Published OnlineFirst September 20, 2016; DOI: 10.1158/1078-0432.CCR-16-1576

Biology of Human Tumors

Clinical Cancer Research

The T-cell Receptor Repertoire Influences the Tumor Microenvironment and Is Associated with Survival in Aggressive B-cell Lymphoma

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Abstract

Purpose: To investigate the relationship between the intratumoral T-cell receptor (TCR) repertoire and the tumor microenvironment (TME) in *de novo* diffuse large B-cell lymphoma (DLBCL) and the impact of TCR on survival.

Experimental Design: We performed high-throughput unbiased TCRβ sequencing on a population-based cohort of 92 patients with DLBCL treated with conventional (i.e., non-checkpoint blockade) frontline "R-CHOP" therapy. Key immune checkpoint genes within the TME were digitally quantified by nanoString. The primary endpoints were 4-year overall survival (OS) and progression-free survival (PFS).

Results: The TCR repertoire within DLBCL nodes was abnormally narrow relative to non-diseased nodal tissues ($P < 0.0001$). In DLBCL, a highly dominant single T-cell clone was associated with inferior 4-year OS rate of 60.0% [95% confidence interval (CI), 31.7%–79.6%], compared with 79.8% in patients with a low dominant clone (95% CI, 66.7%–88.5%; $P = 0.005$). A highly dominant clone also predicted inferior 4-year PFS rate of 46.6% (95% CI, 22.5%–76.6%) versus 72.6% (95% CI, 58.8%–82.4%, $P = 0.008$) for a low dominant clone. In keeping, clonal expansions were most pronounced in the EBV^{+} DLBCL subtype that is known to express immunogenic viral antigens and is associated with particularly poor outcome. Increased T-cell diversity was associated with significantly elevated *PD-1*, *PD-L1*, and *PD-L2* immune checkpoint molecules.

Conclusions: Put together, these findings suggest that the TCR repertoire is a key determinant of the TME. Highly dominant T-cell clonal expansions within the TME are associated with poor outcome in DLBCL treated with conventional frontline therapy. *Clin Cancer Res; 1–9. ©2016 AACR.*

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive B-cell lymphoma (1). Despite the addition of rituximab to frontline CHOP chemotherapy (R-CHOP), one third of patients still die from the disease (1).

Increased T-cell infiltration within the tumor biopsy is associated with improved outcome in CHOP- and R-CHOP–treated patients with DLBCL (2–4). The association between increased T-cell infiltration and improved survival is also seen in patients with solid tumors (5). However, there is a limited assessment of the impact of the T-cell receptor (TCR) repertoires of intra-tumoral T cells on outcome in cancer. There have been several studies examining the dynamics of the TCR repertoire after treatment with immune checkpoint inhibitors (6, 7). Conversely, there are minimal data on the impact of the TCR repertoire on survival after conventional (i.e., non-checkpoint targeting) multi-agent therapy in solid tumors or lymphomas. In DLBCL, the relationship between the intra-tumoral TCR repertoire and the TME and the prognostic impact of the TCR repertoire after R-CHOP remains unclear.

However, detailed analyses of the TCR repertoire have however been performed in the context of chronic viral infections. Here, a diverse repertoire of T cells has long been thought to be critical for effective host resistance to persistent infections (8–12). In longitudinal studies in patients with asymptomatic human immunodeficiency virus (HIV), a dynamic bi-directional adaptive interplay between viral epitope escape mutants and the T-cell clonal repertoire has been demonstrated (8). In this scenario, a large HIV-specific T-cell clone was functionally equivalent to numerous modest sized HIV-specific T-cell clones in preventing disease progression. However, overreliance on a single dominant T-cell clone permits the emergence of virus immune escape mutants that predispose to disease progression (13–15). There may be parallels between these processes and those active in lymphoma; however, the importance of repertoire diversity in controlling human B-cell lymphomas and its impact on survival remains unproven.

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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-16-1576

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Published OnlineFirst September 20, 2016; DOI: 10.1158/1078-0432.CCR-16-1576

Translational Relevance

Several studies have highlighted the importance of various aspects of the tumor microenvironment upon the clinical outcome in lymphoma. However, the role of the intra-tumoral T-cell receptor (TCR) repertoire has not been established. This is the first intra-tumoral TCR repertoire study of a large diffuse large B-cell lymphoma (DLBCL) cohort. There have been several studies in solid cancers examining the dynamics of the TCR repertoire after treatment with immune checkpoint inhibitors. However here, particular attention has been paid to the association with progression-free and overall survival and TCR repertoire after conventional frontline (i.e., non-checkpoint blockade) therapy. Observations in the setting of a non-defined tumor antigen (EBV^{-} DLBCL) were contrasted with those with a well-defined tumor antigen (EBV^{+} DLBCL) and with a non-lymphomatous cancer (melanoma) that is typically associated with a high mutational load. The composition of the TCR repertoire should be factored into the rational design of immune-based therapies.

The diversity of T cells is determined by their TCR. The complimentary determining region 3 (CDR3) of the TCRβ chain is the most variable portion of the TCR and is critical for MHC–peptide complex recognition (16, 17). The range of individual TCR-bearing T-cell clones that comprises the repertoire specific for a particular antigenic MHC–peptide complex varies substantially in terms of TCR frequency and diversity. A consequence of this specificity is that the TCRβ CDR3 sequence can be used as a "molecular tag" to identify each T-cell clone.

In this study, we applied high-throughput sequencing of the CDR3 region of TCRβ to comprehensively interrogate DLBCL nodal tissues. Data were collated with gene expression and survival to clarify the relationship between the clonal complexity of intra-tumoral T cells the TME and outcome after conventional frontline R-CHOP therapy.

Materials and Methods

Study populations

The study was approved by Ethics Committees at participating sites. The cohort comprised 92 adult patients with histologically confirmed DLBCL (and sufficient DNA quantity of at least 500 ng) collected from 2 centres (Princess Alexandra Hospital and Canberra Hospital). The median follow-up was 3.6 years (range, 0.02–8.9 years). All patients received R-CHOP and were otherwise selected solely on the basis of formalin-fixed, paraffin-embedded (FFPE) tissue and clinical annotation (including survival data) availability. Only *de novo* cases of DLBCL were included. Grade IIIB or transformed follicular lymphoma, HIV-positive and post-transplant patients were excluded. Twelve non-diseased, normal lymph node tissues (purchased from ProteoGenex Inc.) obtained from individuals without lymphoma were also analyzed. Twenty-four tumor samples from patients with melanoma from a publically available cohort that were treated with anti-PD1 immune checkpoint therapy were used to compare results obtained from the lymphoma biopsies (7).

High-throughput sequencing

For FFPE samples, DNA was extracted using RecoverAll total nucleic acid extraction kit for FFPE (Ambion, Life Technologies) as per manufacturer's instructions. TCRβ CDR3 regions were amplified from between 500 ng and 2 μg of DNA. All samples had the immuno-SEQ assay performed at Adaptive Biotechnologies. Briefly, this involved a multiplex PCR reaction, with spike-in controls to adjust for primer bias. The reaction contained forward primers annealing to each V segment and reverse primers annealing to each J segment, resulting in the selective amplification of an 87-base pair fragment spanning the CDR3 region. Amplicons were sequenced on an Illumina HiSeq platform with at least 10-fold coverage as calculated from sequencing of an artificial immune system present in the assay that allows identification of any potential primer bias. CDR3 regions were identified as per the International ImMunoGeneTics (IMGT) definitions (18). Sequencing reads that did not match the CDR3 canonical structure were removed. Data were analyzed using in-house immuno-SEQ software. The public cohort of 24 melanoma biopsies from FFPE tissues were sequenced using the same procedure as our lymphoma cohort. This cohort is available via the Adaptive immuno-SEQ Analysis Platform (7).

Assessment of the TCR repertoire

The "total productive sequences" were first calculated. This value is dependent on how many cells carry a particular TCR rearrangement and how many copies (sequences) of each of those cell's genomes are produced because of PCR amplification. The total number of productive (in-frame CDR3) amplification-normalized TCRs was then calculated ("productive TCRs"). This value is a measure of the total number of functional T cells present in the tissue. Only samples with sufficient number of productive TCRs present were included in the analysis (at least 100 normalized productive TCRs) to exclude clonality measures being influenced by a small T-cell population. Four samples were excluded using this cutoff. Then, the percentage of total T cells ("% total T cells") as a proportion of all cells within the biopsy was determined using the following calculation: productive TCRs/[input gDNA (ng) $\times$ 156 diploid genomes/ng DNA].

The numbers of individual productive T-cell clones in the samples were then identified by calculating the number of "productive uniques". This is a measure of the number of functional T cells with a distinct TCR rearrangement (an estimate of how many clones exist in the entire repertoire) and is also termed "richness."

Shannon's entropy (H) was calculated using the frequency of amino acid sequences. Shannon's entropy (or "entropy") measures both the sample richness and the degree of unevenness in clone frequencies. The higher the value of entropy, the more diverse the distribution of the CDR3 clones (19). The clonality metric is the reciprocal of the normalized entropy. It describes the "evenness" of the distribution of TCR clones in the repertoire, that is, how much of the TCR repertoire is composed of expanded clones independent of sample size. A maximally polyclonal cell population is one in which every sequence is represented once and represented by a clonality score of 0, whereas an entirely monoclonal population has a clonality score of 1.

Finally, to quantify the clones that were most highly expanded, we used the "% maximal frequency clone," which is the percentage frequency of the largest dominant clone. The top 10 (or 100) most frequent clones ["% top 10 (or 100) maximal frequency

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clones"] is the frequency of the top 10 (or 100) dominant clones identified in each sample.

Frequency normalizes for the percentage of each clone in a sample and allows a direct comparison of samples with different sampling depths. Therefore, frequency measures such as entropy, clonality, % total T cells, and % maximal frequency clones were used for analyses, so as to enable comparison of samples irrespective of the numbers of T-cell sequencing reads. To make this process more stringent, only samples with at least 100 normalized productive T cells were included. These measures also allowed us to compare samples across melanoma and lymphoma cohorts from different sequencing runs. Full tabulated results for each TCR sequencing parameter in each individual sample analyzed is available in Supplementary Table S1.

nanoString nCounter RNA quantification and flow cytometry

RNA was extracted from FFPE tumor biopsies using RecoverAll total nucleic acid extraction kit for FFPE (Ambion, Life Technologies) as per manufacturer's instructions. Genes were quantified using the nCounter platform (nanoString Technologies), and the cell of origin (COO) was calculated as previously outlined (2). Flow cytometry was performed as published (3).

Statistical analysis

Values between groups of data were tested for statistical significance using the 2-tailed nonpaired Mann–Whitney test. Categorical data were compared using the Fisher exact test or χ^2 test as appropriate. Progression-free survival (PFS) was determined from the date of diagnosis to the date of last follow-up or disease progression, death, or discontinuation of treatment for any reason. Overall survival (OS) was determined from the date of diagnosis to the date of last follow-up or death. Survival analysis was performed using Kaplan–Meier curves and the log-rank test. Tests were 2-sided at $P = 0.05$. Multivariate analysis was performed using Cox regression. Analyses were prepared using GraphPad Prism (version 6), Statistical Package for the Social Sciences version 22 (International Business Machines Corporation), and immuno-SEQ analysis platform (Adaptive Biotechnologies).

Results

The immuno-SEQ assay correlates with other methods of T-cell detection

For stringency, so as to exclude clonality measures being potentially influenced by a small T-cell population, only samples with sufficient number of productive TCRs present were included in the analysis (arbitrarily chosen as at least 100 normalized productive TCRs). Of the 92 initial patient samples, 88 had sufficient levels of productive TCRs to be included for further analysis. Median age was 62 years (range, 27.5–85.8 years), 63% were male, and the international prognostic index (IPI) was: IPI 0, 9.8%; IPI 1–2, 46.3%; and IPI 3–5, 43.9%. In these 88 DLBCL tumors, 75 also had sufficient RNA to permit gene expression performed by nanoString nCounter, an established platform for quantifying mRNA expression on FFPE specimens (20). This showed significant correlation between *CD3* ($r = 0.52$, $P < 0.0001$), *CD4* ($r = 0.56$, $P < 0.0001$), *CD5* ($r = 0.54$, $P < 0.0001$), *CD8* ($r = 0.21$, $P = 0.06$) and the number of T cells detected by sequencing (productive TCRs). In addition, 26 tumors had enumeration of T cells performed by flow cytometry on fresh tissue at diagnosis as previously described (3). The percentage of T cells detected by flow cytometry ($CD5^+/CD19^-$ cells) significantly correlated with productive TCRs detected by sequencing ($r = 0.42$, $P = 0.03$).

DLBCL nodes have a narrower TCR repertoire than non-diseased nodes

The TCR repertoire in the DLBCL biopsies and 12 non-diseased normal lymph nodes was compared, outlined in Table 1. The amount of input DNA and number of total input cells was almost identical between the groups. However, the percentage total T cells was about 7-fold higher in the non-diseased normal lymph nodes than in DLBCL biopsies ($P < 0.0001$). There was a marked increase in clonality, and consistent with this, reduced diversity (entropy) in DLBCL biopsies relative to non-diseased normal lymph nodes (both $P < 0.0001$). There was also an increased percentage of maximal frequency clone within DLBCL biopsies relative to non-diseased normal lymph nodes ($P < 0.0001$). These findings are consistent with an abnormally narrow TCR spectrum of T cells within tumor samples. The distribution of the percentage of top 100 maximal frequency clones is illustrated in Fig. 1. Within patient biopsies, higher diversity (entropy) was present in those in whom the percentage of maximal frequency clone was below the median level ($P < 0.001$). Only FFPE samples were tested. Paired fresh biopsy samples were not available for sorting in T-cell subsets to definitely establish the frequency of T-cell clones within particular T-cell groupings. However, the ratio of CD4/CD8 mRNA measured by nanoString grouped according to level of the percentage of maximal frequency clone, with higher *CD8* levels relative to *CD4* associated with greater T-cell clonal expansions (Fig. 2).

A narrow TCR repertoire associates with adverse outcome

We next investigated the impact of the TCR repertoire on outcome following R-CHOP. To address whether biopsy tissues from patients with particularly skewed (i.e., highly dominant) clonal expansions were associated with outcome, we tested the impact of percentage of maximal frequency clone on survival. This

Table 1. Comparison of DLBCL and non-diseased nodal tissue with regard to TCRβ sequencing

Analyzed parameter	DLBCL biopsies ($n = 88$)	Non-diseased nodes ($n = 12$)	P
Input DNA, median, ng	1,175 (509–1,743)	1,171 (618–1,386)	0.91
Estimated total input cells	183,256 (79,404–271,908)	180,135 (96,408–216,216)	0.9
Productive uniques	2,444 (129–19,689)	17,461 (6,267–32,906)	<0.001
Productive TCR rearrangements	2,796 (130–21,889)	19,289 (6,731–35,730)	<0.001
% Total T cells	1.67 (0.6–12.8)	12.47 (3.3–27.3)	<0.001
Entropy	9.7 (5.6-13.4)	13.6 (11.87-14.59)	<0.001
Clonality	0.097 (0.059-0.4)	0.057 (0.043-0.11)	<0.001
% Maximum frequency clone	2.09 (0.24-35.22)	0.51 (0.29-3.3)	<0.001

NOTE: All values quoted are median (with range).

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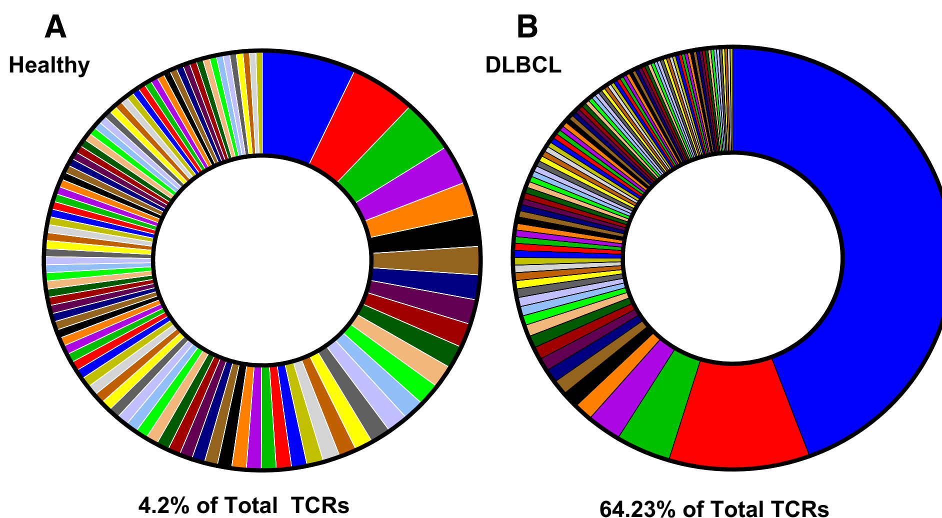

Figure 1.
Percentage of top 100 maximal frequency clones. Percentage of Top 100 maximal frequency clones with the % of total TCR population they represent, in a representative (**A**) non-diseased normal lymph node tissue and (**B**) nodal tissue in a patient with DLBCL.

cutoff was established using the upper 95% confidence interval (CI) for the mean value of the dominant clones of all samples. These highly dominant, that is, a markedly expanded clones, were termed "high % maximal frequency" clones. These were present in 17% of cases, with the remaining 83% classified as having "low % maximal frequency" clones. Interestingly, the presence of a high percentage of maximal frequency in the tumor biopsy in the whole cohort was associated with an inferior 4-year OS rate of 60.0% (95% CI, 31.7%–79.6%), compared with 79.8% (95% CI, 66.7%–88.5%, $P = 0.005$) in patients with a non-dominant or "low % maximal frequency" clone (Fig. 3). PFS was also significantly reduced in patients with high percentage of maximal frequency clones with 4-year PFS rate of 46.6% (95% CI, 22.5%–76.6%) versus 72.6% (95% CI, 58.8%–82.4% $P = 0.008$) for low percentage of maximal frequency clones. To establish whether a broader number of highly expressed clones showed different or similar findings to that seen with a single highly dominant clone, we calculated the percentage of top 10 clones present in the tumor samples and found that patients with a higher proportion of percentage of top 10 clones still had a significantly inferior outcome with 4-year OS rate of 56.2% (95% CI, 27.2%–77.5%) versus 80.7% (95% CI, 69.0%–88.3%, $P = 0.01$) and 4-year PFS rate of 64.2% (95% CI, 36.9%–82.1%) versus 81.0% (95% CI, 73.5%–92.6%, $P = 0.033$), respectively. There was no significant association between the conventional prognosticators COO and R-IPI with any TCR parameter.

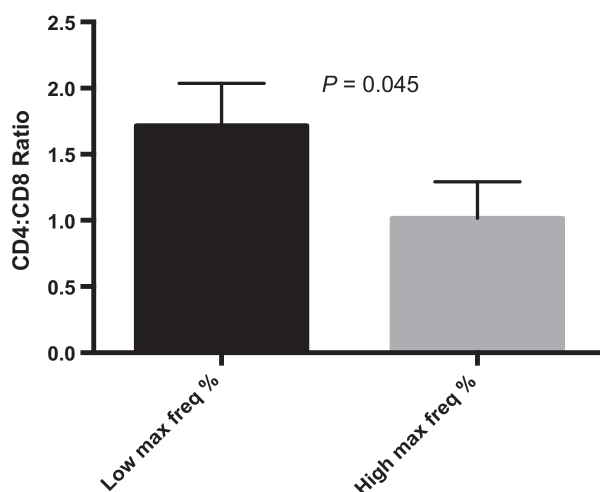

Figure 2.
The ratio of CD4:CD8 RNA is elevated in patients without a high maximal frequency clone.

Comparison of the TCR repertoire between melanoma and DLBCL

We compared our results from patients with DLBCL treated with frontline multi-agent therapy with a publicly available cohort of patients with melanoma treated with anti-PD1 therapy (7). In this cohort, patients with melanoma responsive to anti-PD1 therapy had significantly higher levels of clonality and a trend for a higher maximal frequency clones compared with non-responders (7). Intriguingly, we observed that the TCR environment was different between lymphoma and melanoma. The analysis was restricted to only those parameters not influenced by sequencing or number of input cells. There was no difference in the number of productive T cells identified in each cohort ($P = 0.18$). However, the percentage of maximal frequency clones were significantly higher in melanoma biopsies ($P = 0.0011$, Fig. 4A). In addition, the lymphoma cohort had reduced clonality ($P < 0.0001$) compared with the melanoma cohort (Fig. 4B).

EBV^+ DLBCL is associated with a small number of highly expanded T-cell clones

EBV^+ DLBCL is a subtype of DLBCL that is known to express well-defined immunogenic viral antigens. It is associated with particularly poor outcome to frontline therapy (21, 22). In 7 biopsies, EBV-encoded RNA (EBER) was detectable at high digital gene counts of more than 1,500 digital counts and/or were EBER-*in situ* hybridization–positive, in keeping with a diagnosis of EBV^+ DLBCL (21, 23). These 7 tumor samples were more clonal (Fig. 5), with a median clonality of 0.15 (range, 0.08–0.4), compared with a median of 0.095 (range, 0.06–0.37) for the remaining (EBV^-) DLBCL samples ($P = 0.008$). Interestingly, in EBV^+ tumors, the sum of the percentage of top 10 maximal frequency clones as a percentage of the productive TCR were more than 2-fold higher than in EBV^- DLBCL tumors (median, 18.6%; range, 3.6%–42.6% vs. median, 7.5%; range, 1.46%–44%, respectively,

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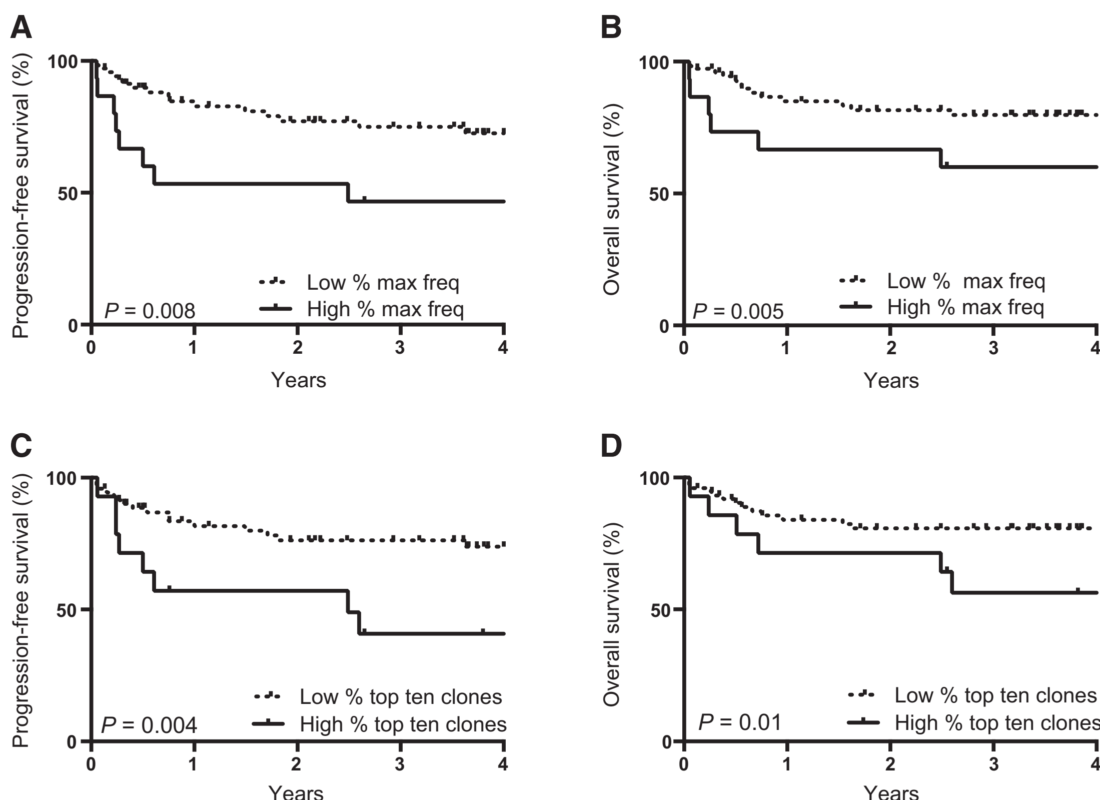

Figure 3.
The influence of the TCR repertoire on survival in *de novo* DLBCL treated with front-line R-CHOP. Kaplan–Meier survival plots showing (**A**) PFS and (**B**) OS comparing patients with a high percentage of maximal frequency with those with a low percentage of maximal frequency clone; (**C**) PFS and (**D**) OS comparing patients with a high percentage of Top 10 maximal frequency clones versus those with a low percentage.

$P = 0.006$). These results suggest that EBV$^+$ DLBCL is associated with small numbers of highly expanded T-cell clones. Consistent with the reported adverse outcome of EBV$^+$ DLBCL, 6 of 7 patients had relapsed/refractory disease and 5 subsequently died from their disease. Taken together, the TCR repertoire findings in EBV$^+$ DLBCL are in keeping with the earlier findings that a narrow TCR repertoire consisting of expanded T-cell clones is associated with an adverse outcome. Interestingly, EBV$^+$ DLBCL had levels of clonality similar to those observed in melanoma biopsies (median clonality, 0.155; range, 0.07–0.43; $P = 0.94$).

Due largely to recombinatorial biases, a small fraction of EBV-specific T cells can have TCRβ chains shared by a number of individuals or "public" TCR. However, clonal overlap between the 7 samples was very low indicative that there was no public TCR of significance found in this small group of patients (<0.012 in all comparisons).

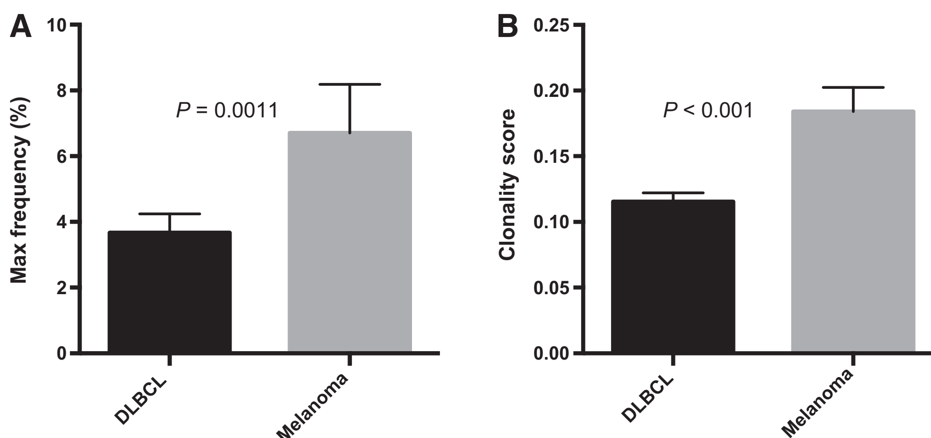

Figure 4.
Comparison of TCR populations between DLBCL and melanoma. Higher levels of (**A**) percentage of maximal frequency clone and (**B**) clonality in pretreatment melanoma compared with DLBCL samples.

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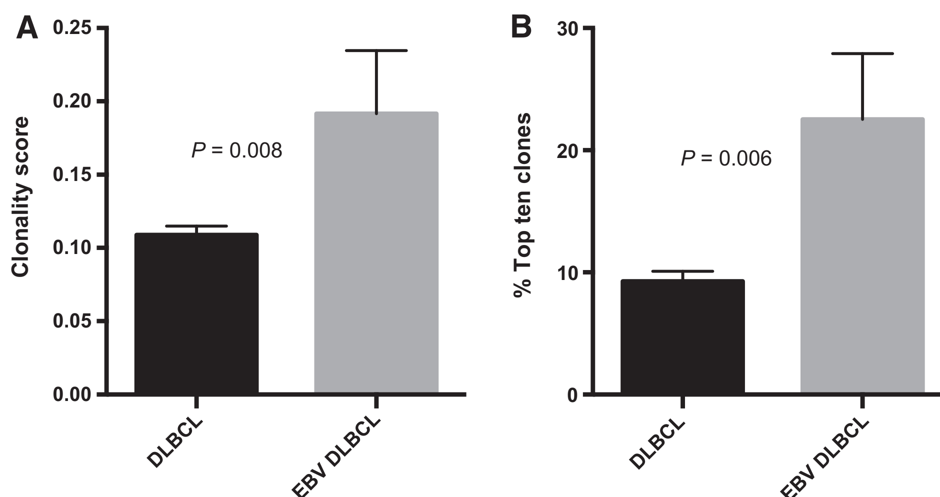

Figure 5.
A comparison of the TCR repertoire in EBV^+ DLBCL and EBV^- DLBCL. A comparison between the (**A**) clonality in EBV^+ DLBCL and EBV^- DLBCL and (**B**) the sum of percentage of top 10 maximal frequency clones present as a percentage of the total productive TCRs in EBV^+ DLBCL and EBV^- DLBCL cases.

The TCR repertoire is a key determinant of the TME

We next tested the relationship between the TCR repertoire and the expression of immune checkpoint molecules (quantified by the nanoString nCounter in the 75 biopsies with sufficient RNA) known to be present within the TME of malignant lymphomas (2). Entropy was dichotomized using a median cut-off, with elevated levels of immune checkpoint molecules significantly associated with higher entropy (*PD-1*: high entropy median = 64 gene counts, range, 11–315; low entropy median = 48 gene counts, range, 6–69, $P = 0.013$; *PD-L1*: high entropy median = 69 gene counts, range, 17–497; low entropy median = 42, range, 14–285, $P = 0.0189$; and *PD-L2*: high entropy median = 82 gene counts, range, 24–1,417; low entropy median = 50 gene counts, range, 23–418, $P = 0.0032$). Consistent with this, there was modest but highly significant positive correlations between entropy and these immune checkpoints (*PD-1*: $r = 0.28$, $P = 0.015$; *PD-L1*: $r = 0.21$, $P = 0.063$; *PD-L2*: $r = 0.25$, $P = 0.03$). Together, this indicates that diversity and immune checkpoints are interrelated. Our data are consistent with an "adaptive immune response," in which the more diverse the TCR repertoire, the more the malignant B cells are required to adapt to counter anti-tumoral immunity by eliciting an immune checkpoint response within the TME. The TCR repertoire therefore appears to be related to the expression of checkpoint molecules within the TME.

Discussion

This study shows the value of assessing the T-cell repertoire in the TME of malignant tumors. TCR sequencing correlated with gene expression and flow cytometry analysis. We showed that the TCR repertoire within DLBCL nodes is abnormally narrow relative to non-diseased lymph nodes. Large clonal T-cell expansions were associated with inferior PFS and OS and were most pronounced in patients with EBV^+ DLBCL. Finally, we showed that within diseased nodes, there are a number of features indicative of an adaptive immune response. When combined, these findings implicate the TCR repertoire as a key parameter of the TME and of survival, which has implications for the monitoring and design of future immune-based therapies.

In our study, there were significant differences in the TCR repertoire between non-diseased and lymphomatous nodes. DLBCL nodes showed reduced levels of percentage of total T-cells but a more clonal and less diverse T-cell population compared with non-diseased nodes. These findings are consistent with the differences in TCR repertoire seen between colon cancer biopsies and adjacent non-diseased mucosa (24). In diseased nodes, elevated checkpoint levels were associated with higher T-cell diversity (entropy). Together, these findings are in keeping with an adaptive immune response, in which malignant B-cells influence (i.e., "adapt") the TME in an attempt to counter an effective anti-lymphoma T-cell response, which is in part influenced by the breadth of the TCR repertoire. This adaptation to immune attack appears to be more marked in the presence of a diverse rather than a clonally restricted repertoire of tumor-infiltrating T cells.

There are minimal data regarding TCR sequencing and response in any histologic lymphoma subtype; however, some interesting findings have emerged from studies of relapsed/refractory solid tumors. In metastatic melanoma, *CTLA-4* blockade diversifies the peripheral T-cell pool (6). However, this was not associated with clinical response and patients losing high-frequency clones had particularly poor survival. Broadly consistent with this, in a study using *PD-1* blockade, a more clonal T-cell repertoire was associated with improved outcome (7). The latter finding is in keeping with our observations in a large cohort of *de novo* patients with DLBCL with long-term follow-up, where high ratios of *PD-1* axis molecules to T-cell molecules were associated with adverse outcome (2). Furthermore, there remains no information on the impact of the TCR repertoire on lymphoma outcomes in the conventional frontline (i.e., not treated with *PD-1* blockade) therapeutic setting. We demonstrate that high percentage of maximal frequency clones are associated with inferior survival. This suggests that a highly clonal TCR repertoire may contain gaps, meaning that the intratumoral T cells do not provide adequate immunosurveillance to target all malignant B-cell subclones.

The prevailing view is that B-cell lymphoma evolves by a process of clonal expansion, during which repeated rounds of cellular replication permit subsequent genetic diversification within neoplastic B-cell sub-clones (25). This can lead to the development of chemo-resistant malignant B-cell subclones. These clones may be sub-dominant at the time of clinical presentation; however, therapeutic intervention provides a potent selective pressure for their expansion, which may also include the survival of sub-clones containing somatic mutations that facilitate immune evasion (26). Mutations in somatic genes have the

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potential to be recognized as "non-self" tumor-specific immunogenic peptides (termed "neo-antigens"). In melanoma, high mutational load associates with increased numbers of candidate neo-antigens (27). Furthermore, for both melanoma and colorectal cancers, patients with high mutational load are more likely to respond to *CTLA-4* and PD-1 blockade, and in non–small cell lung cancer, efficacy to *PD-1* blockade correlates with higher neoantigen burden (28, 29). Consistent with this, it is known that subdominant T-cell clones can potentially target neo-antigens (30). One explanation is that increased numbers of neo-antigens are driving the *in-situ* expansion of high-affinity anti-tumoral T-cell clones, resulting in a skewed, that is, narrow TCR repertoire. In this scenario, the emergence of an immune escape mutant predisposes to disease progression (after conventional first-line therapy). This is in keeping with observations in the setting of chronic viral infections (14, 31). Conversely, a diverse TCR repertoire consisting of fewer highly expanded T-cell clones, which is known to be a hallmark of robust anti-viral T-cell immunity, might enhance broad tumor antigen control, thus making immune escape less likely (8–10). Consistent with this notion, it is known that subdominant T-cell clones can potentially target neo-antigens (6). In DLBCL, it is possible that smaller T-cell clones are more important to response once the effective chemotherapy regimen has significantly debulked the initial tumor load. Furthermore, experimental models indicate radiation therapy increases TCR diversity, which contributes to response (32). It is unclear whether T-cell responses are required to be antigen-specific or can, at least in part, mediate their effector activity through a non-specific bystander effect via local antitumor cytokine production.

Our findings provide interesting insights into a sub-group of DLBCL associated with high levels of EBV expression within the tumor (33). EBV^+ DLBCL has poor outcome (22). TCR sequencing indicates that these patients have particularly large intratumoral T-cell clonal expansions. This is unsurprising, as it is known that EBV has numerous latent antigens that elicit effective T-cell responses (34). The reduced diversity of the TCR repertoire observed with EBV^+ DLBCL (i.e., when external antigens drive clonal expansion) is consistent with our findings in the main DLBCL cohort and also confirms inferior outcomes with clonal T-cell expansions. It is possible that the dominant antigens expressed by EBV^+ tumors elicit too narrow a TCR response in the TME, thus allowing subclone immunoescape, as has been observed in adoptive transfer of EBV-specific T cells for EBV^+ lymphomas (31). TCR repertoire analysis within a larger EBV^+ DLBCL cohort treated with conventional frontline therapy is required to confirm this.

In melanoma, patients responsive to anti-PD1 therapy had significantly higher clonality measures than non responders and a trend for a higher percentage of maximal clone frequency (7). It may be that patients with DLBCL who have a high percentage of maximal frequency clone and inferior outcomes would also be those to benefit most from the addition of anti-PD1 therapy. TCR sequencing might be helpful in identifying patients who would most benefit from these agents. Interestingly, while DLBCL had more clonal T-cell populations compared with non-diseased nodes, melanoma biopsies had significantly higher clonality than our lymphoma samples. This may relate to the markedly higher high mutational load (and hence potential for candidate neo-antigens) observed in melanoma relative to lymphoma (35). This, in turn, results in enhanced immunogenicity, which is more likely to drive the expansion of large dominant T-cell clones. In keeping with this scenario, EBV^+ DLBCL, which is known to express immunogenic viral antigens, had clonality measures strikingly similar to those observed in melanoma. Given that high clonality equated to response in melanoma, it would be interesting to investigate whether poor outcomes in EBV^+ DLBCL could be ameliorated by addition of immune checkpoint therapy. Future studies that incorporate an assessment of the TCR repertoire could stratify patients on the basis of a narrow or broad repertoire, for example, radiation or ipilumumab could be used to broaden the repertoire of a patient with a narrow initial immune response. This knowledge could lead to prediction of which combination of immune checkpoint/effector therapies best suit an individual patient not only on the basis of the TCR repertoire but also incorporating other factors such as mutational load or specific mutations present in an individual's tumor.

There are several limitations of this study. One is that we provide only a snapshot of TCR diversity restricted to the TCRβ CDR3 region. A functional TCR is made up α and β chains, so our findings likely underestimate the degree of diversity within samples. Recent developments may allow characterization of the α and β chains of individual TCRs in a clinical setting (36). This would enable TCRs to be reconstituted for functional analysis and permit modeling of receptor antigen binding. In future, this might potentially assist in the production of tumor-specific T cells for therapeutic use (37). Because of constraints regarding the availability of tissue (deaggregated fresh nodal tissue was not available), we were unable to sort into T-cell subsets. Furthermore, there was insufficient tissue remaining to analyze MHC polymorphisms. These would all be important to consider in future studies. In addition, as this study is limited by the use of FFPE biopsies, it is not possible to input a known quantity of T cells for sequencing. We utilized an approach that focused on percentage of frequency of clones rather than copy counts to allow comparison across samples with different T-cell inputs and thus differing sequence outputs. Also, the nature of the different T-cell subsets in which clones reside could not be determined, except that in general terms that higher *CD8* RNA levels were associated with greater T-cell clonal expansions relative to *CD4*.

To our knowledge, this is the largest TCR sequencing analysis for any human cancer. The data demonstrate that the intratumoral T-cell repertoire in aggressive B-cell lymphoma is less diverse and more clonal than T-cell populations seen in nondiseased nodes. Tumors with large clonal T-cell expansions were associated with inferior outcomes that were independent of conventional prognosticators following conventional first-line therapy with R-CHOP. The TCR repertoire is a key determinant of the TME. Collectively, these results provide important insights into a potential link between the TCR repertoire and efficacy to R-CHOP in DLBCL and should be factored in the rational design of novel immune-based therapies. The predictive utility of the TCR repertoire with checkpoint blockade therapy for DLBCL remains to be determined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Keane, K. Jones, M.K. Gandhi
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Published OnlineFirst September 20, 2016; DOI: 10.1158/1078-0432.CCR-16-1576

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Grant Support

We acknowledge funding support from the Pathology Queensland Study, Education and Research Committee, the Leukaemia Foundation of Queensland, the Leukaemia Foundation of Australia – Bridgestone Award, Metro South Health Private Practice Trust Fund, and the Princess Alexandra Research Foundation.

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Received June 23, 2016; revised August 31, 2016; accepted September 2, 2016; published OnlineFirst September 20, 2016.

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Clin Cancer Res Published OnlineFirst September 20, 2016.

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