3 Pathology and Molecular Pathology of Hodgkin Lymphoma

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Contents

3.1 Subclassification and Pathology

The history of Hodgkin lymphoma (HL) dates back to the first half of the nineteenth century (see Chap. 1), and it has also been an established view for quite some time that HL comprises two different disease entities, namely, classical Hodgkin lymphoma (cHL) and nodular lymphocyte-predominant Hodgkin lymphoma (LPHL) [1]. Both entities have in common that the neoplastic

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cell population, which can be mononucleated or multinucleated, makes up only a small percentage of all cells present in an affected lymph node. However, morphological, clinical, epidemiologic, and molecular evidence strongly support the belief that the pathogenesis of these lymphomas is distinct enough to be considered separate entities. From a diagnostic point of view, morphological details and immunohistochemistry for a selected set of markers almost always allow for a proper classification of a given lymphoma into the group of LPHL or cHL, the latter of which can be further subdivided into nodular sclerosis cHL (NSCHL), mixed cellularity cHL (MCCHL), lymphocyte-depleted cHL (LDCHL), and lymphocyte-rich cHL (LRCHL) [1].

The following sections summarize the key morphological aspects and important immunohistochemical features of HL, as well as key biological and genetic features of the HL tumor cells. For microenvironmental, clinical, and epidemiologic parameters, please refer to the respective other chapters of this book.

3.1.1 Nodular Lymphocyte-Predominant Hodgkin Lymphoma

Although the morphology of the tumor cell population of LPHL can occasionally mimic Hodgkin and Reed-Sternberg (HRS) cells of cHL, in most instances the tumor cells in LPHL, which are termed lymphocyte-predominant (LP) cells according to the current WHO classification (previously called L&H cells, for lymphocytic and/or histiocytic Reed-Sternberg (RS) cell variants), carry one large nucleus that is often multilobated ("popcorn cell") (Fig. 3.1a). In contrast to classic HRS cells, the number of nucleoli is increased, but they are usually less prominent and less eosinophilic. LP cells are found in a nodular or follicular background that is dominated by small B lymphocytes that usually express IgD, but a more diffuse growth pattern can also be encountered, especially during progression. The follicular infiltration pattern is highlighted by the presence of CD21-positive follicular dendritic cells that tend to form a well-developed meshwork in the nodules. Immunohistochemically, LP cells demonstrate a complete B cell phenotype with expression of CD20, CD75, and, frequently, CD79a (Fig. 3.1b; Table 3.1). Moreover, the essential B cell transcription factors BOB.1 and OCT-2 are usually positive, and the expression of BCL6 and activation-induced cytidine deaminase (AID) is well in line with a germinal center (GC) derivation of the tumor cells, although CD10 is generally negative [1–3]. The negativity of the tumor cells for CD30, CD15, and Epstein-Barr virus (EBV) helps to distinguish LP cells from HRS cells in

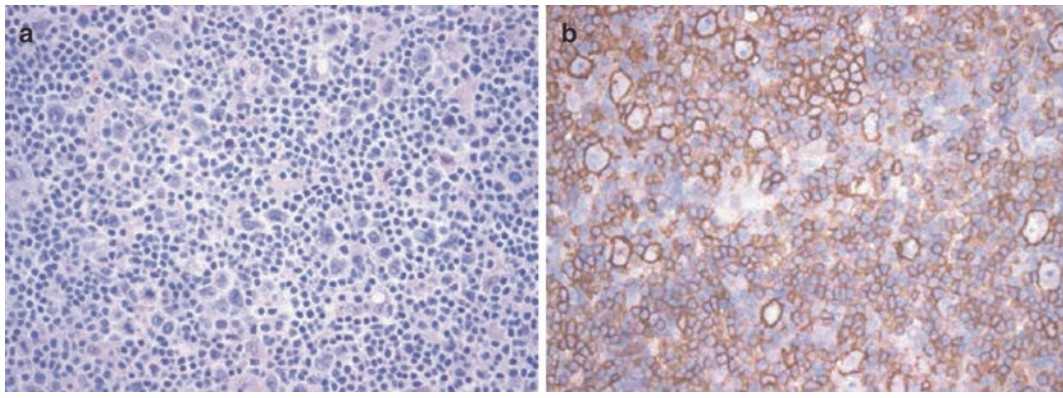

Fig. 3.1 Nodular lymphocyte-predominant Hodgkin lymphoma (LPHL). **(a)** HE-stained lymph node infiltrate showing multiple characteristic, multilobated tumor cells—termed lymphocyte-predominant (LP) cells—in a background of small lymphocytes and histiocytes (×400). **(b)** Strong CD20 expression in LP cells, but also in reactive, small B cells in the background (×400). Note that some of the tumor cells show rosetting by a CD20-negative lymphocyte population. These cells are T cells that often express the follicular T helper cell marker PD-1

Table 3.1 Genetic and phenotypic features of HRS and LP cells

Feature	HRS cells	LP cells
Phenotype		
CD30 expression	Yes	Rare
CD15 expression	Yes (~70%)[a]	No
B cell receptor expression	No	Yes
Loss of most B cell markers	Yes	Modest
Expression of germinal center (GC) B cell markers (e.g., BCL6, activation-induced cytidine deaminase (AID))	Rarely	Yes
Expression of markers for non-B cells (e.g., CD3, granzyme B, CCL17)	Frequently	No
Putative cell of origin	Defective, pre-apoptotic germinal center B cell	Germinal center B cell
EBV positivity	Yes (~40%)	No
Signaling pathways		
NF-κB activation	Yes	Yes
JAK/STAT activation	Yes	Yes
Aberrant expression of multiple RTKs	Yes (~60–100%)	Yes (~40%)
PI3K/AKT activation	Yes	n.a.
AP-1 activation	Yes	Partly
Genetic lesions		
NFKBIA mutations	Yes (~10–20%)	No
NFKBIE mutations	Yes (~10%)	n.a.
TNFAIP3 mutations	Yes (~40%)	No
REL gains/amplifications	Yes (~50%)	No
MAP3K14 (NIK) gains/amplifications	Yes (~25%)	n.a.
BCL6 translocations	Rare	Yes (~50%)
JAK2, PD-L1, PD-L2, JMJD2C gains/amplification	Yes (~30%)	No
STAT6 mutations, gains	Yes (~30%)	n.a.
SOCS1 mutations	Yes (~40%)	Yes (~50%)
PTPN1 mutations	Yes (~20%)	n.a.
GNA13 mutations	Yes (~20%)	n.a.
ITPKB mutations	Yes (~15%)	n.a.
XPO1 mutations	Yes (~20%)	n.a.
B2M mutations	Yes (~30%)	n.a.
MHC2TA translocations	Yes (~15%)	n.a.
SGK1	n.a.[b]	Yes (~50%)
DUSP2	n.a.[b]	Yes (~50%)
JUNB	n.a.[b]	Yes (~50%)

n.a. not analyzed, *RTK* receptor tyrosine kinase

[a]Numbers in brackets refer to the percentage of positive cases

[b]No mutations reported in 2 whole exome sequencing studies of together 44 cases of cHL and an exome sequencing analysis of 6 cHL cell lines [8–10]

cHL, although occasionally a weak positivity for CD30 can be present in LP cells (Table 3.1). Whereas in initial lesions small B cells dominate the background, histiocytes and T cells may become more prominent during the evolution of LPHL, to an extent that LPHL may be hardly distinguishable from T cell/histiocyte-rich large B cell lymphoma (THRLBCL). "Variant histology" (e.g., depletion of small B cells in the background or unusual localization of the LP cells) appears to be associated with an inferior prognosis [4]. A prominent feature of LPHL is the often impressive rosetting of LP cells by T cells that belong to the subset of follicular T helper cells and therefore express CD57 and PD-1 [5–7].

3.1.2 Classical Hodgkin Lymphoma: The HRS Cells

The characteristic tumor cell of cHL, the RS cell, is large and contains at least two nuclear lobes or nuclei, usually with a prominent nuclear membrane (Fig. 3.2a). In contrast to LP cells in LPHL, the nucleoli of RS cells are often eosinophilic. The mononuclear variant of RS cells is termed the Hodgkin cell. However, the morphological spectrum of the tumor cell population in cHL can be broad and includes variants such as lacunar cells and mummified cells. In general, the tumor cells in cHL are called Hodgkin and Reed-Sternberg cells. Immunohistochemically, the HRS cells stain positive for CD30 (Fig. 3.2c), and CD15 is coexpressed in the majority of cases, occasionally with prominent staining of the Golgi area of the tumor cell. However, CD15 is negative in a significant proportion of cHL (20–25%) and therefore not required to establish the diagnosis of cHL [1]. CD45 is usually negative, as are the B cell transcription factors BOB.1 and OCT-2. In the vast majority of cases, the derivation of the tumor cells from the B cell lineage is indicated by a nuclear positivity for the B cell-specific activator protein PAX5/BSAP, but the staining is usually weaker compared to the staining intensity in the small reactive B cell population in the background of the infiltrate [11]. CD20 expression can be observed in HRS cells in 30–40% of cases, but the expression is frequently restricted to a subset of the tumor cell population, and even within one HRS cell, it is of varying intensity in

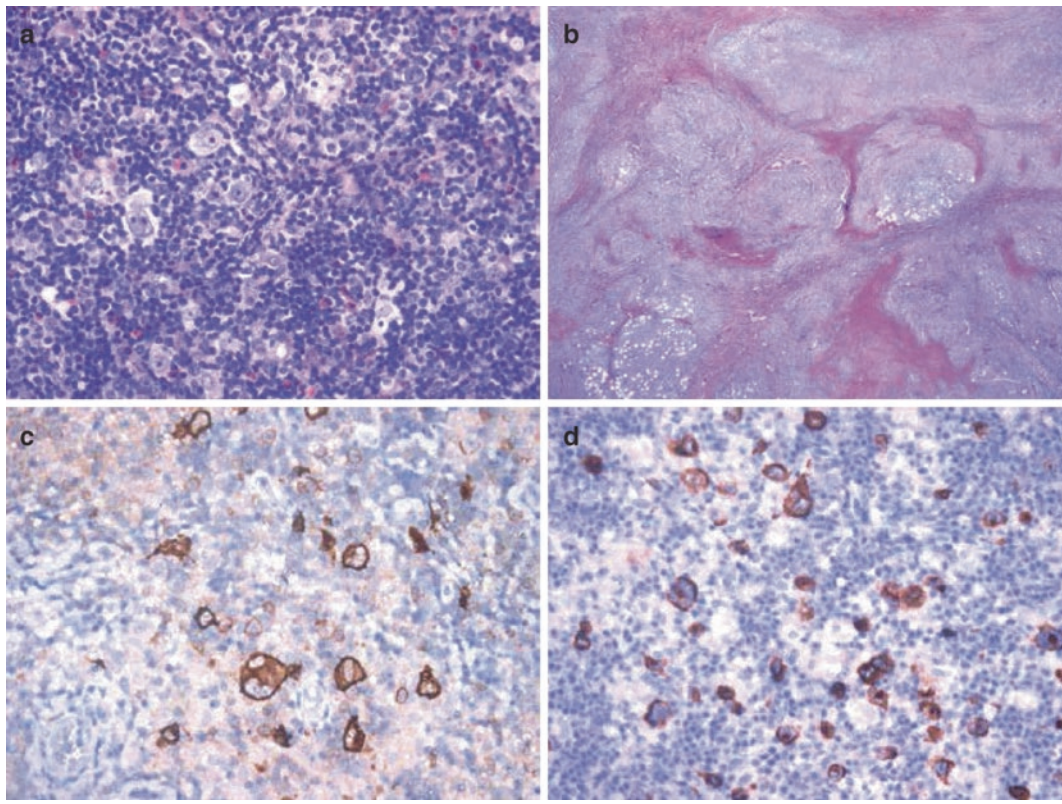

Fig. 3.2 Classical Hodgkin lymphoma (cHL). (**a**) Characteristic Hodgkin and Reed-Sternberg (HRS) cells in a mixed background of small lymphocytes, histiocytes, and eosinophils in a mixed cellularity cHL (MCCHL) (HE, ×400). (**b**) Nodular sclerosis subtype of cHL that demonstrates thick collagen bands surrounding the nodular infiltrates (PAS, ×20). (**c**) CD30 expression in HRS cells (×400). (**d**) Immunohistochemical staining for latent membrane protein 1 (LMP1) shows Epstein-Barr virus (EBV) association of HRS cells (×400)

different parts of the cell membrane. In comparison to CD20 expression, CD79a expression is observed less frequently [12, 13]. An EBV association, either demonstrated by immunohistochemical staining for LMP1 (latent membrane protein 1; Fig. 3.2d) or by EBER in situ hybridization, is found in a significant proportion of cHL, but the frequency varies considerably between different histological subtypes and across geographical areas [1]. Whether cHL cases exist with a *bona fide* derivation from the T cell lineage is currently a matter of debate. Single cases have been reported, in which a T cell receptor rearrangement could be proven in the HRS cells [14, 15], but others argue that such cases might represent only mimics of cHL which are not to be included in a disease entity that—based on fundamental principles of current lymphoma classification schemes—is of B cell derivation [16]. HRS cells reside in a cellular background that varies among the different histological subtypes of cHL which will be discussed in the following sections.

3.1.2.1 Nodular Sclerosis Classical Hodgkin Lymphoma

In NSCHL, affected lymph nodes frequently show a markedly thickened capsule and a nodular infiltrate whereby individual nodules are surrounded by broad collagen bands (Fig. 3.2b). HRS cells are present in a background of small lymphocytes and other nonneoplastic cells such as histiocytes and eosinophils. The number of HRS cells can vary significantly between NSCHL cases and also within a single infiltrated lymph node. Occasionally, HRS cells can form sheets that can be associated with necrosis and an intense fibrohistiocytic reaction. Morphologically, HRS cells in NSCHL often show a retraction artifact of the cytoplasmic membrane that appears to be a consequence of formalin fixation, which has led to the term "lacunar cell variant" of HRS cells. The immunohistochemical phenotype of HRS cells in NSCHL as described above is the classic phenotype; however, association with EBV is less common as compared to other cHL subtypes, especially MCCHL.

3.1.2.2 Mixed Cellularity Classical Hodgkin Lymphoma

HRS cells in MCCHL usually have a classic morphological appearance and are scattered in a background that can contain small lymphocytes, eosinophils, neutrophils, plasma cells, and histiocytes. The infiltration pattern can be diffuse or vaguely nodular; sometimes, the lymph node architecture and especially some B cell areas are partially preserved leading to an interfollicular infiltration pattern. The characteristic features of other histologic cHL subtypes (e.g., the formation of nodular collagen bands) are absent, and, thus, MCCHL is sometimes considered as the "wastebasket" of cHL. The EBV association of HRS cells is the highest among all cHL subtypes and can reach 75% [1].

3.1.2.3 Lymphocyte-Depleted Classical Hodgkin Lymphoma

LDCHL is the rarest histological subtype of cHL (<1% of cases) and probably the most problematic one to define. It is characterized by an increased number of HRS cells present in the infiltrate and/or depletion of small lymphocytes in the nonneoplastic background population. In some cases, HRS cells are of anaplastic appearance, and in other cases, the background is composed of extensive diffuse fibrosis. However, if the pattern of fibrosis is nodular and therefore characteristic of NSCHL, a given case should be classified as NSCHL, regardless of whether there is a high number of HRS cells. Since the definition of LDCHL has changed over the past decades, some of the established clinical and biological features appear outdated in the context of the current definition. Moreover, with the increase in knowledge and the development of additional immunohistochemical markers, some of the cHL cases that were previously assigned to the LDCHL category would nowadays be included into borderline categories or even different entities [1].

3.1.2.4 Lymphocyte-Rich Classical Hodgkin Lymphoma

In LRCHL, the HRS cells are present in a lymphocyte-rich background that can be nodular or, rarely, diffuse. Often, B cell follicles are

partially preserved with recognizable GC, and HRS cells can be found in expanded mantle and marginal zones, thus providing a B cell-rich background. HRS cells in LRCHL may resemble LP cells in LPHL morphologically to such an extent that they are indistinguishable from each other without additional immunohistochemical characterization. It is of significance that eosinophils and neutrophils should be absent from the nodular infiltrates and may only be found in low numbers in interfollicular zones and close to vascular structures. The immunophenotype of the HRS cells is classic, and an EBV association is occasionally observed, though at a lower frequency compared to MCCHL [1].

3.2 Differential Diagnosis

In most instances, the diagnosis of LPHL and cHL is unambiguous on the basis of morphological, clinical, and, especially, immunohistochemical features (Table 3.1). However, a gray area between cHL and diffuse large B cell lymphoma (DLBCL), specifically with primary mediastinal large B cell lymphoma (PMBL), has long been known, and the most recent WHO classification introduced the category of "B cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma" [1]. It is important to note that lymphomas falling into this category are not considered a separate disease entity; rather, it was felt that lymphomas in which there is a discordance between morphological aspects of the infiltrate and the expected immunophenotype should be labeled as "intermediate" to allow a more precise definition of biological and clinical features of these lymphomas in the future. Frequently, these borderline lymphomas present with large mediastinal masses. Morphologically, they consist of large, pleomorphic B cells that grow in a sheet-like pattern in a background of a fibrotic stroma. A subset of the tumor cells may resemble HRS cells, specifically the lacunar variant, and parts of the infiltrate may correspond to the growth pattern of cHL, particularly the nodular sclerosis subtype. Immunophenotypically, there is often a preserved expression program of cHL including expression of CD30 and CD15, while markers of the B cell lineage that are often downregulated in cHL, such as CD20 and CD79a, are equally expressed in the tumor cells [1]. It is important to note that these gray zone lymphomas appear to be more common in male patients, in contrast to NSCHL and PMBL that are more frequent in females [17]. Clinically, these tumors may behave more aggressively than NSCHL and PMBL; it has to be determined in the future whether treatment regimens for aggressive B cell lymphomas or for cHL are more beneficial.

The differential diagnosis between cHL and ALK-negative anaplastic large cell lymphoma (ALCL) of T cell lineage can usually be resolved using an appropriate panel of immunohistochemical markers including T cell, cytotoxic, and other markers. Problems arise when morphological features favor cHL, but tumor cells lack PAX5/BSAP expression while cytotoxic markers are expressed. As discussed above, it is a matter of current debate whether such cases should be grouped into the cHL category or diagnosed as ALCL. Remarkably, a global gene expression study revealed surprisingly few consistent differences in the gene expression of HRS cells and ALK-negative ALCL cells [18].

Finally, EBV-associated lymphoproliferations, e.g., in the context of a coexisting T cell non-HL as well as EBV-associated DLBCL of the elderly, a subgroup of DLBCL introduced in the new WHO classification [1], can harbor HRS or HRS-like cells and therefore mimic cHL [19]. Besides other morphological and immunohistochemical features and information on the clinical setting, the pattern of EBV infection, determined by LMP1 staining or EBER in situ hybridization, might help to distinguish between these tumors.

3.3 Histogenesis of HRS and LP Cells

3.3.1 Cellular Origin of HRS and LP Cells

The unusual immunophenotype of HRS cells, which does not resemble any normal hematopoietic cell, has hampered the identification of the

cellular origin of these cells considerably. Moreover, only few cell lines were available for detailed genetic studies, and the rarity of the HRS cells in the tissue posed a problem for their molecular analysis. Finally, by microdissection of HRS cells from tissue sections and single-cell polymerase chain reaction analysis of these cells, it was clarified that HRS cells derive from B cells in nearly all cases [20, 21]. This is because rearranged immunoglobulin (Ig) heavy (IgH) and light (IgL) chain gene rearrangements were detected in these cells. The detection of identical IgV gene rearrangements in the HRS cells of a given HL case also established the monoclonal nature of these cells, a hallmark of malignant cancer cells. With a few exceptions, somatic mutations were detected in the rearranged V genes of HRS cells [20–23]. As the process of somatic hypermutation, which generates such mutations, is specifically active in antigen-activated mature B cells proliferating in the GC microenvironment in the course of T-dependent immune responses [24], the presence of mutated IgV genes in the HRS cells established their derivation from GC-experienced B cells. A surprising finding was that about 25% of cases of cHL showed destructive IgV gene mutations, such as nonsense mutations or deletions causing frameshifts that rendered originally functional V region genes nonfunctional [20]. When such mutations happen in normal GC B cells, these cells quickly undergo apoptosis. On this basis, it was proposed that HRS cells in these cases derive from pre-apoptotic GC B cells that were rescued from apoptosis because they harbored or acquired some transforming events [20, 25]. It is important to note that crippling mutations, such as those generating premature stop codons, represent only a small fraction of disadvantageous IgV gene mutations that cause apoptotic death of GC B cells, and it is therefore likely that also most or even all other cases of cHL are derived from pre-apoptotic GC B cells. Even a few HL with unmutated IgV genes may derive from these precursors, because GC founder cells proliferating in GC become prone to apoptosis before the onset of somatic hypermutation activity [26]. The GC B cell origin of HRS cells was further supported by the molecular analysis of composite lymphomas, composed of a cHL and a B cell non-HL. Such cases are often clonally related and show an intriguing pattern of shared as well as distinct somatic V gene mutations [27–30]. This pattern supports the assumption that both lymphomas were derived from distinct members of a proliferating GC B cell clone.

A comparison of the transcriptomes of HRS cells and normal GC and extrafollicular $CD30^+$ B cells revealed that HRS cells are in their global gene expression pattern more similar to the normal $CD30^+$ B cells than to bulk GC B cells [31]. However, a direct derivation of HRS cells from $CD30^+$ GC B cells seems unlikely, as $CD30^+$ GC B cells are positively selected GC B cells with functional BCR that are preparing to return to the dark zone of the GC for a further round of proliferation and IgV gene mutation. Perhaps, in the course of their malignant transformation, the HRS cell precursors that managed to escape from apoptosis acquired the gene expression program of the positively selected and proliferation prepared $CD30^+$ GC B cells.

A few cases of cHL appear to originate from T cells, because T cell receptor gene rearrangements were detected in some cases diagnosed as HL and expressing some typical T cell molecules [14, 15]. However, it is debated whether these are true HL (see above). Remarkably, among HL cases with expression of one or more T cell markers, the majority nevertheless derives from B cells [14, 15].

The expression of multiple B cell markers by LP cells of LPHL already indicated a B cell derivation of these cells. Moreover, LP cells express several markers typically expressed by GC B cells, such as BCL6, AID, centerin, and hGAL, and the cells grow in a follicular pattern in close association with typical constituents of normal GC, i.e., follicular dendritic cells and GC-type T helper cells [2, 3, 5, 6, 32, 33]. This pointed to a close relationship between LP cells and GC B cells. This is indeed supported by the detection of clonally related and somatically mutated IgV genes in these cells [21, 34–36]. As opposed to cHL, the V genes are selected for functionality, and a fraction of cases shows ongoing somatic hypermutation during clonal expansion, a hallmark of GC B cells [21, 34, 35]. Thus, these

findings altogether indicate a GC B cell origin of LP cells. A large-scale gene expression profiling of isolated LP cells in comparison to the main subsets of mature B cells has led to a further specification of the derivation of LP cells by showing that the gene expression pattern of LP cells resembles that of GC B cells that have already acquired some features of post-GC memory B cells [37].

3.3.2 Relationship of Hodgkin Cells and Reed-Sternberg Cells and Putative HRS Cell Precursors

The relationship of the mononucleated Hodgkin cells to the multinuclear RS cells and the potential existence of HRS precursor cells has been a matter of debate. Based on the "mixed" phenotype of HRS cells and many numerical chromosomal aberrations in these cells, it has been speculated that HRS cells as such or, specifically, the RS cells may derive from cell fusions of different cells (e.g., a B cell and a non-B cell). However, a detailed study of antigen receptor loci revealed that HRS cells do not carry more than two different alleles of these loci, which strongly supports the assumption that these cells do not derive from cell fusions [38]. Several studies of HL cell lines showed that the mononuclear Hodgkin cells give rise to the RS cells and that the latter have little proliferative activity [39–41]. Long-term time lapse-microscopy analyses revealed that mononucleated Hodgkin cells undergo incomplete cytokinesis and refusion to give rise to the multinucleated RS cells [42, 43].

Two studies reported the existence of a small subpopulation of side population cells among the mononuclear Hodgkin cells. Side population cells extrude the Hoechst dye, because they express multidrug transporters, such as MDR1 and/or ABCG2. In several types of cancers, there is an overlap between side population cells and cancer stem cells. Side population cells of cHL cell lines were $CD30^{+}CD20^{-}$ and showed increased resistance against chemotherapeutic drugs [44, 45]. However, it has not yet been determined whether they have a higher capacity to sustain the HRS cell clone in long term than other mononuclear Hodgkin cells, and the fact that side population cells were not identified in all cHL cell lines analyzed argues against an essential role of these cells for the survival of the HRS cell clone.

Another debated issue relates to the question whether the $CD30^{+}$ typical HRS cells represent the entire tumor clone in HL or whether members of the HRS cell clones exist among small $CD30^{-}$ cells. An initial study for numerical chromosomal abnormalities indeed suggested that such $CD30^{-}$ clone members might exist [46]. However, trisomies of chromosomes as studied in that work are not a stringent clonal marker. Moreover, a molecular analysis of EBV-positive HL cases for members of the malignant clones among small, $CD30^{-}$ EBV^{+} B cells in the HL lymph nodes suggested that the small EBV^{+} B cells rarely, if at all, belong to the HRS cell clones [47]. Two HL cell lines were reported to contain small subpopulations of $CD20^{+}CD30^{-}Ig^{+}$ B cells coexpressing the stem cell marker aldehyde dehydrogenase (ALDH) [48]. These cells had clonogenic potential and gave rise to the typical HRS cells of these lines. It is important to note that $ALDH^{high}$ cells were also detectable in the peripheral blood of most HL patients, and it was reported that these cells were often clonally related to the HRS cells [48]. However, the clonal relationship between the HRS cells and $ALDH^{high}$ peripheral blood B cells was not clearly shown [49], so it remains to be clarified whether $ALDH^{high}$ B cells indeed represent precursors of the HRS cell clones. A previous study using a highly sensitive PCR for HRS cell-specific Ig gene rearrangements failed to detect members of the HRS cell clone in the peripheral blood or bone marrow of two HL patients [50].

3.4 Genetic Lesions

HRS cells have a much higher number of chromosomal aberrations, including multiple numerical as well as structural abnormalities, than most other lymphomas [51]. However, it is still unclear

whether this is mostly a side effect of some type of genetic instability and whether the expression of specific oncogenes or tumor suppressor genes is recurrently affected by these lesions. When the B cell origin of HRS cells became clear, HRS cells were studied for the presence of chromosomal translocations involving the Ig loci, as such translocations are a hallmark of many B cell lymphomas. Fluorescence in situ hybridization (FISH) studies indeed provided evidence for such translocations in about 20% of cases, but most of the translocation partners involved remain to be identified [52, 53]. In a few cases, the translocation partners were *BCL2*, *BCL3*, *REL*, *BCL6*, or *MYC* [52–55]. Recurrent translocations affecting the major histocompatibility complex (MHC) class II transactivator (*MHC2TA*) were detected in about 15% of cHL cases [56]. These translocations appear to cause downregulation of MHC class II expression by HRS cells. In LPHL, translocations of the *BCL6* gene have been found in about 30% of cases [57, 58]. These translocations can involve the Ig loci, but also multiple other partners [59].

Due to the difficulty to analyze the few HRS and LP cells for mutations in oncogenes and tumor suppressor genes, only relatively few of such genes have been analyzed so far in these cells. There was a major interest to understand the apoptosis resistance of HRS cells, but it turned out that mutations in the *CD95* gene, an important death receptor, as well as in members of the CD95 signaling pathway (FADD, caspase 8, caspase 10) were rare or not found at all [60–62]. Likewise, no mutations were found in the BCL2 family member *BAD*, and also *ATM* lesions are very rare [63–65]. The *TP53* tumor suppressor gene was mutated in less than 10% of cases where the exons of *TP53* usually carrying mutations were studied in isolated HRS cells [66, 67]. However, studies of HL cell lines indicate that HRS cells may additionally carry untypical *TP53* mutations and that the frequency of *TP53* mutations may therefore be higher than previously thought [68]. MDM2, a negative regulator of TP53, frequently shows gains in HRS cells, which might contribute to impaired functions of TP53 in these cells [69].

Further candidate gene mutation studies revealed frequent mutations in the exportin 1 gene (*XPO1*) [70], which encodes a nuclear expert receptor for numerous RNAs and proteins, and inactivating mutations in and deletions of *CD58* [71, 72]. CD58 is important for targeting of cells by cytotoxic T cells and NK cells, so that CD58 inactivation may contribute to immune escape of HRS cells from an attack by these cells.

HRS cells show constitutive activity of the NF-κB transcription factor (see below), which is essential for the survival of these cells. The mechanisms of this activation were originally not understood. Consequently, members and regulators of this signaling pathway were studied for genetic lesions (Table 3.1). Inactivating mutations in the main NF-κB inhibitor NFKBIA (IκBα) were found in about 10–20% of HL cases and also in several HL cell lines (Fig. 3.3) [73–76]. Recurrent mutations were also detected in another NF-κB inhibitor, NFKBIE (IκBε) [77, 78]. Inactivating mutations or deletions in two further negative regulators of NF-κB signaling, CYLD and TRAF3, have also been detected in HL cell lines and a few primary cases, but overall these events are rare [79, 80]. Moreover, HRS cells frequently harbor genomic gains or amplifications of the *REL* gene [81–83], encoding an NF-κB family member, and a correlation between such gains and strong REL protein expression was found [84]. The *MAP3K14* gene, which encodes the NIK kinase, a major activating component of the alternative NF-κB pathway, shows gains or amplifications in about 15% of cHL [79, 85]. Also the IκB family member BCL3, which acts as a positive regulator of NF-κB activity, is affected by chromosomal gains or translocations in a small fraction of cHL [86, 87]. Somatic and clonal inactivating mutations were found in the *TNFAIP3* gene in about 40% of cHL [88, 89]. *TNFAIP3* encodes for the A20 protein, which is a dual ubiquitinase and deubiquitinase that functions as a negative regulator of NF-κB. It inhibits signaling from the receptor-interacting protein (RIP) and TNF receptor-associated factors (TRAFs) to the IKK kinases, which are essential mediators of NF-κB signaling. *TNFAIP3* mutations were mainly found in EBV-negative cases.

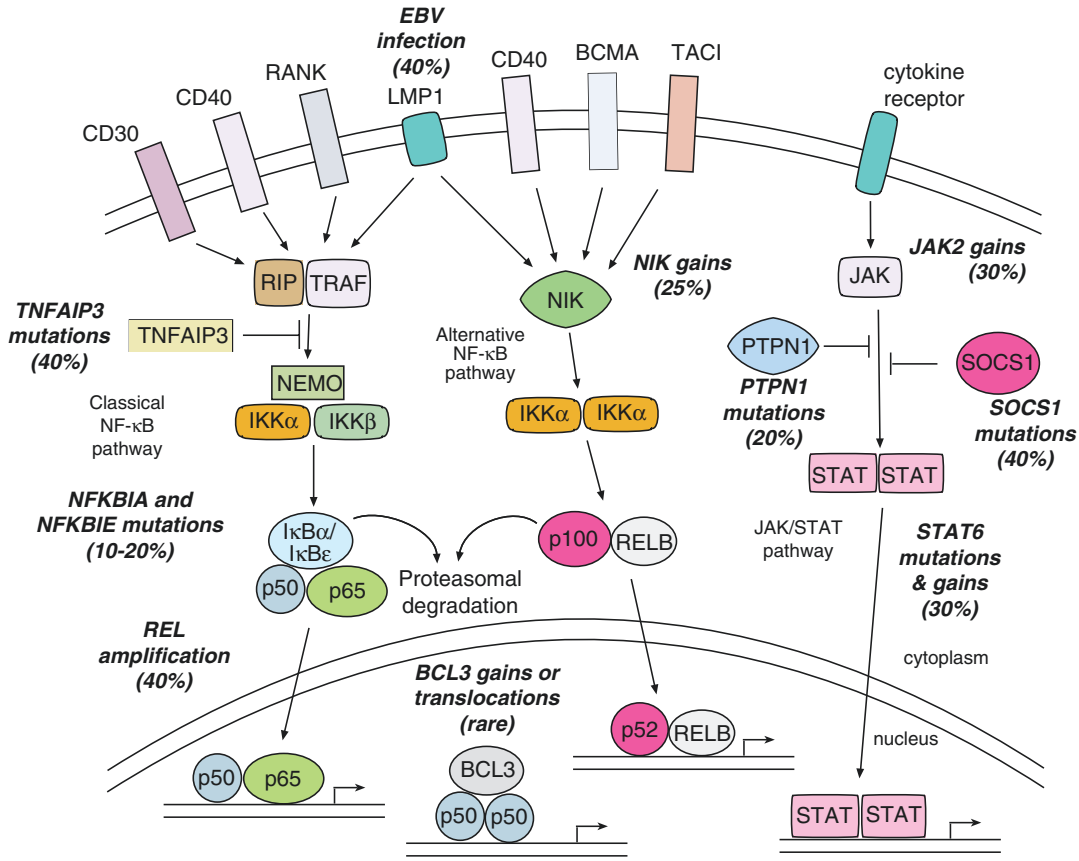

Fig. 3.3 NF-κB and JAK/STAT activity in HRS cells. In the classical NF-κB signaling pathway, stimulation of numerous receptors leads via TNF receptor-associated factors (TRAFs), which are often associated with the receptor-interacting protein (RIP), to activation of the IKK complex, which is composed of IKKα, IKKβ, and NEMO. The IKK complex subsequently phosphorylates the NF-κB inhibitors IκBα and IκBε. This marks them for ubiquitination and subsequent proteasomal degradation. Thereby the NF-κB transcription factors (p50/p65 or p50/REL heterodimers) are no longer retained in the cytoplasm and translocate into the nucleus, where they activate multiple genes. The signal transduction from TRAFs/RIP to the IKK complex can be inhibited by TNFAIP3, which removes activating ubiquitins from RIP and TRAFs and additionally links ubiquitins to these molecules to mark them for proteasomal degradation. In the alternative NF-κB pathway, activation of receptors such as CD40, BCMA, and TACI causes stimulation of the kinase NIK, which then activates an IKKα complex. Activated IKKα processes p100 precursors to p52 molecules, which translocate as active p52/RELB NF-κB heterodimers into the nucleus. HRS cells show constitutive activity of the classical and alternative NF-κB signaling pathway. This activity is probably mediated by diverse mechanisms, including receptor signaling through CD40, RANK, BCMA, and TACI; genomic *REL* and *MAP3K14* (NIK) amplification; destructive mutations in the *TNFAIP3*, *NFKBIA*, and *NFKBIE* genes; and signaling through the EBV-encoded LMP1. The role of CD30 signaling in HRS cells is controversially discussed. HRS cells may also harbor nuclear BCL3/$(p50)_2$ complexes, and in a few cases, the strong BCL3 expression appears to be mediated by genomic gains or chromosomal translocations. The JAK/STAT pathway is the main signaling pathway for cytokines. Upon binding of cytokines to their receptors, members of the JAK kinase family become activated by phosphorylation. The activated JAKs then phosphorylate and thereby activate STAT transcription factors. These phosphorylated factors homo- or heterodimerize and translocate into the nucleus where they activate target genes. Main inhibitors of the JAK/STAT pathway are the phosphatase PTPN1 and SOCS (suppressor of cytokine signaling) factors, which function by binding to JAK molecules and inhibiting their enzymatic activity and additionally by inducing proteasomal JAK degradation. In HRS cells, STAT3, 5, and 6 are constitutively active. Besides activation of cytokine receptors (e.g., IL13 receptor and IL21 receptor) through cytokines, activation of this pathway is mediated by genomic gains or rare translocations of the *JAK2* gene, activating mutations in the *STAT6* gene, and frequent inactivating mutations in the *SOCS1* and *PTPN1* gene. The frequency of genetic lesions and viral infections affecting NF-κB or STAT activity in cHL cases is indicated

Nearly 70% of EBV⁻ cases carried *TNFAIP3* mutations, indicating that EBV infection and A20 inactivation are alternative pathogenetic mechanisms in HL [88, 89]. As LMP1 of EBV, which is expressed in EBV-positive HRS cells, mimics an active CD40 receptor and signals through NF-κB [90, 91], LMP1 may replace the role of A20 inactivation in EBV⁺ HL.

As it was recently revealed that also the LP cells of LPHL show strong constitutive NF-κB activity [37], also these cells were studied for mutations in *NFKBIA* and *TNFAIP3*, but clonal destructive mutations were not found (Table 3.1) [92].

Genetic lesions were also found in members of the JAK/STAT pathway, which is constitutively activated in HRS and LP cells. In about 40% of cases analyzed, both HRS and LP cells showed somatic mutations in the *SOCS1* gene, which encodes a main inhibitor of STAT signaling (Fig. 3.3) [93, 94]. In HRS cells, recurrent mutations were additionally found in the gene of another negative regulator of JAK/STAT signaling, namely, the *PTPN1* gene, which encodes a phosphatase [95]. Furthermore, a fraction of cHL cases show genomic gains or amplifications of the *JAK2* locus, which encodes one of the kinases activating the STAT factors (Table 3.1) [82, 96]. Importantly, the genomic gains at 9p24 do not only affect the *JAK2* locus, but additionally the *PD-L1*, *PD-L2*, and *JMJD2C* genes [97, 98]. PD-L1 and PD-L2 are inhibitory receptors for PD1-positive T cells and may hence inhibit a cytotoxic T cell attack on HRS cells. *JMJD2C* encodes a histone demethylase and plays a role in the epigenetic remodeling of HRS cells. Finally, the *JAK2* gene is in rare instances also deregulated by chromosomal translocations [99]. Activating point mutations in the *STAT6* gene and genomic gains involving this gene were also detected in HRS cells [10, 100]. Thus, multiple types of genetic lesions cause a constitutive JAK/STAT signaling, suggesting an essential role of its deregulated activity for cHL pathogenesis.

With the availability of high-throughput sequencing methods, tumor cells can now be studied for genetic lesions at a genome-wide level. An exome sequencing analysis of six cHL lines and the only LPHL cell line (DEV) revealed over 400 genes mutated in at least two of the lines [8]. This is a valuable database that should be considered when performing functional studies with these cell lines. A first whole exome sequencing study of primary HRS cells used flow-cytometry isolated lymphoma cell from ten cases of cHL [9]. Between 100 and 500 somatic mutations were found per case. A main finding of this analysis was recurrent inactivating mutations in the *B2M* gene. B2M is essential for MHC class I expression, so that the loss of its expression presumably leads to immune evasion from CD8⁺ cytotoxic T cells. Other novel recurrent mutations identified in that work affect several histone genes, the inositol-trisphosphate 3-kinase B (ITPKB), the B cell transcription factor EBF1, and the G protein subunit GNA13 [9]. Tiacci and colleagues performed a whole exome sequencing analysis of pools of HRS cells microdissected from 34 cases of cHL [10]. A median of 47 non-silent somatic mutations in the exomes was found. This study confirmed recurrent mutations in *ITPKB* and *GNA13*, and newly revealed recurrent point mutations in *STAT6*, further adding to the complexity of JAK/STAT deregulation in cHL. Although only four EBV⁺ cases were included in the study by Tiacci et al., it seems that such cases carry considerably fewer somatic mutations than the EBV-negative cases. A mutation study of LP cells of LPHL was based on a whole genome analysis of DLBCL clonally related to LPHL in the same patient, followed by targeted sequencing analysis of microdissected LP cells. In this work, three genes were found to be each mutated in about half of the cases of LPHL (also in cases without co-occurring DLBCL), namely, the genes encoding the kinase SGK1, the AP-1 family member JUNB, and the phosphatase DUSP2 [101].

3.5 Deregulated Transcription Factor Networks and Signaling Pathways

3.5.1 The Lost B Cell Phenotype

Early immunohistochemical studies already revealed that HRS cells usually do not express typical B cell markers, such as CD20, CD79b, or

the BCR [13, 102–104]. This lack of expression of B cell markers was indeed one of the reasons why the B cell origin of HRS cells was not revealed until genetic studies for Ig gene rearrangements unequivocally demonstrated a B cell identity of these cells (see above). Gene expression profiling studies of HRS cells in comparison to normal B cells then showed that there is a global loss of the B cell typical gene expression in HRS cells [105]. This downregulation involved all types of genes with important functions in these cells, for example, cell surface receptors (CD37, CD53), components of signaling pathways (SYK, BLK, SLP-65), and transcription factors (PU.B, A-MYB, SPI-B). As plasma cells also show a downregulation of many B cell-typical genes, it had been speculated that HRS cells lost their B cell gene expression and acquired a partial plasma cell differentiation program [2, 106]. However, a gene expression profiling study of microdissected HRS cells revealed that HRS cells have not acquired a plasma cell phenotype [107].

Remarkably, HRS cells have retained expression of molecules that are involved in antigen-presenting functions and the interaction with $CD4^+$ T helper cells. HRS cells usually express CD40, CD80, and CD86 and often MHC class II [105, 108]. This indicates that an interaction with T helper cells is important for HRS cell survival. In line with this view, HRS cells are typically surrounded by CD40L expressing $CD4^+$ T cells [109].

We are now beginning to understand which factors contribute to the lost B cell phenotype of HRS cells. First, several transcription factors that positively regulate the expression of multiple genes in B cells are downregulated, including OCT-2, PU.1, EBF1, ETS1, and BOB.1 [102, 103, 110–112]. The downregulation of ETS1 may often be due to heterozygous deletions of the gene, which have been observed in over 60% of cHL analyzed [112]. Second, although E2A, a master regulator of the B cell transcription program, is still expressed, HRS cells also show deregulated expression of ID2 and ABF1 [113–115], which bind to E2A and inhibit its function [114]. The physiological role of ABF1 is poorly understood, but ID2 is normally expressed in dendritic cells and natural killer cells, and supports the generation of these cells concomitant with suppression of B cell development [116, 117]. Third, HRS cells express activated NOTCH1, which normally induces T cell differentiation in lymphocyte precursors and suppresses a B lineage differentiation of such cells [118, 119]. Activation of NOTCH1 is probably caused by interaction with its ligand Jagged-1, which is expressed by other cells in the HL microenvironment [119], and by high-level expression of the NOTCH coactivator mastermind-like 2 (MAML2) [120]. Moreover, HRS cells have downregulated the NOTCH1 inhibitor Deltex1 [118]. Fourth, STAT5A and STAT5B are activated in HRS cells and have been reported to induce an HRS cell-like phenotype in normal B cells [121]. Constitutive active STAT5 induced expression of CD30 and of the T cell transcription factor GATA3 in the B cells and led to downregulation of BCR expression. Aberrant GATA3 expression in HRS cells is furthermore mediated by NOTCH1 and NF-κB activity in HRS cells [122]. Fifth, the downregulation of multiple B cell genes in HRS cells is further caused by epigenetic mechanisms, as DNA methylation has been detected for numerous such genes [123–125]. Sixth, HRS cells express several transcription factors that have important roles in hematopoietic stem cells and early lymphoid precursors, including GATA2, BMI1, RING1, and RYBP [126–129]. The expression of these factors may contribute to a "dedifferentiated" phenotype of HRS cells.

Surprisingly, PAX5, the main B lineage commitment and maintenance factor, is still expressed in HRS cells, albeit at reduced levels [11]. As many of its direct target genes are not expressed, it is likely that PAX5 activity is inhibited. NOTCH1 is a candidate for this inhibition [118]. It may also be that PAX5 target genes are not expressed because other transcription factors needed for the efficient expression of these genes are missing.

Expression of the myeloid specific colony-stimulating factor 1 receptor (CSF1R) by HRS cells is a further important example of aberrant

expression of a non-B cell gene in HRS cells [130]. CSF1R expression promotes HRS cell survival. The mechanism of its deregulated expression is remarkable, because this is mediated by derepression of an endogenous long terminal repeat upstream of the *CSF1R* gene that replaces the function of the normal CSF1R promoter [130].

The downregulation of many B cell transcription factors that also suppress the expression of non-B lineage genes, combined with the upregulated expression of genes promoting expression of genes of other hematopoietic cell types (e.g., NOTCH1, ID2), not only explains the lost B cell phenotype of HRS cells but also the heterogeneous expression of genes specifically expressed by dendritic cells, T cells, or other cell types. It is an intriguing question whether the lost B cell phenotype of HRS cells is related to their origin from crippled GC B cells. Perhaps, due to the stringent selection of B cells for expression of a functional BCR (a high-affinity one in the GC), there is a selection in HRS cell pathogenesis downregulating the B cell gene expression program to escape the selection forces that induce apoptosis in GC B cells with unfavorable IgV gene mutations. The observation that enforced re-expression of the B cell transcription factors PU.1, FOXO1, or E2A or the pharmacological restoration of the B cell phenotype in HL cell lines induces apoptosis is in line with this view [131–134]. However, the lost B cell phenotype could also be a side effect of so far unknown transforming events.

3.5.2 Constitutive Activation of Multiple Signaling Pathways

It is obvious that tumor cells need to activate and deregulate signaling pathways and transcription factors that promote their survival and proliferation. Nevertheless, it is striking how many of such pathways are constitutively activated in HRS cells, and cHL appears to be rather unique among lymphoid malignancies in the extent to which multiple signaling pathways contribute to the survival and expansion of HRS cells. It has already been mentioned above that HRS cells show constitutive NF-κB activity. This activity is essential for HRS cell survival [135] and is most likely not only mediated by genetic lesions (see above) but also by signaling through receptors. NF-κB factors of both the canonical pathway (p50/p65) and the noncanonical NF-κB pathway (p52/RelB) are activated (Fig. 3.3). HRS cells express the TNF receptor family members CD30, CD40, RANK, TACI, and BCMA, which activate NF-κB, and cells expressing the respective ligands are found in the HL microenvironment [109, 136–140]. There are, however, conflicting data about the role of CD30 in NF-κB activation [141, 142]. In EBV-positive cases of cHL, the virally encoded LMP1 mimics an active CD40 receptor and hence also contributes to NF-κB activation [143].

Another central signaling pathway, which is like NF-κB activated both by genetic lesions and by ligand-mediated receptor triggering, is the JAK/STAT pathway (Fig. 3.3). This is the main signaling pathway for cytokines. Activation of cytokine receptors causes activation of JAK kinases which in turn phosphorylate and thereby activate STAT transcription factors. The phosphorylated STAT factors dimerize and then translocate into the nucleus where they activate transcription of target genes. HRS cells show activation of STAT3, STAT5, and STAT6 [121, 144–146]. The activation of STAT6 is at least partly mediated by signaling through IL13. As HRS cells express IL13 and its receptor, STAT6 activation can be mediated through an autocrine stimulation loop [147, 148]. Signaling through the IL21 receptor contributes to STAT3 and STAT5 activation in HRS cells, which is also enhanced by the NF-κB activity in the cells [121, 149, 150]. As mentioned above, STAT5 activity may contribute to the lost B cell phenotype of HRS cells. Inhibition of STAT activity in HL cell lines resulted in reduced proliferation of the cells, further supporting an important pathogenetic role of this signaling pathway [144, 145, 147].

Receptor tyrosine kinases (RTKs) are important regulators of cell growth, survival, and proliferation. In multiple cancers, specific RTKs are

activated, often by somatic mutations [151]. In contrast, HRS cells show multiple activated RTKs, and their activation does not appear to be due to activating mutations but at least partly to ligand-mediated stimulation [152]. RTKs that are often expressed in varying combinations in HRS cells include PDGFRA, DDR2, EPHB1, RON, TRKA, TRKB, CSF1R, and MET [130, 152, 153]. The expression of most of these is aberrant, as they are not expressed by normal GC B cells [130, 152]. They are also usually not expressed by other B cell non-HL, showing that this is a specific feature of HL among B cell lymphomas [152, 154]. Expression of multiple RTKs is most pronounced in EBV-negative cases of cHL, suggesting that EBV activates pathways in HRS cells replacing the function of RTKs [155]. For PDGFRA, TRKA, and CSF1R, a growth-inhibitory effect has been shown upon their inhibition in HL cell lines, giving a first indication that the activity of RTKs is important for HRS cell proliferation [130, 152, 156].

Signaling through various receptors is mediated by the mitogen-activated protein kinase (MAPK)/ERK pathway. In HRS cells, the serine/threonine kinases ERK1, ERK2, and ERK5 are activated [157, 158]. Inhibition of their activity has antiproliferative effects on HL cell lines [158]. Signaling through CD30, CD40, and RANK may contribute to the stimulation of this pathway [158].

The transcription factor AP-1 acts as homo- or heterodimers of JUN, FOS, and ATF components. In HRS cells, JUN and JUNB are overexpressed and constitutively active [159]. The overexpression of JUNB is mediated by NF-κB [159]. AP-1 induces many target genes and promotes proliferation of HRS cells. Target genes of AP-1 include CD30 and galectin-1, the latter of which has immunomodulatory functions [160, 161]. HRS cells also show strong expression of BATF3, another member of the AP-1 transcription factor family [162, 163]. BATF3 expression is induced by STAT3 and STAT6 in HRS cells. It forms heterodimers with JUN and JUNB, and the proto-oncogene MYC was identified as one of the direct BATF3 target genes [162]. Importantly, downregulation of BATF3 in HL cell lines is toxic for these cells, revealing an essential role of this factor in cHL pathophysiology [162].

Finally, also the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is a main promoter of cell survival, shows activity in HRS cells [164, 165]. AKT is a serine/threonine kinase that is activated in HRS cells, as evident from its phosphorylated state and phosphorylation of known target proteins [164, 165]. Inhibition of AKT in HL cell lines causes cell death, suggesting an important role of active AKT in HRS cell survival [164, 165]. PI3K may be activated in HRS cells by signaling through CD30, CD40, RANK, and RTK. Moreover, downregulation of the AKT inhibitor INPP5D in HRS cells may further contribute to strong AKT activity in these cells [107].

While we have a relatively detailed insight into signaling pathways active in HRS cells, less is known about signaling pathways constitutively active in LP cells of LPHL. However, LP cells also show a high constitutive activity of NF-κB [37]. RTKs are partly also aberrantly expressed by these cells [152], and activation of the JAK/STAT pathway has been observed [93].

In conclusion, HRS cells are characterized by the deregulated and constitutive activation of multiple signaling pathways and transcription factors that contribute to the survival and proliferation of these cells. The multitude of different stimulated pathways appears to be rather unique among human B cell lymphomas. Often, these pathways are activated by common mechanisms, and they may interact in numerous ways.

3.6 Anti-apoptotic Mechanisms

With a presumed origin from pre-apoptotic GC B cells, it is critical to understand through which mechanisms HRS cells escape from apoptosis. A number of factors contributing to HRS cell survival have already been discussed in the previous section: constitutive activity of NF-κB, STAT, PI3K, NOTCH1, AP-1, RTK, and ERK. Several specific inhibitors of the two main apoptosis pathways deserve specific mentioning. Although HRS cells express the CD95 death receptor of the extrinsic apoptosis pathway as well as its

activating ligand, HL cell lines are resistant to CD95-mediated death induction, suggesting a specific inhibition of this pathway [166–168]. As mentioned above, this resistance is neither due to mutations in the CD95 receptor itself nor in its interaction partners FADD, caspase 8, or caspase 10. However, HRS cells show strong expression of the CD95 inhibitor CFLAR (previously known as cFLIP, cellular FADD-like interleukin 1b-converting enzyme-inhibitory protein), and this factor impairs CD95 signaling in HRS cells [166, 167]. Inhibition of the intrinsic (mitochondrial) apoptosis pathway is probably mediated through strong expression of the anti-apoptotic factors BCLXL and XIAP (X-linked inhibitor of apoptosis) and downregulation of the pro-apoptotic factor BIK [107, 169, 170]. BCLXL inhibits apoptosis at the level of the mitochondrial apoptosis induction, whereas XIAP inhibits activity of caspases 3 and 9, which are downstream executioners of the mitochondrial apoptosis program. Although HRS cells also express pro-apoptotic SMAC, which can inhibit XIAP, the cells show an impaired release of SMAC from the mitochondria into the cytoplasm [171]. As mentioned above, HRS cells express high levels of the pro-apoptotic TP53 factor, but resistance to TP53-mediated apoptosis appears to be rarely due to inactivating mutations in the TP53 gene. An important factor for the inhibition of TP53 activity is MDM2, which is expressed at high levels in HRS cells [172]. The functional role of MDM2 as a TP53 inhibitor in HRS cells is supported by the fact that HL cell lines expressing wild-type TP53 are rendered apoptosis-sensitive toward pharmacological apoptosis inducers upon inhibition of MDM2 by its antagonist nutlin 3 [173, 174].

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