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·论著·

Immunophenotypic Characterization of Normal Peripheral Blood B Lymphocyte by Flow Cytometry: Reference for Diagnosis of Chronic B Cell Leukemia/Lymphoma

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Abstract To establish reference values of various immunophenotypic markers in B lymphocyte population in healthy Chinese adults and build background information for accurate interpretation of B cell immunophenotyping data in clinical practice, peripheral blood from 41 healthy adults were collected separately into test tubes containing EDTA-K_2 and stored in room temperature no more than 24 hours before analysis. Whole blood lysis technique and multiparameter flow cytometry were applied to immunophenotype B cells gated on CD19/SSC dot-plot. The results showed that CD22, CD20, CD62L, CD40, CD24, CD79b, CD79a, and FMC-7 were almost positive in the circulating B cell population, whereas CD11a, CD80, CD103, CD10, CD40L, CD54, CD95L, CD86, and CD95 were almost negative in the perapheral blood B lymphocytes. CD18, CD44, CD23, CD5, CD11c and CD43 were positive in different B cell subpopulations. 78% of B cells were IgD positive and ratio κ/λ was 1.26. The significance of all these markers in the differential diagnosis of lymphoproliferative diseases was discussed. The conclusion is that it is necessary to consider the qualitative and quantitative levels of expression of various markers in normal B cell population in order to accurately interpret the pathologcal immunophenotypic data in clinical practice. It is also important to note the immunotypic differences of B cells between Chinese and Western populations.

Key words B lymphocyte; Immunophenotype; lymphproliferative diseases; B cell leukemia; lymphoma

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健康成人外周血B淋巴细胞流式细胞术免疫表型分析:慢性B细胞白血病/淋巴瘤诊断参考值

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摘要 本研究建立中国健康成人外周血B淋巴细胞各种免疫标志的参考范围,为临床正确诊断B淋巴细胞增殖性疾病提供基础信息。采用全血溶RBC法和SSC/CD19设门技术以三色流式细胞术对来自41名健康成人EDTA-K_2抗凝静脉血进行免疫表型分析。结果表明:外周血B淋巴细胞几乎全部表达CD22,CD20,CD62L,CD40,CD24,CD79b,CD79a和FMC-7分子,几乎不表达CD11a,CD80,CD103,CD10,CD40L,CD54,CD95L,CD86和CD95分子,CD18,CD44,CD23,CD5,CD11c和CD43的阳性率各不相同,78%的B细胞为IgD阳性,κ/λ比例为1.26。结论:为了对患者的免疫分型资料进行准确判读和对B淋巴细胞增殖性疾病作出正确的诊断,必须考虑正常B细胞群体中各种标志的表达情况。同时,在临床工作中不宜直接套用国外的标准,而必须注意中国人和西方人群之间在某些B细胞标志方面可能存在的差异。

关键词 B淋巴细胞;免疫表型;淋巴细胞增殖性疾病;B细胞白血病;淋巴瘤

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During the past decade immunophenotyping of blood lymphocytes has become an important tool in the diagnosis of lymphoproliferative disorders. Accurate interpretation of the immunophenotyping data of individuals with these diseases relies on reliable reference values. Reference ranges were published in some earlier studies, which, however, mainly focused on lymphocyte differential populations and T-cell subpopulations.

With a panel of directly conjugated antibodies and a flow cytometry, we studied the presence and expression levels of different surface molecules on healthy adult peripheral blood B lymphocytes, that may be potentially useful for the differential diagnosis

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of chronic B-cell leukemias and non-Hodgkin's lymphomas.

Materials and methods

Subjects

We only studied on adult healthy volunteers, for chronic B-cell lymphoproliferative disorders only affect adult patients. 4 ml EDTA anticoagulated whole blood was collected by venipuncture of 41 blood donors, including 21 males and 20 females, ranging in age from 27 to 69 years. Blood was analysed not later than 24 hours after collection.

Sample preparation

We performed these multiple labelling as stated by Deneys *et al*[1], with minor modification. Three-color flow cytometry immunophenotyping of B lymphocytes was performed using matched combinations of mouse monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin-cyanin 5 (PE-Cy5). The panel of antibodies applied was listed in Table 1. These antibodies were handled according to the manufacturers' instruction. A lysed whole blood technique was adopted as routine practice. 100 μl whole blood was added into each combination of monoclonal antibodies and gently mixed. After incubation for 30 minutes in dark at room temperature, each tube mixture was added with 100 μl red cell lysing solution CAL-LYSE (Caltag) and mixed gently. After 10 minutes of incubation in dark at room temperature, 1 ml distilled water was added to each tube, gently mixed and incubated for 10 minutes in dark at room temperature. The samples were then washed with PBS (pH 7.4), and centrifuged at 300 ×g for 5 minutes at room temperature. The supernatant was aspirated, and the pellet was resuspended in 500 μl PBS for analysis.

Flow cytometry analysis

Data were acquired on a Coulter Epics XL (Beckman-Coulter) flow cytometer. The instrument set up was checked weekly using Flow-Check Fluorospheres (Beckman-Coulter). Forward scatter and side scatter measurements were made using linear amplifiers, whereas fluorescence measurements were made with logarithmic amplifiers. Two flow cytometric parameters, dot plots and quadrant statistics, were generated by System II software (Beckman-Coulter). Analysis was performed after manual gating around a lymphocyte population on a side scatter versus forward scatter dot-plot. A second gate was subsequently put on a CD19 versus side scatter dot-plot. The mean fluorescence intensity (MFI) values related to channels on a linear scale of 0 - 1024 were considered independently.

In order to estimate and control non-specific or background staining of monoclonal antibodies, we used individual matched isotype control in every experiment. When seperation between negative and positive populations was clear-cut, the determination

Table 1 Combinations of monoclonal antibodies used in this study

Marker(FITC/ PE/ PE-Cy5)	Source(FITC/ PE/ PE-Cy5)	Clone(FITC/ PE/ PE-Cy5)
CD11c/ CD86/ CD19	BU15/ IT2.2/ SJ25-C1	Caltag/ BD/ Caltag
CD95/ CD80/ CD19	DX2/ MEM-233/ SJ25-C1	Caltag/ Immunotech/ Caltag
CD5/ CD11a/ CD19	CD5-5D7/ MEM25/ SJ25-C1	Caltag/ Caltag/ Caltag
CD103/ CD25/ CD19	LF61/ CD25-3G10/ SJ25-C1	Caltag/ Caltag/ Caltag
CD23/ CD79b/ CD19	9P25/ CB3-1/ SJ25-C1	Immunotech/ Immunotech/ Caltag
CD22/ CD10/ CD19	RFB4/ 5-1B4/ SJ25-C1	Caltag/ Caltag/ Caltag
FMC-7/ CD38/ CD19	FMC-7/ LS198/ SJ25-C1	Caltag/ Immunotech/ Caltag
CD20/ CD62L/ CD19	B9E9/ DREG-56/ SJ25-C1	Immunotech/ Caltag/ Caltag
CD43/ CD40L/ CD19	1G10/ TRAP1/ SJ25-C1	BD/ Immunotech/ Caltag
CD18/ CD54/ CD19	CLB-LFA-1/ 1/ MEM-111/ SJ25-C1	Caltag/ Caltag/ Caltag
CD44/ CD40/ CD19	MEM85/ mAb89/ SJ25-C1	Caltag/ Immunotech/ Caltag
CD24/ CD95L/ CD19	SN3/ Alf-2.1/ SJ25-C1	Caltag/ Caltag/ Caltag
CD43/ CD79a/ CD19	1G10/ HM47/ SJ25-C1	BD/ Immunotech/ Caltag
Kappa/ IgD/ CD19	G20-193/ IA6-2/ SJ25-C1	BD/ BD/ Caltag
Lambda/ IgD/ CD19	JDC-12/ IA6-2/ SJ25-C1	BD/ BD/ Caltag

All the above are mouse anti-human monoclonal antibodies

of positive events was unambiguously resolved. When the seperation seems to be less clear, a contour plot was used. The mean channels of fluorescence intensity was determinated on the positive population when the seperation between negative and positive cells was clear, and on the whole population in the cases of continuing staining patterns.

Absolute counting

The absolute number values were evaluated by a dual-platform technique. A complete blood count, including an automated differential count was performed with a Beckman-Coulter Gen's hemolyzer. The absolute cell count of each subpopulation was derived from the percentage of each marker in the B-cell gate within the lymphocyte gate, combined with the measure of the absolute lymphocyte count from the hematology analyzer.

Statistics

Statistic analysis was performed with SPSS 10.0 software.

Results

Percentage of each marker in the B cell population

The mean value and interquartile of each marker presented in the B-cell population are showed in Table 2. CD22, CD20, CD62L, CD40, CD24 CD79b, and FMC7 were present on nearly all B lymphocytes. CD44 was positive on majority of circulating B lymphocytes, however, the expression of CD11a and CD18 was almost discrepant, the former was absent on peripheral B cells and the latter present on 13 % B cells. Some markers such as CD38, CD23, CD5, CD11c, CD43 were present on a small part of B population. 78 % of the B cells were IgD positive. There was slightly more κ positive B cells than λ positive cells (κ/λ ratio = 1.26). CD80, CD103, CD25, CD10, CD40L, CD54, CD95L, CD86 and CD95 were essentially absent from the normal B-cell population, because there was less than 10 % of the CD19 positive lymphocytes.

Mean fluorescence intensity of each marker in the B cell population

These results are shown in Table 3. The values must be interpreted with care and direct comparison should be avoided because these data were derived from logarithmic amplifiers of dual dot-plot of each marker. An example of the distribution of the double labelling in the CD19 positive population was shown in the dot plots of Figure.

Absolute counts

As stated previously in methods and materials, absolute count of each subpopulation was derived from the percentage of each marker flow cytometrically combined with the measurement of the absolute count of lymphocytes from hematologieal analyzer. Results of Table 4. can be assessed in parallel with those of Table 2.

Table 2 Phenotypes of B-lymphocyte subpopulation from 41 healthy subjects

Marker in the B cell population	Percentage of each B cell subpopulation
CD5	17.9(10.2 - 25.6)
CD10	0.6(0.1 - 1.1)
CD11a	1.6(0 - 3.2)
CD11c	16.5(6.2 - 26.8)
CD18	12.6(5.5 - 19.7)
CD20	93.4(89.8 - 97.0)
CD22	88.5(83.4 - 93.6)
CD23	35.4(23.5 - 47.3)
CD24	81.9(71.9 - 91.9)
CD25	1.6(0.2 - 3.0)
CD38	29.3(13.1 - 45.5)
CD40	90.6(83.7 - 97.5)
CD40L	0.5(0.1 - 0.9)
CD44	67.1(52.4 - 81.8)
CD54	1.9(0.2 - 3.6)
CD62L	85.8(77.6 - 94.0)
CD79a	89.0(84.4 - 94.4)
CD79b	84.0(74.8 - 93.2)
CD80	0.5(0 - 1.1)
CD86	5.5(1.6 - 9.4)
CD95	5.4(4.1 - 6.7)
CD95L	0.6(0.2 - 1.0)
CD103	2.8(0.6 - 5.0)
FMC-7	78.7(68.8 - 88.6)
IgD	78.2(69.4 - 87.0)
Kappa	54.6(49.7 - 59.5)
Lambda	43.6(39.8 - 47.4)
κ/λ ratio	1.3(1.2 - 1.4)

Data presented as the mean (and interquartile) percentage of each subpopulation in B cell population

Discussion

Chronic lymphoproliferative diseases (LPD) include different entities characterized by distinct clinicopathological features. B lymphoproliferative disorders with frequent peripheral blood involvement include chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HCL)

Table 3 Mean fluorescence intensity of each marker in the B-lymphocyte population

Marker in the B cell population	MFI of each B cell subpopulation
CD5	4.8(3.3 - 6.8)
CD10	24.4(12.2 - 36.6)
CD11a	17.2(13.2 - 21.2)
CD11c	4.2(3.1 - 5.3)
CD18	4.3(3.3 - 5.3)
CD20	39.9(35.2 - 44.6)
CD22	6.5(5.0 - 8.0)
CD23	3.6(2.9 - 4.1)
CD24	31.3(25.0 - 37.6)
CD25	20.7(11.4 - 30.0)
CD38	69.0(34.2 - 103.8)
CD40	12.2(9.2 - 15.2)
CD40L	46.9(18.2 - 75.6)
CD43	10.694.6 - 16.6)
CD44	7.3(5.3 - 9.3)
CD54	31.7(24.6 - 38.8)
CD62L	104.3(92.1 - 116.5)
CD79a	16.1(11.9 - 20.3)
CD79b	56.8(44.7 - 68.9)
CD80	35.6(25.4 - 45.8)
CD86	8.0(5.8 - 10.2)
CD95	4.0(3.2 - 4.8)
CD95L	61.8(39.8 - 83.8)
CD103	5.0(3.4 - 6.6)
FMC - 7	12.3(9.5 - 15.1)
IgD	39.8(34.4 - 45.2)
Kappa	13.0(8.0 - 18.0)
Lambda	13.7(10.4 - 17.0)
κ/λ Ratio	1.0(0.7 - 1.3)

Data presented as mean (interquartile) of mean fluorescence intensity of each marker in peripheral blood B lymphocyte population

Table 4 Absolute count of each B-cell subpopulation

Marker in the B cell population	Absolute count of each B cell subpopulation (c/μl)
CD5	26(12 - 40)
CD10	1(0 - 2)
CD11a	2(0 - 4)
CD11c	24(7 - 41)
CD18	17(8 - 26)
CD20	128(98 - 158)
CD22	122(92 - 152)
CD23	50(28 - 72)
CD24	112(86 - 138)
CD25	2(1 - 3)
CD38	39(17 - 61)
CD40	124(94 - 154)
CD40L	1(0 - 2)
CD44	92(65 - 119)
CD54	3(1 - 5)
CD62L	117(90 - 144)
CD79a	121(115 - 129)
CD79b	116(85 - 147)
CD80	1(0 - 2)
CD86	8(2 - 14)
CD95	7(5 - 9)
CD95L	1(0 - 2)
CD103	4(0 - 8)
FMC-7	108(81 - 135)
IgD	107(81 - 133)
Kappa	75(58 - 92)
Lambda	60(45 - 75)

Data presented as mean (interquartile) of absolute count of each B-cell subpopulation. The count values are derived from the percentage of each B-cell subpopulation in lymphocyte population multiplied by absolute count of lymphocyte/μl

and its variant (HCL-v), spenic lymphoma with villous lymphocytes (SLVL), mantle cell lymphoma (MCL), and follicular cell lymphoma (FL). At least 70 % of cases presenting with lymphocytosis were identified as B-cell lymphoproferative disorders.

The combination of clinical features, cellular morphology and immunophenotyping characteristics permits an accurate identification of abnormal B cell population in most cases of chronic lymphoproliferative disorders. However, the diagnosis remains uncetain in small part of cases. Flow cytometric analysis of cell surface markers appears to be very important tool in the diagnosis of chronic B cell leukemia/lymphoma[2]. Nevertheless, it is essential to refer to normal ranges of B cell subpopulations from healthy persons. It is valuable to establish reference values of normal B cell subpopulations for Chinese people.

In a CD19 positive gated lymphocyte population, we analyzed each monoclonal antibody on peripheral blood collected from 41 healthy volunteers.

As shown in Table 2, CD20, CD22, CD24, CD44, CD62L (L-selectin), CD79a, CD79b, and FMC-7 were considered as pan-B cell marker because these markers were present on most of circulating B cells. For their application in diagnosis of B cell malignancy, it is essential to know their normal level of expression. FMC-7[3], CD22[4] and CD79b[5] are dimly expressed in B-CLL. CD24 could be helpful for the differential diagnosis between SLVL ($CD24^+$) and HCL, esp. HCL-v ($CD24^-$)[6]. CD40 monoclonal antibodies stain B-cell NHL inconsistantly. Combined measurement of CD23 and FMC-7 expression facilitates accurate and reproducible classification of B-cell lymphoma and has diagnosis usefulness[7]. Compared with normal B lymphocytes, CD79b expression frequency and intensity were significantly lower in all types of B LPDs, and can be applied in

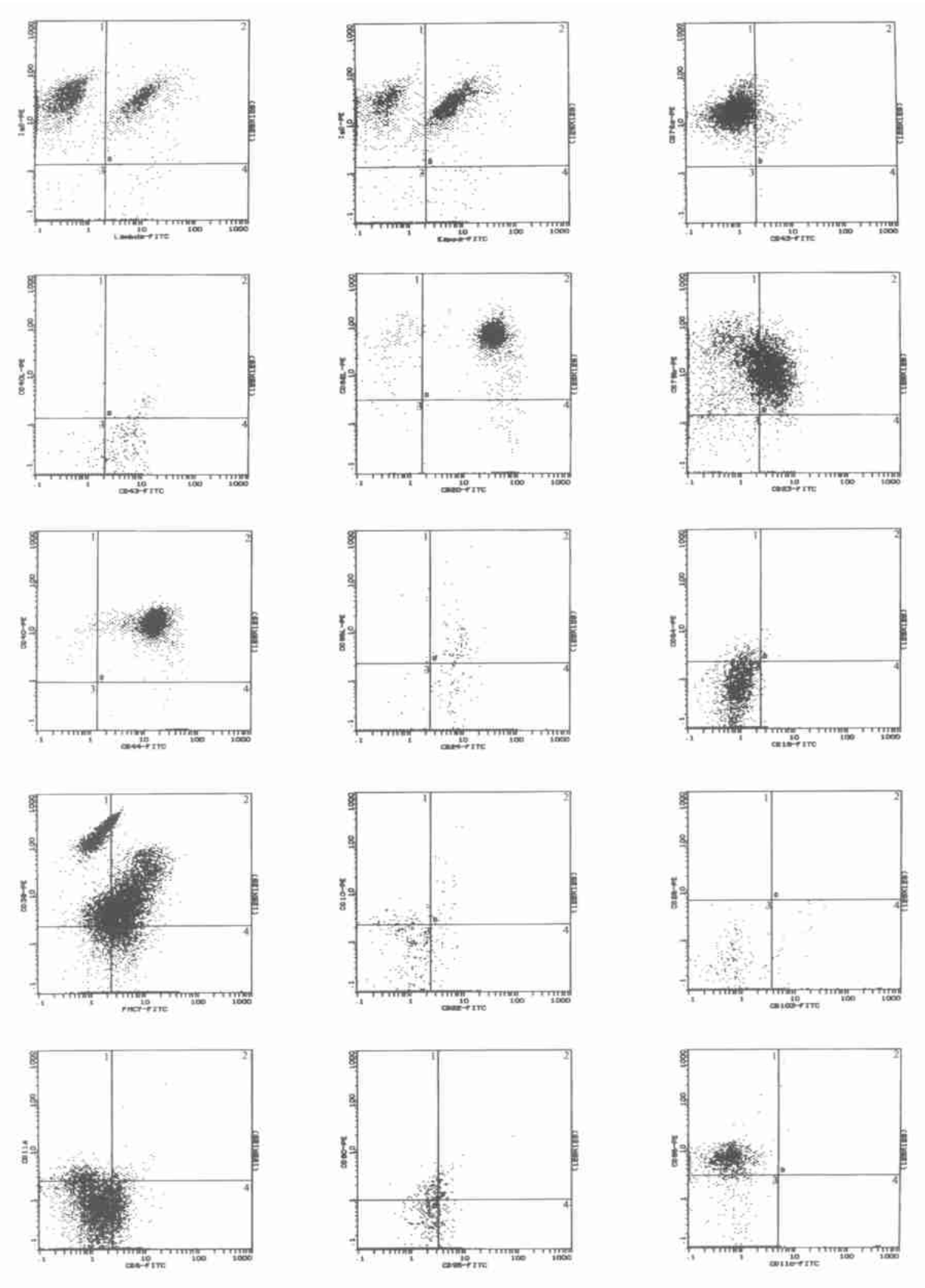

Figure　The distribution of the double labelling in the CD19 positive population

differentiation of CLL from other B-cell disorders[8]. $CD62L^+$ B lymphocytes possibly represent a naive subpopulation. Indeed, in intestinal and mesenteric lymph, naive cells are usually positive for CD62L, whereas memory cells are either positive or negative[9].

In the present study, the expression frequencies of some adhesion molecules eg. CD11a, CD18, and CD44 on normal B lymphocytes are much lower than the results reported by Deneys et al[1]. The discrepancy in results may possibly be attributed to differences in sources of monoclonal antibodies, sample treament, analytic methodology, and instrument alignment and set-up. CD11a has been shown to be present on malignant B cells but was dimly expressed in SLL, FCL and MCL, and very brightly expressed on cells from patients with immunoblastic lymphoma compared to normal B lymphocytes[2]. The intensity of CD44 expression may be helpful for the differential diagnosis between MCL (bright) and SLL (weaker intensity)[10]. CD11c is particularly valuable in the diagnosis of villous lymphocyte proliferations, such as HCL and SLVL[11], and possibly in the differential diagnosis of SLL and MCL[12].

CD5 and CD23 are important parameters for the differential diagnosis of CLL and other types of LPDs. $CD5^+$ B lymphocytes, B1 subpopulation, comprises the majority of neonatal B cells[13]. This proportion decrease to 33 % in adult and even less in elderly subjects[14]. In contrast, CD23 increase with age, being 35 % of the B cells in cord blood[15] and more than 60 % in the adult blood. However, we found CD23 expression only on 35 % of the peripheral blood B lymphocytes. CD38, often considered as an immature or activation marker, is also present at a high level in the plasma cell population, and is present on about 30 % of the peripheral blood B cells. Absence of CD25(IL-2R) on peripheral blood B lymphocytes exclude the possibility that CD38 expression was caused by B cell activation.

CD43 is present on about 16 % of normal peripheral blood B lymphocytes and its presence is independent of CD5 expression even though CD43 and CD5 are frequently coexpressed. CD43 is thought to be involved in cell activation and adhesion[16]. CD43 expression on B cells has been proposed as an immunophenotypic feature suggestive of malignancy[17]. It is present in more than 90 % of MCL, B-cell SLL and CLL, in 20 % to 40 % of nodal and extranodal MZCL and lymphoplasmacytic lymphoma (LPL), and is negative on nearly all splenic MZCL and FL[18]. Accordingly, CD43 is a useful marker for classifying B cell NHL.

CD80 and CD86 are both ligands for CD28 and CD152 (CTLA-4) molecules. CD80 is almost absent on normal B lymphocytes and CD86 expression is very low. CD80 has been found on several B cell malignancies such as CLL, MCL and HCL, usually at an intermediate to high level, whereas CD86 is present at lower levels than CD80[19].

CD95 (APO-1, Fas)-mediated apoptosis plays a major role in normal lymphocyte regulation. CD95 is present on about 5 % of the peripheral blood B cells. Its level of expression differs in different diseases: being low in CLL, but moderate in HCL-v[20]. CD10, CD103, CD95L (FasL), CD54 (ICAM-1) and CD40L (CD154) are undetectable on normal peripheral blood B lymphocytes. CD10, a common acute lymphoblastic leukemia antigen, CALLA, is a major marker of immature B cells. In chronic B-cell lymphoproliferative disorders, its presence is a major phenotypic hallmark of FL[21]. In conjunction with CD11c, CD25 and HC2, CD103 is a very useful marker for the diagnosis and detection of minimal residual disease in HCL[3,22]. In the present study, we couldn't detect CD54 expression on normal B cells. CD54 has been implicated in B cell adhesion to stromal cells[23]. The results obtained with CD11a and CD54 by Jacob et al[2] suggest that in low grade NHL, malignant cells remain to be localized because they are able to adhere to neighbouring cells. Moreever, Horst et al[24] have shown that neoplasmas with a leukemic dissemination pattern are either negative for CD54 or express at low levels. Nevertheless, CD54 is also present on circulating B cells in CLL, but in this cases, the number of positive cells seems to be lower than non-CLL cases[25].

In conlusion, since the morphology of one given disease can sometimes be closely mimicked by a variety of proliferative entities, flow cytometric immunophenotyping has become an important tool in the differential diagnosis of B-cell lymphoproliferative disorders. The aim of this work is to determine some reference values of normal B-cell subpopulation. It is necessary and important to consider the quantitative and quantitative differences of normal B-cell immunophenotypic features between Chinese and Western populations for accurate interpretation of acquired immunophenotyping data.

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