Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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SUPPLEMENTARY APPENDIX

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International Workshop to Standardize Response Criteria for Lymphoma[1]

Responses must last for at least 4 weeks off treatment.

Complete remission (CR): Complete disappearance of all detectable clinical and radiographic evidence of disease and disappearance of all disease-related symptoms if present before therapy and normalization of those biochemical abnormalities (for example, lactate dehydrogenase [LDH]) definitely assignable to the lymphoma. All lymph nodes must have regressed to normal size (≤1.5 cm in greatest diameter if >1.5 cm before therapy). Previously involved nodes that were 1.1 to 1.5 cm in greatest diameter must have decreased to ≤1 cm or by more than 75% in the sum of the products of the greatest diameters (SPD). The spleen, if considered to be enlarged before therapy, must have regressed in size and not be palpable on physical examination. The bone marrow must show no evidence of disease by histology. Flow cytometry, molecular or cytogenetic studies will not be used to determine response. Response must persist for 1 month. For fluorodeoxyglucose (FDG)-avid or positron-emission tomography (PET)-positive lesions prior to therapy, mass of any size is permitted if the current scan is PET negative. For variably FDG-avid or PET-negative lesions, regression to normal size on computed tomography (CT) is required.

Partial response (PR): ≥50% decreased in SPD of six largest dominant nodes or nodal masses. No increase in size of nodes, liver, or spleen and no new sites of disease. Splenic and hepatic nodules must regress by ≥50% in the SPD. Bone marrow is irrelevant for determination of a PR. No new sites of disease should be observed. For FDG-avid or PET-positive lesions prior to therapy, one or more PET positive at previously involved site is permitted. For variably FDG-avid or PET-negative lesions, regression on CT is required.

Progressive disease (PR, non-responders) requires the following: ≥50% increase from nadir in the SPD of any previously identified abnormal node for PRs or non-responders. Appearance of any new lesion during or at the end of therapy.

Stable disease (SD): Defined as less than a PR but not progressive disease. ALL assessment of clinical response will be made according to the non-Hodgkin lymphoma guidelines.

Relapsed disease (CR) requires the following: Appearance of any new lesion or increase by ≥50% in the size of the previously involved sites. Greater than or equal to 50% increase in greatest diameter of any previously identified node >1 cm in its shortest axis or in the SPD of more than one node.

The major criteria for judging response will include physical examination and examination of blood and bone marrow. All laboratory studies that are abnormal prior to study will be repeated to document the degree of maximal response.

Assessment of 9p24.1 Copy Number by Fluorescence *in situ* Hybridization (FISH) and Immunohistochemical (IHC) Staining

Fluorescence *in situ* Hybridization

Five micron tissue sections were mounted on standard glass slides and baked at 60°C for at least 2 hours, then de-paraffinized and digested for 5 minutes as described previously.[2]

The following two bacterial artificial chromosome (BAC) probes were co-hybridized: RP11-599H20 (labeled in Spectrum Orange), which maps to 9p24.1 and includes *CD274* (encoding PD-L1, hereafter *PD-L1*) and RP11-635N21 (labeled in Spectrum Green), which also maps to 9p24.1 and includes PDCD1LG2 (encoding PD-L2, hereafter *PD-L2*). Both BAC clones were obtained from CHORI (www.chori.org). These DNA probes were direct-labeled using nick

translation, precipitated using standard protocols, and hybridized with a final probe concentration of 100 ng/ul. A control centromeric probe, Spectrum Aqua-labeled *CEP9* (maps to 9p11-q11), was obtained from Abbott Molecular, Inc. (Abbott Park, IL) and was hybridized following manufacturer's recommendations.

The tissue sections and probes were co-denatured at 80°C for 5 minutes, hybridized at least 16 hours at 37°C in a darkened humid chamber, washed in 2X SSC at 70°C for 10 minutes, rinsed in room temperature 2X SSC, and counterstained with DAPI (4',6-diamidino-2-phenylindole; Abbott Molecular, Inc.). Slides were imaged using an Olympus BX51 fluorescence microscope. Individual images were captured using an Applied Imaging system running CytoVision Genus version 3.92.

Malignant Hodgkin Reed–Sternberg (RS) cells were identified by nuclear morphology and all RS cells/slide were analyzed. Nuclei with a target:control probe ratio of three or greater were classified as amplified for the target locus. Nuclei with a target:control probe ratio of between one and three were scored as having relative gain of the target locus. A target:control probe ratio of one, but with greater than two copies of each probe, was interpreted as being polysomic for the probes.

Immunohistochemical Staining

Anti-PD-L1 and anti-PAX5. Double staining of PD-L1 (405.9A11, from G. Freeman) and PAX5 (24/PAX-5, BD Biosciences, San Jose, CA) was performed using an automated staining system (BOND-III, Leica Biosystems, Buffalo Grove, IL) following the manufacturer's protocol. Four-μm thick paraffin-embedded sections were pre-baked at 60°C for 1 hour and subsequently loaded onto BOND-III with "Bond Universal Covertiles" (Leica Biosystems). After slides were dewaxed and rehydrated, heat-induced antigen retrieval was performed using ER2 solution (pH8)

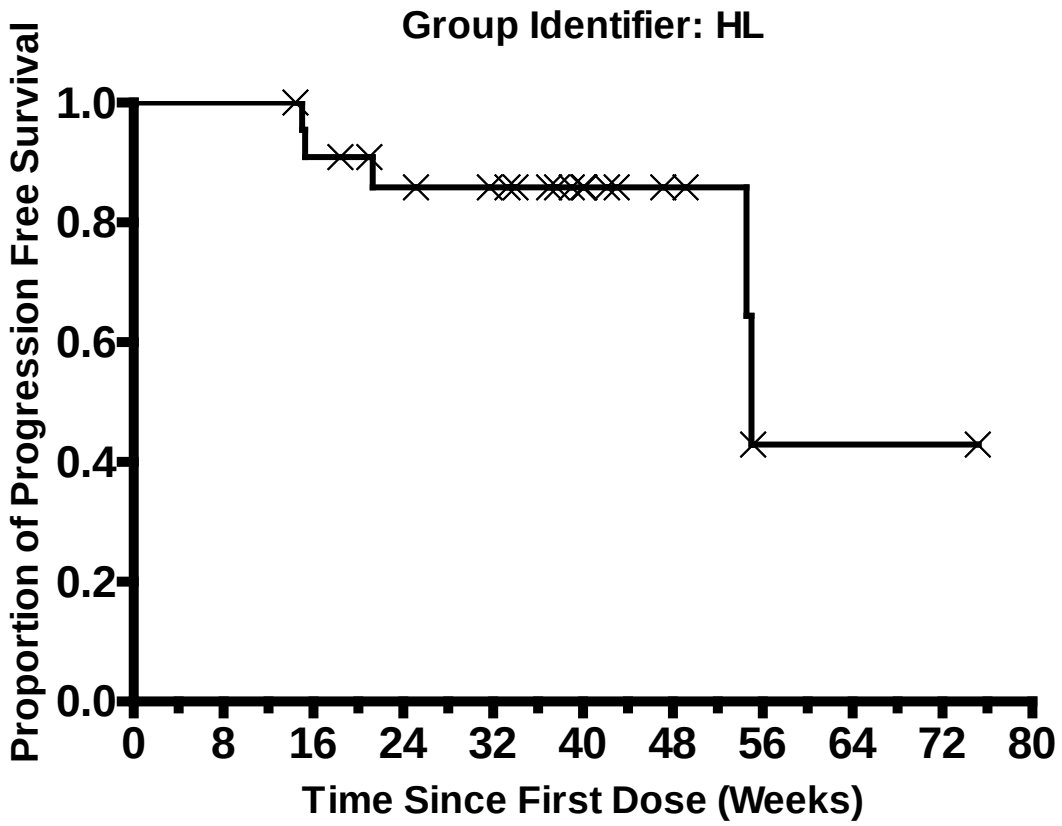
(Leica Biosystems) for 30 minutes. PD-L1 immunostaining was performed first. Primary antibody (1:100 dilution of clone 405.9A11 [final concentration: 13 µg/ml] in Ab Discovery Diluent, Ventana Medical Systems, Tucson, AZ) was incubated for a total of 2 hours with two separate applications, followed by 8 minutes of post-primary blocking reagent, 12 minutes of horseradish peroxidase-labeled polymer, 5 minutes of peroxidase block, and 15 minutes of DAB development. All reagents were components of the Bond Polymer Refine detection system (Leica Biosystems). PAX5 (24/PAX-5) immunostaining was subsequently performed. PAX5 primary antibody (1:100 dilution [final concentration: 2.50 µg/ml] in Bond Primary Antibody Diluent) was incubated for a total of 2 hours with two separate applications followed by 20 minutes of post-primary AP-blocking reagent, 15 minutes of AP-labeled polymer, and 10 minutes of Red substrate development. Slides were then counterstained with hematoxylin for 10 minutes. All reagents were components of the Bond Polymer AP Red detection system (Leica Biosystems). Slides were subsequently dehydrated and coverslipped.

Anti-PD-L2 and anti-phospho STAT3. Double staining of PD-L2 (366C.9E5, from G. Freeman) and pSTAT3 (D3A7; Cell Signaling Technology, Danvers, MA) was performed as described above with the following modifications. PD-L2 (366C.9E5) immunostaining was performed first. Primary antibody (1:6000 dilution of clone 366C.9E5 [final concentration: 0.23 µg/ml] in Discovery Ab diluent) was incubated for a total of 2 hours with two separate applications, followed by 8 minutes of post-primary blocking reagent, 12 minutes of horseradish peroxidase-labeled polymer, 5 minutes of peroxidase block, and 15 minutes of DAB development. The pSTAT3 (D3A7) immunostaining was subsequently performed; pSTAT3 primary antibody (1:125 dilution of clone D3A7 [final concentration: 0.89 µg/ml] in Bond Primary Antibody Diluent) was incubated for total of 2 hours with two separate applications.

Post-primary AP blocking and labeling reagents were applied and slides were counterstained by hematoxylin as above and subsequently dehydrated and coverslipped.

Anti-PD1 and CD3. Immunohistochemistry for PD1 and CD3 was performed as described above with the following modifications. After blocking with serum-free protein block (Dako), the slides were incubated with mouse anti-PD1 monoclonal antibody (final concentration, 1.4 μg/ml, clone EH33, from G. Freeman, Dana Farber Cancer Institute, Boston, MA) or rabbit anti-CD3 polyclonal antibody (final concentration 2.4 ug/ml, A0452, Dako) for 1 hour. Slides were then treated with Dako HRP Envision secondary antibodies for 30 minutes. After further washing, slides were developed using a 3,3'diaminobenzidine (DAB) chromogen (Dako) for 5 min, counterstained with hematoxylin, dehydrated and coverslipped. Stained slides were loaded into an Aperio Scanscope XT and scanned at 20X magnification via a semi-automated method. Images were checked for quality using visual inspection. Quantification analysis of each stained slide was then performed using a membranous Her2 algorithm[3].

Figure S1. Progression Free Survival by Kaplan-Meier Method



Number of patients at risk*

BL	WK 8	WK 16	WK 24	WK 32	WK 40	WK 48	WK 56	WK 64	WK 72	WK 80
23	23	20	17	15	10	5	1	1	1	0

*Number of subjects at risk listed on the plot is the number of subjects at risk before entering the time period

X denotes censored

HL, Hodgkin lymphoma

Figure S2. Analyses of the PD-L1 and PD-L2 Loci and PD-L1 and PD-L2 Protein Expression in Hodgkin Reed–Sternberg Cells. (A) Location and color labeling of bacterial artificial chromosome clones used for the 9p24.1/*PD-L1/PD-L2* fluorescence *in situ* hybridization assay. (B) Representative images of cases with extra copies of 9p (polysomy 9p, 9 copies of *PD-L1*, *PD-L2* and *CEP9* [centromeric probe], first panel), *PD-L1/2* copy gain (second panel, 6 green-red [yellow] signals compared to 3 centromeric signals [aqua]), or *PD-1/2* amplification (third and fourth panels, >3x green-red [yellow] signals compared to centromeric signals [aqua]). (C) PD-L1 (upper panel, brown) and PD-L2 (lower panel, brown) protein expression in Hodgkin Reed–Sternberg cells from the same cases as in (B). PD-L1 is evaluated in conjunction with PAX5 to identify PAX5+ Hodgkin Reed–Sternberg cells (upper panel, red) and PD-L2 is assessed in association with pSTAT3, which reflects JAK-STAT activation (lower panel, red). Scale bar equals 50 μm.

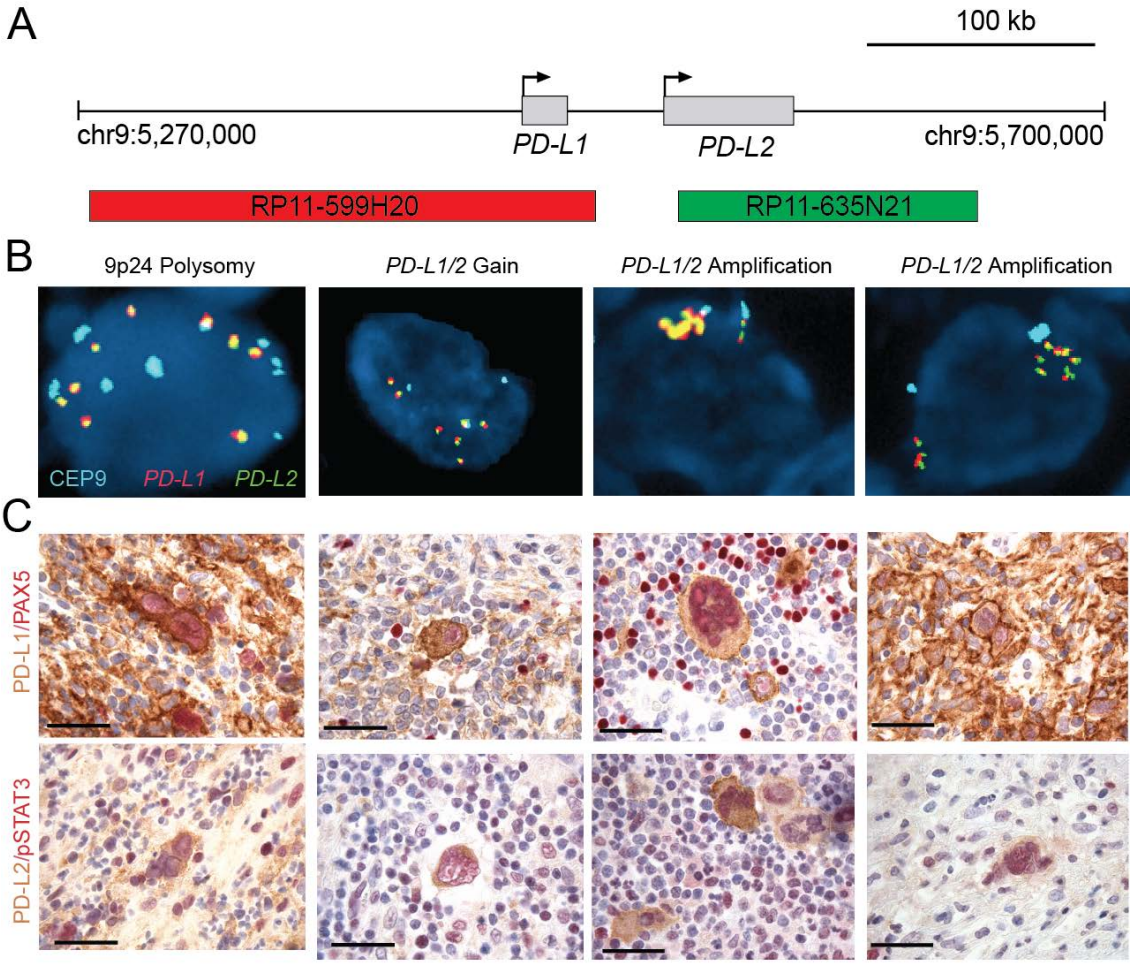

A
100 kb
chr9:5,270,000
PD-L1
PD-L2
chr9:5,700,000
RP11-599H20
RP11-635N21
B
9p24 Polysomy
PD-L1/2 Gain
PD-L1/2 Amplification
PD-L1/2 Amplification
CEP9
PD-L1
PD-L2
C
PD-L1/PAX5
PD-L2/pSTAT3

Figure S3. Analyses of PD-1 and CD3 Expression in Classical Hodgkin Lymphoma Biopsies. Tissue sections stained with anti-PD1 (B, D; F) or anti-CD3 (A, C; E). Tissue biopsies from Subject 7 (A, B), Subject 8 (C, D) and Subject 3 (E, F) showing Hodgkin Reed–Sternberg cells (black arrows) and positive staining (brown coloration) of a subset of lymphocytes for the indicated markers. Insets (B, D) show high-power views of individual lymphoid cells with positive membrane staining for PD-1. All images 1000X original magnification. Scale bar is approximately 50um and is applicable to all images.

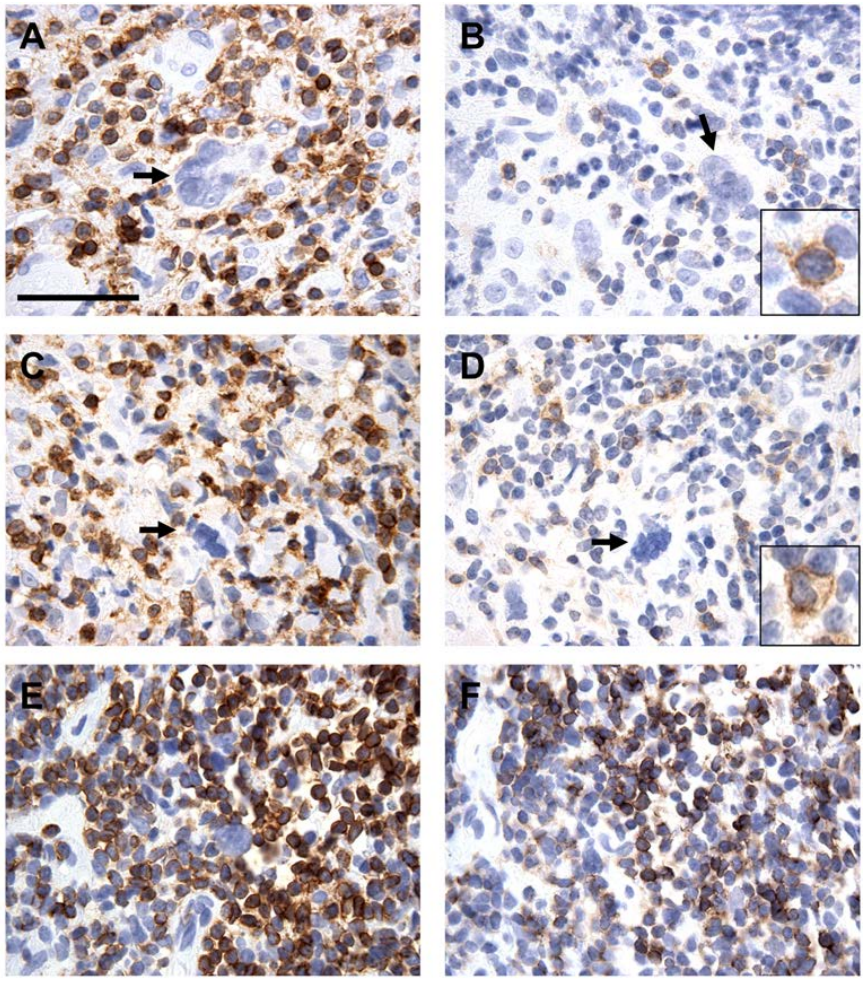


Table S1. Grade 3, 4, or 5 Drug-related and Unrelated Adverse Events

Related Adverse Events	Grade 3
Pancreatitis	1
Myelodysplastic syndrome	1
Thrombocytopenia	1
Lipase increased	1
Pneumonitis	1
Gastrointestinal inflammation	1
Stomatitis	1
Colitis	1
Leukopenia	1
Lymphocyte count decreased	1

Unrelated Adverse Events	Grade 3	Grade 4	Grade 5
Renal failure acute		1	
Back pain	1		
Bacteremia		1	
Blood creatine phosphokinase increased	1		
Diarrhea	1		
Encephalitis	1		
Epistaxis	1		
Fatigue	1		
Febrile neutropenia	1		
Graft vs host disease			1
Hemoptysis	1		
Hypocalcemia	1		
Hypokalemia	1		
Infection	1		
Leukopenia		2	
Lymphopenia		2	
Pneumonia mycoplasma	1		
Neutropenia		2	
Neutrophil count decrease		1	
Platelet count decreased	1		
Skin infection	1		
Small intestinal obstruction	1		
Soft tissue infection	1		
Thrombocytopenia		2	
Thrombotic microangiopathy		1	

Table S2. Genetic Analyses of the *PD-L1* and *PD-L2* Loci

Case	Cytogenetic Alterations			
	Polysomy 9p	***PD-L1/PD-L2* Gain**	***PD-L1/PD-L2* Amplification**	**Signals**
1	+	-	-	3-5F:3-5A
2	+	-	-	3-4F:3-4A
3	+	-	-	3-4F:3-4A
4	+	+	-	3-6F:3-6A
5	+	+	-	3-6F:2-4A
6	+	+	-	3-6F:2-5A
7	+	+	+	3-8F:2-8A
8	+	+	+	3-10+F:2-6A
9	-	+	+	3-9F:3-4A
10	-	-	+	4-15+F:1-4A

All analyzed cases had structural bases for increased 9p24.1/*PD-L1/PD-L2* copy numbers including extra copies of 9p (polysomy 9p), copy gain of *PD-L1*/*PD-L2*, or *PD-L1/PD-L2* amplification; F=fused signals, *PD-L1+PD-L2*;A=*CEP9* signal.

Table S3. Immunohistochemical Analyses of PD-L1, PD-L2 and pSTAT3 Expression in Hodgkin Reed–Sternberg Cells

Case	PD-L1			PD-L2			pSTAT3
	405.9A11 ab			366C.9E5 ab			
	Interp.	% pos	Intensity	Interp.	% pos	Intensity	%pos
1	+	58% (21/36)	2+	+	14% (4/29)	1+	80%
2	+	79% (33/42)	3+	+	29% (8/28)	1+	60%
3	+	69% (18/26)	2+	+	32% (21/66)	1+	60%
4	+	81% (73/90)	3+	+	68% (68/100)	1+	70%
5	+	96% (96/100)	3+	+	79% (63/80)	1+	100%
6	+	34% (21/61)	3+	+	81% (22/27)	2+	5%
7	+	66% (66/100)	3+	+	66% (66/100)	1+	70%
8	+	90% (90/100)	3+	+	61% (61/100)	1+	70%
9	+	98%(98/100)	3+	+	20%(20/100)	1+	80%
10	+	95%(95/100)	2+	+	58%(58/100)	2+	50%

In all analyzed cases, Hodgkin Reed–Sternberg cells expressed PD-L1, detected with antibody 405.9A11 (from G. Freeman), and PD-L2, detected with 366C.9E5 (from G. Freeman). For each antibody, the percent of positive Reed–Sternberg cells and numbers of positive/total Reed–Sternberg cells and staining intensity are noted. In each case, the percent of Reed–Sternberg cells expressing nuclear pSTAT3 (detected with antibody D3A7, Cell Signaling), is shown.

Table S4. Immunohistochemical Analyses of PD-1 and CD3 Expression in Classical Hodgkin Lymphoma Biopsies

Case	PD1-positive cells	CD3-positive cells	PD1/CD3 [%]
1	1,270	42,320	3.0
2	na	na	na
3	46,419	79,562	58.3
4	10,286	261,452	3.9
5	na	na	na
6	5,861	98,783	5.9
7	30,259	463,213	6.5
8	63,934	839,626	7.6
9	na	na	na
10	1126	479,793	0.3

SUPPLEMENTAL REFERENCES

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3. Nassar A, Cohen C, Sally S et al. Trainable immunohistochemical HER2/neu image analysis: A multisite performance study using 260 breast tissue specimens. Arch Pathol Lab Med 2011;135:896-902.