Chapter 6

General Discussion
6.1. A model of IP-DLBCL lymphomagenesis

The data presented in this thesis have been placed in a broader context of immune privilege and immunoediting, resulting in a model that tries to explain why certain DLBCL arise specifically in immune-privileged sites and how these lymphomas interact with their environment, eventually leading to a specific IP-DLBCL phenotype.

**IP-DLBCL are highly immunogenic and survive only in immune-privileged sites**

In Chapter 3 an average frequency of somatic hypermutation of the immunoglobulin heavy chain (IgH) variable region of 18% in testicular DLBCL was reported and in Chapter 4 a frequency of 23% in CNS DLBCL. These frequencies are comparable to those reported in other series of testicular\(^1\) and CNS DLBCL\(^2-4\) and are considerably higher than those in non-IP DLBCL which range from 6% to 11%\(^5,6\). The knowledge that the idiotype of B-cell lymphomas can be recognized as a tumor-associated antigen\(^7,8\) leads to the hypothesis that a high frequency of IgH mutations makes a lymphoma highly immunogenic and that these lymphomas would benefit from the absence of an anti-tumor immune response in an immune-privileged site.

Localization of a lymphoma in an immune-privileged site can be either an active or a passive process. In the active process the lymphoma cell selectively ‘homes’ to the immune-privileged site by means of expression of specific adhesion molecules or chemokines. Expression of integrin LFA-1 and its ligand ICAM-1 has been reported in CNS lymphoma\(^9\) as well as expression of CXCL\(^\text{13}\) (BCA-1) and its receptor CXCR\(^5\)\(^10-12\). However these molecules are also expressed in lymph nodes\(^13\), cutaneous lymphoma\(^14\) and gastric MALT lymphoma\(^15\) and therefore probably have a less specific role in attracting B cells to extranodal sites in general, stimulating the formation of an ectopic lymphoid microenvironment\(^16\) and facilitating local dissemination. Testicular DLBCL lack expression of most adhesion molecules\(^17\) and expression levels of CXCR\(^5\) in testicular DLBCL were significantly lower than in nodal DLBCL (unpublished result of the global gene expression analysis in Chapter 2). Based on these observations, there is no solid evidence supporting selective homing of lymphomas to the CNS or the testis. In the proposed model the localization of a lymphoma in an immune-privileged site is a passive process, in which the highly immunogenic lymphoma cells with a high frequency of IgH mutations are only able to survive in the protective environment of an immune-privileged site while they are eliminated by the immune system everywhere else.

**Progressive growth of IP-DLBCL disturbs immune privilege, leading to an influx of immune cells**

Historically immune privilege was thought to be equal to immune ignorance, caused by physical and physiological barriers that prevented exchange of antigens and immune cells between immune-privileged sites and the systemic immune system. It is now known that
immune privilege is actively maintained and that multiple mechanisms suppress the immune response and promote tolerance in immune-privileged sites.

Both CNS and testis possess a blood-organ barrier. The blood-brain barrier protects the brain from metabolites and toxins and prevents extensive influx of immune cells into the brain. It is however not completely impermeable for such cells, as macrophages and CD4-positive T cells are found in the perivascular spaces under normal circumstances. Also peripherally activated T cells express integrins that enable them to migrate across the blood-brain barrier.¹⁸ The blood-testis barrier protects the sperm cells from the immune system, but the interstitial space, that also is considered immune privileged, is not protected by this barrier.¹⁹ So in both CNS and testis other mechanisms must be present that regulate the immune response. Both organs contain two populations of macrophages/dendritic cells. One is a residential macrophage population, which is rarely replenished, is incapable of stimulating T-cell proliferation and has reduced antigen presenting capacity. This population is thought to have an immunoregulating role, maintaining immune privilege. The other is an ‘inflammatory’ population of macrophages (and in CNS also dendritic cells) that is rapidly replenished from the circulation and has full antigen presenting capabilities. In the CNS, microglia make up the residential population. The composition of the total population of macrophages/dendritic cells determines whether immune privilege can be maintained or is overcome by an inflammatory response. The mechanism regulating this composition is not completely clear, but probably involves local cells. In the CNS astrocytes and neurons regulate the microglial immune function, while in the testis cytokines produced by somatic cells such as Leydig cells, Sertoli cells and peritubular cells possibly play a role. Other mechanisms that can alter the threshold for an inflammatory response are neurodegeneration (pro-inflammatory) and androgen production (anti-inflammatory).

Because immune privilege is such a delicately balanced system, it is very plausible that a growing lymphoma would disturb this balance, leading to a breach of immune privilege. One possible effect is disruption of the blood-organ barrier, facilitating the influx of T cells and APCs. Disruption of the blood-brain barrier could cause an uncontrolled entry of metabolites and toxins into the brain, leading to neuronal stress and activation of the innate immune response.¹⁸ The lymphoma could also cause tissue damage. In the CNS, damage to the neurons will alter the regulation of the immune response. In the testis, tissue damage can result in the release of heat shock proteins that act as ‘danger signals’ and will enhance dendritic cell maturation.¹⁹ To induce its own growth and progression a lymphoma could also release pro-inflammatory cytokines such as IL-6 or TNF.²⁰ The presence of a substantial number of activate T cells in IP-DLBCL provides evidence for a breach of immune privilege.²¹ The percentages of activated T cells as compared to the total number of T cells are also significantly higher in IP-DLBCL (44–64%) than those in non-IP DLBCL (24%), reflecting the postulated higher immunogenicity of IP-DLBCL.
IP-DLBCL undergo immunoediting and escape the immune response

After immune privilege is breached the IP-DLBCL will undergo immunoediting under pressure of the anti-tumor immune response, resulting in an immune escape phenotype. The frequent loss of HLA class I and II expression in IP-DLBCL may act as an immune escape mechanism in these lymphomas, which is supported by the finding that in some cases only part of the tumor cells show loss of expression and/or deletions of the HLA region.\(^22\) In chapter 2 it is shown that loss of expression of HLA class II is associated with downregulation of numerous genes involved in inflammation and with a lower number of tumor-infiltrating T cells. It is important to note that HLA expression in this study was measured as expression of both lymphoma cells and cells from the lymphoma microenvironment together. When in another study T-cell numbers were compared between testicular DLBCL with and without HLA protein expression on the lymphoma cells only, there was no significant difference.\(^21\) This could indicate that a loss of HLA class II expression on tumor cells alone is not sufficient to escape the immune response, because surrounding APCs that express MHC class II can still present the tumor-associated antigens through cross-presentation. The effects of the downregulation of HLA class II were independent from the mechanism causing the downregulation. This observation raises the questions why the IP-DLBCL so frequently have deletions of the HLA region and why the mechanisms of HLA class II downregulation differ between IP-DLBCL and non-IP DLBCL.\(^23\) One hypothesis is that these deletions, in particular the large hemizygous deletions, have an additional effect on the expression of non-HLA genes located in the deleted region that would be beneficial for the specific circumstances of IP-DLBCL. However it is shown in chapter 2 that this is not the case. Another explanation could be that in IP-DLBCL HLA alleles which are genetically more fragile and therefore more susceptible to deletions are overrepresented, although a study on HLA-DR and HLA-DQ alleles in 50 IP-DLBCL, 48 nodal DLBCL and 2400 normal cases failed to find evidence for the significant association of overrepresented alleles with deletions in testicular DLBCL.\(^24\) A third possible explanation, fitting the proposed model, might be that the high immunogenicity of IP-DLBCL requires a more permanent means of HLA downregulation, as opposed to the less immunogenic non-IP DLBCL that often use some form of (reversible) transcriptional control like the downregulation of CIITA.\(^23\)

The case report presented in chapter 4 sheds some more light on the process of immunoediting in IP-DLBCL. The primary CNS DLBCL showed a low number of infiltrating T cells and macrophages, indicating a low immune pressure. This lymphoma had only lost HLA class II expression. There was a much larger number of infiltrating T cells and macrophages present in the testicular biopsy, reflecting a stronger anti-tumor immune response. When the CNS lymphoma clone (which was only adapted to a low immune pressure) arrived in the testis the equilibrium phase started, in which the stronger immune system was able to suppress lymphoma growth for a long period of time. Only after 8 years the immunoediting process resulted in a lymphoma that had adapted to the stronger immune pressure by downregula-
tion of both HLA class I and II and thus could escape the immune response to become clinically manifest.

Also in a larger series of IP-DLBCL the CNS DLBCL showed a lower number of infiltrating T cells and lower frequency of LOH in the HLA region than testicular DLBCL.\textsuperscript{21,25} If the immune pressure in CNS DLBCL is indeed generally lower than in testicular DLBCL this could explain why relapse from the testis to the CNS is more frequent than vice versa.

\textbf{6.2 IP-DLBCL can be considered as a separate subentity of DLBCL}

The first step towards a subclassification of DLBCL into IP- and non-IP DLBCL was made by proposing an IP-DLBCL-specific model of lymphomagenesis. Further arguments for this subclassification are found in \textsc{chapters 3 and 5} of this thesis. Here it is shown that there is a notable difference in the ABC/GCB immunophenotype of IP- and non-IP DLBCL. While in non-IP DLBCL the distribution between the ABC and GCB subtypes is reasonably balanced, in IP-DLBCL the ABC subtype is much more frequent (90% in testicular and 78% in CNS DLBCL). Subtyping of IP-DLBCL using gene expression profiling was unambiguous but a significant proportion of ABC-type testicular DLBCL showed an ambiguous protein expression pattern of both CD10 and MUM1, which is rare in non-IP DLBCL.\textsuperscript{26,27} This illustrates the importance of distinguishing IP-DLBCL from non-IP DLBCL: since the most widely used immunohistochemistry classifier\textsuperscript{26} is based on a population of non-IP DLBCL, it does not accommodate for this expression pattern and would have misclassified these cases as GCB-type DLBCL.

The association of certain genomic aberrations with either IP- or non-IP DLBCL (\textsc{chapter 5}) also supports the proposed subclassification. The presence of aberrations that were associated with only CNS DLBCL or only testicular DLBCL indicates there would still be some heterogeneity in the proposed IP-DLBCL subtype and might reflect the differences in immune pressure as discussed in the previous paragraph.

In conclusion, this thesis shows that IP-DLBCL have specific molecular characteristics that probably underlie their specific localization and clinical behavior. The interaction between the lymphoma and its microenvironment further shapes the IP-DLBCL geno- and phenotype. These findings underwrite the subclassification of DLBCL into IP- and non-IP DLBCL.
6.3 Recommendations for further research

Target gene analysis of the genomic aberrations in Chapter 5 showed that DLBCL use all three means of immune escape: avoiding recognition, suppressing the immune response and resisting apoptosis induced by immune cells. Some aberrations were site-specific, supporting the view that the mechanisms used to accomplish immune escape differ between IP- and non-IP DLBCL. Immunohistochemical analysis of the described candidate genes and their downstream targets is needed to affirm their proposed role in immune escape and fluorescence in situ hybridization (FISH) analysis could confirm the genomic aberrations. Because both techniques are suitable for the analysis of paraffin-embedded tissue, the number of cases can be increased. This would allow analysis of target genes for IP- and non-IP DLBCL separately, to investigate whether immune escape mechanisms indeed differ between IP- and non-IP DLBCL.

Since genomic aberrations are only present in the lymphoma cells and not in the cells of the microenvironment, analysis of gene expression changes associated with these aberrations focuses on the intrinsic properties of the lymphoma cells. In the IP-DLBCL lymphomagenesis model however it is believed that the interactions between the lymphoma and the microenvironment determine the outcome of the immunoediting process and the immune escape mechanism that is chosen. Global gene expression profiling on whole tissue sections includes information on the microenvironment. This analysis was performed in Chapter 5 using gene set enrichment analysis (GSEA) but biologically meaningful differences between CNS, testicular and nodal DLBCL could not be extracted from the large lists of significant genes. If these data are to be used for further analysis a functional reduction of the number of significant genes would probably be beneficial, such as the exclusion of genes that are not related to immunity or the exclusion of genes that are associated with genomic aberrations and thus with lymphoma-intrinsic effects and not with the effect of the microenvironment.

Another, probably more promising approach to investigate the interaction between the lymphoma and its microenvironment would be immunohistochemistry using specific markers for lymphocyte and macrophage subsets and antibodies against immune (escape)-related proteins such as cytokines, costimulatory proteins and proteins involved in apoptosis.

A new area of research that has developed over the past few years investigates the role of microRNAs in B-cell development and lymphomagenesis (reviewed in ref. 28). MicroRNAs are non-coding RNA’s of ~22 nucleotides that function primarily as translational repressors by binding to complimentary target sequences in the 3’ untranslated region of mRNA molecules. A current study on a small set of 15 microRNAs with known involvement in B-cell development or lymphomagenesis in a series of IP- and non-IP DLBCL showed a higher expression of MIRN127, potentially targeting Bcl6,29 in testicular DLBCL and a higher expression of MIRN17-5p, shown to target pro-apoptotic gene E2F1,30 in CNS DLBCL (J.L. Robertus, unpublished data).
As discussed, different techniques are used to investigate different aspects of the interaction between IP-DLBCL and the microenvironment. Combining the results from these techniques will give more insight into the biology of IP-DLBCL. To get an even more complete overview of IP-DLBCL as a separate subgroup of DLBCL, comparisons could be made not only with nodal DLBCL but also with extranodal non-IP DLBCL such as gastric or cutaneous DLBCL.
References


