The T-cell receptor repertoire influences the tumor microenvironment and is associated with survival in aggressive B-cell lymphoma.

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Translational Relevance

Several studies have high-lighted 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 front-line (i.e. non checkpoint blockade) therapy. Observations in the setting of a non-defined tumor antigen (EBV-ve DLBCL) were contrasted with those with a well-defined tumour 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.
Abstract

Purpose: To investigate the relationship between the intra-tumoral T-cell receptor (TCR) repertoire and the tumor microenvironment (TME) in de novo Diffuse Large B-cell Lymphoma (DLBCL), and the TCR’s impact on survival.

Experimental Design: We performed high-throughput unbiased TCRβ sequencing on a population based cohort of 92 DLBCL patients 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 and progression free survival (OS and 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 of 60.0% (95% C.I. 31.7-79.6%), compared to 79.8% in patients with a low dominant clone (95% C.I. 66.7-88.5%, p=0.005). A highly dominant clone also predicted inferior 4-year PFS of 46.6% (95% C.I. 22.5-76.6%) versus 72.6% (95% C.I. 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.

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 is 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, over reliance 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.

The diversity of T-cells is determined by their TCR. The complimentary determining region 3 (CDR3) of the TCR\(^{\beta}\) 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 TCR\(^{\beta}\) 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 TCR\(^{\beta}\) to comprehensively interrogate DLBCL nodal tissues. Data was 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 front-line 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 500ng) collected from two centres (Princess Alexandra Hospital, Brisbane 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 FFPE tissue and clinical annotation (including survival data) availability. Only de novo cases of DLBCL were included. Grade IIIIB or transformed follicular lymphoma, HIV-positive and post-transplant patients were excluded. Twelve non-diseased, normal lymph node tissues (purchased from ProteoGenex Inc. CA) obtained from individuals without lymphoma were also analysed. 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, Carlsbad, CA, USA) as per manufacturer’s instructions. TCRβ CDR3 regions were amplified from between 500ng-2μg of DNA. All samples had the immuno-SEQ™ assay performed at Adaptive Biotechnologies (Seattle, WA, USA). 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 analysed 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) each of those cell’s genomes are produced due to 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 normalised productive TCRs) to exclude clonality measures being influenced by
a small T-cell population. Four samples were excluded using this cut-off. 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 genomic DNA (ng)*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 i.e. 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, while 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 ten (or 100) most frequent clones (‘% top 10 [or 100] maximal frequency clones’) is the frequency of the top ten (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 normalised 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 analysed is available in Supplementary Table 1.

**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, Carlsbad, CA, USA) as per manufacturer’s instructions. Genes were quantified using the nCounter platform (Nanostring™ Technologies, Seattle, WA, USA) 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 non-paired Mann-Whitney test. Categorical data were compared using Fisher’s exact test or Chi-squared 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 two sided at p=0.05. Multivariate analysis was performed using Cox regression. Analyses were prepared using GraphPad Prism (version 6, La Jolla California USA), Statistical Package for the Social Sciences version 22 (International Business Machines Corporation, New York USA) and immuno-SEQ™ analysis platform (Adaptive Biotechnologies, Seattle, USA).

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 normalised 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 % 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 % total T-cells were ~7-fold higher in the non-diseased normal lymph nodes compared to 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 % 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 % top 100 maximal frequency clones is illustrated in Figure 1. Within patient biopsies, higher diversity (entropy) was present in those in whom the % 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 % maximal frequency clone, with higher CD8 levels relative to CD4 associated with greater T-cell clonal expansions (Figure 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 % maximal frequency clone on survival. This cut-off was established using the upper 95% confidence interval for the mean value of the dominant clones of all samples. These highly dominant i.e. 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 % maximal frequency in the tumor biopsy in the whole cohort was associated with an inferior 4-year OS of 60.0% (95% C.I. 31.7-79.6%), compared to 79.8% (95% C.I. 66.7-88.5%, p=0.005) in patients with a non-dominant or ‘low % maximal frequency’ clone (Figure 3). Progression free survival was also significantly reduced in patients with high % maximal frequency clones with 4-year PFS of 46.6% (95% C.I. 22.5-76.6%) versus 72.6% (95% C.I. 58.8-82.4% p=0.008) for low % maximal frequency clones. In order to establish if a broader number of highly expressed clones showed different or similar findings to that seen with a single highly-
dominant clone, we calculated the % top 10 clones present in the tumor samples and found that patients with a higher proportion of % top 10 clones still had a significantly inferior outcome with 4-year OS of 56.2% (95% C.I. 27.2-77.5%) versus 80.7% (95% C.I. 69.0-88.3%, p=0.01), and 4-year PFS of 64.2% (95% C.I. 36.9-82.1%) versus 81.0% (95% C.I. 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.

**Comparison of the TCR repertoire between melanoma and DLBCL**

We compared our results from patients with DLBCL treated with front-line multi-agent therapy, to a publicly available cohort of patients with melanoma treated with anti-PD1 therapy. In this cohort, melanoma patients responsive to anti-PD1 therapy had significantly higher levels of clonality and a trend for a higher maximal frequency clones compared to non-responders. 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 % maximal frequency clones were significantly higher in melanoma biopsies (p=0.0011, Figure 4A). In addition, the lymphoma cohort had reduced clonality (p<0.0001) compared to the melanoma cohort (Figure 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 front-line therapy.\(^{(21, 22)}\) In seven biopsies EBV encoded RNA (EBER) was detectable at high digital gene counts of >1,500 digital counts and/or were EBER-in situ hybridisation positive, in keeping with a diagnosis of EBV+ DLBCL.\(^{(21, 23)}\) These seven tumor samples were more clonal (Figure 5), with a median clonality of 0.15 (range 0.08-0.4), compared to a median of 0.095 (range 0.06-0.37) for the remaining (EBV-ve) DLBCL samples (p=0.008). Interestingly, in EBV+ tumors, the sum of the % top 10 maximal frequency clones as a % of the productive TCR were >2-fold higher than in EBV-ve DLBCL tumors (median 18.6%, range 3.6-42.6% versus median 7.5%, range 1.46-44% respectively, 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, six of seven patients had relapsed/refractory disease and five 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 of 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 seven 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).

**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 24-1417, 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 is 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 % total T-cells, but a more clonal and less diverse T-cell population compared to 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 is minimal data regarding TCR sequencing and response in any histological lymphoma subtype, however some interesting findings have emerged from studies of relapsed/refractory solid-tumors. In metastatic melanoma, \textit{CTLA-4} blockade diversifies the peripheral T-cell pool.\cite{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 \textit{PD-1} blockade, a more clonal T-cell repertoire was associated with improved outcome.\cite{7} The latter finding is in keeping with our observations in a large cohort of \textit{de novo} DLBCL patients with long-term follow-up, where high ratios of \textit{PD-1} axis molecules to T-cell molecules were associated with adverse outcome.\cite{2} Furthermore, there remains no information on the impact of the TCR-repertoire on lymphoma outcomes in the conventional front-line (i.e. not treated with \textit{PD-1} blockade) therapeutic setting. We demonstrate that high % maximal frequency clones are associated with inferior survival. This suggests that a highly clonal TCR-repertoire may contain gaps, meaning that the intra-tumoral T-cells do not provide adequate immune-surveillance to target all malignant B-cell sub-clones.

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.\cite{25} This can lead to the development of chemo-resistant malignant B-cell sub-clones. 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 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 neo-antigen burden. (28, 29) Consistent with this, it is known that sub-dominant 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 i.e. 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 sub-dominant 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 if 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 anti-tumor cytokine production.

Our findings provide interesting insights into a sub-group of DLBCL associated with high levels of EBV expression within the tumor. EBV+ DLBCL has poor outcome. TCR sequencing indicates that these patients have particularly large intra-tumoral T-cell clonal expansions. This is unsurprising, as it is known that EBV has numerous latent antigens that elicit effective T-cell responses. 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 confirm 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 sub-clone immune escape, as has been observed in adoptive transfer of EBV-specific T-cells for EBV+ lymphomas. TCR-repertoire analysis within a larger EBV+ DLBCL cohort treated with conventional front-line therapy is required to confirm this.

In melanoma, patients responsive to anti-PD1 therapy had significantly higher clonality measures compared to non-responders and a trend for a higher % maximal clone frequency. It may be that patients with DLBCL who have a high % 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, whilst DLBCL had more clonal T-cell populations compared to 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 based on a narrow or broad repertoire e.g. radiation or ipilimumab 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 based not only on the TCR repertoire but incorporating other factors such as mutational load or specific mutations present in an individual's tumour.

There are several limitations of this study. One is that we provide only a snapshot of TCR diversity restricted to the TCR\(\beta\) CDR3 region. A functional TCR is made up \(\alpha\) and \(\beta\) chains so our findings likely underestimate the degree of diversity within samples. Recent developments may allow characterization of the \(\alpha\) and \(\beta\) 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) Due to 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 % 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 demonstrates that the intra-tumoral T-cell repertoire in aggressive B-cell lymphoma is less diverse and more clonal than T-cell populations seen in non-diseased 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.

Authors Contributions


Author Disclosure

DH is an employee of Adaptive Biotechnologies. There are no other conflicts of interest.

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References:


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<td>Entropy</td>
<td>9.7 (5.6--13.4)</td>
<td>13.6 (11.87--14.59)</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Clonality</td>
<td>0.097 (0.059--0.4)</td>
<td>0.057 (0.043--0.11)</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>% Max Freq Clone</td>
<td>2.09 (0.24--35.22)</td>
<td>0.51 (0.29--3.3)</td>
<td>&lt;0.00</td>
</tr>
</tbody>
</table>

Table 1. Comparison of DLBLCL and non-diseased nodal tissue with regard to TCRβ sequencing. All values quoted are median (with range)

Figure 1. % Top 100 maximal frequency clones.

% Top 100 maximal frequency clones with the % of total TCR population they represent, in a representative (A) Non-diseased normal lymph node tissue, (B) Nodal tissue in a patient with DLBLCL.

Figure 2:

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

Figure 3. The influence of the TCR-repertoire on survival in de novo DLBLCL treated with front-line R-CHOP.
Kaplan Meier survival plots showing (A) Progression Free and (B) Overall survival comparing patients with a high % maximal frequency to those with a low % maximal frequency clone; (C) Progression free and (D) Overall survival comparing patients with a high % Top 10 maximal frequency clones versus those with a low percentage.

**Figure 4. Comparison of TCR populations between DLBCL and melanoma**

Higher levels of (A) % maximal frequency clone and (B) clonality in pre-treatment melanoma compared to DLBCL samples.

**Figure 5. A comparison of the TCR-repertoire in EBV⁺ DLBCL and EBV⁻ve DLBCL**

A comparison between the (A) clonality in EBV⁺ve DLBCL and EBV⁻ve DLBCL; and (B) the sum of % top 10 maximal frequency clones present as a percentage of the total productive TCRs in EBV⁺ve DLBCL and EBV⁻ve DLBCL cases.
Figure 1

4.2% of total TCRs

64.23% of total TCRs
Figure 2

P = 0.045

CD4:CD8 Ratio

Low Max Freq %

High Max Freq %

P = 0.045
Figure 3
Figure 4
Figure 5

A

Clonality score

P = 0.008

DLBCL | EBV DLBCL

B

% Top Ten Clones

P = 0.006

DLBCL | EBV DLBCL
The T-cell receptor repertoire influences the tumor microenvironment and is associated with survival in aggressive B-cell lymphoma.

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