Proposals of B cells standard immunophenotyping in mature B cell non-Hodgkin lymphomas

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Abstract

Immunophenotyping of leukemia cells plays the crucial role in identification of proliferating cell line, what is very useful for individual treatment monitoring and detection of residual disease. The purpose of this study was to develop standard laboratory procedure for immunophenotyping of leukemia cells in mature B cell non-Hodgkin lymphomas (NHL) to establish the kit of necessary antibodies and to work out strategy of typing in CLL/SLL, MCL and MZL.

Leukocytes from 115 patients with B-cell NHL and 15 blood donors were tested. Immunophenotype determination was carried out by direct antibody staining, three-color immunophenotyping with CD19 gating and analysis with FACS Canto flow cytometer.

Flow cytometry analysis revealed that the first step for proper determination of phenotype of leukemic cells is evaluation of expression of CD5, CD20, CD23, CD79b, CD22, CD10, FMC7, kappa/lambda immunoglobulin light chains, and IgM, IgD as well as the antigen density. We established useful screening kits for three-color flow cytometry. Proposed three color kit are: CD45/CD14, CD5/CD20/CD19, CD23/CD79b/CD19, CD22/CD10/CD19, FMC7, kappa/lambda/CD19, IgM/ IgD/CD19.

Our data indicate that short panel (including markers for CD5, CD19, CD23, CD79b, CD20, kappa, lambda) is sufficient for typical CLL/SLL phenotyping and the panel extended by markers for CD22, FMC7, CD10, IgM and IgD allows for CLL/PLL, MCL and MZL differentiation. Intensity of antigen expression determination, especially CD20, CD79b and light chains, should be integral part of immunophenotyping analysis.

Key words: *immunophenotyping, B-NHL, CLL/SLL, MCL, MZL.*

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Introduction

Flow cytometry (FC) is essential in the laboratory assessment of malignant hematological diseases, since it may support diagnosis, and facilitate classification and monitoring of many diseases such as leukemia, lymphoma and myeloma [1-3]. FC analysis can detect a wide spectrum of membrane, intracytoplasmic or nuclear cell markers. It can help to differentiate between morphologically similar diseases by assessment of antigens expressed on malignant cells.

B-cell chronic lymphoproliferative disorders can be classified on the basis of morphology and immunological markers defined by World Health Organization (WHO) in 2001 for clinical and pathologic use [4-5].

The purpose of this study was to develop standard laboratory procedure for immunophenotyping of leukemia cells in mature B cell non-Hodgkin lymphomas (NHL) to establish the kit of necessary antibodies and to work out strategy of typing in CLL/SLL (Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma), MCL (Mantle Cell Lymphoma) in leukemic phase and MZL (Marginal Zone Lymphoma).

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Material and Methods

Material consisted of leukocytes from peripheral blood or bone marrow from 115 patients with B-cell NHL and 15 blood donors (patients' characteristics in Table 1).

Specimen requirement: 2 mL EDTA whole blood, with no signs of hemolysis, or 2 mL EDTA bone marrow aspirate. Blood or bone marrow specimens were stored at room temperature up to 24 h or 6 h respectively. Immunophenotype assessment was carried out by direct antibody staining. A total of 0.5×10^6 white blood cells were stained. Three-color immunophenotyping with CD19 gating (except FMC7 calculating as percentage of WBC and converting into percentage of B CD19+ cells) for the following markers was performed: CD5, CD20, CD23, CD79b, CD22, CD10, FMC7, kappa and lambda immunoglobulin light chains, IgM, IgD. Appropriate isotype control antibodies were included into each series. 10000 cells were counted and data were recorded in the list mode.

Antigen expression was quantified using the Stain Index (SI), a useful metric to express the relative brightness of a positive signal [6].

$$D : W = [\text{Mean}_{(pos)} - \text{Mean}_{(neg)}] : [2 \times SD_{(neg)}] = SI$$

D = the difference of the populations means
W = the width of negative (at 2 standard deviations)

Mean $_{(pos)}$ = the mean of the positive population
Mean $_{(neg)}$ = the mean of the negative population
SD $_{(neg)}$ = the standard deviation of the negative population

The algorithm normalizes the positive population signal with the degree of spread observed in the negative population or compensated cells from another dye dimension.

Analyses were conducted with FACS Canto flow cytometer and FACS Diva software (Becton Dickinson).

Distinct cell populations present in the specimen were identified using CD19 and SSC. To determine the immunophenotype of B cells (CD19/SSC dot plot) a triple labeling method with CD19-PC5 against each CD-FITC and CD-PE was used. The threshold value for antigen positivity was more than 20% positive B CD19+ cells.

The statistical analysis was made by Mann Whitney U Test ($p < 0.05$ were considered as statistically significant).

Results

The results of B-cell immunophenotyping and antigen expression intensity estimation found in the group of 115 patients are presented in Table 2 and Table 3.

The preliminary diagnosis of CLL/SLL, MCL and MZL was based on clinical and laboratory features. After initial diagnostic tests 11 of the 85 CLL/SLL cases were classified as transforming into prolymphocytic leukemia (CLL/PLL).

Table 1. Patients characteristics

B-cell NHL	N	Age years ± SD	Sex	Samples PB / BM	Leukocytosis G/L + SD	Percentage of B CD19+ cells in PB or BM
CLL/SLL	74	67 ± 11	31 F / 43 M	70 / 4	46,6 ± 44,5	70,3 ± 15,5
CLL/PLL	11	72 ± 10	5 F / 6 M	11 / 0	143,9 ± 111,5	87,8 ± 8,4
MCL	19	68 ± 10	7 F / 10 M	6 / 11	107,9 ± 156,9	56,5 ± 28,7
MZL	11	74 ± 10	3 F / 8 M	3 / 7	71,3 ± 54,8	43,6 ± 30,3

F – female, M male; PB – peripheral blood, BM – bone marrow.

Table 2. Results of immunophenotyping in CLL/SLL, CLL/PLL, MCL, MZL – percentage of positive cells in CD19+ gate (mean ± SD)

	CD5	CD23	CD20	CD79b	Kappa[#]	Lambda[#]	CD22	CD10	FMC7	IgM	IgD
CLL/ SLL	92.5±13.4	88.9±10.9	96.3±6.8	65.7±31.7	71.8±30.1	72.1±33.0	33.9±26.6	0.4±0.5	9.1±8.1	37.5±13.8	47.8±36.5
CLL/ PLL	91.4±18.7	70.5±31.3	99.8±0.5	86.5±27.9	91.1±10.2	97.7±1.7	58.5±28.3	0.2±0.2	36.8±29.2	61.6±26.3	71.5±34.4
MCL	91.8±10.0	5.5±5.1	99.2±2.0	96.6±5.8	96.2±4.7	90.2±18.3	76.1±24.6	4.3±11.6[&]	76.8±18.5	98.6±1.9	21.6±28.6
MZL	7.3±8.3	26.9±18.2	98.8±1.9	96.8±4.1	94.2±8.0	97.5±3.1	80.8±25.1	3.6±5.4[&]	80.7±18.4	99.5±0.3	97.4±3.1
Donors blood	8.7±5.9	41.5±16.1	98.3±1.0	85.7±5.9	54.9±3.6	42.4±3.8	98.3±0.7	<0.5	91.8±7.6	78.9±6.6	77.9±8.8

[#] – kappa or lambda respectively; in 29 of 74 CLL/SLL specimens 31-99% B CD19+ cells without surface immunoglobulin light chains (κ-λ-).
[&] – CD10+ residual „normal" B-cells in bone marrow, usually CD19+ κ-λ-.

Table 3. Antigen expression intensity on B CD19+ cells in CLL/SLL, CLL/PLL, MCL, MZL – in arbitrary unit SI (Stain Index)

	CD5	CD23	CD20	CD79b	Kappa[#]	Lambda[#]	CD22	FMC7	IgM	IgD
CLL/SLL N=20	24±14	21±9	71±51	11±7	9±4	12±9	5±2	17±25	22±33	8±4
CLL/PLL N=11	31±15	14±10	201±198	31±41	29±31	22±11	7±5	19±28	49±55	10±6
MCL N=17	16±9	9±2	343±178	129±98	191±163	200±86	11±9	34±24	107±39	13±11
MZL N=1	No expression	9±3[##]	303±159	67±43	62±29	93±53	18±10	19±9	101±10	51±46
Donors blood	11±5	15±2	313±58	89±13	92±7	224±33	30±9	73±32	103±14	40±3

 # – kappa or lambda respectively.
– weak expression in some cases (19.5-45.5% of B CD19+).

Mandatory antigens for flow cytometry analysis in B-NHL:
- Pan-leukocyte antigen CD45 (+ CD14) for leukocyte subpopulation separation
- B-cell line-specific antigens: CD19, CD20, CD22, CD23, CD79b, FMC7, CD10, kappa and lambda light chains, IgM and IgD heavy chains
- T-cell line-specific antigen: CD5.

Stage of immunophenotyping – proposal of antibody panels:
- Preliminary diagnosis: CLL/Lymphoma (high WBC count, lymphocytosis more than $5 \times$ G/L) – Panel 1:
    1) CD45 FITC /CD14 PE
    2) CD5 FITC /CD20 PE /CD19 PC5
    3) CD23 FITC /CD79b PE /CD19 PC5
    4) Kappa FITC /lambda PE /CD19 PC5
    5) Adequate isotype controls
- Preliminary diagnosis: Lymphoma – Panel 2:
    6) CD45 FITC /CD14 PE
    7) CD5 FITC /CD20 PE /CD19 PC5
    8) CD23 FITC /CD79b PE /CD19 PC5
    9) CD22 FITC /CD10 PE /CD19 PC5
    10) FMC7
    11) Kappa FITC /lambda PE /CD19 PC5
    12) IgM FITC /IgD PE /CD19 PC5
    13) Adequate isotype controls

All investigated B-NHLs express CD19 and furthermore they are characterized by:

CLL/SLL: <u>CD5+ CD23+</u> CD20+^{dim} CD79b+/±^{dim} κ or λ +/±^{dim} or κ-λ- (CD22-/+ ^{dim} CD10- FMC7- IgM±^{dim} IgD±^{dim})

CLL/PLL: <u>CD5+/±/- CD23+/±/- CD10-</u> CD20+$^{bright/medium}$ CD79b+$^{dim/medium}$ FMC7+/ ± CD22+$^{normal/dim}$ κ or λ+ $^{dim/medium}$ IgM±$^{medium/bright}$ IgD±$^{dim/medium}$

MCL: <u>CD5+ CD23- CD10-</u> CD20+$^{bright/medium}$ CD22+ CD79b+$^{bright/medium}$ FMC7+/ ± κ or λ+^{bright} IgM+^{bright} IgD-/+^{dim}

MZL: <u>CD5- CD23- CD10-</u> CD20+$^{bright /medium}$ CD22+/± CD79b+$^{bright/medium}$ FMC7+/± κ or λ+$^{bright/medium}$ IgM+^{bright} IgD+$^{bright/medium}$

Each B-NHL has its own quantitative pattern of surface antigen expression. We suggest cheap method (without additional costs) for antigen density determination. Stain Index (SI) normalizes the positive population signal with the degree of spread observed in the negative population. Results of antigen expression intensity analysis (measured in arbitrary units) indicate antigens important for B-NHL diagnosis: CD20, CD79b and κ/λ surface light chains (see Table 3 and Figure 1).

Taking into consideration antigen expression intensity investigated B-NHLs could be put in following order (increasingly):
- CD20: CLL/SLL < CLL/PLL (p<0.001 vs. CLL/SLL) < MZL (p<0.0001 vs. CLL/SLL, p<0.04 vs. CLL/PLL) < MCL (p<0.0001 vs. CLL/SLL, p>0.4 vs. MZL and p<0.01 vs. CLL/PLL)
- CD79b: CLL/SLL < CLL/PLL (p<0.03 vs. CLL/SLL) < MZL (p<0.0001 vs. CLL/SLL, p<0.02 vs. CLL/PLL) < MCL (p<0.0001 vs. CLL/SLL, p=0.3 vs. MZL, p<0.01 vs. CLL/PLL)
- Light chains (kappa or lambda): CLL/SLL < CLL/PLL (p<0.02 vs. CLL/SLL) < MZL (p<0.001 vs. CLL/SLL, p<0.001 vs. CLL/PLL) < MCL (p<0.001 vs. CLL/SLL, p=0.1 vs. MZL, p<0.001 vs. CLL/PLL)

Discussion

Based on general recommendation for immunophenotyping in B cell non-Hodgkin lymphomas [7-10] and our results and earlier experience we suggest optimization
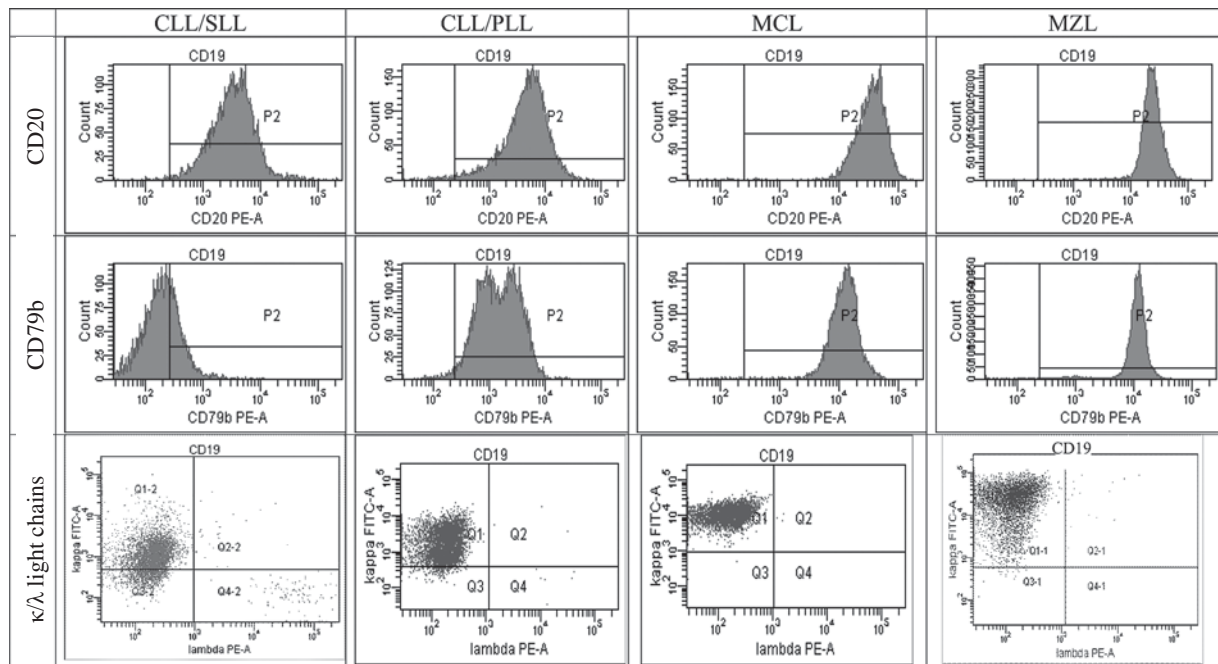
Fig. 1. Antigen expression intensity comparison in B-NHLs (examples): CLL/SLL, CLL/PLL, MCL and MZL. Cells were gated on B CD19+ vs. SSC cytogram. The highest CD20, CD79b (histograms) and light chains (cytograms) antigen expression are visible in MZL and MCL

for selected B-NHL immunophenotyping as it is shown on Figure 2.

There are no significant differences in antigen characteristics between B-CLL cells obtained from peripheral blood and bone marrow (own observations, retrospective analysis) thus peripheral blood B-cells immunophenotyping seems to be sufficient for typical B-CLL cases. CD22 and CD10 antigens analysis in routine CLL/SLL immunophenotyping seems to be unneccessary: CD22 is usually dim or absent, CD10 – absent (in all cases < 0.5% CD10+/ CD19+).

In B-CLL transforming into B-PLL complete blood count is variable (from ~25 × G/L to ~350 × G/L), lymphocyte percentage – about 90%. Peripheral blood B-cells immunophenotyping seems to be sufficient. First symptoms of transformation are: intensification of CD20 and IgM expression (dim to medium-bright), subsequently – CD79b. Expression of FMC7 (significant marker for prolymphocytes in original *de novo* CD5-negative B-PLL) may be variable as regards percentage of B CD19+ cells and expression intensity. Smears may show higher percentage of prolymphocytes in some cases (but correspond with CD20+[bright] B CD19+ cells), especially in early stage of transformation.

For confirmation of MCL diagnosis assessment of cyclin D1 is recommended (using immunohistological

method, FC method is not recommended). It should be taken into consideration that in some MCL cases disease involves nodes, but there is no bone marrow infiltration. In advanced stage of the disease peripheral blood may rarely contain lymphoma cells. Cases with high lymphocytosis (>100 G/L) should be differentiated with CD5+ prolymphocytic leukemia and prolymphocyte transformation of CLL. In such cases type of involvement of lymph nodes and/or bone marrow (immunohistological methods) should be determined. Classic MCL phenotype (CD5+ CD10- CD23-) is seen in 80% cases. CD5-negative MCL comprises of 11% and CD10+ MCL - 6% (CD5+)of cases, CD23+[dim] MCL are seen in 6% (CD5+) [8].

There are 3 types of lymphomas with overlapping antigen characteristics (CD19+ CD20+ CD22+ CD79b+ FMC7+ IgM+ CD5- CD10- CD23-): marginal zone lymphoma MALT type, nodal margin zone lymphoma and splenic marginal zone lymphoma. Often B-cells in MZL show dim to moderate expression of CD11c [8]. MZL in leukemic phase should be differentiated with CD5-negative B-PLL. Final diagnosis should be done on the basis of clinical and histomorphologic features as well as pattern of involvement.

Comprehensive immunophenotyping should include simultaneous analysis of all antigens, taking into consideration expression intensity of the antigens, especially
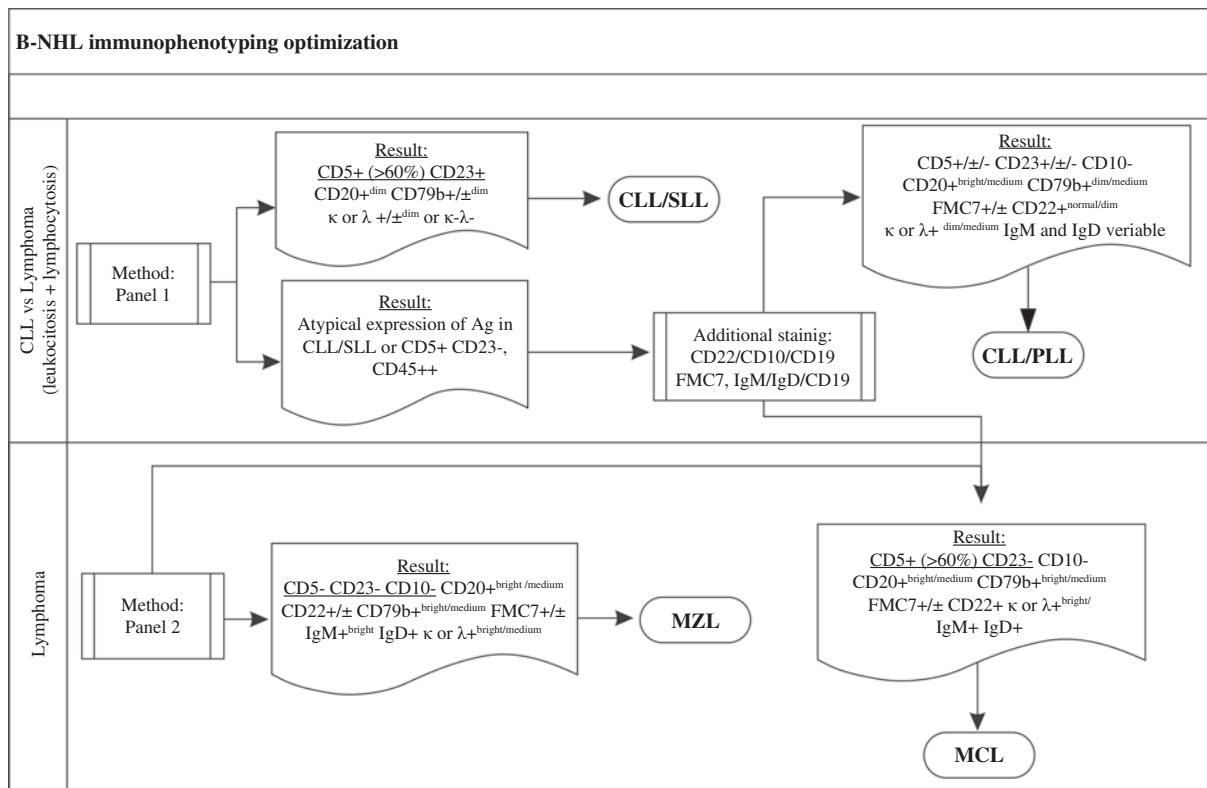
B-NHL immunophenotyping optimization

Fig. 2. Immunophenotyping optimization for selected B-NHLs (CLL/SLL, CLL/PLL, MCL and MZL). Panel 1 and Panel 2 – see in the text. Percentage of B cells with an antigen expression: (+) 75% - 100%; (±) >50% < 75%; (-/+) >20% < 50%; (-) <20%. Designation of antigen expression intensity: dim, normal, medium, bright (in relation to mature B CD19+ cells from donor's peripheral blood)

CD20, CD79b and surface light chains. Intensity of antigen expression may serve as additional characteristics of leukemic cells. It may be presented as relative fluorescence intensity (RFI) or as antibody molecules bound by cell (MESF, ABC) [11, 12]. We propose Stain Index (SI) for antigen density determination, cheap method, with no additional costs. Complete analysis allows to distinguish CLL/SLL (also cases with transformation into PLL), MCL and MZL. Suggested "Panel 2" provides also preliminary detection of another B-NHL: B-cell prolymphocytic leukemia, follicular lymphoma (additional required staining: Bcl-2), Burkitt's lymphoma (additional required staining: Bcl-6, c-MYC), hairy cell leukemia (additional required staining: CD11c, CD103, CD25).

Conclusions

Our data indicate that:

1. Short panel (including markers for CD5, CD19, CD23, CD79b, CD20, kappa, lambda) is sufficient for typical CLL/SLL immunophenotyping.
2. The panel extended by markers for CD22, FMC7, CD10, IgM and IgD allows for CLL/PLL, MCL and MZL differentiation.
3. Determination of antigen expression intensity, especially CD20, CD79b and light chains, should be integral part of immunophenotyping analysis.

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