CHAPTER TWO

THE MICROENVIRONMENT IN CLASSICAL HODGKIN LYMPHOMA: AN ACTIVELY SHAPED AND ESSENTIAL TUMOR COMPONENT

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Classical Hodgkin lymphoma (cHL) is characterized by a minority of tumor cells derived from germinal center B-cells and a vast majority of non-malignant reactive cells. The tumor cells show a loss of B-cell phenotype including lack of the B-cell receptor, which makes the tumor cells vulnerable to apoptosis. To overcome this threat, tumor cells and their precursors depend on anti-apoptotic and growth stimulating factors that are obtained via triggering of multiple membrane receptors. In addition, tumor cells shape the environment by producing a wide variety of chemokines and cytokines. These factors alter the composition of the microenvironment and modulate the nature and effectiveness of the infiltrating cells. The attracted cells enhance the pro-survival and growth stimulating signals for the tumor cells. To escape from an effective anti-tumor response tumor cells avoid recognition by T and NK cells, by downregulation of HLA molecules and modulating NK and T-cell receptors. In addition, the tumor cells produce immune suppressive cytokines that inhibit cytotoxic responses. In this review the relevance of the microenvironment in the pathogenesis of cHL will be discussed.
1 General background on classical Hodgkin lymphoma

Hodgkin lymphoma (HL) is a distinctive disease with a characteristic clinical presentation and it was the first lymphocyte malignancy to be described. It has an incidence rate of 3 per 100,000 person years and is among the most common cancers in adolescents and younger adults. About 50% of the HL patients are diagnosed between ages 15 and 35 years and a second incidence peak can be observed in the elderly. HL primarily involves lymph nodes and has a unique histomorphological presentation with a minority of neoplastic cells, which generally comprise less than 1% of the total cell population, and a large majority of non-malignant reactive immune cells (Figure 2.1). HL has been divided into classical HL (cHL), which accounts for 95% of all cases, and the less common nodular lymphocyte predominant HL form, which is considered to be a different disease entity.

The tumor cells in cHL are named Hodgkin and Reed-Sternberg (HRS) cells and they express diagnostic markers CD30 and usually CD15. The HRS cells are characterized by a very large (sometimes lobated) nucleus with little DNA condensation, one or more very large nucleoli, a large Golgi apparatus and much cytoplasm. This typical morphology indicates that the HRS cells are strongly activated and produce a large amount of proteins.

In 20-40% of cHL cases in the western world, monoclonal infection with Epstein Barr virus (EBV) is present in HRS cells and this is considered to be a tumor-initiating factor. EBV+cHL patients show a latency type II infection pattern that is restricted to expression of latent membrane protein 1 (LMP1), LMP2 and EBV-related nuclear antigen 1 (EBNA1). LMP1 and LMP2 are oncogenes that mimic CD40 activation and BCR signaling respectively.

Based on growth pattern, morphology of the HRS cells and the composition of the background infiltrate, cHL is divided into four histological subtypes, of which nodular
sclerosis (~80%) and mixed cellularity (~15%) are the most common subtypes. Lymphocyte depleted and lymphocyte rich subtypes are relatively uncommon. A nodular growth pattern, sclerotic bands and large HRS cells define the nodular sclerosis subtype. In mixed cellularity HL the growth pattern is diffuse, without sclerosis and somewhat smaller, often binucleated HRS cells. In virtually all cases the microenvironment contains numerous small T-cells with variable numbers of eosinophils, histiocytes/macrophages, B-cells, plasma cells and sometimes neutrophils (Figure 2.1). In mixed cellularity this microenvironment tends to be more mixed, usually with loose or granulomatous collections of histiocytes. Regardless of subtype, the tumor cells are usually in intimate contact with small CD4+ T-cells. This can be seen in tissue sections as a layer of lymphocytes directly surrounding single tumor cells (so-called rosettes)\(^\text{10}\).

cHL can be considered an extreme model of how the tumor microenvironment impacts cancer pathogenesis. Much research has been focused on the cross talk between HRS cells and the microenvironment and its (potential) functional relevance. However, there are no cHL animal models in which cross talk between HRS cells and the microenvironment can be studied. In addition, primary tissue derived HRS cells usually do not survive in culture. This hampers the possibilities to do functional studies that closely mimic the in vivo situation. Functional studies are usually done in cHL cell lines and/or specific subsets of the reactive infiltrate. Caution should be taken in interpreting putative autocrine effects especially for membrane bound factors, as tumor cell-tumor cell contact is very infrequent in cHL tissue. Relevant markers are tested for expression in cHL tissue samples to support these experimental findings. In general, this is the closest approximation possible.

This review focuses on the microenvironment of cHL to show that the reactive infiltrating cells are not innocent bystander cells but an essential component of the tumor. The most accepted mechanisms with respect to enabling tumor cell driving mechanisms, shaping of the microenvironment and disabling anti-tumor immune responses will be discussed.
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2 Tumor cell driving mechanisms

Due to the lack of a functional BCR, HRS cells and their precursors are dependent on anti-apoptotic and pro-survival signals from the microenvironment. This dependence most likely already exists at the initiation of the malignant transformation. Constitutive activation of the NF-κB pathway is one of the hallmarks of the HRS cells and provides the tumor cells with a strong pro-survival signal. Activation of NF-κB is achieved via multiple mechanisms, i.e. mutations of the NF-κB and JAK/STAT pathways\textsuperscript{11} and signaling via the tumor necrosis factor receptor superfamily (TNFRSF), tyrosine kinases (TK) and cytokine receptors (Figure 2.2).

Figure 2.1: Hematoxylin and eosin staining of a cHL case showing the typical tumor cells and the cellular infiltrate seen in cHL. Indicated in the image are typical mononuclear Hodgkin (H) cells and a binucleated Reed-Sternberg (RS) cell. The reactive infiltrate consists of a vast majority of small lymphocytes, usually T-cells (T), eosinophils (E), plasma cells (P) and histiocytes (Hi).
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Figure 2.2: A schematic representation of the tumor cell driving mechanisms in cHL. The picture is focused on the tumor cell driving factors that have been validated in cHL. The TNFRSF family members, RTKs and cytokine receptors and their intracellular signaling pathways are shown. Putative paracrine and/or autocrine activation mechanisms of these signaling pathways as well as the cells that provide the activating signals are depicted.

2.1 Tumor necrosis factor receptor superfamily

CD30, a member of the TNFRSF (TNFRSF5), is abundantly expressed on HRS in virtually all cHL patients and on cHL cell lines. CD30L is expressed on eosinophils and mast cells and these cells can enhance proliferation of cHL cell lines. CD30 over expression as observed in HRS cells can induce NF-κB activation by itself, independent of CD30L. CD40, another member of the TNFRSF (TNFRSF8), is also highly expressed on HRS cells and cHL cell lines. Triggering of CD40 results in activation of the NF-κB pathway in HL via proteolysis of TRAF3. The CD40 ligand (CD40L) is mainly expressed on CD4+ T-cells that are present in the close vicinity of the HRS cells. CD40 stimulation enhances colony formation of cHL cell lines and this effect is enhanced by IL-9 in some cHL cell lines. In EBV+ cHL cases the EBV derived LMP1
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acts as a constitutively activated CD40 receptor\(^{21}\).

Tumor-promoting effects of cells that are present in the reactive infiltrate can be enhanced by certain cytokines. CD30L expression by eosinophils increases in response to IL-5 and GM-CSF produced by the HRS cells\(^{13}\). Stimulation with CD30L and CD40L induces secretion of several cytokines, including IL-6, IL-8 (only with CD40L), TNF and LT-a and it also induces expression of ICAM-1 (CD54)\(^{17,22}\). IL-10 derived from tumor cells and T-cells enhances membrane expression of CD40L on T-cells\(^{20}\). Thus IL-10 enhances the pro-survival CD40-CD40L signaling pathway in HL. In addition to CD30 and CD40, the HRS cells also express several other members of the TNFRSF family, i.e. receptor activator of NF-κB (RANK, TNFRSF11A), CD27 (TNRSF7), FAS (CD95, TNFRSF6), CD120a and CD120b (TNFR type I and II, TNFRSF1A and 1B), CD137 (4-1BB, TNFRSF9)\(^{23,27}\). However, the functional relevance of these receptors for survival of HRS cells has not been studied in detail.

2.2 Tyrosine kinase family members

TKs and receptor tyrosine kinases (RTKs) are important regulators of inter- and intra-cellular signaling and regulate cellular processes such as proliferation, differentiation and survival. CHL cell lines aberrantly express certain RTKs as compared to normal B-cells and B-cell non-Hodgkin lymphoma. HRS cells express PDGFRA in HRS cells in 75% of the patients, whereas DDR2, EPHB1, RON, TRKB and TRKA are expressed in HRS cells in at least 30% of the patients\(^{28}\). These RTKs are activated in HRS cells and can be detected as phosphorylated forms in cHL tissue. RTK activation is probably induced by binding of ligands, since there are no activating mutations in the RTKs in cHL cell lines. Collagen type 1 (ligand of DDR2) and Nerve growth factor (NGF; ligand of TRKA) are expressed by infiltrating reactive cells indicating a possible paracrine activation, whereas PDGFA (ligand of PDGFRA) is expressed by the tumor cells indicating a possible autocrine activation\(^{28}\). EphrinB1 (ligand of EPHB) is also expressed by the HRS cells, but autocrine signaling is unlikely since the receptor and its ligand are both membrane bound. The receptor for hepatocyte growth factor (HGF), c-Met, is a RTK that is expressed by HRS cells in the majority of the cHL patients\(^{29,30}\). Expression of HGF by CD21+ dendritic reticulum cells and in 20% of the patients also by the HRS cells indicates both paracrine and autocrine activation of c-Met+ HRS cells\(^{29,30}\). Inhibition of c-Met suppresses cell growth by blocking the cells in the G2/M phase. Thus, c-Met acts as an oncogene, providing growth advantage for the HRS cells\(^{29,30}\). Activation of
RTKs results in activation of signal transduction pathways, such as the MAPK pathway resulting in pro-survival signals for the HRS cells.

### 2.3 Notch 1

In contrast to several other B-cell non-Hodgkin lymphomas, HRS cells show a strong expression of Notch1. Inhibition of Notch1 activity reduces cell viability and enhances apoptosis in cHL cell lines via downregulation of the NF-κB transcriptional activity. Stimulation of Notch1 via Jagged1, has a strong proliferative effect on cHL cells and reduces the number of apoptotic cells. Jagged1 expression is present on HRS cells and on smooth muscle cells and epithelial cells, two cell types that are not common in the cHL microenvironment.

### 2.4 Cytokine receptors

Another signaling pathway frequently activated in cHL is the JAK/STAT pathway. This pathway can be activated via somatic mutations of SOCS1 and via stimulation of the cytokine receptors expressed on HRS cells. The IL-3R is highly expressed on HRS cells in the vast majority of cHL patients and cell lines. Exogenous IL-3 promotes growth of cHL cells in a dose-dependent manner, and also rescues cells from apoptosis. Co-stimulation with IL-9 can further enhance the proliferative effect of IL-3. Multiple cells present in the microenvironment of cHL produce IL-3 and as such presents a paracrine growth factor for the HRS cells. IL-9 is produced by HRS cells, which also express the receptor IL-9R. Addition of IL-9 increases cell growth in the KM-H2 HL cell line that produces low amounts of endogenous IL-9. Inhibition of IL-9 function or production reduces growth in HDLM-2 cells that produce large amounts of endogenous IL-9. So, IL-9 can act as an autocrine growth factor in HL. IL-13 and IL-13R are both expressed in HRS cells and in cHL cell lines. Treatment of cHL cells with a neutralizing antibody to IL-13 results in a dose-dependent inhibition of proliferation indicating that IL-13 acts as an autocrine growth factor in cHL. Co-expression of IL-7 and its receptor IL-7R is found on HRS cells in a high proportion of the cHL cases. Inhibition of IL-7 inhibits clonogenic growth while addition of IL-7 increases clonogenic growth in HL cell lines. IL-7 is thus another potential autocrine growth factor in HL. HRS cells express the CC-chemokine receptor 5 (CCR5), which is one of the receptors of CC-chemokine ligand 5 (CCL5, Rantes). Treatment of cHL cells with neutralizing anti-CCL5 antibody inhibits proliferation, whereas treatment with recombinant CCL5 increases their clono-
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...genic growth. T-cells present in the microenvironment of HRS cells produce CCL5, indicating a paracrine activation mechanism.

In conclusion, both autocrine and paracrine signaling are proposed in HRS cell activation, survival and proliferation. However, it has to be kept in mind that many of these tumor cell driving mechanisms rely on the use of cHL cell lines, which may introduce a bias toward autocrine stimulation mechanisms as opposed to the more likely paracrine and thus microenvironment dependent mechanisms.

3 Shaping the microenvironment

cHL arises in lymph nodes, but at the time of diagnosis the lymph node architecture and the microenvironment of the HRS cells has altered completely. The vast majority of reactive cells are CD4+ memory T-cells. These cells express a range of activation markers, have a transcription factor profile that fits Th2 and Treg cells and express cytokines that match to anergic Th2 and Treg cells. To create this specific environment HRS cells apply multiple mechanisms that can be divided into those that alter the composition of the reactive infiltrate and those that modulate the nature or effectiveness of the cells that are present (Figure 2.3).

3.1 Chemokines produced by the HRS cells

HRS cells aberrantly express several chemokines and these chemokines represent a mechanism to shape the environment. CCL17 (TARC) is expressed at extremely high levels by HRS cells in a high percentage of patients (85-91%) \(^\text{45,47}\). CCL17 attracts Th2 cells and Treg cells by binding to their CCR4 receptor and is the main driver in the formation of the intensive reactive infiltrate. CCL22 (MDC), the other chemokine that attracts CCR4 positive cells, is also expressed by HRS cells \(^\text{48}\), but at lower levels and with less specificity as it is also found in NLPHL and other B-cell non-Hodgkin lymphomas \(^\text{49}\). Consistent with the known function of CCL17 and CCL22, cHL cell lines and their culture supernatants are able to attract CCR4 positive T-cells including CD25+/FoxP3+ Treg cells. These cells have immunosuppressive potential as they can suppress the activation of effector CD4+ T-cells \(^\text{50}\). CCL20 (MIP-3a) expressed by HRS cells also recruits Tregs and its expression can be induced by EBNA-1 and IL-21 \(^\text{51,52}\). Gene expression profiling confirms higher CCL20 mRNA levels in EBV+ primary HRS cells in comparison to EBV-HRS cells \(^\text{51}\). Nevertheless, there is no difference in Treg cell numbers in EBV+ as...
Figure 2.3: A schematic representation of the factors that determine the composition of the cHL microenvironment. The picture includes chemokines produced by HRS cells and the infiltrating cells that contribute to the composition of the microenvironment. In addition, factors known to modulate the nature or the effectiveness of the infiltrate are depicted. Gal-1, Galectin-1; CTL, cytotoxic T-cell; NK, natural killer cell.

compared to EBV- cHL cases, due to high CCL17 and CCL22 levels in all cHL cases irrespective of EBV status\textsuperscript{53,54}. CHL cell lines and HRS cells also produce chemokines, such as CXC chemokine ligand 9 (CXCL9, MIG) and CXCL10 (IP10)\textsuperscript{49,55,56}, that can attract CXCR3+ activated T-cells and NK cells\textsuperscript{52,57,58}. CXCL9 and CXCL10 are IFN-γ inducible chemokines, and production of IFN-γ by T-cells present in the cHL microenvironment can potentially enhance their production. CXCL9 and CXCL10 expression is associated with EBV+ cHL\textsuperscript{58} and more CD8+ T-cells and NK cells are indeed detected in EBV+ cHL\textsuperscript{59}. Although CXCL9 and CXCL10 supposedly support an anti-tumor immune response, this may be counteracted by the concomitant expression of multiple chemokines that attract high numbers of Th2 and Treg cells. CCL28 (MEC) is weakly expressed by
HRS cells and leads to recruitment of eosinophils, T-cells and plasma cells into cHL affected lymph nodes\textsuperscript{60}.

### 3.2 Chemokines produced by the microenvironment

In addition to the chemokines produced by HRS cells, chemokines produced by the microenvironment may also contribute to the composition of the infiltrate. CCL5 expressed in T-cells surrounding the tumor cells\textsuperscript{40} is a chemoattractant for monocytes, T lymphocytes, eosinophils, basophils and mast cells. CCL5 producing cHL cell lines indeed attract mast cells, CD4+ T-cells and eosinophils\textsuperscript{39,61,62}. Fibroblasts co-cultured with cHL cell lines are stimulated by tumor cell derived TNF-a to produce CCL11 (Eotaxin), which results in the attraction of CCR3+ Th2 cells\textsuperscript{63} and eosinophils\textsuperscript{64}. CXCL8 (IL-8) is mainly produced by macrophages, neutrophils, and mesenchymal cells in the cHL microenvironment, especially in the nodular sclerosis subtype and attracts neutrophils\textsuperscript{65}. An important feature of the infiltrating Th2 cells that directly surround the HRS cells, is their lack of CD26 expression\textsuperscript{41}, an enzyme that can proteolytically process and inactivate several chemokines like CCL5, CCL11 and CCL22\textsuperscript{66}.

### 3.3 Cytokines

A second group of molecules that contribute to shaping of the environment is the cytokine family. M2 macrophages dominate the macrophage population in the cHL microenvironment\textsuperscript{67} and these cells are induced by macrophage migration inhibitory factor (MIF) produced by the HRS cells\textsuperscript{68,69}. In mice MIF can increase the generation of Treg cells from unstimulated T-cells by downmodulation of IL-2 production\textsuperscript{70}. IL-10 secreting Treg cells (Tr-1 cells) are induced by both LMP1 and Galectin-1 produced by the HRS cells\textsuperscript{54,71}. Consistent with these findings, co-culture of an irradiated cHL cell line with naïve CD4+ T-cells results in differentiation toward IL-10 producing CD25+FoxP3+ Treg cells and to a lesser extent CD4+ cytotoxic T-cells\textsuperscript{72}. Moreover, IL-7 production by HRS cells induces proliferation of Treg cells\textsuperscript{38}. Thus, HRS cells apply multiple mechanisms to induce and enhance the number of Tr-1 and Treg cells in the microenvironment. A Th2 cell differentiation can be induced via IL-13, which is secreted by HRS cells\textsuperscript{35} and by T-cells. Eosinophils are attracted to the HL microenvironment by IL-5 and IL-9 produced by the HRS cells\textsuperscript{73,74}. IL-9 also contributes to the proliferation and survival of eosinophils and is involved in mast cell differentiation\textsuperscript{74}. Mast cells promote growth of HRS cells by the production of pro-angiogenic factors and they also induce fibrosis\textsuperscript{75}.  

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In conclusion, HRS cells modulate their microenvironment by actively attracting certain cell types and influencing the differentiation of other cells in such a way that it is favorable for HRS cell survival.

4 Immune escape mechanisms

Despite actively shaping the environment, HRS cells intuitively also need to apply mechanisms to escape from both antigen-dependent and innate immune responses (Figure 2.4). Escape from effective anti-tumor responses seems important especially in EBV+ cHL cases. The latency type II infection pattern observed in EBV+ cases, includes expression of LMP1, LMP2 and EBNA-1. LMP1 and LMP2 derived antigenic peptides can induce CTL responses in healthy individuals, albeit at much lower frequencies than the more immune-dominant peptides derived from EBNA-3A, EBNA-3B, EBNA-3C and the lytic proteins that are not expressed in HRS cells. In cHL, EBV-specific CTL can be detected in blood and biopsy samples of both EBV- and EBV+ cHL patients. In EBV-cHL, expression of the tumor-specific protein MAGE-A4 is expressed in a proportion of the cases. Moreover, cytotoxic T-cell responses recognizing MAGE-A4 expressed by HRS cells can be induced.

4.1 HLA molecules

Loss of HLA class I and II expression is frequently observed in cHL and presents a way to avoid recognition by the immune system. This loss of HLA is most prominent in EBV-cHL patients. The tumor cells in EBV+ cHL generally have normal or even elevated HLA class I expression levels. Besides being positive for HLA, EBV+ cases also have increased numbers of cytotoxic CD8+ and Granzyme B positive T-cells. These cells are usually not observed in close vicinity of the HRS cells, limiting their potential effectiveness. It remains enigmatic why HLA loss is most prominent in EBV- cHL patients and less pronounced in EBV+ cHL. This suggests that EBV derived antigenic peptides are not effectively presented, whereas other antigenic peptides present in EBV- cHL are effectively presented and require HLA downregulation. Around 15% of the cHL patients have translocations involving the MHC class II transactivator (CIITA) gene locus and as a consequence of these translocations HLA class II expression is downregulated, but probably not absent. The mechanism of complete HLA loss has not been studied in cHL, but most likely includes both genetic and epigenetic mechanisms.
The risk of getting EBV+ cHL is associated with genetic variants in the HLA class I region\textsuperscript{82}. More specifically, HLA-A*01 is a risk and HLA-A*02 a protective HLA-A type for the development of EBV+ cHL\textsuperscript{83,84}. It has been shown that HLA-A*01 has a low affinity and HLA-A*02 has a high affinity for LMP1 and LMP2 derived antigenic peptides\textsuperscript{85}. Together, these data indicate that lack of HLA-A*02 contributes to the immune escape of EBV+ HRS cells despite normal or even elevated HLA class I expression levels.

A potential threat for HLA class I negative HRS cells is recognition and killing by NK or CTL mediated responses. Expression of both HLA-G and HLA-E can protect HLA class I negative cells from NK and CTL cell mediated cytotoxicity via binding to
inhibitory receptors expressed by NK and CTL cells. HLA-G is expressed on HRS cells in more than 50% of the patients, and is associated with lack of HLA class I expression and EBV- tumor cell status. HLA-E is expressed on the vast majority of HRS cells in more than 50% of the cHL patients, but it is unclear if HLA-E expression is related to presence of EBV or downregulation of the expression of the classical HLA class I genes. Expression of both HLA-E and HLA-G might thus contribute to the escape of an anti-tumor response that is a potential threat for HLA class I negative HRS cells.

4.2 NKG2D ligands

Low levels of the membranous NKG2D-ligands, i.e. MHC class I related chain-A (MIC-A) and UL16 binding protein 3 (ULBP3), on the HRS cells presents another way to escape from cytotoxic T-cell responses. These low levels are the result of proteolytic cleavage of the NKG2D-ligands by ERp5 and ADAM10 produced by HRS cells and mesenchymal stromal cells. In addition, T-cells in cHL tissue have lower NKG2D receptor expression levels as compared to T-cells in normal lymph nodes. This is caused by TGF-β produced by the mesenchymal stromal and HRS cells, which blocks the IL-15-induced expression of NKG2D receptor on cytotoxic T-cells. Thus, the anti-tumor activity of CD8+ T-cells is blocked by lack of membrane NKG2D-ligands, release of soluble NKG2D-ligands and by reduced NKG2D receptor levels on effector T-cells.

4.3 Tumor necrosis factor receptor superfamily

Two of the TNFRSF members expressed on HRS cells, have a potential function in immune suppression by targeting T-cells. FAS and its ligand FASL are expressed in a high percentage of cHL cases. Expression of FAS could make the HRS cells sensitive to apoptosis, but HRS cells escape from this apoptosis pathway via expression of FLICE inhibitory protein (cFLIP). On the other hand, FASL expressed on the HRS cells can drive activated Th1 and CD8+ T-cells in apoptosis. CD137 (4-1BB, TNFRSF9) and CD137L expression are observed on the tumor cells in the majority of the cHL cases. Co-culture of HRS cells with pre-activated T-cells reduces production of IFN-γ. This effect is most pronounced in CD137low and CD137Lhigh HRS cells. Inhibition of CD137 resulted in a significant upregulation of CD137L indicating a direct regulatory effect by CD137. Moreover, co-culture of wild type HRS cells treated with neutralizing anti-CD137 antibodies with pre-activated T-cells, enhances the CD137L levels on HRS cells.
and induces higher IFN-γ production by the T-cells. Thus low CD137L levels promote escape from immune surveillance by reducing T-cell co-stimulation.

4.4 PD-1 and PD-1-ligand

Another mechanism to escape effective anti-tumor responses is achieved by T-cell exhaustion via continuous stimulation of the programmed death-1 (PD-1) receptor expressed on T-cells by PD-1 ligand (PD-1L) expressed on HRS cells. Functionality of this pathway has been demonstrated by increased IFN-γ production upon inhibition of the PD-1L signaling pathway. Amplification of the 9p24 locus, containing the PD-1L 1 and 2 gene loci, is common in nodular sclerosis cHL and HRS cells in this cHL subtype show a higher PD-1L expression level. The translocation partner of the CIITA chromosomal translocations is the PD-1L gene locus in some cases and these translocations also result in enhanced PD-L1 and PD-L2 expression. The number of PD-1 positive T-cells in cHL tissue samples is low in nodular sclerosis subtype and 9p24 amplification positive cases. Together these studies indicate that enhanced expression of PD-1L is a potential immune escape mechanism in cHL. The low numbers of PD-1 positive cells observed in nodular sclerosis cHL characterized by high PD-1L levels, might indicate an effective apoptosis induction.

4.5 Galectin-1

Galectin-1 is highly expressed in HRS cells in more than 50% of the cHL patients and also in cHL derived cell lines. High expression of Galectin-1 in HRS cells is correlated with a low number of CD8+ T-cells in cHL tissues and with impaired LMP1 and LMP2 specific T-cell responses. T-cells co-cultured with Galectin-1 positive cHL cell lines are skewed toward a Th2 phenotype. Upon downmodulation of Galectin-1, co-culture experiments show a restoration of the Th1/Th2 balance.

4.6 Cytokines

The Th1-polarizing cytokine IL-12 is produced by T-cells surrounding the neoplastic cells in EBV+ cHL. However, its functionality is hampered due to expression of an EBV-induced cytokine called EBI3 by the HRS cells that can antagonize IL-12 effects and thereby block the development of an effective Th1 immune response.

By producing large amounts of immune suppressive cytokines, such as IL-10 and TGF-β, HRS cells protect themselves from being attacked by cytotoxic T-cells. TGF-β...
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β mRNA was found predominantly in HRS cells of the nodular sclerosis subtype of cHL\textsuperscript{100,101}. The L428 cell line produces a high molecular weight active form of TGF-β\textsuperscript{102} and this same active form is also found in the urine of patients with active nodular sclerosis cHL\textsuperscript{103}. CD4+ T-cells in cHL indeed show a genomic TGF-β fingerprint, comparable to a fingerprint established in vitro in which T-cells treated with TGF-β show inhibition of proliferation and IFN-γ production\textsuperscript{104}. Both the percentage of IL-10 positive cases and the percentage of IL-10 positive HRS cells are higher in EBV+ cHL patients as compared to EBV- cHL patients\textsuperscript{105}. Moreover, also the percentage of IL-10 producing lymphocytes is higher in EBV+ cases\textsuperscript{106}. Immunosuppressive regulatory T-cells, both Treg and IL-10 producing Tr-1 cells, are abundant in the reactive infiltrate and contribute to the immune suppressive environment\textsuperscript{107}. Together these studies support a role of both IL-10 and TGF-β in suppressing cytotoxic anti-tumor cell responses. In addition to the production of immunosuppressive cytokines, HRS cells also actively attract Th2 and Treg cells. These cells form a physical layer between the HRS cells and cytotoxic cells in the microenvironment and thereby prevent targeting of the HRS cells.

In conclusion, the immune escape and immune suppressive mechanisms that the HRS cells employ are complex and multifaceted and seem to be redundant in providing immune escape strategies for the HRS cells.

5 Concluding remarks

cHL is a malignancy at the crossroads of oncology and immunology. The characteristic tumor cells have gone a long way from small B-cell to the very large atypical and deregulated HRS cells. Because HL arises in a lymph node, the tumor cells and their precursors have to orchestrate the nature and effectivity of the immune cells constantly. This is not only by escaping from anti-tumor immune responses, but also via their critical dependence on the microenvironment for survival. In the development of cHL the tumor cells and the environment have to develop simultaneously and probably the tumor cells acquire tumor-promoting mechanisms in a step-wise manner. It is therefore not surprising that the cross talk between the tumor cells and the reactive infiltrate is very complex and that multiple mechanisms exist. Many of these mechanisms are redundant and therefore specific mechanisms can usually only be found in a subset of cases, making cHL a very heterogeneous disease. Based on current knowledge it is evident that the microenvironment in cHL is actively shaped and an essential component of the tumor. There is still much to learn about the importance of the microenvironment in malignancies in gen-
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eral and cHL stresses the possible extent of this importance by its highly characteristic phenotype with less than 1% of tumor cells.
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