CD68-positive tumour associated macrophages, PD-L1 expression, and EBV latent infection in a high HIV-prevalent South African cohort of Hodgkin lymphoma patients

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MB ChB (UCT), FCP(SA), MMed (UCT)

Certificate in Clinical Haematology (Phys)

Thesis Presented for the Degree of

MASTER OF PHILOSOPHY

in the Department of Medicine, Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

March 2021

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DECLARATION: INCLUSION OF PUBLICATION

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CONTENTS

ACKNOWLEDGMENTS

The research reported in this thesis was made possible through funding support as follows: i) Discovery Health Academic Fellowship; ii) South African HIV Haematology training award funded by Fogarty International Center and NIH grant D43-TW010345; iii) Fogarty International Clinical Research Scholarship / Fellowship that was funded through NIH grant D43- TW010543 and iv) The Peter Jacobs Bursary Trust

This work for this project was the first research grant that I received and has led to me doing a Ph.D. in the field of lymphoma. I am grateful to Estelle Verburgh for encouraging me to apply for the Discovery Award that started this journey and for her support, encouragement and supervision throughout. I have learnt so much in the process.

CONTRIBUTIONS OF ALL CO-AUTHORS

I thank my co-authors for their contributions. Estelle Verburgh for the supervision, for conceptualising the study with me, and for review of the manuscript. Dharshnee Chetty for pathology review and reporting of the immunohistochemical stains. Zainab Mohammed for sharing her patient clinical records and for review of the manuscript. Jenna Oosthuizen for assisting me in performing the biostatistical analysis. And Lydia Van der Vyfer for assisting me with patient data collection.

Katherine Antel, 11 March 2021

ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
CD	Cluster of differentiation
CI	Confidence Interval
CT	Computed tomography
EBER-ish	EBV-encoded ribonucleic acid in sity hybridisation
EBV	Epstein-Barr virus
ECOG	Eastern European Cooperative Group
FFPE	Formalin-fixed paraffin-embedded
GSH	Groote Schuur Hospital
Hb	Haemoglobin
HIV	Human immunodeficiency virus
HL	Hodgkin Lymphoma
HR	Hazard ratio
HREC	Human research ethics committee
HRS	Hodgkin reed-sternberg cell
IHC	Immunohistochemistry
IPS	International prognostic score
IQR	Interquartile range
JAK-STAT	Janus kinase (JAK)-signal transducer and activator of transcription (STAT)
KM	Kaplan-meier
LDH	Lactate dehydrogenase
MTB	Mycobacterium tuberculosis
NHLS	National health laboratory service
OR	Odds Ratio
OS	Overall Survival
PD-L1	Programmed death-ligand 1
PET-CT	Positron Emission Tomography – Computed Tomography
PFS	Progression-free survival
PLHIV	People Living with HIV
Plt	Platelet count
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TAM	Tumour-associated macrophages
TME	Tumour microenvironment
UCT	University of Cape Town
WCC	White cell count

ABSTRACT

Background and aims

A higher proportion of CD68-positive tumour associated macrophages (TAMs) has been associated with poorer outcomes in HIV-negative patients with Hodgkin lymphoma (HL), but whether this is true in HIV-positive patients with HL is not known. In this study, we investigated the number of CD68-positive TAMs and expression of programmed cell death-ligand 1 (PD-L1) in lymph node specimens from HL patients and correlated expression with clinical features (HIV status, disease severity and survival) and histopathological features (EBV latent positivity and subtype of HL).

Methods

We stained archived lymph node specimens from 77 patients diagnosed with HL for CD68 and PD-L1. Stains were graded as: CD68 low (≤25%), CD68 high (>25%), PD-L1 low (≤50%), and PD-L1 high (>50%). Expression levels were correlated with the clinical and histopathological features using bivariate and multivariate analyses. Survival was analysed by overall and progression-free survival.

Results

Thirty-four of the 77 included patients (44%) were HIV-positive. EBV latency was detected in 97% of HIV-positive HL patients and in 14% of HIV-negative HL patients. A high CD68 score was associated with lower median haemoglobin levels (9.4 vs 11.4 g/dL; p=0.02), platelet numbers (262 vs 424 cells $\times 10^9$/L; p=0.01), and lymphocyte numbers (0.99 vs 1.70 cells $\times 10^9$/L, p=0.01) and a trend towards advanced disease (international prognostic score ≥4; hazard ratio 2.4; confidence interval 0.89–6.47; p=0.08). HIV status did not affect CD68 or PD-L1 expression. A higher proportion of CD68-positive TAMs was found in samples that were EBV-positive. HIV positivity and EBV negativity correlated with poorer survival. CD68 and PD-L1 expression were not predictive of survival.

Conclusions

High CD68 expression was associated with EBV positivity but not HIV positivity and did not predict adverse outcomes. PD-L1 expression was unaffected by HIV status or EBV positivity and did predict adverse outcomes.

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HAEMATOLOGY

CD68-positive tumour associated macrophages, PD-L1 expression, and EBV latent infection in a high HIV-prevalent South African cohort of Hodgkin lymphoma patients

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Summary

A higher proportion of CD68-positive tumour associated macrophages (TAMs) has been associated with poorer outcomes in HIV-negative patients with Hodgkin lymphoma (HL), but whether this is true in HIV-positive patients with HL is not known. In this study, we investigated the number of CD68-positive TAMs and expression of programmed cell death-ligand 1 (PD-L1) in lymph node specimens from HL patients and correlated expression with clinical features (HIV status, disease severity and survival) and histopathological features (EBV latent positivity and subtype of HL).

We stained archived lymph node specimens from 77 patients diagnosed with HL for CD68 and PD-L1. Stains were graded as: CD68 low (≤25%), CD68 high (>25%), PD-L1 low (≤50%), and PD-L1 high (>50%). Expression levels were correlated with the clinical and histopathological features using bivariate and multivariate analyses. Survival was analysed by overall and progression-free survival.

Thirty-four of the 77 included patients (44%) were HIV-positive. EBV latency was detected in 97% of HIV-positive HL patients and in 14% of HIV-negative HL patients. A high CD68 score was associated with lower median haemoglobin levels (9.4 vs 11.4 g/dL; p=0.02), platelet numbers (262 vs 424 cells $\times 10^9$/L; p=0.01), and lymphocyte numbers (0.99 vs 1.70 cells $\times 10^9$/L, p=0.01) and a trend towards advanced disease (international prognostic score ≥4; hazard ratio 2.4; confidence interval 0.89–6.47; p=0.08). HIV status did not affect CD68 or PD-L1 expression. A higher proportion of CD68-positive TAMs was found in samples that were EBV-positive. HIV positivity and EBV negativity correlated with poorer survival. CD68 and PD-L1 expression were not predictive of survival.

High CD68 expression was associated with EBV positivity but not HIV positivity and did not predict adverse outcomes. PD-L1 expression was unaffected by HIV status or EBV positivity and did predict adverse outcomes.

Key words: Hodgkin lymphoma; HIV; Epstein–Barr virus; South Africa; tumour associated macrophage; tumour microenvironment.

Received 29 June, revised 30 September, accepted 2 November 2020
Available online: xxx

INTRODUCTION

Classical Hodgkin lymphoma (HL) is a B-cell lymphoma. Due to immune system dysfunction, HIV-infected persons have a seven-fold increased risk of HL compared with age and gender matched persons from the general population, even in the era of antiretroviral therapy.[1] Differences in tumour histology and biology have been described in HIV-positive and HIV-negative HL patients, but studies in well resourced settings have shown no difference in outcome between these two patient groups.[2] However, in sub-Saharan Africa, survival outcomes are significantly poorer in HIV-positive HL patients.[3,4] In HIV-negative patients, current treatment strategies cure around 80% of patients.[5] In order to improve this, and particularly in the setting of relapsed/refractory disease, targeted biological therapies for HL have been approved. These therapies include the anti-CD30 antibody, brentuximab,[6,7] and programmed cell death-1 ligand (PD-L1) inhibitors.[8] The development and use of these novel agents have been guided by an understanding of the tumour biology and the tumour microenvironment (TME).

HL is unique in that it is characterised by a paucity of tumour cells. HL tumour cells are Hodgkin Reed–Sternberg (HRS) and Hodgkin mononuclear cells. These large cells arise from crippled B cells and occupy a small proportion (1–5%) of the overall tumour. They are enveloped by inflammatory cells (macrophages, CD4-positive and CD8-positive T cells, plasma cells, eosinophils, and other cells) comprising the TME.[9] The TME has been associated with treatment outcomes in HIV-negative HL patients; a high proportion of tumour associated macrophages (TAMs) resulted in treatment failure and poorer outcome,[10–14] although this was not supported by all studies.[15] There are

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2020.11.004

Please cite this article as: Antel K et al., CD68-positive tumour associated macrophages, PD-L1 expression, and EBV latent infection in a high HIV-prevalent South African cohort of Hodgkin lymphoma patients, Pathology, https://doi.org/10.1016/j.pathol.2020.11.004

variations in the tumour biology and TME of HIV-positive and HIV-negative HL patients. These include differences in proportions of mixed cellularity HL,[16] latent Epstein–Barr virus (EBV) infection in HRS cells,[17,18] CD4-positive and CD8-postive T cells (inverted CD4:CD8 ratio), and the pattern of T cells surrounding HRS cells.[19–21] One small study has shown similar proportions of CD68-positive TAMs in HIV-positive and HIV-negative HL patients,[22] but this study did not look at survival outcomes or correlate CD68 expression with EBV positivity.

TAMs and HRS cells express PD-L1, and this is a key mechanism by which HRS cells achieve immune evasion. PD-L1 binds to the PD-1 receptor (CD279) on the surface of antigen-experienced T cells and induces immune tolerance by suppressing T-cell activation. PD-1 inhibitors work by interrupting this interaction, thereby enhancing tumour cell recognition by T cells.[8,23] In HRS cells, increased PD-L1 expression is attributed to copy gains of chromosome 9p24.1, which includes the PD-L1, PD-L2, and JAK2 loci, and directly increases the level of PD-L1 and PD-L2 protein expression. An indirect increase in PD-L1 and PD-L2 protein expression is also achieved through augmented JAK-STAT signalling[24,25] which is likely the predominant mechanism by which EBV-positive HL induces PD-L1 expression.[26]

In this study, using archived lymph node tissue from patients with HL, we correlate the expression of CD68-positive TAMs and PD-L1 expression with histopathological factors (EBV latency and HL subtype) and clinical factors (HIV status, HL stage, and survival).

METHODS

Patients

Formalin-fixed, paraffin-embedded lymph node tissue specimens from 77 patients diagnosed with HL between 2004 and 2018 were obtained from the archives of the National Health Laboratory Service at Groote Schuur Hospital (GSH), Cape Town. GSH is one of two major tertiary referral hospitals in Cape Town and South Africa. Patients were included if there was sufficient tissue available for further immunohistochemical staining. Demographic and baseline clinical characteristics including HIV status, modified Lugano staging,[27] International Prognostic Score (IPS),[28] treatment details, and treatment outcome were extracted by retrospective chart review. The study was approved by the University of Cape Town and hospital ethics review boards (HREC 610/2016). Patient consent was waived in view of the retrospective nature of the study.

Study patients were treated by institutional protocol with ABVD chemotherapy regimen (doxorubicin, bleomycine, vinblastine, dacarbazine). For early stage disease (I and II), ABVD was given for two cycles followed by involved-site radiation therapy (IFRT) or up to six cycles for patients either at risk of long-term complications for radiation therapy or with unfavourable early stage disease (as defined by the German Hodgkin Study Group).[27] For advanced stage disease (III and IV), ABVD was given for six to eight cycles. Response to therapy was assessed after cycle two of ABVD, in most cases using computed tomography (CT), and where available positron emission tomography-CT (PET-CT). Salvage chemotherapy was given to patients with primary progressive or relapsed disease, followed by autologous transplantation in patients with responsive disease.

Morphology and immunohistochemical staining

Tissue sections of 3–4 μm thickness were cut from formalin-fixed, paraffin-embedded tissue blocks. Sections were stained with haematoxylin and eosin stain and visualised using light microscopy. The World Health Organization (WHO) 2017 Classification of Tumours of Haematopoietic and Lymphoid Tissues was used to classify the HL subtype.[29] Tissue sections, original diagnostic reports, and immunohistochemical staining results were reviewed by an expert anatomical pathologist (DC).

CD30 and CD15 staining had been undertaken as part of the routine clinical work-up. For this study, we performed new immunohistochemical stains for CD68 and PD-L1. If EBV-encoded small RNA (EBER) *in situ* hybridisation (EBER-ISH) had not been done during the routine clinical work-up, we tested latent membrane protein-1 (LMP1) expression to assess EBV positivity (because LMP-1 is cheaper than EBER-ISH and detects latent EBV with high sensitivity in HL.[30]) Three-micron sections were cut from the tissue blocks, placed onto silanised slides and heat fixed on a hotplate at 75°C for 30 min. Tissue sections were then dewaxed in xylene, cleared in ethanol, and rehydrated in water. All immunohistochemistry stains were performed with the Envision Detection System on a Dako Autostainer (Universal Staining System; Dako, Denmark) using routine staining protocols and the antibodies listed in Table 1. EBER-ISH was performed using the Ventana ISH iVIEW Blue Plus Detection Kit (Ventana Medical Systems, USA) on the BenchMark ULTRA IHC/ISH System (automated slide stainer).

CD68 and PD-L1 stains were graded only in areas containing tumour cells; areas with fibrosis, medium or large blood vessels, residual reactive lymph nodes, and necrosis or crush artefacts were excluded. The CD68 stain was graded as one (<5% of the TME is positive), two (5–25% of the TME is positive) and three (>25% of the TME is positive) as per Tan *et al.*[13] For regression modelling, categories one and two were combined into a single category ('CD68 low') and compared with category three ('CD68 high'). The PD-L1 stain was graded as low (<50%) or high (≥50%); there was no established method of quantification from the literature we identified. On original analysis the PD-L1 groups groups were: <5%, 5–10%, 11–25%, 25–50%, and >50%; however, there was no association between any of these categories and the variables analysed and the group was simplified with a 50% cut-off for grading the stain.

Statistical analysis

Categorical and continuous variables were summarised as frequencies and percentages or medians and interquartile ranges (IQRs), respectively. Univariate comparisons between categorical variables were made with the chi-squared test. Medians for non-parametric data were compared using the Wilcoxon rank-sum test, or the Kruskal–Wallis test for categorical variables with more than two groups.

For each patient, overall survival (OS) was calculated as the time between the date of diagnosis and the date of death or last follow-up for censored cases. Progression-free survival (PFS) was calculated as the time from the date of diagnosis until date of relapse, progression, or death from any cause. A multivariable Cox proportional hazards model was developed to assess the impact of variables on OS and PFS. Covariates in the Cox proportional

Table 1 Antibodies used

Antibody	Clone	Dilution (PBS)	Antigen retrieval	Control	Supplier
CD30	Ber-H2	1:400	Tris-EDTA	HL lymph node	Dako, Denmark
CD15	MMA	Ready to use	Tris-EDTA	HL lymph node	Roche, Switzerland
LMP-1	Cs.1-4	1:300	Tris-EDTA	Nasopharyngeal carcinoma	Dako, Denmark
CD68	PG-M1	1:50	Protease	Lymph node	Abcam, USA
PD-L1	B7–H1/CD274	1:300	Citric acid	Placenta	Sino Biological, USA

EDTA, ethylenediamine tetraacetic acid; HL, Hodgkin lymphoma.

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hazards model were: HIV status, age, IPS, HL stage, EBV positivity, CD68 expression, and PD-L1 expression. The Kaplan–Meier method was used to estimate survival curves, and differences between survival distributions were determined using the log-rank test. Two-sided p values less than 0.05 indicated statistical significance.

STATA v14 (StataCorp)[31] was used for all descriptive and quantitative analyses.

RESULTS

Clinical features

Of the 77 included patients, 44% were HIV-positive (n=34). HIV-positive participants were older than HIV-negative participants (median age 36 vs 27 years; p=0.04). In HIV-positive patients, the median number of CD4-positive cells was 194 cells/mm^3 (IQR 123–275). Twenty-seven HIV-positive patients (79%) were on antiretroviral therapy at the time of diagnosis, and 19 (56%) had a lower than detectable viral load. A high proportion of patients (56%) had stage IV disease, and 34% had advanced disease indicated by an IPS ≥4 (Table 2).

First-line treatment in all cases was ABVD chemotherapy with or without radiation according to the institutional protocol. Fifteen patients had relapsed or refractory disease of whom only three were HIV-positive. Ten of these 15 patients (nine HIV-negative, one HIV-positive patient) were fit enough to receive salvage chemotherapy with curative intent. Five of these 10 patients went on to have an autologous stem-cell transplant and they were HIV-negative (Table 2).

Histological features

The most frequent histological HL subtype was nodular sclerosing HL in HIV-positive and HIV-negative HL patients. However, the proportion of mixed cellularity HL was higher in HIV-positive HL patients (24% vs 4%, p<0.01). Positivity for EBV latent infection was found in 14% (6/43) of HIV-negative cases and in 97% (33/34) of HIV-positive cases (p<0.01).

The percentage of CD68-positive macrophages was <5% in 17% of patients (13/77), 5–25% in 47% of patients (36/77), and >25% in 28% of patients (36/77). The association of these categories with histological and clinical variables is shown in Table 3. When the groups were categorised as low (<25%) or high (>25%) in univariate analysis, high CD68 expression was more likely to be associated with EBV latent infection [hazard ratio (HR) 6.9; confidence interval (CI) 2.3–20.2; p<0.01], and HIV infection (HR 2.9; CI 1.1–7.5; p=0.03). There was a non-statistically significant association of high CD68 expression with the mixed cellularity HL subtype (HR 3.1; CI 0.8–12.5; p=0.1). In the multivariate model, CD68 expression was still associated with EBV positivity (HR 25; CI, 2.6–256; p=0.06), but the association with HIV infection and mixed cellularity was no longer

Table 2 Demographics and clinical data

Demographics	Total (n=77)	HIV-negative (n=43)	HIV-positive (n=34)	p
	No. (%)	No. (% of HIV-negative)	No. (% of HIV-positive)	
Male sex	38 (49)	21 (49)	17 (50)	0.92
Age, years, median (IQR)	31 (25–43)	27 (24–43)	36 (30–43)	0.03
<30	32 (42)	24 (56)	8 (24)	0.013
30–50	37 (48)	15 (35)	22 (65)	
>50	8 (10)	4 (9)	4 (12)	
Stage				
1	5 (6)	1 (2)	4 (12)	0.089
2	19 (25)	14 (33)	5 (15)	
3	7 (9)	5 (12)	2 (6)	
4	46 (60)	22 (52)	24 (71)	
Blood results, median (IQR)				
Haemoglobin, g/dL, n=77	10.8 (8.9–12.4)	10.8 (8.9–12.6)	10.6 (8.9–12.3)	0.45
Platelet, cells×10^9/L, n=73	398 (215–494)	460 (314–568)	270 (199–424)	0.005
Total white cell count, cells×10^9/L, n=77	9.9 (6.4–14.7)	13.7 (9.3–16.6)	6.5 (4.3–9.0)	<0.01
Lymphocyte count, cells×10^9/L, n=65	1.43 (0.8–2.2)	1.69 (1.4–2.6)	1.01 (0.7–1.9)	0.018
Lactate dehydrogenase, IU/L, n=72	470 (352–609)	477 (365–631)	437 (352–585)	0.5
ESR, mm/h, n=55	78 (39–122)	78 (32–107)	83 (46–127)	0.362
Albumin, g/L, n=74	37 (27–41)	37 (32–42)	36 (24–39)	0.1
IPS ≥4 (high risk)	26 (34)	14 (33)	12 (35)	0.8
EBV positive	39 (51)	6 (14)	33 (97)	<0.01
HL subtype				
Nodular sclerosing	56 (73)	38 (88)	18 (53)	0.003
Mixed cellularity	10 (13)	2 (5)	8 (24)	
Lymphocyte rich	0 (0)	0	0 (0)	
Lymphocyte depleted	2 (3)	1 (2)	1 (3)	
HL unspecified	9 (12)	2 (5)	7 (21)	
Treatment				
Primary treatment				
ABVD chemotherapy +/− radiation	75 (97)	43 (57)	32 (43)	0.32
Died before chemo	2 (3)	0 (0)	2 (100)	0.46
Secondary treatment				
Other therapy with curative intent (i.e., DHAP, IGEV or ICE)	10 (16)	9 (29)	1 (3)	0.04
Autologous stem-cell transplantation	5 (8)	5 (16)	0	0.08

Data are median (interquartile range) or n/N(%).

DHAP, dexamethasone, high dose ara-C, cisplatin; EBV, Epstein–Barr virus; ESR, erythrocyte sedimentation rate; HL, Hodgkin lymphoma; ICE, ifosfamide, carboplatin, etoposide; IGEV, ifosfamide, gemcitabine, vinorelbine; IPS, International Prognostic Score; IQR, interquartile range.

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Table 3 Clinical and histopathological correlations with CD68 and PD-L1 expression

	Total (%)	CD68 <5%	CD68 5–25%	CD68 >25%	*p*	PD-L1 low	PD-L1 high	*p*
HIV status								
Positive	34 (44)	2	15	17	0.02	15	19	0.54
Negative	43 (56)	11	21	11		22	21	
EBV latent status (LMP-1/EBER-ish)								
Positive	38 (49)	2	15	22	<0.00	21	17	0.21
Negative	39 (51)	11	21	6		16	23	
CD15								
CD15–	5 (6)	13	33	26	0.57	2	3	0.71
CD15+	72 (94)	0	3	2		35	37	
PD-L1								
Low	37 (12)	8	17	12	0.53			
High	40 (52)	5	19	16				
HL subtype								
Nodular sclerosing	56 (72)	10	29	17	0.2	26	30	0.88
Mixed cellularity	10 (13)	0	4	6		6	4	
Other	11 (14)	3	3	5		5	6	
IPS ≥4	26 (34)	2	10	14	0.05	10	16	0.23
Stage IV disease	45 (58)	7	19	20	0.19	18	27	0.09
Peripheral blood counts								
Haemoglobin, g/dL	10.8 (8.9–12.4)	10.8 (9.1–12.4)	11.7 (9.5–12.9)	9.4 (8.7–10.9)	0.057	10.8 (8.9–12.6)	10.5 (8.5–12.2)	0.39
Platelets, cells×10^9/L	398 (215–494)	425 (301–603)	424 (290–517)	262 (168–439)	0.032	424 (268–520)	315 (192–486)	0.13
Total white cell count, cells×10^9/L	9.8 (6.4–14.7	12.9 (8.2–15.7)	10.2 (7.1–15.4)	7.25 (4.23–11.4)	0.01	11.2 (7.0–14.9)	8.2 (5.3–13.8)	0.21
Lymphocytes, cells×10^9/L	1.4 (0.8–2.2)	1.7 (1.4–2.6)	1.4 (1.0–2.3)	1 (0.5–1.6)	0.020	1.8 (1.2–3.0)	1.4 (0.7–1.7)	0.08

Data are median (interquartile range) or n/N(%).
EBV, Epstein–Barr virus; HL, Hodgkin lymphoma; IPS, International Prognostic Score.

observed (HR 0.19; CI 0.13–2.68; p=0.15; and HR 1.7; CI 0.37–7.4; p=0.51, respectively). This signifies that HIV infection and the mixed cellularity HL subtype are not independently associated with increased CD68 expression, but rather are a consequence of EBV positivity. High CD68 expression was not associated with high PD-L1 expression (HR 1.38; CI 0.5–3.5; p=0.49).

Patients with a high CD68 score were more likely to show features of bone marrow suppression with a lower median haemoglobin (9.4 vs 11.4 g/dL; p=0.02), lower platelet count (262 vs 424 cells ×10^9/L, p=0.01), and lower lymphocyte count (0.99 vs 1.70 cells ×10^9/L, p=0.01). Patients with a high CD68 score also showed a trend towards more advanced disease (IPS ≥4), but this trend was not statistically significant (HR 2.4; CI 0.89–6.47; p=0.08).

PD-L1 expression was low in 48% (37/77) and high in 52% (40/77) of patients. High PD-L1 expression was not associated with HIV status (HR 1.32; CI 0.5–3.3; p=0.54), disease severity nor survival. In addition, high PD-L1 expression was not associated with EBV status (HR 1.77; CI 0.71–4.3; p=1.25), nor the mixed cellularity HL subtype (HR 1.74; CI 0.45–6.74; p=0.8).

Treatment and survival outcomes

After a median follow-up of 51 months (range 0.65–149 months), the OS at 2 and 5 years was 89% (95% CI 79–94) and 67.8% (95% CI 59–78), respectively; and the PFS at 2 and 5 years was 75% (95% CI 63–84) and 66% (95% CI 53–76), respectively. Univariate analysis showed that the following factors were not predictive for OS nor PFS: HIV status, age >45 years, stage four disease, EBV positivity, CD68 expression, and PD-L1 expression. An IPS ≥4 trended towards a poorer OS (HR 2.2; 95% CI 0.98–4.97; p=0.06) but this trend was not statistically significant. EBV-negative patients had a higher risk of relapse, with a lower PFS (HR 3.3; 95% CI 0.5–3.22; p=0.01). Kaplan–Meier curves show the effect of HIV status, EBV status, CD68 expression, and PD-L1 expression on OS (Fig. 1) and PFS (Fig. 2).

A multivariate model for OS and PFS is shown in Table 4. In the multivariate model, HIV positivity predicted statistically poorer OS and PFS (HR 7.5; CI 1.05–53.7; p=0.05; and HR 8.8; CI 1.26–61.67; p=0.03). EBV negativity also predicted lower OS and PFS (HR 12.5; CI 1.61–100; p=0.02; and HR 33.3; CI 3.8–100; $p \leq 0.01$). The remaining variables were not statistically significant, although the IPS again showed a trend towards poorer OS.

DISCUSSION

In this study, we investigated the expression of CD68 and PD-L1 in lymph node tissue of HL patients, and evaluated correlation of this with clinical and histopathological features. We revealed higher CD68 expression in EBV-positive HL. Neither CD68 nor PD-L1 expression impacted on survival. An interesting finding from the study was that EBV-positive patients had a better overall survival, and given the high rates of EBV positivity in HIV-positive HL this effect might mask the negative impact of HIV on survival.

Almost 100% of HIV-positive HL patients are EBV-positive, whereas approximately 40% of HIV-negative patients are EBV-positive. Regional variation and dissimilarity has also been reported among race groups.[30,32,33] PD-L1 is produced by HRS cells and macrophages. In HIV-negative HL patients, chromosome 9p24 amplification in HRS cells increases PD-L1 and PD-L2 expression.[24,25] In EBV-positive HL patients, PD-L1 expression is increased by direct effects of EBV. LMP-1 is an EBV protein that activates the NF-kB, JAK/STAT, and P12–K pathways, all of which recruit macrophages and increase PD-L1 expression, thereby

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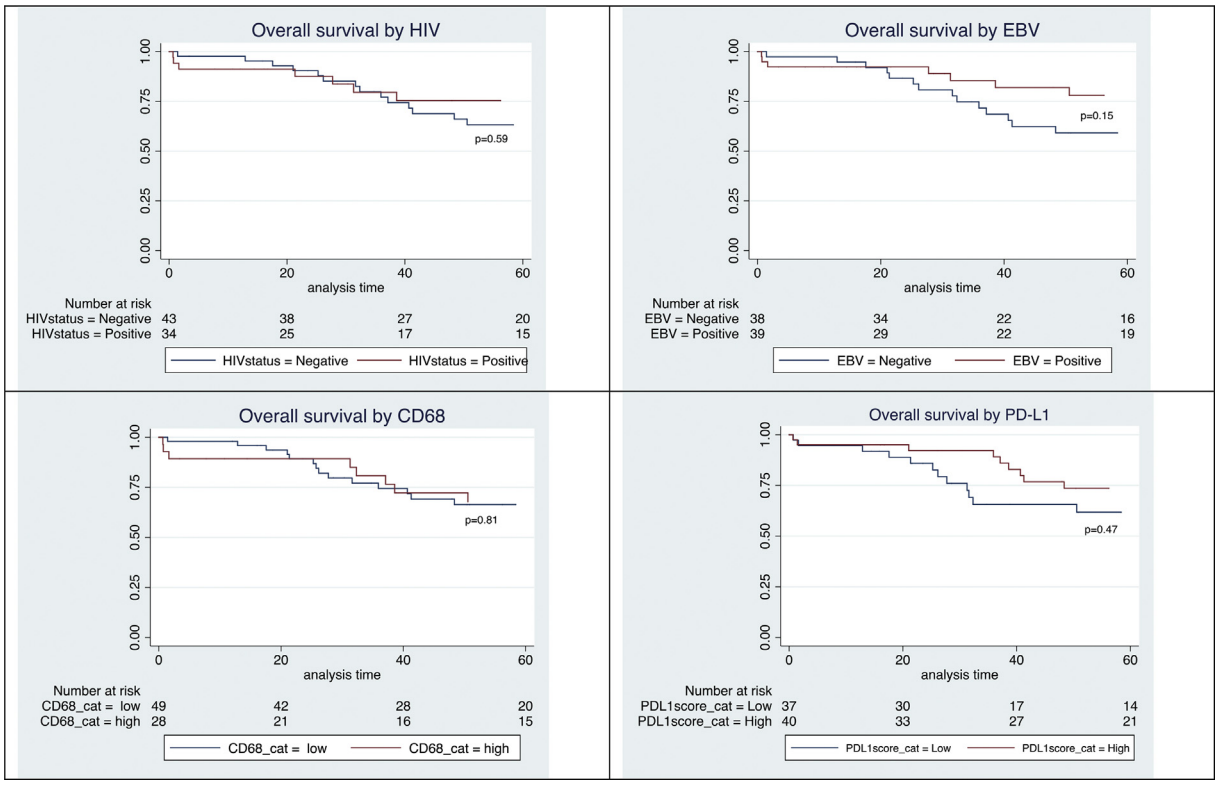

Fig. 1 (A–D) Kaplan–Meier curves for overall survival.

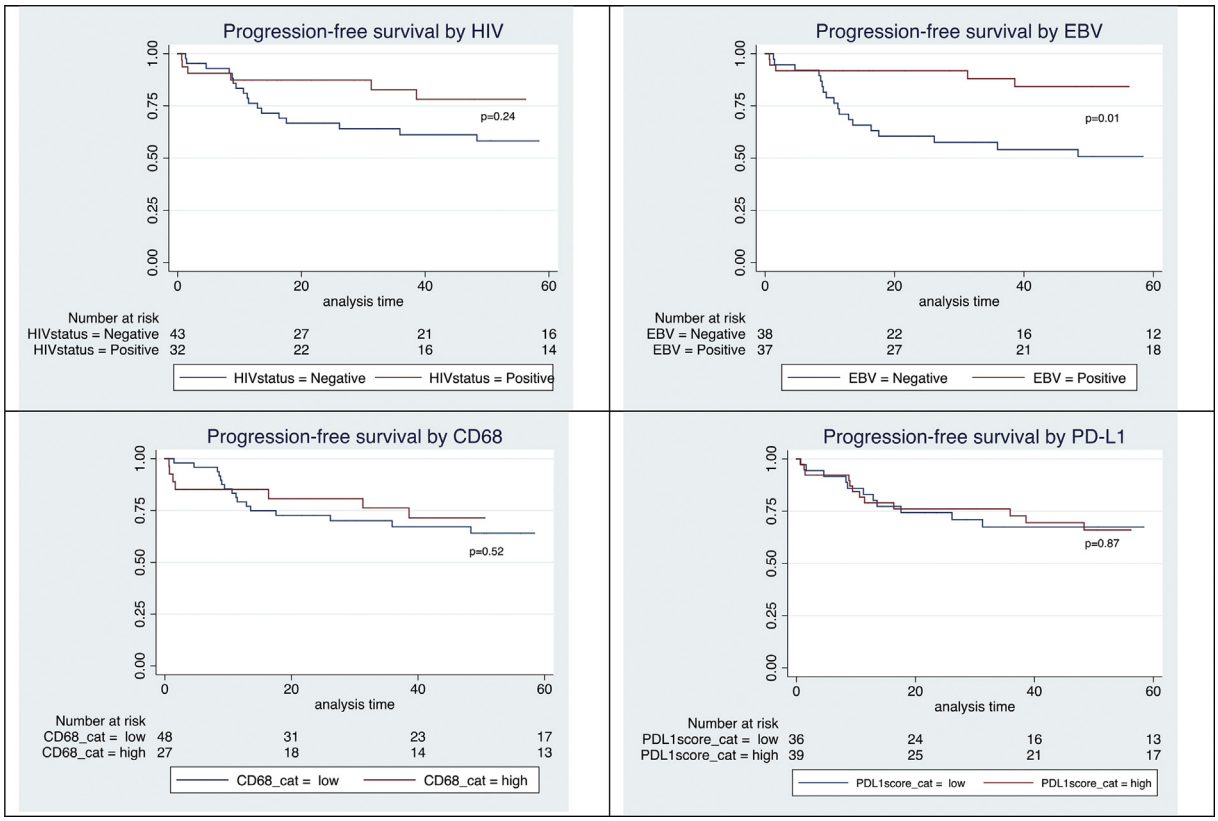

Fig. 2 (A–D) Kaplan–Meier curves for progression-free survival.

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Table 4 Multivariate Cox model for OS and PFS

Variable	HR for OS	*p*	95% CI	HR for PFS	*p*	95% CI
HIV-positive (vs negative)	7.50	0.05	1.05–53.70	8.80	0.03	1.26–61.67
Age >45 (vs ≤45)	1.81	0.24	0.67–4.83	0.76	0.68	0.21–2.78
IPS >4 (vs ≤4)	2.57	0.06	0.97–6.80	2.22	0.11	0.82–5.99
Stage 4 (vs stages 1–3)	1.20	0.73	0.44–3.27	1.59	0.39	0.56–4.50
EBV-negative (vs positive)	12.5	0.02	1.61–100.0	33.3	0.00	3.84–100.0
CD68 high (vs low)	1.40	0.45	0.58–3.39	1.29	0.62	0.48–3.45
PD-L1 high (vs low)	0.68	0.39	0.28–1.66	0.97	0.94	0.40–2.32

CI, confidence interval; EBV, Epstein–Barr virus; HR, hazard ratio; IPS, International Prognostic Score; OS, overall survival; PFS, progression-free survival.

evading T-cell immunity. Differences in the mutational status of chromosome 9 between EBV-positive and negative cases may partly explain why EBV-positive HL had improved survival, this needs further investigation.

PD-L1 is expressed predominately by TAMs and to a lesser degree by HRS.[34] In this study, PD-L1 expression did not correlate with the number of CD68-positive TAMs in the tumour samples, suggesting that CD68-positive TAMs are not the main producers of PD-L1 in EBV-positive HL. Further study of the PD-L1/PD-1 axis in EBV-positive HL is required, using immunohistochemical techniques to elucidate the topography of PD-L1 in relation to the surrounding cells.

The effect of EBV positivity on survival in HL patients is controversial, with some studies showing improved survival,[35–38] some showing poorer survival,[39–42] and others showing no difference.[43–47] EBV-positive HL is associated with extreme ages (<15 and >45 years), the mixed cellularity HL subtype, male sex, and HIV infection, which makes is difficult to accurately quantify the effect of EBV positivity on survival. Almost all HIV-positive HL patients are also EBV-positive, which means the CI in our multivariate model was wide (only a few included patients were HIV-positive and EBV-negative). Despite these limitations, the effect of EBV positivity on improved survival is interesting, and considering the high proportion of EBV latency in HIV-positive HL one does wonder if EBV positivity may have masked the negative impact on OS in other studies. In our study, the 17 HIV-negative patients with disease progression, relapse, or death were all EBV-negative.

Our study has several limitations. Firstly, it would have been preferable to use PET-CT to evaluate the response to therapy, but PET-CT was not consistently available. Secondly, it might have been more accurate to quantify the proportion of CD68-positive cells as a percentage initially before looking for correlations. Furthermore, quantification of immunohistochemical stains may have been improved by having more than one reviewing pathologist, and by using a computer assisted stereology system. Thirdly, we only included patients with sufficient lymph node tissue, which may have introduced selection bias because all patients with a primary diagnosis on other tissues (which may have represented a more aggressive type of HL) were excluded. For example, up to 39% of all HIV-positive patients in our local setting are diagnosed with HL based on analysis of bone marrow samples.[4]

CONCLUSION

In summary, a higher proportion of CD68-positive TAMs was seen in EBV-positive HL and correlated with disease severity but did not affect survival. PD-L1 expression was not affected by HIV and EBV status or by HL subtypes. Further research is needed to elucidate the relationship of different cells with the TME and to identify which cells in EBV-positive and HIV-positive HL predominantly produce PD-L1. The expression of PD-L1 in HIV-positive HL patients supports the use of PD-1 antibodies, but further clinical research is needed. Improved OS in EBV-positive patients with HL may help to explain why HIV-positive HL patients do not have a poorer outcome than HIV-negative counterparts in first world settings and from this research we advise that EBV latency is accounted for within a multivariate model when analysing outcomes in HIV-positive HL. However, the existing literature remains inconclusive and further study is warranted.

Conflicts of interest and sources of funding: The research reported in this publication was supported by the Fogarty International Center of the US National Institutes of Health (grant numbers: D43-TW010345 and D43-TW010543), the Discovery Foundation, and the Peter Jacobs Bursary Trust. EV was supported in part by the Thuthuka grant (TTK14052268878). The authors state that there are no conflicts of interest to disclose. The funders had no role in the design of the study; the collection, analysis, and interpretation of data; or writing the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Appendix 1

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The Editor
Pathology
The journal of the Royal College of Pathologists of Australasia (RCPA)

30 Sep. 20

Dear Prof. Delahunt

Thank you for sharing the reviewers' comments with me, I appreciate the feedback and I hope that my replies and requests for further information are sufficient. Please will you let me know if you need anything else. I've copied the reviews comment below, with my reply to the comment in blue text.

Reviewer comments and author replies:

1. The authors present a clinicopathological correlation study exploring the relationship between CD68 positive TAMs and PDL1 expression with EBV, HIV infection and clinical outcomes in HL. The manuscript is well written, clear and concise. The data is compelling and the limitations of the study are well articulated in the discussion. Thank you for this feedback.

2. The manuscript would benefit from a rationale for the CD68 and PDL-1 stain grading and if this strategy has been validated.

- The reference for the grading of CD68 has been included (line 171)
- For PD-L1 stain grading an explanation has been added to the methods section (line 173-176)

3. The clinical data is limited and the manuscript would benefit from augmenting this if possible. A table of patient characteristics would be useful.

- In table 2 I the patient characteristics, the data given includes: age, stage of disease, full blood count, LDH and the prognostic score (IPS). I am unlikely to be able to augment this further but there if there is something specific, I will be glad to try and add it in.

4. A description of the institutional policy regarding ABVD including cycle number, response assessment methodology and criteria for radiotherapy.

- Added to methods, under 'patients', line 148-156

5. It is somewhat unusual that 10/15 of the relapse/refractory patients received salvage chemotherapy and only 5 proceeded to autologous stem cell transplant with no HIV patients proceeding to transplantation. Particularly with such a young patient cohort. This would influence survival outcomes and the strategies or reasons behind these numbers should be explained.

Appendix 1

- Patients with HIV infection were excluded from transplant until very recently (2017), as per institutional protocol and now included in lines 148-156.
- The selection of patients for salvage chemotherapy also explained in those lines, unfortunately a number of them were either too unfit or were non-compliant and not considered eligible for salvage chemotherapy.

Thank you for your further consideration of our manuscript.

Yours sincerely

Signed by candidate

Katherine Antel