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Expression of immune checkpoint molecules programmed death protein 1, programmed death-ligand 1 and inducible T-cell co-stimulator in mycosis fungoides and Sézary syndrome: association with disease stage and clinical outcome*

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Abstract

Background The relationship between immune checkpoint status and disease outcome is a major focus of research in cutaneous T-cell lymphoma (CTCL), a disfiguring neoplastic dermatological disorder. Mycosis fungoides (MF) and Sézary syndrome (SS) are the two most common types of CTCL.

Objectives The aim was to evaluate the immune checkpoint markers programmed death protein 1 (PD1), inducible T-cell co-stimulator (ICOS) and programmed death-ligand 1 (PD-L1) in skin biopsies from patients with CTCL relative to disease stage and overall survival.

Methods This consecutive case series enrolled 47 patients: 57% had stage IA–IIA disease and 43% had stage IIB–IVA2 disease (including seven with SS).

Results PD1, PD-L1 and ICOS expression was seen in all biopsies. Notably, PD-L1 was predominantly expressed on histiocytes/macrophages, but focal expression on CTCL cells was seen. High expression of either ICOS or PD-L1 was associated with advanced-stage disease ($P = 0{\cdot}007$ for both) and with the appearance of large-cell transformation (LCT), a histopathological feature associated with a poor prognosis (ICOS: $P = 0{\cdot}02$; PD-L1: $P = 0{\cdot}002$). PD1 expression was not significantly associated with disease stage ($P = 0{\cdot}12$) or LCT ($P = 0{\cdot}49$), but expression was high in SS biopsies. A high combined checkpoint marker score (PD1, PD-L1 and ICOS) was associated with advanced-stage disease ($P = 0{\cdot}001$), LCT ($P = 0{\cdot}021$) and lower overall survival ($P = 0{\cdot}014$).

Conclusions These findings demonstrate the existence of a complex immunoregulatory microenvironment in CTCL and support the development of immunotherapies targeting ICOS and PD-L1 in advanced disease.

What is already known about this topic?

- Expression of immune checkpoint markers has been observed in many cancers.
- Immunotherapies targeting one or more immune checkpoint markers can be very effective.
- However, little is known about the immune checkpoint marker status in cutaneous T-cell lymphoma (CTCL), including the potential relationship between checkpoint marker expression and disease stage or survival.

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Conflicts of interest
C.D.R., B.R.-G., D.D.W., X.W., Z.H. and J.Y.S.: nothing to declare; J.P.: advisory board (Gilead Science); J.Z.: speakers bureau (Spectrum), advisory board (Seattle Genetics); S.T.R.: speaker's bureau (Celgene, Global Education Group, Paradigm Medical Communications, AbbVie), consulting (Novartis, PeproMene Bio, Exicure, Apobiologix/Apotex); advisory board (Seattle Genetics, NeoGenomics, Aileron Therapeutics); C.Q.: advisory board (Miragen, Bioniz, Trillium, Kyowa Kirin, Medivir, Helsinn/Actelion, Mallinckrodt), research funding (Celgene).

Data availability
Our datasets have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus [(GEO); https://www.

ncbi.nlm.nih.gov/geo/, #GSE113113] and are available upon request.

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What does this study add?

- We show an association between checkpoint marker expression and disease outcomes in patients with mycosis fungoides and Sézary syndrome, the most common forms of CTCL.
- A combined checkpoint score predicts disease stage and survival better than individual scores.
- These findings support the development of immune checkpoint scores as prognostic indicators for CTCL.
- Further investigation of checkpoint inhibitor immunotherapies for patients with CTCL, especially combination therapies, may have clinical benefit.

Mycosis fungoides (MF) and Sézary syndrome (SS) are the most common types of cutaneous T-cell lymphoma (CTCL).[1] MF ranges from an indolent disorder presenting with patches/plaques to advanced disease with cutaneous tumours, erythroderma and/or systemic involvement, whereas SS is an aggressive erythrodermic variant with leukemic involvement. The current thinking is that CTCL originates from clonally expanded, effector/central memory $CD4^+$ T cells in a background of chronic inflammation, which create a protumorigenic microenvironment as they expand. A subset (~20–50%) of patients with MF undergo large-cell transformation (LCT), a process characterized by the appearance of numerous large tumour cells constituting ≥25% of the infiltrate and an aggressive clinical course.[2–4]

A protumorigenic microenvironment is established through the action of immune regulatory proteins in the CD28 superfamily, such as programmed death protein 1 (PD1) and its ligands (PD-L1/2). PD-L1 expression correlates with negative outcomes across a variety of cancers and has been identified in CTCL biopsies.[5] In most cancers, PD-L1 is expressed on the tumour cells and other cells in the microenvironment. Interaction of PD-L1 with PD1, which is expressed on effector T cells, downregulates T-cell activation and proliferation, thereby attenuating the antitumour immune response.[6] PD1 can also be expressed on tumour cells, especially in SS.[7] Whether PD-L1 is expressed on CTCL cells or in the microenvironment remains unclear.[5] We previously showed that a low percentage of T-cell émigrés from lesional CTCL samples express PD-L1 and that PD-L1 does not co-express with PD1 in tissue sections.[8] Therefore, one goal of this study was to identify the PD-L1-expressing cells in CTCL.

Other immune checkpoints contribute to the protumorigenic microenvironment. The inducible T-cell co-stimulator (ICOS), another member of the CD28 superfamily, is essential for the activation of T cells and is expressed on tumour cells in CTCL.[9,10] ICOS promotes T-cell proliferation and differentiation, and is thought to maintain immunosuppressive T-cell subsets, such as inducible $CD4^+$ regulatory T cells.[11]

Given the success of immune checkpoint blockade in restoring the antitumour functions of effector T cells in a wide variety of cancers, there is considerable interest in understanding how these immunomodulatory molecules contribute to CTCL.[12] Thus, the aims of this study were to investigate the expression of the immune checkpoint markers PD1, PD-L1 and ICOS in skin biopsies from patients with all stages of MF/SS and to correlate the immune checkpoint status with the disease stage and outcome.

Patients and methods

Patients

This prospective consecutive case series enrolled 47 adult patients diagnosed with MF or SS. All study participants were seen at our multidisciplinary clinic at City of Hope National Medical Center between 1 April 2015 and 31 December 2016. The study was performed in accordance with the provisions of the Declaration of Helsinki, the International Conference on Harmonization, and the Guidelines for Good Clinical Practice, and was approved by the City of Hope Institutional Review Board.

Patients were diagnosed according to the revised 2018 World Health Organization–European Organisation for Research and Treatment of Cancer (WHO–EORTC) classification and classified according to the revised staging system for MF/SS based on the tumour–node–metastasis–blood (TNMB) classification system.[13] Skin disease for both MF and SS was classified according to T stage. T1 and T2 are defined by patches and/or plaques involving <10% or ≥ 10% of the body surface area, respectively, whereas T3 is defined by ≥ one tumour and T4 by erythroderma. Relevant demographic and clinical data were collected from the electronic medical record including age, sex, race/ethnicity, assessment of skin tumour burden by the modified severity-weighted assessment tool, MF/SS subtype, and vital status at last contact. MF/SS is treated with individualized therapeutic strategies, leading to significant variability in the current patient population and precluding analysis by treatment type. These data were locked for analysis on 31 December 2016.

Histopathology, immunophenotyping and statistical analysis

The pathological diagnosis was made on formalin-fixed paraffin-embedded (FFPE) tissue sections from 47 lesional skin biopsies stained with haematoxylin and eosin and for routine immunohistochemistry (IHC) markers and was confirmed by two board-certified pathologists (C.Q., J.Y.S.). Tissue sections

of representative specimens were chosen for IHC staining for ICOS (Cat #MA5-16415, Invitrogen, Carlsbad, CA, USA), PD1 (NAT-105; Cell Marque, Rocklin, CA, USA) and PD-L1 (clone SP263, Cat #0709374001, Roche, Basel, Switzerland). PD1, PD-L1 and ICOS expression was graded in four categories. The expression within epidermotropic and dermal lymphoid infiltrates was scored for entire tissue sections by two different pathologists using the following criteria: –, negative (< 5%); +, rare-scattered (5–15%); ++, numerous (> 15–30%); +++, abundant (> 30%) on tumour cells (and nontumour cells if applicable). Any discordant cases were re-reviewed until consensus was reached. The presence of LCT was defined as the presence of large cells comprising >25% of the infiltrate or formation of microscopic nodules. A combined checkpoint marker score for ICOS, PD-L1 and PD1 was generated by converting the expression levels (–, +, ++ and +++) to the values 0–3 and then adding the scores for each marker for a combined score (0–3 is low, 4+ is high). The biopsies were taken from patients who had not been on active treatment at the initial visit and prior to receiving treatment at our hospital, although we cannot rule out the possibility that the patients had received prior treatment elsewhere.

To further characterize the spatial relationship of the immune checkpoint markers ICOS, PD1 and PD-L1 with T cells and macrophages/histiocytes in the tumour microenvironment, seven-colour multispectral images of FFPE sections were obtained in a subset of cases representing patch, plaque and tumour (three each, nine in total) using the Opal™ seven-colour IHC kit.[14] Slides were scanned using the PerkinElmer Vectra system and images were taken at 200× and analysed by two trained pathologists for evidence of colocalization. Two different panels were used and six markers were included in each panel (panel #1: PD1, PD-L1, CD163, CD3, CD4, CD8; and panel #2: ICOS, PD-L1, CD163, CD3, CD4, CD8). Antibody information is given in Appendix S1 (see Supporting Information).

Fisher's exact test was used to evaluate the potential association between low or high immune checkpoint marker expression and clinicopathological features such as LCT, clinical stage and MF subtype. The co-expression profiles (ICOS with PD-L1; ICOS with PD1; and PD1 with PD-L1) were assessed overall and by disease subtype. In the SS subgroup, descriptive statistics were employed given the small sample size ($n = 7$). Overall survival estimates were calculated based on the Kaplan–Meier product-limit method, and 95% confidence intervals were calculated using the logit transformation and the Greenwood variance estimate. Differences between Kaplan–Meier curves were assessed by the log-rank test. Patients who were alive at the time of analysis were censored at the last contact date. Overall survival was measured from the time of study enrolment to death from disease or other causes.

RNA sequencing

RNA sequencing data for the 47 skin biopsies analysed here were previously analysed for markers of immune response and T-cell exhaustion (Gene Expression Omnibus accession number GSE113113).[8] For the current study, the gene expression profiles were analysed for the gene signatures of checkpoint markers PD1 (*PDCD1*), PD-L1 (*CD274*) and *ICOS*, as well as genes associated with tumour-associated macrophage phenotypes (*CD68*, *CD163*, *MRC1*, *CD80*) and T-cell function [*FOXP3*, CD25 (*IL2RA*), granzyme B (*GZMB*)]. The $\log_2$ transformed values were clustered with correlation as a similarity matrix and average linkage using Cluster v3·0 and visualized using Java TreeView.

Results

Clinical features and histopathological findings

The clinical features of the 47 consecutive patients in this study are described in Table 1. Histopathology revealed that most cases (44 of 47) showed a $CD4^+$ T-cell phenotype with an increased CD4 : CD8 ratio of ≥10 : 1, indicating the presence of clonally expanded $CD4^+$ cells. A $CD8^+$ phenotype was identified in patients with hypopigmented MF (two) and granulomatous MF (one). A superficial perivascular or band-like lymphoid infiltrate below the basal layer with epidermotropism and/or tagging of the basal layer by small-to-medium-sized atypical, cerebriform lymphocytes was observed in the patch-plaque MF or SS (erythrodermic) lesions, whereas the tumour lesions showed deep dermal lymphoid infiltrates with partial or complete loss of epidermotropism. Figure 1a–c shows a patient with early-stage MF presenting with a thin erythematous scaly plaque. The skin biopsy revealed a band-like and focally epidermotropic infiltrate composed of atypical lymphocytes interspersed with small lymphocytes and histiocytes. Figure 1d–g shows a patient with advanced-stage MF with tumours within pre-existing plaques, and a skin biopsy demonstrating a deep dermal lymphoid infiltrate and epidermal Pautrier microcollections composed of large, atypical lymphocytes consistent with LCT. Overall, 13 skin biopsies (three plaques/early-stage MF and 10 tumours/advanced-stage MF) showed LCT.

Immune checkpoint marker expression in the cutaneous T-cell lymphoma microenvironment

Within the plaque lesion described in Figure 1a–c, we found focal colocalization of PD-L1 with the T-cell marker CD3 (Figure 2a; Table 2), which was otherwise negative for co-expression of both markers, supporting our prior finding that PD-L1 expression is generally low on the tumour cells.[8] PD-L1 was also not colocalized with PD1 (Figure 2b) and was instead colocalized with the macrophage/histiocyte marker CD163 (Figure 2c). PD1 showed co-expression with the T-cell marker CD3, but not CD163 (Figure 2d), which is consistent with the expectation that PD1 is expressed on T cells.

Similar imaging analysis was performed on the tumour lesion described in Figure 1d–g. As expected, PD1 immunoreactivity colocalized with CD3 but not CD163 or

Table 1 Demographic and clinical characteristics

Category	Total sample n = 47 patients (%)[a]
Age (years), median (range)	59 (26–83)
Sex	
Male	26 (55)
Female	21 (45)
Clinical stage (TNMB)	
Total early stage (IA–IIA)	27 (57)
IA	10 (21)
IA (T1aN0M0B0a)	5
IA (T1aN0M0B0b)	1
IA (T1bN0M0B0a)	4
IB	16 (34)
IB (T2N0MOB0a)	14
IB (T2N0M0B0b)	2
IIA (T2N1M0B0a)	1 (2·1)
Total advanced (IIB–IVA2)	20 (43)
IIB (T3N0M0B0a)	8 (17)
IIIB (T4NxM0B1b)	1 (2·1)
IVA2 (T4N0M0B2b)	11 (23)
Sézary syndrome (stage IVA2)	7 (15)
Large-cell transformation	13 (28)
Early stage (IA–IIA)	3
Advanced stage (IIB–IVA2)	10
Race/ethnicity	
Caucasian	30 (64)
African American	7 (15)
Hispanic	8 (17)
Asian	2 (4)
Mycosis fungoides subtypes	
Classic	40 (85)
Folliculotropic	4 (9)
Hypopigmented	2 (4)
Granulomatous	1 (2)

[a]n (%), unless otherwise noted.
TNMB, tumour–node–metastasis–blood classification of mycosis fungoides/Sézary syndrome. Stage classifications are in parentheses.

PD-L1 (Figure S1a,b; see Supporting Information). Also, as expected, CD3 and CD163 were not colocalized (Figure S1c) and PD-L1 was predominantly co-expressed with CD163 but not CD3 (Figure S1d–f), indicating that PD-L1 is expressed on macrophages/histiocytes and not on T cells. Notably, PD-L1 was positive on a subset of dermal cells that was negative for both CD3 and CD163. The findings could possibly represent different macrophage-like subsets or other immune cell populations such as myeloid suppressor cells, which are generally negative for CD163. ICOS was colocalized with CD3 but not PD-L1 (Figure S1g,h), consistent with the known role for ICOS on T cells and further supporting the lack of PD-L1 expression on T cells. Notably, PD-L1 expression was generally higher in tumour lesions (Figure S1a–h) compared with plaques (Figure 2a–d). In addition, we observed PD1 co-expression with both CD4 and CD8, but there was high variability among skin biopsy specimens. Although the $CD4^+$ population is a mixture of malignant and nonmalignant T cells, there is currently no marker to distinguish these populations.

Relationship of immune checkpoint marker expression to clinicopathological features and overall survival

Unsupervised clustering analysis of the RNA expression profiles for PD1, PD-L1 and ICOS, as well as markers of macrophages/histiocytes and T cells, revealed a qualitative trend towards increased expression of these markers with increasing disease stage of MF/SS (Figure 3).

Immune checkpoint marker expression by IHC was also evaluated relative to the clinicopathological features. High ICOS expression (++/+++) was observed in 11 of 13 biopsies with LCT (84·6%) and in 16 of 20 patients with skin tumours or erythroderma (80%) (Table 2, Figure 4a). Notably, only 10 of 27 skin biopsies (37%) from patch/plaque lesions of early-stage (stage IA–IIA) MF showed either (++) or (+++) ICOS expression, respectively. ICOS expression was positively associated with both the disease stage ($P = 0·007$) and LCT ($P = 0·02$) (Table 2; Figure 4b). High expression of PD-L1 (++/+++) was found in 12 of 13 skin samples with LCT (92%) and in the lesions in 16 of 20 (80%) of patients with advanced-stage MF/SS (Table 2). Thus, high PD-L1 expression was associated with both advanced clinical stage and LCT ($P = 0·007$ and $P = 0·002$, respectively) (Table 2, Figure 4c). PD1 expression did not show any statistical association with LCT or disease stage (Table 2). PD1 was highly expressed (+++) in six of seven SS cases, but no inferential statistics were performed due to the small sample size (Figure S2; see Supporting Information). The combined checkpoint marker score (as described in 'Histopathology, immunophenotyping and statistical analysis') was positively associated with both the disease stage ($P = 0·001$) and LCT ($P = 0·021$) (Table 2, Figure 4d). Notably, none of the samples with a low combined score were associated with advanced clinical stage or LCT.

The co-expression profiles of PD1, ICOS and PD-L1 in the tumour microenvironment were also investigated using IHC. ICOS expression was positively associated with PD-L1 expression ($P = 0·043$) and ICOS-PD-L1 co-expression was associated with LCT ($P = 0·002$) (Table S1; see Supporting Information). There was no significant association between PD1 and ICOS expression or between PD1 and PD-L1 expression.

Stratification of the patient population by immune checkpoint marker status revealed a trend towards increased overall survival for ICOS, PD1 and PD-L1, which did not reach statistical significance ($P = 0·48$, $P = 0·17$ and $P = 0·15$, respectively) (Figure 5a–c). However, stratification of the patient population into high and low combined immune checkpoint marker status revealed a significant association with overall survival ($P = 0·014$, Figure 5d). These results support a model wherein immune checkpoint markers all contribute to a complex protumour microenvironment associated with worse overall survival. We have plotted out the Kaplan–Meier survival function for the SS (seven) vs. all other MF (40) cases,

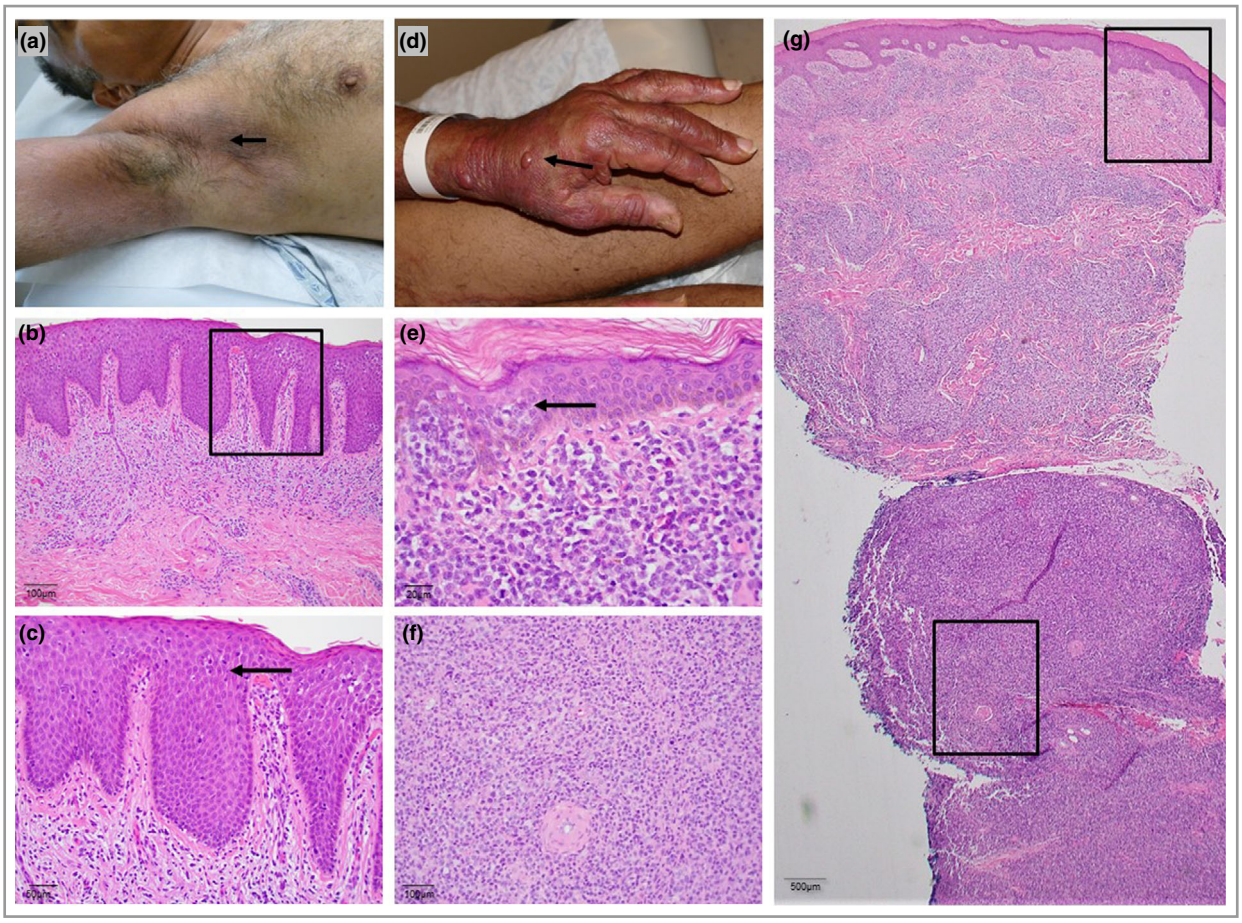

Figure 1 Clinical and histopathological presentation of plaque and tumour lesions of mycosis fungoides. (a) The arrow shows an early mycosis fungoides (MF) lesion clinically presenting as an erythematous thin plaque. (b, c) Haematoxylin and eosin staining: a superficial band-like infiltrate of atypical lymphocytes with epidermotropism (arrow) is present. Scale bars: b, 100 μm; c, 50 μm. (d) The arrow shows a tumour nodule developing within a plaque on the left hand. (e–g) Haematoxylin and eosin staining: a tumour nodule with a dense, diffuse and nodular proliferation of large atypical lymphoid cells consistent with large-cell transformation. (e) Superficial dermis. (f) Nodule in the deep dermis. Epidermal Pautrier microcollections are seen in the epidermis (arrow) along with sheets of large, atypical cells. Scale bars: e, 20 μm; f, 100 μm; g, 500 μm.

which did not show any significant difference in overall survival between SS and MF ($P = 0{\cdot}54$) (Figure S3; see Supporting Information).

Discussion

This study describes the expression profiles of PD1, PD-L1 and ICOS in MF/SS and demonstrates a relationship between high expression of these markers and high disease stage, LCT and poor overall survival. We here expand on our previous analysis that explored permitting checkpoint analysis of T-cell subsets and dendritic cell emigrees from skin explant cultures, which provided insight into the checkpoint expression profile of the entire skin infiltrate, allowing for global expression score and outcome analysis.[8] Furthermore, this study shows that PD-L1 is not expressed on CTCL cells but is expressed on histiocytes/macrophages in the tumour microenvironment. These results highlight the importance of considering the complex interactions among the various cells and multiple immunoregulators in the tumour microenvironment when considering therapeutic targets. They also suggest that a combined checkpoint marker score might have clinical utility for predicting CTCL outcomes.

There is emerging evidence that immunotherapy may be a useful strategy in CTCL, and clinical trials of PD1 inhibitors have shown durable clinical responses in a subset of patients with CTCL.[15,16] However, in this study, PD1 expression did not statistically correlate with disease stage, LCT or survival. Although this result is somewhat surprising, upregulation of PD1 suppresses tumour-infiltrating T-cell activity in various advanced tumours and interpretation of PD1 levels may, therefore, be complicated by changes in the cellular composition of the immune microenvironment. This theory is consistent with previous studies in CTCL, which have demonstrated a lower proportion of cytotoxic $CD8^+$ tumour-infiltrating T cells in skin biopsies of advanced MF compared with early disease,

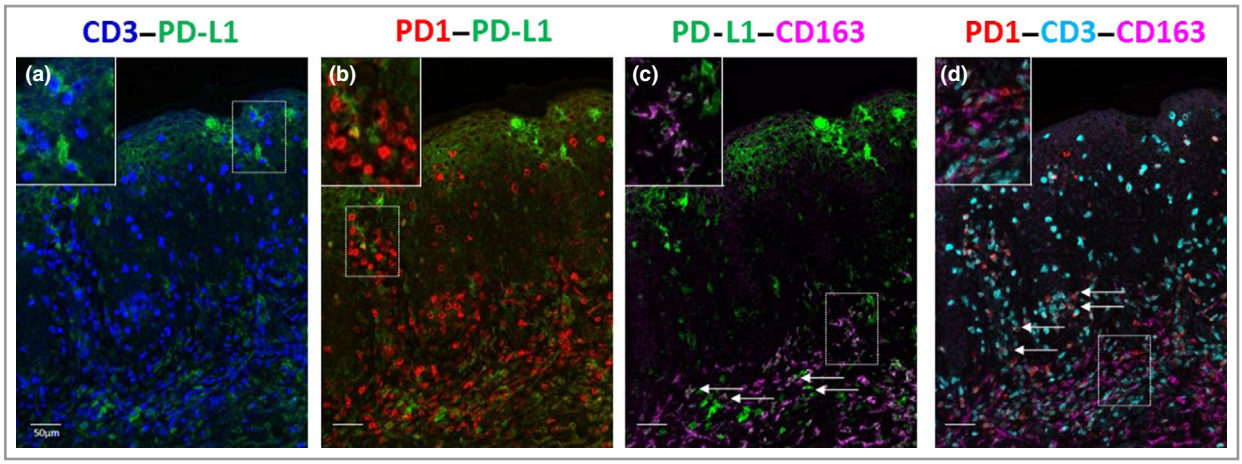

Figure 2 Immune checkpoint marker co-expression in a plaque lesion of mycosis fungoides. (a–d) Multispectral immunofluorescence of the plaque lesion described in Figure 1a–c. (a) CD3 (blue) and PD-L1 (green) expression; (b) PD1 (red) and PD-L1 (green) expression; (c) PD-L1 (green) and CD163 (pink) expression with arrows indicating colocalization; (d) PD1 (red), CD3 (cyan) and CD163 (pink) with arrows indicating colocalization of PD1 and CD3. Scale bars: 50 μm. The white boxes indicate the location of the insets shown in the upper left corners of each panel. PD1, programmed death protein 1; PD-L1, programmed death ligand 1.

Table 2 Relationship between immune checkpoint marker expression by immunohistochemistry and clinical characteristics

		Clinical stage n (%)			LCT n (%)		
		Early N = 27	Advanced N = 20	Total N = 47	No N = 34	Yes N = 13	Total N = 47
ICOS	Low (–/+)	17 (63)	4 (20)	21	19 (56)	2 (15)	21
	High (++/+++)	10 (37)	16 (80)	26	15 (44)	11 (85)	26
			P = 0·007			P = 0·020	
PD-L1	Low (–/+)	17 (63)	4 (20)	21	20 (59)	1 (8)	21
	High (++/+++)	10 (37)	16 (80)	26	14 (41)	12 (92)	26
			P = 0·007			P = 0·002	
PD1	Low (–/+)	12 (44)	4 (20)	16	13 (38)	3 (23)	16
	High (++/+++)	15 (56)	16 (80)	31	21 (62)	10 (77)	31
			P = 0·12			P = 0·49	
Combined score[a]	Low (0–3)	11 (41)	0 (20)	11	11 (32)	0 (0)	11
	High (4+)	16 (59)	20 (100)	36	23 (68)	13 (100)	36
			P = 0·001			P = 0·021	

[a]Combined score for the expression of ICOS, PD1 and PD-L1. ICOS, inducible T-cell co-stimulator; LCT, large-cell transformation; PD1, programmed death protein 1; PD-L1, programmed death ligand 1.

and fewer $CD8^+$ T cells was associated with decreased 5-year survival.[17,18] Although our RNA analysis indicated that T-cell levels increase with disease stage, there may be changes in the occurrence of certain T-cell subsets. Also, because all biopsies were taken from patients on their initial (diagnostic) visit at our institution, we cannot rule out that PD1 expression was influenced by prior or current therapies. Therefore, although PD1 contributes to the tumour microenvironment, the complexity of evaluating PD1 levels suggests that PD1 IHC is not an ideal stand-alone marker for CTCL disease progression or survival.

PD1 expression was high in six of seven patients with SS, similar to previous reports[19–21] and consistent with the possibility that PD1 may be a useful diagnostic marker to distinguish SS from erythrodermic MF. Statistical significance was not reached given the overall small number of patients with SS included in this study.

Despite a lack of association between PD1 and disease stage, there was a robust positive association between expression of PD-L1 and both disease stage and LCT, which is consistent with a prior report.[5] Upregulation of PD-L1 expression on mouse tumour cells has been shown to inhibit antitumour T-cell-mediated responses.[22] In many solid cancers, PD-L1 is highly expressed on tumour cells, antigen-presenting cells, activated T cells and/or other immune cells,[23] and is often associated with an unfavourable prognosis. However, we observed a lack of PD-L1 expression on CTCL cells, similar to our prior report.[8] Instead, PD-L1 staining was observed on

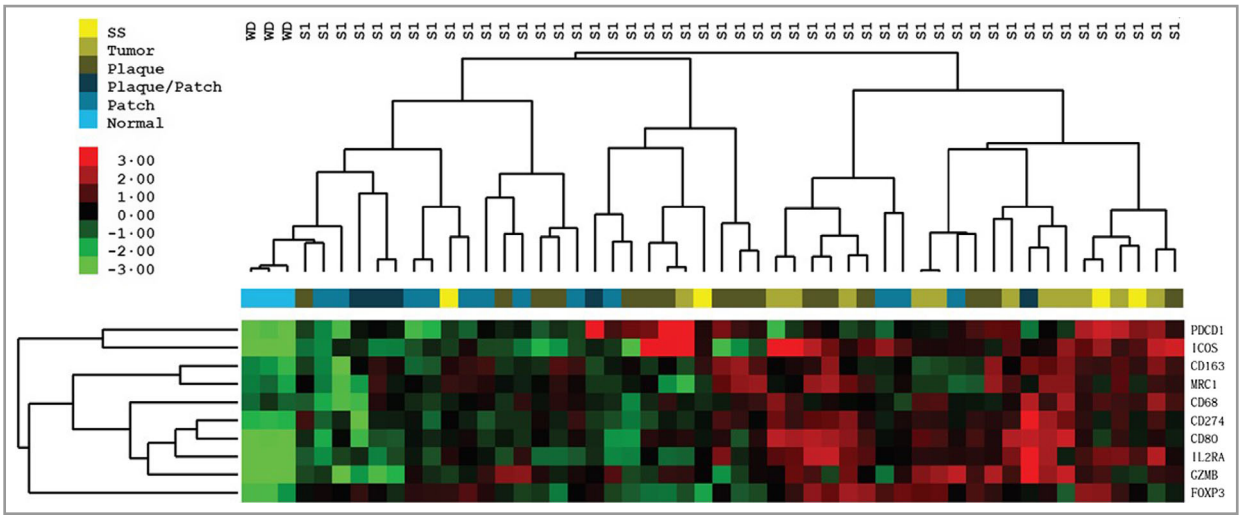

Figure 3 RNA expression of immune checkpoint markers by disease stage. Shown are the normalized RNA expression profiles for PD1 (*PDCD1*), PD-L1 (*CD274*) and *ICOS*, as well as markers of macrophages/histiocytes [*CD68*, *CD163*, mannose receptor C-type 1 (*MRC1*), *CD80*] and T-cell markers [*FOXP3*, CD25 (*IL2RA*), granzyme B (*GZMB*)]. ICOS, inducible T-cell co-stimulator; PD1, programmed death protein 1; PD-L1, programmed death ligand 1.

infiltrating histiocytes/macrophages. This result contrasts with one study, which evaluated the expression of PD-L1 in 26 cases of CTCL by IHC, and asserted that PD-L1 was frequently expressed on tumour and/or transformed CTCL cells.[5] However, others have observed PD-L1 expression predominantly in the tumour microenvironment of other lymphomas.[24,25] We note that some PD-L1-positive cells did not co-express with any of the markers used in this study and it is possible that a CD163-negative histiocyte/macrophage population, or another cell type in the tumour microenvironment, also expresses PD-L1. Further studies are needed to better define other cell populations that express PD-L1 in CTCL.

Little is known about PD-L1 signalling in macrophages and how these signals may affect the growth of CTCL cells.[26,27] In Hodgkin lymphoma and nodal B-cell lymphoma, PD-L1 may promote chemotherapy resistance and correlates with a poor prognosis.[28–30] Macrophages play a critical role in disease progression of various T-cell malignancies.[31,32] Of note, in CTCL, we have shown that PD-L1 is upregulated in macrophages by proinflammatory and immunosuppressive cytokines.[33] Thus, PD-L1-expressing macrophages may be a key element driving CTCL growth and progression and may mediate resistance to treatment by blunting an antitumour response.[34,35]

Similar to PD-L1, ICOS expression was increased in tumour lesions and in LCT. Also, ICOS appears to be strongly expressed by neoplastic $CD4^+$ T cells. Both of these findings are consistent with a prior report.[9] ICOS has been implicated in the regulation of effector T-cell differentiation and the induction and regulation of T-helper 2 immune responses,[36] as well as production of cytokines including interleukin (IL)-4 and IL-10.[37] ICOS is also a diagnostic marker for follicular T-helper cells in T-cell lymphomas, such as angioimmunoblastic T-cell lymphoma, and has also been observed in MF and formerly classified $CD4^+$ small/medium T-cell lymphoma.[38] Recent data suggest that targeting ICOS may be a promising immunotherapy for various lymphoma subtypes.[39] In addition, ICOS expression positively correlated with PD-L1 expression. One possible explanation is that both checkpoint markers are regulated by distinct cytokines or transcription factors involved in the PI3k–Akt pathway, which is aberrantly expressed in CTCL.[40]

A combined checkpoint marker expression score, which combined the scores for PD1, PD-L1 and ICOS, was significantly associated with disease stage, LCT and overall survival. Furthermore, there were no cases with LCT or advanced-stage disease that showed a low combined checkpoint marker score. This finding indicates that, given the complexity of the tumour microenvironment, combining the scores for multiple checkpoint markers may provide a better prognostic indicator than any single marker.

Although this observational study provides novel information about immune checkpoint marker status in CTCL, there are some limitations. Firstly, the number of samples for each disease stage was low, reducing the statistical power to detect associations. Interesting observations, such as the finding that PD1 expression was high in most SS samples, will need further evaluation in a larger cohort. Secondly, although biopsies were analysed from patients without active treatment, we cannot rule out that potential prior treatments had affected the immune checkpoint expression profile. In addition, there was limited information about prior treatment protocols and variability among the treatment approaches that were employed following our initial diagnostic biopsies and investigations, precluding analysis by treatment type. Thirdly, although a combined score predicted disease stage and survival better than expression of the individual markers, it is possible that

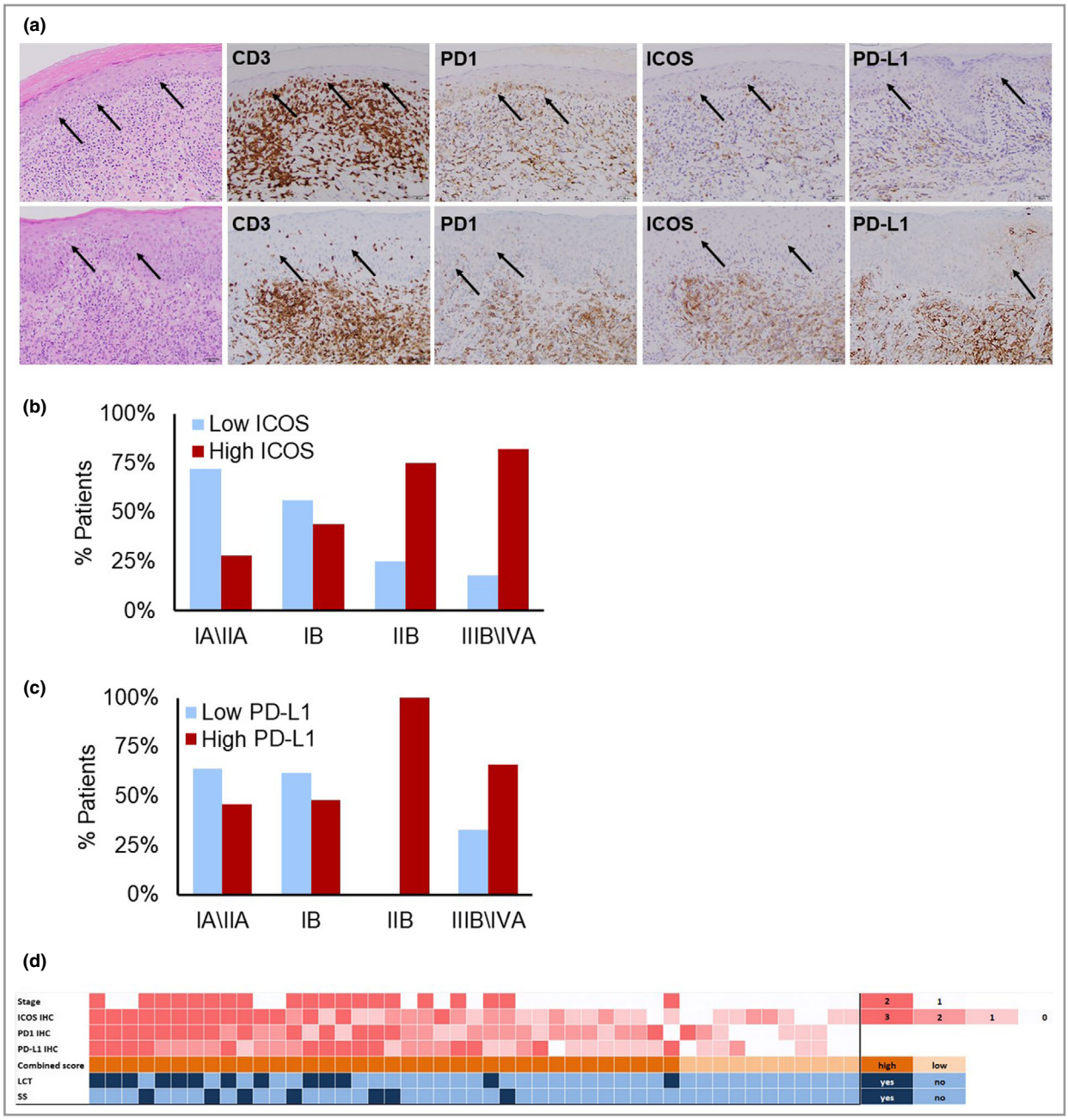

Figure 4 Expression of immune checkpoint markers by disease stage. (a) Representative histopathological features and CD3, PD1, ICOS and PD-L1 expression in a plaque lesion of early-stage MF (upper panel) with prominent epidermotropism (arrows) and with overall low expression of checkpoint markers, and a tumour lesion of advanced MF (lower panel) with mild epidermotropism (arrows) showing high expression of checkpoint markers in the dermal infiltrate. Notably, both upper and lower panels highlight epidermotropic lymphocytes expressing PD1 and ICOS, but not PD-L1 (arrows), which appears to be focally positive in epidermal histiocytes and keratinocytes (arrow) in the lower panel. Distribution of low (–/+) (blue) and high (++/+++) (red) expression of ICOS (b) and PD-L1 (c) is shown across MF/SS stages (IA–IVA). The heatmap (d) shows the protein expression scores of each immune checkpoint marker (0–3+) and a combined score for each lesional skin specimen (low = 0–3; high = 4+) aligned with patient's clinical stage and LCT status. Cases with Sézary syndrome are identified at the bottom of the heatmap. Early-stage MF (IA–IIA) = 1; advanced MF (IIB–IVA2) = 2. ICOS, inducible T-cell co-stimulator; LCT, large-cell transformation; PD1, programmed death protein 1; PD-L1, programmed death ligand 1.

using more markers will improve the predictive power of a combined checkpoint marker score. And while the entire skin infiltrate was assessed for global score and outcome analysis, sections were not compartmentalized for epidermotropic or dermal checkpoint expression profiles due to the variable epidermotropism noted.

In summary, our study found that high expression of immune checkpoint markers in MF/SS, including ICOS and

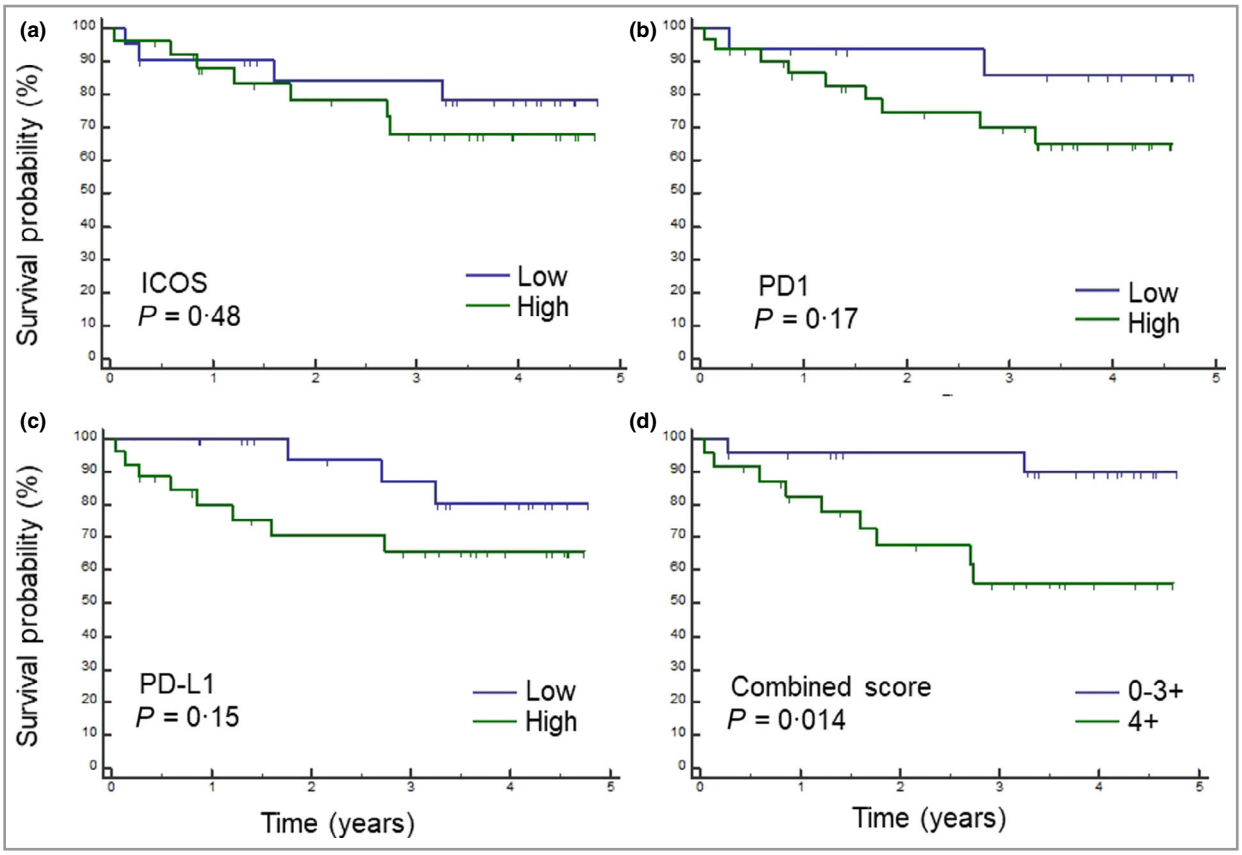

Figure 5 Overall survival stratified by immune checkpoint marker status. Kaplan–Meier analysis of overall survival by expression status of (a) ICOS; (b) PD1; (c) PD-L1 and (d) the combined expression score for ICOS, PD1 and PD-L1. Low expression (blue) represents an expression score of –/+ (a–c) or 0–3 (d), and high expression (green) represents an expression score of –/+ (a–c) or 4+ (d). ICOS, inducible T-cell co-stimulator; PD1, programmed death protein 1; PD-L1, programmed death ligand 1.

PD1 on T-cell subsets and PD-L1 on tumour-infiltrating macrophages/histiocytes, is associated with advanced CTCL and reduced overall survival. The immune checkpoint markers ICOS and PD-L1, as well as PD1, should be considered as complementary immunostains. This work also lays the foundation for future studies to evaluate subgroups and determine the response to checkpoint inhibitor therapies, especially combined therapies. Further investigation is needed to assess the value of combining PD-L1 and ICOS inhibition as a treatment strategy for MF/SS.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1 Supplementary methods.
Table S1 Relationship among immune checkpoint molecules and correlation with large-cell transformation.
Figure S1 Immune checkpoint co-expression in tumour lesion of mycosis fungoides.
Figure S2 Multispectral immunofluorescence for PD1 expression in skin sections of Sézary syndrome.
Figure S3 Overall survival for patients with Sézary syndrome vs. mycosis fungoides.