Slovenian Society for Flow Cytometry (SSC)

Institute of Microbiology and Immunology, Faculty of Medicine, Ljubljana

FLOW CYTOMETRY IN RESEARCH AND DIAGNOSTICS OF PRIMARY IMMUNODEFICIENCY DISORDERS

INTERNATIONAL SCIENTIFIC SYMPOSIUM

October 14, 2016

Mednarodni znanstveni simpozij | International scientific symposium
Uporaba pretočne citometrije v diagnostiki primarnih imunskih pomanjkljivosti in raziskovalnem delu | Flow cytometry in research and diagnostics of primary immunodeficiency disorders

Prizorišče | Venue
Onkološkem inštitutu v Ljubljani, stavba C
Zaloška 2,
1000 Ljubljana

Datum |Dates
14th October, 2016

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Slovensko združenje za pretočno citometrijo
Institute of Microbiology and Immunology, Faculty of Medicine, Ljubljana

Organizacijski odbor simpozija | Members of the organization committee
Andreja Nataša Kopitar (University of Ljubljana, Faculty of Medicine)
Alojz Ihan (University of Ljubljana, Faculty of Medicine)

Znanstveno recenzentski odbor|Board of Scientific Reviewers:
Assoc. Prof. Andreja Nataša Kopitar, Ph.D.
Prof. Alojz Ihan, M.D., PhD

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Glavni urednik | Editor-in-chief
Andreja Nataša Kopitar

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1. Kopitar, Andreja Nataša
287681536

PROGRAMME

Chair: Andreja N. Kopitar

11:00–11-10 Welcome: Andreja N. Kopitar

11:10–11:35 Jirji Sikora: Microvesicles in flow cytometry

11:35–12:00 Špela Konjar: CD8+ T Cells and their metabolic plasticity

12:00–12:25 Mojca Benčina: Single-cell analysis using ratiometric flow cytometry

12:25–12:50 Katarina Černe: Kinetics of ovarian cancer tumor markers sOPN and sCD44-v6

12:50–13:20 Coffee break, snacks

Chair: Alojz Ihan

13:20–13:45 Tadej Avčin: Treatment of patients with T cell deficits

13:45–14:15 Tomas Kalina: The role of flow cytometry in diagnostics of immune deficiencies

14:15–14:40 Andreja N. Kopitar: Analysis of T and B cell subsets in healthy subjects – implications for CVID monitoring.

14:40–15:05 Alojz Ihan: Functional tests for the diagnosis of immune deficiency

15:05–15:35 Coffee break

Chair: Gašper Markelj

15:35–16:00 Štefan Blazina: Treatment of patients with B cell deficits syndrome

16:00–16-25 Gašper Markelj: Treatment of patients with chronic granulomatous disease CGD

16:25–16:50 Nataša Toplak: Diagnosis of periodic fever syndrome

16:50–17:15 Marija Holcar: Systemic lupus erythematosus and antiphospholipid syndrome

MICROVESICLES IN FLOW CYTOMETRY

Jiri Sinkora

Application Specialist, BD Life Sciences for Central Europe, Czech Republic

Extracellular vesicles are generated during and play role in many physiological processes like development, angiogenesis, wound healing, tissue remodelling and transfer of information but they have also been documented to contribute to pathologic conditions, e.g. excessive coagulation and thrombosis, inflammation and vascular dysfunctions. In general, they are smaller than 1 µm and are thus called microparticles (MP). MP in circulation originate from different cell types, most frequently studied are hematopoietic lineages-derived MP as well as their counterparts secreted by endothelia. The quantification and qualification (cellular origin) of MP may be a predictor or marker of a disease; microparticles have been studied in individuals suffering from hypertension, acute coronary syndromes including myocardial infarction, type I and II diabetes, severe trauma and sepsis, cancer and diabetes. Due to their size, MP are difficult to observe and a lot of effort has been put to optimize flow cytometers for detecting as small objects as possible in the attempt to rapidly quantify and characterize objects comparable to platelets (e.g. apoptotic bodies) or smaller – microvesicles in the range of 100 nm to 1 µm or exosomes, the objects that are smaller than 100 µm and are comparable to viruses in size. However, physical limitations, namely the wavelength of the excitation light has made it difficult and, at smaller sizes practically impossible, to detect MP by light scattering. Low angle light scatter (forward scatter, FSC) and right angle light scatter (side scatter, SSC) on the majority of commercially available flow cytometers allow for detecting objects with dimensions of approximately 0.5 and 0.2 µm, respectively. Special or modified cytometers reportedly can detect light scatter from objects with a diameter of 0.1 µm or even smaller. However, even very small objects with relatively bright fluorescence can be easily distinguished from noise, namely on modern devices with low noise electronics. We and others have thus been relying on staining microvesicles using lipophilic non-fluorescent esters of dyes that become fluorescent after cleavage by intracellular/intravesicular esterases. In combination with Annexin V staining and surface/intravesicle immunophenotyping this provides an interesting tool for MP detection and characterization without limitations caused by scattering properties. Different BD instruments will be compared in terms of their capability to see small particles and technical tips and tricks will be discussed to achieve sensitive and reliable data in the submicron region of blood born particles.

Microvesicles in Flow Cytometry

Jiri Sinkora, PhD.

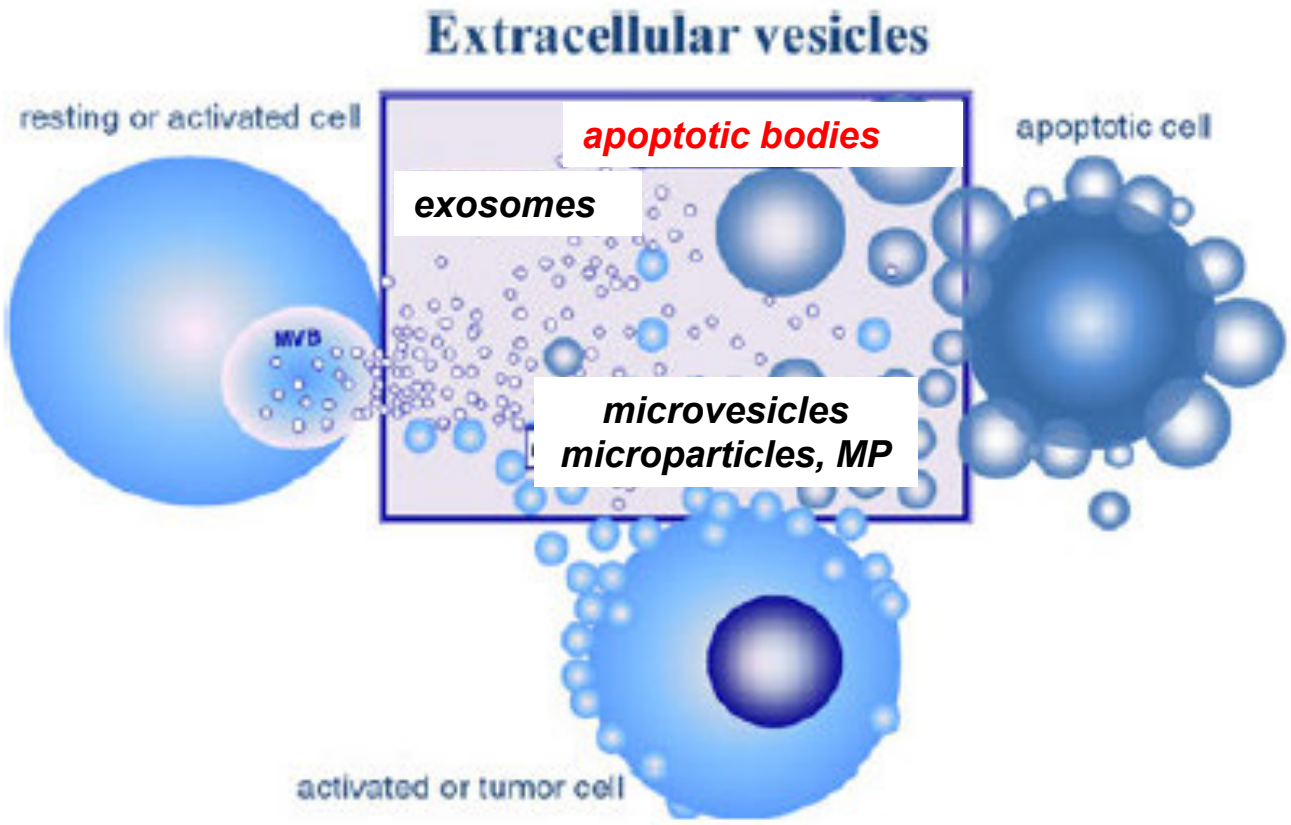
Application Specialist BD Life Sciences

Czech Republic

Slovenian society for Flow Cytometry, Ljubljana 14. 10. 2016

Extracellular vesicles: origins

B. György et al.

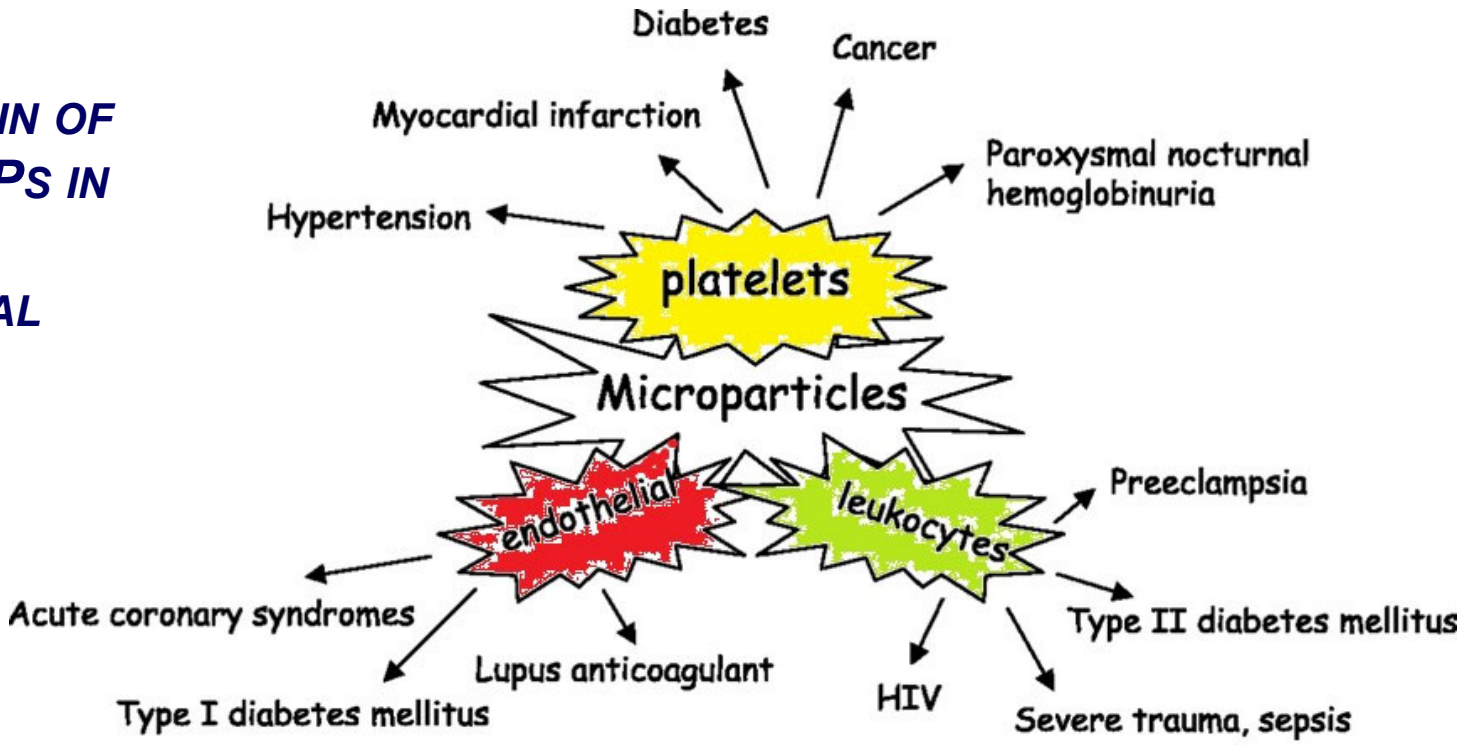


Permeamble for live-dead discriminators
PI, 7-AAD

Intact lipid bilayer

Microparticles: pathological processes involvement

CELLULAR ORIGIN OF CIRCULATING MPS IN DIFFERENT PATHOLOGICAL SETTINGS.



Adapted from Martínez M C et al. Am J Physiol Heart Circ Physiol 2005

Detection of MP cellular origin may be a predictor or marker of the disease

Methods to study MPs

MP activity:

- *Pro-thrombotic activity*
- *Pro-coagulant activity*

MP Numbers and Phenotype:

- *Flow cytometry, **FCM***

Arterioscler Thromb Vasc Biol 2012

High-Sensitivity Flow Cytometry Provides Access to Standardized Measurement of Small-Size Microparticles—Brief Report

Stéphane Robert, Romaric Lacroix, Philippe Poncelet, Karim Harhouri, Tarik Bouriche, Coralie Judicone, Jennifer Wischhusen, Laurent Arnaud, Françoise Dignat-George

Journal of Thrombosis and Haemostasis, 8: 2571–2574 DOI: 10.1111/j.1538-7836.2010.04047.x

OFFICIAL COMMUNICATION OF THE SSC

Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop

R. LACROIX,* S. ROBERT,* P. PONCELET,† R. S. KASTHURI,‡ N. S. KEY‡ and F. DIGNAT-GEORGE* ON BEHALF OF THE ISTH SSC WORKSHOP

*UMR-S 608 INSERM-Aix-Marseille Université, UFR de Pharmacie and Laboratoire d'Hématologie, CHU Conception, Marseille; †Biocytex, Marseille, France; and ‡Department of Medicine, University of North Carolina, Chapel Hill, NC, USA

Sample preparation

Sample manipulation (centrifugation steps, freezing, storage, temperature….) influences results

Platelets, May 2009; 20(3): 225–226 informa healthcare

LETTER TO THE EDITOR

Centrifugation is a crucial step impacting microparticle measurement

FRANÇOISE DIGNAT-GEORGE[1], JEAN-MARIE FREYSSINET[2], & NIGEL S. KEY[3]

[1]*Unité Mixte de Recherche S 608 (UMR-S 608), Institut National de la Santé et de la Recherche Médicale (INSERM), Université de la Méditerranée, Unité de Formation et de Recherche (UFR) de Pharmacie, Marseille, France,* [2]*Université Louis Pasteur, Faculté de Médecine, Institut d'Hématologie et d'Immunologie, Strasbourg, F-67085 France, and* [3]*Department of Medicine, University of North Carolina, Chapel Hill, NC, USA*

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Regular Article

Measurement of circulating cell-derived microparticles by flow cytometry: Sources of variability within the assay

Lisa Ayers[a,*], Malcolm Kohler[b], Paul Harrison[c], Ian Sargent[d], Rebecca Dragovic[d], Marianne Schaap[e], Rienk Nieuwland[e], Susan A. Brooks[f], Berne Ferry[a]

[a] Department of Clinical Immunology, Churchill Hospital, Oxford, UK
[b] Sleep Disorders Centre and Pulmonary Division, University Hospital of Zurich, Switzerland
[c] Oxford Haemophilia and Thrombosis Centre, Churchill Hospital, Oxford, UK
[d] Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford, UK
[e] Department of Clinical Chemistry, Academic Medical Centre, University of Amsterdam, The Netherlands
[f] School of Life Sciences, Oxford Brookes University, Oxford, UK

FCM Sample preparation: Microparticle isolation and staining

Human WB from Healthy subject (Sodium Citrate anticoagulant, BD Vacutainer™) → **Whole Blood (WB)**

Buffy Coat → **Platelet Rich Plasma (PRP)**

***1st Centrifugation** : 1,500 x g 15 minutes R.T.* → **Platelet Poor Plasma (PPP)**

***2nd Centrifugation** : 13,000xg 3 minutes R.T.→ PFP * R.T.* → **Platelet Free Plasma (PFP)**

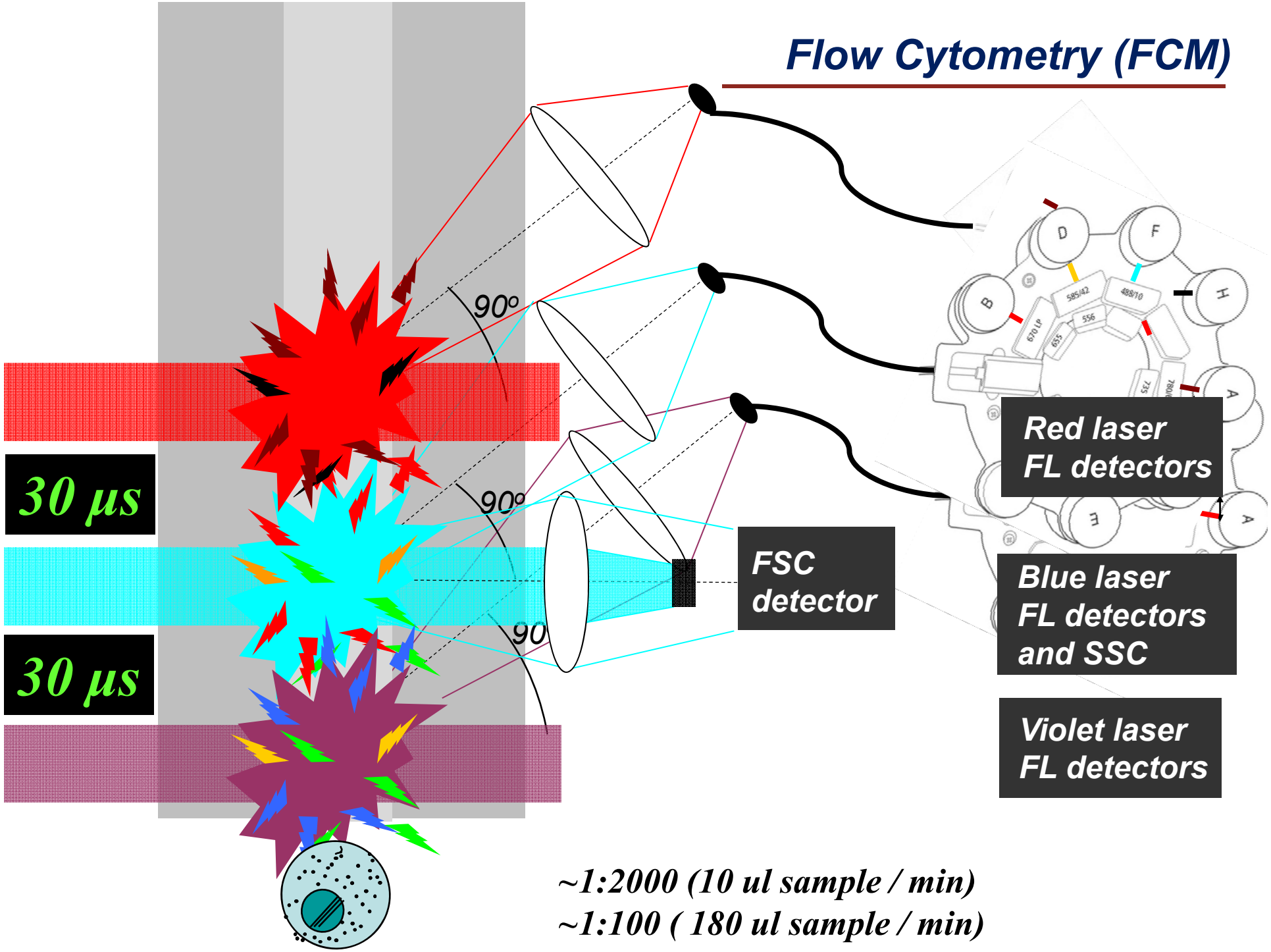
***Staining** with fluorinated Annexin V and monoclonal Abs at 4°C, 0.2 µm filtered sterile buffer containing $CaCl_2$ at the final concentration of 5mM. **Incubation** 1 hour, in the dark.*

No wash!

FCM Acquisition and Analysis

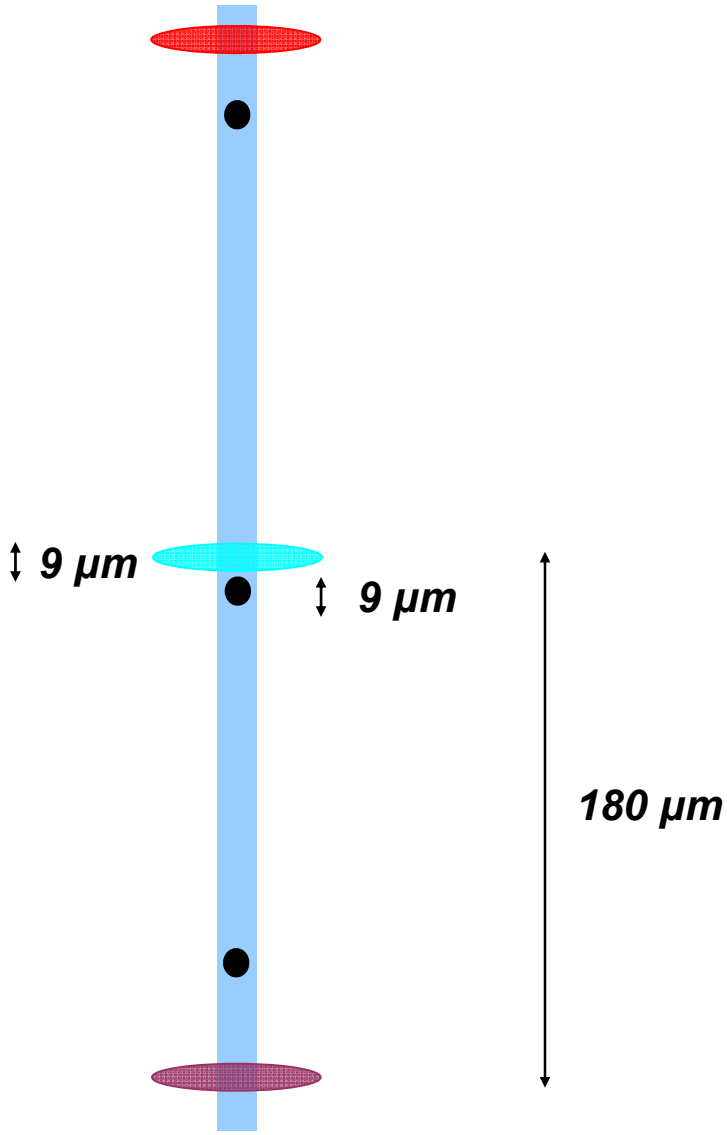
** Adapted from: S. Robert et al., High-Sensitivity Flow Cytometry provides access to standardized measurement of small-size microparticles-Brief report. Arterioscler Thromb Vasc Biol 2012*

Flow Cytometry (FCM)

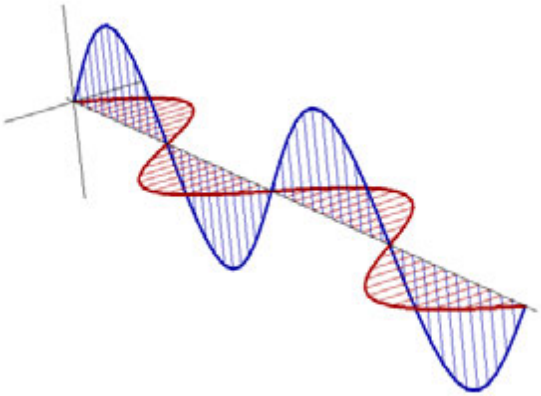


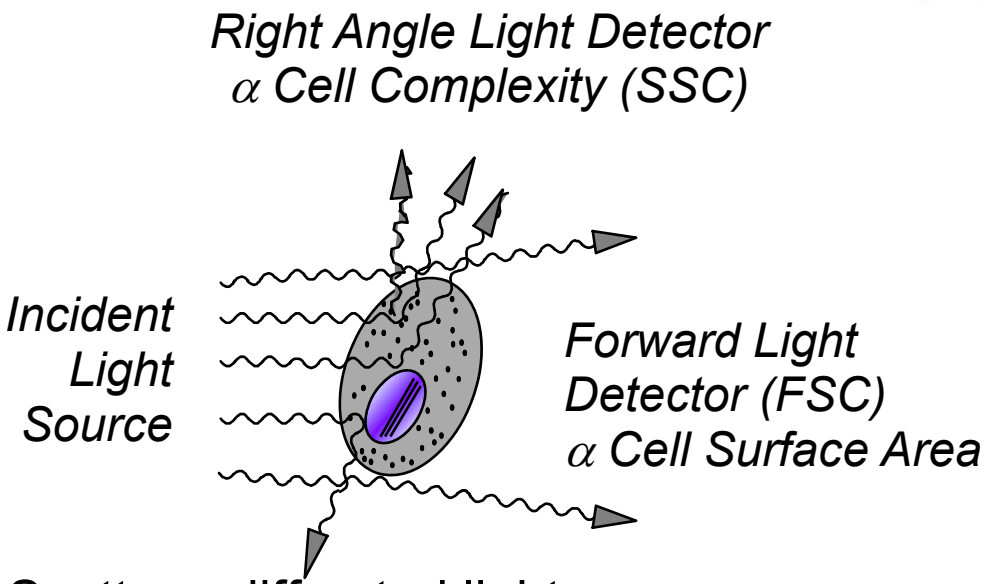
~1:2000 (10 ul sample / min)
~1:100 (180 ul sample / min)

Proportions in Flow Cytometry



Light Scattering





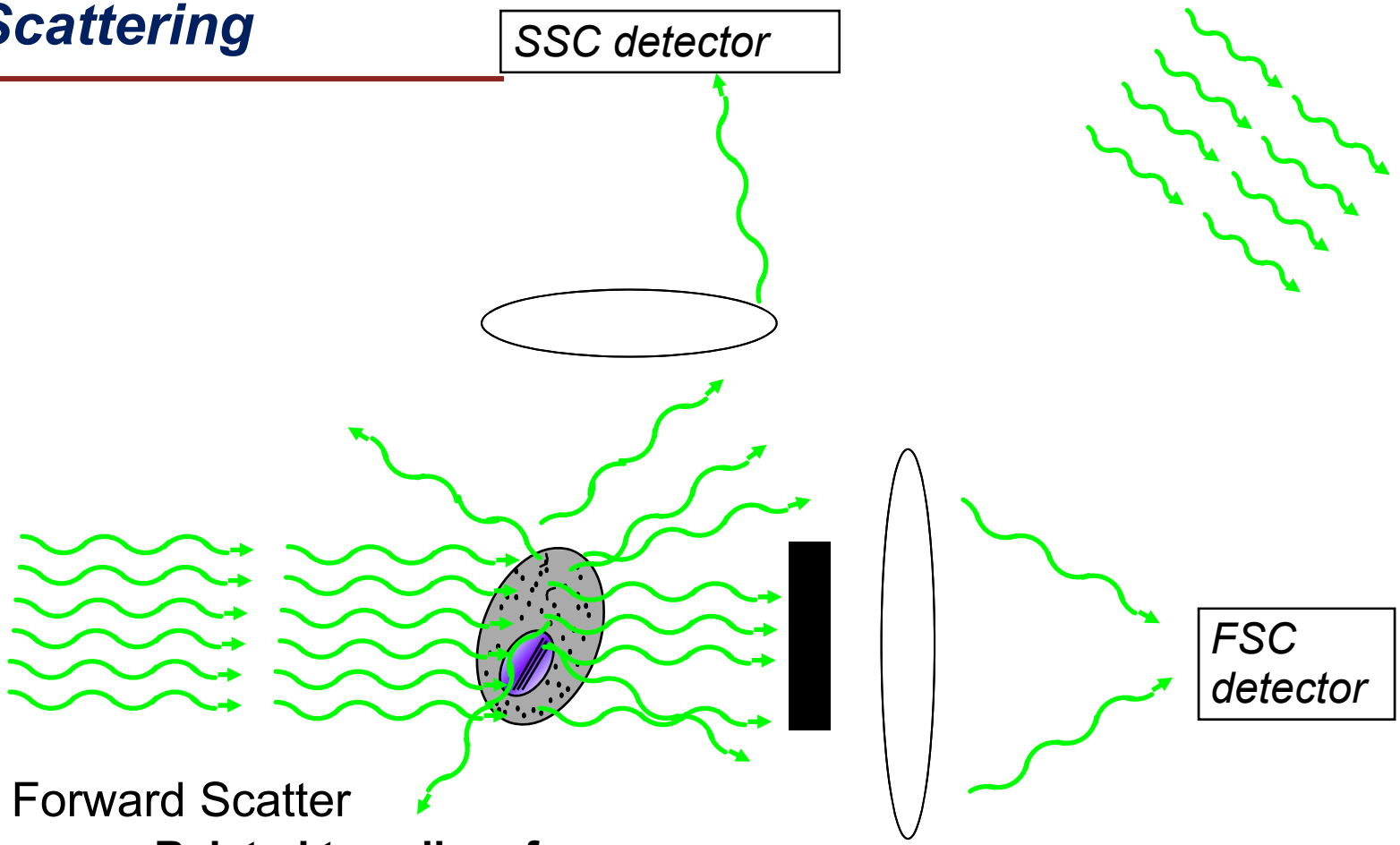
Forward Scatter—diffracted light

- **Related to cell surface area**
- **Detected along axis of incident light in the forward direction**

Side Scatter—reflected and refracted light

- **Related to cell granularity and complexity**
- **Detected at 90° to the laser beam**

Light Scattering



Forward Scatter

- **Related to cell surface area**
- **Detected close to axis of incident light in the forward direction**

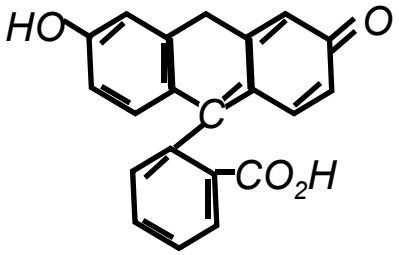
Side Scatter

- **Related to cell complexity**
- **Detected close to 90° to the laser beam**

Fluorescence

λ = 488 nm

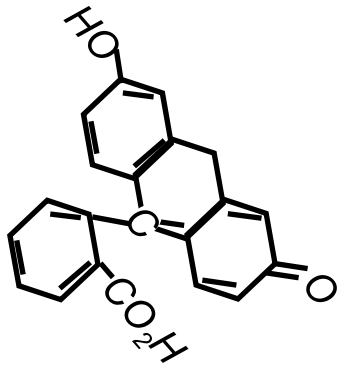
Incident Photon



Fluorescein Molecule

The fluorochrome absorbs a photon (energy) from the laser.

Fluorescence

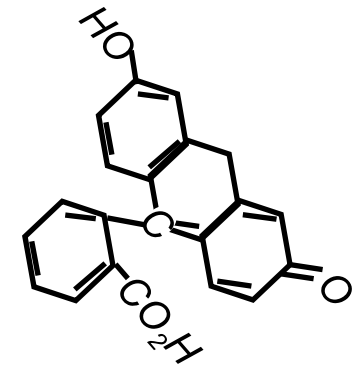


Fluorescein Molecule

Emitted Photon

The fluorochrome rotates and loses a part of excitation energy due to collisions with its microenvironment (solvent) accompanied by decreased vibration a rotation intensity.

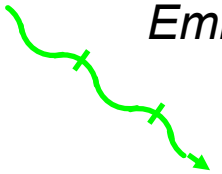
Fluorescence



Fluorescein Molecule

$\lambda \cong$ 520 nm

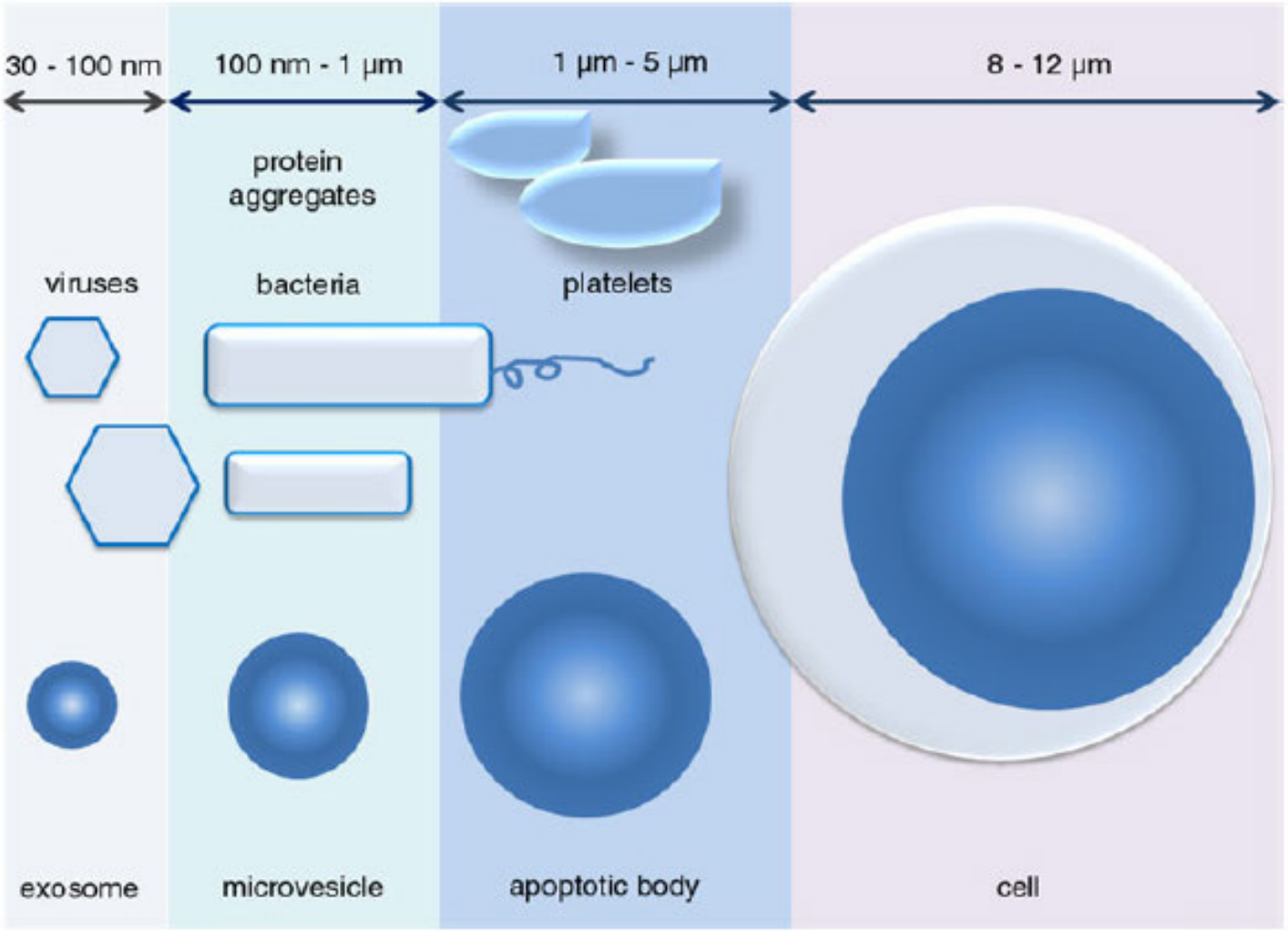
Emitted Photon



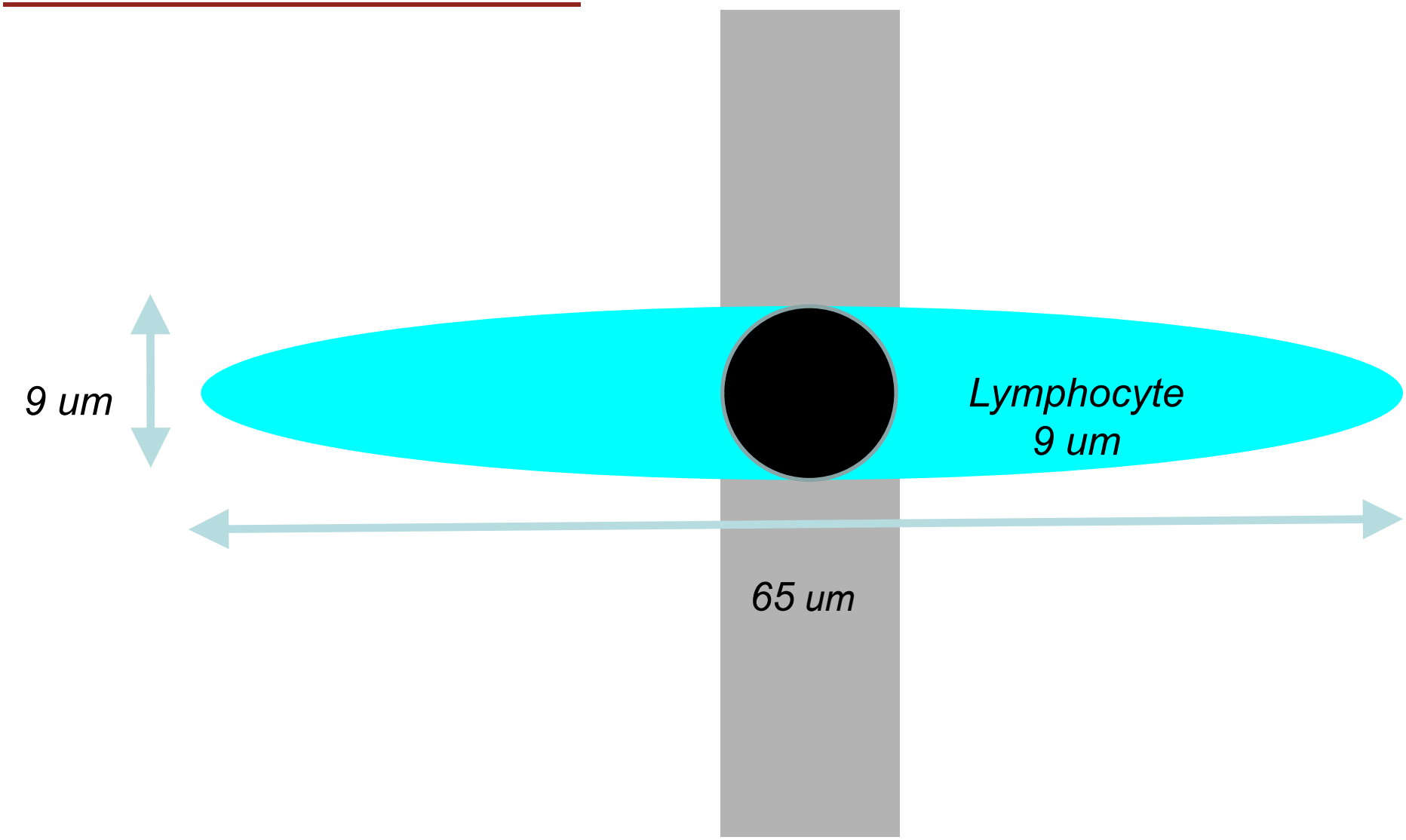
The fluorochrome emits a photon with lower energy (different color).

Extracellular vesicles: size

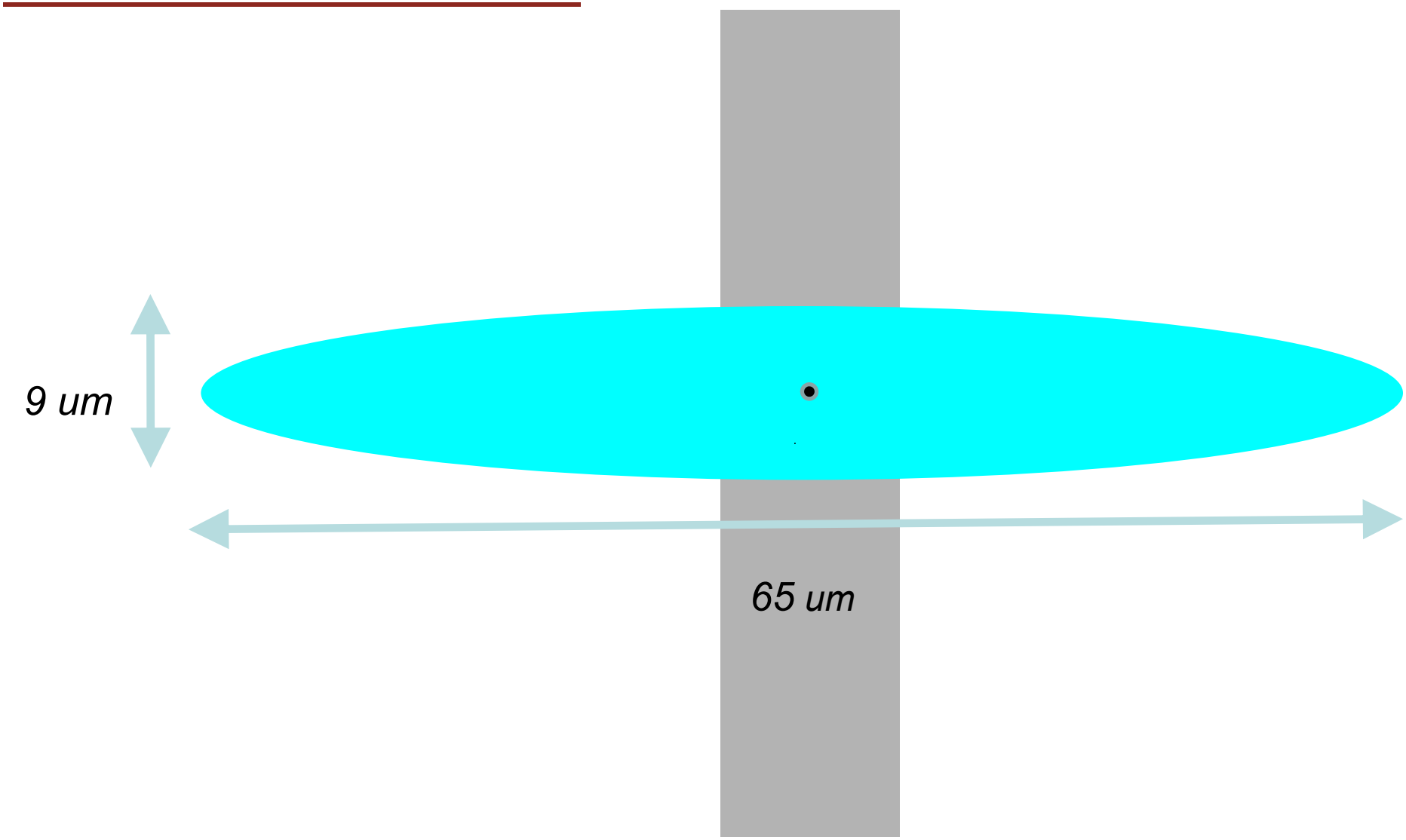
B. György et al.



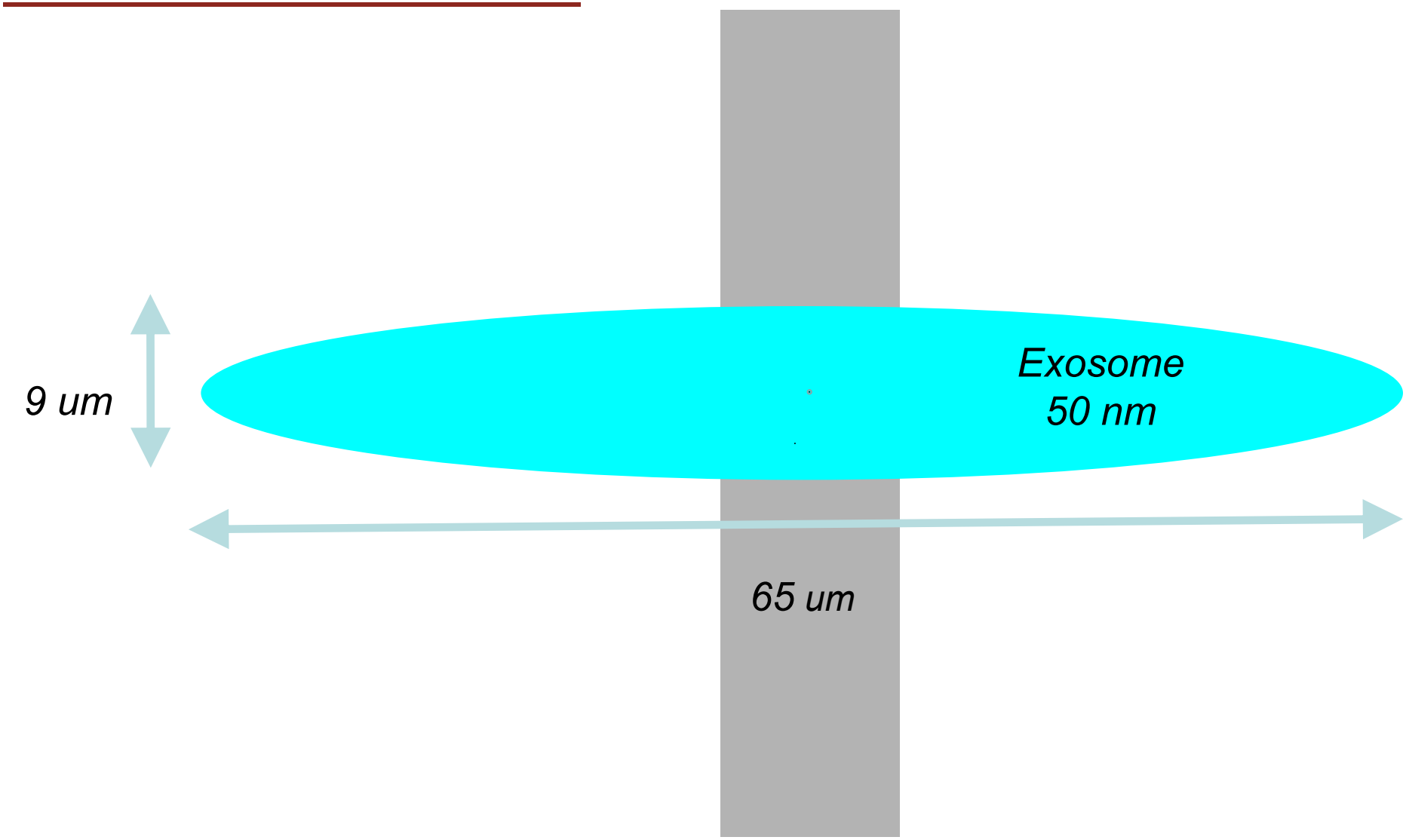
Proportions in Flow Cytometry



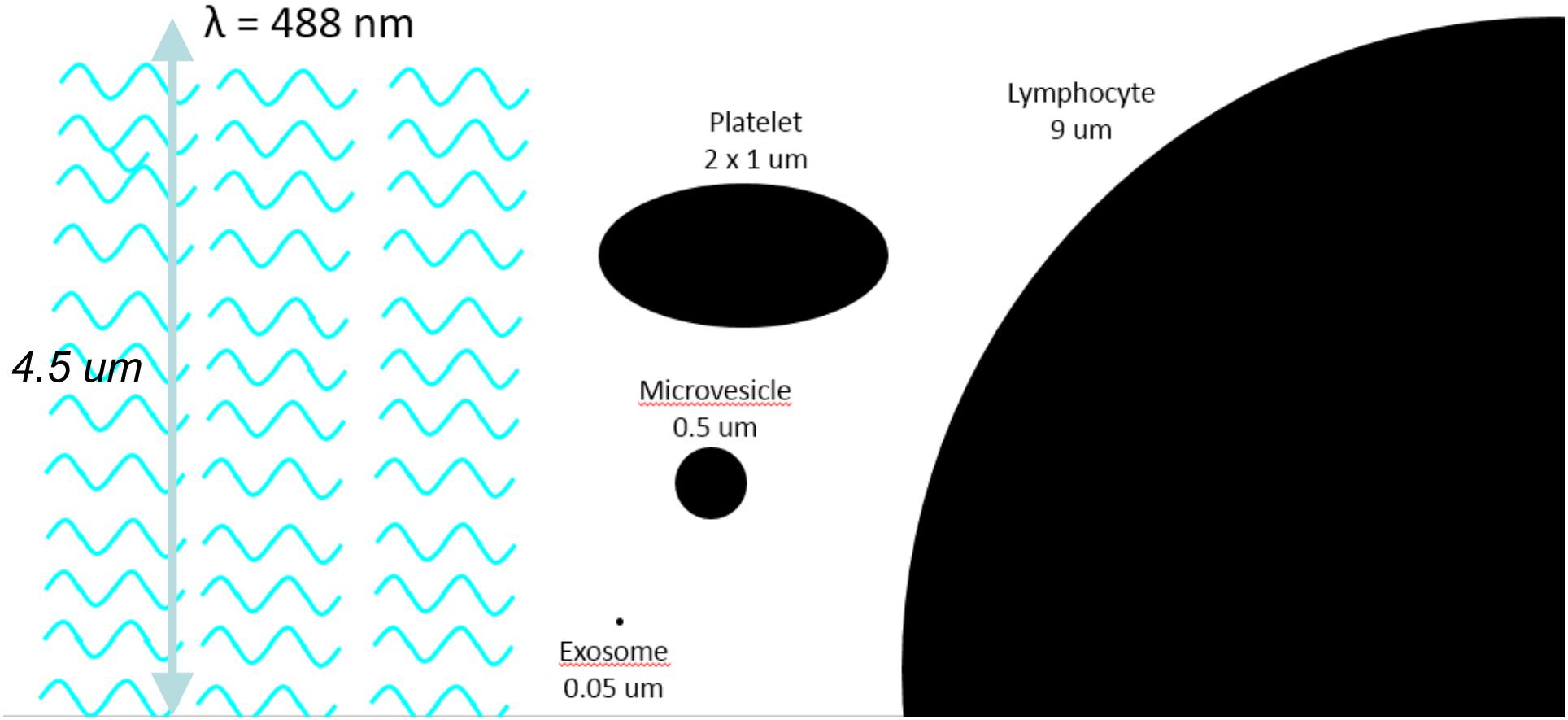
Proportions in Flow Cytometry



Proportions in Flow Cytometry



Proportions in Flow Cytometry

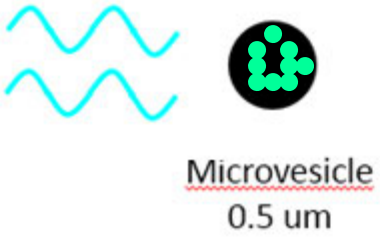


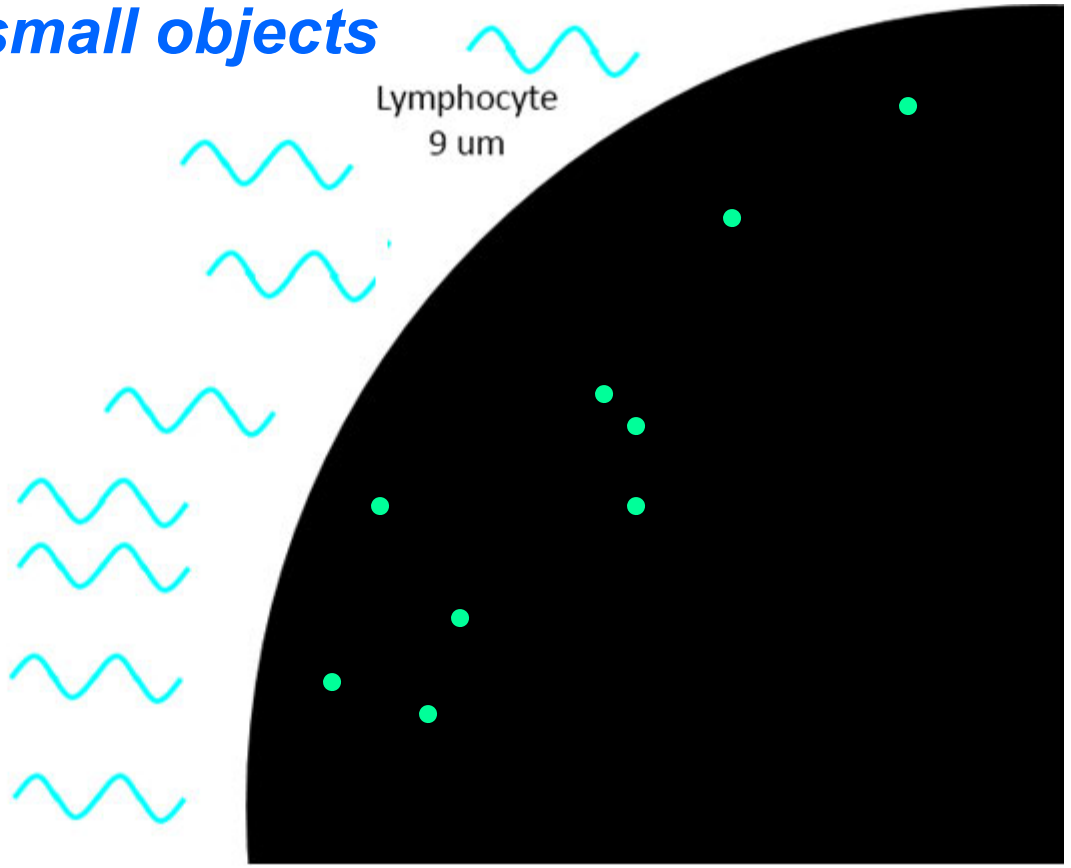
Proportions in Flow Cytometry

Fluorescence signal can be much stronger than scattering in small objects

Want to see them?

- **MAKE THEM BRIGHT**
- **USE THEIR BRIGHTNESS**

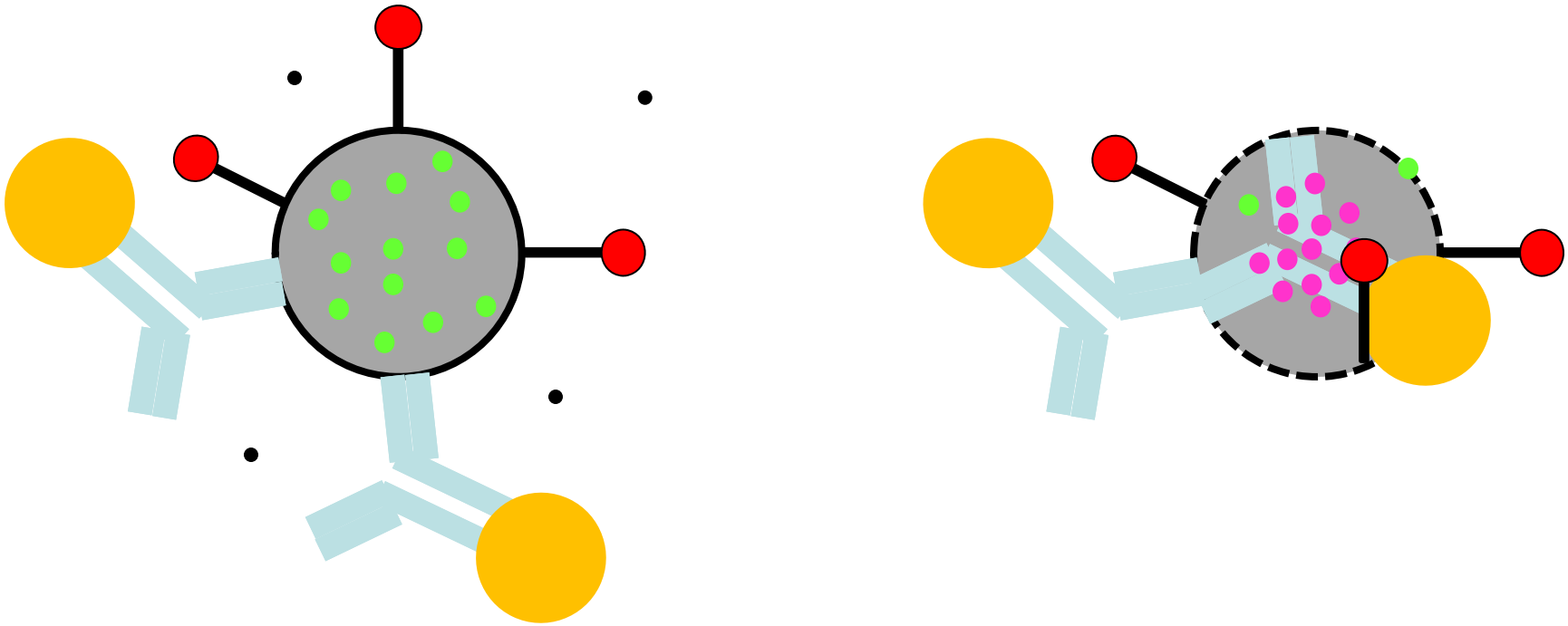




Staining microvesicles

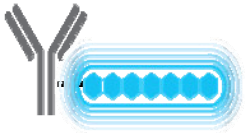
Surface staining: fluorinated Annexin V (pan-marker)
+ conjugated anti-CD antibodies (microvesicle origin, multicolor???)

Intravesicle staining: highly cell permeable lipophilic esters of dyes
that become fluorescent upon cleavage by esterases
covalently bind to amins and accumulate

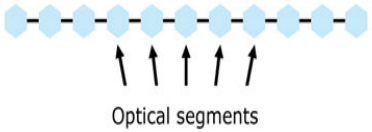


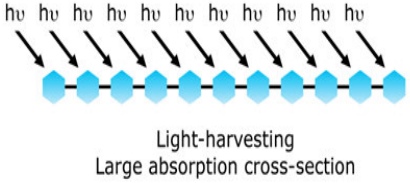
Staining microvesicles

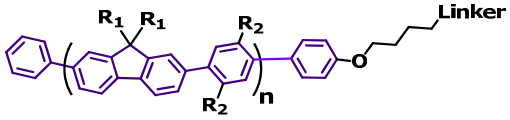
Annexin V and mAbs conjugates: Brilliant polymers



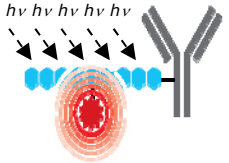
Direct reporters



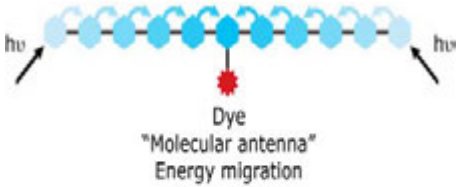


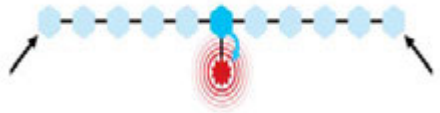


π-conjugated polymers



Tandem reporters





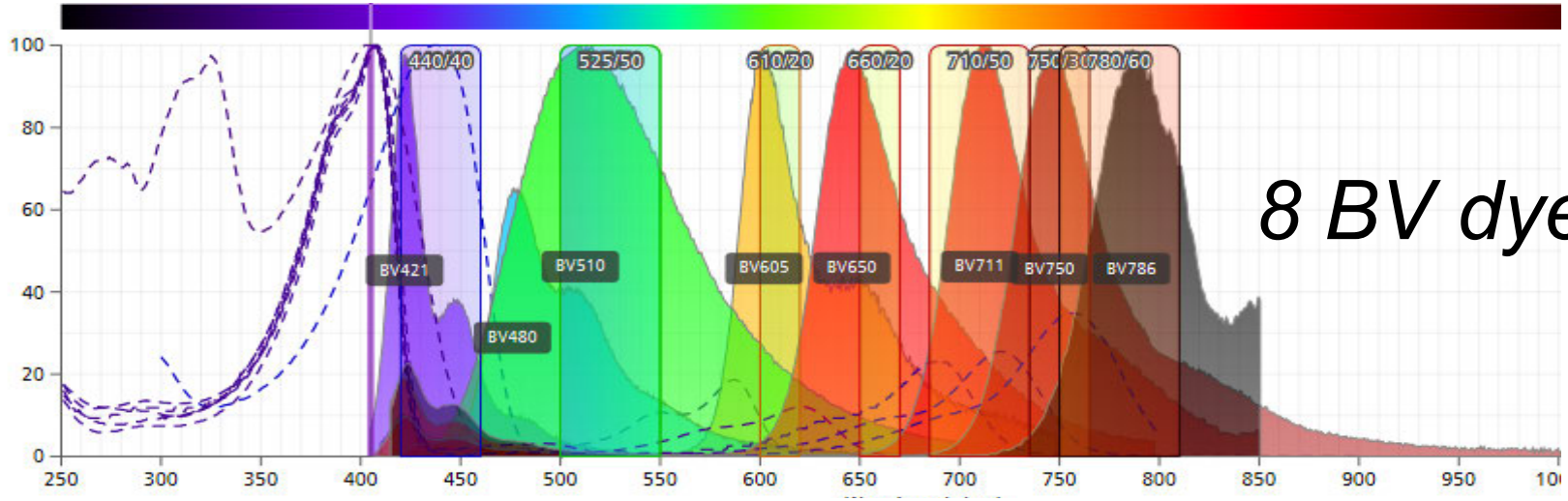
Intense dye emission

- Bright fluorescent materials
- Large collective optical response

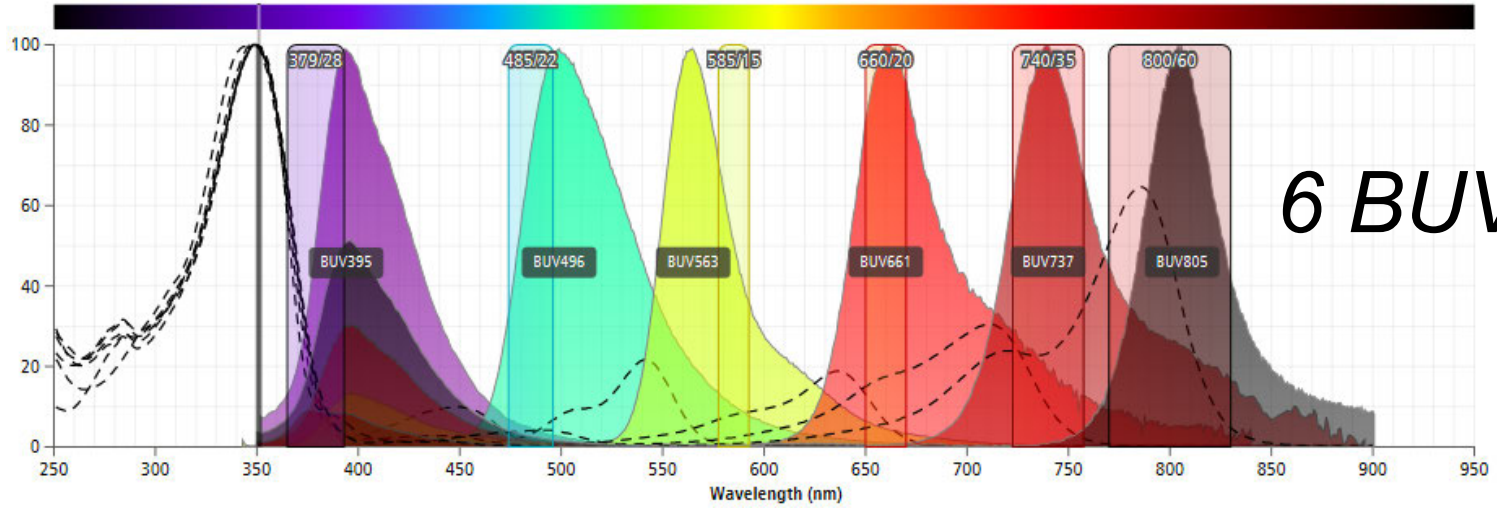
- Efficient energy donors
- Amplified dye emission
- Reproducible synthetic framework

Staining microvesicles

Annexin V and mAbs conjugates: Brilliant polymers



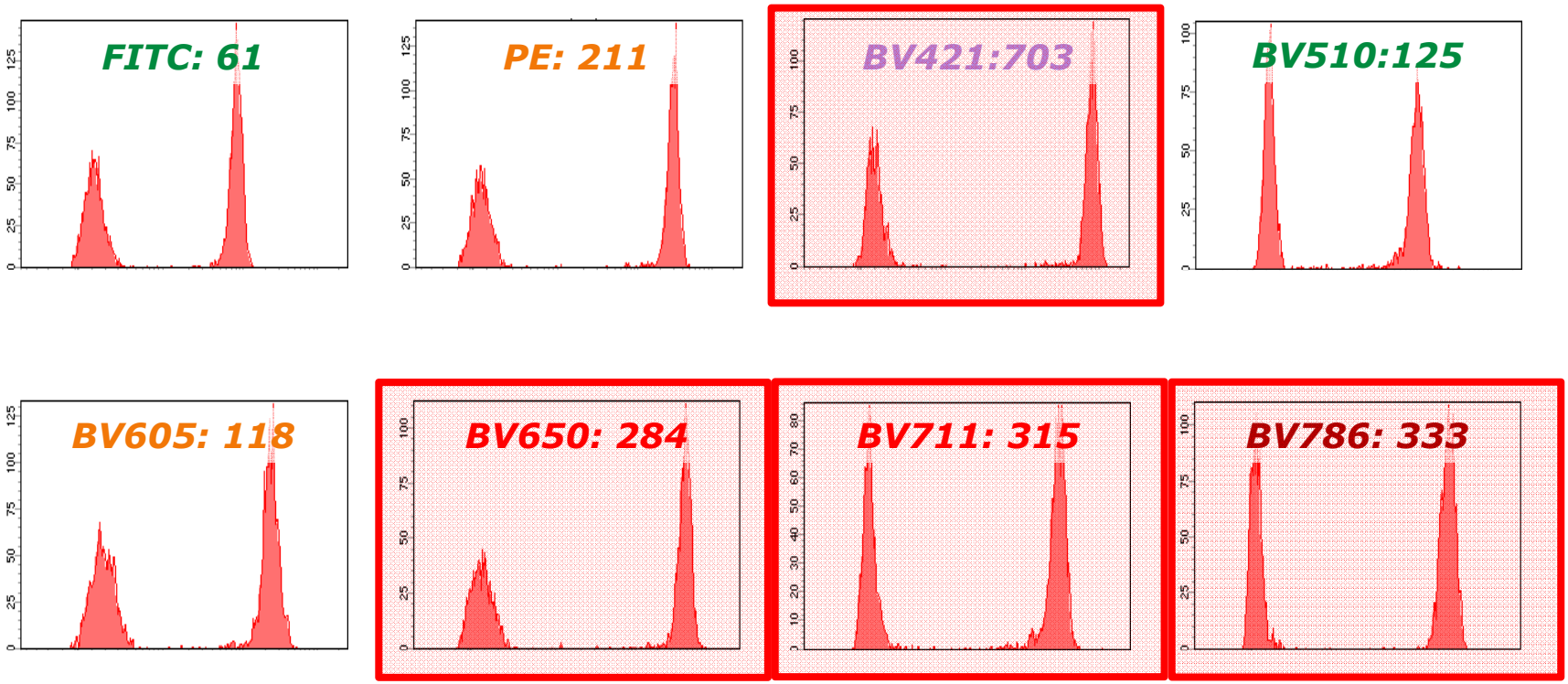
8 BV dyes



6 BUV dyes

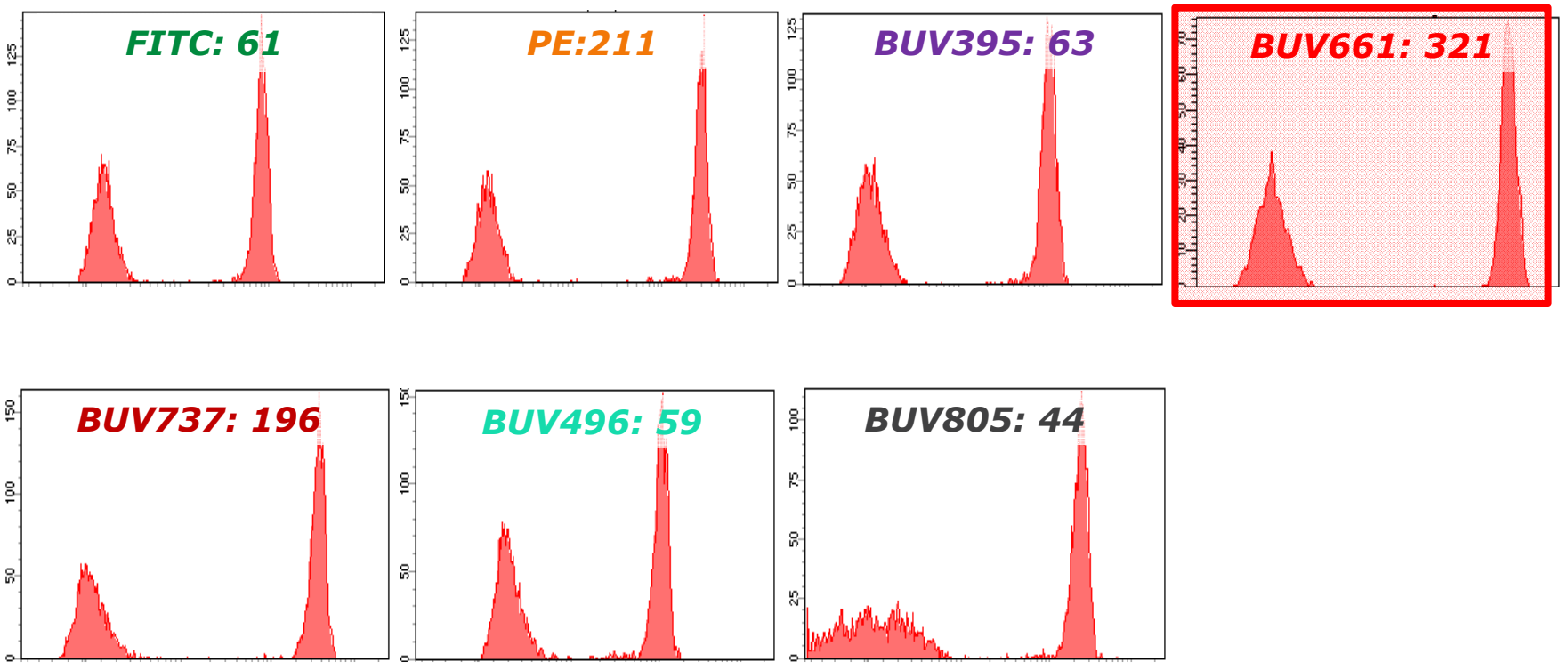
Staining microvesicles

Annexin V and mAbs conjugates: Brilliant polymers



Staining microvesicles

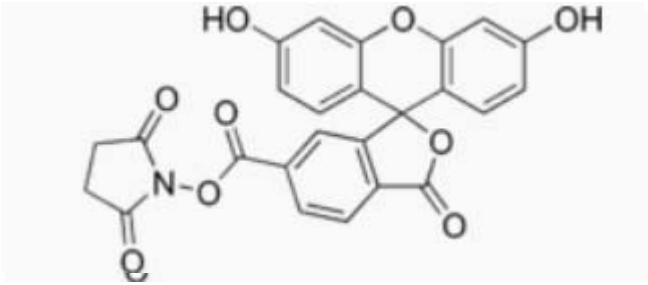
Annexin V and mAbs conjugates: Brilliant polymers



Staining microvesicles

Vesicle permeable dyes

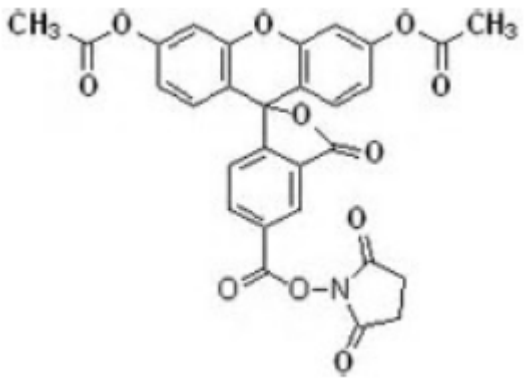
CFSE



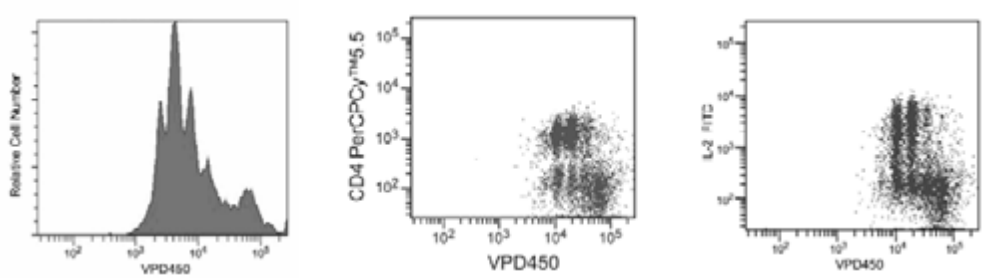
or better

Green FL
in 488 nm

CFDA

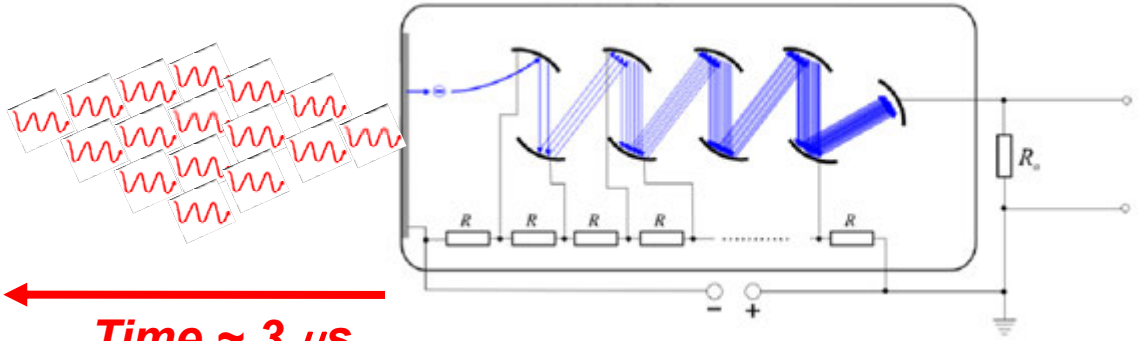


Violet proliferation dye VPD 450

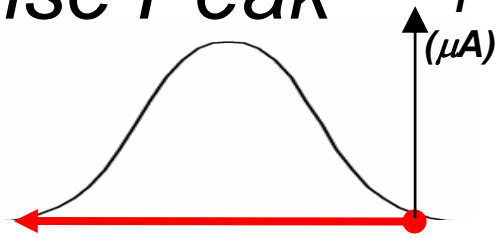


Blue FL
in 488 nm

Signal Processing in FCM

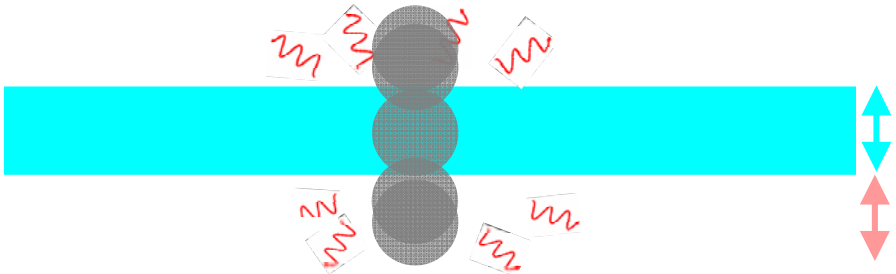


Pulse Peak



Time ~ 3 μs

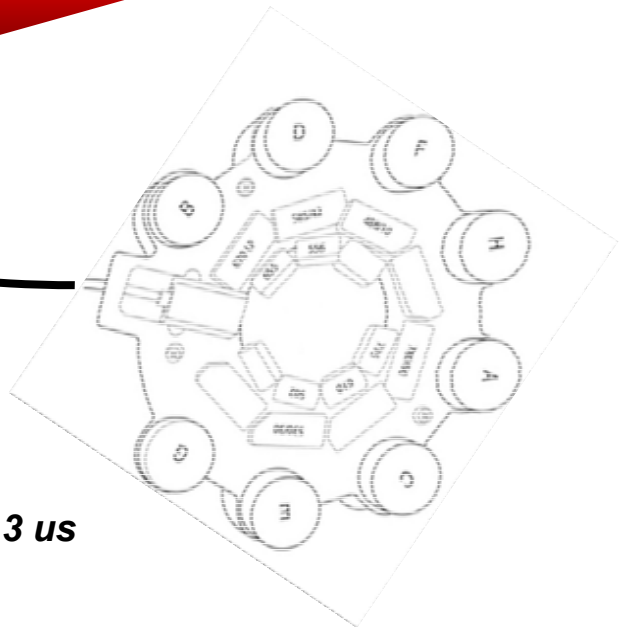
Time ~ 3 μs



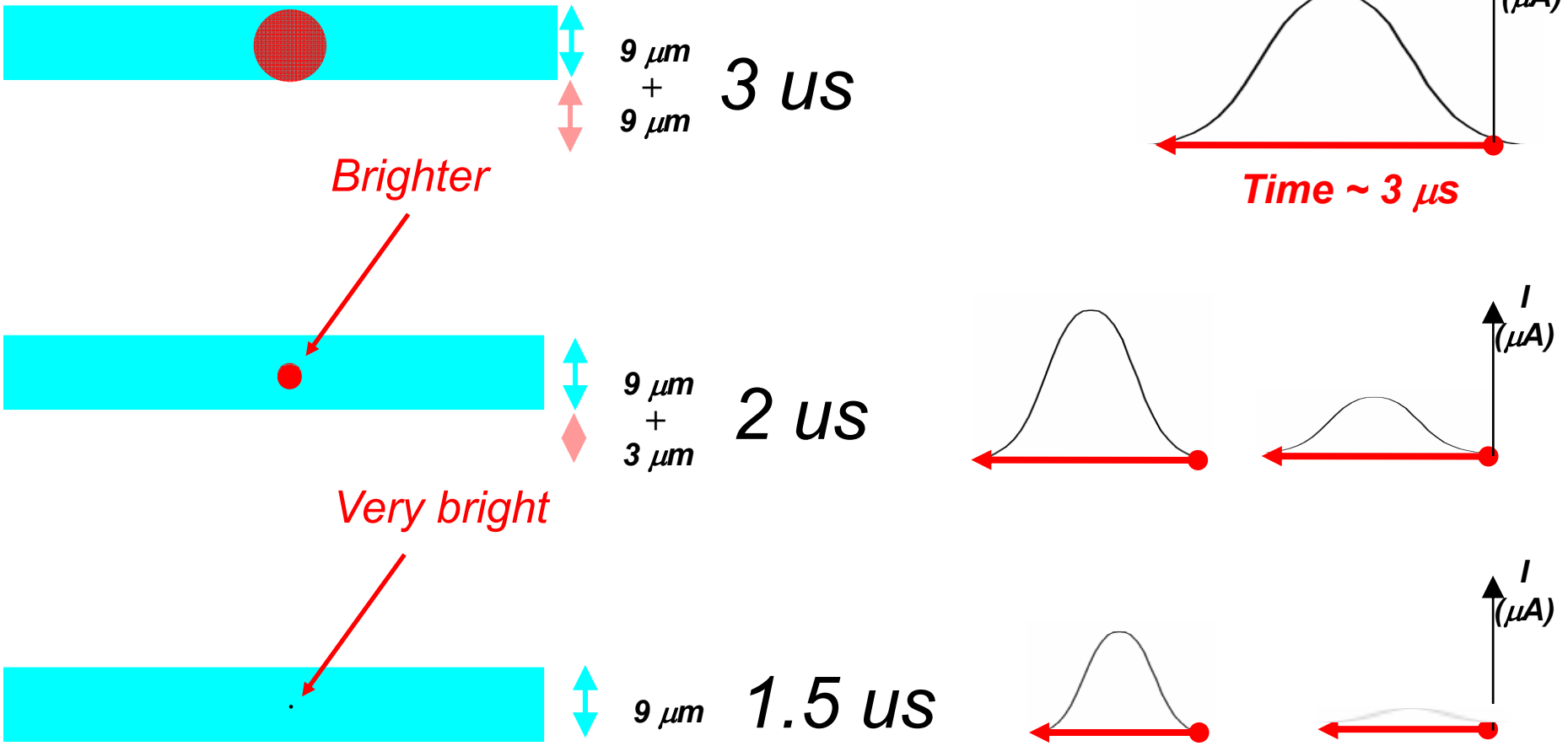
9 μm
+
9 μm

Time ~ 3 μs

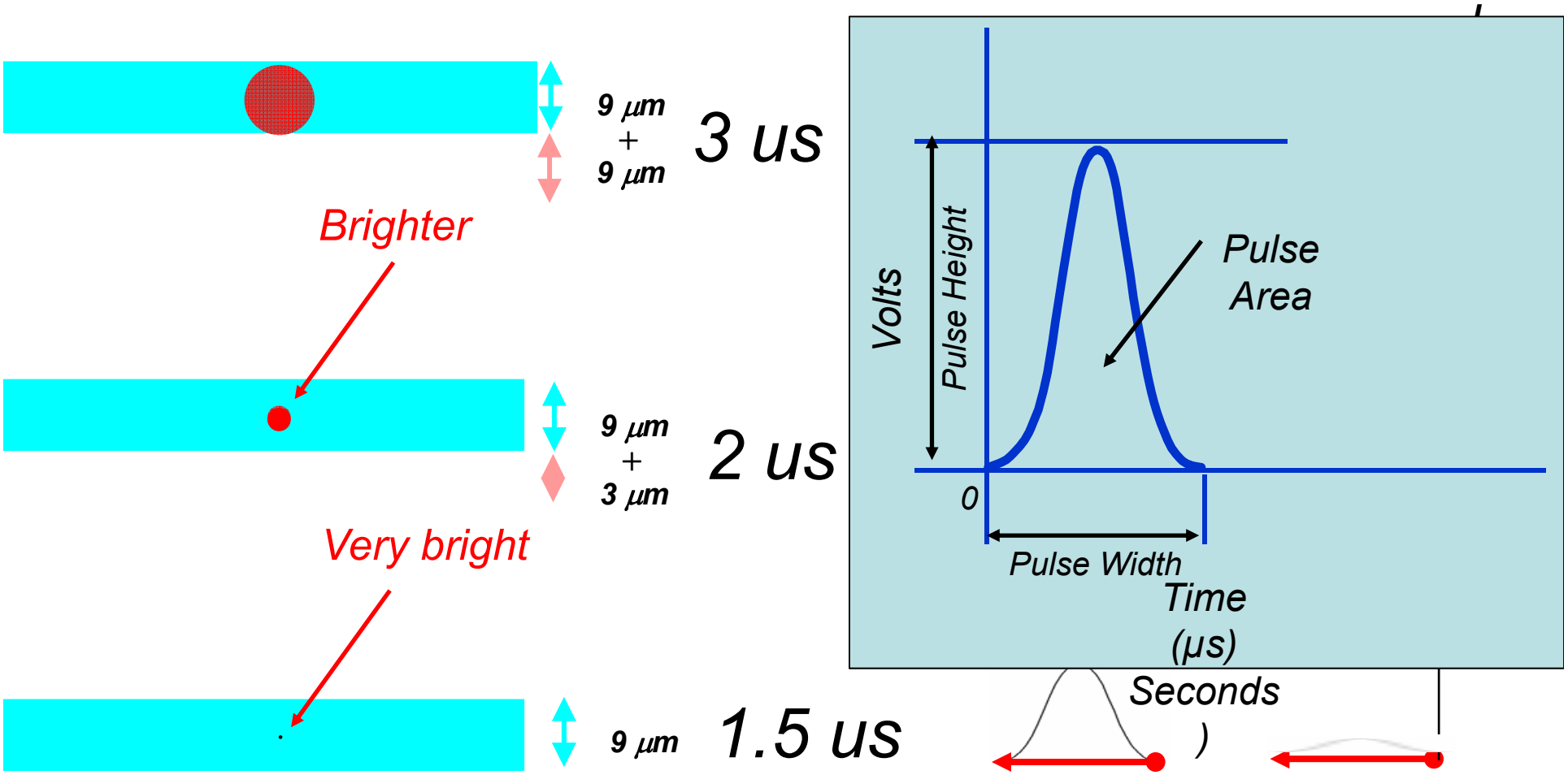
18 μm : 6 um/us = 3 us



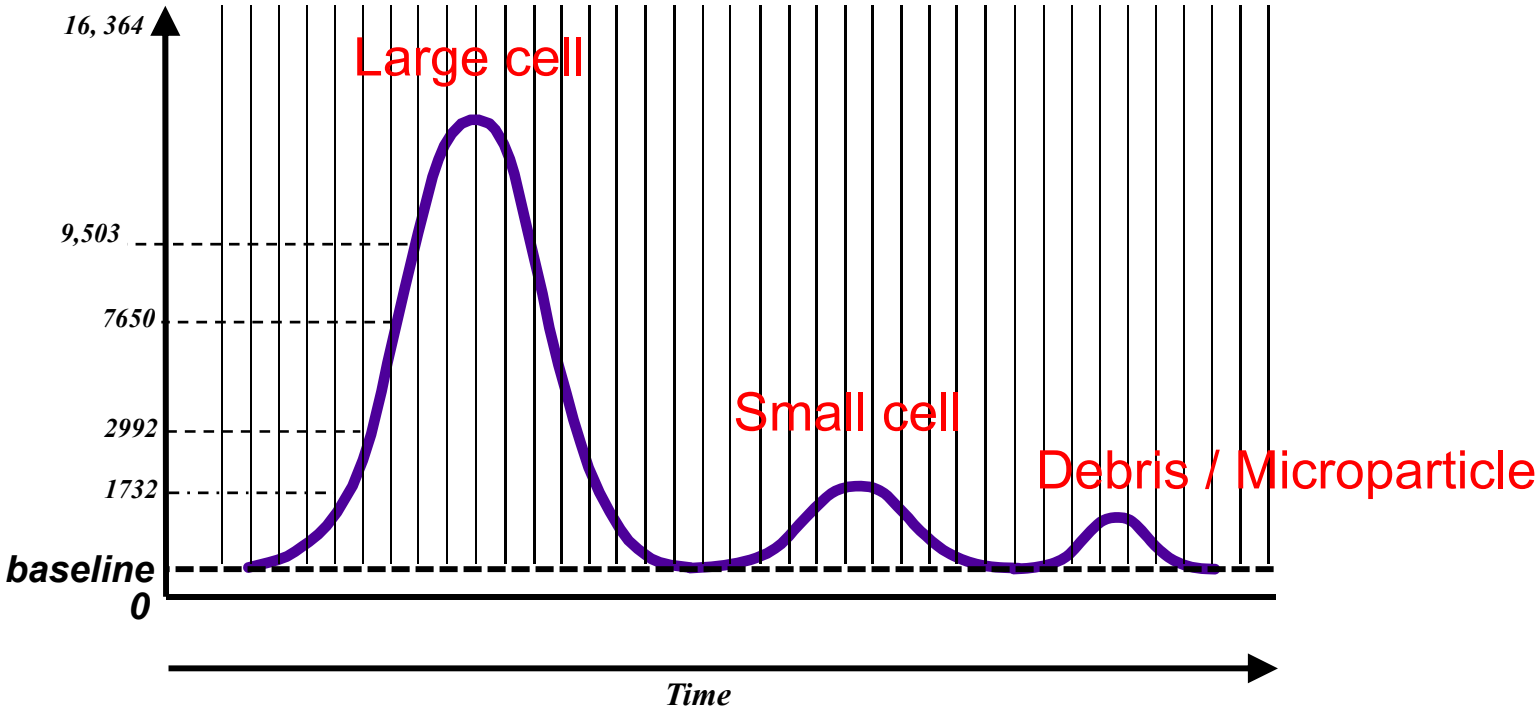
Signal Processing



Signal Processing

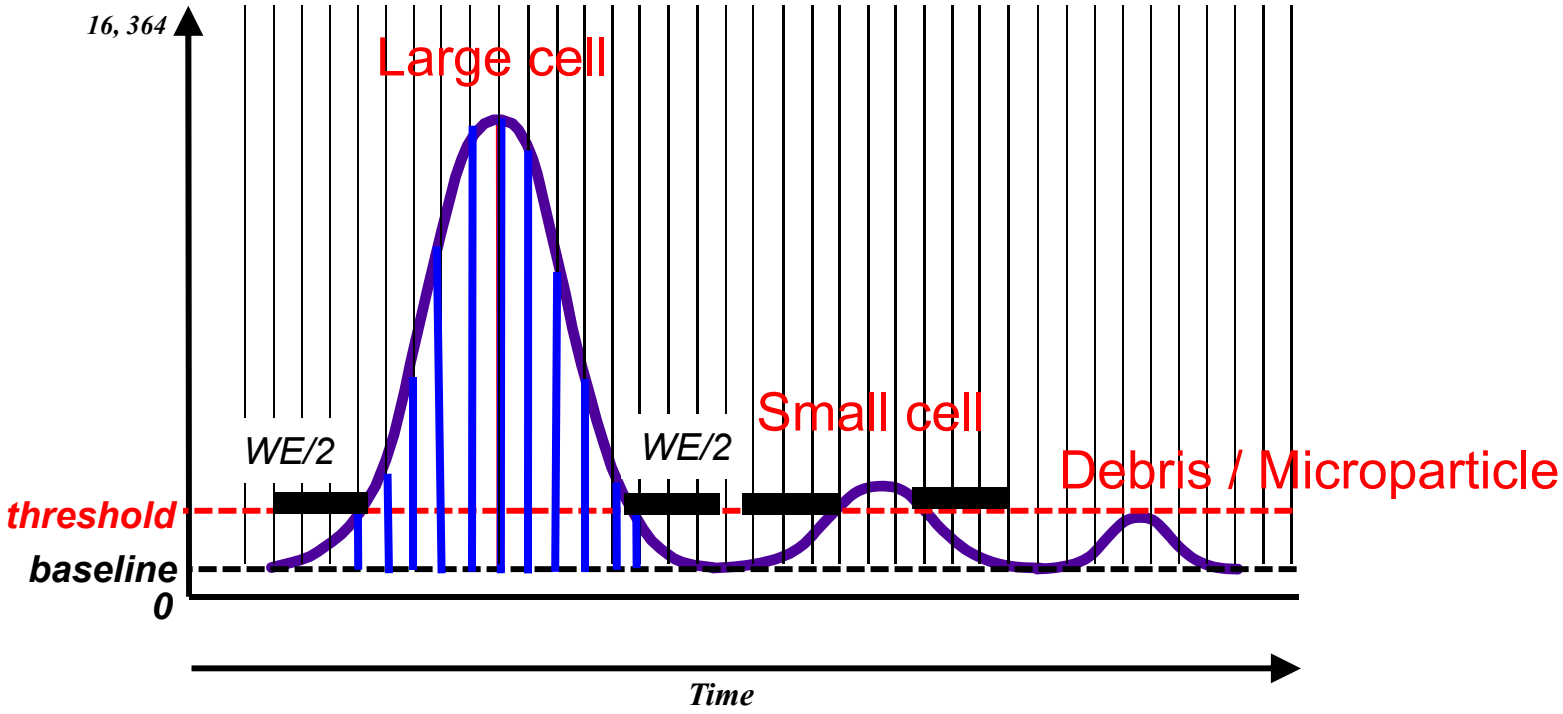


Digital Signal Processing



10 Mhz (10 x per us, 3us = 30 values)

Digital Signal Processing

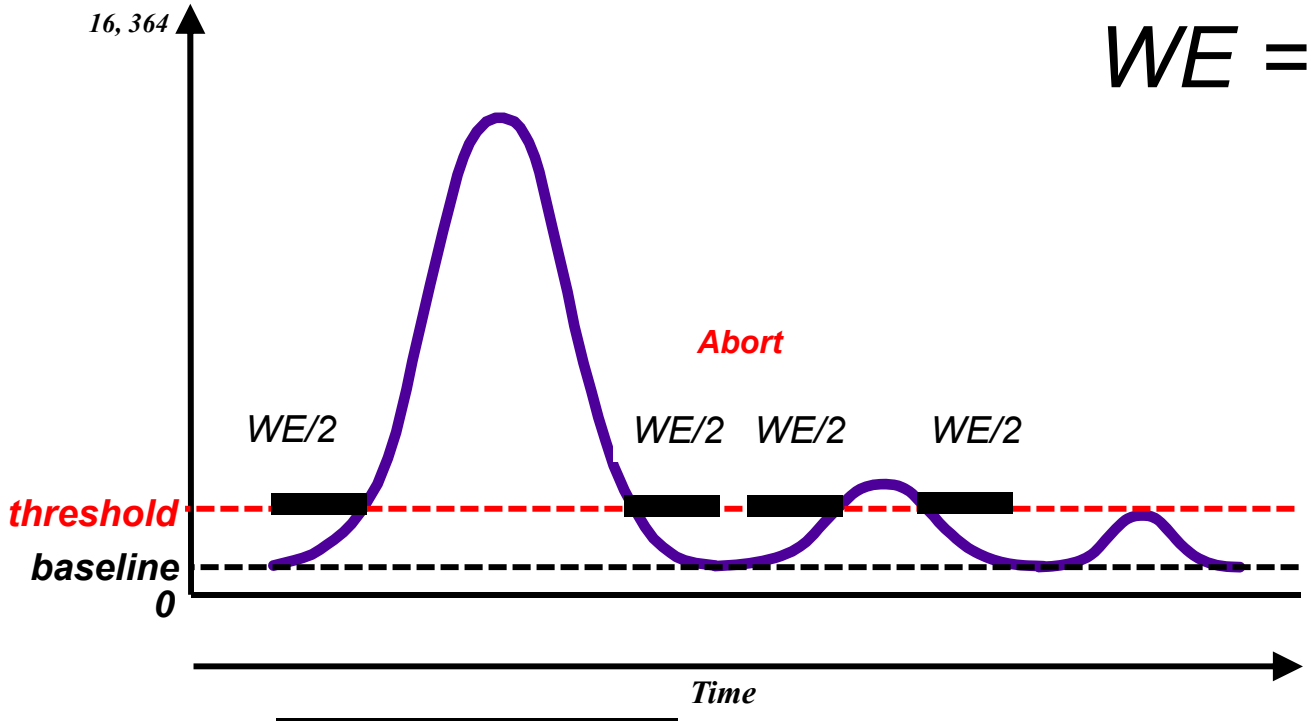


Peak Height (H) is the highest digitized value within the peak

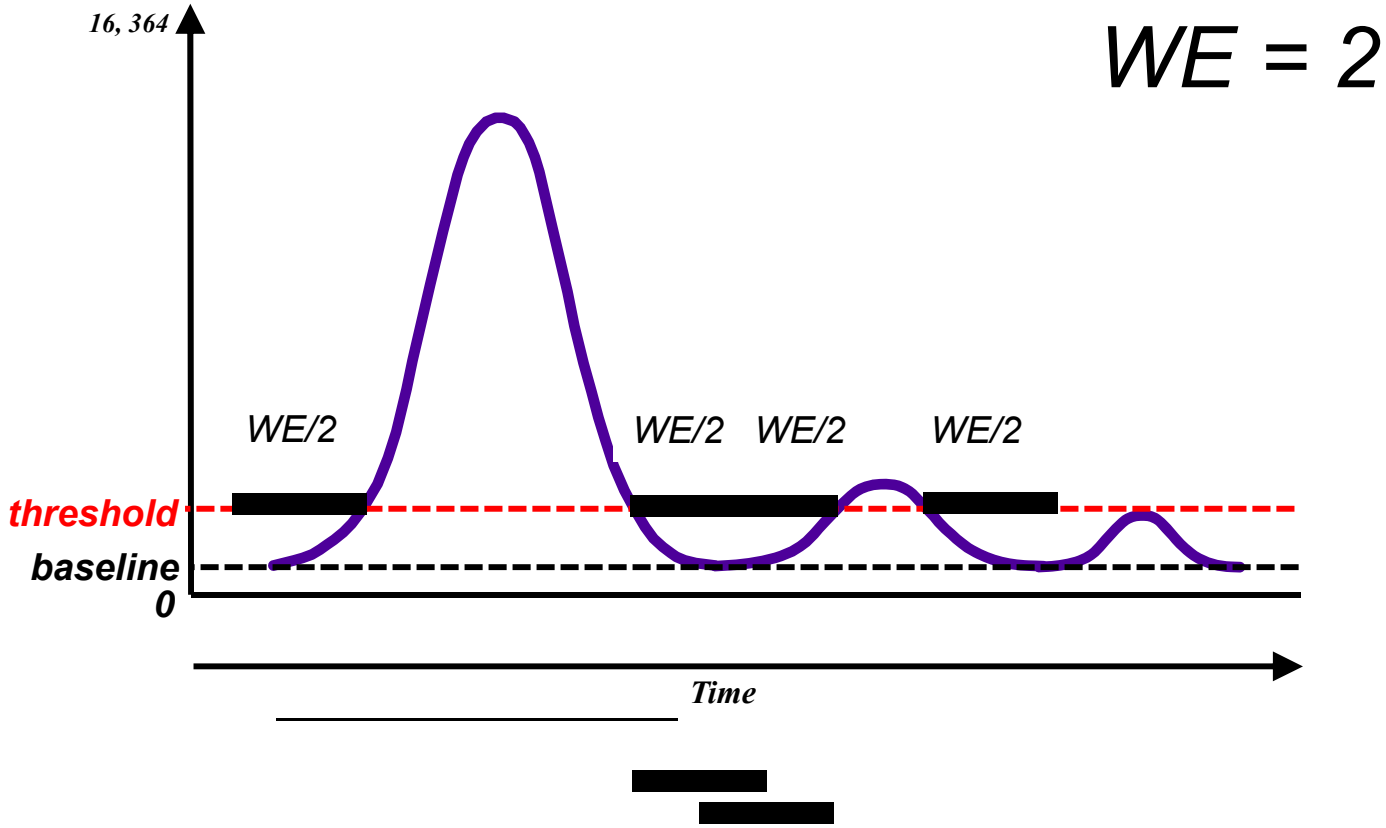
Peak Area (A) is the sum of all measured values between thresholds plus WE

Peak Width (W) is an A / H ratio

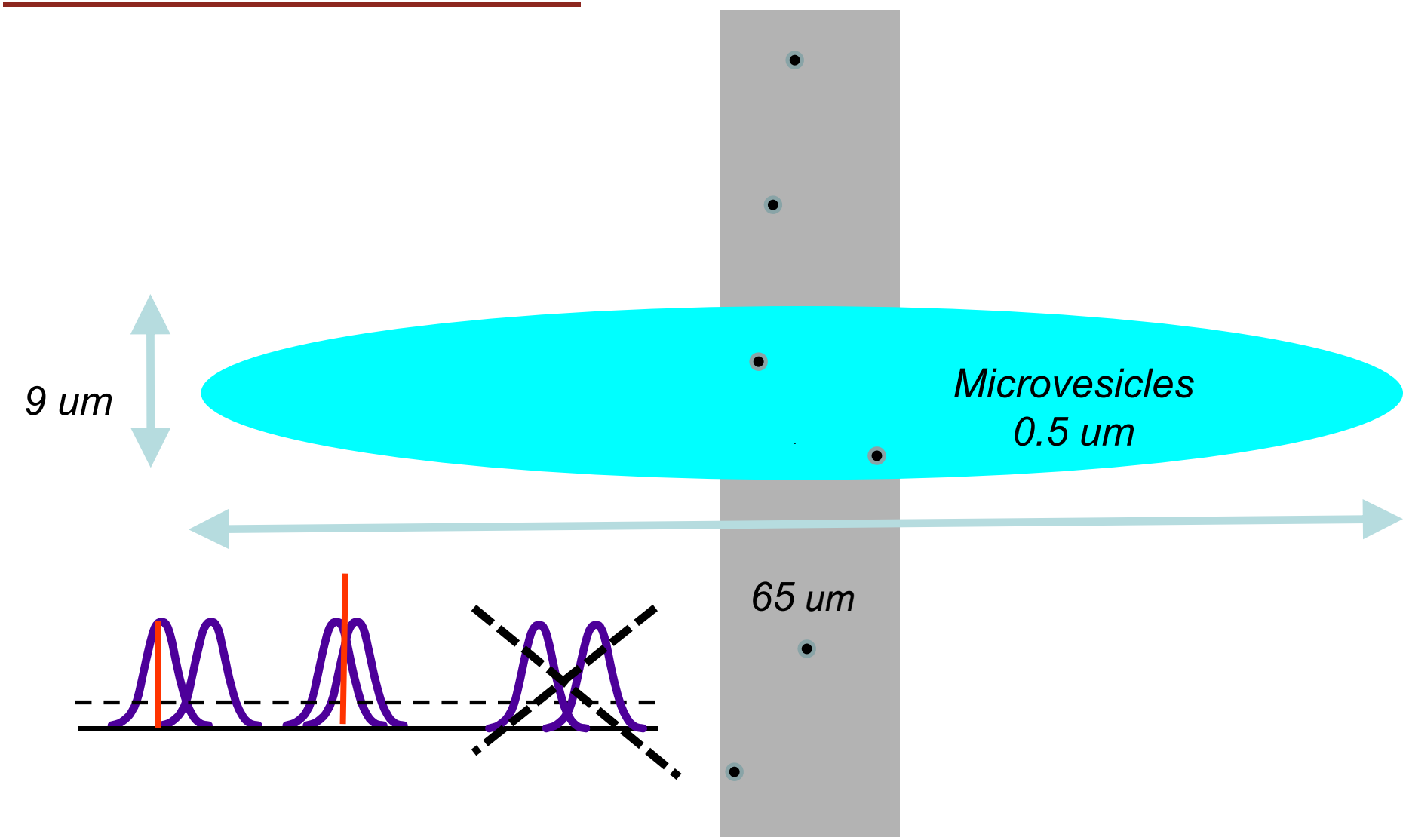
Digital Signal Processing: Electronic Abort



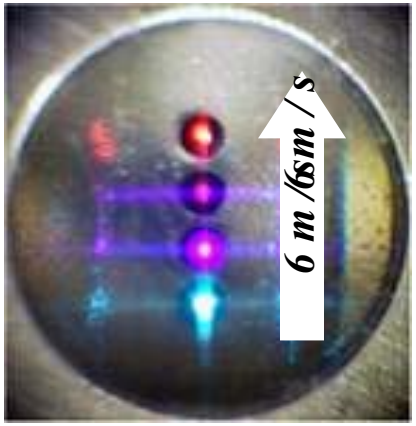
Digital Signal Processing: Electronic Abort

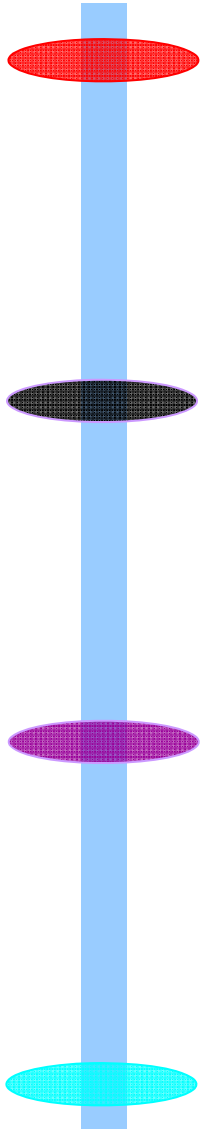


Swarm Effect



Digital Signal Processing: Electronic Abort





120 µm
20 µs

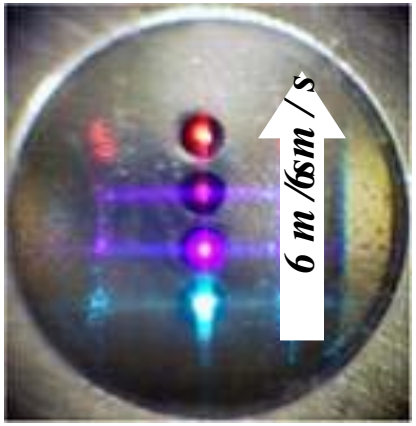
15 µm
2.5 µs

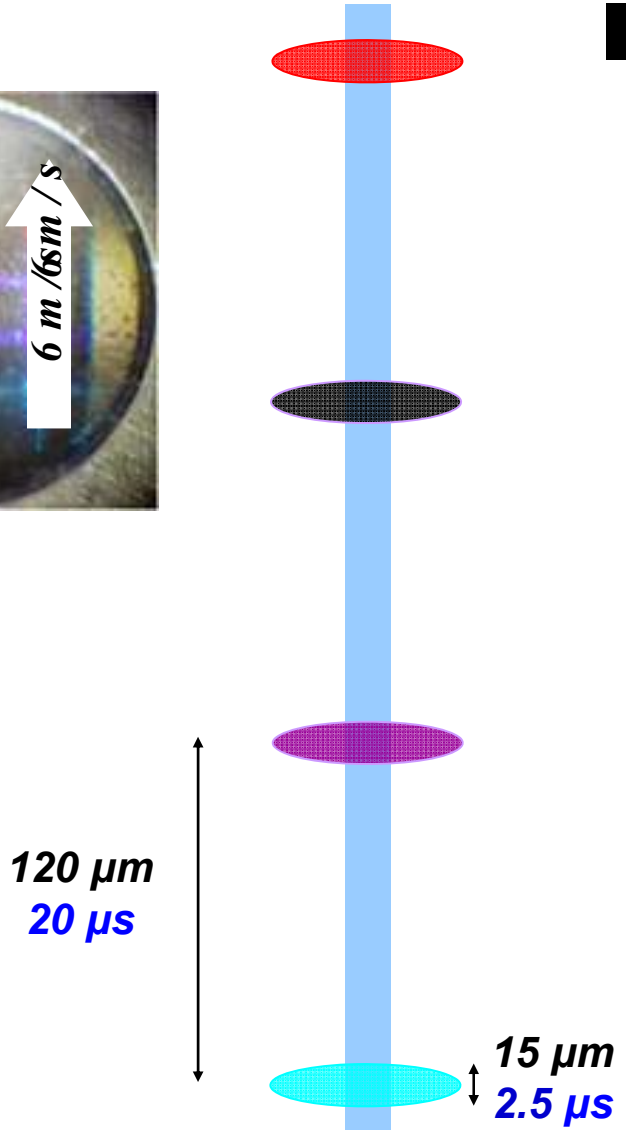
300 µm
50 µs

170 µm
28 µs

85 µm
14 µs

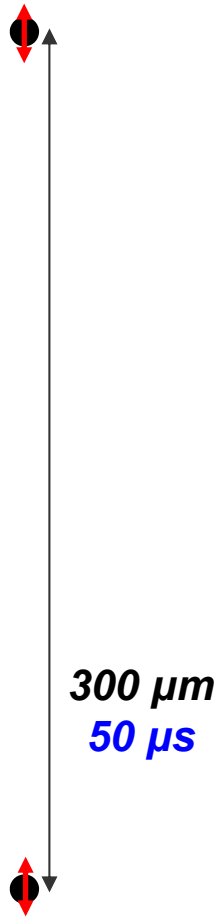
Digital Signal Processing: Electronic Abort

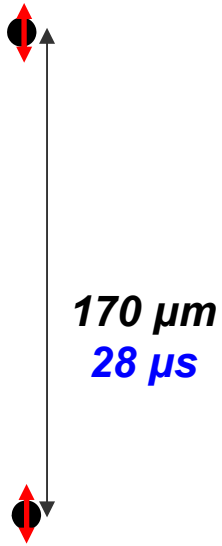


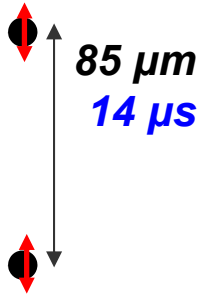


20 000 eps	35 000 eps	70 000 eps

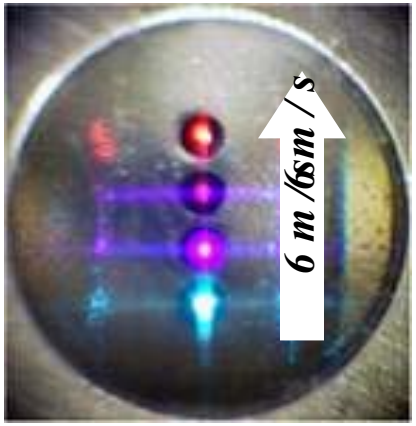
WE = 2

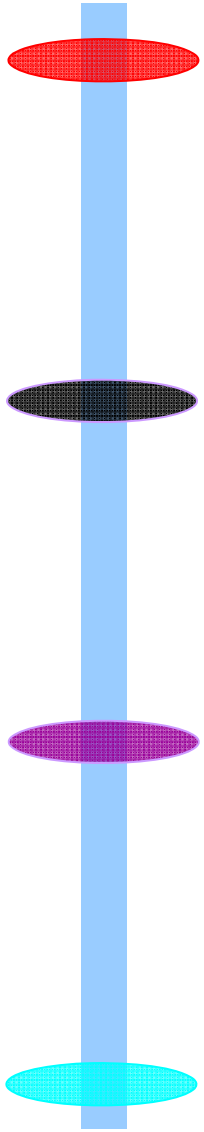






Digital Signal Processing: Electronic Abort





120 µm
20 µs

15 µm
2.5 µs

20 000 eps	35 000 eps	70 000 eps

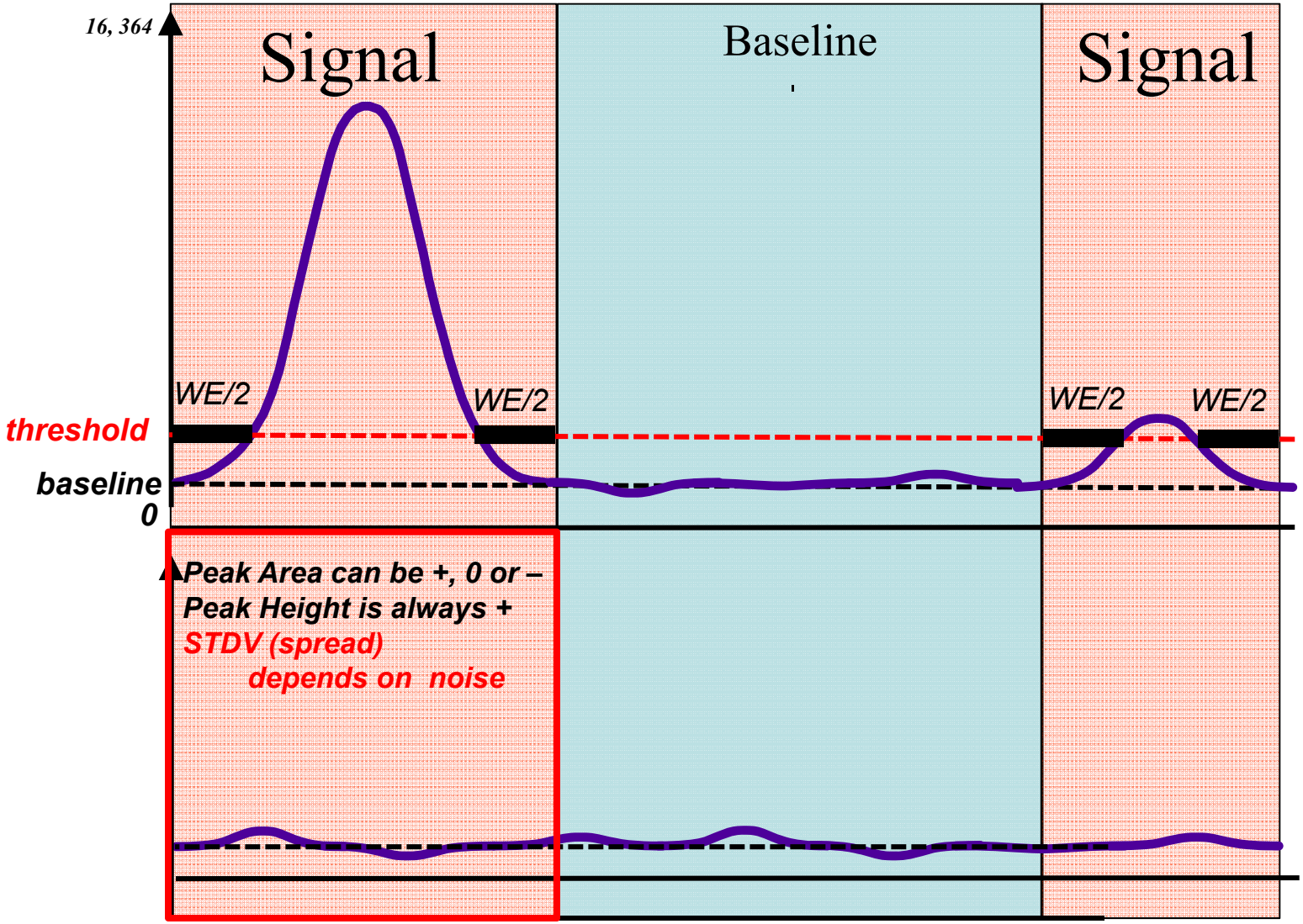
WE = 10

300 µm
50 µs

170 µm
28 µs

85 µm
14 µs

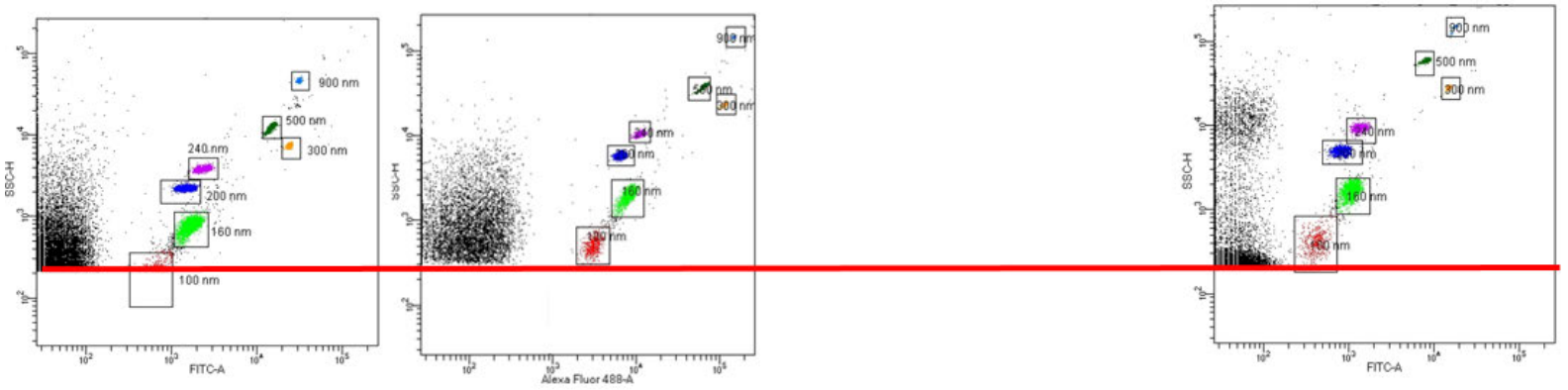
Signal Baseline Definition and Signal Detection



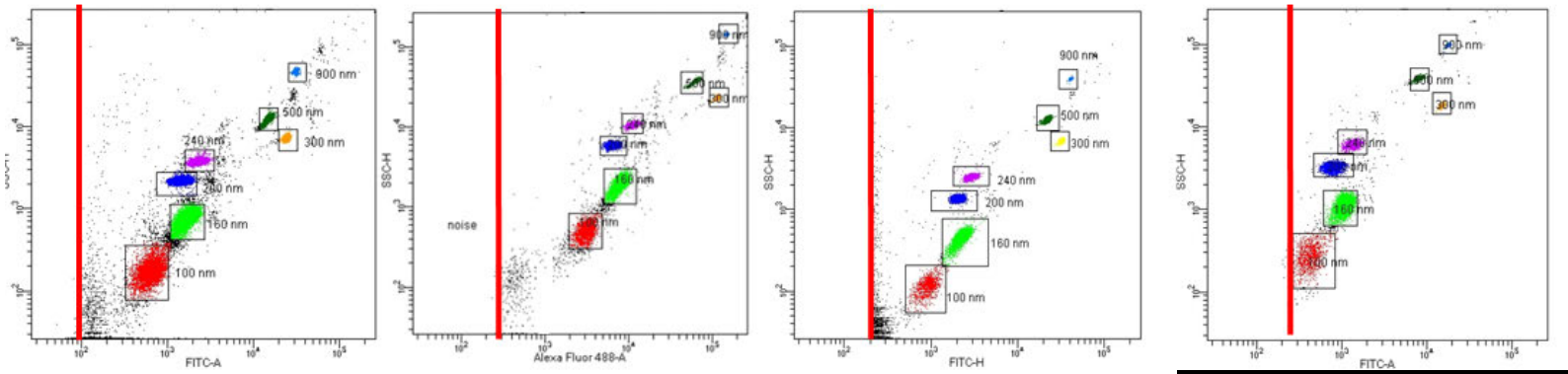
Plastic microparticles on BD Flow Cytometers

FACSAria III *FACSFusion* *LSR Fortessa* *FACSCanto*

SSC



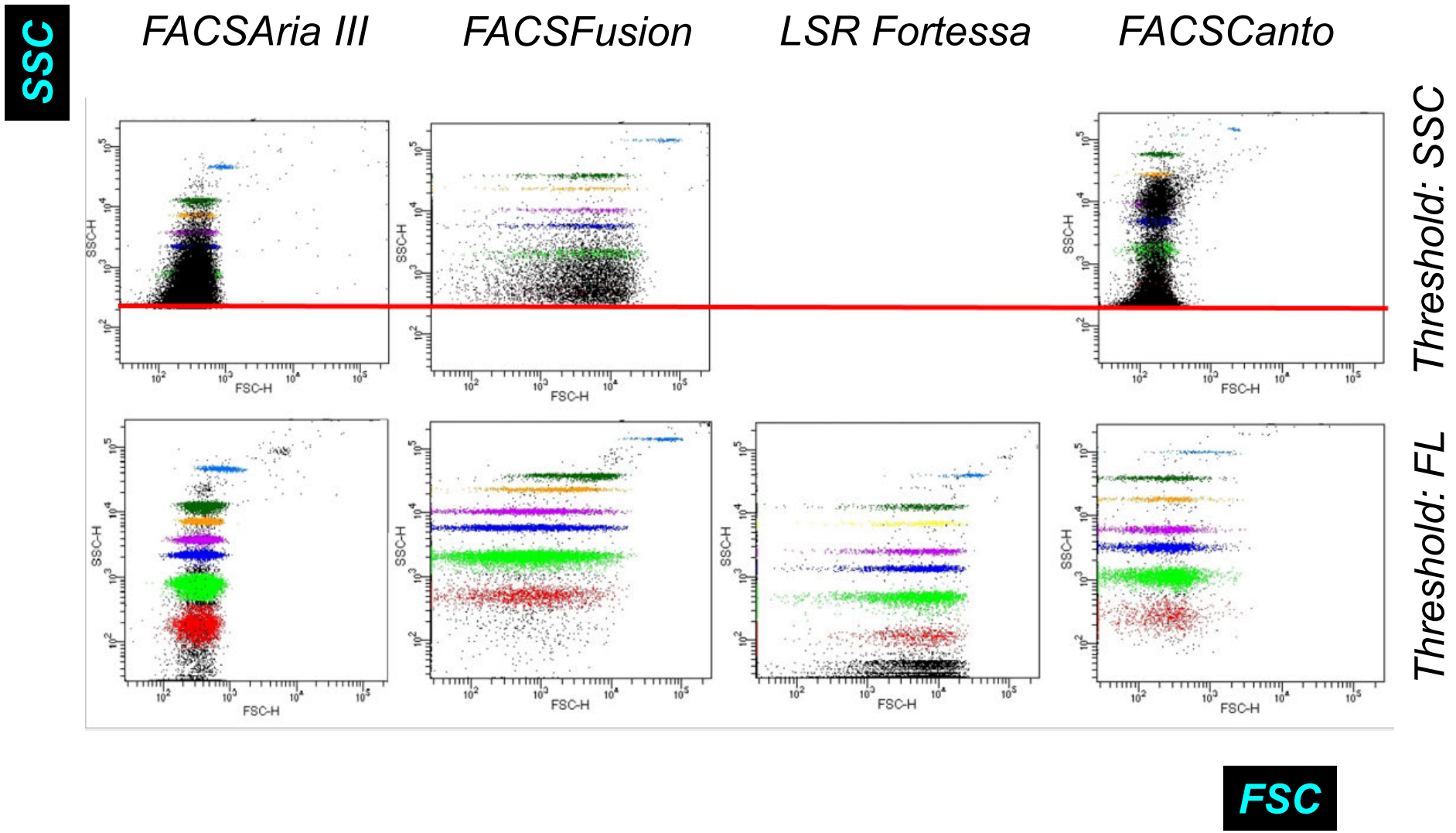
Threshold: SSC



Threshold: FL

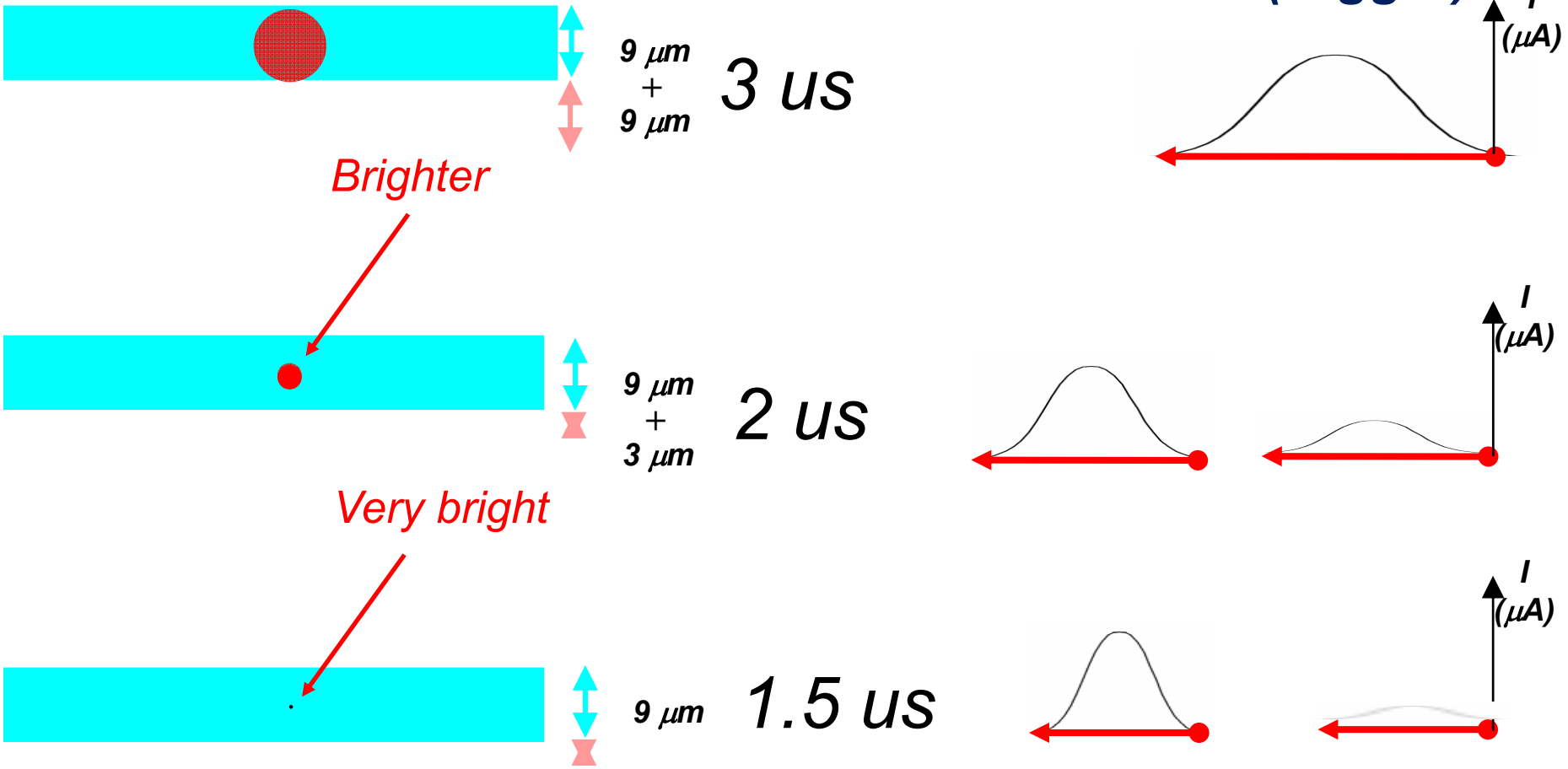
Green FL on 488

Plastic microparticles on BD Flow Cytometers



Signal Processing: Height or Area for small particles?

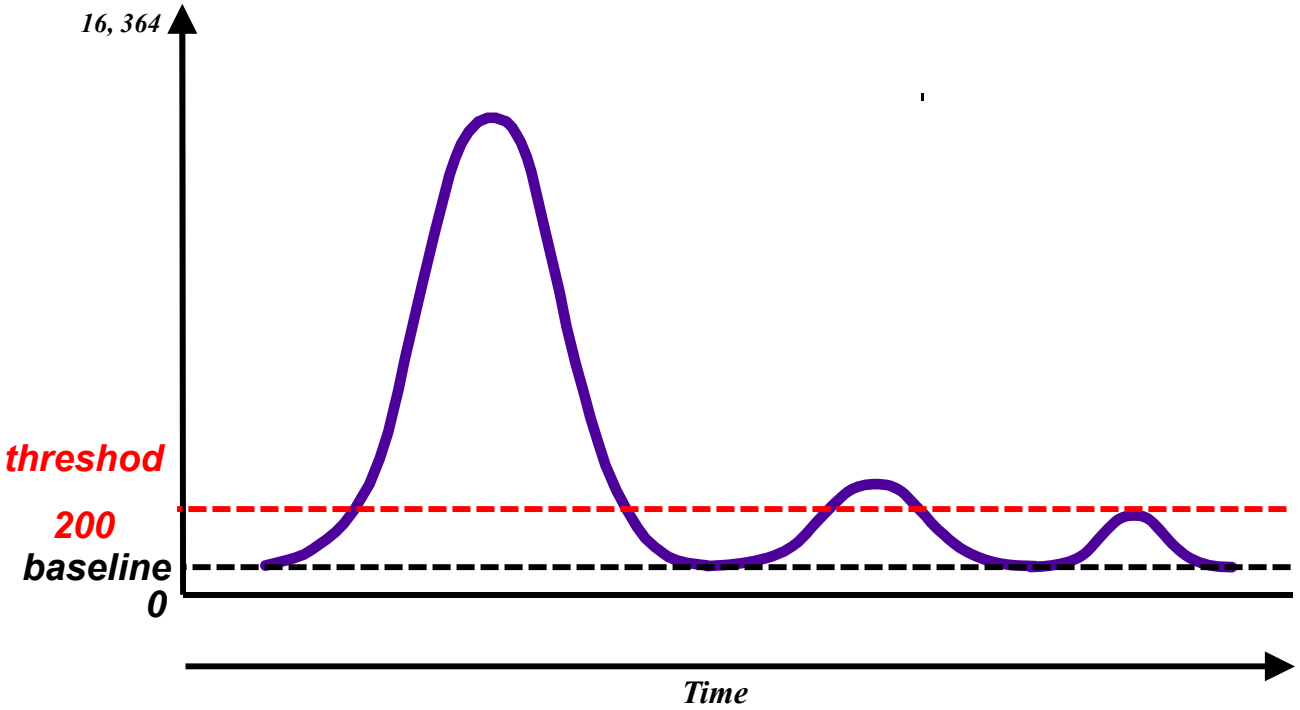
Which threshold (trigger)?



Trigger on fluorescence

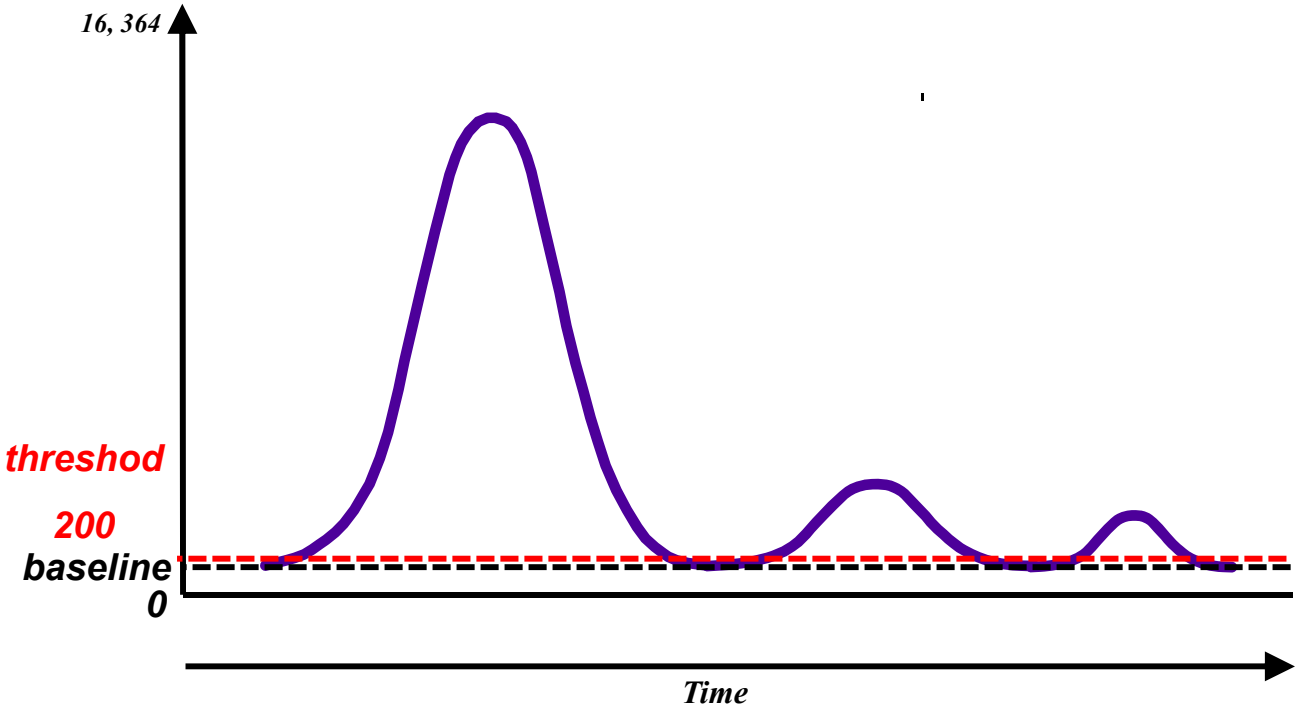
Time ~ 3 μs (18 : 6)

Small Particle Detection: Low Threshold



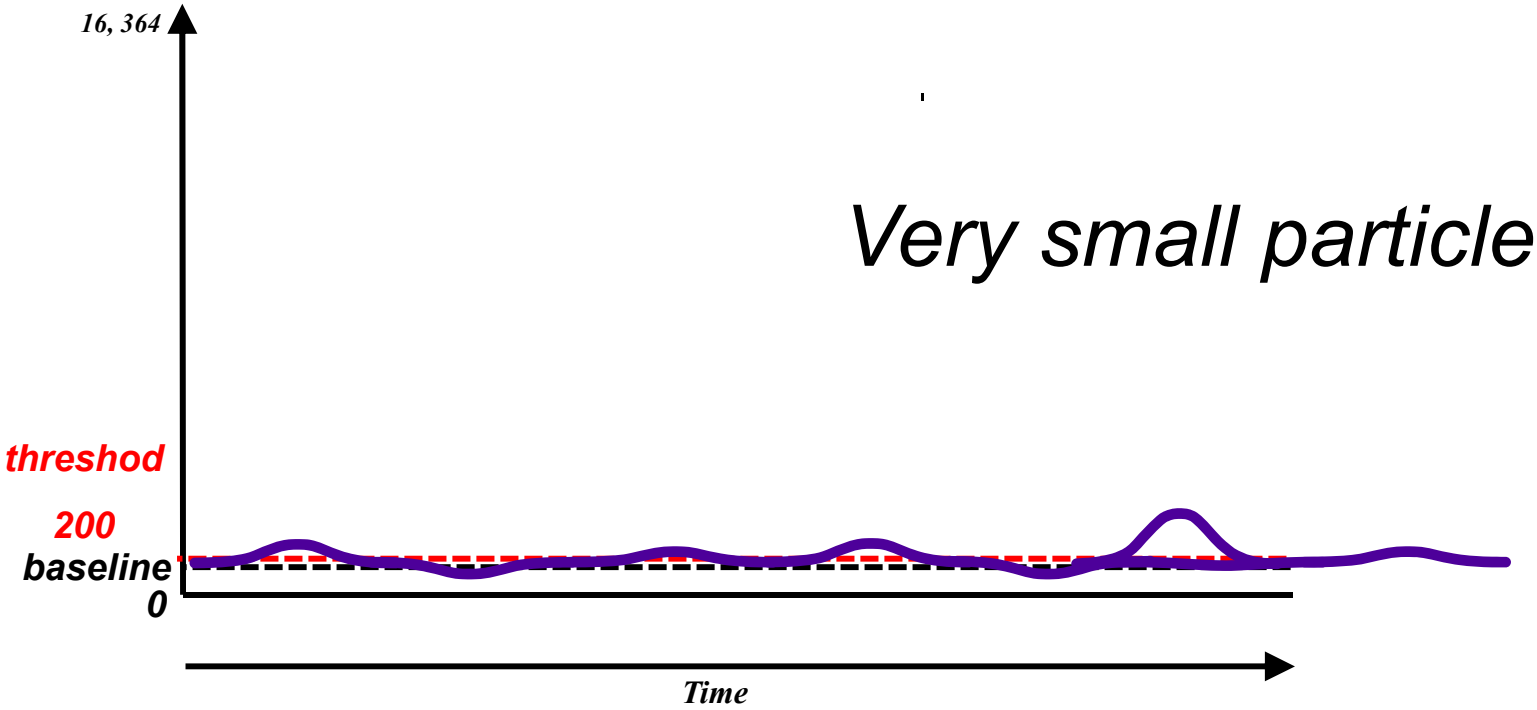
Trigger on fluorescence

Small Particle Detection: Low Threshold



Trigger on fluorescence

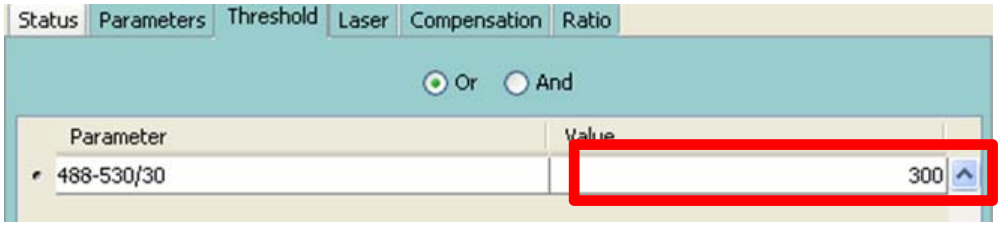
Small Particle Detection: Low Threshold

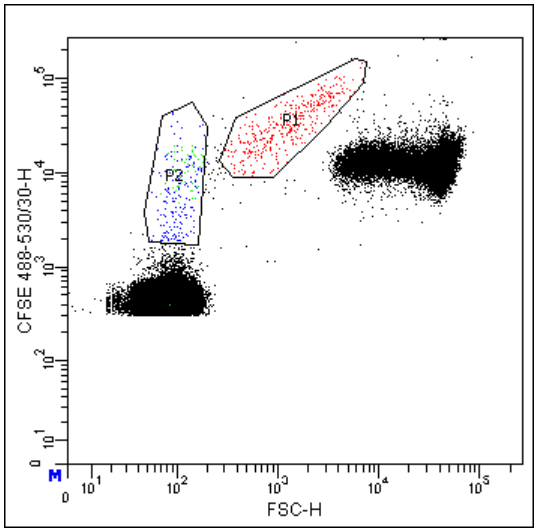


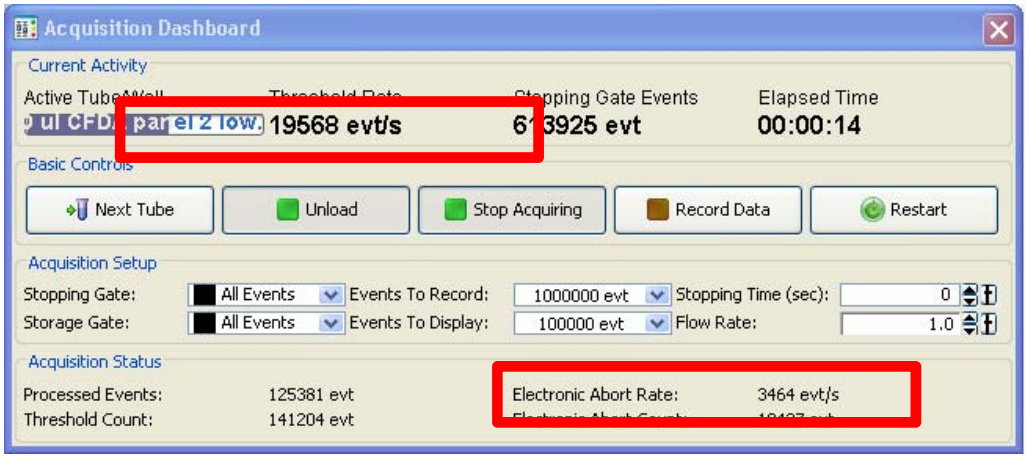
Very small particle

Trigger on fluorescence

Example: FL threshols setup, whole blood analysis

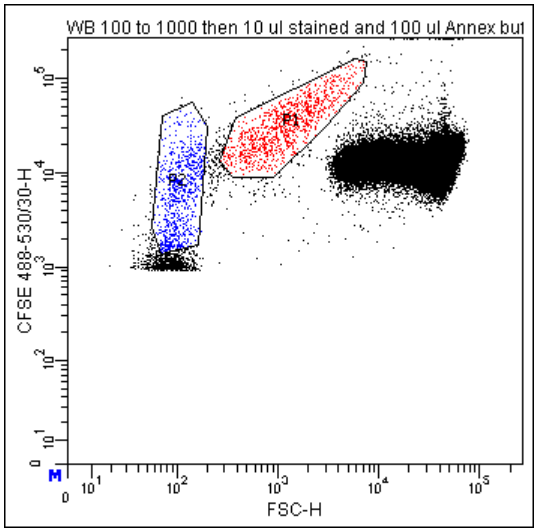


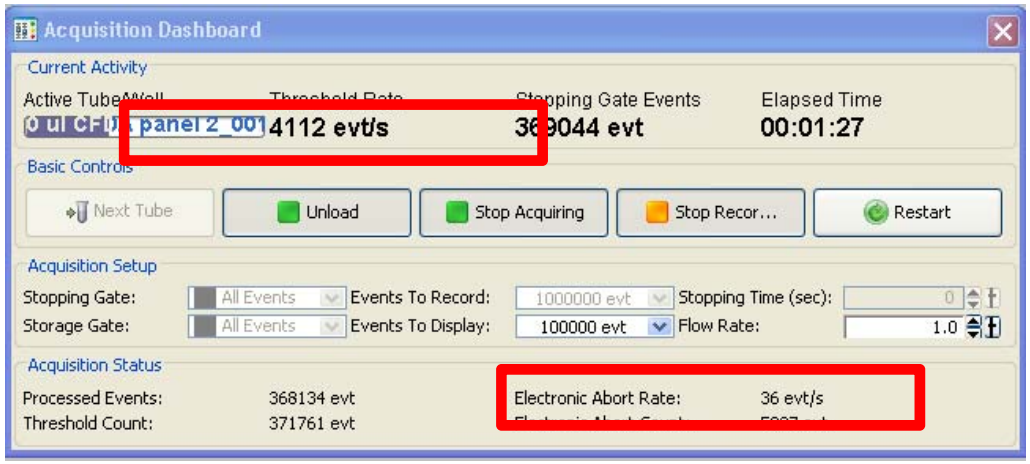




Abort Rate: 3464 / 19568 ~ 17 %

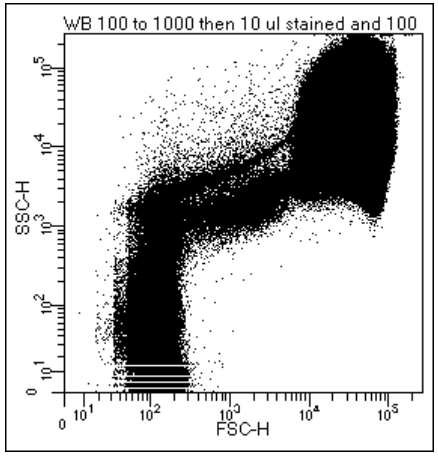
Example: FL threshols setup, whole blood analysis

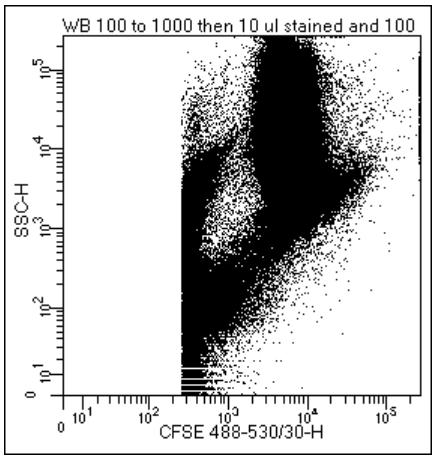


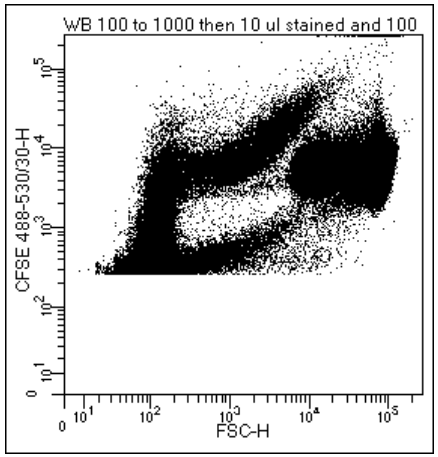


Abort rate: 36 / 4112 ~ 0.8 %

Example: Whole blood analysis

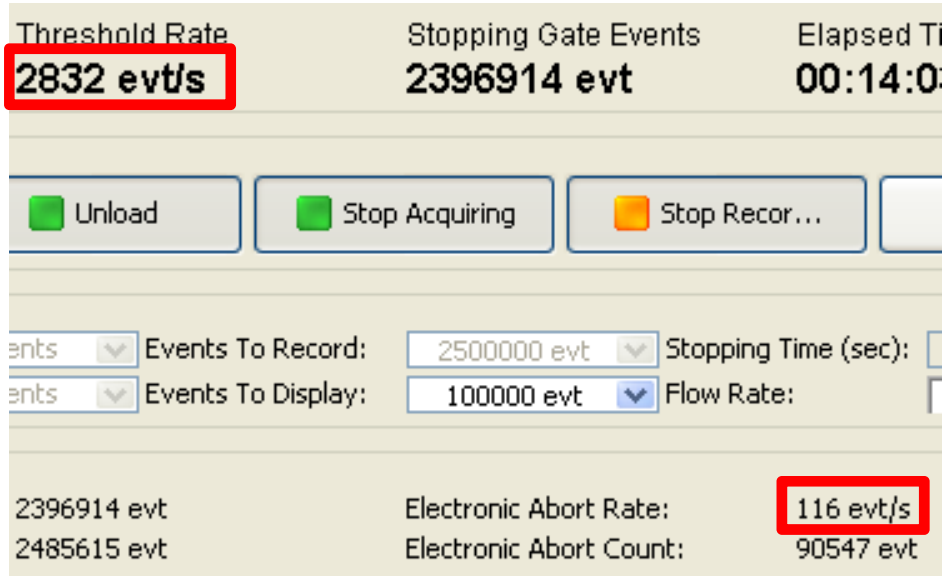






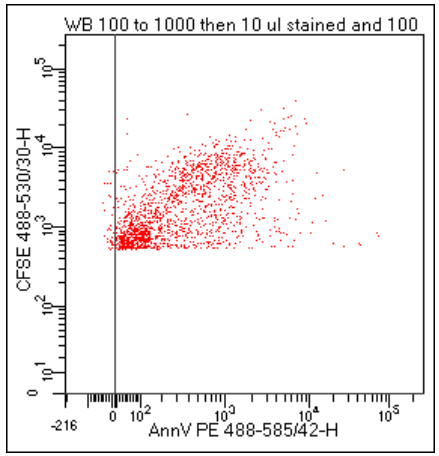
Parameter	Type	Log	Voltage
FSC	A, H	✓	120
SSC	A, H	✓	485
488-530/30	A, H	✓	500
488-585/42	A, H	✓	500

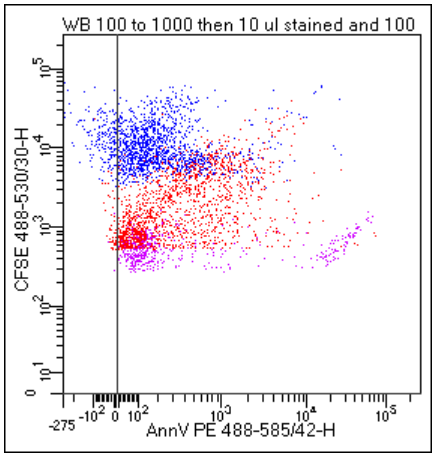
Threshold Operator	Or
Parameter	**Value**
488-530/30	250

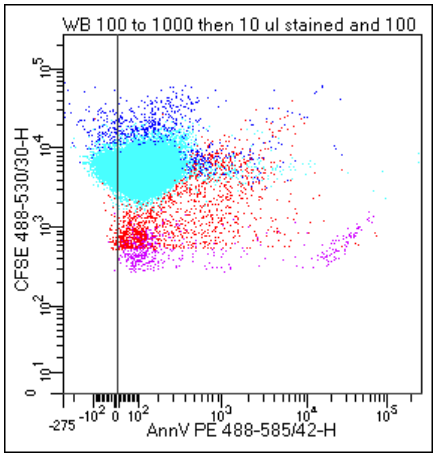


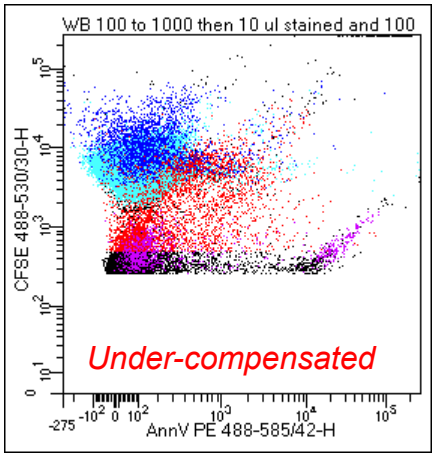
Electronic aborts: 4 %

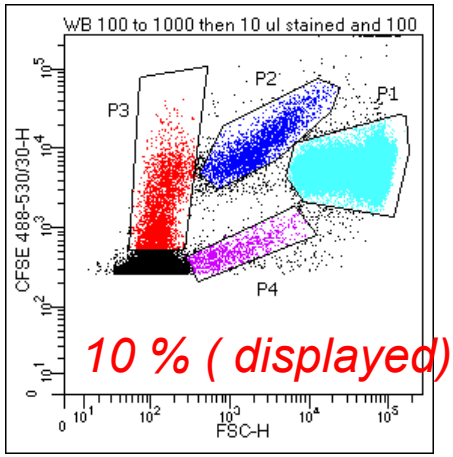
Example: Whole blood analysis

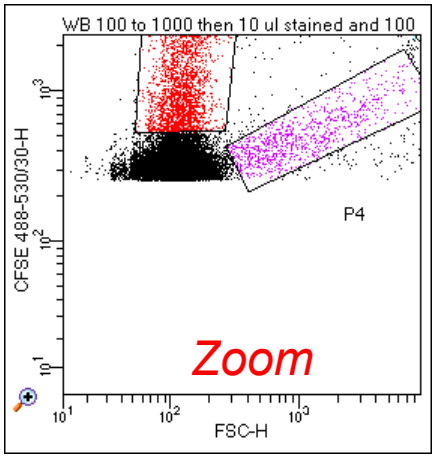


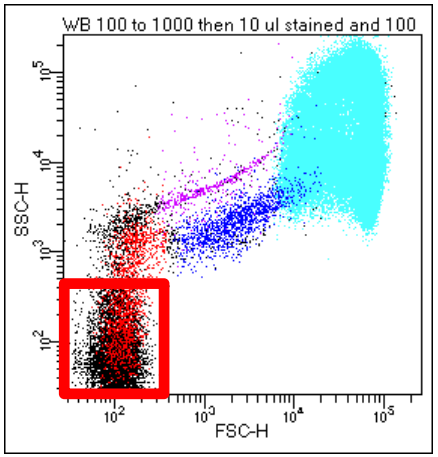




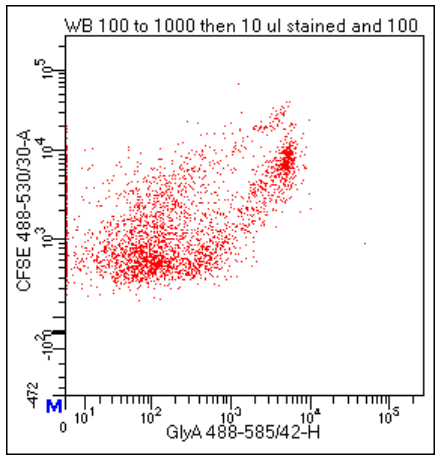


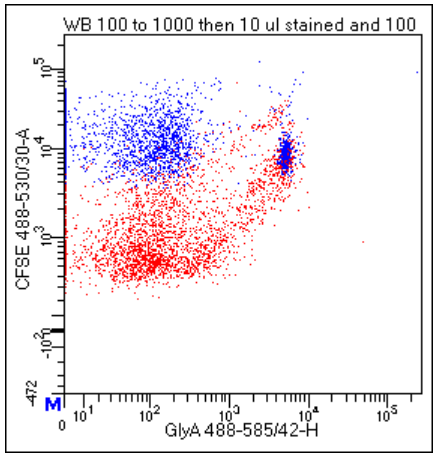


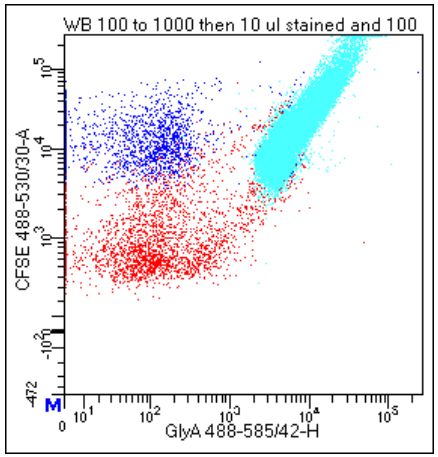


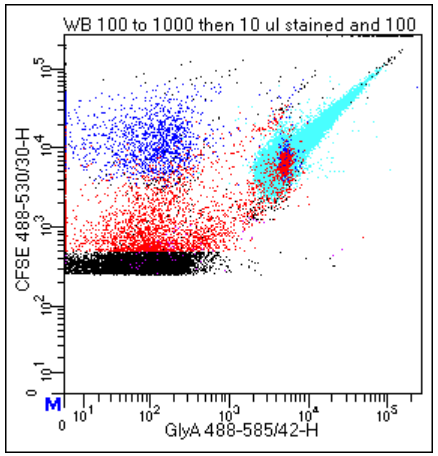


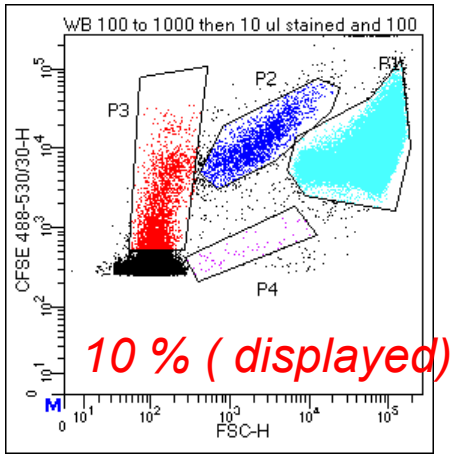
Example: Whole blood analysis

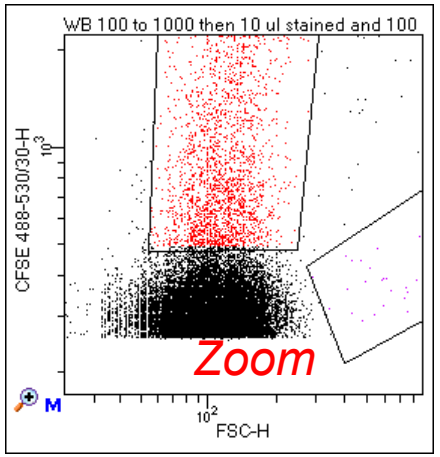


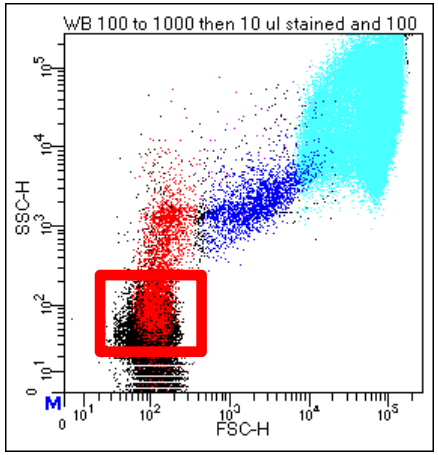








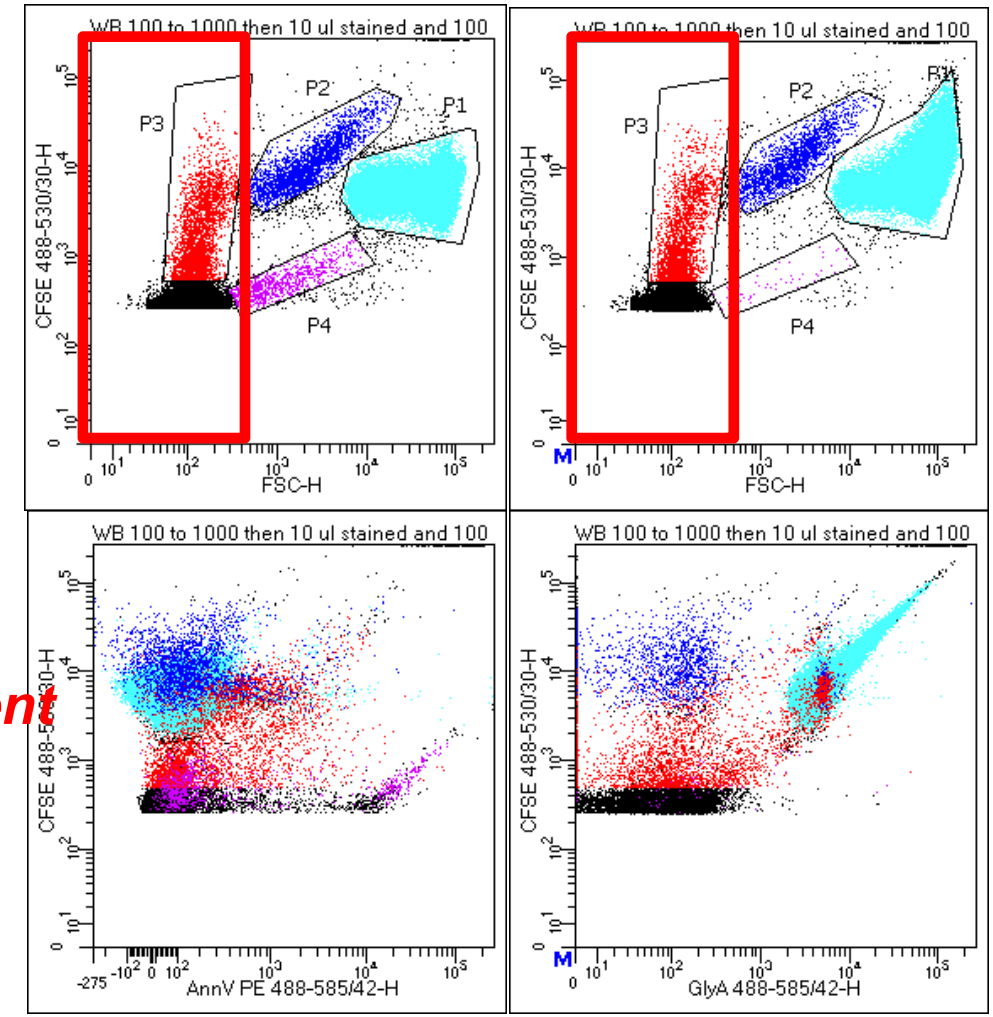




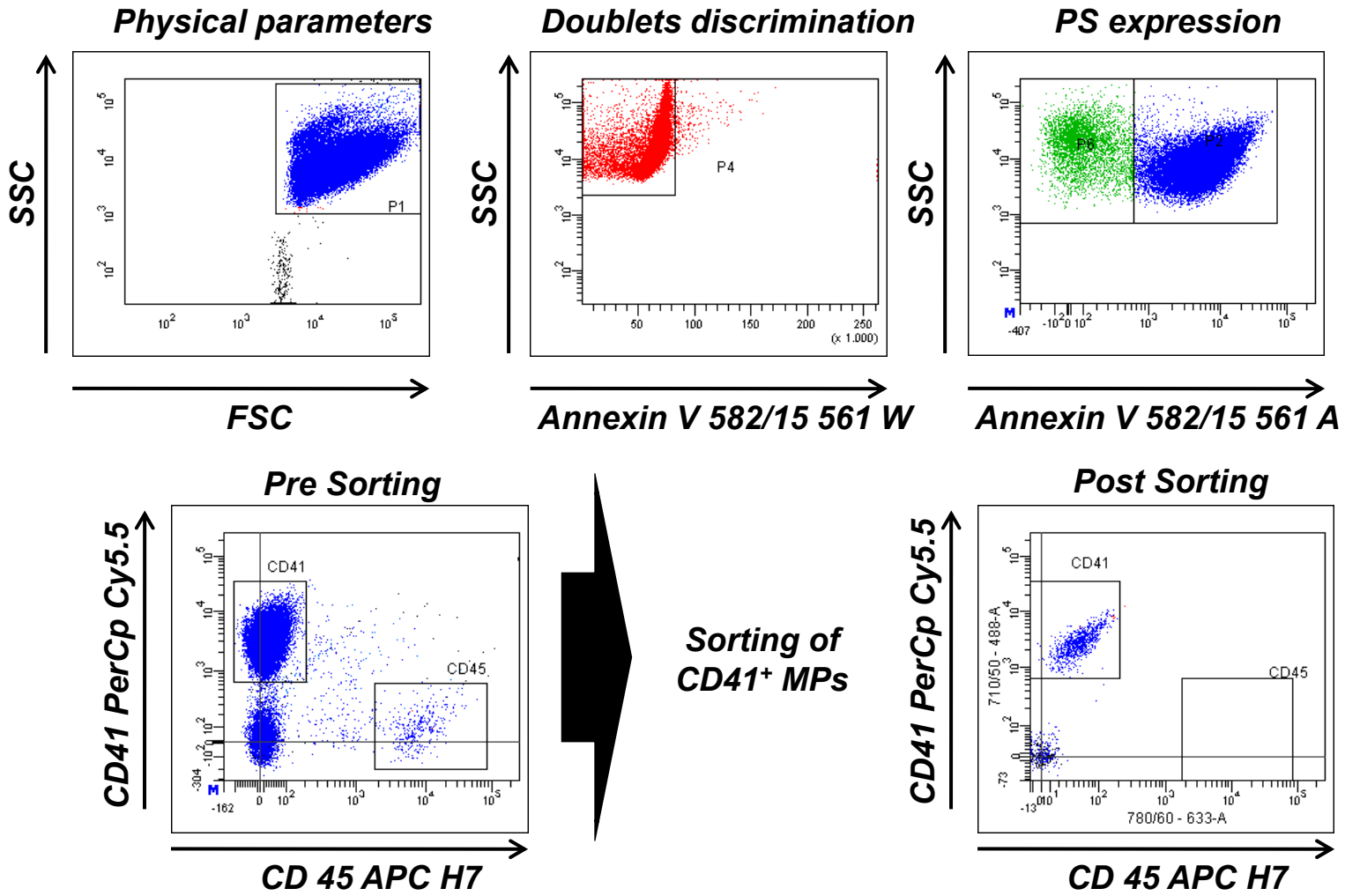
Example: Whole blood analysis

Microparticles, vesicles and exosomes should be Identified as SMALL

Larger (red cell born) MP can be isentified by mcAb within the platelet compartment



MPs sorting: RELATIVELY SLOW due to SWARMING



Tube: 128 - CD41_PRESORT

Population	#Events	%Parent	%Total
All Events	30.721	####	100,0
P1	30.070	97,9	97,9
P3	29.848	99,3	97,2
CD41	26.160	87,6	85,2
CD45	318	1,1	1,0

Tube: 128 - CD41_POSTSORT CD41

Population	#Events	%Parent	%Total
All Events	1.201	####	100,0
P1	1.012	84,3	84,3
P3	1.003	99,1	83,5
CD41	844	84,1	70,3
CD45	0	0,0	0,0

CD8+ T CELLS AND THEIR METABOLIC PLASTICITY

Špela Konjar[1], Silvia Innocentin[1], Nejc Haberman[2], Urška Vrhovšek[3], Marc Veldhoen[1]

[1] Babraham Institute, Lymphocyte signalling and development laboratory, Babraham Research Campus, Cambridge, CB22 3AT, United Kingdom

[2] Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, WC1N 3BG, London, United Kingdom

[3] Food Quality and Nutrition Department, Research and Innovation Centre - Fondazione Edmund Mach, S. Michele all'Adige (TN), Italia

It is becoming increasingly clear that T cell function and differentiation are closely connected with metabolic programs, because of that there is huge interest to develop techniques that could manipulate metabolism of immune cells for immunotherapy. During differentiation, T cells move from a nutrient sufficient environment in the secondary lymphoid organs to sites of inflamed peripheral tissues where there is a different nutritional status, low oxygen pressure and altered levels of other signals needed for immune cell metabolism. These new environmental conditions force T cells to metabolically adapt in order to survive and to perform their primary function. Here we show that CD8+ memory and CD8+ naïve T cells from spleen, but not CD8+ T cells from the small intestine, possess substantial mitochondrial spare respiratory capacity (SRC). This indicates that CD8+ T cells sourced from lymphoid organs exhibit higher reserves of energy which can be used in response to stress or inflammation. We also show that mitochondrial density in CD8+ memory and CD8+ naïve T cells is higher than found in CD8+ and γδ T cells sourced from the small intestine. Our transcriptome analysis of these subtypes of CD8+ T cells highlighted gene candidates that may be able to explain why mitochondrial respiratory capacity of CD8+ memory and CD8+ naïve T cells sourced from spleens is higher than CD8+ and γδ T cells sourced from the small intestine. In conclusion, our findings give insights into how CD8+ T cells adapt to different metabolic capacities depending on their functional requirements and the tissue environment they encounter.

CD8$^+$ T cells and their metabolic plasticity

Špela Konjar

Postdoctoral researcher (Feb. 2012-May 2015)

Babraham Institute

Cambridge, UK

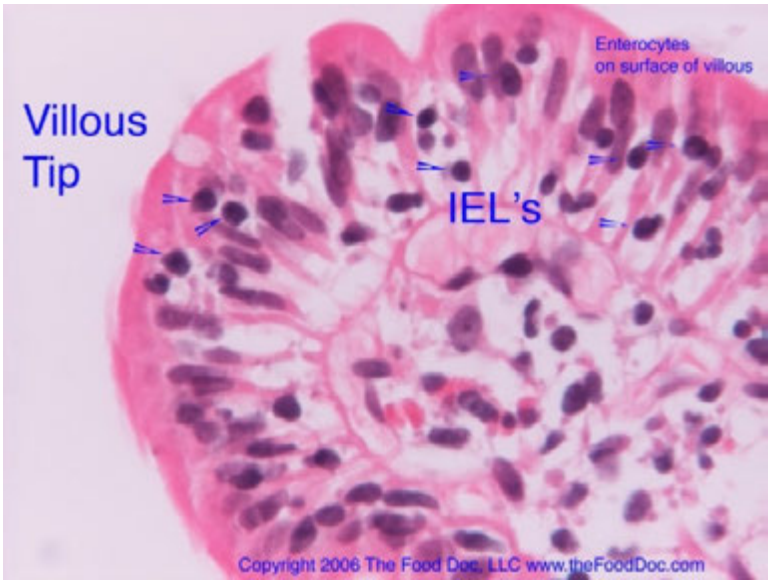
Babraham Institute

BBSRC bioscience for the future

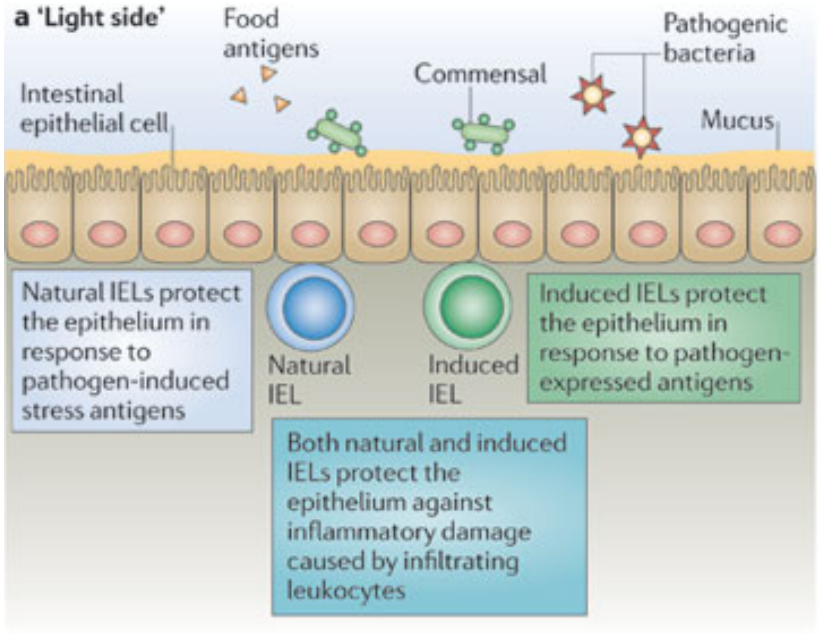
European Research Council erc

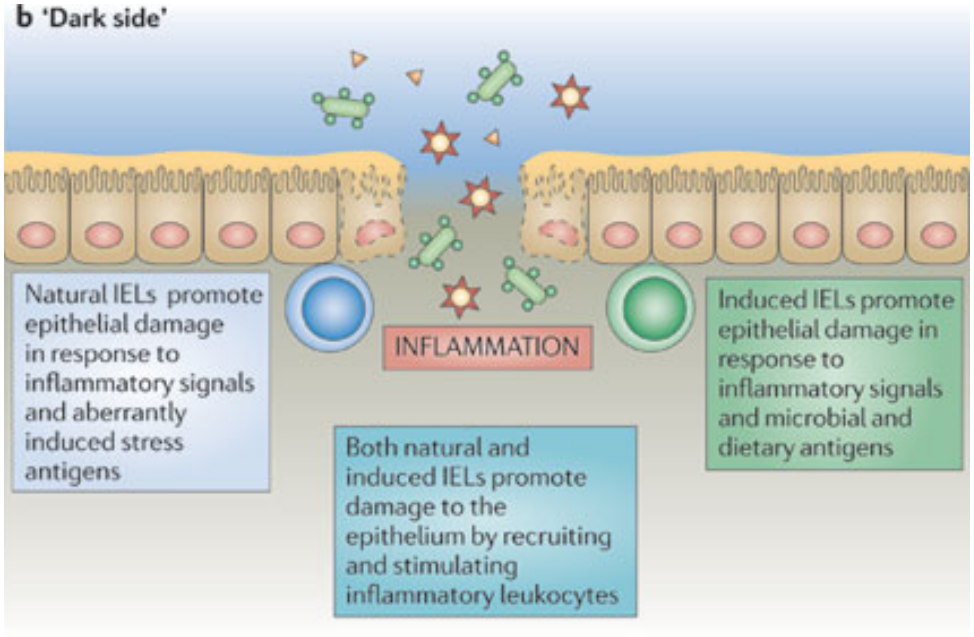
Intraepithelial Lymphocytes (IEL)

- Distinct population of **CD8$^+$T** cells
- T cell receptor; TCR γδ cells in skin and **TCR γδ** and TCRβ$^+$CD8αα$^+$ in the intestine
- In the small intestine 60% of IEL are TCRγδ$^+$
- Abundant cytoplasmic granules: cytotoxic activity, express effector cytokines (IFN-γ)
- Activating and inhibitory NK cell receptors
- Express CD103 (interaction with intestinal epithelial cells)



Light(good) and dark (bad) side of intestinal IELs

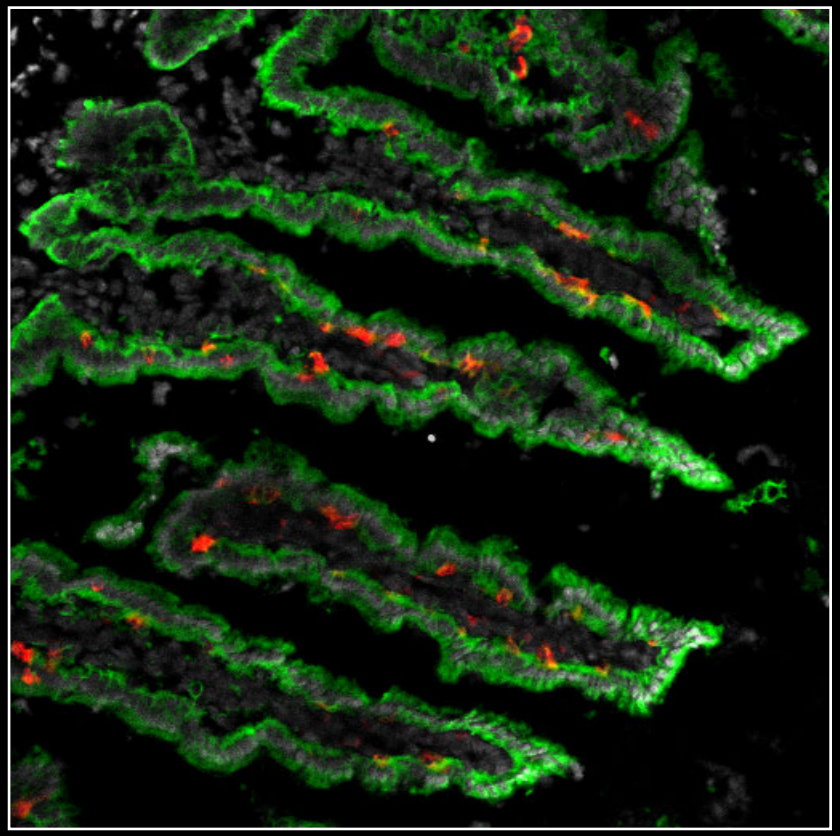




Nature Reviews | Immunology
Cheroutre et al., 2012

IELs are present at all epithelial barrier sites:

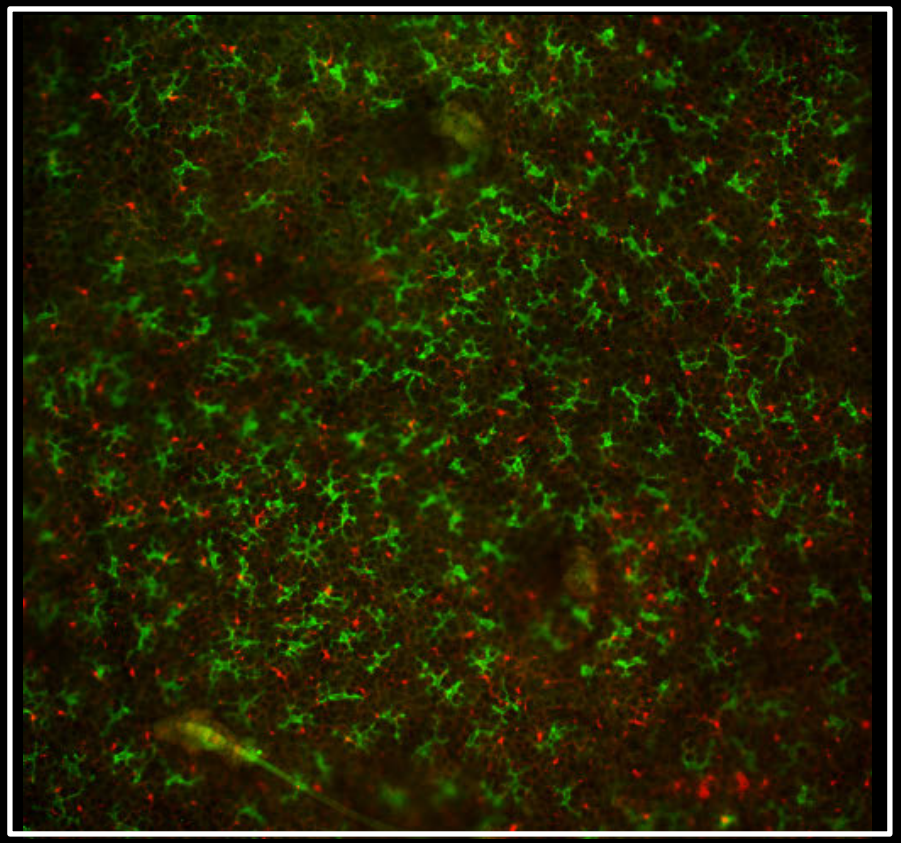
Intestine



Li, Y. *et al*. (2011) Cell

(IELs in red)

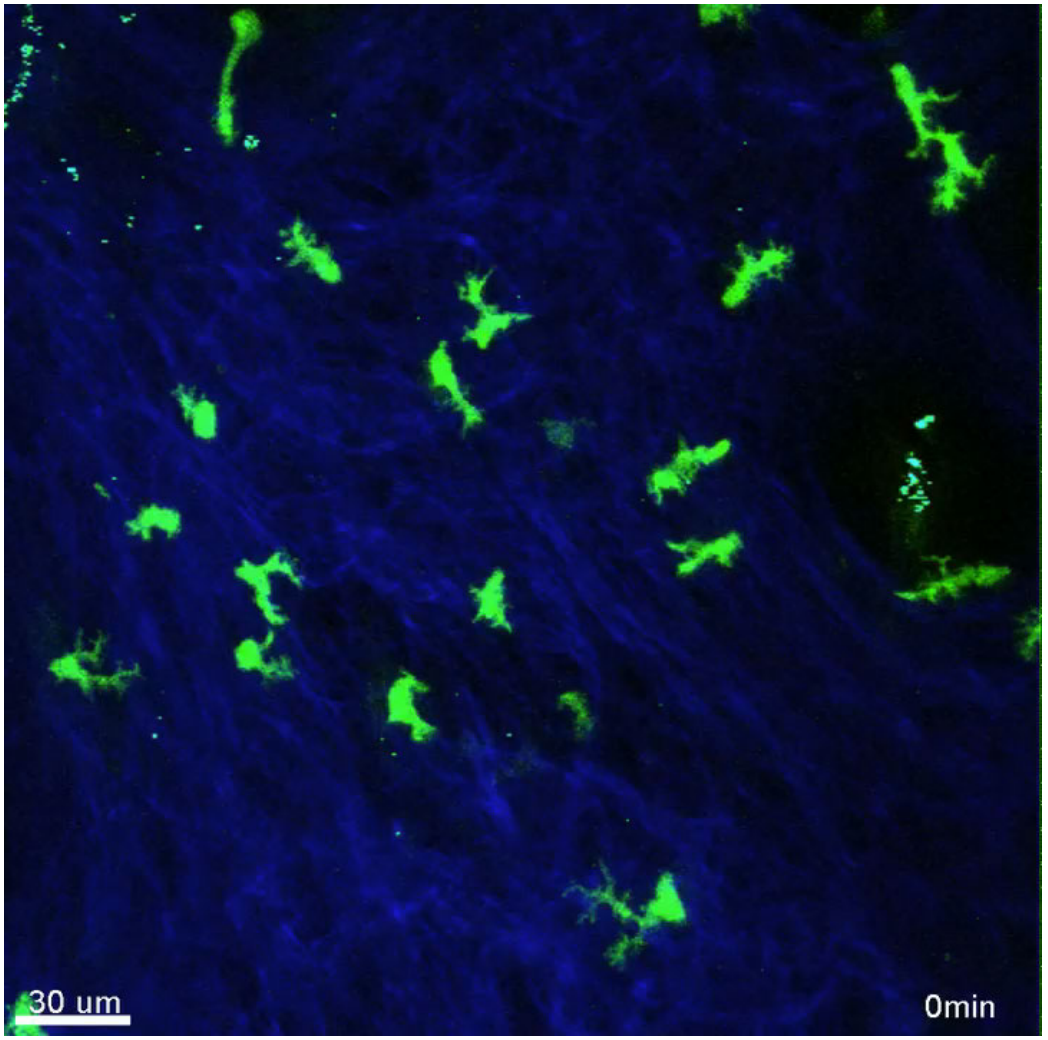
Skin



Yunhua Loo

(DETC in green)

Migration of IELs in the skin



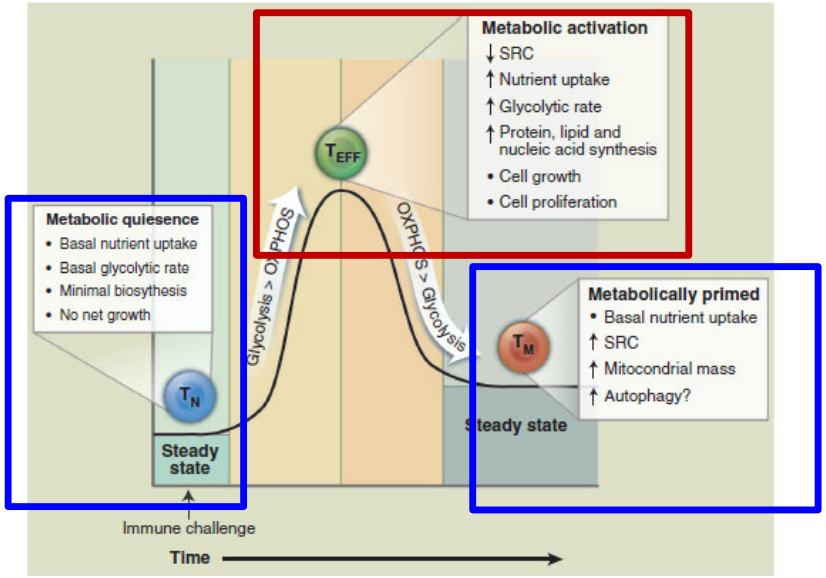
http://www.pnas.org/content/suppl/2014/03/24/1322292111.DCSupplemental/sm01.mov

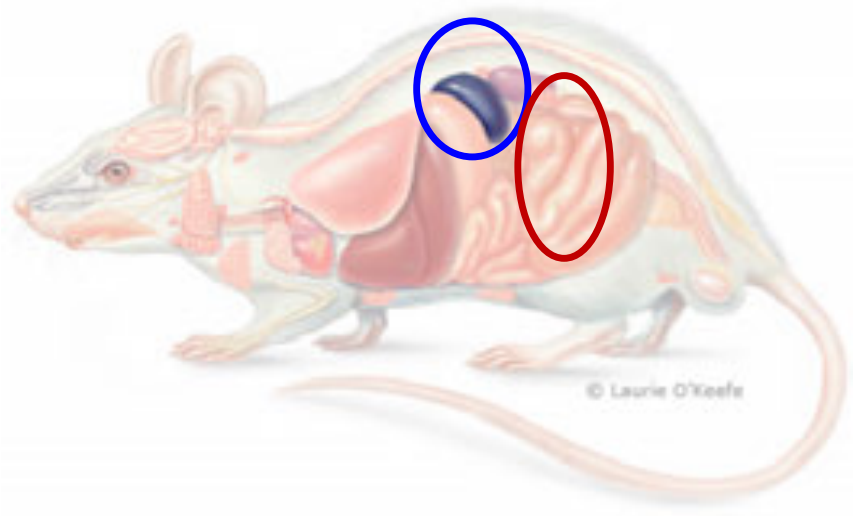
Zaid et al.,(2014) PNAS

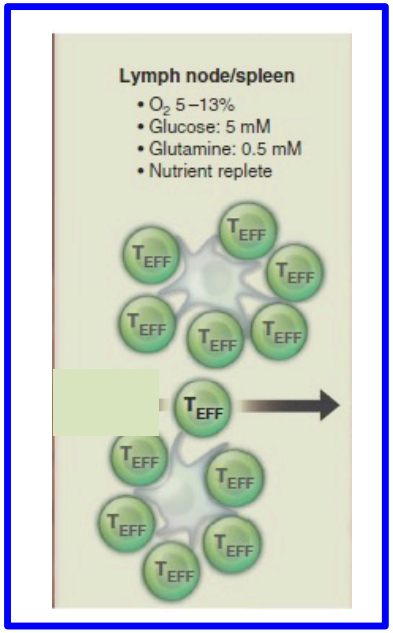
$CD8^+$ T Cells and their metabolic plasticity

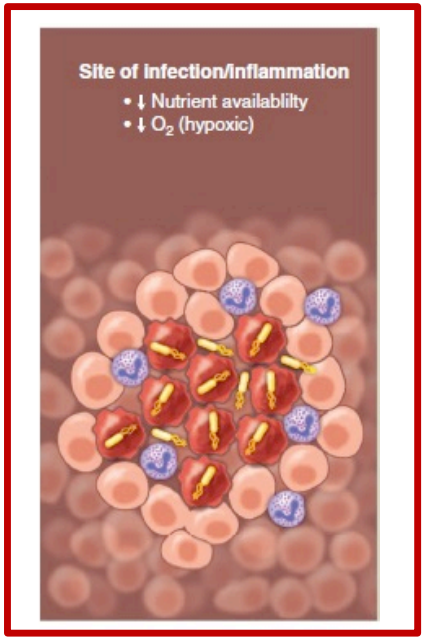
$CD8^+$ memory ($CD8^+CD44high$) and $CD8^+$ naïve ($CD8^+CD44low$) from spleen

IEL; $CD8^+$ T cells (γδ and αβ T cells) from small intestine



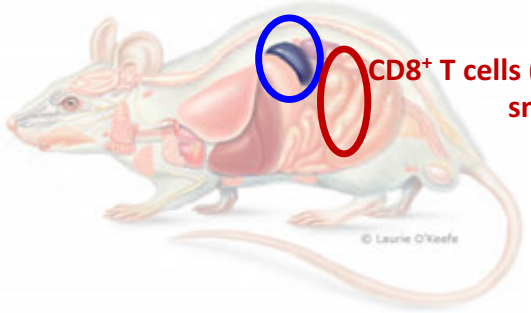






Preparation of CD8⁺ T Cells

CD8⁺ memory (CD8⁺CD44high) and CD8⁺ naïve (CD8⁺CD44low) from spleen



IEL; CD8⁺ T cells (γδ and αβ T cells) from small intestine

Preparation of IELfraction
(labeling with CD8+-apc , TCRβ-pe)
Prepation of spleenocytes
(labeling with CD8+-apc, CD44+-PB)

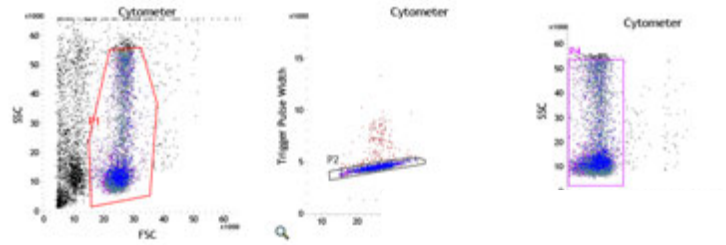
Separation on Automacs,
using APC beads, + selection on CD8+-APC

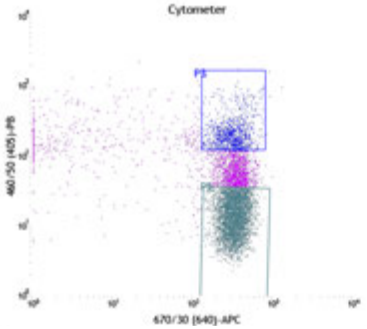
Sorting on Influx

Cells used for:

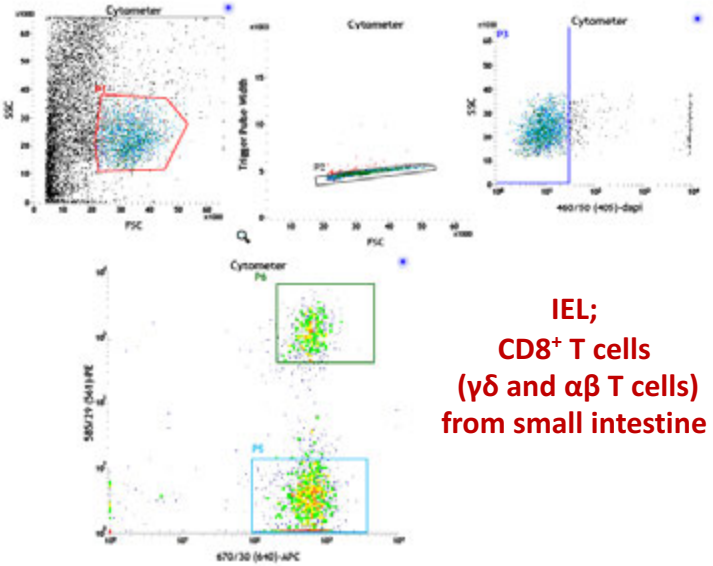
- RNA Seq
- for *in vitro* experiments
- microscopy

Sorting on Influx



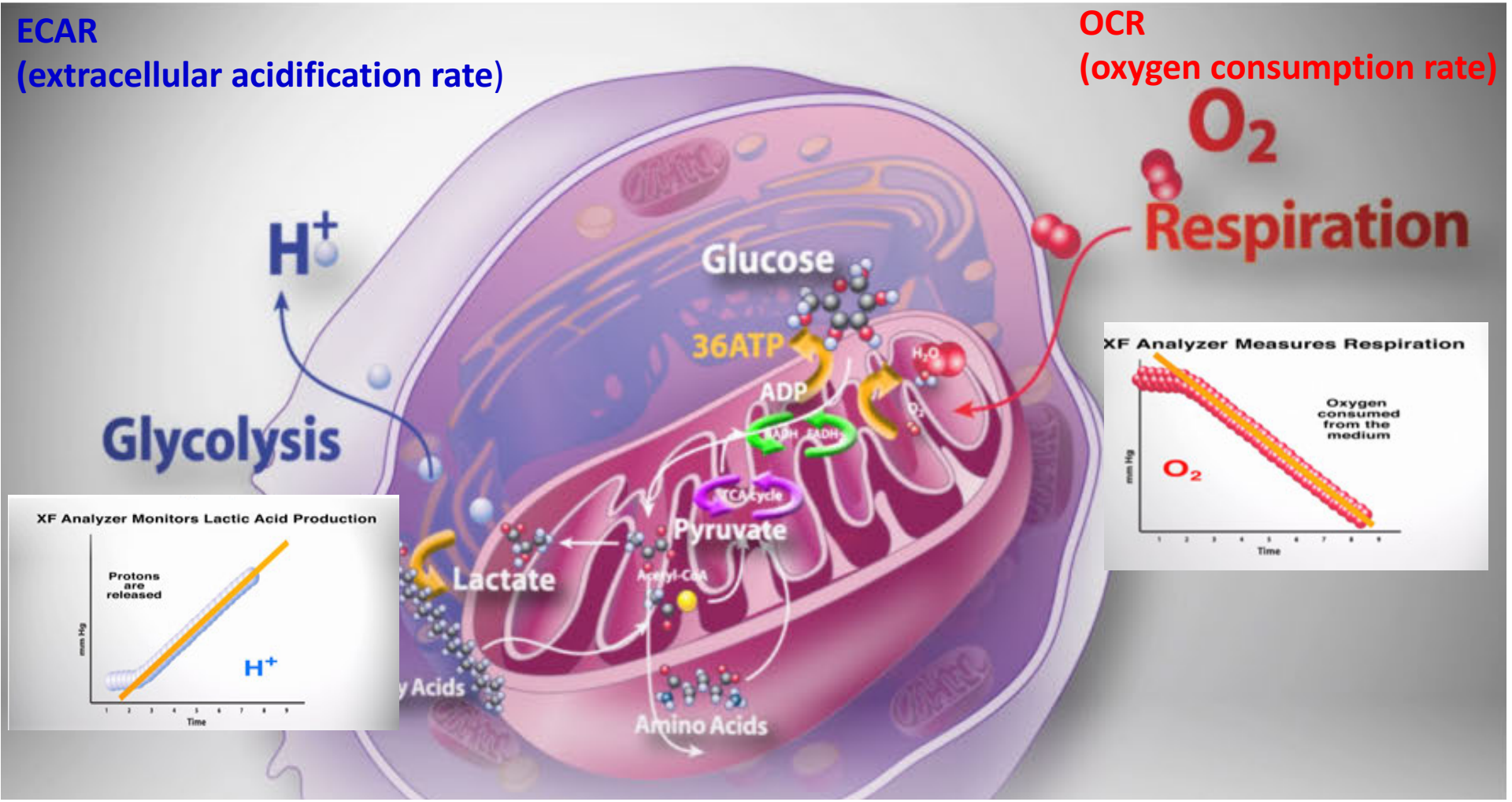


CD8⁺ memory (CD8⁺CD44high) and CD8⁺ naïve (CD8⁺CD44low) from spleen



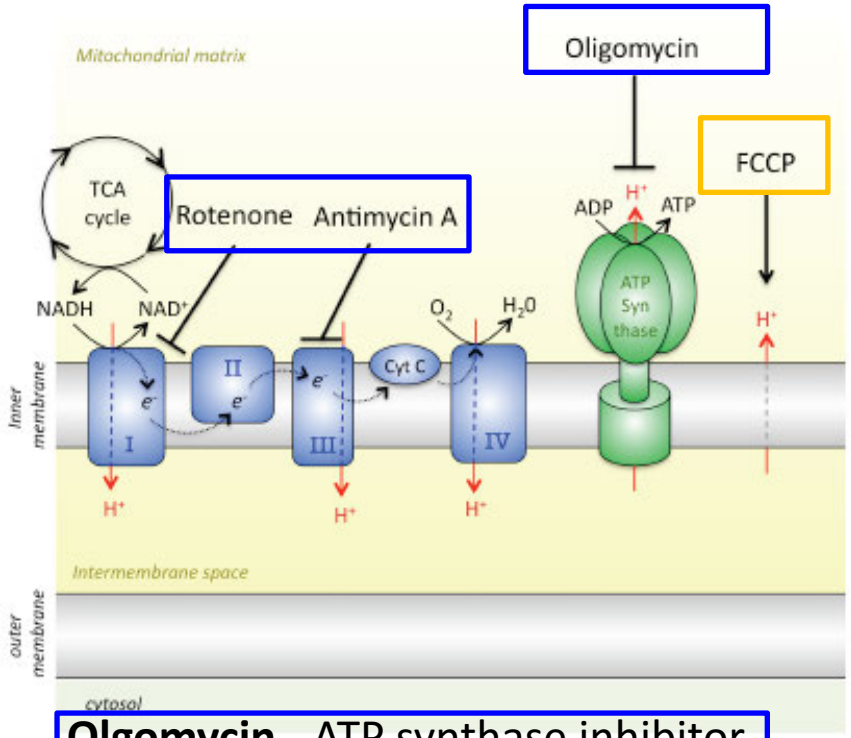
IEL; CD8⁺ T cells (γδ and αβ T cells) from small intestine

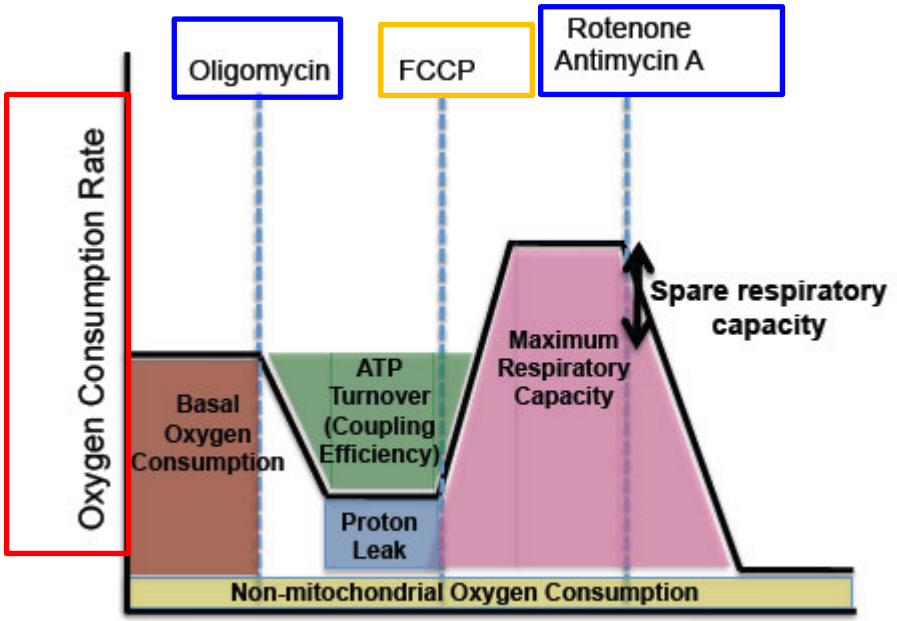
Energy pathways



Mitochondrial respiration

$C_6H_{12}O_6$ (or other substrates as fatty acids, amino acids) + $6O_2$ → $6CO_2$+ $6H_20$+ATP





Olgomycin- ATP synthase inhibitor

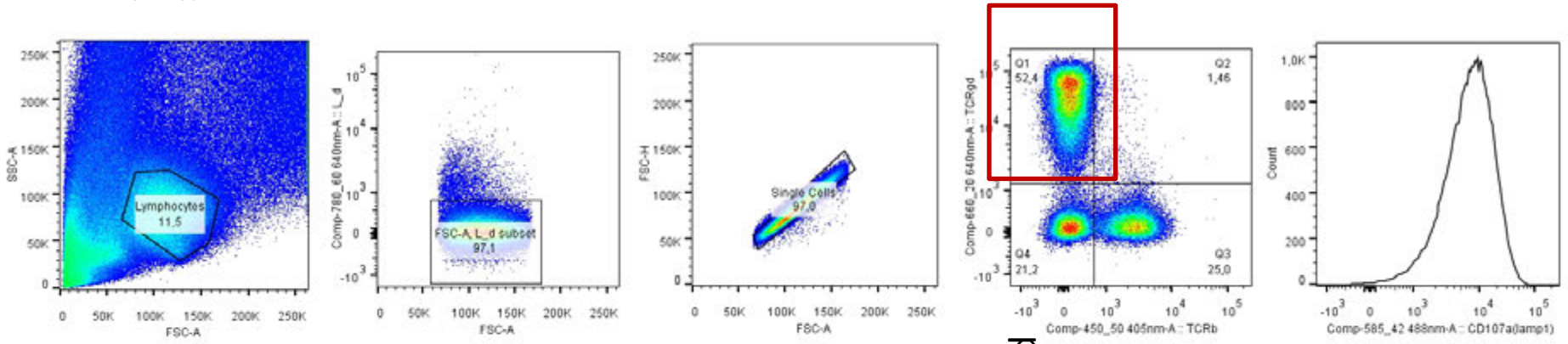
FCCP-protonphore- allows protons to cross lipid bilayers and uncouples ATP synthesis from electron transport chain

Rotenone –complex I inhibitor

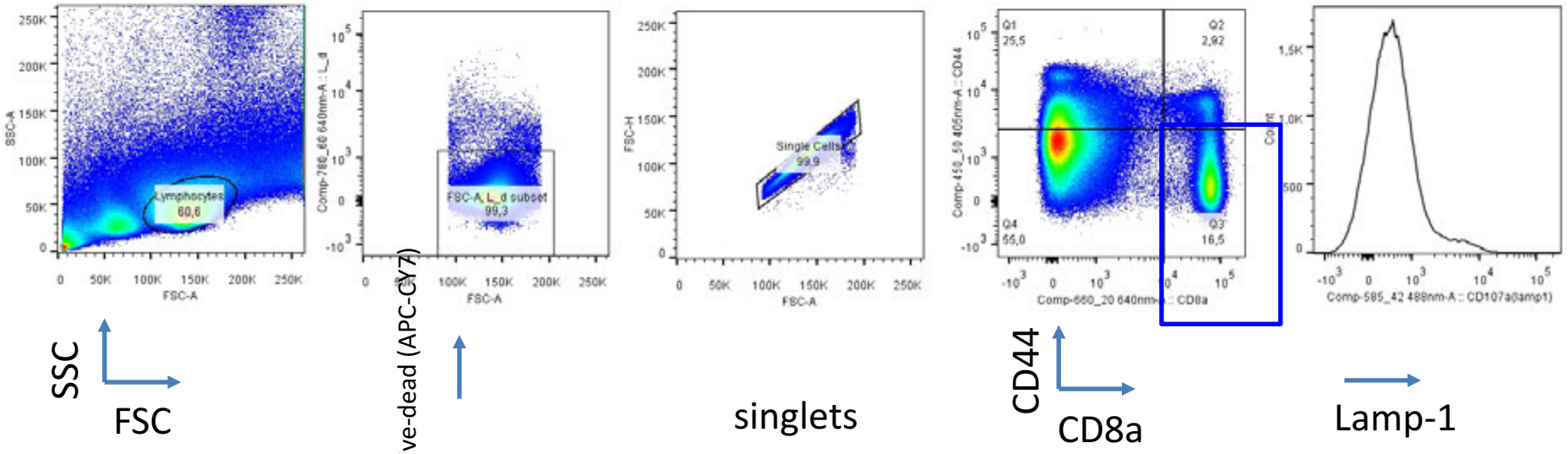
Antimycin A-complex III inhibitor

Comparison of IELs and CD8+nai

IELfr



spleenocytes



SINGLE-CELL ANALYSIS USING RATIOMETRIC FLOW CYTOMETRY

Mojca Benčina

Department of synthetic biology and immunology, National Institute of Chemistry, Ljubljana, Slovenia and Center of Excellence EN-FIST, Ljubljana 1000, Slovenia

Many traditional methods provide information at population level, excluding the fact that cell cultures are very heterogeneous. Single-cell analysis, on the other hand, offers more detailed insight into population variability, thereby facilitating a considerably deeper understanding of cell physiology. Although microscopy methods can address this issue, they suffer from limitations in terms of the small number of individual cells that can be studied and complicated image processing. We developed a noninvasive high-throughput method that employs flow cytometry to analyze large populations of cells that express pHluorin, a genetically encoded ratiometric fluorescent probe that is sensitive to pH. Moreover, we developed a high-throughput method that employs a ratiometric flow cytometry for analyzing large populations of cells that express the FRET-HIV sensor. The methods enable measurements of the intracellular changes (pH or HIV protease activity) of single cells with high sensitivity and speed, which is a clear improvement, compared to previously published methods that either require pretreatment of the cells, measure cell populations, or require complex data analysis. The ratiometric flow cytometry pros and cons will be discussed.

GABER, et al. 2013. Noninvasive high-throughput single-cell analysis of HIV protease activity using ratiometric flow cytometry. Sensors 13, 16330-16346

VALKONEN, et al 2013 Non-invasive high-throughput single-cell analysis of the intracellular pH of yeast by ratiometric flow cytometry. Applied and environmental microbiology 79, 7179-7187

Single-cell analysis using ratiometric flow cytometry

Mojca Benčina

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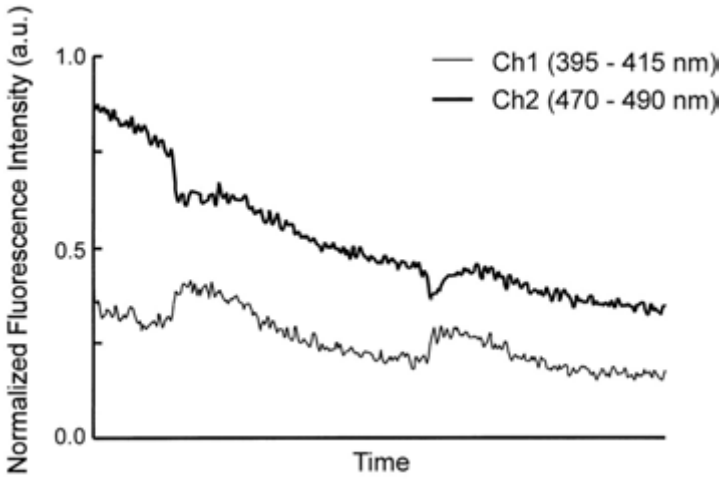
Slovenija

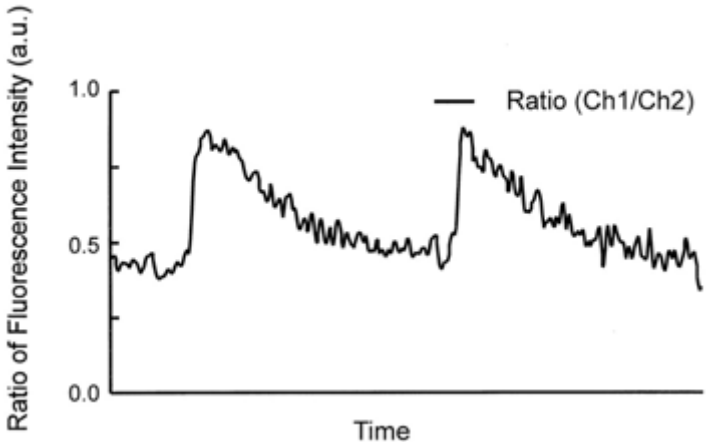
National
Institute of Chemistry
Slovenia

www.ki.si

Ratiometric flow cytometry

National
Institute of Chemistry
Slovenia

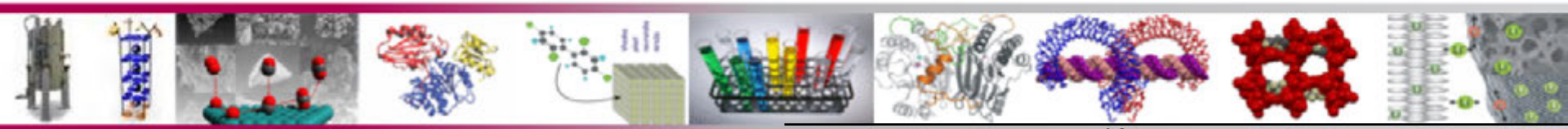




Ratiometric indicators

Advantages of ratiometric over non-ratiometric indicators

Applications and equipment



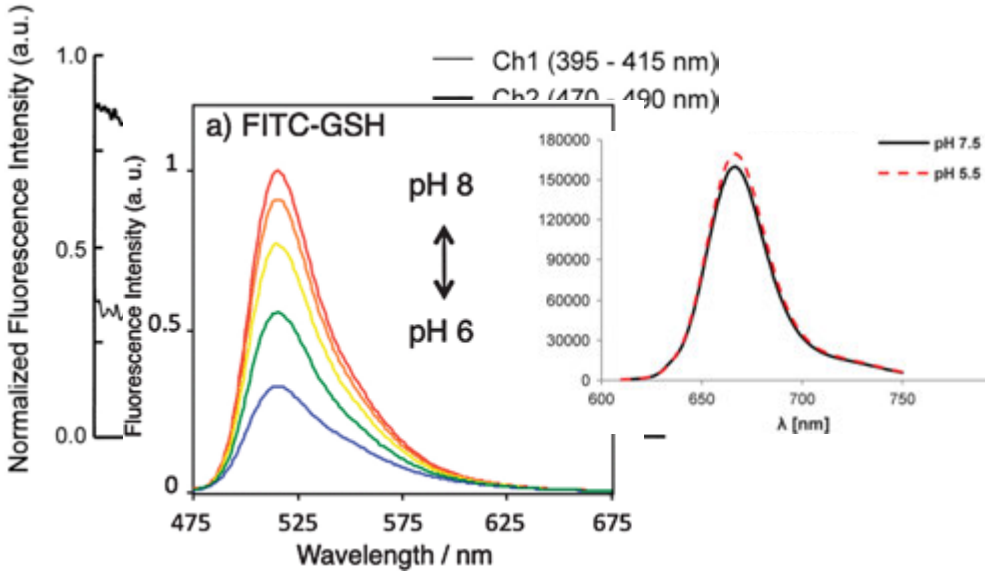
Ratiometric indicators

National
Institute of Chemistry
Slovenia

Dual characteristic of single fluorphore

Mixture of two fluorophores with different behaviour

FRET



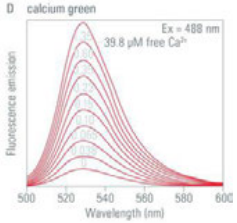
Ratiometric dyes

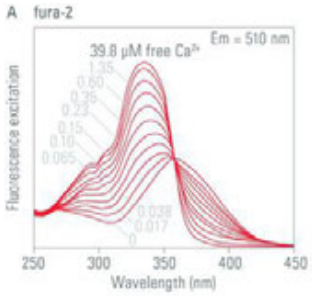
National Institute of Chemistry Slovenia

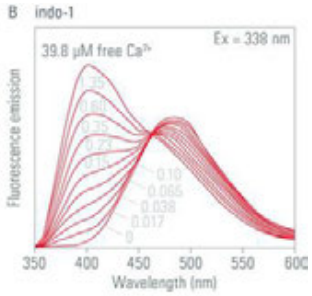
Dual characteristic of single fluorphore

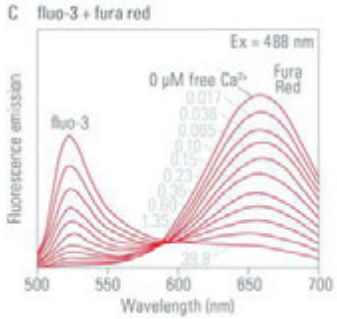
Mixture of two fluorophores with different behaviour

FRET

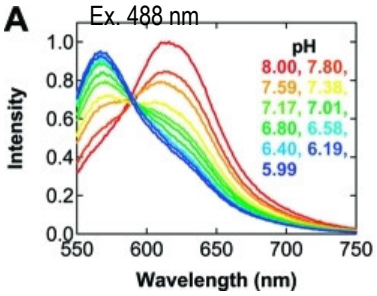


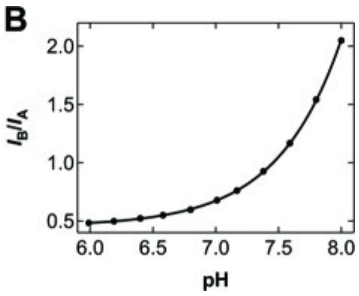


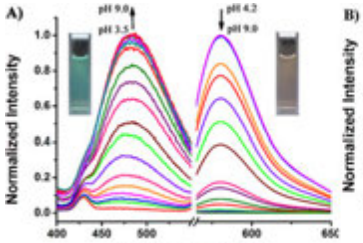




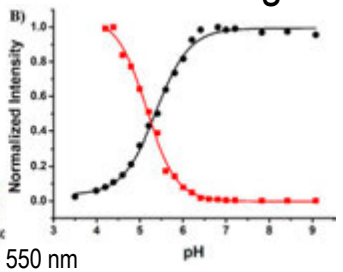
Dyes:
pH sensitive dyes
calcium sensitive dyes
ion sensitive dyes
voltage sensing dyes





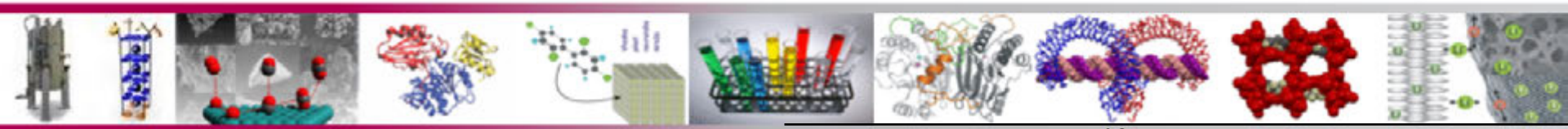


Ex. 375 nm



Ex. 550 nm

Protease, caspase activity indicators



Ratiometric biosensors

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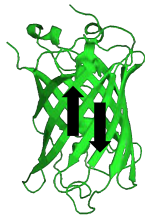
Fluorescent proteins:

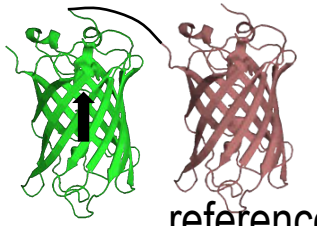
Dual characteristic of single fluorphore

ratiometric pH sensitive fluoresent proteins: pHlourin



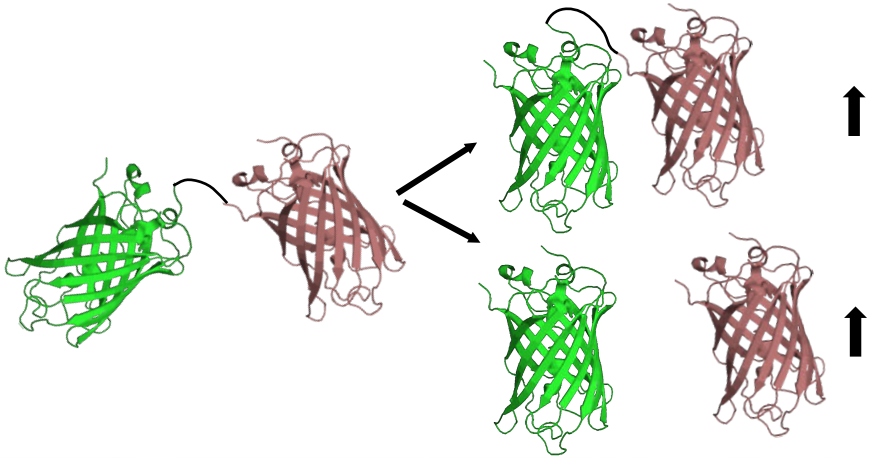
Mixture of two fluorophores with different behaviour

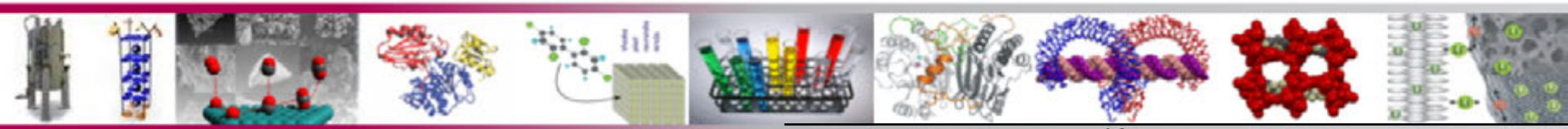
fluoresent proteins sensitive for analyte linked to



reference

FRET





Advantages of ratiometric over intensity-based indicators

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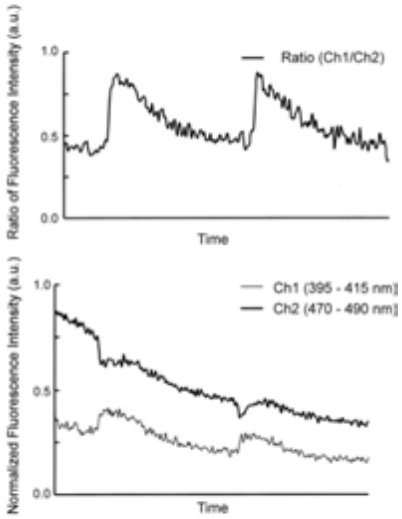
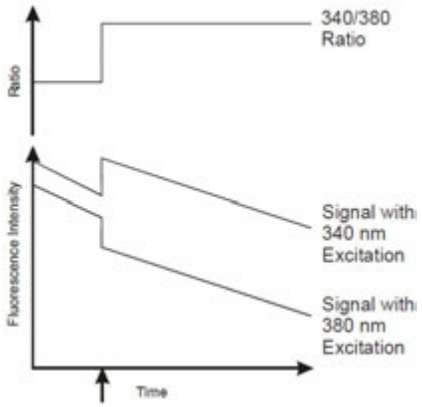
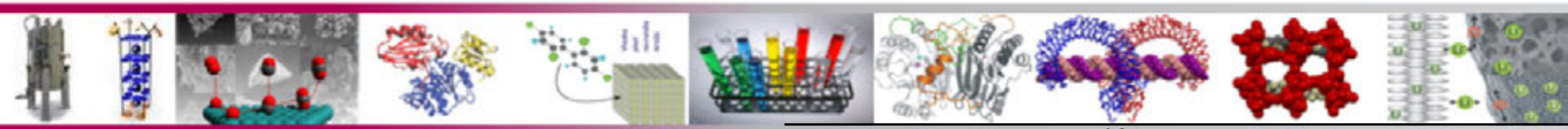
Resistance to photobleaching





Variability of indicator loading and leakage



Applications and equipment

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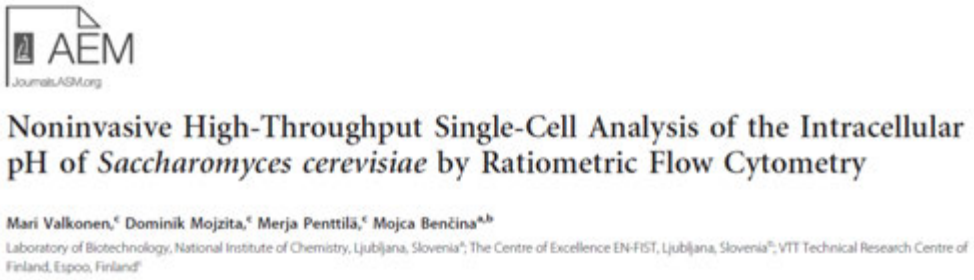
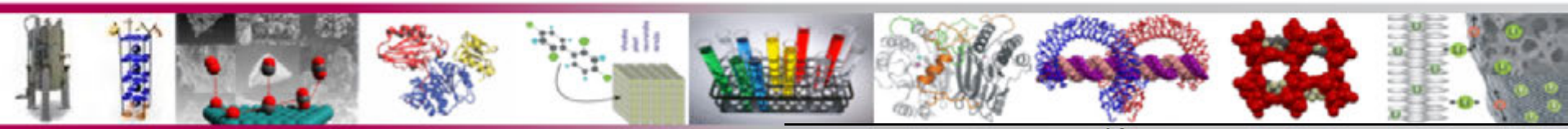
pH measurements using genetically encoded ratiometric pH sensitive fluorescent protein





The optical system of a flow cytometer to analyze ratiometric pH probes

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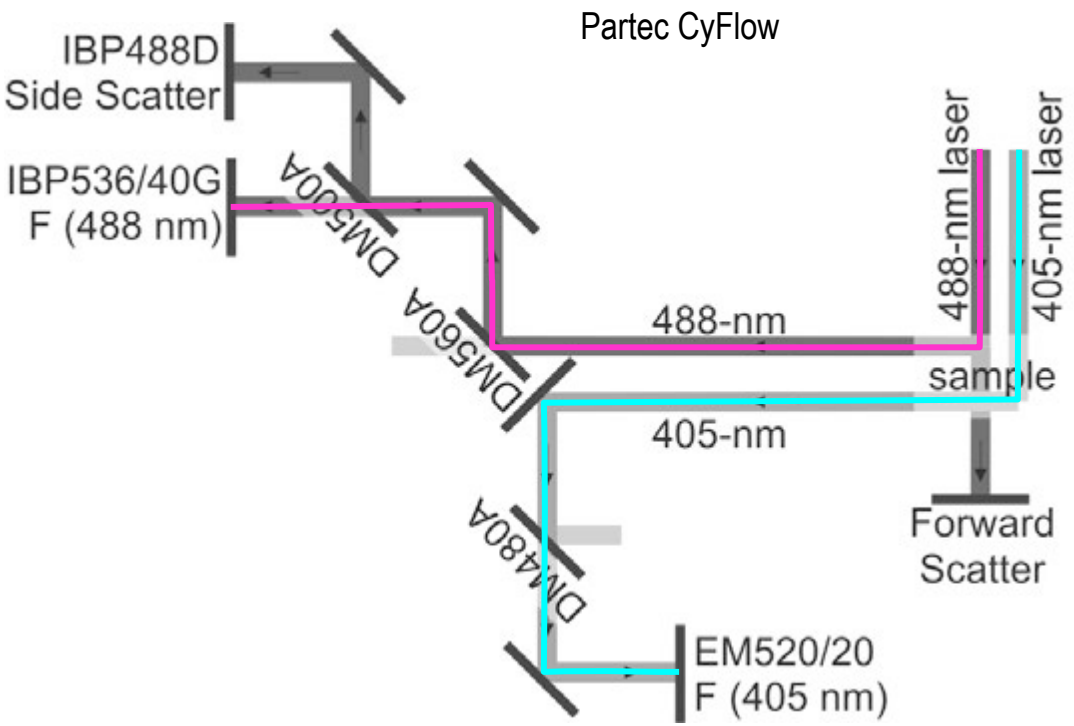
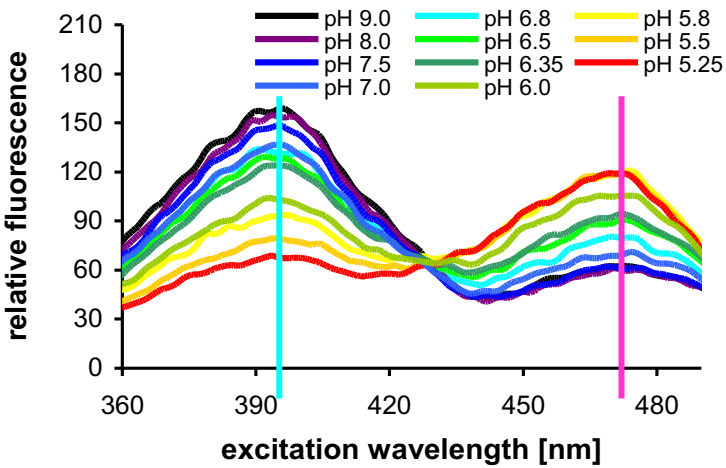
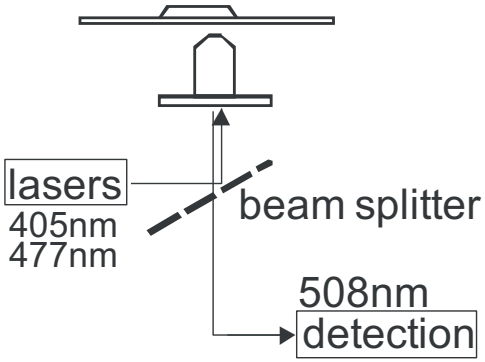
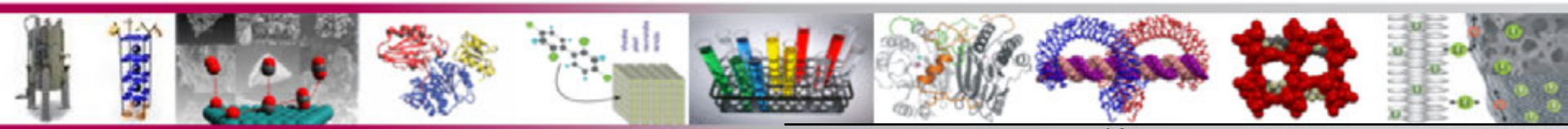




The emission optics after excitation with 405-nm laser and 488-nm laser with dichroic mirrors and filters.
A dichroic mirror (DM480A) and a filter (EM520/20) for the emission light path at 405-nm excitation differ from prefabricated versions.





Ratiometric flow cytometry of pHluorin

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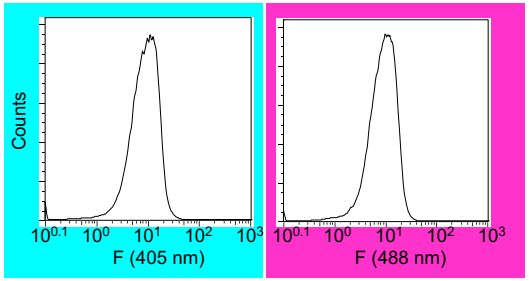
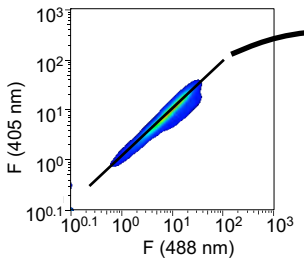
A



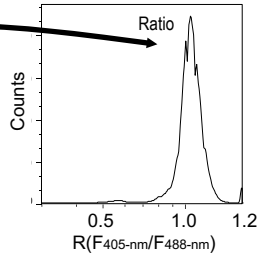
The emission signals after 405- and 488-nm excitation were collected and plotted as pulse height signals.

B



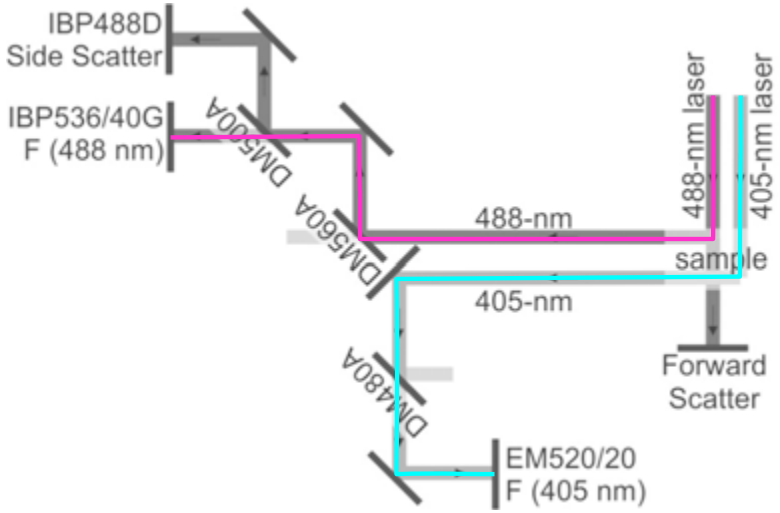
A 2D plot of the fluorescence of $F_{405\text{-nm}}$ versus $F_{488\text{-nm}}$

C



A histogram of the fluorescence intensity ratios of $F_{405\text{-nm}}/F_{488\text{-nm}}$.

Yeast cells were diluted with 2-ml spent medium and analyzed at low rate of 400 cells s^{-1}.



In situ calibration of pHluorin with ratiometric flow cytometry

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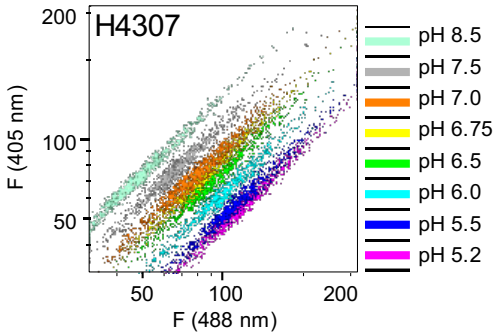
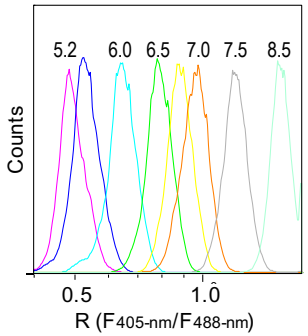
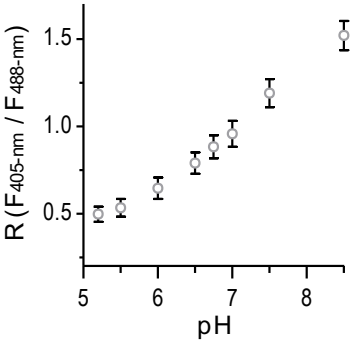


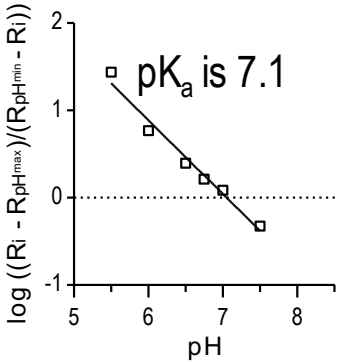
Overlay of 2D plots of $F_{405\text{-nm}}$ versus $F_{488\text{-nm}}$ fluorescence.



Overlay of histogram of the ratios of fluorescence intensities at defined pH.

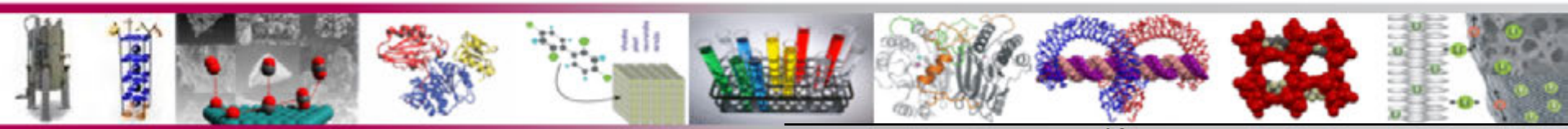


Mean values of fluorescence ratios.



Logs versus buffer pH.

Yeast cells were washed, resuspended in calibration buffers (pH 5.2 to 8.5), and treated with nigericin for 15 min before analysis



Reproducibility of calibrations for pHluorin

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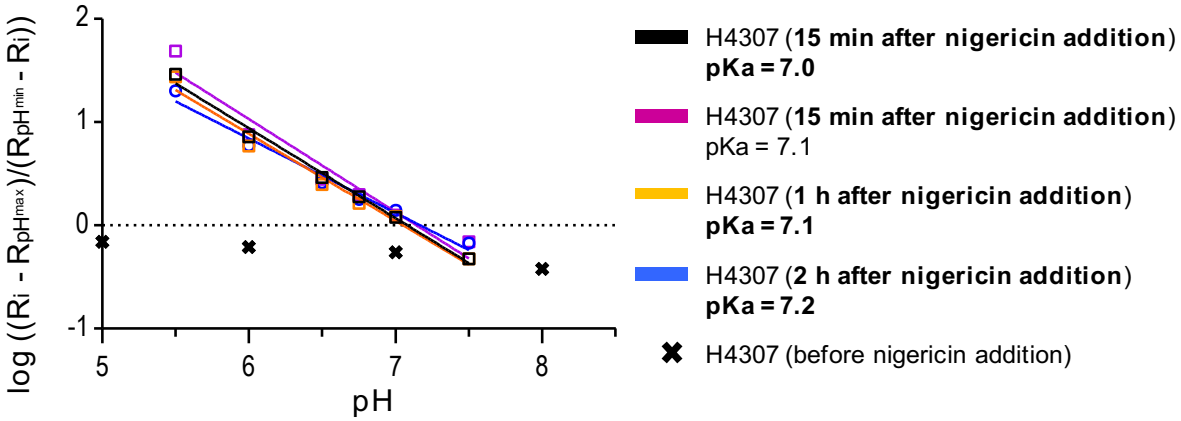
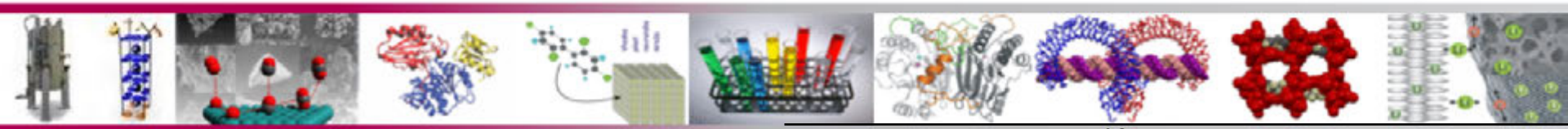


Yeast H4309 cells were washed, resuspended in calibration buffers, and treated with freshly prepared nigericin (10 μM). The cells were then analyzed at low rate of 400 cells s^{-1}. For each point at least 20,000 cells were analysed.

Each calibration curve was calculated from the results of an individual set of experiments with different incubation periods and nigericin addition.



pH_{ex} exerted only a minimal effect on the pH_i of *S. cerevisiae*

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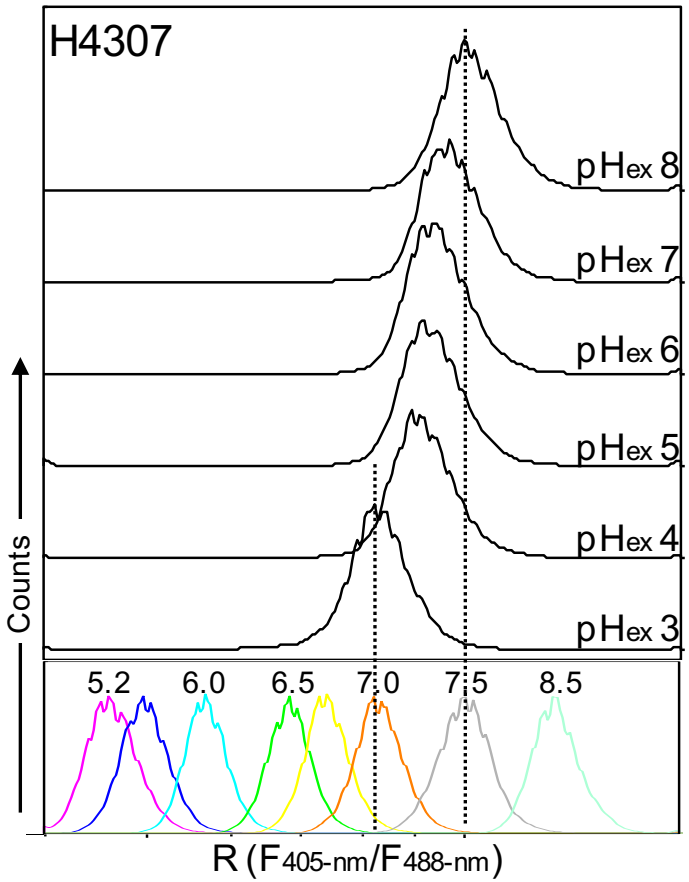
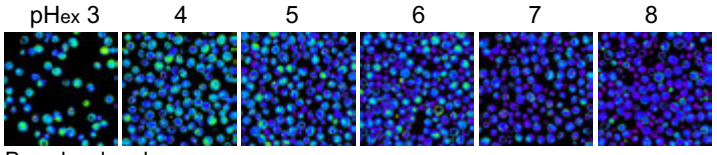
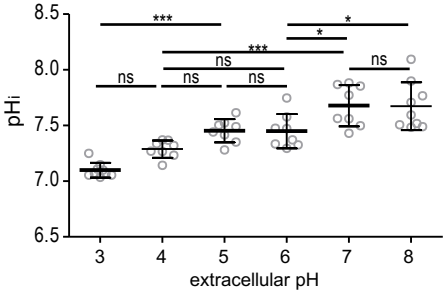


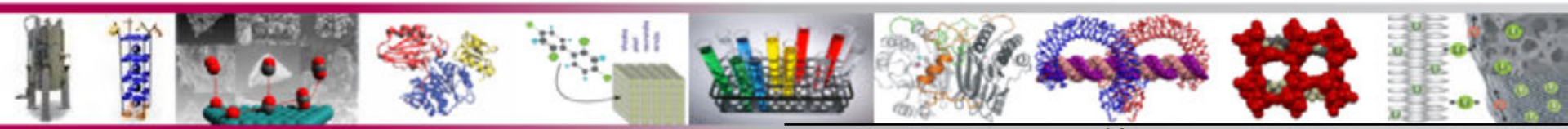
Histograms of fluorescence ratios against calibration generated at the same time.





Pseudocolored images and calculated pH_i.

Cells were harvested, washed and incubated in buffered Verduyin medium with glucose for 30 min to 1 h.



Glucose starvation acidified cells

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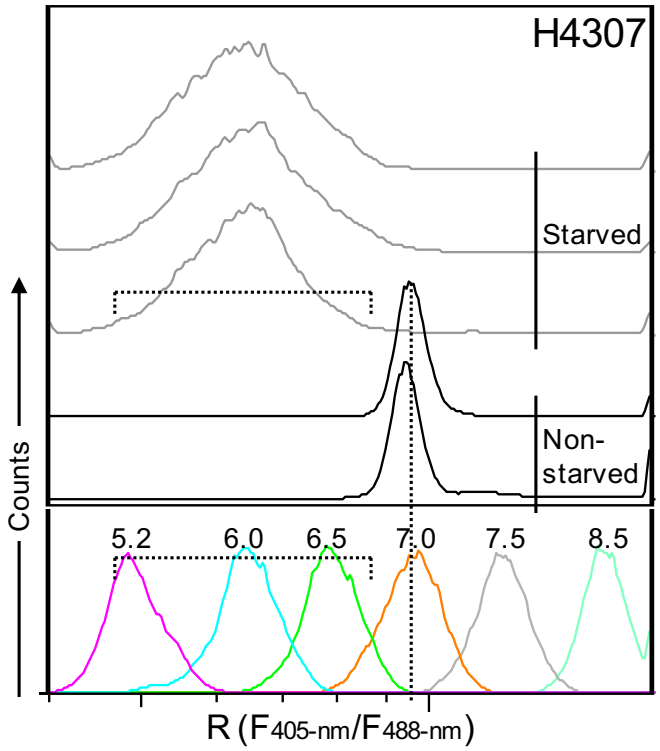
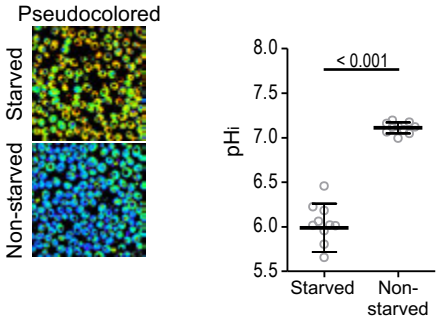


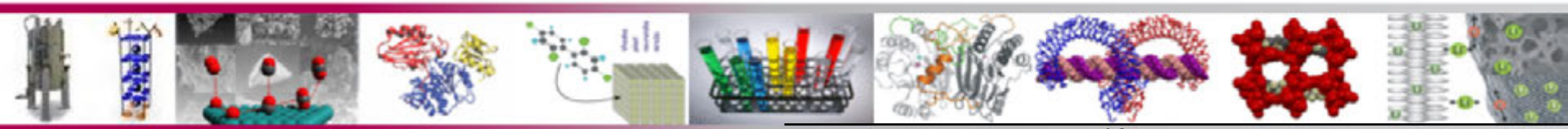
Histograms of ratios plotted against calibration.

Each point on the graphs presents a pH_i calculated from the ratio (R_i) between the emission intensities (collected at 500 to 550 nm) at 405- and 476-nm excitation for each image.



Pseudocolored images and calculated pH_i of glucose-starved and non-starved yeasts.

*Yeast cells were harvested and diluted in SC-Met media with or without 20 g l^{-1} glucose. About 30 min to 1 h after media change. pH_i was analyzed by (**C**) flow cytometry and (**D**) microscopy.*



Dynamic changes in pH_i: the application of glucose to starved cells alkalinized pH_i

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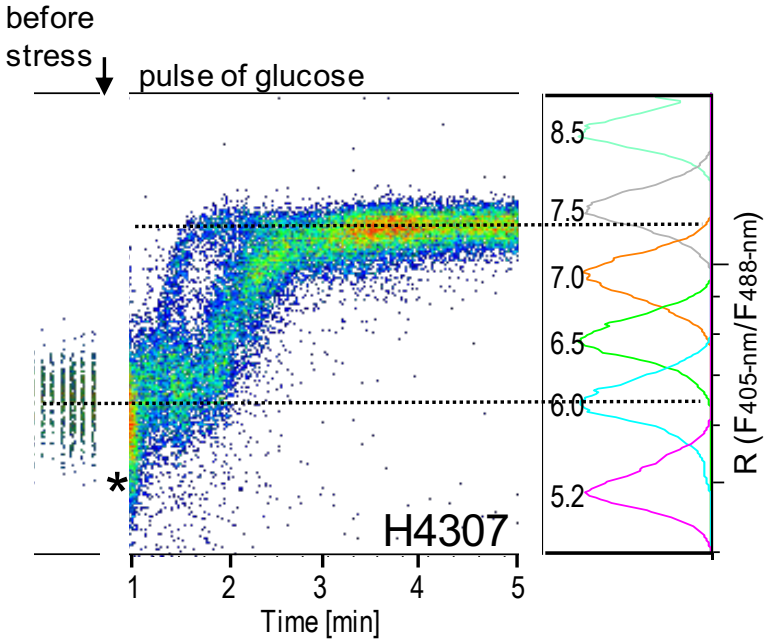
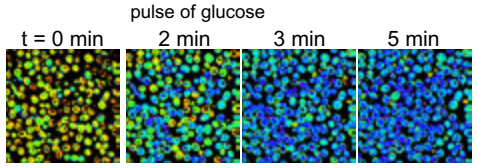
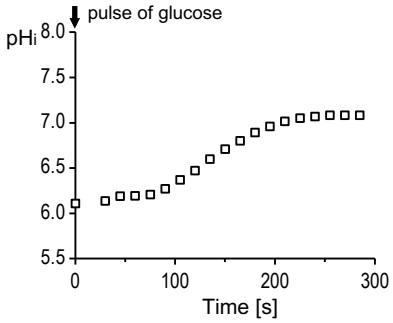


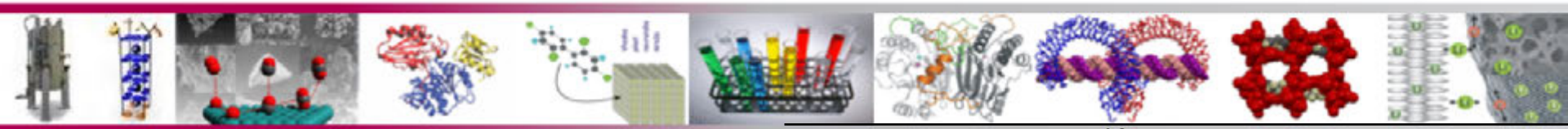
Ratios of $F_{405\text{-}nm}/F_{488\text{-}nm}$ fluorescence over time were plotted against calibration.

*Yeast H4307 grown for 1 h in SC-Met medium without glucose was fed with 20 g l^{-1} glucose. pH_i measurement was followed by (**A**) ratiometric flow cytometry and (**B**) microscopy. At least 50,000 cells over time were analyzed. The arrow indicates the addition of glucose.*



Pseudocolored images at indicated time points after glucose pulse application.





Dynamic changes in pH_i: weak sorbic acid stress acidified cytosol

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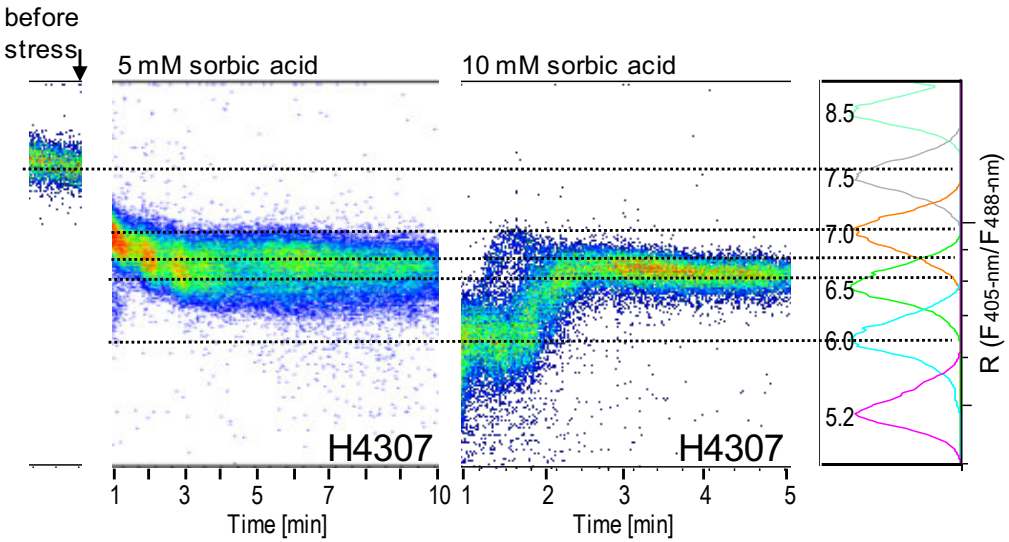
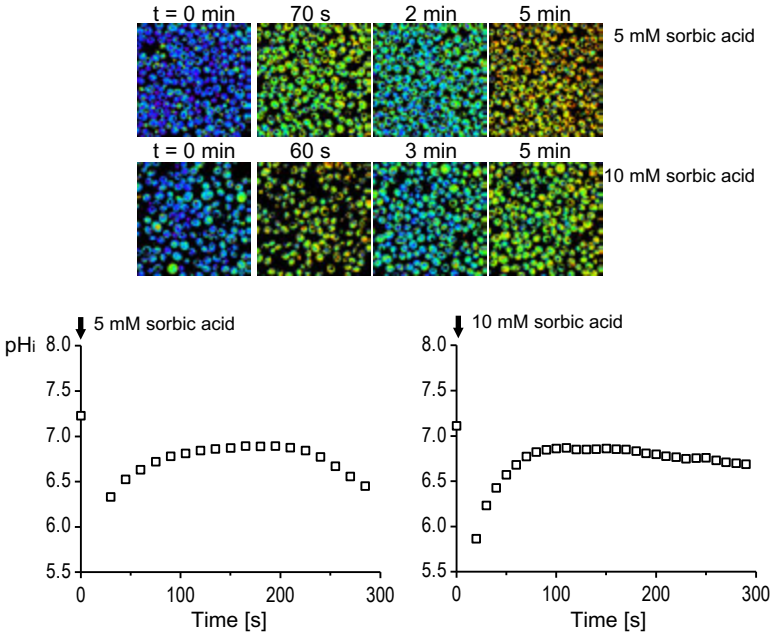
Yeast H4307 cells grown in SC-Met with 20 g l^{-1} glucose were treated with 5 and 10 mM sorbic acid. At least 50,000 cells over time were analyzed. The arrow indicates the addition of sorbic acid.



The analysis of pH_i dynamics was followed with cytometry; fluorescence ratios over time plotted against calibration are shown.



Pseudocolored images calculated from images taken at 405- and 476-nm excitation at indicated time points.

Two distinct populations with different pH_i levels

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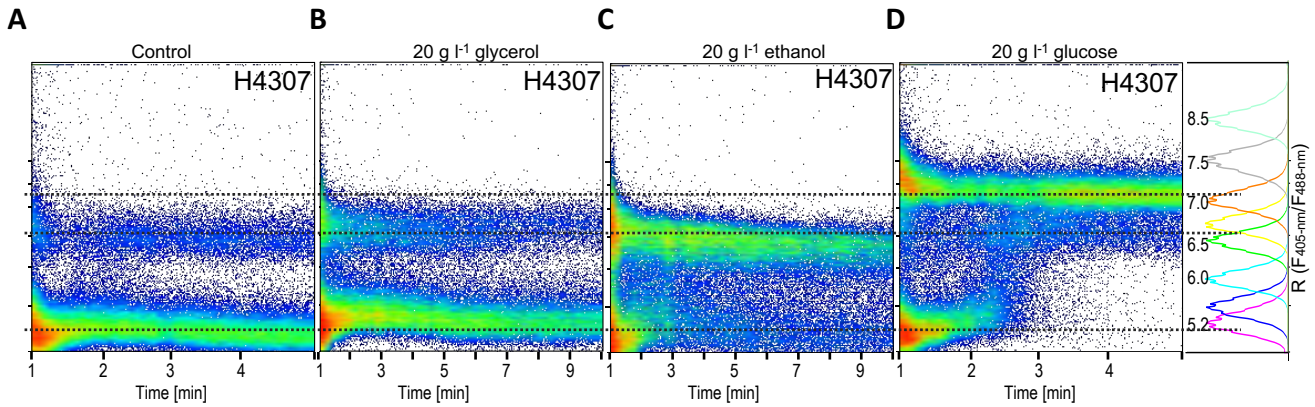
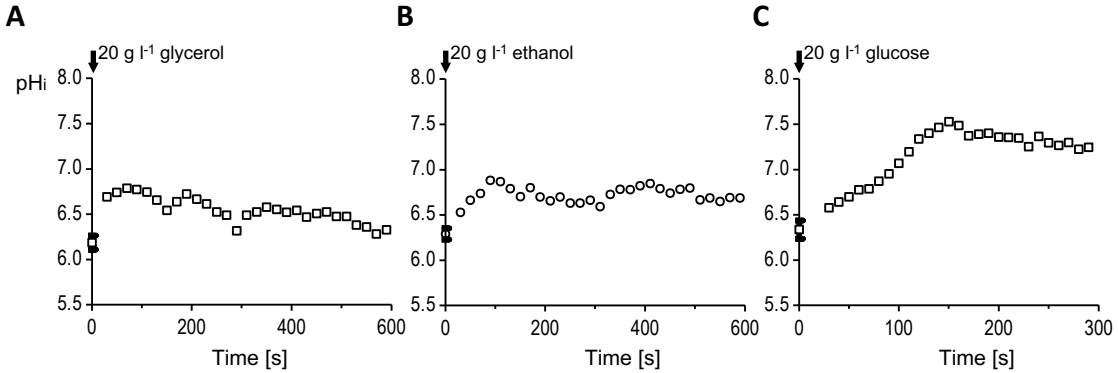


Cells in stationary phase were diluted in spent media to obtain a 2-ml cell suspension and then glycerol, ethanol, or glucose (20 g l^{-1}) was added. Fluorescence changes after addition were monitored by cytometry at a low rate of 200 cells s^{-1}. The fluorescence ratios over time were plotted against calibration. At least 100,000 cells over time were analyzed.

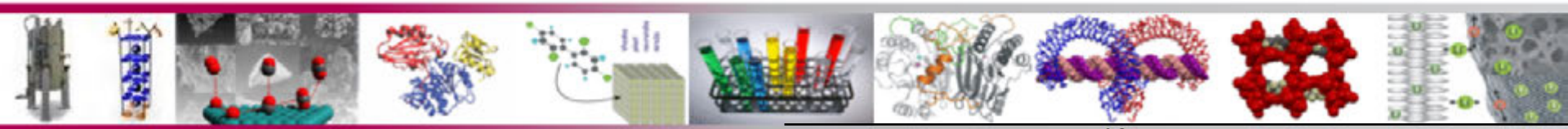
The subpopulations of steady-state cells behaved differently after (**B**) glycerol, (**C**) ethanol, or (**D**) glucose addition.



The pH_i and ratio (R_i) of the emission intensities (collected at 500 to 550 nm) at 405- and 476-nm excitation for each image were calculated. An average value (R_i and pH_i) was calculated from at least 50 cells per image. After nutrient feeding, images at 405- and 476-nm excitation were taken every 20 s.

The dynamics of the pH homeostasis of stationary cells was analyzed by confocal microscopy after feeding with 20 g l^{-1} (A) glycerol, (B) ethanol, or (C) glucose.

The assessed pH_i values present an average pH_i for the populations, which differs from the cytometry-obtained pH_i of the subpopulations.



Applications and equipment

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Protease activity measurements using FRET-based probe

Sensors **2013**, *13*, 16330-16346; doi:10.3390/s131216330

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Article

Noninvasive High-Throughput Single-Cell Analysis of HIV Protease Activity Using Ratiometric Flow Cytometry

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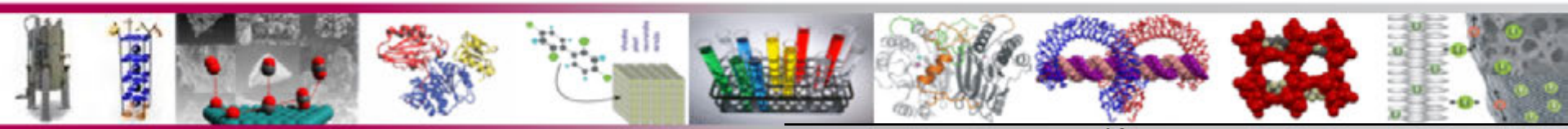
[2] Center of Excellence EN-FIST, Ljubljana 1000, Slovenia



FRET-HIV protease-sensitive sensor

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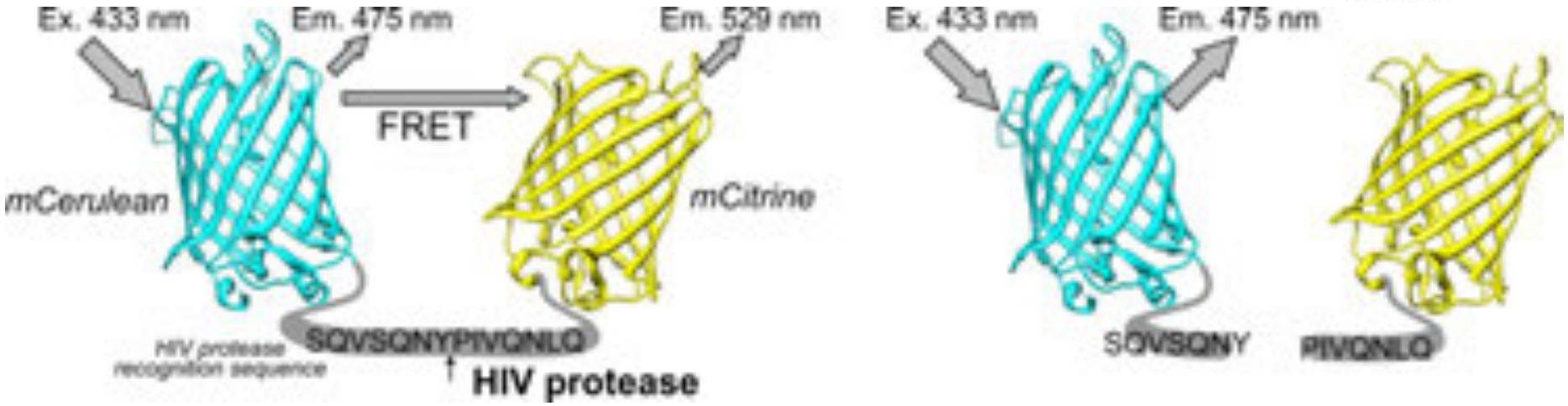

The FRET-HIV sensor is composed of a donor mCerulean protein linked to an acceptor mCitrine with a peptide, which is a target site for the HIV protease.

When excited with 433-nm light, the mCerulean emits light at 475 nm.
In close proximity with the mCitrine, some energy is transferred to mCitrine, which then emits light at 529 nm.
When the fusion protein is cleaved by the HIV protease, mCitrine is no longer in close proximity to mCerulean, resulting in a decrease of acceptor and a concomitant increase of donor fluorescence intensity.

Ratiometric flow cytometry setup

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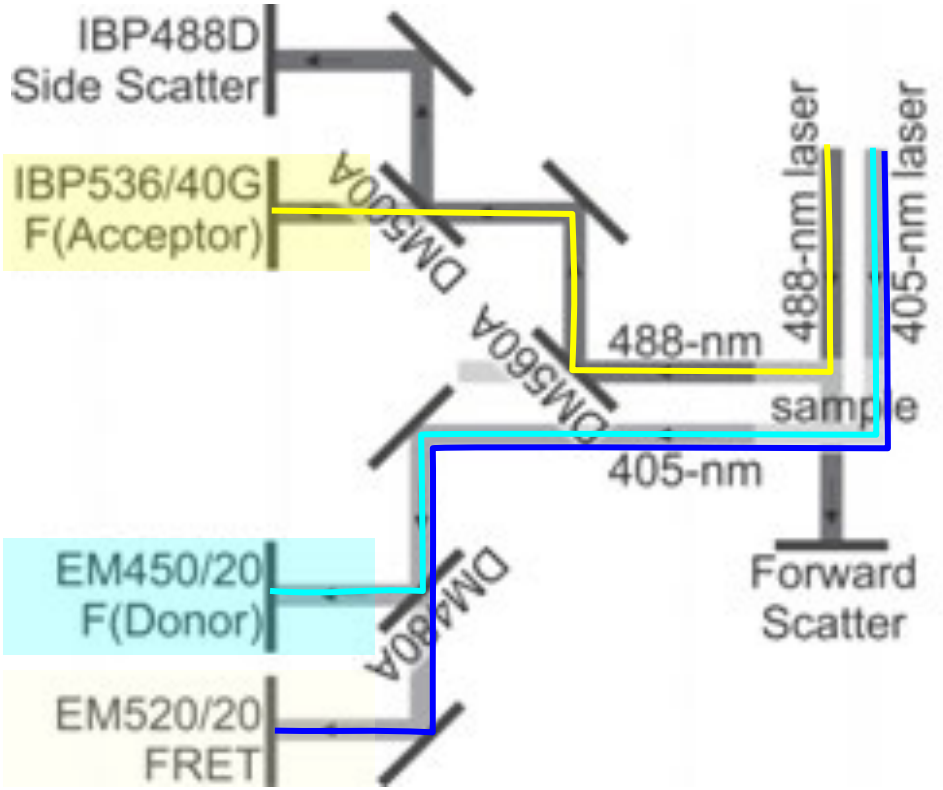

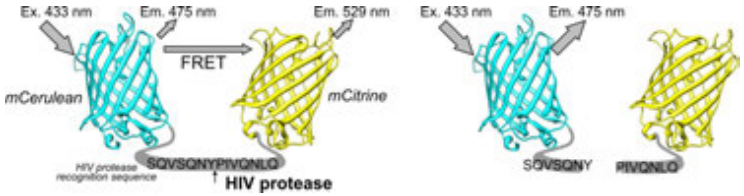

Ratiometric flow cytometry setup with emission optics after excitation with 405-nm and 488-nm lasers with dichroic mirrors and filters.
A dichroic mirror (DM480A) and a filter (EM520/20) for the emission light path at 405-nm excitation differed from the prefabricated version.

Ratiometric flow cytometry and the FRET-HIV sensor

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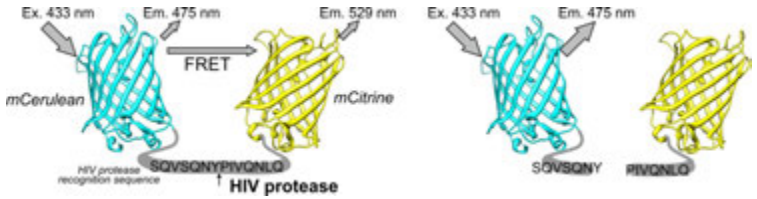
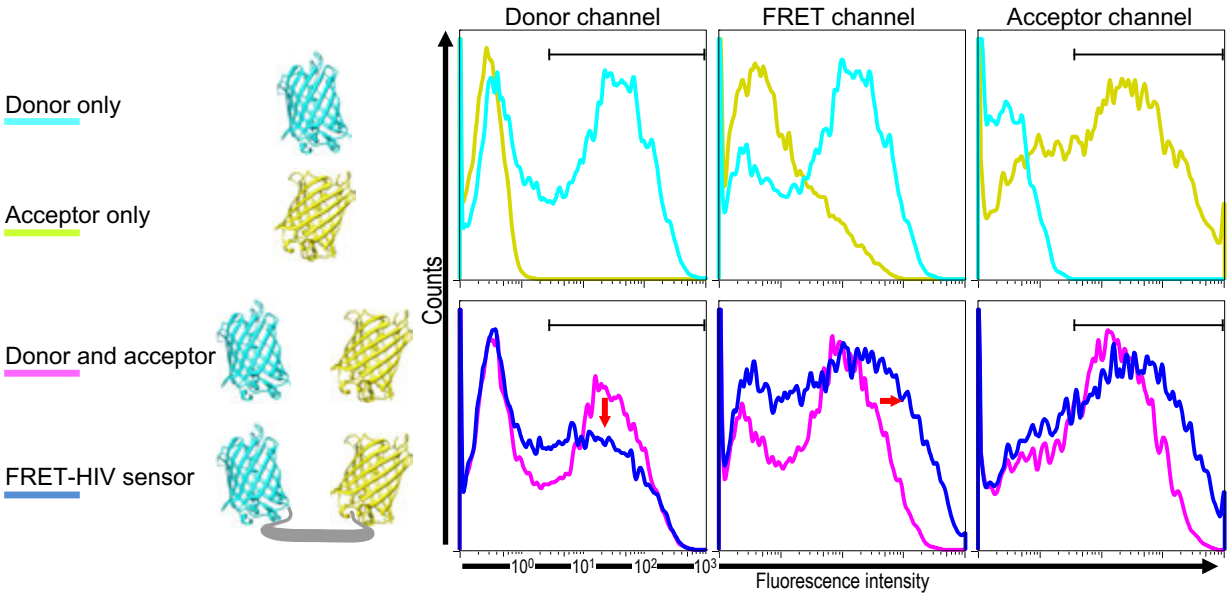
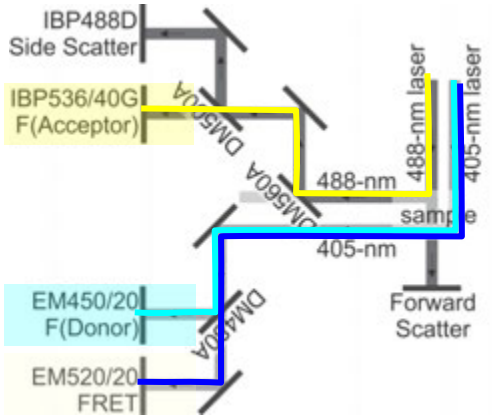
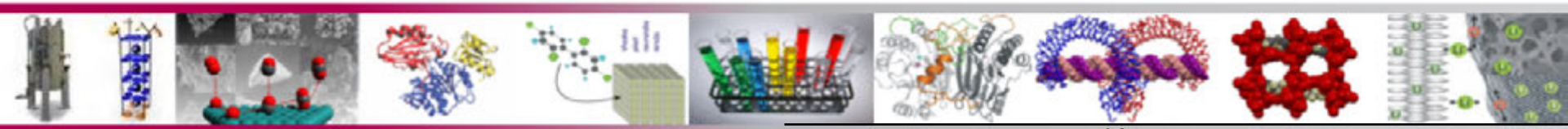




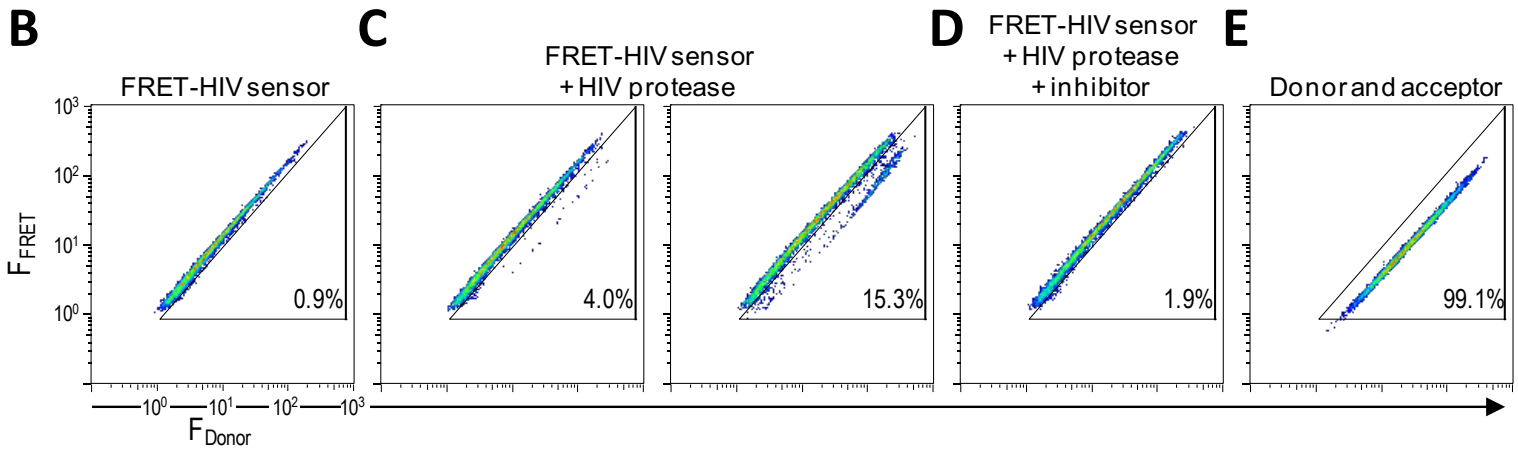


Pulse height emission signals of the donor, FRET, and acceptor of the HEK293T cells transfected with plasmids expressing the FRET-HIV sensor and controls after excitation with 405- and 488-nm lights. The arrows indicate the fluorescence intensity shifts forced by FRET.

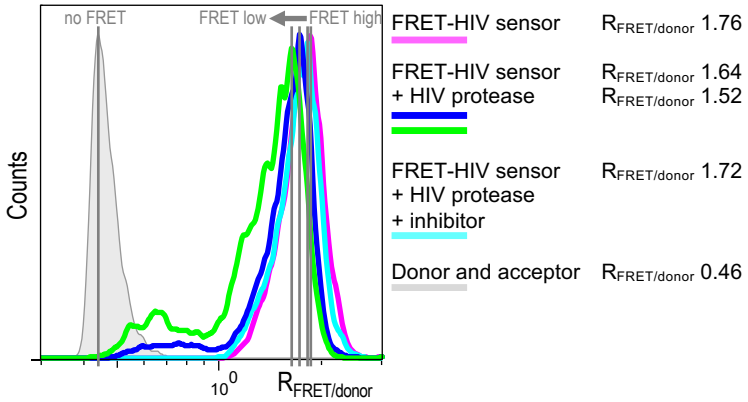


Ratiometric flow cytometry and the FRET-HIV sensor

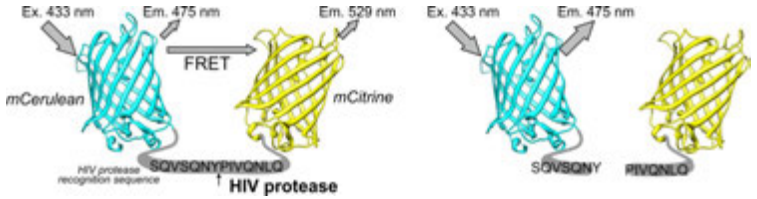
National Institute of Chemistry Slovenia



2D plots of the fluorescence intensities of the F_{FRET} *versus* the F_{donor} of cells transfected with plasmids expressing (**B**) the FRET-HIV sensor, (**C**) the FRET-HIV sensor with the HIV protease without or (**D**) with the protease inhibitor saquinavir (5 μM), and (**E**) the mCerulean and mCitrine expressed separately.



Histogram of the fluorescence intensity (F_{FRET}/F_{donor}) ratios.



Sensitized emission FRET analysis

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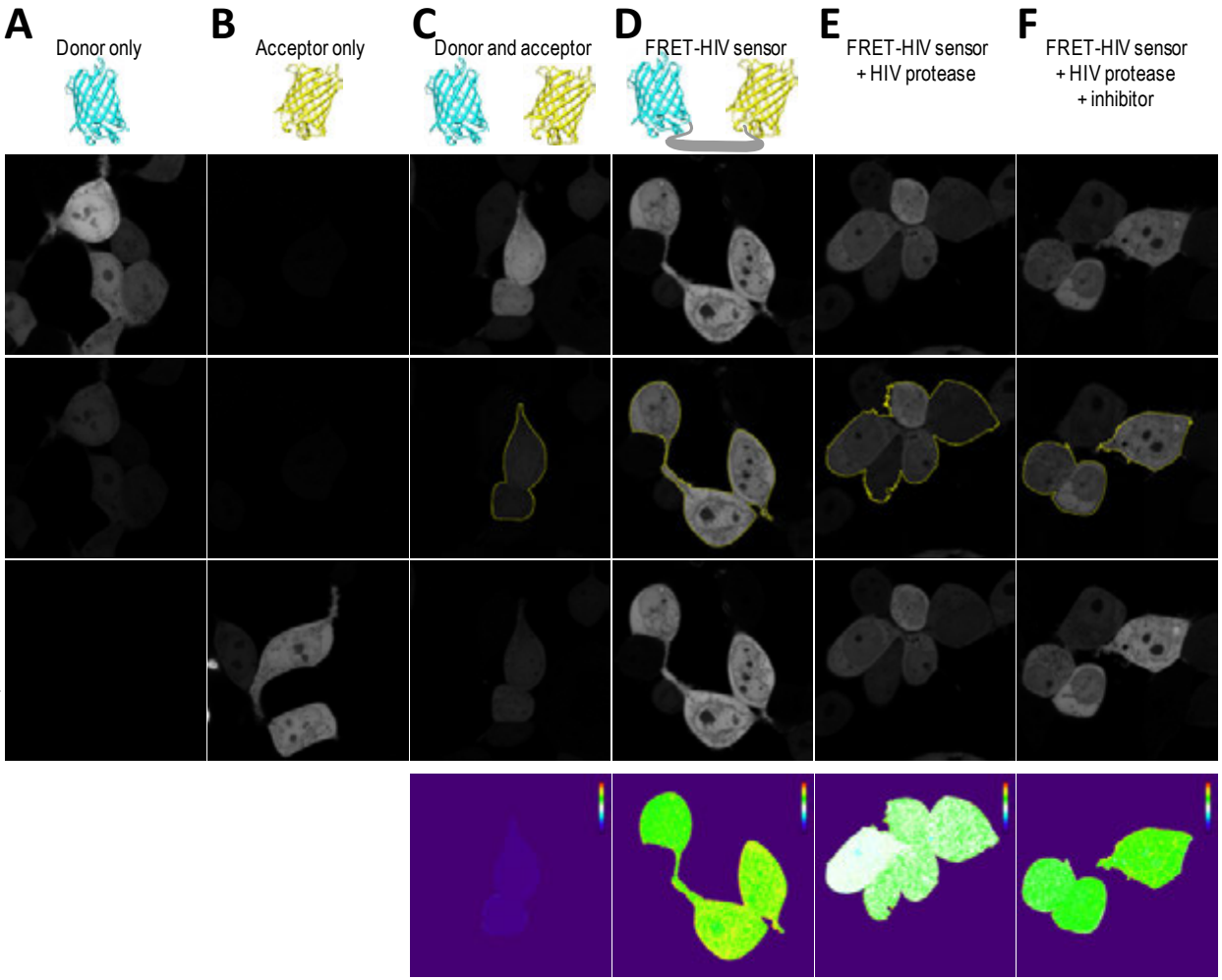

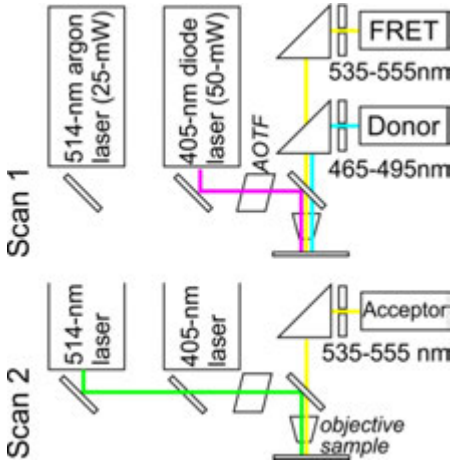

KINETICS OF OVARIAN CANCER TUMOR MARKERS SOPN AND SCD44-V6

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The high mortality of ovarian cancer is largely explained by the fact that majority of cases (81%) present at an advance stage. Extensive research in the field of serum tumour markers for early detection of ovarian cancer is therefore currently underway. However, it is questionable whether sufficient tumor product can reach the peripheral blood for early disease detection with diagnostic tests, taking into account the sensitivity of the blood assay. We can therefore apply a different approach and evaluate the concentrations of ovarian cancer markers in the fluid of the local environment. Therefore, the aim of the study was to evaluate the relationship of ovarian tumour markers osteopontin (sOPN) and splice variant 6 of sCD44 (sCD44-v6) between serum and local environment, represented by ascites or peritoneal fluid (PF)/peritoneal washing (PW), in patients with malignant and non-malignant conditions, respectively. PW is already included in the International Federation of Gynecology and Obstetrics (FIGO) staging classification for ovarian cancer but sampling has not yet been standardized. Thus, standardization of the sampling protocol was a prerequisite to ensure reliable results. Concentrations of sOPN and sCD44-v6 were measured separately using bead-based flow cytometric assay. In the malignant condition, both tumour markers, but sOPN in particular, in spite of their different kinetics, showed a tendency for retention in ascites. Serum sCD44-v6 concentrations positively correlated to those in local fluids in both malignant and non-malignant conditions, although they seem less dependent on the concentration in ascites than in PF. This presentation will outline systematic comparison of sOPN and sCD44-v6 levels between local fluid and serum in patients with ovarian carcinoma and benign disease. Furthermore, the main steps to established standardized protocol for sampling PF and performing washing during laparoscopy will be discussed. In addition to its use in tumour marker research, analysis of the concentrations of ovarian cancer markers in the fluid of the local environment has potential clinical applicability in patients with suspect adnexal masses, where determination of tumour markers not only in blood but also in local fluid, in combination with cytology, may be useful in order to distinguish more accurately between benign and malignant forms of ovarian neoplasm.

Kinetics of Ovarian Cancer Tumor Markers sOPN and sCD44-v6

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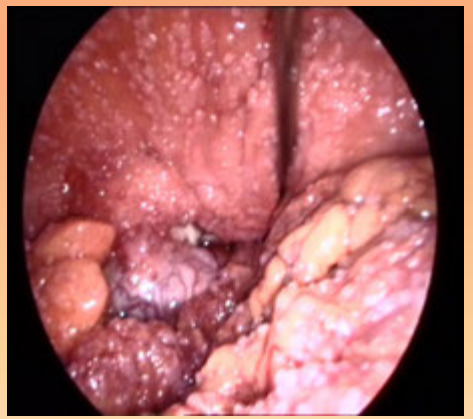
Slovenian society for Flow Cytometry, Ljubljana 14. 10. 2016

OVARIAN CANCER *"SILENT KILLER"*

- The often fatal prognosis by the time of ovarian cancer actual detection resulting from lack of symptoms or from presence of symptoms that mimic other conditions.
- On the other site, ovarian cancer has an excellent prognosis, with a 5-year survival rate exceeding 90% if diagnosed at an early stage.
- For this reason extensive research in the field of serum tumour markers for early detection of ovarian cancer is currently underway.

Most frequently ovarian cancer spread over the peritoneal surface:

- forming a myriad of tiny nodules on visceral and parietal peritoneum
- complete surgical removal is impossible

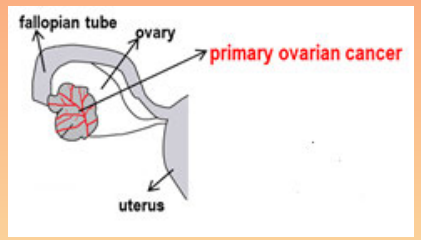


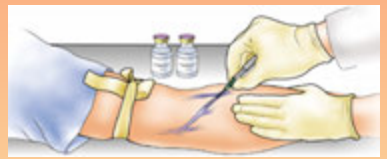
BLOOD TUMOR MARKERS

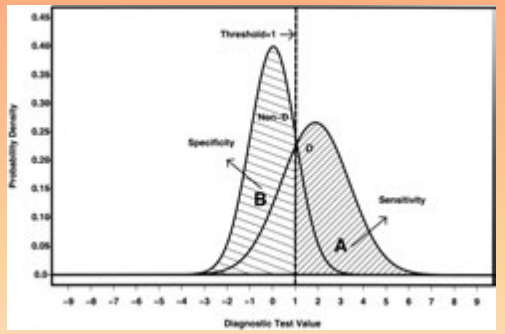
Blood assay for detecting tumour markers is an important non-invasive method for establishing a cancer diagnosis.

However, it is questionable whether sufficient tumour product can reach the peripheral blood for early disease detection with diagnostic tests, taking into account the sensitivity of the blood assay

- ***a range of 0.1 -20 % of secreted or shed protein is assumed***







LOCAL FLUIDS

Evaluation of ovarian cancer markers concentrations in the fluid closer to the origin of disease could help to elucidate the potential of promising blood tumour markers for early-stage disease:

- their changes in concentration, due to higher quantities, can be detected faster
- they are also more specific

As local fluid we could use:

ASCITES

PERITONEAL FLUID (PF)

PERITONEAL WASHING (PW) **"complex procedure involvig many factors"**

Peritoneal washing is already included in the International Federation of Gynaecology and Obstetrics (FIGO) staging classification for ovarian cancer but sampling has not yet been standardized.

OSTEOPONTIN (*sOPN)

Ovarian cancer

- among ten genes differentially over-expressed in ovarian cancer cells, more than 10-fold compared to primary human ovarian surface epithelial cells
- included in a list of top promising blood tumour markers for early detection of disease

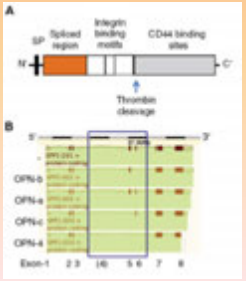
Female reproductive tract

- synthesized by the oviductal epithelium, detected in oviductal fluid, where binds to oocytes, thereby providing sites for attachment between gametes or to the epithelium.
- OPN expression has been demonstrated in:

  1.) surface mesothelial-like cells of the ovary;

  2.) the mucinose epithelium of the endocervix;

  3.) secretory endometrium.



SPLICE VARIANT 6 of CD44 (*sCD44-v6)

Cancer

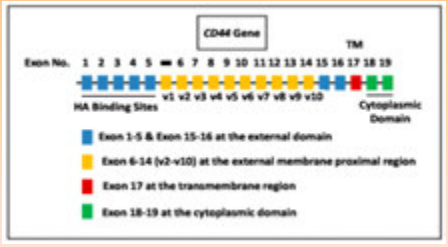
- among CD44v isoforms, CD44-v6 appears to be a key functional tumour marker critically involved in the main features of cancer progression

Ovarian cancer

- expression is increased in tumour tissue at the peritoneal metastasis sites compared with those at the corresponding primary tumour
- In patients with advanced OC is associated with peritoneal metastatic dissemination, tumour resistance to chemotherapy and shortened overall survival

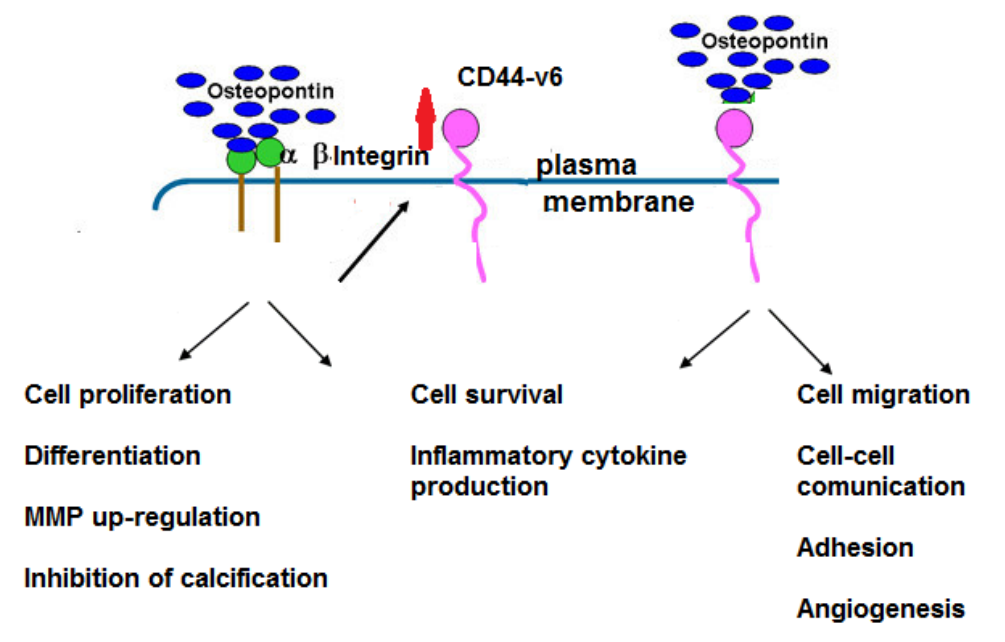
Female reproductive tract

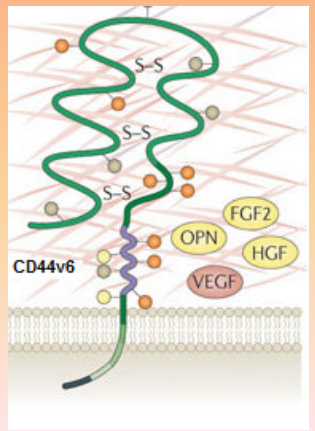
- mediates apoptosis inhibition, which is important in preventing oocytes from succumbing to atresia during follicular maturation.



*s: soluble form of OPN and CD44-v6

Enchensment of metastatic behaviour
sOPN – s CD44-v6



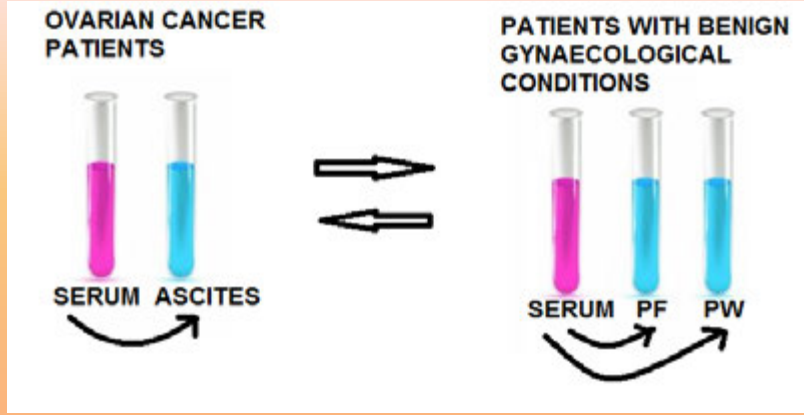


OBJECTIVES

To compare sOPN and sCD44-v6 concentrations between local fluids (represented by ascites, PF, PW) and serum.

To elucidate weather malignant situation could change the relationship of sOPN and sCD44-v6 concentrations between local fluids and serum.

To investigate the relationship between concentrations of sOPN and sCD44-v6 in all types of samples.



To standardise the protocol for sampling peritoneal fluid and performing washing during laparoscopy to ensure reliable results.

Patient characteristics

OVARIAN CANCER

Parameters	Data
Number of patients	33
Age (value ± SEM)	60.03 ± 2.13
Age range	28-83
Elevated CA125	
n (%)	33 (100%)
Value (mean ± SEM)	3745.1 ± 1266.2 U/ml
Hystological type, *n* (%)	
Serous	29 (88%)
Endometrioid	3 (9%)
Serous + clear cell	1(3%)
FIGO stage, *n* (%)	
III B	1 (3%)
III C	22 (67%)
IV	10 (30%)
Local fluid, *n* (%)	
Ascites	33 (100%)

BENIGN GYNAECOLOGICAL CONDITIONS

Parameters	Data
Number of patients	33
Age (value ± SEM)	43 ± 1.82
Age range	21-69
Elevated CA125	
n (%)	0
Value (mean ± SEM)	NA
Benign diagnosis, *n* (%)	
Benign ovarian cyst	10 (30%)
Myoma of the uterus	15 (46%)
Pelvic pain, sterilisation	6 (18%)
Preventive adnexectomy	2 (6%)
Local fluid, *n* (%)	
Peritoneal fluid	26 (79%)
Peritoneal washing*	33 (100%)

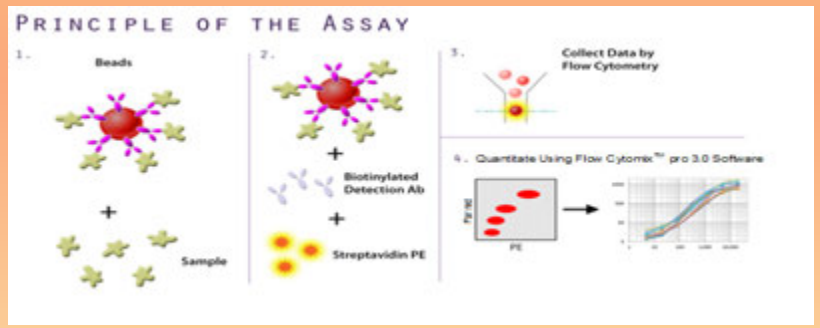
*Peritoneal washing was performed after the aspiration of PF or immediately afterentering the abdominal cavity if the PF was not present.

Patients were operated at the Department of Gynaecology, University Medical Centre Ljubljana.

Analysis of sOPN and sCD44-v6 with bead-based flow cytometric assay

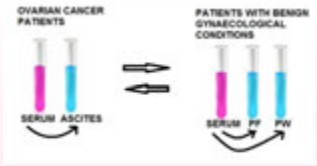
- Cell Lab Quanta™ SC-MPL (Beckman Coulter).
- Flowcytomix™ Pro 3.0 software (eBioscience).

- FlowCytomix Simplex Kit (eBioscience, Vienna) consist of:
- fluorescent beads (diameter: 5 µm; emission wavelength at 700 nm) coated with specific antibodies against each of the analytes.
- biotin-conjugated second antibody
- straptavidine-phycoerythrin emitting at 575 nm.



Seven point standard curve range: sOPN: 0.423 - 200 ng/ml

s CD44-v6: 0.137 - 100 ng/ml

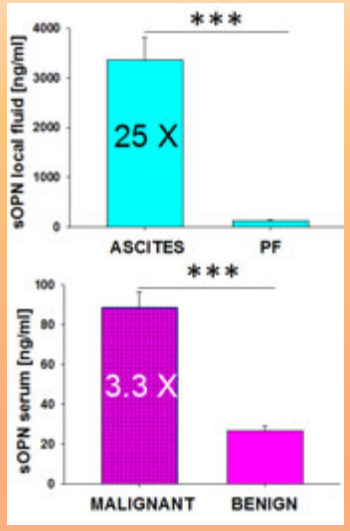


RESULTS sOPN

Local fluid-to-serum ratio

MALIGNANT vs BENIGN

Sample	Malignant [ng/ml]	Benign
serum	88.6 ± 7.8	26.7 ± 2.1
ascites or PF	3355.7 ± 459.4	132.14 ±2.5
PW	NA	12.4 ± 12.2



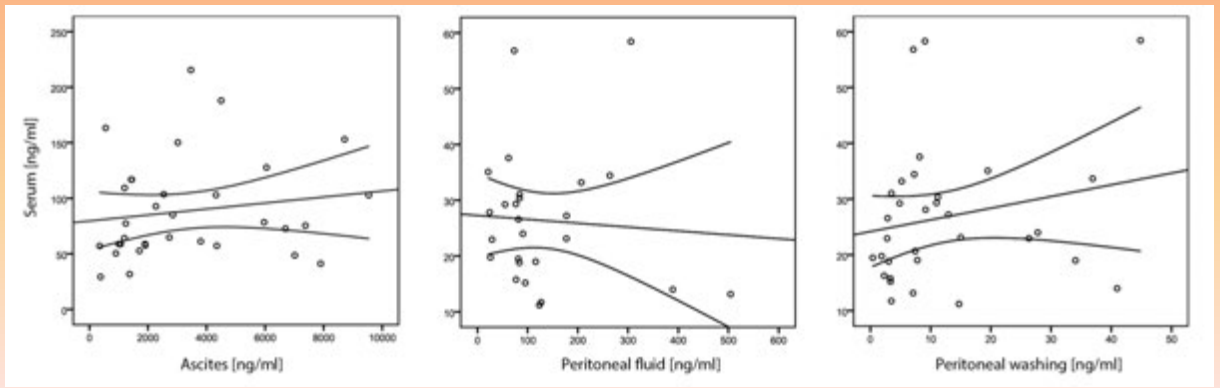
local production / accumulation in local fluids

Volume of different local fluids		
	Average ± SEM [ml]	Range [ml]
Ascites	2917.2 ± 480.1	50 - 9000
PF	7.66 ± 1.4	0.5 – 28.5
PW	15.1 ± 0.5	10.0 – 19.4

Correlations sOPN - volume

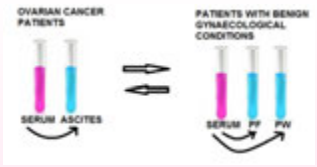
sOPN loc.fluid conc.	Pearson's corr. coefficient	Sig.
	Volume of local fluid	
Ascites	0.431	0.013
PF	- 0,122	0.552
PW	- 0.108	0.601

Correlation of sOPN concentrations between serum and local fluids



Correlations sOPN

	Pearson's corr. coefficient	Sig.
	Serum	
Ascites	0.157	0.376
PF	-0.223	0.261
PW	0.302	0.142



RESULTS sCD44-v6

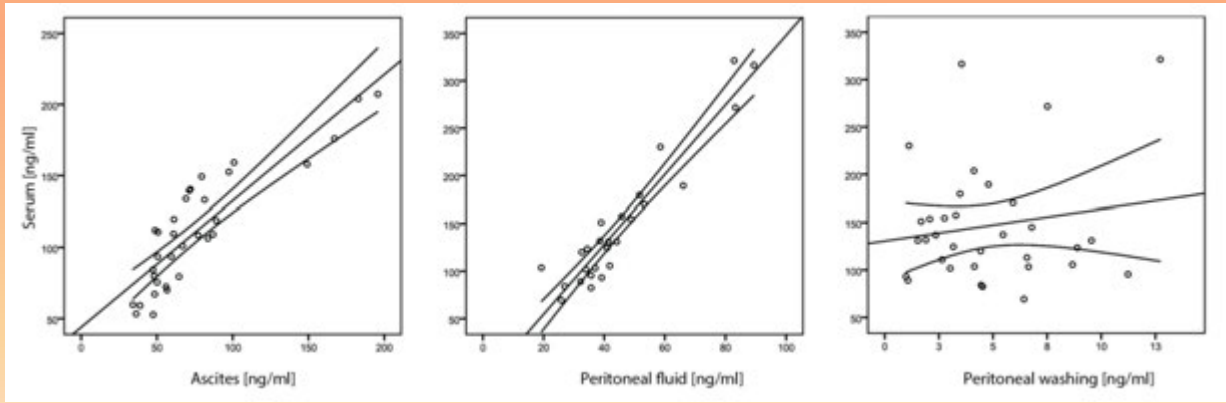
Serum-to-local fluid ratio

Correlations sCD44-v6 - volume

sCd44-v6 loc.fluid conc.	Pearson's corr. coefficient	Sig.
	Volume of local fluid	
Ascites	0.109	0.230
PF	0.580	0.002
PW	0.510	0.029

local production / accumulation in malignant condition

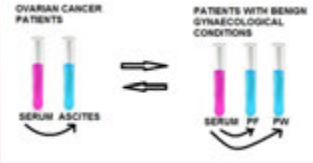
Correlation of sCD44-v6 concentrations between serum and local fluids



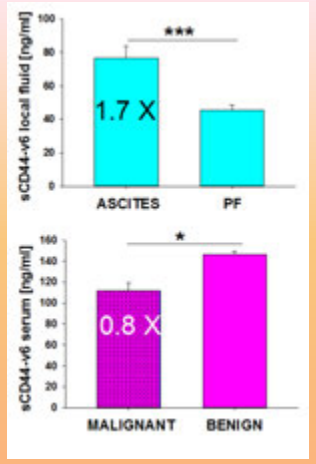
Correlations sCD44v6

	Pearson's corr. coefficient	Sig.
	Serum	
Ascites	0.876	< 0.001
PF	0.949	< 0.001
PW	0.496	0.002

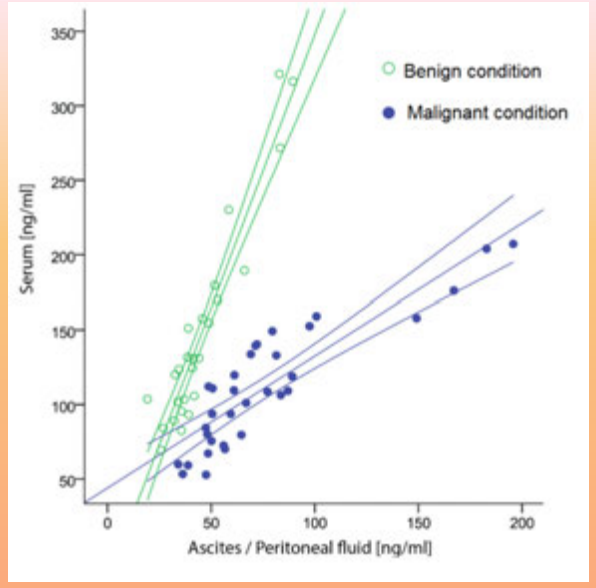
RESULTS sCD44-v6



Comparison of serum and local fluid concentrations between malignant and benign condition



Sample	Malignant	Benign
	[ng/ml]	
serum	111.9 ± 7.1	146.4 ± 10.8
ascites or PF	76.7 ± 7.1	45.3 ± 3.4
PW	NA	4.6 ± 0.5



Comparison of linear regression of sCD44-v6 concentrations between serum and ascites in ovarian cancer patients and between serum and peritoneal fluid in patients with benign conditions..

RESULTS correlation between sOPN and sCD44-v6

Correlations sOPN vs. sCD44-v6

	Pearson's corr. coefficient	Sig.
Ovarian cancer		
Serum	0.124	0.493
Ascites	0.651	0.001
Benign gynaecological conditions		
Serum	0.249	0.162
PF	-0.329	0.100
PW	0.247	0.225

CONCLUSIONS 1

OBJECTIVE
To compare tumour markers concentrations between local fluids (represented by ascites, PF, PW) and serum.

- **sOPN**

1) sOPN mean concentration in local fluids was significantlly higher than in serum (ascites 44-fold, PF 6.5-fold).
2) Serum sOPN levels were not related to concentrations in local fluids in either group, so it would be reasonable to set separate control values of sOPN in the blood, ascites, PF and PW.

- **sCS44-v6**

1) sCD44-v6 mean concentration in serum was significantly higher than in all types of local fluids.
2) A low concentration of sCD44-v6 in PF/PW shows low baseline production of this tumour marker in the local environment and therefore the sensitivity of sCD44-v6 in local fluid.
3) Serum sCD44-v6 levels were related to concentrations in local fluids in both groups.

CONCLUSIONS 2

OBJECTIVE
To elucidate weather malignant situation could change the relationship of tumour markers concentrations between local fluids and serum.

- **sOPN**

The retention of sOPN in local fluid already exists in the non-malignant situation but it is potentiated in malignant disease.

- **sCS44-v6**

Serum sCD44-v6 concentrations were positively correlated to those in local fluids in both malignant and non-malignant conditions, although they seem less dependent on the concentration in ascites than in PF.

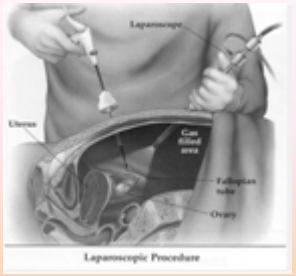
CONCLUSIONS 3

OBJECTIVE
To investigate the relationship between concentrations of sOPN and sCD44-v6 in all types of samples (serum, ascites, PF, PW).

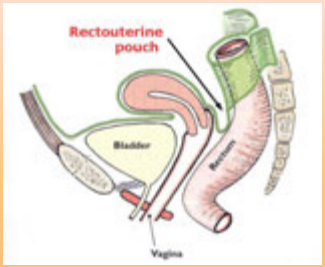
- sOPN concentrations correlated with sCD44-v6 levels in ascites, which indicates the presence of a high metastatic local environment in patients with advanced ovarian cancer.

- Completely different kinetics of sOPN and sCD44-v6 might be an explanation why no correlation was found when we evaluated the association between sOPN and sCD44-v6 concentrations in serum.

Standardised sampling protocol (SSP)
-during laparoscopy-



Aspiration all the available PF from the cavum Douglasi.



WASHING PROCEDURE
standardisation of the main factors

[1.] **SOLUTION VOLUME**
20 ml 0.9% NaCl

[2.] **TIME**
2 min

sOPN
sCD44-v6
sVCAM-1

[4.] **ACCURACY DURING ASPIRATION**
of the whole solution volume
(in an ideal anatomical condition)
back into the syringe.

[3.] **AREAS**
- Uterus
- Ovaries
- Pelvic peritoneum

CONCLUSIONS – STANDARDIZED SAMPLING PROTOCOL

OBJECTIVE
Standardisation of a protocol for sampling peritoneal fluid and performing washing during laparoscopy to ensure reliable results.

- Standardized sampling protocol is necessary to obtain comparable results of tumor markers concentrations among patients.
- For selected tumour markers (sOPN, sCD44-v6 and sVCAM-1) washing can replace PF when PF is absent.
- A smaller volume of washing solution is better than a larger one because of
    1. higher or equal concentrations of markers in samples, which allow their detection without loss of efficiency of washing;
    2. procedure is also technically easier to perform.

Members of the surgical team at Dpt. Gynaecology, University Medical Centre Ljubljana

This work was supported by research grants from the University Medical Centre Ljubljana (Project Number: 20110224) and the Slovenian Research Agency (P3-067).

SSP – SOLUTION VOLUME

In particular we tried to clarifay the influence of **the solution volume** used for performing washing on:

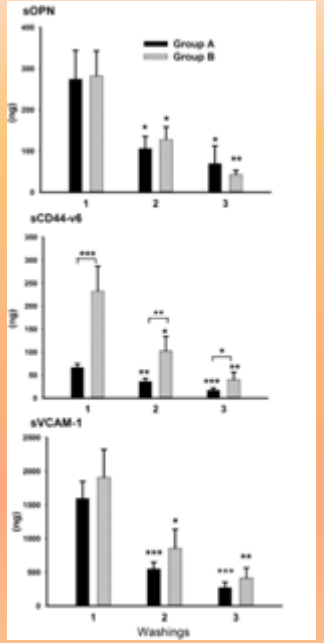
- tumour marker concentrations in washing samples
- the efficacy of the washing procedure

To determine the **efficiency of washing** in relation to the solution volume, the procedure was repeated twice after the first washing.

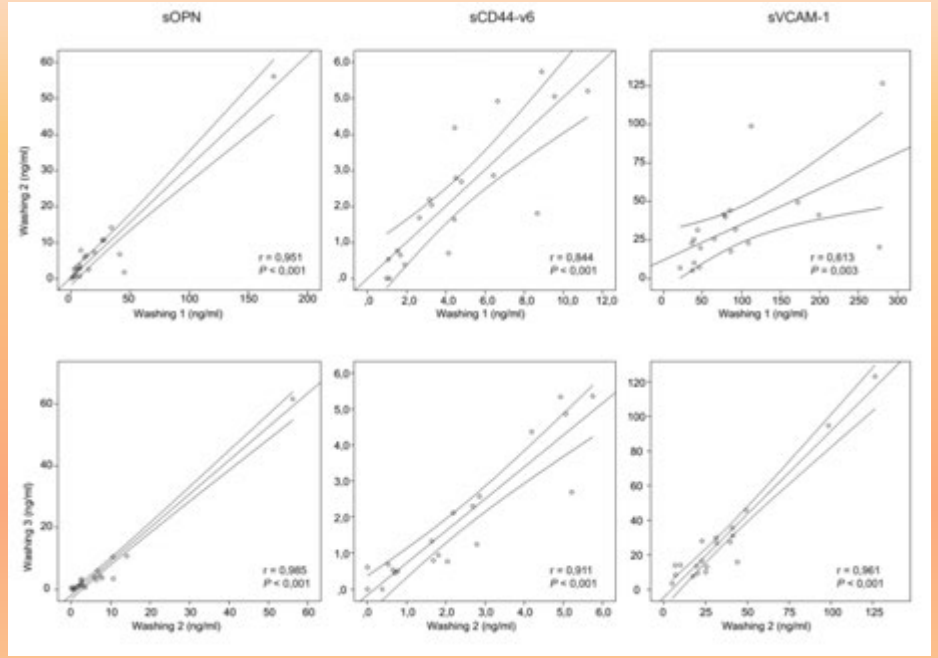
Based on solution volume patients were divided in two groups:

Group A	**Group B**
(smaller solution valume)	(larger solution valume)
20 ml + 20 ml + 10 ml	50 ml + 50 ml + 20 ml

CLARIFICATION OF WASHING PROCEDURE



Comparison of absolute quantity (ng) among three consecutively performed washings, as well as between group A and group B.



Correlation between first and second washings as well as between second and third washings.

TABLE: Concentrations of sVCAM in PW collected without SSP.

Peritoneal washings	sVCAM-1 [ng/ml]
sample 1	653
sample 2	362
sample 3	0
sample 4	36
sample 5	417
sample 6	50
sample 7	0
sample 8	408
sample 9	544
sample 10	123

TABLE: Concentrations of sOPN, sCD44-v6 and sVCAM-1 (average ± SEM) in PF and PW collected with SSP.

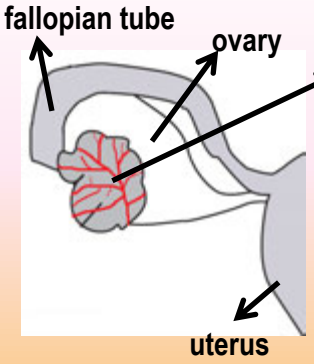
Sample (number of patients)	sOPN	sCD44-v6	sVCAM-1
		(ng/ml)	
Peritoneal fluid (26)	132.1 ± 22.9	45.3 ± 3.4	438.8 ± 11.7
range	21.4 - 483.1	19.3 - 89.3	311.8 - 598.43
Group A (smaler volume of solution for performing washing)			
Washing 1A (26)	20.0 ± 3.3	4.6 ± 0.5	108.2 ± 14.7
Washing 2A (20)	7.2 ± 2.7	2.3 ±0.4	34.4 ± 6.7
Washing 3A (20)	6.0 ± 3.0	1.8 ± 0.4	28.3 ± 6.7
Group B (larger volume of solution for performing washing)			
Washing 1B (7)	6.7 ± 1.7	5.5 ± 1.3	46.2 ± 11.6
Washing 2B (7)	3.0 ± 1.8	2.6 ± 0.9	20.7 ± 6.7
Washing 3B (7)	2.2 ± 0.5	2.1 ± 1.0	22.1 ± 8.5
Concentration ratio			
Peritoneal fluid:washing 1A	6.6	9.8	4,1

- **SSP is a prerequisite to ensuring reliable results.**
- **Smaller volume is more apropriate for washing.**

Correlations PF vs PW

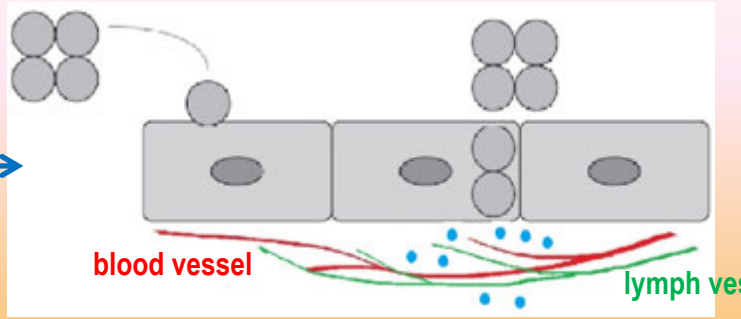
	Pearson's corr. coefficient	Sig.
sOPN	0.447	0.048
sCD44-v6	0.660	0.002
sVCAM-1	0.562	0.017

- **Washing can replace PF when PF is absent**



peritoneal dissemination

peritoneal implantation

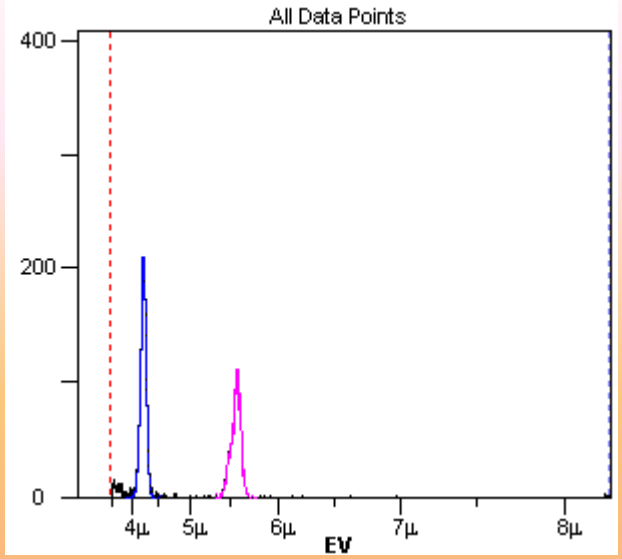


lymphatic obstruction

tumor-secreted factors

Neovascularization: • peritoneal • visceral	Increased capillary permeability. Macromolecular leakage.	Direction of fluid flow into the peritoneal cavity. High protein concentration in ascites.	Decreased efflux from peritoneal cavity.
Increased plasma inflow			Decreased lymphatic outflow

Malignant Ascites


All Data Points
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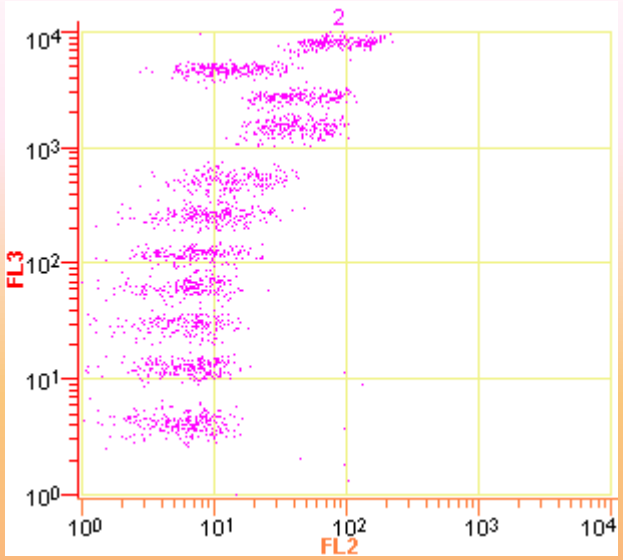
2
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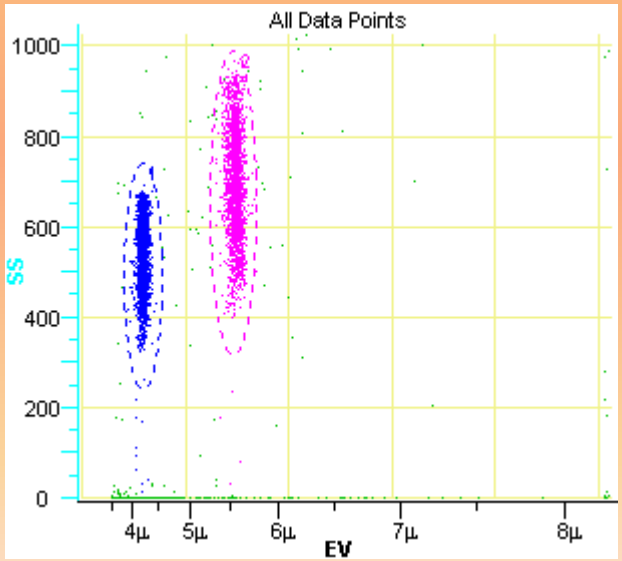
FL2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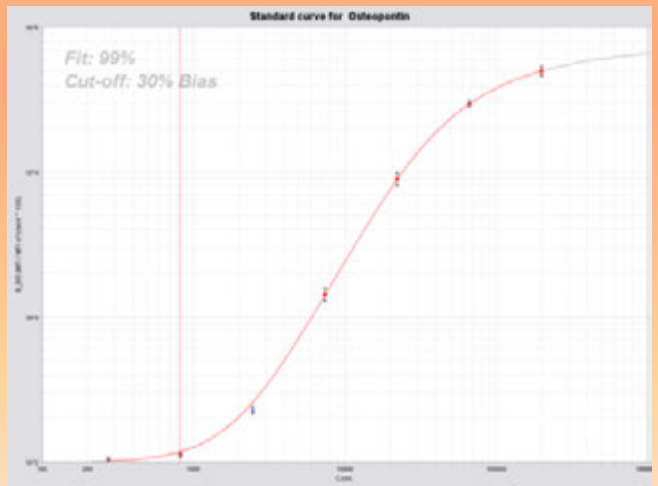

Standard curve for Osteopontin
Fit: 99%
Cut-off: 30% Bias

TREATMENT OF PATIENTS WITH T CELL DEFICITS

Tadej Avčin

Department of Allergology, Rheumatology and Clinical Immunology, Children's Hospital, University Medical Center Ljubljana, Slovenia and Department of Pediatrics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

Primary immunodeficiencies (PIDs) include a heterogeneous group of conditions that affect the development and function of the immune system. Loss of primary immune system function leads to increased susceptibility to infections. Patients with PID frequently develop also other features associated with abnormal regulation of immune responses, including autoimmunity, lymphoproliferation/granulomas, autoinflammation and allergy. In addition, ineffective recognition and removal of transformed self-cells predisposes to malignancy. Defects in T-cell function lead to susceptibility to infections that are more severe than those associated with antibody deficiency disorders. Those affected usually present during infancy with either common or opportunistic infections and without appropriate treatment rarely survive beyond infancy or childhood. The spectrum of T-cell defects ranges from the syndrome of severe combined immunodeficiency (SCID), in which T-cell function is absent, to combined immunodeficiency disorders (CID) and "atypical" SCID in which there is reduced, but not absent T-cell immunity. Most of T-cell defects could be diagnosed by neonatal screening for lymphopenia or for T-cell deficiency in cord blood. Early recognition of patients with T cell deficits is essential to the application of the most appropriate treatments for these conditions at a very early age. Fully defining the molecular defects of such patients is important for genetic counselling of family members and prenatal diagnosis. Treatment for T-cell defects can be divided into two main groups, the prophylactic treatment (i.e. preventative) and curative treatment. The former attempts to manage the opportunistic infections common to SCID patients and the latter aims at reconstituting healthy T-cell function. Absent T cell immunity in patients with SCID provides a clear rationale for hematopoetic stem cell transplantation (HSCT). This treatment strategy is highly successful when an HLA-matched sibling donor or a fully HLA-matched unrelated donor is available. When a related but HLA-mismatched donor is used (e.g., when one of the parents donates to a child), the survival rate is significantly lower. Gene therapy could circumvent significant limitations associated with HSCT and thus represent an attractive therapeutic alternative in certain forms of SCID such as X-linked SCID and adenosine deaminase deficiency. In patients with profound CID associated with infections or autoimmunity the long-term outcome data are insufficient for unambiguous early transplant decisions. Recent discoveries suggest that neither the genetic diagnosis nor basic measurements of T-cell immunity are good predictors of disease evolution in patients with profound CID.

Treatment of patients with T-cell deficits

Tadej Avčin

Department of Allergology, Rheumatology and Clinical Immunology
University Children's Hospital Ljubljana

Flow cytometry in PID, Ljubljana, 14.10.2016

Outline

- Introduction
- T-cell deficits
  - Early recognition & clinical manifestations
  - Treatment
- Management of PID in Slovenia

Primary immunodeficiencies

- A genetically heterogeneous group of disorders that affect distinct component of the innate and adaptive immune system
- Explosive growth of knowledge

Year	No. of recognized PIDs
1997	60
1999	71
2013	200
2016	> 300

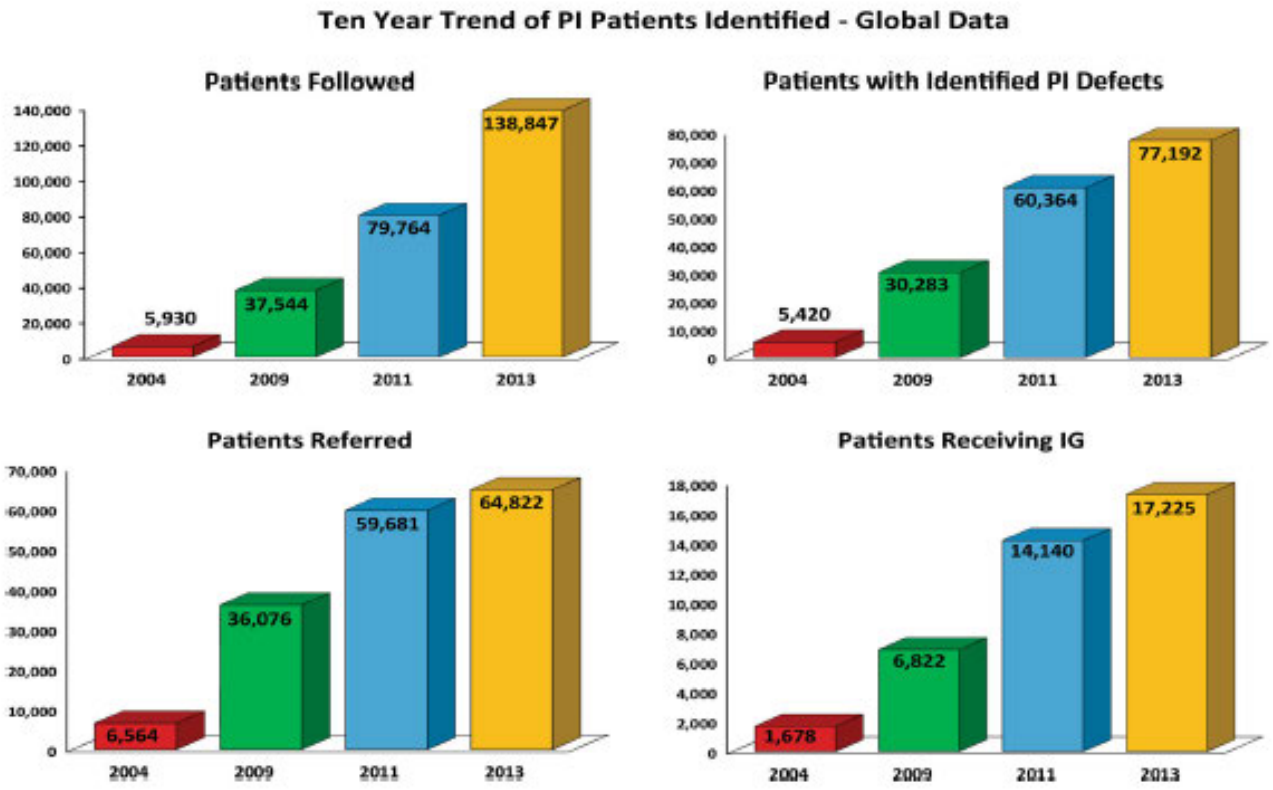
Classification of PIDs based on their known molecular causes

(Al-Herz W, et al. Front Immun 2014; Gathmann B, et al. Clin Exp Immunol 2012)

1. Predominantly antibody deficiencies	55 %
2. Combined T-cell and B-cell immunodeficiencies	8 %
3. Other well defined immunodeficiency syndromes	16 %
4. Diseases of immune dysregulation	4 %
5. Congenital defects of phagocyte number, function, or both	8 %
6. Defects in innate immunity	1 %
7. Autoinflammatory disorders	2 %
8. Complement deficiencies	5 %
9. Phenocopies of PID	?

Increased prevalence of diagnosed PID patients

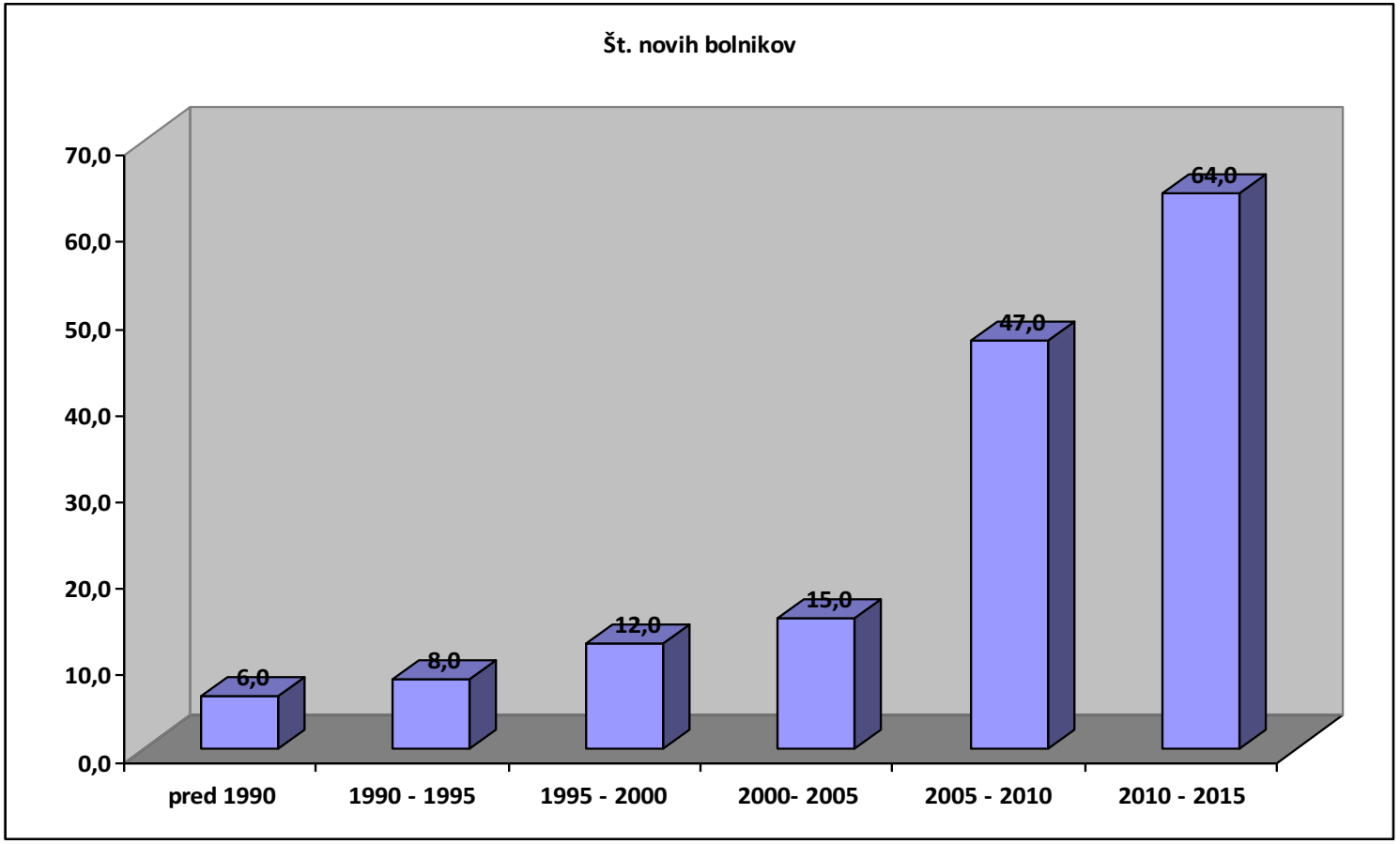
Modell V, et al. Immunol Res 2016; 64: 736-53.



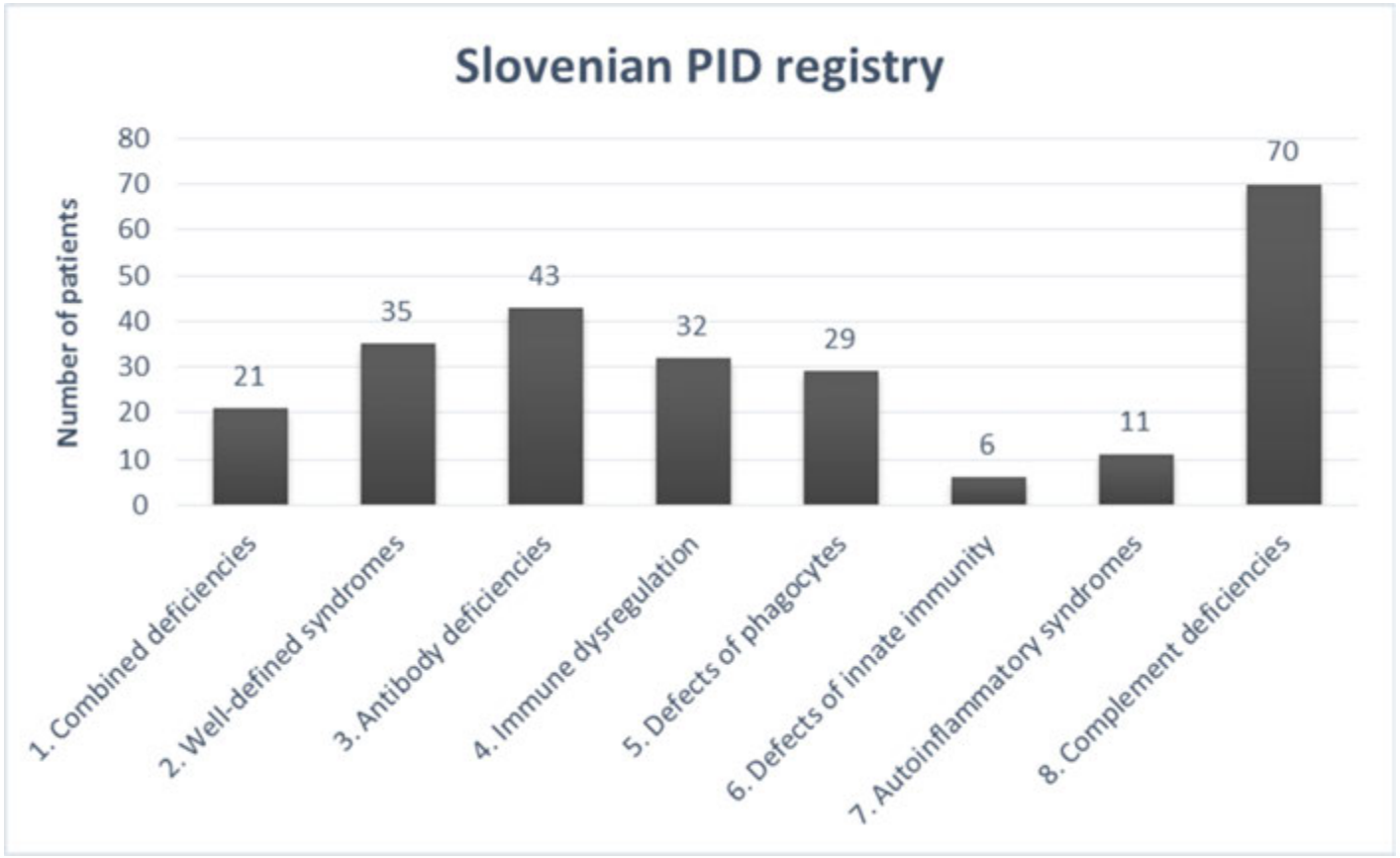
National PID patients registry (Nov 2015)

- Total number of registered PID patients: 252
- 50 different disease entities
- # of Pts according to genetic diagnosis: 49%
- # of adult PID patients: 52 (21%)

Number of newly diagnosed PID patients in 5 year periods



Slovenian PID Registry

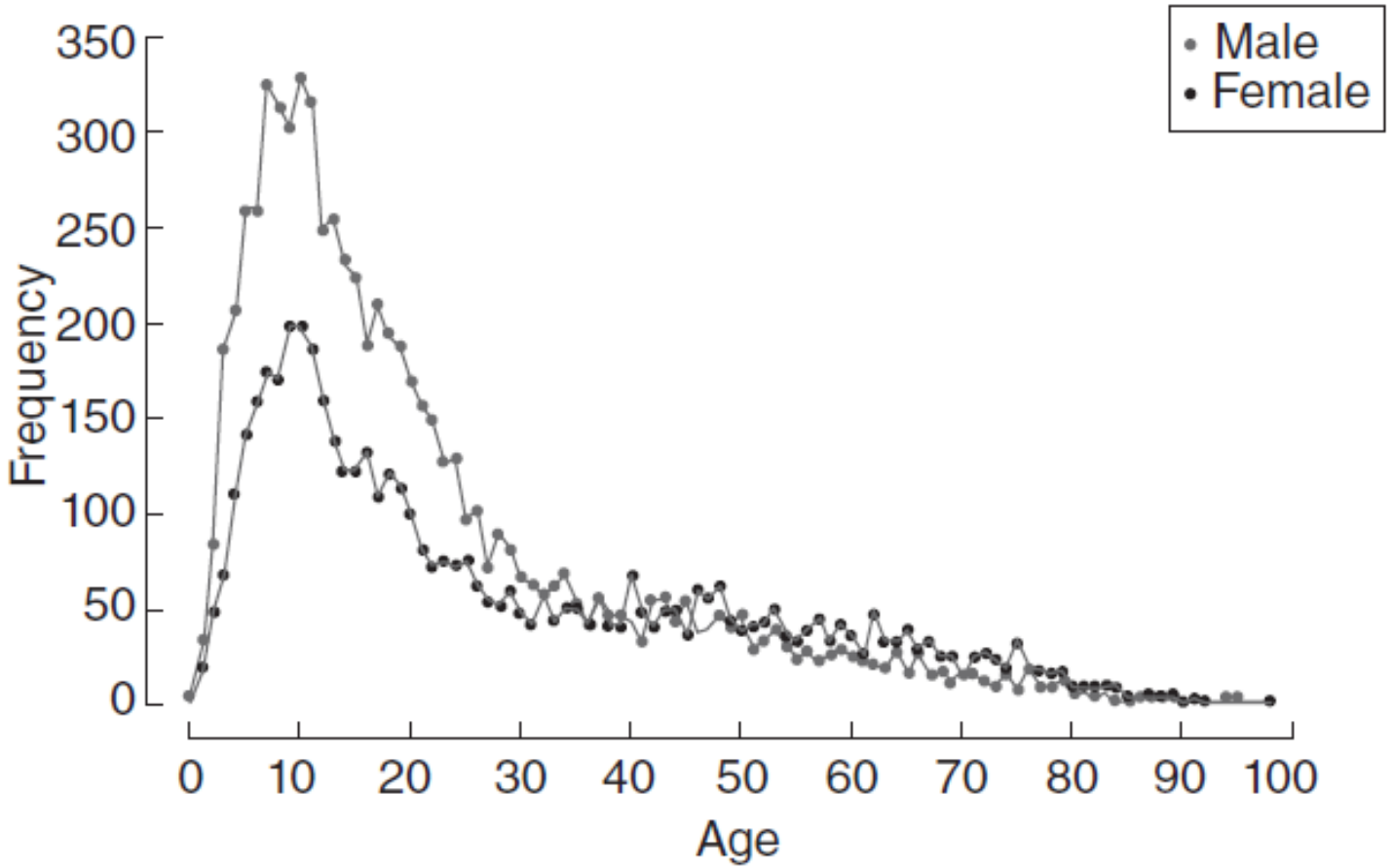


Early recognition / clinical manifestations of PID

- **Impaired antimicrobial host defence**
  - **More frequent, longer and more severe infections**
  - **Opportunistic infections**
- **Impaired surveillance function of immune system**
  - **Autoimmune manifestations**
  - **Granulomatosis**
  - **Hemophagocytic syndrom**
  - **Lymphoproliferation**
  - **Solid tumors**

Age distribution of PID patients

(Gathmann B, et al. Clin Exp Immunol 2012; 167: 479-91.)



PID in adults

- **Increased number of adult PID patients:**
  - **Recognition of milder, atypical forms of PID with clinical presentation in adult**
  - **Improved survival of pediatric PID patients**
- **Wrong beliefs:**

→ PID are very rare diseases

→ PID occur only in children

→ all patients with PID are severely affected at clinical presentation

Outline

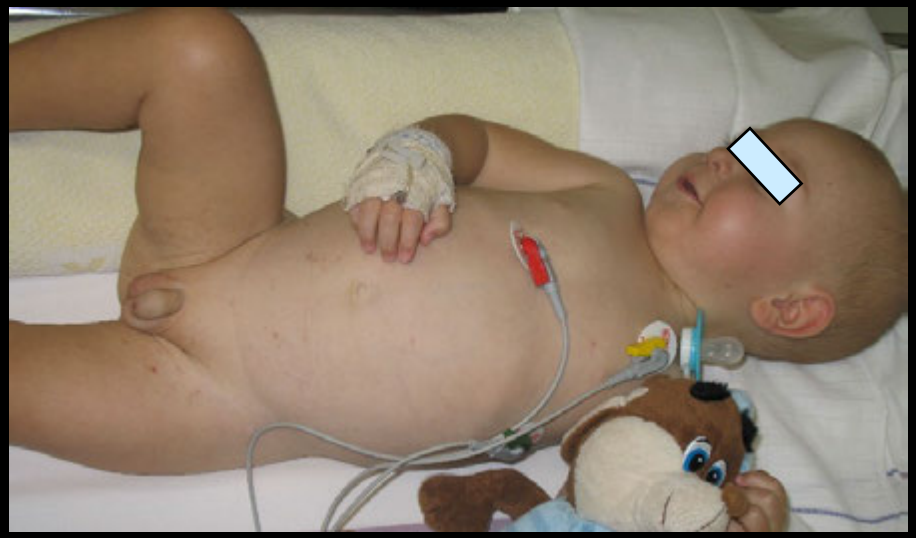
- Introduction
- **T-cell deficits**
  - **Early recognition & clinical manifestations**
  - Treatment
- Management of PID in Slovenia

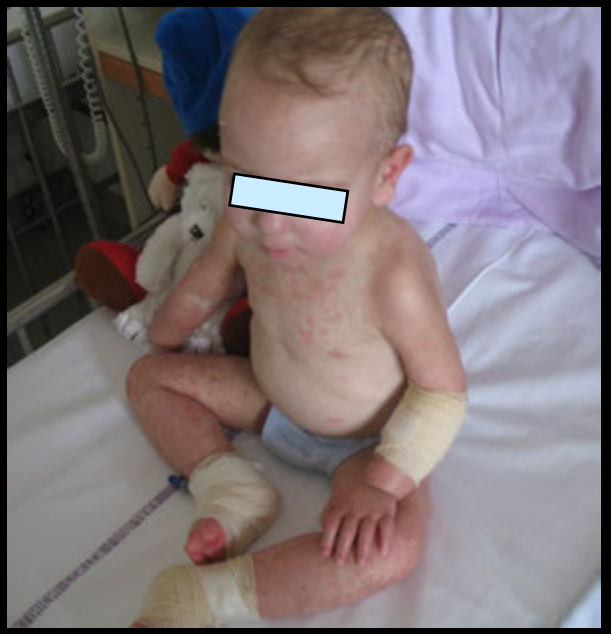
Severe Combined Immunodeficiency (SCID)

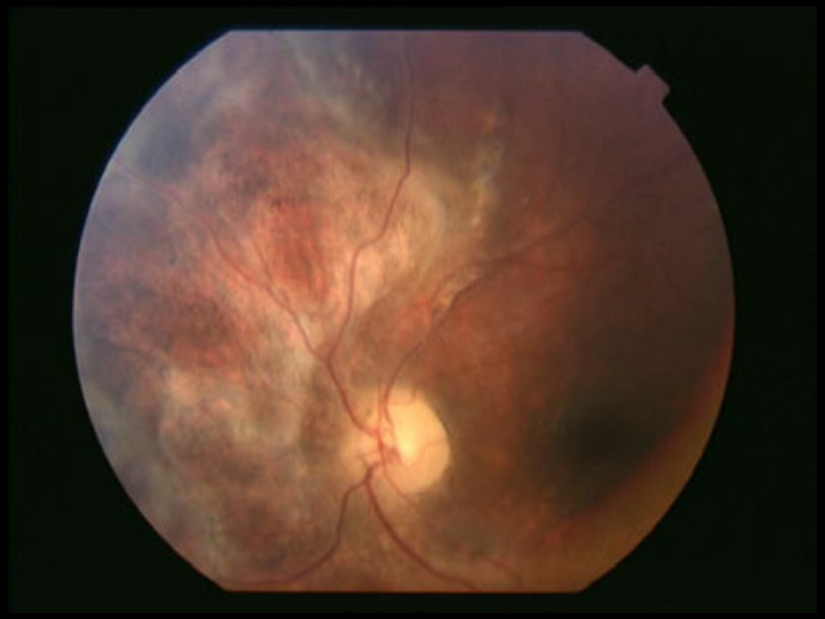
- **A group of disorders with genetic defects of T cell development**
  - **absence of mature T cells (abrogated adaptive immunity)**
  - **variably associated with defective differentiation of other hematopoietic cells**
- **Incidence data from newborn screening:**
  - **1/58.000 births** *(Kwan A et al. JAMA 2014)*
- **Considerable genetic heterogeneity**
  - **inherited as an X-linked or autosomal recessive disorder**
  - **at least 22 molecularly defined SCID disorders**

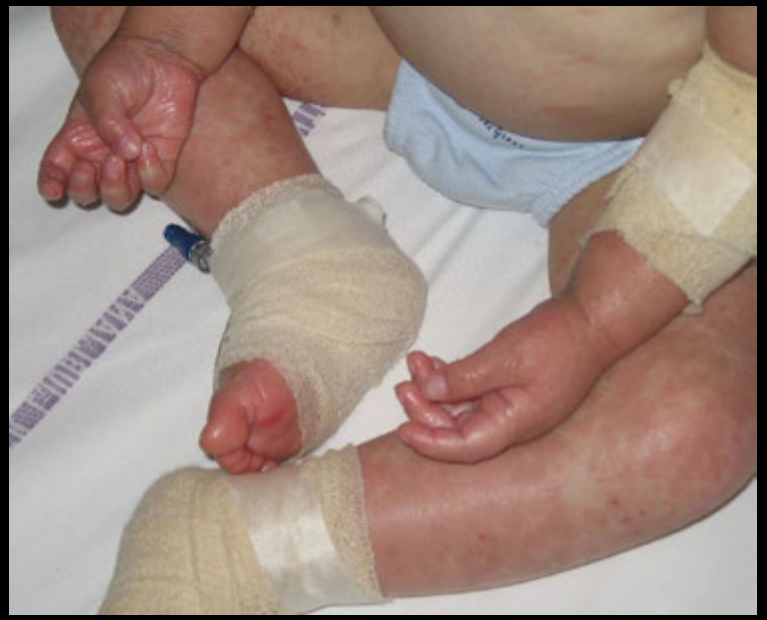
Clinical findings suggestive of SCID

- **Early onset: present during the first 2-7 months of life**
- **Failure to thrive**
- **Oral thrush, candida diaper rash**
- **Absent tonsils**
- **Erythematous skin rashes: from spontaneous or transfusion-related GVHD**
- **Protracted diarrhea**
- **Sepsis, severe bacterial infections**
- **Viral infections: adenovirus, enteric viruses, varicella, herpes, EBV (lymphoproliferation)**
- **Opportunistic infections**
  - ***Pneumocystis jiroveci*, CMV, extensive candidiasis**
  - **systemic BCG infection postvaccination**









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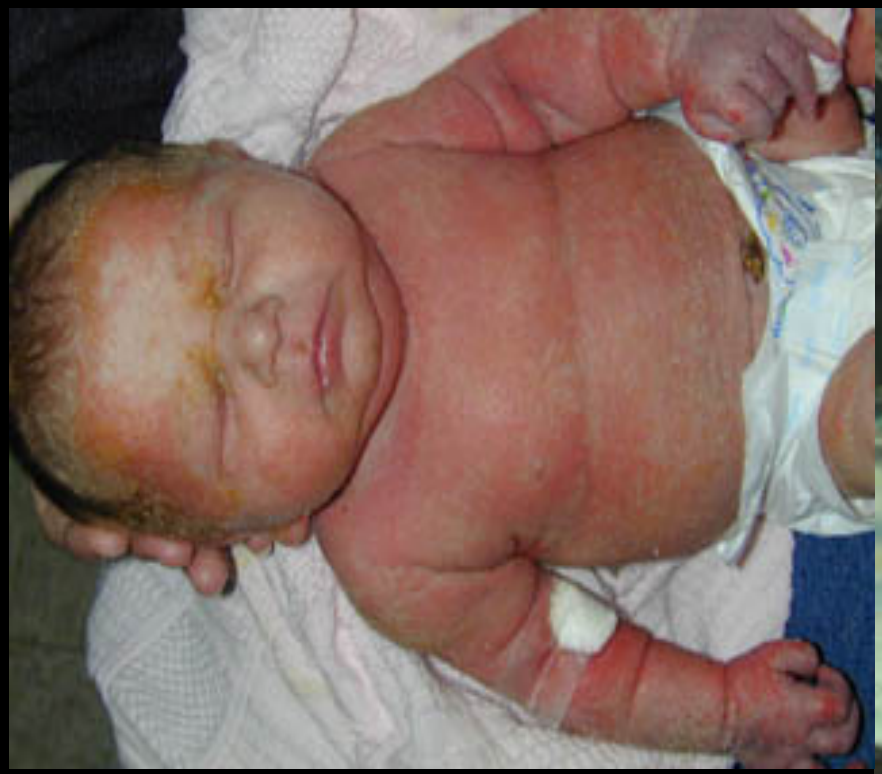
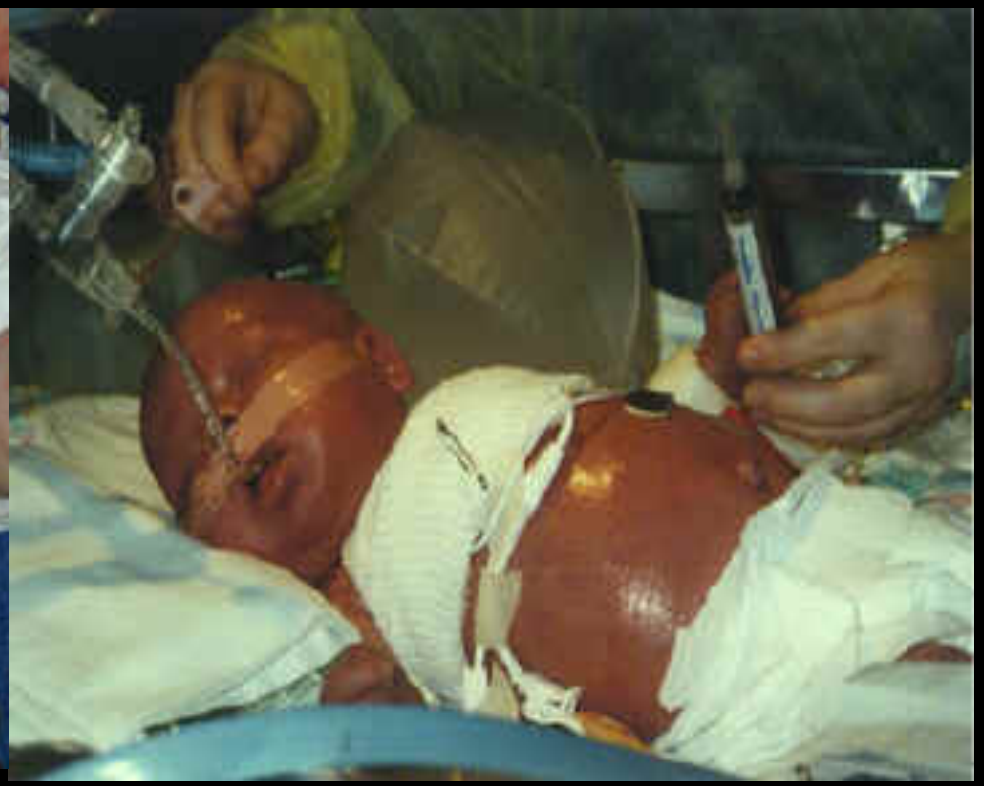
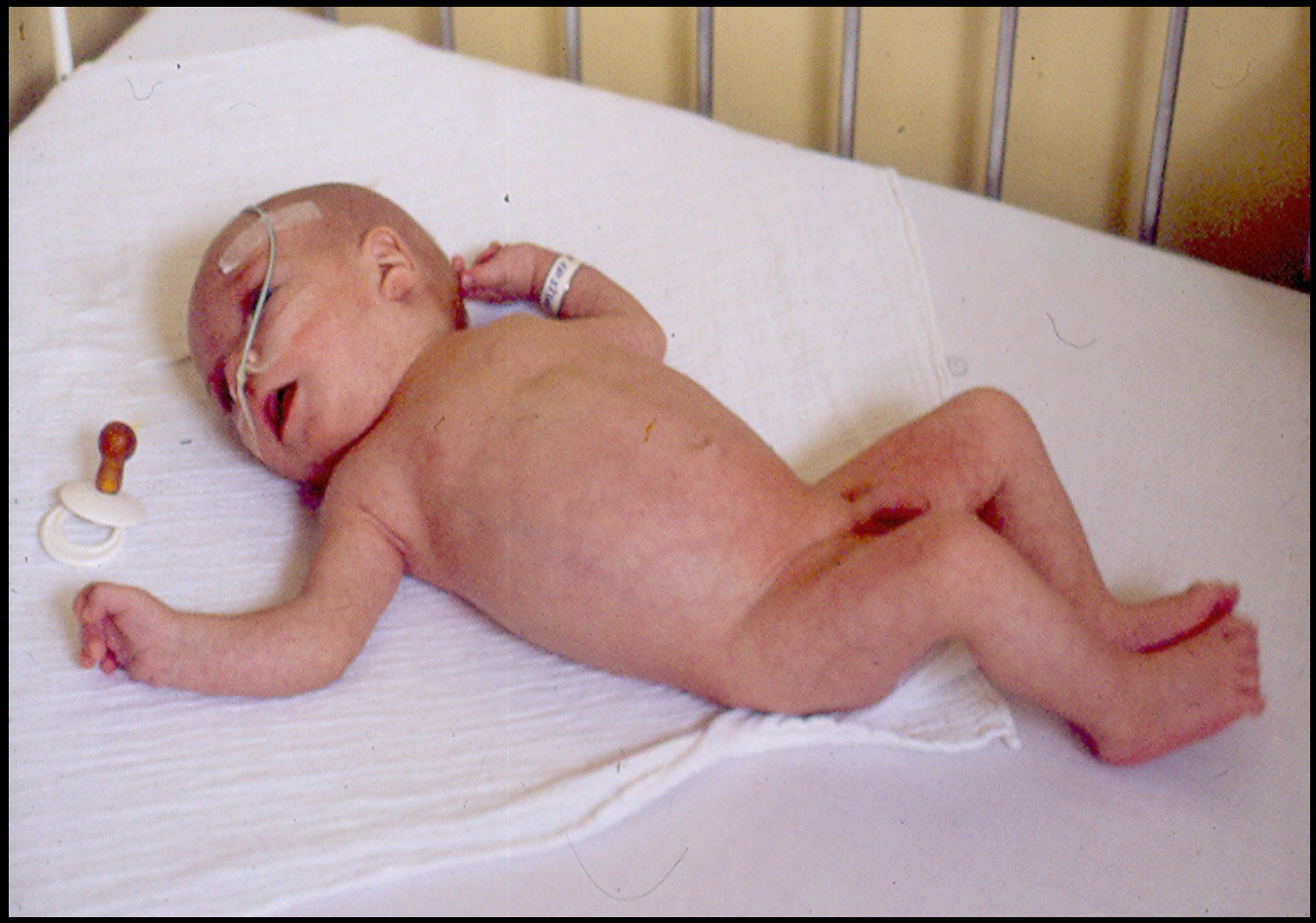




ADA deficiency

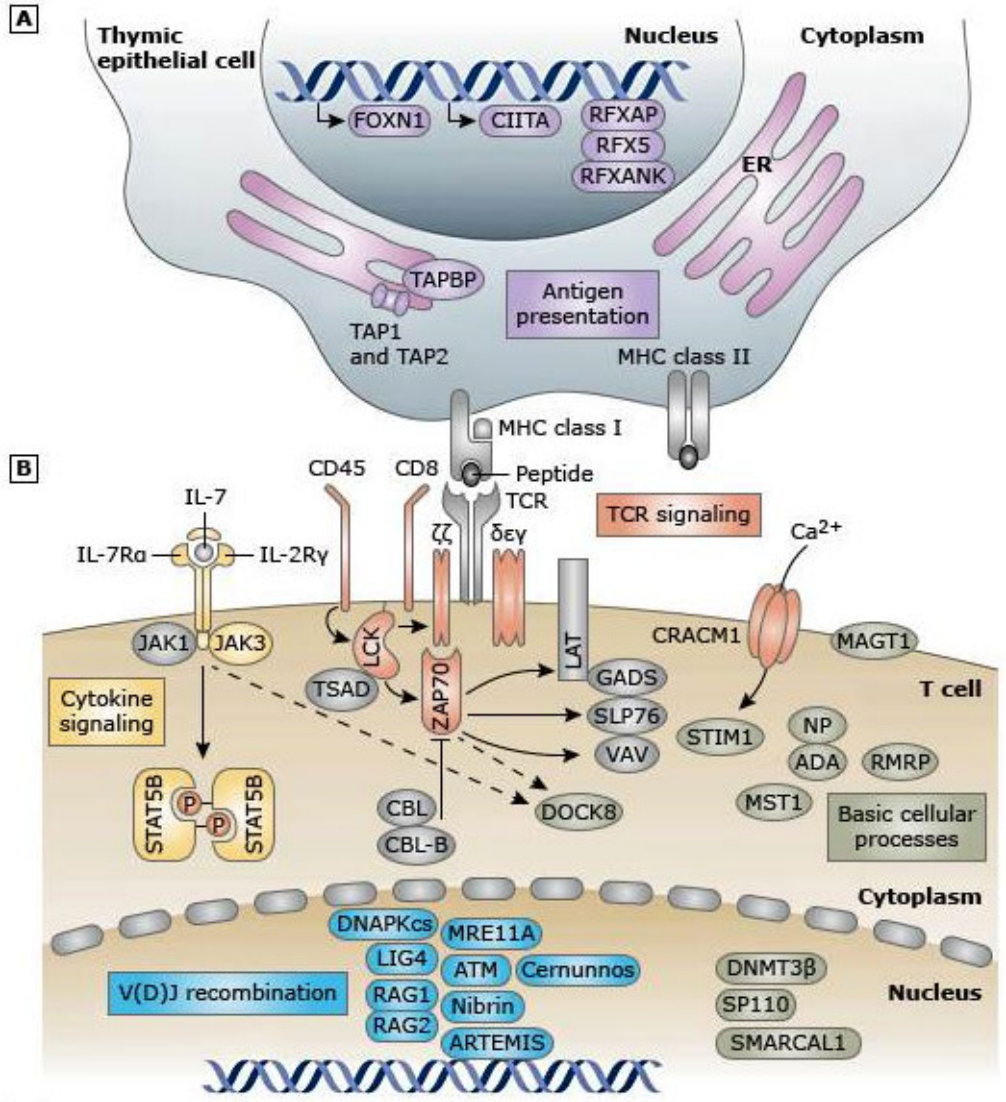


Severe Combined Immunodeficiencies

Liston et al. *Nat Rev Immunol* 2008;8:545.

Categorization of SCID:

- according to the specific molecular defect
- based on the cellular function of the protein encoded by the defective gene



Block in lymphopoiesis caused by SCID

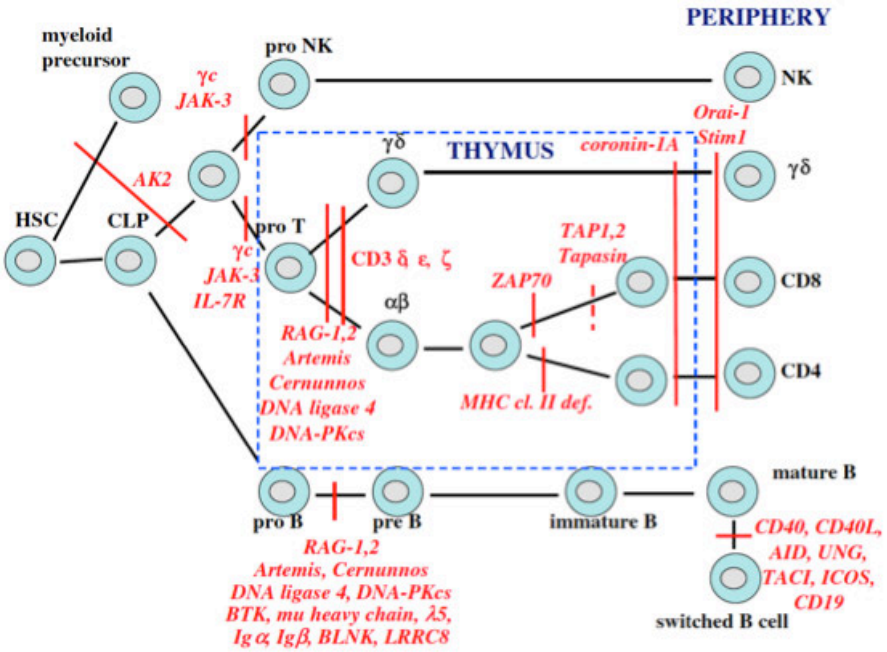


FIG 1. Blocks in T-and B-cell development associated with PIDs.

Notarangelo LD. J Allergy Clin Immunol 2010;125:S182-S194

Defect	Gene Defect	Inheritance	T,B, NK Cells
Cytokine signalling	CγC	XL	- + -
	JAK 3	AR	- + -
	IL7 Rα	AR	- + +
Nucleotide biosynthesis salvage pathway defects	ADA	AR	T_{low} B_{low} NK_{low}
	PNP	AR	$T_{low}B_{low}NK_{low}$
Defects affecting signalling through the T cell antigen receptor	CD45	AR	- + -
	CD3δ	AR	- + -
	CD3ε	AR	- + -
	CD3ζ	AR	- + +
	ZAP70 kinase	AR	+ + + (absent CD8)
VDJ recombination defects	RAG 1 & 2	AR	- - +
	Artemis	AR	- - +
	Cernunnos	AR	T_{low} B_{low} NK+
	DNA ligase 4	AR	T_{low} B_{low} NK+
Thymic defects	22q11	Sporadic/AD	T-B+NK+
	CHD7	Sporadic/AD	T-B+NK+
	FOXN1	AR	T-B+NK+
Other	AK2 (RD)	AR	- - - (+ myeloid dysfunction)
	MHC class II deficiency	AR	+++ (absent CD4)
	ORAI1	AR	Ca-dependent T cell activation
	STIM1	AR	

Diagnostic approach – *infectious work-up*

- **Transfer the patient to a tertiary care center with experience**
- **Surveillance cultures (at diagnosis, when symptomatic, and weekly from start of transplant conditioning until reconstitution)**

Virology:

- NP swab for respiratory viruses, when symptomatic

- Stool for EM

- Urine for CMV

Bacteriology:

- Superficial swabs for C&S and fungus from orifices (ears, nose, throat) and skin

- Stool C&S and fungus

- Urine C&S and fungus

Diagnostic approach – *infectious work-up*

- **Virology**
  - **CMV antigen**
  - **Semi-quantitative EBV titres**
  - **Herpes group PCR**
  - **HIV antigen & PCR**
  - **HBsAg**
- **Bacteriology**
  - **Blood culture if T $\geq$ 38°C axillary**
  - **BAL for Pneumocystis jiroveci (if indicated)**

Diagnostic approach – *immunology work-up*

- **Immunology tests:**
  - **Absolute lymphocyte count**
  - **Assessment of T cell subsets / T cell differentiation 'states'**
  - **Assessment of proliferation in response to mitogens and anti-CD3/CD28**
  - **ADA and PNP**
  - **Total serum immunoglobulins**
  - **Specific antibodies if vaccinated**

Treatment

- **SCID is a pediatric emergency**
  - **vulnerability to infections**
  - **potentially lethal complications from live vaccines and GVHD caused by unirradiated blood transfusions**

    → All blood products must be irradiated with CMV negative
- **Generally fatal unless an immune system reconstituted by:**
  - **Allogeneic hematopoietic stem cell transplantation (HSCT)**
  - **Correction of autologous hematopoietic cells by gene therapy**

Aim of HSCT

- **Stable donor engraftment**
  - **Partial or full ablation of recipient**
- **No graft versus host disease (GVHD)**
  - **GVHD damages thymus**
  - **Stable mixed chimerism can lead to cure in PID**
- **Good quality immune reconstitution**
- **Long-term quality of life**

Treatment – *pre-transplant*

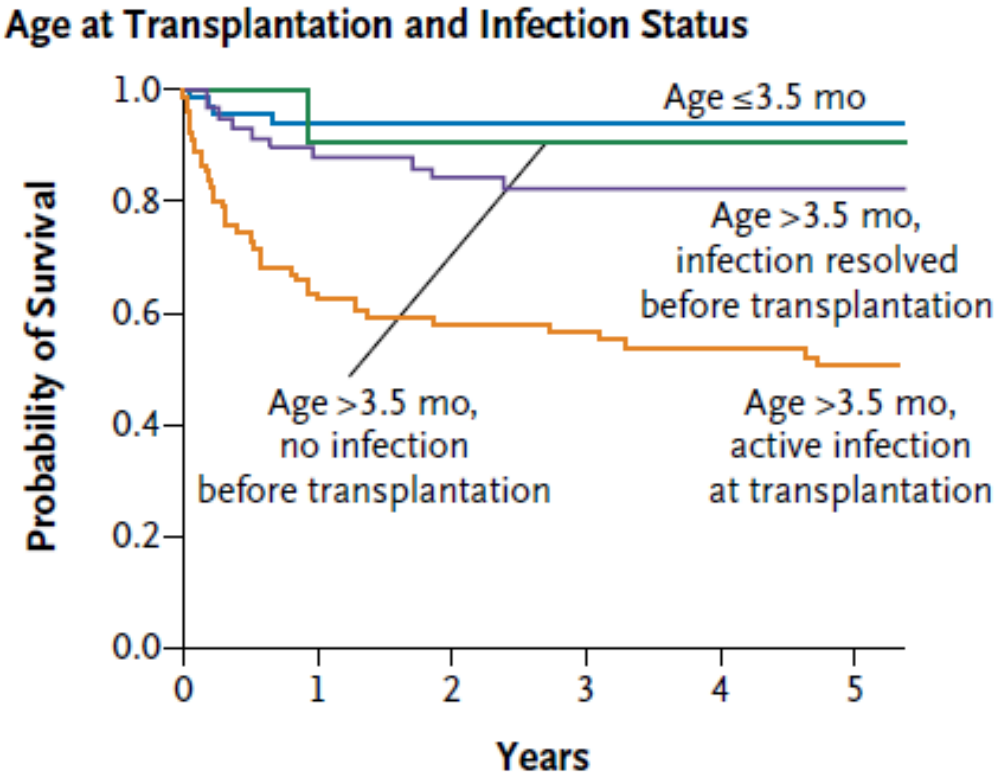
- IV gammaglobulin 600 mg/kg to keep IgG levels > 6 g/l
- TMP/SMX prophylaxis
- Parenteral nutrition, if required
- Developmental assessment
- Transplant preparation
  - HLA typing on patient and family
  - Infectious and immune work-up on donor

Transplantation outcome for SCID

(Pai, et al, NEJM 2014; 371: 434-46)

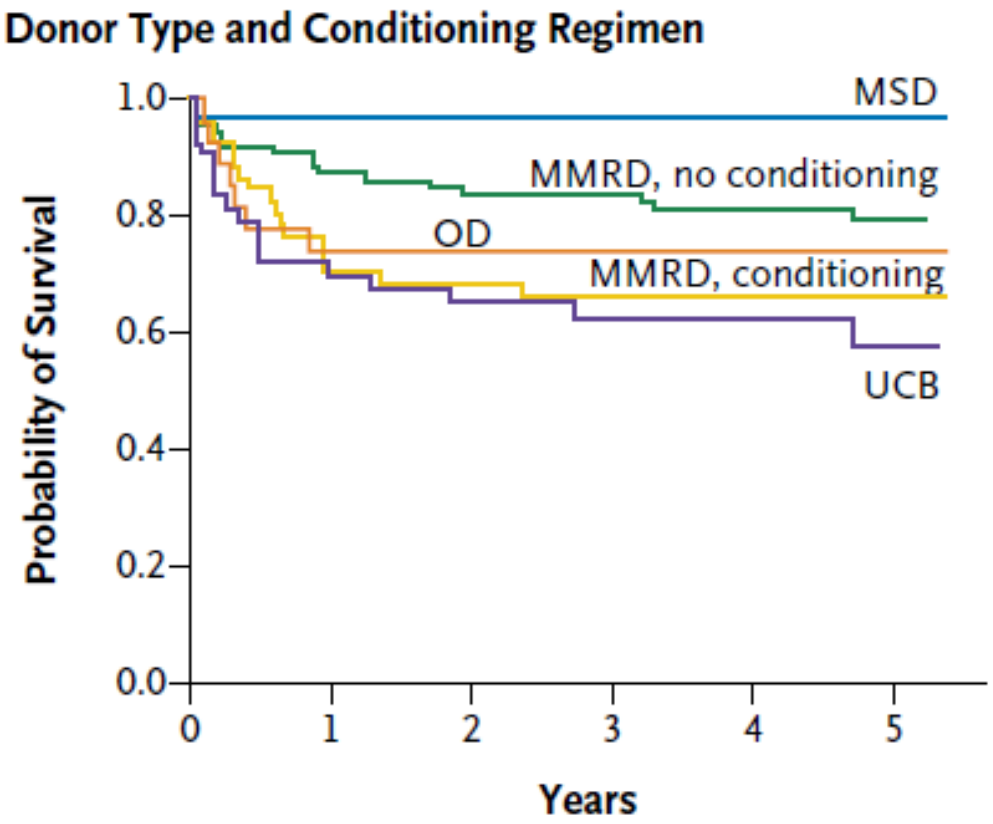
Retrospective data from

240 infants with SCID



Transplantation outcome for SCID

(Pai, et al, NEJM 2014; 371: 434-46)



Complications

- **Chemotherapy toxicity**
- **Infection**
- **Graft versus host disease**
  - most common serious transplant-related complication
  - causing significant morbidity and mortality through secondary infection and organ damage
- **Veno-occlusive disease**

HSCT in Slovenian PID

	Gender	Diagnosis	Age at HSCT	Year	Outcome	Location
1	m	XLP	4 yrs	1997	†	Vienna
2	m	XLP	3,5 yrs	2003	†	Ljubljana
3	m	SCID - OMENN syndrome (RAG1)	3 months	2003	†	Ljubljana
5	m	XCGD	5 yrs	2006		Zürich
6	m	XCGD	25 yrs	2009		Zürich
7	m	SCID - Hypomorphic Rag1 deficiency	4 yrs	2010	† before conditioning	Newcastle
8	f	Osteopetrosis	10 months	2011		Ulm
9	m	APDS (PI3Kδ)	8,5 yrs	2011		Newcastle
10	m	AR CGD	21,5 yrs	2012	Slow reconstitution	Zürich
11	m	fHLH	1,5 yrs	2012	†	Ljubljana
12	m	SCID - OMENN syndrome	17 months	2011	Bronchiolitis obliterans	Ljubljana
13	m	SCID - JAK3	9 months	2012		Ljubljana
14	m	XCGD	19,5 yrs	2013	Slow reconstitution	Zürich
15	m	SCID (CD3E) - sibling A	12 months	2013		Ljubljana
16	m	SCID - OMENN syndrome (RAG1)	5 months	2014		Ljubljana
17	f	MALT 1 deficiency - sibling A	6,5 yrs	2014	CMV, HSV1 reactivation	Ljubljana
18	m	MALT 1 deficiency - sibling B	4,5 yrs	2015	CMV, HSV1 reactivation	Ljubljana
19	f	unknown CID, Monosomy 7	5,5 yrs	2015		Newcastle
20	m	XCGD	7 yrs	2015		Zürich
21	m	XCGD	3,5 yrs	2015		Ljubljana
22	m	SCID (CD3E) - sibling B	4 months	2015		Ljubljana

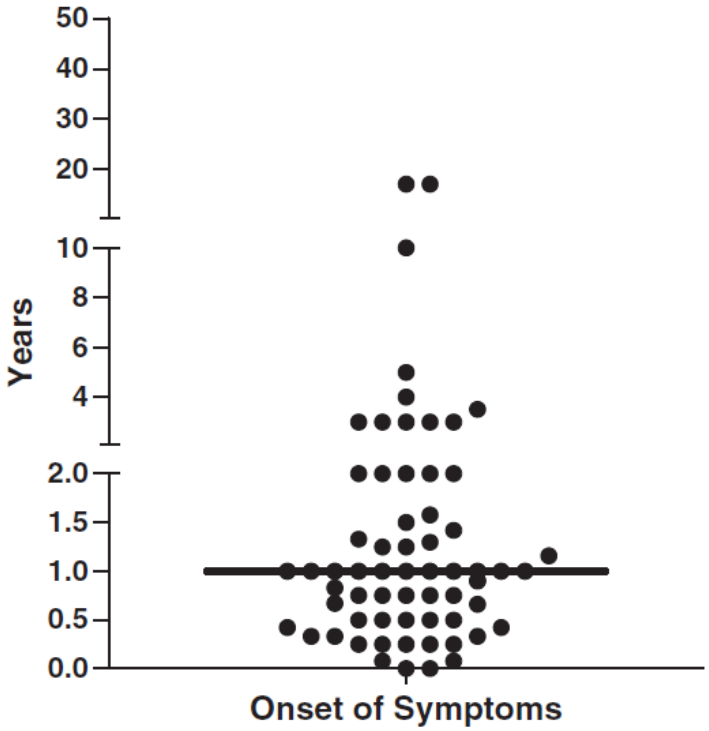
Clinical and immunological manifestations of patients with atypical SCID

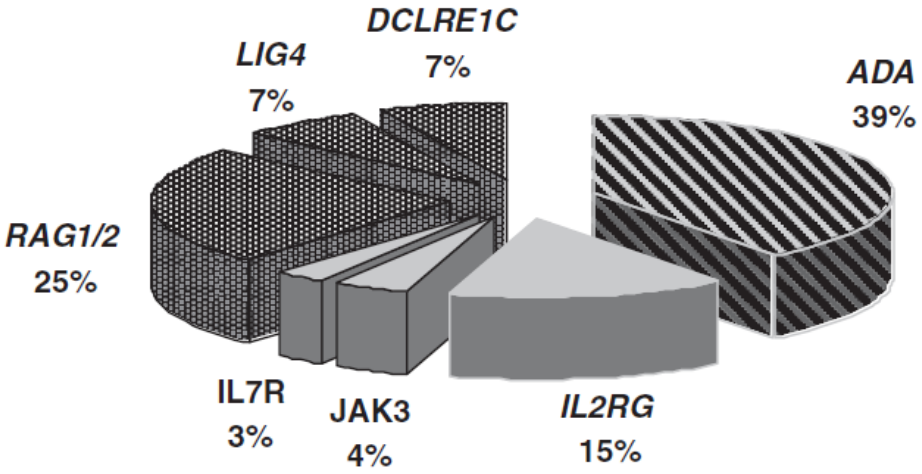
(Felgentreff et al. Clin Immunol 2011; 141:73-82.)

- Analysis of 10 new + 63 patients from the literature with "atypical" SCID
- Case definition for "atypical" SCID:
  - clinical presentation beyond the first year of life
  - mutation in a gene regularly associated with a typical SCID phenotype
  - CD3+ T cell counts above 100/μl at diagnosis

Clinical and immunological manifestations of patients with atypical SCID

(Felgentreff et al. Clin Immunol 2011; 141:73-82.)

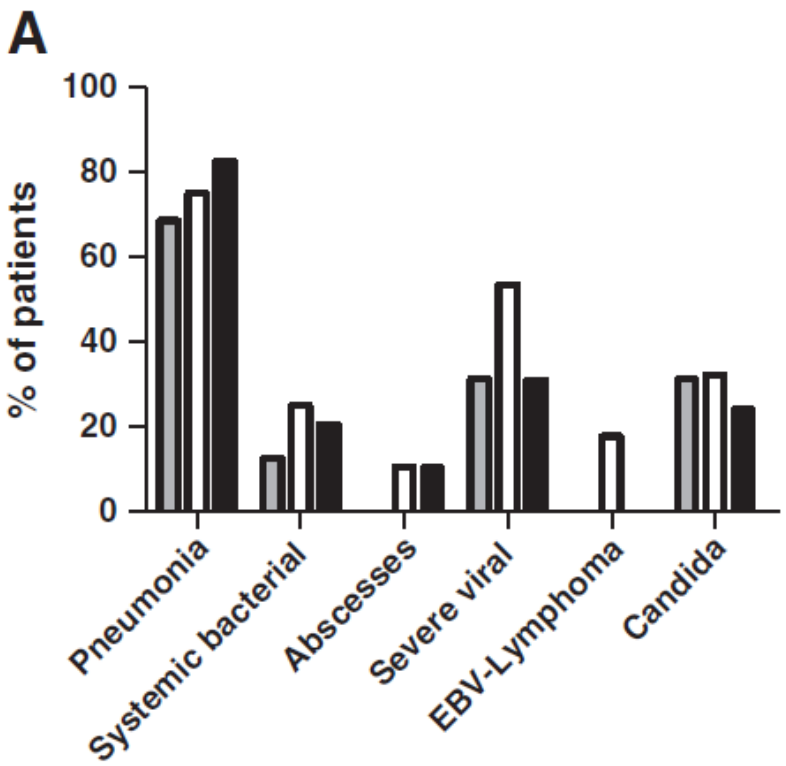




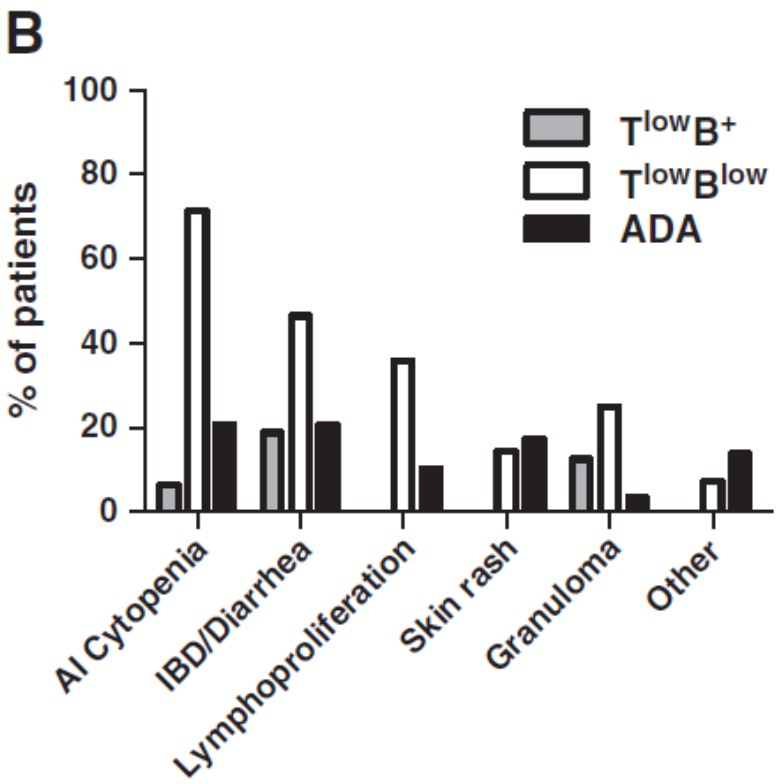
Clinical and immunological manifestations of patients with atypical SCID

(Felgentreff et al. Clin Immunol 2011; 141:73-82.)

Infectious disease manifestations



Manifestations of immune dysregulation



Outcome of patients with profound combined immunodeficiency (P-CID)

(Speckmann C, et al. J Allergy Clin Immunol 2016 (in press)

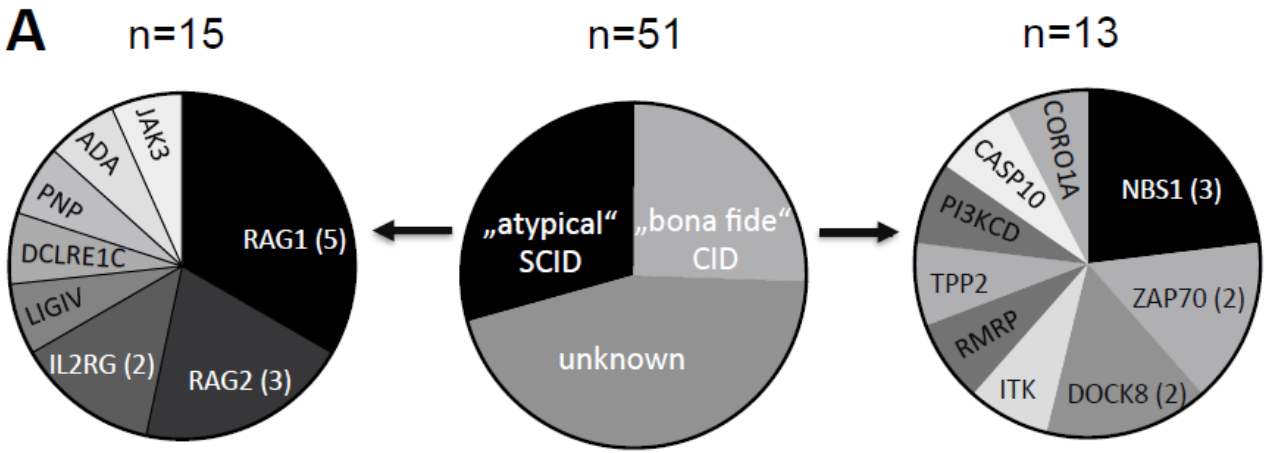
- **Observational study of first 51 patients with P-CID**

Inclusion criteria
I. T-cell criteria (at least 2 of the following 4):
1. Reduced T-cell counts, i.e. CD4 < 700 (< 2y), < 500 (2-4y), < 300 (> 4y) or CD8: < 350 (<2y), < 250 (2-4y), < 150 (> 4y) 2. Reduced thymic function i.e. naïve T cells < 30% (< 2y), < 25% (2-6y), < 20% (>6y) 3. Impaired or absent T cell proliferation i.e. PHA or anti CD3 response < 30% of lower limit of normal 4. Elevated fraction of γ/δ T cells, i.e. > 15% of total CD3+ T cells
II. **At least one severe clinical event*** Infection or immune dysregulation or malignancy

Outcome of patients with profound combined immunodeficiency (P-CID)

(Speckmann C, et al. J Allergy Clin Immunol 2016 (in press)

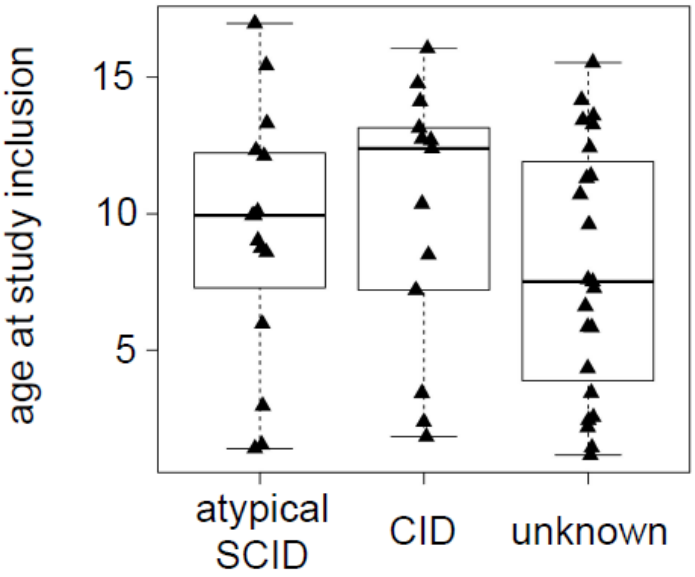
Molecular diagnoses at study entry:



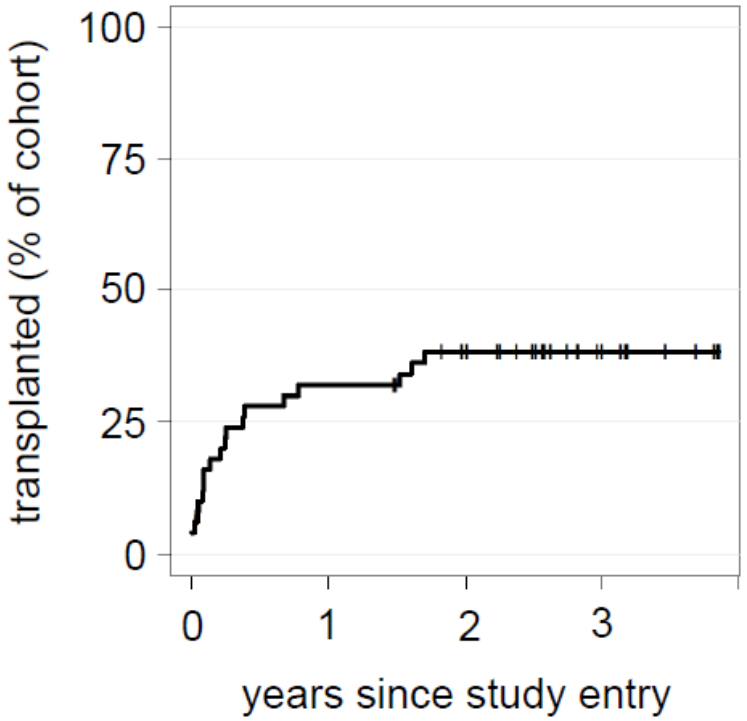
Outcome of patients with profound combined immunodeficiency (P-CID)

(Speckmann C, et al. J Allergy Clin Immunol 2016 (in press)

Age at study inclusion:



HSCT since study inclusion:



Conclusions

- **PIDs one of the most rapidly developing fields in medicine**
  - Significant new knowledge on molecular recognition of new diseases, novel management strategies, newborn screening
- **Care for PID patients multidisciplinary and reflects development and quality of a health care system in the country**
- **Mortality for HSCT in SCID now around 10%**
- **Autoimmune manifestations frequent and often multiple in patients with CID**

THE ROLE OF FLOW CYTOMETRY IN DIAGNOSTICS OF IMMUNE DEFICIENCIES

Tomas Kalina

CLIP – Cytometry, Department of Paediatric Hematology/Oncology, 2nd Medical School, Charles University Prague, Czech Republic

Flow cytometry is widely used as the most appropriate tool for evaluation of the lymphocytic compartment in diagnostics and research of primary immunodeficiency (PID). The field is however moving rapidly and both, the scope of questions and available flow techniques diversifies. Flow cytometry can thus be used for fast screening and diagnostic evaluation of Severe Combined Immunodeficiency (SCID) and Combined Immunodeficiency (CID), including Recent Thymic Emigrants' evaluation; it can be used as readout of functional tests (CD40L deficiency, degranulation deficiency, CTLA-4 deficiency, p-kinase status assessment). It is also employed in translational research leading to disease entity discovery (immunophenotype descriptions of lymphocytes in new mutations), classification (in Common Variable Immunodeficiency), and also in longitudinal follow up of patients' (monitoring of treatment efficacy). I would like to present a lymphocyte directed screening approach to immunodeficiency developed by EuroFlow PID group, where we designed a set of five 8-color tubes that describe subsets of T and B-lymphocytes in detail. I would present typical cases and discuss the complementarity of flow cytometry and NGS. Next, I will show examples of functional tests and discuss their pitfalls. In the case of new and very rare PIDs, so called "diagnosis by research" is necessary to obtain final diagnosis. Classification of known PIDs is currently a matter of intensive research, where I would show an example of algorithmic grouping of disease subcategories in CVID. Lastly, monitoring of patients with PID that receive specific inhibitors or targeted therapies will be discussed. In conclusion, decision about the spectrum and scope of cytometry tests must be tailored to the expected patient population and to the experience, equipment and manpower capacities of each laboratory. Interlaboratory collaboration on higher level is needed to achieve the goals of PID diagnostics, prognostics and monitoring.

Ljubljana, 2016

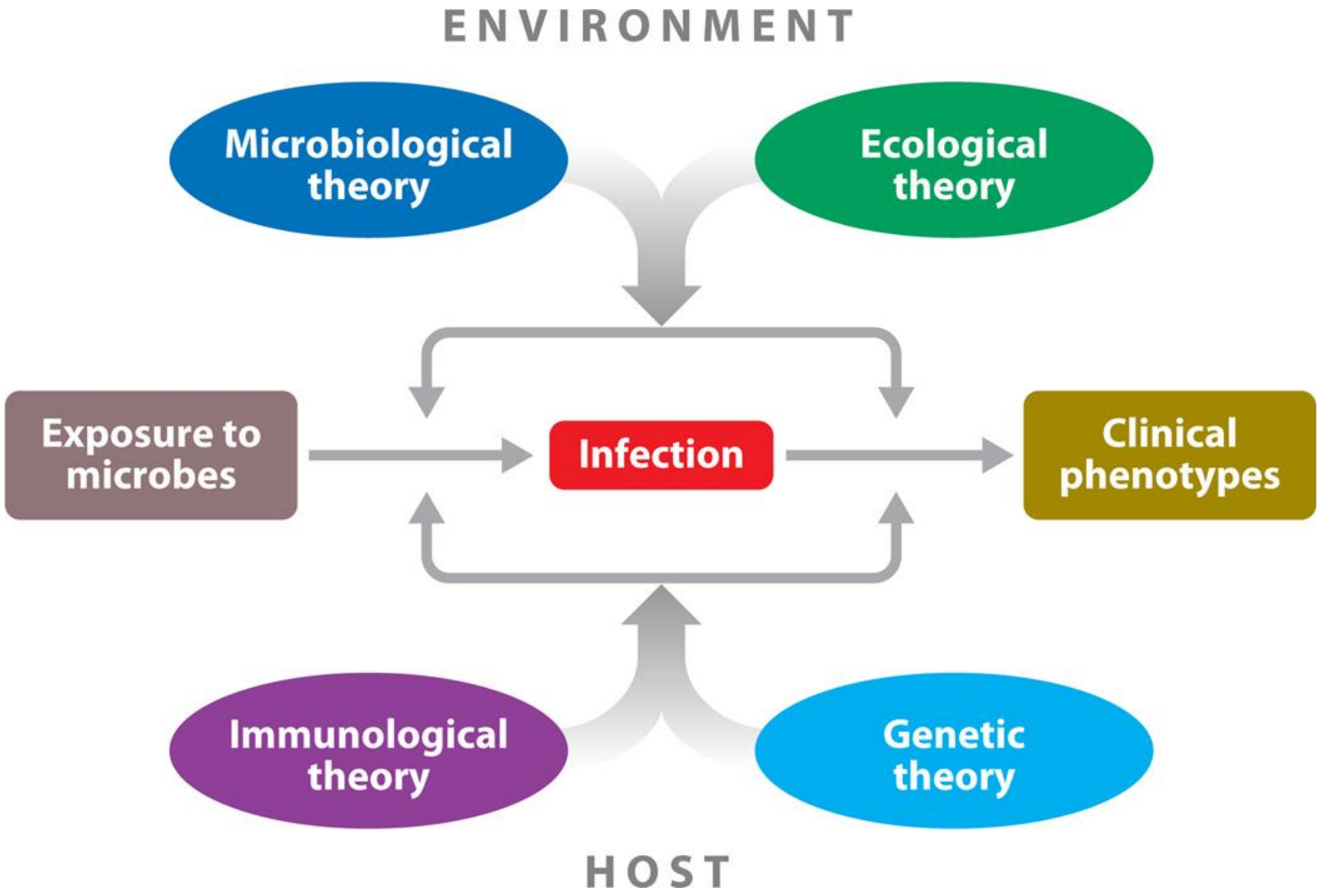
(New) possibilities in flow cytometry for PIDs

Tomáš Kalina

Charles University, 2nd Faculty of Medicine,
Prague, Czech Republic
Dpt. of Pediatric Hematology and Oncology

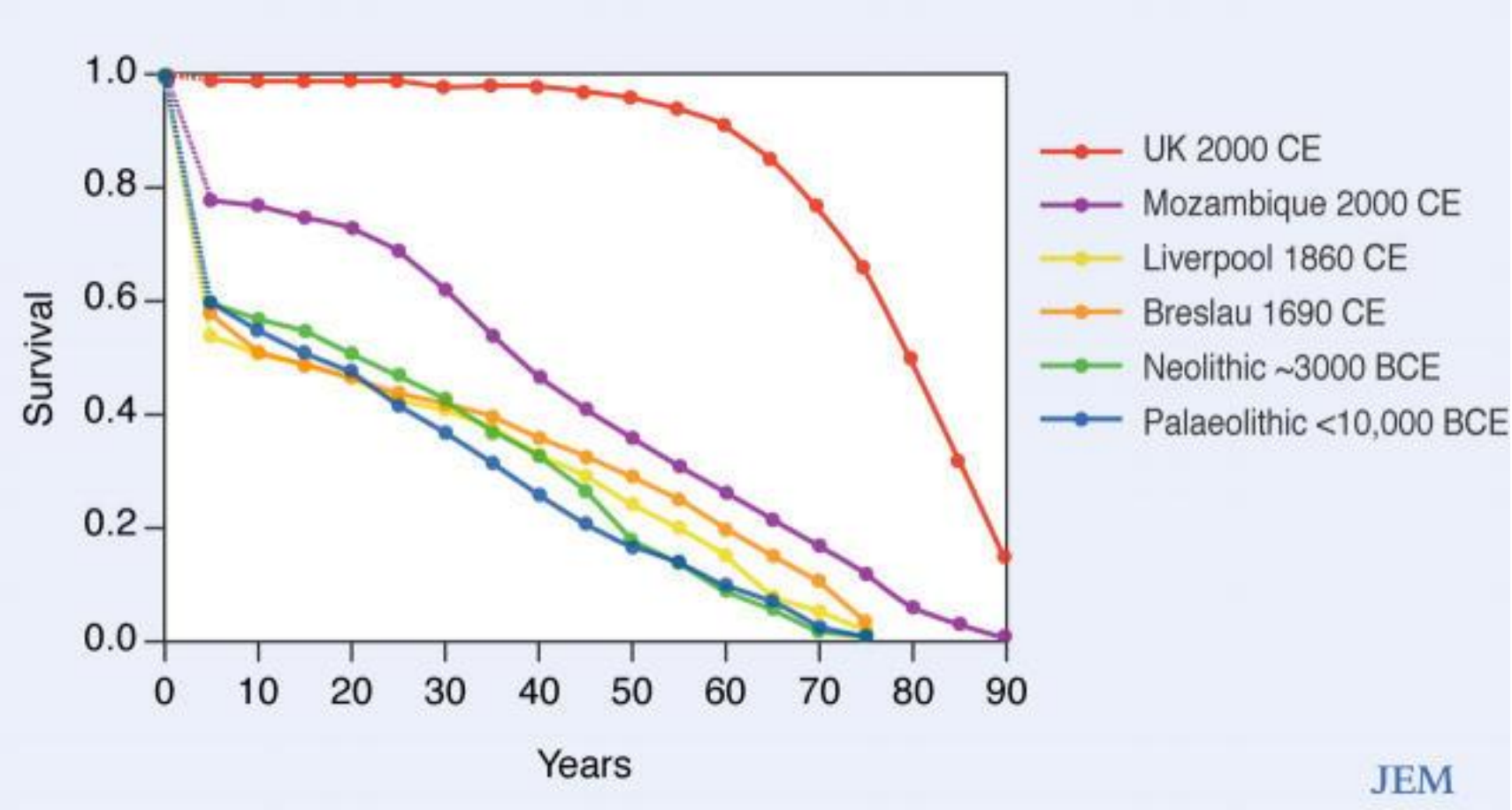
- Childhood Leukemia Investigation Prague

Immunodeficiency is a failure to respond to infection



Casanova, J.-L., and L. Abel. 2013. The Genetic Theory of Infectious Diseases: A Brief History and Selected Illustrations. Annu. Rev. Genomics Hum. Genet 14: 215–43.

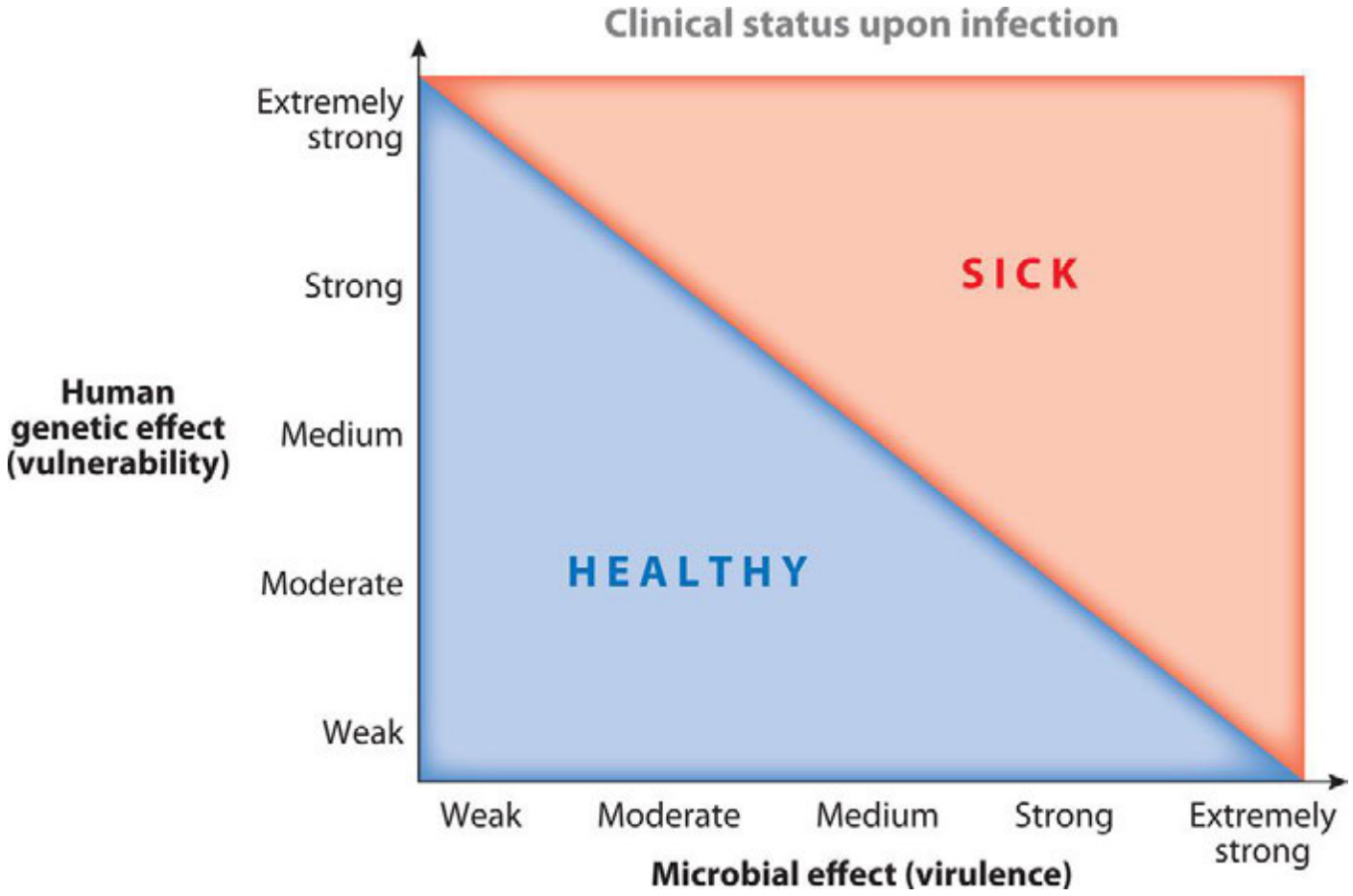
Mortality curves throughout the history



~60% of deaths were due to infectious disease until mid 19th century

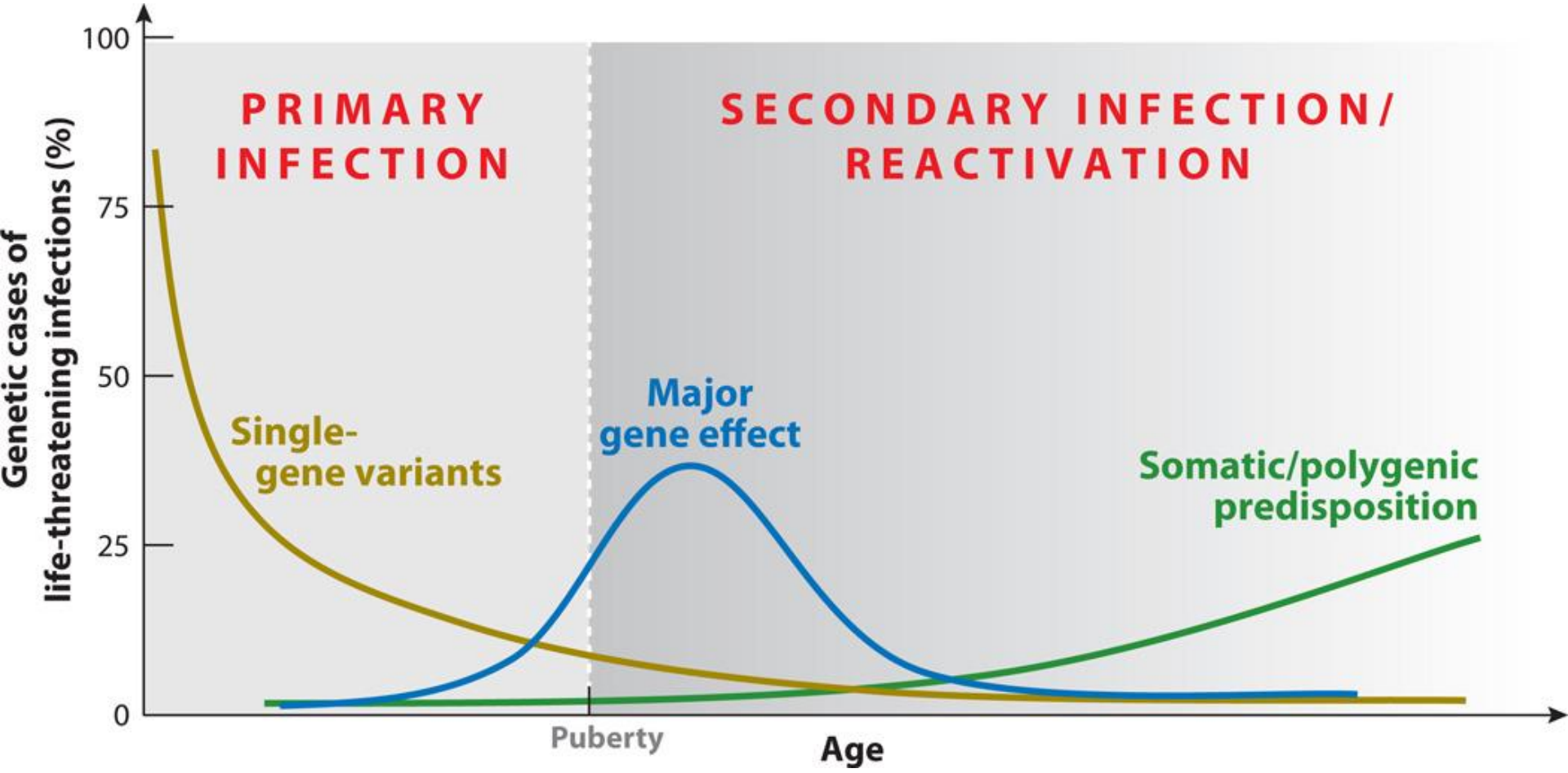
Casanova, J.-L., and L. Abel. 2005. Inborn errors of immunity to infection: the rule rather than the exception. J. Exp. Med. 202: 197–201.

Genes versus Microbes



Casanova, J.-L., and L. Abel. 2013. The Genetic Theory of Infectious Diseases: A Brief History and Selected Illustrations. Annu. Rev. Genomics Hum. Genet 14: 215–43.

PID at a different age



Casanova, J.-L., and L. Abel. 2013. The Genetic Theory of Infectious Diseases: A Brief History and Selected Illustrations. Annu. Rev. Genomics Hum. Genet 14: 215–43.

PID is a Rare disease diagnostics

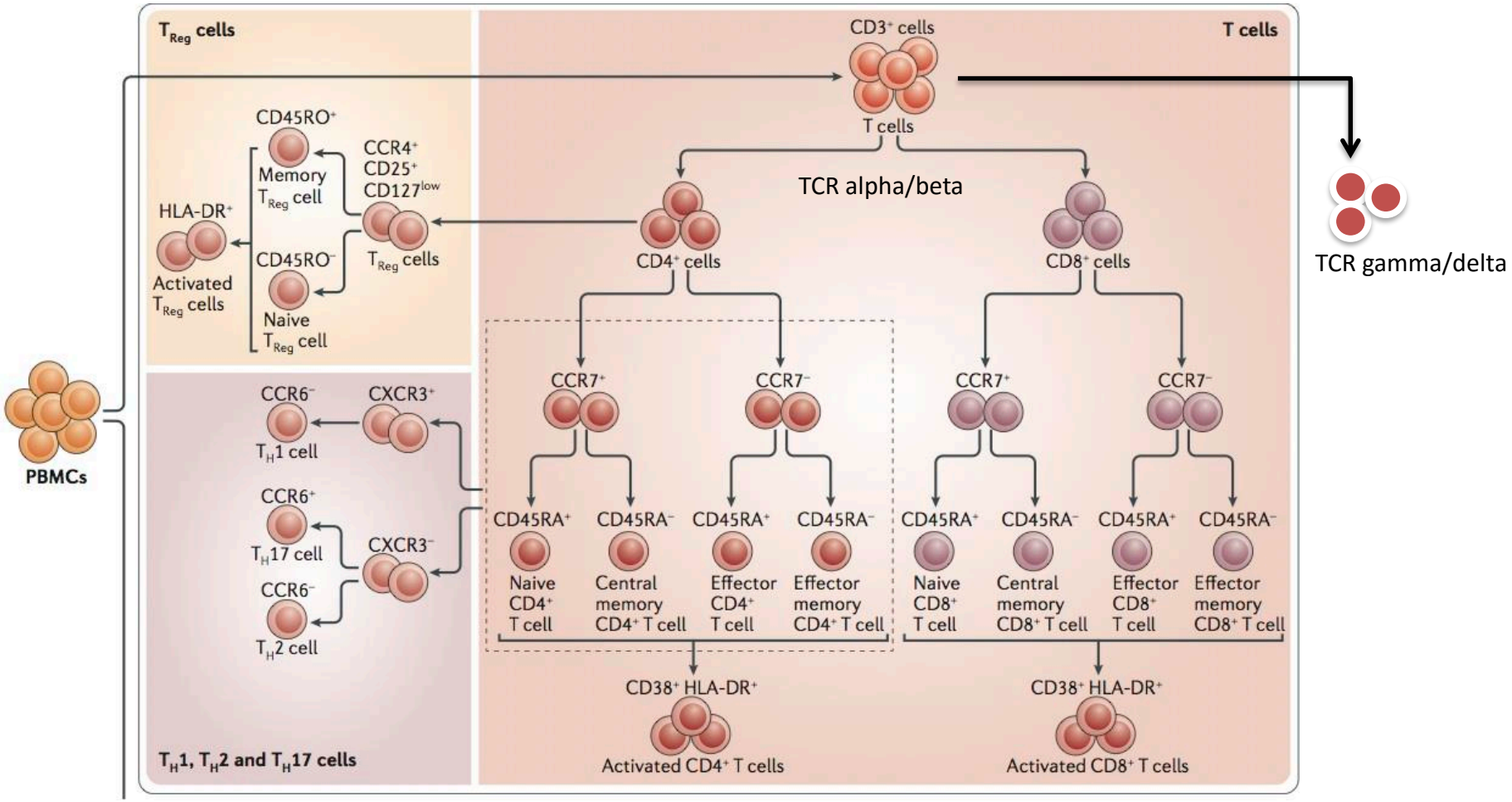
- Rare disease cases are rare
- Frequently non-typical
- Not fully understood
- Diagnostic criteria in development
- “Diagnosis by research”
- How to share the knowledge?

  - Centralized diagnostic
  - Inter-laboratory collaboration
  - Standardized
  - Computer assisted data analysis

Flow Cytometry for cellular detection

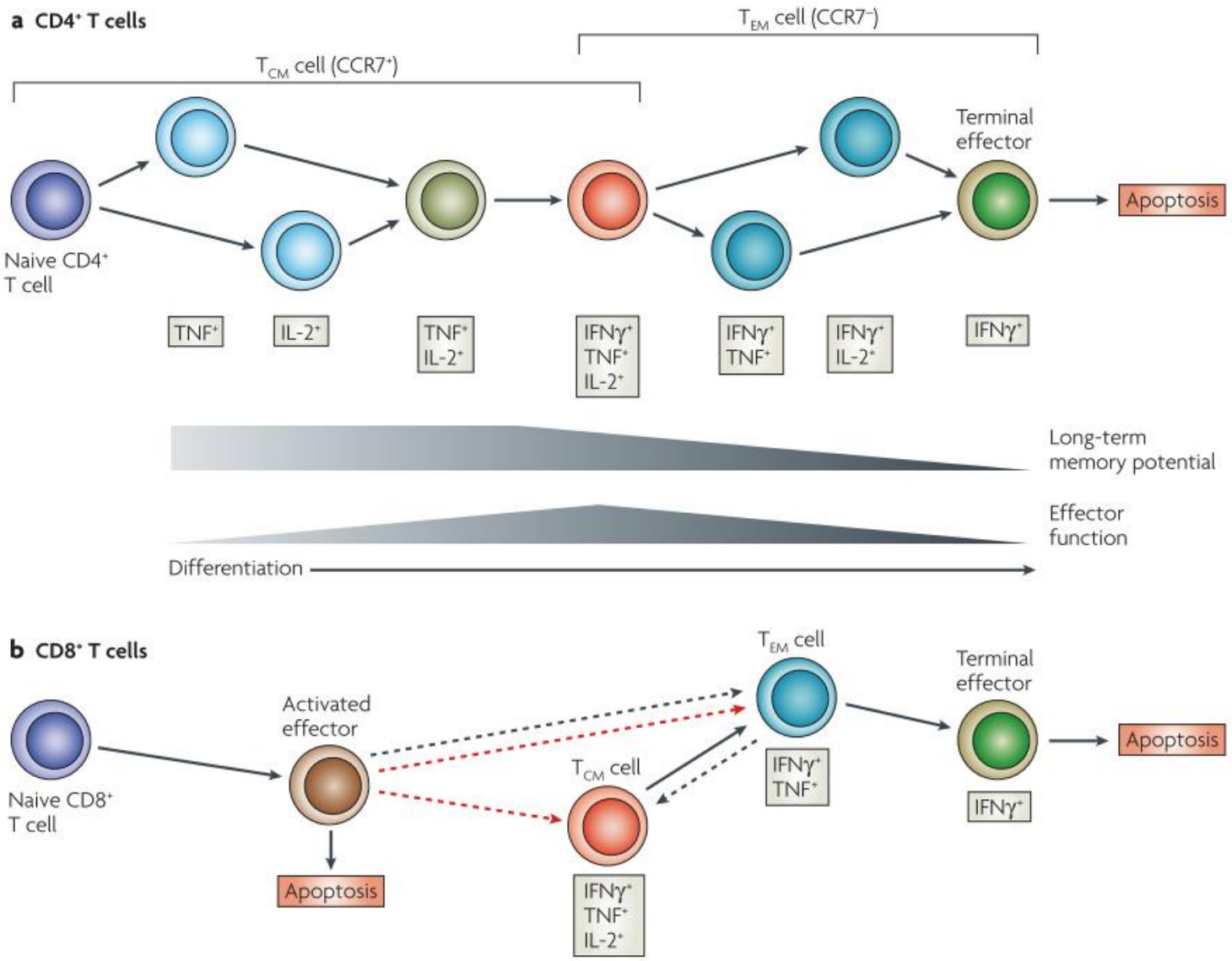
- ✓ Cytometry became extremely powerful
  - ✓ Single cell detection + functional studies
  - ✓ Many colors available
  - ✓ Millions cells per tube
  - ✓ Fast acquisition 20 000/s
- ✓ We need knowledge to employ it correctly
  - ✓ Technology
  - ✓ Education & Experience
  - ✓ Data analysis – statistics / bioinformatics
- ✓ Standardization
  - ✓ Quality Control
  - ✓ Inter-laboratory collaborations
  - ✓ Data sharing

Immunophenotyping T cells in peripheral blood



Maecker HT, et al.
Nat Rev Immunol. 2012

Memory stages of T cells in periphery



Naive

CM = Central Memory

EM = Effector Memory

Terminal effectors

Developmental defects of lymphocytes

Al-Herz, 2014:

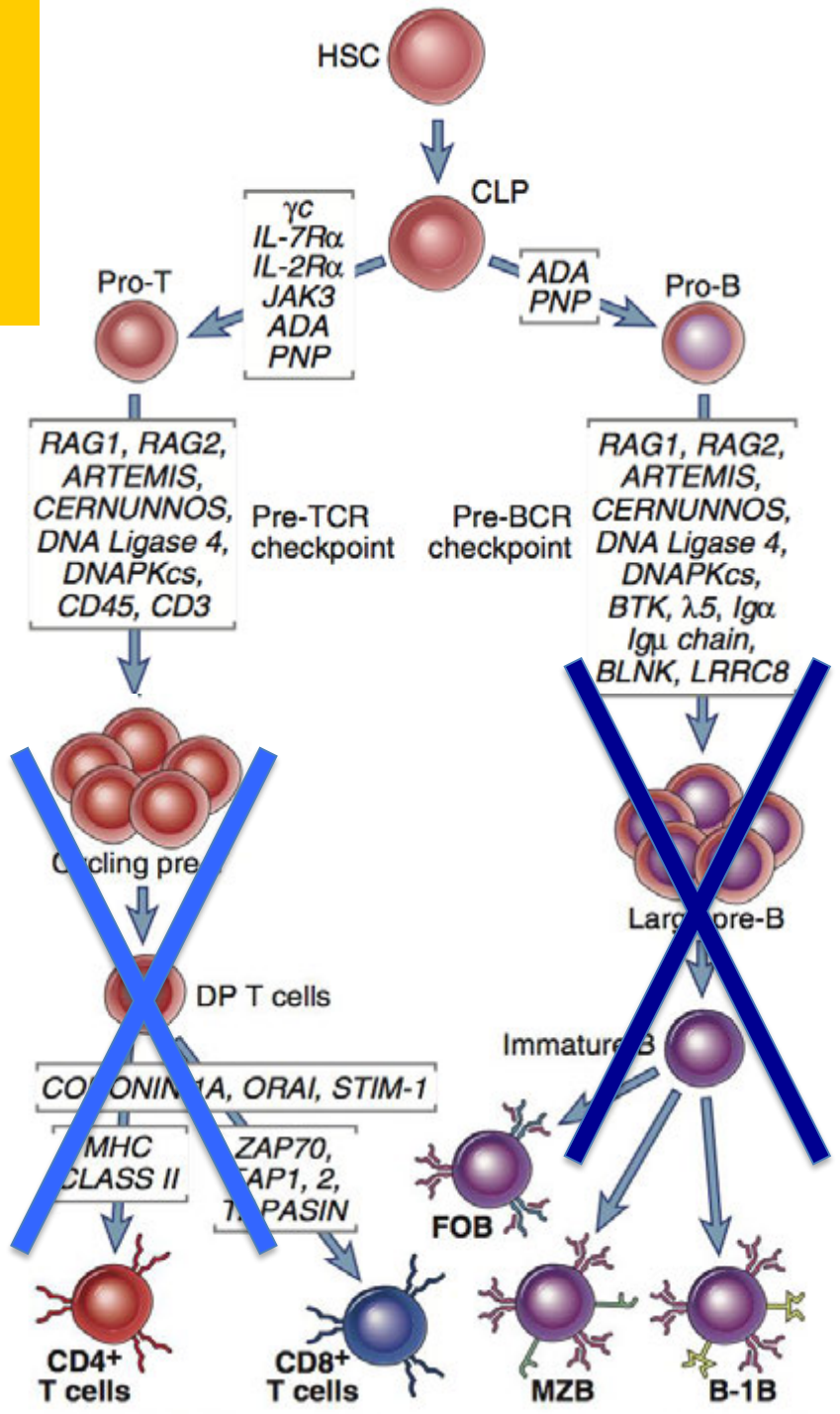
Combined immunodeficiency

1. T− B+ severe combined immunodeficiency (SCID)

(a) γc deficiency
(b) JAK3
(c) IL7Rα
(d) CD45
(e) CD3δ
(f) …..

2. T-B- SCID

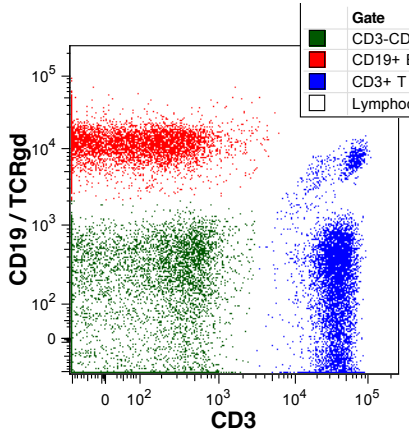
(i) DNA recombination defects (RAG1, RAG2 …)
(ii) Reticular dysgenesis
(iii) Adenosine deaminase (ADA)



Abbas: CELLULAR AND MOLECULAR IMMUNOLOGY, 7th ed., 2012

Lymphocytes

T B NK



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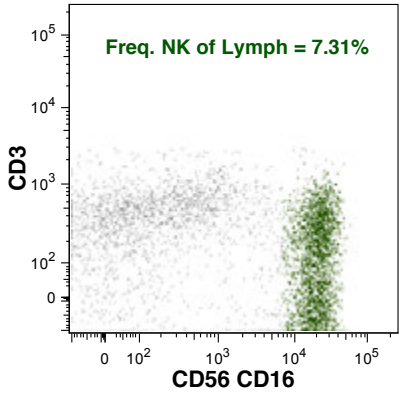
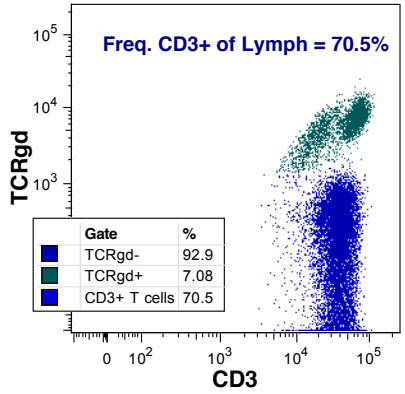
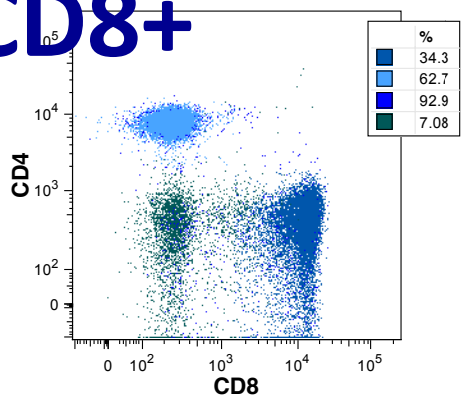
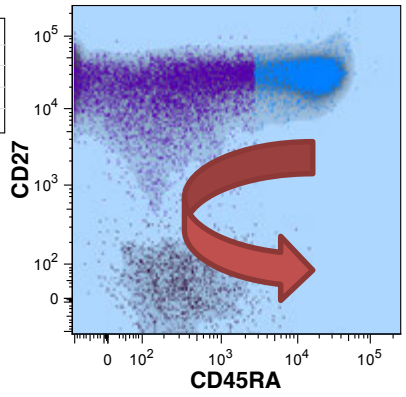
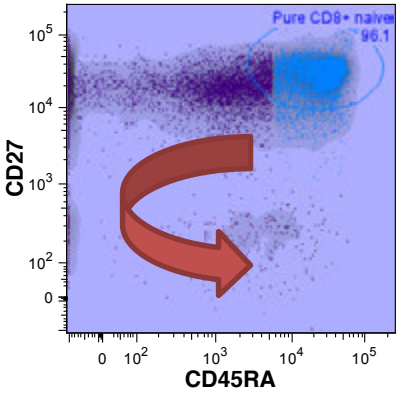




CD4+ CD8+



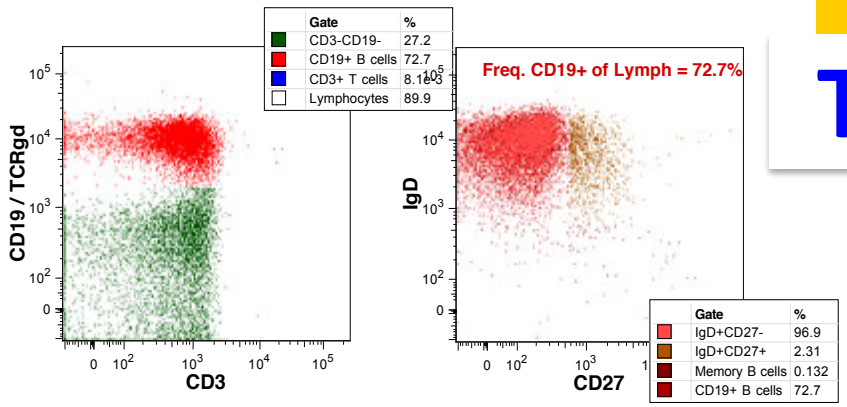




Naïve->CentrMemory->Eff Memory -> Terminal Eff

What is missing?

T B NK



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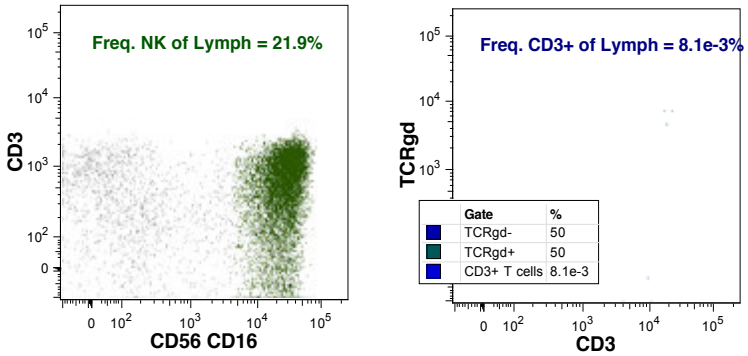
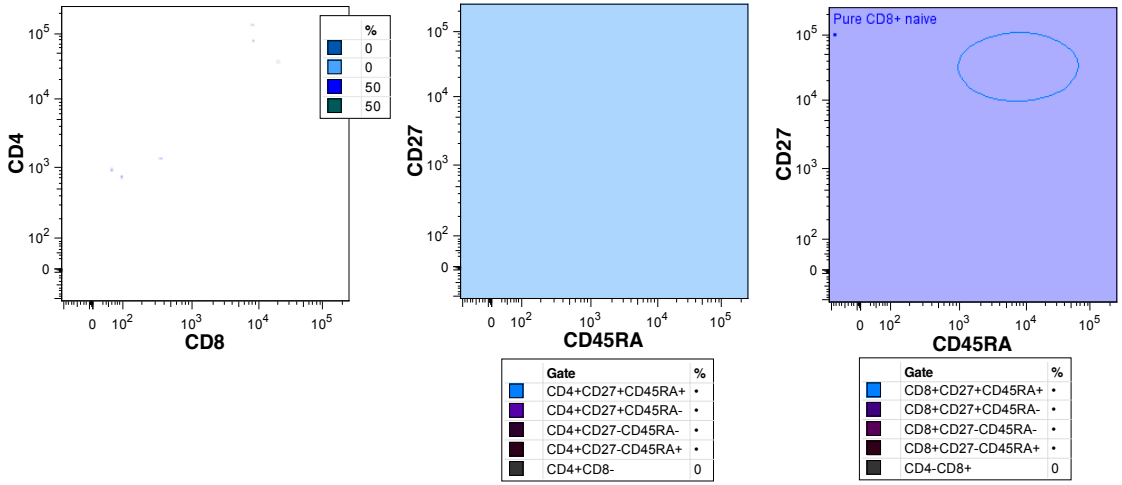
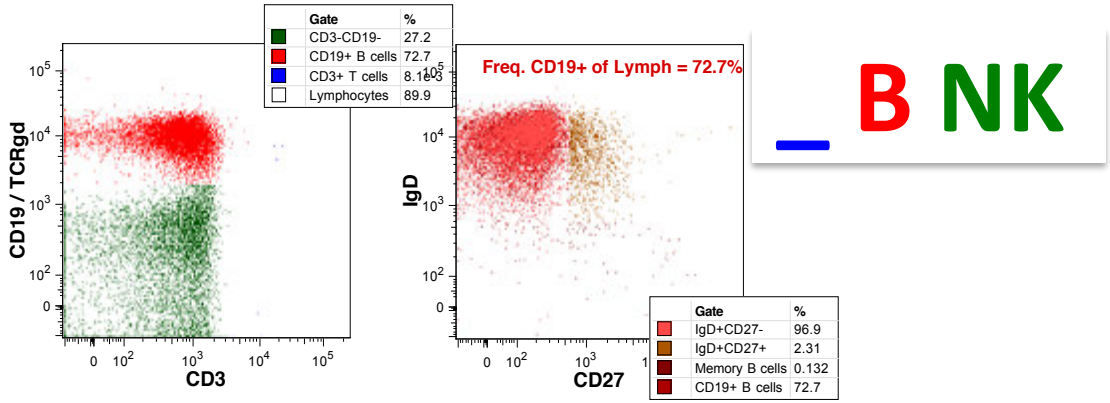
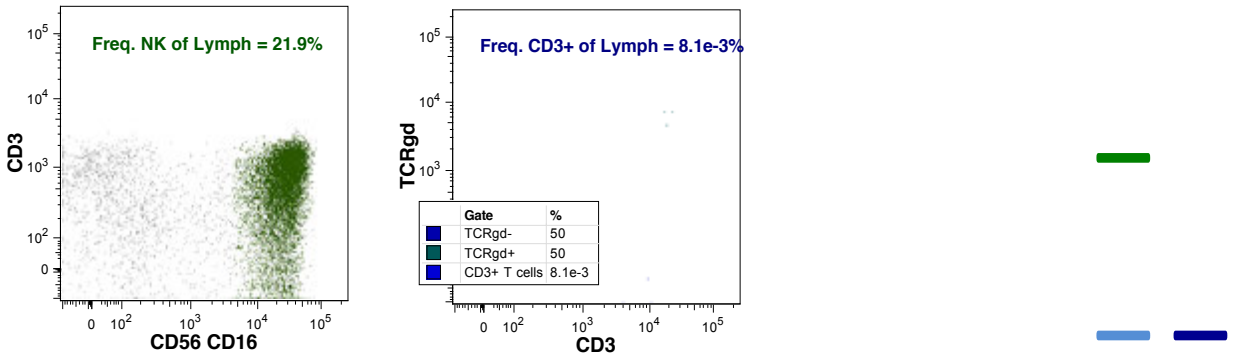


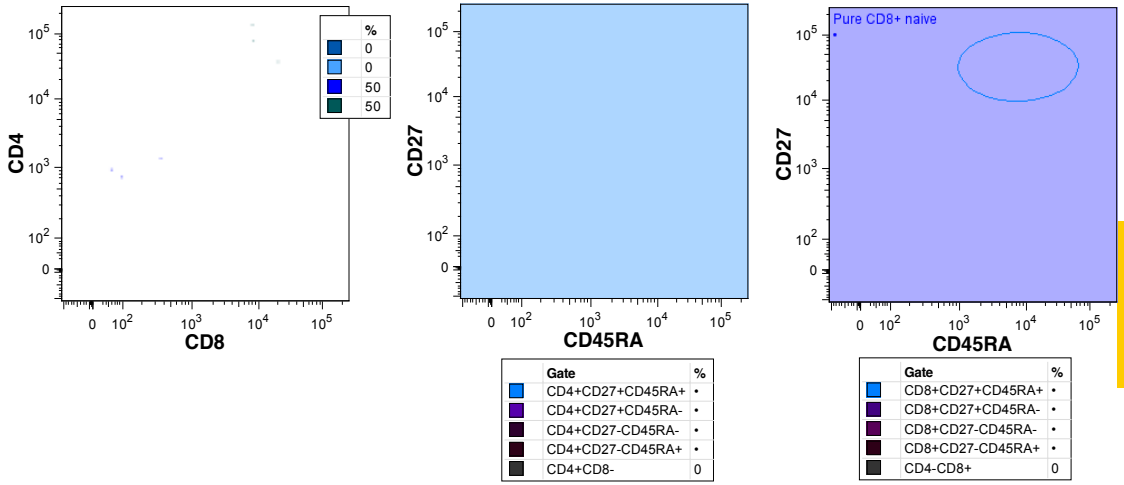
CD4+ CD8+





B NK





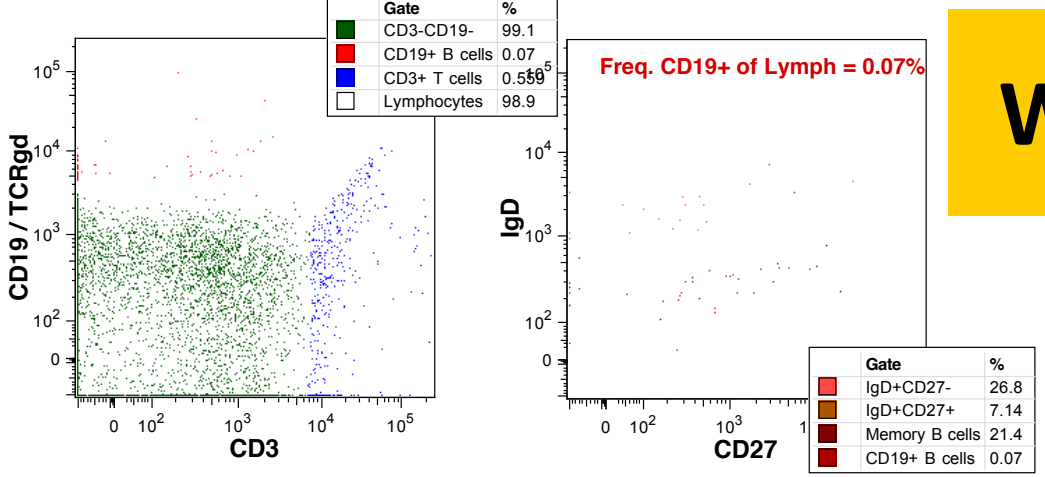
SCID IL7Rdef

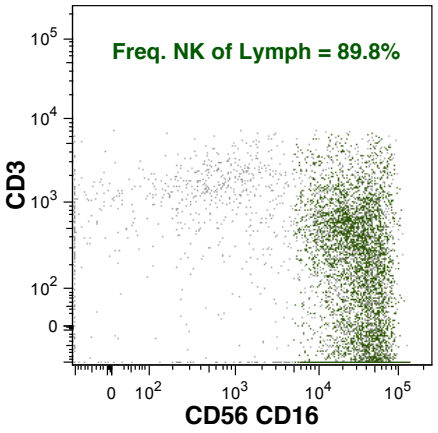
What is missing?

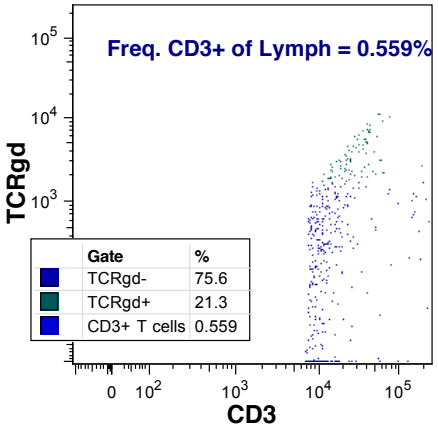
T B NK

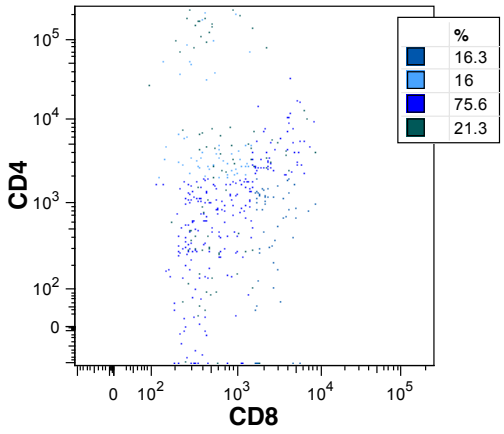
TCRgd

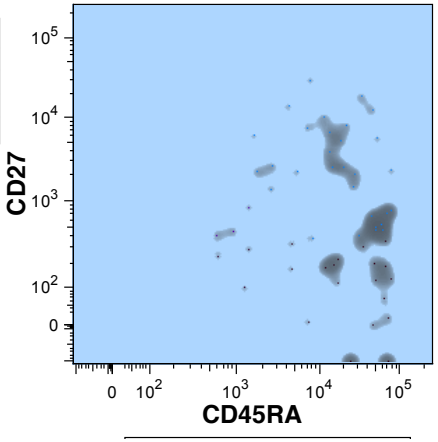
CD4+ CD8+

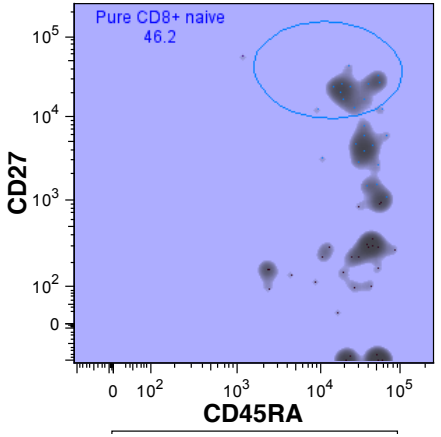


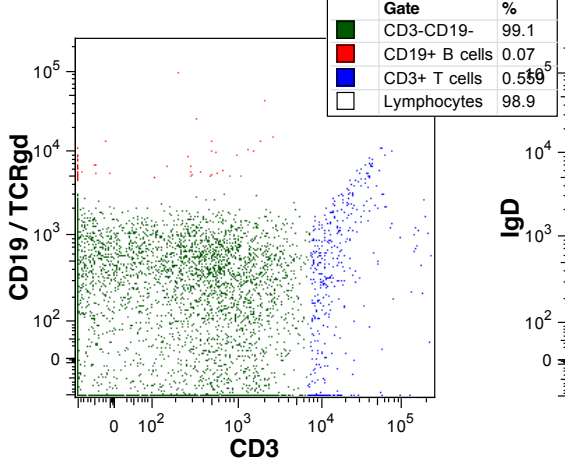


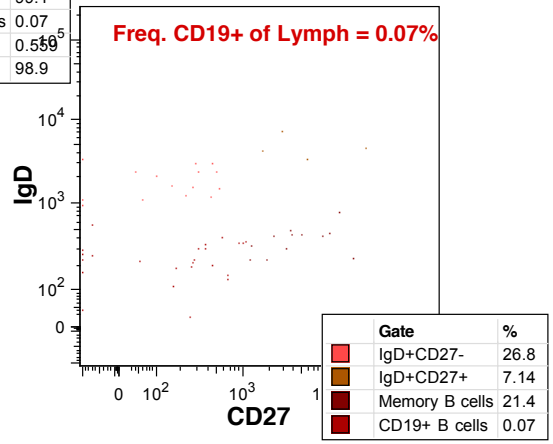




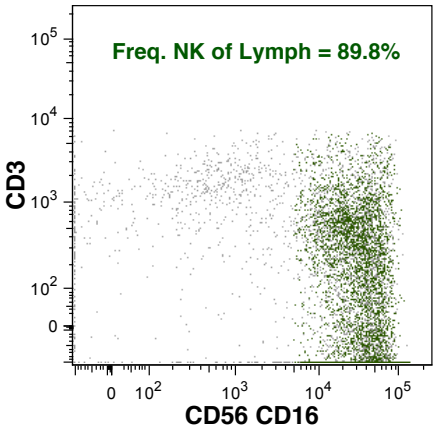


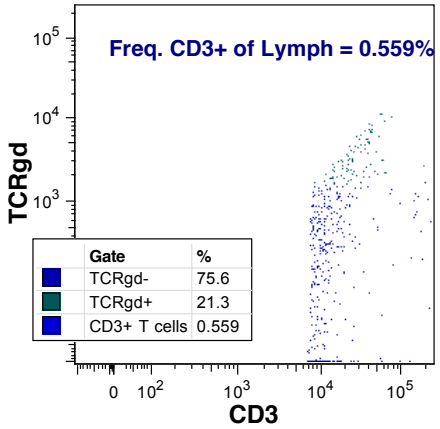


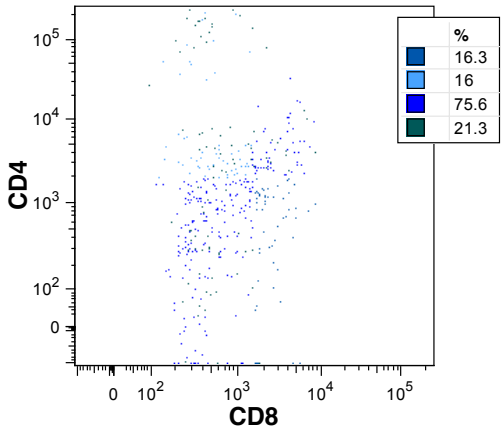


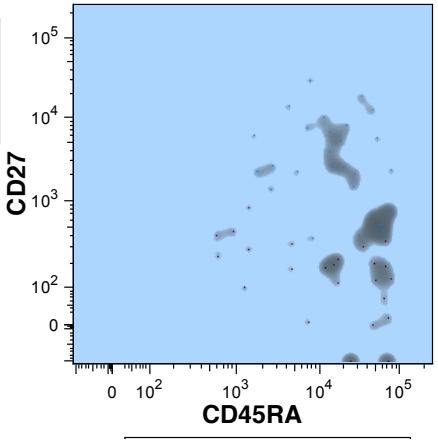


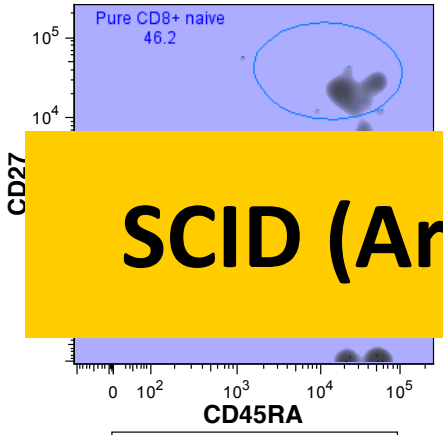
NK







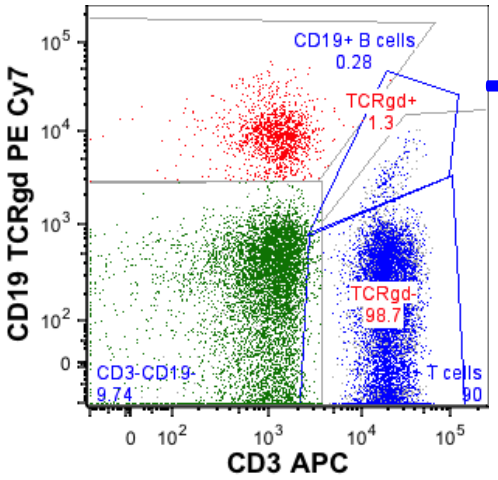


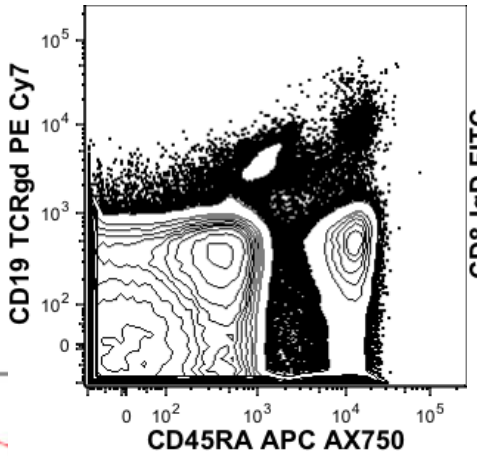


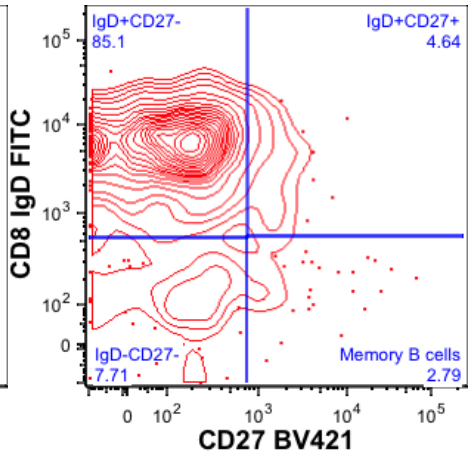
SCID (Artemis)

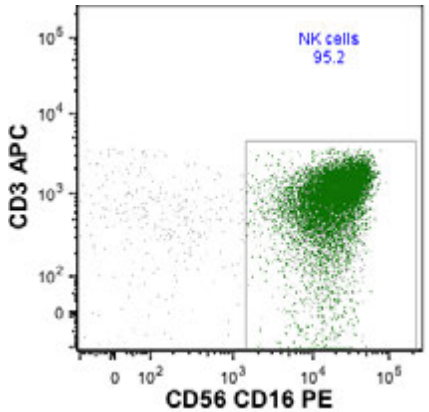
What is wrong here?

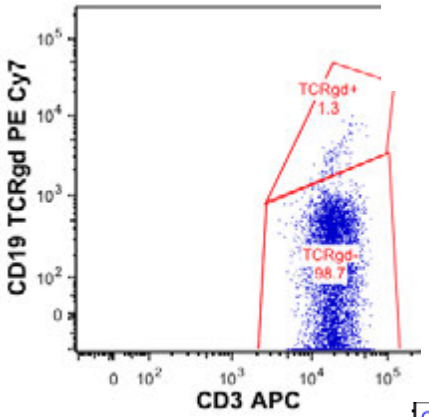
T B NK

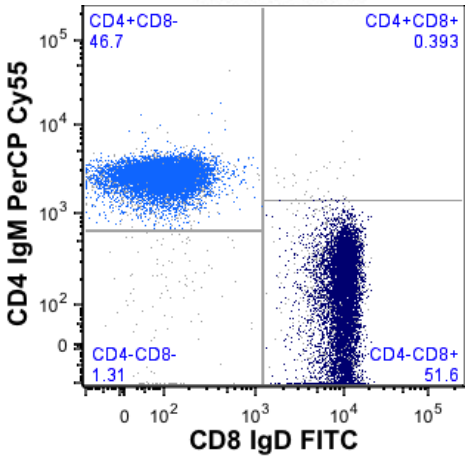


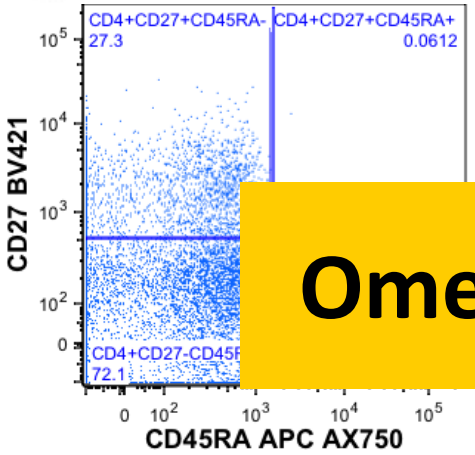


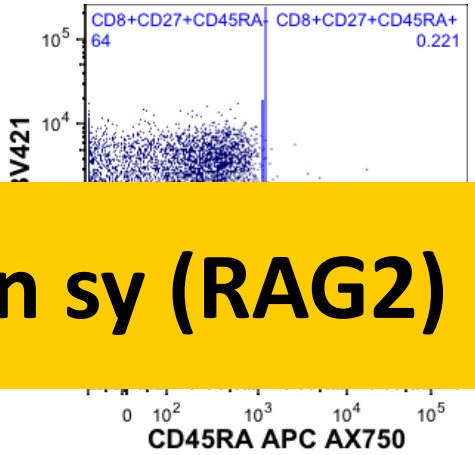








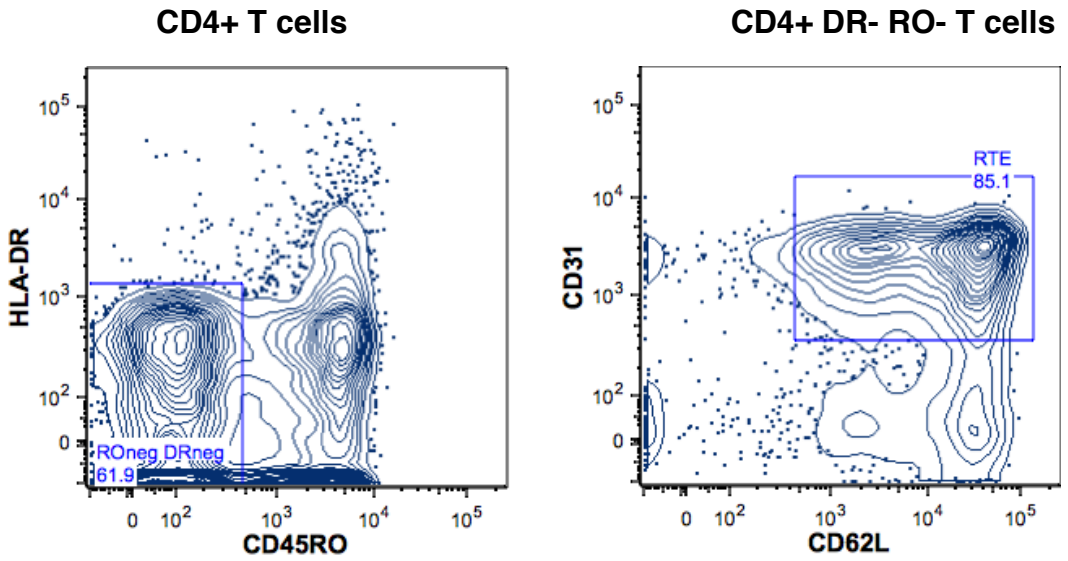




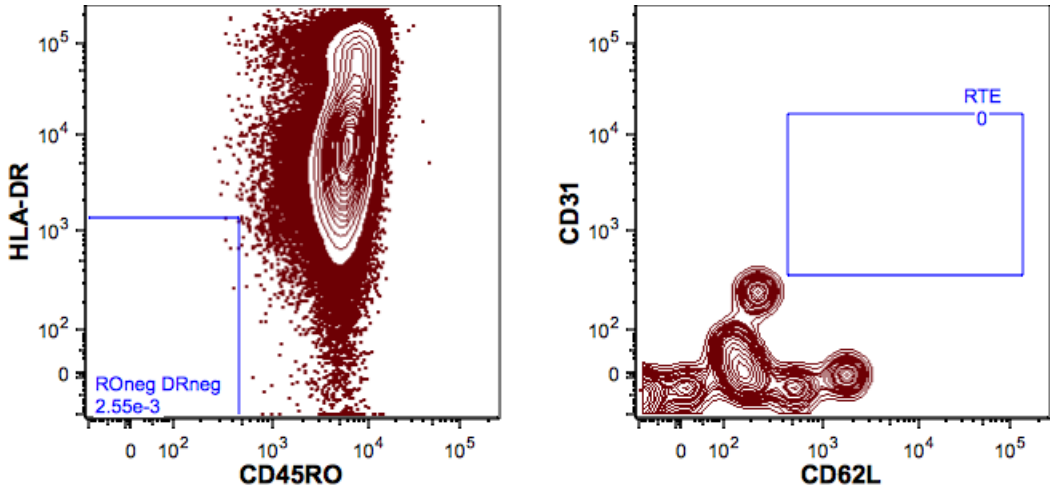
Omen sy (RAG2)

Recent Thymic Emigrants

Control



Pacient : SCID – RAG1



Lack of RTE

EuroFlow PID

Interlaboratory-standardized
8-color cytometry

Two step test:

- PID Orientation tube

- T cell: SCID / RTE tube
- T cell: subset tube
- B cell: PreGC
- B cell: PostGC

Subsets in PID orientation tube

SUBSET			GATE NAME
Leukocytes			CD45+ FSc SSc
Monocytes			$CD4^{med}CD45+SSC^{med}$
Lymphocytes			$CD45^{high}SSC^{low}$
NK cells			CD3-CD19-CD16/56+
CD3+ T cells			CD3+ CD19-CD16/56-
CD4+	Helper T cells (Th)		CD3+TCRgd-CD4+
	CD4+ Naive	Naive Th cells	CD3+TCRgd-CD4+CD27+CD45RA+
	CD4+ Tcm	Central memory Th cells	CD3+TCRgd-CD4+CD27+CD45RA-
	CD4+ Tem	Effector memory Th cells	CD3+TCRgd-CD4+CD27-CD45RA-
	CD4+ Ttd	Terminally differentiated (TEMRA)	CD3+TCRgd-CD4+CD27-CD45RA+
CD8+	Cytotoxic T cells (Tc)		CD3+TCRgd-CD8+
	CD8+ Naive	Naive Tc cells	CD3+TCRgd-CD8+CD27+CD45RA+
	CD8+ Tcm	Central memory Tc cells	CD3+TCRgd-CD8+CD27+CD45RA-
	CD8+ Tem	Effector memorty Tc cells	CD3+TCRgd-CD8+CD27-CD45RA-
	CD8+ Ttd	Terminally differentiated (TEMRA)	CD3+TCRgd-CD8+CD27-CD45RA+
CD4-CD8-	Double negative T cells		CD3+TCRgd-CD4-CD8-
CD4+CD8+	Double positive T cells		CD3+TCRgd-CD4+CD8+
TCRgd+	TCRγδ T cells		CD3+TCRgd+
CD19+ B cells			CD19+CD3-CD16/56-
Naive B cells			CD19+IgM+IgD+CD27-
Natural effectors			CD19+IgM+IgD+CD27+
Memory B cells			CD19+CD27+IgD-
	Switched memory B cells		CD19+IgM-IgD-CD27+
	IgD only Memory B cells		CD19+IgM-IgD+CD27+
Plasmablasts			$CD19+CD45RA+CD27^{high}$

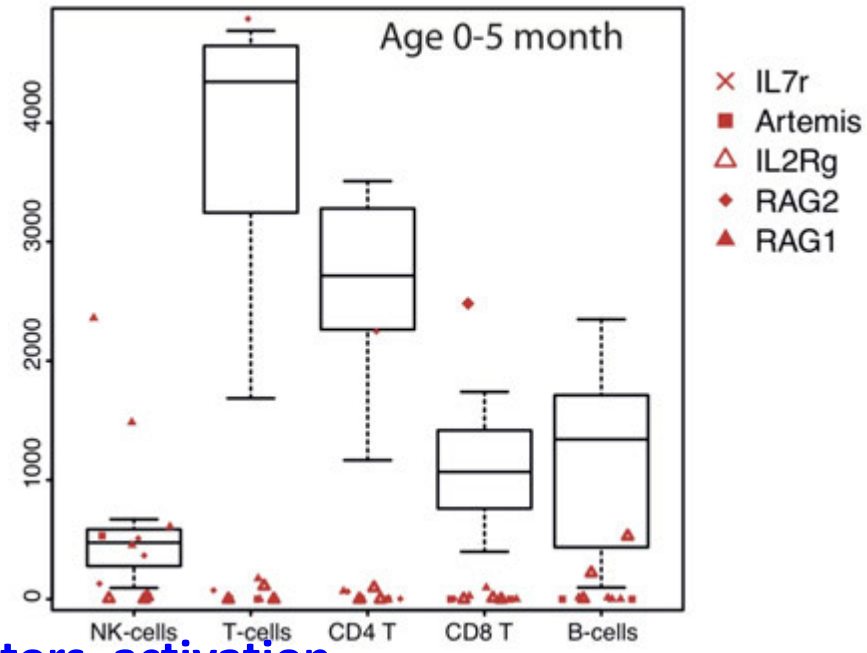
Absolute and relative numbers for all

Flow Cytometry role (in the world of NGS)

✓ Simple lymphocyte screening

T (CD4 CD8) B NK

✓ Extensive lymphocyte screening



van den Burg, Kalina, unpublished

T cell subests incl:

RTE, Naïve, Central memory, Effectors, activation

B cell subsets

✓ Particular tests for protein expression or function

Flow Cytometry role (in the world of NGS)

- ✓ Simple lymphocyte screening
- ✓ RTE detection **- > SCID diagnosis**

- ✓ Lymphocyte phenotyping **-> CID diagnosis**

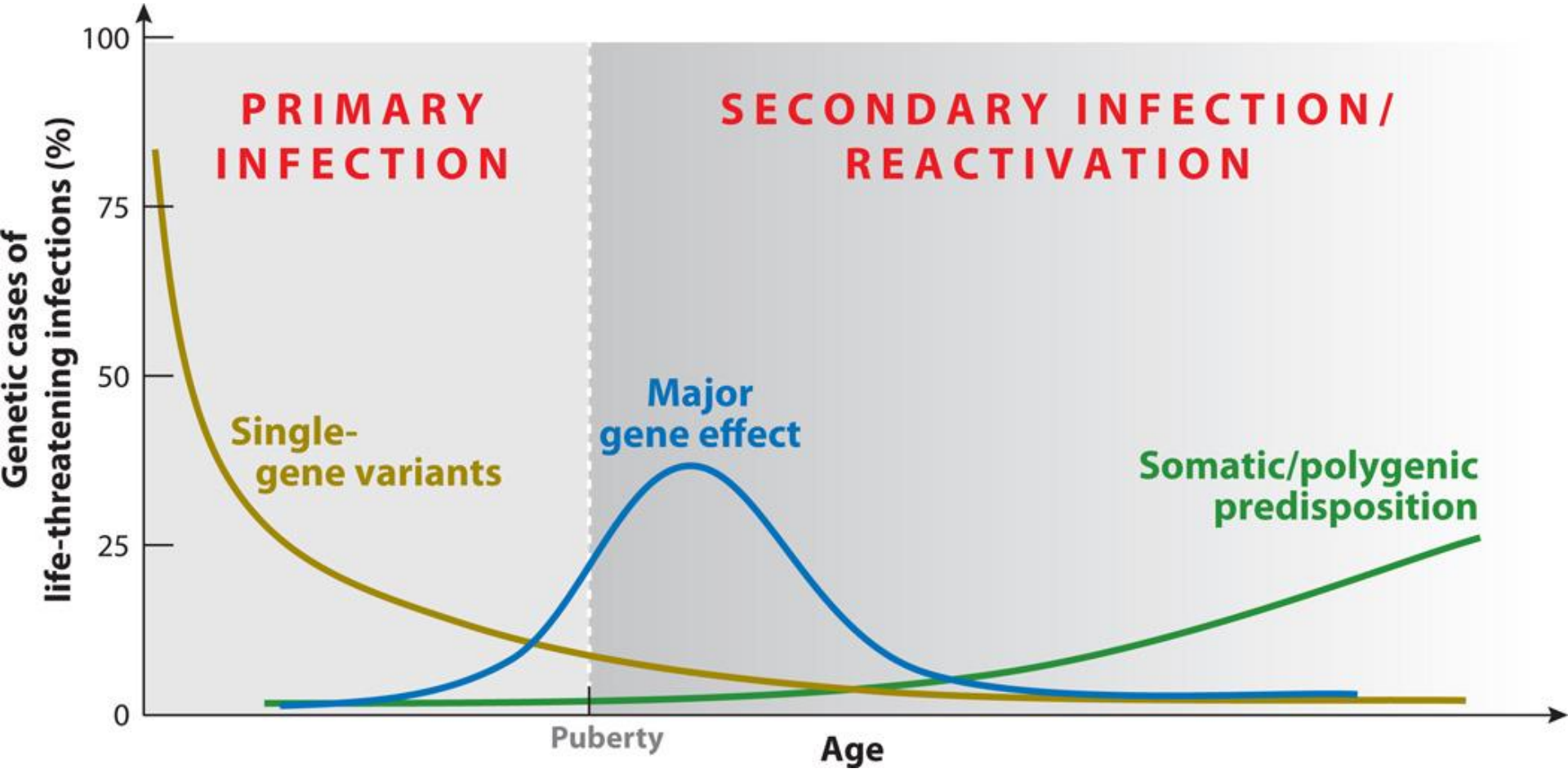
At least one of:
- at least one severe infection (requiring hospitalization)
- one manifestation of immune dysregulation
(autoimmunity, IBD, severe eczema, lymphoproliferation, granuloma)
- malignancy
- affected family member

AND 2 of 4 T cell criteria fulfilled:
- reduced CD3 or CD4 or CD8 T cells (using age-related reference values)
- reduced naive CD4 and/or CD8 T cells
- elevated g/d T cells
- reduced proliferation to mitogen or TCR stimulation

AND HIV excluded

AND exclusion of clinical diagnosis associated with CID (e.g. defined syndromic diseases, DKC, AT, CHH)

PID at a different age



Casanova, J.-L., and L. Abel. 2013. The Genetic Theory of Infectious Diseases: A Brief History and Selected Illustrations. Annu. Rev. Genomics Hum. Genet 14: 215–43.

Flow Cytometry role (in the world of NGS)

Generic tests

- ✓ Simple lymphocyte screening
- ✓ RTE detection **- > SCID diagnosis**
- ✓ Lymphocyte phenotyping **-> CID diagnosis**
  (inc. naïve) -> support for clinical observation -> NGS

Particular tests

- ✓ Detailed phenotyping, protein detection and quantification
  -> support for NGS

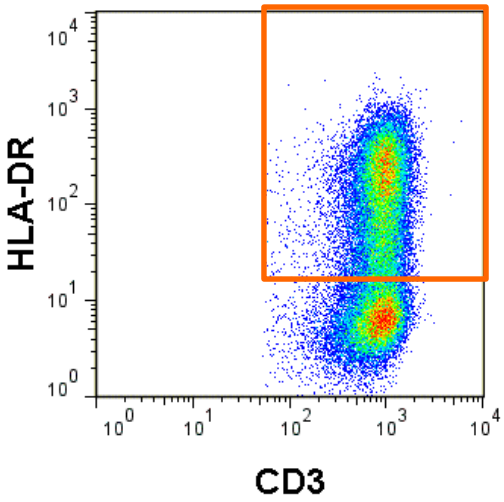
- ✓ Functional assays - > mechanistic proof of the causative role of mutation

Intracellular protein detection

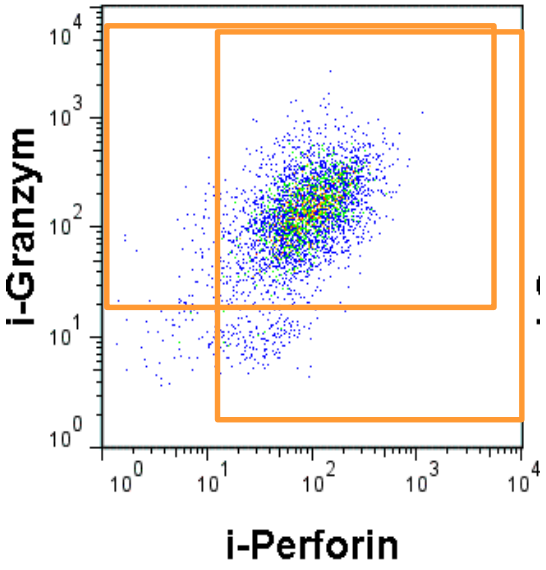
HLH test

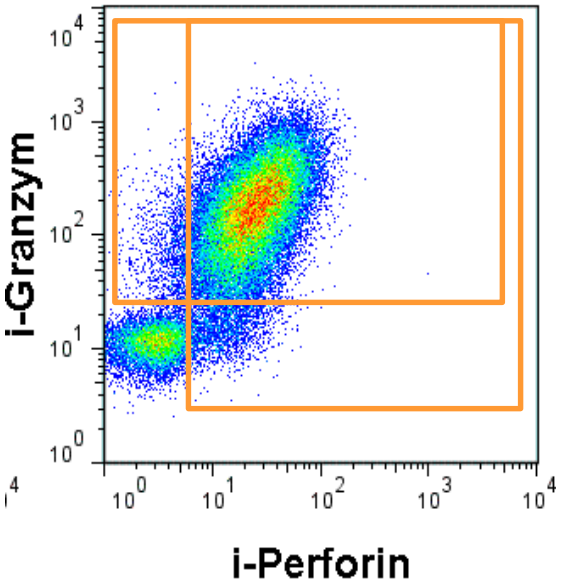
Activated T-cells?

% HLA-DR / CD8



Granzym and perforin expression by T-cells and NK-cells?

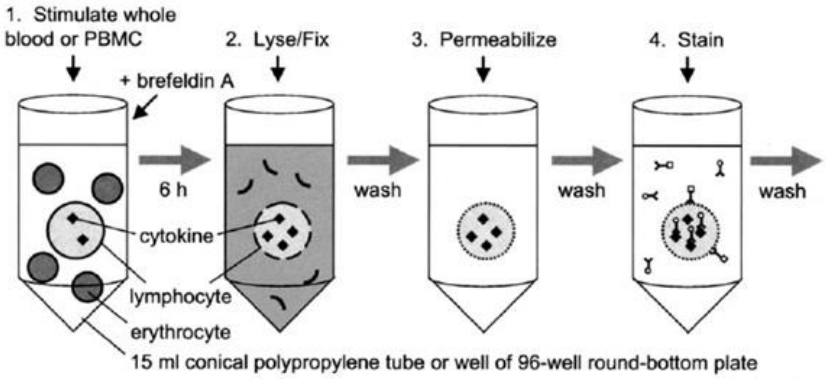




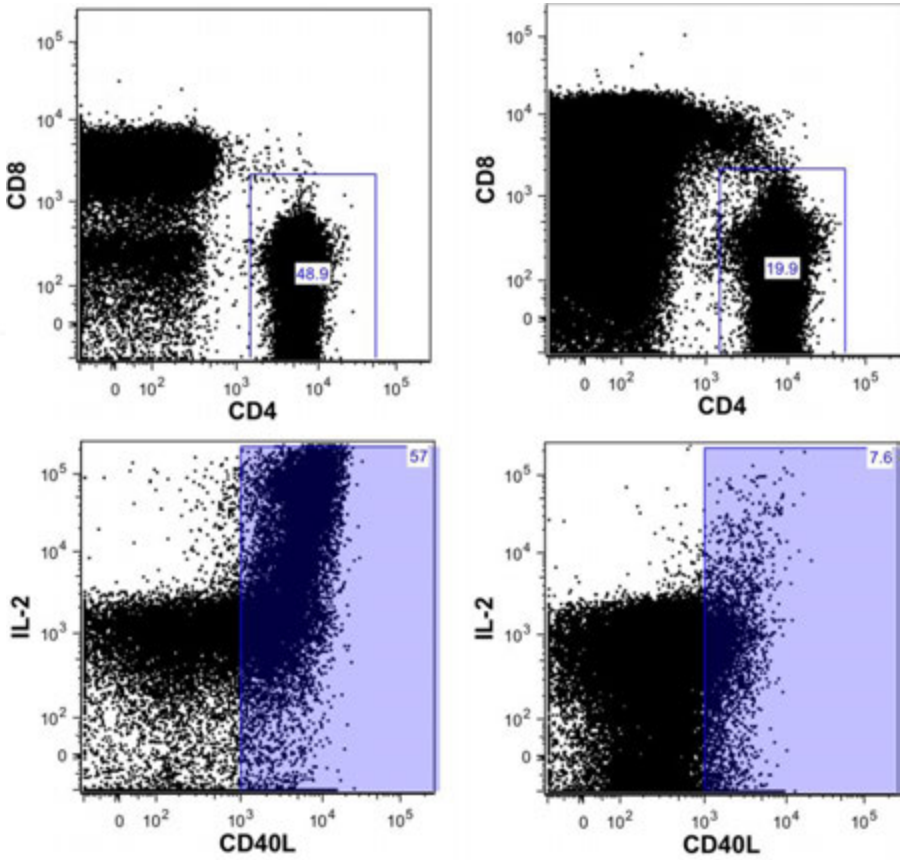
thanks to: Michaela Nováková

Antigen non-specific tests

Response of T-cells to anti-CD3



Maecker and Maino, Analyzing T-cell responses to cytomegalovirus by cytokine flow cytometry, Hum Immunol. 2004



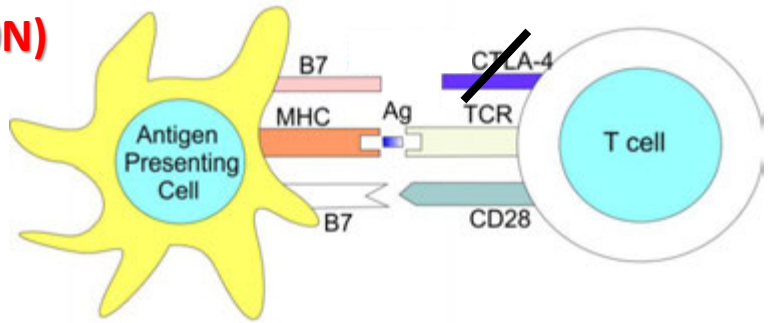
CD40L deficiency

thanks to: Ondrej Pelák

Activation of T regs

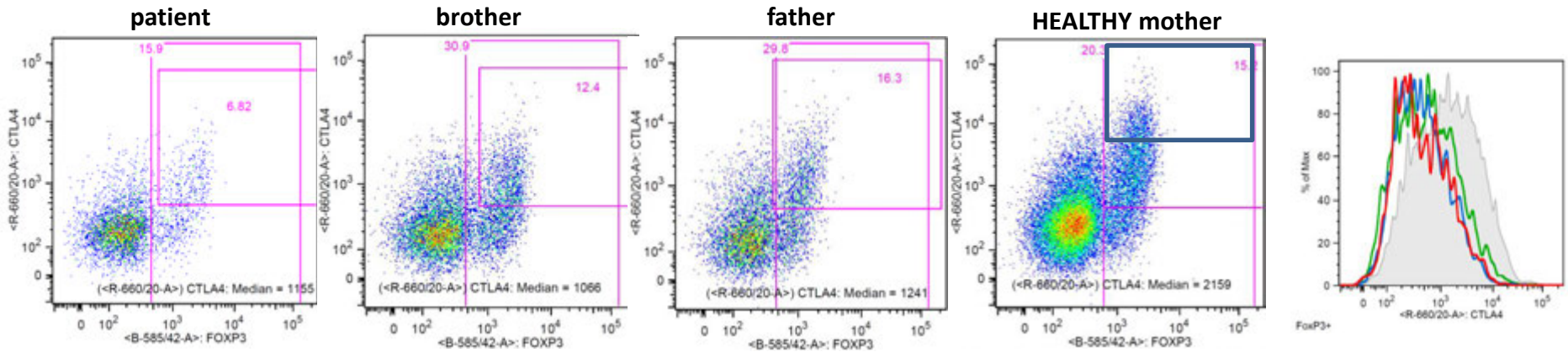
Family with mutation in CTLA-4 (Y60N)

T-cells
anti-CD3/28/49d (24h)
PMA/ionomycine (4h)



patological activation of T-cells (autoimmunity)

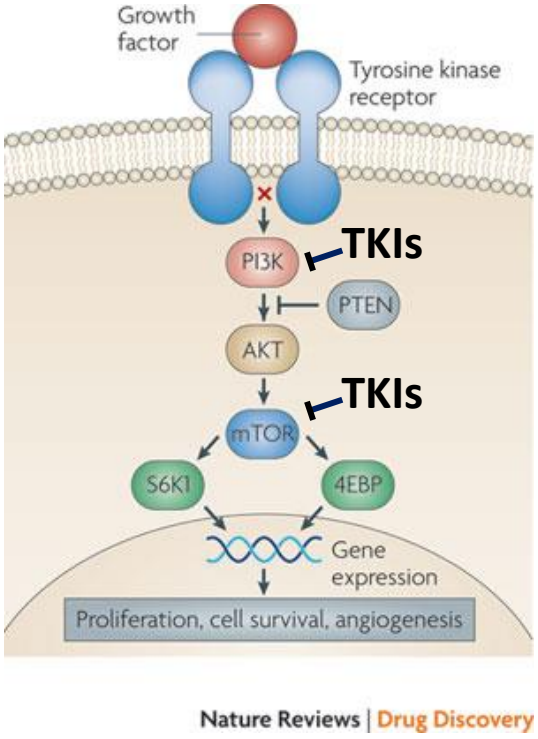
Upregulation of CTLA-4 is impaired in patients



thanks to: Dr. Veronika Kanderová, prof Jaroslav Štěrba, Dr. Tomáš Freiberger
thanks to: VERONIKA KANDEROVÁ, EVA FRONKOVÁ, MICHAL SVATOŇ

Case # 1 (M, 9 years) … susp. APDS

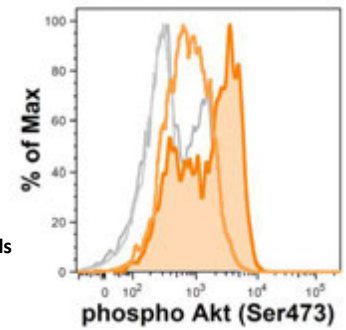
activated PI3K-delta syndrome



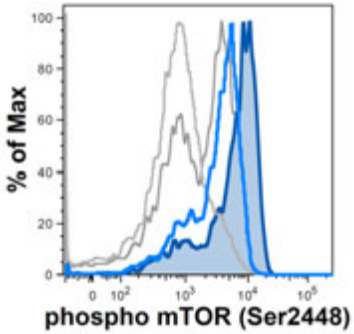
T-cells
anti-CD3 + IL-2 pre-activation (24h)
anti-CD3/28/49d stimulation

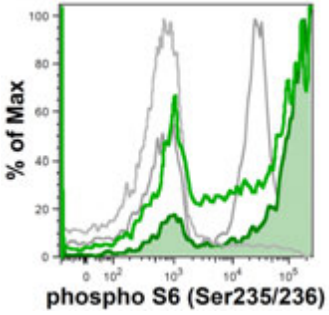
Dg

CD3 T-cells



Patient
Control
Tinted : CD3 stimulation

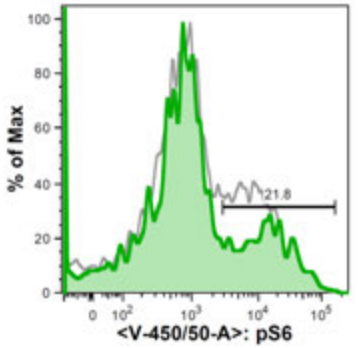




Day 15 (PI3K inhibitor)

Idelalisib *(trade name* ***Zydelig****)*

CD8 T-cells

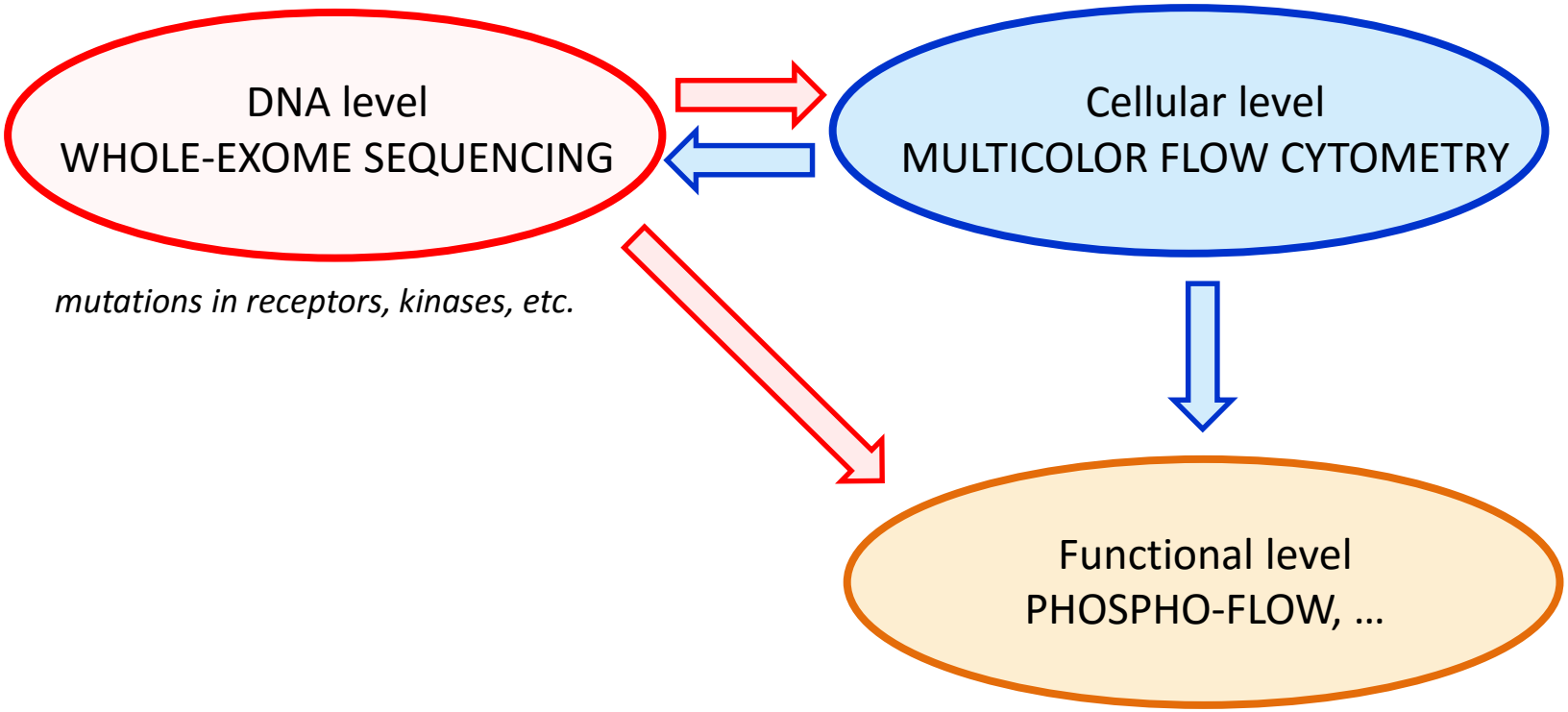


PacBlue	BV510	AL488	PE	ECD	PerCP	PC7	AL700	APCH7
pS6	CD3	pAkt	p-mTOR	CD4	CD45	CD19	CD45RA	CD8

thanks to: Dr. Veronika Kanderová, prof Jaroslav Štěrba, Dr. Tomáš Freiberger

Tests employed in PID

(Clinical suspicion of PID)



Practical: How to send a sample for "flow cytometry"?

- ✓ Specify what investigation do you need
- ✓ Call the lab if in doubts what to request
- ✓ Interpretation is a key
  - ✓ Is your patient on therapy?
  - ✓ -> have your flow lab seen a PID before?
  - ✓ -> do they have a reasonable set of controls for generic tests?

Particular tests

- ✓ Most of the PID tests are rare, the lab does not have reagents on stock -> call them well ahead before sending the sample
- ✓ Tune down your expectation – it is a hit and miss game; Repeat the test
- ✓ We need a fresh sample (beware of logistics, Friday afternoon samples)

Summary

- T and B cell pool in the peripheral blood is heterogeneous and dynamic –> “frozen” in PIDs
- PID can be caused by defect in production, activation, proliferation or maintenance of T or B cells
- T B NK test is crucial, further insight into maturation stages is important
- Several proteins can be directly assessed by flow cytometry (perforin, CD27 …)
- Functional tests can show activation, cytokine secretion, proliferation
- EuroFlow PID orientation tube is useful in all cases of PIDs that involve lymphocytes.

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ANALYSIS OF T AND B CELL SUBSETS IN HEALTHY SUBJECTS - IMPLICATIONS FOR CVID MONITORING

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Common variable immunodeficiency (CVID) is the most often diagnosed primary immune deficiency (PID). This designation encloses a heterogeneous group of disorders that cause hypogammaglobulinemia. Due to the depressed immune system, patients suffer from recurrent bacterial and viral infections. The diagnosis of CVID can be very challenging because CVID symptoms are unspecific and extremely variable. In order to accomplish a solid diagnosis, it is indispensable to hold reliable healthy control baseline values. Our study followed two aims: to implement an eight-color flow cytometry screening system which helps identify and classify PID patients earlier and to improve the diagnostic follow-up management of immunocompromised patients. For the first time in Slovenia we define reference values for a wide range of peripheral blood lymphocyte phenotypes that apply to healthy adult population. The study was done on 26 healthy adult volunteers and 10 well-characterized CVID patients from Slovene national registry. We created three eight color screening panels that detect and differentiate lymphocytes subsets of: 1. B-cells, 2. maturation of all T cell subpopulations and 3. activation as well as regulation of T cell populations. To validate this panels, we compared them with 4 color panels which we already use in our laboratory for the diagnosis of PID. Using the newly introduced eight-color immunophenotypisation we classified our CVID patients according to the EUROclass scheme. Two patients had less than 2% of lymphocytes B and were assigned to the B- group. They are assumed to have severe defects of early B-cell differentiation. From the B+ group (8 patients), 6 patients were assigned to the (switched memory negative) smB- group, which has severely reduced number of class switched memory B cells and indicates defective germinal center development. Three patients from the smB- group were further classified into smB- transitional (Trhi) group based on the expansion of transitional B cells. Three patients from the smB- group were also in the smB-21lo group and all three had splenomegaly. Two patients had more than 2% of class switched memory B cells and were assigned to the smB+ group, one of them had more than 10% of CD21low cells. We present here the reference values of peripheral blood lymphocytes phenotype for the Slovenian adult population. The simultaneous detection of T and B cell subpopulations by our eight color panels makes diagnosis faster, cheaper and more reliable. We therefore are convinced that the use of our multicolour screening system helps identify and classify PID patients earlier and improve the diagnostic follow-up management of immunocompromised patients.

Analysis of T and B cell subsets in healthy subjects - implications for CVID monitoring

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Slovenian Society for Flow Cytometry meeting

14th October 2016

Common variable immunodeficiency (CVID)

- Heterogeneous disease with hypogammaglobulinemia (different causes)
- Most common primary immune deficiency
- Most patients present the disease in early to mid adulthood
- Patients suffer from recurrent bacterial and viral infections
- CVID symptoms are unspecific and extremely variable, diagnosis can be challenging
- Reliable normal healthy control baseline values are indispensable

The aim

- Design and introduce eight-color flow cytometry immunophenotypisation for routine diagnostic to:
  - Help identify and classify PID patients earlier
  - Improve the diagnostic follow-up management of immunocompromised patients
  - Detect most T and B cell subpopulations simultaneously
  - Standardize lymphocyte subsets measurements
- For the first time in Slovenia we define reference values for a wide range of peripheral blood lymphocyte subpopulations that apply to healthy adult population

Methods

- 26 healthy adult volunteers: 15 females and 11 meals, average age 27,1 (from 22-45 years)
- 10 well-characterized CVID patients from Slovene national registry: 5 females and 5 males average age 26,1 (15-35 years)
- We created three eight color screening panels to detect:
  - Lymphocytes subsets of B-cells
  - Differentiation of all T cell subpopulations
  - Activation as well as regulation of T cell populations
- Eight color screening system was validated by comparison with 4 color panels

Eight-color screening panels

- Each antibody was individually titrated to determine the optimal dilution for a given staining volume of 100 µl
- Setup experiments were performed with BD CompBeads
- Cell discrimination using Fluorescence Minus One Controls - FMO controls
- Data acquisition: FACS Canto II flow cytometer.

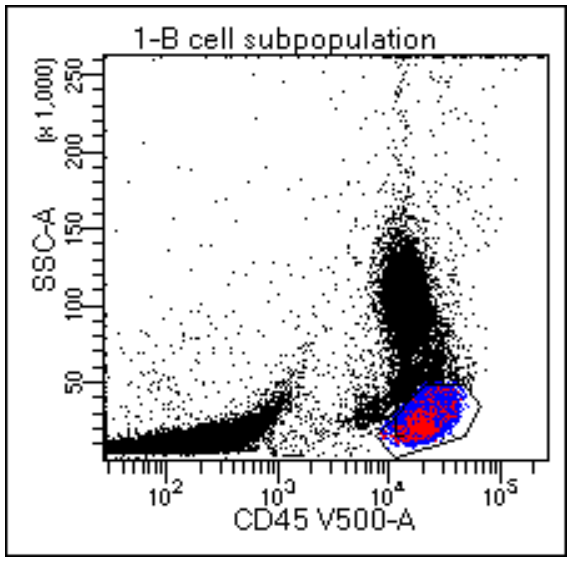
  405 nm violet laser, 488 nm blue laser and 647 nm red laser.
- 10,000 events in the lymphocyte gate (CD45 versus SSClow)
- Analysis with FACS DIVA (BD Biosciences) software.

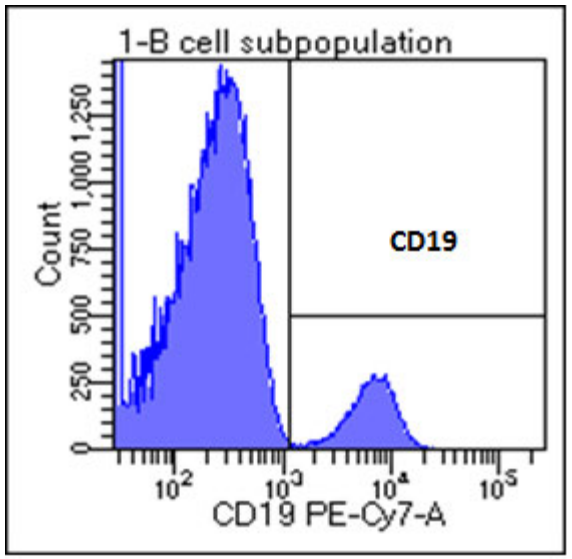
B cell differentiation - panel I

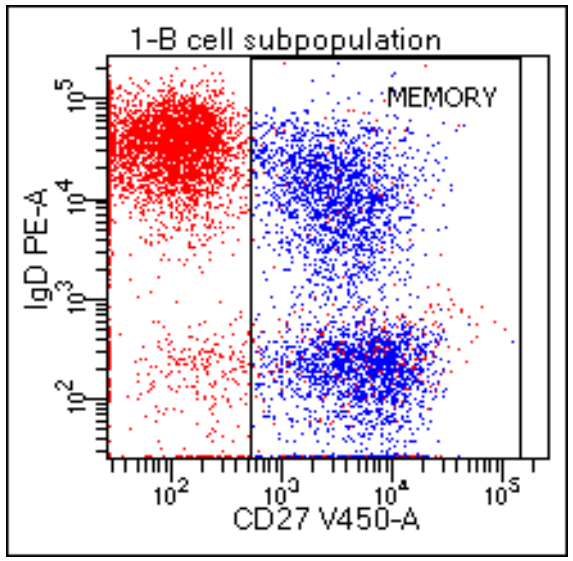
Antigen	Fluorochrome	Antibody/100 µl $2x10^6$ /ml PBMNC
IgM	FITC	15
IgD	PE	10
CD20	PerCP Cy5.5	5
CD19	PE Cy 7	5
CD21	APC	10
CD38	APC H7	5
CD27	BV421	5
CD45	V500	5

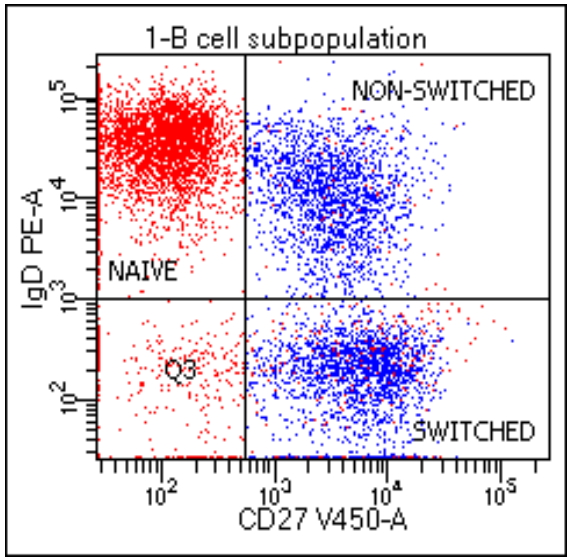
T cell phenotypisation

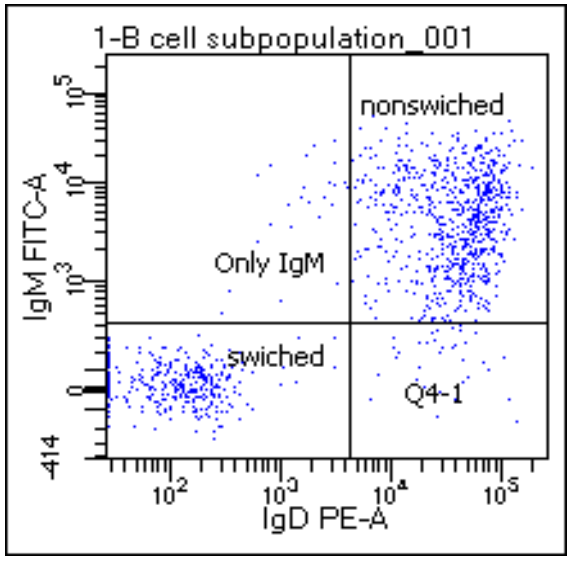
Antigen	Fluorochrome	Antibody/100 µl Whole blood
Panel (II):		
TCRαβ	FITC	10
TCRγδ	PE	10
CD45	PerCP	10
CD3	PE-Cy7	5
CD31	APC	5
CD8	APC-Cy7	5
CD4	V450	5
CD45 RA	BV510	5
Panel (III):		
HLA-DR	FITC	10
CD127	PE	5
CD45	PerCP	10
CD3	PE-Cy7	5
CD25	APC	10
CD8	APC-Cy7	5
CD4	V450	5

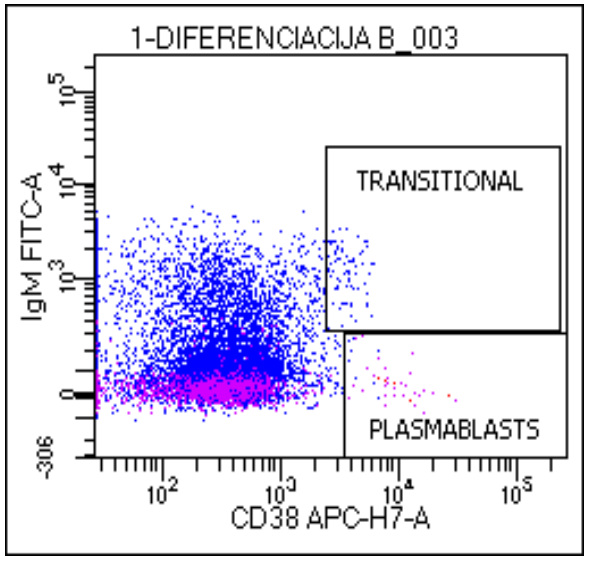

1-B cell subpopulation
SSC-A
(x 1,000)
CD45 V500-A

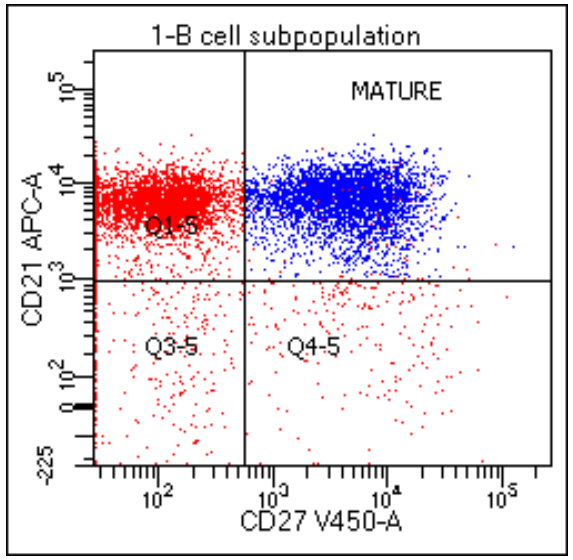

1-B cell subpopulation
Count
CD19
CD19 PE-Cy7-A

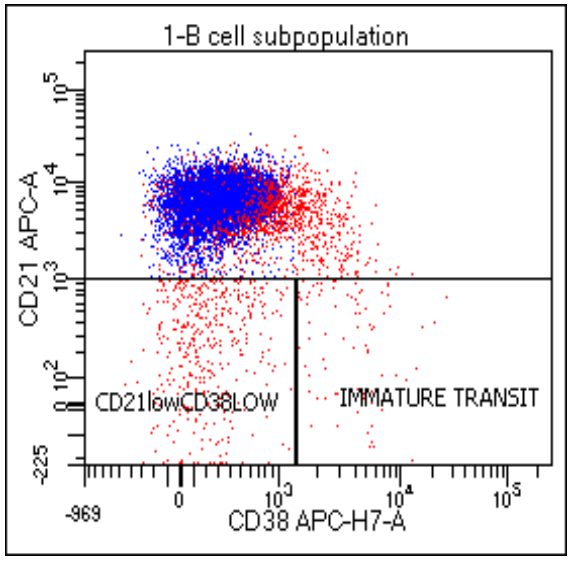

1-B cell subpopulation
MEMORY
IgD PE-A
CD27 V450-A

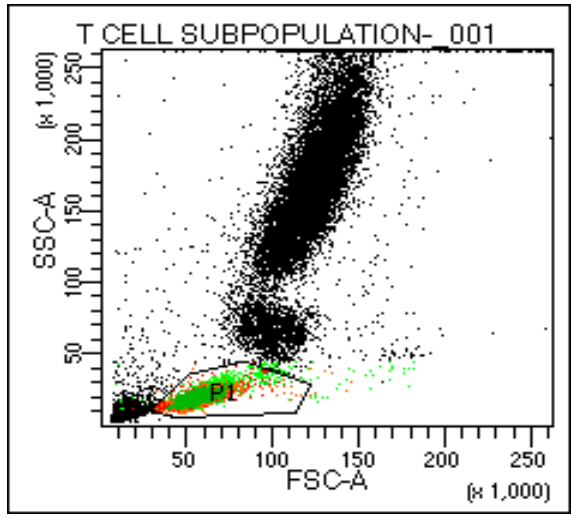

1-B cell subpopulation
NON-SWITCHED
NAIVE
Q3
SWITCHED
IgD PE-A
CD27 V450-A

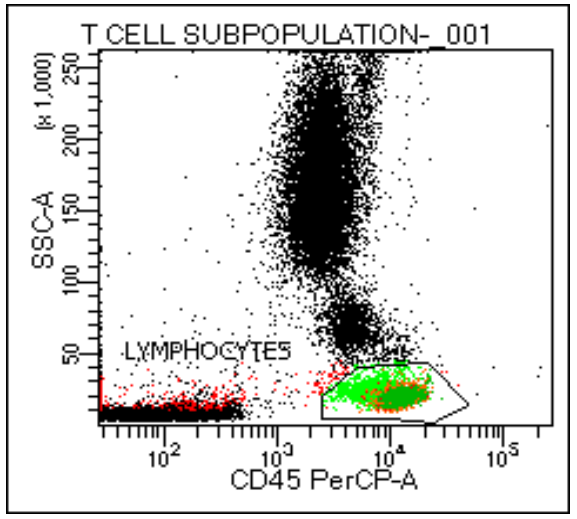

1-B cell subpopulation_001
nonswiched
Only IgM
swiched
Q4-1
IgM FITC-A
IgD PE-A

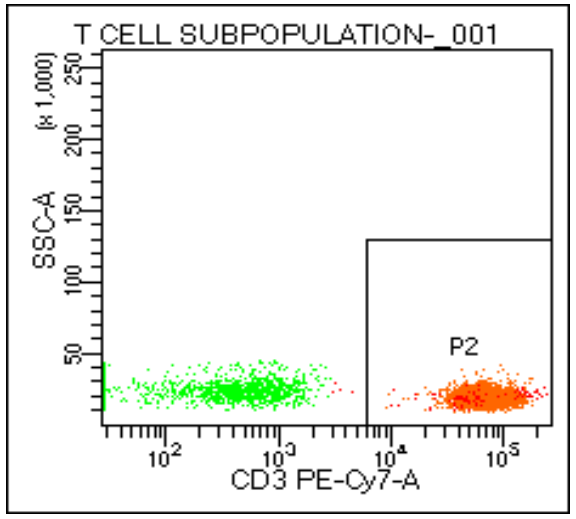

1-DIFERENCIACIJA B_003
TRANSITIONAL
PLASMABLASTS
IgM FITC-A
CD38 APC-H7-A

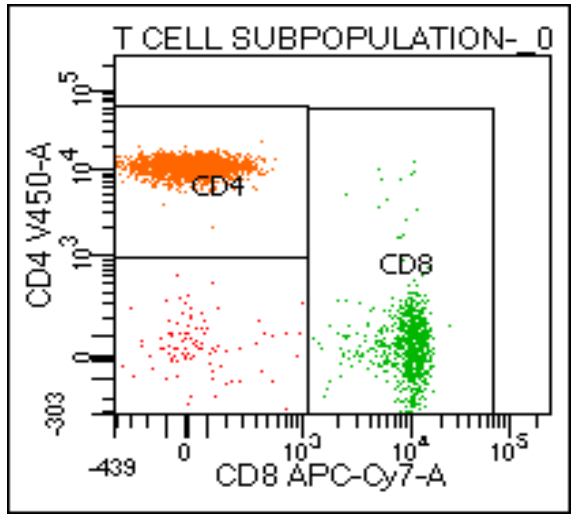

1-B cell subpopulation
MATURE
Q1-5
Q3-5
Q4-5
CD21 APC-A
CD27 V450-A

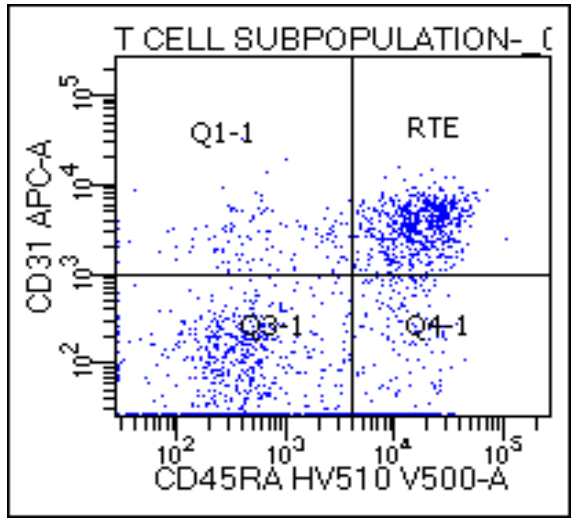

1-B cell subpopulation
CD21lowCD38LOW
IMMATURE TRANSIT
CD21 APC-A
CD38 APC-H7-A

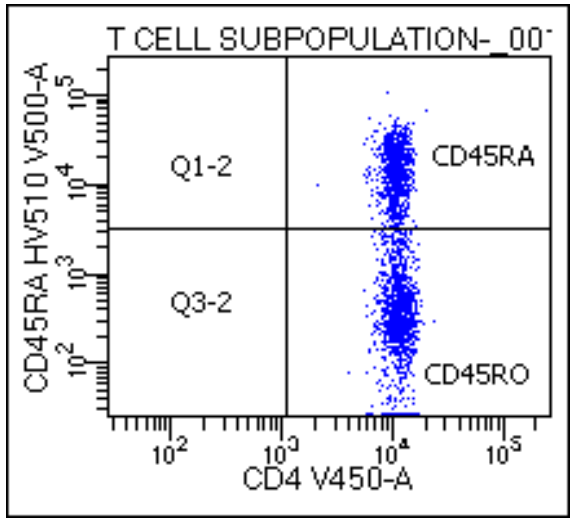


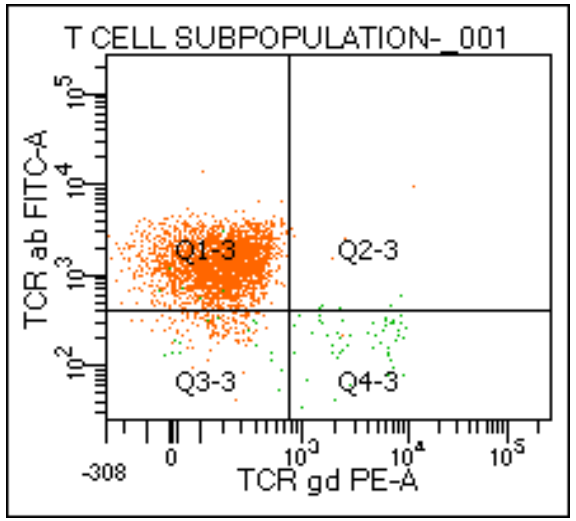


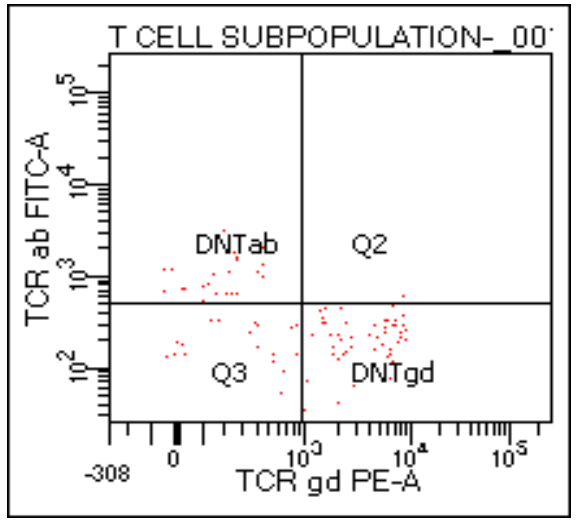




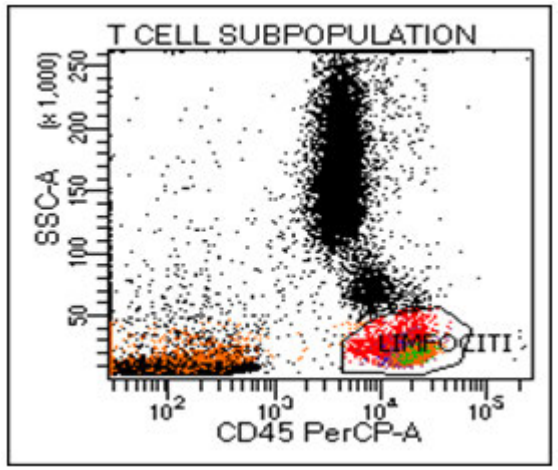


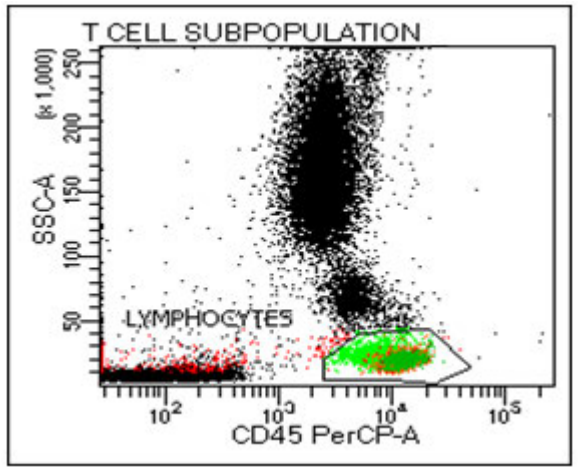






Gated on CD3+, not CD8 or CD4


T CELL SUBPOPULATION
SSC-A (x 1,000)
LIMFOCITI
CD45 PerCP-A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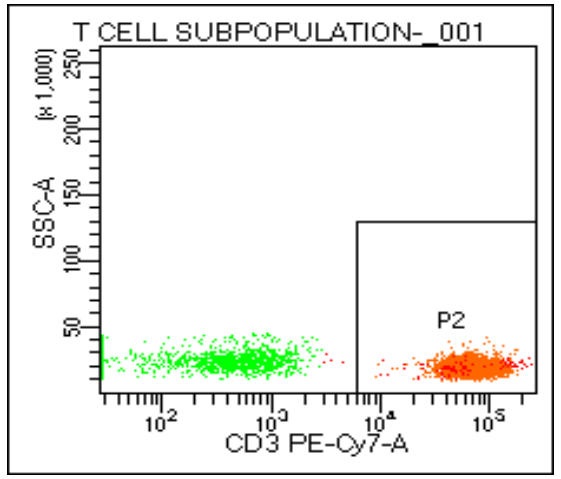
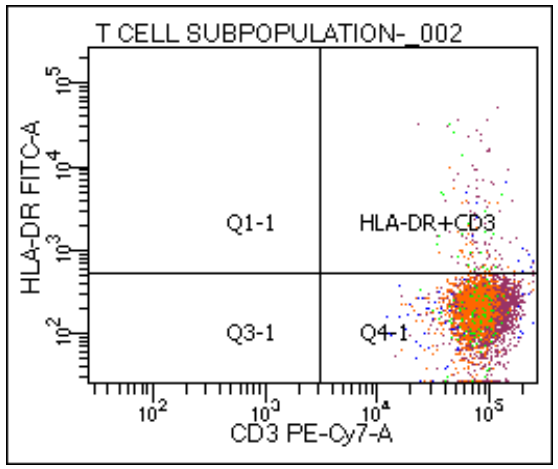
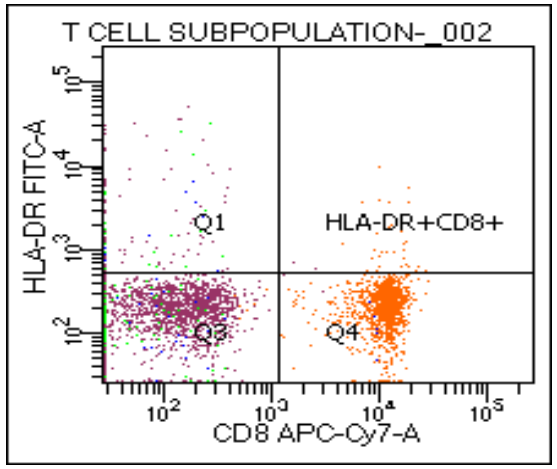
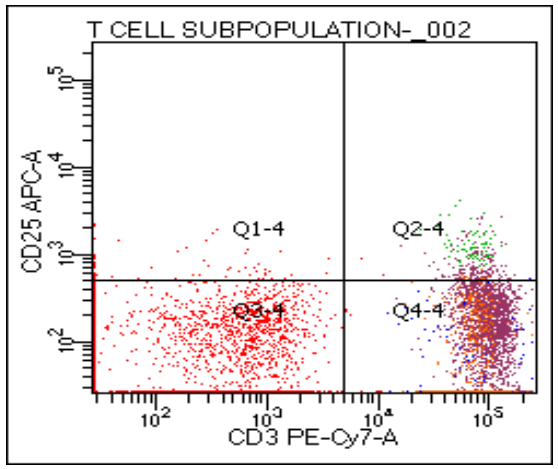
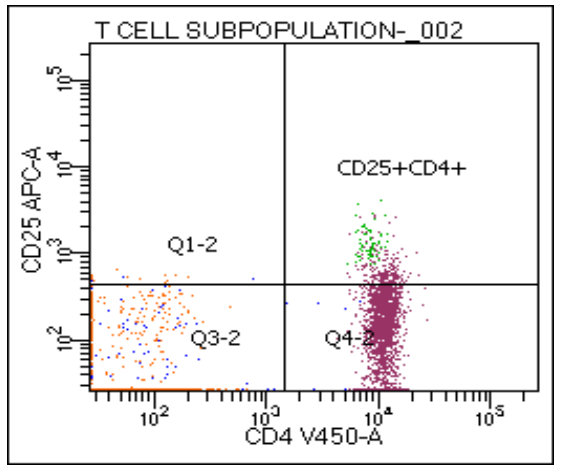
SSC-A (x 1,000)
LYMPHOCYTES
CD45 PerCP-A


T CELL SUBPOPULATION-_001
SSC-A (x 1,000)
P2
CD3 PE-Cy7-A


T CELL SUBPOPULATION-_002
HLA-DR FITC-A
Q1-1
HLA-DR+CD3
Q3-1
Q4-1
CD3 PE-Cy7-A


T CELL SUBPOPULATION-_002
HLA-DR FITC-A
Q1
HLA-DR+CD8+
Q3
Q4
CD8 APC-Cy7-A


T CELL SUBPOPULATION-_002
CD25 APC-A
Q1-4
Q2-4
Q3-4
Q4-4
CD3 PE-Cy7-A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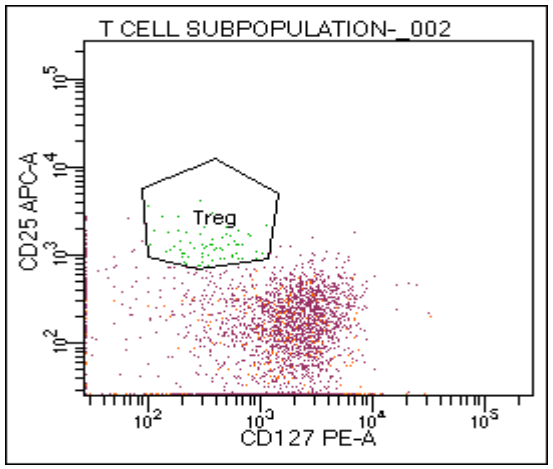
CD25 APC-A
CD25+CD4+
Q1-2
Q3-2
Q4-2
CD4 V450-A


T CELL SUBPOPULATION-_002
CD25 APC-A
Treg
CD127 PE-A

Validation of eight color panels

	Percentage ($\bar{x}$ (5.-95. percentile) (%))		
Phenotype	4 color analysis	8 color analysis	p
CD3+ HLA-DR+	9,0 (4,6-21,3)	9,7 (5,1-17,5)	NS
CD4+CD31+CD45RA+	34,0 (20,3-55,1)	34,5 (21,5-57,9)	NS
CD3+CD4-CD8-TCRαß+	2,2 (0,7-7,2)	2,0 (0,5-8,1)	NS
CD4+CD25hi	5,9 (4,1-8,8)	6,2 (3,9-8,4)	NS
CD19+CD27+	28,5 (14,0-67,2)	25,5 (5,2-41,0)	NS
CD19+CD27+IgM+IgD+	13,8 (5,0-23,6)	13,5 (2,1-24,3)	NS
CD19+CD27+IgM-IgD-	14,3 (5,5-52,4)	12,2 (1,6-21,9)	NS
CD19+CD21lowCD38low	4,0 (1,6-8,8)	5,0 (1,9-9,2)	NS
CD19+CD21+	91,5 (71,3-97,3)	92,5 (88,4-97,2)	NS
CD19+IgM+	18,8 (9,2-30,6)	18,2 (5,0-32,9)	NS
CD19+ CD38hi IgM+	4,1 (1,6-10,7)	4,1 (1,2-8,9)	NS
CD19+ CD38hiIgM-	6,8 (3,2-9,4)	6,1 (2,6-8,5)	*

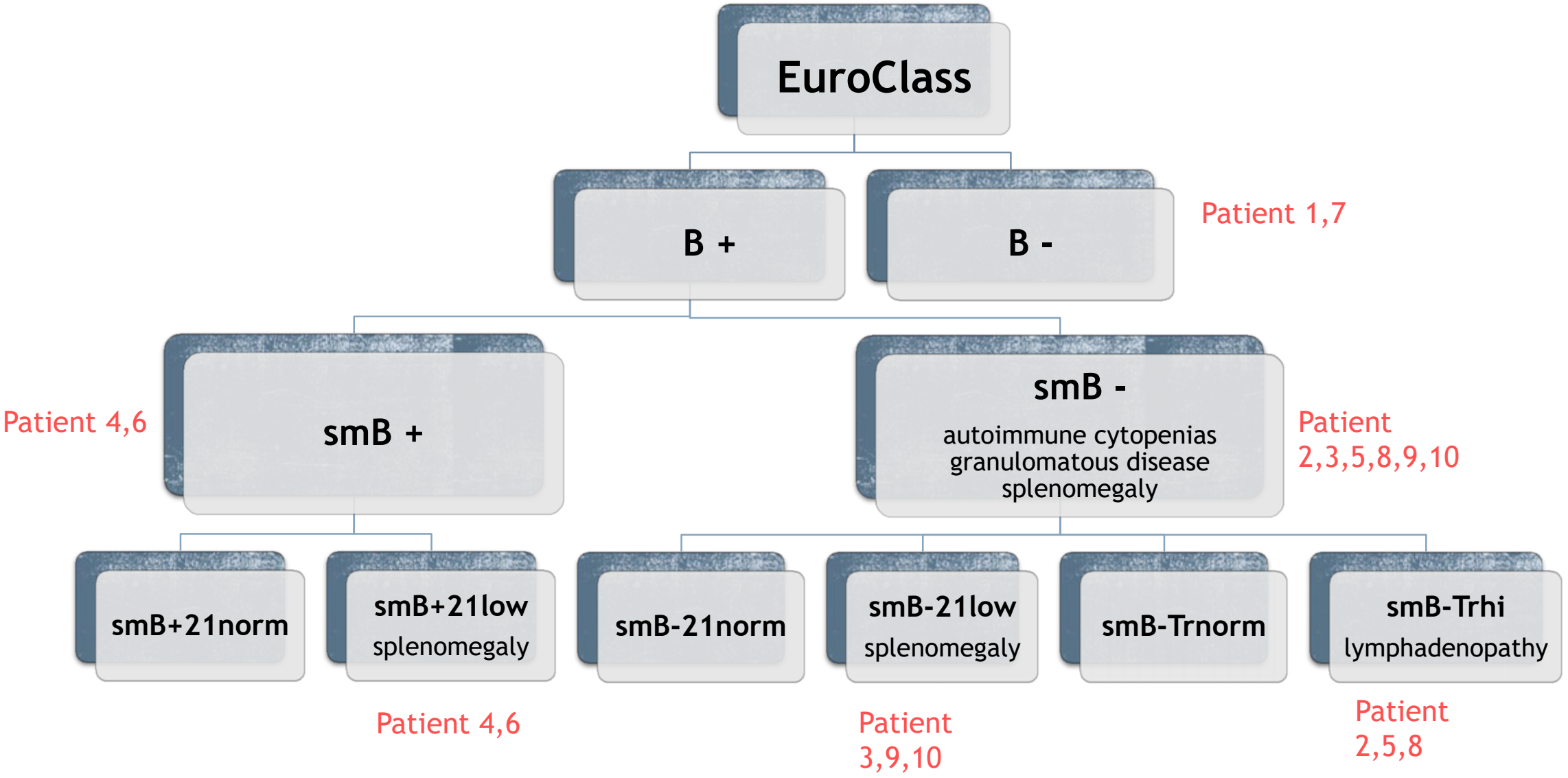
Reference values for B cells

Phenotype	Description	N	Percentage (%)	Concentration (*10^9 cells/L)
CD19+	Lymphocytes B	26	10,0 (6,3-12,7)	0,224 (0,130-0,436)
CD19+CD27+	Memory B cells	26	26,5 (9,4-40,0)	0,052 (0,017-0,107)
CD19+CD27+IgM+IgD+	Non-switched memory B cells	26	15,1 (3,2-22,1)	0,027 (0,006-0,057)
CD19+CD27+IgM+	Only IgM cells between the memory B cells	26	2,0 (0,7-7,7)	0,001 (0,000-0,004)
CD19+CD27+IgM-IgD-	Class-switched memory B cells	26	12,6 (3,7-20,0)	0,023 (0,005-0,054)
CD19+CD21lowCD38low	Activated CD21loCD38lo B cells	26	4,4 (2,4-8,1)	0,010 (0,004-0,018)
CD19+CD21+	Mature B cells	26	93,6 (89,8-96,8)	0,209 (0,122-0,313)
CD19+ IgM+	IgM lymphocytes B	26	18,0 (6,7-27,0)	0,052 (0,011-0,080)
CD19+CD38++ IgM++	Transitional B cells	26	3,6 (1,6-7,6)	0,007 (0,003-0,021)
CD19+CD38++IgM-	Plasmablasts	26	6,2 (3,1-8,8)	0,013 (0,007-0,020)

Reference values for T cells

Lymphocytes	Description	N	Percentage (%)	Concentration (*10^9 cells/L)
CD3+	Lymphocytes T	26	72,8 (65,5 - 80,1)	1,601 (1,140-2,312)
CD3+CD4+	Helper T cells -Th	26	44,0 (32,1-52,1)	0,929 (0,661-1,356)
CD3+CD8+	Cytotoxic T cells	26	26,8 (17,5-40,3)	0,611 (0,297-0,901)
CD3+CD4+CD31+CD45RA+	Recent Thymic Emigrants RTE	26	32,6 (22,9-52,5)	0,326 (0,153-0,567)
CD3+CD4-CD8-TCRαß+	Double Negative αß lymphocytes T	26	1,3 (0,7-4,0)	0,021 (0,011-0,17)
CD3+CD4-CD8-TCRγδ+	Double Negative γδ lymphocytes T	26	3,5 (1,3-11,8)	0,068 (0,018-0,199)
CD3+TCRαß+	αß lymphocytes T	26	66,8 (56,7-76,3)	1,017 (0,681-1,531)
CD3+TCRγδ+	γδ lymphocytes T	26	4,5 (1,7-15,5)	0,082 (0,023-0,225)
CD3+CD45RA+	Naive lymphocytes T	26	57,5 (42,5-69,8)	0,923 (0,578-1,457)
CD3+CD45RO+	Memory lymphocytes T	26	42,6 (30,2-57,5)	0,640 (0,439-1,129)
CD3+CD4+CD45RA+	Naive T_h cells	26	48,5 (36,8-65,6)	0,467 (0,307-0,708)
CD3+CD4+CD45RO+	Memory T_h cells	26	51,5 (34,7-63,2)	0,445 (0,312-0,786)
CD3+HLA-DR+	Activated lymphocytes T	26	9,5 (5,2-15,2)	0,156 (0,073-0,260)
CD3+CD8+HLA-DR+	Activated cytotoxic T cells	26	4,9 (1,2-12,6)	0,077 (0,022-0,215)
CD3+CD4+HLA-DR+	Activated helper T cells	26	4,1 (2,4-6,7)	0,037 (0,020-0,072)
CD3+CD25+	Activated lymphocytes T IL-2R+	26	11,6 (5,6-15,5)	0,158 (0,093-0,320)
CD3+CD4+ CD25+	Activated helper T cells IL-2R+	26	19,3 (10,6-28,8)	0,325 (0,137-0,457)
CD3+CD4+CD25++CD127lo	Regulatory T cells	26	6,2 (4,3-8,0)	0,059 (0,035-0,096)

Data from Slovenian CVID patients



Conclusion

- Introduction of eight color immunophenotypisation of T and B cells, and the establishment of normal value for the Slovenian population
  - provides precise interpretation of lymphocyte subsets.
  - detects most T and B cell subpopulations simultaneously.
  - standardizes lymphocyte subsets measurements.

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FUNCTIONAL TESTS FOR THE DIAGNOSTIC OF IMMUNE DEFICIENCY

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Laboratory testing plays a central role in the evaluation of the immune system. The laboratory evaluation of cellular immunity typically begins with determining the numbers of different types of immune cells and immune molecules in the blood. This is typically followed by more sophisticated tests chosen on the initial test results. An important aspect in determining definite diagnosis of many immune deficiencies depends upon evaluating the function of immune cells in in vitro conditions. Functional *in vitro* tests for evaluation of T-cell immunity may determine many aspects of cytokine and/or T cell receptor function, intracellular signalling and effector functions of T cells (cytokine production, proliferation, cytotoxic function). The simplest test to evaluate possible decreased or absent T-cells is a complete blood count (CBC) and differential to establish the total blood (absolute) lymphocyte count. This is a reasonable method to access for diminished T-cell numbers, since normally about three-quarters of the circulating lymphocytes are T-cells and a reduction in T-lymphocytes will usually cause a reduction in the total number of lymphocytes, or total lymphocyte count. This can be confirmed by using flow cytometry with markers specific for different types of T-cells. The standard screening tests for antibody deficiency starts with measurement of immunoglobulin levels in the blood serum and may be followed by tests for specific antibody production and/or determination of B cell maturation and differentiation steps. The laboratory evaluation of the neutrophil begins by obtaining a series of white blood cell counts (WBC) with differentials. If these initial screening tests of neutrophil numbers were normal, testing would then focus on two possible primary immune disorders: Chronic Granulomatous Disease (CGD) and Leukocyte Adhesion Deficiency (LAD). Laboratory testing to diagnose CGD relies on the evaluation of the creation of reactive oxygen. Flow cytometry can measure the oxidative burst of activated neutrophils using a specific dye (dihydrorhodamine 123 or DHR), referred to as the DHR test. Laboratory tests are also available to measure the function of the various elements of innate immunity. This includes determining the number and activity of lymphocytes such as natural killer cells, as well as the function of various cell surface receptors such as the toll-like receptors.

Functional tests for PID

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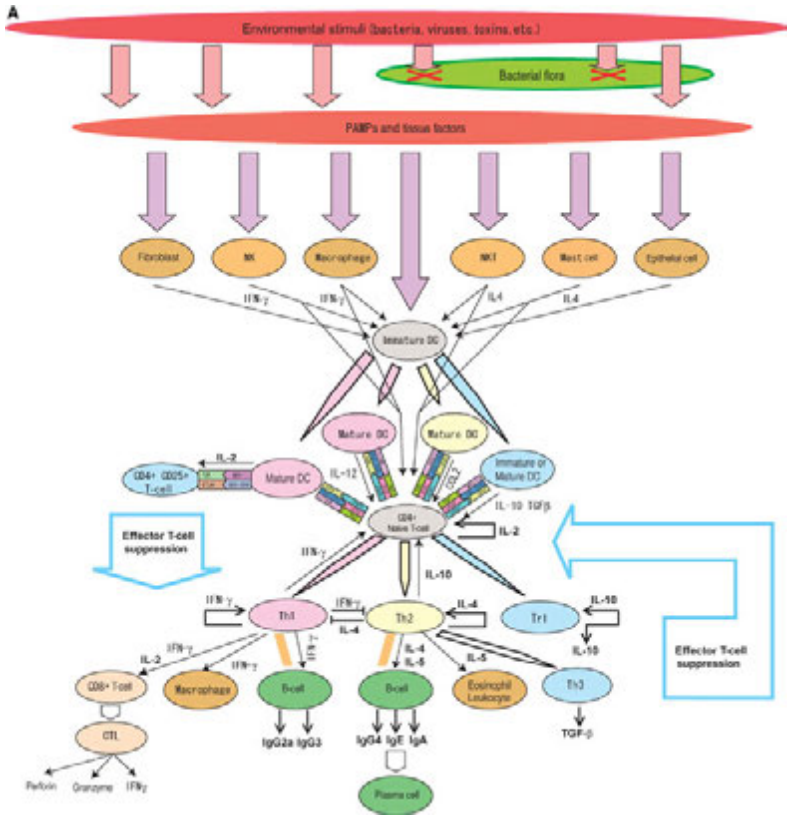
Immunity in function – *in vivo* / *in vitro*



Immune system – a complex network of molecular/cellular interactions, making a system extremely resilient and sustaineble despite harsh external and immunopathological conditions. Immune plasticity makes diagnostic difficulties in discrimination normal vs. pathologic

Likely (clinical) deficiency and investigation

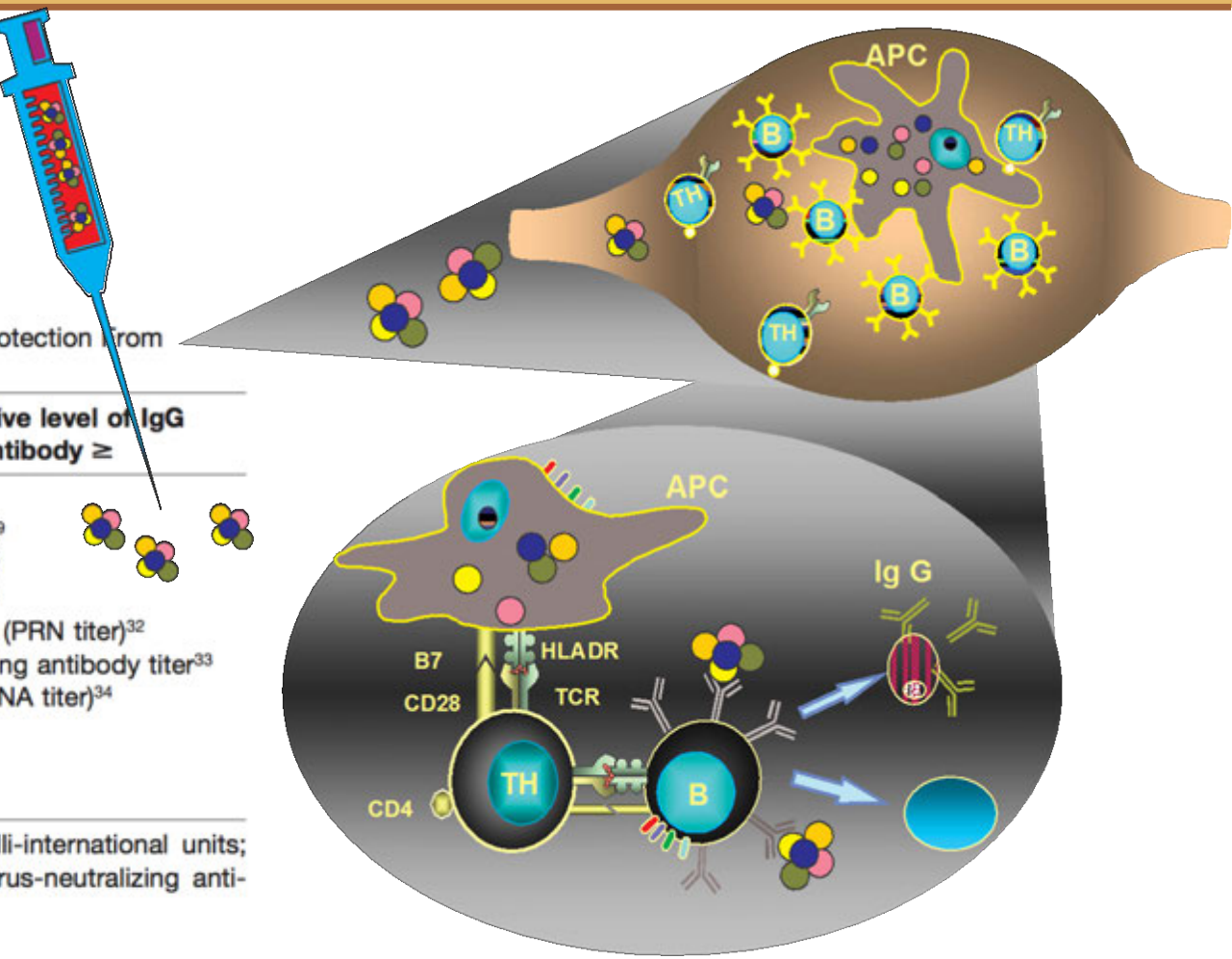
- B cells – Igs, Electrophoresis, (BJP), IgG subclasses, Specific Antibodies; Immunisation challenges; Specialised studies
- T cells – total lymphocyte numbers, phenotyping; genetics; functional analysis
- Neutrophils – numbers; function assays
- Complement – CH50; individual components
- Cytokine defects – specialised studies

Immune function in vivo - antibody responses after immunisation 1

Table 1. Levels of Antibody Associated With Protection From Vaccine-Preventable Diseases

Vaccine	Protective level of IgG antibody ≥
Diphtheria	0.1 IU/mL[11]
Haemophilus influenzae type B	0.15 μg/mL[29]
Hepatitis A	10 mIU/mL[30]
Hepatitis B surface antibody	10 mIU/mL[31]
Measles (rubeola)	120 mIU/mL (PRN titer)[32]
Polio (inactivated)	1:8 neutralizing antibody titer[33]
Rabies	0.5 IU/mL (VNA titer)[34]
Rubella	10 IU/mL[80]
Tetanus	0.1 IU/mL[11]
Yellow fever	0.7 IU/mL[29]

Abbreviations: IU, international units; mIU, milli-international units; PRN, plaque reduction neutralization; VNA, virus-neutralizing antibodies.



Immune function in vivo - antibody responses after immunisation 2

TABLE 2. PEAK ANTIBODY RESPONSES AFTER IMMUNIZATION.*

ANTIBODY ASSAY	PATIENT 1	PATIENT 2	PATIENT 4	PATIENT 5	CONTROLS
Diphtheria toxoid (IU/ml)	3	93	22	<0.1	>0.10
Tetanus toxoid (IU/ml)	3	63	89	<0.1	>0.10
Poliovirus titer					
First	1:640	1:640	1:20	0	>1:40
Second	1:320	1:640	1:80	1:20	>1:40
Third	1:160	1:160	1:40	0	>1:0
Anti-A antibody titer	1:64	1:32	1:8	1:4	>1:8
Anti-B antibody titer	1:32	—	—	—	—
Haemophilus influenzae (%)†	26	16	ND	ND	>10
Streptococcus pneumoniae (μg/ml)	ND	8	ND	ND	>0.3

*Patients were immunized three times with diphtheria toxoid, tetanus toxoid, and poliovirus between month 4 and month 6; they were immunized with *Streptococcus pneumoniae* and *Haemophilus influenzae* one year after gene therapy. Serum antibodies were measured in serum samples drawn every three months thereafter. ND denotes not done.

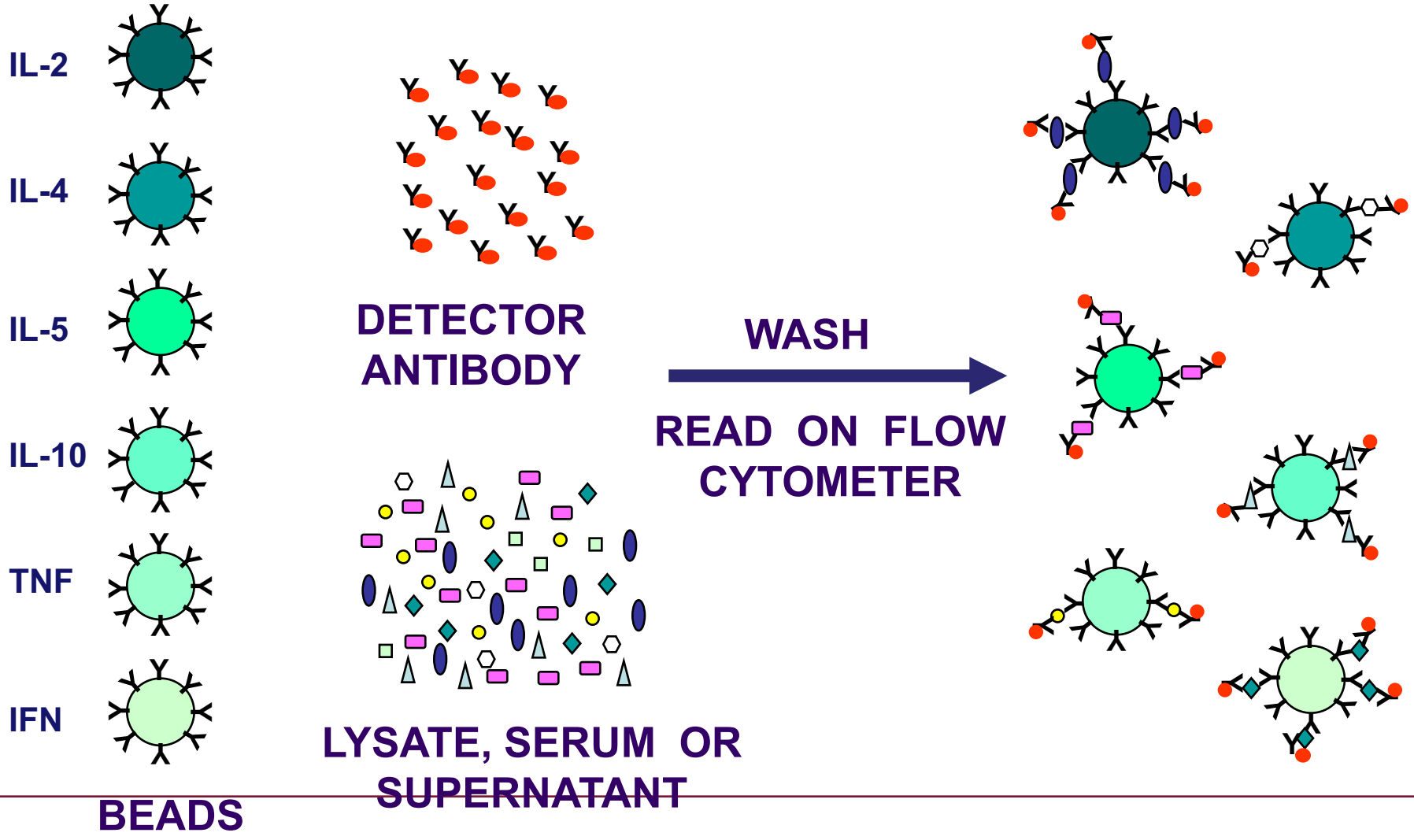
†A positive value is more than 10 percent.

FCM in functional testing of humoral & cellular Immunity

- Humoral Immunity
  - Antibody production: ELISA, CBA

- Cellular Immunity
  - T cell specificity: MHC multimer staining
  - Cytokines: ELISA, CBA, ICS, ELISPOT
  - Degranulation: CD107 staining
  - Cytotoxicity: ^{51}Cr release
  - Proliferation: BrdU incorporation, LPA

CBA (cytometric bead array) assay



ELISA versus CBA assays

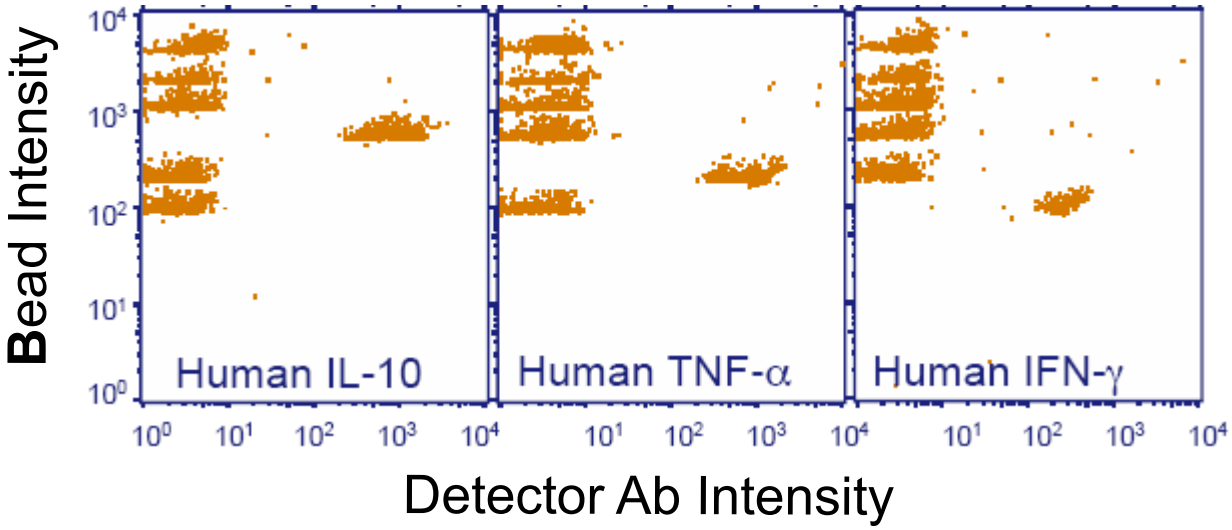
	ELISA	CBA
Types of analytes	antibodies, cytokines	Antibodies, cytokines
Number of simultaneous analytes	One	Up to seven or more
Type of readout	Colorimetric	Flow cytometry

Best use of ELISA or CBA

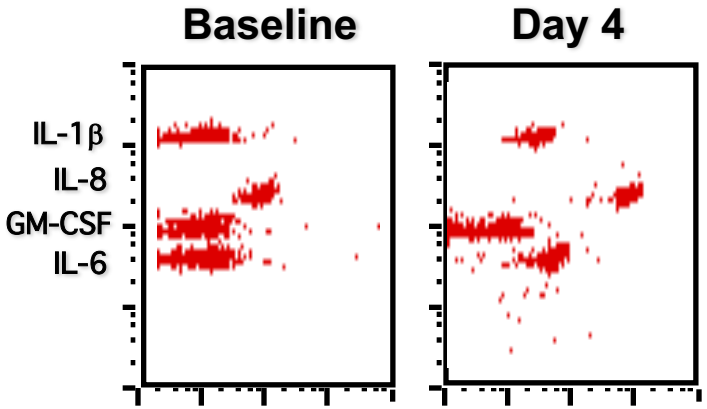
- ELISA: defined system where only one or a few analytes are to be measured
  - Example: testing the effect of various conditions on IL-12 production from purified DC
- CBA: systems in which multiple analytes are of potential interest and the sample is limited
  - Example: intraocular fluid (IL-6 and IL-10 to differentiate intraocular lymphoma from uveitis), CSF, measuring the effect of allergens on cytokines in human tears

Examples of CBA assays

Spiking of single cytokines to show assay specificity:



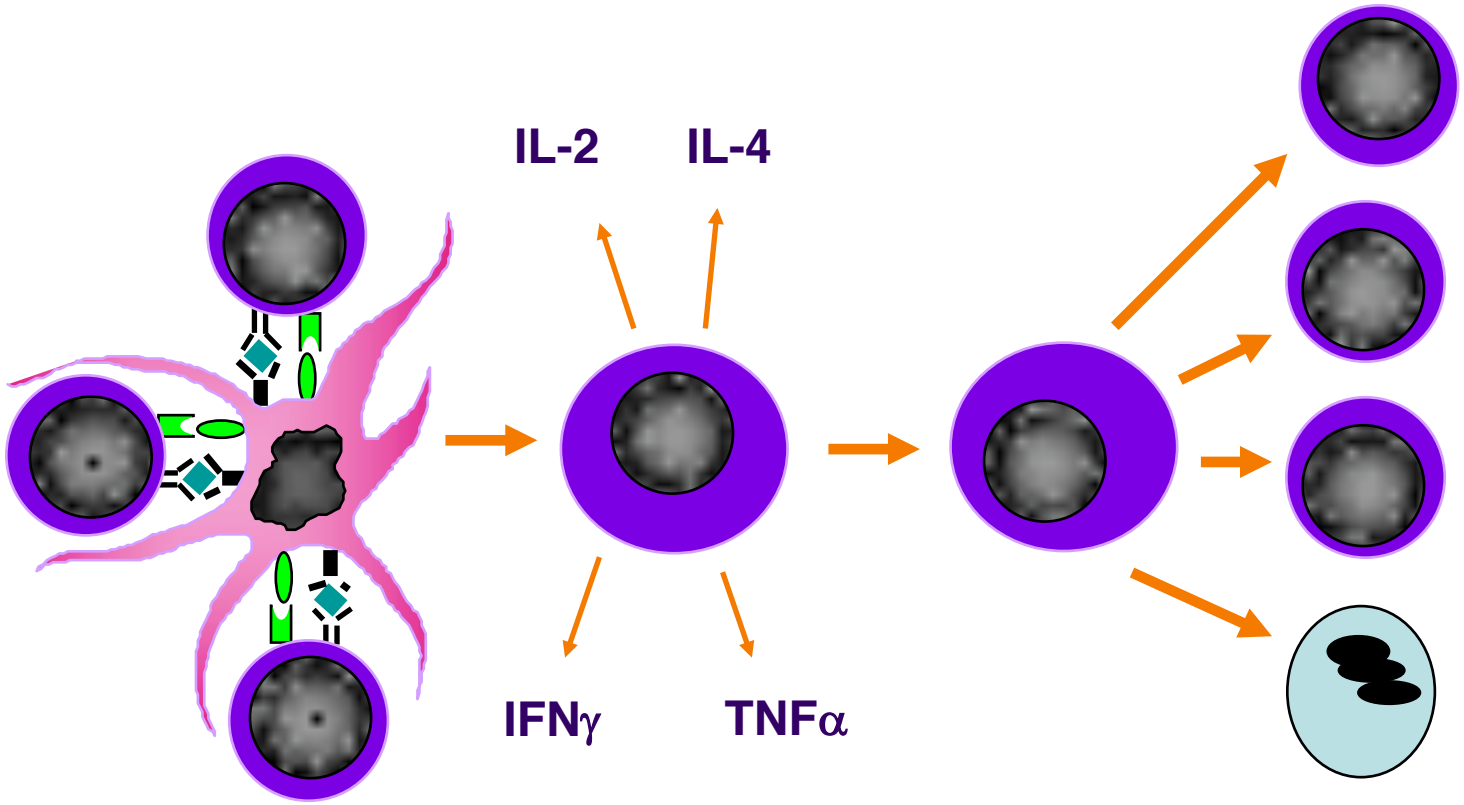
Effect of Rhinovirus inoculation on cytokines in nasal lavage:



General activity of cytokines

- **Cytokines involved in acute inflammation**
  - TNF-α, IL-1, IL-6, IL-8, IL-11
  - And other chemokines, G-CSF, and GM-CSF
- **Cytokines involved in chronic inflammation**
  - They can be subdivided into: -
    - Cytokines mediating humoral responses
      - IL-4, IL-5, IL-6, IL-7, and IL-13
    - Cytokines mediating cellular responses
      - IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferon (IFN), transforming growth factor-ß (TGF), and tumour necrosis factor-α and ß (TNF).

FCM and early & late functions of cellular immunity

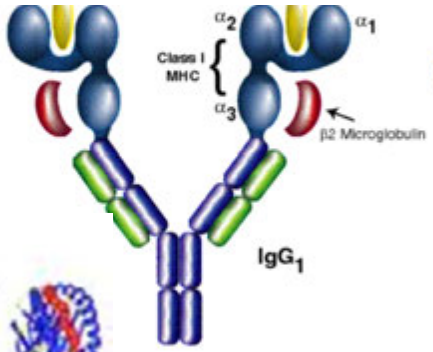


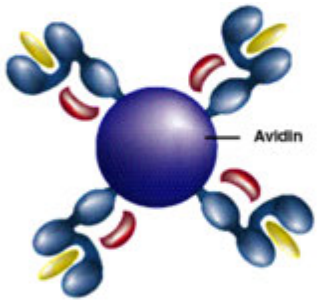
Categories of cellular functional assays

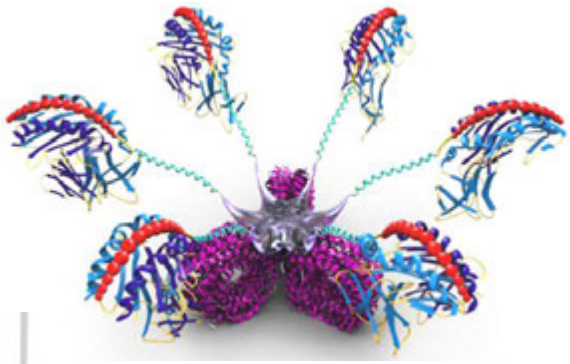
- **Single-cell Assays**
  - **For Specificity:**
    - **MHC-peptide tetramer staining**
    - **MHC-Ig dimer staining**
  - **For Function:**
    - **ELISPOT**
    - **ICS**
    - **CD107 staining**
    - **BrdU incorporation**
    - **CFSE assay**
- **Bulk Assays**
  - **Radioactive:**
    - **^{51}Cr release**
    - **LPA (^{3}H-thymidine incorporation)**
  - **Non-Radioactive:**
    - **ELISA**
    - **CBA**

MHC-peptide dimers and multimers

- Measure binding of T cells to a specific peptide+MHC combination
- Can be used to identify rare populations of antigen-specific T cells without *in vitro* activation



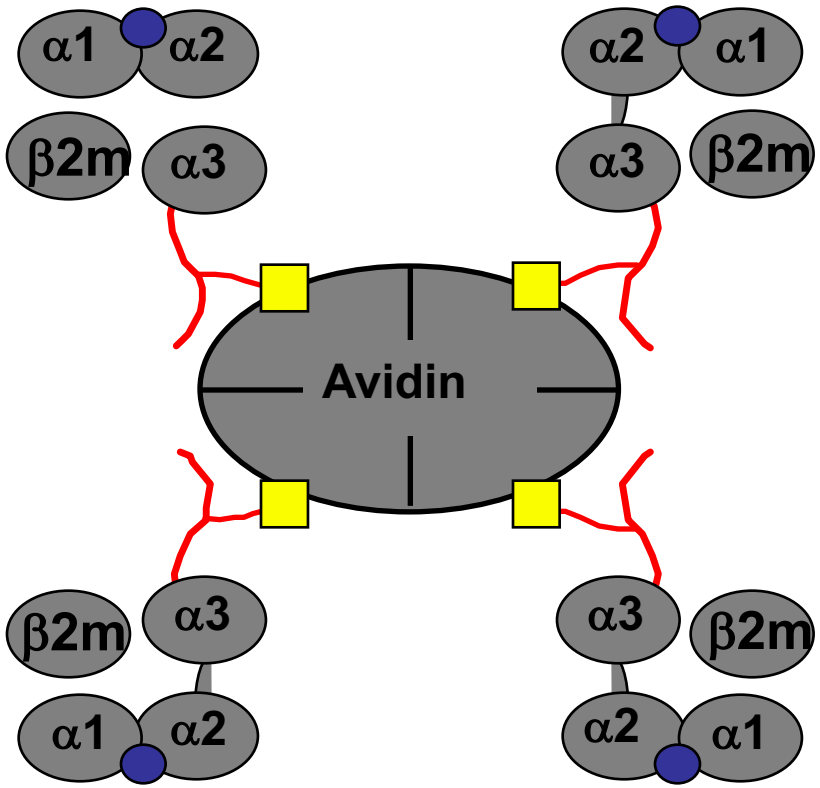




- MHC buckets

MHC Tetramer design features

- Enzymatic Biotinylation
- Oriented T cell epitope
- Single Peptide Ligand
- Specificity Altered at Will



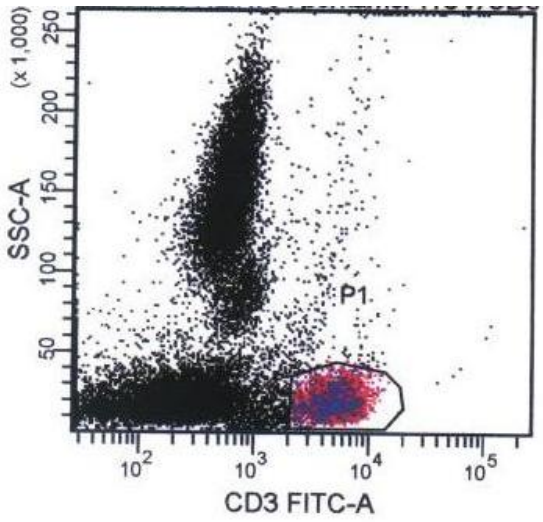
MHC I pentamers - anti-HCV response

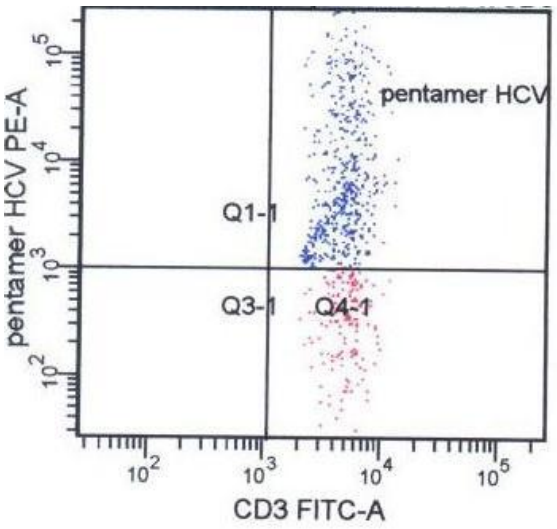
HCV Peptides :

- CINGVCWTV
- DLMGYIPAV
- KLVALGINAV
- RVCEKMALY
- AYSQQTRGL

Other viruses:

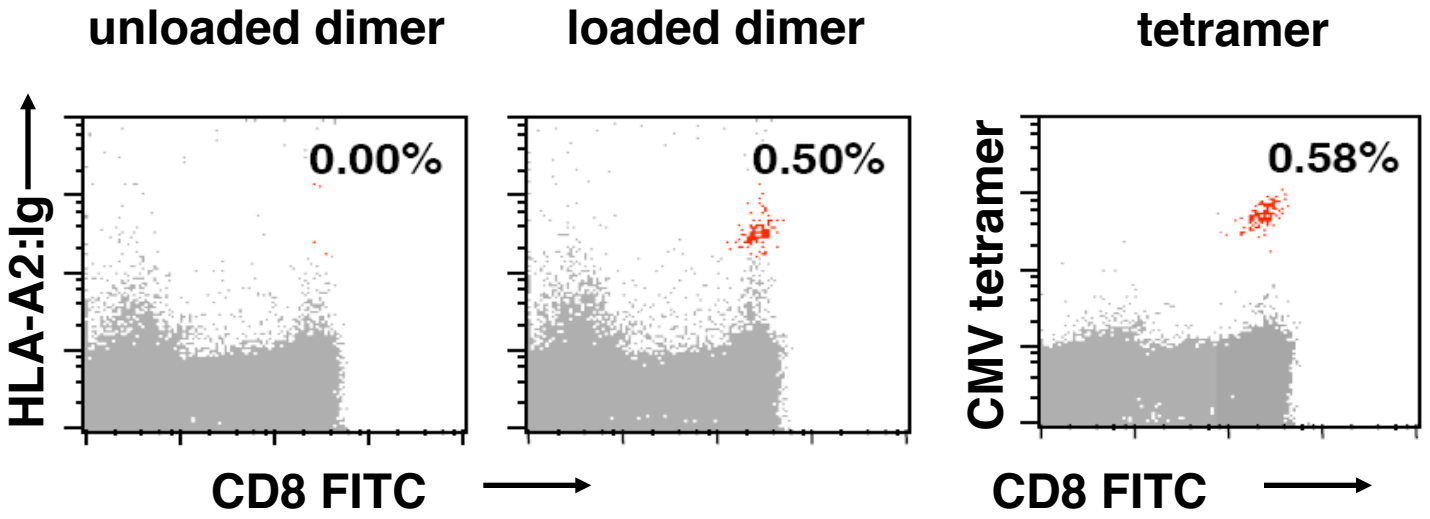
CMV, EBV, HBV, InfluA, HIV, HPV, HSV, ...





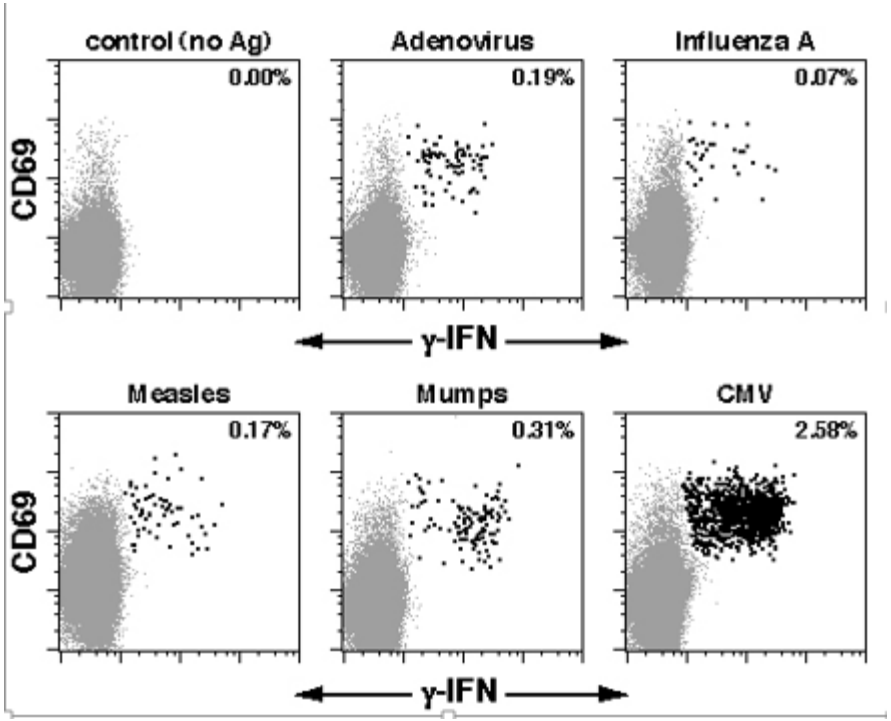
Example of dimer vs. tetramer staining

- Dimers:
  - Investigator can load peptide of interest
  - Can be used to coat plates for antigen-specific cell capture/stimulation
- Tetramers:
  - More MHC alleles commercially available
  - Higher affinity binding in some systems
  - Directly fluorochrome labeled



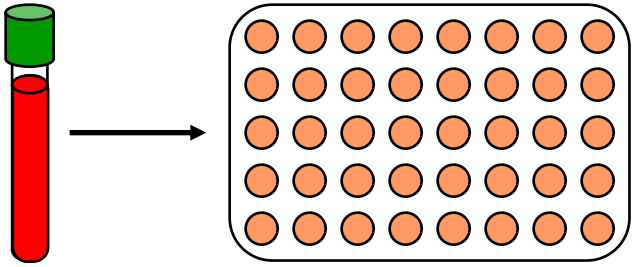
ICS Assays

- Measure production of cytokines in short-term stimulated whole blood, PBMC, etc.
- Can measure multiple cell-surface and intracellular markers in combination, using multiparameter flow cytometry
- Can detect rare events such as antigen-specific T cells



- Quantification of viral specific CD4+ memory cells in a normal subject

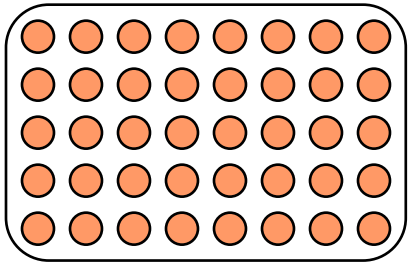
Principle of Plate-Based ICS Assays



20 ml PBMC/
WB sample

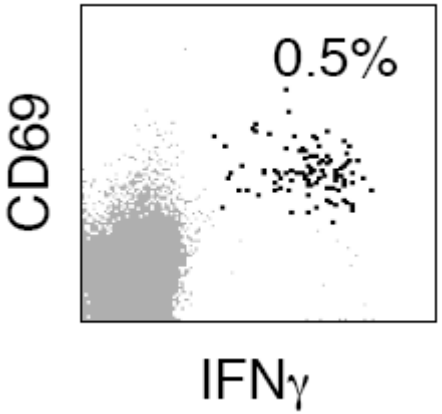
Antigenic stimulus
+ brefeldin A

Incubate 6-24 h

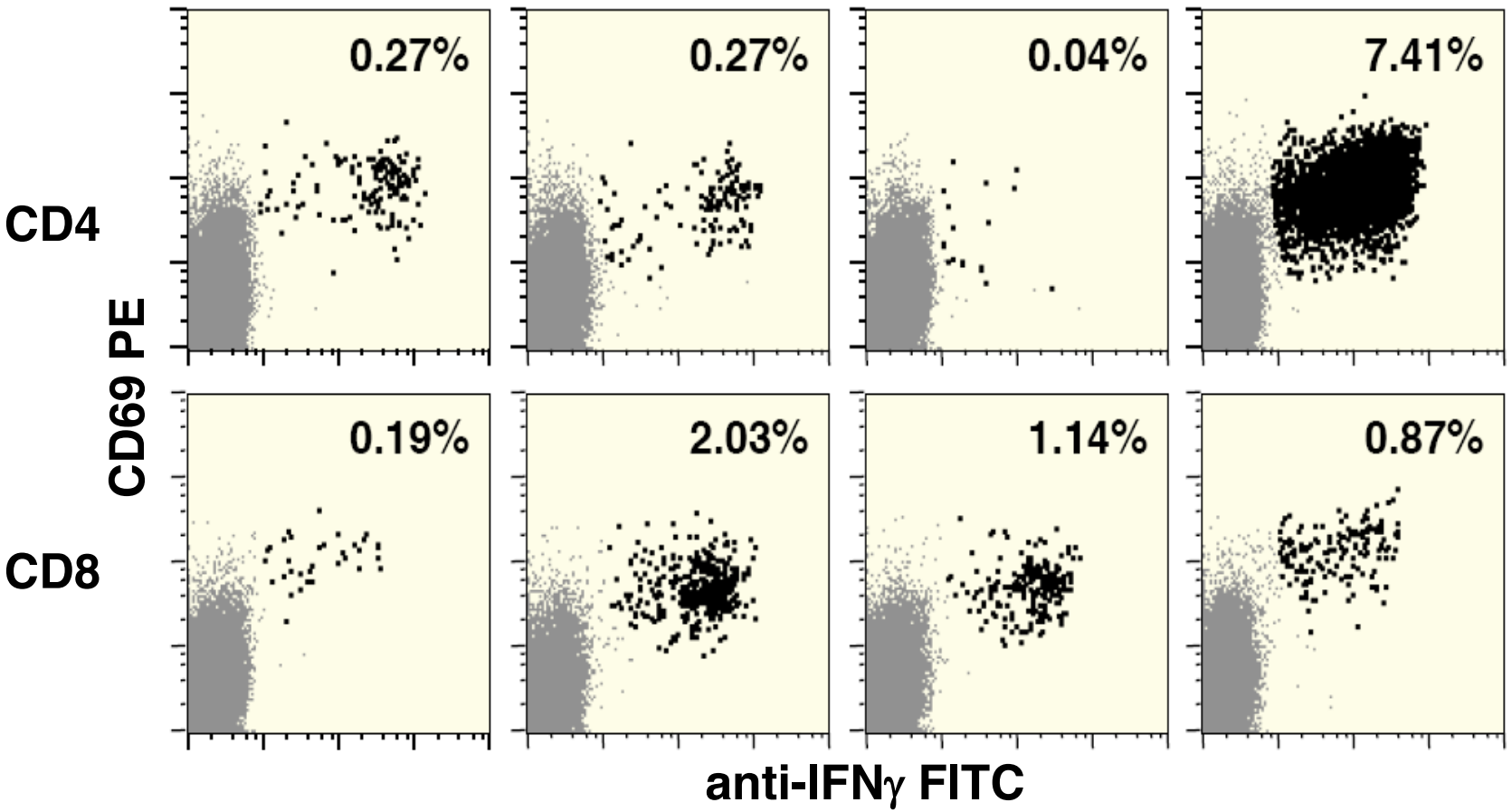


- Fix cells
- Permeabilize
- Stain

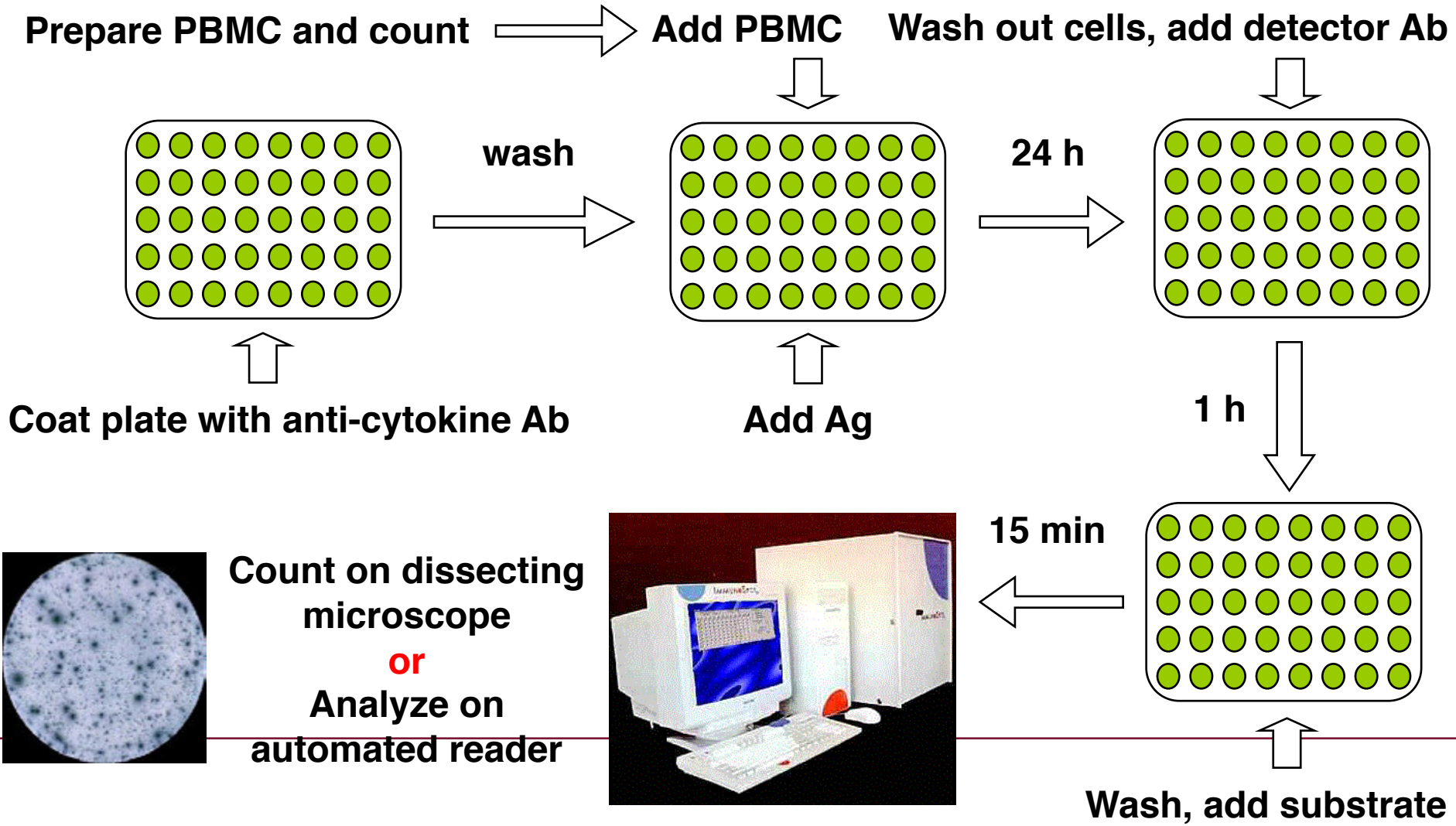
Gate on cells
of interest



Example of ICS Results



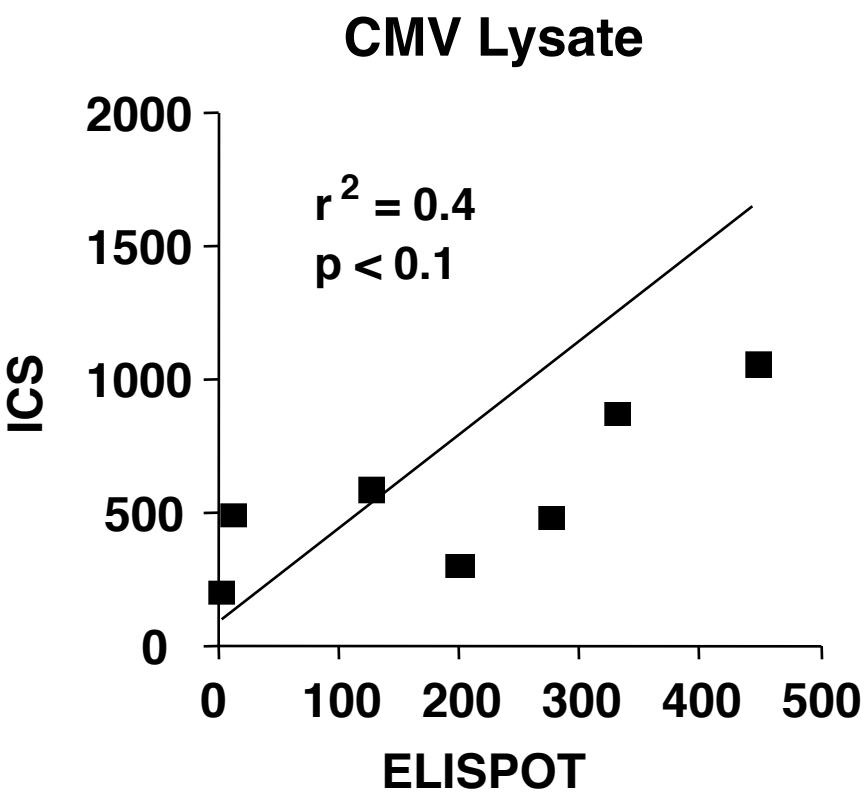
ELISPOT Assay Principle

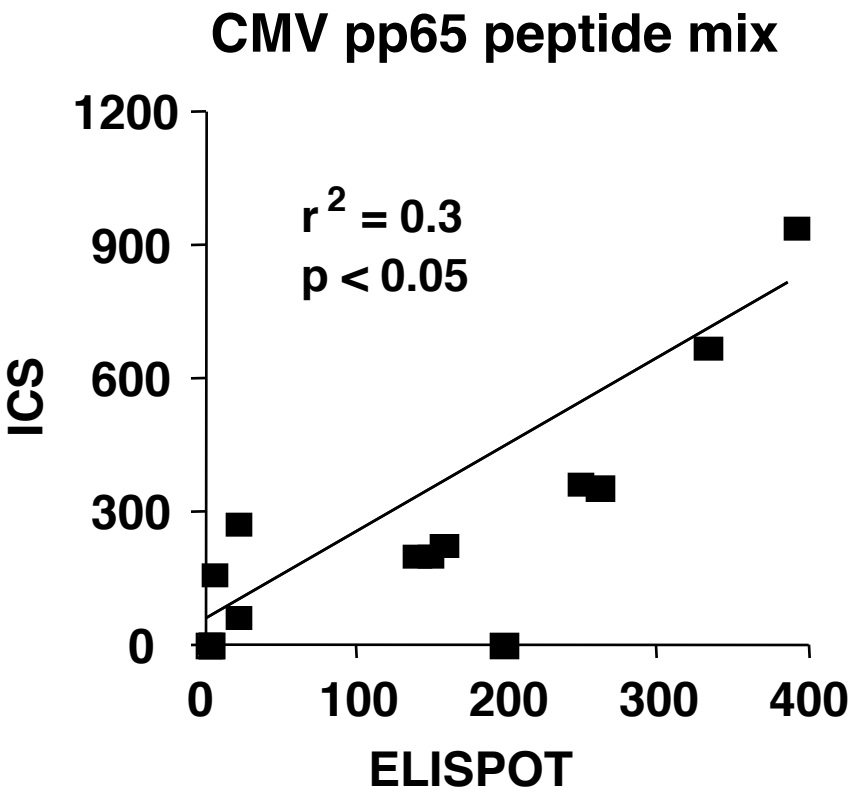


ELISPOT Assays

- PBMC are plated on a filter-bottom 96-well plate coated with anti-cytokine antibody.
- The plate is cultured 24-48 hours to allow cytokine secretion and capture on the plate.
- Cells are washed off and detector antibody is added, followed by enzyme substrate.
- Cytokine-secreting cells are identified as spots of secreted cytokine.

Correlation of ICS and ELISPOT Assays





Cytomegalovirus-specific CD8$^+$ T cells targeting different HLA/peptide combinations correlate with protection but at different threshold frequencies

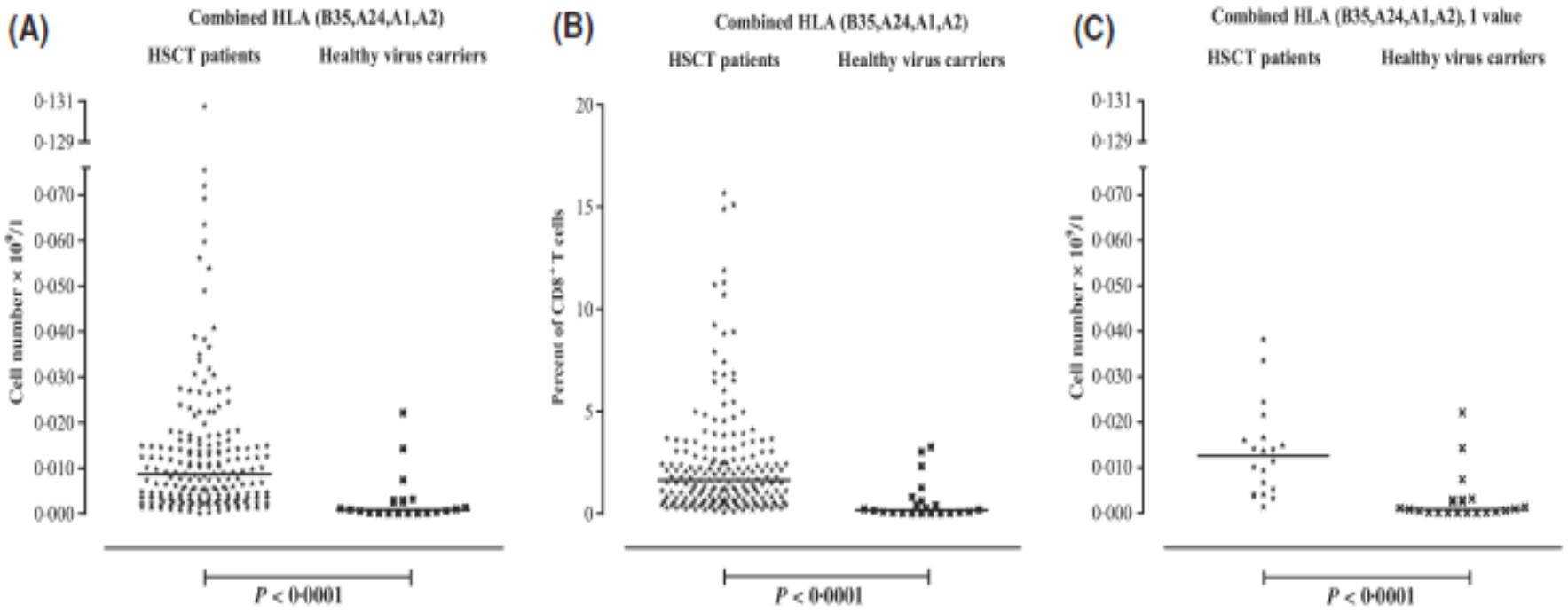


Fig 3. CMV specific CD8$^+$ T cells in HSCT patients *versus* healthy individuals. This figure shows the pooled data from Fig 2B, C. Cell numbers measured in HSCT patients (median 0·00865 × 10^9/l corresponding to 1·63% of CD8$^+$ T cells, n = 190) were compared with those measured in CMV seropositive healthy blood donors (median 0·00094 × 10^9/l, corresponding to 0·18% of CD8$^+$ T cells, n = 22). Panel A shows the absolute numbers of tetramer binding T cells per volume of blood, while panel B shows the frequency of these cells as a proportion of total CD8$^+$ T cells. Panel C is a variation of panel A showing only one median level of response for each patient.

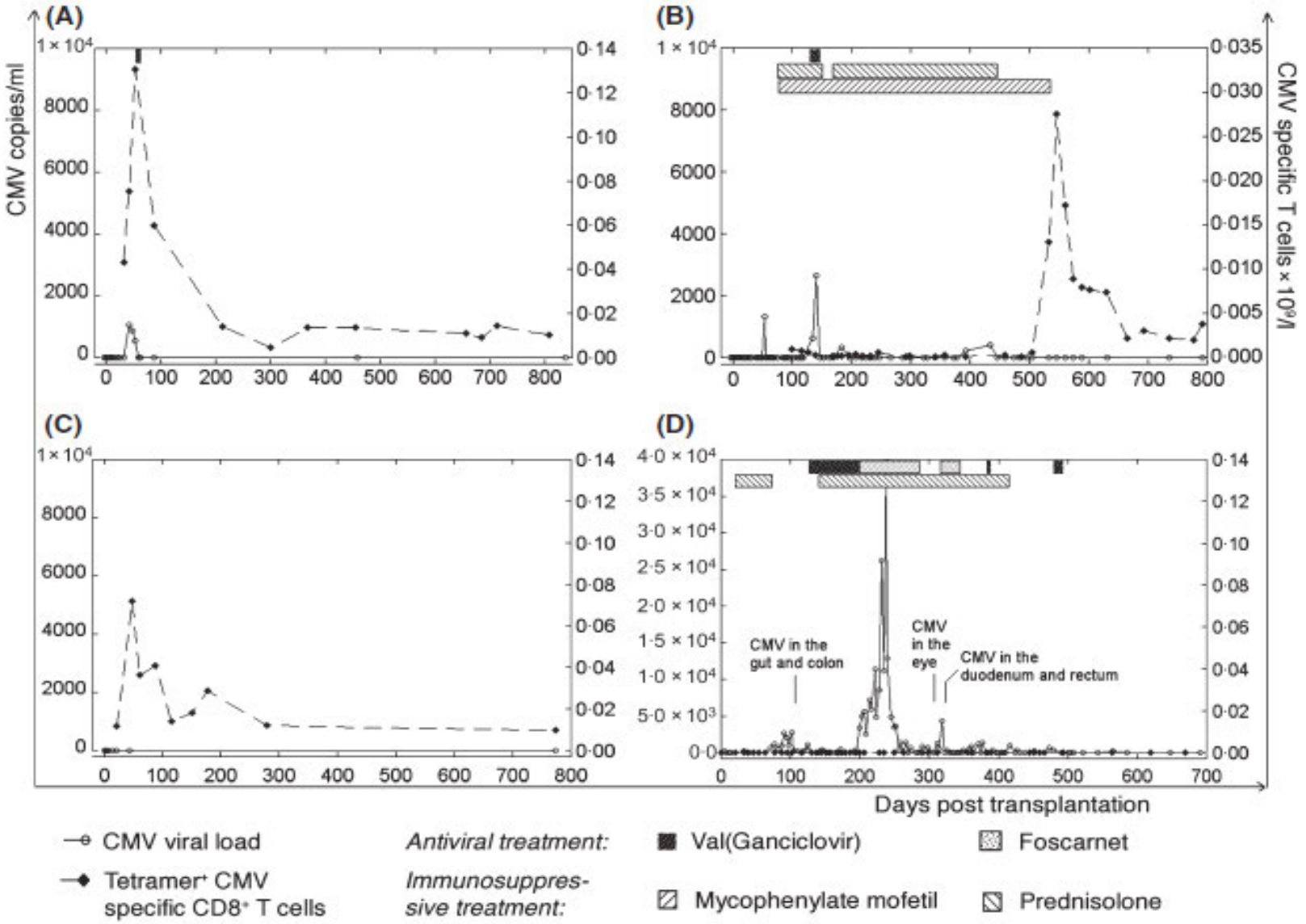
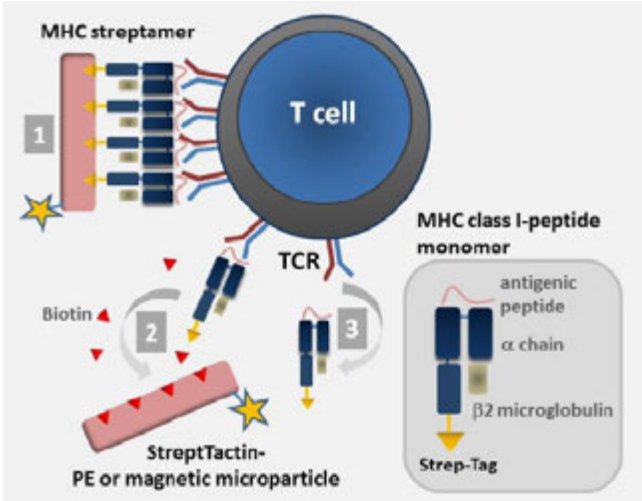
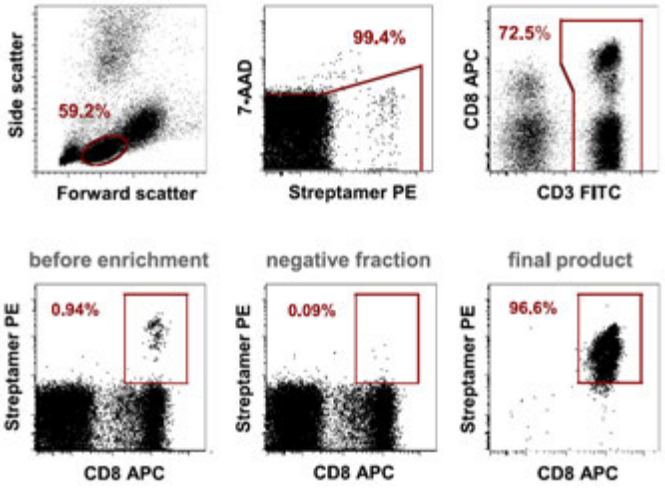


Fig 1. Correlation between functional CMV-specific CD8⁺ T cell numbers and CMV viral load in representative patients. Peripheral blood CMV-specific CD8⁺ T cell numbers (×10⁹/l) are illustrated along with viral load measurements (note different scales for patients) for Patients 9 (A), 11 (B), 13 (C) and 30 (D). Patient characteristics are given in Tables I and III.

Clinical-scale isolation of 'minimally manipulated' cytomegalovirus-specific donor lymphocytes for the treatment of refractory cytomegalovirus disease

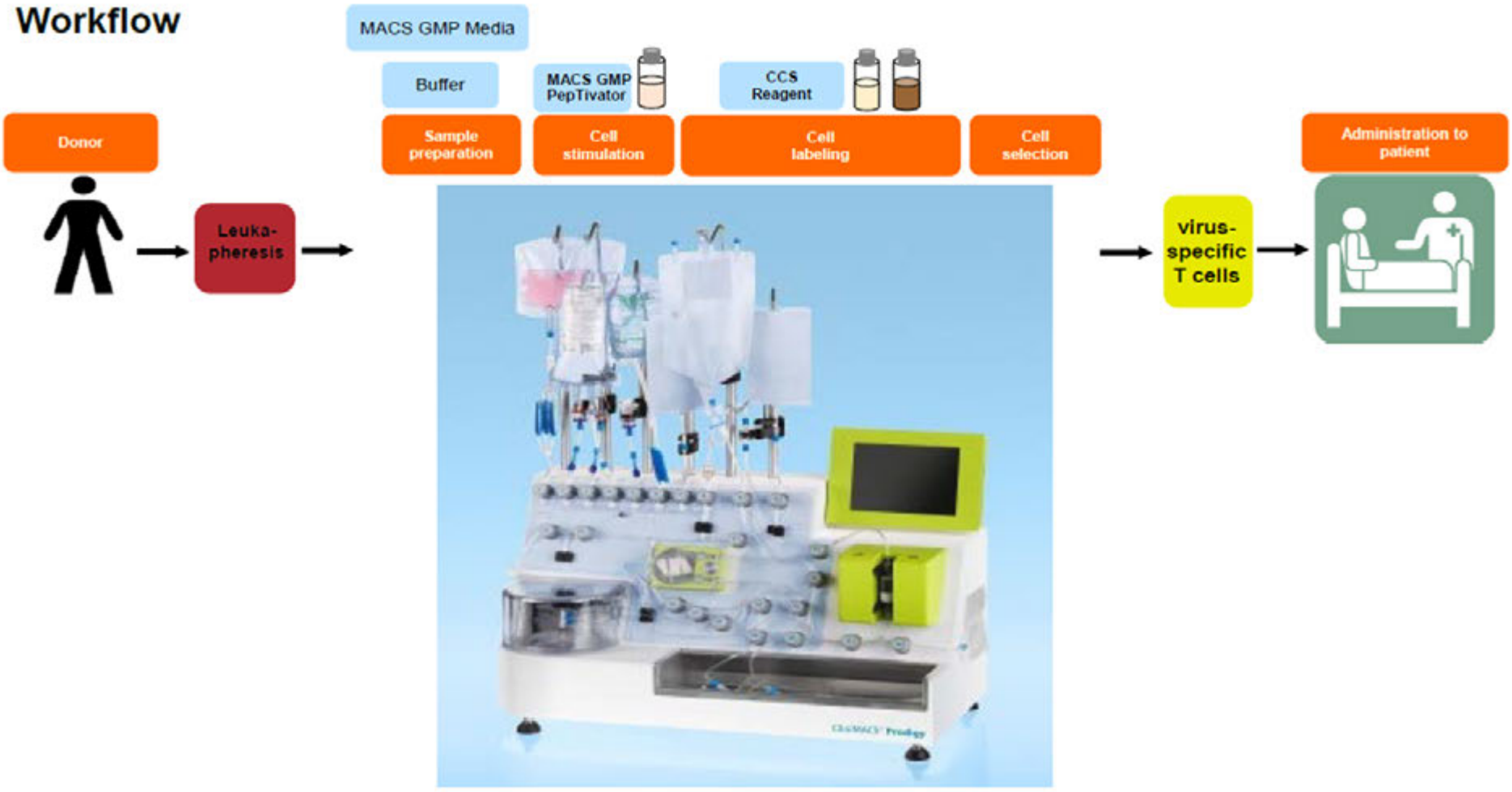
	Mean ± standard deviation	Median (range)
Before enrichment		
WBCs [1 × 10^{10} cells]	1.10 ± 0.48	1.04 (0.07–2.59)
CD3+ T cells [1 × 10^{9} cells]	5.42 ± 3.49	5.64 (0.03–1.61)
CMV-specific T cells [1 × 10^{7} cells]	5.05 ± 11.36	1.14 (0.16–53.28)
CMV-specific T cells [%]	1.21 ± 3.11	0.41 (0.03–14.8)
After enrichment		
WBCs [1 × 10^{7} cells]	3.18 ± 3.08	1.93 (0.11–13.1)
CD3+ T cells [1 × 10^{7} cells]	1.72 ± 2.04	1.25 (0.04–9.03)
CMV-specific T cells [1 × 10^{6} cells]	12.59 ± 18.01	7.77 (0.11–31.00)
CMV-specific T cells [%]	75.1 ± 27.5	90.2 (17.7–99.5)
Viability [%]	92.0 ± 8.11	91.5 (75.0–100.0)
Recovery [%]	100.2 ± 153.7	67.2 (0.9–139.2)
Enrichment factor	4868 ± 7168	2541 (105–27,440)





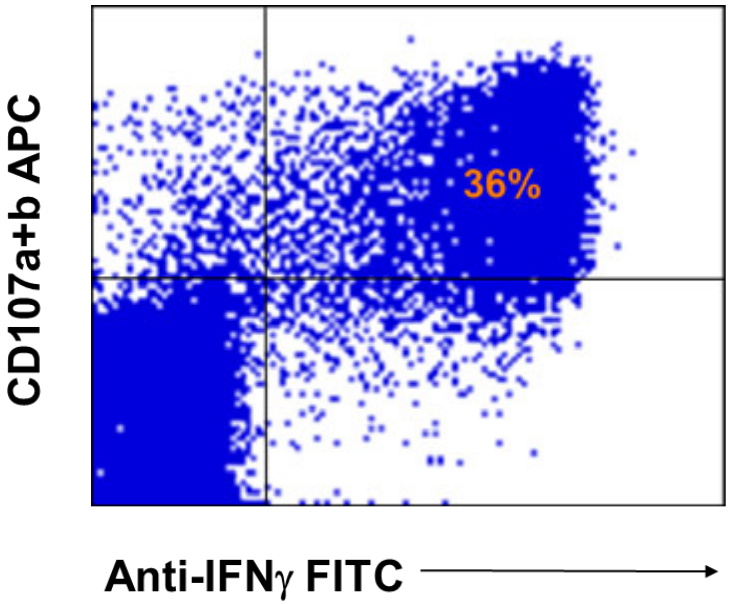
Cytotherapy, 2014; 16: 1245–1256

Aim – adoptive therapy with virus specific T cells



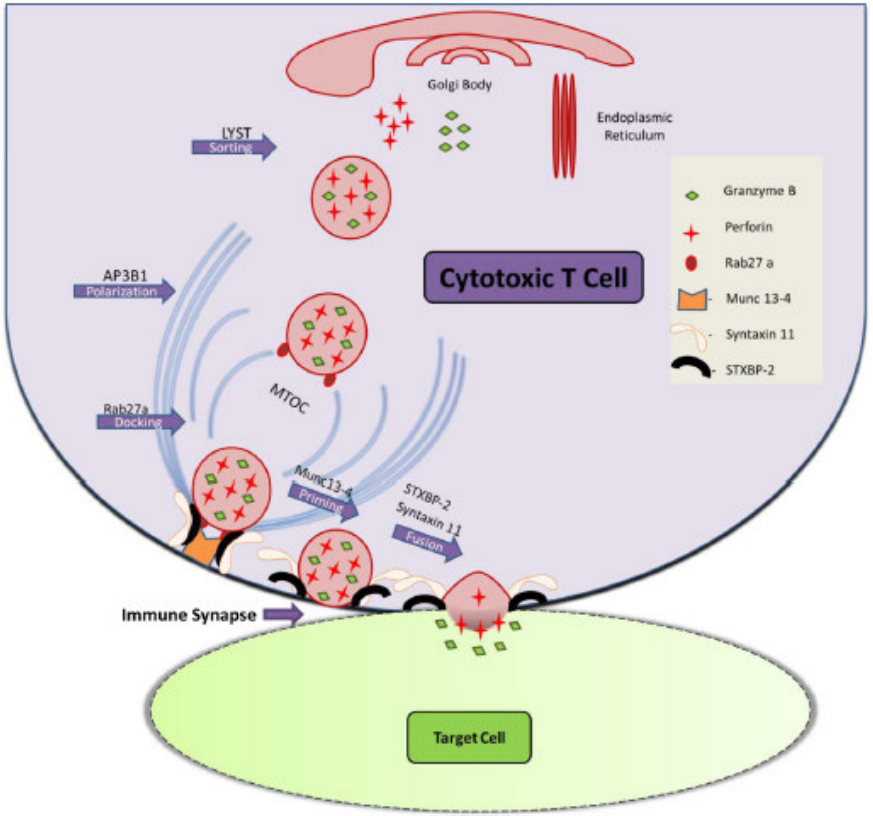
CD107 Assays

- CD107a and CD107b are proteins found in cytotoxic granules of CTL and other cells
- Upon degranulation, CD107a and CD107b are transiently transported to the cell surface
- Using labeled antibodies to CD107a and CD107b during short-term stimulation, the exocytosis of CD107 is captured on degranulating cells.
- Compare BAT – CD63



Genetic defects involved in granule-mediated cytotoxicity leading to Hemophagocytic Lymphohistiocytosis (HLH)

- CTL activation results in microtubule organizing Centre (MTOC) polarization and transport of cytotoxic granules.
- LYST and AP3B1are involved in sorting and transport of cytotoxic granules.
- The granules are then docked to the site of immune synapse by Rab27a.
- Granule priming is mediated by Munc13-4, and membrane fusion by STX11 and STXBP2.
- Granule fusion results in perforin mediated pore formation and release of lysosomal enzymes leading to target cell death.
- Genetic defects in highlighted proteins involved in granule-mediated cytotoxicity leads to HLH.



The Journal of Pediatrics 2013;163:1253–1259

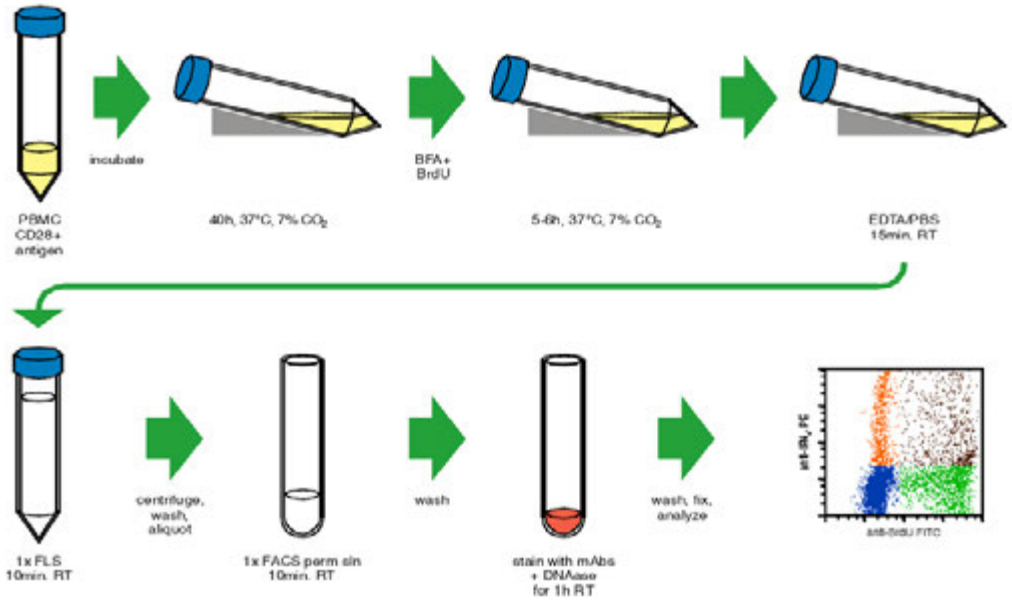
Diagnostic criteria for HLH

- **Diagnosis of HLH is based on the presence of 5 or more of the following**: Fever; Splenomegaly; Cytopenias (affecting 2 of 3 lineages in the peripheral blood); Hemoglobin <90 g/L (<100 g/L in infants aged <4 weeks); Platelets <100 109/L; Neutrophils <1.0 109/L; Hypertriglyceridemia and/or hypofibrinogenemia; Fasting triglycerides >3.0 mmol/L; Fibrinogen <1.5 g/L; Hemophagocytosis in bone marrow, spleen, or lymph nodes; Low or absent NK-cell activity; Ferritin >500 mg/L; sIL-2R >2400 U/mL

HLH type	Defective gene	Function	Notable clinical findings	Rapid diagnosis by flow cytometry
FHLH-2	*PRF1*	Pore formation		Decreased/absent perforin expression
FHLH-3	*UNC13D*	Vesicle priming	Increased incidence of CNS HLH	Decreased CD107a expression
FHLH-4	*STX11*	Vesicle fusion	Mild, recurrent HLH, and colitis	Decreased CD107a expression
FHLH-5	*STXBP2*	Vesicle fusion	Colitis and hypogammaglobulinemia	Decreased CD107a expression
Syndromes				
Griscelli syndrome type II	*RAB27A*	Vesicle docking	Partial albinism and silvery-gray hair	Decreased CD107a expression, abnormal hair shaft examination*
Chediak-Higashi syndrome	*LYST*	Vesicle trafficking	Partial albinism, bleeding tendency, and recurrent pyogenic infection	Decreased CD107a expression, abnormal neutrophil granules†
Hermansky-Pudlak syndrome type II	*AP3B1*	Vesicle trafficking	Partial albinism, bleeding tendency, and immunodeficiency	Decreased CD107a expression
EBV-driven				
XLP-1	*SH2D1A*	Signaling in T, NK, and NK T-cells	Hypogammaglobulinemia and lymphoma	Decreased/absent SAP expression
XLP-2/XIAP‡	*BIRC4*	Signaling pathways involving NF-κB	Mild, recurrent HLH and colitis	Decreased/absent XIAP expression
IL-2–inducible T-cell kinase deficiency	*ITK*	Signaling in T-cell	AR, Hodgkin lymphoma	NA (gene sequencing required)
CD27 deficiency	*CD27*	Lymphocyte costimulatory molecule	AR, combined immunodeficiency	Absent CD27 expression on B cells
XMEN	*MAGT1*	T-cell activation via T-cell receptor	Combined immunodeficiency, chronic viral infections, and lymphoma	Decreased CD4 cells and defects in T-cell receptor signaling

Proliferation assays - BrdU Assay

- Can measure cell proliferation based on incorporation of fluorescently labeled BrdU
- Can be combined with cell-surface and intracellular markers (e.g., cytokines) for multiparameter staining



What do we want to determine with proliferation assays?

Any defect between TCR engagement/cytokine receptor stimulation/ mitogen-induced T cell activation and cell division

What do we actually measure with T cell proliferation assays?

Bulk proliferation in a heterogenous pool of responder cells:
- CD4, CD8, TCRgd T cells
- Naive, activated, different memory subsets

- Proliferation on a per cell basis (cave: net result of proliferation and cell death)
- Differentiation between CD4 and CD8
- analysis of kinetics, number of cell divisions
- Low sensitivity for weak TCR stimuli (eg TT, allo)
- Variability of assays
- Requires more cells than Thymidin uptake assay, cell loss during washing steps
- Quantification/interpretation of results: what is pathological?

Proliferation assays: optimization/standardization

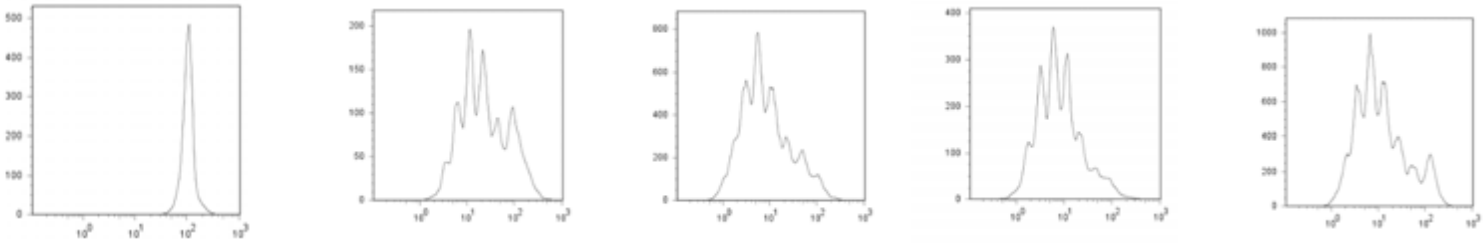
Conditions tested:

- Which stimuli?
  - PHA
  - anti-CD3 (which clone?, soluble vs. platebound)
  - anti-CD3 +|- anti-CD28
  - anti-CD3/CD28 beads
- Roundbottom vs. flatbottom plate?
- Days of culture before flow cytometry?

Final conditions:

- PHA, soluble anti CD3 (OKT3) plus anti CD28
- flatbottom plate
- 4-6 day incubation

Medium **CD3** **CD3+CD28** **PHA** **beads**



Proliferation tests: conclusions

Robust and reproducible protocol?

-Assay performance variable, day control needed

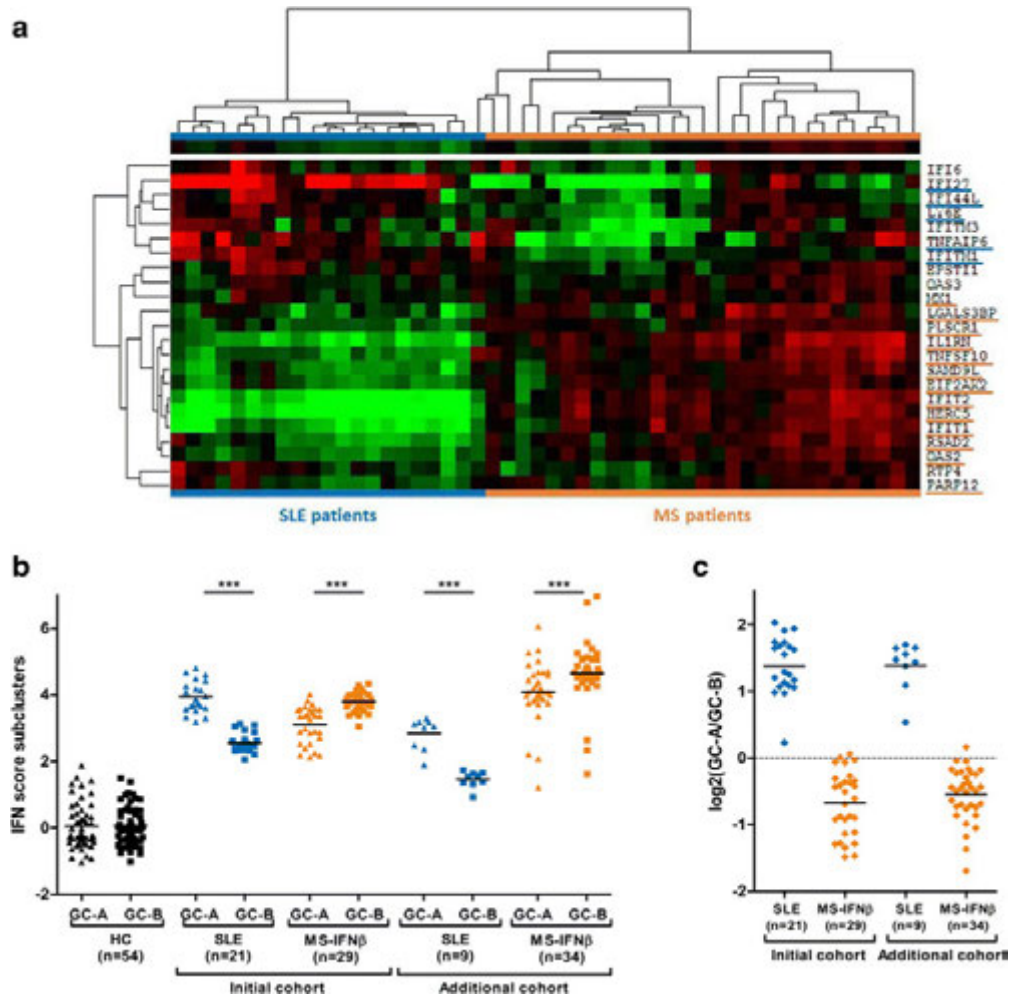
Should allow to investigate antigen-specific proliferation?

- Assay not sensitive enough for tetanus toxoid stimulation

Feasable with limited numbers of PBMC?

- Problems with patients with low T cell counts

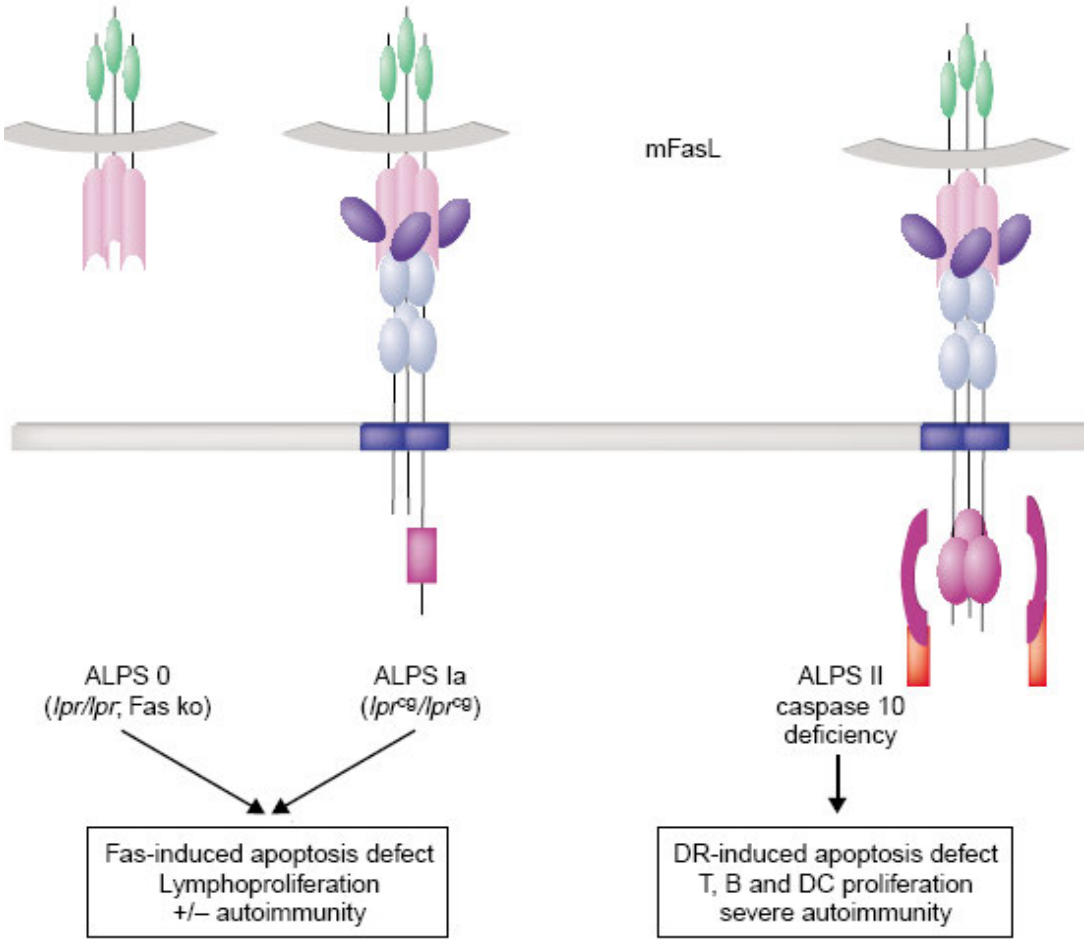
Interferon signature – what is it?



- Part of normal immune response
- Some SLE patients have over-reactive IFN pathway upon stimulation
- Identified by measuring IFN inducible mRNA
- A fingerprint for severe SLE

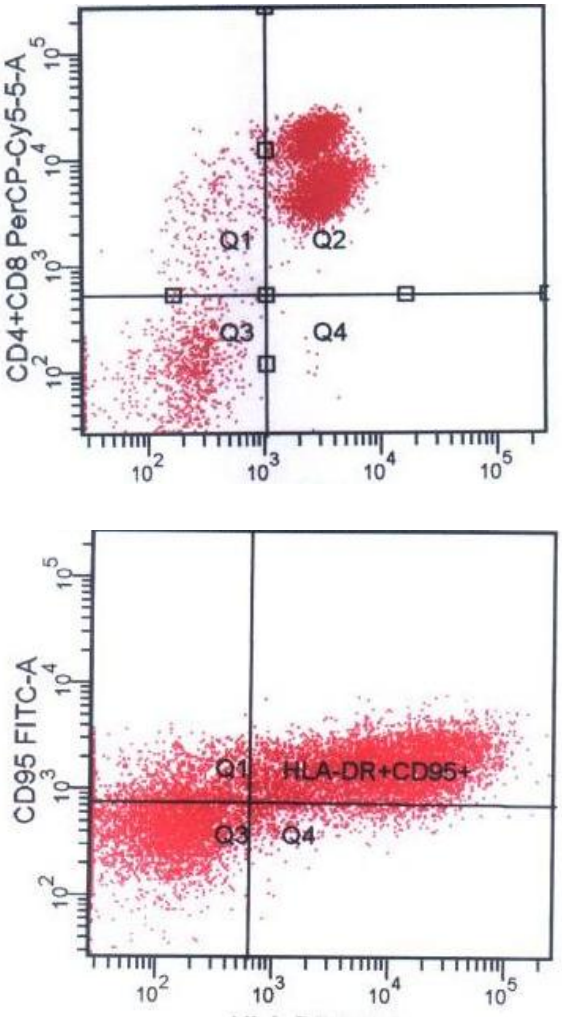
Autoimmune lymphoproliferative syndrome (ALPS)

- Autoimmune lymphoproliferative syndrome (ALPS), is caused by a defect in apoptosis (programmed cell death) of lymphocytes via the Fas pathway leading to the abnormal accumulation of lymphocytes.



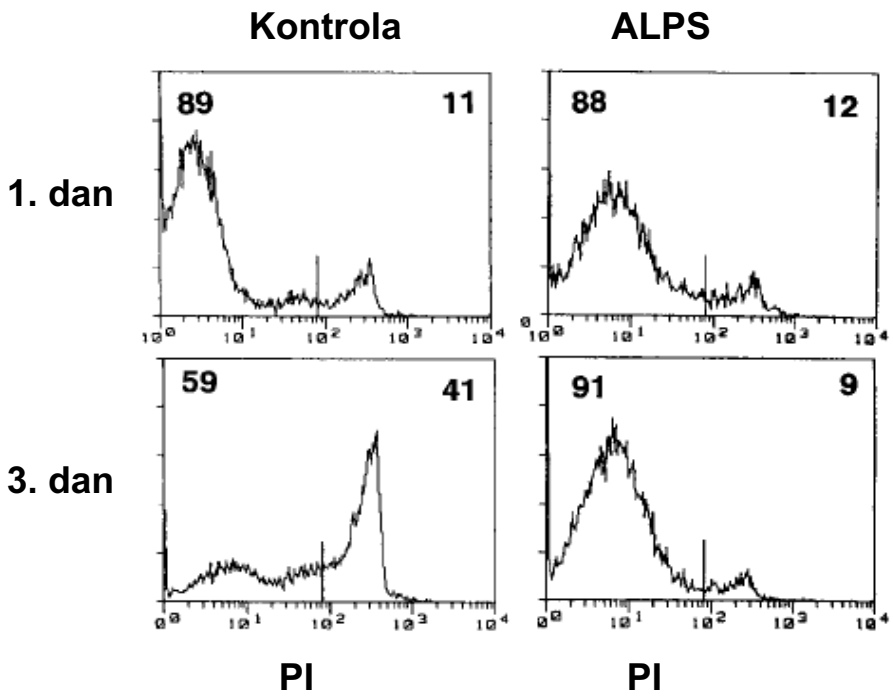
Detection methodology

- Normally, less than 1 percent of T cells that express the T cell receptor alpha and beta chains do not express either the CD4 or the CD8 co-receptors. These T cells are termed double negative T-cells (DNT).
- In ALPS, the number of +DNT cells is increased.



ALPS – apoptosis disfunction

PBL stimulated
with PMA / IL-2



Thank YOU!!

Univerza v Ljubljani

Thank YOU!!

Questions??

TREATMENT OF PATIENTS WITH ANTIBODY DEFICIENCY

Štefan Blazina

Department of Allergology, Rheumatology and Clinical Immunology, Children's Hospital, University Medical Center Ljubljana, Slovenia

Antibody deficiency is either a consequence of genetic defect (primary) or immunosuppressive treatment (secondary). Some genetic defects affect specifically B cells (XL or AR agammaglobulinemia, common variable immunodeficiency – CVID, activated PI3 kinase delta syndrome – APDS, isolated IgA and IgM deficiency). Others affect T cells (severe combined immunodeficiency syndrome - SCID) or communication between T and B cells (class switch defects), which both result in antibody deficiency. Agammaglobulinemia is treated with lifetime subcutaneous or intravenous immunoglobulin (Ig) substitution therapy. Although bone marrow transplant (BMT) would offer a complete cure of the disease the risks of BMT are still too high to recommend BMT to these patients. Reduced concentrations of Ig in patients with CVID are treated with Ig supplementation, however one third to one half of these patients need immunosuppressive treatment (steroids, mycophenolate mofetil, rituximab) for disease complications, including autoimmunity (AI cytopenia, enteropathy etc.) and lymphoproliferation (granulomatous-lymphocytic interstitial lung disease – GLILD, lymphadenopathy, hepatosplenomegaly). Patients with APDS are candidates for BMT because they frequently develop complication despite of Ig supplementation: autoimmunity, chronic EBV and CMV related lymphoproliferation and herpes infections. Patients with isolated IgA and IgM deficiency are not treated with Ig, however they often need treatment for autoimmunity and allergies, respectively. While SCID can only be cured with BMT/genetic/enzyme therapy these patients receive Ig supplementation (sometimes in higher doses and shorter intervals) while waiting for curative treatment. Patients with class switch defects also receive Ig for antibody deficiency, however they also require antibiotic prophylaxis for Pneumocystis. Early BMT was proven to be treatment of choice for patients with class switch defects as it reduces morbidity and mortality. Patients with secondary immunodeficiency from immunosuppressive treatment should have regular evaluation of their immune system. Those with reduced concentration of IgG do profit from Ig supplementation. In addition, patients with severe suppression of T cell number or function have reduced ability to develop specific antibody response, therefore Ig supplementation should be considered in patients with intensive suppression of T cell function. In patients with antibody deficiency the frequency of infections decreases with increasing IgG through level – the recommended IgG through level is above 8 g/L (above 10 g/L for patients with bronchiectasis).

Treatment of patients with antibody deficiency

14.10.2016

Štefan Blazina

Department of Allergy, Rheumatology and Clinical Immunology

University Children‘s Hospital Ljubljana

Antibody def. - etiology

- **Primary (genetic):**
- **B cells** (XL or AR agammaglobulinemia, common variable immunodeficiency - CVID, activated PI3 kinase delta syndrome - APDS, isolated IgA and IgM deficiency).
- **T cells** (severe combined immunodeficiency syndrome – SCID; APDS)
- **Communication** between T and B cells (class switch defects)
- **Secondary (acquired):**
- HIV
- Immunosuppressive therapy

Ab def. - etiology

Primary Immunodeficiency Diseases: an Update on the Classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015

J Clin Immunol (2015) 35:696–726

- **Primary (genetic):**

- **B cells** (XL or AR agammaglobulinemia, common variable immunodeficiency - CVID, activated PI3 kinase delta syndrome - APDS, isolated IgA and IgM deficiency).
- **T cells** (severe combined immunodeficiency syndrome – SCID; APDS)
- **Communication** between T and B cells (class switch defects)

- **Secondary (acquired):**

- HIV
- Immunosuppressive therapy

Ab def. - etiology

- **Primary (genetic):**
- **B cells:**

XL or AR agammaglobulinemia
common variable immunodeficiency - CVID
activated PI3 kinase delta syndrome - APDS
isolated IgA and IgM deficiency

- **T cells** (severe combined immunodeficiency syndrome – SCID; APDS)
- **Communication** between T and B cells (class switch defects)

- **Secondary (acquired):**
- HIV
- Immunosuppressive therapy

Flowcytometric Phenotyping of Common Variable Immunodeficiency

Cytometry Part B (Clinical Cytometry) 74B:261–271 (2008) Klaus Warnatz* and Michael Schlesier

- agammaglobulinemia

- CVID
- APDS
- IgA def.
- IgM def.

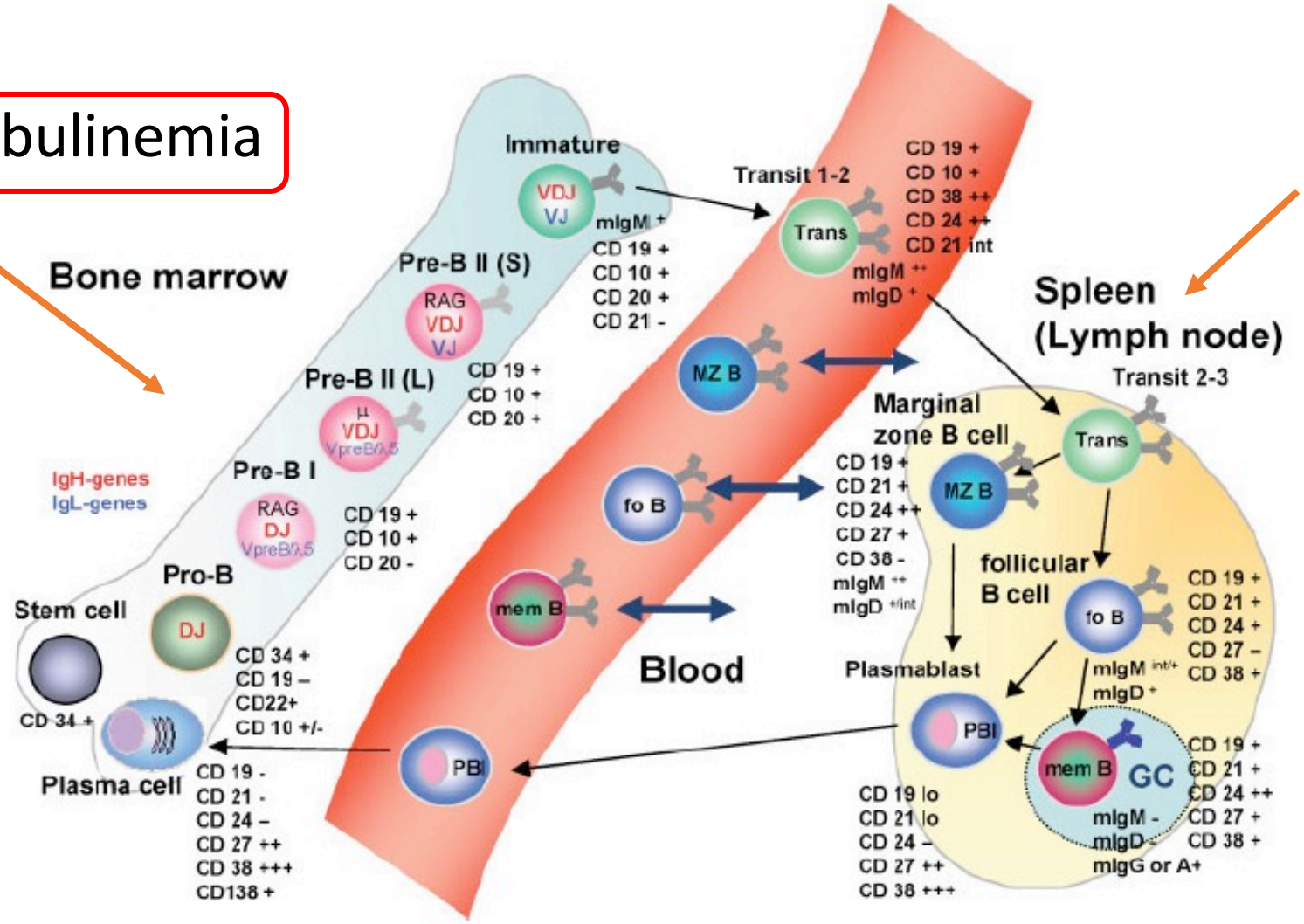


Fig. 1. Scheme of B cell development. The central and peripheral B cell developments are outlined. "Blood" represents circulating B cell populations. Relevant (not all!) surface markers for each differentiational step are listed. Please refer to text for more details. fo B cell, follicular B cell; GC, germinal center; mem B, memory B cells; MZ B cell, marginal zone B cell; Pbl, plasmablast; Trans or Transit, transitional B cell.

B cell defects – agammaglobulinemia

- **Agammaglobulinemia**:

- lifetime subcutaneous or intravenous IgG supplementatiton
- BMT would offer a complete cure of the disease
- risks of BMT are still too high to recommend BMT

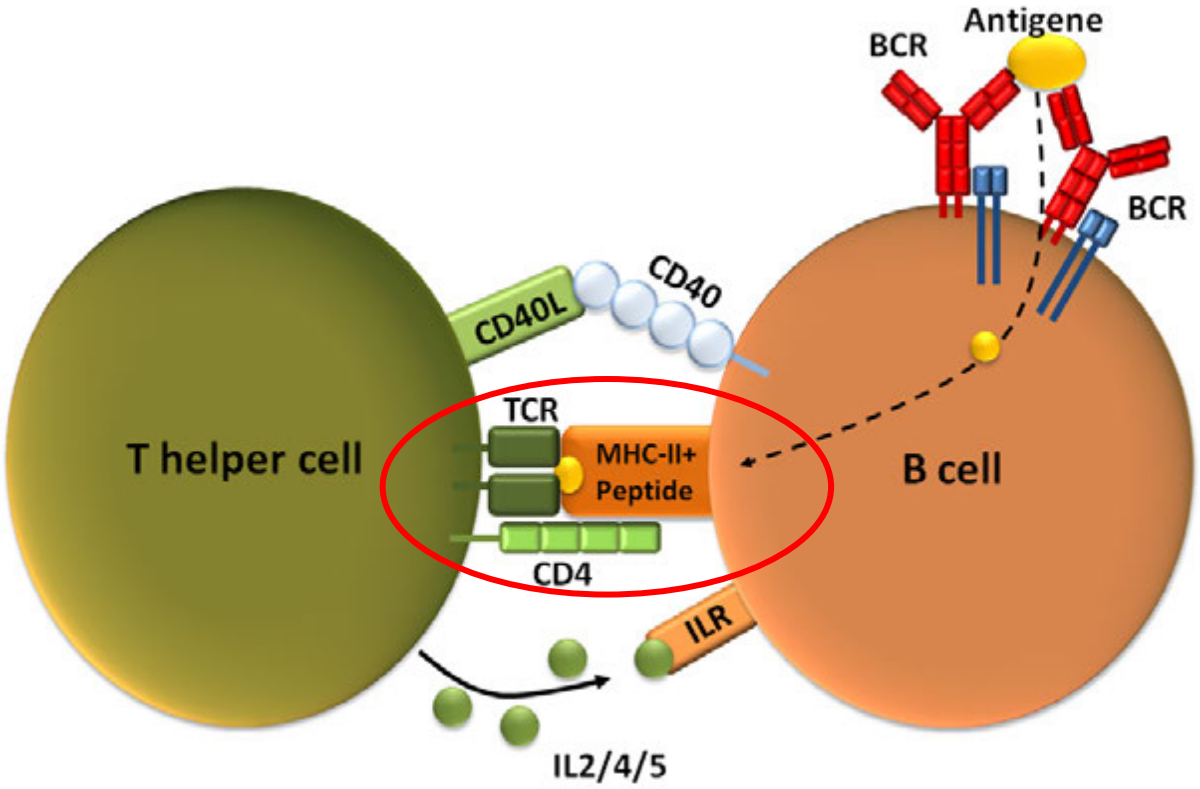
B cell defects - CVID, APDS, IgA def., IgM def.

- **CVID:**
- IgG supplementation
- ⅓ to ½ of these patients need immunosuppressive treatment (steroids, mycophenolate mofetil, rituximab)
- autoimmunity (AI cytopenia, enteropathy etc.)
- lymphoproliferation (granulomatous-lymphocytic interstitial lung disease – GLILD, lymphadenopathy, hepatosplenomegaly).
- **APDS:**
- IgG supplementation
- BMT because they frequently develop complications / enzyme Th
- autoimmunity
- chronic EBV and CMV related lymphoproliferation
- herpes infections.
- **IgA and IgM deficiency:**
- no IgG supplementation
- often need treatment for autoimmunity and allergies

Ab def. - etiology

- **Primary (genetic):**
- **B cells** (XL or AR agammaglobulinemia, common variable immunodeficiency - CVID, activated PI3 kinase delta syndrome - APDS, isolated IgA and IgM deficiency).
- **T cells** (severe combined immunodeficiency syndrome – SCID; APDS)
- **Communication** between T and B cells (class switch defects)
- **Secondary (acquired):**
- HIV
- Immunosuppressive therapy

T cell presenting Ag to B cell



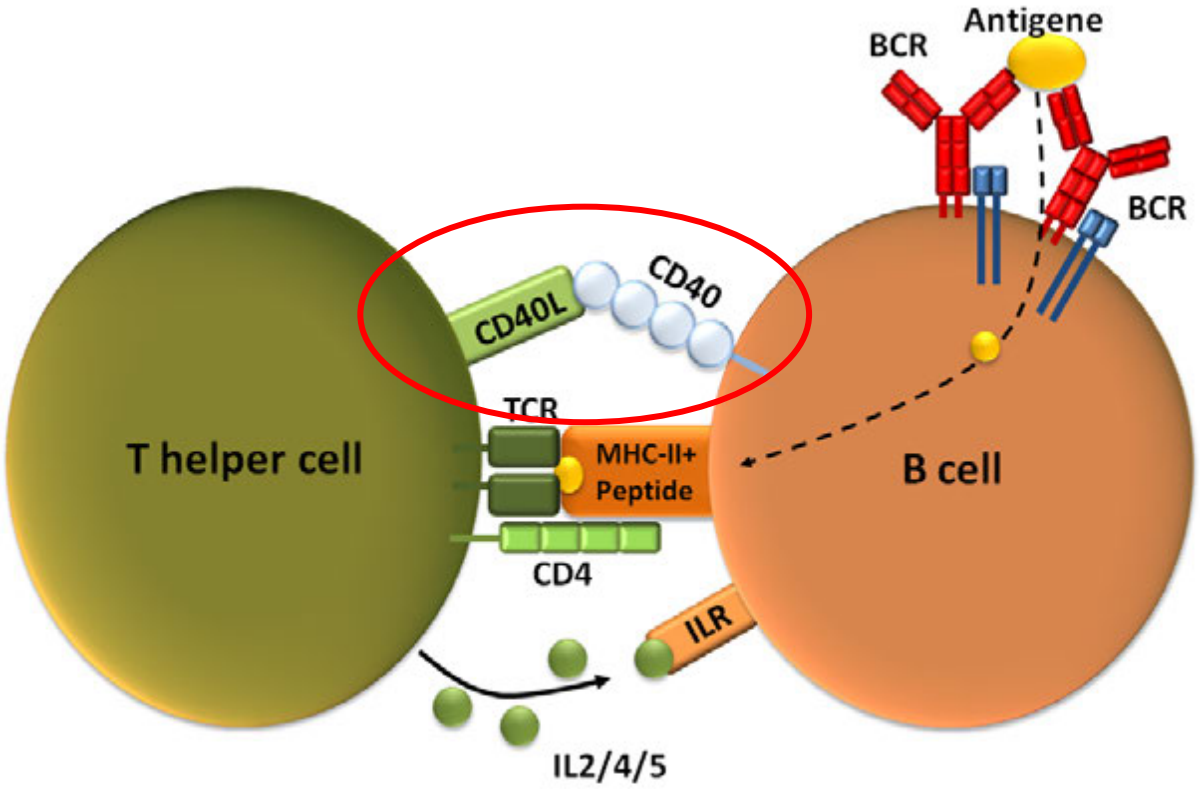
SCID

- only curative treatment: BMT/genetic/enzyme therapy
- receive Ig supplementation (sometimes in higher doses and shorter intervals) while waiting for curative treatment.

Ab def. - etiology

- **Primary (genetic):**
- **B cells** (XL or AR agammaglobulinemia, common variable immunodeficiency - CVID, activated PI3 kinase delta syndrome - APDS, isolated IgA and IgM deficiency).
- **T cells** (severe combined immunodeficiency syndrome - SCID)
- **Communication** between T and B cells (class switch defects)
- **Secondary (acquired):**
- HIV
- Immunosuppressive therapy

T cell dependant B cell activation



Class switch defects

- IgG supplementation for antibody deficiency
- also require antibiotic prophylaxis for Pneumocystis
- Early BMT is treatment of choice (reduced morbidity and mortality)

Ab def. - etiology

- **Primary (genetic):**
- **B cells** (XL or AR agammaglobulinemia, common variable immunodeficiency - CVID, activated PI3 kinase delta syndrome - APDS, isolated IgA and IgM deficiency).
- **T cells** (severe combined immunodeficiency syndrome - SCID)
- **Communication** between T and B cells (class switch defects)
- **Secondary (acquired):**
- HIV
- Immunosuppressive therapy

Immunosuppressive treatment

- regular evaluation of immune system function

=> Ig supplementation if reduced concentration of IgG

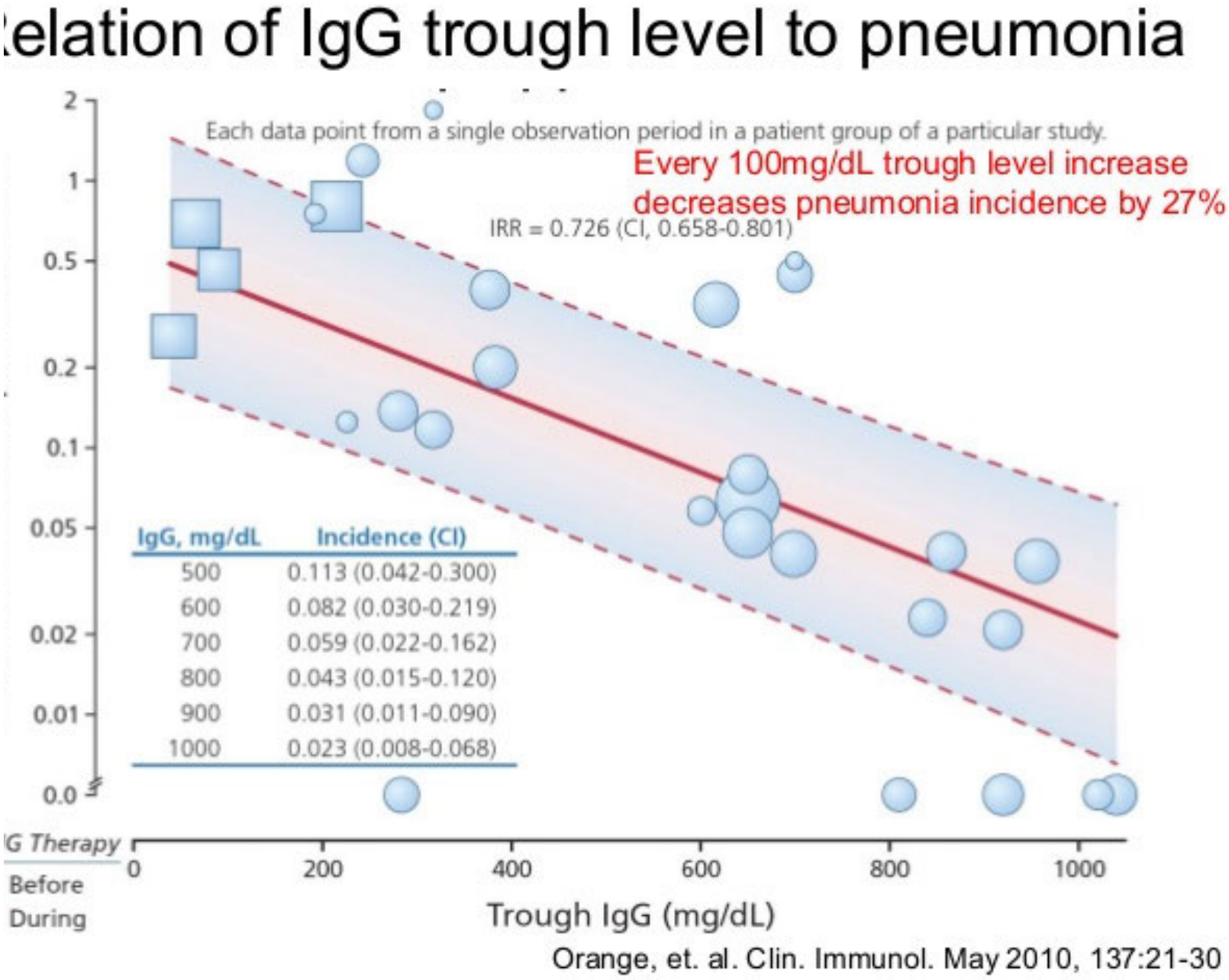
Severe suppression of T cell number or function

$\Rightarrow$ reduced ability to develop specific antibody response

$\Rightarrow$ Ig supplementation should be considered

IgG trough level

- frequency of infections decreases with increasing IgG trough level
- the recommended IgG trough level is above 8 g/L (above 10 g/L for patients with bronchiectasis).



IVIg	SCig
• Generally given once every three to four weeks	• Given biweekly, weekly or more frequently
• Achieves an initial high concentration of IgG, which decreases gradually until the next infusion	• No peak in serum IgG level once steady state is achieved, the IgG level varies little
• Requires IV access and a health care professional to establish access and monitor the infusion	• Does not require IV access and can be self-administered, but still does require one or more needle sticks
• Requires a health care professional to establish access and monitor infusion	• Requires a committed, compliant patient and./or caregiver for administration
• Generally well tolerated by most people, but... • Intrainfusion adverse effects are possible including chills, rigors, nausea, subjective sense of dis-ease, backache • Post infusion adverse effects can include headache, malaise, fatigue	• Systemic side effects are rare, but local reactions including redness, swelling and itching are frequent, but tend to decrease with each infusion
• Pre-medication with acetaminophen, NSAID's, diphenhydramine, and/or short acting corticosteroids may be required to prevent adverse effects.	• As reactions are local, there is seldom a need for systemic pre-medication
• Cost for drug and infusion center/nursing	• Cost for drug and supplies (and nursing only until independent)

Possible issues with IgG supplementation

- **Immune reaction to IgA present**
- Common in IgA def.
- Mediated by IgG anti-IgA and complement
- Usually tolerate s.c. supplementation
- **Allergic reaction to glue/disinfectant**
- Most common complication
- **Incresed loss of IgG**
- Especially proteinuria and protein loosing enteropathy
- **Need for fridge**
- Problems with vacation and planes

TREATMENT OF PATIENTS WITH CHRONIC GRANULOMATOUS DISEASE

Gašper Markelj

Department of Allergology, Rheumatology and Clinical Immunology, Children's Hospital, University Medical Center Ljubljana, Slovenia

Chronic granulomatous disease (CGD) has evolved from a disease with early fatality since its first recognition 60 years ago to the disease with effective management and high survival. CGD is caused by an inherited defect of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex present in phagocyte (neutrophil, monocyte, macrophage, eosinophil) but also in a variety of other cells. Mutation in any of the five proteins composing the NADPH complex leads to defect of generation of reactive oxygen species (ROS) which has different implications. Cells with defective NADPH oxidase are unable to kill microorganisms in phagocytic vacuoles. Defective neutrophils are impaired in mechanisms of extracellular killing through NET (neutrophil extracellular trap) formations. Both mechanisms are responsible for one of the hallmarks of the disease – *deficient antimicrobial defence.* Inefficient ROS formation has also a great role in uncontrolled inflammation in various organs, which is a second disease manifestation – *excessive inflammation.* Introduction of antibacterial prophylaxis with trimetoprim/sulfamethoxazole and antifungal prophylaxis with itraconasole together with early and aggressive treatment of infections has greatly diminished the rate of severe infections and increased survival. Additional judicious use of antiinflammatory drugs has increased the quality of life and decreased the inflammation complications. Nevertheless, bone marrow transplantation (BMT) with various conditioning regiments has in recent years been the successful standard curative treatment in children and in adults. Gene therapy in some patients that were unable to undertake BMT has shown transitory beneficial effects with important safety issues. A new generation self-inactivating (SIN) vector is now being used in selected cases of gene therapy in CGD patients. In addition, laboratory results with gene-editing techniques are also showing the potential for further clinical applications in near future.

Treatment of patients with chronic granulomatous disease (CGD)

Gašper Markelj
Pediatična klinika, UKC Ljubljana, Slovenija

Flow cytometry in research and diagnostics of Primary Immunodeficiency Disorders

14.10.2016, MF, Ljubljana

1. Chronic granulomatous disease (CGD)

2. Treatment (past, present and future)

Chronic granulomatous disease

- Rare inherited disorder of the innate immune system
- prevalence 1/200000-250000
- 85% male patients
- In majority presents in early childhood

History

- 1954 – Janeway and al. – description in first 5 children
- 1957 and 1959 additional descriptions – Fatal granulomatous disease of the childhood

- 1967 Quie and al. - defect in bactericidal activity of phagocytes

- At the end of 60s – Chronic granulomatous disease

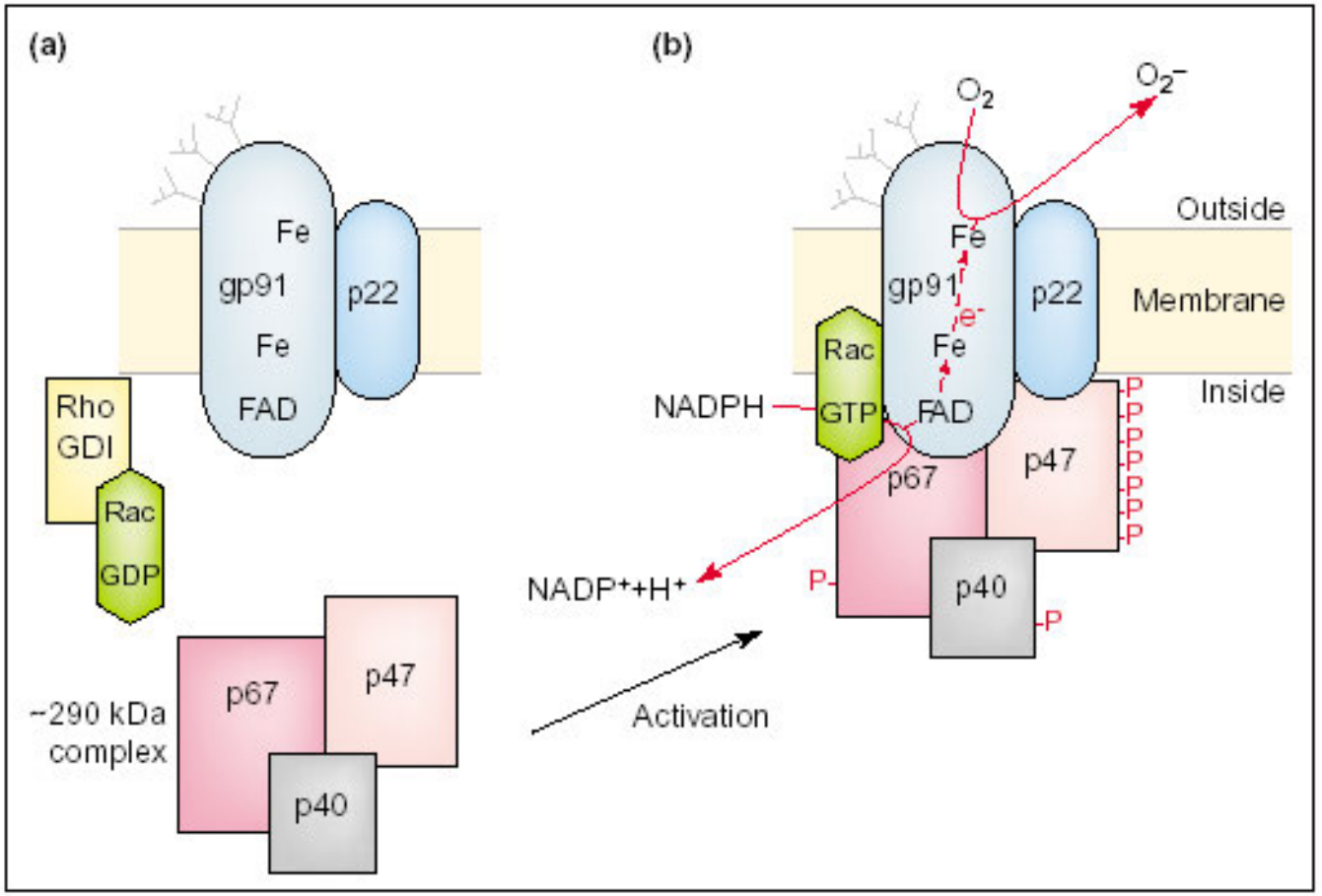
- In next 25 years detailed description of mechanisms of the disease and NADPH Enzyme complex (1987 –NADPH oxidase)

- Specific defect of phagocytes
  - nicotinamide adenine dinucleotidephosphate-(NADPH) oxidase
  - Defective oxidative burst – defective superoxide generation

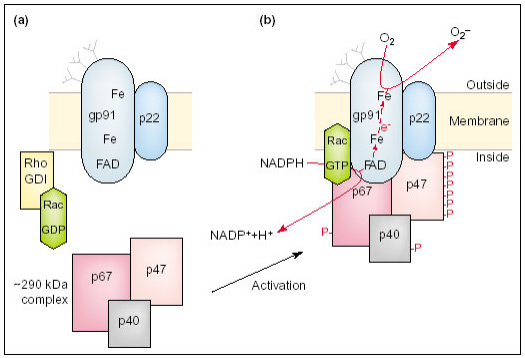
1. ***Deficient Antimicrobial Defense***

2. ***Excessive Inflammation***

Pathogenesis



Genetics



Affected Component	Responsible Gene	Subtype Designation	NBT (%Positive)	O2 production (% Normal)	Cytochrome b (% Normal)	Frequency (%of Cases)
gp91^{phox}	CYBB / Xp21.1	X91^0	0	0	0	70
		X91$^-$	0-Weak	Low	Low	
		X91$^+$	0-Weak	0	100	
p22^{phox}	CYBA / 16q24	A22^0	0	0	0	<5
		A22$^+$	0	0	100	
p47^{phox}	NCF1 / 7q11.23	A47	0	0-1	100	25
p67^{phox}	NCF2 / 1q25	A67	0	0-1	100	<5

Few patients with AR mutation in p40^{phox}

Clinical manifestation

1. ROS (H2O2, HClO) with microbicidial activity

2. Activation of microbicidal granule proteases (elastaza, cathepsin G)

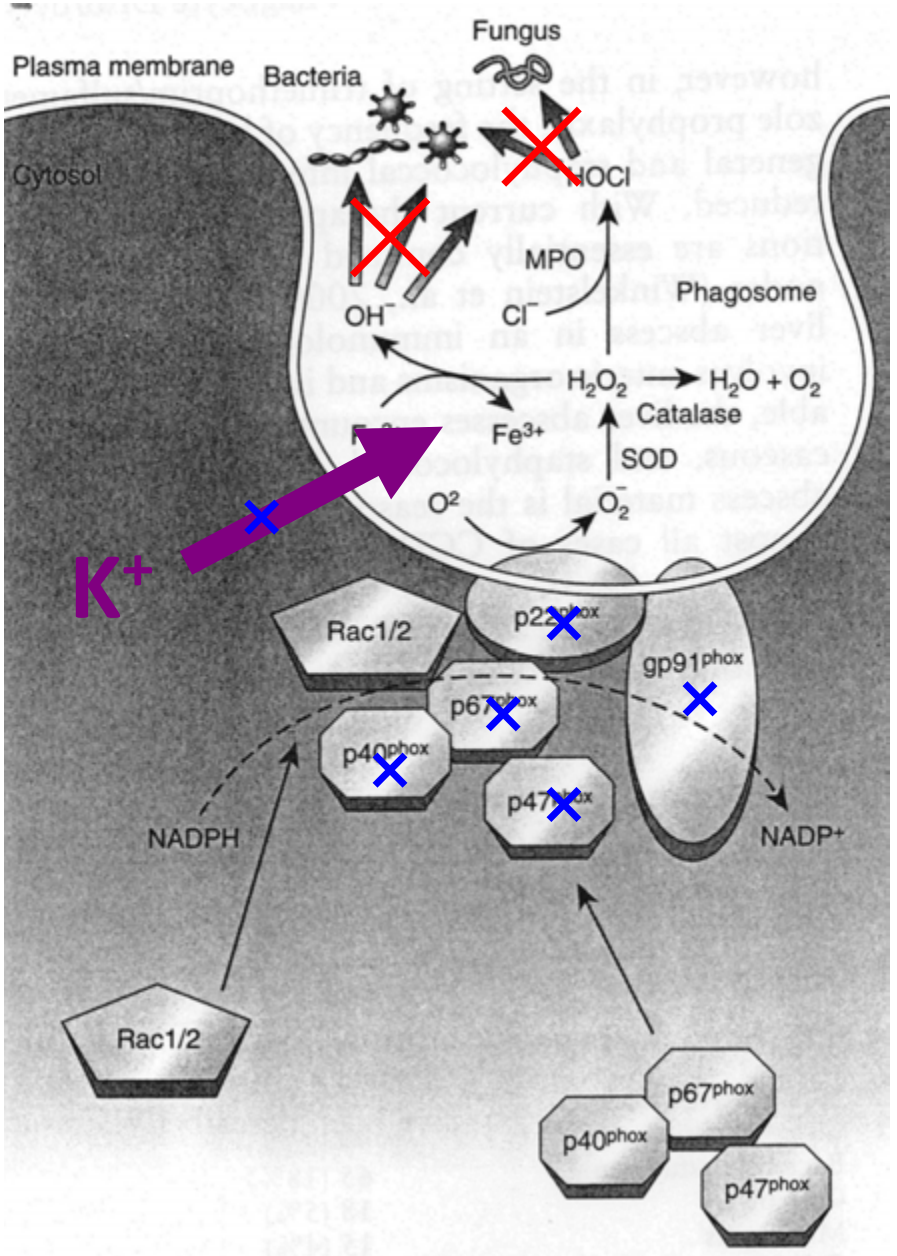
3. Neutrophil extracellular traps (NETs)

Deficient Antimicrobial Defense

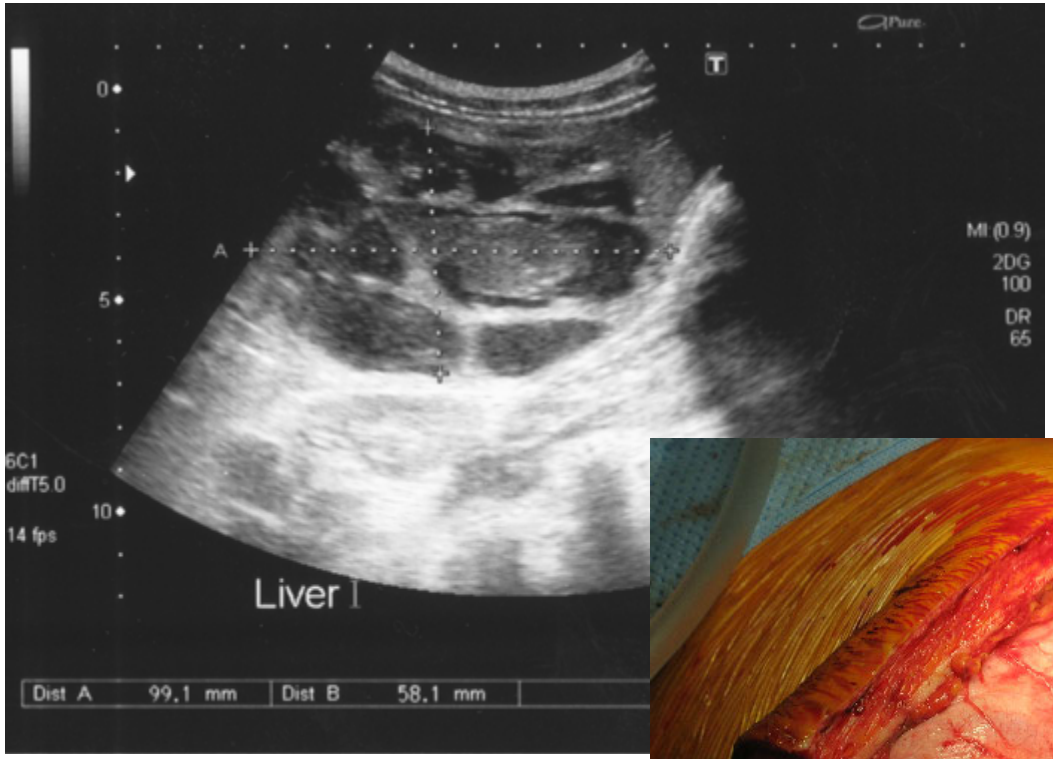
- Repetitive, life-thretening bacterial and fungal infections (catalase +)

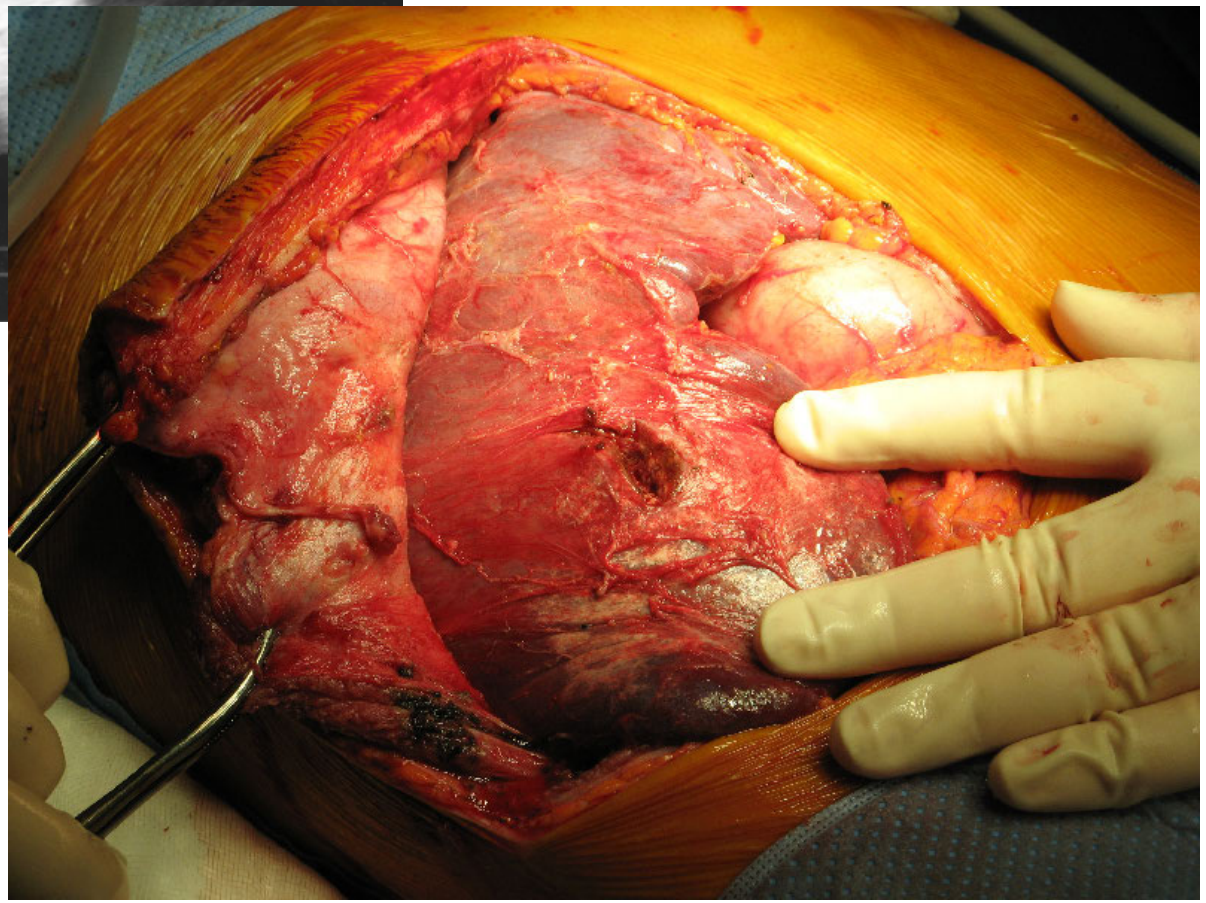
  - *Staphylococcus aureus,*
  - *Burkholderia.(Pseudomonas) cepacia,*
  - *Serratia sp.,*
  - *Nocardia sp.,*
  - *Aspergillus sp.*

  - Salmonella sp.
  - BCG
  - Mycobacteria



Liver abscess in patient with CGD





Clinical picture

Type of Infection	Total (n=368) No. (%)*
Pneumonia	290 (79%)
Abscess (any kind)	250 (68%)
Subcutaneous	156 (42%)
Liver	98 (27%)
Lung	60 (16%)
Perirectal	57 (15%)
Brain	12 (3%)
Other‡	28 (8%)
Suppurative adenitis	194 (53%)
Osteomyelitis	90 (25%)
Bacteremia/fungemia	65 (18%)
Cellulitis	18 (5%)
Meningitis	15 (4%)
Other¶	112 (30%)

Modified from JA Winkelstein et al. *Chronic Granulomatous Disease. Report on a national registry of 368 patients. Medicine 2000*

Merljin van den Berg et al. Chronic granulomatous disease: The European Experience . PLoS ONE 2009

Site of Disease	Number of episodes	Number of patients with ≥1 episode	% of patients with ≥1 episode
Lung	634	284	66%
Skin/ Subcutis	341	229	53%
Lymph node	622	213	50%
Gastro-intestinal	643	208	48%
Liver	240	138	32%
Kidney/ Urinary tract	139	95	22%
Septicaemia	111	85	20%
Ear	84	62	14%
Bone	84	56	13%
Eye	68	46	11%
Joint	35	31	7%
Brain	34	31	7%
Autoimmunity-Rheumatology	26	26	6%

(429 patients)

in 940 y F/u patients in ECE cohort have suffered from 834 different infections (0,9 per year)

	No. of infections	No. of affected patients
RT infections	200 (24%)	94/116 (81%)
Lymphadenitis	183 (22%)	93/116 (80%)
GIT infections with abscesses	150 (18%)	78/116 (67%)
Skin infection with abscesses	117 (14%)	64/116 (55%)
Sepsis	50 (6%)	31/116 (27%)
Osteomyelitis	25 (3%)	21/116 (18%)
other	92 (11%)	

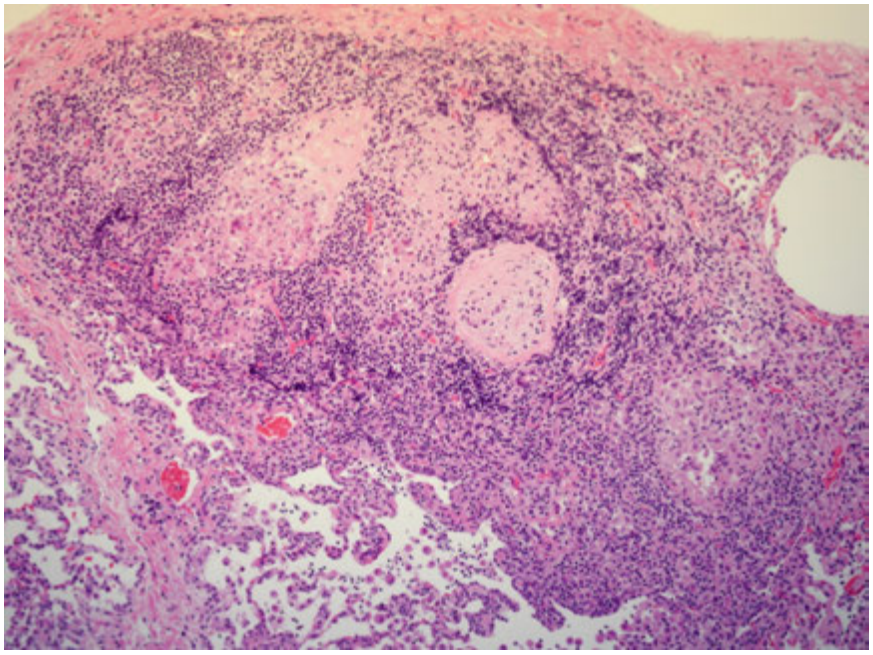
135 pts.

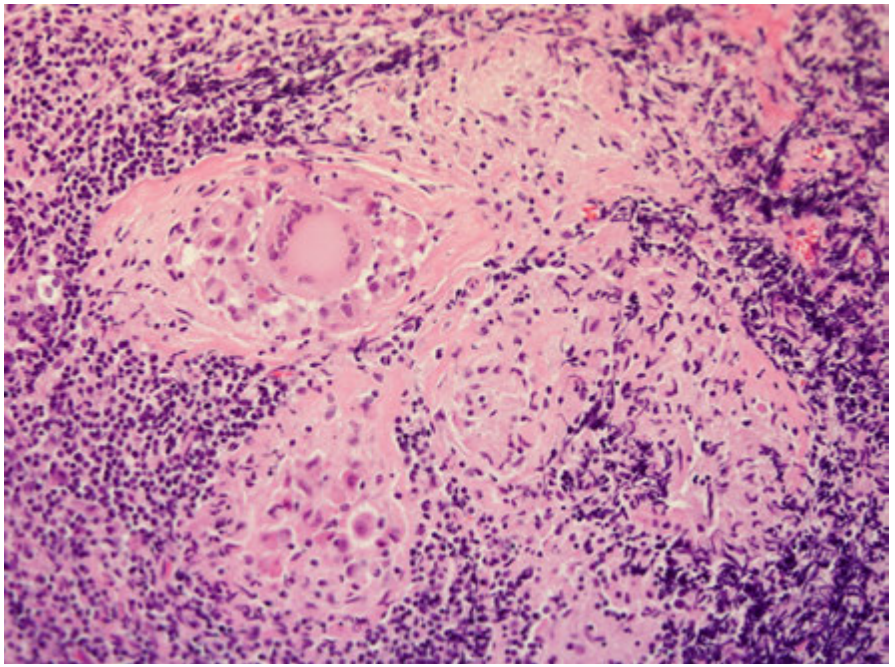
Slovenia - **15** patients

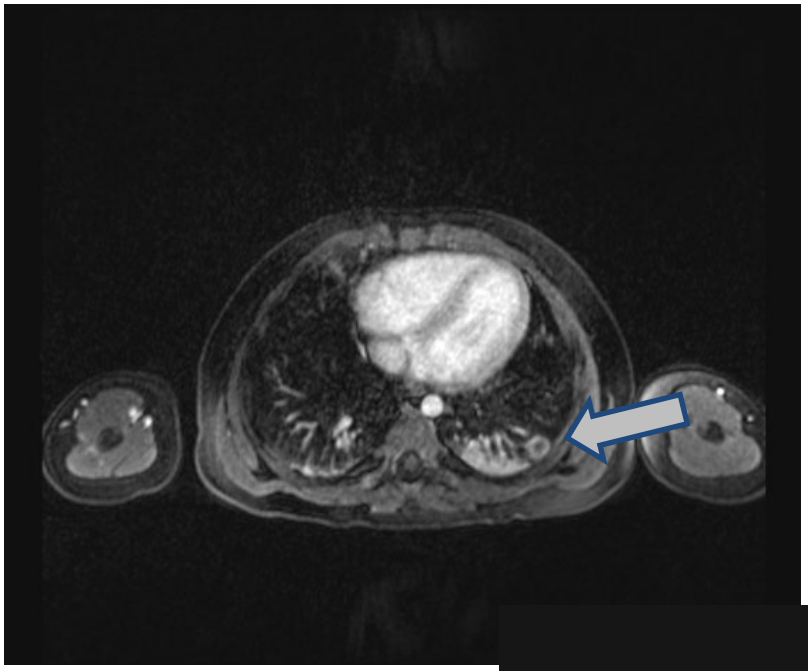
Incidence in last 35 years: **~ 1/61 000**
(916.000 LB)

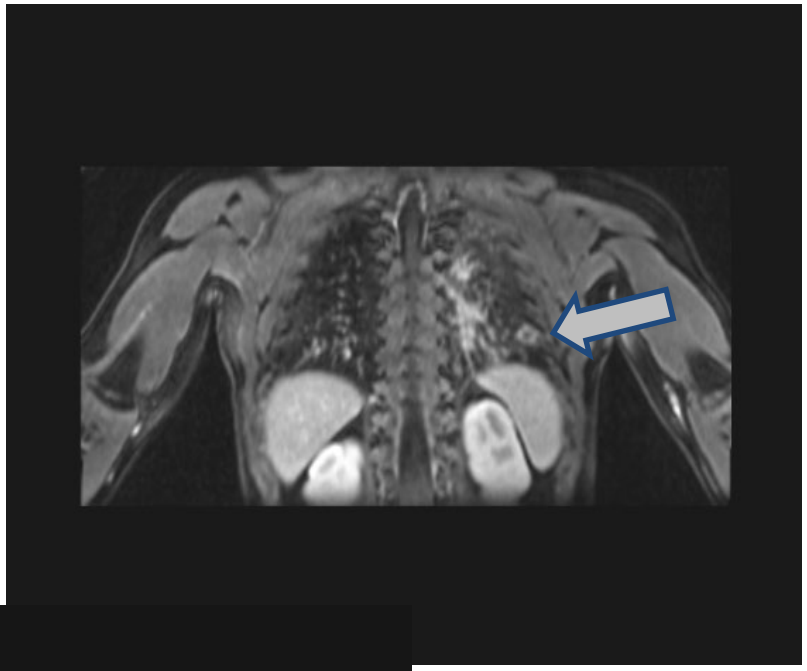
Excessive Inflammation

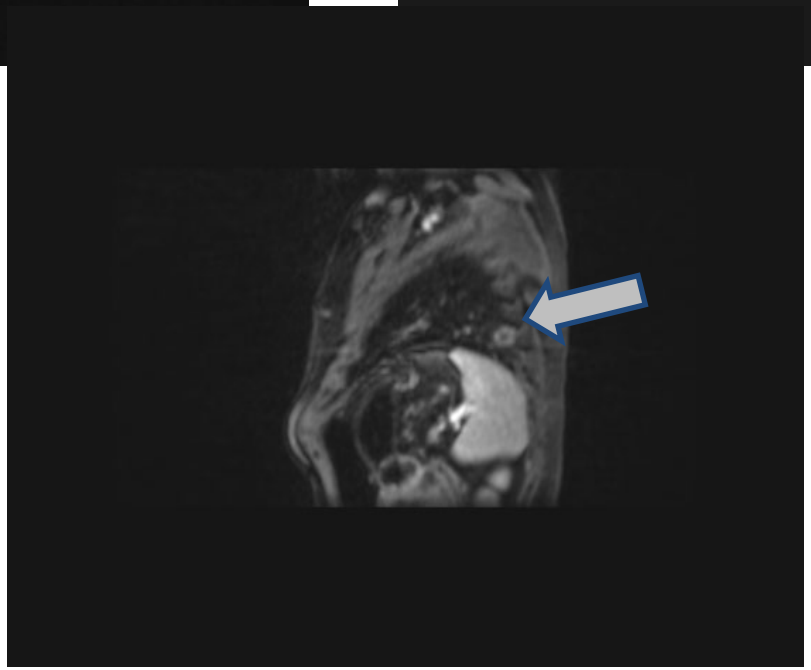
- NADPH oxidase is critical for downregulation of inflammation
  - *ROS-sensitive antiinflammatory transcription factor, Nrf2*
  - Defect in *macrophage mediated clearance of activated and infected neutrophils*.
- Uncontroled inflamation, granulome formation
- GIT and urinary tract











Noninfective complications	No. of affected patients
Lymphadenopathy	42/59 (71%)
Chronic lung disease	22/59 (37%)
Hepatosplenomegaly	34/59 (58%)
Colitis	21/59 (36%)
Low height	20/59 (34%)
Anemia	24/59 (41%)
Hypergamaglobulinemia	22/59 (37%)
Liver granuloma	8/59 (14%)
other	7/59 (12%)

Treatment

Prophylactic treatment

- **Antibacterial : TMP/SMX**
- **Antifungal: itraconazol**

- **+- γIFN**

Agressive treatment of infections

Antiinflammatory treatment

- **Steroids**
- **Antimetabolics (imuran)**

Stem cell transplantation

ACTA PÆDIATRICA
NURTURING THE CHILD

Acta Paediatrica ISSN 0803-5253

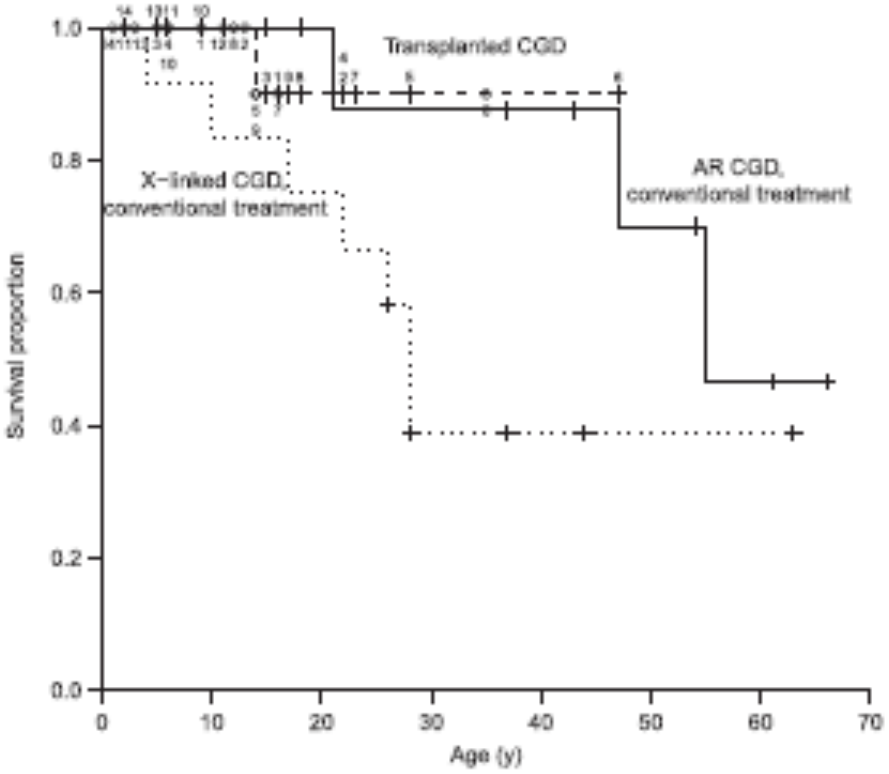
REGULAR ARTICLE

Chronic granulomatous disease – haematopoietic stem cell transplantation versus conventional treatment

Anders Åhlin (anders.ahlin@sodersjukhuset.se)[1], Jakob Fugeläng[1], Martin de Boer[2], Olle Ringden[3], Anders Fasth[4], Jacek Winiarski[5]

©2013 Foundation Acta Paediatrica. Published by John Wiley & Sons Ltd 2013 **102**, pp. 1087–1094

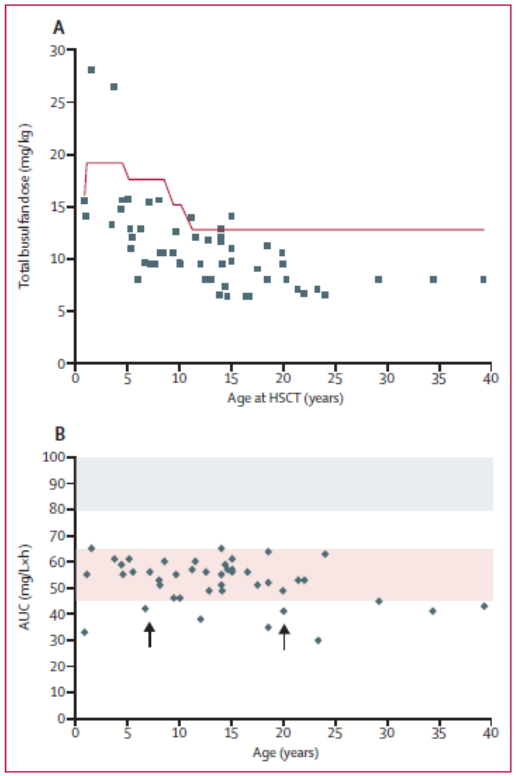
Methods: Forty-one patients in Sweden were diagnosed with CGD between 1990 and 2012. From 1997 to 2012, 14 patients with CGD, aged 1–35 years, underwent HSCT and received grafts either from an HLA-matched sibling donor or a matched unrelated donor.

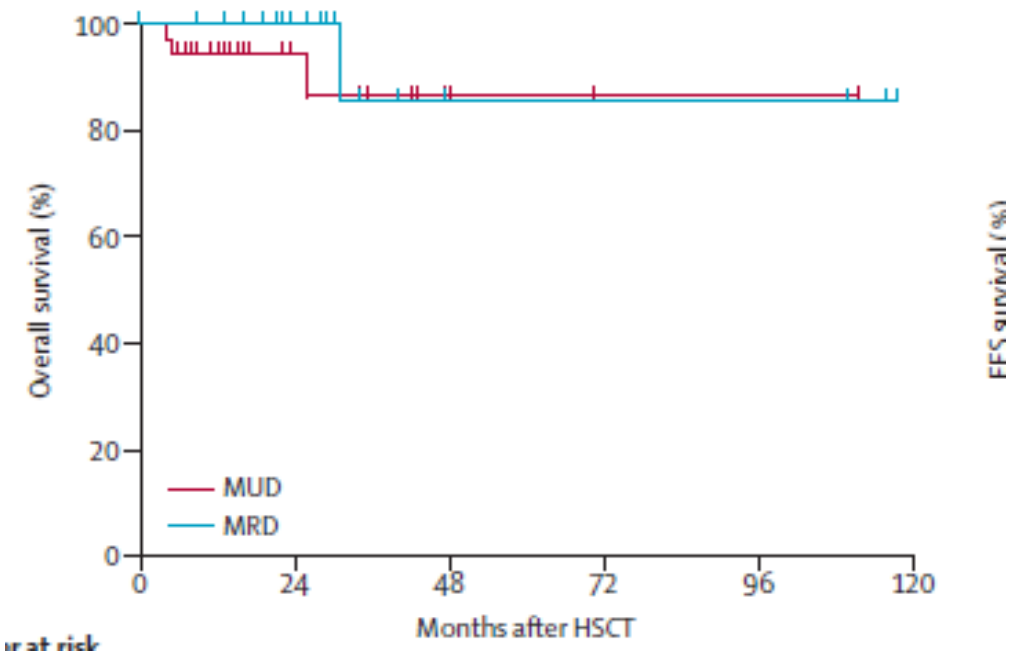


Reduced-intensity conditioning and HLA-matched haemopoietic stem-cell transplantation in patients with chronic granulomatous disease: a prospective multicentre study

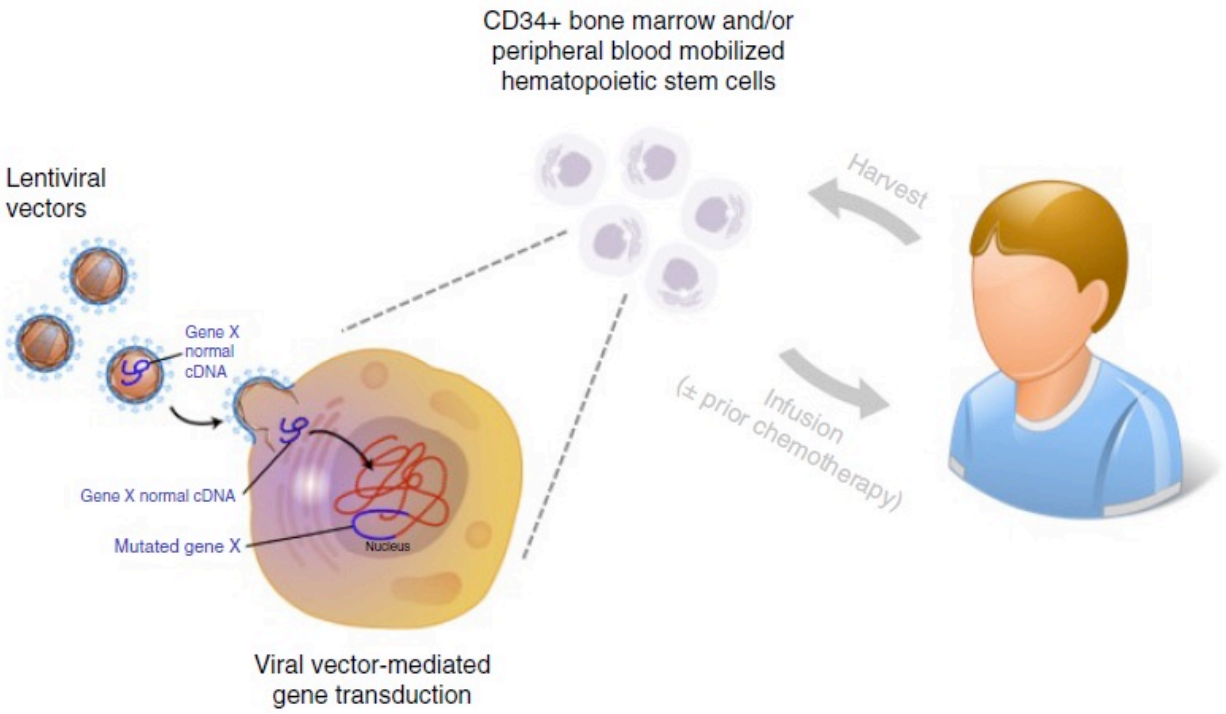
Lancet 2014; 383: 436–48

Tayfun Güngör, Pierre Teira, Mary Slatter, Georg Stussi, Polina Stepensky, Despina Moshous, Clementien Vermont, Imran Ahmad, Peter J Shaw, José Marcos Telles da Cunha, Paul G Schlegel, Rachel Hough, Anders Fasth, Karim Kentouche, Bernd Gruhn, Juliana F Fernandes, Silvy Lachance, Robbert Bredius, Igor B Resnick, Bernd H Belohradsky, Andrew Gennery, Alain Fischer, H Bobby Gaspar, Urs Schanz, Reinhard Seger, Katharina Rentsch, Paul Veys, Elie Haddad, Michael H Albert, Moustapha Hassan*, on behalf of the Inborn Errors Working Party of the European Society for Blood and Marrow Transplantation†*





Gene Therapy

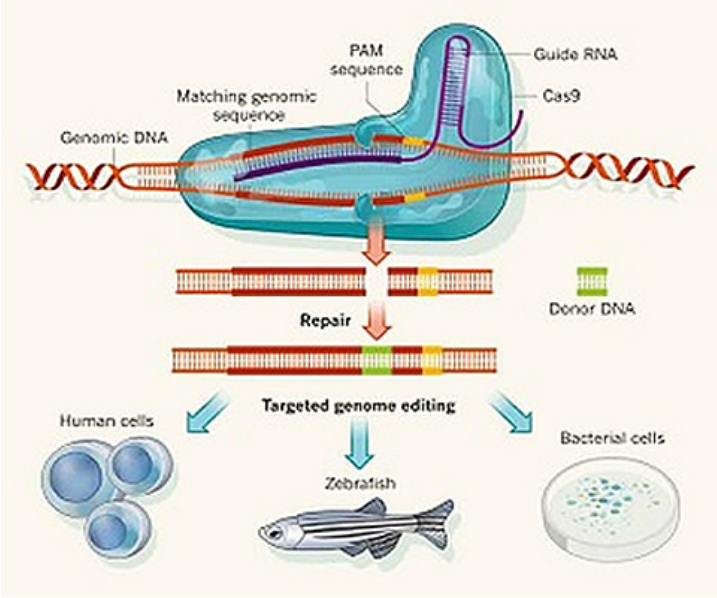


First generation vectors: - transient improvement

- Gene silencing
- Activation of oncogenes – clonal expansion in some patients

Second generation vectors - NET4CGD

Target Genom Editing



ELSEVIER

Experimental Hematology 2015;43:838–848

CRISPR-mediated genotypic and phenotypic correction of a chronic granulomatous disease mutation in human iPS cells

Rowan Flynn[a,b], Alexander Grundmann[b], Peter Renz[b], Walther Hänseler[a,b], William S. James[b], Sally A. Cowley[a,b], and Michael D. Moore[b]

[a]*James Martin Stem Cell Facility, Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom;*
[b]*Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom*

TALEN-mediated functional correction of X-linked chronic granulomatous disease in patient-derived induced pluripotent stem cells

Anne-Kathrin Dreyer [a, 1], Dirk Hoffmann [a], Nico Lachmann [a, b, c], Mania Ackermann [a, b], Doris Steinemann [d], Barbara Timm [e, f], Ulrich Siler [g], Janine Reichenbach [g, h], Manuel Grez [i], Thomas Moritz [a, b], Axel Schambach [a, j, **], Toni Cathomen [a, e, f, *]

ESID6-0342
PARALLEL SESSION IX: NEXT GENERATION STEM CELL THERAPY

CRISPR-MEDIATED SEAMLESS REPAIR OF CYBB MUTATION RESTORES GRANULOCYTE FUNCTION IN X-LINKED CHRONIC GRANULOMATOUS DISEASE HEMATOPOIETIC STEM CELLS

S.S. de Ravin[1], L. Li[2], C. Allen[3], C. Uimook[4], S. Koontz[5], N. Theobald[6], J. Lee[6], A. Viley[7], P. Natarajan[8], X. Wu[9], M. Peshwa[8], H. Malech[10]

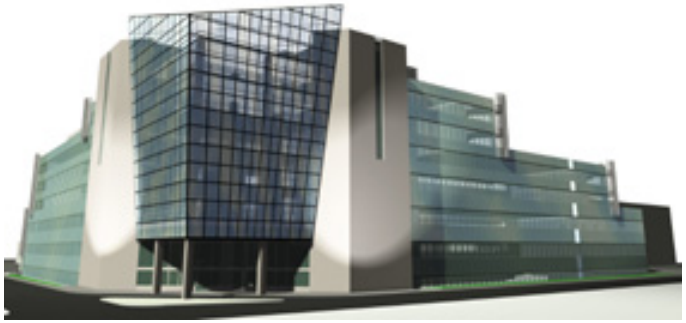
Hvala!

DIAGNOSIS OF PERIODIC FEVER SYNDROMES

Nataša Toplak

Department of Allergology, Rheumatology and clinical Immunology, University Children's Hospital, University Medical Centre Ljubljana, Slovenia and Medical Faculty Ljubljana, University of Ljubljana, Slovenia

Periodic fever syndromes comprise a subset of autoinflammatory diseases, which are a group of rare, genetically heterogeneous diseases. In these diseases no pathogen, autoantibodies or antigen specific T cells can be found. The basic mechanism is abnormal activation of innate immune system. Patients suffer from recurrent attacks of febrile episodes accompanied by involvement of inflammation in several organs. Most commonly, skin, musculoskeletal system, gastrointestinal tract and central nervous system are affected. Approach to a child with periodic fever presents a diagnostic challenge. If a patient had at least 3 febrile episodes in 6 month period, at least 7 days apart, we have to consider periodic fever syndrome after exclusion of infection, autoimmune disease or malignancy. For monogenic autoinflammatory syndromes genetic testing is needed to confirm the diagnosis. Some of these diseases are inherited recessively and others dominantly. The delay in diagnosis may lead to severe complications and end organ damage due to AA-amiloidosis. Recent advances in genetic diagnostic, mainly new technologies such as next generation sequencing (NGS) and whole exome sequencing (WES) led to remarkable progress in the identification of disease- associated genes. Newly discovered mutations brought up a new dimension on genotype-phenotype relationship. Mutations in the same gene can cause a range of phenotypes with a common inflammatory component. This suggests the influence of modifying allels and environmental factors on clinical presentation of disease. Among several new diseases which were recently described is deficiency of adenosine deaminase 2 (DADA 2). These patients have early onset systemic inflammation with recurring stroke and vasculopathy or necrotizing vasculitis polyarthritis nodosa.



Diagnosis of periodic fever syndromes

assoc.prof. Nataša Toplak, MD, PhD

Department of Allergology, Rheumatology and clinical Immunology, University Children's Hospital Ljubljana, Medical faculty, Slovenia

Slovenian Society for Flow Cytometry (SSC) meeting

14th October 2016, Ljubljana

Outline

- **Case presentation-** history, clinical picture, diagnostic approach
- **Background**- Autoinflammation & Autoinflammatory diseases, definition, classification
- **Periodic fever syndromes** / *clinical & genetic aspects & th.*
  - Sporadic conditions ***1. PFAPA***
  - Hereditary syndromes
    2. FMF
    3. MKD / HIDS
    4. TRAPS
    5. CAPS
- ***Case presnetation-*** *diagnosis and tretment*
- ***What is new in the world of autoinflammation?***

Case presentation

Male patient, born on 25th December, 2008

- Ethnicity- Caucasian, Slovene
- FH- neg

- Pregnancy uneventful, gestation age 37 weeks
- Meconial amniotic fluid
- Hypoglycemia
- Neonatal sepsis suspected, not proven, antibacterial treatment

- **Urticarial rash- appeared soon after birth**

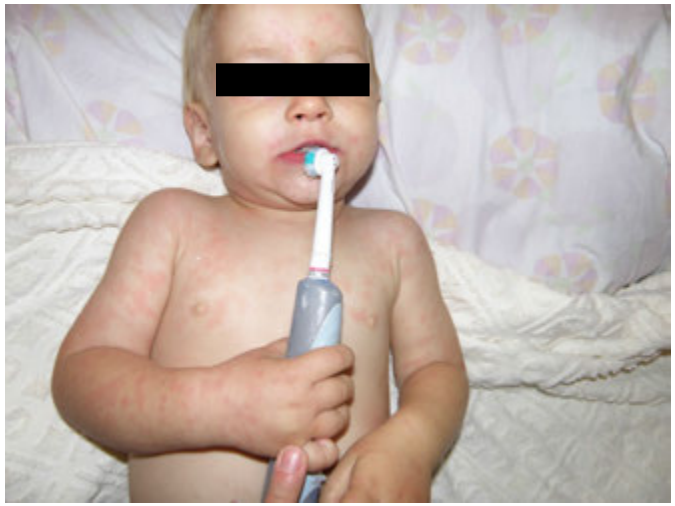
- He had the first episode of **fever with elevated CRP (50)** and no signs of an infection at the age of six months, **fever lasted for four days (no infection was proven)**

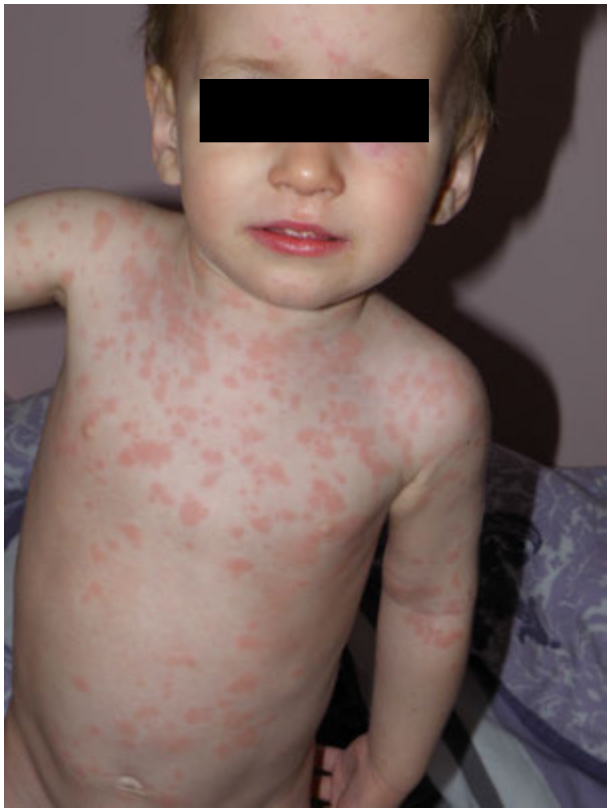
- First episode of **limping at the age of 16 months, arthritis of a hip disappeared in 24 hours**

Case presentation

Urticarial rash-

appeared soon after birth, was never itchy, was more intense during fever, but didn't disappeared between fever attacks





Case presentation

MAIN PROBLEMS

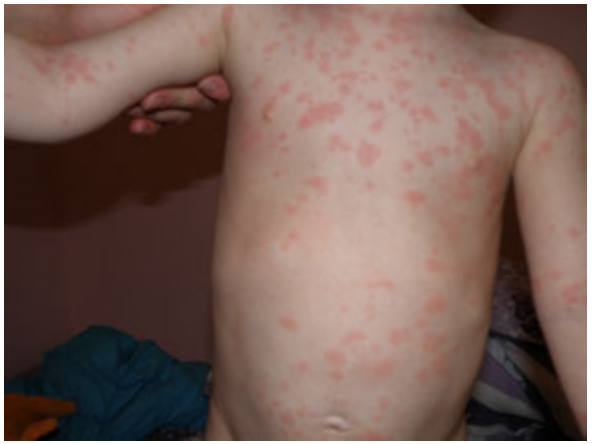
- **periodic fevers** from 6 month of age, **short attacks** 1-2 days, sometimes up to 5 days, every 7-30 days
- **elevated inflammatory parameters** (CRP up to 200), between attacks inflammatory parameters also elevated- CRP from birth never below 40

- **Arthritis** from 16 month of age - last 1-2 days, sometimes red joint as in FMF, fluid in hip joints, knees, ankle- not all of them in every attack

- **Rash-** looks like urticarial, sometimes more macular; rarely disappears completely; in milder form between attacks present all the time; mastocyts found in skin biopsy;

systemic mastocitosis excluded

by bone marrow biopsy



Case presentation/ Diagnostic approach

- Exclusion of infections
- Exclusion of PID

- Exclusion of autoimmune diseases autoantibodies negative: ANA negative, ANCA negative, antiC1 Q- negative
- Exclusion of allergy milk, egg,triptase normal on several occasions
- Exclusion of malignant disease bone marrow biopsy

- Metabolic disease?

Organic acid in urine

2011 - negative 4 times in febrile attacks, **no mevalonic acid**

- Complemet levels- classic, alternative activation normal
- IgG, IgM, IgA normal

IgD 324E/ml (normal up to 90)

- Flow cytometry- normal

- Exclusion of chronic granulomatosis
- Titre of anti-diphteria antibodies 0,292 IU/ml (protected)
- Titre of anti-tetanus antibodies 0,350 IU/ml (protected)

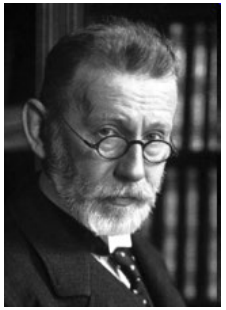
s-SAA ELISA 480 mg/L (N<6,4)

Autoinflammation

McDermott et al.,1999

- "Horror Autotoxicus"

  Ehrlich P. Studies in immunity. London: Wiley; 1910.



- The concept of autoinflammatory diseases- 1999

- ➢ no autoantibodies
- ➢ no antigen specific T-cells
- ➢ predominance of monocytes and neutrophils as EFFECTOR cells/ rather than lymphocites

EXCLUDE INFECTION, AUTOIMMUNE DISEASE AND MALIGNANCY

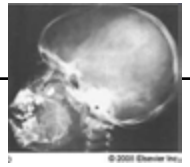
Think about PID…

Horror Autoinflammaticus:

The Molecular Pathophysiology of Autoinflammatory Disease

Seth L. Masters1, **Anna Simon**2, **Ivona Aksentijevich**1, and **Daniel L. Kastner**1

Annu Rev Immunol. 2009

	Provisional molecular/ functional classification of AID	Disease
1	**IL-1β activation disorders (inflammasomopathies)**	Intrinsic: **FCAS, MWS, CINCA / NOMID** Extrinsic: **FMF**, PAPA, CRMO / SAPHO, Majeed sy, **HIDS, DIRA**, recurrent hydatiform mole Complex /acquired: gout, DM II, Schnitzler sy., fibrosing disorders
2	**NF-B activation disoreders**	Crohn's disease, Blau sy, **FCAS2- Guadalupe periodic fever- NALP 12**
3	**Protein folding disorders of the innate immune system**	**TRAPS**, spodyloarthropathies
4	**Complement disorders**	aHUS, AMD- age related macular deg.
5	**Cytokine signaling disorders**	Cherubism 
6	**Macrophage activation**	fHLH, Chediak-Higashi sy.,Griscelli sy., X-linked lymphoproliferative sy., Hermansky-Pudlak sy., secHLH, atherosclerosis

PID classification

frontiers in IMMUNOLOGY

CLASSIFICATION ARTICLE
published: 22 April 2014
doi: 10.3389/fimmu.2014.00162

Primary immunodeficiency diseases: an update on the classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency

Waleed Al-Herz[1,2], Aziz Bousfiha[3], Jean-Laurent Casanova[4,5], Talal Chatila[6], Mary Ellen Conley[4], Charlotte Cunningham-Rundles[7], Amos Etzioni[8], Jose Luis Franco[9], H. Bobby Gaspar[10], Steven M. Holland[11], Christoph Klein[12], Shigeaki Nonoyama[13], Hans D. Ochs[14], Erik Oksenhendler[15,16], Capucine Picard[5,17], Jennifer M. Puck[18], Kate Sullivan[19] and Mimi L. K. Tang[20,21,22]*

CATEGORIES- major groups of PID
1. Combined ID- SCID
2. Combines ID with associated syndromic features
3. Predominantly ab deficiencies
4. Diseases of immune dysregulation / type 1 interpheronopathies
5. Congenital defects of phagocyte number, function or both
6. Defects in innate immunity
7. Autoinflammatory disoreders
8. Complement deficiencies
9. Phenocopies of PID (acquired defects)

Aksentijevich, 2015

Masters SL, et al. Annu Rev Immunol. 2009

NLRP3 inflammasom

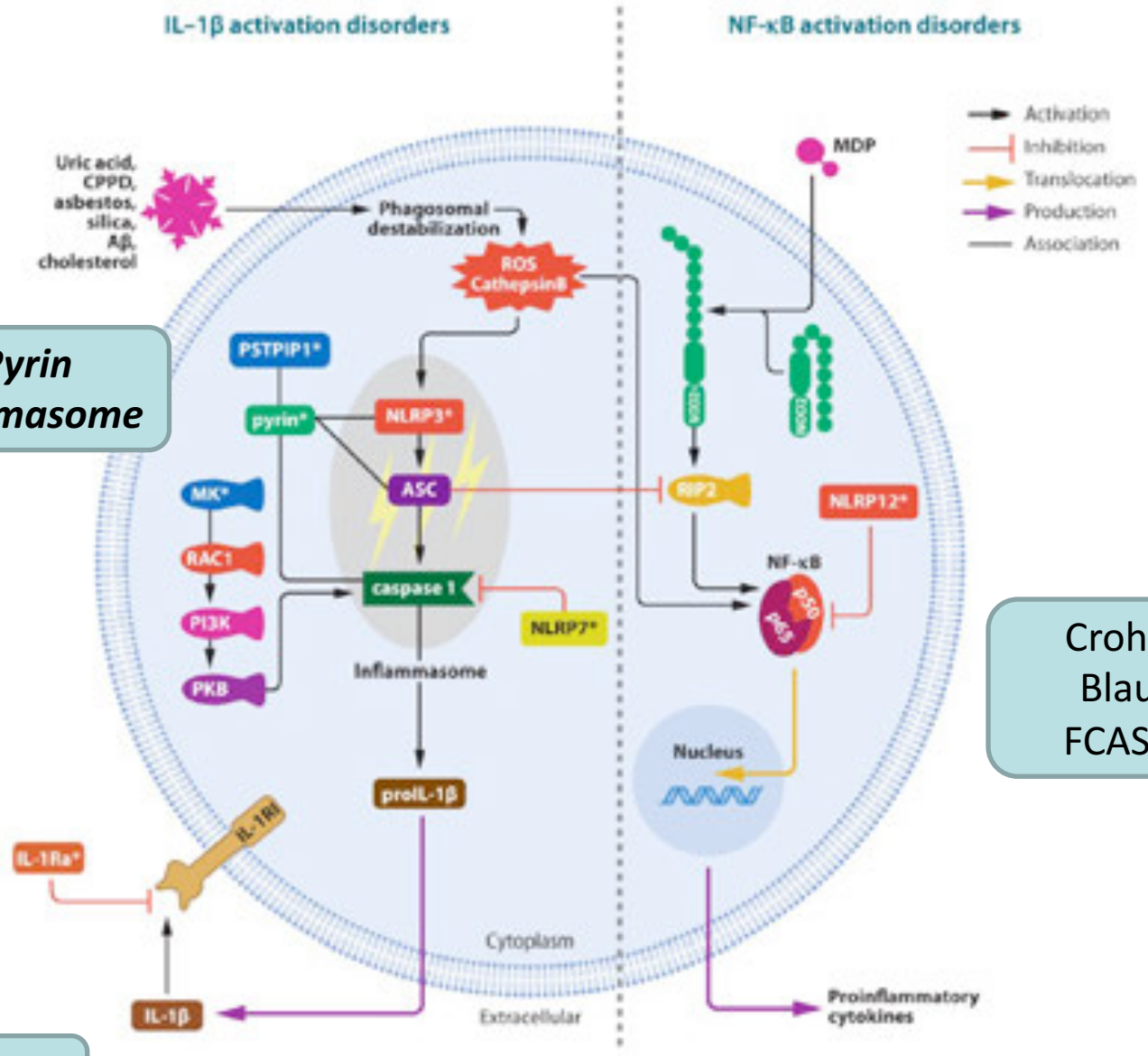
Park JH, et al. Nat Immunol 2016

Pyrin inflamasome

CAPS
FMF
HIDS / MKD

Crohn
Blau
FCAS2

New inflammasomes-
NLRP1, NLRC4, pyrin, AIM 2..



Periodic Fever Syndromes

Sporadic conditions- PFAPA
Hereditary conditions: FMF, MKD/HIDS, TRAPS, CAPS

- Diverse group of diseases- ***3 or more episodes of inflammation in 6 month period, at least 7 days apart, no other cause*** John CC et al. Pediatr Infect 2002

- Recurrent episodes of fever, elevated inflammatory parameters and localized inflammation affecting:

  serosal membranes, joints, skin, eyes, gut

- No signs of inflammation between attacks

Padeh S. Peditr Clin North Am 2005

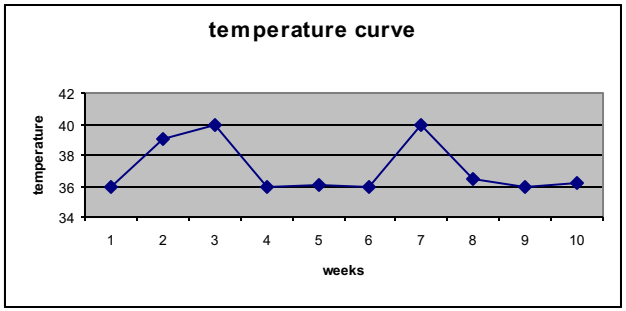
Periodic Fever Syndromes
PFAPA syndrome- sporadic idiopathic condition?

Perko, Debeljak, Toplak, Avčin. Mediators of inflammation 2015 (FH + in 78% PFAPA patients) Marshal 1987

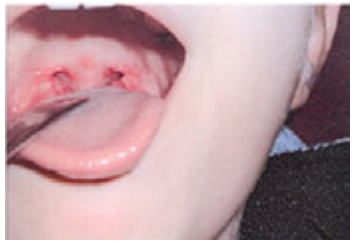
Periodic fever with Aphthous stomatitis, Pharyngitis and Adenitis

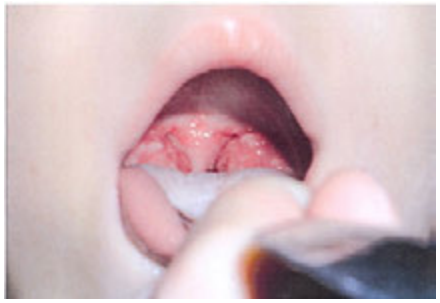
- Attacks of fever start early- 2-4 years of age
- Recur at intervals 3-6 weeks, last 3-5 days
- Temperature raises abruptly to 38-41^{0}C
- Tonsilitis, tender enlarged cervical nodes
- Painful oral aphthous ulcers
- Elevated inflammatory parameters

Hofer M et al. Rev med Suisse, 2008



AA, 4 years old PFAPA patient, Ljubljana

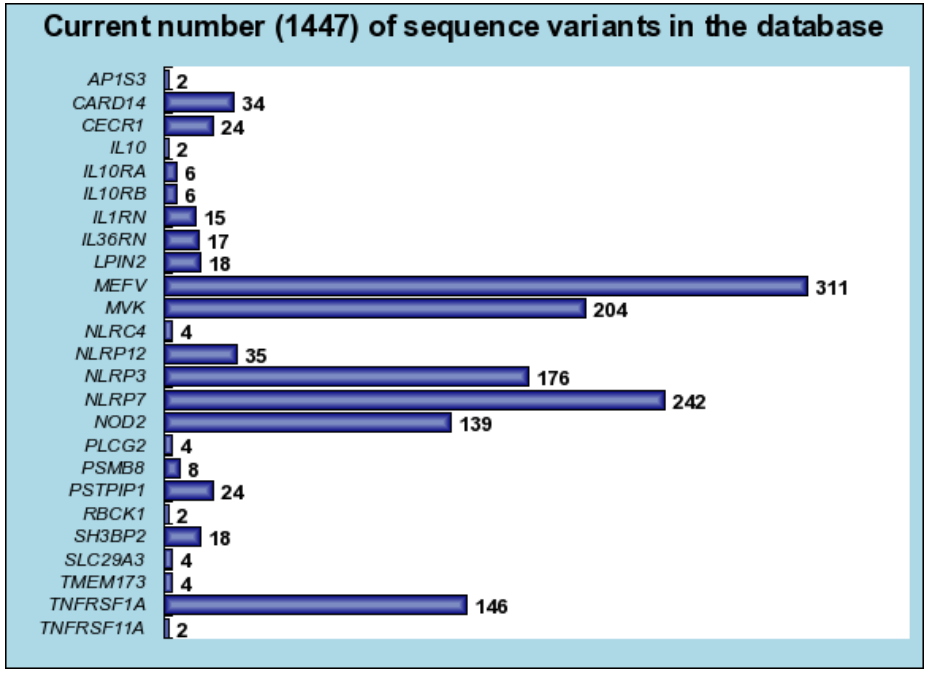




Our cohort- th.: tonsillectomy performed in 35% of patients, succesful in 93 %

Hereditary periodic fever syndromes

- **Familiar Mediterranean fever - FMF**
- Hyperimunoglobulinemia D - HIDS / mevalonate kinase deficiency- **MKD**
- TNFαR periodic fever syndrome - **TRAPS**
- Criopyrin associated periodic fever syndormes- **CAPS**
  - *Familial Cold Autoinflammatory Syndrome-FCAS/ Familial Cold Urticaria Syndrome- FCUS*
  - *Muckle-Wells syndrome-MWS*
  - *Neonatal-Onset Multisystem Inflammatory Disease-NOMID/Chronic Infantile Neurologic, Cutaneus and Articular Syndrome-CINCA*



http://fmf.igh.cnrs.fr/ISSAID/infevers/

Last update: 2014-10-29 3:41 PM

Familial Mediterranean fever- FMF

- first description – 1945 as "benign paroxysmal peritonitis" (Siegal)
- first description of nephropathy in FMF patient - 1950
- Colchicine treatment N Engl J Med 1972

FAMILIAL MEDITERRANEAN FEVER—AN UPDATE*

STEPHEN E. GOLDFINGER**

- first study that confirm effectiveness of colchicine in FMF

ZemerD et al. A controlled trial of colchicine in preventing attacks of familial Mediterranean fever. N Engl J Med 1974

The New England
Journal of Medicine

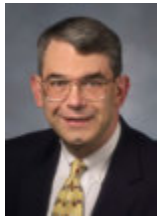
Volume 326 JUNE 4, 1992 Number 23

MAPPING OF A GENE CAUSING FAMILIAL MEDITERRANEAN FEVER TO THE SHORT ARM OF CHROMOSOME 16

ELON PRAS, M.D., IVONA AKSENTIJEVICH, M.D., LUIS GRUBERG, M.D., JAMES E. BALOW, JR., LEANDREA PROSEN, B.S., MICHAEL DEAN, PH.D., ALFRED D. STEINBERG, M.D., MORDECHAI PRAS, M.D., AND DANIEL L. KASTNER, M.D., PH.D.

Cell 90:797, 1997

The French FMF Consortium.
Dr.Isabelle Touitou
INSTITUTE of HUMAN GENETICS, Montpellier, France, 1997



The International FMF Consortium.
Dr. Daniel Kastner
NIH/NIAMS, Bethesda, USA, 1997

AR- gene *MEFV*, 16p13.3

Cardinale features of FMF:

Short painful, recurrent febrile episodes,

12 hours-3 days

Painful manifestation in the abdomen,

chest, joints, muscle, skin

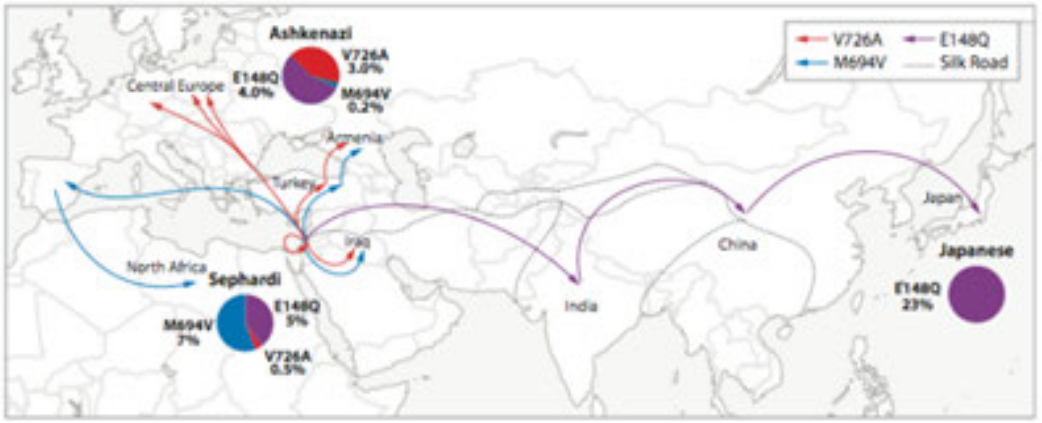
peritonitis (90%)

monoarthritis (>50%) --- red joint!

pleuritis (30%)

skin rash (25%)

muscle pain (10%)



Hyperimunoglobulinemia D- HIDS / mevalonate kinase deficiency- MKD

- First description - 1984

Van der Meer JWM et al. Hyperimmunoglobulinemia D and periodic fever: a new syndrome. Lancet 1984

letter

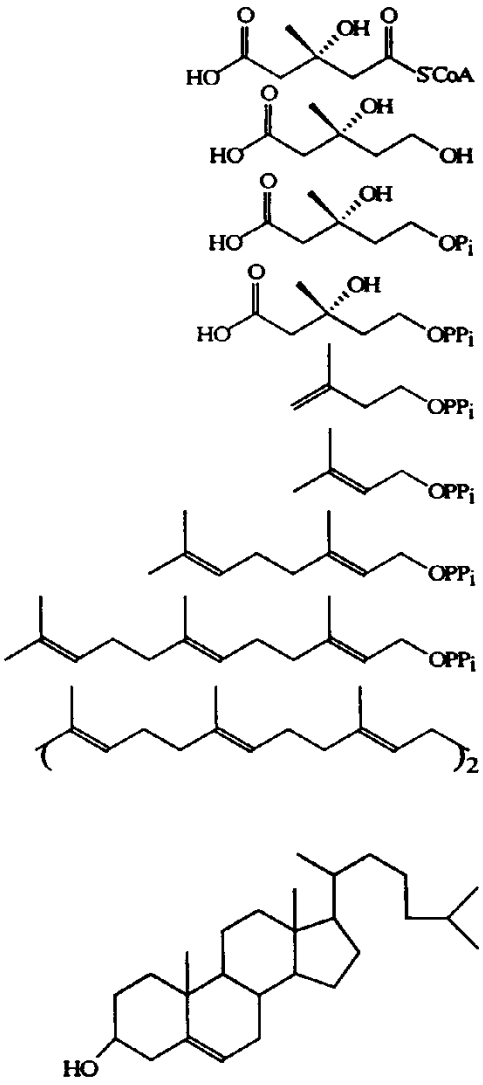
Mutations in the gene encoding mevalonate kinase cause hyper-IgD and periodic fever syndrome

Joost P.H. Drenth[1,2*], Laurence Cuisset[1*], Gilles Grateau[3], Christian Vasseur[1], Saskia D. van de Velde-Visser[4], Jan G.N. de Jong[5], Jacques S. Beckmann[6], Jos W.M. van der Meer[2], Marc Delpech[1]
& contributing members of the International Hyper-IgD Study Group

AR, MVK gene mutations, 12q24
80% of patients- p.V377I,
Other mutations are less frequent

- The majority of patients are Dutch

CHOLESTEROL PATHWAY



Acetyl-CoA + Acetoacetyl-CoA

↓

HMG-CoA

↓ **HMG-CoA reductase**

Mevalonate

↓ **mevalonate kinase**

5-Phosphomevalonate

↓

5-Pyrophosphomevalonate

↓

Isopentenylpyrophosphate → Isopentenyl Adenine (tRNA)

↓

Dimethylallylpyrophosphate

↓

Geranylpyrophosphate

↓

Farnesylpyrophosphate → Dolichol; Haem A; Ubiquinone; Isoprenylated proteins (e.g. RAS, lamin B)

↓

Squalene

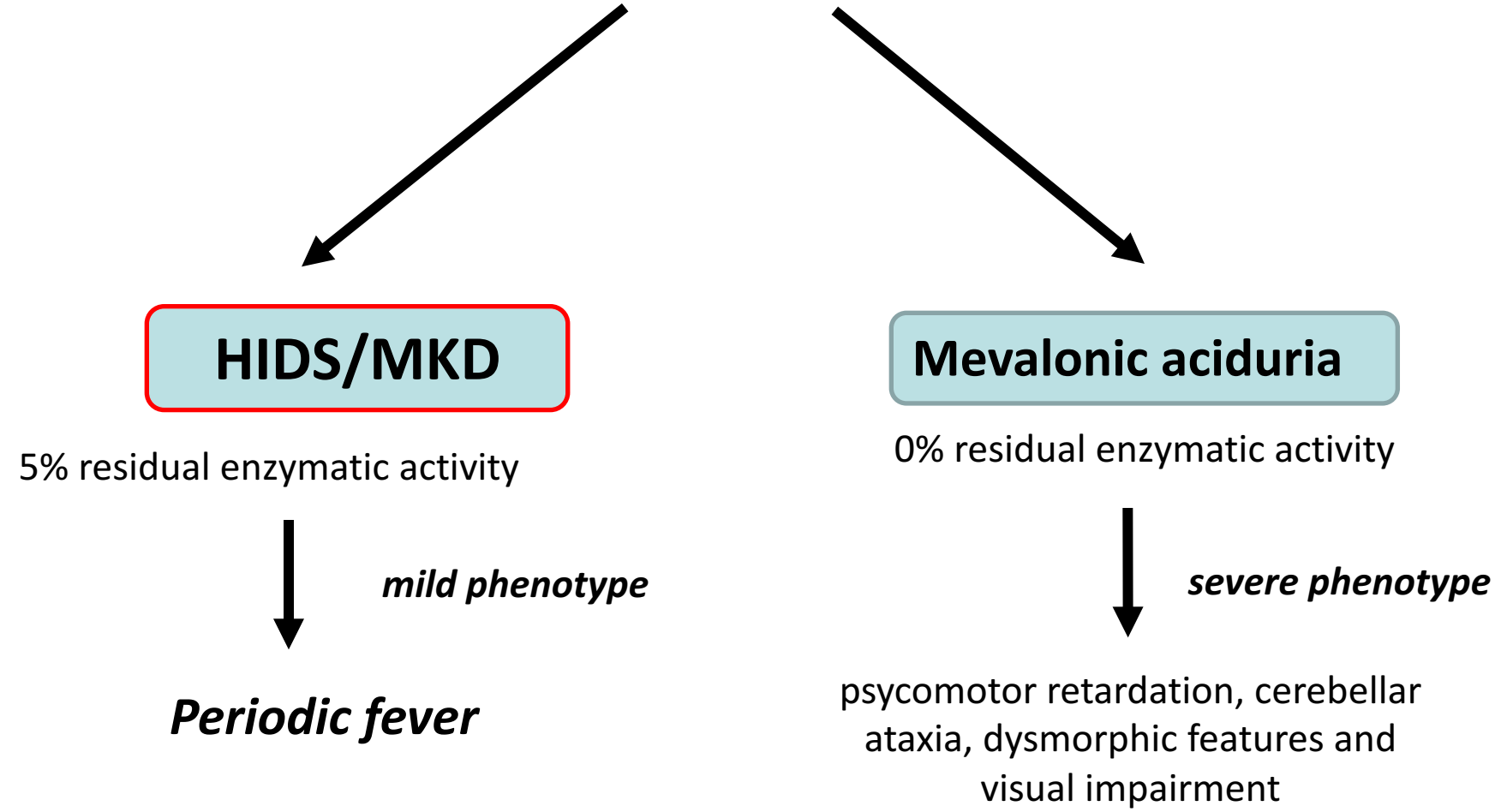
↓

↓

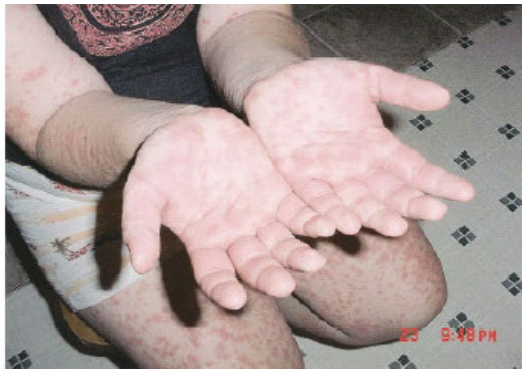
Cholesterol → Steroid Hormones; Vitamin D; Bile Acids; Lipoproteins

↑↑ **inflamatory cytokines**

Residual activity of MVK or complete deficiency



HIDS / MKD: febrile episodes

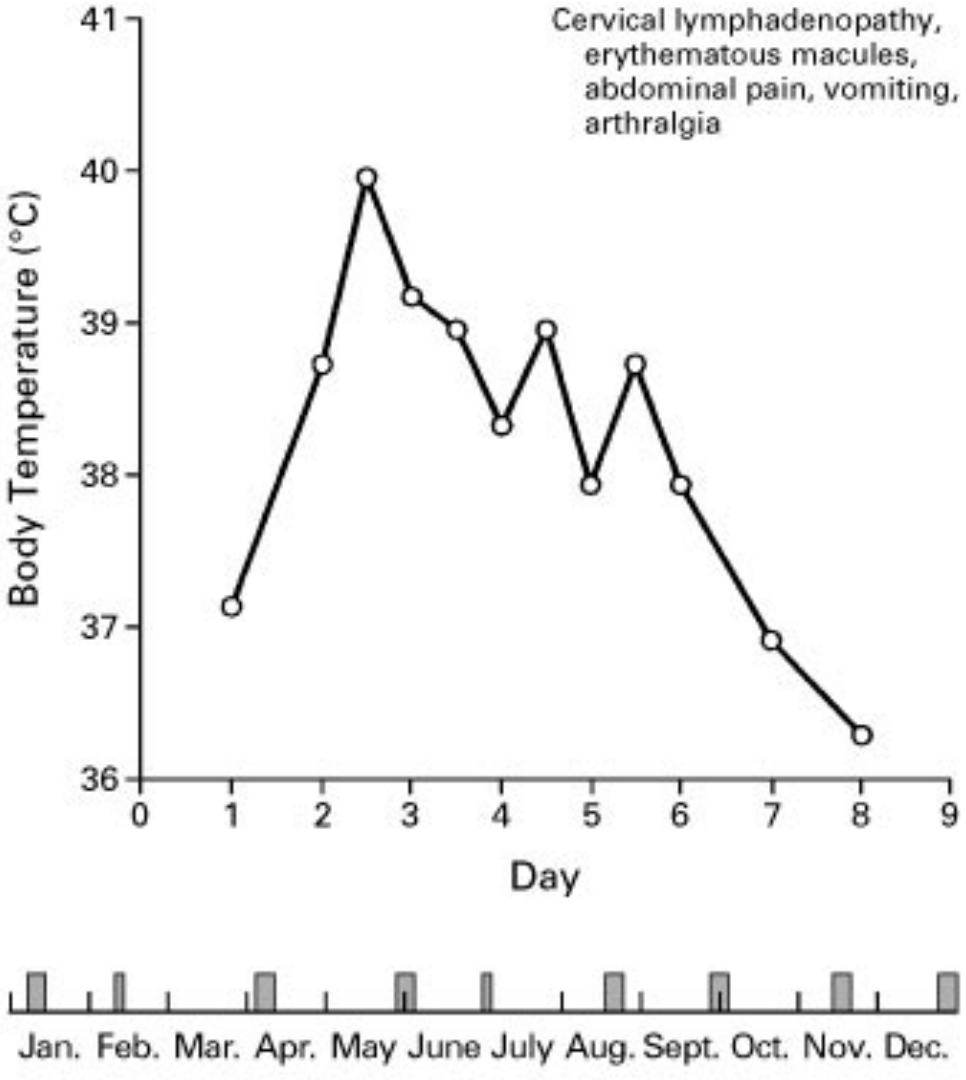


Frenkel, Rheumatology, 2001

Treatment: (EUF registry)

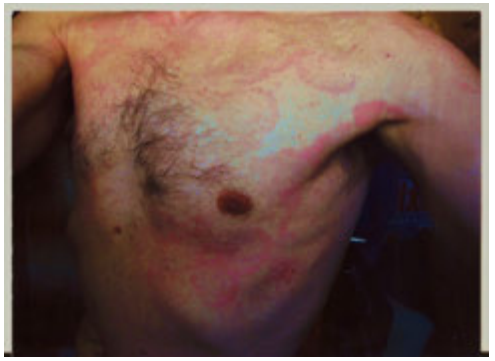
- ***anakinra-*** *antil-L1- used in 27 patients, effective in 24* ***(90%)***

- **etanercept**- anti-TNFα- used in 17 patients, effective in 11 **(65%)**



TNFα receptor associated periodic fever syndrome - TRAPS

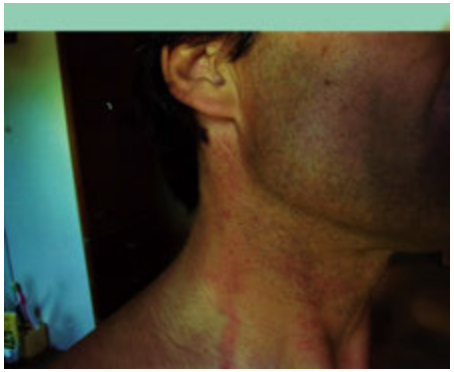
- = familiar hibernian fever (FHF)
- First description- 1982 (Irish, Scottich)
- AD, 12p13.2, *TNFRSF1A* McDermott-Cell 1999
- 146 mutations described http://fmf.igh.cnrs.fr/ISSAID/infevers/
- Eurofever registry- median age at disease onset- 5y, 0-63
- ↓ concentration of soluble TNFα receptor- increased TNF activity



CLINICAL PICTURE
longer duration of episodes (21 days)
severe myalgia, painful periorbital edema, conjunctivitis, rash

Tumor Necrosis Factor Receptor-Associated Periodic Fever Syndrome in a 58-Year-Old Man:
Caution Not to Discount TRAPS as a Diagnosis in Older Patients.
Sinožić D, **Toplak N**, Milotić I. J Clin Rheumatol. 2011 Sep;17(6):325-8.

THERAPY
- methylprednisolone
- etanercept **Drewe et al. Rheumatology, 2004**
- anakinra **data from Eurofever registry, 2012**



Criopyrin associated periodic fever syndormes-CAPS

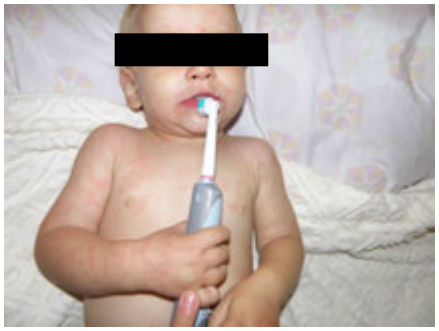
- Familial Cold Autoinflammatory Syndrome-FCAS/ Familial Cold Urticaria Syndrome- FCUS
- Muckle-Wells syndrome-MWS
- Neonatal-Onset Multisystem Inflammatory Disease-NOMID/Chronic Infantile Neurologic, Cutaneus and Articular Syndrome-CINCA

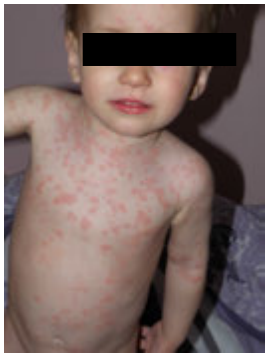
- First description 2001
- AD, 1q44, NLRP3 gene
- 176 mutations described http://fmf.igh.cnrs.fr/ISSAID/infevers/

Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome.
Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Nat Genet. **2001**

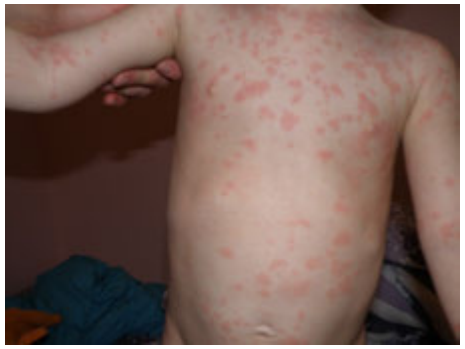
Dr. Hal Hoffman, USDC

CAPS- clinical picture





Phenotype	**FCAS** **Familial cold autoinflammatory syndrome**	**MWS** **Muckle-Wells syndrome**	**CINCA/NOMID** **Chronic infantile neurological, cutaneous and articular syndrome**
Age at onset	Children, adults	Children	Neonatal
Disease course	Recurrent episodes triggered by cold	Recurrent	Chronic
Skin	Urticaria	Urticaria	Urticaria
Joints & bone	Arthralgia	Arthralgia/arthritis	Arthralgia/arthritis/bone dysplasia
Eyes	Conjunctivitis	Conjunctivitis/uveitis	Conjunctivitis/uveitis/ papilledema/optic atrophy
Neurological signs	-	Neurosensorial deafness	Chronic meningitis/ neurosensorial deafness
Amiloidosis	Extremely rare	Often	Rare in childhood, possible in adulthood



Case presentation

- After exclusion of infection, PID, autoimmunity, malignancy……

Is it ***autoinflammation??***

Periodic fever syndrome with rash and arthritis, from birth on---

CAPS??

- Short attacks, sometimes up to 7 days; Elevated IgD, serum amiloid levels

  but neg mevalonic acid in urin

  FMF?MKD??

Case presentation- genetic testings

- **FMF- *MEFV:***

Heterozygous R202Q

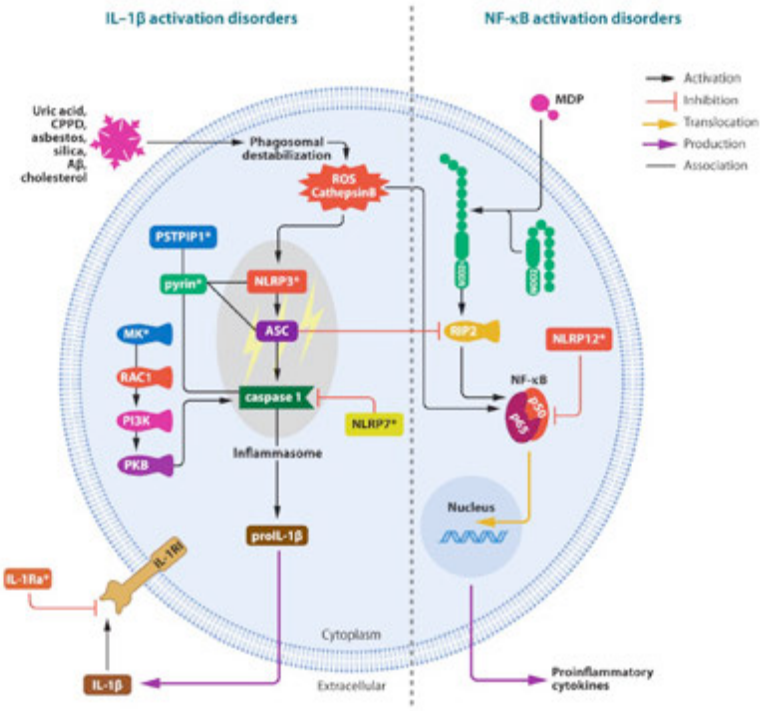
(Genetic lab, University Children's hospital Ljubljana)

- **MKD**
- **TRAPS**

negative

- **CAPS**

NLRP 3 mutation- G326E



Th: anti IL 1 th - anakinra / canakinumab

New diseases emerging

Semin Immunopathol (2015) 37:395–401
DOI 10.1007/s00281-015-0478-4

REVIEW

Update on genetics and pathogenesis of autoinflammatory diseases: the last 2 years

Ivona Aksentijevich[1]

Table 1 Molecular classification of new monogenic autoinflammatory diseases (the diseases are listed in the chronological order as they have been discovered)

Disease/abbreviation	Gene mutated	Protein	Inheritance	References
Deficiency of ADA2/DADA2	*CECR1*	ADA2	Autosomal recessive	[2–4]
STING-associated vasculopathy/SAVI	*TMEM173*	STING/MITA	Autosomal dominant	[15, 16]
TNFRSF11A-associated disease	*TNFRSF11A*	RANK/ODFR	Autosomal dominant	[27]
NLRC4-associated diseases/ NLRC4-MAS, SCAN4, NLRC4-FCAS	*NLRC4*	CARD12/IPAF	Autosomal dominant	[30–32]
Sideroblastic anemia, B-cell immunodeficiency, periodic fevers, developmental delay/SIFD	*TRNT1*	TRNT1	Autosomal recessive	[39, 40]
Monogenic form of systemic juvenile idiopathic arthritis	*LACC1*	LACC1	Autosomal recessive	[45]

New treatment

8th International Congress on FMF and other autoinflammatory diseases, Dresden; Germany, October 2015

- **anti-TNFα agents**

- tretamnet successful in DADA2 treatment (early stroke and vasculopathy); 14 patients were treated with etanercepet or adalimumab

- ***JAK inhibitors- Janus kinase inhibitors-*** interfering with the JAK-STAT signalling pathway

- treatment for SAVI, CANDLE, severe JDM (under investigation)

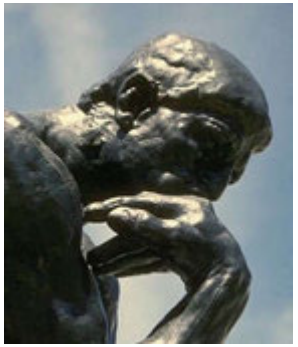
- ***Anti-IFN gama Ab***

- treatment in a patient carrying NLRC4 mutation and severe HLH

Conclusion

- Fever- the most common sign of very common diseases
- Recurrent / periodic fever- the sign of rare and orphan diseases

- The concept of autoinflammation is not that simple any more / nor it ever was
- Everything is infectious until proven otherwise
- Everything is autoimmune until proven otherwise
- ***…when you run out of ideas for diagnosis- think about AUTOINFLAMMATION***
- Well, I guess at the end everything is genetic……



Thank you for your attention

LYMPHOCYTE SUBPOPULATIONS IN PATIENTS WITH CHILDHOOD-ONSET SYSTEMIC LUPUS ERYTHEMATOSUS AND ANTIPHOSPHOLYPID SYNDROME

Marija Holcar[1], Aleš Goropevšek[2,3] , Alojz Ihan[4] , Tadej Avčin[1,5]

[1] Department of Allergology, Rheumatology and Clinical Immunology, University Children's Hospital, University Medical Centre Ljubljana, Slovenia
[2] Department of Laboratory Diagnostics, University Medical Centre Maribor, Slovenia
[3] Faculty of Medicine, University of Maribor, Slovenia
[4] Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia
[5] Department of Pediatrics, Faculty of Medicine, University of Ljubljana, Slovenia

Systemic lupus erythematosus (SLE) and antiphospholypid syndrome (APS) are multisystem chronic autoimmune disorders with variable clinical and laboratory manifestations. Childhood-onset SLE (cSLE) is more agressive disease differing in disease severity and organ involvement, leading to more-rapid damage accrual. APS is characterised by persistent circulating antiphospholipid antibodies, connected to arterial and venous thrombosis and recurrent fetal loss. APS is primary or associated with other autoimmune diseases, mainly SLE, suggesting that diseases may be related. The highest prevalence of SLE is among childbearing women but in 15% SLE begins before the age of 18 years. Pediatric APS (pedAPS) is difficult to diagnose due to heterogeneity and low prevalence (2.8% of patients before the age of 15 years). Most of published studies highlighted the characteristics of SLE and APS in adults, but there is a lot of uncertainty regarding pediatric patients. We analysed cytokine profiles and lymphocyte subsets in the peripheral blood of healthy donors (HD), JIA patients (disease control), and Slovenian patients with cSLE and pedAPS, focusing on effector (Teff), regulatory (Treg) T lymphocytes and STAT1/STAT5 signalling response in helper T lymphocytes (Th), using immunoassay on biochip and flow cytometry. Results demonstrate a significant increase in the percentage of $FoxP3^{+}$nonTregs and $CD25^{-}FoxP3^{+}$Tregs in cSLE patients compared to HD and in the percentage of naïveTregs comparing cSLE to JIA patients, but severe lymphopenia leads to significant decrease in concentrations of activatedTregs as well as total Tregs, comparing cSLE patients to HD. We found no significant differences in percentage of total Teff lymphocytes or their subpopulations between the groups, but there was a decrease of concentration of Th1, Th1Th17, $Th17CD161^{+}$, Th17 and total Teff between cSLE and JIA as well as HD group. Despite lower concentrations of Tregs and Teff lymphocytes in patients with cSLE, we found not significant differences analysing cytokine profile (IL1-a, IL1-b, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α and IFN-γ) between groups. Together with significantly higher Th lymphocyte STAT1, STAT5 expression and basal phosphorylation of STAT1 comparing cSLE to both HD and JIA groups our results indicate cytokine overproduction in patients with cSLE.

klinični center ljubljana
University Medical Centre Ljubljana

LYMPHOCYTE SUBPOPULATIONS IN PATIENTS WITH CHILDHOOD-ONSET SYSTEMIC LUPUS ERYTHEMATOSUS AND ANTIPHOSPHOLYPID SYNDROME

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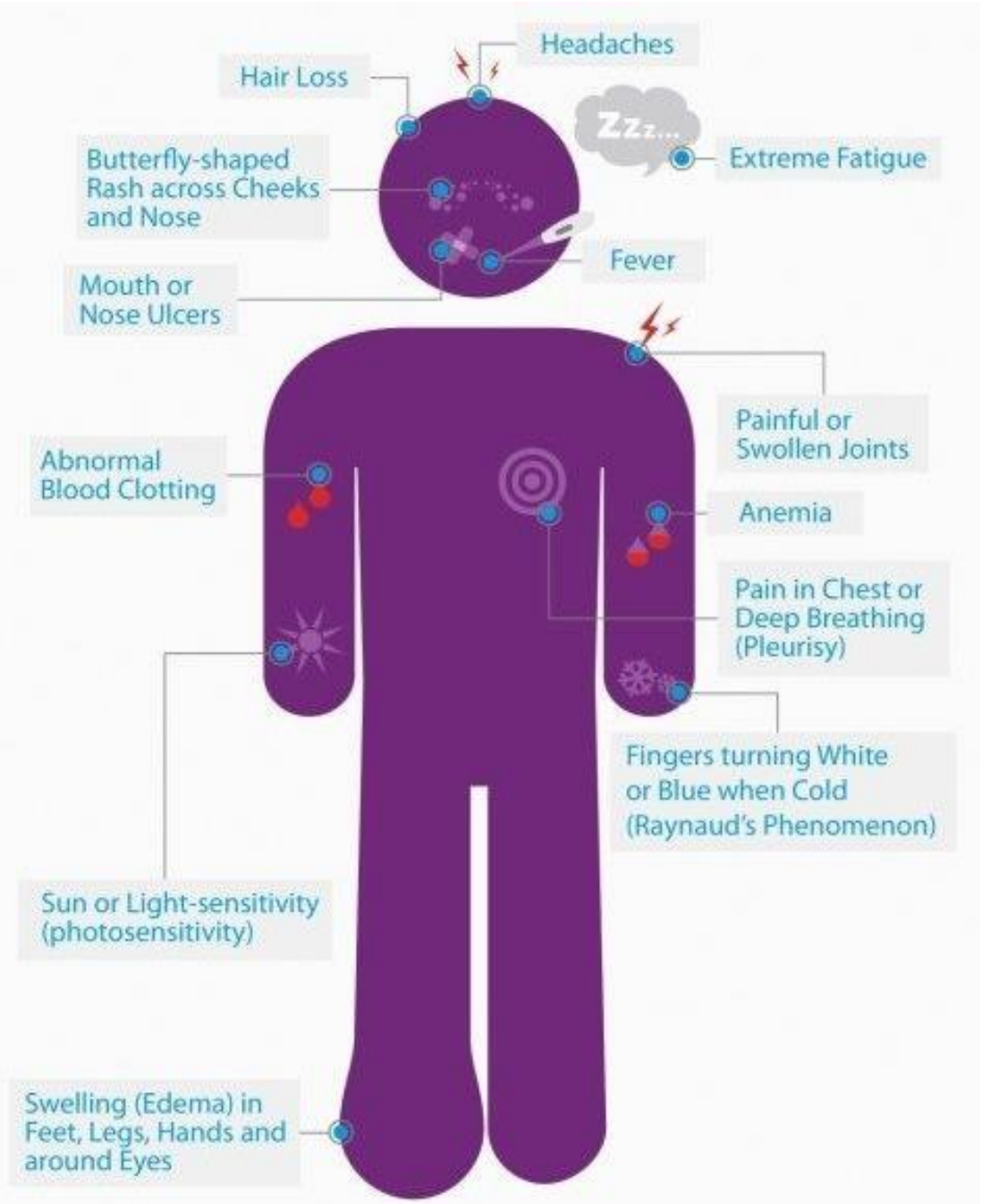
[1]Department of Allergology, Rheumatology and Clinical Immunology, University Children's Hospital , University Medical Centre Ljubljana, Slovenia
[2]Department of Laboratory Diagnostics, University Medical Centre Maribor, Slovenia
[3]Faculty of Medicine, University of Maribor, Slovenia
[4]Faculty of Medicine, University of Ljubljana, Institute For Microbiology and Immunology, Slovenia
[5]Faculty of Medicine, University of Ljubljana, Slovenia

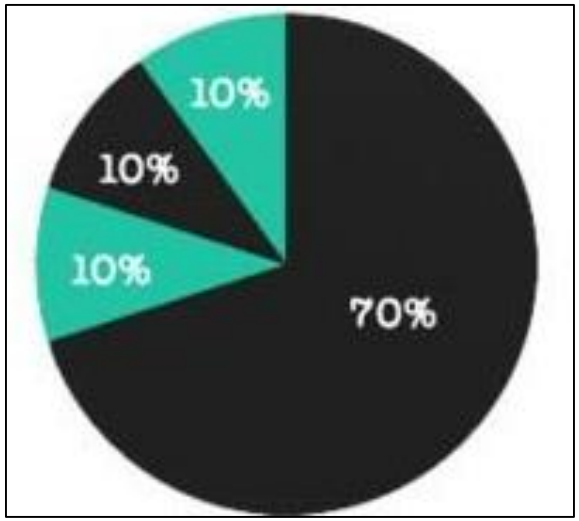
SYSTEMIC LUPUS ERYTHEMATOSUS

- Chronic multisystem autoimmune inflammatory disease
- Loss of B-cell tolerance
  - hyperactive B cells produce **autoantibodies** against different components of **cell nucleus**
- Disposing of immune complexes
- **Inflammation** and breakdown of various tissues and organs

Crispin et al., Trends in Molecular Medicine, 2010; 16:47-57.



- Skin
- Muscles and joints
- Eyes, nose and mouth
- Brain and nervous system
- Heart and lungs
- Blood and circulatory system
- **Kidneys**



70% Systemic lupus erythematosus (SLE)
10 % Cutaneous lupus
10% Discoid lupus
10% Drug induced lupus
<1% Neonatal lupus

- **5 million** worldwide suferers, 500.000 deaths per year
- Average age of onset: **15-45 years**
- Female to male ratio: **9:1** (12:1)
- Flares, remissions; disease activitiy index: **SLEDAI**
- 15% of lupus cases are children (before age 16) – **cSLE** (more aggresive)

Danchenko et al., Lupus, 2006; 15:308-318.

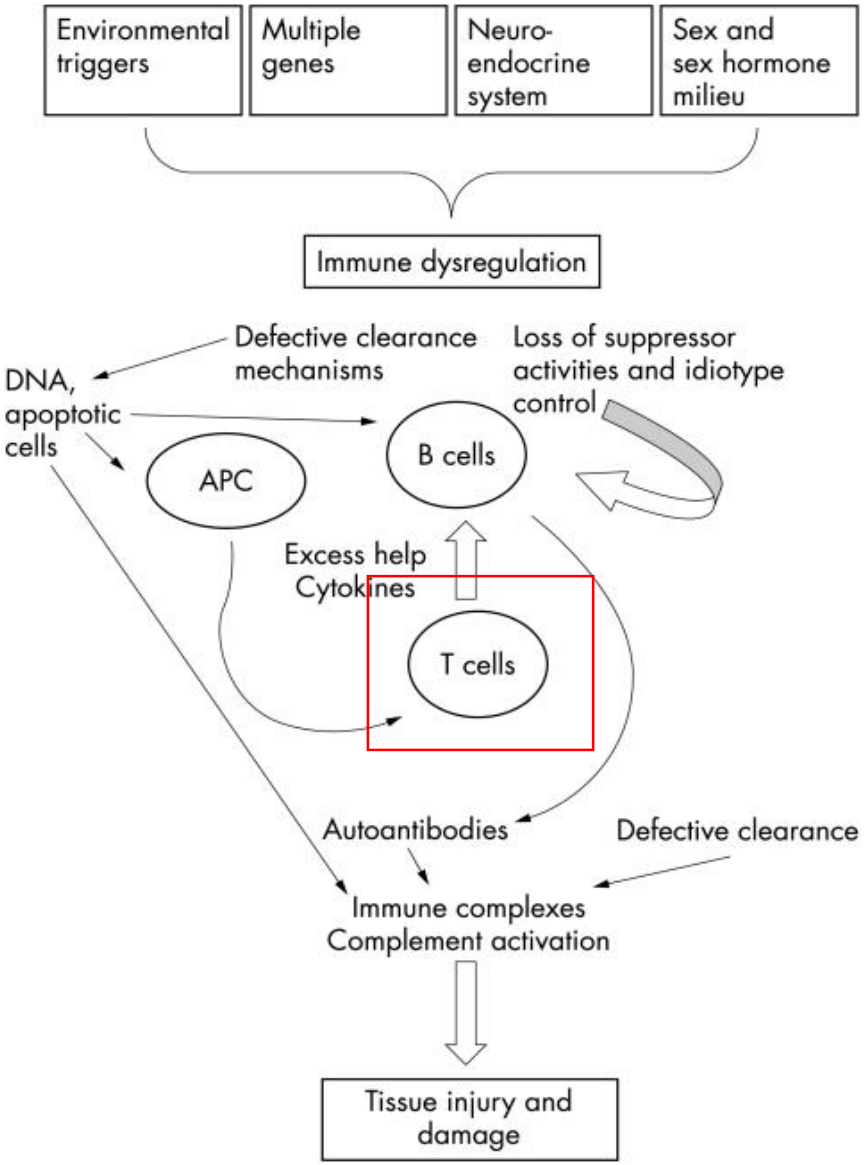
PATHOGESIS OF SLE

- Patho-etiology probably involve complex interactions between different **multi-genetic**, **environmental**, **endocrine** and other factors

- No two cases are alike

- central role:

  T-cells



La Cava, Lupus, 2009; 18:196-201.

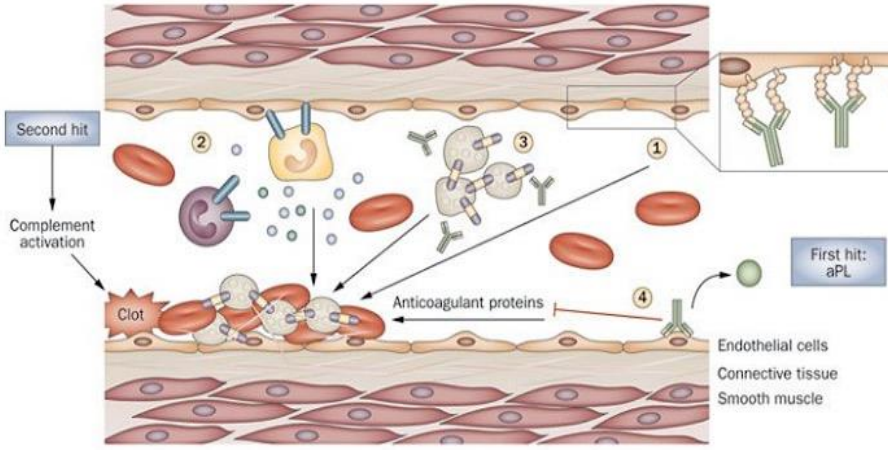
Mok and Lau, Journal of Clinical Pathology, 2003; 56:481-490.

ANTIPHOSPHOLIPID SYNDROME (APS)

Autoimmune disorder, **antiphospholipid** antibodies (aPL)

Clinical Event		Laboratory Test Result
		Cardiolipin antibody (IgG) >40 GPL[a] or >99[th] percentile
Venous thrombosis		Cardiolipin antibody (IgM) >40 MPL[a] or >99[th] percentile
Arterial thrombosis	+	β_2-glycoprotein I antibody (IgG) >20 SGU
Recurrent abortion		β_2-glycoprotein I antibody (IgM) >20 SMU
		Lupus anticoagulant

- altered homeostatic regulation of **blood coagulation**
- mechanisms of thrombosis are not yet defined
  - **1-5%** of healthy individuals have aPL antibodies



Salmon et al., Nature Clinical Practice Rheumatology, 2007;3:140-147

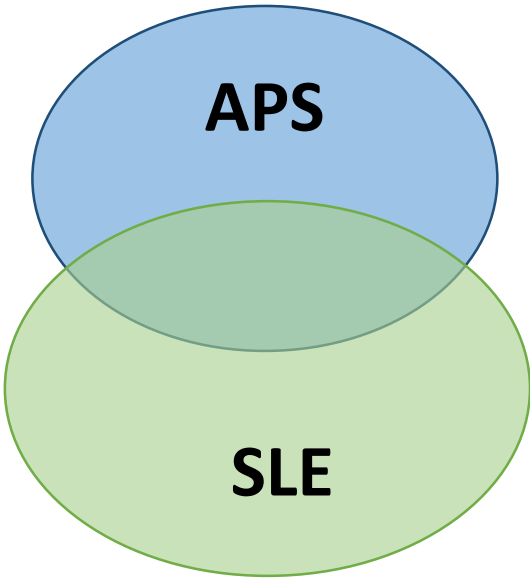
•**Female** predominance, especially for secondary APS

• Low prevalence in children – only 2.8% of patients before age of 15 (**pedAPS**)

no validated criteria

•50% of APS cases associated with another rheumatic disease (mostly SLE)

•aPL antibodies found in 30-40% of SLE cases (secondary APS)



STUDY DESIGN

GROUPS:

- 18 patients with **cSLE:** 18.2 years (range 9-21 years), 3 male, 15 female
  - Average age at the diagnosis: 14.1 years
  - Average disease duration at the study entrance: 3.9 years
  - 5 with SLEDAI = 0
  - 8 with SLEDAI 1-6
  - 5 with SLEDAI >6

- 5 patients with **pedAPS:** 16.6 years (range 14-20 years), 3 male, 2 female
  - Average age at the diagnosis: 15.4 years
  - Average disease duration at the study entrance: 2.6 years

- 10 patients with **jouvenile idiopathic arthritis (JIA):** 12.1 years (range 5 15 years), 1 male, 9 female
  - 7 with JADAS = 0
  - 3 with JADAS > 0

- 20 healthy donors (**HD**): 16.0 years (range 15-21 years), all female

METHODS

peripheral venous blood

- flow cytometry (whole blood)
  - **surface staining** – T_{eff}, basic lymphocyte subpopulations (stain – lyse - wash)
  - **intracellular staining** – STAT, T_{reg} analysis

  -STATs: BD Phosflow (BDBiosciences) protocol
  1)formaldehyde containing red cell **lysing/lymphocyte fixing** solution
  2)methanol based buffer for **permeabilisation**
  3)simultaneous **staining** of surface and intracellular antigens

  -*T_{reg}*: **1)staining** of surface antigens,
  2)fixation and **permeabilisation** using Human FoxP3 Buffer set (BDBiosciences)
  3)staining of intracellular protein FoxP3

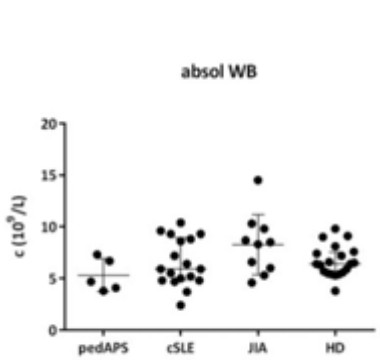
  -Dual-platform approach (WB cell count on Hematology Analyzer)

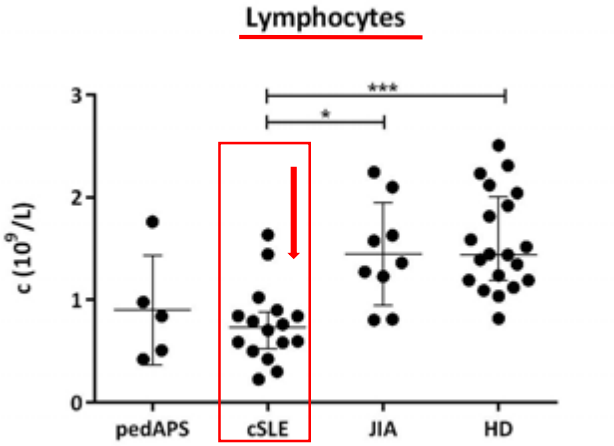
- imunoassay on biochip (plasma)
  - **cytokine profile** (IL1-α, IL1-β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α and IFN-γ)

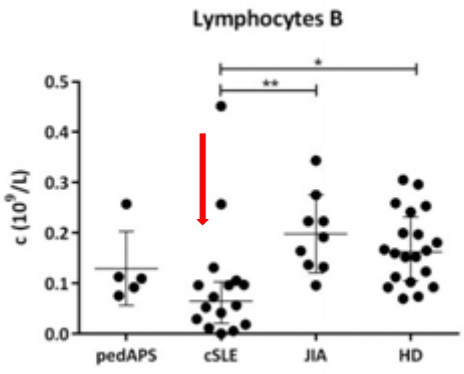
QUESTIONS

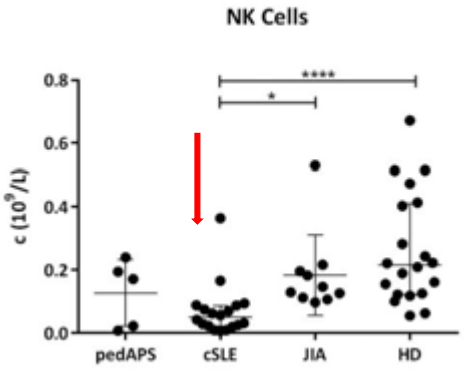
- How do lymphocyte subsets of patients with **cSLE** and **pedAPS compare** to those of HD and DC?
- Do cSLE and pedAPS patients share **common lymphocyte subset aberrances**?
- Is there **difference in STAT** (STAT5 and STAT1) expression/phosphorylation?
- Is there any correlation between SLEDAI and any of markers?
- Do our findings differ from adult-onset SLE population?

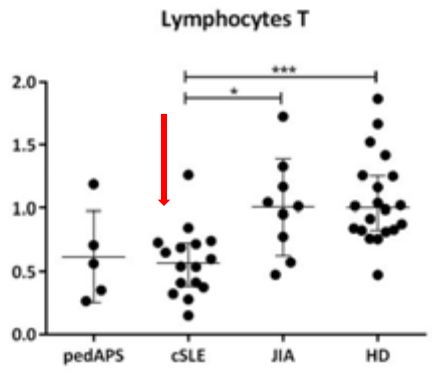
SURFACE STAINING +
FLOW CYTOMETRY

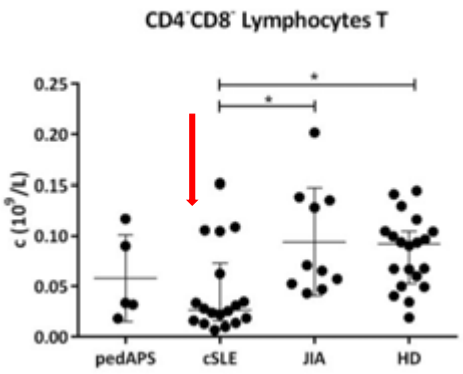


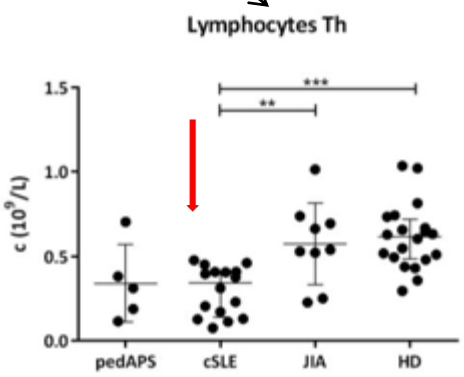










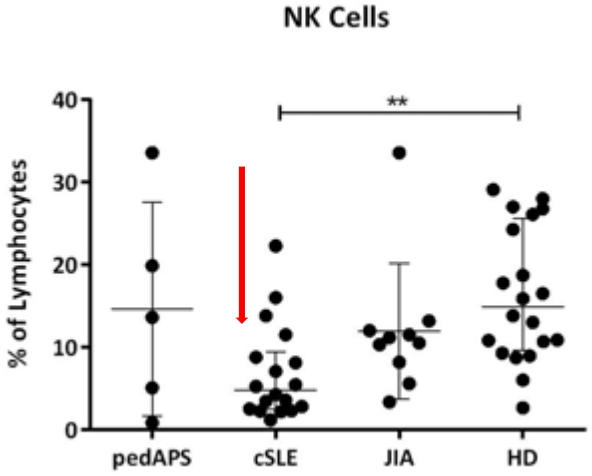


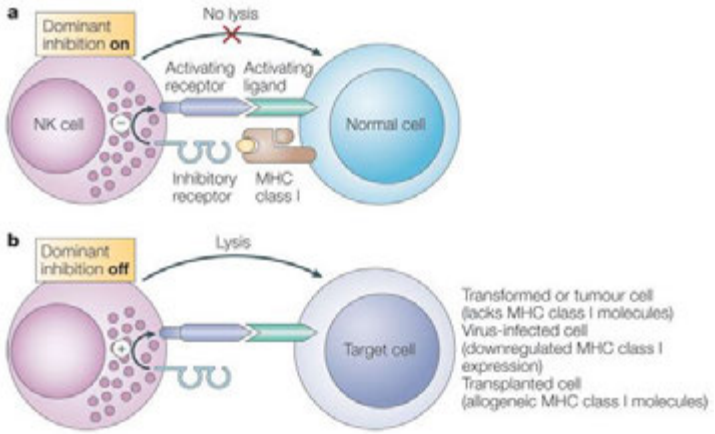
- **lymphopenia**

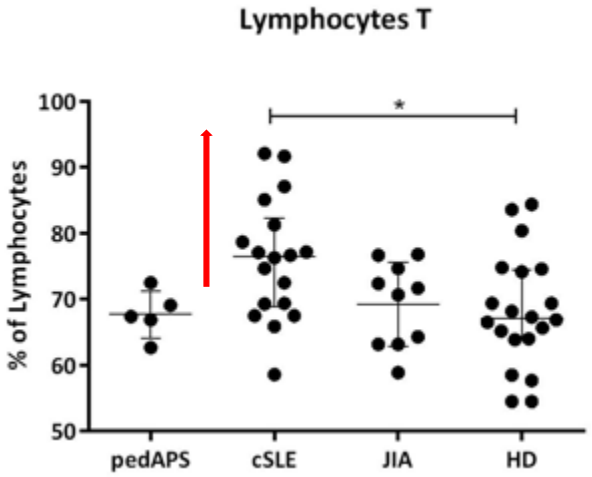
↓

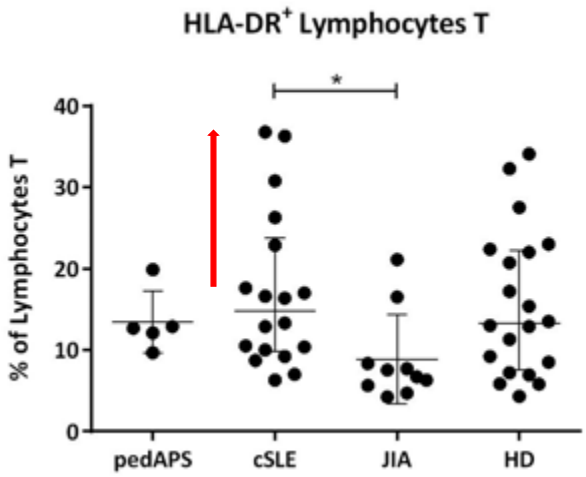
lower concentrations of the major **lymphocyte subsets** in patients with cSLE

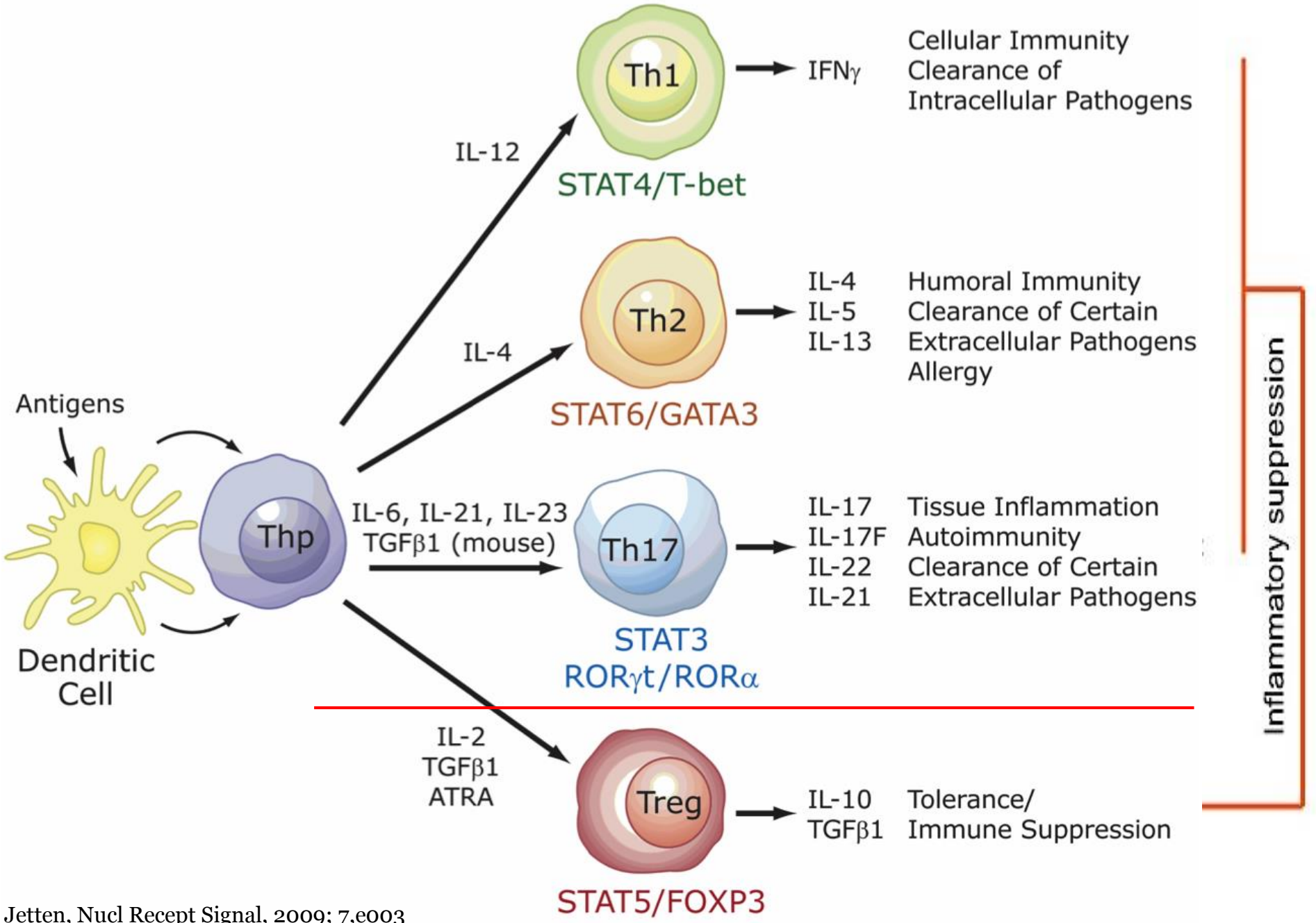
SURFACE STAINING +
FLOW CYTOMETRY





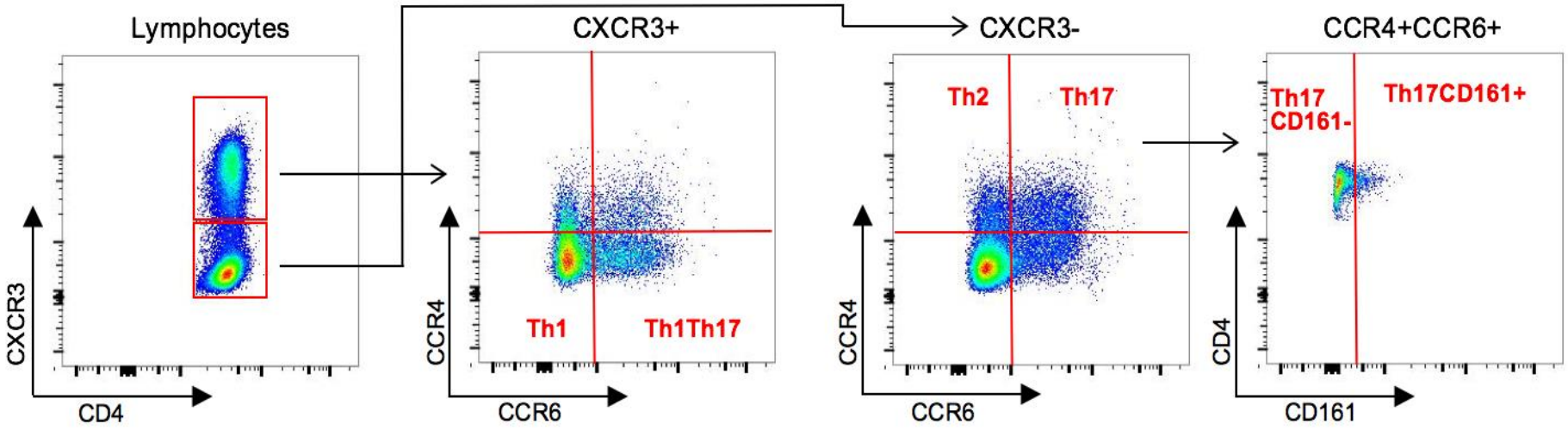






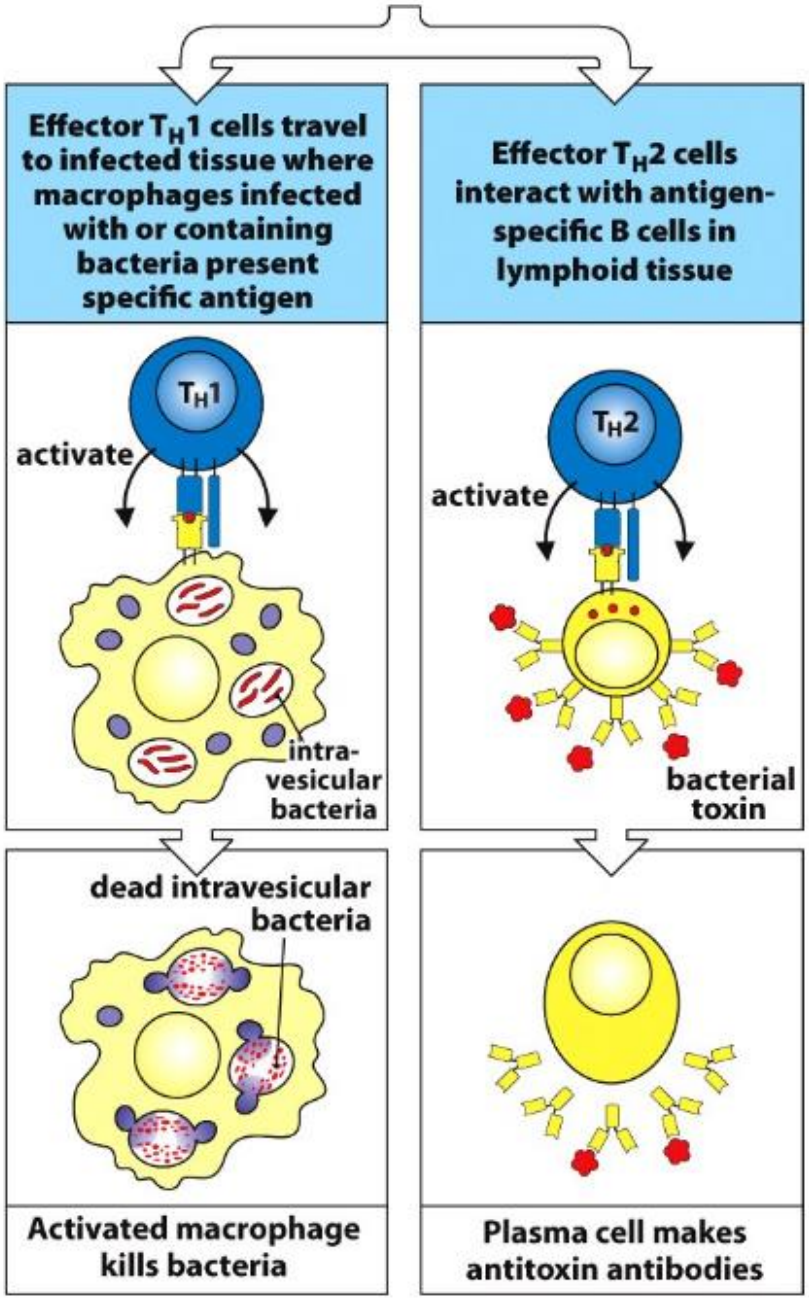
Jetten, Nucl Recept Signal, 2009; 7,e003

INTRACELLULAR STAINING
+ FLOW CYTOMETRY

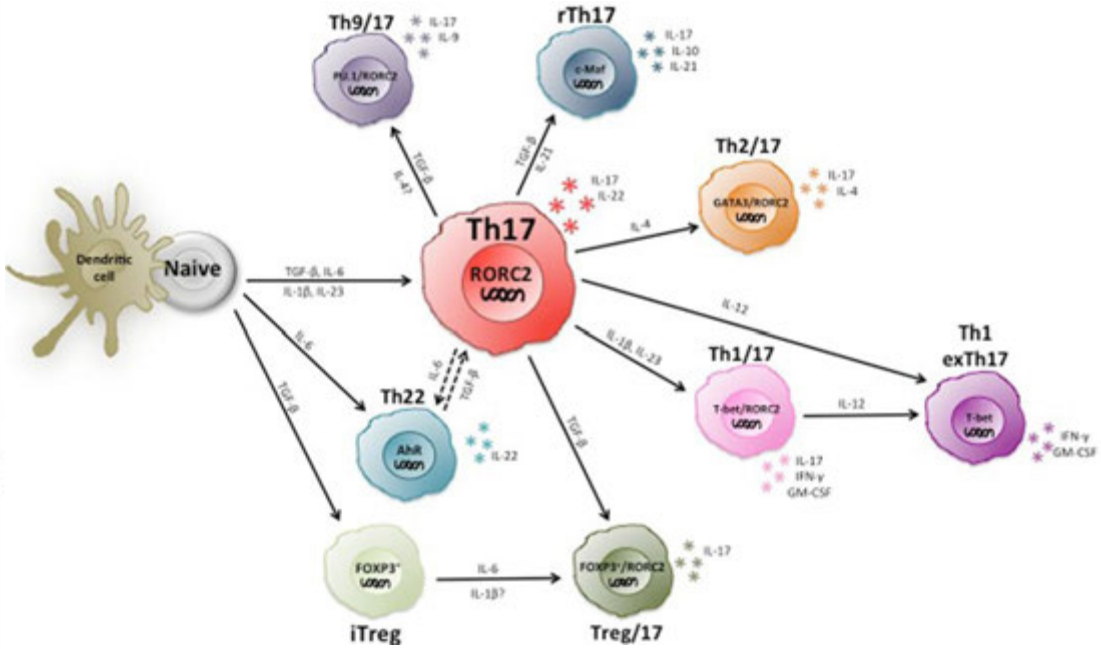


Th1	$CD4^+CXCR3^+CCR4^-CCR6^+$
Th2	$CD4^+CXCR3^-CCR4^+CCR6^-$
Th1Th17	$CD4^+CXCR3^+CCR4^-CCR6^+$
Th17 $CD161^+$	$CD4^+CXCR3^-CCR4^+CCR6^+CD161^+$
Th17 CD161-	$CD4^+CXCR3^-CCR4^+CCR6^+CD161^-$

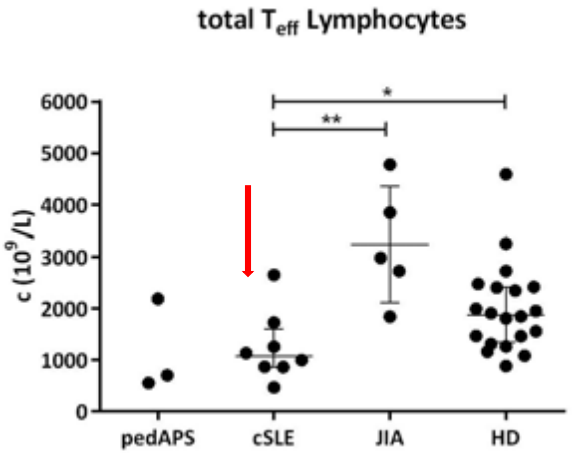
Crome et. al., The Journal of Immunology, 2010;185:3199-3208.

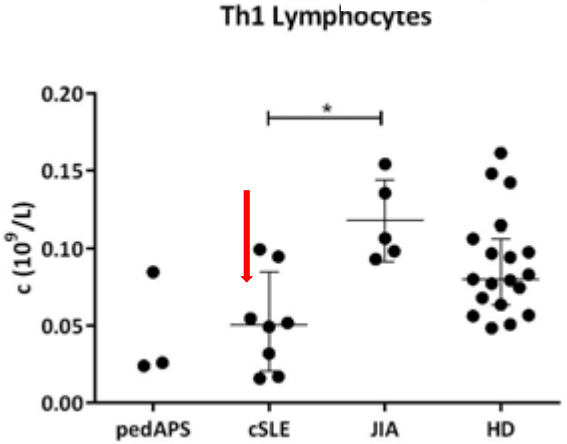


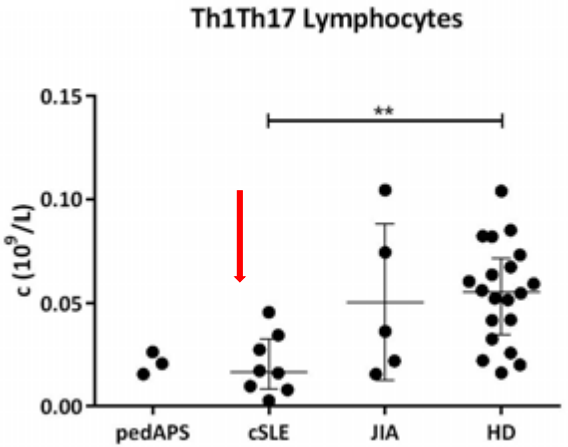
- **no differences in percentages of T_{eff}** between groups

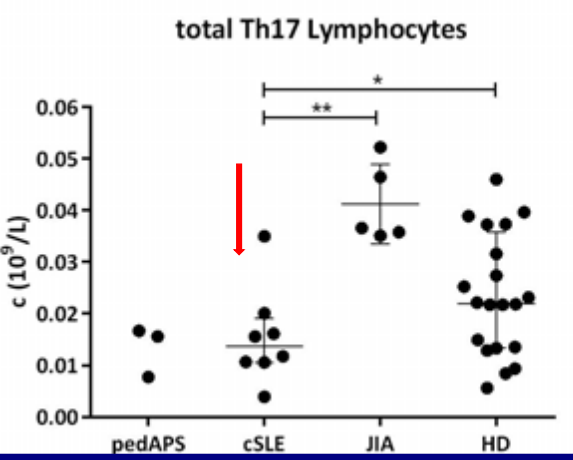


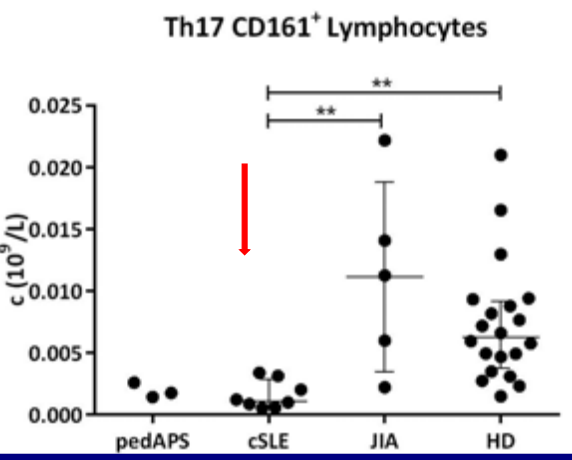
SURFACE STAINING + FLOW CYTOMETRY

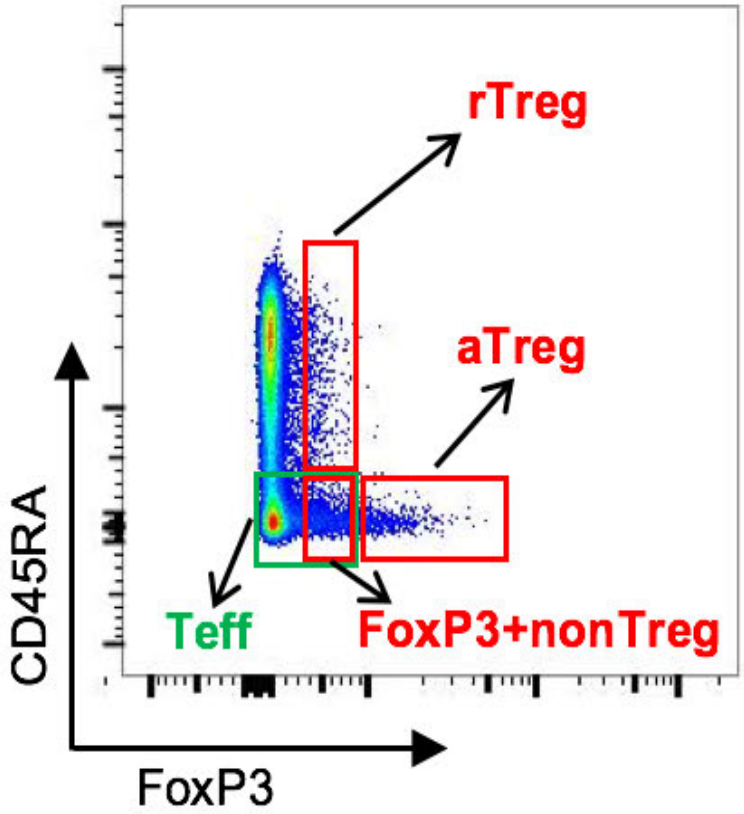


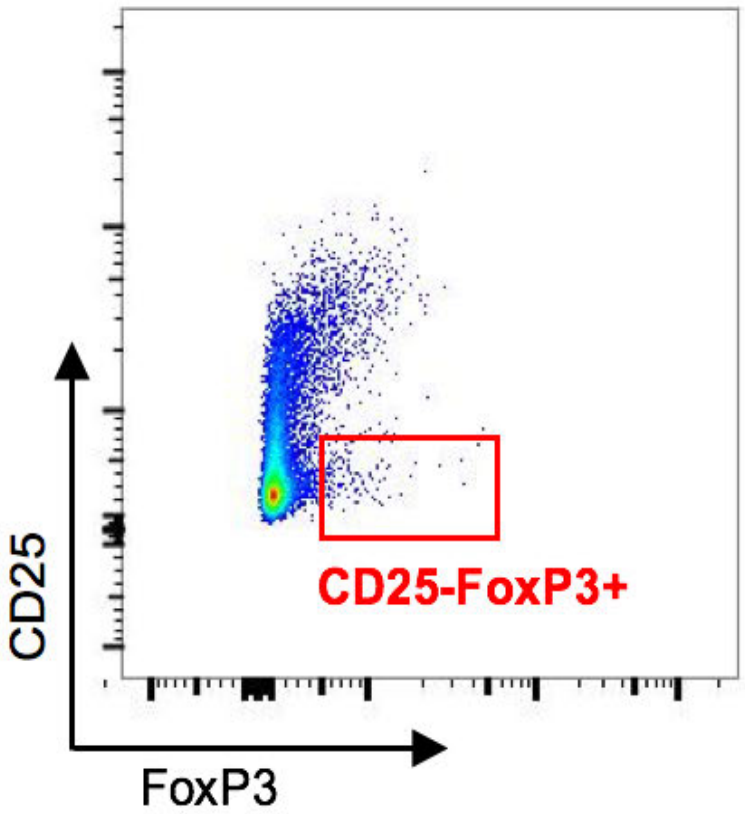








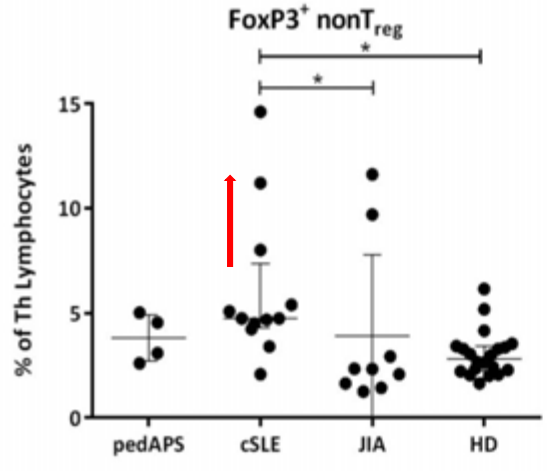


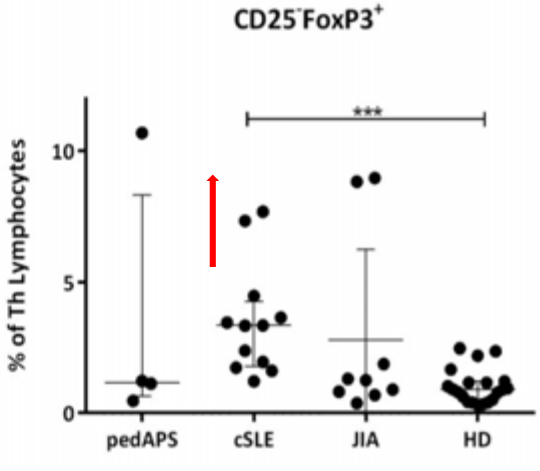


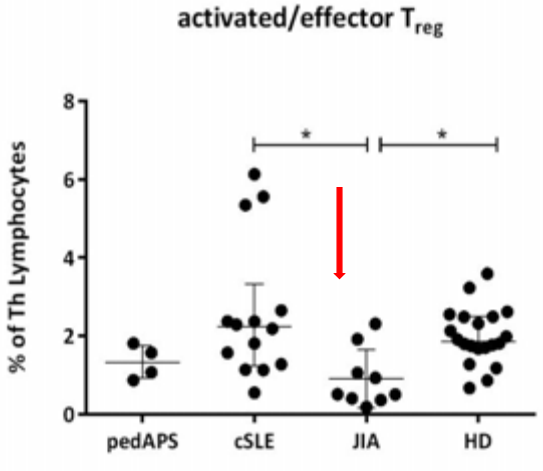
aTreg	$CD4^+CD45RA^-FoxP3^{hi}$
rTreg	$CD4^+CD45RA^+FoxP3^{lo}$
FoxP3+non Treg	$CD4^+CD45RA^-FoxP3^{lo}$
Teff	$CD4^+CD45RA^-FoxP3^{lo}$, $CD4^+CD45^-FoxP3^-$
CD25-FoxP3+	$CD4^+CD25^-FoxP3^+$

Miyara et. al., Autoimmunity Reviews, 2011;10:744–755.

INTRACELLULAR STAINING + FLOW CYTOMETRY

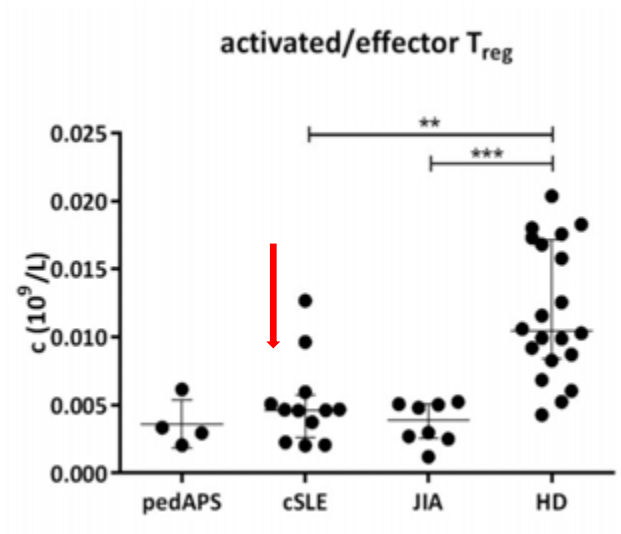


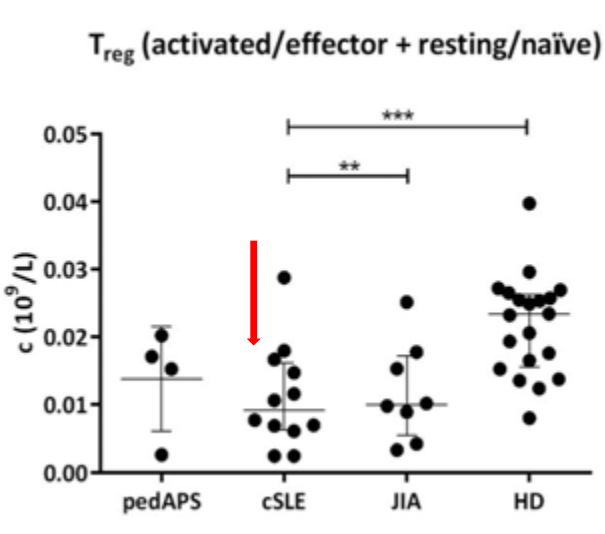


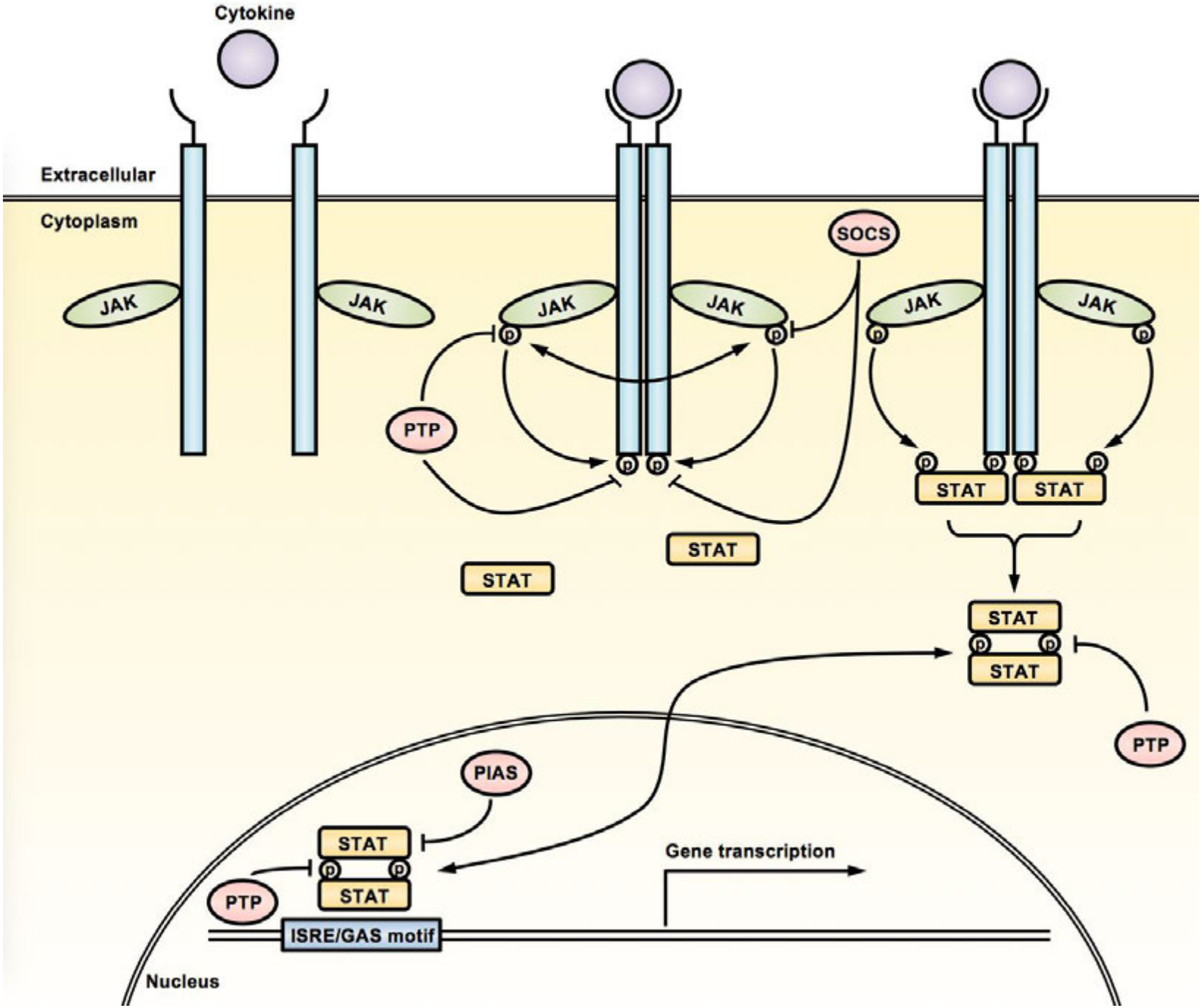


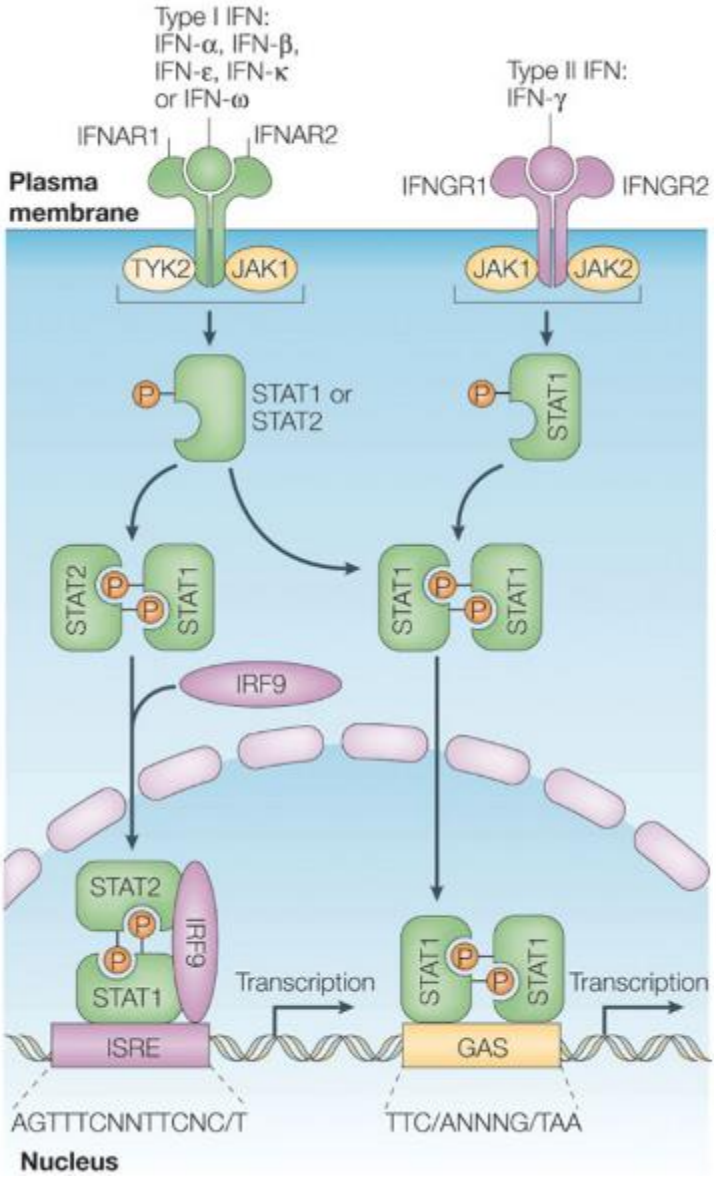
- **higher percentage**

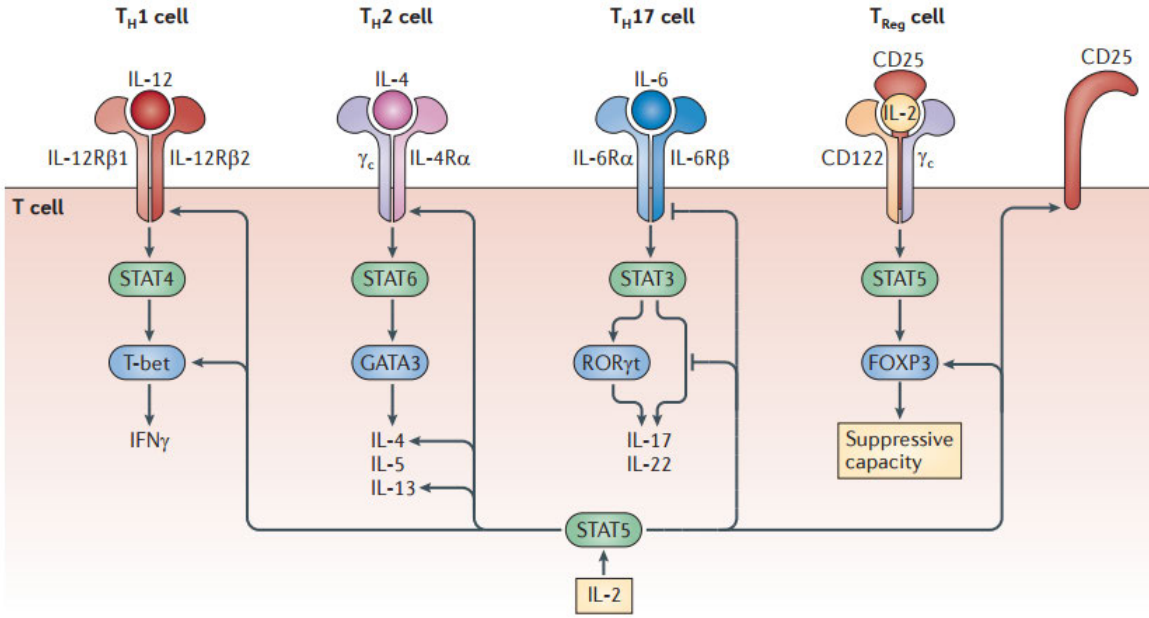
- **lower concentrations**






Cytokine
Extracellular
Cytoplasm
JAK
JAK
JAK
JAK
JAK
JAK
SOCS
PTP
STAT
STAT
STAT
STAT
STAT
STAT
PTP
PIAS
STAT
STAT
PTP
Gene transcription
ISRE/GAS motif
Nucleus

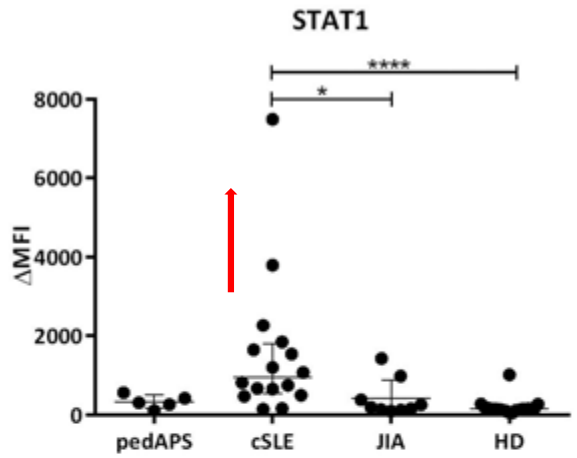


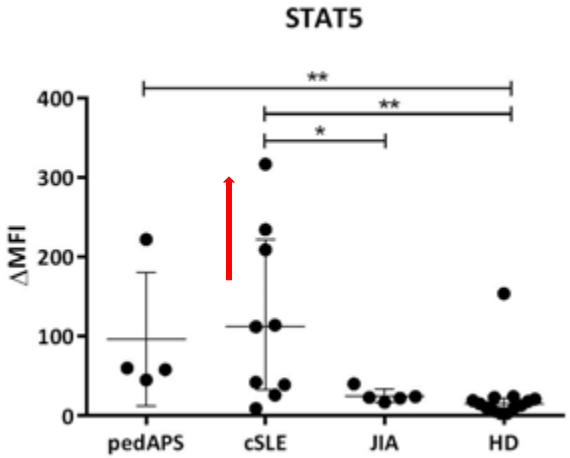


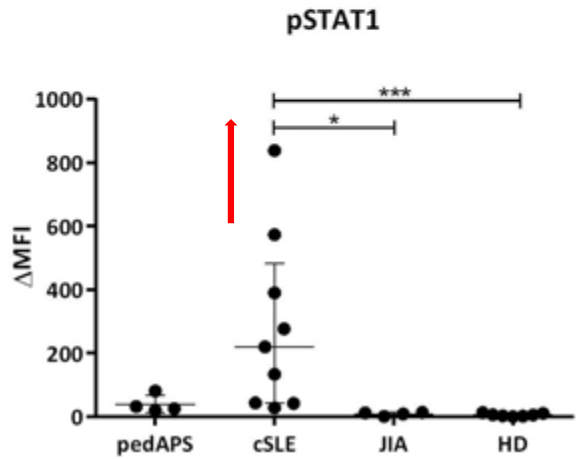
Begitt et. al., Nature Immunology, 2014; 15,168-176

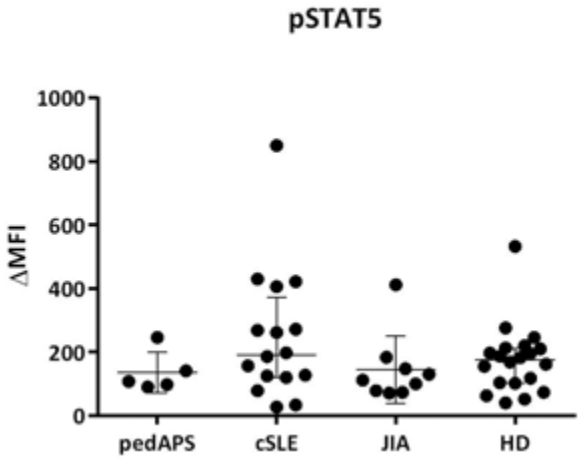
Boyman and Sprent, Nature Reviews Immunology, 2012; 12,80-190

INTRACELLULAR STAINING + FLOW CYTOMETRY (“PHOSFLOW”)

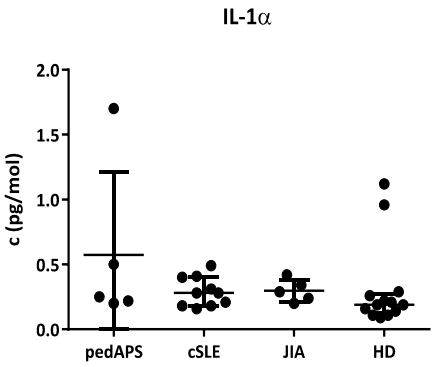


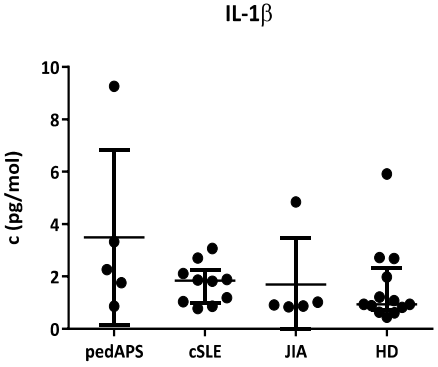


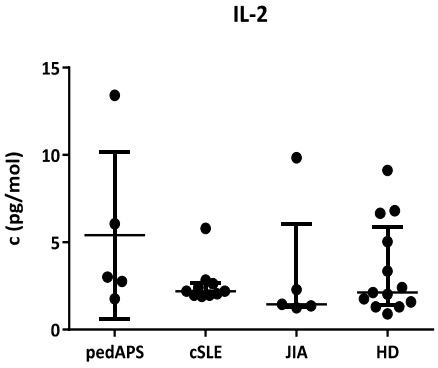


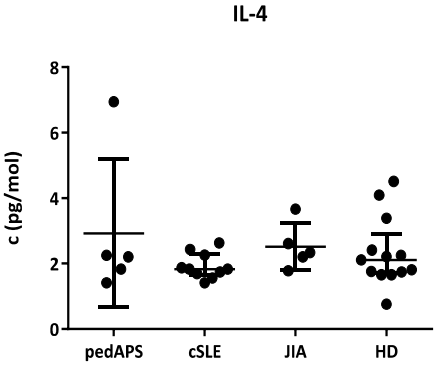


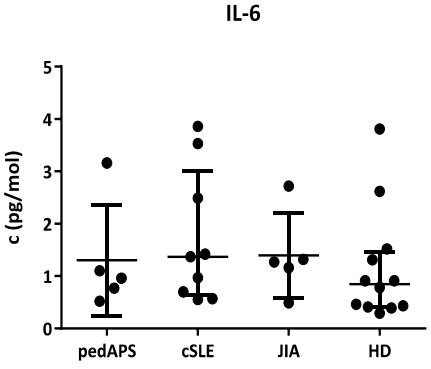
STAT5	$CD3^+CD4^+STAT5^+$
pSTAT5	$CD3^+CD4^+STAT5(pTyr694)^+$
STAT1	$CD3^+CD4^+STAT1^+$
pSTAT1	$CD3^+CD4^+STAT1(pTyr701)^+$

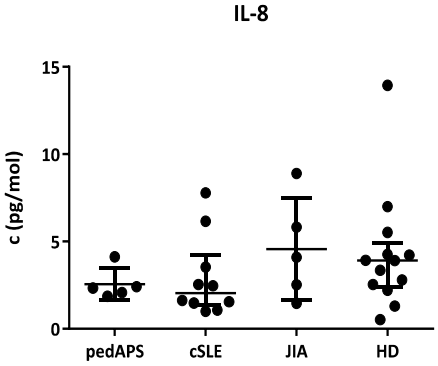


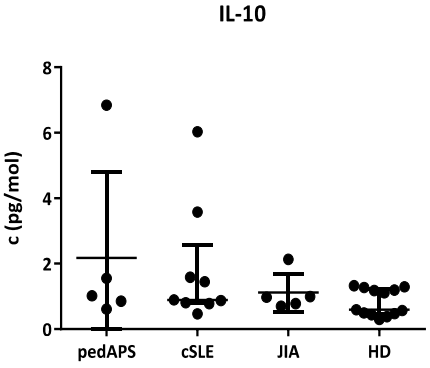


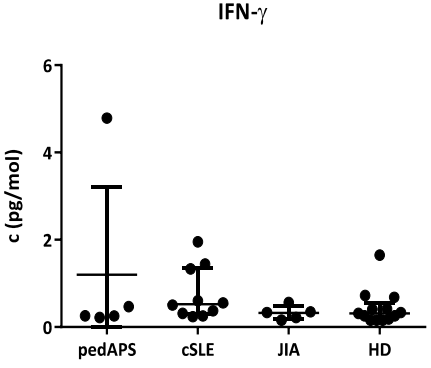


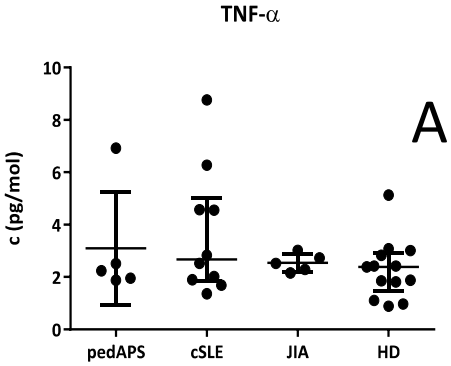












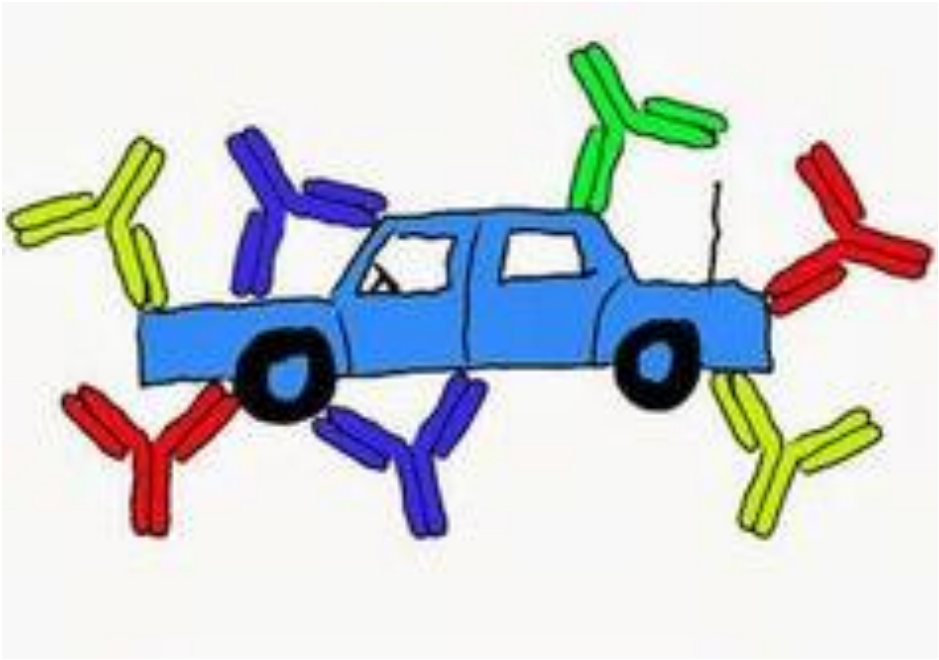
no differences in concentrations of pro-inflammatory or anti-inflammatory cytokines

↓

Autoimmunity driven by other cytokines

- Is there any correlation between SLEDAI and any of marker?
    - Disease severity
    - Organ damage

- Do our findings differ from adult-onset SLE population?
    - Age = immaturity of immune system/organs



Thank you

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