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Precision Molecular Pathology of Hodgkin Lymphoma

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Precision Molecular Pathology of Hodgkin Lymphoma

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Preface

This new multiauthored text is designed to be the most up-to-date authoritative text on the molecular pathology and pathobiology of Hodgkin lymphoma currently available. Chapters have been written by an internationally recognized team of experts. While the emphasis is on molecular pathobiology, the text includes chapters covering all aspects of the disease. All chapters are generously referenced and, where appropriate, illustrated with tables, figures, and histopathologic images.

Chapter topics include clinical features (SM Ansell, Mayo Clinic, USA), histopathology (SD Hudnall, Yale University, USA), pathogenesis and molecular genetics of classical Hodgkin lymphoma (R Küppers, University of Duisburg-Essen, DE), targeting the microenvironment in Hodgkin lymphoma (L Visser, A Diepstra, A van den Berg, University Medical Center Groningen, NL, and C Steidl, BC Cancer Institute, CAN), the role of EBV in classical Hodgkin lymphoma (P Murray and M Ibrahim, University of Birmingham, UK), pathobiology of nodular lymphocyte predominant Hodgkin lymphoma (S Hartmann and M-L Hansmann, Goethe University, Frankfurt/Main, DE), composite lymphomas and the relationship of Hodgkin lymphoma to non-Hodgkin lymphomas (M Weniger and R Küppers, University of Duisburg-Essen, DE), epidemiology and genetic risk factors (W Cozen and T Mack, University of Southern California, USA), treatment and prognosis (F Montanari and CM Diefenbach, NYU Medical Center, USA), and development of targeted therapies (RW Chen, City of Hope, CA, USA).

We hope the text will prove to be of value to students, teachers, clinical practitioners, and research scientists interested in Hodgkin lymphoma.

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Chapter 1
Clinical Features of Hodgkin Lymphoma

Stephen M. Ansell

Presenting Symptoms

The majority of Hodgkin lymphoma patients present with lymphadenopathy at the time of diagnosis (Ansell 2016). For many patients, the site of lymphadenopathy is supradiaphragmatic, and most commonly patients present with cervical, mediastinal, supraclavicular, and axillary lymph node involvement (Kaplan 1971; Kaplan et al. 1973). Less frequently, inguinal lymph nodes and intra-abdominal lymph nodes are the presenting sites of disease (Krikorian et al. 1986). Additional symptoms, which commonly occur at the time of diagnosis, may include fevers, night sweats, and weight loss (Ekstrand and Horning 2002). Many patients may present with a history of chronic pruritus. These symptoms are present in at least one-third of newly diagnosed patients. While mediastinal lymphadenopathy resulting in large mediastinal masses is often seen, this is rarely the only site of disease. More commonly, mediastinal masses occur in conjunction with cervical or supraclavicular lymph nodes. Infradiaphragmatic disease alone is uncommon, and this presentation constitutes only 3% of newly diagnosed patients (Krikorian et al. 1986; Leibenhaut et al. 1987).

Splenomegaly is present in approximately 10% of newly diagnosed patients. Initial studies suggested that clinical splenomegaly may be a nonspecific manifestation, as only half of the patients with splenomegaly were found to have confirmed involvement of the spleen by active Hodgkin lymphoma (Kaplan et al. 1973). These data, however, were generated in an era of staging laparotomies. More recent data has suggested that splenic involvement may be seen in the absence of splenomegaly in approximately 20–30% of patients when computerized tomography (CT) scans or positron emission tomography (PET) scans are used (Hancock et al. 1994).

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Extranodal disease can occur at any site. The most common extranodal presentations include lung, liver, bone, and bone marrow involvement. Extranodal involvement by Hodgkin lymphoma is a relatively uncommon phenomenon at diagnosis and is seen in approximately 5–10% of cases (Musshoff 1971; Rosenberg 1971). Other sites of involvement such as central nervous system involvement or testicular involvement are very rare. Primary extranodal Hodgkin lymphoma is extremely uncommon, the diagnosis of Hodgkin lymphoma should be questioned in these cases, and a pathology review should be requested.

Patterns of Disease Progression

Hodgkin lymphoma is somewhat unique among lymphomas in that the disease tends to spread in a contiguous fashion (Kaplan 1971; Mauch et al. 1993). Most commonly, the disease presents in the neck or chest and spreads to regional lymph nodes. Disease in the supraclavicular region can spread to sites in the upper abdomen in a contiguous fashion via the thoracic duct. Involvement of the hilar lymph nodes is extremely rare in the absence of mediastinal disease, and similarly, involvement of lung parenchyma is very rare if the mediastinal and hilar nodes are not involved (Diehl et al. 1991). Splenic involvement is a relatively common abdominal site of disease and most likely represents hematogenous spread. Liver involvement is very uncommon, particularly in the absence of splenic involvement (Fialk et al. 1979).

Clinical History

An accurate clinical evaluation is important in this disease. The presence of systemic symptoms is associated with a poor prognosis and may be a clue that more advanced disease than suspected is in fact present. The clinical evaluation may also direct the treating physician to include additional testing to exclude other comorbid diseases that may impact the patient's outcome. These include further evaluation for cardiovascular disease, exclusion of immune deficiencies such as those induced by HIV infection, and further evaluation of smoking and autoimmune diseases. Furthermore, a complete history of prior medical conditions is important. Previous medical conditions such as a history of breast cancer or severe asthma may influence decisions to incorporate treatments such as radiation therapy or the inclusion of bleomycin in the treatment regimen.

Constitutional symptoms have been shown to be of prognostic value. These include an unexplained fever of 101 °F, drenching night sweats, or weight loss ≥10% of the patient's weight. These symptoms have been included in the original Cotswolds staging system (see Table 1.1) and have been denoted as a "B" attached to the numeral to identify the presence of these systemic symptoms (Lister et al. 1989;

Table 1.1 Cotswold staging classification for Hodgkin lymphoma

Stage I	
Involvement of a single lymph node region or lymphoid structure (e.g., spleen, thymus, Waldeyer's ring)	
Stage II	
Involvement of two or more lymph node regions on the same side of the diaphragm (the mediastinum is a single site; hilar lymph nodes are lateralized)	
Stage III	
Involvement of lymph node regions of structures on both sides of the diaphragm	
Stage IV	
Involvement of extranodal site(s) beyond the designated *E*	
A	No symptoms
B	Fever, drenching sweats, weight loss
X	Bulky disease, greater than one-third widening of the mediastinum, >10 cm maximum dimension of nodal mass
E	Involvement of a single extranodal site, contiguous or proximal to a known nodal site
CS	Clinical stage
PS	Pathologic stage

Lister and Crowther 1990). Other unique symptoms associated with Hodgkin lymphoma include pain or flushing at the sites of involved lymph nodes with the ingestion of alcohol. The mechanism for the phenomenon is unknown, and thus far no prognostic significance has been attached to these findings. Generalized pruritus is also commonly seen but is not felt to be a constitutional symptom. Pruritus may be present, however, in 10–15% of patients but does not have any known prognostic importance. Other symptoms that are commonly seen include fatigue and weakness, but these symptoms are frequently seen with other diseases and are therefore not incorporated into any staging evaluation.

Evaluation and Recommended Testing

The management of patients is largely dependent on the stage of the disease and the presence of unfavorable characteristics. A complete staging evaluation, as well as the inclusion of specific laboratory tests to allow for prognostication, is critically important. Aside from a comprehensive physical exam and clinical history, additional testing of importance includes a set of baseline imaging studies. Typically, these include PET with integrated CT scanning (PET-CT) as this has in large part supplanted the use of CT scan alone. Studies have suggested the use of PET-CT scan compared to CT alone changes the stage of disease in up to 20–40% of cases and changes the treatment in approximately 5–15% of patients (Jerusalem et al. 2001; Seam et al. 2007; Kostakoglu et al. 2004). If a CT-PET is unavailable, CT scans of at least the chest, abdomen, and pelvis should be used. In rare cases, additional or alternative imaging modalities can be used, and these could include

ultrasound or MRI scan. A bone marrow aspirate and biopsy have typically been used as a part of the staging evaluation in the past. These have been more relevant in patients with advanced-stage disease. Recent data has suggested that early-stage patients with normal blood counts have a very low likelihood of bone marrow involvement (Munker et al. 1995). With the routine use of PET-CT scans, a negative PET-CT with no bone or bone marrow positivity has been associated with a negative bone marrow test, and therefore, in patients with a negative PET scan, a bone marrow aspirate and biopsy could be omitted (Cheson et al. 2014).

Laboratory testing that needs to be included includes a complete blood count with differential count, an erythrocyte sedimentation rate, liver function testing, as well as measurement of the serum albumin. Additional testing for renal function and thyroid function should be considered. Thyroid function testing is needed to serve as a baseline value particularly if radiation to the neck is considered. Serology testing for human immunodeficiency virus (HIV) is appropriate in selected cases. A pregnancy test should always be performed in any female patient of childbearing potential. In view of the fact that many patients are young, fertility preservation should be considered, and the patient should be counseled regarding cryopreservation of sperm or ova. Additional assessments could include an assessment of the ejection fraction by echocardiogram or radio nucleotide study as most regimens incorporate anthracycline chemotherapy. A baseline pulmonary function test is commonly included if the chemotherapy will contain bleomycin. As newer agents such as brentuximab vedotin and others are incorporated into frontline therapy, an assessment of baseline neuropathy should also be undertaken.

Prognostic Factors

Clinical Factors. An accurate assessment of the stage of disease in Hodgkin lymphoma is vitally important to select appropriate treatment. The stage of this disease is a major prognostic factor for Hodgkin lymphoma, based on the fact that it determines the selection of treatment. The staging system for Hodgkin lymphoma is based on sites of lymphadenopathy with specific attention paid to whether the disease occurs on one or both sides of the diaphragm (see Table 1.1) (Lister et al. 1989). Further attention is paid to the number of involved sites and whether the sites of involvement are bulky in size. Additionally, the staging system takes into consideration contiguous extranodal spread or disseminated extranodal disease, and furthermore as mentioned above, it takes into consideration systemic symptoms (B symptoms) that may be present. Further prognostication with prognostic indices takes staging into account, and there are separate prognostic factors for limited-stage and advanced-stage disease.

In patients with limited early-stage Hodgkin lymphoma, clinical prognostic factors of importance are the presence of a large mediastinal mass, an elevated sedimentation rate, involvement of extranodal sites, involvement of multiple nodal sites, age greater than 50 years, and significant splenic enlargement (see Table 1.2). Both

Table 1.2 Unfavorable characteristics for limited-stage Hodgkin lymphoma

Risk factor	GHSG	EORTC
Age		≥50 years
ESR and B symptoms	>50 if A >30 if B	>50 if A >30 if B
Mediastinal mass	MMR > 0.33	MTR > 35
# of nodal sites	>2	>3
E lesions	Any	

GSHG German Hodgkin Study Group, *EORTC* European Organisation for the Research and Treatment of Cancer, *MMR* mediastinal mass ratio (maximal width of mass/maximal intrathoracic width), *MTR* mass to thoracic width at T5–T6 interspace on standing chest radiograph, *E lesions* involvement of a single extranodal site contiguous to a known nodal site

the European Organisation for the Research and Treatment of Cancer (EORTC) and the German Hodgkin Lymphoma Study Group (GHSG) have utilized these prognostic factors and have differentiated patients into favorable and unfavorable subsets with differing approaches to treatment based on these categories (Tubiana et al. 1989; Diehl et al. 2003).

In patients with advanced-stage Hodgkin lymphoma, the disease bulk and other typical prognostic variables have been less predictive of outcome. Therefore, the International Prognostic Factors Project on Advanced Hodgkin Disease developed a different prognostic scoring system (Hasenclever et al. 1998). In this study, seven variables were identified as prognostically relevant. These include age greater than 45 years, the presence of stage IV disease, male gender, white cell count greater than 15,000/μL, lymphocyte count less than 600 cells/μL, albumin less than 4 g/dL, and hemoglobin less than 10.5 g/dL. The outcome of patients with none of these negative prognostic factors is excellent with an 84% likelihood of being free from disease progression at 5 years. In contrast, patients with 5 or more of these poor prognostic factors have a 5-year freedom of progression of only 42%.

PET Scan. A more recent prognostic factor, which has proven to be as important as many of these clinical features, has been the response to treatment as defined by PET scan. An interim PET scan, typically done after two cycles of treatment, has been highly prognostic as far as overall outcome of patients is concerned (Hutchings et al. 2006; Gallamini et al. 2007). Similarly, a PET scan that is negative upon completion of treatment is also being shown to be associated with a favorable overall survival and progression-free survival (Jerusalem et al. 1999; Zinzani et al. 1999). A negative PET scan after two cycles of treatment predicts a favorable progression-free survival and overall survival and is in fact a better predictor of patient outcome than stage of disease, presence of extranodal disease, or other prognostic factors. The prognostic importance of PET scans has resulted in the utilization of PET scan as method to adapt initial treatment based on risk. Patients with positive PET scans after two cycles of treatment are typically receiving a de-escalation in treatment intensity or duration, compared to patients with a positive PET scan after two cycles of treatment who are receiving more intensive or prolonged treatment or the addition of other modalities of therapy.

Specific Presentations

Hodgkin Lymphoma in Pregnancy. Because Hodgkin lymphoma is often seen in women of childbearing potential, it is not surprising that Hodgkin lymphoma can occur in a woman who is pregnant. Studies have been done to access whether the pregnancy affects the course of Hodgkin lymphoma, and these studies have not suggested a different outcome for Hodgkin lymphoma in patients who are pregnant when compared to age-adjusted controls with Hodgkin lymphoma who are not pregnant (Barry et al. 1962). The management of pregnant Hodgkin lymphoma patients however does present some challenges. Staging of a pregnant patient must be modified to avoid risks to the fetus, and the treatment should also be tailored to optimize the care of the mother but avoid additional injury to the child (Doll et al. 1989). The workup is typically tailored to avoid exposure of the fetus to additional radiation. Ultrasound is often used to access abdominal disease, and imaging of the chest should be done with very careful screening of the fetus. The overall plan is to delay treatment until delivery, unless there is bulky disease that would require urgent treatment.

Hodgkin Lymphoma in Immunocompromised Patients. Although Hodgkin lymphoma is not an AIDS-defining illness, the incidence of Hodgkin lymphoma is significantly increased in patients with HIV infection (Shiels et al. 2009; Engels et al. 2006). In contrast to patients without any immunosuppression, Hodgkin lymphoma in patients who are HIV positive is more commonly mixed cellularity subtype and is often associated with advanced-stage and constitutional symptoms (Levine 1998). Bone marrow involvement can also be seen more commonly. HIV-positive patients may have an excellent outcome, but it is important to ensure that the HIV infection is adequately managed by optimizing treatment of the immune deficiency with highly active antiretroviral therapy (Xicoy et al. 2007; Hartmann et al. 2003). Staging and further management thereafter are typically the same as what is administered to patients who are HIV negative (Okosun et al. 2012).

Elderly Patients. Older patients with Hodgkin lymphoma are understudied, particularly because they are uncommonly included in clinical trials. Overall, the outcome of this population is inferior to younger patients primarily due to comorbid conditions that are more common in this population (Evens et al. 2013; Ballova et al. 2005; Boll et al. 2011). Advanced patient age often leads to modifications in dose intensity of treatment, and less therapy often results in poorer outcomes. To combat this, fit elderly patients should be managed in a similar fashion to younger patients. Those with comorbid disease should have their underlying diseases aggressively managed so that they can be treated with similar therapy as to what is administered to younger patients. Specific clinical trials are being developed for frail elderly patients that omit agents such as bleomycin and doxorubicin to avoid the potential heart and lung toxicity that may impact their outcome. This makes it particularly important to evaluate elderly patients for cardiac and pulmonary compromise prior to therapy. Patients with low ejection fractions may require treatments that contain less or no anthracyclines. Furthermore, in patients with compromised

renal or pulmonary function, it may be important to renally adjust the doses of therapy and avoid bleomycin altogether in elderly patients with limited pulmonary reserve.

Patient Follow-Up and Survivorship

Follow-up of Hodgkin lymphoma patients needs to address the risk of disease relapse as well as the potential for late complications of the treatment previously administered. In long-term follow-up studies, the risk of death over time has included an approximately 50% likelihood of dying due to Hodgkin lymphoma and a 22% risk of dying from second malignancies (Aleman et al. 2003). After 5 years, the likelihood of Hodgkin lymphoma recurrence significantly declines, but the incidence of second malignancies and complications, particularly of cardiovascular disease, continually increases beginning 10–15 years from therapy and is increasing further over time.

After completing therapy, patients in clinical follow-up are typically monitored over the first 5 years for the likelihood of relapse (Torrey et al. 1997; Guadagnolo et al. 2006; Thompson et al. 2014). Longer-term follow-up is focused on complications and toxicity of therapy. The likelihood of relapse is most common within the first 2 years, and routine imaging is usually included in the evaluation of patients with follow-up testing. After 2 years, the likelihood significantly declines, and most providers will then do symptom-directed imaging rather than routine surveillance scans. Typical testing done at the time of ongoing follow-up includes a history and symptom-directed evaluation, physical exam, and lab testing. Lab testing commonly includes a complete blood count, serum chemistries, and a sedimentation rate evaluation. The value of routine lab testing is uncertain as only a minority of patients with relapsed disease are detected by lab testing alone. The use of imaging studies in the follow-up setting is primarily restricted to the use of CT scans. PET scans have a significant increased likelihood of false-positive tests requiring additional evaluation, and therefore, routine PET scans after an end of therapy are not recommended. Commonly, testing for hypothyroidism is included in the evaluation, particularly in patients who received radiation to the neck and chest.

Second Malignancies. Secondary malignancies related to Hodgkin lymphoma therapy are a significant concern (Ng et al. 2002a). Second cancers are more commonly encountered in patients receiving combined modality therapy or radiation alone, compared to patients who receive chemotherapy alone (Franklin et al. 2006). However, intensified therapy including high-dose regimens as initial chemotherapy or high-dose therapy at the time of an autologous stem cell transplant significantly increases the risk of leukemia (Goodman et al. 2008). The cumulative risk of developing acute myeloid leukemia is approximately 1.5% for patients with advanced-stage disease, and this likelihood may further increase when patients require additional salvage therapy. The risk of breast cancer, particularly in female patients receiving radiation therapy to the chest and mediastinum, is also increased. Annual

breast mammography is recommended to start at least 8–10 years after completion of treatment or at age 40, whichever occurs first. The risk of secondary breast cancer is associated with a young age at the time of receiving treatment and has increased in patients who receive treatment under the age of 30 (Ng et al. 2002b). Similarly, lung cancer risk is also increased in patients who received mediastinal radiation. Chest imaging during follow-up should be considered in high-risk groups, particularly if patients have a history of smoking (Ng et al. 2010).

Cardiovascular Toxicity. Cardiovascular disease is significantly increased in patients treated for Hodgkin lymphoma (Elbl et al. 2006). This includes an increased risk of coronary artery disease, pericardial disease, cardiomyopathy, as well as valvular disease. Patients receiving combined modality therapy are at particularly increased risk, and the risk begins to increase approximately 5 years after completion of treatment. The optimal screening strategy to manage this issue is unclear, but aggressive management of cardiovascular risk factors such as smoking, diabetes, hypertension, and hyperlipidemia is recommended. Newer testing modalities including echocardiograms with a ventricular strain assessment may be useful in the future (Tsai et al. 2011). An assessment for the risk of stroke is also indicated, particularly in any patients with suspicious symptoms (Krull et al. 2012; De Bruin et al. 2009).

Infertility. A significant concern with the use of chemotherapy is infertility. Studies done on patients receiving ABVD chemotherapy or escalated BEACOPP have shown that intensification of treatment compromises fertility over time (Behringer et al. 2013; Sieniawski et al. 2008). The studies done to assess whether utilization of gonadotropin-releasing hormone analogs or oral contraceptives would be protective and minimize infertility particularly in young women have not shown that ovarian follicle preservation was improved (Behringer et al. 2010). Therefore, fertility preservation prior to initiating treatment is extremely important. Treatment also seems to affect hormone levels particularly in women. Interestingly, the level of testosterone in males typically remains within the normal range despite chemotherapy. In women, however, the likelihood of developing menopausal symptoms if over the age of 30 is very high when chemotherapy is given for Hodgkin lymphoma. A consequence for chemotherapy-induced hormonal deficiency is that the patient may develop osteoporosis, and therefore, bone density needs to be monitored over time (van Beek et al. 2009).

Summary

Hodgkin lymphoma is a disease that is successfully treated with modern chemo- and radiation therapy. While a comprehensive evaluation at the time of diagnosis is critical for managing the patients in an optimal fashion, close monitoring after completion of treatment is equally important in ensuring long-term outcomes are satisfactory by avoiding long-term complications. The success of management for this

disease therefore depends on an adequate staging evaluation, the appropriate choice of therapy, as well as an aggressive management of long-term complications.

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Chapter 2
Pathology of Hodgkin Lymphoma

S. David Hudnall

Abbreviations

ALCL	Anaplastic large cell lymphoma
CHL	Classical Hodgkin lymphoma
cRSC	Classical RS cell
EBER	Epstein-Barr early RNA
EBV	Epstein-Barr virus
HL	Hodgkin lymphoma
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
ISH	In situ hybridization
LDHL	Lymphocyte-depleted Hodgkin lymphoma
LMP	Latent membrane protein
LP	Lymphocyte predominant
LRHL	Lymphocyte-rich Hodgkin lymphoma
MCHL	Mixed cellularity Hodgkin lymphoma
NHL	Non-Hodgkin lymphoma
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
NSHL	Nodular sclerosis Hodgkin lymphoma
PTGC	Progressive transformation of germinal centers
RS	Reed-Sternberg
RSC	Reed-Sternberg cell

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TCHRLBCL	T cell/histiocyte-rich large B cell lymphoma
Tfh	Follicular T helper cell
TGFβ	Tumor-derived growth factor beta
Treg	Regulatory (suppressor) T cells

Introduction

Hodgkin lymphoma is a lymph node-based neoplasm that often presents with asymptomatic lymphadenopathy. Symptoms may include fatigue, weight loss, fever, and night sweats (B symptoms), as well as pruritus, and alcohol-induced pain in affected lymph nodes (Gallamini et al. 2016).

With the exception of the nodular sclerosis subtype, Hodgkin lymphoma is more common in males. In the industrialized world, there is a bimodal age distribution of classical HL, with a peak in adolescents and young adults and a second peak in older adults (Barista et al. 2007). While childhood HL is rarely seen in industrialized countries, a childhood peak of the mixed cellularity subtype of classical HL is noted in the developing world (Ambinder et al. 1993).

Hodgkin lymphoma (HL) is a malignant lymphoid neoplasm composed of large clonal germinal center B cell-derived *Reed-Sternberg (RS) cells* (also known as Hodgkin Reed-Sternberg cells) admixed with variable numbers and types of reactive inflammatory cells and varying degrees of fibrosis (Küppers et al. 1998). Extent of disease is clinically determined by staging with the modified Ann Arbor staging system (Gallamini et al. 2016). Stages range from stage 1 with a single site of nodal disease to stage 4 with extensive disease involving extranodal sites. Stages are modified to signify the presence or absence of symptoms, either A (asymptomatic) or B (fever, weight loss, night sweats) (see Table 2.1).

There are two distinctive forms of HL, termed *classical Hodgkin Lymphoma (CHL)* and *nodular lymphocyte predominant HL (NLPHL)*, that differ not only in clinical behavior but also in morphology and immunophenotype (Nogová et al. 2008). Contrasting features of classical HL and nodular lymphocyte predominant HL are presented in Table 2.2.

Table 2.1 Hodgkin lymphoma staging

Stage	Sites of involvement
I	Single lymphoid region or lymphoid structure (spleen, thymus, Waldeyer's ring)
II	Two or more lymph node regions on the same side of the diaphragm
III	Lymph node regions or structures on both sides of the diaphragm
IV	Extranodal sites other than those designated E

E used to indicate involvement of a single extranodal site or contiguous/proximal to known nodal site of disease
B symptoms: fever, night sweats, and weight loss

Table 2.2 Two types of Hodgkin lymphoma

	Classical Hodgkin lymphoma	Nodular lymphocyte predominant Hodgkin lymphoma
Age distribution	Bimodal (adolescent, adult)	Unimodal (adult)
Symptoms	Often	Rare
Stage	Wide range (subtype specific)	Usually limited stage
Tumor cell type	Classical Reed-Sternberg (RS) cell (CD45− CD30+)	Lymphocyte predominant (LP) cell (CD45+ CD30−)
EBV positivity	10–75% (subtype specific)	0%
Clinical behavior	May be aggressive	Usually indolent
DLBCL progression	1%	2–3%

Classical Hodgkin Lymphoma

CHL most commonly involves cervical lymph nodes, with other common sites including mediastinum and lymph nodes in inguinal, axillary, and para-aortic regions. While splenic and marrow involvement may be seen, primary extranodal presentations are rare. The cut surface of the tumors is typically off-white ("fish flesh") in appearance, with variable degrees of nodularity, fibrosis, and necrosis. In some cases, the node may be obliterated by fibrosis. At low magnification, H&E-stained sections reveal complete to partial effacement of the lymph node architecture by a polymorphous infiltrate of small lymphocytes and histiocytes; variable numbers of lymphocytes, eosinophils, neutrophils, plasma cells, and fibroblasts; and scattered large classical Reed-Sternberg cells with binucleated, multinucleated, or multilobulated nuclei, vesicular chromatin, prominent central nucleoli, and abundant eosinophilic cytoplasm. In most cases, CD4-positive T cells predominate, with an elevated CD4:CD8 ratio (>4:1) (Hudnall et al. 2008). Many of the CD4-positive T cells express FoxP3, a phenotype consistent with *regulatory suppressor T cells (Treg)* (Hudnall et al. 2008). Variations on these histologic patterns give rise to the four well-recognized subtypes of CHL—nodular sclerosis, mixed cellularity, lymphocyte rich, and lymphocyte depleted (Gattringer et al. 1986) (Table 2.3).

Classical RS cell variants include *mononuclear Hodgkin cells*, with monolobated nuclei and prominent central nucleoli; *mummified cells*, with contracted, deeply eosinophilic cytoplasm; *lacunar cells*, mononuclear cells with multilobulated nuclei and prominent nucleoli and surrounded by clear spaces due to retraction artifact; and *pleomorphic RSC*, multinucleated cells with highly irregular hyperchromatic nuclei and prominent nucleoli (Angel et al. 1987). In some cases, RS cells accumulate to form large syncytial aggregates, often associated with focal necrosis.

Classical RS cells express a highly distinctive immunophenotype (Table 2.4; Fig. 2.1), consistently and strongly positive for CD30, Fascin, and MUM1, weakly positive for PAX-5 (nuclear), variably positive for CD15, and negative for CD45, B cell transcription factors (OCT2, BOB1, PU.1), the plasma cell marker CD138, the NK cell marker CD56, monocyte markers CD68 and CD163, the myeloid

Table 2.3 Classical Hodgkin lymphoma subtypes

	Nodular sclerosis	Mixed cellularity	Lymphocyte rich	Lymphocyte depleted
Percentage of CHL cases[a]	70%	25%	5%	<1%
Age[a]	Adolescent/young adults and older adults	Adults (35–40 years)	Adults	Adults (30–40 years)
Gender	Male = female	Male > female	Male > female	Male > female
Symptoms (usual)	Asymptomatic	Symptomatic	Asymptomatic	Symptomatic
Stage (usual)	Low stage	Advanced stage	Low stage	Advanced stage
EBV association[a]	10–40%	75%	50% (est.)	90–100%
RS cells	Common lacunar variants, few classic RS cells	Numerous classic RS cells	Few classic RS cells	Variable number of pleomorphic variants
Fibrosis	Nodular sclerosis	Mild fibrosis	Usually none	Diffuse fibrosis
Background cells	Mixed inflammatory	Mixed inflammatory	Small lymphocytes	Stromal cells

[a]These results are derived from a variety of independent sources and represent only estimates. Also, the demographic information presented here derives entirely from western sources and does not include demographic information about Hodgkin lymphoma in the developing world

Table 2.4 Classic Reed-Sternberg cell immunophenotype

Positive	Negative
CD30	CD45
CD15 (+/−)	CD20 (−/+)
MUM-1	CD3 (−/+ very rare)
FASCIN	J chain
PAX-5 (usually weak)	OCT-2
EBV (+/−)	BOB.1
TARC (CCL17)	IgH/IgL
	EMA
	ALK-1
	CD138
	CD68

marker MPO, and pan-T cell markers (CD2, CD3, CD5, CD7, CD43) (Hugh and Poppema 1992; Pinkus et al. 1985, 1997; Schwering et al. 2003; Hertel et al. 2002; Buettner et al. 2005; Carbone et al. 2002; Kurtin and Pinkus 1985). In some cases, the pan-B cell marker CD20 is expressed by classical RS cells, usually weakly as a subset (Zukerberg et al. 1991), and in rare cases, classical RS cells may express T cell or cytotoxic T/NK markers (Kadin et al. 1988; Müschen et al. 2000; Oudejans et al. 1996). Assuming that no other features of non-Hodgkin lymphoma

are present, expression of CD20 or pan-T cell markers such as CD2 or CD3 is not inconsistent with a diagnosis of classic Hodgkin lymphoma if all other findings are supportive. Classical RS cells also express the chemokine CCL17 (TARC), a molecule that recruits *T helper 2 (Th2)* cells to the microenvironment, forming T cell rosettes around classical RS cells (Peh et al. 2001).

Classical RS cells are hyperdiploid and extensively aneuploid, harbor nonproductive clonal immunoglobulin heavy chain gene rearrangements, and have no immunoglobulin expression (Bräuninger et al. 2006; Küppers 2009). The somatic hypermutation within the immunoglobulin genes is consistent with an origin from germinal center or post-germinal center B cells (Küppers et al. 1998). Although many gene mutations have been described in CHL, no specific "founder" mutation has been discovered. CHL might possibly be characterized instead by a variety of signaling pathway specific gene defects leading to a final common neoplastic phenotype.

In some cases, classical RS cells are latent infected by the *Epstein-Barr virus (EBV)*, a ubiquitous human gamma herpesvirus (Weiss et al. 1987; Anagnostopoulos et al. 1989; Herbst et al. 1991; Herbst 1996). In this setting, there is virtually no viral replication, with viral gene expression limited to latency genes EBNA-1, LMP-1, LMP-2A, EBER-1, and EBER-2, a gene expression pattern referred to as latency II (Deacon et al. 1993; Anderton et al. 2011; Massini et al. 2009). EBNA-1 (EB nuclear antigen-1) protein is critical for maintenance of the circular viral episome within latent infected cells, thus preventing virus loss during cell division (Middleton and Sugden 1994). LMP-1 (latent membrane protein-1) is expressed at an unusually high level in EBV-positive RS cells, mimicking the effects of a constitutively activated TNF (tumor necrosis factor) receptor by activating several oncogenic signaling pathways, leading to cytokine production and inhibition of apoptosis (Izumi et al. 1997). LMP-2A acts to prevent lytic viral activation, thus maintaining viral latency, and inhibits TGFβ-induced apoptosis (Miller et al. 1994). Whereas EBNA-1 and LMP genes encode for viral proteins, the EBERs do not encode for protein, instead producing millions of copies of EBER RNA in each infected cell. This RNA abundance has been exploited to produce a highly sensitive in situ hybridization method for virus detection (see below). EBER RNA likely contributes to viral latency by stimulating production of interleukin-10 (IL-10) and insulin-like growth factor (IGF-1) and blocking alpha interferon-mediated apoptosis (Nanbo et al. 2002). A pathogenic role for EBV in EBV-positive cases of CHL is underscored by the finding of clonal virus, as demonstrated by detection of a single-length fused terminal repeat fragment of viral DNA (Anagnostopoulos et al. 1989). However, since many cases of HL are EBV negative (Hummel et al. 1992), EBV is likely not a necessary cofactor in all cases of CHL.

EBV can be detected by immunohistochemical staining (IHC) for *latent membrane protein-1 (LMP1)* using monoclonal antibodies as probe (Poppema 2005) or by colorimetric in situ hybridization (ISH) with oligonucleotide probes to Epstein-Barr early RNA (EBER1, EBER2) (Wu et al. 1990; Gulley and Tang 2008). Membrane and cytoplasmic positivity is seen with LMP1 IHC, while intranuclear positivity is seen with EBER ISH. While LMP1 positivity is generally confined to RSC, *EBER* positivity may be seen in both RSC and small B cells in Hodgkin biopsies. Only those cases with EBER-positive RS cells are designated as EBV-positive CHL.

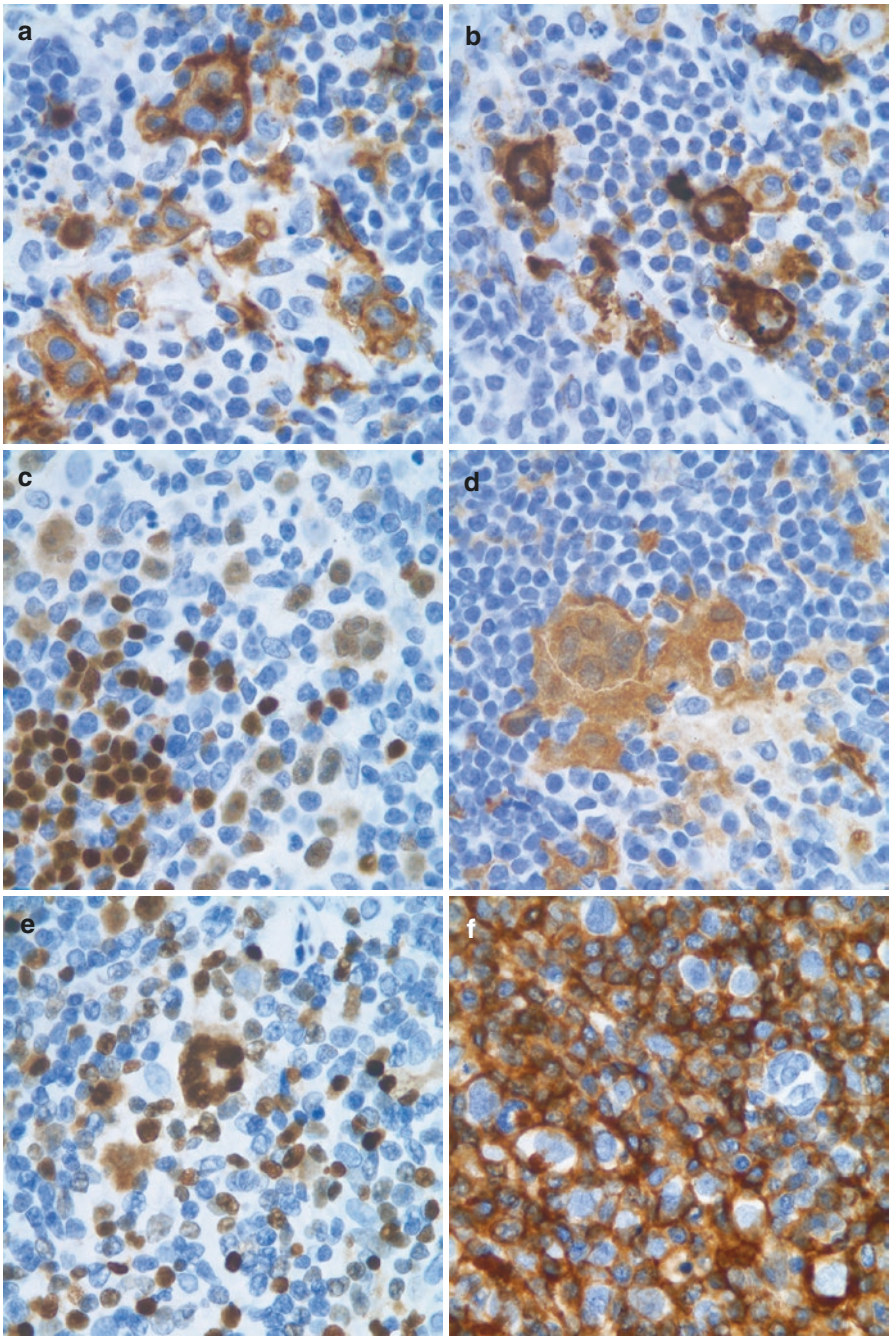

Fig. 2.1 Classical Reed-Sternberg cell immunophenotype. (**a**) CD30 (may also stain mononuclear immunoblasts). (**b**) CD15 (also stains granulocytes). (**c**) PAX-5 (weak staining of RS cells, strong staining of B cells). (**d**) FASCIN (may also stain dendritic cells). (**e**) MUM-1 (also stains plasma cells and activated B cells). (**f**) CD45 (RS cells are negative but surrounded by positive small lymphocytes)

Nodular Sclerosis Hodgkin Lymphoma (NSHL)

NSHL, the most common subtype of HL, accounts for roughly 70% of all cases of CHL. The incidence of NSHL, at least in industrialized countries, follows a bimodal distribution, with two peaks, one in adolescents and young adults (age 15–35) and one in older adults (>50 years). In contrast to the male predominance seen with other CHL subtypes, the male/female ratio of NSHL is nearly equal. Approximately 40% of patients present with fatigue, weight loss, fever, and night sweats, findings that for the sake of clinical staging are referred to as B symptoms. Mediastinal involvement is common, particularly in young females. Bulky disease is often seen. Splenic and marrow involvement is uncommon.

The morphologic diagnosis of NSHL is based upon the presence of nodular sclerosis and distinctive large cells with multilobulated nuclei, vesicular chromatin, and prominent nucleoli that occupy clear spaces (lacunar variants) (Hansmann and Kaiserling 1982) (Fig. 2.2). The lacunar morphology is a formalin fixation-induced retraction artifact that is not seen with metallic fixatives. In many cases of NSHL, classic RS cells are rare. In other cases, lacunar cells form sheets or aggregates, a finding variably referred to as *syncytial variant* or *NSHL grade 2* (Strickler et al. 1986). In some cases, the RS cells are pleomorphic with bizarre anaplastic forms. This variant is often accompanied by necrosis, sometimes associated with granulomatous inflammation.

In most cases of NSHL, the fibrosis is dense, broad, collagenous, poorly cellular (i.e., sclerotic), and accompanied by capsular fibrosis. The fibrotic bands encircle and compartmentalize the lymphoid infiltrate, leading to large irregular lymphoid nodules. In extreme cases, obliterative sclerosis may predominate, with little remaining lymphoid tissue, a pattern of disease sometimes referred to as *lymphocyte-depleted variant* of NSHL (Slack et al. 2009). In other cases, the fibrosis may be delicate, with no broad bands of sclerosis, a form of disease referred to as *cellular phase* of NSHL (Colby et al. 1982; Boiocchi et al. 1992). Rarely, the sclerosis is more cellular, with plump fibroblasts, a pattern sometimes referred to as the *fibroblastic variant* of NSHL (MacLennan et al. 1989). Rarely, classic RS cells are limited to interfollicular regions of the node, often associated with prominent follicular hyperplasia, a finding sometimes referred to as *interfollicular pattern* (Cajaiba and Kahwash 2012; Zanetto et al. 2006; Doggett et al. 1983).

The inflammatory background of NSHL consists primarily of T lymphocytes and histiocytes, with variable numbers of eosinophils and neutrophils. Neutrophilic and eosinophilic abscesses are sometimes seen. In some cases, the inflammatory infiltrate may be quite prominent, mimicking that of mixed cellularity HL. However, in the presence of bands of collagenous fibrosis, the appropriate diagnosis is NSHL. Most NSHL cases are EBV negative, with reported positivity rates ranging from 10 to 40%. In positive cases, the large RS cells (including lacunar variants) are positive for LMP1 by IHC and EBER1-2 by ISH.

The distinctive histopathology of NSHL usually makes diagnosis straightforward. Nevertheless, rare cases of *ALK-positive anaplastic large cell lymphoma*

(ALCL) with a pattern of growth resembling that of NSHL have been described (Vassallo et al. 2006). Helpful diagnostic findings include the presence of *hallmark cells* (Benharroch et al. 1998) in ALCL, large tumor cells with horseshoe-shaped nuclei, and positivity for ALK, EMA, and CD43. Some cases of diffuse large B cell lymphoma, anaplastic variant, may resemble the syncytial variant of NSHL. Correct diagnosis rests upon identification of the tumor cell immunophenotype (CD45 + CD20+ B cells in DLBCL or CD15 + CD30+ RS cells in NSHL). Also, some cases of ALK-negative ALCL with cohesive sheets of large cells and increased eosinophils may resemble syncytial variant NSHL (Ferreri et al. 2013). Once again, the diagnosis is based upon the tumor cell immunophenotype. In contrast to the RS cells of Hodgkin lymphoma, the large CD30-positive cells in ALK-negative ALCL are typically CD15 negative and CD43 positive.

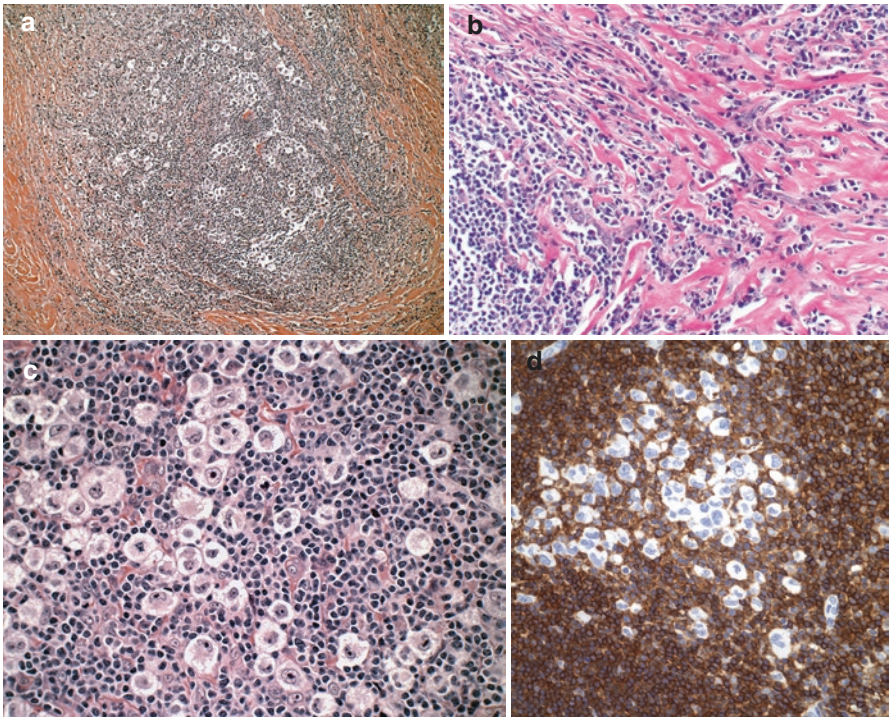

Fig. 2.2 Nodular sclerosis Hodgkin lymphoma. (**a**) Lymphohistiocytic nodule surrounded by broad band of fibrosis and containing large lacunar cells. (**b**) Broad band of fibrosis. (**c**) Numerous scattered lacunar cells. (**d**) Cluster of CD45 negative lacunar cells. (**e**) Weak PAX-5 positive lacunar cells. (**f**) Focal eosinophilia and necrosis. (**g**) Scattered dark mummified cells (degenerating lacunar cells). (**h**) Sheets of lacunar cells with necrosis (type 2, syncytial variant)

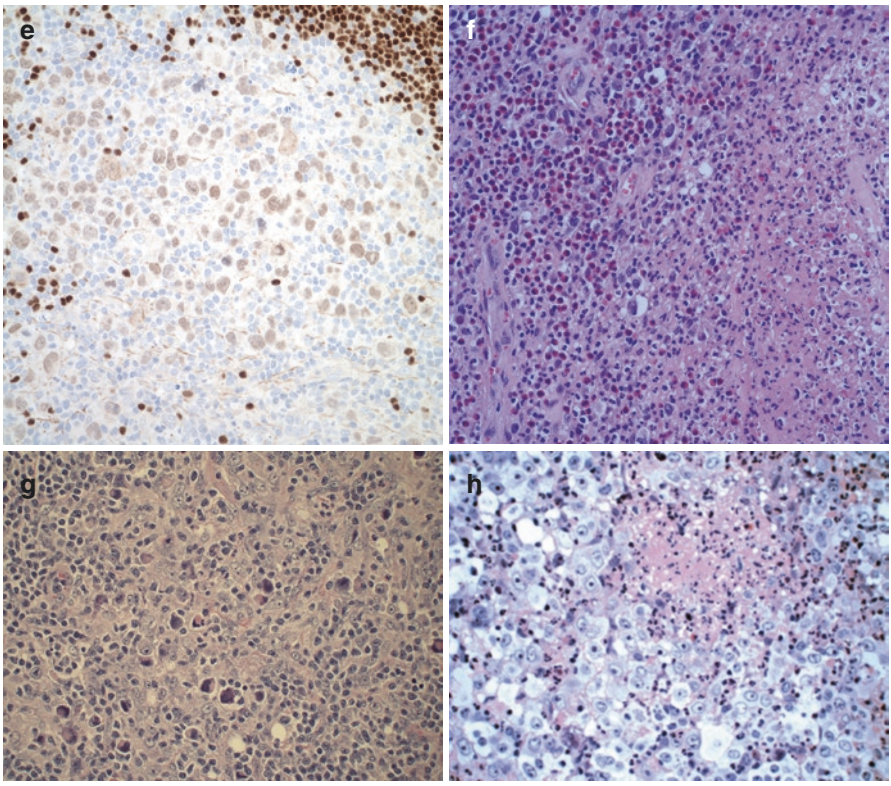

Fig. 2.2 (continued)

Mixed Cellularity Hodgkin Lymphoma (MCHL)

In the industrialized world, approximately 20–25% of CHL cases are of mixed cellularity subtype, with a higher percentage seen in the developing world and in HIV-positive persons. Unlike the bimodal age distribution of NSHL, the age distribution of MCHL in the industrialized world is unimodal, with a single peak in middle-aged adults. In contrast, in the developing world, MCHL is seen in young children. In contrast to NSHL, disease stage is more often advanced, with B symptoms.

Affected lymph nodes are typically effaced by a diffuse to vaguely nodular infiltrate notable for relatively numerous classic RS cells embedded within a mixed (polymorphous) infiltrate of reactive inflammatory cells, including small lymphocytes, histiocytes, eosinophils, neutrophils, plasma cells, and fibroblasts (Fig. 2.3). The presence of highly atypical T cells should raise a concern for peripheral T cell lymphoma. Epithelioid histiocytes may cluster to form small granulomas. Necrosis is typically absent. In some cases, the epithelioid histiocytes predominate, with relatively few admixed small lymphocytes, a form called the *histiocyte-rich variant* (Patsouris et al. 1989). While classic, often pleomorphic, RS cells predomi-

nate, RS cell variants, including mononuclear and lacunar forms, may be seen. Rarely, classic RS cells and inflammatory cells are confined to interfollicular regions of non-effaced lymph nodes with reactive follicular hyperplasia, a pattern referred to as the *interfollicular* pattern (Cajaiba and Kahwash 2012; Doggett et al. 1983). Although delicate interstitial fibrosis may be seen in MCHL, sclerosing bands of collagenous fibrosis should not be seen. In the presence of sclerosing fibrosis, a diagnosis of nodular sclerosis HL is more appropriate. Most cases of MCHL are EBV positive (75%), including those seen in young children from the developing world.

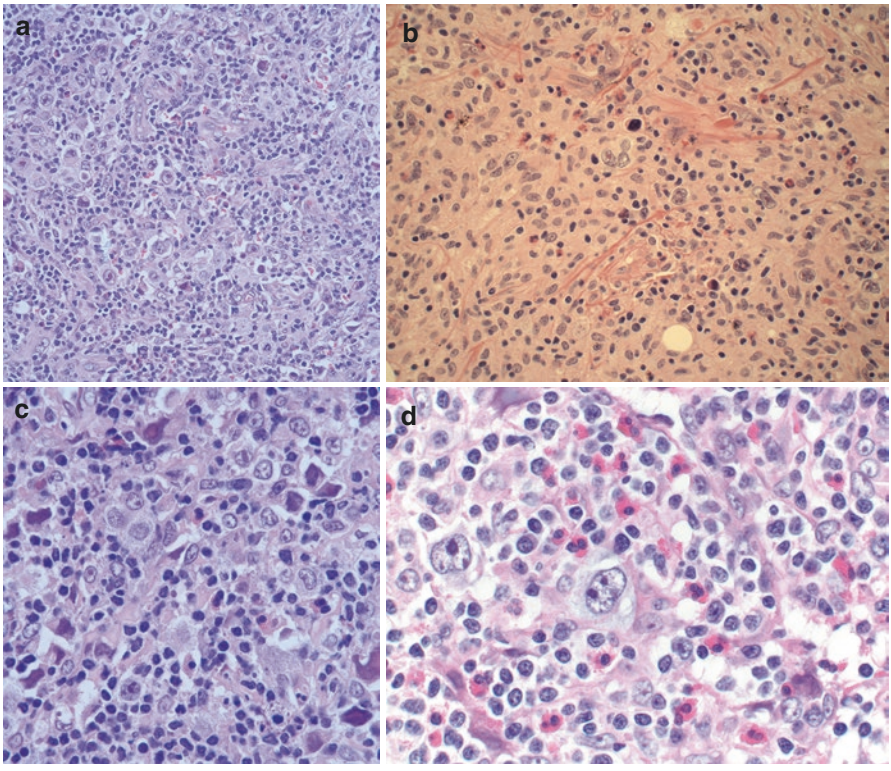

Fig. 2.3 Mixed cellularity Hodgkin lymphoma. (**a**) Diffuse infiltrate with relatively numerous classical RS cells, with a variable admixture of inflammatory cells, including small lymphocytes, histiocytes, eosinophils, granulocytes, plasma cells. (**b**) Histiocyte-rich variant with classic RS cells and few scattered eosinophils. (**c**) Scattered small darkly stained apoptotic Hodgkin cells called *mummified cells* are present. (**d**) Mononuclear Hodgkin cells with numerous small lymphocytes and eosinophils. (**e**) Epstein-Barr virus (EBV) infected RS cells (*dark blue* intranuclear stain by EBER ISH). Most cases of mixed cellularity HL contain EBV-positive RS cells. (**f**) Anaplastic variant of diffuse large cell lymphoma (shown here) may sometimes mimic Hodgkin lymphoma with anaplastic Reed-Sternberg cells

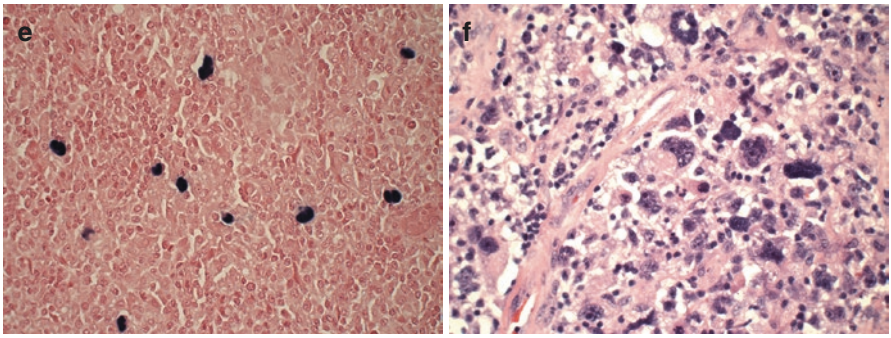

Fig. 2.3 (continued)

Lymphocyte-Rich Hodgkin Lymphoma (LRHL)

Lymphocyte-rich CHL is rare, accounting for about 5% of all cases of CHL, and seen primarily in males (70%), usually without B symptoms. Peripheral lymph nodes are the most common sites of disease, while mediastinal involvement and bulky disease are uncommon. The infiltrate is composed of small lymphocytes, devoid of other inflammatory cells (histiocytes, eosinophils, neutrophils, plasma cells), with few admixed classic RS cells (Fig. 2.4). Necrosis and fibrosis are not seen. There are two histologic patterns of disease, a common nodular form and a rare diffuse form (Nam-Cha et al. 2009; Shimabukuro-Vornhagen et al. 2005).

The more common *nodular form* of LRHL consists of numerous large follicles with expanded mantle zones and small eccentric (sometimes hyperplastic) germinal centers, which in some cases may resemble *Castleman disease* (Zarate-Osorno et al. 1994) (Fig. 2.5). In contrast to the follicles in most cases of LRHL, the hyaline vascular follicles of Castleman disease consist of atretic centrally located and well-defined germinal centers surrounded by tight concentric layers of mantle zone B cells (Cronin and Warnke 2009). To complicate matters, Castleman disease may coexist with Hodgkin lymphoma (Drut 1996).

CD21 and/or CD23 staining of follicular dendritic cell meshworks helps delineate the nodular areas as abnormal follicles. The mantle zone B cells are characterized by dual expression of IgM and IgD. The RS cells, which may include both classic forms and LP-like forms, are found scattered within the expanded mantle zones, often encircled by rosettes of small CD57/PD1-negative T cells. In cases of LRHL with numerous LP-like cells, clear distinction from NLPHL depends upon detection of the classic RS cell immunophenotype (Anagnostopoulos et al. 2000). The rare *diffuse form* of LRHL consists of an interfollicular or diffuse T cell-rich infiltrate with scattered classic RS cells and admixed histiocytes. In contrast to the nodular form, expanded CD21/CD23-positive FDC meshworks are absent. EBV positivity is seen in a variable number of cases, perhaps ranging from 40% to 70%. The diffuse form of LRHL may resemble T cell/histiocyte-rich large B cell lym-

phoma. In these cases, a diagnosis of LRHL rests on the demonstration of the classic RS cell immunophenotype (CD45-CD30 + EBV+/−) in contrast to that of large B cell lymphoma (CD45+ CD30-EBV-). Also, in contrast to LRHL, the background lymphocytes in de novo T cell-rich large B cell lymphoma are typically CD8-positive T cells.

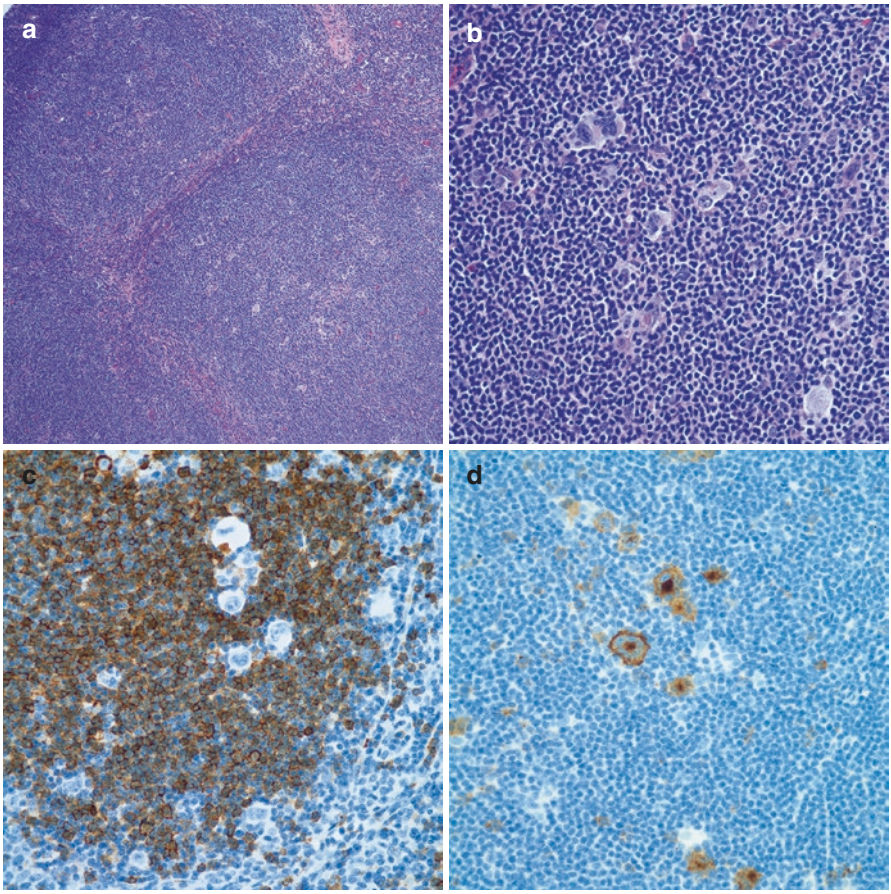

Fig. 2.4 Lymphocyte-rich Hodgkin lymphoma. (**a**) At low magnification (100×), the lymph node is effaced by large vaguely-outlined lymphocyte-rich aggregates without intervening bands of collagenous fibrosis. At this low magnification, one can just make out scattered large cells. (**b**) At higher magnification (200×), a few scattered large abnormal cells are seen. These cells can resemble lacunar cells, mononuclear Hodgkin cells, and/or classical RS cells. (**c**) CD20 highlights the small B cell nodules with few large CD20 negative RS cells. (**d**) CD30 immunohistochemistry highlights the classical Reed-Sternberg cells with the typical membrane and Golgi-zone positivity. (**e**) PAX-5 immunohistochemistry highlights a few large weakly positive Reed-Sternberg cells surrounded by numerous strongly positive B cells (intranuclear stain). In the common nodular variant, B cells predominate. Note that PAX-5-negative T cells closely surround the RS cells. (**f**) CD3 highlights the T cell rosettes that surround the RS cells

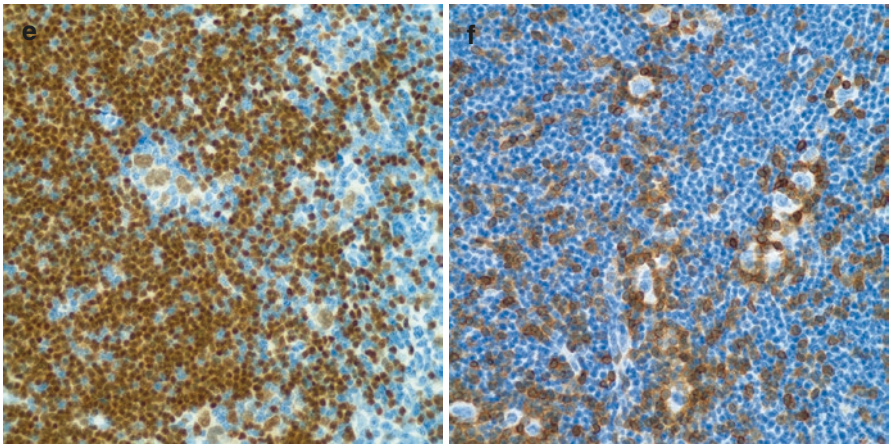

Fig. 2.4 (continued)

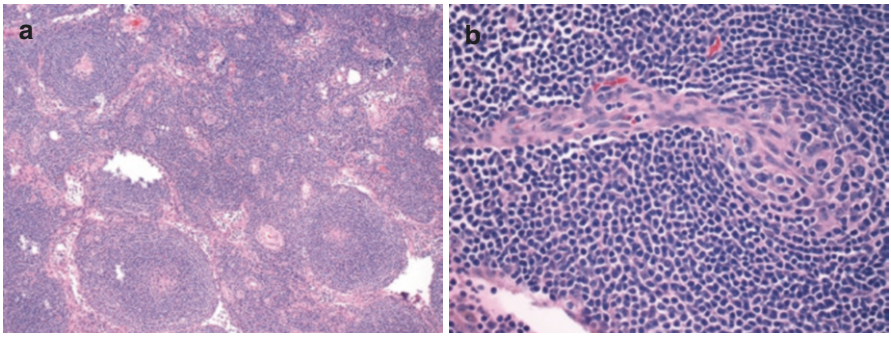

Fig. 2.5 Castleman disease, hyaline vascular variant (**a**) The abnormal follicles of the hyaline vascular variant of Castleman disease (CD) may sometimes resemble the nodular form of lymphocyte-rich HL. However, in CD, the abnormal follicles contain atrophic hyalinized germinal centers largely devoid of centrocytes with dysmorphic follicular dendritic cells. The small germinal centers are surrounded by concentric onion skin-like layers of mantle zone B cells. No Reed-Sternberg cells are present. (**b**) Often, small penetrating arterioles are seen within the atrophic germinal centers, forming a "lollipop" pattern

Lymphocyte-Depleted Hodgkin Lymphoma

LDHL is the least common subtype of CHL, accounting for <1% of all CHL cases in the industrialized world (Slack et al. 2009; Karube et al. 2013; Klimm et al. 2011). In contrast, LDHL is more common in developing countries and in HIV-positive persons. The disease is more common in adult males, age 30–37, who

often present with advanced stage and B symptoms. Common sites of involvement include retroperitoneal nodes, abdominal organs (liver, spleen), and bone marrow.

There are two histologic patterns of disease, reticular and diffuse fibroses (Fig. 2.6). The more common *reticular pattern* of LDHL (Slack et al. 2009) is composed of a diffuse fibrotic infiltrate with numerous admixed classical RS cells with bizarre pleomorphic features and few inflammatory cells. The stromal cell-rich background often contains amorphous fibrillary material and necrosis. The less common *diffuse fibrosis pattern* of LDHL (Slack et al. 2009) is characterized by diffuse fibrosis, marked hypocellularity, and rare classic RS cells. In some cases, the classic RS cells are quite inconspicuous, leading to diagnostic uncertainty. In these cases, the rare RS cells may be detected by positivity for CD30, CD15, Fascin, PAX-5 (weak), and EBV (usually) and negative staining for CD45 and CD20 (usually). The hypocellular background often contains amorphous eosinophilic material and necrosis. LDHL cases with nodular sclerosis are best classified as nodular sclerosis HL, lymphocyte-depleted variant. EBV (EBER) is detected in nearly all cases of LDHL.

The differential diagnosis of the reticular variant includes diffuse large B cell lymphoma; B cell lymphoma, unclassifiable, with features intermediate between DLBCL and Hodgkin lymphoma (mediastinal gray zone lymphoma); and anaplastic large cell lymphoma. In some cases, if RS cells are not immediately apparent, the diffuse fibrosis variant may mimic sarcoma. The identification of multilobulated large cells with prominent nucleoli and the typical RS cell immunophenotype (with absence of B cell markers) is diagnostic for Hodgkin lymphoma.

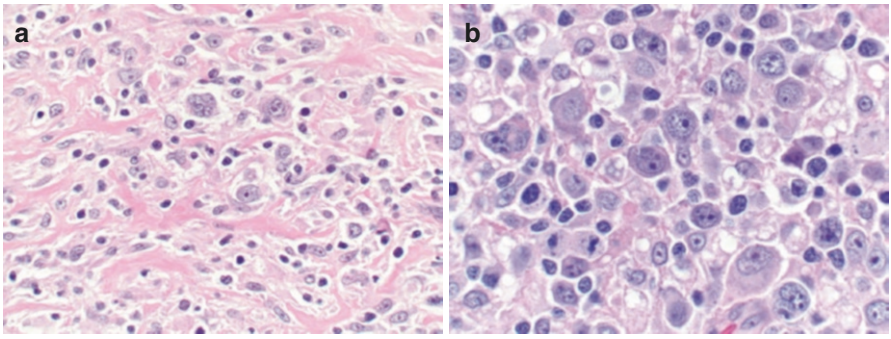

Fig. 2.6 Lymphocyte-depletion Hodgkin lymphoma. (**a**) Diffuse fibrosis variant with numerous stromal cells and scattered RS cells. (**b**) Reticular variant with a predominance of atypical often pleomorphic large RS cells

B Cell Lymphoma, Unclassifiable, with Features Intermediate Between DLBCL and Hodgkin Lymphoma (Mediastinal Gray Zone Lymphoma)

B cell lymphoma, *unclassifiable*, *with features intermediate between DLBCL and Hodgkin lymphoma (mediastinal gray zone lymphoma)* is a rare high-grade lymphoma with morphologic and immunophenotypic features that overlap primary mediastinal large B cell lymphoma (PMBL) and classical Hodgkin lymphoma (CHL) (Traverse-Glehen et al. 2005; Gualco et al. 2012) (Fig. 2.7). This tumor most often presents as a mediastinum mass in middle-aged males. In one study, four histologic patterns were described, CHL-like, PMBL-like, mixed CHL/PMBL-like, and sequential composite (CHL followed by PMBL) (Drut 1996). The CHL-like pattern (seen in one case) resembles nodular sclerosis HL, with a composite B cell/RS cell immunophenotype (positive for CD15, CD20, CD30, CD45, and CD79a). The PMBL-like pattern (seen in two cases) consists of confluent sheets of large cells, with variable sclerosis/fibrosis, admixed inflammatory cells, and tumor cells positive for CD20 and CD30 and variable expression of CD15 and CD45. The mixed pattern (seen in six cases) consists of admixed centroblast-like cells and classic RS cells in a diffuse fibrotic background, with tumor cells positive for CD20, CD30, and CD45 and variable expression of CD15. The sequential composite pattern (described in only one case) first presented as CHL and 1 year later presented as PMBL. The original immunophenotype was compatible with classic RS cells (CD30+, CD20+/−, CD45−), while the later immunophenotype was compatible with B cells (CD30−, CD20+, CD45+). EBV was detected in two of the cases (both mixed type).

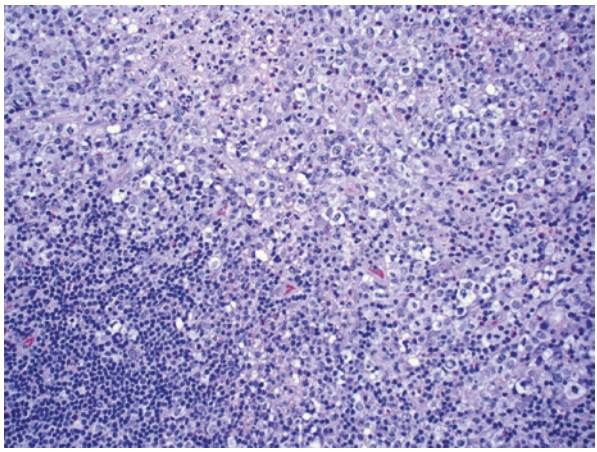

Fig. 2.7 Mediastinal grey zone lymphoma. (**a**) The histology often resembles that of mediastinal large B cell lymphoma with few Reed-Sternberg cells. The large cell immunophenotype is composite, with large cells expressing both B cell markers (CD45, CD20) and classical RS cell markers (CD15, CD30)

Nodular Lymphocyte Predominant Hodgkin Lymphoma (NLPHL)

NLPHL is uncommon, accounting for approximately 5% of all cases of HL. The disease is predominantly seen in young to middle-aged adult males, aged 30–50 years, who usually present with asymptomatic lymphadenopathy (most commonly cervical, axillary, inguinal) of limited extent (stages 1–2). The mediastinum, spleen, and marrow are only rarely involved. Some cases of NLPHL progress to diffuse large B cell lymphoma.

Involved lymph nodes are partially to completely effaced by large expanded follicular nodules, usually devoid of germinal centers, composed primarily of small B lymphocytes, with scattered epithelioid histiocytes, and medium-large mononuclear cells with delicate multilobulated nuclei and multiple distinct nucleoli known as *LP cells* (Fan et al. 2003; Fuchs et al. 2008) (Fig. 2.8). LP cells often resemble popcorn kernels, thus the term "*popcorn*" cells. Care must be taken to avoid mistaking epithelioid histiocytes and follicular dendritic cells as LP cells.

The genotype and immunophenotype of LP cells are consistent with germinal center-derived B cells (Braeuninger et al. 1997) (Table 2.5). Unlike classical RS cells, LP cells harbor productive clonal immunoglobulin gene rearrangements and express immunoglobulin proteins. LP cells consistently and strongly express the pan-leukocyte marker CD45, as well as pan-B cell markers CD20, PAX-5, and CD79a (Herbeck et al. 2011). LP cells are consistently negative for CD15, Fascin, and EBV. In most cases, LP cells are CD30 negative, with weak and variable positivity seen in some cases. Great care should be exercised to differentiate CD30-positive immunoblasts from LP cells.

LP nodules often merge, leading to a vaguely nodular appearance that may require staining with CD21 to help delineate the abnormal follicles by highlighting FDC meshworks. Sclerosis may be seen, but is uncommon in primary lesions. The nodules are composed primarily of small mantle zone B cells (CD20+ IgD+) with few/rare plasma cells, eosinophils, and neutrophils. In some cases, epithelioid histiocytes may cluster to form small granuloma-like aggregates. The B cell-rich nodules also contain CD4/CD57-positive *Tfh cells (follicular helper T cells)* that form rosettes around LP cells (Peh et al. 2001; Kamel et al. 1993; Poppema 1992). These cells co-express other Tfh cell markers, including PD-1 and BCL-6. CD8-positive T cells are typically rare, a finding that may be helpful in distinction of NLPHL from T cell/histiocyte-rich large B cell lymphoma. In some cases of partial effacement, remaining normal follicles may be hyperplastic. In other cases, some reactive well-circumscribed follicles are greatly expanded in size, with germinal centers disrupted by infiltrating mantle zone B cells, a process termed *progressive transformation of germinal centers* (PTGC) (Burns et al. 1984; Hansmann et al. 1990; Hartmann et al. 2015) (Fig. 2.9). PTGC, a reactive process, may precede or follow a diagnosis of NLPHL. The presence of dual CD4/CD8-positive T cells in both PTGC and NLPHL lends further support to a close relationship between PTGC and NLPHL (Rahemtullah et al. 2008). In some cases of NLPHL, the infiltrate may be partially diffuse. If completely diffuse (as judged by absence of CD21-positive FDC meshworks), an alternative diagnosis of non-Hodgkin B cell lymphoma should be considered.

A number of unusual morphologic patterns have been described in NLPHL (Benharroch et al. 1998). One variant is described as resembling *T cell/histiocyte-rich large B cell lymphoma* (*TCHRLBCL-like*), with numerous LP cells located in diffuse T cell-rich areas. In some cases, this histology may represent diffuse progression of NLPHL. Another variant is the *diffuse B cell-rich variant*, without discernible nodules. While the background small lymphocytes in typical NLPHL are largely B cells, the background small lymphocytes in TCRLBCL are usually cytotoxic CD8+ TIA-1+ T cells with only rare small B cells. However, in background lymphocytes in the rare diffuse variant of NLPHL are primarily CD4-positive T cells. The characteristic CD57/PD1+ T cell rosettes of NLPHL are not seen in TCHRLBCL. Detection of clonally rearranged B cells strongly favors a diagnosis of TCHRLBCL over NLPHL.

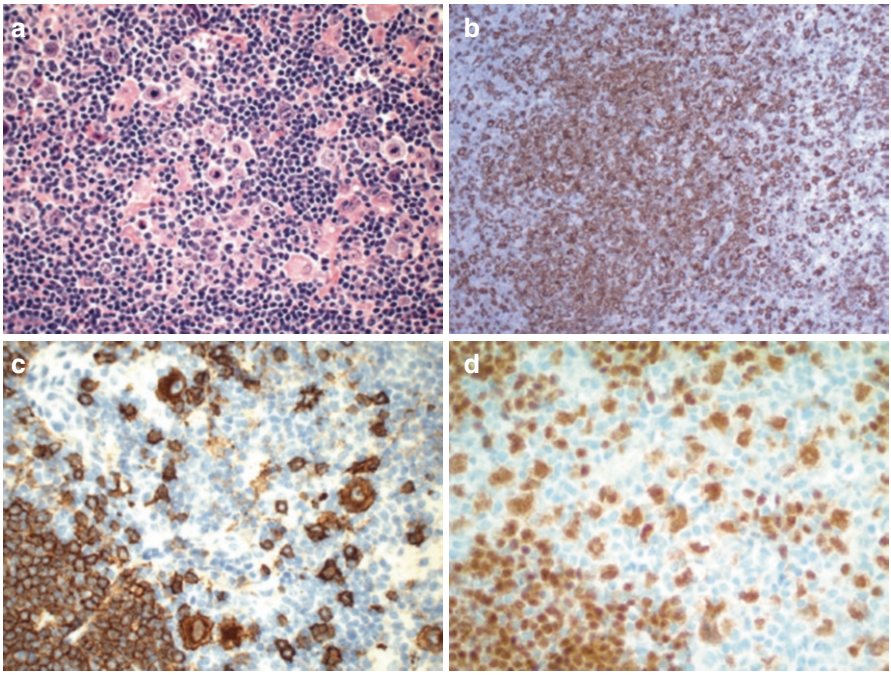

Fig. 2.8 Nodular lymphocyte predominant Hodgkin lymphoma. (**a**) Scattered medium-large mononuclear LP (popcorn cell) RS cell variants within large nodular lymphoid aggregates. Note the presence of few epithelioid histiocytes but absence of eosinophils. (**b**) CD20 highlights the B cell nodules with outward expansion of the large CD20 positive LP cells (low magnification). (**c**) Higher magnification of the CD20 stain showing a small aggregate of small B cells on the lower left with few large CD20-positive LP cells beyond the aggregate. (**d**) PAX-5 staining highlights the cluster of small B cells in the lower left, and scattered larger LP (popcorn) cells with irregular nuclear contours elsewhere. Note that the PAX-5 stain intensity of the LP cells matches that of the small B cells, a finding in contrast to the weak positivity of classic RS cells. (**e**) The B cell marker OCT-2 is positive in the LP cells of NLPHL. In contrast, OCT-2 is negative in the classic RS cells of classical Hodgkin lymphoma. (**f**) EMA is positive in the LP cells of some but not all cases of NLPHL. (**g**) CD57 positive T cells are increased in NLPHL, and form tight rosettes around LP cells. (**h**) In some cases of NLPHL, immunoglobulin light chain expression can be detected. In this case, the LP cells express kappa light chain (shown) while negative for lambda light chain—a finding indicative of a clonal B cell process

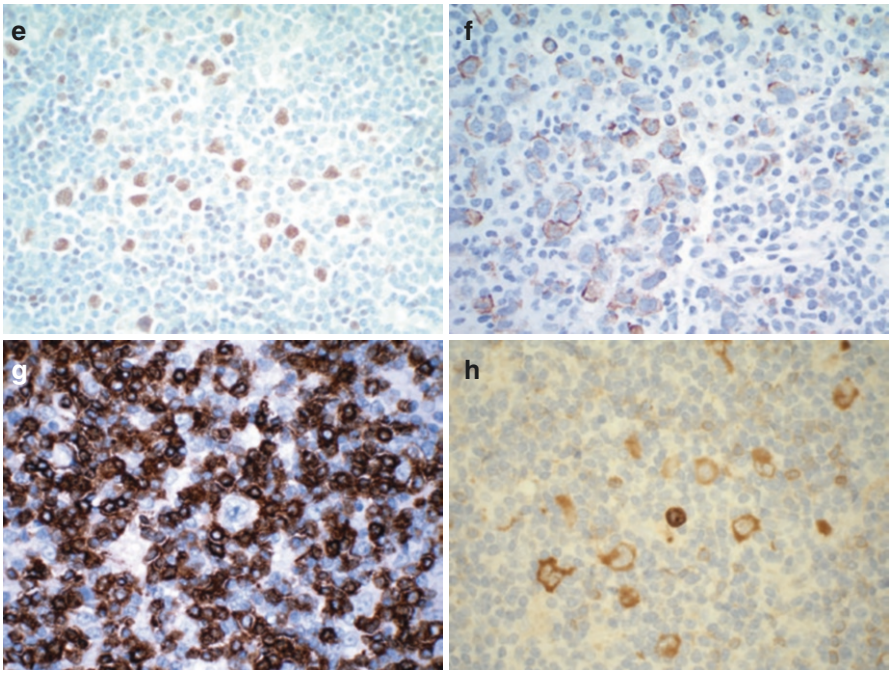

Fig. 2.8 (continued)

Table 2.5 Lymphocyte predominant (LP) cell immunophenotype

Positive	Negative
CD20	CD15
CD79a	CD30*
PAX5 (strong)	FASCIN
CD45	CD10
J chain	BCL-2
OCT-2	EBV
BOB.1	
BCL-6	
IgD (<50%)	
EMA (>50%)	
AID	
PU.1	

*may be weak and variable

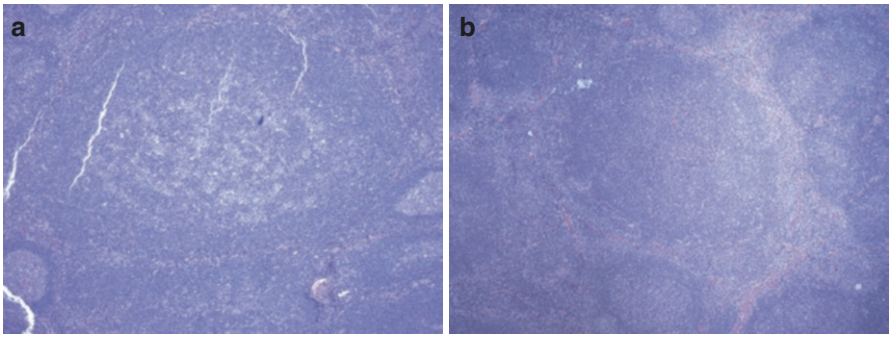

Fig. 2.9 Progressive transformation of germinal centers (PTGC). (**a** and **b**) In PTGC, unlike most cases of Hodgkin lymphoma, the lymph node architecture is not entirely effaced. Instead, there are a few markedly enlarged abnormal lymphoid follicles admixed with normal reactive follicles. The enlarged follicles contain irregular distorted germinal center remnants infiltrated by a greatly expanded population of mantle zone B cells. In contrast to NLPHL, typical LP ('popcorn') cells are absent

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Chapter 3
Pathogenesis and Molecular Genetics of Hodgkin Lymphoma

Ralf Küppers

Abbreviations

cHL	Classical Hodgkin lymphoma
EBV	Epstein-Barr virus
GC	Germinal center
HL	Hodgkin lymphoma
HRS	Hodgkin and Reed/Sternberg
Ig	Immunoglobulin
MHC	Major histocompatibility complex
NHL	Non-Hodgkin lymphoma
NLPHL	Nodular lymphocyte-predominant Hodgkin lymphoma
RTK	Receptor tyrosine kinase
TCR	T cell receptor

Introduction

Hodgkin lymphoma (HL) is one of the most frequent types of lymphomas in the Western world and one of the most frequent tumors in young adults. HL is subdivided into classical HL (cHL), accounting for about 95% of cases, and nodular lymphocyte-predominant HL (NLPHL) (Swerdlow et al. 2016). cHL is further subdivided into the four forms: nodular sclerosis, mixed cellularity, lymphocyte-rich,

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and lymphocyte-depleted HL (Swerdlow et al. 2016). Nodular sclerosis HL is the most frequent subtype (about 60% of cases), followed by mixed cellularity (20%), whereas the two remaining forms are less frequent. The distinction of these subtypes is based on differences in the morphology and phenotype of the tumor cells and differences in the histopathological picture (Swerdlow et al. 2016). In cHL, the tumor cells are named Hodgkin and Reed/Sternberg (HRS) cells and in NLPHL lymphocyte-predominant (LP) cells (until recently, LP cells were called lymphocytic and histiocytic cells, L&H cells). HRS and LP cells are typically present in the involved tissues at a low frequency, often only accounting for about 1% of the cellular infiltrate. The rarity of the HRS and LP cells in the tissue has very much hampered their molecular analysis. Moreover, only a few HL cell lines were established and can be used for molecular and functional studies. No transgenic mouse model of HL has yet been established. Despite these technical challenges and obstacles, major progress has been made in the molecular analysis of HRS and LP cells, so that we now understand the molecular biology of HL much better than 20 years ago, although several key pathogenetic features remain to be clarified (Küppers 2009a; Küppers et al. 2012).

Cellular Origin of HRS and LP Cells

HRS cells in cHL show a very peculiar morphology and immunophenotype that does not resemble any normal cell in the immune system. HRS cells typically express some B cell markers (e.g., PAX5, E2A), but also T cell markers (e.g., GATA3), markers of natural killer cells (ID2), and markers of dendritic cells (e.g., CCL17) and of macrophages (e.g., CSFR1) (Table 3.1) (Küppers 2009a). Because of this aberrant immunophenotype and the initial lack of suitable methods to perform molecular studies with isolated HRS cells, the origin of the cells had remained enigmatic for a long time, and various types of cells of the hematopoietic system

Table 3.1 Features of HRS and LP cells

Feature	HRS cells	LP cells
Diagnostic immunophenotype	CD15$^{+/-}$, CD30^{+}, CD45^{-}, CD75^{-}, EMA^{-}	CD15^{-}, CD30^{-}, CD45^{+}, CD75^{+}, EMA^{+}
B cell marker expression (selection)	CD20$^{-/+}$, CD40^{+}, CD79^{-}, BOB.1^{-}, OCT2^{-}, PAX5^{low}, BCR^{-}, IRF4^{+}, SYK^{-}	CD20^{+}, CD40^{+}, CD79^{+}, BOB.1^{+}, OCT2^{+}, PAX5^{+}, BCR^{+}, IRF4^{low}, SYK^{+}
GC B cell factor expression	BCL6$^{-/low}$,AID^{-},SERPIN9^{-},HGAL^{-}	BCL6^{+}, AID^{+}, SERPIN9^{+}, HGAL^{+}
Non-B-cell marker expression (selection)	CD3$^{-/+}$, CCL17^{+}, CSFR1^{+}, NOTCH1^{+}, granzyme B$^{-/+}$, ID2^{+}	CD3^{-}, CCL17^{-}, CSFR1^{-}, NOTCH1^{-}, granzyme B^{-}, ID2^{-}
Presumed cell of origin	Crippled GC B cell	Selected GC B cell
EBV infection	30–40% of cases	No
Multinuclear cells	Always present	Absent

were proposed as the histogenetic origin of these cells. It was only when methods were established to microdissect HRS cells from tissue sections and analyze single cells for the presence of immunoglobulin (Ig) or T cell receptor (TCR) gene rearrangements that the cellular derivation of HRS cells could be identified about 20 years ago (Kanzler et al. 1996; Küppers et al. 1994). Ig and TCR gene rearrangements occur specifically in developing B and T cells, respectively, and hence represent genetic markers for such cells. When isolated HRS cells were tested for the presence of Ig heavy and light chain gene rearrangements, such rearrangements were indeed identified in nearly all cases, establishing the B cell origin of HRS cells (Kanzler et al. 1996; Küppers et al. 1994; Bräuninger et al. 2003; Irsch et al. 1998; Marafioti et al. 2000). Moreover, HRS cells of a given case carried identical IgV region genes (Kanzler et al. 1996; Küppers et al. 1994; Bräuninger et al. 2003; Irsch et al. 1998; Marafioti et al. 2000). As IgV region genes are highly diverse and represent clonal markers (Rajewsky 1996), the detection of identical IgV genes in HRS cells of a given case was also a firm demonstration of the monoclonality of the HRS cells. The sequence analysis of the rearranged IgV genes carried by HRS cells uncovered further important insights into their specific origin. This is because normal B cells activate the process of somatic hypermutation when they become engaged in T-dependent humoral immune responses and differentiate into germinal center (GC) B cells (Rajewsky 1996; Küppers et al. 1993). GC B cells acquire numerous somatic mutations during their clonal expansion in the GC, so that the presence of somatic mutations is also a hallmark of post-GC B cells, i.e., long-lived memory B cells and plasma cells (Seifert and Küppers 2016). HRS cells of cHL in nearly all cases harbored somatically mutated heavy and light chain IgV genes, indicating that they derive from GC or post-GC B cells (Kanzler et al. 1996; Küppers et al. 1994; Bräuninger et al. 2003; Irsch et al. 1998; Marafioti et al. 2000). An unexpected finding was that about 25% of cases of cHL showed clearly destructive IgV gene mutations that rendered originally functional Ig heavy or light chain gene rearrangements nonproductive (Kanzler et al. 1996; Küppers et al. 1994; Bräuninger et al. 2003; Irsch et al. 1998; Marafioti et al. 2000). Such "crippling" mutations physiologically happen in GC B cells but normally cause the immediate apoptotic death of the cells. This indicates that HRS cells in such cases are derived from the pool of apoptosis-prone GC B cells but were somehow rescued from apoptosis by transforming events. As only some disadvantageous mutations in rearranged IgV genes can be easily identified (e.g., those causing a premature stop codons), we speculated that HRS cells as a rule stem from pre-apoptotic GC B cells (Kanzler et al. 1996; Küppers and Rajewsky 1998). There are a few cases of cHL in which the IgV genes of the HRS cells are unmutated (Müschen et al. 2001). Such cases may potentially originate from pre-GC B cells. However, GC founder cells start to proliferate and become apoptosis-prone even before the onset of somatic hypermutation (Lebecque et al. 1997), so that potentially also these cHL cases might derive from GC B cells.

The expression of T cell or cytotoxic markers by HRS cells of some cHL prompted an analysis whether such cases derive from T cells. In a very few instances, clonal TCR gene rearrangements were indeed amplified from isolated

HRS cells, although most of the cHL with T cell marker expression analyzed carried Ig and not TCR gene rearrangements (Müschen et al. 2000; Seitz et al. 2000; Willenbrock et al. 2002). Thus, it appears that there are rare lymphomas diagnosed as cHL that have a T cell origin. It remains a matter of discussion whether this is sufficient to denote a T cell form of cHL or whether these are unusual "gray zone" lymphomas of mature T cell lymphomas with a histopathological picture indistinguishable from cHL.

In NLPHL the B cell phenotype of the LP cells, with consistent expression of B cell markers, pointed to a B cell origin of these lymphoma cells (Table 3.1). This was indeed confirmed when rearranged IgV genes were amplified from isolated LP cells (Braeuninger et al. 1997; Marafioti et al. 1997; Ohno et al. 1997). As in cHL, the rearrangements were clonal and somatically mutated (Braeuninger et al. 1997; Marafioti et al. 1997; Ohno et al. 1997). However, no instances of crippling mutations were found, and the cells appeared to be well-selected for expression of a functional B cell receptor (BCR) (Braeuninger et al. 1997; Marafioti et al. 1997; Ohno et al. 1997; Küppers et al. 1998). In addition, some cases of NLPHL showed intraclonal diversity in the rearranged IgV genes of the LP cells, which indicates ongoing somatic hypermutation during clonal expansion and therefore a close relationship to GC B cells (Marafioti et al. 1997; Braeuninger et al. 1997). This is indeed also indicated by the histological picture of NLPHL, which is characterized by a nodular growth pattern and a close association of the LP cells with typical constituents of GC B cells, i.e., follicular dendritic cells and T follicular helper cells. Moreover, the immunophenotype of LP cells also points to a GC B cell derivation of these cells, as they express typical GC B cell markers, such as BCL6, AID, HGAL, and SERPINA9 (Carbone et al. 1998; Greiner et al. 2005; Montes-Moreno et al. 2008; Natkunam et al. 2005). Finally, a global gene expression analysis of isolated LP cells in comparison to the main human mature B cell subsets further supported a close link of LP cells to GC B cells and indicated that LP cells have features of late centrocytes at transition to memory B cells (Brune et al. 2008).

Relationship of Hodgkin Cells to Reed/Sternberg Cells

The tumor cell clone in cHL is always composed of a mixture of mononuclear Hodgkin and bi- or multinucleated Reed/Sternberg cells. There has been a longstanding debate about the relationship between these two forms of HL cells. It had been speculated that fusion of cells of different hematopoietic lineages is involved in the generation of the HRS cell clone in general, or the Reed/Sternberg cells in particular, but molecular analyses argued against this (Küppers et al. 2001). Several studies with HL cell lines showed that Reed/Sternberg cells have little proliferative potential and that the Hodgkin cells are the proliferative compartment of the HRS cell clone (Drexler et al. 1989; Ikeda et al. 2010; Newcom et al. 1988). This led to the idea that Reed/Sternberg cells are generated from Hodgkin cells by endomitosis or acytokinetic mitosis. Recent time lapse studies of HL cell lines resolved this issue

and showed that Reed/Sternberg cells are generated from Hodgkin cells by an incomplete cytokinesis followed by refusion of the daughter cells (Rengstl et al. 2013; Xavier de Carvalho et al. 2015). Thus, Reed/Sternberg cells are not generated by fusion of two independent Hodgkin cells but by refusion of two daughter cells that fail to completely separate from each other and refuse again. The molecular causes for this frequent incomplete cytokinesis are not understood.

Studies with HL cell lines indicate that perhaps not the whole population of Hodgkin cells is the main proliferative compartment but that perhaps only a subset of these cells is responsible to maintain the lymphoma clone and hence resemble tumor stem cells (Nakashima et al. 2010; Shafer et al. 2010). Indeed, in some HL cell lines, a small subset of Hodgkin cells express the multidrug receptors MDR1 and ABCG2, and it is these cells that are particularly efficient in reestablishing the HRS cell clone in subcloning experiments (Nakashima et al. 2010; Shafer et al. 2010). However, not all HL cell lines showed such cells, and it is also unclear whether such cells exist in vivo.

Considering that Reed/Sternberg cells do not contribute substantially to the lymphoma clone expansion, one might wonder whether they contribute to the pathophysiology of the disease. This is indeed likely, because the Reed/Sternberg cells are very active in secreting chemokines and cytokines and in this way contribute to the establishment of the microenvironment, which is important for the survival and expansion of the HRS lymphoma clone (Küppers 2011).

HRS Cells and Their DNA or RNA in the Peripheral Blood

Several studies addressed the issue whether HRS cells or their precursors can be found in the peripheral blood of cHL patients. A detailed study of two patients with advanced disease did not detect any HRS cell-specific IgV gene rearrangements in peripheral blood cells of the patients (Vockerodt et al. 1998), but the fact that a few cHL cell lines were established from peripheral blood of end-stage patients argues that HRS cells principally can sometimes be present in the blood (Drexler 1993). It was also reported that $CD20^{+}BCR^{+}CD30^{-}$ HRS cell clone members can be found in the peripheral blood of HL patients (Jones et al. 2009), but the reliability of that study was criticized based on technical concerns (Küppers 2009b). A recent study based on IgV gene sequences thought to derive from HRS cells detected such rearrangements also in peripheral blood cells of a few cHL patients (Oki et al. 2015), further supporting the idea that HRS cells may occasionally enter the blood stream and perhaps metastasize in this way. Importantly, in this latter study, HRS cell-specific IgV gene sequences were frequently identified in cell-free DNA of cHL patients. Further support for the frequent presence of HRS cell-derived DNA stems from an analysis of genomic imbalances in cell-free DNA of serum or plasma (Vandenberghe et al. 2015) and from the detection of *XPO1* mutations in cell-free DNA, as these mutations are frequent somatic events in HRS cells (Camus et al. 2016). There is now a high interest to use the detection of cell-free DNA from HRS

cells in serum or plasma for disease monitoring and prognostification. Notably, besides HRS cell-derived DNA, it has also been reported that exosomes in the plasma of cHL patient are enriched for several miRNAs highly expressed by HRS cells, so that also miRNA monitoring from exosome fractions may be useful for disease monitoring (van Eijndhoven et al. 2016).

Lost B Cell Phenotype of HRS Cells

One of the peculiarities of HRS cells is their unusual immunophenotype and gene expression pattern. Although the detection of rearranged and mostly somatically mutated Ig heavy and light V region genes unequivocally identified these cells as being derived from mature B cells, HRS cells show an immunophenotype that does not resemble any normal B cell, as mentioned above. Genome-wide gene expression studies further extended and validated this observation (Schwering et al. 2003a; Tiacci et al. 2012). In a small fraction of cases, and often only on a subset of HRS cells in a given case, some B cell markers are still expressed, such as CD19, CD20, and BCL6 (Falini et al. 1996; Watanabe et al. 2000). Notably, HRS cells have typically retained expression of a number of surface molecules that play a role in the interaction of B cells with $CD4^+$ T helper cells, including CD40, CD54, CD80, CD86, and partly CD58 (Carbone et al. 1995a; Delabie et al. 1993; Van Gool et al. 1997; Ree et al. 1994; Abdul Razak et al. 2016). This suggests that it is advantageous for HRS cells to be still able to interact with T helper and/or T regulatory cells. Whether this is a cognate interaction is unclear, because HRS cells often lack MHC class II expression, partly due to genetic lesions (Steidl et al. 2011).

The initiating events for the combined loss of B cell gene expression and aberrant upregulation of non-B-cell genes are still poorly understood. However, several factors are known which contribute to this "reprogramming" of the HRS cells (Fig. 3.1). HRS cells have downregulated the expression of several major B cell transcription factors, including OCT2, PU.1, EBF1, and BOB1 (Table 3.1), so that consequently also many of their target genes are downregulated (Schwering et al. 2003a; Bohle et al. 2013; Re et al. 2001; Stein et al. 2001; Torlakovic et al. 2001). PAX5 is still expressed by HRS cells, but at considerably lower level than is seen in normal B cells (Foss et al. 1999). The B cell transcription factor E2A is expressed by HRS cells, but its function is likely impaired by high expression of two E2A inhibiting factors, namely, ABF1 and ID2 (Mathas et al. 2006; Renné et al. 2006). ID2 is normally expressed by NK cells and in early lymphocyte development promotes NK cell development at the expense of B cell development (Yokota et al. 1999). HRS cells show also high expression of NOTCH1, which promotes T cell development and inhibits expression of B cell genes in normal lymphocyte development (Jundt et al. 2002, 2008). High constitutive NOTCH1 activity in HRS cells is likely mediated by expression of its ligand JAGGED1 on cells in the cHL microenvironment, by high expression of its cofactor MAML2 and by low expression of the NOTCH1 inhibitor DELTEX1 in HRS cells (Fig. 3.1) (Jundt et al. 2002, 2008;

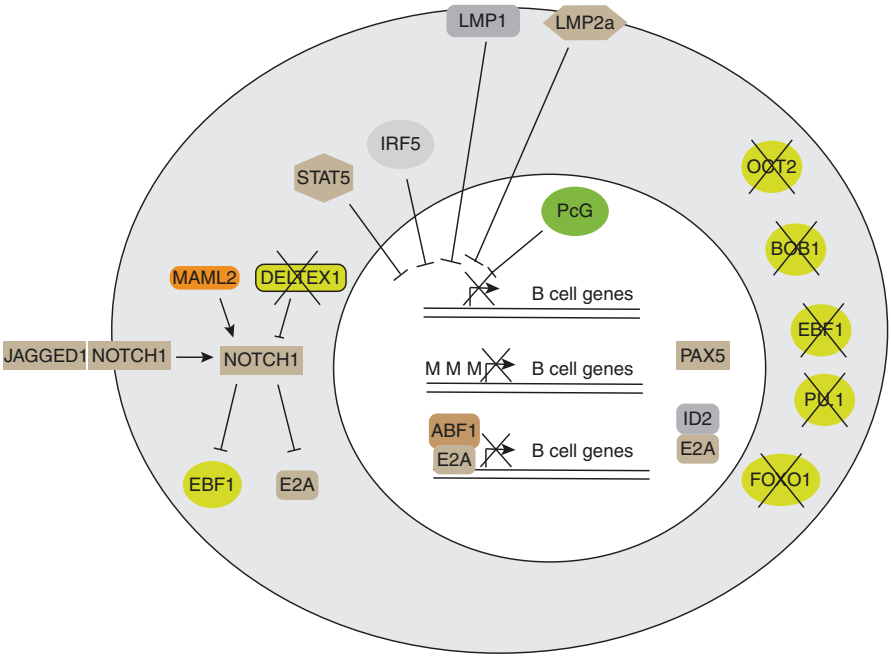

Fig. 3.1 Factors contributing to the lost B cell phenotype of HRS cells. HRS cells have downregulated the expression of several key B cell transcription factors, including OCT2, BOB1, EBF1, FOXO1, and PU.1. PAX5 is still expressed, but at lower level than in normal B cells. The activity of the E2A transcription factor is inhibited by binding to ABF1 and E2A. The T cell transcription factor NOTCH1 is constitutively active in HRS cells and inhibits the B cell factors EBF1 and E2A. NOTCH1 signaling is presumably induced by its ligand JAGGED1 and promoted by high-level expression of MAML2, whereas the NOTCH1 inhibitor DELTEX is downregulated in HRS cells. The activity of many B cell genes is furthermore inhibited by expression of the transcription factors STAT5a, STAT5b, and IRF5, by members of the polycomb group family (PcG), and in EBV-positive cases by the EBV-encoded latent membrane proteins LMP1 and LMP2a. Many B cell genes are epigenetically silenced by methylation of promoter elements (marked with "M"). Updated from ref. Küppers (2009a)

Köchert et al. 2011). Further transcription factors highly expressed in HRS cells and contributing to downregulation of B cell genes are STAT5A, STAT5B, and IRF5 (Kreher et al. 2014; Scheeren et al. 2008). Furthermore, HRS cells show expression of multiple members of the polycomb repressive complexes 1 and 2 (Dukers et al. 2004; Raaphorst et al. 2000; Sanchez-Beato et al. 2004). These complexes have a main role in hematopoietic stem and precursor cells to inhibit expression of differentiation markers, including those of B cells. Polycomb repressive complexes 1 and 2 are also expressed in normal GC B cells but physiologically in separate B cell subsets (Raaphorst et al. 2000). The co-expression of both complexes in HRS cells thus appears to be abnormal.

The downregulation of many B cell-specific genes in HRS cells is not only due to low or absent expression of B cell-specific transcription factors but most likely

functionally linked to this also mediated by epigenetic changes. In several studies, it was revealed that B cell-specific genes often show hypermethylation of their promoter regions, which stabilizes their transcriptional silencing (Ammerpohl et al. 2012; Doerr et al. 2005; Ushmorov et al. 2006).

As mentioned above, we have little insight into the initial events that promote the downregulation of the B cell program in the HRS cells or their immediate precursors. It is, however, known that hypoxic conditions promote in many cell types the dedifferentiation of cells, often linked to an upregulation of factors such as ID2. In vitro studies with human B cells and B cell lymphoma cell lines indeed showed that culturing of these cells under hypoxic conditions resulted in a partial acquisition of an HRS cell-like phenotype (Wein et al. 2015). Thus, one may speculate that perhaps transitory hypoxic conditions in the GC might contribute to the initial downregulation of the B cell program in the precursor cells of HRS cells.

As the lost B cell phenotype is such a consistent feature of cHL and involves a large number of factors, one wonders whether this is only an epiphenomenon or whether it is of major pathogenetic relevance for this malignancy. Perhaps, the lost B cell phenotype is linked to the proposed origin of HRS cells from "crippled" GC B cells: GC B cells are stringently selected for expression of a high-affinity BCR and express a program that causes the immediate death by apoptosis when the selection criteria are not met (Liu et al. 1989). There might therefore be a strong selection in the precursor cells of HRS cells to downregulate the GC B cell gene expression program and thereby escape from the selection pressure to undergo apoptosis if the BCR acquires disadvantageous mutations. In line with this scenario, it was shown that enforced reexpression of the B cell transcription factors FOXO1, PU.1, and E2A is toxic for cHL cell lines (Vogel et al. 2014; Yuki et al. 2013; Guan et al. 2016).

Role of Epstein-Barr Virus (EBV) in cHL Pathogenesis

Most humans worldwide are latently infected by the γ herpes virus family member EBV. EBV resides mostly in memory B cells, and in healthy EBV$^+$ individuals, about 1 in 10^6 B cells carries the viral genome, well controlled by the immune system (Souza et al. 2005). However, EBV is also found in the lymphoma cells of several types of B cell lymphomas, including cHL. About 30–40% of cases of cHL in the Western world show EBV infection of the HRS cells (Kapatai and Murray 2007). In childhood cases of cHL in Latin America, even up to 90% of cases are EBV-associated (Kapatai and Murray 2007). Also cHL developing in AIDS patients and in the posttransplant immunosuppressive setting are in 80–100% of cases EBV-positive (Kapatai and Murray 2007). EBV is more often found in cases of the mixed cellularity subtype of cHL than in the nodular sclerosis subtype. NLPHL is practically always EBV-negative.

In EBV-positive cHL, typically all HRS cells are positive for the virus, and analysis of the variable viral genome circularization site in latently infected cells further supports that EBV infection is an early and clonal event in HL pathogenesis (Weiss

et al. 1989). In EBV$^+$ cHL, the HRS cells show a latency II EBV infection pattern. This means that besides a number of non-coding RNAs, three viral proteins are expressed, namely, EBV nuclear antigen 1 (EBNA1) and the latent membrane proteins 1 and 2a (LMP1 and LMP2a) (Kapatai and Murray 2007). EBNA1 is essential for replication of the episomal viral genome in proliferating cells. LMP1 has a cytoplasmic domain that mimics a signaling domain of the B cell-costimulatory receptor CD40 and causes constitutive activation of the NF-κB and other pathways (Mosialos et al. 1995). It therefore functions as an oncogene. A cytoplasmic domain of LMP2a mimics the immunoreceptor tyrosine-based activation motif (ITAM) of the BCR coreceptors CD79A and CD79b (Mancao and Hammerschmidt 2007). Hence, LMP1 and LMP2a mimic the two main survival signals for GC B cells, i.e., BCR activation and CD40 triggering through T follicular helper cells. It was therefore speculated that EBV might rescue crippled GC B cells from apoptosis. Studies with in vitro EBV-infected human GC B cells indeed validated that EBV can immortalize GC B cells that had acquired destructive BCR mutations in vivo (Bechtel et al. 2005; Chaganti et al. 2005; Mancao et al. 2005). The important role of EBV in the rescue of crippled GC B cells was further supported by the recognition that all cases of cHL analyzed with destructive IgV gene mutations that prevent the expression of a BCR are EBV-positive (Bräuninger et al. 2006). It is therefore likely that in GC B cells, losing the capacity to express the BCR due to destructive IgV gene mutations LMP2a is essential to replace the survival signal of the BCR and to prevent the immediate death of the B cells by apoptosis. The role of LMP2a in the established HRS cell clone is questionable, because in connection with the lost B cell phenotype of the HRS cells, these cells have also downregulated expression of most components of BCR signaling (Schwering et al. 2003a), so that LMP2a may not be able anymore to cause a BCR-like signaling in HRS cells.

Genetic Lesions

HRS cells have an aberrant karyotype with numerous structural and numerical abnormalities (Weber-Matthiesen et al. 1995). There is also evidence for genomic instability at the chromosomal level, as HRS cells frequently show subclonal genomic imbalances and other structural aberrations (Weber-Matthiesen et al. 1995; Martin-Subero et al. 2003). Chromosomal translocations affecting the Ig loci, as they are typical for many other types of B cell lymphomas, were also detected in about 20% of cHL (Martin-Subero et al. 2006a). In a few instances, the translocation partners were identified as *BCL2*, *BCL3*, *BCL6*, or *MYC*, but for the majority of cases, the partner genes are not known (Martin-Subero et al. 2006a, b; Schmitz et al. 2005; Szymanowska et al. 2008). The pathogenetic relevance of these translocations in the established HRS cell clone is unclear, because the Ig loci are typically silenced in HRS cells (Ushmorov et al. 2004, 2006), so that also proto-oncogenes brought under the control of the Ig regulatory elements through the translocation should not show (strong) deregulated expression. Perhaps, the translocations were

important in early steps of HL pathogenesis, when the HRS precursor cells still had a B cell phenotype, so that the deregulated expression of the translocated proto-oncogenes was driven by the Ig enhancers.

The recognition of strong and constitutive NF-κB activity of HRS cells that is essential for their survival (Bargou et al. 1996, 1997) prompted a search for genetic lesions that might cause this activation. Gains or amplifications of the gene encoding the NF-κB factor REL were identified in about 40% of cases of cHL, and gains of *MAP3K4* (*NIK*), encoding a kinase and main factor of the noncanonical NF-κB pathway, are present in about 25% of cHL (Table 3.2) (Barth et al. 2003; Martin-Subero et al. 2002; Steidl et al. 2010). The genes of the principal negative regulators of canonical NF-κB signaling, IκBα and IκBε, i.e., *NFKBIA* and *NFKBIE*, respectively, are affected by deletions and/or inactivating point mutations in 10–20% of cases (Cabannes et al. 1999; Emmerich et al. 2003; Jungnickel et al. 2000; Mansouri

Table 3.2 Recurrent genetic lesions in HRS and LP cells

Cells	Gene	Types of alterations	Approximate frequency (%)
HRS cells			
	NF-κB pathway		
	NFKBIA	SNVs, deletions	10–20
	NFKBIE	SNVs, deletions	10
	TNFAIP3	SNVs, deletions	40[a]
	REL	Gains, amplifications	50
	MAP3K14	Gains, amplifications	25
	JAK/STAT pathway		
	SOCS1	SNVs, deletions	40
	PTPN1	SNVs, deletions	20
	JAK2	Gains, amplifications[b]	40
	STAT6	Gains	30
	Antigen presentation		
	B2M	SNVs, deletions	70
	CIITA	Translocations	15
	CD58	Deletions	20
	Others		
	TP53	SNVs, deletions	5–10
	CD95	SNVs	5–10
	XPO1	SNVs	25
LP cells	BCL6	Translocations	50
	SOCS1	SNVs, deletions	50
	REL	Gains	40
	SGK1	SNVs	50
	DUSP2	SNVs	50
	JUNB	SNVs	50

SNVs single nucleotide variants

[a]Frequency in EBV-negative cases about 70%

[b]JAK2 gains also involve the neighboring genes PDL1, PDL2, and JMJD2C

et al. 2016; Lake et al. 2009). The gene *TNFAIP3*, encoding A20, a more upstream-acting negative regulator of NF-κB activity, carries destructive mutations in about 40% of cHL (Kato et al. 2009; Schmitz et al. 2009a). *TNFAIP3* mutations were mostly found in EBV-negative cases (70% of such cases), indicating that EBV infection and *TNFAIP3* lesions are mostly two alternative and mutually exclusive mechanisms for NF-κB deregulation in HRS cells (Schmitz et al. 2009a). At a lower frequency, there are also mutations in the genes of the negative NF-κB regulators CYLD and TRAF3 (Otto et al. 2012; Schmidt et al. 2010), and some cHL carry gains or translocations affecting the *BCL3* gene (Martin-Subero et al. 2006b; Mathas et al. 2005), which encodes a costimulatory factor of the noncanonical NF-κB pathway. Thus, cHL is characterized by genetic lesions in multiple components of both the canonical and noncanonical NF-κB pathways, all contributing to constitutive NF-κB activity (Weniger and Küppers 2016).

A further pathway for which several recurrent genetic lesions are known in HRS cells is the JAK/STAT pathway, which is the main mediator of cytokine signaling (Table 3.2). HRS cells of about 30% of cHL harbor gains or amplifications of the JAK2 kinase gene (Joos et al. 2000). Importantly, gains of the JAK2 locus typically not only involve this gene but also the neighboring genes *PL-L1*, *PD-L2*, and *JMJD2C* (Rui et al. 2010; Green et al. 2010). JMJD2C is a histone demethylase, and PD-L1 and PD-L2 have an important role in immune evasion, as binding of these ligands to the PD1 receptor on activated T cells leads to impairment of T cell functions. The *STAT6* gene is affected by genomic gains in 30% of cHL (Hartmann et al. 2008). Two main negative regulators of JAK/STAT signaling, SOCS1 and PTPN1, are inactivated by genetic lesions in about 40% and 20% of cHL, respectively (Gunawardana et al. 2014; Weniger et al. 2006). Thus, similar to the NF-κB pathway, multiple components of JAK/STAT signaling are affected by genetic lesions in HRS cells, pointing to an essential role of constitutive STAT activity for cHL pathogenesis.

In a considerable fraction of cases, HRS cells lack expression of major histocompatibility complex (MHC) class I and/or class II (Steidl et al. 2011; Oudejans et al. 1996; van den Berg et al. 2000; Roemer et al. 2016). This may represent an immune evasion strategy of the HRS cells, so that, for example, EBV-positive HRS cells are not attacked by EBV-specific cytotoxic T cells. The loss of MHC class I or II expression is partly mediated by genetic lesions. The gene encoding the MHC class I component β2-microglobulin, *B2M*, is according to one study mutated in about 70% of cHL (Reichel et al. 2015). The MHC class II transactivator (*CIITA*), which is an essential factor for class II expression, is affected by chromosomal translocations with heterogeneous partners in about 15% of cHL (Table 3.2) (Steidl et al. 2011). These translocations seem to cause downregulation of the transactivator and as a consequence reduced MHC class II expression. The loss of MHC class I expression by HRS cells should render these cells vulnerable to an attack by natural killer (NK) cells, because NK cells have the function to eliminate class I-negative cells. However, in a subset of cHL cases and cell lines, HRS cells show mutations or deletions affecting the CD58 gene, which is an important adhesion molecule for the binding of cytotoxic T cells and NK cells to their target genes (Abdul Razak et al. 2016; Schneider et al. 2015).

HRS cells were also analyzed for mutations in a number of other well-known proto-oncogenes and tumor suppressor genes. These studies revealed that mutations in the genes *TP53*, *CD95*, and *ATM* are rare [summarized in (Schmitz et al. 2009b)]. No mutations were found in the genes *BAD*, *FADD*, *CASP8*, *CASP10*, and *NRAS* (Schmitz et al. 2009b). Recently, mutations of the exportin 1 (*XPO1*) gene were detected in about a quarter of cHL (Camus et al. 2016). Exportin 1 regulates the export of numerous RNAs and proteins out of the nucleus. The *XPO1* mutations all cause a replacement at codon 571 (E571K), strongly indicating that this is a gain of function mutation (Camus et al. 2016).

Only a few studies addressed genetic lesions of the LP cells of NLPHL. Translocations of the *BCL6* gene are seen in 40% of cases (Table 3.2) (Renné et al. 2005a; Wlodarska et al. 2003, 2004). *SOCS1* is mutated in 40% of NLPHL, indicating a role for JAK/STAT activity also in this form of HL (Mottok et al. 2007). In spite of the fact that LP cells show a strong NF-κB activity, no mutations were found in LP cells in the *TNFAIP3* and *NFKBIA* genes (Schumacher et al. 2010). However, *REL* gains are recurrent in LP cells (Hartmann et al. 2015). Thus, although there is some overlap, it seems that the mechanisms for NF-κB deregulation are mostly distinct in cHL and NLPHL. A recent genetic analysis of LP cells uncovered three new recurrently mutated genes in LP cells, namely, *SGK1*, *DUSP2*, and *JUNB*. SGK1 is a serine/threonine kinase, DUSP2 a phosphatase, and JUNB a member of the AP1 transcription factor family (Hartmann et al. 2016). *JUNB* mutations in LP cells seem to be inactivating, which fits to the absence of AP1 activity in LP cells (Hartmann et al. 2016).

Several investigations addressed the issue whether also germline mutations or polymorphisms may contribute to HL pathogenesis. There is indeed indication that this might be the case as HL is one of the lymphomas with the strongest familial association (Goldin et al. 2009). A constitutional mutation in the *KLHGC8B* gene was detected in a family with several members affected by HL (Salipante et al. 2009). Although the function of this gene is not known, it is remarkable that downregulation of its expression in a cell line caused the appearance of binucleated cells, indicating a potential role in cytokinesis. A germline mutation in the *NPAT* gene, the function of which is not known, was identified in several patients affected by NLPHL belonging to one family (Saarinen et al. 2011). A further rare germline mutation/polymorphism in this gene was found more frequently in HL patients than in healthy individuals. Genome-wide association studies revealed several risk loci for HL, including polymorphisms linked to the genes *EOMES*, *GATA3*, *TCF3*, and *REL* and to the MHC loci (Cozen et al. 2014; Enciso-Mora et al. 2010; Frampton et al. 2013; Urayama et al. 2012; Diepstra et al. 2005). EOMES has a role in embryogenesis and pluripotency, but its role in HL is unclear. GATA3 is a T cell transcription factor frequently expressed in cHL and contributing to cytokine expression of HRS cells (Stanelle et al. 2010); *TCF3* encodes the E2A transcription factor, which is functionally impaired in cHL (Mathas et al. 2006); and REL is – as already mentioned above – one of the NF-κB factors that is highly active in HRS cells.

Deregulated Signaling Pathways and Transcription Factor Networks in HRS Cells

In the discussion about genetic lesions in HRS cells, it was already mentioned that HRS cells show constitutive activity of the NF-κB and JAK/STAT signaling pathways. However, these pathways are not only activated by somatic mutations but also by autocrine and paracrine signaling events. HRS cells express several members of the tumor necrosis factor receptor (TNFR) superfamily, including CD30, CD40, TNFRSF17 (BCMA), TNFRSF13B (TACI), and TNFSF11A (RANK) (Carbone et al. 1995a; Chiu et al. 2007; Fiumara et al. 2001). Signaling through these receptors activates the canonical and/or noncanonical NF-κB pathway and partly also other pathways. Ligands for these receptors are expressed by other cells in the cHL microenvironment. For example, eosinophils and mast cells express CD30L (Molin et al. 2001; Pinto et al. 1996), activated $CD4^+$ T cells express CD40L (Carbone et al. 1995b), and neutrophils express APRIL, which can bind to TACI and BCMA (Schwaller et al. 2007). Also autocrine stimulation seems to play a role, as HRS cells themselves express the ligand of RANK (RANKL), and BAFF, which binds to TACI and BCMA (Chiu et al. 2007; Fiumara et al. 2001). The essential role of NF-κB activity for cHL pathogenesis is evident from the observation that its inhibition in cHL cell lines is toxic for these cells and induces their death (Bargou et al. 1997; Schmitz et al. 2009a).

In HRS cells, STAT3, STAT5a, STAT5b, and STAT6 are constitutively active in the vast majority of cases (Scheeren et al. 2008; Baus and Pfitzner 2006; Kube et al. 2001; Skinnider et al. 2002). Interference with JAK/STAT signaling in cHL cell lines leads to growth inhibition and cell death. In a similar fashion as described for the NF-κB pathway, also constitutive activation of JAK/STAT signaling in HRS cells is mediated by both genetic lesions and signaling through surface receptors. HRS cells express multiple cytokine receptors, and for IL7, IL13, IL15, and IL21 autocrine stimulation can occur, as HRS cells coexpress the ligands and their receptors (Scheeren et al. 2008; Skinnider et al. 2001; Cattaruzza et al. 2009; Ullrich et al. 2015). STAT activation in HRS cells is not only mediated through cytokine receptors but can also be mediated through a cross talk with other signaling pathways. For example, NF-κB activity augments STAT5 activity (Hinz et al. 2002). Microenvironmental stimulation of cytokine receptors on HRS cells likely involves IL3 secreted by $CD4^+$ T cells, IL7 secreted by fibroblasts, and IL15, which is produced by monocytes, dendritic cells, and endothelial cells (Aldinucci et al. 2016).

Many growth factors signal through receptor tyrosine kinases (RTK), and HRS cells show an unusually broad expression and activation of such receptors, most of which are not expressed by normal B cells. This includes platelet-derived growth factor receptor α (PDGFRA), macrophage stimulating 1 receptor (MST1R, also called RON), discoidin domain receptor tyrosine kinase 2 (DDR2), tyrosine receptor kinase A and B (TRKA and TRKB), colony-stimulating factor 1 receptor (CSF1R), and MET (Lamprecht et al. 2010; Renne et al. 2005b, 2008; Teofili et al.

2001). For several of these receptors, activation in an autocrine or paracrine manner is likely. For example, DDR2 is activated by collagen, which is frequent in the extracellular space, in particular in nodular sclerosis HL (Renné et al. 2005b, 2008). Nerve growth factor, which binds to TRKA and TRKB, is produced by HRS cells (Renne et al. 2008). The RTK MET is also expressed by a subset of normal B cells (Teofili et al. 2001; van der Voort et al. 2000). In HRS cells, MET is presumably stimulated through its ligand hepatocyte growth factor, which is produced by dendritic cells in the cHL microenvironment (Teofili et al. 2001). A remarkable finding concerns the aberrant expression of CSF1R by HRS cells. This RTK is expressed in HRS cells through a mechanism that involves derepression of an endogenous long terminal repeat upstream of the *CSF1R* gene that replaces the function of the normal CSF1R promoter, which is not active in B cells (Lamprecht et al. 2010).

The PI3K/AKT pathway plays a major role in cell survival and the regulation of cell metabolism. It is chronically active in HRS cells, partly mediated through CD30, CD40, RANK, and RTK signaling (Dutton et al. 2005; Georgakis et al. 2006). Furthermore, MAP kinase signaling has been detected in HRS cells. In particular the factors ERK1, ERK2, and ERK5 are active in HRS cells (Watanabe et al. 2005; Zheng et al. 2003; Nagel et al. 2007). As already mentioned above as one of the factors contributing to the lost B cell phenotype, HRS cells show constitutive activation of NOTCH1 signaling, which physiologically has an important role in T lineage specification and differentiation (Jundt et al. 2002, 2008).

AP1 transcription factors function as homo- or heterodimers and encompass members of the JUN, FOS, ATF, and BATF families. JUN, JUNB, and ATF3 are highly expressed in HRS cells and contribute to HRS cell proliferation (Mathas et al. 2002; Janz et al. 2006). The BATF family member BATF3 is also highly expressed by HRS cells (Brune et al. 2008; Rosenwald et al. 2003; Schwering et al. 2003b) and is essential for their survival (own unpublished observation). BATF3 is induced in HRS cells through STAT3 and STAT5, and BATF3 induces MYC expression by HRS cells (own unpublished observation). Among many other target genes, AP1 factors likely are involved in the upregulation of CD30 expression in HRS cells (Watanabe et al. 2003). MAP kinase activity also contributes to AP1 activation (Watanabe et al. 2005). AP1 factors can interact with members of the IRF transcription factor family, and in HRS cells IRF4 and IRF5 are highly expressed (Kreher et al. 2014; Falini et al. 2000). IRF5 orchestrates, together with NF-κB, the typical HRS cell gene expression pattern (Kreher et al. 2014).

Concluding Remarks

cHL is a unique malignancy in several key aspects: The HRS tumor cells are very rare in the tissue; they have a unique phenotype that does not resemble any normal cell type of the hematopoietic system, including a unique downregulation of their B cell gene expression program and an upregulation of numerous genes not normally expressed by B cells but by other immune cells; they derive from a peculiar subset of crippled, apoptosis-prone GC B cells; they consistently show aberrations of

cytokinesis, leading to the generation of bi- and multinucleated Reed/Sternberg cells; and they have constitutively activated a very large number of signaling pathways which are usually only transiently activated in B cells or not normally used by B cells at all. We are now beginning to understand more and more the mechanisms causing these characteristics. For example, numerous factors contributing to the loss of the B cell phenotype have been revealed, and for several of the constitutively activated signaling pathways, we now know genetic lesions and cellular interactions in the microenvironment that cause activation of these pathways. A comprehensive understanding of the genetic lesions involved in the pathogenesis of HL is still missing.

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Chapter 4
Targeting the Microenvironment in Hodgkin Lymphoma: Opportunities and Challenges

Lydia Visser, Arjan Diepstra, Christian Steidl, and Anke van den Berg

Abbreviations

B2M	β2-microglobulin
CC	CC chemokine
CIITA	Class II transactivator
CREBBP	cAMP response element-binding protein-binding protein
CTGF	Connective tissue growth factor
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T cells
CXC	CXC chemokine
DDR	Discoidin domain receptor tyrosine kinase
DHMEQ	Dehydroxymethylepoxyquinomicin
DLBCL	Diffuse large B-cell lymphoma
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EPHB1	Ephrin type-B receptor 1
HDAC	Histone deacetylases
HGF	Hepatocyte growth factor

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HL	Classical Hodgkin lymphoma
HLA	Human leukocyte antigen
HRS	Hodgkin and Reed-Sternberg
HSP	Heat shock protein
HuR	Human antigen R
IL	Interleukin
L	Ligand
Lag3	Lymphocyte-activation protein 3
LFA	Lymphocyte function-associated antigen
LMP	Latent membrane protein
LP	Lymphocyte-predominant tumor
LT	Lymphotoxin
MAGE-A4	Melanoma-associated antigen 4
MAPK	Mitogen-activated protein kinase
miR	MicroRNA
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
NIK	NF-κB-inducing kinase
NK	Natural killer
NKG2D	NK cell receptor D
NLPHL	Nodular lymphocyte-predominant Hodgkin lymphoma
NS	Nodular sclerosis
ORR	Objective response rate
PD-1	Programmed cell death protein 1
PDGFRA	Platelet-derived growth factor receptor alpha
PMBCL	Primary mediastinal large B-cell lymphoma
PRAME	Preferentially expressed antigen in melanoma
PTPN1	Protein tyrosine phosphatase, non-receptor type 1
R	Receptor
RON	Recepteur d'origine nantais
RTK	Receptor tyrosine kinases
SOCS	Suppressor of cytokine signaling
SSX2	Synovial sarcoma X
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TIM3	T-cell immunoglobulin and mucin-domain containing-3
TNF	Tumor necrosis factor
TNFAIP3	TNF alpha-induced protein 3
TNFRSF	Tumor necrosis factor receptor superfamily
Tr-1	T regulatory 1
TRAF	TNF receptor-associated factor
Treg	T regulatory cell
TRK	Tropomyosin receptor kinase

The Microenvironment in Hodgkin Lymphoma

Hodgkin lymphoma can be subdivided into the most common classical Hodgkin lymphoma (HL) and the less common nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL), which both typically involve lymph nodes. The tumor cells derive from germinal center B cells and constitute a minority of the affected tissue, usually less than 1% of the total cell population. The tumor cells in classical HL called Hodgkin and Reed-Sternberg (HRS) cells are characterized by an extremely large size, including presence of big nucleoli. The most common classical HL subtype is nodular sclerosis (NS) in which the microenvironment tends to be organized in large nodules that are surrounded by thick fibrous septa. The mixed cellularity subtype shows a diffuse architecture, without fibrous bands and often with granuloma-like aggregates of histiocytes. The lymphocyte-depleted and lymphocyte-rich subtypes are both relatively rare. The lymphocyte-predominant (LP) tumor cells of NLPHL often have extensively multilobulated nuclei. As the literature is dominated by studies about classical HL, this chapter mainly focuses on this subtype.

Cell Types Present in the Microenvironment

The microenvironment of HL is highly variable in architecture and cell composition. It virtually always contains many T cells (Alvaro et al. 2005; Kelley and Parker 2010; Ma et al. 2008a; Marshall et al. 2004; Schreck et al. 2009) and variable percentages of eosinophils, histiocytes (Ree et al. 1981; Steidl et al. 2010), neutrophils, plasma cells, B cells, fibroblasts, and mast cells. The T cells consist mainly of T regulatory (Treg) cells (Marshall et al. 2004) and T helper (Th) cells, although the balance between Th1 or Th2 remains inconclusive. Mast cells have been found predominantly in the NS subtype (Crocker and Smith 1984; Molin et al. 2002). The number of mast cells was inversely correlated with the number of macrophages ($CD68^+$ and $CD163^+$) and the number of granzyme+ cytotoxic T cells (CTLs) (Andersen et al. 2016). High eosinophilic infiltration is found in 34% of HL patients and is associated with NS subtype. Fewer eosinophils are usually seen in Epstein-Barr virus $(EBV)^+$ HL (Glimelius et al. 2011).

The Th1 vs Th2 Dilemma

Based on the low number of CTLs and the high number of eosinophils, HL was always considered to be dominated by a Th2-type immune response. This is in agreement with the protective role of a Th1-type background suggested by epidemiological studies comparing early oral exposures between the unaffected and

affected sibling in twins (Cozen et al. 2009). Immunohistochemistry for the Th2-specific transcription factor c-Maf confirmed the presence of high numbers of Th2 (c-Maf$^+$) cells (Schreck et al. 2009). However, a recent flow cytometry study suggested predominance of Th1-type cells based on presence of high numbers of CXCR3$^+$ Th cells. This finding was also supported by increased production of the Th1 pro-inflammatory cytokine TNF-α in comparison to CXCR3$^+$ T cells from reactive lymph node (Greaves et al. 2013). Subsequent validation experiments by immunohistochemistry on tissue microarray showed more Tbet$^+$ Th1 cells as compared to c-Maf$^+$ and GATA3$^+$ Th2 cells (Greaves et al. 2013). On the other hand, the level of the Th2/T follicular helper (Tfh) cell cytokine IL-21 was also increased.

To solve this Th2/Th1 dilemma, it is important to look at cells directly rosetting the tumor cells and to discriminate between EBV$^+$ and EBV$^-$ HL cases. In EBV$^+$ HL more CD8$^+$ T cells and natural killer (NK) cells are found (Dukers et al. 2000; Wu et al. 2016; Chetaille et al. 2009). In addition, M1 macrophages (CD68/CD163$^+$ pSTAT1$^+$) are more abundant in EBV$^+$ HL cases fitting a Th1 background. In EBV$^-$ HL cases, M2 macrophages (CD68/CD163$^+$ c-Maf$^+$) are predominant, which is in concordance with a Th2 subtype (Barros et al. 2015). Thus, in EBV$^+$ HL the balance has shifted toward a Th1-type background, while in EBV$^-$ HL a shift has occurred toward Th2.

The question is, however, if the Th1/Th2 balance is the real issue, since the T-cell subsets present in both EBV$^+$ and EBV$^-$ HL cases are suppressed by Treg cells. In EBV$^+$ HL, additionally increased numbers of IL-10-producing Tr-1 cells are found (Marshall et al. 2007; Morales et al. 2014). The suppressive role of the Treg cells is supported by the overall low cytokine levels produced by ex vivo activated T cells isolated from primary HL cell suspensions (Ma et al. 2008a), which suggests an anergic state. Both, FoxP3- and GITR-positive CD25$^+$ Treg cells have been detected in the microenvironment of HL (Marshall et al. 2004; Schreck et al. 2009; Wu et al. 2016). So, no matter whether Th1 or Th2 cells prevail, the end situation is an overall suppressed state of the T cells in the microenvironment of both EBV$^-$ and EBV$^+$ HL.

Therapies Targeting Cells in the Microenvironment

Based on the high numbers and regulatory activity of Treg cells in the microenvironment, targeting CD25, expressed on Tregs, activated T cells, and HRS cells, in part of the HL cases might be effective. Indeed, treatment with ^{90}Y-daclizumab, an anti-CD25 antibody labeled with Yttrium-90, induced an overall response rate (ORR) of 50%. This response was present in patients with and without CD25$^+$ HRS cells indicating that the treatment effectively might be dependent at least in part on targeting CD25$^+$ T cells. Treatment-induced DNA damage was predominantly seen in nonmalignant CD25$^+$ T cells (Janik et al. 2015). Targeting B cells with rituximab showed an ORR of 22% and was not dependent on expression of CD20 on the HRS cells (Younes et al. 2003). This indicates that targeting the nonmalignant infiltrating B cells might also help to disrupt the tumor cell-supporting microenvironment. Functional blocking of the CSF-1 receptor on macrophages with PLX3397 or

JNJ-40346527 resulted in an ORR of 5% (von Tresckow et al. 2015). Treatment of mast cells with bortezomib blocked the effect on growth of HL cell lines (Mizuno et al. 2012) but has not been tested in HL patients.

In summary, inhibition of Treg shows most clinical potential in HL.

Shaping the Composition and Activity of the Microenvironment

The tumor cells in HL actively shape their own microenvironment, and the cells in the environment get signals to further help in this process. Both the procedure of attracting and shaping the cells of the microenvironment as well as the active suppression of cells able to mount an antitumor response play a crucial role (Fig. 4.1).

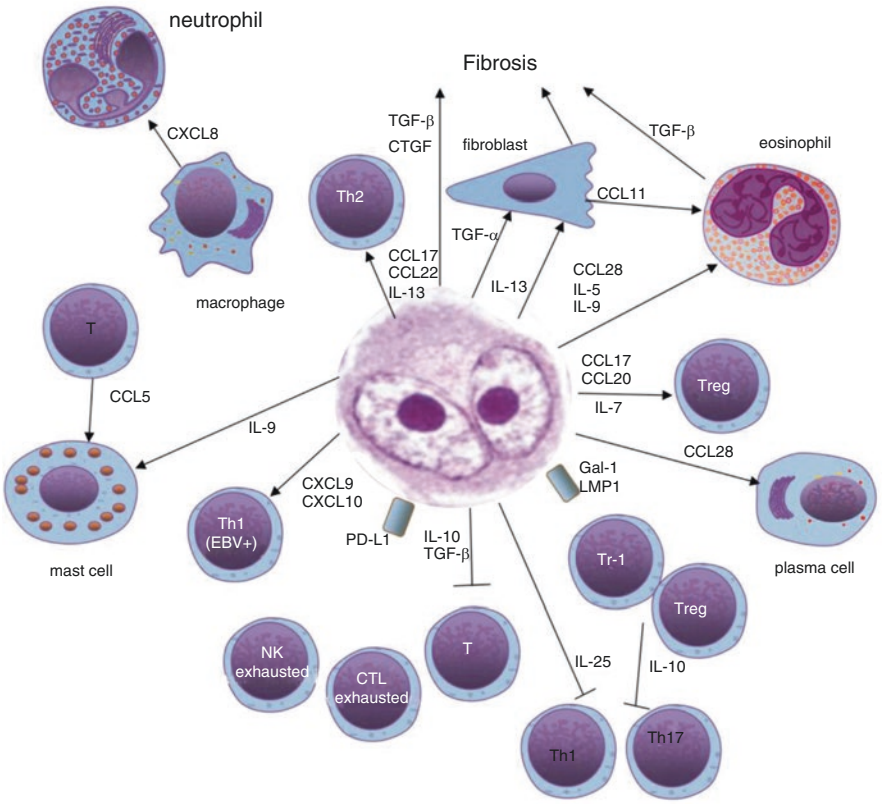

Fig. 4.1 Shaping the composition and the activity of the microenvironment. Several factors produced by HRS cells attract cells to the microenvironment (i.e., CCL17 and CCL28); other factors either shape the environment (i.e., IL-13) or suppress an antitumor response (i.e., PD-L1, Gal-1)

Attracting and Shaping the Microenvironment

The discovery of high expression of CCL17 (TARC) in HRS cells helped to explain the abundant presence of T cells expressing the CCL17 receptor, CCR4 (van den Berg et al. 1999). CCR4 is expressed on Th2 and Treg cells, and CCR4$^+$ cells form rosettes around HRS cells (Ishida et al. 2006). These cells have direct contact with the tumor cells and form a protective layer. IL-25 expressed by HRS cells (Ma et al. 2008b) blocks Th1 and Th17 development, which might fit with the predominance of CCR4$^+$ T cells rosetting the HRS cells rather than seeing Th1 cells in their close vicinity. Presence of Tregs is further supported by CCL20, a chemoattractant of Tregs. CCL20 expression is induced by IL-21 produced by HRS cells (Lamprecht et al. 2008). In addition, IL-7 stimulates proliferation of Tregs (Cattaruzza et al. 2009). Moreover, lymphotoxin α produced by HRS cells may also contribute to the attraction of T cells by activating endothelial cells (Fhu et al. 2014).

Expression of the chemoattractants CXCL9 and CXCL10 (ligands of CXCR3) by HRS cells of EBV$^+$ HL explains the presence of CXCR3$^+$ Th1 cells in the microenvironment (Ohshima et al. 2002). The EBV-derived protein LMP1 induces Tr1 and Tregs (Marshall et al. 2007), while EBNA1 (Baumforth et al. 2008) expression induces CCL20, which is a chemoattractant of Tregs.

The recruitment of eosinophils especially in the NS subtype is most likely due to the Th2-based cytokine environment with IL-5 (Samoszuk and Nansen 1990) and IL-13 (Skinnider et al. 2002a), as well as the presence of CCL28 (Hanamoto et al. 2004) being produced by the HRS cells and CCL11 by fibroblasts. TNFα and IL-13 produced by HRS cells synergize to stimulate the production of CCL11 by fibroblasts (Jundt et al. 1999; Terada et al. 2000). The recruitment of mast cells in HL can be explained by the production of CCL5 by cells in the microenvironment (Buri et al. 2001; Fischer et al. 2003). IL-9 produced by the HRS cells has been associated with the number of mast cells in HL and could play a role in the attraction of IL-9R$^+$ mast cells that are sensitive to IL-9 (Glimelius et al. 2006). Neutrophils are attracted to the microenvironment by CXCL8, which is produced mainly by reactive cells and in a small number of cases by the HRS cells (Foss et al. 1996). Plasma cells are attracted by CCL28 secreted by HRS cells (Hanamoto et al. 2004). A characteristic feature of the NS subtype of HL is the presence of sclerotic bands. TGF-β, produced by HRS cells and eosinophils (Newcom and Gu 1995; Kadin et al. 1993), is likely the main cause of this fibrosis. IL-13 produced by the HRS cells and connective tissue growth factor (CTGF) may also play a role in the fibrotic process. IL-13 can bind to the IL-13R on fibroblasts, which will lead to the production of collagen. IL-13 expressing HRS cells and IL-13R-positive fibroblasts were indeed more commonly found in the NS subtype fitting with the presence of sclerotic bands found in the NS subtype of HL (Ohshima et al. 2001). CTGF, another factor produced by HRS cells, might also play a role in fibrosis and is correlated with the NS subtype and the extent of fibrosis (Birgersdotter et al. 2010).

Suppressing T-Cell Responses

HRS cells (Newcom and Gu 1995) and eosinophils (Kadin et al. 1993) in HL produce TGF-β, which will lead to a suppression of the T-cell responses. The TGF-β protein found in HL has a high molecular weight and is already active at a physiological pH (Newcom et al. 1988). In addition, TGF-β downregulates the activating NKG2D receptor on T cells in the microenvironment. This will prevent recognition of HRS cells by NK cells and CTLs (Zocchi et al. 2012). IL-10 produced by HRS cells especially in EBV$^+$ HL (Herbst et al. 1996a) plays an important role in the suppression of a Th1 response. IL-10 is also produced by CTLA4$^+$ T cells (Marshall et al. 2007) and by Tr-1 cells (Marshall et al. 2004) present in the microenvironment of HL. Galectin-1 expressed by the HRS cells induces apoptosis of Th1 cells and induction of IL-10-producing Tr-1 cells (Juszczynski et al. 2007; Cedeno-Laurent et al. 2012). PD-L1 expressed on the HRS cells induces exhaustion of CTLs and NK cells via inhibitory signaling via PD-1 (Yamamoto et al. 2008).

Therapeutic Approaches to Modulate Factors that Shape the Microenvironment

To counteract the attraction of Treg and Th2 cells by CCL17, an antibody against CCR4 could be used for the treatment of HL patients. The anti-CCR4 antibody, mogamulizumab, has already been used in T-cell lymphomas (Yoshie and Matshushima 2014). In a CCL22$^+$ ovarian cancer xenograft humanized mouse model, anti-CCR4 treatment did indeed enhance antitumor responses (Chang et al. 2016). Therapy aiming to block the factors shaping the microenvironment has the potential to additionally affect tumor cell growth and support conventional treatment. HDAC inhibitors are excellent candidates to disrupt the protective environment but may activate the AKT and mTOR signaling pathways in HRS cells (Lemoine et al. 2012). Treatment of HL patients with the HDAC inhibitors panobinostat, vorinostat, and entinostat reduced for example circulating levels of CCL17, CCL11, CCL5, and TGF-β levels (Harrison et al. 2014; Oki et al. 2014; Buglio et al. 2008). In addition, levels of these cytokines were reduced in HL cell lines upon treatment with these inhibitors (Buglio et al. 2008; Jona et al. 2011). In HL cell lines, the vorinostat-induced reduction of CCL17 production was caused by the inhibition of STAT6 (Buglio et al. 2008). Treatment with HDAC inhibitors also induced protein levels of CXCL8, CXCL10, and IL-13 in patients' serum samples and HL cell lines (Harrison et al. 2014; Oki et al. 2014; Buglio et al. 2008). These cytokines might enhance the numbers of Th1 cells and neutrophils in the microenvironment. However, vorinostat treatment results in increased production of IL-10, which might reduce its beneficial effects by suppressing T-cell responses (Buglio et al. 2008). Similarly, panobinostat might counteract the favorable effect of TGF-β downregulation by decreasing expression of the activating NKG2D receptor on NK

cells (Klein et al. 2013). HDAC inhibitors are effective in treatment of HL, with ORR varying between 22 and 40% for panobinostat (Copeland et al. 2010). Combination of HDAC inhibitors with the mTOR inhibitor everolimus enhanced the antiproliferative effect of panobinostat in HL cell lines (Lemoine et al. 2012). Consistent with these findings, treatment with a combination of vorinostat and the mTOR inhibitor sirolimus in a single patient with advanced refractory HL resulted in clinical remission (Subbiah et al. 2014). Other therapeutic options to block CCL17 are via inhibition of the NF-κB signaling pathway using NF-κB inhibitor DHMEQ (dehydroxymethylepoxyquinomicin) or by using the anti-inflammatory drug auranofin (Celegato et al. 2014a, 2015). Moreover, the PI3Kδ inhibitor GS-1101 blocks the production of CCL5 by HL cell lines (Meadows et al. 2012). The latter drug might be effective in combination with the mTOR inhibitor everolimus.

Overall, the use of HDAC inhibitors in combination with mTOR inhibitors might be the most optimal choice for disrupting the way HRS cells shape their environment.

Cross Talk Between the Microenvironment and the HRS Cells

HRS cells receive signals of various soluble factors produced by cells in the microenvironment and in addition receive signals via direct cell-cell contact with the rosetting T cells. These two types of signals are important for cell growth and survival of the tumor cells in HL.

Activation of Pro-survival Signaling Pathways

HRS cells receive a broad spectrum of pro-survival signals from the cells in the microenvironment via signaling of cytokine receptors, various members of the tumor necrosis factor receptor superfamily (TNFRSF), and several receptor tyrosine kinases (RTK) (Fig. 4.2).

Supporting HRS Cell Growth by Cytokines and Chemokines

HRS cells produce various cytokines and chemokines that serve as autocrine growth and survival factors. A number of these factors are also produced by the cells in the microenvironment and may thus also act as paracrine growth factors.

IL-6 is produced by HRS cells (Jucker et al. 1991) and is upregulated in EBV$^+$ HL (Herbst et al. 1997). The IL-6 receptor is also expressed on HRS cells (Jucker et al. 1991). Based on the known functions of IL-6, it might act as an autocrine growth factor for HRS cells. IL-6 is also produced by T cells and macrophages, enabling

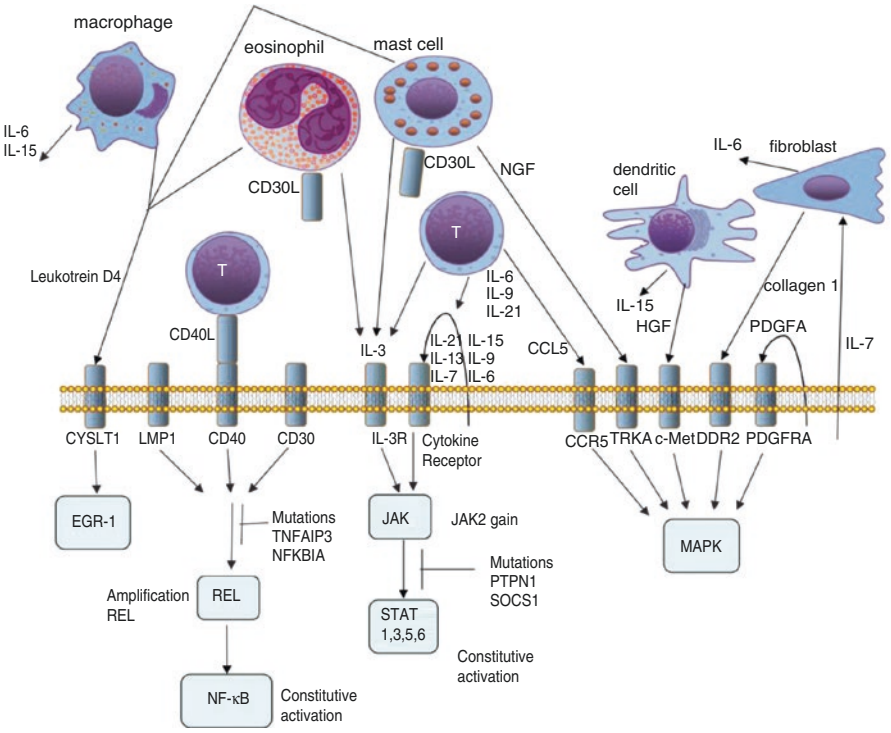

Fig. 4.2 Activation of pro-survival signaling pathways. Several cytokines activate the JAK-STAT pathway in HRS cells in combination with gain of JAK2 and mutations of multiple inhibitory proteins; this results in constitutively activated STATs. NF-κB is constitutively activated via multiple receptors and somatic mutations or amplifications. Several RTKs are activated in HL by ligands expressed by HRS cells or cells in the microenvironment

paracrine signaling as well. Both IL-7 and its receptor are expressed by HRS cells (Cattaruzza et al. 2009; Foss et al. 1995). IL-7 enhances proliferation of HL cell lines and protects them against apoptosis. In addition, IL-7 induces production of IL-6 in fibroblasts cultured from HL-involved lymph nodes, which might further support growth of HRS cells (Cattaruzza et al. 2009). Expression of the IL-9 receptor has been observed in HRS cells in a subset of cases (Glimelius et al. 2006). IL-9 is produced by HRS cells and lymphocytes in about half of the HL cases (Merz et al. 1991a). In HL cell lines, IL-9 inhibition with antibodies inhibited cell growth, while stimulation with IL-9 increased proliferation (Gruss et al. 1992). Together this indicates that at least in a portion of the HL cases, IL-9 may act both as an autocrine and paracrine growth factor for HRS cells. IL-13 and its receptor are both produced by HRS cells, whereas production of IL-13 in the microenvironment is restricted to some rare T cells (Skinnider et al. 2002a). This indicates that IL-13 mainly acts as an autocrine factor in HL (Ohshima et al. 2001). Anti-IL-13 (Kapp et al. 1999) and the soluble IL-13Rα2 decoy receptor (Trieu et al. 2004) can both block growth of HRS

cells, indicating the pathogenic relevance of IL-13 for HL. The most recent cytokine identified in HRS cells is IL-15 (Ullrich et al. 2015). IL-15 expression is also found in monocytes and dendritic cells present in the microenvironment (Ullrich et al. 2015). The IL-15 receptor is expressed on HL cell lines, and based on gene expression studies, the IL-15 receptor is also expressed in primary HRS cells. IL-15 induces proliferation of HL cell lines and protects the cells against apoptosis. Experiments in a single HL cell line revealed increased mRNA levels of IL-1α, IL-6, IL-9, IL-12β, IL2Rα, and CCL3 upon IL-15 treatment (Ullrich et al. 2015). Thus, IL-15 might not only support growth of HRS cells but also shape the cytokine milieu of the HRS cells. IL-21 is expressed in HRS cells and is also produced by T cells in the microenvironment. The receptor of IL-21 is expressed on HRS cells (Scheeren et al. 2008). Based on its known function, IL-21 may also support growth of HRS cells.

HRS cell survival factors specifically produced by cells present in the microenvironment are IL-3 and CCL5. The IL-3 receptor is expressed on HRS cells (Aldinucci et al. 2002), and IL-3 is expressed in a subset of cells present in the microenvironment (Merz et al. 1991b). HL cell lines also express the receptor for IL-3 and show increased proliferation when incubated with IL-3 (Aldinucci et al. 2002). The CC-chemokine receptor 5, a receptor for CCL5, is expressed on HRS cells (Aldinucci et al. 2008), but in primary HL cases, not HRS cells but T cells express CCL5 (Fischer et al. 2003). In HL cell lines, expression of both the receptor and CCL5 has been shown (Buri et al. 2001). Blocking this autocrine signaling loop using CCL5 antibodies inhibits colony growth of HL cell lines by 10–20% (Aldinucci et al. 2008), supporting the potential relevance of CCL5 for growth of HRS cells.

JAK-STAT Signaling

Since several cytokine receptors are present on HRS cells and the corresponding cytokines are produced by the HRS cells and/or cells in the microenvironment, signaling through the JAK-STAT pathway in HL is apparent. STAT1, STAT3, STAT5, and STAT6 are constitutively activated in HL cell lines (Cochet et al. 2006; Kube et al. 2001), and their relevance for HL has been shown in multiple studies. Experimental knockdown of STAT3 or overexpression of the SOCS1 and SOCS5 inhibitors blocks growth of HL cell lines (Baus and Pfitzner 2006), whereas knockdown of STAT1 and STAT6 induces apoptosis (Baus et al. 2009). Phosphorylation of STAT5 induced by binding of IL-21 to its receptor on HL cell lines enhanced proliferation (Scheeren et al. 2008). Phosphorylation of STAT3 induced by IL-21 enhanced production of CCL20 (Lamprecht et al. 2008). STAT3, STAT5, and STAT6 are expressed in HRS cells of primary cases (Hinz et al. 2002; Skinnider et al. 2002b).

Tumor Necrosis Factor Receptor Superfamily

CD30 (TNFRSF8) is abundantly expressed on HRS cells in virtually all HL patients and on HL cell lines (Falini et al. 1987). CD30L is expressed on eosinophils and mast cells (Pinto et al. 1996; Molin et al. 2001), and these cells can enhance proliferation of HL cell lines (Molin et al. 2001). Moreover, CD30 overexpression as

observed in HRS cells can induce NF-κB activation independent of CD30L (Horie et al. 2002; Watanabe et al. 2011). CD40 (TNFRSF5) is also highly expressed on HRS cells and HL cell lines (Gruss et al. 1994; Carbone et al. 1995). In EBV$^+$ HL cases, the EBV-derived LMP1 acts as a constitutively activated CD40 receptor (Jarrett 2002). Triggering of CD40 results in activation of the NF-κB pathway in HL via proteolysis of TRAF3 (Annunziata et al. 2000). The CD40 ligand (CD40L) is mainly expressed on CD4+ T cells that are present in the close vicinity of and rosetting around the HRS cells (Carbone et al. 1995). Stimulation of HRS cells with CD30L and CD40L induces secretion of several cytokines, including IL-6, CXCL8 (only with CD40L), TNF-α, and LT-α (Gruss et al. 1994, 1995). Tumor-promoting effects of cells present in the reactive infiltrate can be enhanced by certain cytokines. IL-5 and GM-CSF produced by the HRS cells enhance the expression of CD30L on eosinophils (Pinto et al. 1996). However, it is unclear if this will lead to enhanced CD30 signaling in HRS cells, as CD30 signaling in HRS cells is not dependent on CD30L. IL-10 produced by tumor cells and T cells enhanced expression of CD40L on T cells (Carbone et al. 1995). Thus cytokines produced by HRS cells augment the pro-survival CD30 and CD40-CD40L signaling pathways in HL.

Receptor Tyrosine Kinase (RTK) Family Members

HRS cells aberrantly express certain RTKs as compared to normal B cells and B-cell non-Hodgkin lymphoma, i.e., PDGFRA in 75% of the patients and DDR2, EPHB1, RON, TRKA, and TRKB in at least 30% of the patients (Renne et al. 2005). The phosphorylated active forms of these RTKs can be detected in HRS cells in HL tissue samples (Renne et al. 2005). Activation of RTKs is probably induced by binding of ligands, since to date there are no activating mutations reported in HL cell lines and HRS cells of primary HL cases (Renne et al. 2005; Reichel et al. 2015). Collagen type 1 (ligand of DDR2) and nerve growth factor (NGF; ligand of TRKA) are expressed by infiltrating reactive cells indicating paracrine activation. PDGFA, the ligand of PDGFRA, is expressed by the tumor cells (Renne et al. 2005). HRS cells express ephrin-B1, the ligand of EPHB, but autocrine signaling is unlikely since the receptor and its ligand are both membrane-bound. The receptor for hepatocyte growth factor (HGF), c-Met, is expressed by HRS cells in the majority of HL patients (Teofili et al. 2001; Xu et al. 2012). HGF is expressed by CD21$^+$ dendritic reticulum cells and in 20% of the HL patients also by the HRS cells (Teofili et al. 2001; Xu et al. 2012). Inhibition of c-Met suppresses growth of HL cell lines by blocking the cells in the G2/M phase (Xu et al. 2012). Thus, HRS cells can receive pro-survival signaling through various mechanisms that activate RTKs and downstream signal transduction pathways, such as the MAPK pathway.

Cysteinyl-Leukotriene (CysLT) Type 1 Receptor

The CysLT type 1 receptor is expressed on HL cell lines and primary HRS cells, while its ligand, leukotriene D4, is produced by eosinophils, macrophages, and mast cells that are present in the microenvironment of the tumor cells. Signaling through

this pathway induces TNFα, IL-6, CXCL8, CCL3, and CCL4 production in HL cell lines, by upregulation of the transcription factor EGR-1 (Han et al. 2015).

Lack of Antigen Presentation

The very strong susceptibility of single nucleotide variants in the HLA locus and the association of specific HLA subtypes have suggested the importance of antigen presentation in the pathogenesis of HL (see below). Loss of both HLA class I and II expression is a common feature of HRS cells and represents an immune escape mechanism for the HRS cells (Diepstra et al. 2008; Murray et al. 1998; Oudejans et al. 1996). This is especially true in EBV^- HL where HRS cells have lost HLA class I in about 85% and HLA class II in about 55% of the cases (Diepstra et al. 2007, 2008). In EBV^+ HL, these percentages are much lower with loss of HLA class I in 30% (Diepstra et al. 2007) and loss of HLA class II in 45% (Diepstra et al. 2008). Loss of HLA class I makes the HRS cells vulnerable to recognition and elimination by NK cells. Expression of HLA-G by HRS cells (Diepstra et al. 2007) in turn might prevent targeting by NK cells.

Since HRS cells are genomically and phenotypically highly aberrant cells, it is expected that the HLA^+ cases produce and present a large number of neo-antigens, i.e., tumor cell-specific antigens. EBV^+ HL will, besides presenting these neo-antigens, also present EBV-derived antigenic peptides. Tumor-specific antigens presented by HLA class I pose a threat to the tumor cells as this physiologically activates CTLs. Presentation of neo-antigens by HLA class II also activates Th1 and Th2 cells, and activation of Th1 cells can amplify cytotoxic antitumor responses. However, cytokines like TGF-β and IL-10 induce an immune-suppressive environment. In addition, Th2 and Treg cells form a protective layer by rosetting around the HRS cells, whereas both CTLs and Th1 cells are often not in close vicinity of HRS cells. Conceptually, HLA class II may even be involved in recruiting the protective Th2 and Treg cells at some point in disease pathogenesis. These rosetting T cells are polyclonal (Trumper et al. 2001), implying that multiple different neo-antigens are being presented and recognized.

Immune Checkpoints

In the past years, a lot of attention has been given to expression of immune checkpoint molecules and their potential as therapeutic targets. Several of the activating and inhibitory checkpoint molecules have been studied in HRS cells and/or in the reactive cells of the microenvironment (Fig. 4.3). These studies have been focused on OX40, CD27, CD137, and CD28 as activating checkpoints and on PD-1, CTLA4, and Lag3, as inhibitory checkpoints.

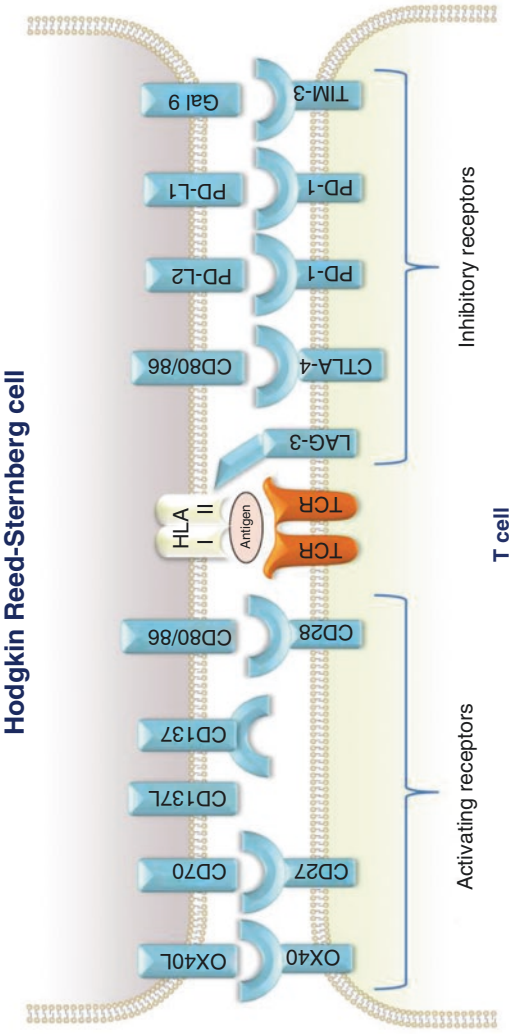

Fig. 4.3 Immune checkpoints. All activating and inhibitory immune checkpoints known to be expressed in (subsets of) HL are shown. The OX40L expression level is low

OX40L is expressed at low levels in HRS cells (Buglio et al. 2011), and OX40 is expressed on CD26$^-$ T cells (Ma et al. 2008a) as well as on eosinophils but not on cells immediately rosetting the HRS cells. The low level of OX40L might prevent activation of CTLs via OX40 and as such be favorable for HRS cells. In addition, the low OX40L levels induce immune-suppressive Tr-1 cells (Buglio et al. 2011).

CD70, the ligand of CD27, is strongly expressed on HRS cells and T cells in the microenvironment (Herbst et al. 1996b). However, it is unclear if CD27 is expressed on T cells rosetting HRS cells. In vitro studies showed expression of CD70 on memory T cells that also express TIM3 and PD-1, indicating T-cell exhaustion. TGF-β induces FoxP3 and CD70 expression in naïve T cells (Yang et al. 2014). This finding suggests that CD70 expression is mainly restricted to exhausted T cells and/or Tregs in HL.

CD137 expression is normally found on CTLs (Ho et al. 2013) and on eosinophils (Pinto et al. 1997). CD137 expressed on HRS cells can bind to CD137L (Anderson et al. 2012), which is also expressed on HRS cells. The resulting complex will be internalized, preventing activation of CD137$^+$ CTLs by CD137L expressed on HRS cells (Pang et al. 2013).

CD80 and CD86 expression on HRS cells is induced by NF-κB activation (Hinz et al. 2001). These ligands may bind to and activate CD28$^+$ T cells in the microenvironment (Munro et al. 1994). Although this might in theory lead to T-cell activation and mediate an antitumor response, it is apparently not effective possibly due to the immune-suppressive environment. Moreover, binding of CD80 and CD86 to CTLA4, which is expressed on the CD26$^-$ T-cell population in HL (Ma et al. 2008a), will provide inhibitory signals to T cells. Using co-culture approaches, HL cell lines were able to induce differentiation of naïve T cells toward CTLA4$^+$ Tregs (Tanijiri et al. 2007).

PD-L1 is highly expressed in HL cell lines and primary HRS cells, while PD-1 is expressed on T cells in the microenvironment (Yamamoto et al. 2008). Virtually all HL cases show increased expression of PD-L1 in HRS cells (Roemer et al. 2016). Although no association with EBV is seen, LMP1 and LMP2 can also induce PD-L1 expression (Yamamoto et al. 2008). PD-L1 expression is also found on infiltrating macrophages. Only 12% of HL patients were found to have more than 20% PD-1$^+$ cells in the microenvironment (Koh et al. 2016). Thus, presence of PD-1$^+$ T cells in the direct vicinity of the HRS cells will be rare, and inhibition of these cells by PD-L1$^+$ HRS cells will only be effective in a limited number of HL cases.

The Treg marker LAG-3 is expressed in T cells in the proximity of HRS cells, and the percentage of LAG-3$^+$ cells is high especially in EBV$^+$ cases (Gandhi et al. 2006). In HLA class II positive cases, these cells will be inhibited upon binding of LAG-3 to HLA class II. LAG-3 and FoxP3 expression levels on tumor-infiltrating cells were associated with impaired functionality of LMP1- and LMP2-specific CTLs (Gandhi et al. 2006).

Galectin-9 produced by HRS cells (Tureci et al. 1997) is an inhibitory ligand of TIM-3 (Zhu et al. 2005), but expression of TIM-3 has not been studied in HL T cells.

Therapeutic Interventions to Modulate Cross Talk

Several therapeutic options are available to inhibit JAK-STAT signaling. The multikinase inhibitor, lestaurtinib, inhibits JAK2. Treatment of HL cell lines with this drug inhibits cell growth and induces apoptosis (Diaz et al. 2011). At a low dose, the JAK inhibitor AZD1480 reduces production of cytokines and chemokines, mildly inhibits proliferation, and induces low levels of apoptosis. At a high dose, it inhibits Aurora kinases and causes G2/M arrest and apoptosis (Derenzini et al. 2011). Inhibition of HSP90 with 17-AAG, a geldanamycin derivate, results in reduced JAK-STAT signaling, reduced phosphorylation levels of STATs, and decreased growth of HL cell lines (Schoof et al. 2009).

NF-κB inhibition reduces expression of CD40 on HRS cells (Celegato et al. 2014a, 2015). Inhibition of NF-κB and treatment with the HDAC inhibitor panobinostat both lower the expression of CD30 on HRS cells thus potentially reducing CD30- and CD40-driven pro-survival signals (Buglio et al. 2008; Celegato et al. 2014a, 2015). In contrast to panobinostat, the HDAC inhibitor MGCD0103 induces activation of NF-κB. Additional inhibition with the proteasome inhibitor bortezomib is required to induce effective tumor cell killing (Buglio et al. 2010). Soluble CD40L decreases the effect of the proteasome inhibitor bortezomib on the induction of apoptosis in HL cell lines (Celegato et al. 2014b). This implicates that expression of CD40L on T cells might also reduce the effectiveness of bortezomib as a therapeutic intervention in HL. Targeting of CD30 is effective only when administered as an antibody drug conjugate, aiming to kill the HRS cells (Younes et al. 2010). So CD30 antibody effects are not related to receptor inhibition but to toxicity of the conjugate. Targeting of CD40 with the antagonistic antibody, lucatumumab, has limited effects in HL with an ORR of 13.5% (Fanale et al. 2014).

The expression of a large number of RTKs in HRS cells indicates the rationale for testing RTK-blocking antibodies or small inhibitory molecules in the treatment of HL patients. In HL cell lines, sorafenib and lestaurtinib, targeting PDGFRα, TRKA, and RON (Holz et al. 2013), were effective in inhibiting several signaling pathways and inducing cell death. Sorafenib in combination with the AKT inhibitor perifosine resulted in an ORR of 28% in HL patients (Guidetti et al. 2014). Auranofin blocks expression of DDR1 in HRS cells (Celegato et al. 2015) but has not been tested in HL patients. Several c-Met inhibitors are available for clinical use and have been tested in solid tumors (Chang et al. 2015), but no studies have been performed in HL patients.

Enhancing the effectivity of NK cells and CTLs to start an antitumor response is a therapeutic option that might be effective. Treatment with the immune modulatory drug lenalidomide increases the activity of NK cells and CTLs and induced an ORR of 19% in HL patients (Fehniger et al. 2011), supporting the clinical potential of this drug. The use of lenalidomide to activate CTLs and NK cells also reactivates EBV lytic proteins and might enhance the immune response in EBV^+ HL but might also cause development of new tumors (Jones et al. 2016). Another approach to enhance NK-cell activity is by linking of HRS cells to NK cells with the AFM13 bispecific

antibody targeting both CD30 and CD16A. This antibody enhanced activation of NK cells in ex vivo experiments (Reiners et al. 2013). In a phase I clinical trial using this antibody, the ORR was 11.5% in a group of 26 HL-relapsed patients. Targeting of IL-12 alone or in combination with IL-2 to HRS cells via an anti-CD30 fusion protein induced IFN-γ secretion and activation of NK cells resulting in an enhanced target cell lysis (Hombach et al. 2005; Jahn et al. 2012; Heuser et al. 2003).

Adoptive T-cell therapy with autologous CTLs specific for the tumor cell-specific MAGE-A4 antigen might increase antitumor response in HL cases with MAGE-A4-positive HRS cells. Generation of multispecific T cells directed against a range of tumor-associated antigens, including MAGE-A4, survivin, SSX2, and PRAME, is another possibility (Gerdemann et al. 2011). To increase the effectivity of these T-cell treatments, patients can be treated with decitabine, which induces MAGE-A4 expression on HRS cells (Cruz et al. 2011). Several strategies have been developed to target EBV^+ HL with autologous EBV-specific cytotoxic cells (CTLs). EBV-specific CTLs transduced with IL-12, to block suppression by TGF-β, overcame the inhibitory effects of the Th2 cells by inducing Th1 cytokines and inhibition of Th2 cytokines (Wagner et al. 2004). Using IL-15 instead of IL-2 for the expansion of EBV-specific T cells favors survival and proliferation of CTLs in the presence of Tregs (Perna et al. 2013). LMP2-specific CTLs were successfully developed by overexpression of LMP2 in dendritic cells followed by initial reactivation of LMP2-specific T cells, which could be further expanded using LMP2 overexpressing LCLs (Bollard et al. 2004). In a clinical study using this latter protocol to expand autologous LMP-specific CTLs, an ORR of 84% was achieved in 25 high-risk or multiple relapsed HL patients. In responders an increase in T cells reacting specifically to other non-viral tumor-associated antigens, such as MAGE-A4, survivin and PRAME, was seen (Bollard et al. 2014).

EBV-specific CTLs overexpressing CD30 as a chimeric antigen receptor (CAR) were able to target autologous EBV^+ cells as well as $CD30^+$ EBV^- HRS cells in a xenograft model (Savoldo et al. 2007). Potentially this approach might be effective in all HL patients.

Targeting immune checkpoints in HL might represent an attractive therapeutic option, given the highly abundant reactive infiltrate. HDAC inhibition induces OX40L expression and inhibited the generation of IL-10-producing Tregs (Buglio et al. 2011). Blocking of CD70 expression by T cells might prevent the induction of exhaustion in T cells and reduce the differentiation of naïve T cells to Treg cells (Yang et al. 2014). As CD70 is also expressed on HRS cells, blocking of CD70 might also affect HRS cells directly. Three blocking CD70 antibodies are currently available (Jacobs et al. 2015), but these have not yet been tested in HL patients. Agonistic antibodies against CD137 have been used in preclinical models to stimulate T cells (Sun et al. 2004). Antagonistic CD137 antibodies targeting the HRS cells might have a contrary effect on the T cells. However, it is unclear whether and how this will affect HRS cells that express both CD137 and CD137L.

Stimulation of T cells using CD3/CD30 and CD28/CD30 bispecific antibodies induced TCRζ expression in a preclinical HL animal model (Renner et al. 1994, 1996). In addition, it improved cytolytic potential of T cells through induction of

perforin, granzyme, and Fas and led to an increase in apoptosis of target cells (Renner et al. 1997). Treatment of 14 HL patients with the CTLA4 inhibitor ipilimumab induced complete remission in two patients (Bashey et al. 2009). Treatment with therapeutic anti-PD-1 antibodies showed remarkable effects in HL patients with ORR of 87% and CR in 17% of the patients for nivolumab (Ansell et al. 2015) and an ORR of 65% and CR in 16% of the patients for pembrolizumab (Armand et al. 2016). In contrast to CTLA-4 and PD-1 knockouts, LAG-3 knockout animals do not develop an autoimmune phenotype indicating a more subtle role in T-cell modulation (Drake 2015). Based on these findings, LAG-3 might represent a more attractive therapeutic target with no or less severe autoimmune side effects as compared to PD-1- and CTLA4-based treatments.

Inherited and Somatic Aberrations Influencing the Microenvironment

Both inherited and somatic aberrations have an impact on the microenvironment of HL. Multiple-targeted and genome-wide studies have shown several genetic risk and protective loci (reviewed in (Kushekhar et al. 2014)), indicating a host-specific role in the development of HL. The most striking finding in these studies was the association with the HLA region, supporting a pathogenic role of certain HLA alleles. In addition, these studies showed multiple non-HLA loci, including genes related to immune responses and T cells. Somatic aberrations influencing the composition and functionality of cells in the microenvironment have been identified in both HL cell lines and primary HRS cells.

Host-Specific Role

HLA type has been associated with the risk of developing HL for a long time. More recently, this has become even more evident by genome-wide association studies that revealed a very strong association of HL risk with the HLA region (Urayama et al. 2012; Cozen et al. 2012). EBV^+ HL is generally associated with HLA class I risk alleles, while EBV^- HL associates with HLA class II risk alleles. In the Caucasian population, HLA-A*01 (Niens et al. 2007; Hjalgrim et al. 2010; Huang et al. 2012a) and HLA-B*37 (Huang et al. 2012a) are risk types in EBV^+ HL, while HLA-A*02 (Niens et al. 2007; Hjalgrim et al. 2010; Huang et al. 2012a, b; Monroy et al. 2011) is a protective type. In the Chinese population, HLA-A*02:07 is a risk allele in EBV^+ HL, while it is protective in EBV^- HL (Huang et al. 2012b). Generally it is thought that HLA-A*02 can more efficiently present antigenic peptides derived of the EBV proteins LMP1 and LMP2, while A*01 and A*02:07 have low affinity for EBV-derived peptides.

Besides HLA, several non-HLA loci have been identified in targeted gene approach studies and by genome-wide screens. SNPs associated with HL susceptibility in targeted gene approach studies that might play a role in the microenvironment include SNPs in the IL4R (Monroy et al. 2011), IL10RA (Nieters et al. 2006), STAT3, STAT4, and STAT6 gene loci (Butterbach et al. 2011). In genome-wide studies, associations with IL-13 (Urayama et al. 2012; Cozen et al. 2014) and GATA3 (Enciso-Mora et al. 2010; Frampton et al. 2013) gene loci have been identified.

In summary, inherited factors are well-established contributors to HL pathogenesis and might shape the microenvironment in a way that it is favorable for malignant transformation of precursor B cells and survival of HRS cells within the abundant reactive infiltrate.

Somatic Aberrations in HRS Cells

In the past decades, several recurrent gene mutations and genomic aberrations have been identified in the tumor cells of HL (Küppers et al. 2012). A large proportion of these aberrations have been linked to survival mechanisms of the tumor cells, such as gains of REL, NIK, and MAP3K14; activation of IκB (Jungnickel et al. 2000); loss of NF-KB inhibitors such as NFKBIA, TNFAIP3 (A20), and TRAF3; and mutations in TNFAIP3 (Reichel et al. 2015; Lake et al. 2009; Schmitz et al. 2009; Nomoto et al. 2012; Otto et al. 2012). All these aberrations result in constitutive activation of the NF-KB signaling pathway, which is commonly observed in HL. Moreover, gain of JAK2 (Green et al. 2010) and mutations in PTPN1 (Gunawardana et al. 2014) and SOCS1 (Mottok et al. 2007; Lennerz et al. 2015; Weniger et al. 2006) are common mechanisms leading to inappropriate activation of the JAK/STAT signaling pathway in HRS cells. Gain of JAK2 has also been identified in circulating cell-free DNA (Vandenberghe et al. 2015; Amant et al. 2015). Both pathways are either constitutively activated by somatic genomic aberrations in about half of the HL cases or in the remaining cases by autocrine or paracrine triggering of TNF or interleukin family receptors (e.g., CD30, CD40, IL-6R, IL-13) expressed on the HRS cells. Activation of these pathways results, besides directly stimulating HRS cell survival, also in induction of a tumor cell favorable microenvironment by inducing expression of cytokines.

Several reports have shown lack of expression of HLA classes I and II by HRS cells (Diepstra et al. 2008; Murray et al. 1998; Oudejans et al. 1996). The mechanisms responsible for loss of HLA have at least in part been elucidated by next-generation sequencing approaches in HL cell lines and primary tissue samples in the past few years. β2-microglobulin (B2M) gene mutations in the start codon have been identified in two of seven HL cell lines (Liu et al. 2014). These mutations resulted in loss of B2M protein and as a consequence also to loss of membranous HLA class I expression. In primary HL cases, mutations in B2M have been found in

seven out of ten cases (Reichel et al. 2015). Enforced expression of B2M in a HL cell line restored membranous HLA class I expression (Reichel et al. 2015). Thus, loss of HLA class I expression can at least in part be explained by mutations affecting B2M.

The major histocompatibility complex class II transactivator (CIITA) locus is rearranged in a HL-derived cell line leading to decreased HLA class II expression (Steidl et al. 2011). Breaks in the CIITA locus were subsequently detected in 15% of HL cases. Using whole-exome sequencing, mutations in *CIITA* were identified in two other HL cell lines (Liu et al. 2014). Mutations in CREB-binding protein (CREBBP) were observed in two of the seven HL cell lines (Liu et al. 2014). CREBBP promotes CIITA-dependent transcription including transcription of HLA class II. Consistent with this function, CREBBP mutations have been associated with a decreased antigen presentation signature including low abundance of HLA class II in follicular lymphoma (Green et al. 2015). Thus, inactivation of CIITA and possibly mutations of *CREBBP* might explain loss of HLA class II expression but do not explain complete lack of HLA class II. Another mechanism of HLA class II loss is the deletion of the HLA class II gene as can be seen in a proportion of immune-privileged site DLBCL (Riemersma et al. 2005).

CD58, also known as lymphocyte function-associated antigen-3 (LFA-3), provides a co-stimulatory signal to T cells via binding to the CD2 receptor (Dengler et al. 1992). This interaction is critical for the regulation of effector functions of T cells (Springer et al. 1987; Springer 1990). CD58 is involved in immune recognition of tumor cells by binding to the CD2 receptor expressed on CTLs. Formation of $CD4^+$ T-cell rosettes around the HRS cells was shown to be dependent on expression of CD2 on T cells and their interaction with CD58 on HL cells (Sanders et al. 1988). This suggests that CD58 expression may be necessary for tumor cell survival (Ellis et al. 1992; Jacob et al. 1999). However, CD58 is also involved in immune recognition of tumor cells by CTLs and NK cells, via binding to the CD2 receptor (Warren and Smyth 1999; Challa-Malladi et al. 2011). *CD58* mutations were found in three HL cell lines, and heterozygous loss of CD58 gene locus was found in HRS cells of 3 out of 13 primary cases (Abdul Razak et al. 2016; Schneider et al. 2015). Together these data suggest that loss of CD58 might provide escape from immune recognition, especially in advanced disease when the tumor cells have become less dependent on the reactive infiltrate and more immunogenic (Schneider et al. 2015).

Among the translocation partners of the abovementioned CIITA translocation were the genomic region of the ligands of programmed cell death 1 (CD274/PD-L1 and CD273/PD-L2) (Steidl et al. 2011). In primary mediastinal B-cell lymphoma (PMBCL), two cluster breakpoint areas have been found, one in CD274 and one in CD273 (Chong et al. 2016). A translocation involving PD-L2 and IGHV7-81 has been found in a HL cell line, and a PD-L2–CIITA translocation was detected in the NLPHL cell line DEV (Twa et al. 2014). Moreover, amplification and overexpression of PD-1L have been reported in HL cell lines and in nodular sclerosis HL cases (Roemer et al. 2016; Green et al. 2010). In a more recent study, PD-L1/2 rearrangements were found in 4 out of 200 HL cases and high-level amplification in 40 cases.

Four of these had a selective amplification of the PD-L1/2 gene region (Van Roosbroeck et al. 2016). Using low-coverage whole-genome sequencing of 500 purified HRS cells of 19 primary cases, gain of the PD-L1/2 region was identified in 8 cases (Salipante et al. 2016). Cases with lower percentages of disomic cells and thus more cells with gain amplifications of the PD-L1/2 loci showed higher PD-L1 protein expression levels (Roemer et al. 2016). Another mechanism leading to overexpression of PD-L1 shown in other cancer types, i.e., stabilization of the transcript by structural aberrations of the 3′-UTR (Kataoka et al. 2016), has not been studied in HL so far. Thus, amplification and overexpression of the PD-1 ligands are common features of HL, and this might impact antitumor immune responses in HL cases.

miRNAs Shaping the Microenvironment

HL tumor cells are characterized by an altered miRNA signature, which includes among others, high expression of miR-9, members of the miR-17~92 cluster, miR-21, and miR-155 (Gibcus et al. 2009; Van Vlierberghe et al. 2009). Many of these miRNAs are directly or indirectly linked to the NF-KB and/or JAK/STAT pathways (Ma et al. 2011; Lui et al. 2015) and might indirectly be relevant for the composition and functionality of the cells in the microenvironment. Analysis of the miRNA profile of total tissue samples indicated marked differences between EBV^+ and EBV^- HL cases, possibly reflecting differences in the microenvironment (Navarro et al. 2008). The most evident example of a miRNA shaping the microenvironment in HL is miR-9. Inhibition of miR-9 resulted in derepression of HuR levels in HL. HuR binds to AU-rich sequences present in transcripts of various cytokines and chemokines, and this results in promotion or inhibition of translation (Khabar 2010). Inhibition of HuR by siRNAs induced enhanced production of TNFα, CCL5, IL-5, and IL-6 (Leucci et al. 2012). Thus high miR-9 levels as seen in HL might be responsible for high cytokine levels produced by HRS cells via downregulating HuR protein levels.

Future Directions to Target the Microenvironment

For the design of new therapeutic strategies based on the currently known tumor cell-supporting mechanisms, it is evident that the microenvironment should be changed from a Th2-/Treg-/Tr-1-dominated environment with exhausted CTLs toward an active Th1 type of environment enhancing CTL- and NK cell-mediated antitumor activity.

To initiate an adaptive antitumor response, expression of HLA class I and class II by the HRS cells is essential. Loss of HLA expression as frequently observed in

HL is likely to reduce or eliminate the efficiency of most T-cell-enhancing treatment strategies. To gain insight in the importance of HLA, it is imperative to determine HLA expression and test its utility as a predictive marker for treatment responses. Similarly, it will be important to determine expression of immune checkpoint receptors and ligands to understand the mode of action of immune checkpoint inhibitors. Ideally, these biomarker approaches should be combined with studying functional HLA expression by tumor cells. In this context, it is tantalizing that the percentage of HL patients with a complete response upon PD-1 treatment (17%) is similar to the percentage of patients with functional expression of both HLA class I and II (15%, personal observation). The potential of immune checkpoint-related biology and HLA expression as biomarkers has to be further explored retrospectively in completed clinical trials (Ansell et al. 2015; Armand et al. 2016), but more importantly, biomarker considerations have to be firmly integrated into the design of new randomized clinical trials.

In the future, biomarker approaches might enable rational selection of patients that are likely to benefit most from these treatments, thus improving overall outcomes in HL (Fig. 4.4).

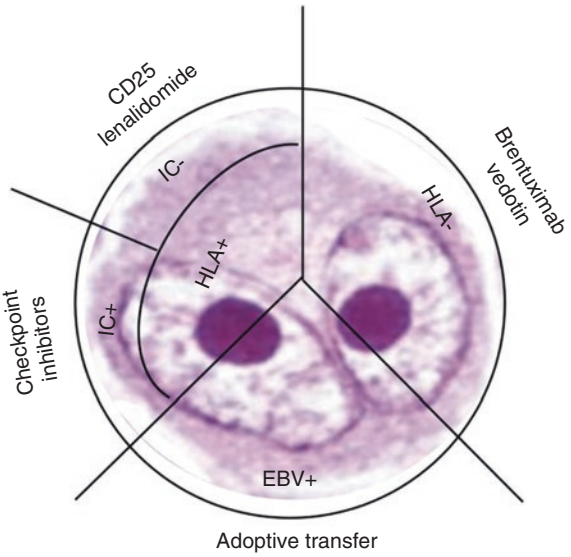

Fig. 4.4 Considerations for selection of targeted therapies. HRS cells in EBV$^+$ HL patients usually are HLA$^+$, and adoptive transfer is, although technically challenging, very effective. Therapeutic approaches for EBV$^-$ HL patients may be selected based on two subgroups, i.e., HLA$^+$ and HLA$^-$ cases. HLA$^+$ HL patients are likely to benefit most from checkpoint inhibitors if immune checkpoint proteins are present or from blocking of Tregs in combination with activation of CTLs and NK cells. HLA$^-$ HL patients may be treated best by targeting CD30 with the chemotoxic brentuximab vedotin

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Chapter 5
Role of EBV in Classical Hodgkin Lymphoma

Paul Murray and Maha Ibrahim

Introduction

The Epstein-Barr virus (EBV) is a gamma-herpesvirus that colonises the B-cell system of its human host, allowing it to persist asymptomatically in the majority of the world's adult population. In most people primary infection goes unnoticed, whereas in a minority of individuals, primary infection results in infectious mononucleosis (IM), a benign condition that almost always resolves after several weeks or months. However, EBV is also causally linked with a number of malignancies, including B-cell lymphomas, such as classical Hodgkin lymphoma (cHL). We begin by considering the evidence for EBV as a transforming virus before reviewing what is known about its contribution to the pathogenesis of cHL.

EBV Is a Transforming B Lymphotropic Virus

If peripheral blood lymphocytes from asymptomatic chronic virus carriers are cultured in vitro, then the minor fraction of EBV-infected B cells present can grow out as EBV-transformed, immortalised cell lines, known as lymphoblastoid cell lines (LCLs), but only if T lymphocytes are first depleted or their activity inhibited with drugs such as cyclosporin A (Rickinson et al. 1984). Direct infection of resting B lymphocytes with EBV derived from producer B-cell lines will also give rise to LCL.

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Publication in 1984 of the complete genome sequence of the B95.8 strain of EBV provided the opportunity not only to define the genes encoded by EBV but also to explore how each contributes to the process of in vitro B-cell transformation (Baer et al. 1984). The EBV genes are separated into the 'latent' genes, expressed in a phase of the virus life cycle in which there is no virus replication (known as latency), and the 'lytic' genes which are expressed during the virus replicative cycle ultimately leading to the assembly and release of infectious virions. The latent genes act collectively and in a coordinated fashion to drive in vitro B-cell transformation. These latent genes include six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and EBNA-LP), two latent membrane proteins (LMP1 and LMP2), the non-coding Epstein-Barr-encoded RNA (EBER1 and EBER2), and a number of viral miRNA (Kerr et al. 1992; Pfeffer 2004). The use of recombinant EBV technology has confirmed the absolute requirement for EBNA2 and LMP1 in the in vitro transformation of B cells and highlighted important contributions of EBNA-LP, EBNA3A, EBNA3C and LMP2A in this process (Young et al. 2016). The form of latency observed in LCL in which all the latent genes are expressed is usually referred to as latency III to distinguish it from other, more restricted, forms of latency observed during the different stages of the 'normal' differentiation of EBV-infected B cells and in EBV-associated malignancies, including cHL.

Asymptomatic Infection of B Cells

Before considering how EBV contributes to the pathogenesis of cHL, it is first important to briefly summarise how the virus colonises B cells in the asymptomatic host.

Although memory B cells were shown to be the major site of EBV persistence (Babcock et al. 1998), how EBV gets into the memory B-cell pool is still a matter of some debate. In one model, proposed by Thorley-Lawson, EBV infection of naïve B cells drives them to proliferate (Fig. 5.1). These EBV-infected B cells express the latency III programme and are considered to be the in vivo equivalent of in vitro transformed LCL. By processes yet undefined, these EBV-infected B cells then enter a germinal centre (GC) reaction and express an alternative form of latency, known as latency II in which there is expression of EBNA1, LMP1 and LMP2, but not of the other EBNAs (Babcock et al. 2000). LMP1 and LMP2 have been shown to provide surrogate CD40 and B-cell receptor (BCR) signals, respectively, and it is their combined expression which probably mediates the survival of EBV-infected B cells in the GC and their subsequent differentiation into memory B cells (Gires et al. 1997; Caldwell et al. 1998). To avoid recognition by the immune system, EBV-infected memory B cells shut down virus gene expression (latency 0), only occasionally switching on EBNA1 expression when they are required to proliferate (latency I) (Babcock et al. 2000). EBV-infected B cells can also differentiate into plasma cells which switch on the lytic cycle ultimately leading to the production of new virions (Laichalk and Thorley-Lawson 2004).

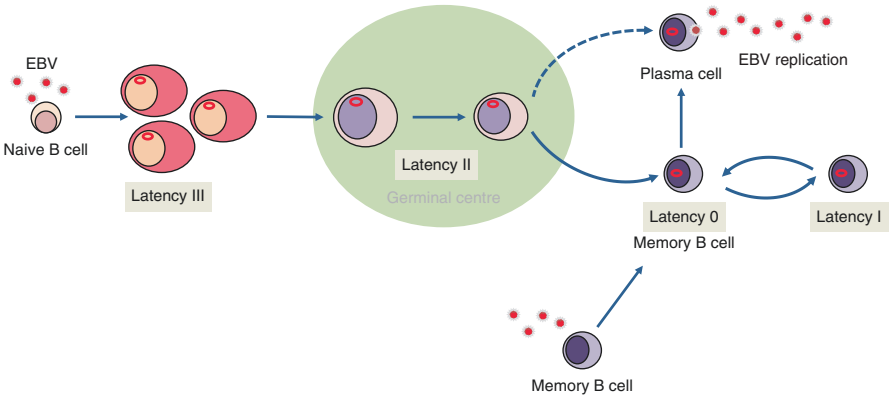

Fig. 5.1 Models of asymptomatic EBV infection. In the germinal centre model of EBV persistence, naïve B cells are infected with EBV and after a proliferative phase in which they express all latent proteins, they then enter a germinal centre, express a more restricted form of latency (latency II) and subsequently differentiate to memory or plasma cells. EBV-infected memory B cells are the site of long-term latency and persistence, whereas virus replication occurs in plasma cells. In the direct infection model, EBV directly infects memory B cells

In other studies the rare EBER-expressing cells isolated from the GCs of patients with IM have been shown to carry somatically mutated immunoglobulin genes without evidence of intra-clonal diversity (Kurth et al. 2000, 2003). In other words, these cells are apparently not undergoing SHM which might be expected if they were participating in the GC reaction. Furthermore, LMP1 has been shown to be expressed mainly by EBV-infected cells outside, but not inside, the GC (Mohamed et al. 2014). Taken together these data suggest the possibility of a different model, one in which EBV directly infects memory B cells (the direct infection model; Fig. 5.1). EBV infection is also detectable in 'non-switched' memory B cells, which unlike conventional memory B cells are apparently not dependent on GC activity, as evidenced by their presence in certain GC-null immunodeficiency states, such as XLP1 (Chaganti et al. 2008; Agematsu et al. 1998; Ma et al. 2006). EBV infection of naïve B cells has been shown to induce somatic hypermutation by inducing expression of activation-induced cytosine deaminase (AID) (Heath et al. 2012). Thus, it seems that EBV may also be able to impose a memory genotype without the requirement for a GC reaction.

Detection of EBV in cHL

The detection of raised antibody levels to EBV antigens in HL patients compared with other lymphoma patients provided the first clues that EBV might be involved in the pathogenesis of cHL (Levine et al. 1971). Furthermore, these raised levels were found to precede the development of cHL by several years (Mueller et al. 1989). In 1974,

two reports were published documenting a significantly increased risk of cHL in individuals with a prior history of IM (Connelly and Christine 1974; Rosdahl et al. 1974). Subsequently, it was shown that a prior history of either self-reported or laboratory-confirmed IM is associated with an increased risk of developing EBV-positive cHL, an association not observed for EBV-negative cHL (Hjalgrim et al. 2002, 2007).

In 1985 Sibrand Poppema detected the 'EBNA' protein in the HRS cells of a single patient using the anti-complement immune fluorescence assay; later this protein was designated EBNA1 (Poppema et al. 1985). EBV DNA was then detected in 20–25% of HL biopsies by Southern blot hybridisation (Weiss et al. 1987). In situ hybridisation for EBV DNA confirmed the existence of the EBV genome in HRS cells (Anagnostopoulos et al. 1989; Weiss et al. 1989), and later, the demonstration of the abundant expression of EBER1 and EBER2 in HRS cells provided a sensitive method for detecting latent infection in situ, a technique which is now the preferred method for detecting EBV in paraffin-embedded tissues (Wu et al. 1990). In EBV-associated cHL, the viral genomes are found in monoclonal form indicating that infection of the tumour cells occurred prior to their clonal expansion (Anagnostopoulos et al. 1989). Furthermore, EBV persists in HRS cells throughout the course of disease and in multiple sites of disease, suggesting that it is required for tumour maintenance in vivo (Coates et al. 1991).

Epidemiology of EBV-Associated cHL

The fraction of patients with cHL harbouring the EBV genome in their tumour cells varies dramatically with factors such as age, gender, histological subtype, ethnicity and country of residence (Glaser et al. 1997; Glaser and Jarrett 1996). Thus, EBV rates in cHL from North America and Europe vary between 20% and 50%, but much higher rates are observed in some underdeveloped countries (Chang et al. 1993; Weinreb et al. 1996). EBV-positive rates have also been shown to be higher in males compared with females, in Asians and Hispanics compared with whites or blacks (Glaser et al. 1997) and in South Asian children compared with non-South Asian children from the UK (Flavell et al. 2001). In developed countries, the proportion of cases with EBV is higher in older people and in children, especially in those under 10 years of age, whereas the lowest rates of EBV-positive disease are found in young adults (Armstrong et al. 1998; Jarrett et al. 1991). Thus, cHL might comprise three distinct entities: paediatric cHL (EBV-positive, mixed cellularity type), cHL of young adults (EBV-negative, nodular sclerosis type) and cHL of older adults (EBV-positive, mixed cellularity type) (Armstrong et al. 1998). EBV-positive classical cHL in older adults has been attributed to an age-related decline in EBV-specific immunity (Armstrong et al. 1998).

In contrast to some other forms of EBV-associated B-cell lymphoma, the incidence of EBV-positive cHL is only modestly increased in patients infected with the human immunodeficiency virus (HIV) (Biggar et al. 2006; Glaser et al. 2003). Furthermore, cHL occurs more commonly in HIV patients with intermediate levels

of immune impairment (Biggar et al. 2006). This is in contrast to the peak in the incidence of BL which occurs early in HIV infection when circulating CD4+ T-cell numbers are normal or only slightly decreased and the peak in the incidence of the 'immunoblastic' form of diffuse large B-cell lymphoma (DLBCL) which occurs when circulating CD4+ T cells are very low and the patient is severely immunocompromised. The incidence of cHL in HIV-positive patients has not fallen during the era of highly active antiretroviral therapy (HAART); indeed, some studies suggest cHL risk may be increased in the first few months following immune reconstitution on HAART (Bohlius et al. 2011; Kowalkowski et al. 2013; Gotti et al. 2013). These findings suggest that although defects in EBV-specific immunity contribute to the development of EBV-positive HL, CD4+ T cells have a critical role in tumour development which is lost when CD4+ T-cell numbers fall below a critical threshold. In BL, both malaria and HIV infection contribute to the risk of tumour development by promoting polyclonal B-cell activation (Rowe et al. 2014a). It is possible that an initial polyclonal B-cell activation induced by HIV might also be involved in the development of EBV-associated cHL.

There are other examples among the B-cell lymphomas in which chronic inflammation is believed to be involved in tumour development. For example, 'DLBCL associated with chronic inflammation' are EBV-positive tumours that occur in the context of long-standing chronic inflammation and are derived from late germinal centre or post-germinal centre B cells. They usually present as a tumour mass involving body cavities. Pyrothorax-associated lymphoma (PAL) is the prototypic form. PAL is associated with a history of chronic pyrothorax or chronic pleuritis due to the initiation of a therapeutic artificial pneumothorax for pleuropulmonary tuberculosis, which was used in the past as a surgical treatment for tuberculosis (Loong et al. 2010). Other EBV-positive DLBCL associated with chronic inflammation with similar features to PAL include those associated with metallic implants in bones and joints (Sanchez-Gonzalez et al. 2013) and chronic osteomyelitis or chronic venous ulcers (Cheuk et al. 2005; Copie-Bergman et al. 1997).

The incidence of EBV-positive cHL is dramatically increased in autoimmune lymphoproliferative syndrome (ALPS), providing another potential example in which a chronic immune stimulus could contribute to cHL development (Price et al. 2014). Most ALPS patients inherit mutations in the FAS gene leading to defective apoptosis and to higher than normal numbers of immature CD4-/CD8-, so-called 'double negative', T cells. These T cells are known to stimulate B-cell proliferation in ALPS patients which in turn may increase the risk of cHL development (McClain 2014).

Contribution of EBV Latent Genes to the Pathogenesis of cHL

EBV-infected HRS cells have been shown to express a restricted pattern of virus latency in which EBNA1 and the two latent membrane proteins are expressed (Fig. 5.2). Many of the studies that have attempted to unravel the contribution of EBV to the pathogenesis of cHL have focussed on these three virus proteins.

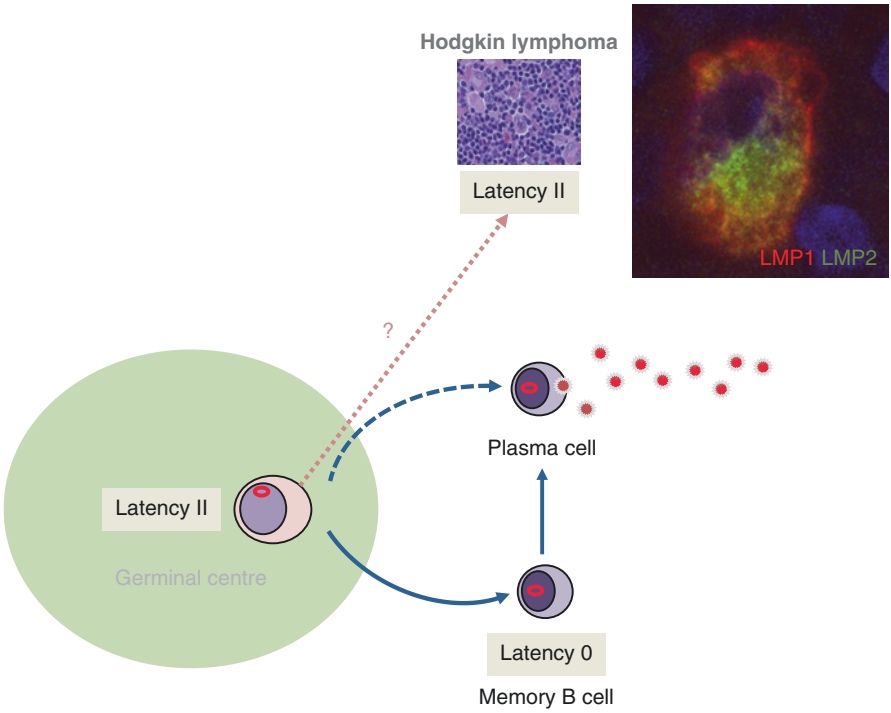

Fig. 5.2 The origin of EBV-positive HRS cells. The precise stage of B-cell differentiation from which HRS cells are derived is not known, but they show evidence of somatic hypermutation and therefore are likely to originate from germinal centre or post-germinal centre B cells. The observation that HRS cells express the latency II form of EBV infection in viral-positive cases has been used as additional evidence of their GC origin. HRS cells show expression of both LMP1 and LMP2, shown here in the same HRS cell by double labelling (LMP1 *red*, LMP2, *green*)

Epstein-Barr Virus Nuclear Antigen-1 (EBNA1)

EBNA1 is necessary for the maintenance of EBV infection as it acts as a viral replication factor and tethers the viral genome to the chromosomes of daughter cells during cell division (Westhoff Smith and Sugden 2013). In addition to its genome maintenance function, EBNA1 is also a transcription factor that regulates both viral and cellular gene expression (Frappier 2012a, b, c). EBNA1 has been shown to inhibit TGFβ signalling, in part through increasing the turnover of SMAD2 (Wood et al. 2007). EBNA1 also promotes the growth and survival of cHL cells by downregulating the TGFβ target gene, PTPRK (Flavell et al. 2008). EBNA1 increases expression of the T-cell chemokine CCL20 which may dampen the immune response to HRS cells by recruiting regulatory T cells to the tumour microenvironment (Baumforth et al. 2008).

Latent Membrane Protein-1

LMP1 is a member of the tumour necrosis factor receptor (TNFR) superfamily and functions as a constitutively active homologue of the cellular CD40 receptor (Lam and Sugden 2003). LMP1 can activate multiple cell signalling pathways that are known to be aberrantly activated in HRS cells. These include the NF-κB, JAK/STAT, AP-1 and phospatidylinositol-3 kinase (PI3K)/AKT pathways (Heath et al. 2012; Bargou et al. 1997; Dutton et al. 2005; Holtick et al. 2005). Indeed, it has been suggested that in EBV-positive HRS cells, LMP-1 might be sufficient to drive these pathways in the absence of cellular mutations. For example, mutations that inactivate the negative regulator of NF-κB, TNFAIP3 are almost always only seen in EBV-negative cHL (Schumacher et al. 2010). Likewise, the aberrant expression of several receptor tyrosine kinases (RTKs) is restricted mainly to EBV-negative cHL (Renne et al. 2007). These observations suggest that EBV and cellular mutations in critical signalling pathways might provide mutually exclusive means to the same pathogenic end point. In keeping with this, it has been shown that compared with EBV-negative tumours, EBV-positive cHL has significantly fewer chromosomal breakpoints and aneuploid autosomes (Montgomery et al. 2016).

The importance of LMP1 in cHL pathogenesis is further emphasised by the finding that LMP1 expression in primary GC cells, the presumed progenitors of cHL, induces many of the changes characteristic of HRS cells. These changes include the downregulation of B-cell transcription factors and BCR signalling components required to maintain B-cell identity and the up-regulation of survival genes such as BCL2 and BFL-1 that protect B cells from apoptosis (Henderson et al. 1991; Vockerodt et al. 2008). LMP1 also induces the expression of FLICE-inhibitory protein (c-FLIP), a negative regulator of Fas-induced apoptosis (Cahir-McFarland et al. 2004), suggesting that one of its major pathogenic roles could be to rescue pre-apoptotic GC B cells from cell death.

Some of the transcriptional changes induced by LMP1 may be mediated through the induction of ID2 (Vockerodt et al. 2008), while others may occur through epigenetic mechanisms involving DNA methyltransferases and protein arginine methyltransferases (Anderton et al. 2011; Leonard et al. 2011, 2012). LMP1 can also regulate cellular gene expression by modifying the H3K27me3 histone mark through poly (ADP-ribose) polymerase 1 (PARP1) activation (Martin et al. 2016). LMP1 can also affect gene transcription and subsequent B-cell lymphomagenesis through the activation of cellular miRNAs, for example, by regulating miR-10b, miR-29b, miR-146a and miR-155 (Motsch et al. 2007). LMP1 might also contribute to the characteristic morphological features of HRS cells. For example, LMP1 downregulates expression of the shelterin proteins RF1, TRF2 and POT1 leading to the formation of multinucleated HRS-like cells (Lajoie et al. 2015).

Latent Membrane Protein-2 (LMP2)

LMP2 exists as two isoforms, LMP2A and LMP2B, which share eight common coding exons but have different 5′ exons. While the 5′ exon of LMP2B is non-coding, the unique N-terminus of LMP2A includes an ITAM motif, which resembles the signalling domain of the BCR. Indeed, in transgenic mice, LMP2A has been shown to function as a BCR mimic, allowing B-cell development in the absence of normal signalling through the BCR (Caldwell et al. 1998; Merchant et al. 2001). In doing so LMP2A engages signalling pathways important for B-cell survival, including the RAS/PI3K/AKT pathway which is implicated in HRS cell survival (Fukuda and Longnecker 2007). Global gene expression studies show that LMP2A can suppress expression of numerous B-cell transcription factors, including EBF1 and E2A, recapitulating many of the gene expression changes seen in HRS cells (Portis et al. 2003; Portis and Longnecker 2003, 2004; Vockerodt et al. 2013). LMP2A also activates the Notch pathway, which might contribute to the loss of B-cell identity in HRS cells (Anderson and Longnecker 2009). However, it should be noted that many of the adaptor molecules necessary for both BCR and LMP2A signalling are absent in HRS cells, and therefore it remains unclear whether LMP2A acts as a BCR surrogate in the context of cHL. One possible explanation for this paradox is that LMP2A provides essential survival signals in the HRS progenitor cells, which retain the downstream BCR signalling molecules, but subsequent transformation events followed by downregulation of B-cell-specific genes replace some of these critical LMP2A functions. However, given that EBV-positive cases of cHL are consistently LMP2A positive, it is likely that LMP2A also has BCR-independent functions that are important for maintenance of the HRS phenotype.

Whereas LMP2A has been shown to induce autoimmunity in transgenic mice (Chang et al. 2012), LMP1 expression in the B cells of transgenic mice can lead to lymphoma development (Kulwichit et al. 1998; Zhang et al. 2012). However, the combined expression of LMP1 and LMP2A in transgenic mice resulted in no significant B-cell abnormalities (Vrazo et al. 2012), suggesting that LMP2A might have tumour suppressor functions. Recently it was shown that LMP1 was dispensable for EBV-induced lymphoma formation in cord blood-humanised mice and that deletion of LMP2A delayed the onset of lymphoma in this model (Ma et al. 2017). Plasma cell differentiation was inhibited even in the absence of LMP1 and LMP2 suggesting that this was important for EBV-induced lymphomagenesis. The apparent contradictory findings from these animal models underscore the need to develop better in vivo models of EBV-associated lymphomagenesis.

Suppression of the EBV Lytic Cycle as a Potential Pathogenic Event in EBV-Positive cHL

Productive EBV infection is associated with the temporal expression of immediate early, early and late viral genes leading ultimately to the production of new virus particles. The switch to virus replication from latency is mediated by two distinct

mechanisms. The first occurs via plasma cell differentiation, the second through the activation of the BCR. Given that the full replicative cycle of EBV leads to cell death, suppression of both routes to virus replication is probably necessary for transformation.

Plasma cell differentiation is dependent on the temporal expression of transcription factors which coordinately induce the transcription of genes required for terminal B-cell differentiation while switching off the germinal centre B-cell programme. A key transcription factor is BLIMP1, which is required for plasma cell differentiation and which can activate the EBV immediate early genes BZLF1 and BRLF1, thus providing a mechanistic link between plasma cell differentiation and EBV reactivation (Reusch et al. 2015). LMP1 has been shown to suppress plasma cell differentiation by inhibiting BLIMP1α expression. EBV can also induce expression of the dominant negative BLIMP1 isoform, BLIMP1β (Vrzalikova et al. 2011, 2012). Thus, disruption of the normal functions of BLIMP1α is likely to be important for the pathogenesis of cHL, preventing not only the terminal differentiation of the tumour cell progenitors but also virus replication. It should be noted that in virus-negative B-cell lymphomas, BLIMP1 can be inactivated by other mechanisms, for example, by mutation (Mandelbaum et al. 2010; Pasqualucci et al. 2006).

The second route to EBV replication occurs via BCR activation. As described elsewhere, EBV-positive HRS cells frequently have non-functional immunoglobulin genes and lack expression of critical downstream molecules of the BCR signalling pathway. The loss of a functional BCR is likely to be an important event in cHL development because it could also prevent entry into the replicative cycle. Almost all cases of cHL bearing destructive immunoglobulin gene mutations are EBV-positive (Brauninger et al. 2006). These data suggest that progenitor cells carrying such mutations can only survive if infected with EBV. Further support for this contention is provided by the observation that EBV can immortalise BCR-negative germinal centre B cells in vitro (Mancao et al. 2005; Chaganti et al. 2005; Bechtel et al. 2005). Importantly, while LMP2A can still induce lytic cycle entry in the absence of a functional BCR, it cannot do so when these downstream BCR components are missing (Vockerodt et al. 2013). Thus, the loss of BCR, as well as of BCR signalling components could prevent both BCR- and LMP2A-induced virus replication.

EBV and the cHL Microenvironment

The non-tumour stroma of cHL is composed of T-cells, B-cells, macrophages, mast cells, eosinophils and fibroblasts, the composition of which appears to be important for patient outcomes (Sanchez-Aguilera et al. 2006; Chetaille et al. 2009; Steidl et al. 2010, 2011a). HRS cells can modify this reactive microenvironment by attracting certain cell types. For example, HRS cells secrete multiple chemokines such as CCL5 (RANTES), CCL17 (TARC), CCL20 and CCL22, which can recruit Th2 helper and FoxP3+ regulatory T cells (Aldinucci et al. 2016; Fischer et al. 2003; Skinnider et al. 2001; Skinnider and Mak 2002). HRS cells also activate fibroblasts to produce CCL11 (eotaxin) and CCL5 which further contribute to the attraction of eosinophils and regulatory T cells (Buri et al. 2001; Jundt et al. 1999). In addition,

HRS cells secrete multiple cytokines including IL5, IL9 and IL13, which influence the recruitment and proliferation of cells in the tumour infiltrate. In EBV-positive cases of cHL, LMP1 contributes to the recruitment and the modification of the tumour microenvironment by stimulating the production of many of these chemokines and cytokines in infected HRS cells (Sueur et al. 2016; Kis et al. 2006; Dukers et al. 2000).

While the tumour microenvironment supports the growth and survival of the HRS cells, it also contributes to the suppression of the host anti-EBV-specific immune responses. Immune evasion is particularly important in the context of EBV-positive cHL, since virus-encoded LMP1 and LMP2A proteins are targets for CD8+ cytotoxic T lymphocytes (CTLs) (Rickinson and Moss 1997). In vitro, cHL cells can process and present epitopes from LMP1 and LMP2A in the context of multiple class I alleles and are sensitive to lysis by EBV-specific CTLs (Khanna et al. 1998; Lee et al. 1993).

In this regard, regulatory T cells (Tregs), attracted by high levels of chemokines including CCL17, CCL20 and CCL22, can inhibit the activity of infiltrating effector CD4+ T cells (Baumforth et al. 2008). EBV-positive cHL also appears to be preferentially infiltrated by regulatory Type 1 cells (Tr1) which express ITGA2, ITGB2 and LAG3 and secrete IL-10 (Morales et al. 2014). Although the infiltrate in EBV-positive cHL is enriched for different subpopulations of Th2 and Treg cells, other studies indicate the presence of a functional Th1 cell infiltrate (Greaves et al. 2013). In fact, it was demonstrated some years ago that infiltrating activated CD8+ T cells are more frequent in EBV-positive cHL and are associated with significantly poorer disease outcomes (Oudejans et al. 1996, 1997). The observation that CD4+ Tregs surround HRS cells, whereas activated CD8+ T lymphocytes do not, may provide a partial explanation for the lack of effective CTL responses in the tumour tissues of cHL (Wu et al. 2016).

EBV-positive cHL has more CD68- and CD163-expressing tumour-associated macrophages (TAMs) than EBV-negative cHL (Barros et al. 2012). Macrophage numbers are also strongly associated with inferior survival in newly diagnosed cHL patients, both in those treated with standard chemotherapy and in those having received autologous stem cell transplant (Tan et al. 2012). Gene expression profiling of the tumour tissues of newly diagnosed cHL patients also showed that a gene expression signature of macrophage infiltration was associated with poor prognosis, a finding which was validated using immunohistochemistry to detect TAMs in an independent patient cohort (Steidl et al. 2010). Another study in paediatric cHL showed that those patients with a higher frequency of tumour-associated M1-like macrophages (defined as CD163 + pSTAT1+) had better overall survival, whereas patients whose tumours were infiltrated with higher numbers of M2-like macrophages (CD163+CMAF+) had significantly worse progression-free survival (Barros et al. 2015). The potential tumour-promoting functions of TAMs may also be explained by their ability to induce angiogenesis mediated by the secretion of soluble angiogenic factors, including VEGF (Mantovani et al. 2006; Murdoch et al. 2008). CD68-positive and CD163-positive TAMs are associated with increased micro-vessel density (MVD) in cHL (Panico et al. 2013; Koh et al. 2014), and MVD

correlates with poor outcome in cHL patients (Korkolopoulou et al. 2005; Mainou-Fowler et al. 2006; Doussis-Anagnostopoulou et al. 2002).

T-cell effector functions in cHL can be abrogated by the engagement of programmed cell death ligand-1 (PD-L1) expressed on HRS cells with its receptor, PD-1, on T cells resulting in functional exhaustion of the T cells (Muenst et al. 2009; Yamamoto et al. 2008). Amplification of the PD-1L1 and PD-1L2 genes at 9p24 is common in nodular sclerosis cHL (Green et al. 2010), while the PD-1L1 gene can also be deregulated in a proportion of cHL cases as a result of a reciprocal translocation that involves CIITA (Steidl et al. 2011b). LMP1 has also been reported to induce PD-L1 expression (Green et al. 2012). The importance of this pathway in the microenvironment of cHL is exemplified by studies which show that many patients with relapsed or refractory cHL respond to PD-1 blockade therapy (Ansell et al. 2015).

Loss of HLA expression is frequently observed in cHL and is another important mechanism that enables the tumour cells to avoid the host immune response. HLA class I loss is more common in EBV-negative cases of cHL (Oudejans et al. 1996; Lee et al. 1998; Murray et al. 1998). Recently, it was shown that most cases of nodular sclerosis HL carry inactivating mutations in the beta-2-microglobulin gene that lead to loss of MHC class I expression, providing a potential mechanistic explanation for the absence of MHC class I in EBV-negative cHL (Reichel et al. 2015). In contrast, EBV-positive cases of cHL express normal or even elevated levels of HLA expression and contain more activated CTLs than EBV-negative cases (Oudejans et al. 1996, 1997; Lee et al. 1998; Murray et al. 1998). These data suggest that EBV must exploit additional mechanisms to avoid recognition by CTLs, for example, by inducing the immune checkpoint ligands described above.

A three- to ninefold increased risk of developing cHL has been reported for first degree relatives of patients (Crump et al. 2012a, b), and a 100-fold increase for monozygotic twins compared with dizygotic twins (Mack et al. 1995). Genetic association studies based on segregation and linkage analysis in families have identified susceptibility loci in the human leukocyte antigen (HLA) region (Kushekhar et al. 2014; Hjalgrim et al. 2010). Thus, an increased and decreased risk of EBV-positive cHL is associated with HLA-A*01 and HLA-A*02 alleles, respectively (Hjalgrim et al. 2010; Niens et al. 2006, 2007). These data have led to an immunological model for the development of EBV-positive cHL in which the levels of circulating EBV-infected lymphocytes regulated by cytotoxic T-cell responses is a critical determinant of disease risk (Farrell and Jarrett 2011).

As well as providing growth-promoting and immunosuppressive functions, the microenvironment of cHL also plays an important role in modulating virus gene expression in HRS cells. For example, LMP1 expression in HRS cells is driven from an alternative STAT-responsive promoter which is stimulated by IL-4, IL-10, IL-13 and IL-21 (Kis et al. 2006, 2011). This provides a mechanism for LMP1 expression in the absence of EBNA2, which is a hallmark of the typical type II latency pattern seen in HRS cells. Activated Notch also inhibits EBV entry into the lytic cycle in lymphoma cells by up-regulating the cellular transcription factor Zeb2, which represses the transcription of BZLF1 (Rowe et al. 2014b). There is also

emerging evidence that the microenvironment can modulate EBV gene functions. Thus, LMP1 induces expression of the discoidin domain receptor 1 (DDR1), a potentially oncogenic receptor tyrosine kinase. However, DDR1 is normally only active in the presence of its ligand, collagen, which is a major constituent of the cHL microenvironment (Cader et al. 2013). Notably, ligation of DDR1 by collagen promotes the survival of lymphoma cells in vitro (Cader et al. 2013). These observations suggest that some of LMP1's oncogenic effects may be dependent on the tumour microenvironment.

Conclusions

A proportion of patients with cHL harbour EBV within their tumour cells. Emerging evidence suggests that while EBV is able to subvert cellular processes to promote the growth and survival of HRS cells or their progenitors, mutations in key cell signalling pathways are probably required to do this when EBV is absent. The challenge is to unravel exactly how EBV and its latent genes contribute to the pathogenesis of cHL particularly with respect to how the virus co-operates with cellular genetic and epigenetic changes to drive transformation. It is hoped that the development of better in vitro and in vivo models of disease will reveal more fundamental aspects of EBV's role in HL pathogenesis and pave the way for targeted therapies for patients with EBV-positive cHL.

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Chapter 6
Pathobiology of Nodular Lymphocyte Predominant Hodgkin Lymphoma

Sylvia Hartmann and Martin-Leo Hansmann

Abbreviations

CGH	Comparative genomic hybridization
cHL	Classical Hodgkin lymphoma
DLBCL	Diffuse large B-cell lymphoma
HLA	Human leukocyte antigen
NF-kappaB	Nuclear factor kappa B
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
PTGC	Progressive transformation of germinal centers
THRLBCL	T cell/histiocyte rich large B-cell lymphoma

History

Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) was first recognized as a special subtype of Hodgkin lymphoma (HL) in the classification by Jackson and Parker in 1947 (Jackson and Parker 1947) and termed lymphocytic and histiocytic variant by Lukes and Butler in 1966 (Lukes and Butler 1966). Lennert distinguished already in 1974 (Lennert and Mohri 1974) between a nodular and a diffuse type of NLPHL. The tumor cells of NLPHL were previously called L and H cells, according to the lymphocytic and histiocytic appearance of the infiltrate. In the WHO classification of 2008 (Swerdlow et al. 2008) their name was revised to "lymphocyte predominant" or LP cells.

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Clinical Characteristics

NLPHL has a predilection for the male gender, which is affected in about 75% of cases (Anagnostopoulos et al. 2000; Jackson et al. 2010). Male gender does not only represent an increased risk for the development of NLPHL but bears furthermore an even sixfold higher risk for relapse in diseased patients (Hartmann et al. 2013a). Usually, NLPHL affects middle-aged patients around 40 years. However, the age range is broad, including pediatric patients from approximately the age of 8 years up to elderly persons. In most cases, NLPHL is diagnosed in early stage, usually stage I or II (Jackson et al. 2010). Axillary and cervical lymph nodes are most frequently affected. Only a small subgroup of patients presents with advanced disease. These patients have often liver and spleen involvement. Although NLPHL is generally an indolently behaving lymphoma, relapses are much more frequently observed than in classical HL (cHL) (Anagnostopoulos et al. 2000). These can occur after a long latency of about 10 years. Some patients even present with multiple relapses, and in long standing disease, there is an important risk of transformation into an aggressive diffuse large B-cell lymphoma (DLBCL) (Biasoli et al. 2010; Al-Mansour et al. 2010), which can be fatal. The histological criteria when to diagnose transformation are not well defined, and small sheets of blasts do not seem to impact the clinical outcome (Hartmann et al. 2013a). Due to this reason, the outcome of patients with NLPHL and transformation into DLBCL is heterogeneous (Biasoli et al. 2010; Al-Mansour et al. 2010; Huang et al. 2004; Sundeen et al. 1988; Hansmann et al. 1989; Hartmann et al. 2014a).

Pathology

Growth Patterns

NLPHL can generally be divided into cases with nodular and diffuse growth patterns (Fig. 6.1a and b), (Hansmann et al. 1991; Boudova et al. 2003). Apart from their growth pattern, these also differ in the composition of the microenvironment. Cases with a

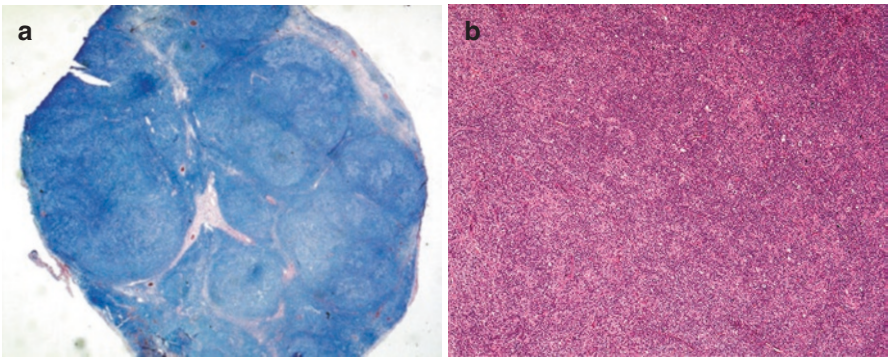

Fig. 6.1 (**a**) NLPHL with a typical nodular growth pattern. Giemsa stain, 5×. (**b**) NLPHL with a predominant diffuse growth pattern. HE, 10×

predominant nodular pattern usually have a high content of reactive B cells, whereas in cases with a predominant diffuse growth pattern, ill-defined follicular dendritic cell meshworks in a T-cell-rich background can be observed (Hansmann et al. 1991). In 2003 a minute analysis of the different growth patterns and their combination was performed by Fan et al. (2003). Six different growth patterns were described (Fig. 6.2) including nodular patterns with a T-cell-rich background as well as a rare purely diffuse

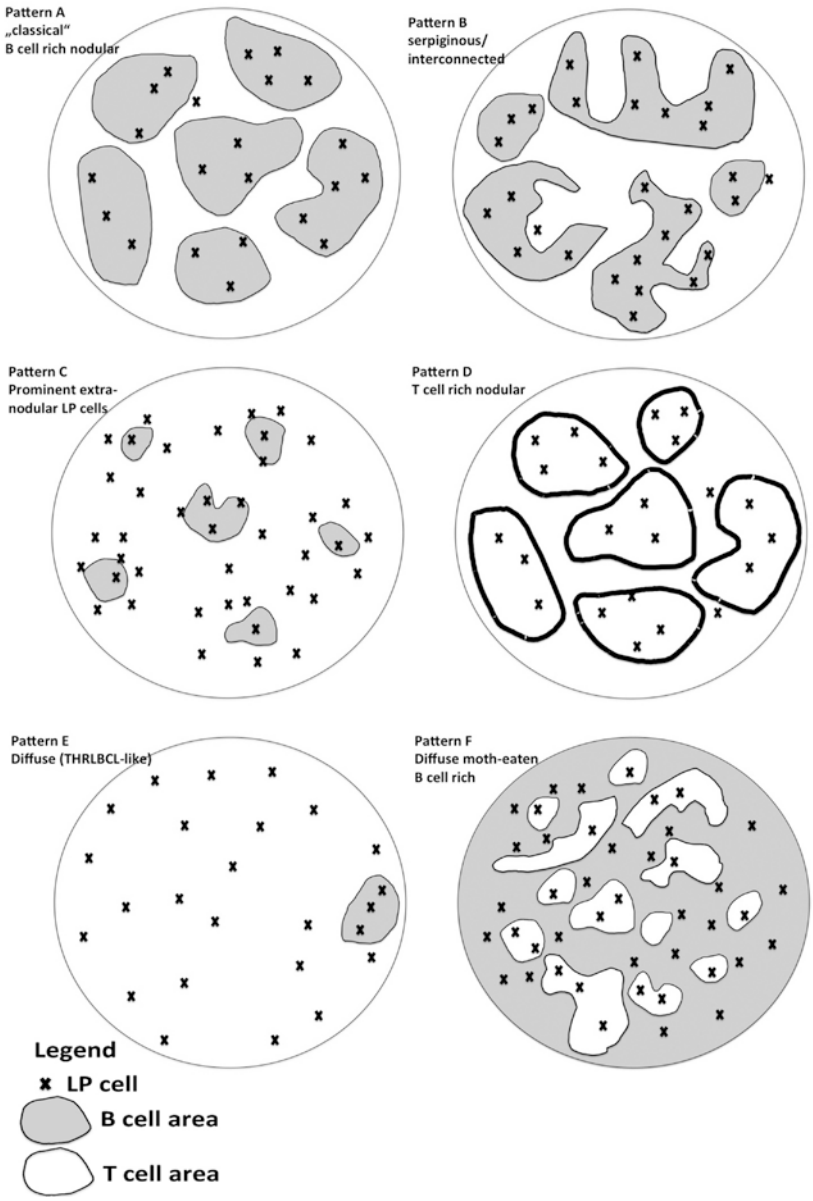

Fig. 6.2 Schematic representation of NLPHL growth patterns (**a**–**f**), modified after Fan et al. (2003)

growth pattern with a B-cell-rich background. It was furthermore noted that the cases with a purely diffuse growth pattern and morphologic features resembling T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL), i.e., diffusely distributed scattered large blasts in a background poor of B cells, had a higher risk of recurrence. This observation was confirmed in a large study involving 413 NLPHL patients from the German Hodgkin Study Group (Hartmann et al. 2013a), in which the cases with an atypical growth pattern presented significantly more frequently with an advanced clinical stage and a higher relapse rate. In some cases, the growth pattern can very closely resemble THRLBCL, and the revised WHO classification from 2016 (Swerdlow et al. 2016) suggests to label these cases NLPHL with THRLBCL-like transformation.

Tumor Cells: The LP Cells

Although the growth pattern in NLPHL can vary, the LP cells, the tumor cells in NLPHL, almost always consist of mononucleated large blasts with folded, popcorn-like nuclei and usually one nucleolus (Fig. 6.3). The cytoplasm is usually a small rim. Tumor cells resembling classical Hodgkin-Reed-Sternberg (HRS) cells can only rarely be found in NLPHL. LP cells generally show a scattered distribution in the tissue with hot spot areas in the nodules of the typical nodular variants. LP cells derive from germinal center B cells and usually show a preserved B-cell phenotype, which can be slightly downregulated. They are positive for CD20, CD79a, OCT2,

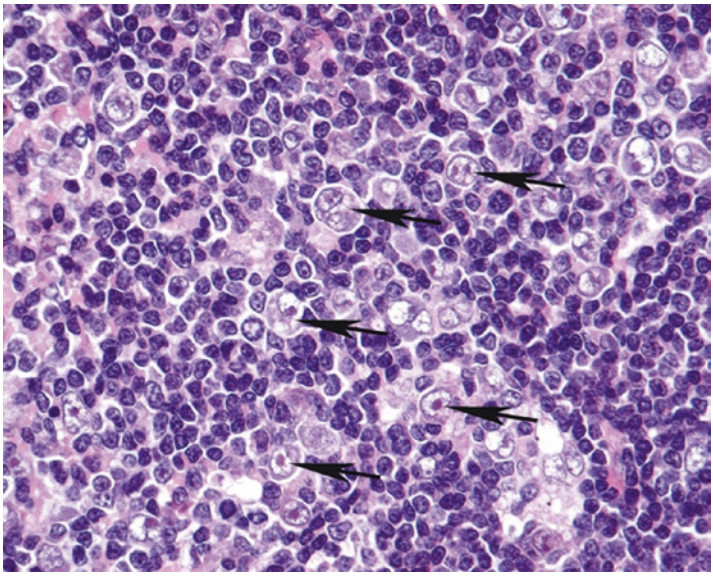

Fig. 6.3 LP cells represent mononucleated large blasts with folded, popcorn-like nuclei and usually one nucleolus (*arrows*). HE, 40×

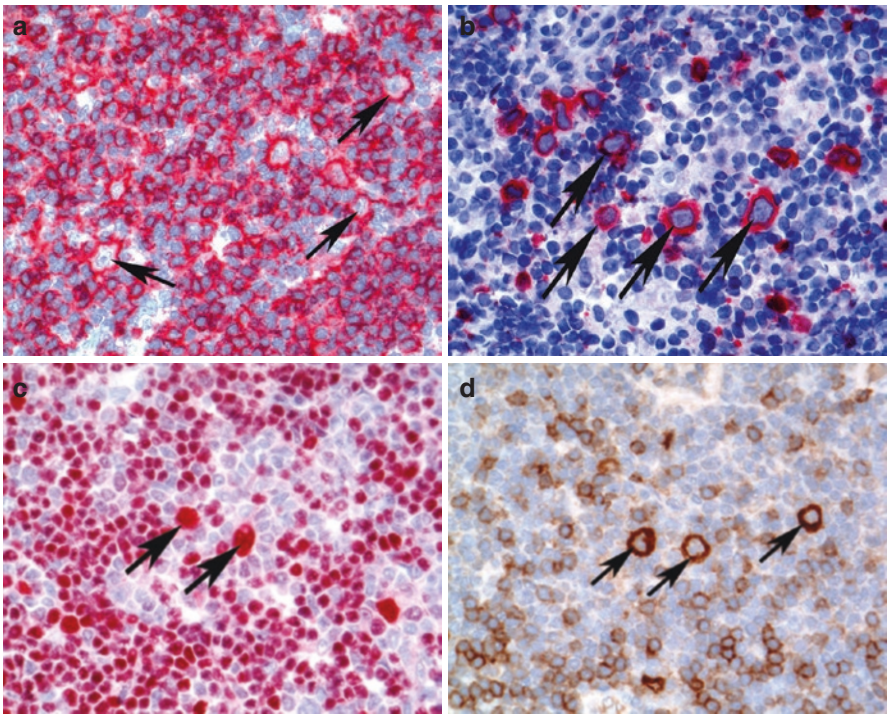

Fig. 6.4 LP cells (*arrows*) are positive for B-cell antigens, including (**a**) CD20, (**b**) CD79a, and (**c**) OCT2 as well as (**d**) IgD. Magnification 40× each

PAX5, BOB.1 (Fig. 6.4), and the germinal center markers BCL6, HGAL, and CD75 (Anagnostopoulos et al. 2000; Natkunam et al. 2005; Carbone and Gloghini 2014; Kraus and Haley 2000). LP cells are furthermore positive for J chain (Stein et al. 1986) and EMA (Delsol et al. 1984) in a fraction of cases (Table 6.1). CD19 expression, which is found by almost all reactive B cells, is lost in the LP cells in more than half of the cases (Nathwani et al. 2013). A subset of NLPHL cases expresses IgD in the LP cells (Fig. 6.4) (Prakash et al. 2006). These are often pediatric male patients (Prakash et al. 2006; Huppmann et al. 2014). Furthermore, LP cells express the immunoglobulin kappa light chains more frequently than lambda light chains (Schmid et al. 1991). Infection by the Epstein-Barr virus (EBV) is only very rarely observed in LP cells (Anagnostopoulos et al. 2000; Huppmann et al. 2014).

Differential Diagnoses

Differential diagnoses to NLPHL include all kinds of lymphomas which present with scattered single B-cell blasts. This includes on the one hand cHL with CD20 expression, which occurs in rare cases. However, in these cases usually CD30 is

Table 6.1 Immunophenotype of LP cells

Antigen	Immunophenotype of LP cells
CD20	++/+
CD79a	++/+
CD19	+/−
OCT2	+++
PAX5	++/+
BOB.1	++/+
CD30	− (+)
CD15	− (+)
IgD	+/−
CD75	++/+
BCL6	+++
EMA	+/−
J chain	+/−
CD10	− (+)
BCL2	−/+
HGAL	++/+
MUM1	+/−

much stronger expressed than CD20, and additionally CD15 is positive. Despite the fact that CD20 can be expressed by HRS cells, the expression intensity is mostly weak, and other B-cell antigens are negative in HRS cells of cHL. Both CD15 and CD30 can be expressed in rare cases of NLPHL, but usually they are not coexpressed (Hartmann et al. 2014a). Moreover, EBV is present in the HRS cells of cHL in around 30–40% of the cases in the Western world. This, however, depends very much on the country of origin, and EBV infection of HRS cells is more frequently encountered in developing nations. Other lymphomas that can be a differential diagnosis to NLPHL are all kinds of nodal peripheral T-cell lymphomas with Hodgkin-like cells (Quintanilla-Martinez et al. 1999; Moroch et al. 2012). Hodgkin-like cells in peripheral T-cell lymphomas usually do not belong to the T-cell lymphoma clone and are frequently EBV-infected B cells with a preserved B-cell phenotype and CD30 expression. Frequently the background infiltrate of nodal peripheral T-cell lymphomas can contain nodular areas of reactive B cells, resembling atypical variants of NLPHL. However, the most striking difference to NLPHL is the aberrant immunophenotype of the T cells, usually of T-helper cell origin and the clonality of the T cells, which helps to confirm an underlying T-cell neoplasia.

Another differential diagnosis to NLPHL is the progressive transformation of germinal centers (PTGC) (Poppema et al. 1979; Hansmann et al. 1990). Like in NLPHL, large nodular areas composed of naive B cells can occur in lymph nodes with PTGC. However, the important difference to NLPHL is that within these nodules still germinal center residues of variable size exist, which are completely

destroyed in NLPHL. Moreover, although in PTGC scattered centroblasts can be found within germinal center residues, LP cells surrounded by rosetting T cells do not occur. PTGC follows exact morphological patterns (Hartmann et al. 2015a). Knowledge of these patterns can help to distinguish PTGC from NLPHL. However, in every lymph node with PTGC, a close workup is necessary, since sometimes NLPHL can coexist with PTGC in the same lymph node.

Cellular Origin

Applying single-cell PCR from micromanipulated LP cells, it was shown that LP cells are clonal and that they have ongoing somatic hypermutation of their immunoglobulin genes (Braeuninger et al. 1997). LP cells furthermore present an aberrant somatic hypermutation of the genes *PIM1*, *PAX5*, *RhoH/TTF*, and *MYC* (Liso et al. 2006) which are also mutated in germinal center B cells. Some of these mutations show intraclonal diversity in the LP cells, consistent with ongoing aberrant somatic hypermutation. All these data suggest that LP cells derive from germinal center B cells. This is furthermore consistent with the observed expression of germinal center B-cell markers in the LP cells. Interestingly, also in THRLBCL the tumor cells have clonal immunoglobulin gene rearrangements and show ongoing somatic hypermutation (Bräuninger et al. 1999), further supporting the close relationship between NLPHL and THRLBCL. When microdissected LP cells were investigated by gene expression profiling (Brune et al. 2008), they showed a similar degree of relationship to germinal center B cells and memory B cells, suggesting that they resemble an intermediate developmental stage in the transition between germinal center and memory B cells.

NLPHL Cell Line DEV

The NLPHL cell line DEV was established in 1985 by Poppema et al. (1985). Originally, it was assumed to be derived from cHL and only later reclassified as NLPHL (Poppema et al. 1989). The DEV cell line expresses B-cell antigens like CD20 and CD19 but is additionally positive for CD30. Moreover, it has an alternative *BCL6* break and complex translocations involving chromosome 3 (Atayar et al. 2006). It furthermore displays a mutation in the start codon of the *B2M* gene, resulting in very low levels of B2M expression (Liu et al. 2014). DEV is negative for both human leukocyte antigens (HLA) classes I and II due to complex genomic rearrangements, including the gene locus of the HLA class II transactivator gene *CIITA* (Liu et al. 2014; Mottok et al. 2015).

Deregulated Transcription Factor Networks and Signaling Pathways

Primary LP cells have an active JAK-STAT signaling related to frequent mutations in *SOCS1* (Mottok et al. 2007), which is a negative regulator of *JAKs*. Mutations in *SOCS1* were usually found in motifs of somatic hypermutation, and they presented intraclonal diversity, in line with ongoing somatic hypermutation and the germinal center B-cell derivation of LP cells. JAK2 is phosphorylated in approximately 39% of NLPHL cases, whereas phosphorylation of STAT6 occurrs in 49% of NLPHL (Mottok et al. 2009). In contrast, phosphorylation of STAT3 and STAT5 was not seen in LP cells. It was furthermore observed by gene expression profiling that LP cells have a constitutive active NF-kappaB signaling, and a subset of cases shows activation of ERK (Brune et al. 2008). NF-kappaB activity in NLPHL is usually not related to mutations in *NFKBIA* or *TNFAIP3*, which are frequent in cHL (Schumacher et al. 2010).

Genetic Lesions

A variety of genetic lesions has been observed in NLPHL (Table 6.2). LP cells are always strongly positive for BCL6, and translocations involving both the *BCL6* locus and the immunoglobulin loci have been identified in up to 30% of NLPHL (Wlodarska et al. 2003; Renné et al. 2005). Additional NLPHL carries *BCL6* translocations with diverse non-Ig locus partners (Wlodarska et al. 2003). By classic comparative genomic hybridization (CGH), a high number of genomic imbalances were detected both in NLPHL and THRLBCL (Franke et al. 2001, 2002).

Table 6.2 Genetic lesions observed in LP cells

Gene	Type of lesion	References
SOCS1	Point mutations/deletions	Mottok et al. (2007) and Hartmann et al. (2016)
BCL6	Translocations	Wlodarska et al. (2003) and Renné et al. (2005)
MYC	Point mutations	Liso et al. (2006) and Hartmann et al. (2016)
PIM1	Point mutations	Liso et al. (2006)
PAX5	Point mutations	Liso et al. (2006)
RhoH/TTF	Point mutations	Liso et al. (2006)
B2M	Point mutations	Liu et al. (2014) (DEV cell line)
CIITA	Translocations	Mottok et al. (2015) and Hartmann et al. (2016)
REL	Copy number gains	Hartmann et al. (2015b)
SGK1	Point mutations/deletions	Hartmann et al. (2016)
DUSP2	Point mutations	Hartmann et al. (2016)
JUNB	Point mutations	Hartmann et al. (2016)
NPAT	Germline deletions	Saarinen et al. (2011)
FAS	Germline mutations	van den Berg et al. (2002)

Surprisingly, in these studies, the number of aberrations was higher in NLPHL when compared with THRLBCL. However, the NLPHL cases investigated were not subtyped according to their growth patterns. Using array CGH, the number of aberrations was higher in THRLBCL and atypical NLPHL variants compared with typical NLPHL (Hartmann et al. 2015b). Despite the differences in the number of aberrations, common genomic events, e.g., gains of the *REL* locus, were recurrently detected in both NLPHL and THRLBCL.

Whole genome sequencing of two DLBCL derived from and clonally related to NLPHL and a subsequent targeted analysis of primary NLPHL revealed frequent mutations in *SOCS1*, *DUSP2*, *JUNB*, and *SGK1* (Hartmann et al. 2016). Whereas JUNB works as an oncogene in classical HL, it was frequently affected by heterozygous stop mutations in NLPHL, leading to a very weak expression of the wild-type allele in the LP cells and suggesting a tumor suppressor function in NLPHL. SGK1 was strongly expressed both in the LP cells of primary NLPHL and the NLPHL cell line DEV. Application of a specific SGK1 inhibitor resulted in a high rate of apoptotic DEV cells, suggesting that SGK1 may act as an oncogene in NLPHL.

Familial NLPHL

There are several reports on families with accumulation of NLPHL (Merli et al. 2013; Saarinen et al. 2011, 2013). In one Finnish study, this was associated with a small germline deletion of serine 724 of the *NPAT* gene (Saarinen et al. 2011). However, there were also family members which were not affected by NLPHL. This small germline *NPAT* deletion was furthermore also observed in non-familial NLPHL patients, suggesting an increased risk for the development of NLPHL. Furthermore, genetic syndromes leading to a defect in the innate and adaptive immune system as well as the autoimmune lymphoproliferative syndrome, which can occur in children, have been associated with the development of NLPHL (Lorenzi et al. 2013; van den Berg et al. 2002).

Microenvironment

The microenvironment is considerably different in NLPHL with a typical nodular growth pattern compared to cases with a predominantly diffuse growth pattern. In typical NLPHL, where LP cells are mainly located within the B-cell nodules, the microenvironment has a strong similarity to that observed in normal germinal centers. Rosetting T cells around the LP cells are frequently observed, and these represent follicular T-helper cells, which are usually PD1 positive (Fig. 6.5) (Nam-Cha et al. 2008; Churchill et al. 2010). Furthermore a high number of CD57-positive cells are observed within the nodules of typical NLPHL (von Wasielewski et al. 1997). These

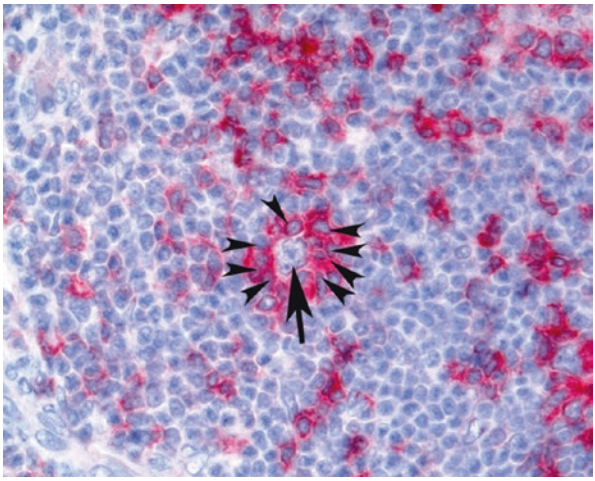

Fig. 6.5 Rosetting PD1-positive T cells (*arrow heads*) around an LP cell (*arrow*). PD1 immunostaining, 40×

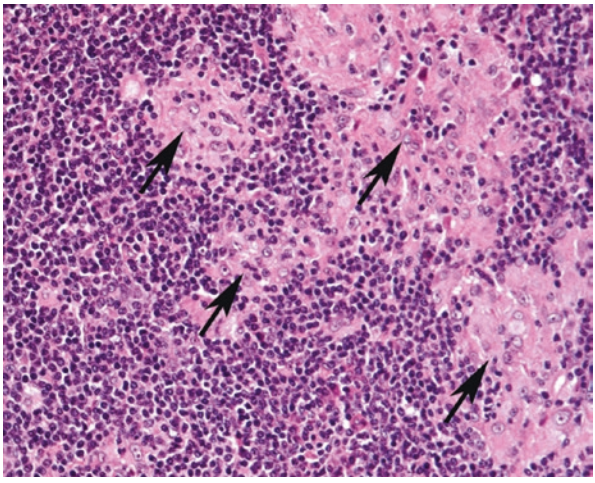

Fig. 6.6 Abundant epithelioid cells (arrows) forming granulomas in NLPHL. HE, 20×

CD57-positive cells are in the majority also CD4 and PD1 positive (Sattarzadeh et al. 2015). Other components in the microenvironment represent epithelioid cells which can be so abundant that they form granulomas (Fig. 6.6). A prominent epithelioid cell reaction was associated with a tendency to show less frequent relapses (Hartmann et al. 2014b).

In NLPHL with mainly diffuse areas, PD1- and CD57- as well as MUM1-positive rosetting T cells were less frequently observed (Churchill et al. 2010; Hartmann et al. 2013b). However, even rare cases of THRLBCL showed PD1-

positive rosetting T cells. The NLPHL cases with predominant diffuse areas resembling THRLBCL present a high content of macrophages, like observed in THRLBCL (Hartmann et al. 2013b). Furthermore, a low lymphocyte-monocyte ratio in the peripheral blood was observed to be an independent risk factor for progression-free and overall survival in NLPHL (Porrata et al. 2012), likely reflecting the composition in the nodal compartment.

A specific double-positive T-cell population was observed in the microenvironment of NLPHL. This population consists of $CD4^+CD8^+$ double-positive T cells and occurs both in NLPHL and in PTGC (Rahemtullah et al. 2006, 2008). It can be detected by flow cytometry and can be helpful in the diagnosis of NLPHL. This double-positive T-cell population usually constitutes a minority of the T cells in the microenvironment (10–38% of the T cells) and probably represents an activated T-cell population.

Relationship to THRLBCL

Already a long time ago, it was noticed that both NLPHL and THRLBCL occur predominantly in middle-aged men, the tumor cells have the same immunophenotype, and NLPHL patients exist, who present with relapses under the morphologic picture of THRLBCL (Rüdiger et al. 2002). Vice versa, it has also been observed that THRLBCL patients developed NLPHL in the relapse situation (Rüdiger et al. 2002). The tumor cells in NLPHL and THRLBCL are both of germinal center origin (Braeuninger et al. 1997) and they have a high similarity in their gene expression patterns (Brune et al. 2008; Hartmann et al. 2013b). Furthermore they share common genetic events (Hartmann et al. 2015b). However, the clinical behavior of THRLBCL is usually aggressive (Achten et al. 2002) and distinct from that of typical NLPHL. Therefore, THRLBCL seems to be a lymphoma entity which is closely related to NLPHL but which may represent a tumor-cell poor transformation like a DLBCL with an abundant microenvironment. To date, the relationship between NLPHL and THRLBCL is not fully understood, and further workup is necessary.

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Chapter 7
Composite Lymphomas and the Relationship of Hodgkin Lymphoma to Non-Hodgkin Lymphomas

Marc A. Weniger and Ralf Küppers

Abbreviations

ALCL	Anaplastic large cell lymphoma
BCR	B-cell receptor
cHL	Classical Hodgkin lymphoma
CLL	Chronic lymphocytic leukemia
DLBCL	Diffuse large B-cell lymphoma
EBV	Epstein-Barr virus
FISH	Fluorescence in situ hybridization
FL	Follicular lymphoma
GC	Germinal center
HL	Hodgkin lymphoma
HRS	Hodgkin and Reed/Sternberg
IgV	Immunoglobulin variable
LMP1	Latent membrane protein 1
LP	Lymphocyte predominant
MCL	Mantle cell lymphoma
MZL	Marginal zone lymphoma
NHL	Non-Hodgkin lymphoma
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
PCR	Polymerase chain reaction
PMBL	Primary mediastinal B-cell lymphoma
SLL	Small lymphocytic lymphoma
TCR	T-cell receptor

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Introduction

Hodgkin lymphoma (HL) is subdivided into a classical form, which accounts for about 95% of cases, and the rare nodular lymphocyte predominant HL (NLPHL). In both types of HL, the tumor cells, which are the Hodgkin and Reed/Sternberg (HRS) cells in classical HL (cHL) and the lymphocyte predominant (LP) cells in NLPHL, are typically rare and account for only about one or a few percent of cells in the tissue. The historical separation of HL from the other types of B- and T-cell lymphomas and leukemias is also kept in the current version of the WHO lymphoma classification (Swerdlow et al. 2016). This separation might imply a strong distinction between HL and non-Hodgkin lymphomas (NHL). cHL is indeed very peculiar and unique among lymphoid malignancies in several key aspects (Küppers et al. 2012). However, both forms of HL are derived from the malignant transformation of mature B cells (Küppers 2009), as are B-cell NHL (B-NHL), so in terms of their cellular derivation, HL and NHL are closely related. On this background, it is relevant to compare the biology and pathogenesis of these lymphomas and in particular compare HL to NHL to which they have a number of similarities. Moreover, in rare instances a HL and a NHL occur concurrently or sequentially in the same patient, and in such instances, it is highly interesting to understand whether these are chance occurrences of two independent lymphoid malignancies or whether they share a common cellular origin. In this chapter, we discuss the current knowledge about the biology and pathogenesis of composite HL and NHL and consider the relationship between HL and related NHL.

Composite HL and NHL

Composite lymphomas are defined as the concurrent occurrence of two histopathologically distinct types of lymphomas in a patient, mostly in the same organ (Kim et al. 1977; Küppers et al. 2014). The transformation of a low-grade (indolent) NHL into an aggressive lymphoma, often a diffuse large B-cell lymphoma (DLBCL), is typically not considered as a composite lymphoma, even if both are present in the same biopsy. Also the sequential occurrence of two distinct lymphomas in a patient is in the strict definition no composite lymphoma, but we will also discuss such cases, as their pathogenesis is often similar to the one of concurrent lymphomas. Various types of combined lymphomas have been described, including two distinct NHL, a combination of a HL and a NHL, and very rarely a cHL combined with a NLPHL (Kim et al. 1977; Küppers et al. 2014), but we will focus here on combined HL and NHL.

It has been estimated that about 1–4% of lymphomas are composite lymphomas (Thirumala et al. 2000), but there are few systematic investigations. A recent study from Japan showed that combined HL and NHL are very rare in younger adult patients, but 20% of patients of at least 40 years of age who were diagnosed with a

cHL also showed concurrent or subsequent NHL (Maeshima et al. 2015). A careful pathological diagnosis is essential to establish the diagnosis of a composite lymphoma, typically also involving immunohistochemical evaluations. For example, the HRS cells should express CD30, often CD15, weak PAX5, IRF4, and mostly no or only weak CD20. If the two lymphomas are present in the same organ, they may be sharply demarcated, but there are also cases where the borders are diffuse, or the lymphomas are even partly intermixed. Some NHL may show solitary HRS-like cells, but these are not defined as composite lymphomas, because for the diagnosis of a HL, presence of the typical cellular microenvironment is essential. In cHL, this involves at least $CD4^+$ T cells and macrophages but typically also granulocytes and numerous other subsets of immune cells. In composite HL and NHL, various types of NHL can be found, including DLBCL, follicular lymphoma (FL), mantle cell lymphoma (MCL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), marginal zone lymphoma (MZL), primary mediastinal B-cell lymphoma (PMBL), and also T-cell NHL.

Clonal Relationship of Composite HL and NHL

For an understanding of the pathogenesis of composite or sequential HL and NHL, it is essential to clarify whether the two lymphomas are clonally related or represent two independent lymphoid malignancies. B-cell lymphomas carry clonally rearranged and highly diverse immunoglobulin variable (IgV) heavy and light chain gene rearrangements. This is because during early B-cell development, each B-cell precursor undergoes a series of somatic V(D)J recombination processes to assemble functional Ig heavy and light chain genes by rearranging diverse V and J segments (and for the heavy chain additionally D_H segments), with additional diversity generated at the joining sites of the rearranging gene segments. IgV region genes therefore represent ideal clonal markers for B-lineage cells. Rearranged IgV genes from the HRS cells and the NHL cells of numerous composite lymphomas have therefore been analyzed in order to clarify the relationship of the two lymphomas. As HRS cells have been found to originate from T cells in very rare instances (Müschen et al. 2000; Seitz et al. 2000), it is also useful to study combined cHL and T-NHL for a potential common T-cell origin by analyzing T-cell receptor (TCR) gene rearrangements. Importantly, IgV gene or TCR studies should be performed with isolated HRS cells. If whole tissue sections or areas enriched with the cHL component are analyzed by amplification for IgV or TCR gene rearrangements, it cannot be excluded that some NHL cells present in the cHL microenvironment give rise to a false interpretation of a clonal relationship of the two lymphomas. Moreover, whole tissue analysis of a HL may not be sensitive enough to detect the IgV genes of the rare HRS or LP cells. We therefore consider only cases in which HRS cells were microdissected or sequential cases in which the cHL occurred several years before the NHL.

Besides B-cell receptor (BCR) or TCR rearrangements, also genetic lesions can serve as markers of clonality. By fluorescence in situ hybridization on tissue sections or polymerase chain reaction, the detection of hallmark chromosomal translocations, such as the t(14;18) *IGH-BCL2* in FL and some DLBCL, can indicate whether the two lymphomas originate from a common precursor cell or distinct precursor cells.

Among 24 composite lymphomas of a cHL and a B-NHL that were analyzed for their clonal relationship and that were informative, 17 cases turned out to be clonally related (Table 7.1). The B-NHL clonally related to the cHL included CLL, FL, DLBCL, and MCL, showing that diverse B-NHL can have a common origin with a cHL. Moreover, in one instance, a cutaneous T-NHL was related to a cHL, representing a rare example in which a lymphoma diagnosed as cHL has a T-cell origin (Willenbrock et al. 2002). Three cases of NLPHL with a concurrent B-NHL at primary diagnosis have been molecularly analyzed, and in all three instances, the LP cells were clonally related to the B-NHL cells (twice a DLBCL and once a T-cell-rich B-cell lymphoma (TCRBL)) (Table 7.1) (Hartmann et al. 2016; Shimodaira et al. 2000; Szczepanowski et al. 2013). Similarly, in two cases of NLPHL combined with cHL, the HRS and LP cells showed a clonal relationship (Hansmann et al. 1989; Miettinen et al. 1983).

Studies on the clonal relationship of consecutive cases of cHL and NHL revealed in 13 of 22 cases a common cell of origin. Also in this setting, the NHL clonally related to the cHL comprised various types of NHL, including CLL, TCRBL, FL, splenic marginal zone lymphoma, and a lymphomatoid papulosis and cutaneous T-cell lymphoma (Table 7.2). The clonally related consecutive lymphomas also included three PMBL. Their clonal relationship to the cHL might not be that surprising, considering the many shared features of these two types of lymphomas, including many similar genetic lesions, as is discussed further below. In seven of the ten clonally related consecutive lymphomas, the HL occurred after the NHL, and in the other three cases, the HL was diagnosed first, demonstrating that there is no strict order in which such consecutive cHL-NHL occur (Table 7.2). Usually, the second lymphoma develops several years after the first one. In the most extreme case of a clonally related consecutive lymphoma reported in the literature, a HL was diagnosed 15 years after the diagnosis of a splenic marginal zone lymphoma (Rosenquist et al. 2004a). Thus, the HRS cells or their premalignant precursor cells must have resided in the body for at least 15 years, before the cHL developed.

Taken together, in a fraction of composite and consecutive HL and NHL, the lymphomas are clonally unrelated; hence, such cases may represent chance occurrences of two unrelated tumors in the same patient. In unrelated consecutive lymphomas, the development of the secondary lymphoma might have been promoted by the therapy the patient received to treat the first lymphoma. Although the detection of different Ig or TCR gene rearrangements defines a combined HL and NHL as being derived from clonally unrelated B or T cells, this does not mean that there is no connection between clonally unrelated composite or consecutive lymphomas. There is accumulating evidence that some genetic lesions in mature B- or T-cell lymphomas, including CLL, hairy cell leukemia and angioimmunoblastic T-cell

Table 7.1 Composite HL and NHL for which the clonal relationship was clarified[a]

Hodgkin lymphoma	Non-Hodgkin lymphoma	Clonal relationship	Remark	Reference
Classical	FL	Yes	Shared and distinct V gene mutations	Bräuninger et al. (1999)
Classical	FL	Yes	Shared and distinct V gene mutations	Küppers et al. (2001)
Classical	FL	Yes	Based on shared t(14;18) BCL2/IgH translocation detected by FISH	Maeshima et al. (2015)
Classical	FL	Yes	Based on shared t(14;18) BCL2/IgH translocation detected by FISH	Maeshima et al. (2015)
Classical	FL	Yes	Based on shared t(14;18) BCL2/IgH translocation detected by FISH	Maeshima et al. (2015)
Classical	FL	Yes	Based on shared t(14;18) BCL2/IgH translocation detected by chromogenic in situ hybridization	Yoshida et al. (2012)
Classical	CLL	Yes	Shared and distinct V gene mutations; initial CLL diagnosis 5 years earlier	Küppers et al. (2001)
Classical	MCL	Yes	Mantle cell lymphoma unmutated, HRS cells with mutations[b]	Tinguely et al. (2003)
Classical	DLBCL	Yes	Shared and distinct V gene mutations	Rosenquist et al. (2004b)
Classical	DLBCL	?	Two different V_H genes rearranged to the same D_HJ_H joint; receptor revision in one clone, or separate development from a common pro-B cell	Bellan et al. (2002)
Classical	CLL and anaplastic DLBCL	Yes	Three lymphomas in one lymph node; identical V_H mutation pattern	van den Berg et al. (2002)
Classical	DLBCL	Yes		Huang et al. (2006)
Classical	DLBCL	Yes	Same D_HJ_H rearrangement	Goyal et al. (2016)
Classical	CLL	No		Mao et al. (2007)
Classical	CLL	No		Mao et al. (2007)
Classical	MCL	No		Caleo et al. (2003)
Classical	MCL	No		Caleo et al. (2003)
Classical	MCL	Yes	Only shared mutations	Schneider et al. (2014)

(continued)

Table 7.1 (continued)

Hodgkin lymphoma	Non-Hodgkin lymphoma	Clonal relationship	Remark	Reference
Classical	MCL, blastoid	Yes	Based on shared t(11;14) CCND1/IgH translocation detected by FISH	Murray et al. (2016)
Classical	Low-grade B-NHL	Yes		Kerl et al. (1999)
Classical	Low-grade B-NHL	Yes		Kerl et al. (1999)
Classical	CLL	No		Kerl et al. (1999)
Classical	High-grade B-NHL	No		Kerl et al. (1999)
Classical	Cutaneous T-cell LPD	Yes	HL in lymph node; T-cell origin of both lymphomas	Willenbrock et al. (2002)
Classical	T-cell NHL	No		Gualco et al. (2009)
NLP	DLBCL	Yes		Szczepanowski et al. (2013)
NLP	DLBCL	Yes	Shared and distinct V gene mutations	Hartmann et al. (2016)
NLP	TCRBCL	Yes		Shimodaira et al. (2000)
NLP and classical		Yes	Clonality based on two identically sized VκJκ joints, not on sequencing	Miettinen et al. (1983)
NLP and classical		Yes	Shared mutations (short sequence)	Hansmann et al. (1989)

CLL chronic lymphocytic leukemia, *DLBCL* diffuse large B-cell lymphoma, *LPD* lymphoproliferative disorder, *FL* follicular lymphoma, *NHL* non-Hodgkin lymphoma, *MCL* mantle cell lymphoma, *NLP* nodular lymphocyte predominant, *TCRBCL* T-cell-rich B-cell lymphoma

[a]Only cases in which at least the HRS or LP cells were microdissected for molecular analysis are considered. Whole tissue analysis might not be sensitive enough to detect clonal rearrangements in the rare HRS or LP cells, and in case of a clonal amplificate from the whole area of the HL part, it remains unclear whether the rearrangement is carried by the HRS or LP cells or by members of the NHL clone present in the HL microenvironment

[b]In this case the HRS cells carried mutated IgV genes, but the MCL harbored unmutated V genes. This suggests that the common precursor of the two lymphoma clones was a pre-GC B cell and that only the HRS cell precursor entered a GC reaction and underwent malignant transformation, whereas the MCL developed without involvement of a GC reaction

lymphoma, occur already in hematopoietic stem or precursor cells (Chung et al. 2014; Damm et al. 2014; Quivoron et al. 2011). It is hence an intriguing possibility that some clonally unrelated HL and NHL nevertheless carry some shared genetic lesions. Although numerous combinations of HL and NHL are unrelated, it is remarkable that the majority of composite and consecutive HL and NHL are clonally related and thus have a common precursor cell. This is also of relevance for our

Table 7.2 Clonal relationship of cHL and NHL developing consecutively in a patient[a]

Non-Hodgkin lymphoma	Clonal relationship	Remark	Reference
TCRBCL	Yes	HL developed 3 years after TCRBCL; shared and distinct V gene mutations	Bräuninger et al. (1999)
FL	Yes	FL developed 2 years after HL; shared and distinct V_H gene mutations	Marafioti et al. (1999)
FL	Yes	HL diagnosed 4 years after FL	Nakamura et al. (2007)
FL	Yes	FL diagnosed 3 years after HL	Yoshida et al. (2012)
DLBCL	No	DLBCL diagnosed 12 years after initial HL diagnosis	Thomas et al. (2004)
DLBCL	No	DLBCL diagnosed 4 months after initial HL; analysis based on numerous somatic mutations in DLBCL, which were not found in enriched HRS cells	Siddiqi et al. (2014)
DLBCL and FL	Yes	HL diagnosed 13 months after NHLs; based on shared t(14;18) *IGH/BCL2* translocation detected by FISH	Maeshima et al. (2015)
PMBL	Yes	HL developed after PMBL	Traverse-Glehen et al. (2005)
PMBL	Yes	PMBL developed after HL	Traverse-Glehen et al. (2005)
PMBL	Yes	PMBL developed after HL; confirmation of clonal relationship mainly based on shared chromosomal lesions	Eberle et al. (2011)
SMZL	Yes	HL developed 15 years after SMZL; unmutated V_H region genes[b]	Rosenquist et al. (2004a)
SMZL and DLBCL	Yes	SMZL diagnosed 1 year before and DLBCL 1 year after HL	Alonso-Alvarez et al. (2016)
MALT lymphoma and anaplastic DLBCL	No	MALT lymphoma 4 years before and DLBCL 2 years after HL; NHL clonally related to each other	Parrens et al. (2002)
Marginal zone lymphoma and T-NHL	No	T-NHL first diagnosed 30 years before the other lymphomas, which occurred concurrently	Steinhoff et al. (2006)
Marginal zone lymphoma	No	Based on detection of MALT1 split by FISH only in NHL	Maeshima et al. (2015)

(continued)

Table 7.2 (continued)

Non-Hodgkin lymphoma	Clonal relationship	Remark	Reference
Small non-cleaved cell B-NHL	No	B-NHL developed 6 years after HL	Ohno et al. (1998)
CLL	No	HL diagnosed 10 years after CLL	Venkatraman et al. (2007)
CLL	Yes	HL developed 4 years after CLL	Fong et al. (2005)
CLL	Yes	HL developed 8 years after CLL	Fong et al. (2005)
CLL	No	HL developed 10 years after CLL	Fong et al. (2005)
CLL	No	HL developed 5 years after CLL	de Leval et al. (2004)
CLL	No	HL developed 4 years after CLL	de Leval et al. (2004)
Lymphomatoid papulosis and cutaneous T-cell lymphoma	yes[c]	HL developed 4 years after lymphomatoid papulosis and was followed 10 years later by a cutaneous T-cell lymphoma	Davis et al. (1992)

CLL chronic lymphocytic leukemia, *FL* follicular lymphoma, *NLP* nodular lymphocyte predominant, *DLBCL* diffuse large B-cell lymphoma, *PMBL* primary mediastinal B-cell lymphoma, *SMZL* splenic marginal zone lymphoma

[a]Only cases in which at least the HRS cells were microdissected for molecular analysis are considered. Whole tissue analysis might not be sensitive enough to detect clonal rearrangements in the rare HRS cells, and in case of a clonal amplificate, it remains unclear whether the rearrangement is carried by the HRS cells or by members of the NHL clone present in the HL microenvironment

[b]In this case, both lymphomas carried unmutated V region genes. Thus, these lymphomas might derive from a pre-GC B cell, and the HRS cell clone might be a direct descendent of the B-NHL clone. However, also in this instance, the HRS clone could principally derive from a GC B cell, because GC founder cells already acquire the phenotype of GC B cells, undergo proliferation, and become apoptosis sensitive before somatic hypermutation becomes active (Lebecque et al. 1997)

[c]Although HRS cells were not microdissected for molecular analysis, this case is included, because at time of HL diagnosis, no T-cell malignancy as a potential source for contamination in the PCR analysis was evident in the patient

understanding of the cellular origin of HRS cells, as there was initially much debate from which cell type HRS cells are derived (Küppers and Rajewsky 1998). The demonstration that cHL in composite lymphomas are often clonally related to the B-NHL with which they co-occur is a further strong argument for the B-cell origin of the HRS cells in these instances.

Cellular Origin of Composite Lymphomas

The sequence analysis of the rearranged IgV genes of composite lymphomas is not only instrumental to determine their clonal relationship but provides also essential information about the differentiation stage of the lymphomas and what their specific

relationship is. This is because in T-cell-dependent immune responses, antigen-reactive B cells activate the process of somatic hypermutation that introduces point mutations and some deletions and insertions at a very high rate into the rearranged IgV genes (Klein and Dalla-Favera 2008; Küppers et al. 1993). Importantly, somatic hypermutation takes place only in the histological structure of the germinal center (GC) in secondary lymphoid organs, where the antigen-activated B cells also undergo very strong proliferation, and where the mutated GC B cells are selected for expression of a high-affinity BCR (Klein and Dalla-Favera 2008; Küppers et al. 1993). GC B cells undergo muliple rounds of proliferation, mutation, and selection, generating large GC B-cell clones with a high intraclonal IgV gene diversity. Throughout the GC reaction, some positively selected GC B cells leave the GC and differentiate either into long-lived plasma cells or memory B cells (Klein and Dalla-Favera 2008). The presence of somatic mutations in the rearragend Ig heavy and light chain V region genes is consequently a genetic marker for GC B cells, memory B cells, and post-GC plasma cells.

Most clonally related composite and consecutive lymphomas carried somatically mutated IgV gene rearrangements (Tables 7.1 and 7.2), demonstrating an origin of these cases from GC or post-GC B cells. Importantly, in many of these instances, the clonally related rearrangements showed both shared mutations as well as mutations present in only one of the lymphomas. Hence, the common precursor of both lymphomas was a mutating GC B cell (Bräuninger et al. 1999; Marafioti et al. 1999; Küppers et al. 2001). Moreover, the frequently observed pattern that the rearrangements of the cHL and the B-NHL both carried unique mutations that are not present in the other lymphomas strongly argues for a scenario in which the lymphomas derived from distinct daughter cells of a mutating GC B-cell clone (Fig. 7.1). This means that it is mostly not the case that one lymphoma represents the transformation from the other lymphoma, because in such a situation, the secondary lymphoma should show all IgV gene mutations already present in the initial lymphoma and perhaps some additional ones. Distinct IgV gene mutations in both lymphomas are not compatible with a parent-child connection. It is remarkable that the scenario of a development of clonally related cHL and B-NHL from distinct daughter cells of a common precursor also applies to four of five informative consecutive lymphomas (Table 7.2). Thus, even in these cases, the secondary lymphoma, which sometimes developed several to more than 10 years after the first lymphoma, is not a transformation from the first lymphoma but developed separately from another descendent of the common, presumably premalignant precursor.

In one sequential combined lymphoma, both the initial splenic marginal zone lymphoma and the later occurring related cHL carried unmutated IgV genes (Rosenquist et al. 2004a). Consequently, in this case, the cHL may represent a transformation from the B-NHL, and the lymphomas may derive from a pre-GC B cell. However, as GC founder cells frequently still carry unmutated IgV genes (Lebecque et al. 1997), even in this case, a GC B-cell origin of both lymphomas is possible.

In two combinations of NLPHL and DLBCL – a composite lymphoma and a case where the DLBCL developed several years after the NLPHL – the already described pattern of shared and distinct IgV gene mutations was present, too

(Hartmann et al. 2016). This shows that also in these lymphomas involving NLPHL and a DLBCL, the DLBCL was not a simple transformation from the NLPHL, but that both lymphomas separately developed from distinct daughter cells of the common precursor.

Genetic Lesions in Composite Lymphomas

For clonally related composite lymphomas, it is likely that the two lymphomas share some transforming events that were present already in the common (premalignant) precursor and that the daughter cells of that common precursor acquired additional separate distinct transforming events that then caused the development of two different types of lymphomas (Fig. 7.1). The molecular analysis of related composite lymphomas for genetic lesions or other transforming events therefore offers the possibility to study the multi-step transformation process during lymphomagenesis and to determine which events were early and which played as later events a role in defining the different lymphomas that developed. Only a few composite HL and B-NHL were so far studied for selected genetic lesions, but these already gave important insights into the development of composite lymphomas.

In two combinations of MCL and cHL, the hallmark chromosomal translocation t(11;14), which brings the *CCND1* gene under control of the regulatory elements of the IgH locus, was not only detected in the MCL but also in the HRS cells, identifying these translocations as early shared events (Table 7.3) (Schmitz et al. 2005; Schneider et al. 2014; Tinguely et al. 2003). Similarly, in several combinations of FL and cHL, the t(14;18), which juxtaposes the *BCL2* proto-oncogene and the IgH locus, was detected in the FL and the HRS cells (Table 7.3) (Maeshima et al. 2015; Schmitz et al. 2005; Nakamura et al. 2007; Yoshida et al. 2012). Sequence analysis of the chromosomal breakpoint validated the identity of the translocation (Schmitz et al. 2005; Nakamura et al. 2007). The observation that the translocations t(11;14) und t(14;18) are shared by the cHL and the B-NHL is not surprising, because they happen as mistakes of V(D)J recombination during early B-cell development as mistakes of V(D)J recombination (Küppers and Dalla-Favera 2001). The major pathogenetic role of the *BCL2* and *CCND1* translocations for FL and MCL, respectively, is beyond any doubt; however, it is less clear which role these translocations play in the fully developed cHL. This is because the IgH locus is typically silenced in HRS cells (Ushmorov et al. 2004, 2006; Stein et al. 2001; Doerr et al. 2005), so that IgH enhancers that drive the constitutive expression of proto-oncogenes brought into the locus are less active in HRS cells. Moreover, HRS cells often express BCL2 also in the absence of a t(14;18) (Gravel et al. 1998), so the translocation is principally not needed for expression of this antiapoptotic factor. Regarding CCND1, in one of the composite lymphomas with the t(11;14) also in the HRS cells, the HRS cells were CCND1 negative (Tinguely et al. 2003), whereas in the second case, CCND1 expression by HRS cells was detected by immunohistochemistry (Schneider et al. 2014). Thus, in this latter case, there was apparently still IgH enhancer activity

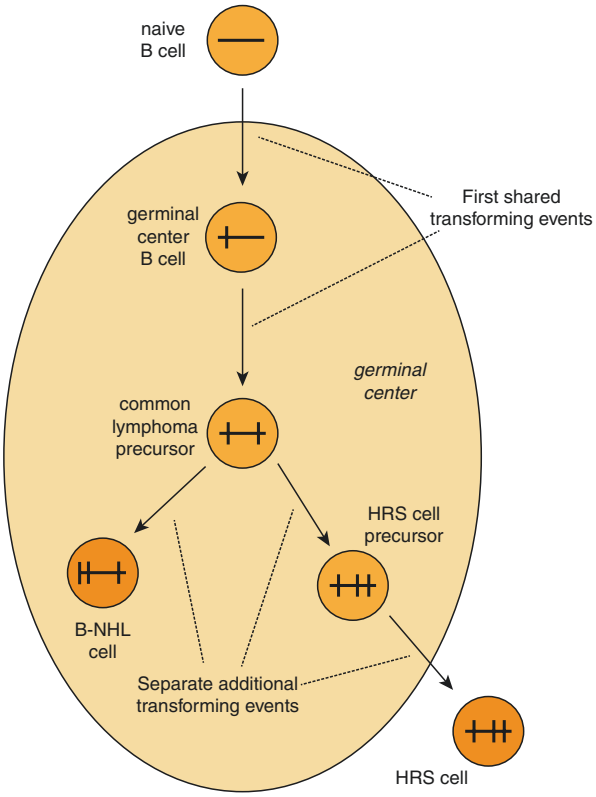

Fig. 7.1 Scenario for the generation of composite lymphomas from GC B cells**.** In a large fraction of clonally related cHL and B-NHL lymphomas, the IgV genes of the two lymphomas carry common somatic mutations as well as mutations present only in one or the other of the two lymphomas. This suggests a scenario for the development of such lymphomas in which both lymphomas derive as separate daughter cells from a common lymphoma precursor that was a mutating GC B cells and that was characterized by the shared mutations. Distinct descendents of that common precursor, characterized by the additional IgV gene mutations present only in one or the other of the lymphomas, then gave rise to the cHL and the B-NHL. It is likely that the common precursor already carried some transforming events and that the descendents of that cell later acquired additional separate transforming events, finally causing development of the cHL and the B-NHL. Horizontal lines in the cells indicate an IgV gene, and vertical lines somatic mutations. Modified from reference Bräuninger et al. (1999)

in the HRS cells, driving expression of the translocated gene. Perhaps, the translocations were of pathogenetic relevance during early stages of the development of the composite lymphomas, but later, when the HRS cell precursor acquired further genetic lesions, the translocated proto-oncogenes became less relevant, so that they could be silenced without impairing cHL development.

In a composite lymphoma consisting of a DLBCL and a cHL, a *TP53* mutation was present only in the DLBCL (Schmitz et al. 2005). Thus, this represents an example of a late genetic lesion that occurred in the precursor of the DLBCL. There

Table 7.3 Shared and distinct genetic lesions and viral infections in clonally related composite or consecutive HL and B-NHL

Case	Lymphoma	Type and presence of transforming event	Remark	Reference
1	Classical HL DLBCL	EBV+ EBV−		Huang et al. (2006)
2	Classical HL Follicular lymphoma	t(14;18), BCL2/IgH+ t(14;18), BCL2/IgH+	Identical translocation	Nakamura et al. (2007)
3	Classical HL Follicular lymphoma	t(14;18), BCL2/IgH+ t(14;18), BCL2/IgH+	Identical translocation	Schmitz et al. (2005)
4	Classical HL Follicular lymphoma	t(14;18), BCL2/IgH+ t(14;18), BCL2/IgH+	Identical translocation	Schmitz et al. (2005)
5	Classical HL Follicular lymphoma	t(14;18), BCL2/IgH+ t(14;18), BCL2/IgH+	Based on FISH analysis	Maeshima et al. (2015)
6	Classical HL Follicular lymphoma	t(14;18), BCL2/IgH+ t(14;18), BCL2/IgH+	Based on FISH analysis	Maeshima et al. (2015)
7	Classical HL Follicular lymphoma	t(14;18), BCL2/IgH+ t(14;18), BCL2/IgH+	Based on FISH analysis	Maeshima et al. (2015)
8	Classical HL Follicular lymphoma	t(14;18), BCL2/IgH+ t(14;18), BCL2/IgH+	Chromogenic in situ hybridization	Yoshida et al. (2012)
9	Classical HL Mantle cell lmyphoma	t(11;14), BCL1/IgH+ Subclone EBV+ t(11;14), BCL1/IgH+ EBV−	Identical translocation, HRS cell subclone with distinct V_H mutations EBV+	Schmitz et al. (2005) and Tinguely et al. (2003)
10	cHL Follicular lymphoma and DLBCL	t(14;18), BCL2/IgH+ t(14;18), BCL2/IgH+	HL diagnosed 13 months after NHLs; based on shared t(14;18) BCL2/IgH translocation detected by FISH	Maeshima et al. (2015)
11	Classical HL DLBCL	TP53 mutations− TP53 mutations+		Schmitz et al. (2005)
12	Classical HL Mantle cell lymphoma	t(11;14), BCL1/IgH+ TP53 deletion and mutation t(11;14), BCL1/IgH+ TP53 deletion and mutation	Identical translocation Identical TP53 point mutation, but presumably distinct deletion of other TP53 allele	Schneider et al. (2014)
13	Classical HL CLL	EBV+ EBV−	HL developed after CLL	Fong et al. (2005)
14	Classical HL CLL	EBV+ EBV−	HL developed after CLL	Fong et al. (2005)
15	Classical HL NLPHL	EBV+ EBV−		Szczepanowski et al. (2013)

Table 7.3 (continued)

Case	Lymphoma	Type and presence of transforming event	Remark	Reference
16	NLPHL DLBCL	DUSP2, SGK1, SOCS1, and others shared; overall 12/21 mutations in DLBCL also present in LP cells	Composite lymphoma	Hartmann et al. (2016)
17	NLPHL DLBCL	DUSP2, SGK1, SOCS1, and others shared; overall 18/22 mutations in DLBCL also present in LP cells	DLBCL developed after NLPHL, but IgV gene analysis argues against transformation, but for parallel development	Hartmann et al. (2016)

are rare examples of *TP53* mutations also in HRS cells, but typically, cHL develops without such mutations (Montesinos-Rongen et al. 1999; Maggio et al. 2001).

In about 30–40% of cHL in the Western world, HRS cells are infected by Epstein-Barr virus (EBV) (Küppers 2003). EBV is a γ herpes virus that latently infects B cells. In cHL a pathogenetic role of EBV is likely, because HRS cells express the oncogenic protein latent membrane protein 1 (LMP1) among a few other EBV-encoded genes (Küppers 2003). It is generally unclear at which stage of B-cell development the precursor cell of an EBV^+ HRS cell clone becomes EBV-infected. Interestingly, in each of five clonally related composite lymphomas of a cHL and a B-NHL or a cHL and a NLPHL with EBV infection of lymphoma cells, the virus was detected only in the HRS cells (Table 7.3). This indicates that EBV infection was a rather late event, presumably in a GC B cell, although detailed IgV gene data are missing for most of these cases. In a combined cHL and MCL, only a subclone of the HRS cells, which was also defined by a distinct IgV gene mutation pattern, carried EBV, strongly suggesting that EBV infection was a late event in a mutating GC B cells (Tinguely et al. 2003).

There is so far only one study of genetic lesions in LP cells of NLPHL in cases combined with a B-NHL. In that study, one composite NLPHL and DLBCL at primary diagnosis and one instance in which the DLBCL developed after the NLPHL are included (Hartmann et al. 2016). Also the latter case is considered here, because the IgV gene analysis of that case revealed that the DLBCL is not a simple transformation from the NLPHL, but that both lymphomas developed separately from a common precursor (Hartmann et al. 2016). Sequencing of isolated LP cells for several mutations identified in the DLBCLs by whole genome sequencing revealed that in the composite and the consecutive lymphoma 12/21 and 18/22 mutations found in the DLBCL were also present in the LP cells, respectively (Hartmann et al. 2016). Thus, in both cases many mutations were shared by the NLPHL and DLBCL,

but the DLBCL also has acquired additional mutations that were not detected in the LP cells.

Overall, the first studies on chromosomal translocations, somatic mutations, and viral infections in composite lymphomas validated the concept that such cases carry both shared as well as distinct transforming events and that such lymphomas develop in a multi-step transformation process.

Relationship of HL to NHL

The distinction between HL and NHL is clinically of high relevance, because these lymphomas show highly variable clinical behavior, and there are major differences in the treatment strategies for HL and the various types of NHL. With the recognition that the lymphoma cells of cHL and NLPHL are derived from mature B cells, as are most NHL (Küppers 2005), it became also biologically highly relevant to compare HL and NHL to understand how pathobiologically and clinically so different lymphomas can develop from the same type of lymphocyte. This comparison of HL and NHL is mainly done at five levels: the morphology and immunophenotype of the lymphoma cells, the histological picture and hence the microenvironmental composition, the global gene expression pattern of the lymphoma cells, the specific cell of origin, and the pattern of genetic lesions that caused the malignant transformation of the cells. In the following sections, we focus on the comparisons of NLPHL and cHL to their most similar NHL, namely, the comparison of NLPHL to FL and the comparison of cHL to PMBL and anaplastic large cell lymphoma (ALCL).

NLPHL and FL

NLPHL shows a high similarity to FL. In both entities the lymphoma cells show an immunophenotype of GC B cells, with expression of markers such as BCL6 and AID (Carbone et al. 1998; Greiner et al. 2005; Smit et al. 2003). Also global gene expression studies revealed a high similarity of the lymphoma cells of FL and NLPHL to normal GC B cells (Brune et al. 2008). Furthermore, the histological picture of both lymphomas resembles normal GC in key aspects, including an association of the lymphoma cells with follicular dendritic cells and T follicular helper cells (Hansmann et al. 1999). Additional support for a GC B-cell origin of NLPHL and FL stems from analyses of the rearranged IgV genes of the malignant cells. The lymphoma cells of both entities carry somatically mutated IgV genes that are selected for functionality, and there is ongoing somatic hypermutation during clonal expansion of the lymphoma cells, a hallmark of GC B cells (Braeuninger et al. 1997; Marafioti et al. 1997; Bahler and Levy 1992). The main differences are seen

at the level of the transforming events. The t(14;18) causing translocation of the *BCL2* gene into the IgH locus is a hallmark of FL, whereas LP cells of NLPHL typically lack this translocation and in a fraction of cases harbor translocations involving the *BCL6* gene and various translocation partners. Genetic lesions frequently found in FL involve the genes *MML2*, *EZH2*, *CREBBP*, *MEF2B*, *EPHA7*, and *TNFRSF14* (Kridel et al. 2012). In NLPHL, a recent study revealed recurrent mutations in *SGK1*, *DUSP2*, and *JUNB* (Hartmann et al. 2016). Overall, it appears that both lymphomas are derived from selected and mutating GC B cells that largely retain the immunophenotype and gene expression pattern of their cells of origin and that still depend on a GC microenvironment. The main distinction between these lymphomas is the very different combination of genetic lesions that caused these malignancies.

cHL and PMBL

PMBL has been recognized as a distinct subtype of DLBCL with a better prognosis than nodal DLBCL (Rosenwald et al. 2003; Barth et al. 2002). PMBL present with a bulk mass in the upper anterior mediastinum and are often sclerotic (Barth et al. 2002). The lymphoma cells can spread to supraclavicular lymph nodes, but systemic spread must be excluded. A common immunophenotypic marker for both cHL and PMBL is the expression of CD30, and not only HRS cells but also PMBL cells seem to lack expression of the BCR (Stein et al. 2001; Pileri et al. 2003; Schwering et al. 2003). Whereas HRS cells of many cHL cases express CD15, PMBL cells are typically negative for CD15 (Table 7.4). Moreover, HRS cells show a global downregulation of the B-cell phenotype, whereas PMBL cells still show B-cell marker expression (Pileri et al. 2003; Schwering et al. 2003). Additionally, HRS cells show an aberrant expression of markers that are typical for other cells of the immune system, such as the T-cell markers NOTCH1 and GATA3, the natural killer markers ID2, the myeloid marker CSF1R, and dendritic cell markers such as CCL7 (Schmitz et al. 2009a). PMBL do not show such a non-B-cell marker expression.

The IgV genes of HRS cells and PMBL cells are somatically mutated, and the malignant cells of both lymphomas often have undergone class-switching (Kanzler et al. 1996; Irsch et al. 2001; Martin-Subero et al. 2006a; Leithäuser et al. 2001), but there are also important differences. HRS cells of approximately one quarter of cHL have crippling IgV gene mutations that rendered originally functional IgV genes nonfunctional and that normally results in the elimination of the respective GC B cells (Küppers 2009; Kanzler et al. 1996). IgV genes in PMBL cells, however, do not have crippling mutations (Leithäuser et al. 2001). Hence, HRS and PMBL cells both derive from GC-experienced B cells, but HRS cells presumably originate from pre-apoptotic GC B cells, whereas PMBL cells originate from positively selected GC B cells.

Table 7.4 Comparison of cHL, PMBL and ALCL

Feature	cHL	PMBL	ALCL
Presumed cell of origin	Pre-apoptotic GC B cell	Positively selected GC B cell, potentially immigrated to the thymus	Mature T cell
Phenotype	Loss of B-cell markers (e.g., CD19, CD20, CD22, BCR, CD79), $CD30^+$, often $CD15^+$,$CSF1R^+$,EMA^-	Undetectable Ig with otherwise retained B-cell phenotype, incl. CD19, CD20, CD21, CD22, CD23; $CD30^+$ (weaker than in HRS cells), $CD23^+$, MAL^+, $CD15^-$, EMA-	Frequent loss of TCR and co-receptor CD3; $CD4^+$, $CD5^+$, $CD30^+$, EMA+, $CD15^-$, granzyme B
EBV status	30–40% positive with latency II; mostly cases of mixed cellularity subtype	Negative	Negative
Intracellular markers, transcription factors	Loss of SYK, LCK, BCMA, BLNK, BLK, SHIP Negative or low for BOB1, OCT2, PU.1, BCL6, PAX5, EBF1, FOXO1 Positive for IRF4, MYC, GATA3, ID2	Positive for IRF4, MYC, AID, BCL6	$PAX5^-$, $GATA3^{low}$ Positive for IRF4, MYC, ID2
Active signaling pathways, transcription factors	NF-κB, AP-1, STATs, NOTCH1, PI3K/AKT, various receptor tyrosine kinases	NF-κB, AP-1, STATs	NF-κB, AP-1, STATs NOTCH1, PI3K/AKT
Genetic lesions	Ig translocations (≤20%); CIITA (20%) NF-κB pathway: Gains of *REL*, *MAP3K14*; losses of/inactivating mutations in *TNFAIP3*, *NFKBIA*, *NFKBIE*, *TRAF3* JAK/STAT pathway: Gains of *JAK2* Gains of STAT6 Inactivating mutations in *SOCS1*, *PTPN1* Others: Gains of *JMJD2C*, *PDL1*, *PDL2* Inactivating mutations in *B2M*, *XPO1*	Ig-BCL2 or Ig-BCL6 rare or absent, CIITA, PDL1, PDL2 NF-κB pathway: Gains of *REL*; Losses of/inactivating mutations in *TNFAIP3*, *NFKBIE* JAK/STAT pathway: Gains of *JAK2* Mutations in *STAT6* Inactivating mutations in *SOCS1*, *PTPN1* Others: Gains JMJD, PDL1/2 Mutations in *XPO1*	NPM-ALK and variant ALK translocations in ALK^+ ALCL, DUSP22-IRF4, NFκB2-TYK2, NFκB2-ROS1 Mutations in *JAK1*, *STAT3* (in ALK^-); deletions of *TP53*, *PRDM1*, *ATG5*

The process of somatic hypermutation sometimes also targets non-Ig genes, and a particular broad targeting of non-Ig genes was originally identified in DLBCL (Pasqualucci et al. 2001). Later studies showed that aberrant somatic hypermutation also occurs in HRS cells and in PMBL cells (Liso et al. 2006; Rossi et al. 2005), hence representing a further genetic similarity of these lymphomas.

In PMBL, Ig locus-associated chromosomal translocations involving *BCL2*, *BCL6*, *MYC*, or other genes are absent or rare (Scarpa et al. 1999; Tsang et al. 1996). Recurrent breakpoints in Ig loci have been detected in HRS cells in approximately 20% of cHL (Martin-Subero et al. 2006a). The translocation partners, however, remained largely unidentified, and the oncogenes *BCL6*, *MYC*, *REL*, and *BCL3* were only rarely involved, and *CCND1* was not affected (Martin-Subero et al. 2006a; Szymanowska et al. 2008).

In about 15% of cHL, chromosomal rearrangements involve the *CIITA* gene encoding the MHC class II transactivator, which is important for MHC class II expression (Steidl et al. 2011). These translocations seem to result in loss of CIITA function and consequently downregulation of MHC class II expression. Structural aberrations involving *CIITA* are also highly recurrent in PMBL, with about 40% of cases showing such events (Steidl et al. 2011; Mottok et al. 2015). A further genetic similarity between cHL and PMBL are alterations affecting the genes encoding programmed death ligand 1 (PDL1) and PDL2 (Green et al. 2010; Chong et al. 2016; Twa et al. 2014). These factors presumably contribute to immune evasion, as they can impair the function of PD1-positive cytotoxic cells. In cHL gains of the *PDL1* and *PDL2* genes predominate (Green et al. 2010), whereas in PMBL chromosomal translocations are more frequent (Chong et al. 2016; Twa et al. 2014). Recently, mutations in the *XPO1* gene, which encodes for a nuclear export factor, were identified in about 25% of cHL and PMBL, thus representing a further shared genetic lesion of these lymphomas (Camus et al. 2016; Jardin et al. 2016).

Constitutive activity of the NF-κB pathway is a hallmark of both cHL and PMBL (Table 7.4). In both entities, genetic lesions contribute to this dysregulated activity. Increased copy numbers of the *REL* gene on 2p16 are highly recurrent in both types of lymphomas (Barth et al. 2003; Martin-Subero et al. 2002; Joos et al. 1996). Moreover, genes encoding inhibitors of NF-κB signaling, i.e., *NFKBIE* and *TNFAIP3*, are often inactivated in HRS and PMBL cells (Emmerich et al. 2003; Schmitz et al. 2009b; Mansouri et al. 2016). For *NFKBIA*, mutations were only found in cHL, but not in PMBL (Takahashi et al. 2006; Jungnickel et al. 2000; Emmerich et al. 1999).

Constitutive activity of the JAK/STAT pathway is a further shared feature of cHL and PMBL, and in both lymphomas, genetic lesions play an important role in this activity. Increased copy numbers of the *JAK2* gene on 9p24.1 are frequently found in HRS and PMBL cells (Green et al. 2010; Rui et al. 2010; Joos et al. 2000). Of note, JAK2 activity has been shown to not only support constitutive STAT phosphorylation but also affect histone H3 phosphorylation in cooperation with JMJD2C, which is co-amplified with JAK2 on 9p24.1 in PMBL and HRS cells

(Green et al. 2010; Rui et al. 2010). Mutations in the *SOCS1* and *PTPN1* genes, encoding inhibitory molecules of the JAK/STAT pathway, are often inactivated or deleted in both types of lymphomas, leading to sustained signaling (Weniger et al. 2006; Gunawardana et al. 2014; Melzner et al. 2005).

HRS cells are characterized by constitutively high activity of AP-1 transcription factors, including JUN, JUNB, and ATF3 (Janz et al. 2006; Mathas et al. 2002). These factors not only support proliferation, growth, and survival of HRS cells (Janz et al. 2006; Mathas et al. 2002), but they also contribute to the induction of immunomodulatory molecules such as galectin(s) and PD L1 in HRS cells and support immune evasion in cHL (Juszczynski et al. 2007; Yamamoto et al. 2008; Rodig et al. 2008; Green et al. 2012). The expression of AP-1 factors has been reportedly associated with lymphomas and other malignancies that are characterized by CD30-positive (malignant) cells (Drakos et al. 2007; Rassidakis et al. 2005). Immunhistochemical analyses of AP-1 factors suggested that JUN, JUNB, and ATF3 are less commonly expressed in PMBL than in HRS cells (Janz et al. 2006; Rodig et al. 2008; Rassidakis et al. 2005).

A clear pathogenetic distinction between PMBL and cHL is the role of EBV. As already mentioned, in approximately 40% of cHL, HRS cells are infected by EBV (Küppers et al. 2012). EBV-encoded genes contribute to the transformation of HRS cells. For example, LMP1 mimics CD40 signaling resulting in NF-κB activation and has been shown to rescue BCR-negative B cells from apoptosis (Küppers et al. 2012). In the pathogenesis of cHL, EBV infection may thus promote survival of the HRS precursor cell. PMBL cells are usually not infected with EBV.

Some histological and immunophenotypical features, the clinical site of manifestation in the upper mediastinum and the identification of a B-cell population in the thymus led to the idea that PMBL originates from a thymic B cell. Recent data showed that thymic B cells immigrated from the periphery, and some were post-GC B cells as they were somatically mutated, and a fraction was class-switched (Yamano et al. 2015). There has been the speculation that cHL of the nodular sclerosis subtype with a primary mediastinal manifestation may also stem from thymic B cells (Shaffer et al. 2012), but there are so far few data to support this idea.

Taken together, HRS and PMBL cells share numerous features, such as the tumor cell phenotype, numerous genetic lesions, and deregulated transcription factors and signaling pathways (Table 7.4). Both HRS and PMBL cells evidently derive from a GC (−experienced) B cell, but the specific cells of origin are most likely distinct.

cHL and ALCL

ALCL is a peripheral T-cell lymphoma and separated in two major subtypes based on the expression of the receptor tyrosine kinase ALK. ALK is not expressed in lymphoma cells of most other T- and B-cell lymphomas or in nonmalignant lymphocytes (Swerdlow et al. 2016). Its overexpression and constitutive activity in ALK-positive ALCL is the consequence of chromosomal translocations, most

frequently the *NPM1-ALK* translocation t(2;5) (p23;q35), but several other translocation partners of *ALK* have also been identified. Presence or absence of ALK expression is critical to not only distinguish between ALK-positive and ALK-negative ALCL, which are otherwise morphologically indistinguishable, but also between ALCL and lymphomas that bear histological and morphological resemblance with ALCL. Cases of cHL with a high proportion of HRS cells and lymphocyte depletion may resemble variants of ALCL. However, HRS cells of cHL do not carry translocations involving the *ALK* gene (Weber-Matthiesen et al. 1996). The distinction between ALK-negative ALCL and cHL requires additional markers such as PAX5, which is expressed by HRS but not by ALCL cells (Foss et al. 1999; Krenacs et al. 1998).

A marker that is defined as immunophenotypic hallmark of HRS and ALCL lymphoma cells is the TNF receptor family member CD30, but the lymphoma cells share numerous additional pathobiological features despite their derivation from different types of lymphocytes (Table 7.4). Similar to HRS cells that lack expression of the BCR, ALCL cells generally lack expression of T-cell-specific markers, including the TCR and CD3 (Swerdlow et al. 2016; Piccaluga et al. 2010). CD8 is generally not expressed, but cytotoxic markers such as perforin and granzyme B (that are occasionally also expressed by HRS cells) are commonly detected (Swerdlow et al. 2016; Piccaluga et al. 2010). While the lymphoma cells in a larger fraction of ALCL express CD4, other have a so-called null phenotype, and a T-cell derivation in these cases is confirmed by detection of rearranged TCR genes in the lymphoma cells (Swerdlow et al. 2016; Piccaluga et al. 2010).

A characteristic feature of HRS cells is their aberrant expression of several non-B-lineage-specific markers, including the T-cell factors NOTCH1 and GATA3, the NK cell marker ID2, and the myeloid cell marker CSFR1 (Küppers et al. 2012; Lamprecht et al. 2010), all of which contribute to the HRS cell phenotype and cHL pathogenesis. Except for GATA3, which is downregulated as a consequence of miRNA-135b overexpression (Matsuyama et al. 2011), all of these factors are also expressed by ALCL cells (Table 7.4). NOTCH1 contributes to proliferation and survival of both HRS and ALCL cells (Jundt et al. 2002, 2008; Kamstrup et al. 2014). Notably, aberrant expression of ID2 contributes to the loss of cell type-specific phenotypic markers both in cHL and ALCL (Mathas et al. 2006, 2009).

Gene expression profiling analyses showed that HRS cells are closer related to ALCL cells than to lymphoma cells of various other B-NHLs, including Burkitt lymphoma, DLBCL, FL, and CLL (Willenbrock et al. 2006). A direct comparison of HRS and ALCL profiles identified numerous differentially expressed genes. For example, CD40, CD80, CD83, CD54, CCL17, LTA, IL6, RAB13, and TNFAIP3 are expressed at higher levels in HRS cells, and ALK, CD45, perforin, and granzyme B are more highly expressed in ALCL cells (Willenbrock et al. 2006). Many of the genes expressed at higher levels in HRS cells than ALCL cells are NF-κB target genes. In ALCL cells, NF-κB activity is also increased in comparison to nonmalignant activated and resting T lymphocytes (Willenbrock et al. 2006; Eckerle et al. 2009; Thorns et al. 2002). The high constitutive NF-κB activity in HRS is partly the consequence of genetic lesions (e.g., gains of *REL*, loss of *TNFAIP3*) that are not

found in ALCL cells, except for genomic gains or translocations of *BCL3*, which can cause its overexpression in HRS as well as ALCL cells (Martin-Subero et al. 2006b; Mathas et al. 2005). Moreover, receptor-mediated signaling via several members of the tumor necrosis factor receptor family or LMP1 in case of EBV-infected HRS cells contributes to NF-κB activity in cHL. EBV infection is not found in ALCL cells.

A further common feature of cHL and ALCL is the constitutive activity of the JAK/STAT signaling pathway. Several highly recurrent genetic lesions in JAK/STAT pathway components as well as autocrine and/or paracrine secretion of cytokines (e.g., IL-13, IL-21) contribute to constitutive activity of this pathway in HRS cells. HRS cells typically show active STAT3, STAT5, and STAT6. In ALK$^+$ ALCL, ALK activity leads to strong constitutive STAT3 activity (Chiarle et al. 2005) that causes a deregulated target gene expression profile on the ALK$^+$ ALCL lymphoma cells (Crescenzo et al. 2015). In ALK-negative ALCL, recurrent activating mutations in the *JAK1* and *STAT3* genes cause constitutive STAT activity, but also in the absence of *JAK1* and *STAT3* mutations, nuclear phosphorylated STAT3 has been detected in ALK-negative ALCL cells, as a consequence of chromosomal translocations, e.g., *NFKB2-TYK2* and *NFKB2-ROS1* translocations (Crescenzo et al. 2015). In addition, JAK/STAT signaling in ALCL cells is supported by autocrine and/or paracrine cytokine secretion (Dien Bard et al. 2009).

The oncogenic signature of ALK$^+$ in comparison with ALK$^-$ ALCL is enriched for HIF1α, IL-10, and H-Ras/K-Ras target genes, while ALK$^-$ ALCL have a slightly enriched PI3K/AKT signaling signature. Nevertheless, also ALK-positive ALCL can have active PI3K/AKT signaling due to the *NPM1-ALK* translocation. The PI3K/AKT pathway is constitutively active also in HRS cells (Dutton et al. 2005; Georgakis et al. 2006). Cross-talk signaling through members of the tumor necrosis factor receptor family (e.g., CD30, CD40, RANK) as well as downregulation of PI3K inhibitory molecules (INPP5D, also known as SHIP1) and RHEBL1 (Tiacci et al. 2012) contribute to PI3K/AKT signaling in HRS cells. In contrast to HRS cells, in about two-third of ALCL cases, PTEN has been reported to be partially or completely lost, supporting active PI3K/AKT signaling in ALCL (Uner et al. 2005).

A further common pathobiological feature of HRS and ALCL cells is their high expression of several members of the AP-1 transcription factor family. Autocrine and/or paracrine stimulation can trigger MAPK/ERK/JNK kinases and contribute to AP-1 activity or alternatively ALK fusions proteins in ALK$^+$ ALCL (Leventaki et al. 2007). Constitutive activity of JUN, JUNB, and ATF3 was found to be critical not only for tumor growth, cell cycle progression, and cell survival (e.g., by regulating cyclins and/or CDK inhibitors) (Janz et al. 2006; Mathas et al. 2002; Staber et al. 2007) but also for modulation of immune responses. AP-1 mediates the expression of galectin-1 in HRS and ALCL cells, which suppresses TH1 cells (Juszczynski et al. 2007; Rodig et al. 2008). Genetic lesions of AP-1 genes are rare in cHL and ALCL. Gene copy numbers of *JUNB* are increased in ALK-positive ALCL; however, JUNB is highly expressed independent of gene copy number in ALK-positive as well as in ALK-negative ALCL lymphoma cells (Atsaves et al. 2014). JUNB is

also a target gene of NF-κB, which can increase its expression. Both HRS and ALCL cells also consistently express IRF4, which is driven by NF-κB and can interact with AP-1 factor at selected promoters (Boddicker et al. 2015; Carbone et al. 2002; Weilemann et al. 2015). It is required for proliferation and survival of the lymphoma cells, and in ALCL cells it regulates the expression of MYC (Boddicker et al. 2015; Carbone et al. 2002; Weilemann et al. 2015).

Taken together, HRS and ALCL cells share many pathobiological features including the loss of phenotypic markers of their cells of origin and the constitutive activities of several signaling pathways and transcription factors. However, the genetic lesions involved in the pathogenesis of these lymphomas are mostly distinct, and the derivation of HRS cells from B cells and of ALCL cells from T cells is a further decisive difference of cHL and ALCL.

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Chapter 8
The Epidemiology of Hodgkin Lymphoma

Wendy Cozen, Behdad Kavianpour, and Thomas M. Mack

Abbreviations

AYAHL	Adolescent and young adult Hodgkin lymphoma
BMI	Body mass index
cHL	Classical Hodgkin lymphoma
CLL	Chronic lymphocytic leukemia
DZ	Dizygotic
EA	Early antigen
EBV	Epstein-Barr virus
GWAS	Genome-wide association study
HIV	Human immunodeficiency virus
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
HRS	Hodgkin-Reed Sternberg
IM	Infectious mononucleosis
LDHL	Lymphocyte-depleted Hodgkin lymphoma
LRHL	Lymphocyte-rich classical Hodgkin lymphoma

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MCHL	Mixed cellularity Hodgkin lymphoma
MM	Multiple myeloma
MZ	Monozygotic
NHL	Non-Hodgkin lymphoma
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
NSHL	Nodular sclerosis Hodgkin lymphoma
OR	Odds ratio
SES	Socioeconomic status
SIR	Standardized incidence rate
VCA	Viral capsid antigen

Introduction

For the epidemiologist, Hodgkin lymphoma (HL) is unique among neoplasms for several reasons. As noted in previous chapters, the malignant giant multinucleated (Hodgkin-Reed Sternberg [HRS]) cells are rare and are surrounded by an infiltrate of nonmalignant lymphocytes and other immune cells. The histopathologic classification, based on the WHO classification from 2001, (Jaffe et al. 2001), divides HL into classical Hodgkin lymphoma (cHL) comprising >95% of the cases, and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). cHL is further subdivided into two common subtypes, nodular sclerosis (NSHL) and mixed cellularity (MCHL), and two uncommon subtypes, lymphocyte rich (LRHL) and lymphocyte depleted (LDHL). Because the HRS cells morphologically resemble giant cells associated with chronic infection and because the symptoms of fever, malaise, and lymphadenopathy are also reminiscent of infection, the condition was originally thought to be an infectious disease. Eventually, the multifocal and progressive nature of the disease with tumors in lymph nodes, spleen, liver, and other organs, resulting in death if untreated, demonstrated its malignant nature.

cHL is unusual because of the epidemiologic risk pattern, with three age-specific modes of incidence that vary by time period, geography, race/ethnicity, gender, and socioeconomic status (SES) (MacMahon et al. 1971; Cozen et al. 1992; Glaser et al. 2008; Correa and O'Conor 1973), all suggesting strong environmental determination. HL is also a highly heritable cancer with an evident genetic contribution (Mack et al. 1995). Progress in understanding this etiologic heterogeneity has been impeded because many investigators initially examined HL as a single disease, with the effect of dampening or misrepresenting subtype-specific associations.

In this chapter, we present an overview of the epidemiology of HL, including descriptive, nongenetic, and genetic epidemiology. We emphasize the unique epidemiologic risk pattern of HL, the importance of early life exposures, the complicated role of Epstein-Barr virus (EBV), the immune-related risk factors, and the strong influence of heritability and genetic factors including that of HLA.

Descriptive Epidemiology: Person, Place, and Time

Understanding the pattern of HL occurrence is essential to understanding fundamental questions about its causation. HL is the most common form of malignant lymphoma affecting people under the age of 30 in economically developed countries, with some variation by race/ethnicity and especially socioeconomic status (SES) (Cozen et al. 1992; Mueller and Grufferman 2006). Figure 8.1 depicts the histologic pattern by age of all cases of HL diagnosed among the roughly 10 million people of Los Angeles County over 20 years. Characteristic of the occurrence in developed countries, especially in North America, is the high incidence of NSHL, the lesser frequency of MCHL, and the relative paucity of cases of the other cHL subtypes and of NLPHL.

Figure 8.2 depicts the age-specific incidence of the two major histologic subtypes, NSHL and MCHL, among males and females in Los Angeles County diagnosed from 1995 to 2014. Note the dramatic prominence of the young adult mode in cases from ages 15 to 35 of NSHL (now labeled adolescent/young adult Hodgkin lymphoma [AYAHL]), the absence of any such young adult mode among cases of MCHL, and the lower incidence of MCHL in females overall and especially at older ages.

In the USA, trends in the incidence of HL over time have been generally stable for at least four decades as reflected nationally by the National Cancer Incidence Surveillance, Epidemiology, and End-Results Program and by the constituent registries in Los Angeles County and California (https://seer.cancer.gov). However, over the last decade, a decrease of no more than 1–2% has occurred in the overall incidence rate per 100,000, but it is not clear whether this represents a true decrease or is a result of misclassification with non-Hodgkin lymphoma (NHL) due to the increasing use of needle biopsies for diagnosis (Glaser et al. 2015).

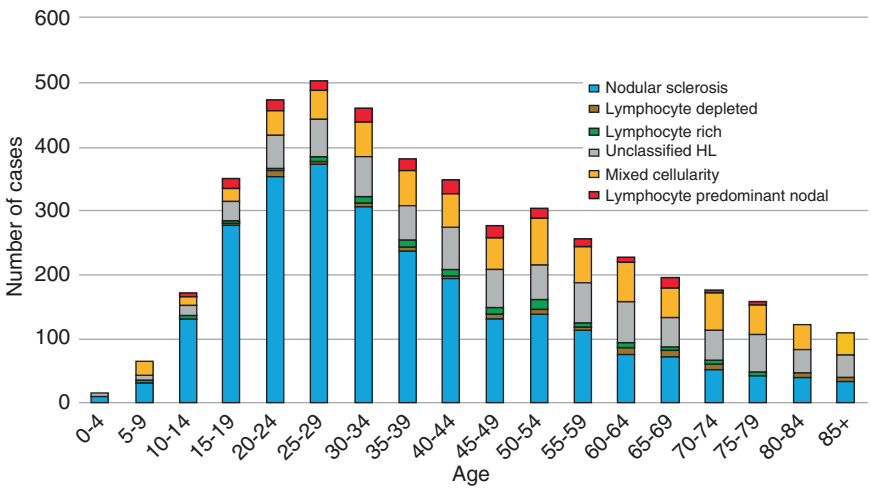

Fig. 8.1 Age-specific number of cases of Hodgkin lymphoma by histologic subtype, Los Angeles County, 1995–2014

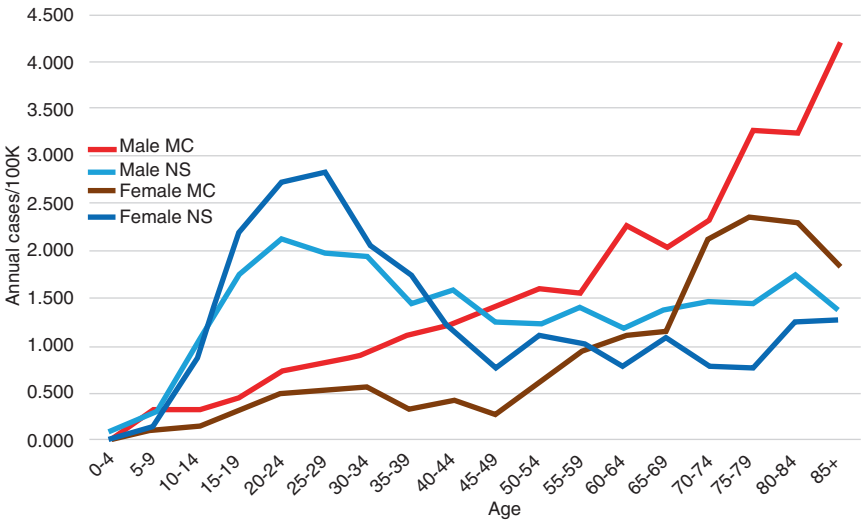

Fig. 8.2 Age-specific incidence rates of nodular sclerosis (NSHL) and mixed cellularity (MCHL) by gender, Los Angeles County, 1995–2014

Figure 8.3 shows gender-specific comparisons in recent age-specific incidence for internationally diverse populations from North and South America, South and East Asia, and Europe (Forman et al. 2014). The AYAHL mode varies by gender and geography/demography and is generally correlated with the level of economic development in the source population, as was first noted by MacMahon (MacMahon 1957). Moreover, as MacMahon also first pointed out, AYAHL cases more often appear first in the mediastinum. These clear differences in the pathology, presentation, and pattern of occurrence are strongly consistent with a substantial difference in etiology (Cozen et al. 1992).

Changes in incidence patterns according to the level of economic development (Fig. 8.3a, b) imply that age-specific incidence of AYAHL should also vary with changes in development over calendar time and place as this demographic characteristic changes. Population-based registration in the USA and Europe began well after the attainment of economic stability, but there are some available pertinent observations. A secular increase in AYAHL was first reported from Connecticut from a cancer registry dating from 1935; records from the first 45 years have been separately published (O'Conor et al. 1973), and those from an additional 35 years are included in the 10-volume International Agency for Research on Cancer series "Cancer in Five Continents" (Forman et al. 2014; Doll et al. 1966) and in the US Surveillance, Epidemiology, and End Results (SEER) Program records (https://seer.cancer.gov). From these data, it appears that the secular increase in incidence rates of AYAHL began in cohorts born in the 1940s.

In middle age, after about age 40, age-specific incidence increases throughout the world (Fig. 8.3a, b), with a pattern similar to that of NHL (Morton et al. 2008) and many solid neoplasms. At advanced ages, the average level of incidence and the rate of increase with age are greater among men than among women and greater among US whites than among Europeans or Asians. They are also greater among

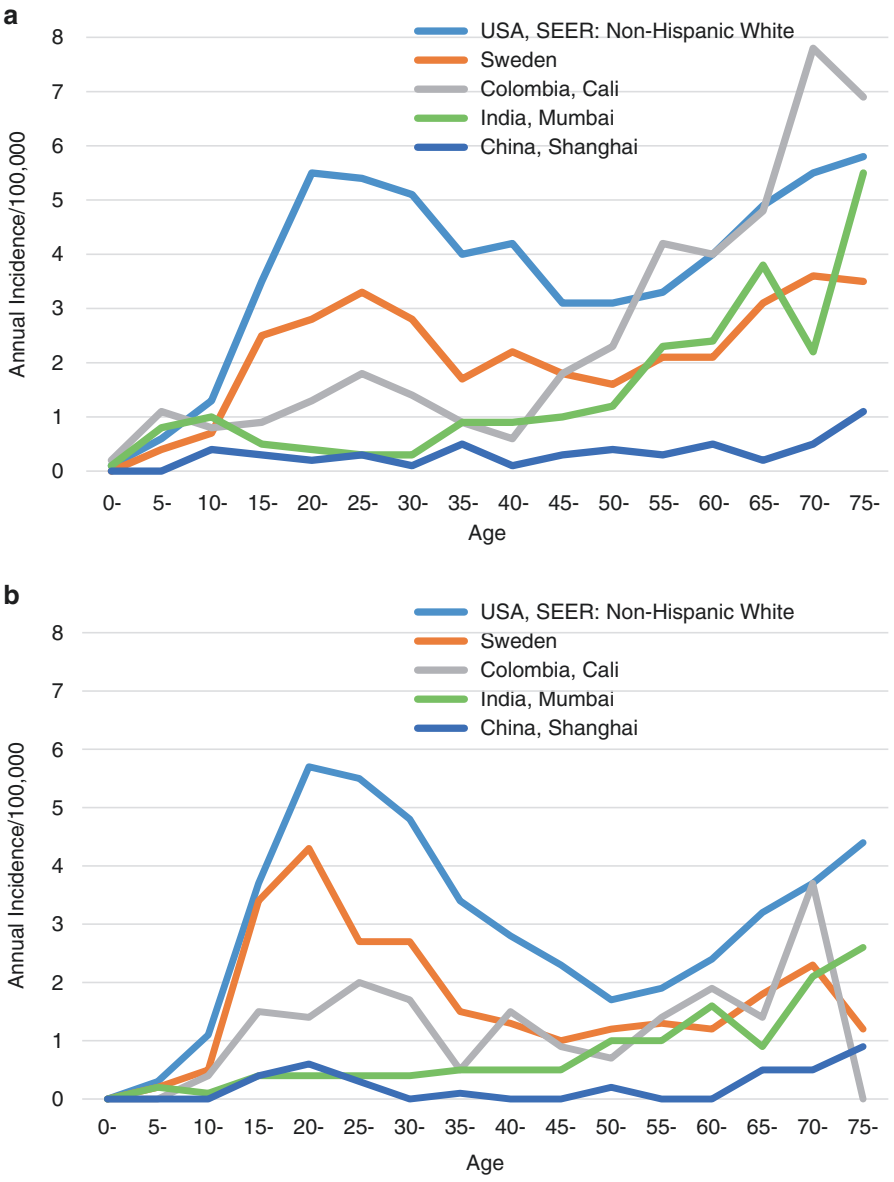

Fig. 8.3 Age-specific incidence rates of Hodgkin lymphoma in males (**a**) and females (**b**) by international population, 2003–2007 (Forman et al. 2014)

non-Hispanic whites than among Hispanic whites or African-Americans (data not shown). Of particular interest is the extremely low rate seen throughout advanced age in residents of Shanghai, a pattern characteristic of other B-cell lymphomas among East Asians (Gale et al. 2000).

At the other end of the age spectrum, the earliest incidence mode has occurred historically in the first decade of life among children in less developed countries

(Correa and O'Conor 1971, 1973). Childhood cases are more similar in histopathologic subtype to those of older persons than to those with AYAHL, usually being MCHL. A hint of the earlier mode is seen among male children from both Cali and Mumbai (Fig. 8.3a, b). Recent reports from Brazil (Ferreira et al. 2012) and Mexico (Rendon-Macias et al. 2016) have contrasted the patterns of childhood and adolescent HL by levels of regional economic development within those countries, with lower rates and a larger proportion of NSHL in the more economically developed regions and higher rates and more MCHL in the less developed regions. As these reports indicate, the early childhood mode of MCHL is gradually diminishing worldwide (MacFarlane et al. 1995), leaving a low baseline frequency (Hjalgrim et al. 2016). In between the age extremes, NSHL predominates, accounting for the large and growing AYAHL mode, with incidence rates roughly equal among males and females.

Also included in IARC volumes are rates from Slovenia, of special interest because, unlike most of the other western European countries, the timing of a sudden social upturn was reflected in the rapid appearance of the AYAHL mode (likely to be mostly NSHL) (Fig. 8.4). The age-specific rates of AYAHL in Slovenia appear flat in 1975 but dramatically increase by 2005, with no such secular increase evident in HL at older ages. Similar increases in AYAHL rates have suddenly occurred in Finland and Estonia (Forman et al. 2014). Over a period of more gradual SES development in Asia, similar young adult incidence modes have been documented, i.e., in Singapore (Hjalgrim et al. 2008), as well as in Japan (Chihara et al. 2014) and Korea (Koh et al. 2013), in spite of the overall lower incidence of B-cell tumors in these populations (Morton et al. 2006). Even among US African-Americans and Hispanics, the beginning of similar changes in AYAHL incidence recently has been observed in Los Angeles (unpublished). These increases over calendar time in relation to SES are presumed largely due to increases in the rate of NSHL.

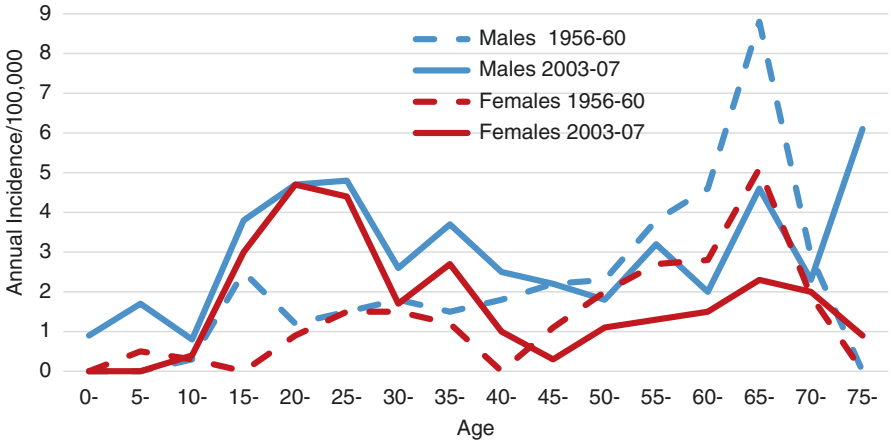

Fig. 8.4 Age-specific incidence rates of Hodgkin lymphoma in Slovenia by gender and period of occurrence

Epstein-Barr Virus

EBV is a ubiquitous DNA virus with many latent, incompletely understood, genes and is a very successful human parasite (Crawford 2001). EBV infects most residents of developing countries as young children, usually without symptoms, but residents of developed countries are often infected a decade or so later, occasionally resulting in the clinical syndrome of infectious mononucleosis (IM), presumably in those who are genetically susceptible (McAulay et al. 2007). IM is familiar to generations of teenagers and can be identified by a simple blood test (Crawford 2001). EBV has a crucial biological association with multiple malignancies, including epithelial carcinoma of the nasopharynx, Burkitt lymphoma, other NHL, and HL (Coghill and Hildesheim 2014). In the case of nasopharyngeal carcinoma, causal interactions with both genetic and behavioral determinants are known (Hildesheim and Levine 1993), and in the case of other lymphomas, the association appears under circumstances of reduced immune competence (MacMahon et al. 1991; Clarke et al. 2013).

During EBV infection, a variable proportion of B lymphocytes become and remain latently infected with the virus. These B-cells can both circulate and remain covertly within the germinal centers of lymph nodes (Mohamed et al. 2014). Following infection, after a short period of early convalescence and recovery, antibodies, to certain viral antigens, such as Epstein-Barr Nuclear Antigen 1 (EBNA1), are maintained more or less permanently (Crawford 2001), and other antibodies, such as those against viral capsid antigen (VCA), are also present. The virus then exists in B lymphocytes in a latent-lytic cycle, with different patterns of latency measured by different EBV gene expression patterns, by corresponding antibodies. T-helper Type 1 (T_H1) immunity keeps EBV latent, but when T_H1 immunity temporarily wanes, the virus can become reactivated leading to a lytic stage, which can promote oncogenesis through a number of mechanisms (Khanna and Burrows 2000). During initial infection, antibodies to EBNA 2 predominate over antibodies to EBNA 1, but with recovery, this ratio reverses. Persistent higher anti-EBNA 2 compared to anti-EBNA 1 antibody levels may indicate chronic infection. When EBV is activated and replicating, the number of EBV copies (viral load) can be measured by PCR in serum (Gallagher et al. 1999), plasma (Ghandi et al. 2006) or whole blood (Gulley and Tang 2010). Thus, both prior infection and current activity of EBV can be measured using a variety of techniques.

In a minority of HL patients (~40% overall), EBV DNA is clonally present as an episome in HRS cell DNA, strongly suggesting that the cells were infected prior to malignant expansion (Weiss et al. 1989, 1991). Tumors demonstrating EBV-positive HRS cells are classified as EBV-positive HL. In general, risk factors for EBV-positive HL correspond to those for MCHL, including a relative increase among males, those lower of SES, and those diagnosed at age extremes (<8 or >50 years old) (Glaser et al. 2008; Flavell et al. 1999a; Chang et al. 2004a). Measures of maternal deprivation (Flavell et al. 1999b) and birthplace outside the USA (among US Hispanic cases) (Glaser et al. 2014) also predict EBV-positive HL (relative to EBV-negative HL). In acquired immunodeficiency conditions such as infection with the human immunodeficiency virus (HIV)/AIDS and transplant recipients, HL is almost always EBV-positive (Glaser et al. 2003a; Quinlan et al. 2010).

Serological evidence of past infection is present in essentially all cases of MCHL and most (but not all) cases of NSHL (some are known to be infected after HL develops) (Gallagher et al. 2003). An important set of studies conducted by Mueller and colleagues demonstrated an association between elevated titers of antibodies to EBV and (years) later risk of developing HL. First, historical samples from a blood bank in Norway were linked to the cancer registry to identify subjects who later developed HL. Higher than average antibody titers to IgG VCA, IgM VCA, and Ig (early antigen) EA strongly predicted future HL (Mueller et al. 1989). The results were later confirmed in a study of 128 cases in a nested case-control study within a cohort of US military active duty personnel (Levin et al. 2012) but only in EBV-positive cases. In a case-control study, higher IgG and IgA antibody titers to VCA and EA and an EBNA1/EBNA2 ratio <1.0 were associated with older age, male sex, lower educational level, smoking, and MCHL (vs. NSHL) (Chang et al. 2004a). The associations were attenuated when the variables were adjusted for each other, suggesting that the variables are correlated.

A number of investigators (Connelly and Christine 1974; Munoz et al. 1978; Miller and Beebe 1973; Rosdahl et al. 1974; Carter et al. 1977; Kvale et al. 1979; Gutensohn and Cole 1981; Bernard et al. 1987; Serraino et al. 1991; Mack et al. 2015) consistently found an association between a history of IM and risk of HL, mainly in the AYA group, the age at which the majority of cases are EBVnegative NSHL. As it became possible to assess the presence of EBV DNA in tumors, Hjalgrim and colleagues (Hjalgrim et al. 2003) conducted a population-based registry linkage study and found up to a 20-fold increased risk for EBV-positive HL within 5 years of documented IM, but no increase in risk for EBV-negative HL. A large well-conducted Scandinavian case-control study by the same group confirmed the lage discrepancy in the association between IM and EBV-positive and EBV-negative HL (Hjalgrim 2007a). Another case-control study in the U.K. (Alexander et al. 2003) found a less clear distinction, with IM conferring a significant 3-fold risk of EBV-positive and a non-significant 1.9-fold risk of EBV-negative HL among young adults. A significant association was observed for EBV-positive and EBV-negative HL separately when all ages were combined. Finally, a U.S. study (Chang et al. 2004a) found no difference in association with IM when EBV-postive and EBV-negative cases were compared to each other, but the study lacked power. In summary, there is strong evidence for an association between IM and EBV-positive cHL specifically, but an association with EBV-negative HL is less certain. This finding is paradoxical because IM occurs in adolescence and young adulthood and reflects delayed primary EBV infection associated with higher SES, factors more commonly associated with EBV-negative compared to EBV-positive HL (Flavell and Murray 2000).

Mueller and colleagues found that HL cases were more likely to have an EBNA1/EBNA2 ratio <1.0 compared to their unaffected siblings (Mueller et al. 2012). Similarly, the unaffected siblings with a history of IM also had a low EBNA1/EBNA2 compared to those who did not report IM. There were too few cases to examine the effect of histological subtype, and EBV tumor status was not provided; nevertheless it is clear from these studies that a dysregulated response to EBV after primary infection is associated with development of HL.

While peripheral blood mononuclear cell, serum, or plasma EBV DNA has been detected significantly more often in EBV-positive compared to EBV-negative cHL

patients (Gallagher et al. 1999; Ghandi et al. 2006), it has not been systematically examined by histological subtype (Gallagher et al. 2003).

There is evidence of familial (Rostgaard et al. 2014) and genetic determinants of IM, including HLA class I genetic variants (McAulay et al. 2007), and of genetic determinants for antibody levels to EBNA1 (Rubicz et al. 2013). Besson and colleagues showed that the correlation between EBV viral load and levels of Ig against VCA was similar in HL patients and their relatives (Besson et al. 2006a). Although there is abundant evidence of HLA-specific risk alleles associated with EBV-positive cHL (reviewed in detail below in the genetic section), these appeared to be independent of IM (Johnson et al. 2015).

In summary, children with HL occurring before 8 years of age have invariably been infected with EBV and usually have the MCHL subtype and EBV-positive disease, similar to HL cases occurring in older adults. There appears to be a strong association between poor control of and/or response to EBV, including IM, and EBV-positive HL. It is still not clear whether this association reflects a more generalized immune dysregulation resulting in an ineffective immune response to EBV and HL as parallel consequences or whether actively replicating EBV itself is the causal agent. In contrast, there is little evidence for a role for EBV in the etiology of AYAHL EBV-negative NSHL (the majority of cases in developed countries).

Socioeconomic Status (SES)

SES can be characterized at the individual or at the population level according to the characteristics of the place of birth or residence at diagnosis based on the average local levels of income and education. It is important to realize that even within a given community, economic development does not impact all residents equally or at the same rate. Therefore, disease risk associated with different SES levels will be distributed through the community and will create a gradient of risk. Higher SES is strongly and positively associated with AYAHL but is inversely associated with HL in childhood and at older ages (Cozen et al. 1992; Gutensohn and Cole 1981; Clarke et al. 2005; Glaser et al. 2002; DeLong et al. 1984). SES is an index so generic that it provides a measure of almost any aspect of human circumstance or lifestyle, indicating the direction of a gradient but not the underlying causal exposure. Individual-level factors reflecting SES, such as family income (Abramson 1974), single family housing and higher maternal education (Gutensohn and Cole 1981), educational attainment (Serraino et al. 1991), intelligence quotient (LeShan et al. 1959), prestigious occupation (Vianna et al. 1974), and military rank (Cohen et al. 1964) also predict AYAHL. Maternal deprivation was associated with a higher risk of EBV-positive HL compared to EBV-negative HL in a UK study (Flavell et al. 1999b).

Body Size and Reproductive Factors

Height and higher body mass index (BMI) in association with HL have been repeatedly investigated, usually with respect to AYAHL, with most studies reporting

modestly elevated levels of association (Mack et al. 2015; Li et al. 2013; Paffenbarger et al. 1977; Murphy et al. 2013). A recent meta-analysis (Larsson and Wolk 2011) provided stronger evidence of a positive relationship. Height alone either in childhood (Keegan et al. 2006; Isager and Andersen 1978) or adulthood (Mack et al. 2015; Murphy et al. 2013) has been more consistently associated with HL than has BMI. There is some heterogeneity of the association by age: Positive associations have generally been found for both BMI and height among younger cases and inverse associations for older cases (Li et al. 2013; Keegan et al. 2006). Birth weight has not predicted subsequent HL diagnosis (Isager and Andersen 1978; Barker et al. 2013; Langagergaard et al. 2008); however a high rate of fetal growth was positively associated in a study of 943 Swedish HL cases diagnosed prior to age 38 (Crump et al. 2012). Strenuous physical exercise was modestly inversely related to risk (Keegan et al. 2006). An association between AYAHL and greater consumption of saturated fat and lower consumption of monounsaturated fat was suggested (Gao et al. 2013). In the same study, HL cases showed a special fondness for sweets compared to controls (Epstein et al. 2015). Both adult height and BMI reflect cumulative nutrition, likely to reflect childhood affluence, although alternatively, each could also reflect increased levels of B-cell activating growth factors. A nested case-control study of male AYAHL cases permitted the adjustment of both BMI and height by measures of childhood family affluence and found that the latter was likely to be the operative determinant (Mack et al. 2015).

Reproductive behavioral measures, including low parity (Kravdal and Hansen 1993), late age at first pregnancy, cumulative breastfeeding experience (by the case), and hormone replacement therapy (Glaser et al. 2003b), predict HL risk. The associations could be explained by greater access to medical care, a biologic endocrine- or immune-related exposure, or higher SES. Maternal breastfeeding has also been found to predict childhood HL (Davis et al. 1988; Grufferman et al. 1998).

Measures of Early Life Microbial Exposure

Family Structure

The number of siblings emerged as a potential explanation for the association with high childhood SES and is often interpreted as a surrogate for microbial exposures in early life (with more exposure corresponding to more, closely spaced, siblings). Even after accounting for SES, sibship size has been repeatedly linked to HL in adolescence and young adulthood, with risk decreasing as the number of siblings increases (Mack et al. 2015; Westergaard et al. 1977; Chatenoud et al. 2005; Bernard et al. 1987; Bonelli et al. 1990; Altieri et al. 2006) (Fig. 8.5). In contrast, among cases occurring in early childhood, the opposite association is seen, with an increasing number of siblings linked to increasing risk (Westergaard et al. 1977). Sibship size has not been carefully studied in relation to HL among the elderly.

In addition to sibship size, AYAHL occurrence seems to be related to birth order and, within birth order, to the interval in age between siblings (although even in the

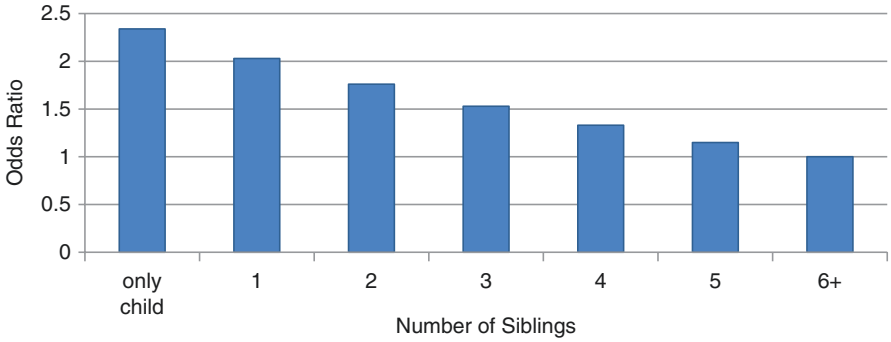

Fig. 8.5 Odds ratio of young adult Hodgkin lymphoma according to sibship size

largest study to examine these factors, sample sizes were not large after adjustment) (Mack et al. 2015). In case-control studies conducted with subjects ascertained since 2004, the association with sibship size has attenuated, but attendance at day care early in life was protective (Chang et al. 2004b, c).

Childhood Infections

Another marker of childhood microbial exposure is the recalled history of common infections of childhood. AYAHL patients have generally reported fewer than expected infections such as measles and mumps at early ages compared to controls (Mack et al. 2015; Paffenbarger et al. 1977; Gutensohn and Cole 1977; Glaser et al. 2005; Alexander et al. 2000). In contrast, HL cases occurring in early childhood have reported more infections compared to controls (Linabery et al. 2014). In AYAHL patients, these observations have been taken to imply a lack of exposure (generally or specifically) to a ubiquitous infection in childhood, with increased susceptibility in adolescence or beyond. In a Jerusalem birth cohort, HL diagnosed at <40 years of age occurred less commonly among infants hospitalized in the first year of life for a respiratory infection, while NHL diagnosed during the same follow-up period was significantly more common (Paltiel et al. 2006).

Tonsillectomy and Appendectomy

A marker of childhood risk that has been frequently reported but is difficult to interpret is a history of tonsillectomy. Studies have been inconsistent (Grufferman and Delzell 1984), but it seems likely (Vestergaard et al. 2010; Mueller et al. 1987) to be a predictor of AYAHL. In a cohort study of >55,000 tonsillectomized patients in Sweden, having a tonsillectomy before age 12 was associated with an increased risk of HL diagnosis prior to age 25 (1.7 cases expected, 7 observed, standardized incidence ratio [SIR]= 4.1) (Liaw et al. 1997). There was a 20-fold increased risk associated with having a tonsillectomy before age 5, based on an even smaller number of cases.

With respect to the significance of this association, it is possible that HL cases have some underlying immune susceptibility resulting in many respiratory infections and are therefore more often candidates for tonsillectomy. This explanation is consistent with findings from the Children's Oncology Group study for HL cases diagnosed under 14 years (Linabery et al. 2014), but not with the observations in the Jerusalem birth cohort study cited above (Paltiel et al. 2006) (i.e., severe respiratory infections were protective for HL occurring up to age 39 years). A higher rate of tonsillectomy could also be correlated with higher childhood SES and therefore more access to medical care, which includes this common childhood surgery that has always had imprecise indications. However, this explanation is less likely to explain the results in the Swedish tonsillectomy cohort study (Liaw et al. 1997) since access to care is available equally to all in Sweden. Finally, regardless of the indication for tonsillectomy, it does have the effect of removing a lymphoid barrier to infections, which could have an impact on susceptibility.

Appendectomy has also occasionally (Henderson et al. 1979) albeit inconsistently (Silingardi et al. 1982) been found to be positively associated with AYAHL. While a Swedish linkage study (Cope et al. 2003) found the expected number of appendectomies preceding HL diagnoses, a study of 188 twin pairs discordant for AYAHL showed that appendectomy was more common among the HL-affected twin compared to their unaffected co-twin, a comparison naturally controlled for genotype and childhood SES (Cozen et al. 2009a).

One possible explanation for a true association with tonsillectomy and/or appendectomy is a tendency for a hyper-inflammatory response in lymphoid tissue, regardless of the trigger, among cases.

In addition to evidence of specific infections or surgeries related to them, evidence of general microbial exposure can be assessed in specific study circumstances. Disease-discordant twins make excellent subjects for this line of research because they can recollect, and therefore validate, each other's childhood exposures. In the aforementioned study of AYAHL-discordant co-twins, the twin who had more oral exposures in infancy or early childhood, indicated by a history of relatively more thumb- and finger- sucking and the use of a pacifier, was significantly less likely to develop AYAHL by a factor of 50–80% (Cozen et al. 2009a). In a follow-up study, the fecal microbiome of the HL-affected twin had fewer unique microbial taxa than that of their unaffected co-twin (Cozen et al. 2013). Lower fecal microbial diversity is linked to a higher level SES in childhood (Björkstén et al. 1999), fewer older siblings (Laursen et al. 2015), a T-helper Type 2 (Th-2) skewed immune response, and a higher risk of atopy (Riiser 2015), factors which overlap to some extent with characteristics of AYAHL.

Adult Lifestyle

A large number of investigators have studied the link between cigarette smoking and HL over the years with mixed results. Since 2005, two hospital-based case-control studies (Monnereau et al. 2008; Gallus et al. 2004) found no increased risk. However, 3 cohort studies (Kroll et al. 2012; Nieters et al. 2008; Lim et al. 2007) found relative risks of 1.5, 2.1, and 2.3, 3 population-based case-control studies

(Hjalgrim et al. 2007b; Briggs et al. 2002; Glaser et al. 2004) found OR's of 1.6 and 1.8, and 2 large multicenter pooled case-control studies (Besson et al. 2006b; Kamper-Jorgensen et al. 2013) found odds ratios (ORs) of 1.4 and 1.2. In the latter, control for known determinants was generally accomplished, a dose-response relationship was observed, and risk was increased in men, in the elderly, and in those with MCHL and/or EBV-positive HL. In some studies, risk diminished with time since last exposure. Two meta-analyses based on 17 (Castillo et al. 2011) and 50 (Sergentanis et al. 2013) reports, respectively, found summary increased ORs of 1.2 and 1.4 and a suggestion that the risk was specific to EBV-positive HL.

Studies of alcohol consumption adjusted for tobacco use are remarkably consistent in showing a substantial protective effect, especially among non-smokers. In most instances, all adult cases were included (Monnereau et al. 2008; Kroll et al. 2012; Lim et al. 2007; Gorini et al. 2007; Klatsky et al. 2009; Deandrea et al. 2007; Tramacere et al. 2012; Ji et al. 2014). Pain experienced in untreated HL after alcohol consumption may impact its use (Atkinson et al. 1976) and thus reverse causality cannot be ruled out. In a study of the long-term use of anti-inflammatory agents, including COX-2 inhibitors, low-dose aspirin was suggestively protective against HL, but significance was marginal (Chang et al. 2010). Also seemingly protective was exposure to solar (UV) radiation, as measured by a history of sunburn and of recalled sun exposure (Smedby et al. 2005). This initial study was followed by others (Petridou et al. 2007; Grandin et al. 2008; Boffetta et al. 2008), using different measures of UV exposure and with disparate results. These were all included in a pooled analysis in an attempt to explain diverse results by examining subgroups of HL that concluded that protection was especially pertinent to EBV-positive HL (Monnereau et al. 2013). More recently, studies of HL and a latitude gradient in Australia (Van Leeuwen et al. 2013) and the U.S. (Bowen et al. 2016) adjusted for age, (and in the U.S.) and race, but not social class, are supportive of a protective effect with a dose-response, protective against NSHL, MCHL and in the U.S., all other cell types.

Clustering

There has long been interest in variation in the frequency of HL in time and space. Geographic differences in the incidence of HL between US cities are substantial (Glaser 1987). An early report of seasonality of the birth dates of HL patients (Langagergaard et al. 2003) was not confirmed by a second investigation (Crump et al. 2014). Several investigators have reported non-random variation in season at diagnosis (Newell et al. 1985; Westerbeek et al. 1998; Douglas et al. 1998; Chang et al. 2005a) generally finding peaks in the late winter or early spring, especially among young adults. This pattern is consistent with seasonality found for IM incidence (Douglas et al. 1996). HL mortality has also been linked to season of diagnosis (Porojnicu et al. 2005). A recent comprehensive report based on SEER data found an association between early spring (March) and latitude and HL incidence and mortality within 3 years of diagnosis (Borchmann et al. 2017). The authors suggest, as have others, that a reduction in Vitamin D production due to the low solar flux in the preceeding months could explain the seasonal effect. Although the observations appear

sound, they must be interpreted with caution, especially with respect to etiology. A long latency between causal exposure and presenting symptom is the norm in cancer, including HL. In the registry linkage study, Hjalgrim and colleagues (Hjalgrim et al. 2003) estimated a median incubation period for EBV-positive HL following IM of 4.1 years (95% CI 1.8-8.3 years), inconsistent with a short latency period. Moreover, a similar early spring excess in diagnoses of etiologically distinct non-Hodgkin lymphoma was found using the same data source and similar methodology (Koutros et al. 2009). Borchmann et al. (Borchmann et al. 2017) and others (Chang et al. 2009) suggest an alternative hypothesis that perhaps the early spring diagnostic peak could be due to infection with seasonal viruses that bring undiagnosed younger patients in contact with medical care, where the HL is then discovered incidentally. However the association between the early spring diagnosis and mortality requires additional investigation and explanation.

Allegations of geographically localized high risk (Vianna et al. 1971) have been unconvincing (Grufferman et al. 1979), but remaining suspicions of an infectious etiology have led to multiple studies of the tendency of HL to cluster in time and space (Alexander et al. 1989; Glaser 1990), as would be produced by a common-source or person-to-person infection (Grufferman 1977). Reports of cases with social connections are to be expected by chance, and some have been more widely publicized than closely scrutinized (Vianna et al. 1971). To result in a time-space cluster, cases would have had to have a common time-space exposure or a time-space link between cases in successive generations. Clustering of a rare condition like HL would be difficult to detect, since the need for statistical power mandates a large geographic and chronological range and this requirement is likely to lengthen the average time-space distance between cases beyond biological credulity. Most of those who have studied clustering have been too willing to assume that the incubation period between putative exposure and clinical onset is short, as it is with most familiar person-to-person viral infections. Malignancy takes time to progress to symptomatic disease, and the longer the incubation period, the more probable that the geographic link between cases will have been lost if presumptively based on a common residence or workplace onset. Most importantly, investigators have failed to recognize that an underlying association with SES will invariably produce an apparent geographic clustering (Alderson 1982; Greenberg et al. 1983). Two studies that did consider potential time-space connections searched for common social contact (Smith et al. 1973; Matthews et al. 1984). These investigators found no difference in the observed frequency of common social contact among case-case, case-control, and control-control pairs. These studies provide some evidence against person-to-person, congenital, or common-source infection.

Occupation and Environment

In a search for environmental determinants of HL, links between occupation and HL have repeatedly been investigated. With person-to-person transmission in mind, a modest increase in risk of HL among physicians (Vianna et al. 1974) was found but

is also consistent with their higher SES (Grufferman et al. 1976; Smith and Kinlen 1974). In that context, no convincing increase in risk has observed among teachers (Grufferman et al. 1976), who also would have increased levels of exposure to cHL by virtue of exposure to students.

Historically, the most frequent finding to be reported was an association with woodworking occupations (Milham and Hesser 1967; Abramson et al. 1978). Less attention has been given to this exposure since an IARC review of the relationship failed to confirm a suggestive pattern (Demers and Boffetta 1998).

A significant inverse association between occupational exposure to allergens and risk of HL was seen in the EPILYMPH multi-country case-control study (Espinosa et al. 2013). With an eye toward the possible effects of immunogenic high molecular weight molecules, occupations involving exposure to materials such as latex were found to convey substantial increased risk (Kogevinas et al. 2004). Another set of studies concentrated on chemicals, with studies of chemists, workers in the chemical industry, and those exposed to a variety of commercial chemicals; however, no individual chemical or class of chemicals were shown to consistently predict occurrence (Grufferman et al. 1976; McCunney 1999). More recently, HL has been found in single reports to be weakly associated with exposure to the pesticide chlorpyrifos, moderately associated with exposure to ionizing radiation from uranium (Karunanayake et al. 2009), and substantially and significantly more common among gas-station workers (Neasham et al. 2011). Childhood MCHL but not NSHL has been found associated with household pesticide use during pregnancy (Rudant et al. 2007). No confirmatory studies have yet appeared for any of these reports.

Comorbid Conditions

Other conditions linked to HL can be grouped into several categories: conditions producing risk of HL by virtue of immunosusceptibility, conditions caused by HL and/or treatment for HL, and conditions likely to share environmental/genetic determinants.

In the first category, the major example is HIV infection (Biggar and Rabkin 1996), which produces a more than tenfold increase in risk of HL (Goedert 2000; Grulich and Vajdic 2015), and is almost always EBV-positive (Rapezzi et al. 2001). Unlike NHL, the risk of HL decreases with decreasing CD4 counts, and this decrease is especially precipitous for NSHL, which does not occur in patients with CD4 counts <50 cells/μL (Biggar et al. 2006). Cases also occur within a few months of first treatment with combination anti-retroviral therapy (cART) (Lee et al. 2016). Patients receiving transplantation have a fourfold increase in risk of HL (Quinlan et al. 2010), particularly if the transplant occurs at an early age (Clarke et al. 2013). The majority histologic subtype in both acquired immunodeficiencies is MCHL, however NSHL is increasing among HIV+ persons treated with cART with higher CD4 counts (Biggar et al. 2006; Jagadeesh et al. 2012).

In the second category, HL and/or treatment for HL increases the risk of other hematological neoplasms and solid tumors, and these risks persist long after treat-

ment (Swerdlow et al. 2011; Castellino et al. 2011). Those cancers which occur in excess include NHL, both lymphoid and myeloid leukemias, viral carcinomas such as that of the cervix, and carcinomas of the lung, kidney, and especially breast and thyroid, probably because of proximity to the radiation therapy field (Swerdlow et al. 2011; Kaldor et al. 1990; Schonfeld et al. 2006). For example, HL patients diagnosed at 25–34 years old and treated with chemotherapy alone have a 33-fold increased risk of leukemia and those treated with both chemotherapy and radiation have a 17-fold increased risk of non-Hodgkin lymphoma (Swerdlow et al. 2011). A Scandinavian sudy estimated excess risk of acute myelocytic leukemia within the first 10 years following HL diagnosis at 7.9%, more pronounced in patients diagnosed prior to 1984 compared to after, probably due to modifications in chemotherapy (Schonfeld et al. 2006) A genetic variant in the gene *PRDM1* is associated with increased susceptibility to radiation-associated second malignancies in HL patients, especially those receiving radiation therapy under age 20 (Best et al. 2010). Nonmalignant comorbidities related to both chemotherapy and radiation therapy include cardiomyopathy, pulmonary fibrosis, esophageal strictures, and reflux (Castellino et al. 2011).

Of more etiological interest is the third category of independent comorbidity. Case-control studies have identified a list of chronic diseases affecting both those with HL and their first-degree relatives more often than would be expected by chance. The descriptive commonality between HL and multiple sclerosis has long attracted attention (Newell 1970; Vineis et al. 2001; Kurtzke and Hamtoft 1976). A modest but significant increase in the frequency of HL and multiple sclerosis (from the population-based Danish registry) in the first-degree relatives of cases of the other condition has been reported, and anecdotal examples of individuals affected by both conditions have been noted (Hjalgrim et al. 2004). An attempt to confirm this association using hospital discharges in both Sweden and Denmark confirmed the Danish findings but found a modest and nonsignificant association in Sweden (Landgren et al. 2005a). More recently, modest evidence of genetic overlap was observed (Khankhanian et al. 2016), discussed in more detail in the genetic section below.

Using the linkage systems of Denmark and Sweden, a Scandinavian case-control study based on serology and personal interview found an association between personal histories and serological evidence of rheumatoid arthritis and HL, especially for EBV-positive and/or MCHL. The risk increased with subgroups of more definitive or more advanced rheumatoid arthritis (Hollander et al. 2015). In 2006, Swedish investigators selected a roster of population-based HL cases, identified their first-degree relatives, and searched hospital discharge registries for cases of autoimmune disease among them (Landgren et al. 2006). Cases of HL themselves had significantly increased lifetime risks of systemic lupus erythematosus, rheumatoid arthritis, sarcoidosis, and immune thrombocytopenic purpura, and among the relatives of cases, significant increased risks were found for sarcoidosis and ulcerative colitis, albeit based on small numbers. In a separate report of the same study design (Landgren et al. 2005b) but based only on the Swedish linkage system, the same investigators found a substantial deficit of type II diabetes mellitus in both HL cases and their first-degree relatives.

In 2014, a second team addressed the same question beginning with Swedish cases of autoimmune disease and identifying the cases of HL occurring among them

and their relatives (Fallah et al. 2013). Again mostly with small numbers, this team found significant increases of NSHL compared to expected, among cases of sarcoidosis, immune thrombocytopenic purpura, rheumatoid arthritis, multiple sclerosis, and psoriasis. MCHL appeared in significant excess in cases of rheumatoid arthritis, sarcoidosis, systemic lupus erythematosus, immune thrombocytopenic purpura, Sjogren syndrome, and polymyositis/dermatomyositis. In addition to persons affected with certain of these same conditions, cases of HL of unspecified histology occurred among cases of Crohn's disease. Having a parent with an autoimmune disease increased the risk of EBV-positive but not EBV-negative HL in children under 15 years old (Linabery et al. 2014). The most recent effort, again from Sweden and Denmark, examined the risk of autoimmune disease in a case-control study of HL and found a strong (>twofold risk) association with rheumatoid arthritis and EBV-positive MCHL (Hollander et al. 2015). Such autoimmune conditions may share genetic determinants, may be commonly influenced by EBV infection, or may share unknown environmental determinants of aberrant immune function.

T-helper Type 2 (T_H2)-related immune abnormalities, such as asthma and hay fever, have been inconsistently linked to HL (Hollander et al. 2015; Martínez-Maza et al. 2010). Exceptions include a strong and significant positive association between a childhood history of eczema and risk of AYAHL in twin pairs discordant for the neoplasm (Cozen et al. 2009b) and between allergy in a parent or sibling and risk of childhood (under age 15) EBV-negative HL (Linabery et al. 2014).

A hallmark of HL is evidence of immunological dysfunction. As above, abnormalities in T-cell function, indicative of atopy or poor T-cell response, were more prevalent in relatives of HL cases compared to unrelated individuals (Merk et al. 1990). A twin study found higher levels of interleukin-6 (IL6) (Cozen et al. 2004) and lower levels of interleukin-12 (IL12) (Cozen et al. 2008) in the unaffected identical co-twins of cases (surrogate cases) compared to controls, suggesting a genetically determined immunophenotype. A recent case-control study nested in a cohort of US military recruits confirmed that higher IL6 levels prior to diagnosis predicted future development of HL, in addition to higher levels of CD30 (Levin et al. 2017). In the same study, detectable (vs. undetectable) levels of interleukin-10 (IL10) years prior to diagnosis predicted risk of EBV-positive HL only. Thus, the association between immune-related abnormalities and risk of HL and HL subsets provides evidence for the highly immunological nature of HL etiology.

Heritability, Familial, and Genetic Risk

Familial Risk

There is general consensus that HL is a heritable cancer. Early studies reported a three- to seven-fold higher risk of HL in relatives of cases (Grufferman et al. 1977; Kerazin-Storrar et al. 1983). and a nine-fold risk of HL in persons with a family history (Bernard et al. 1987). Subsequently, investigators in Sweden (Altieri and Hemminki 2006), Israel (Paltiel et al. 2000) and Iceland (Amundadottir et al. 2004)

made use of cancer registries and/or large populationbased family registries to assess familial risk by comparing observed to modeled expected incidence, and found a three- to six-fold risk for HL individuals with affected first-degree relatives, or conversely, for the risk of HL in first-degree relatives of case. In Utah (Kerber and O'Brien 2005), Sweden (Goldin et al. 2004 ; Chang et al. 2005b; Goldin et al. 2009), and in all 5 Nordic countries combined (Kharazmi et al. 2015), the frequency of HL occurrence among first-degree relatives of cases was compared to the frequency among those of controls, again with risks of HL given an affected relative of three- to nine-fold. (The exception was a risk of 81 for LRHL among persons with an affected relative, based on six cases [Kharazmi et al. 2015]). Another Swedish group (Crump et al. 2012) conducted a birth cohort study to examine risk factors for HL, following the entire popualtion from birth in 1978 to 2008, linking to various Swedish registries to find HL-affected family members.They estimated that having a parent or sibling affected with HL conferred a seven- to nine- fold risk of HL to the cohort member, based on 13 HL-affected relatives identified for the entire cohort (Crump et al. 2012). The only study to examine familiality by EBV tumor status was conducted within the Children's Oncology Group; the authors found that having a first-degree relative with HL conferred a roughly twofold nonsignificant higher risk for both EBV-positive and EBV-negative childhood HL (diagnosed at leass than 14 years) (Linabery et al. 2015).

Finally, a registry-based study conducted in Finland examined the risk of cancers among 4126 relatives of 693 rarely studied NLPHL cases (Saarinen et al. 2013). There were 12 occurrences of NLPHL among the relatives, with a very high risk compared to the expected based on population rates (SIR = 19, 95% CI = 8.8–36) and an even higher risk among those whose relative was diagnosed <30 years old, based on three cases (Saarinen et al. 2013). There was an especially high SIR for familial NLPHL among females (SIR = 48) compared to males (SIR = 17), regardless of the sex of the relative.

Of special interest in distinguishing between environmental and genetic factors is the study of twins. 179 MZ (initially unaffected) co-twins of HL cases had a significant, much higher histopathologically documented risk of developing HL than that expected based on population incidence (10 observed vs. 0.1 expected) , whereas no increased risk was observed among 187 DZ cotwins (0 observed vs. 0.1 expected) (Mack et al. 1995). Moreover, the majority of the originally unaffected MZ co-twins developed HL within an average of 4.5 years after diagnosis in the proband, All co-twins' diagnoses occurred before age 50, and most had EBV-negative NSHL, consistent with the more common prevalence of the subtype at a young age (Mack et al. 1995). MZ-cotwins ascertained prior to their diagnosis and followed prospectively had the same higher risk as those identified in retrospect. In a review of cancer in twins born in England and Wales, HL risk to the likesex co-twins of HL cases was found to be higher than that to the unlike-sex co-twins of cases (Swerdlow et al. 1996), consistent with the higher reported risk to genetically identical MZ twins (Mack et al. 1995). In the largest HL study from a family database (Kharazmi et al. 2015), the risk of developing HL was 57 for co-twins of HL cases (6 co-twins out of 46 like-sex pairs). The age at diagnosis in both members of 4 out of 6 twin pairs occurred before age 30. In summary, family and twin studies indicate that HL, especially, but not exclusively AYAHL, is among the most familial of neoplasms. The relative contribution of genetic vs. environmental factors has not yet been determined.

In the Co-Morbidity section we discussed the occurrence of NHL following an HL diagnosis, presumably related to treatment. However, this association raises the question of other hematological neoplasms in family members of HL patients, suggesting common determinants. A low relative incidence (up to three-fold) has been documented in investigations based on cancer registries linked to family data bases in Israel (Paltiel et al. 2000) and Sweden (Goldin et al. 2005) , and in a case-control study in Scandinavia (Chang et al. 2005b). Further examination of links between HL and NHL subtypes in relatives have been conducted in a pooled international multicenter case-control study conducted within the InterLymph consortium (Wang et al. 2007) and again using the Swedish resources (Goldin et al. 2009a). These studies confirmed a modest association between HL and both diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma. No increased familial risk betweeen HL and chronic lymphocytic leukemia (CLL) (Goldin et al. 2009b) or multiple myeloma (MM) (Schinasi et al.2016) was observed. None of these associations approach the mangnitude of the familiality of HL specific risk described above.

Genetic Risk Factors

Based on the evidence of strong heritability, a decades-long search to find the specific genetic risk factors in question has been ongoing. Different strategies have been employed that focus on either highly penetrant, rare variants associated with a very large risk, or common variants associated with a low risk but possibly a larger public health impact in terms of attributable risk (Manolio et al. 2009). Generally, studies of multiplex families are used to identify high-risk, rare variants, and very large case-control studies are employed to identify common loci at lower levels of risk.

Family Studies

HL clustering in families has been reported numerous times (Robertson et al. 1987; Cragen and Fraumeni 1972; Halazun et al. 1972; Shibuya et al. 1984). Family study designs, including segregation and linkage analyses in multiplex families and case-parent trio studies, are effective at identifying rare variants that explain risk in families using modern genotyping and sequencing technology. A group at the National Cancer Institute has followed HL families for over 40 years. Initially, HLA alleles were examined (Harty et al. 2002) (see HLA section below), and subsequently, an early genome screen using microsatellites in 44 of these families identified a significant peak at chromosome 4p16 flanked by loci D4S2935 and D4S394, with a nonparametric LOD score of 2.6 ($p = 0.0002$), suggesting a recessive model (Goldin et al. 2005). The biological implication of this locus is not known, but it has been associated with a variety of diseases including familial systemic lupus erythematosus (Xing et al. 2007), Wolfram syndrome (Larsen et al. 2004; Ohata et al. 1998), and progression of colon cancer (Al-Mulla et al. 2006).

In another study, a family with three siblings affected with NSHL as young adults and a mother who died of an unspecified mediastinal tumor were found to have an inher-

ited translocation of t(2,3) (q11.2;p21.31) (Salipante et al. 2009). The translocation was localized to a breakpoint carrying the *Kelch domain protein 8B* (*KLHDC8B)* which was under-expressed in the affected family members (Salipante et al. 2009). A C > T substitution in the 5′-untranslated region of this gene was detected in affected probands from 3 out of 52 replication families with at least 2 cHL-affected members compared to 4 out of 307 controls, resulting in an OR of 4.64 (95% CI = 1.01–21.4) (Salipante et al. 2009). The *KLHDC8B* mutation was linked with cHL and lung cancer in the three families. One sporadic (non-familial) cHL patient's malignant HRS cells showed loss of heterozygosity in a region that includes *KLHDC8B.* When the protein level of the gene was halved, there was disruption of cytokinesis resulting in an increase of binucleated cells, suggesting a link between the protein level and development of multinucleated HRS cells. This mutation has not been demonstrated in other sporadic or familial cHL patients but remains a suggestive candidate for follow-up.

In the sequencing era, a single family with three affected out of five siblings provided samples for exome sequencing. All three siblings had EBV-positive tumors; two were diagnosed with NSHL at ages 5 and 12 and the third was diagnosed with MCHL at age 12 (Ristolainen et al. 2015). In a comparison with controls with other phenotypes and with publically available exome data, a single locus with a homozygous deletion and resulting frame shift in the gene *aggrecan* (*ACAN*) was identified that resulted in 19 missing amino acids. *ACAN* is a component of the extracellular matrix of cartilage, including intervertebral disks (Gruber et al. 2011). In addition, the three affected siblings and unaffected father had a single nucleotide polymorphism (SNP) in *KIAA0141*, and a removal of a stop codon in fusion protein *LY75-CD302*, also expressed in HRS cells (Kato et al. 2003).

The Finnish group that demonstrated the strong heritability of NLPHL from registry data (Saarinen et al. 2013) identified a single family from the same registry with four cousins affected with NLPHL. Based on samples from 11 family members, a heterozygous 2 base pair deletion resulting in a frame shift and stop codon in exon 13 of the *nuclear protein coactivator of histone transcription* (*NPAT*) gene was observed in the affected cases and 3 healthy relatives but in none of the unrelated healthy Finnish controls (Saarinen et al. 2011). Furthermore, the deletion corresponded to lower expression of the *NPAT* mRNA. A different *NPAT* mutation appeared to be overrepresented in some sporadic HL cases, but the power was low and therefore results not conclusive. The function of *NPAT* in relation to NLPHL risk may be related to its interactions with the *ataxia-telangiectasia mutated gene* (*ATM*), known to be involved in leukemias and lymphomas (Taylor et al. 1996a). Of note, none of the members in this family had the *KELCH* (Salipante et al. 2009) mutation.

NCI investigators conducted another study using exome sequencing to examine 17 multiplex HL families and found an association with a single locus that replicated in an additional 48 families (Rotunno et al. 2016). The C > T missense mutation (rs56302315) at chromosome 4q12, located in the *kinase insert domain receptor* (*KDR*) gene, was found in one NHL patient and three HL patients in the offspring generation in one family, and in two HL patients in two different generations in the second family (Rotunno et al. 2016). *KDR* is one of two receptors for vascular endothelial growth factor (VEGF) and mediates induction of endothelial proliferation, survival, and migration. It is expressed in endothelial cells in the liver and spleen

among other sites and has been implicated in progression of a number of solid tumors including esophageal, head, and neck and other cancers and presumably facilitates metastasis. Several additional mutations were found in individual families but none replicated (Rotunno et al. 2016). There were no shared variants identified in the previously reported *KELCH* (Salipante et al. 2009), *ACAN* (Hafez et al. 1985) and *NPAT* (Saarinen et al. 2011) high-risk genes.

HLA Type as a Genetic Risk Factor

Overview

The HLA region, located at chromosome 6p21.3, is the most gene-dense region of the entire human genome and includes the genes that encode the HLA molecules, antigen processing genes such as *TAPI* and *TAP2*, and other immune response genes such as *TNFα*. The highly polymorphic HLA receptors present antigen processed from intracellular pathogens, phagocytized extracellular pathogens, endogenous degraded material, tumor antigens, or environmental antigens (such as those causing allergic reactions). HLA class I receptors include A, B, and C types and are expressed on every nucleated cell in the body and present processed antigen to $CD8^+$ T-cells. HLA class II receptors include DR, DQ, and DP, which are expressed only on professional antigen-presenting cells including dendritic cells, monocytes/macrophages, and B-cells and present antigen to $CD4^+$ helper T-cells. The HLA class III region is located between the class I and class II regions and contains genes encoding some other proteins involved in the immune response. The HLA receptors include a single or heterodimer chain and a binding pocket with variable amino acid sequences; they are the most highly variable set of proteins in the organism, conferring variation in antigen-specific binding, presentation, and subsequent T-cell signaling, permitting differential immune response to the antigenic stimulation that varies across individuals. There are over 2600 possible alleles representing up to 17 HLA class I and II genes. The DNA sequences encoding the series of HLA class I, II, and III immune genes are contained within haplotype blocks in very long sequences, sometimes over 1,000,000 DNA base pairs, and are thus highly linked, making it difficult to determine the actual causal set of variants within the haplotype. Furthermore, because of geographic-specific natural selection, genetic drift, and even differential mating preferences, HLA haplotypes vary by genetic origin, and they must be evaluated within ethnic strata. In addition, because of the immense variability of genetic haplotypes and the molecular structure of the various receptors present in any individual, large sample sizes are needed to identify more specific haplotype associations.

HLA alleles were originally studied using antibodies formed against the major HLA molecules with serology and have subsequently been examined using genetic sequencing techniques correlating DNA polymorphisms to HLA receptor proteins. HLA phenotypes and more recently genotypes emerged as early candidates to explain the obvious heritability of HL. An excellent and detailed review of this topic is presented by McAulay and Jarrett (2015). A summary of the major results for HLA class I and class II associations is presented below.

HLA Class I

Because HLA class I proteins present antigen processed from intracellular pathogens such as viruses and because it is suspected that a viral infection may be a trigger in the development of at least some HL, it could be presumed that HLA class I alleles would be more strongly associated than class II alleles. Indeed, the most striking initial finding was an association between HLA-A1 alleles and increased risk of HL, using the serological techniques available prior to sequencing technology (Hafez et al. 1985). The association with the HLA-A*01 alleles has been replicated using both serological and genotyping techniques in nine case-control and secondary analytic studies targeting HLA (Johnson et al. 2015; Hafez et al. 1985; Falk and Osoba 1974; Svegaard et al. 1975; Kissmeyer-Nielsen et al. 1975; Niens et al. 2007; Hjalgrim et al. 2010; Huang et al. 2012a; Hansen et al. 1977), with five of these studies showing that risk was specific for MCHL (Kissmeyer-Nielsen et al. 1975) or EBV-positive HL (Johnson et al. 2015; Niens et al. 2007; Hjalgrim et al. 2010; Huang et al. 2012a). Other HLA class I alleles associated with increased HL risk include HLA-B*05 (Falk and Osoba 1974; Svegaard et al. 1975), HLA-B*08 (Falk and Osoba 1971, 1974; Svegaard et al. 1975; Kissmeyer-Nielsen et al. 1975; Bjorkholm et al. 1975), and HLA-B*37 (Johnson et al. 2015; Huang et al. 2012a; Greene et al. 1979), while HLA-A*02 (Niens et al. 2007; Hjalgrim et al. 2010; Huang et al. 2012a), HLA-A*03 (Falk and Osoba 1974; Enciso-Mora et al. 2010) and HLA-A*11 (Svegaard et al. 1975; Falk and Osoba 1971) have been associated with decreased risk. In more detailed examinations, HLA-A*02 was specifically associated with a decreased risk of EBV-positive but not EBV-negative HL (Niens et al. 2007; Hjalgrim et al. 2010). A study showed that an HLA-A*02*07 allele, common among Chinese, was associated with a decreased risk of EBV-negative HL and an increased risk of EBV-positive HL (Huang et al. 2012b). Using microsatellite markers in a case-family control study in the Netherlands, Diepstra and colleagues (Diepstra et al. 2005) identified two microsatellite markers in the HLA class I region, D6S265 and D6S510, that conferred a high risk of EBV-positive HL. The location of these markers corresponds to the HLA-A*01 and HLA-A*02 mentioned above (McAulay and Jarrett 2015).

A decade later, technology permitted agnostic genome-wide association studies (GWAS) that examined genetic variation in markers spaced across the genome that could be imputed utilizing known linkage disequilibrium to include numbers of markers larger by orders of magnitude. The variation in the markers (single nucleotide polymorphisms, SNPs) among cases is compared to that in controls, adjusting for ancestry because of the differential linkage in different ancestral populations. The resultant *p*-value estimate is roughly 0.05 divided by the total number of independent blocks of linked genes in Europeans, thought to be 1,000,000, thus the genome-wide significance threshold is usually set at 5×10^{-8}. This strategy has been very successful in identifying much of the genetic contribution for some conditions and traits, including prostate cancer (Olama Al et al. 2014) and multiple sclerosis (International Multiple Sclerosis Genetics Consortium et al. 2011). For rare diseases like HL, it can be challenging to compile the very large numbers of subjects needed to find significant associations. In addition, in order to find important causal variants without the dampening effect of misclassification, GWAS should be conducted separately for HL subtypes (EBV/age/histology), decreasing available num-

bers further. When significant associations are identified, the genetic variants must be validated by a more direct genotyping method like quantitative real-time PCR and confirmed in an independent set of cases and controls.

Four main GWAS of HL were conducted from 2010 to 2014, two in Europe (Enciso-Mora et al. 2010; Urayama et al. 2012) and two in the USA (Best et al. 2010; Cozen et al. 2012), each combining subjects from other studies. (The larger US GWAS study was first published with the smaller (Best et al. 2010) in a meta-analysis (Cozen et al. 2012). In spite of the challenges, a small number of highly associated risk variants ("low-hanging fruit") were identified, probably because of the strong heritability of HL.

HLA class II associations were observed in all of these studies (see below); however only one European study (Urayama et al. 2012) identified risk loci in the HLA class I region, specifically associated with EBV-positive and not EBV-negative HL. These loci were confirmed in a meta-analysis that added additional 22 cases (Cozen et al. 2014). Of note, the risk estimates for these HLA-A loci were both >2.0, among the highest of the GWAS associations. HLA typing was available for the majority of the cHL patients (257 EBV-positive and 642 EBV-negative patients), and the two HLA class I variants were in strong linkage with the previously reported HLA-A*01 (rs2734986) and HLA-A*02 (rs6904029) associations (Hjalgrim et al. 2010).

HLA Class II

HLA class II alleles are more complex than HLA class I because there are two chains (α and β) compared to the one chain for HLA, and for one group, DRB genes, the number of genes is variable between individuals and can include a DRB3, DRB4, and/or DRB5, in addition to the universal DQ and DP. Examination of HLA class II alleles began in earnest in the 1990s, mainly in the UK, with the advent of molecular typing. Seven publications from several groups with multiple studies per group found that HLA-DPB*0301 was associated with an increased risk of HL (Johnson et al. 2015; Oza et al. 1994; Alexander et al. 2001; Taylor et al. 1996b, 1999; Tonks et al. 1992; Klitz et al. 1994), with a relative risk of approximately 1.95 in the largest, a multinational study of 741 cases (Oza et al. 1994). Cases with EBV-positive tumors were slightly more likely to be positive for HLA-DPB*03:01 compared to EBV-negative tumors (Johnson et al. 2015; Alexander et al. 2001). The lone US study found an elevated risk of NSHL associated with the HLA-DPB*03:01 allele (Klitz et al. 1994) however no study examined the allele by both histology and EBV status together. Asian patients from Japan and Taiwan were included in a multinational study, and among these patients, the allele DPB1*04:01 was associated with a decreased risk of cHL (Oza et al. 1994). DRB1*15:01 was associated with an increased risk of NSHL in 2 studies (Harty et al. 2002; Klitz et al. 1994), including a study of 16 multiplex families (Harty et al. 2002). Two studies also found that DQB1*06:02 was a risk allele for NSHL and that a haplotype including both DRB1*15:01 and DQB1*06:02 accounted for the same level of risk as either allele alone, possibly explained by a tight linkage (Harty et al. 2002; Klitz et al. 1994). The third study modeled previously generated data from the UK and found DRB1*15:01 and DPB1*01:01 were associated with a decreased risk of EBV-positive cHL, the

opposite effect seen for NSHL (Johnson et al. 2015). Finally, a study conducted in the Netherlands found an inverse association between DRB1*07:01 and cHL (Huang et al. 2012a); the association was confirmed in the UK modeling study, with a suggestion of a stronger inverse association with NSHL (Johnson et al. 2015).

The importance of the HLA class II region was confirmed in the agnostic GWAS mentioned above. In the first published GWAS, a SNP at 6p21.3 (rs6903608) was the most significantly associated locus found to be associated with cHL, with an OR of 1.7 and p-value of 2.84×10^{-50} in the combined discovery and replication sets (Enciso-Mora et al. 2010). The SNP is located near the DRA and DRB1 gene regions (usually assigned to DRA because of proximity). This SNP was confirmed in a combined meta-analysis of the other three GWAS, where it was strongly associated with NSHL ($p = 1 \times 10^{-26}$), EBV-negative cHL ($p = 7 \times 10^{-33}$), and NSHL in young adults from 15 to 35 years old ($p = 6 \times 10^{-27}$), but not with MCHL ($p = 0.19$) or EBV-positive cHL ($p = 0.68$) (Cozen et al. 2014). The UK HLA modeling study also confirmed that rs6903608 was the best predictor for the risk of EBV-negative cHL, accounting for all other common risk alleles (Johnson et al. 2015). In a study combining the two US GWAS (Cozen et al. 2012), a protective haplotype of 5 SNPs mapping to DRB1*07:01 was associated with a ~50% decreased risk of (mainly EBV-negative) NSHL, similar to results in other studies (Johnson et al. 2015; Huang et al. 2012a). Finally, a SNP located near HLA-DPB1 (rs6457715) was identified by one of the European GWAS studies, linked to EBV-positive HL independent of histological subtype (Delahaye-Sourdeix et al. 2015).

The functional significance of the strong HLA associations observed for cHL has yet to be determined, but there are some plausible hypotheses. It is possible that the mechanism underlying increased risk of EBV-positive cHL associated with HLA-A*01:01 may be due to a poor immune response to EBV infection, either by weak binding or downstream signaling, whereas the HLA-A*02 allele, which is protective, may be associated with a stronger immune response resulting in better control of the EBV infection (McAulay and Jarrett 2015; Kushekhar et al. 2014). Interestingly, some of the HLA associations have opposite effects for EBV-positive and EBV-negative/NSHL (Johnson et al. 2015; McAulay and Jarrett 2015). That, combined with the attenuated association between IM and EBV-negative compared to EBV-positive HL (Hjalgrim et al. 2007a), suggests that HLA alleles associated with an effective immune response to EBV may protect against consequences of a poorly controlled EBV infection, which include IM and EBV-positive HL.

There is less evidence for a mechanism that explains the association between HLA class II types and risk of EBV-negative HL and NSHL, but one possibility is a differential ability to present tumor antigens and the propensity for a strong $CD4^+$ T_H2 response in the microenvironment that generally promotes tumor cell survival (Kushekhar et al. 2014). Of note, HLA class II alleles and haplotypes have been among the strongest genetic associations identified from GWAS of any B-cell lymphomas, including CLL (Conde et al. 2010), follicular lymphoma (Smedby et al. 2011), and multiple myeloma (MM) (Beksac et al. 2016).

HL has also been linked to the blood group B antigen, another cell surface receptor (Mack et al. 2015).

Non-HLA Genetic Associations

A large number of genetic associations in non-HLA genes have also been identified and have been classified as relating to the immune response, carcinogen metabolism, DNA repair, or folate metabolism (Sud et al. 2017). A comprehensive review focused on published reports of 13 genetic variants in 10 genes and included meta-analyses of published results and false-positive report probabilities (Sud et al. 2017). The authors concluded that the only risk SNP that maintained a significant association after these considerations was rs17655 located in the DNA repair gene *XPG/ERCC5* ($OR_{meta\text{-}analysis}$ = 2.05, p = 0.046). This meta-analysis highlights the importance of achieving adequate power to appropriately measure the association between polymorphic variants and an increased risk of HL in candidate gene association studies. Additional significant variants in immune-related and DNA repair genes were found by including GWAS data, but these were interpreted with caution.

Harty et al. identified a Ile333Val polymorphism of *TAP1*, an antigen processing gene located within the HLA III region, associated with familial NSHL in the multiplex family study described above (Harty et al. 2002). A microsatellite marker in the same region was also associated with EBV-negative HL in a case-control study from the Netherlands (Diepstra et al. 2005).

Seven additional genome-wide significant associations with risk loci outside of the HLA region have also been identified: 2p16, 3p24, 5q31, 6q23, 8q24, 10p14, and 19p13.3 in or near the genes *REL*, *EOMES*, *IL13*, *HBS1L-MYB*, *PVT1*, *GATA3*, and *TCF3*, respectively (Enciso-Mora et al. 2010; Urayama et al. 2012; Cozen et al. 2014; Frampton et al. 2013). The effect sizes associated with these loci were weaker than those observed for the HLA alleles, ranging from 19 to 50% increases in risk. There was heterogeneity by subtype, with variants in *REL*, *EOMES*, *IL13*, *and GATA3* linked to EBV-negative/NSHL and AYAHL risk, and there was a suggestion of a similar pattern for the genetic variant in *TCF3*. In contrast, the loci at *HBS1L-MYB PVT1* had similar effects and p-values across all cHL subtypes.

There is biological plausibility for at least some of these GWAS-identified loci. *REL* encodes c-REL, a member of the NFκB family of proteins that facilitate activation of the NFκB pathway. NFκB is expressed in HRS cells and promotes proliferation while suppressing apoptosis (Kuppers 2009). The NFκB pathway can be activated by CD40 or alternatively by expressed EBV genes LMP1 or LMP2, but since these risk variants did not appear to be associated with EBV-positive cHL, the significance of EBV as an activator of the pathway is questionable. *EOMES* codes the protein eomesodermin that regulates embryological limb development as well as lymphocyte effector function. It is a member of the TBR1 family of T-box genes and interacts with TBET (Tbx2) to increase differentiation of $CD8^+$ T-cell function, necessary for effective tumor response in cHL (Zhang et al. 2011). The highly variable (but gene desert) 8q24 region harbored a SNP (rs2019960), similarly associated across HL subtypes. This region has been associated with risk of multiple cancer types and is thought to interact with and regulate MYC (Grisanzio and

Freedman 2010). The cHL variant is not in the same LD block as the loci associated with other cancers, but it may have a similar functional significance with respect to interaction with *MYC*, a master oncogene. The SNP is located near *PVT1*, a gene that regulates *MYC* expression, although the role of *MYC* is unclear in HL pathogenesis.

More interesting is the missense SNP (rs201541) located in the interleukin-13 (IL13) gene (rs20541) at chromosome 5q13, moderately associated with EBV-negative HL and NSHL (Urayama et al. 2012; Cozen et al. 2014). The SNP results in an amino acid change from glutamine to arginine, resulting in increased IL13 expression. Another SNP (rs2069757) at the same locus is even more strongly associated, primarily with EBV-negative HL. IL13 is a T_H2 cytokine that is associated with increased IgE levels and atopy and increased production of collagen and sclerosis (wound-healing response) (Wynn 2008). It is also expressed by HRS cells, and the protein is sometimes observed in the nonmalignant tumor microenvironment (Skinnider and Mak 2002). IL13 could function in a number of ways, including promoting a T_H2-mediated immune suppression against cytotoxic T-cell activation, production of sclerosis, and/or as an autocrine growth factor for HRS cells (Kushekhar et al. 2014). Genetic variants in the T_H2 transcription factor *GATA3* at chromosome 10p14 are also associated with the EBV-negative NSHL subset, especially in young adults (Cozen et al. 2014). Like IL13, GATA3 is expressed both in the HRS cells and in the T-cells in the HL microenvironment and can promote a T_H2, T-cell exhausted environment (Steidl et al. 2011; Greaves et al. 2013).

In the largest GWAS meta-analysis that combined 3 of 4 the previous GWAS, with a total of 1816 HL cases and 7877 controls, a novel risk locus (rs1860661) was found on chromosome 19 in exome 2 of the *TCF3/E2A* gene (Cozen et al. 2014). The minor (G) allele was significantly correlated with increased gene expression in controls, and a mutation at the same locus was found in one HL cell line. *TCF3*/*E2A* expression promotes stability of the B-cell phenotype; it is inhibited in HL tumor cells resulting in in the hallmark downregulation of the B-cell receptor and other essential B-cell markers (Mathas et al. 2006). Thus, evidence suggests that the G allele protects against HL by increasing *TCF3/E2A* expression which stabilizes the B-cell phenotype. Similar to most of the other variants, the associations were mainly observed for EBV-negative, NSHL subtypes and not for EBV-positive subtypes.

In summary, a number of genome-wide significant loci have been identified for cHL, with the strongest associations in the HLA-class II region, especially for the

AYA, EBV-negative, NSHL subtype. In addition, there are several associations suggesting an interaction with EBV among patients with EBV-positive tumors. The association of HLA and T_H2-related genetic risk variants with EBV-negative NSHL suggests that deficient T_H1 surveillance in the setting of virally infected B-cells may have a role in susceptibility, but there is currently no candidate virus for this subset. It has been proposed that this same immunophenotype may enhance survival of HRS precursor cells (Zhang et al. 2011) but this seems further down the path of pathogenesis than risk, more related to progression. Thus, at present, the causal mechanisms for these common risk SNPs, identified by GWAS, remain unknown. For a comprehensive discussion on risk mechanisms of the reported susceptibility alleles, see Kushekhar et al. (2014).

Genetic Overlap with Other Diseases

To date, two studies examined GWAS data for cHL in combination with GWAS for other diseases. Law et al. used a method that compares subsets of studies, accounting for shared controls and multiple testing, to examine commonalities in HL, CLL, and MM, all cancers of B-cell origin at different stages of development. Novel loci inversely or positively associated with the three B-cell tumors were identified. An allele in HLA class II DRB1 (Ser37+Phe37) was significantly positively associated with both HL and CLL (and inversely associated with MM), and an allele in HLA class II DQB1 (Gly70) was inversely associated with HL and positively with CLL. A variant at 3q22.2 (rs11715604) was also inversely associated with HL risk and positively associated with CLL risk. This SNP maps to the gene *NCK1* which, among many other functions, increases T-cell proliferation and activation. In addition, risk loci for CLL (*BAK1*, *IRF4*), MM (*ULK4*), and both (*TERC*) were all positively associated with HL.

Because of the previously noted association between cHL and autoimmune disease, a meta-analysis was conducted with a cHL (1816 patients) and a multiple sclerosis (9772 patients) GWAS with a total of 25,255 controls, in order to identify common risk loci. Both diseases had many HLA region risk SNPs in common. Polygenic risk allele scores composed of the MS risk alleles explained ~4.5% of HL risk. In a genetic diseasome network, in which published GWAS data proximity was calculated and plotted, HL was genetically much more closely aligned with autoimmune diseases than with solid tumors (meaning more SNPs and similar loci in common) (Fig. 8.6).

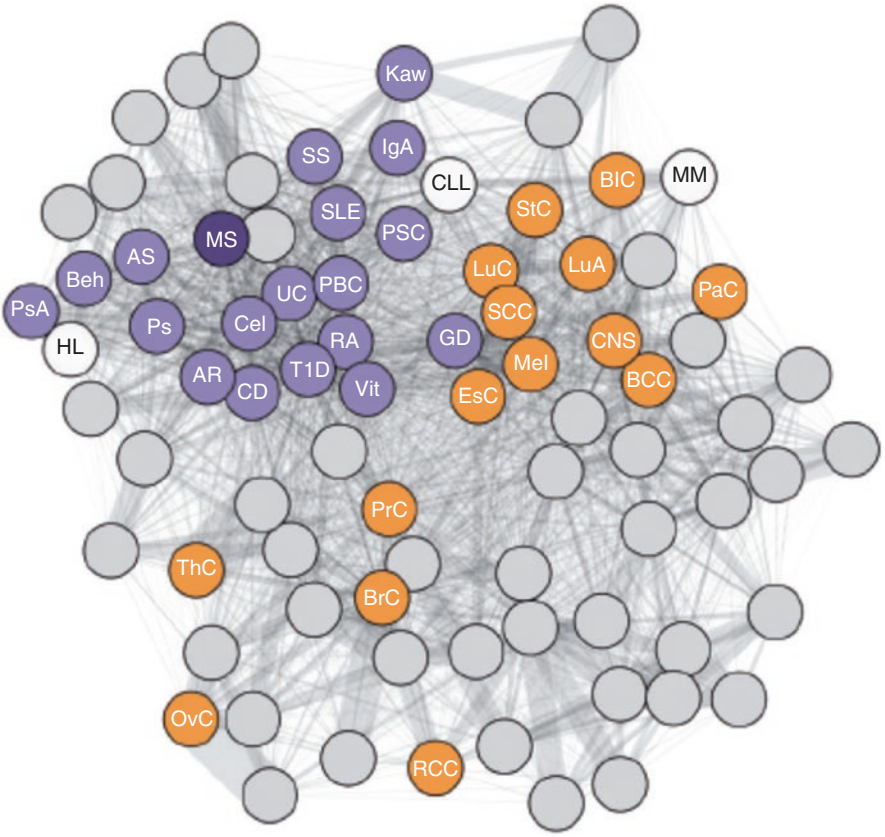

Fig. 8.6 Human disease network shows distinct autoimmune and solid cancer clusters and places hematologic cancers in context. In a network of disease proximity, constructed using systematic GWAS data, autoimmune diseases (purple) tightly cluster. Solid tumors (orange) also form a cluster but exhibit less genetic relatedness to HL compared to autoimmune diseases (from Khankhanian et al. 2016). Autoimmune Diseases: *AR* alopecia areata, *AS* Ankylosing spondylitis, *Beh* Behcet's disease, *Cel* Celiac Disease, *CD* Crohn's disease, *GD* Grave's Disease, *IGA* IgA glomerulonephritis, *KAW* Kawaski disease, *MS* Multiple sclerosis, *PBC* Primary biliary cirrhosis, *PSA* Psoriatic arthritis, *RA* Rheumatoid arithitis, *PSQ* Sclerosing cholangitis, *SLE* Systemic lupus erythematosus, *SS* Systemic scleroderma, *TID* Type 1 diabetes mellitus, *UC* Ulcernative colitis, *Vit* Vitiligo. Hematologic Neoplasms: *CLL* Chronic lymphocytic leukemmia, *HL* Hodgkin lymphoma, *MM* Multiple myeloma. Solid Cancers: *BCC* Basal cell carcinoma, *BLC* Bladder carcinoma, *BRC* Breast cancer, *CNS* Central nervious syst-sem, *OESC* Oesophageal carcinoma, *LUA* Lung adenocarcinoma, *LUC* Lung carcinoma, *MEL* Melanoma, *OVC* Ovarian Carcionoma, *PAC* Pancreatic Carcinoma, *PRC* Prostate Carcionoma, *RCC* Renal cell carcinoma, *SCC* Squamous carcinoma, *STC* Stomach carcinoma, *THC* Thyroid carcinoma

Heritability

Heritability of a trait or phenotype is a theoretical statistical concept that represents the proportion of variation of the occurrence of the disease in a population that is explained by genetic variation. Assumptions about the contribution of

environmental factors and chance undermine the accuracy, but it is a useful measure when comparing the genetic contribution between different diseases Using GWAS data on 906 cases from Northern Europe with replication from the Swedish Family Cancer Registry, Thomsen and colleagues estimated heritability for all HL together at greater than 35%. About 20% of this heritability estimate is attributable to genetic variation in the HLA region. Another heritability analysis again on the Swedish Family Cancer Registry (without genetic data) estimated heritability at 28.4%.

Summary

HL comprises multiple etiological entities that share fundamental immunological dysregulation and a rare neoplastic cell. There are both genetic and environmental determinants that vary by age at diagnosis, SES, histological subtype, and EBV prevalence in the tumor cells. It is difficult to separate the presence of EBV in the HRS cells and the characteristics of the microenvironment (histology) because they are correlated; nevertheless there appears to be distinct determinants for each. NSHL/AYAHL is the most common type, varying greatly in place and time in relation to the social changes that accompany economic development in societies and increasing affluence in individuals. It is highly heritable and is linked to specific HLA class II alleles and genotypes and T_H2-related genes. The behavioral factors responsible for the relatively sudden appearance of this condition at times of affluent social change are unknown; there are suggestions that a deficit of early childhood exposure to microbes may result in a specific or nonspecific a susceptible immune response. EBV-positive HL, like MCHL, is more common at the age extremes and in settings of social deprivation or acquired immunodeficiency. It appears to be associated with ineffective response to EBV infection and specific HLA class I alleles and genotypes. Both genetic and environmental risk factors tend to show opposite gradients with EBV-positive MCHL and EBV-negative NSHL. NLPHL is quite rare and also appears to be highly heritable and is almost never associated with EBV. The other types are too rare to study separately in an epidemiological context.

What must we still learn about the epidemiology of HL? The most pressing epidemiological question is what causes the relatively rapid appearance of AYAHL/NSHL in response to increasing economic development. Could it be related to a general deficit of early childhood microbial exposures mediated by the microbiome or delayed exposure to an, as yet unidentified, specific virus? A related question is why is IM not associated with risk of AYAHL/NSHL since both preferentially affect affluent adolescents? Another important question involves the relationship of EBV to HL. Could EBV be a marker for an immunosusceptible phenotype or is it causal? Some of these important questions may be answerable in the future through large international collaborations providing sufficient sample size to study individual determinants and entities by EBV tumor status, age, and histopathology simultaneously.

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Chapter 9
Treatment and Prognosis

Francesca Montanari and Catherine S.M. Diefenbach

Risk Assessment in HL

Since 1964, with the development of the MOPP (mustard, vincristine, procarbazine, and prednisone) regimen for advanced Hodgkin's lymphoma (HL), major progress has been achieved in the treatment of HL, leading to cure for approximately 80% of patients in the modern era (Canellos et al. 2014). Given the young age of many HL patients (58% are younger than 45 years old at diagnosis) (Howlander et al. 1975), the integration of maximizing cure with minimizing long-term therapy-related toxicities has been a primary goal of clinical research.

For newly diagnosed HL, the selection of the optimal treatment regimen is currently based on clinical staging, with the use of positron emission tomography (PET) and upfront risk stratification tools. For patients with early-stage (i.e., stage I/II) disease, adverse prognostic factors include: the presence of a large mediastinal mass (ratio $\geq$ 1/3 of maximum thoracic diameter), constitutional (B) symptoms such as weight loss or night sweats, an elevated erythrocyte sedimentation rate (ESR) >50 (ESR > 30 if B symptoms), involvement of multiple nodal sites, the presence of extranodal disease, and age older than 50. Based on these risk factors, early-stage patients are categorized as either favorable or unfavorable. These risk definitions have slight regional variation (see Table 9.1); these differences may have some impact on the heterogeneity of this patient population across different clinical trials.

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Table 9.1 Risk factor definitions in favorable early-stage Hodgkin's lymphoma (HL)

GHSG	– No large mediastinal mass (ratio ≥ 1/3 of maximum thoracic diameter) – No extranodal lesion – ESR < 50 (ESR < 30 if B symptoms) – ≤2 nodal areas involved (out of 11 GHSG areas)
EORTC	– No large mediastinal mass (ratio ≥ 0.35 of maximum thoracic diameter) – Age < 50 years – ESR < 50 (ESR < 30 if B symptoms) – <4 nodal areas involved (out of 5 supradiaphragmatic EORTC areas)
NCCN	– No large mediastinal mass (ratio > 1/3 of maximum thoracic diameter) – No bulky sites (>10 cm) – ESR < 50 No B symptoms – <4 nodal areas involved (out of 17 Ann Arbor regions)

GHSG German Hodgkin Study Group, *EORTC* European Organization for Research and Treatment of Cancer, *NCCN* National Comprehensive Cancer Network

For patients with advanced HL, defined as stage III/IV or stage IIB with extranodal and/or bulky disease, the most widely utilized prognostic index is the International Prognostic Score (IPS-7), which includes seven factors: a serum albumin level of less than 4 g/dL, a hemoglobin level of less than 10.5 g/dL, male sex, an age of 45 years or older, stage IV disease, leukocytosis (a white cell count of at least 15,000/mm^3), and lymphopenia (a lymphocyte count of less than 600/mm^3, a count that was less than 8% of the white cell count or both). The IPS-7 was originally developed in 1998 by Hasenclever from the analysis of over 4600 patients treated between 1983 and 1992 with an anthracycline-containing regimen (>75%) or MOPP (20%); the majority of the patients (60%) did not receive radiation therapy. Five-year freedom from progression (FFP) ranged from 84% in patients with no risk factors to 42% in patients with five or more risk factors; overall survival (OS) ranged from 98% to 67%, respectively (Hasenclever and Diehl 1998).

Over the past few decades, advances in therapy and supportive care have resulted in a dramatic improvement in clinical outcomes, even for patients with advanced HL, with over 75% of the patients cured with frontline therapy (Canellos et al. 2014). Given this global improvement in outcome, the IPS-7 was reanalyzed in the modern era in a cohort of HL patients treated between 1980 and 2010, demonstrating that while the IPS-7 remained prognostic, its discriminatory capacity had diminished with the FFP and OS curves considerably narrowed. Five-year FFP and OS ranges were 88–62% and 98–73%, respectively (Moccia et al. 2012).

In an attempt to update the IPS7, to reflect the improved outcomes in HL with modern therapy, a new IPS was developed using data from the 854 patients with locally extensive and advanced-stage HL enrolled in North American intergroup trial E2496 (Gordon et al. 2013). When applied to this series, IPS-7 remained prognostic, although it was again noted that its prognostic range had significantly narrowed. On multivariate analysis, of the 7 IPS factors, only age and stage remained predictive of FFP, while age, stage, and hemoglobin predicted OS. Based on these results, an alternative and simplified prognostic index, the IPS-3, based on three

variables (i.e., age, stage, albumin) and providing four risk groups was proposed; IPS-3 outperformed the IPS-7 for risk assessment of PFS and OS (Diefenbach et al. 2015a).

As our knowledge of the complex crosstalk and interdependence between the malignant Hodgkin and Reed-Sternberg (HRS) cell and its surrounding microenvironment has deepened, many promising biomarkers of lymphomagenesis and biological correlates of treatment failure are under investigation [see (Howlander et al. 1975)]. Reports utilizing gene expression profiling (GEP) and tissue microarrays have identified tumor-associated macrophages (TAMs) as independent negative prognostic factor for clinical outcome (Steidl et al. 2012; Tan et al. 2012; Kamper et al. 2011). The largest of these studies analyzed 287-patient tissue microarray constructed from formalin-fixed paraffin-embedded tumor (FFPET) tissue samples from patients enrolled in the multicenter E2496 Intergroup trial and confirmed that an increase in CD68 and CD163 expression by immunohistochemistry (IHC), typical of TAMs, is an independent predictor of inferior failure-free survival (FFS) and OS (Tan et al. 2012). Similar findings were shown in a second study using GEP on microdissected HRS cells from 29 patient tumor tissues. A macrophage-like signature, characterized by the expression of CSF1R, was found to correlate with treatment failure and, in combination with CD68 expression by IHC, was demonstrated to be an independent predictor for inferior PFS and OS in a validation cohort of 132 patients (Steidl et al. 2012). In a subsequent study, utilizing FFPET biopsies from 290 patients on the E2496 Intergroup trial, a 23-gene outcome predictor was generated by means of NanoString technology, with the ability to identify patients with advanced-stage HL at higher risk of death when treated with standard intensity first-line chemotherapy. This model was validated in a second cohort of 78 patients (Scott et al. 2013).

Epstein-Barr virus (EBV) has been recognized as an important player in the pathogenesis of HL, and latent EBV genomes are found in up to 40% of the cases in the Western countries and in more than 90% of pediatric cases in Central America (Kapatai and Murray 2007). Serum EBV-DNA levels have been shown to correlate with EBV status as determined by viral nucleic acid (EBER) in situ hybridization (ISH) on tissue sections and with response to therapy in patients with EBV-associated HL (Gandhi et al. 2006). In a recent study EBV-DNA levels from 274 patients, included in the E2496 Intergroup trial, were analyzed at different timepoints, before, during, and after the therapy. Despite a concordance of plasma EBV-DNA positivity (i.e., ≥60 copies/100 μL) and EBER-ISH of 96%, FFS was inferior among those patients with positive pretreatment EBV-DNA (hazard ratio (HR) = 2.0; 95% CI 1.2, 3.5; $P = 0.01$) but not in those with EBER-ISH positivity and was independent from other adverse prognostic factors included in the IPS score. Plasma EBV-DNA positivity at month 6 was also associated with particularly poor outcome (Kanakry et al. 2013). If validated, EBV-DNA levels may be integrated into clinical risk assessment tools and may suggest a clinical rationale for investigation of virus-directed therapy paradigms in relapsed patients with persistent elevation of EBV-DNA.

Peripheral blood cytokines and chemokines have also been investigated as biomarkers of response. Levels of thymus and activation-related chemokine (TARC) have been evaluated in 62 HL patients selected from multiple GHSG trials; eleva-

tions in serum TARC levels correlated with decreased freedom from treatment failure (FFTF) and OS (Weihrauch et al. 2005). These findings were confirmed in a larger cohort of 322 patients from GHSG trials, where TARC levels correlated with response to therapy and established risk factors. Interestingly, serum baseline TARC significantly contributed to a multivariate model predicting response to chemotherapy, suggesting a potential use of this biomarker to complement clinical prognostic factors (Sauer et al. 2013). Elevation in pretreatment levels of the inhibitory cytokines IL-6 and IL-2R (Marri et al. 2013) has also been associated with advanced-stage disease and inferior outcome; mutation of the β-2 microglobulin gene in primary HRS cells correlates with nodular sclerosis subtype, younger age, and favorable survival (Reichel et al. 2015). More recently *PD-L1* and *PD-L2* genetic alterations and 9p24.1 amplification were associated with advanced-stage disease and poor outcome in 108 newly diagnosed HL patients treated with the Stanford V regimen (mechlorethamine, doxorubicin, vincristine, bleomycin, vinblastine, etoposide, and prednisone) (Roemer et al. 2016) (Table 9.2).

The significance of PET has emerged, not only as an accurate staging tool but also to assess response during and at the end of the therapy; a potential role for PET in interim risk stratification has been suggested (Gallamini et al. 2007; Hutchings et al. 2006). Clinical trials utilizing early PET assessments as part of response-adapted

Table 9.2 Selected biomarkers in HL

Category	Biomarkers	Prognostic value	Identifies potential therapeutic target
Microenvironment-related	Tumor-associated macrophages: • By IHC CD68 and CD163 (Tan et al. 2012; Kamper et al. 2011) • By gene expression profiling: CSF1R (Steidl et al. 2012)	Unfavorable Inferior FFS and OS	No
	TARC (Weihrauch et al. 2005; Sauer et al. 2013)	Unfavorable Inferior FFTF and OS	No
	IL-6 and IL-2R (Marri et al. 2013)	Unfavorable Advanced stage and inferior outcome	No
	Mutation in β-2 microglobulin gene (Reichel et al. 2015)	Favorable, low risk	No
HRS-cell related	EBV-DNA ≥60 copies/100 μL (Gandhi et al. 2006; Kanakry et al. 2013)	Unfavorable Inferior FFS	Yes
	PD-L1 and PD-L2 (Roemer et al. 2016)	Unfavorable Poor outcome	Yes
Molecular	Gene expression profile for risk group (23 gene predictor by NanoString Technology) (Scott et al. 2013)	Unfavorable Inferior OS	No

therapy strategies have been reported and will be discussed below. More are ongoing and may lead in the future to personalized therapy based on early response patterns.

These scientific advances in HL biology are exciting, yet they must now be validated as risk assessment tools or surrogate endpoints. This will optimally be achieved through translational aims embedded within prospective clinical trials.

Therapy in Early-Stage HL

For patients with early-stage HL, combined modality therapy (CMT) with ABVD chemotherapy (doxorubicin, bleomycin, vinblastine, and dacarbazine) and involved field or involved nodal radiation therapy (IFRT or INRT) or single modality ABVD chemotherapy are both acceptable treatment standards.

The role for CMT has been established by the GHSG HD10 and HD11 trials**.** In the HD10 trial, 1370 patients with early-stage and favorable risk factors were randomized to one of four groups, exploring two different chemotherapy schedules (ABVD for 2 and 4 cycles) and two IFRT doses (30 Gy versus 20 Gy). Two cycles of ABVD plus 20 Gray of IFRT proved to be as effective as and less toxic than 4 cycles of ABVD followed by 30 Gy of IFRT, with a 5-year freedom from treatment failure (FFTF) of 91% and overall survival (OS) of 97% (Engert et al. 2010).

For patient with unfavorable risk, intensified chemotherapy regimens have been explored to improve long-term tumor control. In the HD11 trial, 1395 patients with early-stage and unfavorable risk factors were randomized to one of four groups comparing two different chemotherapy regimens, ABVD for 4 cycles and bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone (BEACOPP) for 4 cycles with two different radiation doses (30 Gy versus 20 Gy IFRT). Of note, patients with bulky disease accounted for 19.5% of all patients included in the study. The study showed no benefit in using a moderate dose escalation (i.e., BEACOPP compared to ABVD) and established ABVD with IFRT 30 Gy as the standard of care with a long-term disease control of 80% (5-year FFTF of 85% and 5-year OS of 95%) (Eich et al. 2010). A higher intensity of chemotherapy was investigated in a subsequent trial, HD14, where 1528 patients with early-stage unfavorable disease were randomized to ABVD for 4 cycles versus escalated dose of BEACOPP (BEACOPPesc) for 2 cycles, followed by 2 cycles of ABVD (2 + 2). Both groups received IFRT 30 Gy. The FFTF favored the 2 + 2 arm, with a difference of 6.2% at 5 years, at the cost of a significantly higher toxicity (grade 3 and 4 hematological toxicity 87% vs 50%). Overall survival was not impacted, likely due to effective salvage regimens. Based on these results, 2 + 2 became a new standard for high-risk patients within the GHSG. Although the study was not powered for subset study analysis, there is a suggestion that the 2 + 2 approach might be particularly beneficial in patients with bulky mediastinal stage I/II disease, typically treated with 6–8 cycles of ABVD plus IFRT 36 Gy, with the advantage of a shorter duration of chemotherapy and lower radiation dose (von Tresckow et al. 2012).

Strategies to reduce chemotherapy intensity and chemotherapy-related toxicity have also been investigated. GHSG HD13 compared 2 cycles of ABVD with 2 cycles of the reduced intensity regimen variants ABV (with the omission of dacarbazine), AVD (with the omission of bleomycin), and AV (with the omission of dacarbazine and bleomycin), in 1502 favorable early-stage HL patients. All treatment arms received IFRT (30 Gy) at the completion of chemotherapy (data from the HD10 study showing equal efficacy of the 20 Gy in this patient population were not available at the time of the study design). Both arms where dacarbazine was omitted were closed early due to a higher rate of patient with progression of disease or relapse, with 5-year FFTF of 81.4% for ABV and 77.1% for AV, compared to 93.1% for ABVD. Omission of bleomycin also resulted in inferior tumor control compared to ABVD, with 5-year FFTF of 89.2%, and non-inferiority with ABVD could not be demonstrated (Behringer et al. 2015).

Data supporting the use of ABVD chemotherapy as a single modality for 4–6 cycles in non-bulky early-stage disease derives primarily from the results of the HD6 study conducted by the National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG) and ECOG. This study was specifically designed to evaluate whether omitting radiation resulted in a survival disadvantage. Four hundred and five patients, with non-bulky stage I-IIA HL, were randomized to ABVD alone for 4–6 cycles or subtotal nodal irradiation (STNI) with or without ABVD. Among those assigned to STNI, favorable risk patients received STNI alone and unfavorable risk patients received STNI plus two cycles of ABVD. The primary end point was late survival. At 12-year follow-up, OS was 94% in the ABVD-alone arm compared to 87% in the STNI arm (p 0.04), with progression-free survival (PFS) of 87% and 92%, respectively (p 0.05). Survival advantage in the ABVD-alone arm has been interpreted to be a consequence of fewer deaths observed due to causes unrelated to progressive HL (Meyer et al. 2005). This data must be interpreted with caution as the STNI, which was the standard of care when the study was designed, has been replaced by modern limited field radiation techniques.

In an effort to compare the data of the HD6 ABVD-alone arm with the data of the CMT trials using more updated radiation techniques, an individual patient-data comparison between the GHSG HD10 and HD11 and the Canadian HD6 studies was conducted. Data from 588 patients who met mutually inclusive eligibility criteria for HD10/HD11 and HD6 were analyzed. The 8-year time to progression (TTP) favored the CMT group (93% in the HD10/11 group versus 87% in the HD6 chemotherapy-alone group); however, there was no difference in 8-year OS (95% both groups). This analysis suggests that CMT results in a superior disease control rate, yet has an equivalent OS rate to chemotherapy alone, likely due to the availability of efficacious salvage chemotherapy options for relapsed patients, and a higher incidence of late toxicity associated with the CMT treated patients (Hay et al. 2013). Both CMT and ABVD chemotherapy remain standard treatment options for early-stage low-risk patients.

Response-adapted therapy platforms, incorporating early assessment of response by PET, are currently under evaluation. The H10 trial, conducted by the EORTC, the Lymphoma Study Association (LYSA), and the Italian Lymphoma Foundation

(FIL), was designed to assess whether chemotherapy alone is as effective as CMT in patients with non-bulky early-stage HL who achieve PET negativity after 2 cycles of ABVD and whether escalation from ABVD to BEACOPPesc could improve outcome in patients who were PET positive after 2 cycles of ABVD. With regard to the early PET-negative group, the planned interim futility analysis, at a median follow-up of 1.1 years with 1137 patients enrolled, demonstrated that the null hypothesis of non-inferiority of chemotherapy alone versus CMT would not be rejected, and the trial was amended so that all the future patients would receive radiation therapy. Radiation therapy was associated to a superior 1-year PFS of 5.1% in patients with early-stage favorable disease per the EORTC criteria (100% vs 95% for chemotherapy alone) and 2.6% in patients with unfavorable early stage (97.3% vs 94.7%). Even though a non-inferiority of chemotherapy alone compared to CMT could not be proved, both arms had excellent early outcomes, and longer follow-up is now needed to assess whether an improvement in the burden of late toxicities related to the omission of radiation therapy could potentially outweigh the difference in disease control observed early on (Raemaekers et al. 2014). The updated results for the early PET-positive group were presented at the 13th International Conference on Malignant Lymphoma. This analysis included the 361 of the 1950 patients with an early positive PET scan; 188 received standard ABVD and 142 received BEACOPPesc; both groups subsequently received INRT. At 5 years, PFS was significantly higher in the BEACOPPesc group (91% vs 77%) but at the cost of an increased incidence of cytopenias and febrile neutropenia (23.9% vs 1.1%), and infections without neutropenia, 11.2% versus 1.1%. There was no difference in 5 years OS (Raemaekers 2015).

Similar results were shown by the United Kingdom National Cancer Research Institute RAPID study, a phase 3 non-inferiority randomized clinical trial where 602 patients with non-bulky early-stage HL received response-adapted therapy based on the results of PET after 3 cycles of ABVD. PET-negative patients were randomized to IFRT versus no further therapy, whereas PET-positive patients received a fourth cycle of ABVD and IFRT. In the PET-negative group, 3-year PFS was 94.5% in the IFRT arm versus 90.8% in the no further therapy arm, with an absolute risk difference of −3.8% (95% CI, −8.8 to 1.3); OS was similar in both arms (97.1% and 99.5%, respectively). Despite the modest improvement in disease control of IFRT, chemotherapy alone did not meet the non-inferiority boundaries set by the study (Radford et al. 2015).

GHSG is currently conducting non-inferiority randomized clinical trials (HD16 and HD17) to further evaluate the feasibility of PET response-adapted therapy for favorable and unfavorable early-stage HL. The HD16 trial includes favorable early-stage HL. In the standard therapy arm, patients receive 2 cycles of ABVD followed by IFRT 20 Gy; in the experimental arm, patients not achieving PET negativity after 2 cycles of ABVD will receive IFRT. The HD17 trial includes unfavorable early-stage HL. The standard therapy arm differs from previous trials, as it includes 2 cycles escalated BEACOPP as initial therapy, followed by 2 cycles of ABVD followed by 30 Gy IFRT. Patients randomized into the experimental arm will receive IFRT only if PET positive after chemotherapy. Results from these trials are eagerly anticipated.

Therapy in Advanced-Stage HL

ABVD chemotherapy has been the standard of care for patients with advanced HL for over 20 years. Initially developed as non-cross-resistant alternative regimen to MOPP chemotherapy in the 1970s (Bonadonna et al. 1975), ABVD became the standard of care based on the results of randomized clinical trials conducted in the 1980s–1990s, which showed higher efficacy and lower toxicity of this regimen compared to MOPP and alternating and hybrid regimens i.e., alternating MOPP/ABVD and MOPP/ABV hybrid (nitrogen mustard, vincristine, procarbazine, prednisone/adriamycin, bleomycin, vinblastine) (Bonadonna et al. 1986; Viviani et al. 1996; Connors et al. 1997; Duggan et al. 2003). In particular ABVD markedly reduces the risks of sterility, secondary myelodysplastic syndromes, and acute leukemias. However, for patients with advanced-stage HL, the cure rate with ABVD remained suboptimal compared to early-stage patients (75% compared to over 90%) (Canellos et al. 2014).

To increase long-term cure for high-risk patients, regimens with increasing dose intensity and dose density such as standard and escalated variants of the BEACOPP regimen were developed. In the HD9 trial, BEACOPPesc demonstrated superiority to standard BEACOPP and cyclophosphamide, vincristine, procarbazine, and prednisone (COPP)-ABVD with 5-year OS of 91%, 88%, and 83% and 5-year FFTF of 88%, 76%, and 68%, respectively. In a recently published 10-year follow-up, the updated FFTF and OS rates continued to favor BEACOPPesc, with overall secondary malignancy rates similar across all the three groups (5.7–6.6% range), but an increase in secondary acute myeloid leukemias and myelodysplastic syndromes (3% compared to 1.5% in BEACOPP and 0.4% in ABVD COPP) and organ-related toxicities, including infertility and cardiopulmonary toxicities in the BEACOPPesc-treated patients (Engert et al. 2009).

Subsequent trials in this population focused on balancing dose intensity with moderating toxicity. The GHSG HD12 trial demonstrated that 4 cycles of BEACOPPesc followed by 4 cycles of standard BEACOPP (4 + 4) did not reduce the number of toxicity-related deaths (27 vs 19) and resulted in significantly more patients with progressive disease (3.3% vs 1%) (Borchmann et al. 2011). The non-inferiority GHSG HD15 trial compared 6–8 cycles of BEACOPPesc and 8 cycles or BEACOPP administered every 14 days (BEACOPP14), followed by radiation therapy in patients with residual end of therapy PET positivity. BEACOPPesc for 6 cycles achieved similar 5-year FFTF rates than 8 cycles of the same chemotherapy regimen and of BEACOPP14 (i.e., 89.3% vs 84.4% and 85.4%) with less toxicity and better OS (95.3% vs 91.9% vs 94.5%, respectively). Based on these results, 6 cycles of BEACOPPesc became the standard of care for advanced HL in Europe (Engert et al. 2012).

Four randomized clinical trials have subsequently compared ABVD with different schedules of BEACOPP (Federico et al. 2009; Merli et al. 2016; Viviani et al. 2011; Carde et al. 2016; Mounier et al. 2014). The Italian trial sponsored by Fondazione Michelangelo randomized 331 patients with unfavorable HL (stage IIB,

III, or IV or an International Prognostic Score of ≥3), to receive either standard BEACOPP or ABVD, each followed by local radiotherapy when indicated. The 7-year freedom from first progression (FFFP) favored the BEACOPP group (85% vs 73%); however, the OS from both groups was equivalent to 89% vs 84% with ABVD ($P = 0.39$). Of note, more patients in the ABVD group (45 vs 20) required high-dose salvage therapies, and more patients in the BEACOPP groups had therapy-related toxicities (Viviani et al. 2011). The HD2000 trial, whose 10-year follow-up was recently published, randomized 307 patients with advanced HL to 6 cycles of ABVD versus 4 BEACOPPesc plus 2 standard BEACOPP cycles (4 + 2) versus 6 cycles of COPP-EBV-CAD (cyclophosphamide, lomustine, vindesine, melphalan, prednisone, epidoxorubicin, vincristine, procarbazine, vinblastine, and bleomycin; CEC). The dose-intense regimens, BEACOPP and CEC, resulted in better tumor control with 10-year PFS of 75% for BEACOPP and 76% with CEC versus 69% for ABVD, but again there was no benefit in terms of OS (84%, 86%, and 85%, respectively) (Merli et al. 2016). The French LYSA H34 and the EORTC 20012 Intergroup phase III trials compared 8 cycles of ABVD to 8 cycles of BEACOPP (4 cycles of BEACOPPesc followed by 4 cycles of standard BEACOPP) in two different subgroups of patients with advanced-stage HL, those with low-risk (i.e., IPS-7 of 0–2) and those with high-risk disease (i.e., IPS-7 of 3 or greater). In the low-risk group, the 5-year PFS favored BEACOPP arm (93% vs 75% for ABVD); however, the differences in 5-year event-free survival (EFS) (77% vs 62%) and 5-year OS (99% vs 92%) did not reach statistical significance. BEACOPP was associated with higher morbidity during treatment, with 35% of patients experiencing febrile neutropenia versus 8% in the ABVD arm. Longer follow-up is needed to assess for long-term toxicity. In the high-risk population, no significant difference in the 4-year EFS (63.7% for ABVD versus 69.3% for BEACOPP) or 4-year OS (86.7% versus 90.3%) was observed, although twice as many patients in the BEACOPP group had early discontinuation of therapy (before cycle 5) (Carde et al. 2016).

As in early stage, interim PET-adapted therapy paradigms have been explored. The interim results of the Response-Adapted Therapy in Advanced Hodgkin Lymphoma (RATHL) study were recently published (Johnson et al. 2016). This trial was designed to investigate whether interim PET results, after 2 cycle of ABVD, could direct further therapy, eliminating potentially unnecessary treatments in PET-negative patients, who were randomized to continue ABVD or omit bleomycin (AVD), and improving the outcome through early escalation of therapy with BEACOPP in PET-positive patients. A total of 1214 patients were registered and 937 (83.7%) achieved PET negativity after 2 cycles. Three-year PFS and OS were 85.7% and 97.2% for the ABVD arm, and 84.4% and 97.6% for AVD group, with the results falling just short of the specified non-inferiority margin, but with lower incidence of pulmonary toxicity in the AVD group (1% vs 3%). Of note, incidence of pulmonary toxicity observed in this study was lower than the 6% historically reported, with higher incidence in older patients, and only two deaths occurred among the patients with negative interim PET. These data support the elimination of bleomycin in patients older than 40 years or patients with negative PET findings

after 2 cycles (Barrington et al. 2016). With respect to the 16% of the patients who were PET positive after 2 cycles of ABVD, treated with 4–6 cycles of BEACOPP, 3-year PFS and OS and PFS were 67.5% and 87.8%. Similar results were observed in the Southwest Oncology Group S0816 (Press et al. 2016) where the 18% of patients who had PET positivity after 2 cycles of ABVD were escalated to BEACOPP; these patients had a 2 year PFS of 64%. Although these rates compare well with historical data, this warrants validation in a prospective randomized comparison (Gallamini et al. 2007). Improved risk stratification tools for these patients may help to further clarify treatment decision making.

The impressive and durable responses induced by brentuximab vedotin (BV), in relapsed HL (Younes et al. 2010) have prompted clinical trials exploring its potential role integrated into the frontline therapy of HL. Data from a phase 1 trial, where BV was administered in combination with ABVD or AVD, showed a significant increase of pulmonary toxicity when BV was administered concurrently with bleomycin; however, tolerability was good with impressive efficacy when BV was combined with AVD. Patients with advanced-stage and unfavorable prognostic features achieved a CR rate of 96% treated with the combination of BV and AVD (Younes et al. 2013). Based on these data, a randomized phase III study is currently ongoing comparing BV in combination with AVD (doxorubicin, vinblastine, and dacarbazine) vs ABVD for frontline treatment of advanced HL (ClinicalTrials.gov #NCT01712490).

Another ongoing phase III trial in Europe (ClinicalTrials.gov #NCT01569204) is investigating BV in combination with a modified version of BEACOPP, BrECADD (BV, etoposide, cyclophosphamide, doxorubicin, dacarbazine, and dexamethasone). This original chemotherapy regimen developed to avoid procarbazine-related gonadal toxicity showed a superior organ toxicity profile compared to a more conservative modified version of BEACOPP, BrECAPP (BV, etoposide, cyclophosphamide, doxorubicin, procarbazine, and prednisone) in a phase II trial presented at the 2015 ASH meeting (Borchmann et al. 2015).

Currently several factors need to be weighted in the choice of therapy including: risk score of patient, patient fitness, short- and long-term toxicities, and the availability of efficacious although intensive salvage therapy.

Treatment of Relapsed/Refractory HL

Autologous stem cell transplantation (ASCT) can induce durable responses in about half of the patients with refractory or relapsed disease who achieve adequate disease control with salvage chemotherapy, and it has been the standard of care for transplant eligible patients for over two decades (Linch et al. 1993; Schmitz et al. 2002). The choice of the salvage chemotherapy is usually institution dependent, as there is no randomized controlled clinical trial comparing regimens. Ifosfamide, carboplatin and etoposide (ICE) (Moskowitz et al. 2001), dexamethasone, high-dose cytarabine, and cisplatin (DHAP) (Josting et al. 2005) and various gemcitabine containing

regimens (Baetz et al. 2003; Bartlett et al. 2007; Hawkes et al. 2014) are the most commonly used. Similar efficacy has been reported in single-arm studies with ORR in the 70–89% range and CR rates in the 20–37% range. More intensive regimens such as BCNU, etoposide, cytarabine, and melphalan (Mini-BEAM) and the modification of it with dexamethasone (Dexa-BEAM) have also been explored, but are not routinely adopted as first-line salvage therapy due to potential impairment and of hematopoietic stem cell collection and higher toxicity (Colwill et al. 1995; Brandwein et al. 1990).

Historically, patients who do not respond to pretransplant salvage therapy or who relapse after ASCT remain largely incurable and until recently had a poor prognosis and a median survival of less than 3 years (Kewalramani et al. 2003). The role of chemotherapy in this setting has historically been palliative, with responses usually of short duration. The efficacy of the cytotoxic drugs, gemcitabine (Santoro et al. 2000) and bendamustine (Moskowitz et al. 2013), has shown a high ORR rate between 39% and 55%, in heavily pretreated HL patients; however, the improvement in outcomes is limited by short PFS duration of 5–6 months.

Over the past 5 years, tremendous advances have been accomplished in the treatment of patients with relapsed and refractory HL. BV, an antibody directed against CD30 conjugated to an anti-microtubule drug, monomethyl auristatin E, has received accelerated approval by the US Food and Drug Administration (FDA) in 2011 for the treatment of HL relapsed after ASCT or after two lines of chemotherapy and ineligible for ASCT. In an early phase I trial of relapsed HL and ALCL, BV demonstrated significant activity, with a tolerable safety profile (Younes et al. 2010; Fanale et al. 2012). In the pivotal phase II study, 102 patients with relapsed or refractory HL after ASCT, who received a median of 3.5 prior therapies, were treated with 1.8 mg/kg of BV every 3 weeks for maximum of 16 cycles with ORR of 75%, including CR 34% (Younes et al. 2012). The median duration of response (DOR) was 6.7 months for responders and 20.5 months for complete responders, with PFS of 5.6 months in the whole group and up to 21.7 months in complete responders. In a recently published update of response and survival outcomes of this study, at a median follow-up of 5 years, durable remissions in a small subset of complete responders (13 of 34, 38%) were confirmed. Of these 13 patients, nine remained in remission without receiving any further anticancer therapy, whereas four received consolidative allogeneic stem cell transplant (Chen et al. 2016a). Favorable prognostic factors in this series were younger age, good performance status, and lower disease burden at baseline. The most common adverse effects (AE) reported was peripheral neuropathy, which occurred in 42% of the patients, and was grade 3 or higher in 8% of them, leading to discontinuation of the drug. The median time to onset of neuropathy was 12.4 weeks, and it was reversible in 80% of patients at a median of 13 weeks after BV discontinuation or dose reduction. Other grade 3 or higher AE included neutropenia (20%), thrombocytopenia (8%), and anemia (6%).

The activity of BV as consolidation therapy after ASCT has also been demonstrated. In a randomized, double-blind, placebo-controlled phase III clinical trial, 329 patients with unfavorable or refractory HL status post-ASCT received 16 cycles

of 1.8 mg/kg of BV or placebo (Moskowitz et al. 2015a). Median PFS was almost doubled in the BV arm compared to placebo (42.9 months vs 24 months), at a cost of a significant increased peripheral sensory neuropathy (56% vs 16%, respectively) and neutropenia (35% vs 12%). Pulmonary toxicity was similar in the two groups (5% and 3%). At the time of analysis, 53 (16%) patients had died, equally distributed in the two treatment groups. While this study demonstrates the efficacy of BV in high-risk patients in the post-ASCT setting, it is not clear if it has equal benefit to lower-risk patients who proceed to ASCT with no residual disease or in patients who had significant prior exposure to BV, who were not included in this study. It also remains to be seen whether this improvement in PFS will translate into an improvement in OS.

To improve the PET negativity rate prior to ASCT, which appears to be the strongest predictor of outcome after ASCT (Moskowitz et al. 2010; Gentzler et al. 2014; Akhtar et al. 2013; Devillier et al. 2012), integration of BV into the salvage regimen has also been investigated**.** In a phase II trial, 45 patients with relapsed or primary refractory HL received 1.2 mg/kg of BV on day 1, 8 and 15 of two 28-day cycles, followed by ASCT if PET negative or augmented ICE and ASCT if PET positive. Overall 12 of 45 patients (26%) achieved PET negativity prior to transplant without additional chemotherapy, and 22 of 45 patients (48%) achieved PET with BV followed by augmented ICE (Moskowitz et al. 2015b). In another phase II study, 37 patients with relapsed/refractory HL received 1.8 mg/kg of BV on day 1 of four 21-day cycles, followed by ASCT. Overall 24 of 37 patients (65%) achieved a CR pretransplant, and 12 patients (32%) achieved a partial remission (PR), four proceeded to ASCT, and eight received additional chemotherapy prior to ASCT. All patients collected an adequate number of hematopoietic stem cells for the transplant and successfully engrafted (Chen et al. 2015). These data suggest that BV is an active and well-tolerated component of the pretreatment salvage regimen, which allows adequate stem cell collection, and may optimize cytoreduction prior to ASCT. Future trials are needed to clarify which would be the best role and timing of BV use, for pre-ASCT cytoreduction or post-ASCT consolidation.

Nivolumab, a fully human immunoglobulin (Ig) G4 anti-programmed death receptor-1 (PD-1) antibody that blocks the interaction between the PD-1 receptor and its ligands PD-L1 and PD-L2, recently obtained accelerated approval by the FDA, on May 17, 2016, for HL that has relapsed or progressed after ASCT and posttransplantation. In a phase I study of 23 heavily pretreated patients with relapsed or refractory HL, nivolumab showed high activity with an ORR of 87%, including 17% CRs and an acceptable toxicity profile, with only 22% grade 3 (including myelodysplastic syndrome, pneumonitis, and colitis) and no grade 4 AEs; PFS at 23 weeks was 86%. Analyses of pretreatment tumor specimens from ten patients revealed copy-number gains in PD-L1 and PD-L2 and increased expression of these ligands in all ten patients (Ansell et al. 2015). A registration trial evaluating nivolumab in patients with HL after failure of ASCT is currently ongoing and recruiting participants (CheckMate-205 NCT02181738).

Similarly promising results were reported for pembrolizumab, a second human anti-PD1 monoclonal antibody. In a cohort of 31 patients with relapsed refractory

HL, most of whom received more than 4 prior lines of therapy including BV, the ORR was 65% including 16% CR, with a DOR not reached at a median follow-up of 10 months. Five of 31 patients experienced grade 3 AE; there were no grade 4 AEs, with the most common toxicities colitis and pneumonitis (Ansell et al. 2015). In an ongoing phase 2 study (NCT02453594), pembrolizumab is being investigated in HL patients: (1) relapsed after ASCT and subsequent BV (cohort 1), 2) ineligible for ASCT due to chemoresistance and BV therapy failure (cohort (2), or (3) relapsed after ASCT but not treated with BV after ASCT (cohort 3). Preliminary results of cohort 1 and 2 patients were presented at the 2016 ASCO meeting. At 12 weeks, ORR among 30 patients in cohort 1 was 70% (95% CI, 51–85) including 20% of CR for 30 pts. in cohort 2 ORR was 80% (95% CI, 61–92) including 27% CR. The most common AEs included fever (13%) and diarrhea (8%) (Chen et al. 2016b). A phase 2 trial of pembrolizumab as consolidation post-ASCT (NCT02362997) and a phase III trial of pembrolizumab versus BV in relapsed refractory HL (NCT02684292) are currently ongoing and recruiting patients.

Another strategy currently under investigation is to combine an immune checkpoint inhibitor with BV potentially overcoming tumor cell resistance and deepening clinical response. The preliminary results of 23 relapsed/refractory HL patients treated in a phase 1 study, investigating the combination of BV and the checkpoint inhibitor ipilimumab, an anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) antibody, were reported at the 2015 ASH meeting. Overall the regimen was extremely well tolerated and active, with primarily grade 1 and 2 AE, such as diarrhea, rash, and peripheral sensory neuropathy, with ORR of 72% and CR 50% (Diefenbach et al. 2015b). This study is currently ongoing and enrolling to subsequent arms evaluating the combinations of BV with nivolumab and with nivolumab and ipilimumab (NCT01896999).

Allogeneic Stem Cell Transplant

Despite an overall improvement in SCT outcomes, primarily mediated by advances in supportive care, HLA typing, and the introduction of reduced intensity conditioning (RIC) regimens, recurrence of disease and treatment-related toxicities remain the major causes of ASCT failure. Additionally the lack of adequate disease control or the availability of a suitable donor, further limits the application of allo-SCT for relapsed HL. The outcome report for RIC allo-SCT from 188 HL patients from the Working Party Lymphoma and the European Group for Blood and Marrow Transplantation described a 20% treatment-related mortality at 1-year and 2-year OS of 50% (Robinson et al. 2002). Results from a large prospective European phase II trial of RIC allo-SCT, which enrolled 78 HL patients reported that almost 20% did not proceed to allo-SCT due to inadequate disease control and died of progressive disease at a median of 10 months. For patients undergoing allo-SCT, PFS at 1 year was 48% and dropped to 24% at 4 years, with significantly superior outcome observed for patients allografted in complete remission (PFS at 1 and 4 years of

70% and 50%, respectively) (Sureda et al. 2012). At this stage, RIC allo-SCT should be considered in selected patients with excellent disease control prior to allo-SCT, a matched donor, and limited alternate treatment options or in the setting of a clinical trial.

HL in Elderly Patients

Age is a negative prognostic factor for HL on IPS-7, and indeed outcomes for elderly patients with HL are significantly inferior to those of younger patients (Jagadeesh et al. 2013). Factors contributing to chemotherapy resistance and poor outcome for elderly patients are myriad and include higher incidence of advanced disease, comorbidities impairing chemotherapy delivery, increased therapy-associated toxicities (i.e., bleomycin-induced lung toxicity), and biologic differences including histology (Halbsguth et al. 2011). Elderly patients are often excluded from randomized trials, so an evidence-based standard of care is lacking. In a recent analysis of 287 patients older than 60 with early-stage favorable HL, enrolled in the GHSG HD10 and HD13 trials, 2 cycles of ABVD or AVD followed by IFRT were equally tolerated with grade 3/4 AE occurring in about 40% of the patients, whereas 4 cycles of ABVD plus IFRT were associated with unacceptable toxicity (including 10% bleomycin pulmonary toxicity) and grade 3/4 AE in up to 65% of the patients, resulting in 3 lethal events. Complete response rates also favored 2 cycles of therapy (AVD or ABVD) compared to 4 cycles of ABVD (96% and 99% versus 88%, respectively), resulting from a higher number of deaths in the latter group (Boll et al. 2016). Based on this study, prior reports on bleomycin-induced lung toxicity in elderly (Evens et al. 2012) and the emerging RATHL data (Barrington et al. 2016) bleomycin should be omitted after 2 cycles in elderly HL patients.

Second-line salvage therapies are challenging in this population, due to limited data and poor tolerability. In a recent retrospective analysis of second-line treatment and survival in 105 relapsed/refractory HL patients with a median age of 66 years, less than 25% received intensified salvage regimens followed by ASCT, less than 50% received conventional poly-chemotherapy and/or salvage radiotherapy with curative intent, and the remainder received palliative approaches. Median OS for the entire cohort was 12 months. Early relapse (<12 months from initial therapy), advanced stage at relapse (III-IV), and anemia were used to risk stratify the patients. In low-risk patients, the impact of therapy on survival favored the conventional poly-chemotherapy/salvage radiotherapy approach, whereas in high-risk patients, OS was low overall and did not differ significantly among treatment strategies, suggesting that intensified therapies do not improve the outcome in frail or high-risk elderly patients (Boll et al. 2013).

A retrospective subgroup analysis of 38 patients with refractory/relapsed HL older than 60 enrolled in several BV clinical trials, showed an ORR of 56%. Toxicity

was manageable but significantly higher than in younger patients with higher rates of grade 3 AE, i.e., anemia 30% vs 10%, peripheral sensory neuropathy 60% vs 46% and fatigue 58% vs 43% (Gopal et al. 2014). A phase 2 clinical trial designed to evaluate the efficacy and safety of BV as a single-agent and in combination with dacarbazine or bendamustine in frontline therapy of HL in adults age 60 and above is currently ongoing (NCT01716806).

Pediatric HL

In children HL is a highly curable disease with long-term survival approaching 90–95%. Toxicities from chemotherapy and radiation constitute a major threat to long-term survival and the preservation of long-term quality of life, and the minimization of late therapy-related toxicity in this population remains a high priority. Modifications of ABVD and of radiation protocols have been investigated, to minimize long-term complications without compromising therapeutic efficacy. Several risk-based and response-adapted strategies have been developed by pediatric oncology groups worldwide; no single standard of care regimen has been adopted.

In North America, the Children's Oncology Group (COG) has developed a modified ABVD regimen, ABVE-PC (doxorubicin, bleomycin, vincristine, etoposide, prednisone, cyclophosphamide) with variations based on risk stratification (Tebbi et al. 2012; Keller et al. 2010; Schwartz et al. 2009). In Europe, the European pediatric and adolescent Hodgkin lymphoma network (Euronet-HD) is currently investigating OEPA (vincristine, etoposide, prednisone, and doxorubicin) for low-risk disease and OEPA with the addition to COPDac (cyclophosphamide, vincristine, prednisone, dacarbazine) for intermediate- and high-risk groups (NCT00433459). All the above regimens were designed to decrease cumulative doses of anthracyclines, alkylating agents, and bleomycin compared with the MOPP and ABVD regimens, which are thought to be involved in most of the long-term complications.

Low-dose IFRT/INRT is the standard of care in children with advanced disease; however, it is omitted in low-risk patients achieving a CR after 2 cycles (Mauz-Korholz et al. 2010) and in intermediate-risk patients achieving a rapid reduction in tumor size by CT after 2 cycles of chemotherapy (Friedman et al. 2014). Pediatric radiation protocols utilize lower doses (15–25 Gy) and smaller fields (with lower heart and lung exposure), to minimize long-term toxicities such as risk of bone and soft-tissue hypoplasia observed in children treated with adult protocols. Longer follow-up is needed to assess the benefits of this approach (Hodgson et al. 2007). Proton therapy, which may allow further reductions in normal tissue exposure, is currently being assessed in a COG trial for high-risk HL (NCT02166463).

For patients with relapsed or primary refractory disease, current therapies include high-dose chemotherapy and ASCT with potential for cure similar to their adult counterpart.

Therapy and Prognosis of Nodular Lymphocyte Predominance Hodgkin's Lymphoma (NLPHL)

There is no established prognostic score in NLPHL; in the largest series reported, which included 8298 patients (394 with NLPHL and 7904 with cHL) enrolled in the GHSG HD 4 and HD 12 trials, negative prognostic factors affecting FFTF in NLPHL were advanced stage ($P = 0.0092$), Hb less than 10.5 g/dL ($P = 0.0171$), and lymphopenia ($P = 0.010$); negative prognostic factors affecting OS were age > or = 45 years ($P = 0.0125$), advanced stage ($P = 0.0153$), and Hb less than 10.5 g/dL ($P = 0.0014$) (Nogova et al. 2008).

There are no prospective randomized clinical trials to guide the therapy of this uncommon subtype of HL; data are extrapolated from subset analyses of NLPHL patients included in cHL trials and single-institution or pooled multi-institutional retrospective analyses. The largest dataset comes from the European Task Force on Lymphoma Project on NLPHL, which included 426 patients, of which only 219 (51%) were confirmed as NLPHL after pathology review. More than 75% of the cases were diagnosed as early stage, peripheral node involvement was more common than mediastinal disease, and B symptoms were rarely reported. Despite the excellent prognosis, high rate of late relapses and propensity to transform into an aggressive form of B cell NHL were noted, indicating the need of long-term follow-up and re-biopsy at relapse. Secondary malignancies and other treatment-related toxicities seem to contribute significantly to overall mortality in this disease (Diehl et al. 1999).

Early-stage disease is usually treated with RT alone. Recommendations regarding the use of radiation in early stage derive from observational studies, which showed excellent outcomes with limited RT and no benefit of more extensive radiation fields or CMT, which were associated with greater toxicity (Nogova et al. 2005; Wilder et al. 2002). A large retrospective study from the Australasian Radiation Oncology Lymphoma Group, including 202 patients with early-stage NLPHL treated with RT alone at a median follow-up of 15 years, reported OS rates of 88% at 10 years and 83% at 15 years, with FFDP of 88% at 10 years and 82% at 15 years, respectively (Wirth et al. 2005). In contrast, in a retrospective analysis of patients with early-stage NLPHL (IA/IB/IIA) from the British Columbia Cancer Agency, 51 treated with ABVD or ABVD plus RT, and 35 treated with RT alone, 10-year PFS favored the use of chemotherapy or CMT (98%) versus RT alone (76%), with a trend toward improved survival (84% versus 93%) (Savage et al. 2011). In the absence of conclusive data, the general recommendations include RT for early-stage NLPHL (NCCN) versus r = RT alone only for stage IA and CMT as in cHL for stage IIA (ESMO). Patients with B symptoms and bulky early-stage disease are extremely rare and usually treated with cHL advanced disease protocols.

Patients with advanced disease account for only 20–25% of the NLPHL patients and have a worse prognosis than their cHL matched counterpart. Treatment usually consists of multi-agent chemotherapy, including ABVD which appears less efficacious in this setting (Xing et al. 2014). A retrospective CALGB study suggests that alkylator-based chemotherapy may be a better option for these patients; among 37 patients with advanced NLPHL, 8 of the 25 (32%) treated with MOPP or MOPP/

ABVD and 9 of the 12 (75%) treated with ABVD/EVA (etoposide, vinblastine, adriamycin) relapsed or had refractory disease (Canellos and Mauch 2010). Finally, chemotherapy regimens used for NHL have been used in NLPHL, based on the immune-phenotypic resemblance and CD20 positivity. In a series from MD Anderson Cancer Center describing the outcome of 52 patients with NLPHL, 15 patients, 11 of whom had stage III/IV disease, were treated with R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy without radiation. At a median follow-up of 42 months, no patients had relapsed suggesting long-term efficacy of this combination (Fanale et al. 2010). Rituximab is often administered along with multi-agent chemotherapy, based on CD20 positivity of NLPHL, although evidence for improved outcome is lacking.

In patients with relapsed disease, a repeat biopsy is crucial to rule out transformation to high-grade NHL. Therapy options range from RT alone in localized recurrences, to combination chemotherapy or CMT, and there are no large series describing outcomes with these strategies. Salvage chemotherapy followed by ASCT in highly selected patients has been reported in small retrospective studies, but it is not standard of care (Karuturi et al. 2013). Small phase 2 trials have been conducted to investigate the role of single-agent rituximab in untreated and relapsed NLPHL (Schulz et al. 2008; Eichenauer et al. 2011; Advani et al. 2014). Despite the high response rate observed across all the studies with ORR ranging from 94 to 100%, the main limitation appears to be the short duration of response and high incidence of transformed lymphoma at recurrence. In one series 9 of the 23 patients who relapsed after single-agent rituximab had evidence of large cell transformation (Advani et al. 2014).

Conclusion

Over the past years tremendous efforts have been carried on to improve the efficacy of therapy and minimize the long-term morbidity and mortality of HL treatment. Future directions include refining clinical prognostic scores with the integration of new molecular prognostic markers and interim PET results, to more precisely identify high-risk patients, and the incorporation of novel agents such as BV and checkpoint inhibitors into frontline and salvage therapy paradigms.

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Chapter 10
Novel Targeted Therapies in Hodgkin Lymphoma

Robert W. Chen

Introduction

Hodgkin lymphoma is a lymphoproliferative disorder distinctly characterized by the presence of Reed-Sternberg (RS) cells in the lymphoma mass. It accounts for approximately 10% of all lymphomas, with approximately 9000 new cases in 2014 and an expected estimate of 1180 resulting deaths (Siegel et al. 2013; Jemal et al. 2009). On the basis of the World Health Organization (WHO) classification, Hodgkin lymphoma has been divided into two main types: classical Hodgkin lymphoma (HL) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL). The development of curative treatments for HL has been voted as one of the top five achievements of oncology in the last 50 years (http://www.asco.org/research-progress/cancer-progress/top-5-advances-modern-oncology). In recent years, further understanding into the molecular biology of HL has given rise to the development of novel targeted therapies. Additional classes of drugs such as antibody drug conjugates, PD-1 and PD-L1 inhibitors, histone deacetylase inhibitors, PI3-Kinase inhibitors, AKT/mTOR pathway inhibitors, and immunomodulating agents are currently undergoing clinical trials. This chapter will focus on the novel agents with demonstrated activities and on those under clinical evaluation.

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Brentuximab Vedotin

The neoplastic classical Hodgkin Reed-Sternberg malignant cells are universally and immunophenotypically defined by CD30 expression (Younes and Kadin 2003). CD30 is a transmembrane glycoprotein and a member of the tumor necrosis factor (TNF) family (Younes and Kadin 2003). The pathological role of CD30 in HL seems to involve cytokine production of IL6 and TNF-alpha and induction of the NF-κB pathway, resulting in expression of anti-apoptotic genes (Aizawa et al. 1997). CD30 expression appears to be a marker of lymphocyte activation and hence is rarely expressed in normal tissues, making it an ideal candidate for targeted anti-tumor therapy.

Brentuximab vedotin (BV) is the first agent approved for the treatment of HL in the last 30 years. It is in a relatively new class of drugs called antibody drug conjugates (ADC). This drug has three components: the CD30-specific chimeric IgG1 monoclonal antibody (mAb) cAC10, a dipeptide linker, and monomethyl auristatin E (MMAE). Once BV binds to CD30, it is internalized inside the cell. The linker is then cleaved, releasing MMAE to bind tubulin and disrupt the microtubule network, resulting in cell cycle arrest and subsequent apoptosis of neoplastic cells expressing CD30 (Newland et al. 2013). The mouse-human chimeric IgG1 anti-CD30 mAb is conjugated via a citrulline-valine peptide linker to MMAE, a synthetic dolastatin 10 analog. This peptide linker is highly stable in the plasma but can be efficiently cleaved by lysosomal proteases after internalization in targeted CD30-positive cells, allowing for minimal toxicity (Doronina et al. 2003). However, a small fraction of free MMAE can leak out of the cells and result in cytotoxicity in the tumor microenvironment, further diminishing the supporting sources of tumor growth (van de Donk and Dhimolea 2012). The half-lives of the ADC and of MMAE are approximately 4–6 days and 3–4 days, respectively.

The first-in-man phase I trial conducted by Younes et al. enrolled 45 patients with relapsed or refractory CD30-positive lymphomas (93% of patients had HL) (Younes et al. 2010). These patients were given escalating doses of BV (from 0.1 mg/kg to 3.6 mg/kg) every 3 weeks. This study reported dose-limiting toxicities to include grade 4 thrombocytopenia, grade 3 hyperglycemia, and febrile neutropenia. Overall the drug was well tolerated and yielded an impressive 50% overall response rate. These notable results prompted an open-label phase II study in 102 patients with relapsed/refractory HL after autologous stem cell transplantation (ASCT).

The investigators administered BV 1.8 mg/kg every 3 weeks by 30-minute intravenous infusion for up to 16 cycles. All 102 patients in this trial had relapsed/refractory HL post-ASCT. The patients were heavily pretreated, with a median of 3.5 prior therapies. In this trial, the objective overall response rate (ORR) was 73%, and the complete response (CR) rate was 34%. The data were first reported by Chen et al. at the 2010 annual meeting of the American Society of Hematology (ASH) (Chen et al. 2010) and later by Younes et al. in the Journal of Clinical Oncology in 2012 (Younes et al. 2012a). In this trial, 42% of patients developed treatment-related peripheral neuropathy, 35% nausea, and 34% fatigue. Eight percent of all patients

developed neuropathy of grade 3 or higher, and this was the most common reason for discontinuation of BV. On the basis of the results of these early trials, BV was granted accelerated approval by the Food and Drug Administration (FDA) in August 2011 for the treatment of patients with HL after failure of ASCT or after failure of at least two prior multi-agent chemotherapy regimens in patients ineligible for ASCT.

Results of a 5-year follow-up of this study were recently published (Chen et al. 2016a). These results demonstrated an ORR of 73%, and 34% of patients achieved a CR with the median duration of response not reached (95% CI of 20.5 months and not reached). At 5 years, the overall survival (OS) rate was 41% and progression-free survival (PFS) rate was 22%. Patients who achieved a CR to brentuximab vedotin (N = 34) had estimated OS and PFS rates of 64% and 52%, respectively. The median OS and PFS were not reached in CR patients, with 13 patients (38% of all CR patients) remaining in follow-up and in remission at study closure. Of the 13 patients, 4 received consolidative hematopoietic allogeneic-SCT (allo-SCT), and 9 (9% of all enrolled patients) remain in sustained CR without receiving any further anticancer therapy after treatment with brentuximab vedotin. Of the patients who experienced treatment-emergent peripheral neuropathy (PN), 88% experienced either resolution (73%) or improvement (14%) in symptoms. This is the first study showing that patients with HL who relapsed post-ASCT may achieve long-term remission or possibly cure with a single agent.

BV in the Transplant Setting

The impressive results from the phase II study have led to the exploration of BV in various phases of the treatment continuum of HL. Two different prospective trials examined the role of BV as a bridge to ASCT for patients refractory to conventional chemotherapy. In a single center phase II trial, Moskowitz et al. implemented a PET-adapted strategy of salvage therapy with BV followed by augmented ifosfamide, carboplatin, and etoposide (ICE) in relapsed and refractory patients. Having previously shown that pre-ASCT PET negativity is a strong predictor of transplant outcome, the investigators enrolled 46 patients to receive BV at 1.2 mg/kg weekly on days 1, 8, and 15 for two 28 days cycles followed by PET evaluation. If they were PET negative, patients proceeded to high-dose therapy (HDT)/ASCT, and if PET positive, patients were assigned to two cycles of augmented ICE. The results are impressive, as overall 34 of the 45 evaluable relapsed/refractory patients achieved PET negativity allowing for a successful bridge to ASCT and improved chance of favorable transplant outcome (Moskowitz et al. 2015a). In a prospective multicenter phase II study, Chen and colleagues accrued 37 patients with relapsed or refractory disease to be treated with BV at 1.8 mg/kg intravenously every 3 weeks for 4 cycles. Their results as presented at the annual ASH meetings in 2014 and 2015 and published in Biology of Blood and Marrow Transplantation (Chen et al. 2015a) illustrated the efficacy of BV as first-line salvage therapy and as instrumental in bridging

to ASCT. The overall best response (CR + PR) among the 36 evaluable patients was 68% with 35% CR and PR in 33% of the patients. Seventy-two percent of the patients were in CR at the time of ASCT, with only half of the patients requiring multi-agent chemotherapy regimens. Patients who were in CR and who received only BV had the best 2-year PFS outcome post-ASCT (Chen et al. 2015b).

BV was also used to prolong the duration of PFS post-ASCT. The phase III AETHERA study evaluated the use of BV for patients with high risk of residual HL following ASCT (Moskowitz et al. 2015b). The investigators reported results on 329 patients who were categorized into three high-risk groups: "refractory to frontline therapy, relapse < 12 months after frontline therapy, and relapse ≥ 12 months after frontline therapy with extra nodal disease." After completion of ASCT, these patients were then given BV 1.8 mg/kg every 3 weeks and best supportive care (BSC) or placebo and BSC for up to 16 cycles. The toxicity profiles were similar to the initial pivotal trial. Patients who received BV had significantly improved median PFS as compared to the placebo group (42.9 months versus 24.1 months, hazard ratio 0.57, 95% CI 0.4–0.81, $p = 0.0013$). The study met the primary endpoint, and BV received an additional FDA approval for use post-ASCT as consolidation therapy.

For patients who relapse after ASCT, allogeneic stem cell transplantation (allo-SCT) remains a potentially curative therapy. As expected, there has been great interest in utilizing BV for cytoreduction prior to allo-SCT. This question was examined in a collaborative effort between City of Hope and Fred Hutchinson Cancer Research Center, retrospectively examining 18 patients who were treated with BV after ASCT failure (Chen et al. 2012). Most of these patients ultimately received a matched unrelated donor graft. Acute graft-versus-host disease (GVHD) occurred in 33.3% in patients, and chronic GVHD in 56.3%. More striking is that overall survival after allo-SCT was 100% and PFS was 92.3% at 1 year. An updated report of these patients has been provided, with a median follow-up of 29.9 months. The investigators of this follow-up analysis have also retrospectively compared outcomes of these patients treated with BV prior to reduced intensity (RIC) allo-SCT to those in a similar historical cohort who also underwent RIC allo-SCT but without pretreatment with BV. Chen et al. showed that BV given prior to allo-SCT can improve disease status at transplantation and minimize peri-transplant toxicities, resulting in a 2-year PFS in the BV-pretreated patients of 59.3%, compared to 26.1% in patients who underwent RIC allo-SCT without BV pretreatment (Chen et al. 2014).

Naturally, interest was raised regarding the introduction of BV in HL patients relapsing after allo-SCT—a group historically with dismal prognosis. Gopal et al. investigated the efficacy of BV given at 1.2 or 1.8 mg/kg every 3 weeks to 25 HL patients with recurrent disease after allo-SCT. In this multicenter retrospective analysis, the ORR was 50%, with a CR rate of 38%, and median PFS of 7.8 months. Again, it was shown that BV could induce durable complete remissions, with over 80% of the patients in CR without evidence of disease at 1 year. Retreatment with BV has also been explored and has also yielded impressive results (Gopal et al. 2012). Bartlett et al. conducted a phase II trial in patients with CD30-positive lymphomas who had previously achieved CR or PR on BV. Notably, the ORR was 60% (30% in CR) in classical HL patients, with a median duration of response of

9.5 months (range 0–28 months) in those with objective response. Impressively, 45% of the patients who achieved CR had response durations over 1 year (Bartlett et al. 2014).

The success of BV in these various settings has led investigators to explore BV with combination chemotherapy in the upfront treatment of advanced HL. The phase III ECHELON-1 trial (NCT01712490) compares standard ABVD with BV and AVD. During the phase I trial lead by Dr. Younes, BV was combined with ABVD, and the study showed excessive pulmonary toxicity (Connors et al. 2014). Bleomycin was then removed from the BV-containing regimen. Long-term outcomes of the phase I trial were presented at the 2014 ASH meeting and were later published, showing a 3-year failure-free survival rate of 83% for ABVD + BV and 96% for AVD + BV and a 3-year overall survival of 92% and 100%, respectively (Younes et al. 2013a).

Other trials of interest involving BV are an elderly upfront trial and a salvage trial adding BV plus bendamustine. Forero-Torres and colleagues presented their data for frontline study of BV monotherapy at the 2014 ASH meeting (NCT01716806). This study was later published in Blood (Forero-Torres et al. 2015). Out of 27 patients treated, the ORR was 92% and CR 73%. The median PFS was 10.5 months. Seventy percent of these patients had stage III–IV disease, and their demographic characteristics included nearly half with creatinine clearance of 30–60 mL/min.

The remarkable response of combination therapy with the ADC and traditional cytotoxic chemotherapy described above has led to the potential addition of bendamustine to BV. This approach is currently being explored in patients with HL who relapse or are refractory to frontline therapy. The interim data have also been presented at the 2014 ASH meeting (LaCasce et al. 2015). Thus far, 45 patients have been enrolled. Twenty-eight of 34 evaluable patients achieved a complete response (82%), and an ORR was seen in 94% (32 of 34 patients). Twenty-four of the 28 patients in CR underwent successful stem cell mobilization and collection. The median duration of remission for patients who obtained a CR had not been reached.

Although BV is efficacious in patients with relapsed/refractory HL, there are rare but serious adverse reactions associated with the drug. There have been two known cases of progressive multifocal leukoencephalopathy (PML) during treatment with BV. There was one report of a patient with Stevens-Johnson syndrome. Finally, a case series of eight patients noted the development of pancreatitis during treatment. Physicians should monitor for these rare but serious adverse reactions during treatment.

HDACi

Histone deacetylase (HDAC) inhibitors are able to exert an anti-lymphoma effect in HL by affecting both the RS cells and the tumor microenvironment. There are a variety of HDACi available with differences in their specificity for particular HDAC isotypes. Vorinostat, a pan-HDAC inhibitor, was studied in a phase II study by

SWOG in patients with relapsed/refractory HL. Kirschbaum et al. showed that this single agent only has a modest activity, with only 1/25 patients achieving a PR (Kirschbaum et al. 2012). Another pan-HDAC inhibitor, panobinostat, was studied in an international phase II trial in patients with relapsed/refractory HL. Twenty-seven percent of patients achieved an ORR, with 4% in CR and 23% in PR (Younes et al. 2012b). The toxicities associated with pan-HDACi are thrombocytopenia, diarrhea, nausea, and fatigue.

Selective HDACi have also been tried to treat patients with relapsed/refractory HL. Entinostat is a class I isoform HDACi that targets HDACs 1, 2, 3, and 11. It was tested in a multicenter phase II trial involving 49 patients. The ORR was 12%, and the disease control rate was 24% (Batlevi et al. 2016). The most frequent grade 3/4 adverse events (AEs) were thrombocytopenia, anemia, neutropenia, leukopenia, hypokalemia, and hypophosphatemia. Mocetinostat is an HDACi specific for class I and IV HDACs. It was evaluated in 51 patients with HL and showed an ORR of 33% (Younes et al. 2011). Little hematologic toxicity was seen with this agent. Overall, as single agents, HDACi are only of modest activity in HL, but the toxicity profile is favorable. Thus, they appear to be suitable candidates for combination therapy with other novel agents.

PI3K/AKT/mTOR Pathway

The PI3K/AKT/mTOR pathway has been implicated in the pathogenesis of HL (Georgakis et al. 2006; Dutton et al. 2005). Idelalisib, a PI3Kd inhibitor, was evaluated in a phase II trial in relapsed/refractory HL. PI3Kd is expressed in B cells and also HL cell lines. In a 25 patient trial, the ORR was 12% with 1 CR, 2 PR, and 9 stable disease (SD) (Younes et al. 2013b). The drug appears to be well tolerated, with some mild liver function elevation. TGR, another PI3K inhibitor, is undergoing evaluation in combination with BV in a phase I/II trial in patients with relapsed/refractory HL (NCT02164006). Results are not available yet at this time. Everolimus, an mTOR inhibitor, has been evaluated in a phase II trial in patients with relapsed/refractory HL (Johnston et al. 2013). In this 57 patient trial, the ORR was 42%, with several patients achieving long-term disease control. The main adverse reactions were grade 3 or 4 thrombocytopenia and anemia occurring in 21% and 12% of patients, respectively. Another study combined HDACi and an mTOR inhibitor. In the 14 patients with HL, the ORR was 43% (Oki et al. 2013). However, thrombocytopenia was a frequent adverse event.

Immunomodulators

Lenalidomide is an immunomodulatory drug with various biological effects, such as increasing IL-2, activating cytotoxic T and NK cells, and degrading IKZF1 and IKZF3 (Kronke et al. 2014). In a phase II study of 36 patients with relapsed/

refractory HL, the ORR was 19%, and an additional 14% achieved stable disease (Fehniger et al. 2011). There are several trials studying the combination of lenalidomide with everolimus, panobinostat, temsirolimus, and bendamustine.

Recent literature has indicated programmed death-1 (PD-1) as a significant component of a pathway used by tumors to evade the immune system. Expression of PD-1 ligands (PD-L1 and PD-L2) on tumor cells inactivates cytotoxic T cells through binding of PD-1 on tumor-infiltrating cells (Keir et al. 2008; Pardoll 2012). PD-L1 is overexpressed on HL RS cells, and PD-1 expressing T cells can be found in the HL tumor microenvironment and circulating blood (Yamamoto et al. 2008). Nivolumab, a PD-1 inhibitor, has been tested in a phase I trial for patients with relapsed/refractory HL, showing an impressive ORR of 87% and CR of 17% (Ansell et al. 2015). This response was confirmed in a phase II trial in patients with HL who progressed after ASCT and BV treatment. The ORR of the phase II trial was 66% (Younes et al. 2016). The drug is well tolerated, with rare occurrences of immunological adverse reactions such as hypothyroidism, diarrhea, and rash. This drug recently received accelerated FDA approval for relapsed/refractory HL post-ASCT and BV. Pembrolizumab, another PD-1 inhibitor, has also been tested in a phase I trial for patients with relapsed/refractory HL, showing an ORR of 65% and CR of 16% (Armand et al. 2016). This response was also confirmed in a phase II trial in patients with relapsed/refractory HL. The ORR in the phase II trial was 73% to 83% depending on the specific cohort, and the CR was 27% to 30% by investigator (Chen et al. 2016b). Avelumab, a PD-L1 inhibitor, is undergoing phase I testing in patients with relapsed/refractory HL (NCT02603419).

Other forms of immunotherapy include recruiting natural killer (NK) cells by using bispecific antibodies and generating cytotoxic immune effector cells to mediate tumor cell lysis. AFM13 is a bispecific anti-CD30/CD16A antibody construct designed to recruit NK cells through CD16A to fight CD30-expressing malignancies. This drug was evaluated in a phase I dose-escalation study of 28 patients with relapsed or refractory HL (Rothe et al. 2015). Adverse events were generally mild to moderate. The ORR was 11.5%, and 50% achieved SD. In 13 patients who received doses of $\geq$1.5 mg/kg AFM13, the overall response rate was 23%, and the disease control rate was 77%. AFM13 treatment resulted in significant NK cell activation and a decrease of soluble CD30 in peripheral blood. A combination study involving AFM13 and pembrolizumab is under way. CAR-T cells, T cells engineered with chimeric antigen receptors, have been tested in various hematological malignancies including acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and non-Hodgkin lymphoma (NHL). Currently a phase I trial is under way to evaluate their efficacy in HL (NCT01192464).

Conclusions

The advances in HL treatment in the last 5 years have been truly exciting. This began with the FDA approval of BV in 2011 and most recently FDA approval of nivolumab. Many patients have benefited from the use of both drugs. The current

landscape of HL treatment is changing rapidly, with BV being incorporated into various settings of treatment. With time, PD-1 inhibitors will soon be combined and used in various settings of HL treatment as well. Precision medicine is the goal of every cancer therapy in the future. Certainly, treatment of HL will also need to be tailored for specific patients with drugs targeting either the RS cells, the tumor microenvironment, or both. Hopefully, the understanding of molecular and cellular biology in HL will keep on advancing, and new drugs will continue to replenish the current pipeline.

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