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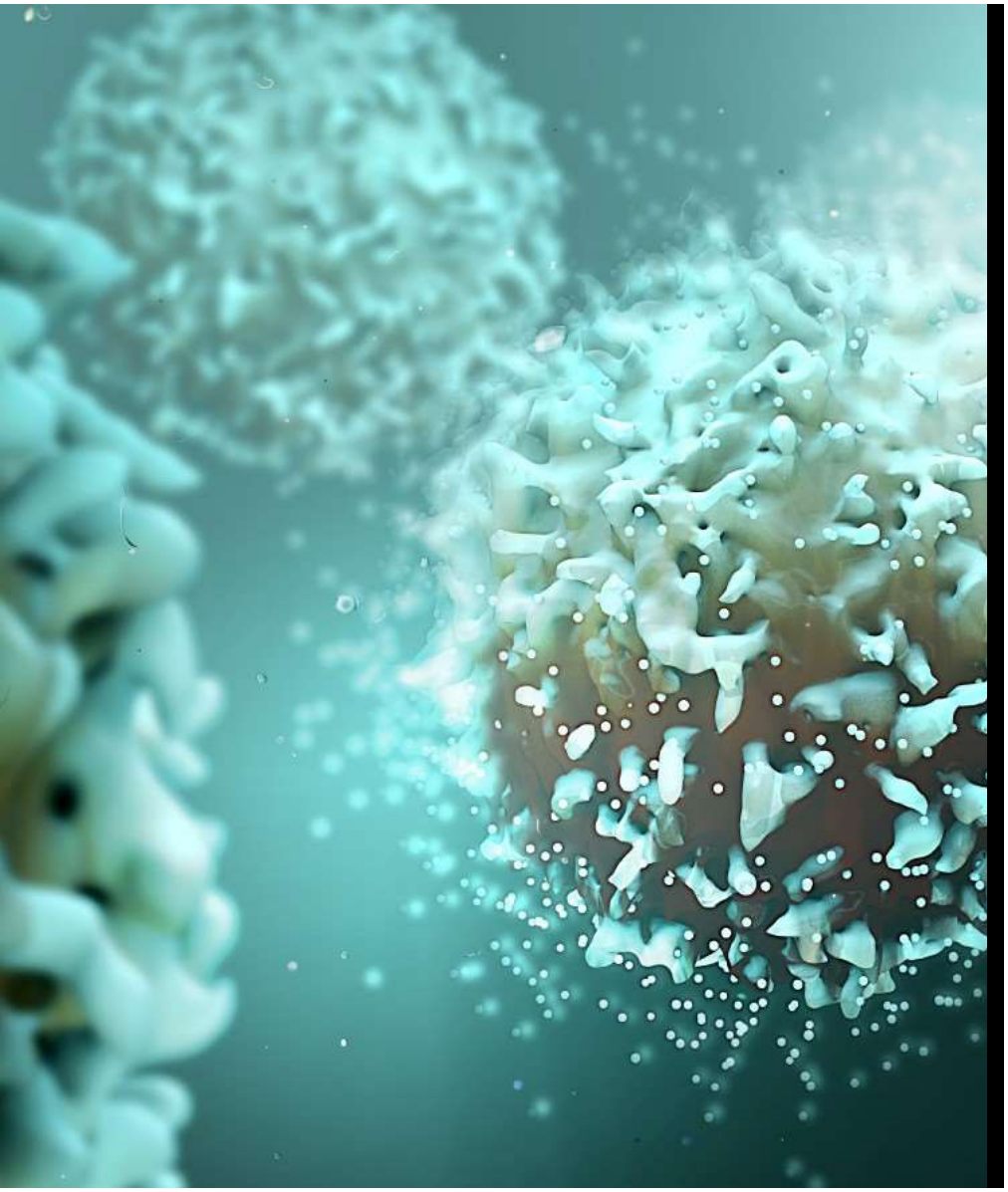
On-demand Training Course

IMMUNOPHENTOYPING

Birgit Fogal, Ph.D. (Boehringer Ingelheim)



Learning Objectives

- Understand what immunophenotyping is and what it can be used for
- Understand basic flow cytometry, one of the technologies used for IP
- Understand basic immunophenotyping markers in different tissues and species and how they compare to humans
- Understand basic interpretation of IP data (as well as challenges, pitfalls, and potential follow ups)
- Have some idea on additional more specific readouts that can be added to basic parameters to answer additional questions

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Introduction

What is Immunophenotyping?

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In general terms:

A technique that uses antibodies to identify cells based on the type of antigens or markers expressed on the surface of a cell.

In immunotoxicological investigations it is used to evaluate changes to the immune cell composition in the blood or lymphoid organs following exposure to pharmaceuticals or toxins

Not a functional readout

Technologies used for Immunophenotyping

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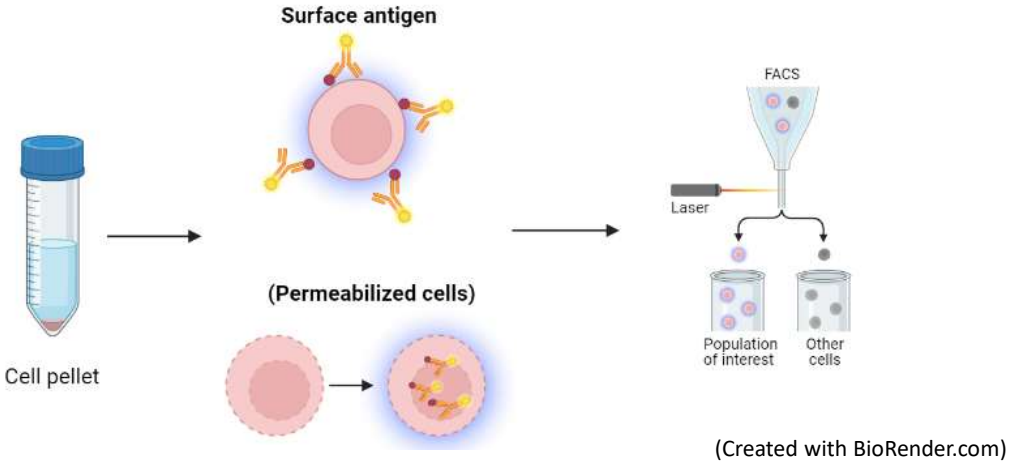
Flow Cytometric Analysis



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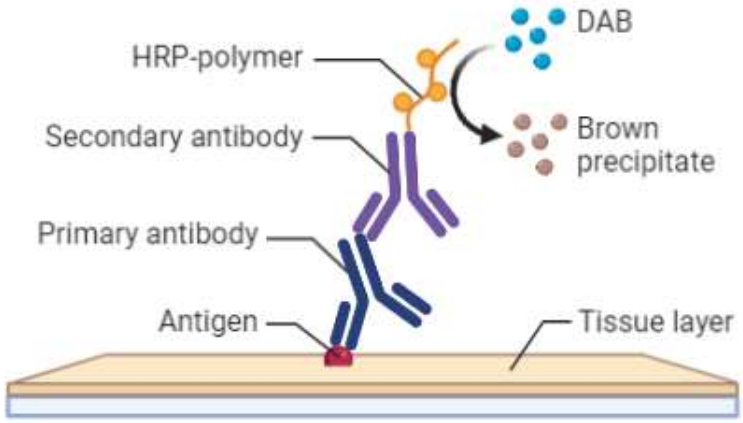
- Can be added to standard tox studies
- Leukocyte enumeration in periphery can be done over time in standard tox studies

Immunohistochemistry



- Can be done retrospectively
- Can examine changes in specific tissue compartments

Regulatory Guidance in ICH S8

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Guidance for Industry

S8 Immunotoxicity Studies for Human Pharmaceuticals

- Provides recommendations on nonclinical testing to identify compounds with the potential to be immunotoxic
- Provides guidance on weight of evidence approach for immunotoxicity testing
- Covers specific section on immunophenotyping

Weight of Evidence De-risking Approach

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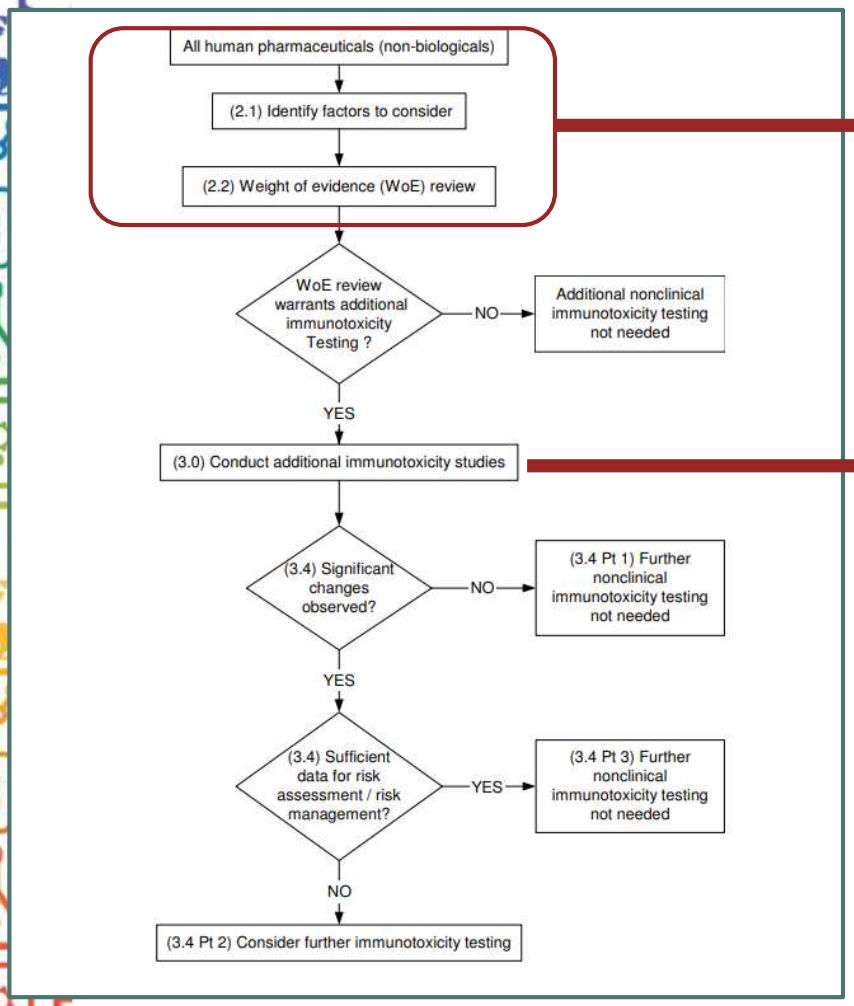

STS
Hematology
Pathology
(IP)

Other Factors
Mechanism of action
Patient population
Disposition of drug
Drug Structure
Clinical Information

Appendix lists additional immunotoxicity studies
T cell dependent antibody response (TDAR)
Immunophenotyping (IP)
Natural Killer cell activity assays
Macrophage/Neutrophil function
Delayed Type Hypersensitivity (DTH)
Host Resistance Assays

Immunophenotyping Guidance

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- Lists both immunohistochemistry and flow cytometry as acceptable approaches (dependent on goal)
- Retrospective analysis listed as an advantage of immunohistochemistry, as well as allowing investigation of specific compartments within the lymphoid tissue
- Quantitative results however are more difficult to obtain with IHC
- Careful consideration should be given to the choice of lymphoid organ and/or peripheral blood to be evaluated (based on observed changes)

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Basic Overview of Immune Cells

Basic Overview of the Immune System

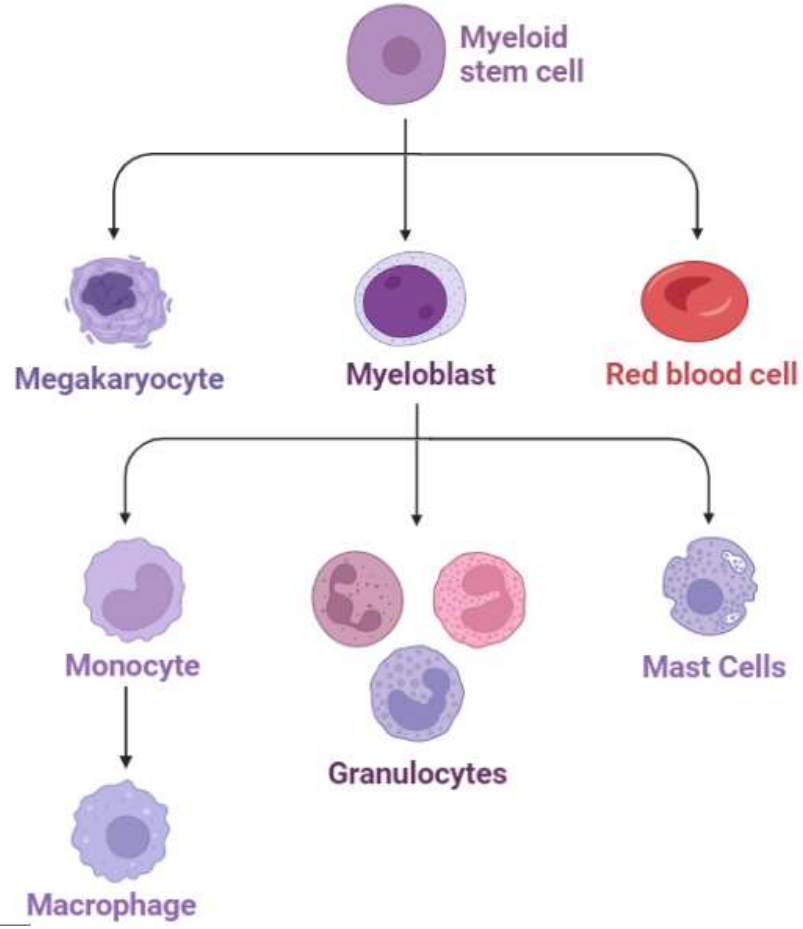
Innate Immune System

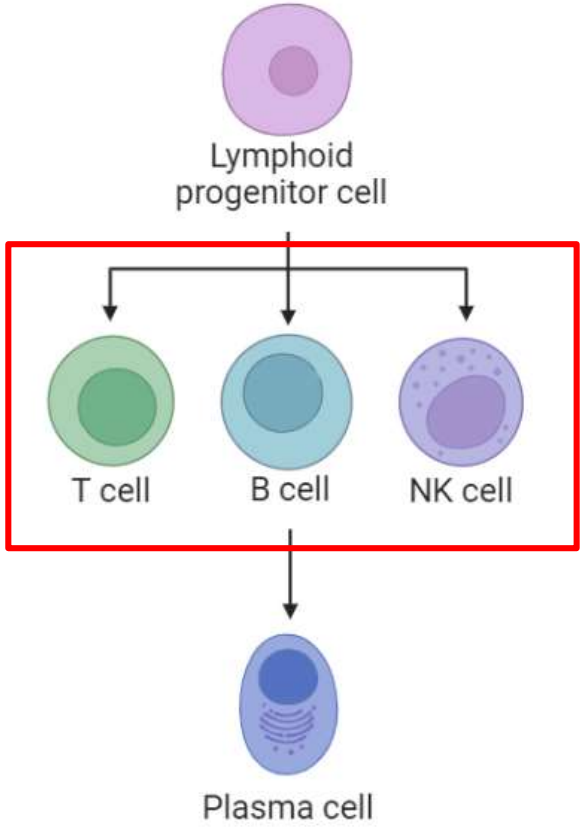
- Immediate response (typically within hours)
- Relatively non-specific
- Lower potency
- No memory (no change in response to second exposure)
- **Innate immune cells: granulocytes, NK cells, monocytes/macrophages**

Adaptive Immune System

- Delayed response (typically days to weeks)
- Specific, pathogen unique responses
- High potency
- Immunological memory (response to second exposure faster, more potent)
- **Adaptive immune cells: T cells and B cells**

Components of the Immune System





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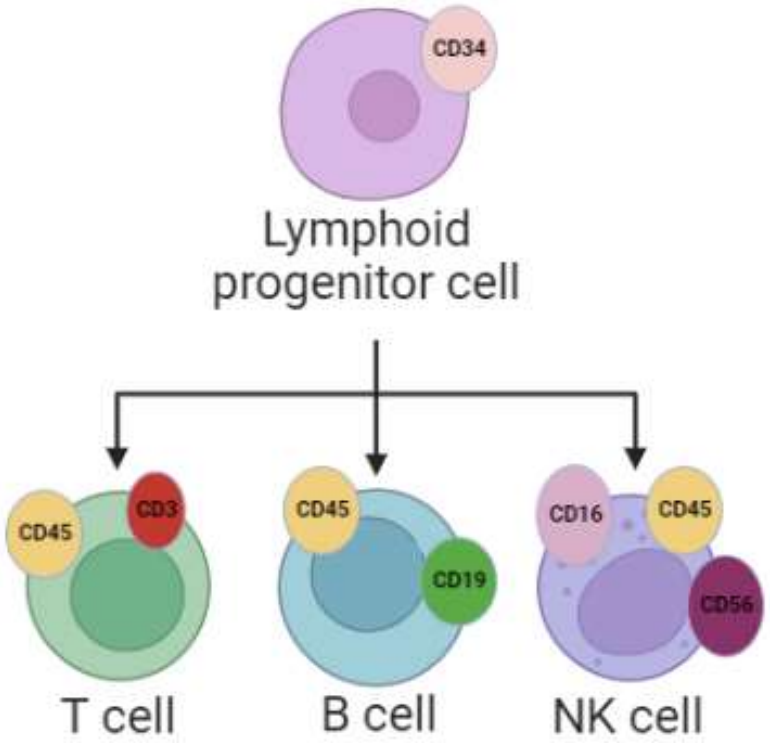
Cluster of Differentiation

- Sometimes referred to as cluster of designation or classification determinant => **in short CD**
- CD nomenclature was originally proposed in 1981 as a classification system for monoclonal antibodies against surface molecules of leukocytes
- CD nomenclature consists of “CD” followed by a number
- As of 2017: CD1 – CD371 (> 400 CD markers due to subsets)
- Commonly used as cell markers => allows cells to be defined based on what molecules are present on their surface

Utilization of CDs in Immunophenotyping



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- For human (clinical studies) the standardization has been promoted by the **Human Immune Phenotyping Consortium:**
  - Maecker, McCoy, and Nussenbaum (2012), Nat Rev Immunol
  - Finak et al. (2016), Scientific Reports

[Essential Markers for Phenotyping (biolegend.com)]

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Introduction to Flow Cytometric Analysis

Flow Cytometer Analysis

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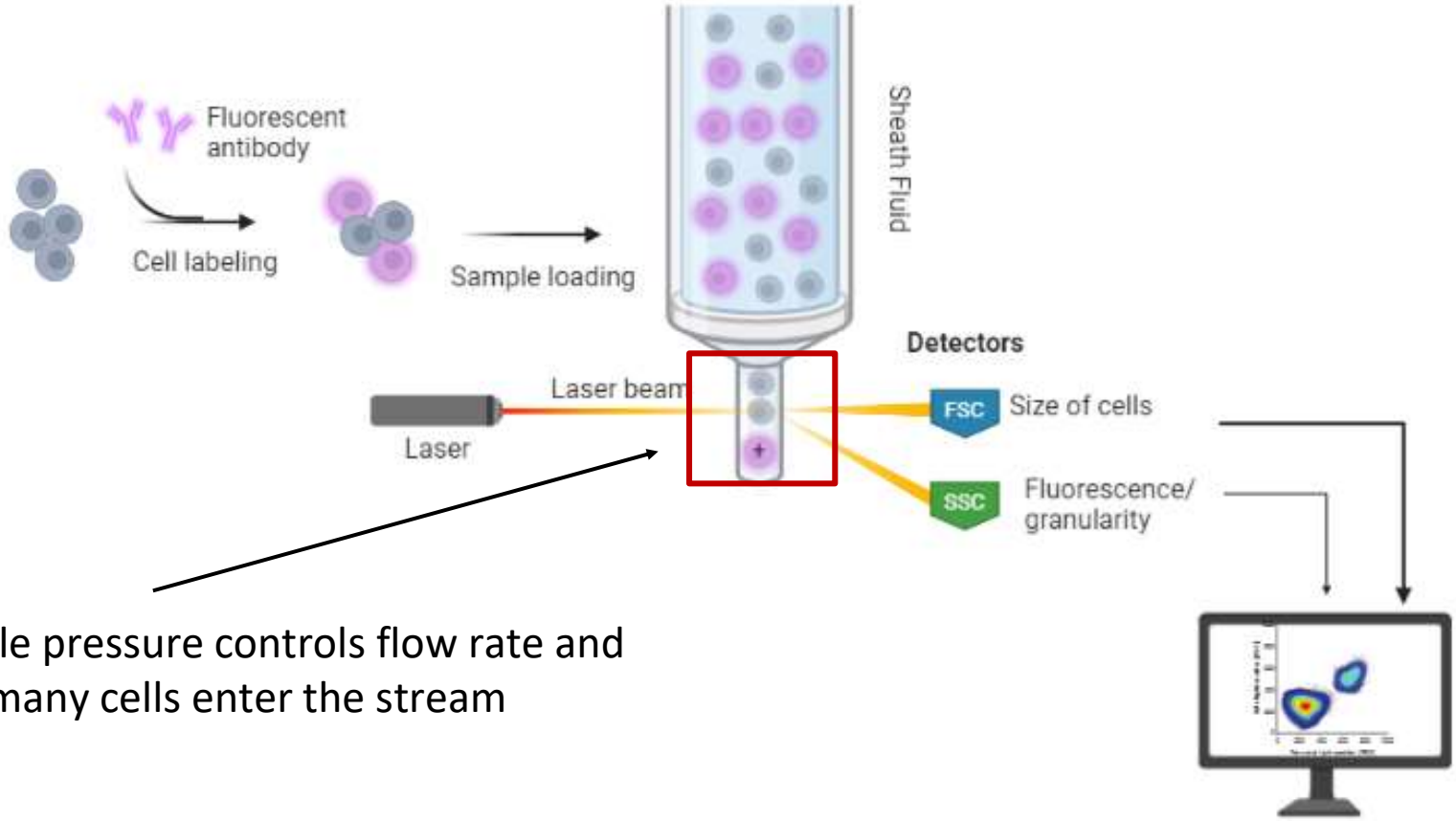

Sample pressure controls flow rate and how many cells enter the stream

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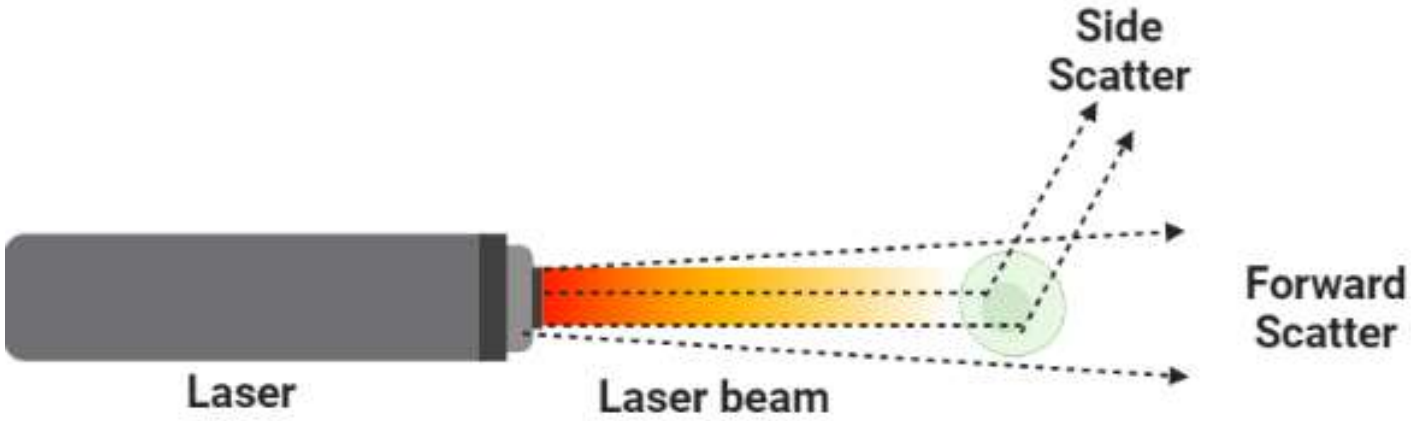
Light Scatter Properties



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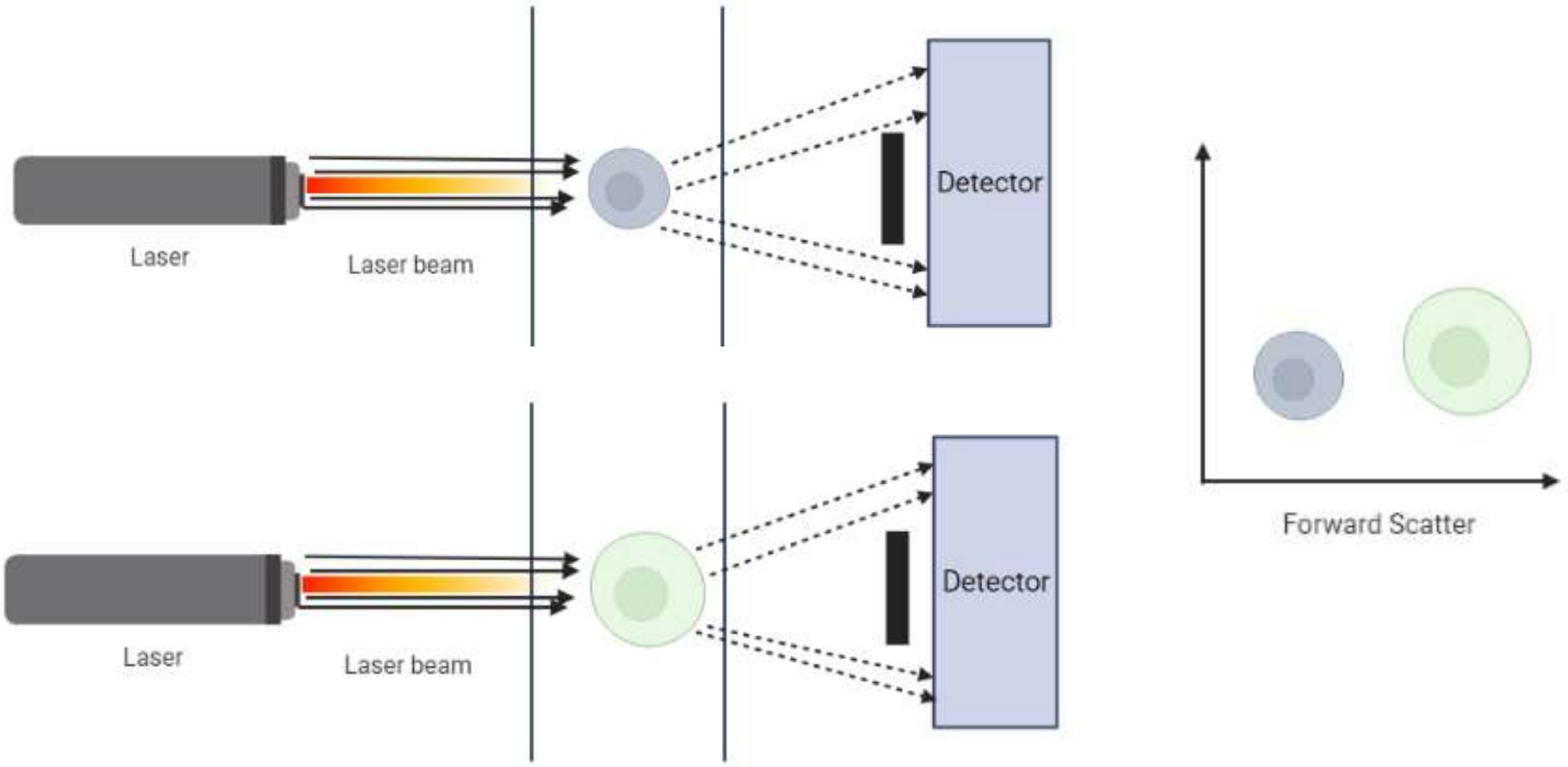
- When laser beam hits the cell, excitation light is scattered in the forward and side direction
- Cell size affects the forward scatter light
- Cell morphology and granularity affects the side scatter light

Forward Scatter



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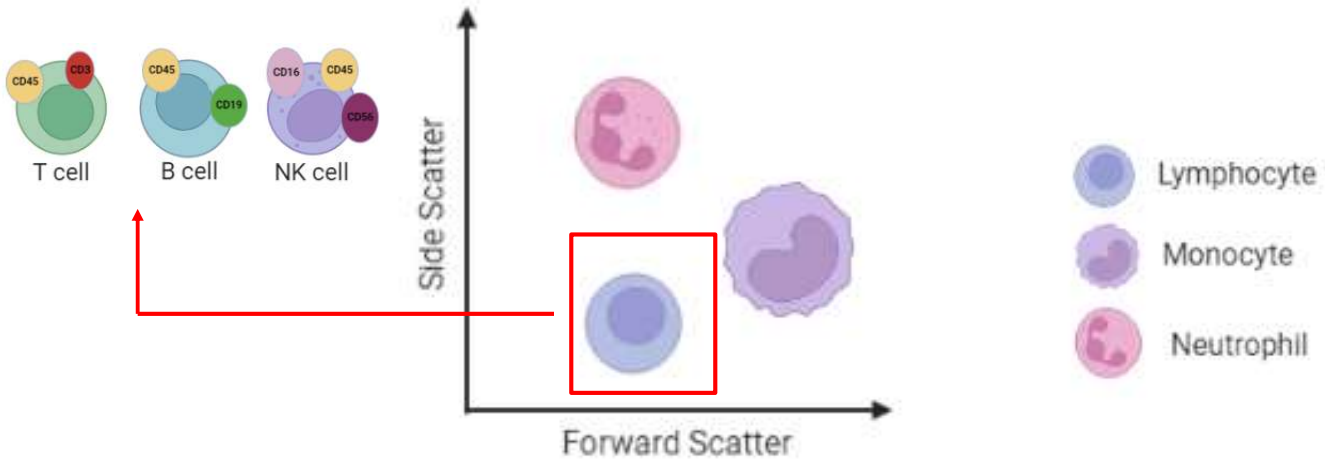
Side Scatter

- The side angle scatter represents the scattering from the internal complexity of the cells being examined.
- Cellular components such as the nucleus, granules, etc. will influence the intensity of the side scatter



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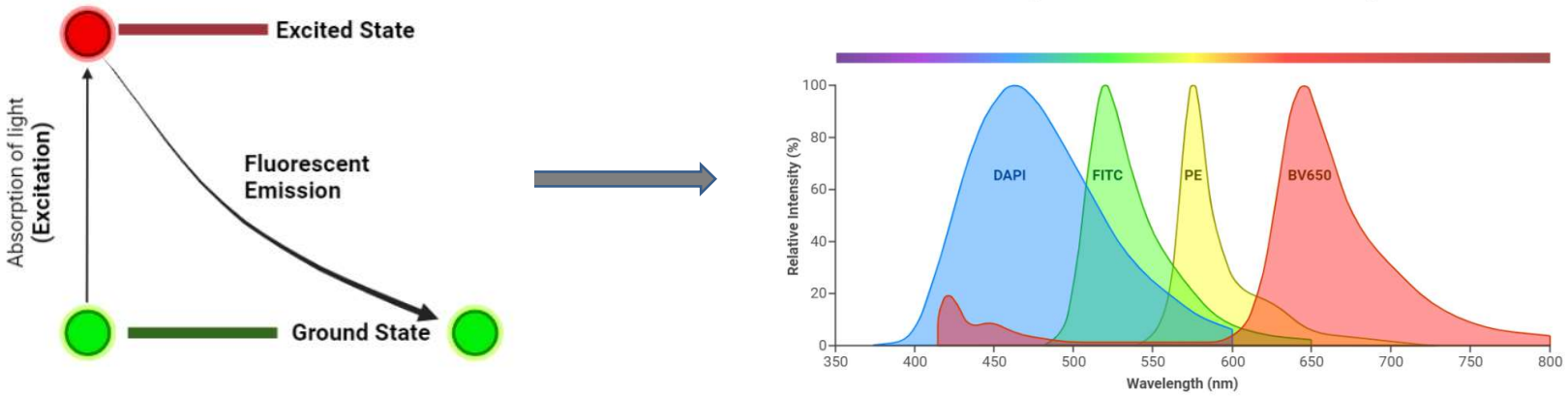
Fluorescence Detection

- Fluorescently labeled antibody absorb light energy over a range of wavelength that is characteristic for that fluorophore (**excitation wavelength**)
- Electron in fluorescent compound moves to higher energy state
- Excited electron quickly decays to ground state and emits energy as a photon of light (**emission wavelength**)



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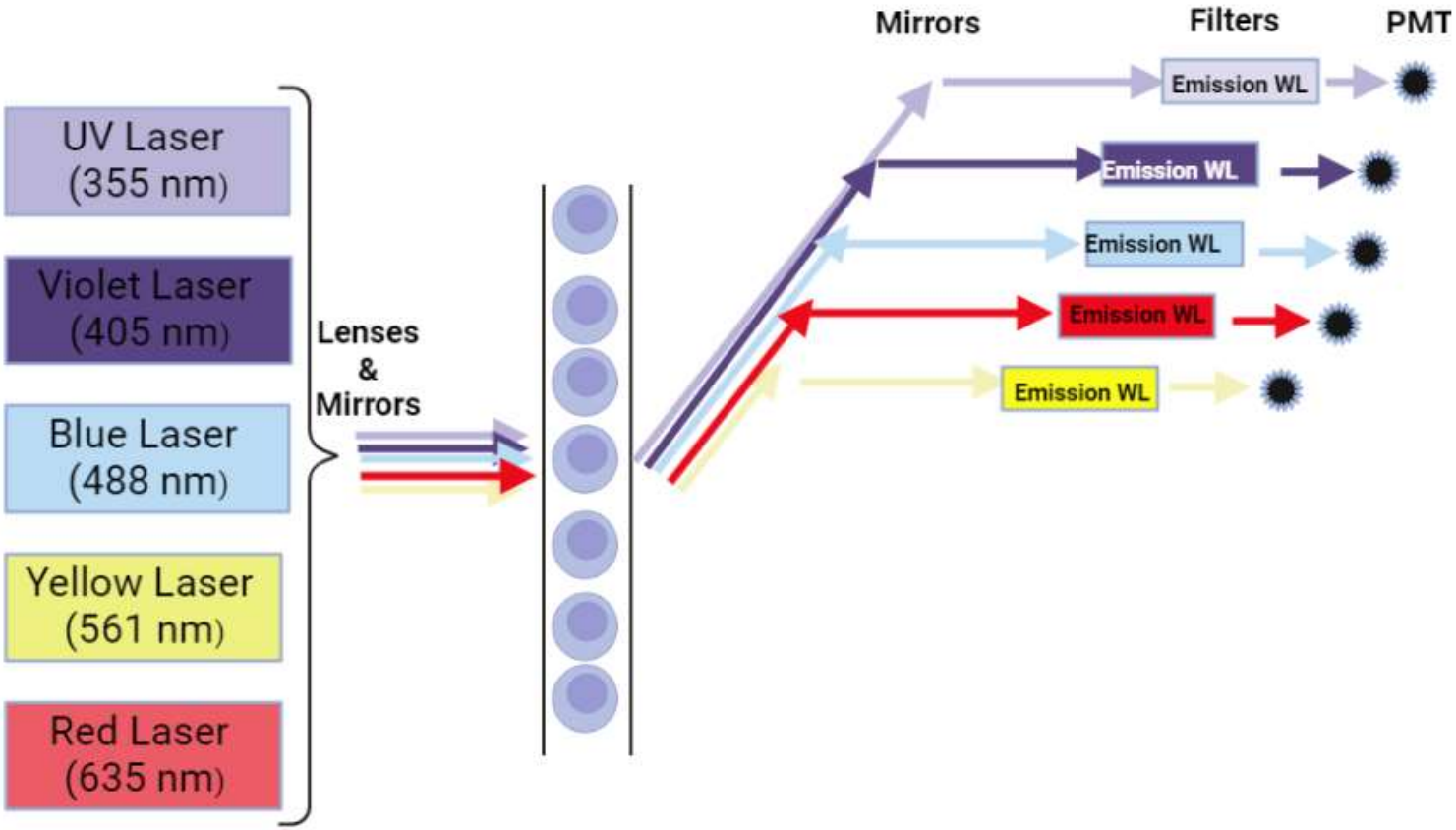
Fluorescence Detection



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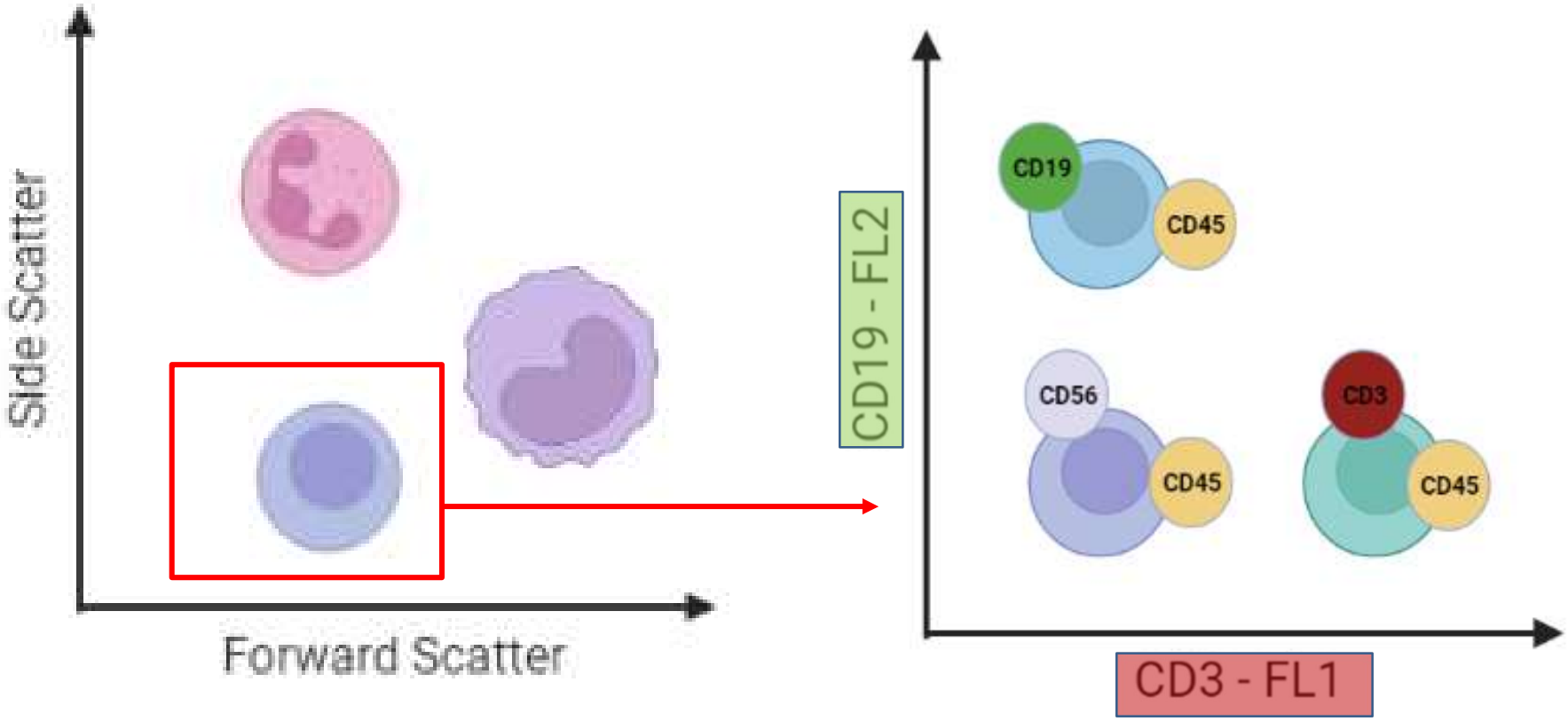
Combine to identify cells of interest



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Basic Immunophenotyping of Blood Cells

Immunophenotyping of Blood cells

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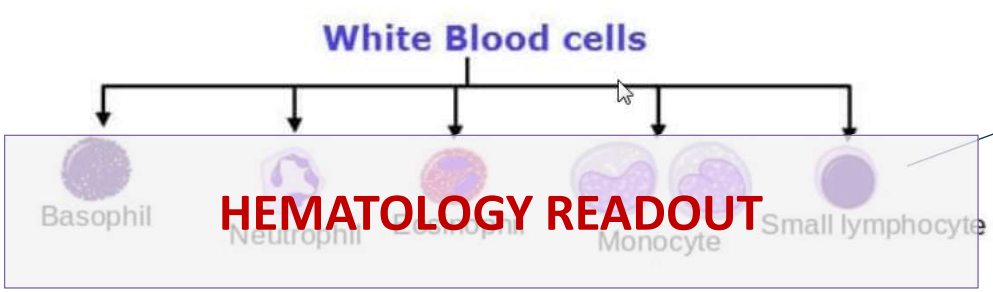
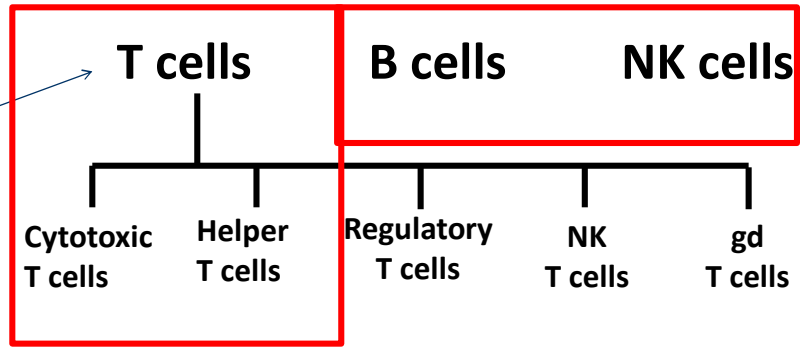
- Basic immunophenotyping of peripheral blood cells generally consists of enumerating T cells, B cells, and Natural Killer cells
- Sometimes monocytes are also included, as well as further characterization of subsets, such as cytotoxic and helper T cells
- Typically, the percentage of each cell type among a subset of cells as well as the total number of cells is measured





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Most Common CD Markers for basic IP

	Human	Rat	Dog	Macaque	Minipig
Total T cells	CD3+	CD3+	CD3+	CD3+	CD3+
Helper T cells	CD3+ CD4+	CD3+ CD4+	CD3+ CD4+	CD3+ CD4+	CD3+ CD4+
Cytotoxic T cells	CD3+ CD8+	CD3+ CD8+	CD3+ CD8+	CD3+ CD8+	CD3+ CD8+
Total B cells	CD3- CD20+	CD3- CD45RA+	CD3- CD21+	CD3- CD20+	CD3- CD21+
NK cells	CD3- CD56+	CD3- CD161a+	-	CD3- CD16+	CD3- CD16+ CD335+

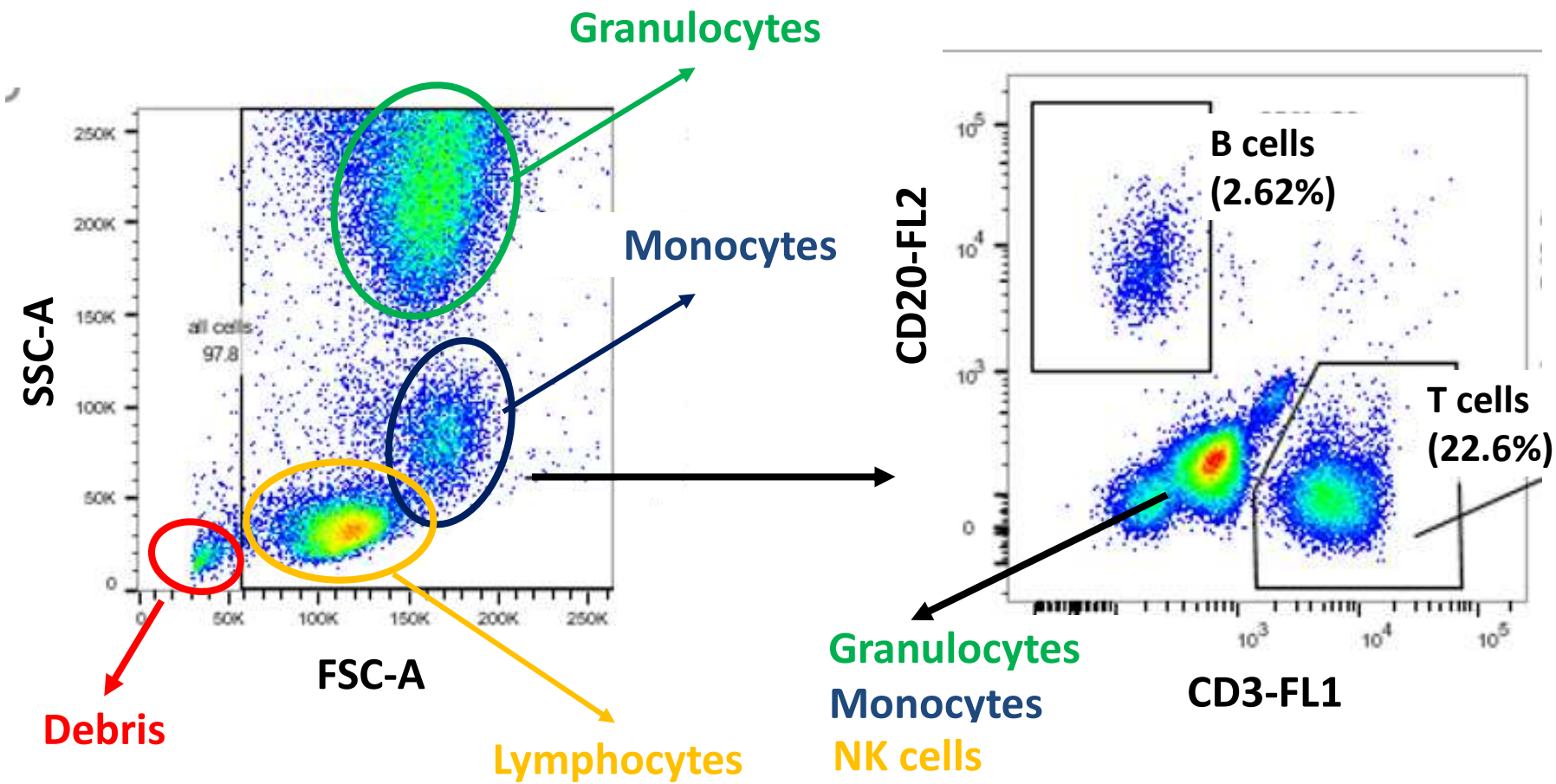
What are these markers?

- CD3: part of the TCR complex, expressed on T cells and NK T cells
- CD4: TCR co-receptor, binds to MHC II, expressed on T helper cells, monocytes/macrophages
- CD8: TCR co-receptor, binds to MHC I, expressed on T cytotoxic cells
  (in minipig, CD8 can be expressed at low levels on T helper cells; minipigs also show relatively high frequency of CD4/CD8 double positive cells)
- CD20: expressed on pre-B cells, resting and activated B cells, some T cells (low) and DCs
- CD56: neural cell adhesion molecule; homophilic binding glycoprotein expressed on NK cells
- CD16: low affinity FcγRIII, expressed on NK cells, activated monocytes
- CD159a: NKG2A, inhibitory NK cell receptor
- CD45RA: leukocyte common antigen, B cell specific isoform in rats
- CD161a: NKR-P1a (C type lectin R), expressed on NK cells, subset of T cells, activated monocyte
- CD21: complement/EBV receptor, expressed on mature B cells, some T cells and DCs
- CD335: also known as NKp46, is a 46 kDa transmembrane receptor expressed on NK cells

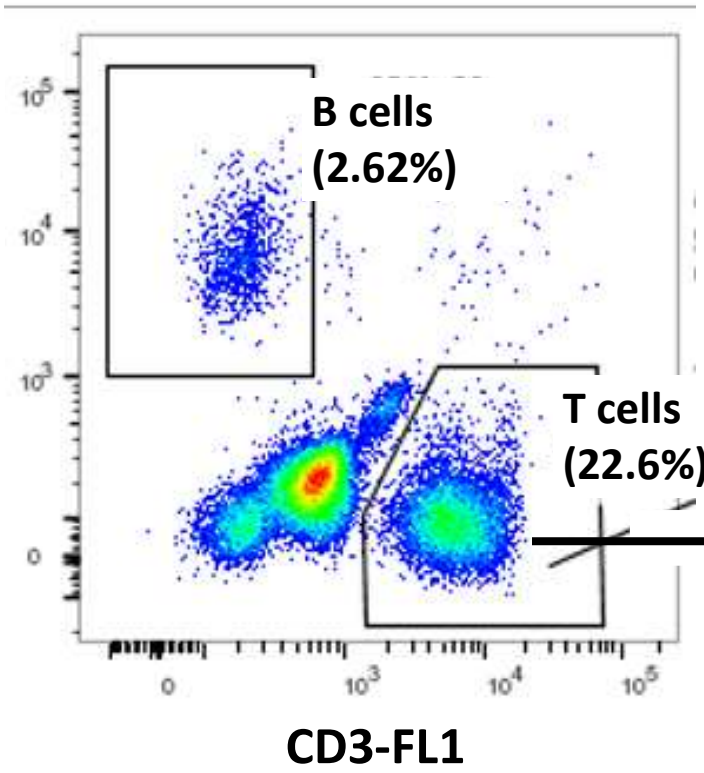
Species Differences

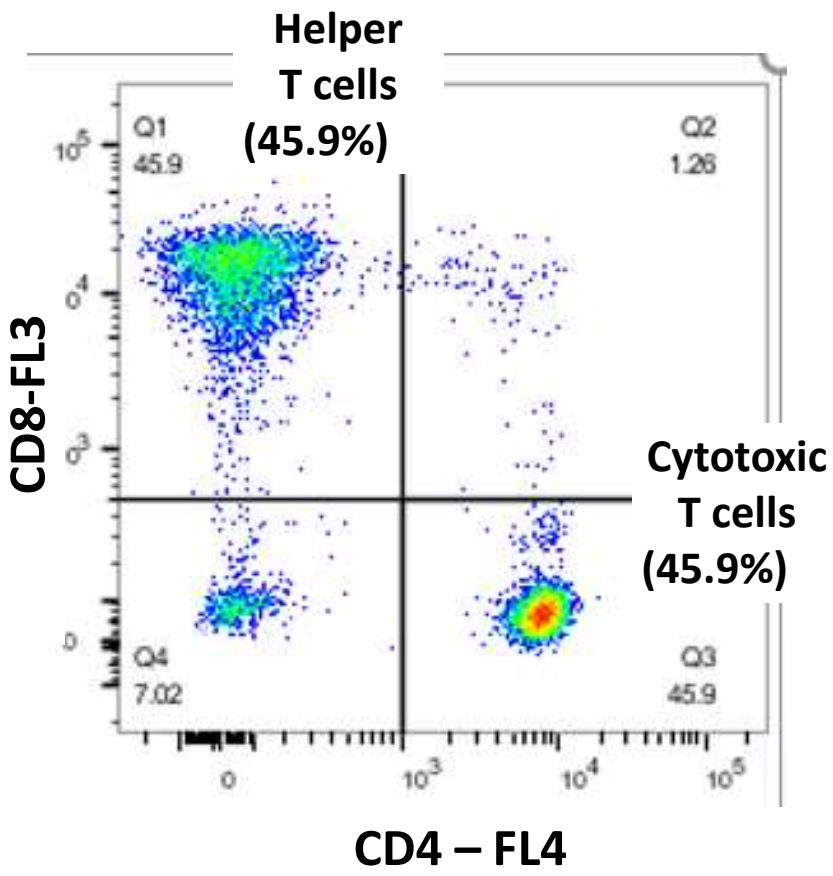
	Human	Rat	Dog	Macaque	Minipig
Total T cells	15-30%	40-60%	60%	17-25%	25-55%
Helper T cells	10-20%	30-40%	37%	7-15%	6-10%
Cytotoxic T cells	3-10%	15-25%	19%	2-6%	10-15%
Total B cells	3-10%	20-40%	11.4%	2-14%	9%
NK cells	2-5%	5-10%	n/a	1-5 %	10-40%

Example – Human Cells

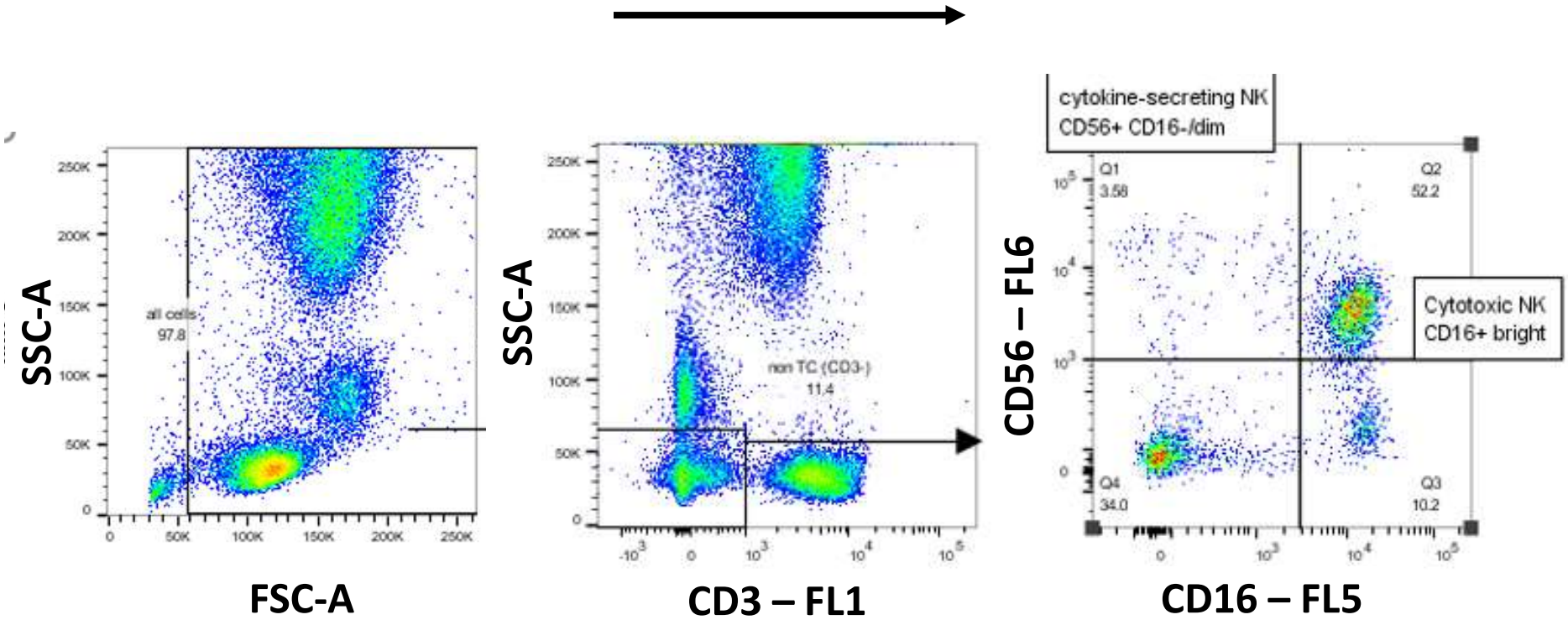


Example – Human Cells (T cells)

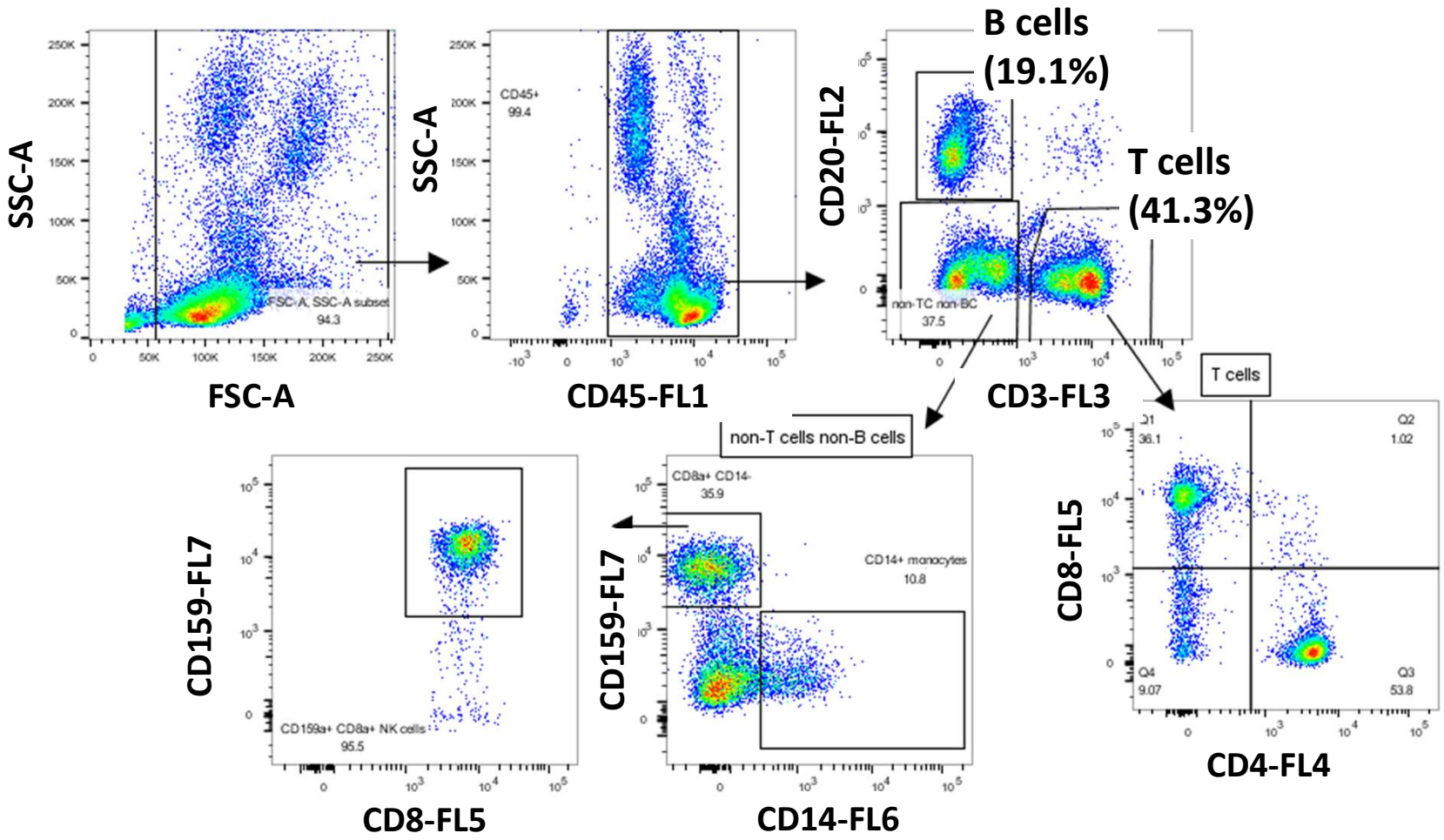




Example – Human Cells (NK cells)



Example – Cynomolgus monkey Cells



How to enumerate cells?

Hematology based (e.g. Advia)

- Hematology data will provide a number of white blood cells/µl or L of blood
- This can be utilized to calculate the number of cells identified by flow cytometry: If 2.62% of WBCs are B cells the number can be calculated as a percentage of WBC #

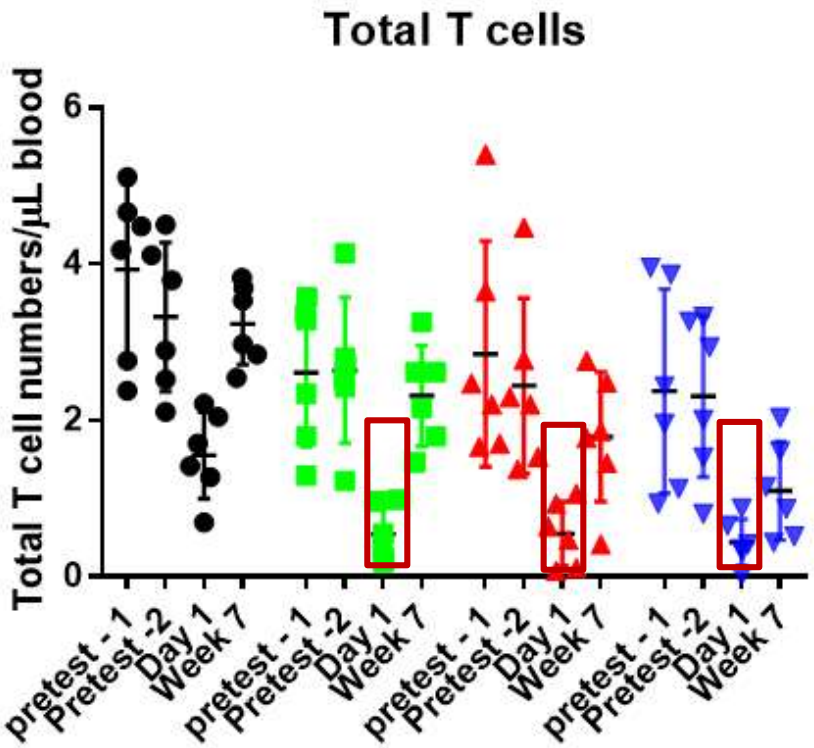
2.6% of WBC (10,000/uL blood), then
0.026 * 10,000 = B cells/ul blood = 260 B cells/uL

Counting Beads based

- Offered by broad # of vendors
- Beads the size to be captured with most cell types, and with broad excitation/emission spectrum
- added to cell solution
- Defined number of particles/µL solution
- Because # of beads added/unit of volume is known, one can calculate absolute # of e.g. WBCs
- Calculations are described in product protocols

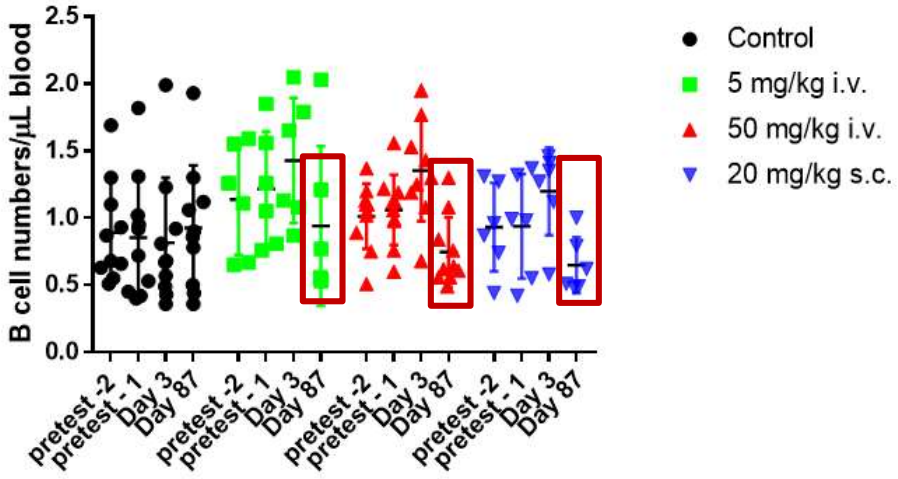
Example of Treatment Effects on T cells

- Treatment of cynomolgus monkeys with T cell engagers (TcE) often results in drop of T cells following dosing
- Increasing concentrations of TcE resulted in decreased numbers of T cells in peripheral blood
- Recovery slower at higher concentrations



Example of Treatment Effects on B cells

- Treatment of cynomolgus monkeys with mAb targeting Receptor expressed on B cells (and some other immune cells)
- Involved in regulation of survival and proliferation of B cells
- Drop in B cell number observed starting at
  5 mg/kg treatment
- TDAR showed decrease in IgG production



To detect 50% change by comparing pre-treatment with post-treatment counts within single given group of animals power analysis indicates 6-8 animals are necessary

Biological Significant Consequences

- Main challenge when performing immunophenotyping analyses:
  - **How small or large of a quantitative change in a parameter assessed by flow cytometry is necessary to predict biologically significant consequences?**
- The reduction in B cells in previous example correlated with reduction in a T cell dependent antigen response, a functional assay that measures antibody production by B cells
- Peripheral analysis may not always be telling by itself (consider inclusion of spleen immunophenotyping when trying to identify hazard)
- Can be helpful to monitor a mechanism-based effect or to focus on the underlying observed effect (e.g. diminished functional response)

Considerations for Study Design in Rats

- **Sex, Age and Strain differences exist** (Morris and Komocsar 1997, Petkova et al. 2008, Pinchuk and Filipov 2008, Yamatoya et al. 2012, Ridge and Finney.,2019):
- While there might be little variation in some populations, changes might exist in subpopulation makedup (e.g. T helper versus T cytotoxic T cell ratio)
- For Immunophenotyping studies typically both genders are included and are analyzed separately similar to analysis of standard hematology data.
- Consider power analysis to understand what differences can be detected and always keep in mind what you are not measuring

Considerations for Study Design in Cynomolgus Monkeys

- **Gender and age** associated effects have been observed in cynomolgus monkeys (Kresja et al 2013)
- Small, but statistically significant difference in average cell counts of total T cells, helper and cytotoxic T cells, B cells and NK cells (males have higher mean counts than females, in all cell types except NK cells)
- For Immunophenotyping studies typically both genders are included and are analyzed separately similar to analysis of standard hematology data.
- Some age-related differences were also observed, but as animals are typically age-restricted for toxicology studies, impact is unlikely
- **Origin of monkeys** (“Island” vs “Mainland” monkeys) can be associated with differences in average cell counts for immune cells

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Additional considerations

Spectral Overlap

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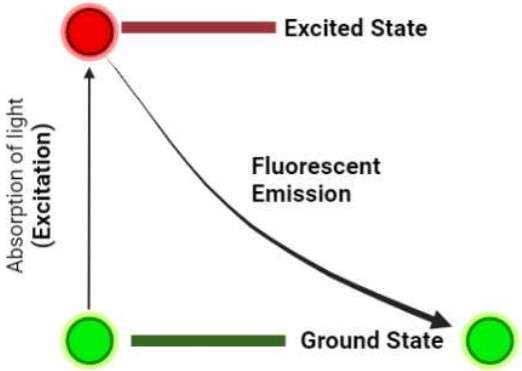
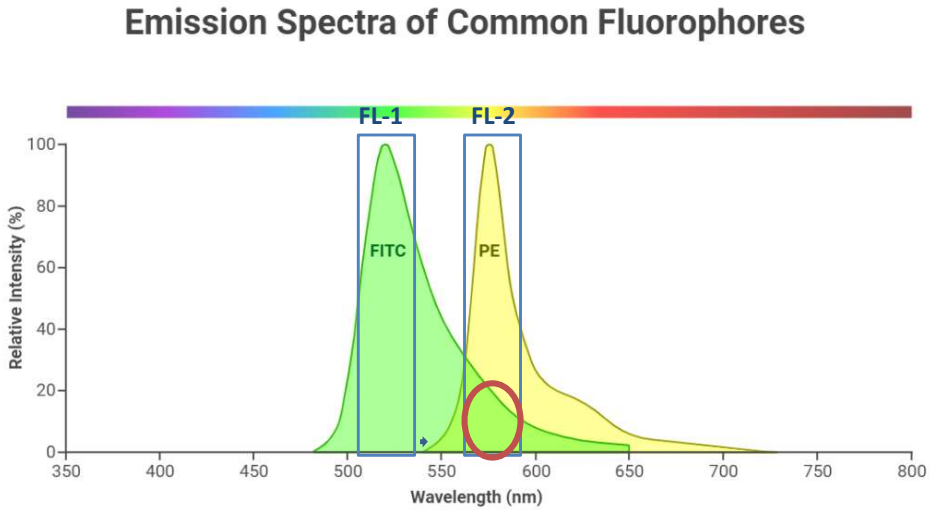
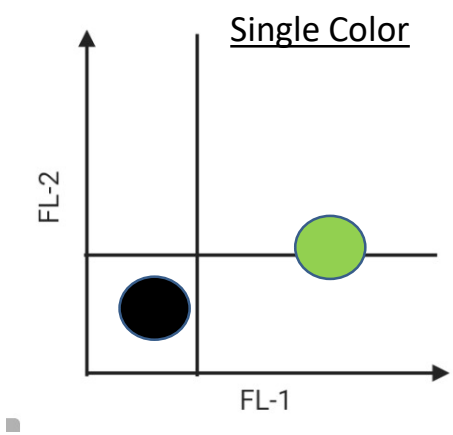
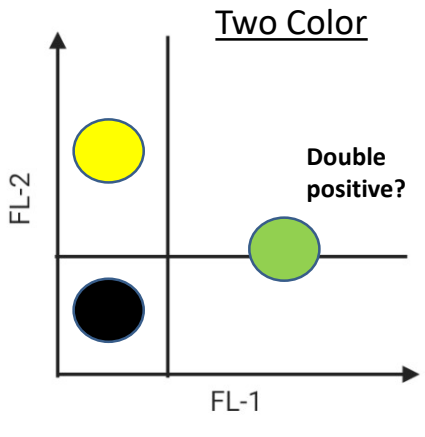
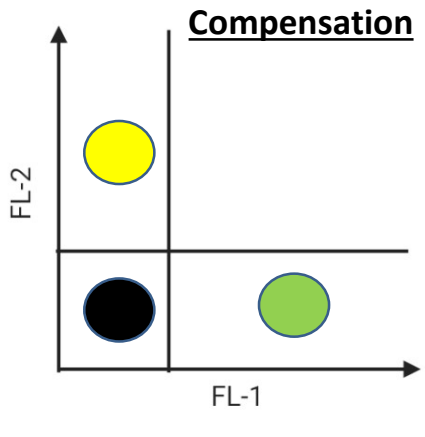






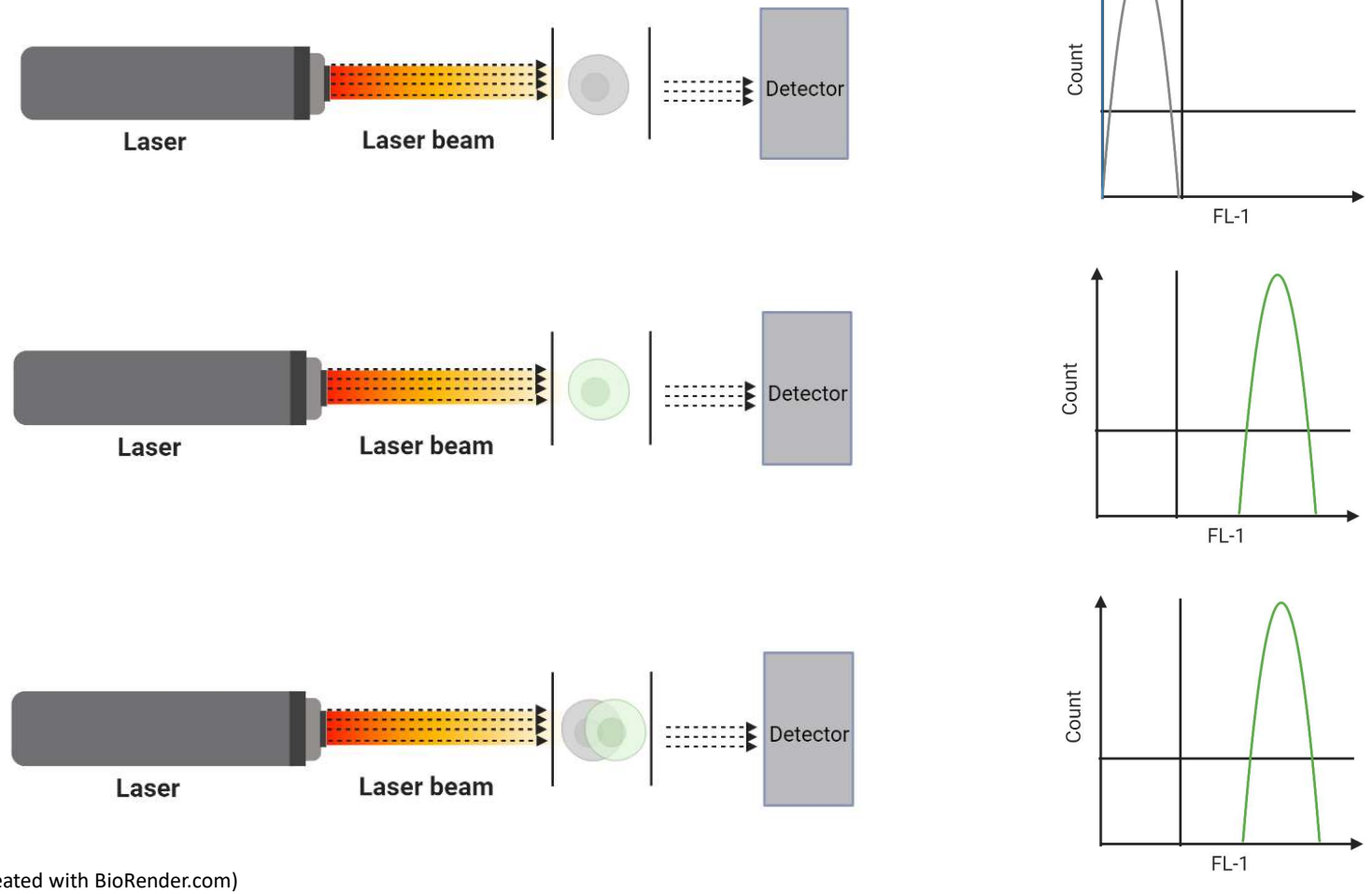




Compensation

- **Depends upon having single-color stained controls, as well as unstained controls:**
  - Can use single color stained cells
  - Also possible to use flow cytometry compensation beads
- **Some important considerations for preparation of single color controls:**
  - Stained compensation control must be as bright (or brighter) than the sample
  - Typically, one fluorophore conjugated antibody can be substitute for another with the exception of tandem dyes
  - Autofluorescence needs to be the same (always need negative control)
  - Cannot substitute one fluorophore for another (e.g. GFP and FITC)
- Software calculates the spillover and applies that to the compensated data
- Compensation need can somewhat be minimized
  - Available excitation lasers
  - Choice of fluorophores
  - Panel design
- The greater the number of fluorophores the higher the need for compensation

Doublet Detection



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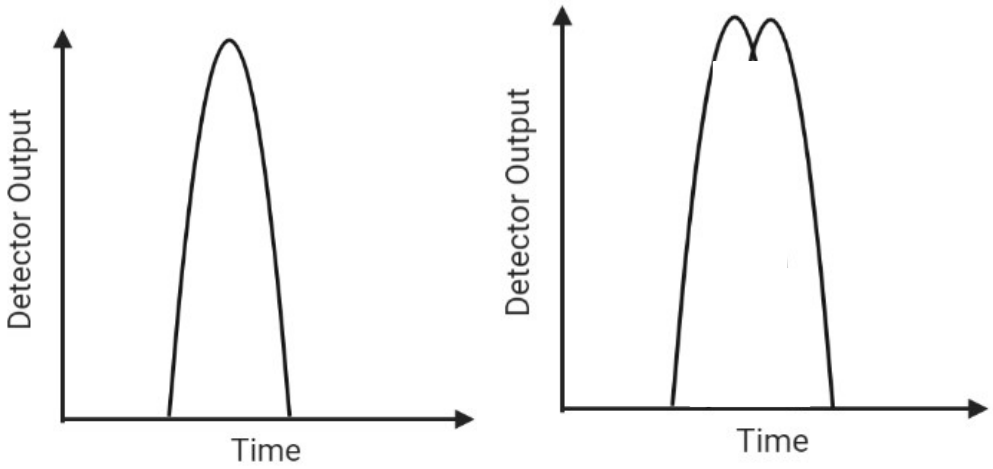
Doublet Discrimination



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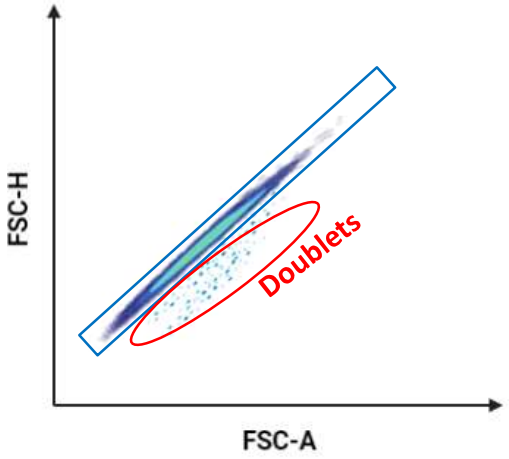
X-Axis	Y-Axis
FSC-Area	FSC-Height
FSC-Area	FSC-Width
FSC-Height	FSC-Width

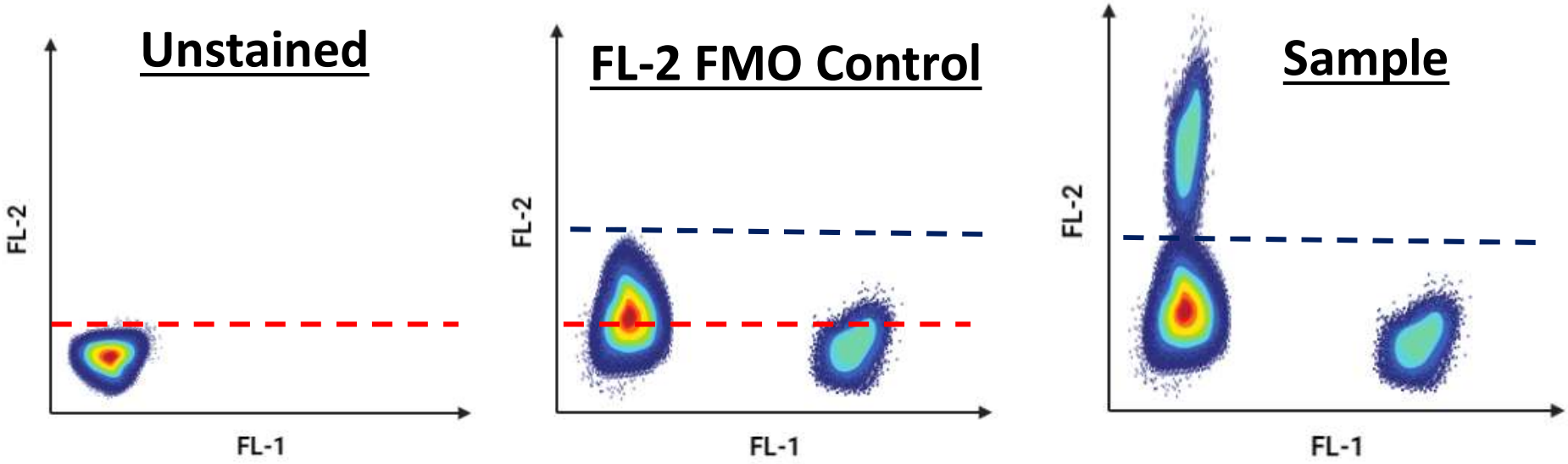
- Two cells take a longer time passing through the laser beam than one
  - The width of the doublet pulse is wider
  - The area of the double pulse is wider
  - BUT, the height remains the same



Fluorescence Minus Once (FMO) Controls

- For multi-color panels, especially complex ones, FMOs are more accurate gating controls than unstained or single stained samples:
  - Take into account the widening of negative population due to the spillover spread
  - Especially important when positive stained cells are not clearly separated from the negatives (e.g. dim populations)
- These samples are stained with all the fluorophores in the panel, minus one of them
- Does not control for non-specific antibody binding (i.e. through FcRs or to off-target antigens)

Gating with FMO controls



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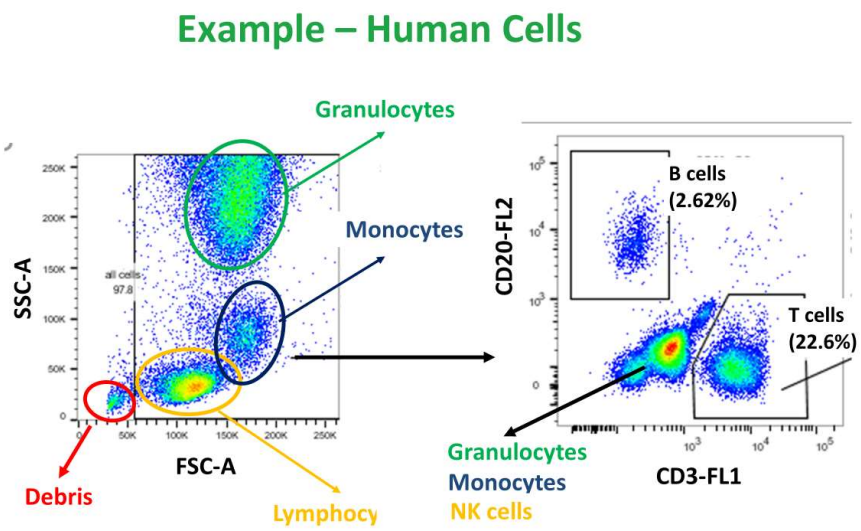
Considerations for Panel Design

- **Understand your instrument:**
  - Know what lasers and filters you have
- Understand the spectral overlap of fluorophores
  - Online tools that allow visualization of spectral overlap
- Use bright fluorophores on antibodies for low abundance proteins and dim fluorophores on antibodies for highly expressed proteins
- Use fluorophores that are spectrally similar for different cell subpopulations that will be gated and analyzed separately
- Titrate and optimize each antibody
- Include a cell viability dye in the panel to exclude dead cells and debris from the data

Panel Design is an art and requires experience and collaboration (technical and biological knowledge)

Automated Flow cytometry gating



- Inherent subjectivity of gating: strategies may differ between analysts, analyzers and laboratories
  => challenges for comparison of data
- Increasing number of parameters included in the flow cytometry panel (> 50 colors are possible now)
  => manual gating can become prohibitive

Need for computational assistance for flow cytometry analysis

Automated Flow cytometry gating

- Freely and commercial software packages have been reported
  - completely automated (without need for guidance by end-user)
  - partially automated (requires tuning)
- Software needs to correctly identify clusters of groups of data points
- FlowCap consortium

K-Means	Model-based	Manual gating
k number of clusters are identified/specified	Does not require a priori input (most use Gaussian model)	End-user pre-defines cell populations manually, using FMO controls
Computational efficiency	Time consuming (computational effort)	Computational efficiency
Restricted to spherically shaped populations	Robust to shape of cell populations	Advantage when looking for rare populations

Considerations for rare populations

- Some cell populations are relatively low in number (< 1%)
  => technical and interpretative challenges
- Enrichment of sample could be considered (positive or negative)
  => can be challenging in a toxicology study
- Try to maximize signal to noise ratio of cells of interest from background
- Ensure instrument is clean (consider background rate of noise)

- To increase precision, increase the number of target events collected
  - For 5% CV, 400 events of interest would need to be collected
    => For a population occurring at 10%, 4,000 total events would suffice
    => For population occurring at 0.1%, 400,000 total events would need to be collected for same precision

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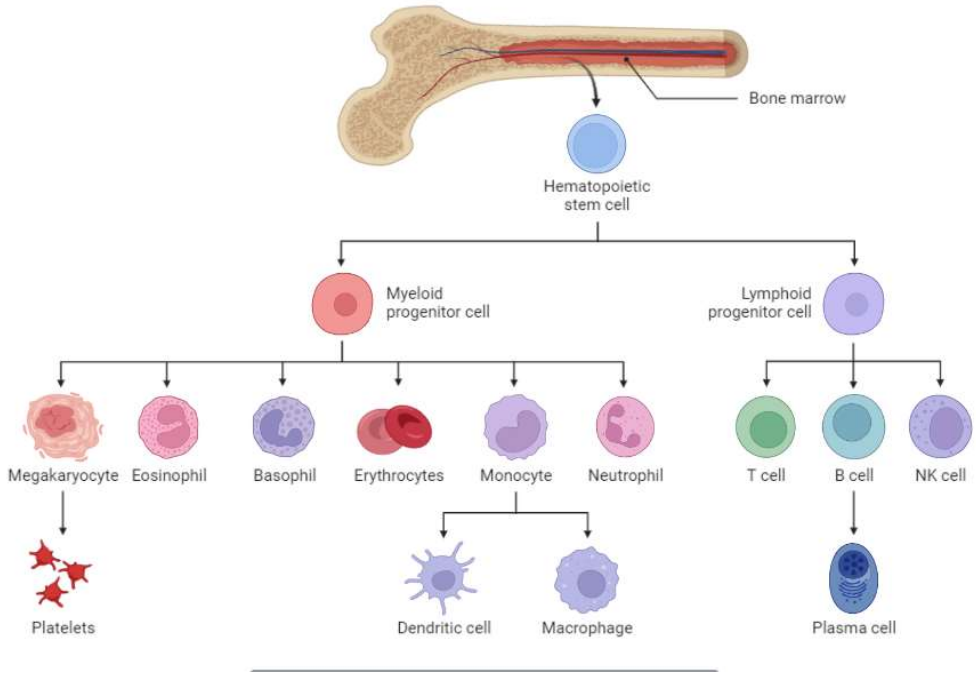
Tissue Immunophenotyping

Bone Marrow

Major hematopoietic organ and primary lymphoid organ



- Production of red blood cells, granulocytes, lymphocytes, monocytes and platelets
- Depends on complex interplay between stem cells, committed progenitor cells and the bone marrow microenvironment
- Changes can be investigated by histopathology, but deeper characterization might be helpful
- Typically considered when changes have been observed

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Flow Cytometry versus Cytology Evaluation

Flow Cytometry	Cytology
Requires fresh samples for analysis	Cytological smears can be analyzed at later time
Cannot assess cellular morphologic changes	Can assess cellular morphologic changes
Can classify major categories of hematopoietic cells (e.g. neutrophilic, granulocytic, lymphocytic and erythrocytic lineages)	Can provide complete classification of all different cell types; development orderliness
Can classify proliferating and non-proliferating cell pools (via addition of additional staining)	Can incorporate understanding of proliferation and differentiation by inclusion of specialized methods
Data can be acquired quickly with high precision	Relatively slow process, requiring special training
Typically analyzes between 10-35,000 cells	Typically analyzes 300-500 cells

Cytological analysis should always be prepared

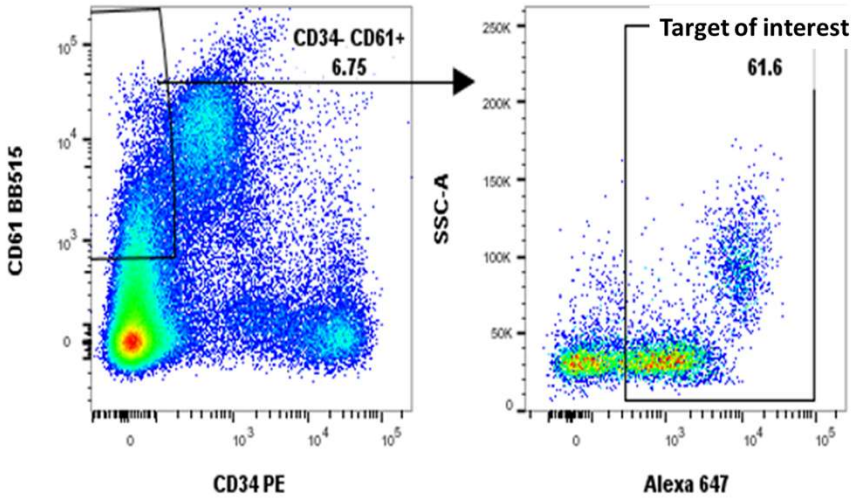
Interest in expression of target in bone marrow (Focus: Megakaryocytes)

	MEP	Mega karyoblast	Promega karyoblast	Mega karyocyte
CD34	++	-/+	-	-
CD38	++	-/+	+	++
CD41	++	+	+	++
CD42	-	-	-/+	+
CD61	-	+	+	++

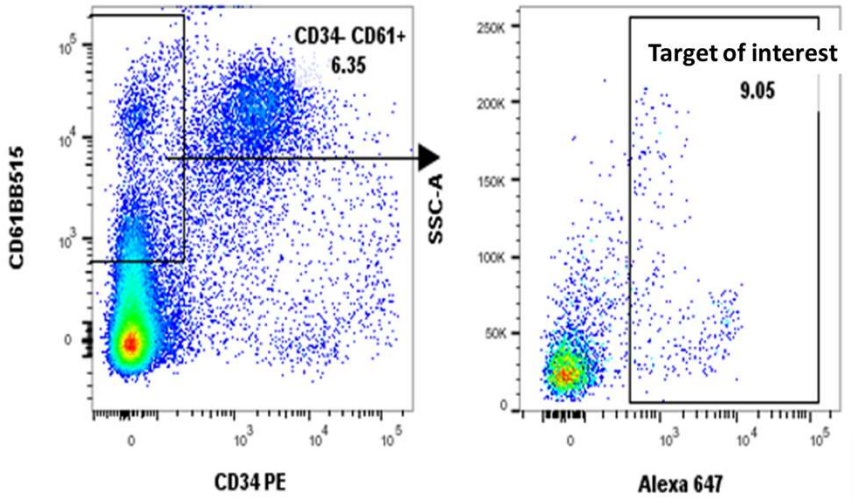
Attar et al 2014

Interest in expression of target in bone marrow (Focus: Megakaryocytes)

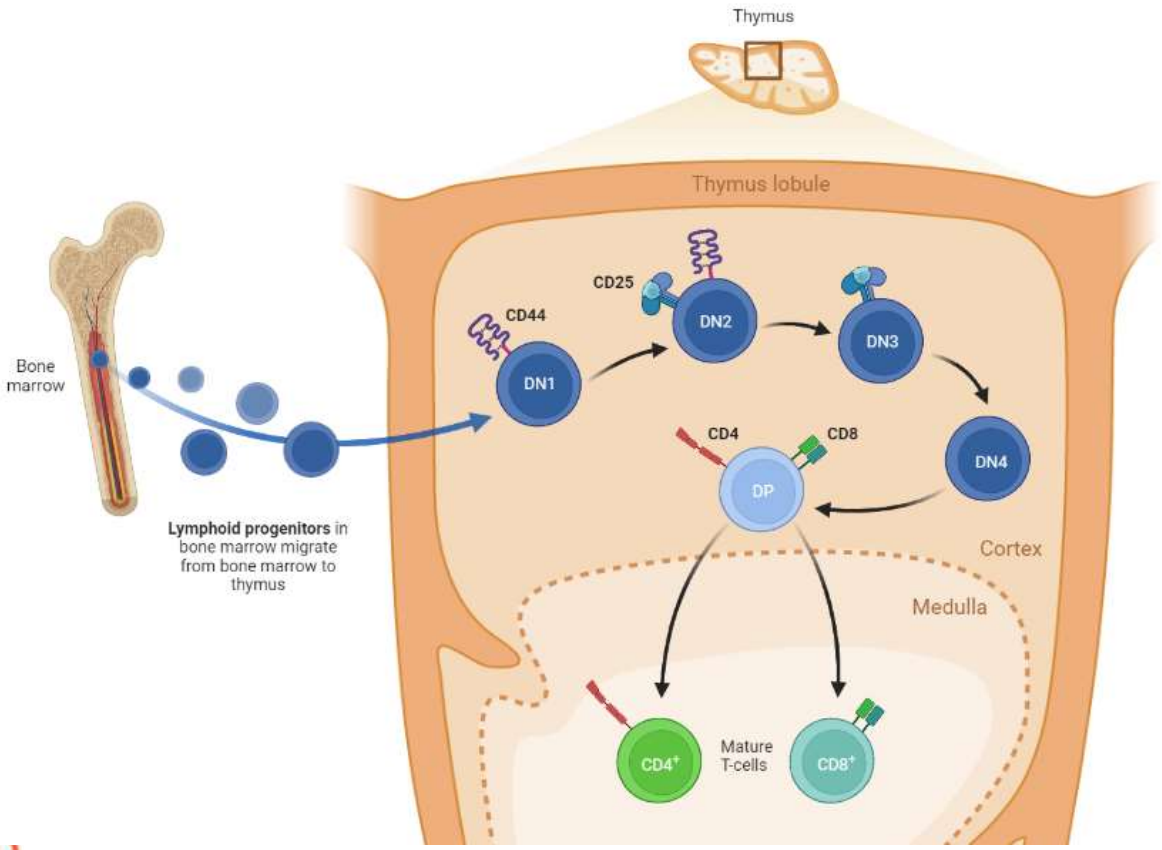
Human Bone Marrow



Cyno Bone Marrow



Thymus



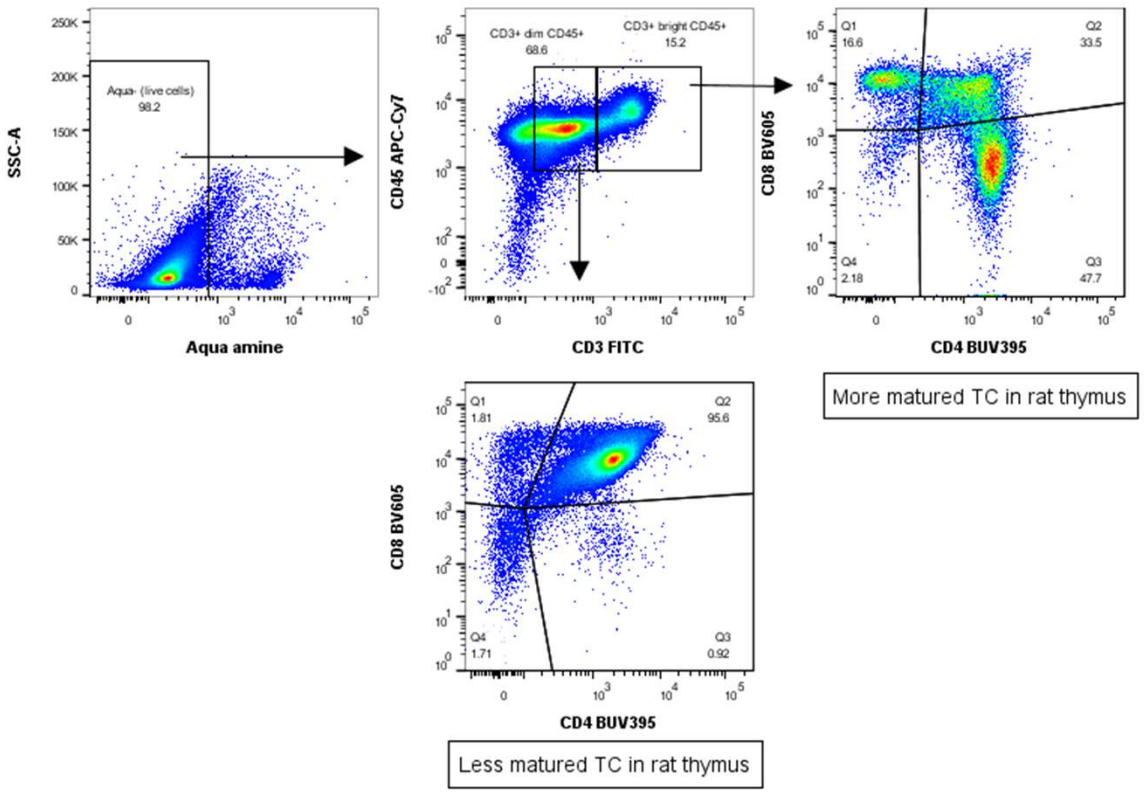
- Primary lymphoid organ where T cells mature
- Starts to shrink following puberty
- Abnormalities in thymus can result in changes in T cell numbers and also in autoimmune disease

Thymus Immunophenotyping

Thymocyte markers:

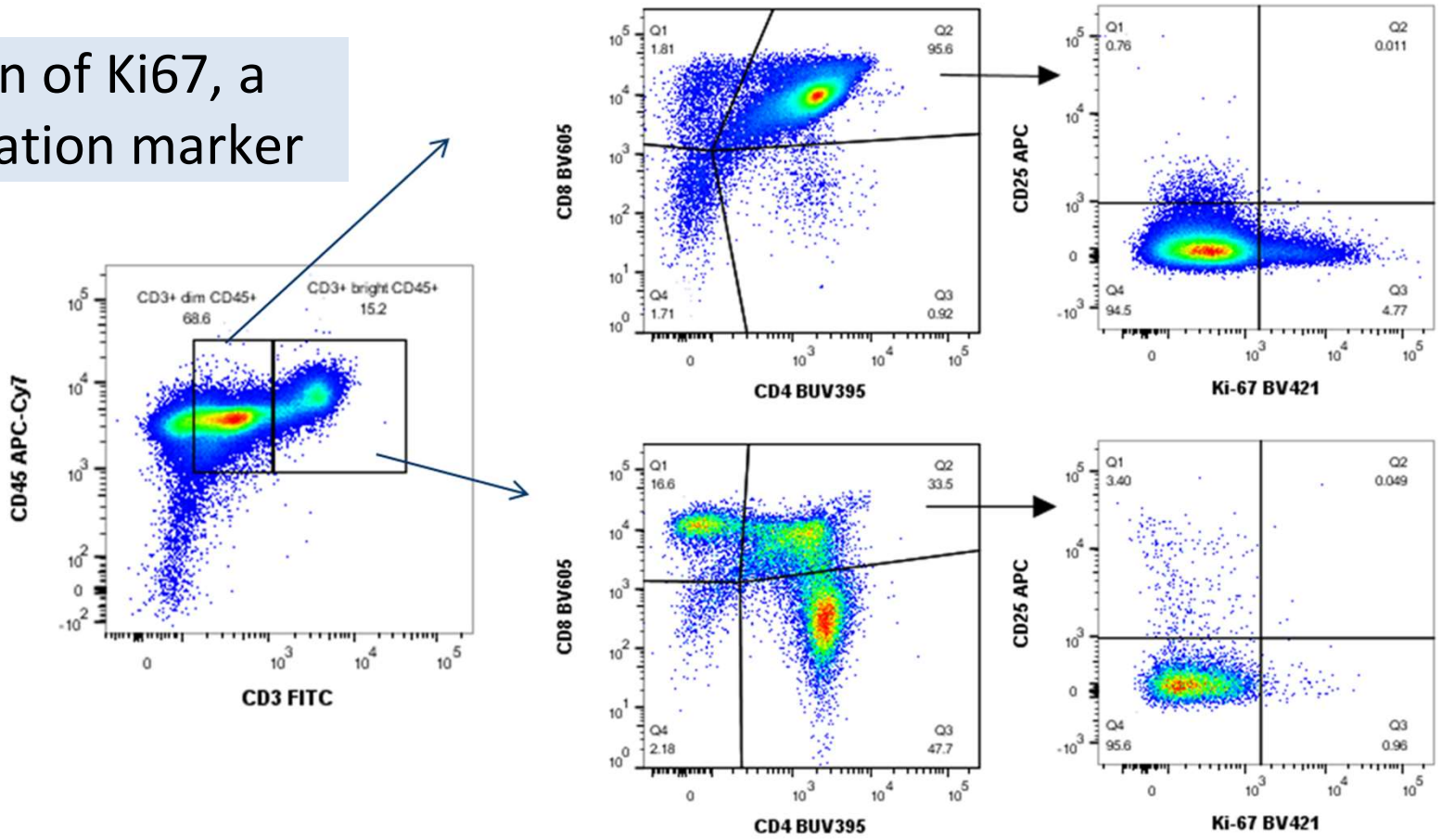
- DN1: CD44+, CD25-
- DN2: CD44+, CD25+
- DN3: CD44-, CD25+
- DN4: CD44-, CD25-, CD3+
- DP: CD3+, CD4+, CD8+
- SP: CD3+, CD4+ and CD3+, CD8+

Addition of cell death markers, e.g. Aqua amine can be helpful for tissue IP



Thymus Immunophenotyping - Proliferation

- Inclusion of Ki67, a proliferation marker



Spleen

- Largest secondary lymphoid organ and primary site of blood filtration
  - Clears red blood cells
  - Filters pathogens and abnormal cells
  - Relevant for the function of innate and adaptive immune cells
- Divided into **red pulp**, **white pulp**, and marginal zone (perifollicular zone in humans)
  - Red pulp: removes red blood cells and pathogens; contains many leukocytes with innate function (**neutrophils, monocytes, dendritic cells, macrophages, γδ T cells**); can change quickly in response to inflammatory insult => shapes adaptive immune response
  - White pulp: compartmentalization separates **T and B cell** areas into distinct zones; cellular border that is primarily made up of innate immune cells
- Some species differences in organization and function
- Review Article: **Lewis et al. (2019)**. Structure and function of the immune system in the spleen. *Cellular Immunology*

Spleen Immunophenotyping

- Cell types included similar to whole blood (can utilize same markers)
  - T cells (20-25%)
  - B cells (40-50%)
  - NK cells
  - Macrophages
  - DCs
- Can add activation markers
- Can distinguish between different type of B cells based on expression markers (not available for all species)

Enumeration of cells in tissues

- Often simply expressed as percentage of a parent population
  => Does not provide full picture (percentage can change due to changes in other cell types)

- Utilization of counting beads to the cell solution can result to defined number of particles/ /µL solution
- **Important to note the weight of the tissue and volume of reconstitution** (keep track of dilution steps)
- Because # of beads added/unit of volume is known, one can calculate absolute # of e.g. T cells/g of tissue
- Calculations are described in product protocols

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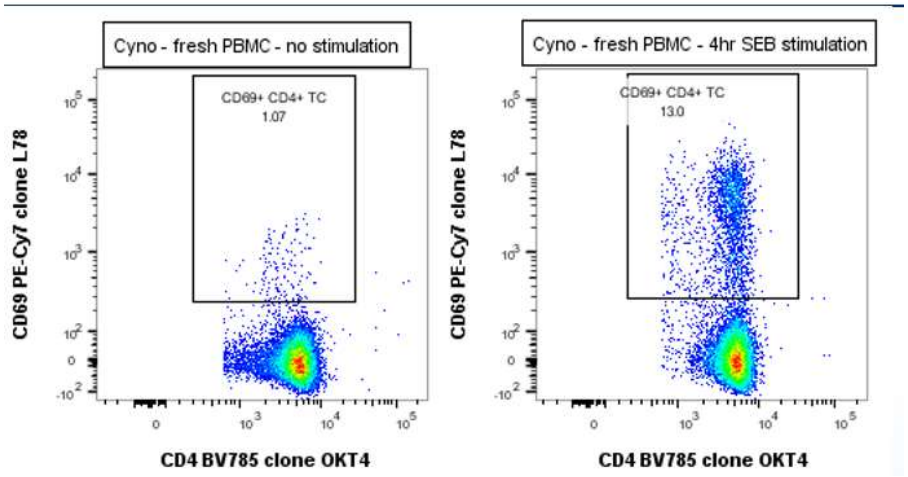
Additional Readouts of Interest

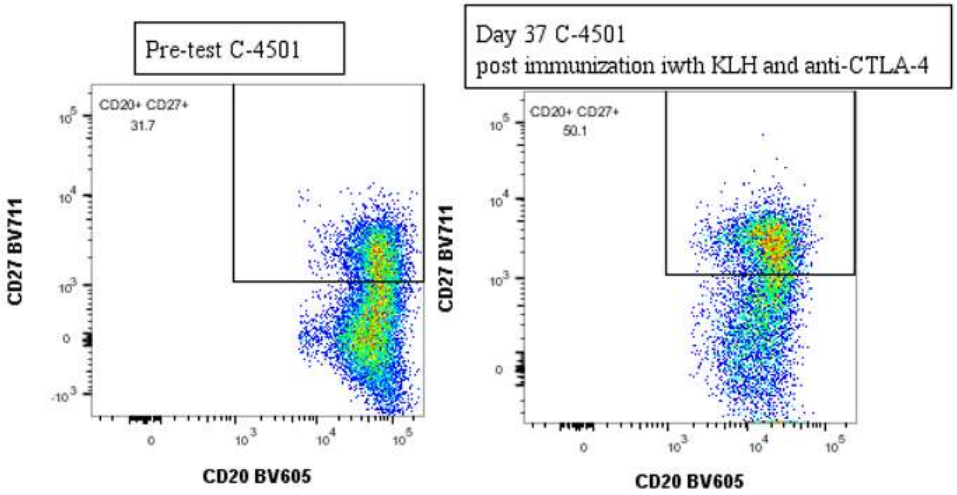
Cell Activation

- Following activation immune cells upregulate certain receptors => can be added as (functional) readout
  - T cell activation markers: CD69 (early: 24h), CD25
  - B cell activation markers: CD27

T Cell Activation

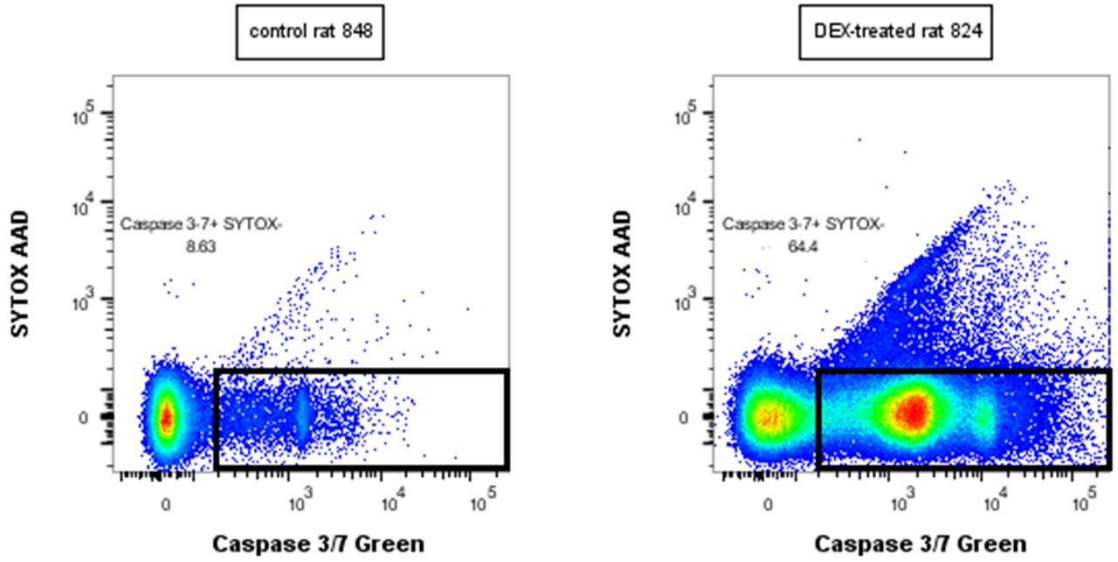


B Cell Activation

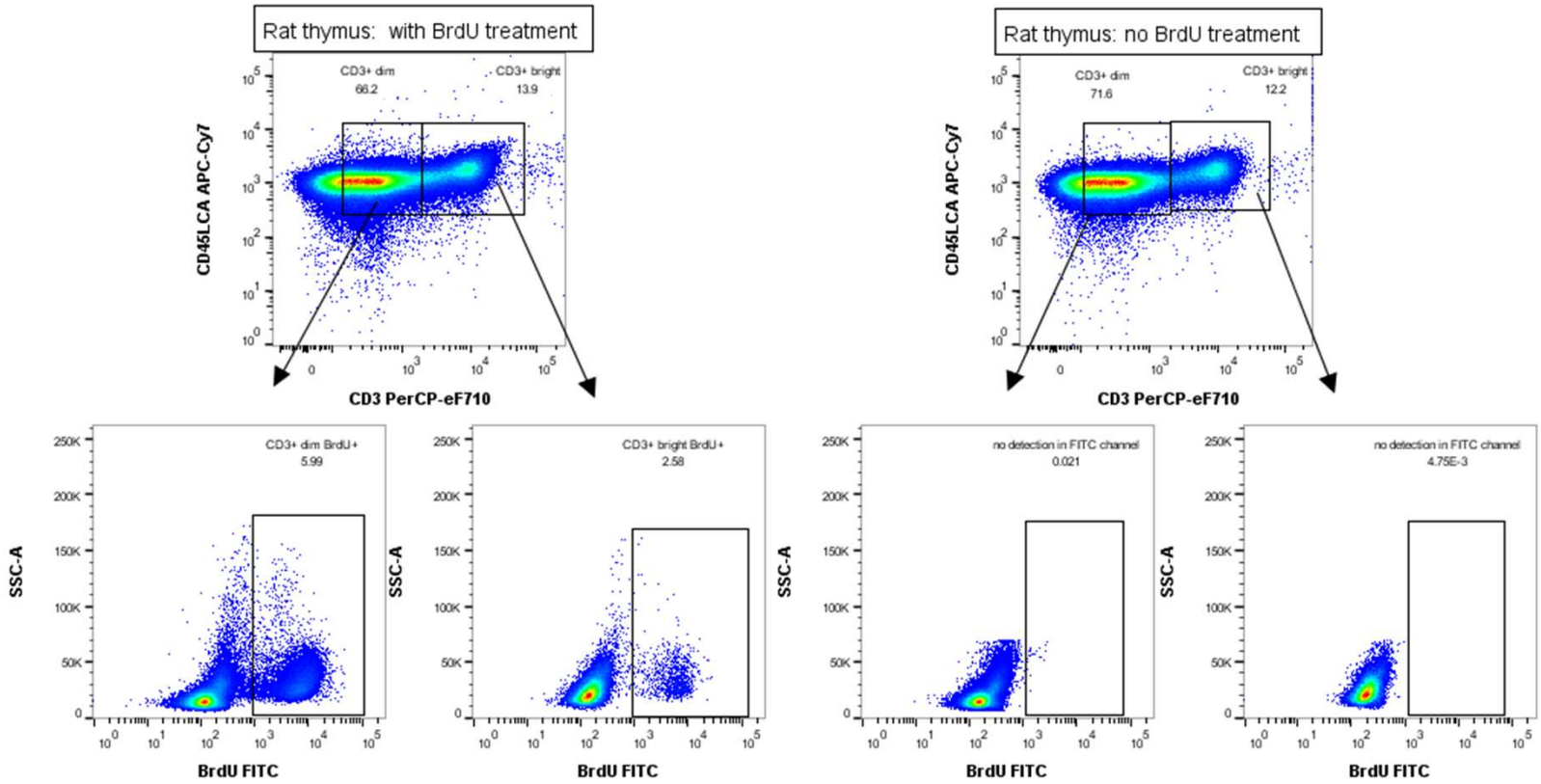


Cell Apoptosis

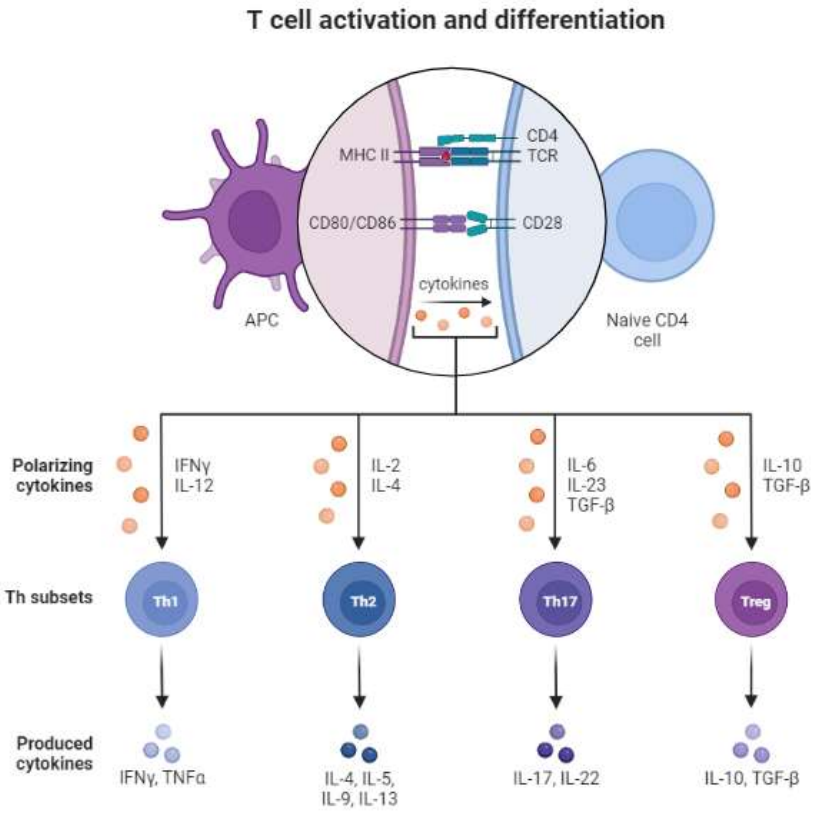
- Cell Apoptosis can be assessed by various markers:
- Annexin V: high affinity for membrane phospholipid phosphatidylserine (translocated to outer membrane during apoptosis)
- Caspase 3/7: caspase enzymes are activated during apoptosis

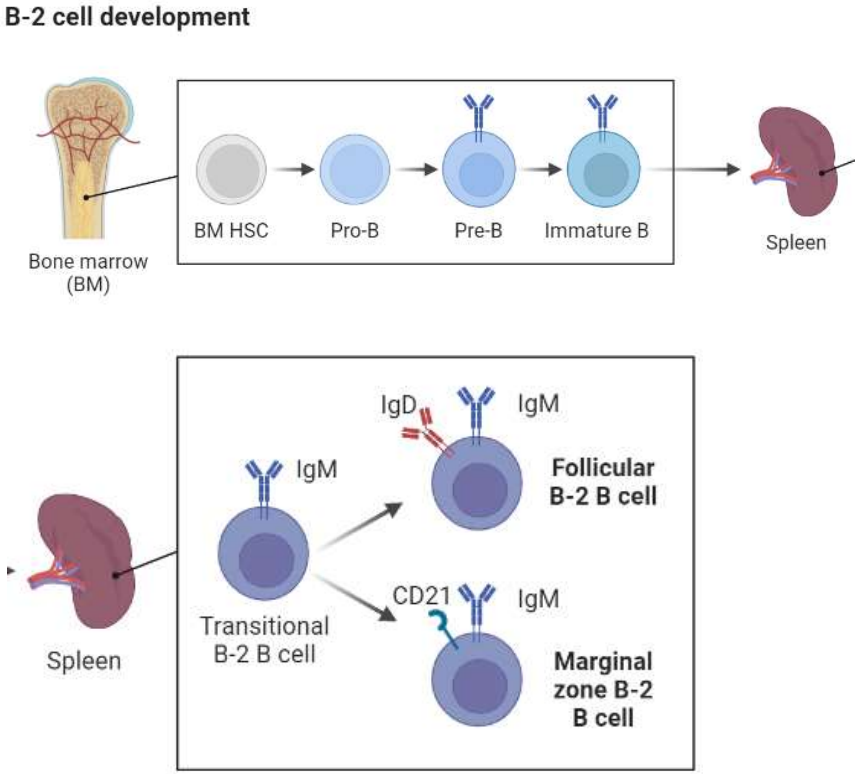


Cell Proliferation



Detailed examination of subpopulations





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Thank you for your interest!

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Literature of Interest

- ICH S8 Immunotoxicity Studies for Human Pharmaceuticals (April 2006): Microsoft Word - 6748fnl.doc (fda.gov)
- Immune System Reviews:
  - Parkin J and Cohen B.(2001). An overview of the immune system. Lancet
  - Sattler S. (2017). The role of the Immune System Beyond the Fight Against Infection. Adv Exp Med Biol
  - Lewis et al. (2019). Structure and function of the immune system in the spleen. *Cellular Immunology*
- Flow Cytometry Reviews (Introduction):
  - Herzenberg L.A. et al. (2006). Interpreting flow cytometry data. A guide for the perplexed. Nature Immunology
  - Lugli E. et al. (2010). Data Analysis in Flow Cytometry: The Future Just Started. Cytomtry A.
  - McKinnon K.M. (2018). Flow Cytometry: An Overview. Curr Protoc Immunol

- IP Standardization:
  - Maecker, H. T., McCoy J. P., and Nussenbaum, R. (2012), Standardizing immunophenotyping for the Human Immunology Project, Nat Rev Immunol
  - Finak G. et al. (2016), Standardizing Flow Cytometry Immunophenotyping Analysis from the Human ImmunoPhenotyping Consortium, Scientific Reports
- Immunophenotyping:
  - Lappin, P. B. and Black, L. E. (2003). Immune Modulator Studies in Primates: Utility of Flow Cytometry and Immunohistochemistry in the Identification and Characterization of Immunotoxicity. Tox Pathol.
  - Krejsa C. M. (2012). An inter-laboratory retrospective analysis of immunotoxicological endpoints in non-human primates: Flow cytometry immunophenotyping. J Immunotox
  - ITC (2001). Application of flow cytometry to immunotoxicity testing: summary of a workshop. Toxicology

- Bjornson-Hooper et al. (2022). A comprehensive atlas of immunological differences between human, mice and non-human primates. Front Immunol.

- Tissue IP publications:
  - Reagan WJ et al. (2011). Best practices for evaluation of bone marrow in nonclinical studies. Toxicol Pathol.
  - Criswell KA et al. (1998). Comparison of Flow Cytometric and Manual Bone Marrow Differentials in Wistar Rats
  - Morris and Komocsar (1997). Immunophenotyping analysis of peripheral blood, splenic and thymic lymphocytes in male and female rats. J Pharmacol Toxicol Methods
- Automated IP analysis:
  - Verschoor CP et al. (2015). An Introduction to automated flow cytometry gating tools and their implementation. Frontiers in Immunology