BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Characterization and Purification of Neoplastic Cells of Nodular Lymphocyte Predominant Hodgkin Lymphoma from Lymph Nodes by Flow Cytometry and Flow Cytometric Cell Sorting

Jonathan R. Fromm,^{*} Anju Thomas,[†] and Brent L. Wood^{*}

From the Department of Laboratory Medicine,^{*} University of Washington; and Phenopath Laboratories,[†] Seattle, Washington

Accepted for publication
October 18, 2016.

Address correspondence to
Jonathan R. Fromm, M.D.,
Ph.D., University of Washington
Medical Center, Box 357110,
1959 NE Pacific St., Seattle, WA
98195. E-mail: jfromm@u.washington.edu.

We report the flow cytometric (FC) identification and characterization of lymphocyte predominant (LP) cells from tissues involved by nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). First, we immunophenotyped the NLPHL cell line (DEV) confirming a germinal center immunophenotype, lack of expression of CD32 and CD58, and expression of CD54. Nineteen of 26 lymph nodes involved by NLPHL demonstrated a population with an LP immunophenotype (73%), which included expression of germinal center markers (CD75/Bcl-6-positive, CD32-weak/negative without CD10), a B-cell immunophenotype (CD19/CD20/CD40⁺), IgD and/or IgM expression (67%), and lack of programmed death-ligand 1/ligand 2. The LP cells demonstrated an adhesion macromolecule expression pattern distinct from Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma (CHL) (uniform CD50 and variable CD58 for NLPHL; minimal CD50, bright CD58 expression for CHL). A two-tube consensus assay identified LP cells in all seven NLPHL cases examined and only one non-NLPHL case (94 cases evaluated). Finally, FC cell sorting studies confirm that FC-defined populations have an LP cytomorphology. Taken together, these findings demonstrate a two-tube consensus assay can be used to immunophenotype NLPHL with high specificity and sensitivity and rapidly purify LP cells for genetic studies. This study also confirms aneuploidy in LP cells, provides antigens that may be helpful in distinguishing NLPHL from CHL, and suggests that T cells interact less avidly with LP cells than with Hodgkin and Reed-Sternberg cells. (*Am J Pathol* 2017, 187: 304–317; <http://dx.doi.org/10.1016/j.ajpath.2016.10.007>)

Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) is an uncommon type of B-cell lymphoma, characterized by the presence of scattered, rare, large neoplastic germinal center B cells [lymphocyte predominant (LP) cells] embedded in a nodular, reactive infiltrate that includes small, non-neoplastic lymphocytes and histiocytes.^{1,2} In immunohistochemical sections, critical for making a pathologic diagnosis, the LP cells express CD20 and Pax-5 (pan-B-cell markers), CD45 (pan-hematopoietic marker), Bcl-6 (marker of germinal center origin) without expression of CD3 (pan-T-cell marker), CD15 (granulocyte marker), or CD30 (lymphoid activation marker).^{1–3} Lack of CD15 and CD30 is particularly important because the neoplastic cells of the related neoplasm, classical Hodgkin lymphoma (CHL), express these antigens.^{4–6}

Flow cytometry is a technique in which a cell suspension is placed in a moving fluid stream.⁷ The resulting individual cells are directed through a flow cell where the cells can be interrogated by laser-excitation of fluorochrome-conjugated antibodies bound to cell surface, cytoplasmic, or nuclear antigens, allowing for measurement of antigen expression.^{7,8} Although analogous to immunohistochemistry, flow cytometry affords the evaluation of multiple antigens on/in cells interpreted in the context of their light scatter properties.⁷ Today, flow cytometry is helpful in making the diagnosis of leukemia and lymphoma. We have shown that

Supported by the Department of Laboratory Medicine, University of Washington.

Disclosures: None declared.

flow cytometry and flow cytometric cell sorting (FCCS) can be used to diagnosis CHL^{9–11} and to purify the neoplastic Hodgkin and Reed-Sternberg (HRS) cells of CHL.^{12,13}

T-cell–Hodgkin cell rosettes are observed in tissue sections of CHL and NLPHL and provide a diagnostic characteristic of these lymphomas.^{1,4,14–18} We and others have demonstrated that the rosettes of CHL are mediated by CD54 and CD58 on the HRS cells binding to their adhesion binding partners (lymphocyte function-associated antigen-1 and CD2) on the T cells.^{12,19} These interactions are useful to help diagnose CHL by flow cytometry and can be directly observed by FCCS.^{10,12,13} However, what mediates the interaction of LP cells to T cells is unknown.

The intent of this study was to provide the identification and characterization of neoplastic LP cells of NLPHL by flow cytometry, affording a novel method to suggest the diagnosis of NLPHL, rapidly purify viable LP cells for further study, and evaluate antigen expression of these cells.

Materials and Methods

Cell Line

The NLPHL cell line DEV^{20,21} was a generous gift from Dr. Anke van den Berg (University of Groningen, the Netherlands). The cell line was cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin at 37°C in 5% CO₂ for use in immunophenotypic studies.

Fluorescently Labeled Antibodies and Controls

For all experiments, flow cytometric studies were performed on a modified 4-laser, 10-color Becton Dickinson (Franklin Lakes, NJ) LSRII flow cytometer using the following laser-fluorochrome combinations: i) 405 nm violet laser (one color) – Pacific Blue or DAPI; ii) 488 nm blue laser (five colors) – fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Texas Red (electron coupled dye/PE-TR), PE-cyanine-5 (PE-Cy5) or PE–cyanine-5.5 (PE-Cy5.5), and PE–cyanine-7 (PE-Cy7); iii) 594 nm yellow laser (one color) – Alexa Fluor 594 (A594); and iv) 633 nm red laser (three colors) – allophycocyanin (APC), APC-Alexa Fluor 700 (APC-A700), and APC-Cy7. The specific fluorescently labeled antibodies used in this study are listed in [Table 1](#). Fluorescence minus one controls were run to determine background staining.

Immunophenotyping of DEV Cells

For surface immunophenotyping (performed at room temperature in the dark), 10,000 to 100,000 DEV cells were incubated with fluorescently labeled antibodies for 15 minutes in 0.1 mL RPMI. Cells suspensions were lysed and fixed with 0.25% paraformaldehyde, 0.15 mol/L ammonium chloride, pH 4.8 for 15 minutes, washed with 3 mL phosphate-buffered

saline and bovine serum albumin (PBS-BSA), and incubated with 0.1 mL of 1:10 DAPI in PBS to exclude nonviable cells before analysis. For combined cell surface and nuclear immunophenotyping, cells were incubated for 15 minutes with surface-reactive labeled antibodies, washed twice, incubated for 15 minutes with 0.1 mL of Invitrogen (Carlsbad, CA) Fix and Perm reagent A, and washed twice. After the addition of 0.1 mL of Invitrogen Fix and Perm reagent B, cells were washed twice, incubated for 60 minutes with antibodies to nuclear antigens (Bcl-6), washed, and analyzed. For DNA index experiments, the cells were incubated with 0.1 mL of DAPI solution (2.5 mg/mL) after the final washing step. DNA indices are calculated using reference normal G₁ and G₂M fractions.

Preparation of Lymph Node Specimens for Flow Cytometry

This study was conducted with approval of the University of Washington Human Subjects Review Committee. Lymph node biopsy specimens were collected from April 2005 to July 2013, and the corresponding paraffin-embedded tissue diagnoses were rendered with a combination of hematoxylin and eosin morphologic examination and immunohistochemistry at the University of Washington/Seattle Cancer Care Alliance. Fresh lymph node suspensions were prepared in a manner identical to that used for clinical studies and is described elsewhere.¹²

Ten-color flow cytometric tubes were prepared after clinical flow cytometric evaluation on morphologically confirmed NLPHL (diagnosed by tissue section morphologic examination and immunohistochemistry^{1,2}). Experiments used 50,000 to 500,000 cells in 0.1 mL per tube and were stained with labeled antibodies in a manner described above (for DEV). The specific combinations in the initial phase of the work varied, based on the amount of cell suspension available. Combinations included backbone antibodies [three to four antibodies that invariantly recognize antigens present on LP cells (CD20, CD40, and CD71)], one pan–T-cell antigen (CD5), one macrophage antigen (CD64), and CD45 to which a variety of other investigational antibodies were added. When a putative LP population was present, it was additionally evaluated for the expression of CD15, CD30, and CD95, using our clinical assay for CHL.^{12,13} Subsequently, consensus tubes were designed for routine immunophenotyping of NLPHL ([Table 2](#)) and evaluated using seven additional NLPHL specimens and 94 tissues (reactive, involved by CHL, or involved by various non-Hodgkin lymphomas).

Criteria for Identifying LP Cells

From the known immunophenotype of LP cells in paraffin section^{1–3} and our previous work with immunophenotyping CHL,^{9–11} the following criteria were required for the identification of an LP population: i) LP cells are large and

Table 1 Antibodies Used in the Study

Antigen	Fluorochrome	Vendor	Clone
HLA-ABC	FITC	Beckman Coulter (Immunotech; Hialeah, FL)	B9.12.1
HLA-DR	FITC	BD Biosciences (San Jose, CA)	L243
Ig light chains κ - λ	FITC/PE	BD Biosciences	TBC-8-2/1-155-2
IgA	PE	Miltenyi (San Diego, CA)	IS11-8E10
IgD	FITC/PE Cy7	BD Pharmingen (San Jose, CA)/BioLegend (San Diego, CA)	IA6-2
IgM	FITC/PB	BD Pharmingen/BioLegend	G20-127/MHM-88
IgG	APC	Miltenyi Biotec (San Diego, CA)	IS11-3B2.2.3
CD1a	APC	BD Pharmingen	HI149
CD2	FITC	BD Biosciences	S5.2
CD3	FITC/PE	BD Biosciences	SK7
CD4	PE	BD Biosciences	SK3
CD5	PE-TR (ECD)	Beckman Coulter	BL 1a
CD7	APC	Thermo Fisher Scientific (Waltham, MA)	CD7-6B7
CD8	PE	Beckman Coulter	SFC121 Thy2D3 (T8)
CD9	FITC	BD Biosciences	M-L13
CD10	A488	Serotec (Oxford, UK)	SB5c
CD10	PE-Cy7/APC	BioLegend/BD Biosciences	HI10a
CD11 c	APC	Miltenyi Biotec	MJ4-27G12.4.6
CD13	PE	BD Biosciences	L138
CD14	PE Cy5.5	Beckman Coulter	RM052
CD15	APC	BD Biosciences	HI98
CD16	A647	BD Pharmingen	3G8
CD18	FITC	BD Biosciences	L130
CD19	PE-TR/PECy7	Beckman Coulter (Immunotech)	J4.119
CD20	V450	BD Biosciences	L27
CD20	Pe-Cy7/APC-H7	Beckman Coulter/BD Biosciences	B9E9/L27
CD21	FITC	Beckman Coulter	BL13
CD22	PE	Beckman Coulter	SJ10.1H11
CD23	PE	BD Biosciences	EBVCS-5
CD24	PE	Beckman Coulter (Immunotech)	ALB9
CD25	PE	BD Biosciences	2A3
CD26	PE	Beckman Coulter (Immunotech)	BA5
CD27	PE	Beckman Coulter (Immunotech)	1A4-CD27
CD28	PE	BD Biosciences	CD28.2
CD30	PE	BD Biosciences	Ber-H83
CD32	PE/APC	BioLegend	FUN-2
CD33	PE-Cy7	BD Biosciences	P67.6
CD34	ECD	Beckman Coulter (Immunotech)	581
CD36	FITC	BD Biosciences	CB38 (NL07)
CD38	PE/A594	BD Biosciences	HB7
CD40	PE Cy5.5	Beckman Coulter	MAB89
CD43	APC	Thermo Fisher Scientific	L10
CD44	FITC	BD Biosciences	L178
CD45	APC-H7	BD Biosciences	2D1
CD45 RO	PE	Beckman Coulter (Immunotech)	UCHL-1
CD45RA/B220	FITC	Beckman Coulter (Immunotech)	ALB11
CD47	FITC	BD Biosciences	B6H12
CD48	PB/FITC	Exbio (Czech Republic)/BD Biosciences	MEM-102/TU145
CD50*	Unlabeled/PeCy7	eBioscience (San Diego, CA)	CBR-IC3/1/M1-14D12
CD50	PE	BioLegend	CBR-IC3/1
CD52	APC	Invitrogen	CD1D12
CD54	PE/APC	Invitrogen/BioLegend	MEM-111/HA58
CD56	PECy7	Beckman Coulter	N901 (NKH-1)
CD57	FITC	BD Biosciences	HNK-1
CD58	PE	Beckman Coulter (Immunotech)	AICD58
CD62L	FITC	Beckman Coulter (Immunotech)	Dreg 56
CD64	FITC	Beckman Coulter (Immunotech)	22

(table continues)

Table 1 (continued)

Antigen	Fluorochrome	Vendor	Clone
CD66b	FITC	Beckman Coulter (Immunotech)	80H3
CD70	FITC	BD Biosciences	Ki-24
CD71	APC A700	Beckman Coulter	YDJ1.2.2
CD74	PE	BioLegend	LN2
CD75 [†]	BIOTIN/V450	BioLegend/BD Biosciences	LN1
CD75	FITC	BD Biosciences	LN1
CD77*	Unlabeled/PE	BD Biosciences	5B5/R6-60.2
CD77	FITC	BD Biosciences	5B5
CD79b	APC	Beckman Coulter	HM47
CD80	PE/PE-CY7	BD Biosciences/BioLegend	L307.4/2D10
CD81	FITC	BD Biosciences	TAPA-1
CD83	PE/APC	BD Biosciences	HB15e
CD86	PE	BD Biosciences	2331 (FUN-1)
CD90	PE	BD Biosciences	5E10
CD94	PE	Beckman Coulter (Immunotech)	HP-3B1
CD95	PB/APC	Invitrogen/BD Biosciences	DX2
CD99	FITC	BD Biosciences	TÜ12
CD102	PE	BioLegend	CBR-IC2/2
CD103	FITC	BD Pharmingen	Ber-ACT8
CD117	PECy5	Beckman Coulter (Immunotech)	95C3
CD123	PE	BD Biosciences	9F5
CD126	PE	BD Pharmingen	M5
CD127	PE	BD Pharmingen	HIL-7R-M21
CD133	APC	Miltenyi	AC133
CD135	PE	BD Pharmingen	4G8
CD138	APC	BD Biosciences	MI15
CD158a	FITC	BD Biosciences	HP-3E4
CD158b	PE	Beckman Coulter	GL183
CD158e	PE	Beckman Coulter	Z27 (Z27.3.7)
CD163	PE	BD Pharmingen	GHI/61
CD166	PE	BD Pharmingen	3A6
CD203c	PE	Beckman Coulter	97A6
CD221	PE	BD Pharmingen	1H7
CD227	FITC	BD Pharmingen	HMPV
CD252	PE	BD Pharmingen	ik-1
CD273	PE	BioLegend	MIH18
CD274	PE-Cy7	eBioscience	MIH1
CD275*	Unlabeled/APC	BD Biosciences/BioLegend	2D3/POLY4053
BCL-6	PE	BD Pharmingen	K112-91
CXCR3	A488	BD Pharmingen	IC6 \ CXCR3
CXCR4	A488	BD Pharmingen/BioLegend	I2G5
CXCR5	FITC	BD Pharmingen	RF8B2
CCR5	PE	BD Pharmingen	2D7 \ CCR5
CCR6	PE	BD Pharmingen	11A9
CCR7	PE	BD Pharmingen	3D12
EPCAM	FITC	BD Biosciences	EBA-1

*Antibody/fluorochrome conjugate was generated using an unlabeled primary antibody and fluorochrome-labeled secondary antibody.

[†]Antibody/fluorochrome conjugate was generated using a biotin-labeled antibody and streptavidin labeled with the indicated fluorochrome.

A488, Alexa Fluor 488; A594, Alexa Fluor 594; A647, Alexa Fluor 647; A700, Alexa Fluor 700; APC, allophycocyanin; Cy5.5, cyanine-5.5; Cy7, cyanine 7; DR, antigen D—related; ECD, electron coupled dye; EPCAM, epithelial cell adhesion molecule; FITC, fluorescein isothiocyanate; HLA, human leukocyte antigen; L, ligand; PB, Pacific Blue; PE, phycoerythrin; TR, Texas Red.

thus should have increased forward and side light scatter compared with small lymphocytes; ii) the immunophenotype should be similar to that observed for DEV and in paraffin section with expression of CD20¹⁻³ and CD40^{22,23}; iii) the cells may be immunoglobulin light chain

restricted^{24,25}; iv) the cells should be of germinal center origin (Bcl-6⁺,¹⁻³ and CD75²⁶) but without expression of CD10^{3,27} and; v) a putative LP population will form a distinct cluster of events in multidimensional flow cytometric space.

Table 2 Combinations Used in NLPHL Consensus Tubes

Fluorochrome	V450	FITC/A488	PE	ECD	PE-Cy5.5	PE-Cy7	A594	APC	APC-A700	APC-H7
Tube 1	CD75	CD64+CD10	CD32	CD5	CD40	CD20	CD38	CD54	CD71	CD45
Tube 2	DAPI	CD64	BCL-6	CD5	CD40	CD10	CD38	CD54	CD71	CD20

A488, Alexa Fluor 488; A594, Alexa Fluor 594; A700, Alexa Fluor 700; APC, allophycocyanin; Cy5.5, cyanine-5.5; Cy7, cyanine 7; ECD, electron coupled dye; FITC, fluorescein isothiocyanate; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; PE, phycoerythrin.

Flow Cytometry Combinations Used for FCCS Experiments

The antibody combinations used in the FCCS sorting experiments are as follows: i) CD75-V450, CD64-FITC, CD77-PE, CD5-PE-TR, CD40-PE-Cy5.5, CD83-APC, CD71-APC-A700, CD20-APC-Cy7; ii) CD95-PB, CD64-FITC, CD32-PE, CD5-PE-TR, CD40-PE-Cy5.5, CD54-APC, CD71-APC-A700, CD20-APC-Cy7; iii–vi) CD75-V450, CD64-FITC, CD32-PE, CD5-PE-TR, CD40-PE-Cy5.5, CD54-APC, CD71-APC-A700, CD20-APC-Cy7.

FCCS of Lymph Nodes Involved by NLPHL

Cells from the lymph node suspension of six NLPHL cases were stained with the antibody combinations (above) in 1 mL of PBS-BSA (15 minutes at room temperature), washed once with PBS-BSA, reconstituted in 1 to 2 mL of RPMI, and sorted using a FACSaria II flow cytometric cell sorter (Becton Dickinson). The sorted cells were concentrated by centrifugation, Wright-Giemsa stained, and examined by light microscopy. For five sorted cases, an aliquot of the sorted cells was run on the cell sorter to measure purity of the sorted LP cell fraction.

Results

Flow Cytometric Studies of DEV

In an effort to identify antigens useful for identification of LP cells from lymph nodes, we immunophenotyped the NLPHL cell line DEV.^{20,21} DEV expressed pan-B-cell antigens (CD19, CD20, CD45R/B220, and CD79b) and brightly expressed CD40 (B-cell-associated antigen); no expression of CD32 (FcγRIIb) was identified. DEV expressed CD75 (germinal center-associated marker), nuclear Bcl-6, but not CD10, in accord the immunophenotype identified in paraffin sections.^{1,3} Likewise, immunoglobulin heavy chains (IgH) were not expressed. T-cell and myeloid antigens (including CD15) were not expressed. Activation antigens (CD30, CD70, and CD71) were expressed, as were adhesion macromolecules (CD48, CD50, CD54, and CD102). However, the adhesion macromolecule CD58 (important in the interaction of HRS cells with T cells^{12,19}) was absent. Finally, given current interest in tumor immunotherapy directed at PD-1,^{28,29} we evaluated expression of PD-L1 (CD274), PD-L2 (CD273), and the related B7

molecule, B7-H2 (inducible T-cell costimulator ligand; CD275); CD273 and CD275 were expressed, whereas CD274 was not present on DEV cells (Table 3).

Identification of LP Cells in Tissues by Flow Cytometry

A total of 26 lymph nodes confirmed by morphologic examination to be NLPHL were immunophenotyped in the initial phase of the study (using a variety of antibody combinations); an LP population was identified in 19 examinations (73%). LP populations were small in size, ranging from 0.0002% to 0.25% of the leukocytes (Table 4, and Figures 1 and 2).

Our prior work with CHL demonstrated that T-cell and HRS cell rosettes can be readily identified on a plot of CD45 versus CD5, with the rosetted fraction of HRS cells being those cells with evidence of CD5 expression.^{10,12,13} With the use of such an approach, we observed rosettes in 50% of cases (9 of 18 cases) of NLPHL examined. For comparison, 20 consecutive CHL cases, previously immunophenotyped as part of routine clinical care, were re-examined, with 90% showing rosette formation, suggesting that LP cells interact with less avidity than HRS cells with T cells (under the conditions of flow cytometric experiments).

B-Cell–Associated Antigens

For NLPHL cases for which a putative LP population were identified, intensity of antigen expression was scored on a semiquantitative scale as follows: 0, negative (no expression); 1, low or dim; 2, moderate; and 3, bright expression. Mean antigen intensity (MAI) was calculated across all the cases in which the antigen was measured. When possible, antigen expression was also compared with normal cells in the specimen. As expected, the putative LP populations expressed B-cell antigens; CD19 and CD20 were expressed in almost all cases, with intensities varying from between weak and moderate for CD19 (MAI = 1.6) and between moderate and bright expression for CD20 (MAI = 2.2). CD19 was at, and CD20 typically above, the expression seen in normal small B cells. CD40 was moderately to brightly expressed on the LP cells (MAI = 2.4) in all 19 cases, usually at a level higher than the small B cells. The germinal center B-cell–associated marker CD75 was weakly to moderately expressed (MAI = 1.8), usually showing a variable pattern, similar to that observed in reactive germinal centers (data not shown). BCL-6 showed nuclear expression at a weak-to-moderate level (MAI = 1.7), whereas CD10

Table 3 Expression of Antigens in Hodgkin Cell Line (DEV) as Determined by Flow Cytometry

Antigen	DEV expression* [†]
B-cell—associated antigens	
HLA-DR	—
Ig light chains (κ/λ)	—
IgA	—
IgD	—
IgM	—
IgG	—
CD10	—
CD19	++
CD20	+ / ++++, v
CD21	—
CD22	++
CD23	— (possible low + on subset)
CD24	—
CD32	—
CD38	+ / —
CD40	+++
CD44	++
CD45R/B220	++ / ++++
CD75	+ / ++++, v
CD77	—
CD79b	+ / ++
CD81	++ / ++++
CD86	+ / ++
CD103	—
CD138	—
CD252	+ / ++, v
Bcl-6 (nuclear)	+
Activation antigens	
CD25	—
CD30	+ / ++
CD70	+ / ++
CD71	+++
T/NK-cell—associated antigens	
CD1a	—
CD2	—
CD3	—
CD4	—
CD5	—
CD7	—
CD8	—
CD26	— (or very low +)
CD27	—
CD43	—
CD56	—
CD57	—
CD94	—
CD158a	—
CD158b	—
CD158e	—
Adhesion molecules	
CD18	— / +
CD48	+++
CD54 (ICAM-1)	+++
CD50	+++
CD58	—

(table continues)

Table 3 (continued)

Antigen	DEV expression* [†]
CD102	++ / ++++
Myeloid/monocyte/macrophage-associated antigens	
CD13	—
CD14	—
CD15	—
CD16	—
CD33	—
CD36	—
CD64	—
CD66b	—
CD117	—
CD123	—
CD163	—
CD203c	—
Miscellaneous antigens	
EPCAM	—
HLA-ABC	—
CD34	—
CD45	++
CD45 RO	—
CD47	++
CD52	— (or low +)
CD62L	+ / —
CD80	++ / ++++
CD83	+ / — (low +)
CD90	—
CD95	++
CD99	—
CD126	—
CD127	—
CD133	—
CD135	—
CD166	++
CD221	—
CD227	—
CD273	++ / ++++
CD274	—
Chemokine receptor	
CXCR3	—
CXCR4	++ / ++++
CXCR5	+
CCR5	—
CCR6	—
CCR7	— (or very low +)
Megakaryocyte antigens	
CD61	—

*Degree of antigen expression relative to isotype matched control (+, ++, +++ is one, two, or three log above isotype-matched control, respectively).

[†]Cell surface expression unless otherwise noted.

DR, antigen D—related; EPCAM, epithelial cell adhesion molecule; HLA, human leukocyte antigen; ICAM-1, intercellular adhesion molecule 1; T/NK, T/natural killer; V, variable.

was not expressed. Finally, CD32 (not expressed or weakly expressed on normal germinal centers, strongly expressed on reactive non-germinal center B cells) (Figure 2) showed very weak expression when evaluated (MAI = 0.4),

Table 4 Antigen Expression of LP Events from Flow Cytometry and Morphology-Positive NLPHL Cases

Antigen	Mean scored LP intensity*	No. of cases	No. of cases positive (percent positive) [†]	Antigen intensity
B-cell antigens				
κ	0.7	10	4 (40)	
λ	0	10	0 (0)	
CD10	0	12	0 (0)	
CD19	1.6	11	10 (91)	Most at level of small B cells
CD20	2.2	19	19 (100)	Mostly at or higher than small B cells
CD32	0.4	9	6 (67)	8 of 9 cases less than small B cells or negative
CD38	1.5	14	14 (100)	
CD40	2.4	19	19 (100)	Mainly higher than small B cells
CD75	1.8	7	7 (100)	Variable expression
Bcl-6	1.7	10	10 (100)	
Adhesion molecules				
CD48	0.9	10	10 (100)	At or slightly lower than small B cells
CD50 (ICAM-3)	1.8	12	12 (100)	At or higher than other lymphocytes
CD54 (ICAM-1)	2.6	12	12 (100)	Invariably higher than all other cells
CD58 (LFA-3)	1.7	12	12 (100)	Some cases lower, some higher than small B cells
B7 molecules				
CD80	0.2	10	2 (20)	
CD273 (PD-L2)	0.4	5	3 (60)	When expressed, weak expression
CD274 (PD-L1)	0.2	5	2 (40)	When expressed, weak expression
CD275 (ICOS-L)	0.1	5	1 (20)	When expressed, weak expression
Other antigens				
CD5	0	18	0 (0)	Evaluated on non-rosetted fraction
CD15	0	14	0 (0)	
CD30	0.2	17	5 (29)	
CD45	2.7	17	17 (100)	
CD64	0	19	0 (0)	
CD71	2.2	18	18 (100)	Usually higher than all other cells
CD95	1.8	16	15 (94)	Variable intensity

*Mean scored intensity is average intensity of antigen expression; each case scored as 0, not expressed; 1, weak; 2, moderate; or 3, bright expression.

[†]Expression at any level.

ICAM, intercellular adhesion molecule; ICOS-L, inducible costimulatory ligand; LFA, lymphocyte function-associated antigen; LP, lymphocyte predominant; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2.

with eight of nine cases showing intensities either less than reactive, non-germinal center B cells or without any expression. The HRS cells of six CHL cases were examined for the expression of CD32 for comparison with NLPHL; for three cases, the expression of CD32 on the HRS cells was slightly lower than that seen on the non-germinal center B cells. For the other three cases, the expression of CD32 on the HRS cells was higher than that seen for the non-germinal center B cells.

A single tube combination was designed to evaluate surface IgH expression (IgA, IgD, IgG, and IgM) on LP cells. The six cases evaluated showed, two cases with bright IgD expression, two cases with no IgH expression, one case with co-expression of IgM and IgD, and one case with low IgM. No IgA or IgG expression was identified in the six cases. Four of 10 cases evaluated showed κ surface light chain restriction (MAI = 0.7), the other 6 cases showed no surface light chain restriction (Figure 3). For five cases for which both IgH and light chain were evaluated, the three cases that were positive for IgH were positive for a light

chain (κ); conversely, two cases that were negative for IgH were negative for light chain.

Miscellaneous Antigens

The T-cell antigen CD5 and the granulocyte marker CD15 (expressed in CHL) were not expressed on putative LP populations. Weak expression of CD30 was, however, occasionally identified (MAI = 0.2; 5 cases positive, 17 cases examined). The activation antigen CD71 (transferrin receptor) was moderately to brightly expressed (MAI = 2.2), often at a level higher than all other cells in the lymph node suspension (and generally higher than that seen on HRS cells from CHL). CD64 (monocyte/macrophage marker) was not expressed, whereas the pan-leukocyte antigen (CD45) was brightly expressed (MAI = 2.7), consistently higher than that seen on the HRS cells of CHL. CD95 (FAS; expressed in CHL) was usually present on LP cells (94% positive in 16 cases tested); intensity of expression varied from very weak to bright (MAI = 1.8) (Table 4).

Figure 1 Flow cytometric studies of a lymph node-involved NLPHL. The large (as determined by increased forward and side light scatter compared with the small lymphocytes) neoplastic LP cells express CD20 (bright), CD40 (intermediate to bright), CD48 (low to absent), CD50 (intermediate to bright), CD54 (intermediate to bright), CD58 (intermediate), CD71 (bright), CD75 (variable), and BCL-6 (low to intermediate) without CD5, CD10, or CD64. The immunophenotype identifies a distinct population in multidimensional space with a germinal center immunophenotype. The flow-sorted cells from this case are shown in Figure 4A. The putative neoplastic LP population is colored in red; all other events are in blue. A488, Alexa Fluor 488; APC, allophycocyanin; Cy5.5, cyanine 5.5; Cy7, cyanine 7; FSC, forward scatter; LP, lymphocyte predominant; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; PB, Pacific Blue; PE, phycoerythrin; SSC, side scatter; TR, Texas Red.

Adhesion Molecules

Because of their importance in mediating the interaction of HRS cells with T cells,^{12,19} we surveyed expression of these macromolecules on the LP cells. The adhesion molecule CD48 was weakly expressed (MAI = 0.9), usually at a level slightly lower than that seen on reactive small B cells. CD50 (intercellular adhesion molecule 3) was moderately expressed (MAI = 1.8), at the level of or higher than the other lymphocytes in the lymph node suspension. CD54 was brightly expressed (MAI = 2.6), invariably at a level higher than all other cells in the lymph node suspension. Finally, CD58 showed weak-to-moderate expression (MAI = 1.7), with some cases showing lower and some cases showing higher expression by the LP cells than the small reactive B cells.

Expression of adhesion molecules (CD48, CD50, CD54, and CD58) was measured in six cases of CHL for comparison with NLPHL. CD54 and CD58 were consistently and brightly expressed on HRS cells of CHL, whereas little to no expression of CD50 and no expression of CD48 were identified.

Light Scatter and DNA Content

LP cells were large (as measured by forward scatter). Side scatter was markedly increased compared with the small lymphocytes such that on a plot showing side scatter on a log scale, the normal lymphocytes and the LP population did not overlap. Nine of 11 cases evaluated for DNA content using DNA binding dye DAPI showed aneuploidy (Figure 3). Of the aneuploid cases, the DNA index for the cells varied from 1.1 to 1.5, with an average of 1.3, consistent with hyperdiploidy.

Expression of B7 Family Molecules

Expression of PD-L1 (CD274), PD-L2 (CD273), and B7-H2 (inducible costimulatory ligand; CD275) was evaluated on LP cells in five cases; absent-to-minimal expression was noted on the LP cells from all tested cases for the three antigens (MAIs = 0.2, 0.4, and 0.1, respectively). Six cases of CHL were run for comparison. All six CHL cases showed expression of CD273, whereas five of six cases showed expression of CD274.

Figure 2 Comparison of a lymph node involved by NLPHL with normal germinal center cells. The putative LP population is identified with arrows (A), in isolation (B) and compared with normal germinal center cells from a tonsil (C). Both the LP cells and germinal center cells have expression of BCL-6 (with increased expression by LP cells relative to germinal center cells) and decreased expression of CD32 (relative to the normal CD20⁺ non-germinal center B cells). The LP cells have increased expression of CD54 and decreased expression of CD58 relative to the normal germinal center cells. The LP (A and B) and germinal center (C) population are in red; all other events are in blue. A700, Alexa Fluor 700; APC, allophycocyanin; Cy5.5, cyanine 5.5; LP, lymphocyte predominant; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; PE, phycoerythrin.

Evaluation of Consensus Tubes for Identification of LP Cells

Two tubes (Table 2) were designed to identify LP cells in lymph nodes with high specificity and sensitivity. Both tubes used CD20, CD38, CD40, CD54, and CD71 to identify the LP cells and CD5, CD10, and CD64 to exclude other events. The first tube also included CD32 and CD75 to evaluate the expression of these antigens on LP cells, and CD5 and CD45 to evaluate for rosette formation. The second tube evaluated DNA content (using DAPI) and nuclear expression of BCL-6. All 7 NLPHL cases evaluated (1 case was not among the 19 described above) demonstrated convincing LP populations in both consensus tubes. Evaluation of 94 tissues diagnosed as CHL (9 cases), reactive (30 cases), or B-cell non-Hodgkin lymphoma (55 cases) (Table 5) did not identify an LP-like population in either tube for 91 cases (sensitivity, 100%; specificity, 96.8%). An LP-like population was present in one of two tubes for two cases and both tubes for one case; the morphologic diagnosis for all three misclassified cases was diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS).

FCCS of LP Cells from Lymph Nodes Involved by NLPHL

To evaluate the morphologic structure of the LP populations identified by flow cytometry and to investigate the preparative utility of this technique, we purified LP cells from six lymph nodes involved by NLPHL with FCCS (Figure 4). Representative photomicrographs of cytocentrifuge preparations of the sorted LP cell populations confirm that the sorted cells have the cytomorphologic features of LP cells (cytomorphology of five of six cases shown). Two cases demonstrated the presence of occasional LP cell–T-cell rosettes. Rosettes were not observed in the four other sorted cases. Sorted cells were evaluated for purity by sorting the previous sorted cells. Purities obtained by FCCS nominally ranged from 56% to 82% (Table 6).

Discussion

This study provides the identification and characterization of LP cells from NLPHL by flow cytometry. Evidence that the cells identified by flow cytometry represent authentic LP cells includes i) cells identified by flow cytometry (Table 4)

Figure 3 Representative examples of clonality and aneuploidy of LP cells by flow cytometry. Examples of κ surface light chain-restricted LP populations (**A** and **B**) and aneuploidy (**C** and **D**) from involved lymph nodes. The LP population was gated with expression of CD20, CD40, and CD71 (**A**), CD20, CD40, CD71, and CD95 (**B**), Bcl-6, CD20, CD40, and increased side light scatter and the absence of expression of CD5, CD10, or CD64 (**C** and **D**). Measured DNA indices for panels **C** and **D** were 1.3 and 1.5, respectively. The LP population is in red; all other events are in blue. APC, allophycocyanin; FITC, fluorescein isothiocyanate; LP, lymphocyte predominant; PB, Pacific Blue; PE, phycoerythrin.

have an immunophenotype that matches (with minor exceptions) that seen with the NLPHL cell line DEV ([Table 3](#)) and by immunohistochemistry of NLPHL¹⁻³; ii) cells are aneuploid as measured by ploidy experiments ([Figure 3](#)); iii) cells purified by FCCS have the morphologic structure expected for LP cells, displaying multilobated frankly malignant popcorn characteristics ([Figure 4](#)); iv) two of the NLPHL cases transformed to large B-cell lymphoma (one case to DLBCL, NOS; one case to T-cell/histiocyte-rich large B-cell lymphoma); in both cases, the transformed lymphoma cells had immunophenotypes identical to the LP populations (data not shown); and v) evaluation of surface light chain expression by flow cytometry suggests these LP populations are abnormal as evidenced by κ light chain restriction ([Figure 3](#)) or lack of surface light chain.³⁰

LP cells in tissues have a unique immunophenotype, being that of an abnormal germinal center B cell with expression of CD20,¹⁻³ CD40,^{22,23} CD75,²⁶ and Bcl-6^{1,3} but without the

Table 5 Diagnoses for Specimens Used in Evaluating Consensus NLPHL Tubes

Diagnosis	No.
Reactive tissues	
26 reactive follicular hyperplasias, 1 case of sarcoidosis, 1 case of reactive tissue with <i>Actinomyces</i> , 1 benign tissue removed for treatment, 1 benign lymph node with focal necrotizing lymphadenitis	30
Follicular lymphoma	19
DLBCL, NOS	18
Classical Hodgkin lymphoma	9
Marginal zone	7
CLL/SLL	4
Mantle cell lymphoma	3
B-cell lymphoma, NOS	2
Splenic marginal zone lymphoma	1
PMBCL	1

CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; NOS, not otherwise specified; PMBCL, primary mediastinal large B-cell lymphoma; SLL, small lymphocytic lymphoma.

germinal center marker CD10^{3,27} or T-cell antigens. Interestingly, in paraffin sections when light chains are expressed, the cells are usually κ light chain restricted.^{24,25} Similar results were seen in the present study, showing κ surface light chain restriction in 4 of 10 cases and absent surface light chain in the other 6 cases. The putative LP cells by flow cytometry showed expression of CD19 and CD20 (usually at the level of the reactive small B cells), bright expression of CD40 (similar to that observed in CHL), and Bcl-6. LP cells did not express CD5, CD10, CD15, or CD64, with only minimal CD30 identified. As expected, the putative LP cells were large (measured by forward light scatter).

For the six cases evaluated, the LP cells of two cases demonstrated bright surface expression of IgD, one case co-expressed IgD and IgM, one case expressed IgM, and two cases were negative for IgH. No surface IgG or IgA expression was identified. Prakash et al³¹ reported that IgD-expressing LP cells (27% of NLPHL in their cohort) may constitute a unique subset of NLPHL with younger age at presentation (average age, 21 years), although no follow-up data were available for these cases. Although our case series is small, the three patients who developed NLPHL with IgD + LP cells in our series were older than that seen in the aforementioned study (average age, 38 years). For our cases for which IgH was evaluated, clinical outcome data were available for five cases. Two cases with transformation to large B-cell lymphoma showed expression of IgD [at 8 (IgD only) and 98.5 (IgM and IgD) months]. One case that recurred after 228 months showed IgM expression. No evidence of progression was noted in one IgD- and one IgH-negative case. Although the number of cases is small, given transformation to large B-cell lymphoma is reported in only 3% to 5% of NLPHL overall,³² these results raise the

Figure 4 Composite of cytomorphology of LP cells from lymph nodes purified by FCCS. Five FCCS experiments from five cases are shown. A sixth case (not shown) was also subjected to FCCS, with the LP cells also showing appropriate cytomorphology. Occasional T-cell-LP cell rosettes were identified with cases **A** (third cell from the left) and **E** (rosettes not shown) with none identified with the other cases. The cells are large compared with normal reactive lymphocytes (rosetted lymphocytes seen in **A**, third cell from the left) and have pol- ylobated nuclear structure consistent with LP cells. Images obtained with Leica DM1000, Leica DFC290 digital microscope camera, and processed with Photoshop CS5 (Adobe Systems, Mountain View, CA) to assemble collage of cells. Flow cytometric combinations used for the FCCS experiments are shown in [Materials and Methods](#). Original magnification, $\times 1000$ (**A–E**). FCCS, flow cytometric cell sorting; LP, lymphocyte predominant.

possibility that IgD or IgD and IgM expression suggests a worse prognosis. Supporting this conclusion, Sohani et al³³ have demonstrated (in a small number of cases) that NLPHL with atypical T cells are more likely to recur and that these

Table 6 Purity and Percentage of LP Cells Collected from Six Lymph Nodes Involved by NLPHL

Experiment no.*	No. of LP cells sorted	Percentage of LP cells sorted†	Purity (%)
1	964	ND	ND
2	1622	0.1	77.1
3	1045	0.04	72.7
4	1560	0.03	81.6
5	2270	0.07	57.1
6	268	ND	55.6

*Experiments 1 to 5 correspond to the displayed cases shown in [Figure 4](#).
†Total number of LP cells sorted/total number of cells sorted.
LP, lymphocyte predominant; ND, not determined; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma.

cases are more likely to show LP cells expressing IgD. In addition, for DLBCL, NOS, IgH expression is predictive of outcome with IgD/IgM cases having a worse prognosis.³⁴ More extensive studies of IgH expression and outcome in NLPHL are warranted.

Nine of 11 cases evaluated demonstrated that the putative LP cells were aneuploid. In all nine cases, the LP cells showed a net gain of DNA (DNA indices varied from 1.1 to 1.5; mean, 1.3). Although few cytogenetic studies of NLPHL have been reported, other workers have found similar findings. In a series of 13 NLPHL cases studied by conventional cytogenetics and fluorescence *in situ* hybridization, Stamatoullas et al³⁵ demonstrated that LP cells were aneuploid and clonal in 12 of 13 cases examined; the neoplastic cells from nine patients were near diploid (46 to 49 chromosomes), whereas hypodiploidy and tetraploidy were identified in the other two cases.

By morphologic examination, LP cells are known to be rosetted by T cells, but the molecular underpinnings of this

phenomena have not been investigated. Interestingly, our data suggest less avidity of T cells for LP cells compared with T cells for HRS cells, because few rosettes are identified in cytopreparations made from sorted LP cells (Figure 4) compared with CHL cells that demonstrated numerous rosettes from similar FCCS experiments.^{12,13} Supporting this conclusion and unlike CHL cell lines,^{12,19} DEV does not spontaneously rosette normal T cells (data not shown). Finally, apparent expression of T-cell antigens is significantly less for NLPHL than for CHL (data not shown), the intensity of which affords a crude estimate of the degree of rosetting in CHL.¹³ We chose to evaluate adhesion molecules that are known to be important in the interaction of HRS with T cells.^{12,19} Our results suggest a role for CD54 (intercellular adhesion molecule 1) in mediating this interaction, because CD54 was consistently and brightly expressed in all cases of NLPHL examined (similar to CHL) and consistently greater than reactive germinal center B cells. CD58 showed weak expression on some cases and may be less important in mediating these interactions; this result is in contrast to reactive germinal centers and to CHL (in six cases) in which CD58 is consistently expressed and clearly plays a role in T cell–HRS cell interactions.^{12,19} Interestingly, although CD50 (intercellular adhesion molecule 3), known to interact with the same ligand (lymphocyte function-associated antigen-1) as CD54, was often overexpressed on LP cells, HRS cells showed only low expression. CD48 (low-affinity receptor of CD2 and high-affinity receptor for 2B4³⁶) showed little expression on either LP or HRS cells. It is tempting to propose that decreased and variable expression of CD58 may be responsible for the decrease in rosetting observed in NLPHL compared with CHL. In prior studies, we and others confirmed the importance of CD54 and CD58 on HRS cells of CHL in mediating the interaction of HRS cells to T cells by using blocking antibodies to disrupt the interactions.^{12,19} Unfortunately, sufficient material was not available to further evaluate these interactions by this approach; consequently, further studies will be required to confirm the importance of these adhesion molecules in contributing to the interaction of T cells with LP cells and to examine how this pattern of adhesion macro-molecule expression affects the immune response to LP cells.

PD-1 is expressed on T cells and binds to PD-L1 and PD-L2 (on the cancer or antigen-presenting cell) resulting in inhibition for the T cells during the effector phase of a T-cell response.³⁷ Blocking PD-1/PD-L1/PD-L2 interactions may augment tumor immunotherapy.²⁹ Although there are conflicting reports regarding PD-L1 expression on LP cells in the literature,^{28,38} the results presented herein suggest such an approach may not be effective in NLPHL because PD-L1 (CD274) and PD-L2 (CD273) are not present on LP cells by flow cytometry^{28,39} and (for at least subset of neoplasms) PD-L1/PD-L2 expression on tumor cells is correlated with anti-PD-1 therapeutic response.^{40,41}

CD32 is a low-affinity Fc receptor and tends to be expressed at lower levels on B-cell non-Hodgkin lymphoma of germinal center origin.⁴² By binding Fc, CD32 may provide a mechanism of resistance to rituximab therapy.⁴² We evaluated 11 cases of follicular hyperplasia and noted that CD32 is not expressed on T cells, is not expressed or weakly expressed by germinal center B cells, and is strongly expressed on non-germinal center B cells (Figure 2). Similarly, LP cells demonstrated decreased expression of CD32 relative to the non-germinal center B cells in eight of nine cases examined. Six CHL cases demonstrated overall higher levels of CD32. Although these results suggest efficacy of rituximab in NLPHL, these studies argue for correlating CD32 expression with rituximab efficacy in NLPHL in future clinical trials.

This work demonstrates that NLPHL can be immunophenotyped by flow cytometry. We identified an LP population by flow cytometry in 19 of 26 cases examined (73%). Furthermore, a set of consensus tubes (Table 2) identified an LP population in both tubes from all seven subsequent test cases of NLPHL (100% sensitivity). Evaluation of 94 tissues diagnosed as B-cell lymphoma (55 cases) (Table 5), reactive (30 cases), or CHL (9 cases) did not identify an LP-like population in 91 cases (96.8% specificity). We envision the consensus tubes to be primarily useful in suggesting a diagnosis of NLPHL in the following two clinical settings: when a reactive CD4/CD8 co-expressing T-cell population suggestive of NLPHL has been identified by flow cytometry^{43,44} and in challenging cases when the morphologic suspicion is NLPHL and additional information is needed to support that impression. Work presented here suggests the consensus tubes would be useful in differentiating NLPHL from CHL and DLBCL, NOS (two entities frequently in the differential diagnosis with NLPHL).

These studies suggest an antibody combination (consensus tube 1) that would be useful for purifying LP cells by FCCS. Because purification of LP cells currently involves the labor-intensive process of microdissection, the method described herein will afford thousands of isolated LP cells in a few hours. In addition, although a formal comparison of microdissection and FCCS has not been described, one study showed twofold to threefold higher yield of single cells with amplifiable DNA from sorted versus microdissected T cells,⁴⁵ suggesting sorted cells may be more amenable to subsequent genetic studies.

Herein, we report the characterization of LP cells from NLPHL by flow cytometry and FCCS. Further studies are required to fully characterize the diagnostic utility of the two-tube consensus tube assay and to determine whether the differences in expression of CD32, CD50, CD273, and CD274 between CHL and NLPHL can distinguish these lymphomas by immunohistochemistry. Another intriguing question is whether the neoplastic cells of T-cell/histiocytic-rich large B-cell lymphoma can be immunophenotyped and purified by flow cytometry and FCCS, given the

immunophenotypic similarity to NLPHL.^{4,46–49} These questions are being investigated in our laboratory.

Acknowledgments

We thank Dr. Lori Soma for her critical review of the manuscript.

J.R.F. conceived of the project; J.R.F. and B.L.W. designed the experiments; A.T. performed all the experiments; J.R.F. and A.T. analyzed the data; and J.R.F. wrote the manuscript. All authors read and approved the manuscript.

References

- Poppema S, Delsol G, Pileri SA, Stein H, Swerdlow SH, Warnke RA, Jaffe ES: Nodular lymphocyte predominant Hodgkin lymphoma. Edited by Swerdlow SH, Campos E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW. In WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press, 2008. pp. 323–325
- Schmitz R, Stanelle J, Hansmann ML, Kuppers R: Pathogenesis of classical and lymphocyte-predominant Hodgkin lymphoma. *Annu Rev Pathol* 2009, 4:151–174
- Uherova P, Valdez R, Ross CW, Schnitzer B, Finn WG: Nodular lymphocyte predominant Hodgkin lymphoma. An immunophenotypic reappraisal based on a single-institution experience. *Am J Clin Pathol* 2003, 119:192–198
- Stein H, Delsol G, Pileri SA, Weiss LM, Poppema S, Jaffe ES: Classical Hodgkin Lymphoma, introduction. Edited by Swerdlow SH, Campos E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW. In WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press, 2008. pp. 326–329
- Harris NL: Hodgkin's lymphomas: classification, diagnosis, and grading. *Semin Hematol* 1999, 36:220–232
- Harris NL: Hodgkin's disease: classification and differential diagnosis. *Mod Pathol* 1999, 12:159–175
- Wood BL, Borowitz MJ: The flow cytometric evaluation of hematopoietic neoplasia. Edited by McPherson RA, Pincus MR. In Henry's Clinical Diagnosis and Management by Laboratory Methods. Philadelphia, PA: Saunders Elsevier, 2011. pp. 656–673
- Givan AL: Flow cytometry: an introduction. *Methods Mol Biol* 2011, 699:1–29
- Fromm JR, Wood BL: A six-color flow cytometry assay for immunophenotyping classical Hodgkin lymphoma in lymph nodes. *Am J Clin Pathol* 2014, 141:388–396
- Fromm JR, Thomas A, Wood BL: Flow cytometry can diagnose classical hodgkin lymphoma in lymph nodes with high sensitivity and specificity. *Am J Clin Pathol* 2009, 131:322–332
- Wu D, Wood BL, Fromm JR: Flow cytometry for non-Hodgkin and classical Hodgkin lymphoma. *Methods Mol Biol* 2013, 971:27–47
- Fromm JR, Kussick SJ, Wood BL: Identification and purification of classical Hodgkin cells from lymph nodes by flow cytometry and flow cytometric cell sorting. *Am J Clin Pathol* 2006, 126:764–780
- Fromm JR, Wood BL: Strategies for immunophenotyping and purifying classical Hodgkin lymphoma cells from lymph nodes by flow cytometry and flow cytometric cell sorting. *Methods* 2012, 57:368–375
- Stein H: Hodgkin lymphoma-introduction. Edited by Swerdlow SH, Campos E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW. In WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press, 2008. pp. 322
- Kadin ME, Newcom SR, Gold SB, Stites DP: Letter: Origin of Hodgkin's cell. *Lancet* 1974, 2:167–168
- Payne SV, Jones DB, Wright DH: Reed-Sternberg-cell/lymphocyte interaction. *Lancet* 1977, 2:768–769
- Payne SV, Newell DG, Jones DB, Wright DH: The Reed-Sternberg cell/lymphocyte interaction: ultrastructure and characteristics of binding. *Am J Pathol* 1980, 100:7–24
- Stuart AE, Williams AR, Habeshaw JA: Rosetting and other reactions of the Reed-Sternberg cell. *J Pathol* 1977, 122:81–90
- Sanders ME, Makgoba MW, Sussman EH, Luce GE, Cossman J, Shaw S: Molecular pathways of adhesion in spontaneous rosetting of T-lymphocytes to the Hodgkin's cell line L428. *Cancer Res* 1988, 48:37–40
- Poppema S, De Jong B, Atmosoerodjo J, Idenburg V, Visser L, De Ley L: Morphologic, immunologic, enzymehistochemical and chromosomal analysis of a cell line derived from Hodgkin's disease. Evidence for a B-cell origin of Sternberg-Reed cells. *Cancer* 1985, 55:683–690
- Drexler HG, Minowada J: Hodgkin's disease derived cell lines: a review. *Hum Cell* 1992, 5:42–53
- Carbone A, Ghoghini A, Pinto A: CD40: a sensitive marker of Reed-Sternberg cells. *Blood* 1996, 87:4918–4919
- Carbone A, Ghoghini A, Gattei V, Aldinucci D, Degan M, De Paoli P, Zagonel V, Pinto A: Expression of functional CD40 antigen on Reed-Sternberg cells and Hodgkin's disease cell lines. *Blood* 1995, 85:780–789
- Schmid C, Sargent C, Isaacson PG: L and H cells of nodular lymphocyte predominant Hodgkin's disease show immunoglobulin light-chain restriction. *Am J Pathol* 1991, 139:1281–1289
- Stoler MH, Nichols GE, Symbula M, Weiss LM: Lymphocyte predominance Hodgkin's disease. Evidence for a kappa light chain-restricted monotypic B-cell neoplasm. *Am J Pathol* 1995, 146:812–818
- Torlakovic E, Torlakovic G: B-cell markers in lymphocyte predominance Hodgkin disease. *Arch Pathol Lab Med* 2002, 126:862–863
- Gruss HJ, Kadin ME: Pathophysiology of Hodgkin's disease: functional and molecular aspects. *Baillieres Clin Haematol* 1996, 9:417–446
- Chen BJ, Chapuy B, Ouyang J, Sun HH, Roemer MG, Xu ML, Yu H, Fletcher CD, Freeman GJ, Shipp MA, Rodig SJ: PD-L1 expression is characteristic of a subset of aggressive B-cell lymphomas and virus-associated malignancies. *Clin Cancer Res* 2013, 19:3462–3473
- Ribas A: Tumor immunotherapy directed at PD-1. *N Engl J Med* 2012, 366:2517–2519
- Li S, Eshleman JR, Borowitz MJ: Lack of surface immunoglobulin light chain expression by flow cytometric immunophenotyping can help diagnose peripheral B-cell lymphoma. *Am J Clin Pathol* 2002, 118:229–234
- Prakash S, Fountaine T, Raffeld M, Jaffe ES, Pittaluga S: IgD positive L&H cells identify a unique subset of nodular lymphocyte predominant Hodgkin lymphoma. *Am J Surg Pathol* 2006, 30:585–592
- Huang JZ, Weisenburger DD, Vose JM, Greiner TC, Aoun P, Chan WC, Lynch JC, Bierman PJ, Armitage JO: Diffuse large B-cell lymphoma arising in nodular lymphocyte predominant Hodgkin lymphoma: a report of 21 cases from the Nebraska Lymphoma Study Group. *Leuk Lymphoma* 2004, 45:1551–1557
- Sohani AR, Jaffe ES, Harris NL, Ferry JA, Pittaluga S, Hasserjian RP: Nodular lymphocyte-predominant hodgkin lymphoma with atypical T cells: a morphologic variant mimicking peripheral T-cell lymphoma. *Am J Surg Pathol* 2011, 35:1666–1678
- Ruminy P, Etancelin P, Couronne L, Parmentier F, Rainville V, Mareschal S, Bohers E, Burgot C, Cornic M, Bertrand P, Lenormand B, Picquenot JM, Jardin F, Tilly H, Bastard C: The isotype of the BCR as a surrogate for the GCB and ABC molecular subtypes in diffuse large B-cell lymphoma. *Leukemia* 2011, 25:681–688
- Stamatoullas A, Picquenot JM, Dumesnil C, Ruminy P, Penhert D, Bertrand P, Courel MN, Maisonneuve C, Francois A, Gaulard P,

- Tilly H, Bastard C: Conventional cytogenetics of nodular lymphocyte-predominant Hodgkin's lymphoma. *Leukemia* 2007, 21:2064–2067
36. Elishmereni M, Levi-Schaffer F: CD48: a co-stimulatory receptor of immunity. *Int J Biochem Cell Biol* 2011, 43:25–28
 37. Saresella M, Rainone V, Al-Daghri NM, Clerici M, Trabattini D: The PD-1/PD-L1 pathway in human pathology. *Curr Mol Med* 2012, 12: 259–267
 38. Dorfman DM, Brown JA, Shahsafaei A, Freeman GJ: Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. *Am J Surg Pathol* 2006, 30: 802–810
 39. Yamamoto R, Nishikori M, Kitawaki T, Sakai T, Hishizawa M, Tashima M, Kondo T, Ohmori K, Kurata M, Hayashi T, Uchiyama T: PD-1-PD-L1 ligand interaction contributes to immunosuppressive microenvironment of Hodgkin lymphoma. *Blood* 2008, 111: 3220–3224
 40. Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, Chen L, Pardoll DM, Topalian SL, Anders RA: Association of PD-1, PD-L1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin Cancer Res* 2014, 20: 5064–5074
 41. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M: Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012, 366: 2443–2454
 42. Lim SH, Vaughan AT, Ashton-Key M, Williams EL, Dixon SV, Chan HT, Beers SA, French RR, Cox KL, Davies AJ, Potter KN, Mockridge CI, Oscier DG, Johnson PW, Cragg MS, Glennie MJ: Fc gamma receptor IIb on target B cells promotes rituximab internalization and reduces clinical efficacy. *Blood* 2011, 118:2530–2540
 43. Rahemtullah A, Reichard KK, Preffer FI, Harris NL, Hasserjian RP: A double-positive CD4+CD8+ T-cell population is commonly found in nodular lymphocyte predominant Hodgkin lymphoma. *Am J Clin Pathol* 2006, 126:805–814
 44. Rahemtullah A, Harris NL, Dorn ME, Preffer FI, Hasserjian RP: Beyond the lymphocyte predominant cell: CD4+CD8+ T-cells in nodular lymphocyte predominant Hodgkin lymphoma. *Leuk Lymphoma* 2008, 49:1870–1878
 45. Roers A, Montesinos-Rongen M, Hansmann ML, Rajewsky K, Kuppers R: Amplification of TCRbeta gene rearrangements from micromanipulated single cells: T cells rosetting around Hodgkin and Reed-Sternberg cells in Hodgkin's disease are polyclonal. *Eur J Immunol* 1998, 28:2424–2431
 46. Pittaluga S, Jaffe ES: T-cell/histiocyte-rich large B-cell lymphoma. *Haematologica* 2010, 95:352–356
 47. Abramson JS: T-cell/histiocyte-rich B-cell lymphoma: biology, diagnosis, and management. *Oncologist* 2006, 11:384–392
 48. Toussey T, De Wolf-Peeters C: T cell/histiocyte-rich large B-cell lymphoma: an update on its biology and classification. *Virchows Arch* 2011, 459:557–563
 49. Fraga M, Sanchez-Verde L, Forteza J, Garcia-Rivero A, Piris MA: T-cell/histiocyte-rich large B-cell lymphoma is a disseminated aggressive neoplasm: differential diagnosis from Hodgkin's lymphoma. *Histopathology* 2002, 41:216–229