Epidemiology and prognosis in classical Hodgkin lymphoma

PETER HOLLANDER
Abstract

Classical Hodgkin lymphoma (HL) is a B cell derived neoplasm with an overall good prognosis. Its etiology and pathogenesis are largely unknown. The tumor microenvironment consists of sparse malignant cells and abundant leukocytes. In paper I we found that patients with rheumatoid arthritis (RA) had an increased risk of developing HL, especially patients with proxies of more severe RA. In addition, patients with RA had an especially increased risk of developing Epstein-Barr virus positive HL. These findings indicate that patients exposed to chronic inflammation have an increased risk of developing HL. We further studied the inflammatory milieu in the microenvironment of HL in paper II by investigating different leukocytes with immunohistochemical markers on diagnostic HL biopsies. We demonstrated that an anergic immune signature with a high amount of immune suppressive regulatory T lymphocytes was associated with inferior time to progression in an age-adjusted analysis. Another mechanism utilized by malignant cells and leukocytes to induce a suppressed antitumor immune response is to upregulate expression of programmed death ligands 1 and 2 (PD-L1 and PD-L2), that induces apoptosis in tumor killing leukocytes by binding to programmed death receptor 1 (PD-1). In paper III, we investigated the prognostic impact of PD-1, PD-L1 and PD-L2 in the tumor microenvironment of diagnostic HL biopsies with immunohistochemistry. We found that high proportions of PD-1+ and PD-L1+ leukocytes were associated with worse outcome in fully adjusted multivariate analyses. However, both PD-1 and PD-L1 are expressed to variable degrees in malignancies. Therefore, in paper IV we wanted to determine how expression of PD-1 and PD-L1 changes in repeated biopsies from both untreated and treated patients with relapsed HL. There were increased proportions of PD-1+ and PD-L1+ leukocytes, and PD-L1+ tumor cells in the relapse biopsies compared to the primary biopsies. These findings indicate that the PD-1 pathway is upregulated due to primary treatment, longer disease duration or altered conditions in the microenvironment at relapse.

Keywords: Epstein-Barr virus, rheumatoid arthritis, tumor microenvironment, immunohistochemistry, immune checkpoints, regulatory T lymphocytes, PD-1, PD-L1

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Dedicated to my family and friends
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*Both authors contributed equally to the work

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### Abbreviations

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</thead>
<tbody>
<tr>
<td>ABVD</td>
<td>Doxorubicine, Bleomycin, Vinblastine, Dacarbazine</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>Allo-SCT</td>
<td>Allogeneic Stem Cell Transplantation</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>ASCT</td>
<td>Autologous Stem Cell Transplantation</td>
</tr>
<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
</tr>
<tr>
<td>BEACOPP</td>
<td>Bleomycin, Etoposide, Doxorubicine, Cyclophosphamide, Vincristine, Procarbazine, Prednisolone</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric Antigen Receptor</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine Receptor type 5</td>
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<tr>
<td>CCL</td>
<td>Chemokine Ligand</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CDL</td>
<td>Cluster of Differentiation Ligand</td>
</tr>
<tr>
<td>CHOP</td>
<td>Cyclophosphamide, Hydroxydaunorubicin, Oncovin, Prednisolone</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
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<tr>
<td>CR</td>
<td>Complete Remission</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal Carcinoma</td>
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<tr>
<td>CT</td>
<td>Computer Tomography</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T Lymphocyte Associated protein 4</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-Free Survival</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DDR1</td>
<td>Discoidin Domain Receptor 1</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse Large B Cell Lymphoma</td>
</tr>
<tr>
<td>DSS</td>
<td>Disease-Specific Survival</td>
</tr>
<tr>
<td>EBER</td>
<td>Epstein-Barr Virus-Encoded small RNA</td>
</tr>
<tr>
<td>EBNA1</td>
<td>Epstein-Barr Virus Nuclear Antigen 1</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EFS</td>
<td>Event-Free Survival</td>
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<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PD-L</td>
<td>Programmed Death Ligand</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RS</td>
<td>Reed-Sternberg</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
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<tr>
<td>SCALE</td>
<td>SCAndinavian Lymphoma Etiology</td>
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<tr>
<td>SCT</td>
<td>Stem Cell Transplantation</td>
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<tr>
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<td>T Cell Receptor</td>
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<tr>
<td>Teff</td>
<td>T effector cells</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
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<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIGIT</td>
<td>T cell Immunoglobulin and ITIM domain</td>
</tr>
<tr>
<td>TIM-3</td>
<td>T cell Immunoglobulin 3</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, lymph Node, Metastasis</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T lymphocytes</td>
</tr>
<tr>
<td>TTP</td>
<td>Time To Progression</td>
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<tr>
<td>WBC</td>
<td>White Blood Cell</td>
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</table>
Introduction

Hodgkin lymphoma (HL) is a malignant neoplasm with approximately 160 new cases each year in Sweden and an overall good prognosis\(^1\). HL is named after the British physician Thomas Hodgkin, who first described the disease in 1832 in his paper “On some morbid appearances of the absorbent glands and spleen”\(^2\). The malignant Reed-Sternberg (RS) cells in HL are named after the American pathologist and pediatrician Dorothy Reed, and the Austrian pathologist Carl Sternberg, who independently conducted histopathological studies of the disease\(^3\). In 1994, HL was proved to be derived from B cells\(^4\). Although many factors that influence the risk of developing HL have been identified, the etiology is highly multifactorial and still largely unknown\(^2\). With more understanding of the etiology and initiating pathogenic mechanisms of HL, patients with high risk of developing HL might be more easily identified. Even though the overall prognosis is good, a proportion of the patients will suffer treatment failure and eventually succumb to the disease, despite several established risk stratification tools\(^1\). The establishment of more accurate prognostic and predictive markers would enable patients to receive more optimized treatment, with low risk of relapse and death due to HL, and few side effects due to treatment.

This thesis will focus on diseases that influence the risk of developing HL, and how the composition of the tumor microenvironment affects the prognosis in HL.

Epidemiology

HL has a bimodal age incidence in the western world. The disease is rare in children, with a rapid increase in teenagers and a peak at 25 years of age. The incidence decreases in middle life but starts to increase again at 50 years of age\(^2\). Proposedly, the etiology for HL in young patients is immunologic immaturity as compared to immune dysregulation in older patients\(^2,5\). The risk of developing HL among children and young adults is associated with environmental factors such as infection with Epstein-Barr virus (EBV)\(^2\).
**Epstein-Barr virus**

Infection with EBV is the most firmly established risk factor for developing HL\(^2\). Approximately one third of patients in the western world with HL harbor EBV in their tumor cells, and it is the probable driving cause of HL in these cases\(^6\). EBV is a lymphotrophic virus of the gamma herpes family and spreads mainly via saliva. Approximately 95% of adults worldwide are EBV seropositive. Infection typically results in latent infection, but a proportion develops infectious mononucleosis (IM)\(^7\). IM is associated with an elevated risk of developing HL and the average time between IM and HL diagnosis is 4 years\(^8\). Children (<14 years) and adults (>45 years) are more prone than young adults to harbor EBV positive tumor cells in HL\(^2,5\). Since only one third of the HL cases express EBV, it cannot solely explain the etiology for HL. The “hit-and-run hypothesis” proposes that EBV is the cause of all HL, but the tumor cells have lost expression of the viral proteins during the pathogenic process\(^8,9\). The risk of EBV positive HL is associated with genetic variants in the Human Leukocyte Antigen (HLA) class I region. HLA-A*01 is associated with an increased risk, and HLA-A*02 is associated with a decreased risk of developing EBV positive HL\(^10\). In epidemiological studies, cases are often stratified into EBV positive and EBV negative HL.

**Human immunodeficiency virus**

Infection with human immunodeficiency virus (HIV) is associated with an increased risk of HL\(^11\), and in almost all patients with acquired immune deficiency syndrome (AIDS) the tumor cells are EBV infected\(^12\). This is compatible with the knowledge that immunocompromised patients are at an increased risk of developing HL, possibly due to increased susceptibility to infections\(^13\), loss of immunologic control of EBV infected B cells, and impaired T cell immunosurveillance\(^11\). Interestingly, patients with AIDS with restored cluster of differentiation 4 positive (CD4+) T cell counts due to treatment with highly active antiretroviral therapy have an increased risk of HL, while the risk of other lymphomas is decreased\(^14,15\).

**Other infections**

Individuals with delayed exposure to infectious agents as children with no or few siblings or playmates, early birth order, or raised in single-family households of high socioeconomic status had in earlier investigations an increased risk of developing HL\(^9,13,16\). Supposedly, patients not exposed to previous pathogens have an immature immune system unable to properly handle infections, and are more prone to develop HL\(^2\).
Autoimmune diseases

Autoimmune diseases comprise a heterogeneous group of chronic disorders with the common ability to produce self-reactive antibodies that results in inflammation of engaged tissues\(^{17}\). Rheumatoid arthritis (RA) is one of the most common autoimmune disease in Sweden\(^{18}\) and most often affects joints, but may also involve other organs such as heart, lungs and kidneys\(^{19}\). Patients with RA, systemic lupus erythematosus, Sjögrens syndrome, psoriasis and sarcoidosis have, in a few studies, an elevated risk of developing HL\(^{20-22}\). Other autoimmune diseases such as multiple sclerosis, diabetes mellitus type 1 and celiac disease are not associated with risk of HL\(^{21}\). It is unknown whether patients with autoimmune diseases have an equally increased risk of developing EBV positive and EBV negative HL. The mechanism of an increased risk of HL in patients with autoimmune diseases is probably multifactorial. Firstly, the autoimmune disease is associated with increased chronic inflammation that increases the probability of neoplastic transformation\(^{19,23}\). Secondly, autoimmune diseases and HL have common genetic aberrations and the association might be due to shared genetic susceptibility\(^{2,24,25}\). Thirdly, patients with autoimmune diseases are often treated with non-biologic disease modifying anti-rheumatic therapy, which might increase the risk of HL\(^{22,26}\).

Atopic diseases

The atopic diseases allergic rhinitis, atopic eczema and asthma are caused by production of immunoglobulin E (IgE) by plasma cells, specific to one particular allergen\(^{27,28}\). IgE attach to mast cells, and when exposed to the allergen, mast cells release their contents which results in inflammation\(^{29}\). Serologic analysis for specific IgE reactivity against allergens (Phadiotop test) is commonly used to diagnose IgE-mediated atopic diseases\(^{30}\). Atopic diseases are a disputed risk factor considering risk of developing HL. One study found that patients with asthma had a reduced risk of HL\(^{31}\), the same association was reported in a study on allergic rhinitis\(^{32}\). Hypothetically, a hypersensitive immune system aimed to focus on atopic conditions would contribute to improved immune surveillance and a decreased chance of permitting neoplastic cells to proliferate\(^{33}\). In another study, patients with a history of eczema had an elevated risk of developing HL\(^{34}\). Also, there are several studies that report no association between atopic diseases and risk of HL\(^{35-37}\).

Other risk factors

A familial association has pointed to a genetic susceptibility to HL, with an increased risk observed especially in identical twins and first-degree relatives\(^{38}\). Genetic predispositions for HL is also implied by patterns of inci-
idence in different ethnic groups with different HLA subtypes. Finally, cigarette smoking is associated with a slightly increased risk of developing EBV positive, but not EBV negative HL. Several other risk factors are established in HL.

Most of the factors associated with an increased risk of HL involve the immune system. Proliferation and survival of HL is highly dependent on recruitment of immune system-derived leukocytes into the tumor microenvironment, which contributes to HL distinct histopathologic morphology.

Pathology and pathogenesis

Based on the histologic morphology, classical HL is classified into nodular sclerosis (NS), mixed cellularity (MC), lymphocyte rich (LR) and lymphocyte depleted (LD) subtype. In the western world, NS is the most common and represents 2/3 of the cases, MC represents about 25%, LR represents 5% and LD constitutes <1% of the cases. All of the papers in this thesis only include patients with classical HL. The separate HL entity nodular lymphocyte-predominant HL will not be further discussed. The diagnosis of HL should preferably be made on excisional biopsies, since core needle biopsies often contain inadequate tumor material.

Hodgkin and Reed-Sternberg cells

In HL, the malignant cells are mononucleated Hodgkin and multinucleated Reed-Sternberg (HRS) cells. RS cells are large, with abundant basophilic cytoplasm and at least two nuclear lobes. The nuclei are often prominent, with an irregular nuclear membrane, and usually one nucleolus. With immunohistochemical markers, HRS cells are positive for CD30 in nearly all cases and CD15 in the majority of the cases. The B cell transcription factor paired box protein 5 (PAX5) is weakly expressed by HRS cells in 95% of the cases. EBV-harboring HRS cells are demonstrated with immunohistochemistry to detect latent membrane proteins (LMPs) or in situ hybridization to detect Epstein-Barr virus-encoded small ribonucleic acids (EBERs). EBV infection is more prevalent in MC compared to other HL subtypes.
Pathogenesis

In 1994, the cell of origin for HRS cells was demonstrated to be post germinal center (GC) B cells with clonal and somatic mutations in Ig heavy and light chains. HRS cells show a downregulation of B cell genes, and the initiating event for this reprogramming is unknown. Critical steps in the pathogenesis most likely occur in the GC, where HRS cell precursors escape from apoptosis. However, HL development is a multistep process and key transforming events might occur before the GC, with the final events occurring after the GC. Hypothetically, GC B cells that lose part of their B cell identity, i.e. through loss of their B cell receptors (BCR) are prone to escape from apoptotic stimuli and develop into HRS cells. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor with constitutive activity in HL. Favorable mutations lead to enhanced NF-κB signaling with transcription of genes that contribute to proliferation and anti-apoptotic properties of the HRS cells. NF-κB may also be activated by CD30 and CD40 expressed by HRS cells, with corresponding ligands expressed by leukocytes in the vicinity of the HRS cells.

EBV and pathogenesis

In HRS cells where EBV is present, the EBV-encoded proteins EBV nuclear antigen 1 (EBNA1), LMP1 and LMP2 are expressed. EBNA1 is essential for replication of the EBV genome, LMP1 imitates an active CD40 receptor.
and constitutively activates the NF-κB pathway, and LMP2 carries a cytoplasmic motif that resembles the signaling module of the BCR$^{51,52}$. LMP-1 upregulates discoidin domain receptor 1 (DDR1), which allows HRS cell precursors to proliferate upon interaction with collagen$^{49}$. This suggests that EBV rescues GC B cells from apoptosis, and therefore participates in the pathogenesis of HL$^{53}$.

Figure 2. Putative pathogenesis of EBV positive and EBV negative HL. Figure adapted from Carbone et al$^{49}$ with permission.

In addition, HRS cells secrete chemokines and cytokines which results in tumor progression in an autocrine manner$^{54,55}$, and recruitment of leukocytes. HRS cells and leukocytes together form the tumor microenvironment$^{40}$.

Hodgkin lymphoma microenvironment

The HL microenvironment is characterized by only containing a few percent of malignant HRS cells. The rest of the microenvironment consists of eosinophils, neutrophils, lymphocytes, plasma cells, dendritic cells (DCs), macrophages, mast cells and fibroblasts$^{40}$. Leukocytes are attracted to the microenvironment by cytokines and chemokines secreted by both HRS cells and other cells$^{56}$. Compared to other lymphomas, HL is highly dependent on the recruitment of leukocytes in order to survive, proliferate and evade antitumor immune responses$^{57}$.
Figure 3. A simplified schematic view of how HRS cells proliferate, recruit and inhibit leukocytes in the HL microenvironment.

**Innate and adaptive immune system**

The human immune system is divided into the innate and the adaptive immune system\(^5^8\). The innate immune system serves as the initial immune defense and consists of the complement system and leukocytes including macrophages, granulocytes, DCs, mast cells and natural killer (NK) cells. DCs and macrophages serve as antigen presenting cells (APCs) and present peptide fragments on the cell surface on Major Histocompatibility Complex (MHC) class I or II\(^5^9\). APCs provide an interface to the adaptive immune system with T and B cells with somatically generated, clonally expressed repertoires of antigen receptors. The adaptive immune system allows for specific antigen recognition and the mounting of a specific response against pathogens or altered cells of the host\(^6^0\).

**Eosinophils**

Besides being mediators in allergic diseases, eosinophils are also recruited to the microenvironment in neoplastic diseases\(^6^1\). Chemokine ligand 5 (CCL5), IL-5, CCL28 and granulocyte-macrophage colony stimulating factor secreted by HRS cells recruit eosinophils to the microenvironment\(^5^6,6^2,6^3\). CCL5 also stimulates the HRS cells to proliferate in an autocrine loop, by engaging
the C-C chemokine receptor type 5 (CCR5) on the HRS cells. In two earlier studies, a high amount of eosinophils was associated with an inferior prognosis in HL. Eosinophils express CD40 ligand (CD40L) and CD30L that provide the HRS cells with proliferative stimuli, which could explain the adverse outcome. However, eosinophilic cationic protein secreted by eosinophils is cytotoxic to HRS cells in HL cell lines, indicating that eosinophils may be able to kill HRS cells. Two other studies report no prognostic impact of eosinophils in the HL microenvironment. It is unknown whether eosinophils affect prognosis in a modern treatment setting.

Figure 4. HE staining with abundant eosinophils in HL.

**Mast cells**

Mast cell precursors arise from myeloid stem cells and circulate in the blood before they enter various tissues and mature into mast cells. Mast cell degranulation induces a pro-inflammatory response with increased vascular permeability and vasodilation. Mast cells may promote inflammation by inhibiting regulatory T lymphocytes (Tregs) by the binding of histamine to H1 receptors on Tregs. CCL5 secreted by HRS cells recruits mast cells to the HL microenvironment. Several previous studies report that high numbers of mast cells are associated with an inferior prognosis in HL. However, one previous study reported no association between mast cells and prognosis. The association between high numbers of mast cells and inferior prognosis is probably due to CD30L expressed by mast cells, which stimulates the HRS cells to proliferate.
Macrophages

Macrophage precursors are released into the circulation as monocytes. When they migrate into tissues they differentiate into macrophages or DCs. Macrophages display a spectrum of phenotypes and are roughly divided into pro-inflammatory M1 and anti-inflammatory M2 macrophages. With immunohistochemistry, CD68 stains both M1 and M2 macrophages, while CD163 is more specific for M2 macrophages. Macrophages defend the host from infectious pathogens by phagocytosis, and support adaptive immunity by secreting IL-12 that helps naïve T cells to differentiate into pro-inflammatory T helper lymphocytes (Th). However, macrophages may also downregulate Th cytokines, and recruit Tregs. Macrophages are recognized to be involved in tumor progression and metastasis in numerous malignancies. Macrophages are attracted by macrophage migration inhibitory factor (MIF) produced by HRS cells. Several studies report that high numbers of macrophages are associated with an adverse prognosis in HL, both when CD68 and CD163 were studied. However, numerous studies do not find an association between macrophages and outcome in HL. The adverse outcome with a high amount of macrophages is probably due to suppression of adaptive immune responses, leading to immune evasion by HRS cells and an inferior prognosis.
T cells

T cell precursors migrate into the thymus to generate a unique T cell receptor (TCR) and mature. CD4+ or CD8+ T cells move out of the thymus as naïve T cells\(^9\). Naïve CD4+ T cells are able to engage specific peptides presented by MHC II on APCs, and mature into Th cells\(^9\). The main function for Th cells is to provide stimuli to other leukocytes by releasing different cytokines\(^9\). Naïve CD8+ T cells are able to engage specific peptides presented by MHC I, and mature into cytotoxic T lymphocytes (CTL)\(^9\). Activated CD4+ and CD8+ T cells, as well as Tregs are sometimes referred to as T effector cells (Teff)\(^9\). In order to become fully activated, a second stimulatory signal is provided by CD80 or CD86 expressed by APCs to the T cell’s co-stimulatory receptor CD28\(^9\). However, the immune checkpoint receptor cytotoxic T lymphocyte associated antigen 4 (CTLA-4) is also expressed by T cells to variable degrees and binds to CD80 or CD86 with higher affinity than CD28, which leads to inhibition of T cells\(^9\). Besides CTLA-4, several other immune checkpoint pathways are able to inhibit T cells\(^9\). Galon et al studied different T cell markers and introduced the “immunoscore” in colorectal carcinoma (CRC), where high numbers of CD3+, CD8+ and memory T cells were superior predictors of outcome compared to the TNM (Tumor, lymph Node, Metastasis) classification\(^9\). In HL, none have identified a prognostic immune signature analogous to Galons “immunoscore”. T cells constitute the major component of leukocytes in the HL microenvironment, and are mostly of the Th or Treg phenotype\(^9\).
Regulatory T lymphocytes

Tregs are generated in the thymus, where expression of the transcription factor forkhead box P3 (FoxP3) is induced in immature CD4+CD8-CD25- T cells. Tregs generated in the thymus are designated as natural Tregs (nTreg). They are attracted to tissues by cytokines and chemokines, including IL-2 secreted by Th9. Tregs may also be induced from naïve T cells in peripheral tissues and are then designated as induced Tregs (iTreg). iTreg are induced by MHC peptide stimulation and cytokines such as IL-2 and Transforming Growth Factor Beta (TGF-β), and also by programmed death ligand 1 (PD-L1).

Tregs principal immunologic function is to suppress destructive immunologic responses by other leukocytes, conducted via several mechanisms:

1. Tregs express CTLA-4, Lymphocyte activation gene-3 (LAG-3) and PD-L1, which reduce activation of naïve T cells by DCs.
2. Tregs consume IL-2 that is required for activation of naïve T cells, and also directly disrupts other T cells interaction with DCs.
3. Tregs secrete IL-10 and TGF-β that results in apoptosis of CTLs and DCs, and further recruits Tregs.
4. Tregs induce cytolysis in other T cells by production of perforins and granzymes.
5. Tregs induce metabolic disruption in other T cells.

Figure 7. Different mechanisms that Tregs utilize to suppress adaptive immune responses. Figure adapted from Caridade et al. with permission.

Suppression of leukocytes by Tregs may result in a disadvantageous immune response in malignant diseases. A high amount of Tregs is associated with an inferior prognosis in several malignancies, e.g. ovarian carcinoma.
chronic lymphocytic leukemia (CLL). Tregs are recruited to the HL microenvironment by CCL5 and galectin-1 secreted by HRS cells.

Figure 8. FoxP3 staining to detect Tregs in HL.

Several studies report that high proportions of Tregs in the microenvironment of HL are associated with superior outcome. This indicates that Tregs suppress the HRS cells, or other tumor-promoting leukocytes in HL, however, evidence for such a mechanism is lacking. One previous study reported an inferior outcome in patients with a high proportion of Tregs in HL, while another study found no association between outcome and amount of Tregs. In addition, a recent study showed that Tregs often distribute in close vicinity to the HRS cells, suggesting that Tregs protect HRS cells and inhibit antitumor immune responses.

Table 1. Previous studies investigating the prognostic impact of Tregs in HL.

<table>
<thead>
<tr>
<th>Study by (year)</th>
<th>Patients included (n)</th>
<th>Patients diagnosed (year)</th>
<th>Age of patients</th>
<th>Results</th>
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<td>Schreck (2009)</td>
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<td>Unknown</td>
<td>Mean age 39</td>
<td>High Tregs: inferior DFS</td>
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<tr>
<td>Chetaille (2009)</td>
<td>146</td>
<td>Unknown</td>
<td>Unknown</td>
<td>High Tregs: superior EFS</td>
</tr>
</tbody>
</table>
Koreishi (2010) 111
Greaves (2013) 62
123  1972-2005  15-80  High Tregs: superior OS and DSS

DFS=Disease-free survival (time to relapse or death from lymphoma or acute toxicity of treatment), EFS=Event-free survival (time to failure of treatment or death from any cause), OS=Overall survival (time to failure of treatment or death from any cause), DSS=Disease-specific survival (time to death from lymphoma), FFS=Failure-free survival (time to failure of treatment or death from lymphoma)

**Activated lymphocytes**

Lymphocytes, referred to as activated lymphocytes in this thesis, include CTL and NK cells. CTL are activated by interaction between their TCR and MHC I expressed by APCs, while NK cells recognize cells with a series of activating and inhibitory receptors. Activated lymphocytes induce apoptosis in their target cells either via secretion of perforins and granzymes, or via FAS-ligand interaction. Activated lymphocytes are the main leukocytes capable of killing malignant cells. Thus, a high number in the tumor microenvironment would hypothetically result in superior outcome. This has been observed in CRC and breast carcinoma. Activated lymphocytes are scarce in HL and their cytotoxic abilities are crippled via several mechanisms:

1. Activated lymphocytes are suppressed by Tregs.
2. HRS cells secrete IL-10, TGF-β and galectin-1 that inhibits activation and function of activated lymphocytes.
3. HRS cells are able to evade FAS-ligand induced apoptosis by activated lymphocytes by expressing inhibitory proteins.
4. CD4+ T cells form a physical barrier around HRS cells, preventing activated lymphocytes from mediating their functions.
5. HRS cells downregulate MHC class I to avoid CTL.

Studies have reported that a high amount of activated lymphocytes in HL is associated with an inferior prognosis. However, no impact on outcome by the amount of activated lymphocytes is also shown. Due to the exhausted state of the T cells, CTL might paradoxically provide survival factors to the HRS cells and/or the microenvironment. However, the mechanism for this proposition has not been substantiated. In addition, activated lymphocytes are inhibited via immune checkpoint pathways such as the programmed death receptor 1 (PD-1) pathway, further discussed below.
Immune checkpoint pathways

There are numerous different immune checkpoint pathways that are able to downregulate the antitumor immune response in malignancies\textsuperscript{126}. The PD-1, CTLA-4 and LAG-3 pathways were mentioned in the previous section. The immune checkpoint pathway of most interest for this thesis is the PD-1 pathway, which will be discussed in detail. A few additional immune checkpoint pathways of potential future clinical interest will be briefly presented.

PD-1

PD-1 is a member of the CD28/CTLA-4 family of receptors and is expressed on activated CD4+ and CD8+ T cells, NK cells, B cells, macrophages, and DCs\textsuperscript{100}. PD-1 is also upregulated due to cytokine stimulation via IL-2, IL-7, IL-15, IL-21 and interferon β\textsuperscript{127,128}, and due to persistent antigen exposure\textsuperscript{128}. Activation of PD-1 inhibits cell proliferation and survival, cytokine production and protein synthesis\textsuperscript{59}. However, activation of PD-1 in Tregs induces development and enhanced suppressive activity\textsuperscript{100}. In addition, activation of PD-1 induces naïve T cells into iTreg, and may even convert Teff into iTreg, leading to expansion of Tregs\textsuperscript{100}. PD-1 is highly expressed by follicular helper T cells, which probably provides inhibitory signals to prevent an excess of T cells in the GCs\textsuperscript{129}. Macrophages with the M2 phenotype mostly express PD-1, rather than macrophages with the M1 phenotype\textsuperscript{130}. 

Figure 9. Granzyme B staining to detect activated lymphocytes in HL.
PD-1 ligands

PD-1 has 2 ligands; PD-L1 is expressed by CD4+ and CD8+ T cells, NK cells, B cells, macrophages, DCs and mast cells, and also by non-hematopoietic cells; PD-L2 is more restricted and only expressed by macrophages, DCs and epithelial cells. In malignant diseases, PD-1 ligands become upregulated on leukocytes in the tumor microenvironment by various cytokines, e.g. interferon γ, IL-10, and tumor necrosis factor α, produced by tumor cells (e.g. HRS cells) and other leukocytes such as T and NK cells. The PD-1 pathway inhibits self-reactive T cells and protects from tissue damage induced by autoreactive T cells in autoimmune diseases. However, expression of PD-1 ligands by malignant cells and leukocytes also inhibits antitumor immune responses by interaction with cells that express PD-1.

Other immune checkpoint pathways

LAG-3, T cell immunoglobulin-3 (TIM-3), and T cell immunoglobulin and ITIM domain (TIGIT) are three other immune checkpoint pathways. Like the PD-1 pathway, they all inhibit CD4+ T cells, CD8+ T cells and NK cells, promote Tregs function and proliferation, and their ligands are expressed by APCs and malignant cells. Inhibition of these pathways suppresses Tregs and stimulates other T cells to proliferate. These immune checkpoint pathways perform similar immunological functions to maintain immunologic tolerance and downregulate ineffective or destructive immune responses. Anergic and exhausted T cells are often used synonymously, but there are distinct differences between the terms. T cell anergy is an induced hyporesponsive state in naïve T cells with low IL-2 production or incomplete activation due to low CD28 expression and/or high activity of PD-1, LAG-3 or CTLA-4. Exhausted T cells are activated T cells with decreased cytokine expression, resistance to reactivation, and expression of multiple inhibitory receptors, e.g. PD-1, TIM-3, LAG-3 and TIGIT. Apparently, the immune system has a functional excess to ensure homeostasis and self-tolerance in the event of other pathways being compromised. Anergic and exhausted T cells coexist in the tumor microenvironment in malignancies.
PD-1 in Hodgkin lymphoma

PD-1 is infrequently expressed by leukocytes in HL\textsuperscript{132}. The HRS cells frequently have copy number alterations involving chromosome 9p24.1, resulting in expression of PD-1 ligands\textsuperscript{132}. A large proportion of HL cases have increased expressions of PD-L1 and PD-L2 on the HRS cells and leukocytes\textsuperscript{137-139}. Macrophages constitute the majority of the PD-L1 expressing cells in the HL microenvironment\textsuperscript{132}. PD-L1+ HRS cells more often have PD-L1+ rather than PD-L1- macrophages in their close vicinity. Also, PD-L1+ T cells are more likely to be localized in close proximity to PD-L1+ macrophages. Thus, PD-L1+ macrophages surround HRS cells and inhibit T cells and enhance the already crippled antitumor immune response in HL\textsuperscript{132}.

Figure 10. Comparison between anergic and exhausted T cells.
Figure 11. Interaction between Tregs and activated lymphocytes with APCs and HRS cells via the PD-1 pathway in HL.

**Prognostic implications of PD-1 in Hodgkin lymphoma**

Several studies have investigated the prognostic significance of the PD-1 pathway in HL. A high proportion of PD-1+ leukocytes in the microenvironment was associated with an inferior OS in most studies\textsuperscript{123,140,141}. No study has reported an association between PD-1+ leukocytes and survival outcomes involving HL relapse and progression. There are also studies that report no prognostic impact of PD-1+ leukocytes in HL\textsuperscript{110,142}. One study found that 9p24.1 gene amplifications in the HRS cells were associated with increased expression of PD-1 ligands and an inferior prognosis\textsuperscript{143}. However, most studies that used immunohistochemical markers to determine the prognostic impact of PD-L\textsubscript{1}\textsuperscript{141,144} and PD-L\textsubscript{2}\textsuperscript{141} on HRS cells found no association with prognosis. No previous study has reported that PD-L1+ leukocytes are associated with outcome in HL.
Figure 12. Immunohistochemical staining with PD-1+ (brown) leukocytes to the left, and double staining with PD-L1 (brown) and PAX5 (red) to the right in HL. PD-L1 is expressed by both leukocytes (arrowheads) and HRS cells (arrows).

**PD-1 in solid malignancies**

High expressions of both PD-L1 on tumor cells and PD-1 on leukocytes are associated with inferior outcome in several solid malignancies (e.g. renal cancer, non-small cell lung cancer (NSCLC) and urothelial carcinoma)\(^{145}\), and high expression of PD-L1 on tumor cells is associated with inferior outcome in e.g. breast cancer, gastric cancer, and melanoma\(^{146}\). PD-L2 has been less studied than PD-L1, a few studies found that a high proportion of PD-L2 on tumor cells is associated with worse outcome in CRC\(^{147}\) and breast cancer\(^{148}\). Tumor cell expression of PD-L1 in NSCLC is an established predictive marker for response to PD-1 blockade\(^{149}\), while the results are more inconclusive in other malignancies\(^{149,150}\). However, there are studies where high expression of PD-L1 by both tumor cells and leukocytes correlated with favorable response to PD-1 pathway inhibiting drugs in melanoma, renal cancer, prostate cancer and CRC\(^{151,152}\). These findings suggest that not only PD-L1 expressed by tumor cells, but also PD-L1 expressed by tumor-associated leukocytes mediates suppression of antitumor T-cell responses and may be activated upon blockade of the PD-1 pathway\(^{152}\). In addition, high infiltration of PD-1+ and CD8+ T cells in the tumor microenvironment was predictive of response to PD-1 blockade in melanoma\(^{153}\), suggesting that tumors with a high infiltration of PD-1+ T cells will respond better to PD-1 blockade\(^{149,153,154}\).

**PD-1 in hematologic malignancies**

In diffuse large B cell lymphoma (DLBCL), high expression of PD-L1 on tumor cells was associated with inferior outcome\(^{155,156}\), while expression of
PD-L1 on leukocytes did not affect outcome\(^\text{155}\). Increased numbers of PD-1\(^+\) leukocytes in DLBCL were associated with superior OS in two studies\(^\text{157,158}\), while a trend for worse OS was observed in another study\(^\text{159}\). In CLL, a high proportion of PD-L1\(^+\) tumor cells and PD-1\(^+\) T cells was associated with poor prognosis\(^\text{160}\). Evidence that PD-1 and/or PD-L1 are suitable as predictive markers in lymphomas is lacking\(^\text{161}\). When evaluating the PD-1 pathway markers, one should be aware that these markers might change due to longer disease duration and following anti-cancer treatment.

**PD-1 in paired tumor samples**

Leukocytes are induced to express PD-1 and PD-1 ligands in the microenvironment of malignancies due to the cytokine milieu\(^\text{127,133}\), this suggests that expression of PD-1 and PD-L1 may vary depending on whether the biopsy is taken in an early or a later stage of the disease in HL. There is a limited number of studies that have investigated to what extent expression of the PD-1 pathway changes over time in malignant tumors. In a study on paired melanoma samples, patients tended to have a higher expression of PD-L1 on tumor cells in the second sample compared to the first sample\(^\text{162}\). Another study reported that expression of PD-1 on tumor-associated macrophages accumulated over time in a mouse model on CRC, and correlated with higher disease stage in humans with CRC\(^\text{130}\). As to whether PD-1 and PD-L1 varies in repeated biopsies from patients with untreated HL, this has never been studied.

**PD-1 in paired tumor samples following treatment**

There have been a few reports on expression of the PD-1 pathway following neoadjuvant cancer treatment. In two studies on NSCLC, patients were treated with chemoradiation therapy followed by surgery, and the proportion of PD-L1\(^+\) tumor cells significantly decreased after treatment\(^\text{163,164}\). While the opposite was found in another study on patients with NSCLC and treated with neoadjuvant chemotherapy followed by surgery\(^\text{165}\). It was also shown on a CRC cell line that 5-fluoracil upregulated tumor cell expression of PD-L1\(^\text{166}\). In a mouse model, radiotherapy (RT) upregulated expression of PD-L1 on DCs and tumor cells\(^\text{167}\). A couple of studies have investigated how PD-1 and PD-L1 is expressed when primary and relapsed tumor samples are compared. In NSCLC, there was a higher proportion of PD-L1\(^+\) tumor cells in the relapsed tumor than in the primary tumor\(^\text{168}\). In multiple myeloma (MM), patients with relapse as well as persistent minimal residual disease (MRD) showed a higher proportion of PD-1\(^+\) T cells. There was also a higher expression of PD-L1 on tumor cells in patients with positive MRDs after treatment than before treatment\(^\text{169}\). In summary, expression of PD-1 and its ligands changes following anti-cancer therapy. This might be explained by
genetic alterations due to treatment, longer disease duration, and alterations of the immune microenvironment at relapse. It is unknown whether the PD-1 pathway changes in paired samples including primary and relapsed HL.

**Immune checkpoint inhibitors**

CTLA-4 was the first immune checkpoint to be medically targeted with the antibody ipilimumab, which was approved by the U.S. Food and Drug Administration (FDA) for patients with advanced melanoma in 2011\(^{170}\). Blockade of the PD-1 pathway with the monoclonal antibodies pembrolizumab and nivolumab became registered treatments for advanced melanoma\(^{171}\) in 2014 and 2015, respectively. Since then, PD-1 blockade is a registered treatment in several other solid malignancies such as renal carcinoma, NSCLC, and urothelial carcinoma\(^{126,172}\), and relapsed and refractory HL\(^{173}\). For other hematologic malignancies, PD-1 blockade is partially effective in DLBCL, follicular lymphoma and T cell lymphomas. For acute myeloid leukemia, PD-1 blockade in combination with 5-azacitidine has shown promising results\(^{174}\). However, PD-1 blockade as single therapy for MM\(^{174}\) and CLL showed limited clinical response\(^{175}\).

**Mechanism of action for immune checkpoint inhibition**

PD-1 inhibitors reactivate the cytotoxic activity of anergic and exhausted CD8+ T cells toward the malignant cells\(^{135,173}\). However in HL, the tumor-associated lymphocytes often express PD-1 to low degrees\(^{132,138}\), and MHC class I on the HRS cells is frequently downregulated\(^{176,177}\), suggesting that CD8+ T cells are not solely responsible for the cytotoxic activity toward the HRS cells. Other lymphocytes that are able to target cells independent of MHC class I might mediate cytotoxic activity towards HRS cells, such as NK cells and \(\gamma\delta\) T cells\(^{178}\). PD-1 blockade also reduces the induction of Tregs, which contributes to reactivation of the suppressed immune response\(^{179}\). In addition, PD-1 inhibitors increase the phagocytic ability of macrophages toward tumor cells\(^{130}\). Hence, PD-1 blockade reactivates the antitumor immune response by interaction with several leukocytes in the tumor microenvironment. Side effects related to PD-1 blockade are most commonly immune related, such as pneumonitis, colitis, hepatitis and hypoo- or hyperthyroidism\(^{175}\). PD-1 blocking drugs are often used as monotherapy\(^{126}\), and resistance to treatment has been observed\(^{172}\). In mouse models on NSCLC, cases that progressed on PD-1 blockade upregulated other immune checkpoints, especially TIM-3 in PD-1 antibody bound T cells, and also LAG-3 and CTLA-4. Addition of TIM-3 blockade resulted in superior prognosis in these cases\(^{172}\). Antibodies that inhibit the LAG-3, TIM-3 and TIGIT pathways are currently being investigated in clinical trials\(^{96}\).
The introduction of immune checkpoint inhibitors, particularly PD-1 inhibitors offers new treatment options in HL\textsuperscript{173}.

**Clinical aspects**

**Symptoms and staging**

HL most often manifests as lymph node enlargements in the cervical region, although any lymphoid region may be the site of engagement. About 1/3 of the patients present with at least 1 of the B-symptoms; night sweats, unexplained weight loss, and unexplained persistent or recurrent fever\textsuperscript{173}. The modified Ann Arbor staging system by Cotswold is used to stage HL\textsuperscript{173}. The original classification was developed in 1971 in Ann Arbor, Michigan\textsuperscript{180}. Stage I-IV indicates the distribution of the disease. In 1989, the Cotswold modifications added bulky tumor to the classification\textsuperscript{181}, defined as a tumor measuring $>$10 cm in diameter. A and B indicate presence (B) or absence (A) of B-symptoms\textsuperscript{182}.

**Prognostic factors**

Patients are further grouped into limited (I-IIA) and advanced stage (IIB-IV)\textsuperscript{182}. Limited stages are stratified into favorable and unfavorable limited stage\textsuperscript{183}. The International Prognostic Score (IPS) was established to risk stratify patients with advanced stages. Factors associated with poorer prognosis are age $>$45 years, male gender, stage IV, hemoglobin (Hb) $<$105 g/L, S-albumin $<$40 g/L, white blood cell count (WBC) $>$15 x $10^9$/L, and lymphocyte count $<$0.6 x $10^9$/L or $<$8% of total WBC\textsuperscript{184}. The IPS is still widely used to risk stratify patients, even though the study population that the risk factors were established on were generally treated before 1990, and treatment regimens used today have changed\textsuperscript{185}.

**Radiological examination**

To determine the extent of HL, radiological examination with positron emission tomography (PET), combined with computer tomography (CT) is used. Radioactive labeled fluorodeoxyglucose (FDG) is used as a tracer to visualize tissues with high glucose metabolism, such as HL engaged tissues\textsuperscript{186}. PET/CT is also performed after 2 courses of chemotherapy for advanced stage patients to assess early treatment response, and at the end of treatment to determine remission status\textsuperscript{186}. A positive PET/CT after 2 courses of chemotherapy is a superior prognostic indicator of adverse prognosis compared to the IPS score in patients with advanced stages of HL\textsuperscript{187}.
Prognosis

Therapy is chosen based on stage of disease, presence of B-symptoms, bulky tumor, and prognostic factors. With combined chemotherapy regimens and RT, the prognosis steadily improved during the 20th century. However, 10% of patients with limited stage, and 20-30% of patients with advanced stage will be refractory or relapse. There is a need for additional prognostic markers to identify which patients that will be refractory to primary treatment. Treatment is mostly given with curative intent, but with the mildest regime possible in order to spare patients from complications such as reduced physical performance, reduced fertility, cardiovascular disease, and secondary malignancies.

Front-line treatment

In Sweden, limited stages (I-IIA) are treated with 2-4 courses of doxorubicine, bleomycin, vinblastine, and dacarbazine (ABVD) and RT. Patients with advanced stages (IIB-IV) and <3 risk factors according to IPS are treated with 6-8 courses of ABVD, and escalated to bleomycin, etoposide, doxorubicine, cyclophosphamide, vincristine, procarbazine, and prednisolone (BEACOPPescalated) if the patient is PET/CT positive after 2 courses. In advanced stages with ≥3 risk factors according to IPS, BEACOPPescalated is used and de-escalated to ABVD if the patient is PET/CT negative after 2 courses. Patients aged >70 years were generally treated with cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisolone (CHOP) in Sweden, which has now been replaced by AVD. Different front-line chemotherapy regimens are preferred in different countries, ABVD or Stanford V are used in North America, and BEACOPP is more frequently used in Germany compared to the United Kingdom where ABVD is preferred.

Treatment of relapsed and refractory patients

Relapsed or refractory patients are treated with salvage chemotherapy, and those who respond are treated with high dose chemotherapy and autologous stem cell transplantation (ASCT). ASCT is associated with better outcome compared to patients treated with conventional chemotherapy in relapsed and refractory HL. Allogeneic stem cell transplantation (allo-SCT) is reserved for selected patients, mostly those that relapse after ASCT. Allo-SCT relies primarily on introducing a new immune system to induce a graft-versus-tumor (GVT) effect by generating antigen-specific T cells that attack the malignancy. However, compared to ASCT, there is a higher risk of toxicity, infections, and graft-versus-host disease (GVHD). In HL patients treated with allo-SCT, the 3-year OS was 60% in studies conducted.
in the year 2000 or later\textsuperscript{198}. For patients that relapse after stem cell transplantation (SCT) or are not suitable for this treatment, new therapies have been approved in recent years\textsuperscript{173}.

**Novel therapies**

Brentuximab vedotin is an antibody drug conjugate, targeting CD30 on the surface of HRS cells and delivers the cytotoxic drug monomethyl auristatin E into the HRS cell, causing disruption of microtubules and cell death\textsuperscript{199}. An overall response rate (ORR) of 75% and complete remission (CR) of 34% was reported in post-ASCT patients with HL\textsuperscript{189}. It was approved for treatment of CD30+ HL by the FDA in 2011\textsuperscript{173}. Ipilimumab was the first immune checkpoint inhibitor used in a phase I study in 14 patients with relapsed or refractory HL after allo-SCT, where 2 patients achieved durable CR\textsuperscript{200}. There are ongoing clinical trials with ipilimumab in patients with relapsed and refractory HL\textsuperscript{201}, but recent research has mostly focused on PD-1 blockade\textsuperscript{202}. As described in the previous section, PD-1 inhibitors reactivate the antitumor immune response toward the HRS cells\textsuperscript{173,182}. In patients with relapsed or refractory HL, nivolumab had an ORR of 87% and 17% reached CR\textsuperscript{138}. In 2016, the FDA approved nivolumab for treatment of patients with relapsed or refractory HL after brentuximab vedotin and ASCT treatment\textsuperscript{173}. Pembrolizumab has similar response rates as nivolumab\textsuperscript{173}. An essential clinical question is to identify which patients that benefit most from these new treatment options. One study found that patients with high expression of PD-L1 on HRS cells had the best response to treatment with nivolumab, however, patients with a low PD-L1 expression also responded with partial remission, and further investigations are needed to establish if PD-1 and/or PD-1 ligands are predictive markers of response to PD-1 blockade in HL\textsuperscript{203}. Although targeted therapies in relapsed and refractory HL result in high response rates, their long-term efficacy outcomes and toxicities remain to be determined\textsuperscript{202}.

**Novel therapies and stem cell transplantation**

At what point the drugs presented in the last paragraph are to be used in relation to SCT in relapsed or refractory patients with HL is a matter to be elucidated\textsuperscript{202}. Brentuximab vedotin has been used successfully in patients before allo-SCT with high response rates\textsuperscript{204}, as well as in patients that relapse after allo-SCT and it does not seem to enhance GVHD\textsuperscript{205}. PD-1 blockade may enhance the GVT effect if given after allo-SCT\textsuperscript{206}. In a study where PD-1 blockade was administered after allo-SCT it resulted in more frequent GVHD-related deaths\textsuperscript{207} compared to a study where PD-1 blockade was administered before allo-SCT\textsuperscript{207,208}. Supposedly, PD-1 blockade results in a potent activation of graft-derived T cells in the thymus, and/or a more potent inhibition in peripheral organs by affecting the cytokine milieu by blocking
the PD-1 pathway\textsuperscript{207}. Further studies on allo-SCT in combination with immune checkpoint blockade are needed to determine for which patients this is a favorable approach.

**Future therapies**

Another experimental approach to target the HRS cells is to use Chimeric Antigen Receptor (CAR) T cells. CTL from the patient are engineered to recognize a desired receptor. A chimeric gene that encodes a specific antibody coupled to the intracellular signaling part of the T-cell receptor is retrovirally transduced in vitro, then the CTL are reintroduced to the patient to exert their cytotoxic activity\textsuperscript{173}. Promising results were observed with CAR T cells targeting the EBV proteins LMP1 and LMP2 in a cohort on both HL and Non-Hodgkin lymphoma\textsuperscript{209}. There are ongoing clinical trials that use CD30 as the target receptor for treatment of HL\textsuperscript{173}. Histone deacetylase inhibitors target multiple epigenetic mechanisms, including histone acetylation and chromatin condensation, inducing cell cycle arrest and apoptosis. It may also affect the microenvironment by downregulating PD-1 on T cells\textsuperscript{173}. There are several ongoing trials where targeted therapy (e.g. brentuximab vedotin or nivolumab) is used as part of front-line treatment\textsuperscript{173,201}. Other possible targets include blockade of immune checkpoints other than the PD-1 pathway, such as the TIM-3, LAG-3 and TIGIT pathways\textsuperscript{96}. Also, blockade of multiple immune checkpoint pathways might be beneficial\textsuperscript{96,210}.

Figure 13. Novel therapies in HL and their mechanisms. Figure adapted from Glimelius et al\textsuperscript{173} with permission.
Aims

Overall aim
To study the etiology of HL with reference to autoimmune diseases and the prognostic significance of tumor-infiltrating leukocytes and immune checkpoints pathways.

Specific aims
I To investigate the impact of autoimmune and atopic diseases on the risk of developing HL by histologic subtypes and EBV status.

II To evaluate the immune signatures in HL and the prognostic impact of eosinophils, mast cells, macrophages, Tregs and activated lymphocytes in the tumor microenvironment and its relation to clinical characteristics.

III To explore the prognostic impact of PD-1, PD-L1 and PD-L2 in the tumor microenvironment of HL, and determine if these markers are independent prognostic factors when adjusted for other established prognostic criteria.

IV To compare the expression of PD-1 and PD-L1 in paired biopsies from untreated and treated patients with HL to determine how these markers change due to disease progression and relapse.
Materials and methods

Patients

In papers I\textsuperscript{211}, II\textsuperscript{212}, and III\textsuperscript{213}, patients were part of the Scandinavian Lymphoma Etiology (SCALE) study, a population-based case-control study on risk factors for lymphomas. Adult patients from Denmark and Sweden were included from 1999 to 2002. A total of 585 cases were included in paper I. On the same cohort as paper I, 459 cases where tumor material was available were included in paper II. A pooled cohort of 387 cases was used in paper III, with 128 Swedish patients from the SCALE study and 259 adult patients diagnosed with HL at Aarhus University Hospital, Denmark, from 1990 to 2007. In paper IV, 11 patients diagnosed at three Swedish pathology departments (Uppsala, Umeå and Lund) with at least two paired biopsies with HL prior to treatment start were included in the untreated cohort, and 30 patients with paired primary and relapse biopsies with HL were included in the treated cohort. Clinical information was obtained from medical records. Patients were treated according to national guidelines, mostly with ABVD or ABVD-like chemotherapy regimens. BEACOPP was used in Swedish patients with advanced stage and ≥3 IPS criteria. A few patients were treated with other chemotherapy regimens or RT only\textsuperscript{1,74}.

Evaluation of risk factors

For paper I, patients and controls were interviewed about possible risk factors for lymphomas. Information included family characteristics (sibship size and birth order), parental and personal education, childhood housing conditions, tobacco smoking history, history of IM, and autoimmune and atopic diseases. Questions regarded specific autoimmune and atopic diseases as diagnosed by a physician. Controls were sampled using computerized population registers in each country. Controls were sampled every six months during the study period, and matched within each country on the expected age and sex distribution of the cases. For paper I, blood samples were provided before initiation of treatment in patients, and as soon as possible after the interview for controls. Analyses were carried out for specific IgE reactiv-
ity against allergens using an enzyme-linked immunosorbent assay (Phadiotop® test, Phadia, Uppsala)\textsuperscript{214}.

Immunohistochemical stainings

Tumor tissue used in paper II, III and IV were formalin-fixed and paraffin-embedded and slides were prepared on 3-4 μm thick sections. Immunohistochemical antibodies used are displayed in table 2.

Table 2. Immunohistochemical antibodies used in paper II, III and IV

<table>
<thead>
<tr>
<th>Cell(s) visualized</th>
<th>Antibody; stains</th>
<th>Developer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paper II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mast cells</td>
<td>G3; Tryptase</td>
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<td>Macrophages</td>
<td>PG-M1; CD68</td>
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<td>Treg</td>
<td>MbAbcam-22509; FoxP3</td>
<td>Abcam, Cambridge, MA, USA</td>
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<td>Activated lymphocytes</td>
<td>NCL-L-GRANB; Granzyme B</td>
<td>Novocastra, Newcastle, UK</td>
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<td><strong>Paper III</strong></td>
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<td></td>
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<tr>
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<td><strong>Paper IV</strong></td>
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*Double stains for PD-L1/PAX5 and PD-L2/PAX5 were used in paper III, and PD-L1/PAX5 and CD30/PAX5 were used in paper IV.

**For PAX5, two different antibodies were used depending on if it was double stained with PD-L1 and PD-L2, or CD30. With PD-L1 and PD-L2, M7307/DAK-Pax5 stained PAX5; with CD30, ab140341 stained PAX5.
Evaluation of tissue material

In paper II, each marker was evaluated manually on a microscope. Eosinophils were evaluated on slides stained with HE. The number of mast cells and eosinophils were counted in 10 high-power fields (HPF). The proportions of macrophages, Tregs and activated lymphocytes in relation to the overall leukocyte infiltrate were evaluated in 3-6 HPF. The cut-off for a high proportion of macrophages, Tregs and activated lymphocytes, and a high number of mast cells was set at the upper quartile. The cut-off for a high number of eosinophils was set at 200. Part of the material (n=105) was evaluated on tissue microarrays (TMAs). In paper III, the proportions of PD-1+ and PD-L1+ and PD-L2+ leukocytes were calculated using an image analysis software (Visiomorph, Visiopharm®). The proportions of PD-L1+ and PD-L2+ HRS cells were manually calculated on a microscope. The cut-off for a high proportion of PD-1+ leukocytes was set at \( \geq 10\% \) and high proportions of PD-L1+ and PD-L2+ leukocytes were \( \geq 5\% \). The material in paper III was evaluated on TMAs. In paper IV, the proportions of PD-1+ and PD-L1+ leukocytes, and PD-L1+ HRS cells were manually counted in 5 HPF. The material in paper IV was evaluated on tissue sections. Areas with HRS cells were chosen, while fibrosis and GCs were avoided. Eosinophils and mast cells were evaluated prior to my studies. I evaluated macrophages, Tregs, activated lymphocytes, PD-1, PD-L1 and PD-L2.

Epstein-Barr virus classification

Tumor biopsies were also analyzed for presence of EBV by immunohistochemical staining with LMP-1 and/or in situ hybridization for EBERs\textsuperscript{16,74}. Tumor EBV status was available for 498 (85\%) of 585 cases in paper I, 441 (96\%) of 459 cases in paper II, 385 (99\%) of 387 cases in paper III, and 11 cases in the untreated group in paper IV. EBV status was determined prior to my studies in papers I, II and III, and I evaluated the EBER stainings in paper IV.

Statistical methods

In paper I, multiple logistic regression to calculate Odds Ratios (OR), with 95\% confidence intervals (CI) as estimates of relative risk of HL in association with studied exposures was used. HL was also analyzed by histological subtype and age. Statistical significance of independent variables was tested by the likelihood ratio test. All statistical analyses were adjusted for matching variables. In paper II, the material was divided into separate immune
signatures. An “active” immune signature was defined as a high amount of activated lymphocytes and low amount of Tregs, an “anergic” immune signature as a high amount of Tregs, and an “innate” immune signature as a high number of eosinophils, mast cells and macrophages. Cases unable to be designated into any of these groups were assigned to the referral group “mixed” immune signature. In papers II, III and IV, survival was analyzed based on infiltration of high versus low amount of each cell type, and each immune signature (paper II) using Cox proportional hazard regression to calculate hazard ratios (HR) with 95% CI as estimates of risk, and Kaplan-Meier method compared with the log-rank test. Survival outcomes were time to progression (TTP) and OS in paper II, and event free survival (EFS) and OS in paper III. TTP was defined as time from diagnosis to progression, relapse or death from HL. OS was defined as time from diagnosis to death from any cause. EFS was defined as time from diagnosis to relapse or progression of HL, or death from any cause. In paper IV, time to death from relapse was defined as time from diagnosis of relapse confirmed by biopsy, to death from any cause. In paper II, correlations between potential prognostic factors were analyzed with Spearman Rank Order Correlation test. Chi-square or Fischer’s exact tests were used to compare tabulated values in paper III. Wilcoxon signed rank test was used to compare differences between groups in paper IV. Statistical analyses for papers I and II were performed by a statistician. I carried out the statistical analyses for papers III and IV.

Ethical considerations

All studies were ethically approved; reference number 99-154 and 2007/619-32 for the SCALE study in papers I, II and III, reference number 2012-41-1190 (The Swedish Data Protection Agency), and 20070067 (Regional Ethical Committee) for Danish cases in paper III, and reference number 2014/020, 2014/020/1 and 2014/233 in paper IV.
Results and discussion

Paper I

There was a higher proportion of autoimmune diseases among cases (3.6%) than among controls (2.8%). Regarding EBV status for histologic subtypes, 50% of MC cases and 22% of NS cases were EBV positive.

Patients with RA had an increased risk of developing HL. This study further substantiates the association between RA and risk of HL as previously reported\textsuperscript{20,21,26,215}. Novel findings compared to previous studies include a notably increased risk to develop EBV positive HL. Fallah et al\textsuperscript{21}, reported an increased risk of all histological subtypes of HL in patients with RA, while we found an increased risk especially for MC subtype. We also found that patients with proxies of more severe RA had an even higher risk of developing HL, including having ever received daily RA medication, RA duration of 6-20 years, surgery for RA, and having ever been hospitalized for RA.

The increased chronic inflammation induced by autoimmune diseases might lead to less control by T cells of EBV infected B cells, with an increased rate of replication of EBV and a higher risk of developing HL\textsuperscript{23,216}. In addition, shared genetic susceptibility\textsuperscript{2,24} and treatment with antirheumatic drugs\textsuperscript{22,26} might contribute to the pathogenesis of HL in patients with RA.

None of the other autoimmune and atopic diseases was associated with risk of HL. Some studies report a decreased risk of HL in patients with atopic diseases\textsuperscript{31}, while others are in line with our findings and find no association between atopic diseases and risk of HL\textsuperscript{36}. Atopic diseases and HL might be related to socioeconomic protective factors during childhood, such as number of siblings, childhood infections, and housing density\textsuperscript{2}, which we controlled for in our study.
Paper II

The tumor microenvironment in HL was characterized by studying separate leukocytes with immunohistochemical markers and HE. During follow-up, 49 patients suffered disease relapse or progression, and 78 patients died.

Patients with a high proportion of Tregs had an inferior TTP compared to patients with a low proportion of Tregs in an age-adjusted analysis. However, the results were not significant when additionally adjusted for advanced stage, low albumin and bulky tumor. Hence, Tregs are not an independent marker of inferior outcome. The anergic immune signature was also associated with inferior TTP when compared with mixed immune signature in an age-adjusted analysis.

![Figure 14. Kaplan-Meier estimate of TTP for Tregs. A shorter TTP was seen for cases with a high proportion of Tregs (blue line) compared with cases with a low proportion of Tregs (red line) (log-rank test, p=0.03).](image-url)

Tregs contribute to creating an exhausted and anergic state in the tumor microenvironment, and a subsequent increased risk of relapse, progression and death due to HL. Most previous studies report a superior prognosis in patients with a high amount of Tregs in the tumor microenvironment of HL\(^{82,107-111,113}\), however there are other studies that support our findings\(^{112}\). Several previous studies included cases diagnosed over a long period of time (often several decades)\(^{82,108,109,113}\), resulting in different treatment regimens used in the same cohort. Also, various survival outcomes were studied and
defined differently between the studies. To be able to compare different studies, a homogenous definition of survival outcomes is important.\textsuperscript{217}

None of the other leukocytes were of prognostic significance. Several studies report an inferior outcome in patients with a high amount of macrophages.\textsuperscript{81,83,218} We found that macrophages were associated with several clinical factors associated with adverse prognosis, e.g. bulky tumor, low Hb, advanced stage, and bone marrow involvement. Previous results on the prognostic implications of tumor-infiltrating leukocytes might partly have been due to correlations with other clinical prognostic factors, and not due to the biologic effects of the leukocytes.

![Figure 15](Image)

Figure 15. Leukocytes studied in paper II, a high proportion of Tregs indicated an inferior outcome (circled), while none of the other leukocytes affected outcome (crossed over).

**Paper III**

In paper III we investigated the prognostic impact of the PD-1 pathway in primary HL. During follow-up, 133 patients suffered treatment failure and 79 patients died.

Patients with high proportions of PD-1+ and PD-L1+ leukocytes had a worse outcome compared to patients with low proportions of PD-1+ and PD-L1+ leukocytes, respectively. In multivariate analyses adjusted for putative prog-
nostic factors for EFS, high proportions of PD-1+ and PD-L1+ leukocytes were significantly associated with inferior EFS. In multivariate analyses adjusted for putative prognostic factors for OS, a high proportion of PD-L1+ leukocytes was associated with inferior OS, while a high proportion of PD-1+ leukocytes was not. High proportions of PD-L2+ leukocytes and HRS cells, and PD-L1+ HRS cells were not associated with outcome.

![Event-free survival by proportion of PD-1](image)

![Event-free survival by proportion of PD-L1](image)

Figure 16. Kaplan-Meier estimates according to (A) ≥10% (red line) and <10% (black line) PD-1+ leukocytes (log-rank test, p=0.002), and (B) ≥5% (red line) and <5% (black line) PD-L1+ leukocytes (log-rank test, p=0.01).

High proportions of PD-1+ and PD-L1+ leukocytes are markers of an exhausted and anergic immune system, with reduced ability of leukocytes to exert their cytotoxic abilities toward the HRS cells. Our results are in line with several previous studies that report an inferior OS in patients with a high amount of PD-1\textsuperscript{123,141}. One previous study found that a high amount of
PD-L1+ leukocytes and HRS cells, in combination with a high amount of PD-1+ leukocytes was associated with inferior OS, however not in a multivariate analysis\textsuperscript{219}. Expression of PD-1 ligands on HRS cells was not associated with outcome in our cohort, in contrast to one\textsuperscript{143}, but in line with two other studies\textsuperscript{141,144}. This is the first study to report inferior EFS due to high proportions of PD-1+ and PD-L1+ leukocytes, and inferior OS due to a high proportion of PD-L1+ leukocytes in fully adjusted multivariate analyses. Our findings suggest that it is insufficient to only evaluate expression of PD-1 ligands on tumor cells; leukocytes in the microenvironment should also be evaluated since they contribute by means of tumor-protective immune responses. This is probably especially important in malignancies with a pronounced inflammatory infiltrate in the tumor microenvironment, such as HL.

![Diagram of immune cells with PD-L1 and PD-1 ligands](image)

Figure 17. Receptors and ligands studied in paper III. Circled receptors and ligands are associated with inferior outcome, while crossed over ligands are not associated with outcome.

**Paper IV**

In paper IV, we compared expression of PD-1 and PD-L1 in paired biopsies from untreated and treated patients with HL.

The proportions of PD-1+ and PD-L1+ leukocytes, and PD-L1+ HRS cells differed between the primary and the relapse biopsy to statistically signifi-
cant degrees in the treated group, where the majority of the cases had an increased proportion in the relapse biopsy compared to the primary biopsy. In the treated group, PD-L1 was expressed to some degree (>0%) in the HRS cells in all relapse biopsies, while it was expressed in 87% of the primary biopsies. There was an indication of increased expression of PD-L1+ leukocytes in the second biopsy compared to the first biopsy in the untreated group, however not to a statistically significant degree. Expression of high vs low proportions of PD-1+ and PD-L1+ leukocytes, and PD-L1+ HRS cells in the relapse biopsies did not affect survival after relapse.

To our knowledge, this is the first study to report that PD-1 and PD-L1 are upregulated in relapsed cases with HL, and an indication of a higher proportion of PD-L1+ leukocytes if HL is able to progress without treatment. Previous studies on solid malignancies have found that PD-L1 is upregulated on tumor cells and leukocytes following chemotherapy and RT. PD-1+ macrophages tended to be more numerous in the later course of the disease in CRC. Supposedly, longer disease duration, primary treatment, or changed conditions in the microenvironment at relapse results in upregulations of PD-1 and PD-L1 in HL.

PD-1 blockade is currently only used in relapsed and refractory patients with HL, and patients with high expression of PD-L1 on HRS cells responded more favorably to PD-1 blockade. These findings, in combination with our
observation of an upregulation of the PD-1 pathway in relapsed HL, should be recognized by ongoing clinical trials that use PD-1 blockade as part of combination therapy in primary HL. Treatment with PD-1 blockade might be useful in certain patients with primary HL at high risk of treatment failure, however identification of predictive markers is probably needed.

Strengths and limitations

Limitations in paper I include the use of self-reported interviews rather than medical journals, as they can represent a source of bias and risk of incorrect diagnosis. Since autoimmune diseases are rare, the number of autoimmune conditions analyzed was limited. There are several strengths. Firstly we did not only include hospitalized patients. Hospitalized patients with autoimmune diseases might have an exceptionally increased risk of lymphoma due to expected pronounced inflammation or more extensive treatment with antirheumatic drugs. Secondly, we were able to control for known risk factors for HL that register-based studies were unable to include, such as smoking and socioeconomic status. Another strength is the review of tumor cases to confirm the HL diagnosis, which almost all (except one) previous studies have not performed thus risking of misclassification of HL as other lymphomas. Limitations and strengths in papers II and III are similar. Limitations include multiple testing that may have generated significant results by chance, and since these are retrospective studies, we were not able to fully adjust for potential residual confounders. Despite the fact that both papers included a large cohort of patients, there were few events that resulted in low precision of our risk estimates. Multivariate analyses are associated with limitations when several variables are included with a limited number of events. However, tests for interaction between independent significant variables were performed and none were significant in paper III. Strengths include the population-based setting with histopathological confirmation of most of the cases and the large and homogenously treated cohorts, in contrast to several other studies. One strength of immunohistochemistry is that we were able to separately evaluate the expression of PD-L1 and PD-L2 on HRS cells and leukocytes, which proved to be crucial since only PD-L1+ leukocytes proved to be associated with outcome. In addition, we were able to evaluate PD-1+ leukocytes that were close to the HRS cells, and exclude areas with fibrosis and GCs. Also, the wide array of different leukocytes studied in paper II adds credibility to this study. The low number of cases studied in paper IV is a drawback that limited the statistical power. However, the design and uniqueness of the material both represent strengths in paper IV.
Summary of results

I Patients with RA had an elevated risk of developing HL, the risk of developing EBV positive HL was especially increased. Atopic diseases did not affect the risk of developing HL.

II Patients with a high proportion of Tregs in the microenvironment of HL had a tendency for inferior outcome. Macrophages were associated with several clinical factors of adverse prognostic outcome.

III Patients with high proportions of PD-1+ and PD-L1+ leukocytes in the microenvironment of primary HL had worse outcome.

IV The proportions of PD-1+ and PD-L1+ leukocytes, and PD-L1+ HRS cells were increased in relapse biopsies compared to primary biopsies with HL.
General discussion and future perspectives

A great deal of research has been put into the etiology and prognostic implications of the tumor microenvironment in HL, but there is still a great deal to be further explored.

The findings of paper I add knowledge to the etiology of HL. It would be interesting to investigate the mechanism for the association between autoimmune diseases and EBV positive HL. As discussed, the association is probably multifactorial and due to inflammation induced by the autoimmune disease, shared genetics, and treatment given. The determination of how autoimmune diseases induce inflammation that favors survival of pre-neoplastic B cells needs to be further addressed. It has been a concern that treatment with modern anti-rheumatic drugs might increase the risk of lymphomas. Since more patients are treated with these drugs compared to when the material for my first study was gathered, new studies with similar design as that of paper I would clarify this matter. With more understanding of the pathogenic process, patients with high risk of HL could be identified, and perhaps even preemptive strategies could be employed.

For paper II, the contradicting results compared with previous studies on the prognostic impact of Tregs were intriguing and novel findings. It would be of interest to elucidate the mechanism that Tregs has in the HL tumor microenvironment, to find out if they truly possess HRS-killing abilities, or if they act as proposed in this thesis and are responsible for downregulating the antitumor immune response. Given the success with PD-1 blockade with its known mechanism to inhibit Tregs, it is to some degree evident that Tregs are probably unfavorable cells in the microenvironment of HL. The other leukocyte of particular interest to further study in the microenvironment of HL is macrophages. Macrophages have a wide variety of phenotypes and express different markers depending on which function they possess, including both PD-1 and the PD-1 ligands. Some macrophage subtypes might possess a particular tumor-promoting phenotype, while some phenotypes might be associated with favorable outcome due to preserved phagocytic abilities.

This thesis proves that the expression of immune checkpoint pathways has prognostic implications in HL. Naturally, these markers have to be compared with other markers of inferior prognosis in HL to determine their applicabil-
ity in a clinical setting. PET/CT is used after two courses of chemotherapy and a positive result is a superior prognostic marker of dismal prognosis compared to the IPS criteria. Patients with a negative PET/CT are thus expected to have a favorable prognosis. Further investigation is of interest to identify factors that are associated with unfavorable outcome in patients with a negative PET/CT. One previous study indicated that a high proportion of PD-1+ leukocytes and macrophages was associated with inferior outcome in PET/CT negative cases\textsuperscript{221}, while another study found no prognostic impact of macrophages in PET/CT negative cases\textsuperscript{92}. A thorough panel of tumor markers and other clinical parameters to elucidate whether any additional factors could be identified in this subgroup of patients should be further studied.

The IPS criteria will probably be used for as long as contemporary chemotherapy regimens are used as front-line treatment. However, prognostic markers tend to change as treatment modalities change. As to whether IPS is also applicable to risk-stratify patients when other front-line treatments are introduced remains to be explored. Perhaps, evaluation of the tumor microenvironment with regards to different immune checkpoint pathways to determine which pathway is most vulnerable will guide us towards which front-line treatment that should be used. Future front-line therapies in HL will probably include brentuximab vedotin and additional immune checkpoint pathway blockade, probably even as combination therapies. One exceptionally interesting aspect to further investigate in the era of immune checkpoint blockade is to study cases that do not respond to these therapies. It is important to obtain tumor material from these relapsed and refractory cases to be analyzed with different methods, e.g. immunohistochemical antibodies and gene sequencing techniques. This is important in order to determine predictors of response to immune checkpoint blockade. Further studies of the tumor microenvironment to identify new pathways of prognostic and predictive impact, and possibly identification of new treatment options in order to achieve higher cure rates and fewer side effects are the primary objectives in HL.
Hodgkins lymfom (HL) är en elakartad tumörsjukdom som utgår från en viss sort av vita blodkroppar (B lymfocyter). I Sverige insjuknar ca 160 nya personer per år i sjukdomen. Vad som orsakar HL är till stora delar okänt. Epstein-Barr virus (EBV) påvisas i en tredjedel av fallen av HL i tumörcellerna och bidrar förmodligen till sjukdomsutvecklingen i dessa fall. Vad som orsakar sjukdomsutvecklingen i de resterande två tredjedelarna som är negativa för EBV är mer oklart. Patienter med reumatoid artrit (RA) har i tidigare studier visat sig ha en ökad risk för att utveckla HL, dock är det oklart om risken är lika stor att utveckla både EBV positiv och EBV negativ HL för patienter med RA. Det är dessutom oklart om patienter med allergiska sjukdomar har en ökad eller minskad risk att insjukna i HL.

HL behandlas med olika kombinationer av cellgifter och strålterapi. Chansen för långvarigt bot uppgår till drygt 80 %, men det finns idag inga fullgoda verktyg för att identifiera de patienter som kommer att drabbas av återfall. Därför finns det ett behov av att finna ytterligare faktorer för att identifiera vilka patienter som har sämst förväntad prognos. Patienter med stor risk för återfall bör få mer aggressiv behandling så att de inte återfår sin sjukdom.

Ett utmärkande drag för HL är dess utseende i mikroskopet. Där många andra typer av elakartade tumörer till större delen består av tumörceller, består HL till större delen av olika godartade inflammatoriska celler (leukocyter) och bara några få procent tumörceller. Tumörcellerna attraherar de godartade leukocyter och skapar tillsammans en så kallad tumörmikromiljö. I tidigare studier har det visat sig att olika leukocyter i tumörmikromiljön påverkar prognosen för patienterna. Aktiverade leukocyter har normalt till uppgift att döda tumörcellerna i HL. Dock har tumörcellerna (och andra leukocyter) i HL möjlighet att uttrycka proteiner på cellytan (programmerad celldöd ligand 1 och 2 (PD-L1 och PD-L2)) som binder till en receptor på de aktiverade leukocyterna (programmerad celldöd 1 (PD-1)) vilket har en hänneskande effekt på de aktiverade leukocyterna. Det är känt att PD-1 och PD-L1 uttrycks till följd av lokal utsöndring av signalsubstanser i tumörmikromiljön, och förekommer i varierande grad i olika typer av elakartade tumörsjukdomar. Eftersom ett vävnadsprov från en lymfknuta med HL enbart ger en ögonblicksbild av en dynamisk och varierande tumörmikromiljö
är det misstänkt att uttrycket av PD-1 och PD-L1 skiljer sig beroende på när i sjukdomsförloppet som vävnadsprovet är taget.

Det övergripande målet med min avhandling har varit att finna faktorer som påverkar uppkomsten av HL, samt att studera tumörmikromiljön för att finna faktorer som påverkar prognosen för patienterna.

Delarbete I

I första studien undersökte jag risken att insjukna i HL hos individer med reumatiska och allergiska sjukdomar. Vi fann att patienter med RA har en ökad risk att utveckla främst EBV positiv HL, vilket ingen tidigare studie påvisat. Dessutom fann vi att patienter med tecken på svårare RA har en speciellt ökad risk att insjukna i HL, nämligen patienter som medicinerats för RA, haft en längre sjukdomsduration och patienter som opererats för RA. Inga övriga reumatiska eller allergiska sjukdomar påverkade risken att utveckla HL. Vi misstänker att RA medför en ökad inflammation vilket ökar risken för att EBV ska återaktiveras och bidra till utveckling av HL.

Delarbete II

I andra studien undersökte jag hur tumörmikromiljön i HL påverkar prognosen. Vi samlade in tumörmaterial och undersökte fem olika inflammatoriska celler (eosinofiler, mastceller, makrofager, regulatoriska T lymfocyter (Tregs) och aktiverade lymfocyter). Vi fann att en hög andel Tregs i tumörmikromiljön medförde en sämre prognos. Detta förklaras troligtvis av att en hög andel Tregs hämmar andra inflammatoriska celler vars uppgifter normalt är att döda tumörcellerna. Inga av de övriga undersökta inflammatoriska cellerna påverkade prognosen.

Delarbete III

I tredje studien undersökte jag också hur tumörmikromiljön i HL påverkar prognosen. Tumörmaterial undersöktes för tre olika markörer som tyder på ett hämmat immunförsvar (PD-1, PD-L1 och PD-L2). Vi visade att patienter med en hög andel inflammatoriska celler som uttrycker PD-1 och PD-L1 har en sämre prognos. Dessa resultat var statistiskt signifikanta när alla övriga riskfaktorer som kan ha påverkat prognosen för patienterna i studien togs i beaktande. Ett högt uttryck av både PD-1 och PD-L1 medför att tumören är mer benägen att undgå immunförsvarets tumördödande celler, vilket förmod-
ligen också medför att tumören är mer kapabel att stå emot behandling med cellgifter och strålaterapi.

Delarbete IV

I min fjärde studie undersökte jag hur uttrycker av PD-1 och PD-L1 skiljer sig hos samma patient där det tagits upprepade vävnadsprover med HL. Tumörvävnad från både obehandlade, och behandlade patienter som senare återfått sin sjukdom färgades med PD-1 och PD-L1. Vi såg att PD-1 och PD-L1 uttrycks till en högre grad i tumörvävnad med återfall, jämfört med de primära tumörearna. Vi såg även en tendens till en högre andel av inflammatoriska celler som uttryckte PD-L1 i det senare vävnadsprovet jämfört med det tidigare i den obehandlade gruppen. Fynden tyder på att ju längre tid tumören tillåts att växa obehandlad, alternativt på grund av förändrade förutsättningar för tumörmikromiljön vid återfallet så organiseras en tumörmikromiljö som skyddar tumörcellerna.
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