

## Serum CD163 and TARC as Disease Response Biomarkers in Classical Hodgkin Lymphoma

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### Abstract

**Purpose:** Candidate circulating disease response biomarkers for classical Hodgkin lymphoma (cHL) might arise from Hodgkin-Reed-Sternberg (HRS) cells or nonmalignant tumor-infiltrating cells. HRS cells are sparse within the diseased node, whereas benign CD163<sup>+</sup> M2 tissue-associated macrophages (TAM) are prominent. CD163<sup>+</sup> cells within the malignant node may be prognostic, but there is no data on serum CD163 (sCD163). The HRS-specific serum protein sTARC shows promise as a disease response biomarker. Tumor-specific and tumor-infiltrating circulating biomarkers have not been compared previously.

**Experimental Design:** We prospectively measured sCD163 and sTARC in 221 samples from 47 patients with Hodgkin lymphoma and 21 healthy participants. Blood was taken at five fixed time-points prior, during, and after first-line therapy. Results were compared with radiological assessment and plasma Epstein-Barr virus DNA (EBV-DNA). Potential sources of circulating CD163 were investigated, along with immunosuppressive properties of CD163.

**Results:** Pretherapy, both sCD163 and sTARC were markedly elevated compared with healthy and complete remission samples. sCD163 better reflected tumor burden during therapy, whereas sTARC had greater value upon completion of therapy. sCD163 correlated with plasma EBV-DNA, and associated with B symptoms, stage, and lymphopenia. Circulating CD163<sup>+</sup> monocytes were elevated in patients, indicating that sCD163 are likely derived from circulating and intratumoral cells. Depletion of cHL CD163<sup>+</sup> monocytes markedly enhanced T-cell proliferation, implicating monocytes and/or TAMs as potential novel targets for immunotherapeutic manipulation.

**Conclusion:** The combination of circulating tumor-infiltrate (sCD163) and tumor-specific (sTARC) proteins is more informative than either marker alone as disease response biomarkers in early and advanced disease during first-line therapy for cHL. *Clin Cancer Res*; 19(3); 731–42. ©2012 AACR.

### Introduction

Classical Hodgkin lymphoma (cHL) is associated with high cure rates (1). Those with a rapid response to initial treatment have the best outcomes, and may benefit from truncated treatment regimens. Paradoxically, there remains a significant minority with refractory disease in whom prolonged exposure to first-line agents induce chemo-resistance and unnecessary toxicity. The challenge remains to

tailor treatments to eradicate malignancy with minimal side-effects, and to simultaneously identify those patients in whom alternate strategies should be instituted early.

Radiological imaging modalities such as computed tomography (CT) or positron emission tomography (PET) scans are typically used to assess treatment efficacy. Although combined functional-anatomical imaging modality (PET/CT) has a high negative predictive value, its positive predictive value is more modest (2, 3). Outside of the context of a clinical trial, PET/CT scanning is typically available only before and at the end of, but not during, therapy. Unlike blood tests, it is impractical to conduct PET/CT before each follow-up visit (4). Furthermore, PET/CT interpretation can be confounded by concurrent inflammation or infection, and influenced by the timing of prior therapy.

Ideal biomarkers must be specific, sensitive, easy to assay, and interpret, with rapid turnaround, high reproducibility, and comparability between laboratories. Serum protein estimation by ELISA exploits an established technology used in the diagnostic laboratory. Furthermore, while tissue markers could be applied routinely to the diagnostic biopsy

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-12-2693

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### Translational Relevance

The challenge in treating classical Hodgkin lymphoma (cHL) remains to tailor therapies to eradicate malignancy with minimal side-effects, and to simultaneously identify those patients in whom alternate strategies should be instituted early. There are currently no circulating disease response biomarkers for Hodgkin lymphoma. Blood biomarkers may assist interpretation of positron emission tomography/computed tomography, and can also be conducted at each consultation to assess disease response and detection of early relapse. We perform the first comparison of HRS-specific (TARC) and tumor-infiltrate-specific (CD163) serum markers in cHL and show that both serum CD163 and serum TARC by ELISA are cHL disease response biomarkers in early- and advanced-stage disease. Furthermore, functional analysis suggests that CD163<sup>+</sup> monocyte-mediated immunosuppression of T-cells occurs in cHL, providing potential targets for novel therapeutics.

to provide prognostic information, they have no applicability in monitoring disease response. In contrast, blood-based biomarkers have potential to provide information on disease response and detection of early relapse. It is known that indirect blood markers of biologic activity, such as erythrocyte sedimentation rate, lymphocyte count, hemoglobin, and albumin have prognostic value at baseline. However, once therapy has commenced, their lack of specificity precludes their clinical value as a measure of disease response. A validated circulating measure of tumor burden, that is informative in the majority of patients, and accurately reflects disease response during first-line therapy would be of great benefit.

In cHL, the malignant Hodgkin–Reed–Sternberg (HRS) cells account for a minority of the diseased nodal cell population. The affected node is primarily composed of nonneoplastic, tumor-infiltrating cells which are important in pathogenesis (5). It is yet to be determined whether disease response in patients with cHL will be best reflected by HRS-specific or tumor-infiltrate specific biomarkers. Biomarkers that originate from HRS cells hold the promise of high specificity, whereas those that reflect the benign tumor-infiltrate may be more sensitive. The relative kinetics of circulating tumor-specific versus tumor-infiltrating biomarkers during therapy have not been compared.

The cysteine-cysteine thymus and activation related chemokine (TARC, CCL17) is elevated in HRS cell-lines, primary cHL tissues, and pretherapy cHL patient serum (irrespective of EBV-tissue status) and holds promise as a tumor-specific disease response marker in cHL (6–11). A recent study of 60 cHL patients with serial samples throughout therapy found that sTARC reflected disease response in both early- and advanced-stage cHL and in relapsed disease (11).

cHL nodes are enriched with anti-inflammatory M2 macrophages. CD163 is an M2 macrophage marker that is

also expressed in a subset of monocytes, and is highly expressed within tumor-infiltrating cells of the malignant node. Tissue levels of CD163 within the cHL node may be associated with survival, but results are conflicting (12–18). A study of serum CD163 (sCD163) in patients with stage I/II melanoma found that pretherapy sCD163 was an independent predictor of survival (19). To our knowledge, no study of serum CD163 (sCD163) in Hodgkin lymphoma has been conducted.

We prospectively evaluated sCD163 and sTARC in patients with Hodgkin lymphoma (43 cHL and 4 nodular lymphocyte predominant: NLPHL) at 5 fixed time-points: pretherapy, during (immediately before the second and the third cycles), one month, and 6 months after completion of therapy. Results were compared with disease response as determined by radiological assessment. In patients with EBV-related cHL, plasma cell-free EBV-DNA was quantified. cHL tissue CD163 immunohistochemistry was correlated to matched serum samples. Potential sources of circulating CD163 were investigated, along with the immunosuppressive properties of CD163 on global T-cell proliferation.

### Materials and Methods

#### Patients

Forty-seven newly diagnosed patients with Hodgkin lymphoma were prospectively enrolled. One patient with atopic dermatitis was excluded from analysis and will be discussed separately (excluded from Table 1). Of the remaining 46 patients, 42 had cHL and 4 were NLPHL patients. Patient characteristics are provided in Table 1. Patients with NLPHL and cHL were analyzed separately. Exclusion criteria were limited to HIV positivity, active Hepatitis B, or C infection. All patients were enrolled before commencement of therapy and serial blood samples were taken at 5 fixed time-points: pretherapy, immediately presecond, and prethird therapy, one month and 6 months after therapy. Peripheral blood mononuclear cells (PBMC), plasma, and sera were cryopreserved, thawed, and tested in batches as previously outlined (20). Tissue from diagnostic tumor biopsies was tested when available. Clinical parameters including the Hasenclever prognostic score were prospectively recorded (21, 22). Early-stage disease was defined according to the Southwest Oncology Group (SWOG) and Cancer and Leukemia Group B (CALGB) previously published definition (Ann Arbor stage I or II without any B symptoms, infradiaphragmatic presentations, or mediastinal masses more than one third the maximum thoracic diameter; ref. 23).

This was a multi-center, Australia-wide, observational (non-interventional) study conducted under the auspices of the Australasian Leukaemia & Lymphoma Group (ALLG). Therapy was applied as per clinician's preference (Table 1). The majority of our patient cohort (85%) was treated with "ABVD" (adriamycin, bleomycin, vinblastine, and dacarbazine) combination chemotherapy (24). Other regimens used were "BEACOPP" (bleomycin, etoposide, adriamycin, cyclophosphamide, procarbazine and prednisolone; ref. 25), ABVD followed by BEACOPP and

**Table 1.** Patient characteristics

Patient characteristics	No. of Patients (N = 46 <sup>a</sup> )	% Total
Age median (range)	35.6 (18–79)	
Male	25	54%
Ann Arbor Stage III–IV	22	48%
Serum Albumin, less than 40 g/L	32	70%
Haemoglobin, less than 105 g/L	9	20%
Lymphocyte Count, at least $0.6 \times 10^9/L$	42	91%
White blood cell count, at least $15 \times 10^9/L$	7	15%
Hasenclever score, at least 3	16	35%
B symptoms	17	37%
EBER-ISH Positive	12	26%
EBER-ISH Unavailable	3	7%
<sup>b</sup> Early stage	10	22%
Advanced stage	36	78%
Histologic subtype		
Nodular sclerosing	26	57%
Mixed-cellularity	4	9%
Lymphocyte-rich	4	9%
Lymphocyte depleted	1	2%
cHL, unspecified	7	15%
NLPHL	4	9%
Treatment	cHL Patients (N = 42 <sup>a</sup> )	
ABVD	36	85%
BEACOPP	2	5%
ABVD + BEACOPP	2	5%
ChIVPP	2	5%

<sup>a</sup>Excluded patient with atopic dermatitis.

<sup>b</sup>Early stage is defined Ann Arbor stage I or II without any B symptoms, infradiaphragmatic presentations or mediastinal masses greater than one third the maximum thoracic diameter. Patients not fulfilling this criteria were defined as advance stage.

'ChIVPP' (chlorambucil, procarbazine, prednisolone, and vinblastine; 26).

Initial staging and restaging one month after completion of therapy was by PET and CT scans. Interim disease response (generally after the third cycle of therapy), and restaging at the 3 to 6 months after completion of therapy was assessed by CT, typically in combination with PET (70% had interim treatment restaging PET). Complete response (CR) and partial response (PR) were defined as per the International Harmonization response criteria (27) or, when applicable (i.e., patients that had interim treatment CT scans only), the International Working Group response criteria was used (28). Refractory disease was defined as

patients with persistent Hodgkin lymphoma at one month posttherapy, which was either residual disease (if there was stable or reduced tumor burden as compared with pretherapy), or progressive disease (if there was an increase in tumor burden). Relapse was defined as Hodgkin lymphoma reappearing in a patient who had previously been in CR at the interim therapy or one month posttherapy time-point. Relapsed/refractory disease was based on clinical and radiological assessment and in some cases by repeat biopsy.

Twenty-one healthy age- and gender-matched participant blood samples were used. This study conformed to the Declaration of Helsinki and written informed consent was provided by all participants and was approved by all participating hospitals/research institute Human Research Ethics Committees.

#### Serum ELISA analysis

CD163 and TARC were quantified in 221 serum samples using Quantikine Human CD163 ELISA kits (R&D Systems) and RayBio Human TARC ELISA kits (RayBiotech) as per manufacturer's instructions. A 1:20 dilution was used for sCD163.

#### Tissue mRNA quantification

RNA was extracted from all available formalin-fixed, paraffin-embedded (FFPE) tumor biopsies (36 Hodgkin lymphoma in total: 32 cHL and 4 NLPHL; 29 of the 32 cHL had paired slides for immunohistochemical staining available) using RecoverAll kit (Ambion) as per manufacturer's instructions. RNA was stored at  $-80^{\circ}C$ . Superscript III reverse transcriptase (Invitrogen) was used to produce cDNA primed by random hexamer as per manufactures instructions. TARC, CD163, CD68 and  $\beta$ -2-microglobulin ( $\beta$ 2M) mRNA levels were quantified using real-time PCR (TARC forward primer: 5'-CTTCTCTGCAGCACATCCAC-3', TARC reverse primer: 5'-AGTACTCCAGGCAG-CAC-TCC-3', CD68 forward primer: 5'-CCACACAGGGGT-CTTTGG-3', CD68 reverse primer: 5'-TGATGAGAGGCAG-CAAGATG-3', CD163 forward primer: 5'-CAATGGGG-TGGACTTACCTG-3', CD163 reverse primer: 5'-AAC-CAGCTCTGGGTTCCCTGT-3',  $\beta$ 2M forward primer: 5'-ACTCTCTTTCTGGCCTGGAG-3',  $\beta$ 2M reverse primer: 5'-CATTCTCTGCTGGATGACGTGAG-3'). All primers were synthesized by Sigma-Aldrich and used at 0.2  $\mu$ mol/L. Rotorgene 3000 real-time PCR machine (Corbett Research) was used to run 20  $\mu$ L reactions with A&B SYBR Green (Applied Biosystems). Results were normalized to  $\beta$ 2M and comparative quantification was used for analysis. Healthy participant PBMC cDNA was used as the standard control for CD68 and CD163 and the Hodgkin lymphoma cell line HDML2 cDNA was used for TARC.

#### Circulating plasma EBV-DNA quantification

EBV-tissue positivity was determined by EBV encoded RNA *in situ* hybridization (EBER-ISH) in conjunction with hematoxylin and eosin staining (29). EBV-DNA was quantified in plasma of EBV-related patients with cHL by real-time PCR using primers for the single copy EBV gene BALF5

as previously described (20). A threshold of 200 EBV genomes/mL was used.

### Immunohistochemistry

CD163 (mouse anti-human CD163 clone: 10D6, 10 mg/ml, Biocare Medical) staining was conducted on 36 Hodgkin lymphoma tumor biopsy FFPE Superfrost Plus Adhesive slides (32 cHL, 4 NLPHL; 29/32 cHL had paired FFPE tumor slices for RNA extraction available). CD163 positivity was scored by 2 independent hematopathologists (C. Keane and L.A. Seymour) using a standardized protocol and the mean value recorded. Samples were delineated as high or low based on a previously published cutoff of 25% (16–18). In addition, dual staining of CD163 and CD68 (mouse anti-human CD68 clone: PG-M, 40 µg/mL, DAKO) was done on 12 samples to confirm CD163 specificity. The number of HRS cells per 5 fields at ×40 magnification (hematoxylin and eosin staining) was quantified in the 32 available cHL slides, and tested for correlation with sTARC.

### CD163 flow cytometry

CD163<sup>+</sup>CD14<sup>+</sup> monocytes and monocytic myeloid-derived suppressor cells (mo-MDSC, defined as CD14<sup>+</sup>HLA-DR<sup>low/-</sup>) were quantified in PBMC from 10 healthy participant and 10 cHL patient at 2 time-points: pretherapy and 6 months posttherapy. Briefly, PBMCs were surface stained for CD14-PE (Miltenyi), CD163-Alexa-647 (Biolegend), HLA-DR-PerCP (BD Biosciences), and intracellular stained for CD68-FTIC (Invitrogen). Samples were run on FACSCanto II (BD Biosciences) and analyzed using FlowJo version 9.2 (Treestar).

### Effect of monocyte depletion on global T-cell proliferation

Eight pre-therapy cHL patient (3 nodular sclerosing, 1 mixed cellularity, 1 lymphocyte rich, and 3 cHL unspecified) and 6 healthy participant samples were randomly selected from our cohorts. PBMC were carboxyfluorescein succinimidyl ester (CFSE) stained (1.5 µmol/L), washed, and then divided into 2. One half was depleted of CD14<sup>+</sup> cells using Easysep Human CD14 positive selection kit (Stem Cell Technologies), as per manufacturer's instructions. A total of  $2.5 \times 10^5$  cells from both the depleted and nondepleted populations were surface stained for CD14-PerCP-Cy5.5 (BD Pharmingen) and CD163-Alexa-647 (Biolegend) and the proportions of CD163<sup>+</sup>CD14<sup>+</sup> cells determined by flow cytometry.

To account for a higher proportion of T-cells in the depleted population, depleted and nondepleted cells were cultured at a concentration of  $2.0 \times 10^6$  cells/mL and  $2.5 \times 10^6$  cells/mL, respectively. Both populations were stimulated with Dynabeads Human T-activator CD3/CD28/CD137 (1:10, Invitrogen) and expanded for 7 days in RPMI 1640 with penicillin/streptomycin, 10% FBS and 20 U/mL interleukin (IL)-2. On day 7, cells were surface stained for CD3-APC-Cy7, CD4-APC, and CD8-PerCP (BD Pharmingen). Samples were run on FACSCanto II (BD Biosciences) and analyzed using FlowJo version 9.2 (Treestar).

### Statistical analysis

Paired *t* tests were used to compare matched serum samples. Wilcoxon-matched pairs tests were used to compare matched monocyte-depleted and nondepleted T-cell expansion samples. Otherwise, the unpaired *t* test was used and Welch correction was included when variances were significantly different. Correlations were determined using the Pearson test with the exception of plasma EBV-DNA (which had non-Gaussian distribution), in which the Spearman test was used. Receiver operating curve (ROC) analysis was used to determine the sensitivity and specificity of sCD163 and sTARC. Statistical analysis was conducted using GraphPad Prism 5.0 (GraphPad Software Inc).

## Results

### Patient characteristics

Forty-seven patients with Hodgkin lymphoma were accrued (mean age: 36 years, range: 18–79; female:male ratio, 21:26; 42 cHL, 4 NLPHL, one cHL with atopic dermatitis). Posttherapy samples were available for 37 of 42 patients with cHL. Of these 37 patients with cHL, 32 remained in CR by the 6-month posttherapy time-point, whereas 5 had relapsed/refractory disease. Two of 5 had biopsy proven relapsed/refractory disease, whereas 3 were confirmed by clinical and radiological assessment. In addition, 21 healthy participant blood samples were used (mean age: 36 years; range: 22–68; female:male ratio, 9:12).

### Specificity and sensitivity of sCD163 and sTARC as biomarkers in cHL

First, we conducted ROC analysis to determine the sensitivity and specificity of sCD163 and sTARC at defined time-points. We found that both sCD163 and sTARC are highly sensitive and specific markers for delineating pretherapy cHL from healthy participants [sCD163: AUC, 0.8776;  $P < 0.0001$ ; 95% confidence interval (95% CI), 0.7877–0.9674; sTARC: AUC, 0.9138,  $P < 0.0001$ ; 95% CI, 0.8440–0.9837; Supplementary Fig. S1A and S1C). Pretherapy sCD163 and sTARC cHL samples were also highly sensitive and specific when compared with CR samples at 6 months posttherapy (sCD163: AUC, 0.7333;  $P = 0.001$ ; 95% CI, 0.6072–0.8594; TARC: AUC, 0.8793,  $P < 0.0001$ ; 95% CI, 0.7492–0.9613; Supplementary Fig. S1B and S1D). Cutoff levels were defined by 2 criteria; a value greater than the healthy participant mean plus one SD and a value with more than 80% sensitivity and specificity from the ROC analysis of pretherapy versus healthy participant values. A cutoff level of 500 ng/mL for sCD163 resulted in 86% sensitivity and 81% specificity at pretherapy and 62% sensitivity and 75% specificity at 6 months posttherapy. For sTARC, the cutoff value was defined as 300 pg/mL with 90% sensitivity and 86% specificity at pretherapy and 70% sensitivity and 84% specificity at 6 months posttherapy.

### sCD163 and sTARC as disease response biomarkers in cHL

Second, we conducted a match-paired analysis of sCD163 and sTARC at all time-points in all patients with

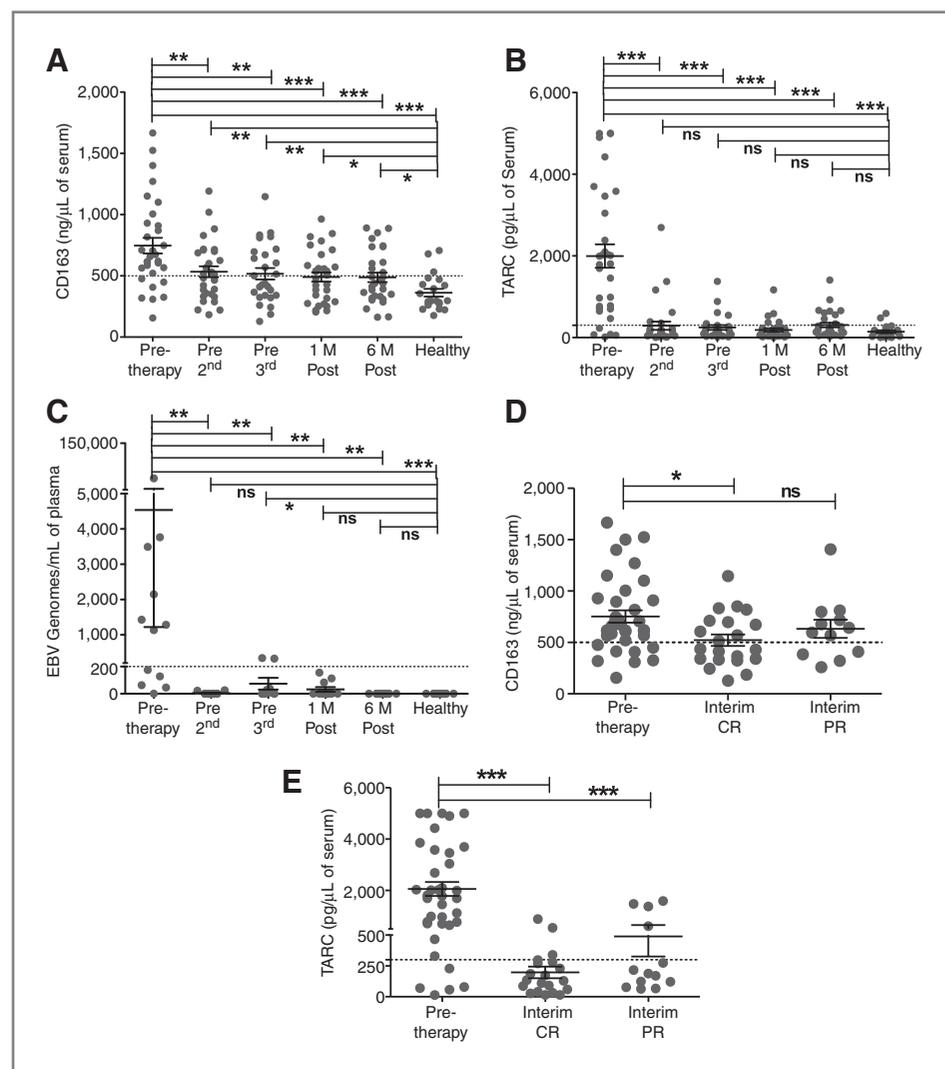
cHL attaining CR and remaining in CR at the 6-month posttherapy time-point. Results were compared with healthy control participants. Both sCD163 and sTARC were elevated in pretherapy cHL patients compared with healthy participants (sCD163  $P < 0.0001$  and sTARC  $P < 0.0001$ , Fig. 1A and B). In addition, both sCD163 and sTARC were significantly elevated in pre-therapy cHL patients compared with paired samples taken one month posttherapy (sCD163  $P = 0.0008$ , sTARC  $P < 0.0001$ ) and 6-month posttherapy (sCD163  $P = 0.0005$ , sTARC  $P < 0.0001$ ). Levels of sTARC was equivalent to healthy participants by presecond therapy, whereas levels of sCD163 approached but did not reach equivalence by 6 months posttherapy. These results show that sCD163 and sTARC are disease response biomarkers in patients with cHL attaining and remaining in CR.

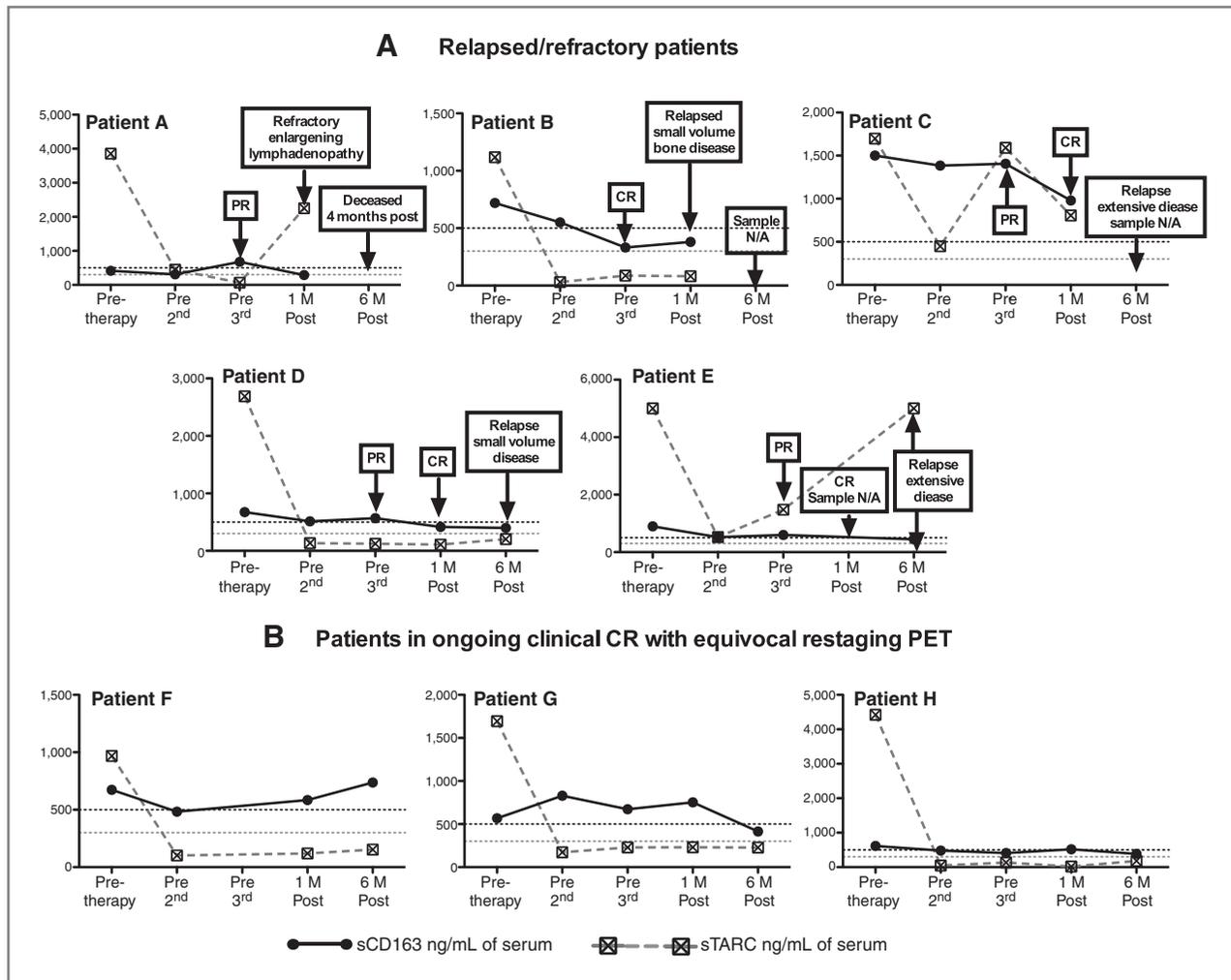
Next, we analyzed patients with relapsed/refractory disease. One patient had biopsy proven refractory disease at one month posttherapy (Fig. 2A), another patient who had resolution of disease at the interim time-point relapsed

(confirmed by biopsy) at one month posttherapy, (Fig. 2B) and 3 patients had clinical- and radiological-confirmed relapse after one month post-therapy (Fig. 2C–E). The time course of sCD163 and sTARC are shown in Fig. 2, in association with the tumor burden. In 3 patients, sTARC was well above threshold at time of refractory/relapsed disease (Fig. 2A, C, and E). In one of these cases (Fig. 2C), both sCD163 and sTARC were elevated, whereas in 2 of these cases (Fig. 2A and E) sCD163 was not (in the case shown in Fig. 2A, sCD163 was not elevated pretherapy). In the remaining other 2 cases (Fig. 2B and D), both serum biomarkers were elevated at pretherapy, but neither were elevated at time of diagnosis of low volume relapsed/refractory disease.

We then analyzed whether sCD163 and/or sTARC were useful in the setting of equivocal PET scans (i.e., low-grade avidity of uncertain clinical significance). Out of our cHL cohort, 4 patients had persistent clinical CR despite an equivocal restaging PET result. We will discuss

**Figure 1.** sCD163 and sTARC as biomarkers in cHL. A–C, comparison of sCD163, sTARC, and plasma EBV-DNA levels in cHL patients with CR by one month posttherapy and who remained in CR at 6 months posttherapy. sCD163 levels (A), sTARC levels (B), plasma EBV-DNA levels (C). Comparison of interim-therapy treatment response. cHL patients, restricted to those with paired interim radiological assessment. D, sCD163 levels show a significant difference between paired pretherapy and CR interim-therapy ( $P = 0.0152$ ), whereas no significant difference was seen between paired pretherapy and PR interim-therapy ( $P = NS$ ). E, sTARC levels show a significant difference between paired pretherapy versus CR interim-therapy ( $P = 0.0001$ ) and paired pretherapy versus PR interim-therapy ( $P = 0.0001$ ). Error bars represent mean with SEM. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; NS,  $P > 0.05$ .





**Figure 2.** Kinetics of serum CD163 and TARC in relapsed/refractory cHL and in patients in ongoing CR with equivocal restaging PET. The dashed gray lines represent sTARC and the solid black lines sCD163. The dotted gray and black lines are the sTARC and sCD163 thresholds, respectively. The arrows represent the timing of the radiological assessment. Limited small volume disease was defined as disease confined to a single site and 2 cm or less. Patient A was a 31-year-old female with EBV nonrelated nodular sclerosing cHL stage IIIA and a Hasenclever score of 3, with refractory disease at one month following completion of ABVD. sTARC but not sCD163 was elevated at the one month posttherapy time-point. The patient died of progressive disease at 4 months posttherapy. Patient B was a 22-year-old female with EBV nonrelated nodular sclerosing cHL stage IVB and a Hasenclever score of 3, with resolution of disease by interim PET/CT prior to third cycle of BEACOPP. One month following completion of BEACOPP limited small volume bony disease was present by PET/CT. Neither sCD163 nor sTARC were above threshold at the one-month posttherapy time-point. The patient went on to have salvage chemotherapy, and no further research blood samples were obtained. Patient C was a 54-year-old male with EBV nonrelated nodular sclerosing cHL stage IVB with a Hasenclever score of 5 who entered CR at one month following completion of ABVD, but had a PET/CT confirmed relapse at 5 months posttherapy. Both sCD163 and sTARC were elevated at one month posttherapy. Patient D was a 27-year-old male with EBV nonrelated nodular sclerosing cHL stage IIIA with a Hasenclever score of 3 who entered CR at one month following completion of ABVD, but small volume relapse at 6 months posttherapy confirmed by PET-CT. Neither sCD163 nor sTARC were elevated at this time-point. Patient E was a 34-year-old male with EBV nonrelated nodular sclerosing cHL stage III with a Hasenclever score of 2 who entered CR at one month following completion of ABVD, but relapsed with extensive PET/CT positive disease at 6 months posttherapy. sTARC, but not sCD163, was elevated at 6 months posttherapy. Patients F–H had persistent clinical CR despite low-grade PET avidity of uncertain significance at restaging.

each of these patients in turn. The first patient was not informative as both sCD163 and sTARC were below threshold before therapy and remained below during and after therapy (data not shown). In the remaining 3 patients (Fig. 2F and G), the HRS-specific marker sTARC remained below threshold consistent with ongoing CR. However, values of the tumor infiltrate-specific marker sCD163 ranged from either modestly above or borderline

below threshold, indicating that sTARC was more informative in patients with equivocal restaging PET scans that remain in clinical CR.

#### sCD163 levels decline gradually in cHL patients, whereas sTARC levels decline rapidly

Figure 1A–C illustrates that sCD163 levels decline gradually during treatment. In contrast, levels of the

tumor-specific biomarker sTARC dropped rapidly, with levels presecond therapy comparable with healthy participants. Interestingly, a similar pattern to sTARC was observed for plasma EBV-DNA in patients with EBV-related cHL (Fig. 1C). Circulating cell-free EBV-DNA is an established tumor-specific biomarker in EBV-related cHL (20, 29–31).

Our next aim was to determine the association between each serum biomarker and the interim radiological assessment. The serum sample taken closest to the interim radiological assessment (typically prethird therapy) was compared with its paired pretherapy sample. Interestingly, for sCD163 levels, there was no significant difference between pretherapy samples and those in PR at the interim time-point. However, there was a significant difference for sCD163 between pretherapy and those in CR at the interim time-point ( $P = 0.0152$ ; Fig. 1D). At pretherapy, 21% of patients were below the sCD163 threshold, rising to 33% and 48% in those attaining PR and CR respectively by the interim time-point.

In contrast, there was a significant difference between pretherapy sTARC levels and both PR and CR samples at the interim time-point ( $P = 0.0001$  for both; Fig. 1E). For sTARC at pre-therapy, 14% of patients were below threshold, and the proportions below threshold in those attaining PR and CR were similar to each other at 75% and 86% respectively by the interim time-point. This is consistent with a more rapid decline in the tumor-specific circulating biomarker as compared with the tumor-infiltrate biomarker.

We then determined whether the kinetics of the biomarker (stratified by CR/PR) at the interim time-point was associated with outcome once chemotherapy was completed. All patients in CR at the interim time-point maintained ongoing CR at one and 6 months posttherapy. Interestingly, of those PR patients below the sCD163 threshold at the interim time-point, all achieved and maintained ongoing CR, whereas 4 of the 8 patients above the sCD163 threshold went on to have refractory/relapsed disease. However, for sTARC, the results were less clear-cut. There were 4 patients above the sTARC threshold at the interim therapy time-point. Two remained in ongoing CR, whereas one had refractory disease and one relapsed by 6 months posttherapy. Similarly, 2 (of 9) patients below the sTARC threshold had relapsed disease by 6 months posttherapy.

#### **sCD163 levels correlate with tissue CD163 protein levels**

Biopsy tissue was available in 32 cHL patients from our cohort. Tissue CD163 protein levels (quantified by immunohistochemistry) were modestly correlated to matching pretherapy sCD163 levels ( $r = 0.4005$ , 95% CI, 0.05373–0.6611,  $P = 0.0256$ ). The number of HRS cells did not correlate with pretherapy sTARC levels ( $P = \text{NS}$ ). Tissue CD163 protein levels were associated with the Hasenclever prognostic score ( $P = 0.0332$ , score  $<3$ : 31.3%  $\pm$  3.9%,  $\geq 3$ : 51.4%  $\pm$  6.5%) and with advanced stage disease ( $P = 0.0025$ , early: mean 21.2%  $\pm$  SEM 2.5, advanced: mean

37.8%  $\pm$  SEM 4.3; refs. 21–23). No other associations were found between tissue CD163 protein levels and any other clinical characteristics. In addition to measuring CD163 protein levels, real-time reverse transcription PCR was used to measure tissue CD163, CD68, and TARC mRNA levels. Tissue CD163 mRNA levels weakly correlated with tissue protein levels ( $r = 0.3898$ , 95% CI, 0.02703–0.6618,  $P = 0.0366$ ); however, neither CD163 nor TARC tissue mRNA levels correlated with paired pretherapy serum protein levels and TARC tissue mRNA levels did not correlate with number of HRS cells. No associations were found between any of the clinical parameters and mRNA levels of CD163, CD68, and TARC. CD163 and CD68 mRNA levels moderately correlated ( $r = 0.3851$ , 95% CI, 0.04202–0.6470,  $P = 0.0295$ ). Twelve cHL tissue samples were dual stained for CD163 and CD68. In these, CD163 cells were universally positive for CD68, whereas the proportion of CD68 cells that were CD163 positive was variable.

#### **cHL patients have a higher proportion of total circulating CD163<sup>+</sup>CD14<sup>+</sup> monocytes, suggesting that not all of the elevated circulating CD163 is tumor derived**

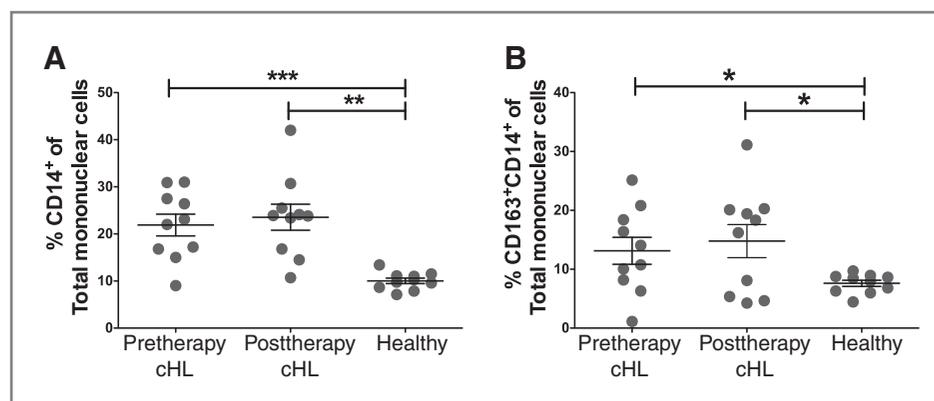
Pretherapy cHL patients ( $n = 10$ ) had a higher proportion of circulating CD14<sup>+</sup> monocytes within the mononuclear gate compared with 10 healthy control participants ( $P = 0.0006$ ; Fig. 3A). The proportion of circulating CD163<sup>+</sup>CD14<sup>+</sup> cells were also higher in patients with cHL compared with healthy control participants ( $P = 0.0437$ , Fig. 3B). All these patients remained in CR at 6 months posttherapy. They continued to have an elevated proportion of total monocytes and CD163<sup>+</sup> monocytes ( $P = 0.0010$ , Fig. 3A and  $P = 0.0332$ , Fig. 3B, respectively). Levels of sCD163 did not correlate with the proportion of circulating CD163<sup>+</sup>CD14<sup>+</sup> cells in cHL. mo-MDSCs were not elevated in cHL compared with healthy control participants.

#### **sCD163 levels are associated with B symptoms, Ann Arbor stage, lymphocyte count, and plasma EBV-DNA in cHL**

Pretherapy sCD163 and sTARC levels were associated with patient characteristics as outlined in Table 2. Higher sCD163 levels were significantly associated with B symptoms ( $P = 0.0351$ ), Ann Arbor stage ( $P = 0.0328$ ), lower lymphocyte counts ( $P = 0.0297$ ), and (in EBV-related cHL) with higher plasma EBV-DNA ( $P = 0.0197$ ). sTARC levels were inversely associated with age ( $P = 0.0250$ ). We then conducted a correlation analysis between plasma EBV-DNA and sCD163 and sTARC. sCD163 was strongly correlated with plasma EBV-DNA ( $r = 0.791$ ,  $P = 0.001$ ), whereas sTARC was not.

#### **Depletion of CD163<sup>+</sup> monocytes enhances proliferation of T cells in cHL**

To further investigate the inverse association between sCD163 and lymphocyte count, we assessed global CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation in the presence or absence of CD163<sup>+</sup> monocytes. We randomly selected patients with



**Figure 3.** Comparison of total circulating CD163<sup>+</sup>CD14<sup>+</sup> monocytes in patients with cHL versus healthy participants. Error bars represent mean with SEM. A, percentage of monocytes (defined as CD14<sup>+</sup>) within the total population of viable mononuclear cells (pre-therapy cHL vs. healthy  $P = 0.0006$ , posttherapy cHL vs. healthy  $P = 0.0010$ ). B, percentage of CD163<sup>+</sup> monocytes within the total population of viable mononuclear cells (pretherapy cHL vs. healthy  $P = 0.0437$ , posttherapy cHL vs. healthy  $P = 0.0332$ ).

8 cHL and 6 healthy participants. As the majority of CD14<sup>+</sup> monocytes are CD163<sup>+</sup> (pretherapy cHL patient mean = 65%), we compared *in vitro* proliferation of CD14<sup>+</sup>-depleted PBMC with nondepleted PBMC in the pretherapy cHL and healthy participants. CFSE staining was used to quantify CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation following stimulation by T-cell proliferation beads. Total percent proliferation and extent proliferation (top ten percentile) were compared. *In vitro* depletion of monocytes from pretherapy cHL samples led to enhancement of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation, indicating that monocyte-dependent T-cell immunosuppression occurs in patients with cHL (total proliferation CD4<sup>+</sup>  $P = 0.0078$ , CD8<sup>+</sup>  $P = 0.0078$ , extent of proliferation CD4<sup>+</sup>  $P = 0.0078$ , CD8<sup>+</sup>  $P = 0.0078$ , Fig. 4). Monocyte depletion from healthy participants also enhanced proliferation; however, this only reached significance for CD8<sup>+</sup> T-cell total proliferation ( $P = 0.0313$ ).

#### CD163 but not TARC levels are elevated in the serum of patients with NLPHL

TARC is expressed by the HRS cells of cHL, as well as the thymus, subsets of dendritic cells, endothelial cells, and activated PBMC (6, 32). HRS cells are not present in NLPHL and it is known that TARC is not expressed by primary NLPHL tissues (6, 10). Consistent with this, sTARC was not elevated in all 4 pretherapy NLPHL patients compared with healthy participants and were significantly lower than pretherapy cHL samples ( $P < 0.0001$ ). This indicates that sTARC is specific for HRS cells and is not a useful circulating biomarker for NLPHL.

Using the previously published cutoff, CD163 protein was present at low levels in 3 of the 4 NLPHL tissues (mean 17%, range 12–31%; refs. 16–18). Interestingly, sCD163 levels were elevated in all 4 pretherapy NLPHL patients compared with healthy participants ( $P = 0.001$ ) at a similar level compared with pretherapy cHL samples. Further investigation into the use of sCD163 as a disease response biomarker for NLPHL is warranted.

#### sTARC is not a disease response biomarker in cHL patients with concomitant severe allergic disease

One patient with stage IVB EBV-related nodular sclerosing cHL in the setting of long standing methotrexate therapy for severe atopic dermatitis was censored from all analysis. The dermatitis remained poorly controlled for the duration of the study. This patient was treated with ABVD, which resulted in radiological PR at the interim time-point and CR at the end of therapy. This patient's sCD163 was elevated (646 ng/mL) at pretherapy and borderline at the interim time-point (493 ng/mL). EBV-DNA was present pretherapy, but could not be detected at any subsequent time-point. At one month posttherapy, sCD163 fell below the cutoff to 340 ng/mL. Interestingly, sTARC was grossly elevated at all time-points with the mean more than 10 times our threshold value (mean 3163 pg/mL, range 2383–3920 pg/mL). Serum TARC level is known to be elevated in a range of allergic disease such as atopic dermatitis (32, 33).

#### Discussion

We present the first study comparing tumor-infiltrate versus tumor-specific circulating disease response biomarkers in Hodgkin lymphoma patients. Both sCD163 and sTARC are sensitive and specific disease response biomarkers in the majority of cHL. However, when results are stratified by interim radiological assessment, sCD163 seems to more accurately reflect disease response than sTARC during therapy in cHL. Reflecting the relative abundance of tumor-infiltrating cells compared with HRS cells, sCD163 levels were 350-fold higher at pretherapy compared with sTARC, and then declined less rapidly (than sTARC and plasma EBV-DNA) once chemotherapy had commenced. However, in patients completing first-line therapy who went on to have persistent clinical CR despite low-grade PET avidity of uncertain significance at restaging, sCD163 was frequently (albeit only modestly) above threshold, whereas sTARC values remained below threshold. sTARC appeared more frequently elevated than

**Table 2.** cHL patient characteristics and pretherapy serum protein levels

Patient characteristics	Total (N = 42)	% Total	Mean ± SEM serum CD163 (ng/μL)	CD163 P	Mean ± SEM serum TARC (pg/μL)	TARC P
Age				NS		0.0250
Less than 45 years	30	71%	725.9 ± 52.61		2442 ± 295.4	
At least 45 years	12	29%	816.8 ± 137.7		816.8 ± 137.7	
Gender				NS		NS
Female	20	48%	731.9 ± 53.66		1912 ± 376.6	
Male	22	52%	770.1 ± 91.57		2240 ± 354.1	
Ann Arbor Stage				<b>0.0328</b>		NS
I-II	20	48%	632.6 ± 61.04		1748 ± 327.4	
III-IV	22	52%	860.3 ± 80.92		2389 ± 383.4	
B symptoms				<b>0.0351</b>		NS
No	26	62%	647.8 ± 43.18		1968 ± 325.3	
Yes	16	38%	921.0 ± 112.4		2272 ± 424.4	
Hasenclever score				NS		NS
At least 3	27	64%	670.8 ± 45.55		1996 ± 330.6	
Less than 3	15	36%	897.8 ± 120.1		2242 ± 411.8	
Lymphocyte count				<b>0.0297</b>		NS
At least $1 \times 10^9/L$	15	36%	933.8 ± 110.1		2621 ± 467.6	
Less than $1 \times 10^9/L$	27	64%	650.8 ± 48.73		1785 ± 293.1	
Stage				NS		NS
Early	9	21%	609.8 ± 86.72		1763 ± 554.9	
Advanced	33	79%	790.6 ± 63.15		2171 ± 291.3	
Histology				NS		NS
Nodular sclerosing	26	62%	793.6 ± 67.99		2140 ± 326.9	
Other specified cHL	9	21%	651.0 ± 95.64		1141 ± 517.8	
cHL, unspecified	7	17%				
EBER-ISH				NS		NS
Negative	27	64%	808.1 ± 66.62		2357 ± 314.4	
Positive	12	29%	611.4 ± 103.1		1286 ± 464.1	
Unavailable	3	7%				
Plasma EBV-DNA <sup>a</sup>				<b>0.0197</b>		NS
At least 200 genomes/mL	5	12%	344.7 ± 74.42		583.3 ± 326.6	
Less than 200 genomes/mL	7	17%	801.9 ± 127.5		1788 ± 724.8	
NA	29	69%				

NOTE: Bold type indicates significant *P* values.

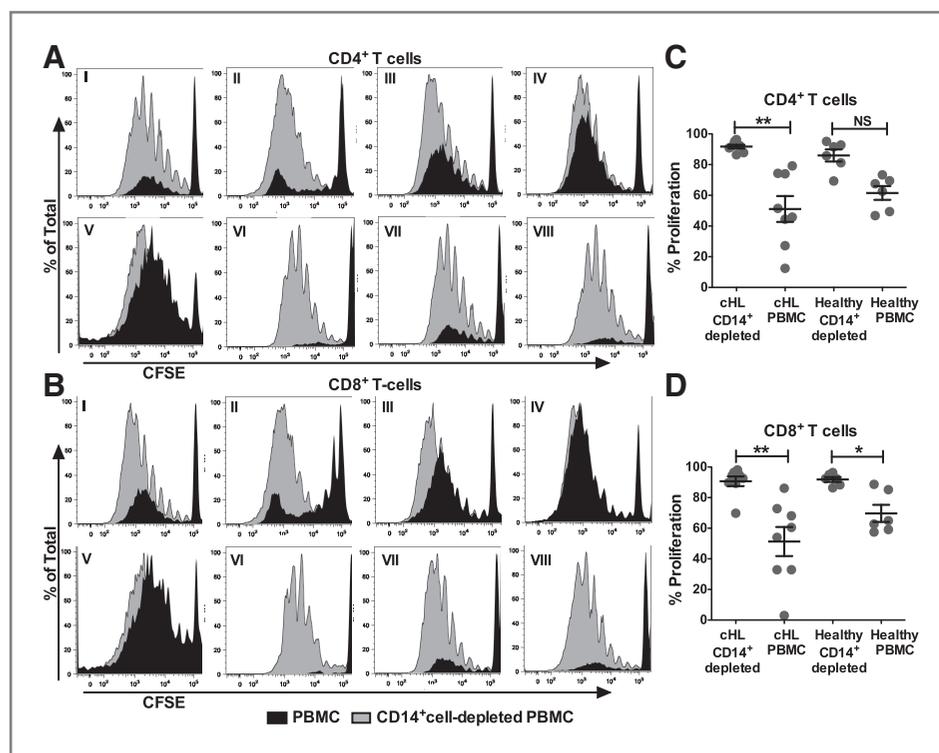
Abbreviations: NS, not significant; SEM, standard error of the mean; Other specified cHL, mixed-cellularity, lymphocyte-rich, and lymphocyte-depleted cHL subtypes.

<sup>a</sup>In patients with EBV-related cHL.

sCD163 in those with relapsed/refractory disease. However, in a patient with active atopic dermatitis sTARC was persistently elevated despite attaining CR. Thus the combination of sCD163 and sTARC seem more informative than either biomarker alone. Finally, we found that patients with cHL had increased total circulating CD163<sup>+</sup>CD14<sup>+</sup> monocytes, suggesting that the diseased node is not the only source of sCD163. Depletion of circulating CD163<sup>+</sup>CD14<sup>+</sup> monocytes in cHL enhanced both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation.

Tissue-associated macrophages (TAM) are potentially one of the most powerful independent markers for clinical

prediction in cHL, (12–18) as well as potentially representing a novel therapeutic target (34). The best markers of TAMs are still a matter of debate (35). Results from studies are conflicting, with some, but not all, finding that high protein expression (by immunohistochemistry) of the monocyte/macrophage markers CD68 and CD163 correlated with adverse outcomes (12–15). Although our study was not designed to test the prognostic ability of tissue CD163, we did find (in line with some but not all prior reports), that values were associated with both Hasenclever score and the presence or absence of early-stage disease. The inconsistency between reported studies may in part reflect



**Figure 4.** Monocyte depletion enhances T-cell proliferation in patients with cHL. Histograms show (A) CD4<sup>+</sup> and (B) CD8<sup>+</sup> T-cell proliferation from eight patients with cHL (labeled I–VIII). T-cell proliferation from CD14<sup>+</sup> cell-depleted PBMC in gray and from nondepleted PBMC in black. CD4<sup>+</sup> (C) and CD8<sup>+</sup> (D) T-cell proliferation from cHL patient and healthy participant CD14<sup>+</sup> cell-depleted PBMC and nondepleted PBMC represented as total percent proliferation above background. Error bars represent mean with SEM. \*\*,  $P < 0.01$ ; NS,  $P > 0.05$ .

the different populations tested and their retrospective nature, and also the intrinsic variability that is inherent in quantifying protein by immunohistochemistry. In contrast, quantification of proteins by ELISA is readily standardized between laboratories. We observed a modest but significant correlation between tissue and sCD163, and also (consistent with previous studies of tissue CD163) that sCD163 correlated with B symptoms and Ann Arbor stage (13, 14).

Previous studies have implied that EBV<sup>+</sup> HRS cells modulate the tumor microenvironment (36–38). Intriguingly, we show a strong positive correlation with sCD163 and plasma EBV-DNA levels, suggesting a possible interaction with EBV proteins in HRS cells and CD163<sup>+</sup> monocyte/macrophages. Monocytes have been shown to enhance proliferation and LMP1 expression of tumor cells in nasal natural killer/T-cell lymphoma (NNKT), which is also an EBV latency type II-associated malignancy (39). Consistent with this, 4 studies (with sample sizes ranging from approximately 100–300 tissues) have found that tissue CD163 protein does correlate with the presence of EBV-positive HRS cells (13–16). Cell-free EBV-DNA was not tested in these studies. Although we found no association between tissue CD163 protein levels and EBV-related cHL, our results are likely underpowered to show this association. Further studies are required to investigate the mechanistic basis for the association between CD163 and EBV in cHL.

Interestingly, sCD163 correlated with low lymphocyte count. At present, it is not fully understood how HRS cells recruit monocyte/macrophages, and once recruited to the tumor microenvironment, how these cells impact host systemic circulating immunity. HRS cells may result in

increased CD163<sup>+</sup> monocytes/macrophages. These, in turn, may mediate impaired T-cell proliferation, resulting in the inverse association between sCD163 and circulating lymphocyte count numbers that we observed. It has been shown in diffuse large B-cell lymphoma and follicular lymphoma that mo-MDSC inhibit T-cell proliferation (40). Notably, we found that mo-MDSC were not elevated in cHL, whereas circulating CD163<sup>+</sup>CD14<sup>+</sup> monocytes were high compared with healthy participants. In functional assays, we show that monocyte depletion enhances T-cell proliferation in both cHL and healthy participants, albeit to a greater extent in cHL. Understanding why monocytes are elevated in cHL and the mechanism by which monocytes limit T-cell proliferation may be critical to understanding the pathogenesis of cHL and have therapeutic implications, particularly as inhibition of TAMs with blocking molecules has been shown to slow tumor growth in other cancers (34).

The elevated circulating CD163<sup>+</sup>CD14<sup>+</sup> monocytes in cHL suggest that the diseased node is not the only source of sCD163. Consistent with this, although sCD163 was greatly reduced at 6 months posttherapy in CR cHL patients, sCD163 remained modestly elevated compared to healthy participants. Circulating CD163<sup>+</sup>CD14<sup>+</sup> monocytes also remained elevated at 6 months posttherapy in CR cHL compared with healthy participants, possibly accounting for the remaining sCD163 levels.

This is the first study of sCD163 as a marker of disease response and the first to compare tumor-specific and tumor-infiltrate markers in cHL. Both sCD163 and sTARC were markedly elevated in pretherapy cHL samples and significantly declined in complete responders. Our sTARC results

are comparable with the recent study by Platell and colleagues (11). In both cases, sTARC was elevated pretherapy and dropped rapidly to normal levels following the first cycle of therapy in the majority of responding patients. A similar trend was also observed in a phase II study of patients receiving panobinostat (41). In both the Platell and colleagues' study and our study (11), there was little difference between CR and PR sTARC levels at the mid-treatment time-point. In our study, the gradual decline of sCD163 throughout treatment and elevated levels of sCD163 in PR versus CR at the mid-treatment time-point suggests that the tumor-infiltrate marker may have higher specificity during treatment. However, although our numbers of relapsed/refractory patients were limited, sCD163 was elevated in only one of the 5 patients at time of relapse/refractory disease. In contrast, sTARC was elevated in 3 of 5, suggesting sTARC may be a more sensitive marker of relapse. Notably, the 2 relapsed patients with nonelevated levels of sTARC had low tumor volume, one with ongoing disease confined to the bone. The work of Platell and colleagues found that sTARC was elevated in all 12 of their relapsed cHL patients, however, sCD163 was not measured (11). The different levels of sTARC and sCD163 may reflect a change in the relative proportions of tumor and tumor-infiltrating cells in the diseased nodes of refractory/relapsed patients. Biopsies were not taken at refractory/relapse presentation, otherwise a comparison of tumor composition between biopsies taken at initial presentation and at relapse may have been informative.

We show for the first time that sCD163 is elevated in patients with cHL. Circulating CD163 seems to arise from both tumor tissue and CD163<sup>+</sup> monocytes. sCD163 correlated with cell-free EBV-DNA, and was associated with B symptoms, stage, and low lymphocyte count. Depletion of CD163<sup>+</sup> monocytes enhanced T-cell proliferation, implicating monocytes and/or TAMs as potential novel targets for immunotherapeutic manipulation. Finally, our results suggest that the combination of tumor-infiltrate (CD163) and tumor-specific (TARC) serum proteins are more informative than either marker alone as disease response biomarkers in

early- and advanced-stage disease during first-line therapy for cHL. It is likely that circulating disease response biomarkers will vary according to the regimen and dose schedule. Future studies are needed to evaluate the differences in kinetics of serum disease response biomarkers in patients treated with commonly used first-line regimens such as ABVD, escalated BEACOPP and BEACOPP-14.

#### Disclosure of Potential Conflicts of Interest

D. Gottlieb has a commercial research grant from Celgene and honoraria from speakers' bureau from Gilead. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

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#### Acknowledgments

This was an Australasian Leukaemia & Lymphoma Group sponsored study. The authors thank Susan Arnold for her assistance with data collection and Clay Winterford for immunohistochemistry staining.

#### Grant Support

Work in the Clinical Immunohaematology laboratory is sponsored by the National Health and Medical Research Council (Australia), Leukaemia Foundation (Australia), Cancer Council of Queensland, and Queensland Office of Health and Medical Research. K.L. Jones is supported by the Leukaemia Foundation (Australia), the Australian Centre for Vaccine Development, and QIMR.

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Received August 15, 2012; revised November 21, 2012; accepted November 28, 2012; published OnlineFirst December 5, 2012.

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*Clin Cancer Res* 2013;19:731-742. Published OnlineFirst December 5, 2012.

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