CHAPTER 1

Introduction and scope of the thesis
General introduction of Hodgkin lymphoma

Since its first description in 1832, the biology of Hodgkin lymphoma (HL) continues to intrigue scientists with the broad variety of mechanisms applied by the malignant cells to survive. HL has a heterogeneous etiology with the highest incidence in Caucasians, followed by African Americans and Hispanics, and the lowest incidence in Orientals\(^1\). The annual incidence of HL in Europe and North America has been estimated to be 1.3 to 4.0 per 100,000 in males and 0.9 to 3.1 per 100,000 in females, in Asian countries the incidence is 0.1 to 1.3 per 100,000 in males and less than 0.7 per 100,000 in females\(^2\). In general, HL accounts for about 1% of all cancers and ~30% of the lymphoid malignancies worldwide\(^1\).

Early stage HL is curable in the vast majority of patients. The treatment of patients with advanced disease stages, primary refractory disease after initial standard treatment, and relapses, is still challenging\(^3\). A considerable proportion of the patients suffer from treatment-related long-term toxicity, including cardiovascular diseases and treatment-related secondary malignancies, even 20 years after treatment\(^4\). So there is a need to deepen our understanding of the biological basis of HL.

Pathology of HL

HL is currently classified as two different disease entities: classical Hodgkin lymphoma (cHL) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL). Both HL subtypes are characterized by a minority of malignant cells that usually represent about 1% of cells in the tumor mass. The tumor cells in cHL are called Hodgkin Reed-Sternberg (HRS). The mononuclear Hodgkin cells generate the multinucleated Reed-Sternberg cells by incomplete cytokinesis and cell re-fusion of daughter cells\(^5\). The Reed-Sternberg cells have a limited proliferative ability \(^6, 7\). HRS cells are surrounded by reactive cells including lymphocytes, plasma cells, eosinophils and histiocytes\(^8\). Based on morphology of the HRS cells and the composition of the background cells cHL can be classified into 4 different subtypes: nodular sclerosis
(NS) 60-80%, mixed cellularity (MC) 15-30%, lymphocyte depleted (LD) 5% and lymphocyte rich (LR) 5%. The tumor cells in NLPHL are called lymphocytic predominant (LP) cells.

Despite extensive efforts in the last years, the pathogenesis of HL is not completely understood. HRS cells show crippling somatic hypermutation of the immunoglobulin (Ig) genes, indicating a germinal centre antigen-experienced B cell origin. In the germinal centre reaction naïve B cells differentiate into memory and plasma B cells via positive selection, whereas auto-reactive B cells and B cells lacking functional Ig expression are eliminated. HRS cells escape from apoptosis by a variety of pro-survival mechanisms (see below). In contrast, LP cells of NLPHL usually have somatically mutated functional Ig gene rearrangements.

In general, B cell malignancies including NLPHL, retain key B cell markers. HRS cells, on the contrary, have lost expression of most of the B cell lineage-specific gene expression program, including expression of Ig. HRS cells retain B-cell features related to antigen-presentation and factors involved in the cross talk with T helper (Th) cells, such as MHC II, CD40 (TNFRSF5) and CD80. At the same time non-B cell lineage proteins are upregulated. The mechanisms for the loss of B cell phenotype include methylation of the promoter regions of the B cell associated genes, loss of E2A function, upregulation of NOTCH1, a negative regulator of the B cell program and activation of STAT5.

HRS cells consistently express non-B cell proteins CD15 and CD30. These two markers are used as diagnostic markers of cHL. The CD15 and CD30 staining pattern is characterized by a membranous staining combined with staining of the Golgi apparatus. CD30 or tumor necrosis factor (TNF) receptor superfamily (TNFRSF8) was originally identified as a HRS cell marker almost 30 years ago. CD30 can mediate activation of the nuclear factor kappa-B (NF-KB) pathway and the extracellular signal-regulated kinase (ERK) signaling pathway and prevent induction of apoptosis of HRS cells. In addition, HRS cells often express markers of cytotoxic T cells (granzyme B) and dendritic cells (Fascin).

A number of genomic aberrations have been identified in HRS cells. These include...
gains or amplifications of the gene loci for JAK2, FGFR3, REL and ID2\textsuperscript{28, 29}. Especially increases of the gene locus for REL and JAK2 locus were reported as recurrent alterations in primary HRS cells\textsuperscript{28}. A gene fusion involving the major histocompatibility complex (MHC) class II transactivator CIITA has also been reported in 15\% of cHL\textsuperscript{30}. In addition to these structural aberrations, mutations in HRS cells frequently carry mutations in members of the NF-KB family, such as A20/TNFAIP3, NFKBIA and NFKBIE\textsuperscript{31}.

**EBV**

30-60\% of cHL cases contain monoclonal copies of the Epstein-Barr virus (EBV) genome in the HRS cells\textsuperscript{32}. There are two known mechanisms that support the relevance of EBV in the pathogenesis of cHL: EBV latent membrane protein LMP1 can mimic CD40 resulting in activation of the NF-kB transcription factor\textsuperscript{33, 34} and LMP2a can mimic immunoglobulin (Ig) and may rescue HRS cells that lack Ig expression\textsuperscript{35}. Based on the function of LMP1 and LMP2a proteins and the clonal infection pattern, EBV is considered to be a causal event in EBV+ cHL\textsuperscript{32, 36}. The prevalence of EBV infection in cHL is highly variable between racial groups and geographic locations\textsuperscript{37, 38}. The proportion of EBV involvement is almost 100\% in Hispanic cHL patients\textsuperscript{39}, intermediate in the Asian population and around 30\% in Caucasians\textsuperscript{37, 40-42}. EBV-associated cHL is more common in mixed cellularity subtype, males, children and in the elderly\textsuperscript{43-45}. In EBV+ cHL patients response to therapy and 2-year failure-free survival of young patients (<50 years of age), is significantly better than in older patients\textsuperscript{46-48}. In older patients treatment failure is associated with a positive tumor cell EBV status\textsuperscript{49}.

The effectiveness of the immune reaction to EBV derived antigenic peptides depends on the HLA-restricted T cell responses. The T cell response is dependent on the affinity and immunogenicity of the antigen presented in the context of specific HLA alleles. Two microsatellite markers, D6S265 and D6S510, located in the HLA class I region were associated with the risk of EBV+ cHL in a Dutch population\textsuperscript{50}. Follow-up
studies indicated that HLA-A*01 and HLA-A*02 were responsible for this association, with HLA-A*01 giving an increased and HLA-A*02 a decreased risk for developing EBV+ cHL\(^5^1\). The possible reason for this is that HLA-A*02 molecules are able to effectively present antigenic EBV-derived peptides, while HLA-A*01 molecules are not.

In EBV+ cases HRS cells may survive the anti-tumor immune response by downregulating both class I and class II HLA antigens. Interestingly, in EBV- HL patients HLA class I was present on HRS cells in only 30% of cases while in EBV+ cHL HRS cells in 79% of the cases retained HLA class I expression. Downregulation or lack of HLA class II expression was observed in HRS cells in 40-50% of the cHL, with no significant differences between EBV+ and EBV- cHL\(^5^2\). So, immune escape by downregulation of HLA antigens is not an obvious mechanism applied by HRS cells in EBV+ HL.

Survival strategies of HRS cells and function of the microenvironment

Normally B cells that fail to produce high affinity immunoglobulin (Ig) undergo apoptosis during the germinal center reaction\(^5^3-5^5\). Survival strategies applied by the HRS cell precursors include a combination of aberrant growth signals provided by ligation of TNF receptor (TNFR) family members CD30, CD40 and receptor activator of NF-kB (RANK)\(^5^6,5^7\) and in addition, activation of the activator protein 1 (AP-1)\(^5^8\), members of the STAT signaling pathway and deregulation of NOTCH1 signaling\(^5^6\). Overexpression of c-Jun and JunB leads to constitutive activation of AP-1, which plays a role in proliferation\(^5^8\). Overexpression of CD30 can induce JunB overexpression\(^5^9\). HRS cells harbor active STAT3, STAT5, and STAT6, and IL-13 and IL-21\(^6^0\) contribute to the activation of these pathways. Notch1 and Notch2 are aberrantly expressed in HRS cells, and the Notch ligand Jagged1 stimulates Notch signaling in HRS cells\(^2^1\). Together this leads to a strong and constitutive activity of the NF-kB pathway which is essential for survival and growth of HRS cells\(^6^1\). In EBV+
cases, LMP1 also activates this pathway, independent of ligand binding\textsuperscript{34}, whereas mutations of the negative regulator of NF-KB, A20, are observed predominantly in EBV- cases, which might enhance the NF-KB activation state\textsuperscript{31}. In addition, over 80\% of the cHL cases show constitutive expression of c-FLIP which protects the HRS cell precursor cells from Fas/CD95 triggered apoptosis without the need for CD40L survival signals\textsuperscript{62, 63}. Another mechanism to escape from FAS induced apoptosis, might involve triggering of the IL-21 receptor with IL-21, both produced by the HRS cells. This leads to activation of the STAT3 pathway and protects HRS cells from CD95 induced apoptosis\textsuperscript{64}.

Several studies indicate that it is difficult to grow HRS cells in culture or in immunodeficient mice\textsuperscript{65}. This supports a crucial role of the microenvironment for HRS cell survival. Indeed, there is a complex cytokine network between tumor and inflammatory cells. HRS cells secrete TARC and MDC, which attract Th2 cells and T regulatory (Treg) cells\textsuperscript{66, 67}. Through upregulation of MIP-3\(\alpha\), IL-21 attracts Treg cells\textsuperscript{64}. A large proportion of the infiltrating cells in cHL indeed consist of CD4+ Th and Treg cells. These cells have an immunosuppressive activity on infiltrating cytotoxic T cells which is favorable for HRS cells. Besides attraction by chemokines these cells can also be obtained by promoting differentiation of naive CD4+T cells into Th2 and Treg cells\textsuperscript{68}. HRS cells may further orchestrate the microenvironment by changing a cellular Th1 response to a humoral Th2 response, which has tumor cell survival effects\textsuperscript{69}. HRS cells produce immunosuppressive cytokines IL-10 and TGF\(\beta\), and other factors such as galectin-1, to inhibit T cell function\textsuperscript{70-72}. Moreover, HRS cells express programmed cell death protein 1 (PD1) ligand to inhibit T-cell effector functions by binding of PD1 on T cells\textsuperscript{73, 74}. In some cases CIITA chromosomal translocations involving the Programmed cell death 1 ligand (PD-L) 1 or PD-L2, gene loci, lead to overexpression of PD-1 ligand by HRS cells. High PD-1 ligand expression levels results in exhaustion of PD-1 positive cytotoxic T cells. Expression of Galectin-1 and PD-1L can be regulated through AP-1 dependent enhancers\textsuperscript{70, 75}. Moreover, HRS cells trigger IL-3 secretion by activated T cells \textsuperscript{76}, which has growth- and survival-promoting effects on HRS cells by binding to the IL-3R.
Introduction and scope of the thesis

Receptors tyrosine kinase pathways

Figure 1: Receptor tyrosine kinases expressed in HL. Triggering of these kinases leads to activation of PI-3K- AKT and MAPK-ERK pathways, which promote survival and proliferation of HRS cells.

Many receptor tyrosine kinases (RTK) have emerged as key regulators of critical cellular processes, such as proliferation, differentiation, survival, metabolism and migration\(^\text{77}\). In humans there are 58 RTKs belonging to 20 subfamilies. All RTKs have a similar molecular structure: an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic domain that contains the tyrosine kinase (TK), carboxy (C-) terminal and juxtamembrane regulatory regions. Nowadays RTK abnormalities have been found in a variety of cancer types\(^\text{78}\). In HL, several RTKs have been shown to be expressed and possibly contribute to survival of HRS cells.

Renne et al. reported aberrant expression of 6 RTKs in HL, i.e.: platelet-derived growth factor receptor A (PDGFRA), discoidin domain receptor tyrosine kinase 2 (DDR2), ephrin type-B receptor 1 (EPHB1), recepteur d’Origine nantais (RON), neurotrophic tyrosine kinase receptor, type 1 (TRKA) and neurotrophic tyrosine kinase receptor, type 2 (TRKB). Aberrant RTK expression was most pronounced in the NS subtype\(^\text{79}\). There was an inverse correlation between expression of RTKs and EBV
status, indicating that the RTK pathway might represent a complementary mechanism for the survival of HRS cells in EBV negative cases of cHL\textsuperscript{80}. The ligand for DDR2, collagen 1, is present at high levels in the sclerotic bands of NS-HL. Nerve Growth factor, the ligand for TRKA is produced by granulocytes in HL. The ligands for PDGFRA and EPHB1 are expressed by the tumor cells and not by the microenvironment and may thus act as autocrine factors. However, phosphorylation of the receptor was found in HRS cells of only 25-30\% of the patients expressing PDGFRA and TRKA. In general, HRS cells showed higher phosphorylation level than non-Hodgkin lymphomas, these high phosphorylation levels were observed especially in NS-HL\textsuperscript{79}.

Hepatocyte growth factor or scatter factor (HGF/SF) is produced by cells of mesenchymal origin. HGF activity is mediated by binding to its receptor, a receptor tyrosine kinase (RTK) encoded by the proto-oncogene c-Met. After binding to HGF, c-Met undergoes auto-phosphorylation and this leads to activation of several cellular targets involved in a variety of biological processes, including proliferation, survival and migration.

c-Met expression has been reported in HRS cells in 33\% to 100\% of cHL tissue samples\textsuperscript{81-83}. HGF was observed in the HRS cells of 8\% (10/121) of the cases\textsuperscript{83}. In addition, HGF expression was observed in infiltrating cells, especially in dendritic cells. c-Met expression correlated with a good 5-year freedom from tumor progression. In functional studies activation with HGF did not affect cell growth, while the c-Met inhibitor SU11274 suppressed cell growth by inducing G2/M cell cycle arrest\textsuperscript{83}.

In contrast to the above mentioned RTKs, there are limited or no publication on the role of other potentially interesting RTKs. IGF-1R has been implicated in survival and cell cycle progression in many cancers. A unique feature of IGF-1R is that at least three PI3K molecules can be recruited by one IGF-1R molecules. PI3K is constitutively activated in HRS cells and promotes their survival. Together these data support a potential role of IGF-1R In HL pathogenesis, but this remains to be studied. The Ephrin family, which is the largest TK family, is deregulated in various tumor types including hematopoietic malignancies, but the family has not been studied in HL.
Scope of the thesis

The aim of this thesis is to further investigate tumor cell survival mechanisms. In Chapter 2 we evaluated the expression, functionality and prognostic significance of IGF-1R in cHL. In chapter 3 we explored the Ephrin family expression patterns of both the tumor cells and the normal cells in the microenvironment in both cHL and NLPHL. As stimulation of Toll-like receptors (TLRs) by microbial pathogen associated ligands results in the activation of NF-κB, which is one of the hallmarks of cHL, we reviewed current knowledge on TLR in B cell lymphoma in chapter 4. In chapter 5 we analyze the expression pattern of TLRs in HL and perform a preliminary functional study. Downregulation of HLA class I and II is one of the mechanisms to escape from an anti-tumor response in EBV+ tumor cells. In HL, downregulation is more common in EBV- cases, which indicates that EBV+ tumor cells use alternative mechanisms. In chapter 6 we evaluated the expression of HLA class I, HLA class II, CLIP, HLA-DM and HLA-DO in EBV positive cHL and undifferentiated nasopharyngeal carcinoma (NPC), to explore if the peptide binding groove of HLA class II is blocked binding to EBV-derived antigenic peptides by retention of CLIP in the binding groove.
References:


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