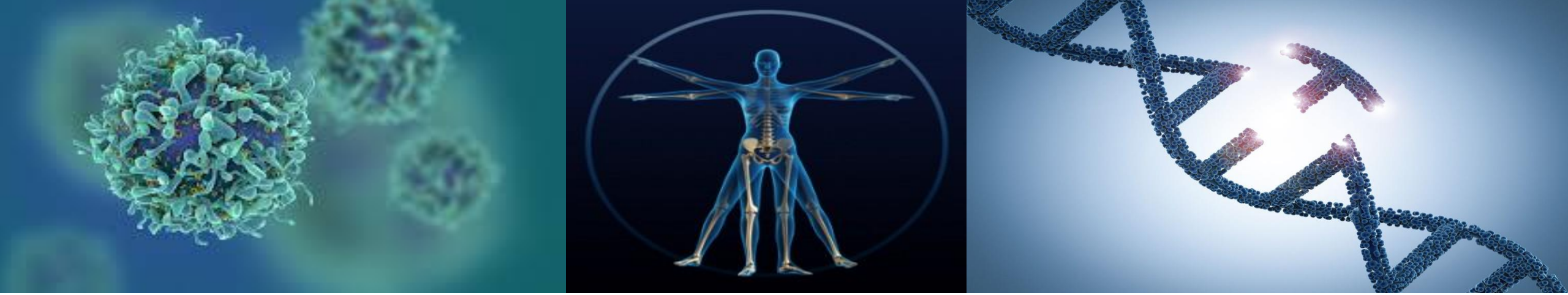

Recommendations for immune monitoring

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the Netherlands

March 12th 2026

Content

I. **Case studies**

II. **Recommendation for B- and T-cell subset analysis**

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Case 1

A 5-year-old boy presents with recurrent infections and frequent nosebleeds. Examination reveals hepatosplenomegaly and bilateral cervical lymphadenopathy. Laboratory tests show severe thrombocytopenia and moderate neutropenia, with the patient exhibiting hematomas

Genetic analysis identified a heterozygous c.848A>G (p.Gln283Arg) variant in the *FAS* gene (VUS classification)

Key points include:

- The variant is located in a region with multiple previously reported pathogenic mutations
- It is absent from a control population of ~140,000 individuals
- The variant has not been reported in patient databases
- In silico predictive analyses suggest it may be disease-causing

=> Suspicion of **Autoimmune Lymphoproliferative Syndrome (ALPS)**

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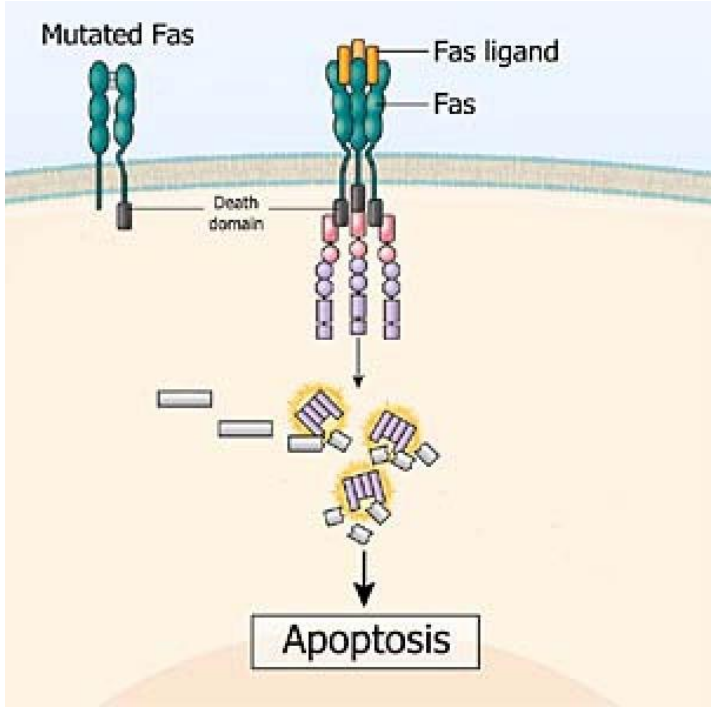
Autoimmune lymphoproliferative syndrome

- A rare immune disorder marked by chronic, benign lymphocyte proliferation and autoimmune cytopenias. It involves impaired apoptosis of activated lymphocytes and is associated with mutations in genes such as *FAS*, *FASL*, and *CASP10*

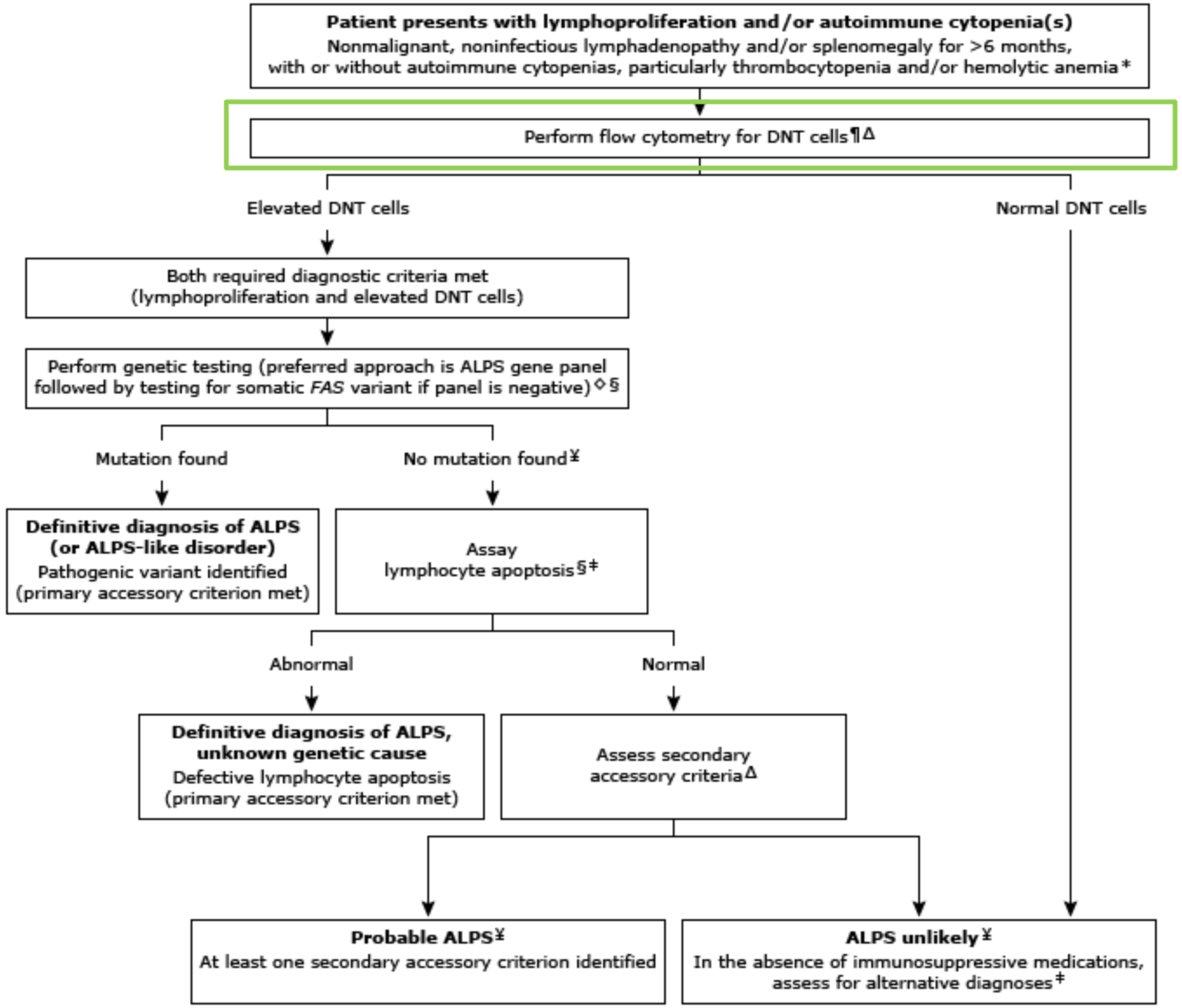
- Impaired apoptosis leads to the accumulation of activated lymphocytes in lymphoid organs and the loss of CD4 and CD8 surface markers.
- The presence of TCRαβ-positive double-negative T cells is considered pathognomonic for ALPS

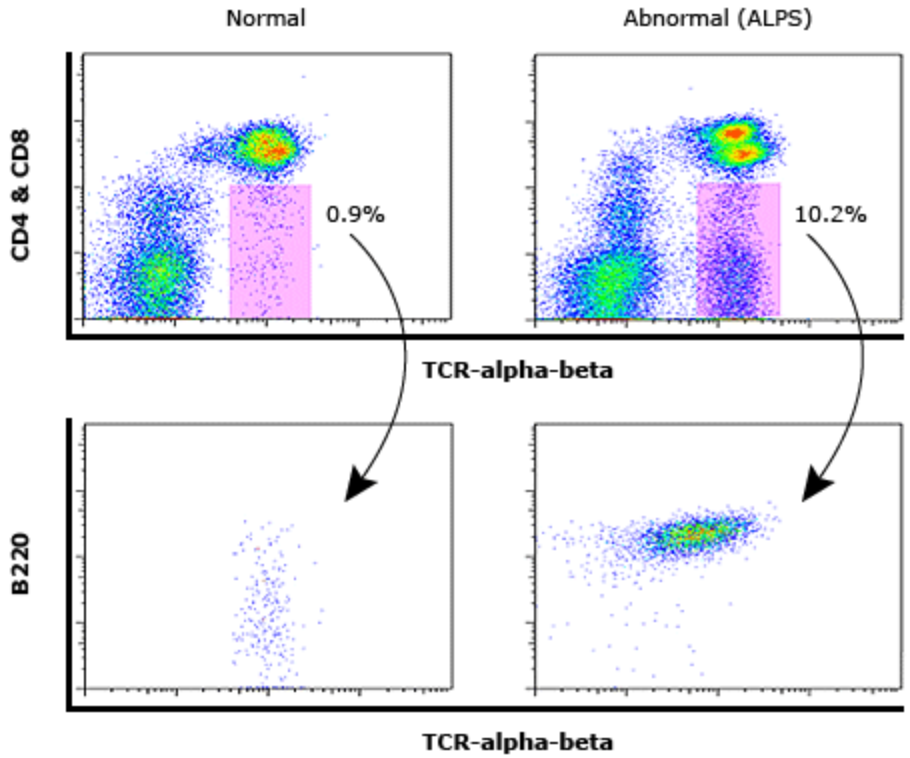


Source: NIAID

Price et al. Blood 2013

Autoimmune lymphoproliferative syndrome diagnostic algorithm





Source: UpToDate

ESID Registry – working definitions for clinical diagnosis of ALPS

Disease and OMIM number for disease entry (examples)	IUIS category	OMIM number for disease-associated genes (examples)	ORPHA number for disease entry (examples)	HPO terms (examples)	Contributors	Clinical criteria for a probable diagnosis (= working definitions for clinical diagnosis classification)	Suggestions for alternative diagnosis (i.e., if the criteria are not completely fulfilled)
						Does not fulfil the criteria for Omenn syndrome	

Autoimmune lymphoproliferative syndrome (ALPS)
 	
601859, 603909, 607271, 616100, 615559, 614470
	4. Diseases of immune dysregulation	134637, 134638, 601762, 601763, 602457, *123890, 176977, 164790*	ORPHA:3261
			ORPHA:436159
			ORPHA:275517
				ALPS	David Edgar, Stephan Ehl, Frederic Rieux-Laucat, Benedicte Neven	**At least one of the following:**
						• splenomegaly
						• lymphadenopathy (>3 nodes, >3 months, non-infectious, non-malignant)
						• autoimmune cytopenia (>/= 2 lineages)
						• history of lymphoma
						• affected family member
						AND at least one of the following:
						• TCRab+CD3+CD4-CD8- of TCRab+CD3+ T cells > 6%
						• elevated biomarkers (at least 2 of the following):
						  • sFASL > 200pg/ml
						  • Vitamin B12 > 1500ng/L
						  • IL-10 > 20pg/ml
						  • Impaired FAS mediated apoptosis
							For patients with lymphoproliferation and/or autoimmunity who do not fulfil these criteria, please consider the following diagnoses:
							• CVID
							• Combined immunodeficiencies
							• Unclassified disorders of immune dysregulation

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Case 1

Significantly increased percentage of TCRαβ-positive double-negative T cells, increased levels of IL-10 and sFASL in serum

Test	Ref. waarde	Materiaal	28-12-2021 10:24 KCL	Eenheid
Bloed				
T-Cellen	0.7-2.1	Bloed	0.64	10^9/L
CD4+T-Cellen	0.3-1.4	Bloed	0.26	10^9/L
CD8+T-Cellen	0.2-0.9	Bloed	0.29	10^9/L
B-Cellen	0.1-0.5	Bloed	0.12	10^9/L
NK-cellen	0.09-0.6	Bloed	0.14	x10^9/L
Dubbel negatieve ab+ T-cellen	<2.5	Bloed	17.3	%

Test	Ref. waarde	Materiaal	28-12-2021 10:24 KCL	Eenheid
Bloed				
IL-10	<10	Bloed	>400	pg/mL

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Case 1

- The patient has been diagnosed with autoimmune lymphoproliferative syndrome

- This is a congenital disorder for which treatment is limited to symptom management. Lifelong follow-up is required, as all individuals carrying *FAS* mutations have a significantly increased risk of developing lymphoma. Family members of children with ALPS may be asymptomatic or have only mild manifestations; for example, the patient's father carries the same mutation but shows no clinical symptoms

- Treatment: start prednisone and sirolimus (rapamycin)

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Case 2

After a complex medical history (starting in 1995, 1 year of age) that included recurrent respiratory infections, lymphadenopathy, and hepatosplenomegaly, a patient was diagnosed with **activated PI3K delta syndrome (APDS)** due to a gain-of-function mutation c.3061G>A p.(Glu1021Lys)

The activated PI3K delta syndrome in this patient is associated with hypogammaglobulinemia, systemic lupus erythematosus (SLE)-like autoimmune disease, and autoimmune hepatitis

The autoimmune hepatitis is complicated by

- liver cirrhosis with portal hypertension leading to fluid accumulation in the abdomen (ascites)
- Scans show multiple suspicious lesions in the liver, one of which is more strongly suspicious for liver cancer

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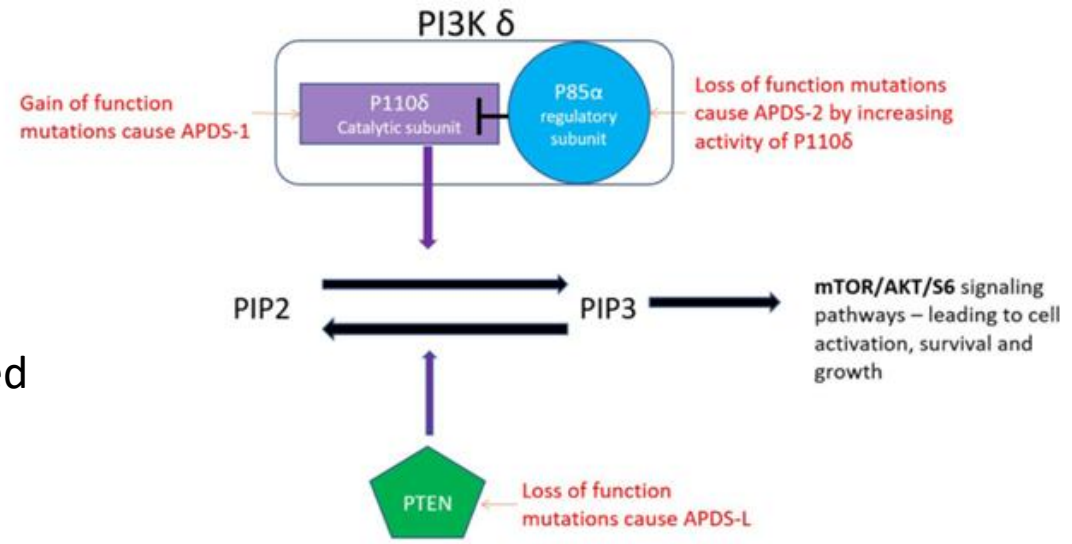
Activated PI3K delta syndrome (APDS)

- Since its identification in 2013, over 300 cases of APDS have been reported worldwide
- Gain-of-function mutations in *PIK3CD* affect the p110δ catalytic subunit of PI3Kδ (APDS1)
- Loss-of-function mutations in *PIK3R1* affect the p85α regulatory subunit (APDS2)
- Patients commonly present with recurrent respiratory infections, lymphadenopathy, and hepatosplenomegaly
- Cytopenias frequently occur such as thrombocytopenia, anemia, and neutropenia
- There is an increased risk of lymphoma and autoimmune manifestations, including thyroid dysfunction, inflammatory joint disease, and chronic gastrointestinal inflammation
- No consensus clinical diagnostic criteria for activated PI3K delta syndrome have been published



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The immunological phenotypes are heterogeneous with both B and T cell abnormalities

PIK3CD gain-of-function mutation

- Impaired class switch recombination (CSR) in B lymphocytes
- IgA and IgG levels show variability, indicating that CSR may be partially or fully disrupted
- Reduced memory B cell counts, often accompanied by elevated IgM levels
- Frequently, transitional B cell numbers are increased
- Decreased naïve T cell populations with a corresponding increase in terminally differentiated T cells, such as effector memory T cells, indicating T-cell senescence

A patient with a heterozygous missense variant (c. 3061 G>A p. Glu 1021 Lys) in the PIK3CD gene (gain-of-function)

Immunologie cellulair				
T-Cellen	0.7-4.2	Bloed	1.69	10^9/L
CD4+T-Cellen	0.3-2.0	Bloed	0.45	10^9/L
CD8+T-Cellen	0.3-1.8	Bloed	1.13	10^9/L
B-Cellen	0.2-1.6	Bloed	0.82	10^9/L
Transitioneel B	11-77	Bloed	715	cellen/µL
naief matuur B	111-486	Bloed	68	cellen/µL
natural effector B	15-88	Bloed	12	cellen/µL
memory B	13-100	Bloed	2	cellen/µL

Case 2

The patient received a liver transplant for cirrhosis secondary to autoimmune hepatitis, complicated by a hepatocellular carcinoma.

Post-transplant, ongoing immunological and hepatic follow-up is critical to optimize outcomes and monitor for potential complications related to both APDS and immunosuppressive therapy

He was treated with immunoglobulins, antibiotics, and immunosuppressive therapy, taking into account his symptoms, along with the immunosuppression needed after liver transplantation.
Approximately 5 years after transplantation, treatment was initiated with leniolisib, a selective PI3Kδ inhibitor

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Targeted therapy for APDS, a selective PI3Kδ inhibitor

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

A randomised, placebo-controlled, phase III trial of leniolisib in activated phosphoinositide 3-kinase delta (PI3Kδ) syndrome (APDS): Adolescent and adult subgroup analysis

V. Koneti Rao [a,*], Anna Šedivá [b], Virgil A.S.H. Dalm [c], Alessandro Plebani [d], Catharina Schuetz [e], Anna Shcherbina [f], Antonino Trizzino [g], Yulia Zharankova [h], Alanvin Orpia [a], Elaine Kulm [i], Sharon Webster [a], Julia Körholz [e], Vassilios Lougaris [d], Yulia Rodina [f], Niall Conlon [j], Tanya Coulter [k], Jason Bradt [l], Anurag Relan [l], Gulbu Uzel [a]

Leniolisib inhibits the active site of the PI3Kδ enzyme, reducing its overactivity and helping to normalize the development and function of white blood cells, including B and T lymphocytes

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Longitudinal follow-up evaluation

Test	Ref. interval	Materiaal	18-12-2024	22-1-2025	5-3-2025	23-4-2025	4-6-2025	3-9-2025	Eenheid
Bloed									
IgG2	1.5 - 6.4	Bloed							g/L
T-Cellen	0.7-2.1	Bloed	0.09	0.28	0.18	0.40	0.44	0.51	10^9/L
CD4+T-Cellen	0.3-1.4	Bloed	0.05	0.12	0.10	0.23	0.21	0.27	10^9/L
CD8+T-Cellen	0.2-0.9	Bloed	0.04	0.15	0.08	0.14	0.2	0.23	10^9/L
B-Cellen	0.1-0.5	Bloed	0.01	0.02	0.01	0.01	0.01	0.01	10^9/L
NK-cellen	0.09-0.6	Bloed	0.04	0.15	0.07	0.06	0.10	0.13	x10^9/L
Transitioneel B	3-50	Bloed	0.00	5.00	1.00	2.00	1.00	1.00	cellen/µL
naief matuur B	57-447	Bloed	0.00	4.00	2.00	2.00	3.00	2.00	cellen/µL
natural effector B	9-88	Bloed	0.00	1.00	<1	<0.1	<1	<1	cellen/µL
memory B	13-122	Bloed	0.00	2.00	<1	1.00	1.00	<1	cellen/µL
naief CD4	74-1173	Bloed	4	4	6	11	12	20	cellen/uL
cm CD4	117-886	Bloed	45	109	98	231	230	285	cellen/uL
em CD4	14-500	Bloed	3	9	7	14	14	22	cellen/uL
naief CD8	29-737	Bloed	11	17	19	33	45	57	cellen/uL
cm CD8	54-453	Bloed	13	79	32	68	92	71	cellen/uL
em CD8	2-515	Bloed	11	45	18	25	33	37	cellen/uL

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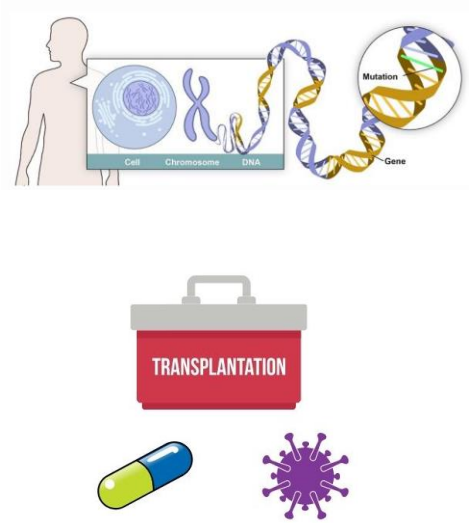
Analysis of B- and T-cell subsets

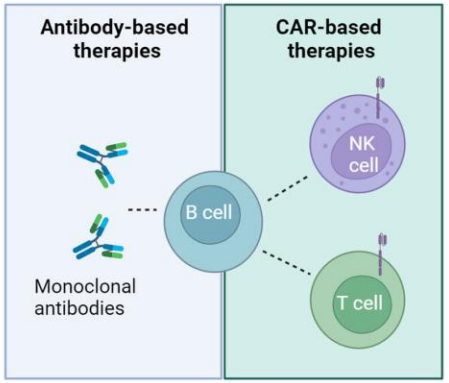
Indications

- Performed when immune deficiency is suspected
- Applied for immune monitoring – longitudinal analysis of a patient's immune system
- Used to monitor immune reconstitution following stem cell transplantation

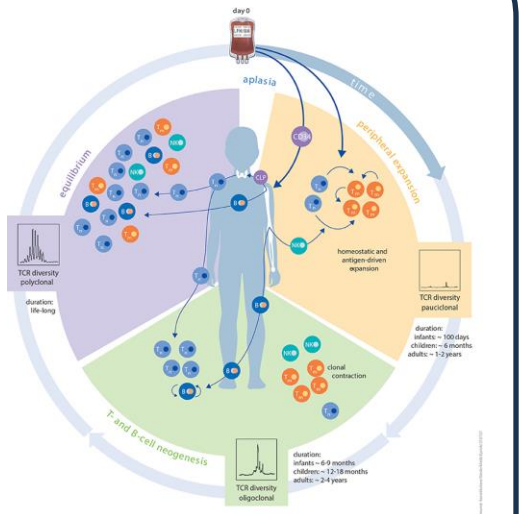
Immune deficiency



Immune monitoring



Immune reconstitution



Yanir et al. 2021

Lymphocyte subsets included in PID/IEI criteria

Lymphocyte population	Markers in PID/IEI criteria	Disease
Ig-switched memory B cells, $CD4^+$ T-cells (count/µL), % naive $CD4^+$ T-cells	Not specified	CVID
$CD19^+$ ($CD20^+$) B cells, $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cells	CD19, CD20, CD3, CD4, CD8	Agammaglobulinemia
Double-negative T cells ($TCR\alpha\beta^+$ $CD3^+$ $CD4^-$ $CD8^-$)	TCRαβ, CD3, CD4, CD8	ALPS
$CD4^+$ T-cells (count/µL), % naive $CD4^+$ T-cells	Not specified	HIGM, HIES
FOXP3 expression in $CD4^+CD25^+$ T-cells	CD4, CD25, FOXP3	IPEX
$CD3^+$, $CD4^+$, $CD8^+$, naive T-cells, $CD56^+/16^+$ NK cells, $CD19^+$ B-cells	CD3, CD4, CD8, CD56/CD16, CD19, CD45RA/CD45RO	SCID newborn screening (abnormal TREC result)

**CVID: common variable immunodeficiency; ALPS: autoimmune lymphoproliferative syndrome; HIGM: hyper-IgM syndrome; HIES: hyper-IgE syndrome; IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked; TREC: T-cell receptor excision circles; SCID: severe combined immunodeficiency*

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External Quality Assessment for B- and T-cell subset analysis

National External Quality Assessment:

- An interlaboratory comparison is conducted for B- and T-cell subset analysis in the Netherlands
- Participating laboratories use different markers and panels, which makes it challenging to compare results across laboratories

Goal:
harmonized approaches across laboratories

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Harmonization of B- and T-cell subset analysis ensures uniform interpretation of results

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Dutch Society for Cytometry (NVC) workgroup immune monitoring TBNK subsets 2021 - present

Objectives and key topics of the workgroup:

- Standard panel for routine B- and T-cell subset analyses
- Extended panel for detailed subset characterization
- Harmonized terminology for subset identification
- Recommended monoclonal antibodies for B- and T-cell subsets
- Gating strategy - define how cell populations are identified using flow cytometry
- Interpretation of possible findings
- Normal ranges for lymphocyte subsets

Questions related to hematological malignancies are beyond the scope of the workgroup.

Other panels are used for this purpose in the Netherlands

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Standard B-cell panel

Marker	Clone
CD19*	J3-119, SJ25C1, HIB19
CD20*	B9E9 (HRC20), 2H7
CD24	ALB9, ML5
CD27	M-T271, 1A4CD27, O323, L128
CD38	HB7, LS198-4-3, HIT2
CD45	J33, HI30, 2D1
IgD	IA6-2
IgM	MHM-88, SA-DA4, G20-127

Identifying B-cells

- B-cells can be selected using CD19 *or* CD20
- CD20 absent on plasmablasts
- CD19 is essential especially during anti-CD20 therapies

Plasmablasts, transitional, naive mature and memory B cells

- CD38 and CD24 can be used to discriminate transitional B-cells and plasmablasts
- CD27 is essential to separate naive mature from memory B-cells
- IgD/IgM differentiate non-switched from switched memory B-cells

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Standard T-cell panel

Identifying T-cells

- T-cell markers: TCRαβ/γδ, CD3, CD4, CD8
- All T cells: $CD3^+$ / Helper T cells: $CD4^+$ / Cytotoxic T cells: $CD8^+$
- Double-negative (CD4-CD8-) T cells within $TCR\alpha\beta^+$ population
- Naive T cells can be identified using CD45RA/RO, CD27/CD28

* TCRαβ or TCRγδ, CD45RA or CD45RO, CD27 or CD28

Marker	Clone
CD3	SK7, UCHT1
CD4	OKT4, 13B8.2, SFCI12T4D11, SK3
CD8	B9.11, SK1
CD27*	M-T271, 1A4LDG5.3.1, O323, L128
CD28*	CD28.2
CD45	J33, HI30, 2D1
CD45RA*	2H4LDH11LD89, HI100, L48
CD45RO*	UCHL1
TCRαβ*	IP26A, WT31, BMA031
TCRγδ*	11F2, immuno 510, 11F2

Extendend B- and T-cell panel

B-cell panel	T-cell panel
CD3	CD25
CD5	CD127
CD10	CD31
CD21	CD38
IgA	CD197
IgE	HLA-DR
IgG	Intracellular Foxp3

B-cells

- Use CD3, CD5, CD10 for accurate B cell identification
- Isotype-switched memory B cells: membrane-bound IgE, IgG, IgA
- CD21: identifies $CD38^{-}/CD21low$ B cells, elevated in infection, CVID, or autoimmune disease (≤10% in healthy controls)

T-cells

- Naive: $CD45RA^{+}/CD197^{+}$ Central memory: $CD45RA^{-}/CD197^{+}$ Effector memory: $CD45RA^{-}/CD197^{-}$ Terminally differentiated: $CD45RA^{+}/CD197^{-}$
- $CD4^{+}$ recent thymic emigrants: $CD45RA^{+}/CD31^{+}$
- Activated T cells: $HLA\text{-}DR^{+}$ and/or $CD38^{+}$
- Regulatory T cells: $CD25hi/CD127^{-}$
- For IPEX diagnosis: intracellular FOXP3 staining required

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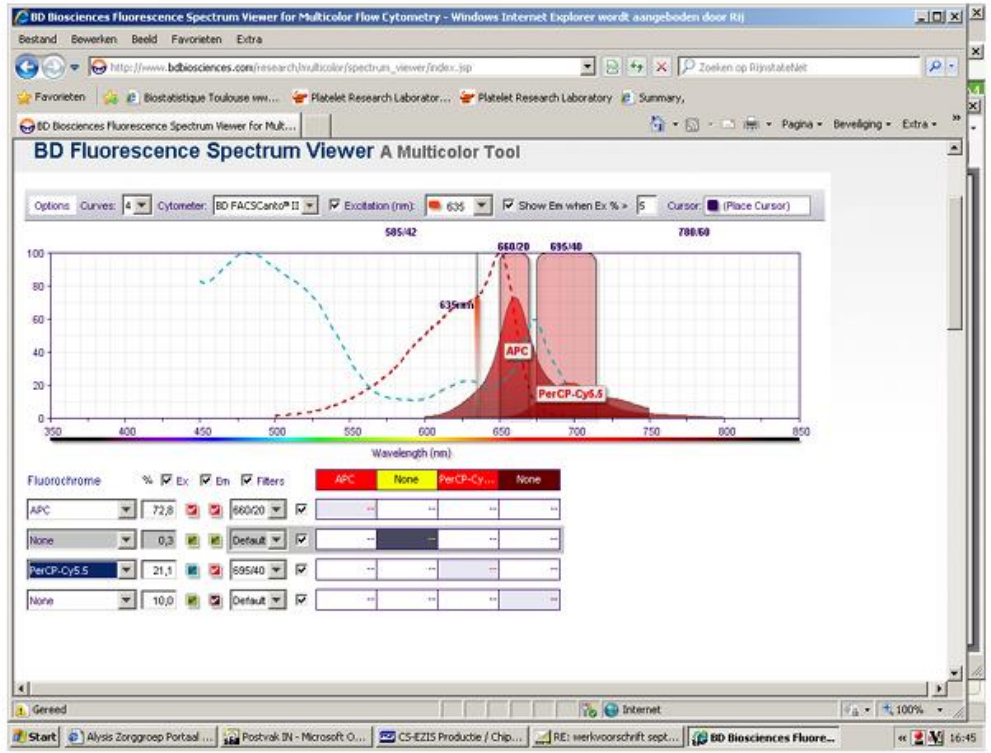
Selection of fluorochromes for flow cytometric immunophenotyping

https://cytometrie.nl/aanbevelingen/

Keuze van fluorochromen voor flowcytometrische immuunfenotypering, een handreiking

Relative Brightness	Reagent	Filter
BRIGHTEST	Brilliant Violet™ 421	450/50
	PE	575/26
	Brilliant Violet 605	610/20
	BD Horizon PE-CF594	610/20
	PE-Cy5	670/14
	APC	660/20
BRIGHT	PE-Cy7	780/60
	Alexa Fluor® 647	660/20
	PerCP-Cy5.5	695/40
MODERATE	Alexa Fluor® 488	530/30
	FITC	530/30
	BD Horizon V450	450/50
	Pacific Blue™	450/50
DIM	Alexa Fluor® 700	730/45
	PerCP	695/40
	APC-Cy7	780/60
	AmCyan	525/20
	BD Horizon V500	525/20
	BD APC-H7	780/60



Figuur 1 Voorbeeld van spectrale overlap tussen PECy5.5 en APC

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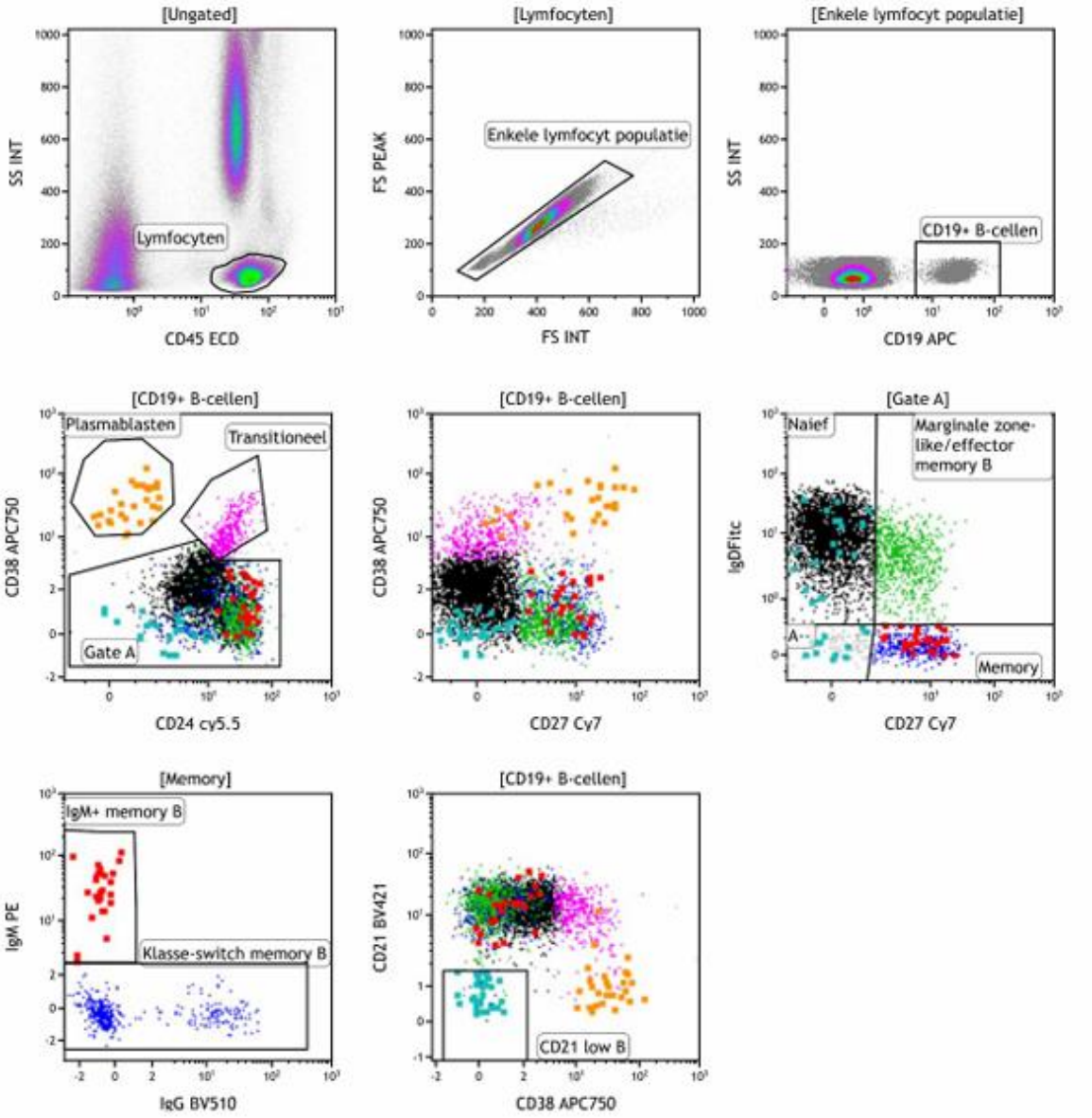
Definitions of B- and T-cell subsets

- To report absolute numbers of the various B and T cell subsets, it is necessary to perform a quantitative determination for total lymphocytes and/or B and T cells.
- Examples of CE-marked available tests: TBNK BD Multitest with BD TruCount tubes, TBNK with Aquios Tetra-1/Tetra-2 kit.

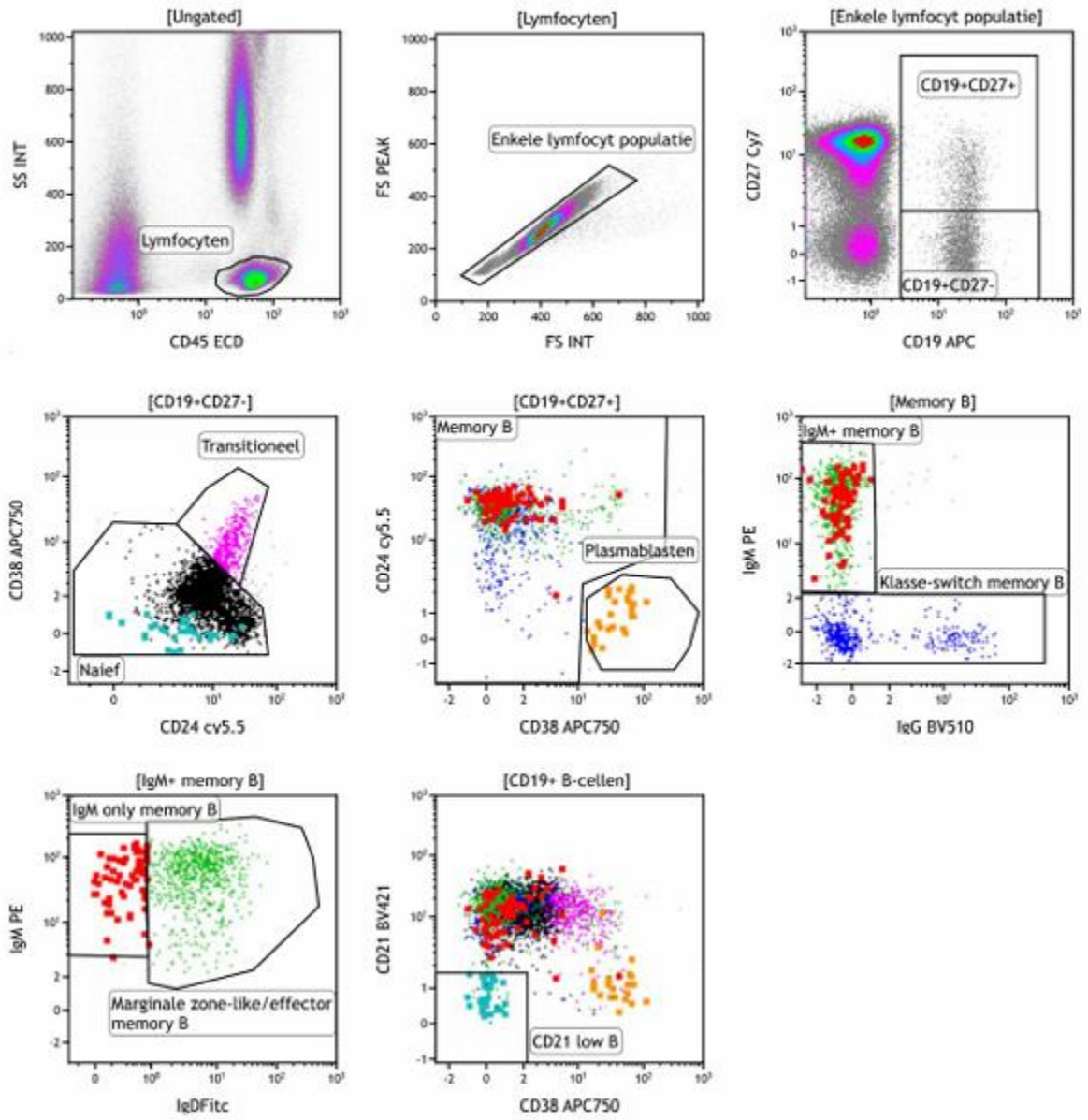
Subset	Markers
Within CD3+ T-cells	
CD4+CD8− within CD3+ TCRαβ+ T-cells	CD45, CD3, CD4, CD8, TCRαβ or TCRγδ
TCRγδ+ T-cells	CD45, CD3, CD4, CD8, TCRαβ or TCRγδ
TCRαβ+ T-cells	CD45, CD3, CD4, CD8, TCRαβ or TCRγδ
Within CD4+ T-cells	
Naive T-cells	CD45RO− or CD45RA+, with CD27+ or CD28+
Within CD8+ T-cells	
Naive T-cells	CD45RO− or CD45RA+, with CD27+ or CD28+
Within CD19+ (or CD20+) B-cells	CD27− IgD+ IgMhi CD24hi CD38+ CD24hi CD38+
Transitional B-cells	CD27− IgD+ IgM+ CD24int CD38int CD27− IgD+
Naive mature B-cells	CD27+ IgD+ IgMhi CD38int
Non-switched memory B-cells*	CD27+ IgD− IgM− CD38int
Isotype switched memory B-cells	CD27− IgD− IgM− CD38lo
Double negative B-cells	CD27hi IgM− IgD− CD24− CD38hi
Plasmablasts	CD27hi IgM− IgD− CD24− CD38hi

* Marginal zone or natural effector B cells

B-cell gating strategy #1



B-cell gating strategy #2



Interpretation – sample cases (1 of 2)

B-cell related findings

- Reduced memory B-cells – defect in differentiation (common in CVID)
- Low switched memory B-cells – class-switch recombination defect
- Low naive mature + memory B-cells – impaired B-cell proliferation/survival
- Low transitional B-cells – impairement in B-cell development in bone marrow

Additional B-cell patterns

- High transitional + low switched memory – APDS or regeneration
- Increased CD21low B-cells – infection, CVID, autoimmunity

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Interpretation – sample cases (2 of 2)

Key T-cell abnormalities

- CD45RO+CD45RA+ memory T cells – may reflect CD45 polymorphism present in ~1% of the population
- Increased double-negative TCRαβ T-cells – ALPS
- Low FOXP3 expression in CD4+CD25+ T cells – IPEX (immune dysregulation, polyendocrinopathy, X-linked)
- High terminally differentiated CD8 T-cells – latent CMV infection, otherwise possible immunodeficiency

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Reference values

- Various reference values are used, based on literature or in-house reference values

  Saule et al. Mechanisms of Ageing and Development 2006;127: 274281

  van der Burg et al. Frontiers Immunol. 2019;Mar 4:10:246

  Driessen et al. Haematologica. 2013 Oct;98(10):1617-23

  van Gent et al. Clin. Immunol. 2009 Oct;133(1):95-107

  Comans-Bitter et al. J. Pediatr. 1997 Mar;130(3):388-93

  aan de Kerk et al. J. Immunol. 2013 May 15;190(10):5012-9

  Oras et al. Clin. Exp. Immunol. 2020;202: 363-378.

- A key feature of the reference values is that they vary with age

- It is recommended that reference values for lymphocyte subset measurements, established according to the recommendation, be re-evaluated in a multi-center study

- This approach will enable a better understanding of the variability in test results and help assess the feasibility of establishing universal reference values

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Recommendation

- Standard panel for routine B- and T-cell subsets analyses
- Extended panel for detailed subset characterization
- Harmonized terminology for subset identification
- Recommended monoclonal antibodies for B- and T-cell subsets
- Gating strategy - define how cell populations are identified using flow cytometry
- Interpretation of possible findings
- Normal ranges for lymphocyte subsets

https://cytometrie.nl/aanbevelingen-werkgroep-tbnk/

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NTVH RICHTLIJNEN 64

Harmonisatie van B- en T-cel-subset-analyses in Nederland

Harmonization B- and T-cell subset analysis in the Netherlands

dr. J.J.B.C. van Beers[1,2,5*], dr. H.J. Bontkes[1,6*], dr. J.G.M.C. Damoiseaux[1,5], dr. W. Hobo[1,7], dr. T. Hutten[2,8], dr. C.A. Koelman[1,9], dr. H.J.P.M. Koenen[3,7], dr. A.J.A. Lambeck[1,10], dr. J. Leuvenink[2,11], dr. E.G. van Lochem[1,12], dr. M.M. van Ostaijen-ten Dam[4,13], dr. S. Veenbergen[1,14],
namens de Nederlandse Vereniging voor Cytometrie (NVC) Werkgroep Immuunmonitoring TBNK-subsets

(NED TIJDSCHR HEMATOL 2026;23:64-73)

Additional publication in Dutch Journal for Allergy, Asthma and Clinical Immunology

Erasmus MC

Thank you!

NVC workgroup immune monitoring TBNK subsets

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Carin Koelman (Meander MC)

Hans Koenen (Radboudumc)

Annechien Lambeck (UMC Groningen)

Jacqueline Leuvenink (Jeroen Bosch Ziekenhuis)

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