CHAPTER ONE

INTRODUCTION AND SCOPE OF THE THESIS

1 General Introduction

1.1 The immune system

The human immune system consists of a wide variety of different types of cells and organs. Immune responses start upon recognition and end in eradication or limitation of a pathogen. Immune responses are not limited to pathogens, but also include immune responses towards antigenic peptides derived from infected, defective, premalignant and malignant cells. The immune system includes several layers, starting from a physical barrier such as the epithelial barrier, to innate and adaptive immunity. Innate immunity provides the first immune response against pathogens that pass the physical barriers. Macrophages, neutrophils, eosinophils and natural killer (NK) cells\(^1\) together with the complement system and inflammatory cytokines provide an initial general response against such pathogens. NK cells play a critical role in the initial innate immune responses by the induction of apoptosis of virally infected cells. The innate immune system excites the adaptive immune response. The adaptive immunity is divided into two types of immune responses, i.e. humoral and cell mediated immunity. In contrast to the general response induced by the innate immunity, the adaptive immunity induces an antigen specific immune response. Humoral immunity is based on production of specific antibodies directed against a specific epitope or protein of a microbial agent,
while cell mediated immunity mainly targets intracellular antigens. These two forms of adaptive immunity are mediated by B-cells and T-cells.

B-cells mature in the bone marrow and express a specific B-cell receptor (BCR), i.e. a specific immunoglobulin (Ig) molecule. After emerging from the bone marrow B-cells enter the circulation and the secondary lymphoid organs such as lymph nodes and spleen. When B-cells encounter foreign antigens in the lymph nodes or spleen, presented by follicular dendritic cells a germinal center (GC) reaction is initiated. During this process, B-cells proliferate and undergo somatic hypermutation (SHM) of Ig heavy and light genes, to produce high affinity antibodies against the antigens. This is followed by Ig class switch recombination (CSR) resulting in conversion of IgM+ B-cells to other isotypes such as IgG. B-cells that produce high affinity antibodies are positively selected and mature to plasma cells or memory B-cells. Plasma cells are professional producers of a specific antibody in high amounts. Memory B-cells mediate secondary immune responses upon re-infection of the same pathogen. The majority of the GC B-cells fail to produce high affinity antibodies and will undergo apoptosis.

T-cells mature in the thymus and express a specific T-cell receptor. Based on expression of CD4 and CD8, T-cells are further subdivided into T-helper (Th) cells and cytotoxic T-cells (CTLs), respectively. Th cells provide assistance to B-cells and macrophages, while CTLs recognize and eradicate damaged cells through binding to HLA class I. Th cells are further subdivided into T-helper 1 (Th1), T-helper 2 (Th2), T follicular helper (TFH) cells, regulatory T (Treg) and natural killer T (NKT) cells (Table 1). TFH cells reside in secondary lymphoid tissues and play a critical role in the maturation of B-cells during the GC reaction. Selection of high affinity antibody producing B-cells and their conversion to memory B-cells or plasma cells is mediated by TFH cells.

Both Th and CTLs can be subdivided in naïve and memory T-cells. There are three subpopulations of memory cells that can be distinguished by expression of CCR7 and CD45RA: central memory cells (TCM) CD45RA-CCR7+, effector memory cells (TEM) CD45RA-CCR7-, and terminally differentiated effector cells (TEMRA) CD45RA+CCR7-. TCM cells mainly reside in the secondary lymphoid organs and upon re-infection, proliferate and differentiate into effector cells. TEM cells immigrate into inflamed tissues and have a direct effector function against antigens. TEMRA cells arise late during the immune response and, although they are still functional, have limited proliferative capacity.
### Introduction and scope of the thesis

#### Table 1.1: Different sub-types of T-cells

<table>
<thead>
<tr>
<th>Type of Cell</th>
<th>Th1</th>
<th>Th2</th>
<th>TFH</th>
<th>Treg</th>
<th>CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main Function</strong></td>
<td>Activation of macrophages and CTLs</td>
<td>Help B cells and switch antibody isotype production</td>
<td>Maturation of B cells</td>
<td>Regulation of immune responses</td>
<td>Kill virus-infected cells, tumor cells</td>
</tr>
<tr>
<td><strong>Membrane Markers</strong></td>
<td>CD4, CXCR3</td>
<td>CD4, ST2L, CCR4</td>
<td>CD4, PD-1 ICOS, CXCR5</td>
<td>CD4, CD25</td>
<td>CD8</td>
</tr>
<tr>
<td><strong>Transcription Factors</strong></td>
<td>T-bet, Stat1, Stat6</td>
<td>GATA3, Stat5, Stat6</td>
<td>Bcl-6, c-MAF</td>
<td>FoxP3, Stat5</td>
<td>Eomes, T-bet, Id2, BATF</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td>IFN-γ, LT-α, TNF</td>
<td>IL-4, IL-5, IL-6, IL-13</td>
<td>IL-21</td>
<td>TGF-β, IL-10</td>
<td>IFN-γ, TNF, LT-α</td>
</tr>
</tbody>
</table>

#### 1.2 Lymphoma

Lymphomas originate from malignant transformation of lymphocytes, usually B-cells. T-cell and NK cell derived lymphomas are less common. The first lymphoma type that was recognized is Hodgkin lymphoma (HL). All the other lymphoma types have been collectively called non-Hodgkin lymphomas (NHL) and comprise various subtypes (like diffuse large B-cell lymphoma, follicular lymphoma and small lymphocytic lymphoma) as well as various T-cell lymphomas.11

#### 2 Hodgkin lymphoma

HL was originally described by Thomas Hodgkin in 1832.12 It is a unique type of lymphoma with a low number of neoplastic cells that comprise less than 1% of the total cell population. Neoplastic cells are found in a background of immune cells that consists of T-cells, eosinophils, neutrophils, plasma cells, fibroblasts and histiocytes. Despite their abundance, these immune cells are not able to induce an effective anti-tumor response. Instead the microenvironment protects neoplastic cells from anti-tumor immune responses and provides survival signals for the neoplastic cells.13,14 Based on differences in histopathology, morphology, epidemiology and phenotype of the neoplastic cells, HL is divided into classical HL (cHL) and nodular lymphocyte predominant HL (NLPHL).11
2.1 Classical Hodgkin Lymphoma

CHL accounts for 95% of all HL cases. It often presents with painless enlarged lymph nodes in the cervical region or the mediastinum along with fever. Over time the disease can gradually spread to other lymph nodes. The incidence of cHL is 3 per 100,000 per year, with \( \sim 30\% \) Epstein Barr virus (EBV) positive cases in western countries. The age incidence curve is bimodal with one peak in young adults around the age of 25 years and a second peak in people above 55 years. EBV+ HL is found especially in patients below 10 years of age and patients older than 60 years.

CHL is divided into four subtypes based on differences in histopathology: nodular sclerosis (NS), mixed cellularity (MC), lymphocyte depleted (LD) and lymphocyte rich (LR). The NS type accounts for two third of all cHL cases and these cases are characterized by nodules separated by fibrotic bands. The microenvironment is composed of lymphocytes, eosinophils and neutrophils. Approximately 14\% of NS cases are EBV positive. MC is the second most common type of cHL with 10-20\% of all cases in western countries. The microenvironment of MC is characterized by a heterogeneous mixture of small lymphocytes, neutrophils, histiocytes and plasma cells. The MC subtype has the highest frequency of presence of EBV with \( \sim 70\% \). LD is a rare form of cHL, which along with an obvious depletion of lymphocytes, shows reticular and/or diffuse fibrosis. LR is also rare, and is distinguishable from the other types by low numbers of HRS cells in a background of small mature lymphocytes. In some cases of LR cHL, small GC with HRS cells in the mantle zones have been found.

CHL patients are treated with chemotherapy alone or in combination with radiotherapy. The 5- and 10-year overall survival rates of cHL patients are quite good with 81\% and 74\%. Disease-specific survival rates at 5, 10, and 15 years were 86\%, 82\% and 80\%. Younger patients and early stage patients are more likely to respond to treatment as compared to older and late stage (III, IV) patients.

Several lines of evidence indicate a strong genetic risk component in the development of cHL. Siblings of HL patients have an increased risk of development of cHL with a much higher risk in monozygotic as compared to heterozygotic twins. Specific HLA-A alleles have been associated with susceptibility of Epstein Barr virus (EBV) positive cHL, whereas several HLA class II alleles have been associated with susceptibility of cHL and EBV-negative cHL. In addition, GWAS studies in cHL indicated that besides multiple associations with variants at the HLA locus, there are associations with specific non-HLA variants, i.e. rs1432295 (REL), rs2019960 (PVT1), rs501764 (GATA3) and rs1860661 (TCF3).
The neoplastic cells of cHL are large cells with one or more nuclei originating from crippled GC B-cells. The mononucleated cells are called Hodgkin cells and the multinucleated cells are called Reed-Sternberg cells. These Hodgkin and Reed-Sternberg (HRS) cells have lost expression of common B-cell markers, such as CD20 and BCR. Instead, HRS cells express non B-cell markers such as CD30 and CD15.

Constitutive activation of NF-κB is a hallmark of HRS cells. In addition, these cells frequently show amplification of the tyrosine kinase gene JAK2. HRS cells in EBV+cHL are characterized by expression of EBNA1, LMP1 and LMP2. LMP1 contributes to survival and proliferation of HRS cells by mimicking the active CD40 receptor. LMP2 mimics the BCR and is critical for survival of GC B-cells that lack expression of the BCR.

Microenvironment of cHL

HRS cells are well known for the ability to shape their microenvironment by production of specific chemokines and cytokines. The cells in the microenvironment can provide survival signals for the HRS cells through production of cytokines and/or through expression of specific membrane ligands. The T-cells that are in the close vicinity of the HRS cells are mainly CD4+CD26- with an anergic phenotype or a suppressive function. The T-cells in direct contact with the HRS cells, i.e. the so-called rosetting T-cells, are anergic cells and provide a physical barrier for active immune cells. The other cells of the microenvironment such as Treg cells protect HRS cells from immune responses through suppression of the immune system.

2.2 Nodular lymphocyte predominant Hodgkin Lymphoma

NLPHL is a rare type of HL comprising approximately 5% of all cases. NLPHL starts with enlargement of lymph nodes in the upper part of the body, which gradually extends to other secondary lymphoid organs. NLPHL occurs at all ages with a peak incidence at the age of 30-40 years. The 5- and 10-years OS rates are 91% and 83%. Disease-specific survival rates are 95%, 93%, and 88% at 5, 10, and 15 years respectively. Younger age and early stage patients show a better response to treatment.

The neoplastic cells of NLPHL are called lymphocyte-predominant (LP) cells. These cells originate from GC B-cells and express known markers of B-cells such as CD20 and BCR. LP cells, also known as popcorn cells, have a broad cytoplasm and a lobulated appearance of the nuclei. Constitutive activation of the NF-KB pathway is a common
feature in LP cells and it contributes to their survival\textsuperscript{39}. About 3\% of all NLPHL cases transform into diffuse large B-cell lymphomas\textsuperscript{40}.

NLPHL has two classic morphologic patterns: nodular and diffuse\textsuperscript{41}. In the nodular pattern LP cells settle in a small B-cell rich microenvironment, while in the diffuse pattern LP cells reside in a T-cell rich environment\textsuperscript{41}.

**Microenvironment of NLPHL**

The microenvironment of LP cells consists mainly of B-cells and CD4+ T-cells which are present around LP cells. LP rosetting cells probably play a critical role in the microenvironment. These cells show expression of CD57 and transcription factors c-Maf\textsuperscript{42}, BCL6\textsuperscript{43} and MUM1\textsuperscript{44}. These CD57+CD4+ rosetting cells have increased expression of INF\textsubscript{γ} and lack expression of IL-2 and IL-4\textsuperscript{42}. In addition, CD4+ T-cells present in the microenvironment are characterized by expression of CD45RO and low expression of CD45RB, which indicates a memory T-cell phenotype\textsuperscript{45}. The other cells population present in the microenvironment of NLPHL in high levels, are CD4+CD8+ double positive T-cells\textsuperscript{46}.

### 3 Scope of the thesis

Although several studies on immune cells of the microenvironment in HL have been reported, the overall picture is not yet complete. A main knowledge gap in our current understanding of the role of the microenvironment is the functional capacity of the cells observed in the microenvironment. The rosetting cells located within the tumor cell area might be regarded as survivors of the suppressive nature of the tumor cells and might very well behave different from the non-rosetting cells outside the tumor cell area.

In this thesis we focused on the characterization of the composition of the microenvironment of cHL and NLPHL. In **chapter 2**, we reviewed the literature and present an overview of the currently known interactions of cells in the microenvironment with the HRS cells in cHL. In **chapter 3** we studied the composition of the microenvironment of both EBV+ and EBV- cHL by flowcytometry. We focused on the main immune cell types and on specific T-cell subpopulations, such as Th1, Th2, Treg, TFH, cytotoxic CD4+ cells and CD8+ cells, along with NK cells and macrophages. In addition, we studied expression of specific activation and differentiation markers. To allow distinction of T-cells in and outside the tumor cell areas, we used co-expression of CD26.

In **chapter 4** we studied the composition of the microenvironment of NLPHL using the same approach as indicated for cHL. In **chapter 5**, we specifically studied the
CD4+PD-1+CD57+/- rosetting cells in the microenvironment of NLP HL. Our aim was to define if these cells are TFH cells using a combination of markers by flow and by immunohistochemistry and immunofluorescent staining. In chapter 6 in addition to a general summary and discussion, the microenvironment of cHL and NLP HL are compared to each other.
References


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