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RESEARCH ARTICLE

Lymphocyte predominant cells of nodular lymphocyte predominant Hodgkin lymphoma interact with rosetting T cells in an immunological synapse

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Abstract

Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) is a subtype of Hodgkin lymphoma with a preserved B-cell phenotype and follicular T helper (T_{FH}) cells rosetting around the tumor cells, the lymphocyte-predominant (LP) cells. As we recently described reactivity of the B-cell receptors of LP cells of some NLPHL cases with *Moraxella* spp. proteins, we hypothesized that LP cells could present peptides to rosetting T cells in a major histocompatibility complex class II (MHCII)-bound manner. Rosetting $PD1^+$ T cells were present in the majority of NLPHL cases, both in typical (17/20) and variant patterns (16/19). In most cases, T-cell rosettes were $CD69^+$ (typical NLPHL, 17/20; NLPHL variant, 14/19). Furthermore, both MHCII alpha and beta chains were expressed in the LP cells in 23/39 NLPHL. Proximity ligation assay and confocal laser imaging demonstrated interaction of the MHCII beta chain expressed by the LP cells and the T-cell receptor alpha chain expressed by rosetting T cells. We thus conclude that rosetting T cells in NLPHL express markers that are encountered after antigenic exposure, that MHCII is expressed by the LP cells, and that LP cells interact with rosetting T cells in an immunological synapse in a subset of cases. As they likely receive growth stimulatory signals in this way, blockade of this interaction, for example, by PD1-directed checkpoint inhibitors, could be a treatment option in a subset of cases in the future.

1 | INTRODUCTION

Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) is a type of Hodgkin lymphoma (HL) that differs from the classic types of HL in terms of morphology, immunophenotype, pathogenesis and clinical presentation.[1] The tumor cells, that is, the lymphocyte predominant (LP) cells, present a preserved B-cell phenotype and active B-cell receptor (BCR) signaling.[2-4] Both nodular growth patterns with a germinal center-like environment as well as several variants of diffuse histopathological growth patterns with a T-cell-rich microenvironment can occur.[5,6] Histopathological growth patterns have been classified into six pattern A-F by Fan et al.[6] A subgroup of NLPHL that includes mainly young and male patients, presents immunoglobulin D (IgD) expression in the LP cells.[7,8] IgD^+ NLPHL cases more frequently show variant histopathological growth patterns.[7,9] We noted recently that the BCRs of IgD^+ LP cells, which have a genotype with overrepresented VDJ gene segments, high mutation frequency, and an extraordinarily long complementarity-determining region 3 (CDR3), recognize antigens of *Moraxella* spp.,[10] gram-negative bacteria that can cause infections of the upper respiratory tract. As these patients also showed light chain-restricted serum antibodies against the specific *Moraxella* antigens, an infection with *Moraxella* spp. probably

precedes the development of NLPHL in these patients. In contrast to IgD^+ NLPHL patients, who frequently present with high-titer serum antibodies against the *Moraxella*-derived antigen RpoC, no such antibodies were found in patients with T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL), a lymphoma entity closely related to NLPHL. As NLPHL patients with IgD^+ LP cells had a slightly higher relapse rate,[9] relapses in these patients could potentially be triggered by reinfection with *Moraxella* spp. The LP cells in NLPHL are characteristically surrounded by rosetting follicular T helper (T_{FH}) cells, strongly expressing PD1[11-13] and less frequently by rosetting $CD57^+$ cells.[14-16] Little is known about the nature and pathogenesis of these rosetting T_{FH} cells.

When a lymph node is involved in infection, the vast majority of antigen recognition occurs within the lymphocyte-antigen-presenting cell (APC) interfaces. Thus, T_{FH} differentiation is a multistep process. Normal T_{FH} differentiation usually requires two APCs: dendritic cells and B cells.[17] Dendritic cells are generally required for early T_{FH} differentiation, while B cells are required for later events and full germinal center T_{FH} maturation.[18] Antigen-specific B cells undergo exponential expansion and thus APCs become more abundant; activated B cells also upregulate major histocompatibility complex class II proteins (MHCII). The T_{FH} cells depend on B cells in most contexts, and germinal center B cells depend on T_{FH} cells.[18] The T-cell receptor (TCR) binds to its peptide ligands if they are presented by MHCII proteins on the surface of, for example, B cells.[19] When a B cell binds an antigen, it internalizes the antigen by receptor-mediated endocytosis and then undergoes a series of phenotypic changes.[20] Activated B cells present proteolyzed antigenic peptides in complex with MHCII for recognition by cognate T helper cells.[21] So, T cells that recognize the peptide-MHC complexes provide cognate help to B cells in the form of costimulatory signals and cytokines.

Keeping in mind that IgD^+ NLPHL is triggered by infection with *Moraxella* spp. and that these patients frequently present the same MHCII haplotypes, our hypothesis was that LP cells present peptides derived from antigens in an MHC-restricted way to rosetting T_{FH} cells and thus obtain stimulatory and growth-promoting signals from the rosetting T_{FH} cells. The aim of the present study was therefore to further elucidate the interaction and contact between rosetting T_{FH} cells and LP cells.

2 | MATERIALS AND METHODS

2.1 | Patients

A set of 39 NLPHL and six THRLBCL cases were retrieved from the archives of the Dr. Senckenberg Institute of Pathology, Frankfurt. All cases were reviewed by S.H. and M.-L.H.; diagnoses were confirmed and NLPHL cases were classified into growth patterns according to the classification by Fan et al[6] (Table 1). Informed consent was obtained from all patients according to the Declaration of Helsinki. The local ethics committee of Frankfurt University Hospital approved the study (157/17). A tissue microarray (TMA) with 1-mm cores in triplicate was established.

2.2 | Immunohistochemical staining and proximity ligation assay

Immunohistochemical staining for CD20 (clone L26, RTU, Agilent-DAKO, Santa Clara, CA, USA), PD1 (clone EP239, RTU, DCS-Diagnostics, Hamburg, Germany), IgD (IR517, RTU, Agilent-DAKO), MHCII alpha chain (mouse monoclonal, 1:500 dilution, HLA-DR, clone TAL1.B5, Agilent-DAKO), MHCII beta chain (rabbit monoclonal, 1:25 dilution, HLA-DPB1, clone SP229, LS-C210442, Lifespan Biosciences, Seattle, WA, USA), TCR alpha chain (mouse monoclonal, 1:100 dilution, clone 3A8, NB100-65265, Novus Biologicals, Wiesbaden, Germany), PD-L1 (1:100 dilution, ZR3, Zeta Corporation, Arcadia, CA, USA), OCT2 (dilution 1:100, clone MRQ-2, Cell Marque, Merck, Darmstadt, Germany) and CD69 (1:50 dilution, HPA050525, Atlas Antibodies, Sigma-Aldrich, Munich, Germany) was performed in TMA format as previously described.[22] In brief, after deparaffinization and antigen demasking at pH 6 or pH 9, slides were incubated with the primary antibody for 2 hours, and visualized using the Envision-FLEX kit (DAKO). For double staining of the MHCII beta chain and TCR alpha chain, the TMA was incubated for 2 hours with the anti-MHCII beta chain antibody; after detection with the Permanent HRP Green Kit (ZUC070-100, Zytomed Systems, Berlin, Germany), the TMA was incubated with the anti-TCR alpha chain antibody overnight at 4°C. The results were visualized using the K5005 Detection Kit (Agilent-

TABLE 1 Cases and their characteristics

	Number of cases	Major pattern according to Fan et al[6]	IgD expression in tumor cells (%)	$PD1^+$ rosetting T cells (%)	$CD69^+$ rosetting T cells (%)	MHCII alpha chain expression in tumor cells (%)	MHCII beta chain expression in tumor cells (%)	Proximity ligation assay[a]: mean number of tumor cell specific signals/case (range)
NLPHL, typical pattern	20	19x A, 1 x B	4 (20)	17 (85)	17 (85)	18 (90)	15 (75)	9 (1-24)
NLPHL, variant pattern	19	10x C, 3x D, 6x E	7 (37)	16 (84)	14 (74)	16 (84)	11 (58)	12 (5-21)
THRLBCL	6	—	0 (0)	1 (17)	1 (17)	4 (67)	3 (50)	12 (3-21)

[a]Antibodies against MHCII beta chain and TCR alpha chain used in this assay.

DAKO). The VectaFluor Duet Immunofluorescence Double Labeling Kit (DK-8188, Vector Laboratories, Burlingame, CA, USA) was used for fluorescent double staining of the MHCII beta chain and TCR alpha chain.

The proximity ligation assay (DUOLINK In Situ Brightfield, Sigma-Aldrich) was carried out according to the manufacturer's protocol with the antibodies against the MHCII beta chain (rabbit monoclonal, 1:25 dilution) and TCR alpha chain (mouse monoclonal, 1:100 dilution). A melanoma metastasis with interdigitating dendritic cells and T cells was used as the positive control.

Alexa Fluor 488-labeled phalloidin staining (Thermo Fisher, Waltham, MA, USA) was performed according to the manufacturer's instructions on 10 NLPHL cases with available frozen tissue, which had easily identifiable LP cells.

For IgD and MHCII immunostaining, a case was considered positive when >80% of the tumor cells presented expression of the antigen. For $PD1^+$ and $CD69^+$ rosetting T cells, a case was considered positive when at least one typical complete rosette around an LP cell was identified. For the analysis of the proximity ligation assay, signals located to 10 tumor cells per case were counted and summed.

3 | RESULTS

3.1 | $PD1^+$ and $CD69^+$ rosetting T cells show similar frequencies in typical and variant NLPHL and are rare in THRLBCL

All cases were analyzed for the presence of rosetting T cells in PD1 and CD69 immunostaining; PD1 is highly expressed in T_{FH} cells when they are stimulated by a cognate antigen,[23] and CD69 is a T cell activation marker that is highly expressed on the surface of T lymphocytes after TCR/CD3 engagement.[24] Thus, both markers are expressed in T_{FH} cells that have been stimulated by cognate antigen-presenting B cells. Both PD1 and CD69 were expressed in the majority of NLPHL cases with typical and variant growth pattern (PD1, 85% vs 84%; CD69, 85% vs 74%, Table 1, Figure 1). Albeit the number of THRLBCL cases investigated was small, there was only one THRLBCL case that presented $PD1^+$ and $CD69^+$ rosetting T cells (Fisher's exact test, PD1, $P = .002$; CD69, $P = .005$, when compared with all NLPHL).

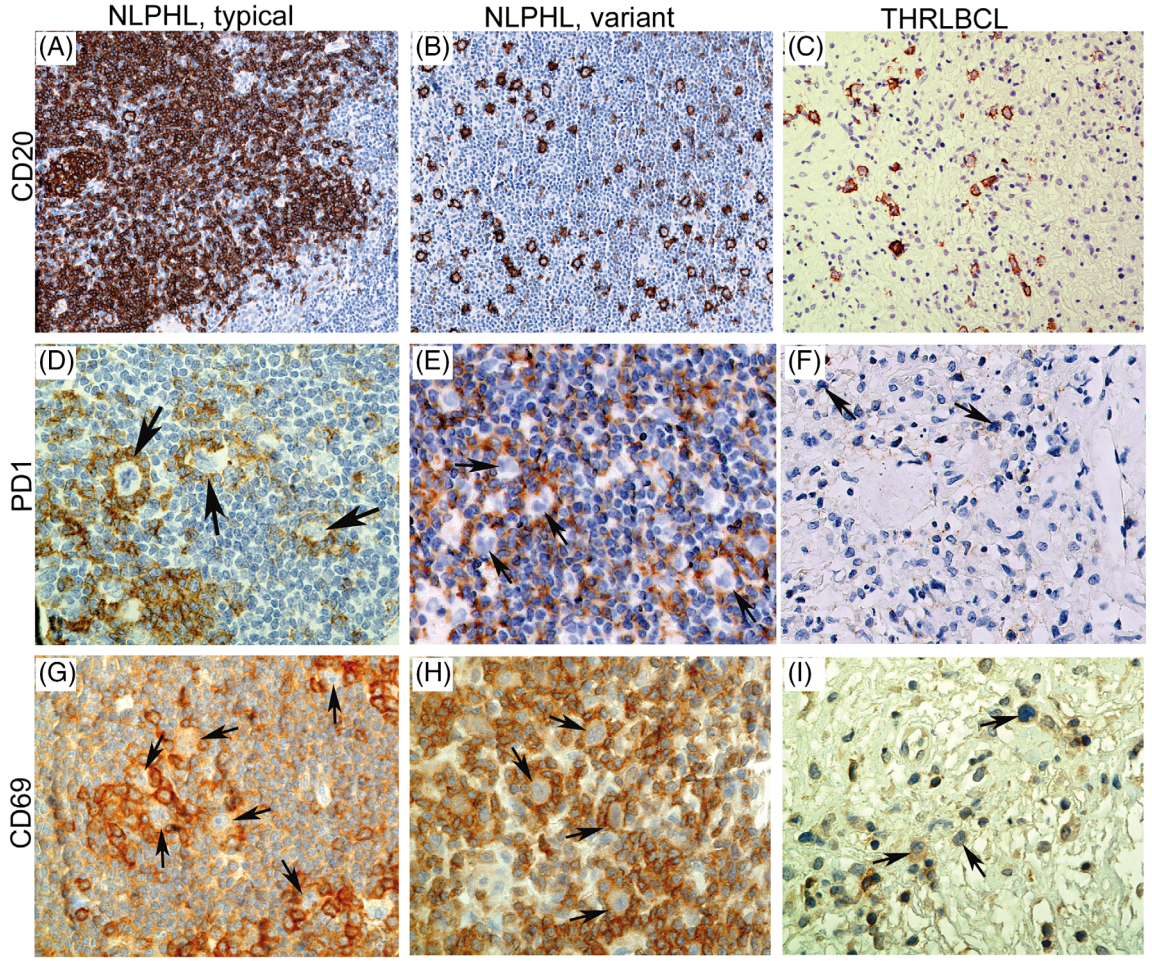

FIGURE 1 Rosetting T cells in typical and variant NLPHL and THRLBCL. A, CD20 immunostainings (200×) of a case of typical NLPHL, B, NLPHL variant and C, THRLBCL. D, PD1 immunostainings (400×) of the same cases of typical NLPHL E, NLPHL variant and F, THRLBCL showing prominent T_{FH} cell rosetting in typical and variant NLPHL. LP cells are highlighted by arrows. G, CD69 immunostainings (400×) of the same cases of typical NLPHL, H, NLPHL variant and I, THRLBCL showing prominent CD69-positive T cell rosetting in typical and variant NLPHL. LP cells are highlighted by arrows.

CD69$^+$ rosetting T cells occurred mostly in the cases with PD1$^+$ rosettes (85%, 29/34 cases with PD1$^+$ rosettes vs 27%, 3/11 cases without PD1$^+$ rosettes, P = .001, Fisher's exact test, Supplementary Figure 1). While there was no difference in the occurrence of PD1$^+$ rosettes between IgD$^+$ and IgD$^-$ cases (73% vs 76% of cases), CD69$^+$ rosettes were significantly more frequent in IgD$^+$ cases (100%, 11/11 IgD$^+$ cases vs 62%, 21/34 IgD$^-$ cases [NLPHL and THRLBCL], P = .020, Fisher's exact test).

3.2 | MHCII is frequently expressed in LP cells

The majority of cases presented strong membrane-bound expression of MHCII, alpha and beta chain, in the LP cells (Figure 2). The LP cells were slightly more frequently positive in typical NLPHL (MHCII alpha chain, 18/20; MHCII beta chain, 15/20; Table 1) than in variant-pattern NLPHL (MHCII alpha chain, 16/19; MHCII beta chain, 11/19). A total of 13/20 typical NLPHL cases (65%) expressed both MHCII

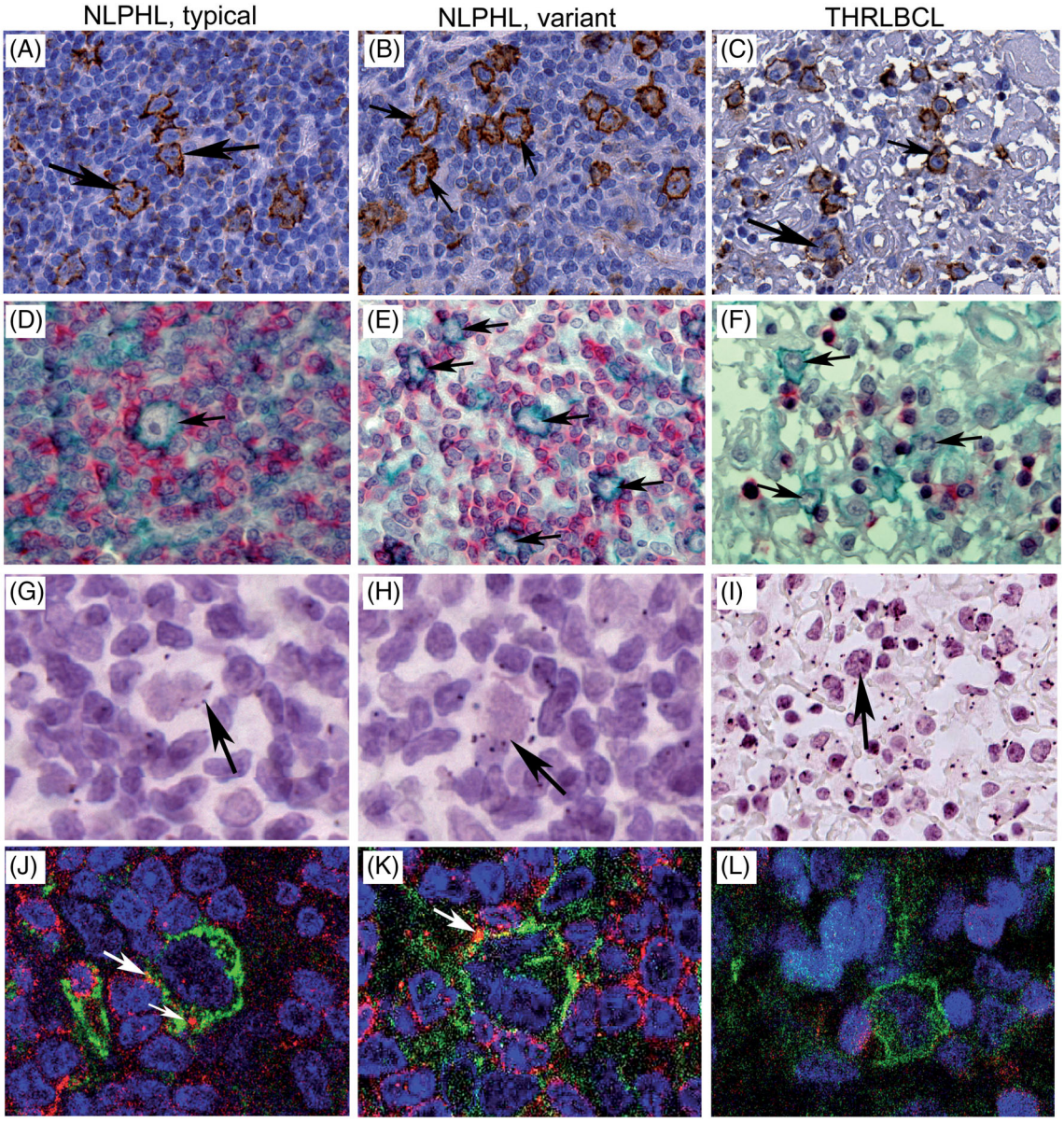

FIGURE 2 Close spatial localization and interaction of MHCII and TCR alpha in NLPHL A, Immunostainings for MHCII alpha chain (400×) of typical NLPHL, B, NLPHL variant and C, THRLBCL showing a membrane bound expression in the tumor cells (highlighted by arrows). D, Brightfield double immunostainings (400×) of typical NLPHL, E, NLPHL variant and F, THRLBCL showing a close localization of membrane bound MHCII beta chain (green) on LP cells (D and E, arrows) and TCR alpha (red) on rosetting T cells. G, Duolink proximity ligation assay (400×) on MHCII beta chain and TCR alpha antibodies with brown signals of close proximity around LP cells (arrows) of typical and H, variant NLPHL and I, abundant signals among different cells in THRLBCL. J, Confocal laser images of fluorescent double immunostainings (630×) of typical NLPHL, K, NLPHL variant and L, THRLBCL showing a close localization of membrane bound MHCII beta chain (green) on LP cells (J and K, arrows) and TCR alpha (red) expressed on T cells. L, No such close association was found in THRLBCL

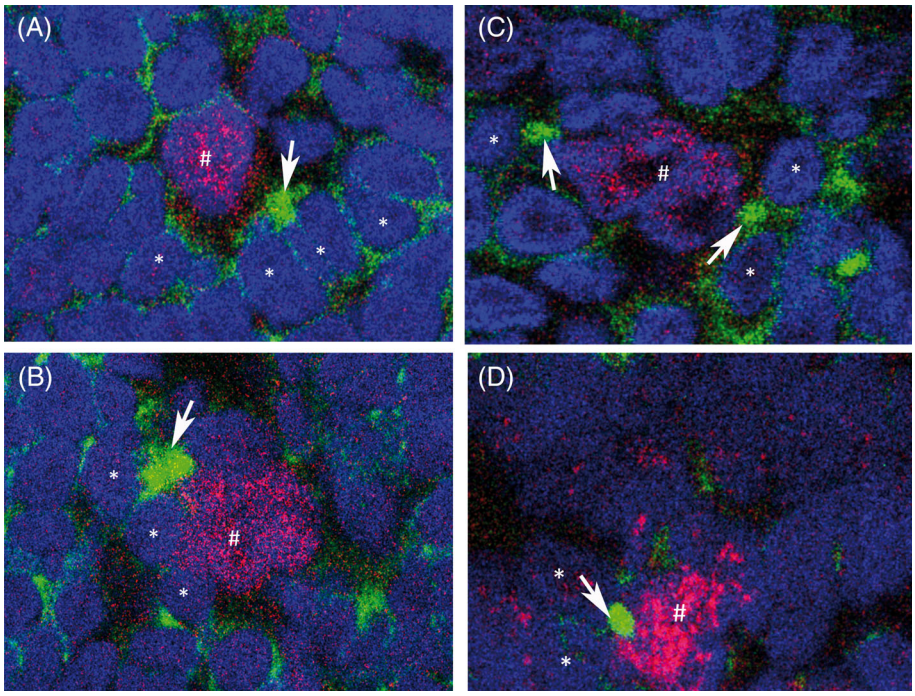

FIGURE 3 Phalloidin staining highlights Actin filament accumulations at the borders between LP cells and T cells. A-D, Phalloidin/OCT2 double staining: Examples of four LP cells (#) from four individual cases identified by OCT2 staining (Alexa-Fluor-594) and chromatin structure with an accumulation of F-actin filaments (green, arrows) in phalloidin staining (confocal image, 630×) in direction towards surrounding lymphocytes (*)

chains, as did 10/19 variant NLPHL cases (53%). THRLBCL tumor cells were positive for both MHCII chains in 50% of the cases (MHCII alpha chain, 4/6; MHCII beta chain, 3/6). IgD^+ NLPHL showed membrane-bound expression of both MHCII chains (82%, 9/11 cases) slightly more frequently than IgD^- NLPHL (50%, 17/34 cases of NLPHL and THRLBCL, not significant). The presence of $CD69^+$ T cell rosettes was significantly more frequent in cases expressing both MHCII chains (85%, 22/26 cases) than in cases expressing one MHCII chain only or completely lacking MHCII expression (53%, 10/19 cases, $P = .043$, Fisher's exact test, Figure S1).

3.3 | MHCII beta chain expressed by LP cells and TCR alpha chain of rosetting T cells have a tight interaction in several NLPHL cases

A proximity ligation assay allows visualization of the interaction of proteins in a tissue with respect to localization to specific cells. A signal is generated only if the proteins of interest are localized within 40 nm, therefore detecting interaction of the respective proteins. In the proximity ligation assay for the MHCII beta chain and TCR alpha chain, signals located to the LP cells were found in typical NLPHL (mean = 9 LP cell-specific signals/case) and in NLPHL variants (mean = 12 LP cell-specific signals/case). In the THRLBCL cases, abundant signals were observed between histiocytes and T cells, and were also closely located to the tumor cells (mean = 12 tumor cell-specific signals/case, Figure 2). In addition, confocal images of exemplary NLPHL cases double-stained with the same antibodies showed a close association of MHCII beta chain on the LP cell membrane and TCR alpha chain proteins (Figure 2). In a total of 10 cases with frozen tissue, we performed phalloidin staining, which highlights intracellular actin filaments, and observed an accumulation of signals between LP cells and rosetting lymphocytes in five cases (two cases IgD^+, three cases IgD^-, Figure 3), as it is expected for an immunological synapse.

4 | DISCUSSION

Rosetting T cells in HL have been known for many years.[25,26] However, their nature was mostly enigmatic. Both the possibility of antigen presentation from Hodgkin-Reed-Sternberg cells to the T cells,[27,28] as well as a host-anti-lymphoma response have been discussed.[29-31] When T_{FH} markers such as PD1 became available, it became clear that the rosetting T cells in NLPHL and the lymphocyte-rich classic HL differ from the other subtypes of HL, as they represent T_{FH} cells.[11] In the present study, we confirmed the presence of $PD1^+$ rosetting T cells at similar frequencies in both typical and variant growth pattern NLPHL, and additionally observed that these T cells frequently express CD69. In contrast, PD-L1, a ligand for PD1, was only inconsistently expressed in LP cells, with only 3/39 NLPHL cases showing PD-L1 expression in the LP cells in the present series (data not shown) and with heterogeneous frequencies in the literature.[32,33] It has further been specified that a proportion of $PD1^+$ and $BCL6^+$ rosetting T cells also express CD57.[34] The $CD57^+$ $CD4^+$ cells have been described as cells that are unable to proliferate due to chronic antigen exposure.[35] Visser et al[13] observed a high number of $CD69^+$ T cells in the nodules of NLPHL via flow cytometry. Here, we report that, in particular, the T-cell rosettes in direct contact with the LP cells are $CD69^+$ and strongly overlap with the $PD1^+$ rosetting T-cell compartment in NLPHL. Thus CD69 is induced on the surface of T lymphocytes after TCR/CD3 engagement.[24] Anti-CD69 monoclonal antibody treatment attenuated the T-cell exhaustion and tumor

progression in tumor-bearing mice.[36] Thus, with respect to their phenotype, there is a strong likelihood that the rosetting T cells in NLPHL are activated by antigen presentation from the LP cells. Furthermore, we demonstrate MHCII interaction with the TCR alpha chain and an accumulation of actin filaments in this region as observed in an immunological synapse in a remarkable number of cases, which would be a further argument for this hypothesis. The majority of LP cells also express ICAM1 and CD58 (data not shown), which interact with LFA1 and CD2 expressed by the rosetting T cells.[25] Whereas LFA1-ICAM1 are found in the peripheral supramolecular activation clusters of the immunological synapse,[37] CD2-CD58 interactions are of the correct size for co-localizing with the TCR in the center of the immunological synapse.[37] Generally, our results strongly suggest that there is a true immunological synapse between LP cells and rosetting T cells and that intact interaction between T_{FH} cells and LP cells is required for the development of NLPHL. Consistent with this hypothesis, NLPHL is hardly ever observed in HIV-infected individuals, who probably do not have sufficient $CD4^+$ T_{FH} cells, or in patients receiving immunosuppressive treatment. In contrast, there is a link between autoimmunity and NLPHL, as patients with germline FAS mutations causing autoimmune lymphoproliferative syndrome both show various manifestations of autoimmunity and have an increased risk to develop NLPHL.[38,39] Additionally, the human leukocyte antigen (HLA) haplotype HLA-DRB1-04 that is frequently observed in NLPHL[10] was described to occur also in a wide range of autoimmune diseases.[40] Thus, already activated T cells may pave the way for the development of NLPHL.

The strong constitutive expression of BCL6 due to genomic aberrations in LP cells[41-43] probably hampers LP cell precursors exit from the germinal center and prevents LP cell differentiation towards memory B or plasma cells. Thus, the deregulated *BCL6* expression in the LP cells may be a major factor contributing to this prolonged antigen presentation by the LP cells. In light of the recent finding that LP cells of a subset of NLPHL cases react with *Moraxella* spp. antigens, it becomes even more plausible that the LP cells retain antigen presentation and derive proliferation stimuli from interaction with the respective T cells. Based on this, we have observed a strong prevalence of certain MHCII haplotypes among patients with NLPHL with *Moraxella*-reactive LP cells.[10] However, in the present study, we did not observe a restriction of this phenomenon to IgD^+ cases. Therefore, stimulation by rosetting $PD1^+$ T cells may be a more general phenomenon in NLPHL pathogenesis and is probably not restricted to IgD^+ NLPHL. BCR of IgD^- LP cells may react with autoantigens or other so far unknown bacterial agents. In the present study, the number of cases with $PD1^+$ and $CD69^+$ rosetting T cells did not differ between typical and variant NLPHL. In contrast, we rarely observed rosetting T cells in THRLBCL, which points either to a different pathogenesis or speaks in favor of a transformation process, meaning that the tumor cells are no longer dependent on help from T cells. The same holds probably true for NLPHL variants lacking $PD1^+$ rosetting T cells which may also show a higher frequency of somatic mutations in the LP cells.[44,45] The expression of MHCII was slightly less frequent in the NLPHL variant cases, which however did not completely match with positivity in the proximity ligation assay. This may be due to technical reasons, as the cases were of different age and fixation, only a limited number of cases was studied in TMA format, and an unknown number of MHCII-TCR synapses may be missed on 2-D sections. The loss of MHCII expression in NLPHL variant cases could be an indicator of tumor progression, as many aggressive lymphomas lose MHCII expression, which is frequently related to translocations involving the CIITA locus.[46] This is also relevant for NLPHL, as the only available NLPHL cell line DEV[47,48] is MHCII-negative and has a *CIITA* translocation,[46] and we were able to observe the same structural variant in a diffuse large B-cell lymphoma transformed from NLPHL, but not in the originating NLPHL component.[44] Thus, CD58, which is also part of the immunological synapse, is frequently lost in aggressive B-cell lymphomas and classic HL,[49,50] as it can also be a target of cytotoxic T cells; thus, the loss of CD58 can be protective for tumor cells unless they require its expression for a functional immunological synapse. Therefore, our present results strongly suggest that LP cells of a remarkable number of NLPHL cases benefit from their interaction with rosetting T_{FH} cells in an immunological synapse. A functional model as final proof of this interaction would be desirable. However, as the only available NLPHL cell line DEV,[47] established from progressive disease, has lost its MHCII expression[22,46] and the tumor cell content in primary NLPHL suspensions is usually <1%, a functional proof seems impossible. However, the presence of an immunological synapse between LP cells and rosetting T_{FH} cells could be a potential therapeutic target in the future. Albeit there is no specific drug targeting immunological synapses in general, PD1-specific antibodies may also act in NLPHL, targeting the $PD1^+$ T_{FH} cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with the content of this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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