The impact of EBV and HIV infection on the microenvironmental niche underlying Hodgkin lymphoma pathogenesis.

Short title: The impact of EBV and HIV on cHL pathogenesis

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Abstract

The pathogenesis of classical Hodgkin lymphoma (cHL) is still enigmatic, largely because its tumor cells, the so-called Hodgkin and Reed-Stenberg (HRS) cells, invariably reside in a prominent reactive microenvironment, are rare and therefore difficult to analyze. On the other hand, the broadly investigated cHL-derived cell lines are not unequivocally considered as suitable and representative models for this puzzling disease. Based on current knowledge, it appears that the cross talk between the tumour cells and the reactive infiltrate of the microenvironment is complex and that multiple mechanisms occur, making cHL a very heterogeneous disease. In 20 to 40% of cHL cases, HRS cells carry a monoclonal infection by Epstein Barr virus (EBV), which is considered a tumour-initiating factor. In these cases, EBV shows a latency type II infection pattern with the expression of latent membrane protein-1 (LMP-1), a viral oncoprotein that mimics CD40 activation. This scenario is particularly intriguing for the pathogenesis of cHL arising in HIV-infected patients, which, for still obscure reasons, is invariably EBV-associated with LMP-1 expression in HRS cells. Recent evidences are consistent with the occurrence of different pathogenic pathways variably triggered by virus infections (EBV and HIV), genetic alterations, and interactions with critical microenvironmental components. This review focuses on the different microenvironmental niches that characterize cHL of the general population as well as cases of HIV-infected patients. A more comprehensive understanding of the complex interplay existing between HRS and tumour microenvironment is pivotal for the development of more effective treatments, particularly for relapsed or refractory diseases.

Key words: Hodgkin lymphoma; Classical Hodgkin lymphoma; Pathogenesis; Microenvironment; EBV-associated Hodgkin lymphoma; HIV-associated Hodgkin lymphoma.

General background

Hodgkin Lymphoma (HL) has been classified into classical HL (cHL),^{1,2} which accounts for 95% of all HL cases, and the nodular lymphocyte predominant HL (NLPHL), which is a separate entity.³⁻⁵ cHL is a neoplastic disease with heterogeneous epidemiological, clinical, pathological, and virological features. It accounts for 10% of all lymphomas and is the most common cancer in patients under 20 years.^{1,2}

cHL morphologically includes the following four subtypes: nodular sclerosis (NS), mixed cellularity (MC), lymphocyte-rich (LR) and lymphocyte-depletion (LD) subtypes. In cHL, microenvironmental cell types include reactive T- and B-lymphocytes, eosinophils, granulocytes, histiocytes/macrophages, plasma cells, and mast cells.^{3,4} In addition, a high number of fibroblast-like cells and a prominent fibrosis are frequently found.⁶ Cellular components of the cHL microenvironment show complex interactions with Hodgkin Reed-Sternberg (HRS) cells and express molecules involved in cancer cell growth and survival (such as CD30L or CD40L), and in immune escape (programmed death 1 (PD-1)).⁶⁻⁸

Molecular studies of isolated tumour cells have shown that LP cells of NLPHL are derived from antigen-selected germinal center (GC) B cells, whereas other studies carried out on microdissected HRS cells have shown that they derive from GC or post-GC B cells carrying somatically mutated immunoglobulin (Ig) V genes. The occurrence of destructive IgV gene mutations suggests a derivation of HRS cells from GC B lymphocytes that normally would undergo apoptosis. Since Epstein Barr virus (EBV) may be capable to rescue pre-apoptotic GC B cells from apoptosis, a significant role for EBV in HL pathogenesis has been hypothesized in cHLs in which HRS cells are infected by EBV.⁹⁻¹³

As shown by gene expression profiling (GEP) studies, HRS cells have lost the expression of most B cells genes. Silencing of B-cell genes and down-regulation of B-cell transcription factors (OCT2, BOB1, and PU1) seem to contribute to the loss of the B-cell phenotype in HRS cells.^{14,15} Moreover, multiple signaling pathways, mainly including nuclear factor kappa-B (NF- κ B), Janus kinase-signal transducer and activator of transcription signaling (JAK/STAT), PI3K-Akt and ERK, have deregulated activity in HRS cells. Coherently, recurrent genetic alterations detected in HRS cells of cHL frequently affect members of the NF- κ B or JAK/STAT signaling pathways. These alterations include inactivating mutations in NFKBIA, NFKBIE, TNFAIP3, and SOCS1, genomic gains of REL and JAK2, and rare BCL3 translocations. TNFAIP3

mutations are found in EBV-negative cases of cHL. Mutations have been found in the tumour suppressor genes CD95 and TP53.¹⁶⁻¹⁹

Despite the significant progress in the understanding of the HRS cell biology, the pathogenesis of cHL is still enigmatic. There are unanswered questions on how the tumour microenvironment impacts cHL pathogenesis: which is the lymphomagenic role of the complex cross talk between tumour cells and the reactive infiltrate characterizing the surrounding microenvironment? Is cHL heterogeneity dependent on the heterogeneity of underlying pathogenic mechanisms? Why monoclonal EBV infection of HRS cells, which may be a tumour-initiating factor, rarely occurs in NS cHL, is found in 20-40% of the MC cHL cases of the general population, whereas, for still unclear reasons, the virus is invariably detectable in HIV associated cHL? (Table 1 and Figure 1).

In an attempt to provide answers to these questions, this review focuses on the genome level features of HRS cells investigated on cHL-derived cell lines and microdissected cells. This review also focuses on microenvironmental tumour cell driving factors recently highlighted in cHL and discuss the role of their intracellular signaling pathways.

Methods for analyzing Hodgkin and Reed-Sternberg cells

Genome level studies of microdissected HRS cells from pathologic cHL tissues have been performed using different technologies.^{15,20-22} Single cells retrieved by laser capture microdissection (LCM) have been used to evaluate chromosomal imbalances using comparative genomic hybridization (CGH) and to perform transcriptional analysis using whole-genome expression arrays.²³ To overcome the limitations of LCM, especially due to the small number of single HRS cells commonly retrieved, flow cytometric cell sorting (which yields thousands of purified HRS cells from primary biopsy samples) was combined with a refined exome sequencing library construction methodology that obviates the need for biased whole genome amplification techniques.²⁴ Moreover, gene expression assays in formalin fixed paraffin embedded tissues have been proposed for the determination of cell of origin and clinical subgroups of patients affected by cHL.²⁵

Whole-tissue RNA analysis was valuable in characterizing the cHL microenvironment but not the HRS cells.^{22,26,27} In the last decade, the application of proteomic techniques, particularly those allowing the study of secretome and surfaceome,

proved to be powerful tools for the identification of novel biomarkers involved in HL pathogenesis.^{28,29}

Genome level studies

The study of copy number changes in microdissected HRS cells with the use of conventional CGH revealed that cHL shows a characteristic profile of recurrent copy number gains and losses, including gains of chromosomes 2p, 9p, 16p, and 17q and losses of 13q, 6q, and 11q.^{30,31} In more recent studies in which the investigators used oligonucleotide arrays, novel copy number changes were identified, including amplification of STAT6, NOTCH1, and JUNB.³² Another study of microdissected HRS cells from 53 cHL cases identified at high resolution new and recurrent chromosomal gain or loss of genes potentially involved in HL pathogenesis, including IKBKB, CD40, MAP3K14, and TNFRSF14²² (Table 2). Overall, genomic imbalances identified by CGH studies included gains of IKBKB, CD40 and MAP3K14 that are regulators of NF- κ B signaling.^{16,18,19,22,32,33}

Genome-wide expression studies on cHL cell lines identified genes aberrantly expressed by HRS cells and uncovered a loss of the B-cell transcriptional program in cHL.^{15,20} An approach to perform genome-wide expression profiling of cHL cell lines and primary HRS cells laser-microdissected from frozen biopsies²³ showed that cHL cell lines, although retaining key features of primary HRS cells, have undergone major gene expression changes to survive in suspension,²³ which must be taken into consideration when studying these cell lines. The analysis also demonstrated that the loss of B-cell identity of HRS cells is not the result of the acquisition of a transcriptional plasma cell signature²³ and that EBV infection has a minor influence on the gene expression pattern of HRS cells.²³ Two major subgroups of cHL emerged that were not defined by the histologic subtype or EBV status but were associated with a differential signature strength of the proto-oncogenes MYC, IRF4, and NOTCH1.²³ Importantly, the study also explored the transcriptional relatedness of HRS cells among the various cHL subtypes to large B-cell lymphomas. NS and LD cHL appeared more similar to primary mediastinal large B-cell lymphoma cells than MC and LR cHL did.²³

Interestingly, it has been shown that HRS cells show aberrant somatic hypermutation of several proto-oncogenes (PIM1, Rho/TTF, MYC, PAX5) in a considerable fraction of cases (Table 2).^{18,19,34}

Microenvironmental tumor cell driving factors and their intracellular signalling pathways.

Pathways activation in HRS cell through membrane-bound signaling. NF- κ B, JAK-STAT, and MAPK/ERK pathways are activated by molecules released by reactive cells of cHL microenvironment. HRS cells have constitutive activity of both canonical and alternative NF- κ B pathways (Figure 2). In the canonical NF- κ B signaling pathway, activation of the IKK complex, targeting the NF- κ B inhibitors I κ B α and I κ B β for ubiquitination and proteasomal degradation, is produced through stimulation of various receptors. They include CD30, CD40, RANK, OX40L, which complex with TNF receptor-associated factors (TRAFs) and the receptor interacting protein (RIP). Degradation of NF- κ B inhibitors leads to translocation into the nucleus of NF- κ B transcription factors that activate multiple genes. In the alternative NF- κ B pathway, stimulation of the kinase NIK (MAP3K14), which activates an IKK α complex, results in activation of receptors such as CD40 and TACI. Activated IKK α processes the p100 NF- κ B precursor to p52, which translocates as p52/RELB heterodimers into the nucleus (reviewed in ^{8,13,18,19}). HRS cells have constitutive activity of STAT3, -5, and -6. The JAK/STAT pathway is activated by cytokines usually present within cHL microenvironment. Activation of this pathway is also mediated by genomic gains or translocations of the JAK2 gene. The constitutive activity of the JAK/STAT pathway and the NF- κ B transcription factors may be also the result of autocrine and/or paracrine loops (Figure 2) (reviewed in ^{8,13,18,19}).

Pathways activation in HRS cell through extracellular matrix and HL-associated fibroblasts. Since NS cHL microenvironment contains a collagen-rich extracellular matrix (ECM), and a large number of fibroblasts, defined as HL-associated fibroblasts, a lymphomagenic role for discoidin domain receptor1 (DDR1), a receptor tyrosine kinase (RTK),^{35,36} has been proposed. Interactions between HRS cells and HL-associated fibroblasts produce collagen-rich reticular fibers composed of collagen I, III and IV covered by the ECM. Importantly, collagen IV, which is recognized by DDR1 in turn induces DDR1 up-regulation and activation.³⁷ After binding to collagen, DDR1 phosphorylation triggers the activation of downstream signaling pathways, including NF- κ B³⁸ (Figure 2).

Microenvironmental impact of EBV infection

The immunophenotypic and genetic features of HRS cells are identical in the different histologic subtypes of cHL. Conversely, the association with EBV shows marked differences: EBV is found in HRS cells preferentially in cases of MC and LD cHL, and less frequently in NS and LR cHL. Notably, the virologic characteristics of cHL vary according to the immunocompetence status of the host and cHL subtype³⁹ (Table 1 and Figure 1), with EBV being found in HRS cells in nearly all cases of cHL occurring in patients infected with HIV.^{1,40} Available evidence is consistent with a pathogenic role for this herpesvirus in EBV-positive cases, probably as an early event in HL development. In fact, the demonstration of monoclonal EBV genomes in HRS cells indicates that EBV infection occurred prior to clonal expansion. EBV-positive HRS cells express a relatively restricted set of viral genes, the so-called type II latency pattern, including EBNA-1, LMP-1, and LMP-2 latent proteins, together with EBERs and BARTs RNAs.⁴¹ Despite the multiplicity of functions ascribed to these EBV-encoded genes, genome-wide transcriptional analysis of microdissected HRS cells disclosed only a minor impact of EBV in the transcriptional programme of clonal HRS cells.²³ It should be however considered that the same transcriptional changes imposed by EBV in infected HRS cells can be induced by other mechanisms in EBV-negative HRS cells. In fact, the NF- κ B activation promoted by LMP-1 expression in EBV-positive cases may be similarly induced by inactivating mutations affecting the TNFAIP3 NF- κ B inhibitor, which are significantly more prevalent in EBV-negative cHL⁴² (Figure 3). Moreover, the aberrant co-expression of several receptor tyrosine kinases (RTKs) is largely restricted to EBV-negative cHLs, suggesting that the signaling mediated by these RTKs can be at least partially replaced by EBV in HL pathogenesis.⁴³ These findings again point to a prevalent contribution of EBV to the early steps of cHL pathogenesis and clearly indicate that, although morphologically indistinguishable, EBV-positive and EBV-negative cHL cases arise through distinct pathogenic routes. The main role of EBV is probably to functionally substitute for cellular genetic changes that are critical for HL development.

Convincing evidence indicates that HRS cells are derived from pre-apoptotic GC B lymphocytes expressing non-functional BCRs, being therefore cells committed to die by apoptosis. Intriguingly, the large majority of cases of cHL carrying inactivating immunoglobulin gene mutations are EBV-positive,¹² suggesting that EBV infection of a HRS progenitor cell within GC microenvironment may promote survival and further malignant evolution of these pre-apoptotic precursors. Indeed, EBV is able

to immortalize BCR-negative B cells *in vitro*⁴⁴⁻⁴⁶ and has the capacity to rescue from apoptosis B lymphocytes missing functional BCR, mainly thanks to the ability of LMP-1 and LMP-2 to mimic CD40- and BCR-mediated signaling.^{47,48} Nevertheless, the restricted latency II pattern is unable to convey a strong growth-promoting stimulus in B lymphocytes, an effect that is probably due to the inadequate differentiation state of the precursor cells, which lack the B-cell specific transcription factors that are required for the expression of EBNA-2, the main driver of B-cell proliferation. In fact, experiments carried out in early cultures of *in vitro* EBV-infected lymphocyte populations demonstrated that latency II cells are overgrown by those expressing the broader latency III pattern.⁴⁹ In EBV-positive cHL, HRS cells invariably express high levels of LMP-1 expression, and are endowed with a highly efficient interaction with the cells of the tumour microenvironment, being able to attract a variety of cellular populations and respond to several autocrine and paracrine signals. Survival and low proliferation capacity of HRS cells are critically dependent on these interactions. Consistently, *in vitro* establishment of representative cHL-derived cell lines proved to be very difficult, and only one EBV+ cell line (L591) is available, although it does not show the HRS cell-typical latency II.⁵¹

Recent evidence indicates that the expression of critical EBV-encoded proteins may be modulated by factors released or expressed by stromal cells that may be present within cHL tumour microenvironment. In particular, the T cell-derived cytokines IL-21 and CD40L were shown to down-regulate EBNA-2 expression in latency III EBV-positive lymphoblastoid cell lines, whereas IL-4, IL-10, IL-13, and IL-21 can induce LMP-1 expression in some malignant B-cell lines.⁵¹⁻⁵⁴ Notably, expression of LMP-1 in GC B lymphocytes, the putative precursors of HRS cells, upregulates DDR1 thereby allowing the binding of these cells to collagen, an interaction resulting in protection from apoptosis.^{37,55}

Notably, gene profiling of a large series of cHL revealed that the signature of EBV-positive cHL tissues is enriched in genes characteristic of T-cell and antiviral responses,⁵⁶ further supporting the notion that EBV has a major role in influencing the tumour microenvironment, whereas its impact on the infected HRS cells is minor and probably limited to the early stages of the disease. The cellular infiltrate of EBV-positive cHL is largely composed of immune cells that are probably attempting to eliminate EBV-positive HRS cells together with inflammatory cells that provide local support for the growth and survival of the neoplastic clone. Although EBV-specific cytotoxic T lymphocytes can be isolated from cHL patients and have been shown to

specifically kill LMP-1 and LMP-2 expressing targets *ex vivo*, these effectors are unable to eliminate EBV-carrying HRS cells *in vivo*.⁵⁷⁻⁵⁹ This is probably the result of a microenvironment blunting antitumor immune responses. Indeed, high numbers of natural CD4+CD25+ regulatory T cells (Tregs) have been detected in the peripheral blood and tumour tissues of cHL patients.⁶⁰⁻⁶² The viral protein EBNA1 has probably a direct role in attracting natural Tregs through the up-regulation of the CCL20 chemokine,⁶³ and LMP-1 epitopes were shown to promote Treg cell infiltration of cHL tissues.⁶⁴ More recently, it has been demonstrated that EBV-positive cHL are preferentially infiltrated by regulatory Type 1 cells (Tr1) expressing ITGA2, ITGB2 and LAG3 and producing the immunosuppressive IL-10 cytokine.⁶⁵ Notably, the alpha and beta chains of the IL-10 receptors are significantly up-regulated in EBV-positive cHL, suggesting that these cases have an enhanced sensitivity to IL-10 allowing therefore Tr1 cells to exert more pronounced immunosuppressive effects.⁶⁵ Recruitment of Tr1 cells in EBV-positive cHL was also associated with overexpression of several chemokines, such as CCL17, CCL19, CCL20, CCL21, CCL22, and CXCL9, known to attract T-helper 2 (Th2) and Treg cells thus contributing to the local immune suppression. Globally, these findings are consistent with the co-existence in EBV-positive cHL of different subpopulations of Th2 and Treg cells endowed with various functional properties. Nevertheless, the notion that the tumour microenvironment of cHL is largely enriched in poorly effective, senescent Th2 cells has been recently challenged by the demonstration of a prevalent functional Th1 cell infiltrate in these cases.⁶⁶ This may be particularly true for EBV-positive cHL cases, which were also shown to carry significantly higher numbers of infiltrating activated CD8+ T cells and natural killer cells.⁶⁷ Therefore, available data indicate that, in EBV-positive cHL, activated CD8+ T cells, probably specific for viral epitopes, and various subpopulations of Treg cells coexist in a puzzling microenvironmental scenario. This could be at least in part explained by a different localization and contact of immune effectors with respect to HRS cells, although this issue is still a matter of debate. Recently, HRS cells were found mainly surrounded by CD4+ Treg cells, whereas activated CD8+ T lymphocytes were not present in tumour-cell-rich areas, suggesting therefore that they are probably ineffective in terms of immune control of the disease.⁶⁷ The features of tumor microenvironment may however show relevant variations in cHLs occurring at different ages, as shown for pediatric, EBV-positive cases that are characterized by a predominantly cytotoxic/Th1 profile.⁶⁸ The HLA background of cHL patients may also have a role in this respect and, more importantly, in dictating the inherent risk to develop an EBV-positive cHL. Indeed,

several studies have shown that the HLA-A*02 allele is protective against EBV-positive cHL,⁶⁹⁻⁷² and this association has been recently substantiated by the demonstration that the subdominant T-cell responses to LMP-1 and LMP-2 epitopes is largely confined to patients carrying this allele.⁷³

Tumour microenvironment of EBV-positive cHLs is also characterized by a more pronounced infiltration by macrophages as compared to EBV-negative cases. In fact, a prominent signature including the overexpression of macrophage-related genes was identified in EBV-positive cHLs by gene expression profiling of whole tumour tissues.⁵⁶ Moreover, all immunohistochemistry studies carried out so far consistently detected significantly higher numbers of CD68⁺⁷⁴⁻⁷⁶ and CD163⁺^{74,76} cells in EBV-positive cHLs. Depending on availability of different microenvironmental signals, macrophages may undergo polarized activation towards two functional states: the M1 macrophages with a pro-inflammatory phenotype, the ability to promote Th1 responses and kill tumour cells and the M2 macrophages with potent tumor-promoting activity, regulatory functions in tissue repair and remodeling and promotion of Th2 responses.⁷⁷ Considering that the distinction between M1 and M2 macrophages using immunophenotypic markers is not unequivocal, it has been recently proposed to combine the analysis of macrophage markers such as CD68 or CD163 together with transcription factors differentially expressed in polarized macrophages: STAT1 in M1 and CMAF in M2 cells.⁷⁸ In keeping with a predominant Th1 microenvironment of EBV-positive cHLs, this approach disclosed a main M1 polarization of macrophages infiltrating pediatric EBV-positive cHL.⁷⁹ Therefore, available data indicates that the EBV status of cHL heavily influences tumor microenvironment, which is similar to that of Th1-predominant inflammatory disorders, albeit with a less prominent magnitude.

The ability of EBV to impose a Th1-skewed microenvironment in cHL is however counterbalanced by different functional activities mediated by the same virus that inhibit host immune responses against EBV-carrying lymphocytes. Particular relevance in this respect has the ability of EBV to hijack the exosome pathway to excrete viral and cellular components that can modulate its microenvironment.^{80,81} The major EBV oncoprotein LMP-1 can be detected in EBV exosomes and may inhibit both T-cell and natural killer cell responses *in vitro* through the strong immunosuppressive properties of a conserved region within the first transmembrane domain of LMP-1.⁸² EBV-infected B cells were also shown to produce and release FasL⁺ exosomes that have the capacity to kill antigen-specific T

cells,^{83,84} an effect that could contribute to keep immune effectors away from tumour cells. EBV exosomes also contain deoxyuridine triphosphate nucleotidohydrolase (dUTPase), a protein encoded by the BLLF3 EBV gene expressed during abortive or lytic replication, which may interfere with both innate and adaptive immune responses.⁸⁵ Notably, dUTPase-containing EBV exosomes are able to interact with dendritic cells through Toll-like receptor-2 to enhance the production of inflammatory cytokines and induce NF- κ B activation.⁸⁵ Moreover, the observation that EBV dUTPase may also promote the secretion of various chemokines⁸⁵ suggests that exosomes carrying this viral protein may modulate cell trafficking and attract a variety of cells that may favor not only the induction of a persistent EBV infection, but also a local milieu permissive for EBV-driven lymphomagenesis. Whether the BLLF3 EBV gene is expressed at some time during cHL lymphomagenesis remains to be elucidated. Exosomes containing EBV-encoded miRNAs may also promote a local immunosuppression, as shown for the EBV BHRF1-3 miRNA, which targets and suppresses the interferon-inducible T-cell attracting chemokine CXCL-11/I-TAC, thus inhibiting immune responses to lymphoma cells.⁸⁶ Exosomes released by EBV-carrying cells can also directly convey biologically active signal transduction components potentially able to activate pathways that are critical for growth and survival of recipient B cells,⁸⁰ although their potential role in cHL pathogenesis has been poorly investigated.

LMP-1 may directly contribute to the generation of an immunosuppressive microenvironment through its ability to induce/enhance the production of immunosuppressive cytokines such as IL-6, IL-8, and IL-10.⁸⁷⁻⁸⁹ Moreover, Both LMP-1 and LMP-2 proteins enhance the expression of Galectin-1 (Gal-1),⁹⁰ a glycan-binding protein that has been identified as a critical mediator of the immunosuppressive activity of cHL due to its ability to generate a Th2/regulatory T cell-skewed microenvironment.⁹¹⁻⁹² The inflammatory milieu of EBV-positive cHL was shown to be enriched in histiocytes, dendritic cells and endothelial cells expressing the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), a potent local suppressor of T-cell immunity.⁹³ Indeed, EBV is able to induce IDO expression and enzymatic activity in monocyte-derived macrophages, an effect mediated by IL-6- and TNF- α -dependent mechanisms via the p38/MAPK and NF- κ B pathways.⁹⁴ These findings are consistent with a role of EBV-mediated IDO expression in tumour stroma of cHL.

As for other tumours, also cHL has been carefully investigated for the expression of immunomodulatory molecules such as PD-1 and its ligands PD-L1 and PD-L2, which can enable tumour cells to evade host immune system by usurping immune checkpoint pathways. Anti-PD1 antibodies have been recently tested in clinical studies that included patients with hematological malignancies and showed remarkable efficacy in cHL.^{95,96} In this malignancy, PD-L1 expression may be the result of two main mechanisms: 9p24.1 amplification and EBV infection.^{97,98} Indeed, expression of PD-L1 may be up-regulated by LMP-1 in EBV-immortalized lymphoblastoid B-cell lines through activation of JAK3 and STAT5 phosphorylation or the engagement of c-Jun.⁹⁸ EBV-positive and EBV-negative cHL cases were recently shown to have a similar frequency of 9p24.1 gains/amplifications.⁹⁹ No significant correlation was found between EBV and the expression of PD-1, PD-L1, or PD-L2,¹⁰⁰ although EBV-positive cases were shown to have higher PD-L1 expression levels consistently with a further up-regulation of PD-L1 by viral infection.⁹⁹ The finding that EBV-specific T lymphocytes may express PD-1 further strengthens the possibility that treatments targeting this receptor may successfully restore therapeutically relevant immune responses against EBV-carrying HRS cells.¹⁰¹

The microenvironmental niche of HIV-related cHL

In addition to a predominance of more unfavorable histological subtypes, namely MC and LD,^{102,103} HIV-related HL shows several other morphologic differences as compared with cases of the general population. Among them, the occurrence of large confluent areas of necrosis is a distinctive feature of HL occurring in HIV-positive patients and underlies the presence of a pro-inflammatory activity.¹⁰⁴ A “sarcomatoid” pattern, although not specific, is also observed more frequently in these cases and it has been associated with increased numbers of CD163+ spindle-shaped macrophages.¹⁰⁴ Intriguingly, a significant increase in spindle-shaped cells was observed after differentiation of monocytes into M1 compared with M2 macrophages.¹⁰⁵ Nevertheless, available data on the absolute numbers and functional polarization of macrophages infiltrating HIV-related HL are scarce and controversial. Unlike what it could be expected in an immune compromised setting such as HIV infection, similar numbers of HRS cells are usually found in HIV-related and HIV-unrelated cHLs.¹⁰⁴ This could be the result of a partially retained ability of host immune system to control the expansion of the tumour cell pool, consistently

with the observation that cHL generally occurs in HIV-positive patients with a moderate level of immune deficiency, as indicated by CD4+ T-cell count.¹⁰⁶⁻¹⁰⁷

Perhaps the most common and relevant microenvironmental abnormality of HIV-related cHL is the marked reduction in the number of CD4+ T cells and an inverted CD4/CD8 T-cell ratio in involved tissues.^{104,108,109} This is the likely consequence of the declining of circulating CD4+ T-cell counts preceding the diagnosis of cHL, an effect observed also in patients responding to cART.^{107,110} In contrast, the absolute number of CD8+ T lymphocytes is generally stable in these cases, although a decrease in infiltrating GrB+ cells (activated cytotoxic cells) and an increase in infiltrating TIA+ T cells (mainly non-activated cytotoxic cells) were observed.^{104,109} The reduced number of CD4+ T cells probably accounts for the less frequent detection of HRS cells surrounded by “rosetting” CD4+ T lymphocytes in HIV-related cHL as compared to cases of immunocompetent patients. More frequently, HRS cells from HIV-related cases show resetting with cytoplasmic protrusion of CD163+ macrophages, an effect verified also in *in vitro* cultures.¹⁰⁴ The functional consequences of this abnormal pattern of cell aggregation are still obscure, but it can be hypothesized that these rosettes may behave as a shield protecting HRS cells from the attack of tumour-specific immune effectors.

The numbers of CD56+ (functional NK cells) and CD57+ (terminally differentiated T lymphocytes and mature NK) cells infiltrating HIV-related cHL tissues are usually lower than those detected in HIV-negative cases,¹¹¹ indicating in particular that innate immune effectors such as NK cells have probably a negligible impact on tumor microenvironment. A reduced number of infiltrating CD20+ B cells was also observed in HIV-related cHL and it has been suggested that this could contribute to the aggressive behaviour of this tumour.¹¹¹ It remains however to be elucidated if these alterations reflect differences in cell distribution between peripheral blood and lymphoid tissues or if they are the result of the cytokine/chemokine profile of HIV-related cHL.

One of the main features characterizing HIV-associated cHL is its strong association with EBV infection. In fact, virtually all these cases show HRS cells harboring EBV genome and intensely expressing the LMP-1 viral oncoprotein, whereas only 20-50% of HIV-unrelated cases carry EBV infection. Although the pathogenic mechanisms underlying the close association of HIV-related cHL with EBV are still unknown, it is likely that the development of this tumour and the distinctive features of its microenvironment are the result of the complex interplay

between HIV and EBV, as in the case of most HIV-associated non-Hodgkin lymphomas.¹¹² Viral cooperation is a mechanism by which different viruses co-infecting human tissues have synergistic or regulatory effects on carcinogenesis.¹¹³ In HIV-related cHL, cooperation between EBV and HIV probably occurs mainly through indirect interactions mediated by cellular dysregulation/immunodeficiency or chronic antigenic stimulation/inflammation. In this respect, it is well known in that HIV itself as well as several HIV-encoded proteins, including gp120 and Tat,^{114,115} may promote and sustain B-cell hyperactivation, a relevant component of the more general chronic antigenic stimulation observed in HIV-positive patients. Together with the abnormal production of B-cell growth-promoting cytokines such as IL-6 and IL-10,¹¹⁶ these effects may lead to an uncontrolled poly- or oligo-clonal expansion of dysregulated B lymphocytes, which are at increased risk of acquiring critical genetic alterations, ultimately leading to lymphoma development. Intriguingly, HIV virion budding from activated CD4+ T cells allows these viral particles to include CD40 ligand (CD40L),^{117,118} a costimulatory molecule whose interaction with the cognate CD40 receptor is critical for the growth and survival of HRS cells.¹¹⁹ It should be considered in this respect that LMP-1 may substitute for some of the effects of CD40 activation in HRS cells,¹²⁰ leaving open the possibility that the stimulation provided by CD40L-bearing HIV virions might complement the effects induced by LMP-1 on the CD40 pathway. B cells hyperactivated by a variety of stimuli, induced either directly or indirectly by HIV, may express activation-induced cytidine deaminase (AID), a DNA editing enzyme mediating immunoglobulin class switch recombination, somatic hypermutation and the development of chromosomal translocations.^{121,122}

Nevertheless, cHL is an infrequent event in HIV-positive individuals, indicating that EBV infection and HIV-related B-cell hyperstimulation *per se* are not sufficient to drive lymphomagenesis. Recent findings suggested a new possible scenario in which distinct HIV-encoded protein variants of potential pathogenic relevance could be generated by the well-known HIV genome hypervariability. In particular, it has been shown that, unlike the prototype HIV clade B p17 and p17 proteins derived from HIV-positive individuals without lymphoma, p17 variants derived from an Ugandan HIV-1 A1 strain or from HIV-related non-Hodgkin lymphomas are able to activate the PI3K/Akt signaling pathway and enhance B-cell proliferation and clonogenicity.^{123,124} Notably, EBV infection or the expression of the LMP-1 oncoprotein in primary B lymphocytes were shown to up-regulate CXCR2, a functional cellular receptor for HIV p17,¹²⁵⁻¹²⁸ mirroring thus the effects of CD40

activation. On these grounds, stimuli physiologically found within GC microenvironment, such as soluble or cell-bound CD40L, as well as EBV infection may render B lymphocytes permissive to HIV p17-mediated effects through CXCR2 expression. This may be of pathogenic relevance for HIV-related cHL considering that p17 accumulates within GCs of lymphoid tissues of HIV-positive individuals, even after cART.^{40,128} Intriguingly, clonogenic HIV p17 variants may strongly upregulate LMP-1 expression, thus creating a pathogenic loop (Figure 4).¹²⁷ This mechanism may be highly relevant for the pathogenesis of HIV-related cHL considering that LMP-1 may prevent apoptosis of HRS cell precursors that are committed to die being unable to produce functional B-cell receptors or carrying a high load of pre-lymphomatous genetic alterations in their genome.¹⁸ It remains to be elucidated, however, if HIV p17 variants endowed with the property to promote clonogenicity and enhance LMP-1 expression are enriched also in cHL tissues of HIV-positive patients, as shown for HIV-related non-Hodgkin lymphomas. If confirmed, this hypothesis could at least partly explain the almost complete EBV association of HIV-related cHL on the basis of a complex pathogenic interplay between the two viruses.

Concluding remarks

In conclusion, different pathogenic pathways are variably triggered by interactions of HRS cells with critical microenvironmental components, by inherent genetic alterations, and concomitant viral infections (EBV and HIV). As a final result, the cross talk between tumour cells and microenvironmental factors plays a lymphomagenic role through the activation of critical intracellular signaling, mainly including the NF- κ B, JAK/STAT, and MAP/ERK pathways. An important role is also played by ECM and cHL-associated fibroblasts of the microenvironment through DDR1 upregulation and activation. Therefore, cHL heterogeneity may depend on the heterogeneity of the underlying pathogenic mechanisms.

The different frequency of association with EBV infection is probably related to the different level of patient immunosuppression, both locally in the tumour tissue and at the systemic level. Notably, however, a role for HIV beyond immunosuppression is emerging as supported by recent finding indicating that HIV infection may contribute to lymphomagenesis also through viral cooperation. In particular, a microenvironmental interplay between HIV-released products and EBV-infected HRS

cell precursors may play a crucial pathogenic role in the development of cHL in the HIV setting. A better understanding of the different pathogenic interactions between HRS cells and microenvironment in both HIV-unrelated and HIV-associated cHL will provide the rationale to improve current treatment strategies for this puzzling disease.

Conflict of Interest

The authors declare no competing financial interests.

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Figure Legends.

Figure 1. Morphologic and virologic characteristics of classical Hodgkin lymphoma (cHL) according to the immunocompetence status of the host. Panel A: Nodular sclerosis (NS) (Top) and mixed cellularity (MC) (Bottom) cHL subtypes of the general population. Lacunar cells are the characteristic tumour cells of NS (top left). The inset shows a lacunar cell at higher magnification. Inter-cellular-perivascular sclerosis may be observed in the early phases of the disease (top center). Lacunar cells express CD30 (top right). Hodgkin Reed-Sternberg cells are the characteristic tumour cells of MC (bottom left). In this picture HRS cells are infected by EBV as demonstrated by EBER in situ hybridization (ISH) (bottom center). EBV-infected tumour cells express latent membrane protein-1 (LMP-1) (bottom right). **Panel B:** MC (Top) and lymphocyte depletion (LD) (Bottom) cHL subtypes in patients with HIV-infection (PWHA). HRS cells are the characteristic tumour cells of MC (top left). HRS cells are infected by EBV in all MC cases as demonstrated by EBER ISH (top center). EBV-infected tumour cells express LMP-1 (top right). HRS cells are numerous in LD (bottom left). They express CD30 (inset). HRS cells are infected by EBV in all LD cases as demonstrated by ISH (bottom center). EBV-infected tumour cells express LMP-1 (bottom right).

Figure 2. The schematic figure highlights pathways activation in Hodgkin Reed-Sternberg (HRS) cell through membrane-bound signaling. Different pathogenic pathways are variably triggered by interactions of HRS cells with critical microenvironmental components. Abbreviations: discoidin domain receptor1, DDR1; latent membrane protein -1, LMP-1.

Figure 3: Proposed model of the pathogenesis of EBV-associated and EBV-unrelated classical Hodgkin lymphoma (cHL) lymphomas of the general population. EBV infection may rescue from apoptosis B cells committed to die due to the presence of mutations inactivating BCR. Among other pathogenically relevant functions (NFkB activation, IL-6, IL-8, IL-10 production, etc.), latent membrane protein-1 (LMP-1) upregulates discoidin domain receptor1 (DDR1) that allows Hodgkin Reed-Sternberg cells (HRS) cell precursors to survive and proliferate upon interaction with collagen. In EBV-negative cases, HRS cell precursors may acquire a

growth and survival advantage as a consequence of mutations leading to NFkB activation. In both EBV-associated and EBV-unrelated cases, HRS cells activate a network of growth-promoting and immunosuppressive cytokine network. Additional genetic alterations also contribute to the malignant phenotype of HRS cells.

Figure 4: Proposed model of the pathogenesis of classical Hodgkin lymphoma (cHL) arising in HIV-infected patients. In a systemic background of immune suppression variably mitigated by cART, B lymphocytes are chronically activated by persistent antigenic stimulation, HIV virions bearing CD40L, HIV-released proteins (gp120, p17, Tat, etc), and various cytokines. EBV infection may allow the survival of pre-apoptotic B cells and concomitantly upregulate CXCR2, an IL-8 receptor that may also serve as cellular receptor for HIV p17 and its variants. Triggering of CXCR2 by HIV p17 protein accumulated within nodal or extranodal lymphoid tissues may enhance B-cell clonogenicity and growth, thus increasing the likelihood of critical genetic alterations. In EBV-infected Hodgkin Reed-Sternberg (HRS) cell precursors, p17 proteins could upregulate latent membrane protein-1 (LMP-1), the major EBV oncoprotein contributing to the development of HIV-associated cHL. As for HIV-unrelated cases, HRS cells activate a network of growth-promoting and immunosuppressive cytokine network.

Table 1. Morphologic and virologic characteristics of Hodgkin lymphoma according to the immunocompetence status of the host.

Host	Hodgkin lymphoma subtype	EBV infection
HL of the general population	Nodular lymphocyte predominance	-
	cHL, nodular sclerosis	Usually -*
	cHL, mixed cellularity	Usually +*
	Rare types	
	cHL, lymphocyte rich	Variably +
	cHL, lymphocyte depleted	Variably +
Immunodeficiency-associated HL		
HIV-associated HL		
	cHL, lymphocyte depleted	+
	cHL, mixed cellularity	+
	Less frequent	
	cHL, lymphohistiocytoid	+
	cHL, nodular sclerosis	+
Post-transplant (cHL type PTLD)		
	Similar to other cHL	+
Iatrogenic (methotrexate)		
	cHL, mixed cellularity	Variably + (usually +)

Abbreviations. cHL, classical Hodgkin lymphoma; PTLD, post-transplant lymphoproliferative disorder; -, negative; +, positive.

*Association with EBV is less frequent in NS (10-40%) than in MC cHL (approximately 75% of cases).

Table 2. Relevant signaling pathways showing deregulated activity in Hodgkin Reed-Sternberg cells.^{16,17,19,20}

Deregulated pathways	Genetic alterations
NFkB	Gains and amplification of <i>REL</i>
NFkB	Point mutations and deletions of <i>TNFAIP3</i>
NFkB	Point mutations and deletions of <i>NFKBIA</i>
NFkB	Point mutations and deletions of <i>NFKBIE</i>
NFkB	Gains and translocations of <i>BCL3</i>
NFkB	Gains of <i>IKBKP</i> , <i>CD40</i> , <i>MAP3K14</i>
JAK/STAT	Gains and amplification of <i>JAK2</i>
JAK/STAT	Point mutations and deletions of <i>SOCS1</i>
FAS-mediated apoptosis	Point mutations of <i>CD95</i>

Abbreviations: JAK2, Janus kinase 2; JAK-STAT, Janus kinase/signal transducers and activators of transcription; NFkB, nuclear factor KB inhibitor; SOCS, suppressor of cytokine signaling; *TNFAIP3*, tumor necrosis factor- alpha induced protein.

Figure 1. Morphologic and virologic characteristics of classical Hodgkin lymphoma (cHL) according to the immunocompetence status of the host. Panel A: Nodular sclerosis (NS) (Top) and mixed cellularity (MC) (Bottom) cHL subtypes of the general population. Lacunar cells are the characteristic tumour cells of NS (top left). The inset shows a lacunar cell at higher magnification. Intercellular-perivascular sclerosis may be observed in the early phases of the disease (top center). Lacunar cells express CD30 (top right). Hodgkin Reed-Sternberg cells are the characteristic tumour cells of MC (bottom left). In this picture HRS cells are infected by EBV as demonstrated by EBER in situ hybridization (ISH) (bottom center). EBV-infected tumour cells express latent membrane protein-1 (LMP-1) (bottom right). Panel B: MC (Top) and lymphocyte depletion (LD) (Bottom) cHL subtypes in patients with HIV-infection (PWHA). HRS cells are the characteristic tumour cells of MC (top left). HRS cells are infected by EBV in all MC cases as demonstrated by EBER ISH (top center). EBV-infected tumour cells express LMP-1 (top right). HRS cells are numerous in LD (bottom left). They express CD30 (inset). HRS cells are infected by EBV in all LD cases as demonstrated by ISH (bottom center). EBV-infected tumour cells express LMP-1 (bottom right).

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HIV-negative setting

Naïve B cell

LEGEND

- EBV
- LMP-1
- DDR1
- Collagen
- Activated NF-κB

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HIV-positive setting

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