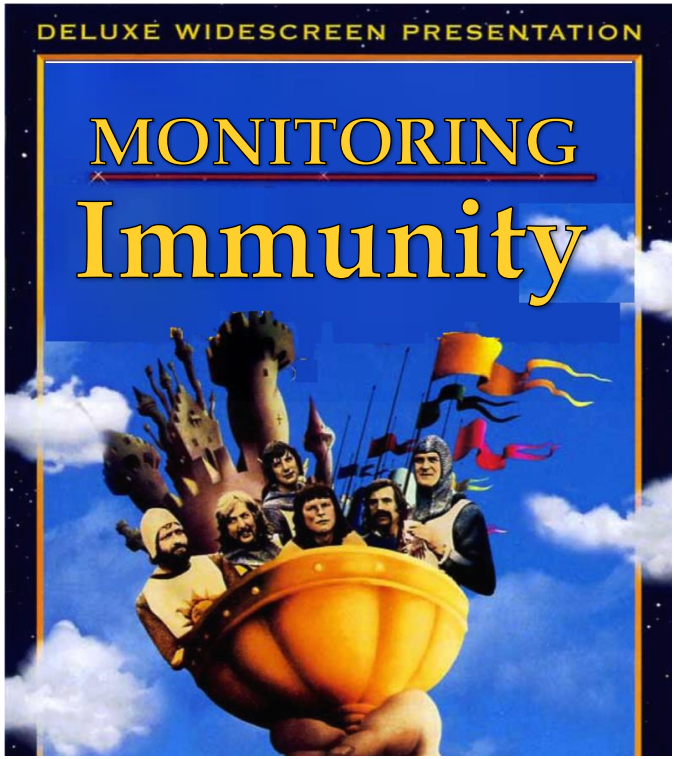
Immune monitoring in health and disease



(Can it be done?)

Florian Kern
Division of Medicine
BSMS
Brighton, UK

Note

- Talk sponsored by Beckman Coulter
- I hold an appointment as product developer with JPT Peptide Technologies

Introduction

‘Immune monitoring’ means measuring any parameter or function of the immune system and repeating the measurement(s) across a population and/or over time.

General considerations of any immune monitoring:

1) Is the measurement reproducible?

Different values in different people should reflect **biological differences** rather than **intra-assay or inter-assay variations** of the method. Changes over time should reflect biological changes (same criteria as above).

2) Is the measurement clinically relevant?

If the monitoring is meaningful, detected biological changes will have consequences for health (for example rejection in a transplant patient). The result of the monitoring test could lead to an intervention (for example, anti-rejection therapy in that transplant patient)

This presentation will focus on **immune monitoring by flow-cytometry** and related assays.

Example 1: Organ transplantation

The main questions will be:

- Immune activation/infection
- Graft rejection
- Too much or not enough Immunosuppression
- CMV-specific T-cells, other Ag-specific T-cells (BK virus, EBV, Adenovirus, HHV-6…)

Example 2: General screening

The main questions will be:

- Presence of immunodeficiency
- Presence of chronic immune activation (chronic inflammation)
- Immune status to confirm someone’s good health

Example 3: Immunosenescence/reduced immune responsiveness in older people

The main questions will be:

- Immunocompetence
- Presence of chronic inflammation
- Autoimmunity
- Expected responsiveness to vaccines
- CMV- and other antigen-specific T-cells

This presentation will focus on immune senescence

Examples of changing immunity in senescent (older) people…

- **Decreased response to vaccines** (typically the flu vaccine is being studied)
- **More chronic inflammatory/autoimmune disease**
- **Increased incidence of Tumors**
- **Constitutive low-grade inflammation** (‘inflammaging’), i.e. increased baseline CRP
- **Increased susceptibility** to infections (e.g. gut, lung, urinary tract)
- Higher incidence of **sepsis**
- **Reactivation** of **latent infections** (e.g. tuberculosis)

Immunosenescence and biological age

The ‘age’ of the immune system is generally linked to the ‘biological age’ of the rest of the organism. Many tissues not considered to be part of the immune system still act as part of the overall immune defense. Consider for example

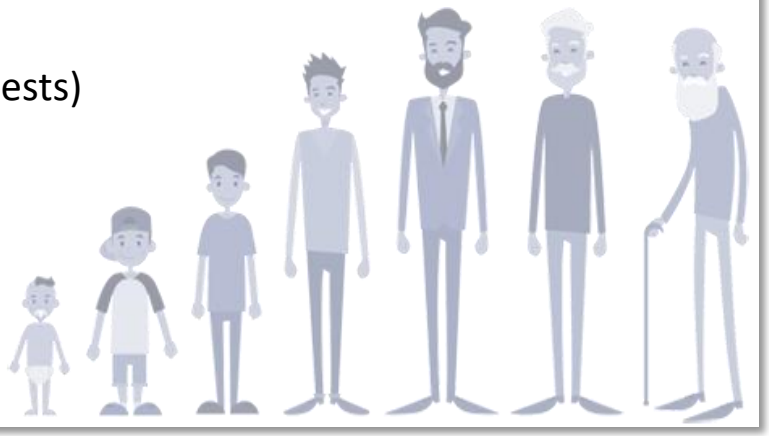
- skin and mucosal barriers
- Changes in nutrient absorption and metabolism and their consequences
- Changes in microbiota and subsequent changes to metabolic products
- Cognitive changes and increased risk exposure, sense of taste and smell

Can we measure biological age?

(pubmed returns 127,915 entries upon searching ‘biological age’)

Measurements taken at the whole organism level include

- Physical/mental performance tests
  (Activities of daily life, deficit accumulation, cognitive tests)
- Vascular status
  (e.g. vascular compliance, carotid sonography or MRI)
- How young or old does someone look?
- Grip strength



Observable effects of ageing on the **adaptive immune system**

T-cells	
Process/Mechanism	**Effect**
Involution of thymus	about 80% Decrease in **thymocytes** and **thymic hormones**, increase in memory vs. naïve CD4 cells; accumulation of dysfunctional memory cells
Impaired T-cell proliferation	Little clinical correlation, even when adjusted for relative sensitivity to mitogen; less effect when fixed antibody used to stimulate T cells
T-cell Cytokines, Th1 response	**Decrease in IL-2 and IL-2R, variable IFN-g** (variable study outcomes)); impaired upstream events: IL-2R expression, decreased activation of AP-1/NF-AT, decreased phosphorylation of MAP kinase, calcium signaling
Cytokines, Th2 response	Increase in PGE2 and IL-10, variable IL-4; **decreased costimulatory molecule expression** (CD28), cytokine shift reversed by IL-12

Impaired activation & impaired proliferation may be related to decreased co-stimulatory molecules on APC including MHC II and CD40

Adapted from Castle et al. 2002

Senescent people versus senescent cells

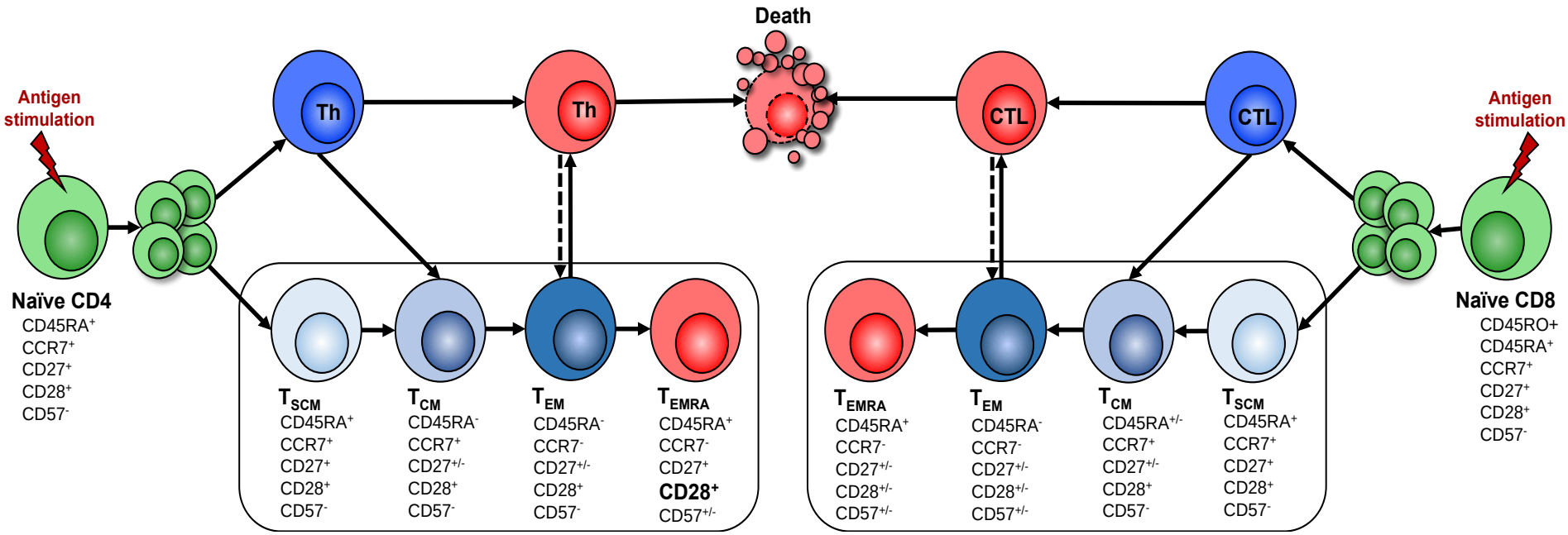
- Senescent people may have fully functional cells
- Senescent cells may be found in young people
- Older people are more likely to have more senescent cells

Part I: Monitoring immunosenescence

Possible approaches to testing/monitoring immunosenescence

- Immunophenotyping (T cells, B-cells, APCs…)
  - T cell markers of memory (CD45RA/RO, CD27, CCR7, etc.), terminal differentiation ($CD28^{null}$), exhaustion (PD1, KLRG1), etc….
- In vitro proliferation studies (CFSE, PKH, BrdU…)
  - Response to polyclonal stimuli, specific stimuli, etc.
- 'Test vaccination' and measurement of antibody titers
  - Flu vaccine
- Telomer length of immune cells
- DNA methylation age of immune cells
- Global functional tests with broad antigen pools, e.g. a CEFX pool

Phenotype changes in (immune) ageing



Aging is associated with an increase of T-cell populations of an antigen-experienced, phenoptype commensurate with advanced differentiation

Exemplar immune monitoring panel

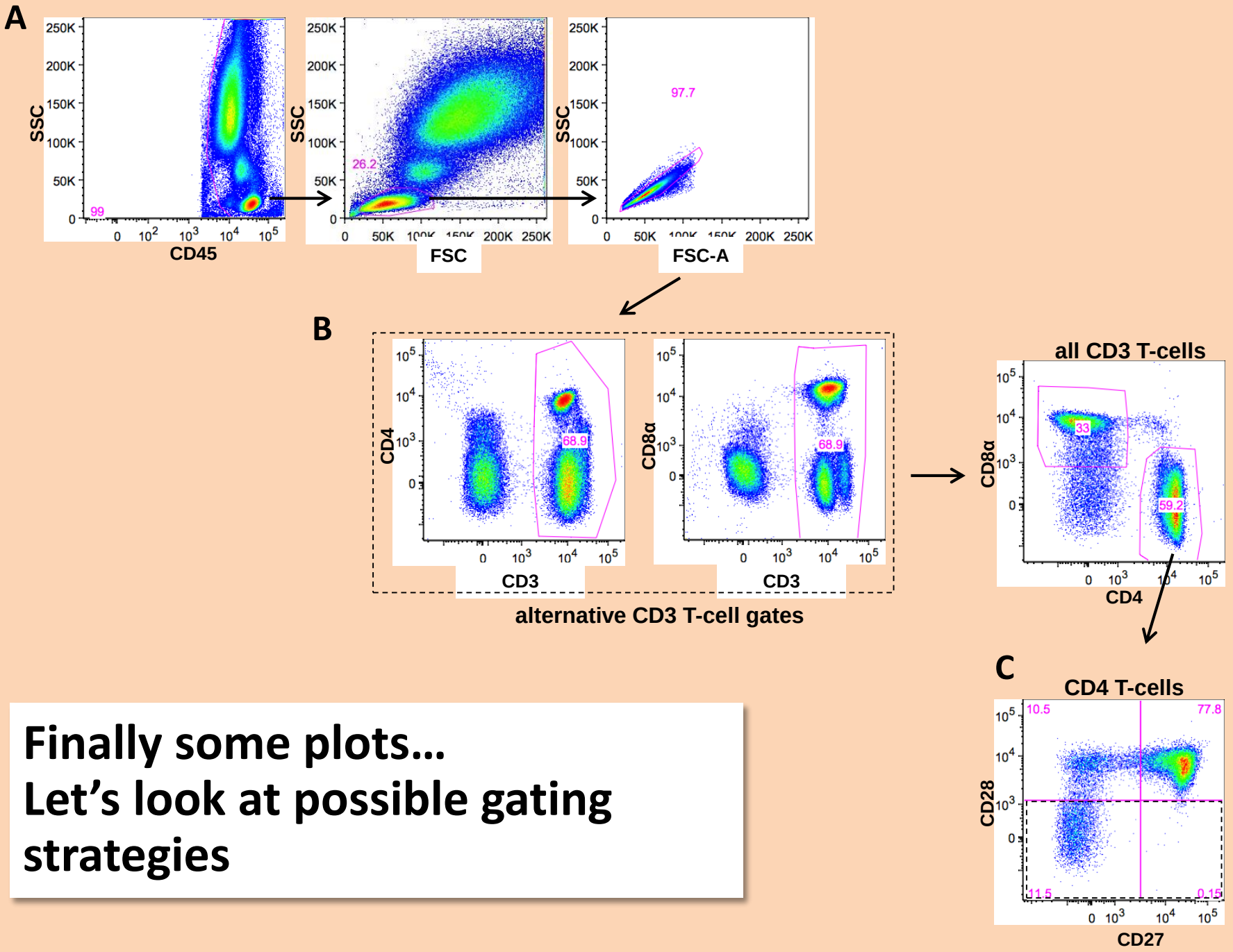
Fluorochrome	T□memory
PB	CD3
BV510	CD4
BV605	CCR7
APC	CD27
Alexa□700	CD45
APC□H7	CD8a
FITC	CD45RA
PE	CD57
PETR/ECD□etc.	optional
PerCP(Cy5.5)	CD45RO
PECy7	CD28

Use of markers in a flow-panel

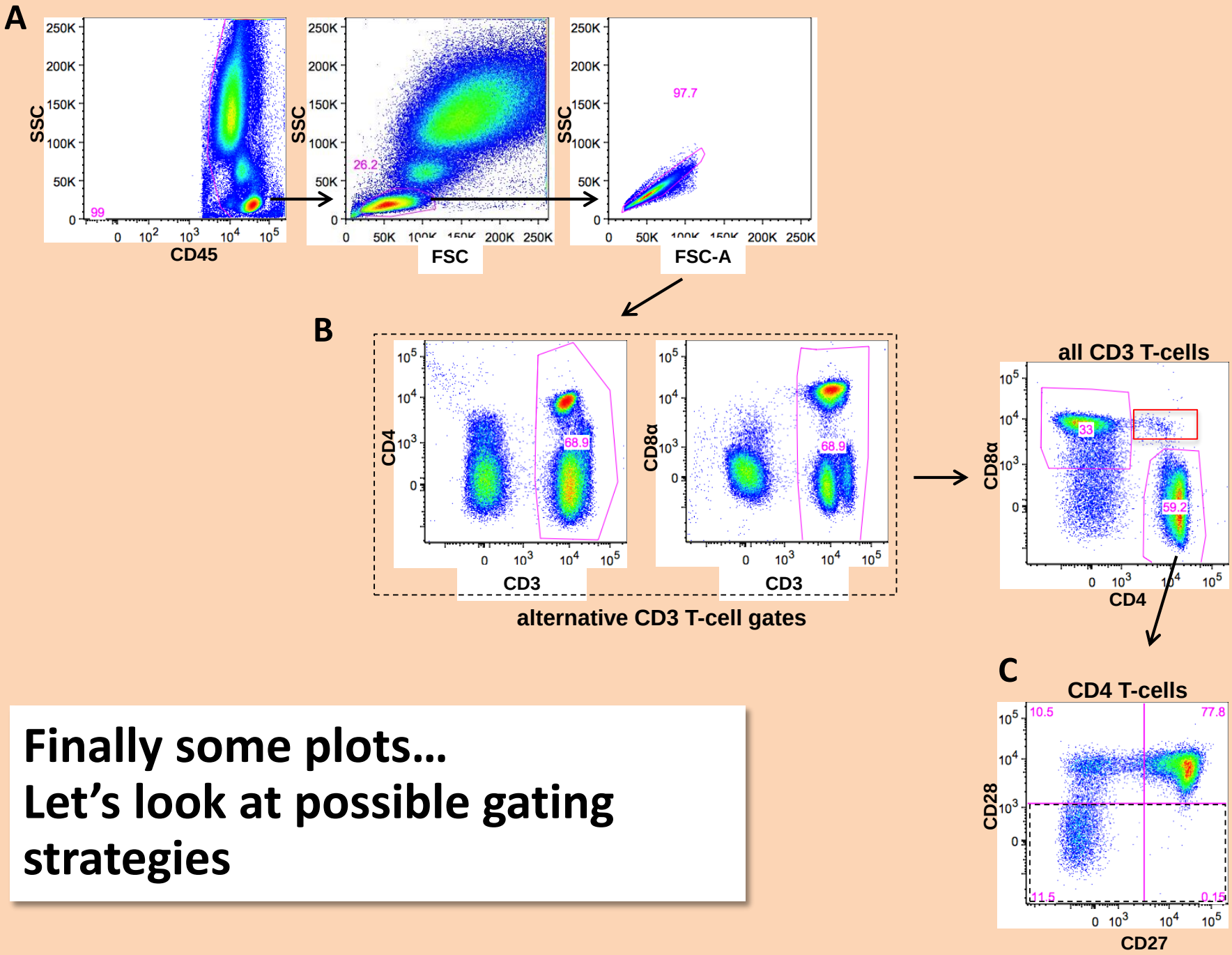
CCR7: naïve/memory
CD27: naïve/memory
CD45RA/RO – memory
CD57: CMV and immunosenescence
CD28: works with CD57
CD28/CD27: costimulation/memory

Defining memory populations by marker pairs
CCR7 vs CD45RA
CD27 vs CD45RA
CD28 vs CD45RO

It is vital to any monitoring panel that its composition does not change over time. The best solution to achieve consistent staining is producing a master mix that can be used for all samples. However, there are some issues with the stability of fluorescent dyes over time, and therefore, solutions like Beckman Coulter's Duraclone tubes are ideal.



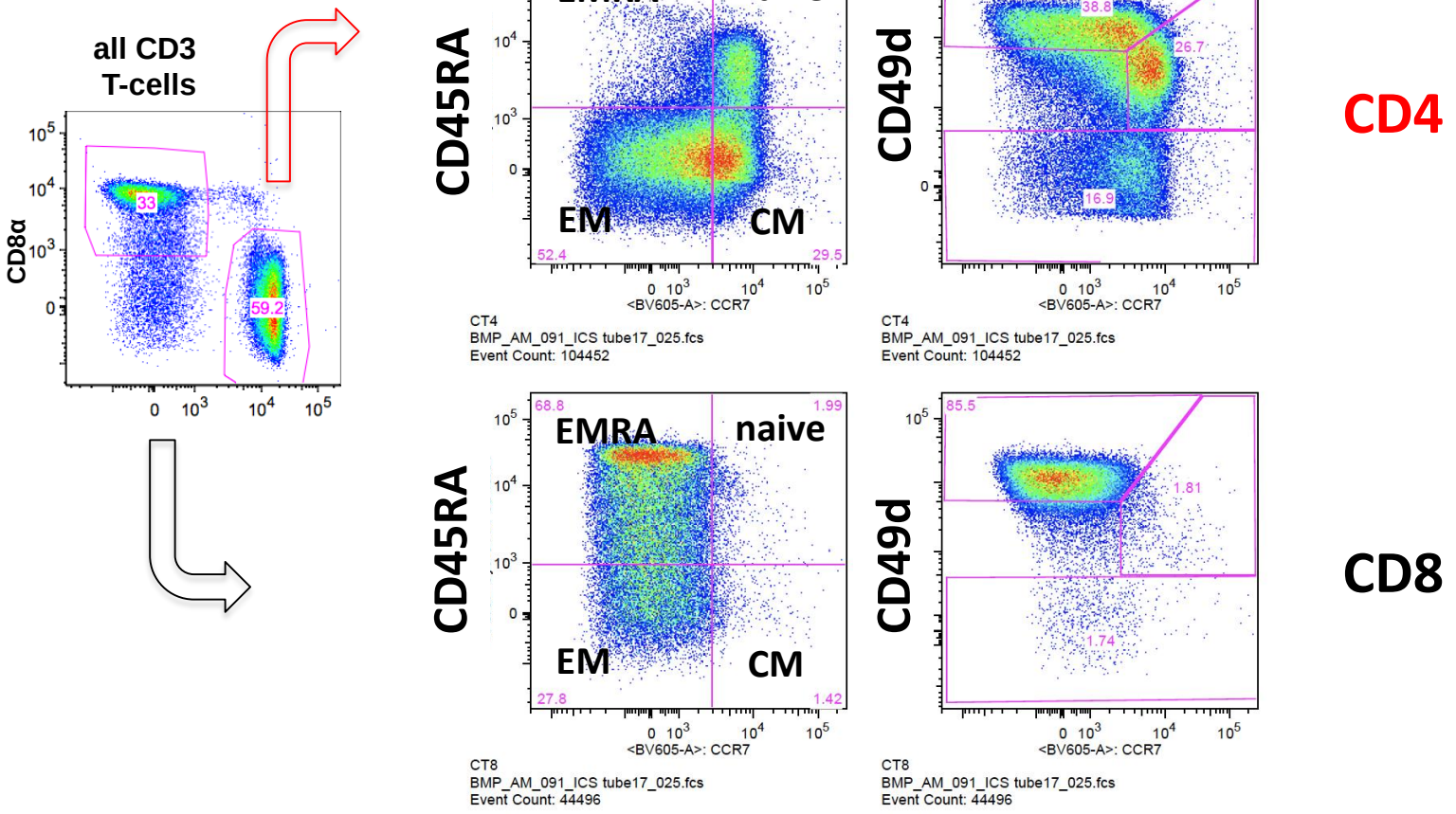
Finally some plots…
Let's look at possible gating strategies



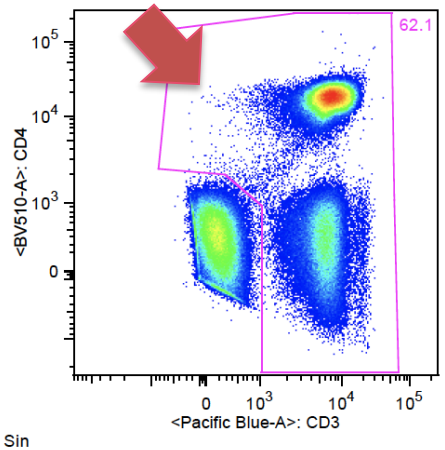
Finally some plots…
Let's look at possible gating strategies

Phenotype changes in (immune) ageing

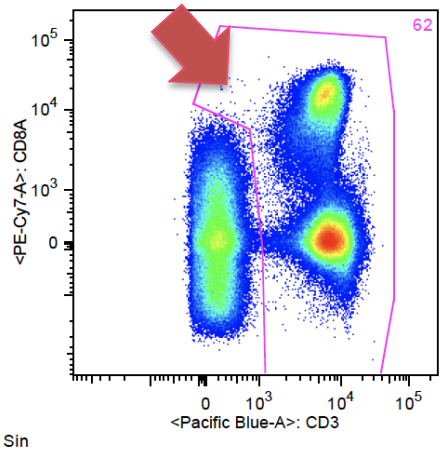
Memory subsets defined by marker pairs: **CD45RA and CCR7**



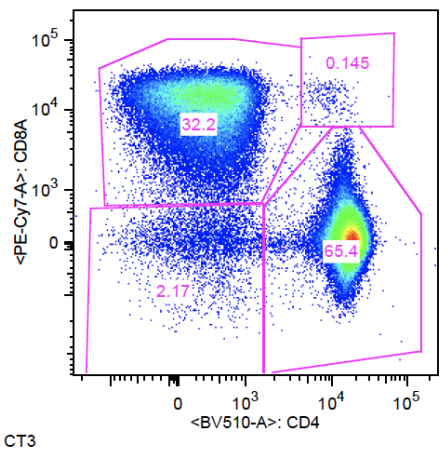
Phenotype changes during cell activation



Sin
BMP_AM_091_ICS tube1_010.fcs
Event Count: 265941

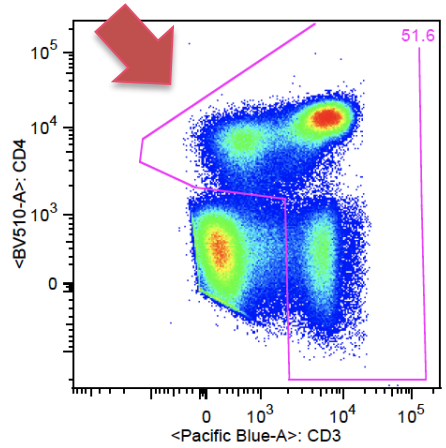


Sin
BMP_AM_091_ICS tube1_010.fcs
Event Count: 265941



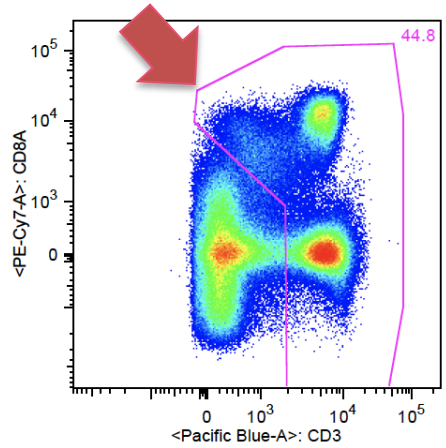
CT3
BMP_AM_091_ICS tube1_010.fcs
Event Count: 165234

unstim



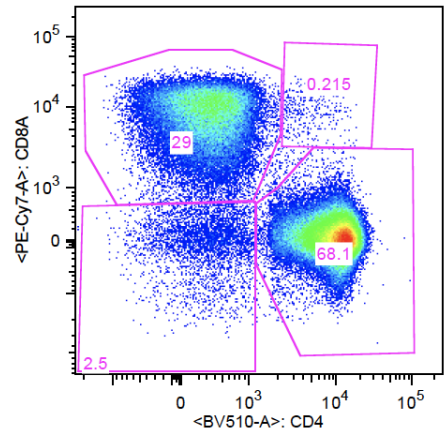
Sin
BMP_AM_091_ICS tube17_025.fcs
Event Count: 271737

CD3



Sin
BMP_AM_091_ICS tube17_025.fcs
Event Count: 271737

CD3

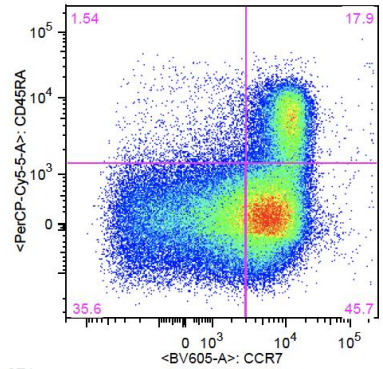


CT3
BMP_AM_091_ICS tube17_025.fcs
Event Count: 153361

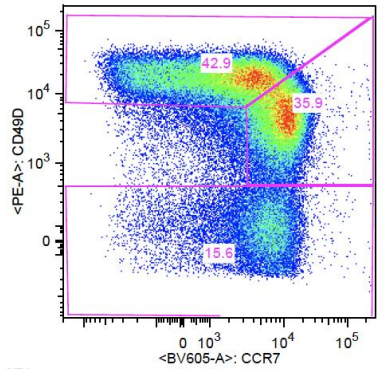
CD4

SEB

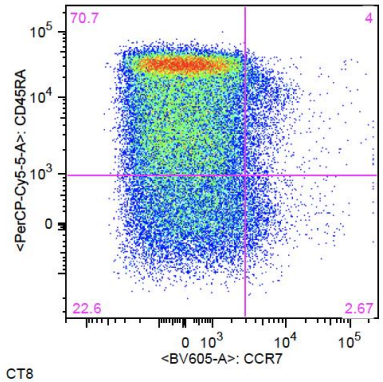
unstim → **SEB**



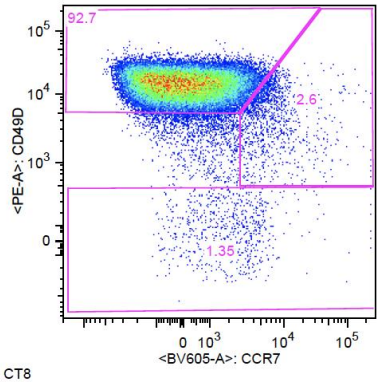
CT4
BMP_AM_091_ICS tube1_010.fcs
Event Count: 108001



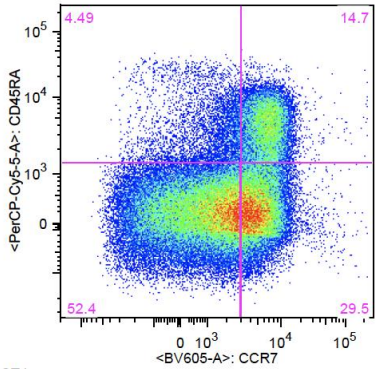
CT4
BMP_AM_091_ICS tube1_010.fcs
Event Count: 108001



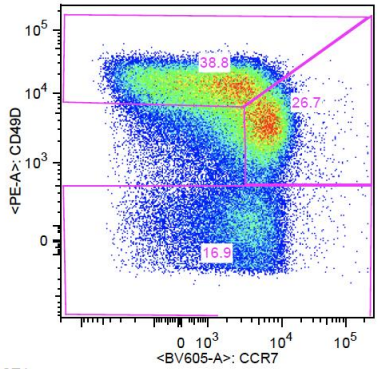
CT8



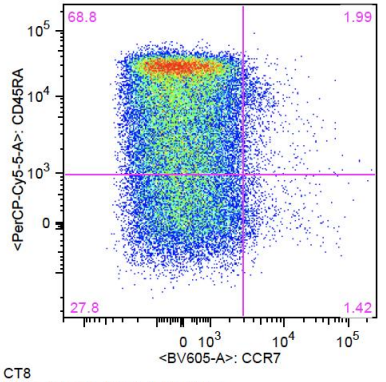
CT8



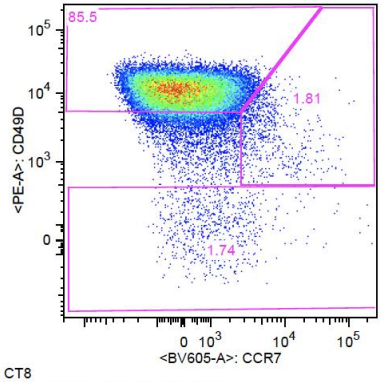
CT4
BMP_AM_091_ICS tube17_025.fcs
Event Count: 104452



CT4
BMP_AM_091_ICS tube17_025.fcs
Event Count: 104452

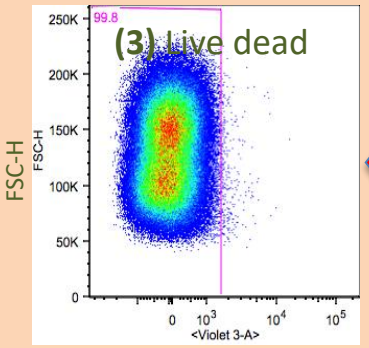


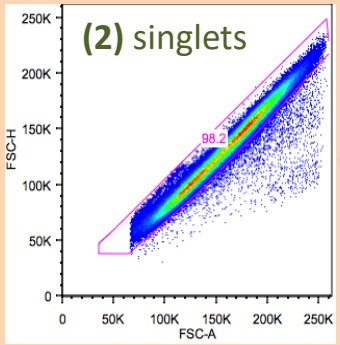
CT8
BMP_AM_091_ICS tube17_025.fcs
Event Count: 44496

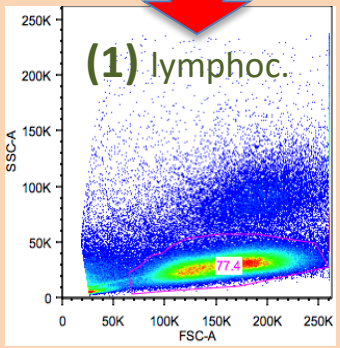


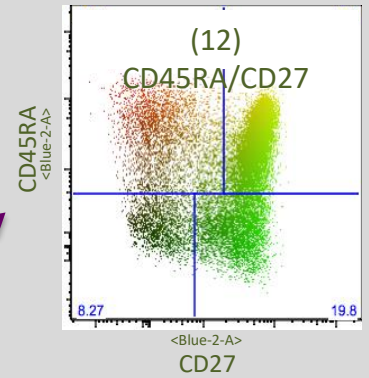
CT8
BMP_AM_091_ICS tube17_025.fcs
Event Count: 44496

Does activation (16h) change your phenotype distribution?

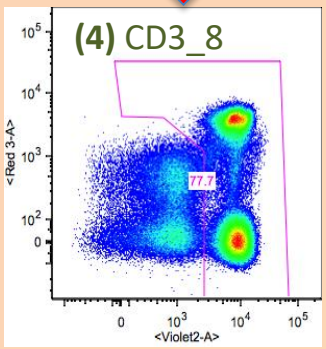


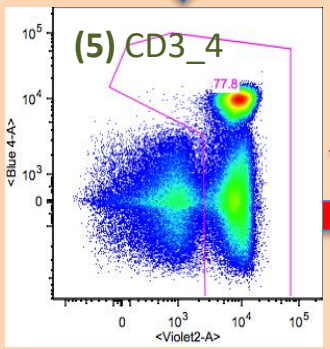




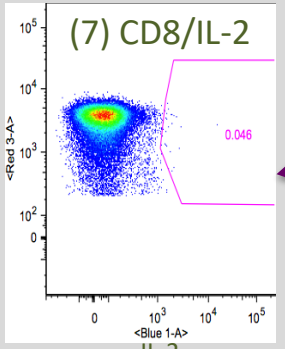


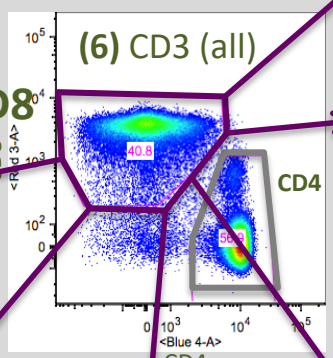
Combine with (7) – (11)

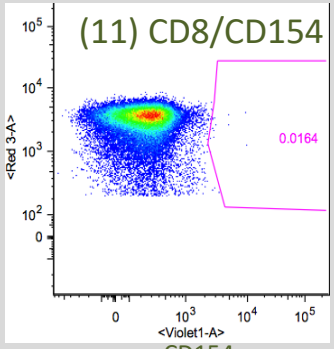




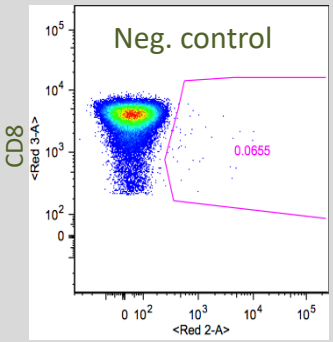
Boolean Gate Combination: '(4) OR (5)'

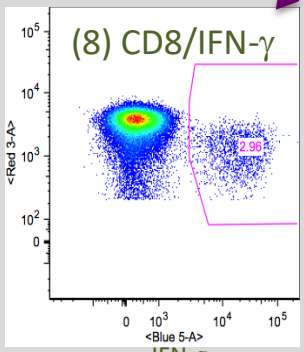


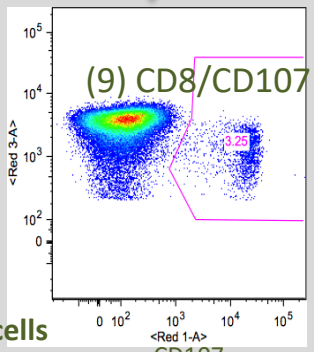


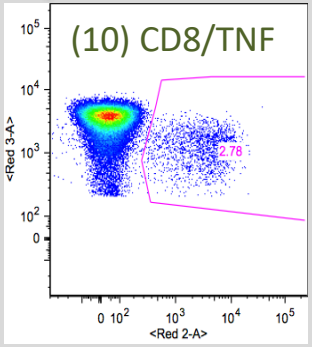


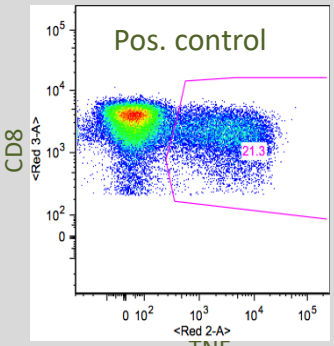
Example for Assay Controls (TNF)











The strategy for CD4 T-cells is the same in principle

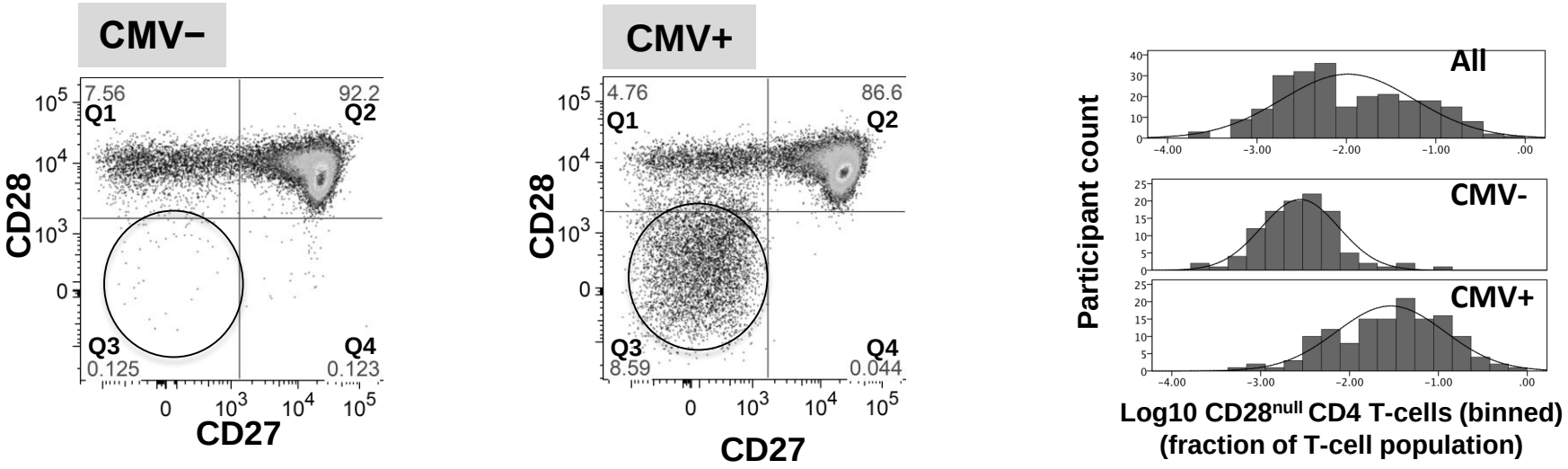
Monitoring immunosenescence by flow?

No generally accepted and validated panels to measure immunosenescence by flow exist to date. However, many panels have been designed to capture parameters related to immunosenescence….

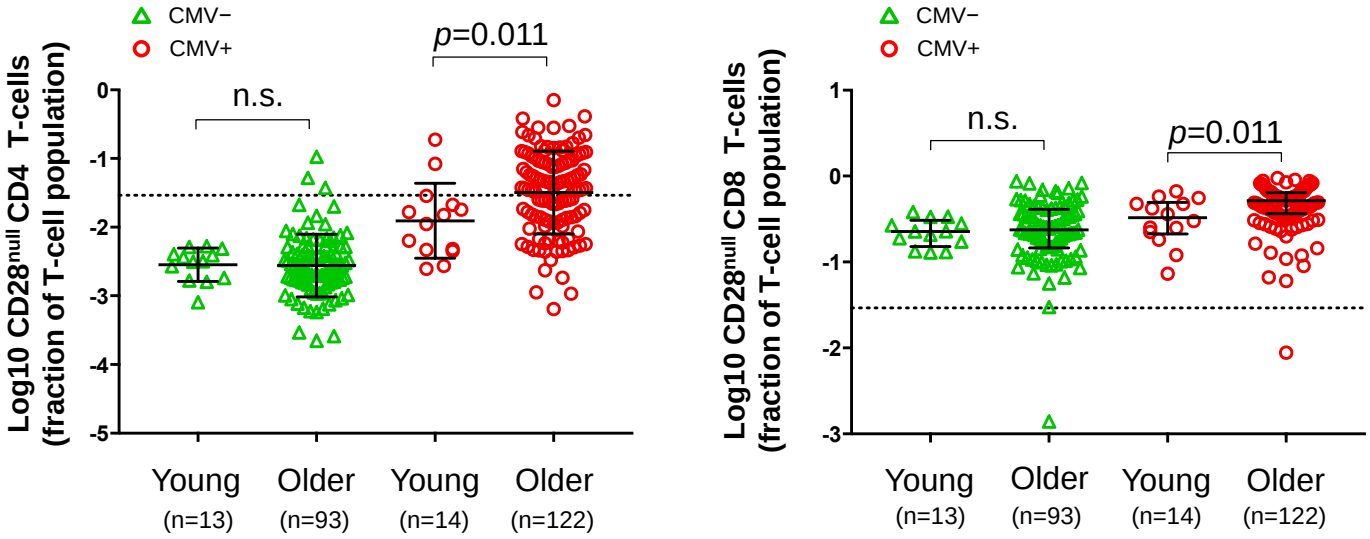
- In a recent study about 100 ‘generally healthy’ older CMV+ and CMV- people were recruited
- We obtained a detailed vascular status (compliance) and extensive health data (questionnaire) (as a correlate of biological age)
- General blood pathology
- CMV-specific immunity (T cell responses to 19 different proteins)
- Two year clinical follow-up
- 4 Flow-cytometry panels (3 x T cells, 1 x Monocyte/Macrophage, 1 x B cells)

Results will provide insight into the spectrum of immune markers expression and profiles in this population, CMV-related changes, gender-related changes. These are pre-requisites for monitoring at a population level.

CD28^{null} CD4 T-cells as a sign of immunological age? Or CMV infection?



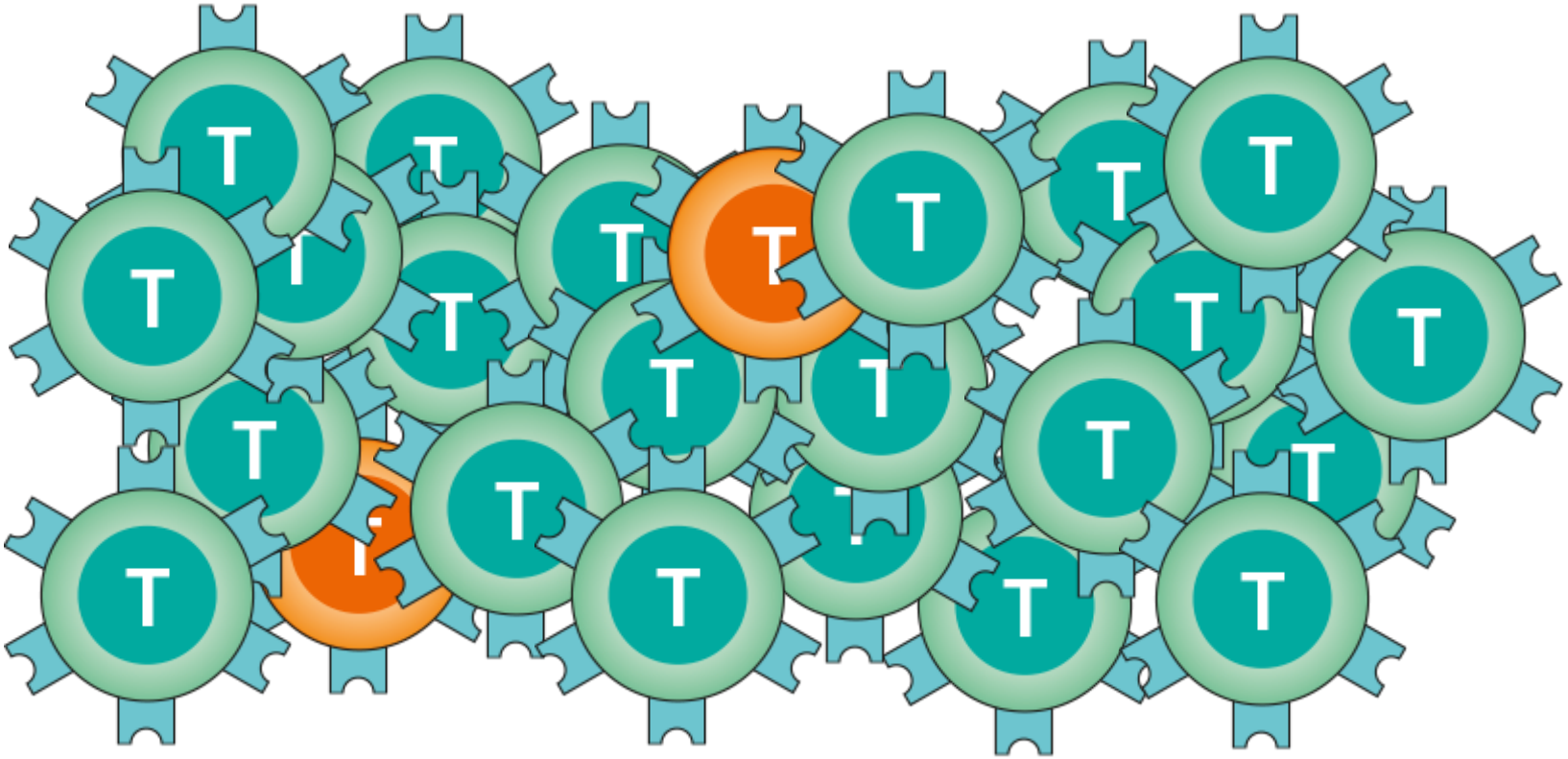
CD28^{null} CD4 and CD8 T-cells



WHOLE BLOOD PANELS CMV/ageing – Laboratory Protocol (Brighton Immunology Lab)
Overview, Version 2, 03/10/2014

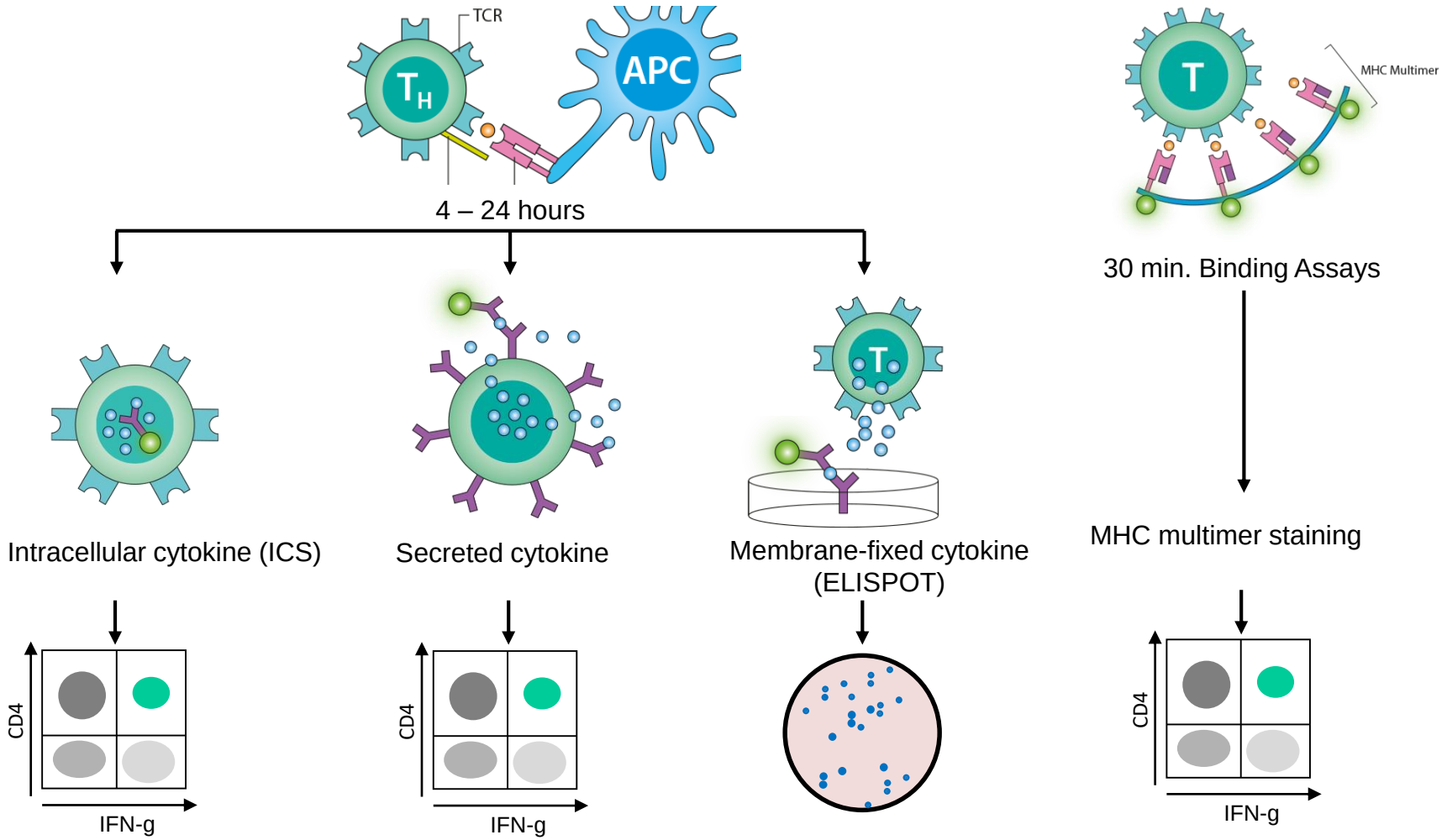
Detector (LSRII)	LP	BP	Fluorochrome	T cells	Treg	Th	TCR
Violet 1		450/50	PB	CD3	CD3	CD3	CD3
Violet 2	505	525/50	BV510	CD4	CD4	CD4	CD4
Violet 3	595	610/20	BV605	CCR7	CCR7	CCR7	CCR7
Red 1		660/20	APC	CD27	CD127	CCR6	TCR gd
Red 2	710	730/45	Alexa 700	CD45	CD45	CD45	CD45
Red 3	755	780/60	APC H7	CD8a	optional	CD8a	CD8a
Blue 1	505	530/30	FITC	CD45RA	CD45RA	CD45RA	CD45RA
Blue 2	550	575/26	PE	CD57	CD25	CCR10	Vdelta2
Blue 3			PETR/ECD etc.	optional	optional	optional	optional
Blue 4	635	670/14	PerCP(Cy5.5)	CD45RO	CD45RO	CCR4	CD16
Blue 5	755	780/60	PECy7	CD28	CD39	CXCR3	TCR ab

Part II: Monitoring antigen-specific T-Cells uses a variety of methods



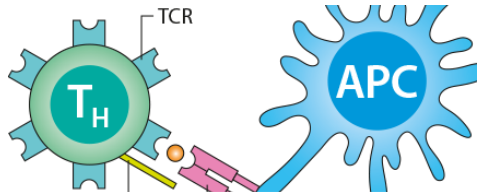
To detect antigen-specific T-cells, a variety of sensitive immunological assays is used, eg. **Elispot, intracellular cytokine assays by flow-cytometry, or MHC-multimer staining**

Each of these assays has **methodolical characteristics** that make it more or less susceptible to variation

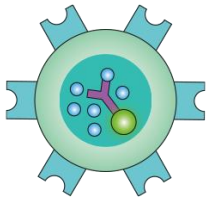


brighton and sussex medical school

Each of these assays has methodolical characteristics that make it more or less susceptible to variation

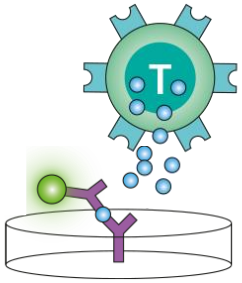


Stimulation time
& conditions
Permeabilization
Antibody concentration
& staining duration
etc….

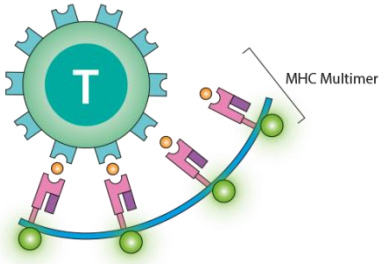


Intracellular cytokine (ICS)

Secreted cytokine

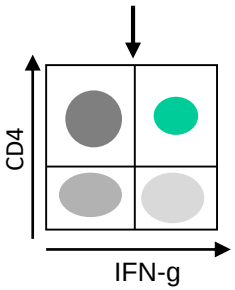


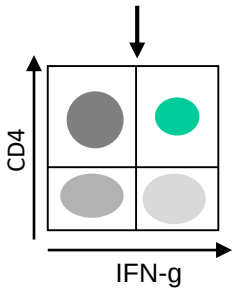
Membrane-fixed cytokine
(ELISPOT)

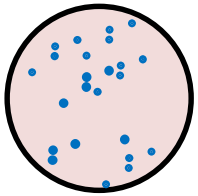


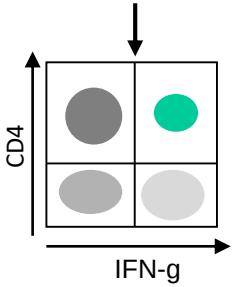
30 min. Binding Assays

MHC multimer staining



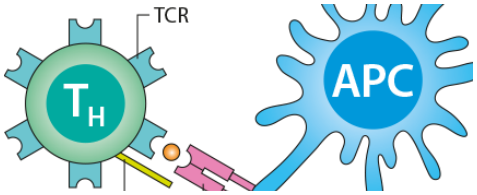


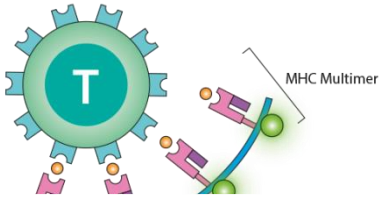




brighton and sussex
medical school

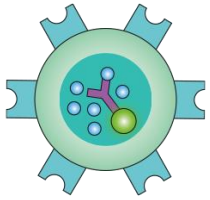
Each of these assays has methodolical characteristics that make it more or less susceptible to variation





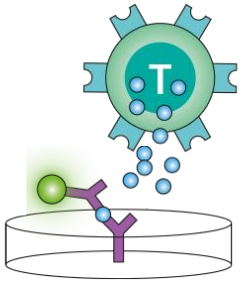
Stimulation time
& conditions
Permeabilization
Antibody concentration
& staining duration
etc….

Staining duration, dead cells, TCR affinity,…



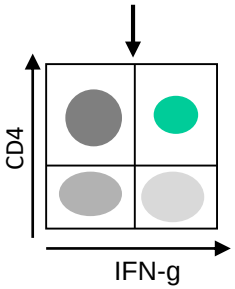
Intracellular cytokine (ICS)

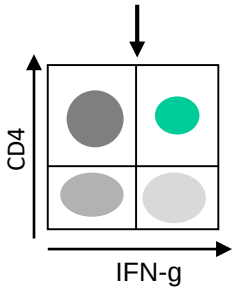
Secreted cytokine

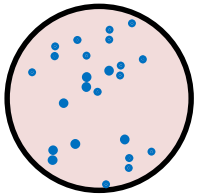


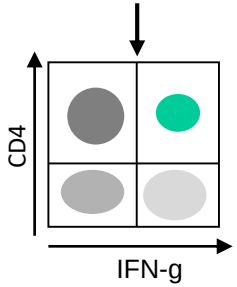
Membrane-fixed cytokine (ELISPOT)

MHC multimer staining



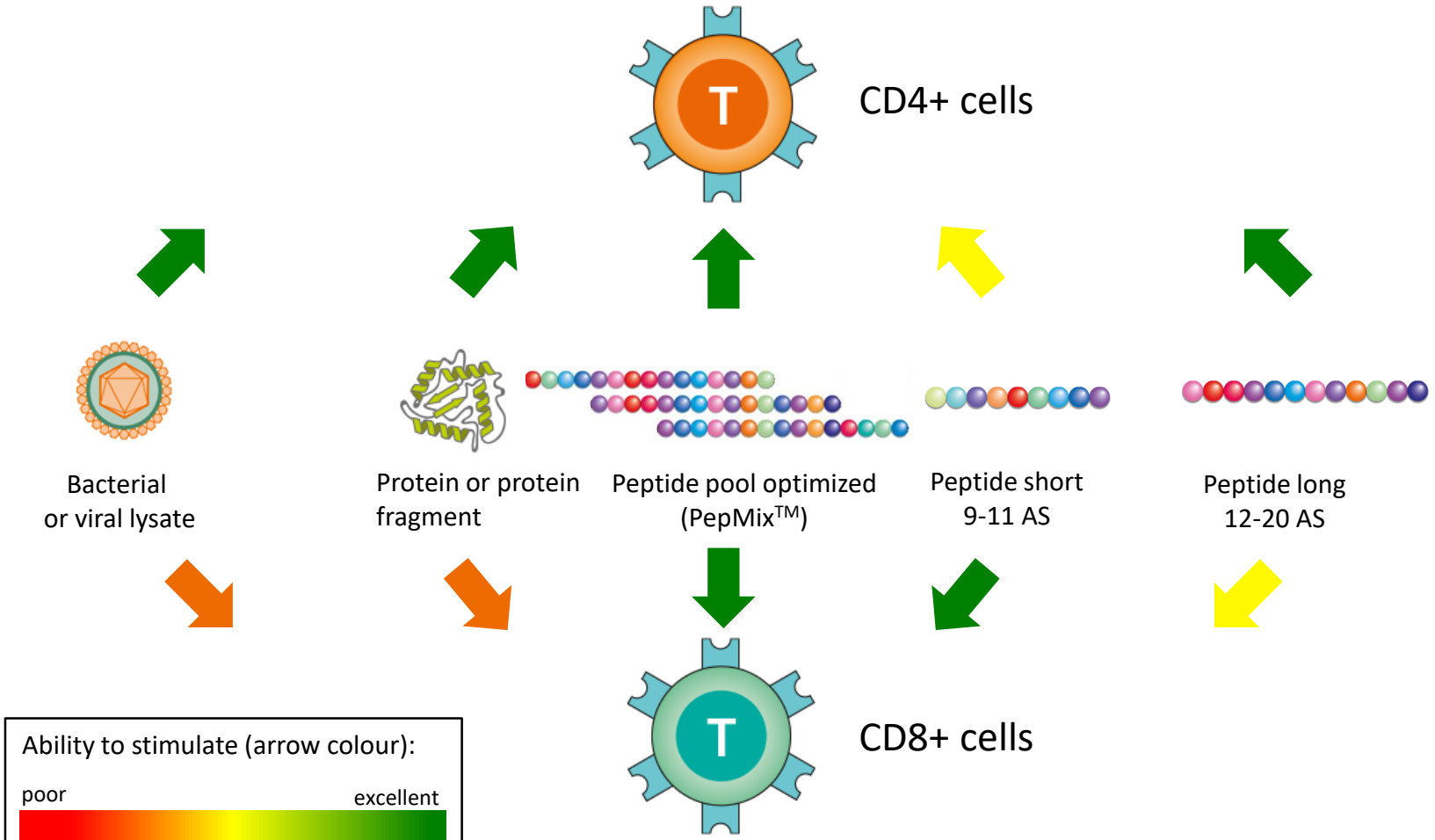






brighton and sussex medical school

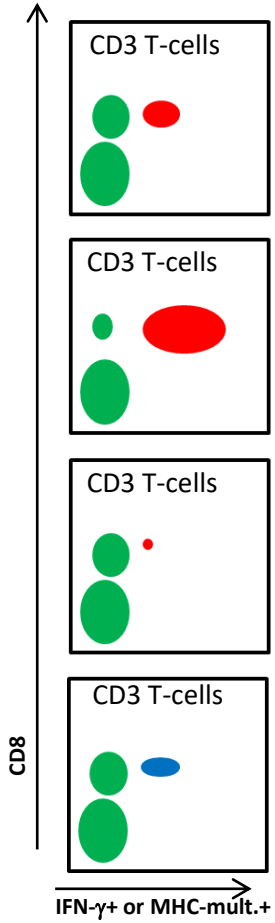
Antigen Formats and *in vitro* T-Cell Stimulation may also contribute to variability, e.g. lot changes



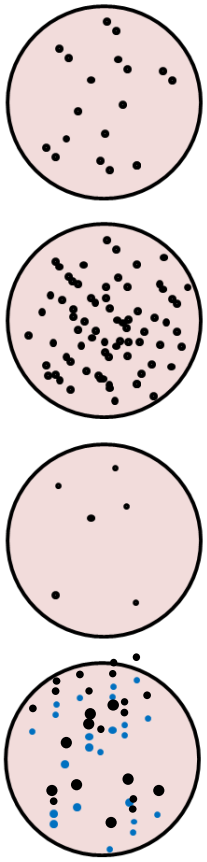
brighton and sussex medical school

What do assay controls do?

Flow cytometry
(ICS or MHC-multimer staining)



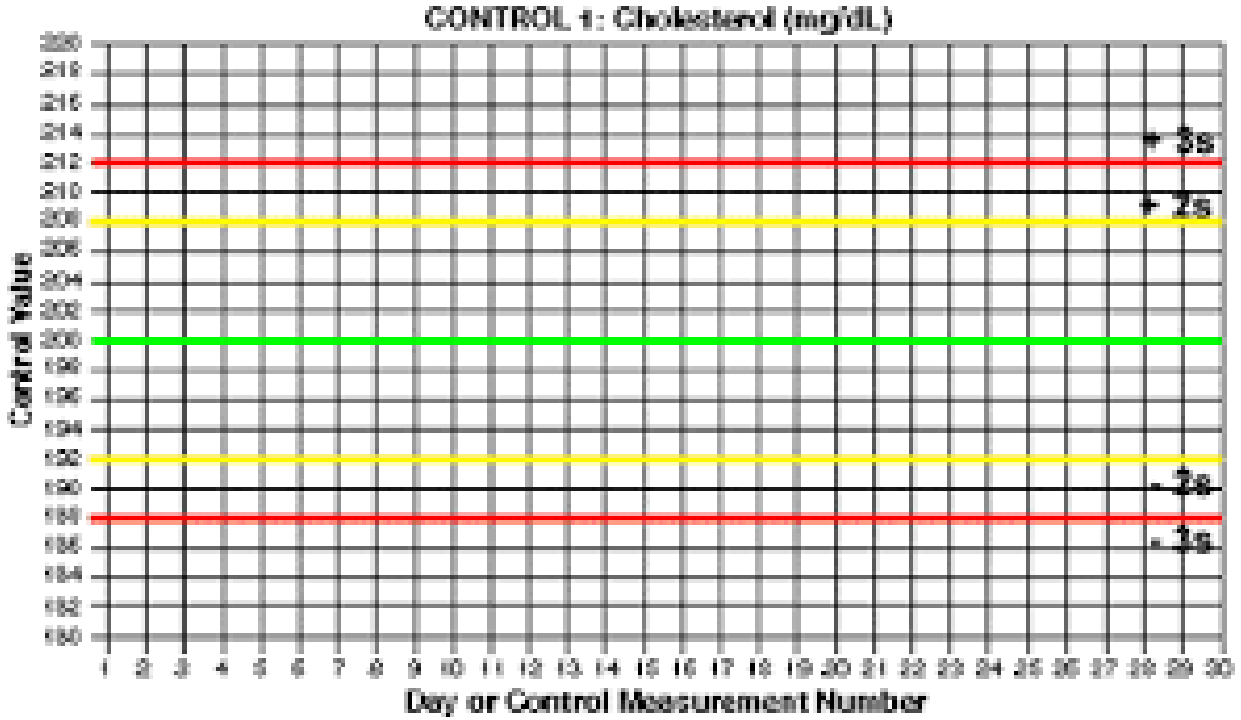
Elispot



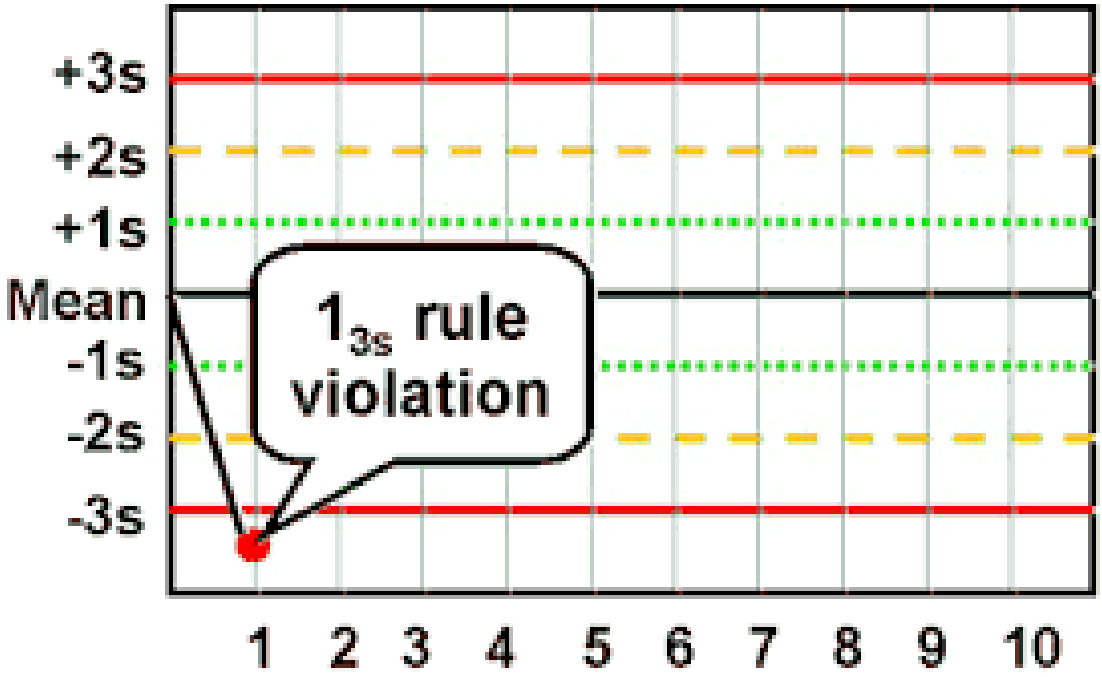
Task/role

Patient sample	Needs to be quantified
Pos Control (e.g. SEB, PHA)	Confirms that cytokine can be induced by sufficient stimulation under the applied conditions
Neg Control (e.g. DMSO)	Shows the extent to which cytokine is induced in the absence of external stimulation
Standard? usually missing	Shows if a known frequency of antigen-specific cells is determined correctly

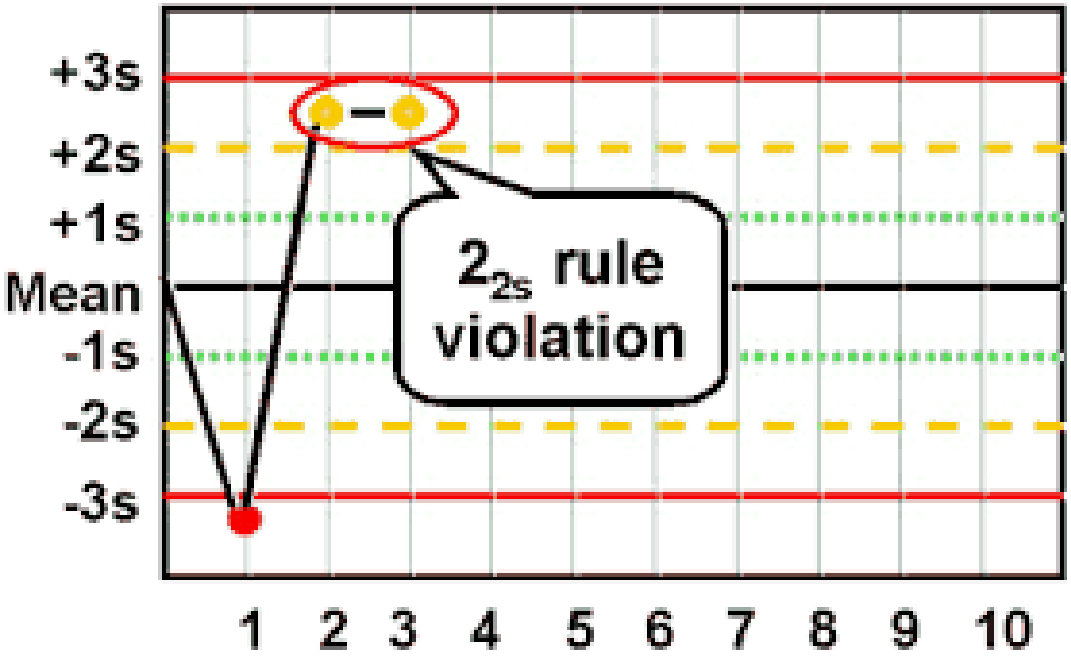
Laboratory Quality control: Levey-Jennings Plot & Westgard Rules



Laboratory Quality control: Levey-Jennings Plot & Westgard Rules



Laboratory Quality control: Levey-Jennings Plot & Westgard Rules



In order to set up something similar for antigen-specific T-cells you need a standard, ideally a T-cell suspension with known responsiveness to the same or a similar stimulus you use in your study.

T-cell engineered reference samples as (external) reference standards

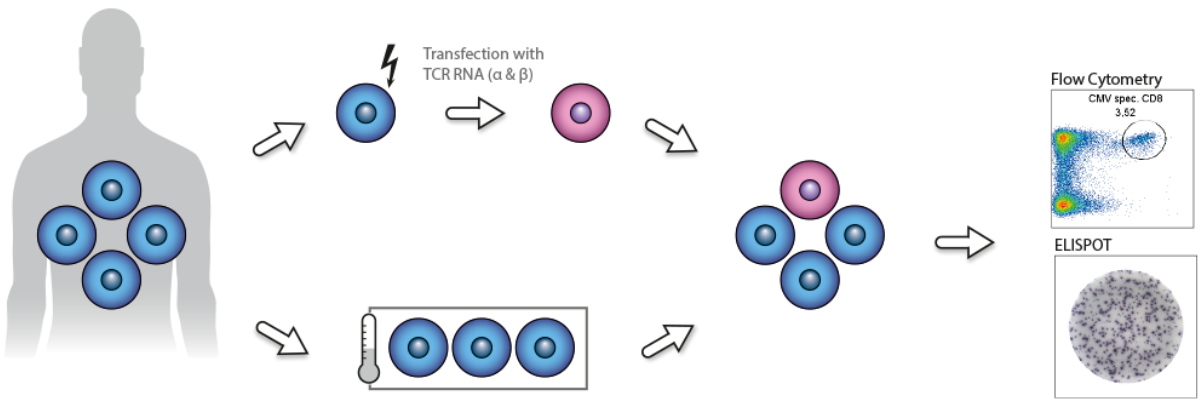
- **A TCR of interest** identified (from a clone), sequenced, and presented as a linear RNA.
- The linear RNA leads to a transient but fully functional expression of the TCR in PBMC.
- Standard suspensions can be adjusted in terms of cell frequencies (any you wish) and many aliquots (like hundreds) can be frozen. You can generate a high and a low control (e.g. 1% and 0.01%).

TERS – How standards are produced

Method

RNA is transfected into PBMC and transiently expressed in various cell types

Multimer staining identifies the proportion of transfected CD8 T-cells (=population of interest)



PBMCs from buffy coat donor

Cultivation and storage of transfected and native cells

Pooling as required

Performance control of assays

Native PBMC are stored for subsequent dilution of transfected cells

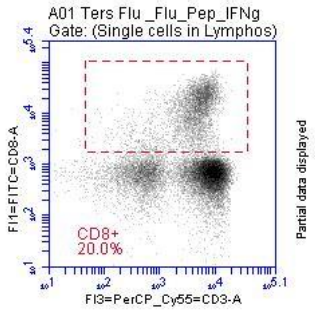
Transfected cells are mixed with non-transfected cells to create a sample with a defined frequency of Ag-specific CD8 T-cells

The standard samples are used in antigen-specific T-cell assays

TERS – How standards are produced

Following transfection, cells are activated or stained with tetramer

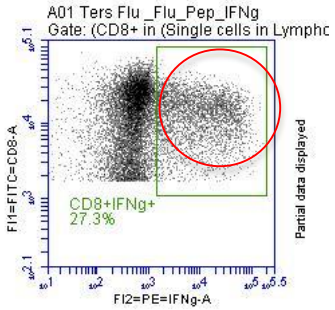
CD8+



CD8

CD3

IFNg+

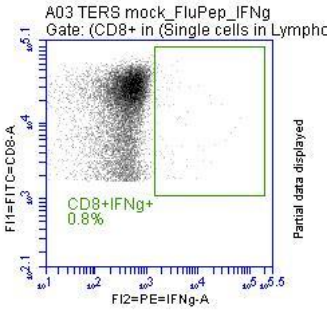


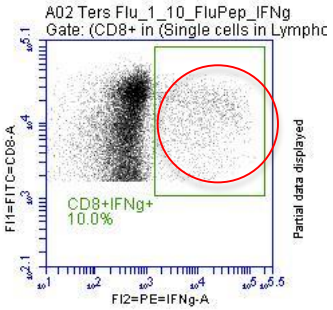
CD8

activation

Dilution 1:2.8 with untouched cells

Mock control (untouched cells stimulated with peptide)

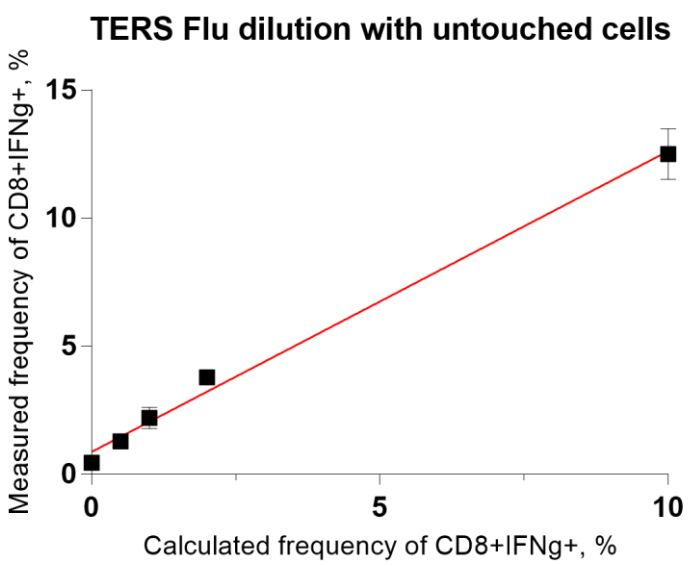




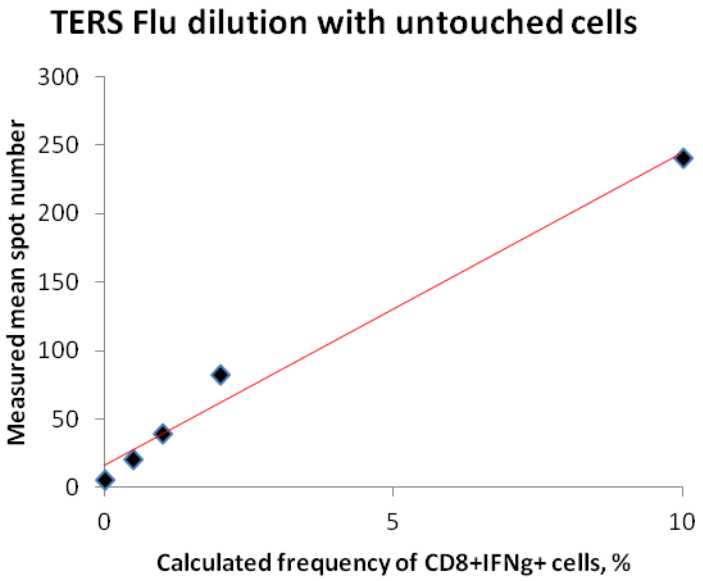
confirmation

Calculated versus measured frequencies

ICS

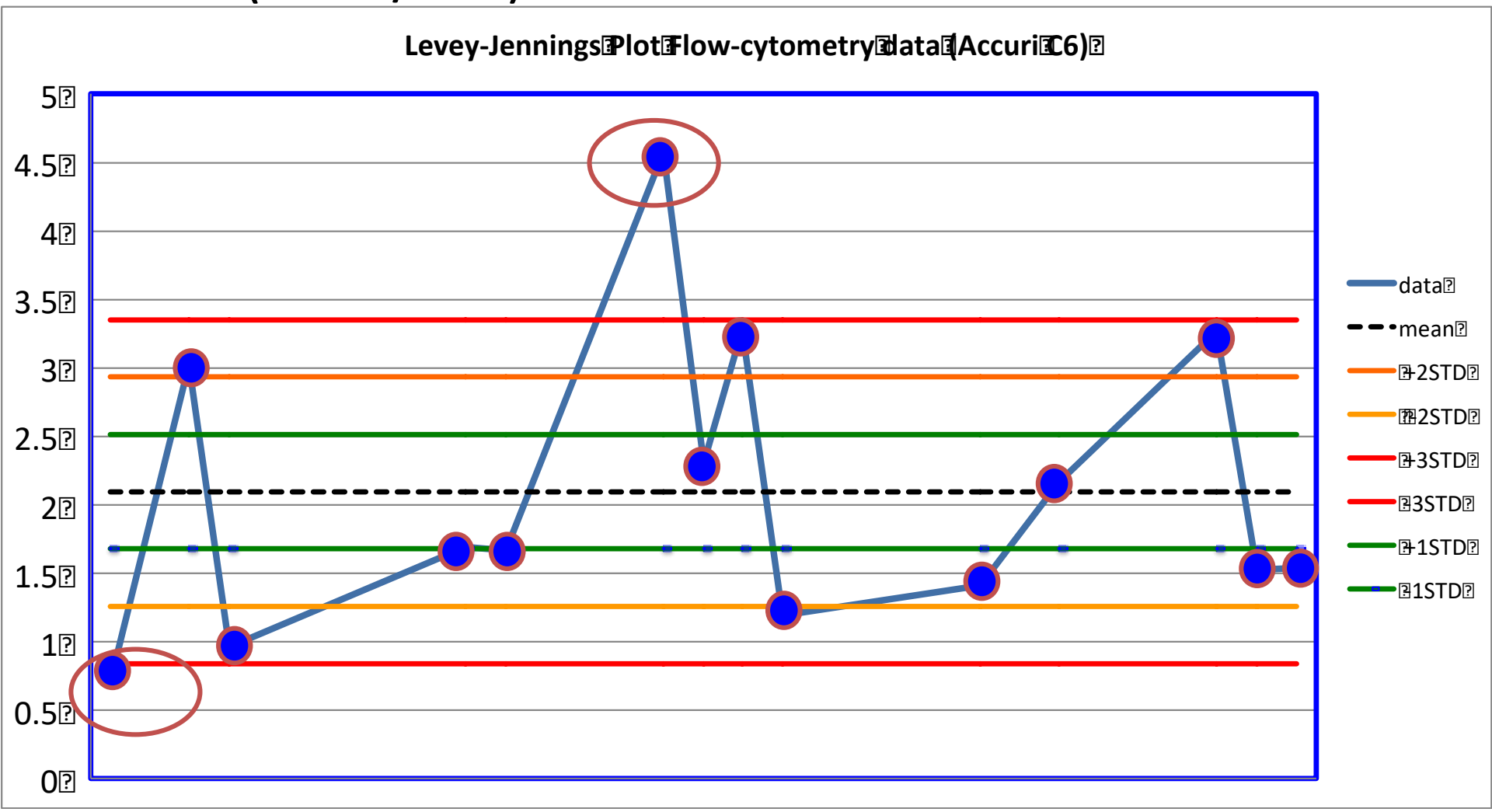


ELISPOT



Running TERS like a normal assay standard in a clinical lab

Flowcytometry – Flu epitope - Intracellular cytokine staining, first attempts, CV set to 20% (CV=STD/mean)



14 measurements spaced over 4 weeks

Current availability of TERS

- HLA-A2*0201
- CMV
- Influenza
- Tyrosinase
- NY-ESO

- In order to monitor your assay performance in general, you can use any specificity. If you can use the same specificity as in your monitoring study, you obtain an additional level of control.

Factors that will change the peripheral blood lymphocyte distributions

Non ill health-related

- **Age (very significant increase of late memory subsets)**
- **Exercise (CD8↑CD4↓)**
- **CMV infection (increase in late memory subsets, CD4+CD28-T-cells, CD8+CD57+ T-cells)**
- **Pregnancy (changes in Treg, other changes?)**
- **Acute Mental Stress (e.g. doing arithmetic)**
- **Diurnal (always draw blood at the same time; what about shift workers?)**
- **Environment?**
- **Seasonal?**

Ill-Health-related:

- **Chronic Stress**
- **Smoking**
- **inflammation**
- **Disease**
- **CMV infection status (CD4+CD28-/CD8+CD57+CD28-)**

These lists are certainly not complete, since we discover new factors all the time and will continue doing so as we compile more ambitious panels and increase the levels of standardization)

Factors of variation and how to avoid them

Sample tube and anti-coagulant	Stability, anticoagulant volume (may affect absolute counts!)
Environmental factors, e.g. temperature	Stable environment
Time to processing	As short as possible, but always the same
Staining protocol	Always exactly the same
Reagent stability	Dried/freeze dried, pre-titered and pre-mixed antibodies
Instrument set-up and calibration	automated
Cell numbers acquired	A minimum number to determine percentages/counts
Instrument	Always the same type of instrument
Analysis strategy	Always the analysis strategy
Operator bias	Same operators, central analysis, automated analysis

The One study

- Six panels covering **lineage**, **TCR**, **T-cell activation**, **T-effector/T-regulatory cells**, **B-cells**, **and mDC/pDC subsets**.
- Centralized formulation and supply of antibody panels (Beckman Coulter)
- All samples were measured on the same type of **10 color**, **3 laser** Navios flow cytometer (Beckman Coulter) = identical instruments (inasmuch as this is possible)

- **Centralized optimization** of settings transferred to all instruments
- Extensive standardization, intra&interassay, between labs
- Centralized analysis by the same operator

This represents probably the highest possible level of instrumentation standardization. The setup addresses some of the other issues identified as causing variablity in the HIPC study (in particular the advantage of centralized manual analysis). A potential improvement might have been the use of (now available) Beckman Coulter Duraclone™ preparations for the antibody cocktails (dried antibodies providing extreme reagent stability).

The One study

- Six panels covering **lineage**, **TCR**, **T-cell activation**, **T-effector/T-regulatory cells**, **B-cells**, **and mDC/pDC subsets**.
- Centralized formulation and supply of antibody panels (Beckman Coulter)
- All samples were measured on the same type of **10 color**, **3 laser** Navios flow cytometer (Beckman Coulter) = identical instruments

Streitz *et al. Transplantation Research* 2013, **2**:17
http://www.transplantationresearch.com/content/2/1/17

TRANSPLANTATION RESEARCH

RESEARCH Open Access

Standardization of whole blood immune phenotype monitoring for clinical trials: panels and methods from the ONE study

Mathias Streitz[1], Tewfik Miloud[2], Michael Kapinsky[3], Michael R Reed[3], Robert Magari[3], Edward K Geissler[4], James A Hutchinson[4], Katrin Vogt[1], Stephan Schlickeiser[1], Anders Handrup Kverneland[1], Christian Meisel[1], Hans-Dieter Volk[1,5] and Birgit Sawitzki[1,5*]

THANKS TO

Brighton and Sussex Medical School

Alejandra Pera, Stefano Caserta, Nadia Terrazzini, Pinar Blowers, Martha Bajwa, Serena Vita, and many more…

JPT Peptide Technologies Cellular Lab (Berlin)

Pavlo Holenya, Maren Eckey, Tatjana Teck