

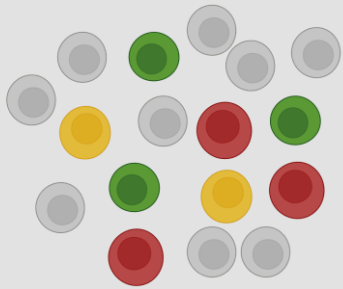
**13rd International Conference Analytical
Cytometry
6th October 2025**

**Advanced immunophenotyping of
peripheral blood leukocytes – Part I.**

**Aleš Efenberk
EXBIO Praha, a.s.**

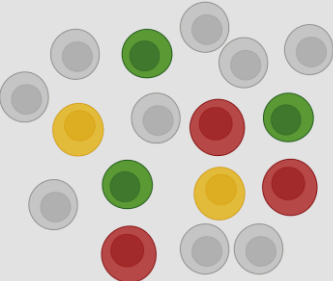
Immunophenotyping

A

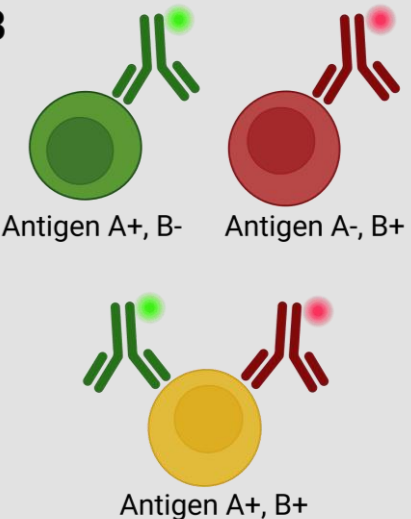


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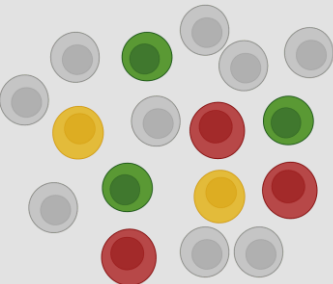


B

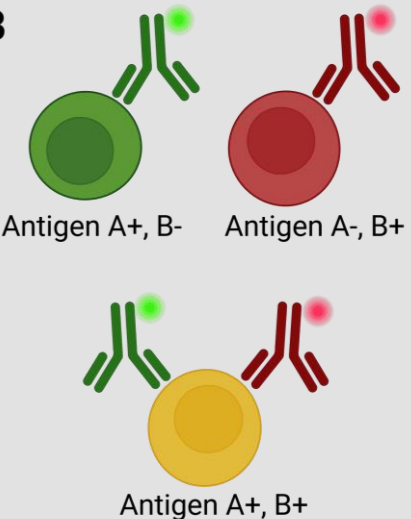


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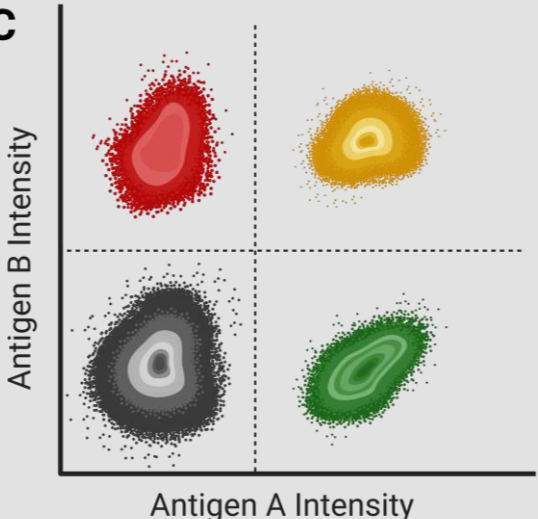
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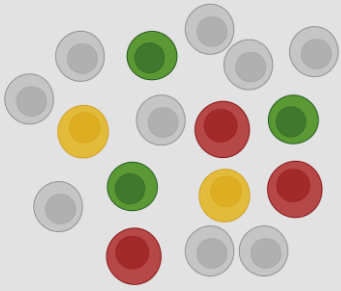


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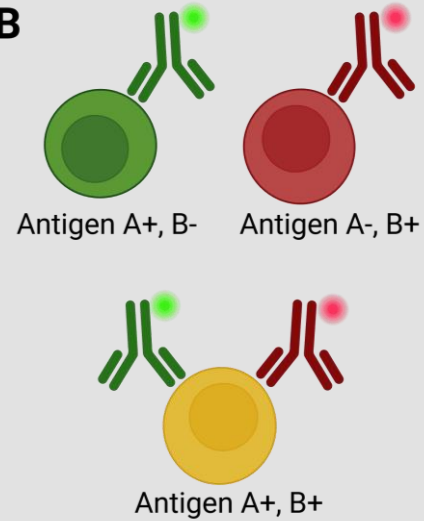


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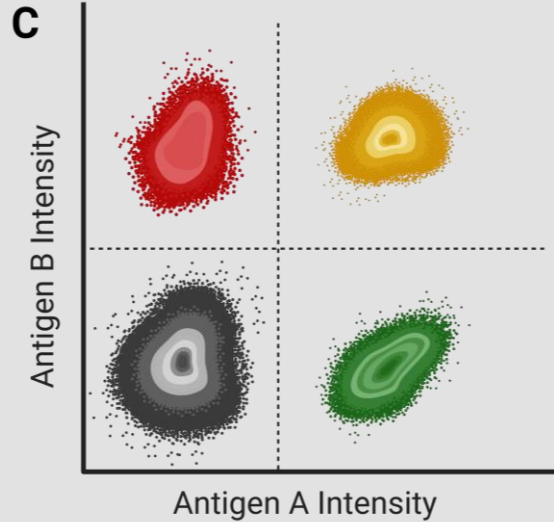
A



B



C



Utilization:

- Diagnosis of hematological malignancies (e.g. leukemias, lymphomas)
- Identification of immune system cells
- In research and development of new treatments
- In veterinary medicine, etc.

Immunophenotyping

Advantages:

- Highly specific method - Identifies markers that are unique to certain cell types
- Sensitive method - Can detect even a small number of abnormal cells
- Multi-color analysis option - Allows simultaneous evaluation of multiple markers on a single cell
- Quantification option - Allows you to quantify the amount of different cell types in a sample

Immunophenotyping - clinical meaning

Basic Immunophenotyping in clinical = **TBNK**

- Identification and characterization of T-lymphocytes, B-lymphocytes and NK cells
- In the "six-color" version, it also allows the differentiation of cytotoxic (Tc) and helper (Th) T-lymphocytes.

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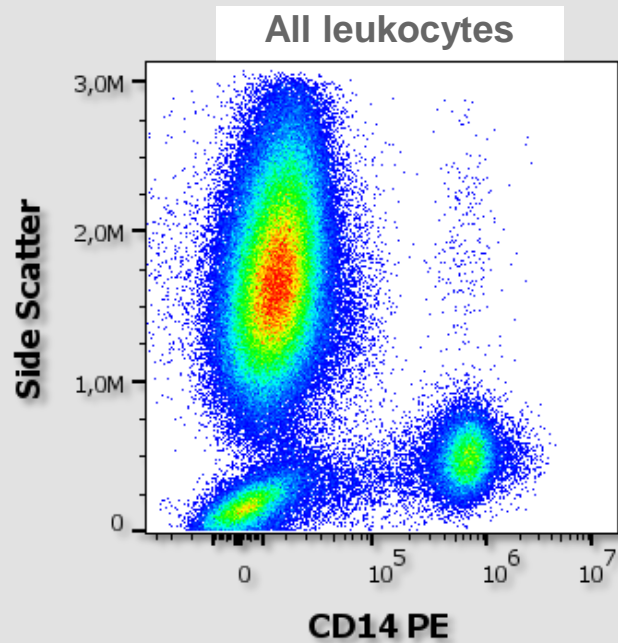
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Advanced Immunophenotyping in clinical = **TBNK + other markers**

- CD14
- HLA-DR
- and many others...

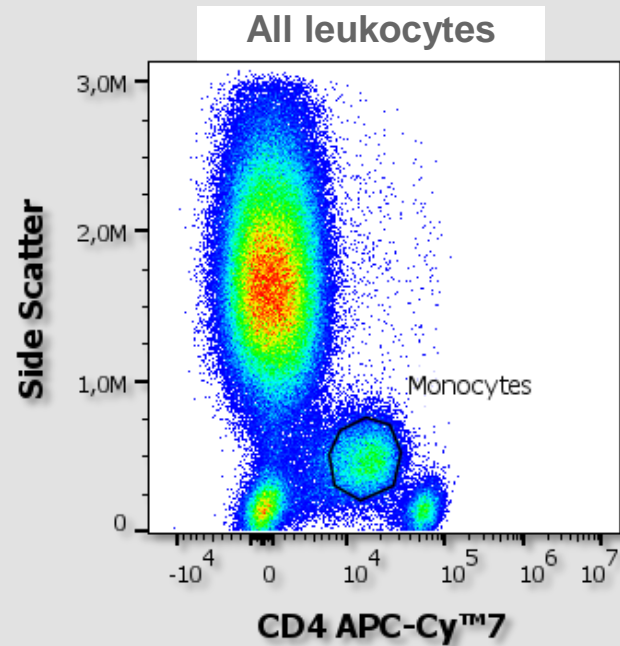
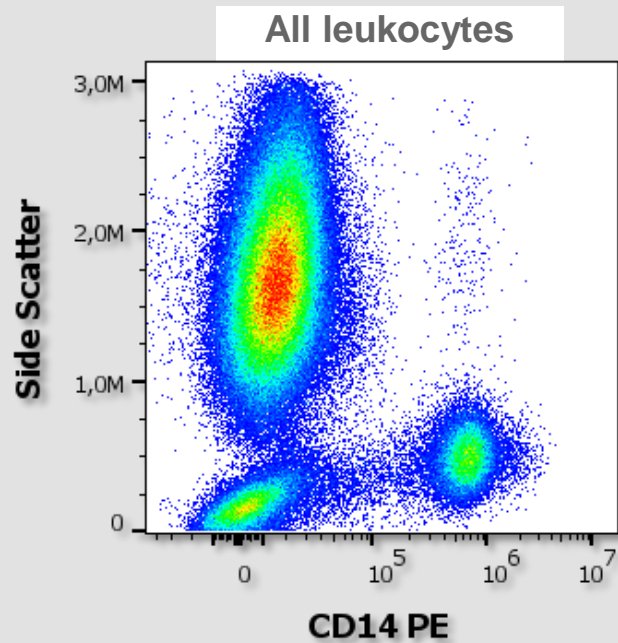
Advanced Immunophenotyping – CD14

Would the CD14 marker help?



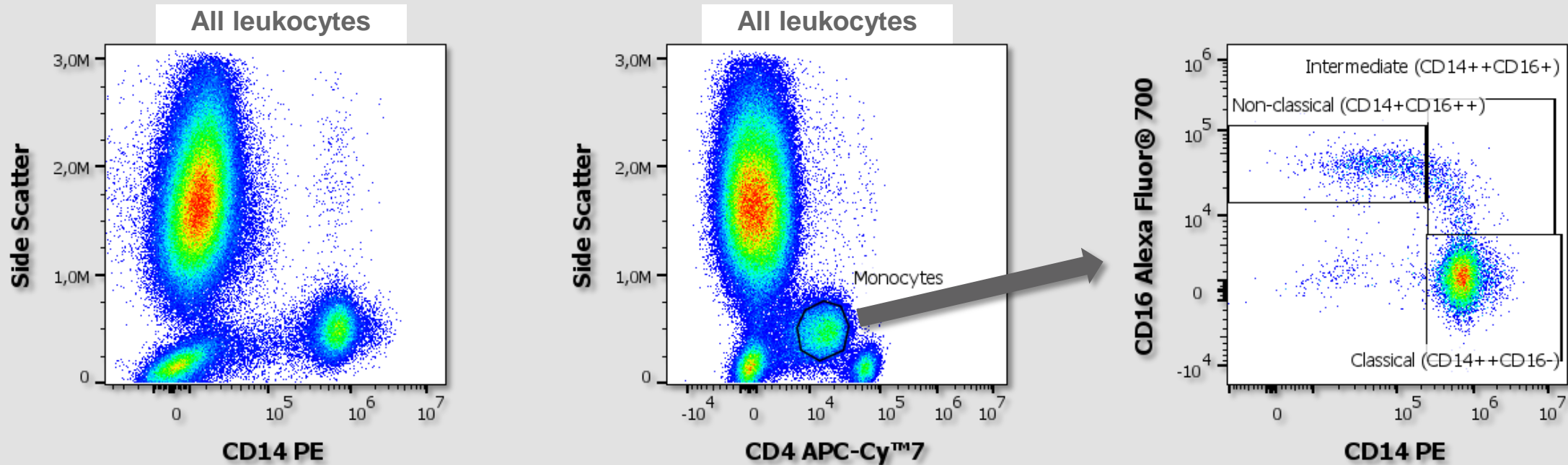
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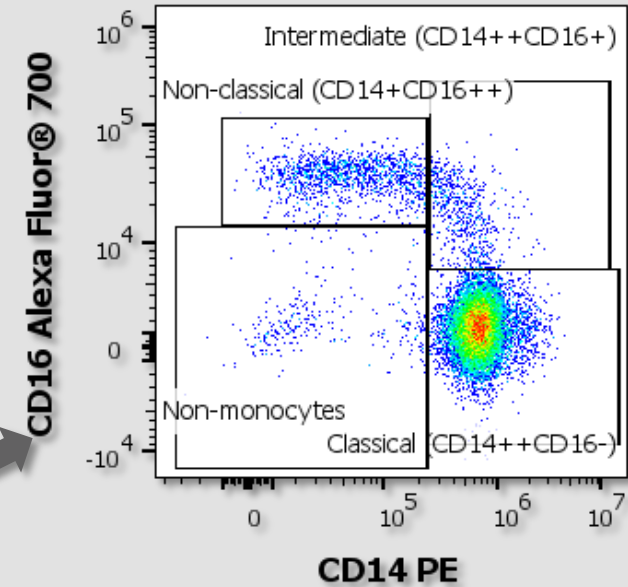
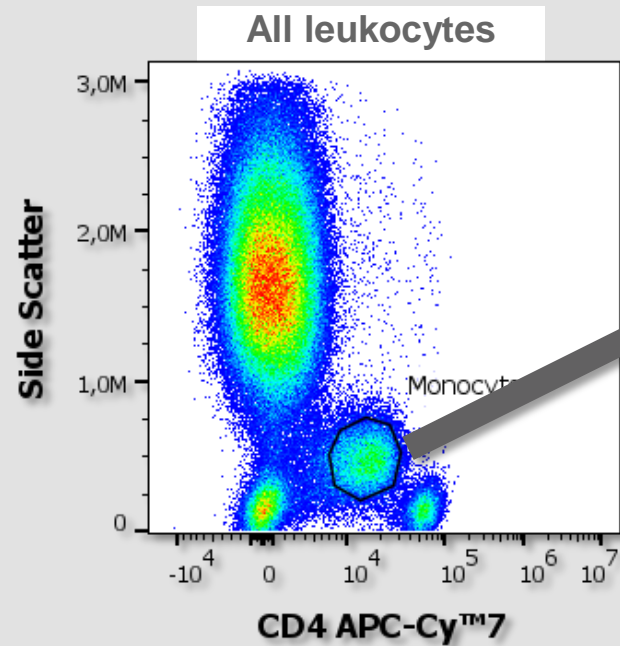
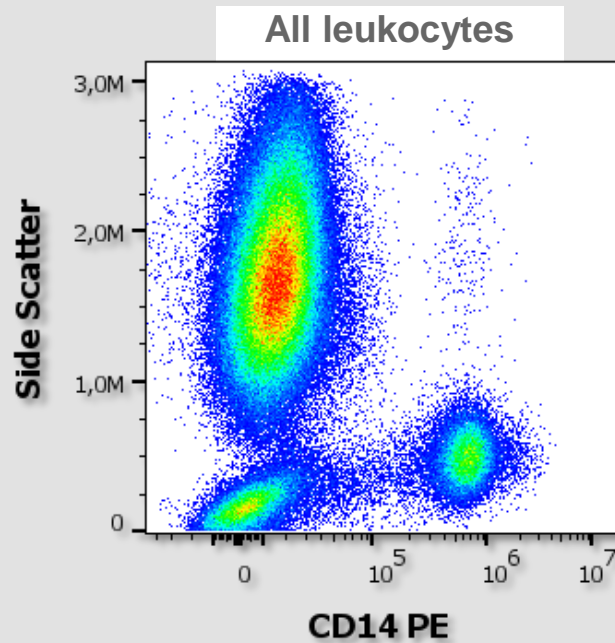
Advanced Immunophenotyping – CD14 addition

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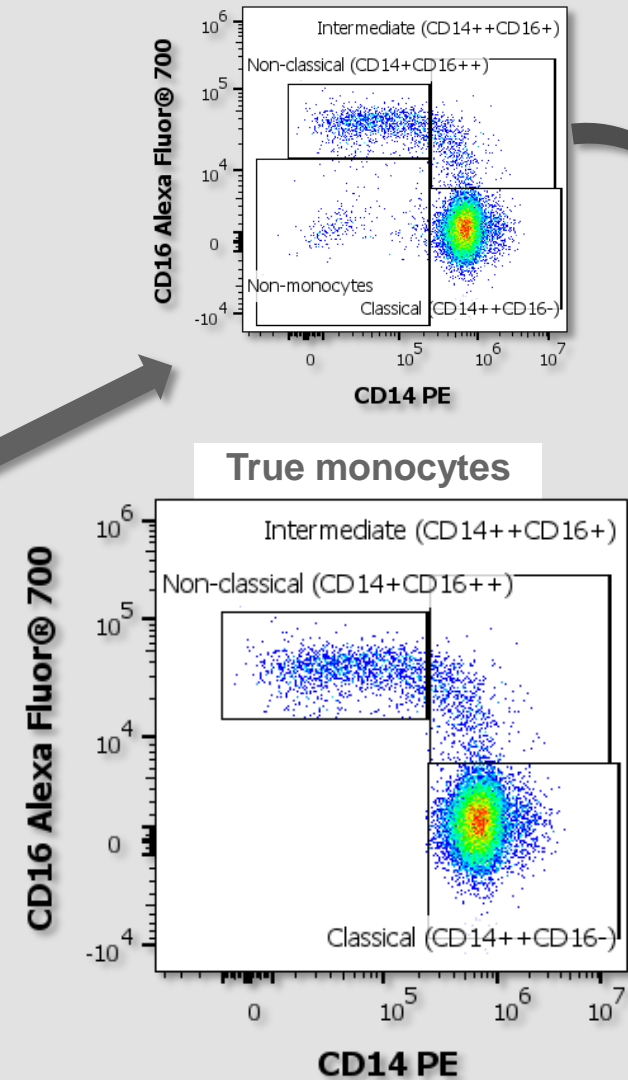
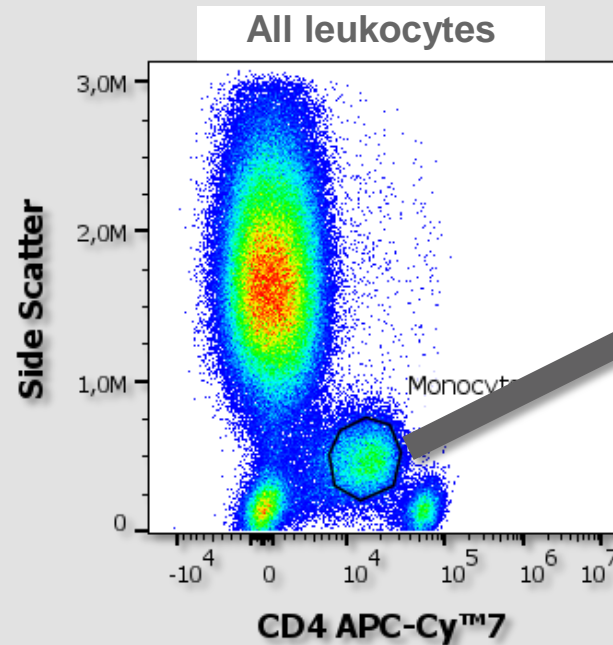
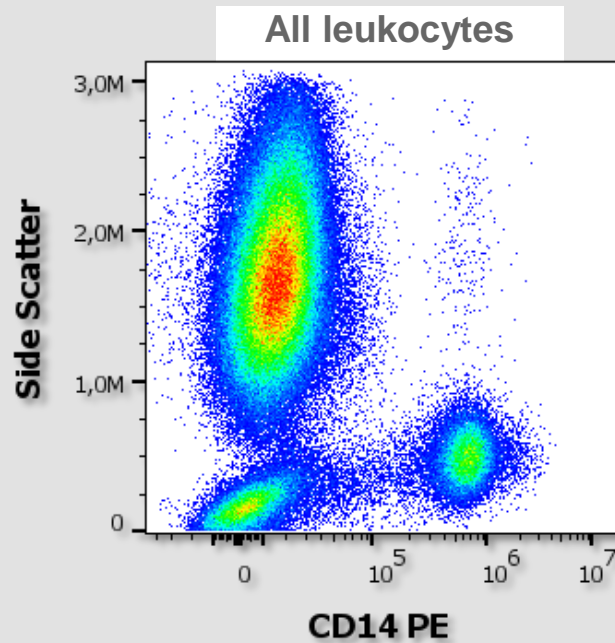
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Advanced Immunophenotyping – CD14 addition

Utilization in clinical diagnostics:

The proportions of monocyte subsets are altered in various diseases, making them potential biomarkers for disease severity, progression, and treatment response.

- Higher levels of intermediate and non-classical monocytes are often seen in sepsis, chronic inflammation, or autoimmune disease.
- Non-classical monocytes are implicated in atherosclerosis and vascular damage
- Monitoring subsets helps understand rejection risk or immune reconstitution
- Altered monocyte subset distribution was associated with disease severity of some viral infections

However, the complex equilibrium and differentiation pathways among monocyte subsets mean that a single marker or simple count may not fully capture their role in a disease.

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However, the complex equilibrium and differentiation pathways among monocyte subsets mean that a single marker or simple count may not fully capture their role in a disease.

Reference Intervals:

Reference intervals for these three monocyte subsets are not clearly defined.

The Czech laboratories most often report total monocytes in healthy individuals as ~3-8 % of leukocytes in the peripheral blood.

Advanced Immunophenotyping – CD14 addition

Commercial options:

Mixing the entire antibody cocktail from single-color reagents

Drop-in anti-CD14 antibody to an existing TBNK cocktails

DuraClone IM Phenotyping BASIC Tube (8 color / 8 markers)

Newly developed product (9 color / 9 markers) from EXBIO for assessing an individual's immune status (very soon on the market)

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Important notice:

Density gradient centrifugation of PBMC changes surface expression of CD14 and CD16 on monocytes.

Purification in PBMCs led to a significant decrease in CD14+/CD16- monocytes with a concurrent increase in CD14dim/CD16+monocytes⁽¹⁾. Classical and non-classical monocytes can therefore **ONLY** be determined on fresh blood.

1) Mukherjee, R., Kanti Barman, P., Kumar Thatoi, P. et al. Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematosus. Sci Rep 5, 13886 (2015). <https://doi.org/10.1038/srep13886>

Advanced Immunophenotyping – HLA-DR

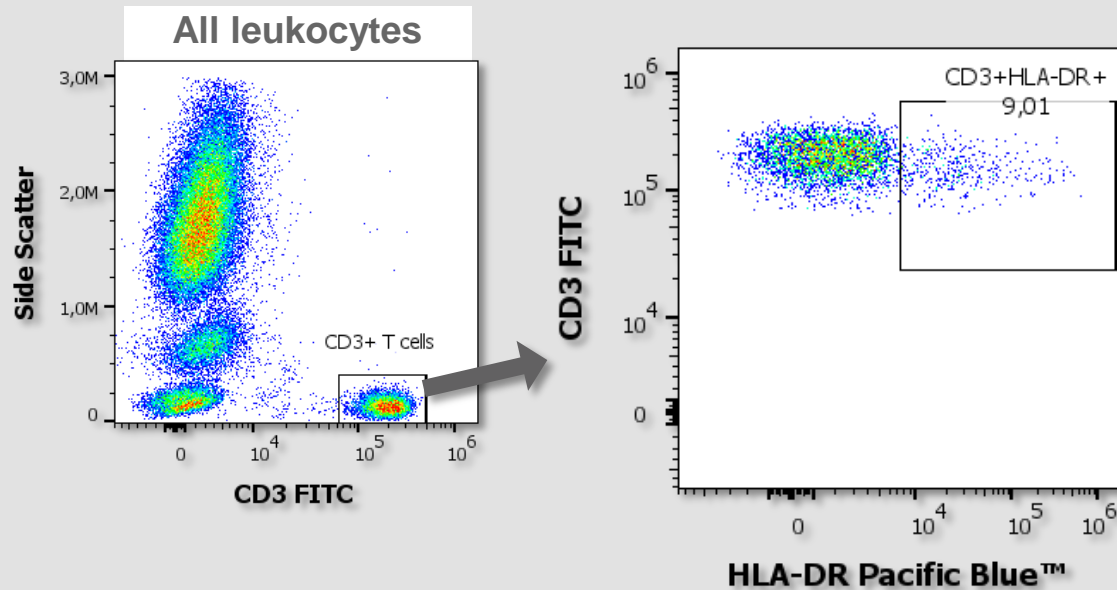
What if we added the HLA-DR marker?



Advanced Immunophenotyping – HLA-DR

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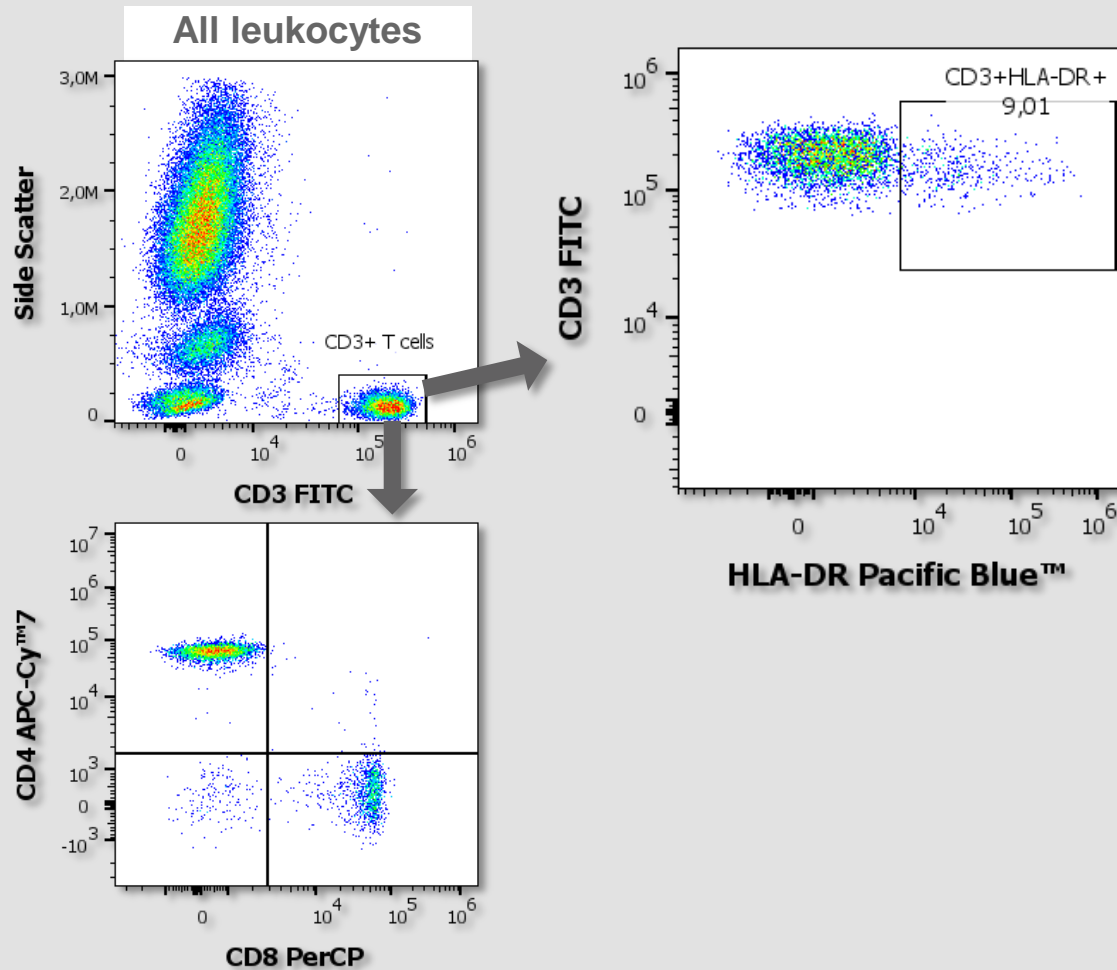
We have the ability to detect the activated state of the T-lymphocyte population.



Advanced Immunophenotyping – HLA-DR

What if we added the HLA-DR marker?

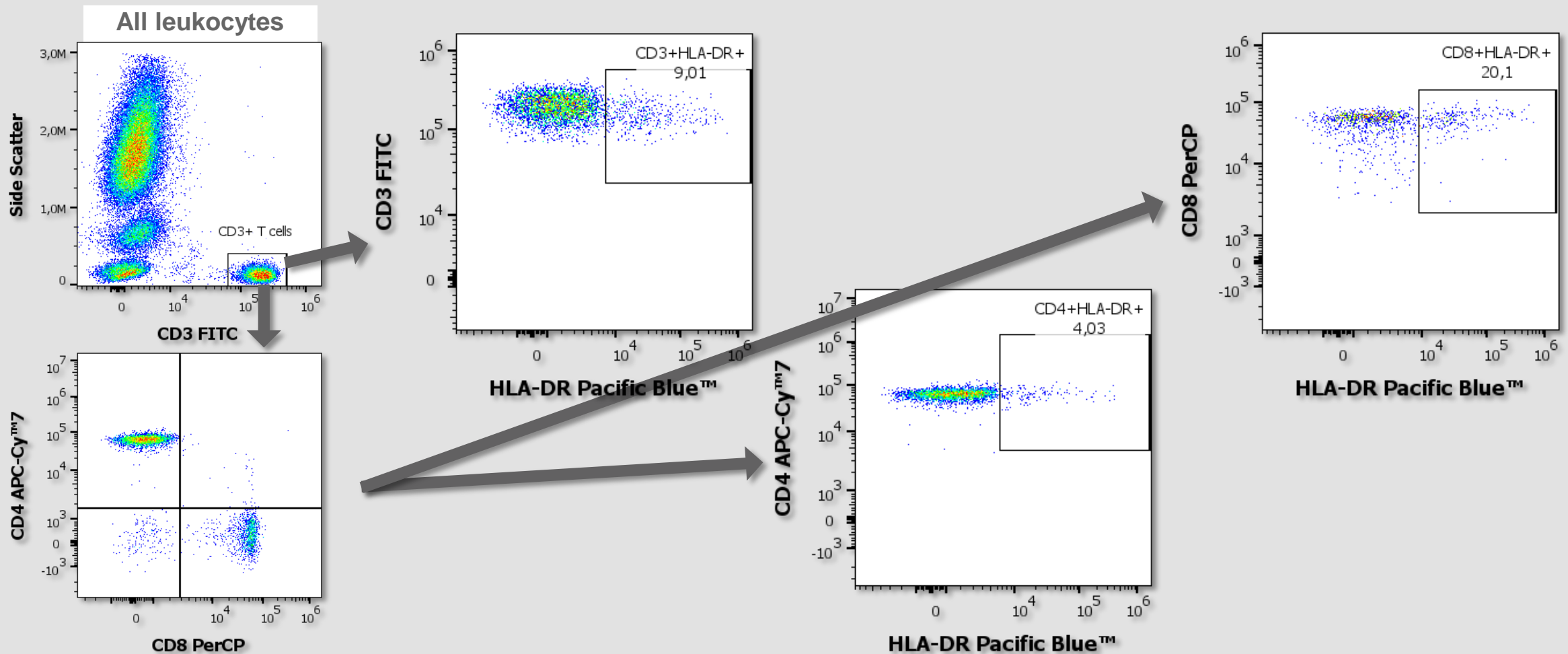
We have the ability to detect the activated state of the T-lymphocyte population.



Advanced Immunophenotyping – HLA-DR

What if we added the HLA-DR marker?

We have the ability to detect the activated state of individual T-lymphocyte subpopulations.



Advanced Immunophenotyping – HLA-DR

Reference Intervals (T-lymphocytes):

In healthy individuals, HLA-DR expression on T-lymphocytes is typically low because resting T cells do not express HLA-DR. It is only upregulated upon activation.

T Cell Subset	% HLA-DR⁺ (Healthy Adults)
Total CD3 ⁺ T-lymphocytes	~1–15% (typically 3–8%)
CD4 ⁺ T helper cells	~1–10% (typically 2–5%)
CD8 ⁺ Cytotoxic T cells	~1–20% (typically 5–10%)

Advanced Immunophenotyping – HLA-DR

Utilization in clinical diagnostics (T-lymphocytes):

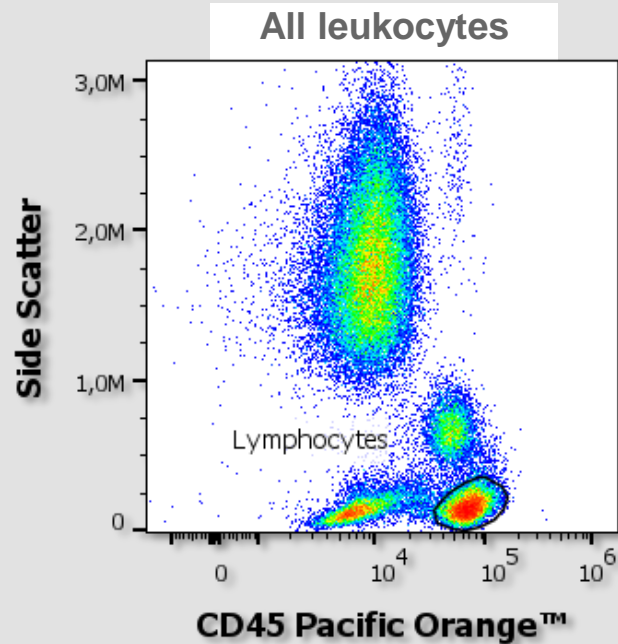
HLA-DR expression on activated T-lymphocytes (CD4⁺ and CD8⁺ T cells) does vary significantly depending on the immune context:

Condition	T Cell Type	HLA-DR Expression
Viral infection (e.g., HIV, EBV, CMV, influenza, COVID-19)	CD8 ⁺ > CD4 ⁺	High
Bacterial infection (e.g., sepsis)	CD4 ⁺ mostly	Moderate/Variable
Autoimmune disease (e.g., Rheumatoid arthritis, Multiple sclerosis, Systemic lupus erythematosus)	CD4 ⁺ ± CD8 ⁺	Moderate–High

HLA-DR expression significantly above the reference intervals, especially >20% of CD8⁺ T cells or CD4⁺ T cells, may suggest recent immune activation.

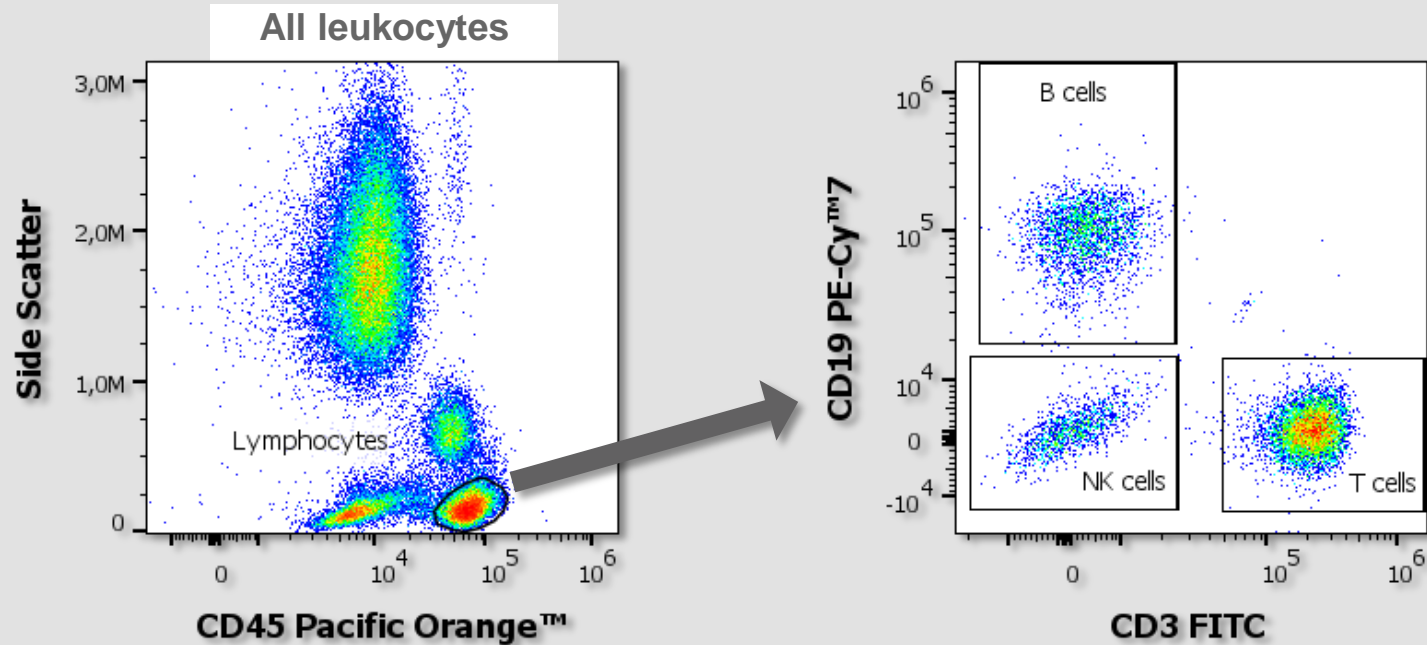
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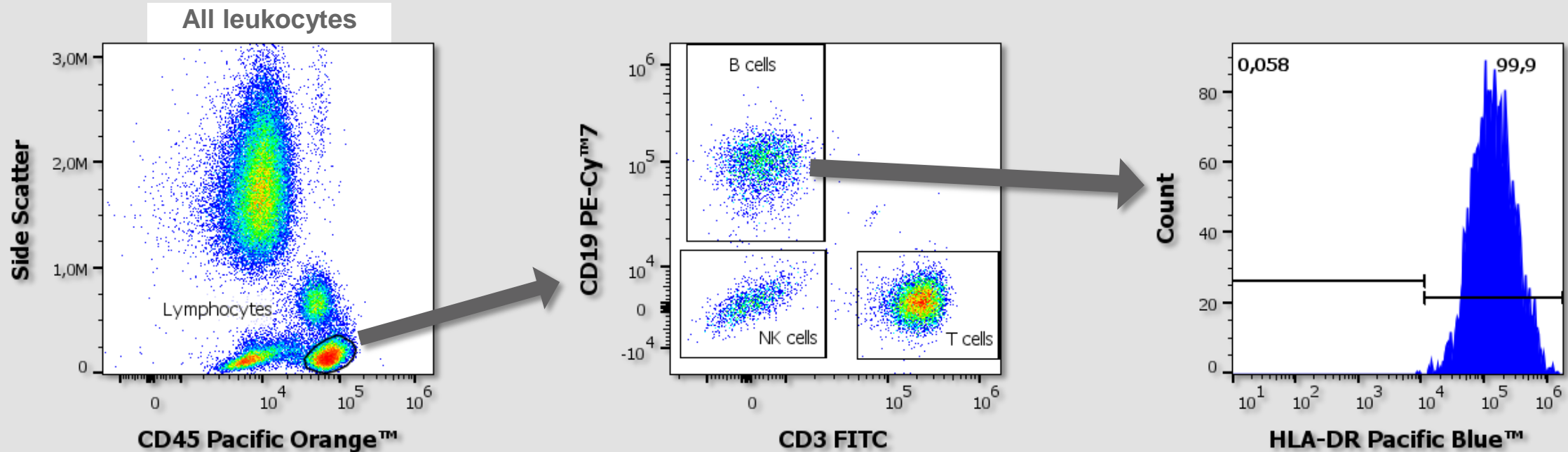
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Advanced Immunophenotyping – HLA-DR

What if we added the HLA-DR marker?

We can obtain information about HLA-DR expression on B-Lymphocytes.



Advanced Immunophenotyping – HLA-DR

Reference Intervals (B-lymphocytes):

In healthy individuals, HLA-DR is constitutively expressed on all B-lymphocytes, since B-lymphocytes are professional antigen-presenting cells (APCs).

Percentage of HLA-DR⁺ B cells (from total CD19⁺ B cells): normal range: >95%; typically 98–100%

Advanced Immunophenotyping – HLA-DR

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Utilization in clinical diagnostics (B-lymphocytes):

Reduced expression of HLA-DR on B-Lymphocytes may be seen in e.g.:

- Certain primary immunodeficiencies

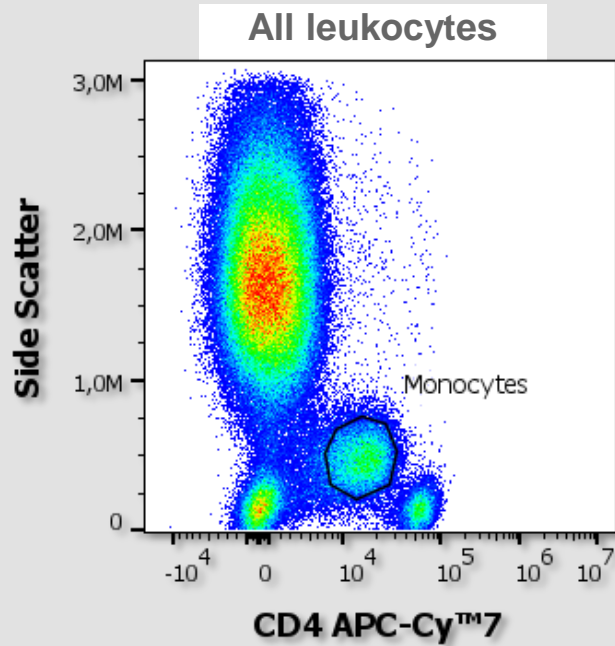
- Profound B cell immaturity or depletion

- Severe immunosuppressive therapy

- Late-stage hematologic malignancies (e.g., some B-cell leukemias/lymphomas)

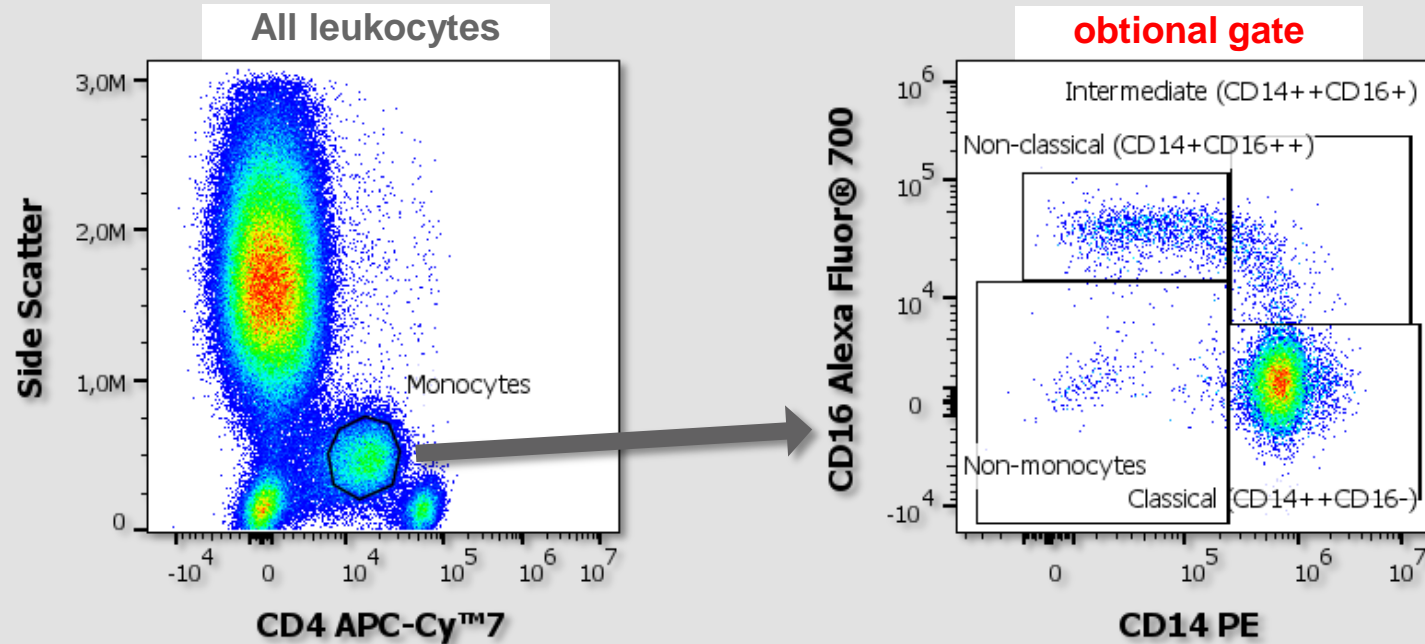
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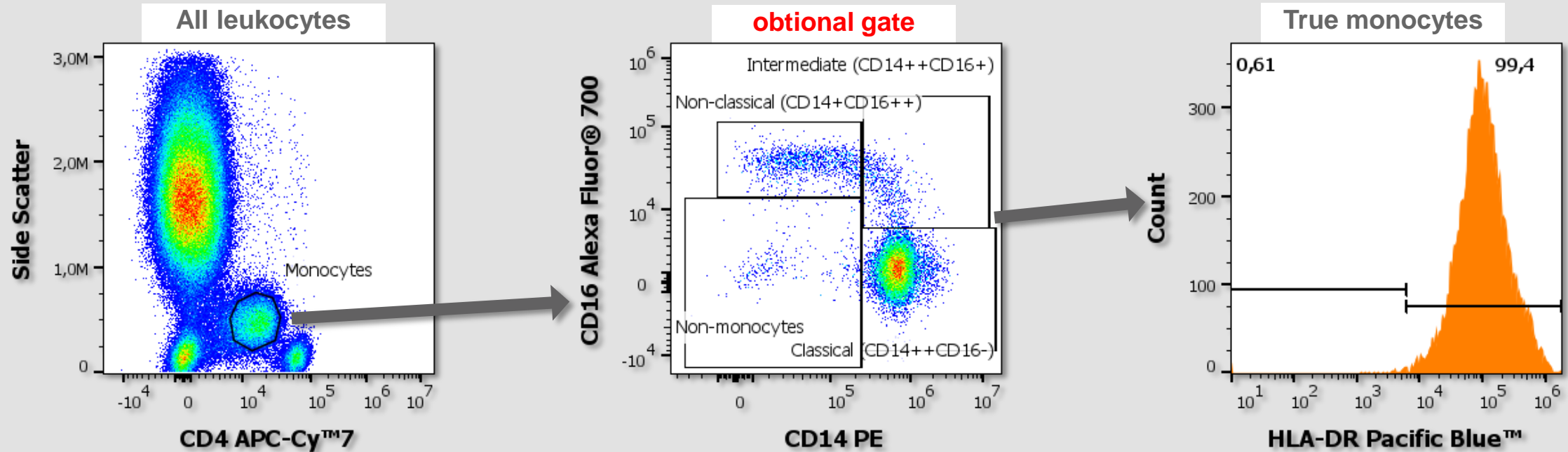
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Advanced Immunophenotyping – HLA-DR

What if we added the HLA-DR marker?

We can obtain information about HLA-DR expression on monocytes.



Advanced Immunophenotyping – HLA-DR

Reference Intervals (Monocytes):

> 80% of monocytes are HLA-DR⁺ (commonly 85–98%; median/mean in healthy controls 90–95%)

Advanced Immunophenotyping – HLA-DR

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> 80% of monocytes are HLA-DR⁺ (commonly 85–98%; median/mean in healthy controls 90–95%)

Utilization in clinical diagnostics (Monocytes):

< 80% HLA-DR⁺ monocytes → considered as abnormal, especially:

- < 70%: early sign of immune suppression

- < 30%: severe immunoparalysis

Lower expression of HLA-DR on monocytes is a well-established marker of immune dysfunction e.g.:

- Sepsis/Septic Shock

- Trauma/Surgery

- Immunosuppressive Therapy (Especially corticosteroids, calcineurin inhibitors)

- Severe Viral Infections (e.g., COVID-19)

Advanced Immunophenotyping – HLA-DR

Commercial options:

Mixing the entire antibody cocktail from single-color reagents

Drop-in anti-HLA-DR antibody to an existing TBNK cocktails

BD Simultest™ CD3 FITC/Anti-HLA-DR PE

BD Multitest™ Anti-Human CD4 (or CD8) FITC/CD38 PE/CD3 PerCP/HLA-DR APC

CD3-FITC/HLA-DR-PE Antibody Cocktail – Beckman Coulter

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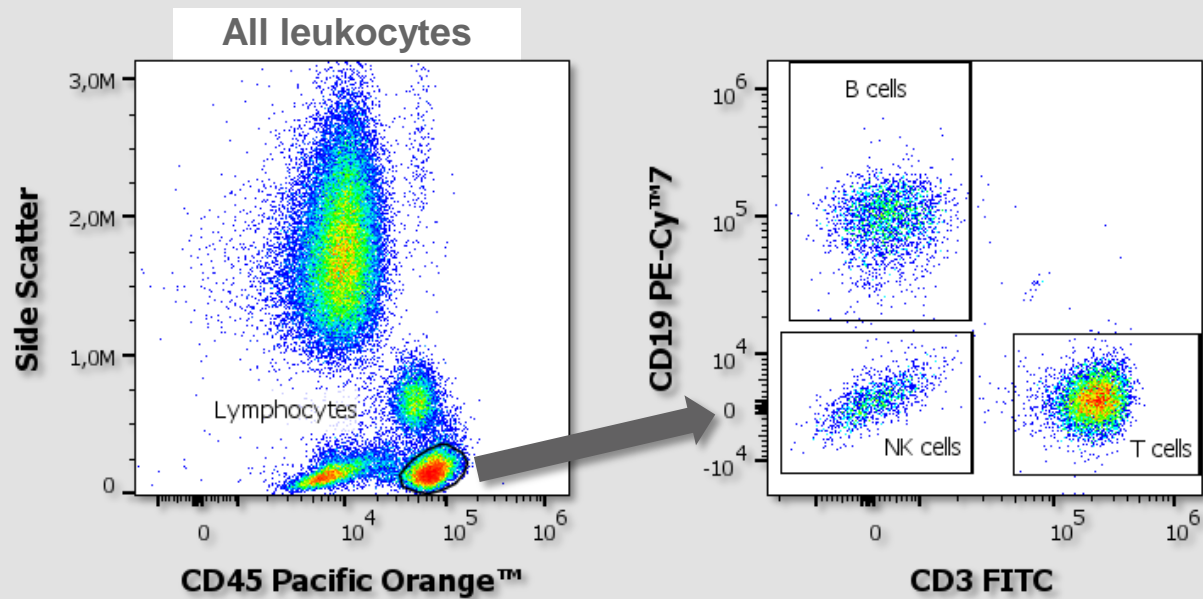
Advanced Immunophenotyping — CD16/CD56 decomposition

What if we used different fluorochromes for the anti-CD16 and anti-CD56 antibodies?



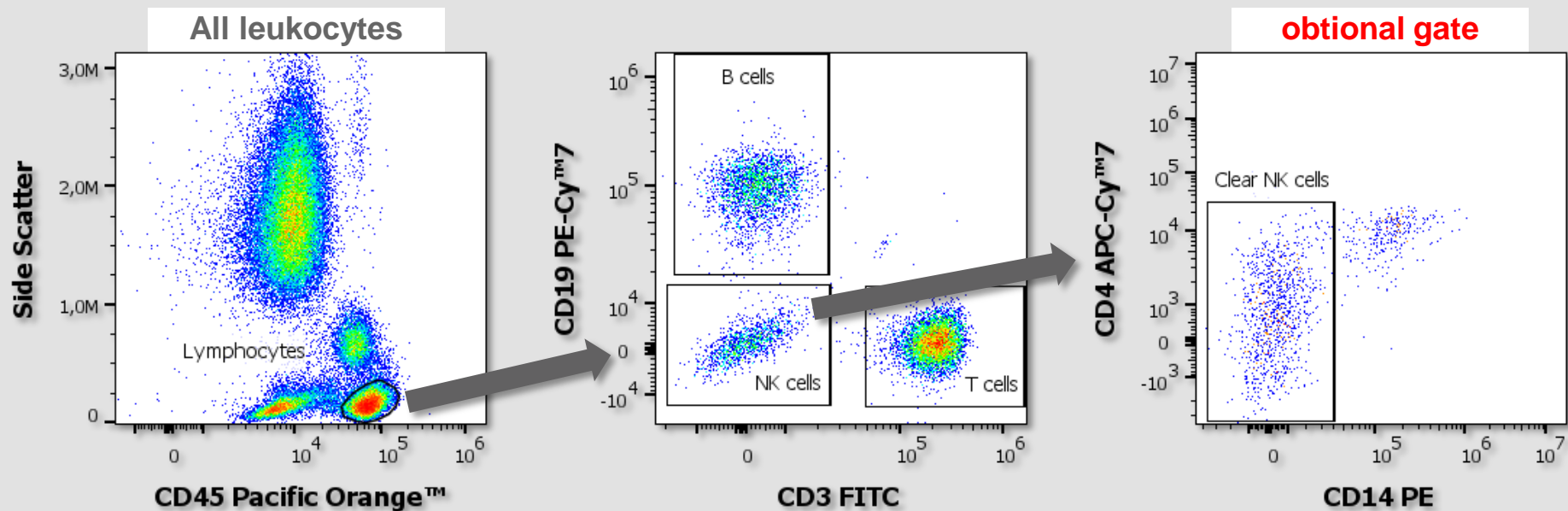
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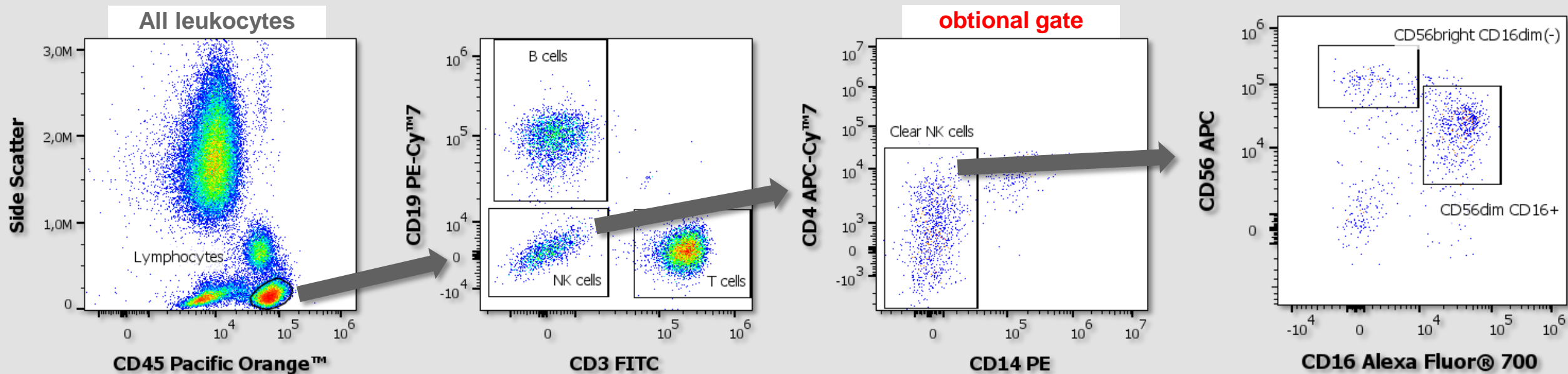
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Advanced Immunophenotyping – CD16/CD56 decomposition

Possibility of detailed subtyping of NK cells [CD56bright/CD16dim(-) vs. CD56dim/CD16+].



Advanced Immunophenotyping – CD16/CD56 decomposition

Utilization in clinical diagnostics:

NK cells are heterogeneous, and dividing them into CD56bright and CD56dim/CD16+ subsets has both immunological and clinical significance

CD56dim/CD16+ - ~ 80–98% of NK cells, perform antibody-dependent cellular cytotoxicity; more differentiated, short-lived effector cells

CD56bright/CD16dim(-) - ~ 0–23% of NK cells, poor cytotoxicity (low perforin, granzyme B); regulators and immunomodulators (cytokine producers)

Tracking is important in e.g.:

Primary immunodeficiencies – some patients have a lack of CD56bright NK cells, leading to impaired cytokine-driven immune regulation

Viral infections - loss or dysfunction of CD56bright in severe viral disease

Therapeutic monitoring - NK subsets are used as biomarkers in trials of cytokine therapies, checkpoint inhibitors and other immunotherapies

Advanced Immunophenotyping – CD16/CD56 decomposition

Reference Intervals:

Reference intervals for NK cell subsets are not clearly defined.

Some recent population studies produced age-stratified reference intervals:

- Oras A, Quirant-Sanchez B, Popadic D, et al. *Comprehensive flow cytometric reference intervals of leukocyte subsets from six study centers across Europe. Clin Exp Immunol. 2020;202(3):363-378. doi:10.1111/cei.13491*
- Zhang L, Chen X, Xing R, Lu Y. *Reference intervals for peripheral blood natural killer cell, monocyte, and dendritic cell subsets in healthy adults from Zhejiang province, China. BMC Immunol. 2025;26(1):47. Published 2025 Jul 4. doi:10.1186/s12865-025-00731-6*

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**Acknowledgements
to the entire team of employees of EXBIO Praha**



Thank you for your attention!