**Functional Cytometry: Definition**

**Level 1** (not a functional experiment):
Assessment of the presence of molecules characteristic for functional properties (e.g. level of differentiation, migratory potential, cytotoxic molecules including granzymes and perforin).

**Level 2**
Assessment of functional consequences after exposure of cells to reagents that modify cellular processes (e.g. Ca flux, proliferation, activation, release of cytotoxic granules, apoptosis, signal transduction).

**Level 3**
Cell-cell interactions, such as immune-tumor cell interactions, including cytotoxicity, can be assessed and classified as cell-cell engagement, target-cell apoptosis, and necrosis.
T cells
1st generation T cell assays: low sensitivity

GraB, perforin

TCR

MHC I

MHC II

51CR release

Proliferation
2nd generation T cell assays: „ex vivo“ quantification

ELISPOT

Tetramer

TCR

MHC I

GraB, perforin

Cytokines

ICC
3rd generation T cell assays: specific T cell functions

- TCR
- MHC I
- FAS-L
- FAS
- TRAIL
- TRAIL-R
- CD107
- GrB, perforin
- Induction of Apoptosis
- Vital dye-exclusion
- CFSE
- Migration
Example: Antigen-specific T cell responses

- Cytokine profile?
  - IFN$_\gamma$ vs. IL-2, TGF$\beta$, IL-10, etc.
- Memory/effector markers?
  - CD27, CD28, CD45RA, etc.
- Functional avidity?
  - Response to limiting Ag conc.
- Degranulation capacity?
  - CD107a+b
- Presence of anergic cells?
  - Tetramer vs. cytokine
Functional characterization of vaccine-induced T cells by flow cytometry

- Differentiation subsets (effector/memory)
- Cytotoxic potential
- Proliferative capacity
- Type 1/type 2 T cells
- Migratory potential
Functional characterization of vaccine-induced T cells

• T cell differentiation subsets
Simplified CD8+ T Cell Differentiation

Naïve: CD45RA+ CD27+ CD28+

Memory: CD45RA- CD27+ CD28+

Effector: CD45RA+ CD27- CD28-

Increasing cytotoxic potential
Decreasing proliferative potential
Differentiation subset of tyrosinase-specific T cells in peripheral blood following vaccination

C Pat 2: peripheral blood- tetramer

1.6 %

CD8

Tyr- Tetramer

CD45RA

Effector

CCR7
Vaccine-induced tyrosinase-specific central memory T cells reside in bone marrow

Peripheral blood

Bone marrow

Tyrosinase-specific CD8+ IFNγ+ T cells

Tn  Tcm  Tem  Teff

Tyrosinase-specific CD8+ IFNγ+ T cells

Tn  Tcm  Tem  Teff
Functional characterization of vaccine-induced T cells

- Cytotoxic potential
  - granzyme B/perforin
  - CD107 mobilization (Betts MR, JIM, 2003)
CD107 : A new marker for CTL

SEB-activated PBMC

CD107a+b APC

Anti-IFNγ FITC

36%
A subpopulation of tyrosinase-specific T cells mobilizes the cytotoxic membrane protein CD107

+ irrelevant peptide
+ Tyrosinase.368-376 peptide

However, degranulation occurs in the absence of cytotoxicity!

(Wolint P, JEM, 2004)
Functional characterization of vaccine-induced T cells

- Cytotoxicity
  - necrosis (propidium iodide)
  - apoptosis (Annexin V, anti-caspase)
Propidium iodide assay with specific CTL

HIV-peptide

FLU-peptide

30 %

60 %
Flow Cytometric Techniques for Characterizing Proliferating T Cells

Ex-vivo

CFSE
BrdU
Ki-67

in-vitro

Ki-67

Multi-color Combinations
CFSE Example - anti-CD3 Stimulation

Day 0  →  Day 4 - IL-4⁺ cells

No CD81 costim.

+ CD81 costim.
Ki-67 Expression: Correlation With BrdU Labeling Time

In-vivo administration of BrdU, monkey, SIV-gag restim. in vitro
WT1.126-specific, vaccine-induced PB T cells proliferate in response to IL-2, IL-7 and WT1.126-134.
Functional characterization of vaccine-induced T cells

• Type 1/type 2 T cells
Type 1/type 2 T cell response to tyrosinase A2-peptide in 2 melanoma patients before and after treatment with IL-2

before

after 2 cycles

IFN\(\gamma\) - FITC

IL-13 - PE
9-Plex bead assay for detecting P-proteins in activated T cells

1. Itk (Y511)
2. ERK (T202/Y204)
3. JNK (T183/Y185)
4. P38 (T180/Y182)
5. PLCγ (Y783)
6. ZAP70 (Y319)
7. LAT (Y171)
8. c-Jun (S63)
9. RSK (S380)
Functional characterization of vaccine-induced T cells

Therapeutic vaccination:
• ability to migrate into the tumor

Adjuvant vaccination:
• ability to migrate into many compartments
Flow cytometry facilitates direct assessment of the functional characteristics of vaccine-induced T cells including:

- production of type 1/type 2 cytokines
- Migratory potential
- Cytotoxic potential
- proliferative capacity
- differentiation into distinct T cell subsets

Correlation with clinical efficacy?
Differences between various vaccines and adjuvants?
Tumor cells
Tumor cells

- Apoptosis induction
- Signal transduction pathways
- Antigen presentation machinery
- Migratory potential
Low Frequency Measurement and Validation of T Cell and Tumor Cell Characteristics in Vaccine Trials

Areas to cover in breakout session
Technical issues

• Sample Prep/Processing
• Analysis Methods
• Determining Assay Performance
• Management of data from multiple immune assays
Low Frequency Measurement and Validation of T Cell and Tumor Cell Characteristics in Vaccine Trials

Areas to cover in breakout session

Application

• Prioritize assays per question
• Judge assay development stage
• Compile examples of successful applications and reasons for failures