Several immune escape patterns in non-Hodgkin’s lymphomas

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Keywords: immune escape, lymphoma, PD-1/PD-L1 axis, TMA, transcriptomes

Abbreviations: ABC subtype DLBCL, activated B cell origin of DLBCL; DLBCL, diffuse large B cell lymphoma; ECDF, empirical cumulative distribution function; FFPE, formalin-fixed paraffin-embedded; FL, follicular lymphoma; FLIP, Follicular Lymphoma International Prognostic Index; GC subtype DLBCL, germinal center B cell origin of DLBCL; GELF, Groupe d’Etude des Lymphomes Folliculaires; HLA, human leucocyte antigen; IHC, immunohistochemistry; KS, Kolmogorov–Smirnov; NA, Not available; R-CHOP, rituximab cyclophosphamide, doxorubicin, vincristine and prednisone; TAMs, tumor associated macrophage; TMA, tissue microarray

Follicular Lymphomas (FL) and diffuse large B cell lymphomas (DLBCL) must evolve some immune escape strategy to develop from lymphoid organs, but their immune evasion pathways remain poorly characterized. We investigated this issue by transcriptome data mining and immunohistochemistry (IHC) of FL and DLBCL lymphoma biopsies. A set of genes involved in cancer immune-evasion pathways (Immune Escape Gene Set, IEGS) was defined and the distribution of the expression levels of these genes was compared in FL, DLBCL and normal B cell transcriptomes downloaded from the GEO database. The whole IEGS was significantly upregulated in all the lymphoma samples but not in B cells or other control tissues, as shown by the overexpression of the PD-1, PD-L1, PD-L2 and LAG3 genes. Tissue microarray immunostainings for PD-1, PD-L1, PD-L2 and LAG3 proteins on additional biopsies from 27 FL and 27 DLBCL patients confirmed the expression of these proteins. The immune infiltrates were more abundant in FL than DLBCL samples, and the microenvironment of FL comprised higher rates of PD-1+ lymphocytes. Further, DLBCL tumor cells comprised a higher proportion of PD-1+, PD-L1+, PD-L2+ and LAG3+ lymphoma cells than the FL tumor cells, confirming that DLBCL mount immune escape strategies distinct from FL. In addition, some cases of DLBCL had tumor cells co-expressing both PD-1, PD-L1 and PD-L2. Among the DLBCLs, the activated B cell (ABC) subtype comprised more PD-L1+ and PD-L2+ lymphoma cells than the GC subtype. Thus, we infer that FL and DLBCL evolved several pathways of immune escape.

Introduction

In order to develop within immunocompetent hosts, it is imperative that tumors evolve an immune escape strategy.1-3 This Darwinian selection may operate not only through emergence of somatic mutations which protect against immunity, but also by upregulation of genes mediating immune escape. Indeed, genetic instability stands on a passive and random basis while regulation of gene expression represents an active evolution strategy. Different mechanisms leading to immune-evasion have been identified, and include the impairment of immune infiltration through endothelial defects, the inhibition or blockade of immune activation by galectin 3 or CTLA4 ligands,4-7 metabolic depletion by indoleamine dioxygenase8-10 or arginase, the secretion of suppressive mediators such as PGE2, TGFβ, IL10 and adenosine,11 the local recruitment of immunosuppressive cells such as Tregs, TAMs or myeloid-derived suppressor cells (MDSC), and the impairment of functional responses from pre-activated lymphocytes through apoptotic depletion by FasL, PD-L1 or PD-L2, as well as exhaustion or anergy, to name but a few. Among these different options, tumor cells particularly need to develop protection against T-cell-mediated immune responses, and indeed some aggressive B cell lymphomas frequently delete HLA class I or β2-microglobulin genes accordingly.12 Inhibitory
Programmed death-1 (PD-1, CD279) is an immune-suppressive molecule that is upregulated in a large proportion of tumor-infiltrating lymphocytes (TILs) in many different tumor types, but it is also upregulated in other immune cells. Intracellular PD-1 signaling is activated upon PD-1 binding to its ligands PD-L1 (B7-H1, CD274) or PDL2 (PDCD1LG2, CD273), which induce a reduction in the T cell activation cascade. Thus, by expressing PD-1 ligands on the tumor cell surface and engaging PD-1-positive infiltrating lymphocytes, tumors utilizing the PD-1 pathway can therefore evade an immune response. Accordingly, a blockade of the PD-1/PD-L1 interaction has led to good clinical responses in several but not all cancer types, and the heterogeneity observed in the cellular expression of these immune checkpoint proteins efficiently allows the immune-evasion of tumor cells.

**Table 1. List of 54 genes of IEGS**

| GENE ID | Protein, alias names |
|---------|----------------------|
| ARG1    | Arginase 1           |
| BTLA    | CD272, B and T lymphocyte associated |
| CCL2    | Chemokine (CC motif) ligand 2, MCP1 |
| CCL22   | Chemokine (CC motif) ligand 22 |
| CD163   | CD163                |
| CD80    | CTLA4 ligand, B7.1   |
| CD86    | CTLA4 ligand, B7.2   |
| COL17A1 | Collagen, type XVII, α1 |
| CSF1    | Colony stimulating factor 1 M-CSF1 |
| CTLA4   | Cytotoxic T-lymphocyte-associated protein 4, CD152 |
| ENTPD1  | Ectonucleoside triphosphate diphosphohydrolase 1, CD39 |
| FOXP3   | Forkhead box P3      |
| GDF15   | Growth differentiation factor 15 |
| HAVCR2  | TIM3                 |
| HGF     | Hepatocyte growth factor |
| ICOS    | CD278, Inducible T-cell co-stimulator |
| IDO1    | Indoleamine 2,3-dioxygenase 1 |
| IDO2    | Indoleamine 2,3-dioxygenase 2 |
| IL10    | Interleukin 10       |
| IL13    | Interleukin 13       |
| IL23    | Interleukin 23 A p19 |
| IL4     | Interleukin 4        |
| IL6     | Interleukin 6        |
| IL6ST   | Interleukin 6 signal transducer, CD130 |
| JAK2    | Janus kinase 2       |
| KIR2DL1 | CD158A, NK cell inhibitory receptor p58 |
| KIR2DL2 | CD158B1, NKAT1       |
| KIR2DL3 | CD158B2, NKAT2       |
| LG3     | Lymphocyte-activation gene 3, CD223 |
| LAG3    | Leukocyte-associated Ig-like receptor 1 |
| LGALS1  | Galectin 1           |
| LGALS3  | Galectin 3           |
| MCL1    | Myeloid cell leukemia 1, BCL2-related |
| MRC1    | Mannose receptor C type 1, CD206 |
| MSR1    | Macrophage scavenger receptor 1, CD204 |
| MYC     | c-Myc                |
| NT5E    | S’ ectonucleotidase, CD73 |
| PDCD1   | PD-1, programmed cell death 1, CD279 |
| CD274   | PDCD1LG1, PD-L1, PD-L1, B7H1 |
| PDCD1LG2| CD273,PD-L2, PD-L2, butyrophilin B7DC |
| PIM1    | PIM1 oncogene        |
| PTGS1   | Prostaglandin-endoperoxide synthase 1 |
| PTGS2   | Prostaglandin-endoperoxide synthase 2 |
| PVR     | Poliovirus receptor, CD155 |
| SOCS3   | Suppressor of cytokine signaling 3 |
| STAT3   | Signal transducer and activator of transcription 3 |
| STAT5   | Signal transducer and activator of transcription 5 |
| TGFβ1   | Transforming growth factor β 1 |
| TIGIT   | T cell immunoreceptor with Ig and ITIM domains |
| TIMP1   | Tissue inhibitor of metalloprotease 1 |
| TNFRSF14| HVEM, ligand of BTLA |
| VEGFA   | Vascular endothelial growth factor A |
| C100RSF4| VISTA, B7H5, PD-1H |
| VTCN1   | B7H4, B7S1          |

Results

FL and DLBCL upregulate the expression of immune escape genes

By collating the literature, we were able to define a non-exhaustive set of 54 genes involved in different pathways of cancer immune escape (Immune Escape Gene Set, IEGS) (Table 1). We analyzed the distribution of these genes in transcriptomes from lymphoma samples. First, we ranked the IEGS genes from each transcriptome in decreasing order of expression, and then analyzed the distribution of these ranks by plotting and
testing their empirical cumulative distribution function (ECDF).
For a subset extracted from a random permutation (null hypothesis), the ECDF approaches the identity function, and the plot is close to the diagonal. Hence, genes in the IEGS are revealed to be collectively upregulated when the ECDF plot is above the diagonal or downregulated when below the diagonal. We also needed to compare two ECDF’s, and test whether they were significantly different, without reference to the identity function. For statistical procedures, a homogeneous choice was made for both cases: either the one-sample Kolmogorov–Smirnov (KS) test for the first case (comparison of one ECDF with the identity function) or the two-sample KS test for the second case (comparison of two ECDFs). We then downloaded a series of transcriptomes from 38 samples of FL and 20 samples of the corresponding normal B cell controls from the NCBI GEO dataset GSE1219529. The mean of transcriptomes from each group of samples (control B cells, FL) was calculated as arithmetic mean of each gene expression value. In a preliminary control test, a set of 54 randomly chosen genes was defined and its distribution was tested as above for the mean of control B cell group and the mean of FL cell group (blue and red, respectively, Fig. 1A). There was no significant difference between these two ECDFs.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** ECDFs of the (normalized) orders of immune escape genes in transcriptomes from FL and DLBCL biopsies. Each plot represents the appearance order of the IEGS genes in transcriptomes from the specified cell type (depicted as ECDF). The ECDFs of a random gene set show similar random distributions in transcriptomes from normal B cell controls (blue: mean from \( n = 20 \) samples) or FL cells (red: mean from \( n = 38 \) samples) (A), unlike the ECDFs of the IEGS which evidence a significantly up-regulated expression in the FL samples (red) compared to the normal B cell controls (blue) (B). The distribution of a random gene set is uniform and not different in transcriptomes from normal B cells (blue) and DLBCL cells (red) (C), while that of IEGS shows significant upregulation in the transcriptomes from DLBCL samples when compared to the normal B cell controls (red curve: mean from \( n = 73 \) DLBCL samples) from the same study (D) (\( p \) values for lymphoma vs. normal group comparisons) (data for A-D comes from the NCBI GEO dataset GSE1219529). The ECDF plots for control human tissues show no upregulation of the IEGS (E) in lymph nodes, tonsils, spleen, skin, kidney, pancreas, colon and breast (means from \( n = 3–9 \) samples, \( p \) values for tissue vs. diagonal), (NCBI GEO data set GSE730731). ECDFs of the IEGS from individual samples (F) of normal B cell controls (\( n = 20 \)), FL biopsies (\( n = 38 \)), DLBCL biopsies (\( n = 73 \)) (GEO dataset GSE1219529), multiple myeloma (MM, \( n = 12 \)) (NCBI GEO data set GSE669133), and chronic lymphocytic leukemia (CLL, \( n = 188 \)) (NCBI GEO dataset GSE3104832).
indicating no upregulation for a random gene set in these two groups. The same KS test involving the same samples as above was then applied to the IEGS instead of the random gene set. In contrast to the previous result, the expression of the IEGS was significantly upregulated in the FL group when compared to the control B cell group \((p = 0.0075, \text{Fig. 1B})\). The IEGS genes that were significantly upregulated in FL versus normal B cells were: TIMP1, CCL2, TIGIT, CTLA4, IDO1, VEGFA, CD163, CSF1, IL6ST, LGALS3, MRC1, LGALS1, ICOS, IL10, PDCD1, SOCS3, CCL22, MCL1, CD274(PDCL1LG1), JAK2, HAVCR2, LAG3, IDO2, PDCD1LG2, PTGS2 and GDF15 \((p\) values from \(5.10^{-16}\) to \(2.10^{-2}\)).

Similar results were obtained when we likewise tested 73 samples of DLBCL and 20 normal B cell controls, both downloaded from the same GEO data set. The random gene set was uniformly distributed along both groups of transcriptomes (Fig. 1C), whereas the IEGS distribution was not uniform for the DLBCls and revealed significant upregulation in the DLBCL group relative to the B cell control group \((p = 0.025, \text{Fig. 1D})\). The IEGS genes significantly upregulated in DLBCL vs. normal B cells were, in decreasing order of magnitude: TIMP1, LGALS3, CCL2, IL6ST, LGALS1, CD163, IDO1, VEGFA, CSF1, IL10, LAG3, MRC1, CTLA4, CD274(PDCL1LG1), TIGIT, SOCS3, PDCD1LG2, PVR, HGF, GDF15, IDO2, HAVCR2, MSR1, JAK2, LAIR1, CCL22, MCL1, PDCD1, and PIM1 \((p\) values from \(5.10^{-12}\) to \(3.10^{-2}\)).

The above results evidencing significant upregulation of IEGS in FL and DLBCL samples from the Compagno study’s cohort were confirmed when testing as above another cohort of FL samples \((p = 0.032)\) and DLBCL \((p = 0.032)\). Since both groups of lymphomas showed a significant upregulation of the IEGS, we wondered whether this gene set was also enriched in unrelated samples of lymphoid and non-lymphoid control tissues. Transcriptomes from normal human tissues were downloaded from the NCBI GEO dataset GSE730731 and the IEGS was analyzed as above. The uniform distribution of the IEGS ECDF indicated that the IEGS genes were not upregulated in normal lymph nodes, tonsils, spleen, colon, breast, kidney skin and pancreas (Fig. 1E).

We then asked whether the pattern of IEGS upregulation was homogeneous among the individual FL and DLBCL samples. The individual IEGS ECDF plots of all the normal B cell samples were below the diagonal, indicating that none of these had upregulated expression of the IEGS. In contrast, the same type of analysis indicated that most of the individual FL and DLBCL samples had upregulated expression. Outliers were scarce in these two lymphoma cohorts, indicating that the upregulation of immune escape genes is a common occurrence in these diseases (Fig. 1F). Analysis of an independent DLBCL cohort from The Cancer Genome Atlas confirmed that 78% of individual DLBCL samples had upregulated mRNA expression of genes from IEGS. The analysis of additional cohorts from other cancers evidenced an individual variation of IEGS expression among patients with chronic lymphocytic leukemia (CLL) (NCBI GEO data set GSE31048\(^{35}\)) and no significant upregulation of the IEGS in samples from multiple myeloma patients (NCBI GEO dataset GSE66913\(^{35}\)) (Fig. 1F). In contrast to FL and DLBCL, the most upregulated IEGS genes from other cancers comprised CTLA4, TIGIT, KIR2DL1, KIR2DL2, KIR2DL3, BTLA and IL4 in CLL, and FOXP3, TIMP1, COL17A1, PVR, PDCD1, IDO2 and VEGFA in lung carcinomas (not shown).

In summary, the upregulation of 26–30 IEGS genes in FL and DLBCL is likely to reflect selection of multiple mechanisms comprising the CTLA4 and PD-1/PD-L1–2 inhibitory axes, the LAG3 and TIM3/galectins exhaustion axis, the production of the immunosuppressive molecules IDO, IL10, GDF15 and CSF1, and the recruitment of immunosuppressive cells by CCL2, CCL22 and CSF1, among others. We thus speculate that the gene and protein expression patterns of FL and DLBCL indicates these malignancies evolved a combination of several immune escape pathways.

**Distribution of the PD-1, PD-L1, PD-L2 and LAG3 proteins in biopsies of FL and DLBCL tumors**

In the above analyses, the mRNA expression of most IEGS genes was significantly upregulated. For example among these genes, PDCD1, encoding PD-1, was up-regulated 1.4-fold in FL samples relative to controls \((p < 10^{-7})\) and 1.1-fold in DLBCL relative to controls \((p < 0.05)\); PDCD1-LG1, encoding PD-L1/CD274, was upregulated 1.3-fold in FL samples relative to controls \((p < 10^{-5})\) and 1.7-fold in DLBCL relative to controls \((p < 10^{-7})\); PDCD1-LG2, encoding PD-L2, was upregulated 1.5-fold in FL samples relative to controls \((p < 10^{-10})\) and 2.5-fold in DLBCL relative to controls \((p < 10^{-8})\) and 3.4-fold in DLBCL relative to controls \((p < 10^{-13})\); and the LAG3 gene was upregulated 2.1-fold in FL samples relative to controls \((p < 10^{-5})\) and \(10^{-8}\) in DLBCL. This slight-but-significant upregulation could result from the increased expression of these immune checkpoints by malignant cells as well as by immune cells from the lymphoma samples.

To formally validate the actual presence of these immune checkpoints in FL and DLBCL samples in an independent cohort, we analyzed the expression of the PD-1, PD-L1, PD-L2 and LAG3 proteins using specific antibodies and IHC analysis of tissue microarrays (TMA) comprising 54 biopsy samples obtained from 27 DLBCL and 27 FL patients recently diagnosed at Institut Universitaire du Cancer-Oncopole de Toulouse (Table 2). FL samples were divided into two groups according to the WHO classification: those graded 1–2 FL (85% of FL samples) and those graded 3 FL (15% of FL samples). The two main subtypes of DLBCL, namely GC and ABC (26% and 74% of DLBCL cases, respectively) were classified using the Hans algorithm. Expression of the PD-1, PD-L1, PD-L2 and LAG3 proteins was visually inspected on each of the tumor cells from the 27 DLBCL and 27 FL samples and was quantified in terms of percentage of stained cells and intensity of staining (scored 1+, 2+, 3+). In FL samples, tumors harbored a predominantly follicular pattern, and the neoplastic follicles were composed of small- to medium-sized centrocyte-like cells associated with a variable number of large centroblasts (Fig. 2A, B). None of the FL cases expressed PD-1 (Fig. 2C, D), PD-L1 (Fig. 2E, F) or LAG3 (Fig. 2H) on the lymphoma cells themselves. However, most FL samples contained a rich immune infiltrate of PD-1+ cells, as illustrated in Figure 2C, D. These cells were mostly located in
Table 2. Clinical characteristics of FL and DLBCL patients

|                     | FL       | DLBCL    |
|---------------------|----------|----------|
| Median age (range)  | 63 (26–85) | 65.5 (40–84) |
| Sex                 | Male: Female 16:11 | 22:5 |
| Elevated LDH        | 35% | 74% |
| Stage               | I:II 55% | 44% |
|                     | III:IV 45% | 56% |
| FLPI                | Low risk (0–1) 35% | - |
|                     | Intermediate risk (2) 25% | - |
|                     | High (≥ 3) 40% | - |
| GELF (active disease) | No 55% | - |
|                     | Yes 45% | - |
| FL grade            | 1–2 85% | - |
|                     | 3 15% | - |
| DLBCL subtypes      | - | 7 |
| GC subtype          | - | 20 |
| ABC subtype         | - | 20 |
| Treatment           | Rituximab alone 5% | - |
|                     | Wait and Watch 35% | - |

As depicted above, FL tumor cells did not express PD-1, PD-L1 and LAG3. Only the PD-L2 protein was detected in 75% of the FL samples, in which it was expressed on average by 27% of tumor cells (range: 5–60%) with a staining intensity score of 1+.

In DLBCL, tumor cells expressed these immune checkpoint proteins at different levels. We found that 22% of the DLBCL cases expressed PD-1, and in these cases it was expressed on average by 80% (range: 60–100%) of the tumor cells with a staining intensity score of 3+. We also observed that 61% of DLBCL cases were PD-L1+, and this was expressed on average by 46% of tumor cells (range: 10–100%) with a staining intensity score of 1+. Further, 81% of the DLBCL cases expressed PD-L2, which was expressed on average by 68% of tumor cells (range: 30–90%) with a staining intensity score of 2+. Finally, 85% of the DLBCL cases were LAG3+, which was expressed on average by 20% of tumor cells (range: 5–100%) with a staining intensity score of 1+.

We then analyzed immune infiltrates in the non-Hodgkin’s lymphomas (NHL) samples comparatively to those of samples of tonsil controls. In most FLs, the immune infiltrates expressed PD-L1, PD-L2 and LAG3 at normal levels (i.e., in 5 to 10% of cells), while PD-1 was notably overexpressed (80% of FL cases were stained with an intensity score of 3+) and more intensely stained than in extra-follicular area of tonsils. This staining was also more frequent and more intense than in DLBCL samples (25% of immune cells in FL vs. 10% of immune cells in DLBCL) (Fig. 5). Besides, the immune infiltrates in DLBCL were mostly similar to FL in terms of PD-L1, PD-L2 and LAG3 expression, with nearly 10% of positive immune cells in both lymphomas. Of note, the rate of PD-L1+ immune cells was significantly higher in DLBCL than in FL and in control samples (Fig. 5).

These figures indicate that the proportion of cancer cells expressing immune checkpoint genes was significantly higher in DLBCL than in FL, while their immune infiltrates were similar in both cases except for a higher PD-1 expression in FL lymphocytes. These conclusions validate the above transcriptome data-mining results and extend them by delineating the cellular nature of their respective expression patterns.

Differential expressions of PD-1, PD-L1, PD-L2 and LAG3 in ABC vs. GCB subtypes of DLBCL

We next compared the expression of PD-1, PD-L1, PD-L2 and LAG3 between the ABC and GC subtypes of DLBCL samples. As shown in Figure 6A and D, ABC and germinal center B cell origin (GCB) DLBCL were roughly similar in terms of PD-1 and LAG3 expression. Of note, the aforementioned few cases of PD-1+ DLBCL cells were present in both groups.

In contrast, the mean rates of PD-L1+ and PD-L2+ lymphoma cells were significantly higher in the ABC subtype (p = 0.04 for each marker) (Fig. 6B, C). This trend for PD-L1 expression was in agreement with a previous study of DLBCL.36 We found that the percentage of PD-L1+ tumor cells per sample was 8-fold higher in ABC DLBCL than in GCB DLBCL: there were on average 36% PD-L1+ tumor cells in the ABC DLBCL samples versus only 4% in GC DLBCL samples. In addition, half
Figure 2. IHC of immune escape markers expressed by FL. (A and B) Classical H&E stainings of FL biopsies (A: FL grades 1–2, ×100, B: FL grade 3, ×100) reveal small and atypical lymphoid cells with a round nucleus and an irregular membrane. (C–H) Representative IHC of FL samples stained for: PD-1 (C: ×100, D: ×200), PD-L1 (E: ×100, F: ×40), PD-L2 (G: ×100), and LAG3 (H: ×100).
Figure 3. IHC of immune escape markers expressed by DLBCL. (A and B) Classical H&E stainings of DLBCL biopsies (A: ×200, B: ×100) reveal large-sized and atypical lymphoid cells harbouring a round nucleus and an irregular membrane. (C-H) Representative IHC of DLBCL samples stained for: PD-1 (C: ×100, D: ×200); PD-L1 (E: ×100; F: ×150); PD-L2 (G: ×100); and LAG3 (H: ×100).
the PD-L1 stained cells from the ABC DLBCL cases had a staining intensity rated 1+, while only 15% of PD-L1+ cells from the GCB DLBCL samples had this intensity. Likewise, the percentage of PD-L2+ tumor cells per sample in ABC DLBCL was double that of GCB DLBCL, with an average of 60% PD-L1+ tumor cells in the ABC DLBCL samples vs. 26% in the GCB DLBCL. However, both subtypes of DLBCL had the same pattern of staining for PD-L2, with two-thirds of the PD-L2+ tumor cells showing 1+ staining intensities.

We finally compared mRNA expression levels of the PD-1 gene PDCD1, the PD-L1 gene PDCD1LG1, the PD-L2 gene PDCD1LG2, and the LAG3 gene in samples of both subgroups of DLBCL taken from the same independent cohort used for the transcriptome data sets. These data confirmed the IHC results, namely that the ABC DLBCL subgroup significantly overexpresses the PD-L1 gene relative to the GCB DLBCL subgroup (Fig. 6E).

**Discussion**

The present combination of transcriptome data-mining and IHC analysis has revealed that both DLBCL and FL tumor cells as well as their infiltrating immune cells upregulate several immune checkpoint genes and critical proteins in a distinct pattern of several immune escape strategies. The expression of genes involved in pathways of cancer immune escape has been already explored in solid tumors.1-3 Here, the data mining of a large series of tumor samples from lymphoma patients evidenced the
upregulation of 26–30 genes involved in different mechanisms of immune escape. This conclusion is based on the analysis of the expression of a non-exhaustive group comprising only 54 genes, regardless of additional genes that could be relevant for immune escape and the additional contribution of mutational and epigenetic events. Here, we found that the overexpression of 25 immune escape genes was shared by both FL and DLBCL. These genes are involved in mechanisms as varied as the CTLA4 and PD-1/PD-L1–2 T cell inhibitory axes, the LAG3 and TIM3/galectin T cell exhaustion axes, the production of the immunosuppressive molecules IDO, IL10, GDF15 and CSF1, and the recruitment of immunosuppressive cells by CCL2, CCL22 and CSF1, among others.

We speculate that this expression pattern might result from immune selection in the lymphoma niche, which imposed to evolve several immune escape pathways. Multiple immune escape pathways were recently proposed to take place in solid tumors. However, their occurrence in FL and DLBCL had not been reported previously, and their combination might differ from those evolved by other cancers.

Indeed, transcriptomes from biopsies do not identify which cells in these samples are expressing the immune checkpoint

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**Figure 6.** Proportion of tumor cells expressing immune escape markers in ABC- and GC-type DLBCL. (A–D) Shown are the rates of tumor cells found to be positive for the specified stainings upon visual examination of the ABC (n = 20) and GC (n = 7) DLBCL samples: PD-1⁺ (A), PD-L1⁺ (B), PD-L2⁺ (C), LAG3⁺ (D). * indicates significant differences between groups (p = 0.7; p = 0.04; p = 0.04; p = 0.29, respectively), Wilcoxon–Mann–Whitney tests. (E) Box-plot for normalized mRNA expression of the PD-1 gene PDCD1, the PD-L1 gene CD274, the PD-L2 gene PDCD1LG2, and the LAG3 gene in published transcriptomes from GC and ABC subtypes of DLBCL samples, p values for ABC vs. GC obtained from Wilcoxon–Mann–Whitney tests.
molecules. Therefore, our IHC study not only confirmed the expression of immune- evasion at the protein level, but also specified their respective distribution pattern, whether in tumor cells themselves or in their immune infiltrates. However, IHC on formalin-fixed paraffin-embedded (FFPE) slides is poorly suited to detecting extracellular proteins like secreted cytokines and it does not detect small metabolites like the immunosuppressive PGE2, adenosine or the indoleamine dioxygenase (IDO) product N-formyl-kynurenine. Therefore, combining these two approaches represented a better strategy for a more exhaustive exploration of immune-evasion pathways in lymphomas.

Our study suggests that FL and DLBCL evolve several patterns of immune escape. In these malignancies, the expression of PD-L1 by the tumor cells of DLBCL and by the immune infiltrates of both is consistent with recent reports in various human cancers. The proportion of PD-L1+ DLBCL cases (found in 61% of cases) is higher than in other solid tumor types, in which only 30% of melanoma cases and 25–36% of non-small cell lung cancer cases, respectively, were positive for PD-L1.39,40 Moreover, the ABC subtype of DLBCL prominently expressed not only PD-L1,36,41,42 but also PD-L2. This ABC DLBCL subtype is associated with an inferior survival prognosis compared to the GCB subtype.43 Although over-activation of NFKB and BCR represent major cell-intrinsic determinants of the ABC DLBCL aggressiveness, we propose that by mediating immune escape, the co-expression of PD-L1 and PD-L2 contributes further to giving ABC the worst prognosis of the DLBCL subtypes.44 Although the modest size of the cohort depicted here did not allow us to investigate correlations with clinical data, further studies from our group are currently ongoing to evaluate the impact of immune escape checkpoints on patients’ survival. Indeed, recent studies have indicated that PD-L1 expressed on tumor cells not only protects them from antitumoral immunity,2,24,25,45 but also enhances the tumorigenicity in vivo.44 The co-expression of both PD-1 and PD-L1 we detected on some DLBCL tumor cells might unveil a positive autocrine feedback loop of unknown significance in these tumors. Likewise, we found that DLBCL tumor cells express other immune-evasion proteins such as LAG3, a marker involved in inducing lymphocyte anergy. Thus, in DLBCL, the high proportion of cases with immune checkpoint-expressing tumor cells suggests that evolving immune escape pathways is mandatory for this disease. In contrast to DLBCL, most FL cases lacked tumor cells expressing LAG3, PD-1 and its ligands but these markers were instead harbored by the immune microenvironment.36,47 Such immune infiltrates were frequently and strongly stained for PD-1 (reported in48–52 and this study). Most FL-infiltrating lymphocytes which expressed PD-1 had lost cytokine responsiveness and had impaired antitumor immunity.50 Therefore, the PD-1/ PD-L1 immunosuppression axis is an important component of the immune escape armament of FL, as was recently demonstrated by the therapeutic benefits of PD-1 blockade in phase 2 FL trials.53 The PD-1/PD-L1,2 pathway is of special concern in Hodgkin’s lymphoma as well, where 9p24 chromosomal alterations result in JAK-STAT activation and subsequent overproduction of the ligands PD-L1 and /PD-L2.19,21 As recently demonstrated by trials of anti-PD-1 in Hodgkin’s lymphomas,54 this prominence extends beyond of NHL up to non-hematopoietic tumors such as melanoma and lung carcinomas.55,56

In conclusion, we report here that FL and DLBCL evolved several immune escape pathways, suggesting that escape to antitumor immunity was critical in these aggressive lymphomas. Accordingly, NHL tumor cells expressed more of the immune checkpoint genes and proteins on DLBCL than FL. The overexpression of PD-L1 depicted here by DLBCL tumor cells extended previous reports of soluble PD-L1 in circulating blood which is associated with poor outcome for DLBCL patients.57 In accordance with these earlier studies, the PD-1/PD-L1 axis was almost confined to the ABC subtype of DLBCL which presents the worst prognosis of the DLBCL subtypes.

Indeed, the landscape of somatic mutations such as HLA-I deletions and activation of neoantigens and endogenous retroviruses is a major driver to the multiplicity of immuno-escape pathways in human cancer.29,37 We postulate that together with genetic instability, multiple gene expression strategy reflects a strong and sustained immune pressure on lymphomas in line with the cancer immunoediting concept.58 Since NHL cells emerge and grow despite permanent exposure to distinct mechanisms of surveillance by various immune effectors in their lymphoid tissues, we suggest that the several escape pathways of NHLs reflect their tumor’s age, aggressiveness and the local stringency of immune pressure.

Clearly, much remains to be done to determine to what extent immune checkpoints impact on lymphoma patients survival. However, our report provides a rationale for clinical trials investigating multi-targeted combinations of immune checkpoint inhibitors.

Materials and Methods

Patients
Twenty-seven FL and 27 DLBCL patients were diagnosed in the Department of Hematology, CHU Toulouse, France, between 2009 and 2013, according to the World Health Organization 2008 classification34 (Table 2). The median age of the FL cohort was 63 yr (range: 26 to 85 years) and the male/female ratio was 16/11. The median age of the DLBCL cohort was 65.5 yr (range: 40 to 84 years) and the male/female ratio was 23/5. According to Ann Arbor staging, 27 patients (50%) were in the advanced stages (stages III–IV). Institutional ethical approval from INSERM and informed consent were obtained in compliance with the Helsinki protocol.

TMA and IHC staining
We constructed a TMA from nine control tonsil samples, 27 DLBCL and 27 FL tissues using duplicate 2 mm cores from FFPE blocks. IHC was then performed on 3 μm TMA sections using routine protocols on a fully-automated platform (Ventana Benchmark XT).

The following antibodies were used: CD20 (1:250; DAKO GA604 clone L26), CD10 (1:100; Novocastra-Leica microsystems ORG-8941 clone 56G6), BCL6 (1:30; DAKO GA625...


Statistical analysis

KS test for IEGS distribution analysis

This analysis of transcriptomes from human FL and DLBCL biopsies as well as B cell controls was based on relevant datasets downloaded from NCBI GEO (http://www.ncbi.nlm.nih.gov/gds). Since several IEGS genes were missing from some of these data sets however, we focused our work to the most recent and exhaustive dataset GSE12195. All sets of data from this study were used, except the five cell lines which were excluded because cultured in vitro. The data set was annotated (mapping from probe-ids to gene symbols) using the R/Bioconductor package hgu133plus2.db. Then, the duplicate gene symbols were removed, by keeping the row with the maximal interquartile range (reduction). Thus, a matrix of 16,845 rows for genes and 136 columns for samples was obtained. It comprised 20 normal B cell controls, 38 FL and 73 DLBCL. For each of these three groups the row means were computed, producing three vectors of length 16,845 which were then ranked in decreasing order.

We defined the immune escape gene set (IEGS) as a non-exhaustive list of 54 immune escape genes (Table 1) as already reported elsewhere for different sets of genes. This list of genes was selected from a literature (Pubmed)-based search using the keywords: immunosuppressive cytokines, immunosuppressive mechanisms, chemokines, tumor-supportive myeloid genes, immunosuppressive myeloid and monocyte-related genes, immunosuppressive signaling pathway genes, synthesis of immunosuppressive metabolite, lymphocyte exhaustion marker, lymphocyte inhibitory receptor genes, Tregs genes, tumor immune escape, tumor immune evasion, tumor immune suppression. This search yielded the 54 relevant gene symbols which were selected for the IEGS (Table 1). As a control, we also defined a random gene set composed of 54 randomly chosen genes.

The positions of the IEGS and the random gene set in the three vectors, once divided by the total number of genes, were calculated and their respective ECDFs were plotted (Fig. 1) using R script for auxiliary functions depicted below. The curves corresponding to the samples of normalized orders computed from healthy cells appear in blue and those from cancer cells appear in red. For both the random gene set and the IEGS gene set, a two-sample KS test was applied to evaluate if the two samples of their normalized orders in the vector from healthy cells and from cancer cells were significantly different or not. As a control, we simulated 1,000 sets of 54 randomly chosen genes, tested them in the control B cells and NHL samples, applied the two-sample KS test and obtained 1,000 p values. The p values so obtained were checked to be uniform as they should be for random gene sets. We also considered the possibility that non-relevant genes such as olfactory receptors randomly reaching the random set might be upregulated in the NHL samples. There were 114 olfactory receptor genes (i.e., gene symbols ORnXm) out of the 21,229 analyzed genes in GSE12195. Since such genes are expected to represent <1 of any random set of 54 genes, they might not suffice to impact the over-expression the whole random set. Actually, only eight genes out of these 114 were upregulated (p < 0.05, fc >1), while 54 were downregulated and 52 unchanged in FL relative to control B cells. In the DLBCL samples, 24 were upregulated (p <0.05, fc >1), 20 were downregulated and 35 were unchanged relative to the control cells. So non-relevant genes such as the ORnXm cannot significantly impact on upregulation of the random sets in the NHL samples.

The above-depicted procedure was applied to the GEO dataset GSE7307, where the samples corresponding to normal colon, breast, kidney, skin, pancreas, lymph nodes, tonsils and spleens were considered separately. Here, the orders of the IEGS genes in the five ranked lists, normalized accordingly, were computed. The one-sample KS test was applied to test whether the IEGS genes are randomly distributed throughout the ranked vector or primarily found at the top of it in each of the five cases. Normal B controls (blue) and lymphoma samples (red) are shown in Figure 1A–D; a two-sample KS test was applied to compare these groups. For normal samples of lymph nodes, tonsils, spleen, skin, kidney, pancreas, colon and breast (Fig. 1E), the random distribution of ECDF of IEGS was tested by one-sample KS test. The same procedure was applied to other samples of NHL ; ABC and GC samples of DLBCL from the study GSE12195, MM samples of the study GSE669133 and CLL samples of the study GSE3104832 (ECDFs shown in red, Fig. 1F).

R script for auxiliary functions of KS test for IEGS distribution analysis downloaded of GEO data set, treatments of annotations and reduction were done using functions that have been encoded as R script in61.

Additional R scripts used for the present study are as follows:

```r
# Auxiliary functions
colline <- "black"  # color for lines
cexa <- 1.1  # axes size
cexm <- 1.3  # title size
lty <- 2  # line type
lth <- 1.5  # line thickness
#v <- scan("IEGS.txt",what="char")
order.matching <- function(v1,v2){
  # returns the indices of those entries of v1 found in v2,
  # v1 and v2 being any two vectors of character chains
  return(which(v1 %in% v2))
}
```
Figures 4 and 5
cated in

Comparison of gene expression in ABC and GC subtypes of

DLBCL

The GEO dataset GSE1219529 contained 73 DLBCL samples with 31 ABC DLBCL samples and 34 GC DLBCL samples. In each of the two subtype matrices, column values were replaced by ranks and further normalized, by dividing by the total number of rows. The normalized ranks of the genes $PDCD1$, $CD274$, $PDCD1LG2$ and $LAG3$ in the transcriptomes from GC and ABC subtypes of DLBCL samples are shown as boxplots, and their ranks compared by using the Wilcoxon–Mann–Whitney test (Fig. 6E).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Laurence Jalabert, Gabrielle Perez and François Xavier Frenois for the TMA and whole-imaging (Plate-formes d’immunohistochimie et d’imagerie, département de Pathologie du Pr Brousset, IUCT Oncopole, France). We acknowledge Fabien Soldevilla (Roche-Ventana, France) for kind gifts of anti PD-L1 antibodies and Pierre Ferre (Institut de Recherche Pierre Fabre, France). We thank Kelly Thornber for English editing of the text.

Funding

This work was supported in part by institutional grants from the Institut National de la Santé et de la Recherche
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Authors' Contributions

CL, KC, and PG performed experiments and analyzed data; CL, MT, and CF analyzed the data; CL, BY, AB, and JJF designed experiments; BY, CL, and JJF supervised the study and wrote the manuscript. AB is an employee of the Laboratoires Pierre Fabre.

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