

Reciprocal T-cell and myeloid remodeling at relapse in diffuse large B-cell lymphoma

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Abstract

Despite advances in immune-based therapies, most patients with relapsed/refractory diffuse large B-cell lymphoma (rrDLBCL) have poor outcomes. Focusing on immune changes during therapy, we performed a large integrated analysis of gene expression profiling, T-cell receptor (TCR) sequencing, spatial profiling and whole exome sequencing (WES) across 140 clinically annotated rrDLBCL patients, including 87 with matched diagnostic and relapse biopsies. Targeted immune gene profiling demonstrated coordinated downregulation of T-cell abundance and functional immune programming at relapse, alongside increasing myeloid-based immunosuppression and enrichment for a fibroblast/macrophage (FMAC) archetype. Late relapsing patients contrasted with early-relapsing/refractory patients, showing relative preservation of T-cell infiltration and diversity, less immunosuppressive macrophage polarization, and improved overall survival. T-cell abundance correlated with salvage chemotherapy response, and low TCR diversity at relapse predicted inferior overall survival. Cell of origin showed higher concordance across relapse than either DLBCLclass or LymphGen. COO stability was independent of relapse timing; however genetic categorization switching did not correlate with relapse timing or TME archetype. These findings underscore the importance of tumor microenvironment (TME) evolution in DLBCL relapse, and support further interrogation of the TME in prospective trials to guide possible new combinatorial approaches and sequencing of T-cell-directed strategies.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive lymphoma¹ and while approximately 60% of patients are cured with standard chemo-immunotherapy, most patients with relapsed or refractory DLBCL (rrDLBCL) experience poor outcomes². Traditional intensive salvage chemotherapy followed by autologous stem-cell transplant (ASCT) is feasible in only a subset of patients, and fewer than half of those achieve durable remission². In recent years, chimeric antigen receptor (CAR) T cells and bispecific antibodies have improved patient outcomes and largely replaced intensive chemotherapy at relapse however durable remission is still only achieved in fewer than 50% of patients³⁻⁵. As both CAR-T and bispecific antibodies continue to be evaluated not only in relapse but also as components of upfront therapy, the role of the tumor microenvironment (TME) *at each disease timepoint* becomes central to treatment response and relapse biology. Furthermore, although recent genomic studies demonstrate that genetic changes at relapse are relatively modest^{6,7}; the marked difference in efficacy of checkpoint inhibitors at relapse (very low response rates)⁸ compared to encouraging results as immune priming prior to first-line therapy⁹ suggests that the TME may undergo substantial reprogramming during therapy for DLBCL. This may create a relapse immune milieu resistant to reinvigoration, consistent with pan-cancer evidence that the trajectory of TME remodeling during immunotherapy, rather than the pre-treatment state alone, determines outcome¹⁰.

Late relapsing patients have traditionally demonstrated better outcomes and higher rates of chemosensitivity^{11,12}, and ASCT remains an option for these patients, with minimal evidence to support CAR-T therapy superiority at second-line in this population¹³. The cell-of-origin (COO) and genetic profile of late relapsing patients has been recently well described by Hilton et al.⁶, however an equivalent in-depth analysis of the tumor microenvironment in relation to relapse timing has yet to be reported. Further defining the biological distinctions between early- and late-relapsing disease and how these relate to evolving immune dysfunction will be crucial for refining the sequencing of immune-based treatments.

Studies of the DLBCL TME have historically yielded conflicting results, reflecting marked disease heterogeneity, small cohort sizes and variable analytical platforms, however, the expansion of technologies including bulk gene expression deconvolution, public dataset computational analysis, single-cell sequencing and spatial platforms have created a clearer and more reproducible understanding of the DLBCL TME^{14,15}. Building on earlier work identifying host-response and stromal gene-expression signatures as biologically distinct, prognostically relevant subsets of DLBCL^{16,17}, several large-

scale computational frameworks including Kotlov et al.'s four lymphoma microenvironment categories¹⁸, the EcoTyper atlas of malignant cell states and tumor ecosystems¹⁹, and the LymphoMAP archetype classification¹⁵, have demonstrated the prognostic significance of TME composition at diagnosis. There has been limited assessment of the DLBCL TME in paired cohorts, with focus predominantly on newly diagnosed patients and to a lesser extent, relapse cohorts without paired biopsies assessed.

Here, we have assembled one of the largest paired-biopsy cohorts of rrDLBCL with longitudinal immune profiling using targeted gene expression analysis, T-cell receptor repertoire assessment, spatial profiling and whole exome sequencing (WES) to evaluate the evolution of the TME and the malignant B-cell compartment. We identify progressive T-cell and macrophage/myeloid changes as a recurrent, clinically relevant feature of relapse with implications for response in the era of T-cell therapy.

Methods

Patient Samples

Retrospective review identified 197 adult patients with relapsed or refractory DLBCL treated with front-line immunochemotherapy with archived diagnostic +/- relapse/refractory biopsies from 6 Australian sites between 2004–2021. Patients with HIV-associated DLBCL and post-transplant lymphoproliferative disorder were not included. Patients were excluded from analysis for insufficient clinical annotation or failure to obtain NanoString gene expression profiling for at least one timepoint, creating an rrDLBCL cohort of 140 patients (Fig. 1a). Eighty-seven patients had paired diagnostic and relapse biopsies, including 16 with three or more serial samples (Fig. 1b). Staging and response were recorded as per Lugano staging and response classification²⁰. Histological diagnosis was harmonized with the WHO 2017 classification system²¹ with MYC, BCL-2 and BCL-6 rearrangement results obtained from clinical records where performed. Relapse or primary refractory disease was histologically and/or radiologically confirmed. Late relapse was defined as relapse occurring greater than 12 months from diagnosis. Patients were categorized as primary refractory (n = 42, 30.0%), early relapse (n = 43, 30.7%) and late relapse (n = 55, 39.3%). This distribution was mirrored within the 87-patient paired-biopsy cohort (primary refractory (n = 25, 28.7%), early relapse (n = 25, 28.7%) and late relapse (n = 37, 42.5%)) (Fig. 1b). The availability of each assay per patient is summarised in Fig. 1c.

This study was approved by the Metro South Health ethics committee (AM/2025/QMS/61849), which granted a waiver of informed consent, and conducted in accordance with the Declaration of Helsinki.

RNA and DNA quantification

Archived formalin-fixed paraffin-embedded (FFPE) blocks of diagnostic and rrDLBCL biopsies were correlated with histology reports to select blocks with maximal tumor content and optimal preservation. RNA and DNA extraction was performed on DNase/RNase-free scrolls using Maxwell RSC FFPE (Promega, Madison, WI, USA) and Qiagen Allprep DNA/RNA FFPE extraction kits (Qiagen, Hilden, Germany) as per manufacturer instructions. Quantification was performed with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Gene expression profiling

Immune gene expression was successfully quantified using the NanoString PanCancer Immune panel (NanoString Technologies, Seattle, WA, USA) of 770 immune related genes on 249 biopsies across 140 patients. COO status was assigned using the Lymph2Cx score as previously described²². Lowly expressed genes with normalized expression count less than 20 in more than 90% of samples were excluded. Normalisation, cell abundance scores and pathway signature

scores were determined on the nCounter platform advanced analysis module. Paired differential gene expression analysis was conducted using the limma R package and Wilcoxon matched-pairs signed-rank test; comparison of unpaired samples was made using Mann-Whitney U testing. Correction for false discovery rate was performed using the Benjamini-Hochberg procedure. Two-sided p value < 0.05 was used for significance. Gene set enrichment was assessed by over-representation analysis (ORA) using the clusterProfiler package in R (version 4.4.2) and the Gene Ontology (GO) Biological Process dataset, with the background restricted to the genes represented on the panel. LymphoMAP tumor microenvironment categorization was predicted from normalized NanoString gene counts, as described in the author's methods for analysis of ZUMA-7 trial patient biopsies¹⁵.

Whole exome sequencing

DNA exome libraries were prepared from 103 FFPE tumor samples using the Integrated DNA Technologies (IDT; Coralville, IA, USA) xGen™ DNA Library Prep Kit, followed by exome capture with the Agilent SureSelect Human All Exon V8 probe set (Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced on the Illumina NovaSeq X platform (Illumina, San Diego, CA, USA). As matched germline DNA was not available, tumor-only sequencing was performed. Reads were aligned to the GRCh38 reference genome and variants were identified using the Nextflow Sarek workflow incorporating Mutect2, Strelka and FreeBayes, supported by a panel of normals comprising 64 germline samples. Variants were retained when detected by Mutect2 and at least one additional caller, and filtered according to predicted functional impact, sequencing depth, variant allele frequency and population frequency. Molecular classification was performed using DLBclass and LymphGen with subtype switching assessed using McNemar's exact test. Association between DLBclass cluster and LymphoMAP archetype was assessed using Fisher's exact test. Additional details are provided in the Supplementary Methods.

Five samples were excluded due to low coverage, resulting in 98 analyzable samples (59 diagnosis and 39 relapse) from 74 patients, including 23 patients with paired biopsies. Mean coverage was 87x.

T-cell receptor sequencing

TCR sequencing was performed on 22 pairs of diagnostic and rrDLBCL biopsies using the Archer Immunoverse™ CDR3 Beta/Gamma Kit (IDT) following the manufacturer's protocol. Target enrichment and library preparation were carried out using Archer's proprietary Anchored Multiplex PCR (AMP™) technology. Libraries were sequenced on an Illumina platform to a minimum read depth of 1.5 million reads per sample. Sequencing data were processed using the Archer Analysis Platform. TCR repertoire clonality was assessed using four measures (Shannon entropy, clonotype frequency, clonotype size distribution and clonal space homeostasis), which are defined in Supplementary Methods. Shannon entropy and clonotype frequency were calculated within the Archer Analysis software, and clonotype size distribution and clonal space homeostasis were analysed using the scRepertoire R package (v2.5.1) to assess clonal expansion.

Digital spatial profiling

Spatial Whole Transcriptome Atlas (WTA) and immuno-oncology protein profiling was performed on tissue microarrays (TMA) constructed from duplicate 1.5mm tumor cores using the NanoString GeoMx® Digital Spatial Profiler (DSP) platform following manufacturer workflow. Three fluorescent morphology markers segmented three cell types of interest (CD20-AF488 clone REA1087:tumor marker, CD8a-AF647 clone OTI3H6:CD8 T-cells, CD68-AF594 clone KP0:macrophage (NanoString)) with DAPI as a nuclear stain. Region of interest (ROI) selection and segmentation were performed by two reviewers selecting tumor dense areas with associated highest presence of CD8 and CD68 staining to obtain sufficient nuclei count within segmentation masks. Data were processed and analyzed in the GeoMx DSP Data Analysis Suite for quality control (QC) and normalization. The standR package was used to perform QC, normalization and batch correction.

Paired protein data were analysable for nine patients and paired WTA data for six, reflecting sample attrition from inadequate remaining tissue, particularly at relapse.

Statistical Analysis

Descriptive statistics were used to present patient and disease characteristics. Continuous variables were compared using the Kruskal-Wallis test, and categorical variables were compared using the Fisher-Freeman-Halton exact test, with variables restricted to patients with a known result. Overall survival (OS) was measured from diagnosis of relapse, or confirmation of refractory disease, through to date of last follow up or death. Survival analysis was performed using Kaplan-Meier curves and the log-rank test.

Results

Late relapse is enriched for favourable disease characteristics and associated with improved overall survival

Baseline characteristics are reported in Table 1. Median age at diagnosis was 64 with slight pre-ponderance to male sex, consistent with prior descriptions of DLBCL epidemiology²¹. Median follow-up from diagnosis was 34 months (range, 3-202 months). Late relapse was associated with more favourable disease at diagnosis; with late relapsing patients more likely to have lower IPI (0–2) at diagnosis ($p = 0.005$). Furthermore, no late relapsing patients harbored concurrent MYC-rearrangement with BCL-2 rearrangement, more commonly referred to as ‘double hit/triple hit’ (DH/TH), compared with 14.3% of refractory patients and 11.6% of early relapse patients ($p = 0.006$). The histological classification of High-Grade B cell lymphoma not otherwise specified (HGBL, NOS) accounted for less than 5% of cases across the entire cohort.

Most patients ($n = 119$, 85%) received traditional Rituximab-Cyclophosphamide-Doxorubicin-Vincristine-Prednisone (R-CHOP) or R-CHOP-like therapy at diagnosis. Twenty-one patients (15%) received an intensified regimen as per physician discretion, either due to high-risk features such as CNS/marrow/peripheral blood involvement, DH/TH/HGBL-NOS or prior R-CHOP exposure ($n = 3$) for low grade lymphoma treatment. Nine patients (6.4%) had a prior diagnosis of low-grade B-NHL, and 17 patients (12%) were diagnosed with concurrent low-grade B-NHL histology at DLBCL diagnosis, with no statistically significant differences based on relapse timing.

At second-line treatment, most patients received intensive salvage regimens ($n = 106$, 75.7%), however, only 45% of these patients (34% of the entire cohort) proceeded to ASCT, consistent with historical rates of ASCT delivery². Notably there was a trend to higher ASCT rates in the late relapsing group (45.5%), compared with the refractory and early-relapsing groups (28.6% and 25.6% respectively, $p = 0.084$). Fifty-four patients proceeded to systemic third-line treatment (38.6%) with only 13 patients (9.3%) proceeding to 4 + lines of treatment. Twelve patients in this cohort received CAR-T at 3rd line therapy (or beyond), and only 5 received bispecific antibody therapy, reflecting Australian practice prior to the wider availability of T-cell-engaging therapies.

Patients with late relapse had significantly superior overall survival compared with those with early-relapsing ($p = 0.0005$) and refractory disease ($p = 0.0002$) (Fig. 1d), consistent with data from previous retrospective studies^{6,11,12}. Median overall survival (from relapse) for late relapsing patients was 71 months, compared to 12 months and 10 months for early-relapsing and refractory patients respectively.

Relapse in DLBCL is characterized by progressive T cell depletion and immunosuppressive myeloid influence through first relapse and beyond

Immune gene expression analysis of 87 paired diagnostic and relapsed biopsies identified 57 differentially expressed genes corrected for multiple testing (Fig. 2a) dominated by downregulation of key T-cell genes (*CD3E*, *CD5*, *CD7*, *CD8A*,

adj. $p < 0.044$), genes associated with immune-cell recruitment (*CCL19*, *CXCL9*, adj. $p < 0.0003$) and immune signaling (*IRF3*, *IKBKE*, *JAK2*, *RELA*, *MYD88*, *STAT6*, adj. $p < 0.037$). Relapse was further characterized by upregulation of markers suggestive of polarization toward a scavenger-receptor-driven, immunosuppressive tumor-associated macrophage (TAM) phenotype^{23–25}, including *SPP1*, *MSR1* and *TREM2* (adj. $p < 0.016$) (Fig. 2b).

Gene Ontology Biological Process (GO:BP) enrichment analysis, using the genes on the panel as background, demonstrated coordinated suppression of pathways governing T-cell activation, co-stimulation, and immune response at relapse (Fig. 2c). Confirmatory analysis of predefined nCounter pathway signature scores showed broad down-regulation of T-cell function, cytotoxicity, antigen processing, and interleukin signalling at relapse (Fig. 2d). Cell abundance scores demonstrated a marked reduction in overall T-cell abundance, particularly total CD8 and exhausted CD8 T-cell subtypes at relapse ($p = 0.015$) (Fig. 2e). Digital spatial profiling of protein (9 patients with paired biopsies) and whole transcriptome (6 patients with paired biopsies) supported the bulk gene expression profiling (GEP) with overall dampening of T-cell activation and signalling (Supplementary Fig. 1).

Analysis of 16 patients with three serial biopsies available demonstrated ongoing reduction in overall T-cell and exhausted CD8 T-cell nCounter abundance scores ($p = 0.0125$) across treatment episodes (Fig. 2f). *SPP1* and *MSR1* continued to increase through to subsequent relapse timepoints ($p < 0.0016$) (Fig. 2g), despite no significant change in macrophage abundance.

Late relapse is immunologically distinct: T-cell preservation and stromal remodeling, not macrophage polarization

Stratifying by relapse type revealed that the T-cell/immune-signalling reduction seen at relapse was statistically robust in refractory and early-relapse patients but not significant in late-relapse patients, indicating that late relapsers largely preserve their T-cell compartment rather than undergoing the depletion characteristic of early treatment failure (Fig. 3a). This was corroborated by NanoString cell abundance scores, with late-relapsing patients demonstrating preserved T-cell abundance across timepoints compared with refractory and early-relapsing patients (Fig. 3b).

The myeloid/stromal signature also diverged dramatically by relapse timing. Increases in *SPP1*, *MSR1* and *TREM2* were confined to refractory and early-relapsing patients (Fig. 3c). Consistent with this, the *CXCL9:SPP1* ratio was significantly reduced from diagnosis to relapse overall ($p = 0.0001$), but this was driven by refractory ($p = 0.0023$) and early-relapsing ($p = 0.0016$) patients, with no significant change in late-relapsing patients ($p = 0.26$; Supplementary Fig. 2). Instead, late-relapsing patients showed upregulation of stromal markers (including *FN1* and *PDGFRB*) consistent with an extracellular-matrix/fibroblast-associated program rather than immunosuppressive macrophage polarization, a pattern that parallels the favourable stromal signatures described by Lenz et al. (Stromal-1)¹⁶ and Kotlov et al. (Mesenchymal LME)¹⁸ at diagnosis, rather than the adverse macrophage remodeling seen in early treatment failure.

Acquisition of an FMAC-like microenvironment is a feature of DLBCL relapse but agnostic of relapse timing

LymphoMAP archetypes¹⁵ (lymph node, LN; fibroblast/macrophage, FMAC; and T-cell exhausted, TEX) were assigned for diagnosis and relapse biopsies from NanoString immune gene profiling, with a significant switch to the FMAC archetype at relapse ($p = 0.0015$) (Fig. 4a). Of the 87 patients with archetype calls at both timepoints, 44% (38/87) changed classification between diagnosis and relapse (Fig. 4b). Switching was directional, with 25% of the cohort (22/87) acquiring an FMAC classification at relapse that they did not have at diagnosis, versus 7% (6/87) switching to LN and 11% (10/87) to TEX. FMAC acquisition occurred in every relapse timing group (early relapse 32%, 8/25; primary refractory 24%, 6/25; late relapse 22%, 8/37), indicating that this switch is not restricted to a particular relapse timing. Because these groups showed divergent gene expression changes at relapse, the shared shift toward FMAC may reflect distinct macrophage-associated and stromal-associated programs converging on a common archetype, as FMAC integrates fibroblast and macrophage modules. Santiago et al. applied the same archetype classifier to paired diagnosis-relapse

DLBCL biopsies from 65 patients, and similarly found that switching was common (48%) and unrelated to relapse timing²⁶, though they reported no directional trend, unlike in our cohort. Overall survival among patients treated with intensive therapy at relapse did not differ based on LymphoMAP archetype at relapse (Fig. 4c).

T-cell quantity and clonality are associated with clinical outcome

Among intensively salvaged patients who had GEP results at relapse, complete response (CR) was achieved in 32 out of 70 patients (46%), with 38 patients (54%) achieving less than CR. Achieving CR was associated with a higher total T-cell abundance, CD8 T-cell abundance and exhausted CD8 T-cell abundance at relapse (Fig. 5a). Paired T-cell receptor sequencing in 22 patients demonstrated concordance of overall T-cell repertoire across disease timepoints, independent of relapse timing (Fig. 5b), however, low TCR diversity at relapse was associated with inferior overall survival ($p = 0.014$) (Fig. 5c), mirroring observations previously reported in newly diagnosed DLBCL^{27,28}. Analysis of clonal composition revealed that patients with primary refractory or early-relapsing disease were more likely to harbor hyper-expanded clones (clones representing > 10% of the total repertoire) at diagnosis, with this proportion increasing at relapse. In contrast, late-relapsing patients exhibited few hyper-expanded clones at either timepoint and instead showed an enrichment of small clones (0.01–0.1% of the repertoire) at relapse (Fig. 5d).

COO is highly concordant through to first and subsequent relapse events

Among 123 relapsed/refractory patients with evaluable data from diagnosis, 63 (51%) were classified as germinal-center B-cell-like (GCB), 41 (33%) as activated B-cell-like (ABC), and 19 (15%) as unclassified. Within the 87 patients with paired biopsies, COO was highly concordant between diagnostic and relapse samples (Fig. 6a), with only two patients (2%) demonstrating definitive class switching. Both were very late-relapsing cases, occurring 54 months and 38 months after initial diagnosis. This pattern mirrors that reported by Hilton et al. and is consistent with their analysis demonstrating that late relapse may arise from genetically distinct clones⁶.

COO was not prognostic at relapse (Fig. 6b). Concordance of COO persisted through subsequent disease events: in 16 patients with three or more serial timepoints, including two with four, the Lymph2Cx classifier remained stable from diagnosis through to the final biopsy (Fig. 6c), underscoring the stability of COO biology even in multiply relapsed DLBCL.

Molecular classification shows less concordance than COO at relapse and does not predict TME archetype

DLBclass and LymphGen assignment was performed on 74 patients using WES (98 samples: 59 diagnostic biopsies, 39 relapse biopsies) and MYC-R/BCL2-R/BCL6-R results where available. Relative proportions of DLBclass and LymphGen classifications are shown in Fig. 7a, with no significant cluster increase evident at relapse. Paired data were analysable for 23 patients, with 57% DLBclass concordance. There was no statistically significant evidence of directional switching ($p > 0.25$), though the cohort is underpowered to rule out a modest effect (Fig. 7b). Highest mutational concordance was seen in primary refractory patients (Fig. 7c), excluding 2 cases of T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL) given the low proportion of the malignant B-cell clone. Neither DLBclass nor LymphGen cluster assignment predicted LymphoMAP archetype at diagnosis, consistent with previous findings¹⁵. This was also demonstrated at relapse (Supplementary Fig. 3a).

As a cohort, KMT2D and CREBBP were the most frequently mutated genes at diagnosis (56% and 37%); TP53, though less frequent (29%), was also recurrently mutated (Supplementary Fig. 3b). Unpaired analysis showed no significant change in mutation frequency from diagnosis to relapse. Paired biopsies showed consistent mutation of commonly mutated genes (Fig. 7d), with many genes mutated in both samples from the same patient, although private mutations at

either timepoint were also evident, and the number of paired patients (n = 23) was not powered to detect mutational acquisition or loss.

Discussion

In this study, we conducted a multiomics analysis of a large, real-world, clinically annotated rrDLBCL cohort and demonstrated significant longitudinal remodeling of the TME at relapse. Relapse was associated with divergent immune programs: a coordinated reduction in T-cell abundance and functional T-cell programming, occurring alongside an increase in myeloid-based immunosuppressive signatures. Importantly, relapse timing defined distinct immune trajectories, with late-relapsing disease retaining T-cell functionality and lacking the immunosuppressive macrophage polarization seen with refractory and early-relapsing disease, a distinction that aligned closely with clinical outcome. These findings support the view that TME evolution, rather than genetic divergence alone, is a key feature of relapse biology, with potential direct implications for treatment selection and sequencing in the current era of T-cell-engaging therapies.

To our knowledge, this is the largest paired-biopsy rrDLBCL cohort profiled for the microenvironment to date, and it adds to literature that has focused primarily on genetic changes at relapse^{6,7,29}. Across 87 patients with paired biopsies, relapse was characterized by reduced expression of genes associated with T-cell identity, TCR signalling and effector function, with concordant suppression of T-cell activation pathways, lower T-cell function signature scores and reduced T-cell abundance, particularly of CD8 + and exhausted CD8 + subsets. Serial biopsies showed that T-cell loss was progressive across treatment episodes, supporting cumulative immune attrition, and complementing the decline in TME favourability and rise in immunosuppressive signalling across successive prior therapy lines reported by Locke et al.³⁰.

T-cell changes in the TME occurred alongside an increasingly immunosuppressive myeloid compartment, but differed by relapse timing. T-cell changes were largely confined to primary refractory and early-relapsing disease, whereas late relapsing patients maintained relatively preserved T-cell abundance and function. Myeloid/stromal changes occurred in all timing groups, but the underlying programs diverged sharply: upregulation of immunosuppressive macrophage markers (*SPP1*, *MSR1* and *TREM2*) was restricted to primary refractory and early-relapsing patients, while late relapsing patients instead upregulated stromal/extracellular-matrix-associated genes, including *FN1* and *PDGFRB*. In keeping with this, the *CXCL9:SPP1* ratio fell from diagnosis to relapse only in primary refractory and early-relapsing patients. Although derived from bulk gene expression and therefore not attributable to macrophages specifically, this ratio was described by Bill et al. across solid tumors as a measure of macrophage polarity, with *SPP1*-high macrophages associated with immunosuppressive, tumor-promoting programs and *CXCL9*-high macrophages with T-cell-inflamed microenvironments³¹, supporting a shift toward *SPP1*-associated polarization in these groups, although single-cell or spatial validation in DLBCL is required. The late relapsing pattern resembles the prognostically favorable stromal and mesenchymal programs described at diagnosis by Lenz et al.¹⁶ and Kotlov et al.¹⁸, rather than the adverse myeloid remodeling characteristic of early treatment failure.

Applying broader LymphoMAP archetype assignment, FMAC acquisition was the dominant direction of archetype switching but occurred independent of relapse timing. As the FMAC archetype is defined by both a cancer-associated fibroblast component (FAP+/FN1+) and a tumor-associated macrophage component (including TREM2+)¹⁵, we hypothesize that this timing-independence reflects two distinct routes converging on the same archetype call: an immunosuppressive macrophage-dominant trajectory in primary refractory and early-relapsing disease, and a stromal/fibroblast-dominant trajectory in late relapsing disease. FMAC acquisition may therefore represent divergent underlying biology depending on relapse timing, rather than a single convergent mechanism.

These findings indicate that early treatment failure is enriched for a T-cell-depleted, myeloid-dominant TME that is expected to be least responsive to current immunotherapy approaches, consistent with mechanisms of CAR-T resistance

recently synthesised by Cashman and Turtle³². Locke et al. demonstrated that an immunosuppressive myeloid/stromal gene expression signature was associated with inferior outcomes following CAR-T therapy³⁰, using a mix of archival diagnostic and treatment-proximal biopsies, underscoring the added value of the longitudinal, paired biopsy design used in the present study. Similarly, Li et al. showed that the LN archetype derived the greatest CAR-T benefit over salvage chemotherapy compared with FMAC and, especially, TEX¹⁵. The DLBCL-IQ molecular and immune classification framework associated a “hot” immune phenotype with significantly improved response to the bispecific antibody mosunetuzumab but not to CAR-T across two independent cohorts, possibly reflecting non-TME determinants of CAR-T response such as tumor bulk, LDH and inflammation³³. These TME findings mirror an emerging pan-cancer paradigm in which the direction of immune microenvironment remodeling during checkpoint inhibitor therapy determines response, with persistence in or transition toward myeloid dominance consistently associated with resistance across ten solid-tumor types¹⁰.

In contrast to this substantial immune evolution, changes in the malignant B-cell compartment through relapse were comparatively modest. COO remained highly concordant through relapse, whereas DLBclass concordance was more limited (57% vs 74% reported by Calabretta et al.²⁹), with no predominant direction of cluster switching, and molecular cluster identity was not associated with relapse timing or TME archetype at either timepoint. Notably, molecular concordance was highest in primary refractory disease, suggesting the malignant clone is genetically most stable precisely when the surrounding microenvironment is undergoing extensive remodeling. COO was not prognostic at relapse in our cohort, in keeping with the loss of prognostic significance at relapse of other malignant B-cell features, including DH/TH status and molecular classification³⁴; even the significance of TP53 for CAR-T outcomes remains unresolved³². By contrast, T-cell abundance and TCR diversity were associated with response and survival in our cohort. Similar to previous findings, KMT2D, CREBBP and TP53 were frequently altered genes at diagnosis; however, gene-level mutation frequencies at relapse remain inconsistent across published series^{6,7,35,36}, likely reflecting cohort heterogeneity. Taken together, these findings suggest that genetic classification alone is insufficient to capture relapse biology, and that TME evolution should be accounted for alongside molecular classification in future studies.

This study has several limitations. Use of the NanoString PanCancer Immune Profiling panel rather than whole-transcriptome sequencing restricted analysis to 770 immune-related genes, with limited coverage of stromal and extracellular matrix markers that constrained characterization of the stromal changes observed in late relapse. However, its low RNA input requirement allowed more FFPE biopsies to be analyzed than bulk RNA sequencing would have permitted. Copy number data could not be reliably derived from FFPE-derived tumor-only exomes and were not incorporated into DLBclass or LymphGen classification, which may reduce sensitivity for clusters defined in part by copy number alteration, particularly those characterized by TP53 inactivation and aneuploidy (DLBclass C2 and LymphGen A53). Paired TCR sequencing, DSP and DNA sequencing numbers were all limited, reflecting real-world attrition despite an initial 197-patient cohort collected over almost two decades. Nonetheless, we consider paired biopsy analysis fundamental to characterizing DLBCL evolution given its biological heterogeneity. Paired analysis was limited mainly by absent relapse biopsies or core-only sampling, highlighting the need to collect adequate tissue at relapse. Finally, this cohort predates the T-cell-engaging therapy era in Australia, a trade-off necessary to achieve adequate paired biopsy numbers given relatively late local approval of these therapies.

In summary, these findings position the TME as a dynamic and clinically relevant axis of DLBCL relapse biology, distinct from and complementary to genetic evolution. Building on prior work from our group demonstrating the prognostic relevance of host immunity at diagnosis, including T-cell effector:checkpoint gene ratios²² and TCR repertoire narrowing²⁷, this study extends those observations longitudinally, showing that the trajectory of immune remodeling, not simply its endpoint, distinguishes relapse phenotypes with divergent clinical behavior. The retention of T-cell function and stromal-dominant remodeling in late-relapsing disease, in contrast to the progressive immunosuppressive myeloid

polarization seen in early treatment failure, raises the possibility that relapse timing itself may serve as a clinically accessible proxy for underlying TME biology, a hypothesis warranting prospective evaluation. As molecular subtyping is increasingly incorporated into biomarker-directed trials, immune profiling should also be considered, particularly at relapse and in studies of T-cell-engaging therapies and myeloid-targeting agents. Furthermore, as T-cell-engaging therapies and other novel agents become increasingly incorporated into first-line treatment trials, prospectively evaluating their impact on relapse timing and associated TME trajectories will be critical to further informing the treatment of relapsed DLBCL.

Declarations

Authorship

Contribution: FS and CK designed the research and wrote the paper; FS, MB, MS, ZH, NT, RC, LB, LML, SB and PW undertook laboratory work; SK and AM provided bioinformatics support; AH, JS, JW, HM, EG, DT, MKG and EH provided samples and collected clinical data; all authors reviewed and approved the paper.

Competing Interests

FS has received honoraria from Gilead. AH has received consulting, speaker and honoraria fees from Kite Gilead, Astellas, AstraZeneca, Caribou, STBiopharma, The Limbic and Research Review, and research funding from the Gilead Australia Research Fellowship. EH has received research funding (paid to her institution) from Bristol Myers Squibb/Celgene, Merck KGaA, AstraZeneca, TG Therapeutics and F. Hoffmann-La Roche; has acted as a consultant or advisor for F. Hoffmann-La Roche, Antengene, Bristol Myers Squibb, AstraZeneca, Novartis, Merck Sharp & Dohme, Specialised Therapeutics, Sobi, Regeneron and Gilead; has acted as a speaker for Roche, AstraZeneca, Janssen, Regeneron, AbbVie and Genmab; and has received travel expenses from AstraZeneca, AbbVie and Genmab. CK has received honoraria from Gilead, Merck and Roche. The research funding received by AH and EH had no role in this work. All other authors declare no competing interests.

Supplementary Material

Supplementary information is available at Blood Cancer Journal's website

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Data Availability:

The datasets generated and analysed during this study contain patient-level clinical and genomic data and are not publicly available because of patient confidentiality and the terms of ethics approval. De-identified data are available from the corresponding author (Colm Keane, c.keane@uq.edu.au) on reasonable request, subject to ethics approval and a data sharing agreement.

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Tables

Table 1: Baseline Characteristics

	Whole Cohort	Primary Refractory	Early Relapse	Late Relapse	p Value
	(N=140)	(N=42)	(N=43)	(N=55)	
Age at Diagnosis					
Median (Min, Max)	64.0 (22-85)	62.5 (25-85)	66.0 (31-84)	62.0 (34-82)	0.378
Sex					
Male	80 (57.1%)	27 (64.3%)	21 (48.8%)	32 (58.2%)	0.362
Female	60 (42.9%)	15 (35.7%)	22 (51.2%)	23 (41.8%)	
Stage at Diagnosis					
I-II	9 (6.4%)	4 (9.5%)	1 (2.3%)	4 (7.3%)	0.303
III-IV	81 (57.9%)	22 (52.4%)	28 (65.1%)	31 (56.4%)	
Missing	50 (35.7%)	16 (38.1%)	14 (32.6%)	20 (36.4%)	
IPI at Diagnosis					
0-2	32 (22.9%)	9 (21.4%)	4 (9.3%)	19 (34.5%)	0.005
3-5	85 (60.7%)	27 (64.3%)	33 (76.7%)	25 (45.5%)	
Missing	23 (16.4%)	6 (14.3%)	6 (14.0%)	11 (20.0%)	
Histological Classification					
DLBCL	126 (90.0%)	36 (85.7%)	36 (83.7%)	54 (98.2%)	0.006
DH/TH	11 (7.9%)	6 (14.3%)	5 (11.6%)	0 (0%)	
HGBL NOS	3 (2.1%)	0 (0.0%)	2 (4.7%)	1 (1.8%)	
FISH for DH/TH					
Positive	11 (7.9%)	6 (14.3%)	5 (11.6%)	0 (0%)	0.018
Negative	81 (57.9%)	26 (61.9%)	23 (53.5%)	32 (58.2%)	
Not done/failed/missing	48 (34.3%)	10 (23.8%)	15 (34.9%)	23 (41.8%)	
Other					
Prior low grade B-NHL	9 (6.4%)	3 (7.1%)	3 (7.0%)	3 (5.5%)	1.000

Concurrent low grade B-NHL	17 (12.1%)	4 (9.5%)	5 (11.6%)	8 (14.5%)	
First Line Treatment					
R-CHOP/R-CHOP like	119 (85.0%)	35 (83.3%)	37 (86.0%)	47 (85.5%)	0.913
Intensified regimen	21 (15%)	7 (16.7%)	6 (14%)	8 (14.5%)	
Second line Treatment					
Intensive salvage regimen	106 (75.7%)	33 (78.6%)	32 (74.4%)	41 (74.5%)	0.908
– Autologous stem cell transplant	48 (34.3%)	12 (28.6%)	11 (25.6%)	25 (45.5%)	0.084

Figures

Figure 1.

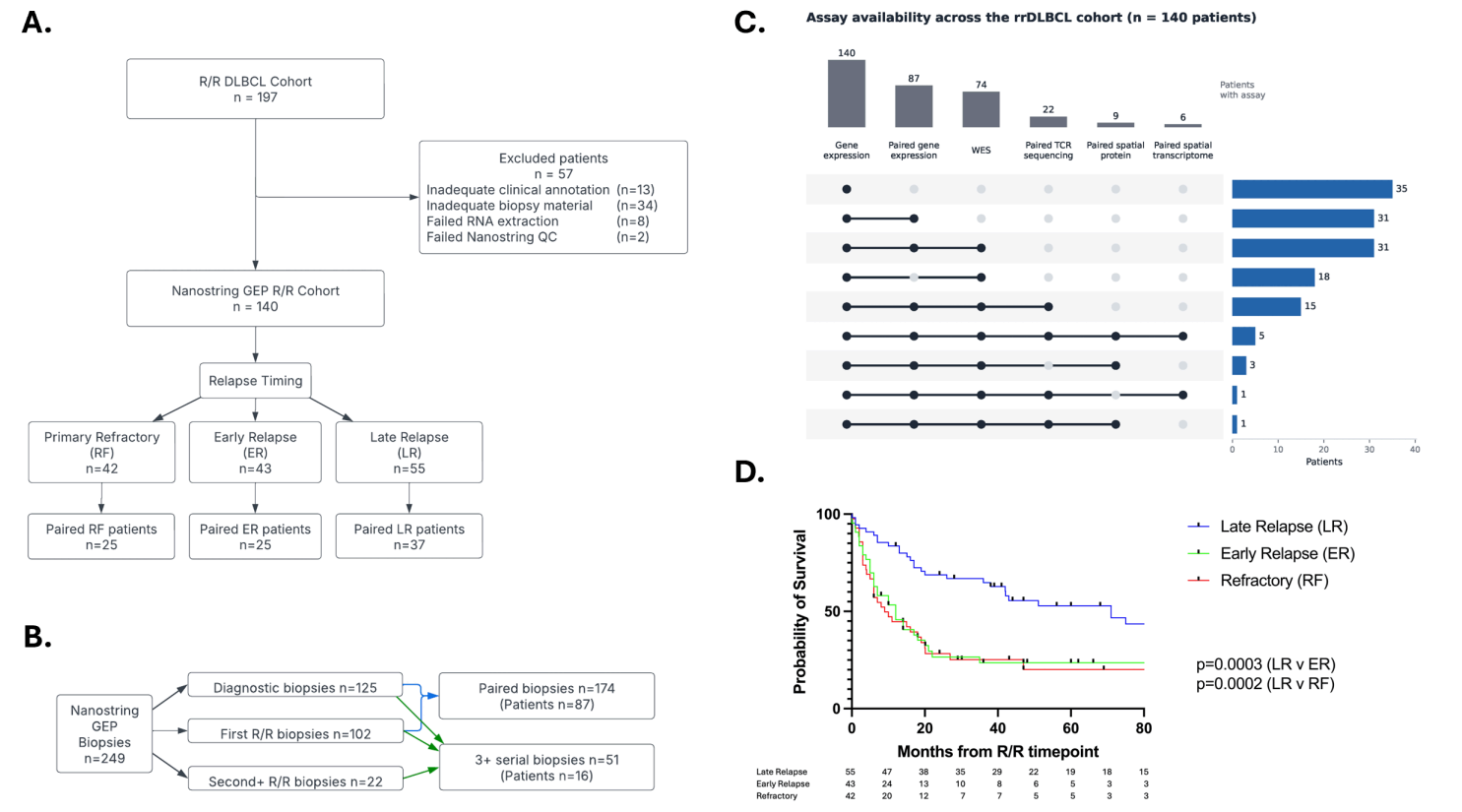


Figure 1
Relapsed/refractory DLBCL cohort characteristics and assay availability.

(A) Flow diagram of cohort selection: 197 patients identified with relapsed/refractory DLBCL (rrDLBCL), yielding 140 patients with NanoString gene expression profiling (GEP); categorised by relapsing timing into primary refractory (RF), early relapse (ER) and late relapse (LR)

- (B) Overview of total rrDLBCL biopsies with NanoString GEP, categorised by disease timepoint and showing patients numbers for paired or serial (3+) timepoints
- (C) UpSet plot of assay availability across the 140 patient cohort (gene expression, paired gene expression, whole exome sequencing (WES), paired T-cell receptor (TCR) sequencing, paired spatial protein and paired spatial whole transcriptome with patient counts for all assay combinations
- (D) Kaplan-Meier overall survival from relapse/refractory timepoint based on relapse timing; late relapse associated with improved survival versus early relapse ($p=0.0003$) and refractory disease ($p=0.0002$). Numbers at risk shown.

Figure 2.

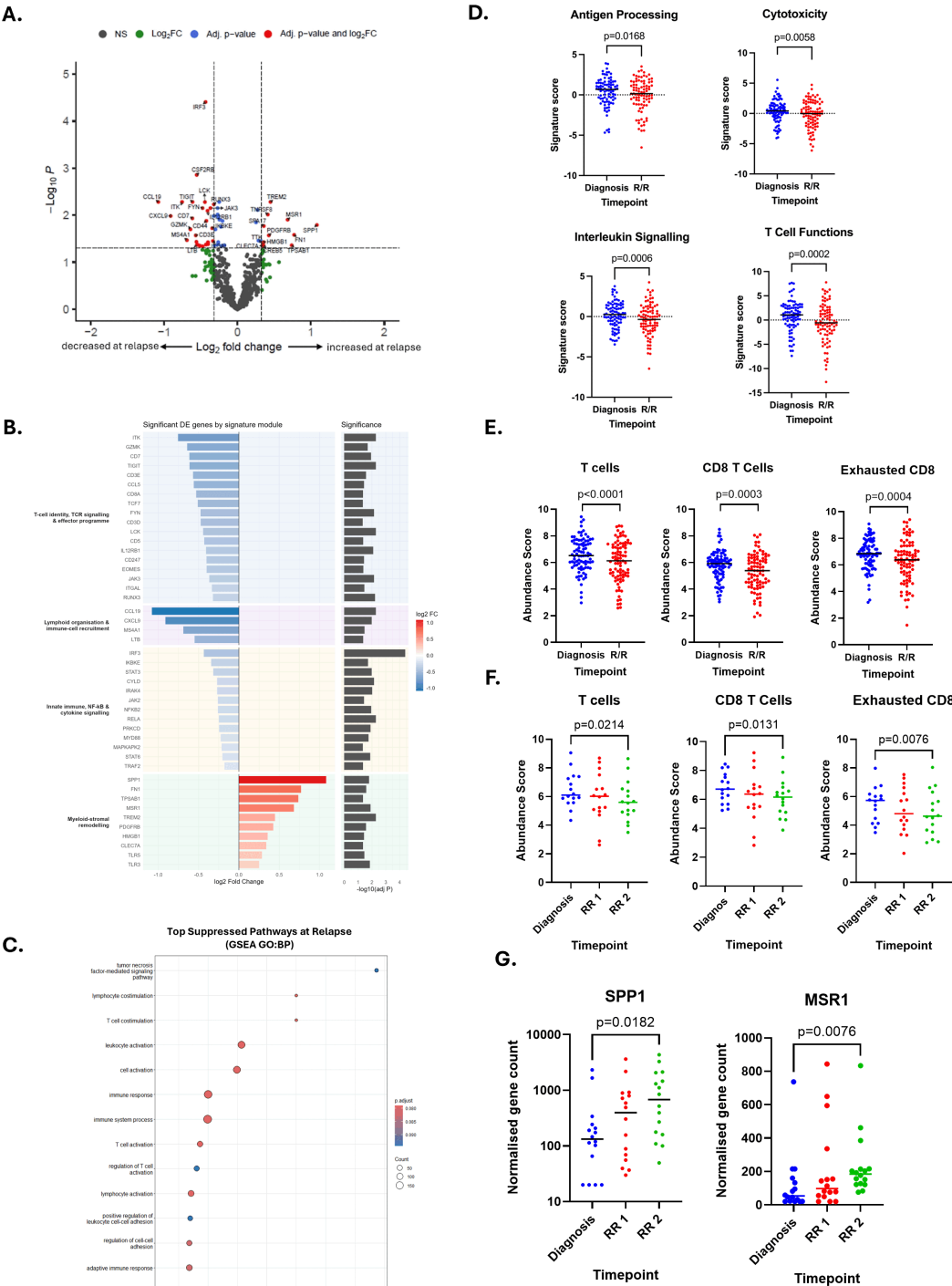


Figure 2

Immune gene expression remodeling from diagnosis to relapse (paired samples).

(A) Volcano plot of paired differential gene expression (NanoString PanCancer Immune panel, n=87 pairs) showing log₂ fold change (FC) against -log₁₀p (p value) with gene expression decreased (left) or increased (right) at relapse, colored by significance of adjusted (adj.) p value and log fold change.

(B) Significantly differentially expressed genes (adjusted p<0.05), grouped by functional modules with log₂ fold change and significance (-log₁₀ adj. p) shown per gene.

(C) Gene Ontology Biological Processes (GO:BP) terms significantly suppressed at relapse by over representation analysis (ORA), ranked by gene ratio; dot size = gene count, color = adj. p value

(D) nCounter pathway signature scores at diagnosis versus relapse/refractory (RR) timepoint. P-values in (D-G) from Wilcoxon matched-pairs signed-rank test.

(E) T-cell, CD8 T-cell and exhausted CD8 T-cell abundance scores at diagnosis versus R/R timepoint

(F) T-cell, CD8 T-cell and exhausted CD8 T-cell abundance scores across 3 serial timepoints; diagnosis, first RR timepoint (RR 1), second R/R timepoint (RR 2)

(G) Normalised *SPP1* and *MSR1* gene counts across diagnosis, RR 1 and RR 2

Figure 3.

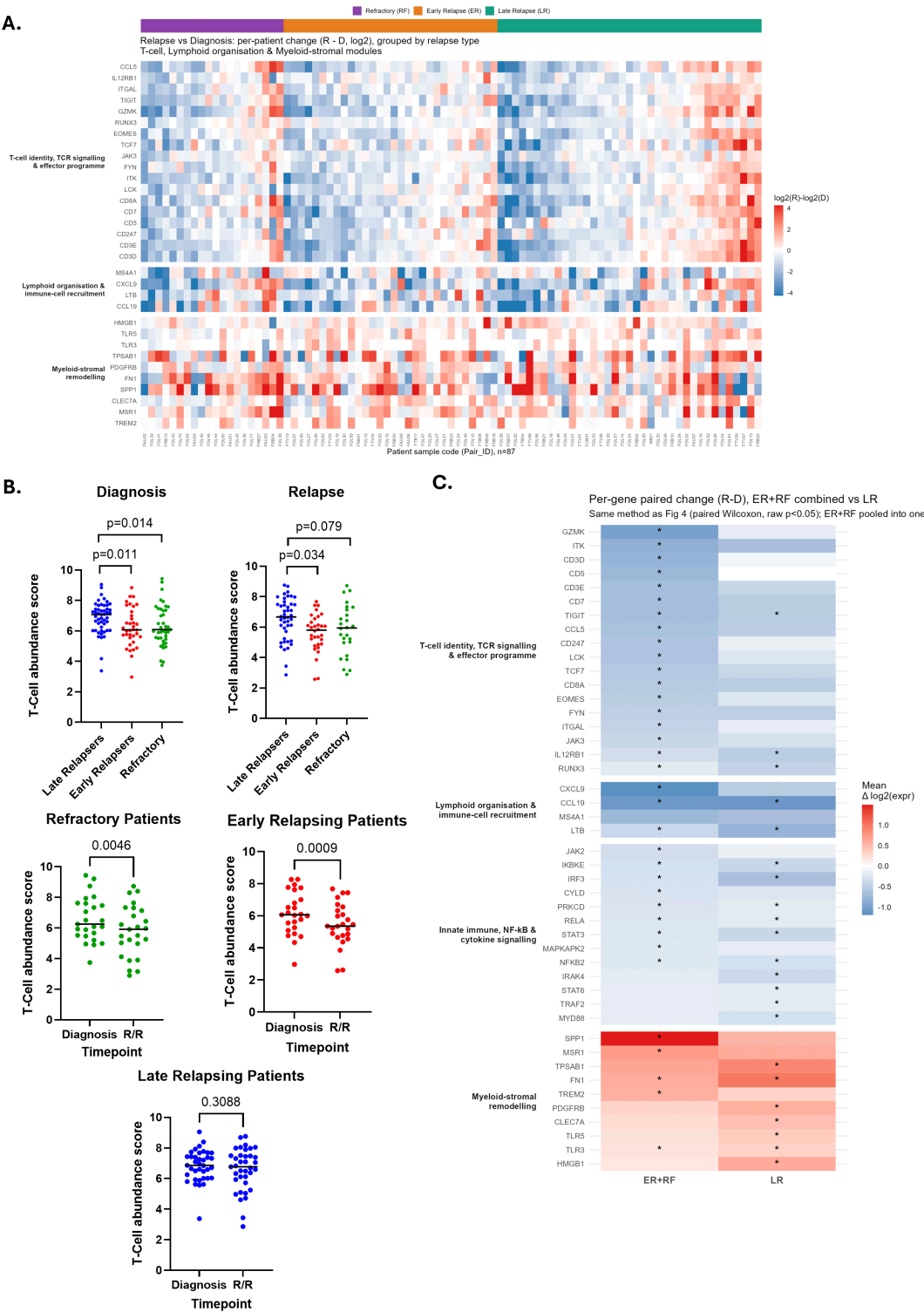


Figure 3

Relapse timing defines distinct immune trajectories.

(A) Heatmap of per-patient change in expression (relapse – diagnosis, log2) grouped by functional module and separated by relapse timing

(B) T-cell abundance scores from diagnosis to relapse compared across relapse-timing groups (refractory, early relapsing, late relapsing). Between group adj. p values from Mann-Whitney U test, across paired biopsies from Wilcoxon matched-

pairs signed-rank test.

(C) Heatmap of per-gene paired change in expression (relapse – diagnosis) comparing early relapse/refractory patients (ER+RF) against late relapsing patients (LR); asterisks denote raw $p < 0.05$ (paired Wilcoxon)

Figure 4.

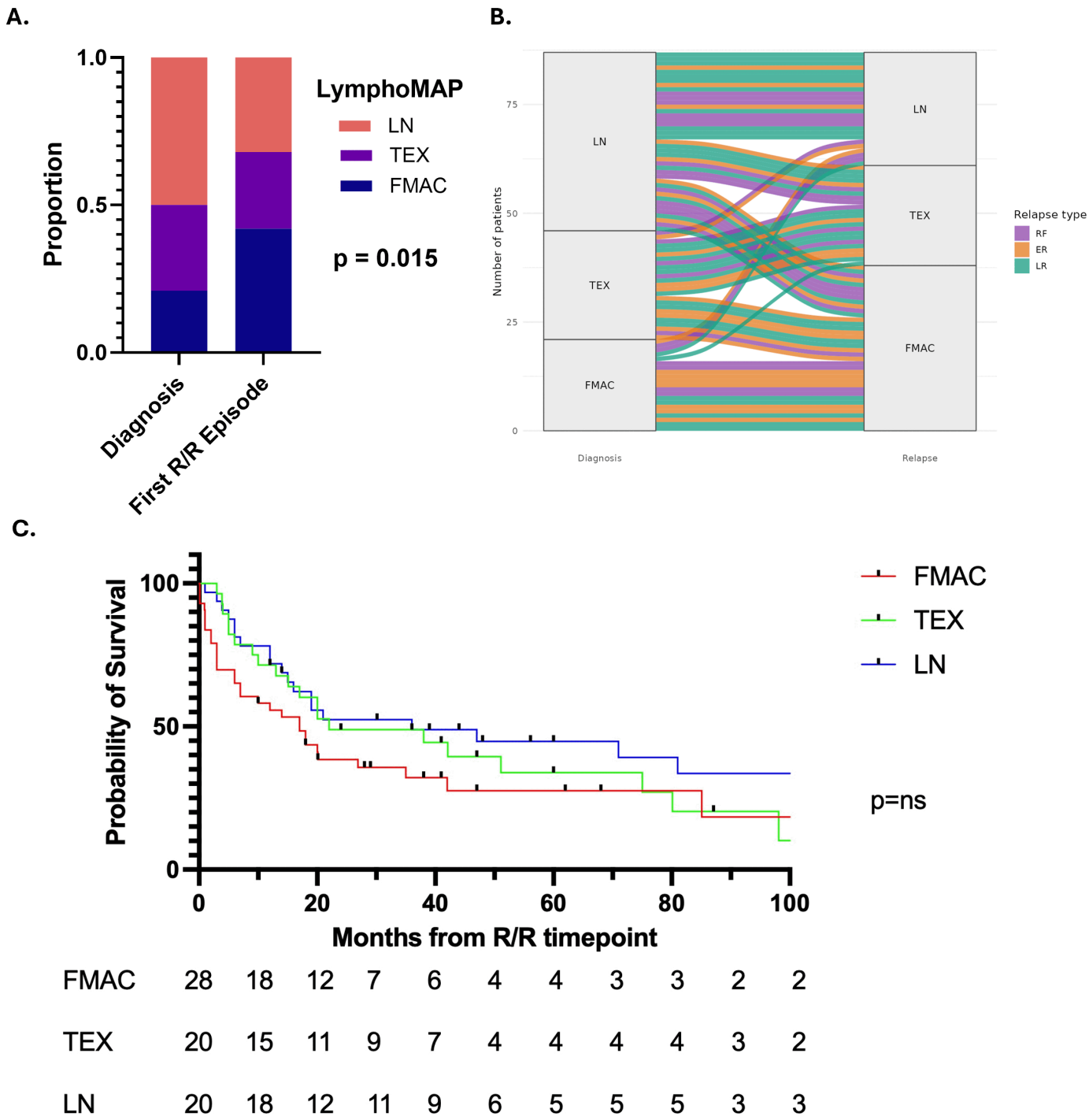


Figure 4

LymphoMAP archetype switching at relapse is dominated by FMAC acquisition, independent of relapse timing

(A) Proportion of patients assigned to LymphoMAP archetype (LN, TEX, FMAC) at diagnosis and first R/R episode (p=0.015)

(B) Alluvial plot of patient archetype from diagnosis to relapse, colored by relapse timing

(C) Kaplan-Meier overall survival for intensively salvaged patients stratified by LymphoMAP archetype at relapse

Figure 5.

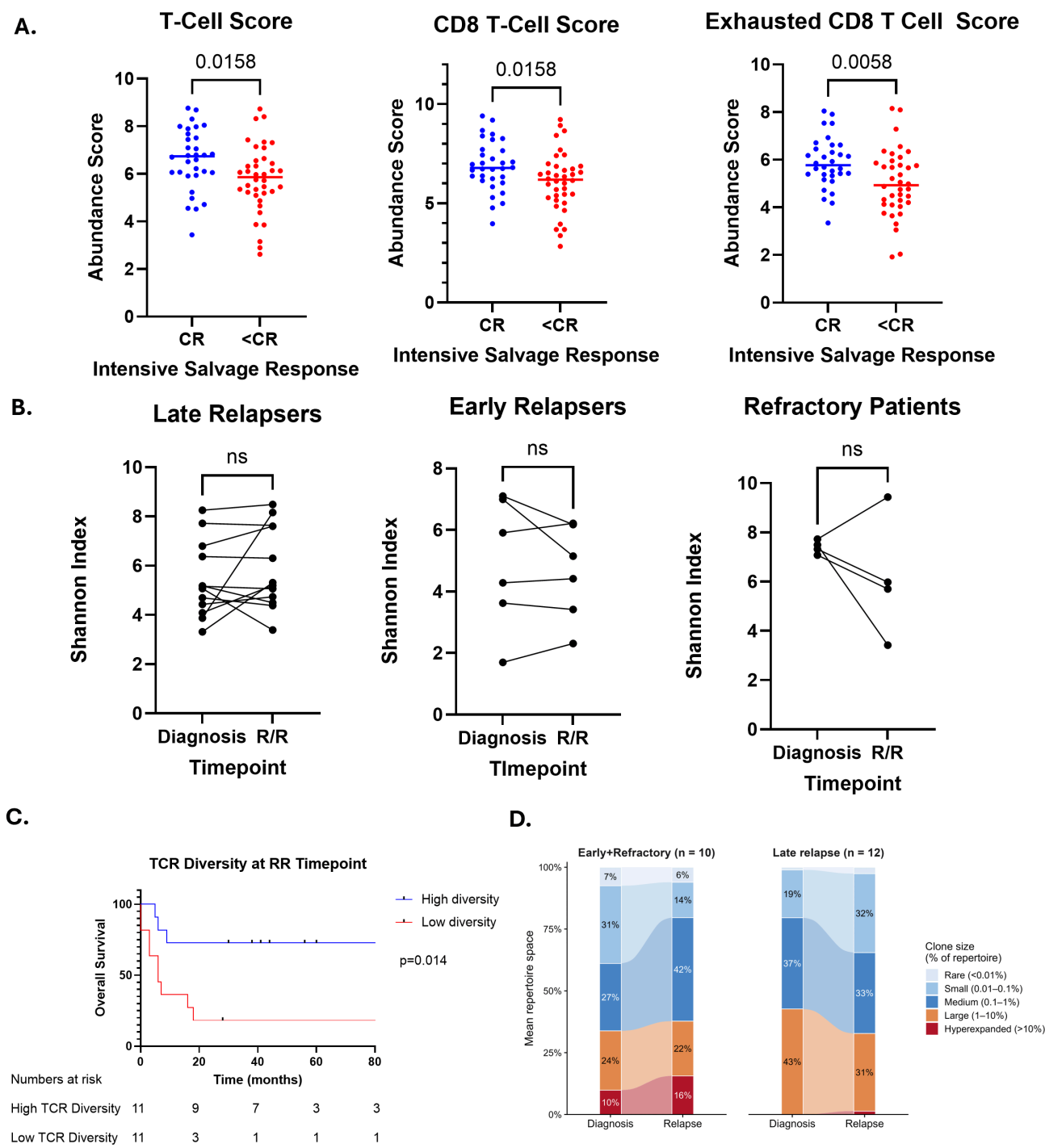


Figure 5

T-cell quantity and clonality associate with treatment response and survival

- (A) T-cell, CD8 T-cell and exhausted CD8 T-cell abundance score at relapse comparing intensive salvage treatment response, complete response (CR) versus less than CR (<CR); Mann-Whitney U test.
- (B) Paired Shannon diversity index calculated from T-cell receptor (TCR) sequencing, from diagnosis to R/R timepoint separated into relapse timing groups (n=22 patients); ns, Wilcoxon matched-pairs signed-rank test.
- (C) Kaplan-Meier overall survival from R/R timepoint showing high versus low TCR diversity measured at R/R timepoint (p=0.014, log-rank test). Numbers at risk shown below.
- (D) Clonal composition of the TCR repertoire from diagnosis to relapse based on relapse-timing group.

Supplementary Files

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