ORIGINAL ARTICLE

WILEY Cancer Science

Clinical significance of tryptophan catabolism in Hodgkin lymphoma

Ayako Masaki[1,2] | Takashi Ishida[1] iD | Yasuhiro Maeda[3] | Asahi Ito[1] |
Susumu Suzuki[4] | Tomoko Narita[1] | Shiori Kinoshita[1] | Hisashi Takino[2] |
Takashi Yoshida[1] | Masaki Ri[1] iD | Shigeru Kusumoto[1] | Hirokazu Komatsu[1] |
Hiroshi Inagaki[2] | Ryuzo Ueda[4] | Ilseung Choi[5] | Youko Suehiro[5] | Shinsuke Iida[1] iD

[1]Department of Hematology and Oncology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

[2]Department of Anatomic Pathology and Molecular Diagnostics, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

[3]Laboratory of Hospital Pharmacy, Nagoya City University Graduate School of Pharmaceutical Sciences, Nagoya, Japan

[4]Department of Tumor Immunology, Aichi Medical University School of Medicine, Nagakute, Japan

[5]Department of Hematology, National Kyushu Cancer Center, Fukuoka, Japan

Correspondence
Takashi Ishida, Department of Hematology and Oncology, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku, Nagoya, Japan.
Email: itakashi@med.nagoya-cu.ac.jp

Indoleamine 2,3-dioxygenase 1 (IDO) is an enzyme catabolizing tryptophan (Trp) into the kynurenine (Kyn) pathway. The purpose of the present study was to determine the clinical significance of Trp catabolism in newly diagnosed Hodgkin lymphoma (HL) patients. We quantified serum Trp and Kyn in 52 HL patients, and analyzed their associations with different clinical parameters including serum soluble CD30 concentration. The IDO expression was evaluated in the patients' affected lymph nodes. The cohort comprised 22 male and 30 female patients (age range, 15-81 years; median, 45 years), with a 5-year overall survival (OS) of 88.6%. The OS was significantly shorter for patients with a high Kyn/Trp ratio (OS at 5 years, 60.0% vs 92.2%), for those with stage IV disease, and for those with lymphocytopenia (<600/mm^3 and/or <8% white blood cell count). The latter two parameters are components of the international prognostic score for advanced HL. In contrast, there were no significant differences in OS according to age, serum albumin, hemoglobin, sex, white blood cell count, or serum soluble CD30 (≥ or <285.6 ng/mL). Multivariate analysis using the three variables stage, lymphocytopenia, and serum Kyn/Trp ratio showed that only the latter significantly affected OS. Indoleamine 2,3-dioxygenase 1 was produced by macrophages/dendritic cells, but not by HL tumor cells, and IDO levels determined by immunohistochemistry had a significant positive correlation with the serum Kyn/Trp ratio. In conclusion, quantification of serum Kyn and Trp is useful for predicting prognosis of individual HL patients.

KEYWORDS
Hodgkin lymphoma, indoleamine 2,3-dioxygenase, innate immunity, kynurenine, tryptophan

1 | INTRODUCTION

Hodgkin lymphoma (HL) has a unique pathological manifestation consisting of a small number of tumor cells in a rich environment of T and B cells, macrophages, and other inflammatory cells.[1-3] This characteristic finding could be due to an equilibrium between host immune responses aiming to eradicate HL tumor cells and opposing immune responses supporting their survival. With respect to the latter, we have previously reported that HL tumor cells produced chemokine (C-C motif) ligand 17 (CCL17) and/or CCL22, and that migratory CC chemokine receptor 4-expressing regulatory T cells induced by HL cells suppressed host immune attack on the tumor cells, and created a favorable environment for their survival.[4] In addition, the recent great success of programmed death 1 (PD-1) antibody treatments showed the importance of the interaction between the PD-1 ligand expressed on HL tumor cells and PD-1-expressing reactive T cells in the microenvironment.[5,6] Furthermore, it has been reported by many investigators that macrophages present in the tumor microenvironment are associated with unfavorable prognosis in HL.[7-9] Accordingly, here, we have focused on indoleamine 2,3-dioxygenase 1 (IDO1, here referred to as IDO), an enzyme catabolizing tryptophan (Trp) into the kynurenine (Kyn) pathway, which can be produced by tumor cells themselves, or by tumor-associated cells such as dendritic cells or macrophages. Tryptophan catabolism in malignant tumors is increasingly being recognized as an important microenvironmental factor that suppresses antitumor immune responses, and creates a favorable environment for tumor cells to escape from host immunity.[10-15] Choe et al. reported that, in HL, IDO was expressed to varying degrees by histiocytes, dendritic cells, and some endothelial cells, but not by HL tumor cells themselves. In addition, they reported that histologically determined high IDO expression was associated with inferior survival in HL patients.[16] Although they reported these important observations, details of Trp catabolism in HL have not yet been fully explored. Therefore, the aim of the present study was to determine the clinical significance of Trp catabolism in HL patients.

2 | MATERIALS AND METHODS

2.1 | Patients and control subjects

This study included 52 previously untreated HL patients. Fifty healthy volunteers participated as control subjects, and their samples were anonymized. All donors provided written informed consent at blood sampling according to the Declaration of Helsinki, and the present study was approved by the Institutional Ethics Committees of Nagoya City University Graduate School of Medical Sciences (Nagoya, Japan), and the National Hospital Organization Kyushu Cancer Center (Fukuoka, Japan). Diagnoses and assignment of histological subtypes of HL were made according to the WHO criteria for classification of tumors of hematopoietic and lymphoid tissues.[1,3] The clinical characteristics of the HL patients analyzed in this study comprised histological subtype, serum soluble CD30 (sCD30) level,

and the seven components of the international prognostic score (IPS) for advanced Hodgkin lymphoma,[17] namely: age ($\geq$45 vs <45 years), Ann Arbor stage[18] (IV vs I-III), sex, hemoglobin level (Hb) (<10.5 vs $\geq$10.5 g/dL), serum albumin (Alb) (<4.0 vs $\geq$4.0 g/dL), white blood cell (WBC) count ($\geq$15 000 vs <15 000/mm^3), and lymphocytopenia (<600/mm^3, <8% of WBC, or both vs others). Pretreatment blood samples of all HL patients were obtained and we used the clinical characteristics recorded at that time. Most patients enrolled in the present study were initially treated according to the doxorubicin, bleomycin, vinblastine, and dacarbazine regimen.[19]

2.2 | Measurement of serum Trp and Kyn

L-Tryptophan and L-Kynurenine were determined according to our method using ultra-high performance liquid chromatography (UPLC)–tandem mass spectrometry system (MS/MS) reported previously.[20,21] The calibration curves were prepared using L-Tryptophan-d$_5$ as internal standard. After Trp and Kyn were extracted from serum using an Oasis MCX 30 mg/L cc solid-phase extraction cartridge (Waters Corporation, Milford, MA, USA), the extracted solution was injected into an Acquity UPLC BEH C18 column (2 $\times$ 100 mm; Waters Corporation). Tryptophan and Kyn were detected by the multiple reaction monitoring mode of MS/MS in the positive ion mode.

2.3 | Soluble CD30 measurement

The concentration of human sCD30 in serum was measured by ELISA using the Human CD30 Platinum ELISA (BMS240; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.4 | Immunohistochemistry

The 20 affected lymph nodes of HL patients available at the time of blood sampling for serum Trp and Kyn measurement were examined using immunohistochemistry. Hematoxylin–eosin staining and immunostaining analysis for IDO (mouse anti-human IDO1/IDO mAb, clone 1A3, LS-C338105; LifeSpan Biosciences, Seattle, WA, USA) was carried out on formalin-fixed, paraffin-embedded sections. Three $\times$400 high power fields in the HL-affected lymph node (LN) were selected by a hematopathologist (A.M.) and two hematologists (T.I. and A.I.) according to each investigator's judgement that IDO-positive cells were rich in those areas. Subsequently, IDO-positive cells were independently counted by these three investigators and averaged. Finally, IDO expression levels were expressed as the number of IDO-positive cells/0.25 mm^2.

2.5 | Flow cytometry analysis

The HL cell lines L-428, L-1236, HDLM-2, and KM-H2[4] were incubated with or without γ-interferon (IFN-γ) (SRP3058; Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 100 ng/mL, at 37°C

in 5% CO_2 for 72 hours. The IDO expression was evaluated using a phycoerythrin-conjugated anti-IDO mAb (eyedio; 12-9477-42) and appropriate isotype control (Thermo Fisher Scientific). Intracellular staining was undertaken using the Intracellular Fixation and Permeabilization Buffer Set (88-8824-00; Thermo Fisher Scientific) according to the manufacturer's instructions. The cervical cancer cell line HeLa[22] served as the positive control. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with the aid of FlowJo software (Tree Star, Ashland, OR, USA).

2.6 | Statistical analysis

Correlations between two variables were assessed using Spearman's rank correlation coefficient (Rs). Differences between two groups were examined with the Mann–Whitney U-test or Fisher's exact test. The probability of progression-free survival (PFS) and overall survival (OS) was estimated by the Kaplan–Meier method, and both survival times were compared using the log–rank test. The starting date for the survival analyses was the day when serum was obtained. Progression-free survival was defined as the time from the starting date to progression, relapse, or death resulting from any cause, whichever occurred first. Clinically meaningful cut-off values for serum concentrations of Kyn, Trp, the Kyn/Trp ratio, and sCD30 in these patients have not been determined. Thus, we attempted to divide HL patients into two groups according to their serum levels of Kyn, Trp, the Kyn/Trp ratio, and sCD30. Cut-off values for each in the HL patients were tested at 11 values between the median ± SD (ie, median − SD, median − 0.8 SD, median − 0.6 SD, median − 0.4 SD, median − 0.2 SD, median, median + 0.2 SD, median + 0.4 SD, median + 0.6 SD, median + 0.8 SD, and median + SD). Univariate analyses for PFS and OS was carried out using Cox proportional hazards regression for each parameter at each of the 11 cut-off points. In the present study, the cut-off point yielding minimum P-values was chosen as the most clinically meaningful value for PFS and OS. If two different cut-off points resulted in the same stratification of the patients, we adopted the larger one. Multivariate analysis by Cox proportional hazards regression was then used to evaluate variables potentially affecting OS. All analyses were carried out with SPSS Statistics 17.0 (IBM, Armonk, NY, USA). In this study, $P < .05$ (two-sided) was considered significant.

3 | RESULTS

3.1 | Characteristics of the HL patients

Of the 52 HL patients enrolled in this study, 30 were male and 22 were female (range, 15-81 years; median, 45 years). They included 2 patients with predominant nodular lymphocytes (NLP) and 49 classical HL patients, comprising 29 nodular sclerosis (NS), 1 lymphocyte-rich, 16 mixed cellularity (MC), 3 lymphocyte-depleted, and 1 unclassified HL patient (Table 1).

TABLE 1 Clinical characteristics of patients with Hodgkin lymphoma ($n = 52$)

Characteristic	Number (%)
Age, years	
Median	45
Range	15-81
Sex	
Female	22 (42)
Male	30 (58)
Histological subtype	
Nodular LP	2 (4)
Nodular sclerosis	29 (56)
Lymphocyte-rich	1 (2)
Mixed cellularity	16 (31)
Lymphocyte-depleted	3 (6)
Unclassified	1 (2)
Stage	
I	3 (6)
II	23 (44)
III	14 (27)
IV	12 (23)
Serum Alb, g/dL	
Median	3.7
Range	2.1-4.8
WBC, /μL	
Median	8710
Range	3150-21 700
Lymphocyte count, /μL	
Median	1280
Range	160-2860
Hb, g/dL	
Median	12.2
Range	7.5-16.1
Serum sCD30, ng/mL	
Median	147.7
Range	23.4-462.0
Serum Kyn, μmol/L	
Median	1.17
Range	0.46-4.56
Serum Trp, μmol/L	
Median	60.7
Range	17.9-101.3
Serum Kyn/Trp $\times 10^3$	
Median	20.49
Range	5.60-125.11

Alb, albumin; Hb, hemoglobin; Kyn, kynurenine; LP, lymphocyte-predominant; sCD30, soluble CD30; Trp, tryptophan; WBC, white blood cell count.

3.2 | Concentrations and correlations of serum Kyn and Trp levels and the Kyn/Trp ratio in HL patients and healthy volunteers

The concentration of serum Kyn in HL patients was 1.29, 1.17, and 0.46-4.56 μmol/L (mean, median, range). The corresponding values in healthy volunteers were 1.09, 1.09, and 0.69-1.74. There was no significant difference in serum Kyn concentrations between HL patients and healthy volunteers (P = .268) (Figure S1a). The concentration of serum Trp in HL patients was 58.8, 60.7, and 17.9-101.3 μmol/L (mean, median, range). The corresponding values in healthy volunteers were 66.3, 63.4, and 43.6-110.7 μmol/L. The serum Trp concentration was significantly lower in HL patients than in healthy volunteers (P = .039) (Figure S1b). Finally, the serum Kyn/Trp ratio (Kyn [μmol/L]/Trp [μmol/L] × 10^3) in HL patients was 26.1, 20.5, and 5.6-125.1 (mean, median, range) and the corresponding values in controls were 16.8, 16.6, and 8.8-29.3. Thus, the serum Kyn/Trp ratio was higher in HL patients than in healthy volunteers (P = .050) (Figure S1c).

3.3 | Serum Kyn and Trp levels, and the Kyn/Trp ratio according to HL histological subtype

The mean concentration of serum Kyn in HL patients with NS was 0.99, median value 0.93, and range 0.46-1.52 μmol/L. The corresponding values in patients with MC were significantly higher (1.63, 1.35, and 0.74-4.56, respectively; P = .001). The concentration of serum Trp in HL patients with NS was mean 61.8, median 62.5, and range 29.1-87.5 μmol/L. The corresponding values in patients with MC were not significantly different (54.5, 53.5, and 26.5-98.7, respectively; P = .075). The mean serum Kyn/Trp ratio was 17.07, median 15.13, and range 5.60-36.11 in HL patients with NS but significantly higher at 33.53, 27.80, and 11.03-100.41, respectively, in those with MC (P = .003).

3.4 | Progression-free survival in HL patients according to serum Kyn and Trp levels, Kyn/Trp ratio, and serum sCD30 levels

The 5-year PFS of HL patients in this study was 79.2% (95% confidence interval, 67.0%-91.4%) (Figure 1A). Cut-off values for serum Kyn, Trp, and the Kyn/Trp ratio for PFS were set at 1.30 μmol/L, 57.1 μmol/L, and 20.49, respectively (Table S1). No significant differences between patients with high or low serum Kyn were seen (5-year PFS 61.4% vs 89.5%; P = .054) (Figure 1B), or between those with a high or low serum Trp (84.6% vs 69.9%; P = .050) (Figure 1C). However, PFS was significantly shorter in patients with a high serum Kyn/Trp ratio than in those with a low ratio (61.7% vs 92.1%; P = .017) (Figure 1D).

In the present study, the cut-off value for serum sCD30 for PFS was set at 285.6 ng/mL (Table S2). There was no significant difference in the PFS of patients with a high or low serum sCD30 level (5-year PFS 55.0% vs 87.3%; P = .062) (Figure 2A).

3.5 | Overall survival of HL patients according to serum Kyn and Trp levels, Kyn/Trp ratio, and serum sCD30 levels

The 5-year OS of HL patients is shown in Figure 1E and was 88.6% (95% confidence interval, 77.4%-99.8%). In the present study, the cut-off values for serum Kyn and Trp, and the Kyn/Trp ratio for OS analysis were set at 1.83 μmol/L, 57.1 μmol/L, and 38.16, respectively (Table S3). There were no significant differences in the OS between patients with a high or low serum Kyn level (5-year OS 75.0% vs 89.9%; P = .204) (Figure 1F), but OS was significantly shorter in patients with a low serum Trp level than in those with a high level (5-year OS 81.6% vs 93.8%; P = .019) (Figure 1G). Overall survival was also significantly poorer in patients with a high serum Kyn/Trp ratio than in those with a low ratio (5-year OS 60.0% vs 92.2%; P = .018) (Figure 1H).

In the present study, the cut-off value for serum sCD30 for OS was set at 285.6 ng/mL (Table S4). There was no significant difference in the OS between a high and a low serum sCD30 level (5-year OS 72.7% vs 94.4%; P = .125) (Figure 2B).

## 3.6 | Correlations of serum Kyn, Trp, the Kyn/Trp ratio, and sCD30 in HL patients

There was a significant positive correlation between serum Kyn and sCD30 (Rs = 0.535, P < .001; Figure 2C) and a significant inverse correlation between Trp and sCD30 (Rs = −0.374, P = .006; Figure 2D). There was also a significant positive correlation between the Kyn/Trp ratio and serum sCD30 concentration (Rs = 0.576, P < .001; Figure 2E).

3.7 | Clinical characteristics of HL patients according to serum Kyn and Trp levels, and the Kyn/Trp ratio

A high serum Kyn (>1.83 μmol/L) level was significantly associated with a low Hb level (P = .029), whereas a low serum Trp (≤57.1 μmol/L) was significantly associated with a low serum Alb (P = .038), as well as a low Hb level (P = .007), lymphocytopenia (P < .001), and a high serum sCD30 level (P = .027). Finally, a high serum Kyn/Trp ratio (>38.16) was significantly associated with a low Hb level (P = .007), lymphocytopenia (P = .004), and a high serum sCD30 level (P = .003) (Table 2).

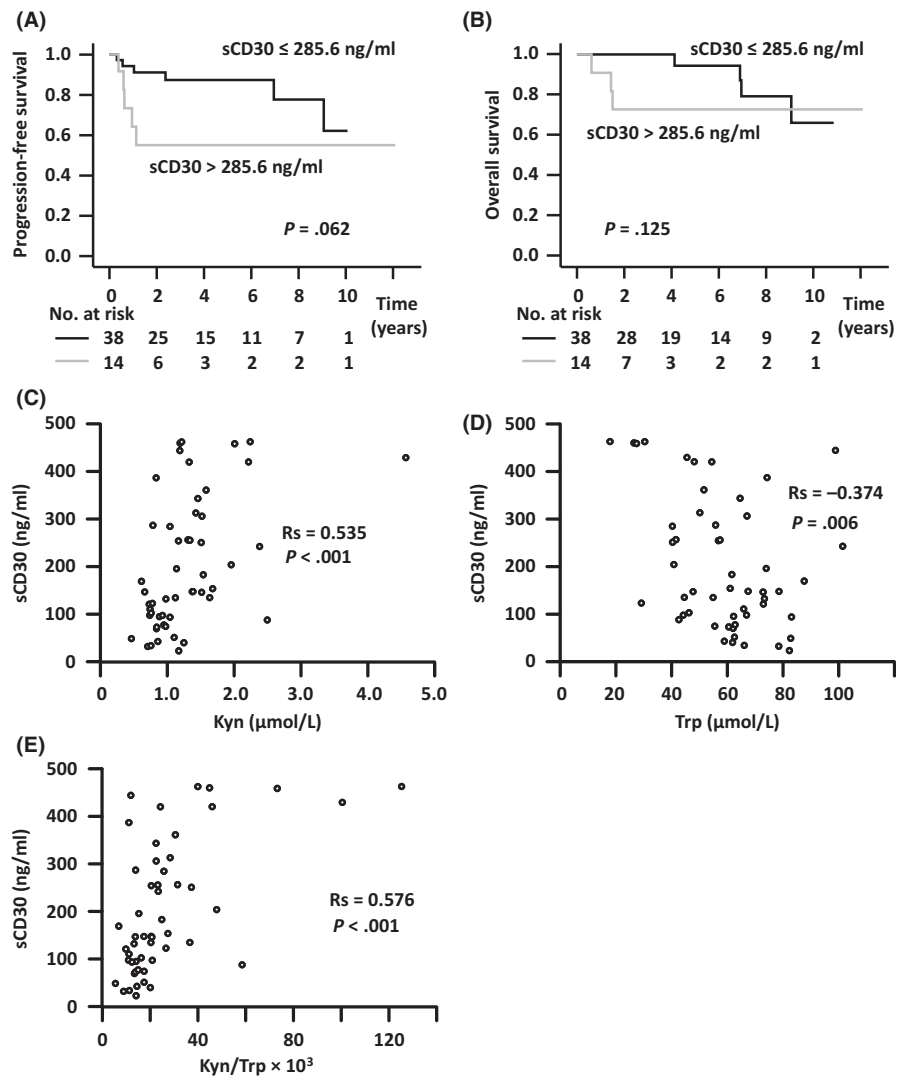## 3.8 | Progression-free survival and OS of HL patients according to component factors of IPS for advanced HL

There were no significant differences in either PFS or OS between older (≥45 years) and younger (<45 years) HL patients (P = .228 and P = .119, respectively). The PFS and OS rates at 5 years in the older HL patients were 76.1%, and 80.6%, respectively, and those in the younger patients 82.0% and 95.2%, respectively (Figure S2a,b). There
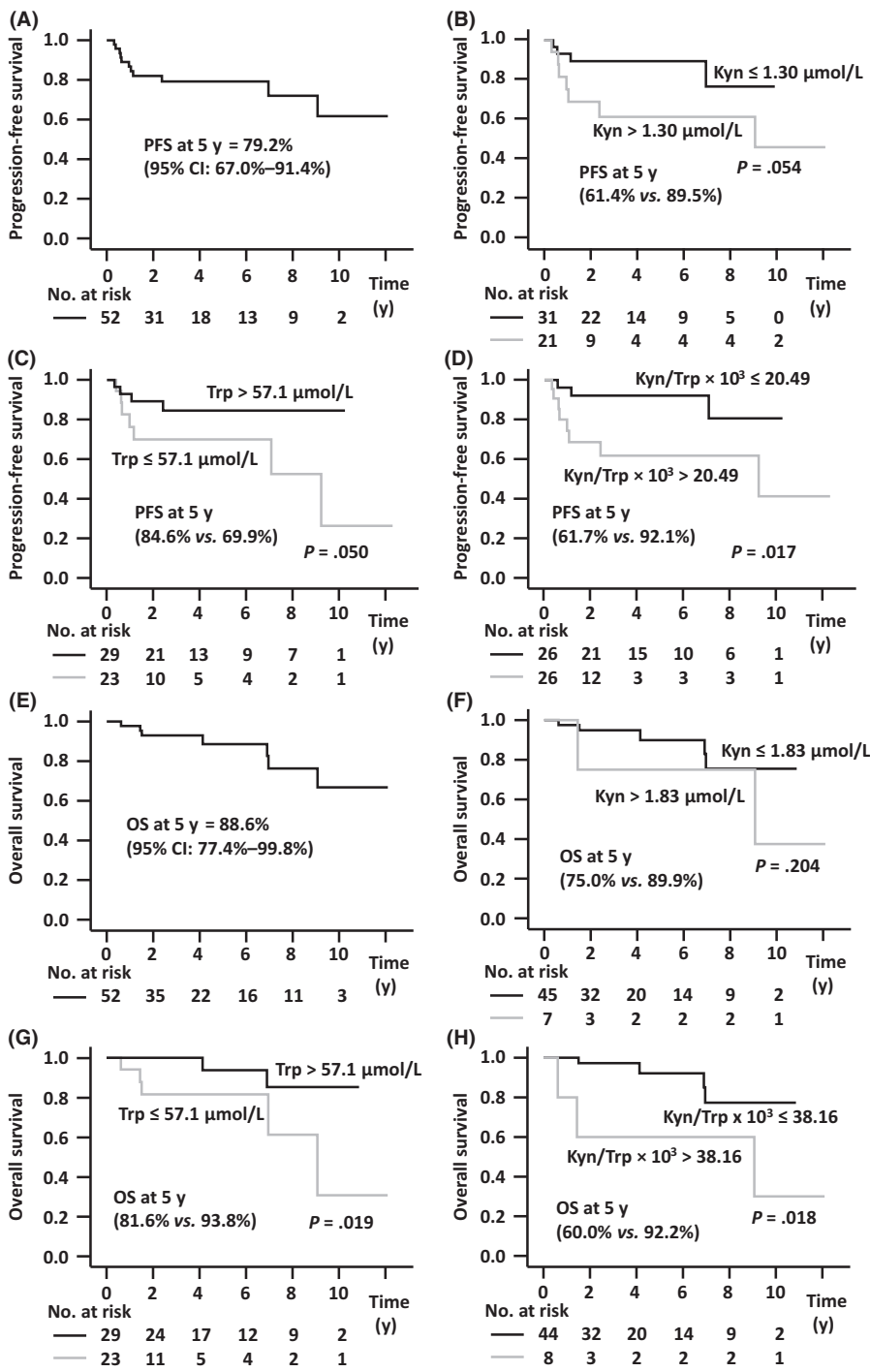
FIGURE 1 Progression-free survival (PFS) and overall survival (OS) of Hodgkin lymphoma (HL) patients. (A) PFS of all HL patients enrolled in the study (n = 52). (B) PFS of the HL patients according to serum kynurenine (Kyn) level. (C) PFS according to serum tryptophan (Trp) level. (D) PFS according to the serum Kyn/Trp ratio (Kyn [μmol/L]/Trp [μmol/L] × 10^3). (E) OS of all HL patients enrolled in the study (n = 52). (F) OS according to serum Kyn level. (G) OS according to serum Trp level. (H) OS according to serum Kyn/Trp ratio. Survival curves were compared using the log–rank test, and the P-value is indicated in each panel. PFS or OS rates at 5 years for each curve are indicated in each panel. CI, confidence interval; No., number

were also no significant differences in PFS in patients at Ann Arbor stage IV or stage I–III (P = .075); 5-year PFS in the former was 57.7% vs 84.9% in the latter (Figure S2c). However, OS was significantly shorter in stage IV patients than in stage I–III patients. Thus, the 5-year OS in the former was 78.8%, but 91.9% in the latter (P = .015) (Figure S2d). Neither were there any significant differences in PFS and OS between female and male HL patients (P = .954 and P = .394, respectively). The PFS and OS rates at 5 years in women were 78.6% and 95.0%, respectively, vs 79.9% and 83.3%, respectively, in men (Figure S2e,f). There were also no

significant differences in either PFS or OS between patients with a high or low Hb level (P = .377 and P = .131, respectively); 5-year PFS and OS in patients with a high Hb were 80.3%, and 91.1%, respectively, and those in patients with a low Hb were 72.9% and 71.4%, respectively (Figure S2g,h). Again, there were no significant differences in either PFS or OS between patients with high or low serum Alb levels (P = .440 and P = .547, respectively), the 5-year rates being 71.3% and 86.6%, respectively, in the high Alb group and 84.8% and 92.1%, respectively, in the low group (Figure S2i,j). The same was true for patients with or without leukocytosis

FIGURE 2 Progression-free survival (PFS) and overall survival (OS) of Hodgkin lymphoma (HL) patients according to the serum soluble CD30 (sCD30) level and correlations of serum kynurenine (Kyn), tryptophan (Trp), the Kyn/Trp ratio, and sCD30. (A) PFS of HL patients according to serum sCD30 level. (B) OS according to serum sCD30 level. Survival curves were compared using the log–rank test, and the P-value is indicated in each panel. No., number. (C) Serum Kyn concentration is plotted on the x-axis and serum sCD30 concentration is indicated on the y-axis. (D) Serum Trp concentration is plotted on the x-axis and serum sCD30 concentration is indicated on the y-axis. (E) Serum Kyn/Trp ratio is plotted on the x-axis and serum sCD30 concentration is indicated on the y-axis. Each dot plot in panels (C–E) represents a different HL patient. Spearman's rank correlation coefficient (Rs) between serum Kyn (C) and Trp (D) concentrations and the Kyn/Trp ratio (E) vs serum sCD30. P-values are indicated in each panel

($P = .654$ and $P = .998$, respectively), in which PFS and OS at 5 years were 80.0% and 100.0%, respectively, in patients with leukocytosis and 79.0% and 86.9%, respectively, in those without (Figure S2k,l). This was also the case for lymphocytopenia ($P = .164$), where 5-year PFS was 71.4% and 80.5% for patients with or without lymphocytopenia (Figure S2m). However, here a difference was seen in OS, which was significantly shorter in patients with lymphocytopenia. Thus, 5-year OS in patients with lymphocytopenia was 71.4%, whereas it was 92.0% in those without ($P = .010$) (Figure S2n).

3.9 | Prognostic significance of serum Kyn, Trp, and the Kyn/Trp ratio in HL patients

Multivariate analysis of factors influencing OS in the cohort of 52 HL patients studied here was carried out using the following three variables: Ann Arbor stage (I–III or IV), lymphocytopenia (presence or absence), and serum Trp (≤ 57.1 or >57.1 μmol/L). Of these, none was found to significantly affect OS (Table S5). Multivariate analysis for OS in the 52 HL patients was also carried out using three

different variables, namely, the Ann Arbor stage, lymphocytopenia, and the Kyn/Trp ratio (≤ 38.16 or >38.16). Of these, only the serum Kyn/Trp ratio significantly affected OS (Table 3).

3.10 | Indoleamine 2,3-dioxygenase 1 expression in affected LNs of HL patients

Expression of IDO was detected in macrophages/dendritic cells, but not in the HL tumor cells themselves, as shown in Figure 3A. Although IDO expression assessed by immunohistochemistry (positive cells/0.25 mm^2) varied among the cases, IDO levels did have a significant positive correlation with the serum Kyn/Trp ratio ($Rs = 0.565$, $P = .009$) (Figure 3B).

3.11 | Indoleamine 2,3-dioxygenase 1 expression by flow cytometry analysis

Expression of IDO in HeLa and HL cell lines stimulated by IFN-γ are shown in Figure 3C. The HL cell lines L-428, L-1236, HDLM-2, and KM-H2 were all negative for IDO even after stimulation with IFN-γ.

TABLE 2 Characteristics of patients with Hodgkin lymphoma according to serum kynurenine (Kyn), tryptophan (Trp), and Kyn/Trp ratio

	Serum Kyn, μmol/L			Serum Trp, μmol/L			Serum Kyn/Trp ×10³		
Characteristics	≤1.83	>1.83	P-value	>57.1	≤57.1	P-value	≤38.16	>38.16	P-value
Total patients, n (%)	45	7		29	23		44	8	
Serum Alb, g/dL									
≥4.0	17 (38)	1 (14)	.399	14 (48)	4 (17)	.038	17 (39)	1 (13)	.236
<4.0	28 (62)	6 (86)		15 (52)	19 (83)		27 (61)	7 (87)	
Hb, g/dL									
≥10.5	38 (84)	3 (43)	.029	27 (93)	14 (61)	.007	38 (86)	3 (38)	.007
<10.5	7 (16)	4 (57)		2 (7)	9 (39)		6 (14)	5 (62)	
Sex									
Female	21 (47)	1 (14)	.216	14 (48)	8 (35)	.403	20 (45)	2 (25)	.442
Male	24 (53)	6 (86)		15 (52)	15 (65)		24 (55)	6 (75)	
Age, years									
<45	24 (53)	1 (14)	.101	17 (59)	8 (35)	.103	24 (55)	1 (13)	.051
≥45	21 (47)	6 (86)		12 (41)	15 (65)		20 (45)	7 (87)	
Stage									
I–III	36 (80)	4 (57)	.331	24 (83)	16 (70)	.329	35 (80)	5 (62)	.366
IV	9 (20)	3 (43)		5 (17)	7 (30)		9 (20)	3 (38)	
WBC, /mm³									
≥15 000	7 (16)	0 (0)	.574	2 (7)	5 (22)	.219	6 (14)	1 (13)	1.000
<15 000	38 (84)	7 (100)		27 (93)	18 (78)		38 (86)	7 (87)	
Lymphocytopenia[a]									
Present	7 (16)	3 (43)	.120	0 (0)	10 (43)	<.001	5 (11)	5 (62)	.004
Absent	38 (84)	4 (57)		29 (100)	13 (5)		39 (89)	3 (38)	
sCD30, ng/mL									
≤285.6	35 (78)	3 (43)	.075	25 (86)	13 (57)	.027	36 (82)	2 (25)	.003
>285.6	10 (22)	4 (57)		4 (14)	10 (43)		8 (18)	6 (75)	

Alb, albumin; Hb, hemoglobin; sCD30, soluble CD30.
[a]Lymphocyte count <600/mm³, or <8% of white blood cell count (WBC), or both.

As a control, HeLa cells were positive for IDO, as reported previously.[22] There was no IDO expression in HeLa cells or in L-428, L-1236, HDLM-2, or KM-H2 cells when they were not stimulated by IFN-γ (data not shown).

4 | DISCUSSION

It has been reported that, relative to healthy controls, serum Trp levels are significantly lower in several types of cancer such as colorectal cancer,[23] ovarian carcinoma,[24] and adult T-cell leukemia/lymphoma.[25] This might be due to accelerated Trp catabolism mediated by the IDO produced by tumor cells and/or non-tumor cells in the microenvironment. Consistent with these reports, we found that serum Trp levels were significantly lower in patients with HL than in healthy controls. In this context, the serum Kyn/Trp ratio was also higher in patients with HL than in healthy controls. It must be stated that, in the present study, serum Kyn and Trp levels and the Kyn/Trp ratio in healthy controls were not identical with those of our

**TABLE 3** Multivariate analysis for overall survival in patients with Hodgkin lymphoma (n = 52)

Variable	n	Hazard ratio	95% CI	P-value
Stage				
I–III	40	1.000	—	Reference
IV	12	4.899	0.743-32.295	.099
Lymphocytopenia				
Absent	42	1.000	—	Reference
Present	10	4.015	0.670-24.064	.128
Serum Kyn/Trp (×10³)				
≤38.16	44	1.000	—	Reference
>38.16	8	7.577	1.362-42.160	.021

CI, confidence interval; Kyn, kynurenine; Trp, tryptophan.

previous study,[21] although the methods and the machine used (UPLC–MS/MS) were the same. Compared to the previous studies, some unavoidable variables, such as an error of calibration curve due to using different batches of reagents or components of the
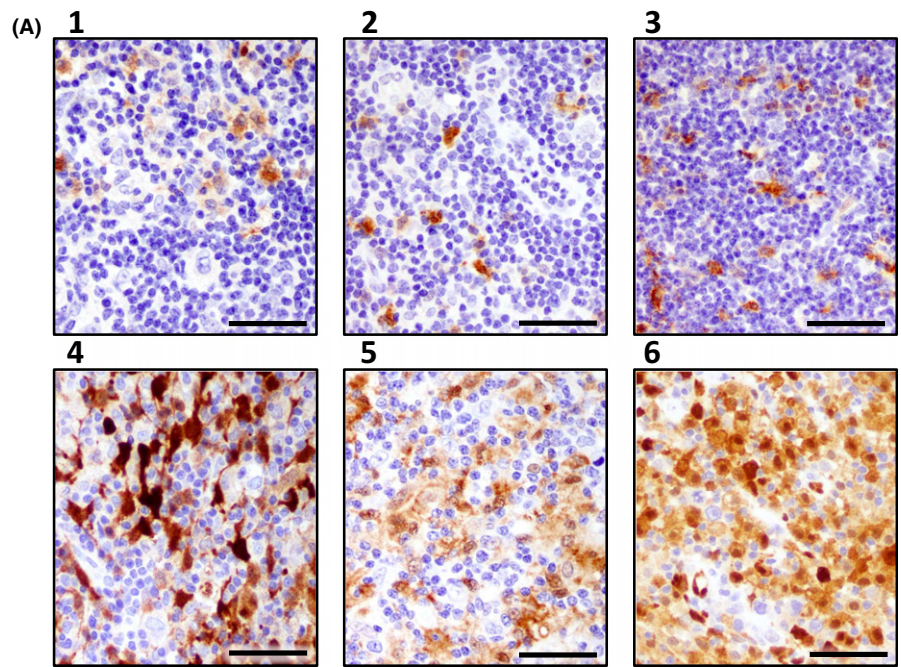
FIGURE 3 Indoleamine 2,3-dioxygenase 1 (IDO) expression in the affected lymph nodes of patients with Hodgkin lymphoma (HL). (A) Immunostaining for IDO in the affected lymph node lesions from six individual HL patients. IDO expression can be seen in macrophages/dendritic cells, but not in HL tumor cells. Scale bar = 50 μm. (B) Patients' serum kynurenine (Kyn)/tryptophan (Trp) ratios are plotted on the x-axis and IDO expression levels determined by immunohistochemistry (IDO-positive cells/0.25 mm^2) on the y-axis. Spearman's rank correlation coefficient (Rs) between serum Kyn/Trp ratios and IDO expression levels; P-values are indicated in the panel. The dot plots labeled 1-6 in the panel correspond to photographs 1-6 in (A). (C) Cells were stimulated by γ-interferon and stained with anti-IDO mAb (blank histograms) or isotype control mAb (filled histogram)
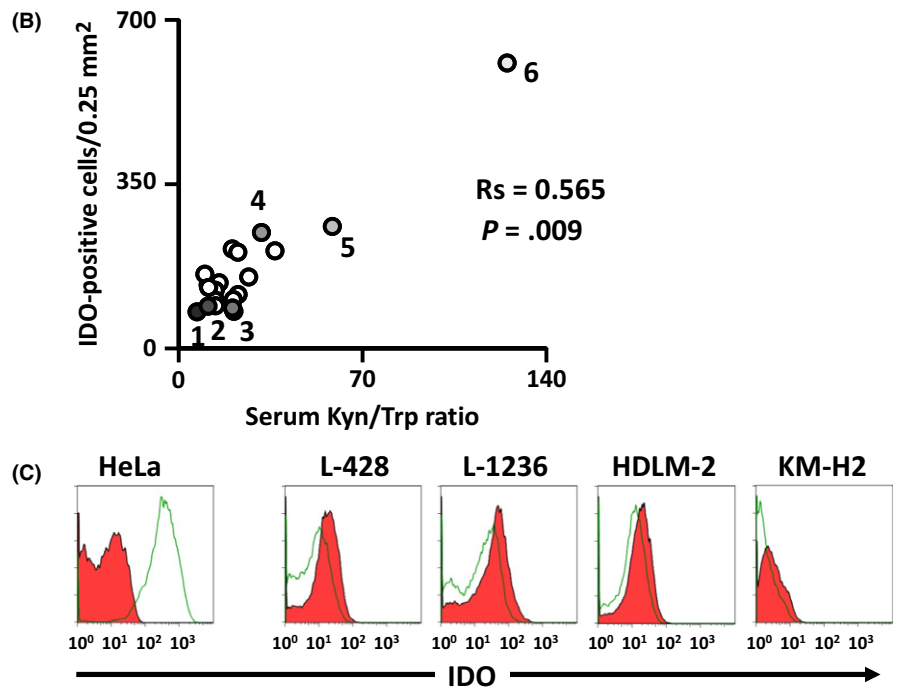
machine, may have changed. However, importantly, in the present study, we measured serum Kyn and Trp levels, and the Kyn/Trp ratio of both healthy controls and HL patients almost at the same time and under the same conditions as previously, so both should have been equally affected by any changes.

Because very few patients with nodular lymphocytes, lymphocyte-rich, or lymphocyte-depleted HL were included in the present study (two, one, and three patients, respectively), we analyzed the serum Kyn and Trp levels and the Kyn/Trp ratio for the two histological subtypes, NS and MC. A statistically significant elevation of serum Kyn and the Kyn/Trp ratio in patients with MC relative to those with NS is consistent with previous findings from Kamper et al.[8] They reported that macrophages present in the HL tumor microenvironment correlated with Epstein–Barr virus status in the HL tumor cells,[8] and it is generally accepted that Epstein–Barr virus is usually positive in MC, but negative in NS.[1,3]

A significant positive correlation between serum Kyn and sCD30 levels, and between the Kyn/Trp ratio and sCD30 levels, as well as a significant inverse correlation between serum Trp and sCD30 levels, all emphasize the importance of Trp catabolism in HL patients, because it has been reported that sCD30 is an important biomarker predicting therapeutic efficacy and outcome in HL patients.[26-30]

Tryptophan catabolism in HL patients did not seem merely to reflect HL tumor burden, because neither serum Kyn and Trp levels, nor the Kyn/Trp ratio, were significantly associated with Ann Arbor stage. However, they did seem to be associated with a chronic inflammatory state, because Kyn and Trp levels and the Kyn/Trp ratio were all significantly associated with anemia. In addition, there was an association with immune disorders, because the Trp level and the Kyn/Trp ratio were significantly associated with lymphocytopenia.

Regarding the survival parameters, we found that the Kyn/Trp ratio was significantly associated with PFS, whereas none of the seven component factors of the IPS for advanced HL,[17] nor the serum sCD30 level,[26-30] was significantly associated with PFS. This observation that only the serum Kyn/Trp ratio was significantly associated with shorter PFS should attract attention because it indicates the importance of Trp catabolism in the pathogenesis of HL. Additionally, low serum Trp and a high Kyn/Trp ratio were significantly associated with shorter OS. Among the seven IPS factors, advanced stage and lymphocytopenia were significantly associated with shorter survival, but subsequent multivariate analyses showed that only a high serum high Kyn/Trp ratio was an independent significant unfavorable prognostic factor for OS. This also emphasizes the importance of Trp catabolism in the pathogenesis of HL, which is likely to be associated with serious immune dysfunction leading to poorer OS. These findings also provide novel insights for understanding the immunopathogenesis of HL through Trp catabolism. That is to say, the IDO-rich tumor microenvironment facilitates HL tumor cell survival in the face of host immune responses, even though the tumor cells are very few in number.

Most research in this area to date has focused on IDO as the central and immunobiologically relevant enzyme that catalyzes the conversion of Trp to Kyn. However, there are two other enzymes, tryptophan 2,3-dioxygenase and IDO2, that also catalyze the same enzymatic step.[11,13-15] In addition, this pathway is also responsive to non-specific inflammation. Therefore, in general, the serum Kyn/Trp ratio is merely one surrogate marker of IDO activity, and does not exclusively reflect all IDO activity. However, in the present study, there were significant positive correlations between IDO expression levels in the affected LN, as determined by histology, and the serum Kyn/Trp ratio. In addition, the immunohistochemistry stainings indicated that the sources of IDO were tumor-infiltrating macrophages/dendritic cells, and not the HL tumor cells themselves. These observations are consistent with the IDO expression profiles in the HL tumor cell lines tested in the present study, and in an earlier investigation by Choe et al.[16] Therefore, the serum Kyn/Trp ratio in patients with HL is likely to be dependent on IDO and probably not on tryptophan 2,3-dioxygenase or IDO2, produced by macrophages/dendritic cells in the tumor microenvironment. In addition, interactions between colony stimulating factor-1 produced by HL tumor cells and the colony stimulating factor-1 receptor expressed by tumor-associated macrophages/dendritic cells should play an important role for the present observations.[31-33]

Although the present study offers novel and important insights into the immunopathogenesis of HL, a significant limitation should also be borne in mind. The number of HL patients analyzed in the present study was relatively small, thus a definitive conclusion requires validation in another larger study. Further investigation of Trp catabolism in much larger numbers of HL patients is warranted to confirm the present observations.

We conclude that macrophages/dendritic cells in the HL tumor microenvironment produce IDO, leading to a high Kyn/Trp ratio and a low Trp level not only in the tumor microenvironment, but also in the blood. A high serum Kyn/Trp ratio was a significant detrimental prognostic factor in HL. The present study provides novel insights for a better understanding of the immunopathogenesis of HL. That is to say, understanding how HL tumor cells can survive in the face of host immune responses, even though they are present in very small numbers, and understanding how they create immunocompromised conditions in the host, leading to an unfavorable prognosis. In addition, we believe that measurement of serum Kyn and Trp concentrations will be useful for predicting prognosis of the individual HL patient. Furthermore, IDO has now become a very attractive target for developing novel anticancer agents, and several IDO inhibitors are currently being investigated.[34,35] Hodgkin lymphoma, especially in patients with a high serum Kyn/Trp ratio, is an appropriate disease for testing these novel cancer immunotherapies targeting IDO.

ORCID

Takashi Ishida [ID] http://orcid.org/0000-0002-1060-0777
Masaki Ri [ID] http://orcid.org/0000-0002-9617-486X
Shinsuke Iida [ID] http://orcid.org/0000-0002-4951-960X

REFERENCES

1. Stein H, Poppeman S, Swerdlow SH, et al. Hodgkin lymphoma. In: Swerdlow SH, Campo E, Harris NL, et al., eds. *WHO Classification of*

Tumours of Haematopoietic and Lymphoid Tissues (4th ed., pp. 321-334). Lyon, France: International Agency for Research on Cancer (IARC); 2008.

2. Vardhana S, Younes A. The immune microenvironment in Hodgkin lymphoma: T cells, B cells, and immune checkpoints. *Haematologica*. 2016;101:794-802.

3. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127:2375-2390.

4. Ishida T, Ishii T, Inagaki A, et al. Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. *Cancer Res*. 2006;66:5716-5722.

5. Ansell SM, Lesokhin AM, Borrello I, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*. 2015;372:311-319.

6. Younes A, Santoro A, Shipp M, et al. Nivolumab for classical Hodgkin's lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: a multicentre, multicohort, single-arm phase 2 trial. *Lancet Oncol*. 2016;17:1283-1294.

7. Steidl C, Lee T, Shah SP, et al. Tumorassociated macrophages and survival in classic Hodgkin's lymphoma. *N Engl J Med*. 2010;362:875-885.

8. Kamper P, Bendix K, Hamilton-Dutoit S, Honore B, Nyengaard J, d'Amore F. Tumor-infiltrating macrophages correlate with adverse prognosis and Epstein-Barr virus status in classical Hodgkin's lymphoma. *Haematologica*. 2011;96:269-276.

9. Tan KL, Scott DW, Hong F, et al. Tumor-associated macrophages predict inferior outcomes in classic Hodgkin lymphoma: a correlative study from the E2496 Intergroup trial. *Blood*. 2012;120:3280-3287.

10. Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. *J Clin Invest*. 2007;117:1147-1154.

11. Löb S, Königsrainer A, Rammensee HG, Opelz G, Terness P. Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? *Nat Rev Cancer*. 2009;9:445-452.

12. Godin-Ethier J, Hanafi LA, Piccirillo CA, Lapointe R. Indoleamine 2,3-dioxygenase expression in human cancers: clinical and immunologic perspectives. *Clin Cancer Res*. 2011;17:6985-6991.

13. Platten M, Wick W, Van den Eynde BJ. Tryptophan catabolism in cancer: beyond IDO and tryptophan depletion. *Cancer Res*. 2012;72:5435-5440.

14. Zhai L, Spranger S, Binder DC, et al. Molecular pathways: targeting IDO1 and other tryptophan dioxygenases for cancer immunotherapy. *Clin Cancer Res*. 2015;21:5427-5433.

15. Munn DH, Mellor AL. IDO in the tumor microenvironment: inflammation, counter-regulation, and tolerance. *Trends Immunol*. 2016;37:193-207.

16. Choe JY, Yun JY, Jeon YK, et al. Indoleamine 2,3-dioxygenase (IDO) is frequently expressed in stromal cells of Hodgkin lymphoma and is associated with adverse clinical features: a retrospective cohort study. *BMC Cancer*. 2014;14:335.

17. Hasenclever D, Diehl V. A prognostic score for advanced Hodgkin's disease. International prognostic factors project on advanced Hodgkin's disease. *N Engl J Med*. 1998;339:1506-1514.

18. Lister TA, Crowther D, Sutcliffe SB, et al. Report of a committee convened to discuss the evaluation and staging of patients with Hodgkin's disease: Cotswolds meeting. *J Clin Oncol*. 1989;7:1630-1636.

19. Canellos GP, Anderson JR, Propert KJ, et al. Chemotherapy of advanced Hodgkin's disease with MOPP, ABVD, or MOPP alternating with ABVD. *N Engl J Med*. 1992;327:1478-1484.

20. Maeda Y, Ito T, Ohmi H, et al. Determination of 3-hydroxyisovaleryl-carnitine and other acylcarnitine levels using liquid chromatography-tandem mass spectrometry in serum and urine of a patient with multiple carboxylase deficiency. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2008;870:154-159.

21. Masaki A, Ishida T, Maeda Y, et al. Prognostic significance of tryptophan catabolism in adult T-cell leukemia/lymphoma. *Clin Cancer Res*. 2015;21:2830-2839.

22. Robinson CM, Shirey KA, Carlin JM. Synergistic transcriptional activation of indoleamine dioxygenase by IFN-gamma and tumor necrosis factor-alpha. *J Interferon Cytokine Res*. 2003;23:413-421.

23. Huang A, Fuchs D, Widner B, Glover C, Henderson DC, Allen-Mersh TG. Serum tryptophan decrease correlates with immune activation and impaired quality of life in colorectal cancer. *Br J Cancer*. 2002;86:1691-1696.

24. Sperner-Unterweger B, Neurauter G, Klieber M, et al. Enhanced tryptophan degradation in patients with ovarian carcinoma correlates with several serum soluble immune activation markers. *Immunobiology*. 2011;216:296-301.

25. Hoshi M, Ito H, Fujigaki H, et al. Indoleamine 2,3-dioxygenase is highly expressed in human adult T-cell leukemia/lymphoma and chemotherapy changes tryptophan catabolism in serum and reduced activity. *Leuk Res*. 2009;33:39-45.

26. Pizzolo G, Vinante F, Chilosi M, et al. Serum levels of soluble CD30 molecule (Ki-1 antigen) in Hodgkin's disease: relationship with disease activity and clinical stage. *Br J Haematol*. 1990;75:282-284.

27. Gause A, Pohl C, Tschiersch A, et al. Clinical significance of soluble CD30 antigen in the sera of patients with untreated Hodgkin's disease. *Blood*. 1991;77:1983-1988.

28. Nadali G, Vinante F, Ambrosetti A, et al. Serum levels of soluble CD30 are elevated in the majority of untreated patients with Hodgkin's disease and correlate with clinical features and prognosis. *J Clin Oncol*. 1994;12:793-797.

29. Plattel WJ, Alsada ZN, van Imhoff GW, Diepstra A, van den Berg A, Visser L. Biomarkers for evaluation of treatment response in classical Hodgkin lymphoma: comparison of sGalectin-1, sCD163 and sCD30 with TARC. *Br J Haematol*. 2016;175:868-875.

30. Marri PR, Hodge LS, Maurer MJ, et al. Prognostic significance of pretreatment serum cytokines in classical Hodgkin lymphoma. *Clin Cancer Res*. 2013;19:6812-6819.

31. Paietta E, Racevskis J, Stanley ER, Andreeff M, Papenhausen P, Wiernik PH. Expression of the macrophage growth factor, CSF-1 and its receptor c-fms by a Hodgkin's disease-derived cell line and its variants. *Cancer Res*. 1990;50:2049-2055.

32. Koh YW, Park C, Yoon DH, Suh C, Huh J. CSF-1R expression in tumorassociated macrophages is associated with worse prognosis in classical hodgkin lymphoma. *Am J Clin Pathol*. 2014;141:573-583.

33. Dammeijer F, Lievense LA, Kaijen-Lambers ME, et al. Depletion of tumor-associated macrophages with a CSF-1R kinase inhibitor enhances antitumor immunity and survival induced by DC immunotherapy. *Cancer Immunol Res*. 2017;5:535-546.

34. Brochez L, Chevolet I, Kruse V. The rationale of indoleamine 2,3-dioxygenase inhibition for cancer therapy. *Eur J Cancer*. 2017;76:167-182.

35. Vacchelli E, Aranda F, Eggermont A, et al. Trial watch: IDO inhibitors in cancer therapy. *Oncoimmunology*. 2014;3:e957994.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.