SECOND FACULTY OF MEDICINE
CHARLES UNIVERSITY

Diagnostic antibodies: CD nomenclature and beyond

Tomáš Kalina

IUIS Immunology without Borders
International Union of Immunological Societies

Standardization and CD nomenclature sub-committees

HCDM

Human Cell Differentation Molecules (HCDM)

CLIP - *Childhood Leukemia Investigation Prague*

ESCCA 2022, Belfast

All immunophenotyping relies on antibodies

HCDM and CD nomenclature

Human **C**ell **D**ifferentiation **M**olecules:

- *Independent, academic organisation which runs HLDA (Human Leucocyte Differentiation Antigens) Workshops and names and characterizes CD molecules.*
- *Nomenclature committee of the International Union of Immunological Societies (IUIS)*

HLDA Workshops I-X (1982-2014)

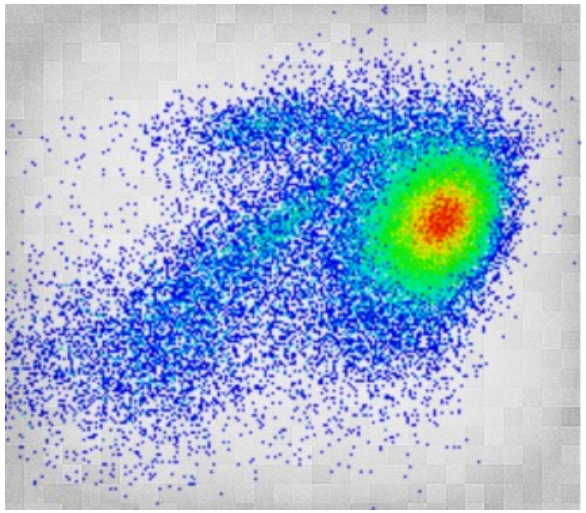
Workshop		CDs assigned
I. Paris	1982	CD1-CDw15
II. Boston	1984	CD16-CDw26
III. Oxford	1987	CD27-CD45
IV. Vienna	1989	CD46-CDw78
V. Boston	1993	CD79-CDw109
VI. Kobe	1996	CD110-CD166
VII. Harrogate	2000	CD167-CD247
VIII. Adelaide	2004	CD248-CD339
IX. Barcelona	2009	CD340-CD364
X. Wollongong	2014	CD365-CD371

Kalina, Engel, Lundsten:
Relevance of Antibody Validation for Flow Cytometry, ***Cytometry A, 2020***

http://hcdm.org/

Engel et al:
CD Nomenclature 2015: Human Leukocyte Differentiation Antigen Workshops as a Driving Force in Immunology, ***J Immunol, 2015***

Monoclonal antibodies and flow cytometry



The happy marriage of monoclonal antibody and multi-parameter flow cytometry

Outline

- Antibody failures -> Ab validation
- Mapping CDs expression – CD Maps
- Ab benchmarking

The problem

HLDA Workshops experience indicates that nearly 50% of the submitted antibodies failed to function for the recommended application, or their staining patterns were inconsistent with the previous literature or presented unexpected cross-reactivity, or even failed the most fundamental tests of activity or specificity

It has been estimated that there are more than 300 antibody suppliers providing >2.000.000 antibodies for the research and clinical markets

Monoclonal antibodies that do not bind or stain properly pose a huge problem ..

NEWS FEATURE

BLAME IT ON THE ANTIBODIES

Antibodies are the workhorses of biological experiments, but they are littering the field with false findings. A few evangelists are pushing for change.

BY MONYA BAKER

In 2006, things were looking pretty good for David Rimm, a pathologist at Yale University in New Haven, Connecticut. He had developed a test to guide effective treatment of the skin cancer melanoma, and it promised to save lives. It relied on antibodies — large, Y-shaped proteins that bind to specified biomolecules and can be used to flag their presence in a sample. Rimm had found a combination of antibodies that, when used to 'stain' tumour biopsies, produced a pattern that indicated whether the patient would need to take certain harsh drugs to prevent a relapse after surgery. He had secured more than US$2 million in funding to move the test towards the clinic.

But in 2009, everything started to fall apart. When Rimm ordered a fresh set of antibodies, his team could not reproduce the original results. The antibodies were sold by the same companies as the original batches, and were supposed to be identical — but they did not yield the same staining patterns, even on the same tumours. Rimm was forced to give up his work on the melanoma antibody set. "We learned our lesson: we shouldn't have been dependent on them," he says. "That was a very sad lab meeting."

Antibodies are among the most commonly used tools in the biological sciences — put to work in many experiments to identify and isolate other molecules. But it is now clear that they are among the most common causes of problems, too. The batch-to-batch variability that Rimm experienced can produce dramatically differing results. Even more problematic is that antibodies often recognize extra proteins in addition to the ones they are sold to detect. This can cause projects to be abandoned, and waste time, money and samples.

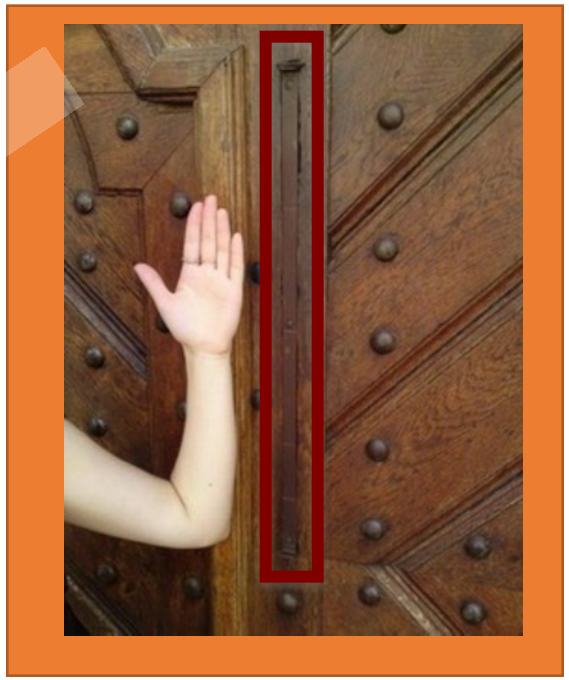
Many think that antibodies are a major driver of what has been deemed a 'reproducibility crisis', a growing realization that the results of many biomedical experiments cannot be reproduced and that the conclusions based on them may be unfounded. Poorly characterized antibodies probably contribute more to the problem than any other laboratory tool, says Glenn Begley, chief scientific officer at TetraLogic Pharmaceuticals in Malvern, Pennsylvania, and author of a controversial

274 | NATURE | VOL 521 | 21 MAY 2015

Baker, Nature, 2015

What is the unit of “goodness” of Ab?

Hradcany Townhall
Loretánská street 173/1



“Prague cubit”
est 1228

“Prague cubit”
est 1228
“Viennese cubit”
est 1765

What do we need to know about Ab

- Does it recognize its intended target?
- Does it cross-react with another target?
- How reproducible is the staining pattern?

What is the evidence?

- is the evidence good enough?
- how can we independently validate it?

Is this valid on all cells or just some (transfectants?)

How good is good enough?

- sensitivity
- method used

Main reasons for antibody failure

Cross-reactivity
Reactivity with other proteins with which they share sequence identity.
Antibodies can also exhibit cross-reactivity to epitopes that are not predictable based on sequence analysis

Lack of binding to the endogenous or natural protein
Some mAbs specifically recognize recombinant proteins or transfected cells, over-expressing the target antigen, do not recognize the antigen on cell lines or normal primary cells

Lack of specific recognition of the same target in different species
Species cross-reactivity must be validated experimentally

Batch-to-batch variability
Even the same monoclonal antibody (clone) from different suppliers may exhibit variability in performance

Wrong application
Most of the antibodies are not effective across all techniques

Improper dilution of the antibody
Using the recommended dilution of the antibody by the vendor is not a guaranty of specificity and selectivity

What is antibody validation?

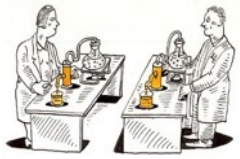
Proof that the Ab is specific, selective, and reproducible in the context for which it is used.

Specificity + **Selectivity** + **Reproducibility**

capability to bind specifically to one unique epitope

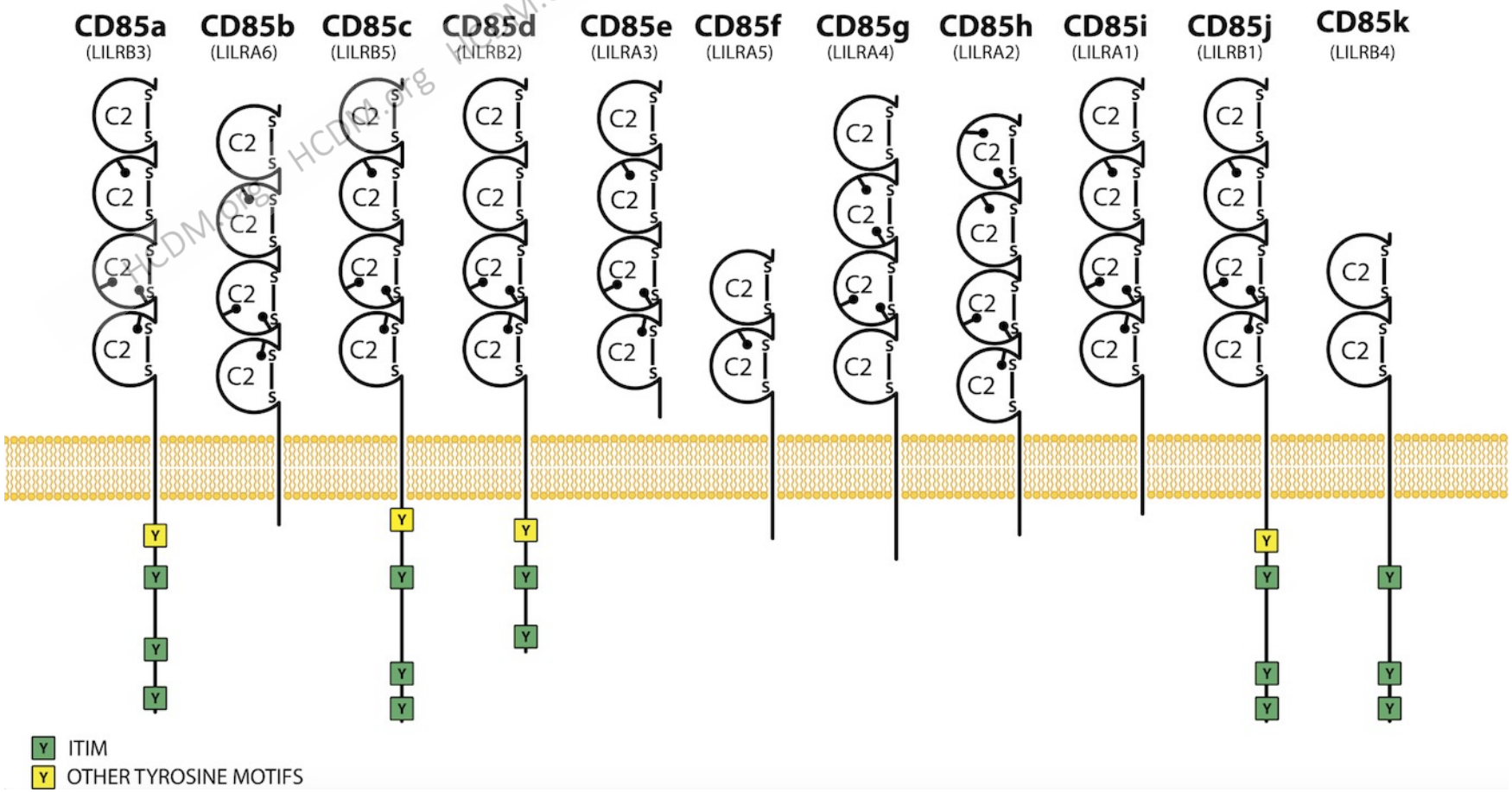
ability of an Ab to react only with one antigen

ability to duplicate results over long periods of time by different laboratories



Example cross-reactivity CD85 (LILR family)

CD85/LILR is a family of 11 cell-surface molecules.
Some function as activators (A) and other as inhibitors (B) of leukocyte function.
They present a high sequence homology (52 to 97%).



P. Engel

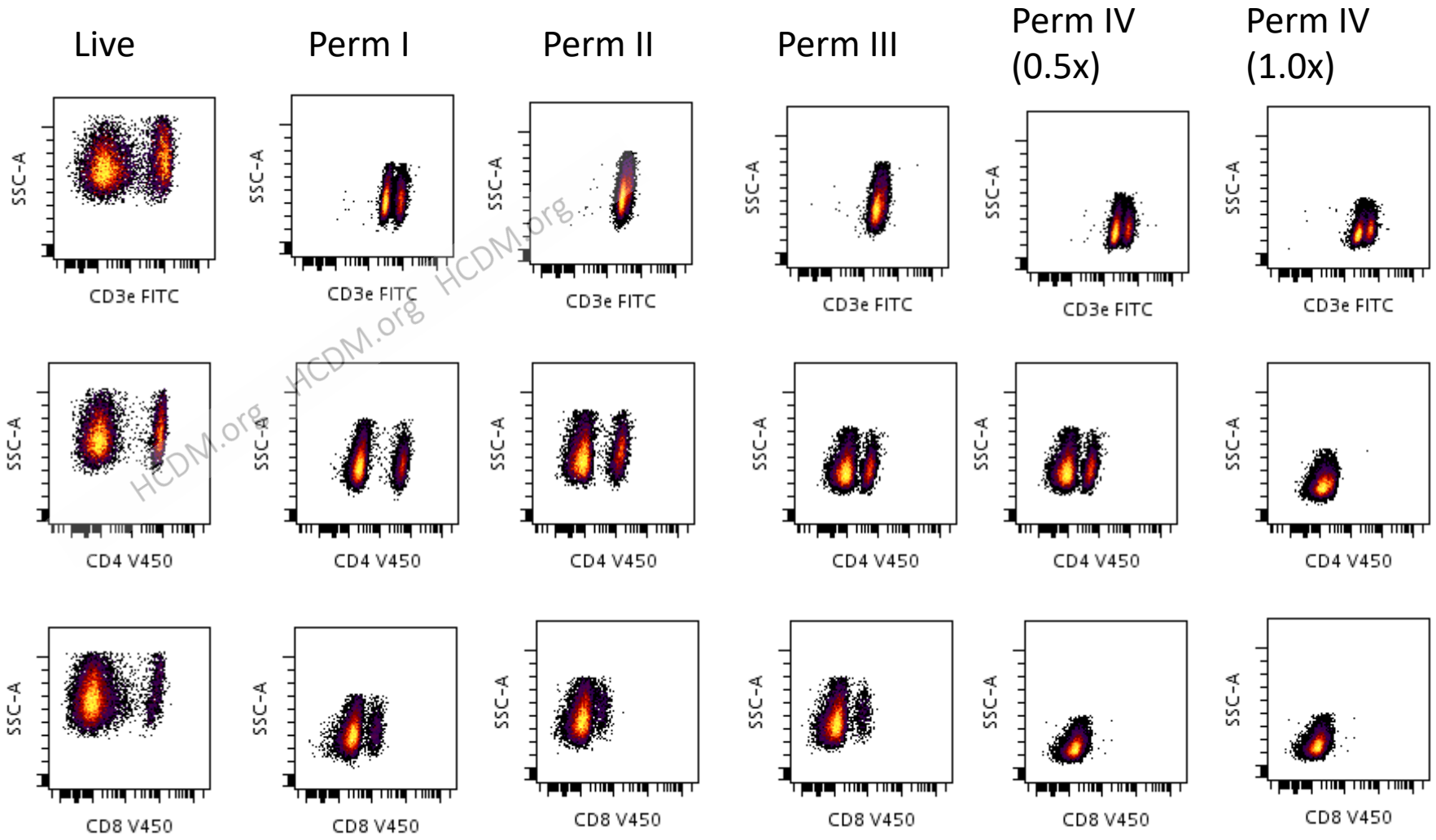
Example cross reactivity CD85 (LILR family) mAbs

Reactivity of several commercial mAbs with COS cells transfected with cDNA transfected cells

Ab/Cells	COS	COS-hCD85a	COS-hCD85b	COS-hCD85c	COS-hCD85d	COS-hCD85f	COS-hCD85h
CD85a (LILRB3) MKT5.1	-	++	++	-	-	++	-
CD85a (LILRB3) 222821	-	++	++	-	-	++	-
CD85b (LILRA6) 921330	-	++	++	-	-	-	-
CD85c (LILRB5) Polyclonal	-	+	-	++	-	-	-
CD85d (LILRB2) 42D1	-	-	-	-	++	-	-
CD85d (LILRB2) 287219	-	++	++	-	++	-	+
CD85d (LILRB2) 27D6	-	-	-	-	++	-	-
CD85e (LILRA3) Polyclonal	-	++	+	-	++	-	+
CD85f (LILRA5) 711828	-	-	-	-	-	++	-

P. Engel

Reactivity is affected by the fixation/permeabilization protocol



Data from BD www.cytobank.org/facselect/

Some solutions to this problem

User should test/validate the monoclonal antibodies before using them in the lab

Description of suppliers datasheets should present validation data of the monoclonal antibodies (including images)

Journals should implement antibody validation requirements for their published articles

Create antibody validation guidelines and structures of independent validation

http://hcdm.org/

Monoclonal antibodies as reagents – what do we need to know before purchase ?

Identity and published history – Ab clone name

Was it „HLDA workshopped"?

Specificity (=target) , immunogen, epitope, cross blocking

Native or denatured immunogen ?

Reactivity (anti-human), **Selectivity** (e.g. CD66, CD85)

Cross reactive with other species, other proteins ?

Application (flow cytometry, IP, WB)

Does the epitope withstand sample prep conditions (denaturation) ?

REVIEW ARTICLE

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

Relevance of Antibody Validation for Flow Cytometry

Tomas Kalina,[1*] Kelly Lundsten,[2] Pablo Engel[3]

Antibody validation …. a good practice

Reagents:
Antibody validation
CD352
SLAMF6

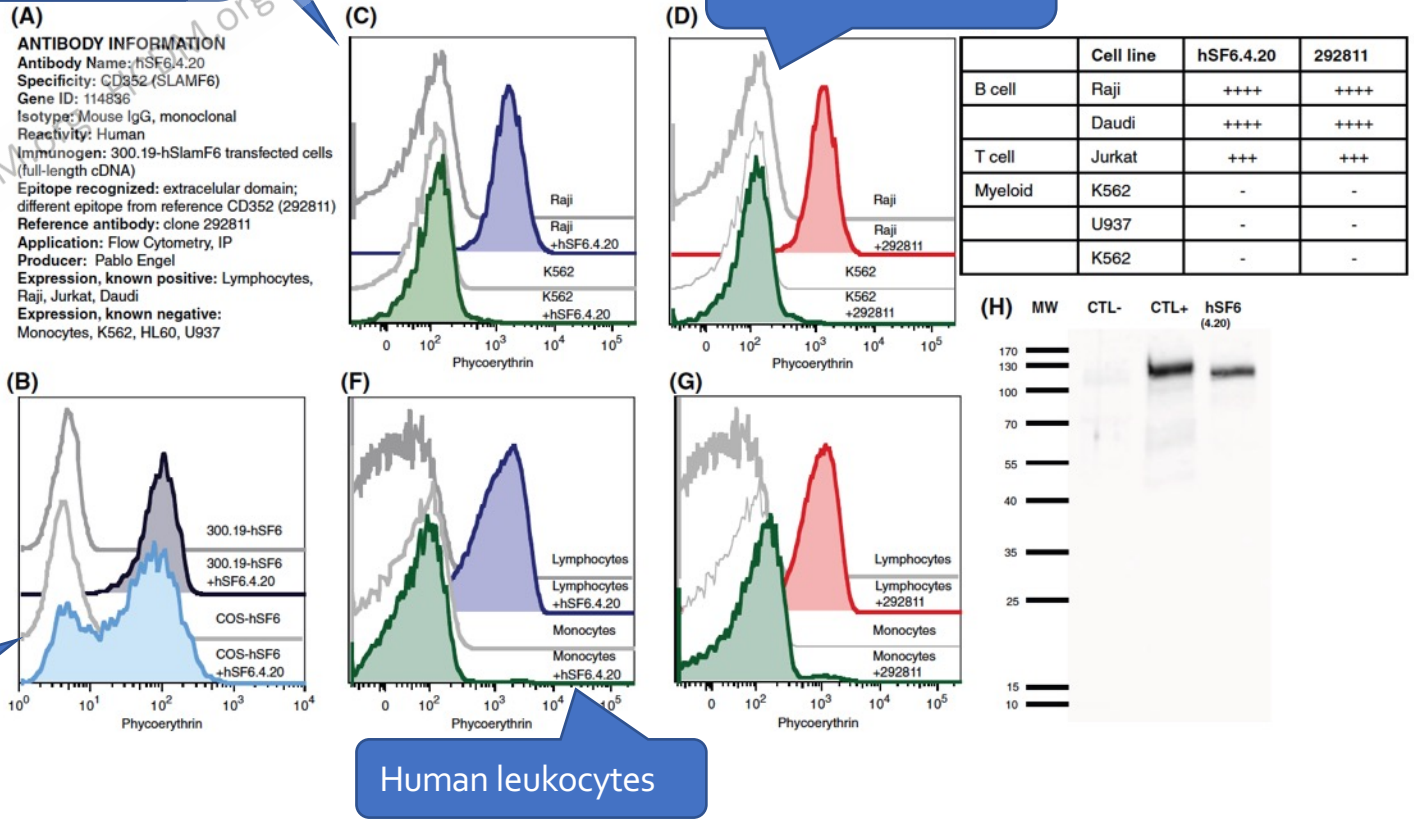
Know positive cell line Raji (blue) and known negative cell line K562 (green)

Reference clone

Ab info

Transfected cell lines

Human leukocytes



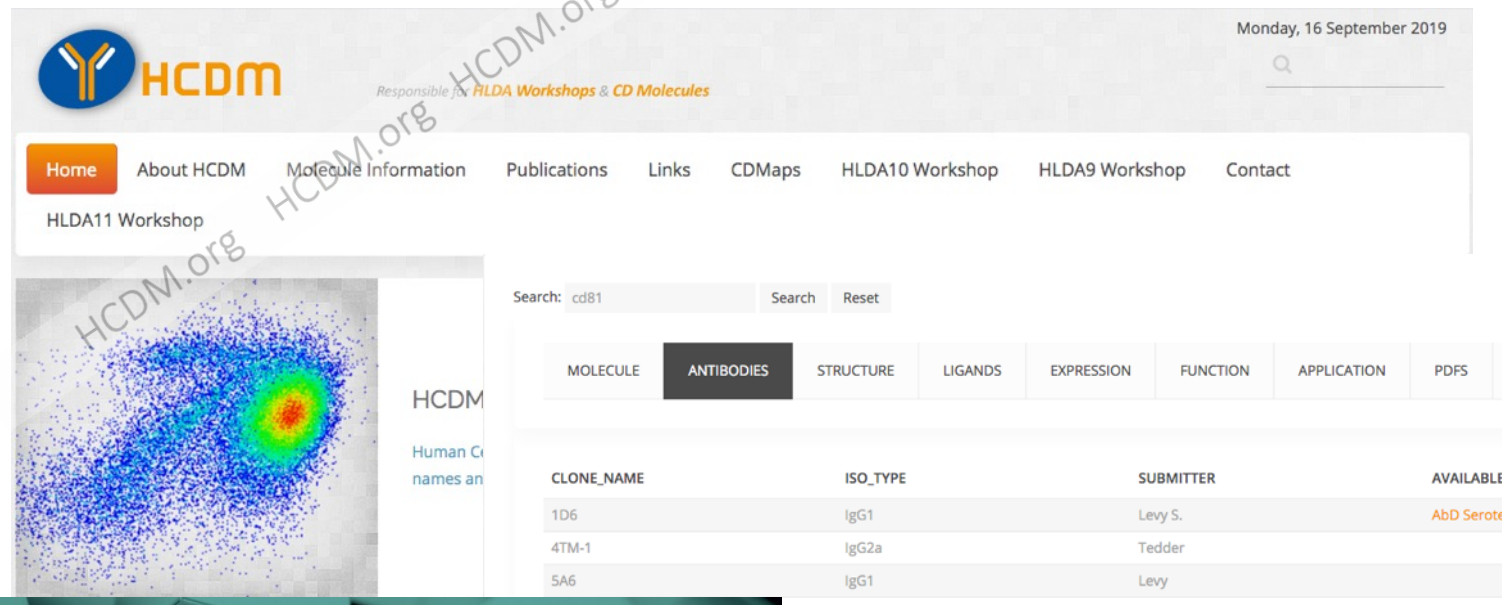
	Cell line	hSF6.4.20	292811
B cell	Raji	++++	++++
	Daudi	++++	++++
T cell	Jurkat	+++	+++
Myeloid	K562	-	-
	U937	-	-
	K562	-	-

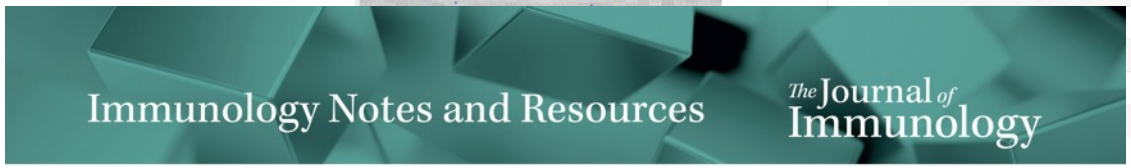
Kalina, Engel, Lundsten:
Relevance of Antibody Validation for Flow Cytometry, Cytometry A, 2020

HCDM .. A resource of HLDA validated clones

Specificity – HLDA workshops – panel of Antibodies evaluated by panel of expert labs

HCDM.org





CD Nomenclature 2015: Human Leukocyte Differentiation Antigen Workshops as a Driving Force in Immunology

Pablo Engel,* Laurence Boumsell,† Robert Balderas,‡ Armand Bensussan,§ Valter Gattei,¶ Vaclav Horejsi,‖ Bo-Quan Jin,# Fabio Malavasi,** Frank Mortari,†† Reinhard Schwartz-Albiez,‡‡ Hannes Stockinger,§§ Menno C. van Zelm,¶¶ Heddy Zola,‖‖ and Georgina Clark##

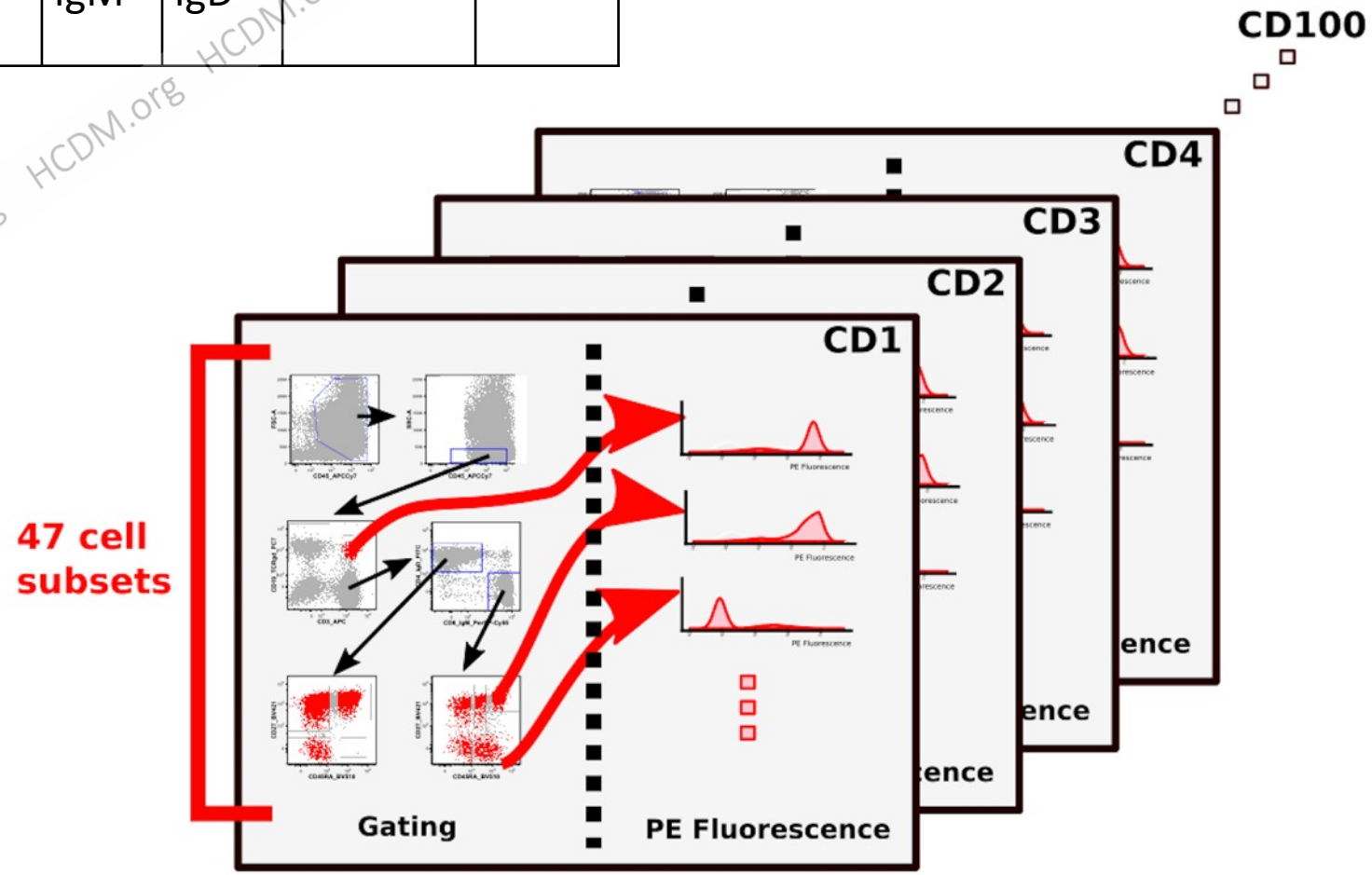
CD Maps – phase I:
HCDM project to map CDs' expression

- CD Maps pilot project (CD1-CD100)
- mapping the expression of CD1–CD100 (n = 110) on 47 immune cell subsets from blood, thymus, and tonsil

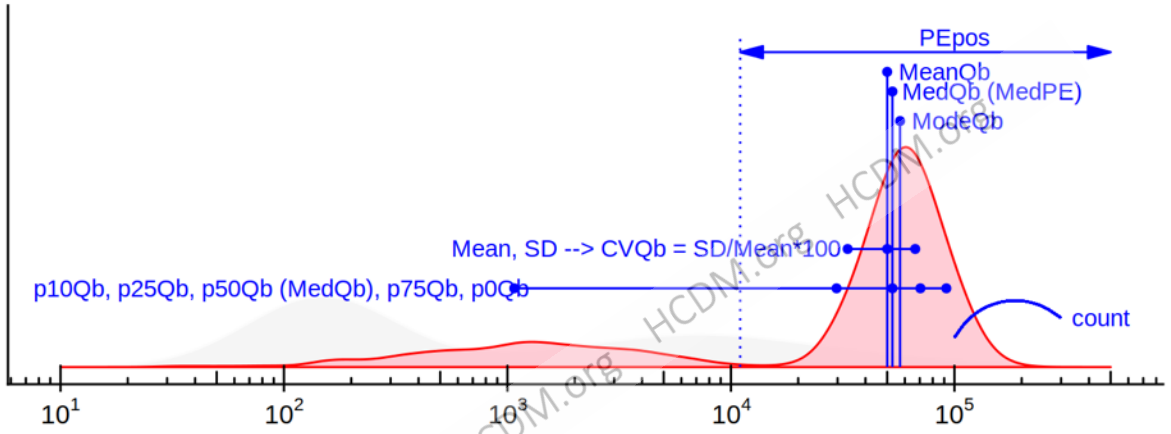
CD Maps I: Blood B- and T-cell tube

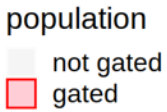
1	2	3	4	5	6	7	8
PE marker	CD45	CD3	TCRgd CD19	CD4 IgM	CD8 IgD	CD45RA	CD27

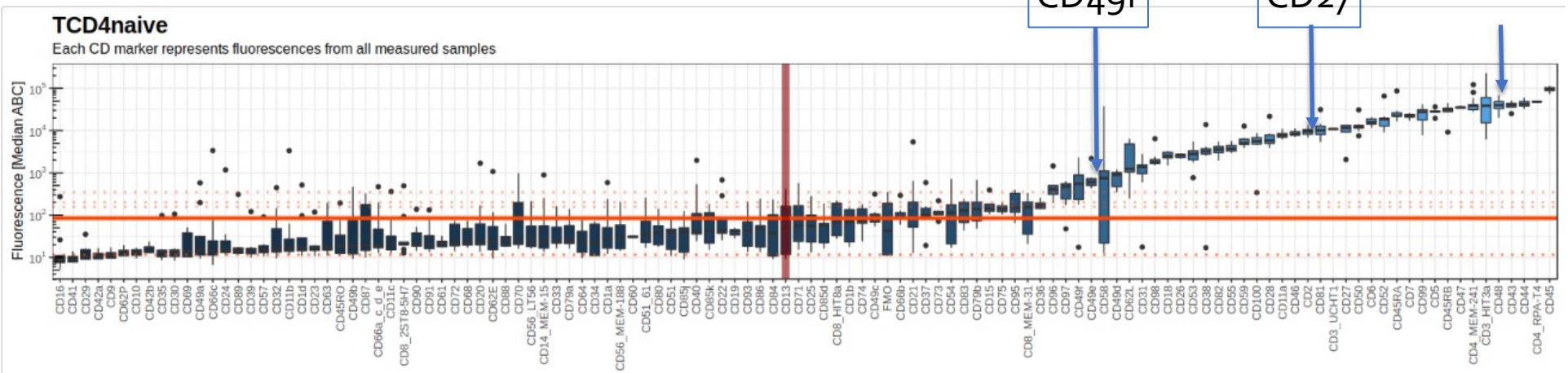
(example of one tube of four)

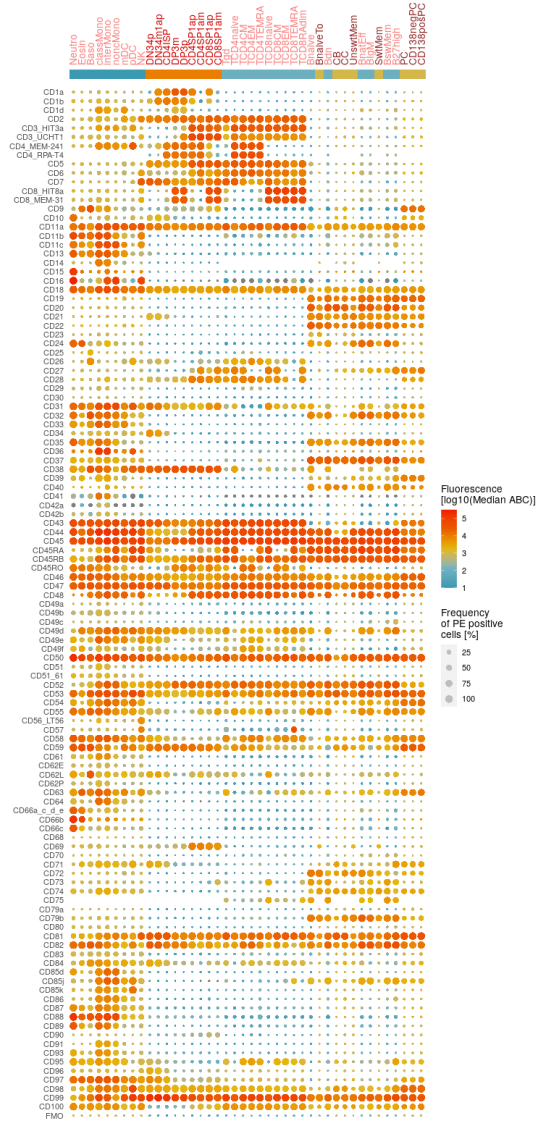


CD Maps – quantity of expression







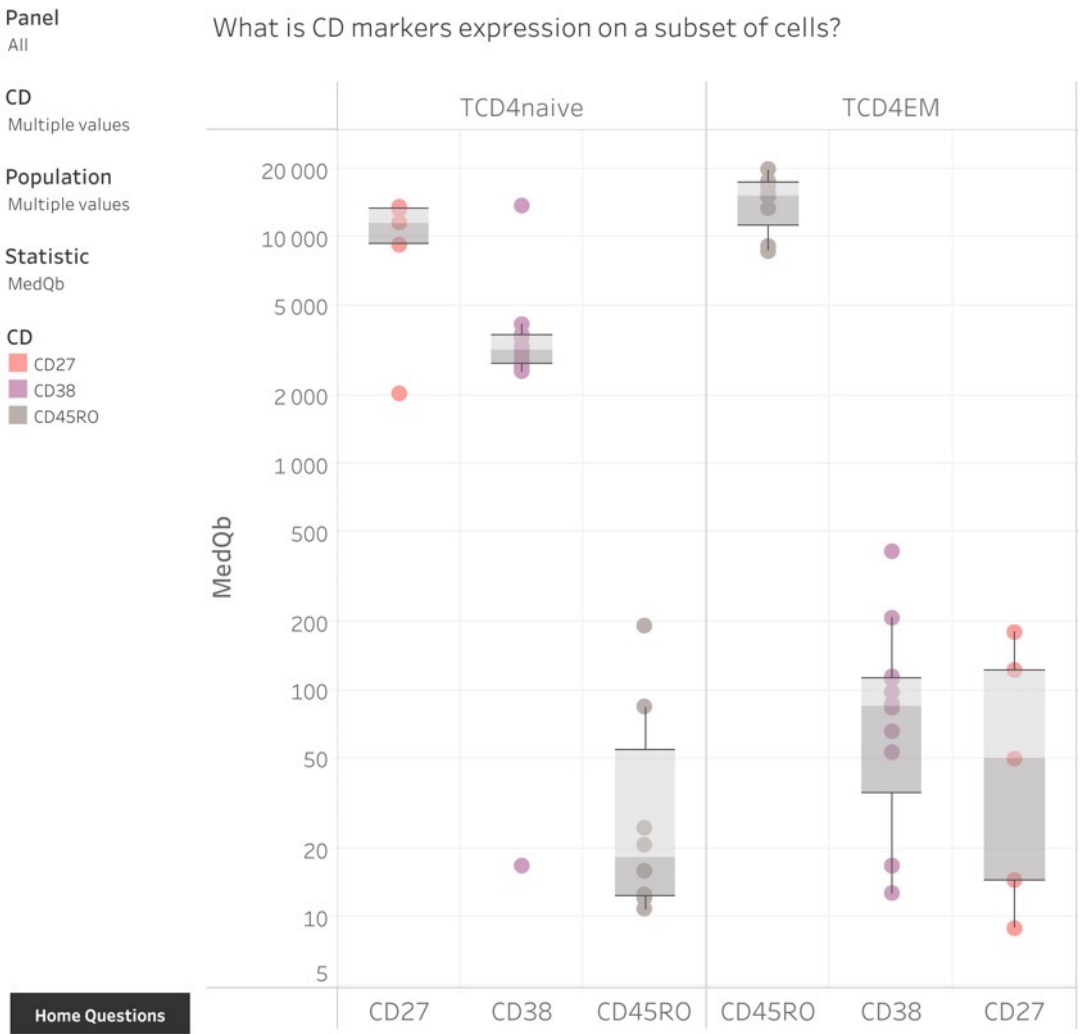


CD Maps – phase I:
HCDM project to map CDs' expression

- Dynamic resource on the web

www.hcdm.org /CDMaps application

https://public.tableau.com/app/profile/fanny2212



Kalina et al: CD Maps-Dynamic Profiling of CD1-CD100 Surface Expression on Human Leukocyte and Lymphocyte Subsets. Frontiers in Immunology, 2019

CD Maps – phase II:
HCDM project to map CDs' expression

- Methods and standardization improvements

  Kužílková D, Puñet-Ortiz J, Aui PM, Fernández J, Fišer K, Engel P, van Zelm MC, Kalina T. Standardization of Workflow and Flow Cytometry Panels for Quantitative Expression Profiling of Surface Antigens on Blood Leukocyte Subsets: An HCDM CDMaps Initiative. *Front Immunol* (2022) **13**:1–15. doi:10.3389/fimmu.2022.827898

- CD Maps (**CD1-CD371**) – in progress
- CD Maps on HLDA 11 workshop – in progress
- Dynamic resource on the web – in progress

Fanny Hedin
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- Business intelligence tools for data interaction
  - Luxembourg Institute of Health

Antonio Cosma
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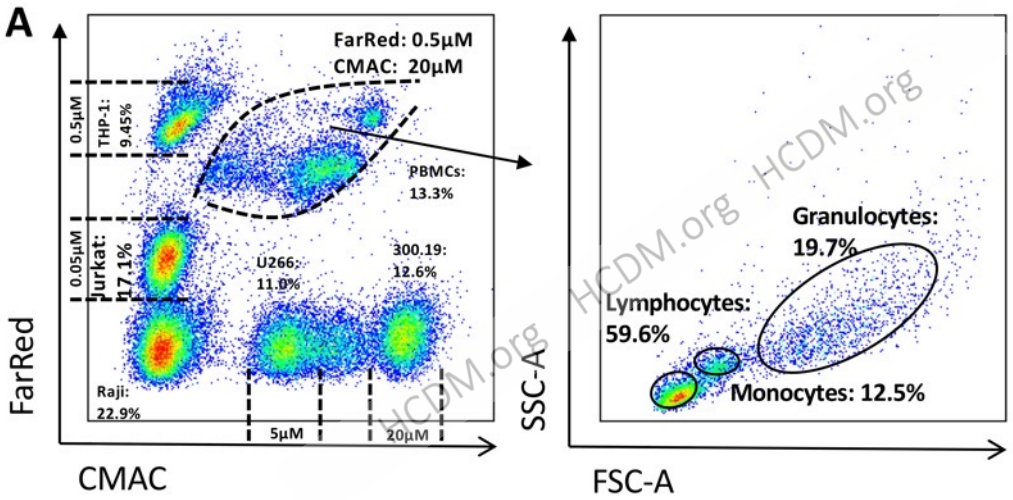
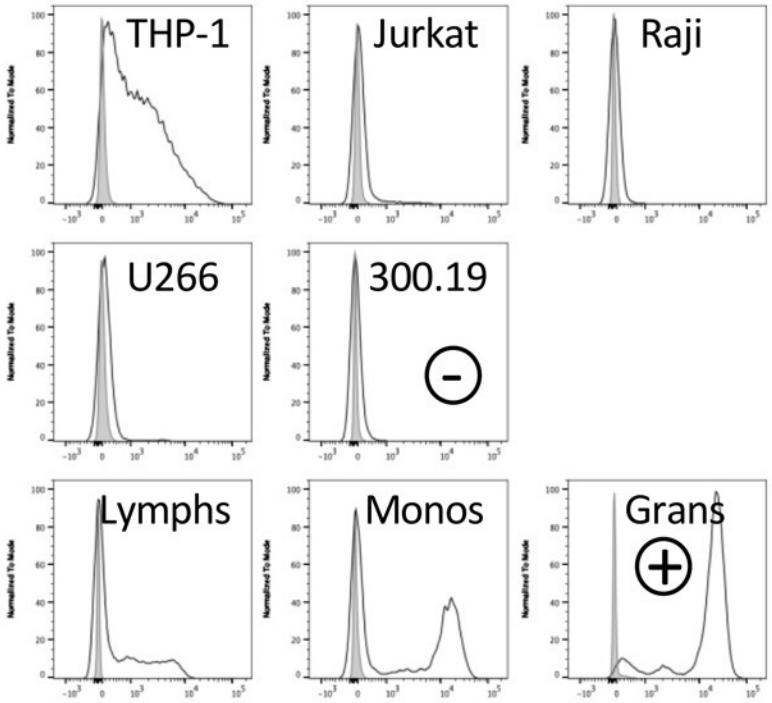
Hedin F, et al. Data integration and visualization techniques for post-cytometric analysis of complex datasets. *Cytom Part A* (2021)

https://public.tableau.com/app/profile/fanny2212

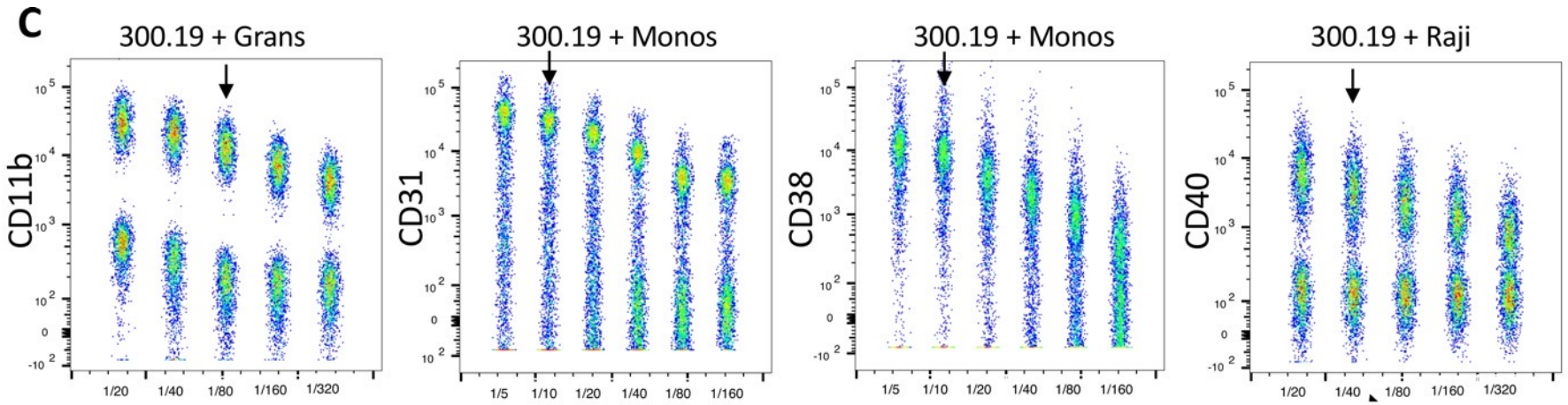
CD Maps II - titration



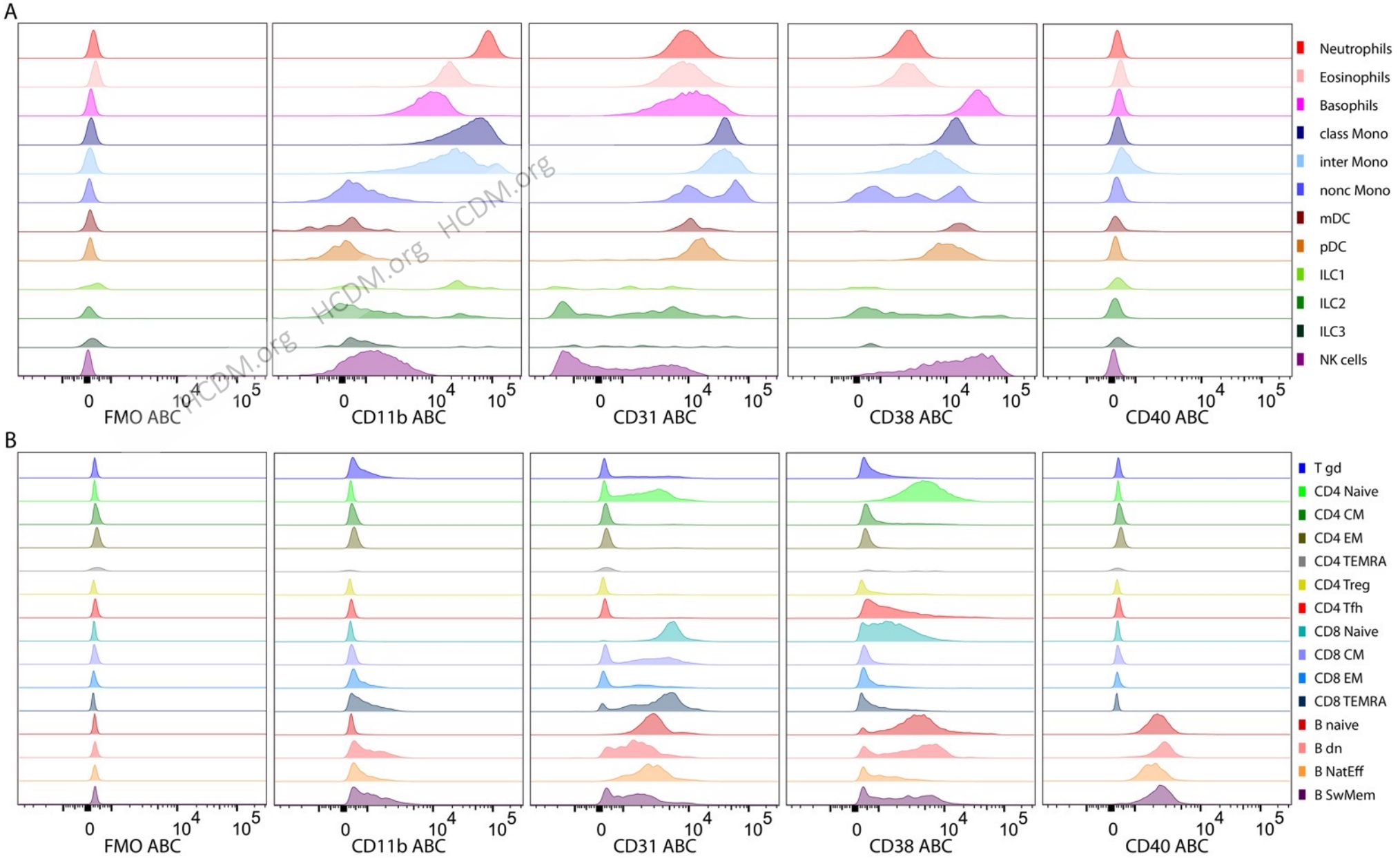
PBMC + Barcoded cell lines



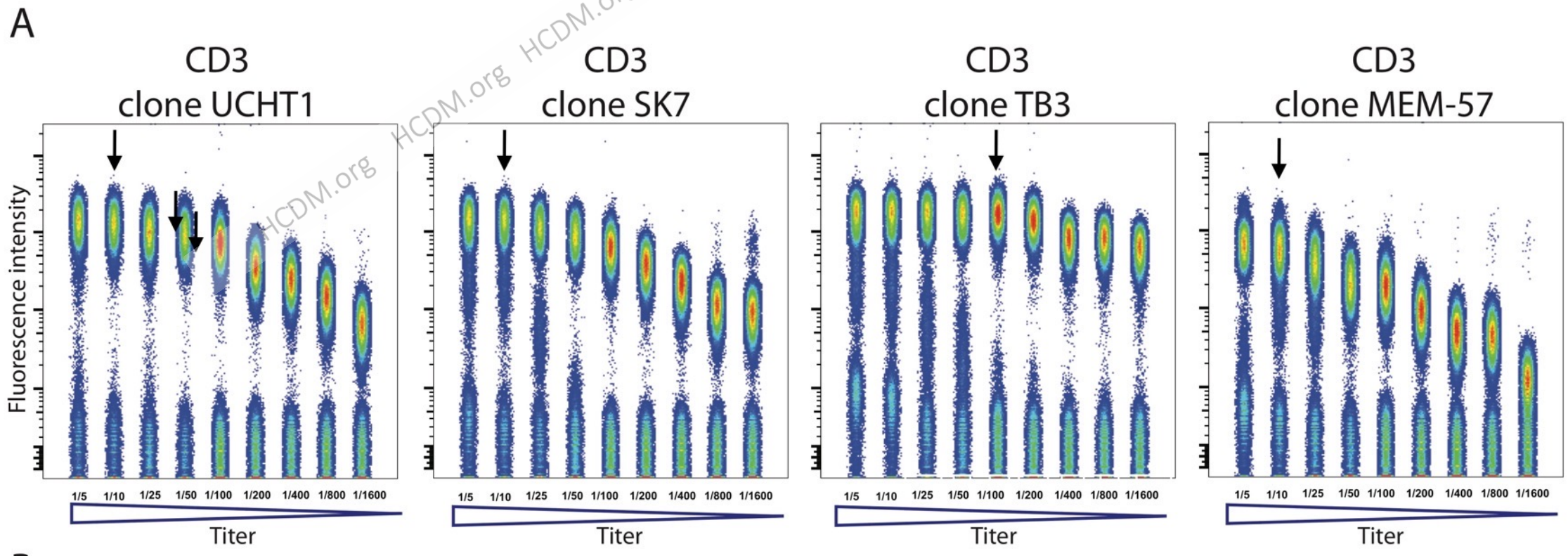
Pos & Neg subsets



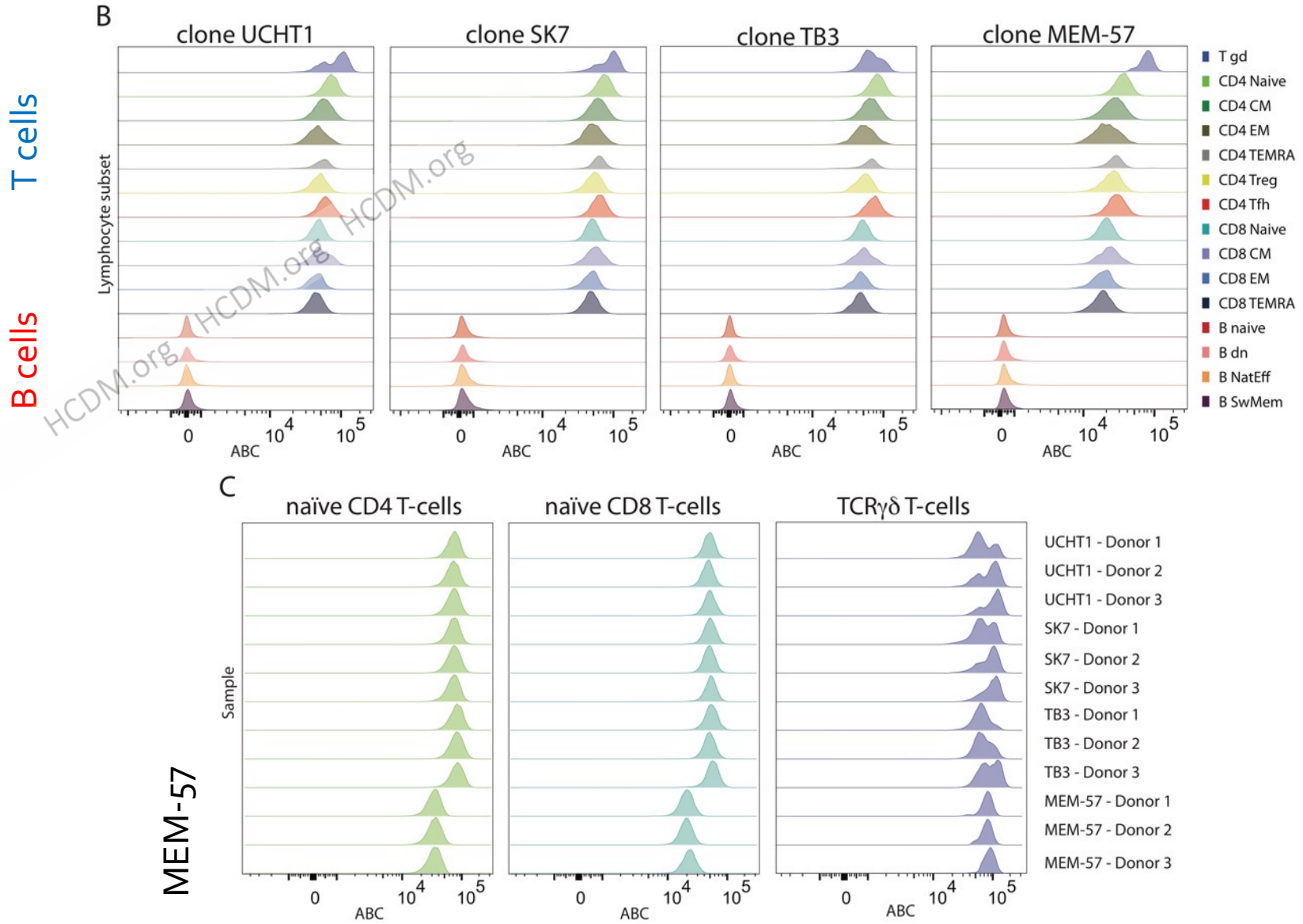
CD Maps II – expression levels per subset



CD Maps – benchmarking CD3 clones



CD Maps – benchmarking CD3 clones



Summary

Ab validation is essential

HLDA validated clones at HCDM.org

Expression quantity (CD1-CD100 at HCDM.org) / CDMaps application

.... Beyond CD100 - in progress

.... New CD markers in HLDA 11 – in progress

Robust CD Maps method building

Ab clone characterisation and benchmarking feasible

Future perspectives

Reagent benchmarking

Detailed expression & performance resource

Thank you

CLIP

Cytometry lab

Daniela Kužílková

Karel Fišer

Pablo Engel

Menno C. van Zelm

Sophinus J. W. Bartol, Pei Mun Aui
Martin Perez Andres, Elena Blanco Álvarez
Marta Cuenca, Javier Fernández Calles, Joan Puñet Ortiz

EXBIO Antibodies

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Ministry of Health of the Czech Republic
project no. 15-26588A, NU20-05-00282.