Microenvironment, Crosstalk, and Immune Escape Mechanisms

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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 a component 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)

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	0	Nodular (+diffuse)	Th2, CD57+ Treg, CD4+/8+	Histiocytes, B cells
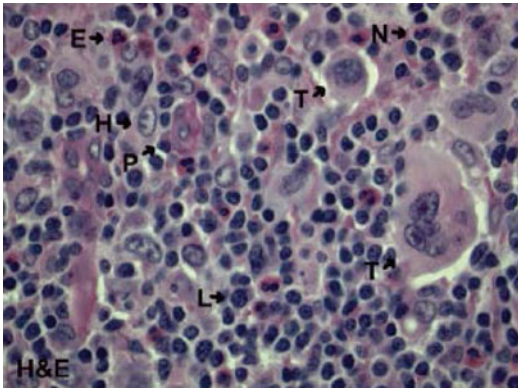
Fig. 4.1 The microenvironment in mixed cellularity classical Hodgkin lymphoma. *T* tumor cell, *L* (T–) lymphocyte, *H* histiocyte, *E* eosinophil, *N* neutrophil, *P* plasma cell. Hematoxylin and eosin staining

HL is considered a separate entity. 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. However, the nature of the tumor cells and the T cells is different. In the cHL subtypes, the HRS cells are transformed post germinal center B cells with a loss of B cell phenotype, while in LPHL the lymphocyte-predominant (LP) cells have a germinal center B cell phenotype. The T cells in cHL have features of paracortical T cells, while those in LPHL are similar to germinal center T cells [3, 4].

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 cellularity subtype (~75 % EBV+) and by definition is absent in LPHL. Depending on the geographic locale, EBV is present in the HRS cells in 10–40 % in nodular sclerosis cases. The percentage of EBV+ classical lymphocyte-rich 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 B cells and usually prevent EBV-driven malignant transformation in immunocompetent individuals. Accordingly, EBV-associated cHL cases contain slightly more CD8+ cytotoxic T cells in the reactive background compared to non-EBV-associated cHL cases [5].

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 [6]. 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 indicates 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, because an increase in HIV-associated cHL incidence has been observed after the introduction of highly active antiretroviral therapy (HAART) [7] (Fig. 4.4).

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 amounts 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, 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 can be divided into naive (CD45RA+) and memory (CD45RO+) subsets depending on whether they have previously been stimulated by antigen. 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 other responses) cells. The Treg cells can be further divided into Th3 (transforming growth factor-β (TGF-β)-producing), Tr1 (IL-10-producing), and
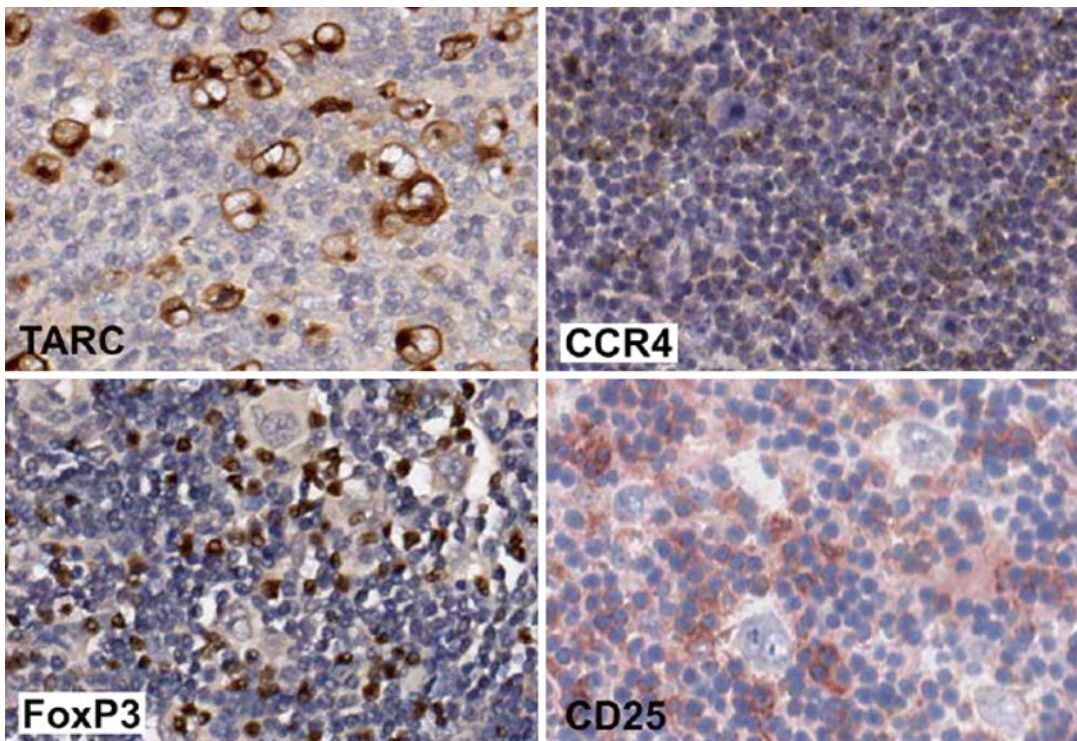
Fig. 4.2 Shaping the microenvironment in classical Hodgkin lymphoma (HL). Immunohistochemistry of classical HL cases. In the *upper panel*, *left*, strong and specific staining of Hodgkin/Reed–Sternberg (HRS) cells for chemokine CCL17 (TARC). This chemokine attracts CCR4+ lymphocytes (*upper panel*, *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*)

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 [8]. 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) [9]. CD26 is also associated with adenosine deaminase (ADA) and with CD45RO and, when interacting with anti-CD26 antibodies, leads to enhancement of T cell activation through the T cell receptor [10]. CD26 is preferentially expressed on CD4+CD45RO+ cells and is normally upregulated after activation. However, CD26 cannot be upregulated on the CD26-negative cells from cHL lesions. In general, a high CD26 expression level correlates with a Th1 subtype of cells.

The transcription factor expression pattern indicates that the CD4+ T cells in cHL are predominantly Th2 (c-Maf) and Treg (FoxP3) [3, 11]. 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 [12]. 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 the 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 [12]. 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, and CTLA-4 shows a more diffuse presence [12]. Likewise, a small number of scattered IL-17-positive cells can be found in the reactive infiltrate. Anergy in T cells is normally induced by lack of costimulation through CD80/CD86, activation by superantigens, or the effect of cytokines like TGF-β and IL-10. The anergic state in cHL is probably not caused by the lack of costimulatory molecules since CD80 and CD86 as well as several other costimulatory molecules are highly expressed on the HRS cells [13, 14]. However, besides CD28, the surrounding lymphocytes express CTLA-4, and HRS cells frequently produce TGF-β and IL-10 which can cause anergy of the surrounding T cells. Although the vast majority of studies indicate that the CD4+ T cells in cHL are (anergic) Th2 cells and Treg cells, a single recent study by flow cytometry of whole lymph nodes showed a predominant Th1-type pattern. In this study there were high numbers of T-bet (Th1-type)-positive cells in tissue by immunohistochemistry, with increased levels in EBV+ cHL [15].

4.1.5 T Cell Subsets in LPHL

The CD4+ T cells in LPHL resemble the CD4+ T cells in cHL, regarding the expression of CD45RO, CD69, CTLA4, and CD28 and lack of CD26. However, these T cells do not express CD40L, and a significant proportion of the cells that immediately surround the LP cells express CD57 and PD-1 [16]. Similar to the Th2 cells in cHL, the rosetting cells in LPHL strongly express the Th2-associated transcription factor c-Maf (Fig. 4.3; [3]).

Characterization 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 the tonsil. Stimulation of these cells fails to induce upregulation of IL-2 and IL-4 mRNA levels [17], which is similar to the lack of cytokine induction upon
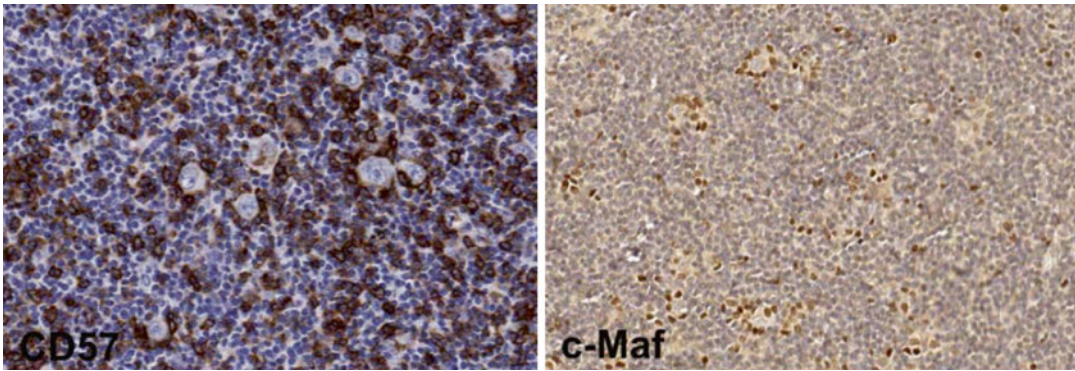
Fig. 4.3 T cells in nodular lymphocyte-predominant Hodgkin lymphoma (LPHL). Immunohistochemistry of a case of LPHL. A variable but usually high amount of reactive T cells express CD57, and as in this case these cells can encircle the tumor cells (*panel*, *left*). The CD57+ T cells also express transcription factor c-Maf, indicating a Th2-type nature (*panel*, *right*)

stimulation of the CD26– T cells in cHL. The normal counterpart of CD4+CD57+ T cells is found almost exclusively in the light zone of reactive germinal centers. These CD57+ T cells 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.

In LPHL, a population of CD4+CD8+ T cells has been reported in more than 50 % of patients. The function of these cells in LPHL is currently unknown, but in other settings these cells have immunoregulatory properties [18].

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 [19]. 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 cause fibrosis in cHL. In a mouse model for fibrosis, the simultaneous application of TGF-β and bFGF causes persistent fibrosis [20]. Both TGF-β and bFGF are produced by the HRS cells as well as the reactive background [21, 22]. TGF-β and bFGF are both produced more prominently in nodular sclerosis than in mixed cellularity cHL [23], which is consistent with this concept. 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, and its ligand 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

The 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, IL-5 and IL-9 have been reported to be expressed by the HRS cells [24]. 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 [25]. 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 [26]. 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 [27]. The stimulation and recruitment of eosinophils in cHL can be illustrated in bone marrow biopsies that often

show reactive enhancement of granulopoiesis with many eosinophils in the absence of bone marrow HRS cells. IL-6 has been shown to be produced by HRS cells in some cases of cHL, and this may explain the presence of variable amounts of plasma cells [28]. B cells that express CD20, CD21, IgM, and bcl-6 can be found in cHL [29]. It is possible that these cells are remnants of the original lymph node B cell areas.

4.2 Crosstalk 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 crosstalk between Hodgkin/Reed–Sternberg (HRS) cells and the microenvironment. Hodgkin lymphoma tumor cells attract specific cell subsets by chemokines, are dependent on growth factors, and use mechanisms of immune suppression 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 grow 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 in cHL cases express the IL-3 receptor, and exogenous IL-3 promotes cell growth in cHL cell lines. Costimulation of IL-3 with IL-9 results in further enhancement of cell growth [30]. There is no evidence for the production of IL-3 by HRS cells themselves, so this signaling pathway depends on the reactive infiltrate. 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 [31]. Moreover, fibroblasts isolated from cHL tissue are able to produce IL-7 [32]. cHL cell lines produce very little IL-7 themselves, but anti-IL-7 has some effect on cell growth. Addition of IL-7 results in an increase in proliferation and protection against apoptosis. Other growth factors important for HRS cells are IL-9, IL-13, and, possibly, IL-6. IL-9 is expressed by the tumor cells and not in the infiltrate, and the IL-9 receptor is expressed on the tumor cells and mast cells. IL-9 supports tumor growth in cell lines and is an autocrine factor in cHL tissue [27]. IL-13 produced by HRS cells as well as the surrounding T cells drives proliferation and is mostly autocrine [33]. IL-6 is mainly produced by the HRS cells and occasionally by the infiltrating cells [28]. In general, IL-6 is found at higher levels in EBV+ cases [34]. IL-6 might have an autocrine effect although neutralizing antibodies have no effect on the growth of cHL cell lines.

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 [35] and mast cells [36] that are present in the cHL infiltrate. Circulating eosinophils in cHL patients also have increased expression levels of CD30L [35]. Binding of CD30L to CD30 causes enhanced secretion of IL-6, TNFα, and lymphotoxin-α; increased expression of ICAM-1 and B7; and, possibly, increased clonogenic growth and protection against apoptosis [37]. 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+ lymphocytes around HRS cells is mediated through the CD40L adhesion pathway [38]. 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 [37].

Several receptor tyrosine kinases (RTKs) are expressed by HRS cells and can have a role in cell growth. Their ligands are expressed in the microenvironment or by the HRS cells themselves. PDGFRA has a role in cell growth, since inhibition of PDGFRA signaling by imatinib blocks proliferation. Its ligand, PDGFA is also produced by the HRS cells [39]. DDR1 [40] and DDR2 [39] can protect HRS cells from cell death by binding to collagen, which is present in the immediate surroundings of the HRS cells. Knockdown of DDR1 decreases survival of the L428 cHL cell line [40]. TRKA is the receptor for NGF which is expressed by granulocytes [39], and TRK inhibition can decrease survival of cHL cell lines [41]. EPHB1 and its ligand ephrin-B1 are both expressed by the HRS cells [39]. HGF receptor c-Met is expressed on HRS cells, and inhibition causes G2/M cell cycle arrest. HGF is produced by the tumor cells in a small group of patients and by dendritic reticulum cells [42]. PDGFRA, DDR2, EPHB1, RON, TRKA, and TRKB are found especially in EBV– HL [43], while DDR1 is upregulated by LMP1 [40].

Another receptor, Notch1 is an upstream regulator of NFκB [44]. It is strongly expressed by HRS cells, and stimulation via Jagged1 induces proliferation and survival of cHL cells [45].

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 the chemokines and thereby modulate the chemotaxic effects exerted by the HRS cells. The attraction of a specific population 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 the vast majority of cHL patients and not in LPHL or non-Hodgkin lymphomas [46, 47]. CCL17 levels can be measured in serum and are a sensitive and specific marker reflecting cHL tumor burden [48–51]. 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; [47, 52]). In turn, Th2-type cytokines (IL-4, IL-13) can induce the production of CCL17 by HRS cells. CCR4-positive lymphocytes are found especially in the rosettes immediately surrounding the HRS cells [12, 53]. 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 LPHL and non-HL patients [54–57]. CCL22 production can also be stimulated by Th2 cytokines, IL-4 and IL-13, and may serve to reinforce the attraction of Th2 and Treg lymphocytes, 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 [58]. HRS cells express both IL-21 and the IL-21 receptors, indicating the presence of an autocrine signaling loop. The expression of some chemokines is more pronounced in EBV+ cHL (i.e., CXCL9 and CXCL10), and perhaps as a result the composition of the reactive background is somewhat different from that in EBV– cHL, with a slightly higher proportion of CD8+ T cells in EBV+ cases.

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 [33]. The production of IL-7 by HRS cells and fibroblasts can induce proliferation of Tregs [32]. 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 cells (expressing granzyme B and TIA-1) that can kill tumor cells directly, suggesting that CD4+ CTLs have the potential to attack tumor cells in vivo [59].

4.2.3 Immune Suppression

Because 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+ CTLs are relatively scarce. Moreover, HRS cells have gained the capacity to prevent CTLs 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 [21, 22], whereas IL-10 is more frequently found in EBV+ (mixed cellularity) cHL [60, 61]. In normal cells, TGF-β is produced in an inactive form, which can be activated by acidification. TGF-β produced by cHL cell line L428 is active at a physiological pH and has a high molecular weight [62]. The same high molecular weight form of TGF-β can also be found in the urine of

cHL patients [63] indicating that in patients HRS cells are able to produce the active TGF-β form.

The Tregs that are 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 [64]. In addition, HRS cells express galectin-1, an animal lectin, which can cause apoptosis in activated T cells and contributes to the elimination of an effective antitumor response in cHL [65]. HRS cells also express FAS and the FAS ligand. 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 [66]. Presumably, activated Th1 and CD8 cells expressing FAS are driven into apoptosis by the FAS ligand expression on the HRS cells. Also, HRS cells were found to express PD-1 ligand, induced by a selective amplification of 9p24.1 [67], AP-1 activation, the presence of LMP-1 [68], or chromosomal translocations involving the CIITA locus [69]. The rosetting lymphocytes are rarely PD-1 positive, and their numbers are significantly lower in cases with PD-1 ligand gain [70]. In LPHL, the rosetting lymphocytes express PD-1 [16], but LP cells do not express PD-1 ligand. In EBV+ 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 [71]. However, an EBV-induced IL-12-related cytokine called EBI3 can block this Th1 response and is produced by HRS cells [72].

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 [73, 74]. A three- to sevenfold increased risk has been observed in first-degree relatives and siblings. In monozygotic twins, the cotwin has an approximate 100-fold increased risk of developing cHL compared to dizygotic twins [75]. 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+ cHL and the HLA class II region with EBV– cHL [76, 77]. At present, three independent genomewide association studies have confirmed that the HLA region is the strongest genetic susceptibility locus in cHL [78–80]. In EBV+ 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 [81]. However, subdominant immune responses to LMP2 and to a lesser extent LMP1 are present in the healthy EBV-infected population [82]. In fact, adoptive immunotherapy in relapsed EBV-associated cHL has been used in some small studies with success, although limited. In these studies, peripheral blood from cHL patients was used to create EBV-specific cytotoxic T cell lines in vitro, and these were reinfused. Some objective responses were observed (3/11 and 5/6), with better responses if the CTLs were specifically targeted to LMP2 [83, 84] (Fig. 4.4).

Interestingly, the genetic association of the *HLA-A* gene with EBV+ cHL is attributed to the presence of the HLA-A*01 type and absence of the HLA-A*02 type [85]. 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+ 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.

4.3.2 HLA Expression

Paradoxically, HLA class I and class II expression by HRS cells is usually retained in EBV+ cHL patients, whereas in EBV– cHL patients these molecules are frequently downregulated. Defects in the antigen-presenting pathways are very common in solid malignancies (HLA class I), as well as in many B cell lymphomas (HLA class I and class II), and are an obvious mechanism to escape from antitumor immune responses. Especially downregulation of HLA class I is a common immune escape mechanism in EBV– cHL, with less than 20 % of cases still expressing cell surface HLA class I on the HRS cells at the time of diagnosis [86]. Different mechanisms are involved in this downregulation because immunohistochemistry has shown complete absence of HLA class I or retention of HLA class I heavy chains within the cytoplasm. This retention in the cytoplasm is usually accompanied by an absence of β2-microglobulin expression, which is necessary for HLA class I assembly and transport to the cell surface. The 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 associated 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 a nonclassical HLA class I–like molecule known as HLA-G. In about two thirds of HLA class I–negative cHL cases, the HRS cells indeed express HLA-G [87]. 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 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 [88].

4.3.2.1 HLA Class I Expression

In contrast to EBV– cHL, 70–80 % of EBV+ cHL patients show cell surface expression of HLA class I and β2-microglobulin at the time of diagnosis. This expression is usually particularly strong in mixed cellularity subtype cases [5, 86]. Upregulation of HLA class I has been attributed to LMP1, but the function of this upregulation is enigmatic, since it should make latent EBV-infected B cells more susceptible to immune recognition. In fact, in primary lytic EBV infection, the HLA class I antigen-presenting pathway is strongly inhibited by EBV proteins [89]. When the virus goes into latent infection, this immune escape mechanism is no longer available. As the lytic gene products are switched off, the expression and function of HLA class I and class II are restored. Importantly, the cHL-associated EBV latent gene products LMP1, LMP2, and EBNA1 are necessary for EBV-infected B cells to go through the germinal center reaction. At that time HLA class I and class II antigen-presenting functions might also be essential for B cell survival. It is generally accepted that HRS cells derive from germinal center B cells, and in EBV+ cHL it is likely that the tumor cell precursor expresses LMP1, LMP2, EBNA1, HLA class I, and HLA class II.

4.3.2.2 HLA Class II Expression

HLA class II cell surface expression on HRS cells is lost in approximately 40 % of all cHL patients [86]. In addition, translocations involving CIITA have been found in 15 % of cHL patients and may result in subtotal downregulation of HLA class II expression [69]. 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 [86]. 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 association of downregulation of HLA class II with extranodal disease [86].

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 [90]. These CD68-positive cells reflect tumor-associated macrophages and probably also other cell types in the microenvironment. 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 [35, 36].

Large numbers of Th2 cells in the microenvironment, as determined by c-Maf expression, correlate with improved disease-free survival [9]. Also, increased numbers of infiltrating Treg cells seem to correlate with improved survival as this effect was observed in two out of three studies [11, 91, 92]. Accordingly, a high percentage of activated CTLs (CD8+/granzyme B+ T cells) is a strong indicator of unfavorable clinical outcome [93]. A high ratio of FoxP3 to CTL markers, granzyme B [92] or Tia-1 [91], gives the best predictive value for a good prognosis. These results are unexpected since in other malignancies the presence of Tregs and the absence of CTLs are associated with adverse prognosis. One explanation 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 immunoevasive strategies. This theory fits with the adverse prognostic impact of the absence of HLA class II expression.

Conclusion

The microenvironment is a fundamental component of the tumor mass and an essential pathogenetic factor in cHL and LPHL. It supplies the tumor cells with growth factors and inhibits antitumor immune responses. In fact, it could be stated that "the infiltrate consists not of 'innocent bystanders' but of guilty opportunists" [27]. As the tumor cells and the reactive infiltrate grow up together, there is an extensive crosstalk 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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