Defining the normal: new approaches for harmonized subset definitions and gating procedures

Ester Mejstříková, Veronika Kanderová
CLIP Cytometry
Department of Pediatric Hematology and Oncology
2nd Faculty of Medicine
University Hospital Motol

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Disclosure commercial conflict of interest

x	No, nothing to disclose
	Yes, as specified below:

Flow cytometry in immunology and hematology

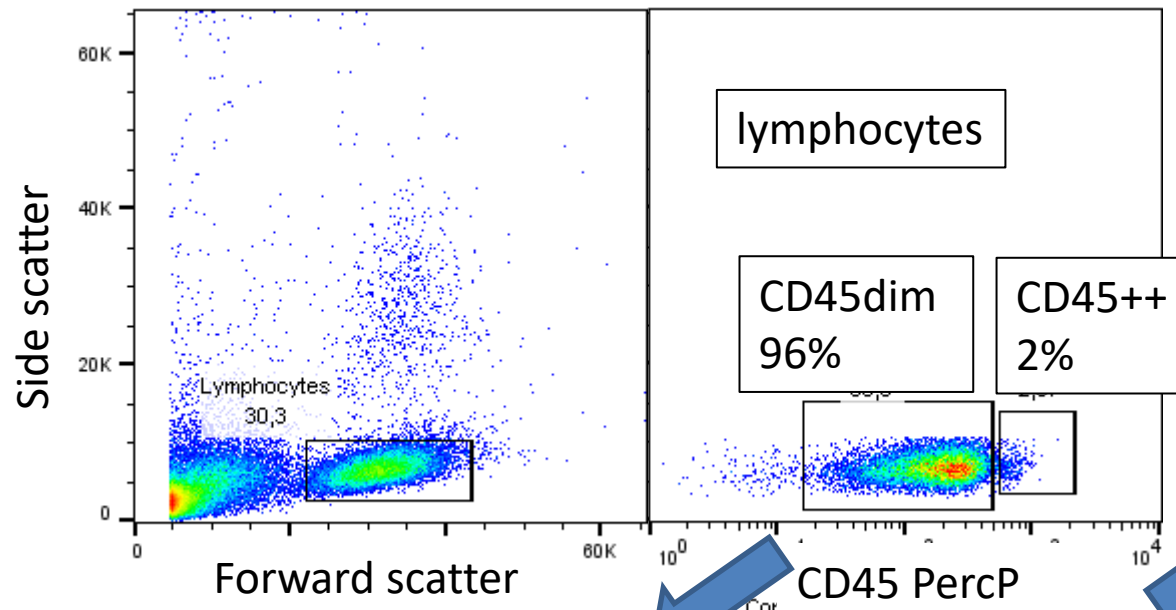
We ask whether the sample is:

- normal (composition of subsets and its phenotype)=diagnosis of primary immunodeficiency (PID)
- reactive (i.e.because of infection)
- abnormal (presence of atypical suspect cells)

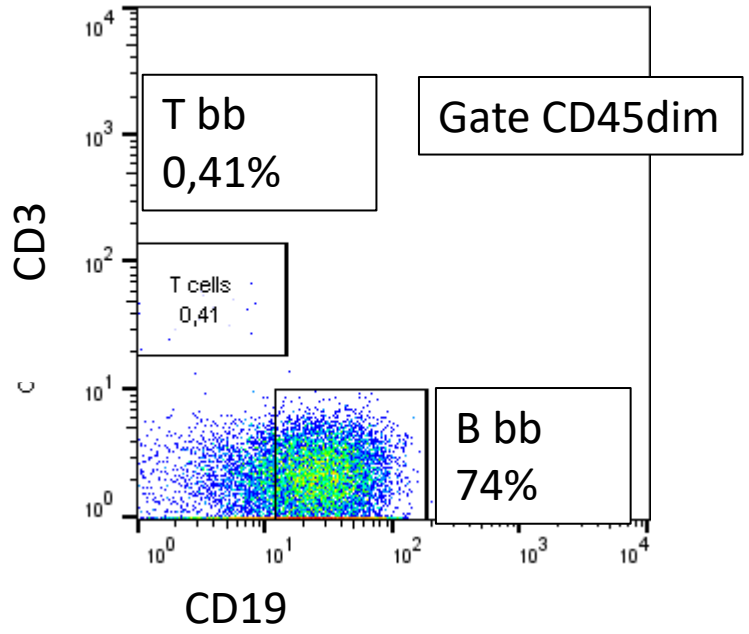
Immunology report
% and absolute count listed as values and compared with reference ranges

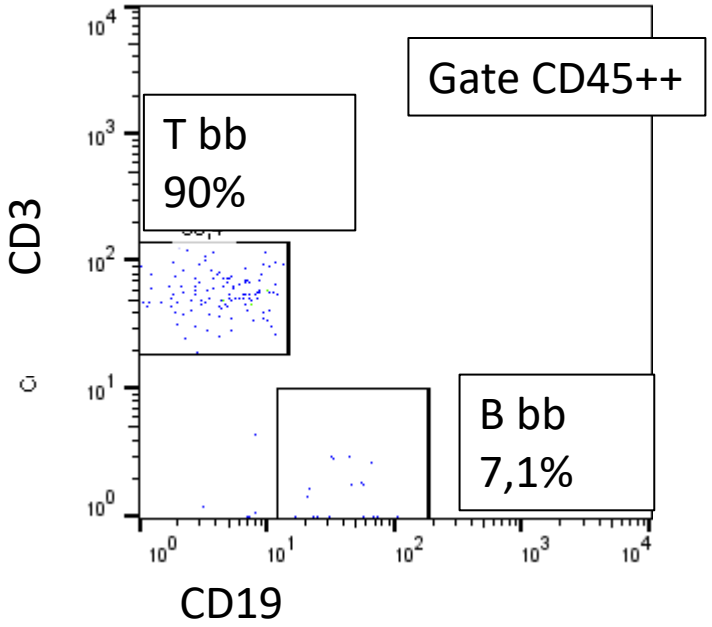
Hematology
Identification of populations (normal/abnormal)
Commentary almost always available

SELECTION OF APPROPRIATE GATE – in PID we focus on CD45++ low Ssc cells



EBV LPD after cord blood SCT basic lymphocyte subsets d+28





PID DIAGNOSIS algorithm:

- Clinical examination
- Laboratory investigations
- Analysis of lymphocyte subpopulations

- Frequently we use experience generated in individual single lab

The composition of immune cells is influenced by age

What should be (ideally) same in sample processing and is distinctly different

- How we gate the cells
- How we define the subset (i.e. plasmablast, RTE)
- What is the normal frequency of rare subsets: Vα24Vβ11, TREGs..
- Which antibody clones are used
- Which fluorochromes are used
- How we prepare sample (bulk lysis. stain-lyse-wash)

Gating strategies

- 2D graph approach = standard in the diagnostic work up
- Backgating strategy
- increasing amount of parameters = becomes impossible to check all 2D combinations of parameters
- Inclusion of bioinformatic tools
- Consider **non PID disease** (especially haematological malignancy)

Spectrum of potential clinical diagnoses according age

neonatal period

- SCID/HIV
- B cell deficiency
- Di George syndrome
- reactive/viral infection
- HLH
- transitory myeloproliferative disorder (TMD)
- hemoblastosis

childhood

- SCID/CID/HIV
- B cell deficiency/CVID
- ALPS
- reactive/viral infection
- (secondary) HLH
- hemoblastosis/lymphoma
- monoMac syndrome (GATA2 deficiency)

adulthood

- HIV
- B cell deficiency/CVID
- ALPS
- reactive/viral infection
- (secondary)HLH
- (B)CLPD
- plasma cell disorders
- hemoblastosis/lymphoma
- monoMac syndrome (GATA2 deficiency)

Lymphoid organogenesis in mammals

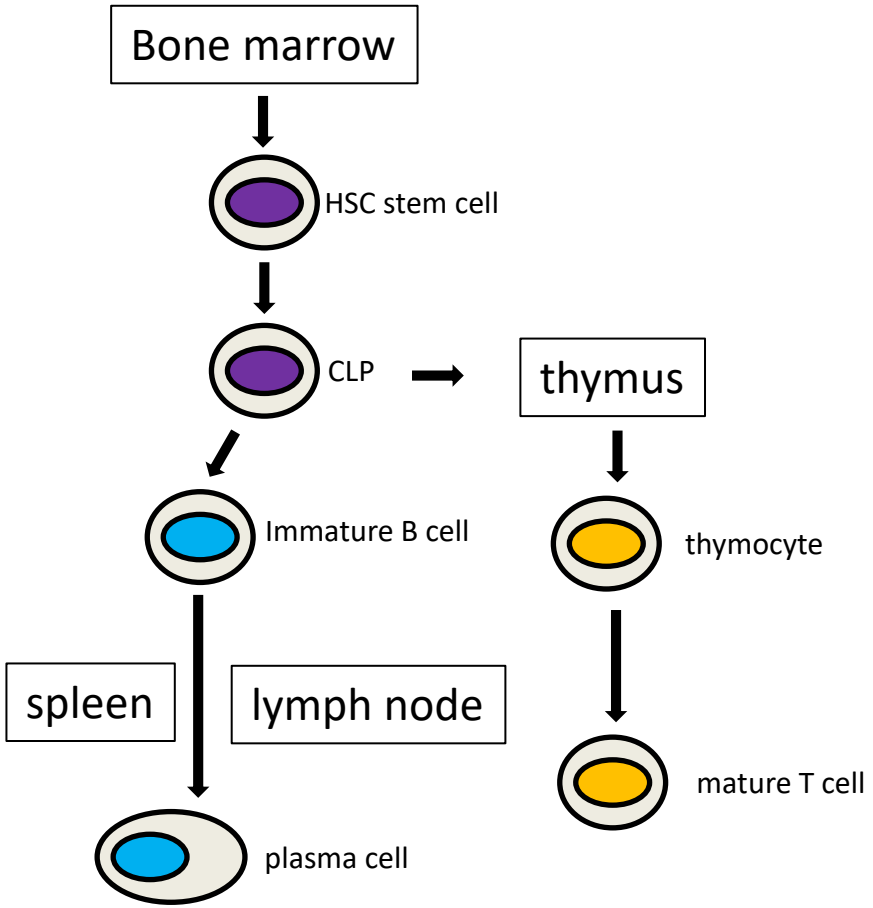
1) genesis of **primary** lymphoid organs: **bone marrow** and **thymus**

2) development of **secondary** lymphoid organs (SLOs): **lymph nodes**, **Peyer patches** and **spleen**

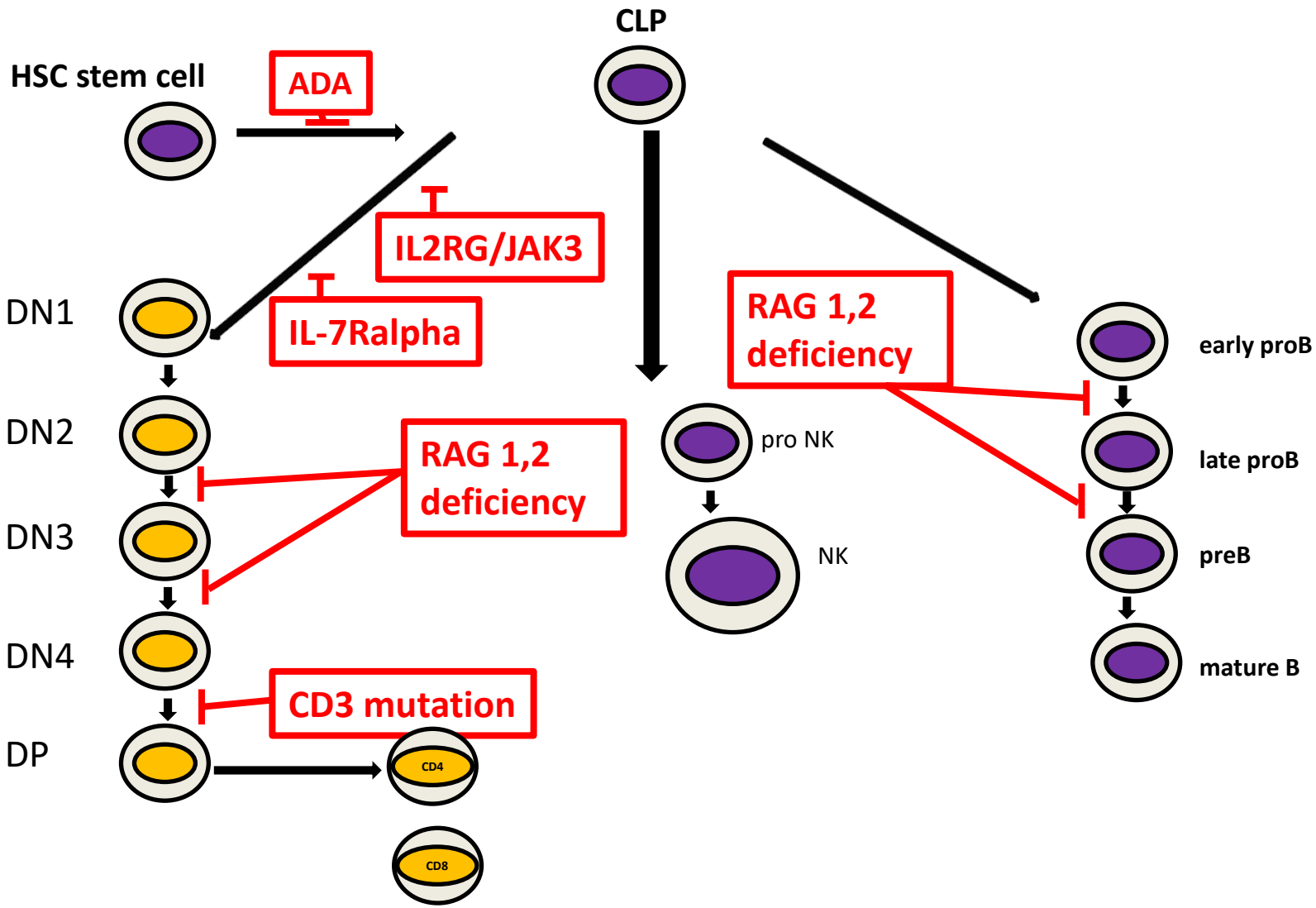
3) development of **terciary lymphoid organs** (TLOs): within tissues after the initiation of immune response

- Role of lymphoid tissue inducer (LTi) cells in generation of most SLOs

In imunology, we analyze mostly **<u>peripheral blood</u>**



Severe combined deficiency learns us about normal lymphocyte development



Dynamics of lymphocyte subsets during aging

- most lymphocytes are naive at birth and have not yet encountered a foreign antigen

- „Redundancy“ in production of lymphocytes to increase the probability of succesful elimination of the pathogen

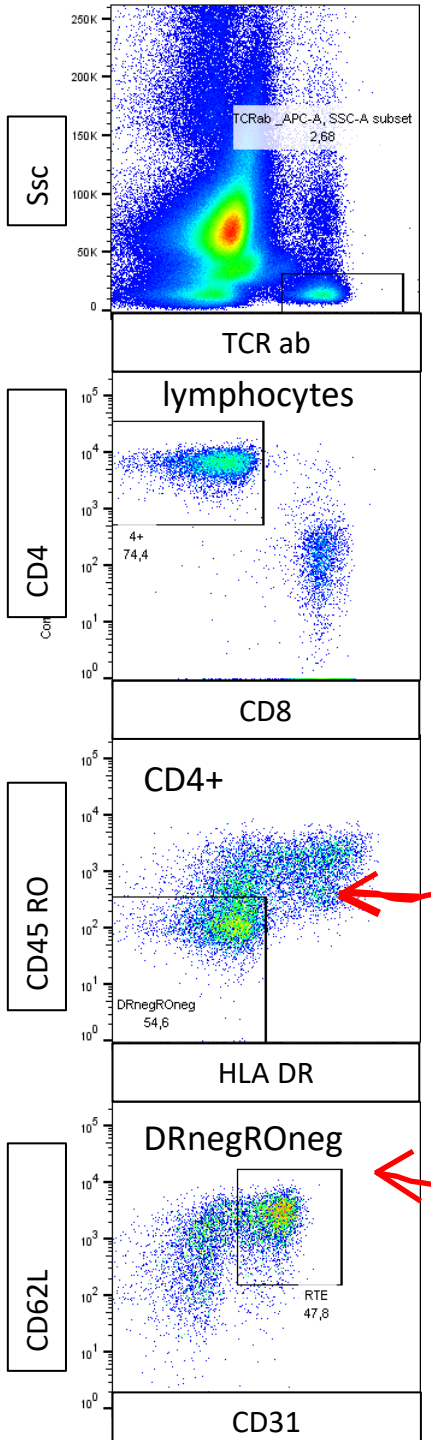
T cell composition and maturation

Development starts in thymus (almost never available for diagnostic purposes, except for patients undergoing cardiac surgery)

- RTE essential parameter indicating thymic function (defined as CD62L+CD45RO-HLA-DR-**CD31**+ Kalina et al.)
- population shifts during infection (incease of CD8, HLA DR expression..)
- T-cell activation

- Replicative senescence, and oligoclonal expansions

Recent thymic emigrants

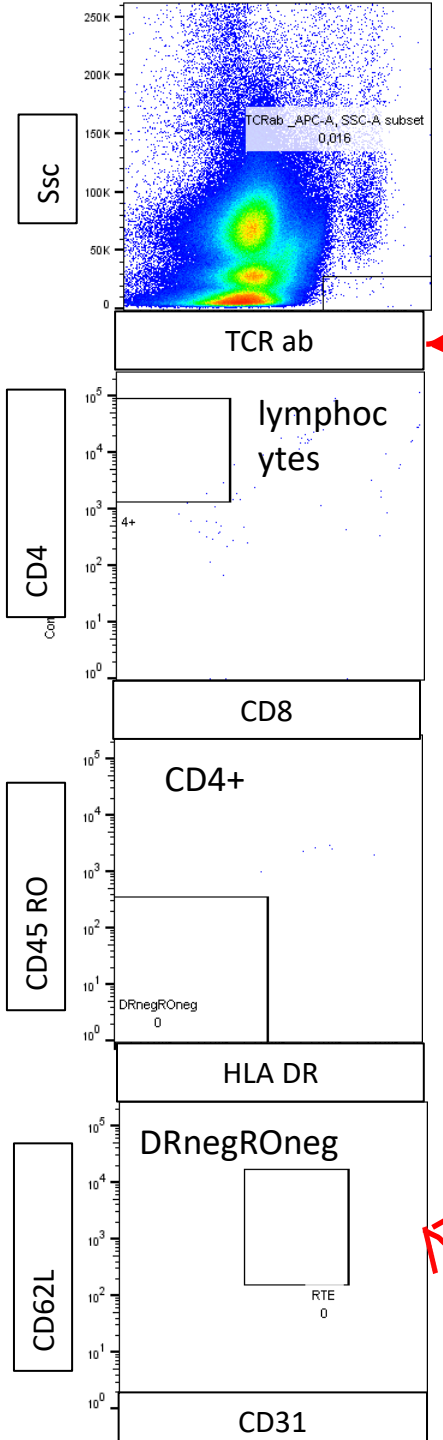


3 months old girl
WBC 49x109/L, abs lymphs 3.9
no clear blasts on smear
presented as respiratory failure
Pneumocystis jirovecii in BAL
TREC/KRECs after birth not analyzed
RTE decreased 25% out of all CD4+

reactive activated mature CD4+

RTE

Final diagnosis:
KMT2Ar lymphoma/leukemia infiltrating lungs



neonate, boy
positive new born screenig program
TRECs 0
no T cells

Absent T cells including RTE

Final diagnosis:
Thymus aplasia due to Diabetic fetopathy

B cell composition

Maturation starts in bone marrow

At birth mostly naive B cells are present in blood

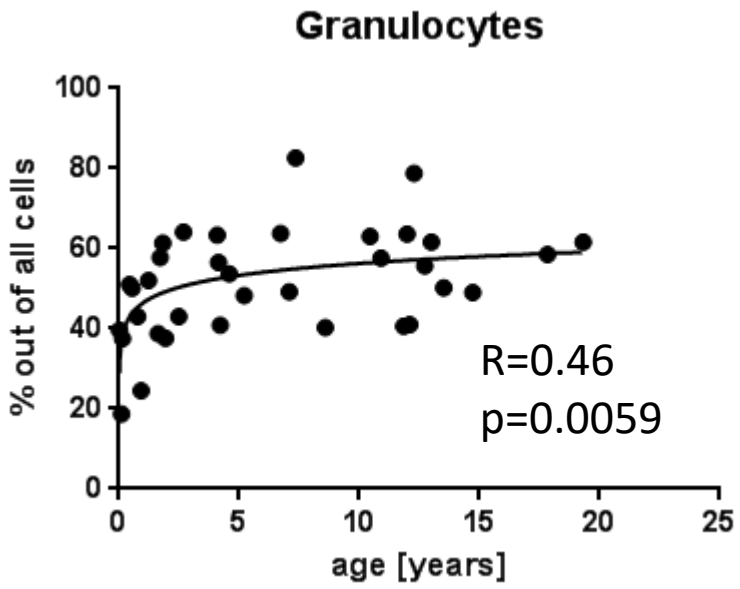
Categorisation of CVID (based on adult reference ranges its applicabilty in children is questionable)

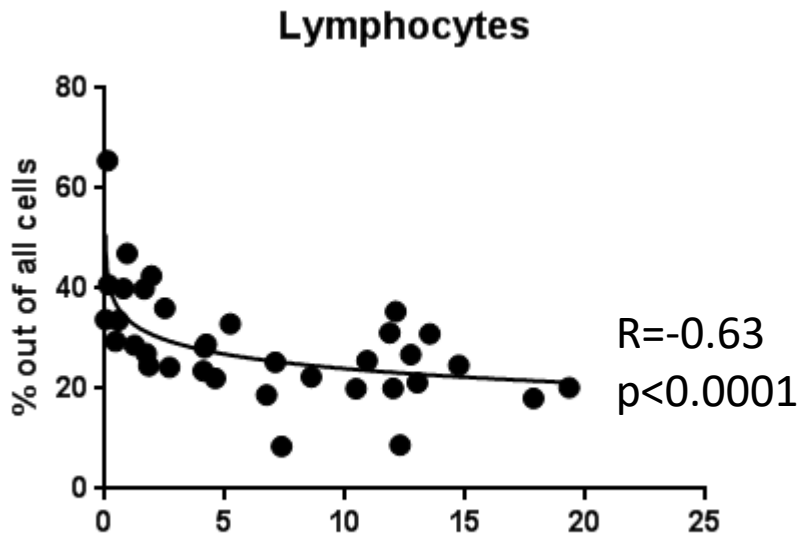
Piatosa et al (Clin Cytometry 2010):

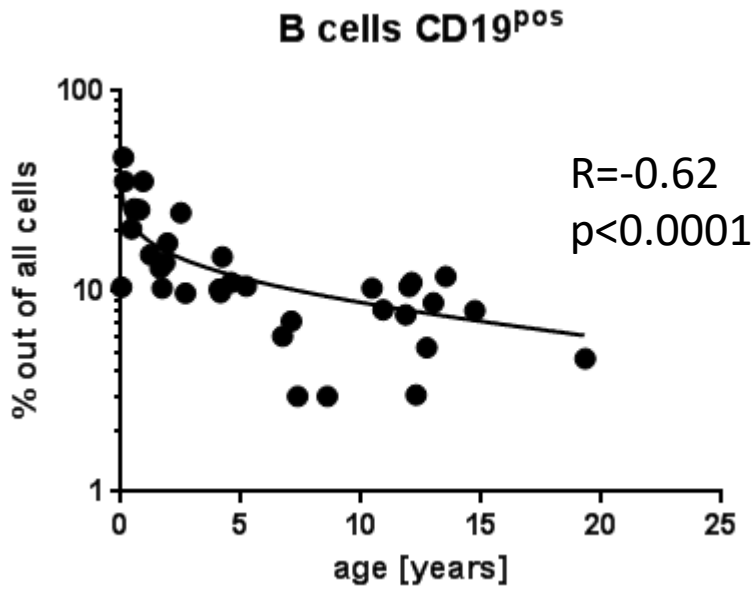
- definition of plasmablasts (Piatosa defines them as IgM^{neg} $CD38^{high}$ – in some age subgroups normal values decrease to 0)
- definition of transitional B cells

Duchamp et al.: French reference values, missing info about plasmablasts and transitional B cells

Bone marrow composition depends on age







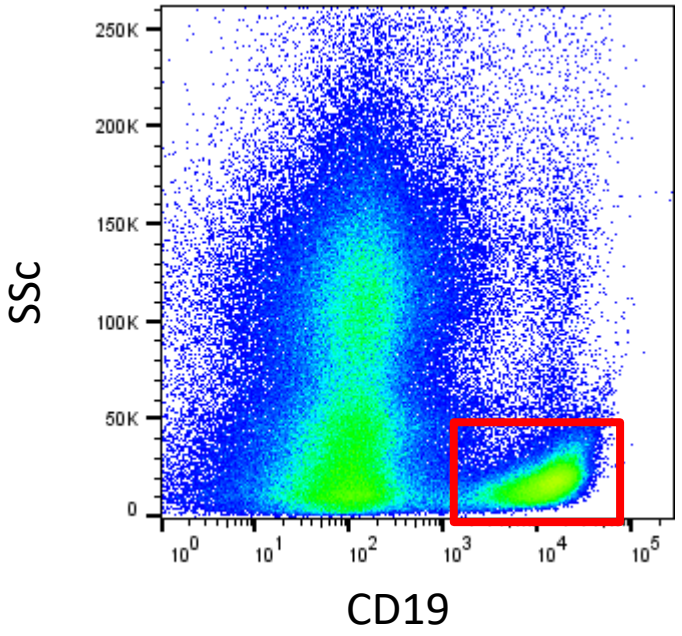
CLIP
Childhood Leukaemia Investigation Prague

Michaela Reiterova

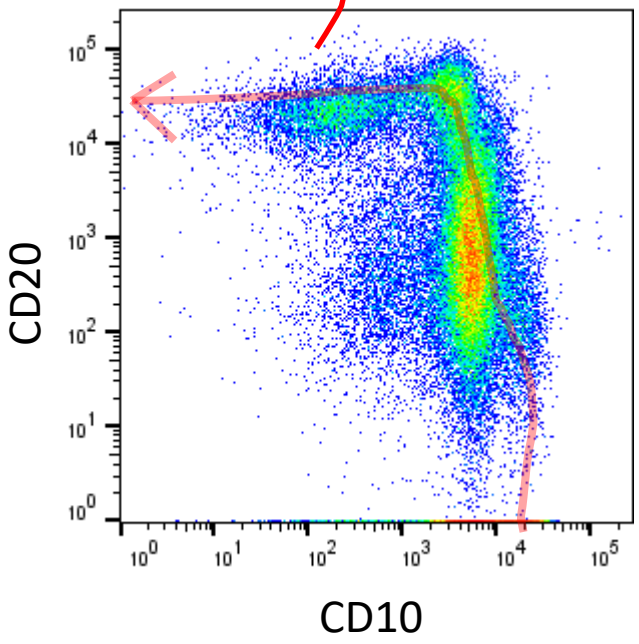
B cell maturation marrow and blood

- 4 yrs old, neutropenia 2.92 WBC, 2.08 lymphocytes, probably immune-mediated

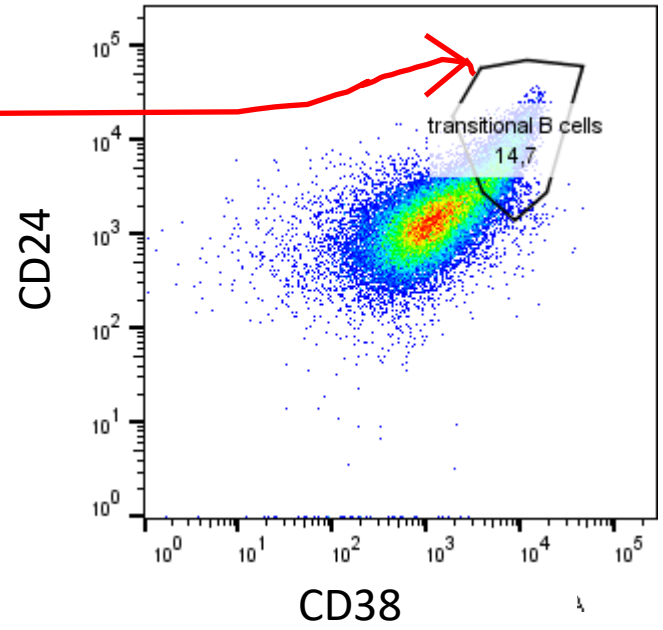
Bone marrow (CD19 21%)



Gate CD19



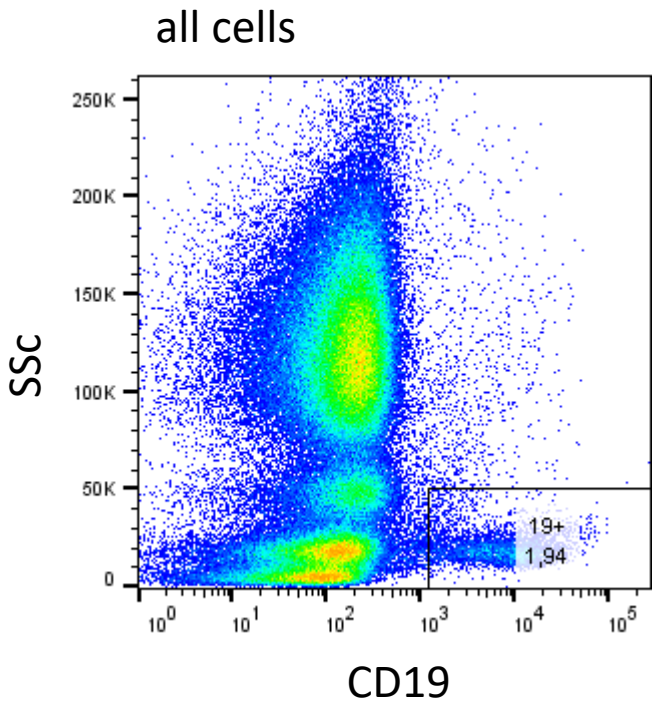
CD19+CD27neg

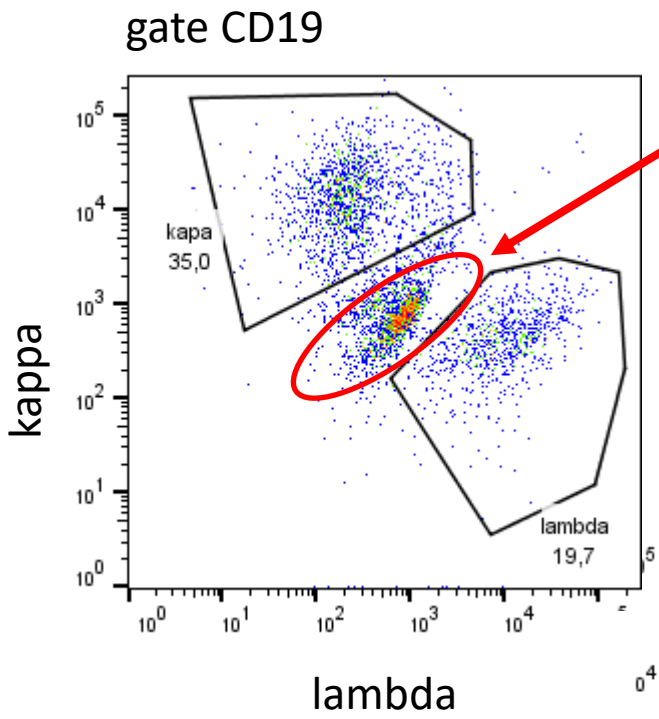


Strong B cell regeneration in bone marrow is frequently reflected by high amount of transitional B cells in blood

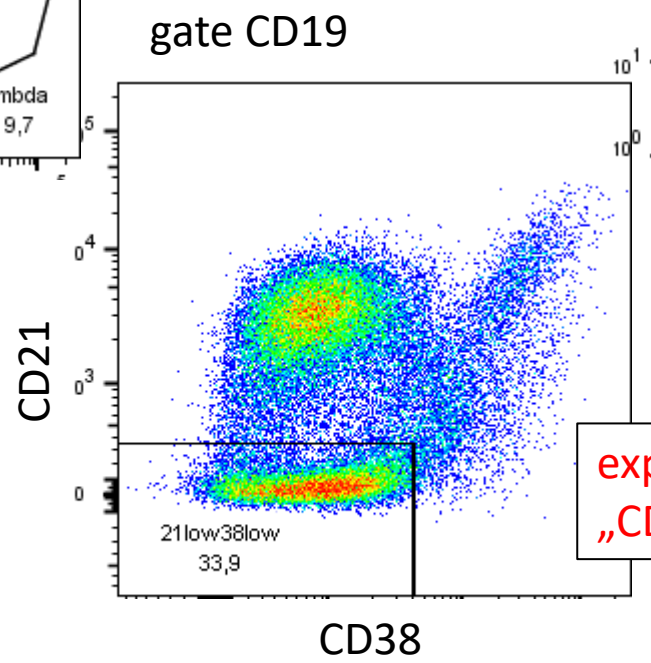
CD19 positive NK cells

- physiological rare variance, prevalence in healthy individuals unknown
- patient followed after liver transplant, EBV reactivation

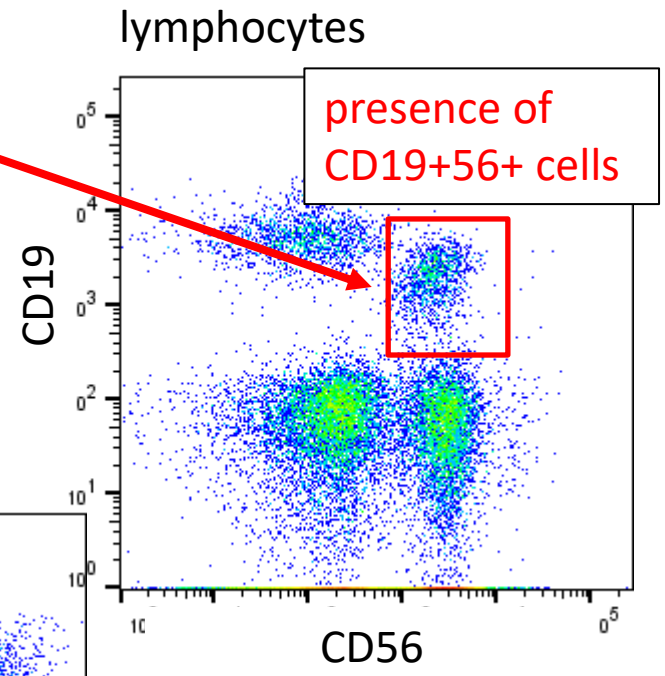




Kappa neg/lambda neg B cells



expansion of „$CD21^{low}CD38^{low}$“



presence of CD19+56+ cells

Brief Communication

Apparent CD19 Expression by Natural Killer Cells: A Potential Confounder for Minimal Residual Disease Detection by Flow Cytometry in B Lymphoblastic Leukemia

Lorinda Soma,* David Wu, Xueyan Chen, Kerstin Edlefsen, Jonathan R. Fromm, and Brent Wood
Department of Laboratory Medicine, University of Washington, Seattle, Washington

Cytometry Part B (Clinical Cytometry) 88B:358–360 (2015)

Letter to the Editor

NK Cells Expressing the B Cell Antigen CD19: Expanding the Phenotypical Characterization and the Potential Consequences from Misinterpretation of This Subset Population

Korol et al.: 44 cases out of 1002 cases with suspect immunodeficiency analyzed for lymphocyte subpopulations
No other B cell specific markers on $CD19^{pos}$ NK cells

How to read immunological report and what does mean shift from normal

Lymphocyte gate

CD19 : ↓ absence BTK deficiency, B- SCID, CVID, B cell targeted therapy, GATA 2def

CD3: ↓ T- SCID , immunosuppression, infection (HIV)

CD3neg16.56+: ↓ absent in NK- SCID, ↓ GATA2def

CD3+ CD4+ CD45RA+ CD27+ (Naive CD4 T): decrease during viral infections, almost absent in SCID

CD3+ CD8+ CD45RA+ CD27+ (Naive CD8 T): decrease during viral infections, almost absent in SCID

CD4 : ↓ T- SCID (cave maternofetal engraftment), infection (HIV), immunosuppresion

CD8 : ↓ T- SCID (cave maternofetal engraftment)

TCRγδ: ↑ some infections (francisella tularensis, EBV), IBD, hypomorphic RAG deficiency, CID

RTE: ↓ absent in SCID, decrease after corticosteroids, infection

HLA DR out of CD3+4+ : increased especially in viral infections, HLH

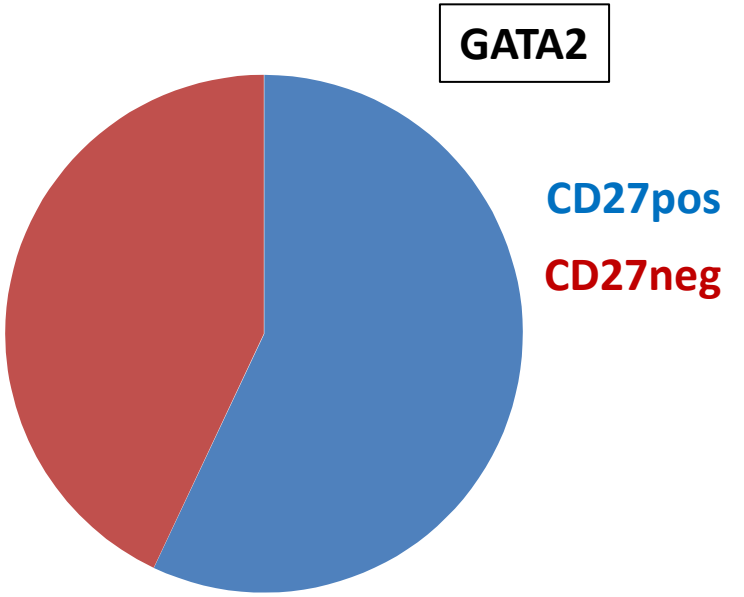
HLA DR out of CD3+8+: increased in viral infections, HLH

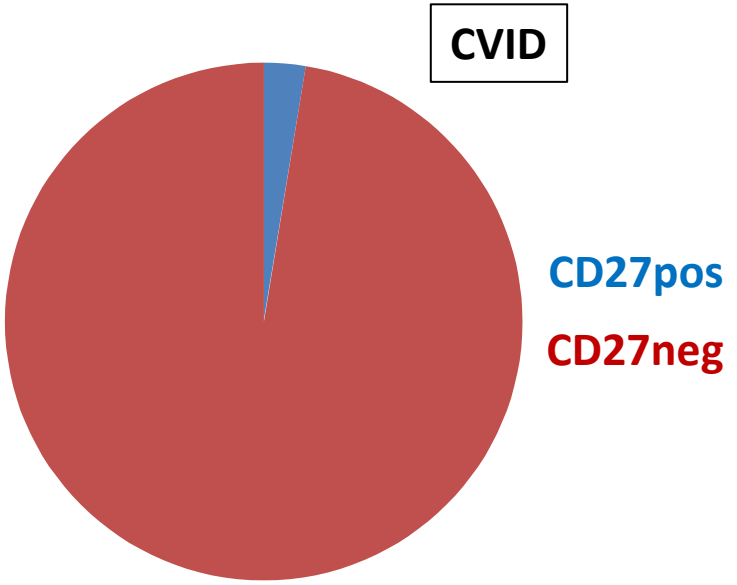
How to read immunological report and what does mean shift from normal

Gate CD19+

CD27+ Memory: ↓ disturbed peripheral B cell maturation (CVID, corticosteroids, ALPS),↑ reactive during the infection, GATA2 deficiency
IgD- IgM- CD27+ Class switched: ↓ disturbed peripheral B cell maturation (CVID, corticosteroids, ALPS)
IgD+ CD27+ Marginal zone like: ↓ disturbed peripheral B cell maturation
IgD+ CD27- Naive: ↑ disturbed peripheral B cell maturation (CVID, corticosteroids, ALPS)

CD21low CD38low: ↑ autoreactivity, autoimmunity especially in CVID, consider CD19+ NK cells
CD24++ CD38++ CD27- Transitional: ↓ B cell suppression in bone marrow
CD38++ CD27++ Plasmablast: ↑ increased during infection (EBV, bacterial), EBV LPD in immune suppressed patients, GATA2 deficiency





Example of case with suspect SCID

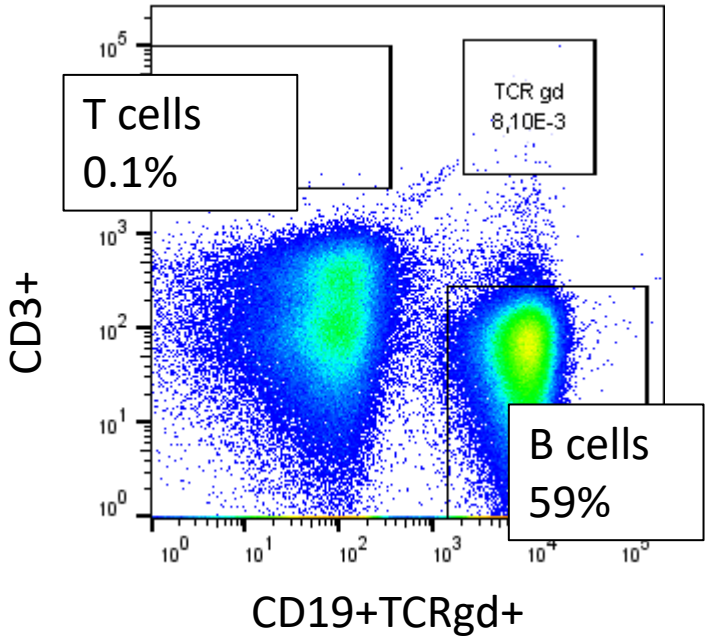
patient (boy) captured by neonatal screening program. T cell excision circles (TRECs are missing)

- diagnosed through newborn screen program (TREC 0. KREC normal range)
- so far no signs of immunodeficiency. no clinical history of immunodeficiency
- in family known methemoglobinemia. abnormal hemoglobin (heterozygous mutation HBB: c.190C>T (His-Tyr). Hb M Saskatoon
- born 22.2.22. first immunological check up 15.03.22

WBC: 7.5 x10^9/L. LY: 2.680 x10^9/L. identified heterozygous mutation HBB

Lymphocyte screening tube CD45RA/CD27/CD4/CD19+TCRgd/CD3/CD16/CD8//HLADR/CD45/CD56

lymphocyte gate



no **T cells (<0.1%).**
B cells dominate **(60% out of lymphocytes)**
NK cells (35%).
B cells are mostly naive 98%. (half are transitional). Marginal zone like 0.9 and switched memory only 0.2%. Plamablasts almost missing (0.02%).

Conclusion: SCID T-B+NK+

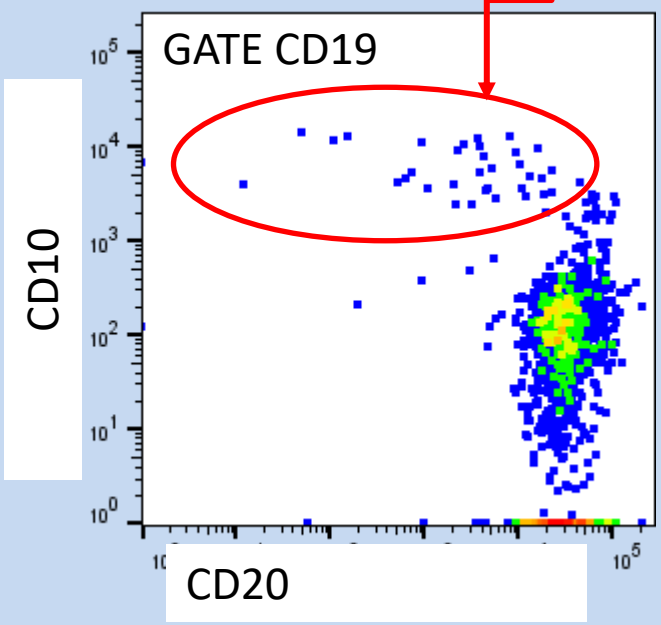
mutation c.353-10C>G (CD3 epsilon)

suspect CVID (marrow, blood, lung biopsy)

- 10 yrs old boy, failure to thrive, 3 weeks respiratory infection not responding to antibiotics/antimycotics. more frequent respiratory infection since early childhood, alopecia spontaneously resolved 2 years ago
- hepatosplenomegaly
- IgG 3.3, IgA 13, IgM 0,27 (g/L)

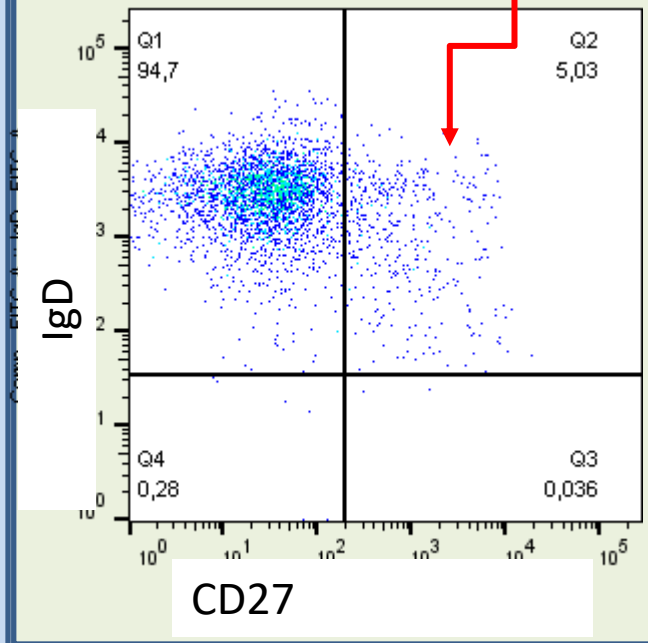
BONE MARROW (CD19: 0.82% out of all cells)

Low precursors

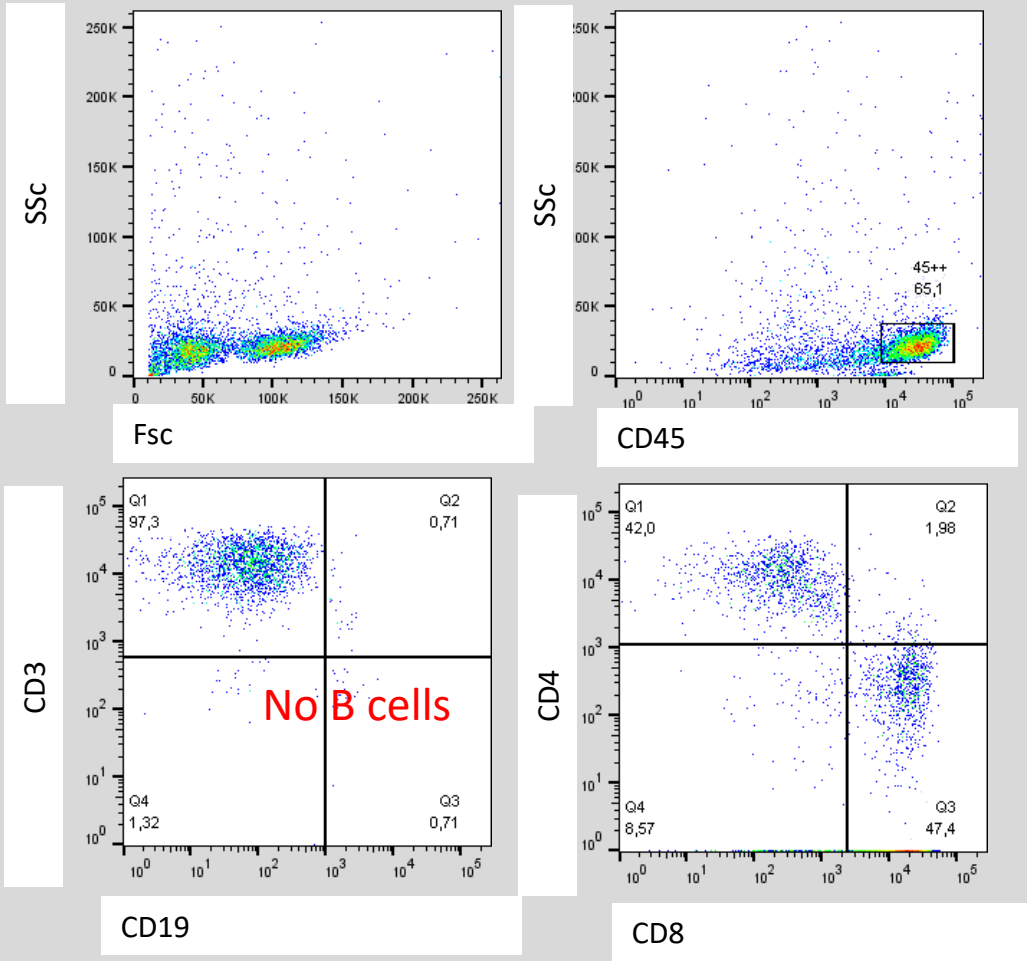


BLOOD
(CD19: 8% out of lymphocytes, abs. 0.103x10^9/L)

Decreased memory and transitionals



LUNG
Susp GLILD syndrome



Example of suspect immunodeficiency

- 01/2022 Boy 9 year old
- Since 02/2021 . repeated infections. recurent fever with unclear focus. fluctuating neutropenia
- Between 07/2021 and 11/2021 clinically doing well
- Autumn 2021 again recurrent fevers

Blood count 01/2022

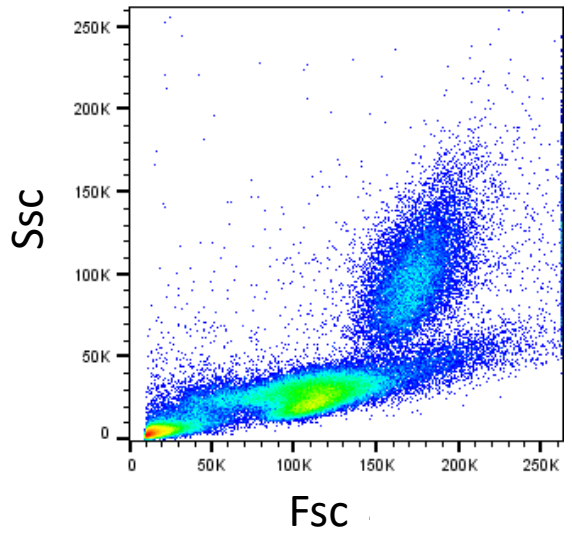
WBC: **1.6 x10^9/l** RBC: 3.97 x10^12/l HGB: 113 g/l HCT: 0.317 l/l MCV: 79.8 fl MCH: 28.5 pg MCHC: 356.5 g/l RDW: 14.7 % PLT: 178 x10^9/l **NEU(Se+T)#: ! 0.469 x10^9/l**

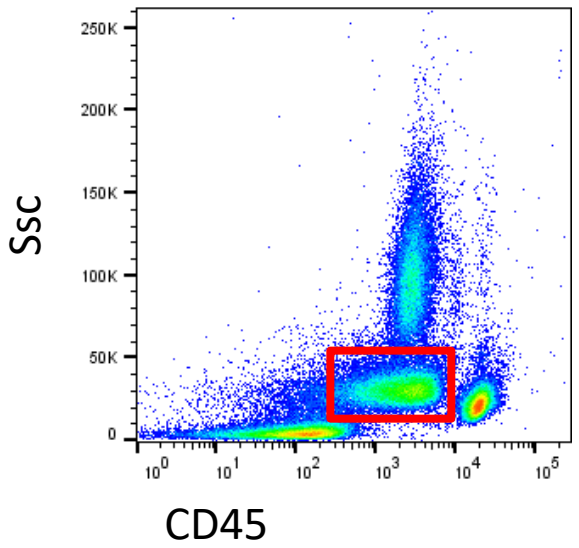
PID panel conclusion:

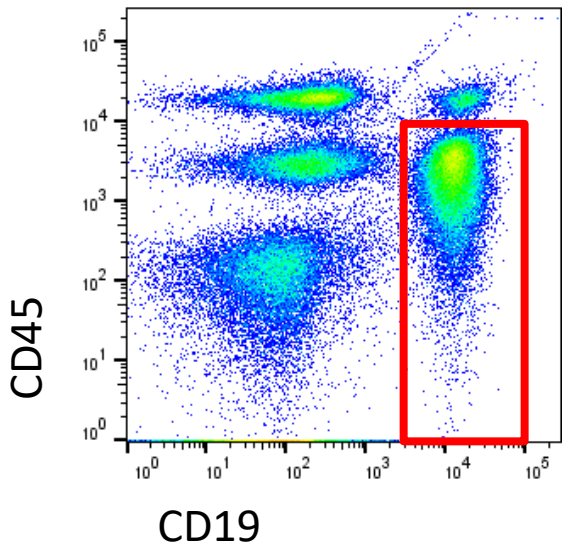
- Lymphopenia, % decreased B cells (5.8%), low transitional B cells (0.3%, probably decreased production in bone marrow), no significant T cell activation

Follow up

- Moderate improvement in blood count parameters within 6 weeks. But still tired, repeatedly subfebrile. During regular check blasts detected on blood smear.



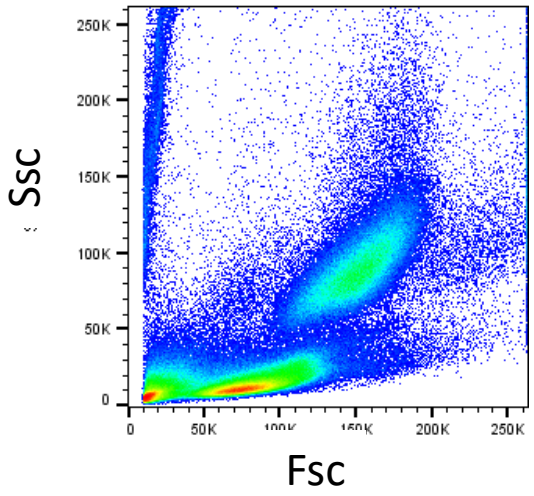


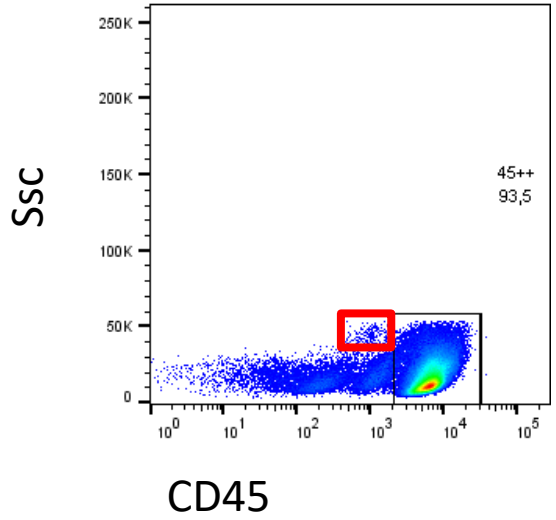


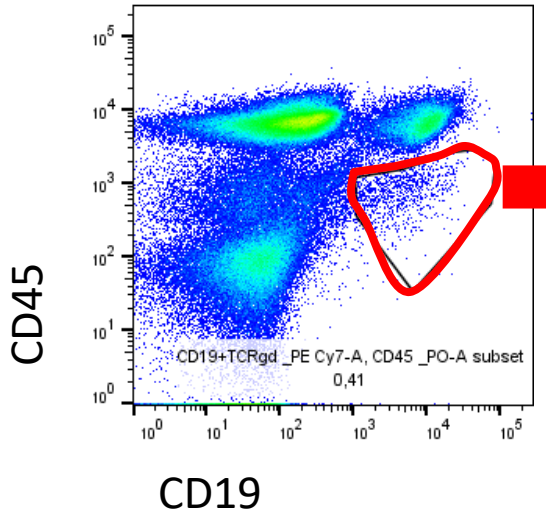
Conclusion BCP ALL

suspect malignant B cells CD19+45dim

Retrospective reanalysis of initial PID panel
Lymphocyte screening tube
CD45RA/CD27/CD4/CD19+TCRgd/CD3/CD16/CD8//HLADR/CD45/CD56







typically we do not focus on this graph during manual analysis <1% out of all cells

Conclusion

- Reference ranges never ending story = we need them not only for accreditation
- Immunology report is a list of values which make complex picture usually about one part of immune system
- Information about patient history is essential but frequently not available
- In routine practise bioinformatic tools would be especially helpful in examination of sample quality, potential role in identification of suspect unexpected population
- In PID algorithm is useful to combine information from the various compartments when the samples are available

CLIP – Childhood Leukemia Investigation Prague

Veronika Kanderova

Michaela Nováková

Barbora Cabalkova

Eva Froňková

Markéta Kubričanová Žaliová

Jan Trka Eva Froňková

Tomáš Kalina Ondřej Hrušák

Alberto Orfao
Jacques van Dongen
Giuseppe Gaipa
Vincent van der Velden
Miriam van der Burg

DPHO:
Lucie Šrámková
Jan Starý
Martina Suková
Petr Smíšek
Markéta Racková

Department of Immunology

Anna Sediva

Marketa Bloomfield

Adam Klocperk