Encountered challenges during the standardization of the ICS assay

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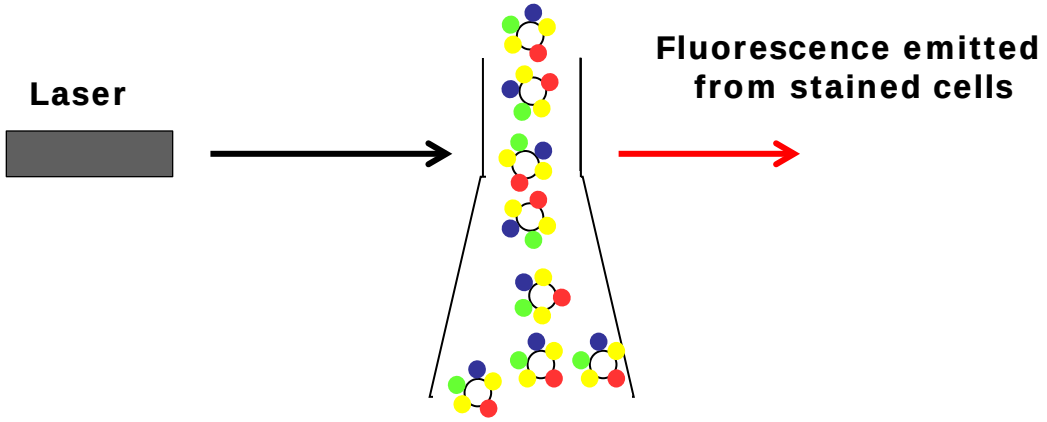
➢Immune responses after vaccination

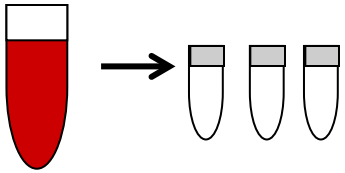
T cell responses (CD8 and CD4 T cells)

Cytokine responses (IFNγ, TNFα, IL-2)

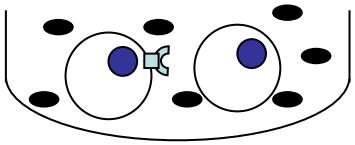
➢ Flow cytometry:

Intracellular cytokine staining (ICS) assay

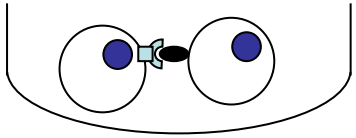




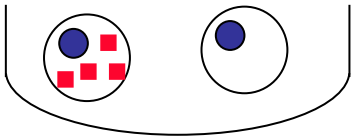
Blood is collected and PBMCs are isolated at the clinical site



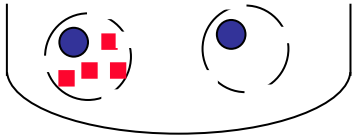
T cells are incubated with antigenic peptides ⬬ in solution



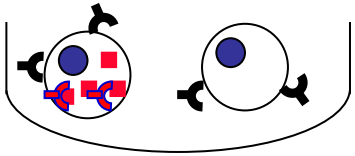
T cells recognize specific peptide antigen and are activated



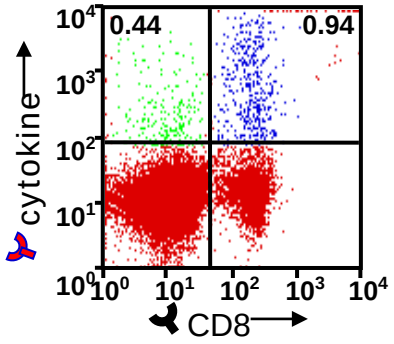
Activated antigen-specific T cells produce cytokines ▬ and cytokine secretion is blocked



Cells are fixed and permeabilised

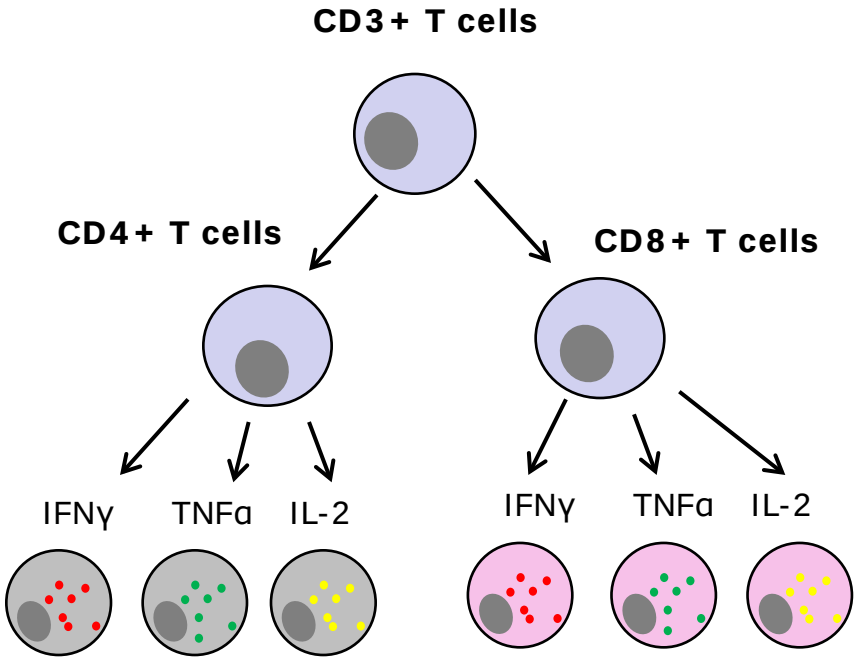


Cells are stained with cytokine- and phenotype-specific antibodies



FACS analysis determines percentage of cytokine+ CD8+ or CD4+ T cells

➢Multifunctional T cells in clinical trials



CD3 – **APC-H7**

CD8 – **PerCP-Cy5-5**

CD4 – **Horizon V450**

IFNγ - **APC**

TNF - **FITC**

IL-2 – **PE**

Live/dead cells –

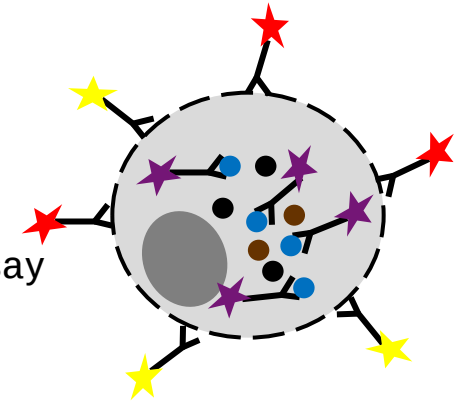
Aqua Fluorescent Dye

ICS assay:

➢Lot of information –

multifunction can be detected at a single cell level

study T cell properties needed for correlate of protection



➢Keep good quality of ICS assay –

Qualified ICS assay
Continuously check the performance of assay
QC
Start trouble shooting immediately
End goal: High quality data

➢ Control of different parameters

Trained operators – optimal sample preparation

Set up beads –day to day performance of the flow cytometry

Compensation beads – optimal compensations

Optimal panel – minimal overlap between fluorochromes

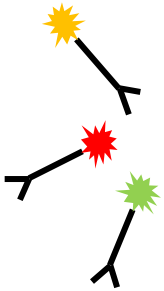
QC

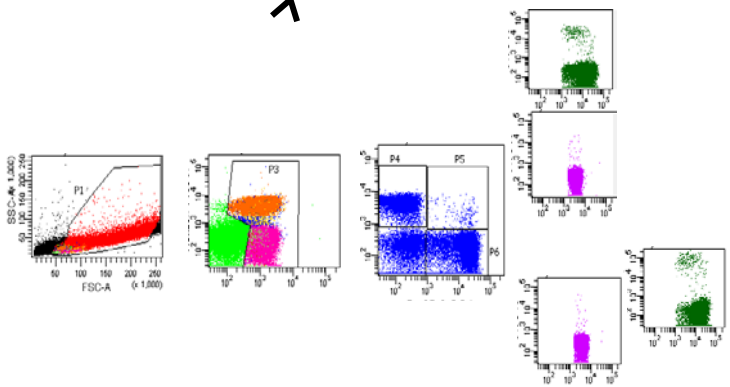
Gating of cell populations

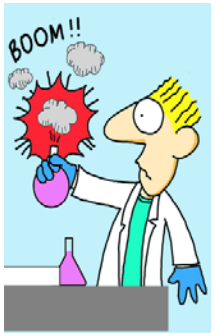
Reagents

Analyze data – gates checked, number of events

Data handling







- **Operators – sample preparation**

Training at site of PBMC isolation

Frozen PBMC

Shipment of samples

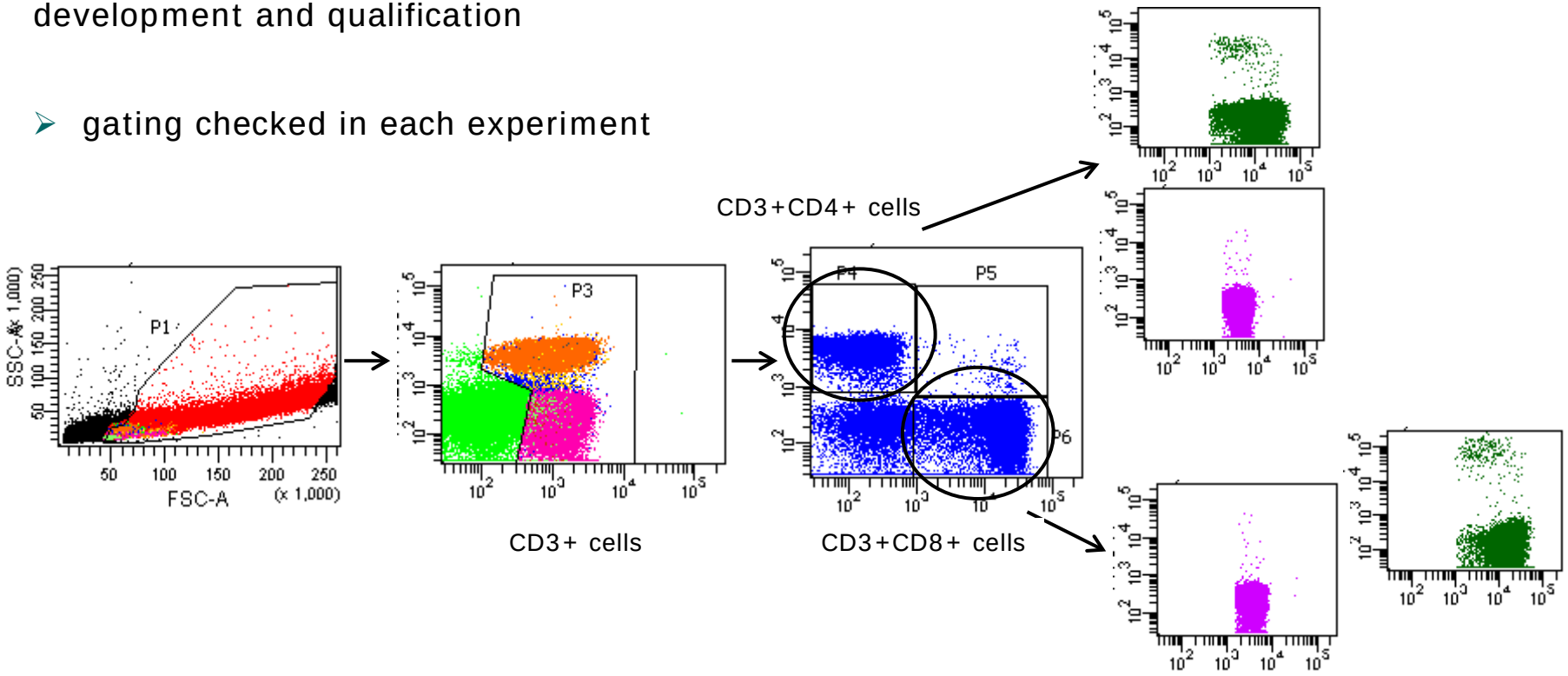
Dry runs (viability and recovery) viability > 80%

PBMC isolation within 8 hours

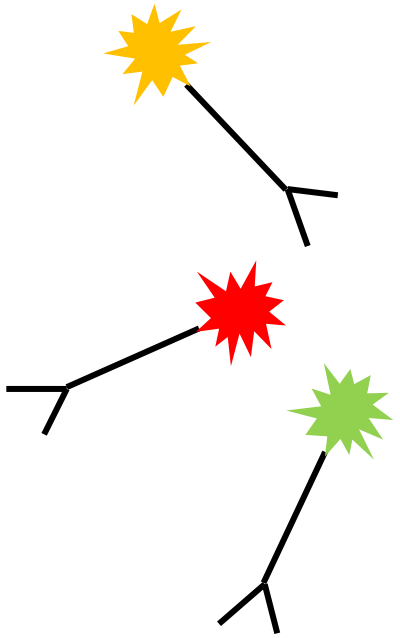
Challenges at site (temperature)

- PBMC batch, included in each assay
- valid/invalid assay run
- range calculated from cytokine responses after CMV or CEF stimulation of T cells
- cytokine response ~ 0.5 -1%, viability >80%
- many PBMC aliquots (last through a clinical trial)
- range for QC
  - at least 6 experiments performed by different operator
  - based on the operators analyzing clinical samples
  - experiments spread over time
  - CD8 IFNγ+ responses
- Trending of QC

- standardized gating used
- gating often set on healthy donors (PBMC batches) during development and qualification
- gating checked in each experiment



- correct storage and handling
- tandem dyes
- correct combinations of antibodies (fluorochromes)
- optimization of antibodies
- if possible use the same lot through the whole trial



No involvement in operator training

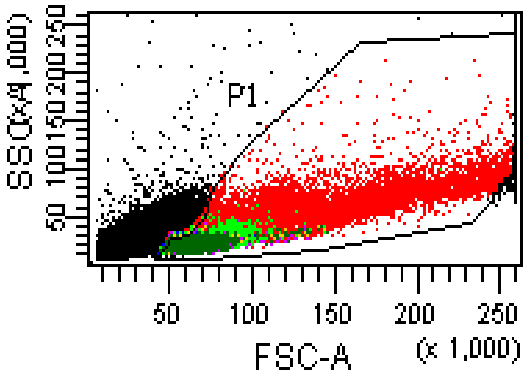
Challenge: Bad quality of samples

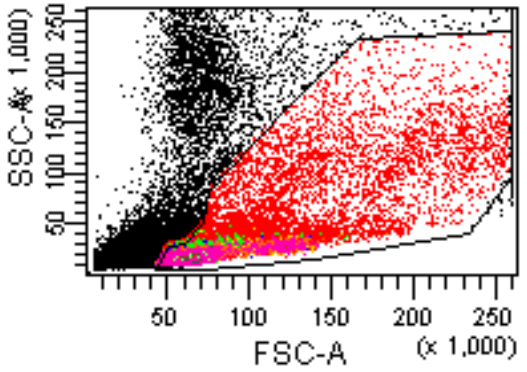
Lower cytokine responses in samples with high debris

Consequence:

training of operators required in future trials

Bad quality samples not reported





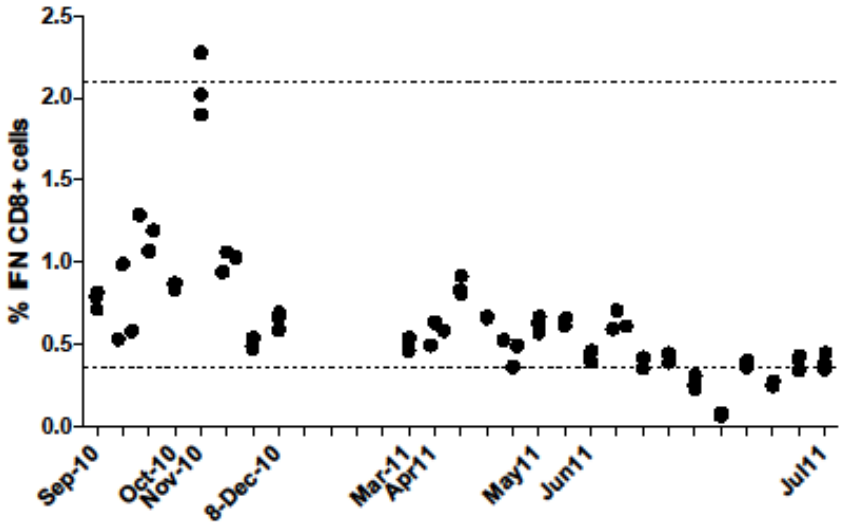
Challenge: Lower CD8 IFNγ responses of QC (invalid or just in range)

Why lower responses of the QC?

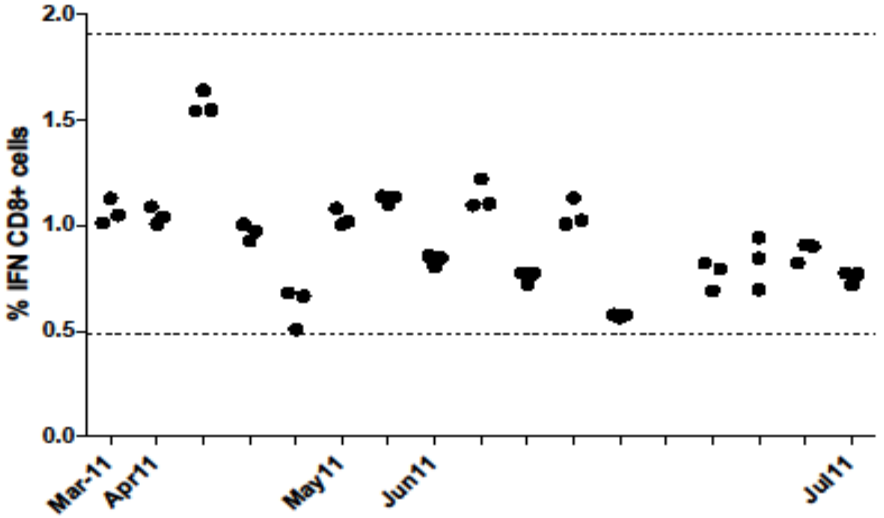
Root cause analysis:
QC bad quality?
Antibodies? Tandem dyes...
Other reagents?
Gating?
Cleaning

Solution:
CD3 cell population not completely fitting the gate - all samples re-gated and QC ranges re-calculated

Original gating



Improved gating



A success story

....however,

Challenge: High background of IL-2+ T cells

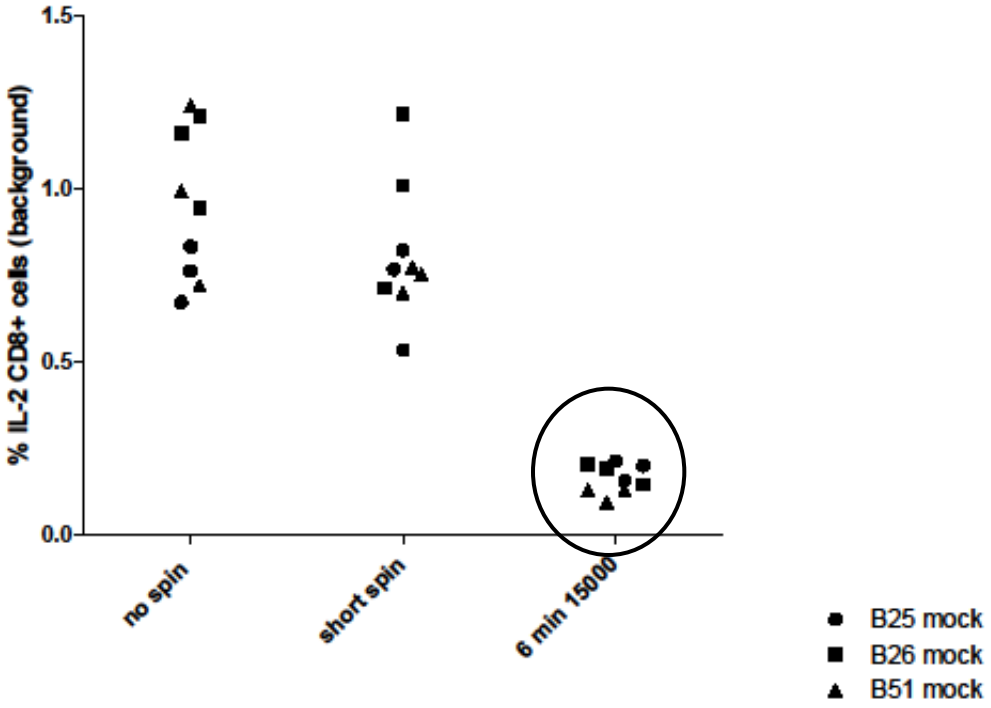
- Same lot used during the whole trial
- Quickly solved by using new vial of IL-2 (same lot)
- IgG2a, IL-2 – **PE** antibody

High background in all samples in assay or no samples in assay

Root cause analysis:

Testing of other vials (same lot number)
Short spinning of vials
IgG1, IL-2 antibodies tested
Gating?
Compensation – spillover from other channels
Thorough cleanings done
Other reagents?

Solution:
Spinning of αIL-2 – **PE** vial at 15000 rpm before use



- Trained operators
- Keep track of gating at all times
- Although same lot number, reagents can perform differently