9.1. Summary

In this thesis, the genetic features of different LBCL subtypes were studied with the goal to identify targetable genetic lesions with a specific focus on immune evasion mechanisms.

PCNSL and PTL are two poor-prognosis LBCLs with insufficient responses to current therapies. For this reason, we sought to determine targetable genetic lesions in these LBCL subtypes. In chapter 2, we comprehensively characterized CNAs, mutations and chromosomal rearrangements in the LBCL subtypes, PCNSL and PTL, and compared these genetic signatures to those of DLBCL and PMBL. We defined CN alterations by using HD-SNP analyses and identified the putative driver genes of these CN alterations by integrating CN and gene expression data in a discovery cohort of 21 PCNSLs, 7 PTLs and 11 PMBLs. In addition, we evaluated the mutational spectrum by performing WES on the subset of PCNSLs with available paired normal specimens (n=5). To increase sample size, we performed RNA-Seq on an additional 9 PCNSLs. We also studied the mutational spectrum of 6 PTLs from the discovery cohort with RNA-Seq. Furthermore, we extended the WES bait set with custom hybrid capture probes covering 49 candidate genetic loci frequently implicated in chromosomal rearrangements in hematologic malignancies and performed targeted sequencing on 24 PCNSLs and 7 PTLs from the discovery cohort. Given the relatively small discovery cohort, we obtained additional FFPE samples of 43 EBV negative PCNSLs, 7 EBV positive PCNSLs and 43 PTLs and validated the most frequently found CN alterations with qPCR and FISH. Mutations were validated with qPCR and Sanger sequencing and protein expression was examined with IHC.

Our CN analyses of the PCNSL and PTL discovery cohort showed frequent CN gains of 3q12.3, 18q21.33 and 19q13.42 and CN losses of 6p21.33, 6q21 and 9p21.3. CN gain of 9p24.1 was more frequent in PTL and PMBLs. Integrative analysis revealed BCL2 as putative driver of 18q21.33 CN gain and FIZ1 as a potential driver of 19q13.42 CN gain. The frequency of these CN gains was validated in the PTL validation cohort. In total, 23/50 (46%) and 33/50 (66%) PTL patients had CN gains of BCL2 and FIZ1, respectively. The HLA class I loci, HLA-B and HLA-C, were most tightly linked to the 6p21.33 CN loss.

We showed frequent, often high-grade, CN loss of 9p21.3/CDKN2A as a genetic feature in 15/21 (71%) and 44/50 (88%) of PCNSLs and PTLs, respectively, resulting in cell cycle deregulation. The overall complexity of the CNA pattern of PCNSL and PTL was similar to that in DLBCL. In contrast, PMBLs showed a paucity of CNAs.
Gain-of-function MYD88L265P mutations were among the most common mutations in PCNSL and PTL, found in 33/55 (60%) and 38/49 (78%) of cases, respectively. Of note, we identified NFKBIZ as additional oncogenic modulator of the TLR/NF-κB pathway. The gene most closely associated with 3q12.3 CN gain was NFKBIZ, encoding for IκB-ζ. IκB-ζ is induced by TLR signaling and activates the NF-κB pathway. We assessed the functional consequences of NFKBIZ gain using a panel of informative LBCL cell lines. We genetically depleted IκB-ζ in cell lines with MYD88 mutation alone (TMD8), MYD88 mutation and NFKBIZ copy gain (HBL1) or NFKBIZ copy gain alone (Ly4). This genetic depletion decreased cellular proliferation, induced apoptosis and reduced expression of the IκB-ζ target genes, IκB-α and BCL-xL. We also overexpressed IκB-ζ in cell lines with wild-type MYD88 and no NFKBIZ copy gain. This enforced expression of IκB-ζ conferred a growth advantage, suggesting that NFKBIZ copy gain functions as an alternative mechanism to activate oncogenic TLR signaling in these LBCLs.

In addition to MYD88L265P mutations, frequent CD79BY196 mutations were seen in PCNSL and PTL in 19/50 (38%) and 22/45 (49%), respectively. These mutations were mostly concurrent with MYD88 mutations (~90% in both lymphoma subtypes). This prevalence of co-occurring CD79B and MYD88 mutations is significantly higher in PCNSLs and PTLs than in systemic DLBCLs.

We also identified a genetic basis of immune evasion through deregulated PD-1 ligand expression by 9p24.1/PD-L1/PD-L2 CN gains in 33/63 (52%) and 26/50 (54%) of the PCNSL and PTL patients, respectively. Also, infrequent chromosomal rearrangements were found in 4/66 (6%) and 2/50 (4%) of PCNSLs and PTLs, respectively. We confirmed the role of viral infection in upregulation of the PD-1 ligands; increased PD-1 ligand expression in EBV positive PCNSLs was largely CN independent. These findings are of interest given the response rates of cHL to PD-1 blockade and suggest that PCNSLs and PTLs may be amenable to targeted treatment with antibodies targeting PD-1.

These findings led us to investigate PD-1 ligand deregulation in different LBCL subtypes, including TCRBCL and immunodeficiency-related LPDs. In chapter 3, we described the incidence of PD-L1/PD-L2 genetic alterations and PD-1 ligand expression in the morphologic DLBCL subtype, TCRBCL. We performed FISH of the 9p24.1/PD-L1/PD-L2 loci and in each case, 50 nuclei were counted per patient, allowing the assessment of clonality of 9p24.1/PD-L1/PD-L2 genetic alterations in each sample. Nuclei with a target:control probe ratio of 1:1 but more than two
copies of each probe were defined as polysomic for chromosome 9p, those with a probe ratio of 1:1 but <3:1 were classified as relative copy gain and those with a probe ratio of >3:1 were assigned as amplified. Cases were classified by the highest observed level of 9p24.1/PD-L1/PD-L2 genetic alteration. Normal CNs (disomy) of these loci were seen in 2/28 (7%), polysomy in 8/28 (39%), CN gain in 10/28 (36%) and amplification in 6/28 (21%) of primary TCRBCLs. Chromosomal rearrangements of the PD-1 ligands were seen in 2/28 (7%) cases. In relapsed TCRBCL, CN gain was seen in 4/6 (66.7%) and amplification in 2/6 (33.3%) cases. Increased PD-L1 expression was associated with higher-level 9p24.1 genetic alterations. These findings suggest that TCRBCL patients might be susceptible to PD-1 blockade.

In chapter 4, we examined 9p24.1/PD-L1/PD-L2 genetic alterations and PD-1 ligand expression in immunodeficiency-related LPDs. A total of 94 cases spanning features of the morphologic entities DLBCL, TCRBCL and cHL across different immunodeficiency settings were collected. PD-L1 and PD-L2 IHC was performed on 59 B-cell LPDs and expression of PD-L1 was shown in 22/22 (100%) DLBCLs, 13/13 (100%) cHLs, 4/4 (100%) Hodgkin-like LBCLs, 7/8 (88%) plasmablastic lymphomas and 9/12 (75%) polymorphic B-cell LPDs. PD-L1 expression was seen across all immunodeficiency settings, including autoimmune/iatrogenic, HIV, primary immunodeficiency and posttransplant. PD-L2 expression was only sporadically observed. We then performed 9p24.1/PD-L1/PD-L2 genetic analyses with the FISH assay on a representative subset of 23 cases. Of these cases, 19 were classified as DLBCL, 2 cases were composite cHL/DLBCL and 2 cases were classified as TCRBCL. A spectrum of the different above-mentioned immunodeficiency settings was included and 16/23 (70%) of the cases were EBV positive. We showed disomy in 5/23 (22%), polysomy in 6/23 (26%), CN gain in 8/23 (35%) and amplification in 2/23 (9%). Two cases had chromosomal rearrangements of the PD-1 ligands. 9p24.1/PD-L1/PD-L2 genetic alterations were observed across all immunodeficiency settings and in both EBV positive and EBV negative cases. These findings indicate that the various morphologic appearances of LBCLs in immunodeficient patients have a shared genetic basis of immune evasion through PD-1 ligand deregulation.

Genetic studies in cHL have been challenging, given the unique cellular composition of this disease. For this reason, the precise incidence, nature and prognostic significance of 9p24.1/PD-L1/PD-L2 genetic alterations were unknown. In chapter 5, we studied chromosome 9p24.1/PD-L1/PD-L2 genetic alterations in primary cHL and determined its prognostic significance. In collaboration with Stanford University, a uniformly treated cHL cohort of 108 cases with long-term follow-up was assembled. The PD-L1/PD-L2 FISH assay was used and in each case, the percent
and magnitude of 9p24.1 amplification, copy gain, polysomy, and normal CNs (disomy) were noted. In this study, we showed that 9p24.1/PD-L1/PD-L2 genetic alterations are a near-universal feature of cHL, occurring in 97% of diagnostic biopsy specimens. We revealed a range of genetic alterations from polysomy 5/108 (5%) to relative copy gain 61/108 (56%) and amplification 39/108 (36%). We showed that in addition to the highest-level alteration, by which these cases are classified, lower-level alterations were present in residual HRS cells within the same patient. In patients with amplification, lower percentages of residual disomic cells were seen and patients with decreased residual disomic cells had higher PD-L1 expression. In addition, we explored the association of 9p24.1/PD-L1/PD-L2 alterations with clinical parameters. As expected, PFS was shorter in advanced stage patients. By exploring PFS in the different 9p24.1/PD-L1/PD-L2 genetic categories, we demonstrated that PFS was significantly shorter for cHL patients with 9p24.1 amplification. We also showed that the incidence of 9p24.1 amplification was increased in advanced stage patients. Both advanced stage disease and 9p24.1/PD-L1/PD-L2 amplification retained prognostic significance in respective univariate models and by adding the clinical factor advanced stage disease and 9p24.1/PD-L1/PD-L2 amplification together in a multivariable model, this genetic alteration further delineated PFS. These near-universal 9p24.1/PD-L1/PD-L2 genetic alterations likely explain the remarkable activity of PD-1 blockade in this disease.

In chapter 6, we analyzed 9p24.1/PD-L1/PD-L2 genetic alterations and PD-1 ligand expression in the multi-center, multi-cohort phase II registration trial of relapsed/refractory cHL patients treated with nivolumab (anti-PD1). We showed that all patients had detectable 9p24.1/PD-L1/PD-L2 genetic alterations and associated increased PD-1 ligand expression. The level of 9p24.1/PD-L1/PD-L2 genetic alterations and PD-L1 H-scores (percentage of malignant cells with positive staining [0% to 100%] multiplied by the average intensity of positive staining in HRS cells [1 to 3+]) was significantly linked to best overall responses and more favourable PFS; patients with amplification and higher PD-L1 H-scores had better overall responses and a longer PFS following PD-1 blockade, suggesting that these factors may play a role in predicting responses to PD-1 blockade.

In chapter 7, we explored additional immune evasion strategies in the uniformly treated cHL cohort. We assessed β2M, MHC class I and MHC class II expression in these cases. Cases were classified as positive, decreased or negative for each marker. HRS cells were positive for β2M in 17/108 (16%), decreased in 29/108 (27%), negative in 57/108 (53%) and not evaluable in 5/108 (5%) of cases. Positive MHC class I expression was seen in 19/108 (18%), decreased expression in
34/108 (31%), absent expression in 51/108 (47%) and 4/108 (4%) of cases was not evaluable. The association between $\beta_2$M and MHC class I expression was highly significant, indicating that $B2M$ alterations are the most common mechanism for MHC class I loss. MHC class II expression on HRS cells was positive in 34/108 (31%), decreased in 40/108 (37%), negative in 32/108 (30%) and not evaluable in 2/108 (2%) of cases. After expression levels were determined, we studied the association of defined clinical and biological factors. We showed that patients with decreased/absent $\beta_2$M/MHC class I expression had significantly shorter PFS. In a multivariable model with the known adverse prognostic factors: advanced stage and 9p24.1 amplification, decreased/absent $\beta_2$M/MHC class I expression retained adverse prognostic significance. In contrast, decreased/absent MHC class II was unrelated to outcome. These findings highlight the biological importance of MHC class I-mediated antigen presentation by HRS cells to cytotoxic T-cells for optimal clinical response to standard induction therapy.

Overall, the findings in this thesis suggest that certain LBCL subtypes have shared genetic bases to evade an effective anti-tumor immune response that may be amenable to targeted treatment. In chapter 8, the findings of these studies are discussed and the clinical implications and future studies are outlined.