Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response

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Abstract

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease with recognized variability in clinical outcome, genetic features, and cells of origin. To date, transcriptional profiling has been used to highlight similarities between DLBCL tumor cells and normal B-cell subtypes and associate genes and pathways with unfavorable outcome. To identify robust and highly reproducible DLBCL subtypes with comprehensive transcriptional signatures, we utilized a large series of newly diagnosed DLBCLs, whole genome arrays and multiple clustering methods. Tumors were also analyzed for known common genetic abnormalities in DLBCL. Three discrete subsets of DLBCLs – “Oxidative Phosphorylation”, “B-cell Receptor/Proliferation” and “Host Response” (HR) were identified, characterized using gene set enrichment analysis and confirmed in an independent series. HR tumors had increased expression of T/NK-cell receptor and activation pathway components, complement cascade members, macrophage/dendritic cell markers and inflammatory mediators. HR DLBCLs also contained significantly higher numbers of morphologically distinct CD2+/CD3+ tumor-infiltrating lymphocytes and interdigitating S100+/GILT+/CD1a-/CD123- dendritic cells. The HR cluster shared features of histologically defined T-cell/histiocyte-richBCL, including fewer genetic abnormalities, younger age at presentation and frequent splenic and bone marrow involvement. These studies identify tumor microenvironment and host inflammatory response as defining features in DLBCL and suggest rational treatment targets in specific DLBCL subsets.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults, comprising almost 40% of all lymphoid tumors. Although a subset of DLBCL patients can be cured with standard adriamycin-containing combination chemotherapy, the majority die of their disease. Robust clinical prognostic models such as the International Prognostic Index can be used to identify patients who are less likely to be cured with standard therapy [1]. However, such models do not provide specific insights regarding tumor cell biology, novel therapeutic targets or more effective treatment strategies. Furthermore, recent studies suggest that subsets of DLBCL may differ with respect to normal cell of origin and genetic bases for transformation as well as clinical outcome.

DLBCLs are thought to arise from normal antigen-exposed B-cells that have migrated to or through germinal centers (GC) in secondary lymphoid organs [2]. Like normal GC B-cells and their descendents, DLBCLs have somatic mutations of immunoglobulin receptor variable (v)-region genes [2]. These tumors also exhibit genetic changes that may be related to normal GC functions. For example, normal GC B-cells undergo vigorous clonal expansion and editing of the immunoglobulin receptor via processes that require DNA strand breaks. In small subsets of DLBCL, several translocations into the immunoglobulin locus have been described, including t(8;14), t(3;14) and t(14;18) [3]. A subset of DLBCLs also exhibits aberrant somatic hypermutation of genes that are not targeted by this editing process in normal GC B-cells [4]. However, a significant percentage of DLBCLs lack known genetic abnormalities.

Given the documented clinical and genetic heterogeneity of DLBCLs, it would be useful to have comprehensive molecular signatures of tumors that share similar features. In addition to highlighting potential pathogenetic mechanisms, such signatures might identify promising subtype-specific targets and pathways for therapeutic intervention. With the advent of gene expression profiling, it is now possible to obtain signatures of DLBCL subtypes.

To date, transcriptional profiling of DLBCLs has been used to highlight similarities between subsets of tumors and normal B-cells and to identify features associated with unfavorable responses to empiric combination chemotherapy. For example, a series of molecular models have been described which relate DLBCL subsets to normal GC B-cells, *in vitro* activated peripheral blood B-cells or an unspecified, third group [5,6]. In these studies, DLBCLs with features common to normal GC B-cells responded more favorably to standard empiric combination chemotherapy. In additional profiling studies, the molecular signatures of DLBCLs with different responses to standard chemotherapy were examined [7]. Two of the pathways associated with poor responses to current regimens have already been credentialed and targeted for possible therapeutic intervention ([8] and P. Smith, personal communication).

However, DLBCLs are not a homogeneous group of tumors that only differ with respect to outcome or possible cell of origin. Given the genetic heterogeneity in DLBCL, there are likely to be subsets of tumors with different pathogenetic mechanisms and possible treatment targets. With a more extensive series of primary tumors and arrays with increasing genome coverage, it is now possible to identify robust subsets of large cell lymphoma with unique, comprehensive transcriptional profiles. For example, we and others recently found that the molecular signature of primary mediastinal large B-cell lymphoma (MLBCL) differs from that of DLBCL and shares important features with that of a clinically similar disorder, classical Hodgkin lymphoma (nodular sclerosis subtype) [9,10]. In the current study, we address the more difficult question of unrecognized biological heterogeneity within DLBCLs, using multiple clustering methods and comprehensive genetic analyses to identify discrete subsets of tumors.

Materials and Methods

Case selection and Histologic classification

Tumor specimens and retrospective clinical data from 176 DLBCL patients were analyzed according to an Institution Review Board-approved protocol. All tumor specimens were nodal biopsies from newly diagnosed, previously untreated patients. The histopathology and immunophenotype of each DLBCL was reviewed by expert hematopathologists to confirm the diagnosis. Clinical variables included in the full International Prognostic Index (IPI) (age, stage, number of extranodal sites, LDH and PS) were obtained; an IPI score was available for 144 patients (Supplementary Information). Overall survival (OS) and freedom from progression (FFP) were determined by the Kaplan-Meier method in 130 study patients who received full-dose CHOP-based (cyclophosphamide, adriamycin, vincristine, prednisone) therapy (eg. 3-4 cycles +XRT for localized disease or minimum of 6 cycles for advanced disease) and had long-term clinical follow-up or disease progression during or following induction therapy.

Target cRNAs of oligonucleotide microarrays

Target cRNAs were prepared as previously described [7]. For 17 randomly selected tumors, 2 separate aliquots of RNA were used for target preparation and analysis. Samples were hybridized to Affymetrix U133A and U133B oligonucleotide microarrays (Affymetrix, Santa Clara, CA) which include probe sets from ~ 33,000 genes. Arrays were subsequently developed and scanned as previously described ([7] and Supplementary Information).

Gene expression analysis

A statistical analysis of the duplicate samples was used to identify genes with high reproducibility within duplicates and high variation across patient tumors (Supplementary Information). Genes were ranked using a robust modification of the F statistic and the

top 5% (2118 genes) were included in the final gene set. Similar analyses were performed using the top 10% of ranked genes (Supplementary Information).

Unsupervised analysis by consensus clustering

Three unsupervised clustering algorithms were used in the analysis: hierarchical clustering (HC) [11], self-organizing maps (SOM) [12] and model-based probabilistic clustering (PC) [13] (Supplementary Information). The stability of the identified clusters (i.e., sensitivity of the cluster boundaries to sampling variability) was assessed using consensus clustering [14]. With this method, perturbations of the original dataset are simulated by resampling techniques. The clustering algorithm of choice is applied to each of the perturbed datasets and the agreement, or consensus, among multiple runs is assessed and summarized in a consensus matrix (Supplementary Information).

Data-set perturbations were obtained by randomly selecting 80% of the samples (141/176 tumors) at each iteration. Two hundred sub-sampling iterations were performed for each clustering algorithm (HC, SOM and PC). Consensus matrices were built and evaluated for partitions including 2 to 9 clusters (Supplementary Information). Confusion matrices were used to measure the agreement between clusters produced by different algorithms and to determine the number of samples assigned to similar clusters by any 2 algorithms. A meta-consensus was used to identify the tumors that were similarly assigned by all 3 clustering algorithms (Supplementary Information).

Gene expression differential analysis

From the top 5% (2118-gene) pool, genes associated with each of the DLBCL clusters were identified using the binary distinction “cluster X *vs*. NOT cluster X”. Genes were ranked according to the signal-to-noise ratio (SNR) (Supplementary Information).

Gene Set Enrichment Analyses (GSEA)

GSEA was performed as previously described [15] using a total of 281 gene sets from 4 independent sources: 1) Biocarta, an internet resource (www.biocarta.com) that includes

169 biological pathways involved in adhesion, apoptosis, cell activation, cell cycle regulation, cell signaling, cytokines/chemokines, developmental biology, hematopoesis, immunology, metabolism, and neuroscience; 2) GenMAPP (Gene MicroArray Pathway Profiler), a set of web-accessible pathways (www.GenMAPP.org) and gene families including 45 gene sets involved in metabolic and cell signaling processes; and 3) 64 manually curated pathways involved in mitochondrial function and metabolism and additional gene sets that are co-regulated in normal murine tissues [15] (Supplementary Information); and 4) 3 recently described co-regulated gene sets in DLBCL [5].

Enrichment was assessed by: 1) ranking the 2118 genes in the top 5% pool with respect to the phenotype “cluster X vs. not cluster X”; 2) locating the represented members of a given gene set within the ranked 2118 genes; 3) measuring the proximity of the gene set to the overexpressed end of the ranked list with a Kolmogorov-Smirnoff (KS) score (with a higher score corresponding to a higher proximity); and 4) comparing the observed KS score to the distribution of 1000 permuted KS scores for all gene sets (Supplementary Information). A $p < .005$, corrected for multiple hypothesis testing (MHT-p), was used to identify highly significant associations between specific gene sets and DLBCL clusters.

Fluorescence in-situ hybridization (FISH)

Air-dried touch preparations were prepared from fresh frozen tumor specimens. Interphase nuclei were hybridized to commercially available probes flanking or spanning the *IGH*, *BCL2* and *BCL6* loci: LSI *IGH/BCL2* Dual Color, Dual Fusion Translocation Probe for detection of t(14;18) and LSI *BCL6* Dual Color, Break Apart Rearrangement Probe for detection of any rearrangement involving 3q24 (t(3;), Vysis, Drowner’s Grove, IL). Translocations were detected by fluorescence microscopy after nuclear counterstaining with DAPI.

Morphologic analysis of tumor infiltrating lymphocytes (TILS).

All study DLBCLs with available hematoxylin and eosin (H&E)-stained diagnostic specimens (119 tumors) were independently assessed for the presence of TILS by an expert morphologist (MM) who had no previous information regarding the DLBCL transcriptional profiles. For the majority of tumors, anti-CD2 stained specimens were also available for review. Tumors were initially scanned at high power (640X) to identify morphologically normal, CD2+ lymphocytes with round or oval nuclei and delicately dispersed chromatin; such lymphocytes were only scored when they directly infiltrated the tumor (TILS, [16]. Twenty-30 representative fields of the tumor were independently scored for TILS at 400X and an average TILS/400X score was obtained. DLBCLs were classified as having either less than or greater than 20 TILS/400X field.

Immunohistochemistry (IHC)

Two representative 0.6mm cores were obtained from diagnostic areas of available paraffin- embedded, formalin- or B5-fixed DLBCLs (80 tumors) and inserted into a tissue array. Tissue array sections were analyzed using mouse monoclonals anti-CD2 (LFA-2) (Novocastra Laboratories LTD, Newcastle upon Tyne, UK), anti-CD123 (Bioscience, San Diego, CA) and anti CD1a (Dako, Carpintera, CA), and rabbit polyclonal anti-CD3 and anti-S100 (Dako), and anti-gamma interferon-induced lysosomal transferase (GILT, Gift from Peter Cresswell, Yale University School of Medicine, New Haven, CT [17]) (Supplementary Information) and horseradish peroxidase-conjugated secondary antibodies (anti-mouse or rabbit, Envision detection kit, DAKO). Slides were developed with diaminobenzidine (DAB) (DAKO), counterstained with harris hematoxylin and analyzed in blinded fashion by two expert hematopathologists, without information regarding cluster designations.

The numbers of CD2+ and CD3+ cells/core were separately recorded for duplicate samples and represented in 5 categories: 1) <50 cells/core; 2) 50-150 cells/core; 3) 150-250 cells/core; 4) 250-500 cells/core; 5) >500 cells/core. Separate analyses of GILT-stained dendritic cells and tumor cells were performed. The number of GILT+ dendritic

cells/core was assessed in duplicate samples and represented in 3 categories: 1) 0-25 cells/core; 2) 25-100 cells/core; and 3) >100 cells/core. The number of S100+ dendritic cells/core was assessed in duplicate samples and represented in 4 categories: 1) 0-25 cells; 2) 25-50 cells; 3) 50-100 cells; and 4) > 100 cells.

Cluster validation

An independent group of 221 newly diagnosed DLBCLs with available cDNA microarray ("lymphochip") profiles [5] was used for cluster validation. This dataset represented the originally described 240 tumors [5] following removal of 19 subsequently identified primary MLBCLs (A. Rosenwald and L. Staudt, personal communication). Seven hundred and three of the top 5% (2118) genes were also represented on the lymphochip platform. These overlapping lymphochip probes were used in HC, SOM, PC and meta-consensus to identify the dominant structure in the independent DLBCL dataset (Supplementary Information).

The level of agreement between the consensus clusters in our dataset and the independent series was determined by comparing the gene markers for each of the respective clusters. Cluster markers were defined as the set of genes with the highest SNR for the corresponding one-vs-all distinction (Supplementary Information). The overlap between respective pairs was represented in a 2-dimensional contingency table and assessed with a Fisher exact test. Similar analyses were also performed using the entire set of genes represented on the lymphochip (7K+) or the top 50% of genes selected with a MAD filter (Supplementary Information).

Cell-of-origin signature

DLBCLs from our dataset were sorted according to the most recent COO signature [6] (germinal center B-cell [GCB], activated B-cell [ABC] and other [not otherwise specified]), using *linear predictive scores* and the 23 (of 27) COO probes represented on the oligonucleotide assays (Supplementary Information). Confusion matrices were used to measure the agreement between the LPS-defined COO signatures and our meta-consensus defined comprehensive clusters (Supplementary Information).

Results

Identification of DLBCL consensus clusters

To identify biologically meaningful subsets of DLBCL with similar transcription profiles, we utilized a large series of tumors from highly representative, newly diagnosed patients (Supplementary Information). We were interested in DLBCL subsets that were sufficiently robust to be captured by multiple methods. For this reason, we used three different clustering algorithms (hierarchical clustering (HC), self-organizing maps (SOM), and probabilistic clustering (PC)) and the top 5% of genes with the highest reproducibility across duplicate samples and largest variation across patient tumors. In addition, we utilized a resampling-based method (consensus clustering) that automatically selects the most stable numbers of clusters with each algorithm.

With all 3 clustering algorithms, the most robust substructure included 3 discrete clusters (Fig. 1A, left panel). There was a high level of agreement between clusters produced by the individual algorithms, with more than 84% of DLBCLs assigned to the same clusters by any two algorithms (Fig. 1A, right panel). A meta-consensus confirmed that 141 of the 176 tumors were assigned to the same clusters by all 3 methods (Fig. 1B). We predicted the cluster membership of the remaining 35 tumors using a naïve-Bayes model trained on the 141 DLBCLs with concordant cluster labels (Supplementary Information). Similar results were obtained when the clustering analysis was performed with the top 10%, rather than the top 5% of genes, indicating that the results were not dependent upon the initial gene selection. The top 50 genes associated with each DLBCL group are visually represented in Fig. 1C.

Characterization of the DLBCL consensus clusters

Having defined the expression profiles of 3 discrete DLBCL clusters, the next challenge was to interpret them objectively. We first asked whether previously characterized, co-regulated sets of genes were more abundant in specific clusters using GSEA (Methods [15]).

The first DLBCL cluster was significantly enriched in genes involved in oxidative phosphorylation, mitochondrial function and the electron transport chain (Table 1A). More detailed analysis of this DLBCL cluster, termed “OxPhos”, revealed increased expression of members of the NADH dehydrogenase complex and cytochrome c/cytochrome c oxidase (COX) complex as well as ATP synthase components and additional mitochondrial membrane enzymes (Table 2A) [[18]]. OxPhos tumors also had higher levels of the anti-apoptotic *BCL2* related family member, BFL-1/A1 [19]. Given the known consequences of mitochondrial membrane perturbation – cytochrome release and caspase-mediated apoptosis – and the regulation of mitochondrial membrane potential and cytochrome c release by *BCL2* family members, these results are of particular interest. OxPhos tumors also had increased expression of multiple components of the 26S proteasome and general and mitochondrial ribosomal subunits (Table 2A). [20].

The second DLBCL cluster was enriched in cell-cycle regulatory genes (Table 1A), including CDK2 and MCM (minichromosome maintenance deficient) family members (Table 2B) [21]. These tumors also had increased expression of DNA repair genes including postmeiotic segregation increased 2 (PMS2) family members [22], H2AX [23], PTIP [24] and p53 (Table 2B). This DLBCL cluster also had higher levels of many components of the BCR signaling cascade (CD19, Ig, CD79a, BLK, SYK, PLCγ2 and MAP4K) and additional B-cell specific or essential transcription factors (including PAX5, OBF-1, E2A, *BCL6*, STAT6 and MYC) (Table 2B) [25,26]. For this reason, this subset of DLBCLs was termed “BCR/Proliferation”.

Unlike the other 2 DLBCL subsets, the third DLBCL cluster had a signature that was largely defined by the associated host response rather than the tumor itself (Table 1). By GSEA, this cluster was enriched for markers of T-cell mediated immune responses and the classical complement pathway (Table 1A). These tumors also had increased expression of an overlapping set of co-regulated inflammatory mediators and connective tissue components (C7, Table 1A and Supplementary Information).

Detailed analysis of the third cluster, termed “Host Response (HR)”, revealed increased expression of multiple components of the T-cell receptor (TCR) (TCRα and β and CD3 subunits), CD2, and additional molecules associated with T/NK-cell activation [27] and the

complement cascade (Table 2C). HR tumors also had more abundant monocyte/macrophage and dendritic cell transcripts, molecules required for efficient antigen processing and certain HLA class I antigens [28-35] (Table 2C). Consistent with the signature of an ongoing inflammatory/immune response, HR tumors had increased expression of interferon-induced genes, certain tumor necrosis family (TNF) ligands and receptors, cytokine receptors, adhesion molecules and extracellular matrix components [36-38] (Table 2C).

Of note, patients in the 3 consensus clusters had similar 5-year survivals (OxPhos 53%, BCR/proliferation 60% and HR 54%, p = .53), suggesting that the clusters may be more useful for identifying potential pathogenetic mechanisms and cluster-specific rational therapeutic targets than predicting responses to empiric combination chemotherapy.

Genetic abnormalities and clinical features in the newly identified DLBCL clusters

Having identified 3 subclasses of DLBCL, we asked whether these subgroups differed with respect to known chromosomal translocations in the disease (t(14;18) and t(3;), involving the *BCL6* locus) (Table 3). The distribution of t(14;18) and t(3;) was examined in the 116 tumors with available data and no more than one translocation (one OxPhos tumor with both translocations was omitted from the analysis). There was an association between cluster membership and the examined genetic abnormalities (p = 0.059, Fisher exact test, Table 3). *BCL2* translocations were more common in the Oxphos cluster whereas *BCL6* translocations were more frequent in the BCR/proliferation cluster. Translocations of either type were uncommon in the HR cluster (Table 3).

The increased incidence of t(14;18) in OxPhos tumors was of particular interest given this cluster's oxidative phosphorylation/mitochondrial gene expression signature and overexpression of additional anti-apoptotic *BCL2* family members (Tables 1 and 2A).

The near absence of known cytogenetic abnormalities and the prominent inflammatory/immune infiltrate in HR DLBCLs prompt speculation regarding other, as yet uncharacterized, mechanisms of transformation in these tumors. In this regard, it is noteworthy that patients with HR DLBCLs were significantly younger than those with OxPhos or BCR/proliferation tumors (p = 0.04, Kruskal-Wallis test, Supplementary

Information). Patients with HR tumors also had a significantly higher incidence of splenic and BM involvement (p = 0.02 and 0.03, respectively).

Immunohistochemical and morphological analysis of HR tumors

The unique characteristics of the HR cluster – fewer known genetic abnormalities and prominent host immune and inflammatory cell transcripts – prompted us to assess host immune cells in study tumors using morphologic and immunohistochemical approaches. Hematoxylin-and-eosin and CD2- stained slides of study DLBCLs were evaluated for the presence of tumor infiltrating lymphocytes (TILs) by an expert morphologist who had no information regarding the DLBCL transcriptional profiles. HR tumors contained significantly higher numbers of TILS than DLBCLs in the other clusters (< 0.0001, Fisher exact test, Supplementary Information).

Since HR tumors had more abundant CD2 and CD3ε transcripts (Table 2C), we also used CD2 and CD3 immunostaining to quantify infiltrating T-cells in study DLBCLs. HR tumors contained significantly higher numbers of CD2+ and CD3+ T-cells than DLBCLs in the other clusters (p = .005 and .003, respectively, Kruskal-Wallis exact test, Fig. 2A). Consistent with these observations, 8 of the 10 tumors initially diagnosed as T-cell-histiocyte-rich DLBCLs [39] were included in the HR cluster (49 tumors total). Additional components of the HR signature – ZAP70 and its substrate, LAT (linker for the activation of T-cells, [40], the TH_2 transcription factors, GATA3 and c-MAF [41], the TH_1 and TC_1 cytokine receptor, CXCR6 [42], the natural killer cell (NK) triggering receptor, LST (NKp30) [43], perforin 1 and the CD28 co-stimulatory molecule [44] – suggest that these tumors include a mixed population of activated T/NK-cells (Table 2C).

In addition to having higher numbers of infiltrating T and NK cells, HR tumors had increased levels of likely macrophage and dendritic cell transcripts, including the gamma interferon-induced lysosomal thiol reductase, GILT [17,34] (Table 2C). Since GILT is required for effective peptide processing and optimal antigen presentation [34,45,46], we used GILT immunostaining to both identify and characterize the dendritic cells in study tumors. When compared to the other clusters, HR tumors contained increased numbers of GILT+ dendritic cells (p = .06, Kruskal-Wallis test, Figs. 2A and B).

For this reason, we further characterized tumor dendritic cells (DC) with S100, CD1a and CD123. These markers distinguish interdigitating DC (S100+ CD1a – CDC123-) that interact with antigen-specific T-cells in secondary lymphoid organs from other DC subtypes [47,48]. There was no detectable CD1a expression in study DLBCLs and only 2 tumors (non-HR) contained CD123 positive cells. In marked contrast, S100+ DC were readily detectable and significantly more abundant in HR tumors than DLBCLs from other clusters ($p = .009$, Krushal Wallis test). In addition, the numbers of CD2+/CD3+ infiltrating T-cells and GILT+/S100+ DC were highly correlated in individual tumors ($p < 0001$, Jonckheere-Terpstra test) (Fig. 2B and Supplementary Information). Therefore, HR tumors contain interdigitating DC and associated infiltrating T-cells, likely capable of participating in a coordinated immune response. Consistent with this interpretation, the HR signature also includes adhesion molecules like LFA-1 that strengthen T-cell/DC contact and T-cell surface molecules, such as SEMA4D/CD100 and LAG3/CD223, that promote DC maturation and activation (Table 2C) [44,49-51].

Validation of DLBCL consensus clusters in an independent dataset

After defining 3 consensus clusters in our own DLBCL series, we asked whether there were similar clusters in an independent group of newly diagnosed DLBCLs with available gene expression profiles [5]. Using the overlapping set of highly reproducible/highly variable genes (703 common genes), our clustering procedure subdivided the independent DLBCL series into 2, rather than 3, major groups (Fig.3, right panel and Supplemental Information). The signature for one of the independent clusters was highly enriched for HR transcripts (overlap P-value $<2.2 \times 10^{-16}$) (Fig. 3A, top left panel). We further analyzed the “non-HR” tumors by clustering this group in the space of non-HR markers. “Non-HR” tumors separated into 2 discrete clusters with highly significant enrichment for either BCR/proliferation or OxPhos transcripts (overlap P-value < 0.0009) (Fig. 3B, bottom panels).

Similar structure was also identified when tumors were clustered using less restricted sets of genes (either the top 50% of genes ranked by a MAD-based variation filter or all genes), indicating that the structure was not dependent upon a highly selected gene set (Supplementary Information). Taken together, these results confirm the presence of similar consensus clusters in an independent DLBCL dataset.

Relationship of consensus clusters to the cell-of-origin signature

Recent studies suggest that subsets of DLBCL share elements of the transcriptional profile of normal purified germinal center B-cells (GCB) or *in vitro*-activated peripheral blood B-cells (ABC) while other DLBCLs lack these features (Other) [5,6]. To compare the newly defined consensus clusters (CC) with these cell-of-origin (COO) subsets, we first classified our tumors with respect to COO ([6], Methods and Supplementary Information). Of note, tumors identified as GCB were associated with significantly longer overall survivals ($p = .003$).

Comparison of the CC and COO assignments indicates that the two classification schema are capturing largely different aspects of DLBCL biology (Fig.4 and Supplementary Information). Although 53% of tumors in the BCR/proliferation cluster and 46% of tumors in the OxPhos cluster were classified as GC-like, the remainder were designated ABC or Other (Fig. 4). In the HR cluster, there were relatively more unspecified (Other) DLBCLs (Fig.4), likely because unspecified (Other) DLBCLs have less striking B-cell signatures and HR tumors have prominent inflammatory infiltrates.

In DLBCLs, additional sets of co-regulated genes (Proliferation, MHC class II and Lymph Node) were previously reported to be expressed independently of the COO signature [5]. For these reasons, we asked whether these additional co-regulated gene sets contributed to consensus cluster signatures using GSEA. Not surprisingly, the BCR/proliferation signature had some evidence of enrichment with the previously described proliferation genes [5] (MHT $p = 0.06$, Table 1B). There was also highly significant enrichment of the LN gene set in our HR signature ($p = < 0.001$, Table 1B). Given the composition of the LN gene set -- T/NK activation antigens, complement components, monocyte markers, interferon-inducible genes, HLA class I molecules, additional cytokines and connective tissue components [5] -- these results are in keeping with the broader definition of a DLBCL cluster characterized by a concomitant host immune/inflammatory response.

Discussion

Using 3 different clustering methods and whole genome arrays, we identified three robust subsets of DLBCL and confirmed their presence in an independent series. The characteristics of these clusters – OxPhos, BCR/proliferation and HR – suggest that these tumors may have novel pathogenetic mechanisms and possible treatment targets. In addition, the signatures identify the tumor microenvironment as a defining feature.

The current study indicates that additional, non-overlapping information can be obtained by sorting DLBCLs with respect to consensus clusters and putative COO. In fact, other features of DLBCLs that track independently of COO ("proliferation" and " lymph node signature" [5]) were captured by the comprehensive clusters. The updated COO signature identifies a subset of "GC-like" DLBCLs that responded more favorably to empiric combination chemotherapy. Although the comprehensive consensus clusters were less predictive of response to empiric combination chemotherapy, the clusters reproducibly defined major groups of tumors that may be amenable to targeted intervention. For example, OxPhos tumors have increased expression of proteosomal subunits and molecules regulating mitochondrial membrane potential and apoptosis. These DLBCLs may be particularly sensitive to proteosome blockade [20] or *BCL2* family inhibition. In contrast, HR tumors may be more sensitive to immunomodulatory approaches.

Thus far, the HR cluster has been most extensively characterized. HR tumors were largely defined by their inflammatory/immune cell infiltrate, including CD2+/CD3+ TILs and interdigitating S100+/GILT+ CD1a-CD123- dendritic cells and suggesting a coordinated immune response. HR tumors had less frequent genetic abnormalities and occurred in younger patients, prompting speculation regarding an alternative pathogenetic mechanism. Patients with HR tumors also had unique clinical features, presenting more commonly with splenomegaly and bone marrow involvement.

The T-cell/dendritic cell infiltrates in HR tumors resemble those of a smaller provisional (WHO) subtype of DLBCL, T-cell/histiocyte-rich B-cell lymphoma (T/HRBCL), which includes abundant non-neoplastic T-cells and associated macrophages ("histiocytes")

[39,52-55]. Like HR DLBCLs, T/HR BCLs are reported to have fewer known genetic lesions and occur in slightly younger patients who often have splenomegaly and bone marrow involvement [53,54]. However, histologically defined T/HRBCLs represent a smaller subset of DLBCL than our HR cluster. It is likely that the comprehensive transcriptional profiles identify additional DLBCL patients with more subtle, related signatures.

In addition to providing insights regarding the nature of the associated immune response in HR tumors, the newly identified molecular and immunohistochemical features of these DLBCLs may increase diagnostic accuracy. For example, histologically defined T/HRLCL is a "grey zone" lymphoma that may resemble lymphocyte predominant Hodgkin's lymphoma, a more indolent disease with different recommended therapy [56,57].

The current HR signature contains more information regarding the infiltrating immune cells and associated inflammatory response than the associated malignant B-cells. Microdissected tumor cells from T/HRBCL were previously shown to have clonal Ig gene rearrangements, somatic hypermutation, and a mutation pattern suggestive of antigen selection [52]. In the current study, HR tumors expressed higher levels of Notch 2, a molecule implicated in specific stages of mature B-cell development [58]. HR tumors also expressed higher levels of TNF receptors and additional TNF co-stimulatory molecules (such as APRIL) known to protect malignant B-cells from apoptosis [59,60]. At present, the antigen specificity of HR malignant B-cells remains undefined. It is possible that HR malignant B-cells and the associated infiltrating T-cells are directed against the same antigen; if so, the TILs and interdigitating dendritic cells may actually support tumor growth [61]. Alternatively, TILs might be directed against the malignant B-cells in HR tumors. However, patients in the HR cluster did not have better outcomes following empiric chemotherapy, suggesting that their immune responses were ineffective and/or inhibited by counter-regulatory mechanisms [62] or their tumors were inherently less responsive to CHOP-based treatment.

For these reasons, it will be important to identify HR tumors with pre-existing abundant T- and dendritic-cell infiltrates and further characterize their associated underlying immune response. Such directed approaches to HR tumors and the other newly

identified DLBCL consensus clusters will likely define more rational treatment targets in this heterogeneous disease.

Figure Legends

Fig. 1 Identification of consensus clusters

A. Left panels). Consensus matrices produced by hierarchical clustering (HC, K=3), self-organizing maps (SOM, K=3) and probabilistic clustering (PC). Right panels). Comparisons of the cluster assignments of the different algorithms (PC vs. HC, HC vs. SOM and PC vs. SOM, respectively). More than 84% of DLBCLs were assigned to the same clusters by any two algorithms.

B. Left panel). Consensus matrix comparing the assignments made by all three clustering algorithms ("Meta Consensus" [PC vs. HC] vs. [PC vs. SOM]). Right panel). Comparisons of the "meta-consensus" cluster assignments. 141 of the 176 tumors were assigned to the same clusters by all three algorighms.

C. Expression profiles of the three DLBCL clusters. The top 50 genes associated with each DLBCL cluster are shown. Each column is a sample, each row a gene. Color scale at bottom indicates relative expression and standard deviations from the mean. Red indicates high-level expression; blue, low-level expression.

Fig. 2 T- and dendritic cell infiltrates in study DLBCLs.

A) Numbers of normal infiltrating CD2+ and CD3+ cells and GILT-positive dendritic cells in primary DLBCLs in each cluster. HR tumors included significantly higher numbers of CD2+ and CD3+ T-cells than DLBCLs in the other clusters ($p = .005$ and .003, respectively, Kruskal-Wallis exact test). HR tumors also contained higher numbers of GILT+ dendritic cells ($p = .06$, Kruskal-Wallis exact test). B) Hematoxylin-and-eosin staining and CD3 and GILT immunostaining of a representative HR tumor.

Fig.3 Validation of DLBCL consensus clusters in an independent dataset.

Application of consensus clustering and meta-consensus (as in Fig. 1B) to the independent DLBCL series (top right panel). One of the identified consensus clusters was highly enriched for HR transcripts ($p < 2.2 \times 10^{-16}$, top left panel). Application of consensus clustering and meta-consensus to the "non-HR" cluster (bottom right panel). The "non-HR" tumors sorted into two discrete clusters with

highly significant enrichment for either BCR/proliferation or OxPhos transcripts (P = < 0.0009, bottom left panel).

Fig. 4 Relationship of consensus clusters to cell-of-origin (COO) signature. Comparison of study DLBCLs sorted into consensus clusters with the same tumors classified by COO. The lack of a clear correlation between the two clustering systems is reflected by the absence of a matrix diagonal structure (ie. large numbers along the diagonal and numbers close to 0 in the off -- diagonal entries).

Table 1. Gene Set Enrichment Analysis of the DLBCL Consensus Clusters[a]

	Ox Phos		BCR/Proliferation		Host Response	
A.	**KS**	**MHT p**	**KS**	**MHT p**	**KS**	**MHT p**
Mitochondrial pathways						
PGC	130.9	0.004	13.2	0.763	3.8	0.931
VOXPHOS	156.1	0.001	13.3	0.760	2.9	0.948
Human mito DB	152.6	0.002	11.8	0.790	0.6	0.987
Mitochondrial	157.5	0.001	16.2	0.703	0.4	0.991
OXPHOS	141.0	0.003	13.7	0.753	2.9	0.946
Gen MAPP						
Electron transport	148.1	0.000	14.4,	0.641	3.1	0.839
Cell cycle	33.4	0.298	104.8	0.004	0.8	0.873
Complement activation - classical	11.9	0.685	7.4	0.766	105.9	0.004
Bio Carta						
Complement	12.0	0.745	6.4	0.846	91.7	0.004
T cytotoxic	8.5	0.809	7.0	0.835	113.5	0.000
T helper	8.5	0.809	7.0	0.835	113.5	0.000
T ob1	27.0	0.440	2.8	0.902	103.2	0.002
Co-regulated gene sets						
C7	26.2	0.494	7.4	0.870	164.6	0.001
C10	130.5	0.004	1.4	0.98	19.7	0.62
B.						
Additional DLBCL gene sets						
Proliferation	120.4	0.103	142.0	0.064	3.1	0.854
Lymph node	24.3	0.577	1.7	0.902	279.7	0.000

[a]GSEA was performed using gene sets from: 1) Biocarta; 2) GenMapp; and 3) a series of manually curated pathways involved in mitrochondrial function and metabolism (Mitrochondrial pathways) [15]. Additional co-regulated gene sets from normal murine tissues (Co-regulated gene sets) [15] and DLBCLs (DLBCL gene sets) [5] were also used. (B). KS is Kolmogorov-Smirnoff score and MHT p connotes a p value corrected for multiple hypothesis testing.

Table 2. DLBCL Consensus Cluster Signatures[a]

A. OxPhos Cluster

Function	Gene
NADH dehydrogenase complex	NADH dehydrogenase (ubiquinone)1: α/β subcomplex 1, 8 kDa; β subcomplex 1, 7 kDa; β subcomplex 2, 8 kDa Ubiquinol-cytochrome c reductase hinge protein
Cytochrome c/ cytochrome oxidase (COX) complex	Cytochrome c COX 5b, 6a1, 7a2L, 7b, 7c, 11
ATP synthase, mitochrondrial	F0 complex, subunit c F1 complex β polypeptide, γ polypeptide 1 and O subunit
ATP other	ATP binding protein ATP binding cassette subfamily D (ALD, member 3) ATPase H+ transporting, lysosomal: 21 kDa, VO subunit c and 9 kDa
Other, Mitochondrial	Translocases of inner mito. memb. (TIMM): #8B and 23 Translocases of outer mito. memb. (TOMM) 7 and 20 Diazepam binding inhibitor
Apoptosis	BFL-1/A1 MIHC (BIRC3) TNFA1P8 (SCC-S2) TNFRSF6 (FAS) Apoptosis related protein (APR-3)
Proteosome	Proteosome subunits: α types 2, 5, 6 and 7; and β type 4 Proteosome 26s subunits: ATPase 2 and non-ATPase 4
Ribosome, mitochondrial	L3, L15, L39, S17, S31 and S36
Ribosome, other	L3, L4, L5, L10, L13a, L15, L17, L27, L30, L31, L36A, L36, large PO, S12, S17, S20 and S21
Other	X-ray repair complementing defective repair 5 (XRCC5) Superoxide dismutase 1 (SOD1) Jumping translocation breakpoint CDW52 (CAMPATH) H2AZ PTEN

B. BCR/Proliferation Cluster

BCR signaling cascade	CD22 CD19 Igμ CD79a BLK SYK PLC γ 2 Inositol 1, 4, 5 triphosphate receptor type 3 Inositol 1, 4, 5 triphosphate 3 kinase B Inositol polyphosphate-5-phosphatase 145 kDa MAP4K1
Class II molecules	CD74 (invariant polypeptide MHC class II)
Transcription factors	PAX5

	FOXO1A
	BCL6
	POU2AF1 (BOB-1/OBF-1/OCAB)
	STAT6
	TCF3 (E2A)
	NFAT
	SPI-B
	ETS-1 (E26 homolog)
	Ikaros
	MYC
Other B-cell markers	CD37
	BC11A
Proliferation	KI67
	CDK2
	Signal-induced proliferation-associated 1 like 3
Replication/repair	DEAD/H box polypeptides 11 and 39
	Postmeiotic segregation increased (PMS) 2-like 2, 6 and 9
	Minichromosome maintenance deficient (MCM) 2, 4, 5 and 7
	p53
	H2AX
	PAX transactivation domain-interacting protein (PTIP)
	MUTL homolog 6
Motility/cytoskeletal	Villin 2 (Ezrin)
Transcription modulators	SMARC A4, B1 and F1
Post-transcriptional Modification	HDAC1
	MYST histone acetyltransferase 4
	Ubiquitin-activating enzyme E1
Other	Heat shock protein 90 beta

C.Host Response Cluster

T/NK cell	T-cell receptor α and β
	CD2
	CD3 δ, ε and γ
	CD6
	CD28
	GATA3
	cMAF
	CXCR6
	LST (NKp30)
	Zap 70
	Linker for activation of T-cells (LAT)
	FYN
	FYN binding protein (SLAP)
	Lymphocyte activation gene 3 (LAG)[b]
	CD100 (Sema 4D)
	Perforin
	NK transcript 4[b]
	T-cell immune regulator (TIRC7)
	Leukocyte-associated Ig-like receptor 1 (LAIR-1)
Complement	Complement 1qB, 1S and 4A
	Complement 3α receptor
	Clade G (C1 inhibitor)
Monocyte/macrophage	CD14[b]
	CD163
	B lymphocyte activator, macrophage expressed (BLAME)
	FGR
	SHPS-1 (BIT/SIRPα)
	Granulin

	Allograft inflammatory factor
Antigen processing	Lysosomal-associated membrane protein 1 (LAMP1)[b] Cathepsins B and D IFN γ inducible protein 30 (GILT)[b]
Interferon (IFN)	IFN-induced transmembrane proteins 1 and 2 Guanylate binding proteins 1** and 2, IFN -inducible STAT1[b] Interferon Regulatory Factors (IRFs) 1 and 7
MHC Class I	HLA A, C, E and F
TNF family members	TNFRSF 1A and B TNFSF10 (TRAIL) TNFSF13 (APRIL)[b]
Cytokine receptors	IL 2 receptor γ IL 6 receptor IL 15 receptor α[b] TGF β receptor II CSF-1 receptor[b]
ECM/Adhesion	LFA-1 (Integrins α L and β 2)[b] PECAM1 (CD31) P-selectin glycoprotein ligand (PSGL-1) Collagens: type XVIII α 1 and type IV α 2 Stromal-derived factor 1
Apoptosis	Caspase 4, apoptosis-related cysteine protease[b]
Other	Notch 2 Disabled homolog 2 (DAB2) FOXO3A MAF B Prostaglandin E receptor 4 S100A4

[a]Gene lists derived from the top 250 genes with higher levels of expression in the indicated concensus cluster.
[b]Genes included in previously described LN signature [5]

Table 3. Genetic Abnormalities in the DLBCL Consensus Clusters

A.

Genetic abnormality[a]	OxPhos		BCR/Prolif		HR		Total	
	n=37	%	n=50	%	n=29	%	n=116	%
t(14;18)	8	(22)	5	(10)	1	(3)	14	(12)
t(3;…)	2	(5)	8	(16)	1	(3)	11	(9)
None	27	(73)	37	(74)	27	(93)	91	(78)

[a]116 tumors had available data and no more than one translocation. One OxPhos tumor with both translocations was omitted from the analysis. The distribution of genetic abnormalities across clusters was represented in a 3X3 contingency table and analyzed with a Fisher exact test (p = 0.059).

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