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ORIGINAL ARTICLE

EuroFlow antibody panels for standardized *n*-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes

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Most consensus leukemia & lymphoma antibody panels consist of lists of markers based on expert opinions, but they have not been validated. Here we present the validated EuroFlow 8-color antibody panels for immunophenotyping of hematological malignancies. The single-tube screening panels and multi-tube classification panels fit into the EuroFlow diagnostic algorithm with entries defined by clinical and laboratory parameters. The panels were constructed in 2–7 sequential design–evaluation–redesign rounds, using novel Infinicyt software tools for multivariate data analysis. Two groups of markers are combined in each 8-color tube: (i) backbone markers to identify distinct cell populations in a sample, and (ii) markers for characterization of specific cell populations. In multi-tube panels, the backbone markers were optimally placed at the same fluorochrome position in every tube, to provide identical multidimensional localization of the target cell population(s). The characterization markers were positioned according to the diagnostic utility of the combined markers. Each proposed antibody combination was tested against reference databases of normal and malignant cells from healthy subjects and WHO-based disease entities, respectively. The EuroFlow studies resulted in validated and flexible 8-color antibody panels for multidimensional identification and characterization of normal and aberrant cells, optimally suited for immunophenotypic screening and classification of hematological malignancies.

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INTRODUCTION

For more than two decades, immunophenotyping has been providing relevant information for the diagnosis, classification and monitoring of hematological malignancies.[1,2] Together with cyto/histomorphology and molecular (cyto)genetics, immunophenotyping is crucial for the identification, enumeration and characterization of leukemia and lymphoma cells. Consequently, it has acquired a prominent position in the current World Health Organization (WHO) classification of hematological malignancies.[3] Preferably, the immunophenotypic profiles of suspected cells should be compared with those of normal hematopoietic cells. Immunophenotypic similarities between the suspected cells and their potential normal counterparts allow the assignment of such cells to a given hematopoietic cell lineage and maturational stage, as well as the identification of aberrant phenotypes, such as leukemia-associated immunophenotypes.[4–14] Such immunophenotyping requires careful selection of unique combinations of individual markers based on their degree of specificity for the identification of a given cell lineage, maturation stage and aberrant phenotype, as well as the selection of appropriate antibody clones and fluorochrome conjugates to be used in multicolor combinations; the performance of these marker combinations is even more relevant than that of the individual markers. Consequently, such careful selection of reagents is essential for the design of standardized multicolor antibody combinations that provide unique staining patterns for each normal or aberrant cell population in a given sample.[6,15,16]

Each marker combination should be designed to answer one or multiple relevant clinical questions, through the identification, enumeration and characterization of the relevant cell populations in a sample. As the target cell population may not be known in advance or might have been defined previously, a different strategy is required in each situation. In the former situation, a rapid screening step based on a limited number of antibodies (preferably in a single tube) directed at differential identification of all relevant cell subsets in the sample is generally most

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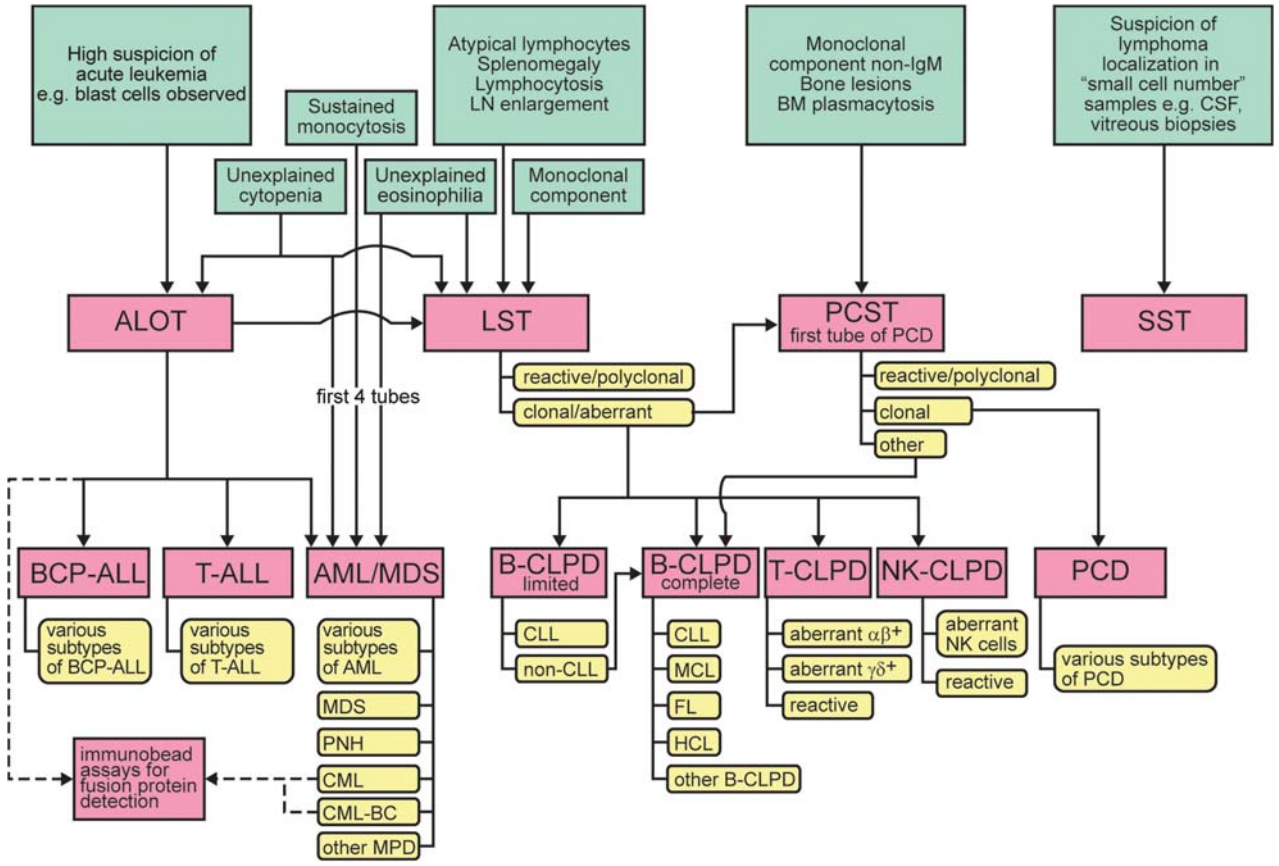

Figure 1. Flowchart diagram of the EuroFlow strategy for immunophenotypic characterization of hematological malignancies. On the basis of several entries of clinical and laboratory parameters, hematological malignancies are screened using a limited screening panel (i.e., typically one single tube) prior to appropriate and comprehensive characterization using extended antibody combinations. Abbreviations: ALOT, acute leukemia orientation tube; AML, acute myeloid leukemia; BC, blast crisis; BCP, B-cell precursor; BM, bone marrow; CLL, chronic lymphocytic leukemia; CLPD, chronic lymphoproliferative disorders; CML, chronic myeloid leukemia; CSF, cerebrospinal fluid; FL, follicular lymphoma; HCL, hairy cell leukemia; LN, lymph node; LST, lymphoid screening tube; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrome; MPD, myeloproliferative disorders; PCD, plasma cell disorders; PCST, plasma cell screening tube; PNH, paroxysmal nocturnal hemoglobinuria; SST, small sample tube.

efficient[7,17–20] (Figure 1). In the latter situation, usage of a full antibody panel is recommended for the characterization and diagnostic classification of the suspected cells, using several (common) backbone markers for reliable and reproducible identification of the target cells in each multicolor combination of the antibody panel[13,14,20–24] (Figure 1). Preferably, the single-tube screening antibody panels and the multi-tube disease-classification antibody panels fit in a diagnostic algorithm with entries defined by clinical and laboratory parameters. Such a diagnostic algorithm is proposed here by the EuroFlow Consortium (Figure 1) as a critical prerequisite in the design of optimal antibody panels for diagnostic immunophenotyping.[25,26]

Multiple consensus panels have been proposed in the last two decades,[12–14,21–23,26–29] but they typically included largely overlapping lists of cluster of differentiation (CD) markers per disease category. Virtually all consensus proposals lack information about reference antibody clones for the proposed CD markers and they only provide limited information on the most appropriate combinations of relevant markers in multicolor antibody panels for immunophenotypic diagnosis and classification of hematological malignancies.[12–14,21–23,26–29] Given the number of CD antigens, the broad range of antibody clones and the variety of fluorochrome-conjugated reagents currently available for each individual CD marker, selection of optimal panels of reagents cannot be based exclusively on experience and expert opinions. In contrast, this requires extensive prospective testing in multicentric studies. The evaluation of the performance of a given antibody conjugate may be based on absolute measures (for example, fluorescence intensity and stain index (SI) obtained for a given control cell population),[30] but these criteria may not apply once the marker is combined with other reagents in a single-tube combination or a multi-tube antibody panel. Therefore, each reagent needs to be evaluated for its unique staining pattern obtained for the distinct cell populations in a multidimensional space defined by all parameters of the newly designed multicolor tube. Consequently, evaluation of the immunophenotypic profiles of leukemia cell populations should preferably be based on a detailed comparison of the phenotypes of individual cells for all markers together, rather than on subjective interpretation of arbitrary mean fluorescence levels (for example: negative versus positive, dim versus strong, homogeneous versus heterogeneous patterns) of a list of single markers. Visualization of such multidimensional spaces and selection of the most relevant parameters for optimal discrimination between the relevant cell populations require new software tools. Such tools were developed by the EuroFlow group and they proved to be essential for the critical evaluation and (re)design of the antibody combinations.[31–33] Among the new tools, reference data files of normal and distinct disease entities were built and the panels evaluated through paired multidimensional statistical comparisons of such normal versus disease-specific data files, as well as of data files corresponding to different well-characterized leukemia/lymphoma entities (Figure 2). In this way we could

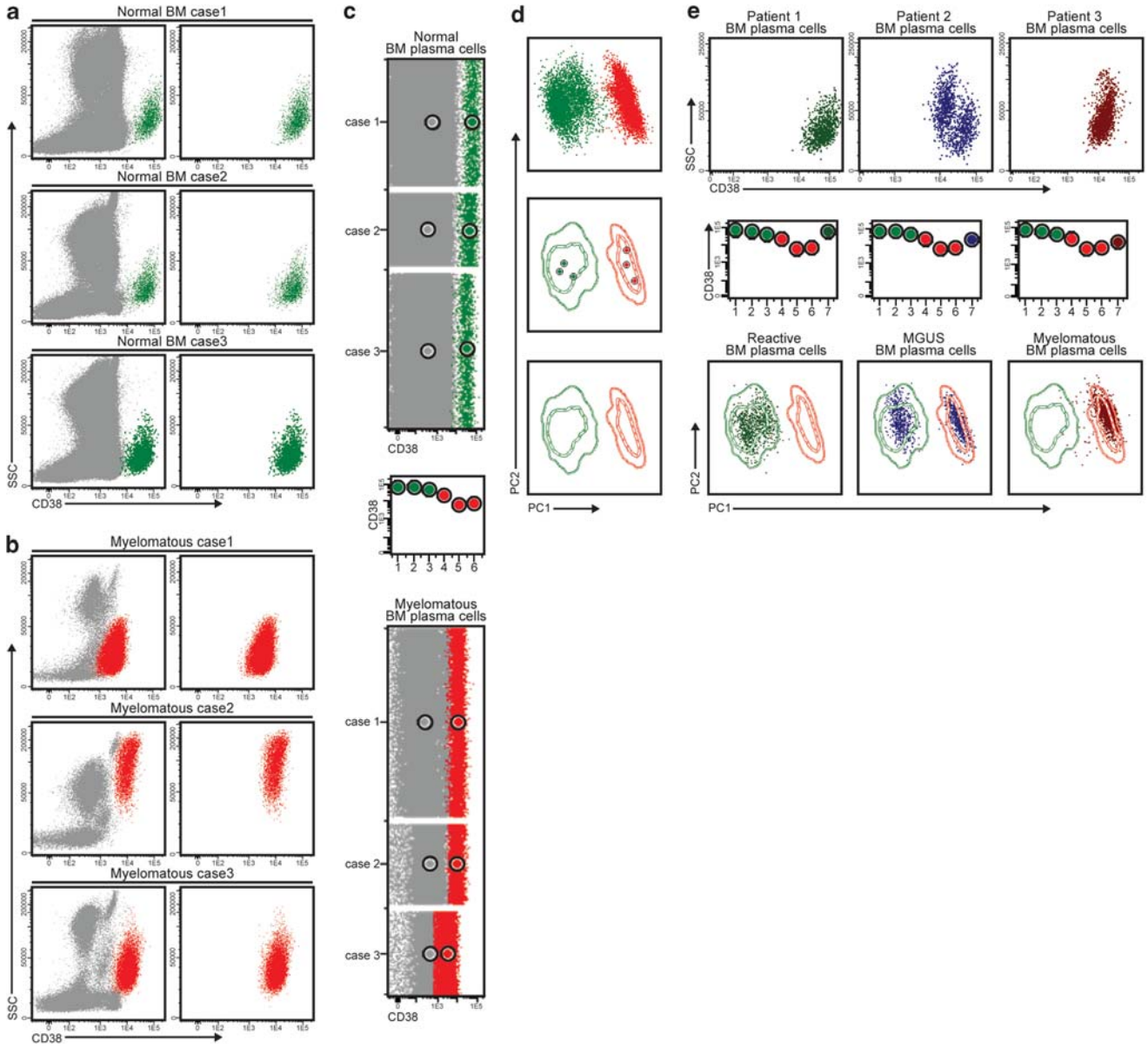

Figure 2. Schematic illustration of how reference data files of normal and leukemia/lymphoma cells were built and used for evaluation of antibody panels and software-guided comparison of individual cell populations from a new interrogated sample. In panels **a** and **b** it is shown how normal (green events in **a**) and tumoral plasma cells (red events in **b**) derived from six different normal ($n = 3$) and myelomatous ($n = 3$) bone marrow samples stained with the plasma cell disorders (PCD) EuroFlow panel (12 different immunophenotypic markers grouped in two 8-color tube combinations) were selected and merged to create a new reference data file (**c**). In (**d** and **e**), it is shown how the PCD panel allows clear discrimination between both types of plasma cells using principal component analysis (**d**) and prospective comparison and classification of plasma cells from new independent bone marrow samples corresponding to a reactive plasmocytosis (green dots in the left column of (**e**) clustered in the normal green plasma cell area in the lower plots), a multiple myeloma (MM) patient (brown dots in the right column of (**e**) clustered in the aberrant plasma cell area in the lower plots) and an MGUS (monoclonal gammopathy of undetermined significance) patient (blue dots in the middle column of panel **e** clustered into two distinct populations localized in the lower plots in the normal and aberrant reference plasma cell areas, respectively). Each individual large and small circle represents median values for single immunophenotypic parameters of the plasma cell populations shown in dot plot diagrams and the median fluorescence expression value for all immunophenotypic parameters measured in the principal component (PC)1 versus PC2 plots for individual samples, respectively; contour lines in these plots represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively).

objectively evaluate the overall performance of the proposed panels for answering the specific clinical questions.

Here we describe the EuroFlow antibody panels developed for comprehensive immunophenotypic diagnosis and classification of hematological malignancies. The proposed panels were initially designed based on the experience and knowledge accumulated in the literature as well as in the individual EuroFlow laboratories. Subsequently, they were optimized in multiple successive evaluation rounds using large numbers of patient samples in the participating laboratories, taking advantage of the reference data files of normal and leukemia and lymphoma samples and the new software tools. The panels are designed in a flexible way to fit the needs of distinct diagnostic laboratories and they can be applied in one or multiple sequential steps. For each

antibody panel, detailed information is provided about the design procedure and about the utility, based on results derived from prospective evaluation of large series of well-defined patient samples.

MATERIALS AND METHODS

Patient and control samples

Multiple types of biological samples were collected from healthy volunteers and patients with suspicion or diagnosis of different types of hematological malignancies and other non-clonal hematological and non-hematological disorders, as specified later in each section of this paper. The collected samples concerned peripheral blood (PB), bone marrow (BM), fine needle aspirates (FNA), biopsies from lymphoid and non-lymphoid tissues, cerebrospinal fluid (CSF) and vitreous samples. For all patients with a hematological malignancy, the diagnosis was established according to the WHO criteria.[3]

Informed consent procedures and forms were proposed and approved at the first EuroFlow meeting (see the Editorial in this *Leukemia* issue). Informed consent was given by donors or their guardians (for example, parents) in case of children according to the guidelines of the local Medical Ethics Committees and in line with the Declaration of Helsinki Protocol. All participants obtained approval or no-objection from the local Medical Ethics Committees for secondary use of remaining diagnostic material for the EuroFlow studies, which also allows the inclusion of anonymized flow cytometric results into a central (public) database to define reference values for normal, reactive, regenerating and malignant cell samples.

Immunophenotypic studies

For immunophenotypic studies, all samples were systematically processed in parallel with the EuroFlow protocol versus the local routine procedures. Accordingly, the EuroFlow standard operating procedures (SOP) for instrument setup, instrument calibration, sample preparation, immunostaining and data acquisition[16] were used at individual centers in parallel to the corresponding local protocols and techniques used for routine diagnosis and classification of hematological malignancies according to the WHO criteria. For data analysis, the Infinicyt software (Cytognos SL, Salamanca, Spain) was used in parallel to the local data analysis software programs and procedures.

For multivariate analysis of samples measured with the EuroFlow SOP and antibody panels, the Infinicyt software was used. For this purpose, the merge and calculation functions were applied for multi-tube panels prior to the analysis, as described elsewhere.[31,32] Briefly, prior to multivariate analyses, the populations of interest were selected and stored each in a distinct data file. Data files corresponding to the same cell population from an individual sample but stained with a different antibody tube of a multi-tube panel were merged into a single data file containing all information measured for that specific cell population. In a second step, 'missing' data in one tube about markers only stained in the other tubes were calculated using previously described algorithms and tools implemented in the Infinicyt software.[32] Consequently, the generated final data file contained data about each parameter measured in the multi-tube panel for each of the events composing the cell population in that data file (Figure 2). This data file was further merged with the data files of other samples either to create a reference pool of a population of normal, reactive or malignant cells or to compare it with one or more of such reference pool data files, through multivariate analysis, for example, principal component analysis (PCA).[31]

SECTION 1. ACUTE LEUKEMIA ORIENTATION TUBE (ALOT)

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BACKGROUND

Acute leukemias comprise a heterogeneous group of malignant diseases characterized by clonal expansion of immature hematopoietic precursor cells. Current international classifications that are used for therapeutic stratification categorize acute leukemias mainly on the basis of the lineage of the blast cells and the type of additional cytogenetic/molecular lesions and, to a lesser extent, detailed immunophenotype.[3] Two major categories of acute leukemias are recognized: (i) lymphoid precursor neoplasms, which are subdivided into B- and T-cell precursor acute lymphoblastic leukemia/lymphoma (BCP-ALL and T-ALL, respectively),[34,35] and (ii) acute myeloid leukemia (AML) and related precursor neoplasms.[3] A small number of cases do not fit into these two major groups because they either show no clear evidence of differentiation along a single lineage or express differentiation antigens highly specific of more than one lineage, making assignment to a single lineage difficult.[36] These cases represent less than 5% of all acute leukemia cases[36–38] and they are categorized separately in the current WHO classification as acute leukemias of ambiguous lineage, including both acute undifferentiated leukemia (AUL) and mixed phenotype acute leukemia (MPAL).[36]

Flow cytometry has an essential role in the diagnosis and classification of acute leukemias.[24,35,39] Together with cytomorphology and cytochemistry, immunophenotyping is crucial for the detection and lineage assignment of blast cells in suspected samples, including the definition of acute leukemias of ambiguous lineage.[37,38,40] Comparison of the immunophenotypic features of blasts cells versus normal hematopoietic precursors and immature cells contributes to the definition of the stage of maturation arrest of the blast population within the B- and T-lymphoid lineages as well as the neutrophilic, monocytic, megakaryocytic or erythroid lineages. In addition, specific immunophenotypic profiles have been associated with prognosis and/or unique cytogenetic and molecular abnormalities.[41–46] Finally, flow cytometric immunophenotyping has also proven to be of great utility for sensitive detection of low levels of residual blast cells and their distinction from normal regenerating immature cells in the BM of acute leukemia patients during treatment.[47]

During the past 5 years, the EuroFlow group has designed and evaluated a set of 8-color antibody panels for the diagnosis and classification of acute leukemias, which can be used in combination with novel software tools in order to optimize flow cytometric *n*-dimensional immunophenotypic characterization of blast cells. As for most EuroFlow protocols, the acute leukemia panels were designed in such a way that they can be applied in two consecutive steps (Figure 1). Depending on the precise clinical question associated with a sample suspected of containing blast cells, the first step includes a single 8-color tube, the ALOT, complemented by a multi-tube panel designed for full characterization of the malignancy. The choice of the second panel depends on the results obtained with the ALOT, that is, the antibody panel for confirmation and classification of BCP-ALL, T-ALL, or the antibody panel for non-lymphoid acute leukemia, the so-called AML/myelodysplastic syndrome (MDS) panel. Rare cases of ambiguous lineage leukemias are identified with the ALOT as requiring the use of more than one complementary panel (for example, the BCP-ALL and AML/MDS panels). This section focuses on the design and evaluation of the ALOT, whereas the subsequent characterization panels are described below in other sections: BCP-ALL (Section 5), T-ALL (Section 6) and AML/MDS (Section 7).

General principle for the design of the ALOT as an 'orientation' tube for blast cells

The ALOT was designed for initial assessment of the nature of immature populations of hematopoietic cells in acute leukemia samples (B- or T-lymphoid versus non-lymphoid lineage or mixed phenotype) in order to allow appropriate orientation towards the complementary BCP-ALL, T-ALL and AML/MDS antibody panels.

More specifically, ALOT was designed for rapid and efficient analysis of a sample, known to contain blasts according to cytomorphology or when the clinical and/or laboratory data are indicative for infiltration of the sample, for example, high white blood cell (WBC) count in PB co-existing with one or multiple cytopenias. However, screening for minimal numbers of blast cells reflecting disseminated disease or exclusion of a hematological malignancy in a systematic way is not possible with the ALOT screening tube alone.

Selection of antibodies

The criteria for antibody selection of the ALOT in order to optimally orientate the acute leukemia sample depend on lineage-associated specificity and sensitivity of the recognized antigens. An ideal orientation marker is constantly expressed by all cells of a single lineage and shows no cross-lineage reactivity. With the potential exception of cytoplasmic (Cy)CD3 in T-ALL patients,[48] if we exclude the exceptional cases of natural killer (NK)-cell acute leukemia,[49] such a marker has not been identified. Because of the inclusion of this antigen (CyCD3), the consequent need for intracytoplasmic staining within the ALOT was assumed to be essential. Very few membrane-bound target antigens are lineage specific; for example, CD7 and CD19 are T- and B-lineage associated markers, respectively, which are expressed in a significant number of AML cases.[50–55] Conversely, myeloid antigens such as CD13 and CD33 can be frequently found in BCP-ALL or T-ALL.[45,56–58] The very few membrane-bound antigens that are *stricto sensu* lineage-specific (for example, antigen receptors in lymphoid lineage) often appear late at the cell surface membrane during physiological ontogeny and, as such, they are expressed in only a minor subset of acute leukemia cases, thus lacking sensitivity. Most lineage-specific markers expressed at early stages of maturation are intracellular markers (for example, CyMPO, CyLysozyme, CyCD3). We therefore opted for a limited selection of intracellular and membrane-bound markers to fit into a single 8-color tube.

Based on previous reports[27] and on our knowledge and experience, all potentially valuable markers were considered. These included, among others, CD7, CD5, CD10, CD13, CD19, CD33, CD117, nuclear (Nu)TdT, HLADR, CyCD3, CyCD79a, CyMPO, CyLysozyme and potentially also mixtures of markers (for example, CD13 + 33). Noteworthily, virtually none of these markers perfectly matched the aforementioned criteria. As lineage commitment has to be assessed by flow cytometry on well-identified immature cells, markers that could contribute to the definition of immaturity and the identification of blast cells were considered independently (for example, CD34, CD45, CD117 and NuTdT). We then designed a list of markers that would be comparatively evaluated following the criteria and rationale described below.

Differentiation of hematopoietic cells comes with a specific pattern of $CD45^{lo}$ intensity correlated with both lineage and maturation stage. Thus, CD45 was a major marker for (i)

Table 1. Design of ALOT in five consecutive rounds with inclusion of backbone markers in common with the acute leukemia panels[a]

Version (no. of cases[b]*)*	*Fluorochromes and markers*							
	PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*AF700*
1 ($n = 35$)	SmCD3	CD45	CyMPO	CyCD79a	CD34	CD19	CD7	CyCD3
2 ($n = 55$)	SmCD3	CD45	CyMPO	CyCD79a	CD34	CD19	CyCD3	CD7
	PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
3 ($n = 102$)	SmCD3	CD45	CyMPO	CyCD79a	CD34	CD19	CD7	CyCD3
4 ($n = 35$)	SmCD3	CD45	CyCD79a	CyMPO	CD34	CD19	CD7	CyCD3
5 (Final)[c] ($n = 158$)	$CyCD3^{T}$	$CD45^{B,T,M}$	CyMPO	CyCD79a	$CD34^{B,M}$	$CD19^{B}$	CD7	$SmCD3^{T}$

Abbreviations: AF700, alexa fluor 700; ALL, acute lymphoblastic leukemia; ALOT, acute leukemia orientation tube; AmCyan, *Anemonia Majano* cyan fluorescent protein; AML, acute myeloblastic leukemia; APC, allophycocyanin; AUL, acute undifferentiated leukemia; BCP, B-cell precursor; Cy, cytoplasmic; Cy5.5, cyanin5.5; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, hilite7; MPAL, mixed phenotype acute leukemia; PacB, pacific blue; PacO, pacific orange; PE, phycoerythrin; PerCP, peridinin–chlorophyll–protein; Sm, surface membrane. [a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 385 acute leukemia cases were evaluated: 190 BCP-ALL, 57 T-ALL, 132 AML, 6 AUL/MPAL. [c]T = backbone markers in common with T-ALL antibody panel; B = backbone markers in common with BCP-ALL antibody panel; M = markers in common with AML/MDS antibody panel. Highlighted boxes: changes as compared to previous version.

Table 2. Utility of ALOT markers

Target antigen	*Maturation markers*	*Lineage markers*		*Shared backbone markers*
		1st level	*2nd level*	
CyCD3		T		T-ALL
CD45	Immature			BCP-ALL/T-ALL/AML-MDS
CyMPO		My		
CyCD79a			B[a]	
CD34	Immature			BCP-ALL/AML-MDS
CD19		B[b]		BCP-ALL
CD7			T[b]	
SmCD3	Mature[c]			T-ALL

Abbreviations: ALL, acute lymphoblastic leukemia; ALOT, acute leukemia orientation tube; AML, acute myeloblastic leukemia; B, B lineage; BCP, B-cell precursor; Cy, cytoplasmic; MDS, myelodysplastic syndrome; My, myeloid lineage; Sm, surface membrane; T, T lineage. [a]Also present in some T-ALL. [b]Also present in some AML. [c]SmCD3 is used as a maturity marker for T-lineage, as identification of suspected immature T-cells relies on detection of $CyCD3^{+/lo}$ $SmCD3^{-}$ $CD34^{+/-}$ blasts.

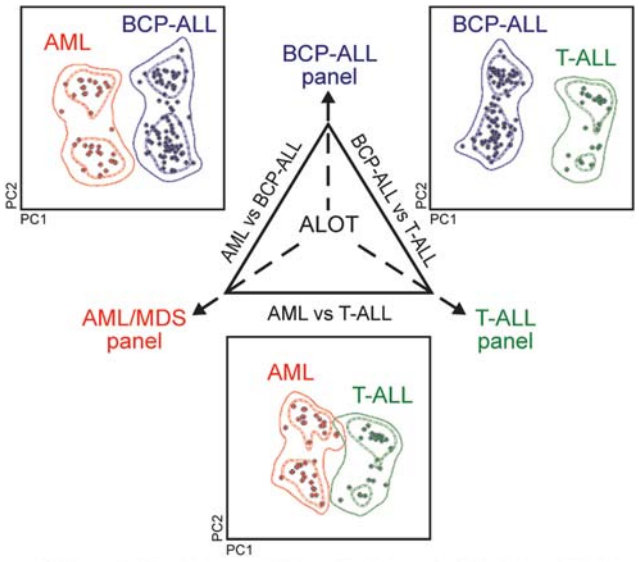
identification of the suspected cell population on the basis of its dim expression and, (ii) exclusion of normal residual cells.[59–61] To refine the blast cell gating and further confirm the immature nature of the pathological population, CD34 was selected, as it is expressed by a significant number of acute leukemias from any lineage. Lineage-associated markers were then added for final blast cell gating and assessment of blast cell lineage. CyMPO was selected as a highly specific myeloid marker. CyCD3 was included as a specific T-lineage marker, which should be interpreted in combination with surface membrane (Sm)CD3 in order to identify a CyCD3^{+}/SmCD3$^{-/lo}$ pattern, the most frequent phenotype of T-ALL. CD19 was selected as a very sensitive B-lineage marker, which is expressed during the early stages of B-cell commitment as well as in virtually all BCP-ALL cases. However, CD19 lacks specificity as it is also expressed in a subset of AML cases, albeit at lower and more heterogeneous levels. Consequently, CyCD79a was added to improve B-lineage assignment, even though it is also expressed in t(8;21)$^{+}$ AML cases[50,51,54,55] and at low levels in a significant number of T-ALL cases, particularly the immature and T-cell receptor (TCR)$\gamma\delta^{+}$ T-ALL subgroups.[62–64] Of note, CyCD79a was preferred to CyCD22 because CD22 is not lineage specific as it is also expressed at high levels in normal basophils, mast cells and some dendritic cells.[65–67] Finally, CD7 was selected because it is positive in virtually all cases of T-ALL and in a subset of, usually CyMPO-negative, AML. The other markers listed above were discarded because they were felt to be not specific enough or they have been found to be redundant with other markers. The choice between NuTdT and CD34 was discussed extensively. For this purpose, 34 cases were analyzed using TdT/CD34 in parallel, including 17 BCP-ALL, 11 AML and 6 T-ALL; the value of each marker was individually appreciated for gating of blast cells for all cases. NuTdT and CD34 were found to be positive and informative in 88 and 82% of BCP-ALL, 83 and 33% of T-ALL, and 18 and 55% of AML cases, respectively. The utility of each marker was overall strictly similar in this series (65% of CD34 or NuTdT positivity over the different disease categories), supporting no evidence in favor of one marker or the other. However, the contribution of each marker did not affect the same cases as the expression of TdT and CD34 was not systematically correlated (15% of cases CD34^{+}/NuTdT^{-}, 15% CD34^{-}/NuTdT^{+}). This highlighted a certain complementarity of CD34 and TdT in this perspective. Owing to constraints of 8-color cytometry, it was chosen to include only one additional immaturity marker. CD34 had the advantage of identifying immature cells of all lineages and was finally selected in order to leave free the fluorescein isothiocyanate (FITC) channel in favor of MPO staining in the ALOT, and to serve as a valuable backbone marker in the BCP-ALL panel, avoiding intracellular staining.

Design of the configuration of the ALOT antibody combination

Once the eight individual markers had been selected, the labeling of antibodies and the specific fluorochrome configuration of the ALOT had to be set. The final configuration of the ALOT (Table 1) was designed to meet two criteria: (i) optimal detection of antigen expression, which may be fluorochrome dependent, and (ii) choice of backbone markers compatible with the different subsequent acute leukemia characterization panels so that information from the ALOT could be integrated with them in a final multiparameter analysis of the whole blast cell phenotype. This meant that the ALOT had to be developed in close synergy with the design of the backbones of the three independent acute leukemia characterization panels (BCP-ALL, T-ALL and AML/MDS panels), and had to be re-tested following each modification proposed for the characterization panels. Consequently, the CD45, CD34 and CD19 markers of the ALOT also serve as backbone markers when information regarding the ALOT is combined (using the merge and calculation functions of the Infinicyt software) with the BCP-ALL panel (see Section 5), whereas the CyCD3, CD45 and SmCD3 markers serve as backbone markers when information regarding the ALOT is combined with the T-ALL panel (see Section 6). CD45 and CD34 are also used as part of the backbone marker set in the AML/MDS panel (see Section 7) (Table 1). The final combination designed with selected target antigens is given in Table 1, whereas the utility of the included markers is summarized in Table 2. Details of the antibody clones are given in the Appendix.

Evaluation of the ALOT antibody combination

The final ALOT antibody combination is the result of multiple evaluation rounds performed to optimize the choice of target antigens, antibody clones, fluorochrome conjugates and combinations of antibody reagents. In total, the ALOT underwent five rounds of evaluation and redesigning with repeated testing on both control and patient samples, including the full spectrum of acute leukemias (Table 1). After analysis of a total of 385 acute



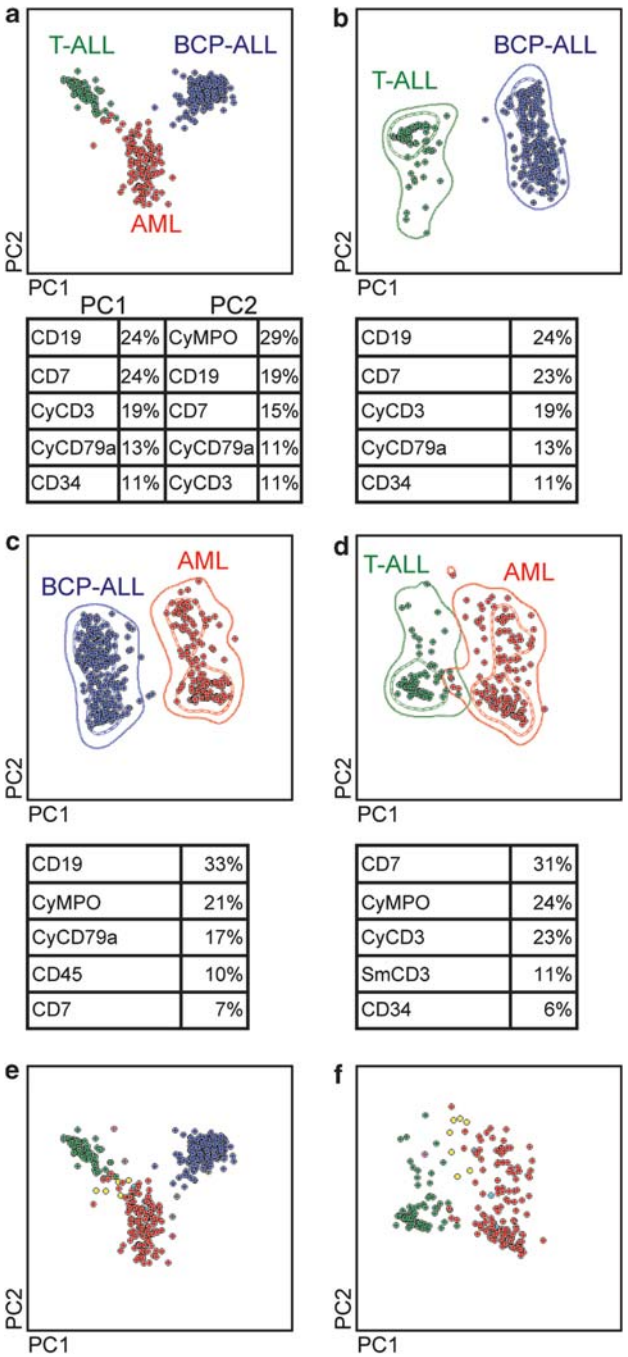
AML vs BCP-ALL	
CD19	32%
CyMPO	27%
CyCD79a	20%
CD45	8%
CyCD3	6%

AML vs T-ALL	
CyMPO	26%
CD7	26%
CyCD3	26%
CD34	15%
SmCD3	6%

BCP-ALL vs T-ALL	
CD19	23%
CyCD3	22%
CD7	22%
CyCD79a	14%
CD34	9%

Figure 3. Results of the preliminary study aimed at classification of 158 acute leukemia samples—89 B-cell precursor (BCP)–acute lymphoblastic leukemia (ALL), 27 T-ALL, 37 acute myeloid leukemia (AML) and 5 acute undifferentiated leukemia (AUL)/mixed phenotype acute leukemia (MPAL)—stained with the final acute leukemia orientation tube (ALOT) combination, using principal component (PC) analysis implemented in the automated population separator (APS) software tool. Comparison of well-defined entities (BCP-ALL, blue circles; T-ALL, green circles; AML, orange circles) shows proper classification based on the expression of the eight antigens, evaluated in the ALOT. Light scatter characteristics were excluded from APS analysis, despite their utility, because standardization had not been achieved at the time those samples were analyzed. Each individual circle represents a single case expressed as median fluorescence expression for all immunophenotypic parameters measured in the PC1 versus PC2 plot, and contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively). The five most informative markers contributing to the best discrimination between each diagnostic entity are displayed at the bottom in a decreasing order of percentage contribution to the discrimination.

leukemia samples, this tube was considered to be in its 'final' configuration. In every evaluation round, variants of the final combination were tested in a multi-centric setting in parallel to in-house procedures. Briefly, initial testing ($n = 35$) included CD45 – *Anemonia Majano* cyan fluorescent protein (AmCyan) and CD3-Alexa Fluor 700 (AF700) fluorochrome-conjugated antibodies (version 1 of the ALOT) (Table 1). A CD7-AF700 antibody was tested ($n = 55$) in order to leave the bright Allophycocyanin (APC) fluorochrome position for the CyCD3 reagent (version 2 of the ALOT), but the obtained results were not satisfactory (Table 1). We then compared the performance of CD45 – Pacific Orange (PacO)/CD3 – APC Hilite7 (APCH7) (version 3 of the ALOT) with that of CD45 – AmCyan/CD3 – AF700 reference conjugates and validated the switch of fluorochromes to these new conjugates ($n = 102$ samples) to get rid of spectral overlap and to improve the brightness of the stainings. Additional evaluations were performed to evaluate alternative clones and antibody labeling, such as the CD79a and MPO FITC and phycoerythrin (PE) conjugates ($n = 35$) (version 4 of the ALOT) (Table 1). Given that CD3 antibodies are conjugated to almost all new fluorochromes, we also checked which fluorochrome was optimal for CyCD3 detection and whether the fluorochrome-conjugated CD3 antibodies were affected by fixation and permeabilization reagents (for example, Fix&Perm, An der Grub, Vienna, Austria). To address this issue, 26 acute leukemia samples (including 3 T-ALL) were analyzed with both SmCD3-APCH7/CyCD3-PacB and CyCD3-APCH7/SmCD3-PacB combinations in parallel. The average SI of residual T-cells measured was 14.9/19.9 with the SmCD3-APCH7/CyCD3-PacB combination, and 8.7/45.6 for the CyCD3-APCH7/SmCD3-PacB combination. In addition, the mean SI of blast cells from T-ALL samples was improved with CyCD3-PacB staining as compared to CyCD3-APCH7 (27.2 versus 9.1 for CyCD3-PacB and CyCD3-APCH7, respectively). We concluded that the results were in favor of a CyCD3-PacB/ SmCD3-APCH7 combination for optimal detection of immature T-cells. This allowed us to fix the final configuration, which was validated on 158 acute leukemia samples (version 5 of the ALOT): CyCD3 – PacB/CD45 – PacO/CyMPO – FITC/CyCD79a – PE/CD34 – peridinin chlorophyll protein complex-Cyanin5.5 (PerCPCy5.5)/CD19 – PECyanin7 (Cy7)/CD7 – APC/SmCD3 – APCH7 (Table 1). Overall, data obtained with the final ALOT version performed similarly to local immunostaining protocols and the stainings were of good quality, with low background signal when fresh and well-preserved samples (within 48 h of sample collection) were analyzed. Deviation from this criterion was notably associated with increasing background for CyCD3 and CD45, and with unspecific SmCD3 staining that could generally be solved by discarding dead cells.



PC1		PC2	
CD19	24%	CyMPO	29%
CD7	24%	CD19	19%
CyCD3	19%	CD7	15%
CyCD79a	13%	CyCD79a	11%
CD34	11%	CyCD3	11%

CD19	24%
CD7	23%
CyCD3	19%
CyCD79a	13%
CD34	11%

CD19	33%
CyMPO	21%
CyCD79a	17%
CD45	10%
CD7	7%

CD7	31%
CyMPO	24%
CyCD3	23%
SmCD3	11%
CD34	6%

Conventional and multivariate analysis of acute leukemia samples

The ALOT configuration was fixed based on conventional analysis of an initial series of 158 acute leukemia samples (89 BCP-ALL,

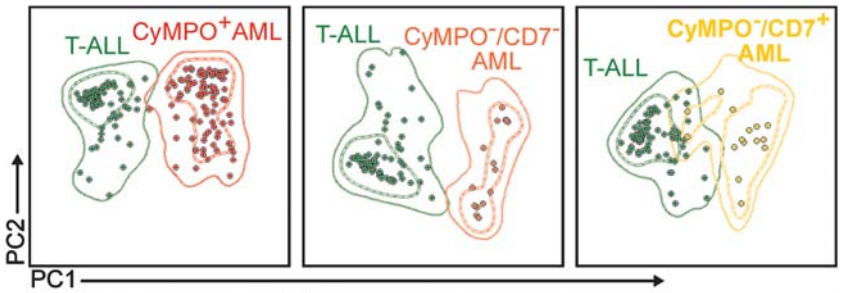
Figure 4. Automated population separator (APS) results of the multicentric evaluation of the acute leukemia orientation tube (ALOT) in its final configuration ($n = 466$ acute leukemia patients). The orientation tube was applied routinely to any fresh acute leukemia sample in all eight EuroFlow laboratories. Results are shown as APS plots of the eight fluorescence parameters with exclusion of light scatter parameters—B-cell precursor (BCP)–acute lymphoblastic leukemia (ALL), blue circles; T-ALL, green circles; acute myeloid leukemia (AML), *orange circles*. (**a**) APS classification of the three well-defined entities; the principal component (PC)1-axis (horizontal) displays B- versus T-discrimination, while the PC2-axis (vertical) highlights lymphoid versus myeloid separation. (**b**–**d**) Pairwise APS analyses of the same well-defined acute leukemia samples. The PC1 axis (horizontal) highlights intergroup differences, while PC2 axis (vertical) displays intragroup heterogeneity. Classification is optimal between BCP-ALL and T-ALL and between BCP-ALL and AML, whereas some overlap is seen between T-ALL and AML, mainly reflecting the intrinsic biological proximity of certain cases ($n = 8/466$; 1.7%) of these diseases. (**e**, **f**) Overlay of unusual acute leukemia samples on the previously defined classification APS plots. Noteworthily, most of these mixed phenotype acute leukemia (MPAL), T + Myeloid (My), My + B and T + B cases map in between the two groups they belong to phenotypically, while acute undifferentiated leukemia (AUL) cases fall together with the non-lymphoid AML cluster. MPAL T + My, yellow; MPAL B + My, grey; MPAL B + T, brown; AUL, blue. Each individual circle represents a single case expressed as median fluorescence expression for all immunophenotypic parameters measured in the PC1 versus PC2 plot, and contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively). The five most informative markers contributing to the best discrimination between each diagnostic group are displayed at the bottom of the corresponding APS plot, in a decreasing order of percentage contribution to the discrimination.

27 T-ALL, 37 AML and 5 AUL/MPAL). Testing of these 158 cases demonstrated a good correlation between the information provided by the ALOT and both in-house procedures and the final WHO diagnosis of the disease. Further confirmation of the ALOT efficiency was obtained when processing the data using PCA (Infinicyt software). For this purpose, each ALOT data file was computed to specifically extract the information relative to the blast cell population. All blast populations derived from the different patients were merged into a single data file and plotted together on an automated population separator (APS) view for automated separation by PCA.[16] Pairwise analysis of well-defined acute leukemia subgroups (BCP-ALL versus T-ALL versus AML) demonstrated excellent discrimination of each disease category, thus validating the approach (Figure 3). The dispersion observed within each reference group resulted mainly from the wide spectrum of CD34 expression in all acute leukemia subgroups.

The approved ALOT antibody combination was then prospectively applied to an independent cohort of 483 freshly collected acute leukemia cell samples (see Supplementary Table 1), mainly PB ($n=89$) and BM ($n=387$), but also some pleural effusions ($n=5$) and other body fluids ($n=2$) obtained from well-characterized patients at diagnosis: 259 BCP-ALL, 131 AML, 76 T-ALL and 17 AUL/MPAL patients. An additional set of 41 samples were analyzed, consisting of other hematological malignancies ($n=21$), such as diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma (BL), chronic mature B-cell lymphoproliferative disorders (B-CLPD) and MDS as well as reactive BM samples ($n=20$). Each ALOT data file contained information on 6000 blast cells per patient and was analyzed with the Infinicyt software, through direct PCA comparison with each pair of acute leukemia groups (BCP versus T-ALL, T-ALL versus AML and BCP-ALL versus AML), as described above.

Analysis of the 466 well-characterized BCP-ALL, T-ALL and AML cases, excluding AUL/MPAL samples ($n=17$), highlighted excellent discrimination of BCP-ALL from both AML and T-ALL (Figure 4). Conversely, T-ALL and AML groups showed a slight overlap. Of note, overlapping cases (8 out of 466; 1.7%) mostly corresponded to immature T-ALL that fit the criteria for the recently described Early T-cell Progenitor T-ALL[68–71] with unusually dim CyCD3 expression and immature CD34$^+$/CyMPO$^-$/CD7$^+$ AML (Figure 5). This overlap may be the consequence of both the biological nature of immature blast cells in some malignancies arrested at a stage of early T/Myeloid (My) development, and the high phenotypic heterogeneity of non-lymphoid acute leukemia cases. Consequently, the few cases falling into this area should optimally benefit from further evaluation with both the T-ALL and AML antibody panels for complete characterization. Acute leukemia of ambiguous lineage (17 out of 483; 3.5%) represented a heterogeneous category, which comprised AUL ($n=4$; 0.8%), MPAL with populations from two distinct lineages ($n=2$; 0.4%) and MPAL with mixed lineage cell populations, either T + My or B + My ($n=11$; 2.3%) (Figure 4). When analyzed in the multidimensional PCA view with supervision based on well-defined entities, MPAL from T + My lineages and also B + T cases clustered as expected in between T-ALL and AML and in between BCP and T-ALL clusters (Figure 4). By contrast, MPAL from B + My lineages appeared more heterogeneous, as two out of five patients clustered in between AML and BCP-ALL, whereas two other clustered together with BCP-ALLs, and one was close to T-ALL, owing to strong expression of CD7. Noteworthily, three out of four AUL cases fall together with non-lymphoid acute leukemia reference cases (Figure 4). These preliminary data suggest that multicenter collection of a large number of ambiguous lineage cases combined with multivariate comparison of harmonized results will be useful to significantly increase the number of these rare cases in order to delineate relevant individual clusters within the AUL/MPAL category that will pinpoint cases that require specific characterization. Until such an extensive database becomes available, cases with suggestive phenotypic criteria of belonging to AUL/MPAL groups according to the WHO classification should be analyzed with appropriate combinations of characterization panels (for example, T-ALL and AML/MDS antibody panels).

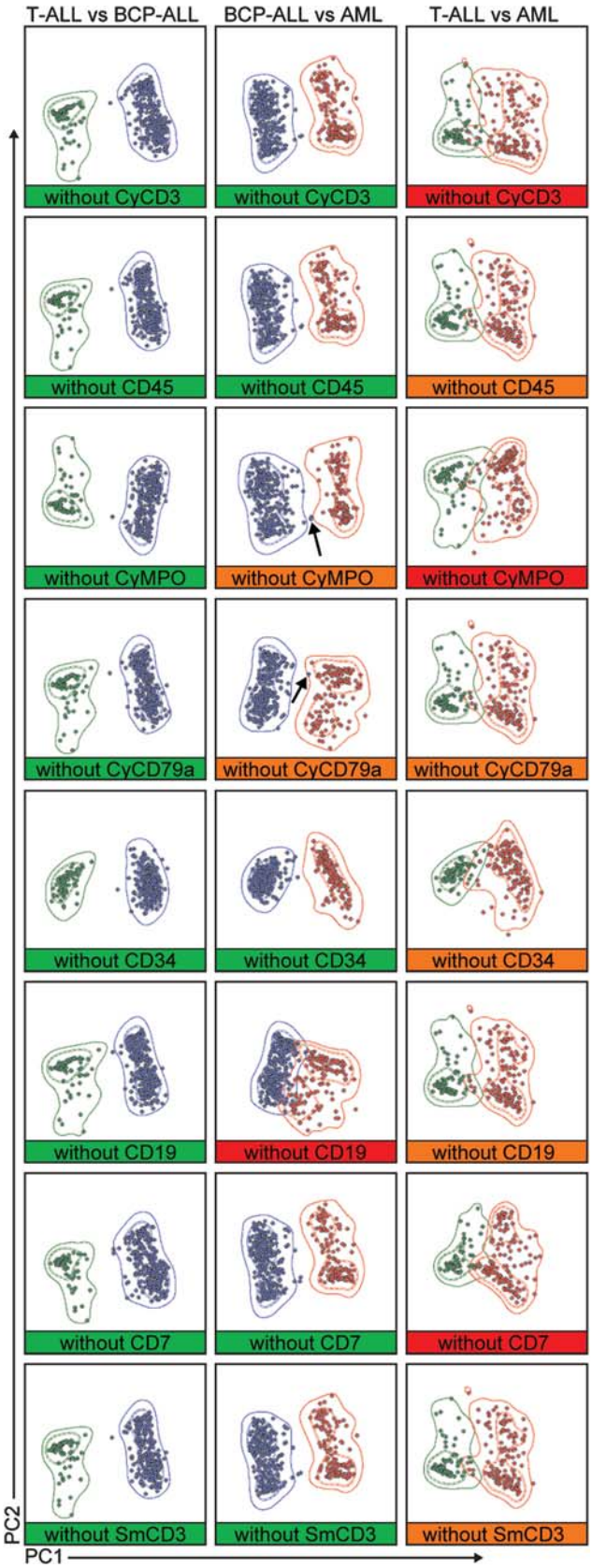
In order to further appreciate the value of each individual marker included in the ALOT the analyses were repeated leaving out each marker one by one. Results showed that most of the seven-antibody combinations led to decreased discrimination of the well-defined entities (Figure 6). For instance, exclusion of CD19 significantly impaired separation of BCP-ALL and AML



CD7	30%	CD7	35%	CD34	34%
CyMPO	26%	CyCD3	26%	CyCD3	27%
CyCD3	22%	CD34	20%	CD7	19%
CD34	10%	CyMPO	8%	SmCD3	8%
SmCD3	6%	SmCD3	7%	CyMPO	7%

Figure 5. Pairwise analysis of the T-acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) cases after AML subsetting based on CD7 and CyMPO expression. AML are known to harbor major phenotypic heterogeneity. Pairwise analysis of AML phenotypic subsets demonstrates that the overlap is mainly observed with CyMPO$^-$ CD7$^+$ AML cases and immature forms of T-ALL (T-ALL, green; CyMPO$^+$ AML, red; CyMPO$^-$/CD7$^-$ AML, orange; CyMPO$^-$/CD7$^+$ AML, yellow). Each individual circle represents a single case expressed as median fluorescence expression for all immunophenotypic parameters measured in the principal component (PC)1 versus PC2 plot, and contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively). The five most informative markers contributing to the best discrimination between each entity group are displayed at the bottom of the corresponding automated population separator (APS) plot, in a decreasing order of percentage contribution to the discrimination.

entities, as expected, while not much affecting discrimination between the T-ALL and BCP-ALL groups. Comparable results were obtained for all markers left out, except for CD45 and SmCD3, whose exclusion had less impact on the quality of the separation of all the well-characterized acute leukemias. These markers contributed less to disease category discrimination but were not excluded from the combination and replaced by alternative markers because they were considered as essential for blast cell identification and gating, as well as for exclusion of residual non-malignant cells.



Finally, the non-acute leukemia samples ($n = 41$) were directed with the ALOT towards the expected panels for further investigation. For example, mature B cell disorders were assigned to a BCP-ALL panel, which allowed exclusion of BCP-ALL and orientation towards the appropriate B-CLPD panel. It should be noted that the ALOT alone using conventional or multivariate analyses did not allow direct re-orientation towards the mature B-CLPD panel as the $CD45^+/CyCD79a^+/CD19^+/CD34^-$ phenotype may correspond to both $CD34^-$ BCP-ALL or a mature B-cell malignancy, regardless of the level of CD45 expression.[72,73] The BCP-ALL panel, however, contains sufficient markers for re-orientation towards the mature B-CLPD panel, such as surface immunoglobulin (Ig) light and heavy chains as well as numerous maturation markers. As expected, non-AML myeloid neoplasias (for example, MDS) clustered with the AML group and were consequently oriented to be further analyzed with the AML/MDS panel.

CONCLUSION

Here we propose an extensively tested single tube with an 8-color combination of antibodies for fast and efficient orientation of acute leukemias towards a full BCP-ALL, T-ALL and/or AML characterization panel. An unprecedented orientation efficiency of 98.3% for non-ambiguous lineage cases was shown for the ALOT combination with a series of 483 newly diagnosed acute leukemia cases, tested prospectively at different centers. In addition, the combination of ALOT and the PCA-based data analysis facilitates standardized orientation to subsequent BCP-ALL, T-ALL and/or AML panel testing. However, the classification efficiency using APS remains to be assessed with an independent validation cohort. Interestingly, in a small fraction of cases an early T/My precursor acute leukemia phenotype is likely to be identified using multivariate analysis, which requires evaluation by more than one of the three acute leukemia multitube antibody panels. Phenotypic clustering of unusual MPAL/AUL cases will require collection of a vast number of acute leukemia cases in order to achieve relevant characterization. Altogether, the ALOT tube with an integrated analysis of only eight markers appeared to be an unprecedentedly strong tool for acute leukemia diagnosis.

Figure 6. Utility of each individual marker of the acute leukemia orientation tube (ALOT) for acute leukemia classification. Classification results using all possible seven-parameter combinations show the importance of the contribution of the eighth marker for disease category discrimination. The quality of separation between classical entities is demonstrated using a traffic-light code: optimal separation (>2s.d.), green; minimal overlap (1–2s.d.), orange; major overlap (<1s.d.), red. Automated population separator (APS) plots illustrate the corresponding pairwise comparisons (B cell precursor (BCP)–acute lymphoblastic leukemia (ALL) cases are plotted as blue circles; T-ALL as green circles; and acute myeloid leukemia (AML) as orange circles). Each individual circle represents a single case expressed as median fluorescence expression for all immunophenotypic parameters measured in the principal component (PC)1 versus PC2 plot, and contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively).

SECTION 2. LYMPHOID SCREENING TUBE (LST)

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BACKGROUND

Detection of phenotypically aberrant and clonal mature lymphocytes is the diagnostic hallmark of CLPD. Clonogenic events lead to the expansion and accumulation of mature-appearing lymphocytes, which carry a proliferative and/or survival advantage over their normal counterparts.[3,74,75] This translates into progressive accumulation of clonal cells and their products causing PB lymphocytosis, BM lymphoid infiltrates, enlargement of one or multiple other tissues (for example, lymphadenopathy, splenomegaly or other organomegalies), or emergence of a serum monoclonal component. Clinical manifestations and laboratory findings related to the increased number of lymphocytes initiate the diagnostic process (Figure 1).[18,25] In addition, functional impairment of tissues involved may also lead to other diagnostic information such as cytopenias, unexplained neurological symptoms or serous effusions. Such findings demand for assessment of the potentially clonal or neoplastic nature of the mature lymphoid cells in the various types of samples.[25,76] Identification of the abnormal lymphocytes and their discrimination from normal and reactive cells are key steps in establishing a final diagnosis. For decades now, flow cytometric immunophenotyping is an essential tool for the diagnostic screening of CLPD and for the specific identification and characterization of the expanded aberrant lymphocytes.[6] Over the years, refined approaches have been developed and technical improvements implemented, which have progressively increased the efficiency of the diagnostic screening of CLPD in clinical and laboratory settings.[17,20]

Identification of aberrant lymphocytes currently not only relies on their absolute or relative numerical distribution among all lymphocytes (or their subpopulations) present in the sample, but also mainly searches for more specific 'abnormal' immunophenotypic profiles. These aberrant phenotypes can clearly be distinguished from normal and reactive patterns in the multidimensional space generated by unique combinations of fluorochrome-conjugated antibodies.[17,18,77–81]

At present, different screening protocols, antibody panels and immunophenotypic strategies are used in individual laboratories for the diagnostic screening of CLPD. Overall, it is well established that in a diagnostic screening step the informative markers have to be combined whenever possible in a single antibody combination that can easily and rapidly be evaluated. The EuroFlow group has designed and evaluated an 8-color, 12-marker combination of antibodies aiming at the detection of phenotypically aberrant populations of mature B-, T- and NK-cells in PB, BM, lymph nodes (LN) and other types of body tissues and fluids, which can be used in the diagnostic screening of CLPD. The use of multiple markers conjugated with the same fluorochrome has previously been shown to be highly efficient in this regard.[17] The antibody combination in this so-called lymphoid screening tube (LST) can guide the need for further immunophenotyping with appropriate antibody panel(s) for accurate diagnosis and classification of lymphoid malignancies, through the EuroFlow panels for B-CLPD (Section 8), T-CLPD (Section 9), and NK-CLPD (Section 10).

Selection of antibodies for the LST

The selection of antibodies for the LST aimed at dissecting lymphoid cells into their major subsets with a set of reagents that can simultaneously define aberrant and clonal phenotypes. Initially CD45 was selected for the definition of the compartments of mature versus immature lymphocytes, CD3 for the identification of T-cells, and both CD19 and CD20 for the selection of B-cells; these two later markers combined with CD45 would allow subsetting of B-cells into mature B-lymphocytes ($CD19^+$, $CD20^{hi}$ and $CD45^{hi}$) and B-cell precursors ($CD19^+$, $CD20^{-/lo}$, $CD45^{lo}$). NK-cells should fulfill the criteria for mature lymphocytes ($CD45^{hi}$, SSC^{lo}) in the absence of CD19 and SmCD3 expression and they would typically show reactivity for CD56. Additional markers selected for further subsetting of B, T and NK-cells included (i) SmIgκ and SmIgλ, (ii) CD4, CD8 and CD56 and (iii) CD56 and CD8, respectively. These antibody reagents were arranged into a first version of an 8-color LST (Table 3). Additional markers were incorporated later in the LST, as described below.

Design and construction of the LST

The proposed LST resulted from a seven-round process of design–evaluation–redesign of successive LST versions (Table 3). Each LST version was evaluated in a large but variable number of normal, reactive and neoplastic patient samples obtained at diagnosis, as well as at other disease time-points (for example follow-up or relapse) in parallel to the routine approaches applied for the same purpose in each participating center (6 EuroFlow centers).

Version 1 of the LST (Table 3) proved to be a robust combination that allowed identification of the major populations of normal B-, T- and NK lymphocytes and their dissection into up to 13 normal lymphoid subsets: (1) $SmIg\kappa^+$ and $SmIg\lambda^+$ mature B-cells, plus B-cell precursors; (2) $CD4^+$, $CD8^{hi}$, $CD4^+/CD8^{lo}$ and $CD4^-/CD8^{-/lo}$ T-lymphocytes (further divided according to CD56 expression into (i) $CD4^+/CD56^-$ and $CD4^+/CD56^+$; (ii) $CD8^{hi}/CD56^-$ and $CD8^{hi}/CD56^+$; (iii) $CD4^+/CD8^{lo}/CD56^-$ and $CD4^+/CD8^{lo}/CD56^+$; and (iv) $CD4^-/CD8^{-/lo}/CD56^-$ and $CD4^-/CD8^{-/lo}/CD56^+$ T-cells, respectively) and both $CD56^+/CD8^{-/lo}$ and $CD56^{hi}/CD8^{-/lo}$ NK-cells.

Preliminary evaluation of version 1 of the LST showed comparable results with the local approaches for the identification of the aberrant or clonal populations of mature lymphocytes ($n = 9/9$). However, this LST version also showed some unwanted features. First, some antibody conjugates performed below the expectations, for example, CD19-AmCyan and CD3-APC Cyanin7 (Cy7) showed suboptimal performance (low resolution) for positive identification of the B- and T-cell populations (data not shown). Second, the relatively high degree of overlap observed between the PE and PerCPCy5.5 fluorochrome conjugates pointed out the need for a more careful selection of the markers included at these two fluorochrome positions. Finally, inclusion of CD38 was proposed to provide complementary information about the normal versus abnormal populations of lymphocytes, to increase the accuracy in discriminating B-cell precursors, particularly in BM samples, and to evaluate additional subsets of B-cells (for example, plasma cells).

The reagents selected to solve these issues were included in version 2 of the LST, evaluated in 29 samples (Table 3). New CD19 and CD20 conjugates were selected and subsequently incorporated in the LST owing to poor resolution of CD19 – PerCPCy5.5 and CD20 – APC, which were replaced in version 3 by CD19 – PECy7 and CD20 – PacB. In version 4, CD38 – AF700 was replaced by a custom-made CD38 – APCH7 conjugate (Table 3). These fluorochrome replacements resulted in a better discrimination between the different populations of normal lymphoid cells. With this version only CD45 – AmCyan showed suboptimal performance owing to relatively high fluorescence spillover into the FITC channel. Additionally, inclusion of an anti-TCRγδ antibody was proposed for version 5 of the LST (Table 3). This 'matured' LST

Table 3. Design of LST in seven consecutive rounds[a]

Version (no. of cases)[b]	*Fluorochromes and markers*							
	PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCCy7*
1 ($n=9$)	CD45	CD19	SmIgλ	SmIgκ	CD8	CD56	CD4 and CD20	SmCD3
	PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*AF700*
2 ($n=29$)	SmCD3	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD19	CD4	CD20	CD38
3 ($n=97$)	CD20	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD4	CD19	SmCD3	CD38
	PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
4 ($n=26$)	CD20	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD4	CD19	SmCD3	CD38
5 ($n=19$)	CD20	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD4	CD19 and TCRγδ	SmCD3	CD38
	PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
6[c,d] ($n=12$)	CD4 and CD20	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD5	CD19 and TCRγδ	CD10	SmCD3
7 (Final) ($n=271$)	CD4 and CD20	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD5	CD19 and TCRγδ	SmCD3	CD38

Abbreviations: AF700, alexa fluor 700; AmCyan, *Anemonia Majano* cyan fluorescent protein; APC, allophycocyanin; BM, bone marrow; Cy7, cyanin7; FITC, fluorescein isothiocyanate; FNA, fine needle aspirate; H7, hilite7; LNB, lymph node biopsy; PacB, pacific blue; PacO, pacific orange; PB, peripheral blood; PB-MNC, PB mononuclear cells; PE, phycoerythrin; PerCPCy5.5, peridinin–chlorophyll–protein–cyanin5.5; Sm, surface membrane. [a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 463 samples (292 PB, 13 PB-MNC, 127 BM, 20 LNB, 8 FNA, 3 other types of samples) was evaluated. Among them, 384 abnormal lymphoid populations were detected: 266 B (1 showed an immature B-cell phenotype), 95 T, 23 NK. No abnormal lymphoid population could be detected in 90 cases (healthy donors and non-infiltrated samples). [c]Samples included for evaluation of this version were stained in parallel with version 5. [d]One center tested this combination in an additional group of 1285 cases. Results were always concordant with local routine diagnostics. Single cases might include more than one abnormal population. One sample showed abnormal lymphoplasmocytoid cells and four cases displayed abnormal plasma cell populations. Highlighted boxes: changes as compared to previous version.

combination (version 5; Table 3) was further fine-tuned by replacing CD45 − AmCyan by CD45 − PacO and including CD5 as a consequence of the direct comparison performed between versions 5 and 6 of the LST. As a result, a final configuration (Table 3) was proposed with two options where either CD10 (version 6) or CD38 (version 7) were alternatively used. Addition of CD10 was proposed for achieving a more B-cell-oriented approach in laboratories in which B-CLPD represent the major fraction of the screened samples.

In summary, major modifications introduced after the evaluation of each version of the LST (versions 1–7) aimed at (i) inclusion of markers that could improve the characterization of the lymphoid cell populations (for example, CD38), (ii) increasing the number of lymphocyte populations and subsets that could be identified (for example, positive discrimination of plasma cells with CD38 or discrimination of TCRγδ$^+$ from other CD8$^{-/lo}$ T-cells with the anti-TCRγδ antibody) and/or (iii) providing a more sensitive and/or robust orientation on subsequent analyses, when lymphocyte populations with aberrant or clonal phenotypes had been detected (for example, CD5 and CD38 in B- and/or T-cell CLPD). Further description of the utility of each marker included in the final version is shown in Table 4. In addition, redistribution of informative markers into other fluorescence channels and substitution of specific fluorochromes showed improved performance.[16] These fluorochrome changes also aimed at keeping compatibility with other EuroFlow panels designed in parallel, for example, markers and conjugates used as backbone markers for the B-CLPD panel were kept in the LST at the same fluorochrome positions (see Section 8).

As already mentioned above, in some fluorescence channels two different markers were placed to overcome the limited number of fluorescence detectors with respect to the number of antibody reagents required. Of note, each pair of markers placed in the same fluorescence channel was arranged in such a way that their expression is typically restricted to different (normal or abnormal) cell populations that could be positively identified and selected using other markers present in the LST. Based on this strategy, any potential misinterpretation of their differential reactivity in normal and neoplastic lymphoid cells can be avoided, if all major and minor phenotypes of normal lymphocytes are identified (for example, CD20^{lo} cytotoxic T-cells). [82]

Evaluation of the LST, version 6

Version 6 of the LST was designed to address the potential need for a more B-cell-oriented approach in some laboratories by including slight modifications with respect to version 5. These modifications aimed at increasing the sensitivity of the combination using relevant markers for B-cell subsetting in combination with SmIgκ and SmIgλ. Inclusion of CD5 and CD10 in version 6 (versus version 5) was considered appropriate because of the kinetics of expression of the two markers during normal maturation and their association with specific B-CLPD entities;[74] CD38 was taken out to leave room for these markers. Version 6 of the LST was extensively evaluated for the screening of abnormal lymphoid cells at one center in a total of 1285 samples of which 504 harbored one or two aberrant or clonal B-cell populations. Of the 504 infiltrated samples, 19 (5%) demonstrated a double clonal B-cell population, one of which was CD10$^+$ (Figure 7), and 5 cases (1%) had a minor CD10$^+$ clonal B-cell population (SmIg light-chain-restricted expression). Without CD10, 5 of these 24 cases would not have been detected at the initial screening step (5/1285; 0.4%). Version 6 of the LST was also evaluated against version 5 by the USAL group in 12 additional samples (7 PB and 5 BM). In this evaluation, both LST versions showed infiltration in 4/12 samples (2 abnormal B- and 2 abnormal T-cell populations). Overall, it was concluded that the combined usage of CD5 and CD10 in version 6 might result in a higher sensitivity for detection of small CD10$^+$ B-cell clones, especially when minor clones composed of a mixture of phenotypically (CD5$^+$ and/or CD10$^+$)

Table 4. Utility of LST markers for identification of lymphoid cells in patients with CLPD

Marker	*Main normal population(s) identified*[a]	*Positive diagnosis*	*Population subsetting*	*Diagnostic subclassification*	*Potential minimal disease value*	*Prognostic relevance*
CD45	Mature lymphocytes and B-cell precursors	X	X			
CD19	B-cells, T- and NK-cells by exclusion	X		X	X	
CD20	B-cells, T- and NK-cells by exclusion	X	X	X	X	
SmIgλ and κ	SmIg^{+} B-cells	X	X		X	
CD38	Plasma cells and B-cell precursors	X	X	X	X	X
SmCD3	T-cells, B- and NK-cells by exclusion	X		X	X	
CD4	CD4^{+} T-cells	X	X	X	X	
CD8	CD8^{hi} T-cells and CD8^{lo} NK-cells	X	X	X	X	
CD56	NK-cells	X	X		X	
TCRγδ	TCRγδ^{+} T-cells	X	X		X	
CD5	T-cells	X		X	X	

Abbreviations: CLPD, chronic lymphoproliferative disorders; LST, lymphoid screening tube; Sm, surface membrane. [a]Some markers may be aberrantly expressed in other abnormal lymphoid populations.

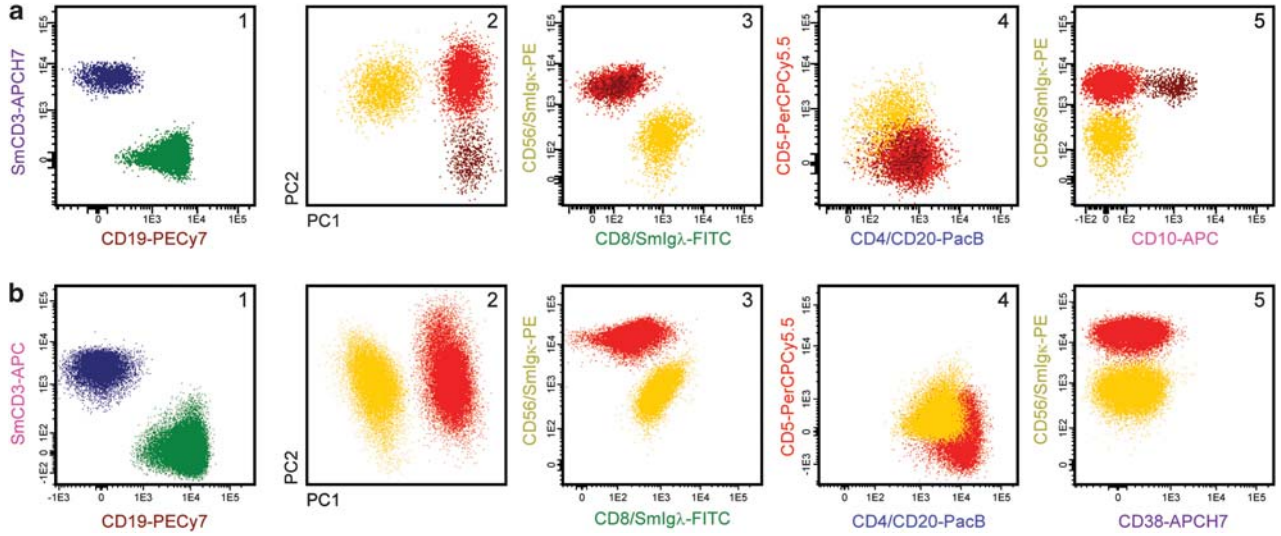

Figure 7. Evaluation of the performance of versions 6 (**a**) and 7 (**b**) of the lymphoid screening tube (LST). Illustrating example of a peripheral blood sample stained with LST version 6 and LST version 7. (**a.1**, **b.1**) Show the presence of a numerically increased B-cell population (46.7% and 50.1%, respectively) (green dots). Principal component analysis (PCA) of the B-cells evidenced the presence of up to three different B-cell populations (**a.2**) with version 6 of the LST tube, while with version 7 only two populations could be discriminated (**b.2**). Conventional bivariate dot plot analysis showed that the two major B-cell populations (red and yellow dots) corresponded to two different B-cell clones displaying distinct surface membrane immunoglobulin (SmIg) light-chain phenotypes: SmIgκ^{+}/SmIgλ^{-}/CD20^{hi}/CD5^{-} (red dots; 32.8% and 33.5% in **a** and **b**, respectively) and SmIgκ^{-}/SmIgλ^{+}/CD20^{+}/CD5^{lo} (yellow dots; 13.9% and 16.6% in **a** and **b**, respectively). Both B-cell populations were clearly detected with the two LST versions (**a.3, a.4** and **b.3**, **b.4**). Further phenotypic characterization of these two B-cell populations evidenced the presence of a minor CD10^{+} B-cell (sub)population (brown dots) within the SmIgκ-restricted B-cells (3.94% of all B-cells; **a.5**). In this case, CD38 expression (**b.5**) added no further information; however, it should be noted that the abnormal phenotypic profile of the two major B-cell clones granted their further immunophenotypic characterization by the application of the B-CLPD panel, which finally evidenced the CD10^{+}/SmIgκ^{+} restricted (sub)population.

distinguishable B-cell populations coexist and sample availability for further stainings is limited, hampering full immunophenotypic characterization with the B-CLPD panel.

Evaluation of the LST, version 7

The aim of the LST was to detect any mature lymphoid malignancy in a diagnostic screening phase. In line with this aim, CD5 has added value for the immunophenotypic evaluation of T- and NK-cells. CD10 may contribute to the screening of T-cell malignancies, particularly for the detection of CD4^{+}/CD10^{+} cells in the diagnosis of some cases of angioimmunoblastic lymphoma, but such lymphomas more frequently show other phenotypic aberrancies such as coexistence of a CD4^{hi}/SmCD3$^{-/lo}$ T-cell phenotype.[83] In turn, CD38 would further allow positive identification of plasma cells and it provides valuable information when evaluating a wide range of lymphoid malignancies including large cell lymphomas with *MYC* translocations (for example, BL and transformed DLBCL), follicular lymphoma, plasmablastic lymphoma, plasma cell disorders (PCD) and other B-CLPD with plasmacytoid differentiation.[3] Consequently, versions 5 and 6 were merged

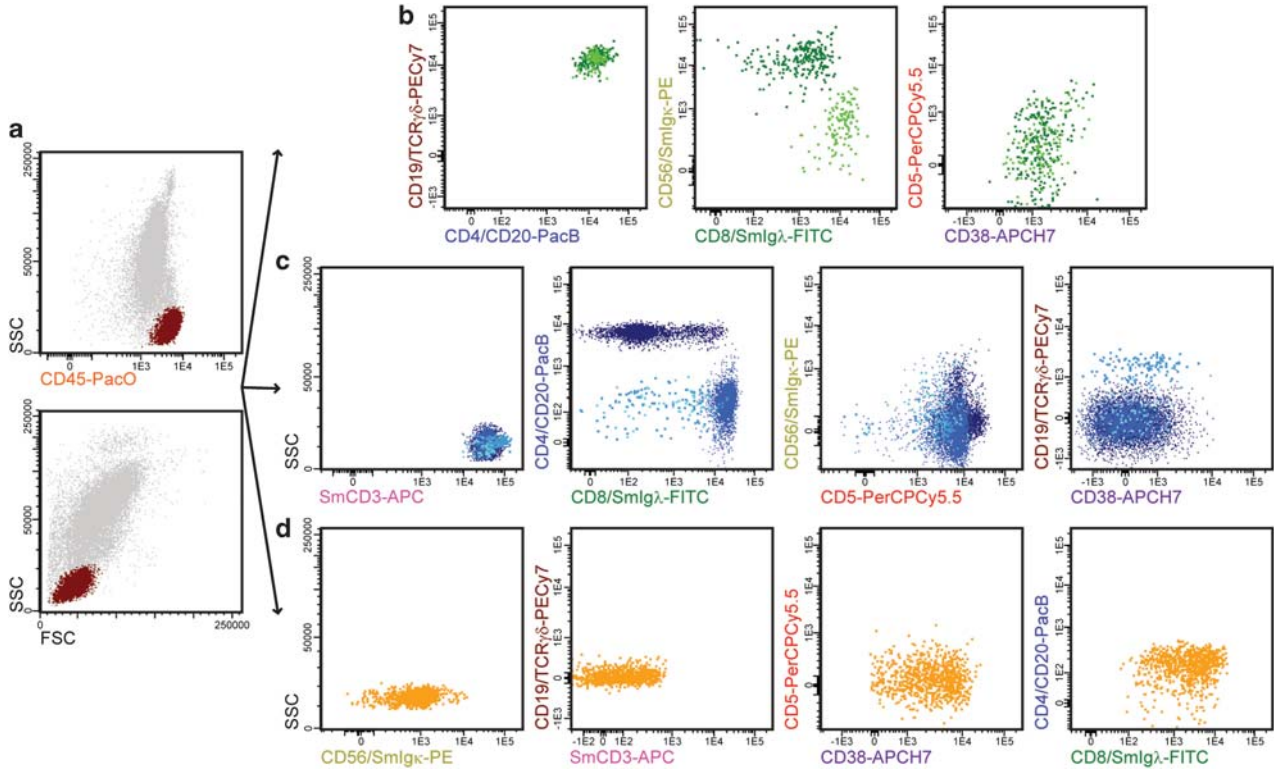

Figure 8. Illustrative example of the immunophenotypic profile of the lymphocyte populations present in normal peripheral blood stained with the lymphoid screening tube (LST) (version 7). (**a**) Typical profile of mature lymphocytes (brown dots) for light scatter parameters and CD45. (**b**) Phenotype of normal mature B-cells for the B-cell-associated markers in the LST combination with a normal distribution according to surface membrane (Sm) light-chain expression (SmIgκ^+ B-cells are painted as dark green dots and SmIgλ^+ B-lymphocytes as light green dots). (**c**) The phenotypic features of normal mature T-cells as defined by the expression of relevant markers in the combination ($CD4^+$ T-cells: dark blue dots; $CD8^{hi}$ T-cells: blue dots; $CD4^-/CD8^{-/lo}/TCR\gamma\delta^-$ T-cells: light blue dots; and $CD4^-/CD8^{-/lo}/TCR\gamma\delta^+$ T-cells: cyan dots). (**d**) Phenotypic pattern of normal peripheral blood NK-cells (yellow dots) for SSC, CD56, CD19/TCRγδ, SmCD3, CD38, CD5, CD8 and CD20/CD4 with version 7 of the LST.

into a final LST version, which incorporated CD5 and CD38 but not CD10 (Table 3): version 7 of the LST.

In a final stage, version 7 of the LST underwent evaluation in all participating EuroFlow centers and included 271 samples (175 PB, 75 BM, 14 LN biopsies, 5 LN FNA, 1 pleural fluid and 1 ascitic fluid). In 228/271 samples (84%) an aberrant lymphoid population was detected with a median percentage of infiltration of 31% of the total number of leukocytes (range: 0.04 – 98%). A 100% concordance was obtained when compared with routine approaches in each participating center, with sensitivity down to 0.1% for detection of small aberrant lymphoid cell populations.

In summary, version 7 enabled a complete phenotypic evaluation of all relevant mature lymphoid compartments in samples most frequently used to screen for mature lymphoid malignancies such as PB, BM and LN tissues. Version 7 includes markers that identify and quantify the populations of interest, allows their detailed subsetting and also detects the most frequent phenotypically aberrant patterns for the markers included. Additionally, version 7 showed an optimal efficiency in guiding the selection of complementary antibody panels for a more complete immunophenotypic characterization of any abnormal lymphoid population present in the sample, at frequencies $\geqslant$0.1%. Finally, usage of CD45, CD19 and CD20 in common to the B-CLPD panel and careful avoidance of redundant stainings further facilitates full integration of the LST as tube 1 of the B-CLPD panel (see Section 8) (Figure 1).

Multivariate analysis of normal lymphocyte cell populations

All samples that were obtained from normal healthy donors ($n = 22$, 19 PB and 3 BM; mean age: 36 ± 14 years, 9 males and 13 females) were analyzed using conventional approaches for the identification of all major normal lymphoid populations contained in them, such as B- (SmIgκ^+, SmIgλ^+); T- ($CD4^+$, $CD8^{hi}$, $CD4^+/CD8^{lo}$, $CD4^-/CD8^{-/lo}/TCR\gamma\delta^-$ and $CD4^-/CD8^{-/lo}/TCR\gamma\delta^+$) and NK-cells (in addition to the positive identification of plasma cell) for a total of 10^5 cells measured per sample (Figure 8). Among the normal PB samples ($n = 19$), lymphoid populations were distributed as follows—mean (range): total lymphocytes, 23.9% (11–42.2%); T-cells, 19.1% (8.2–36%); $CD4^+$ T-cells, 10.1% (4.1–18%); $CD8^{hi}$ T-cells, 7.3% (2.3–17.2%); $CD4^-/CD8^{-/lo}/TCR\gamma\delta^-$, 0.4% (0.1–1%); $CD4^-/CD8^{-/lo}/TCR\gamma\delta^+$, 1% (0.01–3.1%); B-cells, 2.2% (0.4–5%); SmIgκ^+ B-cells, 1.3% (0.2–3.2%); SmIgλ^+ B-cells, 0.9% (0.2–2.4%); κ/λ ratio, 1.5 (1.1–2.2); total NK-cells, 2.6% (1.3–5.7%); and plasma cells: 0.06% (0.01–0.45%).

Afterwards, the lymphoid cell populations from the different normal PB samples were gated and data stored in a separate data file for each population.[32] Subsequently, the data files were merged to create a pool of normal reference cells for each of the above-listed cell populations. PCA showed that when individually considered, all normal lymphocyte populations clustered together in the APS (principal component (PC)1 versus PC2) view of the Infinicyt software according to their normal phenotypic profile in the 10-dimensional space generated by the light scatter and fluorescence emissions measured with the LST (Figure 9).

Multivariate analysis of suspicious lymphoid populations

Once the normal B-, T-$CD4^+$, T-$CD8^+$ and NK-cell reference databases were constructed, multiparametric comparison of each corresponding B-, T- and NK-cell population from each individual sample suspected of carrying a CLPD ($n = 249$) versus the normal

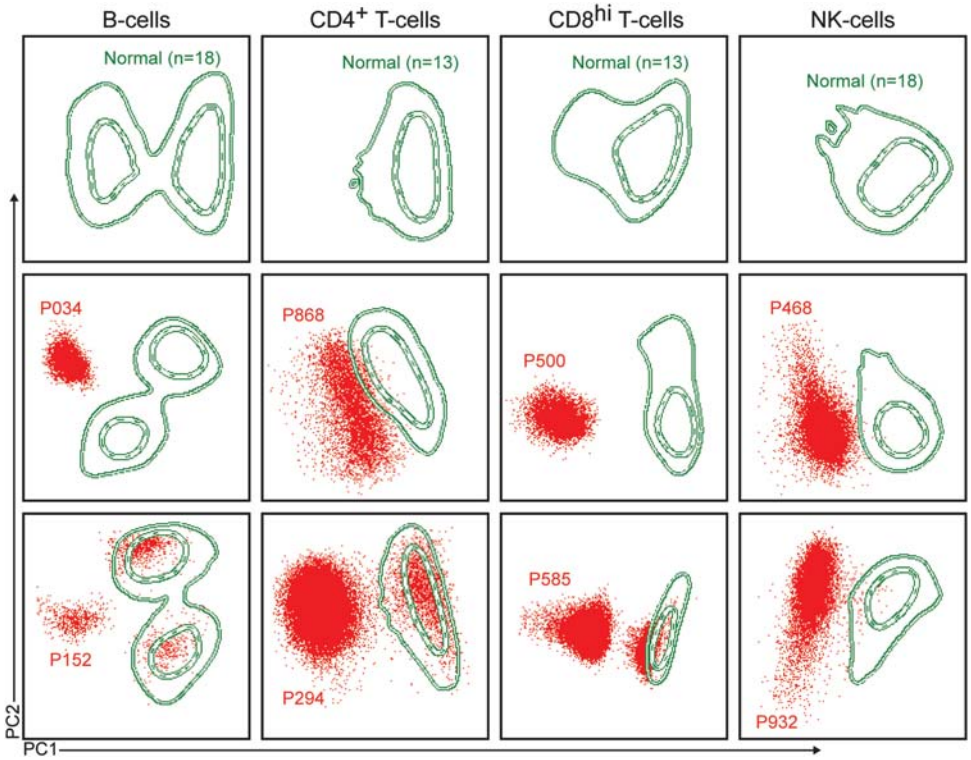

Figure 9. Illustrative automated population separator (APS)—principal component (PC)1 versus PC2 views of B-, CD4$^+$T-, CD8^{hi} T- and NK-cells, defined by their immunophenotypic profile obtained with the markers included in the lymphoid screening tube (LST) (version 7). As illustrated, normal B-, CD4$^+$ T-, CD8^{hi} T- and NK-cells from different samples clustered together on either single or bimodal distribution (green lines in the upper row). In this row, the bimodal distribution of normal B-cells reflects the differential expression of surface membrane immunoglobulin (SmIg) κ versus λ light chains. The middle row illustrates examples of cases (red dots) in which all cells within the leukemic cell population were phenotypically aberrant and clearly separated from the corresponding pool of normal B-, T CD4$^+$, T CD8^{hi} and NK-cells, respectively (green lines). In the lower row, examples of cases in which variable numbers of normal and aberrant or clonal lymphoid cells (both depicted as red dots) coexist in the same sample are shown. Note that part of the red dots in the lower row corresponding to normal cells tend to fall within the normal reference pool (cluster of red dots inside the green lines) resembling their distribution, while clonal/aberrant cells (cluster of red dots outside the green lines) are clearly separated from the normal cell cluster. Contour green lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively from normal B-, T- or NK-cell phenotypes).

reference pool was performed using the APS tool (Infinicyt software). Overall, our results showed an overlap for most cell populations present in every sample with their corresponding pool of normal reference cells. However, 149/150 aberrant B-cell populations were also detected (99.4%) in samples showing the presence of clonal B-CLPD populations. In one case, the phenotypic profile of the neoplastic B-cells overlapped with that of normal B-cells and their presence could only be confirmed when light-chain-restricted B-cell subsets were evaluated because of an altered SmIgκ/SmIgλ B-cell ratio.

Similarly, aberrant T-cell populations were detected in 61/65 T-CLPD (94%). In detail, phenotypically abnormal CD4$^+$/CD8$^{-/lo}$ T-cells were identified in 33/36 cases with CD4$^+$/CD8$^{-/lo}$ T-CLPD infiltrated samples; in the other three samples in which CD4$^+$/CD8$^{-/lo}$ clonal T-cells could not be easily discriminated from normal cells based on their phenotype, an abnormally increased number of CD4$^+$ T-cells was found (CD4$^+$ T-cells represented 99.2 and 19.6% of the total number of lymphocytes in two PB samples and 85.1% of the total number of lymphocytes in a LN biopsy sample), which should also lead to further evaluation of CD4$^+$ T-cells with the T-CLPD EuroFlow panel (Section 9). In addition, aberrant CD8^{hi}/CD4$^-$ T-cells were clearly found outside the normal CD8^{hi} T-cell cluster in 13/14 cases (93%) with monoclonal expansions; the other case showed abnormally high CD8$^+$ T-cell numbers. Finally, abnormal populations of CD4$^-$/CD8$^{-/lo}$/TCRγδ$^-$ T-cells ($n=3$), CD4$^-$/CD8$^{-/lo}$/TCRγδ$^+$ T-cells ($n=11$) and CD4$^+$/CD8$^+$ T-cells ($n=1$) were detected in all cases carrying neoplastic infiltration by these typically minor T-cell populations.

Regarding NK-cells, abnormal populations could be clearly discriminated from normal NK-cell phenotypes in 17/18 cases with NK large granular lymphocytic (LGL) leukemia. In the remaining sample, the altered NK-cell population showed only partial overlap with the normal NK-cell phenotype; such phenotypic differences also required further evaluation.

In summary, the LST detected aberrant B-, T- or NK-cell immunophenotypes in 149/150 (99.4%) of B-CLPD and in 78/83 (94%) of T/NK-CLPD, with an overall frequency of 97.4%. The remaining six cases displayed either partially aberrant phenotypes ($n=1$) or increased numbers of the suspicious lymphoid population, all of which would lead to further confirmatory phenotypic evaluation.

CONCLUSION

In this section a single 8-color LST was designed, evaluated, redefined and re-evaluated for a total of seven sequentially improved versions. The proposed final EuroFlow LST version (version 7) showed 100% concordance with routine approaches. Furthermore, our results confirm the utility of the proposed LST combination to discriminate between normal and abnormal lymphoid cells, based on the detection of aberrant immunophenotypic characteristics in the LST combination in virtually all abnormal B-cell populations (>99%) and most (⩾94%) LGL

NK-cells and CLPD T-cells, with an overall efficiency of 97.4% (sensitivity of down to 10^{-3}). In the other few CLPD cases ($n = 6$; 2.6%), a typically altered numerical distribution would also require further confirmatory testing.

SECTION 3. SMALL SAMPLE TUBE (SST)

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BACKGROUND

Central nervous system (CNS) localization is a relatively rare complication of a systemic non-Hodgkin lymphoma, although the exact frequency varies per histological subtype (from $<5\%$ in low-grade B-cell lymphomas to $\sim 20\%$ in aggressive B-cell lymphomas).[84–89] CNS localization of lymphoma is associated with an unfavorable outcome.[90] In turn, some patients present with neurological symptoms that could be compatible with a CNS lymphoma, but without evidence for lymphoma elsewhere in the body. Primary CNS lymphoma (PCNSL) is even less frequent than secondary CNS localization of a systemic lymphoma, but has a similar poor clinical course.

Intra-ocular lymphomas (IOL) can present as a primary event in the eye (PIOL), or as a clinical manifestation in parallel to or following CNS lymphoma (reviewed by Chan[91] and Coupland[92]). The majority (60–85%) of PIOL patients develop CNS lymphoma within 2–2.5 years after initial diagnosis.[92] The two types of lymphomas thus form a spectrum of the same disease termed oculo-cerebral lymphomas (PIOL/PCNSL). IOL can also present as secondary localizations of a systemic lymphoma (secondary IOL). PIOL are estimated to account for up to 1–2% of all extranodal lymphomas.[92] PIOL are mostly of the B-cell type (DLBCL), whereas T-cell type IOL are often associated with cutaneous T-cell lymphomas or other systemic T-cell lymphoma.[92] Over the last two decades an increase in PCNSL, and potentially also PIOL, has been seen in immunocompromised individuals. PIOL occur in adult individuals (average age 50–60 years) and typically present as chronic, often refractory, uveitis or vitritis, the so-called masquerade syndrome.[91,92] This masquerade syndrome is the reason for a considerable delay (8–21 months) in establishing a correct diagnosis.[92]

In the diagnostic process of patients with neurological and/or ophthalmologic symptoms that could be indicative of CNS lymphoma or IOL, lumbar puncture and vitrectomy are performed to obtain CSF and vitreous biopsy material, respectively. In contrast to most other materials, CSF and vitreous biopsies pose difficulties to the diagnostic process owing to the fact that both types of samples almost systematically present with low cell numbers. In such pauci-cellular or 'small' samples it is of utmost importance to obtain maximal information from minimal numbers of cells. Traditionally, cytomorphology has been the gold standard technique for the detection of malignant leukocytes in both CSF and vitreous material for diagnostic purposes.[93,94] Even though positivity can be highly specific for a CNS lymphoma or IOL diagnosis, the risk of false-negativity in cytomorphology is high.[87,88,95] This is related to the poor cell viability in these materials, as well as the often troublesome discrimination between benign and malignant cells on top of the low cell numbers.[87,96,97] Other studies exploit Ig and/or TCR gene analysis,[91,98,99] but the pitfall of coincidental, non-reproducible, amplification in case of small cell numbers (pseudoclonality) has to be considered.

Multiparameter flow cytometric immunophenotyping combines high specificity with good clinical sensitivity. Several studies indeed underline the importance of multiparameter flow cytometric immunophenotyping for efficient and reliable diagnosis versus exclusion of CNS lymphoma or IOL localizations.[87–90,100–105] For CSF analysis multiple 3-, 4-, 6- or even 8-color single-tube screening panels and protocols have been described (see Kraan *et al.*[106] for an extensive review and description of labelings and methods). In this section we discuss the design and evaluation of the 8-color EuroFlow SST and related sample preparation protocols aimed to evaluate CSF and vitreous biopsy samples, in patients suspected of lymphoma.

Design of the EuroFlow small sample screening tube

The EuroFlow group designed an 8-color flow cytometric labeling aimed at screening for lymphoma in 'small samples' from CSF and vitreous material obtained from suspected cases. As the SST should enable optimal detection and identification of all possible cell types in pauci-cellular materials, a series of antibodies had to be included in a single tube. Thus, the markers that were selected included the pan-leukocyte marker CD45, as well as markers for positive identification of mature B-cells (CD19, CD20) and their SmIg light chain subsets (anti-SmIgκ, anti-SmIgλ), and both T-cells and NK cells (SmCD3, CD4, CD8, CD56). Additional markers like CD14 (monocytes) and CD38 (plasma cells) were selected to identify all possible cell types in a more complete way. To accommodate all these markers in one tube, at three different fluorochrome positions two antibodies were selected that appear on different cell types in a mutually exclusive manner: (a) CD8 and SmIgλ expressed by T- and B-cells, respectively, (b) CD56 and SmIgκ present on T/NK- and B-cells, respectively, and (c) SmCD3 and CD14 markers specific for T-cells and monocytes/macrophages, respectively (Table 5). In this way a single 8-color/11-antibody tube was developed that allows complete typing of all relevant cell types in small samples. In combination with the forward and side scatter (FSC, SSC, respectively) features, 13 parameters are available to characterize cells present in CSF and vitreous biopsy. Through appropriate gating strategies, SmIgκ and SmIgλ positivity can be evaluated within the $CD19^+$ B-cell fraction, and CD4 and CD8 reactivity within the $SmCD3^+$ T-cell fraction, as also described above in Section 2 for the LST (see also Figure 10 for an illustration of the gating strategy). For most positions, the fluorochrome-conjugated antibodies from the LST were selected. In addition, CD14 was placed in combination with

Table 5. Composition of SST for detection of lymphoid cells[a]

Fluorochromes and markers							
PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
CD20	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD4	CD19	SmCD3 and CD14	CD38

Abbreviations: APC, allophycocyanin; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, hilite7; PacB, pacific blue; PacO, pacific orange; PE, phycoerythrin; PerCPCy5.5, peridinin–chlorophyll–protein–cyanin5.5; Sm, surface membrane; SST, small sample screening tube. [a]Further information about markers and hybridomas is provided in the Appendix.

Table 6. Normal and reactive leukocyte populations in 120 CSF and 21 vitreous biopsies without evidence for lymphoma

Group of samples	*% B-cells*	*SmIgκ/SmIgλ ratio*	*% T-cells*	*CD4/CD8 ratio*	*% Monocytes*	*% Neutrophils*[a]	*% Other undefined events*
CSF ($n=120$)	2.0 (0-32)	1.4 (1.0–3.0)	50.8 (0–100)	1.8 (0.1–10.9)	2.1 (0–17)	15 (0–99)	31.7 (0–100)
CSF (MS cohort) ($n=15$)	1.1 (0–4)	1.3 (1.0–2.5)	48.2 (0–89)	2.8 (1.7–7)	3.6 (0–14)	23 (0–99)	21.9 (0–100)
CSF (other HM suspicion) ($n=9$)	0.2 (0–1)	NA	40.8 (0–100)	1.2 (0.2–3.5)	0	9 (0–14)	49.8 (0–100)
CSF (lymphoma suspicion) ($n=96$)	4.8 (0–32)	1.4 (1.0–3.0)	63.4 (0–100)	1.7 (0.1–10.9)	2.6 (0–17)	13 (9–59)	23.4 (0–100)
Vitreous fluid ($n=21$)	0.5 (0–4)	NA	51.7 (3–100)	2.1 (0.3–49)	2.6 (0–14)	18 (0–53)	14.1 (0–100)

Abbreviations: CSF, cerebrospinal fluid; HM, hematological malignancy; MS, multiple sclerosis; NA, not applicable due to lack of B-cells in most samples; Sm, surface membrane. Results are expressed as mean value and range between brackets. [a]Cells that cluster based on $FSC^{int}/SSC^{high}/CD45^{lo}$ can be considered as neutrophilic granulocytes (see also Discussion). NA, not applicable because of too few B-cells.

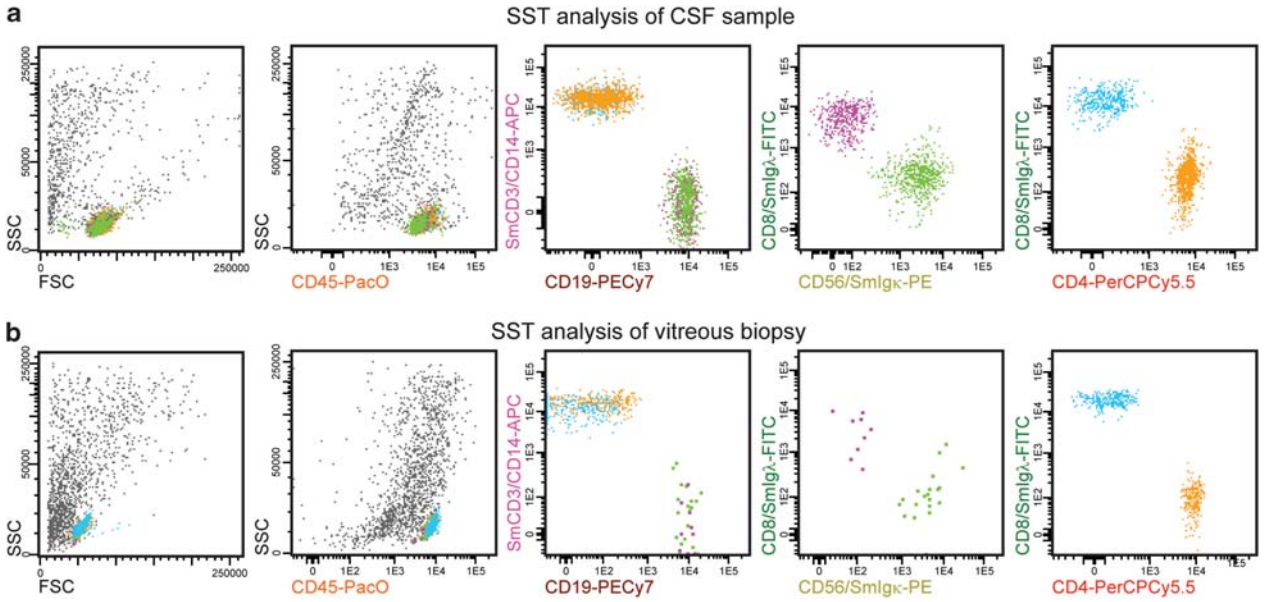

Figure 10. EuroFlow small sample tube (SST) analysis of a cerebrospinal fluid (CSF) (**a**) and a vitreous biopsy (**b**) sample with a normal composition of B- and T-lymphocytes. Based on a FSC/SSC/CD45 gating strategy, $CD19^+$ B-cell and $SmCD3^+$ T-cell populations are identified. Even though the surface membrane immunoglobulin (SmIg) κ and λ markers are both present in combination with other antibodies with the same fluorochrome, $SmIg\kappa^+$ (green dots) and $SmIg\lambda^+$ (purple dots) cells can be discerned by gating on the $CD19^+$ B-cell population. In both samples the $SmIg\kappa^+$ and $SmIg\lambda^+$ B-lymphocytes show a normal ratio (1.5). In a similar way, a normal distribution of $CD4^+$ (orange dots) and $CD8^+$ (blue dots) T-lymphocytes (ratio 2.3 and 2.1 in **a** and **b**, respectively) was detected within the $CD3^+$ T-cell population.

SmCD3 as a CD14 – APC conjugate, which proved to perform in optimal conditions.

Evaluation of the SST

The EuroFlow SST has been tested on a cohort of 164 samples that have been collected within the EuroFlow Consortium. These included 23 vitreous biopsies with a clinical suspicion of IOL and 141 CSF samples. Both groups of samples typically contained low numbers of cells, ranging from <100 to tens of thousands. As controls, 11 CSF samples from patients with an initial diagnosis of acute leukemia or Hodgkin lymphoma were studied, plus 15 CSF samples from patients with a clinical suspicion of (early) multiple sclerosis. In the remaining 115 CSF samples and the 23 vitreous biopsies, lymphoma localization was suspected. CSF and vitreous samples were collected in tubes with a small volume (a few ml) of culture medium plus 0.2% bovine serum albumin (BSA, SIGMA-ALDRICH, St. Louis, MO, USA) or 10% fetal calf serum (FCS, Invitrogen, Carlsbad, CA, USA) for a better cell viability, or in tubes prepared with 0.2 ml of Transfix (Cytomark, Buckingham, UK) (Tubes&Transfix, Immunostep SL, Salamanca, Spain). Upon arrival in the laboratory, cells were centrifuged and the pellet was resuspended in 200 μl phosphate buffer saline +0.5% BSA. Out of this cell suspension, one-third of the volume was initially used for the EuroFlow SST labeling. For sample preparation the EuroFlow protocols were adapted to previously described[89] consensus recommendations using a wash, stain, lyse and wash procedure.

In the CSF control group (multiple sclerosis, acute leukemia, Hodgkin lymphoma samples), the majority of the CSF cells that could be identified with the SST labeling corresponded to T-lymphocytes, whereas the remaining cells were monocytes and B-lymphocytes (Table 6). Of note, no $CD45^{lo}$ blasts could be identified in the CSF samples of acute leukemia patients. In turn, no aberrant B- or T-cell populations were seen in the vast majority (104/115) of the CSF samples that were taken because of a clinical suspicion of lymphoma (Figure 10). In 96 (out of these 104) samples that showed enough viable cells for evaluation, the majority of cells identified with the SST labeling also concerned T-lymphocytes (Table 6). Likewise, in the majority (21/23) of vitreous biopsies no aberrant B- or T-cell populations were found either (Table 6); notably, the number of cells in these vitreous

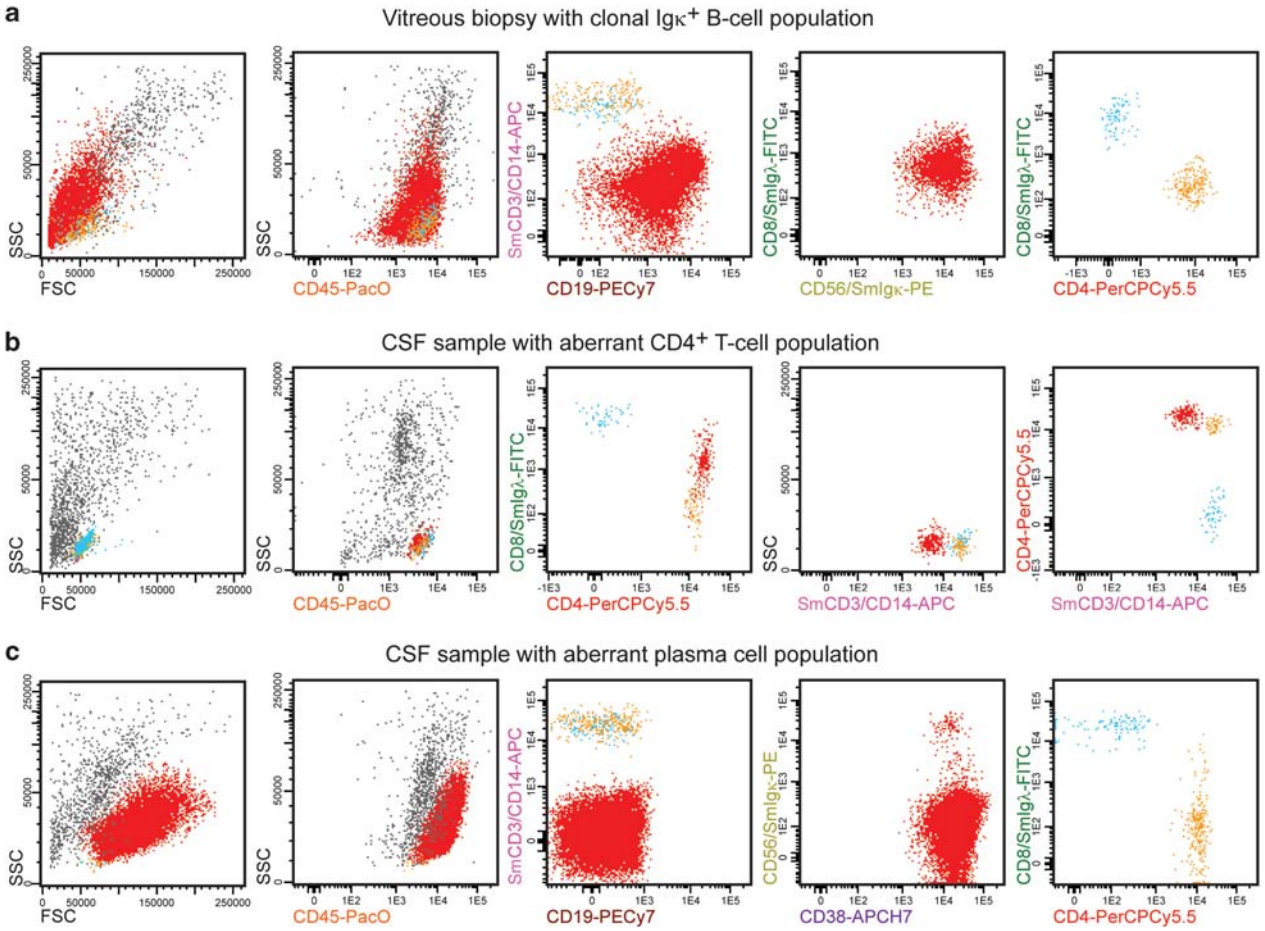

Figure 11. EuroFlow small sample tube (SST) analysis of a vitreous biopsy with prominent clonal B-cell population (**a**) and of two cerebrospinal fluid (CSF) samples with prominent aberrant T-cell (**b**) and plasma cell (**c**) populations. In **a**, following FSC/SSC/CD45 gating, B- and T-cell populations are identified; the B-cell population shows a heavily skewed surface membrane immunoglobulin (SmIg) κ/SmIgλ ratio (>10), in line with an aberrant, monoclonal large B-lymphocyte population (red dots); residual $CD4^+$ (orange dots) and $CD8^+$ (blue dots) T-lymphocytes show a normal distribution (ratio 2.5). In **b**, upon FSC/SSC/CD45 gating, a T-cell population is identified, which consists of $CD4^+$ (orange dots) and $CD8^+$ (blue dots) T-lymphocytes, as well as an aberrant T-cell population (red dots) characterized by a $SmCD3^{lo}$/$CD4^{hi}$/$CD8^{lo}$ phenotype; the presence of this aberrant T-cell population could be caused by a contaminating blood cell population, given the fact that neutrophil granulocytes can be discerned based on FSC versus SSC and CD45 versus SSC features. Based on a FSC/SSC/CD45 gating strategy, a T-cell population with a normal CD4/CD8 ratio (1.6) can be identified (but no B-cell population) in **c**. In addition, in this panel a rather large population of $CD19^-$/$CD3^-$ cells (red dots) is seen that upon further analysis appeared to be $CD38^{hi}$ and $CD56^-$, in keeping with a plasma cell origin; further diagnostic work-up of this patient indeed showed an aberrantly similar plasma cell population in the bone marrow (data not shown).

biopsies was generally rather low, although the relative composition was similar to the CSF samples with a predominance of T-lymphocytes (Table 6). None of these cases was diagnosed as CNS lymphoma or IOL.

In nine CSF samples with an initial suspicion of lymphoma, clonal B-cell populations were observed, in keeping with a primary B-cell lymphoma or a localization of a systemic B-cell lymphoma/B-cell leukemia. Out of the 23 vitreous biopsies two cases also showed an aberrant B-cell population; in one sample this concerned a $SmIg\kappa^+$ B-cell population (Figure 11a), whereas the other contained a suspect SmIg-negative B-cell population that had also been seen in a previous vitreous biopsy of the same patient. Furthermore, in two other CSF samples from patients with lymphoma suspicion, aberrant T-cell populations ($CD3^{lo}$/$CD4^+$/$CD8^+$) were identified (Figure 11b), compatible with their diagnosis of T-cell lymphoma and post-transplant lymphoproliferative disorder. Finally, two CSF samples in the control group without lymphoma suspicion showed clear infiltration by plasma cells ($CD38^{hi}$-expressing cells) (Figure 11c), indicative of a systemic plasma cell malignancy that was indeed found upon further diagnostic work-up. Overall, the results in all analyzed samples underline the power of this 13-parameter flow cytometric SST strategy to reliably confirm or exclude lymphoma localization (Table 7). All these cases (13/13) lacked a final cytological diagnosis of CNS disease or IOL. Despite this, they all had a final diagnosis of CNS lymphoma or IOL based on histopathology, image analysis or the clinical behavior of the disease.

Detection of aberrant lymphoid populations by PCA

Multivariate analysis of the B- and T-cell populations contained in individual CSF and vitreous biopsy samples was performed using PCA implemented in the Infinicyt software APS1 view (PC1 versus PC2) to evaluate the rate of detection of aberrant B- and T-cell phenotypes in cases showing CNS lymphoma or IOL. To this end, we used several of the clonal CSF cases from the current cohort as well as newly obtained clonal CSF cases. In the APS view we found a clear separation of clonal B-cells from normal B-cells for 2/4 $SmIg\kappa^+$ cases and 5/5 $SmIg\lambda^+$ cases, and for the one case in which SmIg expression could not be determined (overall: 8/10

Table 7. SST results of 149 pauci-cellular samples

Suspected lymphoma localized in CSF (n = *115)*	
Aberrant/clonal B-cell populations[a] (1 CLL, 1 BL, 1 FL, 1 MCL, 2 DLBCL, 1 B-cell lymphoma, 2 unknown)	9/115 (7.8%)
Aberrant T-cell populations (T-cell lymphoma, PTLD)	2/115 (1.7%)
Suspicious of other hematological malignancies in CSF (n = *11)*	
Aberrant plasma cell populations	2/11 (18.1%)
Vitreous biopsy (n = *23)*	
Aberrant/clonal B-cell populations	2/23 (8.7%)
Total	15/149[b] (10.0%)

Abbreviations: BL, Burkitt lymphoma; CNS, central nervous system; CLL, chronic lymphocytic leukemia; CSF, cerebrospinal fluid; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; FNA, fine needle aspirate; IOL, intraocular lymphoma; PTLD, post-transplant lymphoproliferative disease; SST, small sample tube. [a]In one case a parallel FNA brain biopsy was analyzed next to the CSF sample, showing the same aberrant B-cell population; [b]All positive cases had a final diagnosis of CNS lymphoma or IOL based on histopathological analysis, imaging techniques and/or the clinical behavior of the disease, while none of the negative cases by the SST labeling was diagnosed as having CNS or IOL.

cases; data not shown). The parameters that contributed most to the APS-based discrimination were FSC and CD38, and to a lesser extent also CD20 and SSC. In contrast, the two other SmIgκ$^+$ cases were hardly or not discernible from control B cells or from SmIgκ$^+$ B-cells unless an increased ratio between SmIgκ$^+$/SmIgλ$^+$ B-cells was considered to be an informative parameter for lymphoma diagnosis. The two aberrant T-cell cases could easily be discerned from control CD4$^+$ and CD8$^+$ T-cells in the APS view, with CD4 and CD8 as the most discriminative markers. Of note, cytomorphological analysis was not informative in any of these cases ($n = 12/12$), as already described above.

CONCLUSION

The EuroFlow SST and sample preparation protocol is a powerful diagnostic tool for the evaluation of CSF and vitreous samples with a clinical suspicion of primary lymphoma. The 13-parameter SST labeling allows for a complete typing of the most relevant leukocyte populations in these samples. While in the majority of cases the presence of aberrant B-, T- or plasma cell populations can be confirmed or excluded using the APS view of the Infinicyt software, in the remaining cases an aberrant B-cell population was more difficult to establish, unless an altered SmIgκ/SmIgλ B-cell ratio was considered as indication for a diagnosis of B-cell lymphoma. A more detailed characterization of the aberrant B- or T-cell populations requires the use of even more markers in additional labelings, which are currently in progress.

SECTION 4. PLASMA CELL DISORDERS (PCD) PANEL

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BACKGROUND

PCD are a group of diseases most frequently characterized by the presence of clonal (neoplastic) plasma cells in the BM capable of secreting a clonal Ig that can be detected in serum and/or urine.[107] It includes different disease entities, among which multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) are the most prevalent and representative entities.[108] Additionally, other less frequent clinical conditions associated with predominant extramedullary plasma cell localizations and organ failure due to the accumulation of the clonal Ig (for example, amyloidosis) are also included in this group of diseases.[107,109]

Multiparameter flow cytometric immunophenotyping, together with other clinical, radiological, biochemical and hematological data, provides relevant information for the diagnosis and classification of PCD. At the same time, flow cytometry contributes to prognostic stratification and minimal residual disease (MRD) monitoring of myeloma patients after therapy.[9,110] The most relevant clinical information provided by flow cytometry relies on the identification and enumeration of aberrant versus normal/polyclonal BM plasma cells: a higher ratio between both populations is associated with malignancy (for example MM versus MGUS). Similarly, the presence of $<5\%$ of normal plasma cells within the total population of BM plasma cells is also associated with a poor outcome in symptomatic MM and a higher risk of progression in both MGUS and smoldering MM.[111] In turn, several individual markers and immunophenotypic profiles (for example, CD28 and CD117 expression) have been associated with specific genetic changes and disease outcome.[112]

Over the years, an increasing number of markers have been identified that provide relevant immunophenotypic information on plasma cells.[9,110,112–122] Current consensus recommendations include CD38, CD138 and CD45 (together with light scatter characteristics) as the best combination of backbone markers for the identification and enumeration of plasma cells.[9,110,114] In addition, expression of CD19, CD56, CD117, CD20, CD28, CD27 and CD81, together with CyIg light-chain restriction, is associated with unique phenotypic patterns that allow clear discrimination between normal/reactive versus clonal plasma cells.[9,110,113,118] More recently, it has also been shown that the plasma cell surface expression of β2 microglobulin negatively correlates with its serum levels and a better outcome, becoming an additional potentially attractive prognostic marker.[121]

On the basis of existing data and consensus recommendations, the EuroFlow Consortium has designed and evaluated a two-tube, 12-marker panel of antibodies devoted to: identification of plasma cells, discrimination and enumeration of coexisting normal/reactive and aberrant plasma cell populations and detailed characterization of additional cell surface markers that contribute to the definition of aberrant phenotypes or disease prognosis.

Selection of antibody reagents

Four distinct backbone markers (CD38, CD138, CD45 and CD19) were selected for efficient identification of plasma cells (CD38 and CD138) and to distinguish between normal/reactive and clonal plasma cell compartments based on their most frequent aberrant phenotypes (CD38, CD19 and CD45). Another eight markers were additionally selected for the characterization of plasma cells with the PCD panel configured as two-tube, 12-marker combination (Table 8). The overall panel of markers aimed at: (i) positive identification of plasma cells in BM, and other less frequent types

Table 8. Design of PCD tubes in six consecutive testing rounds[a]

Version (no. of cases)[b]	*Tube*	*Fluorochromes and markers*							
		PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCCy7*
1 ($n=7$)	1	CD45	CD19	Cylgλ	Cylgκ	CD38	CD56	CD27	[c]
	2	CD45	CD19	β2 micro	CD81	CD38	CD117	CD28	[c]
		PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*AF700*
2 ($n=68$)	1	CD19	CD45	Cylgλ	Cylgκ	CD138	CD56	CD27	CD38
	2	CD19	CD45	β2 micro	CD81	CD138	CD117	CD28	CD38
3 ($n=5$)	1	CD19	CD45	β2 micro	Cylgλ	CD138	CD56	Cylgκ	CD38
	2	CD19	CD45	CD27	CD81	CD138	CD117	CD28	CD38
		PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
4 ($n=29$)	1	CD19	CD45	β2 micro	Cylgλ	CD138	CD56	Cylgκ	CD38
	2	CD19	CD45	CD27	CD81	CD138	CD117	CD28	CD38
5[d] ($n=5$)	1	CD19	CD45	CD138	Cylgλ	β2 micro	CD56	Cylgκ	CD38
	2	CD19	CD45	CD138	CD81	CD27	CD117	CD28	CD38
		PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
6 (Final) ($n=100$)	1	CD45	CD138	CD38	CD56	β2 micro	CD19	Cylgκ	Cylgλ[e]
	2	CD45	CD138	CD38	CD28	CD27	CD19	CD117	CD81

Abbreviations: AF700, alexa fluor 700; AmCyan, *Anemonia Majano* cyan fluorescent protein; APC, allophycocyanin; BM, bone marrow; Cy, cytoplasmic; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, hilite7; MGUS, monoclonal gammopathy of undetermined significance; β2 micro, β2 microglobulin; MM, multiple myeloma; NonPCD, non-plasma cell-related diseases; PacB, pacific blue; PacO, pacific orange; PB, peripheral blood; PCD, plasma cell disorders; PE, phycoerythrin; PerCPCy5.5, peridinin–chlorophyll–protein–cyanin5.5. [a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 214 samples (212 BM, 2 PB) was evaluated. Among them 117 MM, 47 MGUS and 50 other conditions were detected. The other conditions included: 1 amyloidosis; 2 plasma cell leukemias; 6 non-infiltrated samples from patients with suspected PCD that showed no infiltration both in routine and EuroFlow diagnostic approaches (suspected disease category: 2 MGUS, 2 MM, 1 plasmocytoma, 1 IgM paraproteinemia); 3 B-chronic lymphoprolipherative disorders with no involvement of the plasma cell compartment; 10 normal healthy donors; 15 reactive BM samples from patients with NonPCD; 13 regenerating BM samples after chemotherapy from patients with NonPCD. [c]CD138 was proposed for this position but no conjugate was commercially available at the time of evaluation. [d]All samples were evaluated using tube 2 only. Highlighted boxes: changes as compared to previous version. [e]Testing of Cylgλ-APCC750 is ongoing to increase the stain index of Cylgλ$^+$ cells.

of samples evaluated for PCD; (ii) accurate discrimination between normal/reactive and aberrant plasma cells, because of their distinct immunophenotypic profiles; and (iii) confirmation of their clonal nature, as evidenced by a restricted Cylg light-chain expression or expression of $\geqslant 2$ aberrant markers (Table 9). The final proposed configuration of the panel (version 6; Table 8) resulted from evaluation of six sequential versions of distinct two-tube combinations of the above-listed backbone ($n=4$) and characterization markers ($n=8$). Interestingly, no inclusion or exclusion of markers was required during this process, as from initial testing the proposed markers proved to accomplish the tasks they were selected for. However, modifications were required with regard to the specific combination of fluorochrome-conjugated reagents of the selected antibodies because of their technical features and performance. Technical issues that had an impact on the performance of specific fluorochrome-conjugated reagents included high plasma-cell baseline autofluorescence levels, the amount of expression of individual markers (for example, CD38, β2 microglobulin, CD56) and the availability of high-quality fluorochrome-conjugated antibodies for specific fluorochrome positions. In turn, particular attention was also paid to the light scatter positioning of plasma cells (SSC$^{int/hi}$ and FSC^{hi}) with the PCD panel that should completely fall inside the pre-established FSC and SSC window of analysis, influencing the reference values set for these parameters.

Table 9. Utility of PCD markers

Tube	*Target antigen*	*Identification of plasma cells*	*Aberrant markers*	*Assessment of plasma cell clonality*
BB markers	CD38	X	C	
	CD138	X		
	CD45	X	C	
	CD19	X	C	
Tube 1	Cylgλ			X
	Cylgκ			X
	CD56		C	
	β2 micro		S	
Tube 2	CD27		S	
	CD28		S	
	CD117		S	
	CD81		S	

Abbreviations: BB, backbone; C, common aberrant marker; Cy, cytoplasmic; β2 micro, β2 microglobulin; PCD, plasma cell disorders; S, second diagnostic level marker.

Design of the PCD EuroFlow panel

Overall, we analyzed a total of 214 samples from 10 healthy subjects and 204 patients. The samples included monoclonal gammopathies studied at different time points of the disease (diagnosis, relapse and follow-up) ($n=173$), B-CLPD patients studied at diagnosis who showed no involvement of the plasma cell compartment ($n=3$) and patients with non-plasma cell-related diseases (Non-PCD) ($n=28$). In brief, 114 BM were analyzed with versions $1-5$ (Table 8) and 100 samples (98 BM and two PB) with the final version 6 of the PCD panel (Table 8).

Evaluation of version 1 (Table 8) of the PCD EuroFlow panel highlighted the problems caused by the high levels of expression of both CD38 and β2 microglobulin on normal plasma cells, when PerCPCy5.5- and FITC-conjugated reagents were used, respectively. Such high fluorescence intensity levels were associated with inappropriate fluorescence compensation profiles and fluorescence intensity levels falling out of the window of analysis. Therefore, attempts were made to accommodate CD38 and β2 microglobulin in other fluorescence channels for which they show lower fluorescence intensity and lower fluorescence spillover into other channels. For this purpose, CD38-AF700 (versions 2 and 3 of the PCD EuroFlow panel), CD38-APCH7 (versions 4 and 5) and CD38-FITC (version 6) conjugates were further evaluated. Overall, the best performance was obtained with the CD38-FITC reagent (version 6). In turn, β2 microglobulin was posted as a candidate for the PerCPCy5.5 channel. Owing to the need for a custom conjugate, it could only be evaluated in version 5 of the panel, with acceptable performance. Final tuning of the CD38-FITC and β2 microglobulin-PerCPCy5.5 was required in version 6 of the PCD panel for both reagents (Table 8) to further decrease the fluorescence intensity obtained for plasma cells. For these markers, such fine-tuning was achieved by mixing each reagent with the corresponding unconjugated reagents of the same CD38 and β2 microglobulin clones. With this strategy, the overall performance of the two markers improved and matched the desired fluorescence intensity profiles. The mixture of unconjugated and conjugated reagent ensures saturating conditions and avoids unexpected variations in the staining patterns because of changes in cell concentrations, which would otherwise be observed when attempts to reduce fluorescence intensity are just based on reducing the concentration of the fluorochrome-conjugated antibody.[123] The optimal CD38 (clone LD38) staining was obtained with a 3/2 mixture of the conjugated and of the purified antibody, respectively. The optimal β2 microglobulin (clone TÜ99) staining was obtained with a 19/1 mixture of the conjugated and of the purified antibody reagents, respectively.

Similarly, the reagent initially selected for cytoplasmic detection of Igλ light chains—anti-Igλ(polyclonal)-FITC (DAKO, Glostrup, Denmark)—also resulted in positive cells falling out of the window of analysis used for the FITC-associated fluorescence channel, and beyond the linearity range of the corresponding fluorescence detector. Thus, anti-Igλ was also evaluated as a PE-conjugated reagent (versions 3–5 of the PCD panel; Table 8) with similar limitations and as an APCH7-conjugated antibody reagent (version 6), which was finally selected. Noteworthy, the Cylgκ-APC and Cylgλ-APCH7 staining resulted in adequate discrimination of Cylgκ$^+$ versus Cylgλ$^+$ normal plasma cells and identification of Cylg light-chain restriction by clonal plasma cell populations. However, the staining pattern for both reagents on other Smlg$^+$ B-cells was not as discriminative.

Another marker for which different reagents were tested was CD45. CD45 – PacO conjugates were initially selected and evaluated as a potential alternative to CD45 – AmCyan in order to have uniformity across all EuroFlow panels (see other sections of this manuscript). However, in the PCD panel, CD45 – PacO resulted in poor discrimination between CD45$^-$ and CD45^{lo} plasma cell populations. This was due to the relatively high plasma cell baseline autofluorescence levels in the PacO fluorescence channel, which exceeded the resolution power of this reagent for discrimination of CD45$^-$ versus CD45^{lo} plasma cells. AmCyan was then preferred and kept for subsequent versions of the PCD EuroFlow panel (versions 2–5). However, this fluorochrome conjugate was associated with suboptimal results due to fluorescence spillover into the FITC channel,[16] forcing further evaluation and selection of an alternative reagent: CD45–PacB.

CD138 was then selected as the marker to occupy the PacO position when CD45 was removed from this fluorochrome position. CD138-conjugated reagents were absent from version 1, due to the lack of a conjugate to fit the position proposed

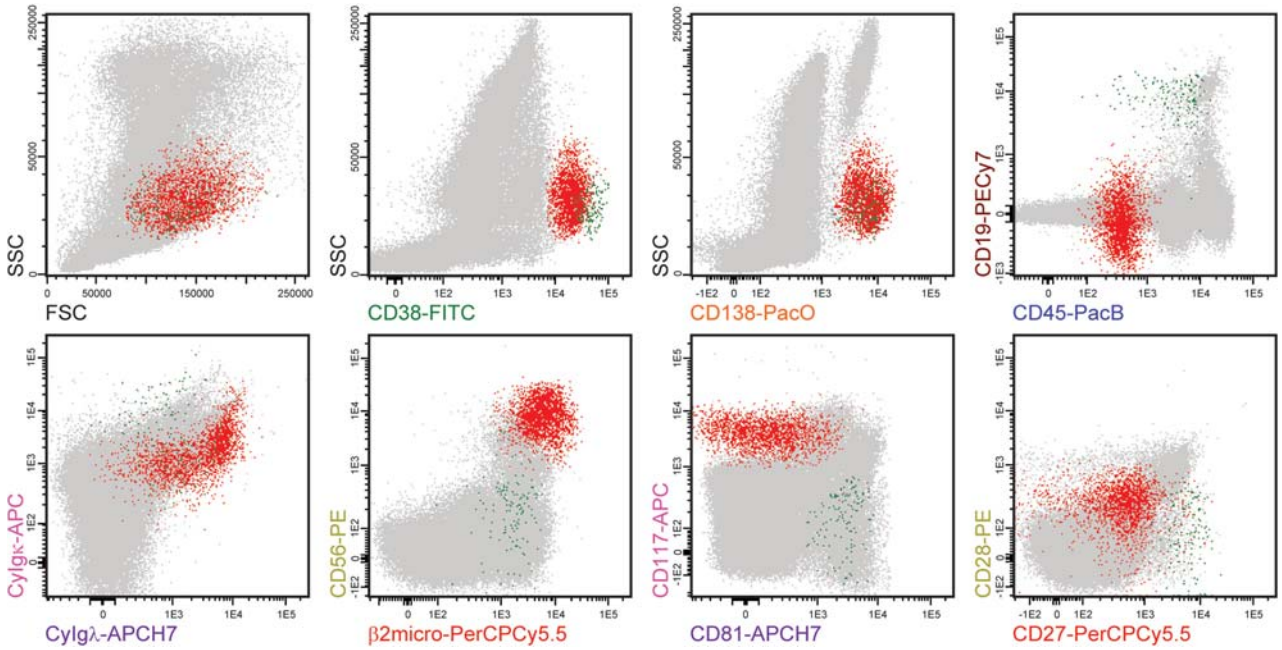


Figure 12. Example of a bone marrow (BM) sample from a monoclonal gammopathy of undetermined significance (MGUS) patient stained with the final version (version 6) of the PCD EuroFlow panel illustrating its power for the identification of plasma cells and discrimination between their normal/polyclonal and clonal counterparts. Normal plasma cells (green dots) show a typically normal immunophenotypic profile and coexist in this sample with a clonal population of plasma cells (red dots), which show multiple aberrant phenotypes—CD38^{lo}, CD45$^-$, CD19$^-$, CD56^{hi}, CD117$^+$, CD81^{lo}, CD28^{lo} and CD27$^-$—together with high expression of β2 microglobulin. The polyclonal versus (mono)clonal nature of both plasma cell populations is confirmed by their pattern of expression of cytoplasmic immunoglobulin (Cylg) κ$^+$ and Cylgλ$^-$ (normal Cylgκ/Cylgλ ratio versus Cylgλ$^+$ restricted expression, respectively).

(for example, APCH7) and it was further evaluated in other positions such as PerCPCy5.5 (versions 2–4), and FITC (version 5), which subsequently had to be assigned to other problematic reagents (β2 microglobulin and CD38, respectively). CD138 was considered as a less problematic marker whose utility relies on the distinction between CD138$^+$ plasma cells and other CD138$^-$ cells and it was finally included in the panels as a CD138 – PacO reagent (version 6), ordered as a custom conjugate. Unexpectedly, when this marker was introduced, decreased fluorescence intensity was observed when sample processing included only surface markers (using FACS Lysing solution, BD Biosciences, San Jose, CA, USA) versus intracellular staining for the detection of Cylg chains (using the Fix&Perm reagent) (data not shown). Because of this, it was decided that both tubes of the panel should be processed similarly with Fix&Perm, until this issue would be solved in order to reduce intra-sample variability due to use of different sample preparation procedures for the two tubes included in the PCD EuroFlow panel. Relocation of other markers in the combination was mainly caused by the modifications required to set the CD45, CD138, CD38 and β2 microglobulin markers at an optimal position, as explained above.

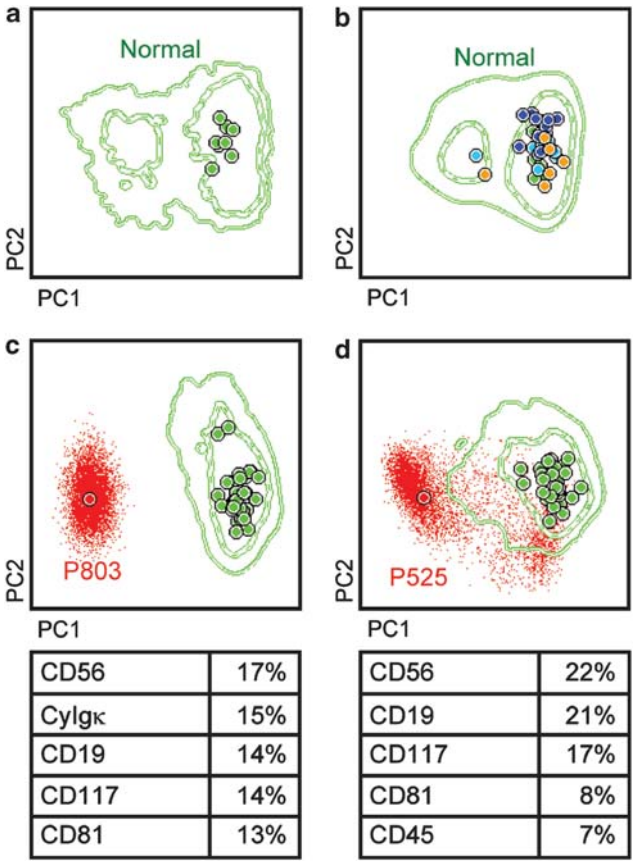
Evaluation of the PCD EuroFlow panel

Among the 100 samples analyzed with version 6 of the PCD EuroFlow panel, 38 corresponded to patients with MM, 23 to patients with MGUS, 2 to plasma cell leukemia patients and 6 to diagnostic samples from patients with suspected PCD (2 MGUS, 2 MM, 1 plasmacytoma and 1 IgM paraproteinemia), but no infiltration by aberrant plasma cells as confirmed by local routine approaches. The other BM samples were from 10 patients with non-PCD who underwent BM evaluation at diagnosis, 13 from regenerating BM after chemotherapy indicated for non-PCD and 8 normal adult BM samples from healthy subjects. In every sample, total BM plasma cells were identified based on their CD38$^{int/hi}$, CD138$^+$ phenotypic profile in association with intermediate to high FSC and SSC characteristics, after excluding cell doublets in a FSC area versus FSC height dot plot.

Further multiparameter flow cytometry gating of the distinct plasma cell populations and analysis of their phenotypic features showed that with version 6 of the PCD EuroFlow panel we were able to detect and fully characterize the immunophenotype of aberrant plasma cells versus their normal/reactive counterparts (Figure 12) in every sample. This included samples that contained aberrant plasma cells ($n = 63/63$ samples) and/or normal/reactive plasma cells ($n = 88/88$) as assessed by routine diagnostic protocols performed in parallel in the eight participating centers. In 49/49 samples coexisting clonal and normal plasma cells were detected with the two approaches. Noteworthily, in all cases evaluated, tube 1 could distinguish between the two plasma cell compartments based on their distinct phenotypes.

These individual data files were then merged to create a reference pool of normal plasma cells. PCA showed that all normal plasma cell populations clustered together in the APS1 (PC1 versus PC2) view of the Infinicyt software. This indicates that normal plasma cells from different healthy subjects show an overlapping phenotypic profile as defined by the 12-dimensional space, generated by all markers evaluated (Figure 13).

Similarly, normal/reactive plasma cells from patients suspected of carrying a PCD in which no abnormal plasma cells were detected ($n = 6$), patients evaluated for a non-PCD at diagnosis



CD56	17%
Cylgκ	15%
CD19	14%
CD117	14%
CD81	13%

CD56	22%
CD19	21%
CD117	17%
CD81	8%
CD45	7%

Figure 13. Automated population separator (APS) views of illustrating principal component analyses (PCA) of the distinct immunophenotypic profiles of plasma cells from healthy donors and two different multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) patients, based on the expression of the 12 markers included in the plasma cell disorders (PCD) EuroFlow panel (Version 6). (**a**) Simultaneous analysis of bone marrow (BM) plasma cells from healthy donors ($n = 8$; green circles). (**b**) Overlapping profiles of plasma cell populations from the same healthy donors ($n = 8$; green circles) when compared to those of BM plasma cells from non-infiltrated patients with distinct PCD at diagnosis ($n = 6$; orange circles), non-plasma cell-related diseases (non-PCD) at diagnosis ($n = 10$; light blue circles) and BM plasma cells from non-PCD patients studied after chemotherapy ($n = 13$; dark blue circles); noteworthily, polyclonal plasma cells from all these cases phenotypically overlapped with BM plasma cells from healthy subjects (**b**), although polyclonal plasma cells from two samples (one PCD and one non-PCD patient studied at diagnosis, who showed no BM infiltration by clonal/aberrant plasma cells) showed overlapping phenotypes with clearly shifted median values, appearing as separated from the main cluster due to increased numbers of CD19$^-$ plasma cells with a normal phenotypic profile. The two lower panels (**c**, **d**) show illustrating examples of the distinctly aberrant immunophenotypic profiles of clonal plasma cells in two different patients (red circles and dots) with MM (**c**) and MGUS (**d**). Noteworthily, in **c** a single group of clonal plasma cells is observed, which clusters separately from the normal/reactive plasma cell cluster, while in **d** two groups of plasma cells were present in the MGUS patient BM: a polyclonal (phenotypically normal) plasma cell population that clustered together with the reference pool of normal plasma cells and a clonal plasma cell population that clustered separately from normal plasma cells (red dots). The five most informative markers contributing to the best discrimination between each of the two clonal plasma cell populations and the corresponding normal plasma cell reference pool are displayed at the bottom of each plot, in a decreasing order of percentage contribution to the discrimination (**c**, **d**). Each circle represents one single case (median expression observed for all phenotypic parameters evaluated), while contour lines represent s.d. curves (dotted and broken lines represent 1s.d. and 2s.d., respectively); dots correspond to single BM plasma cell events from the MM (**c**) and MGUS (**d**) patients represented.

($n = 10$) and patients after chemotherapy ($n = 13$), also clustered together upon the same analytical approach as described above. When analyzed together, plasma cell populations from these reactive/regenerating samples, clustered together with the reference pool of normal plasma cell populations, which confirms that they have a very similar and overlapping immunophenotypic profile (Figure 13b). Noteworthily, in two of these samples (one corresponding to a non-infiltrated PCD sample studied at diagnosis and one from a patient with a non-PCD) polyclonal BM plasma cells clustered slightly apart from the main cluster of normal plasma cells due to an increased percentage of $CD19^-$ plasma cells with an otherwise normal polyclonal phenotype (Figure 13b). Notably, no clear discrimination could be made between samples stained at different centers.

Conversely, when each abnormal plasma cell population from individual patient samples was processed and analyzed as described above, and compared to both the normal and the reactive/regenerating plasma cell reference pools, it emerged as being clearly different from both clusters of polyclonal plasma cells in the APS diagrams, for all infiltrated PCD patient samples tested (illustrated for two patients in Figures 13c and d). Noteworthily, a clear discrimination could already be obtained for every patient with the eight markers of tube 1, the other four markers contributing to improve the separation between normal and aberrant plasma cells in a significant percentage of cases. Interestingly, when data files with information about clonal plasma cells from all altered samples were analyzed and displayed together in the APS view, two clearly different clusters of abnormal plasma cell populations were observed; these clusters reflected different phenotypic profiles, which mostly corresponded to cases with $CD56^{hi}$ versus $CD56^{-/lo}$ aberrant plasma cells.

CONCLUSION

Multiparameter flow cytometric immunophenotyping proved to be an efficient approach for the detection of normal/reactive versus aberrant plasma cells, the calculation of their relative distribution in a sample and the detailed characterization of their immunophenotypic profiles in patients with different subtypes of monoclonal gammopathies. Such information has previously proven to contribute to the diagnosis, classification and prognostic stratification of this clinically heterogeneous group of disorders. Here we propose a panel of 12 distinct markers based on two 8-color tubes, for accurate identification of plasma cells, specific detection and quantification of phenotypically aberrant versus normal/polyclonal plasma cell populations and detailed characterization of their immunophenotypic profiles. The two proposed tubes contain four backbone markers in common (CD38, CD138, CD45 and CD19) and eight additional markers that were equally distributed in tube 1 (CD56, β2 microglobulin, Cylgκ and Cylgλ) and in tube 2 (CD27, CD28, CD81 and CD117). As tube 1 proved sufficient for the specific identification, enumeration and discrimination between normal/reactive and aberrant plasma cells, it can be used as a standalone tube for the initial screening of PCD in a two-step diagnostic approach (Figure 1). In such case, tube 2 could be optional and used for further evaluation and characterization of the altered plasma cells, only when this is indicated.

SECTION 5. ANTIBODY PANEL FOR B-CELL PRECURSOR ALL (BCP-ALL)

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BACKGROUND

The EuroFlow BCP-ALL antibody panel aims at recognition and classification of all immature B-lineage malignancies, that is, the neoplasms of precursor B-cells.[34] Immunophenotypically, BCP-ALL resemble normal precursor B-cells with B-lineage antigen expression (for example, CD19, CyCD79a, CyCD22) and other phenotypic characteristics, mimicking to a certain extent the phenotypic stage of maturation arrest.[124] Conversely, expression of some antigens differs significantly from normal by being asynchronous, ectopic or aberrant. This information can be assessed by multiparameter flow cytometry and is critical for the distinction between normal and leukemic cells and to make (or exclude) the diagnosis of BCP-ALL. It is also useful to establish a phenotypic signature at diagnosis for subsequent MRD monitoring by flow cytometry.[125]

Flow cytometry has a critical role in BCP-ALL diagnosis based on lineage assignment and the phenotypic features of maturation arrest.[27,34] Most of this information relies on combinations of multiple markers. As an example, the European Group for the Immunological Classification of Leukemias (EGIL) uses a combination of four markers (CD10, Cylgμ, SmIgM or Ig light chains) for this purpose.[27,34] Some phenotypic profiles correlate with recurrent molecular abnormalities, but they are complex and do not rely on the expression of a single marker.[36,45] For example, the E2A-PBX1 rearrangement is suspected in a $CD19^+/CD10^+/CD20^{-/lo}/CD34^-/CD9^+/Cylg\mu^+$ phenotype.[126] A series of phenotypic patterns associated with prognosis, genotypic aberrancies or maturation stage of arrest have been reported based on expert and consensus experiences and mainly qualitative (presence or absence of expression) analyses of a relatively limited number of markers.[27,45] This undoubtedly does not reflect the whole phenotypic complexity of BCP-ALL. Deciphering the heterogeneity of BCP-ALL requires integration of the precise expression levels of a wide range of antigens, collected from the leukemic cells of many different BCP-ALL patients. Consequently, harmonized analysis of BCP-ALL cases including all potentially relevant markers for subclassification of BCP-ALL is needed. The aim of the EuroFlow Consortium was to establish a standardized procedure for the immunophenotypic diagnosis and classification of BCP-ALL based on a well-defined set of antibody reagents that can be used in combination with newly developed software tools to perform multiparameter analysis of antigen profiles. The BCP-ALL panel developed consists of a comprehensive 8-color four-tube antibody panel. More precisely, the BCP-ALL panel was designed to reach the following goals: (i) to identify normal B-cell precursors; (ii) to detect phenotypic aberrations allowing distinction between normal and regenerating B-cell precursors and BCP-ALL; (iii) to document the phenotype of leukemic cells versus their normal counterparts, including identification of both common and divergent patterns to determine the stage of maturation arrest and propose relevant leukemia-associated phenotypes (LAP) for MRD assessment, respectively; (iv) to detect immunophenotypic profiles associated with recurrent oncogenic abnormalities; and (v) to provide prognostic information.

Design of the EuroFlow BCP-ALL panel

The BCP-ALL panel was designed to be applicable with both conventional data analysis procedures and innovative data analysis tools to take optimal advantage of n-dimensional flow

cytometry. In order to allow linkage of the data from the different BCP-ALL tubes, an optimal combination of backbone markers (present in every tube) and characterization markers was used as the basic strategy to construct the panel.

Selection of backbone markers. An optimal combination of backbone markers should allow identification and subsequent gating of B-cell blasts in every BCP-ALL case. The backbone markers should also provide unique positions for individual blast cells inside the n-dimensional space detected in common in every tube in the panel, in order to allow accurate calculations of data for each BCP-ALL cell.[16] As for all other EuroFlow panels, the backbone should include a limited number of markers in order to enable inclusion of a maximum of characterization markers within a limited number of tubes.

Overall, three backbone markers were found to be sufficient. The same three backbone markers were used in ALOT to allow the combination of data obtained with both protocols. Up front, candidate backbone markers were limited, and most of them effectively met the afore-mentioned criteria. These included CyCD79a, CyCD22, CD19, CD45, CD34 and CD10. CyCD79a and CyCD22 were discarded in order to avoid intracellular staining and because these antibodies did not demonstrate a clear added value over CD19 for the identification of B-cell precursors. CD19 was chosen for the backbone as it is expressed at an early stage of B-cell commitment and in virtually all BCP-ALL cases. CD45 was also retained as a particularly efficient marker in BCP-ALL for blast cell gating and exclusion of residual normal cells, especially CD45^{hi} mature B cells.[61] In addition, CD45 displays increasing levels of expression during B-cell development, thus also contributing to deciphering immature subpopulations among normal B-lineage cells.[5,11,124]

The two selected markers, however, were not enough, and so a third backbone marker was required with two candidates: CD10 and CD34. CD10 is frequently but variably expressed in BCP-ALL, and represents both an immaturity and B-cell lineage-related marker with a variable expression pattern along normal B-cell development.[5,11,124] Conversely, CD34 is a marker of immaturity, which is not lineage-related and is frequently (but not always) expressed in BCP-ALL. When expressed, CD34 is useful for identification of the blast cells. However, CD34 expression does not reflect normal B-cell development as good as CD10, as its expression is restricted to the earliest stages of B-cell development and is rapidly lost in later stages.[5,11,124] Evaluation of the patterns of expression of both markers showed that BCP-ALL has bimodal or partial expression pattern of CD10 and CD34 in around 1% and 8% of cases, respectively. Consequently, CD34 best reflects the intraclonal heterogeneity of malignant B-cell precursors. On top of this, many CD34 antibody conjugates that work well are currently commercially available, whereas CD10 detection is highly dependent on the specific labeling used, with decreasing levels of sensitivity from PE to APC, PECy7 and FITC conjugates. Accordingly, we decided to add CD34 to the CD19 and CD45 backbone markers.

Comparative testing showed that CD45 – AmCyan should be replaced by CD45 – PacO, which did not negatively affect the FITC channel. CD34 – PerCPCy5.5 was selected based on its excellent staining properties and a sensitive CD19 – PECy7 conjugate was chosen because of the brightness of this fluorochrome, and the better performance observed versus CD19 – PacB (custom-conjugated; data not shown). The final set of backbone markers selected for the BCP-ALL panel therefore consisted of CD45 – PacO, CD34 – PerCPCy5.5 and CD19 – PECy7, and it proved to allow gating of the entire tumor B-cell population in every BCP-ALL case out of the 52 tested; noteworthily, the same antibody conjugates were shared with the ALOT to enable linkage of data from both panels.

Selection of additional characterization markers. Once the backbone markers were set, a list of characterization markers was made, based on previous reports and the experience of the EuroFlow laboratories. These markers were then prioritized into first- and

Table 10. Design of BCP-ALL panel in four consecutive testing rounds[a]

Version (no. of cases)[b]	*Tube*	*Fluorochromes and markers*							
		PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*AF700*
1 ($n = 14$)	1	CD20	CD45	CD58	CD66c	CD34	CD19	CD10	CD38
	2	SmIgκ	CD45	SmIgM	CyIgμ	CD34	CD19	CD123	SmIgλ
	3	CD21	CD45	NuTdT	CD13	CD34	CD19	CD22	CD24
	4	CD81	CD45	CD15 and CD65	NG2	CD34	CD19	CD33	CD9
2 ($n = 17$)	1	CD20	CD45	CD58	CD66c	CD34	CD19	CD10	CD38
	2	SmIgκ	CD45	CyIgμ	CD123	CD34	CD19	SmIgM	SmIgλ
	3	CD21	CD45	NuTdT	CD13	CD34	CD19	CD81	CD24
	4	CD9	CD45	CD15 and CD65	NG2	CD34	CD19	CD33	CD22
		PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
3 ($n = 35$)	1	CD20	CD45	CD58	CD66c	CD34	CD19	CD10	CD38
	2	SmIgκ	CD45	CyIgμ	CD33	CD34	CD19	SmIgM and CD117	SmIgλ
	3	CD21	CD45	NuTdT	CD13	CD34	CD19	CD22	CD24
	4	CD9	CD45	CD15 and CD65	NG2	CD34	CD19	CD123	CD81
4 (Final) ($n = 149$)	1	CD20	CD45	CD58	CD66c	CD34	CD19	CD10	CD38
	2	SmIgκ	CD45	CyIgμ	CD33	CD34	CD19	SmIgM and CD117	SmIgλ[c]
	3	CD9	CD45	NuTdT	CD13	CD34	CD19	CD22	CD24
	4	CD21	CD45	CD15 and CD65	NG2	CD34	CD19	CD123	CD81

Abbreviations: AF700, alexa fluor 700; ALL, acute lymphoblastic leukemia; AmCyan, *Anemonia Majano* cyan fluorescent protein; APC, allophycocyanin; BCP, B-cell precursor; BM, bone marrow; Cy, cytoplasmic; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, hilite7; Nu, nuclear; PacB, pacific blue; PacO, pacific orange; PB, peripheral blood; PE, phycoerythrin; PerCPCy5.5, peridinin–chlorophyll–protein–cyanin5.5; Sm, surface membrane. [a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 215 samples (172 BM, 36 PB and 7 other types of samples) was evaluated. Among them, 193 corresponded to BCP-ALL patients and 18 and 4 BM and PB from healthy donors, respectively. [c]Testing of CyIgλ-APCC750 is ongoing to increase the stain index of CyIgλ cells. Highlighted boxes: changes as compared to previous version.

Table 11. Utility of BCP-ALL markers

Tube	*Target antigen*	*Identification of blasts*	*Additional markers, 1st level*			*Additional markers, 2nd level*		*Aim per tube*
			Positive diagnosis	*Differential diagnosis*[a]	*Classification (e.g. maturation stage)*	*Classification (molecular aberrancy)*	*LAP identification*	
Backbone Markers	CD34	X	X			X		Identification of blasts
	CD19	X	X					
	CD45	X		X				
Tube 1	CD20		X				X	Diagnosis
	CD58						X	Classification
	CD66c					X	X	LAP markers
	CD10		X		X		X	Molecular
	CD38			X		X	X	subtypes
Tube 2	SmIgκ			X	X			Diagnosis Classification
	CylgμCylgμ		X		X			
	CD33			X				
	CD117			X				
	SmIgM			X	X			
	Smlgλ			X	X			
Tube 3	CD9					X	X	Diagnosis Classification LAP markers Molecular subtypes
	NuTdT		X					
	CD13			X				
	CD22		X				X	
	CD24		X			X	X	
Tube 4	CD21						X	Classification LAP markers Molecular subtypes
	CD15			X		X		
	CDw65			X		X		
	NG2					X		
	CD123			X			X	
	CD81						X	

Abbreviations: ALL, acute lymphoblastic leukemia; AUL, acute undifferentiated leukemia. BCP, B-cell precursor; Cy, cytoplasmic; LAP, leukemia-associated phenotypes; MPAL, mixed phenotype acute leukemia; Nu, nuclear; Sm, surface membrane. [a]Main differential diagnoses considered: mature B-cell malignancies, normal immature B-cells (hematogones), other acute leukemias with B-cell marker expression, MPAL/AUL.

second- level markers, based on their relevance for both adult and pediatric BCP-ALL. Noteworthily, few antibodies were conjugated with the new fluorochromes at the time the panel was designed. This required multiple custom conjugations, testing of the new antibody conjugates in comparison to the standard fluorochrome conjugates using reference samples, and evaluation of the newly designed antibody panel on a series of BCP-ALL samples. Each modification within the antibody panel had a significant impact on its configuration, so that a new round of testing was needed. The final configuration of the BCP-ALL panel is thus the result of a carefully thought-out design and multiple rounds of testing to optimize technical aspects, for example target antigens, fluorochrome conjugates and antibody combinations (Table 10).

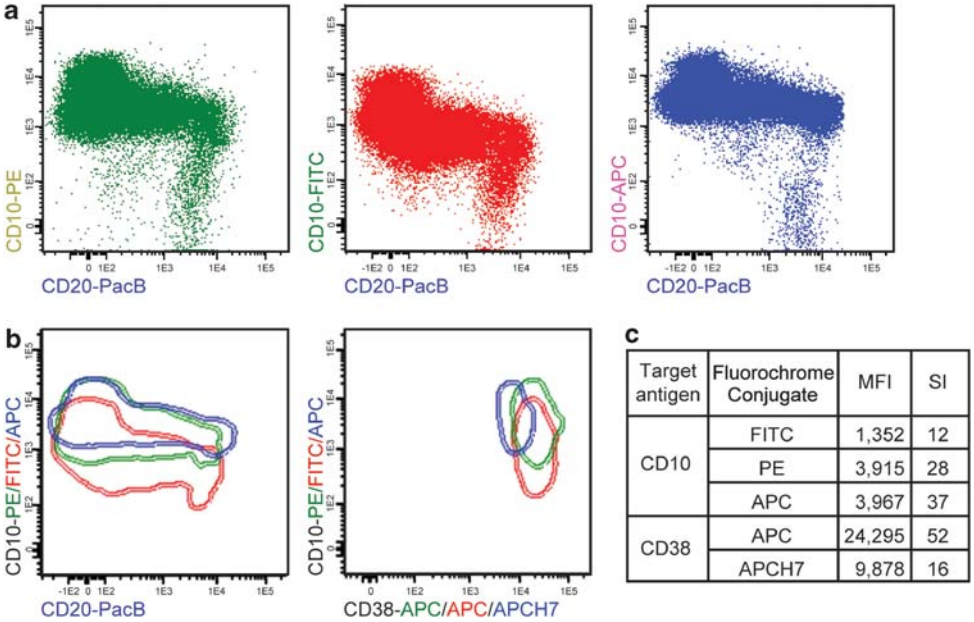
Most EuroFlow panels are designed in such a way that each tube is disease-oriented, lineage-related or aims at a specific goal. The BCP-ALL panel could not be easily built in this way, as many markers are associated with multiple aims. For instance, CD22 and CD10 are both useful for positive diagnosis of BCP-ALL and the identification of LAP for MRD assessment. In addition, CD10 is also useful for subclassification of BCP-ALL. Nevertheless, each tube in the final panel has an underlying rationale that covers one or multiple aims as summarized in Table 11. The way in which the markers were combined should enable usage of both conventional data analysis procedures and software-based link of all information obtained with the panel.

Markers for positive and differential diagnosis of BCP-ALL. The first goal of the BCP-ALL panel is to make a diagnosis of BCP-ALL. For this purpose, the following specific aims should be reached: (1) confirmation of the immaturity of the suspected or abnormal cell population, (2) confirmation of the B-lineage origin, (3) exclusion of overlap with other cell lineages, and (4) demonstration of the malignant versus normal/reactive immunophenotype of the gated cell population. Asynchronous expression of antigens (for example, co-expression of $CD20^{hi}$ and $CD34^{hi}$), aberrant or ectopic antigen expression (for example, $CD33^{hi}$), and disappearance of normal phenotypic maturation kinetics, are all evidence in favor of the malignant nature of immature lymphoid B-cells.

The BCP-ALL panel included B-lineage-associated markers (CD22, CD24, CD10, CD20, Cylg, Smlg), markers for differential diagnosis with other acute leukemias (for example, CD13, CD33, CD117, CD15, CD65 to exclude AML) and other markers useful for comparison with and distinction from normal B-cell development patterns (NuTdT, CD10, CD38, CD20, CD123).

CD22 is strongly associated with the B-lineage, but is not B-cell specific, as it is also expressed on normal basophils, mast cells and plasmocytoid dendritic cells (pDC).[65–67] Within lymphoid cells, its expression indicates B-cell commitment and it appears at a very early stage of normal B-cell development. CD22 was at first conjugated with AF700, but staining was too weak (mean SI on mature B-cells of 3.7) and so an APC conjugate was preferred (mean SI of 85.4) and selected. Similarly to CD22, CD24 is also expressed on B-cells at very early stages, but it is also present on mature granulocytic cells. The first CD24 reagent evaluated was conjugated with AF700 and gave an acceptable staining (mean SI of 6.6) despite being weaker than PE conjugates (mean SI of 12.7). AF700 was later compared with and replaced by a CD24-APCH7 reagent, which gave slightly brighter staining (mean SI of 15.2), in line with other EuroFlow antibody panels that contain CD24.

Myeloid antigens (CD13, CD33, CD117, CD15, and CD65) were added in order to detect CD19-expressing AML, which might not



c

Target antigen	Fluorochrome Conjugate	MFI	SI
CD10	FITC	1,352	12
	PE	3,915	28
	APC	3,967	37
CD38	APC	24,295	52
	APCH7	9,878	16

Figure 14. Performance of the distinct combinations of CD10, CD20 and CD38 used to identify B-cell precursors and evaluate normal B-cell maturation in the bone marrow. Representative dot plots of the different fluorochrome conjugates tested are shown. Thirteen bone marrow samples from reactive or regenerating bone marrows at various time points after chemotherapy were stained using the three combinations in parallel and analyzed together after merging. (**a**) Resolution of individual normal precursor B-cells is optimal using a CD10–PE reagent (green dots). Similar discrimination is reproduced using CD10–APC (blue dots) whereas discrimination is worsened when using CD10–FITC (red dots). (**b**) Virtual merging of the three previous configurations demonstrates that CD10–APC (blue line, left panel) provides equivalent discrimination. CD38–APCH7 (blue line, right panel), despite generating significantly weaker signals than CD38–APC (red line, right panel), correctly recapitulates resolution of the distinct subpopulations and places the most immature population close to the bright 10^4 level. (**c**) Mean fluorescence intensity (MFI) and stain index (SI) of the different reagents tested.

have been positively identified by the ALOT. Given that SmIgμ cannot be expressed independently of CyIgμ and that BCP-ALL virtually never shows CD117 expression, CD117 and SmIgμ were combined in the same tube in the same fluorochrome position. In case of CyIgμ negativity and SmIgμ + CD117 positivity, this latter signal can be attributed to CD117 and a SmIgμ$^-$ CD117$^+$ immunophenotype. CD15 and CD65 were also pooled in the same position, as the information they provide in BCP-ALL cases is similar and overlapping. In fact, both myeloid antigens may also represent cross-lineage markers, supporting the malignant nature of the BCP-ALL population, and to a certain extent they provide information that may contribute to subclassification of BCP-ALL (see below).

When immature lymphoid cells (also referred to as hematogones) are identified by cytomorphology, the primary clinical question may be reduced to the distinction between malignant and normal/regenerating B-cell precursors. Normal/regenerating B-cell precursors display dynamic and reproducible immunophenotypic maturation patterns with sequential and coordinated expression of multiple antigens,[5,11,124] while BCP-ALL cell populations are far more homogeneous. The normal precursor-B-cell maturation pattern is mainly characterized by decreasing expression of both CD38 and CD10 and increasing expression of CD20.[5,11,124] Consequently, these three markers were combined in a single tube and were compared in several combinations, for example CD10 – PE/CD38 – APC/CD20 – PacB, CD10 – FITC/CD38 – PE/CD20 – PacB and CD10 – APC/CD38 – APCH7/CD20 – PacB, together with the three backbone markers (Figure 14). The second combination was discarded because of the dim staining intensity of the CD10 – FITC conjugates (Figure 14c), while the first and the third combination were most efficient for assessment of normal B-cell development (Figure 14a and b). We opted for the latter in order to keep the FITC and PE channels free for other characterization markers. NuTdT is an immaturity marker which is mainly expressed in B and T lymphoid lineage cells but that is also found at a variable percentage in >25% of AML cases.[52,127–129] Consequently, this marker lacks lineage specificity but was found to be useful to (i) confirm the immaturity of B-cells and (ii) to contribute to the distinction between normal and malignant profiles, as NuTdT expression is normally restricted to very early B-cell precursors.

Noteworthily, the selected characterization markers can also contribute to re-orientation towards the LST/ B-CLPD protocol in cases of mature B-CLPD, which were initially misinterpreted on morphological grounds as compatible with an acute leukemia. Such re-orientation can be based on SmIgM, SmIgκ and SmIgλ in addition to CD45, CD34, NuTdT and CD38, a set of markers that enables distinction between mature and immature neoplastic B-cells.

Markers for subclassification of BCP-ALL. Information on the maturation stage of BCP-ALL generally relies on the expression of CD10, CyIgμ, SmIgM, CyIgκ and CyIgλ.[27,34] Consequently, we aimed to obtain this information from a single tube. For this purpose Igκ and Igλ were labeled with novel fluorochromes (PacB and APCH7, respectively). The two Igμ antibodies were initially placed in the FITC and PE positions, but Igμ-PE staining appeared to be unreliable (for example, high levels of unspecific staining). As Igμ-APC only worked well as surface staining, we finally opted for the CyIgμ-FITC/SmIgM-APC configuration.

Many antigens have been reported to be aberrantly expressed in BCP-ALL cases in association with specific recurrent molecular abnormalities.[45] Such markers were also selected albeit that they are positioned in different tubes, but the new software and data analysis tools can combine the information for subclassification of BCP-ALL. BCR-ABL-positive cases are associated with frequent expression of myeloid markers such as CD13 and CD33 in addition to CD34^{hi} and CD38^{lo}, typically without expression of CD117.[43,130] CD66c (KOR-SA3544) has also been reported to be preferentially

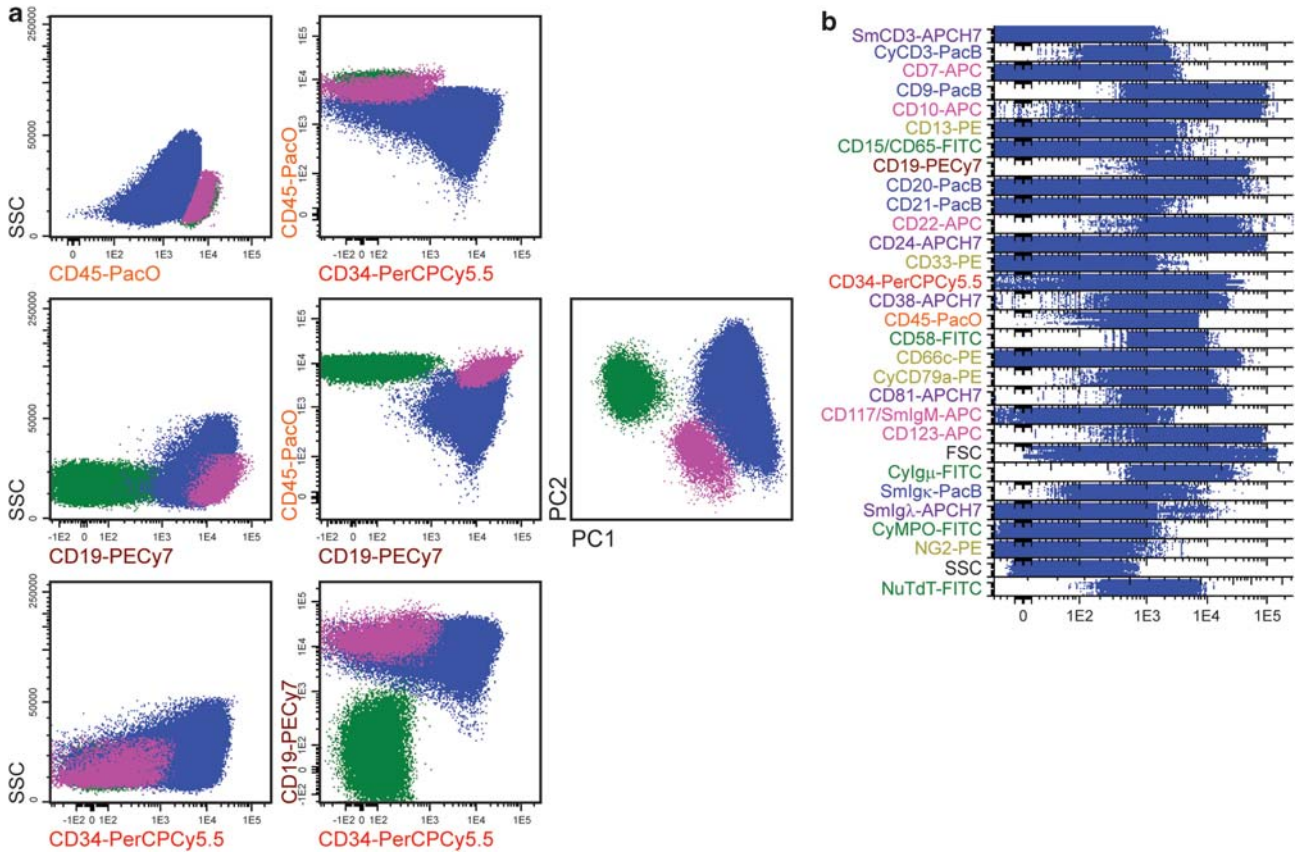


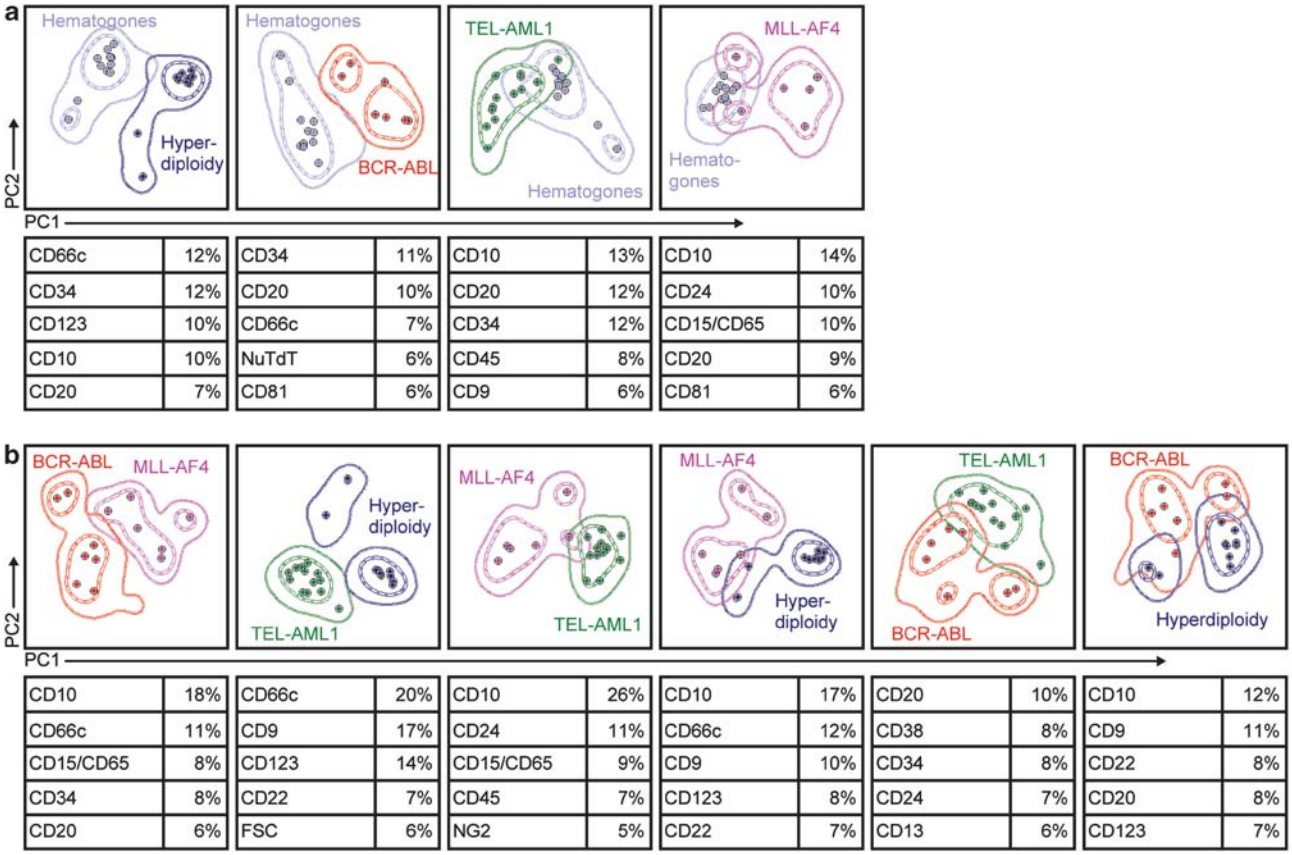
Figure 15. An illustration of the strategy used to gate B cell precursor (BCP)–acute lymphoblastic leukemia (ALL) blasts (**a**) and to evaluate their whole immunophenotypic profile using a band dot plot from the Infinicyt software (**b**). The BCP-ALL blast cell population is depicted as blue dots, while normal residual B- and T-cells are shown as purple and green dots, respectively.

expressed in *BCR-ABL* fusion gene and in hyperdiploid BCP-ALL cases.[131–133] Given the potential impact of CD66c, the antibody was placed in the strong PE channel. *MLL*-rearranged cases generally show a $CD19^{+}/CD34^{+}/NuTdT^{+}/CD10^{-}/CD15^{+/-}/CD65^{+/-}/CD9^{+}/CD24^{-/partially+}$ immunophenotype[134,135] with characteristic expression of NG2, although this is not specific.[136] NG2 (7.1) was introduced with the bright PE labeling. TEL-AML1 BCP-ALL are known to exhibit a $CD9^{-}/CD20^{-}/CD66c^{-}$ pattern in addition to CD19, CD10, NuTdT and CD34 positivity and $CD20^{-/lo}$.[42,137,138–140] CD9 was placed in the PacB channel and found to perform satisfactorily. Lastly, *E2A-PBX1* gene rearrangement is typically associated with a $CD19^{+}/CD10^{+}/CD34^{-}/CyIg\mu^{+}/SmIgM^{-}$ pre-B immunophenotype associated with negativity or partial positivity for CD20 and strong positivity for CD9.[126] Most other molecular abnormalities do not show clear phenotypic associations.

Markers for the identification of LAP and MRD assessment. MRD assessment by flow cytometry is increasingly used in the management of hematological malignancies, including BCP-ALL.[141–145] The CD19, CD34, CD45, CD10, CD38, CD20 combination (tube 1 of the BCP-ALL panel) is highly efficient in discrimination of normal/regenerating versus malignant immature B-cells and could represent an MRD-oriented combination.[140,141,146–148] LAP markers can further contribute to the discrimination between normal/regenerating and malignant B-cell precursors. Accordingly, in addition to the aforementioned CD22, CD24, CD66c and CD9 antigens, CD123, CD21, CD81 and CD58 were selected. CD123 is a major LAP marker, but is not consistently overexpressed by blast cells;[149] in the proposed BCP-ALL panel this antibody is conjugated with the bright APC fluorochrome. CD21 can be expressed by $CD19^{+}/CD34^{+}$ malignant B-cells while absent in their normal counterparts;[150] because of its satisfactory labeling a CD21-PacB reagent was chosen. CD81 is a tetraspanin molecule that is highly expressed on normal B-cell precursors, but is found at abnormally low levels in blast cells from a substantial percentage (>80%) of BCP-ALL patients.[151] CD58 is often overexpressed by blast cells versus regenerating normal precursor B-cells (for example, hematogones).[152,153] Because of design constraints, an FITC labeled CD58 reagent was selected. Although the CD58-FITC signal was weaker than the reference PE conjugate, the discrimination between normal and malignant cells was still satisfactory.

It is worth noting that the markers finally chosen for the BCP-ALL antibody panel were selected from an extensive list of candidate-antigens cited in the literature or considered to be relevant based on the experience of EuroFlow members. Each marker was discussed and many were not chosen (for example, CD52, ZAP-70, HLADR, CD135, CD44, CD11a, CD14, CyBcl-2, CD7, CD25, CD5, CD79b), because their utility was concluded to be of limited value compared to the selected markers.

BCP-ALL immunostaining protocol. Use of the BCP-ALL panel will usually be dictated by results obtained with the ALOT. In case of high suspicion of BCP-ALL (in children for instance), the ALOT and the BCP-ALL panel can be run at the same time, but the ALOT should always be interpreted first to ensure that another panel is not indicated. If the clinical suspicion and cytomorphological information from BM smears merely requires discrimination between hematogones and leukemic cells, application of the ALOT together with the first BCP-ALL tube will be sufficient to provide the correct answer.



CD66c	12%	CD34	11%	CD10	13%	CD10	14%
CD34	12%	CD20	10%	CD20	12%	CD24	10%
CD123	10%	CD66c	7%	CD34	12%	CD15/CD65	10%
CD10	10%	NuTdT	6%	CD45	8%	CD20	9%
CD20	7%	CD81	6%	CD9	6%	CD81	6%

CD10	18%	CD66c	20%	CD10	26%	CD10	17%	CD20	10%	CD10	12%
CD66c	11%	CD9	17%	CD24	11%	CD66c	12%	CD38	8%	CD9	11%
CD15/CD65	8%	CD123	14%	CD15/CD65	9%	CD9	10%	CD34	8%	CD22	8%
CD34	8%	CD22	7%	CD45	7%	CD123	8%	CD24	7%	CD20	8%
CD20	6%	FSC	6%	NG2	5%	CD22	7%	CD13	6%	CD123	7%

Figure 16. Illustrating principal component analysis (PCA) bivariate dot plot representations (automated population separator (APS) views) of the clustering obtained in the pairwise multivariate PCA of B-cell precursor (BCP)–acute lymphoblastic leukemia (ALL) immunophenotypes associated with distinct molecular subgroups of BCP-ALL cases and normal/regenerating hematogones (hematogones, gray circles; hyperdiploid cases, violet circles; BCR-ABL$^+$ cases, red circles; TEL-AML1$^+$ patients, green circles; and MLL-AF4$^+$ cases, pink circles). Each circle represents median values of individual cases for all immunophenotypic markers in the EuroFLow BCP-ALL panel contributing to principal component (PC)1 and PC2. Contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively). The five most informative markers contributing to the best discrimination between each pair of disease subgroups are displayed at the bottom of each plot, in a decreasing order of percentage contribution to the discrimination.

The BCP-ALL panel should be run using the EuroFlow SOP for instrument settings and immunostaining in combination with the Infinicyt software to allow appropriate linkage between the tubes and the ALOT. Of note, the ALOT and BCP-ALL panel includes tubes with only surface staining (tubes 1 and 4 of the BCP-ALL panel) and tubes with both surface and intracellular staining (tubes 2 and 3 of the BCP-ALL panel and ALOT). Each tube should be treated with the appropriate sample preparation protocol for staining of surface markers or surface plus intracellular markers. The different impact of the pre-analytical steps on light scatter parameters and fluorochrome intensities of backbone markers is automatically recognized and adjusted by the software (harmonization process),[33] allowing merging and calculation of marker expression based on parameters measured under different preanalytical conditions. This approach has been validated in B-CLPD[33] as well as on a series of 9 BCP-ALL samples using the BCP-ALL panel (data not shown).

Evaluation of the BCP-ALL panel using conventional versus multivariate data analysis approaches

In order to evaluate the BCP-ALL panel, its final configuration was run on 149 freshly collected cell samples from adult ($n = 37$) and childhood BCP-ALL cases ($n = 112$) derived from different tissues. Testing was carried out by seven laboratories, always in combination with the ALOT. Relevant clinical and biological annotations were collected whenever available, including the most relevant molecular abnormalities (for example, BCR-ABL, TEL-AML1, E2A-PBX, *MLL* rearrangements). To date molecular annotations were completed for 65/149 cases.

Data were analyzed locally using the Infinicyt software including specific gating of the blast cell population based on backbone markers. Noteworthily, the backbone markers selected proved to be highly efficient in specifically selecting all BCP-ALL blast cells in every case analyzed. Interpretation could be then performed in a conventional manner assisted by data analysis tools such as histogram band plots that instantly provided a phenotypic profile of the leukemic population (Figure 15). Through this approach, conventional subclassification of BCP-ALL such as EGIL classification was obtained; BI 18 cases (12%), BII 108 cases (73%) and BIII 23 cases (15%) with an identical distribution to that derived from local panels.

A key challenge for flow cytometry is to identify phenotypic features that could facilitate subclassification of BCP-ALL. Current WHO classification stratifies BCP-ALL according to molecular abnormalities.[34] Consequently, it will be important to determine to what extent flow cytometry immunophenotyping is able to identify genotypic subsets among the phenotypically heterogeneous group of BCP-ALL. Interestingly, multivariate PCA of the whole immunophenotype of BCP-ALL blast cell populations obtained with the ALOT plus BCP-ALL panel demonstrated recognizable phenotypic patterns that could distinguish different molecular-associated entities from both normal and reactive hematogones (Figure 16a) and between other molecular

subgroups (Figure 16b). Based on this strategy, we may be able to define phenotypic subsets of BCP-ALL, which are enriched for specific molecular abnormalities, potentially allowing orientation of genetic screening. It should be emphasized that the currently identified phenotypic profiles do not represent surrogate markers for detection of genetic aberrations. Further investigation of BCP-ALLs with extensive annotations and clinical correlations is required before further conclusions can be made.

CONCLUSION

The BCP-ALL antibody panel proved to be highly efficient in combination with the ALOT, in: (i) identifying BCP-ALL blast cells in a highly sensitive and specific way, (ii) distinguishing normal from leukemic B-cell precursors and (iii) subclassifying BCP-ALL into conventional maturation-associated subtypes of disease; additionally, preliminary data also suggest that the panel could contribute to the orientation of further molecular genetic screening for rapid identification of molecular subgroups of BCP-ALL.

SECTION 6. ANTIBODY PANEL FOR T-ALL

L Lhermitte[1], V Asnafi[1], S Böttcher[2], M Brüggemann[2], L Sedek[3], T Szczepański[3], E Mejstrikova[4], T Kalina[4], A Mendonça[5], P Lucio[5], VHJ van der Velden[6], H Wind[6], J Flores-Montero[7], JJ Pérez[8], M Muñoz[9], AS Bedin[1], JJM van Dongen[6], A Orfao[7] and E Macintyre[1]

[1]AP-HP, Paris, France; [2]UNIKIEL, Kiel, Germany; [3]SUM, Zabrze, Poland; [4]DPH/O, Prague, Czech Republic; [5]IPOLFG, Lisbon, Portugal; [6]ERASMUS MC, Rotterdam, The Netherlands; [7]USAL, Salamanca, Spain; [8]HUS, Salamanca, ES and [9]Cytognos SL, Salamanca, Spain

BACKGROUND

The EuroFlow T-ALL antibody panel aims at the (immunophenotypic) diagnosis and characterization of T-ALL. T-ALL includes a heterogeneous group of malignancies defined by the expansion of immature T-cell precursors blocked at specific stages of maturation, typically deviated from normal differentiation pathways.[56,154–158] Although T–ALL and T-cell lymphoblastic lymphoma (T-LBL) present differently, primarily with respect to involvement of BM and blood relative to tissue involvement,[159] they are very closely-related diseases. In particular, there are no significant immunophenotypic differences and the WHO 2008 classification considers them to be one disease entity.[35]

The T-ALL EuroFlow panel has been designed to be applied when the ALOT orientates towards an expansion of T-lineage precursors. In a first step, the panel aims at recognition of immature T-cells, which is in itself strong evidence in favor of T-ALL. T-cell development occurs primarily in the thymus, and identification of immature T-cells in PB, BM or tissues other than the thymus is *per se* abnormal. However, when immunophenotyping mediastinal biopsies, the panel should also be able to distinguish malignant from normal thymocytes. Furthermore, the panel aims at subclassification of T-ALL according to the T-cell lineage maturation stages and potentially also according to the underlying genetic subgroup. A large variety of somatic genetic markers contribute to T-ALL oncogenesis.[154,157,158,160] Some co-exist (for example, *NUP214-ABL1* and overexpression of *TLX1/TLX3*[161–163]) and may occur in subclones, suggestive of sequential acquisition. This genetic complexity is likely to result in a variety of deregulated transcriptional networks, which impact differently on immunophenotypic profiles. So far, relatively few immunophenotypic profiles in T-ALL have been associated with recurrent molecular abnormalities, compared to BCP-ALL or AML. However, some specific correlations do exist, for example, frequent CALM-AF10 rearrangements in CD2$^-$ and/or TCR$\gamma\delta^+$ T-ALL,[164] but this association is not constant or specific. Conversely, a significant number of T-ALL molecular abnormalities are associated with a specific T-cell maturation arrest[165] or gene expression profile.[154,166,167] Overexpression of TLX1 is typically associated with a cortical immunophenotype.[165,168] Despite phenotypic similarities between T-ALL and normal thymic development,[165] detailed multiparameter immunophenotyping also allows identification of protein expression patterns, which distinguish T-ALLs from their normal thymic counterparts.

Overall, the 8-color T-ALL antibody panel allows for positive and differential diagnosis of T-ALL and subclassification of the disease according to different classification systems and at the same

Table 12. Design of the T-ALL panel in four consecutive testing rounds[a]

Version (no. of cases)[b]	*Tube*	*Fluorochromes and markers*							
		PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*AF700*
1 ($n=7$)	1	SmCD3	CD45	NuTdT	CD99	CD5	CD10	CD4	CyCD3
	2	SmCD3	CD45	CD7	CD117	HLADR	CD8	CD1a	CyCD3
	3	SmCD3	CD45	TCR$\alpha\beta$	TCR$\gamma\delta$	—	CD56	—	CyCD3
	4	SmCD3	CD45	CD44	CD13	CD45RA	CD33	CD123	CyCD3
2 ($n=12$)	1	SmCD3	CD45	NuTdT	CD99	CD5	CD10	CD4	CyCD3
	2	SmCD3	CD45	CD2	CD117	HLADR	CD8	CD1a	CyCD3
	3	SmCD3	CD45	TCR$\alpha\beta$	TCR$\gamma\delta$	—	CD56	CyTCRβ	CyCD3
	4	SmCD3	CD45	CD44	CD13	CD33	CD45RA	CD123	CyCD3
		PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
3 ($n=29$)	1	SmCD3	CD45	NuTdT	CD13	CD5	CD10	CD1a	CyCD3
	2	SmCD3	CD45	CD2	CD117	CD4	CD8	CD7	CyCD3
	3	SmCD3	CD45	TCR$\gamma\delta$	TCR$\alpha\beta$	HLADR	CD56	CyTCRβ	CyCD3
	4	SmCD3	CD45	CD44	CD99	CD33	CD45RA	CD123	CyCD3
4 (Final) ($n=64$)	1	CyCD3	CD45	NuTdT	CD99	CD5	CD10	CD1a	SmCD3
	2	CyCD3	CD45	CD2	CD117	CD4	CD8	CD7	SmCD3
	3	CyCD3	CD45	TCR$\gamma\delta$	TCR$\alpha\beta$	CD33	CD56	CyTCRβ	SmCD3
	4	CyCD3	CD45	CD44	CD13	HLADR	CD45RA	CD123	SmCD3

Abbreviations: AF700, alexa fluor 700; AmCyan, *Anemonia Majano* cyan fluorescent protein; APC, allophycocyanin; BM, bone marrow; Cy, cytoplasmic; Cy5, cyanin5; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, hilite7; Nu, nuclear; PacB, pacific blue; PacO, pacific orange; PB, peripheral blood; PE, phycoerythrin; PerCP, peridinin–chlorophyll–protein; Sm, surface membrane; T-ALL, T-cell acute lymphoblastic leukemia. [a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 112 samples were evaluated (72 BM, 26 PB, 9 pleural effusions and 5 other types of samples); among them, 102 were from T-ALL patients and 10 from healthy donors. Highlighted boxes: changes as compared to previous version.

Table 13. Utility of T-ALL markers

Tube	*Target antigen*	*Identification of blasts*	*Additional markers, 1st level*			*Additional markers, 2nd level*		*Aim of tube*
			Positive diagnosis	*Differential diagnosis*[a]	*Classification (EGIL)*	*Classification (including TCR-chain and ETP-ALL)*	*LAP markers (MRD/MDD)*	
BB markers	CyCD3	X	X	X			X	Identification of blasts
	CD45	X	X				X	
	SmCD3	X			X		X	
Tube 1	NuTDT		X				X	Diagnosis
	CD99		X				X	Classification
	CD5		X	X	X	X	X	Maturation stage
	CD10			X			X	LAP markers
	CD1a		X		X	X	X	Assessment of minimal disseminated disease
Tube 2	CD2		X		X		X	Diagnosis Classification Maturation stage LAP markers
	CD117			X		X		
	CD4		X					
	CD8		X		X	X		
	CD7		X		X		X	
Tube 3	TCR$\gamma\delta$		X	X	X		X	Diagnosis Classification Maturation stage LAP markers
	TCR$\alpha\beta$		X		X			
	CD33			X		X		
	CD56			X		(x)		
	CyTCRβ					X		
Tube 4	CD44					(x)		Classification
	CD13			X		X		
	HLADR					X		
	CD45RA					(x)	X	
	CD123			X		X		

Abbreviations: ALL, acute lymphoblastic leukemia; AUL, acute undifferentiated leukemia; BB, backbone; Cy, cytoplasmic; ETP, early T progenitor; LAP, leukemia-associated phenotype; MDD, minimal disseminated disease; MPAL, mixed phenotype acute leukemia; MRD, minimal residual disease; Nu, nuclear; Sm, surface membrane; TCR, T-cell receptor. [a]Main differential diagnoses considered: mature T-cell malignancies, other acute leukemias with T-lineage marker expression, MPAL/ AUL; (x) to be assessed.

time it provides additional information that reflects T-ALL heterogeneity.

Design of the T-ALL EuroFlow panel

The T-ALL protocol was designed in parallel to the ALOT and the BCP-ALL antibody panel. As a consequence, the rationale for its design (including the choice of backbone markers) was similar to that used for these two other protocols. Here we present the specificities of the T-ALL antibody panel and refer to Sections 1 and 5 for general design concepts. Table 12 summarizes the design of the T-ALL panel in four consecutive steps, while the specific aims of the individual markers are summarized in Table 13.

Selection of backbone markers

In order to link data from ALOT and the different tubes of the T-ALL panel, we aimed to identify a fixed combination of backbone markers capable of identifying all blast cells in every case with minimal contamination by other residual cells. In contrast to other EuroFlow panels, eligible markers for backbone selection were limited because they had to closely match the two criteria of: (i) broad expression, and (ii) efficiency for blast cell identification. Candidate markers included CD7, CD5, CyCD3 and CD99.[35] CD99 was not universally expressed, especially in more mature T-ALL, and its signal was considered to be too weak to allow clear gating of blasts from residual normal T-cells. CD5 was usually expressed but it may be dim or negative, especially in a subset of immature T-ALL.[71] CD7 was virtually always expressed, but it is not lineage-specific.[35] CyCD3 fits perfectly with the above-referred criteria, as by definition it is constantly expressed in T-ALL[35,48,169] and usually at high levels, allowing easy gating of blast cells. However, CyCD3 must be interpreted in combination with SmCD3 to exclude normal mature residual (or reactive) T-cells, which requires the combined assessment of CyCD3 and SmCD3. Finally, blast cell selection was improved by adding CD45 to discard non-lymphoid cells, even if CD45 is most often expressed at a high intensity on T-ALL blasts, thus making them overlap with mature lymphoid cells.[170] Based on these considerations, we finally set the T-ALL backbone as CD45 – PacO, CyCD3 – PacB and SmCD3 – APCH7. The choice of fluorochromes resulted from the strategic decision to use less common fluorochromes for gating markers so that the more common fluorochromes could be used for characterization markers in a more flexible way. Importantly, three T-ALL backbone markers were also included in the ALOT to benefit from the ALOT information on CyMPO,[36] CyCD79a[62–64] and CD34[49] expression using the merge function of the Infinicyt software.[16]

Selection of additional markers

Once the set of backbone markers was defined, we then completed the panel with a wide variety of T-lineage-related markers in order to strengthen and specify the T lineage affiliation of the leukemic cells, subclassify T-ALL according to maturation

stage and distinguish blast cells from other residual cells in the sample.

Markers for differential diagnosis of T-ALL. To specify the T-lineage maturation stage of blast cells, we chose CD2, CD4, CD5, CD7, CD8, CD10, CD99, TCRαβ and TCRγδ to be included in the panel.[2] Collective experience and additional testing allowed identification of appropriate combinations of antibodies and fluorochromes. For example, although PerCPCy5.5 may be a relatively weak fluorochrome, CD5 – PerCPCy5.5 worked remarkably well in T-ALL. This is also true for CD2 coupled to FITC. On the other hand, a CD10 – FITC conjugate was considered unsatisfactory and was consequently replaced by a bright CD10 – PECy7 reagent. It was logical to place CD4 and CD8 in the same tube and the CD4 – PerCPCy5.5/ CD8 – PECy7 combination performed well upon testing. For the TCR, we initially chose TCRαβ – FITC/ TCRγδ – PE in order to optimize detection of TCRγδ$^+$ T-ALL, but our results showed no advantage of the signal intensity for TCRγδ – PE relative to TCRγδ – FITC and detection of TCRαβ with FITC was less satisfactory than with PE, as was detection with the WT31 antibody relative to the IP26A clone. We consequently chose the combination TCRγδ – FITC (IMMU510) and TCRαβ – PE (IP26A).

To indicate the precursor nature of T-cells, we also added TdT, CD1a, CD34 and CD99 to the panel.[35,171] Detection of NuTdT can be difficult, but the TdT – FITC reagent selected gives reliable and robust staining. As this antibody was used concomitantly with staining for CyCD3 we initially compared permeabilization with Fix&Perm and FACS Lysing solution as used for the AML panel (see Section 7), and we finally opted for the Fix&Perm protocol because of optimal CyCD3 staining. Expression of CD99 is often relatively weak and it was consequently important to use a bright fluorochrome, such as PE. CD1a performed well with FITC, PE and APC. In order to group immature T lymphoid markers in one tube, CD1a was placed in the APC channel, with satisfactory results. As CD34 was in the ALOT tube, it was not repeated in the T-ALL panel.

We also introduced myeloid antigens (CD13, CD33, CD117), in addition to MPO that is assessed in the ALOT, but it is worth noting that these antigens are not sufficient for classification as MPAL as they are only considered as evidence of cross-lineage expression within the 2008 WHO criteria.[35,36] However, they are mainly expressed in very immature T-ALL, especially among those cases that fit with the early T-cell progenitor (ETP) entity.[68,70,71] With respect to the differential diagnosis, T-lineage-related antigens such as CD2, CD7, and even CD5 and CyCD3 may be expressed by so-called NK-cell precursor lymphoblastic leukemia/ lymphoma (NK-ALL).[35] CD56 is present in the panel and as such can help to diagnose this exceptional and provisional entity. It may, however, be difficult to distinguish NK-ALL and immature T-ALL,[35,36] since bona-fide, but predominantly immature, T-ALL cases can express CD56.[36,172–176] Whether these are distinct entities remains unclear.[35,36] The expression of myeloid markers in immature T-ALL but not in NK-ALL may be helpful in this situation.[36] Difficulties with differential diagnosis from plasmacytoid dendritic cell leukemias, which can express T-cell-associated markers (for example, CD2, CD7), can be partly solved with the profiles of CyCD3, CD4, CD123 and CD56 expression and to a lesser extent also with HLADR and CD45RA.[177–179]

Markers for subclassification of T-ALL. T-ALLs are classified according to their maturation arrest and on the basis of differential expression of certain markers.[27,35,180] Four distinct stages were classically defined by the EGIL group based on CD2, CD3, CD5, CD7, CD8, CD1a, and TCR expression.[27] Application of this classification is possible with the EuroFlow T-ALL antibody panel because all markers are present. We felt, however, that other markers were required in order to identify the stage of maturational arrest of T-ALL relative to normal thymopoiesis. In particular, classification of T-ALL according to their TCR expression, including CyTCRβF1, SmCD3, TCRαβ and TCRγδ expression, was considered relevant[165] in order to identify three stages of (i) immature (CyCD3$^+$/CyTCRβF1$^-$/SmTCR$^-$), (ii) pre-αβ (CyCD3$^+$/ CyTCRβF1$^+$/SmTCR$^-$) and (iii) mature (CyCD3$^+$/SmTCR$^+$) T-ALL blasts, discriminating among TCRγδ$^+$ and TCRαβ$^+$ T-ALL, which have been previously demonstrated to have prognostic impact.[181] The CyTCRβF1 antibody was custom-conjugated with APC, titrated and validated on CyTCRβ positive cell lines (DND41, Jurkatt, RPMI, CEM) or negative cell lines (SIL-ALL, Kasumi). This antibody was then placed within the *TCR-tube*, along with TCRγδ, TCRαβ and both CyCD3 and SmCD3, thus allowing both EGIL and TCR maturation-associated classifications with the T-ALL antibody panel although it provides a dim staining. Additional markers (CD44, CD45RA, HLADR, CD13, CD33 and CD123) were added in order to contribute to maturational staging.[69,182–185] Some assess immaturity (e.g. CD123[186]) or allow detection of the recently identified (very immature) ETP-ALL cases.[68,70,71] Prognosis of patients with the later is poor and requires appropriate therapeutic stratification.[70,71] The EuroFlow T-ALL panel allows recognition of this entity by co-expression of CyCD3$^+$, CD5^{lo}, CD1a$^-$, CD8$^-$ with stem-cell/myeloid markers including notably, CD34, CD117, HLADR, CD13 and CD33.[70,71]

LAP markers for MRD assessment. As for the BCP-ALL panel, evaluation of LAP already at diagnosis is essential for MRD assessment by flow cytometry.[141,142,144,146,187,188] Given the fact that MRD is assessed in PB or BM samples where virtually no immature T-cells exist, LAP markers are mainly represented by markers that determine the precursor nature of T-cells (immaturity markers). Such markers include CD34 (in ALOT) and NuTdT, CD1a and CD10 in tube 1 of the T-ALL antibody panel.[189,190] Interestingly, distinction between normal and leukemic T-ALL cells could be achieved with a single tube, that is, tube 1 of the T-ALL antibody panel. Of note, the T-ALL panel was designed based on markers listed from the literature and our collective experience. Other potential target antigens (for example, CD52, CD65, CD15, Bcl-2, CD20, CD24, CD64, CD14, CD15, CD65, NG2 and CD38) were not included, as they were considered to provide little added value as compared to those that were selected (Table 13).

Evaluation of the T-ALL panel using conventional and multivariate analysis

To evaluate the utility and efficiency of the T-ALL panel in making the diagnosis and identifying T-ALL subgroups (64) T-ALL cases were prospectively analyzed using both the ALOT and T-ALL panels versus local panels. Data from each T-ALL case were compiled within a single 29-dimensional data file, as described in previous sections. It was possible to confirm the T-ALL diagnosis in virtually all cases and to simultaneously classify T-ALL according to the existing WHO 2008, EGIL and TCR-based immunophenotypic classifications.[27,35,165] In fact, malignant immature T-cells could be distinguished from normal mature T-cells and, more significantly, even from normal human thymocytes, as illustrated in Figure 17. Multiparameter analysis of groups of T-ALL cases that are based on the overall immunophenotypic features of blast cells cluster apart from each other and their correlation with genetic and clinical characteristics will probably allow identification of specific subgroups. However, this will require prospective immunophenotypic analysis of a much larger number of T-ALL cases with full genetic and clinical annotations, given the oncogenetic and clinical heterogeneity of T-ALL. This needs acquisition within an appropriate anonymized database. Such an anonymized database is currently being developed by the EuroFlow group.

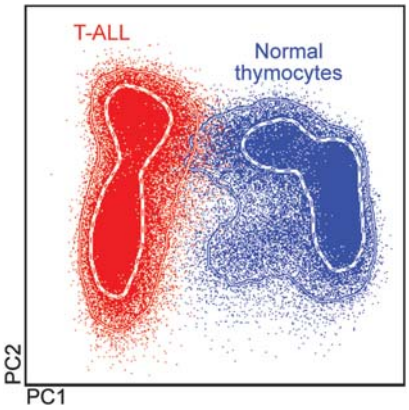


Figure 17. Overall immunophenotypic profile of T-acute lymphoblastic leukemia (ALL) blast cells (red dots) versus normal human thymocytes (blue dots) as assessed by the EuroFlow T-ALL panel. Of note, principal component analysis (PCA) showed a clear separation between the phenotypic profiles of normal versus leukemic T-cell precursors based on a principal component (PC)1 versus PC2 representation (automated population separator (APS)1 view of the Infinicyt software). Contour lines represent s.d. curves (dotted and broken lines represent 1s.d. and 2s.d., respectively).

CONCLUSION

The 4-tube T-ALL antibody panel in combination with the ALOT promises to be highly efficient for the diagnosis and phenotypic subclassification of T-ALL. At the same time it provides information about the presence of LAP that can be used for MRD monitoring. The panel is composed of a set of three backbone markers that allow simultaneous evaluation of the whole phenotypic profile of blast cells plus a set of markers devoted to the classification of T-ALL according to the EGIL and other alternative maturation-associated classification systems, together with the distinction between leukemic blasts and normal T-cell precursors. The markers are combined in such a way that the tubes facilitate flexible usage of the panel depending on the specific aim. For example, the EGIL classification of T-ALL is possible with tube 1, while definition of maturation stages according to TCR can be achieved with tube 3, and distinction between leukemic and normal cells is possible with solely tube 1.

SECTION 7. ANTIBODY PANEL FOR AML AND MDS

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BACKGROUND

The EuroFlow antibody panel for AML and MDS aims at the detection and classification (lineage assignment and definition of maturation profile) of AML and MDS. AML and MDS are both heterogeneous diseases, which, in contrast to T-ALL and BCP-ALL, can affect multiple cell lineages and multiple maturation stages. The EuroFlow AML/MDS antibody panel therefore focuses on all major myeloid lineages. This particularly concerns the neutrophilic lineage, monocytic lineage and erythroid lineage, but also the less frequent pDC lineage, basophilic lineage, mast cell and megakaryocytic lineages are covered. In addition to establishing the involved cell lineage(s) and maturational arrest(s) of the neoplastic cells, the EuroFlow AML/MDS panel also aims at the detection of aberrant expression of lymphoid-associated markers and abnormal lymphoid maturation profiles. The overall resulting immunophenotypic profile of the blast cells will allow classification of the malignancy and in some cases may be related to genetic abnormalities. For example, acute promyelocytic leukemia (APL) with t(15;17) typically has the immunophenotype of a promyelocyte (CyMPO$^+$, HLADR$^-$, heterogeneous CD13$^+$, homogeneous CD33$^+$, CD117$^+$) with abnormally negative to low CD15 expression,[41] while blast cells in AML with t(8;21) frequently co-express CD19[191] and in AML with inv(16) frequently are CD2$^+$.[192]

In patients with MDS, the role for flow cytometric immunophenotyping is not fully established, but recently immunophenotyping was included as one of the co-criteria for the diagnosis of MDS.[193] Several studies indeed have shown that immunophenotypic abnormalities can be detected in the vast majority of MDS patients, including abnormalities in lineages (or cases) with normal cytomorphological appearance.[194,195] However, as many immunophenotypic abnormalities are not specific for MDS, flow cytometric scoring systems are needed, but so far they have not been standardized.[10,196–203] International harmonization of flow cytometry MDS diagnostics is currently in progress.[14,204]

For patients with a suspicion of an acute (myeloid) leukemia, in the first instance the ALOT tube should be performed (see Section 1). If the results of the ALOT tube point towards a non-lymphoid acute leukemia (AML, ambiguous lineage acute leukemia or even pDC neoplasm), the AML/MDS panel should subsequently be performed. For patients with a clinical or cytomorphological suspicion of an MDS or an unexplained cytopenia, one may directly perform the AML/MDS panel (Figure 1).

Many antibodies have been reported to be useful for immunophenotyping of AML/MDS patients and several consensus reports have been published.[12,21,23,205] On the basis of these reports and the knowledge and experience of the EuroFlow members, several antibodies were selected and initially tested in the EuroFlow laboratories. After evaluation of the results during multiple EuroFlow meetings, positions of antibodies were changed or new antibodies were included/excluded in the antibody panel. The resulting antibody panel was subsequently tested again, and this cycle of testing–evaluation–optimization–retesting was repeated until the final EuroFlow AML/MDS panel was approved. The approved panel was also prospectively evaluated. In contrast to previous consensus reports, the EuroFlow AML/MDS antibody panel therefore is not solely based on knowledge, experience and opinions, but is also based on extensive testing and re-testing of many antibody combinations.

Design of the AML/MDS antibody panel

Design of the AML/MDS panel was based on a strategy that would search for the unequivocal identification of blast cells in every tube in the panel using the same set of backbone markers. In addition, a second group of markers was combined with the backbone markers, devoted to the characterization of the identified blast cells as well as of the maturation profile of BM precursors into the three major myeloid lineages (neutrophil, monocytic and erythroid lineages) and also several minor myeloid compartments less frequently involved in the disease (for example, basophil, mast cell, pDC and megakaryocytic precursors).

The combination of the backbone markers and new software tools allows a true multiparameter analysis of complete blast cell phenotypes with the EuroFlow AML/MDS antibody panel. With regard to the characterization markers, this antibody panel was designed in such a way that the more mature cell compartment (for example, CD34$^-$ precursors) of the three main myeloid cell lineages (granulocytic, monocytic, erythroid) could be optimally evaluated in single tubes. Using the backbone markers,

information from other tubes can easily be added to these 'lineage-oriented' tubes for the more immature (for example, $CD34^+$) precursor cells.

Selection of backbone markers. To optimally use the *n*-dimensional possibilities of the Infinicyt software including the merge and calculation functions, each tube needs to contain a number of identical antibodies that can be used to link the data from the other tubes. These so-called backbone markers should allow easy identification of the malignant population among remaining normal cells. Furthermore, they should reflect the potential heterogeneity of the malignant population in order to allow appropriate calculation of data based on the nearest-neighbor principle implemented in the Infinicyt software.[32] On the basis of knowledge and experience, CD34 and CD45 were directly selected as backbone markers for the AML/MDS panel. Other potential backbone markers, that is, CD117, HLADR, CD33 and CD11b, were evaluated on BM or PB samples from 96 AML patients. For this testing, the combination of HLADR(L243)–FITC, CD117(104D2)–PE, CD45(2D1)–PerCP, CD34(8G12)–APC, CD33(p67.6)–PECy7 and CD11b(ICRF44)–APCCy7 was used.

In 61/96 samples (63%), CD45 and CD34 were already sufficient for easy recognition of the heterogeneous leukemic population. In the remaining 25 patients, the additional value of the other markers was evaluated. The percentage of patients in which addition of CD117, HLADR, CD33 or CD11b allowed an easier gating of the leukemic cells was 51%, 40%, 20% and 23%, respectively. Inclusion of a fourth backbone marker gave best results for the combination CD34, CD45, CD117 and HLADR (9% extra patients). Addition of a fifth or sixth backbone marker hardly improved these results. Thus, by using CD34, CD45, CD117 and HLADR as backbone markers, efficient (sensitive and specific) gating of all leukemic blast cells was possible in 84/96 patients (88%; see Figure 18). In the remaining 12 patients gating of the leukemic cells was also possible, but it was less straightforward; these cases mainly concerned patients with monocytic leukemias. Most interestingly, combined expression of HLADR and CD117 is typically associated with distinct maturation (phenotypic) profiles in different myeloid lineages, for example, sequential loss of CD34 and CD117 in $HLADR^+$ monocytic and DC precursors versus CD34 and HLADR on $CD117^+$ maturing neutrophils/erythroid precursors.

To maintain optimal flexibility for the remaining antibodies, it was decided to preferentially select the less commonly available fluorochromes for the backbone markers. Initially CD34–PerCPCy5.5, CD117–PECy7, HLADR–PacB and CD45–AmCyan were tested. In addition, CD45–AmCyan was replaced by CD45–PacO, to be consistent with the other EuroFlow antibody panels (Table 14).

Selection of other characterization antibodies. As mentioned above, selection and combination of characterization markers in individual tubes was accomplished for detailed characterization of the main hematopoietic BM cell compartments (maturing neutrophils, monocytic and erythroid precursors) in the first three tubes of the AML/MDS panel. The other tubes were designed for assessment of cross-lineage and aberrant antigen expression profiles together with the evaluation of B-cell precursors and immature CD34 cells

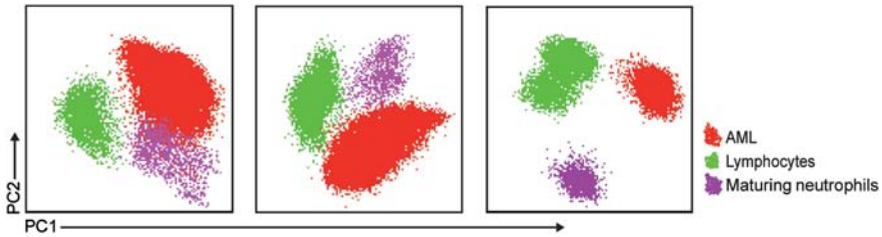

Figure 18. Identification of acute myeloid leukemia (AML) blast cells using the EuroFlow AML/myelodysplastic syndrome (MDS) panel backbone markers (CD34, CD117, HLADR and CD45). Automated population separator (APS) (principal component (PC)1 versus PC2) plots of three different AML patients are shown. Color codes are as follows: red dots, AML cells; green dots, mature lymphocytes; purple dots, maturing neutrophils.

Table 14. The EuroFlow AML/MDS antibody panel[a,b]

Tube	*PacB*	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*	*Aim*
1	HLADR	CD45	CD16	CD13	CD34	CD117	CD11b	CD10	Diagnosis and classification, neutrophilic maturation, PNH
2	HLADR	CD45	CD35	CD64	CD34	CD117	CD300e (IREM2)	CD14	Diagnosis and classification, monocytic maturation, PNH
3	HLADR	CD45	CD36	CD105	CD34	CD117	CD33	CD71	Diagnosis and classification, erythroid maturation
4	HLADR	CD45	NuTdT	CD56	CD34	CD117	CD7	CD19	Aberrant expression of lymphoid markers, abnormal B lymphoid maturation
5	HLADR	CD45	CD15	NG2	CD34	CD117	CD22	CD38	Aberrant marker expression, stem cells
6	HLADR	CD45	CD42a and CD61	CD203c	CD34	CD117	CD123	CD4	Diagnosis and classification of AML Megakaryocytic, basophilic, and plasmacytoid dendritic cell lineages
7	HLADR	CD45	CD41	CD25	CD34	CD117	CD42b	CD9	Characterization of megakaryoblastic leukemia, and systemic mastocytosis

Abbreviations: AML, acute myeloid leukemia; APC, allophycocyanin; BB, backbone; BM, bone marrow; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, hilite7; MDS, myelodysplastic syndrome; Nu, nuclear; PacB, pacific blue; PacO, pacific orange; PE, phycoerythrin; PerCPCy5.5, peridinin–chlorophyll–protein–cyanin5.5; PNH, paroxysmal nocturnal hemoglobinuria. [a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 96 BM samples were evaluated for selection of the BB markers. An additional 84 BM AML samples were evaluated with this version (final) of the panel.

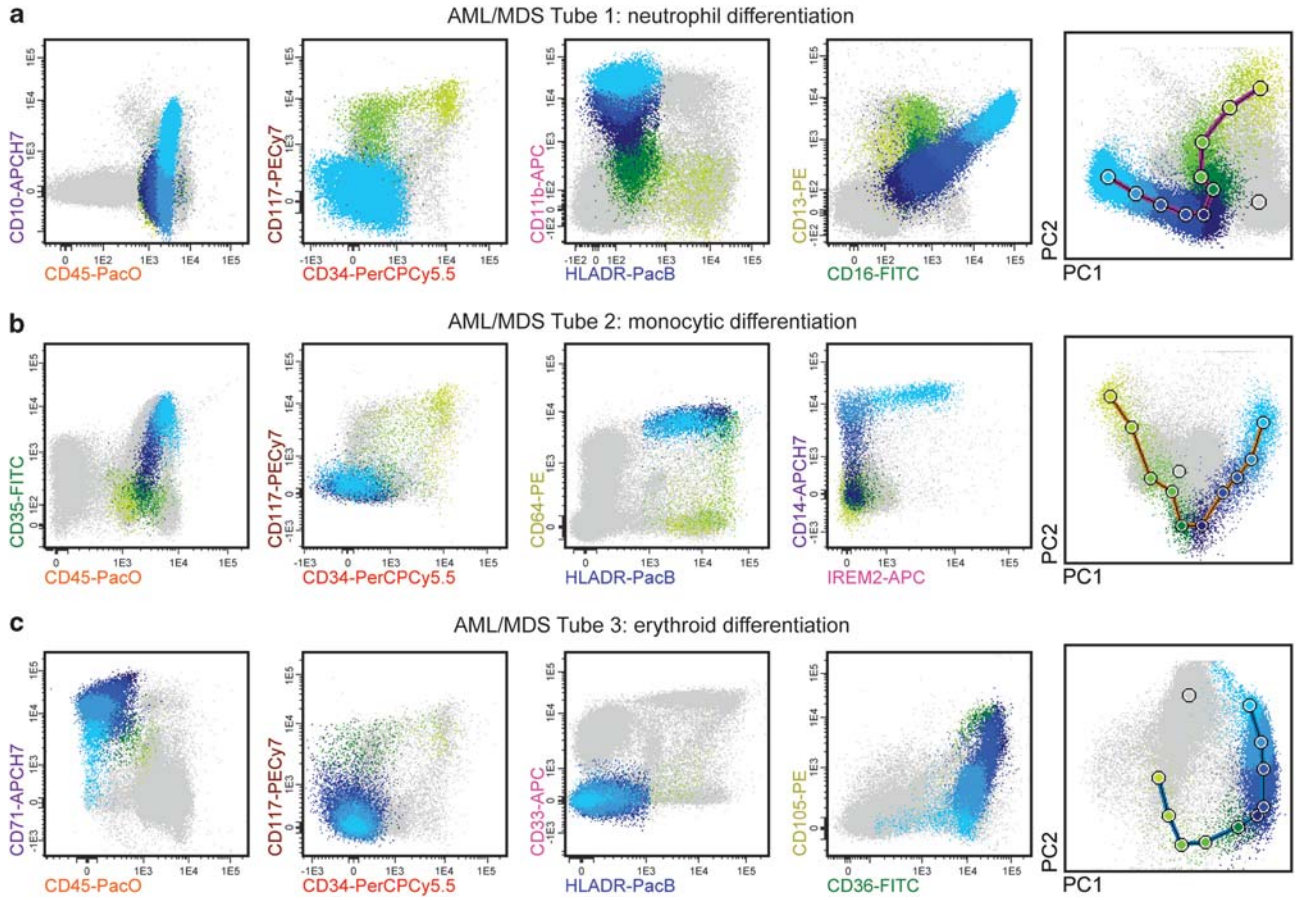

Figure 19. Neutrophil, monocytic and erythroid differentiation in bone marrow from a healthy subject as determined using tube 1 (maturing neutrophils), tube 2 (monocytic cells) and tube 3 (erythroid precursors) of the EuroFlow acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS) antibody panel. The different colors reflect distinct differentiation stages from the more immature CD34$^+$ precursors (light green events) to mature (light blue dots) population of each distinct cell line.

(tubes 4 and 5), assessment of minor compartments of basophils, mast cells, pDC (tube 6) and megakaryocytic lineage cells (tubes 6 and 7).

Tube 1: antibody combination for neutrophil maturation

Within the normal neutrophil lineage, several markers like CD10, CD11b, CD13 and CD16 are expressed in a maturation-stage-specific manner. In combination with the backbone markers, these markers allow detailed characterization of the neutrophil maturation pathway from the most immature myeloid blast cells till the mature polynuclear neutrophilic granulocytes.[11] Abnormalities in the expression of these markers are frequently seen in patients with both AML and MDS.[194,195,203] CD16–FITC and CD13–PE conjugates were selected based on experience, , initially together with CD10–APC and CD11b–AF700. However, testing of this antibody combination on BM samples from AML and MDS patients showed that the fluorescence intensity of CD11b–AF700 was insufficient. Therefore, CD11b–AF700 was replaced by CD11b–APC and CD10–APC was replaced by CD10–APCH7. This resulted in satisfactory stainings for all markers; an example of the staining pattern of tube 1 is shown in Figure 19a.

Tube 2: antibody combination for monocytic maturation

Regarding the monocytic lineage, CD14 is considered to be the typical marker for monocytes, but this marker is only expressed during the intermediate to last stages of monocytic maturation. In contrast, CD64 (and to a lesser extent CD36) are already expressed during the earlier stages of monocytic differentiation. Therefore, usage of at least one of these two markers (preferentially CD64) is required for identification of early monocytic cells. The expression of CD33 increases early during monocytic development to levels higher than those of granulocytic cells.[11] This CD33 expression pattern also facilitates distinguishing monocytic and granulocytic cells. CD11b is absent on immature monocytic cells but it is expressed during later stages. Initially, the combination of the CD36/CD64/CD11b/CD14 characterization markers was evaluated. Because CD11b is also included in tube 1, this marker was later replaced by CD300e (IREM-2) in the monocytic lineage-oriented tube 2. CD300e (IREM-2) is a glycoprotein whose expression on normal hematopoietic cells is restricted to cells of the monocytic and myeloid dendritic cell lineages.[206] During normal monocytic development, CD300e is expressed in the last stages of maturation after CD14 is highly expressed. In AML, CD300e is almost exclusively found on leukemic cells from patients with monocytic leukemia, its reactivity increasing from monoblastic to monocytic AML and CMML. Noteworthily, in a subgroup of these patients CD300e is already expressed before CD14. Because CD36 is also informative for the erythroid differentiation, and it appears on CD64^{hi} monocytic precursors (that can be easily distinguished from other myeloid precursors based on its higher intensity of expression), it was decided to use CD64 in the monocyte-oriented tube and CD36 in the erythroid-oriented tube (see tube 3). In tube 2, CD36 was therefore replaced by CD35(CR1), which is expressed by erythrocytes, granulocytes, monocytes and dendritic cells, as well as by the leukemic cells in a subset of AML

patients.[207] Preliminary data (data not shown) suggest that immature monocytic cells could first express CD35, followed later by CyMPO, while in immature neutrophilic cells, expression of CyMPO clearly precedes that of CD35. CD35 can therefore contribute to distinguishing early monocytic and neutrophil differentiation stages. On the basis of the expression patterns and the availability of antibody conjugates, CD14, CD35, CD64 and CD300e were finally selected for the monocyte-oriented tube. As expression of CD14 is relatively strong, the relatively weak APCH7 conjugate was chosen. The relatively bright PE and APC fluorochromes were selected for CD64 and CD300e, respectively. An example of the immunophenotypic patterns that can be observed with this labeling is shown in Figure 19b.

Tube 3: antibody combination for erythroid differentiation

For evaluation of the erythroid differentiation, markers like CD235a (glycophorin A), CD71 and CD36 are informative. As CD235a is expressed not only on immature erythroid cells but also on mature erythroid cells, this marker appeared to be less suitable for use on whole BM or whole PB. We therefore evaluated several other markers that may characterize erythroid cells: CD233 (band-3 protein), CD238 (Kell) and CD105 ('epithelial cellular adhesion molecule', Ep-CAM) (in several combinations). No satisfactory results were obtained with CD233, even after additional titration experiments. CD238 worked well, but the intensity of the staining on erythroid cells was low. CD36 and CD105 are already expressed during early stages of erythroid differentiation, before CD235a becomes positive.[208,209] Expression of CD105 remains present after the CD71 expression is increased and subsequently disappears shortly after CD117 expression is lost, so that more mature erythroid cells no longer express CD105. Conversely, CD36 expression remains at relatively high levels throughout the maturation of normal red blood cells. Little is known about CD105 expression in AML, but aberrant CD105 expression has been reported in patients with MDS.[199] Testing of CD105 on BM samples from healthy subjects and AML patients showed optimal and specific staining of early erythroid cells at intermediate stages of erythroid maturation. Therefore, CD105, CD36 and CD71 were selected for the erythroid-oriented tube. Given the expression levels and the availability of antibody conjugates, CD36–FITC, CD105–PE, and CD71–APCH7 were chosen. For the fourth antibody position, CD33–APC was chosen, as CD33 expression is absent during the erythroid differentiation and could be used as an exclusion marker. At the same time CD33 also provides relevant information for the granulocytic (tube 1) and monocytic lineages (tube 2), particularly once evaluated in the context of AML. An example of the immunophenotypic patterns that can be observed with this labeling is shown in Figure 19c.

Tube 4: antibody combination for aberrant cross-lineage antigen expression and altered B-cell precursors

The fourth tube in the EuroFlow AML/MDS antibody panel does not focus on a particular cell lineage but aims at the detection of aberrant expression of lymphoid-associated markers on myeloid cells. Frequently occurring cross-lineage markers are CD19 in AML with t(8;21), CD7, and CD56.[4,210,211] Because cross-lineage expression can be weak, CD7–APC and CD56–PE were selected. NuTdT is expressed in many AML, but has limited added value for AML diagnosis.[212] However, NuTdT provides information on precursor B-cells, particularly in patients with MDS when combined with CD19.[10,213] We initially evaluated the antibody combination TdT–FITC, CD19–PE, CD22–APC and CD38–APCCy7, since this combination allows detailed characterization of B-cell differentiation. Although this combination worked well, this combination was later on replaced by TdT–FITC, CD56–PE, CD22–APC and CD19–APCH7. CD56 was initially evaluated as an AF700 conjugate in tube 5, but this resulted in a dim staining and therefore the AF700 conjugate was replaced by the CD56–PE reagent that showed optimal staining. Although CD19–APCH7 also stains relatively weak with respect to other CD19-fluorochrome conjugates, the intensity obtained is sufficient for identification of B-cells. In this regard, it should be noted that CD19 is also present in the ALOT tube as a strong PECy7-conjugated reagent. Finally, CD22–APC was switched over with CD7–APC originally tested in tube 5. The reason for this switchover was that only the first four tubes are needed in case of suspicion of MDS (see below), and that analysis of CD7 on myeloid blast cells is more relevant than CD22 in MDS patients.[195]

Tube 5: antibody combination for stem cells and other markers

Tube 5 contains several markers that are informative in AML but that are less suited for evaluation of differentiation pathways. NG2 is a protein typically absent in normal hematopoietic cells, but its expression has been associated with the presence of *MLL* gene rearrangements and pDC lineages, which occur in approximately 10 and $<1\%$ of AML patients, respectively.[214,215] NG2 expression can be relatively dim and therefore the PE conjugate was chosen, as was also done for tube 4 of the BCP-ALL antibody panel. The CD15 antigen is already expressed during the early stages of granulocytic development and is also dimly expressed on mature monocytes. For this purpose, the CD15–FITC conjugate provided satisfactory staining intensities. CD38 is strongly expressed on most myeloid cells but is absent on early stem cells ($CD34^+/CD38^-$). As the frequency of (leukemic) stem cells may have prognostic significance,[216] CD38 was included in the EuroFlow AML/MDS antibody panel. Given the intensity of the staining, the APCH7 conjugate was selected with a satisfactory pattern. CD22 is a typical B-cell-associated marker, which is also expressed by basophils, mast cells and a fraction of pDC.[66,67,217] Expression of CD22 on AML is very infrequent ($<5\%$), and it may well be that these cases in fact reflect (precursor) basophilic and/or mast cell blast cells or DC leukemia.[215] The main reason to include CD22 in the AML/MDS panel was its use as an additional B-cell marker, next to CD19 and CyCD79a in the ALOT tube and CD10 in tube 1, to exclude a potential MPAL.[36] Initially, we evaluated the antibody

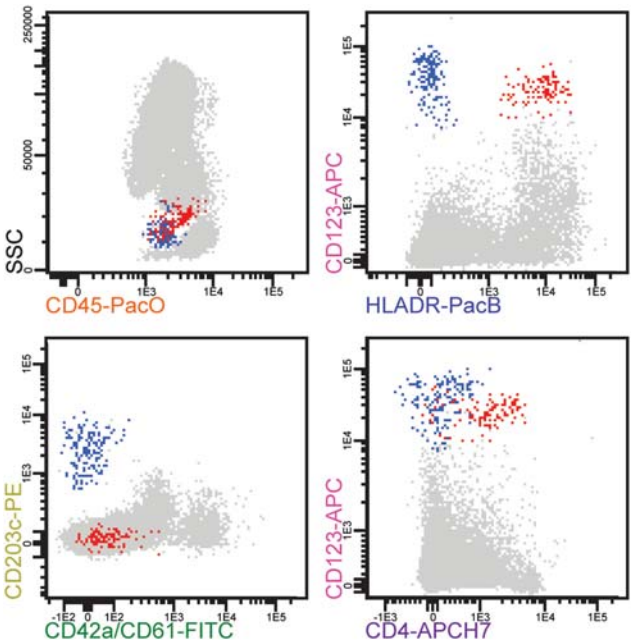

Figure 20. Identification of basophils (blue events) and plasmocytoid dendritic cells (red events) in a representative bone marrow from a healthy subject using tube 6 of the EuroFlow acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS) antibody panel.

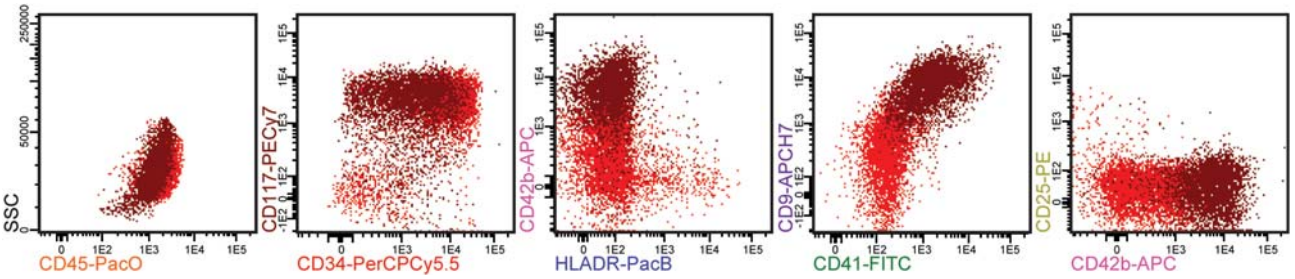

Figure 21. Characterization of an acute megakaryoblastic leukemia using tube 7 of the EuroFlow acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS) antibody panel. The AML cells (brown and red dots) are positive for CD34 and CD117 and they partly express the megakaryocytic-lineage-associated markers CD42b, CD41 and CD9. HLADR and CD25 were both negative on blast cells.

combination CD15–FITC, NG2–PE, CD7–APC and CD56–AF700. The staining intensity obtained with CD56–AF700 was, however, unsatisfactory and therefore this conjugate was replaced by CD56–PE and switched over with CD38 from tube 4. Finally, CD7–APC was switched with CD22–APC in tube 4 (see explanation above).

Tube 6: antibody combination for mast cells, basophils, pDC and megakaryoblastic/megakaryocytic cells

For recognition of the less frequent mast cells, basophils, pDC and megakaryocytic cells as well as their malignant counterparts, markers allowing identification of these cell populations were selected for inclusion in tube 6 of the EuroFlow AML/MDS panel. For identification of megakaryoblastic/megakaryocytic cells, a combination of CD42 and CD61 in the same fluorochrome was selected. Both markers are already expressed during early stages of megakaryocytic differentiation.[218] As megakaryoblastic leukemias can be single positive for CD42 or CD61, a combination of both markers was chosen. If leukemic cells are positive for CD42a and/or CD61, the megakaryoblastic nature of AML cells can be confirmed in an additional tube fully focused on the megakaryocytic lineage (tube 7, see below). CD203c is one of the few markers suitable for identification of $CD117^{hi}$ mast cells, and it is also expressed on $CD117^{-/lo}$ basophils.[219,220] CD123 can be used in combination with the backbone marker HLADR to identify pDC ($CD123^{+}$/$HLADR^{+}$) and basophils ($CD123^{+}$/$HLADR^{-}$) (Figure 20).[221] CD4 is expressed by plasmacytoid dendritic cells as well as by monocytic cells. On the basis of the intensity of expression and the availability of antibody conjugates, CD42a–FITC, CD61–FITC, CD203c–PE, CD4–APCH7 and CD123–APC were selected for this tube combination.

Tube 7: antibody combination for megakaryocytic differentiation

The megakaryocyte-oriented tube aims at confirmation of acute megakaryoblastic leukemia (AML-M7), if the results of tube 6 raise such suspicion. Next to the four backbone markers, CD41 and CD42b are included in this tube, as both markers are specific for the megakaryocytic lineage. CD9 is expressed on a wide variety of cells; within the megakaryocytic lineage CD9 is already expressed during the early stages and therefore can contribute to the diagnosis of AML-M7.[222,223] For the last antibody position, CD25 was chosen, as some data suggest that this marker is expressed during the early stages of megakaryocytic development.[224] Furthermore, CD25 can be used to detect immunophenotypic aberrant mast cells when a systemic mastocytosis or chronic eosinophilic leukemia (possibly in combination with an AML or MDS) is suspected.[225] An example of the immunophenotypic results obtained in AML-M7 with this tube is shown in Figure 21.

In addition to the markers indicated above, several other markers have been reported as consensus markers for the flow cytometric diagnosis of AML patients.[12,21,23,205] This includes CD65, CD66, lysozyme, eosinophilic peroxidase (EPO) and CD2. Although these markers can be informative, some (for example, CyEPO) were extensively evaluated and they proved not to be essential. At the same time, the EuroFlow AML/MDS antibody panel already contains sufficient markers for appropriate characterization of all myeloid lineages and relevant prognostic subgroups. Nevertheless, evaluation of the potential utility of other markers (for example, lysozyme) requires further evaluation.

THE EUROFLOW AML/MDS ANTIBODY PANEL

The final EuroFlow AML/MDS antibody panel is summarized in Table 14. An example of the results obtained with this antibody panel is shown in Figure 22. This antibody panel should be used in combination with the standard EuroFlow immunostaining protocol.[16] Although the Fix&Perm reagents were selected within EuroFlow for intracellular stainings, it was decided to use FACS Lysing Solution for tube 4 of the AML/MDS panel. TdT works well using FACS Lysing Solution and usage of FACS Lysing Solution has the advantage that all tubes can be processed in a similar manner, thereby retaining identical scatter characteristics in all tubes and facilitating the merge and calculation options in the Infinicyt software.

During the EuroFlow studies all evaluated AML cases were analyzed with the first six tubes, whereas tube 7 was also applied if an AML-M7 was suspected. This allowed detailed classification of all evaluated cases. Preliminary evaluation of large series of AML patients suggests that the first four tubes of the EuroFlow AML/MDS panel in combination with the ALOT tube might be sufficient for the diagnosis and classification of most AML patients. Based on the immunophenotyping results of the first four tubes or if complete phenotypic classification of AML is required, tubes 5 and 6 might be added in $\leqslant$10% of cases. Tube 7 should be used (together with tubes 1–6 and ALOT) if a megakaryocytic leukemia or a transient myeloproliferative disorder is suspected (either forehand or based on the results of tube 6).

In case of MDS suspicion, the first four tubes of the EuroFlow AML/MDS panel are sufficient to identify abnormalities in the differentiation of the various (major) myeloid lineages (for example, neutrophil, monocytic and erythroid lineages). Tubes 5–7 do not yet seem to provide relevant additional information for MDS diagnosis.

In addition to evaluation of patients with suspicion of AML or MDS, the EuroFlow AML/MDS panel may also provide relevant information for patients with a suspicion of paroxysmal nocturnal hemoglobinuria (PNH) in the context of BM aplasia and MDS (PNH: detection of $CD10^{+}$/$CD16^{-}$ mature neutrophils in tube 1 and of $CD14^{-}$ monocytes in tube 2), mastocytosis ($CD25^{+}$/$CD117^{hi}$ mast cells in tube 7) and chronic myeloid leukemia (CML, tubes 1 and 6). It should, however, be noted that the EuroFlow tubes were not primarily designed for these diseases and therefore other antibody combinations may be more appropriate, in case of primary suspicious of such diseases.[226,227]

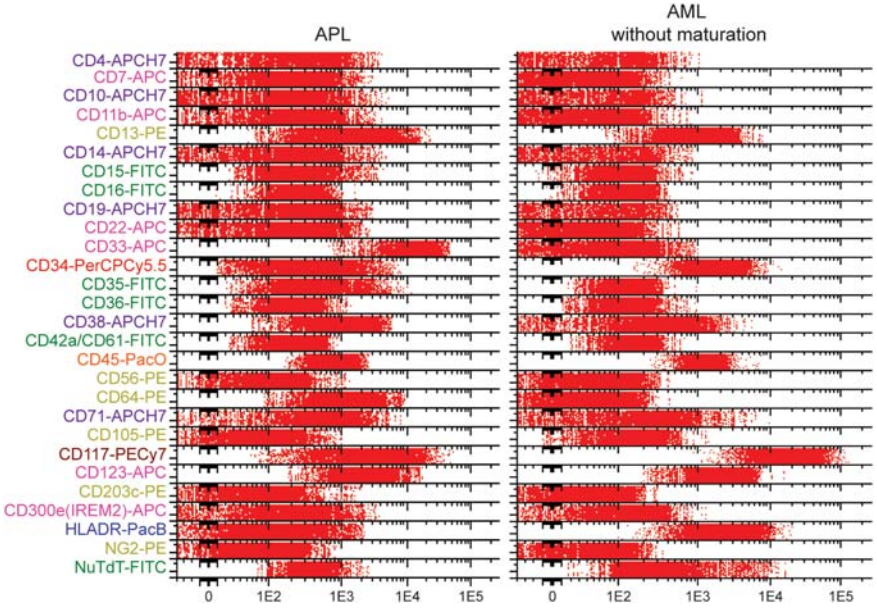

Figure 22. Band plot examples of two acute myeloid leukemia (AML) patients analyzed according to the EuroFlow AML/myelodysplastic syndrome (MDS) antibody panel. The AML blast cells (red dots) are gated based on the backbone markers and their immunophenotype is shown in a multiparameter band-dot plot. A patient with APL (*PML-RARA*-positive) and a patient with an AML without maturation are shown in the left and right panels, respectively.

Univariate and multivariate analysis of AML subgroups

To evaluate whether the EuroFlow AML/MDS antibody panel appropriately characterizes AML subgroups, data were collected from 84 AML patients.[225,228] Univariate analysis identified several markers that were differentially expressed among the various subtypes. Monocytic markers like CD36, CD14, CD4 and CD11b were primarily observed on blast cells from patients with an acute monocytic leukemia, t(9;11)$^+$ AML, or AML with mutated *NPM1*. CD42a/CD61 were only expressed on megakaryoblastic leukemias, while these leukemias did not express CD123. Also, erythroid leukemia did not express CD123. The pan-myeloid marker CD33 was expressed on the vast majority of AML, but not on erythroid leukemias. Based on single markers, leukemias originating from different myeloid lineages could therefore be distinguished, while characterization of genetically-defined subgroups was not straightforward.

In multivariate analysis (APS view) several WHO-defined subgroups could be distinguished from each other (Figure 23). Particularly morphologically-defined subgroups could be distinguished, while separation of genetically-defined subgroups was generally less clear. This may be explained, at least in part, by the fact that immunophenotyping (like morphology) focuses on cell lineages and differentiation stages, while genetically-defined subgroups may be more heterogeneous, owing to different secondary hits that may have a different impact on the differentiation potential of the affected cells.[229,230]

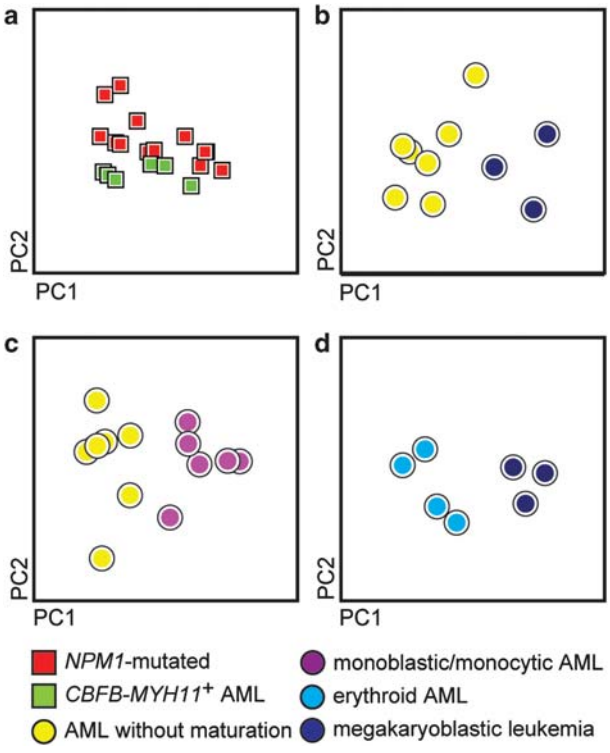

Figure 23. Multivariate principal component analysis (PCA; principal component analysis (PC)1 versus PC2, automated population separator (APS)-1) view of various WHO-defined subgroups of AML. (**a**) NPM1-mutated (red squares) versus CBFB-MYH11$^+$ AML (green squares). (**b**) AML without maturation (yellow circles) versus megakaryoblastic leukemia (dark blue circles). (**c**) AML without maturation (yellow circles) versus monoblastic/monocytic AML (pink circles). (**d**) Erythroid AML (cyan circles) versus megakaryoblastic leukemia (dark blue circles). Each square/circle represents the overall mean/median position of an individual AML patient in the PC1 versus PC2 representation of the whole immunophenotypic profile of the AML blast cells, respectively.

CONCLUSION

The AML/MDS EuroFlow panel allows detailed characterization of the distinct myeloid lineages, including the evaluation of maturation pathways and aberrant immunophenotypes of myeloid cells. This panel should be performed for patients with suspicion of AML or MDS in combination with the ALOT; however, it also provides essential information to rule out PNH and systemic mastocytosis with BM involvement. Of note, it was not possible to use the same backbone markers in the AML/MDS panel and the ALOT, which prevents software linkage of markers present in the ALOT with markers in the AML/MDS panel. The AML/MDS panel was built so that it can be applied in a flexible way, the first four tubes being essential for the phenotypic characterization of both AML and MDS.

SECTION 8. ANTIBODY PANEL FOR B-CELL CHRONIC LYMPHOPROLIFERATIVE DISEASES (B-CLPD)

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BACKGROUND

Mature (peripheral) B-cell malignancies represent the malignant counterpart of normal mature B-cells that have differentiated into naive B cells or their progeny. The WHO subclassification of mature B-cell leukemias and lymphomas, also known as B-CLPD, follows the concept of normal (benign) counterparts and attempts to distinguish the B-cell malignancies in relationship to the germinal center reactions and other B-cell activation and maturation processes.[231] Nevertheless, the exact normal counterparts of several B-CLPD (for example, hairy cell leukemias, HCL) have not been discovered. Thus, integration of a complex set of immunophenotypic, morphological, clinical and cytogenetic information is currently essential for the correct subclassification of mature B-cell malignancies. The reproducible diagnosis of specific B-CLPD WHO entities based on morphology remains a serious challenge, as even agreement between expert hematopathologists on particular disease entities ranges between 53 and 94% of cases.[232] However, already 15 years ago it has been shown that the inclusion of immunohistological assessments could increase the agreement rate by up to 14% in mature B-cell malignancies.[232]

It is clear that precise and reproducible diagnosis of B-CLPD subtypes has high clinical relevance, as the disease group comprises largely indolent entities that are best treated by watchful waiting (for example, certain subgroups of chronic lymphocytic leukemia, CLL[233]) as well as extremely aggressive diseases with a median survival of few months only if left untreated (for example BL). Furthermore, the advent of novel therapeutic options has greatly improved the prognosis for many patients with a mature B-cell neoplasm, for example, for patients with mantle cell lymphoma (MCL).[234] Modern treatment regimens are tailored for the specific B-CLPD subtype, so that obtaining the correct diagnosis has gained additional importance for optimal patient care. Immunophenotyping has proved essential for the diagnostic classification of many B-CLPD cases, but the current immunophenotyping strategies also face several difficulties. For example, the rising number of immunophenotypic markers used for the classification of B-CLPD as well as the heterogeneous and overlapping immunophenotypic profiles among distinct entities have increased the complexity of data interpretation. Clearly, a more objective and more integrated approach is needed with selection of the most informative markers and deletion of redundant makers, which provide more confusion than solution.

The EuroFlow Consortium embarked on a systematic evaluation of the immunophenotype's contribution to the diagnostic classification of mature B-cell malignancies, aiming at a more integrated as well as a fully standardized and reproducible immunophenotypic diagnosis of B-CLPD subgroups. The complete set of morphological, cytogenetic and immunophenotypic information required by the WHO 2008 classification served as the diagnostic 'gold standard' in each patient. We developed an optimized and non-redundant combination of markers in order to reproduce as far as possible that 'gold standard' solely based on flow cytometry (EuroFlow B-CLPD panel). Noteworthily, we decided to separate PCD from the remaining mature B-cell neoplasms as they usually present with a clearly distinguishable clinical picture and immunophenotype that requires different screening and classification strategies (Figure 1). The EuroFlow B-CLPD panel was designed to work in all cases in which the malignant B-cell clone could be separated by gating on backbone markers. The resulting (gated) population was required to systematically contain $<10\%$ contaminating non-clonal B-cells. The B-CLPD panel is intended to be applied subsequent to the detection of a clonal B-cell population using the LST. For reasons of efficiency and cost reduction, the relevant immunophenotypic information of the LST is also used for the classification of the distinct B-CLPD entities. In cases with a very high pre-test suspicion of a B-CLPD, it is recommended to stain the LST simultaneously with the B-CLPD panel (or parts of it) in order to minimise the number of steps required to obtain a definite immunophenotypic diagnosis (Figure 1).

Design of the EuroFlow B-CLPD panel

In line with the EuroFlow strategy on the identification of target populations in different tubes of an antibody panel via common backbone markers, the first step in the design of the B-CLPD panel was to define a minimal set of markers to identify all B-cells between the other leukocytes in PB, BM and LN samples from patients with B-CLPD. The second step focused on the selection of antigens with different expression between B-CLPD entities, aiming at appropriate characterization of each B-CLPD entity.

Selection of backbone markers. Several B-lineage-specific antigens (for example, CD19 and CD20) were obvious candidates for backbone markers, as they are assumed to be useful for identification of all B-cells in a sample. However, aberrantly low expression of CD20,[235] CD19,[236,237] CD22[238] and CD37[150] was known to be a common phenomenon in several mature B-CLPD entities. When we started the project it was not known to what extent such under-expression occurs simultaneously for several B-cell antigens in a single patient and how this would affect the ability to distinguish malignant B-cells from the remaining leukocytes. Therefore we first tested the relative contributions of CD19, CD20, CD22 and CD37 to the identification of malignant B-cells in a B-CLPD sample.

To address the relative contributions of pan-B-cell markers to the identification of malignant B-cells in B-CLPD, a 6-color combination was initially evaluated at two different centers (Backbone panel 1, Table 15).

The analysis included samples from 49 B-CLPD patients: 18 CLL, 4 DLBCL, 7 follicular lymphoma (FL), 4 MCL, 2 marginal zone lymphoma (MZL) and 14 unclassified B-CLPD. The background fluorescence was set to include 95% neutrophils in the sample. B-cells were defined to comprise any lymphocytes with expression of at least one of the four B-cell markers CD19, CD20, CD22 or CD37 above the background fluorescence level. The malignant clone was identified as a subpopulation of mature B-cells showing either light-chain restriction or complete lack of light-chain expression. The mean fluorescence intensity (MFI) of the malignant clone and the percentage of B-cells expressing CD19, CD20, CD22 and CD37 with higher intensity than background was analyzed.

Expression levels of the investigated B-cell antigens were significantly correlated for all 1×1 marker combinations. However, the degree of correlation decreased in the following order: CD19 versus CD37 (Spearman $r = 0.68$, $P < 0.0001$), CD37 versus CD20 ($r = 0.59$, $P < 0.0001$), CD37 versus CD22 ($r = 0.55$, $P < 0.0001$), CD22 versus CD20 ($r = 0.52$, $P < 0.0001$), CD19 versus CD20 ($r = 0.41$, $P = 0.004$), and CD19 versus CD22 ($r = 0.35$, $P = 0.01$). The percentage of undetected neoplastic B-cells with respect to the markers applied is visualized in Figure 24. Overall, none of the investigated antibodies was on its own sufficient to identify all malignant B-cells in every patient. There were individual B-CLPD patients in whom the vast majority of malignant B-cells could not be distinguished from background using one of the markers alone. As expected, low-level CD20 expression was particularly common in CLL, whereas

under-expression of CD19 most often occurred in FL and DLBCL patients (data not shown). The most efficient combination of two of these four markers evaluated to detect neoplastic B-cells comprised CD20 plus CD19, with at most 11% undetected malignant cells per case. The remaining B-cells were most efficiently identified by additionally relying on CD22 (Figures 24b and c).

An alternative set of backbone markers was tested in a total of 10 B-CLPD patients (4 CLL, 1 DLBCL, 3 FL, 1 MZL, 1 non-classified B-CLPD) in one center to study the utility of CD20-PacB instead of the CD20-APCCy7 reagent (backbone panel 2, Table 15). This change in the backbone CD20 antibody clone and conjugate further improved the performance of the CD20 plus CD19 combination with a maximum percentage of undetected lymphoma cells of only 2%. All other correlations were identical to those described for the panel tested at the other two centers (data not shown). Consequently, CD20-PacB was selected for subsequent testing.

As all panels were designed to work not only in blood but also in BM and cell suspensions prepared from tissue sections, we agreed to additionally include CD45–PacO as the third backbone marker. This marker facilitates the distinction of leukocytes from erythroid precursors and non-hematopoietic cells, as well as the identification of the major subpopulations of the leukocytes.

Subsequently, the EuroFlow Consortium tested the combination of CD20–PacB, CD45–PacO, CD19–PECy7 and CD22–AF700 (clone IS7, EXBIO, Prague, Czech Republic) as potential backbone markers, to definitively check the performance of CD20–PacB and evaluate the need for the third B-cell marker for the identification of all B-cells of interest in every case. CD22–AF700 replaced CD22–APC, so that other B-cell characterization markers could be introduced into the APC channel. This configuration of backbone markers was tested in the context of the first version of the full 8-color EuroFlow B-CLPD antibody panel (Table 15) in a total of 63 samples (17 CLL, 6 DLBCL, 13 MCL, 4 FL, 1 HCL, 2 lymphoplasmacytic lymphomas (LPL), 3 MZL, 12 non-classified B-CLPD, 1 normal BM, 4 normal PB). We found that CD19–PECy7 identified all B-cells in all cases except some FL ($n = 2$) and MCL ($n = 4$) patients, whereas CD20–PacB detected all B-cells in

Table 15. Design of the B-CPLD panel in seven consecutive testing rounds[a]

Version (no. of cases)[b]	*Tube*	*Fluorochromes and markers*							
		PacB	*PacO*	*FITC*	*PE*	*PECy5*	*PECy7*	*APC*	*APCCy7*
BB 1 ($n = 49$)	1			SmIgκ	CD37	SmIgλ	CD19	CD22	CD20
BB 2 ($n = 10$)	1	CD20		SmIgκ	CD37	SmIgλ	CD19	CD22	
		PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*AF700*
1 ($n = 63$)	1	CD20	CD45	SmIgκ	SmIgλ	CD5	CD19	SmIgM	CD22
	2	CD20	CD45	CD103	CD10	CD5	CD19	CD43	CD22
	3	CD20	CD45	CD81	CD79b	CD5	CD19	CD23	CD22
	4	CD20	CD45	CD31	CD63	CD5	CD19	CD185 (CXCR5)	CD22
	5	CD20	CD45	CD24	CD305(LAIR1)	CD5	CD19	CD11a	CD22
	6	CD20	CD45	CD38	CD25	CD138	CD19	CD11c	CD22
		PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
2 ($n = 43$)	1	CD20	CD45	SmIgλ	SmIgκ	CD22	CD19	CD23	CD81
	2	CD20	CD45	CD103	CD25	CD11c	CD19	SmIgM	CD24
	3	CD20	CD45	CD31	CD305(LAIR1)	CD5	CD19	CD43	CD185 (CXCR5)
	4	CD20	CD45	CyBcl2	CD10	CD79b	CD19	CD38	CD49d
3 ($n = 37$)	1	CD20	CD45	SmIgλ	SmIgκ	CD22	CD19	CD23	CD81
	2	CD20	CD45	CD103	CD25	CD11c	CD19	SmIgM	
	3	CD20	CD45	CD31	CD305(LAIR1)	CD5	CD19	CD43	
	4	CD20	CD45	CyBcl2	CD10	CD79b	CD19	CD38	CD49d
	5	CD20	CD45	CD24	CD95		CD19	CD200	
4 ($n = 31$)	1	CD20	CD45	SmIgλ	SmIgκ	CD22	CD19	CD23	CD81
	2	CD20	CD45	CD103	CD25	CD11c	CD19	SmIgM	CD43
	3	CD20	CD45	CD31	CD305(LAIR1)	CD5	CD19	CD43	CD24
	4	CD20	CD45	CyBcl2	CD10	CD79b	CD19	CD38	CD49d
	5	CD20	CD45	CD24	CD95		CD19	CD200	CD31
	6	CD20	CD45				CD19	CD185 (CXCR5)	CD103
5 (Final) ($n = 151$)	1 = LST	CD20	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD5	CD19 and TCRγδ	SmCD3	CD38
	2	CD20	CD45	CD23	CD10	CD79b	CD19	CD200	CD43
	3	CD20	CD45	CD31	CD305(LAIR1)	CD11c	CD19	SmIgM	CD81
	4	CD20	CD45	CD103	CD95	CD22	CD19	CD185 (CXCR5)	CD49d
	5	CD20	CD45	CD62L	CD39	HLADR	CD19	CD27	

Abbreviations: AF700, alexa fluor 700; APC, allophycocyanin; BB, backbone; BL, Burkitt lymphoma; BM, bone marrow; B-NHL NOS, B non-Hodgkin lymphoma not otherwise specified; CLL, chronic lymphocytic leukemia; CLPD, chronic lymphoproliferative disorder; Cy, cytoplasmic; Cy5, cyanin5; Cy5.5, cyanin5.5; Cy7, cyanin7; DLBCL, diffuse large B-cell lymphoma; FITC, fluorescein isothiocyanate; FL, follicular lymphoma; HCL, hairy cell leukemia; H7, hilite7; LN; lymph node; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; PacB, pacific blue; PacO, pacific orange; PB, peripheral blood; PE, phycoerythrin; PerCP, peridinin–chlorophyll–protein; Sm, surface membrane. [a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 384 cases (194 PB, 144 BM, 42 LN and 4 tissue biopsies) was evaluated. Among them 98 were CLL, 65 MCL, 55 FL, 40 DLBCL, 35 MZL, 32 B-NHL NOS, 21 LPL, 20 HCL, 7 BL and 11 from healthy donors. Highlighted boxes: changes as compared to previous version.

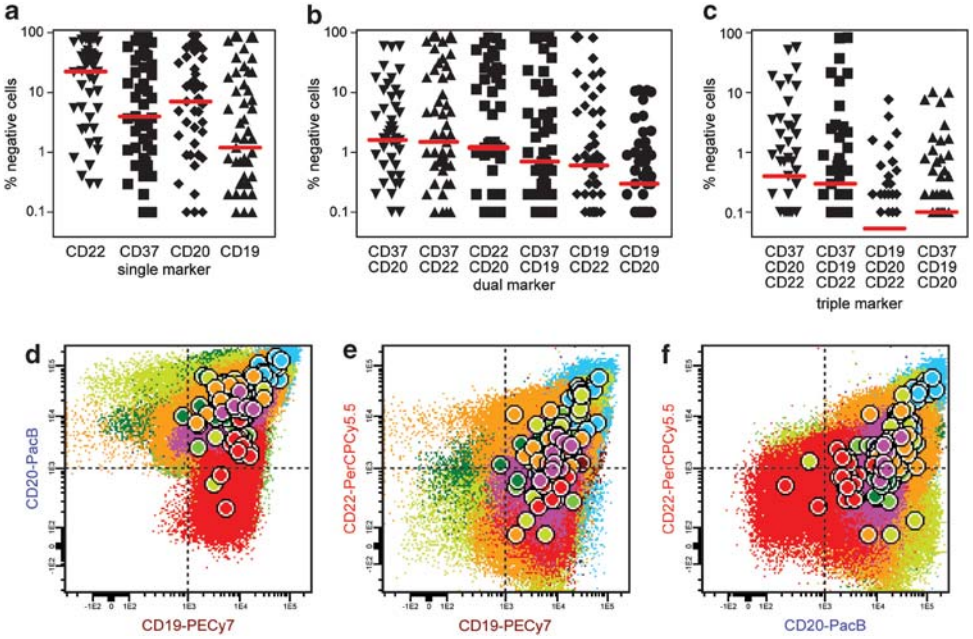


Figure 24. Percentage of clonal B-cells that are not detected using one (**a**), two (**b**) and three (**c**) pan-B-cell markers, respectively ($n = 49$ samples from 49 patients; black symbols represent individual patients, red lines depict the medians), and CD19–PECy7 versus CD20-PacB (**d**), CD19–PECy7 versus CD22-PerCPCy5.5 (**e**) and CD20–PacB versus CD22-PerCPCy5.5 (**f**) dot plots showing the distribution of individual neoplastic B-cells (small dots) and median values of each population in individual cases (circles) from a series of 151 B-cell chronic lymphoproliferative diseases (B-CLPD) patients. Circles are colored according to diagnoses of individual patients: red, chronic lymphocytic leukemia (CLL); dark green, follicular lymphoma (FL); green, marginal zone lymphoma (MZL); light green, $CD10^-$ diffuse large B-cell lymphoma (DLBCL); dark blue, mantle cell lymphoma (MCL); light blue, hairy cell leukemia (HCL); orange, $CD10^+$ DLBCL; pink, Burkitt lymphoma (BL); brown, lymphoplasmacytic lymphoma (LPL).

all patients with the exception of six CLL patients. The combination of CD20–PacB and CD19–PECy7 distinguished all light-chain-restricted B-cells from background in every case analyzed, thus further extending our earlier observation. Conversely, our findings showed that CD22–AF700 did not significantly contribute to the identification of clonal B-cells in addition to the selected markers, as fluorescence overlap from brightly expressed antigens detected by APC-labeled antibodies made CD22-AF700-positive populations undistinguishable from the background.

Based on the above experiments, the EuroFlow Consortium concluded that the combination of CD20–PacB plus CD19–PECy7 was not only required but also sufficient to identify all clonal cells in mature B-lineage malignancies. At diagnosis, we did not observe cases underexpressing simultaneously both antigens to an extent that they would go undetected by the brightly labeled CD19–PECy7 and CD20–PacB antibodies. In fact, 262 patient samples analyzed with subsequent versions of the EuroFlow B-CLPD classification panel never showed a B-cell population that was simultaneously negative for CD19–PECy7 and CD20–PacB (see Figure 24d). Nonetheless, all subsequent B-CLPD panel versions included CD22–PerCPCy5.5 as an additional B-cell characterization marker (Table 15). We conclude that if B-cell CLPD expressing neither CD20 nor CD19 exist, they are extremely rare (that is, $<0.5\%$ of all cases).

Mature data are now available on the correlation of B-cell antigens in B-lineage CLPD, which confirm our initial data: a high correlation between CD20 and CD22 expression ($r = 0.67$, $P < 0.0001$), whereas CD19 expression was less closely correlated to CD20 ($r = 0.47$, $P < 0.0001$) and CD22 levels ($r = 0.43$, $P < 0.0001$). Figure 24 shows the correlation in a total of 151 B-CLPD cases assessed with the final version of the B-CLPD EuroFlow panel (6 BL, 26 CLL, 21 DLBCL, 26 FL, 15 HCL, 15 LPL, 22 MCL and 20 MZL).

Selection of characterization markers for differential diagnosis between distinct B-CLPD entities. In parallel to the last part of the backbone marker testing, we started the selection of the set of characterization markers for the differential diagnosis of B-CLPD entities (Table 15). We used markers that were (i) already known to be of diagnostic value for the differential diagnosis of mature B-cell malignancies; (ii) known to be associated with normal B-cell developmental and maturational steps, thus following the concept of the WHO classification; and (iii) associated with B-cell homing, hypothesizing that different homing receptors might regulate the propensity of lymphoma cells to infiltrate BM, LN or spleen. Marker selection was based on published data and unpublished evidence from the EuroFlow laboratories. Supplementary Table 2 provides an overview on all 33 markers tested within the EuroFlow B-CLPD project and the selected references suggesting their potential utility for the classification of B-CLPD. The table also includes scientific work that was published after the EuroFlow project started.

Detailed analysis of the results of version 1 of the B-CLPD antibody panel (Table 15) showed that the CD11a, CD138 and CD63 antigens did not contribute to the differential diagnosis of mature B-cell malignancies. Furthermore, we identified that the APCH7 fluorochrome is a good alternative to AF700, with an overall good performance and lower fluorescence compensation needs. In line with the development of the other EuroFlow antibody panels, the Consortium decided to use the APCH7 conjugate in further testing. To improve the differential diagnosis among different B-CLPD entities, we additionally introduced CyBcl-2 in a second version of the panel (Table 15), which also contained several custom-conjugated antibodies. Assessment of 43 new B-CLPD cases revealed that the newly introduced custom-conjugated CD185(CXCR5)–APCH7 antibody did not yield the expected results, most likely due to suboptimal fluorochrome labeling of the antibody. Consequently, CD185(CXCR5)–APCH7 was not used in the third panel version. Furthermore, CD95 and CD200 were additionally included into the panel to improve the

identification of germinal center diseases and to distinguish MCL cases from the other B-CLPD entities, respectively. The resulting panel (version 3 in Table 15) was prospectively applied to 37 new cases, yielding promising preliminary results for the differential diagnosis among distinct B-CLPD entities. Nevertheless, owing to the unavailability of antibody reagents in suitable fluorochromes, four fluorochrome positions (APCH7 in three tubes and PerCPCy5.5 in one tube) were left open until custom-conjugated reagents became available (version 4 of the B-CLPD in Table 15). This next version (version 4) of the panel included APCH7-labeled antibodies to the CD43, CD31 and CD103 antigens, which were tested against reference antibodies in the panel in a series of 31 cases representing the major B-CLPD entities. This assessment revealed differences in correlation in the following order: CD103 FITC versus CD103 APC-H7 ($r = 0.39$, $P = 0.02$), CD43 APC versus CD43 APC-H7 ($r = 0.77$, $P < 0.0001$) and CD31-FITC versus CD31 APC-H7 (0.83, $P < 0.0001$). We concluded that CD31 and CD43 could be potentially applied in the APCH7 format.

PCA as integrated into Infinicyt software was applied to the samples acquired with version 4 of the B-CLPD antibody panel. Based on PCA, CyBcl-2, CD25 and CD24 were found not to have added value to the diagnostic power of the panel, once the other markers were present (data not shown). In particular, while CyBcl2 was expectedly[239] underexpressed in BL cases compared to all other mature B-cell lymphomas and leukemias, the remaining immunophenotype of BL was sufficiently unique to identify the disease without CyBcl2. CD25 was helpful for identification of HCL, but this was readily possible without this antigen using markers such as CD305 (LAIR1), CD11c and CD103. The differential diagnosis between HCL and HCL variants was not intended in this panel, as HCL variant is a very rare disease. If that differential diagnosis is in question, CD25 has to be stained after the B-CLPD panel. Finally, CD24 did not show a significant contribution to the classification of B-CLPD in the presence of the other markers. Furthermore, this analysis also showed that most B-CLPD subtypes could unequivocally be classified using the panel. Only the differential diagnosis between subgroups of LPL, MZL, FL and DLBCL cases remained difficult. In an attempt to improve the resolution between those entities, we additionally included CD62L, CD39, HLADR and CD27 into the panel (version 5; Table 15).

In the meantime, the development of the LST was sufficiently mature, so that the information obtained using both the LST and the B-CLPD antibody panel could be combined in a unified single data file for further multivariate analysis.[16]

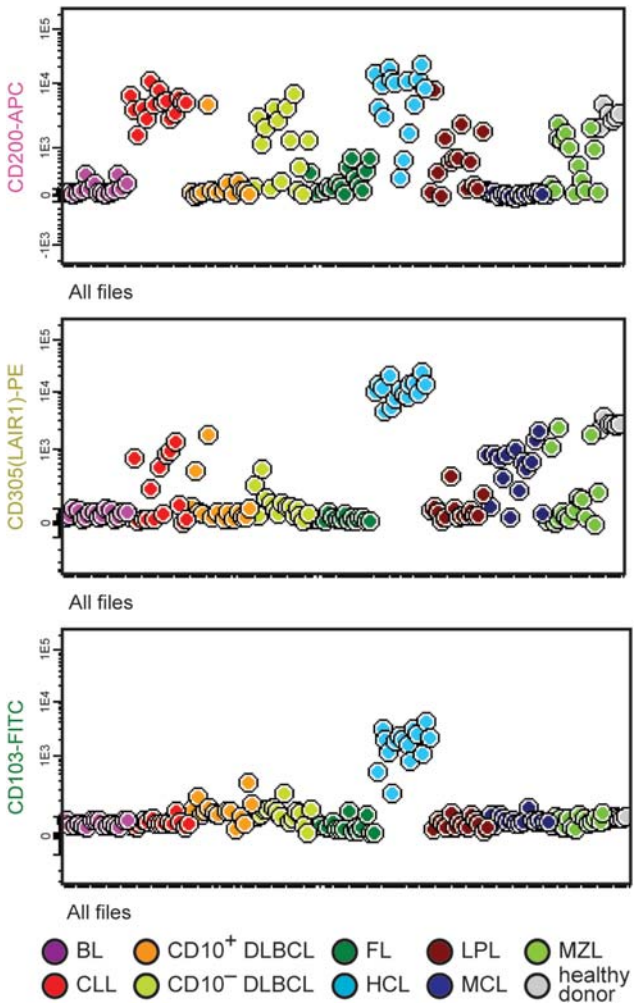


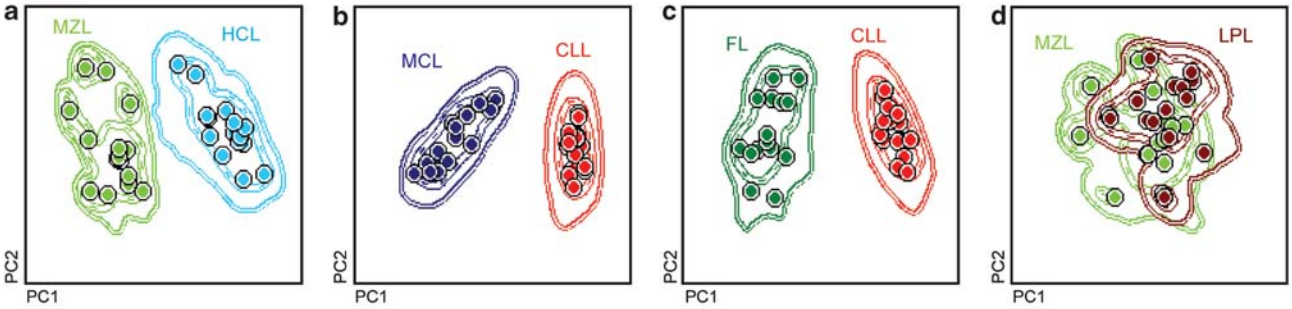
Figure 25. Illustrating examples of the median fluorescence intensity (MFI) of CD200, CD305(LAIR1) and CD103 detected among different major diagnostic categories of B-cell chronic lymphoproliferative diseases (B-CLPD). Circles correspond to median values of individual patient B-cells for each marker. BL, Burkitt lymphoma; 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.

Evaluation of the B-CLPD EuroFlow antibody panel

The final version of the B-CLPD panel (version 5) was then tested in 151 patients in six EuroFlow centers. We analysed the utility of this panel by comparing means of all MFIs per disease category for all evaluated markers (univariate analyses). This analytic step demonstrated a good concordance of mean MFIs in different disease categories to published data (for an overview on relevant literature see Supplementary Table 2; for examples of observed expression levels see Figure 25). These observations suggested that the multi-color panel indeed yielded the expected results. However, this assessment was limited by the lack of published immunophenotypic information for many markers in many mature B-cell malignancies.[240] We also observed that all disease categories included cases that showed overlap in expression levels for individual markers to other disease categories. Even markers considered to be specific for particular diseases (for example, CD103 for HCL) showed some overlap with other disease categories (Figure 25). We also confirmed that newly introduced markers such as CD200 and CD305 (LAIR1) provided improved separation between WHO-defined B-CLPD entities (Figure 25).

Owing to the heterogeneity of marker expression in individual cases within a given WHO entity, we decided to evaluate a novel immunophenotype-based diagnostic approach to mature B-cell malignancies: instead of identifying diseases using the typical expression of a marker or a sequential combination of markers, entities are now identified by their characteristic position within the multidimensional space created by the combination of all measured marker expressions.[241] The total number of markers within a panel defines the number of dimensions of this space. PCA is applied to find the vector of the multidimensional space with the highest variation of all events measured for two given diseases. In general, this vector allows the best discrimination between the two diseases, visualized with the automated population separation (APS) tool of the Infinicyt software.[16]

The novel approach allowed establishing typical immunophenotypic patterns for most of the eight major B-CLPD entities: BL, CLL, DLBCL, FL, HCL, LPL, MCL, and MZL. To distinguish between these eight entities a total of 28 diagnostic decisions have to be made (1×1 comparisons). It is currently possible to make all these decisions, albeit that it remains difficult to distinguish DLBCL from (i) FL and from (ii) MZL and LPL. Also, the unequivocal differential diagnosis between MZL and LPL is not possible with our panel of markers (Figure 26d). We speculate that a lack of separation



CD305(LAIR1)	12%
CD11c	11%
CD103	8%
CD200	8%
CD22	7%

CD79b	15%
CD200	14%
SmIgM	13%
CD23	9%
CD20	9%

CD79b	10%
CD200	9%
CD43	8%
CD23	7%
CD31	7%

Figure 26. First principal component (PC1) versus second principal component (PC2) bivariate dots plots of the complete immunophenotype of a series of marginal zone lymphoma (MZL) versus hairy cell leukemia (HCL) (**a**), chronic lymphocytic leukemia (CLL) versus mantle cell lymphoma (MCL) (**b**), follicular lymphoma (FL) versus CLL (**c**) and MZL versus lymphoplasmacytic lymphoma (LPL) (**d**) cases. Colored circles represent median values of individual cases for all those immunophenotypic markers in the EuroFlow B-cell chronic lymphoproliferative disorder (CLPD) panel contributing to PC1 and PC2. Contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively). The five most informative markers contributing to the best discrimination between each disease entity are displayed at the bottom of each plot, in a decreasing order of percentage contribution to the discrimination.

between those two entities might also be related to difficulties of hematopathologists to distinguish them on histopathological grounds. In fact, expert reference pathologists agreed on these two disease categories in 56% and 63% of all cases, respectively.[232] Individual aberrant cases additionally showed clustering in the border zone of two B-CLPD entities (DLBCL versus BL, 1 case; DLBCL versus MCL, 2 cases; FL versus LPL, 1 case; FL versus MCL, 1 case; and FL versus MZL, 4 cases). Thus, 24/28 differential diagnoses can objectively be made for the vast majority of cases through combined use of the EuroFlow B-CPLD antibody panel and the new Infinicyt software tools.

An example of the differential diagnostic power of the EuroFlow strategy is given in Figure 26. MZL cases are clearly separated from HCL cases when the first (x-axis) and second (y-axis) principal components are simultaneously considered (Figure 26a). The 5 (out of 25) parameters contributing most significantly to the first principal component are, in descending order: CD305 (LAIR1), CD11c, CD103, CD200 and CD22. The diagram of first versus second principal component for CLL and MCL cases (Figure 26) shows again that most patients can unequivocally be classified into one of the disease categories using flow cytometry data only. The five markers that contributed the most to the first principal component were CD79b, CD200, SmIgM, CD23 and CD20.

On the basis of these results, we investigated which markers contributed the most to the differential diagnosis of the most frequent mature B-cell malignancy, CLL, versus all other mature B-cell malignancies. Overall, a combination of 10 relevant antigens (CD20, CD45, CD19, CD5, CD38, CD23, CD10, CD79b, CD200 and CD43) yielded an almost equally good separation between CLL and all other B-CLPD entities (Figure 26b). Therefore, the B-CPLD panel was designed so that those markers were included into tubes 1 (LST) and 2 (of the panel) (Table 15). Consequently, it is possible to unequivocally diagnose this disease using tube 1 (LST) and tube 2 of the B-CLPD antibody panel only.

CONCLUSION

The B-CPLD EuroFlow antibody panel consists of four tubes plus the LST, which combines a total of 27 different B-cell associated markers. Each tube in the panel contains three backbone markers (CD19, CD20 and CD45), which proved to provide efficient positive identification of all clonal B-cells in every B-CPLD patient tested. Backbone markers combined with the characterization markers provide an efficient differential diagnosis among the majority of B-CPLD entities (24/28 diagnostic decisions), using multivariate analysis of complete phenotypes of individual cells. The panel was designed to work in a modular way, so that the first two tubes allow efficient diagnosis of CLL versus all other disease entities.

Future diagnostic strategies in mature B-cell malignancies will take advantage of using the best discriminating immunophenotypes in multivariate analysis, for each possible differential diagnosis. EuroFlow is currently constructing a reference database containing complete immunophenotypic information from well-defined mature B-cell malignancies. Unknown new cases will be evaluated for diagnostic purposes by comparing their immunophenotype to the immunophenotypic information along the vectors in the EuroFlow reference database for best differential diagnosis.

SECTION 9. ANTIBODY PANEL FOR T-CELL CHRONIC LYMPHOPROLIFERATIVE DISEASES (T-CLPD)

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[1]USAL, Salamanca, Spain; [2]HUS, Salamanca, Spain; [3]HSA-CHP, Porto, Portugal; [4]Erasmus MC, Rotterdam, The Netherlands; [5]AP-HP, Paris, France; [6]UNIKIEL, Kiel, Germany; [7]IPOLFG, Lisbon, Portugal; [8]UNIVLEEDS, Leeds, UK; [9]SUM, Zabrze, Poland; [10]DPH/O, Prague, Czech Republic and [11]Cytognos SL, Salamanca, Spain

BACKGROUND

T-cell CLPD, also termed peripheral (mature) T-cell neoplasms (PTCL), are relatively uncommon lymphoid malignancies (~10% of all CLPD in Western countries) derived from post-thymic mature T-cells.[3,242,243] Despite their low frequency, T-CLPD comprise a

highly diverse and heterogeneous group of entities,[3,244] much less understood than their B-cell counterpart owing to their rarity and biological heterogeneity and the lack of distinctive genetic markers for most disease categories.[244,245] Consequently, subclassification of major groups of T-CLPD still remains a challenge. As an example, the most common WHO 'category' of T-CLPD in Western countries (~30% of all T-CLPD) is that of PTCL not otherwise specified (PTCL-NOS),[3] which is mostly defined by exclusion criteria (for example, lack of specific features of other better-defined WHO diagnostic categories of PTCL).[3,246,247] Nevertheless, some progress has been made in recent years in our understanding of the pathogenesis of several subtypes of T-CLPD. On one side, this has been due to the identification of the normal counterparts of specific PTCL groups. For example, angioimmunoblastic T-cell lymphoma (AITL) has emerged as a distinctive subtype of T-CLPD derived from follicular T helper cells[3,247–249] and $CD4^+/CD25^+/CyFoxp3^+$ regulatory T-cells are known to be the closest normal counterpart of adult T-cell leukemia/lymphoma (ATLL) cells.[3,245] On the other side, such advances are related to the identification of unique biological features among specific subgroups of patients that contribute to a better prognostic stratification of patients. For instance, anaplastic large cell lymphoma (ALCL) is composed of two distinct entities with different prognosis, based on the presence or absence of expression of the anaplastic lymphoma kinase (*ALK*) gene;[250] similarly, T-cell prolymphocytic leukemia (T-PLL) cells specifically overexpress the kinase coactivator Tcl1 in a substantial proportion of cases, due to chromosomal rearrangements involving the *TCL1* gene.[251,252]

With the exception of mycosis fungoides (MF), primary cutaneous ALCL and T-cell LGL leukemia,[3] PTCL are among the most aggressive lymphoid neoplasms.[246,253] This means that most patients present with clear signs and symptoms of a CLPD, frequently associated with PB involvement, in addition to infiltration of LN and skin, among other tissues.[3] The application of the EuroFlow T-CLPD panel on samples from patients known to have increased numbers of (aberrant) T-cells as identified/suspected by the LST tube (see Section 2) aims at accurate confirmation of the presence of clonal T-cells in the different samples analyzed regardless of their nature—PB, LN or other tissues—and precise classification of the chronic T-cell disease into a specific WHO PTCL category.

The design and development of a standardized 8-color antibody T-CLPD panel to classify T-CLPD comprised the selection of combinations of both backbone markers and characterization markers for the identification and characterization of the T-cell population(s) of interest, as described below.

Selection of backbone markers

Four antigens (CD45, SmCD3, CD4 and CD8) were chosen as backbone markers for the T-CLPD panel, in order to identify normal mature T-cells ($CD45^{hi}/SmCD3^+$ events) and the major T-cell subsets defined by the expression pattern of CD4 and CD8 ($CD4^+/CD8^-$; $CD4^+/CD8^+$; $CD4^-/CD8^{-/lo}$; and $CD4^-/CD8^{hi}$). Initially, the following fluorochrome-conjugate monoclonal antibodies were tested as backbone reagents (BB1 in Table 16): CD8–PacB, CD45–AmCyan, CD3–PerCPCy5.5 and CD4–AF700. The proposed combination was initially tested by one center on eight PB samples from healthy adults. While the performance of the CD3–PerCPCy5.5 conjugate was optimal, the following limitations were found for the other three backbone markers: (i) interference of CD45-AmCyan in the FITC channel[16]; (ii) very poor (5/8 cases) or poor (3/8 cases) ability of CD4-AF700 for clear cut identification of $CD4^+$ T-cells; and (iii) poor resolution of the CD8-PacB reagent to discriminate $CD8^{lo}$ T-cells (7/8 cases). As a result, a second alternative combination of backbone markers for the T-CLPD panel was designed (BB2 in Table 16): CD4–PacB, CD45–PacO, CD3–PerCPCy5.5 and CD8–APCH7.

Preliminary testing with this second combination of backbone markers was initially done at one center in three PB samples, with clear improvement as regards the identification of normal T cells, in the absence of significant technical issues. Consequently, the combination underwent further evaluation at two distinct EuroFlow sites for a total of 12 PB samples. The new combination of backbone markers proved to be useful in every case tested for the identification of T-cells and the major T-cell subsets, as well as for their discrimination from the remaining leukocyte populations, in both normal/reactive ($n = 9$) and pathological samples ($n = 6$). Notably, this combination of backbone markers was also robust enough to

Table 16. Design of the T-CLPD panel in two consecutive testing rounds[a]

Version (no. of cases)[b]	*Tube*	*Fluorochromes and markers*								
		PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*AF700*	*Aim (phenotypic characterization)*
BB 1	1	CD8	CD45			SmCD3			CD4	
		PacB	PacO	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7	
BB 2	1	CD4	CD45			SmCD3			CD8	
1 ($n = 19$)	1	CD4	CD45	CD7	CD26	SmCD3	CD2	CD28	CD8	SS
	2	CD4	CD45	CD27	CD197(CCR7)	SmCD3	CD45RO	CD45RA	CD8	Maturation stage
	3	CD4	CD45	CD5	CD25	SmCD3	HLADR	CyTcl1	CD8	T-PLL
	4	CD4	CD45	CD57	CD30	SmCD3	TCRγδ	CD11c	CD8	Cytotoxic phenotype; ALCL
	5	CD4	CD45	CyPerforin	CyGranzyme B	SmCD3	CD16	CD94	CD8	Cytotoxic phenotype; T-LGL
2 (Final) ($n = 67$)	1	CD4	CD45	CD7	CD26	SmCD3	CD2	CD28	CD8	SS
	2	CD4	CD45	CD27	CD197(CCR7)	SmCD3	CD45RO	CD45RA	CD8	Maturation stage
	3	CD4	CD45	CD5	CD25	SmCD3	HLADR	CyTcl1	CD8	T-PLL
	4	CD4	CD45	CD57	CD30	SmCD3		CD11c	CD8	Cytotoxic phenotype; ALCL
	5	CD4	CD45	CyPerforin	CyGranzyme B	SmCD3	CD16	CD94	CD8	Cytotoxic phenotype; T-LGL
	6	CD4	CD45		CD279[c]	SmCD3			CD8	AITL

Abbreviations: AF700, alexa fluor 700; AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; AmCyan, *Anemonia Majano* cyan fluorescent protein; APC, allophycocyanin; BB, backbone; BM, bone marrow; CLPD, chronic lymphoproliferative disorder; Cy, cytoplasmic; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, hilite7; LGL, large granular lymphocytic leukemia; PacB, pacific blue; PacO, pacific orange; PB, peripheral blood; PE, phycoerythrin; PerCPCy5.5, peridinin–chlorophyll–protein–cyanin5.5; Sm, surface membrane; SS, Sézary syndrome; T-PLL, T-cell prolymphocytic leukemia.
[a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 86 samples (78 PB, 7 BM and 1 other type of sample) was evaluated: 20 normal samples and 66 pathological samples (37 clonal expansions of $CD4^+$ T cells, 18 of $CD8^+$ T cells and 11 clonal samples of $CD4^-/CD8^{-/lo}$ T cells). [c]Testing of a CD279-PECy7 conjugate reagent is ongoing to avoid tube 6 by placing this marker in the empty position in tube 4. Highlighted boxes: changes as compared to previous version.

allow gating, merging and calculating strategies with the Infinicyt software for aberrant T-cell populations, as detailed below.

Selection of characterization markers

The major aim of the EuroFlow T-CLPD panel is (i) to detect phenotypically aberrant T-cells and (ii) to allow precise classification of T-CLPD into the current WHO diagnostic categories.[3] Therefore, once the optimal backbone reagents had been definitively established, antibody reagents for further characterization and classification of T cells were then selected from a large list of T-cell related markers.[3,78,242,243,245,250–259] These included antibodies against classical pan-T-cell associated antigens known to be aberrantly expressed in many T-CLPD disease categories (for example, CD2, CD5 and CD7),[3,242,257] T-cell maturation-associated markers (for example, CD27, CD197 (CCR7), CD45RA and CD45RO),[245] co-stimulatory molecules (for example, CD26 and CD28),[3,242,243] activation-associated markers (for example, CD25—also constitutively expressed by $CD4^+/CD25^+$ regulatory T-cells[245]—CD38, CD69 and HLADR),[256,257] interleukin-2 receptors other than CD25 (CD122), cytotoxic-related molecules expressed by effector T-LGL,[256,258,259] such as CD11c, CD16, CD56, CD57, killer-cell Ig-like receptors (for example CD158a/b/e/j/k and NKB1), and lectin-type (for example, CD94 and CD161) receptors, as well as intracytoplasmic proteins (for example, perforin, granzymes and TIA-1). In addition, markers reported to be expressed in specific WHO subtypes of T-CLPD such as CD30,[3,260] CyTcl1[251,252] and follicular $CD4^+$ helper T-cell-associated markers[245] (for example, CD10 and CD279, among other markers) were also considered as candidates to be included in the panel. Based on existing data about all these markers, and the experience of the EuroFlow members, the following criteria were used to select individual reagents for further testing: (i) its ability to identify immunophenotypically aberrant T-cells in a significant proportion of T-CLPD or their specific WHO disease categories and (ii) its contribution to the definition of the maturation stage (naive versus memory versus terminally differentiated or effector cells) of the expanded T-cells; the final goal was to classify the different T-CLPD entities according to the more recent version of the WHO classification.[3]

It is currently well known that monoclonal T cells from T-CLPD frequently show downregulation of pan-T-cell markers, such as CD2, CD5 and CD7, in addition to SmCD3 and CD4.[3,242,243] Therefore, reagents recognizing these molecules were considered as mandatory to be selected for further testing (*first diagnostic level* markers in Table 17) and hence included in the initial EuroFlow T-CLPD panel. Other markers (or combinations of markers) were selected also as *first-level* markers based on their contribution to a more precise subclassification of T-CLPD into particular WHO categories.[3] CD26 and CD28 are useful markers for the identification of Sézary cells, as those $CD2^{lo}/CD4^{lo}/SmCD3^{lo}$ T-cells showing a typical $CD26^-/CD28^+$ phenotypic pattern.[255,261,262] Similarly, CD30 is typically, but not exclusively,[263] expressed in systemic ALCL (ALK^- and ALK^+) and primary cutaneous $CD30^+$ T-cell lymphoproliferative disorders.[264] Preliminary studies reported that T-cell neoplasms other than T-PLL could also express CyTcl1,[264] but more recent reports have shown that within T-cell malignancies, CyTcl1 expression is restricted to most (around 70–80%) T-PLL cases,[251,252] while it is absent in $CD30^{-/+}$ ALCL, T lymphoblastic lymphoma, nodal PTCL, MF[265] or any other mature T-cell tumor types.[251,252] Inclusion of the CD11c, CD16 and CD57 *first-level* markers and the CD94, granzyme B and perforin *second-level* cytotoxic-related markers was based on their ability to assess cytotoxic-associated phenotypes and hence to identify T-LGL,[3,258,259] although

Table 17. Utility of T-CLPD markers

Tube	*Target antigen*	*Identification of T-cells and T-cell subsets*	*Diagnostic level*		*Sub-classification*
			1st	*2nd*	
BB markers	CD3	X			
	CD4	X			X
	CD8	X			X
	CD45	X			
Tube 1	CD7		X		
	CD26		X		X
	CD2		X		X
	CD28		X		X
Tube 2	CD27			X	
	CD197 (CCR7)		X		X
	CD45RO			X	
	CD45RA		X		
Tube 3	CD5		X		
	CD25			X	X
	HLADR			X	
	CyTcl1		X		X
Tube 4	CD57		X		X
	CD30		X		X
	CD11c		X		X
Tube 5	CyPerforin			X	X
	CyGranzyme B			X	X
	CD16		X		X
	CD94			X	
Tube 6	CD279		X		X
Other	TCR Vβ families		X		

Abbreviations: BB, backbone; CLPD, chronic lymphoproliferative disorders; Cy, cytoplasmic.

T-CLPD cases other than T-LGL have also been found to express cytotoxic molecules.[3,245] The CD56 cytotoxic molecule was not included in the EuroFlow T-CLPD panel, as it was already present in the LST tube; in contrast, at this time anti-TCRγδ had not yet been included in the EuroFlow LST, and therefore it was considered as a mandatory marker to assess the TCRγδ versus TCRαβ nature of the disease. Other markers selected at this stage included the CD27, CD197(CCR7), CD45RA and CD45RO maturation-associated markers and the CD25 and HLADR activation-related proteins; the inclusion of all these latter groups of markers was based on the fact that the phenotype of pathological cells from many T-CLPD correlates with distinct maturation-associated subsets of T-cells, and therefore the differential expression of the CD45 isoforms, CD27 and CD197(CCR7) could be useful not only for the definition of the maturation stage of tumor cells,[245] but also for a more refined classification of T-CLPD cases into the distinct WHO entities. In this regard, previous studies have shown that Sézary cells typically display a $CD4^+$ memory T-cell phenotype,[255] while T-PLL cells display a phenotype consistent with a naive/central memory T-cell[3] and the phenotype of T-LGL leukemia cells overlaps with that of normal activated effector T-cells.[256]

Once the target molecules were selected, monoclonal antibodies against them were chosen among the FITC-, PE-, PECy7- and APC-fluorochrome conjugates, which were commercially available; the first version of the EuroFlow T-CLPD panel was then designed (Table 16) and evaluated. Shortly after the design of version 1, the second and definitive version of the EuroFlow T-CLPD panel was developed, with the following two major improvements: (i) exclusion of the anti-TCRγδ reagent, to avoid redundancy with the LST (note that version 1 of T-CLPD panel was developed in parallel with the first four versions of the LST, when anti-TCRγδ was not yet included in it); and (ii) inclusion of CD279 as a characteristic marker of follicular $CD4^+$ helper T-cells, to increase the diagnostic power of the panel for the identification of AITL.[3,247–249] The recent availability of new CD279 reagents (for example, CD279–PECy7 from BDB) would contribute to improve the current version of the EuroFlow T-CLPD panel, as the CD279–PE monoclonal antibody reagent used until now might be replaced by the new PECy7 conjugate of the same CD279 clone in the corresponding PECy7 position of tube 4 (Table 16), resulting in a five (instead of six) 8-color combinations of monoclonal antibodies.

The description of the precise role for each marker in the panel of all reagents included in the T-CLPD panel (version 2) is shown in Table 17.

Evaluation of the EuroFlow T-CLPD panel

Testing of the EuroFlow T-CLPD panel (version 2) was performed by seven EuroFlow centers involved in the study of T-CLPD. A total of 67 samples were stained with the full panel (version 2) (Table 18); these included 11 PB samples from healthy adults (mean age of 33 ± 6 years; 7 males and 4 females), 34 samples from patients with clonal expansions of $CD4^+$ T-cells (mean age of 62 ± 12 years; 20 males and 14 females, distributed in the distinct WHO subtype diseases[3] as shown in Table 18), 11 samples containing clonally expanded $CD8^{hi}$ T-cells (mean age of 60 ± 17 years; 3 males and 8 females), 10 samples of $TCR\gamma\delta^+$ T-CLPD (mean age of 59 ± 14 years; 8 males and 2 females) and 1 sample from a 67-year-old male with $CD4^-/CD8^{-/lo}$ $TCR\alpha\beta^+$ T-cell leukemia. In 30/56 patients (54%) T-cell clonality was confirmed by *TCR* gene rearrangement analyses according to the BIOMED-2 multiplex PCR protocols[266] performed either on whole samples or on highly-purified T-cell fractions.

In order to evaluate the utility of the EuroFlow T-CLPD panel for the purposes referred above, pairwise unsupervised discrimination

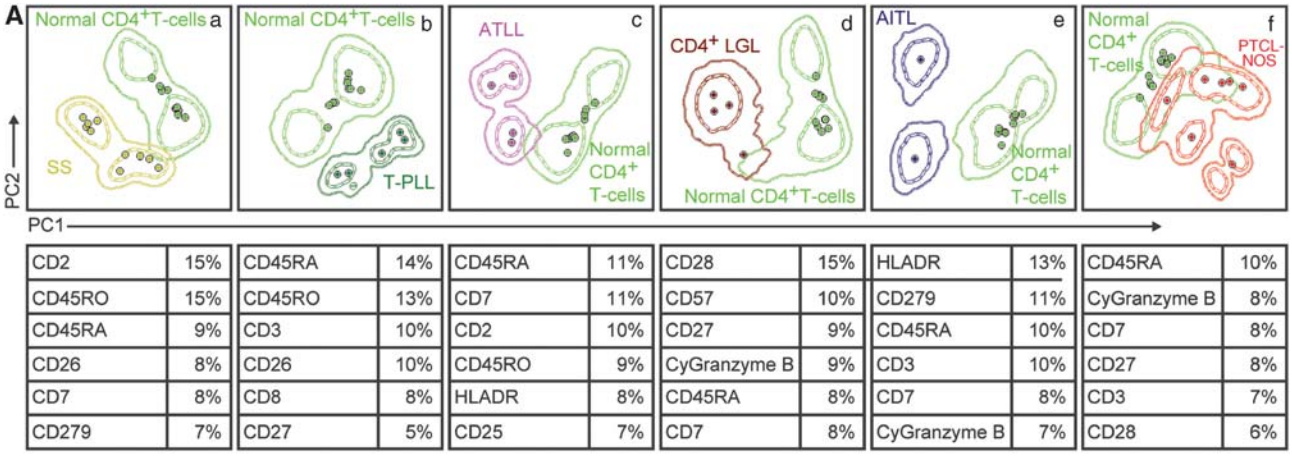
Table 18. Phenotypic patterns and numbers of pathological T-cells detected with the final version of the EuroFlow T-CLPD panel ($n = 67$)

Diagnostic group[a]	*Aberrant T-cells*[b] *(no. of cases/total cases)*	*No of T-cells*	
		% of T-cells (from WBC)[c]	*No of T-cells*[d] *($\times 10^3/\mu l$)*
Healthy adult donors ($n = 11$)	0/11	$CD4^+/CD8^-$: 12.3 (7.4–21.8) $CD8^{hi}/CD4^-$: 7.2 (2.8–14.4) $CD4^-/CD8^{-/lo}$: 0.6 (0.2–3.7) $CD4^+/CD8^{hi}$: 0.34 (0.0–2.1)	700 (400–1900) 400 (170–1000) 50 (10–200) 20 (7–100)
Clonal expansions of $CD4^+$ T-cells[e] (n = *34)*			
Sézary syndrome ($n = 9$)	9/9	24 (4.4–88.7)	3560 (170–5300)
T-PLL ($n = 5$)	5/5	90 (61–96)	214 000 (63 000–528 000)
ATLL ($n = 4$)	4/4	82 (39–85)	23 600 (3200–34 900)
$CD4^+$ LGL ($n = 4$)	4/4	25 (10–60)	2300 (1500–3200)
AITL ($n = 2$)	2/2	5.6 (3–8.3)	1100
PTCL-NOS ($n = 10$)	10/10	14 (3.3–65)	3000 (1900–6100)
Clonal expansions of $CD8^{hi}$ T-cells (n = *11)*			
T-LGL ($n = 10$)	9/10	27 (1–56)	1500 (110–9500)
$CD8^{hi}$ PTCL-NOS ($n = 1$)	1/1	17	6010
Clonal expansions of $CD4^-/CD8^{-/lo}$ T-cells (n = *11)*			
$TCR\gamma\delta^+$ T-LGL ($n = 9$)	8/9	19 (1.3–33)	1500 (30–5900)
$TCR\gamma\delta^+$ hepatosplenic T-cell lymphoma ($n = 1$)	1/1	24	ND
$TCR\alpha\beta^+$ T-CLPD ($n = 1$)	1/1	75	NA

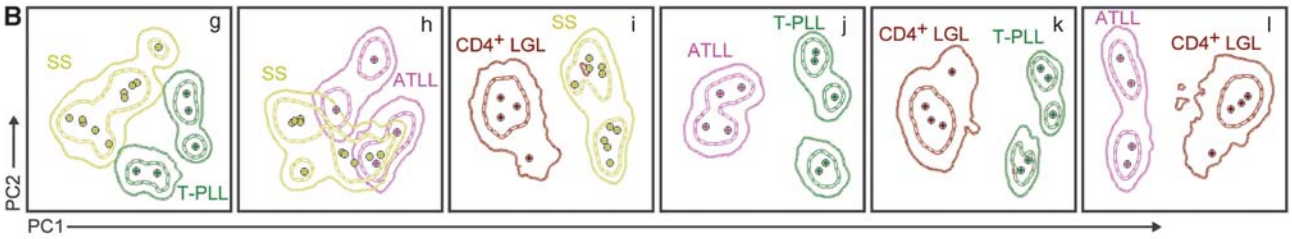
Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; ATLL, adult T-cell leukemia–lymphoma; BM, bone marrow; CLPD, chronic lymphoproliferative disorders; LGL, large granular lymphocyte leukemia; NA, not applicable; ND, not available; PB, peripheral blood; PTCL-NOS, peripheral T-cell lymphoma not otherwise specified; TCR, T-cell receptor; T-PLL, T-cell prolymphocytic leukemia; WBC, white blood cells. [a]A total of 67 cases was evaluated, which corresponded to 59 PB, 7 BM and 1 ascitic fluid sample. [b]According to previously reported studies (References[78,243,245,254,258,260-264,266]). [c]Results expressed as median % of T-cells from WBC (range). For the different patient groups, only data from the expanded/ aberrant T-cell population is shown. [d]Results expressed as median absolute number of circulating T-cells $\times 10^3/\mu l$ (range) for the PB samples analyzed; for the different patient groups, only data from the expanded/ aberrant T-cell populations are shown. [e]Regardless of CD8 expression (either negative or dimly positive).

by PCA with the APS function provided with the Infinicyt software program was used.[16,241] Regarding clonal CD4$^+$ T-cell cases, samples from all T-CLPD categories but PTCL-NOS included in the multicenter testing phase (Sézary syndrome (SS), T-PLL, ATLL, CD4$^+$ T-LGL and AITL) clearly clustered separately from normal CD4$^+$ T-cells (Figure 27A, plots 'a' to 'f'). The most informative markers responsible for such separation included maturation-associated molecules such as CD45RA, CD45RO, CD27 and CD28—probably reflecting the fact that normal CD4$^+$ T cells are heterogeneous, in contrast to the more homogeneous maturational patterns displayed by clonal T cells—and markers expected to be relevant for such a discrimination between normal CD4$^+$ T cells and clonal cells from SS (CD2, CD26 and CD7), T-PLL (CyTcl1), ATLL (HLADR and CD25), CD4$^+$ T-LGL (CD28, CyGranzyme B and CD7) and AITL (CD279, HLADR and SmCD3) (Figure 27). Similarly, CD4$^+$ T-CLPD cases from different WHO disease categories also expressed distinct phenotypic profiles versus the other groups, as illustrated in Figure 27B (plots 'g' to 'l') for several of the most relevant two-by-two comparisons. The precise markers mostly contributing to discriminate each CD4$^+$ T-CLPD group from other CD4$^+$ reference groups are also displayed in this figure (Figure 27B, plots 'g' to 'f'); interestingly, these discriminating markers included most of the markers found to be also relevant for the discrimination between each WHO disease group and normal CD4$^+$ T-cells. PTCL-NOS cases were not included in such comparisons since, as expected, they formed a highly heterogeneous group. However, when each PTCL-NOS case was individually compared with normal CD4$^+$ T-cells and the cases from the other WHO disease categories, no overlap was observed, except for 4/10 PTCL-NOS cases that showed partial overlap with Sézary cells. Noteworthily, no CD30$^+$ cases have been analyzed so far with the EuroFlow panel, but data derived from previous experience and immunohistochemical analyses support its utility in the T-CLPD panel proposed here.

Regarding the CD8^{hi} (10/11) and CD4$^-$/CD8$^{-/lo}$ (10/11) T-CLPD groups, all but two cases also showed clearly different immunophenotypic profiles versus their corresponding normal T-cell counterparts, as illustrated in Figure 28a and c for representative cases from both groups. In only one T-LGL case in each of the CD8^{hi} and CD4$^-$/CD8$^{-/lo}$ T-CLPD groups (Figure 28b and d) was marked overlap between the phenotypic profiles of clonal and normal T-cells observed. Markers contributing the most to the discrimination between each clonal CD8^{hi} T-CLPD case and the reference group of normal CD8^{hi} T-cells included CD45RO, CD27, CyGranzyme B, CD28, CD57 and CD45RA; similarly, CD28, CyGranzyme B, CD45RA, CD45RO, CD16, CD11c and CD27 were



CD2	15%	CD45RA	14%	CD45RA	11%	CD28	15%	HLADR	13%	CD45RA	10%
CD45RO	15%	CD45RO	13%	CD7	11%	CD57	10%	CD279	11%	CyGranzyme B	8%
CD45RA	9%	CD3	10%	CD2	10%	CD27	9%	CD45RA	10%	CD7	8%
CD26	8%	CD26	10%	CD45RO	9%	CyGranzyme B	9%	CD3	10%	CD27	8%
CD7	8%	CD8	8%	HLADR	8%	CD45RA	8%	CD7	8%	CD3	7%
CD279	7%	CD27	5%	CD25	7%	CD7	8%	CyGranzyme B	7%	CD28	6%



CD2	13%	CD45RO	22%	CD28	15%	CD7	12%	CD28	14%	CD28	14%
CD26	12%	HLADR	15%	CD45RA	11%	CD26	11%	CD26	10%	CD45RA	13%
CD7	11%	CD45RA	14%	CD57	11%	HLADR	9%	CD197(CCR7)	9%	CD57	10%
CD45RO	10%	CD2	12%	CD2	9%	CD197(CCR7)	8%	CD7	8%	CD45RO	9%
CD8	8%	CD25	9%	CD27	9%	CD2	8%	CD45RA	8%	CD2	8%
CD197(CCR7)	7%	CD5	5%	CD197(CCR7)	7%	CyTcl1	8%	CD57	7%	CD27	7%

Figure 27. Comparative principal component (PC)1 versus PC2 views of CD4$^+$ T-chronic lymphoproliferative disorders (CLPD) cases. **A** (plots a–f) shows the APS (automated population separator, PC1 versus PC2) views for the comparisons of each CD4$^+$ T-CLPD WHO diagnostic subgroup—Sézary syndrome (SS), light green; T-cell prolymphocytic leukemia (PLL), dark green, adult T-cell leukemia/lymphoma (ATLL), pink; CD4$^+$ large granular lymphocytic (LGL) leukemia, brown; angioimmunoblastic lymphoma (AITL), dark blue; and peripheral T-cell lymphoma not otherwise specified (PTCL-NOS), red—versus normal CD4$^+$ T-cells (green), while **B** (plots g– l) shows two-by-two PCA comparisons between different diagnostic categories of CD4$^+$ T-CLPD. Each circle represents one single case (median expression observed for all phenotypic parameters evaluated), while contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively). The six most informative markers contributing to the best discrimination between CD4$^+$ T cells from the different cases are displayed at the bottom of each plot, in a decreasing order of percentage contribution to the discrimination.

the most informative markers for the distinction between normal and clonal CD4$^-$/CD8$^{-/lo}$ T-cells. No clear phenotypic differences were observed among the distinct WHO disease categories either within the CD8^{hi} (TCR$\alpha\beta^+$ T-LGL versus CD8^{hi} PTCL-NOS) or within the CD4$^-$/CD8$^{-/lo}$ (TCR$\gamma\delta^+$ T-LGL versus hepatosplenic T-cell lymphoma) T-CLPD groups (data not shown); however, owing to the limited number of cases included in some of these groups, further studies are required in the future to confirm these very preliminary findings.

CONCLUSIONS

Although the series of T-CLPD patients analyzed is still relatively limited ($n = 56$), particularly within some WHO disease categories, our results clearly show that the 8-color EuroFlow T-CLPD panel contributes to the diagnostic classification of T-CLPD. Accordingly, this panel allows distinction between clonal T-cells and their corresponding normal counterparts in the vast majority of cases (50/56); at the same time, it provides useful information for the subclassification of the disease. In this regard, the panel proposed allowed unequivocal classification of CD4$^+$ T-CLPD into specific WHO subtypes, particularly SS, T-PLL, ATLL, CD4$^+$T-LGL and AITL, for which markers like CD2, CD7, CD26, CD28 (for SS), CD25, HLADR (for ATLL), cytotoxic-associated markers (for CD4$^+$ T-LGL), CyTcl1 (for T-PLL) and CD279 (for AITL) were particularly informative. The precise value of CD30 in this panel needs to be further investigated in future studies, as no CD30$^+$ T-CLPD cases have been analyzed so far. Regarding CD8^{hi} and CD4$^-$/CD8$^{-/lo}$ T-CLPD, our data also confirm the value of the panel in discriminating between clonal cytotoxic effector T-cells and their normal counterparts, except for a few T-LGL leukemia cases, where clonal T-cells overlapped with their normal/reactive counterpart; however, the value of the marker combinations used here for the identification of specific CD8$^+$ and CD4$^-$/CD8$^{-/lo}$ T-CLPD WHO disease categories seems to be more limited than in CD4$^+$ T-CLPD cases and deserves further investigation in larger series of patients, both by the EuroFlow Consortium and by other groups.

SECTION 10. ANTIBODY PANEL FOR NK-CELL CHRONIC LYMPHOPROLIFERATIVE DISEASES (NK-CLPD)

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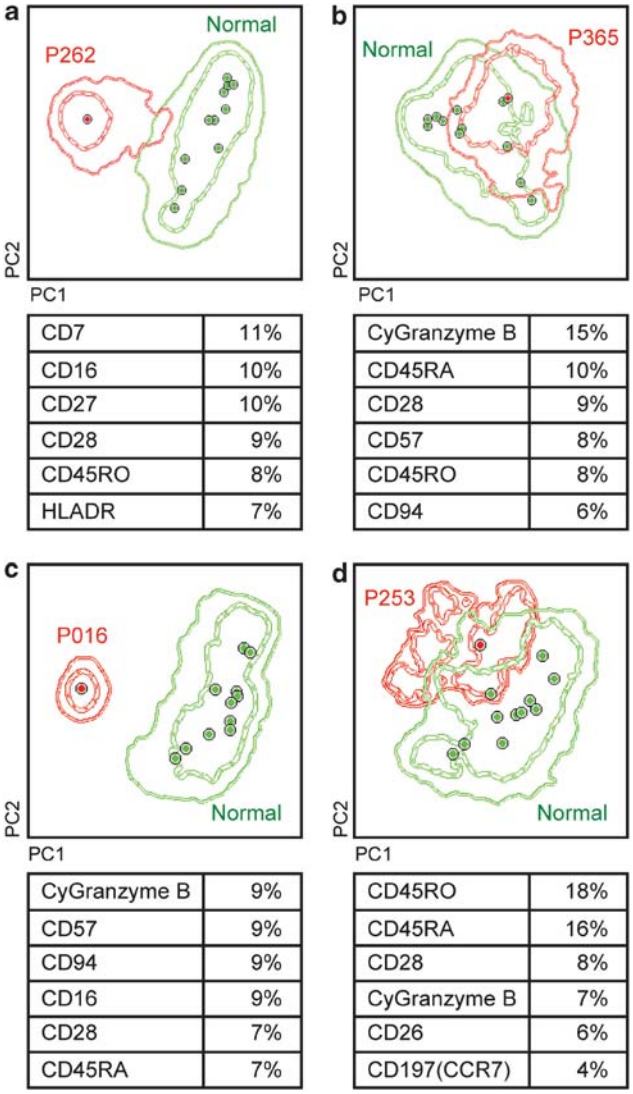
[1]USAL, Salamanca, Spain; [2]AP-HP, Paris, France; [3]UNILEEDS, Leeds, UK; [4]Erasmus MC, Rotterdam, The Netherlands; [5]HUS, Salamanca, Spain; [6]HSA-CHP, Porto, Portugal; [7]IPOLFG, Lisbon, Portugal; [8]UNIKIEL, Kiel, Germany; [9]SUM, Zabrze, Poland; [10]DPH/O, Prague, Czech Republic and [11]Cytognos SL, Salamanca, Spain

BACKGROUND

NK-cell neoplasms are rare hematological disorders, which represent $<1\%$ of all lymphomas and chronic lymphoid disorders in Western countries.[267] In addition to their rarity, they are also rather heterogeneous, including distinct clinicopathologic disease entities. According to the most recent WHO classification,[3] three distinct malignancies derived from mature NK-cells are recognized: aggressive NK-cell leukemia, extranodal (nasal type) NK/T-cell lymphoma and CLPD of NK cells. The former two entities are strongly associated to infection by the Epstein–Barr virus (EBV) and they are characterized by an aggressive clinical course and poor survival. Conversely, CLPD of NK cells (NK-CLPD) represent a new provisional category, which includes cases previously designated as chronic NK-cell lymphocytosis, chronic NK-large granular leukemia and NK-cell LGL lymphocytosis, among other terms.[3,268] Indeed, NK-CLPD comprises a broad spectrum of NK-cell proliferations,[3,268] from reactive to neoplastic expansions, difficult to distinguish because of lack of a universal NK-cell marker for clonality. Noteworthily, NK-CLPD is the most common mature NK-cell proliferation in Caucasians; it is not systematically associated to EBV infection[3] and the clinical course of patients is usually indolent. In these patients the number of NK cells



CD7	11%
CD16	10%
CD27	10%
CD28	9%
CD45RO	8%
HLADR	7%

CyGranzyme B	15%
CD45RA	10%
CD28	9%
CD57	8%
CD45RO	8%
CD94	6%

CyGranzyme B	9%
CD57	9%
CD94	9%
CD16	9%
CD28	7%
CD45RA	7%

CD45RO	18%
CD45RA	16%
CD28	8%
CyGranzyme B	7%
CD26	6%
CD197(CCR7)	4%

Figure 28. Comparative principal component (PC)1 versus PC2 views of CD8^{hi} and CD4$^-$/CD8$^{-/lo}$ T-cells in four T-cell chronic lymphoproliferative disease (T-CLPD) cases. (**a**, **b**) show APS (automated population separator, PC1 versus PC2) views of two CD8^{hi} T-CLPD cases versus normal CD8^{hi} T-cells, while **c** and **d** show comparisons of two CD4$^-$/CD8$^{-/lo}$ T-CLPD cases versus normal CD4$^-$/CD8$^{-/lo}$ T-cells. (**a**) and (**c**) show representative cases of most CD8^{hi} and CD4$^-$/CD8$^{-/lo}$ T-CLPD patients who had a phenotypic pattern clearly different from that of their normal T-cell counterpart (10/11 cases for each T-CLPD group), while (**b**) and (**d**) show the only two cases for which clonal T-cells displayed an overlapping phenotype with that of normal T-cells. In all panels, T-CLPD samples are depicted as red circles, while normal/reference T-cells are shown as green circles. Each circle represents one single case (median expression observed for all phenotypic parameters evaluated), while contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively). The six most informative markers contributing to the best discrimination between normal and clonal T-cells from each individual case displayed are listed at the bottom of each plot, in a decreasing order of percentage contribution to the discrimination.

Table 19. Design of the NK-CLPD panel[a]

Version (no. of cases)[b]	*Tube*	*Fluorochromes and markers*								*Aim*
		PacB	*AmCyan*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*AF700*	
BB 1	1	CD19	CD45			SmCD3			CD56	
		PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*	
BB 2	1		CD45			SmCD3	CD56		CD19	
Final ($n = 38$)	1	CD2	CD45	CD7	CD26	SmCD3	CD56	CD5	CD19	Aberrant NK-cells
	2	CD16	CD45	CD57	CD25	SmCD3	CD56	CD11c	CD19	Aberrant NK-cells
	3	HLADR	CD45	CyPerforin	CyGranzyme B	SmCD3	CD56	CD94	CD19	Aberrant NK-cells, cytotoxic phenotype

Abbreviations: AF700, alexa fluor 700; AmCyan, *Anemonia Majano* cyan fluorescent protein; APC, allophycocyanin; BB, backbone; CLPD, chronic lymphoproliferative disorder; Cy, cytoplasmic; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, hilite7; PacB, pacific blue; PacO, pacific orange; PB, peripheral blood; PE, phycoerythrin; PerCPCy5.5, peridinin–chlorophyll–protein–cyanin5.5; Sm, surface membrane. [a]Further information about markers and hybridomas is provided in the Appendix. [b]A total of 38 PB samples was evaluated, which corresponded to 10 normal and 28 pathological samples. Highlighted boxes: changes as compared to previous version.

typically remains stable for many years without therapy and sometimes it may even show spontaneous regression,[268] which could reflect in such cases the reactive nature of the expansion; however, rare cases of NK-CLPD have been reported that transformed into an aggressive NK-cell leukemia.[269,270]

As in European countries, most NK-cell proliferations correspond to NK-CLPD; the most common reason for consulting in such cases is lymphocytosis detected in a routine WBC count in association or not with cytopenias and in the absence of other associated symptoms or physical signs of disease. According to the stepwise diagnostic workflow recommended by the EuroFlow Consortium (Figure 1), in a first step the LST (see Section 2) should be applied in these cases and when the NK-cell lineage is suspected to be responsible for the lymphocytosis—absolute or relative expansion of either $CD56^{+}$ or $CD56^{-/lo}$/ $CD45^{hi}$ cells in the absence of expression of SmCD3, CD4, TCR$\gamma\delta$ and CD19 in the LST—the NK-CLPD panel is then applied in a second step, for full characterization. Therefore, the EuroFlow NK-CLPD panel aims at further characterization of the expanded NK-cells, to discriminate between aberrant and normal/reactive NK-cells, and to establish the precise diagnosis.

Selection of backbone markers

Four backbone markers (CD45, SmCD3, CD56 and CD19) were selected for the NK-CLPD panel, to unequivocally identify NK cells, as those $CD45^{hi}$/$SmCD3^{-}$ events showing reactivity for CD56; inclusion of CD19 and SmCD3 as backbone markers is supported by the fact that in around one-third of NK-cell expansions NK cells show absence or dim expression of CD56;[79] therefore, in these latter cases NK cells are identified by exclusion of the remaining cells included in the, for example, $CD45^{hi}$/SSC^{lo} 'lymphocyte gate' (T- and B-cells). In the first testing phase, the following fluorochrome conjugates were used as backbone reagents (BB1 in Table 19): CD19–PacB, CD45–AmCyan, CD3–PerCPCy5.5 and CD56–AF700. This proposed combination was initially tested at one center in eight PB samples from healthy adults. This showed that while the performance of the CD19–PacB and CD3–PerCPCy5.5 conjugates was optimal—in terms of, for example, brightness, SI, limited spectral overlap—to exclude mature B- and T-cells, respectively, two technical limitations existed: (i) interference of CD45-AmCyan in the FITC channel[16] and (ii) low resolution of the CD56–AF700 conjugate to identify $CD56^{lo}$ NK cells. Consequently, the following changes were introduced in the backbone markers of the NK-CLPD panel: (i) the AmCyan fluorochrome was replaced by PacO for evaluation of CD45, and (ii) the CD56–AF700 conjugate was planned to be substituted by an anti-CD56–APCH7 reagent. However, owing to the limited availability of APCH7-conjugated reagents at the time of testing (November 2007), a second alternative combination of backbone markers for the NK-CLPD panel was designed (BB2 in Table 19): CD45–PacO, CD56–PECy7, CD3–PerCPCy5.5 and CD19–APCH7. Of note, the CD56–PECy7 reagent included in this second version of the NK-CLPD backbone was selected after testing in parallel two different commercially available CD56–PECy7 reagents: CD56–PECy7 from BD Biosciences (clone: NCAM16.2) and CD56-PECy7 (clone: N901/NKH1); this comparison was performed at one center in a total of three PB samples from healthy adults, and it showed a better performance for the latter reagent, with a greater SI versus the former conjugate (median SI of 9.8 versus 5.2, respectively).

Testing of this second combination of backbone markers proposed for the NK-CLPD panel was initially done at one center in three PB samples. Preliminary results showed a clear

Table 20. Utility of NK-CLPD markers

Tube	*Target antigen*	*Identification of NK-cells*	*Diagnostic level*	
			1st	*2nd*
BB markers	SmCD3	X		
	CD19	X		
	CD45	X		
	CD56	X		
Tube 1	CD2		X	
	CD7		X	
	CD26			X
	CD5			X
Tube 2	CD16		X	
	CD57			X
	CD25			X
	CD11c			X
Tube 3	HLADR		X	
	CyPerforin			X
	CyGranzyme B			X
	CD94		X	

Abbreviations: BB, backbone; CLPD, chronic lymphoproliferative disorders; Cy, cytoplasmic; Sm, surface membrane.

improvement as regards identification of NK cells, in the absence of significant technical issues. Consequently, the combination underwent further evaluation at two EuroFlow sites for a total of 12 PB samples. The new combination of backbone markers proved to be useful for the identification of NK-cells, and their discrimination from the remaining leukocyte populations in every case tested, both in normal/reactive ($n = 8$) and in NK-CLPD ($n = 4$) PB samples. Importantly, this combination of backbone markers was also robust enough to allow gating, merging and calculating strategies with the Infinicyt software, as detailed below.

Selection of characterization markers

Once the optimal backbone reagents were definitively established, antibody reagents for further characterization of NK cells were selected from a large list of NK-cell related markers, previously tested by our[79,80,271] and/or other[258,272–276] groups. Initially, this included a large list of monoclonal antibodies directed against classical NK-cell-associated antigens, including signaling molecules (for example, CD2, CD5, CD7 and CD8), the CD16 low-affinity FcγRIII, activation-related markers (for example, CD26, CD38, CD45RO, CD69 and HLADR), interleukin-2 receptors (CD25 and CD122), cytotoxic molecules (for example, CD11c and CD57) and intracytoplasmic enzymes (for example, perforin, granzymes and TIA-1), together with antibodies against killer-cell Ig-like receptors (KIR; for example, CD158a/b/d/e/l and NKB1), leukocyte Ig-like receptors (LIR; for example, CD85j (LIR1/ILT2)), C-type lectin-like receptors (for example, NKG2A/D, CD94 and CD161) and natural cytotoxicity receptors (NCR), such as CD335 (NKp46), CD336 (NKp44) and CD337 (NKp30), among others. Based on existing data about all these markers, the following criteria were used to select reagents for further testing: the ability to better distinguish normal/reactive versus immunophenotypically aberrant NK-cells and to assess a cytotoxic effector phenotype, and therefore determine the maturation stage of the expanded NK-cells.

Regarding the first purpose, it has been recurrently shown that aberrant or clonal NK cells display unique and altered patterns of expression of CD2, CD7, HLADR and CD94 versus both normal and reactive NK cells,[3,79,80,271,272,277] although a relatively high degree of overlap has been observed. Therefore, reagents recognizing the above referred molecules were considered as mandatory to be selected for further testing (*first diagnostic level* markers in Table 20) and hence included in the NK-CLPD panel, together with CD16; this latter marker was selected as a confirmatory marker typical of $CD56^{lo}$ NK-cells, but also as a useful marker to identify some (rare) $CD16^{-/lo}$ NK-cell malignancies.[270] In order to assess a cytotoxic effector phenotype and the maturation stage of the expanded NK-cells, markers known to be expressed on terminally differentiated cytotoxic effector cells such as CD11c, CD57, perforin and granzyme B[80,271,278,279] were selected to be included in the NK-CLPD panel (Table 19). As these molecules are also expressed by normal/reactive NK-cells, and are therefore not useful for their distinction from clonal/aberrant NK-cells, antibodies recognizing cytotoxicity-related molecules were considered as *second-level* markers (Table 20). Additional antibodies against molecules not usually present in most normal NK-cells under baseline conditions, were also selected for testing with the NK-CLPD panel (CD5, CD25 and CD26), as they have been found to be expressed by pathological NK-cells in some (rare) cases. Other proteins that have been claimed to potentially serve as surrogate markers for NK-cell clonality, such as restricted or absent expression of a single isoform of the NK-cell-associated antigen families (for example, KIR), were finally not included in the EuroFlow NK-CLPD panel, based on the lack of conclusive results reported so far in this regard[258,273–276,280] and the experience of the EuroFlow groups. Previous studies in short series of NK-CLPD cases (between 3[(ref. 273)] and 15[(ref. 280)] cases) pointed out that NK-cell expansions frequently show a skewed NK-receptor expression (for example, homogeneous expression of one or more KIR, particularly CD158a and CD158b),[273,274,280] in contrast to normal NK-cells, which typically display a diversified repertoire of NK-cell receptors. Despite this, both the sensitivity and specificity of this approach to unequivocally identify clonal NK-cells is currently poor (unless a relatively extensive set of antibodies against the repertoire of NK-cell receptors is used), particularly when a background of normal residual NK-cells co-exists with a clonal population of NK-cells (data not shown).

Once the target molecules were selected, monoclonal antibodies against them were chosen from among the PacB-, FITC-, PE- and APC-fluorochrome conjugates commercially available (Table 19). The precise role for each marker included in the NK-CLPD panel is summarized in Table 20.

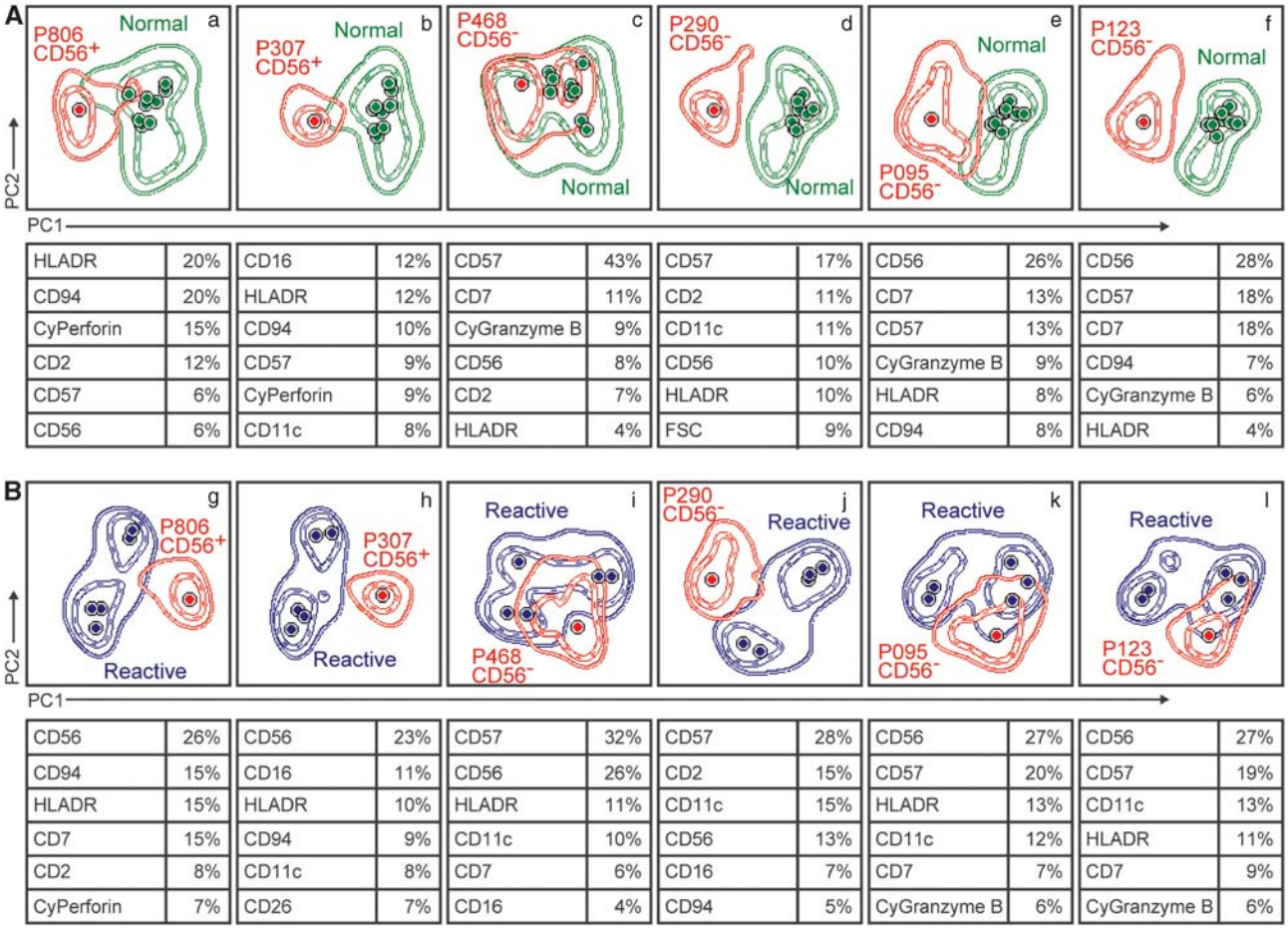
Multicenter testing of the EuroFlow NK-CLPD panel

Testing of the EuroFlow NK-CLPD panel was carried out by seven EuroFlow centers involved in the study of NK-CLPD. A total of 38 PB samples were stained with the full panel: 10 samples from healthy adults (mean age of 36 ± 6 years; 5 males and 5 females); 11 female patients (64 ± 21 years) with expanded circulating

Table 21. Phenotypic patterns and percentage of NK-cells detected with the EuroFlow NK-CLPD panel

Diagnostic group	*Aberrant NK-cells*[a] *(no. of cases/total cases)*	*% of NK-cells (from WBC)*[b]	*No. of PB NK-cells*[c] *(× $10^3/\mu l$)*
Healthy adult donors	0/10	2.1 (1.5–4.5)	130 (60–290)
Cases with expanded polyclonal (reactive) NK-cells[d,e]	0/5	14.5	2020
Clonal NK-CLPD[f]	6/6	30 (16–65)	3280 (1480–7800)
Cases with expanded NK-cells not tested for clonality[g]	9/17	44 (30–62)	4710 (290–7500)

Abbreviations: CLPD, chronic lymphoproliferative disorders; HUMARA, human androgen receptor X-chromosome inactivation assay; PB, peripheral blood; WBC, white blood cells. [a]According to previously reported studies.[79,80,271,277]. [b]Results expressed as median % of NK-cells from WBC (range). [c]Results expressed as median absolute number of circulating NK-cells $\times 10^3/\mu l$ (range). [d]In these cases, diagnosis (assessment of the clonal versus polyclonal nature of the NK-cell expansions) was established by the HUMARA assay,[278,279] performed on highly purified PB NK-cells. [e]In 1 case, NK-cells showed a borderline altered phenotype (virtually all NK-cells, representing 46% of WBC and 7220 cells $\times 10^3/\mu l$, were $CD56^{-/lo}$/ $CD7^{-/lo}$/$CD2^+$/$CD11c^+$/$CD57^{-/+}$/$CD94^{lo}$/ $HLADR^{-/+}$). [f]In 1/6 cases (showing 65% NK-cells), the expanded cells showed overlapping features with reactive NK-cells ($CD56^{-/lo}$/$CD7^{-/lo}$/$CD2^+$/$CD11c^+$/ $CD57^{+/hi}$/CyPerforin^{hi}/ CyGranzyme B^{hi}/$CD94^{lo}$/$HLADR^{-/+}$). [g]Seven of the 9 cases with phenotypically abnormal/aberrant NK-cells were classified by the Infinicyt software as clonal cases (2 were clinically diagnosed with CLPD), one case as reactive and one case could not be classified; from the remaining 8 cases, 7 cases showed a reactive NK-cell phenotype, while one case showed a normal NK-cell phenotype in the manual analysis; from the 7 cases showing a reactive NK-cell phenotype, 4 cases were classified by principal component analysis (PCA) as normal/reactive, 2/7 as clonal (one of them corresponded to the case referred in footnote f) and 1/7 could not be classified; the case considered to have a phenotypically normal NK-cell phenotype could not be classified by PCA and it showed a borderline altered $CD56^+$/$CD7^-$/$CD2^+$/$CD11c^-$/$CD57^{-/+}$/$CD94^+$/$HLADR^-$ phenotype.



HLADR	20%	CD16	12%	CD57	43%	CD57	17%	CD56	26%	CD56	28%
CD94	20%	HLADR	12%	CD7	11%	CD2	11%	CD7	13%	CD57	18%
CyPerforin	15%	CD94	10%	CyGranzyme B	9%	CD11c	11%	CD57	13%	CD7	18%
CD2	12%	CD57	9%	CD56	8%	CD56	10%	CyGranzyme B	9%	CD94	7%
CD57	6%	CyPerforin	9%	CD2	7%	HLADR	10%	HLADR	8%	CyGranzyme B	6%
CD56	6%	CD11c	8%	HLADR	4%	FSC	9%	CD94	8%	HLADR	4%

CD56	26%	CD56	23%	CD57	32%	CD57	28%	CD56	27%	CD56	27%
CD94	15%	CD16	11%	CD56	26%	CD2	15%	CD57	20%	CD57	19%
HLADR	15%	HLADR	10%	HLADR	11%	CD11c	15%	HLADR	13%	CD11c	13%
CD7	15%	CD94	9%	CD11c	10%	CD56	13%	CD11c	12%	HLADR	11%
CD2	8%	CD11c	8%	CD7	6%	CD16	7%	CD7	7%	CD7	9%
CyPerforin	7%	CD26	7%	CD16	4%	CD94	5%	CyGranzyme B	6%	CyGranzyme B	6%

Figure 29. Comparative principal component (PC) 1 versus PC2 views of clonal versus both normal and reactive NK-cell reference cases. The APS (automated population separator, PC1 versus PC2) views of each CD56^{+} (Panel **A**, plots a and b, and Panel **B**, plots g and h) and CD56$^{-/lo}$ (Panel **A**, plots c–f, and Panel **B**, plots i–l) clonal NK-cell case (different red circles), versus the reference groups of normal (green circles) and reactive/polyclonal NK-cells (blue circles). Each circle represents one single case (median expression observed for all phenotypic parameters evaluated), while contour lines represent s.d. curves (dotted and continuous lines represent 1s.d. and 2s.d., respectively). The six most informative markers contributing to the best discrimination between each clonal NK-cell CLPD case and the corresponding reference group are displayed at the bottom of each plot, in a decreasing order of percentage contribution to the discrimination.

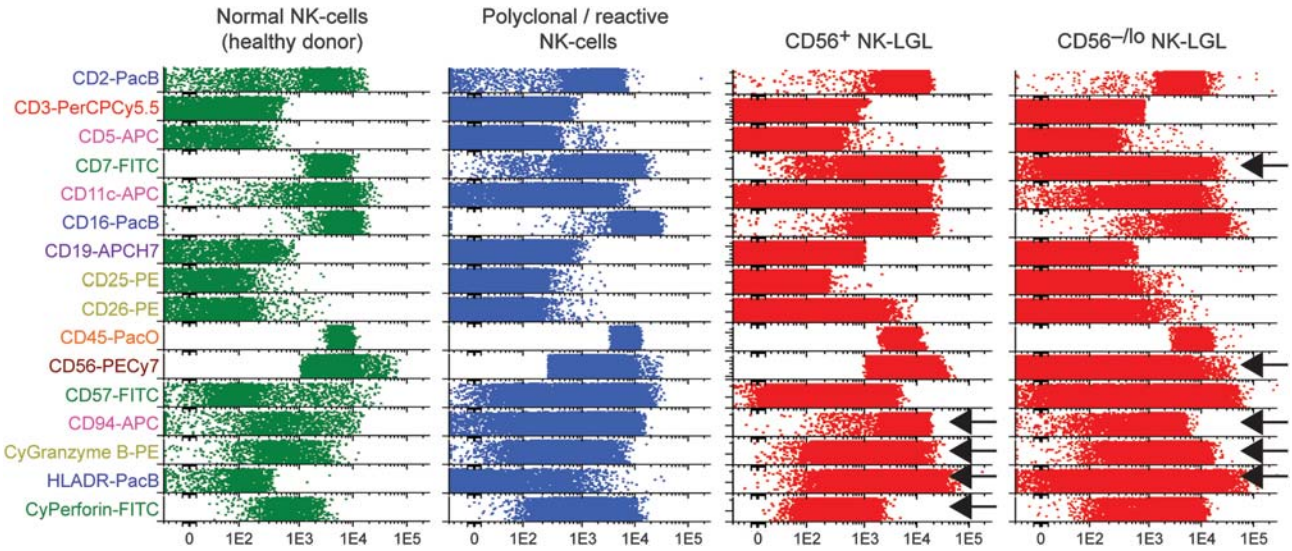


Figure 30. Illustrative phenotypic patterns of NK-cells from healthy donors, and patients with expanded polyclonal, clonal CD56^{+} and clonal CD56$^{-/lo}$ NK-cells. Arrows point to the most informative markers in the distinction between each clonal NK-cell case versus their normal and reactive NK-cell counterparts.

NK-cells found to be suitable (heterozygous) for the HUMARA assay–six clonal and five polyclonal cases—and 17 samples from patients with expanded PB NK-cells (mean age: 66 ± 12 years) in whom the HUMARA assay was not performed (13 males and 3 females) or it was not informative to assess NK-cell clonality (one homozygous female for the HUMARA assay) (Table 21). A case was considered to be monoclonal by the HUMARA assay when the corrected allele ratios were $\leqslant 0.33$ or $\geqslant 3$ for both NK-cells and NK-cells/control cells, indicating that one of the parental alleles was represented at an excess of $\geqslant 50\%$ versus the other allele[278,279] (paired normal purified T-cells or neutrophils were used as control cells). In 2/6 cases in which the presence of monoclonal NK cells could be confirmed (both showing a $CD56^{-/lo}$ phenotype), T-cell clonality was ruled out by PCR TCR gamma gene analysis.

In order to evaluate the utility of the EuroFlow NK-CLPD panel for the distinction between normal/reactive and clonal NK-cells, pairwise unsupervised discrimination by PCA with the APS function provided with the Infinicyt software program was used. In order to be sure about the polyclonal versus clonal nature of the expanded NK-cells from patients, only samples from healthy subjects ($n = 10$) and those female patients ($n = 11$) found to be heterozygous for the HUMARA assay–six clonal and five polyclonal cases—were included in the PCA-based comparisons. As shown in Figure 29A (plots 'a' to 'f'), cases stained with the EuroFlow NK-CLPD panel, from both the $CD56^{+}$ ($n = 2$) and the $CD56^{-/lo}$ ($n = 4$) clonal NK-cell groups, showed clearly different immunophenotypic profiles versus normal NK-cells ($n = 10$). Similarly, cases from the $CD56^{+}$ clonal NK-cell group ($n = 2$) also showed distinct phenotypic profiles as compared to the group of polyclonally expanded reactive NK-cells (Figure 29B, plots 'g' to 'l'). However, in 3/4 cases with $CD56^{-/lo}$ clonal NK-cells, partial overlap between the phenotypic profiles of clonal and normal or reactive NK-cells was observed (Figure 29A, plot 'c' and Figure 29B, plots 'i', 'k' and 'l'). Despite this, it should be noted that each of these three cases was clearly different from normal and reactive NK-cells, when comparisons were made separately for these cases versus each subset of $CD56^{+}$ and the $CD56^{-/lo}$/polyclonal NK-cells (data not shown).

The precise markers mostly contributing to discrimination of each clonal case from the reference groups of both normal and reactive/polyclonal NK cells included CD56, CD57, HLADR and CD94; in addition, CD7 was also an informative marker in the distinction between normal versus clonal NK-cells (mainly $CD56^{-/lo}$), and CyGranzyme B and CyPerforin—and to a lesser extent also CD2—were informative in the distinction between reactive/polyclonal versus clonal NK-cells (Figure 29). Illustrative phenotypic patterns of NK-cells from healthy donors and patients with expanded polyclonal and clonal NK-cells are shown in Figure 30.

Using this Infinicyt-based strategy, expanded NK cells from those additional 17 samples for which clonality could not be determined showed an aberrant phenotypic profile in nine cases (from which seven cases were also found to have phenotypically abnormal NK cells, according to previously reported studies[79,80,271,277]). In five cases, overlapping profiles with normal ($n = 3$) and reactive NK-cells ($n = 2$) were found, while in the remaining three cases the software did not allow precise classification into a specific reference group. Comparisons between the conventional and the new PCA-based classification strategies for these 17 samples are described in a footnote in Table 21.

CONCLUSIONS

Our results obtained in a relatively limited number of patients show that the EuroFlow NK-CLPD panel, consisting of three 8-color combinations of monoclonal antibodies, would contribute to improve the diagnosis of NK-cell CLPD, as it allows the identification of immunophenotypic profiles that distinguish clonal versus polyclonal NK-cells. As a major challenge within NK-cell CLPD is to distinguish between reactive and clonal proliferations of NK cells (because of lack of universal markers for NK-cell clonality), the EuroFlow panel and strategy would facilitate the distinction between them and therefore would be of great help in routine diagnostic settings. However, confirmation of these preliminary results in larger series of patients is necessary, which is a future goal of the EuroFlow activities.

GENERAL DISCUSSION

Immunophenotyping is an essential tool in the diagnostic work-up and classification of hematological malignancies.[1–3] Although other immunophenotypic techniques may also be used for evaluation of tissues, multiparameter flow cytometry is most frequently indicated. Despite the objectivity of flow cytometric measurements and the apparent simplicity of the technique, multiple problems and limitations have emerged over time.[281] Multiparameter flow cytometry is currently regarded as a costly and complex approach, because of increased reagent costs related to the progressively higher number of markers used for answering clinical questions, and because of the exponentially increased need for expertise in data analysis and interpretation. This, together with technical variations between different laboratories around the world (for example, number and type of reagents and instruments available), has resulted in many different strategies and antibody panels, almost as many strategies as individual flow cytometry laboratories. This diversity has hampered real standardization and reproducibility of flow cytometric diagnostics. In addition, interpretation of flow cytometry data slowly evolved from an objective measurement of the percentage of positive or negative cells to highly subjective interpretations of two-dimensional dot-plot pictures, which are interpreted in an 'experience-based' manner, as done for routine histomorphology and cytomorphology. Such shortcomings have pushed the need for standardization in the field and multiple initiatives have been made in the last two decades.[7,9,12–14,22,23,25–29,106,193,204,282–284] However, such 'standardization' initiatives are usually limited because they only address a part of the whole process, without true validation of the 'consensus proposals' in prospective studies. For example, most recommendations and guidelines only provide lists of informative markers without discussing reference reagents, fluorochrome conjugates and the most appropriate multicolor combinations. Even more, the proposed lists of markers are frequently grouped according to one or a few disease categories or cell lineages, which do not necessarily answer the clinical questions, particularly those raised in a diagnostic screening phase. As a consequence, excessively high numbers of markers are being used, which are frequently different between laboratories. Consequently, discrepant results might be obtained for the same patient samples.

Comparable to such consensus groups, also the EuroFlow Consortium started the EuroFlow project with a first *consensus* proposal of potentially informative markers and technical flow cytometry procedures.[16] However, the antibody panels were designed per set of related medical indications and clinical questions (Figure 1). As described in this report, validation of the proposed marker combinations and antibody panels showed multiple shortcomings. Consequently, for optimization purposes, all antibody panels required inclusion of additional markers, replacement of antibody clones and/or fluorochrome conjugates (many of which were even not commercially available) over 2 to 7 successive cycles of panel redesign and panel evaluation with exclusion of redundant markers while retaining the essential ones. Noteworthily, such cycles of panel redesign-and-evaluation aimed at final validation of the total set of EuroFlow antibody panels, which required analysis of more than 2000 informative samples as described in this paper. Of note, the end-points for validation of the EuroFlow panels were a 100% sensitivity with an optimized

specificity or a 100% specificity with a maximized sensitivity. Only under such conditions can we avoid the gray zone of uncertainty associated with approaches that aim at the greatest efficiency and that almost always include both false-negative and false-positive cases, and consequently have disturbing levels of uncertainty for individual (for example, atypical) cases. The EuroFlow validation process required new analytical tools for objective multivariate analysis of flow cytometry data that were not available as ready-to-use software functions and had to be designed and implemented into the user-friendly environment of the Infinicyt software.[16,31] The application of the new analytical tools in the multi-centric setting of the EuroFlow Consortium was made possible by extensive standardization of instrument settings and sample preparation protocols.[16] As an overall result, the validated EuroFlow antibody panels can be integrated in the diagnostic screening and classification strategies (Figure 1). These antibody panels are flexible and can be adapted to fit the needs of different diagnostic laboratories or clinical treatment protocols as discussed below, while serving as validated benchmark.

Acute leukemia orientation tube (ALOT)

The first section of this report describes a single 8-color immunostaining combination (ALOT), which is optimized for recognition of all major types of classical acute leukemias (BCP-ALL, T-ALL and AML) as well as of acute leukemias of ambiguous lineage and allows optimal choice of the most appropriate characterization panel for further phenotypic classification.

The WHO 2008 criteria for classification of acute leukemias emphasize the value of a limited number of lineage-related antigens (CyCD3, CD19 and CyMPO), diagnosis of MPAL predominantly relying on these three markers plus a few monocytic markers.[36,37] For the recognition of B-cell lineage, CD19 expression combined with CyCD79a or CyCD22 is proposed, whereas the sole expression of CyMPO or CyCD3 is regarded by the WHO criteria as sufficient to recognize myeloid and T-cell lineage-positive cases, respectively; CyCD3 would also be systematically necessary for the assignment of blasts to the T-cell lineage. In contrast to previous classifications, there is no diagnostic value for additional myeloid-associated markers such as CD13, CD33, CD117 and others, because they are not sufficiently lineage-specific and therefore they are not considered as definitive evidence for a specific lineage. Despite the fact that the ALOT was designed prior to the publication of the 2008 WHO criteria, the three major lineage-associated markers are all included in ALOT. Other markers considered to be of secondary importance for lineage-assignment were placed in the disease-oriented characterization panels (BCP-ALL, T-ALL and AML/MDS).

In previous versions of the WHO classification[285] only the percentage of cells expressing a marker was taken into account, whereas in the more recent 2008 version[3] the intensity of antigen expression is also regarded to be important. Combination of the ALOT with the EuroFlow instrument settings and sample preparation SOP, as well as the new software tools developed by the EuroFlow group, allows direct integration of quantitative data about the intensity of expression of each individual marker in single leukemic cells in the evaluation of the overall blast cell phenotype for the eight ALOT markers: the four lineage-associated markers (CD19, CyCD79a, CyCD3, CyMPO) plus CD7, CD45, CD34 and SmCD3. Multivariate analysis (for example, PCA) showed that all markers except those used for positive identification of blast cells (CD45 and CD34) or exclusion of mature cells (CD45 and SmCD3) were essential for obtaining optimal results (Figure 6). In this regard, it should be emphasized that ALOT was designed and validated for orientation of an acute leukemia sample towards further characterization panels and thus, it should not be used on its own for definitive exclusion of a hematological malignancy. Nevertheless, when the ALOT is combined with the LST and tubes 1–4 of the AML/MDS panel, virtually all hematological malignancies can be excluded or detected, albeit not definitively classified. In turn, when combined with the appropriate complementary panel, the precise diagnosis of acute leukemia, ambiguous lineage acute leukemia (for example, mixed phenotype and undifferentiated acute leukemias) can be obtained (Figures 3–5). In specific situations that only require distinction between malignant and normal lymphoid B-cell precursors (excluding MRD situations), the combination of ALOT with the first tube of the BCP-ALL antibody panel is sufficient, as discussed below.

Although the acute leukemia panels include two sequential steps (ALOT plus the BCP-ALL, T-ALL and/or AML/MDS panels), in some specific situations they can be performed simultaneously. For example, in case of high suspicion of AML, the ALOT and AML panels can be run in parallel. It should be noted that the ALOT is always required for complete phenotypic characterization of acute leukemias, because it includes essential markers required for the detection of AUL/MPAL. Although the ALOT alone showed promising results with respect to the orientation and identification of acute leukemias of ambiguous lineage among typical single-lineage acute leukemias, the cohort needs to be expanded to include a significant number of cases of these rare disease subcategories and determine whether optimal subclassification of AUL and MPAL cases may benefit in the future from the systematic application of one or two specific acute leukemia panels. The combined application of the ALOT and the new software tools (for example, PCA) of the Infinicyt software will undoubtedly assist acute leukemia diagnosis and promote true standardization in multicenter settings.

Lymphoid screening tube (LST)

Screening for the presence of aberrant mature B-, T- and NK-cell populations in the clinical diagnostic setting remains one of the most frequent requirements for flow cytometric immunophenotyping of leukemias and lymphomas. At present, a wide variety of approaches is used in individual laboratories, which frequently rely on a combination of (inter)national recommendations and local experience. In this report, a single 8-color, 12-marker LST is proposed for the diagnostic screening of CLPD in a wide variety of human samples including PB, BM and lymphoid tissue specimens.

The LST was designed to evaluate the overall lymphoid compartment of such samples in different clinical situations and medical indications. Accordingly, the LST allowed identification of lymphoid populations with aberrant or clonal phenotypes in virtually all infiltrated samples; in the remaining few cases, an altered numerical distribution of the specifically altered lymphocyte populations was observed, indicating that further assessment of the potential clonal nature of the expanded cells is required. Moreover, the information obtained with the LST efficiently points to the precise panel required for full characterization of the altered cell population, that is, PCD, B-CLPD, T-CLPD and/or NK-CLPD. Importantly, the data obtained in the screening step (LST) can be integrated with the data of the full characterization panels, particularly the B-CLPD antibody panel.

Noteworthy, an interesting alternative to the LST combination proposed (version 7) was designed to be well-suited to predominantly B cell CLPD-oriented laboratories (LST version 6). It takes optimal advantage of multiparameter flow analysis using a mixture of 12 antibodies for highly sensitive detection and classification of B-cell clones, particularly among patients with different subtypes of B-cell lymphoma in the absence of absolute or relative lymphocytosis. The major difference between the two LST versions relies on the substitution of CD38 in version 7 by CD10 in version 6. It should be noted that LST version 7 allows full integration with the B-CLPD panel and at the same time it provides a clear advantage for those diseases involving the

plasma cell compartment together or not with mature B-cells (for example, Waldenstrom macroglobulinemia[286]). Conversely, LST version 6 is better suited for cases of CD10$^+$ B-CLPD, specifically in FL patients with low levels of infiltration. However, it should be noted that the LST is not designed for MRD investigations. This requires specific combinations, which are currently being designed and evaluated by the EuroFlow group, aiming at the detection of minimal disease in the different WHO diagnostic subgroups of CLPD, including FL.

Extensive testing in multiple EuroFlow centers showed that the LST combination is a cost-effective protocol when compared to currently used approaches.[18,20] Accordingly, in the five evaluated EuroFlow laboratories the LST reduced the time required for sample processing and data analysis by around one-fourth (mean of 40 min versus 55 min) and reduced reagent costs (1 tube with 12 reagents versus a mean of 3 tubes and 13 markers), while resulting in a higher diagnostic efficiency. In this regard, it should be noted that individual markers in LST version 7 showed independent values. For example, TCR$\gamma\delta$ appeared to be useful for the screening of rare TCR$\gamma\delta$ lymphomas and other clonal expansions of TCR$\gamma\delta$ T-cells in 11/249 (4.4%) cases tested. However, because of the relatively low frequency of TCR$\gamma\delta$ expansions, usage of this marker could be optional at a screening stage and more suited for a later step in the classification of specific T-CLPD WHO entities (see Section 9). More importantly, the LST was highly efficient in distinguishing between normal and clonal/aberrant lymphoid populations, as confirmed through the multivariate analysis tools included in the Infinicyt software (for example, PCA). These findings suggest that automated approaches for recognition of immunophenotypic profiles may be used in the future for specific gating of lymphoid cell populations and its comparison with a pool of reference (for example, normal, reactive or neoplastic) data files for further detailed analysis (Figure 9). Finally, whereas the LST was designed for a diagnostic screening step when increased counts of clonal lymphocytes are typically present, the approach proved to have a good sensitivity, as it detected neoplastic cells at low levels (10^{-3}). Nevertheless, the LST is not optimally suited to search for malignant lymphoma cells in paucicellular samples like CSF or vitreous biopsies.

Small sample tube (SST)

Body fluids that contain low numbers of cells, such as CSF and vitreous biopsies (here collectively referred to as 'small samples'), pose a diagnostic challenge in case of clinical suspicion of lymphoma. Both single and multicenter studies have recently demonstrated that multiparameter flow cytometric analysis is the optimal strategy to establish the cellular composition of these low cell number samples and to confirm or exclude the presence of aberrant cell populations. Even though 4- and 6-color flow cytometric screening protocols have proven their value, the here-presented 8-color/13-parameter EuroFlow SST labeling is currently the most complete flow cytometry assay for clinically suspect CSF and vitreous samples. The main diagnostic contribution of the EuroFlow SST labeling concerns the screening for a primary lymphoma in those situations with a clinical lymphoma suspicion but without evidence for a systemic lymphoma in (earlier) analyses of other cell materials and tissues.

The results of the 141 CSF samples and 23 vitreous biopsies clearly underline the diagnostic value of the SST in that it allows for a complete typing of the most relevant leukocyte populations in these samples with positive identification of all normal subsets of hematopoietic cells present in CSF, except neutrophils (Figure 10). Based on FSC/SSC/CD45 gating neutrophils could be identified, although possible inclusion of, for example, CD11b or CD16 in a future version of the SST for example in the same fluorochrome position as SmCD3 and CD14 should help to define neutrophils in a more direct way. In addition, the SST can confirm or exclude the presence of aberrant B-, T- or plasma cell populations. In this respect, it has been suggested that a SmIgκ/SmIgλ ratio of <0.25 or >4 should be considered as a deviation from normal, and hence an indication for a B-cell lymphoma.[106] However, it should be noted that a skewed ratio per se is not sufficient for the diagnosis of B-cell lymphoma. Apart from clinical features and possibly also morphological indications, the entire flow cytometric profile should give more clues on the true aberrant immunophenotype of the involved cell population. Hence, a combined evaluation of all 13 parameters of the SST labeling using PCA clearly enhanced the diagnostic possibilities to identify aberrant B- and T-cell clones. Nevertheless, the set of markers is apparently still too limited to allow a detailed characterization of the aberrant B- or T-cell populations in all cases for further diagnostic subclassification into specific WHO disease categories. For this reason, we are currently evaluating additional B-cell and T-cell small sample labelings for further typing the aberrant cell population on the remaining cells of the CSF or vitreous material. An alternative option to confirm the aberrant character of the suspect B- or T-cell population could be to use the remaining cells for a multiplex PCR-based Ig/TCR clonality-testing strategy.[266]

Even though, the EuroFlow SST labeling can also be used for small sample analysis in cases with a systemic lymphoma, in such cases it may be better to choose a more targeted approach to identify the lymphoma population in CSF or vitreous biopsies using a limited set of markers specific for the lymphoma (so-called '*minimal disseminated disease*' approach). Finally, localization of acute leukemia cells in, for example, CSF is not a main application of the EuroFlow SST labeling, but would also most probably benefit from a more targeted approach.

Despite its high specificity and sensitivity, the exact diagnostic value of the EuroFlow SST labeling for small samples is greatly dependent on the number of cells that can be studied and the viability of these cells. Cell viability can be increased by collection of the cells in tubes with culture medium plus 10% FCS or 0.2% BSA and a short transport time between clinic and laboratory (optimally within 1 h). Alternatively, so-called TransFix collection tubes are also currently commercially available, which result in optimal stabilization of the cells for a few days (for example, 48 h) and improved cell detection.

Plasma cell disorders (PCD) panel

Interestingly, among all EuroFlow antibody panels, the PCD panel was the only one where the initial list of markers proposed to be included in the panel remained unchanged till the last version of the panel. This indicates that matured consensus already existed in the literature about the most informative and relevant phenotypic markers for evaluation of clonal plasma cells in PCD.[9,110] Nonetheless, because of technical factors and plasma cell-associated biological variables, fine adjustments of specific fluorochrome-conjugated reagents were required to reach optimal performance in the final configuration of the PCD panel (version 6). For several markers (for example, CD38 and β2 microglobulin) dim fluorochrome-conjugated antibody reagents associated with lower fluorescence intensities and lower fluorescence spillover to other channels were preferred. For these two reagents, final adjustments with a mixture of fluorochrome-conjugated and purified (unconjugated) antibodies was required to get optimal fluorescence staining patterns under saturating conditions. In turn, other reagents such as CD45–PacO, used in all other EuroFlow panels, could not be kept in the PCD combination at the same fluorochrome position due to the relatively low discrimination power between CD45$^-$ and CD45$^+$ plasma cells, mainly caused by the increased PC baseline autofluorescence levels in this channel. Last, but not least, the

limited availability of good-quality CD138 fluorochrome-conjugated reagents in the positions left by other markers forced its inclusion as a custom conjugate of PacO. Further testing of new commercially available good-quality conjugates for this marker, which work similarly under Sm only or intracellular markers staining protocols (for example, Horizon V500) is ongoing at the different EuroFlow sites.

As an end result a two-tube, 12-marker, 8-color PCD panel is proposed for accurate identification of plasma cells, specific detection and quantification of phenotypically aberrant or clonal versus normal/reactive/polyclonal plasma cell populations and detailed characterization of their immunophenotypic profiles. As tube 1 (CD38, CD138, CD45, CD19, CD56, β2 microglobulin, CyIgκ and CyIgλ) proved sufficient for the specific identification, enumeration and discrimination between normal/reactive and aberrant plasma cells, it can be used as a stand-alone tube for the initial screening of PCD in a two-step diagnostic approach (Figure 1). In such case, tube 2 could be optional and used only when indicated, for example, for assessment of additional aberrant markers for monitoring MM patients after therapy. Finally, this two-tube PCD antibody panel in combination with the EuroFlow LST and B-CLPD panels may also contribute to the diagnosis and classification of other B-CLPD categories, which involve the plasma cell compartment in addition to clonal B-lymphocytes, for example LPL, which includes Waldenström's macroglobulinemia.

Although the aim of the PCD EuroFlow panel was not directly focused on MRD investigation after therapy, the evaluation of the proposed panel in multiple centers showed a sensitivity of $\leqslant 10^{-3}$ (routine detection of 100 aberrant plasma cell in 100 000 cells acquired).

Antibody panel for B-cell precursor ALL (BCP-ALL)

The four tubes of the BCP-ALL panel in combination with the ALOT enable the diagnosis and detailed immunophenotypic characterization of BCP-ALL, including discrimination from normal and regenerating precursor B-cells. In addition, all phenotypic information required for subclassification of BCP-ALL according to the WHO classification is included.[34] The four tubes also contain antibodies for detection of LAP markers and phenotypes associated with genetic aberrations, such as CD66c and NG2. If immunophenotypic profiles show coexpression of B-lineage markers and myeloid-associated markers (for example, CD13, CD33, CD117, CD15 + 65) as well as CD34 and CD38 positivity, the AML-MDS tube 2 (with monocytic markers CD14, CD36, CD64) should be run in parallel to detect exceptional cases of monoblastic-MPAL.

The BCP-ALL protocol is designed in a flexible way so that only one or two tubes need to be used for specific clinical questions. For example, tube 1 of the BCP-ALL panel is excellently suited to distinguish normal or regenerating precursor B-cells (hematogones) from BCP-ALL blast cells. Tubes 1 and 2 are sufficient for full maturation-based classification of BCP-ALL according to EGIL criteria or WHO guidelines. Furthermore, the BCP-ALL antibody protocol can be run using conventional data analysis tools, although this provides less information than exploiting the potential of innovative multivariate analysis-based software tools after staining for the whole panel. For example, the maturation-associated subclassification of BCP-ALL according to conventional criteria can efficiently be achieved. However, given the subtle variations in the expression levels of a wide range of antigens, we anticipate that multivariate analysis of all markers in the panel will contribute to an improved subclassification of BCP-ALL (Figure 16). Nevertheless, this requires further investigation in larger series of BCP-ALL cases, which is under development by the EuroFlow group.

Antibody panel for T-cell ALL (T-ALL)

We designed an antibody panel for diagnosis and detailed characterization of T-ALL. The application of the T-ALL panel is guided by the results of the ALOT tube. Fusion of the results obtained with the ALOT and the T-ALL panel is based on three common backbone markers. As discussed in the ALOT section, a small number of immature or ETP-ALL and MPAL cases were found to be at the interface between T-ALL and AML with the ALOT tube. These cases should ideally be analyzed with both T-ALL and AML panels, while awaiting the potential development of a specific panel for ambiguous lineage acute leukemias.

The first tube of the T-ALL EuroFlow panel was designed to detect both major and minor T-ALL populations including those that may be missed by microscopic detection, either at diagnosis or during follow-up. As such, this tube can be used for detection of minimal disseminated disease (MDD) at diagnosis in T-LBL.[287,288] However, initial analysis of tumor material with the complete panel is required at diagnosis in order to allow reliable identification of the pathological phenotype(s) and of coexisting intraclonal heterogeneity. In many cases, tube 1 will suffice for MDD detection, essentially when there is co-expression of T-cell-associated (for example, CyCD3 + / − CD5) and immature (NuTdT, CD1a, CD10, $CD99^{hi}$) markers.[287]

The T-ALL panel is flexible, as it allows classification of T-ALL according to the WHO 2008 system or other alternative classifications using both conventional and new multivariate-analysis-based software tools. Usage of the new multivariate analysis tools has the advantages of comparing many phenotypic profiles, which have been acquired in different laboratories in a standardized manner and of integrated analysis of a large number of T-ALLs within the context of prospective clinical trials. In addition to its diagnostic contribution, it can also become a discovery tool for identification of new phenotypic profiles, which might allow improved therapeutic stratification and individual management of T-ALL patients. These profiles should ideally be compared in an *n*-dimensional immunophenotyping model in which neoplastic T-cells are plotted against normal thymic T-cell differentiation for comparison as well as for distinction. With the future help of the EuroFlow T-ALL database, it will be possible to study to what extent T-ALLs differ from single subpopulations of thymocytes, that is, it will become possible to search for the nearest-phenotypic neighbor of T-ALL blasts and to infer the normal T-cell counterpart for each leukemia and more precisely define the LAP. Such new phenotypic classification of T-ALL would potentially be much more accurate than current classifications as it is based on simultaneous analysis of a larger number of parameters versus sequential analysis of multiple individual markers. Further studies are needed to determine the potential correlation of such classification with specific molecular genetic T-ALL subgroups. If this proves to be the case, such information will be available to the clinician within a few hours after cell sample collection.

Antibody panel for acute myeloid leukemia/myelodysplastic syndrome (AML/MDS)

The here-presented AML/MDS antibody panel allows detailed characterization of all myeloid lineages as well as aberrant immunophenotypes of the myeloid cells. For patients with suspicion of AML and/or MDS, this antibody panel should be performed together with the ALOT tube. It was not possible to use the same backbone markers for the AML/MDS antibody panel and the ALOT tube. Consequently, the markers present in the ALOT tube cannot be linked to the markers in the AML/MDS antibody panel. This is not a problem for markers like CD19, CD34, CD45 and CD7, which are also present in the AML/MDS antibody panel, and for CyCD79a, CyCD3 and SmCD3, which are considered not informative for AML/MDS. Merging of the AML/MDS antibody panel with CyMPO would, however, be informative. Although merging based on the common backbone markers CD45 and

CD34 may be possible in some AML patients, this likely will not be appropriate in the vast majority of AML cases.

In contrast to T-ALL and BCP-ALL, patients with AML frequently have a heterogeneous leukemic cell population. As it is not always clear which populations belong to the leukemia, appropriate gating of the leukemic population can be difficult. In our comparison of WHO-defined subgroups, we focused on the immunophenotypic data of the immature cells defined by the CD34, CD117 and HLADR backbone markers. It may, however, well be that inclusion of more mature populations will improve the flow cytometric classification of AML patients. In turn, the utility of other markers associated with immaturity of hematopoietic cells (for example, CD90 and CD133) in AML and MDS remains unclear and should be further evaluated.

To determine which populations are normal and which populations are immunophenotypically abnormal, appropriate analysis of differentiation pathways is crucial. Therefore, the panel was designed in such a way that the multivariate approach, possible by the use of the here-presented 8-color antibody panel in combination with the new Infinicyt software-associated tools, will facilitate this process (Figure 19). Nevertheless, there is still an urgent need for a more objective analysis of such data. In this regard, we are now evaluating a new software module developed for a more objective analysis of maturation pathways. This is based on dissecting the differentiation pathways in multiple segments (for example, stages) in a multidimensional space (for example, based on PCA), generated by the phenotypic parameters analyzed. The patterns observed in healthy individuals subsequently can serve as a reference for the patterns in AML and MDS patients. In such patients, a similar segmentation can be made and for each segment it can be determined with which normal segment it more closely clusters and what markers are aberrant. These software tools are currently being developed and evaluated and will greatly facilitate the analysis of maturation-associated defects, which is crucial for better classification of AML and MDS patients.

Antibody panel for B-cell chronic lymphoproliferative diseases (NK-CLPD) (B-CLPD)

The B-CPLD EuroFlow antibody panel proposed here aims at diagnostic classification of BCLPD into the major WHO subtypes, based on the immunophenotypic profiles of individual malignant B-cells. The panel consists of four tubes, which should be stained sequentially or in parallel to the LST. It is advised that LST and the complete B-CLPD panel are stained in parallel in cases with a high pre-test probability of a mature B-cell malignancy. Cases presenting with suspicion of CLL should be primarily evaluated using the LST and tube 1 from the B-CLPD panel only. The three backbone markers (CD19, CD20, and CD45) common to all four tubes and the LST were designed to allow efficient positive selection of the B-cells in all subtypes of mature/peripheral B-cell malignancies. We demonstrated that this was systematically achieved using the prospective evaluation of several hundreds of cases. The other 24 markers are devoted to the characterization of the identified aberrant clonal B-cell. They included both markers already known to contribute to specific differential diagnoses among distinct WHO B-CLPD entities and novel markers. Inclusion of a relatively high number of markers that are not currently used in many routine diagnostic panels (for example, CD200, CD305(LAIR1), CD31, CD62L) was based on their independent contribution to pairwise differential diagnoses performed through multivariate analyses, particularly between disease entities where previous panels have proven to be of limited utility (for example, atypical CLL). In turn, exclusion of some traditional markers (for example CyBcl2) was due to redundancy with other more informative combinations already included in the panel, as assessed by multivariate analysis.

At a first glance, it might appear that the proposed panel is a rather extensive panel. Nonetheless, it should be noted that it provides additional and more robust information for the diagnosis of specific WHO disease entities compared to the more traditional panels. In fact, the overall panel of backbone and characterization markers proposed here was tested for up to 28 differential diagnosis, by multivariate analysis of individual malignant B-cells from paired WHO diagnostic groups. Overall, the panel provided an efficient differential diagnosis among the major B-CPLD entities in all but four comparisons, which involved MZL versus LPL and DLBCL versus MZL, LPL and FL. Noteworthily, these four differential diagnoses are not only difficult on immunophenotypic grounds but they have also been shown to have a rather limited reproducibility among hematopathology experts when applying the currently used WHO diagnostic criteria (for example, degree of agreement ranging from 56% to 63% for LPL and MZL, respectively).[232]

Comparison to the performance of more traditional 4-color panels used at individual centers which included a slightly lower number of markers, shows that the new markers included in the EuroFlow B-CLPD panel provides more robust diagnosis of the distinct WHO categories of BCLPD, even when similar multivariate analyses are used.[31] In addition, the EuroFlow B-CLPD panel was also designed in a flexible way, so that the relevant immunophenotypic information of the LST is also used for the classification of the distinct B-CLPD entities and subsets of tubes can be applied for a specific set of differential diagnoses. Altogether, this allows comprehensive usage of different combinations of tubes from the panel, for specific differential diagnosis of frequent disease categories such as CLL, limiting reagent and personal costs to a reasonable level.

Antibody panel for T-cell chronic lymphoproliferative diseases (T-CLPD)

In the proposed diagnostic work-up of PTCL, the EuroFlow T-CLPD panel is scheduled to be applied on samples from patients showing aberrant and/or increased numbers of T-cells, as identified by the LST. Overall, this panel aims at confirmation of the presence of aberrant T cells and further classification of PTCL into specific WHO PTCL categories.

Similar to B-CLPD, PTCL represent a rather heterogeneous group composed of multiple disease entities.[3] However, in contrast to mature B-cell neoplasms, T-CLPD derive from multiple different T-cell populations/lineages (for example, TCR$\gamma\delta^+$ versus TCR$\alpha\beta^+$ CD4$^+$ CD8$^-$ and TCR$\alpha\beta^+$ CD4$^-$ CD8^{hi} T lymphocytes), evaluation of clonal excess requires a much higher number of antibody reagents (for example, 24 TCRVβ family antibodies versus κ and λ), whereas they are much less frequent disorders.[3] Because of this, more T- than B-cell associated markers, are required for a diagnostic screening phase, and for the composition of the set of backbone markers in the EuroFlow T-CLPD panel versus the B-CLPD panel. However, for reasons of costs, most clinical flow cytometry laboratories try to keep the overall number of antibodies against T-cell markers lower, according to the relatively lower frequency of PTCL versus B-CLPD. Based on all the above reasoning, the strategy proposed by the EuroFLow Consortium for immunophenotyping of T-CLPD consists of up to three sequential steps. In the first step, the LST tube should be applied. In case the TCR$\gamma\delta^-$/SmCD3$^+$ T-cells are expanded (absolute or relative expansion), show aberrancies (for example, abnormally low expression of SmCD3, CD4, or CD5) or the CD4/CD8 T-cell distribution is altered,[78,254,289] which are not fully conclusive about the clonal nature of such cells, then the immunophenotypic analysis of the TCR-Vβ repertoire should be performed, for confirmation of T-cell clonality.[290–292] Finally, in the third step the T-CLPD panel should be used, aimed at the classification of T-CLPD into specific WHO disease categories[3] in cases suspected of TCR$\alpha\beta^+$,

TCR$\gamma\delta^+$ and TCR$^-$ T-cell CLPD. In Section 9 of this report we focus on the process and steps involved in the design of the T-CLPD panel aimed at final classification of T-CLPD, while the development of the LST tube is detailed in Section 2 of this report. The approach proposed to assess T-cell clonality by flow cytometry has been previously reported in the literature,[290–294] which shows that in cases in which expanded or aberrant TCR$\gamma\delta^-$/SmCD3$^+$ T-cells are detected with the LST, the predominance of a single TCR-Vβ family within a T-cell population (TCR-Vβ restriction) is highly suggestive of a clonal T-cell disorder.[292–294] In cases where phenotypic abnormalities involve TCR$\gamma\delta^+$ T-cells, the TCR-Vγ and TCR-Vδ repertoire may also be investigated, but definitive demonstration of the clonal nature of the infiltrating cell population may not be achieved, due to the more restricted repertoire of normal TCR$\gamma\delta^+$ T-cells, their imbalanced distribution in normal subjects[295] and the relatively limited availability of TCR-Vγ and TCR-Vδ family-specific reagents for flow cytometry.

Regarding the overall performance of the EuroFlow T-CLPD panel, our results clearly show further contribution of the marker combinations used to distinguish between normal/reactive and aberrant/malignant T-cells, except for a few CD8$^+$ T-LGL cases. At the same time it allowed unequivocal classification of T-CLPD into most WHO entities, including SS, T-PLL, ATLL, T-LGL, and AITL. Other rare entities that were not specifically evaluated, like CD30$^+$ T-CLPD, require further prospective studies, although they will most likely be identified with the panel, based on the immunophenotypic features described in the literature for such disease entities and the experience accumulated at several EuroFlow sites.

Antibody panel for NK-cell chronic lymphoproliferative diseases (NK-CLPD)

One of the major challenges in establishing the diagnosis of NK-cell neoplasms is to distinguish between normal, reactive and (mono)clonal proliferations of NK cells, because of the lack of clonal markers in the expanded NK cells. Many studies have clearly shown that clonal pathological cells from most hematological malignancies display aberrant phenotypes that allow their distinction from their normal counterparts.[9,10,14,47,77,237,296,297] Based on this background, the EuroFlow NK-CLPD panel aimed at further characterization of the expanded NK-cells, to discriminate between aberrant and normal/reactive NK-cells, and subsequently define a more precise diagnosis of clonal NK-CLPD. As a result, we propose a panel of three 8-color tubes containing 16 different markers, four of which are backbone markers (SmCD3, CD19, CD45 and CD56), to identify NK cells, and the remaining 12 markers are devoted to distinguish aberrant from normal/reactive/polyclonal NK cells. Upon applying multivariate analysis tools provided with the Infinicyt software, we show that this panel facilitates the distinction between aberrant and normal/reactive NK cells in most cases presenting with NK-cell-associated lymphocytosis.

Consequently, the EuroFlow NK-CLPD panel will be of great help in routine diagnostic settings in this regard. However, partial overlap was observed for some cases, which points out the need for further efforts to improve such discrimination. On the other hand, it has to be taken into account that the rarity of this group of diseases may be an obstacle for some laboratories to perform a relatively large panel of markers to diagnose NK-CLPD. In such cases, a potentially efficient alternative to the implementation of the EuroFlow NK-CLPD panel in all clinical flow cytometry laboratories is centralization in reference centers of this part of the diagnostic work-up of NK-CLPD.

Therefore, in the absence of universal markers of NK-cell clonality, the precise definition of the immunophenotypic profiles associated with clonal versus reactive cells becomes crucial in the diagnosis of NK-CLPD (Figure 29).

The EuroFlow reference database as a tool for immunophenotypic classification of hematological malignancies

Finally, an additional relevant contribution of the work done is the generation of a reference database containing information about large numbers of cases within each WHO disease category. Such database is currently being prospectively expanded by including even more typical and atypical new, fully characterized cases. In the near future, when such database becomes available, it can be used to prospectively classify new individual cases at any clinical flow cytometry laboratory around the world, whenever the EuroFlow SOP for instrument settings and sample preparation are used in combination with the EuroFlow panels and the new multivariate data analysis software tools here described.

CONCLUSION

After 5 years of extensive testing, we present a complete set of antibody panels for standardized *n*-dimensional flow cytometric immunophenotyping of hematological malignancies. The stepwise application of the single-tube screening panels and multitube classification panels fits into the EuroFlow diagnostic algorithm with entries defined by clinical and laboratory parameters. The proposed antibody panels are designed to diagnose and classify the various types of leukemias and lymphomas according to the WHO-defined disease categories. Multiple successive rounds of design–evaluation–redesign were needed to reach the final versions of the antibody panels. The initially designed consensus panels were first optimized by inclusion of additional informative markers and exclusion of redundant markers as identified by novel software tools for multivariate data analysis. In parallel, the composition of the antibody panels was improved by testing the various combinations of antibody clones and fluorochrome conjugates. The final version of the proposed panels was extensively validated on 2031 informative samples, using the novel Infinicyt software tools and the EuroFlow SOP for sample preparation, data acquisition and data analysis, as described by Kalina *et al.*[16] This validation showed that the EuroFlow antibody panels are highly efficient and at the same time they are flexible and can be adapted to fit the different needs of the different laboratories. Altogether, these results indicate that combined usage of the EuroFlow antibody panels and EuroFlow SOP can be considered as the most extensive and validated approach for standardized multidimensional flow cytometric immunophenotyping for diagnostic screening and classification of hematological malignancies. The presented EuroFlow tools may also serve as the basis for future improvements in the field, particularly when the EuroFlow database becomes available for comparing newly diagnosed cases with the many cases in the database.

CONFLICT OF INTEREST

The EuroFlow Consortium is an independent scientific consortium that aims at innovation and standardization of diagnostic flow cytometry. All acquired knowledge and experience will be shared with the scientific and diagnostic community after protection of the relevant Intellectual Property, for example by filing of patents. The involved patents are collectively owned by the EuroFlow Consortium and licensed to companies. The results presented in this manuscript are included in the patent application, entitled 'Methods, reagents n kits for flow cytometric immunophenotyping' (PCT/NL2010050332). The revenues of the patents are exclusively used for EuroFlow Consortium activities, such as for covering (in part) the costs of the Consortium meetings, the EuroFlow Educational Workshops and the purchase of custom-made reagents for collective experiments. M Martin-Ayuso, J Hernández, M Muñoz, are employees of Cytognos SL, which is a pro-profit company.

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APPENDIX

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Table A1. Composition of ALOT and technical information on reagents.

	PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
	CyCD3	CD45	CyMPO	CyCD79a	CD34	CD19	CD7	SmCD3

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
CyCD3	PacB	UCHT1	BD Biosciences	558117	7
SmCD3	APCH7	SK7	BD Biosciences	641397	3
CD7	APC	124-1D1	eBioscience	17-0079-42	2
CD19	PECy7	J3-119	Beckman Coulter	IM3628	5
CD34	PerCPCy5.5	8G12	BD Biosciences	347222	7
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CyCD79a	PE	HM57	Dako	R7159	5
CyMPO	FITC	MPO-7	Dako	F0714	3

Table A2. Composition of LST and technical information on reagents

	PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
	CD20 and CD4	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD5	CD19 and TCRγδ	SmCD3	CD38

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
SmCD3	APC	SK7	BD Biosciences	345767	2.5
CD4	PacB	RPA-T4	BioLegend	300521	0.5
CD5	PerCPCy5.5	L17F12	BD Biosciences	341109	15
CD8	FITC	UCH-T4	Cytognos	CYT-SLPC-50	Part of LST mixture (20)
CD19	PECy7	J3-119	Beckman Coulter	IM3628	5
CD20	PacB	2H7	BioLegend	302320	1
CD38	APCH7	HB7	BD Biosciences	646786	3
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD56	PE	C5.9	Cytognos	CYT-SLPC-50	Part of LST mixture (20)
SmIgκ	PE	polyclonal	Cytognos	CYT-SLPC-50	Part of LST mixture (20)
SmIgλ	FITC	polyclonal	Cytognos	CYT-SLPC-50	Part of LST mixture (20)
TCRγδ	PECy7	11F2	BD Biosciences	649806	1

Table A3. Composition of SST and technical information on reagents

	PacB	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
	CD20	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD4	CD19	SmCD3 and CD14	CD38

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
SmCD3	APC	SK7	BD Biosciences	345767	2.5
CD4	PerCPCy5.5	SK3	Cytognos	CYT-SLPC4-50	Part of SST mixture (20)
CD8	FITC	UCH-T4	Cytognos	CYT-SLPC4-50	Part of SST mixture (20)
CD14	APC	MφP9	BD Biosciences	345787	5
CD19	PECy7	J3-119	Beckman Coulter	IM3628	5
CD20	PacB	2H7	BioLegend	302320	1
CD38	APCH7	HB7	BD Biosciences	646786	3
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD56	PE	C5.9	Cytognos	CYT-SLPC4-50	Part of SST mixture (20)
SmIgκ	PE	polyclonal	Cytognos	CYT-SLPC4-50	Part of SST mixture (20)
SmIgλ	FITC	polyclonal	Cytognos	CYT-SLPC4-50	Part of SST mixture (20)

Table A4. Composition of PCD and technical information on reagents

Tube	*PacB*	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
1	CD45	CD138	CD38	CD56	β2micro	CD19	Cylgκ	Cylgλ
2	CD45	CD138	CD38	CD28	CD27	CD19	CD117	CD81

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*	
CD19	PECy7	J3-119	Beckman Coulter	IM3628	5	
CD27	PerCPCy5.5	L128	BD Biosciences	649805	10	
CD28	PE	L293	BD Biosciences	348047	20	
CD38	FITC	LD38	Cytognos	CYT-38F	3	5[a]
CD38	Pure	LD38	Cytognos	CYT-38P1	2	
CD45	PacB	T29/33	Dako	PB986	5	
CD56	PE	C5.9	Cytognos	CYT-56PE	5	
CD81	APCH7	JS-81	BD Biosciences	646791	5	
CD117	APC	104D2	BD Biosciences	333233	5	
CD138	PacO	B-A38	Exbio	PO-520	4	
β2micro	PerCPCy5.5	Tü99	BD Biosciences	646781	4.75	5[a]
β2micro	Pure	Tü99	BD Biosciences	555550	0.25	
Cylgκ	APC	Polyclonal rabbit serum	Dako	C0222	2.5	
Cylgλ	APCH7	1-155-2	BD Biosciences	646792	4	

[a]Mixture of fluorochrome-conjugated and -unconjugated antibodies is used to reduce signal intensity, while retaining saturating conditions to avoid unpredictable variation in staining patterns. The 19:1 ratio for the two β2micro antibodies is caused by the five fold higher antibody concentration of the unconjugated antibody.

Table A5. Composition of BCP-ALL panel and technical information on reagents

Tube	*PacB*	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
1	CD20	CD45	CD58	CD66c	CD34	CD19	CD10	CD38
2	SmIgκ	CD45	Cylgμ	CD33	CD34	CD19	SmIgM and CD117	Smlgλ
3	CD9	CD45	NuTdT	CD13	CD34	CD19	CD22	CD24
4	CD21	CD45	CD15 and CD65	NG2	CD34	CD19	CD123	CD81

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
CD9	PacB	MEM-61	Exbio	PB-208-T100	4
CD10	APC	HI10A	BD Biosciences	332777	5
CD13	PE	L138	BD Biosciences	347406	7
CD15	FITC	MMA	BD Biosciences	332778	10
CD19	PECy7	J3-119	Beckman Coulter	IM3628	5
CD20	PacB	2H7	Biolegend	302320	1
CD21	PacB	LT21	Exbio	PB-306-T100	4
CD22	APC	S-HCL-1	BD Biosciences	333145	5
CD24	APCH7	ML5	BD Biosciences	646785	5
CD33	PE	P67.6	BD Biosciences	345799	5
CD34	PerCPCy5.5	8G12	BD Biosciences	347222	7
CD38	APCH7	HB7	BD Biosciences	646786	3
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD58	FITC	1C3	BD Biosciences	555920	7
CD65	FITC	88H7	Beckman Coulter	IM1654U	7
CD66c	PE	KOR-SA3544	Beckman Coulter	IM2357U	10
CD81	APCH7	JS-81	BD Biosciences	646791	5
CD117	APC	104D2	BD Biosciences	333233	5
CD123	APC	AC145	Miltenyi Biotec	130-090-901	7
Cylgμ	FITC	Polyclonal rabbit serum	Dako	F0058	10
NG2	PE	7.1	Beckman Coulter	IM3454U	10
SmIgκ	PacB	A8B5	Exbio	PB-504-T100	4
Smlgλ	APCH7	1-155-2	BD Biosciences	646792	4
SmIgM	APC	G20-127	BD Biosciences	551062	10
NuTdT	FITC	HT-6	Dako	F7139	10

Table A6. Composition of T-ALL panel and technical information on reagents

Tube	*PacB*	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
1	CyCD3	CD45	NuTdT	CD99	CD5	CD10	CD1a	SmCD3
2	CyCD3	CD45	CD2	CD117	CD4	CD8	CD7	SmCD3
3	CyCD3	CD45	TCR$\gamma\delta$	TCR$\alpha\beta$	CD33	CD56	CyTCRβ	SmCD3
4	CyCD3	CD45	CD44	CD13	HLADR	CD45RA	CD123	SmCD3

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
CD1a	APC	HI149	BD Biosciences	559775	5
CD2	FITC	RPA-2.10	BD Biosciences	555326	5
CyCD3	PacB	UCHT1	BD Biosciences	558117	7
SmCD3	APCH7	SK7	BD Biosciences	641397	3
CD4	PerCPCy5.5	SK3	BD Biosciences	332772	7
CD5	PerCPCy5.5	L17F12	BD Biosciences	341109	15
CD7	APC	124-1D1	eBioscience	17-0079-42	2
CD8	PECy7	SFCI21Thy2D3	Beckman Coulter	737661	5
CD10	PECy7	HI10A	BD Biosciences	341112	5
CD13	PE	L138	BD Biosciences	347406	7
CD33	PerCPCy5.5	P67.6	BD Biosciences	333146	10
CD44	FITC	L178	BD Biosciences	347943	7
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD45RA	PECy7	L48	BD Biosciences	337186	5
CD56	PECy7	N901	Beckman Coulter	A21692	5
CD99	PE	Tü12	BD Biosciences	555689	5
CD117	PE	104D2	BD Biosciences	332785	5
CD123	APC	AC145	Miltenyi Biotec	130-090-901	7
HLADR	PerCPCy5.5	L243	BD Biosciences	552764	10
TCR$\alpha\beta$	PE	IP26A	Beckman Coulter	A39499	7
CyTCRβ	APC	8A3 (βF1)	Cytognos	CYT-BF1AP	3
TCR$\gamma\delta$	FITC	IMMU510	Beckman Coulter	IM1571U	10
NuTdT	FITC	HT-6	Dako	F7139	10

Table A7. Composition of AML/MDS panel and technical information on reagents

Tube	*PacB*	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
1	HLADR	CD45	CD16	CD13	CD34	CD117	CD11b	CD10
2	HLADR	CD45	CD35	CD64	CD34	CD117	CD300e	CD14
3	HLADR	CD45	CD36	CD105	CD34	CD117	CD33	CD71
4	HLADR	CD45	NuTdT	CD56	CD34	CD117	CD7	CD19
5	HLADR	CD45	CD15	NG2	CD34	CD117	CD22	CD38
6	HLADR	CD45	CD42a and CD61	CD203c	CD34	CD117	CD123	CD4
7	HLADR	CD45	CD41	CD25	CD34	CD117	CD42b	CD9

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
CD4	APCH7	SK3	BD Biosciences	641398	5
CD7	APC	124-1D1	eBioscience	17-0079-42	2
CD9	APCH7	M-L13	BD Biosciences	646782	5
CD10	APCH7	HI10A	BD Biosciences	646783	5
CD11b	APC	D12	BD Biosciences	333143	5
CD13	PE	L138	BD Biosciences	347406	7
CD14	APCH7	MφP9	BD Biosciences	641394	5
CD15	FITC	MMA	BD Biosciences	332778	10
CD16	FITC	CLB FcR gran/1, 5D2	Sanquin	M1604	20
CD19	APCH7	SJ25C1	BD Biosciences	641395	5
CD22	APC	S-HCL-1	BD Biosciences	333145	5
CD25	PE	2A3	BD Biosciences	341011	10
CD33	APC	P67.6	BD Biosciences	345800	10
CD34	PerCPCy5.5	8G12	BD Biosciences	347222	5
CD35	FITC	E11	BD Biosciences	555452	5
CD36	FITC	CLB-IVC7	Sanquin	M1613	5
CD38	APCH7	HB7	BD Biosciences	646786	3
CD41	FITC	CLB-tromb/7, 6C9	Sanquin	M1674	1
CD42a	FITC	GRP-P	Serotec	MCA1227F	1
CD42b	APC	HIP1	BD Biosciences	551061	1
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD56	PE	C5.9	Cytognos	CYT-56PE	5
CD61	FITC	RUU-PL7F12	BD Biosciences	347407	4
CD64	PE	10.1	Serotec	MCA756PE	10
CD71	APCH7	M-A712	BD Biosciences	646789	5
CD105	PE	1G2	Beckman Coulter	A07414	10
CD117	PECy7	104D2D1	Beckman Coulter	IM3698	5
CD123	APC	AC145	Miltenyi Biotec	130-090-901	10
CD203c	PE	97A6	Beckman Coulter	IM3575	10
CD300e	APC	UP-H2	Immunostep	IREM2A-T100	5
HLADR	PacB	L243	Biolegend	307624	1 (1:5 dilution)
NG2	PE	7.1	Beckman Coulter	IM3454U	10
NuTdT	FITC	HT-6	Dako	F7139	10

Table A8. Composition of B-CLPD panel and technical information on reagents

Tube	*PacB*	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
1	CD20 and CD4	CD45	CD8 and Igλ	CD56 and Igκ	CD5	CD19 and TCRγδ	SmCD3	CD38
2	CD20	CD45	CD23	CD10	CD79b	CD19	CD200	CD43
3	CD20	CD45	CD31	CD305	CD11c	CD19	SmIgM	CD81
4	CD20	CD45	CD103	CD95	CD22	CD19	CD185	CD49d
5	CD20	CD45	CD62L	CD39	HLADR	CD19	CD27	

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
SmCD3	APC	SK7	BD Biosciences	345767	2.5
CD4	PacB	RPA-T4	BioLegend	300521	0.5
CD5	PerCPCy5.5	L17F12	BD Biosciences	341109	15
CD8	FITC	UCH-T4	Cytognos	CYT-SLPC-50	Part of LST mixture (20)
CD10	PE	ALB1	Beckman Coulter	A07760	20
CD11c	PerCPCy5.5	B-Ly6	BD Biosciences	646784	5
CD19	PECy7	J3-119	Beckman Coulter	IM3628	5
CD20	PacB	2H7	BioLegend	302320	1
CD22	PerCPCy5.5	S-HCL-1	BD Biosciences	649804	2
CD23	FITC	MHM6	Dako	F7062	2.5
CD27	APC	L128	BD Biosciences	337169	2.5
CD31	FITC	WM59	BD Biosciences	555445	10
CD38	APCH7	HB7	BD Biosciences	646786	3
CD39	PE	TÜ66	BD Biosciences	555464	10
CD43	APCH7	IG10	BD Biosciences	646787	5
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD49d	APCH7	9F10	BD Biosciences	646788	2
CD56	PE	C5.9	Cytognos	CYT-SLPC-50	Part of LST mixture (20)
CD62L	FITC	SK11	BD Biosciences	347443	2.5
CD79b	PerCPCy5.5	SN8	BD Biosciences	646790	5
CD81	APCH7	JS-81	BD Biosciences	646791	5
CD95	PE	DX2	BD Biosciences	555674	20
CD103	FITC	Ber-ACT8	BD Biosciences	333155	2
CD185	APC	51505	R&D Systems	FAB190A	10
CD200	APC	OX104	eBioscience	17-9200	1.25
CD305	PE	DX26	BD Biosciences	550811	10
HLADR	PerCPCy5.5	L243	BD Biosciences	552764	10
SmIgκ	PE	Polyclonal	Cytognos	CYT-SLPC-50	Part of LST mixture (20)
SmIgλ	FITC	Polyclonal	Cytognos	CYT-SLPC-50	Part of LST mixture (20)
SmIgM	APC	G20-127	BD Biosciences	551062	10
TCRγδ	PECy7	11F2	BD Biosciences	649806	1

Table A9. Composition of T-CLPD panel and technical information on reagents

Tube	*PacB*	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
1	CD4	CD45	CD7	CD26	SmCD3	CD2	CD28	CD8
2	CD4	CD45	CD27	CD197	SmCD3	CD45RO	CD45RA	CD8
3	CD4	CD45	CD5	CD25	SmCD3	HLADR	CyTcl1	CD8
4	CD4	CD45	CD57	CD30	SmCD3		CD11c	CD8
5	CD4	CD45	CyPerforin	CyGranzyme B	SmCD3	CD16	CD94	CD8
6	CD4	CD45		CD279	SmCD3			CD8

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
CD2	PECy7	S5.2	BD Biosciences	335821	2
SmCD3	PerCPCy5.5	SK7	BD Biosciences	332771	10
CD4	PacB	RPA-T4	BioLegend	300521	0.5
CD5	FITC	L17F12	BD Biosciences	345781	10
CD7	FITC	4H9	BD Biosciences	347483	10
CD8	APCH7	SK1	BD Biosciences	641400	5
CD11c	APC	S-HCL-3	BD Biosciences	333144	2
CD16	PECy7	3G8	BD Biosciences	557744	2
CD25	PE	2A3	BD Biosciences	341011	10
CD26	PE	L272	BD Biosciences	340423	10
CD27	FITC	L128	BD Biosciences	340424	10
CD28	APC	CD28.2	BD Biosciences	559770	10
CD30	PE	BerH8	BD Biosciences	550041	10
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD45RA	APC	HI100	BD Biosciences	550855	10
CD45RO	PECy7	UCHL1	BD Biosciences	337168	2
CD57	FITC	HNK-1	BD Biosciences	333169	10
CD94	APC	HP-3D9	BD Biosciences	559876	5
CD197	PE	3D12	eBioscience	12-1979	10
CD279	PE	MIH4	BD Biosciences	557946	20
CyGranzyme B	PE	CLB-GB11	Sanquin	M2289	15
CyPerforin	FITC	δG9	BD Biosciences	556577	10
CyTCL1	APC	eBio1-21	eBioscience	17-6699	2
HLADR	PECy7	L243	BD Biosciences	335830	2.5

Table A10. Composition of NK-CLPD panel and technical information on reagents

Tube	*PacB*	*PacO*	*FITC*	*PE*	*PerCPCy5.5*	*PECy7*	*APC*	*APCH7*
1	CD2	CD45	CD7	CD26	SmCD3	CD56	CD5	CD19
2	CD16	CD45	CD57	CD25	SmCD3	CD56	CD11c	CD19
3	HLADR	CD45	CyPerforin	CyGranzyme B	SmCD3	CD56	CD94	CD19

Marker	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*(μl/test)*
CD2	PacB	TS1/8	Biolegend	309216	1
SmCD3	PerCPCy5.5	SK7	BD Biosciences	332771	10
CD5	APC	L17F12	BD Biosciences	345783	2.5
CD7	FITC	4H9	BD Biosciences	347483	10
CD11c	APC	S-HCL-3	BD Biosciences	333144	2
CD16	PacB	3G8	Biolegend	302032	5
CD19	APCH7	SJ25C1	BD Biosciences	641395	5
CD25	PE	2A3	BD Biosciences	341011	10
CD26	PE	L272	BD Biosciences	340423	10
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD56	PECy7	N901	Beckman Coulter	A21692	5
CD57	FITC	HNK-1	BD Biosciences	333169	10
CD94	APC	HP-3D9	BD Biosciences	559876	5
CyGranzyme B	PE	CLB-GB11	Sanquin	M2289	15
CyPerforin	FITC	δG9	BD Biosciences	556577	10
HLADR	PacB	L243	Biolegend	307624	1 (1:5 dilution)

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)