ACS Hobart
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Utility of B cell subset analysis by Flow cytometry – Beyond B cell malignancies

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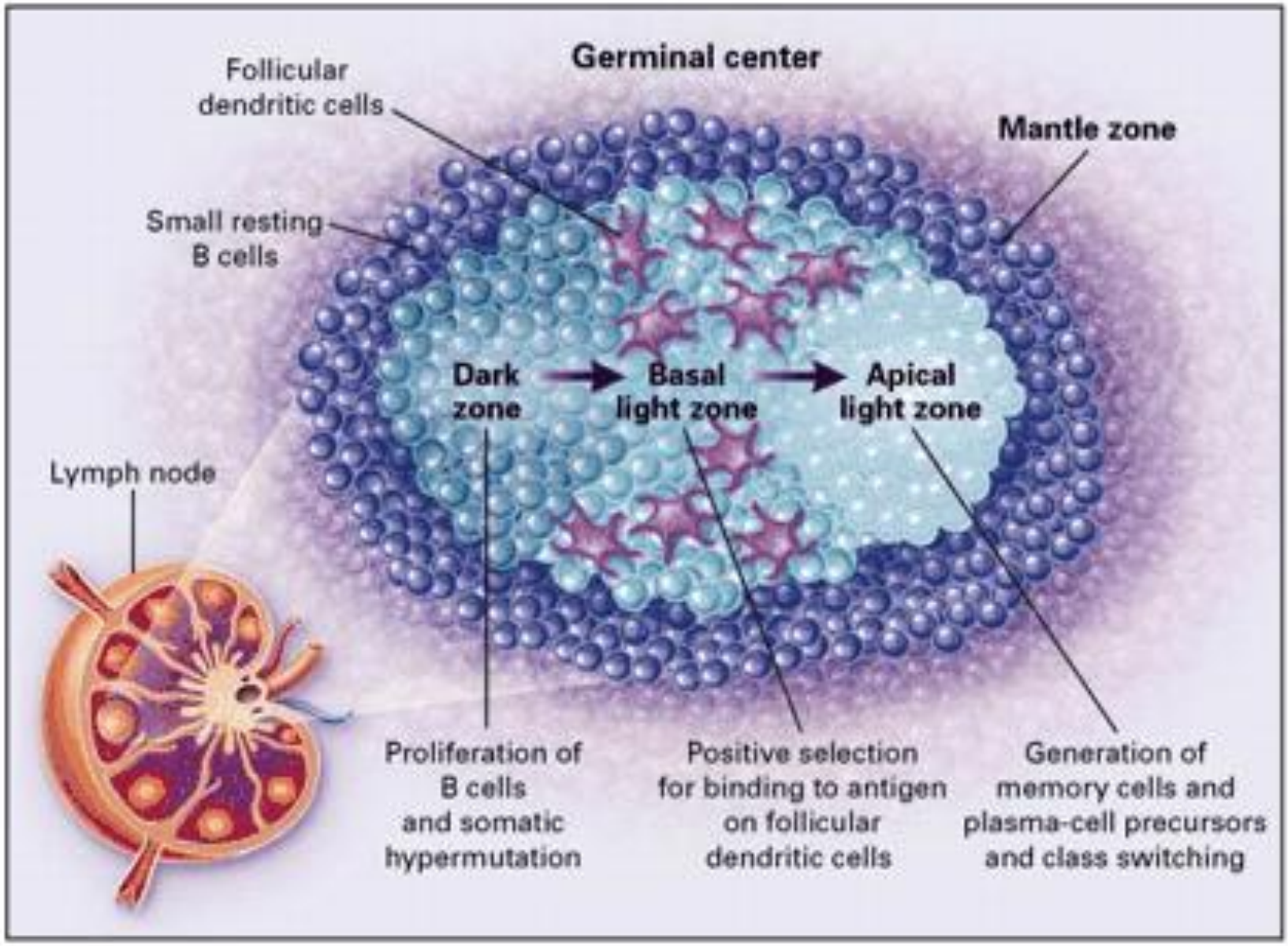
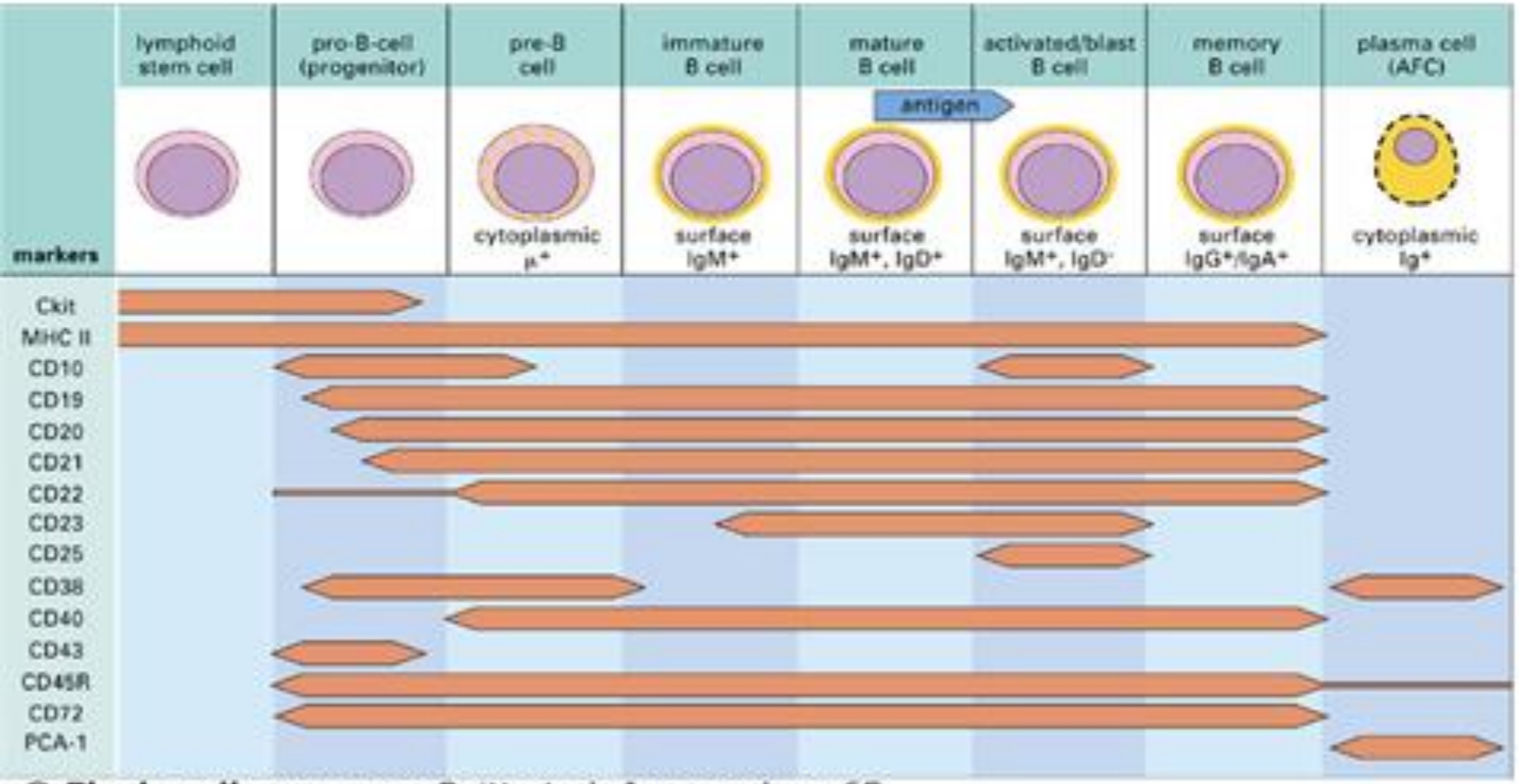
B cell populations

- Bone marrow
  - B cell progenitors- Pre-B, Pro-B
  - Undergo recombination to develop individual Ig
- Pre-GC
  - Transitional B cell
  - Naïve B cell- sIg but no Ag contact
- Post-GC
  - Affinity maturation
  - Class switch


Germinal center
Follicular dendritic cells
Mantle zone
Small resting B cells
Dark zone
Basal light zone
Apical light zone
Lymph node
Proliferation of B cells and somatic hypermutation
Positive selection for binding to antigen on follicular dendritic cells
Generation of memory cells and plasma-cell precursors and class switching

B Cell Differentiation



Identification of B cell subsets on Flow

- Naïve B cell
  - CD19 + CD20 + IgD + CD27- (IgM)
- Transitional B Cell
  - CD24, CD38, IgM or CD10 (T1, T2 and T3 based upon CD21)
- Memory B cells
  - CD19 + CD20 + IgD-CD27+
- Switched Memory
  - CD19 + CD20 + IgD-CD27+
- Antibody Secreting B cells
  - CD19 + CD20-CD38bright
- CD21 Low B cells

Flow cytometry Memory B Gating Strategy – SA Pathology

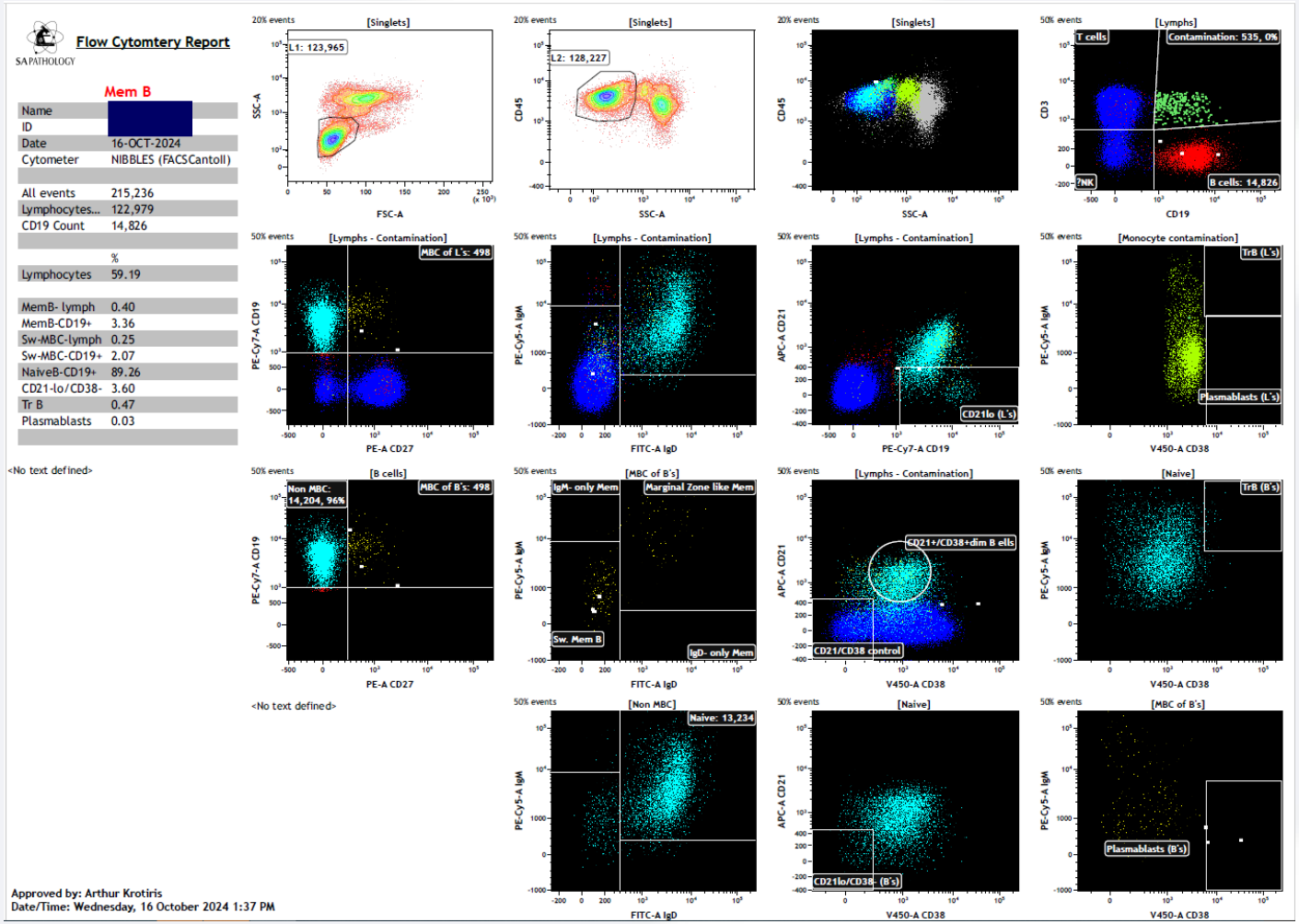
- 8 Colour panel
  - IgD FITC
  - CD27Pe
  - IgM Pe-Cy5
  - CD19 Pe-Cy7
  - CD21 APC
  - CD45 APC H7
  - CD38 V450
  - CD3 BV510
- Acquisition – 1 million events
- B cells – at least 5000

- Gating Strategy:
  - CD45 v Time – remove any air run
  - FSC-H v FSC-A – remove any doublets
  - FSC-A v SSC-A – gate lymphocytes
  - CD45 v SCC-A – gate lymphocytes to further purify
  - CD3 v CD19 – remove any contamination (CD3/CD19 double pos) of lymphocytes
  - CD38 v SSC-A – gate monocytes to remove any monocyte contamination
  - CD3 v SSC-A – Gate T cells to remove any T cell contamination
  - CD19 v CD27– Separate Memory B cells from Non-Memory B cells
  - IgM v IgD
    - Separate memory B cells into IgM only, Marginal Zone like memory B, Switch memory B and IgD only memory B cells.
    - Separate non-memory B cells into naïve
  - CD21 v CD19 – gate CD21los
  - CD21 v CD38 –gate CD21los/CD38- B cells of naïve B cells
  - IgM v CD38
    - naïve B cells – gate transitional B cells
    - Memory B cells : Plasma blasts

22 month old

Patient Switch Memory B = 2.07% of B cells

Age specific switch memory B range: (1.5-4.1)% of B cells

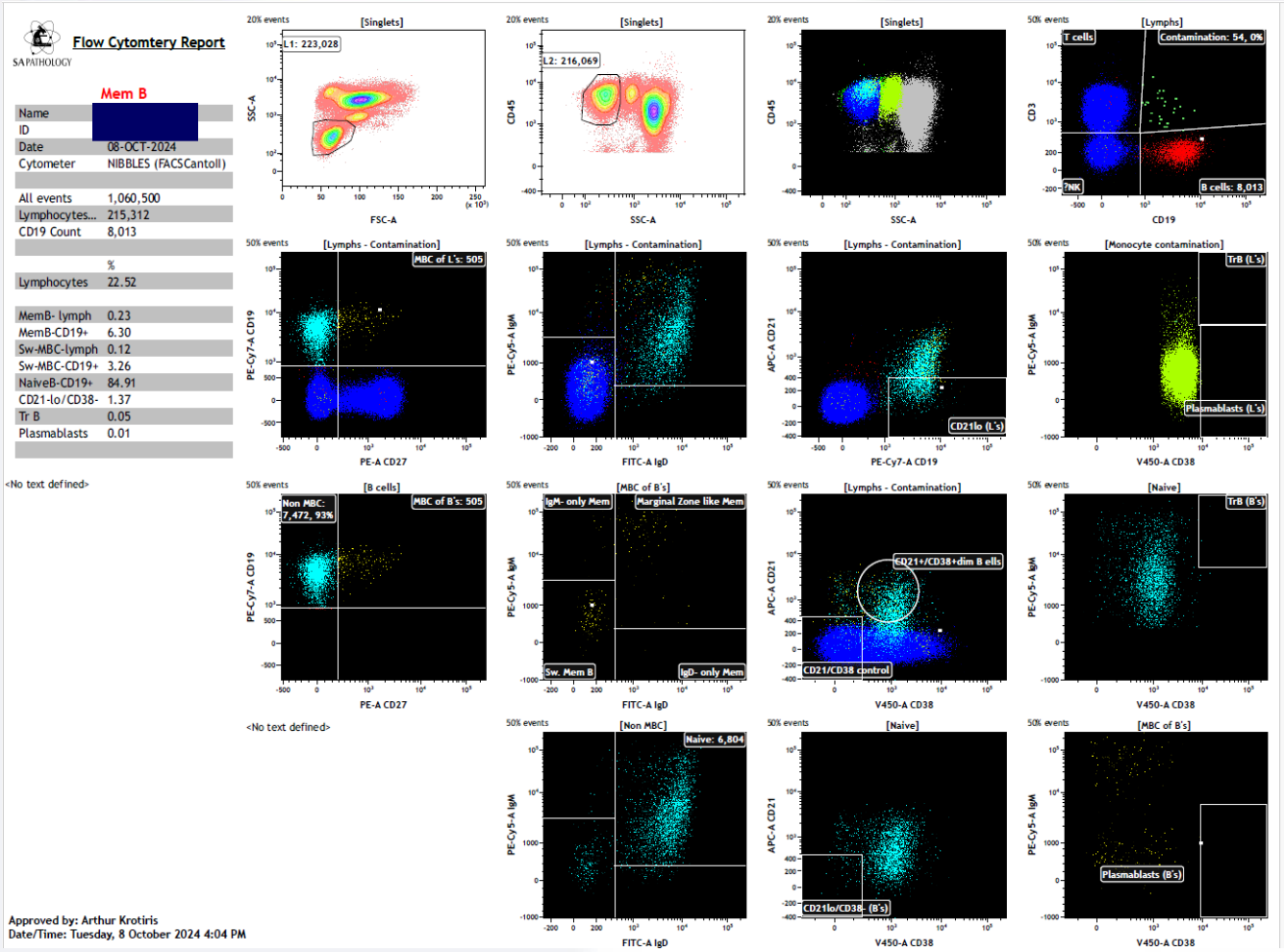


Clin Exp Immunol. 2010 Nov;162(2):271-9. doi: 10.1111/j.1365-2249.2010.04206.x.

68 year old

Patient Switch Memory B = 3.26% of B cells

No age specific switch memory B range
26-50 years old range: 9.2-18.9% of B cells



Clin Exp Immunol. 2010 Nov;162(2):271-9. doi: 10.1111/j.1365-2249.2010.04206.x.

Table 1. Frequency of B cell subsets in distinct age groups.

Age (years) No. of individuals*	0-1 (n = 31)	2-3 (n = 29)	4-5 (n = 19)	6-10 (n = 28)	11-18 (n = 51)	19-25 (n = 31)	26-50 (n = 32)
Lymphocytes	60·9 53·0-68·1	54·7 50·2-61·1	44·0 32·3-50·4	38·2 29·5-43·3	35·3 29·8-39·6	32·1 25·5-39·4	32·9 29·8-45·6
CD19+	13·3 10·2-18·5	20·8 16·5-25·8	16·1 13·4-21·1	12·2 9·8-17·7	13·3 10·2-15·4	9·1 6·6-10·8	9·2 7·2-11·2
CD27-IgD+	93·7 90·9-96·2	85·9 83·4-90·1	81·3 76·3-84·9	75·4 69·4-80·4	80·8 75·2-86·7	74·7 65·6-79·6	65·1 58·0-72·1
CD27+IgD+	2·5 1·6-4·1	5·4 4·2-6·9	6·5 4·1-9·0	10·0 7·5-12·4	7·3 4·6-10·2	11·7 7·4-13·9	15·2 13·4-21·4
CD27+IgD-	1·0 0·1-1·9	2·6 1·5-4·1	5·6 3·3-7·4	6·5 5·2-12·1	5·4 3·3-9·6	9·4 7·2-12·7	13·2 9·2-18·9
CD27-IgD-	1·5 0·9-2·4	2·5 1·6-3·6	4·5 3·4-6·1	5·0 3·5-6·6	3·7 2·3-5·5	3·2 2·1-4·4	3·3 2·1-5·3
CD24++CD38++	10·9 8·3-15·8	8·7 5·1-10·7	7·3 5·4-9·2	6·0 4·5-9·2	5·6 3·9-7·8	4·7 3·0-5·9	2·0 1·0-3·6
CD21lowCD38low	1·7 0·3-4·0	2·6 1·8-3·6	3·7 1·8-5·2	2·3 0·9-3·5	2·4 0·9-3·3	2·7 0·9-3·1	2·4 1·8-4·7
CD24-CD38++	0·4 0·2-1·0	1·1 0·6-2·3	1·4 0·8-2·7	1·5 0·7-3·5	1·0 0·3-1·7	1·2 0·6-1·6	1·0 0·6-1·6

The frequency of lymphocytes (as percentage of all leucocytes), total CD19+ B cells (as percentage of all lymphocytes) as well as B cell subsets (as percentage of all CD19+ B cells) is shown for distinct age groups as medians (upper line) and as the corresponding interquartile ranges (25th and 75th percentiles, lower line).
*Frequencies of CD21lowCD38 low B cells were analysed in an age-stratified subgroup of patients (n = 64).

Clin Exp Immunol. 2010 Nov;162(2):271-9. doi: 10.1111/j.1365-2249.2010.04206.x.

B cell subsets in Primary IEIs

		Circulating B cells	Disturbances of B cell maturation/differentiation steps
SCID B+	X-linked SCID	Normal	Decreased memory B cells and absence switched memory B cells
	JAK3	Normal or increased	Decreased memory B cells and absence switched memory B cells
	IL7R	Normal or increased	Decreased memory B cells and absence switched memory B cells
	CD45 deficiency	Normal	Decreased switched memory B cells
	CD3δ deficiency	Normal	Decreased switched memory B cells
	CD3ε deficiency	Normal	Decreased switched memory B cells
	CD3ζ deficiency	Normal	Decreased switched memory B cells
	LAT deficiency	Normal	Decreased memory B cells, increased transitional B cells
	IkBa GOF (NFKBIA/IkB)	Elevated B cells	Low memory B cells
SCID B-	RAG1	Significantly decreased	Not detectable
	RAG2	Significantly decreased	Not detectable
	Artemis deficiency	Significantly decreased	Not detectable
	DNA-PKcs deficiency	Significantly decreased	Not detectable
	NHEJ1 deficiency	Absent	Not detectable
	DNA ligase IV deficiency	Significantly decreased	Not detectable
	AK2 deficiency (Reticular Dysgenesis)	Decreased	Not detectable
	ADA deficiency	Absent	Not detectable
	BLNK deficiency	Absent	Not detectable
	BTK deficiency	Absent	Not detectable
	IKAROS deficiency	Significantly decreased or absent	B cell acute lymphoblastic leukemia (B-ALL)
	BAFF receptor deficiency	Decreased	Decreased switched memory B cells
	Wiskott Aldrich syndrome (WAS)	Normal or decreased	Decreased memory B cells and increased transitional B cells
	CVID	Normal or decreased	Absence of memory B cells
	TACI deficiency	Normal or decreased	Decreased switched memory B cells
	ICOS deficiency	Normal (children)/ Decreased (adults)	Decreased switched memory B cells and naive B Cells
	Hyper IgM syndromes	Normal	Absence of switched memory B cells
	IgA deficiency	Normal	Absence of IgA memory B cells
	CD19 deficiency	Normal	Decreased switched memory B cells
	AT (gene ATM/ATM)	Normal	Low naive B cells, transitional B cells and memory B cells, increased atypical memory B cells
	CD40 L deficiency	Normal	Absence of switched memory B cells
	CD40 deficiency	Normal	Absence of switched memory B cells
	Di George syndrome	Normal	Decreased switched memory B cells
	DOCK8 deficiency	Normal	Significant reduction of memory B cells; switched memory B cells low
	Fischer Evans syndrome	Normal	Increased transitional B cells and atypical memory B cells, reduction of memory B cells

Cytometry. 2022;101:140–149

Common Variable Immune Deficiency - ESID Criteria 2014 for access to IVIG through NBA

- At least one of the following:
  increased susceptibility to infection
  autoimmune manifestations
  granulomatous disease
  unexplained polyclonal lymphoproliferation
  affected family member with antibody deficiency.

- AND

  A marked decrease of immunoglobulin G (IgG) and marked decrease of IgA with or without low IgM levels (measured at least twice; less than the normal reference range for their age).

  AND

  At least one of the following:
  poor antibody response to vaccines (and/or absent isohemagglutinins); i.e. absence of protective levels despite vaccination where defined
  low switched memory B-cells (less than 70 percent of age-related normal value).

- AND

  Secondary causes of hypogammaglobulinemia have been excluded.

CVID classification

- **Freiburg classification**
- Group 1 – reduced $CD19^{+}/IgD^{-}/CD27^{+}$
- 1a - >20% CD21- peripheral B cells
- 1b – normal level of CD21+ B cells

- Group 2 – Normal $CD19^{+}/IgD^{-}/CD27^{+}$

- Group 1a patients have increased incidence of splenomegaly or autoimmune disease

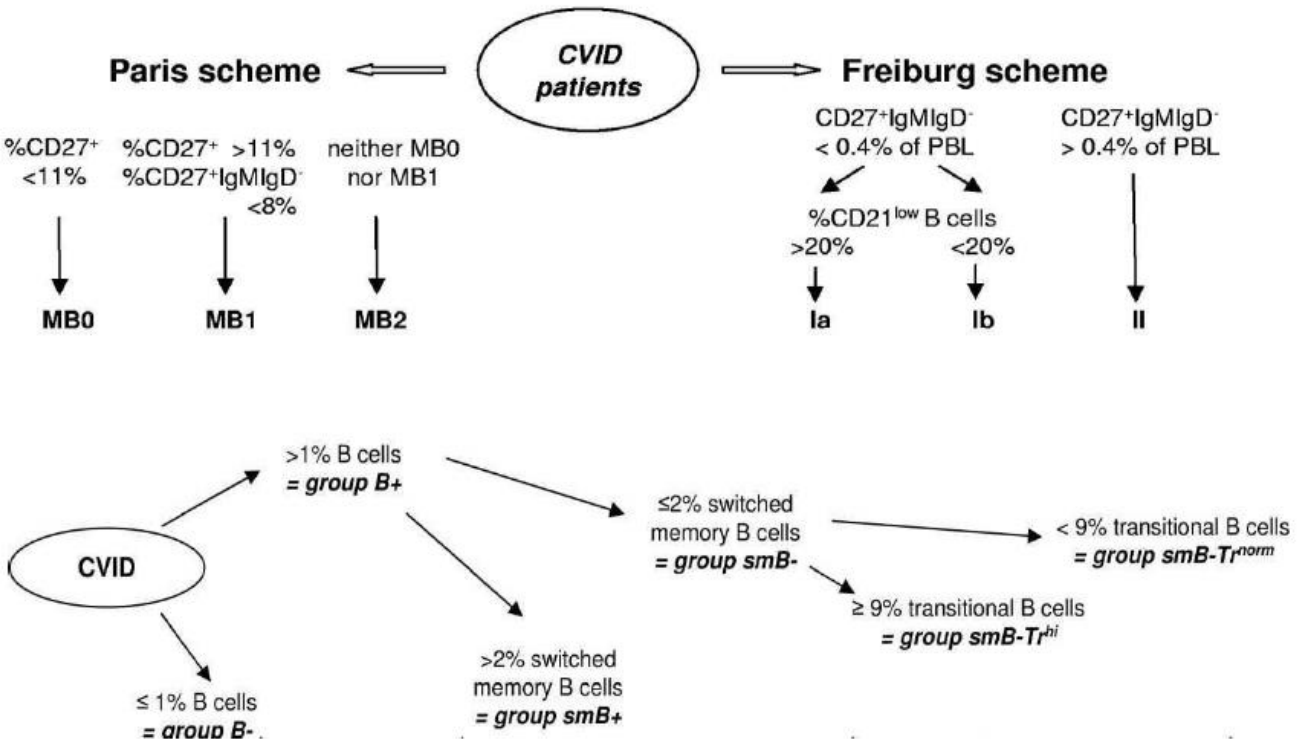
Warnatz et al Blood 2000;99:1544

CVID classification (contd)

- **Paris Classification**
- **MB0** – reduced no. of peripheral memory B cells (<11% of total B cells) Switched or non switched ie. $CD19^{+}/IgD^{+/-}/CD27^{+}$
- **MB1** – Reduced (<8% of total B cells) switched momory B cells only
- **MB2** – Normal levels of memory B cells
- Autoimmune diseases – MB0 & MB1
- Granulomatous disease and splenomegaly more common in MB0
- MB0- bacterial pneumonia and structural lung damage

Piqueras et al, J Clin Immunol 2003; 23: 385

CVID Classification Systems



Euroclass scheme

Wehr C et al. The Euroclass trial : Blood 2008; 111:77-85

Clinical Associations

- Freiburg:
  - **Ia :** 58 % Splenomegaly, 20 % Granuloma
- Paris
  - **MB0:** 47 % Splenomegaly, 17 % Granuloma
- Euroclass
  - **smB-**: 52 % Splenomegaly, 17 % Granuloma
  - **smB-Tr^{hi}** : 57 % lymphadenopathy
  - **smB+21^{lo}**: 50 % Splenomegaly, 14 % Granuloma
  - **smB-21^{lo}** : 60 % Splenomegaly, 20 % Granuloma

Wehr C et al. The Euroclass trial : Blood 2008; 111:77-85
Warnatz K et al . Cytometry Part B 2008; 74B: 261-271.

Comparative Study > Clin Exp Immunol. 2012 Feb;167(2):275-81.
doi: 10.1111/j.1365-2249.2011.04507.x.

Utility of peripheral blood B cell subsets analysis in common variable immunodeficiency

M Al Kindi [1], J Mundy, T Sullivan, W Smith, F Kette, A Smith, R Heddle, P Hissaria

Characteristics of Patients (n=53)	
Female : Male	2.5:1
Median Age (Yrs)	57
Median age at diagnosis (Yrs)	48
Infective Sinusitis	35 (66%)
Bronchiectasis	23 (43%)
Allergy	18 (34%)
Autoimmune diseases	12 (23%)
Granulomas	6 (11%)
Lack of Pneumovax response	12/20 (60%)
Baseline IgG (g/l)	< 3 (26%) 3-6 (74%)

B cell subsets- Qualitative analysis

	High	Normal	Low	P value
B cell (n 4.9-8.4 %)	32	9	11	< 0.0001
MBC (n 26.6-36%)	8	7	37	< 0.0001
SwMBC (n 6.5-29.1%)	2	15	35	< 0.0001
Transitional (n 0.6-3.4%)	30	16	6	0.0002
Plasmablast (n 0.4-3.65%)	5	26	21	0.001
CD 21 lo (n 0.9-7.6%)	25	25	2	< 0.0001

MBC : Memory B cells, SwMBC : switched Memory B cells

All patients with no Pneumovax response had low MBC & SwMBC

No association with clinical features

B cell subsets- Quantitative assessment

Clinical manifestation	P value for different B cell subsets
Sinusitis	NS
Bronchiectasis	NS
Allergy	NS
Autoimmune diseases	**Low B cells *P= 0.07***
Granulomas	**Low B cells & MBC *P <0.05***
Pneumovax response	NS

B cell subsets: Total B cells, MBC, SwMBC, Plasmablasts & Transitional B cells

Using logistic regression model for predictors of binary outcome eg. For every 0.01 unit increase in the number of B cells, the odds for granulomas decreased by 23 % (odd ratio= 0.77, $p < 0.05$)

Association of clinical features with classification systems

	Granulomatous Diseases (n=6)	Autoimmune diseases (n=12)
MB0 (n=27)	4 (15%) *P = 0.7*	6 (22%) *P = 1*
MB1 (n=15)	0 *P = 0.27*	5 (33%) *P = 0.5*
MB2 (n=11)	2 (18%) *P = 0.7*	1 (9%) *P = 0.5*
Ia (n=5)	**3 (60%)** ***P = 0.0015***	0 *P = 0.4*
Ib (n=29)	3 (10%) *P = 1*	9 (31%) *P = 0.3*
II (n=19)	0 *P = 0.15*	3 (16%) *P = 0.7*
Euroclass	*NS*	*NS*

We have stopped reporting the Classification systems on our reports

B cell subsets in Immune mediated inflammatory diseases

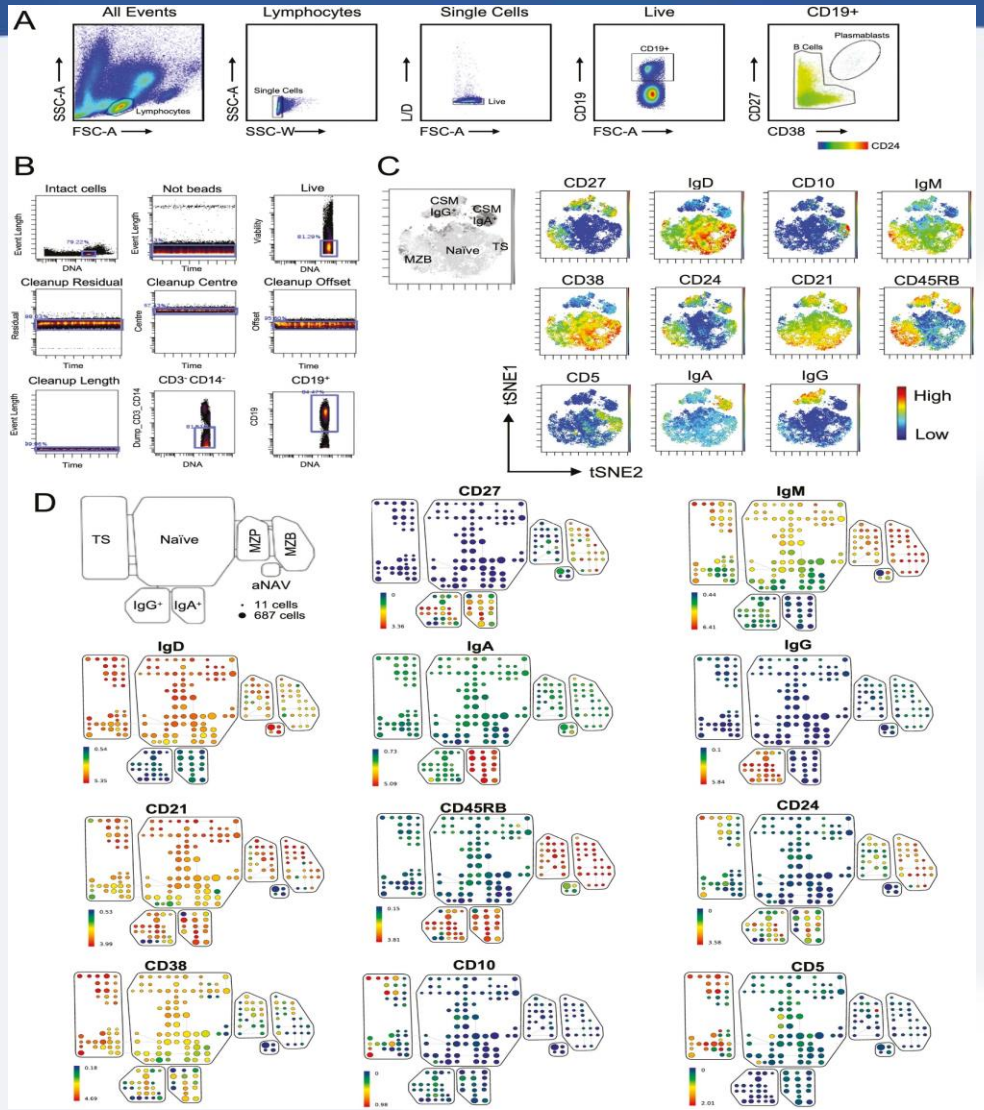
- B cells are known to play important roles in a variety of inflammatory diseases
  - production of antibodies
  - Secretion of cytokines
  - antigen presentation

- Many inflammatory conditions respond to B cell depletion therapies

B cell subsets in inflammatory diseases

- Still a research tool
- Earlier studies used mainly CD21lo cells
- Mass Cytometry – Biaxial, viSNE, UMAP
- ViSNE, a visualization tool for high-dimensional single-cell data based on the t-Distributed Stochastic Neighbour Embedding algorithm and uniform manifold approximation and projection (UMAP)
- spanning tree progression of density normalized events (SPADE) run on ViSNE coordinates can be a useful tool to separate subpopulations existing in phenotypic continuity

Identification of B cells in peripheral blood – Flow cytometry and Mass Cytometry



Clin Exp Immunol, Volume 210, Issue 3, December 2022, Pages 201–216, https://doi.org/10.1093/cei/uxac104

Disease	B subset	Stage	Extrinsic and/or intrinsic mechanism	Relevance to the diseases	
SLE	$CD21^{low}$ subsets ↑	Immature and activated B cells		Correlates with lupus nephritis activity	
SLE	IL-6-producing transitional B cells ↑	Transitional B cells	Type I IFN overactivation with NF-κB activation and reduced Bax	Correlates with disease severity	
SLE	$CD19^{hi}CD21^{-}CD38^{low}IgM^{low}CD23^{-}$ B cells ↑	Activated naïve B cells		Possible precursors of plasma cells	
SLE	$CD23^{-}IgD^{+}$ $CD27^{-}$ activated naïve cells ↑	Activated naïve B cells		Correlates with disease severity	
SLE	$CD19^{hi}CXCR3^{hi}$ B cells ↑	Naïve B cells, memory B cells, ASCs	High basal levels of phosphorylated (spleen tyrosine kinase) Syk and ERK1/2 CXCR3 may mediate migration to the sites of inflammation	Poor clinical outcomes following RTX treatment	
SLE	$CD11c^{hi}$ B cells ↑	Unique memory B cells	Lower CD40 and CD27 expression; increased IL-21R expression; activates IL-21 signaling and drives differentiation	Differentiates into autoreactive plasma cells; correlates with disease severity; negatively associated with C3 and C4; can migrate to target tissue	
SLE	TLR-9 expressing B cells ↑	Memory and plasma B cells	Activated TLR-9 signaling	Correlates with anti-dsDNA antibodies.	
SLE	$CD27^{-}IgD^{-}CD95^{+}$ memory B cells ↑	Memory B cells	Higher levels of CD86, CXCR3, HLA–DR, and CD71	Correlates with disease severity and serological abnormalities	
SLE	$CD27^{-}$ memory like B cells with high SYK ↑	Memory B cells	High expression of p-SYK; enhanced differentiation into $CD27^{++}$ IgG^{-}secreting cells; somatically mutated BCR	Correlates with disease severity; candidate source of plasma cells	
SLE	$IgD^{-}CD27^{-}$ memory B cells ↑	Memory B cells	Hypermutation in rearranged VH Abs	Correlates with disease severity, active renal disease, and autoantibodies	
RA	$CD21^{-/low}$ B cell ↑	Naïve and memory B cells	Increases B cell activation	Correlates with lymph proliferation	[25, 86]
RA	$CD86^{+}$ B cells ↑	Activated B cells	Possible association with ICOS+ Tfh cells and serum IL-21	Elevated levels associated with disease severity	[87]
RA	$IgD^{-}CD27^{+}$ memory B cells ↑	Memory B cells			[88]
RA	$IgD^{+}CD27^{+}$ memory B cells ↓	Memory B cells	Impaired IgM-production capacity and altered BCR repertoire	Correlates with disease activity and the anticyclic citrullinated protein antibodies	[88, 89]
MG	MuSK-specific $CD27^{hi}CD38^{hi}$ B cells ↑	Autoreactive ASCs		Present during relapse but not remission	[90]
MG	$AChR^{+}CD21^{+}$ B cells ↑	Precursors of ASCs?		Elevated levels associated with disease; correlates and anti-AChR antibodies	[5]
MG	$CD19^{-}CD138^{-}$ASCs ↑	Plasmablasts	May associate with follicular helper T cells and IL-21	Elevated levels associated with disease severity	[85]

SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; MG: myasthenia gravis.

Journal of Immunology ResearchVolume 2020, Article ID 9518137,

Comparative Study > Int J Rheum Dis. 2015 May;18(4):421-32. doi: 10.1111/1756-185X.12325. Epub 2014 Mar 2.

Changes in peripheral blood B cell subsets at diagnosis and after treatment with disease-modifying anti-rheumatic drugs in patients with rheumatoid arthritis: correlation with clinical and laboratory parameters

Jeremy McComish [1 2], Joy Mundy [1], Tom Sullivan [3], Susanna M Proudman [4 5], Pravin Hissaria [1 6]

- B cell homeostasis is disturbed in RA
- Circulating non-switched memory B cells are low where CCP is high
  - May reflect homing to synovial lymphoid tissue
- CD21lo cells rise progressively with treatment
  - Potential for a marker

B Cell depletion therapies

- Increasing use in treatment of various Inflammatory diseases
- Even those autoimmune diseases traditionally conceptualised as “T cell mediated”, such as multiple sclerosis, are now recognised to develop from more complex and reciprocal interactions between B and T cells
- CD19 directed CAR-T cells have shown promise in treatment of various refractory autoimmune diseases
- Relapse/Recurrence of autoimmune disease is associated with B cell repopulation

Autoimmunity Reviews
Volume 16, Issue 5, May 2017, Pages 542-547

Review

The utility of monitoring peripheral blood lymphocyte subsets by flow cytometric analysis in patients with rheumatological diseases treated with rituximab

Jessica Day [a b], Vidya Limaye [b d], Susanna Proudman [b d], John D. Hayball [a e], Pravin Hissaria [b c]

- There are no guidelines regarding the timing of retreatment with rituximab as maintenance therapy to prevent relapse of autoimmune disease as data on this issue is lacking
- Monitoring total B cell numbers does not help predict relapse of autoimmune disease following rituximab therapy
- Levels of individual B cell and T cell subpopulations during immune reconstitution following rituximab therapy may correlate with disease relapse in autoimmune conditions
- Specific B cell subpopulations or T cell parameters at baseline may predict response to rituximab therapy in rheumatoid arthritis

Article | PDF Available | Literature Review

European flow cytometry quality assurance guidelines for the diagnosis of primary immune deficiencies and assessment of immune reconstitution following B cell depletion therapies and transplantation

June 2024 · Cytometry Part B Clinical Cytometry

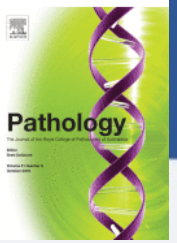
- Run within 4 hours.
- Bulk lysis, monitoring of the time parameter, and doublet discrimination is recommended to acquire sufficient high quality CD45+ lymphocyte events to assess B cell subset proportions and count
- Dual Platform approach for lymphocyte count
- at least 1x 10^6 CD45+ white cells (ideally up to 5x 10^6) should be acquired. Analysis of B cell subsets is possible and clinically relevant when at least 200–300 clean B cell events are acquired at counts greater than 0.5 cells/µL. The lowest level of quantitation for high sensitivity FC lies in the range of 0.002% or 0.2– 0.3 cells/µL:
- Complete B cell depletion is defined at counts below 0.1 cells/µL
- Unlike most chemical analytes, the large number of events acquired using FC means that the laboratory need only perform up to 5 replicates to determine sample variance
- UOM should be defined

FULL TEXT ARTICLE

Recommendations for the reporting of B cell populations in the context of common variable immunodeficiency disorder (CVID)

Louise Wienholt, Michael Lane, Alice Grey and Tiffany Hughes

Pathology, 2019-10-01, Volume 51, Issue 6, Pages 640-641, Copyright © 2019 Royal College of Pathologists of Australasia



- Recommendation 1: Sample type Samples should be collected in ethylenediaminetetraacetic acid (EDTA).
  - The flow cytometric enumeration of B cell populations has been validated using both fresh peripheral blood and isolated PBMC methods, with good agreement.
  - B cell subsets should not be performed in the context of CVID when the total B cell count is <1% of PBL
- Recommendation 2: Populations and units reported To provide clinically useful information for the clas
- Recommendation 3: Reference ranges and interpretive commentary should be written on the report

Table 2 Minimum recommendation of phenotype and units reported in laboratories performing B cell subsets

Subset	Phenotype	Unit(s) reported
Total B cell	$CD19^+$	% of PBL
Naïve B cell	$CD19^+IgD^+ IgM^+CD27^-$	% of PBL and B cells
Marginal zone (non-switched memory B cell)	$CD19^+IgD^+ IgM^+CD27^+$	% of PBL and B cells
Transitional B cell	$CD19^+CD38^{++}IgM^{high}$	% of PBL and B cells
Switched memory B cell	$CD19^+IgD^-IgM^-CD27^+$	% of PBL and B cells
$CD21^{low}$	$CD19^+CD38^{low}CD21^{low}$	% of B cells

Limitations

- No standardised antibody cocktails available for defining subsets
- Limited markers used for defining subsets – No functional or in-depth characterisation
- Interpretation is context dependent
- Normal ranges not validated locally
- Role of Longitudinal monitoring is unknown (except for B cell reconstitution scenarios)

Questions ????