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ORIGINAL REPORT

PD-L1 and *PD-L2* Genetic Alterations Define Classical Hodgkin Lymphoma and Predict Outcome

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ABSTRACT

Purpose

Classical Hodgkin lymphomas (cHLs) include small numbers of malignant Reed-Sternberg cells within an extensive but ineffective inflammatory/immune cell infiltrate. In cHL, chromosome 9p24.1/*PD-L1/PD-L2* alterations increase the abundance of the PD-1 ligands, PD-L1 and PD-L2, and their further induction through Janus kinase 2–signal transducers and activators of transcription signaling. The unique composition of cHL limits its analysis with high-throughput genomic assays. Therefore, the precise incidence, nature, and prognostic significance of *PD-L1/PD-L2* alterations in cHL remain undefined.

Methods

We used a fluorescent in situ hybridization assay to evaluate *CD274/PD-L1* and *PDCD1LG2/PD-L2* alterations in 108 biopsy specimens from patients with newly diagnosed cHL who were treated with the Stanford V regimen and had long-term follow-up. In each case, the frequency and magnitude of 9p24.1 alterations—polysomy, copy gain, and amplification—were determined, and the expression of PD-L1 and PD-L2 was evaluated by immunohistochemistry. We also assessed the association of 9p24.1 alterations with clinical parameters, which included stage (early stage I/II favorable risk, early stage unfavorable risk, advanced stage [AS] III/IV) and progression-free survival (PFS).

Results

Ninety-seven percent of all evaluated cHLs had concordant alterations of the *PD-L1* and *PD-L2* loci (polysomy, 5% [five of 108]; copy gain, 56% [61 of 108]; amplification, 36% [39 of 108]). There was an association between PD-L1 protein expression and relative genetic alterations in this series. PFS was significantly shorter for patients with 9p24.1 amplification, and the incidence of 9p24.1 amplification was increased in patients with AS cHL.

Conclusion

PD-L1/PD-L2 alterations are a defining feature of cHL. Amplification of 9p24.1 is more common in patients with AS disease and associated with shorter PFS in this series. Further analyses of 9p24.1 alterations in patients treated with standard cHL induction regimens or checkpoint blockade are warranted.

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INTRODUCTION

Patients with newly diagnosed classical Hodgkin lymphoma (cHL) are currently treated with empirical combination chemotherapy regimens, such as ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine). An alternative combined modality regimen, Stanford V (doxorubicin, vinblastine, mechlorethamine, vincristine, bleomycin, etoposide, prednisone) and modified involved field radiation, is equally effective.[1-3] Although many patients respond well to these regimens, 20% to 30% experience a relapse after treatment or fail to respond to induction therapy.[4,5] For these patients, new therapies that are based on the unique biology of cHL are urgently needed.

Primary cHLs include small numbers of malignant Reed-Sternberg (RS) cells surrounded by an extensive but ineffective inflammatory/immune cell infiltrate.[6-8] In cHL, chromosome 9p24.1/*CD274(PD-L1)/PDCD1LG2(PD-L2)* alterations have been shown to increase the abundance of these PD-1 ligands. The 9p24.1 amplicon also contains *JAK2*, and copy number–dependent Janus kinase 2–signal transducers and activators of

transcription (JAK2-STAT) signaling further increases PD-1 ligand expression.[6] Less frequent chromosomal rearrangements of the PD-1 ligand loci have also been described.[9] Furthermore, Epstein-Barr virus (EBV) infection can increase expression of PD-1 ligands in EBV-positive Hodgkin lymphomas (HLs).[10]

PD-1 ligands engage the PD-1 receptor on T cells and induce PD-1 signaling and T-cell exhaustion by reversible inhibition of T-cell activation and proliferation.[11] Tumor cells expressing PD-1 ligands on their surface use the PD-1 pathway to evade an effective antitumor immune response.[12]

The genetic bases of PD-1 ligand deregulation and overexpression in cHL suggest the potential vulnerability of cHL to PD-1 blockade. For this reason, PD-1 blockade with nivolumab was evaluated in a phase I/Ib study of 23 heavily pretreated patients with relapsed/refractory cHL. In this pilot study, the overall response rate was 87%, and the median duration of response was not reached at 88 weeks.[13,14] All 10 evaluable patients had *PD-L1/PD-L2* copy number alterations (CNAs), increased expression of the PD-1 ligands, and active JAK-STAT signaling.[13] In another phase Ib study of relapsed/refractory cHL, PD-1 blockade with pembrolizumab resulted in an overall response rate of 65% without serious adverse events.[15] These pilot studies showed that PD-1 blocking agents were well tolerated in relapsed/refractory cHL and associated with high response rates and long-lasting remissions.[13-15]

The unique cellular composition of primary cHL limits its analysis with high-throughput genomic assays. Therefore, the precise incidence, nature, and prognostic significance of *PD-L1* and *PD-L2* alterations in cHL remain undefined. We use a recently developed fluorescent in situ hybridization (FISH) assay to characterize 9p24.1/*PD-L1*/*PD-L2* alterations in a cohort of 108 patients with newly diagnosed cHL who were treated with the Stanford V regimen and have long-term outcome data.

METHODS

Patients

This study was an institutional review board–approved collaborative effort among Stanford University, Brigham and Women's Hospital, VU University Medical Center, and the Dana-Farber Cancer Institute. Formalin-fixed paraffin-embedded (FFPE) tumor samples and clinical data from 108 patients with newly diagnosed cHL were obtained from Stanford University. The pathology on all cases was reviewed and diagnoses confirmed independently by two expert hematopathologists (Y.N. and S.J.R.). Study patients were treated on three concurrent clinical protocols of the Stanford V chemotherapy regimen plus modified involved field radiation (IFR) for clinically defined risk groups as previously described.[16,17] Patients with Ann Arbor early stage (I/II) nonbulky disease and no B symptoms were treated with 8 weeks Stanford V and 30 Gy IFR (early stage favorable [ES-F] G4 protocol). Patients with Ann Arbor early stage (I/II) disease and unfavorable clinical risk factors—bulky disease $\geq$ 10 cm or mediastinal mass ratio of $\geq$ 0.33x and/or B symptoms—were treated with 12 weeks Stanford V for 12 weeks and 36 Gy IFR to sites $>$ 5 cm (early stage unfavorable [ES-U] G2 protocol). Patients with advanced stage (AS) III/IV disease were also treated with 12 weeks Stanford V and 36 Gy IFR to sites $>$ 5 cm and the spleen, if involved (AS G3 protocol). One asymptomatic patient with early-stage nonbulky disease was treated on the G2 protocol because of an elevated erythrocyte sedimentation rate and involvement of more than three nodal sites. Three additional patients with early-stage bulky disease on physical examination were treated on the G4 protocol. Long-term follow-up (median, 9 years) and detailed clinical information were available on all patients.

FISH

FISH was performed as previously described.[13,18] In brief, bacterial artificial chromosome (BAC) clones were selected from the UCSC Genome Browser and ordered from BACPAC Resources Center at Children's Hospital Oakland Research Institute in Oakland, California (https://bacpac.chori.org/home.htm). BAC DNA was extracted from Luria broth cultures by using the Qiagen Large-Construct Kit (Hilden, Germany) according to the manufacturer's recommendations and nick labeled with standard protocols (Abbott Molecular, Des Plaines, IL). Probes included Spectrum Orange–labeled RP11-599H20, which maps to 9p24.1 and includes *CD274/PD-L1*; Spectrum Green–labeled RP11-635N21, which also maps to 9p24.1 and encompasses *PDCD1LG2/PD-L2*; and Spectrum Aqua–labeled CEP9, a control centromeric probe that maps to 9p11-q11 (Abbott Molecular). An additional probe, Spectrum Green–labeled RP11-610G2, which maps upstream of *PDCD1LG2*, was used to confirm a possible chromosomal translocation.

Hematoxylin and eosin–stained FFPE tissue sections were reviewed, RS cells identified by their nuclear morphologic features, and areas with the highest density of RS cells circled by an expert hematopathologist (S.J.R.). Thereafter, slides were hybridized according to the manufacturer's recommendations (Abbott Molecular). Approximately 50 RS cells per case were analyzed. Nuclei with a target:control probe ratio of $\geq$ 3:1 were defined as amplified, and those with a probe ratio of $>$ 1:1 but $<$ 3:1 were classified as relative copy gain. In certain instances, cells with aggregated target signals that were tightly clustered around the control signal were classified as amplified by an expert cytogeneticist (A.H.L.). Nuclei with a probe ratio of 1:1 but more than two copies of each probe were defined as polysomic for chromosome 9p. In each case, the percent and magnitude of 9p24.1 amplification, copy gain, polysomy, and normal copy numbers (disomy) were noted. Cases were classified by the highest observed level of 9p24.1 alteration. Specifically, cases with 9p24.1 copy gain lacked amplification, and cases with 9p polysomy lacked 9p24.1 copy gain or amplification.

Double Immunohistochemistry Staining

Double staining of PD-L1 (clone 405.9A11; G.J.F.[19]) and PAX5 (24/Pax-5; BD Biosciences, San Jose, CA) and of PD-L2 (clone 366C.9E5; G.J.F.) and pSTAT3 (D3A7; Cell Signaling, Danvers, MA) was performed with an automated staining system (Bond III; Leica Biosystems, Buffalo Grove, IL) as previously described.[13] Stained slides were scored by an expert hematopathologist (S.J.R.), and average intensity of staining (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) was reported. PD-L1 expression in PAX5 dim-positive malignant RS cells and PAX5-negative infiltrating normal cells was assessed separately. For PD-L1, 50 RS cells were counted, the number of malignant cells with positive staining (average intensity, 1 to 3+) was determined, and the percentage of positive cells was calculated (0% to 100%). For PD-L2 and pSTAT3, the percentage of malignant and nonmalignant cells with positive staining for PD-L2 within the tissue section was estimated (0% to 100%), and the average intensity of staining scored (1 to 3+). A modified H-score was generated by multiplying the percentage of malignant cells with positive staining (0% to 100%) and average intensity of positive staining in RS cells (1 to 3+).

EBV-Encoded Small RNA In Situ Hybridization

EBV-encoded small RNA (EBER) in situ hybridization was performed with an automated staining system (Bond III; Leica Biosystems) while following the manufacturer's protocol. Briefly, 4-μm-thick paraffin-embedded sections were prebaked at 60°C for 1 hour. Slides were loaded on the Bond III, dewaxed, rehydrated, and pretreated with a diluted enzyme solution for 15 minutes (Enzyme 1; Leica Biosystems). Thereafter, slides were incubated with a fluorescein-conjugated oligonucleotide EBER probe

(600 ng/mL; Leica Biosystems) at 42°C for 2 hours. Subsequently, an antifluorescein antibody (Leica Biosystems) was applied to the slides for 15 minutes, followed by 8 minutes of postprimary blocking reagent, 8 minutes of horseradish peroxidase–labeled polymer, and 5 minutes of peroxidase block. Slides were then developed with 3,3-diaminobenzidine (10 minutes), counterstained with hematoxylin (5 minutes), dehydrated, and coverslipped. The aforementioned reagents were all components of the Bond Polymer Refine Detection system (Leica Biosystems).

Statistical Analysis

Analyses of 9p24.1 alterations, PD-L1 and PD-L2 protein expression, and EBER status were performed while blinded to the clinical data. Clinical characteristics of the patients with cHL were assessed by using descriptive statistics. Associations between variables were evaluated with Fisher exact test for categorical data and Wilcoxon or Kruskal-Wallis rank sum test for continuous data that compared two or more groups, respectively. The modified H-score for PD-L1 and PD-L2 protein expression was divided into four equally sized groups (quartiles), and locally weighted polynomial smoothing was used to fit a trend line over the data. All *P* values were two-sided. Progression-free survival (PFS) was defined from the date of diagnosis until the date of relapse or death in the absence of relapse or was censored at the date of last contact. Time-to-event analyses were performed by using the Kaplan-Meier method, and errors were calculated by Greenwood formula. Differences in survival curves were assessed with log-rank tests. Multivariable Cox proportional hazards models were fit and evaluated by using likelihood ratio tests. Two-sided $P < .05$ was considered statistically significant, and no corrections for multiple comparisons were performed.

RESULTS

Patient Characteristics

The characteristics of the 108 patients with newly diagnosed cHL are summarized in Table 1. The median age was 30 years, and the majority of patients (93 of 108 [86%]) had nodular sclerosis HL, 11% (12 of 108) had mixed-cellularity HL, and 3% (three of 108) had cHL not otherwise specified. Patients were classified on the basis of disease stage and the presence or absence of B symptoms and/or bulky disease as ES-F (no bulky disease or B symptoms, n = 33), ES-U (bulky disease and/or B symptoms, n = 41), or AS (n = 34).

Genetic Analyses of the PD-L1 and PD-L2 Loci

A recently developed FISH assay was used to characterize 9p24.1/*PD-L1*/*PD-L2* alterations in diagnostic FFPE tumor specimens from each patient (Fig 1A).[13,18] RS cells were scored as having 9p24.1 disomy, polysomy, copy gain, or amplification, and the magnitude of 9p24.1 gain and percentage of cells with each alteration was noted (representative images in Fig 1B). Cases were classified by the highest observed level of 9p24.1 alteration.

Frequency of the 9p24.1 Alterations in cHL

Almost all of the 108 patients in this series had concordant alterations of the *PD-L1* and *PD-L2* loci in their diagnostic biopsy specimens. Only one patient (1%) had normal 9p24.1 copy numbers (disomy), and five (5%) had polysomy of 9p. In marked contrast, 56% (61 patients) had 9p24.1 copy gain, and 36% (39 patients) had 9p24.1 amplification (Table 2). There was an association between PD-L1 and PD-L2 protein expression and 9p24.1 genetic alterations in the RS cells (Fig 1C). RS cells also expressed pSTAT3, indicative of active JAK-STAT signaling (Fig 1C). Of note, two of the cHL cases had a chromosomal rearrangement of 9p24.1 detected by a split of the red and green FISH signals (Table 2; Appendix Fig A1, online only).

Spectrum of 9p24.1 Alterations in cHL

By analyzing each case with FISH, we were able to assess the full spectrum of 9p24.1 alterations in each tumor. In cases classified by the highest observed level of 9p24.1 alteration, we also identified RS cells with lower-level 9p24.1 CNAs (Table 2; Appendix Table A1; Fig 2A). Specifically, all cases classified as having 9p24.1 amplification had additional RS cells with 9p24.1 copy gain (2% to 82% of cells), 9p polysomy (2% to 52% of cells), and/or 9p24.1 residual disomy (2% to 35% of cells;

Table 1. Clinical Characteristics and Pathologic Subtypes

Characteristic	All (N = 108)	Early Stage Favorable (n = 33)	Early Stage Unfavorable (n = 41)	Advanced Stage (n = 34)
Age, years				
Median (range)	30 (18-69)	30 (18-56)	29 (18-66)	29 (19-69)
Stage, No. (%)				
I	9 (8)	8 (24)	1 (2)	—
II	65 (60)	25 (76)	40 (98)	—
III	22 (20)	—	—	22 (65)
IV	12 (11)	—	—	12 (35)
Bulky, No. (%)				
> 10 cm	16 (15)	—	12 (29)	4 (12)
≥ 0.33x mediastinal mass ratio	19 (18)	—	11 (27)	8 (24)
Both	15 (14)	—	8 (20)	7 (21)
B symptoms, No. (%)	39 (36)	—	18 (44)	21 (62)
Histologic subtype, No. (%)				
Nodular sclerosis	93 (86)	25 (76)	39 (95)	29 (85)
Mixed cellularity	12 (11)	8 (24)	1 (2)	3 (9)
cHL, not otherwise specified	3 (3)	—	1 (2)	2 (6)

Abbreviation: cHL, classical Hodgkin lymphoma.

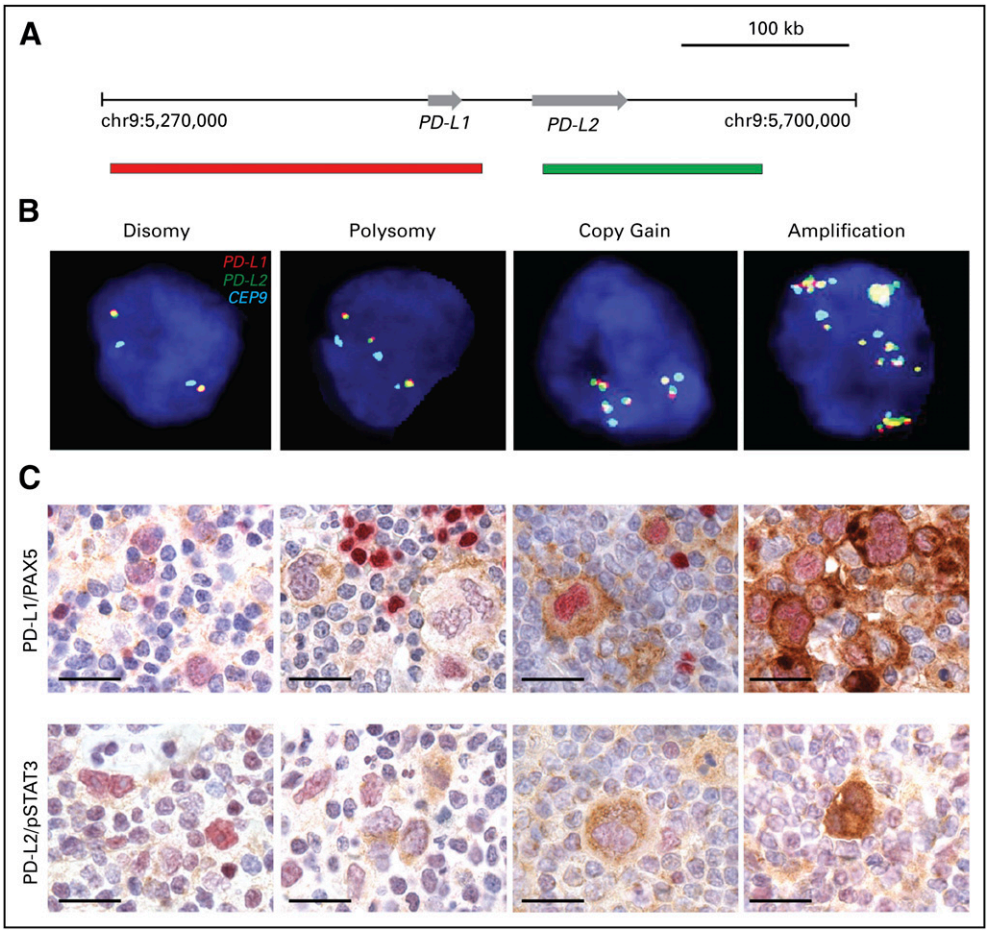

Fig 1. Genetic and immunohistochemical analyses of the *PD-L1* and *PD-L2* loci and PD-1 ligand expression. (A) Location and color labeling of the bacterial artificial chromosome (chr) clones on 9p24.1 used for fluorescent in situ hybridization (FISH). RP11-599H20 including PD-L1, labeled red. RP11-635N21 including PD-L2, labeled green. (B) Representative images of FISH results for the various categories. PD-L1 in red, PD-L2 in green, fused (F) signals in yellow, and centromeric probe (CEP9) in aqua (A). In these images, disomy reflects 2A:2F; polysomy, 3A:3F; copy gain, 3A:6F; and amplification, 15+F. (C) The top panel shows PD-L1 (brown)/PAX5 (red) immunohistochemistry (IHC) in the classical Hodgkin lymphoma (cHL) cases with 9p24.1 disomy, polysomy, copy gain, and amplification from (B). The bottom panel shows PD-L2 (brown)/pSTAT3 (red) IHC in the same cHL cases. Scale bar = 50 μm.

Table 2; Appendix Table A1; Fig 2A). Similarly, cases identified as having 9p24.1 copy gain included additional RS cells with 9p polysomy (4% to 78% of cells) and/or 9p24.1 residual disomy (2% to 86% of cells; Table 2; Appendix Table A1; Fig 2A). In cases classified as polysomic for chromosome 9p, additional RS cells were disomic for 9p24.1 (66% to 93% of cells; Table 2; Appendix Table A1; Fig 2A).

As shown in Figure 2A, there was a spectrum of 9p24.1 alterations in the evaluated cHL series that ranged from low-level polysomy (6% polysomic RS cells) to near-uniform 9p24.1 amplification (92% amplified RS cells). Consistent with the ordered spectrum of 9p24.1 alterations in this series (Fig 2A), the percentage of residual 9p24.1 disomic cells was highest in cases classified as polysomic for 9p, intermediate in tumors with 9p24.1 copy gain, and lowest in tumors with 9p24.1 amplification ($P < .001$, Kruskal-Wallis test; Fig 2B).

9p24.1 Alterations and PD-1 Ligand Expression

After characterizing the spectrum of 9p24.1 CNAs in the cHL series, we assessed the relationship between these alterations and expression of the PD-1 ligands. Given the inverse relationship between 9p24.1 alterations and residual 9p24.1 disomy (Figs 2A

Table 2. *PD-L1* and *PD-L2* Genetic Alterations

				Additional Alterations							
				Disomy		Polysomy		Copy Gain		Amplification	
Cytogenetics	Patients (N = 108), No. (%)	% Positive Cells per Case	Median No. of Copies	No. of Cases/ Total (%)	% Cells	No. of Cases/ Total (%)	% Cells	No. of Cases/ Total (%)	% Cells	No. of Cases/ Total (%)	% Cells
Disomy	1 (1)	100	2	NA	NA	—	—	—	—	—	—
Polysomy	5 (5)	6-34	3	5/5 (100)	66-94	NA	NA	—	—	—	—
Copy gain	61 (56)	4-100	6	58/61 (95)	2-86	57/61 (93)	4-78	NA	NA	—	—
Amplification	39 (36)	2-92	10+	35/39 (90)	2-35	25/39 (64)	2-52	39/39 (100)	2-82	NA	NA
Translocation	2 (2)	72-88	NA	2/2 (100)	4-22	2/2 (100)	6-8	—	—	—	—

Abbreviation: NA, not applicable.

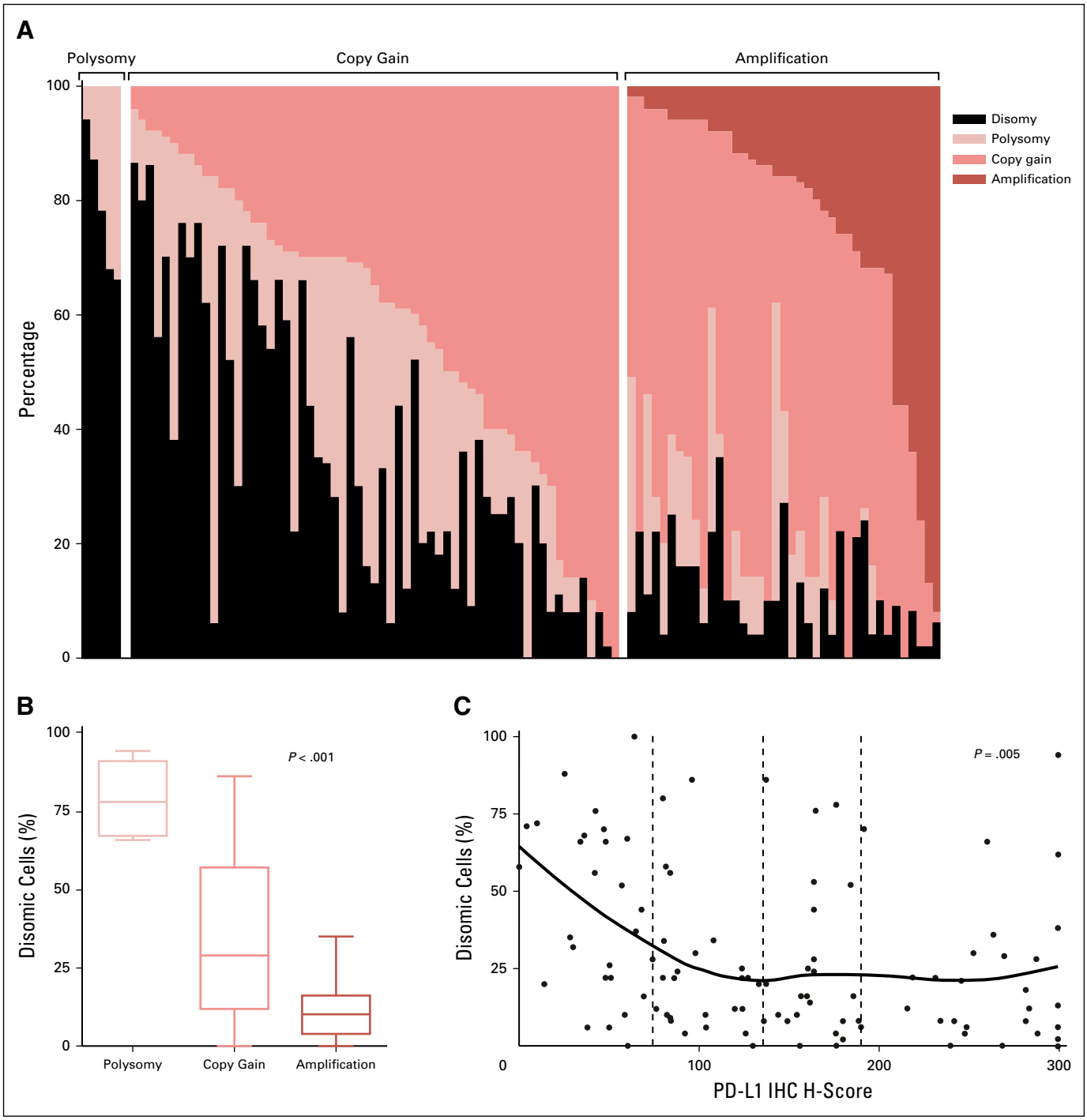

Fig 2. The spectrum of 9p24.1 alterations in classical Hodgkin lymphoma (cHL). (A) 9p24.1 alterations in evaluated cHLs. The cHLs are classified by the highest observed level of 9p24.1 alteration in Reed-Sternberg (RS) cells: polysomy, copy gain, or amplification (top). Individual tumors are depicted as columns on the *x*-axis. In each cHL, the percentage of RS cells with 9p24.1 disomy (black), polysomy (light pink), copy gain (pink), and/or amplification (red) is shown on the *y*-axis. (B) Percentage of RS cells with residual 9p24.1 disomy in cHLs classified by 9p24.1 alterations, as represented as box-and-whisker plots, showing minimum, first quartile, median, third quartile, and maximum. cHLs with 9p24.1 polysomy, copy gain, and amplification have significantly different percentages of residual RS cells with normal (disomic) 9p24.1 copy numbers. $P < .001$, Kruskal-Wallis test. (C) Association of PD-L1 protein expression and 9p24.1 copy number alterations. Residual 9p24.1 disomy is depicted on the *y*-axis; PD-L1 immunohistochemistry (IHC) H-score (in quartiles) is shown on the *x*-axis. Quartiles are indicated by dashed lines. A locally weighted polynomial regression line is shown in black. A highly significant decrease in percentage of residual 9p24.1 disomic cells in cHLs with a higher PD-L1 IHC H-score is shown. $P = .005$, Kruskal-Wallis test.

and 2B), we used residual 9p24.1 disomy and the PD-L1 H-score (percentage of malignant cells with positive staining multiplied by the average intensity of positive staining, divided into quartiles) for these analyses. A highly significant association was found between decreased residual 9p24.1 disomy and increased PD-L1 expression ($P = .005$, Kruskal-Wallis test; Fig 2C; Appendix Fig A2A). Similar

results were obtained for PD-L2 protein expression (Appendix Figs A2B and A2C).

The distribution of genetic alterations in patients with EBV-negative and EBV-positive cHL was similar in this series (Appendix Fig A3). However, EBV-positive cHLs were more likely to have high PD-L1 H-scores (Appendix Fig A3), indicating further induction of PD-L1 expression by viral infection.

9p24.1 Alterations, Clinical Risk Factors, and Outcome

After characterizing the *PD-L1/PD-L2* alterations in this series of patients, we assessed potential associations among these genetic lesions, clinical risk factors, and outcome. First, PFS was assessed for patients with ES-F, ES-U, and AS disease. The outcome for patients with ES-F and ES-U disease was comparable possibly partly due to the more-aggressive treatment of ES-U disease (Fig 3A). As expected, patients with AS disease had a significantly inferior outcome compared with those with ES disease ($P = .002$, log-rank test; Fig 3A). We next assessed the PFS for patients by 9p24.1 genetic alteration and identified significant differences in outcome that were most striking for patients with 9p24.1 amplification ($P < .001$, log-rank test; Fig 3B).

The significantly decreased PFS in patients with AS disease (Fig 3A) and 9p24.1 amplification (Fig 3B) prompted us to assess the distribution of 9p24.1 alterations in the clinical risk groups (Figs 3C [all patients] and 3D [risk groups]). The incidence of 9p24.1 amplification increased by clinical risk group (ES-F, 24%; ES-U, 34%; AS, 50%; $P = .024$, Kruskal-Wallis test; Fig 3D). We next determined the effect of 9p24.1 amplification on PFS in the various clinical risk groups. Although the small numbers limited the statistical analysis, there was a trend toward worse outcome in patients with ES-U and AS disease who had 9p24.1 amplification (Appendix Fig A4).

We next assessed the independent prognostic significance of the clinical risk factors, ES-U and AS, and 9p24.1 amplification in Cox univariable and multivariable models. In respective univariable models, the clinical risk factor, AS disease, and 9p24.1 amplification had independent prognostic significance ($P = .017$ and .02, respectively; Table 3 [top and middle panels]). Despite the association of 9p24.1 amplification with AS disease (Fig 3D), the

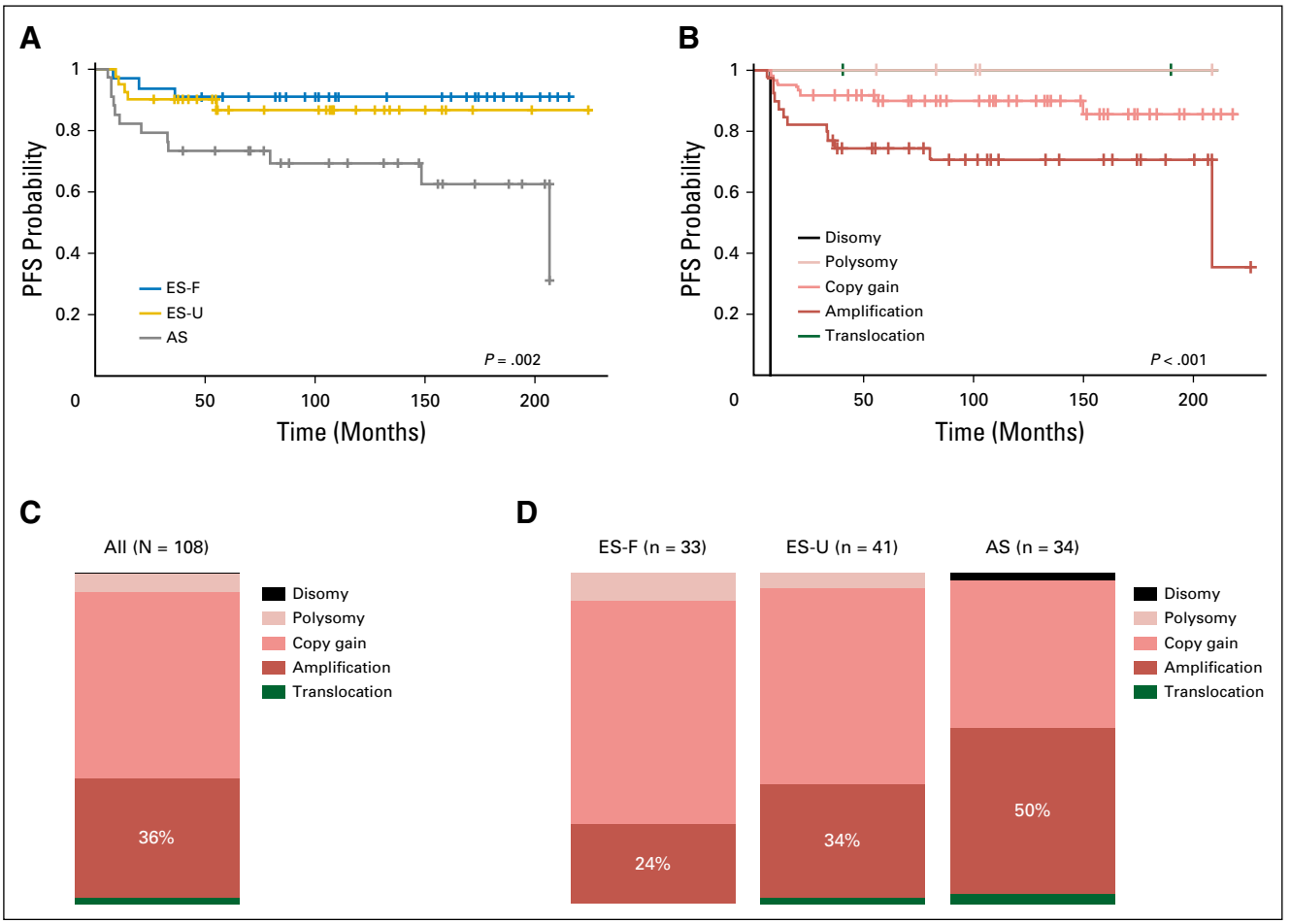

Fig 3. Clinical and genetic predictors of progression-free survival (PFS). (A) PFS by clinical stage in patients with classical Hodgkin lymphoma (cHL), early stage favorable (ES-F; n = 33), early stage unfavorable (ES-U; n = 41), and advanced stage (AS; n = 34). $P = .002$, log-rank test. (B) PFS by 9p24.1 alterations in patients with cHL (disomy, n = 1; polysomy, n = 5; copy gain, n = 61; amplification, n = 39; translocation, n = 2; $P < .001$, log-rank test). (C) Percentage of patients with 9p24.1 disomy (1%), polysomy (5%), copy gain (56%), amplification (36%), and translocation (1%) in the current series. (D) Frequency of 9p24.1 alterations (polysomy, copy gain, amplification, translocation, or disomy) by clinical stage (ES-F, ES-U, and AS) in this series. The incidence of 9p24.1 amplification is significantly different in clinically staged patients (ES-F, 24%; ES-U, 34%; AS, 50%; $P = .024$, Kruskal-Wallis test).

Table 3. Clinical and Genetic Univariable and Multivariable Risk Models

Variable	HR	Lower 95% CI	Upper 95% CI	*P*
Clinical factors*				
Stage I/II, B symptoms and/or bulky disease	1.59	0.377	6.703	.53
Stage III/IV	4.68	1.319	16.616	.017
Presence of amplification†				
Yes	2.9	1.186	7.106	.02
Full model‡				
Stage I/II, B symptoms and/or bulky disease	1.43	0.339	6.041	.63
Stage III/IV	3.76	1.035	13.662	.044
Amplification	2.292	0.919	5.721	.075

Abbreviation: HR, hazard ratio.
*The clinical stages (early stage favorable, early stage unfavorable, advanced stage [AS]) were used to classify patients into risk groups for progression-free survival. The HR estimate for patients with AS (III/IV) disease was significant ($P = .017$).
†The presence or absence of 9p24.1 amplification was used to classify patients into risk groups for progression-free survival. In a univariable Cox model, 9p24.1 amplification was significant ($P = .02$).
‡The multivariable Cox model includes the risk factors with independent prognostic significance (9p24.1 amplification and AS disease) and the additional clinical risk factors that influenced treatment (stage I/II, B symptoms, and/or bulky disease). In this multivariable model, the HR for AS retains significance ($P = .044$; P for amplification = .075).

genetic alteration further delineated PFS in a multivariable model ($P = .075$; Table 3).

DISCUSSION

We use a 9p24.1 FISH assay to determine the incidence, nature, and prognostic significance of *PD-L1* and *PD-L2* alterations in a series of patients with cHL who were uniformly treated and have long-term outcome data. Almost all the cases in this series had concordant alterations of the *PD-L1* and *PD-L2* loci, which included copy gain and amplification in the majority of tumors (56% and 36%, respectively). There was a spectrum of 9p24.1 alterations in the analyzed cases that ranged from low-level polysomy to near-uniform 9p24.1 amplification. Of note, PFS was significantly shorter for patients with 9p24.1 amplification who were also more likely to have AS disease.

The current studies indicate that *PD-L1*/*PD-L2* CNAs are a defining feature of cHL, seen with near uniformity in patients evaluated with the FISH assay. Previous genetic analyses of cHL were hampered by the rarity of RS cells in primary tumors. As a consequence, analyses of genetic alterations in cHL required laser capture microdissection of malignant RS cells.[6,20,21] In these studies, which relied on array-based comparative genomic hybridization or quantitative polymerase chain reaction, the frequency of *PD-L1*/*PD-L2* CNAs was approximately 40% to 50%.[6,20,21] In such analyses, laser capture microdissected RS specimens included surrounding residual normal tissue, which likely caused an underestimate in *PD-L1*/*PD-L2* copy numbers.

The current studies also indicate that 9p24.1 alterations are subclonal in a subset of primary cHLs. Specifically, these tumors exhibited the full spectrum of 9p24.1 alterations, which ranged from low-level polysomy (6% polysomic RS cells) to near-uniform 9p24.1 amplification (92% amplified RS cells). Consistent with these findings, the percentage of residual 9p24.1 disomic cells was inversely related to *PD-L1*/*PD-L2* CNAs and highest in cHLs classified as polysomic for 9p, intermediate in tumors with 9p24.1 copy gain, and lowest in tumors with 9p24.1 amplification. In cHLs with significant residual disomy, platform-based approaches, such as assay-based comparative genomic hybridization or high-density single nucleotide polymorphism array, or quantitative polymerase chain reaction analyses are likely to underestimate the frequency of 9p24.1 alterations.[6,20,21] These findings may also explain the lower incidence of 9p24.1 CNAs in a small series of flow-sorted $CD30^+$ RS cells evaluated by whole-exome sequencing.[22]

Although 9p24.1 alterations were identified in almost all patients with cHL in this series, the highest-level lesion—amplification—was more common in patients with AS disease. This finding suggests that PD-1–mediated immune evasion may limit local containment and foster tumor spread. In addition, the data provide the rationale for evaluating PD-1 blockade in the frontline setting in patients with AS cHL who may have less-favorable outcomes with standard empirical combination chemotherapy. Given these findings, further analyses of the 9p24.1 alteration in patients treated with standard cHL induction regimens or checkpoint blockade are warranted.

The high frequency of 9p24.1 alterations in cHL prompted further assessment of the *PD-L1* and *PD-L2* loci in other lymphoid malignancies. Several lymphomas have been found to have frequent *PD-L1*/*PD-L2* CNAs and additional chromosomal translocations of the same loci. Like cHL, certain large B-cell lymphoma subtypes, including primary mediastinal large B-cell lymphoma, primary CNS lymphoma, and primary testicular lymphoma, often have *PD-L1*/*PD-L2* CNAs or chromosomal rearrangements and increased expression of the PD-1 ligands.[6,18,23,24] In marked contrast, systemic diffuse large B-cell lymphomas rarely exhibit 9p24.1/*PD-L1*/*PD-L2* CNAs and infrequently express the associated PD-1 ligands.[18,25,26]

Taken together, these data support a strategy for identifying lymphoid malignances with genetic bases for PD-1–mediated tumor immune evasion. In cHL, the near-uniform alterations of the *PD-L1*/*PD-L2* loci likely explain the remarkable activity of PD-1 blockade in this disease.[13]

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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GLOSSARY TERMS

copy number alteration (CNA): a structural variation in the genome with an increased (amplification) or decreased (deletion) number of copies of a gene or region.

Epstein-Barr virus (EBV): virus belonging to the herpes family of viruses. EBV is also called human herpes virus 4 and is an oncogenic virus that is responsible for B-cell transformation. It is associated with Hodgkin lymphoma, immunoblastic B-cell lymphomas, Burkitt's lymphoma, and nasopharyngeal carcinoma.

FISH (fluorescent in situ hybridization): in situ hybridization is a sensitive method generally used to detect specific gene sequences in tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. FISH uses a fluorescent probe to increase the sensitivity of in situ hybridization.

PD-1: programmed cell death protein 1 (CD279), a receptor expressed on the surface of activated T, B, and NK cells that negatively regulates immune responses, including autoimmune and antitumor responses.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

_PD-L1_ and _PD-L2_ Genetic Alterations Define Classical Hodgkin Lymphoma and Predict Outcome

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Appendix

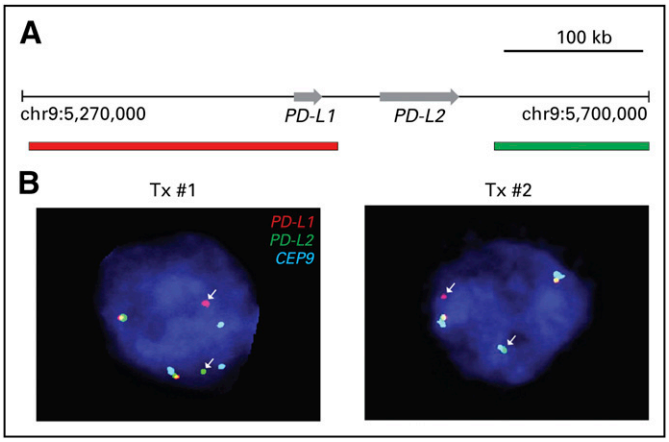

Fig A1. Chromosomal rearrangements in classical Hodgkin lymphoma (cHL). (A) Location and color labeling of the bacterial artificial chromosome (chr) clones on 9p24.1 used for fluorescent in situ hybridization (FISH) in translocation 2 (Tx #2). RP11-599H20 including *PD-L1*, labeled red; RP11-610G2 downstream of *PD-L2*, labeled green. For labeling of bacterial artificial chromosome clones used for translocation 1 (Tx #1), see Figure 1A. (B) FISH analyses of the cHL cases with chromosomal rearrangements. *PD-L1* in red, *PD-L2* in green, and centromeric probe (CEP9) in aqua. Arrows indicate the rearranged allele.

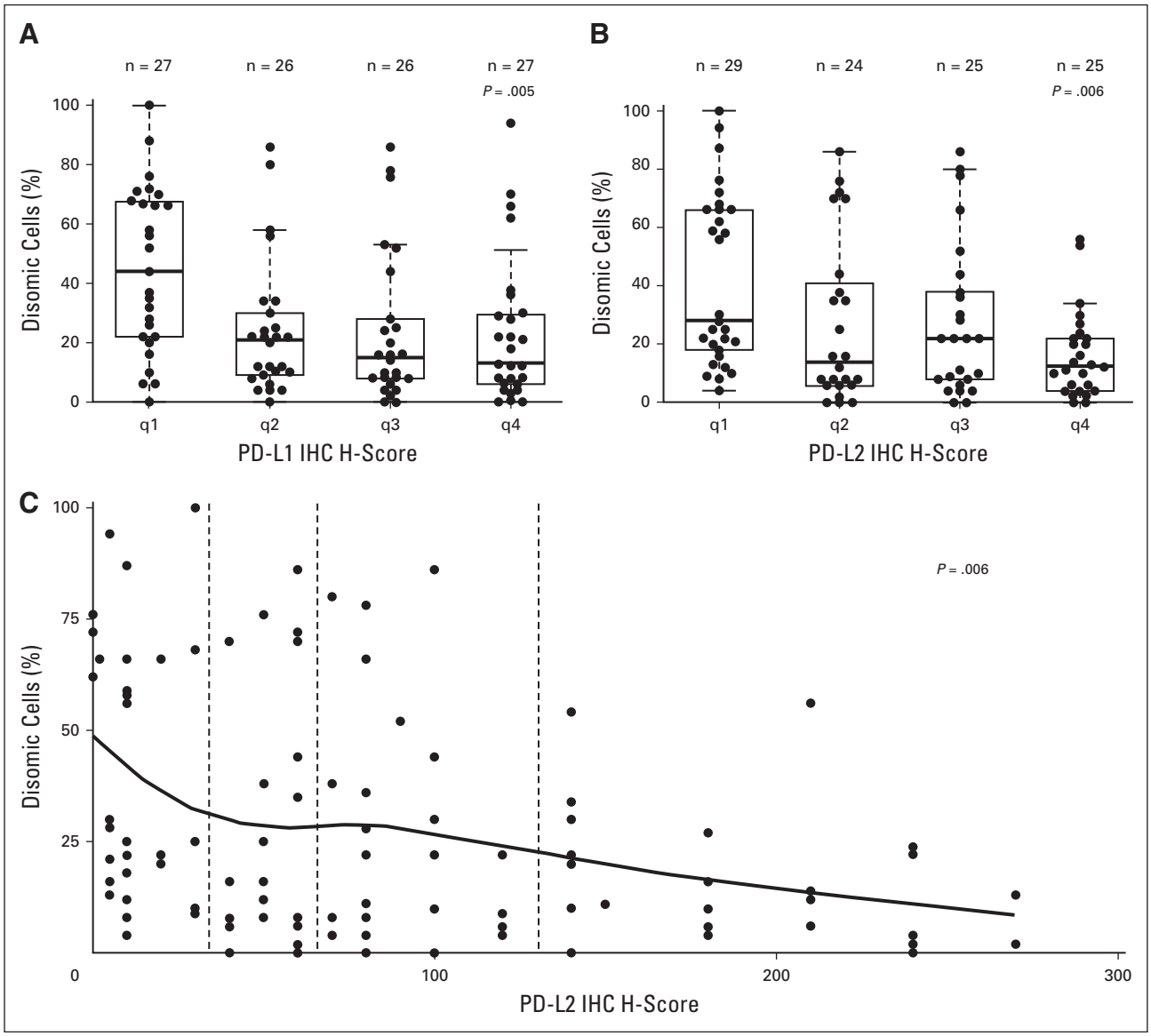

Fig A2. Association of PD-L1/PD-L2 protein expression and 9p24.1 copy number alterations. (A) Percentage of 9p24.1 disomic cells in each of the PD-L1 immunohistochemistry (IHC) H-score quartiles. The *y*-axis shows the percentage of residual 9p24.1 disomic cells; the *x*-axis shows PD-L1 IHC H-score in quartiles. A statistically significant decrease in the percentage of normal (disomic) cells in the H-score quartiles was found. $P = .005$, Kruskal-Wallis test. (B) Percentage of residual 9p24.1 disomic cells in each of the PD-L2 IHC H-score quartiles. The *y*-axis shows the percentage of residual 9p24.1 disomic cells; the *x*-axis shows the PD-L2 IHC H-score in quartiles. The percentage of residual 9p24.1 disomic cells is statistically different in the quartiles. $P = .006$, Kruskal-Wallis test. (C) Percentage of residual 9p24.1 disomic cells (*y*-axis) and PD-L2 IHC H-score (*x*-axis) plotted for individual cases. Quartiles are indicated with dashed lines, and a trend line (locally weighted polynomial regression line) is shown in black. q, quartile.

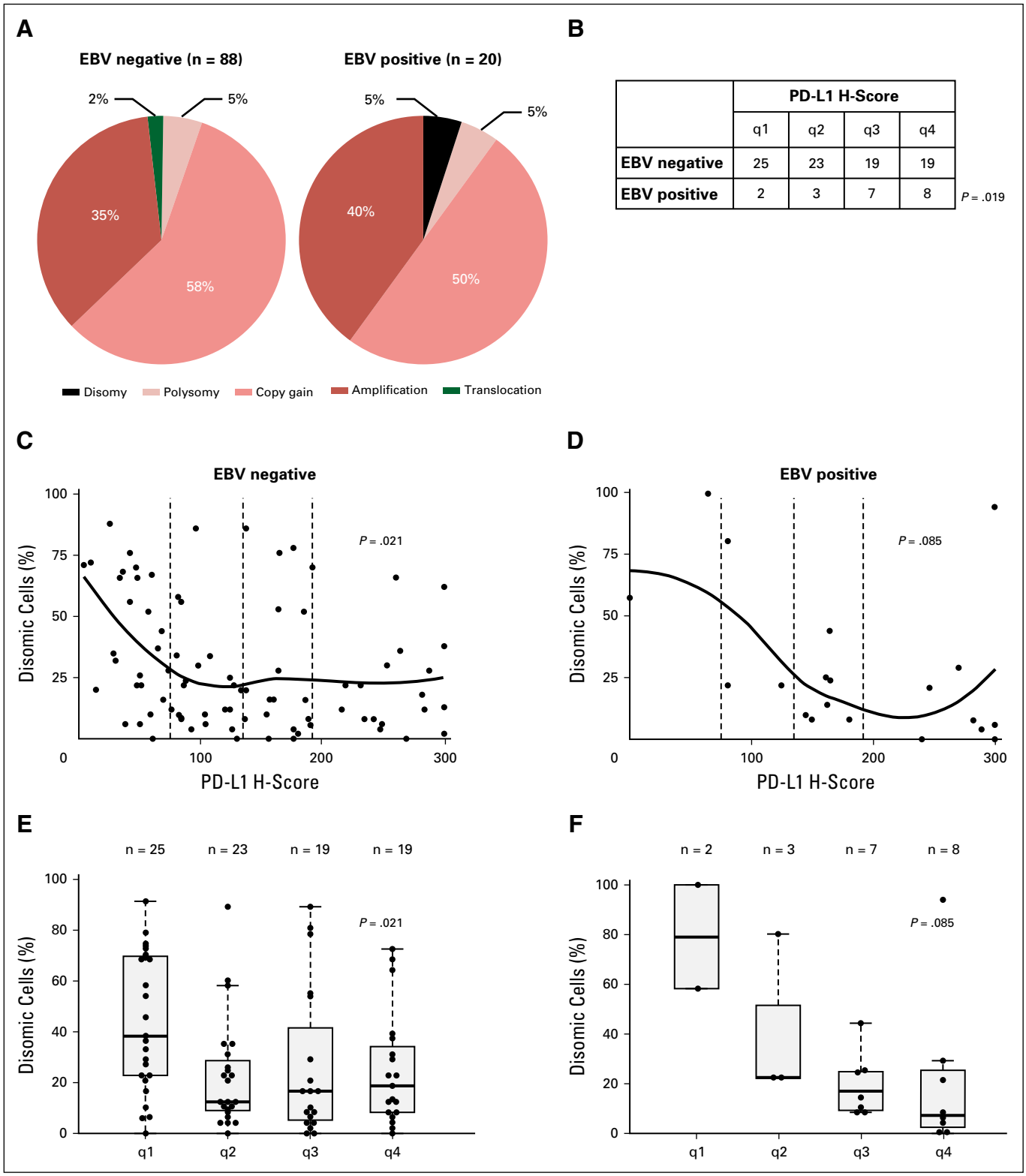

Fig A3. Distribution of genetic alterations in patients with Epstein-Barr virus (EBV) –negative and EBV-positive classical Hodgkin lymphoma (cHL). (A) The status of *PD-L1* and *PD-L2*—disomy, polysomy, copy gain, amplification, and translocation—in EBV-negative (n = 88) and EBV-positive (n = 20) cHLs is visualized with a pie chart. (B) Distribution of EBV-negative and EBV-positive cases in the various PD-L1 immunohistochemistry (IHC) H-score quartiles. The proportion of EBV-positive cases increases as the H-score quartile category increases. $P = .019$, Kruskal-Wallis test. (C) and (D) Percentage of 9p24.1 residual disomic cells (*y*-axis) and PD-L1 IHC H-score (*x*-axis) plotted for individual EBV-negative and EBV-positive cHLs, respectively. Quartiles are indicated with dashed lines, and a trend line (locally weighted polynomial regression line) is shown in black. (E) and (F) Percentages of residual 9p24.1 disomic cells in EBV-negative and EBV-positive cases in the respective PD-L1 IHC H-score quartiles from (C) and (D) are plotted. The percentage of residual 9p24.1 disomic cells is significantly different in the respective H-score quartiles in EBV-negative cHLs in (E). $P = .021$, Kruskal-Wallis test. q, quartile.

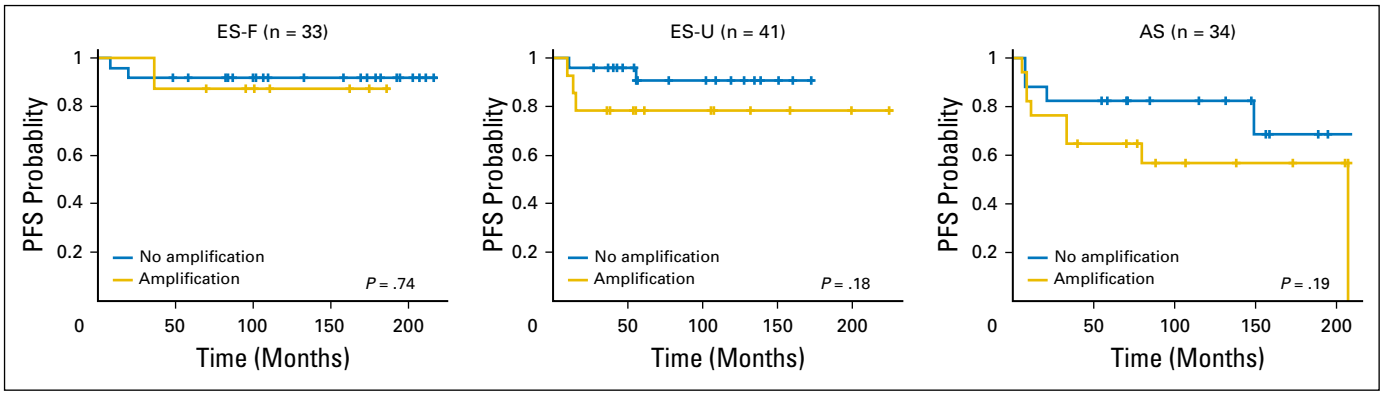

Fig A4. Progression-free survival (PFS) curves for the clinical risk groups with (gold) or without (blue) 9p24.1 amplification: early stage favorable (ES-F; n = 8 and n = 25, respectively), early stage unfavorable (ES-U; n = 14 and n = 27, respectively), and advanced stage (AS; n = 17 and n = 17, respectively).

Table A1. 9p24.1 Fluorescent In Situ Hybridization Analyses in the 108 Patients With Classical Hodgkin Lymphoma

Study ID	No. of RS Cells	Disomy (%)	Polysomy (%)	No. of Copies	Copy Gain (%)	No. of Copies	Amplification (%)	No. of Copies	Translocation (%)	No. of Copies
1	40	87	13	3	—	—	—	—	—	—
2	46	78	22	3	—	—	—	—	—	—
3	50	68	32	3-5	—	—	—	—	—	—
4	50	94	6	3	—	—	—	—	—	—
5	50	66	34	3-4	—	—	—	—	—	—
6	51	86	10	3	4	3-4F:2-3A	—	—	—	—
7	50	80	14	3	6	3F:2A	—	—	—	—
8	50	56	36	3-5	8	3-5F:2-4A	—	—	—	—
9	50	76	12	3	12	3-4F:2-3A	—	—	—	—
10	50	72	6	3-4	22	3-6F:2-5A	—	—	—	—
11	49	59	12	3-5	29	3-5F:2-4A	—	—	—	—
12	49	22	49	3-5	29	3-5F:2-4A	—	—	—	—
13	51	30	39	3-6	31	2-5F:1-4A	—	—	—	—
14	48	13	52	3-6	35	3-8F:2-5A	—	—	—	—
15	49	33	29	3-5	38	3-7F:2-4A	—	—	—	—
16	41	44	17	3	39	3-4F:2-3A	—	—	—	—
17	50	20	38	3-6	42	3-10+F:2-8A	—	—	—	—
18	50	22	28	3-7	50	3-8F:1-6A	—	—	—	—
19	47	9	38	3-6	53	1-9F:1-6A	—	—	—	—
20	54	28	11	3-4	61	3-6F:1-4A	—	—	—	—
21	50	20	12	3	68	3-5F:1-3A	—	—	—	—
22	50	8	22	3-4	70	3-10+F:2-7A	—	—	—	—
23	52	11	6	3	83	3-7F:1-4A	—	—	—	—
24	50	8	6	3	86	3-6F:2-4A	—	—	—	—
25	50	8	6	3	86	3-5F:2-4A	—	—	—	—
26	50	2	—	—	98	3-10+F:2-8A	—	—	—	—
27	51	—	—	—	100	4-10+F:2-8A	—	—	—	—
28	50	10	12	3-4	66	3-9F:1-4A	12	7-9F	—	—
29	50	6	8	3-4	74	3-9F:2-7A	12	10+-15+F	—	—
30	48	4	10	4-6	73	1-10F:1-8A	13	5-10+F	—	—
31	49	27	16	3-4	41	3-5F:1-4A	16	3-7F	—	—
32	50	—	—	—	74	3-8F:2-6A	26	6-10+F	—	—
33	50	24	2	3	42	3-8F:2-5A	32	3-10+F	—	—
34	50	10	—	—	58	3-9F:1-4A	32	3-10+F:1-6A	—	—
35	50	—	—	—	44	3-7F:2-3A	56	6-10+F	—	—
36	50	86	6	3	8	3-4F:2A	—	—	—	—
37	44	70	21	3-4	9	3-4F:2A	—	—	—	—
38	50	70	18	3-4	12	3F:2A	—	—	—	—
39	50	76	10	3-4	14	3-6F:2-3A	—	—	—	—
40	50	6	78	3-7	16	3-7F:2-6A	—	—	—	—
41	50	72	10	3	18	3-4F:1-3A	—	—	—	—
42	33	52	30	3-4	18	3-5F:2-4A	—	—	—	—
43	54	30	50	3-5	20	3-9F:2-6A	—	—	—	—
44	38	58	18	3	24	3-4F:2-3A	—	—	—	—
45	37	54	19	3	27	3-5F:2-4A	—	—	—	—
46	50	66	4	3	30	3-4F:1-2A	—	—	—	—
47	50	44	26	3-4	30	3-6F:2-3A	—	—	—	—
48	50	34	36	3-5	30	3-8F:3-5A	—	—	—	—
49	50	28	42	3-5	30	3-6F:2-5A	—	—	—	—
50	50	16	52	3-6	32	3-7F:2-5A	—	—	—	—
51	50	6	56	3-10	38	4-9F:2-7A	—	—	—	—
52	49	12	49	3-6	39	3-8F:2-6A	—	—	—	—
53	50	52	8	3-4	40	3-7F:2-4A	—	—	—	—
54	50	18	36	3-5	46	3-6F:2-4A	—	—	—	—
55	40	25	15	3-5	60	3-5F:1-3A	—	—	—	—
56	55	25	15	3	60	3-6F:2-4A	—	—	—	—
57	50	20	16	3	64	3-6F:1-4A	—	—	—	—
58	50	—	10	3F and 10+F	90	4-15+F:3-10+A	—	—	—	—
59	50	8	—	—	92	3-8F:2-5A	—	—	—	—
60	49	8	41	3-5	49	3-7F:2-5A	2	10+F	—	—
61	51	22	—	—	76	3-6F:2-6A	2	7F	—	—
62	50	22	6	3	68	3-5F:3-4A	4	8F	—	—
63	49	25	14	3	55	3-4F:1-3A	6	5 and 8F:2A	—	—
64	49	16	19	3-6	59	3-10+F:2-7A	6	10+-15+F	—	—
65	51	22	39	3-7	31	3-9F:2-5A	8	3-10+F	—	—
66	51	35	4	3-4	53	3-8F:2-4A	8	3-10+F	—	—
67	50	10	—	—	82	3-5F:2-3A	8	6-10+F	—	—
68	50	10	—	—	76	3-7F:2-4A	14	6-9F	—	—
69	50	12	16	3-5	50	3-15+F:2-6A	22	3-15+F	—	—
70	49	4	6	3	67	3-7+F:2-5A	23	5+F-7+F	—	—
71	50	4	12	3-7	52	3-9F:2-7A	32	6-15+F	—	—
72	43	9	—	—	35	4-9F:2-4A	56	5-10+F	—	—
73	46	2	—	—	11	3-4F:2A	87	5-10+F	—	—

(continued on following page)

Table A1. 9p24.1 Fluorescent In Situ Hybridization Analyses in the 108 Patients With Classical Hodgkin Lymphoma (continued)

Study ID	No. of RS Cells	Disomy (%)	Polysomy (%)	No. of Copies	Copy Gain (%)	No. of Copies	Amplification (%)	No. of Copies	Translocation (%)	No. of Copies
74	52	4	8	3	—	—	—	—	88	One allele rearranged
75	50	100	—	—	—	—	—	—	—	—
76	50	38	52	3-6	10	4-5F:3A	—	—	—	—
77	50	62	22	3-4	16	3-4F:2-3A	—	—	—	—
78	51	66	10	3-4	24	3-5F:1-4A	—	—	—	—
79	50	66	6	3-5	28	3-6F:2-4A	—	—	—	—
80	49	35	35	3-6	30	3-5F:2-4A	—	—	—	—
81	50	8	62	3-8	30	5-7F:3-6A	—	—	—	—
82	48	56	13	3-4	31	3-4F:1-3A	—	—	—	—
83	49	22	33	3-6	45	3-7F:1-5A	—	—	—	—
84	50	12	38	3-4	50	3-7F:2-5A	—	—	—	—
85	50	36	12	3-5	52	3-6F:1-5A	—	—	—	—
86	37	38	8	3-4	54	3-6F:1-4A	—	—	—	—
87	50	28	12	3-5	60	3-5+F:1-4A	—	—	—	—
88	50	—	36	3-5	64	3-9F:2-5A	—	—	—	—
89	50	30	4	3-4	66	3-7F:2-3A	—	—	—	—
90	49	14	—	—	86	3-6F:2-5A	—	—	—	—
91	48	11	35	3-8	50	4-10+F:3-7A	4	4-9F	—	—
92	46	4	16	3-4	76	3-7F:1-5A	4	10+F	—	—
93	50	16	20	3-6	58	3-9F:2-5A	6	6-9F	—	—
94	50	16	8	3-4	70	3-8F:2-4A	6	6F	—	—
95	49	6	6	3	82	3-9F:2-5A	6	9-15+F	—	—
96	49	4	10	3-4	72	3-10+F:2-8A	14	8-10+F	—	—
97	50	10	52	3-10+	22	3-15+F:2-10+A	16	6-15+F	—	—
98	50	—	18	3-7	66	4-8F:2-6A	16	3-10+F	—	—
99	53	13	9	3-10+	61	4-10+F:2-9A	17	5+F-10+F	—	—
100	50	6	8	3	68	3-8F:2-4A	18	6-10+F	—	—
101	50	—	14	3-7	66	1-10+F:2-9A	20	6-15+F	—	—
102	50	22	—	—	52	3-5F:1-2A	26	3-7F	—	—
103	52	21	—	—	50	3-9F:2-4A	29	3-10+F	—	—
104	51	4	—	—	63	3-8F:2-5A	33	6-10+F	—	—
105	50	8	—	—	28	3-5F:1-3A	64	6-15+F	—	—
106	50	2	—	—	22	4-5F:2A	76	6-10+F	—	—
107	50	6	—	—	2	3F:2A	92	3-9F	—	—
108	50	22	6	3	—	—	—	—	72	One allele rearranged

NOTE. The data are color coded by clinical stage: early stage favorable, light blue; early stage unfavorable, medium blue; advanced stage, dark blue.
Abbreviations: A, aqua centromeric signal; F, fused red/green *PD-L1*/*PD-L2* signal; RS, Reed-Sternberg.

bjh review

The translational science of hodgkin lymphoma

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Summary

Hodgkin Lymphoma (HL) is an unusual B-cell lymphoma because the malignant cells exist as a minority population in a densely cellular microenvironment. The microenvironment is comprised predominately of inflammatory and immune cells with fibrosis in some cases. There are multiple dysregulated signalling pathways that sustain HL within this microenvironment, such as the Nuclear factor-κB and Janus kinase/signal transducers and activators of transcription pathways. Advances in genomic medicine have enabled a better characterisation of the rare tumour cells and improved our understanding of the signalling mechanisms that exist between the malignant cell and its microenvironment. Current therapy for HL produces excellent clinical outcomes in most younger patients. However, problems with current treatment approaches include poorer outcomes in the elderly, toxicity of highly-effective combination chemotherapy regimens and relapse in high-risk patients. Better understanding of disease biology aids in upfront prognostication of patients, defines new methods for treatment monitoring and assists in the recognition of novel targets for therapy. Biology-driven therapies, including anti-CD30 antibody conjugates, cellular immunotherapies and immune modulation, particularly with checkpoint inhibitors, have changed treatment algorithms for relapsed/refractory patients. Future challenges exist in incorporating immune-based therapies earlier in treatment algorithms to reduce toxicity and prevent relapse for patients with HL.

Keywords: hodgkin lymphoma, prognosis, microenvironment, immunotherapy, lymphoma.

In 1832, Thomas Hodgkin described a case series of enlarged lymph nodes, later to be known as Hodgkin Lymphoma (HL) and approximately 60 years later, Dorothy Reed and Carl Sternberg first characterized the abnormal mononucleated Hodgkin and multinucleated Reed-Sternberg cells (HRSC) that are pathognomonic for this disease (Küppers, 2009). The cell of origin was identified 150 years after Thomas Hodgkin published his case series. Current classification for HL denotes classical HL (cHL), with 4 morphological sub-types, and nodular lymphocyte predominant HL (NLPHL) (Swerdlow *et al*, 2008). The pathology of cHL is peculiar, being a B-cell lymphoma with loss of the B-cell phenotype, and being classified by a predominately by the inflammatory tumour microenvironment.

HL has a stable incidence of 1–3 cases per 100 000 persons with a slight male predominance in wealthier countries for which data exists (Engert & Younes, 2015). Its bimodal age distribution has been recognized since the 1960s, with peaks in both young adults and the elderly (Engert & Younes, 2015). HL exhibits a seasonal pattern of incidence with an increased incidence in winter and spring compared to summer and autumn (Borchmann *et al*, 2017). The current treatment of HL is overwhelmingly successful, with 80–90% of patients being cured irrespective of stage (Borchmann *et al*, 2016; Sasse *et al*, 2017). Elderly patients do not have the same excellent outcomes however, probably due to a combination of a more aggressive disease biology and lesser tolerance for the toxic therapies needed to cure younger patients (Sjöberg *et al*, 2012; Borchmann *et al*, 2018b).

Emerging techniques in genomic medicine have significantly advanced our understanding of disease biology. This should lead to better prognostication and more targeted therapies, particularly in high risk patients. The goal of this review is to summarize current literature with respect to the biology of HL. It is not intended to be a systematic review of all publications, rather an overview of disease biology with a focus on avenues that might lead to improved prognostication, targeted treatment and post-therapy monitoring.

Cell of origin and oncogenesis

The difficulty in determining the cell of origin of HRSC was due to their mixed immunophenotype with expression of markers across multiple haematopoietic lineages (Küppers

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et al, 2012). Propagation of HRSC cell lines and development of animal models also created research challenges. To date, only a few cell lines exist that were cultivated from very advanced, multiply relapsed patients, and this should caution the representativeness of data collected from cell lines for most newly diagnosed HL patients. HRSC are dependent on activating, growth-supporting signals from their deregulated microenvironment. Similarly, animal models used to evaluate novel treatment modalities in the past rely on xenografting HL cell lines into immunodeficient mice and ignore the complex interplay between the malignant tumour cells and their microenvironment (Küppers & Rajewsky, 1998). Prior to the availability of microdissection, it was problematic to isolate sufficiently pure tumour-derived DNA from the malignant HRSC or lymphocyte predominant (LP) cells, hampering genomic investigations. Once isolation of malignant cells was possible, clonal immunoglobulin gene rearrangements in HRSC were identified, proving their B-cell origin (Küppers *et al*, 1994; Bräuninger *et al*, 2003). Somatic hypermutation in the rearranged variable (V) region of HRSCs leads to the conclusion that HL is of germinal centre (GC) B-cell origin (Klein *et al*, 1998; Brune *et al*, 2008). Additionally, there is evidence that the HRSC undergoes class-switch recombination (Martin-Subero *et al*, 2006). Interestingly, many HRSCs contain mutations that lead to a non-functional V region that functionally 'cripples' the cell, which in normal B-cell development should lead to apoptosis, but in HL additional oncogenic events rescue these pre-apoptotic cells (Kanzler *et al*, 1996; Bräuninger *et al*, 2003). There is emerging evidence from gene expression analysis that HRSC may be related to a rare normal CD30+ B-cell population, that has a preserved, functional B-cell receptor (BCR) (Weniger *et al*, 2018). This is one peculiarity of the HRSC, which can persist despite its non-functional BCR and evade the usual apoptotic fate similar B cells face.

The HRSC appears to respond to ligands within the microenvironment to constituently activate signalling pathways that promote their survival and proliferation. Central to these processes are the Nuclear factor (NF)-κB and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways. The HRSC express multiple receptors which activate NF-κB signalling via the tumour necrosis factor (TNF) receptor family (including CD30 [TNFRSF8], CD40, RANK [TNFRSF11A], TACI [TNFRSF13B] and BCMA [TNFRSF17]), for which the ligands are expressed within the HL microenvironment shown in Fig 1 (Engert & Younes, 2015). The main mechanism for cytokines signalling in HL is via the JAK/STAT pathway, including phosphorylation and activation of multiple STAT transcription factor,s such as STAT3, STAT5 (STAT5A) and STAT6 in HL (Skinnider *et al*, 2002; Scheeren *et al*, 2008). Signalling via the interleukin 21 receptor (IL21R), demonstrated in HL cell lines, enhances and activates STAT3 and STAT5 transcription (Scheeren *et al*, 2008). Interestingly, this study also reports STAT5A and STAT5B activation in normal B cells can induce an HRS-like immunophenotype (a *loss of B-cell* phenotype). The IL13 receptor at least partly mediates STAT6 expression via an autocrine signalling loop (Skinnider *et al*, 2002). Receptor tyrosine kinase (RTK) activation in HL also appears to be ligand-mediated and their expression profile is distinct from normal GC B cells and other B-cell non-Hodgkin lymphomas (Renne *et al*, 2005). Sphingosine-1 phosphate (S1P) is a recently described ligand of relevance to HL because of its activation of the phosphatidylinositide 3-kinase (PI3K) pathway, mediated by its receptors (S1PRs 1–5). S1P activation in HL leads to increased expression of basic leucine zipper transcription factor, ATF-like 3 (BATF3) in a *feed-forward* loop via an elevated S1PR1 expression compared to lowered S1PR2 (Vrzalikova *et al*, 2018). BATF3 expression is known to be increased in primary HRSC (Brune *et al*, 2008; Lollies *et al*, 2018; Vrzalikova *et al*, 2018). However, in another newly described oncogenic feedback loop, BATF3 expression also appears to be induced by STAT3/6 transcription factors (JAK/STAT pathway) and, in turn, regulates lymphomagenesis via MYC activation (Lollies *et al*, 2018). Hence the constituent activation of multiple signalling pathways (including NF-κB, JAK/STAT, PIK3) allows for HRSC survival and immune evasion.

The anti-apoptosis armour of HRSC remains an area of investigation. Several mechanisms in addition to JAK/STAT, NF-κB and RTK pathways have been described. HRSC demonstrate resistance to CD95 (FAS)-ligand mediated death (via the CD95 inhibitor cFLIP [CFLAR]) (Re *et al*, 2001). Other anti-apoptotic mediators described in HL include BCLXL (BCL2L1), BIK, XIAP and MDM2 (Chu *et al*, 1999; Kashkar *et al*, 2006; Kochert *et al*, 2011; Tiacci *et al*, 2012). Small molecule targets to overcome these anti-apoptotic signals could be important for future studies.

There has been debate about the origin of the pathognomonic, multinucleated phenotype of Reed-Sternberg cells. As HRSC have a mixed phenotype of different haematopoietic cell lineages, with high chromosomal copy number complexity, there was speculation that they are derived from fusion of unrelated cells (e.g. a B cell and a non-B cell). However, HRSC have a maximum of two rearranged immunoglobulin alleles providing evidence against fusions between distinct malignant clones (Ikeda *et al*, 2010). A process of re-fusion has been demonstrated to occur between mononucleated (Hodgkin) daughter cells to create multinucleated (Reed-Sternberg) cells after incomplete cytolysis (Rengstl *et al*, 2013). Multinucleated Reed-Sternberg cells also have little proliferative capacity (Newcom *et al*, 1988) and hence the Hodgkin cells are thought to be the main source of tumour clones.

In NLPHL, mutations in the immunoglobulin gene preserves partial BCR function, with only partial loss of the B-cell phenotype and preserved CD20 (MS4A1)/BCL6 expression (Brune *et al*, 2008). The immunophenotype in NLPHL is in keeping with antigen-selected GC B-cell origin (Bräuninger *et al*, 2003). Rare cases reporting T-cell origin of

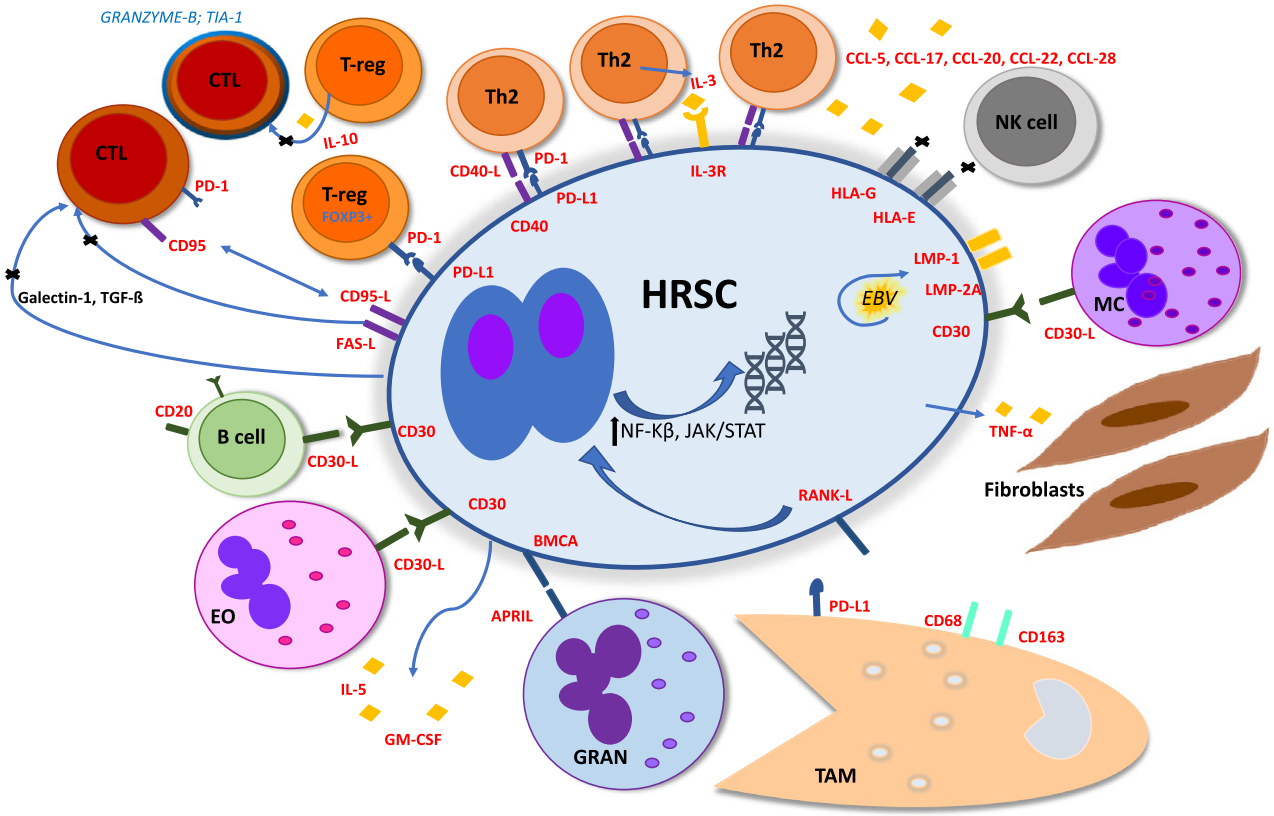

Fig 1. Schema of HRSC interaction with the microenvironment. The Hodgkin-Reed-Sternberg cells (HRSC) secrete cytokines and chemokines (reflected by yellow diamonds) that recruit an inflammatory cell infiltrate into the tumour and help sustain them. The microenvironment is rich in lymphocytes (particularly Th2 and T-regs). Granulocytes (GRAN), eosinophils (EO), mast cells (MC), tumour-associated macrophages (TAM) and fibroblasts are also recruited by the HRSC. Upregulation of NF-κB, JAK/STAT and PI3K pathways leads to altered signalling that is an essential part of HRSC survival mechanisms. Common comparative copy number increases of a locus located at 9p24.1 can lead to overexpression of PD-L1 and PD-L2 and increased JAK/STAT signalling. The presence of FOXP3+ T-regs and CD20+ B-cells incurs a favourable prognosis. Cytotoxic lymphocytes (CTL) expressing granzyme-B or TIA1, and elevated TAM are associated with an inferior prognosis.

HL have been reported by proving clonal T-cell receptor (TCR) gene rearrangements in the absence of immunoglobulin rearrangement. However, debate exists as to whether these isolated cases represent a true diagnosis of HL or a mimic for this disease (Engert & Younes, 2015).

The genomic landscape

Cytogenetic analysis of HL is hampered by the paucity of malignant cells, limited metaphases and chromosomal instability. Microdissection of HRSC enables the use of genomic techniques, such as comparative genomic hybridisation (CGH), by providing an enriched population of tumour cells. Multiple chromosomal imbalances in tumour biopsies (Ohshima *et al*, 1999; Hartmann *et al*, 2008) and HRSC cell lines (Joos *et al*, 2003) have been reported. In summary, HRSC harbour tremendous cytogenetic complexity with frequent, reoccurring chromosomal imbalances affecting multiple oncogenic pathways (Borchmann & Engert, 2017). This can lead to constituent activation of pathways normally only transiently active in B cells (Küppers, 2009). Common examples include copy number increases in 2p12-16 (*REL* gene, NF-κB pathway) and 9p24 (*JAK2* and *CD274*/*PDCD1LG2* [*PD-L1*/*PD-L2*] genes, JAK/STAT pathway). Table I provides a summary of copy number changes in HL and NLPHL. For a discussion of genomic landscape in NLPHL see Data S1.

Chromosomal translocations occur in HRSC less frequently than copy number aberrations. Translocation of the immunoglobulin gene loci is reported in about 20% of HL samples, with partner genes also known to be recurrently mutated in other B-cell malignancies (*MYC*, *BCL6* and *REL*) identified (Martin-Subero *et al*, 2006). In this study, 76% of cHL had at least partial deletions in *IGH* constant regions, suggesting class switch recombination. This finding supports the previous description of HRSC origin.

Somatic mutations in *B2M* are reported to be common in Nodular Sclerosis (NS) HL. By whole exome sequencing, the prevalence of *B2M* mutations was shown to be 70% in NSHL, with loss of β2-microglobulin (B2M) (major histocompatibility complex [MHC] class I) expression correlated with lower

Table I. Summary of comparative copy number changes in cHL and NLPHL.

Location	cHL (All subtypes)	NLPHL	Gene/Pathway
GAINS			
1p	56%	37%	
1q		58%	
2p	28–54%		*REL* (NF-κB pathway)
2q		37%	
3p		53%	
3q		48%	*BCL6* (proto-oncogene, Ig transcription factor)
4q		48%	
5q		53%	
6p		37%	
6q		47%	*ROS1* (oncogene)
7q	56%		
8q		47%	
9p	24–42%		*JAK2* (JAK/STAT pathway), PD-L1 and PD-L2
11q		37%	
12q	30–40%	42%	*MDM2* (cell cycle, apoptosis)
14q	7–30%		
16p	24–30%		*ABCC1* (multidrug resistance gene)
17p	25–40%		
17q	20–70%		*MAP3K14* (NF-κB pathway)
19p	44%		
19q	25–44%		
20q	15–23%		*CD40* (NF-κB pathway)
21q	30%		
LOSSES			
1q	42%		*TNFRSF14* (TNF receptor, immune response)
4q	7–25%		
6p		21%	
6q	5–50%		*TNFAIP3* (NF-κB pathway, apoptosis)
8p		21%	
10p		21%	
11q	3–25%	26%	
13q	22–35%		
16p		26%	
16q	67%		
17p/17		42%	*TP53* (tumour suppressor gene)
21q	30%		
22q	44%		

Sources: (Ohshima *et al*, 1999; Franke *et al*, 2001; Joos *et al*, 2002; Chui *et al*, 2003; Hartmann *et al*, 2008; Steidl *et al*, 2010b; Reichel *et al*, 2015; Salipante *et al*, 2016).

cHL, classical Hodgkin lymphoma; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma.

stage disease and better overall and progression-free survival in one cohort (Reichel *et al*, 2015). *TNFAIP3* mutations were also identified in this study at a higher frequency than prior reports (60% in NSHL) (Reichel *et al*, 2015). *TNFAIP3* encodes an ubiquitin-converting enzyme of the NF-κB pathway, and loss of function mutations was recently linked to lower efficacy of chemotherapy (Yang *et al*, 2018). By whole exome sequencing, Tiacci *et al* (2018) found that over 80% of patient samples carry mutations in the JAK/STAT pathway. In this study, the two most commonly mutated genes identified were *STAT6* (32%) and *SOCS1* (59%) (Tiacci *et al*, 2018). Another somatic mutation in a gene with regulatory function on the JAK/STAT pathway, *PTPN1*, is also described. *PTPN1* mutations were identified after whole genome (and whole transcriptome) sequencing of two cases of Primary Mediastinal B-Cell Lymphoma (PMBCL), and due to known similarities in the molecular signatures of PMBCL and cHL, these mutations were further investigated in HL (Gunawardana *et al*, 2014). The authors described *PTPN1* mutations in 20% of dissected HRSC (from cHL tumours) and in two-thirds of examined HL cell lines (Gunawardana *et al*, 2014). The *PTPNP1* gene is an inhibitory regulator of the JAK/STAT pathway, and the resulting loss of functional PTPN1-B protein in mutated cases leads

to increased activation of the JAK/STAT pathway with phosphorylation of multiple kinases. Taken together, these studies suggest a pivotal role for the JAK/STAT pathway in the pathogenesis of cHL. Other lower frequency somatic mutations, including *GNA13*, *XPO1* and *ITPKB* have also been described (Camus *et al*, 2016; Tiacci *et al*, 2018), with further research ongoing.

Gene expression has also been examined in cHL. One group published a large cohort of 290 patients using the NanoString platform from formalin-fixed, paraffin-embedded (FFPE) tissue samples (Scott *et al*, 2013). Their gene expression data was used to design a predictive model, incorporating survival end-points, and then applied to a validation cohort. NanoString can easily be applied to FFPE tissue, making it an attractive platform for use at diagnosis or relapse. However, external validation of the NanoString platform on an independent cohort is yet to be published. This data will be necessary to judge the clinical usefulness of this platform. Another study identified HL-associated micro-RNA expression patterns that differ from healthy patients and normalize when monitored in extracellular vesicles in patient's plasma after successful treatment (van Eijndhoven *et al*, 2016). Hence, this approach could be used to monitor treatment. Similarly, monitoring patients after therapy with a *liquid biopsy* of cell free DNA (cfDNA) could replace or be an adjunct to interim radiology guidance and/or be used to monitor a patient's response to therapy. One small study identified *XPO1* mutations from cfDNA in serial patient samples and detected a residual mutational load in patients who went on to relapse (Camus *et al*, 2016). Altogether, this data provides proof of concept that cfDNA samples could be used for minimal residual disease monitoring.

The microenvironment in HL

Lymph node tissue involved by HL contains only very few neoplastic HRSC but abundant non-neoplastic cells. The majority of non-neoplastic cells represent a lymphoma-specific response, creating a highly abundant inflammatory tumour microenvironment. HRSC secrete cytokines to attract inflammatory cells, including IL5, CCL5, CCL17; CCL20, CCL22 and CCL28 (Liu & Shipp, 2017). These lead to a microenvironment comprised of predominantly T cells (T-helper [Th] and T-regulatory phenotype), as well as macrophages, eosinophils, neutrophils, mast cells, B cells and plasma cells (Liu & Shipp, 2017) and prominent connective tissue components, such as fibroblasts and collagen fibres. Herein we discuss the microenvironment features relevant to cHL, however a description of the tumour microenvironment in NLPHL is provided in Data S1.

The composition of the tumour microenvironment in cHL is not entirely unique and certain components may be observed in other entities, such as T-cell lymphoma, and other B-cell lymphomas, such as sclerosis in follicular lymphoma and mediastinal large B-cell lymphoma. However, it is the *extent* of the non-malignant compartment within the tumour microenvironment that is an outstanding feature of HL. The non-malignant cells recruited into the HL microenvironment also secrete chemokines/cytokines promoting HRSC proliferation and survival (Liu *et al*, 2014; Aldinucci *et al*, 2016). An example of this interaction is the secretion of IL3 by T cells recruited into the microenvironment, leading to eosinophil upregulation of the ligands for CD40 and CD30, both of which promote HRSC growth and proliferation (Pinto *et al*, 1997). Fibrosis is thought to be mediated by HRSC expression of IL13 and transforming growth factor-β (TGF-β, TGFB1(Ohshima *et al*, 2001). One of the most abundant, highly cHL-specific cytokines in the microenvironment of cHL is CCL17, also known as thymus- and activation-regulated chemokine (TARC). Its relative specificity for cHL has led to its investigation as a disease burden and disease response biomarker (Sauer *et al*, 2013). Other examples of feedback mechanisms within the microenvironment have recently been reviewed elsewhere (Liu *et al*, 2014; Aldinucci *et al*, 2016).

HRSC produce immunosuppressive cytokines (e.g. IL10, TGF-β, Galectin-1) that can regulate and inhibit CD8+ (cytotoxic) T-cell populations and facilitate immune escape by T-cell exhaustion (Liu & Shipp, 2017). Additionally, HRSC can down-regulate MHC class I or II molecules on their surface via loss of function mutations (e.g. B2M) or translocations (e.g. CIITA) (Steidl *et al*, 2011a; Reichel *et al*, 2015). This can make them 'invisible' to T-cells, but potentially vulnerable to natural killer (NK) cell-mediated toxicity. Therefore, additional immune escape mechanisms might be important. HRSC can express human leucocyte antigen (HLA)-G, HLA-E or MHC class I chain-related protein A (MIC-A) that protect them from NK cells and T-cell cytotoxicity (Engert & Younes, 2015). HRSC also express CD95 ligand, which induces apoptosis of activated Th1 and CD8+ T-cells (Poppema *et al*, 1998). Histologically, HRSC are often surrounded by a rosette of T cells. This morphological feature may function as a source of survival, promoting T cells (mostly Th2 cells) but also form a physical barrier against activated CD8+ cytotoxic T cells. Hence, the microenvironment appears to be a *maligned* assembly of immunological cells that build a protective and supportive surrounding for HRSC rather than contributing to an active anti-lymphoma response. See Fig 1 for a schematic overview of interactions within the HRSC microenvironment.

The composition of this tumour microenvironment displays striking intra- and inter-tumoural heterogeneity. Assessing these features has become a focus of research with regard to prognostic information and potential therapeutic targets. Associations between the cellular infiltrate and prognosis have been studied using immunohistochemistry (IHC) and gene expression profiling (Steidl *et al*, 2010a, 2011b). Figure 2 illustrates the characteristic immune populations within the microenvironment, by IHC. Here, we will look in more detail into their role and summarize the most

important prognostic findings for T cells, B cells and macrophages as participants in the microenvironment.

T cells and NK cells

In the majority of cHL subtypes, T cells are the most abundant cell population in the microenvironment, including T-regulatory cells (T-regs), T helper cells (Th1, Th2) and cytotoxic T-lymphocytes (Wein & Küppers, 2016). CD4+ T cells have a supportive effect on HRSC by both directly shielding and inhibiting them from cytotoxic T lymphocytes (CTLs) and NK(T) cells. Interestingly, human immunodeficiency virus (HIV)-infected patients have an up to 10-fold higher risk of developing cHL, similar or, in some cases, lower compared to their additional risk of developing other lymphomas. However, in contrast to other lymphomas, incidence of cHL might even increase in HIV-infected patients after successful highly active antiretroviral therapy (HAART). This is in stark contrast to the incidence of other lymphomas, for which the risk declines with HAART. These observations are consistent with the supportive role of CD4+ T-cells in cHL pathogenesis after an initial malignant transformation (e.g. Epstein–Barr virus [EBV]-infection) has taken place.

The polarization of Th1 and Th2 cells within the microenvironment is subject to conflicting reports. The Th1 transcription factor, T-bet (TBX21), is detectable in the microenvironment T cells of both cHL and NLPHL by IHC. In one study, the Th2 transcription factor c-MAF (MAF) and T-reg transcription factor FOXP3 were shown by IHC to be expressed at much higher densities in the tumour microenvironment than T-bet (Schreck *et al*, 2009). Interestingly, in this study, low numbers of T-regs and Th2 infiltrating T cells were associated with inferior survival. In a more recent report, tissue microarray IHC was applied to a large cohort of cHL samples and Th1 T-bet expression was demonstrated to be more abundant than Th2-associated c-MAF/GATA3 expression (Greaves *et al*, 2013a). We consider this an open question as to the association between Th1 and Th2 populations in the immune response to the malignant HRSC cell. The explanation for this discrepancy may relate to T-regs within the microenvironment and their immunosuppressive

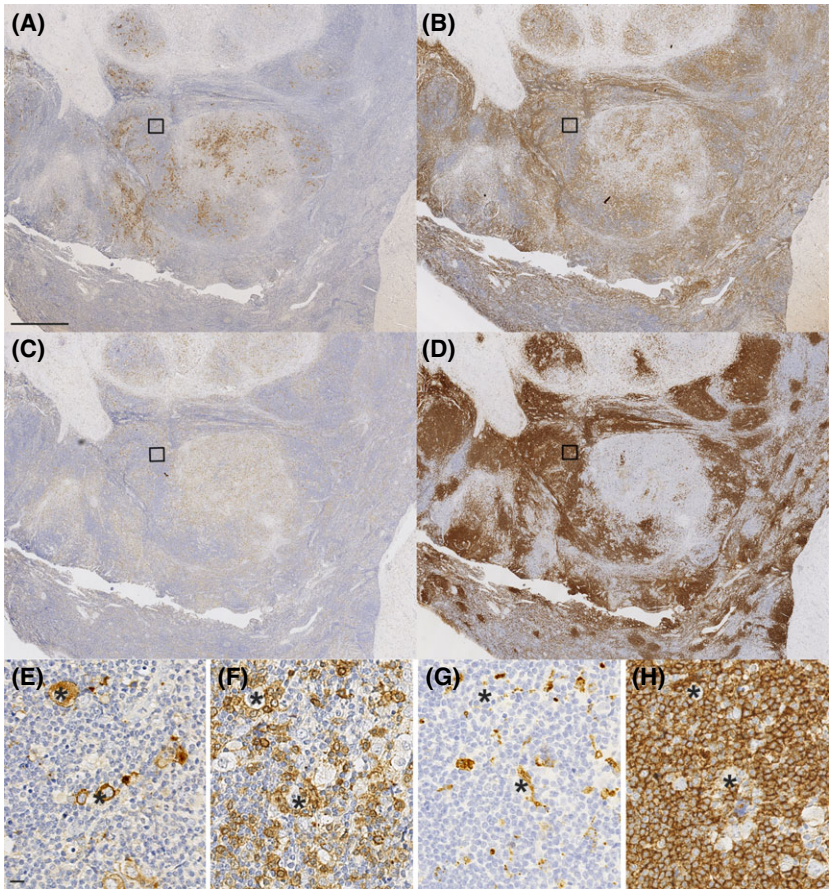

Fig 2. Histology of the microenvironment in cHL. Immunohistochemical staining of formalin-fixed paraffin-embedded tissue from nodular sclerosis Hodgkin lymphoma. (A, E) Anti-CD30 staining to identify Hodgkin-Reed-Sternberg cells (HRSC); (B, F) Anti-CD3 staining to identify T cells; C, G) Anti-CD68 staining to identify macrophages; (D, H) Anti-CD20 staining to identify B cells. Framed areas in (A–D) are shown in a higher magnification in (E–H). Black asterisks in (E–H) indicate HRSC. (A–D): scale bar = 1000 μm; (E–H): scale bar = 10 μm

potential, limiting the activation of different effector T-cell populations (Ishida *et al*, 2006). An increase in T-regs has been shown to be a favourable bio-marker in cHL (Alvaro *et al*, 2005; Greaves *et al*, 2013b), although, some controversary exists (Hollander *et al*, 2018). However, the mechanism of this effect is unclear, as T-regs can inhibit the humoral immune responses mediated via Th2 cells and have immunosuppressive activity on cytotoxic lymphocytes.

The ratio of T helper cells (CD4+) and T effector cells (CD8+) may also correlate with prognosis in cHL, although reports are not consistent. High CD8+ and low CD4+ T-cells were associated with a favourable outcome in one study (Alonso-Álvarez *et al*, 2017). In a larger cohort, low CD8+ T cells in early stage disease showed poorer outcomes (Alvaro-Naranjo *et al*, 2005). In this study, an elevated number of CTLs (by expression of TIA1 and granzyme B) was also correlated with shortened survival. The negative prognostic effect of high numbers of CTLs has been confirmed in other studies (Alvaro *et al*, 2005; Vari *et al*, 2018). A recent study reported increased CD56-bright, CD16-dim NK cells with high PD-1 (also termed PDCD1) expression in pre-treatment blood samples from patients with cHL, a finding that reverts to normal after chemotherapy (Vari *et al*, 2018). In an *in vitro* model, the same study showed that monocytes with a tumour-associated macrophage phenotype suppress the activation of NK cells, a feature that was partly reversed by use of an anti-PD-1 antibody (Vari *et al*, 2018). The findings have several implications: firstly, CD56-bright NK cells are likely to be players in the immune evasion of cHL due to less potent cytotoxicity and altered cytokine production (IFN-γ, IL-10) (Poli *et al*, 2009) as compared to CD56-dim, CD16-bright NK cells from normal controls; secondly, this study identifies an interaction between monocyte/macrophages and NK cells via their respective PD-L1 and PD-1 expression, and thirdly because NK cells might play, at least in part, a role in the sensitivity of cHL to anti-PD-1 therapy (Vari *et al*, 2018). Detailed immunophenotyping of T cells and NK cells within the microenvironment may provide further prognostic information. Here, novel technologies, such as multiplex IHC, single-cell transcriptomics and mass cytometry, might be useful.

B cells

B-cell abundance in cHL is associated with a favourable prognosis. The topology of B cells is relevant, as one group demonstrated only non-follicular arranged CD20+ B-cells confer an advantageous prognosis (Greaves *et al*, 2013b). Other groups observed improved survival in patients with high numbers of aggregated (lymph follicles) or dispersed B-cells (Panico *et al*, 2015). The mechanism of this observation remains uncertain. Anti-tumour activity of Bcells has been proposed, however such B-cell populations could also reflect remnants of pre-existing lymph nodes. Consequently, the high number of B cells might just be a proxy for a less aggressive disease biology causing less destruction of the physiological lymph node architecture. In clinical trials, anti-CD20 antibodies for B-cell depletion initially showed favourable results (Younes *et al*, 2003), but this was not confirmed in larger trials (Borchmann *et al*, 2018a), a finding that may be consistent with the protective association of B-cell abundance.

Macrophages

Tumour-associated CD68+ macrophages (TAM) can be identified by gene expression profiling (Steidl *et al*, 2010a) or IHC and are a predictor of overall survival. High TAM content in tumour biopsies was associated with inferior survival in several studies (Touati *et al*, 2015; Gotti *et al*, 2017). A meta-analysis of almost 3000 patients found that high density of TAM (identified by CD68+ or CD163+ expression) was a robust predictor of adverse outcomes in adults (Guo *et al*, 2016). However, several negative studies, demonstrating no association with elevated TAM have also been published. Macrophages can be quantified by IHC (CD68 and/or CD163) using manual visual inspection (and quantified by counting cells per high powered field), or digital image analysis. Methodological discrepancies between studies is noted; such as the target of the antibody used (CD68 or CD163), the antibody clone or the method of quantification (Klein *et al*, 2014). Heterogeneity in the reporting means that clinical application is not straight-forward, and no guidelines exist for translation of this research outside of clinical trials.

The mechanism of this association between macrophages and aggressive disease biology is not well understood. However, an association between TAM and B cells has been observed. A favourable risk group can be identified by high CD20+ (B cell) background populations and low TAM content (Panico *et al*, 2015). The reverse combination of low CD20+/high TAM was significantly associated with lower overall survival in multivariate analysis (Panico *et al*, 2015). An association between TAM and NK cells has also been reported (Vari *et al*, 2018) to be related to their PD-L1 and PD-1 expression, respectively (see Section on T cells and NK cells). Further studies are required to establish a suitable method for macrophage quantification in routine clinical use.

Histopathological characteristics, subtypes and diagnosis

HL is classified into two major subgroups: cHL (95% of patients) and NLPHL (5% of patients) (Swerdlow *et al*, 2008). The diagnostic characteristics of NLPHL is described in Data S1. In cHL, HRSC are identified as large cells with a slightly basophilic cytoplasm and multiple large, rounded nuclei with usually one prominent eosinophilic nucleolus. Reed-Sternberg cells must have at least two nucleoli in two

separated nuclear lobes to be diagnostic (Swerdlow *et al*, 2008). The smaller mononuclear variant morphologies include Hodgkin cells (with similar cytological features), lacunar cells (with more abundant clear/slightly acidophilic cytoplasm that is sensitive to fixation) and mummified cells (with condensed dark chromatin and dark cytoplasm) (Swerdlow *et al*, 2008).

The subtyping of cHL is primarily based on features of the microenvironment (Fig 3). In a simplified view, the dominant or characteristic feature of the microenvironment determines the histology subtype (Nodular Sclerosis – abundant connective tissue; Lymphocyte Rich – abundant B cells; Mixed Cellularity – T cells and macrophages; Lymphocyte Depleted – lack of B cells and T cells). We have provided a more detailed description of the histopathological subtypes of cHL in Data S1.

The malignant phenotype

HRSC have a characteristic immunophenotype co-expressing CD30 and CD15, although 20–30% of cases will be CD15 negative (Engert & Younes, 2015). They have lost the typical B-cell phenotype. It is currently not known if this is just a side effect of other events in the process of HL tumorigenesis or if this phenotype is selected for to evade the apoptosis a regular B cell would undergo had it incurred non-functional immunoglobulin mutations. HRSC do not express surface BCR and rarely express GC B-cell markers (e.g. BCL6, AID [AICDA]) or surface B-cell proteins (e.g. CD20, CD37, CD53, CD79a) (Swerdlow *et al*, 2008). Markers of haematopoietic origin (e.g. CD45 [PTPRC]) and B-cell transcription factors (e.g. OCT-2 [POU2F2], BOB1 [POU2AF1], PU.1 [SPI1], A-MYB [MYBL1], SPIB) are also typically not detectable due to their downregulation in HL (Engert & Younes, 2015). B-cell lineage can be detected by weak nuclear staining of the B-cell activator protein PAX5 on a background of small B cells. HRSC can express markers typically of T cells (e.g. CD3) and cytotoxic lymphocytes (granzyme B). Interestingly, many molecules involved in antigen presentation and the interaction with T cells, such as CD40, CD80 and CD86 are also expressed in HRSCs (Engert & Younes, 2015). See Data S1 for a description of the malignant phenotype of NLPHL.

EBV signalling

EBV infection occurs in up to 40% of HL in western countries (Küppers *et al*, 2012). The presence of clonal HRSC with EBV infection (i.e. all the HRSC are derived from a single virus-infected B cell) suggests that EBV may be an early event in disease pathogenesis (Engert & Younes, 2015). The same EBV viral strain has also been shown to persist at disease relapse, providing further evidence for the crucial pathogenetic relevance of EBV in these cases. EBV association with HRSC is usually demonstrated by either IHC staining for latent membrane protein 1 (LMP1) or EBV-encoded small RNA (EBER) *in situ* hybridisation. Despite the important role of EBV infection in HL pathogenesis, the EBV-association of a patient's HL currently does not affect treatment algorithms.

Latent EBV infection may be a factor in preventing the HRSC from undergoing apoptosis after *crippling* somatic hypermutation occurs, leading to immune evasion. Hence, latent EBV infection may be crucial to the survival of HRSC (Küppers *et al*, 2012). LMP1 mimics constituently active CD40 on B cells and is linked to NF-κB activation in GC cells and to transcriptional reprogramming (Engert & Younes, 2015). Several studies have demonstrated that latent membrane protein 2A (LMP2A) allows for survival of non-functional B cells within the GC (Portis *et al*, 2003; Anderson & Longnecker, 2009). The LMP2A protein appears to be a functional mimic for the BCR (Engert & Younes, 2015) and its expression in B cells creates a gene expression profile similar to HL cell lines (Portis *et al*, 2003). LMP2A expression is also linked to constituent activation of the NOTCH1 pathway (Anderson & Longnecker, 2009), although downregulation of many BCR signalling molecules suggests the pathogenesis of HL is probably more complex and multifactorial.

Preliminary data exists that translates this research to clinical practice. The use of autologous, manufactured, EBV-specific (LMP1/2) expanded CTLs has demonstrated efficacy in high-risk and multiply relapsed EBV+ lymphomas (Bollard *et al*, 2014). Their results showed a 50% complete response rate for patients with active EBV+ lymphoma (HL and non-Hodgkin lymphoma) with minimal toxicity. This study builds on an earlier report (by the same group) of EBV-specific T cells as potentially effective in EBV+ HL. A potential advantage of virus-specific T cells over other cellular immunotherapies is to avoid the need for lymphodepletion (as in many chimeric antigen receptor T [CAR-T] cell protocols) and obviates the risk of cytokine release syndrome (which is not reported with virus-specific T cells).

EBV-associated microRNAs remain an area of investigation as specific signatures are thought to inhibit B-cell apoptosis (Klinke *et al*, 2014). In addition, gene expression profiling suggests that EBV may only have a small influence on the transcription profile of HRSC (Tiacci *et al*, 2012). In young adults EBV status does not appear to affect prognosis. However, in patients aged over 50 years, inferior outcomes are observed. This may relate to differences in disease biology and other host factors also contributing to the bi-modal incidence of this disease.

Biology-driven treatment in cHL

Antibody directed therapy; antibodies, conjugates, bi-specific antigen therapies and chimeric antigen receptors

Anti-CD30 antibodies (aCD30-Ab) were shown to have modest efficacy in Hodgkin cell lines and mouse models

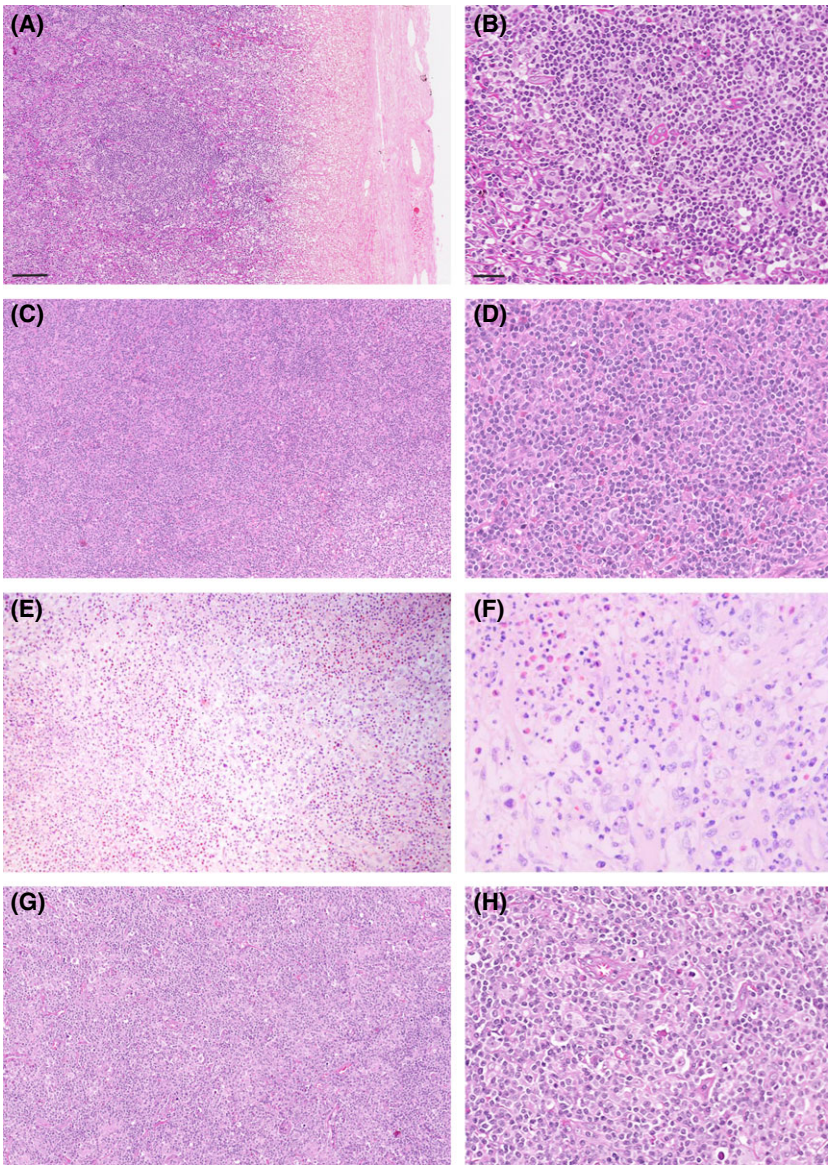

Fig 3. Histological subtyping of cHL. Haematoxylin and Eosin staining of formalin-fixed paraffin-embedded tissue of different subtypes of classical Hodgkin lymphoma. (A, B) nodular sclerosis; (C, D) mixed cellularity; (E, F) lymphocyte-depleted; (G, H) lymphocyte-rich. (B, D, F) and (H) show a higher magnification of an area from (A, C, E) and (G). (A, C, E) and (G): scale bar = 100 μm; (B, D, F) and (H): scale bar = 30 μm.

(Borchmann *et al*, 2003). However, the translation to clinical trials has been disappointing. SGN-30, a chimeric aCD30-Ab, was tested in a small phase 2 dose escalation study for relapsed/refractory CD30+ lymphoma (Forero-Torres *et al*, 2009). There were no responders in the HL arm, hence trials have not progressed for HL.

Immunotoxin conjugates linked to aCD30-Ab provide direct drug delivery to HRSC. Preliminary trials of aCD30-immunotoxin constructs were hampered by low clinical response rates and major toxicity as well as the emergence of neutralizing antibodies. A humanised aCD30-Ab (5F11) was trialled in a phase 1/2 study but complete results remain unpublished and the interim response rate was limited (Renner & Stenner, 2018).

Brentuximab vedotin (BV) is an aCD30-Ab conjugated to the microtubule-disrupting agent, monomethyl auristatin E. BV incorporates the chimeric aCD30-Ab SGN-30 into a novel compound (Renner & Stenner, 2018). The pivotal phase 2 study showed an overall response rate of 75% in relapsed/refractory HL patients(Younes *et al*, 2012a). Application of BV in clinical practice now extends to post-transplant consolidation for high risk HL (AETHERA trial) (Moskowitz *et al*, 2015) and is thought by some investigators to have a role in upfront treatment for advanced stage

disease (ECHELON-1) (Connors *et al*, 2018). The clinical benefit of upfront BV supplementing doxorubicin, vinblastine and dacarbazine (BV-AVD) compared to other, highly effective, conventional treatment regimens, such as the combination of bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone and procarbazine (BEACOPP) is an open question and a direct comparison is yet to be completed. However indirect comparison suggests that BV-AVD is less effective than a positron emission tomography-guided BEACOPP treatment (Borchmann *et al*, 2018a). The excellent efficacy of BV is leading to many concurrent new trials that incorporate its' use earlier in contemporary treatment algorithms.

A bispecific aCD30-Ab/CD16A construct (AFM13) has also been trialled in a phase 1 study (Rothe *et al*, 2015). This compound recruits NK cells to the HRSC for targeted killing. In this dose escalation study, modest activity in relapsed/refractory patients was observed, despite heavy pre-treatment (Rothe *et al*, 2015). Preliminary data from a phase 1b study combining AFM13 with pembrolizumab was recently reported, suggesting combination therapy will be tolerable to patients (Ansell *et al*, 2017). The use of AFM13, a bi-specific antigen therapy, is an exciting translation from other malignancies but further studies are needed.

Another antibody target within the microenvironment is CD123 [IL3RA], a component of the IL3 receptor with moderate expression on HRSC. It is rarely expressed in other B- or T-cell lymphomas and has limited expression in the normal lymph node. CD123 is present on myeloid-derived suppressor cells (MDSC) and correlates with CD68+ TAM that exist within the HL microenvironment (Ruella *et al*, 2017). Targeting CD123 has been tested using anti-CD123 CAR-T cells in a murine model of HL (Ruella *et al*, 2017). CAR-T cell therapy is a promising avenue for therapy in HL because the cells represent a newly sensitized immune compartment, primed to overcome HRSC immune 'evasion'. There are several clinical trials currently examining aCD30 CAR-T cell therapy in relapsed/refractory HL and results are awaited (RELY-30: NCT02917083; CART30: NCT02259556; ICAR30 T-cells: NCT03383965; ATL-CAR.CD30 cells: NCT03602157; Anti-CD30-CAR T-cells: NCT03049449).

Immunomodulation

Lenalidomide, an immunomodulatory agent, has been trialled in a phase 2 study of relapsed refractory HL (Fehniger *et al*, 2011). The grade 3/4 haematological toxicity rate was almost 50% (predominately neutropenia) with overall response rate less than 20%, but one-third of patients achieved complete/partial response or stable disease. Further trials were proposed as combination therapy, however other agents have become available in the interim that demonstrate higher efficacy making this approach less attractive.

JAK/STAT pathway inhibition

Amplification of the 9p24 locus in HRSC provides biological plausibility for JAK/STAT pathway inhibition to be effective. A phase 1 study of SB1518 (an oral JAK2 kinase inhibitor) showed some response in relapsed HL (Younes *et al*, 2012b). However, a phase 2 study of ruxolitinib in relapsed/refractory HL showed an overall response rate <10% and adverse events occurred in >40% of patients (all grades) with anaemia, lymphopenia and infection being the most common grade 3/4 adverse events (Van Den Neste *et al*, 2018). Interestingly, this study reported patients having an improvement in B-symptoms and pruritis despite progressive disease. Based on this study, JAK-STAT pathway inhibition is unlikely to be clinically useful as a monotherapy but might play a role in combination regimens.

Checkpoint inhibition

A phase 1 study of nivolumab (a PD-1 blocking antibody) showed response rates of >70% in heavily pre-treated HL (Ansell *et al*, 2015) with drug-related immune-mediated toxicity reported in a quarter of patients. Phase 2 trials of nivolumab in relapsed/refractory HL have continued to show high response rates (~70–80%) with a tolerable and manageable side effect profile (Younes *et al*, 2016; Maruyama *et al*, 2017). Nivolumab has also been successfully applied in post-allogeneic transplant patients at relapse (Herbaux *et al*, 2017). Pembrolizumab is an alternative anti-PD-1 antibody with good efficacy in phase 1 and 2 trials in heavily pre-treated patients (Armand *et al*, 2016; Chen *et al*, 2017). The response rates are similar to that observed with nivolumab (~70%), with a similarly acceptable side effect profile. Both agents are likely to be equivalent in their efficacy and safety. The success of PD-1 inhibitors has prompted further trials incorporating these agents earlier in treatment algorithms.

The mechanism for such high efficacy of checkpoint inhibition is not entirely clear. Although many cases of HL have increased copy numbers of the 9p24 loci detected, and there is some association between the copy number or PD-L1 expression in IHC and outcome to immune checkpoint inhibition, this relationship is not clear enough to base treatment decisions upon. One biomarker recently reported as prognostic for HL patients treated with checkpoint inhibition is the eosinophil count in peripheral blood. Higher counts are associated with improved responses and longer remissions (Hude *et al*, 2018). High levels of *CD274* expression by HRSC leads to continuous stimulation of the PD-1 receptor on T cells, inhibiting their anti-tumor activity (Roemer *et al*, 2016). There is evidence to support re-sensitizing an exhausted Th1 effector population as the mechanism of PD-1 antibody effectiveness, but further studies are needed (Cader *et al*, 2018). Furthermore, it is still unclear how the microenvironment of HL changes when patients are treated with immune checkpoint inhibition. More studies examining

potential changes over time are needed to understand potential mechanisms of resistance. Recently, it emerged that PD-1 expression on T cells in the microenvironment is markedly upregulated in biopsies of patients developing resistance to immune checkpoint inhibition with antibodies directed against PD-1 (Sasse *et al*, 2018).

Conclusions

The progress in genomic medicine has given us new insights into the biology of HL. The complex signalling between malignant HRSC/LP cells and the microenvironment defines new biomarkers and therapy targets. We predict progress in molecular monitoring of disease response to be a useful adjunct to standard radiology post-therapy.

As immune escape is one the key features of HL disease pathogenesis, further recognition of mechanisms of immune evasion will lead to new avenues for therapeutic targeting. Logical targets for treatment would 're-programme' the cellular (non-malignant) infiltrate within affected lymph nodes to overcome its anergy and limit its support for HL growth. The application of immunotherapy treatments, such as antibody-targeted therapy and checkpoint inhibition, have improved outcomes for high risk and multiply relapsed patients. Furthermore, a major clinical challenge in contemporary HL treatment is the fact that many patients are overtreated due to a lack of early, high-sensitive disease response biomarkers. Advances in the molecular understanding of HL coupled with the emergence of highly sensitive genomic technologies could form the basis of new minimal residual disease monitoring technologies that could help to prevent overtreatment of patients that are cured quickly and therefore prevent treatment-related morbidity and improve patients' long-term outcomes.

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MC, SR, WK and SB performed literature search, wrote the review and critically revised the manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. The Translational Science of Hodgkin Lymphoma; a focus on diagnosis, disease subtyping and the specific entity of Nodular Lymphocyte Predominant Hodgkin Lymphoma.

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Signaling pathways and immune evasion mechanisms in classical Hodgkin lymphoma

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Classical Hodgkin lymphoma (cHL) is an unusual B-cell–derived malignancy in which rare malignant Hodgkin and Reed-Sternberg (HRS) cells are surrounded by an extensive but ineffective inflammatory/immune cell infiltrate. This striking feature suggests that malignant HRS cells escape immunosurveillance and interact with immune cells in the cancer microenvironment for survival and growth. We previously found that cHLs have a genetic basis for immune evasion: near-uniform copy number alterations of chromosome 9p24.1 and the associated PD-1 ligand loci, CD274/PD-L1 and PDCD1LG2/PD-L2, and copy number–dependent increased expression of these ligands. HRS cells expressing PD-1 ligands are thought to engage PD-1 receptor–positive immune effectors in the tumor microenvironment and induce PD-1 signaling and associated immune evasion. The genetic bases of enhanced PD-1 signaling in cHL make these tumors uniquely sensitive to PD-1 blockade. (*Blood*. 2017;130(21):2265-2270)

Introduction to Hodgkin lymphoma and tumor microenvironment

Classical Hodgkin lymphomas (cHLs) include rare malignant Reed-Sternberg cells within an extensive inflammatory/immune cell infiltrate. In cHLs, less than 2% of the cells are Hodgkin and Reed Sternberg (HRS) cells; the remainder include macrophages, eosinophils, neutrophils, mast cells, and T cells.[1]

What causes the influx of T cells into the cHL microenvironment? HRS cells produce chemokines such as CCL5, CCL17/TARC, and CCL22/MDC, whereas $CD4^+$ T-cell subsets express receptors for these factors.[2,3] As a result, HRS cells attract these T-cell subsets into the cHL microenvironment. Additionally, HRS cells secrete CCL5 to attract macrophages and mast cells[4] and interleukin-8 (IL-8) to attract neutrophils.[2] The extensive but ineffective immune/inflammatory cell infiltrates in cHL suggest that HRS cells have developed mechanisms to escape immunosurveillance while relying on microenvironmental signals for survival and growth. Indeed, HRS cells secrete CCL17 and CCL22 to attract immunosuppressive $CCR4^+$ Tregs into the cHL microenvironment to evade immune attack.[5] Moreover, HRS cells and Tregs in the cHL microenvironment secrete immunosuppressive IL-10 to inhibit the function of infiltrating natural killer cells and cytotoxic T cells.[2]

Key pathways used by HRS cells for survival and growth

HRS cells are derived from crippled germinal center B cells that have lost expression of certain B-cell surface proteins, including the B-cell receptor (BCR).[1,6] Mature B cells devoid of BCRs would normally die by apoptosis. Therefore, HRS must rely on alternative deregulated signaling pathways for survival and growth, as discussed later.

NF-κB

The canonical and noncanonical NF-κB signaling pathways are constitutively activated in HRS cells to promote their survival and proliferation. The robust NF-κB activity in HRS cells is mediated by dual mechanisms: (1) inactivation of the negative regulators of NF-κB (eg, *TNFAIP3*, *NFKBIA*, *NFKBIE*, *TRAF3*, and *CYLD*) by point mutations or deletions,[7,8] and (2) amplification of the positive regulators or the components of the NF-κB pathway (eg, *NIK* and *REL*) by copy number gains.[9,10]

JAK/STAT

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, which is vital to signaling by many cytokine transmembrane receptors, transmits survival, proliferation, and differentiation cues. STAT3, STAT5, and STAT6 are highly expressed and activated in cHL.[11,12] Activation of the JAK/STAT pathway promotes tumor cell proliferation and survival in cHL. The *JAK2* gene is frequently amplified in cHL.[9,13] Moreover, negative regulators of JAK/STAT signaling pathway (eg, *SOCS1* and *PTPN1*) in HRS cells are recurrently inactivated by mutations or deletions.[14,15]

AP-1

Activator protein-1 (AP-1) is a dimeric transcription factor composed of proteins from the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra1, and Fra2), and activating transcription factor family members. AP-1 is constitutively activated in HRS cells, which express high levels of c-Jun and JunB.[16]

TNF receptor family

HRS cells express tumor necrosis factor (TNF) receptor family proteins, such as CD30, CD40, CD95, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B-cell mutation antigen (BCMA), and RANK.[1] Engagement of these cell surface receptors by their respective ligands activates downstream signaling pathways and augments canonical and alternative NF-κB activity.[10]

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Additional perturbed pathways

In HRS cells, aberrant Notch1 signaling also modulates alternative NF-κB pathway activity.[17] Despite the loss of proximal BCR signaling, HRS cells also exhibit constitutive activation of phosphatidylinositol 3-kinase/AKT and RAF/MEK/extracellular signal-regulated kinase signaling pathways.[18-21]

Immunosuppressive mechanisms

Multiple stratagems leveraged by cHL to escape immunosurveillance include (1) reduction or loss of antigen presentation through *B2M* inactivating mutations/deletion (perturbing major histocompatibility complex [MHC] class I) and/or *CIITA* inactivating alterations (perturbing MHC class II)[22,23]; (2) secretion of soluble factors, such as IL-10, transforming growth factor β1, prostaglandin and galectin-1, to kill or inhibit the activation of cytotoxic T lymphocytes and/or professional antigen-presenting cells (APCs)[2,24-27]; (3) recruitment of abundant immunosuppressive Tregs and myeloid-derived suppressor cells into the cHL microenvironment[28]; and (4) enhanced PD-1 signaling via interaction of HRS cells expressing the PD-1 ligands with PD-1 receptor+ immune effectors.[29,30]

PD-1/PD-L1 coinhibitory pathway

Activation of T cells requires 2 signals. Signal 1 (stimulation by a specific antigen) is mediated by the interaction of the T-cell receptor (TCR) with a MHC-bound antigen presented on the surface of APCs. Signal 2 (costimulation by coreceptors) is mediated by binding of B7-1 (CD80) or B7-2 (CD86) on the surface of the APC to CD28 on the surface of the T cells.[31,32] The strength and duration of T-cell activation is modulated by signaling pathways of coinhibitory receptors, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death protein-1 (PD-1).[33]

PD-1 is expressed on activated T cells, but not on resting T cells.[33] In addition, PD-1 is also expressed on natural killer cells, B cells, macrophages, Tregs, and follicular T cells.[33,34] PD-1 has 2 ligands, programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2). PD-L1 is highly expressed on the surface of tumor-infiltrating macrophages, dendritic cells (professional APC), and malignant cells of certain solid tumors and lymphomas, including cHL (Figure 1).[29,33] Binding of PD-1 by its ligands, PD-L1 or PD-L2, results in crosslinking of the antigen-TCR complex with PD-1. This event leads to phosphorylation of the tyrosine residue in the immunoreceptor tyrosine-based switch motif (TxYxxL/I) of PD-1 and recruitment of the tyrosine phosphatase SHP-2, which dephosphorylates and inactivates ZAP70 in T cells (Figure 1).[31-33,35,36] The final outcome is the attenuation or shutdown of TCR-associated downstream signaling including phosphatidylinositol 3-kinase/AKT and RAS–MEK–extracellular signal-regulated kinase pathways, downregulation of cytokine production (eg, TNF-α and IL-2), and inhibition of T-cell proliferation.[31-33] Furthermore, PD-L1 competes with CD28 for binding to its ligand, CD80 (B7-1), and inhibits CD28 costimulation (signal 2 in T-cell activation).[37] PD-1/PD-L1 signaling results in T-cell exhaustion/energy, which is a temporary and reversible inhibition of T-cell activation and proliferation. PD-1 signaling also shifts the metabolism of activated T-cells from glycolysis and glutaminolysis to fatty acid oxidation, limiting T effector cell differentiation and function.[38]

Recent studies reveal an additional role for the PD-1 pathway in modulating CD28 signaling.[39,40] Specifically, PD-1–recruited SHP-2 preferentially dephosphorylates CD28 over the TCR intrinsic subunit CD3ζ and its signaling components, including TCR-associated ZAP70 and its downstream adaptors LAT and SLP76 (Figure 1). These studies suggest that (1) CD28 is the preferential immediate downstream target of PD-1 signaling, and (2) PD-1 suppresses T-cell functions primarily by inhibiting signal 2 (costimulation by CD28) rather than signal 1 (TCR stimulation by antigen).

Physiologic roles of PD-1 negative signaling include (1) balancing positive signals transduced through TCR and costimulatory receptors; (2) downregulating the immune response after elimination of disease; (3) limiting the strength of an immune response to prevent tissue damage, autoimmune diseases, and allergic diseases; and (4) maintaining immune tolerance.[31-33] Various cancers and viruses have exploited and hijacked coinhibitory signaling pathways such as PD-1 to escape destruction by the immune system. These inhibitory effects can be reversed by blocking PD-1/PD-L1 signaling at the level of the PD-1 receptor or the PD-1 ligand.[33,41]

Genetic basis of increased PD-1 signaling in cHL

In previous studies, we integrated high-resolution copy number data with transcriptional profiling information to identify *PD-L1* and *PD-L2* as the major targets of 9p24.1 copy gain in cHL cell lines and laser capture microdissected HRS cells (Figure 2, left panel).[29] In cHL cell lines and primary tumors, there was a 9p24.1 copy number–dependent increase in PD-L1 protein expression.[29] In cHL, the extended 9p24.1 amplicon almost always includes *JAK2* (Figure 2, left panel). This finding is noteworthy because *JAK2* copy gain leads to increased JAK2 protein expression and JAK/STAT signaling, which further induces PD-1 ligand expression (Figure 2, right panel).[29] Epstein-Barr virus latent membrane protein 1 (LMP 1) also induces PD-L1 expression via AP-1 and JAK/STAT pathways, highlighting an additional viral basis for PD-L1 upregulation in Epstein-Barr virus + cHL.[42,43]

Analyses of PD-1/PD-L1 pathway alterations in newly diagnosed cHL

Due to the rarity of neoplastic HRS cells in primary cHL, it is not feasible to perform comprehensive genomic analyses of intact biopsy specimens. For this reason, we recently developed a fluorescence in situ hybridization (FISH) assay to directly and precisely characterize copy number alterations (CNAs) of *PD-L1* and *PD-L2* in formalin-fixed paraffin-embedded biopsy specimens from patients with newly diagnosed cHL who were treated with a current induction regimen (Stanford V) and had long-term follow-up (Figure 3A-B).[30] We found that 107/108 (97%) of all evaluated cHLs had alterations of the *PD-L1* and *PD-L2* loci, including polysomy (ie, nuclei with a target:control probe ratio of 1:1 but more than 2 copies of each probe) in 5% of cases, copy gain (ie, target:control probe ratio of $>$1:1 but $<$3:1) in 56%, and amplification (ie, target:control probe ratio of $\geq$3:1) in 36% of tumors.[30] In addition, we used dual PD-L1/PAX5 immunohistochemical staining to demonstrate copy number–dependent increased expression of PD-L1 in PAX5$^+$ HRS cells in these tumors (Figure 3C). In this clinically annotated series of cHL patients treated with standard induction therapy, those with the highest level 9p24.1 alterations (amplification) had significantly shorter progression-free

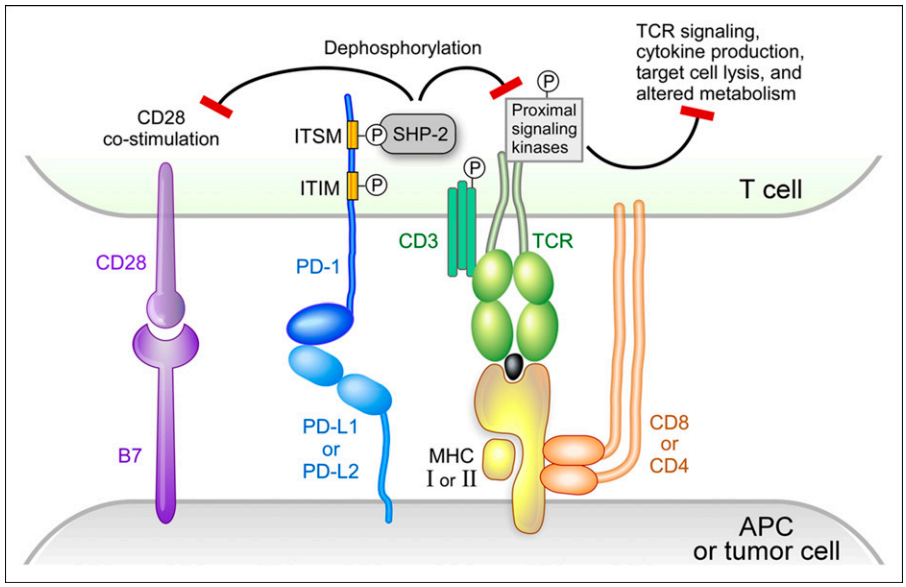

Figure 1. PD-1 signaling. Modified version reprinted with permission from Baumeister, SH et al, 2016; *Annu. Rev. Immunol.* 34:539-73.

survival.[30] In addition, patients with advanced stage disease were more likely to have 9p24.1 amplified tumors.[30]

PD-L1$^+$ tumor-associated macrophages in cHL

Nonmalignant tumor-associated macrophages in the cHL microenvironment also express PD-L1. We recently used multiplex immunofluorescence and digital image analysis to define the relationship between normal and malignant PD-L1$^+$ cells in the cHL tumor microenvironment.[44] In these studies, we found that PD-L1$^+$ tumor-associated macrophages physically colocalize with PD-L1$^+$ HRS cells to form an extended immunoprotective microenvironmental niche.[44] The unique topology of cHL, in which PD-L1$^+$ tumor-associated macrophages surround PD-L1$^+$ HRS cells, likely augments local PD-1 signaling in this disease.[44]

Clinical applications of PD-1 blockade in relapsed or refractory cHL

Although many cHL patients are cured with current treatment regimens, 20% to 30% fail to respond or experience a relapse after treatment.[45] Therefore, there is an unmet medical need to develop alternative therapies for patients with relapsed/refractory cHL. The reversibility of T-cell coinhibitory signaling pathways exploited by cHL for immune evasion suggests that PD-1 blockade may augment antitumor immunity. As CNAs of 9p24.1 increase expression of both PD-1 ligands, PD-L1 and PD-L2, PD-1 receptor blockade may be a better treatment strategy than selective inhibition of PD-L1.

Pilot studies and subsequent registration trials of 2 PD-1–blocking antibodies, nivolumab and pembrolizumab, in patients with multiple relapsed/refractory cHL were associated with response rates of ~70% and long-lasting remissions in a subset of

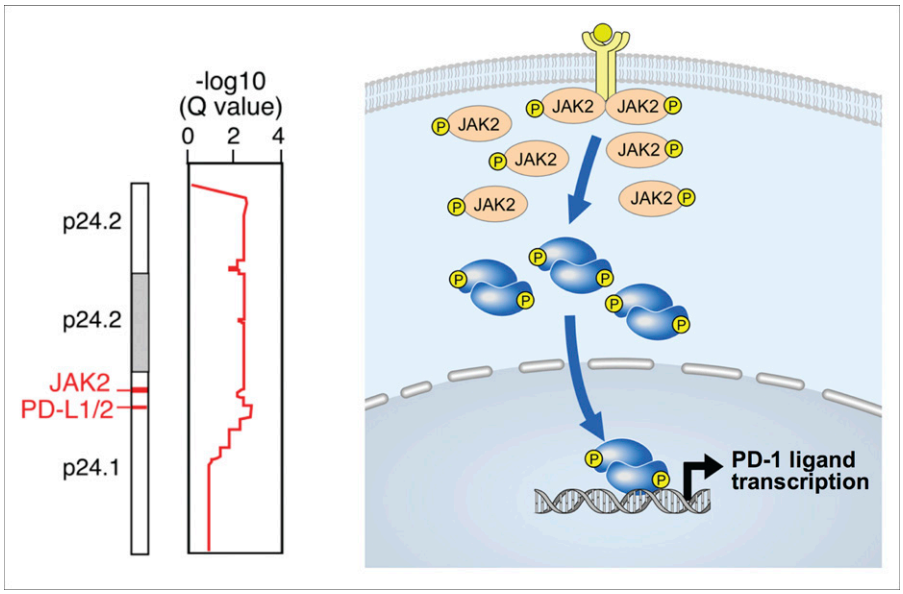

Figure 2. *PD-L1* and *PD-L2* copy gain and further induction via JAK2/STAT signaling. (Left) The 9p24.1 amplicon in cHL includes *PD-L1*, *PD-L2*, and *JAK2*. (Right) *JAK2* copy gain leads to increased JAK2 protein expression and enhanced JAK/STAT signaling, which further induces PD-1 ligand expression.

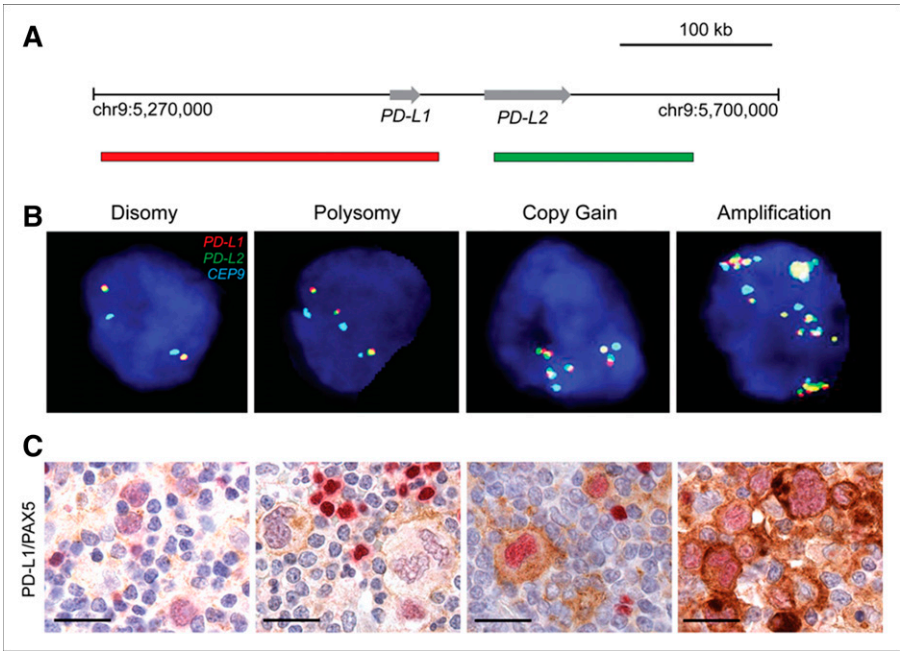

Figure 3. Genetic and immunohistochemical analyses of the *PD-L1* and *PD-L2* loci. (A) Location and color-labeling of the BAC clones on 9p24.1 used for FISH. RP11-599H20 including *PD-L1*, labeled red. RP11-635N21 including *PD-L2*, labeled green. (B) Representative images of FISH results for the different categories. *PD-L1* in red, *PD-L2* in green, fused (F) signals in yellow and centromeric probe (CEP9) in aqua (A). In these images, disomy reflects 2A:2F; polysomy, 3A:3F; copy gain, 3A:6F; and amplification, 15+F. (C) PD-L1 (brown)/PAX5 (red) immunohistochemistry in the cHL cases with 9p24.1 disomy, polysomy, copy gain, and amplification from panel B. Scale bar indicates 50 μm. Reprinted with permission © (2016) American Society of Clinical Oncology. All rights reserved. Roemer, MG et al: *J Clin Oncol* Vol. 34 (23), 2016 2690-7.

patients.[46-49] These clinical trials and additional studies of PD-1 blockade at earlier time points in the therapy of cHL will be discussed in the following chapter. In the pilot study and subsequent registration trial of nivolumab in patients with relapsed/refractory cHL, HRS cells in all available biopsy specimens exhibited 9p24.1 alterations and increased expression of the PD-L1 protein[46,47]; tumor-infiltrating T cells largely expressed low/intermediate levels of PD-1.[46]

Mechanisms of response and resistance to PD-1 blockade

Why do some relapsed/refractory cHL patients respond well to the PD-1 blockade, but others do not? Understanding the molecular mechanisms underlying response and resistance to PD-1 blockade is important for the development of optimal treatment strategies.

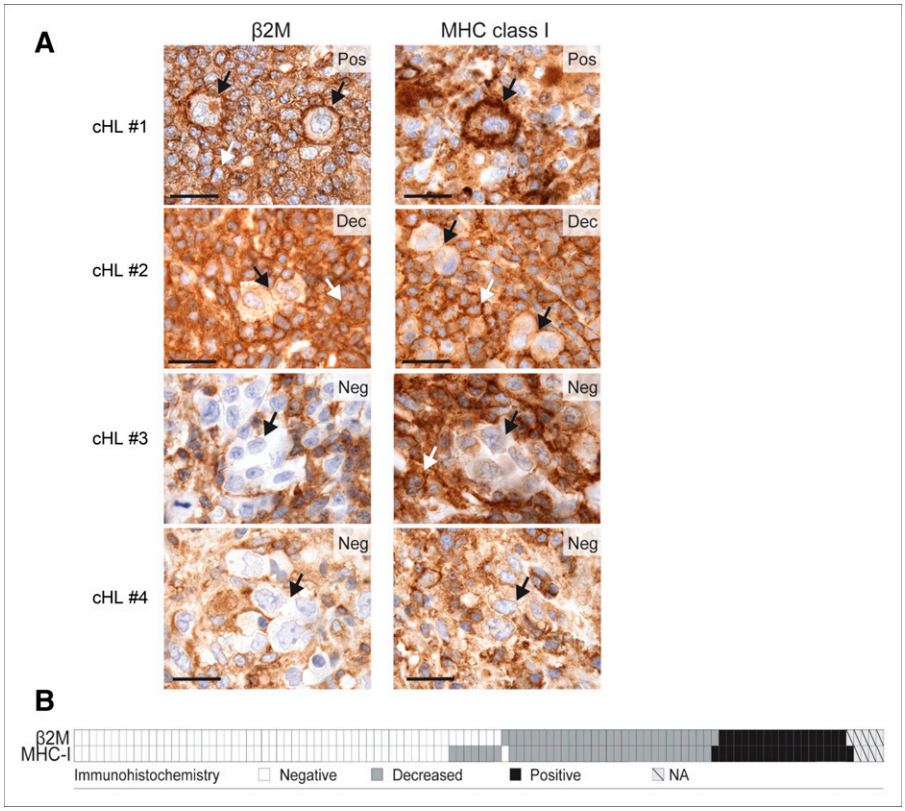

Figure 4. β2M, MHC class I, and MHC class II expression in cHL patients. (A) β2M and MHC class I immunohistochemical staining in 4 representative cHL patients: #1, positive for both markers (Pos); #2, decreased for both markers (Dec); #3 and #4, negative for both markers (Neg). Individual HRS cells are depicted with a black arrow. The white arrows indicate expression on surrounding, nonmalignant inflammatory cells. Scale bar, 50 μm. (B) Heat map representing the distribution of β2M and MHC class I (MHC-I) expression in the 108 cHL patients. White, negative; gray, decreased; black, positive; hatched, not assessable (NA). Reprinted with permission from *Cancer Immunology Research* 2016; 4: 910.

In certain solid tumors, PD-1 ligand expression has been associated with responsiveness to PD-1 blockade.[33] In cHLs, in which there is a genetic basis for PD-1 ligand overexpression and almost all tumors are PD-L1$^+$, the range of 9p24.1 alterations and levels of PD-L1 protein may be more predictive for response to PD-1 blockade. In recent analyses of tumor biopsy specimens from patients with relapsed/refractory cHL treated with nivolumab, we found that patients whose tumors exhibited the lowest-level 9p24.1 alterations and PD-L1 expression were less likely to respond to PD-1 blockade.[47]

An additional potential mechanism of resistance to PD-1 blockade might be ineffective tumor antigen presentation by cell surface MHC proteins. CD8$^+$ cytotoxic T cells recognize tumor antigens presented in the context of cell surface MHC class I proteins. MHC class I proteins are transported to the cell surface in association with β2 microglobulin (β2M). Previous reports suggest that cell surface MHC class I expression may be perturbed by inactivating mutations or copy loss of *B2M* in cHL.[22] For these reasons, we recently evaluated β2M and MHC class I expression in the aforementioned clinically annotated cHL series; 79% of cHLs had decreased/absent HRS cell surface expression of β2M and MHC class I (Figure 4A-B).[50] In this series, there was a highly significant association between the levels of β2M and MHC class I expression in individual cHLs (Figure 4B, $P < .001$).[50] These data support the hypothesis that alterations of β2M may be a major mechanism of deficient MHC class I expression in cHL. Others have also described deficient MHC class I expression in cHL.[51]

The paucity of β2M/MHC class I expression on HRS cells suggests that there may be alternative mechanisms of action of PD-1 blockade in cHL beyond CD8$^+$ cytotoxic T cells. Despite the focus on CD8$^+$ T-cell–mediated mechanisms of PD-1 blockade,[40,52,53] recent studies also suggest a role for MHC class II–associated antigen presentation and CD4$^+$ infiltrating T cells in certain solid tumors[54] and define tumor neoantigens that are largely recognized by CD4$^+$ T cells.[55,56] These observations are particularly interesting given the predominance of CD4$^+$ T cells in cHL inflammatory/immune cell infiltrates and the selective association of PD-L1$^+$ HRS cells with PD-1$^+$ CD4$^+$ T cells.[44] We anticipate that ongoing multiparametric analyses of intact cHL tumor biopsies[44] and cell suspensions and assessments of tumor biopsies from cHL patients treated with PD-1 blockade will be highly informative.

Lessons for other lymphoid malignancies

The principles we elucidate for cHLs may be applicable to other lymphoid malignancies with genetic bases for PD-1 ligand expression, including primary mediastinal large B-cell lymphoma, primary central nervous system lymphoma, and primary testicular lymphoma.[29,57-60] Mediastinal large B-cell lymphoma shares certain molecular features with cHL, including recurrent chromosome 9p24.1 alterations and copy number–dependent increased expression of PD-L1 and PD-L2.[29,57] Although the genetic signature of cHL is quite different from that of primary central nervous system lymphoma and primary testicular lymphoma,[58] these diseases all exhibit recurrent 9p24.1 CNAs and increased PD-1 ligand expression. Early clinical trials suggest that PD-1 blockade may also be active in these additional lymphoid malignancies.[59,60]

In conclusion, cHLs use complementary genetic bases of immune evasion, including PD-1 signaling, to survive within a T-cell and inflammatory cell–rich microenvironment. These features also make cHL uniquely sensitive to PD-1 blockade and establish a paradigm for the identification of PD-1–dependent lymphoid malignancies.

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Authorship

Contribution: W.R.L. and M.A.S. developed and wrote the paper.

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