Primary diffuse large B-cell lymphoma associated with clonally-related monoclonal B lymphocytosis indicates a common precursor cell

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SUPPLEMENTAL DATA

Supplemental Methods

1. Histology

Hematoxylin and eosin-stained sections of formalin-fixed lymphoma tissue and of zinc-formalin-fixed and formic acid-decalcified bone marrow trephine biopsies were reviewed. Paraffin blocks were cut at 4–6 μm, dried overnight at 60°C and dewaxed in xylene prior to immunohistochemical staining. The following antibodies were used: antibodies against CD20, MUM1, BCL6, Ki67 (all from Dako Cytomation, Glostrup, Denmark), CD5, CD21, CD23, CD10, BCL2 (all from Novocastra, Newcastle, U.K.), CD3, cyclin D1 (Lab Vision/NeoMarkers, Fremont, CA), CD138 (Serotec, Kidlington, U.K.) and PAX-5 (Becton Dickinson, Franklin Lakes, NJ). Visualization was performed using the EnVision® detection system (Dako Cytomation) according to the manufacturer's instructions. Appropriate positive and negative controls were used.

2. Flow cytometry of blood and bone marrow samples

Anti-CD56, anti-CD5, anti-CD3 and anti-CD79b were purchased from Becton-Dickinson (San José, CA, USA); anti-CD23 from Dako; anti-CD200 from eBioscience (San Diego, CA); anti-CD8, anti-Igκ and anti-Igλ from Cytognos (Salamanca, Spain) and the remaining of the antibodies (anti-CD4, anti-CD19, anti-CD20, anti-CD38, anti-CD43, anti-CD45 and anti-TCRγ from Beckman Coulter (Brea, CA).

3. Fluorescent activated cell sorting (FACS) of MBL/MSBC from blood or bone marrow

Mononuclear cell suspensions were made of all bone marrow and blood samples using Leucosep® tubes (Greiner Bio-One North America, Inc.) according to manufacturer's recommendations. Cells were resuspended in PBS supplemented with 1% FCS and 10% DMSO and were subsequently frozen using an isopropanol chamber and stored in liquid nitrogen until FACS analysis. Although all blood and bone marrow samples contained monoclonal B-cells as determined by prior flow cytometry analysis, cells were FACS-sorted

from either bone marrow (patients 1, 2, 4 and 6) or from blood (patients 3 and 5) depending on whichever sample volume was largest to be able to sort as many cells as possible. For FACS analysis, the mononuclear cell suspensions were thawed and divided in aliquots of 0,5-1,0 x 10^6 cells/tube. The cells were washed with 2000 µl PBS with 0,5 % BSA (PAA laboratories GmbH, Austria) and stained for surface antigens with the following antibodies: anti-CD45 (clone J.33, Beckman Coulter), anti -CD20 (clone B9E9(HRC20), Beckman Coulter), anti-CD19 (clone J3-119, Beckman Coulter), anti-CD5 (clone L17F12, Becton-Dickinson (San Jose, CA)) and anti-CD10 (clone HI10a, Becton Dickinson) anti-λ and anti-κ (polyclonal antibodies, Cytognos (Salamanca, Spain)). Antibodies were conjugated to either fluoresceine thyocyanate (FITC), phycoerythrine (Pe), peridinin chlorophyll protein-cy5.5 (PerCP-Cy5.5), phycoerythrine cyanine 7 (PeCy7), allophycocyanin (APC), Pacific Blue or Krome Orange. After staining, the cell suspensions were incubated for 15 minutes in the dark at room temperature and washed with 2000 µl PBS supplemented with 0,5 % BSA. Aliquots with stained cell suspensions from each patient, respectively, were pooled and filtered through a 70 µm filter to remove cell clumps.

Stained samples were sorted with high-pressure settings using a FACS Aria Ilu High speed sorter (Becton Dickinson) equipped with 408nm, 488nm and 633nm lasers. Selection of MBL/MSBC for sorting was performed using Becton Dickinson FACSDiva software, starting with the gating of viable cells using the forward scatter versus side scatter dot plot. Subsequently, CD45 bright, low side scatter events (i.e. lymphocytes) were selected. Then, CD5 positive and CD19 negative events (i.e. T cells) were gated out using a CD5 versus CD19 dot plot leaving only B cells. Finally, MBL/MSBC were separated from the polyclonal B cells taking advantage of the aberrant B-cell immunophenotype identified by prior flow cytometry analysis. The marker combination used for the latter is indicated for each patient in Table 1 of the main manuscript.

4. DNA extraction and whole genome amplification

DNA from sorted MBL/MSBC was extracted using Qiagen AllPrep DNA/RNA Micro kit (Qiagen, Hilden, Germany) according the instructions of the manufacturer. Genomic DNA

was subsequently amplified using Illustra Ready-To-Go GenomiPhi V3 DNA Amplification Kit (GE Healthcare Life Sciences, U.K.).

DNA from either formalin-fixed paraffin-embedded tissue or fresh frozen tissue of DLBCL samples, the latter only available for one case, was extracted using appropriate kits from Qiagen according to manufacturer's recommendations. The concentration of all extracted nucleic acid was measured using a NanoDrop 2000 spectrometer (Thermo Scientific, Waltham, MA).

Legend to the supplemental figures

Supplemental figure 1

Representative H&E-stained sections of the diffuse large B cell lymphoma biopsies of patients 3, 4 and 5 (panels A,B and C, respectively; 400x). Large atypical lymphoid cells are seen in all panels, with areas of necrosis in panels A and B. The scale bar indicates 50 micrometer.

Supplemental figure 2

Representative H&E-stained and anti-CD20 stained sections of bone marrow trephine biopsies of patient 4 (panels A,B), patient 5 (panels A,B) and patient 6 (panels A,B), respectively (400x). The sections illustrate infiltrates of small B lymphocytes. The infiltrates are in the intertrabecular parenchyma in patients 4 and 5. By contrast, a paratrabecular infiltrate is seen in patient 6. Histiocytes can be seen in the stroma of the lymphoid infiltrates in patients 4 and 5 (panels A,C). Since immunohistochemical analysis involves deeper sectioning and since the lymphoid infiltrates are small, the size and content of histiocytes differs between the H&E sections and the immunohistochemically-stained sections. The scale bar indicates 50 micrometer.

Supplemental figure 3

Analysis of small monoclonal B cells/monoclonal B cell lymphocytosis by flow cytometry for patients 1 to 6. The cells with an abnormal immunophenotype are highlighted in blue, polytypic B cells are highlighted in red. Panels A show the forward and side scatter of the cells, panels B show the staining with CD45. Forward and side scatter illustrate that the small monoclonal B cells/monoclonal B cell lymphocytosis share the same scatter characteristics of polytypic B lymphocytes present in the samples, consistent with the small

size of the monoclonal B cells. The gating strategy for isolation of MBL/MSBC by FACS is given at the bottom of the figure. Note that the yield in percentage of MBL/MSBC from sorted samples derived from mononuclear cell suspensions is different, usually higher, than the percentage of the respective cells detected by flow cytometry on lysed samples, still containing granulocytes, given in Table 1.

Supplemental Tables

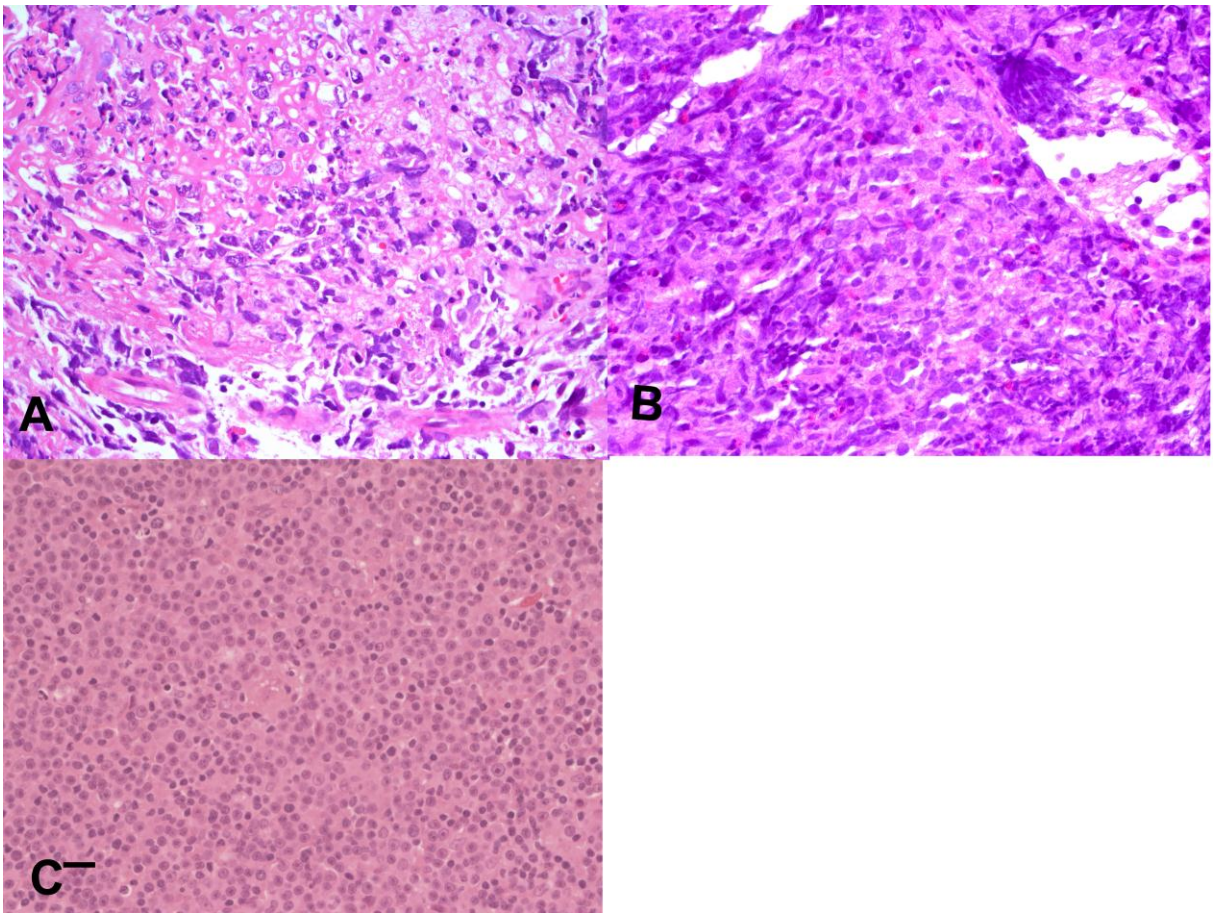
Supplemental Table 1. Patient characteristics

Patient	Age	LDH[1]	Number of extranodal sites	Stage	WHO performance status	IPI[2]	Biopsy site	Blood lymphocyte count (x 10^9/L)	MBL[3] (% of all CD45+ cells)	MSBC[4] (% of all CD45+ cells)
1	84	0.69	1	IEA	1	1	Rectal mucosa	0.9	0.3%	0.9%
2	60	0.89	1	IEA	1	0	Gastric mucosa	1.0	1%	0.4%
3	82	0.91	1	IIEA	1	1	Gastric mucosa	2.6	0.4%	1%
4	68	0.96	1	IVA	1	2	Gastric mucosa	2.7	1%	1.2%
5	80	1.2	1	IVA	1	3	Lymph node	0.7	0.12%	<1%
6	75	1.18	2	IVA	0	4	Gingiva	1.7	1%	0.9%

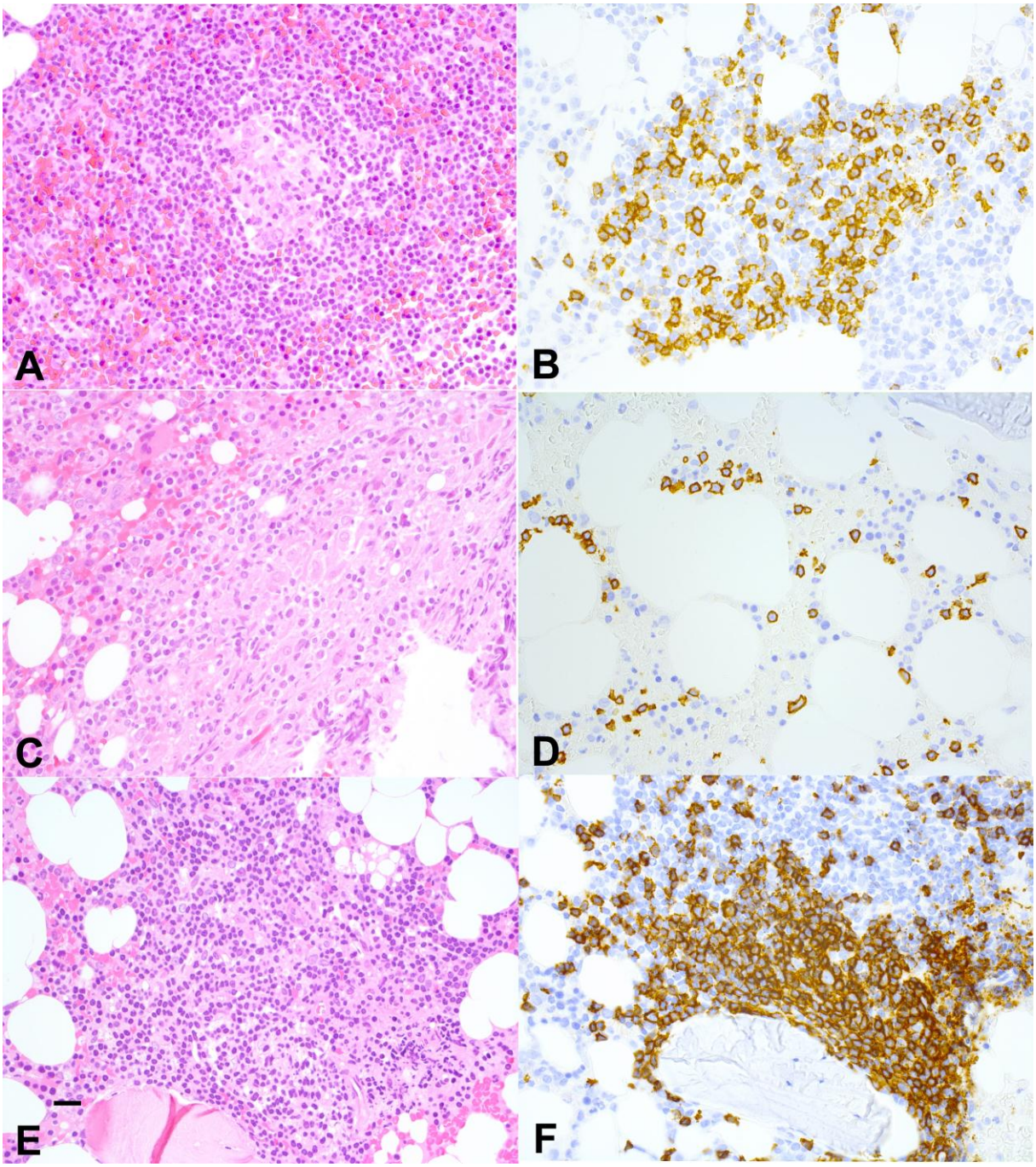
[1]fraction of upper normal limit; [2]IPI: International Prognostic Index; [3]MBL: monoclonal B lymphocytosis (blood); [4]MSBC: monoclonal small B cells (bone marrow)

Supplemental Figures

Supplemental figure 1

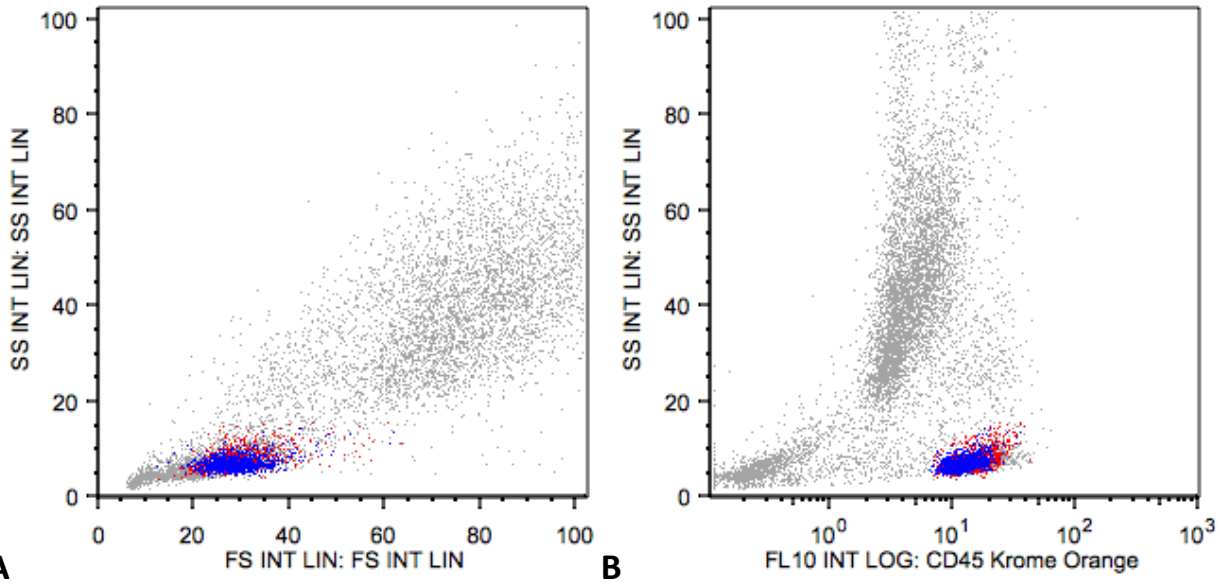


Supplemental figure 2



Supplemental figure 3

PATIENT 1



A **B**

Gating strategy for FACS of MBL/MSBC and cell yields

Mononuclear cell suspension of bone marrow (100%)

Step 1: Lymphocytes (72,8%) – based on the forward scatter versus side scatter plot

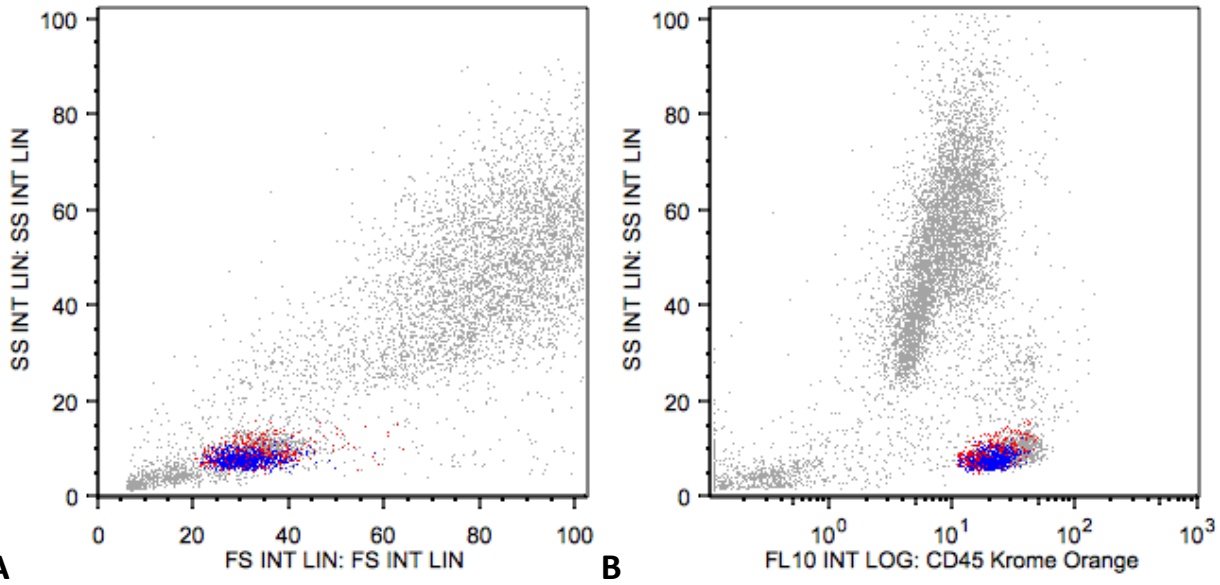
Step 2: CD19+ B cells (6,8%) – based on the CD19 versus CD5 plot

Step 3: CD20+ B cells (6,1%) – based on the CD19 versus CD20 plot

Step 4: CD5+ B cells (2,3%) – based on the CD5 versus CD20 plot

Step 5: Igλ+ B cells (2,1%) – based on the Igκ versus Igλ plot

PATIENT 2



A **B**

Gating strategy for FACS of MBL/MSBC and cell yields

Mononuclear cell suspension of bone marrow (100%)

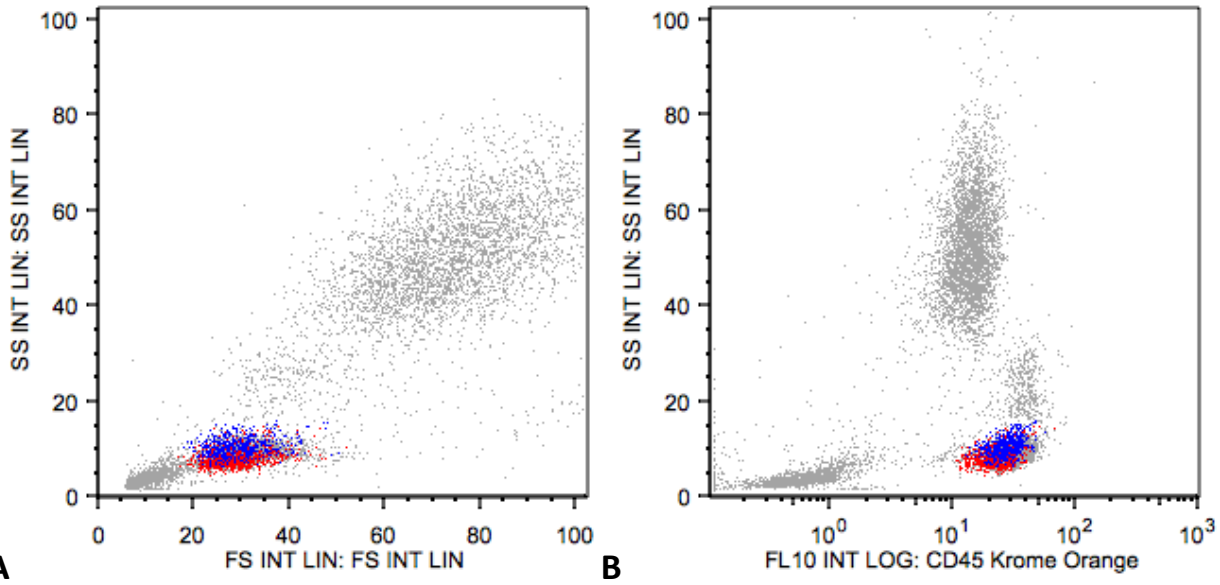
Step 1: Lymphocytes (82,3%) – based on the forward scatter versus side scatter plot

Step 2: CD19+ B cells (4,7%) – based on the CD19 versus CD5 plot

Step 3: CD20+ B cells (3%) – based on the CD19 versus CD20 plot

Step 4: Igκ+ B cells (1,7%) – based on the Igκ versus Igλ plot

PATIENT 3



A **B**

Gating strategy for FACS of MBL/MSBC and cell yields

Mononuclear cell suspension of blood (100%)

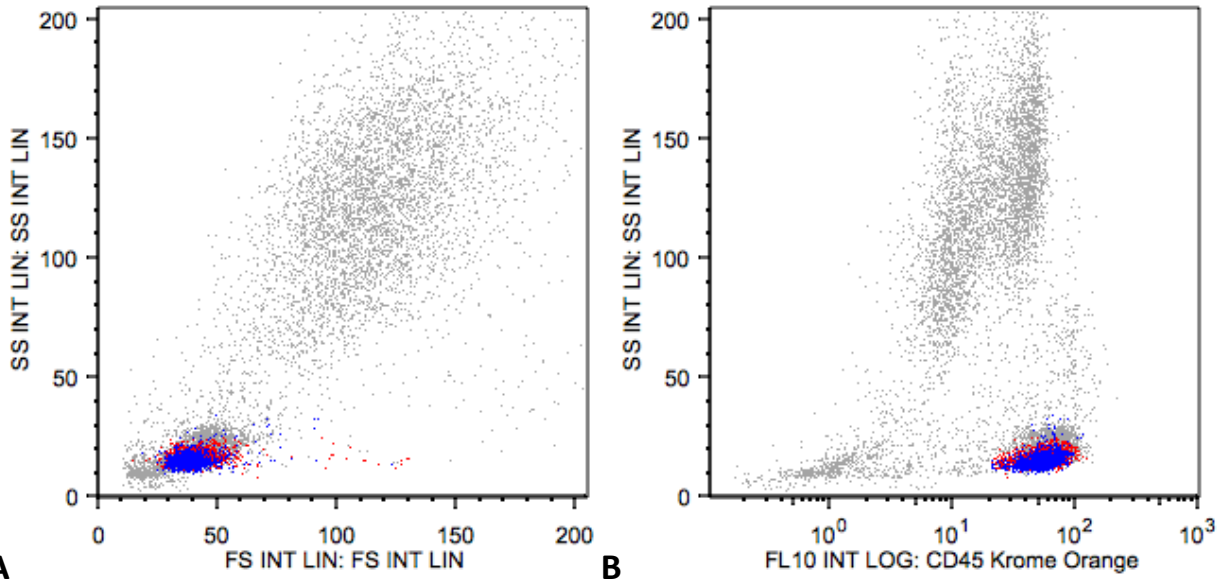
Step 1: Lymphocytes (72,3%) – based on the forward scatter versus side scatter plot

Step 2: CD19+ B cells (4,5%) – based on the CD19 versus CD5 plot

Step 3: CD20+ B cells (0,9%) – based on the CD19 versus CD20 plot

Step 4: Igλ+ B cells (0,4%) – based on the Igκ versus Igλ plot

PATIENT 4



A **B**

Gating strategy for FACS of MBL/MSBC and cell yields

Mononuclear cell suspension of bone marrow (100%)

Step 1: Lymphocytes (83,0%) – based on the forward scatter versus side scatter plot

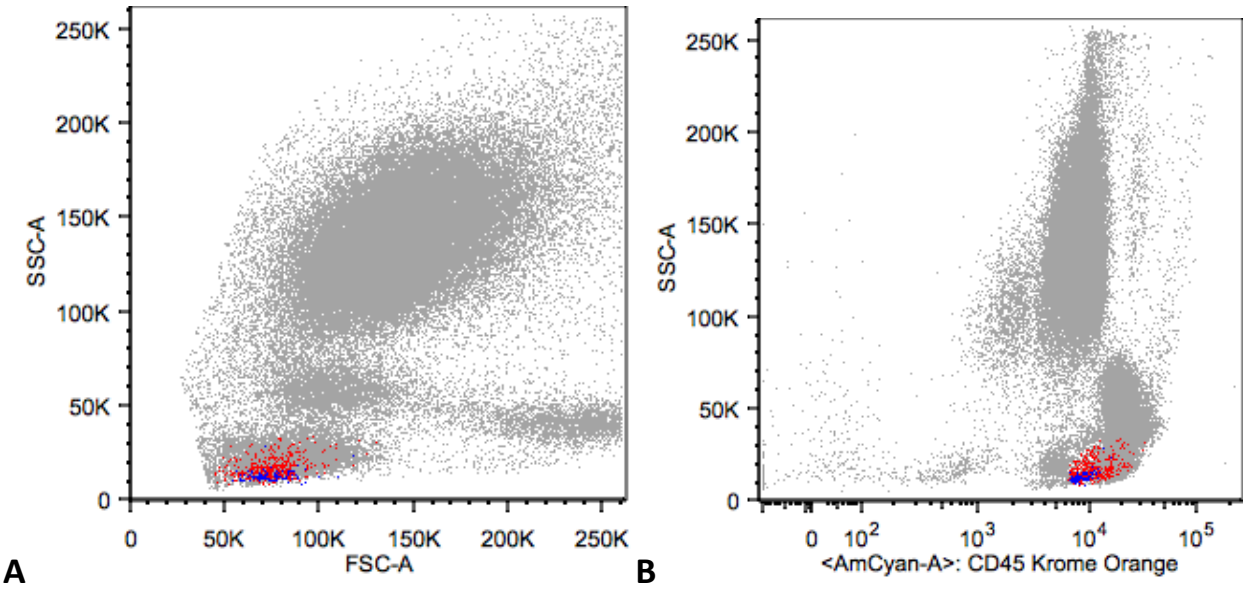
Step 2: CD19+ B cells (8,2%) – based on the CD19 versus CD5 plot

Step 3: CD20+ B cells (7,8%) – based on the CD19 versus CD20 plot

Step 4: CD5+ B cells (2,6%) – based on the CD5 versus CD20 plot

Step 5: Igκ+ B cells (2,6%) – based on the Igκ versus Igλ plot

PATIENT 5



Gating strategy for FACS of MBL/MSBC and cell yields

Mononuclear cell suspension of blood (100%)

Step 1: Lymphocytes (52,4%) – based on the forward scatter versus side scatter plot

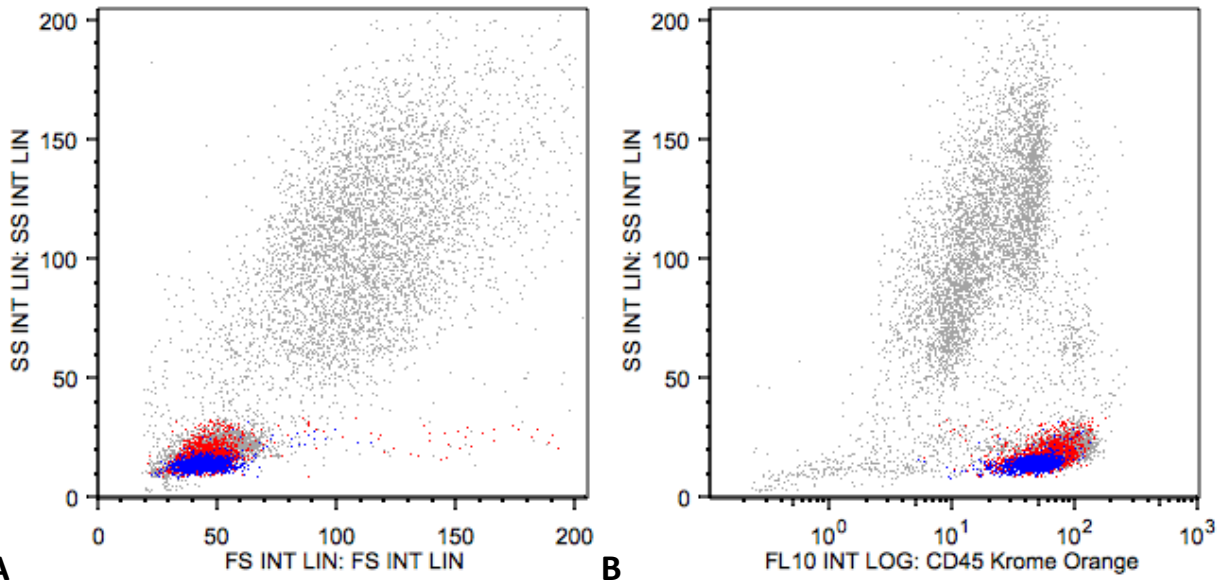
Step 2: CD19+ B cells (3,7%) – based on the CD19 versus CD5 plot

Step 3: CD20+ B cells (3,7%) – based on the CD19 versus CD20 plot

Step 4: CD5+ B cells (0,7%) – based on the CD5 versus CD20 plot

Step 5: Igκ+ B cells (0,7%) – based on the Igκ versus Igλ plot

PATIENT 6



A **B**

Gating strategy for FACS of MBL/MSBC and cell yields

Mononuclear cell suspension of bone marrow (100%)

Step 1: Lymphocytes (84,3%) – based on the forward scatter versus side scatter plot

Step 2: CD19+ B cells (3,5%) – based on the CD19 versus CD5 plot

Step 3: CD20+ B cells (3,2%) – based on the CD19 versus CD20 plot

Step 4: CD10+ B cells (1,3%) – based on the CD10 versus CD20 plot

Step 5: Igκ+ B cells (1,1%) – based on the Igκ versus Igλ plot