Genetic basis of PD-L1 overexpression in diffuse large B-cell lymphomas

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KEY POINTS

• Translocations between PD-L1 and the IGH locus, represent a genetic mechanism of PD-L1 overexpression in DLBCL.

• Genetic alterations in the PD-L1/PDL-2 locus are mainly associated with the non-GCB subtype of DLBCL.
ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is one of the most common and aggressive types of B-cell lymphoma. Deregulation of proto-oncogene expression following a translocation, most notably to the immunoglobulin heavy chain locus (IGH), is one of the hallmarks of DLBCL. By whole genome sequencing analysis, we have identified the PD-L1/PD-L2 locus as a recurrent translocation partner for IGH in DLBCL. PIM1 and TP63 were also identified as novel translocation partners for PD-L1/PD-L2. Fluorescence in situ hybridization was furthermore used to rapidly screen an expanded DLBCL cohort. Collectively, a subset of samples was found to be affected by gains (12%), amplifications (3%) and translocations (4%) of the PD-L1/PD-L2 locus. RNA sequencing data coupled with immunohistochemistry revealed that these cytogenetic alterations correlated with increased expression of PD-L1 but not of PD-L2. Moreover, cytogenetic alterations affecting the PD-L1/PD-L2 locus were more frequently observed in the non-germinal center B-cell like (non-GCB) subtype of DLBCL. These findings demonstrate the genetic basis of PD-L1 overexpression in DLBCL and suggest that treatments targeting the PD-1–PD-L1/PD-L2 axis might benefit DLBCL patients, especially those belonging to the more aggressive non-GCB subtype.
INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the one of the most common and aggressive forms of B-cell lymphomas. At least 2 subtypes of DLBCL can be distinguished by gene expression analysis: the germinal center B-cell like (GCB) and the activated B-cell like (ABC) subtypes, with the latter being characterized by increased disease aggressiveness and worst clinical outcomes. In recent years, the incorporation of anti-CD20 therapeutic antibodies into the standard Cyclophosphamide, Doxorubicin, Vincristine, Prednisolone (CHOP) treatment has contributed to an overall improvement of patient survival. In spite of this, disease relapse is often refractory to the currently available therapies, which marks the necessity for the development of new therapeutic approaches for DLBCL.

Recently, the genomic characterization of DLBCL has been performed in a number studies employing next generation sequencing (NGS) technologies. These studies, along with earlier ones, contributed to the identification of a set of genes/pathways that are frequently targeted by mutations in DLBCL. Moreover, whole genome sequencing (WGS) and RNA sequencing (RNAseq) enabled the identification of novel structural variations (SVs) in DLBCL involving the TP63 and CIITA genes. These translocations may contribute to tumorigenesis in addition to the few well-described, recurrent SVs such as rearrangements involving the proto-oncogenes BCL6, BCL2 and MYC and the immunoglobulin heavy chain (IGH) gene locus.
Translocations between \textit{IGH} and proto-oncogenes usually result in upregulation of the latter by bringing them under the control of the potent \textit{IGH} Eμ and 3’ enhancers\textsuperscript{19}. These events, especially in GC-related B cell lymphomas, have been suggested to be mainly triggered by aberrant, off target activity of activation-induced cytidine deaminase (AID)\textsuperscript{20,21}. The physiological role of AID is to deaminate cytidine residues within the variable (V) or switch (S) regions of the \textit{IGH} locus, which is essential for the somatic hypermutation (SHM) or class switch recombination (CSR) processes respectively\textsuperscript{22,23}. During CSR, AID-induced mismatches lead to double-strand breaks (DSBs) in the S regions that are resolved by one of the two major DSB repair mechanisms, the non-homologous end-joining (NHEJ) pathway\textsuperscript{24}. We have previously suggested that defects in the NHEJ pathway might be associated with the formation of translocations involving the \textit{IGH} locus\textsuperscript{25}.

PD-L1 and PD-L2 are signaling molecules expressed on the surface of antigen-presenting cells\textsuperscript{26}. Upon interaction with their receptor PD-1 on effector T-cells, they transmit a negative regulatory signal that leads to functional anergy of the T-cells\textsuperscript{27}. Various malignancies have been shown to overexpress PD-L1 in order to escape T-cell mediated killing\textsuperscript{28-30}.

In recent years, the introduction of drugs targeting the PD-1–PD-L1/PD-L2 immune-modulatory pathway has shown promising results in the treatment of aggressive malignancies such as melanoma, renal cancer and lung cancer\textsuperscript{31}. A study on Hodgkin lymphoma (HL) demonstrated that up to 87% of patients with refractory disease responded to PD-1 blockade\textsuperscript{32}. Accordingly, the chromosome 9p24.1 cytoband, which includes the adjacent \textit{PD-L1} and \textit{PD-L2} genes, was found to be rearranged to the V regions of the \textit{IGH} locus in a HL derived cell line\textsuperscript{33}. In non-Hodgkin lymphomas, alterations affecting the 9p24.1 cytoband were observed in a subset of DLBCLs\textsuperscript{33}, but more frequently, in several specific types of large B-
cell lymphomas, including primary mediastinal large B-cell lymphomas (PMBCL), primary testicular lymphoma (PTL) and primary nervous system lymphoma (PNSL) \(^{33-35}\). \(PD-L1\) overexpression was furthermore observed in the aggressive ABC/non-GCB subtype of DLBCL \(^{36,37}\). Moreover, a recent study performed on DLBCL-derived cell lines, demonstrated that PD-1 blockade restores T-cell function \textit{in vitro} \(^{38}\). Together, the PD-1–PD-L1/PD-L2 axis may constitute a target for immunotherapy in refractory and aggressive DLBCL.

To investigate the incidence of structural variants affecting the \(PD-L1/PD-L2\) locus in DLBCL and to further understand the genetic mechanisms of \(PD-L1\) deregulation in these tumors, we performed WGS, RNAseq and cytogenetic analysis in samples from several cohorts of DLBCL patients. Copy number gains, amplifications and translocations targeting the \(PD-L1/ PD-L2\) locus were observed in a subset of tumor samples, which resulted in overexpression of the \(PD-L1\) gene.
METHODS

Patient cohorts

Chinese cohort

Frozen material corresponding to tumor biopsies from 176 Chinese DLBCL patients were obtained from the Sun Yat-Sen University Cancer Center and the Tianjin Medical University Cancer Institute and Hospital. Matching peripheral blood DNA samples were available for 76 patients. The Epstein-Barr virus (EBV) active infection status was assessed in a subset of samples using Chromogenic in situ hybridization. Additionally, a tissue microarray (TMA) with 100 Chinese DLBCLs was purchased from US Biomax inc (Rockville, MD).

Swedish cohort

The work described in this manuscript has been approved by the Institutional Review Board and the Swedish Central Ethical Review Board. Samples from 64 Swedish DLBCL patients were collected at the Uppsala University Hospital. Formalin-fixed paraffin-embedded (FFPE) tissue was available and tumor tissue cores were organized in TMAs. DNA and RNA from 3 samples were extracted from the FFPE blocks.

American cohort

FFPE tissue sections were available from 26 DLBCL samples from the database of the Herbert Irving Comprehensive Cancer Center. Frozen tissues from 2 tumors were used for the extraction of DNA and RNA.
These three cohorts of samples were reviewed by experienced pathologists in their respective centers. PMBCLs were excluded based on site of involvement, morphology and in some cases an additional immunostaining against CD30. Samples were further classified as GCB and non-GCB by immunohistochemistry (IHC) according to the Hans algorithm. For a subset of American samples, GCB and ABC classification based on gene expression analysis was also available. A summary of the experiments performed, results and available data is provided in Fig. S1.

**DNA extraction, WGS and analysis of SV**

DNA was isolated either with the DNeasy Tissue and Blood Kit (Qiagen, Venlo, Netherlands) or the Recoverall Total Nucleic Acid Isolation kit (Ambion-Life technologies, Carlsbad, CA). WGS was performed using either the Illumina HiSeq 2000 or Hiseq X10 platform (Illumina, San Diego, CA). SeekSV, an in-house method was used for the detection of SVs. Further details are provided in the supplemental materials and methods.

**RNA extraction, RNAseq and quantitative real-time PCR (qPCR)**

Total RNA was extracted either with the RNeasy kit (Qiagen) or the Recoverall Total Nucleic Acid Isolation kit. Library preparation and the RNAseq method on the Illumina HiSeq 2000 platform have been described before. The number of fragments per kilobase of transcript per megabase of mapped reads (FPKM) was used to determine relative gene expression levels. Detection of fusion transcripts was enabled by SOAPfuse. RNAseq data was also used for the classification of the two disease subtypes, GCB and ABC based on a published set of genes. Information about qPCR is provided in supplemental materials and methods.
Detection of cytogenetic alterations by fluorescence in-situ hybridization (FISH)

Fluorescent probes flanking the PD-L1/PD-L2 locus were generated from bacterial artificial chromosome clones RP11-963L, RP11-12D24, RP11-207C16 and RP11-845C2, labeled with spectrum green and orange dUTPs (Enzo lifesciences, Farmingdale, NY). Vysis LSI IGH (Abbott Molecular, Abbott Park, IL) break-apart rearrangement probes were used for analysis of the IGH locus. Treatment and probe hybridization were performed as previously described. Further details are provided in the supplemental materials and methods.

Detection of PD-L1, Human leukocyte antigen (HLA) class I and CD8 by IHC

The antibodies used for IHC were a PD-L1 monoclonal antibody (#13684, clone E1L3N, Cell signaling, Beverly, MA), a PD-L2 polyclonal antibody (SAB3500395, Sigma-Aldrich, St. Louis, MO), a CD8 monoclonal antibody (M7103, clone C8/144B, DAKO) and the HLA I assessing antibodies HCA2 and HC10 (Nordic MUbio, Susteren, Netherlands). Further details are provided in the supplemental materials and methods.
RESULTS

WGS reveals rearrangements involving the PD-L1/PD-L2 locus

WGS was performed on DNA from 20 Chinese DLBCL biopsies and their respective paired normal samples in two sets of experiments (Fig. S2A, B). The mean sequencing depths were 39.5x and 35.1x for the first and second set respectively. The percentage of bases covered by at least 10 reads was 97.7% for the first and 97.9% for the second set. Structural rearrangement analysis demonstrated the presence of balanced, somatically acquired translocations between the IGH and the PD-L1 loci in one of the samples, DL48. This is, to our knowledge, the first time that PD-L1 is identified as a fusion partner for IGH in DLBCL. The finding of WGS was subsequently validated by breakpoint-specific PCR analysis using primers surrounding the fusion points between the two loci (Fig. 1A, B). The breakpoint within the IGH locus was situated in the Sμ region, which was already rearranged to the Sγ1 region through CSR, whereas on chromosome 9 the breakpoint was located in the intron separating the 6th and 7th exons of the main transcript of PD-L1 (NM_014143). Of note, AID-targeting motifs (WRC/GYW) were furthermore identified on both sides of the breakpoints, suggesting that illicit activities of AID during CSR might be responsible for the initiation of this translocation event (Fig. S3). FISH analysis on FFPE slides derived from the DL48 tumor showed the presence of split signals of PD-L1 and IGH loci in 52% and 46% of the nuclei counted, respectively. This supports the presence of the rearrangement in the main tumor clone. An additional FISH experiment employing four-color probes further demonstrated the fusion between the two loci and the balanced nature of the rearrangements (Fig. 1C).
The fusion transcript that resulted from the translocation of the *PD-L1* and *IGH* loci was subsequently identified by RNAseq, and confirmed by PCR and Sanger sequencing. This transcript contained the first 6 exons of *PD-L1* together with the 3rd exon of *IGHG1*. The last exon of *PD-L1*, which was missing from this fusion transcript, encodes for the final 7 amino acids of the peptide chain, located in the intracellular domain, without any known function. RNAseq reads for each *PD-L1* exon (Fig. S4A) and an allele-specific qPCR approach further illustrated that the majority of *PD-L1* expression in this tumor originated from the fusion transcript, suggesting that the translocation is a driver event for *PD-L1* expression (Fig. S4B).

The WGS analysis of the 20 Chinese DLBCLs furthermore discovered a second translocation juxtaposing the *PD-L1/PD-L2* locus to *TP63*. This translocation was also balanced and the breakpoints were located between the 5th and 6th exons of *PD-L1*, and the 1st and 2nd exons of *TP63* (Fig. S5A, B). Moreover, a number of copy number alterations (CNA) were discovered and are summarized in Fig. S6.

**Cytogenetic analysis on extended DLBCL cohorts**

We employed a FISH assay in order to identify additional cytogenetic alterations affecting the 9p24.1 cytoband where the *PD-L1* and *PD-L2* genes are located adjacent to each other. A total of 179 samples, derived from 3 cohorts of patients (Fig. S1), were analyzed. Of those, 23 cases (13%) presented with copy number gains in the *PD-L1/PD-L2* locus, 3 cases (2%) with amplifications and 6 cases (3%) with split signals indicative of translocations. Among the latter 6 samples, split signals were also observed at the *IGH* locus in 3 samples (including
Representative examples of cytogenetic alterations identified by FISH are presented in Fig. 2A. The collective results from the 190 samples of which the status of the PD-L1/PD-L2 locus was analyzed by FISH and/or WGS identified 23 gains (12%), 5 amplifications (3%) and 7 translocations (4%) (Fig. 2B). In samples that underwent both FISH and WGS, the data concerning the status of the PD-L1/PD-L2 locus was concordant (Fig. S6).

Information about the molecular subtype was available for 141 of the samples analyzed by FISH and/or WGS. Results from all cohorts showed that cytogenetic alterations affecting the PD-L1/PD-L2 locus, including copy number gains, amplifications and translocations, are more frequent in the non-GCB subtype (n=24/88, 27% vs. 3/53, 6% in GCB-type cases; Fisher’s exact test, \( P=0.0016 \)), Fig. 2C). Notably, translocations and amplifications in the PD-L1/PD-L2 locus were exclusively found in non-GCB samples (Fig. 2C) or ABC samples in the American cohort where GCB/ABC classification based on gene expression array was available.

**WGS of selected samples in the expanded cohort**

To further characterize the additional samples presenting a break or amplification of the PD-L1/PD-L2 locus identified by the FISH analysis, WGS was performed on tumor samples from 4 of the 7 affected patients, for which DNA was available (SL24, SL55, 2168 and 2171). A mean sequencing depth of 32.2X was achieved and 96.4% of the genome was covered by at least 10 reads (Fig. S2C). WGS and breakpoint specific PCR confirmed the existence of translocations in the PD-L1/PD-L2 locus in two of these samples (2168 and SL55) and identified the translocation partners. Sample 2168 had a breakpoint in the intergenic region...
between PD-L1 and PD-L2. Interestingly, the translocation partner here was PIM1, a proto-oncogene that is recurrently mutated in DLBCL and constitutes a target of aberrant SHM (Fig. S5C, D). The translocation was balanced, resulting in 2 fusion chromosomes joining PD-L1 with the telomeric half and PD-L2 with the centromeric part of PIM1 respectively. In sample SL55, the location of the breakpoint in the PD-L1/PD-L2 locus was at the 3’ end of PD-L2 (Fig. S5E). The translocation partner was IGH (Sμ), thus bringing the total number of PD-L1/PD-L2 – IGH translocations to 2. In the rearrangements identified in samples 2168 and SL55, the entire PD-L1 and/or PD-L2 genes are being juxtaposed to their translocation partners and thus no fusion transcripts are generated (confirmed by RNAseq analysis). In one additional sample, SL24, breaks in the PD-L1/PD-L2 and IGH loci were identified by WGS, in accordance with the FISH data. However, we were not able to validate it by breakpoint specific PCRs, probably due to DNA degradation in this sample. Copy number variation analysis from the WGS data confirmed the amplification of the PD-L1 locus in sample 2171.

**PD-L1/PD-L2 gene expression analysis**

RNAseq was performed on 85 samples including all samples that underwent WGS and together with cytogenetic analysis. The status of the PD-L1/PD-L2 locus was known in 39 samples that underwent RNAseq. The median expression level was 9.3 FPKM for PD-L1 and 7.4 FPKM for PD-L2. In two of the three samples with proposed translocation between IGH and PD-L1/PD-L2, DL48 and SL24, the expression of PD-L1 was increased to 41.1 and 41.7 FPKM respectively. In sample DL509, where PD-L1 and TP63 were juxtaposed, the expression levels of both PD-L1 (13.8 FPKM) and TP63 (9.8 vs. a median expression level of 2.2 FPKM) were increased. In sample 2168, where the translocation between the PD-L1/PD-L2 and PIM1 loci was observed, the expression of PD-L2 and PIM1 was upregulated (Fig.
S7). A significantly higher expression of PD-L1 in samples harboring cytogenetic alterations in the PD-L1/PD-L2 locus was furthermore observed (Mann-Whitney test, P=0.0062, Fig. 3A). No association was found between PD-L2 expression and cytogenetic alterations (Fig. 3B). High expression of PD-L1 or PD-L2 does not appear to coincide or be mutually exclusive. These results were furthermore validated by complementary qPCR in 166 samples from the Chinese cohort (data not shown).

**PD-L1 and PD-L2 protein expression**

PD-L1 IHC analysis was performed in DLBCL samples derived from the American and Swedish cohorts as well as for 73 samples from the Chinese cohort. Forty-three out of 163 (26.4%) of the assessed tumors were positive for PD-L1 expression, including all samples harboring translocations or amplifications in the PD-L1/PD-L2 locus. Notably, the protein levels were very high in sample SL55 carrying the translocation between the PD-L1/PD-L2 and IGH loci (Fig. S8), whereas the mRNA levels of PD-L1 measured by RNAseq appeared to be low (Fig. S7). This may reflect the low quality of RNA of this particular sample (prepared from FFPE). By pooling together all samples that evaluated by both IHC and genetic/cytogenetic analyses, a strong association between PD-L1 protein expression and alterations in the PD-L1/PD-L2 locus was observed (Fisher’s exact test, P<0.0001; Fig. 4). Furthermore, the PD-L1 expression was more frequently observed in non-GCB as compared to GCB samples (Fig. S9). PD-L2 expression was assessed in 84 samples and in concordance with RNA expression data, no association was observed between cytogenetic alterations and PD-L2 protein expression (Fig. S10).
**HLA class I expression**

Since HLA class I expression is indispensable for antigenic recognition by the immune system, we assessed HLA class I expression in 94 DLBCL samples for which material was available. The prevalence of HLA class I loss was 45% in the Chinese-, 44% in the American- and 36% in the Swedish-cohort. Notably, 4 out of 5 samples with translocations or amplifications affecting PD-L1/PD-L2 were negative for HLA class I expression. However, no correlation was observed between the expression of HLA class I and PD-L1 across the cohorts. Loss of HLA class I expression in DLBCL is often associated with inactivation of the B2M gene, which encodes for the β chain of the HLA class I heterodimer 53,54. In 9 of the HLA class I negative samples, we were able to cross the IHC data with RNA and genomic sequencing results and found that 7 of those samples either express B2M in levels lower than the sample median, or harbor B2M mutations (identified by WGS), or both (Fig. S11). This may explain the loss of HLA class I expression in these samples.
DISCUSSION

We report here that genetic/cytogenetic aberrations involving the PD-L1/PD-L2 locus can be identified in about 20% of DLBCLs and that these alterations occur mostly in the non-GCB subtype of this disease. We further demonstrated that, to the base pair resolution, PD-L1 is a novel fusion partner of IGH in DLBCL whereas PIM1 and TP63 are previously unappreciated translocation partners for the PD-L1/PD-L2 locus. Samples with cytogenetic changes in the PD-L1/PD-L2 locus, especially those with translocations or amplifications, were more likely to overexpress PD-L1 at the mRNA and/or protein level. Previous reports of rearrangements in the PD-L1/PD-L2 locus were mainly focused on HL, PMBCL, PTL and PNSL 33-35. The latter two forms of large B cell lymphomas are located in the immune privileged sites that are subjected to a different and unique immunological context 55. Our study demonstrated the genetic basis for PD-L1 overexpression in DLBCL, no otherwise specified.

The expression of PD-L1 protein has previously been associated with rapid progression of disease in various cancers 56-58 including DLBCL 37. Our results are in general agreement with those reports and indicate that patients with a more aggressive form of disease (non-GCB) could potentially benefit from treatments targeting the PD-1–PD-L1/PD-L2 axis. Previous work also suggested that preexisting infiltration by CD8+ T-cell is a prerequisite for a successful response to PD-1 blockade therapy 59. Furthermore, the presence of neo-antigens resulting from an increased mutation load seems to enhance the T-cell infiltration 60,61. Moreover, studies from colorectal carcinoma showed that patients with impaired mismatch repair (MMR) respond better to the anti-PD-1 therapy 62. Although the mechanisms
responsible for this relation are not clear, it is hypothesized that the improved responsiveness to immunotherapy is due to an increase in the number of neo-antigens as a result of the higher mutational load associated with the impaired MMR \(^{25,63}\). Aberrant SHM as well as mutations in the MMR pathway contribute to an increase of the mutation load in DLBCL \(^{9,52}\). Notably, sample DL48, where the original \textit{PD-L1–IGH} translocation was identified, carries a somatic deleterious mutation in the \textit{MSH2} gene, a major component of MMR and shows an increased number of somatic mutations in the coding genome \(^{25}\). In addition, histological examination confirmed CD8\(^+\) T-cell infiltration in the sample. Taken together, a subset of DLBCLs, represented by DL48, is likely to respond well to the anti-PD-1 – PD-L1 therapy.

The effect of PD-1–PD-L1 blockade therapy is likely to be dependent on the expression of HLA class I molecules on the tumor cells, which are engaged by T-cell receptors on T-cells. Lack of expression of the HLA class I is thus often serving as a potent escape mechanism from the immune system \(^{64}\). HLA class I IHC in our cohort demonstrated that nearly half of DLBCL samples, including those with \textit{PD-L1/PD-L2} translocations or amplifications, have lost the expression of HLA class I. This raises the question whether anti PD-1–PD-L1/PD-L2 therapies would produce any effect on tumor cells that lack HLA class I expression. Intriguingly, a recent study performed on HL demonstrated an impressive result where the majority of patients showed lasting responses to anti-PD-1 treatment \(^{32}\), despite the loss of HLA class I being common in HL \(^{65}\).

Furthermore, we observed in our cohort that some tumors have employed two immune evasion mechanisms, loss of HLA class I expression and PD-L1 overexpression, where the
first should have already provided tumor cells with a full protection from the cytotoxic T-cell killing. However, we must take into account that DLBCLs arise from B-cells, which as professional antigen-presenting cells, also express the HLA class II. The latter presents antigenic determinants to CD4+ helper T-cells, which act by stimulating the response by other immune cells including macrophages and B-cells. Thus, the dynamics of PD-L1 expression in DLBCL could be different from that in most other tumor types due to the unique physiological function of their normal B-cell counterparts. The loss of expression of HLA class II in DLBCL has been described and was associated with increased aggressiveness and reduced immunosurveillance. Thus, the upregulation of PD-L1, even in the absence of HLA class I, may further shield DLBCL from the immune system in an HLA class II dependent manner.

Expression of PD-L1 protein was observed in all samples with translocations or amplifications. In a number of samples, however, we observed expression of the protein without any cytogenetic alterations, indicating that there are other underlying mechanisms leading to the expression of PD-L1 in DLBCL. An association between PD-L1 expression and an active Epstein-Barr virus (EBV) infection in malignant B-cells has previously been reported. We have compared the RNA and protein expression levels of PD-L1 and PD-L2 between EBV positive and negative samples and observed a trend for higher expression of PD-L1 protein in EBV positive tumors (Fig. S12). Among the 24 samples from which both EBV infection information and PD-L1 IHC data were available, 9 were positive for PD-L1, of which four harbored cytogenetic alterations. Locus status information lacked for one sample, but among the four with normal PD-L1/PD-L2 locus, only one case was EBV positive.
Therefore, although EBV infection can explain the expression of PD-L1 in some cases, it does not account for all the positive cases without cytogenetic alterations.

The cytogenetic alterations as well as protein expression of PD-L1/PD-L2 were more frequent in the non-GCB subtype. Non-malignant B-cells require activation in order to express PD-L1 \(^{69}\). Previous studies suggest that genes/pathways expressed on non-GCB samples show a similarity to those in activated B-cells \(^{2,69}\), thus these conditions may be favorable for the constitutive expression of PD-L1. Stimulation of PD-L1 expression in non-GCB DLBCL can also be the result of active \(JAK/STAT3\) signaling \(^{70}\). \(MYD88\) mutations have been found in approximately 30% of non-GCB cases and they are associated with the constitutive expression and activation of the \(JAK2\) kinase that in turn stimulates the expression of PD-L1 \(^{71,72}\). Regardless of the mechanism, the preference of expression of PD-L1 in the non-GCB/ABC disease subtype suggests that immunotherapies blocking PD-1 and PD-L1 may be promising in patients suffering from this aggressive subtype of disease. Of note, we also attempted to classify our samples into the GCB and ABC disease subtypes by RNAseq based on the set of genes described previously \(^{45}\). However, approximately one quarter of the samples could not be grouped to either GCB or ABC, as they displayed an intermediate expression pattern (Fig. S13A), suggesting that the current classification for DLBCL might still be oversimplified. In addition, we also observed a distinctive RNA expression signature in tumors overexpressing \(PD-L1\), which might also reflect an altered tumor microenvironment of these tumors (Fig. S13B). Further integration of genomic, transcriptomic and clinical data would be expected to provide us a more comprehensive picture of DLBCL.
In summary, our data indicate that genetic alterations affecting the PD-L1/PD-L2 locus, especially translocations and amplification, lead to the overexpression of the immune-modulatory factor PD-L1. In addition, our data suggest that patients with an aggressive subtype of disease may benefit from therapies blocking the PD-1–PD-L1/PD-L2 interaction. However, the factors that can predict the efficacy of these therapies are still not fully elucidated.
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AUTHORSHIP CONTRIBUTIONS

K.G. – Involved in sample preparation, performed cytogenetic analysis, Sanger sequencing, qPCR and IHC. Collected, analyzed and interpreted the data and wrote the manuscript.

L.C. – Performed bioinformatics analysis.

M.B. – Involved in sample preparation and acquisition. Involved in IHC analysis.

N.M. – Involved in IHC analysis and the writing of the manuscript.

S.L. – Performed the cytogenetic analysis.

M.F. – Performed the IHC analysis. Involved sample preparation.


C.S. – Performed IHC analysis.

M.N. and M.T. – Supervised cytogenetic analysis.

Q. P.-H. – Designed and supervised the study, interpreted the data and wrote the manuscript.
DISCLOSURE OF CONFLICTS OF INTEREST

The authors have no conflicting financial interests.
REFERENCES


FIGURE LEGENDS

Figure 1  Identification of the *PD-L1–IGH* translocation in sample DL48. A, B: The positions of the breakpoints in the two loci are indicated. The translocation is balanced, resulting in 2 distinct fusion chromosomes. Breakpoint specific PCR and Sanger sequencing confirm the WGS results. The red box indicates the PCR product with expected size. The 100bp plus ladder from Life Technologies was used. C: FISH analysis of sample DL48 shows the colocalization of the probe signals of *PD-L1* and *IGH*. The 5’ end of *IGH* appears in green whilst the 3’ end in red. The probe targeting the 5’ end of the *PD-L1/PD-L2* locus was labelled in cyan and the probe targeting the 3’ end in gold.

Figure 2  Screening for cytogenetic alterations in the *PD-L1/PD-L2* locus by FISH and WGS. FISH was performed on 179 samples and WGS on 24 samples. A number of samples underwent both analyses. In total, the status of the *PD-L1/PD-L2* locus was made known across 190 DLBCL samples. A: FISH probes and examples of cytogenetic alterations. The probes targeting the 5’ end of the *PD-L1/PD-L2* locus were labelled in red and those targeting the 3’ end in green. A split signal indicative of a translocation is characterized by the lack of colocalization of the green and red probes within a nucleus. Gain is defined as the presence of 3-4 target loci within a cell whereas amplification corresponds to 5 or more copies of the loci within a cell. B: Distribution of *PD-L1/PD-L2* translocations, gains and amplifications across the different cohorts investigated. Data acquired by FISH and WGS. C: Distribution of alterations in the *PD-L1/PD-L2* locus in the two disease subtypes across the different cohorts. Fisher’s exact test was used for comparison of the frequency of these alterations between GCB and non-GCB samples.
**Figure 3** mRNA expression data in relation to cytogenetic alterations. A, B: Expression levels of *PD-L1* and *PD-L2* measured by RNAseq, presented in FPKM values. The Mann-Whitney test was used to calculate statistical significance. **, P<0.01. The error bars represent standard error of the mean.

**Figure 4** PD-L1 IHC on 163 DLBCLs. Correlation of PD-L1 protein expression and the occurrence of cytogenetic alterations in the different cohorts expressed as a percentage of the total number of samples in each column. The Fisher’s exact test was used for statistical analysis. *, P<0.05; ***, P<0.001; ****, P<0.0001.
Figure 1.
Figure 2.

A) RP11-963L3
RP11-12D25
PD-L1
PD-L2
RP11-845C2
RP11-207C16

PD-L1/PD-L2 split signal
Paired IGH split signal

PD-L1/PD-L2 copy gain
PD-L1/PD-L2 amplification

B) Percentage of samples

0 50 100
Chinese n=100
Swedish n=64
American n=26
Total n=190

Translocation
Amplification
Copy gain
Normal locus

C) Percentage of samples

0 50 100
GCB n=16
non-GCB n=35
Chinese

GCB n=50
non-GCB n=34
Swedish

GCB n=7
non-GCB n=19
American

GCB n=53
non-GCB n=88
Total

P=0.1764
P=0.0575
P=0.2782
P=0.0016
Figure 3.
Figure 4.
Genetic basis of PD-L1 overexpression in diffuse large B-cell lymphomas

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