The microenvironment of Hodgkin lymphoma
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The most intriguing characteristic of Hodgkin lymphoma (HL) is the very low number of tumor cells in an extensive background of immune cells. This feature is present in both classical HL (cHL) and Nodular Lymphocyte Predominant HL (NLPHL). The neoplastic cells influence the nature and functionality of the cells in the microenvironment to create a favorable environment\(^1\). Characterization of the microenvironment will thus provide insight in the pathogenesis of HL.

This PhD thesis is dedicated to studying the microenvironment of both cHL and NLPHL. We specifically aimed to determine the nature of the infiltrating immune cells present in the microenvironment of HL in comparison to reactive lymph nodes. To achieve this goal we determined the abundance of the main and specific subpopulations of immune cells relevant for the interaction with the tumor cells. We focused on cells located in the tumor cell rich areas as compared to cells located outside the tumor cell areas.
Summary and discussion

1 Microenvironment of cHL

In chapter 2 we reviewed current knowledge about the microenvironment of cHL with the aim to characterize the currently known interactions of the microenvironment and HRS cells. In the first part we describe the role of survival pathways activated via signals of the immune cells in the microenvironment. Activation of pathways such as NF-κB and JAK/STAT are mediated by interactions of ligands, receptors and cytokines. Ligands such as CD30L, expressed by eosinophils and mast cells and CD40L, expressed by T-cells, interact with CD30 and CD40 receptors on the membrane of HRS cells resulting in activation of the NF-κB pathway. Secretion of interleukins such as IL-3 by Th2 cells, eosinophils and mast cells, and IL-7 by HRS cells, results in activation of the JAK/STAT pathway through IL-3R and IL-7R. Activation of these pathways along with some other pathways results in induction of survival and proliferation of HRS cells. Next, we describe how HRS cells shape the microenvironment. One main mechanism used by HRS cells is attraction of immune cells via production of chemokines. For instance CCL17 and CCL22, two chemokines expressed by HRS cells, attract Treg and Th2 cells. In addition, also cells in the microenvironment secrete chemokines that lead to attraction of other cells. For example CCL5, expressed by T-cells, recruits mast cells and macrophages. In the final part we discuss the interactions that contribute to the escape from an effective anti-tumor immune response. Examples of such interactions are PD-L1 and FASL, both expressed by HRS cells. PD-L1 stimulates the PD-1 receptor on T-cells and results in exhaustion of activated T-cells. FASL induces apoptosis in Th1 and CD8+ cells. Moreover, HRS cells have often lost HLA class I and/or II expression, which results in lack of antigen presentation to immune cells and consequently avoidance of adaptive immune responses. In addition, Treg cells present in the microenvironment of cHL as well as production of IL-10 by the HRS cells results in an immunosuppressive environment, which favors escape from effective anti-tumor immune responses.

Together, it is evident that interactions between the cells of the microenvironment and HRS cells are an essential component of HL. In other words, in the pathogenesis of cHL not only the HRS cells, but also the microenvironment plays an essential role.

Recently it has been suggested that Th1 cells are the dominant cells of the microenvironment of cHL based on expression of T-bet and chemokines receptors such as CXCR3 and cytokines such as IL-21. Others suggested Th2 cells to be the major component of the microenvironment of cHL. Therefore the subtype of involved CD4+ cells is still a matter of debate. In chapter 3 we focused on the composition of the microenvironment in cHL as compared to reactive lymph node (RLN). We focused on putative differences.
between EBV+ and EBV- cHL and also analyzed potential differences in the composition of the microenvironment in and outside the tumor cell area. Analysis of the main populations of RLN and cHL revealed no significant differences in the percentages of B-cells, T-cells, NK, NKT-cells and macrophages. The percentages of Th1, Th2 and TFH cells are similar in cHL cases compared to RLN. The percentages of CD25+Foxp3+CD4+ and CD25+GITR+CD4+ Treg cells, and cytotoxic CD8+ cells, including GranzymeB+CD8+ and TIA+CD8+cells are all increased in cHL cases.

The percentages of NK cells and CTLs were lower in EBV- as compared to EBV+ cHL. The lower percentage of CTLs is interesting as there are only very few ‘foreign proteins’ known in HRS cells of EBV- cHL, while in EBV+ cHL antigenic peptides of LMP1, LMP2 and EBNA viral proteins can be presented. Moreover, loss of HLA class I expression is uncommon in EBV+ cHL and quite common in EBV- cHL. The increased number of CTLs in EBV+ cHL suggests effective triggering of the immune response, which might not be effective due to production of cytokines like IL-10, which is more pronounced in EBV+ cHL cases. In addition, in EBV+ cHL cases, CCL20 is expressed by HRS cells and is involved in migration of Treg cells into the tumor cell area, which might protect the HRS cells against an anti-tumor immune response.

In order to discriminate between the cells around the HRS cells from the cells that are not in the tumor cell rich areas, we included CD26 as a marker in our flow analysis. CD26 is expressed predominantly by the cells outside of the tumor cell area, while it is absent on the cells in close vicinity to the HRS cells. This CD26 pattern is observed for all cHL subtypes, with the exception of mixed cellularity subtype of cHL. Therefore, we excluded the mixed cellularity from these analyses. CD4+ T-cells expressing the early activation marker CD69 and regulatory CD4+ T-cells are observed predominantly in the tumor cell area, whereas CD8+ T-cells expressing the late activation marker CD25 are present predominantly outside the tumor cell area. Presence of a higher level of early activated CD4+ T-cells and Treg cells in the tumor cell area suggests a model in which Treg cells prevent complete activation of CD4+ T-cells. The high numbers of late activated CD8+ T-cells located outside the tumor cell area have no access to HRS cells and also fail to evoke an effective anti-tumor immune response.

2 Microenvironment of NLPHL

In chapter 4 we studied the composition of the microenvironment of NLPHL. Analysis of the main cell populations revealed decreased levels of B-cells and NK cells, and a
higher level of CD4+ T-cells. TFH cells and CCR7-CD45RA-CD4+ effector memory T (TEM) cells are increased in the microenvironment of NLPHL, whereas the percentages of Treg cells and activated NK cells are decreased compared to RLN.

Similar to our study in cHL, we also used expression of CD26 to discriminate the cells around the LP cells from those that are not in the tumor cell areas. We show that the percentages of early-activated CD4+ and CD8+ T-cells are increased in the tumor cell area compared to the percentages out of the tumor cell areas. On the other hand CD25+ Treg cells and late activated CD25+CD8+ T-cells are increased outside the tumor cell area. The early activation marker CD69 also is a marker for tissue-resident memory T-cells. These tissue resident memory T-cells play a role in immune suppression. The absence of Foxp3+ Treg cells, which are regulators of the germinal center reaction, results in enlargement of germinal centers. Consistent with this, we indeed found increased numbers of PD1+ TFH cells in the tumor cell areas. TFH cells 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. Absence of NK cells and presence of late activated CD8+ T-cells outside of the LP cell area along with increased Treg cell percentages will attenuate immune responses. The flow results were partly validated by immunohistochemistry (IHC) of NLPHL and RLN tissue sections. This revealed presence of CD8+ cells to be equal inside and outside the tumor area, while expression of CD26 and CD25 is mainly outside the tumor. Foxp3+ Treg cells are totally absent in the tumor area. These data indicate lack of late activation or cytotoxic cells in close vicinity to LP cells.

In chapter 5 we further studied the location and the type of TFH cells in NLPHL using IHC. We also included the CD57 marker in these analyses as CD4+CD57+ T-cells have been reported to form rosettes around the LP cells. In addition, these rosetting T-cells also express PD-1 and BCL6. Our study revealed a high number of CD4+PD-1+Bcl6+ cells in the LP cell rich area, with expression of CD57 in part of these cells. These cells specifically form rosettes around LP cells. These data suggest a microenvironment with increased levels of TFH cells, especially as LP rosetting cells. Therefore we concluded that TFH cells have a crucial role in the microenvironment of NLPHL.

Overall, TFH cells and tissue-resident memory cells are both involved in the protection of LP cells by forming a protective layer precluding direct cell-cell contact of the LP cells with CTLs, and by downregulation of the immune responses. As a consequence reduced numbers of NK cells and CTLs residing in the tumor cell areas that only express early and not late activation markers are found.
3 The comparison of the microenvironments of cHL and NLPHL

The findings as indicated above, show several differences between the microenvironment of cHL and NLPHL. To gain a more complete picture we performed a direct comparison between these two entities. In total we found significant differences in eight cell populations, including naïve CD4+ cells, Foxp3+CD4+, PD-1+CD57+-/ TFH, CXCR5+ICOS+CD4+TFH, CD25+CD4+, CXCR3+CD8+ and CD56+CD3- NK cell sub-sets (Figure 6.1). In cHL we found more CD45RA+CCR7-CD4+ cells, naïve CD4 cells than in NLPHL. There are more Foxp3+CD4+ and CD25+CD4+ cells in cHL than in NLPHL. These cells control the anti-tumor immune response. The microenvironment of NLPHL is full of PD-1+CD4+ and PD-1+CD57+CD4+ TFH cells. TFH cells are known for providing survival signals for germinal center B-cells. Their presence as LP rosetting cells suggest that they provide survival signals for LP cells. This might be achieved by production of IL-21, since LP cells have been shown to have increased expression of the IL-21 receptor\textsuperscript{13}. In cHL, the percentage of ICOS+CXCR5+CD4+ TFH cells is increased, this seems contra-intuitive since germinal center structures in cHL are completely lost. Moreover, HRS cells themselves produce IL-21\textsuperscript{14,15}, so they are not depended on TFH cells for IL-21 production. Maybe because the environment contains IL-21 there are more TFH cells formed\textsuperscript{9}. In NLPHL, the TFH cell population appears to have lost ICOS and only PD-1+CD4+ TFH cells are present. ICOS is necessary for the expression of CXCR5 and BCL6 and plays a role in T-cell activation and IL-4 production.

CHL also contains more CXCR3+CD8+ cells and CD56+CD3-cells. CXCR3+CD8+ show enhanced immunity to cancer after stimulation with IL-15\textsuperscript{16} and presence of these cells correlates with improved survival in melanoma patients\textsuperscript{17}. CD56+CD3- NK cells were found increased in EBV+ cHL, suggesting that the presence of EBV antigens triggers NK cells.

For the analysis including the CD26 marker (see chapters 3 and 4), we observed higher level of CD25+ Treg cells outside the tumor cell area in NLPHL while Treg numbers in cHL are higher inside the tumor cell area. In NLPHL, especially the percentage of Foxp3+CD25+CD4+ are very low in the tumor cell area. It has been shown that in the absence of Treg cells germinal centers are enlarged\textsuperscript{18}, which is associated with an increased number of TFH cells. Early activated CD8+ T-cells are higher in the LP cell area in NLPHL, but show no difference in cHL.
Some populations show a similar pattern in cHL and NLPHL. For example the presence of CD69+CD4+ cells is higher in the tumor area of both HL subtypes. In general, CD69+ CD4 T-cells are able to downregulate immune responses. CD69+CD4+CD25-cells suppress CD4+ T-cell proliferation in a cell-cell contact dependent manner and in addition support growth of the tumor in mice models of melanoma, liver and lung cancer. CD69+CD4+ cells might play a similar role in cHL and NLPHL cases. Another similarity is the high number of late activated CD25+CD8+ cells outside the tumor cell area in both types of HL. Expression of CD25, part of the IL-2 receptor, results in proliferation and differentiation of CD8+ T-cells into effector cells which have cytotoxic activity. Since the late-activated CD25+CD8+ T-cells do not have access to the tumor cells, no CTL-mediated immune responses will be directed against the tumor cells. Presence of early-activated CD69+CD8+ T-cells in tumor cell areas implies that these cells are not able to convert to the late-activated form and therefore are not able to evoke cytotoxic activity against neoplastic cells.

Together, these data suggest that there are both differences and similarities in the mechanisms by which cHL and NLPHL tumor cells manage to escape from an effective anti-tumor response (Figure 6.2). In cHL TH1 and/or TH2 along with Treg cells and early activated cells and CCR7-CD45RA-CD4+ TEM cells form the main population of cells in the HRS cell area. While in NLPHL, CD57+/CD57- TFH cells are the dominant cells around LP cells along with early-activated CD69+CD4+, CD69+ CD8+ cells and CCR7-CD45RA-CD4+ TEM cells. Out of the tumor cell area of cHL, the cells are mainly late-activated CD8+, Treg cells, CCR7-CD45RA-CD4+TEM cells and NK cells. In NLPHL the cells which are located out of the tumor area are mainly Treg cells, late activated CD8+ cells and CCR7-CD45RA-CD4+ TEM cells.
Figure 6.1: Overview of cell populations that show significant differences between NLPHL and cHL. The line indicates the median percentage of the indicated cell population as measured by flow cytometry. EBV+ cHL are shown in black and EBV- cHL in red. Percentages of (A) CCR7+CD45RA+ in CD4+ cells, (B) Foxp3+ in CD4+, (C) CD25+ in CD4+, (D) PD-1 in CD4+, (E) PD-1+CD57+ in CD4+, (F) CXCR3+ICOS+ in CD4+, (G) CXCR3+ in CD4+, (H) CD56+ in CD3-. *: $P < 0.01$, **: $P < 0.001$. P-values < 0.01 are considered to be statistically significant. The Mann-Whitney U test was applied for assessing the difference of two groups. The line indicates the median.
**Summary and discussion**

**Figure 6.2:** Schematic representation of the microenvironments of cHL (left) and NLPHL (right). HRS cell and LP cell are shown in center left and right respectively. The microenvironment of both cHL (left) and NLPHL (right) is divided into two areas: The area close to neoplastic cells is mentioned by “in tumor cell area” and area far from tumor cell area is called “out of tumor area”. Only cell types that show differences are shown. Early activated CD4+ T-cells (CD69+CD4+), TH1 cells (CXCR3+CD4+), TH2 (ST2L+CD4+), Treg (Foxp3+GITR+CD25+CD4+), CD4+ TFH (ICOS+/−), CTL (CXCR3+CD8+), NK (CD56+CD3−) cells.
Chapter 6

4 Future perspectives

The interaction between neoplastic cells and the cells in the microenvironment is mediated by two main mechanisms: direct cell-cell contact and interactions via soluble proteins secreted by either neoplastic cells or cells of the microenvironment. Studying these mechanisms in HL could provide novel insights in its biology. A comparative model including both normal and HL involved tissue may provide insight on how the microenvironment is shaped and/or controlled by neoplastic cells.

To gain further insight into the interactions relevant for the pathogenesis of NLPHL, we suggest to further characterize TFH cells in NLPHL, with a specific focus on the functionality of these cells. Studying subsets of PD-1+ TFH subsets based on presence of CD57 and/or ICOS could help to understand how these cells contribute to the survival of LP cells. As a first step we could sort TFH cells of NLPHL, cHL and RLN tissue cell suspensions and generate gene expression profiles. In a follow-up experiment, we could specifically sort CD26+ and CD26- TFH cells. After determining the differentially expressed genes, a validation might be done at the protein level using IHC or WB techniques. A similar approach could be followed for the Treg cell subsets sorted from cHL cell suspensions. Treg cells might be sorted based on expression of known markers of Treg cells such as FOXP3, GITR and CTLA4. Regarding the low number of Treg cells in total cell population, studying the gene expression of these cells before and after activation might be a proper suggestion for determining the probable differences in these cells compared to control cells from RLN Treg cells. Using the list of differentially expressed genes, we can identify alterations in specific pathways or gene ontologies in NLPHL or cHL as compared to each other or in comparison to RLN. Possible follow-up experiments might include IHC of ligands, receptors or cytokines related to the identified pathways/gene ontologies. This type of experiments will yield further insight on the nature of the interactions between the microenvironment and the neoplastic cells in HL.

To gain further insight in the functionality of TFH cells, a co-culture experiment with germinal center B-cells or neoplastic cells might yield information on survival and proliferation effects on the neoplastic cells. These experiments will indicate whether the main normal function of TFH cells is still intact. In case TFH cells of NLPHL are not or less functional as compared to RLN derived TFH cells, this might indicate that the LP cells have modulated their effectivity. To determine if these changes are reversible we can activate the TFH cells of NLPHL and RLN with anti-CD3 antibodies and then co-culture them with germinal center B-cells. In case activated TFH cells indeed show
higher effectivity as compared to the non-activated TFH cells, we can try to identify the responsible genes by gene expression profiling.

Another cell type of interest to study in more detail in NLPHL are the CTLs. The fact that the CTLs are located in an area consisting of TFH cells, suggests that these cells might have been influenced by the TFH cells. Therefore it would be worthwhile to study the interaction of TFH cell and CTLs. Co-culturing of CTLs of NLPHL with TFH cells from the same case or from RLN, will reveal the putative TFH cell-induced effects on the activation of CTLs and vice versa the putative effects of the CTLs on the TFH cells.

Progressively transformed germinal centers (PTGC), as often present in NLP involved lymph nodes. PTGCs sometimes precede the diagnosis of NLPHL in prior lymph node biopsies. PTGC is a benign disorder of RLN and is associated with enlargement of germinal centers with small B-cells and CD4+CD57+ cells\textsuperscript{22,23}. Presence of CD4+CD57+ TFH cells in combination with enlarged germinal centers, and the association with NLPHL suggest PTGC as a pre-stage of NLPHL. This suggests that there might be similarities in functionality and nature of the TFH cells and germinal center B-cells in NLPHL and PTGC. In order to determine the probable similarities between PTGC and NLPHL, TFH cells, Treg cells and germinal center B-cells might be sorted from NLPHL, PTGC and RLN. Then as first step the gene expression profile of the cells from NLPHL and PTGC might be compared with RLN and later on with each other. Similarities in gene expression of germinal center B-cells from PTGC and LP cells from NLPHL might indicate that under some circumstances germinal center B-cells of PTGC might convert into LP cells. In addition, differences might also suggest some candidate genes that might act as drivers for LP precursor cells as neoplastic cells. Studying the gene expression of TFH cells would also provide valuable information about the role of TFH cells in NLPHL. Since TFH cells have specific T-cell receptors and recognize antigen in the context of HLA\textsuperscript{24}, it would be interesting to analyze if specific T-cell clones are present in NLPHL. This might provide an explanation for the enlargement of germinal center and appearance of high numbers of TFH cells. Gene expression profiling of Treg cells might explain inability of Treg cells in performing their role in controlling germinal center reaction.

One of the candidate cell types that might be considered for functional follow-up studies in cHL are the Treg cells. Several studies have shown that the percentage of Tregs is different compared to RLN\textsuperscript{3,22}. This implies that these cells are specifically attracted by the neoplastic cells in cHL, by production of certain chemokines and cytokines such as CCL17 and IL-21\textsuperscript{14,23}. The main functionality of Treg cells is to suppress immune responses. To study functional differences, it might be of interest to determine
their capacity to suppress the activation of CD4+ and CD8+ T-cells upon co-culture experiments. Changes in activation levels of CD4+ and CD8+ cells might be measured by checking relevant membrane markers such as CD25, CD69, CD154 and CD137, in addition to production of cytokines such as IFN-gamma, IL-2 and TNF-alpha.

Since there are controversial results about the nature of the CD4+ T-cells in the microenvironment of cHL\textsuperscript{1,2}, it might be of interesting to further characterize these cells by extending the number of variables in flowcytometry from 4 to 10 to gain more information of different markers. In addition, co-culture of RLN derived naïve CD4+ T-cells with HRS cells followed by gene expression profiling, might provide insight into the mechanism HRS cells apply to shape the nature of the CD4+ T-cells.

To further characterize the cHL microenvironment future studies should also include the different histological subtypes, i.e. nodular sclerosing (NSHL), mixed cellularity (MCHL), lymphocyte rich (LRHL) and, lymphocyte depleted LDHL\textsuperscript{25}. As EBV is present in a proportion of the NS and MC subtypes, it would be advisable to further separate these two entities. This will be challenging especially for the non-NS subtypes as the number of cases are limited. Therefore we should explore collaborative studies to get sufficient cases in each of the subgroups. In case we can include sufficient patient numbers, we can also try to determine if increases or decreases of specific cell populations is associated with disease outcome. Another potential interesting aspect that might be studied in more detail is the composition of the blood cells of HL patients with active disease. For some diseases changes in the blood composition has been linked with response to treatment\textsuperscript{26}. The level of TEM cells, one of the cell populations increased in NLPHL, is increased in the blood of patients with gastric cancer\textsuperscript{26}. These or other cell types may be altered and possibly associated to prognosis in HL.
References


Chapter 6


