Dissecting CD47 expression in lymphoid neoplasms to inform precision immunotherapy with anti-CD47 phagocytic checkpoint blockade

CD47 is a glycosylated cell surface molecule expressed by a variety of cell types that engages signal regulatory protein α (SIRPα) on macrophages, inhibiting phagocytosis.[1] CD47 is upregulated on tumor cells in several types of cancer, including myeloid[1,2] and lymphoid neoplasms,[3,4] and is associated with aggressive tumor progression and poor prognosis.[1] Targeting CD47-SIRPα phagocytic checkpoint has shown efficacy against lymphomas,[4] however, a comprehensive analysis of the CD47 antigen density across histological subtypes of lymphoid neoplasms is yet to be conducted. In this respect, accurate identification of lymphoma entities with higher CD47 expression on tumor cells is crucial to identify which patients may benefit most from anti-CD47 blockade. To that end, we performed an immunohistochemical (IHC) study to investigate the expression of the CD47 immunoregulatory molecule in diagnostic biopsies taken from patients with mature B-cell and T-cell neoplasms.

Primary diagnosis of lymphoma cases included in the study cohort (N=344) was made by expert hematopathologists, according to updated World Health Organization (WHO) classification of tumors of hematolymphoid tissues.[5] Formalin-fixed paraffin-embedded (FFPE) tissue blocks were retrieved from i) University College Hospital, London, UK, ii) Spanish National Cancer Institute, CNIO, Spain, iii) Department of Clinical Pathology, Robert Bosch Hospital, Stuttgart, Germany. Tissue microarrays sections from marginal zone lymphomas (N=66) were also included. For the IHC study, 2-4-micron tissue sections from the paraffin blocks were cut and transferred on electrically charged slides to subject to IHC. Single IHC was performed according to a previously described protocol using a recombinant rabbit monoclonal anti-CD47 antibody, EPR21794 clone (Abcam, ab218810).[2] CD47 expression was also assessed in normal lymphoid and hematopoietic tissues (*Online Supplementary Figure S1*; *Online Supplementary Table S1*), and in reactive Toxoplasma and non-necrotising granulomatous lymphadenitis (*Online Supplementary Figure S1*). The CD47 immunohistochemical expression and antigen intensity was scored from 0 to 3 (0: absent membranous staining; 1: weak; 2: moderate: 3: strong) and independently assessed by three investigators (AM, AR, and TM). This study was carried out under the Ethical Approval provided to TM by NHS Health Research Authority (REC reference: 09/H0715/64). For bioinformatic analyses of diffuse large B-cell lymphoma (DLBCL), the tumor transcriptome and patients' clinical/genetic correlates were retrieved from the GDC NCICCR-DLBCL dataset (https://xenabrowser.net/).[6,7]

The study cohort comprised 258 cases of B-cell non-Hodgkin lymphoma (B-NHL), 34 cases of Hodgkin lymphoma (HL), ten cases of multiple myeloma, and 42 cases of T-cell lymphoma (Table 1). Most small B-NHL showed positive staining for the CD47 molecule on tumor cells. In particular, all of the 11 cases of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) were positive (Figure 1A), as were all nine cases of nodal mantle cell lymphoma (MCL; Figure 1B), and nine of ten cases of lymphoplasmacytic lymphoma (LPL). However, there was no expression of CD47 on leukemic cells in all cases (10/10) of *BRAF* V600E^{+} hairy cell leukemia (HCL) examined. Approximately 40% (19/46) of adult classic follicular lymphoma (FL) cases were positive for CD47 (Figure 1C), with the majority of these cases being classified as grade 1 or 2 (13/19, 68%; Table 1). All five cases of pediatric-type FL were CD47 negative (Figure 1E). In marginal zone lymphoma (MZL), the lymphoma cells displayed strong positivity for the CD47 marker in all 126 cases, either nodal (Figure 1F) or extra-nodal (Figure 1G, H). In HL, CD47 expression was detected, with variable antigenic intensity, on CD30^{+} Hodgkin/Reed-Sternberg (HRS) cells in 78% (11/14) of cases from the classical histotype (cHL) (Figure 2A, B), but was absent in all nodular lymphocyte predominant HL (NLPHL) cases (0/20) (Figure 2C). In cHL, CD47 was also expressed by reactive small lymphocytes and macrophages infiltrating the exuberant tumor microenvironment (TME). The pattern of expression of the CD47 marker on non-tumor cells was similar in the NLPHL TME, despite lower abundance of macrophages. Amongst high-grade B-cell tumors, the majority (90%, 9/10) of Burkitt lymphomas showed strong positive expression of the CD47 marker on the tumor B cells (Figure 2D). In DLBLC (N=35) (Figure 2E, F), we noted that CD47 upregulation was prevalent in the non-germinal center (~70%, 9/13; Figure 2F) compared to the germinal-center (GC) (~13%, 2/15; Figure 2E) subtype. Furthermore, CD47-positive staining on tumor B cells was detected in one of seven double-hit *MYC-/BCL2*-rearranged DLBCL cases (14%). Six of seven of the "double hit" cases were GC-type by IHC, including the one showing positive staining for CD47. The only one "double hit" non-GC-type DLBCL case was CD47 negative.

In multiple myeloma, the neoplastic plasma cells stained positively for CD47 in all ten cases analyzed. Consistent with a previous report,[8] we observed marked heterogeneity of CD47 expression on tumor cells in T-cell lymphomas. In our study cohort, nodal T follicular helper (TFH) cell lymphoma,

angioimmunoblastic-like and peripheral T-cell lymphomas not otherwise specified (PTCL-NOS), according to WHO-HAEM5 classification,[5] exhibited the highest frequency of CD47$^+$ lymphoma cells (100%, and 64%, respectively), while CD47 was upregulated on neoplastic T cells in 38% and 30% of ALK-positive and ALK-negative anaplastic large cell lymphoma, respectively (Table 1). However, it should be noted that our investigation of CD47 expression in T-cell neoplasms remains preliminary, as only the most common subtypes have been examined, and cannot fully capture the high heterogeneity of these tumors, which persists even within individual histotypes (i.e., PTCL-NOS).

From the perspective of precision medicine, our comprehensive characterization of CD47 expression across lymphoid tumors may inform immunotherapeutic strategies exploiting the CD47/SIRPα phagocytic checkpoint, and provide the rationale for combination therapies in specific settings. Amongst small B-NHL, we observed that only a fraction (~40%) of FL cases displayed positive CD47 immunostaining on neoplastic B cells by IHC. This prompted us to investigate the clinical and biological correlates of CD47 expression in FL. To that end, we surveyed a recently published cohort of newly diagnosed untreated FL cases.[9]

Table 1. Expression of the CD47 immunoregulatory molecule in lymphoid neoplasms.

Lineage	Histology	CD47-positive cases/total cases
B-cell neoplasms N=302	Non-Hodgkin B-cell lymphoma	
	Low grade	
	Chronic lymphocytic leukemia/small lymphocytic lymphoma	11/11
	Mantle cell lymphoma	9/9
	Lymphoplasmacytic lymphoma	9/10
	Hairy cell leukemia	0/10
	Follicular lymphoma	19[a]/46
	Nodal marginal zone lymphoma	115/115
	Extranodal marginal zone lymphoma	11/11
	Follicular lymphoma with marginal zone differentiation	0/1
	High grade	
	Burkitt lymphoma	9/10
	Diffuse large B-cell lymphoma	
	Germinal center	2/15
	Non-germinal center	9/13
	Double-hit (*MYC-/BCL2*-rearranged)	1/7
	Hodgkin lymphoma	
	Classical Hodgkin lymphoma	11/14
	Nodular lymphocyte predominant	0/20
	Hodgkin lymphoma	
	Plasma cell neoplasm	
	Multiple myeloma	10/10
T-cell neoplasms N=42	Nodal TFH cell lymphoma, angioimmunoblastic-like	10/10
	Peripheral T-cell lymphoma, not otherwise specified	9/14
	ALK$^+$ anaplastic large cell lymphoma	3/8
	ALK$^-$ anaplastic large cell lymphoma	3/10[b]

[a]Thirteen of 19 were follicular lymphoma grade 1-2 and 6 of 19 were follicular lymphoma grade 3A. [b]One case showed focal positivity in a proportion of tumor cells. TFH: T follicular helper.

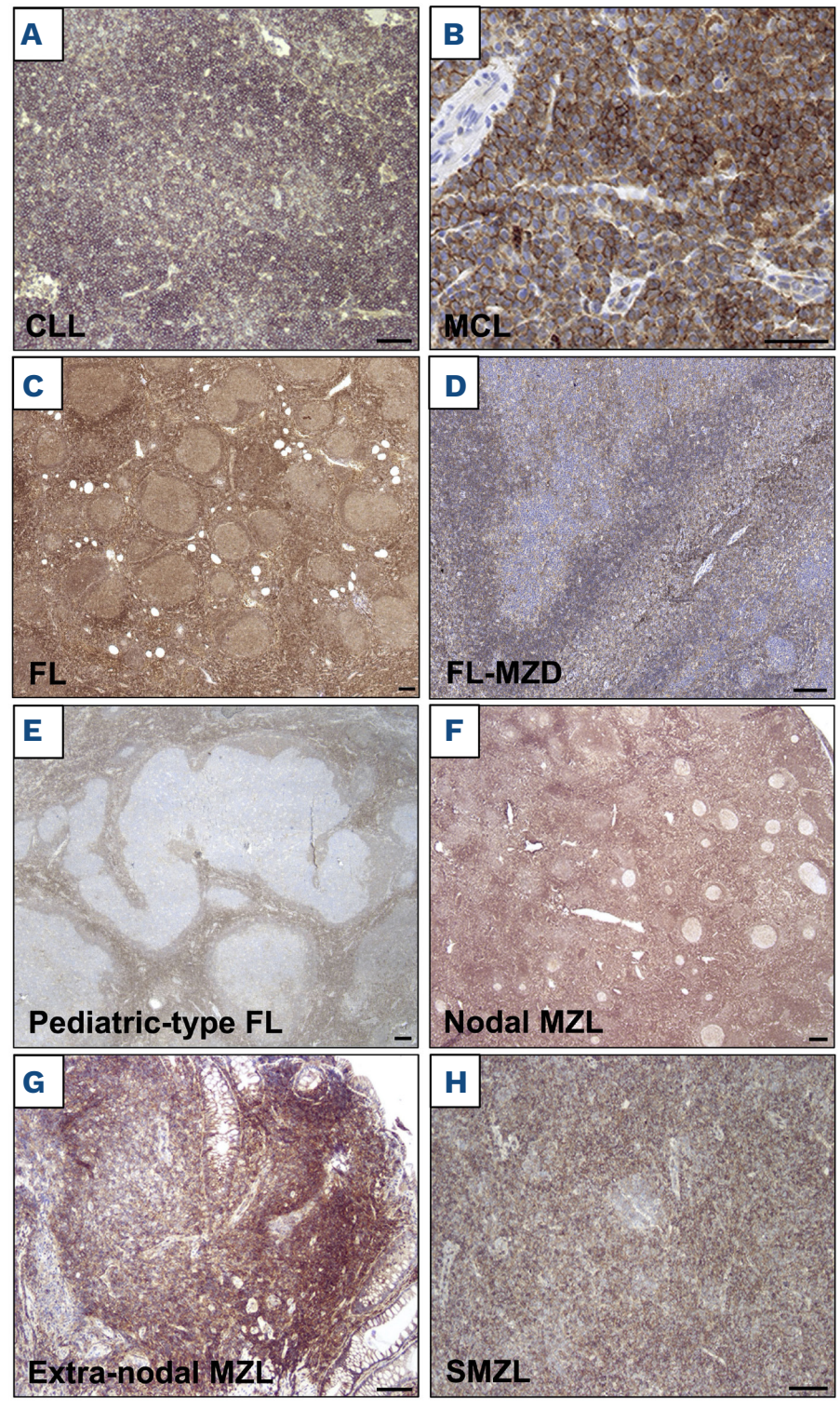

Figure 1. CD47 expression in small B-cell non-Hodgkin lymphomas. CD47 is consistently expressed on neoplastic B cells in chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (A), and mantle cell lymphoma (MCL) (B); about 40% of follicular lymphomas (FL) are positive for CD47, as the case here shown (C); FL with marginal zone differentiation (MZD) shows positive CD47 staining of the marginal zone compartment, while CD47 is negative on neoplastic B cells (D); in pediatric-type FL, neoplastic B cells are negative for CD47 expression (E). In marginal zone lymphoma (MZL) (F-H), strong CD47 expression is detected on neoplastic B cells within the lymph node (F), or extra-nodal sites (G), including the spleen (H). Scale bars, 100 μm.

This study reported that FL B-cells can be distinguished in three distinct transcriptional states, respectively enriched with enhanced inflammation, proliferation or chromatin remodeling gene signatures. Analysis of the *CD47* and *SIRPα* transcripts expression revealed that both these phagocytic checkpoint molecules contributed to the FL group 1, an inflamed memory-like (INFM) subset most sustained by type I interferon response, IL-6 signaling, MYD88-NFkB and

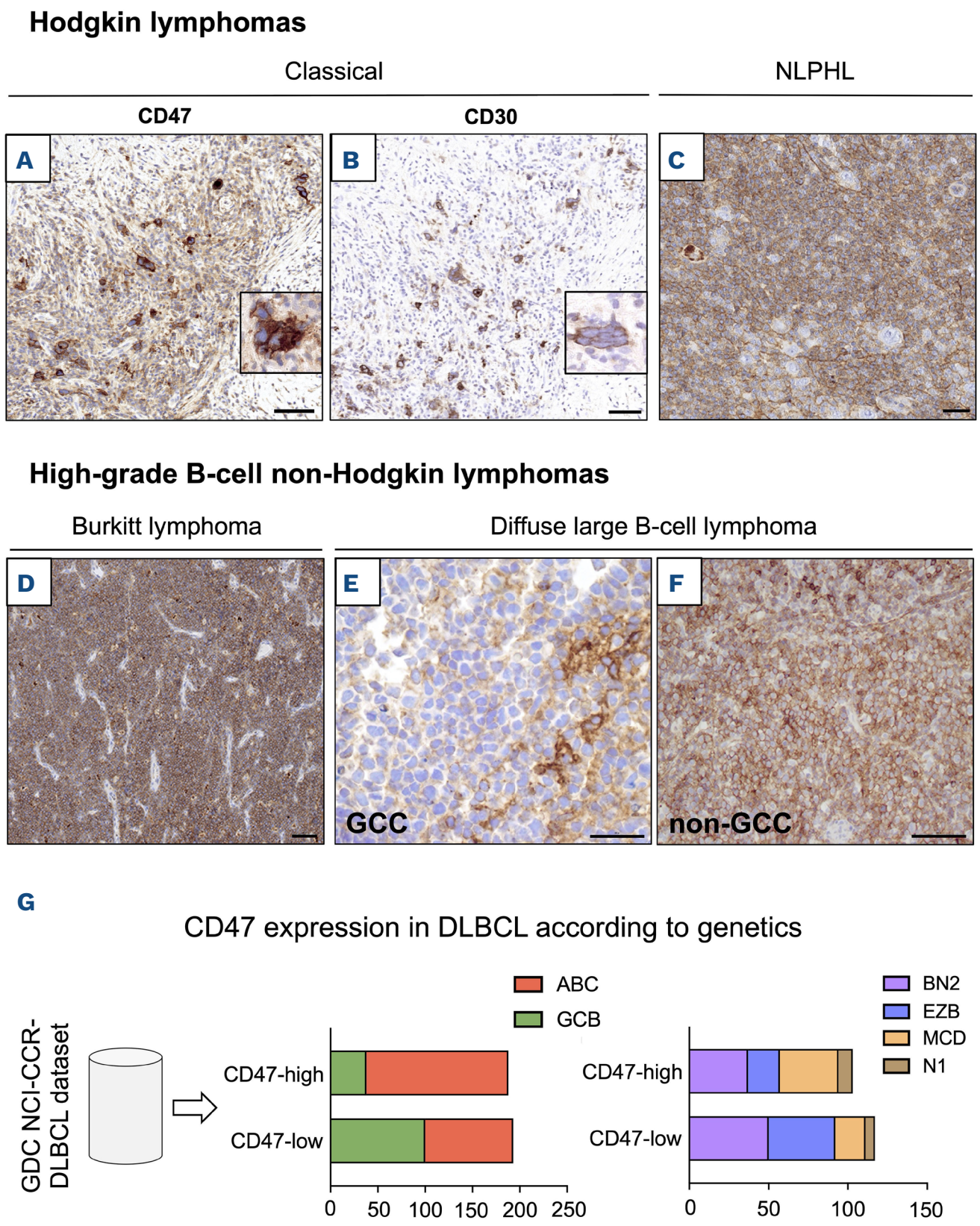

Figure 2. CD47 expression in Hodgkin and high-grade B-cell non-Hodgkin lymphomas. Strong positive membranous/cytoplasmic staining of CD47 in classical Hodgkin lymphoma (HL) (A). In classical HL, CD47 is expressed both on Hodgkin/Reed-Sternberg cells (A), identified by CD30 expression (B), and on reactive small mature lymphocytes and macrophages infiltrating the exuberant tumor microenvironment (A). In nodular-lymphocyte predominant HL, CD47 expression is absent on sparse lymphocyte predominant tumor cells (“popcorn” cells), characterized by large, lobulated nuclei and variable sized nucleoli, but can be detected on background small mature lymphocytes and scattered macrophages in the tumour microenvironment (C). CD47 expression on tumor B cells in Burkitt lymphoma (D), germinal center (GC) (E) and non-germinal-center (non-GC) (F) subtypes of diffuse large B-cell lymphoma (DLBCL). Genetic and clinical data from patients with DLBCL were retrieved from the GDC NCI-CCR-DLBCL dataset (https://xenabrowser.net/) and dichotomized after median *CD47* mRNA expression (G): left histogram, distribution of activated B-cell-like (red) and GC-like (green) genetic subtypes within the CD47-high and CD47-low subsets of DLBCL; right histogram, distribution of *BCL6* fusions and *NOTCH2* mutations (BN2, purple), *EZH2* mutations and *BCL2* translocations (EZB, blue), *MYD88*^{L265P} and *CD79B* mutations (MCD, orange) and *NOTCH1* mutations (N1, brown) seeds according to median *CD47* expression in DLBCL. Scale bars, 100 μm.

repressed cell cycle molecular programs. Consistent with our IHC study which has evidenced that CD47-positive FL cases were primarily of grade 1 or 2, INFM FL demonstrated a less aggressive phenotype. This FL subset was characterized by lower mutational burden and higher abundance of pro-inflammatory cells infiltrating the lymphoma microenvironment, including effector-memory CD4+ T-helper 1 and CD8+ T cells.[9] Overall, *CD47* and *SIRPα* genes expression is enriched within INFM FL, that, because of its tumor B-cell intrinsic programs and microenvironmental landscape, might be more sensitive to immunotherapy-based interventions,[9] including anti-CD47 blockade. Indeed, a pro-inflammatory T-cell enriched microenvironment has been shown to improve the tumor control upon SIRPα-CD47 pathway inhibition.[10] Furthermore, we reported that CD47 was completely absent on leukemia cells in all *BRAF* V600E+ HCL cases examined, which suggests a potential resistance to anti-CD47 blockade. Thus, we propose that *BRAF* V600E-mutated HCL might benefit from a therapeutic strategy combining the anti-CD47 blockade to BRAF or MEK inhibitors. In this respect, both small drugs employed in the treatment of relapsed-/refractory HCL[11] can increase the expression of the CD47 molecule and "eat me" signal calreticulin on tumor cells and synergize with anti-CD47 blockade, as previously reported in *BRAF* V600E-mutated melanoma.[12] In DLBCL, our study expanded previous findings,[3,13] showing that CD47 overexpression was prevalent in non-GC compared to the GC subtype. By querying tumor genetic programs from DLBCL patients included in the GDC NCICCR-DLBCL dataset,[6,7] we showed that CD47 higher expressors preferentially clustered with the activated B-cell-like subtype (GCB-like *vs*. ABC-like DLBCL, Fisher's exact test $P<0.0001$; Figure 2G, left histogram) and were enriched for *MYD88*-L265P/*CD79B* gene mutations (MCD-DLBCL) (MCD *vs*. non-MCD DLBCL, Fisher's exact test $P=0.0011$; Figure 2G, right histogram). Given that MCD-DLBCL might be more sensitive to the inhibition of the BCL2 anti-apoptotic protein or Bruton's tyrosine kinase,[6] we propose that combinatorial approaches enhancing the efficacy of anti-CD47 blockade should be explored in such high-risk DLBCL subtype. Consistent with this latter, in preclinical models of CD47-high DLBCL, the BCL2 inhibitor venetoclax combined with the anti-CD47 blockade, synergistically enhances phagocytic-mediated killing of tumor B cells.[14] In HL, we observed that most cases (78%) of the classical histotype showed positive CD47 staining (with variable intensity across samples) on HRS cells, aligning with a recent IHC study.[15] In addition, we found that CD47 was unequivocally absent on malignant lymphocyte predominant (LP) cells in NLPHL, suggesting a potential resistance to anti-CD47 blockade. In this context, the absent expression of CD47 on tumor cells might be explained by the low abundance of macrophages infiltrating the TME of this rare B-cell lymphoma histotype.

From a diagnostic viewpoint, strong positivity for CD47 was detected on neoplastic B cells in all cases of MZL, irrespective of their nodal or extra-nodal origin. Conversely, ~60% of adult FL cases were CD47 negative. Based on these findings, we propose that CD47 may serve as a diagnostic biomarker for distinguishing (CD47+) MZL from adult FL cases, including those uncommon FL cases showing marginal zone differentiation (FL-MZD). This latter scenario can represent a diagnostic challenge in the routine hematopathology practice. In FL-MZD, the marginal zone cells usually display an immunophenotype distinct from that of GC cells which are typically CD10, BCL6 and BCL2 positive. In FL-MZD, the cells making up the marginal zones are frequently CD10 negative and only weakly BCL6 positive, and so resemble the cells of MZL.[5] To confirm this, we stained one case of FL-MZD, and found that CD47 was completely absent on FL B cells, while the marginal zone component exhibited weakly positive staining (Figure 1D). In addition, cases of higher-grade adult FL, that might be negative for CD47, could lose CD10 expression and show positive staining with MUM1/IRF4 marker.[5] In this latter scenario, the differential diagnosis with MZL becomes particularly challenging because MUM-1/IRF4 is expressed by post-GC B cells, and can be found in nodal MZL cases.[5] In such atypical forms of FL, the diagnosis can be further complicated by the frequent absence of the prototypical FL associated *BCL2/IGH* translocation.[5] However, further studies with larger sample cohort and molecular insights are required to determine whether CD47 expression could aid in resolving this diagnostic conundrum.

In summary, our study provides a comprehensive atlas of CD47 expression across B-cell and T-cell neoplasms, offering clinically-relevant insights to inform precision approaches with the anti-CD47 phagocytic checkpoint blockade in lymphomas. These data also highlight the importance of assessing CD47 expression in each lymphoma patient undergoing anti-CD47 immunotherapy to optimize treatment outcomes. Further studies are required to explore CD47 expression in conjunction with pro-phagocytic molecules (e.g., calreticulin) and other 'don't eat me' signals (e.g., CD24, PD-L1) on lymphoma cells,[16] aiming to optimize precision immunotherapy strategies using phagocytic checkpoint inhibitors. Notably, phagocytic checkpoint inhibitors demonstrate greater efficacy in tumours that exhibit elevated 'eat me' signals and a restricted co-expression pattern of 'don't eat me' molecules.[16]

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https://doi.org/10.3324/haematol.2024.286864

Received: November 14, 2024.
Accepted: March 17, 2025.
Early view: March 20, 2025.

Disclosures

No conflicts of interest to disclose.

Contributions

AM contributed to data curation, formal analysis, and methodology, and wrote the original and revised version of the manuscript. AR, SCK, GS, SPa, SPo, IP, AC, AMu and TM selected the cases and reviewed the staining. MC, VMF and BF critically revised and edited the manuscript. GO, MPi and MPo provided anonymized tissue sections and revised the manuscript. AM and TM conceived, directed and supervised the study, and wrote and revised the manuscript with input from all the authors. All authors read and approved the final version of the manuscript.

Acknowledgments

We are thankful to Ayse U. Akarca for the initial involvement to this study and to Josep Linares for assisting with CD30 staining of Hodgkin lymphoma.

Funding

This work was supported by the UK National Institute of Health Research University College London Hospital Biomedical Research Center grant (to TM).

Data-sharing statement

Original data are available upon email request to the corresponding authors.

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