Supplemental data

Expert-independent classification of mature B-cell neoplasms using standardized flow cytometry: a multicentric study

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Supplemental methods

Patients and eligibility

The diagnosis of a particular mature B-cell neoplasm in each individual patient was based on WHO criteria and confirmed by a predefined minimum set of ancillary methods, which always included compatible cytology and/or histology findings. There was no central pathology review of the submitted cases. MCL and BL cases were required to harbor the CCDN1 and MYC translocations, respectively, while the diagnosis of DLBCL, FL, MZL, and LPL were primarily based on histology. Cytology plus basic flow cytometry were required for the CLL diagnosis. The HCL diagnosis relied on typical histology and/or cytology. Additional features, such as BRAF V600E mutations in HCL, MYD88 mutations in LPL, and presence of t(14;18) in FL were recorded based upon availability. Deletions (del) 17p, del11q, and del13q, trisomy 12 (+12) and the IGHV mutational status were recorded in CLL when available. Information on clonal serum IgM was optionally collected, except in LPL where it was mandatory.

Samples were collected locally in all participating EuroFlow centers based upon availability of left-over sample material and completeness of required ancillary test to establish a diagnosis according to WHO. Unselected patient samples were included provided the purity of the malignant B-cell clone exceeded 90% after gating and results from the ancillary methods allowed for the diagnosis of a particular mature B-cell neoplasm.

Immunophenotypic studies

Samples were processed within 24 hours from collection. A total of 100,000 cellular events per tube from samples stained with the EuroFlow B-CLPD antibody panel (supplemental Table 2) and using EuroFlow SOP were acquired on instruments calibrated according to EuroFlow specifications[1]. Appropriate instrument performance and laboratory procedures were confirmed by results obtained in EuroFlow QA rounds [1, 2].

Data analysis

Flow cytometric analyses were performed locally by an expert using a predefined gating strategy in order to identify the aberrant B-cell clone (supplemental Figure 1). Compensated flow cytometry standard (.fcs) files were pseudonymized and uploaded on a secured server. Prior to inclusion into this study, cases were checked by a second expert (for correct acquisition, gating, purity of the malignant clone, fluorescence compensation and required annotations) following a standardized, previously published workflow.[3] A total of 161 additional cases, that were submitted by the local EuroFlow laboratories, could not be accepted for the study. Lacking annotations to allow an unequivocal diagnosis according to WHO represented the most frequent reason for ineligibility (supplemental Table 8).

The malignant clone of each case was electronically separated from the other cellular events via CD45, CD19, CD20, forward (FSC), and side scatter (SSC) (supplemental Figure 1). As a rule all malignant B-cells within a sample were represented by a total of 6,000 randomly selected clonal events in further analyses. In a minority of cases, fewer total clonal B-cells were available (supplemental Table 1).

Prior to further analysis the nearest neighbor algorithm[4] as implemented into Infinicyt software (Cytognos SL, Salamanca, Spain) was applied to CD20, CD45, CD19; FSC, and SSC, so that a value for CD20, CD45, CD19, immunoglobulin(Ig)λ, Igκ, CD5, CD38, CD23, CD10, CD79b, CD200, CD43, CD31, CD305, CD11c, IgM, CD81, CD103, CD95, CD22, CD185, CD49d, CD62L, CD39, HLA-DR, and CD27 was assigned to each B-CLPD cell in a sample. Scatter parameters from the aberrant B-cell clone were normalized against the median scatter values of $CD4^+$ T-cells from the same sample using the following formula: BT ratio=$FCS_{B\text{-}cells}$ x $SSC_{B\text{-}cells}$ / FCS_{CD4+T} x SSC_{CD4+T}. Thus, 26 quantitative fluorescence and scatter parameters were utilized for further lymphoma classification.

CD3 background expression levels were used as indicator for the level of unspecific background signal in a disease category assuming that this antigen is not expressed in B-cell

neoplasms (supplemental results). The expression level of each parameter was accordingly classified as informative vs. predominantly background signal in a given disease category (supplemental Table 9).

Selection of training cases

Sixteen (in case of BL) to 20 (all other categories) cases per entity were randomly included into the training set (total n=176). We used a robust variant of Mahalanobis distance for all 26 flow cytometric parameters[5] to determine the degree to which a single case represented that class. We confirmed that training set cases were proportionately sampled from typical and atypical cases for a disease category (supplemental Figure 6 and data not shown). The remaining 486 cases formed the independent validation cohort.

T-cell subpopulations as QA

T-cell subpopulations identified in tube 1 were evaluated as potential within-sample QA. Specifically, median fluorescence intensities (medFIs) of the $CD8^+CD3^+$ T-cell subpopulation for CD8-FITC and of the $CD4^+CD3^+$ T-cell subpopulation for CD4-PacB, CD45-PacO, CD5-PerCP-Cy5.5, and CD3-APC were extracted for all cases with at least 200 events of the respective subpopulations.

Statistical methods

Infinicyt software (developmental version 2.0.3 a.B-CLPD_S3) was used to analyze the flow cytometry data, to apply the nearest neighbor algorithm [4], to select the training cases, to build the database, to plot CCA-based two-dimensional projections and to classify the test cases according to the algorithm. We performed 1,000 bootstraps of the validation set (1,000x random selection with replacement from each entity until the numbers of the original validation set for each entity were reached) as initially described by Efron et al.[6] and applied e.g. by Hoster et al.[7] This approach allowed us to approximate mean and distribution of specificity, sensitivity, positive (PPV) and negative predictive values (NPV) per disease category when the fixed training set was used (Table 4). The stability of the model was

investigated using Monte Carlo cross-validation [8, 9]. We randomly selected the same number of training cases per entity as in the initial model (16 BL training cases, 20 training cases for other B-CLPD entities), created 36 CCA-based projections per iteration, adapted the SD lines to create non-overlapping decision criteria for automated diagnosis and performed the classification of the remaining cases as validation set. Based on 1,000 iterations (i.e. 1,000x random split of training and validation cohorts) mean and distribution for sensitivity, specificity, PPV, and NPV per disease category were calculated. R (v. 4.0.2) was used for data analysis and box-plot figures. Significance of single parameter differences between entities was assessed by the Kruskal-Wallis test followed by the Dunns post-hoc test with the Holms correction in $\log_{10}$ transformed data. Intra- and inter-center coefficients of variation (CV) were compared using t-test with Bonferroni correction. $P < 0.05$ was considered statistically significant.

Supplemental results

Using antigens on T-cell subpopulations for in-sample QA

The expression levels of T-cell antigens on residual T-cells were used to quantify technical variation of the method and to establish an in-sample QA. Of note, we report herein very similar mean medFIs for T-cell antigens assessed on bystander T-cells in B-cell lymphoma cases compared to previously reported medFI from EuroFlow QA rounds with normal donors[2], thus validating the robustness of our technical standardization. The quality of individual measurements can therefore be estimated in the future by comparing medFI of bystander T-cell subpopulations in a newly acquired sample to the reference values provided in Table 2. Considering the maximum inter-center CV (32.8%) we recommend that measurements with medFI for the T-cell antigen on T-cell subpopulations outside a range between 34.4% and 165.6% of the mean medFI reference values (equals +/- 2-fold CV) to be considered technical outliers.

General principles on the utility of markers using univariate analysis for B-CLPD classification

(1) Each of the 26 parameters shows significant differences between the peripheral B-cell lymphoma entities. The distribution of medFI per entity suggests that for some markers (e.g. CD103 in DLBCL, Figure 3A) significant differences might be caused by autofluorescent and/or unspecific binding of antibodies to larger lymphoma cells.

(2) Expression levels provide more important diagnostic information compared to a purely qualitative analysis (i.e. positive vs negative). In general, autofluorescence can be seen up to roughly 200 fluorescence channels using EuroFlow standardization (data not shown), i.e. ‘positivity’ of individual cases can be usually defined as medFI above 200 channels. For example, almost all cases analyzed showed at least partial CD20 expression (for exceptional $CD20^-$ cases see Figure 3A), however, median

CD20 expression levels in CLL are roughly 6 fold lower than in MCL. Moreover, median CD20 expression was almost 4fold greater in HCL than in MCL.

(3) Differences between entities in expression levels clearly exceeded technical variation observed for T-cells, e.g. an almost 18fold difference between FL and MCL for median CD305 (Figure 3 B). These differences therefore reflect biological differences between entities that cannot be attributed to technical variation (CV in T-cells ≤ 32.2%).

(4) Expression levels commonly overlap between entities. For example, in spite of under-expression of CD20 in CLL, there are rare DLBCL and MCL cases with similarly low expression (Figure 3 A). Markers with expression ranges virtually specific for a particular entity were observed in HCL only (Figure 3A: CD103, CD11c, Figure 3B: CD305). Reliable flow cytometric diagnostic approaches therefore as a rule require the combined information from different markers.

(5) Expression levels of markers are frequently heterogeneous within disease entities, e.g. some FL cases lack CD10 almost completely, whereas that marker shows some expression in a subgroup of HCL cases. A reliable diagnostic strategy has to account for such heterogeneity and has to focus on markers with a low intra-disease variance.

Significance of CD200 and CD305 example antigens

We confirm published observations [10-14] that CD200 rarely, if at all, is expressed in MCL, while CLL and HCL patients express the antigen at high density. Data on CD200 in FL ranged from negative in one study [13] to negative to moderate in other evaluations[12, 14]. Using a large set of FL patients we confirm a negative to moderate CD200 expression in this type of lymphoma. Whereas there are currently only anecdotic reports on CD200 in BL available [12-14] we consistently found very low CD200 expression in our cohort of 29 BL cases. Finally, median CD200 expression was much higher in $CD10^-$ compared to $CD10^+$ DLBCL, again emphasizing the biological heterogeneity of DLBCL.

CD305 (LAIR-1) was utilized in mature B-cell lymphoid neoplasms so far as a prognostic marker in CLL[15-17] and in an attempt to monitor minimal residual disease (MRD) in MCL[18]. We herein (Figure 3B) confirm the broad expression range in CLL. CD305 indeed shows a bimodal pattern (data not shown) suggestive of its utility as prognostic classification marker within this leukemia. However, due to heterogeneous, dim to moderate expression its general applicability as MRD marker in MCL[18] remains questionable. Nevertheless, we regard CD305 as a very valuable diagnostic marker in mature B-cell lymphomas for its homogeneously high level expression in HCL and very low expression level in FL. The latter feature contributes to differential diagnosis between FL on the one hand vs LPL, MCL, and MZL taken as a group on the other hand.

Contribution of background signal to marker expression

Literature describes CD103 expression as an almost specific feature for HCL. Accordingly, we observed low level CD103 expression in diseases other than HCL. However, the expression levels differed significantly by diseases entity (with e.g. median medFI in $CD10^+$ DLBCL almost 3fold higher than in FL, supplemental Table 6 and Figure 3A). We found a significant correlation between CD3 (Supplementary figure 7) and CD103 expression levels ($r=0.43$, $p<0.001$) for all entities except HCL.

In summary, low level differences in expression levels are unlikely to be caused by specific fluorescence from the fluorochrome-labelled antibody. These variations are likely due to unspecific staining and autofluorescence as a function of cell size. Fluorescence signals originating from autofluorescence or unspecific antibody binding only (background signal) in a given entity were estimated as the 90^{th} percentile of the CD3 medFI, assuming that there is no CD3 expression in B-cell lymphomas. Parameters for which the medFI of less than 20% of training set cases exceeded that background signal were considered non-informative (supplemental Table 9).

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197 **Supplemental references**

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Supplemental Tables

Supplemental Table 1. Detailed biological and demographic features of patients. (*two CLL cases had del13q plus +12)

Disease category	Parameter		Database (n=176)	Validation Cohort (n=486)
BL	N		16	13
	Age (years)	Median (min-max)	6.5 (2-66)	12 (3-73)
	Gender (n)	F	3	3
		M	13	10
	Sample type (n)	PB	0	1
		BM	8	6
		LN	6	2
		other	2	4
	Genetic aberrations	t(8;14)	12	10
		t(8;22)	2	0
		t(2;8)	2	0
		Partner unknown	0	3
CLL	n		20	125
	Age (years)	Median (min-max)	73.5 (53-93)	68 (35-92)
	Gender (n)	F	3	45
		M	17	80
	Sample type (n)	PB	17	115
		BM	1	6
		LN	2	4
		other	0	0
	Genetic aberrations*	del13q	5	28
		del11q	1	5
		del17p	0	4
		+12	0	9
		NT	12	69
	IGVH mutational status	mutated	1	19
		unmutated	3	12
		NT	16	94
DLBCL	n		40	64
	Age (years)	Median (min-max)	65.5 (21-86)	68 (12-95)
	Gender (n)	F	18	36
		M	22	28
	Sample type (n)	PB	3	1
		BM	12	20
		LN	17	24
		other	8	19
FL	n		20	109
	Age (years)	Median (min-max)	72.5 (30-86)	62 (35-87)
	Gender (n)	F	11	63
		M	9	46
	Sample type (n)	PB	3	21
		BM	6	31

		LN	8	48
		other	3	9
	Grade	I	11	34
		II	2	13
		III	3	13
		I-II	0	13
		unknown	4	36
	t(14;18)	positive	14	63
		negative	1	11
		NT	5	35
HCL	n		20	38
	Age (years)	Median (min-max)	67.5 (35-85)	60 (36-79)
	Gender (n)	F	6	10
		M	14	28
	Sample type (n)	PB	13	18
		BM	7	20
		LN	0	0
		other	0	0
	BRAF V600E	mutated	1	14
		unmutated	0	3
		unknown	19	21
LPL	n		20	54
	Age (years)	Median (min-max)	76 (41-88)	66.5 (38-87)
	Gender (n)	F	7	14
		M	13	40
	Sample type (n)	PB	3	5
		BM	17	49
		LN	0	0
		other	0	0
	MyD88	mutated	5	22
		unmutated	3	2
		unknown	12	30
MCL	n		20	56
	Age (years)	Median (min-max)	70 (50-82)	66.5 (34-85)
	Gender (n)	F	5	13
		M	15	43
	Sample type (n)	PB	11	31
		BM	5	19
		LN	3	5
		other	1	1
MZL	n		20	27
	Age (years)	Median (min-max)	69 (46-83)	68 (38-88)
	Gender (n)	F	12	14
		M	8	13
	Sample type (n)	PB	5	9
		BM	9	12
		LN	5	4
		other	1	2

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Abbreviations: BL, Burkitt lymphoma; BM, bone marrow; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; F, female; FL, follicular lymphoma; HCL, hairy cell leukemia; LN, lymph node; LPL, lymphoplasmacytic lymphoma; M, male; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; NT, not tested; PB,peripheral blood.

Supplemental Table 2: Composition of the EuroFlow B-CLPD panel [19] In 71 samples the Cytognos Lyo LST kit was used. In 17 samples the following alternative reagents were used: CD45 OC, CD81 APC C750. Please note that tube 1 of the B-CLPD panel equals the Lymphoid Screening Tube. For regularly updated information on clones and titres refer to www.euroflow.org

Tube	Pac Blue	Pac Orange	FITC	PE	PerCP-Cy5.5	PECy7	APC	APC-H7
1= LST	**CD20** / CD4	**CD45**	**Igλ**/ CD8	**Igκ**/ CD56	**CD5**	**CD19** / TCRγ/δ	CD3	**CD38**
2	**CD20**	**CD45**	**CD23**	**CD10**	**CD79b**	**CD19**	**CD200**	**CD43**
3	**CD20**	**CD45**	**CD31**	**CD305**	**CD11c**	**CD19**	**IgM**	**CD81**
4	**CD20**	**CD45**	**CD103**	**CD95**	**CD22**	**CD19**	**CD185**	**CD49d**
5	**CD20**	**CD45**	**CD62L**	**CD39**	**HLA-DR**	**CD19**	**CD27**	

Marker	Fluorochrome	Clone	Source	Catalogue number	(µl/test)
CD3	APC	SK7	BD Biosciences	345767	**2.5**
CD4	PacB	RPA-T4	BioLegend	300521	**0.5**
CD5	PerCP-Cy5.5	L17F12	BD Biosciences	341109	**15**
CD8	FITC	UCH-T4	Cytognos	Cyt-8F8	**1**
CD10	PE	ALB1	Beckmann Coulter	A07760	**20**
CD11c	PerCP-Cy5.5	B-ly6	BD Biosciences	658330	**10**
CD19	PECy7	J3-119	Beckmann Coulter	IM3628	**5**
CD20	PacB	2H7	BioLegend	302320	**1**
CD22	PerCP-Cy5.5	S-HCL-1	BD Biosciences	658329	**25**
CD23	FITC	MHM6	Dako	F7062	**2.5**
CD27	APC	L128	BD Biosciences	337169	**2.5**
CD31	FITC	WM59	BD Pharmingen	555445	**10**
CD38	APC H7	HB7	BD Biosciences	656646	**3**
CD39	PE	TU66	BD Pharmingen	555464	**10**
CD43	APC H7	1G10	BD Biosciences	655407	**2.5**
CD45	PacO	HI30	life technologies	MHCD4530	**5**
CD49d	APC H7	9F10	BD Biosciences	658332	**1**
CD56	PE	C5.9	Cytognos	Cyt-56PE	**2**
CD62L	FITC	SK11	BD Biosciences	347443	**2.5**
CD79b	PerCP-Cy5.5	SN8	BD Biosciences	656644	**10**
CD81	APC H7	JS-81	BD Biosciences	656647	**5**
CD95	PE	DX2	BD Pharmingen	555674	**20**
CD103	FITC	Ber-ACT8	BD Biosciences	333155	**2**
CD185	APC	51505	R&D Systems	FAB190A	**10**
CD200	APC	OX104	life technologies	17-9200	**1.25**
CD305	PE	DX26	BD Pharmingen	550811	**10**
HLA-DR	PerCP-Cy5.5	L243	BD Biosciences	339216	**10**
IgM	APC	G20-127	BD Pharmingen	551062	**10**
Igλ/Igκ	FITC/PE	polyclonal	Cytognos	CYT-LF-KPE-100	**2.5**
TCRγδ-1	PE-Cy7	11F2	BD Biosciences	655410	**3**

Abbreviations: APC, Allophycocyanin; CV, coefficient of variation; FITC, Fluorescein Isothiocyanate; MedFI, median fluorescence intensity; PacB, Pacific Blue; PacO, Pacific Orange; PE, Phycoerythrin; PerCP, Peridinin-Chlorophyll-Protein

Supplemental Table 3. Overview on the data analysis strategy within the scope of the main study. Data was analyzed using Infinicyt software unless stated otherwise. For the validation of the modular design, steps 8 to 10 were repeated using the parameters from tubes 1 and 2 only. *Background signal cannot be identified for the BT parameter. The software algorithms of the Infinicyt software correspond to the following mathematical algorithms: calculate data: nearest neighbor algorithm, Robust Curve: Robust Mahalanobis distance.

	Aim	Specification	Category individually analyzed, No.	Details / results
1	Identification of the malignant B-cell clone	• exclusion of T-cells in tube 1 merge of tubes 1 to 5 • gating on back-bone markers: CD19, CD20, CD45, FSC, SSC	Patient samples, n=662	Suppl. Fig.1
2	In-sample QA and normalization of FSC/SSC	• gating of $CD8^+CD3^+$ and $CD4^+CD3^+$ T-cell subpopulations in tube 1	Patient samples, n=662	
3	Assignment of all fluorescence parameters	• Calculate Data function using CD19, CD20, CD45, FSC, SSC as common parameters to each malignant B-cell	Patient samples, n=662	
4	Transformation of parameters	• virtual parameter Igκ+Igλ to reflect light chain expression level • Normalization of FSC and SSC vs. $CD4^+CD3^+$ cells	Patient samples, n=662	
5	Univariate analysis	• Export of medFI per sample • Statistical analysis and graphs (R software)	Patient samples, n=662 9 B-CLPD entities	Fig.3
6	Checking representativeness of training set cases	• Ordering cases according to 1D Robust Curve within an entity Checking training cases to represent the distribution of 1D Robust Curve of the entity	9 B-CLPD entities	
7	Identification of background signal parameters	• Comparison of medFI to apparent CD3 expression of entity (R software) in training set cases	9 B-CLPD entities and 25 fluorescent markers,*	Suppl. Table 9
8	Dimension reduction	• CCA projections of training set cases after removal of background signal parameters per differential diagnosis	36 B-CLPD pair-wise differential diagnosis	Fig. 2, Suppl. Fig. 2
9	Definition of decision criteria for diagnosis	• Creation of non-overlapping SD lines of training set cases	36 B-CLPD pair-wise differential diagnosis	Suppl. Table 5. Fig. 2.
10	Independent validation of the analysis strategy	• Inclusion into all diagnostic criteria of a single entity required	Validation set of B-CLPD cases (n=486)	Table 4, Fig. 4

285 Abbreviations: CCA, Canonical Correlation Analysis; FSC, forward scatter; medFI, median
286 fluorescence intensity; SD, standard deviation; SSC, sideward scatter; QA, quality
287 assessment.

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Supplemental Table 4. Canonical coefficients for CA1 and CA2. Significance of contribution of individual parameters to the canonical axes CA1 and CA2 by differential diagnosis. Values derived from the training cases. Please refer to separate EXCEL supplemental file. T1-T5 refers to the analyses from the full data set, T1-T2 provides information after restriction to tubes 1 and 2.

Abbreviations: BL, Burkitt lymphoma; BT ratio, scatter ratio between malignant B-cell clone and residual T-cells; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; CA, canonical axis; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma

Supplemental Table 5. SD lines utilized as decision criterion per pair-wise differential diagnosis. Numbers in brackets represent non-overlapping SD lines when only the information from tubes 1 and 2 was used. Greater SD lines correspond to better separation

	BL	CD10$^-$ DLBCL	CD10$^+$ DLBCL	CLL	FL	HCL	LPL	MCL
CD10$^-$ DLBCL	2.0 (2.0)							
CD10$^+$ DLBCL	1.0 (1.0)	1.5 (1.0)						
CLL	3.0 (3.0)	2.0 (1.5)	2.5 (2.5)					
FL	1.5 (1.5)	1.0 (1.0)	0.5 (0.5)	2.5 (2.0)				
HCL	3.0 (2.5)	2.0 (0.5)	2.0 (1.5)	3.0 (2.5)	2.5 (1.5)			
LPL	2.0 (2.0)	1.0 (1.0)	2.0 (1.5)	2.5 (2.0)	1.5 (1.0)	2.5 (1.5)		
MCL	2.5 (2.5)	2.0 (1.0)	2.0 (1.5)	2.0 (2.0)	2.0 (1.5)	2.5 (2.0)	1.5 (1.0)	
MZL	2.0 (1.5)	0.5 (0.5)	1.5 (1.0)	2.0 (1.5)	1.0 (1.0)	2.0 (0.5)	1.0 (0.5)	2.0 (1.0)

Abbreviations: BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBC, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma.

Supplemental Table 6. Medians (10th – 90th percentile) of medFIs and of BT ratio, respectively, by parameter and entity (see Figure 3 for corresponding box plots).

Para-meter	BL	CD10- DLBCL	CD10+ DLBCL	CLL	FL	HCL	LPL	MCL	MZL
CD10	2347*** (1379 - 5674)	67*** (27 - 140)	1848*** (513 - 5149)	**26** (10 - 57)	1390*** (361 - 4436)	91*** (35 - 3889)	40 (20 - 78)	35 (18 - 88)	48* (16 - 79)
CD103	76 (49 - 132)	125*** (45 - 249)	148*** (61 - 308)	61 (44 - 84)	**55** (23 - 131)	1728*** (792 - 3707)	77* (46 - 117)	71 (47 - 117)	89** (57 - 188)
CD11c	86 (44 - 194)	163*** (69 - 1262)	119** (58 - 580)	190*** (63 - 496)	**68** (37 - 227)	10547*** (3760 - 21628)	113 (46 - 206)	69 (28 - 165)	183* (72 - 776)
CD185	6689*** (2417 - 14898)	5710*** (846 - 18060)	5349*** (1156 - 20635)	13244*** (2962 - 23431)	3650*** (868 - 10776)	**782** (209 - 3691)	2010 (517 - 9215)	9258*** (1658 - 23500)	4126** (907 - 14833)
CD19	9666*** (4801 - 19572)	9023*** (3281 - 26399)	4885 (2214 - 13832)	9017*** (5326 - 14139)	**4747** (1839 - 10535)	29035*** (15656-54521)	9055*** (4204-16529)	6747 (3008 - 12169)	13784*** (5679 - 22462)
CD20	16260*** (10329-30281)	15346*** (2800 - 48402)	19693*** (5256 - 53113)	**3229** (1607 - 8081)	16590*** (8307 - 32409)	75760*** (43013-124973)	17096*** (5721-29211)	19458*** (7736 - 36902)	24327*** (8062 - 48735)
CD200	109 (51 - 275)	783*** (86 - 3613)	136** (49 - 1535)	4396*** (2395 - 7743)	215*** (73 - 1247)	7306*** (661 - 19275)	1125*** (185 - 3435)	**42** (-6 - 165)	559*** (88 - 3065)
CD22	1276 (560 - 3536)	2655*** (270 - 13924)	3096*** (621 - 7377)	**850** (376 - 1951)	2093*** (465 - 6625)	24089*** (6811 - 38209)	1181 (459 - 3212)	1438 (372 - 4508)	3815*** (934 - 9959)
CD23	**92** (52 - 148)	215** (85 - 668)	174* (70 - 523)	1230*** (384 - 3183)	160* (50 - 1005)	210** (117 - 409)	123 (78 - 275)	107 (68 - 244)	160 (97 - 425)
CD27	747** (150 - 3942)	329 (44 - 2038)	791*** (15 - 5690)	1910*** (858 - 3962)	258 (23 - 2235)	**108** (1 - 764)	443 (51 - 1358)	1105*** (206 - 2846)	758** (93 - 4191)
CD305	75* (38 - 258)	103*** (46 - 338)	74** (32 - 464)	172*** (15 - 1426)	**32** (17 - 85)	9577*** (4454 - 17809)	73*** (28 - 752)	568*** (42 - 1437)	75** (30 - 1576)
CD31	172 (91 - 410)	259* (140 - 541)	215 (89 - 587)	730*** (399 - 1273)	**120** (56 - 304)	1641*** (615 - 2924)	552*** (256 - 1282)	513*** (231 - 908)	307** (105 - 959)
CD38	10451*** (5817 - 15501)	755*** (48 - 6689)	3253*** (375 - 7854)	**114** (-45 - 561)	504*** (162 - 2249)	425** (-117 - 1534)	276** (-23 - 2149)	1044*** (88 - 3110)	287 (36 - 958)
CD39	**70** (32 - 133)	851*** (131 - 4635)	137** (65 - 7410)	857*** (327 - 1951)	120 (37 - 540)	1024*** (287 - 3917)	408*** (138 - 1052)	944*** (275 - 2181)	662*** (125 - 2073)
CD43	1670*** (781 - 3818)	490*** (104 - 2750)	275 (101 - 1287)	3638*** (1207 - 6288)	**131** (34 - 453)	850*** (139 - 2392)	295 (37 - 932)	626*** (170 - 2015)	363 (74 - 937)
CD45	**2501** (1352 - 3867)	4193** (1769 - 6679)	4028* (1592 - 5714)	2796 (1948 - 4272)	3593* (1688 - 5591)	6681*** (4591 - 10191)	4534*** (3326 - 6319)	3397 (2121 - 4588)	4239** (2697 - 6501)
CD49d	540** (325 -	537* (104 -	359 (116 -	**256** (89 - 735)	424* (96 -	1354*** (570 -	856*** (413 -	574*** (223 -	804*** (257 -

	1223)	1501)	1274)		1074)	2788)	1849)	1125)	1504)
CD5	89 (-1 - 255)	220* (70 - 692)	153 (51 - 528)	3685*** (976 - 7045)	**80** (3 - 364)	240 (117 - 589)	177 (40 - 692)	2285*** (923 - 5835)	159 (64 - 552)
CD62L	**96** (53 - 158)	216** (100 - 549)	226** (73 - 625)	534*** (132 - 2185)	111 (40 - 475)	289*** (139 - 916)	291*** (123 - 1742)	117 (66 - 305)	156* (99 - 1004)
CD79b	3696*** (203 - 15497)	773** (116 - 5833)	3100*** (199 - 15032)	**271** (94 - 667)	3095*** (325 - 12047)	2405*** (446 - 11397)	6239*** (1286- 20394)	4577*** (907 - 16636)	1217*** (227 - 9340)
CD81	13133*** (5128 - 20995)	3075*** (868 - 8988)	5457*** (1385 - 17621)	**579** (326 - 1082)	2946*** (978 - 6291)	1108** (209 - 3572)	1638*** (766 - 3945)	1693*** (840 - 3348)	2018*** (771 - 4689)
CD95	201 (112 - 478)	885*** (131 - 8888)	1845*** (192 - 9065)	122 (62 - 268)	526*** (145 - 2122)	656*** (258 - 1427)	293*** (104 - 852)	**118** (75 - 230)	412*** (111 - 3094)
HLA-DR	12286 (4475 - 71589)	13059 (971 - 84012)	17425* (1270 - 84266)	12948 (3133 - 32925)	**5512** (816 - 76359)	9560 (2881 - 37063)	6462 (2205- 20169)	10124 (3742 - 27124)	9638 (4723 - 35978)
IgM	2071** (78 - 13812)	666** (78 - 4639)	238** (40 - 8034)	**131** (48 - 490)	336** (30 - 9372)	344* (80 - 2675)	3117*** (333 - 11164)	3512*** (539 - 14785)	664*** (72 - 6744)
Igκ + Igλ	12118*** (2445 - 28428)	6279*** (1037 - 16087)	9094*** (1606 - 30317)	**1805** (557 - 4614)	6960*** (785 - 29414)	10617*** (1797 - 31089)	9920*** (1989- 52827)	8078*** (1037 - 38262)	5248** (831 - 23938)
BT Ratio	2.52*** (1.59 - 3.56)	2.34*** (1.22 - 4.38)	2.89*** (1.31 – 6.66)	**0.80** (0.52 - 1.18)	1.05** (0.48 - 2.41)	3.86*** (1.99 - 5.20)	1.03 (0.65 - 1.51)	0.98 (0.68 - 2.06)	1.35*** (0.81 - 2.91)

Abbreviations: BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBC, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma. Asterisks indicate a significant difference as compared to the entity with the lowest median MedFI (bold font). Total data set (662 cases). * p<0.01, ** p<0.001, *** p<0.0001.

Supplemental Table 7. Monte Carlo cross-validation results. The upper half of the table describes the results when tubes 1 to 5 of the B-CLPD panel are utilized, the bottom half tabulates the results using tubes 1 and 2 of the B-CLPD panel only. Mean±SD of sensitivity, specificity, NPV, PPV were calculated by 1,000-fold random selection of training cases (numbers per entity are the same as for the original model), adjustment of SD lines in order to obtain maximal but non-overlapping areas and performing the classification of the validation cases (i.e. cases that remain per disease category once the training cases are taken out).

	WHO diagnosis	n	Sensitivity	Specificity	PPV	NPV
T1 to T5	**BL**	**13**	55.8±17.7	99.6±0.3	82.9±12.1	98.8±0.5
	CD10$^-$ DLBCL	**31**	9.7±6.3	99.4±0.4	51.2±23.9	94.2±0.4
	CD10$^+$ DLBCL	**33**	13.5±7	98.5±0.8	40.5±15.8	94.0±0.4
	CLL	**125**	86.9±5.6	99.8±0.2	99.4±0.6	95.7±1.7
	FL	**109**	25.4±7.1	99.7±0.3	95.7±3.8	82.2±1.4
	HCL	**38**	91.6±5.5	99.9±0.1	98.5±1.4	99.3±0.5
	LPL	**54**	22.6±10.4	99.5±0.4	85.6±11.9	91.2±1.1
	MCL	**56**	54.9±9.8	99.8±0.2	97.2±3.1	94.5±1.1
	MZL	**27**	11.0±6.6	99.4±0.4	52.6±22.4	95±0.3
T1 + T2	**BL**	**13**	48.9±14.0	99.5±0.3	74.5±13.5	98.6±0.4
	CD10$^-$ DLBCL	**31**	4.6±4.0	99.5±0.4	39.4±30.1	93.9±0.2
	CD10$^+$ DLBCL	**33**	17.2±6.7	98.3±0.8	43.4±14.8	94.2±0.4
	CLL	**125**	82.1±6.3	99.9±0.2	99.6±0.5	94.2±1.9
	FL	**109**	25.2±7.5	99.7±0.3	96.0±3.4	82.2±1.5
	HCL	**38**	29.3±10.9	100.0±0.1	99.5±2.8	94.3±0.8
	LPL	**54**	13.5±7.2	99.6±0.4	81.7±15.3	90.2±0.7
	MCL	**56**	36.1±13.4	99.7±0.2	95.3±4.5	92.3±1.5
	MZL	**27**	5.9±5.5	99.5±0.4	40.8±29.3	94.7±0.3

Abbreviations: BL, Burkitt Lymphoma; CLL, chronic lymphocytic leukemia; DLBC, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; NPV, negative predictive value; PPV, positive predictive value.

Supplemental Table 8. Cases rejected prior to study inclusion.

Cases submitted by the local EuroFlow centers that were rejected upon review by the second expert and reasons for exclusion. Typical examples for lacking annotations included: FISH confirmed translocation (BL, MCL), WBC to be able to differentiate CLL from SLL/MBL, as well as histology in DLBCL, FL, LPL, and MZL. Double submissions refer to cases for which blood and bone marrow samples were submitted simultaneously. A purity of at least 90% after back-bone gating (CD45, CD19, CD20, SSC, FSC) could not be reproduced in 19 cases, as benign B-cells contaminated the gates. 9 cases submitted as CLL turned out to represent high count MBL or SLL. Technical failures frequently included suboptimal compensation, differences of the back-bone markers between the tubes, and instability of the acquisition likely caused by air bubbles.

Submitted diagnosis	**Reason for exclusion**					**total**
	Annotations missing / inconclusive	**double submission**	**purity**	**SLL/MBL**	**technical**	
> 1 diagnosis	7				1	**8**
B-cell lymphoma NOS	11					**11**
BL	2	1				**3**
CLL	20	1	3	9	3	**36**
DLBCL	17	4	5		3	**29**
FL	6	2	6		3	**17**
HCL	3		1		1	**5**
LPL	10		2		4	**16**
MCL	18				1	**19**
MZL	14		2		1	**17**
total	**108**	**8**	**19**	**9**	**17**	**161**

Abbreviations: BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MBL, Monoclonal B-cell lymphocytosis; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; NOS, not otherwise specified; SLL, small lymphocytic lymphoma.

Supplemental Table 9: Markers representing predominantly background signal (BS) by entity. Markers showing background signal for both entities of a given differential diagnosis were not considered in CCA for that particular differential diagnosis.

marker	BL	CD10$^-$ DLBCL	CD10$^+$ DLBCL	CLL	FL	HCL	LPL	MCL	MZL
CD10		BS		BS			BS	BS	BS
CD103	BS	BS	BS	BS	BS		BS	BS	BS
CD11c	BS	BS	BS		BS		BS	BS	BS
CD19									
CD20									
CD200	BS		BS					BS	
CD22									
CD23	BS	BS	BS		BS	BS	BS	BS	BS
CD27		BS							
CD3	BS	BS	BS	BS	BS	BS	BS	BS	BS
CD31	BS	BS	BS		BS				
CD38									
CD39	BS				BS				
CD43			BS		BS				BS
CD45									
CD49d	BS		BS						
CD5	BS	BS	BS		BS	BS			BS
CD62L	BS	BS	BS		BS			BS	BS
CD79b									
CD81									
CD95	BS			BS				BS	
CD185									
HLADR									
IgM									
Igκ+Igλ									
CD305	BS	BS	BS		BS		BS		BS

Abbreviations: BL, Burkitt lymphoma; BS, background signal; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma.

Legends to Supplemental Figures

Supplemental Figure 1. **Gating strategies for identification of the lymphoma clone using a CLL (A-E) and a DLBCL (F-J) case respectively as examples.** T-cells are identified and removed from further analysis in CD3 vs SSC dot plots representing tube 1 only (light blue, A, F). A combination of scatter, CD19, CD20, and CD45 (i.e. the common markers between the tubes) is used to identify the malignant clone in tubes 1 to 5 (red gates are combined by Boolean 'AND', B-D and G-I). If needed, additional combinations of common markers and corresponding gates are used to describe the malignant clone. The purity of the identified clone is checked by Igκ-Igλ light chain restriction (E, J). Gates were optimized for maximum clone size while preserving the purity of the malignant clone (by definition, required to exceed 90%). Please note low level Igκ expression in CLL (E) and the Igκ expression comparable to normal B cells in DLBCL (J). The light blue line in (E) and (J) represents the second standard deviation of background Igκ and Igλ expression from $CD4^+CD3^+$ T-cells of the same sample as internal negative reference. Abbreviations: CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FSC, forward scatter; SSC, side scatter;

Supplemental Figure 2. CA1 and CA2 for the differential diagnoses of CLL vs MCL (A,E), FL vs MCL (B,F) BL vs FL (C,G) and BL vs $CD10^-$DLBCL (D,H). Immunophenotypic information of tubes 1 to 5 (A-D) and tubes 1 plus 2 only (E-H), respectively, were used. The x- and y-axes of each plot represent CA1 and CA2. CA1 is the projection that captures most of the information for maximum separation between two mature B-cell lymphoma entities, CA2 is the projection that provides the second greatest amount of independent information for separation. Numbers in the upper right corner of each plot represent the x fold SD of the immunophenotype shown. Numbers in brackets denote the relative contribution of markers to CA1 and CA2, respectively (cf. supplemental Table 4 for a full list of markers and

coefficients). Note that the separation for MCL vs FL and for $CD10^-$DLBCL vs BL is poorer when only the information of tubes 1 and 2 is used, so that lower fold SD lines completely separate the entities (F, H). Abbreviations: BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; CA, canonical axes; MCL, mantle cell lymphoma; SD, standard deviation

Supplemental Figure 3. MedFIs of CD4 PacB (A), CD3 APC (B), CD45 PacO (C), CD5 PerCP Cy5.5 (D) on $CD4^+CD3^+$ T-cells and of CD8 FITC (E) on $CD8^+CD3^+$ T-cells by center. Bars represent means, whiskers represent 1 SD. Abbreviations: APC, Allophycocyanin; FITC, Fluorescein Isothiocyanate; MedFI, median fluorescence intensity; PacB, Pacific Blue; PacO, Pacific Orange; PE, Phycoerythrin; PerCP, Peridinin-Chlorophyll-Protein, SD, standard deviation

Supplemental Figure 4. MedFIs of CD4 PacB (A), CD3 APC (B), CD45 PacO (C), CD5 PerCP Cy5.5 (D) on $CD4^+CD3^+$ T-cells and of CD8 FITC (E) on $CD8^+CD3^+$ T-cells by sample material. Bars represent means, whiskers represent 1 SD. Abbreviations: APC, Allophycocyanin; BM, bone marrow; CNS, central nervous system; FITC, Fluorescein Isothiocyanate; LN, lymph node; MedFI, median fluorescence intensity; PacB, Pacific Blue; PacO, Pacific Orange; PB, peripheral blood; PE, Phycoerythrin; PerCP, Peridinin-Chlorophyll-Protein; SD, standard deviation; TM, tumor mass

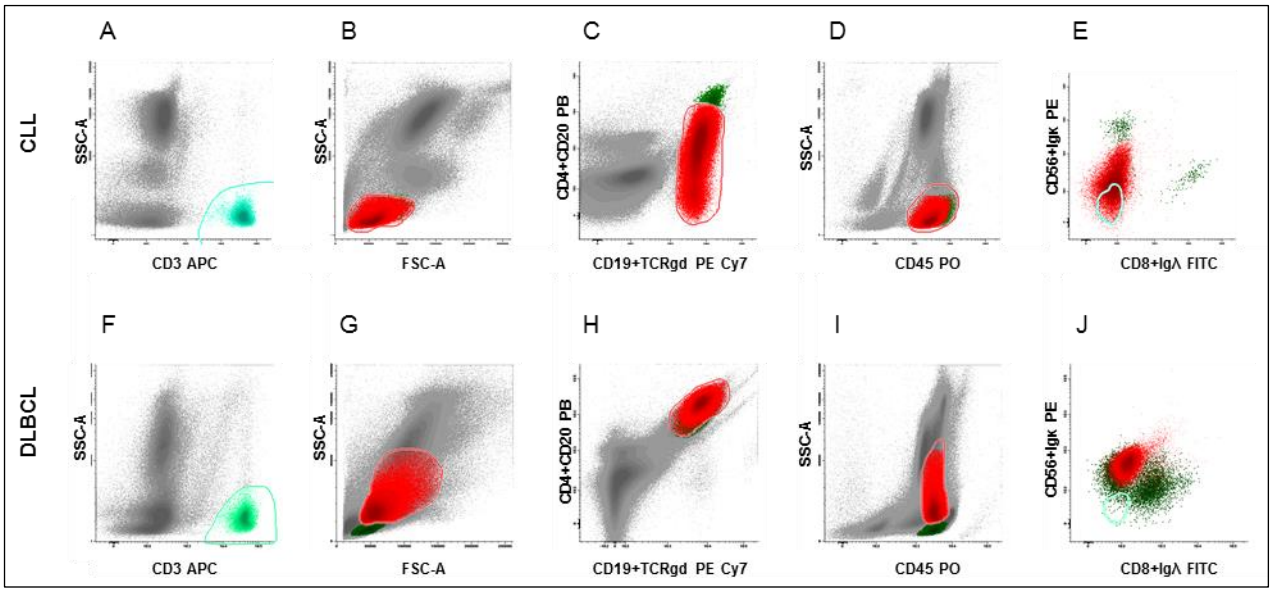
Supplemental Figure 5. **MedFIs of CD4 PacB (A), CD3 APC (B), CD45 PacO (C), CD5 PerCP Cy5.5 (D) on $CD4^+CD3^+$ T-cells and of CD8 FITC (E) on $CD8^+CD3^+$ T-cells by year of acquisition.** Bars represent means, whiskers represent 1 SD. Abbreviations: APC, Allophycocyanin; FITC, Fluorescein Isothiocyanate; MedFI, median fluorescence intensity; PacB, Pacific Blue; PacO, Pacific Orange; PE, Phycoerythrin; PerCP, Peridinin-Chlorophyll-Protein, SD, standard deviation

Supplemental Figure 6. Robust variant of Mahalanobis distance (y-axis) to check representativeness of training set cases for total cohort using CLL as an example. Cases are ordered by Mahalanobis distance, i.e. most typical CLL cases appear on the left. Each individual cases` median is represented by a circle. Numbers on X-axis refer to unique patient identifiers. Training set cases are shown in purple, validation cases are depicted green. Abbreviations: CLL, chronic lymphocytic leukemia

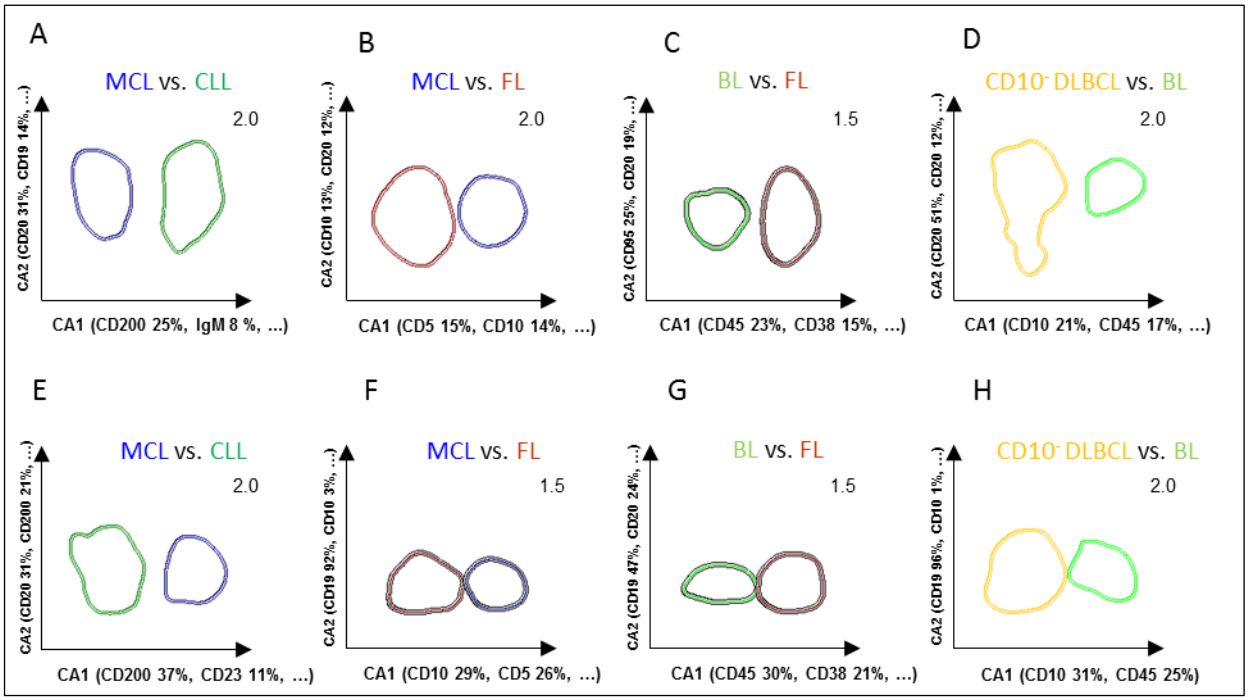
Supplemental Figure 7. Apparent CD3 medFI values by B-cell lymphoma entity. Marker expression in log scale. Horizontal lines indicate medians, boxes show interquartile ranges and whiskers extend to largest/smallest value within the median +/- 1.5x interquartile range. Dots show cases out of the interquartile range. Each case is represented by its medFI (n=662). Abbreviations: BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; medFI, median fluorescence intensity; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma

Supplemental Figure 8. CA1 and CA2 for the differential diagnoses of classical HCL (pink) vs HCL variant (green). The x- and y-axis represent CA1 and CA2. 2 SD are shown. Each dot represents the median of a case. Numbers in brackets denote the relative contribution of markers to CA1 and CA2, respectively. Please note complete separation between those entities. Abbreviations: HCL, hairy cell leukemia; HCLv, hairy cell leukemia variant.

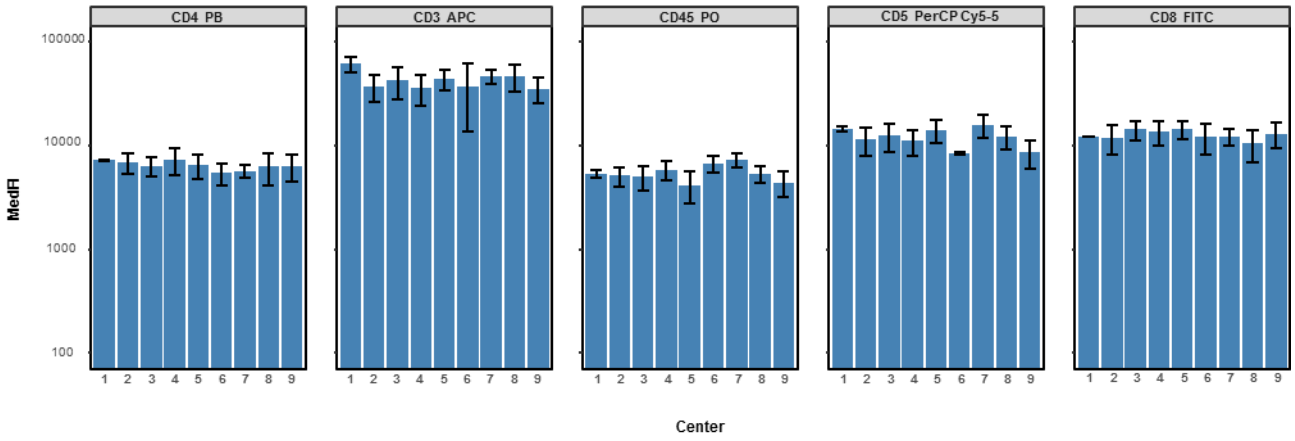
Supplemental Figures



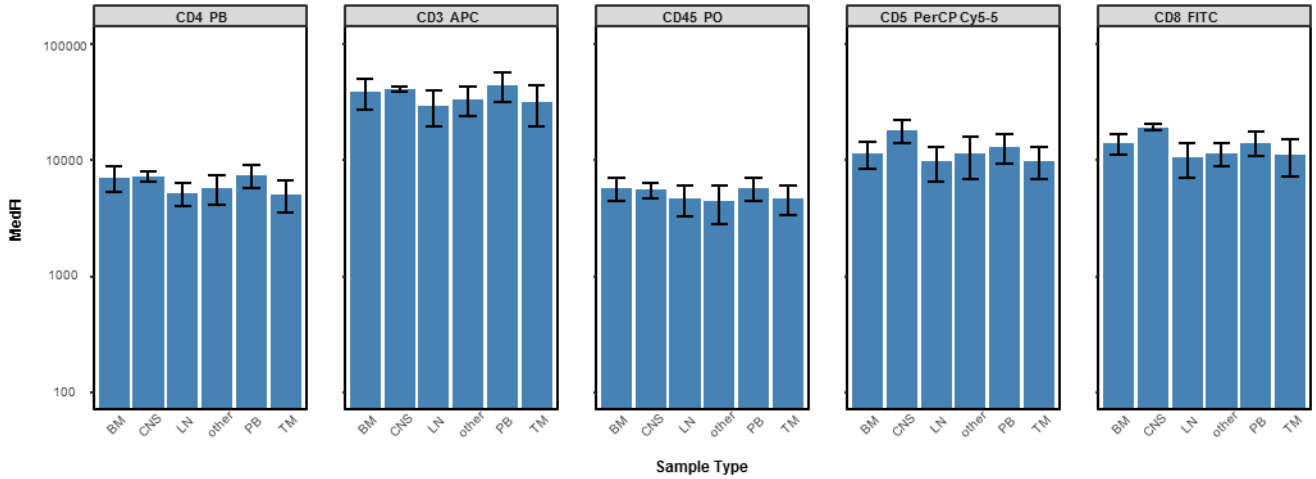
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



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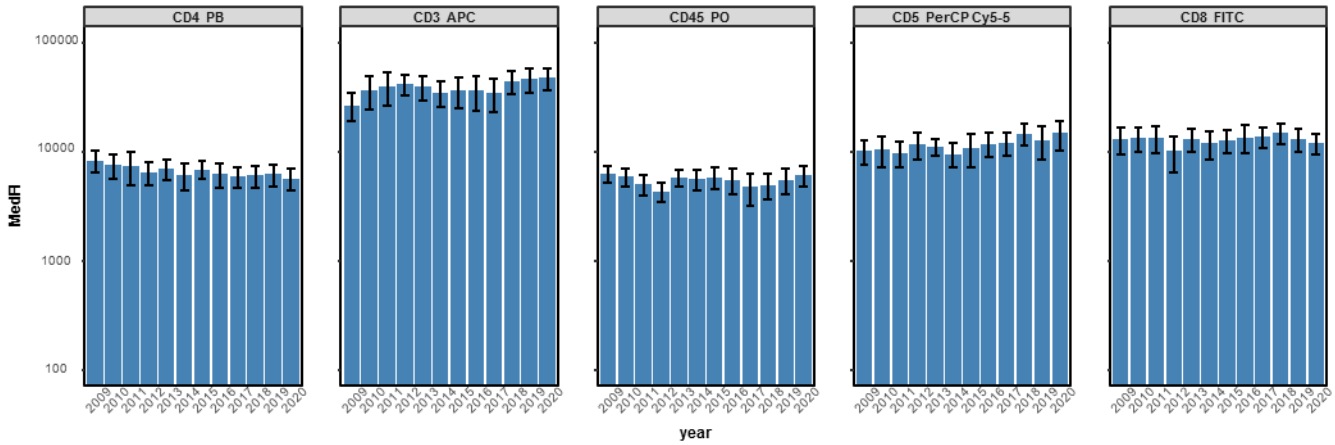
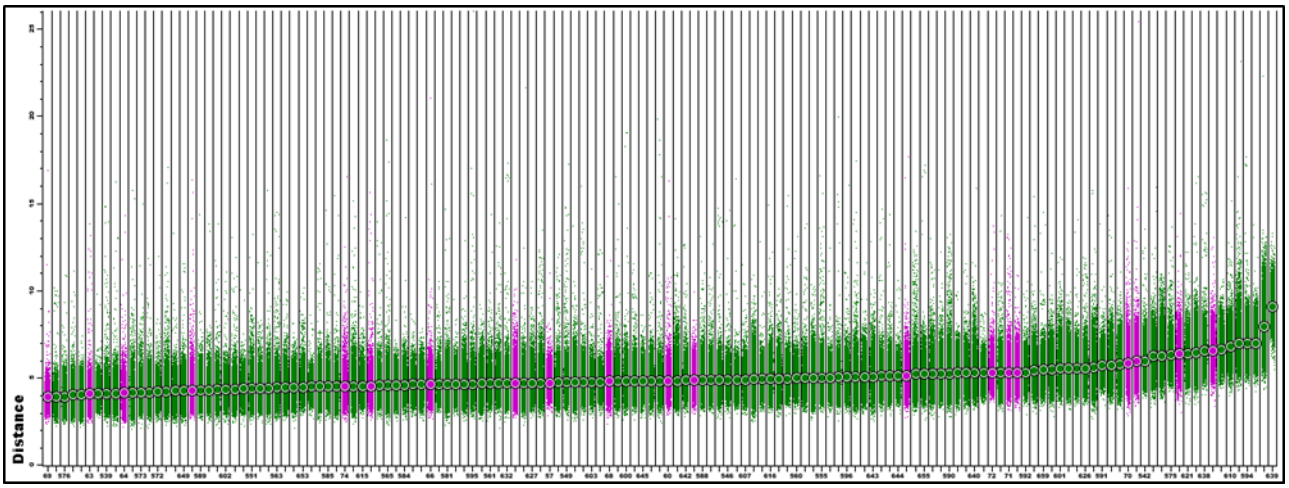


Supplemental Figure 5



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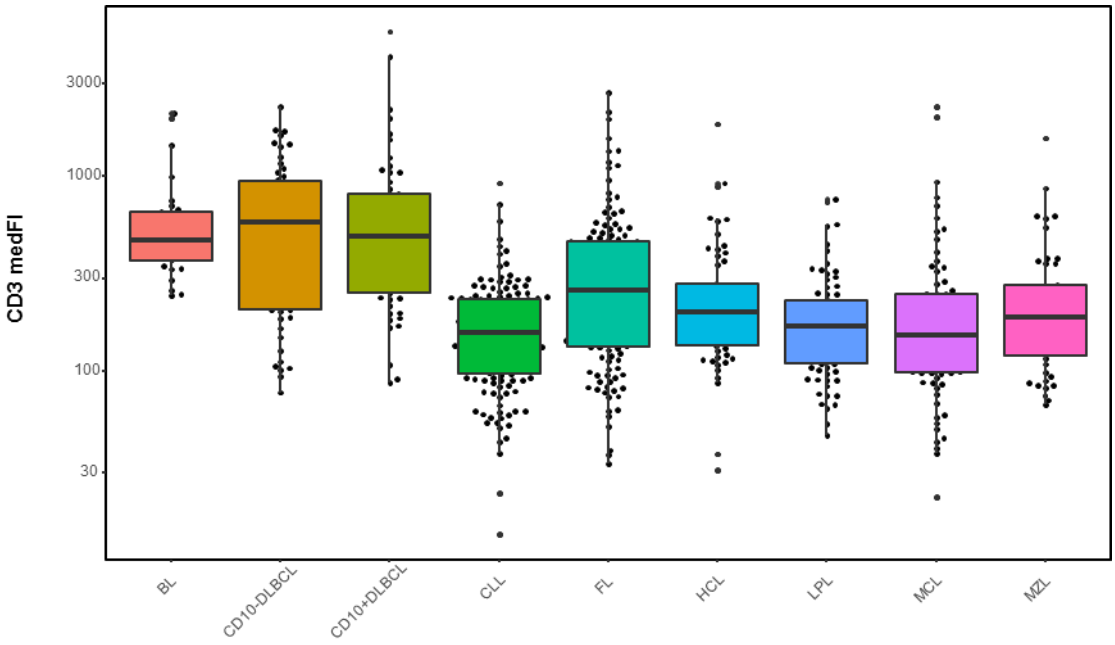
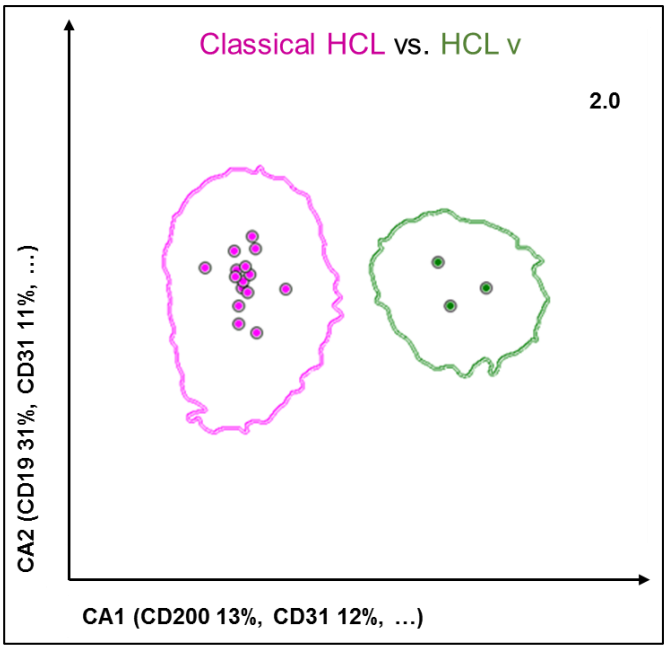


Supplemental Figure 7



Supplemental Figure 8