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4 Microenvironment, Cross-Talk, and Immune Escape Mechanisms

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Contents

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4.1 Microenvironment

4.1.1 Hodgkin Lymphoma Subtypes

When discussing the microenvironment in Hodgkin lymphoma (HL), it is important to recognize the different HL subtypes described by the WHO classification [1, 2]. The classical HL (cHL) subtypes are defined in large part by the composition of the reactive infiltrate (Table 4.1). The most prevalent subtype is the nodular sclerosis type that consists of a nodular background with thick fibrotic bands, usually with a thickened lymph node capsule. In addition to the lacunar type of Hodgkin/Reed-Sternberg (HRS) cells, there is a microenvironment consisting of T cells, eosinophils, and histiocytes, with a variable admixture of neutrophils, plasma cells, fibroblasts, and mast cells. The second most common subtype is mixed cellularity, which is defined by the presence of typical HRS cells and a diffuse infiltrate of T cells, eosinophils, histiocytes, and plasma cells, sometimes with the formation of granuloma-like clusters or granulomas (Fig. 4.1). Lymphocyte-rich cHL also comprises typical HRS cells in a nodular or diffuse microenvironment and small B and/or T lymphocytes dominating the background, sometimes with admixture of histiocytes. Granulocytes are not present in this subtype. The rare lymphocyte-depleted subtype harbors a high percentage of HRS cells in a background consisting of fibroblasts and a low number of T cells. Nodular lymphocyte predominance (NLP) HL is an entity that is fundamentally different from cHL. The morphology may closely resemble that of the nodular variant of the classical lymphocyte-rich subtype, both involving follicular areas with many small B cells, but NLPHL can also show other growth patterns [3]. The characteristics of the tumor cells and the T cells in NLPHL are different from cHL. HRS cells show a loss of B cell phenotype, while in NLPHL the lymphocyte-predominant (LP) tumor cells share many markers with germinal center B cells. The T cells in cHL have features of paracortical T cells, while those in NLPHL are similar to germinal center T cells (T follicular helper cells) [4–6].

4.1.2 Epstein-Barr Virus

The presence of latent Epstein-Barr virus (EBV) genomes in HRS cells appears to influence the composition of the microenvironment. Positive EBV status is strongly associated with the mixed

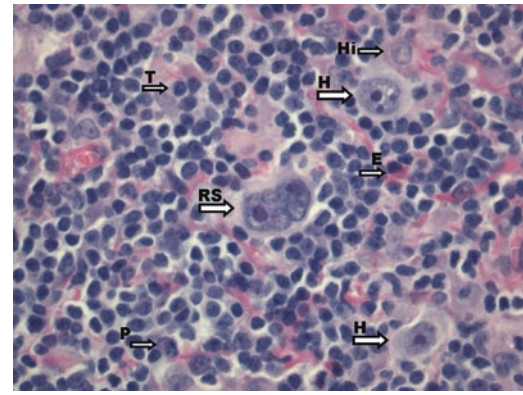

Fig. 4.1 The microenvironment in mixed cellularity classical Hodgkin lymphoma. *RS* classical Reed-Sternberg cell, *H* mononuclear Hodgkin tumor cell, *T* T lymphocyte, *Hi* histiocyte, *E* eosinophil, *P* plasma cell. Hematoxylin and eosin staining

Table 4.1 Composition of the microenvironment in different Hodgkin lymphoma (HL) subtypes

Subtype	EBV (%)	Background	T cells	Other cells
Nodular sclerosis	10–40	Nodular + fibrosis	CD4 > CD8, Th2, Treg > Th1	Eosinophils, histiocytes, fibroblasts, B cells, mast cells (neutrophils)
Mixed cellularity	75	Diffuse	CD4 > CD8, Th2, Treg > Th1	Eosinophils, histiocytes, plasma cells, B cells
Lymphocyte rich	40–80	Nodular or diffuse	CD4 > CD8	Histiocytes
Lymphocyte depleted (including HIV+)	80–100	Diffuse	–	Fibroblasts
Nodular lymphocyte predominant	~2	Nodular (+diffuse)	Th2, PD1+/CD57+ Tfh, CD4+/8+	Histiocytes, B cells

cellularity subtype (~75% EBV-positive) and is almost always absent in NLPHL [7]. Depending on the geographic locale, EBV is present in the HRS cells in 10–40% in nodular sclerosis cases. The percentage of EBV-positive lymphocyte-rich cHL cases is not very clear, but is probably between 40% and 80%. EBV infects more than 90% of the world population and establishes a lifelong latent infection in B cells in its host. Potent cytotoxic immune responses keep the number of EBV-infected B cells at approximately 1/100,000 and usually prevents EBV-driven malignant transformation in immunocompetent individuals. Accordingly, EBV-positive cHL cases contain slightly more CD8+ cytotoxic T cells in the reactive background compared to EBV-negative cHL cases [8].

4.1.3 Human Immunodeficiency Virus

In patients with an impaired immune response, cHL occurs more frequently. After solid organ transplantation, there is a small increase in the incidence of cHL that can largely be attributed to EBV-positive cHL. Human immunodeficiency virus (HIV)-infected individuals have an approximate 10 times increased risk of developing cHL [9]. In comparison to non-HIV-associated cHL, these tumors are more often EBV-associated, mixed cellularity and lymphocyte depletion subtypes, and usually contain more tumor cells. This may reflect a functional defect in the immune response, in particular to EBV, presumably caused by the impairment of CD4+ T cells by HIV. On the other hand, the importance of CD4+ T cells for supporting the growth of HRS cells is also illustrated in HIV-positive patients, in which an increase in the incidence of HIV-associated cHL has been observed after introduction of highly active antiretroviral therapy (HAART) [10].

4.1.4 T Cell Subsets in cHL

A unifying feature of the reactive infiltrate in virtually all cHL subtypes is the presence of large numbers of CD4+ T cells. Besides being widely distributed in the background, these CD4+ T cells form a tight rosette around the tumor cells. T cells within these rosettes often have a distinct phenotype, which is different from the phenotype of the T cells that are located further away from the cHL tumor cells (Fig. 4.2).

In general, CD4+ T cells are divided into naive (CD45RA+) and memory (CD45RO+) subsets based on whether they have previously been stimulated by an antigen or not. A large subset of CD4+ T cells consists of the so-called helper T (Th) cells; these cells play an important role in helping other cells to induce an effective immune response. Th cells can be further divided into Th0 (naive), Th1 (cellular response), Th2 (humoral response), Th17 (IL-17 producing), and Treg (regulating responses of other cells) cells. The Treg cells can be further divided into Th3 (transforming growth factor-β (TGF-β) producing), Tr1 (IL-10 producing), and CD4+CD25+ Treg (originating from the thymus) subpopulations. Some, but not all, Treg cells express the transcription factor FoxP3.

The T cells in cHL consist mainly of CD4+ T cells that have a memory phenotype (CD45RO+) and express several activation markers including CD28, CD38, CD69, CD71, CD25, and HLA-DR, as well as markers like CD28, CTLA-4, and CD40L. However, these T cells lack expression of CD26 [11]. This lack of CD26 expression is most striking in the areas surrounding the tumor cells. CD26, dipeptidyl peptidase IV, regulates proteolytic processing of several chemokines, e.g., CCL5 (Rantes), CCL11 (Eotaxin), and CCL22 (MDC) [12]. CD26 is also associated with adenosine deaminase (ADA) and CD45RO and when interacting with anti-CD26 antibodies leads to enhanced T cell activation through triggering of the T cell receptor [13]. CD26 is preferentially expressed on CD4+CD45RO+ cells and is normally upregulated after activation. However, CD26 cannot be upregulated by ex vivo activation of the CD26-negative cells from cHL lesions. In general, a high CD26 expression level correlates with a Th1 subtype.

The transcription factor expression pattern indicates that the CD4+ T cells in cHL are predominantly Th2 (c-Maf) and Treg (FoxP3) [4, 14].

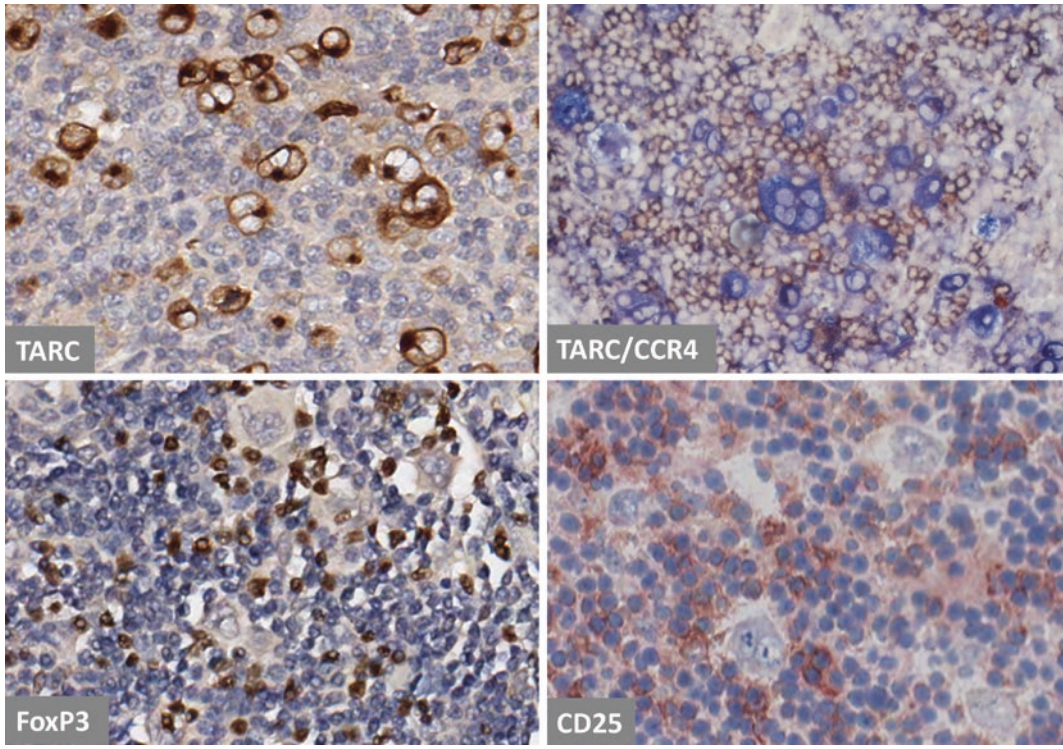

Fig. 4.2 Shaping the microenvironment in classical Hodgkin lymphoma (HL). Immunohistochemistry of classical HL cases. In the upper panel left, strong and specific staining of Hodgkin/Reed-Sternberg (HRS) cells for chemokine CCL17 (TARC). This chemokine attracts CCR4+ lymphocytes (upper panel right). A large proportion of reactive T cells are Treg cells, as shown by positive staining for transcription factor FoxP3 (lower panel left) and activation marker CD25 (lower panel right)

The CD4+CD26− T cell subset in cHL has reduced mRNA levels of Th1- and Th2-associated cytokines in comparison to the CD4+CD26+ T cells from cHL and CD4+ T cells (both CD26− and CD26+) in reactive lymph nodes [15]. Based on much higher mRNA expression levels of IL-2RA (CD25), CCR4, FoxP3, CTLA4, TNFRSF4 (OX-40), and TNFRSF18 (GITR) observed in the CD4+CD26− T cells from cHL, it has been postulated that these cells have a Treg phenotype (Fig. 4.2). In addition, mildly enhanced IL-17 levels can be observed both in CD4+CD26− and CD4+CD26+ T cells from cHL in comparison to the T cells from tonsil. Upon stimulation, the CD4+CD26− T cells fail to induce expression of cytokines, suggesting that the T cell population rosetting around the HRS cells or located in the direct vicinity of the HRS cells have an anergic phenotype (i.e., do not respond to stimulation) [15]. Immunohistochemistry for several Treg-associated molecules demonstrates that the rosetting T cells in cHL express GITR, CCR4, and CD25, but not FoxP3. Scattered FoxP3-positive cells are present in the infiltrate, but only rarely in the direct vicinity of the HRS cells, whereas CTLA-4 shows a more diffuse presence [15]. Likewise, a small number of scattered IL-17-positive cells can be found in the reactive infiltrate. Th17 cells are generally pro-inflammatory, but given the abundance of TGF-β and IL-6 in the Hodgkin microenvironment, the observed IL-17-positive cells might be yet another type of regulatory cells, termed Treg17 cells. Although the vast majority of studies indicate that the CD4+ T cells in cHL are (anergic) Th2 cells and Treg cells, some studies showed a predominant Th1-type pattern in whole lymph node cell suspensions, with a mild increase in EBV-positive cHL [16]. These findings

are not contradictory, as these Th1-like cells are located mainly outside the areas where R-S cells and T cell rosettes are found [17, 18].

4.1.5 T Cell Subsets in NLPHL

The CD4+ T cells in NLPHL resemble the CD4+ T cells in cHL, regarding the expression of CD45RO, CD69, CTLA4, and CD28 and lack of CD26. However, the T cells in NLPHL do not express CD40L, and a significant proportion of the T cells that immediately surround the LP cells express CD57 and PD-1 [19, 20]. Similar to the Th2 cells in cHL, the rosetting cells in NLPHL strongly express the Th2-associated transcription factor c-Maf (Fig. 4.3; [4]).

Characterization of the cytokine profile of the CD4+CD57+ T cell subset shows lack of IL-2 and IL-4 mRNA, but elevated interferon-γ (IFN-γ) mRNA levels in comparison to CD57+ T cells from tonsils. Stimulation of these cells fails to induce IL-2 and IL-4 mRNA levels [21], which is similar to the lack of cytokine induction upon stimulation of the CD26− T cells in cHL. In normal tissue, CD4+CD57+ T cells are found almost exclusively in the light zone of reactive germinal centers and also lack CD40L expression. CD57 is known as an activation marker, but it has also been demonstrated to be a marker for senescent cells. Senescence is the phenomenon by which normal diploid cells lose the ability to divide, normally after about 50 cell divisions. PD-1+ T follicular helper cells are present in NLPHL;

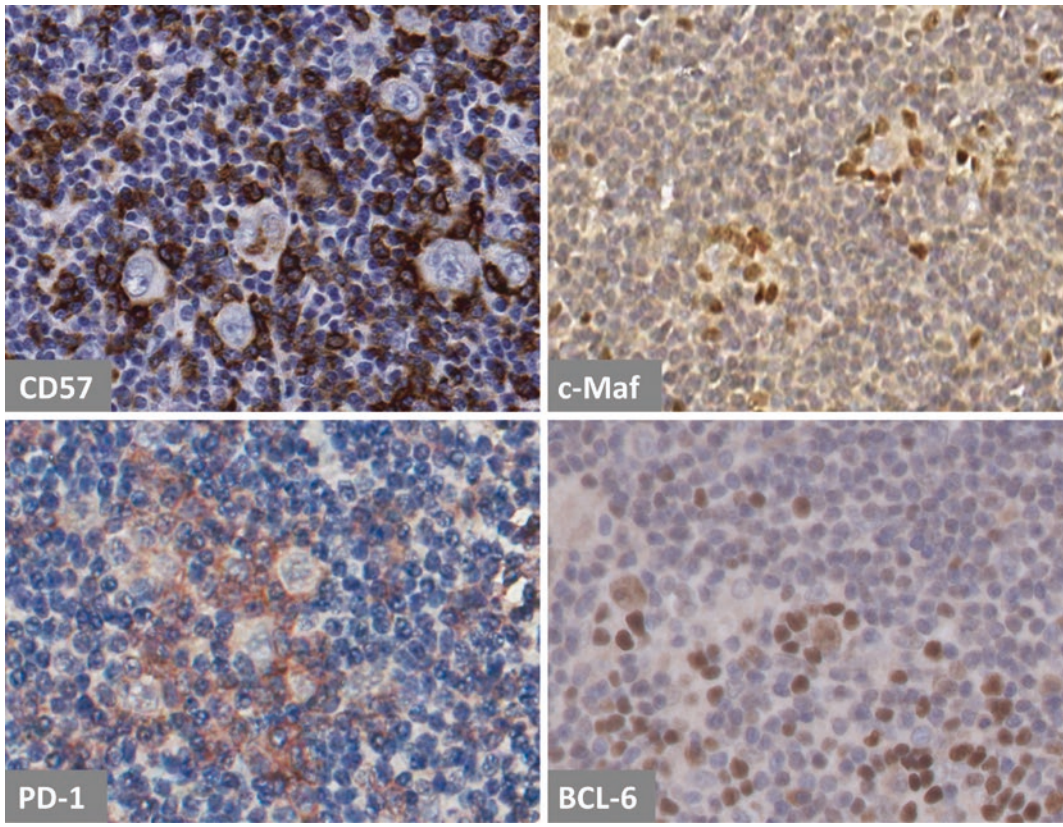

Fig. 4.3 T cells in nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL). Immunohistochemistry of an NLPHL case showing a variable but usually high number of reactive T cells that express CD57. In this case these T cells form a rosette around the tumor cells (upper panel left). The CD57+ T cells also express the transcription factor c-Maf, indicating a Th2-type nature (upper panel right). In addition, these cells express the T follicular helper cell-associated markers PD-1 (lower panel left) and BCL-6 (lower panel right)

these cells normally provide help to B cells during the germinal center reaction. Another cell population, consisting of CD4+CD8+ dual positive T cells, has been reported to be present in more than 50% of NLPHL tumors. A similar population was found in reactive lymph nodes with progressively transformed germinal centers, which can also be seen in conjunction with NLPHL. In normal peripheral blood, they constitute 1–2% of T cells. The function of these cells is ill defined, and currently they are considered to be potent immunosuppressors and/or to have high cytotoxic potential [22].

4.1.6 Fibrosis and Sclerosis

The presence of bands of collagen surrounding nodules and blood vessels is typical of the nodular sclerosis subtype. Several factors can induce the activation of fibroblasts and the subsequent deposition of extracellular matrix proteins. The Th2 cells in cHL might provide a profibrogenic microenvironment by the production of the Th2 cytokine IL-13. IL-13 is expressed at a higher level in nodular sclerosis than in mixed cellularity cHL. Moreover, the percentage of IL-13 receptor-positive fibroblasts is increased in nodular sclerosis cHL cases [23]. IL-13 stimulates collagen synthesis in vitro and also stimulates the production of TGF-β, another potent stimulator of fibrosis. TGF-β can interact with basic fibroblast growth factor (bFGF) to induce formation of fibrosis in cHL. In a mouse model for fibrosis, the simultaneous application of TGF-β and bFGF causes persistent fibrosis [24]. Both TGF-β and bFGF are produced by HRS cells as well as the reactive background [25, 26]. They are produced more prominently in nodular sclerosis than in mixed cellularity cHL [27]. The third factor that stimulates fibroblasts in cHL is the engagement of CD40. CD40, a member of the tumor necrosis factor receptor (TNFR) superfamily, can be upregulated on fibroblasts by IFN-γ. The ligand of CD40 (CD40L) is present on activated T cells, mast cells, and eosinophils present in the cHL microenvironment.

4.1.7 Eosinophils, Plasma Cells, Mast Cells, and B Cells

Presence of eosinophils in the reactive infiltrate can be promoted by both IL-5, produced by Th2 cells, and by IL-9. In cHL patients with eosinophilia in the peripheral blood, HRS cells produce IL-5 and IL-9 [28]. In addition, eosinophils are attracted to cHL tissues by the production of the chemokine CCL11, especially in nodular sclerosis cHL. CCL11 levels can be enhanced by the production of tumor necrosis factor-α (TNF-α) by the HRS cells, which in turn can induce CCL11 production in fibroblasts. This process is specific for cHL since other lymphomas with tissue eosinophilia show no expression of CCL11 [29]. HRS cells also produce CCL28 (MEC), and expression of CCL28 correlates with the presence of eosinophils and plasma cells in cHL. CCL28 attracts eosinophils by signaling through the chemokine receptor CCR3 and attracts plasma cells through CCR10 [30]. CCL5 is produced at high levels by the reactive infiltrate in cHL and can attract eosinophils as well as mast cells. CCL5 and IL-9 may both contribute to the attraction of mast cells in cHL [31]. The stimulation and recruitment of eosinophils in cHL can be illustrated in bone marrow biopsies that often show enhanced granulopoiesis with many eosinophils in the absence of HRS cells. IL-6 produced by HRS cells in some cases of cHL may explain the presence of variable numbers of plasma cells [32]. B cells that express CD20, CD21, IgM, and bcl-6 can be found in the microenvironment of cHL [33]. It is possible that these cells are remnants of the original lymph node B cell areas. Plasma cells, mast cells, and eosinophils are generally absent in NLPHL.

4.2 Cross-Talk between HRS Cells and Microenvironment (Fig. 4.4)

4.2.1 Factors Supporting Tumor Growth

It is likely that HL tumor cells originate from a precursor B cell that has become addicted to

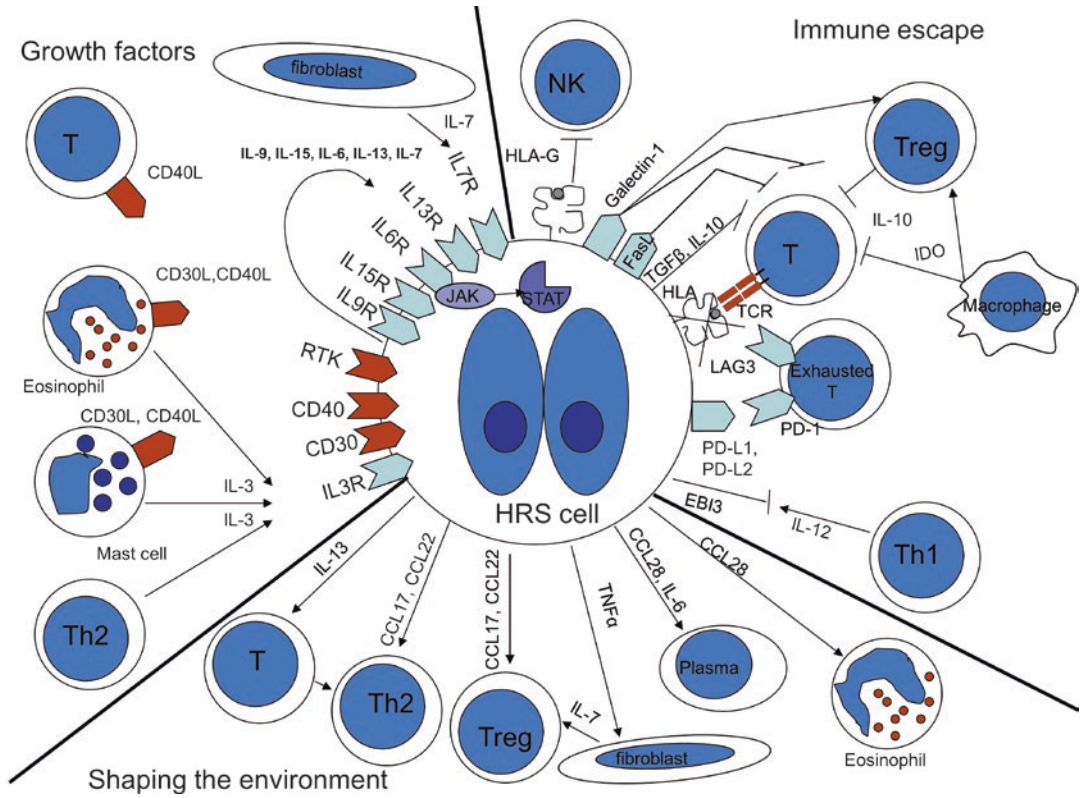

Fig. 4.4 Schematic overview of the cross-talk between Hodgkin/Reed-Sternberg (HRS) cells and the microenvironment. HRS cells attract specific subsets of cells by producing chemokines, are dependent on growth factors, and use mechanisms of immunosuppression and immune escape. Arrows indicate stimulating effects; the other lines indicate inhibitory effects

activating and growth-supporting stimuli during a deregulated immune response. Many additional events are needed to account for the highly deregulated malignant phenotype of HRS and LP cells. Although the tumor cells attain multiple alternative mechanisms to circumvent the dependence on growth-stimulating signals from the reactive infiltrate, they usually are not self-sufficient at the time of diagnosis. This is reflected by the inability to generate cell lines from primary HL cell suspensions.

IL-3 can function as a growth factor for B cells and is produced by activated Th2 cells, mast cells, and eosinophils. Its functions include protection against apoptosis and stimulation of proliferation. Most HRS cells express the IL-3 receptor, and exogenous IL-3 promotes growth of cHL cell lines. Costimulation of HL cell lines with IL-3 and IL-9 results in a further enhancement of cell growth [34]. There is no evidence that HRS cells produce IL-3, so this signaling pathway depends on IL-3 produced by the reactive infiltrate. In contrast, IL-7 is most likely an autocrine as well as a paracrine growth factor for HRS cells, since HRS cells express both the IL-7 receptor and produce IL-7 [35]. cHL cell lines also produce IL-7, albeit at very low levels, and anti-IL-7 treatment has some effect on cell growth. Addition of IL-7 to HL cell line cultures increased proliferation and protected against apoptosis. Moreover, fibroblasts isolated from cHL tissues are able to produce IL-7 [36]. Other growth factors important for HRS cells are IL-9, IL-13, IL-15, and, possibly, IL-6. IL-9 is expressed by the tumor cells and not by the infiltrating cells, and the IL-9 receptor is expressed on cHL tumor cells and mast cells. IL-9 supports tumor growth in cell lines and functions as an autocrine factor in cHL tissue [31]. IL-13 produced by HRS cells as well as the

surrounding T cells drives proliferation and is mostly autocrine [37]. Both IL-15 and the IL-15R are expressed by HRS cells. IL-15 induces proliferation of HL cell lines and protects them against apoptosis [38]. IL-6 is mainly produced by HRS cells and occasionally by the infiltrating cells [32]. In general, IL-6 is found at higher levels in EBV-positive cases [39]. IL-6 might have an autocrine effect although neutralizing antibodies have no effect on the growth of cHL cell lines.

The signaling of cytokines upon binding to their receptors leads to activation of the JAK-STAT pathway. This pathway is constitutively activated in HL cell lines [40, 41], and several of the STAT family members are expressed in HRS cells of primary cHL cases [42, 43]. In addition to the presence of cytokines, amplifications of 9p24.1, including the JAK2 gene locus found in part of the cHL cases [44], can further enhance constitutive activation of this pathway. Functional studies in cHL cell lines have shown that STAT3 is involved in proliferation [45], while STAT1 and STAT6 play a role in protection against apoptosis [46]. Binding of IL-21 to the IL-21R expressed on HL cell lines causes phosphorylation of STAT5 and induces proliferation [47].

HRS cells express several members of the TNFR superfamily including CD30, which has been used as a marker for cHL since the early 1980s. The CD30 ligand (CD30L) is expressed on eosinophils [48] and mast cells [49] that are present in the cHL infiltrate. Circulating eosinophils in cHL patients also have increased expression levels of CD30L [48]. Binding of CD30L to CD30 causes enhanced secretion of IL-6, TNFα, lymphotoxin-α, increased expression of ICAM-1 and B7, and, possibly, increased clonogenic growth and protection against apoptosis in cHL cell lines [50]. Another TNFR expressed on HRS cells is CD40. CD40 is generally found on B cells, and B cells can be activated through CD40. In vitro rosetting of activated CD4+ T cells around HRS cells is mediated through the CD40L adhesion pathway [51]. Engagement of CD40 is important for the prevention of apoptosis. Similar to stimulation of CD30, stimulation of HRS cell lines with CD40L causes enhanced secretion of several cytokines and upregulation of costimulatory molecules [50].

Several receptor tyrosine kinases (RTKs) are expressed by HRS cells and can have a role in cell growth. Their ligands are expressed on cells present in the microenvironment or by the HRS cells themselves. Inhibition of PDGFRA, expressed by the HL tumor cells, by imatinib blocks proliferation. Its ligand, PDGFA, is also produced by the HRS cells indicating autocrine signaling [52]. DDR1 [53] and DDR2 [52] can protect HRS cells from cell death by binding to collagen, which is present in the immediate surrounding of HRS cells. Knockdown of DDR1 decreases survival of the L428 cHL cell line [53]. TRKA, the receptor for NGF, is expressed by granulocytes [52], and TRK inhibition decreases growth of cHL cell lines [54]. EPHB1 and its ligand ephrin-B1 are both expressed by HRS cells [52]. The HGF receptor c-Met is expressed on HRS cells and inhibition causes G2/M cell cycle arrest in HL cell lines. HGF is produced by the tumor cells in a small group of patients and by dendritic reticulum cells [55]. Insulin-like growth factor receptor (IGF-1R) is expressed in 55% of cHL patients, and inhibition of IGF-1R decreases cell growth and induces G2/M cell cycle arrest in HL cell lines [56]. Its ligand IGF-1 is expressed by cells in the microenvironment [57]. PDGFRA, DDR2, EPHB1, RON, TRKA, and TRKB are found especially in EBV-negative HL [58], while DDR1 is upregulated by LMP1 [53].

The Notch1 receptor is an upstream regulator of NFκB [59]. It is highly expressed by HRS cells and stimulation via Jagged1 induces proliferation and survival of cHL cell lines [60].

4.2.2 Shaping the Environment

In addition to the production of several growth factors, HRS cells also produce large amounts of chemokines to attract specific beneficial or non-reacting cells. The lack of CD26 on the T cells surrounding the HRS cells may result in an incapability to cleave chemokines and thereby modulates the chemotactic effects exerted by the HRS cells. The attraction of specific populations of cells is an important immune escape mechanism exerted by the tumor cells.

The most abundant and cHL-specific chemokine is CCL17 (TARC); it binds to CCR4 on Th2 cells, Treg cells, basophils, and monocytes. CCL17 is highly expressed by HRS cells in ~95% of cHL patients but not in NLPHL and most non-Hodgkin lymphomas [61, 62]. CCL17 can be measured in serum and plasma and is a sensitive and specific marker reflecting cHL tumor burden [63–67]. High expression levels of CCL17 might explain the influx of lymphocytes with a Th2- and Treg-like phenotype, and CCL17-positive cases are indeed associated with a higher percentage of CCR4-positive cells (Fig. 4.2; [62, 68]). In turn, Th2-type cytokines (IL-4, IL-13) can induce the production of CCL17 by HRS cells. CCR4-positive T cells are found especially in the rosettes immediately surrounding the HRS cells [15, 69]. CCL22 is another chemokine that has a similar function as CCL17. High CCL22 protein expression levels were found in the cytoplasm of HRS cells in 90–100% of cHL patients and also in tumor cells in the majority of NLPHL and non-HL patients [70–73]. CCL22 production can also be stimulated by Th2 cytokines, IL-4 and IL-13, and may reinforce the attraction of Th2 and Treg cells, initiated by CCL17. Stimulation of the IL-21 receptor on HRS by IL-21 activates STAT3, which can induce CCL20 (MIP3α) production. CCL20 in turn attracts memory T cells and Treg cells [74]. HRS cells express both IL-21 and the IL-21 receptor, indicating presence of an autocrine signaling loop. The expression of some chemokines is more pronounced in EBV-positive cHL (i.e., CXCL9 and CXCL10), and as a result the composition of the reactive background is somewhat different from that in EBV-negative cHL, with a slightly higher proportion of CD8+ T cells in EBV-positive cases and more T cells with a Tr-1 phenotype (expressing LAG3, ITGA2, and ITGB2) [75]. T cell recruitment is also enhanced by the upregulation of adhesion molecules on endothelial cells, induced by LTα [76] produced by HRS cells [77].

In addition to attracting specific cell subsets by chemotaxis, HRS cells also shape their environment by inducing differentiation of specific T cell subsets that are favorable for HRS cell survival and growth. The expression of IL-13 by the HRS cells stimulates differentiation of naïve T cells to Th2 cells [37]. The production of IL-7 by HRS cells and fibroblasts can induce proliferation of Tregs [36]. Also, cHL cell lines with antigen-presenting functions like KMH2 and L428 have been shown to promote the differentiation of Treg like cells in vitro (expressing CD4, CD25, FoxP3, CTLA4, and GITR and producing large amounts of IL-10). Interestingly, these cell lines can also induce the formation of CD4+ cytotoxic T cells (expressing granzyme B and TIA-1) that can kill tumor cells directly, suggesting that CD4+ cytotoxic T cells have the potential to attack tumor cells in vivo [78].

4.3 Immune Escape Mechanisms (Fig. 4.4)

4.3.1 Antigen Presentation

The importance of antigen presentation in the pathogenesis of cHL has been suggested by the association of specific HLA subtypes with increased cHL incidence. cHL is more common in Caucasians as compared to Asians and about 4.5% of cHL cases occur in families [79, 80]. A three- to sevenfold increased risk has been observed in first-degree relatives and siblings. In monozygotic twins, the co-twin has an approximate 100-fold increased risk of developing cHL compared to dizygotic twins [81]. From the 1970s, a number of serological HLA types have been associated with the occurrence of cHL. More recently, a genetic screen of the entire HLA region showed a strong association between the *HLA-A* gene and EBV-positive cHL and the HLA class II region with EBV-negative cHL [82, 83]. Four independent genome-wide association studies have confirmed that the HLA region is the strongest genetic susceptibility locus in cHL [84–87]. In EBV-positive cHL, it can be hypothesized that this association is related to insufficient presentation of EBV antigenic peptides. These antigenic peptides most likely are derived from the latency type II genes that are expressed in cHL, i.e., LMP1, LMP2, and EBV-related nuclear antigen 1 (EBNA1). EBV partially escapes cytotoxic

immune responses by downregulating immunodominant latent genes (*EBNA2* and *EBNA3*). In addition, the glycine-alanine repeat in EBNA1 largely prevents its presentation by HLA class I by blocking its degradation into antigenic peptides through the proteasome [88]. However, subdominant immune responses to LMP2 and to a lesser extent LMP1 are present in healthy EBV-infected individuals [89]. In fact, adoptive immunotherapy in relapsed EBV-positive cHL has been used in some small studies with success. In these studies, peripheral blood from cHL patients was used to generate EBV-specific cytotoxic T cells in vitro, and these were reinfused. Some durable complete responses were observed, with better responses if the cytotoxic T cells were specifically targeted to LMP2 [90–92] (Fig. 4.4). Interestingly, the genetic association of the *HLA-A* gene with EBV-positive cHL is attributed to the presence of the HLA-A∗01 type and absence of the HLA-A∗02 type [93]. HLA-A∗01 is known to have a low affinity for LMP2- and LMP1-derived antigenic peptides, while HLA-A∗02 can present these peptides very well. This suggests that EBV-positive cHL is more likely to occur after primary EBV infection if an individual's set of HLA class I molecules cannot properly present LMP2 and LMP1 to the immune system [94].

4.3.2 HLA Class I Expression

Defects in the antigen-presenting pathways are very common in solid malignancies, as well as in many B cell lymphomas, and are an obvious mechanism to escape from antitumor immune responses. In EBV-negative cHL, less than 20% of cases retain expression of cell surface HLA class I on the HRS cells at the time of diagnosis. Paradoxically, HLA class I expression by HRS cells is retained in ~75% of EBV-positive cHL patients [95–97]. One common mechanism of HLA class I loss is presence of somatic mutations in the β2-microglobulin gene. This leads to loss of β2-microglobulin protein, which is necessary for HLA class I assembly and transport to the cell surface. Other mechanisms also appear to be involved as immunohistochemistry has shown cytoplasmic β2-microglobulin expression in part of the cases that lost HLA class I heavy chain expression [98]. These different mechanisms may indicate that downregulation of HLA class I is based on clonal selection by continuous cytotoxic immune responses. This may be related to the presence of antigenic peptides that are related to malignant transformation or disease progression. However, downregulation of HLA class I generally induces activation of natural killer (NK) cells. These cells contain HLA class I-specific inhibitory receptors and are sparse in the reactive infiltrate of cHL. The inhibitory receptors can also be engaged by the nonclassical HLA class I-like molecule known as HLA-G. In about two thirds of the HLA class I-negative cHL cases, the HRS cells indeed express HLA-G [98]. Besides NK cell inhibition, HLA-G might also induce Treg cells and inhibit cytotoxic T cell responses. Another immune escape mechanism consists of the proteolytic cleavage of MHC (HLA) class I-related chain-A (MIC-A) by ERp5 and ADAM10, which are both expressed by HRS cells. MIC-A is a membranous ligand for the activating NKG2D receptor present on cytotoxic T cells. In addition, the NKG2D receptor expression by these cytotoxic T cells is reduced in the presence of TGF-β [99].

4.3.3 HLA Class II Expression

HLA class II cell surface expression on HRS cells is lost in approximately 40% of all cHL patients [95]. In addition, translocations involving CIITA have been found in 15% of cHL patients and may result in partial downregulation of HLA class II expression [101]. The absence of HLA class II is weakly related to extranodal disease, EBV-negative status, and absence of HLA class I cell surface expression. Lack of HLA class II expression has been associated with adverse failure-free survival and relative survival and is independent of other prognostic factors [95]. It can be hypothesized that antigen presentation in the context of HLA class II is involved in recruitment and activation of CD4+ T cells early in cHL pathogenesis. Under the influence of immunomodulating mechanisms, these T cells are important in providing trophic factors for HRS cells

and also have a role in inhibiting Th1 responses. In the initial stages of cHL pathogenesis, HRS cells are probably highly dependent on the reactive infiltrate and expression of HLA class II, but as the lymphoma develops, this dependency may weaken because of alternative trophic and immunosuppressive strategies. Thus, downregulation of HLA class II without loss of viability of HRS cells might occur when the HRS cells have grown less dependent on the reactive infiltrate. This is supported by the finding that downregulation of HLA class II is associated with extranodal disease [95].

4.3.4 Immune Checkpoints

Immune checkpoint molecules have gained much attention due to their use as treatment targets. Both CTLA-4 and PD-1 blockade have shown remarkable results in cHL patients in clinical trials.

CTLA-4 is expressed exclusively on T cells upon activation. It gives an inhibitory signal early after T cell activation, by binding to CD80/CD86 with a higher avidity than the costimulatory molecule CD28. This limits T cell activation and proliferation [101, 102]. Interestingly, CTLA-4 is present on the characteristic CD26− T cells in HL [15]. Moreover, HL cell lines are able to induce differentiation of naïve T cells into CTLA4+ Tregs [78]. So far, two clinical trials have exploited the use of a monoclonal antibody targeting CTLA-4 in relapsed and refractory HL after allogeneic hematopoietic cell transplantation. Objective response rates were observed in 2 out of 14 and 1 out of 7 cases [103, 104].

The interaction partner of PD-L1, PD-1, is present on activated T cells, B cells, macrophages (which also express PD-L1), and NK cells within the microenvironment [105, 106]. PD-L1 is highly expressed on HRS cells [105, 107], due to a selective amplification of the PD-L1 region on 9p24.1 [44], activation of AP-1 and LMP-1 [107], or chromosomal alterations involving the CIITA locus [100]. Anti-PD-1 therapy in relapsed and refractory HL patients, using the monoclonal antibodies nivolumab or pembrolizumab, showed objective response rates in 65–87% of the cHL patients [108–111]. The mechanism of action of PD-1 blockade in HL remains unknown, but multiple mechanisms have been studied. In contrast to solid tumors where CD8+ cytotoxic T cells seem to be the main effector cells [112], CD4+ T cells might have an important role in mediating the antitumor immune response in HL. CD8+ T cells recognize the tumor cells through (neo)antigens presented in the context of HLA class I, which can ultimately lead to eradication of the tumor cells. However, HLA class I is often absent on HRS cells, making a central role for CD8+ T cells in immune checkpoint efficacy unlikely [96]. In HL, the inflammatory infiltrate is mainly dominated by CD4+ T cells, which are more often in direct contact with the HRS cells when compared to CD8+ T cells. In addition, CD4+PD-1+ T cells are more frequently bound to PD-L1+ HRS cells [113]. The majority of complete responders to nivolumab lack membranous HLA class I expression, while being positive for membranous HLA class II expression. Also, presence of HLA class II is predictive for a prolonged progression-free survival in patients treated with nivolumab >12 months after myeloablative autologous stem cell transplantation, in contrast to presence of HLA class I [114]. Although many studies on PD-1 blockade focused on T cells, expression of PD-1 on T cells in direct contact with HRS cells is rare, and their numbers are significantly lower in cases with PD-L1 gain [115]. Interestingly, exhausted PD-1+ CD3+CD56^{hi}CD16− NK cells are enriched in HL, and their activation can be inhibited by PD-L1+CD163+CD14+ tumor-associated macrophages, which are also increased in HL. This inhibition was effectively reversed by PD-1 blockade [106]. This indicates the importance of other cell types in responses to PD-1 blockade that are currently less well characterized.

An interesting molecule with regard to the role of CD4+ T cells in responses to anti-PD-1 therapy is LAG-3. LAG-3 is an immune checkpoint molecule expressed on activated T cells, NK cells, B cells, and plasmacytoid dendritic cells [116]. The main interaction partner for LAG-3 is HLA class II, to which LAG-3 binds with higher affinity than CD4 [117]. LAG-3 is upregulated on Tregs, and LAG-3-positive lymphocytes are enriched in the

proximity of HRS cells [118, 119]. Increased LAG-3 expression was observed especially in EBV-positive cHL cases [118, 120]. Interestingly, the percentage of LAG-3-positive cells was enriched in CD4+ T cells that express a high intracellular CTLA-4 and GITR, but not FOXP3+ [118]. The $GITR^{hi}$ CD4+ T cells are frequently in direct contact with HRS cells [15]. Moreover, CD4+LAG-3+ cells are significantly expanded in patients with active disease, which is in concordance with the ability of HL cell line supernatant to increase expansion of CD4+LAG-3+ regulatory T cells within PBMCs. In addition, higher FOXP3 and LAG-3 expression on tumor-infiltrating lymphocytes is associated with decreased LMP1- and LMP2-specific CD8+ T cell function [118]. Altogether this implicates an important role for LAG-3 in inhibiting (EBV) mediated cellular immunity in HL and points to LAG-3 as an interesting treatment target in combination with PD-1 blockade.

Currently, more and more immune checkpoint molecules are emerging as potential targets for therapy. Some of these are less well studied in the context of HL. For example, HRS cells express HVEM and CD200/CD200R [121] in addition to the earlier mentioned checkpoints, whereas TIM-3+ T cells are present within the inflammatory infiltrate [120], indicating the complexity of immunosuppressive mechanisms within the HL microenvironment.

4.3.5 Immunosuppression

As normal B cells are professional antigen-presenting cells, HRS cells are expected to present antigens to the immune system, at least early in disease pathogenesis. Indeed, most components of the HLA class I and HLA class II antigen-presenting pathways have been detected in the HRS cells at the time of diagnosis. However, Th1 cells are not actively attracted by the HRS cells and CD8+ T cells are relatively scarce. Moreover, HRS cells have gained the capacity to prevent CD8+ T cells from attacking by producing high amounts of the strongly immunosuppressive cytokines TGF-β and IL-10. TGF-β is produced by HRS cells in nodular sclerosis cHL [25, 26], whereas IL-10 is more frequently found in EBV-positive (mixed cellularity) cHL [122, 123]. In normal cells, TGF-β is produced in an inactive form, which can be activated by acidification. TGF-β produced by cHL cell lines is active at a physiological pH and has a high molecular weight [124]. The same high molecular weight form of TGF-β can also be found in the urine of cHL patients [125] indicating that in patients HRS cells are able to produce the active TGF-β form.

Tregs present in the microenvironment of cHL are highly immunosuppressive and contain Tr1 (IL-10-producing Tregs) as well as CD4+CD25+ Tregs. IL-10, cell-cell contact, and CTLA4 play a main role in executing their immunosuppressive function [126]. In addition, HRS cells express galectin-1, an animal lectin, which can cause apoptosis in activated T cells, induce differentiation into Treg cells, and contribute to the elimination of an effective antitumor response in cHL [127]. Galectin-1 expression blocks CD8+ T cell responses against LMP1 and LMP2 in EBV-positive cHL [128]. HRS cells express FAS and the FAS ligand. However, there are some mechanisms protecting the HRS cells from apoptosis induction, such as FAS mutations in a small proportion of cases and c-FLIP overexpression in all cases [129]. Presumably, activated Th1 and CD8+ T cells expressing FAS are driven into apoptosis by the FAS ligand expression on the HRS cells. Also, indoleamine 2,3-dioxygenase (IDO), a known immunosuppressor, is expressed by histiocytes, dendritic cells, and endothelial cells in the microenvironment of cHL. IDO is found more often in EBV-positive HL, upregulates the number of Tregs [130], and potentially blocks the CD8+ T cell response [131]. In EBV-positive cHL, the Th1-inducing cytokine IL-12 is expressed in T cells surrounding the HRS cells, and its presence suggests that these T cells have the potential to induce antitumor activity [132]. However, an EBV-induced IL-12-related cytokine called EBI3 can block this Th1 response and is produced by HRS cells [133].

4.4 Prognostic Impact of the Microenvironment

Several research groups studied the cHL reactive infiltrate in relation to prognosis. Gene expression profiling of whole tissue and subsequent validation by immunohistochemistry showed that high numbers of CD68-positive cells are related to adverse outcome [134]. In a meta-analysis of almost 3000 patients, a high density of CD68+ tumor-associated macrophages predicted overall survival, shorter progression-free survival, and poor disease survival in adult cHL [135]. Similar findings were obtained for CD163+ macrophages in this study. Analysis of 100 pediatric cHL revealed that especially M2 macrophages, characterized by co-expression of CD163 and c-Maf, are associated with poor survival, while M1 macrophages are associated with better survival [136]. In some studies tumor-associated macrophages were associated with EBV-positive tumors [135, 137] and presence of other cell types in the microenvironment, such as cytotoxic T cells [136] and mast cells [138]. Patients with a higher degree of mast cell infiltration or with tissue eosinophilia have an adverse failure-free survival, probably because the CD30L expression by these cell types is advantageous to the HRS cells [48, 49, 138].

Large numbers of Th2 cells in the microenvironment, as determined by c-Maf expression, correlate with improved disease-free survival [14]. Also, increased numbers of infiltrating Treg cells seem to correlate with improved survival as this effect was observed in two out of three studies [14, 139, 140]. Accordingly, a high percentage of activated CD8+ granzyme B+ T cells is a strong indicator of unfavorable clinical outcome [141]. More recently, a high proportion of Treg cells and the associated anergic phenotype of the microenvironment has been associated with a shorter time to progression [137]. A high CD4/CD8 T cell ratio was associated with treatment failure [142]. A high ratio of FoxP3 to cytotoxicity markers granzyme B [140] or Tia-1 [139] gives the best predictive value for a good prognosis and has also been correlated to the presence of macrophages [136, 138], which might—in part—explain these effects. In other malignancies the presence of Tregs and the absence of CD8+ T cells have been associated with adverse prognosis. One explanation of this opposite effect might be that HRS cells are expected to behave more aggressively as they develop a stronger independency from the reactive infiltrate. In this situation a hostile microenvironment is allowed, because the HRS cells have acquired alternative immune evasive strategies. This theory fits with the adverse prognostic impact of absence of HLA class II expression.

4.5 Conclusion

The microenvironment is a fundamental component of the tumor mass and an essential pathogenetic factor in cHL and NLPHL. It supplies the tumor cells with growth factors and inhibits antitumor immune responses. In fact, it could be stated that the infiltrate does not consist of 'innocent bystanders' but contains 'guilty opportunists' [31]. As the tumor cells and the reactive infiltrate grow up together, there is an extensive cross-talk between these two components. The tumor cells actively attract and shape their environment for their own benefit and make use of a number of mechanisms to fend off antitumor immune responses.

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