molecule VCAM1-expressing mesenchymal cells (green arrows) form a denser meshwork in BL cases rich in CD68-expressing macrophages (black arrows) as compared with cases showing scattered CD68-expressing macrophages. (VCAM and CD68 immunohistochemical stain performed with streptavidin-biotin peroxidase complex system, original magnification 400x).

cytotoxic T-cell infiltrate spatially associated with CD21-expressing FDC meshwork has been correlated with better survival and a higher complete remission rate in high-risk DLBCL [26], thus suggesting a favourable influence of effector T cells populating the lymphoma environment. However, other studies have shown that dense infiltrates of activated cytotoxic T cells in nodal DLBCL correlate with poor survival [27] indicating that the actual outcome of T-cell infiltration is indeed puzzling and variable.

If the interpretation of the contribution of infiltrating T cells to the composition of the lymphoma microenvironment appears rather problematic, this is particularly true for Tregs [4, 28, 29]. In DLBCL, the prognostic significance of infiltrating Tregs has proved quite controversial, since the amount of Tregs associated with lymphoma infiltrates has been found to independently correlate with a good [30, 31] or dismal prognosis (or found unrelated with prognosis) [28, 31] by different Authors. The controversial results of Treg prognostic influence in DLBCL has been interpreted in light of the heterogeneity of settings and methods adopted for Treg assessment [28], yet they probably have a true biological explanation.

Tregs modulate the activity of both CD4+ and CD8+ effector populations through the release of IL-10 and TGF-β1. Therefore, they can contribute to the immune escape of the neoplastic clone producing a detrimental influence on outcome while, at the same time, depriving neoplastic cells from beneficial proinflammatory stimuli induced by other lymphoma-infiltrating cells. Moreover, the above-mentioned analyses of Treg influence in the lymphoma-associated environment are intrinsically flawed by the assumption that Tregs are "functionally stable" which means not considering their plasticity. In their activity of quenching immune responses by interfering with the activation status of innate (e.g., MCs) and adaptive (e.g., T cells) immune effectors, Tregs are exposed to the pressure of the inflammatory milieu, by which they can be skewed towards other functional fates. We have recently demonstrated that activated MCs can induce contrasuppression of Tregs through the OX40/OX40L axis and IL-6 release in a TGF-β1-rich environment towards the generation of proinflammatory Th17 cells [32, 33]. In this light, the different prognostic value of FOXP3+ Tregs in GC-type DLBCL where their presence is related with a positive prognostic influence and non-GC DLBCL where, by contrast, an increase of FOXP3+ Tregs directly correlates with an adverse outcome, could be at least partially explained by the marked inflammatory environment engendered in the latter by the abundancy of IL-6- and TNF-producing Ms and MCs [34–36]; this could inhibit the function of Tregs which, in turn, could even boost inflammation favouring Th17 generation [28].

Overall, it is hard to identify a *leitmotiv* in the functional interactions between neoplastic cells and immune bystander cells of aggressive B-cell lymphomas as the final outcome of such interplay can profoundly vary at discrete stages of the disease course and can be significantly affected by therapy. Nonetheless, aggressive B-cell lymphoid clones, despite their striking proliferative and invasive capabilities, are not disengaged by the influence of the immune microenvironment, which actually represents a reasonable focus for chasing the improvement of treatment efficacy.

3. The Matter of Vasculogenesis

Among the different aspects functional to tumour progression characterizing the cross talk between neoplasms and stroma, a remarkable role is played by vasculogenesis. Neo-angiogenesis has been the focus of extensive scientific investigation in the field of cancer research. The generation of new blood vessels not only provides a dedicated blood supply to the tumour, but also represents the hinge of its dissemination, being the most direct route for the colonization of secondary organs.

In aggressive B-cell lymphomas, as in several other cancers, Neo-angiogenesis is the result of a play of forces between neoplastic and stromal elements involving the axis of vascular endothelial growth factors (VEGFs) and their receptors, known to play a central role in this process (Figure 3). The synthesis of VEGFs along with the expression of their receptors has been extensively described in DLBCL [37],

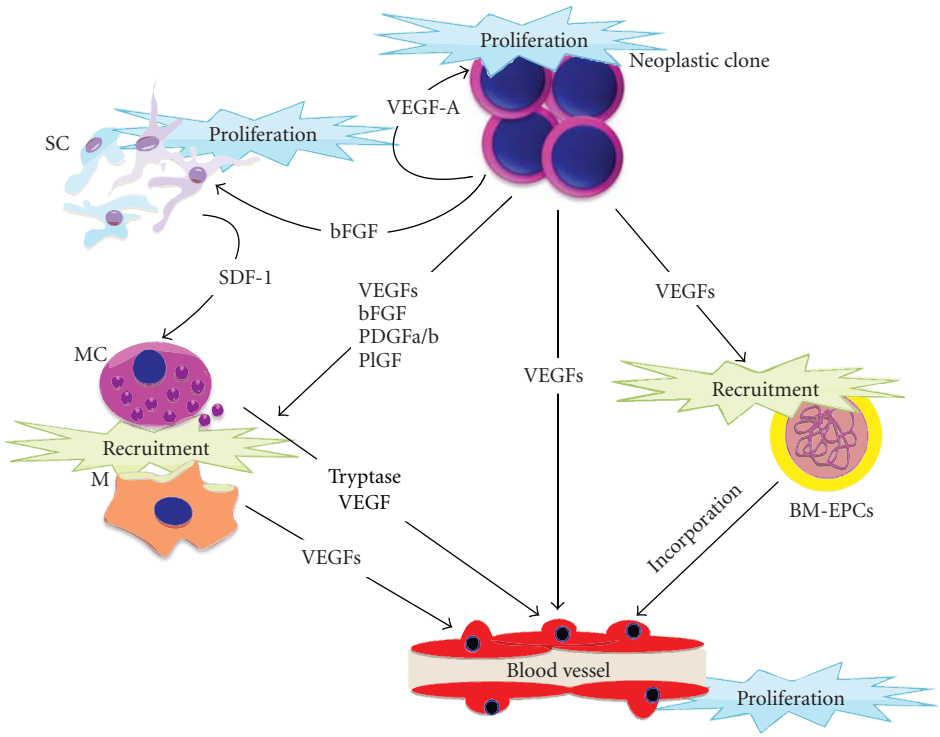

Figure 3: Vasculogenesis plays a key role in clone dissemination and represent a the route for organ colonization. (M: monocyte/macrophage; MC: mast cell; SC: stromal cell; BM-EPC: bone marrow derived endothelial progenitor cell. Black arrows indicate activation pathways).

Mantle cell lymphoma (MCL) [38], BL [39], and grade-3 FL [40], being reported as variably associated with the disease aggressiveness.

In such B-cell lymphomas, neoangiogenesis is regulated by an intricated and redundant network of interactions based on the production of growth factors deriving from malignant cells and from accessory cells of the microenvironment. Production of VEGFs (VEGF-A, VEGF-C, VEGF-D) and other angiogenic factors such as the basic fibroblasts growth factor (bFGF), placental growth factor (PlGF), platelet-derived growth factors (PDGF-a and PDGF-b) and stromal-derived factor-1 (SDF-1) by cancer and stromal cells initiates neoangiogenesis through the contemporary involvement and recruitment of different cytotypes [41]: mature resident endothelial cells receiving direct mitogenic signals stemming from Raf-1-MEK-MAP kinase cascade after interaction with VEGFs [42]; CD68+ monocyte-macrophages and other accessory cells such MCs [43, 44], further enriching the microenvironment by the release of bFGF, PDGF, members of the VEGF family, proteases (e.g., MC-tryptase, matrix metalloproteases), and proinflammatory mediators (IL-1, IL-6, IL-8, and TNF) [45–47]; BM-derived endothelial progenitor cells and BM mesenchymal stem cells recruited by VEGF and SDF-1 gradients, amplifying the synthesis of proangiogenic mediators and eventually incorporating into the growing tumour neovasculature [5, 48–50]. Furthermore, neoplastic B lymphocytes directly perceive pro-angiogenic stimuli through the expression of VEGF receptors (VEGFR-1 and VEGFR-2), which enables them to receive, in an autocrine fashion, proliferation and/or survival signals [50]. A high expression of VEGF therefore directly links the remodelling of the stromal vascular microenvironment to clone-intrinsic B-cell lymphoma progression. Several studies have been so far focused on the relationship between microvascular density (MVD) of lymphomatous infiltrates, prognosis, and clinical outcome in B-cell lymphomas; in general, MVD scores trend higher in aggressive histotypes including BL and DLBCL, compared with indolent ones [51].

The correlation between MVD and the clinical course of different lymphoma subtypes, however, is not straightforward. In DLBCL, for instance, multiple studies have demonstrated that there are no significant differences in the MVD counts between the long- and short-surviving patients and that MVD score does not correlate with overall survival [52, 53]. Conversely, other experimental evidences have suggested a negative impact of vasculogenesis on DLBCL clinical outcome by demonstrating that MVD increases paralleling tumour progression [46, 54].

Differently from DLBCL, in BL the degree of neoangiogenesis is more homogeneously related to the disease progression. BL aggressive behaviour depends on the overexpression of the oncogene *MYC*, which also acts as an essential promoter of early and persistent growth of blood and lymphatic vessels during tumour progression [55]. Moreover, as demonstrated in a murine model of BL, c-MYC-expressing B cells are the major source of the vascular endothelial growth factor [56].

The mechanism through which c-MYC regulates the VEGF axis has not yet been clearly elucidated. c-MYC may conceivably act as a VEGF transcriptional factor [57], and

recent data suggest that it could control the expression of several mRNAs (such as the one encoding for VEGF) by regulating a broad range of microRNAs [58].

Along with BL, also MCL is characterized by a strong and active neoangiogenetic process. In MCL, neoplastic B cells give rise to an autocrine positive feedback mediated by the VEGF-A/VEGFR-1 axis, which is also sensed by stromal cells of blood and lymphatic vessels expressing VEGFR-2 and VEGFR-3 [59].

The established leading role played by neoangiogenesis in aggressive B-cell lymphomas paved the way to several clinical trials targeting the formation of new blood and lymphatic vessels. Both consolidated and ongoing approaches are based on the administration of different combinations of drugs capable to interfere with the VEGF axis either in a direct or an indirect manner. Examples of these strategies are the use of anti-VEGF-A antibodies (e.g., bevacizumab) in association with chemoimmunotherapy, and the administration of endostatin and anti-CXCR-4 monoclonal antibodies in the prospect of blocking circulating endothelial cell progenitors [60, 61].

Neoangiogenesis is intimately involved in the arousal and progression of B-cell neoplastic clones but dissecting its relative contribution to these processes is problematic owing to the strong correlations it displays with almost every other microenvironment-centred process. This apparent limit to the understanding of the true influence of angiogenesis in the setting of aggressive B-cell lymphomas actually represents a precious advantage for antiangiogenic treatments, which are able to interfere with multiple vicious dynamics of the lymphoma-associated microenvironment, including recruitment of accessory cells, recruitment, and integration of mesenchymal and endothelial progenitors as well as with neoplastic B-cell dissemination.

4. The Extracellular Matrix: More Than Scaffolding

Extracellular matrix (ECM) has been considered, for many years, an inert scaffold composed by a complex mixture of proteins, proteoglycans, and in some cases of bone mineral deposits, aimed at providing support and anchorage to cells and regulating intercellular communication.

Synthesized by stromal cells, the ECM represents a reservoir for many growth factors and can be digested by enzymes like serine and threonine proteases and matrix metalloproteases to favour homeostatic processes like tissue remodelling and repair [62].

Similarly, the tumour-associated microenvironment undergoes continuous remodelling, and the ECM components, produced and released by tumour and nonneoplastic stromal cells, represent a major vehicle for the tumour-stroma crosstalk. Accordingly, ECM components have been implicated in tumor growth, progression, and metastasis both in solid and lymphoid malignancies [63–66].

One notable attempt to investigate the influence of the ECM on aggressive lymphoma behaviour has been performed in the DLBCL setting [65] following the identification of different DLBCL prognostic categories based on the expression of tumour-related genes [67]. The study performed by Lenz et al. [65] explored DLBCL from a stromal perspective and highlighted a group of cases showing a "stromal signature" enriched in ECM genes coding for collagens, laminin, metalloproteases, and matricellular proteins. This signature was related with a more favourable prognosis as compared with that of another group of DLBCL cases that was enriched in genes promoting the "angiogenic switch."

Matricellular proteins are a class of nonstructural ECM proteins endowed of regulatory function during tissue remodelling [68] and cancerogenesis [69]. Among them, SPARC (secreted protein acidic rich in cysteine), thrombospondins, and osteopontin (OPN) have been reported to play a pivotal role in providing proliferative and antiapoptotic signals to cancer cells, influencing their binding to structural matrix components or directly triggering tumour cell surface receptors [70]. Through the engagement of specific integrin receptors or CD44, OPN exerts its pleiotropic function in cancer cell survival, ECM remodelling, cell migration, and metastasis in solid cancers as well as in aggressive B-cell lymphomas [71–75]. One of the mechanisms hypothesized as responsible for the promotion of neoplastic cell survival by OPN relies on its binding to CD44. OPN-mediated CD44 engagement can prevent cell death by activation of the phosphatidylinositol 3-kinase/Akt signaling axis and by inhibition of Fas-induced signals [76, 77]. In breast cancer, OPN is also able to modulate the expression of specific CD44 isoforms [78] such as the CD44v6 and v9 variants, which endorse a negative prognostic significance. Tissue microarray analyses performed in DLBCL cases, demonstrated that the expression of CD44v6 variant was predominant in the activated type of DLBCL and, in CD44 negative cases, correlated with a worse prognosis [79].

Besides interacting with CD44 and integrins expressed on neoplastic cell surface, OPN contributes to moulding the cancer-associated immunological microenvironment by directly inducing Ms recruitment and activation towards amplification of the inflammatory milieu rich in TNF, IL-1b, and IL-6 [80, 81]. These dynamics, which have been extensively investigated in many solid tumours like soft tissue sarcomas [82] and breast cancer [83], could also take place in haematological malignancies owing to the critical role played by OPN in regulating normal and aberrant hematopoiesis [84].

Another matricellular protein, whose multifaceted influence in cancer microenvironment has been progressively delineating, is SPARC. SPARC, also known as osteonectin or BM-40, is a secreted, matricellular glycoprotein exerting an homeostatic function in tissue remodelling, being capable of regulating biological processes like angiogenesis, cell proliferation, collagen deposition, and inflammation [85]. The tissue-normalizing function of SPARC could be extended to cancer with implications for tumour growth, invasion, and metastasis. However, SPARC expression and functions are greatly tissue and context dependent, and their investigation often ingenerates ambiguous results. For example,

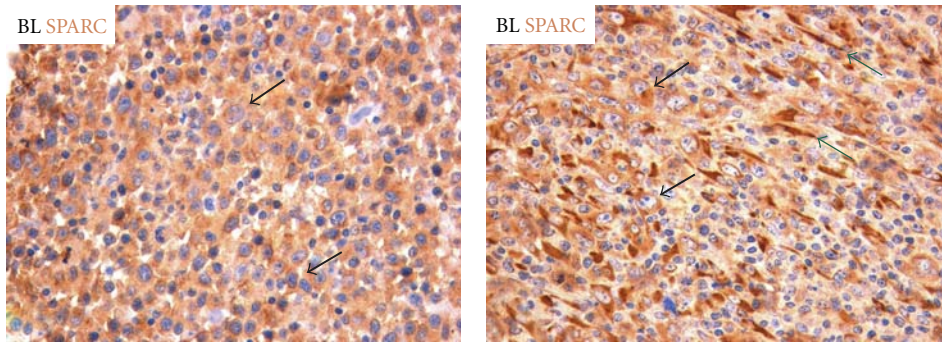

Figure 4: SPARC immunohistochemical analysis of BL cases reveals rather homogeneous expression in neoplastic cells (black arrows) in spite of a variable stroma SPARC reactivity (green arrows). (SPARC immunohistochemical stain performed with streptavidin-biotin peroxidase complex system, original magnification 400x).

in some solid cancers, conspicuous SPARC expression by neoplastic and stromal cells can either promote epithelial-to-mesenchymal transition (EMT), and favour tissue invasion and metastasis, or exercise an antiproliferative effect on neoplastic cells, thus limiting cancer progression [86, 87]. Actually, SPARC function in the tumour microenvironment is deeply influenced by its cellular source. SPARC produced by stromal and immune cells may exert diversified influences over the neoplastic clone, the former contributing to cancer stromatogenesis and stromal remodelling while the latter normalizing the inflammatory milieu by negatively regulating immune cell infiltration and activation (e.g., through suppression of the NF-kB pathway) [75, 88]. SPARC has a paramount importance in the regulation of structural ECM composition [85], in which it participates as a collagen chaperon. The valency of SPARC in regulating the stromatogenesis triggered by neoplastic clones has also a considerable degree of ambiguity. Indeed, SPARC is required for the correct assembly of the collagen meshwork that provides adhesive substrate to cancer cells, yet, it might also inhibit integrin-mediated adhesion and the generation of signals stemming from the integrin-linked kinase activation [89]. Recently, some of us have reported that *SPARC* gene is highly expressed as part of the GC-related signature of BLs, where it specifically characterizes the endemic BL (eBL) subgroup [90]. Notably, SPARC-, TGF-β-, and other EMT-inducer-derived signals, including those stemming from Notch receptors, converge at the Ras-MAPK pathway, which was found upregulated in BLs in spite of a biased BCR signal initiation [90]. This picture is in line with the cellular program of BL oriented towards proliferation, migration, and ECM invasion, and poorly reliant on extracellular signals. SPARC protein expression in BL samples consistently marked neoplastic cells but also variably characterized stromal cells of the microenvironment (Figure 4) suggesting a potential involvement of this molecule in stroma-centred dynamics of BL and other GC-associated neoplasms, which haven't been so far explored [90]. In this regard, a role for SPARC produced by FDCs in orchestrating GC T-cell trafficking towards the establishment of Th-17-mediated responses has been recently demonstrated [91].

Besides Osteonectin and SPARC, many other molecules take part to the complex network created by neoplastic cells and ECM components. In BL cell lines, it has been demonstrated that the ECM protein fibronectin, following binding of alphavbeta3-integrin expressed on neoplastic cell surface, activates signal transduction pathways leading to BL cell proliferation by phosphorylation of the MAP kinase ERK-2 [92]. Similar interactions between integrins and multiple ECM binding partners, namely, vitronectin, laminin, type I and type IV collagen, have been reported to occur in different solid cancer settings and can be also predicted in aggressive lymphomas [93–96].

Matrix metalloproteases (MMPs) were at first identified as mere ECM-regulating components but their involvement in the interplay with factors other than ECM derived, such as growth factors and their receptors, cytokines and chemokines, adhesion receptors, cell surface proteoglycans, and a variety of enzymes has progressively come into evidence [97, 98]. The expression and production of different MMP subtypes in aggressive B-cell lymphomas may depend not only on the different biology of the neoplastic clone, but could be also determined by the surrounding environment [99]. IL-6 produced by reactive lymphocytes, Ms, endothelial cells, and fibroblasts induces MMP-9 and MMP-2 production that, in aggressive B-cell lymphomas, may lead to a more aggressive clinical behaviour [100].

IL-6 produced in the lymphoma microenvironment also acts as a positive regulator of tissue inhibitor of metalloproteinase (TIMP) expression by neoplastic and stromal cells. TIMPs are capable of inhibiting the activity of MMPs thus keeping the balance between ECM deposition and degradation processes; however, multifaceted and apparently paradoxical actions of TIMPs (i.e., TIMP-1 and TIPM-2) have been recently reported, suggesting their direct contribution to lymphoma progression [101].

In fact, TIMP-1 produced by neoplastic B lymphocytes, fibroblasts, and endothelial cells [102] has been shown to

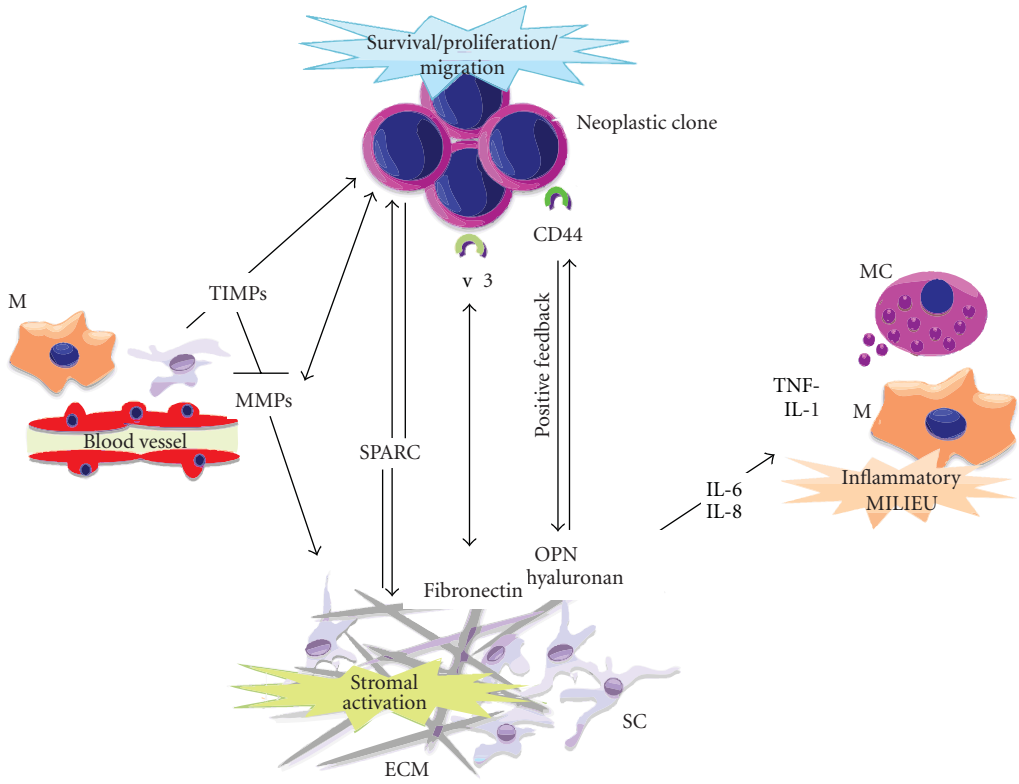

Figure 5: Extracellular matrix orchestrates a functional network through its structural and secreted elements. (M: monocyte/macrophage; MC: mast cell; SC: stromal cell; ECM: extracellular matrix. Black arrows indicate activation pathways. Red lines indicate inhibitory pathways).

inhibit germinal centre B-cell apoptosis and promote cancer cell survival in aggressive B-cell lymphomas. The antiapoptotic effect of TIMP-1, which could be considered one of the causes contributing to the poor survival evidenced in some of these cases, is not to ascribe merely to MMP inhibition and/or cell-matrix interactions, but also to the binding of TIMP-1 to other cell-surface receptors, independent of MMP inhibitory function [103].

In BL cells in which TIMP-1 promotes postgerminal centre B-cell differentiation by upregulating MUM-1 and CD138 and downregulating BCL6, its overexpression leads to the activation and expression of STAT3, and to the upregulated expression of cyclin D2, CD44 and BCL-XL, the latter being a target protein of STAT3 with prominent antiapoptotic function [104].

ECM thus emerges as much more than inert scaffolding for lymphomatous cells, representing a major source of direct "rescue" signals and also critically influencing several aspects of the lymphoma-associated environment, such as the trafficking and activation of immune cells (Figure 5). Although strategies aiming at inducing modifications in the ECM components are hardly plausible, owing to the elevated redundancy of the cellular dynamics leading to ECM regulation, ECM should be considered as a precious source of information regarding the biology of the underlying neoplasm, and ECM-related cues (such as miRNAs) should be taken into account as potential cancer-related markers for risk stratification and prognostication [105].

5. Conclusions

By delineating the main microenvironmental dynamics that take place in aggressive B-cell lymphomas, we aimed to convey the message of a leading role played by the nonneoplastic lymphoma-associated immunological and stromal elements in influencing the natural history of these highly malignant neoplasms, so far classically considered poorly reliant on the environment. The mutual influence between neoplastic B lymphocytes and their microenvironment results in the enhancement of the proliferative and invasive capabilities of the neoplastic clone and in the concurrent reshaping of the infiltrated tissues.

A deeper understanding of such relationship, through the dissection of its complex dynamics, could prove a successful enterprise for the establishment of multitargeted therapeutic approaches and for the identification of new prognostic factors reflective of the clone-extrinsic biology of these B-cell lymphomas.

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Review Article

Murine Models of B-Cell Lymphomas: Promising Tools for Designing Cancer Therapies

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Human B-cell lymphomas, the fourth most common hematologic malignancy, are currently the subject of extensive research. The limited accessibility of biopsies, the heterogeneity among patients, and the subtypes of lymphomas have necessitated the development of animal models to decipher immune escape mechanisms and design new therapies. Here, we summarize the cell lines and murine models used to study lymphomagenesis, the lymphoma microenvironment, and the efficacy of new therapies. These data allow us to understand the role of the immune system in the fight against tumors. Exploring the advantages and limitations of immunocompetent versus immunodeficient models improves our understanding of the molecular and cellular mechanisms of tumor genesis and development as well as the fundamental processes governing the interaction of tumors and their host tissues. We posit that these basic preclinical investigations will open up new and promising approaches to designing better therapies.

1. Introduction

Lymphomas are highly heterogeneous diseases, varying by both the type of malignant cell and the tumor location. They most frequently originate from B cells, and the two main groups of B-cell lymphomas, B-cell non-Hodgkin lymphomas (NHL) and Hodgkin lymphomas, account, respectively, for about 80% and 15% of all lymphomas. Of the NHL, half are diffuse large B-cell lymphomas, followed in prevalence by follicular lymphomas, marginal zone lymphomas, Burkitt's lymphomas, and mediastinal lymphomas. This heterogeneity makes it difficult to collect human samples in sufficient quantities for statistical analyses. Moreover, these samples are not easy to classify in the absence of clear discriminative parameters. In addition, some tumors, such as primary central nervous system (CNS) lymphomas, are located deep within delicate tissues, which complicates the collection of biopsy samples and complete tumor analysis [1]. Studying these tumors is thus quite challenging. Animal models are very useful, because they let us work on very homogeneous materials. They are also essential for preclinical studies and allow us to perform kinetic analyses together with detailed investigation of the tumors' characteristics and microenvironments. Here, we will review the spontaneous and induced B-cell lymphoma models that can occur in transgenic mice, or by various types of transfer of tumor cells into

wild-type mice (Figure 1). We will summarize the known categories of B-cell lymphoma mouse models and discuss their experimental and translational values. Finally, we will examine how the tumors regulate their microenvironment in different tissues and how this knowledge could be translated into practical applications for tumor therapies.

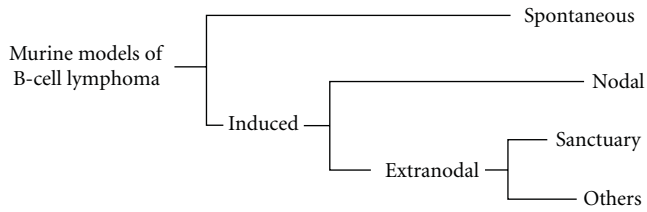


Figure 1: Schematic representation of the major subtypes of B-cell lymphoma murine models. Murine models can be either spontaneous and develop in genetically engineered mice or can be induced by implantation of a tumor cell line. In this case, it is possible to inject cells in lymph nodes (nodal location) or outside of them (extranodal location). Immune sanctuaries such as the brain or the eyes provide information about that particular situation, but many other sites can be envisaged.

2. Models for Studying Lymphomagenesis

One of the key questions about tumor development concerns the origin and the mechanisms responsible for malignant phenotypes. Various spontaneous tumor models have been developed to study how B-cell lymphomas arise and mature in different tumor environments. Observations and experiments with human tissue samples have provided some indications about the possible genetic events that might be responsible for uncontrolled B-cell proliferation. Recent advances in genetic engineering have made it possible to develop transgenic mouse models recapitulating major known modifications of the genome and to infect mice with viruses that can induce B-cell lymphomas.

The myc oncogene is the gene most frequently studied: its translocation behind an enhancer or promoter region specific for B lymphocytes can give rise to B-cell lymphomas (Table 1). The involvement of such a translocation in lymphomagenesis is studied in the most used mouse model, Eμ-Myc. In this transgenic experimental tumor model, when the myc gene is inserted into the IgH locus, B-cell lymphomas develop at a 100% incidence rate. Nonetheless, disease onset is, as in humans, highly variable (from day 32 to day 600), as is the phenotype of the tumors in different mice. More precisely, Mori and colleagues [14] have described two principal tumor phenotypes: the first type arises during an early time window and is composed mainly of immature B cells, thus resembling Burkitt's lymphoma. The second type develops very late (after day 400) and is composed of mature B cells; it is similar to diffuse large B-cell lymphomas [14]. Interestingly, if the myc gene is placed under the enhancer region of the Ig light chain genes, it results in a disease very similar to Burkitt's lymphoma in humans [7] (Table 1). Sheppard and colleagues [4] generated another transgenic mouse with the translocation of the N-myc gene under the IgH enhancer and with only a subtle modification of the endogenous myc expression level. This resulted in an indolent disease and only 25% incidence after 9 to 12 months. After infection with the murine Moloney leukemia virus, however, both the incidence and speed of tumor development were far greater. Following this idea, others developed a model that uses the Brd2 gene and can induce B-cell lymphoma in some mice after translocation but is not sufficient to obtain a high incidence. Modification of these mice by infection with a retrovirus expressing the ras oncogene also aggravated and accelerated lymphomagenesis [10] (Table 1). These results point out that translocation of the oncogene by itself is not sufficient to create a malignant phenotype. The added value of these transgenic models is that tumors develop on a syngeneic background that makes it possible to analyze the tumor microenvironment and its influence on tumor growth. For example, by studying the influence of Bcl2 overexpression in the hematologic compartment in generating a follicular B-cell lymphoma, Egle and collaborators [8] were able to determine that $CD4^+$ T cells were crucial in the proliferation of germinal center B cells and therefore in lymphomagenesis.

To understand the mechanisms linked to lymphomagenesis in more detail, researchers have introduced more strategies to transfer variously modified tumor cells into immunodeficient or immunocompromised hosts (models listed in Tables 2 and 3). These approaches introduce the potential bias of tumor injection to specific tissue sites and are unable to follow the progressive induction and development of tumors from a few malignant cells. Despite these weaknesses, tumor injection models are very useful because they let us study the impact of different mutations on tumor aggressiveness (models listed in Table 2). For example, the potential role of pax5 in lymphomagenesis was studied with cell lines deficient for this gene [17]. Using the same idea, Yu and collaborators [18] developed a strategy to determine the influence of different genes in enhancing the tumor-inducing potential of the myc gene translocation (Table 3). By mixing bone marrow from p53 null mice with a packaging cell line producing the myc-encoding retrovirus, they demonstrated that p53 inactivation together with myc overexpression was sufficient to induce B-cell lymphomas. These models could easily and rapidly be adapted to help define the effect of other genes and gene interactions on lymphomagenesis without the need to develop transgenic mice.

The diversity of the animal models listed in Tables 1–3 makes it very challenging to study the influence of different characteristics on the development of specific types of tumors in experimental animals and to draw significant conclusions about human B-cell lymphoma development. Some models are heterogeneous [14] or can only be classified depending on the differentiation stage of the tumor cells used [47], while some transgenic mice are clearly associated with specific B-cell lymphoma phenotypes. Some models are nonetheless very useful for studying human disease. For example, because NFS.V mice develop tumors that are very similar to marginal zone lymphomas [5], they provided a basis for defining its stages of progression (Table 1). Another highly relevant tumor model is the blastoid variant of mantle

Table 1: Spontaneous models of B-cell lymphoma. B-NHL: non-Hodgkin B-cell lymphoma; CLL: chronic lymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; DTG: double transgenic mice; n.d.: not determined.

Name	Lymphoma subtype/orgin	Strain (haplotype)	Major reference
B10 H-2^a H-4^bp/Wts	CLL	C57Bl/10 (H-2^b)	[2]
SL/KH	Pre-B lymphoma	SL/KH (H-2^{11})	[3]
Eμ-N-myc	Indolent B-NHL	C57Bl/6 x DBA/2 (H-$2^{b/d}$)	[4]
NFS.V+	Marginal zone lymphoma	NFS.V+ (H-2^{sq4})	[5]
NMRI/RFB-MuLV	n.d.	NMRI (H-2^q)	[6]
B6-l-MYC	Burkitt-like lymphoma	C57Bl/6 (H-2^b)	[7]
VavP-Bcl2	Follicular lymphoma	C57Bl/6 (H-2^b)	[8]
Lig4/p53 KO	Pro/Pre-B lymphoma	C57Bl/6 × sv129	[9]
Eμ-BRD2	DLBCL	FVB (H-2^q)	[10]
Bcl6 Knock in	Germinal center, DLBCL	C57Bl/6 × sv129	[11]
Bcl6/Myc transgenic	Post germinal center, DLBCL	C57Bl/6 × sv129	[11]
IL-14aTGxc-Myc TG (DTG)	Blastoid variant of mantle-cell lymphoma	C57Bl/6 (H-2^b)	[12]
Myc/BCR^{HEL}/HEL	Burkitt-like lymphoma	C57Bl/6 (H-2^b)	[13]
Eμ-myc	From follicular to DLBCL (time dependant)	C57Bl/6 × sv129	[14]
RzCD19Cre	NHL, hepatitis C induced	129/Sv (H-2^{bc}); BALB/c (H-2^d); C57Bl/6 (H-2^b)	[15]
UVB induced	Mature B-cell lymphoma	C57Bl/6 p53$^{+/-}$ (H-2^b)	[16]

cell lymphoma, developed by Ford and colleagues [12] by generation of double-transgenic mice for the IL-14a and myc genes (Table 3). A more recent model with the full genome of the hepatitis C virus introduced into CD19-expressing cells spontaneously develops human diffuse large B-cell lymphoma [15]. A nontransgenic strategy also led to the development of a model of mucosa-associated lymphoid tissue lymphoma, shown to recapitulate most human disease characteristics [48].

The major advantage of the after mentioned models is that they develop through multiple spontaneous genetic events that will help us to discover novel mechanisms of tumorigenesis. At the same time, they are also associated with some experimental limitations. First, time to onset of disease varies enormously within and between models (from day 32 to day 600 for some models), precluding the assessment of new therapies in these conditions. Moreover, as described previously, tumor incidence is high but disparate, up to 100% in some cases, but not higher than 25% in others. Furthermore, in a given model system, these tumors can vary greatly in tumor location and phenotype, which makes it difficult to compare different animals in the same experiment. While it is clear that spontaneous B-cell lymphoma models provide unique insight into tumor development, we must nonetheless bear in mind that the *in vivo* tumor generation process is quite complex. These models, together with the adoptive tumor transfer models, will be critical to our understanding of lymphomagenesis.

3. Models to Study the Lymphoma Microenvironment

The tumor microenvironment is an essential and complicating aspect of a tumor that must be better understood if more targeted treatments are to be developed [49]. Studying all the features of a malignancy requires working on syngeneic models. Moreover, reproducible models with well-characterized tumor development are important for the analysis of immune response, which remains impossible with spontaneous models. Two main questions must be addressed in developing such models: the tumor cells to be injected and the site of tumor injection, that is, nodal or extranodal, in the peripheral or central nervous system. The tumor cells are of human origin and therefore implanted into immunodeficient mice, or, more often, they are syngeneic to their host, so the tumor-induced immunity can be studied (Table 2). Lymphomas can invade many different organs in humans, especially secondary lymphoid organs and the central nervous system. Tumor cells may be injected into these organs in the mouse or directly into the blood; the latter allows spontaneous tumor colonization to different locations. The advantages of targeting a specific tissue include the possibility of comparing tumor growth between different microenvironments and determining the relative roles in tumor development of the tissue characteristics and the intrinsic tumor cell characteristics [50].

To study the microenvironment of B-cell lymphomas in different tissues, we implanted a tumor cell line derived from the well-known A20 tumor into different tissue locations, including the spleen, brain, and eyes, in syngeneic mice [45, 50]. As Figure 2 illustrates, T cells infiltrated the tumor at each location, even immune-privileged tissues, and represented up to 15% of all live cells in these sites. Moreover, antigen-presenting cells also infiltrated into the A20.IIA-GFP tumor, particularly in the brain where CD11c$^+$ dendritic cells and CD11b$^+$CD11c$^-$ macrophages accounted for a higher proportion of cells than in the tumor-bearing eye or spleen. Innate immune cells were also found in the tumor microenvironment, especially in the brain and spleen (Figure 2). The absence of spontaneous tumor rejection in these experiments

Table 2: Most common B lymphoma cell lines. B-NHL: non-Hodgkin B-cell lymphoma; DLBCL: diffuse large B-cell lymphoma; N/A: not attributable.

Name	Orgin	Strain (haplotype)	Reference
L1210	Ascitic fluid of 8 months mouse, lymphocytic leukemia cells	DBA/2 (H-2^d)	[19]
Raji	Burkitt lymphoma from an 11-year-old child (maxilla)	N/A	[20]
Jijoye	Lymphoblastic cell line derived from a 7-year old boy with Burkitt lymphoma EBV^+	N/A	[21]
Daudi	16-year-old black male with Burkitt lymphoma, orbital tumor	N/A	[22]
Ramos	Burkitt lymphoma	N/A	[23]
BJAB	Burkitt lymphoma	N/A	[24]
SU-DHL-4	DLBCL patient (peritoneal effusion of woman with B-NHL)	N/A	[25]
38C13	Carcinogen induced	C3H/HeN (H-2^k)	[26]
BCL1	Spontaneous	BALB/c (H-2^d)	[27]
A20	Spontaneous reticulum cell sarcoma of an old mouse	BALB/c (H-2^d)	[28]
CA46	Ascite fluid of a patient with Burkitt lymphoma	N/A	[29]
MC116	Undifferenciated B cell lymphoma	N/A	[29]
4TOO	Plasmacytoma originating from MPC-11 cells	BALB/c (H-2^d)	[30]
B6 spontaneous model	Spontaneous	C57Bl/6 (H-2^b)	[31]
L3055	Burkitt's lymphoma of germinal center origin	N/A	[32]
SC-1	Burkitt lymphoma	N/A	[33]
CH44	Follicular center cell lymphoma derived from B10.H-2a/H-4bp/Wts, large cell type	B10.H-2^aH-4^bp/Wts (H-2^a-4^b)	[34]
DoHH-2	Pleural fluid of 60-year old man with centroblastic non-Hodgkin lymphoma	N/A	[35]
S11	From Gammaherpesvirus-68-infected mice	BALB/c (H-2^d)	[36]
LY-ar / LY-as	Derived from the spontaneous LY-TH tumor	C3Hf/kam	[37]
Granta 519	Peripheral blood at relapse of high grade B-NHL	N/A	[38]
Pi-BCL1	Prolymphocytic, foetal liver derived	BALB/c (H-2^d)	[39]
38C13 Her2/neu	Carcinogen induced	C3H/HeN (H-2^k)	[40]
Myc5-M5	Derived from a tumor induced in p53 null mice infected with myc encoding retrovirus	C57Bl/6 (H-2^b)	[18]
Mouse lymphosarcoma cell line	Nitrosomethylurea induced	CBA (H-2^k)	[41]
FL5.12 transfected by Bcl2	IL-3-dependant BALB/c pro-B cell line	BALB/c (H-2^d)	[42]
38C13 $CD20^+$	Carcinogen induced	C3H/HeN (H-2^k)	[43]
Z138	Mantle cell lymphoma with blastoid transformation	N/A	[44]
A20.IIA-GFP / IIA1.6-GFP	Reticulum cell sarcoma	BALB/c (H-2^d)	[45]
HKBML	Brain lymphoma	N/A	[46]

indicates an immunosuppressive environment. Strikingly, nonetheless, even immune-privileged sites such as the eye or the brain were able to induce an immune response, with cellular and molecular environments similar to those of peripheral tissue sites. These findings suggest that the primary regulator of the tumor microenvironment is the tumor itself rather than the local tissue structure.

Even so, we note some features specific to CNS tumors, in particular the delay in the infiltration of T cells, especially $CD8^+$ T cells, into the eye and the brain. Moreover, we observed among these infiltrated T cells a large proportion of $CD4^+CD25^+Foxp3^+$ regulatory T cells, accounting for up to 40% of all $CD4^+$ T lymphocytes, compared with no more than 20% in the spleen [50]. In related findings, Elpek and colleagues [74], using the parental A20 cell line implanted subcutaneously, highlighted the importance of regulatory T cells in the early phase of tumor growth. Curti's group [60] used the same cell line in an intrasplenic tumor model to study the accumulation of regulatory T cells in the spleen and showed that the IDO enzyme is critical for the local conversion of conventional T cells into regulatory ones. Serafini's team [75] showed that a robust expansion of specific regulatory T cells follows intravenous injection of A20 cells and demonstrated that a population of myeloid-derived suppressive cells is responsible for this expansion. Others have also suggested that myeloid-derived cells can contribute

Table 3: Induced models of B-cell lymphoma. (m): murine origin; (h): human origin; (i): syngeneic models; (ii): syngeneic models with murine tumor cells engineered to express human antigens; (iii): xenogenic models; (iv): humanized models; CLL: chronic lymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; MALT: mucosa associated lymphatic tissue; n.d.: not determined; PCL: primary cerebral lymphoma; PCNSL: primary central nervous system lymphoma; PIOL: primary intraocular lymphoma; SCID: severe combined immune deficiency.

Injection site	Name	Lymphoma model	Recipient mice			Major reference
			Strain (haplotype)	MHC compatibility	Immune status	
Intravenous	B6 spontaneous model (m)	High-grade B lymphoma	C57Bl/6 (H-2^b)	Syngeneic (i)	Immunocompetent	[51]
	Pi-BCL1 (m)	DLBCL	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[52]
	38C13 (m)	Non-Hodgkin lymphoma	C3H/HeN (H-2^k)	Syngeneic (i)	Immunocompetent	[53]
	FL5.12 transfected by Bcl2 (m)	Non-Hodgkin lymphoma	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[42]
	A20 (m)	DLBCL	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[54]
	4TOO (m)	n.d.	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[55]
	BCL1 (m)	CLL	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[56]
	38C13 Her2/neu (m)	Non-Hodgkin lymphoma	C3H/HeN (H-2^k)	Syngeneic (ii)	Immunocompetent	[40]
	Z138 (h)	Human mantle cell lymphoma	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[57]
	BJAB (h)	Burkitt lymphoma	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[58]
	SU-DHL-4 (h)	DLBCL	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[59]
Intrasplenic	A20 (m)	DLBCL	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[60]
	A20.IIA-GFP (m)	DLBCL	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[50]
Intraperitoneal	CH44 (m)	Non-Hodgkin lymphoma	B10.H-2^aH-4^bp/Wts	Syngeneic (i)	Immunocompetent	[34]
	BCL1 (m)	DLBCL	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[53]
	38C13 (m)	Non-Hodgkin lymphoma	C3H/HeN (H-2^k)	Syngeneic (i)	Immunocompetent	[53]
	Tonsillar lymphocytes and EBV infection (h)	Viro-induced lymphoma	BNXhum (humanized)	Allogenic (iv)	Immunocompetent	[61]
Subcutaneous	LY-ar or LY-as (m)	n.d.	C3Hf/kam (H-2^k)	Syngeneic (i)	Immunocompetent	[37]
	S11 (m)	Burkitt lymphoma	BALB/c nude (H-2^d)	Syngeneic (i)	T-cell deficiency	[62]
	LMycSN-p53null (m)	Non-Hodgkin lymphoma	C57Bl/6 (H-2^b)	Syngeneic (i)	Immunocompetent	[18]
	A20 (m)	DLBCL	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[63]
	38C13 Her2/neu (m)	Non-Hodgkin lymphoma	C3H/HeN (H-2^k)	Syngeneic (ii)	Immunocompetent	[40]
	Myc5-M5 (m)	n.d.	SCID mice (H-2^d)	Allogenic	Immunodeficient	[17]
	Splenic Hodgkin lymphoma cells (h)	Hodgkin disease	Nude mice (H-2^b)	Xenogenic (iii)	T-cell deficiency	[64]
	Human hodgkin cell line (h)	Hodgkin disease	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[47]
	Ramos (h)	Burkitt lymphoma	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[58]
	BJAB (h)	Burkitt lymphoma	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[58]
	SC-1 (h)	Follicular lymphoma	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[58]
	DoHH-2 (h)	Follicular lymphoma	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[58]
	SuDHL-4 (h)	DLBCL	C.B-17 SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[65]
	Granta 519 (h)	Mantle cell lymphoma	C.B-17 SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[65]
	HKBML (h)	Brain DLBCL	C.B-17 SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[46]

Table 3: Continued.

Injection site	Name	Lymphoma model	Recipient mice Strain (haplotype)	Recipient mice MHC compatibility	Immune status	Major reference
	Daudi (h)	Burkitt lymphoma	SCID/beige mice (H-2^d)	Xenogenic (iv)	Partially rebuilt	[66]
	Jijoye (h)	Burkitt lymphoma	SCID/beige mice (H-2^d)	Xenogenic (iv)	Partially rebuilt	[66]
Intramuscular	MSV-MuLV-M induced	Waldenstrom's macroglobulinemia	C57Bl/6 (H-2^b)	Syngeneic	Immunocompetent	[67]
	Mouse lymphosarcoma cell line (m)	Non-Hodgkin lymphosarcoma	CBA (H-2^k)	Syngeneic (i)	Immunocompetent	[41]
Stomach	Helicobacter felis	MALT lymphoma	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[48]
Intracerebral	A20.IIA-GFP (m)	PCL (PCNSL)	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[50]
	38C13 CD20$^+$ (m)	PCL (PCNSL)	C3H/HeN (H-2^k)	Syngeneic (ii)	Immunocompetent	[68]
	Raji (h)	PCL (PCNSL)	Nude mice (H-2^b)	Xenogenic (iii)	T-cell deficiency	[69]
	Patient's cells (h)	PCL (PCNSL)	Nude mice (H-2^b)	Xenogenic (iii)	T-cell deficiency	[70]
	MC116 (h)	PCL (PCNSL)	Nude rats (RT1^u)	Xenogenic (iii)	Immunodeficient	[71]
Cisterna magna	L1210 (m)	Leptomeningeal metastases	DBA/2 (H-2^d)	Syngeneic (i)	Immunocompetent	[72]
Intraocular	A20.IIA-GFP (m)	PIOL	BALB/c (H-2^d)	Syngeneic (i)	Immunocompetent	[45]
	38C13 CD20$^+$ (m)	PIOL	C3H/HeN (H-2^k)	Syngeneic (ii)	Immunocompetent	[68]
	CA46 (h)	PIOL	SCID mice (H-2^d)	Xenogenic (iii)	Immunodeficient	[73]

to the tumor immune response, as when M2 macrophages infiltrate the B-cell lymphoma growing in the brain after implantation of human Raji cells into nude mice [76].

All these results show that B-cell lymphomas induce immunosuppressive cells, although how these cells contribute to tumor growth remains unknown. It is assumed that suppressive myeloid cells can influence the molecular milieu of the tumor. We and others have shown the production of various anti-inflammatory mediators, depending on the experimental setting, including IL-10 [74], IL-4 [76], and soluble receptor for IL-2 [15]. Additionally, as Figure 2 shows, we found that T cells from tumor-bearing mice do not produce the proinflammatory cytokines IFNγ or GM-CSF without stimulation, except in the spleen where resident T cells might be responsible for this secretion. After polyclonal stimulation we observed an unbalanced Th1/Th17 profile, with high levels of IFNγ, GM-CSF, and IL-17 and low levels of IL-2, IL-4, and IL-10 [45, 50].

In summary, it is clear that tumor cells closely regulate the microenvironment of B-cell lymphomas. It is advantageous for the tumor to generate a suppressive environment for optimal tumor growth. Understanding the balance between pro- and anti-inflammatory mediators that can contribute to or control tumor growth is essential for designing novel tumor therapies.

4. Models to Analyze the Efficacy of New Therapies

4.1. Assessment of Treatment Efficacy. In recent years, tumor therapies have achieved substantial but still incomplete success. It is generally accepted that well-characterized human tumor cell lines and *in vivo* animal models are required to develop novel antitumor treatments. Basically, four types of models have been developed (Table 3): (i) syngeneic murine models, (ii) syngeneic models with murine tumor cells engineered to express human antigens, (iii) human cells implanted into immunodeficient mice, and (iv) humanized mice, that is immunodeficient mice reconstituted with human immune system and then implanted with human tumor cells.

The standard treatment for aggressive B-cell malignancies is the combination of four chemotherapy agents (i.e., cyclophosphamide, doxorubicin, vincristine, and prednisolone) with rituximab, also called R-CHOP therapy [77]. Because B-lymphoma cells express the CD20 antigen, they are a suitable target for anti-CD20 monoclonal antibodies (mAb) such as rituximab. The use of this chimeric mAb has enhanced the survival of patients with different B-cell malignancies, after various studies confirmed its therapeutic potential. Most of those studies used murine models of human tumor cell lines implanted into immunodeficient mice. For example, the efficacy of rituximab against disseminated Burkitt lymphoma Daudi cells and against DLBCL SU-DHL4 cells was assessed in SCID mice, and the therapeutic advantages varied with the cell line [59]. Hernandez-Ilizaliturri and colleagues [78] also used SCID mice, with the Raji cell line, and obtained better results: rituximab treatment enabled 60% of the animals to reject their tumors. This great potential has led many laboratories to seek to improve this efficacy by designing new engineered antibodies, such as EMAB-6 [79] or the humanized GA101 used against the human SU-DHL4 tumor implanted into SCID/beige mice [80]. Coupling rituximab with other therapies has also been evaluated

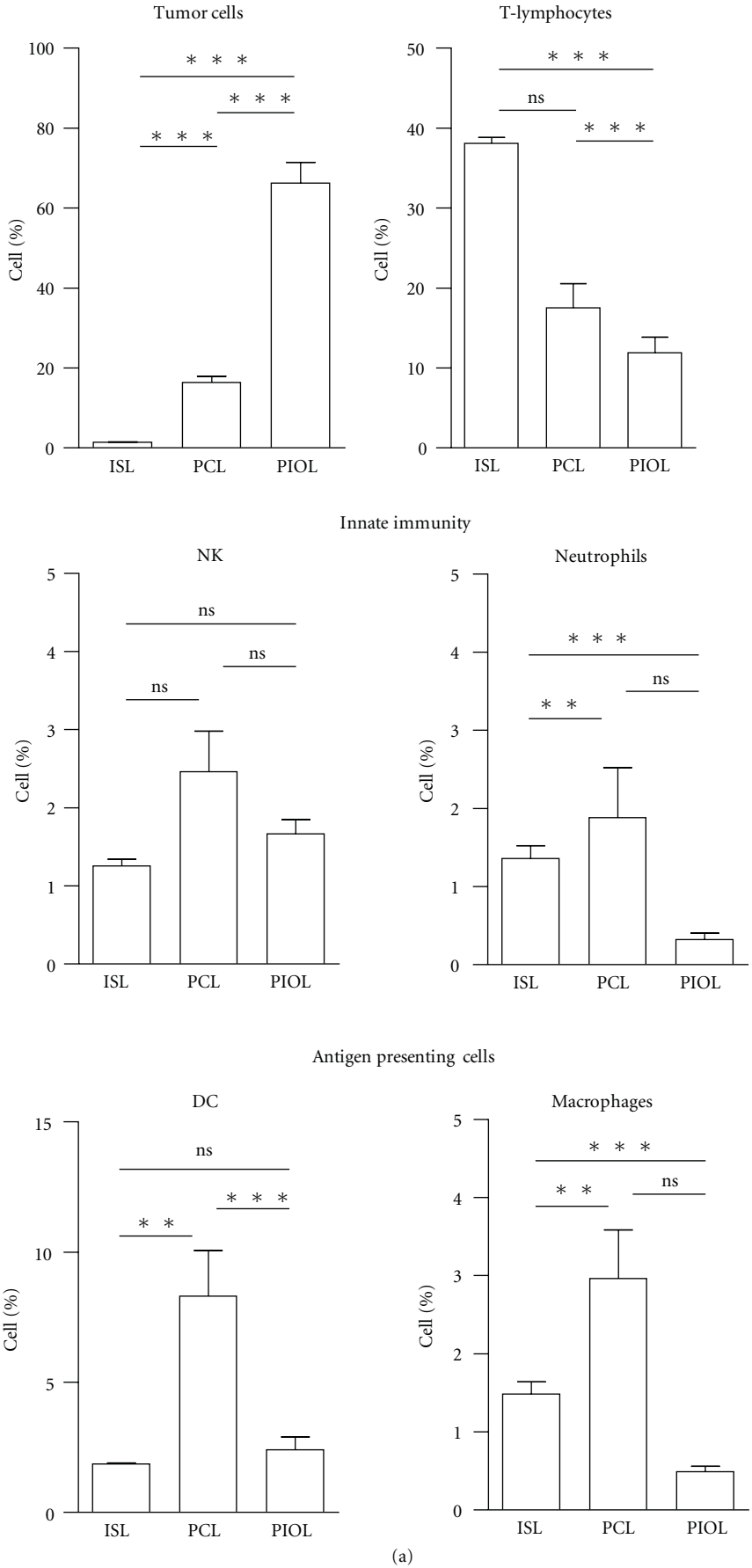
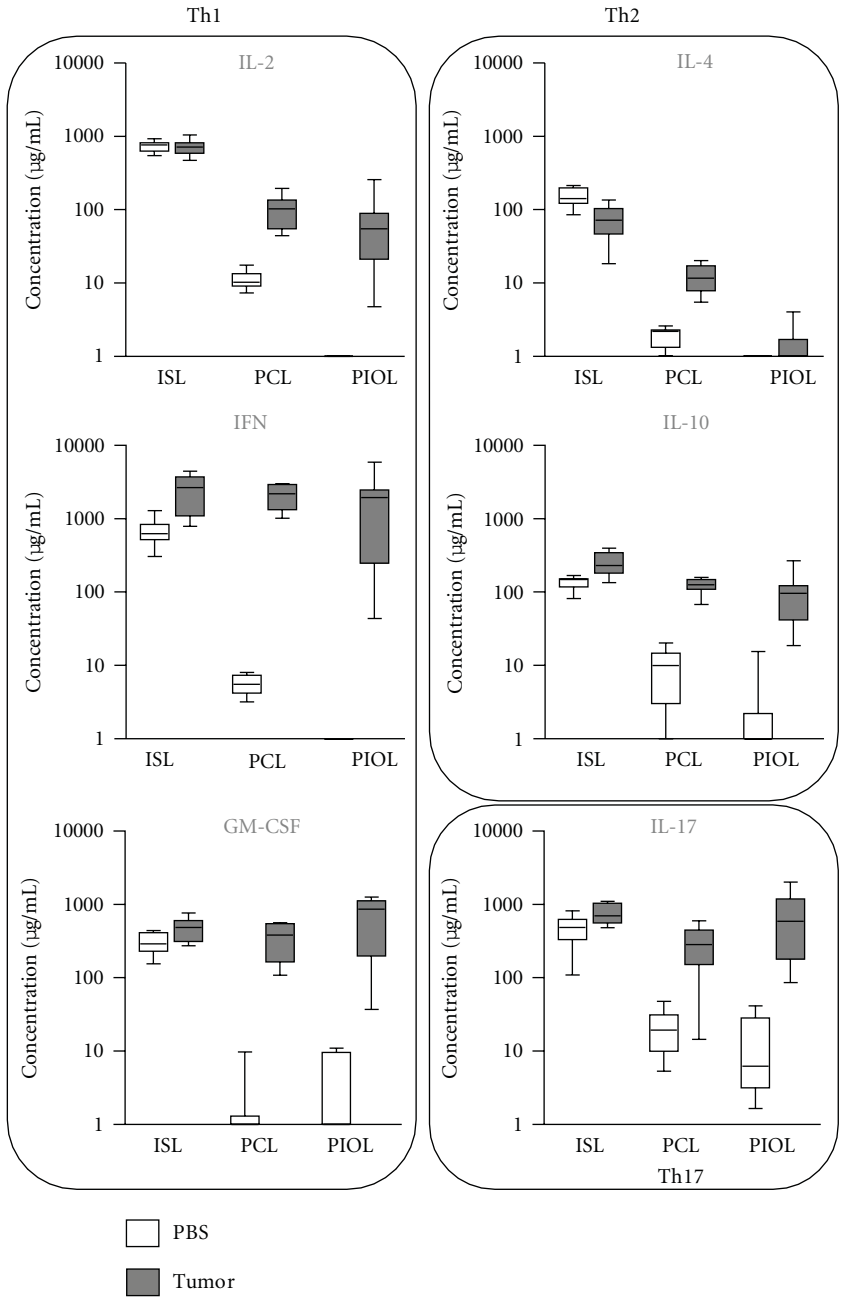


Figure 2: Continued.



(b)

Figure 2: Comparison of the cellular and molecular immune environment of a B cell murine lymphoma implanted in the spleen, in the brain or in the eye. (a) A20.IIA-GFP cells were implanted in immunocompetent syngeneic mice in the spleen (intrasplenic lymphoma model: ISL), in the brain (primary intracerebral lymphoma model: PCL), or in the eye (primary intraocular lymphoma model: PIOL). 21 days after injection, tumor-bearing organs were analyzed by flow cytometry for the presence of GFP^+ tumor cells, $CD3^+$ T lymphocytes, $NKp46^+$ NK cells, $Gr1^+$ neutrophils, $CD11c^+$ dendritic cells, and $CD11b^+CD11c^-$ macrophages. Results are represented as the proportion of the different populations among total living cells ($n = 10$). (b) 21 days after lymphoma (gray boxes) or PBS (white boxes) injection, cells were isolated from appropriate tissues and stimulated for 36 h with anti-CD3/CD28-coated Dynal beads. Secretion of IL-2, IFNγ, GM-CSF, IL-4, IL-10, and IL-17 in the culture supernatant was evaluated by cytokine bead arrays (BD Biosciences) ($n = 10$). Animal studies were conformed to European Union guidelines and were approved by the Charles Darwin Ethics Committee in Animal Experiment, Paris, France.

in these murine models of human tumors, as, for example, with R-CHOP therapy [81], or the TLR9 agonist CpG against Daudi cells [82]. Advances in understanding the biology of tumor cells and the tumor microenvironment have led to the design of new therapies, combined or not with preexisting ones (for review, see [83]). All of these strategies have been evaluated by implanting human cell lines into immunodeficient mice. They include the application of immunomodulatory drugs ([84]: subcutaneous Raji cells into SCID mice), inhibition of antiapoptotic signals ([65]: different cell lines into SCID or SCID/beige mice), or inhibition of specific metabolic or signaling pathways ([69]: intracerebral Raji cells into nude mice; [46]: subcutaneous HKBML cells into SCID mice). New strategies are also evaluated in this way, including the use of immunoconjugates ([73]: intraocular CA46 cells into SCID mice; [85]: intravenous Raji cells into SCID mice), or reoviruses, which target cells expressing high levels of the ras oncogene ([86]: subcutaneous Daudi and Raji cells into SCID/nod mice).

The major disadvantage of the experimental models described in this subsection is that they involve immunodeficient hosts that lack the adaptive immunity present in tumor microenvironment in humans, that do not reflect the complexity of human diseases.

4.2. Assessment of Immune System Involvement in Tumor Rejection. The role of the immune system is essential in tumor rejection. Syngeneic models, besides their utility in analysis of the tumor microenvironment, are particularly helpful for studying how specific treatments modulate the immune system or particular components of it. One of the indicators studied most frequently after treatment is the quantity of T lymphocytes, especially $CD8^+$ T cells, infiltrating the tumor [87]. In an A20 B-cell lymphoma model implanted subcutaneously, treatment by survivin or an idiotype-binding peptide, is correlated with increased $CD8^+$ T-cell infiltration [63, 88]. Some therapies are clearly designed to boost immune response. For example, in the 4TOO intravenous model, administration of a vaccine composed of a fusion between dendritic cells and tumor cells increases T-lymphocyte proliferation and promotes the secretion of IL-2, IL-6, and IFNγ (model listed in Table 3). In this model, significant amounts of IL-17 are found, which suggests that Th17-expressing cells contribute to tumor rejection [55]. Houot and Levy [87], in a two-site subcutaneous A20 model, tried to design an antitumor therapy based on the intratumoral inoculation of CpG and two T-cell-modulating mAbs. They obtained strong response rates and were able to demonstrate the involvement of $CD4^+$ T cells in controlling the primary tumor site and the role of $CD8^+$ T cells in controlling distant tumors.

Another significant advantage of murine models is the abundance of different mutant mice, deficient for well-characterized molecules or cell populations. For example, a subcutaneous Raji lymphoma model implanted into Fcγ-receptor-deficient mice demonstrated the importance of Fc receptors in the therapeutic efficacy of cytotoxic antibodies and the abolition of the anti-CD20 rituximab antitumor effect [89]. Additionally, Flynn and Stockinger [90] studied the role of specific $CD4^+$ populations in the subcutaneous LK35 tumor model with $Rag^{-/-}\gamma c^{-/-}$ mice that lack all lymphocytes and NK cells. They demonstrated that memory T cells were capable of controlling tumor growth initially, without the help of other components of the immune system. However, immune pressure eventually led to selection of tumor cells unable to present antigens, which resulted in turn in paralysis of the T-cell response.

Another potential way to obtain data more relevant to human tumors is to use murine cell lines expressing specific human tumor antigens, which would make it possible to test monoclonal antibodies against specific antigens. For example, the murine 38C13 cell line expressing the human CD20 antigen has been used to evaluate the therapeutic potential of rituximab against intravenously injected disseminated tumors [43] and against central nervous system tumors [68]. The authors showed in the first case [43] that depletion of neutrophils, NK cells, and macrophages did not influence antibody efficacy, but complement inhibition abolished this effect. More recently, others have used the EL4 thymoma cell line transfected with this same human CD20 antigen and the luciferase gene to monitor tumor regression after rituximab therapy [91, 92]. For now, the best system for studying tumor rejection appears to involve the reconstitution of immunodeficient mice with human immune cells to generate humanized mice, followed by the implantation of human tumor cells into these experimental animals. Sato and collaborators [93] developed this model to define the role of the complement system in the efficacy of an optimized variant of rituximab. In another example, human Daudi cells were implanted into SCID mice that were reconstituted with human peripheral blood leukocytes to assess the efficacy of a vaccine composed of immature dendritic cells and antihuman CD40 mAb [94]. This study demonstrated the rate at which activated $CD8^+$ cytotoxic T lymphocytes infiltrate Burkitt lymphomas and the high level at which these cells secrete IFNγ after the injection of this vaccine. More recently, a model was developed to test the efficacy of an agonistic anti-CD40 antibody against different subcutaneous B-cell tumor cell lines, such as Daudi, Raji, and Jijoye, implanted into SCID/beige mice. Although this type of treatment by itself reduced tumor size, it was much more effective after reconstitution of the mice with human T cells and dendritic cells [66].

In summary, antitumor immune responses hold great promise for boosting tumor therapy. More appropriate models are needed to explore the possibility of dendritic cell vaccination therapies in combination with specific tumor cell targeting.

5. Conclusion

Recent experimental animal models that allow us to study the induction and development of human tumors are important achievements. Only with a deeper understanding of the molecular and cellular mechanisms leading to tumor genesis and development of tumor microenvironments can we design better therapies. Despite the advances with the animal models described in this paper, several questions remain open.

Given the difficulty in comparing murine tumors and human malignancies, more relevant models are needed. As this paper shows, there are many different models that seek to mimic human disease, but no consensus exists for any given model. Most tumor cells and spontaneous models require better characterization from histologic, phenotypic, genetic, and immunologic perspectives. Parallels with human diseases are also complicated by the absence of a clear classification of B-cell lymphomas. One interesting future challenge will be to develop a humanized murine model that can be implanted with human tumors and reconstituted with a complete human immune system for each main subclass of B-cell lymphomas. The remarkable impact that these models have had on the development of novel tumor therapies justifies the aggressive pursuit of basic and preclinical investigations to develop more appropriate animal models and unravel the fundamental processes governing the interaction of tumors with host tissues.

Abbreviations

B-NHL: B-cell non-Hodgkin lymphomas
CLL: Chronic lymphocytic leukemia
CNS: Central nervous system
DLBCL: Diffuse large B cell lymphoma
MALT: Mucosa associated lymphatic tissue
PCL: Primary cerebral lymphoma
PCNSL: Primary central nervous system lymphoma
PIOL: Primary intraocular lymphoma
SCID: Severe combined immune deficiency.

Author's Contribution

S. Donnou, C. Galand and V. Touitou performed experiments and analysed the data; S. Fisson conceived the study; S. Donnou wrote the paper; C. S. Fridman, Z. Fabry and S. Fisson contributed to the writing and to the critical reading of the paper.

Conflict of Interests

The authors declare that they have no financial conflict of interests.

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Review Article

AIDS-Related Non-Hodgkin's Lymphoma in the Era of Highly Active Antiretroviral Therapy

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In economically developed countries, AIDS-related lymphoma (ARL) accounts for a large proportion of malignances in HIV-infected individuals. Since the introduction of highly active anti-retroviral therapy (HAART) in 1996, epidemiology and prognosis of ARL have changed. While there is a slight increase in the incidence of Hodgkin's lymphoma in HIV-infected individuals, use of HAART has contributed to a decline in the incidence of non-Hodgkin's lymphoma (NHL) and also a decrease in the overall incidence of ARL. Strategies that employ HAART, improved supportive care, and the use of Rituximab with multi-agent chemotherapy have contributed to improved rates of complete remission and survival of patients with ARL that rival those seen in stage and histology matched HIV negative NHL patients. Most recent clinical trials demonstrate better outcomes with the use of rituximab in ARL. Tumor histogenesis (germinal center vs. non-germinal center origin) is associated with lymphoma-specific outcomes in the setting of AIDS-related diffuse-large B cell lymphoma. High-dose chemotherapy (HDCT) and autologous stem cell rescue (ASCT) can be effective for a subset of patients with relapsed ARL. HIV sero-status alone should not preclude consideration of ASCT in the setting of ARL relapse. Clinical trials investigating the role of allogeneic hematopoietic stem cell transplant in ARL are currently underway.

1. Introduction

Non-Hodgkin's lymphoma (NHL) has been associated with human immunodeficiency virus (HIV) infections since the beginning of the acquired immune deficiency syndrome (AIDS) epidemic. The initial case definition of AIDS by the US Centers for Disease Control and Prevention (CDC) in 1982 included AIDS-defining malignancies such as Kaposi's sarcoma and primary central nervous system lymphoma (PCNSL), with subsequent inclusion of peripheral intermediate and high-grade B-cell NHL [1]. People living with HIV/AIDS (PLWA) are also at a significantly greater risk of developing Hodgkin's disease compared to the general population but this has not yet been added to the CDC case definition of AIDS [2].

HIV seropositivity increases the risk of developing NHL by 60–165-fold [3, 4]. AIDS-related lymphomas (ARLs) tend to present with high-grade B-cell histology, advanced-stage disease, and an aggressive clinical course. Prior to the advent of highly active antiretroviral therapy (HAART) in 1996, ARL was associated with a dismal prognosis, particularly in those patients who had compromised performance status, advanced immune dysfunction, and limited hematopoietic reserve. With the introduction of HAART, the survival of patients with ARL has improved substantially and appears to be comparable to that of their HIV-negative NHL counterparts. Advances in chemotherapy regimens, antiretroviral drugs, and supportive care have led to more aggressive management of ARL compared to the pre-HAART era. Several ARL chemotherapy trials have incorporated the anti-CD20 monoclonal antibody rituximab with multiagent chemotherapy; however, optimal therapy of ARL is still not clearly defined [5–12].

In this paper, we highlight several current strategies for treatment of AIDS-related non-Hodgkin's lymphoma including specific systemic chemo-biologic therapies. We

also briefly review various prognostic tools and factors which influence therapeutic outcomes and potential for treatment-related toxicities. We do so through a systematic review of peer-reviewed publications identified through searches of MEDLINE/PubMed from July 2005 to June 2011. The ongoing phase II and phase III trials for ARL were searched from the US National Institute of Health's web resource, http://clinicaltrials.gov/, a registry of clinical trials conducted in the United States and worldwide. Keywords were used alone and with the modifiers of *treatment, novel therapies, AIDS-related lymphoma, prognostic and biomarkers,* and *HIV/AIDS*. Bibliographies from these references were reviewed. Criteria used for study selection included study design, English language, and relevance to clinicians.

2. Pathobiology of ARL

ARL are comprised of a narrow spectrum of histologic types consisting almost exclusively of aggressive B-cell tumors, derived from either germinal centers or postgerminal centers. While majority of tumors are diffuse large B-cell lymphomas (DLBCLs), a few ARL are B-cell immunoblastic lymphomas and Burkitt's or Burkitt's-like small noncleaved lymphoma. Even more rare are PCNSLs. The World Health Organization has classified HIV-related lymphomas into three categories: lymphomas also occurring in immunocompetent patients, those specifically occurring in HIV-positive patients, and those occurring in other immunodeficiency states (Table 1) [13].

HIV creates a milieu of combined immune suppression and chronic antigenic stimulation in lymph nodes [14]. This environment with dysregulated cytokine release and impaired dendritic function, along with presence of concomitant infection (e.g., Epstein Barr Virus [EBV], Human Herpes Virus type 8 (HHV-8), and cytomegalovirus) may promote a permissive environment for HIV-induced polyclonal B-cell expansion and impaired T-cell immunosurveillance, culminating in lympho-proliferative disorders [15].

The cytokine profile of HIV-infected individuals at risk for ARL demonstrates increased markers of B-cell activation such as interleukin- (IL-) 6, IL-10, and soluble CD-30 compared to HIV-positive controls who do not develop ARL [16]. Although the molecular mechanisms responsible for B-cell transformation associated with ARL have not been completely elucidated, many different ARL-associated molecular lesions have been described, including chromosome translocation and activation of the C-*MYC* oncogene, inactivation of p53 tumor-suppressor gene, somatic mutations in Bcl-6, and overexpression of EBV oncoprotein 4 (e.g., latency membrane proteins 1 and 2). These chromosome breaks and/or molecular lesions likely have significant downstream effects which lead to impaired lymphocyte differentiation and cell-cycle control [15, 17]. Recently, preclinical studies and genome-wide DNA profiling of ARL demonstrated that B-cell-receptor-related signaling is frequently disrupted in DLBCL tumor tissues of PLWA compared to DLBCL in immunocompetent patients. This suggests HIV-associated B-cell dysregulation, and aberrant tumor-specific intracellular signaling may be important in promoting a subset of ARLs [18, 19]. Use of HAART has been associated with a significant reduction in ARL risk [4, 20, 21]. HAART may modify lymphomagenic stimuli in several ways, such as improving responsiveness of EBV-specific cytotoxic T-cell lymphocytes, increasing EBV-specific antibodies, and stabilizing immune perturbations that may contribute to B-cell proliferation [19, 22].

Table 1: Who Classification of lymphoid malignancies associated with HIV infection.

Lymphomas also occurring in immunocompetent patients
Burkitt and Burkitt-like lymphomas
Diffuse large B-cell lymphomas
Centroblastic
Immunoblastic (including primary CNS lymphoma)
Extranodal MALT lymphoma (rare)
Peripheral T-cell lymphoma (rare)
Classical Hodgkin lymphoma
Lymphoma occurring more specifically in HIV-positive patients
Primary effusion lymphoma
Plasmablastic lymphoma of the oral cavity
Lymphoma occurring in other immunodeficiency states
Polymorphic B-cell lymphoma (PTLD-like) (rare)

MALT: marginal zone lymphoma of mucosa-associated lymphoid tissue; PTLD: posttransplant lymphoproliferative disorder; CNS: central nervous system.
Source: [13].

3. Prognostic Factors for AIDS-Related Lymphoma in the Pre-HAART and HAART Era

Early in the AIDS epidemic the clinical course of ARL was dominated by advanced stage disease, concomitant and life-threatening opportunistic infections (OIs), and poor response to treatment. Efforts to treat patients with ARL using aggressive and complex chemotherapy regimens led to unacceptable toxicity and early death while low-dose chemotherapy regimens yielded modest benefit—only 10% of patients survived for 2 years [6]. Various treatments led to dismal outcomes with the majority of patients succumbing to advancing NHL or superimposed OIs [7, 23–25]. Adverse prognostic factors for ARL included a CD4+ count of less than 100 cells/μL, age older than 35, history of injection drug use, poor performance status, elevated serum lactate dehydrogenase (LDH), advanced stage of disease, and prior AIDS diagnosis [26]. Expectations for survival were dismal with a median overall survival (OS) of 46 weeks in "good prognosis" patients with a score of 0 or 1 and just 18 weeks in "poor prognosis" patients with a score of 3 or 4.

Control of HIV viral replication through HAART has emerged as a major positive prognostic factor for patients with ARL with several studies showing dramatic improvements in OS among those who received HAART [27–30]. The international prognostic index (IPI) which incorporates

age, performance status, tumor state, serum LDH, and the number of sites of extranodal disease is a useful means of stratifying risk in aggressive lymphoma in both the non-HIV and HIV settings [31, 32]. In the HAART era, further refinements to prognostic factors have incorporated CD4+ cell counts to IPI as well as use of effective HAART. A multivariate analysis in patients with ARL showed that those with the most favorable IPI and a CD4+ count of >100 cells/μL had a predicted 1-year survival of 82% compared to 15% in those with an unfavorable IPI and CD4+ count <100 cells/μL [33]. Furthermore, in a retrospective study of 192 patients, the complete response rates and the median OS for ARL had improved to 57% and 43.2 months, respectively, in the HAART era compared to 32% and 8.3 months in the pre-HAART era [30]. The importance of HAART and CD4+ cell count was also underscored in a prospective study of 485 ARL patients undergoing risk-adaptive intensive chemotherapy [24]. A time-dependent covariates analysis showed that the significant factors for OS were HAART therapy, HIV score (based on performance status, prior AIDS diagnosis, and CD4+ count <100 cells/μL), and the IPI [24]. More recently, a report by the Collaboration of observational HIV epidemiological research Europe (COHERE) study group showed that risk factors for death in ARL included low nadir CD4+ cell counts and a history of injection drug use [4].

4. Treatment of Newly Diagnosed ARL

ARLs are aggressive lymphomas and roughly 80% of patients present with advanced stage disease. Half of these patients will have gastrointestinal involvement and 30% will have bone marrow involvement [3, 14]. In addition, approximately 5 to 20 percent of patients with ARL have spread to the central nervous system (CNS) at the time of presentation, typically in the form of lymphomatous meningitis [34]. It is not clear that patients with HIV infection are inherently at higher risk for leptomeningeal involvement. It is more likely that the high incidence of CNS disease is related to the greater frequency of extranodal disease and Burkitt histology [35]. Lymphomatous meningitis may also present at the time of disease recurrence, particularly in patients at high risk for occult leptomeningeal involvement not treated with intrathecal prophylactic therapy during initial treatment of the lymphoma.

Several prospective studies have reported using regimens such as cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP); methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, and dexamethasone (m-BACOD); infusional cyclophosphamide, doxorubicin, and etoposide [6, 7, 23, 36]. These studies have combined the high-grade DLBCL and Burkitt or Burkitt-like lymphoma subtypes with little difference in outcome among the various histologic subtypes. Patients who were most likely to achieve a complete remission with chemotherapy were those who had less disease burden, no bone marrow or CNS involvement, no prior AIDS-defining illness, and an adequate performance status.

The introduction of HAART has led to dramatic improvement s in morbidity and mortality for PLWA. Such individuals now have an OS that is near or comparable to that of HIV seronegative individuals [21, 37].

The use of HAART has facilitated the use of standard-dose and even dose-intensive chemotherapy regimens with reasonable safety to patients with ARL. With use of HAART, leading to improvements in HIV viral suppression and patients' immune status, more recent studies have demonstrated that patients with ARL can be treated with standard-dose lymphoma protocols without experiencing undue toxicity [10, 23, 38, 39]. In addition, patients with ARL who receive chemotherapy now achieve a median OS comparable to the outcome in the non-immunosuppressed population [21, 23, 24, 40].

The United States National Cancer Institute (NCI)-sponsored AIDS Malignancy Clinical Trials Consortium (AMC) compared dose-reduced or full-dose cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) with concomitant HAART with complete remission (CR) of 30% and 48% in the reduced dose and full-dose groups, respectively [33]. The German ARL study group also reported results of full-dose CHOP in patients with ARL stratified into standard- (0-1 factors) and high- (2-3 factors) risk based on CD4+ count <50 cells/μL, prior OI and performance status $\geq$3. Standard-risk group patients had a CR of 79%, similar to that achieved in non-HIV infected patients with similar histologically aggressive lymphomas, while the CR was 29% in the high-risk group [32]. A more recent study by Groupe d'Etude des Lymphomes de l'Adulte (GELA) and Gruppo Italiano Cooperativo AIDS e Tumori (GICAT) reported on results of risk-adapted intensive chemotherapy in ARL patients [30]. A total of 485 patients were randomly assigned to chemotherapy after stratification according to "HIV score" based on performance status, prior AIDS diagnosis, and CD4+ count of <100 cells/μL. A total of 218 good-risk patients (HIV score 0) received ACVBP (doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisolone) or CHOP; 177 intermediate-risk patients (HIV score 1) received CHOP or low-dose CHOP (Ld-CHOP); 90 poor-risk patients (HIV score 2-3) received Ld-CHOP or VS (vincristine and steroid). The 5-year OS in the good-risk group was 51% for ACVBP versus 47% for CHOP; in the intermediate-risk group, 28% for CHOP versus 24% for Ld-CHOP; in the poor-risk group, 11% for Ld-CHOP versus 3% for vs. The only significant factors for OS were HAART therapy, HIV score, and the IPI score, but not chemotherapy regimen, suggesting that in ARL patients, HIV score, IPI score, and HAART affect survival but not the dose intensity of the CHOP-based chemotherapy.

Infusional therapy may overcome some drug resistance and high tumor proliferation in aggressive lymphomas. A study of infusional dose-adjusted EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin) in 39 newly diagnosed ARL reported a CR in 74% of patients, and at a median followup of 53 months, disease-free survival (DFS), and OS of 92% and 60%, respectively [9]. An alternative infusional regimen which incorporated CDE (cyclophosphamide, doxorubicin, and etoposide) was employed by the US Eastern Cooperative Oncology Group (ECOG). Study (E1494) enrolled 98 patients with ARL. At

two-year followup a CR of 45% was achieved and failure-free survival (FFS) and OS were 36% and 43%, respectively [7].

A number of recent clinical trials have further sought to combine anti-CD20 chimeric monoclonal antibody (rituximab) with ARL-based chemotherapy based on its superior efficacy when added to treatment of lymphoma in non-HIV infected patients. In a pooled analysis of three ARL prospective phase II studies evaluating rituximab in combination with infusional CDE, the CR was 70% and the estimated 2-year FFS and OS rates were 59% and 64%, respectively [39]. An increased risk of life-threatening infections was noted; 14% of patients were diagnosed with OIs during or within 3 months of completion of R-CDE, and 8% of patients died from infectious complications. In the multicenter AMC 010 phase III trial, 150 ARL patients were randomized at a ratio of 2 : 1 to receive CHOP and rituximab or CHOP only [10]. Results were provocative—with a median followup of 137 weeks there was a non-statistically significant trend towards improvement in time-to-progression (TTP: 125 weeks versus 85 weeks), progression-free survival (PFS: 45 weeks versus 38 weeks), and OS (139 weeks versus 110 weeks). Survival was significantly influenced by CD4+ count and IPI score. Although there was a trend towards a higher CR rate for those patients treated in the rituximab arm (58% versus 47%, $P = 0.147$), there were also substantially more deaths due to treatment-related infections in the rituximab arm (14% versus 2%, $P = 0.027$) with the majority of those deaths occurring in patients with CD4+ counts <50 cells/μL at study entry.

In contrast to AMC 010, a phase II study from France did not show an increase in the risk of life-threatening infections while treating newly diagnosed ARL patients with R-CHOP [10]. In this cohort of 61 patients, R-CHOP produced a CR of 77% and a 2-year OS of 75% with only 1 patient death attributed to infection.

Rituximab has also been evaluated in conjunction with infusional EPOCH. In a randomized phase II AMC trial 034, rituximab was given either concurrently just before each infusional EPOCH chemotherapy cycle or sequentially (weekly for 6 weeks) after completion of all chemotherapy (4–6 cycles) in ARL [12]. CR was 73% in the concurrent arm, compared to 55% in the sequential arm. Toxicity was comparable in the 2 arms, although patients with a baseline CD4+ <50 cell/μL had a high infectious death rate in the concurrent arm. The investigators concluded that concurrent rituximab and infusional EPOCH is an effective regimen for ARL that merits further evaluation, but that caution is needed for those patients with severe immunodeficiency.

To summarize, rituximab has been shown to improve response rates and overall survival for patients with aggressive NHL who were HIV negative and treated with CHOP, without an increased risk of infectious complications. Rituximab, when given sequentially with chemotherapy, is associated with improved control of ARL; however, in a single clinical trial, the use of rituximab accounted for a higher risk of severe infection than has been seen for patients with NHL who were HIV negative. Several more recent ARL studies combining chemotherapy with rituximab have not, however, led to an increased risk of death due to infectious complications.

Most oncologists favor administration of rituximab with chemotherapy, although caution should be exercised for using rituximab in severely immunosuppressed patients with CD4+ counts of less than 50 cells/μL. Mature results from an ECOG study which randomizes non-HIV-infected patients with DLBCL to R-EPOCH or RCHOP will likely influence the way ARL is treated [41]. R-CHOP is more commonly offered in community settings because of ease of administration, because of physician familiarity with this regimen, and because no other regimen has, in a randomized and prospective study, outperformed R-CHOP for the treatment of DLBCL. However, oncologists who have substantial experience with infusional therapy may reasonably choose R-EPOCH based on favorable phase II data.

Several ongoing clinical trials are evaluating novel chemotherapeutic combinations with biologic agents in this setting of ARL as listed in Table 2.

The appropriate timing of institution of HAART in relationship to multiagent chemotherapy has been evaluated in several clinical trials but with no clear answers. In a small study involving ARL patients, antiretroviral control was a significant factor in the ability to attain CR [38]. In a more recent trial evaluating safety and efficacy of liposomal doxorubicin when substituted for doxorubicin in the CHOP regimen effective HIV viral control during chemotherapy was associated with significantly improved survival, but CRs were attained independent of HIV viral control [23]. The AMC 010 study reported the feasibility of combining R-CHOP with concomitant HAART in 65 patients. Only one OI occurred during chemotherapy administration. Cyclophosphamide clearance was reduced compared with historical controls, without clinical significance. Additional studies of CHOP-based chemotherapy and HAART have yielded median survival periods of approximately 2 years [27, 29, 42]. For patients who continue HAART while receiving chemotherapy, CD4+ cell counts decline by 50%, but these values gradually improve and typically return to baseline within 6 months to 1 year of completing lymphoma treatment [43]. For patients who continue to receive effective HAART during chemotherapy, HIV viral replication tends to remain suppressed below the limits of detection using commercial-based polymerase chain reaction assays.

Chemotherapy without concomitant antiretroviral therapy also has been studied in the HAART era. Reasons for HAART omission include concerns of drug interactions with chemotherapy and poor patient adherence because of nausea or vomiting. This could lead to heightened toxicity and the emergence of multidrug-resistant HIV quasispecies. In the NCI EPOCH study, HIV viral load increased and CD4+ cell counts decreased while patients received chemotherapy, but both parameters improved rapidly following the reintroduction of HAART at the completion of chemotherapy [12]. In the hands of experienced investigators, the temporary discontinuation of HAART for 4 to 6 months, while patients received chemotherapy did not lead to persistent and deleterious immunologic consequences. It is acceptable, therefore, to withhold HAART for a brief period of several months

Table 2: Active clinical trial protocols evaluating chemotherapy in ARL.

Study identifier	Phase	Study regimen	Start date	Primary endpoint
NCT00006436	II	EPOCH and Rituximab in ARL	October 2000	PFS
NCT00598169 (AMC 053)	I/II	Bortezomib, Ifosfamide, Carboplatin, and Etoposide ± Rituximab in relapsed ARL	November 2007	ORR and safety
NCT01092182	II	Dose-adjusted R-EPOCH in Burkitt or c-MYC+ DLBCL	February 2010	PFS, EFS, and OS
NCT01193842 (AMC 075)	I/II	R-CHOP or R-EPOCH ± Vorinostat in AIDS-related DLBCL	October 2010	ORR, MTD, and toxicity

EPOCH: etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin; ARL: AIDS-related lymphoma; PFS: progression-free survival; AMC: AIDS Malignancy Consortium; ASCT: autologous stem cell transplant; PET: positron emitting tomography; CT: computed tomography; ABVD: doxorubicin, bleomycin, vinblastine, dacarbazine; BEACOPP: bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone; HL: Hodgkin's lymphoma; OS: overall survival; ORR: overall response rate; DLBCL: diffuse large B cell lymphoma; EFS: event-free survival; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; MTD: maximum tolerated dose.
Source: http://clinicaltrials.gov/.

while patients receive ARL chemotherapy. In the absence of a head-to-head trial of administering or withholding HAART, both approaches are reasonable. HAART may, however, be particularly attractive to offer concurrently with chemotherapy for those patients who have a depleted CD4+ cell count at the time of ARL diagnosis, a history of opportunistic infections or other AIDS-related complications, appear likely to adhere to taking multiple oral medications, and for those patients who would likely achieve a nondetectable HIV viral load with HAART.

5. Treatment of Burkitt and Burkitt-Like NHL

Prospective studies of ARL generally have combined the high-grade DLBCL and Burkitt lymphoma/Burkitt-like lymphoma histologies, with little difference in outcome between the subtypes. In contrast, two retrospective reviews demonstrated substantially inferior outcomes for AIDS-associated Burkitts lymphoma in the HAART era in comparison with HIV-associated DLBCL when CHOP-like treatments were used [44]. Median survival was 5.7 months and 8 months for those with Burkitts lymphoma compared with 43.2 months and 22 months for those with DLBCL who received CHOP-like chemotherapy in the HAART era. Small retrospective and phase II studies of AIDS-associated Burkitt lymphoma reported the feasibility of using dose-intensive protocols with or without HAART treatment with response rates of approximately 70% [25, 45]. In addition, the NCI recently presented preliminary findings of abbreviated (three) cycles of dose-adjusted R-EPOCH in conjunction with prophylactic intrathecal CNS therapy for eight patients with AIDS-related Burkitt lymphoma [46]. All patients achieved a CR and remained in remission with a median followup of 4 years. These preliminary but encouraging results have led investigators to reconsider how best to treat HIV-infected patients with Burkitt lymphoma. AMC Study 048 recently completed accrual of patients with AIDS-related Burkitt lymphoma into a phase II study of high-dose, short-course chemotherapy regimen consisting of rituximab, cyclophosphamide, doxorubicin, vincristine, intrathecal cytarabine and methotrexate, ifosfamide, and etoposide (R-CODOX-M/IVAC). Final results from this study have yet to be reported. For patients with AIDS-related Burkitt lymphoma who have an adequate Karnofsky performance status an adequate CD4+ cell count and well-controlled HIV viremia, an intensive chemotherapy regimen similar to what is offered to patients with Burkitt lymphoma who are HIV negative is recommended.

6. Treatment of Relapsed ARL

Optimal chemotherapy for patients with relapsed/refractory ARL has not been defined and such patients are usually treated with regimens that are used for non-HIV-infected patients with relapsed/refractory aggressive lymphoma, such as ifosfamide, cisplatin and etopside (ICE), or etoposide, solumedrol, high-dose cytosine arabinoside, platinum (ESHAP) [47, 48]. These patients can also be considered for high-dose chemotherapy (HDCT) and peripheral autologous stem cell transplant (ASCT) if they have chemosensitive disease and have not exhausted HAART options. HAART therapy is feasible throughout the transplant process. In one report, 16 of 19 patients remained in remission after 2 years of followup [49]. Infectious complications were comparable with those that have been reported in the HIV-negative NHL patients who underwent ASCT and no long-term deterioration in immune function was noted. Several studies have reported success in the treatment of relapsed ARL using second-line therapy followed by HDCT and ASCT [50–52]. Among 68 ARL patients who underwent HDCT/ASCT from 20 institutions across Europe between the years 1999 and 2004, the PFS and OS were 56.5% and 61%, respectively, at a median followup of 32 months [53]. In a prospective study of HDCT/ASCT conducted by the GICAT group, 27 of the 50 enrolled patients underwent ASCT [54]. Intention-to-treat analysis showed a superior median OS for those who underwent ASCT (44 versus 33 months) compared to those who did not. Response to therapy significantly affected OS. In addition, AMC investigators used a preparative regimen of reduced-dose oral or intravenous busulfan in conjunction with cyclophosphamide as a preparative regimen for ASCT in patients with recurrent ARL and Hodgkin lymphoma [52]. The mean time to achievement of an absolute neutrophil count of greater than

Table 3: Active clinical trial protocols evaluating hematopoietic stem cell transplant in ARL.

Study identifier	Phase	Study	Start date	Primary endpoint
NCT00345865	II	Cyclophosphamide + TBI versus carmustine, cyclophosphamide and etoposide conditioning followed by ASCT in AIDS-related NHL or HL	November 2005	DFS and OS
NCT00641381	II	Carmustine, Etoposide, Cyclophosphamide and ASCT in ARL	March 2000	Feasibility and toxicity
NCT00858793	I/II	HDT and transplantation of gene-modified ASCT for high-risk ARL	October 2008	Adverse events
NCT00968630	II	Immune response after HSCT in HIV-positive patients with hematologic cancer	August 2009	HIV-specific immune response
NCT01045889	II	R-CHOP followed by HDT and ASCT	January 2007	OS
NCT01141712 (AMC 071)	II	HDT (BEAM) and ASCT in ARL	February 2011	OS
NCT01410344	II	Allogeneic HSCT for hematological cancers and myelodysplastic syndromes in HIV-infected individuals	September 2011	NRM

TBI: total body irradiation; NHL: non-Hodgkin's lymphoma; HL: Hodgkin's lymphoma; DFS: disease-free survival; OS: overall survival; HDT: high-dose therapy; ASCT: autologous stem cell transplant; HSCT: hematopoietic stem cell transplant; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; BEAM: carmustine, etoposide, cytarabine, melphalan; NRM: nonrelapse mortality.
Source: http://clinicaltrials.gov/.

0.5×10^9/L was 11 days (range, 9 to 16 days). The median time to achievement of an unsupported platelet count of at least 20×10^9/L was 13 days (range, 6 to 57 days). One patient died on day +33 after ASCT as a result of hepatic and renal occlusive disease and multiorgan failure. No other fatal regimen-related toxicity occurred and 10 (53%) of 19 evaluable patients were in complete remission at day +100.

Recently, investigators from the City of Hope compared their experiences treating 29 consecutive patients with relapsed or progressive ARL who underwent ASCT to age, gender, histology, and stage-matched NHL controls who also underwent ASCT [55]. The median ARL patient age was 42 years and DLBCL/anaplastic large cell (16 patients) and Burkitt (11 patients) histologies predominated. The conditioning regimen most commonly consisted of CBV (cyclophosphamide 100 mg/kg, carmustine 450 mg/m^2, and etoposide 60 mg/kg). The median time to engraftment was 10 days (range, 5–19 days). Treatment-related toxicities between the groups were comparable and 2-year overall survival at 75% was the same for both groups.

European investigators have also reported results involving 53 HIV-positive and 53 HIV-negative matched lymphoma patients who underwent ASCT [20]. Incidence of PFS and OS were similar in both cohorts. The authors concluded that HIV-infected patients should be considered for ASCT according to the same criteria adopted for HIV-negative NHL patients. Furthermore, long-term followup data of ARL patients undergoing ASCT showed the nonrelapsed mortality (11% versus 4%, $P = 0.18$), 2-year DFS (76% versus 56%, $P = 0.33$) and the 2-year point estimates of OS (74% versus 75%, $P = 0.93$) were comparable to matched HIV-negative NHL controls [55]. These studies are preliminary but published experience with ASCT for ARL is accumulating [20, 52, 56–59]. Larger studies (some of which are currently underway, Table 3) with longer followup are needed to better define the optimal ARL conditioning regimen and to better delineate patient selection criteria.

Gene therapy is an emerging technology that holds promise for patients with relapsed ARL. Recently, a stable expression of a lentiviral vector encoding anti-HIV RNAs in blood stem cells was transplanted into four such patients who were undergoing HDT followed by ASCT [60]. They each received gene-modified hematopoietic progenitor cells expressing 3 RNA-based anti-HIV moieties (tat/rev short hairpin RNA, TAR decoy, and CCR5 ribozyme). The gene-modified cells showed no differences in their hematopoietic potential compared with nontransduced cells; by day +11 all four patients were successfully engrafted with a persistent expression of the vector and the introduced small interfering RNA and ribozyme.

7. Supportive Care and Late-Treatment Complications

Attention to supportive care measures is essential for patients with ARL. Judicious use of hematopoietic stimulants including the newest formulation of pegylated granulocyte-colony stimulating factor (G-CSF) may help ameliorate chemotherapy-induced neutropenia.

The US Food and Drug Administration (FDA) has issued a warning to medical providers that erythropoiesis-stimulating agents (darbepoetin alfa and epoetin alfa) are associated with a high rate of thrombosis for patients with

cancer [61]. For these patients, the use of erythropoiesis-stimulating agents also may lead to a heightened risk of cancer progression and death [62]. Until these concerns are more fully evaluated, it is best to transfuse packed red blood cells when patients with ARL develop symptomatic anemia.

To minimize cardiac toxicities, pegylated anthracyclines have been used for the treatment of ARL, and liposomal anthracyclines also may offer some of the pharmacokinetic benefits of infusional doxorubicin but without the need for patients to be connected to cumbersome infusion pumps for several days' duration. Single institution studies suggested that substituting liposomal anthracyclines for doxorubicin in multiagent lymphoma protocols was both safe and effective [23, 63]. AMC Study 047 substituted liposomal doxorubicin for doxorubicin in the R-CHOP regimen for patients with ARL [64]. However, a disappointing CR rate of only 37% was achieved.

Infectious complications associated with ARL may be minimized by using prophylactic fluroquinolone antibiotics and azoles during periods of protracted neutropenia. All patients should receive Pneumocystitis *jiroveci* prophylaxis (e.g., dapsone, inhaled pentamidine, atovaquone) regardless of initial CD4+ cell count. Trimethoprim-Sulfamethoxazole is infrequently used because of its potential to exacerbate myelosuppresasion.

With longer life expectancy following chemotherapy, late-treatment-related complications are emerging as a serious concern after treatment for ARL. Chemotherapy-related acute myelogenous leukemia (11q21 deletion with 2 additional copies of MLL gene at 10q) has been reported in an HIV-positive individual 48 months after therapy with R-EPOCH for ARL [65]. Similarly, three cases of secondary malignancy in the setting of ASCT have been reported [53]. It remains unknown whether the rate of secondary malignancies is intrinsically increased in the setting of HIV infection.

8. Concluding Remarks

Epidemiologic reports clearly indicate that the incidence of ARL has decreased since the introduction of HAART among patients with AIDS. Chemo-immunotherapy for the most common subtype of ARL, DLBCL, is improving, with several phase II clinical trials showing good outcomes employing R-CHOP or R-EPOCH. CR rates for those patients with good prognosis features rival those of similarly matched non- HIV infected patients with DLBCL. Better supportive care, employing the use of G-CSF, prophylactic antibiotics, and HAART either during or shortly after completion of chemotherapy may ameliorate the increased toxicity of concurrent immunotherapy, thereby improving survival of patients with ARL. Clinical trials investigating the role of autologous and allogeneic HSCT in relapsed/refractory ARL are currently underway. With effective treatments and longer life expectancy following chemotherapy, late-treatment-related complications are emerging, and how best to mitigate and manage these complications is an emerging challenge.

Abbreviations

AIDS: Acquired immunodeficiency syndrome
HAART: Highly active antiretroviral therapy
ARL: AIDS-related lymphoma
HIV: Human immunodeficiency virus.

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Review Article

The Role of Autologous Stem Cell Transplantation in the Treatment of Diffuse Large B-Cell Lymphoma

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Diffuse large B-cell non-Hodgkin's lymphoma (DLBCL) accounting for approximately 30% of new lymphoma diagnoses in adult patients. Complete remissions (CRs) can be achieved in 45% to 55% of patients and cure in approximately 30–35% with anthracycline-containing combination chemotherapy. The ageadjusted IPI (aaIPI) has been widely employed, particularly to "tailor" more intensive therapy such as high-dose therapy (HDT) with autologous hemopoietic stem cell rescue (ASCT). IPI, however, has failed to reliably predict response to specific therapies. A subgroup of young patients with poor prognosis exists. To clarify the role of HDT/ASCT combined with rituximab in the front line therapy a longer follow-up and randomized studies are needed. The benefit of HDT/ASCT for refractory or relapsed DLBCL is restricted to patients with immunochemosensitive disease. Currently, clinical and biological research is focused to improve the curability of this setting of patients, mainly young.

1. Introduction

Diffuse large B-cell non-Hodgkin's lymphoma (DLBCL) is the commonest histological subtype of non-Hodgkin's lymphomas (NHL) accounting for approximately 30% of new lymphoma diagnoses in adult patients. Because their incidence increases in old age, this epidemiological pattern might explain, at least in part, the rapid rise in the number of new diagnoses observed over the last decades of the 20th century [1, 2] in which an increase of median age of population has also been registered.

Complete remissions (CRs) can be achieved in 45% to 55% of patients and cure in approximately 30–35% with anthracycline-containing combination chemotherapy [3].

The International Prognostic Index (IPI) proposed in the 1993 [4] has been used in the risk stratification for patients with DLBCL for more than a decade. The age-adjusted IPI (aaIPI) has also been widely employed, particularly to "tailor" more intensive therapy such as high-dose therapy (HDT) with autologous hemopoietic stem cell rescue (ASCT). IPI, however, has failed to reliably predict response to specific therapies. This, in part, reflects the inherent biological heterogeneity of DLBCL and highlights the need for more precise, patient-specific, and biologically based risk factors. Despite these criticisms, the IPI has proved valuable for stratification of patients in clinical trials and remains the prognostic system more widely employed in clinical research and daily practice.

The development of rituximab, a chimeric anti-CD20 monoclonal antibody, has represented a revolutionary advance in the therapy of hematologic malignancies [5]. The addition of rituximab to cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) combination has produced significant survival benefits in elderly patients with untreated DLBCL compared to CHOP alone [6, 7]. Similarly, the same immunochemotherapy regimen has determined an improved outcome in young low-risk DLBCL patients [8], as defined by aaIPI. Thus, first line chemotherapy with CHOP or CHOP-like regimens in combination with rituximab has become standard care for CD20+ DLBCL patients.

Despite the striking advances in the outcome of DLBCL patients, a subgroup of young patients with poor prognosis still exists [9, 10]. Currently, clinical and biological research is focused to improve the curability of this setting of patients, mainly young.

2. HDT with ASCT in Front-Line Treatment of DLBCL

In the prerituximab era, HDT/ASCT has proven effective as salvage treatment in patients with chemosensitive relapsed aggressive NHL [11]. These results suggested the possibility of improving the outcome of aggressive NHL patients by including HDT/ASCT in the first-line therapy. After some phase I/II trials supporting the use of this strategy, HDT/ASCT appeared a promising option for frontline treatment of young patients. However, the results of prospective randomized trials [12–25] have generated conflicting results and several problems have hampered the comparison of data (Tables 1 and 2).

Firstly, trials had different remission status requirements for HDT/ASCT [12–25]. In particular, only patients in PR or CR, after induction therapy (Table 1), were randomized to receive HDT/ASCT or conventional therapy [12–16]. Secondly, in other trials, patients were randomized at diagnosis (Table 2), and HDT/ASCT was employed as part of initial treatment after shortened [19–21, 25] or full course of induction therapies [17, 18, 22–24]. Furthermore, high-dose sequential (HDS) therapy, a type of induction treatment based on a different "philosophy" from the rationale underlying the conventional one, was administered up-front followed by HDT/ASCT in three studies [17, 23, 24]. HDS therapy consists in the administration of several non-cross-resistant drugs, each given at the maximal tolerated dose mainly as single agent within the shortest possible interval. The purpose of this regimen was to prevent the emergence of drug-resistant tumor clones. Thirdly, a great variety of therapeutic regimens, both among conventional or high-dose treatments, were employed. In fact, conventional CHOP regimen or CHOP-like combinations were employed in 3 and 6 trials, respectively, while, in the other studies, MACOP-B or VACOP-B were used [17, 18, 21, 22]. Although the combination of carmustine, etoposide, cytarabine, and melphalan (BEAM) was the most frequently employed conditioning regimen [15, 16, 18–20, 22, 25], other myeloablative treatments [12–14, 17, 21, 23, 24] were also used in several trials. Fourthly, because several of these studies were designed before the introduction in the clinical practice of both the IPI prognostic score [4] and the REAL-WHO histological classification [26], trials included varying proportions of patients with different risk categories and different histological subtypes, not all of which were DLBCL. Despite the poor comparability of these trials, a statistically significant prolongation of PFS or EFS was documented in four trials [15, 19, 22, 25], but none demonstrated a significant improvement of OS associated with HDT/ASCT with the exception of a retrospective subgroup analysis [14]. In summary, in the prerituximab era, HDT/ASCT, employed in front-line therapy, failed to improve the outcome of aggressive NHL patients.

In the rituximab era, HDT/ASCT for intermediate-high (I-H) or high-risk (H) aaIPI patients is still a matter of debate. However, the combination of rituximab with an intensified treatment strategy has resulted in encouraging results in phase II studies (Table 3). Tarella et al. [27] used rituximab in combination with modified HDS chemotherapy delivered with multiple ASCT followed by a consolidation phase consisting of mitoxantrone (Mito) and melphalan (L-PAM) with ASCT. In this study, 93 of the 112 patients enrolled completed the planned therapy. At conclusion of treatment, the CR rate was 80%. At a median followup of 48 months, the estimated 4-year OS projection was 76% (CI: 68–85%), and at median followup of 46 months, the 4-year EFS projection was 73% (CI: 64–81%). Vitolo et al. [28] employed 4 cycles of dose-dense (110 mg/mq epirubicin, 1200 mg/mq ciclofosfamide, 1.4 mg/mq vincristina, and 40 mg/mq prednisone orally days 1 to 5 given every two weeks) CEOP regimen as induction phase, followed by the 2 cycles of mitoxantrone, cytarabine, and dexamethasone (MAD) as intensification phase. The third phase of study design consisted of BEAM with ASCT. A total of six rituximab doses were given, 4 and 2 during induction and intensification phases, respectively. Seventy six of the 94 patients completed treatment and underwent HDT/ASCT. The CR rate was 82% (CI: 73–88%). With a median followup for censored patients of 49 months, the 4-year FFS rate was 73% (CI: 63, 5–82, 5%) and the 4-year OS rate was 80% (CI: 71, 6–88,4%). Dilhuydy et al. [29] reported an overall response (OR) rate of 67%. With a median followup of 66 months, the estimated rates (±SD) of 5-year OS and EFS rates were 74% ± 4% and 55% ± 5%, respectively. Fitoussi et al. [30] treated 208 patients with rituximab combined with cyclophosphamide, vindesine, bleomycin, and prednisolone (ACVBP) for 4 cycles. This induction therapy was followed by BEAM with ASCT in 155 responding patients (CR or PR). A total of 32 patients did not receive HDT/ASCT. Twenty five were withdrawn during induction therapy, 6 because of insufficient response before consolidation and one because of sudden death. With a median followup of 45 months, the 4-year PFS and OS were estimated at 76% (CI: 69–81%) and 78% (72–83%).

In both the Vitolo and the Fitoussi studies, the results achieved with the immunochemotherapy strategy were compared with those obtained in their historical groups of patients treated with similar sequence of chemotherapy program, but not including rituximab. Despite the limitations intrinsic to retrospective analyses, these comparisons showed a clear therapeutic advantage of immunotherapy over chemotherapy in both the two major end points PFS and OS.

Recently two randomized studies conducted by the SWOG [31] and FIL [32] have tested the role of HDT/ASCT in the front line therapy of unfavorable (I-H/H) patients with aggressive NHL. In particular, in the SWOG study, patients responsive to the CHOP or R-CHOP induction therapy were randomized to receive one more cycle of R-CHOP followed by TBI or BCNU-based regimens and ASCT or three additional cycles of R-CHOP [31]. In this trial, the 2 yr PFS was 69% and 56% in the experimental arm compared to the standard one (95% CI: 1.18–2.51) $P = 0.05$, while no significant difference was documented in the 2-year OS. The authors conclude that HDT/ASCT improves PFS for responders, including those induced with R-CHOP, with a stronger outcome seen for those with H IPI grade. The FIL study, a multicenter randomized trial with a 2×2 factorial

Table 1: Phase III trials of HDT/ASCT in CR or PR unfavorable NHL patients.

Author	Year	*n*	Histological classification	DLCL (%)	Immunological phenotype (%)	aaIPI ≥ 2 (%)	Disease status HDT/ASCT	Therapy	Shorten induction Yes/No	PFS/EFS (%)	*P*	OS (%)	*P*
Verdonk [12]	1995	35 34	W.F.	26 33	B: 77 B: 79	44 44	PR	CHOP × 8 versus CHOP × 4 + HD-CTX-TBI/ASCT	Yes	4y: 53 4y: 41	N.S.	4y: 85 4y: 56	N.S.
Martelli [13]	1996	27 22	W.F./Kiel	62 40	B: 70 B: 45	N.R.	PR	DHAP[1] × 6 versus BEAC[1]/ASCT	No	5y: 52 5y: 73	N.S.	59 73	N.S.
Haioun [14]	2000	111 125	W.F.	61 56	B: 63 B: 60	90 80	CR	ACVB versus ACVB + CBV/ASCT	No	8y: 39 8y: 55	0.02	8y: 49 8y: 64	0.04
Kluin-Nelemans [15]	2001	56 49	REAL	58 50	B: 55 B: 66	29 31	CR, PR	ChVmP/BV[1] × 8 versus ChVmP/BV[1] × 6 + BEAM/ASCT	Yes	5y: 56 5y: 61	N.S.	5y: 77 5y: 68	N.S.
Milpied [16]	2004	99 98	W.F.	74 77	B: 74 B: 77	49 57	PR	ACBVP[2] versus CEOP + ECVBP[2] + BEAM/ASCT	No	5y: 37 5y: 55	0.037	5y: 56 5y: 71	N.S.

[1] Plus radiotherapy at bulky disease.

[2] Plus radiotherapy at bulky disease and intrathecal prophylaxis in very high-risk patients.

W.F.: working formulation—NHL classification; Kiel: Kiel classification of NHL; CR: complete response; PR: partial response; CHOP: cyclophosphamide, doxorubicin, vincristine, and prednisone; HD-CTX: high-dose cyclophosphamide; TBI: total body irradiation; DHAP: cisplatin, cytarabine, and high-dose dexamethasone; BEAC: carmustine, etoposide, cytarabine, and cyclophosphamide; ACBV: doxorubicin, cyclophosphamide, vindesine, and bleomycin; CBV: cyclophosphamide, vinblastine, and bleomycin; ChVmP/BV: cyclophosphamide, doxorubicin, teniposide, prednisone, bleomycin, and vincristine; BEAM: carmustine, etoposide, cytarabine, and melphalan; CEOP: cyclophosphamide, epirubicin, vincristine, and prednisone; ECVBP: epirubicin, cyclophosphamide, vindesine, bleomycin, and prednisone; N.S.: not significant.

Table 2: Phase III trials of HDT/ASCT in unfavorable NHL patients.

Author	Year	n	Histological classification	DLCL (%)	Immunological phenotype (%)	aaIPI ≥ 2 (%)	Disease status HDT/ASCT	Therapy	Shorten induction yes/no	PFS/EFS (%)	P	OS (%)	P
Gianni [17]	1997	58 40	W.F.	88 91	N.R.	74 94	CR, CRu, PR, SD, MR, PD	MACOP-B[2] versus HDS[#] + mito-L-PAM[2]/ASCT	No	7y: 49 7y : 76	<0.001	7y: 55 7y : 81	0.09
Santini [18]	1998	61 63	W.F.	72 77	B: 75 B: 83	59 54	CR, CRu, PR, SD, MR, PD	VACOP-B[1] versus VACOP-B[1] + BEAM/ASCT	No	6y: 48 6y: 60	N.S.	6y: 65 6y : 65	N.S.
Gisselbrecht [19]	2002	181 189	Kiel/WHO 1999	62.5 60	B: 79 B: 75	97 99	CR, CRu, PR, SD, MR, PD	ACBVP[3] versus CEOP[3] + ECVBP + BEAM/ASCT	Yes	5y: 52 5y: 39	0.01	5y: 60 5y : 46	0.007
Kaiser [20]	2002	154 158	REAL	61 58	B: 79 B: 73	75 73	CR, CRu, PR, SD, MR, PD	CHOEP[1] × 5 versus CHOEP[1] × 3 + BEAM/ASCT	Yes	3y: 49 3y: 59	N.S.	3y: 63 3y: 62	N.S.
Martelli [21]	2003	75 75	REAL	84 78	B: 81 B: 70	100 100	CR, CRu, PR, SD, MR, PD	MACOP-B versus MACOP-B + BEAC/ASCT	Yes	5y: 49 5y: 61	N.S.	5y: 65 5y: 64	N.S.
Olivieri [22]	2005	106 116	W.F.	78 75	B: 83 B: 80	68 72	CR, CRu, PR, SD, MR, PD	VACOP-B[1] × 12 weeks versus VACOP-B[1] × 8 weeks + HD-CTX + HD-VP16 + BEAM/ASCT	No	7y: 44.9 7y: 40.9	N.S.	7y: 60 7y : 57	N.S.
Vitolo [23]	2005	66 60	REAL	90 80	B: 96 B : 90	80 87	CR, CRu, PR, SD, MR, PD	Mega CEOP[2] × 6–8 versus HDS[#] + mito-L-PAM[2]/ASCT	No	6y: 48 6y: 45	N.S.	6y: 63 6y: 49	N.S.

Table 2: Continued.

Author	Year	n	Histological classification	DLCL (%)	Immunological phenotype (%)	aaIPI ≥ 2 (%)	Disease status HDT/ASCT	Therapy	Shorten induction yes/no	PFS/EFS (%)	P	OS (%)	P
Betticher [24]	2006	59 70	REAL	69 76	B: 74 B: 93	88 72	CR, CRu, PR, SD, MR, PD	CHOP[2] × 8 versus HDS[#] + mito-L-PAM[2]/ASCT	No	3y: 33 3y: 39	N.S.	3y: 53 3y: 46	N.S.
Lynch [25]	2010	234 233	W.F.	N.R.	N.R.	98 98	CR, CRu, PR, SD, MR, PD	CHOP[1] × 6–8 versus CHOP[1] × 3 + BEAM/ASCT	Yes	5y: 38 5y: 44	N.S.	5y: 50 5y: 50	N.S.

[1] Plus radiotherapy at bulky disease.
[2] Plus radiotherapy at bulky disease and intrathecal prophylaxis in very high-risk patients.
[3] Plus intrathecal prophylaxis in very high-risk patients.
[#] See [17, 23, 24].
W.F.: working formulation-NHL classification; Kiel: Kiel classification of NHL; WHO: World Health Organization classification of NHL; CR: complete response; CRu: unconfirmed complete response; PR: partial response; MR: minor response, SD: stable disease, PD: progressive disease; MACOP-B: methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin; HDS: high-dose sequential chemotherapy; mito-L-PAM: mitoxantrone and melphalan; VACOP-B: etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin; BEAM: carmustine, etoposide, cytarabine, and melphalan; ACBVP: doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone; (mega) CEOP: cyclophosphamide, epirubicin, vincristine, and prednisone; ECVBP: epirubicin, cyclophosphamide, vindesine, bleomycin, and prednisone; CHOEP: cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisone; BEAC: carmustine, etoposide, cytarabine, and cyclophosphamide; HD-CTX: high-dose cyclophosphamide; HD-VP16: high-dose etoposide; N.R.: not reported; N.S.: not significant.

Table 3: Studies of HDT/ASCT in unfavorable DLBCL patients.

Author	Year	n	Pathological phenotype	DLCL (%)	Immunological phenotype (%)	aaIPI ≥ 2 (%)	Therapy	Shorten induction yes/no	PFS/EFS (%)	OS (%)
Tarella [27]	2007	112	REAL	79	B. 100	100	Modified R-HDS[#1]	No	4y: 73	4y: 76
Vitolo [28]	2009	97	REAL	86	B. 100	100	R-mega CEOP14 × 4 + R-MAD[2] × 2 + BEAM/ASCT	No	4y: 73	4y: 80
Dilhuydy [29]	2010	42	REAL	N.R.	B. 100	100	R × 4 + CEEP × 2 + R-MTX/R-MC + BEAM/ASCT	Yes	5y: 55	5y: 74
Fitoussi [30]	2011	209	WHO	N.R.	B. 100	100	R-ACVBP × 4 + BEAM/ASCT	Yes	4y: 76	4y: 78

[1] Plus radiotherapy at bulky disease.
[2] Plus radiotherapy at bulky disease and intrathecal prophylaxis in very high-risk patients.
[#] See [26].
REAL: revised European-American lymphoma classification; WHO: World Health Organization classification of NHL; R: rituximab; (mega) CEOP: cyclophosphamide, epirubicin, vincristine, and prednisone; MAD: mitoxantrone, cytarabine, and dexamethasone; BEAM: carmustine, etoposide, cytarabine, and melphalan; CEEP: cyclophosphamide, epirubicin, vindesine, and prednisone; MTX: methotrexate; MC: methotrexate and cytarabine; ACBVP: doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone; N.R.: not reported.

design, compared two rituximab dose-dense treatments (R-CHOP14 versus R-megaCHOP14), followed or not by BEAM with ASCT [32]. With a median followup of 23 months, 2-year PFS was 65% (CI: 59–70%), for the entire group of enrolled patients and 59% (CI: 51–57%) versus 72% (CI: 64–78%) for no HDT/ASCT versus HDT/ASCT respectively. So far, the advantage in PFS does not translate in OS benefit. However, a longer followup will clarify the role of HDT/ASCT as first-line treatment of aaIPI 2-3 DLBCL patients. These and other randomized studies will define whether HDT/ASCT combined with rituximab in the front line therapy is associated with increased cure rate of unfavorable DLBCL patients.

3. HDT with ASCT as Salvage Therapy

In the prerituximab era, the Parma trial established HDT/ASCT as standard therapy in relapsing aggressive NHL patients responding to salvage therapy [11].

The parameters affecting the results of HDT/ASCT are identified in responsive disease to conventional dose salvage therapy before myeloablative treatment, relapse defined as a time less than twelve months from diagnosis to recurrence (early), and the presence of prognostic factors at relapse, as defined by IPI or secondary aaIPI (saaIPI) [33–35].

At present, the emphasis of recent clinical research in HDT/ASCT is focused on three therapeutic aspects. The first consists in the evaluation of the potential benefit of adding rituximab to salvage therapy, followed by HDT/ASCT, in relapsed rituximab naïve patients. Overall, the available data in this setting of patients, although not completely concordant, are in favour of the use of immunochemotherapy (Table 4). In first three studies reported in Table 4, no patient [36] or only a minority of cases [37, 38] had received rituximab before enrollment, while, in the fourth, 25% of patients were treated with rituximab in the first line therapy or during salvage treatment or both at diagnosis and after relapse [39]. In the Kewalramani et al. study, the PFS rates of patients who underwent HDT/ASCT after ICE in combination with rituximab (RICE) were marginally improved compared to those observed in the historical control patients who received salvage therapy alone [36]. The difference was not statistically significant, but the study was not powered to detect minor improvements in survival rates. However, in this study, the addition of rituximab to ICE doubled the percentage of CRs. Sieniawski et al., in their study reported improved OR rate, freedom from second failure (FF2F), and OS in the patient group treated with DHAP plus rituximab, compared to the historical control group treated with the same chemotherapy [37]. In both groups, patients in CR or PR after salvage therapy received HDS therapy followed by BEAM with ASCT. Improved FFS and PFS were documented also by Vellenga et al. [38] in relapsed patients when rituximab was added to DHAP-VIM-DHAP reinduction therapy. The modest impact of rituximab on OS was amplified when the analysis was repeated adjusting for prognostic factors such as time elapse since upfront treatment, saaIPI score, age, and WHO PS. Furthermore, the addition of rituximab increased the group of responders on reinduction therapy from 54% to 74% and therefore the number of patients who might benefit from HDT/ASCT. In this study, as well, only patients in CR or PR after salvage therapy were eligible for HDT/ASCT. In the Mounier et al. study [39], after HDT/ASCT, the 5-year OS was 63% (95 CI, 58–67%), and the 5-year DFS was 48% (95 CI, 43–53%) for the entire population. Statistical analysis indicated a significant increase in DFS after ASCT compared with duration of CR I (median, 51 months versus 11 months; $P < .001$). This difference remained highly significant in patients with previous exposure to rituximab (median, 10 months versus not reached; $P < 0.01$). The second aspect regards the role of rituximab in salvage treatment of patients previously treated with immunochemotherapy (Table 5). In fact, at present, almost all patients with aggressive B-cell NHL are initially treated with rituximab in association with CHOP or CHOP-like regimens. In these patients, the role of rituximab in further salvage treatment remains to be

Table 4: Rituximab-based salvage therapy in rituximab-naïve relapsing/refractory DLBCLs.

Author	Year	*n*	Pathological phenotype	DLCL (%)	Therapy	Conditioning regimen	PFS/EFS (%)	*P*	OS (%)	*P*
Kewalramani [36]	2004	36 147	WHO	100	R-ICE ICE	*	2y: 54 2y: 43	N.S.	2y: 67 2y: 56	N.S.
Sienawski [37]	2007	19 19	WHO	80	R-DHAP[1] DHAP[1]	BEAM	2y: 57 2y: 18	0.0051	2y: 77 2y: 37	0.0051
Vallenga [38]	2008	113 112	WHO	80.5 78.6	R-DHAP-VIM-DHAP[1] DHAP-VIM-DHAP[1]	BEAM	2y: 52 2y: 31	0.002	2y: 59 2y: 52	N.S.
Mounier [39]	2011	470	WHO	100	N.R.	BEAM and others[#]	5y: 48	0.001**	5y: 63	N.R.

[1] Plus radiotherapy at bulky disease.
*The choice of conditioning regimen depended on the patient's age, the extent of previous therapy and the clinical trials active at the time of transplantation (see [30]).
[#]See [39].
**Each patient was assessed as his or her own control.
WHO: World Health Organization classification of NHL; R: rituximab; ICE: ifosfamide, carboplatin, and etoposide; DHAP: cisplatin, cytarabine, and dexamethasone; VIM: etoposide, ifosfamide, and methotrexate; BEAM: carmustine, etoposide, cytarabine, and melphalan; N.R.: not reported; N.S.: not significant.

Table 5: Salvage therapy in relapsing/refractory DLBCLs previously exposed to rituximab.

Author	Year	Kind of study	*n*	Pathological phenotype	DLCL (%)	Therapy	Conditioning regimen	PFS/EFS (%)	*P*	OS (%)	*P*
Martín [40]	2008	Retrospective	94 69	WHO	100	R-ESHAP (prior R) R-ESHAP (no prior R)	*	3y: 17 3y: 57	0.008	3y: 38 3y: 67	0.004
Fenske [41]	2009	Retrospective	818 176	WHO	100	R-CT (no prior R) R-CT (prior R)	*	3y: 50 3y: 38	0.008	3y: 57 3y: 45	0.006
Gisselbrecht [42]	2010	Perspective	194 202	WHO	100	R-DHAP R-ICE	BEAM	3y: 42 3y: 31	N.S.	2y: 51 2y: 47	N.S.

*The choice of conditioning regimen depended on the patient's age, the extent of previous therapy, and the clinical trials active at the time of transplantation (see [40, 41]).
WHO: World Health Organization classification of NHL; R: rituximab; ESHAP: etoposide, methylprednisolone, cisplatin, and cytarabine; CT: multiple variable regimes; ICE: ifosfamide, carboplatin, and etoposide; DHAP: cisplatin, cytarabine, and dexamethasone; BEAM: carmustine, etoposide, cytarabine, and melphalan; N.S.: not significant.

determined. In the GEL/TAMO report by Martín and colleagues [40], no significant differences in response rates were documented in multivariate analysis between patients treated with R-ESHAP and previously exposed or not to rituximab. However, patients who had received prior rituximab had a significantly worse PFS and OS than rituximab naïve patients. Furthermore, prior treatment with this monoclonal antibody was also an independent adverse prognostic factor for both PFS and OS. In the experience of Fenske et al. [41], the administration of rituximab given with first-line or salvage therapy prior to HDT/ASCT was associated with PFS and OS at 3 years superior to that observed when this monoclonal antibody was not employed during the entire therapeutic patient history. In the CORAL trial [42], the response rates after salvage therapy were affected by several independent factors. These include saaIPI score, short relapse time from diagnosis (<12 months), and prior rituximab treatment. These same independent factors negatively influenced the 3-year EFS, PFS, and OS. However, patients relapsing after more than 12 months from diagnosis benefited from the introduction of rituximab into their salvage regimen and showed 3-year EFS ranging from 40% to 50%. In conclusion, at present, the optimal second-line regimen is not defined, and the benefit of the inclusion of standard dose of rituximab in salvage therapy for patients previously exposed to this agent is also unclear although known risk factors might be useful in choosing salvage therapeutic strategy. These factors include saaIPI, response (CR versus PR refractory) to upfront therapy disease status (early versus late relapse) at the time of salvage therapy. The third aspect regards the development of resistance to rituximab. One possibility in overcoming this resistance consists in using high-dose (HD) of this antibody. This therapeutic aspect was evaluated by Khouri et al. [43]. HD-rituximab (HD-R) was employed after mobilization chemotherapy and again on day 1 and day 8 after HDT/ASCT. In this study, the HDT consisted of standard BEAM. Fifty-nine patients (88%) were exposed before to rituximab during salvage chemotherapy. The median time from last rituximab dose to study enrollment was 38 days. The results of this experience indicate that HD-R combined with HDT/ASCT is feasible and effective treatment in relapsed patients previously treated with immunotherapy.

An attempt to develop more effective therapeutic strategy for relapsed DLBCL patients consists in the combination of radioimmunotherapy (RIT) with the standard chemotherapy conditioning regimens. After Press et al. [44], first established the feasibility of high-dose RIT with ASCT, several studies have used myeloablative RIT with promising results.

The RIT combined with high-dose chemotherapy was superior compared to historical data especially in the salvage of patients with high IPI scores and residual PET-avid disease [45]. To further increase the therapeutic potential of RIT, Winter et al. [46] tested dose-escalated ^{90}Y-ibriyumumab tiuxetan combined with BEAM and ASCT. In this study, 30% and 36% of the 44 treated patients had achieved less than a PR to their most recent treatment or never had obtained CR. Thus, respectively, 30% of cases would not have been eligible for HDT/ASCT at most centers. The estimated 3-years PFS and OS reported in this unfavorable series of patients were 43% and 60%, respectively. Careful dosimetry rather than weight-based strategy for dose escalation was required to avoid toxicity and under treatment.

Finally, one relevant prognostic factor associated with DLBCL consists of the cell origin of malignant cells [47–51]. In fact, the gene expression profile (GEP) resembling that of germinal center B cells (GCB) is predictive of better patient outcome than a profile resembling that of activated B cells (ABC). Cell-of-origin (COO) algorithms [52, 53] can also translate GEP data into practical applications. In the prerituximab era, studies using conventional dose therapy or HDT/ASCT concluded in favour of predictive prognostic value of COO [48, 51]. In contrast, the clinical significance of DLBCL subtyping, as defined by COO, is more controversial in patients treated at diagnosis with immunochemotherapy [53–56]. At relapse, few data regarding the clinical impact of COO-subsets are available. Recently a subanalysis of Coral trial [57] has indicated that COO retains its prognostic value in relapse/refractory DLBCL patients. In addition, a better response to R-DHAP was documented in GCB-like DLBCL cases. In contrast with these findings, in the study by Gu et al. [58], COO failed to predict survival in DLBCL patients, either with chemosensitive or chemoresistant disease, treated with HDT/ASCT. Further studies are needed to clarify the predictive value of DLBCL subtyping in the setting of patients with refractory/relapsing disease.

In conclusion, the benefit of HDT/ASCT for refractory or relapsed DLBCL is restricted to patients with immunochemosensitive disease. In fact, the response to second-line treatment seems to predict patient outcome after HDT/ASCT.

Different therapeutic approaches are required to salvage patients with disease resistant to rituximab and chemotherapy. New agents such anti-CD20 antibodies therapeutically more active than rituximab, radiolabeled-antibodies, histone deacetylase inhibitors, various molecules which target mTOR, inhibitor of protein Kinase Cβ, and other types of target therapy might be effective in controlling refractory-relapsing DLBLC.

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Review Article

Towards Curative Therapy in Burkitt Lymphoma: The Role of Early African Studies in Demonstrating the Value of Combination Therapy and CNS Prophylaxis

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This paper describes the treatment of Burkitt lymphoma (BL) from the time of its discovery in Africa up to the present. Pioneer investigators explored the value of chemotherapy since surgery and radiation were not effective modalities. Complete response was observed with many drugs used as single agents, but Ziegler and colleagues showed that patients resistant to one drug could achieve cure and potentially long-term survival with other drugs. Subsequently, a combination of cyclophosphamide (CTX), vincristine (VCR), and methotrexate (MTX) was shown to be active, but a survival advantage compared to CTX alone could not be demonstrated because effective CNS prophylactic therapy, in the form of intrathecal therapy, was not given. A recent re-evaluation of this regimen in Africa with multiple doses of intrathecal therapy compares favourably with recent studies of single agent CTX, and other drugs have been shown to be non-cross resistant. Optimal results for patients with extensive disease probably require 5 or 6 effective drugs along with intrathecal therapy, using MTX and Ara-C. In Africa, doses must be lower, because of limitations in supportive care, but in technically advanced countries cure rates in excess of 90% can be obtained. Rituximab may improve the results in some patient groups and allow less intensive therapy without a reduction in survival in others.

1. Introduction

Burkitt lymphoma (BL), in spite of its low incidence throughout most of the world, has had a major impact upon the understanding and treatment of lymphomas and doubtless upon many other cancers. The fact that it was discovered in equatorial Africa and much of the groundwork for the evolution of its treatment was conducted in equatorial Africa in the 1960s and 70s, but little since, is an indication of the great loss to research that results from the enormous differences in resources that exist among the world's countries. The rich variety of lifestyles, diets, and environmental exposures in the world have probably never been greater, yet the vast bulk of research is conducted in the high income countries and in particular the OECD countries. Much of this research is not relevant, at least, in the immediate future, to the low- and many middle-income countries, which have not yet built strong enough infrastructures to carry out their own scientific investigations. Moreover, in the realm of treatment, regimens have constantly increased in complexity and cost with often questionable advantage, certainly when seen from the perspective of population medicine. Classification and staging systems become more and more complex such that diagnosis and staging comprise significant elements of the cost of managing a patient, without necessarily resulting in improved survival. In the case of Burkitt lymphoma, Burkitt himself showed many years ago that a small fraction of patients could be cured with a single dose of chemotherapy and that the extent of disease was perhaps the most important determinant of outcome, yet while patients with limited disease do receive limited treatment, much of the more recent research into the treatment of patients with extensive disease has been focused on determining which elements of very intensive, albeit successful therapies devised in recent years, can be removed

with impunity. Such studies have revealed a number of superfluous elements, indicating that the first highly successful therapies were also excessively and, doubtless, therefore, more toxic and more expensive. This is a very different approach from that taken by pioneer chemotherapists in Africa, where it was felt that the host response provided an important part of therapy, and too much chemotherapy could, by abrogating immunological defences against the tumor, lead to a negative result. Unfortunately, this approach often led to patients being undertreated, and since every patient is different, precisely how intensive the management of patients needs to be is not easy to determine. Nonetheless, today, most patients with BL can be cured with intensive conventional chemotherapy by accepting the fact that some will be overtreated, but directing research towards trying to identify such patients and minimizing therapy to the extent possible. This approach has led to approximately 90% of children, and a somewhat lower percentage of adults, being cured of their disease.

With the advent of highly active antiretroviral therapy, it has proved possible to use intensive therapy in AIDS patients, with a similar outcome to that achieved in non-AIDS patients. In Africa, there should be little difficulty in achieving reasonable survival rates in AIDS-related BL as long as patients also receive HAART, although the disease burden is likely to remain an important determinant of outcome.

2. Discovery of the Tumor and Early Therapeutic Studies

Dennis Burkitt was a surgeon who had been practicing at the Mulago Hospital in Kampala, Uganda for almost ten years before he saw his first case of the lymphoma which subsequently bore his name. Burkitt had reported a clinical syndrome of jaw tumors with or without tumor at other sites (particularly the abdomen) in 1958 [1]. Shortly afterwards, O'Conor and Davis published a review of childhood cancer in Uganda in which they reported that approximately 50% of all malignant diseases in children had similar pathology with clinical features consistent with those reported by Burkitt, although not all had jaw tumors. They identified the disease as a poorly differentiated lymphocytic lymphoma [2]. Surgery had little to offer these children, and radiation therapy was not then available in equatorial Africa, but, in 1960, David Burchenal, who had been using methotrexate (MTX) in patients with leukemia at the Sloane Kettering Institute in New York, visited Mulago Hospital, bringing some of the drug with him. Burkitt was persuaded to try the drug in two children. Both had remarkably rapid responses after a single dose of MTX, and although the first child subsequently relapsed, the second had a prolonged remission. In the pioneering days of chemotherapy, when many skeptics viewed cytotoxic therapy as merely a means of delaying the inevitable, this was a critically important result and triggered a great deal of interest in further treatment studies. In the course of the next several years, Burkitt in Uganda, Clifford and Oettgen in Kenya, and Ngu in Nigeria, assisted by collaborators in the USA and UK and drug donations, examined responses to many of the drugs then available (Table 1) [3–7]. This was a less than systematic process, and the drugs were used in various doses, schedules, and even routes of administration, but given the absence of any prior data on treatment response, the demonstration of clear diminution in tumor size, complete clinical remission, and over time, prolonged survival, even, on occasion, with single doses of drug, clearly demonstrated the high degree of chemosensitivity of the African lymphoma [8–11], particularly, but not exclusively, to cyclophosphamide (CTX), methotrexate (MTX), and vincristine (VCR) [3–8]. Anthracyclines (initially daunomycin) were not developed until the second half of the 1960s so were not assessed in these early studies. Indeed, single agent data is not available for the anthracyclines although they were informally given to some patients who relapsed (daunomycin or hydroxydaunomycin) with minimal responses being seen, such that these drugs were not further pursued at that time. Interestingly, in contrast to the approach to acute lymphoblastic leukemia in, for example, the USA, and related to both the extraordinarily rapid response to therapy and difficulty for African patients to stay at or near the hospital for prolonged periods, most patients were treated with only one or two doses of the drug being tested—in retrospect, sufficient to cure only a relatively small fraction of patients, mostly with limited disease.

Table 1

Drug	No. of patients	CR	CR + PR	%OR
Cyclophosphamide	163	43	132	81
Orthomerphalan	14	UK	14	100
Chlorambucil	12	3	10	83
Nitrogen mustard	61	10	44	72
Melphalan	26	8	16	61
Procarbazine	6	0	0	0
BCNU	5	0	4	80
Vincristine	21	10	17	81
Vinblastine	2	0	0	0
Methotrexate	45	11	26	58
6-Mercaptopurine	3	0	0	0
Cytosine arabinoside	3	2	2	2
Epipodophyllotoxin	2	2	2	2
Actinomycin D	4	1	4	4

CR: complete response; PR: partial respo2nse; OR: overall response.
Various doses and schedules were used, even for the same drug.
Adapted slightly from [12], UK = unknown.

In Burkitt's series of 90 jaw tumors treated at the Mulago Hospital Uganda with CTX, MTX, or VCR [9], 82% had complete (CR) or partial responses (PR), those with small tumors being much more likely (10 of 10) that those with large tumors (10 of 40) to achieve a CR, suggesting a relationship between response and tumor size, which was subsequently confirmed [10, 11]. Indeed, it seems likely that the extent of tumor is the single most important determinant of treatment outcome, since even patients with central nervous system disease (CNS) can be cured, particularly

when the total tumor burden is small—a situation relatively common in Africa, where many patients with CNS disease have either a jaw or orbital tumor, or a small extradural mass, all of which create major symptomatology and therefore early diagnosis—but rare elsewhere, where CNS disease is usually associated with extensive disease, particularly bone marrow involvement. Burkitt also pointed out that complete remissions occur within a few weeks or not at all and that relapse is very uncommon after a year of remission. Such "very late" relapses may, in fact, be second, clonally discrete tumors occurring in patients at very high risk for BL [13].

Longer followup in Uganda suggested that approximately 20% of patients could be expected to achieve long duration remissions after (by today's standards) minimal therapy [14–16], and Burkitt noted that recurrence could occur at previously uninvolved sites, a finding later confirmed and expanded by Ziegler, who reported that early relapses (within 10 weeks of first treatment) were more likely to recur at the original site of disease and/or in the central nervous system (CNS), while those with late relapse (after 10 weeks, and with a median remission duration of 26 weeks) were more likely to relapse at previously uninvolved sites of disease and only rarely in the CNS. The latter patients were also much more sensitive to further chemotherapy than the former [17, 18]. Burkitt noted that, in 12 patients known to have died after a complete or almost complete remission, 6 were due to CNS lesions—at that time, no preventative intrathecal chemotherapy was given to any of the patients.

Ngu at the University College Hospital in Ibadan, Nigeria, and Clifford, at the Kenyatta Hospital, in Nairobi, also reported dramatic responses and long-term survivors after treatment with CTX as well as, in Clifford's hands, melphalan or orthomelphalan [4, 7, 8, 15], although patients with extensive disease rarely achieved long-term survival. Ngu brought attention to the fact that serum uric acid levels were often raised in patients with extensive tumors and sometimes became even more elevated following therapy, in which case oliguric renal failure and death from electrolyte disturbances were the result. Thus, Ngu appears to be the first to describe what would now be called the acute tumor lysis syndrome (serum uric acid on the day of death was 54 mg per 100 mL) and reported that this and other complications such as perforation of the bowel may ensue from rapid dissolution of tumor following therapy. Knowledge of the severe consequences of untreated massive tumor lysis syndrome led to approaches to its management—initially massive hydration with allopurinol to ensure that the tumor-derived uric acid load was excreted. Alkalinization can be used during this phase, since uric acid is more soluble in an alkaline urine. But care should be taken not to "overshoot" since once the uric acid level is sufficiently low, the major problem becomes excretion of phosphate, which is less soluble in an alkaline urine and if insufficient fluids are given will precipitate in the renal tubules, causing obstructive, oliguric renal failure which is rapidly fatal in the absence of hemodialysis or filtration, which was not then, and is rarely now, available in most hospitals in low income countries. Thus, the key to the management of tumor lysis syndrome in patients with high tumor burdens is hyperhydration, allopurinol, to decrease uric acid formation and allow more oxypurine to e excreted as xanthine and hypoxanthine with careful observation of the patient to ensure that a good urine flow is sustained.

At this time, the relationship between BL and acute lymphoblastic leukemia, a rare disease in equatorial Africa, was frequently debated [19]. Clift et al. described a group of 4 children with cytologically typical BL who had a leukemic blood picture and/or diffuse bone marrow involvement at either relapse or presentation [20] as rarities—overt leukemia was rarely seen in African patients and although bone marrow examinations were infrequently performed in life, bone marrow involvement was present in less than 10% of children who died from BL [21, 22]. Figures on incidence of acute lymphoblastic leukemia (ALL) remain imprecise, but it does seem to be becoming more frequent, as one would expect, particularly in urban regions. Two of the children reported by Clift were initially treated with prednisone (40 mgs per day) and mercaptopurine, then standard therapy for acute lymphoblastic leukemia (ALL). Neither responded but one of the children was subsequently treated with nitrogen mustard and there was marked, although temporary, tumor regression [19]. Many years later, a comparison in children of ALL-like therapy versus repeated courses of a drug combination including CTX, MTX, VCR, and prednisone, carried out in the USA, showed a clear advantage of the latter regime in patients with BL [23], although the role of prednisone is unknown [20]. Similarly, anthracyclines have not been tested as single agents in first-line therapy of BL, but are included in most treatment regimens because of their successful introduction into the therapy of lymphomas in adults. It remains possible that they add little but toxicity to treatment regimens for BL.

2.1. Non-Cross-Resistant Drugs. In the late 1960s and early 1970s, a collaboration between the Uganda Cancer Institute (UCI) of Makerere University in Kampala and the National Cancer Institute (NCI) of the USA resulted in several other important observations. Of particular importance were the demonstrations that CTX, VCR, and MTX were in large part non-cross-resistant. Comparison of a single dose of CTX with 6 doses (patients who achieved CR after one dose being randomized to no more CTX or 5 more doses) [10] showed that, among stage III patients treated with a single dose of CTX, relapses occurred in the same site as the original tumor, sometimes with concomitant CNS disease and nearly always within 2 to 8 weeks of randomization. Such patients responded to further CTX, 7 of 7 achieving a second CR, indicating that a single dose of CTX represented, for the vast majority of patients, inadequate therapy. Stage III patients randomized to receive multiple doses of CTX tended to relapse after 3–5 doses of CTX and all failed to respond to additional CTX. Ten of the total of 24 patients who relapsed after a CR following single-agent CTX, either during or shortly after the completion of therapy were treated with a combination of VCR and MTX followed by Ara-C, a regimen known as BIKE. Nine achieved a CR, 8 remaining free of disease for 30–102 weeks (i.e., most were probably cured). These data suggested that drug combinations would

be likely to result in improved treatment outcome. This was an important finding.

2.2. Early Drug Combinations. The efficacy of combination chemotherapy was tested in a second clinical trial conducted at the LTC in which patients with stage III or IV disease, who were randomized at the time of presentation to receive either 6 doses of cyclophosphamide (40 mgs/kg every 2-3 weeks—24 patients) or a sequential combination regimen (TRIKE) consisting of CTX followed by VCR and MTX followed by Ara-C (18 patients). These therapy components were administered at approximately 2 weekly intervals until 2 cycles of TRIKE had been given [24]. Although the overall relapse rate between the two arms of the study were quite similar (with also no difference between stage III and IV patients), there was a trend in favor of TRIKE. Patients who received CTX alone invariably recurred at the same anatomical location (often with CNS disease), and 7 of 8 who relapsed on treatment failed to respond to continuation of CTX. In contrast, relapse in the TRIKE arm tended to occur earlier, and, of 9 patients who relapsed on therapy, 6 responded to continuation of TRIKE (involving different agents), consistent with the notion that the drugs were non-cross-resistant. Since at least some of the patients treated in the CTX alone arm could be salvaged with BIKE and because of the small numbers of patients available for comparison, there appeared at first to be no clear advantage in terms of overall survival, although later followup did indicate a survival advantage to patients initially treated with TRIKE [25], again consistent with the value of combination chemotherapy.

The early results achieved in the TRIKE trial suggested that the use of a simultaneous drug combination from the beginning would be advantageous. Accordingly, a randomized study was performed in which patients were initially treated with CTX alone (2 doses 2 weeks apart) or with two cycles of COM (a combination of CTX, VCR, and MTX repeated after two weeks) [26]. Since an effective means of preventing CNS disease had still not been identified, no IT therapy was given unless CNS disease was present (at presentation or relapse). However, patients who achieved remission were randomized to receive no further therapy or craniospinal irradiation in Nairobi, where a radiation therapy unit had recently been established. A dose of 20–24 Gy, in 30 fractions over two weeks, was given, but this dose failed to prevent CNS recurrence [27] and did not influence the outcome of therapy. The proportion of relapses treated with CTX alone or with COM was similar over the follow-up period, but 7 of the 8 recurrences among 19 patients treated with CTX alone involved both systemic and CNS disease, while 8 of the 10 recurrences in the 21 patients who received COM were confined to the CNS. Later followup indicated a survival advantage for patients treated with COM from the outset [25], but the study also emphasized, once again, the need for effective prevention of CNS spread. Although inadequate by modern standards, these data all pointed towards combinations of drugs being more effective, but, in the absence of effective CNS treatment, their advantage could not be realized.

In a similar time period, Nkrumah and Perkins, conducted a series of studies in Accra, Ghana, initially supporting the conclusion that the BIKE regimen was non-cross-resistant and also that simultaneous combination therapy (in Ghana, a combination of CTX, Ara-C, and vincristine was used, along with intrathecal methotrexate, during each course of therapy) was likely to be more effective [28, 29]. Very few patients developed CNS relapse compared to a previous group of patients treated with CTX alone, and the frequency of systemic relapse was also much lower in patients randomized to combination therapy. Unfortunately, the high toxic death rate in the latter arm prevented the demonstration of a survival advantage. This study did suggest, however, that the reason that intrathecal therapy had not been effective previously is that it was not sufficiently prolonged—one cycle is not enough.

2.3. Prevention and Treatment of CNS Disease. Further confirmation of the need for multiple cycles of CNS therapy had been suggested by early studies at the LTC in which IT chemotherapy was given only to patients with overt CNS disease. Various regimens, ranging from single weekly doses to (remarkably) 10 consecutive days of therapy with either methotrexate or Ara C, had been used but little success was achieved. In contrast, a fraction of patients with CNS disease at the time of relapse—for example, almost a third of those with isolated CNS relapse—achieved long-term survival when given multiple doses of IT therapy in conjunction with additional systemic therapy. Moreover, some patients with CNS recurrence after intrathecal methotrexate had durable remissions when treated with multiple-dose IT Ara-C [30], indicating the value of both drugs and suggesting the need for more than a single course of therapy for effective CNS prophylaxis. Such a conclusion is taken as self-evident today, although it did not seem obvious at that time. Remarkably, however, (approximately 50%) of patients with CNS disease occurring at any time in their disease achieved prolonged survival following multiple doses of intrathecal therapy, again, entirely consistent with the likelihood that many patients were simply being inadequately treated with intrathecal therapy.

Interestingly, unlike BL in the USA and Europe, a significant fraction of patients presenting with CNS disease in Africa have limited disease—for example, a jaw tumor or isolated CNS relapse and many achieved prolonged survival, although patients who had simultaneous CNS and systemic relapse had a worse outcome [31]. This strongly suggested that total tumor burden was more important than the mere presence or absence of CNS disease in predicting outcome. In contrast, patients in North America or Europe, where CNS disease tends to occur in patients with systemically advanced disease, particularly when there is bone marrow involvement, have a poor prognosis—doubtless primarily due to the overall disease burden, but interpreted for some years as indicating that CNS disease *per se* was a poor prognostic indicator—its presence is still sufficient to classify a patient as stage IV [32].

2.4. The Role of Other Therapeutic Modalities. Chemotherapy constitutes the primary therapy for BL. Surgery may have a limited role in countries where sufficiently intensive therapy is not available—for example, when patients have access only to CTX, but very few patients, apart from those in rural Africa fall into this category. A retrospective study conducted in Uganda showed a survival advantage to patients in whom the bulk (estimated to be at least 90%) of all abdominal tumor was resected prior to the commencement of chemotherapy [33]. Surgery may be disadvantageous in patients who are to receive high intensity therapy but, in patients presenting with insussusception due to localized small-volume bowel disease, total resection and limited chemotherapy, without intrathecal therapy, results in an almost 100% survival rate.

Radiation therapy was also studied in Africa after radiation became available in Nairobi. Single daily dose radiation therapy was shown to be quite ineffective for the control of local disease (with remission occurring in only 1 of 9 irradiated tumors), although hyperfractionation appeared to be of some benefit [34]. No new evidence contradicting these data has been produced, either in Africa or elsewhere, and radiation is not a component of standard therapy for BL. These findings are very important to equatorial Africa, for the rare need for surgery, and poor results of radiation therapy indicate that this most common of pediatric cancers requires little more than expert knowledge, a few drugs, and a minimum of equipment for patients to be successfully treated. This should not be interpreted to mean that patients can be treated at any district hospital. It is essential that the treating physician is familiar with the characteristics of the disease, the side effects, and the complications that can arise from therapy and that there are adequate facilities for hydration—and if necessary dialysis.

3. The Evolution of Therapy in the USA, Europe and Adults

The COM regimen was adopted at the US National Cancer Institute in the 1970s [35] and formed the backbone on which modern intensive chemotherapy regimens used firstly in children with BL and subsequently in adults [36–41] have been developed. The regimens have become increasingly intensive and have led to survival rates of approximately 90%. In fact, the primary goal of therapeutic studies today is to define risk groups as accurately as possible, such that patients receive the minimum of therapy consistent with the best possible outcome. Initially, these more intensive protocols were more intensive than they needed to be, and it has taken some years and a number of studies to demonstrate this. It is even possible that anthracycline, added when this drug was felt to be of major importance in the treatment of adult non-Hodgkin's lymphoma, adds nothing but toxicity to childhood Burkitt lymphoma. Late cardiac toxicity occurs at much lower cumulative doses than acute toxicity and is a particular problem in children whose cardiac development may be impaired and should, if at all possible, be avoided. A randomized study is currently being conducted to explore this. In addition, the advent of rituximab has led to the possibility of obtaining the same excellent results with less toxicity. Once again, this drug is being tested in children with Burkitt lymphoma, although the excellent results achieved with modern intensive chemotherapy mean that, initially, subgroups of patients with a somewhat worse prognosis must be identified for clinical trials of the efficacy of ritixumab, or use made of phase II windows [42]. One such study did show efficacy of rituximab, and, in adults, where the outcome of therapy has been generally somewhat worse than in children, it has been simpler to add ritixumab to therapy and demonstrate an advantage [43, 44].

The intensive treatment approaches used in the western world are not suitable for use in equatorial Africa, where facilities for supportive care remain extremely limited—the paucity of appropriate treatment facilities coupled to poverty have a marked impact on access to care. Recently, therefore, several groups, including the International Society of Pediatric Oncology, the Groupe Francophone pour Oncologie Pediatrique, and the International Network for Cancer Treatment and Research, have worked with colleagues in Africa and other low- and middle-income countries to develop suitable protocols for African countries (including North Africa, where facilities are better) [45–48].

3.1. Therapy of African Burkitt Lymphoma Today. An initial study in Malawi with therapy based on the French LMB studies but much less intensive, in which patients with St Jude stage IV were excluded, showed that event-free survival was 57% for all patients (10 stage I, 5 stage II, and 29 stage III) with a stable survival curve at 500 days [45]. Cyclophosphamide monotherapy was also retrospectively utilized in Malawi in the late 1990s at a dose of 40 mgs per Kg every two weeks. Patients received between 1 and 12 doses [46]. Only 72 of 90 patients were evaluable, but the possibility of cure of patients with limited facial disease using therapy was confirmed (63% survival in patients with disease in the head only), and some patients with more extensive disease also survived (33% survival in 21 children with tumor involving the abdomen or other sites).

In 2000, a SIOP study was initiated in Malawi consisting of a combination of cyclophosphamide (O), vincristine (V), prednisone (P), and methotrexate (M) given as COP1, COP2, COMP1, and COMP2 [47]. A total of 30 boys and 12 girls were entered in study, but 14 patients died during or shortly after the initiation of therapy, and projected event-free survival at one year is not promising, being 50% in stage I and II, 33% in stage III, and 25% in stage IV (33% overall). Although morbidity and mortality was high, only 300 mgs of cyclophosphamide was used, being increased to 500 mgs in the COMP cycles. The authors felt that the likeliest explanation for the poor results was the low cumulative dose of cyclophosphamide. A small protocol (40 patients) of higher-dose cyclophosphamide (40 mgs per M2), IT methotrexate, and hydrocortisone conducted in Malawi in 2006 gave a result of approximately 48% survival for an average cost of less than $50 [49]. The ability to "rescue" patients with high-dose cyclophosphamide and methotrexate [50], suggests that many of these patients, as in the 1960s and 1970s, were undertreated.

A much larger study being conducted by the INCTR in several equatorial African countries is in progress, and initial results look promising. This protocol uses higher doses of cyclophosphamide (1200 mgs/m^2) with vincristine and methotrexate as the first-line therapy, and has a salvage regimen (second line), which has induced complete responses in approximately a third of patients resistant to the first-line therapy. This approach, in which future higher risk patients will receive both the first- and second-line therapy (rituximab remains a possibility but may have to be excluded because of cost), seems likely to produce very acceptable results for equatorial Africa as it already has in India, Egypt, and the USA, albeit with higher doses in the USA [40, 41, 51, 52].

In countries where resources are less limited, results are better since more intensive therapy can be safely given to patients with extensive disease; for example, somewhat modified versions of the highly effective French and German protocols. Depending upon the level of development, results approach those being achieved in high-income countries. Sadly, therefore, one is forced to conclude that a large number of children in equatorial Africa still receive inadequate therapy, and outside formal clinical trials, the advances made 30–40 years ago (for the most part) [53, 54] have led to only limited gains, in the ensuing period in terms of the cure of patients with African Burkitt lymphoma, largely because of the major resource limitations and poor access to care.

4. Conclusions

African BL, discovered and initially investigated in equatorial Africa, has provided an important model for the epidemiology, pathogenesis, and treatment of many other lymphomas. In particular, the value of combination therapy and intrathecal prophylaxis were strongly suggested in the early clinical trials in African Burkitt lymphoma although numbers were small. Collaboration between resource-rich countries and African countries should both improve the care of patients with BL in Africa, where treatment needs to be intensified, and lessen the side effects of therapy outside Africa, where treatment is probably still more intensive than it need be. The use of newer agents such as rituximab may prove to be prohibitively expensive for the time being but is likely eventually to be introduced into the treatment of African patients for reasons of lower toxicity and, eventually, overall lower cost (the treatment of toxic complications can be costly).

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Review Article

Molecular Profiling of Aggressive Lymphomas

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In the last years, several studies of molecular profiling of aggressive lymphomas were performed. In particular, it was shown that DLBCL can be distinguished in two different entities according to GEP. Specifically, ABC and GCB subtypes were characterized by having different pathogenetic and clinical features. In addition, it was demonstrated that DLBCLs are distinct from BL. Indeed, the latter is a unique molecular entity. However, relevant pathological differences emerged among the clinical subtypes. More recently, microRNA profiling provided further information concerning BL-DLBCL distinction as well as for their subclassification. In this paper, the authors based on their own experience and the most updated literature review, the main concept on molecular profiling of aggressive lymphomas.

1. Introduction

Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL) are the commonest aggressive B-NHL worldwide and represent distinct entities in the World Health Organization (WHO) classification [1, 2].

BL is listed in the WHO classification as a single genetic and morphologic entity with variable clinical presentation. It accounts for 30–50% of lymphomas in children, but only 1-2% in adults. In particular, the WHO classification recognizes 3 clinical subsets of BL: endemic (eBL), sporadic (sBL), and immunodeficiency-associated (ID-BL) [1].

The endemic form is the commonest type, being the most frequent childhood cancer in equatorial Africa [1, 3–5]. eBL is almost invariably associated with Epstein-Barr virus (EBV) infection, although local environmental toxics (i.e., *Euphorbia tirucalli*) and coinfection with arbovirus or malaria also appear to be important for its pathogenesis [6–8].

sBL is the most commonly recorded form in the USA and Europe. Contrary to eBL, only ~20% of cases are correlated to EBV [9].

Immunodeficiency-associated BL occurs more commonly in patients infected with HIV (HIV-BL). Intriguingly, because HIV-BL can occur in patients with relatively high CD4 counts, immunosuppression *per se* is not sufficient to explain the relatively high prevalence of BL in this setting [10, 11].

The diagnosis of classical BL rests on the presence of a monotonous infiltrate of medium-sized blastic lymphoid cells that show round nuclei with clumped chromatin and multiple, centrally located nucleoli. The tumor cells have a high proliferation rate and intermingled macrophages containing apoptotic debris lead to the morphological aspect of a "starry sky" pattern [1]. Immunophenotypic features of BL include positivity of tumor cells for CD20 and CD10 (and BCL6), negativity for BCL2, and a proliferation fraction measured by Ki-67 immunohistochemistry of nearly 100%. On the basis of morphology, phenotype, and genetics, BL is currently regarded as a germinal-center- (GC-) derived neoplasm [1]. Notably, according to the somatic hypermutation (SH) patterns and the expression of specific EBV-related molecules, in the WHO classification, a different origin for the endemic and sporadic forms has been suggested [1, 12, 13].

The molecular hallmark of BL is a chromosomal rearrangement of MYC, in form of reciprocal translocation, juxtaposing MYC to the immunoglobulin heavy chain (IGH locus [(t(8; 14)(q24; q32)]) or the κ or λ light chain loci

(t(2; 8)(p12; q24) and t(8; 22)(q24; q11), resp.). Different breakpoints on chromosome 14 and different mutation pattern of the 5′-region of MYC have been recorded between eBL and sBL [14]. In addition, although all BLs have similar phenotype and MYC translocation, it has been demonstrated that the 3 subtypes may have different pathogenetic mechanisms and a key role for EBV has been proposed [13].

Diffuse large B-cell lymphoma (DLBCL) is the most common form of lymphoma and accounts for 30–40% of all lymphomas in adults [2]. Clinical outcome is extremely various, with 5-year survival rates between 30% and 80%, widely dependent on clinical risk factors and biological heterogeneity [2]. DLBCL is characterized by the proliferation of large neoplastic B cells, with nuclear size equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte, that has a diffuse growth pattern and comprises centroblastic, immunoblastic, T-cell/histiocyte-rich and anaplastic morphological variants [2]. Morphological, biological, and clinical studies have subdivided diffuse large B-cells lymphomas into morphological variants, molecular and immunophenotypical subgroups, and distinct disease entities.

Of note, the differential diagnosis between BL and DLBCL is sometimes unclear, as both entities show overlapping morphological, immunophenotypic, and genetic features [15]. This distinction is also often critical for the different management of these two diseases. In fact, relatively low-dose chemotherapy regimens such as cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) are typically used to treat DLBCL, but they can be inadequate for BL [16, 17], for which intensive chemotherapy regimens are required [18–23]. Moreover, the characteristic t(8;14) translocation of BL [24–26] also occurs in 5 to 10 percent of cases of DLBCL [27, 28] and chromosomal breakpoints at the *MYC* locus are recurrently associated with non-IG partner loci and complex chromosomal alterations [24–32]. Because DLBCL is more than 20 times as common as BL [29], a lymphoma with a t(8;14) translocation could present a diagnostic problem. Based on the last WHO classification, B-cell lymphomas with features intermediate between DLBCL and BL have been defined [15] as aggressive lymphomas with morphological and genetic features of both DLBCL and BL, but for biological and clinical reasons, should not be included in these categories. Some of these cases were previously classified as Burkitt-like lymphoma. Most of these cases have intermediate morphological features between DLBCL and BL, with some cells that are smaller than typical DLBCL, resembling BL, and some cells that are larger than typical BL, resembling DLBCL, with a high proliferation fraction, starry-sky pattern, and an immunophenotype consistent with BL. Some cases may be morphologically more typical of BL but have an atypical immunophenotype or genetic features that preclude a diagnosis of BL. The diagnosis of this type of unclassifiable B-cell lymphoma category should not be made in cases of morphologically typical DLBCL that have a *MYC* rearrangement or in otherwise typical BL in which a *MYC* rearrangement cannot be demonstrated. This is a heterogeneous category that is not yet clearly considered a distinct disease entity, but is useful in allowing the classification of cases not meeting criteria for classical BL or DLBCL [15, 30].

2. Gene Expression of DLBCL and BL

Even with the use of current diagnostic criteria, the distinction between DLBCL and BL is not always precise; for this reason, in the last years, different molecular studies were performed to further characterize DLBCL and BL.

The first gene expression profiling (GEP) studies revealed that DLBCL is composed of at least two molecularly and clinically distinct diseases [30, 32, 33]. The first subgroup of DLBCL, called germinal center B-cell-like (GCB) DLBCL, expresses genes that are characteristic of normal germinal center B cells and is characterized by frequent REL amplifications, BCL2 translocations, and ongoing somatic hypermutation of the immunoglobulin genes [30, 34, 35]. The second DLBCL subgroup, called activated B-cell-like (ABC) DLBCL, misses expression of germinal center B-cell-restricted genes and resembles mitogenically activated blood B cells. GEP allowed to identify individual genes that predict overall survival in DLBCL, the majority coming from gene expression signatures that reflect the cell of origin, proliferation rate, and host immune response to the tumor. In another couple of studies, a third subgroup of DLBCL has been defined by GEP, termed primary mediastinal B-cell lymphoma (PMBL) [31, 36]. ABC-DLBCL and PMBCL have a constitutive activation of the nuclear factor KB (*NFKB*) pathway that they require for survival, which is not a feature of GCB-DLBCL [31, 36, 37].

These three DLBCL subgroups should be possibly considered different entities since they correspond to different B-cells stages of differentiation, use different oncogenic pathways, and have distinct clinical behaviors [38].

These three subgroups of DLBCL are associated with a different clinical outcome with 5-year survival rates of 59% in GCB-DLBCL, 30% in ABC-DLBCL, and 64% PMBCL [30, 31, 33].

In the last years, two DNA-microarray studies investigated whether global GEP might help to discern BL from DLBCL on the molecular level [39, 40]. In the first [40], 220 mature aggressive B-cell lymphomas that included classical BL, "atypical BL," and DLBC were studied. A "core group" of eight BLs was defined on the basis of WHO criteria (histological classification of classic or "atypical" BL, CD20+, BCL6+, CD10+, BCL2−, CD5−, KI67 ≥ 95%, and *MYC* translocation). The remaining cases were compared with the BL core group and were labeled with a "BL similarity index" according to gene expression value of 58 genes. On the basis of this index, aggressive B-NHL were classified into molecular Burkitt lymphoma (mBL; 22%), intermediate cases (20%), and nonmolecular Burkitt lymphoma (non-mBL; 58%). The distinctive mBL signature was characterized by 58 genes, including several target genes of the *NFKB* pathway (i.e., *BCL2A1, FLIP, CD44, NFKBIA, BCL3, and STAT3*) that are known to differentiate ABC or GCB lymphomas [30, 32, 33]. These genes were expressed at lower levels in mBL than in cases of GCB DLBCL. This mBL

signature was extended also to cases with morphological characteristics of DLBCL and BCL2 positive. Not all cases with a morphologic and immunophenotypical features of BL were classified as mBL. All patients with mBL had a favourable prognosis (5-year survival rate, 75%). Moreover, mBL cases had very few genetic alterations, detected by CGH, in addition to the *MYC* translocation (low genetic complexity); conversely, intermediate and non-mBL cases carried a higher number of chromosomal imbalances (high genetic complexity). Furthermore, non-mBL (DLBCL) cases with the *MYC* translocation had inferior overall survival as compared with DLBCL cases without *MYC* translocation in a retrospective analysis of patients receiving mostly a CHOP/CHOP-like therapy [40].

In the second study [39], 303 cases with the diagnosis of BL, "atypical" BL, and DLBCL were profiled for gene expression. Aggressive B-NHL was first divided into cases in which a MYC target gene signature was identifiable in the total gene expression profile and those in which such a signature was not evident. Expression profiles of the cases that contained a *MYC* target gene signature were compared with the signatures of germinal center B-cell-like (GCB) DLBCL, activated B-cell-like (ABC) DLBCL, and primary mediastinal lymphoma (PMBL). The gene expression-based classifier for BL includes the expression of *MYC* target genes, a particular subgroup of germinal center B-cell-associated genes, and a low expression of major histocompatibility (MHC) class I genes and *NFKB* target genes. Only when all four pairwise comparisons of the gene expression profiles were in favor of the diagnosis of BL, was the diagnosis "molecular BL" assigned. By using this methodology, some cases that were diagnosed as DLBCL or high-grade lymphoma were classified as BL on the basis of gene-expression profiles. This molecular classifier provided a superior quantitative and reproducible diagnosis of BL to the best actual diagnostic method.

Both gene expression studies [39, 40] confirmed that a subset of aggressive B-NHL with evident morphological features of DLBCL showed indeed a gene expression profile of BL. Therefore, both studies help to improve the molecular distinction between BL and DLBCL, but, at the same time, extend the spectrum of molecular BL to some cases that would currently be classified as DLBCL [41].

On the basis of GEP data [30, 32, 33], GCB-DLBCL and ABC-DLBCL have a different cellular counterpart and a distinct clinical behavior, as patients with ABC-DLBCL show a poorer outcome than patients with a GCB-DLBCL [30]. Moreover, the molecular classification may be helpful in choosing treatments that can be effective only in specific subtypes of DLBCL. Therefore, GEP can also be an important indication in the management of the patients in the near future. But, GEP analysis is not feasible in routine clinical practice. For this reason, different studies have been made to confirm GEP results using a more easy technical approach such as immunohistochemistry (IHC). IHC can be effected in archival, formalin-fixed, paraffin-embedded tissues and is operable at any pathology laboratory. Different algorithms have been created using the expression of well-known antigens [42–46].

None of the actual immunohistochemical algorithms is able to exactly predict the GEP subtype or to perfectly stratify molecular groups with prognostic value. As a consequence, stratification based on immunohistochemical algorithms for leading therapy is possible only with great caution [47].

Importantly, the 2 mentioned GEP studies on BL and DLBCL [39, 40] did not include all BL subtypes. In a most recent paper from our group [48], GEPs of all BL subtypes were studied for the first time and were compared with those of a large panel of B-cell-derived malignancies and normal B lymphocytes. This paper showed that BL was a distinct entity, supporting the current WHO classification [1]. In fact, the GEP of all BL subtypes was quite homogeneous and distinct from those of other lymphomas. Moreover, BL molecular signature could effectively distinguish not only sBL but also eBL and HIV-BL from DLBCL. eBL and HIV-BL shared a common GEPs, whereas sBL cases were relatively more different. The principal differences between eBL and sBL regarded significant pathways such as BCR, TNF/NF-KB, and interleukin-dependent intracellular cascades and probably reflected the different clinical contests. In fact, GEP of eBL, that undergoes a chronic antigen stimulation (e.g., EBV, malaria, and arbovirus infections), had a molecular signature including many genes involved in immune response regulation. Similar considerations could be then applied to HIV-BL. Moreover, a significant difference between eBL and sBL also consisted in the enrichment in genes belonging to the RBL2 network [49, 50].

Furthermore, all BL subtypes were definitely related to GC B lymphocytes. In fact, all BL molecular profiles were closer to those of GC lymphocytes than to those of memory cells. However, it cannot be excluded that EBV + BL may origine from a later developmental stage.

Piccaluga et al. [48] also found a large set of genes differentially expressed in BL and normal GC cells, as a result of malignant event. This set included genes related to immune response, cell cycle regulation, and BCR signalling. These differences may reflect the arrest of differentiation of BL neoplastic cells and the neoplastic transformation [51].

Finally, to further validate gene expression data, immunohistochemistry (IHC) was executed to evaluate the expression of proteins corresponding to genes overexpressed in BL versus normal GC cells and according to potential biologic interest. The 2 molecules CYR61 and SPARC tested by IHC were strongly expressed by the neoplastic cells, suggesting a significant role in BL pathobiology. In fact, CYR61 expression has been related to malignant transformation in different settings, including human lymphomas, being also related to aggressive clinical behavior and drug resistance [52–54], and SPARC contributes to the acquisition of migratory and invasive properties that are recapitulated by malignant tumor cells [42].

3. microRNA in BL

Recently, miRNAs have been identified as posttranscriptional regulators of gene expression and involved in physiological and pathological differentiation and maturation processes

[55]. They represent a novel mechanism that allows cells to regulate many events such as control cell growth, differentiation, apoptosis, and morphogenesis [56]. Several studies have reported the involvement of miRNAs in cancer [57–59].

In particular, in BL, several studies tried to explain some unexplained differences between BL subtypes, for example, *MYC* translocation-positive and *MYC* translocation-negative cases, EBV-positive and EBV-negative cases [60–64]. This is a really important aspect because the *MYC* translocation-negative BL cases may represent a challenging diagnosis to discriminate them from DLBCL and from cases with intermediate features between DLBCL and BL cases (DLBCL/BL) [15, 40, 65, 66].

By using web-available resources (Mirnaviewer, Pic-Tar, Tarbase [67], and miRBase [68]), it was possible to search miRNA directed against a specific target, for example, *MYC*. By using this methodology, Leucci et al. [63] identified hsa-mir-34b. Hsa-mir-34b was found to be downregulated only in BL cases that were negative for *MYC* translocation, suggesting that this event might be responsible for *MYC* deregulation in such cases. Hsa-mir-34b is a member of the mir-34 family, which has been identified as a direct target of p53 and has been observed in human cancers associated with a p53 lack [69]. The hsa-mir-34b downregulation observed in *MYC* translocation-negative cases was not associated with mutations in the hsa-mir-34b gene sequence and probably might be explained with other molecular mechanism. *In vitro* experiments demonstrated that hsa-mir-34b had an impact on MYC regulation. In fact, using a synthetic hsa-mir-34b, a significant dose-dependent decrease of MYC was observed in lymphoblastoid cell lines and using an hsa-mir-34b inhibitor an increase of MYC expression was detected. These findings provided evidence for a novel mechanism of MYC overregulation in BL cases without the *MYC* translocation, as the more common aberrant control exercised by the immunoglobulin enhancer locus. Conversely, the overexpression of hsa-mir-34b in cases with the *MYC* translocation might be due to the loss of regulation on MYC by hsa-mir-34b itself in this condition. In fact, hsa-mir-34b could not be effective in regulating MYC expression, as MYC is under transcriptional control of the immunoglobulin gene promoters. These results are very interesting because they provide a molecular significance of BL *MYC* translocation-negative cases that are often treated as DLBCL. This study showed that MYC upregulation, due to an alternative mechanism, represents a key role in BL pathogenesis also in *MYC* translocation-negative cases.

Interestingly, MYC itself is able to activate the expression of specific miRNAs [55, 56], suggesting the existence of a feedback loop between MYC and specific miRNAs that reciprocally control their expression. MYC overexpression might induce a specific miRNA pattern that, in turn, might be the cause of a differential gene expression and of functional alterations of neoplastic cells [61].

In a paper by Onnis et al. [61], a strong upregulation of hsa-miR-17-5p and hsa-miR-20a, which correlates with high levels of MYC expression in BL, was found. Hsa-miR-9* was the only miRNA strongly downregulated only in BL *MYC* translocation-negative cases.

The hsa-miR-9* expression was tested also in DLBCL and Intermediate DLBCL/BL cases to verify that it is a specific molecular marker for BL cases lacking the typical translocation.

DLBCL cases showed a strong overexpression of hsa-miR-9*, whereas intermediate DLBCL/BL cases showed a heterogeneous expression, suggesting that the latter subgroup may be considered a heterogeneous category; additional studies are necessary to demonstrate whether hsa-miR-9* may be used as a specific marker to differentiate BL from DLBCL and to identify cases that may benefit from a more aggressive therapy.

An interesting target of hsa-miR-9* is E2F1, which is essential for the G1-S1 phase passage, which expression is known to be induced by c-MYC, and in turn controls c-MYC expression [70, 71].

Interestingly, an inverse correlation between hsa-miR-9* and E2F1 was found. In *in vitro* studies, E2F1 regulation by hsa-miR-9* upregulation reduced E2F1 levels; conversely, miR9* silencing induced E2F1 expression. MYC expression was affected both at the mRNA and protein levels. Thus, hsa-miR-9* inactivation may determine E2F1 upregulation and consequent MYC overexpression in BL lacking *MYC* translocation. The downregulation of hsa-miR-9* in *MYC* translocation-negative cases is caused by an epigenetic mechanism; in fact, an aberrant methylation of hsa-miR-9-1 gene was observed in *MYC* translocation-negative cases. The same epigenetic mechanism was observed in human breast cancer [72].

Therefore, this miRNA downregulation may have an important role in the *MYC* negative-translocation BL pathogenesis and may become a molecular signature of these cases, proposing it as a novel candidate for a more careful diagnosis and therapy.

A recent paper by Robertus et al. [64] performed an miRNA expression profile of paediatric t(8; 14) positive and high MYC expressing BL in comparison with *MYC* translocation-negative mantle cell lymphoma (MCL), follicular lymphoma (FL), and chronic lymphocytic leukaemia (CLL). A normal B-cell subset was included to the study. Unsupervised hierarchical clustering analysis showed a unique miRNA profile in BL that was a dominant MYC-induced miRNA profile with most miRNAs being downregulated. The authors observed a significant downregulation for hsa-let7e in BL compared to the three other NHL subtypes. Hsa-miR-150 was also significantly downregulated in BL and targets MYB, which has an essential role in haematopoietic and lymphoid development and apoptosis [73]. Downregulation of hsa-miR-150 in BL compared to the other 3 NHL subtypes might thus result in enhanced MYB levels in BL. The low expression of hsa-miR-26a, that targets EZH2 (a member of the polycomb group of genes) [74], is thus consistent with the previously observed high expression of EZH2 in BL. Also, hsa-miR-155 was found downregulated in BL. Hsa-mir-155 regulates activation-induced cytidine deaminase (AID) expression; in fact, low expression of hsa-mir-155, by the presence of a point mutation in the miR-155 binding site, resulted in increased levels of AID and a higher frequency of MYC translocations [75], consistently

with MYC translocation-positive BL. Finally, several other MYC-regulated miRNAs were found to be downregulated in BL, for example, miR-23a/b targeting glutaminase, hsa-miR-125b targeting IRF4 and PRDM1 (BLIMP1), hsa-miR-146a targeting IRAK1 and TRAF6, and hsa-miR-223 targeting LMO2. This study showed a specific miRNA profiling for BL.

Another paper by Leucci et al. [60] showed that hsa-miR-127, which is directed against B-cell regulators, was differentially expressed between EBV-positive and EBV-negative BL cases. In particular, it was highly upregulated only in EBV-positive BL samples, whereas EBV-negative cases showed the same levels of expression with normal controls. Thus, this upregulation may specifically depend on the presence of EBV, though actually the mechanism of hsa-miR-127 regulation by EBV remains unknown. The authors proposed that hsa-miR-127 overexpression in EBV-positive BL may determine downregulation of PRDM1 and XBP-1, and the consequent persistence of BCL6 and germinal center phenotype in a B cell that is already differentiated in terms of surface immunoglobulin and mutation pattern toward a postgerminal center B cells. This further confirms the concept of a different pathogenetic mechanism between EBV-positive and EBV-negative BL. In fact, the upregulation of hsa-miR-127 may be a key event in the pathogenesis of EBV-positive BL, by inhibiting the B-cell differentiation step.

4. microRNA in DLBCL

In the last years, several studies were performed to found some differentially expressed miRNAs between DLBCL subtypes.

Lawrie et al. [76] demonstrated that 3 miRNAs were highly expressed in ABC-type but not in GCB-type cell lines; these miRNAs (hsa-miR-155, hsa-miR-21, and hsa-miR-221) were then confirmed to be highly expressed also in *de novo* DLBCL, transformed DLBCL, and follicular lymphoma cases compared to normal B cells. According to the cell line model, microRNAs expression levels were greater in ABC immunophenotype DLBCL cases than in GCB-type immunophenotype DLBCL ($P < 0.05$). Of note, deregulation of miR-155 appears to be a recurrent feature of many malignancies including breast cancer [77], thyroid carcinoma [78], and a range of solid tumors [59]. Moreover, high-grade lymphomas were spontaneously developed by transgenic mice expressing miR-155 targeted to B cells [79].

miR-221 has been shown to inhibit normal erythropoiesis through downregulation of c-kit expression [80], but probably this gene is not a significant target for hsa-mir-221 in DLBCL, as no evidence of c-KIT expression was detected by IHC in DLBCL [76].

Overexpression of hsa-miR-21 resulted to be an independent prognostic indicator from IPI status in *de novo* DLBCL, and this high expression was associated with a better prognostic outcome ($P < 0.05$). These 3 microRNAs were investigated also in different developmental stages of B and T cells from healthy individuals; both hsa-miR-155 and hsa-miR-21 were found more highly expressed in ABC than GCB cells. This finding advanced that high levels of these microRNAs in DLBCL are not closely tumor associated but may rather be correlated to a characteristic of their cell of origin [76].

In a paper by Roehle et al. [81], the comparison of miRNA profiles between DLBCL and reactive LN revealed 15 differentially expressed miRNAs. Most differentially expressed miRNAs were downregulated (11 of 15). Hsa-mir-210, hsa-mir-155, and hsa-mir-106A were upregulated; conversely, expression of hsa-mir-149 and hsa-mir-139 was reduced. As previously described, mir-155 seems to play a critical role in B-cell maturation and lymphocyte activation [82, 83]. Moreover, a correlation between mir-155 and NFKB expression was found in DLBCL cell lines and patients [84]. On the other hand, hsa-mir-210 has an important role in cell cycle [85].

A comparison between DLBCL and FL cases showed 10 differentially expressed miRNAs. The expression of hsa-mir-150, hsa-mir-17-5p, hsa-mir-145, hsa-mir-328, hsa-mir-99A, hsa-mir-10A, hsa-mir-95, hsa-mir-151, and hsa-mir-let7e was characteristic of specific DLBCL signature. Hsa-mir-150 was highly downregulated in DLBCL and was found to regulate B-cell development [73, 86]. It has been described that hsa-mir-10A in AML and hsa-mir-17-5p and as hsa-mir-145 were deregulated in various other cancer types [59, 87–90].

In addition, in a paper by Roehle et al. [81], miRNAs with a prognostic relevance were identified. In particular, 8 miRNAs were identified by a multivariate Cox regression analysis including IPI factors. Of these, 6 miRNAs (hsa-mir-19A, hsa-mir-21, hsa-mir-23A, hsa-mir-27A, hsa-mir-34A, and hsa-mir-127) were downregulated and associated with poor EFS and/or OS. Conversely, 2 miRNAs (hsa-mir-195 and hsa-mir-let7g) were correlated to a better prognosis. However, only hsa-mir-127 significantly influenced both OS and EFS in a multivariate analysis and correlated with poor survival prognosis. Hsa-mir-127, reported to be methylated in neoplastic cells and inversely proportional to the expression of BCL6, plays as tumour suppressor [91]. On the contrary, hsa-mir-195 and hsa-mir-let7g, a lower expression of which was associated to a better prognosis, probably function as an oncomiR. Interestingly, in this paper, hsa-mir-155 was not identified as a prognostic marker for survival, although this miRNA was exclusively overexpressed in ABC-DLBCL [76, 92].

Malumbres et al. [93] provided experimental evidence that hsa-miR-125b simultaneously downregulated expression of IRF4 and PRDM1 and had an important role in the GC reaction and post-GC differentiation. In fact, hsa-miR-125b expression was higher in GC centroblasts than in memory B cells and was reciprocal to the expression of IRF4 and PRDM1. Moreover, PRDM1 and IRF4 have a complementary binding sites for hsa-miR-125b in the 3′ UTR region and are able to translationally repress expression of both genes through specific interactions with this locus.

Furthermore, the authors showed that the expression of hsa-miR-223 was low in GC lymphocytes, high in naive and memory B cells, and reciprocal to the expression of LMO2, that is exclusively expressed in GC lymphocytes and has a clinical significance in DLBCL since its expression level is

a powerful predictor of survival in DLBCL patients [94, 95]. Noteworthy, hsa-miR-223 was shown to be able to directly regulate LMO2 expression.

Finally, the authors identified 9 miRNAs whose expression was most different between the DLBCL subtypes; 2 of them (hsa-miR-155 and hsa-miR-21) were previously reported to be differentially expressed between ABC and GCB DLBCL subtypes [76, 96]. Overexpression of hsa-miR-21 was confirmed to be associated with a longer relapse-free survival in a small cohort of 35 not uniformly treated *de novo* in DLBCL cases. The expression level of hsa-miR-21, hsa-miR-155, and hsa-miR-222 was also studied in 106 R-CHOP-treated patients of DLBCL, but only the overexpression of hsa-miR-222, associated with ABC-DLBCL, correlated with shorter OS and PFS. It is unknown if this correlation is caused by a specific function in DLBCL or reflects the ABC subtype [93].

5. Conclusion

In conclusion, recent GEP and miRNA profiling studies provided notable information on the molecular pathogenesis of BL and DLBCL. In addition, they provided the basis for a clear distinction of the 2 entities when morphology and phenotype are not conclusive. Finally, the recognition of specific molecular profiles was useful for a more refined subclassification of these lymphomas, with relevant prognostic implication. Certainly, to date, it is difficult to propose global GEP in the routine workup; however, new technologies, including next generation sequencing, will hopefully improve our ability in the molecular managing of lymphoma patients offering adequate surrogates for diagnosis and prognostication.

Conflict of Interests

The authors have no conflict of interests to declare.

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