

Simultaneous correlation of cytokine production with Treg and Th17 cell proliferation

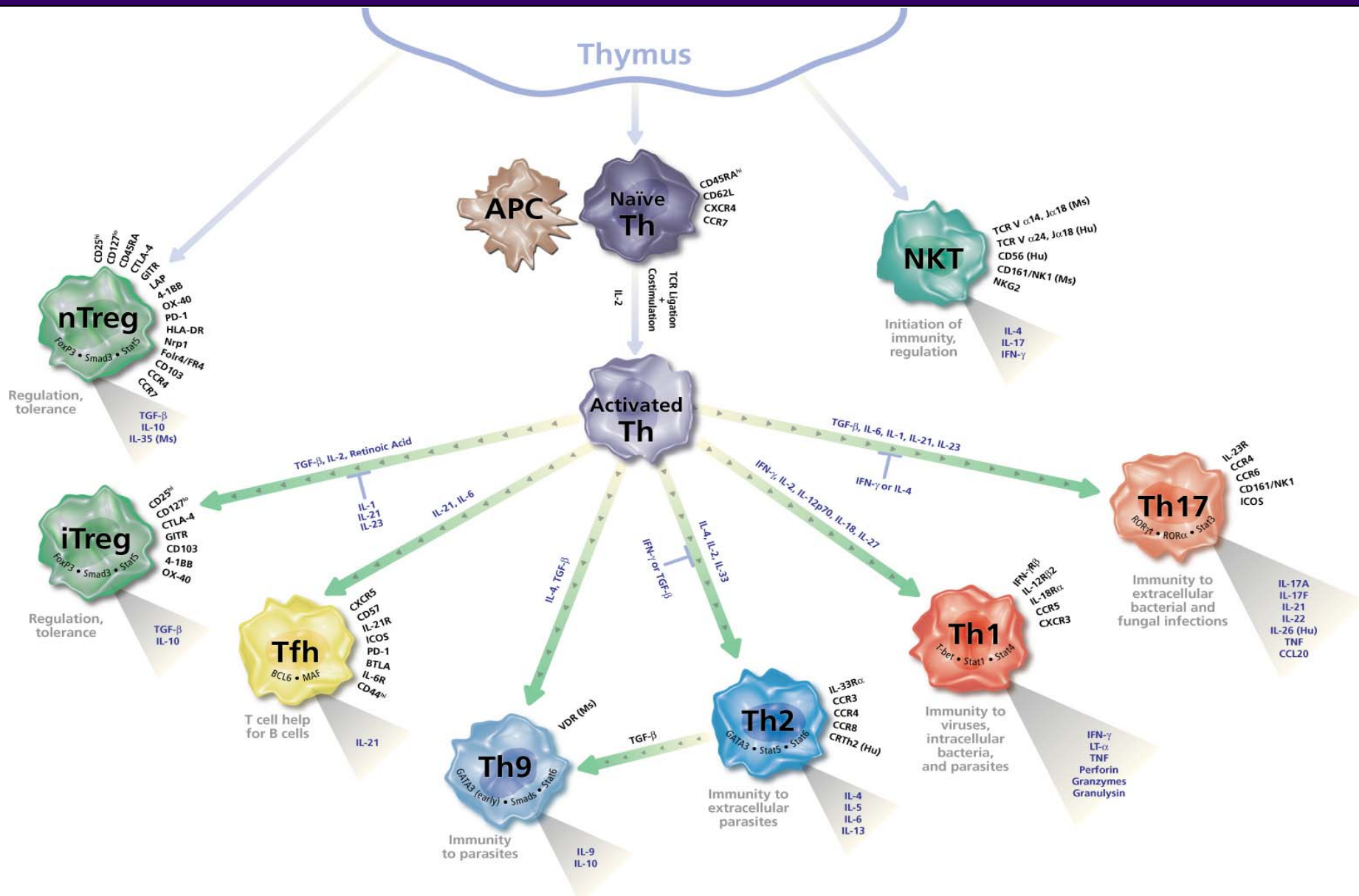
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Director, R&D
BD Biosciences



Overview

- T helper (Th) cell overview
- Experimental setup
- Data analysis
- Conclusions

Introduction to Th biology



Th17 cells

- Developmentally distinct from Th1 and Th2 cells
- Immunity against bacterial and fungal infections
- Play a key role in autoimmune diseases (tissue injury)
- Controlling Th17 activity could aid in the treatment of autoimmune diseases
- TGF- β , IL-6, IL-21, IL-1 β , and IL-23 appear to drive Th17 development
- Produce IL-17A, IL-17F; also IL-21, IL-22, IL-26, and less TNF and IL-6

Treg cells

- Actively suppress T cell proliferation, crucial for T cell homeostasis
- FoxP3, transcription factor is a specific marker for Treg
- FoxP3 is necessary for both development and function of Treg
- nTreg develop in the thymus, iTreg require TGF β , IL-2 and RA
- Produce TGF β and IL-10 and express high levels of CD25 and low levels of CD127
- Dampening Treg activity could improve anti-tumor responses and responses to vaccinations and chronic infections
- Boosting Treg activity could be useful in the treatment of T cell induced diseases

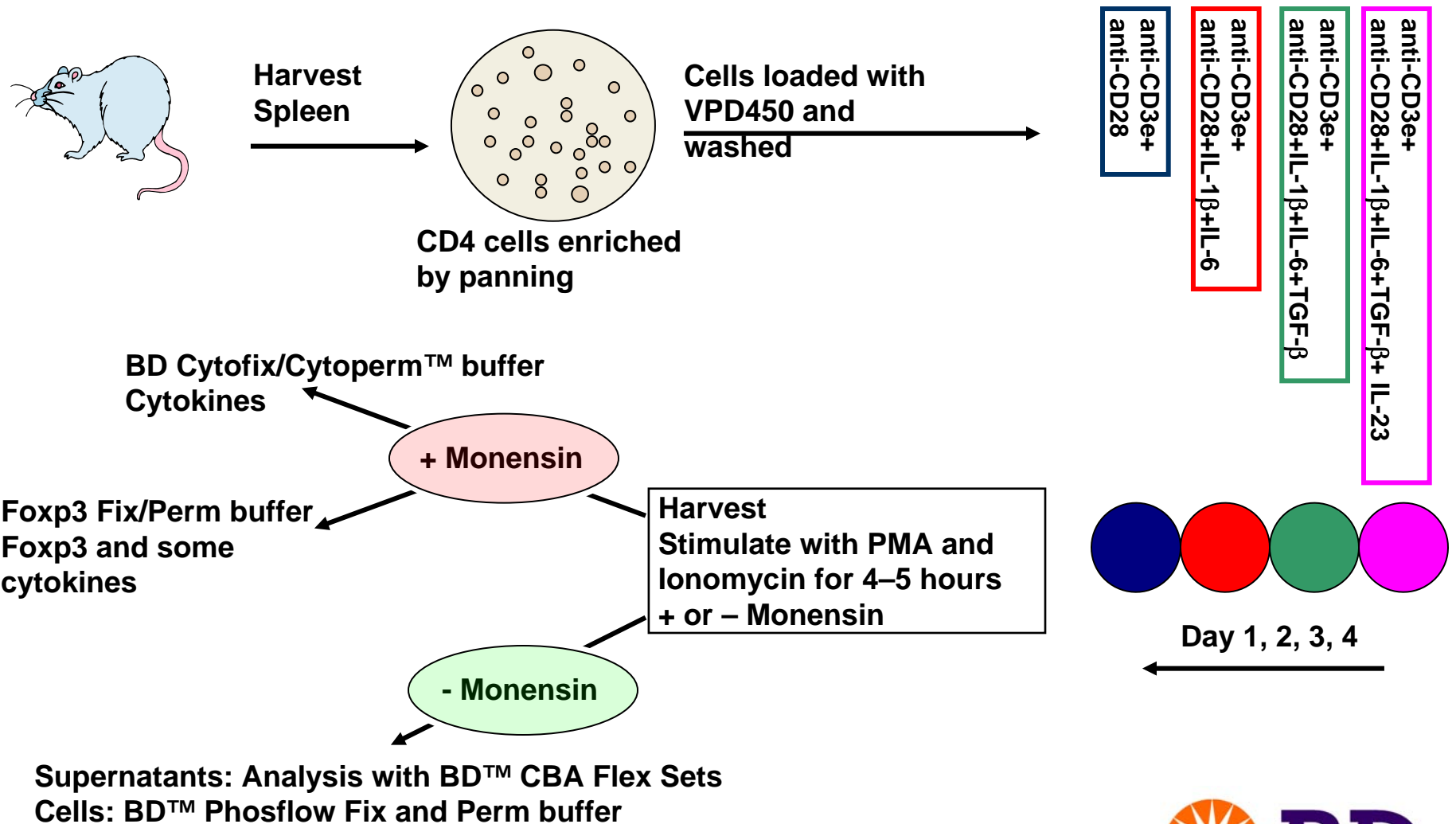


Experimental model

- Enrich Balb/c splenocytes by positive selection via CD4⁺ panning
- Load isolated cells with VPD450 1 μ M, 10 minutes
- Set up cultures as follows:
 - CD3/CD28
 - CD3/CD28/IL-6/IL-1 β
 - CD3/CD28/IL-6/IL-1 β /TGF β
 - CD3/CD28/IL-6/IL-1 β /TGF β /IL-23
- Harvest cells at 1, 2, 3, and 4 days
- Fix/perm and stain cells for IL-17A, Foxp3, IL-4, IL-2, and interferon- γ (IFN- γ)



Experimental setup



Fluorescein Diacetate Derivative

VPD450 Dye



Non-Fluorescent

Enters cells, esterases cleave ECM to give fluorescent product



Fluorescent

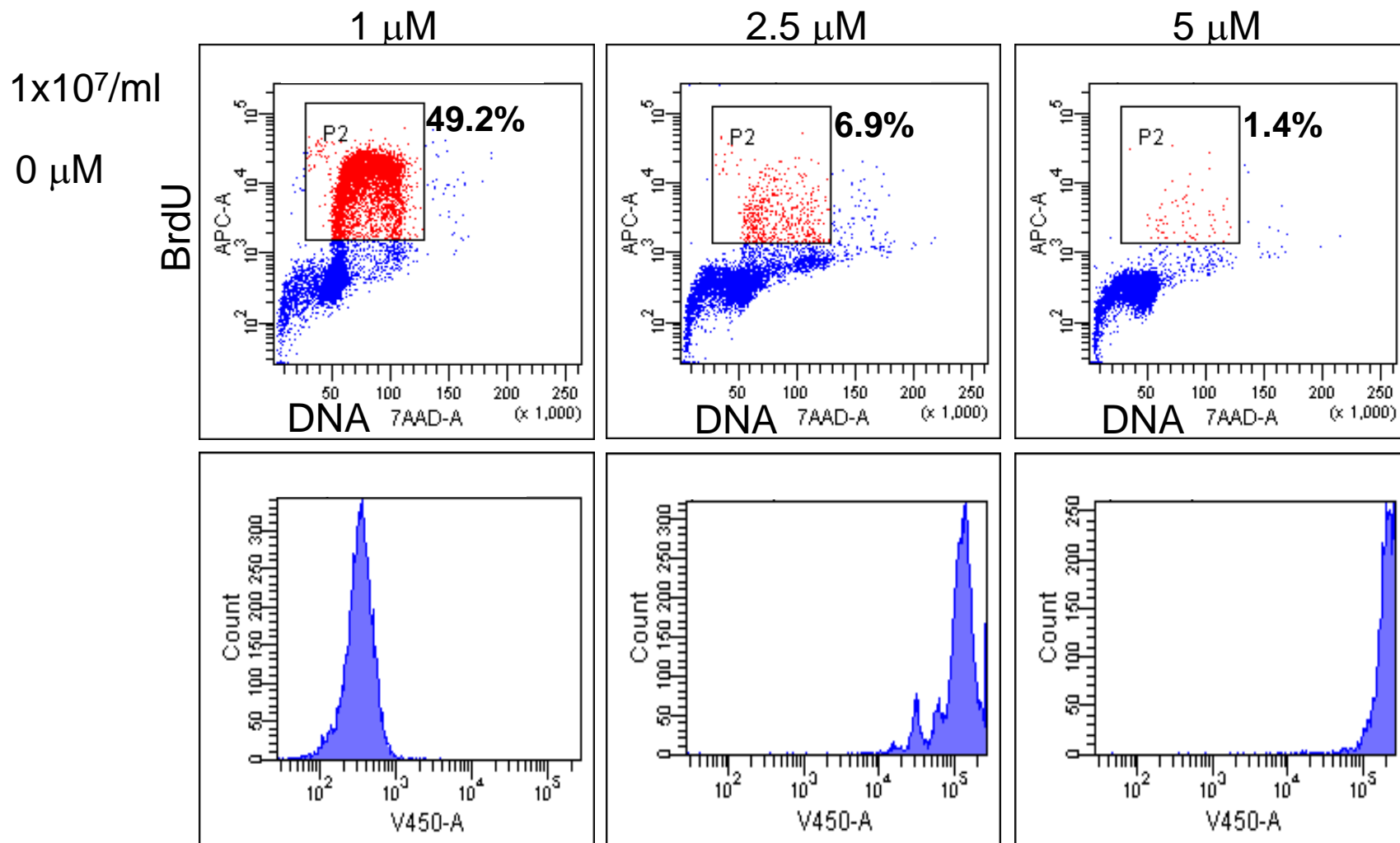
Reacts with cell components to give VPD450 adducts retained inside cells



Fluorescent and Cell-retained

ARM = amino-reactive moiety
ECM = esterase-cleavable moiety
MFM = masked fluorophore moiety
IACB = Intracellular amino-containing biopolymer

Spleen CD3/28 Day 2 - [VPD450]

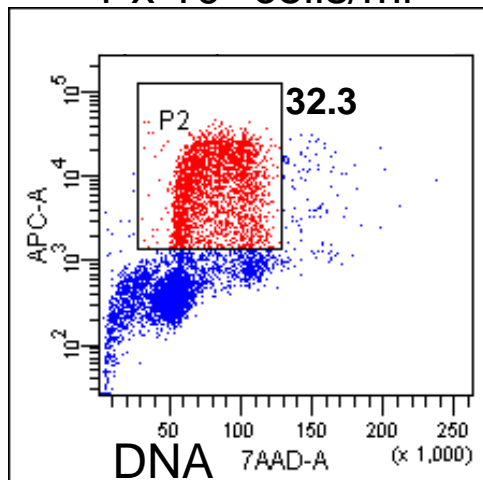


Spleen CD3/28 Day 2 - [Cell]

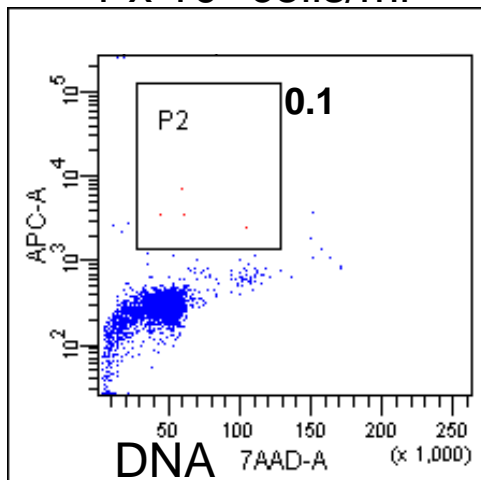
1 μ M

BrdU

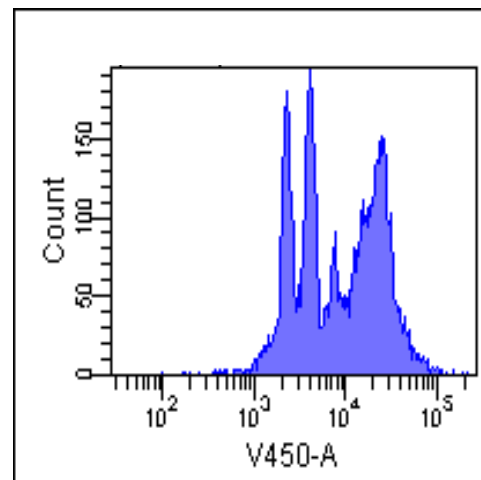
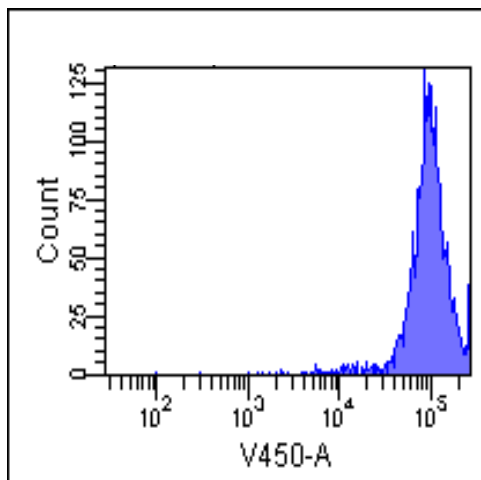
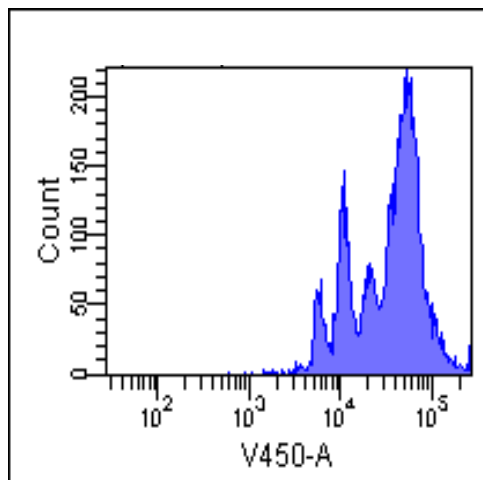
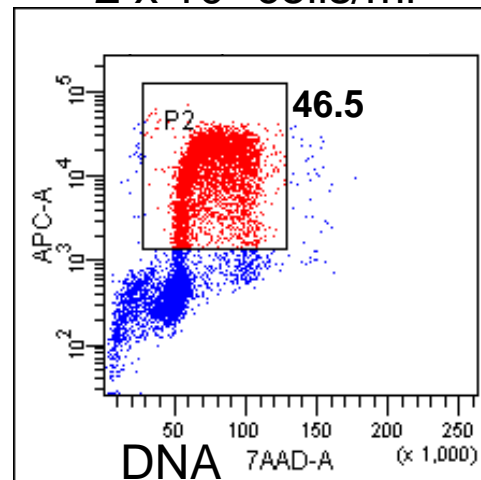
1 x 10⁷ cells/ml



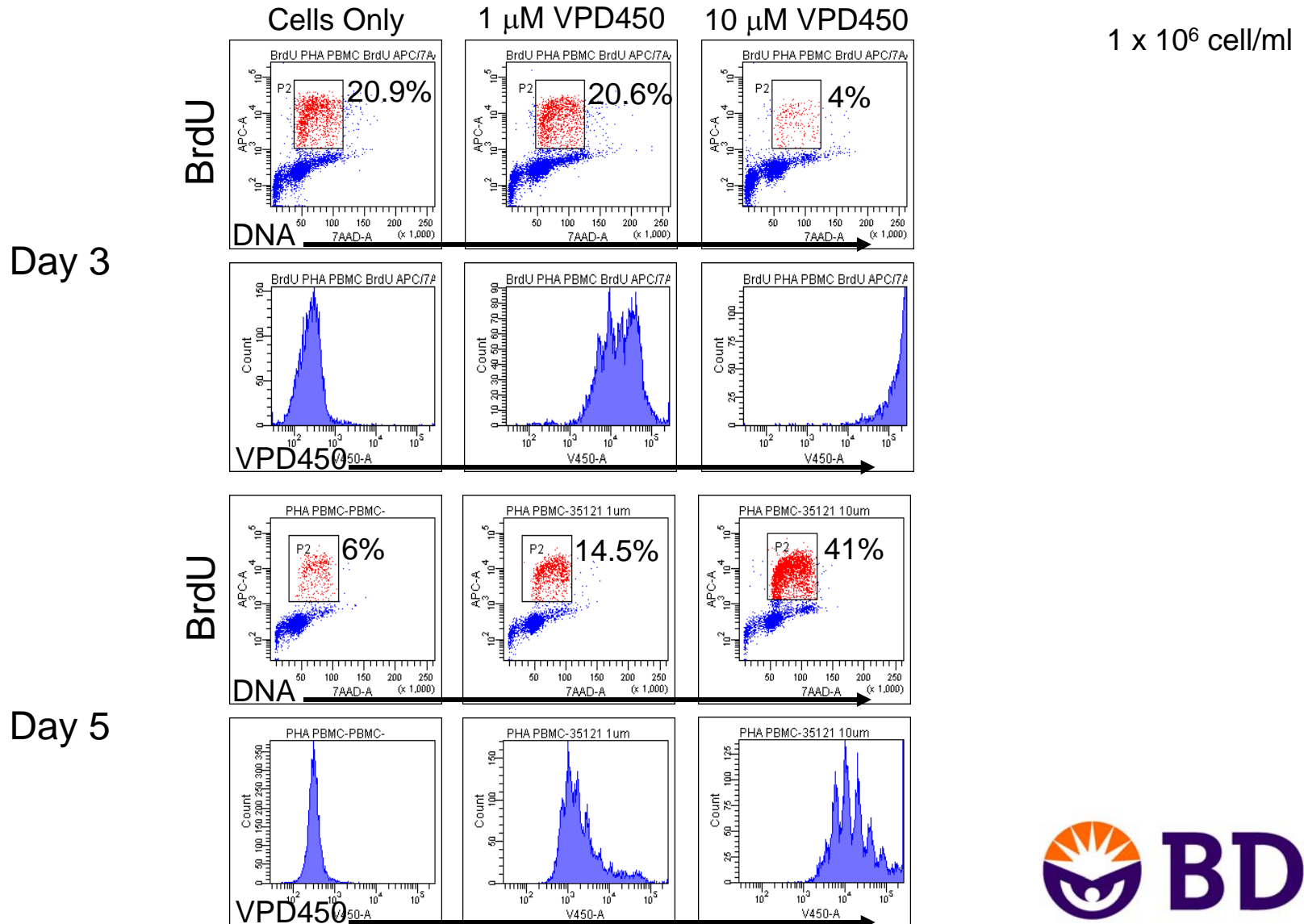
1 x 10⁶ cells/ml



2 x 10⁷ cells/ml



Human PBMC PHA Stimulation [VPD450]



VPD450 histograms

Condition:

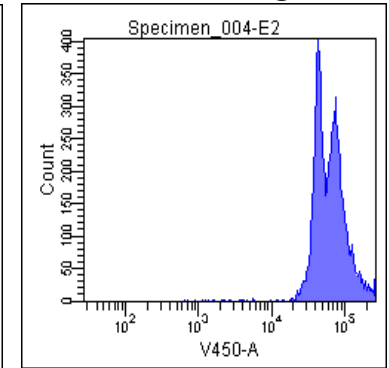
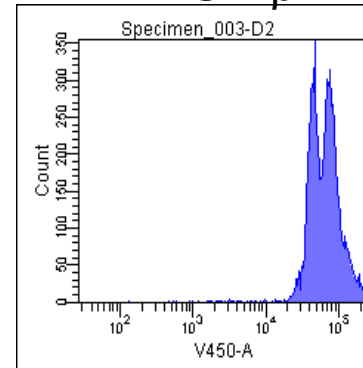
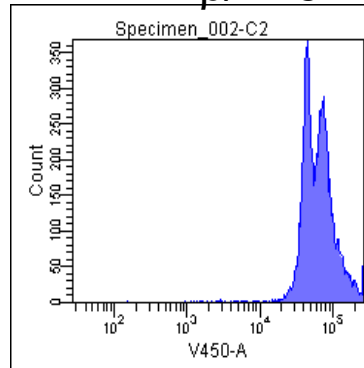
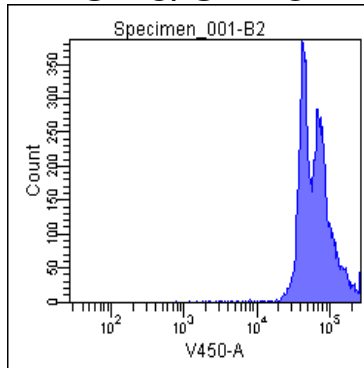
CD3/CD28

+IL-1 β /IL-6

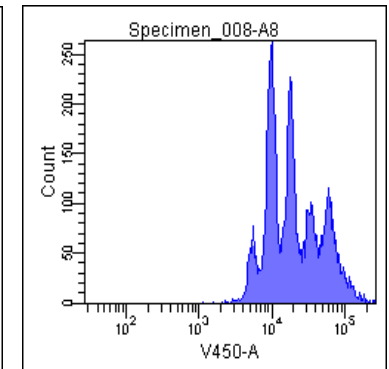
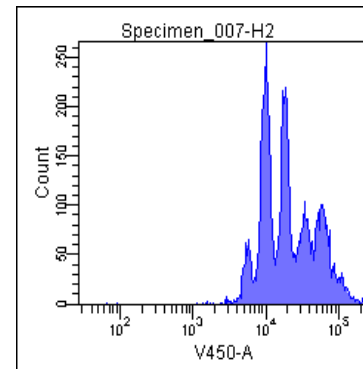
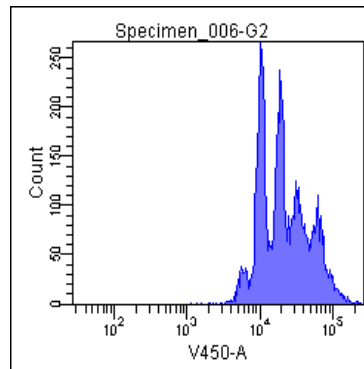
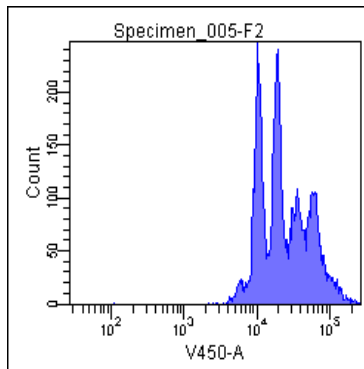
+TGF- β

+IL-23

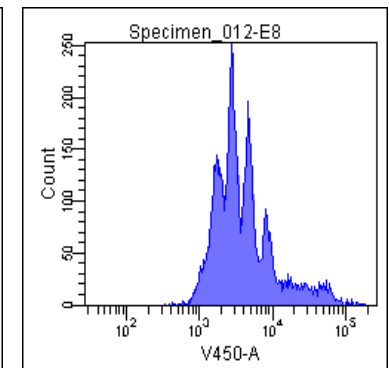
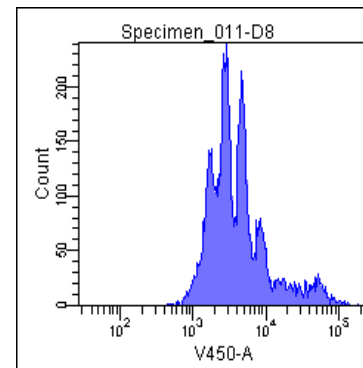
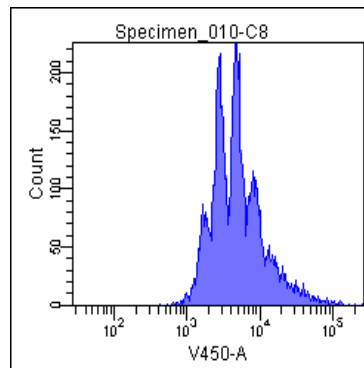
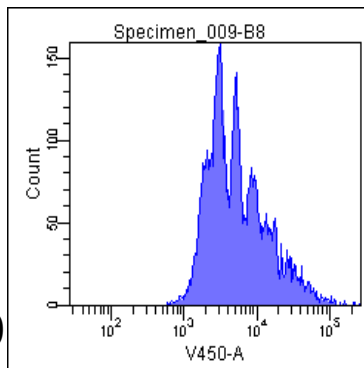
Day 1



Day 2



Day 3

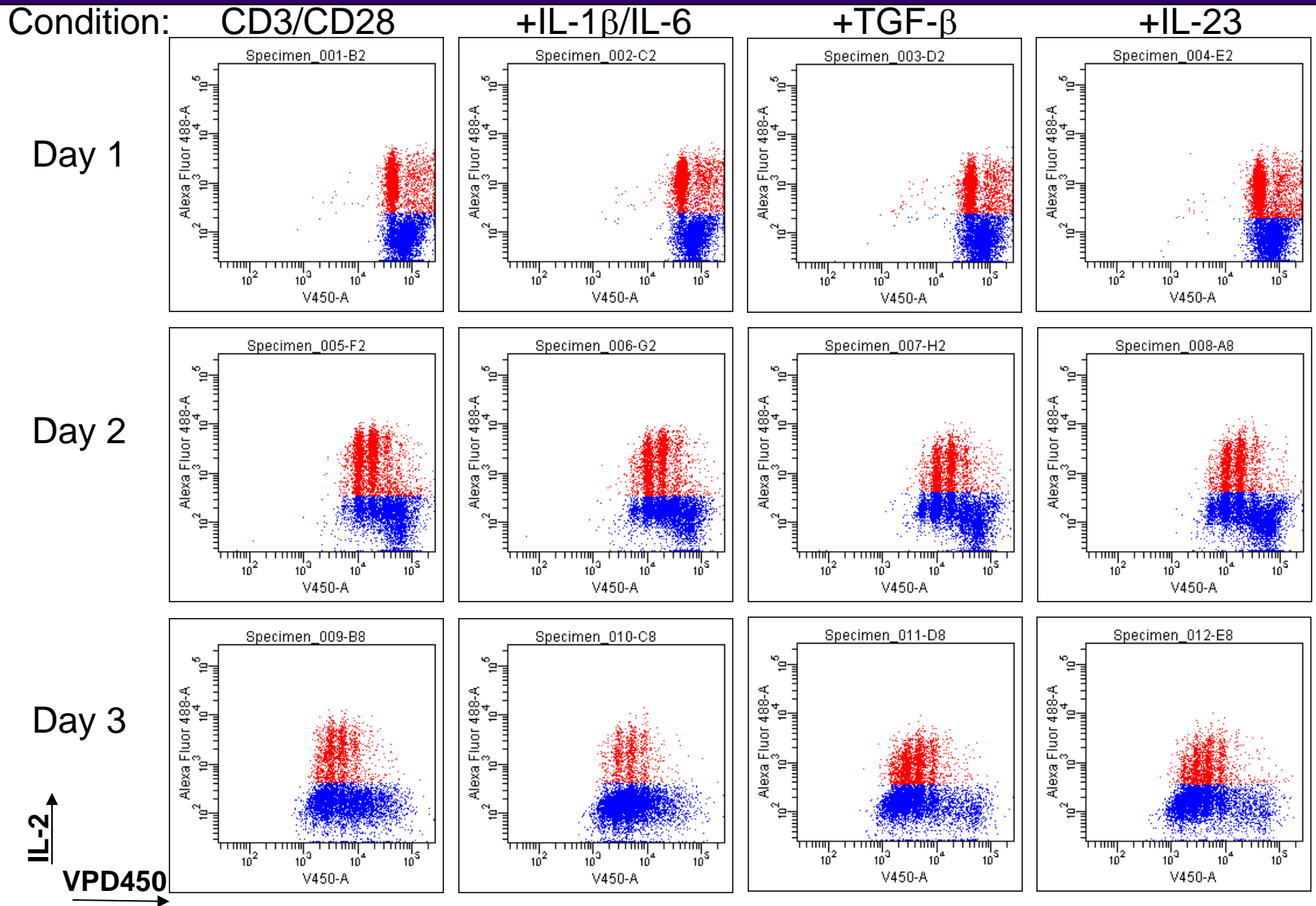


VPD450

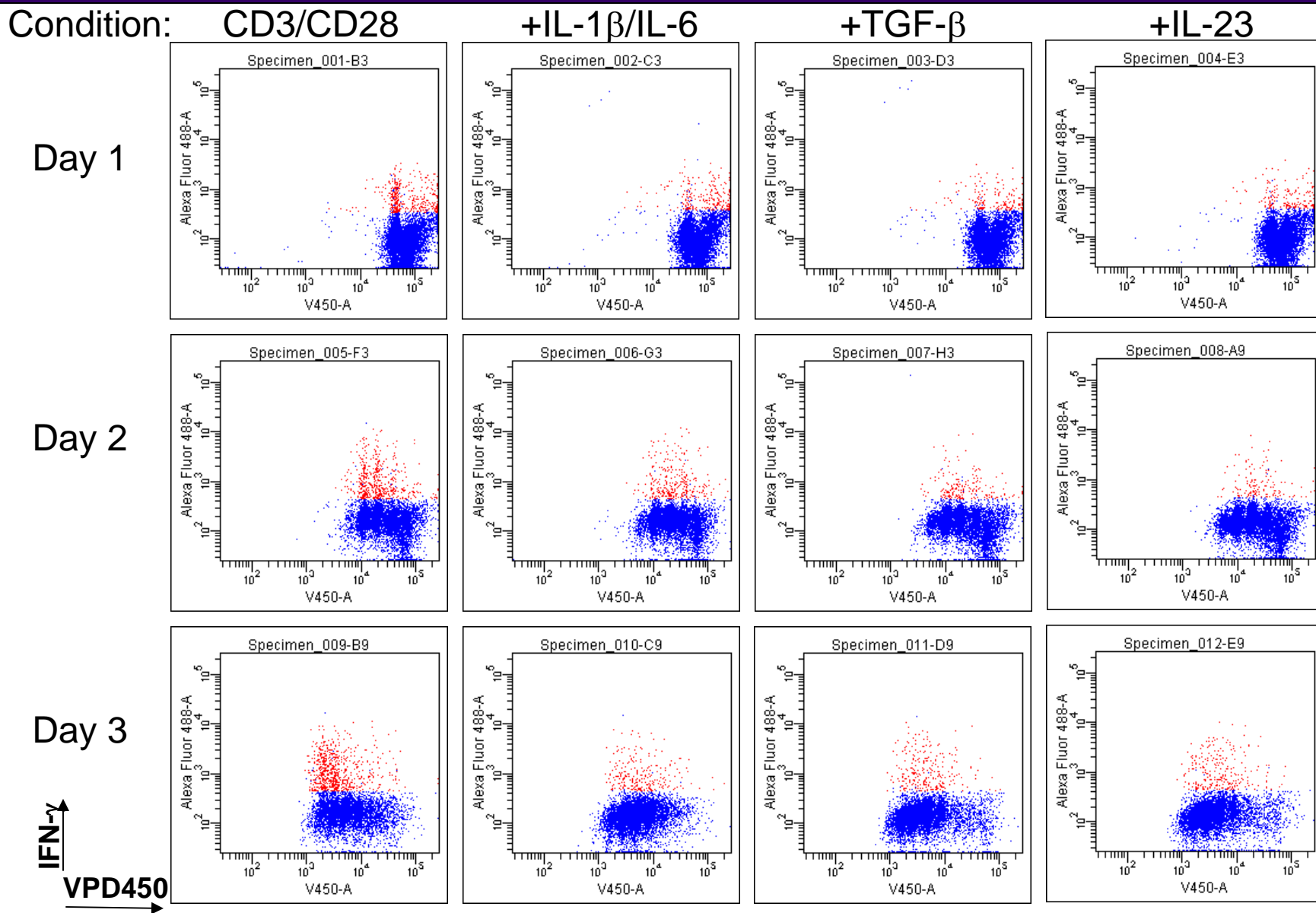
Which conditions for which cytokines

- All conditions result in proliferation of cells to essentially equal extents.
- Which cytokines are being produced under which conditions?
- Which cell types are producing which cytokines?

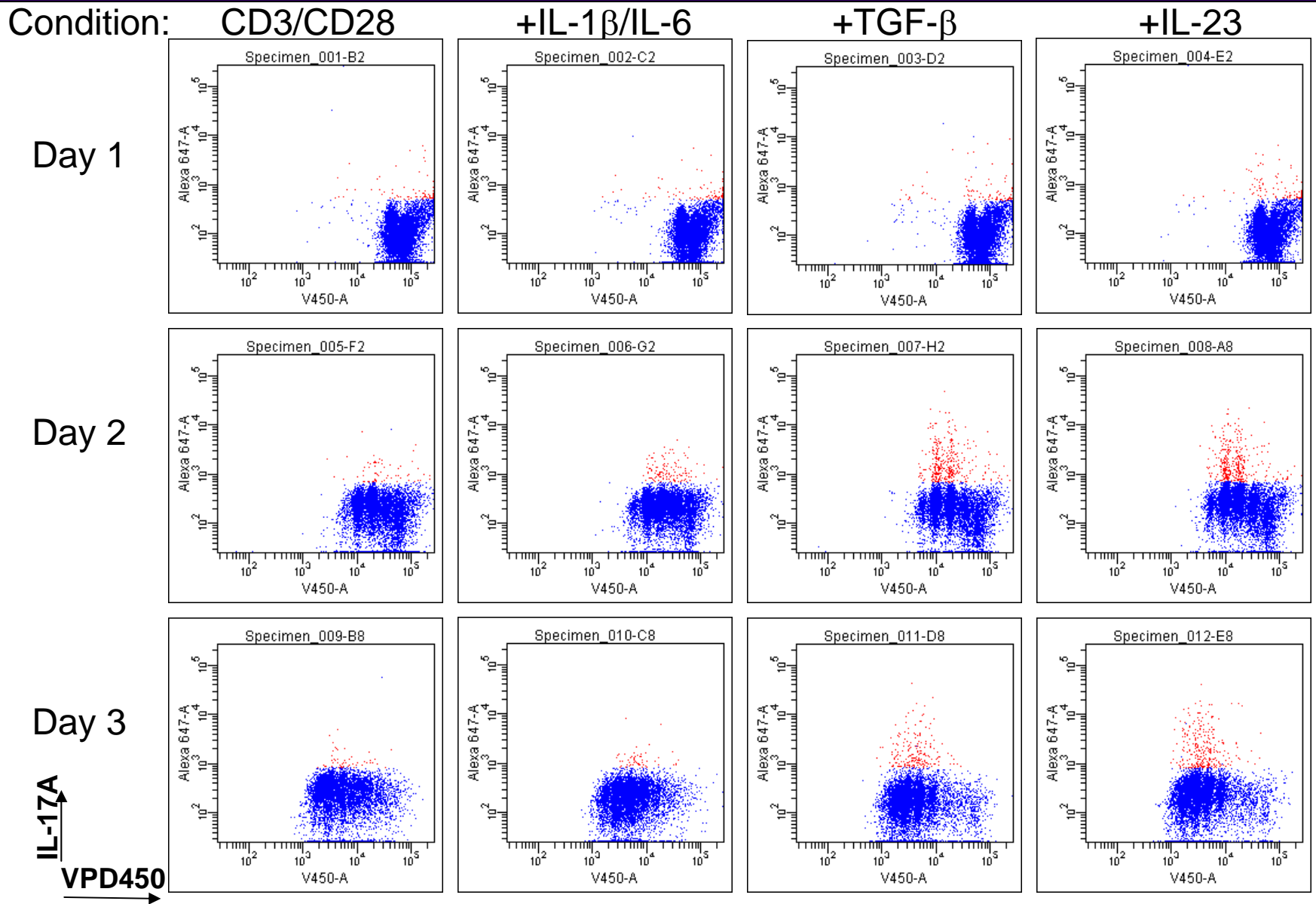
VPD450 vs IL-2 data



VPD450 vs IFN- γ data



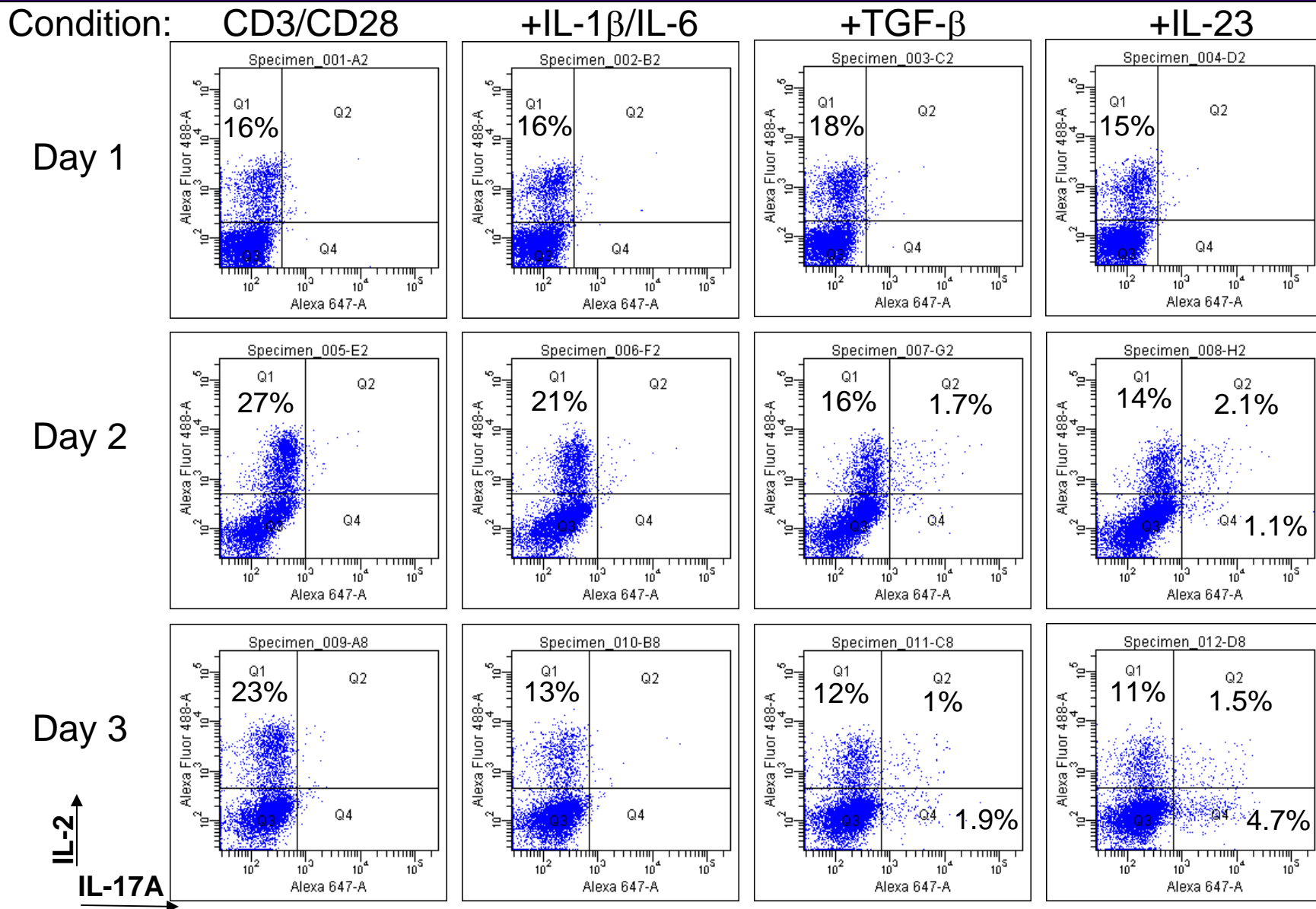
VPD450 vs IL-17A data



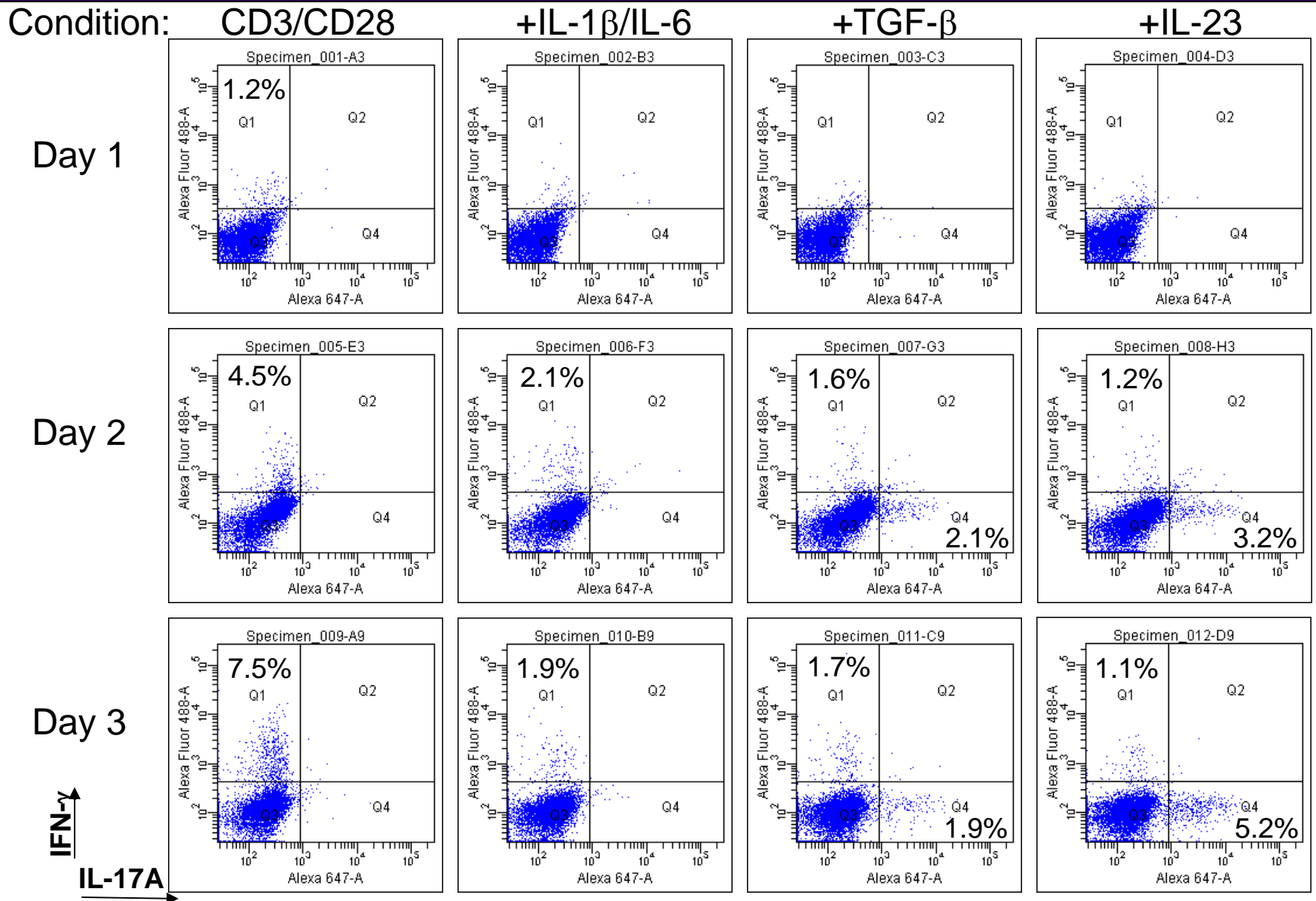
Cytokine co-expression

- IL-2 is expressed under all conditions
- IFN- γ is produced more under condition 1
- TGF- β is required for expression of IL-17A
- Which cytokines are co-expressed?

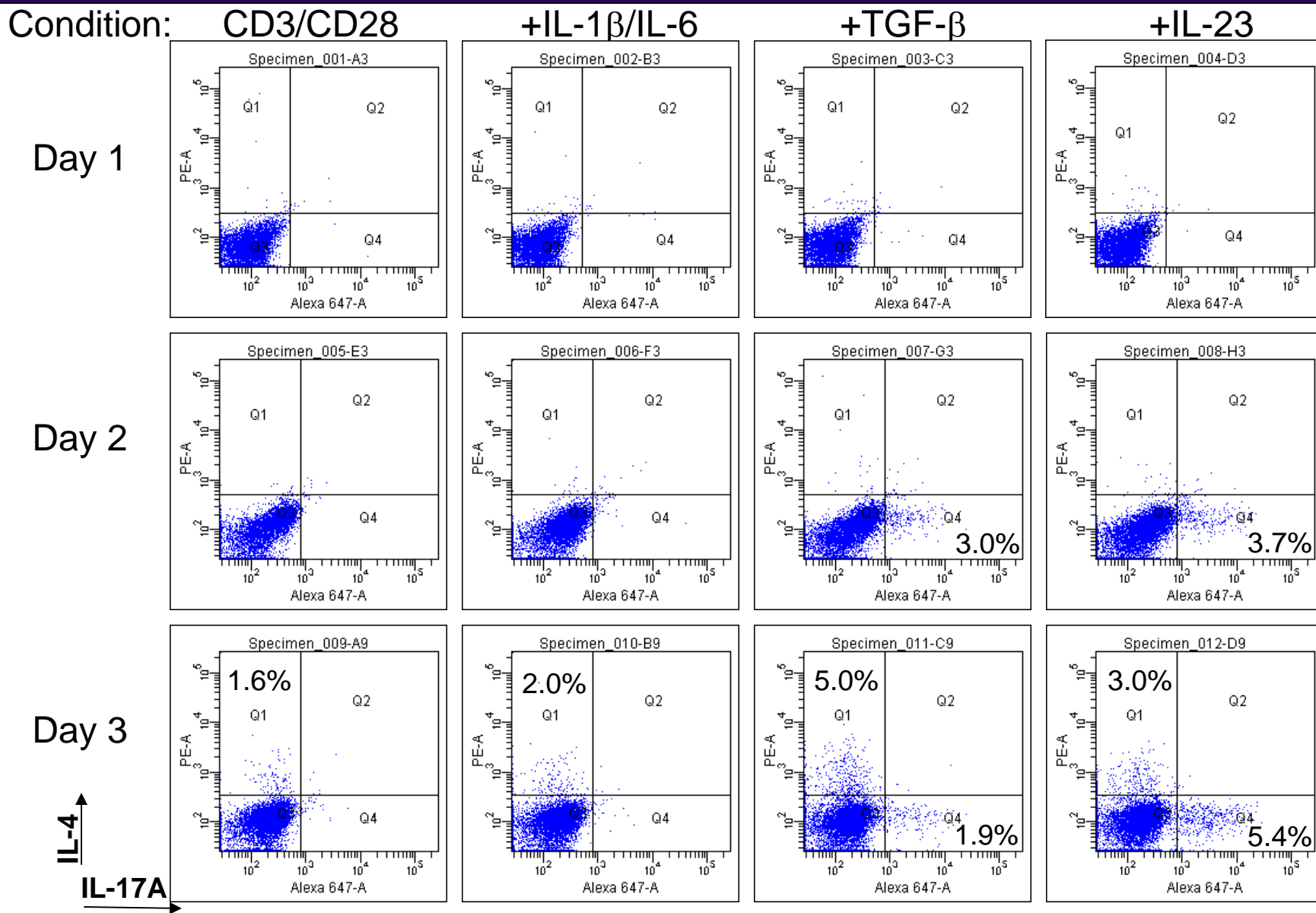
Co-expression of IL-17A vs IL-2



Co-expression of IL-17A vs IFN- γ



Co-expression of IL-17A vs IL-4

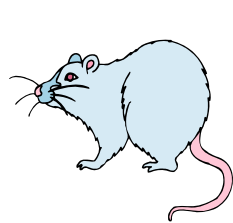


Tracking FoxP3

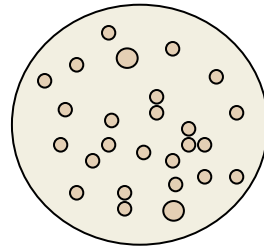
- IL-17A expression is boosted by addition of IL-23.
- Earlier on IL-17A expressing cells co-express IL-2, but over time the two become mutually exclusive.
- IL-4 expression increases as IFN- γ expression decreases.
- What are the FoxP3+ cells doing?



Experimental setup



Harvest
Spleen



CD4 cells enriched
by panning

Cells loaded with
VPD450 and
washed

anti-CD3e+
anti-CD28

anti-CD3e+
anti-CD28+IL-1 β +IL-6

anti-CD3e+
anti-CD28+IL-1 β +IL-6+ TGF- β

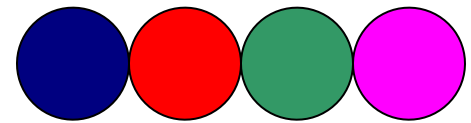
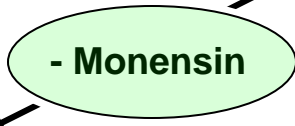
anti-CD3e+
anti-CD28+IL-1 β +IL-6+ TGF- β + IL-23

BD Cytofix/Cytoperm™ buffer
Cytokines



Foxp3 Fix/Perm buffer
Foxp3 and some
cytokines

Harvest
Stimulate with PMA and
Ionomycin for 4–5 hours
+ or – Monensin



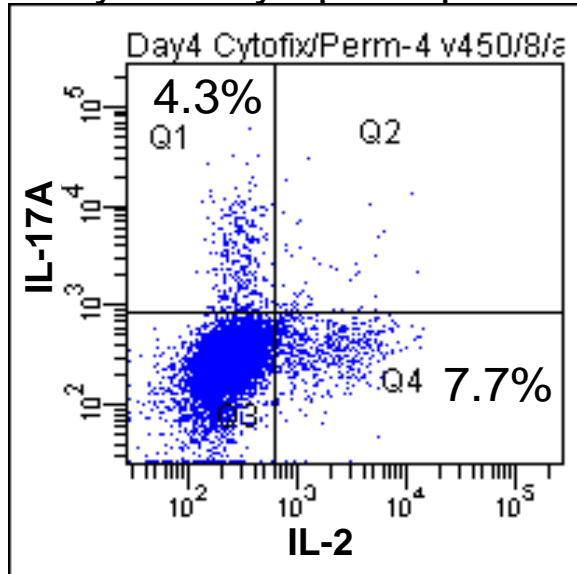
Day 1, 2, 3, 4

Supernatants: Analysis with BD™ CBA Flex Sets
Cells: BD™ Phosflow Fix and Perm buffer

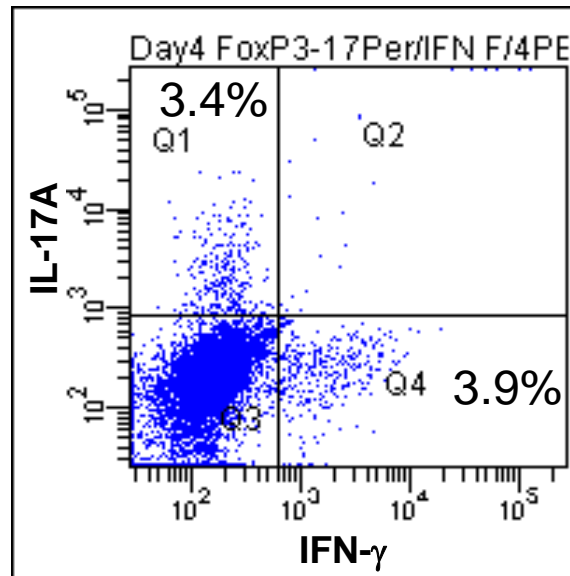
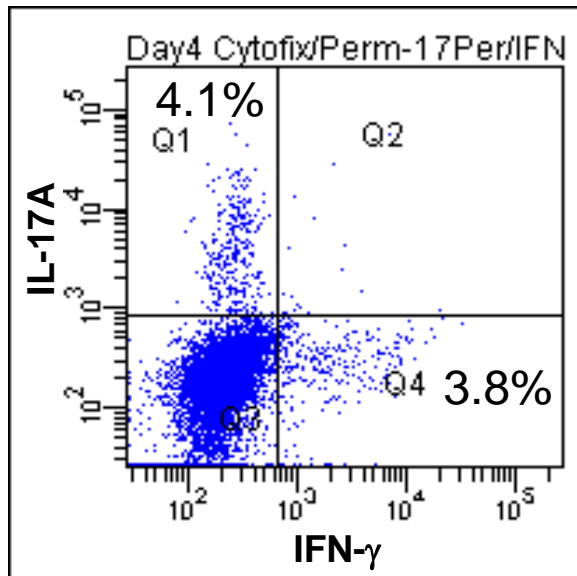
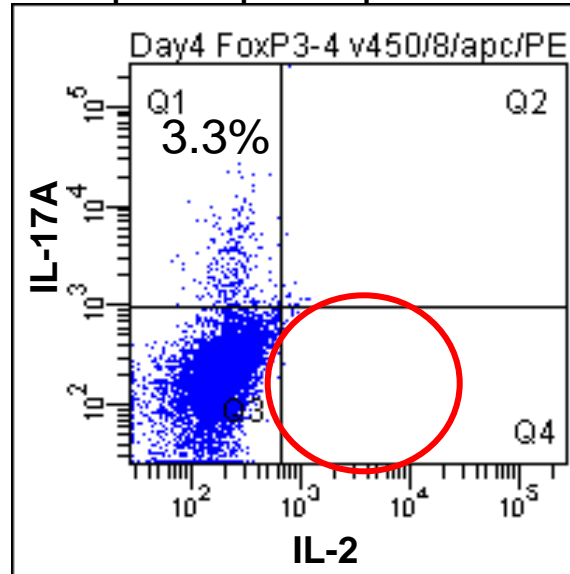


Comparison of two fix/perm protocols

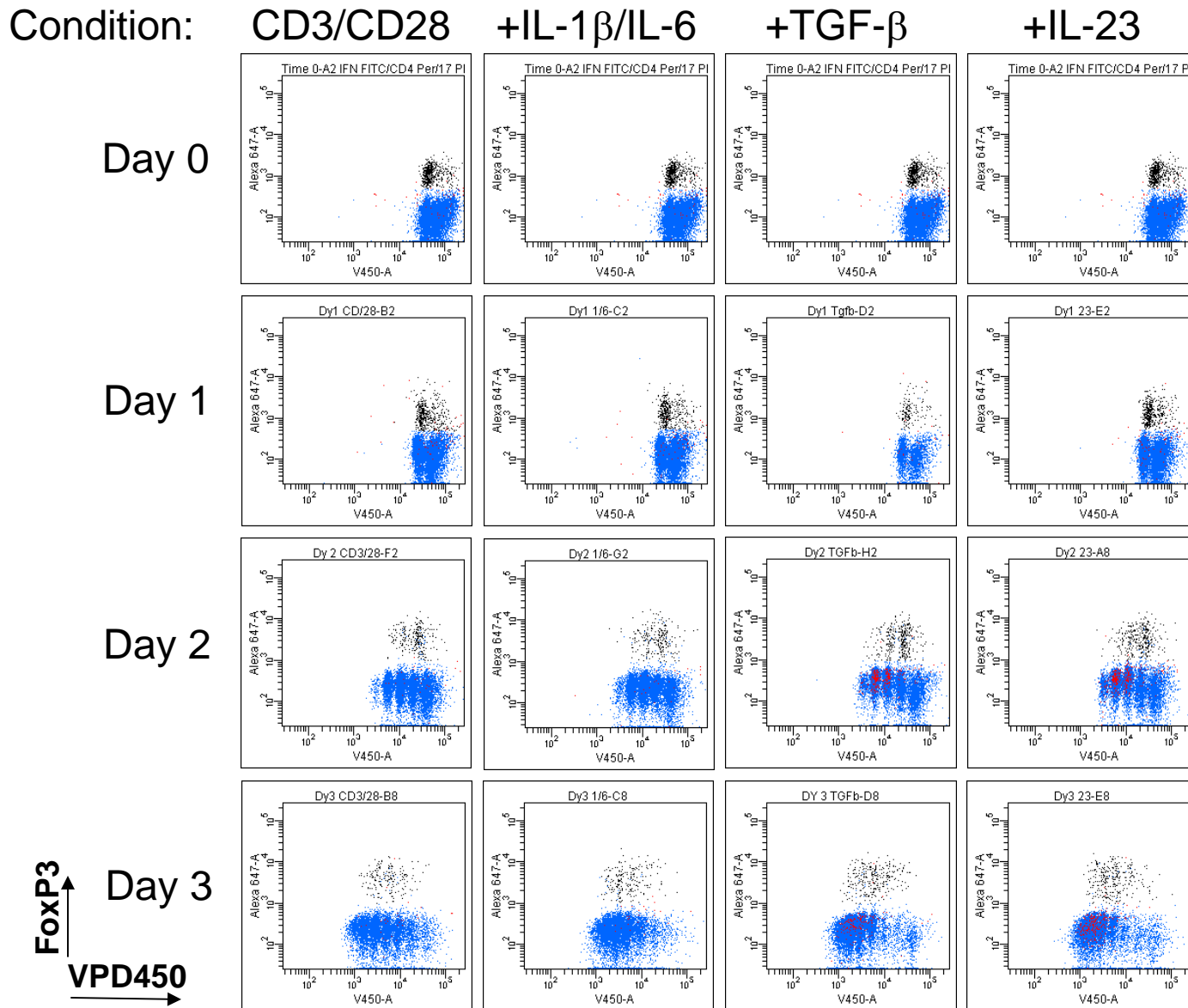
BD Cytfix/Cytoperm protocol



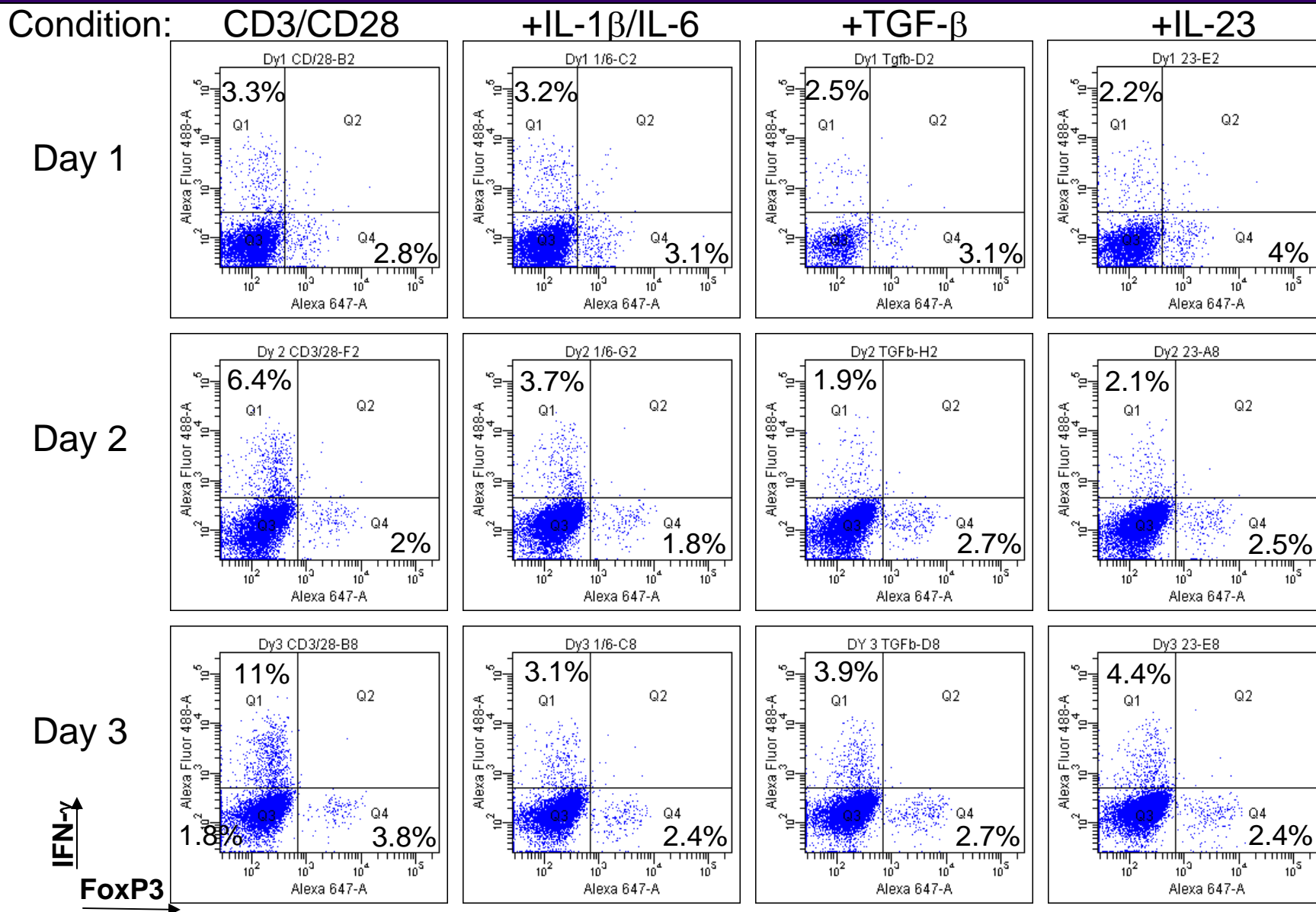
Foxp3 fix/perm protocol



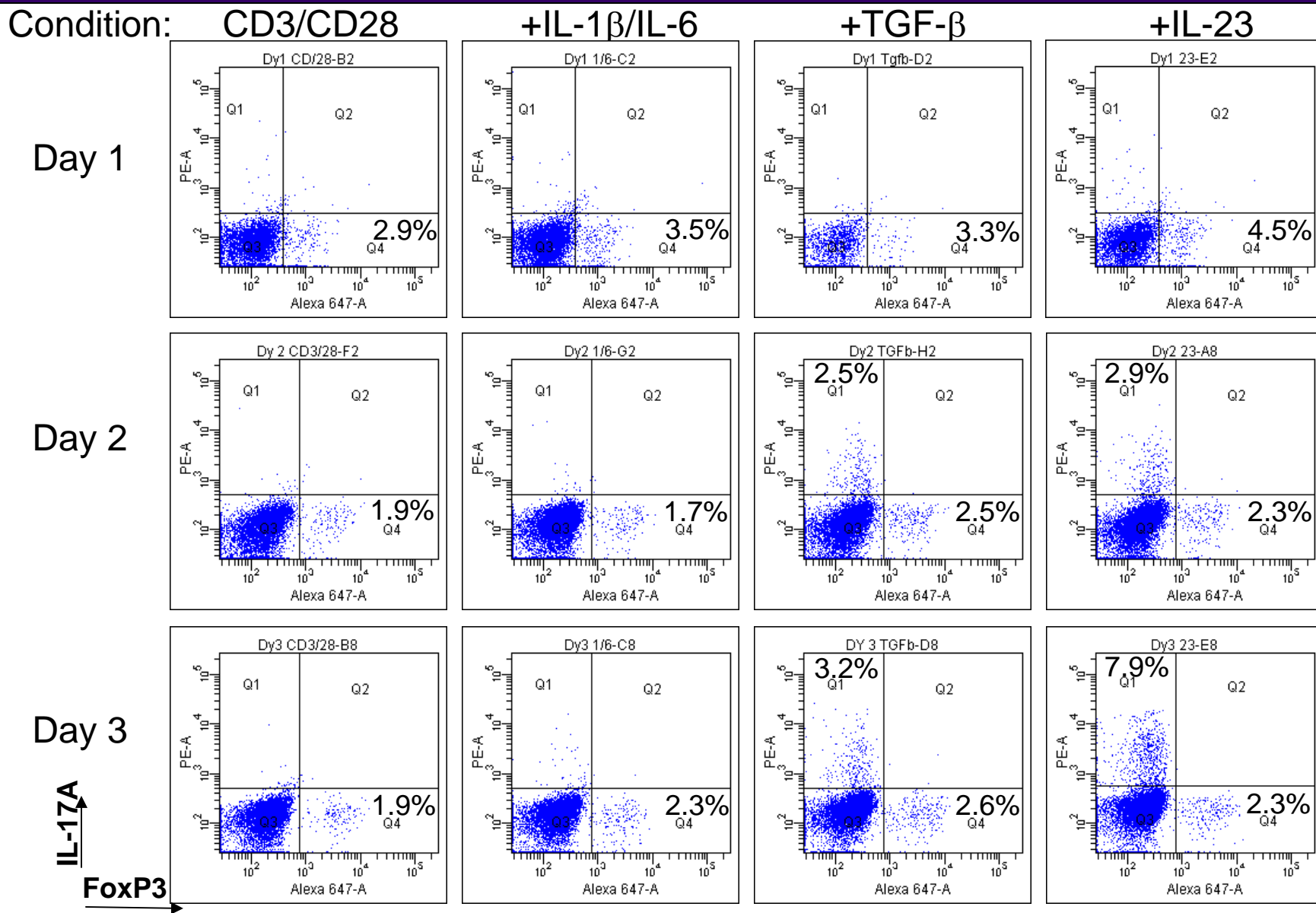
Proliferation of Treg and Th17 cells



Co-expression of *Foxp3* vs *IFN- γ*



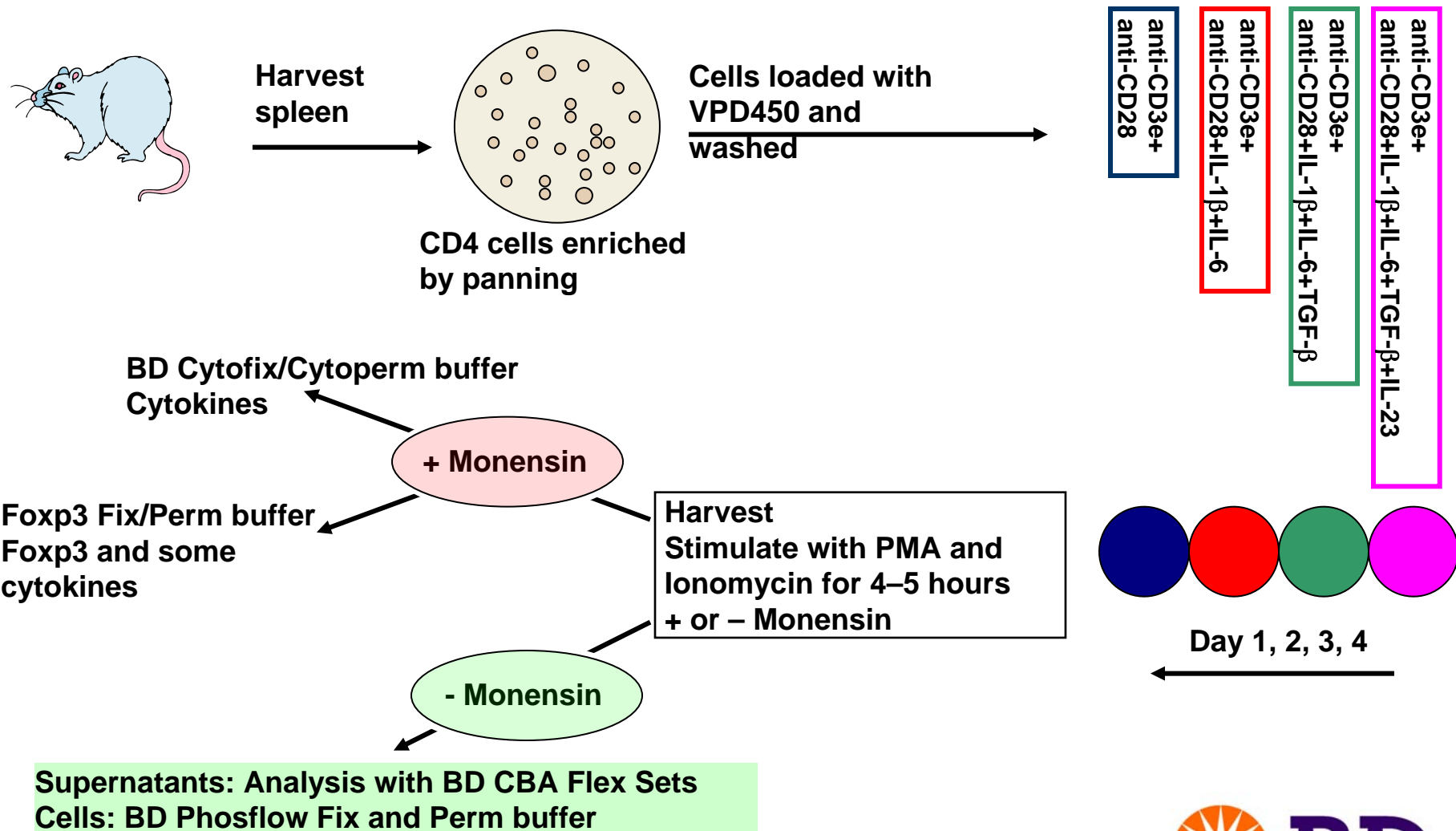
Co-expression of Foxp3 vs IL-17A



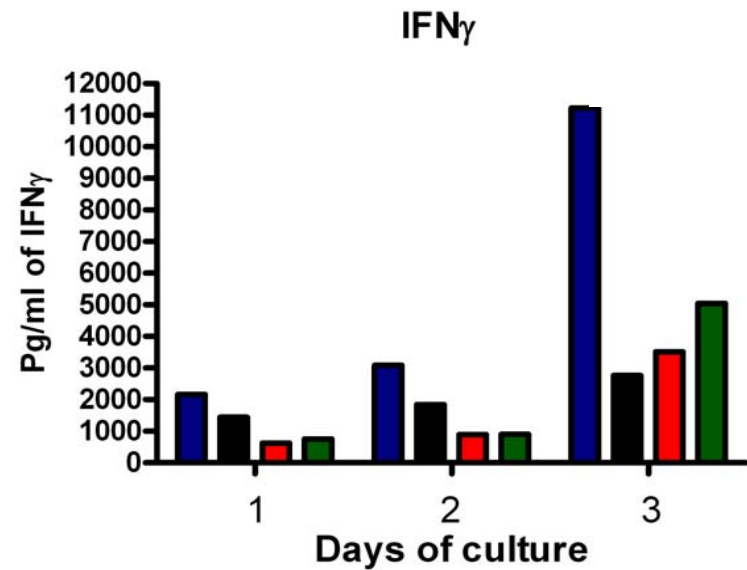
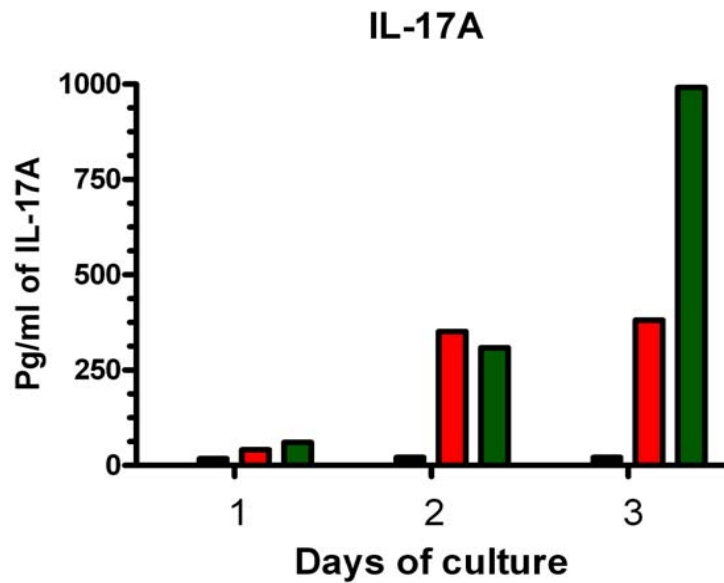
Cytokines in culture supernatants

- FoxP3 expression maintained throughout culture period.
- FoxP3+ Treg cells divide more slowly than other CD4 t cells.
- Expression of IFN- γ and IL-17A not found in Treg.
- Does cytokine expression detected in the cells correlate with cytokine detected in culture supernatants?

Experimental setup

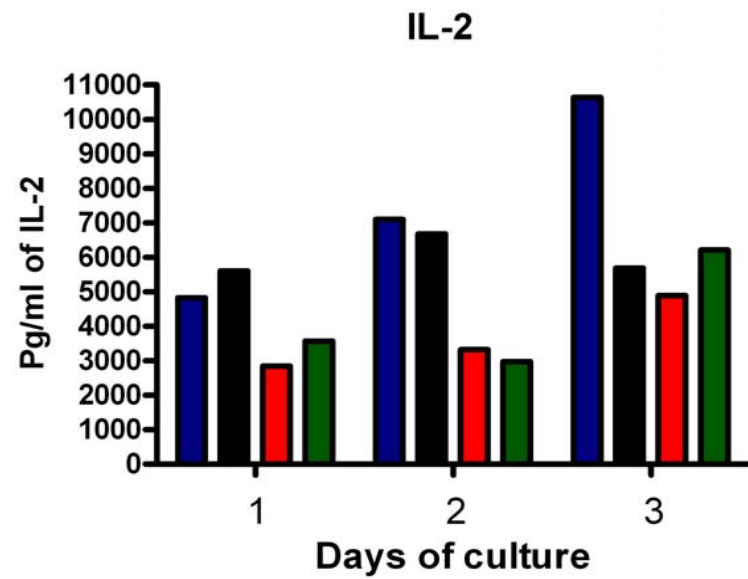
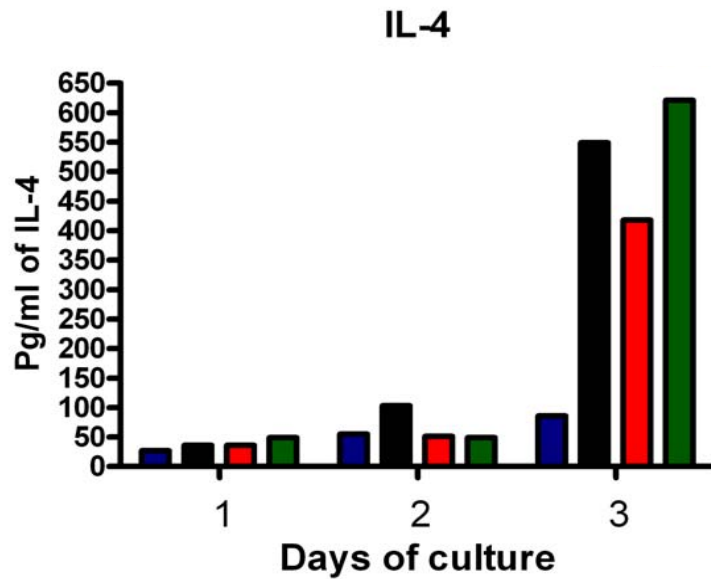


IL-17A and IFN- γ production



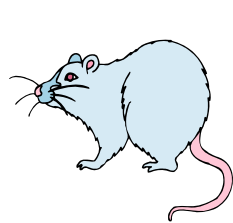
- CD3 /CD28
- CD3 /CD28/IL1 β /IL-6
- CD3 /CD28/IL1 β /IL-6/TGF β
- CD3 /CD28/IL1 β /IL-6/TGF β /IL-23

IL-4 and IL-2 production

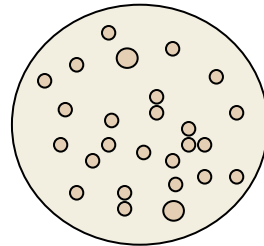


- CD3 /CD28
- CD3 /CD28/IL1 β /IL-6
- CD3 /CD28/IL1 β /IL-6/TGF β
- CD3 /CD28/IL1 β /IL-6/TGF β /IL-23

Experimental setup



Harvest spleen



CD4 cells enriched by panning

Cells loaded with VPD450 and washed

anti-CD3e+
anti-CD28

anti-CD3e+
anti-CD28+IL-1 β +IL-6

anti-CD3e+
anti-CD28+IL-1 β +IL-6+TGF- β

anti-CD3e+
anti-CD28+IL-1 β +IL-6+TGF- β +IL-23

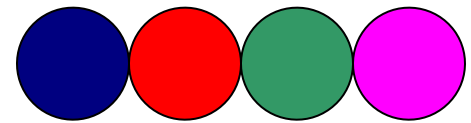
BD Cytofix/Cytoperm buffer
Cytokines

+ Monensin

Foxp3 Fix/Perm buffer
Foxp3 and some cytokines

Harvest
Stimulate with PMA and Ionomycin for 4–5 hours
+ or – Monensin

- Monensin



Day 1, 2, 3, 4

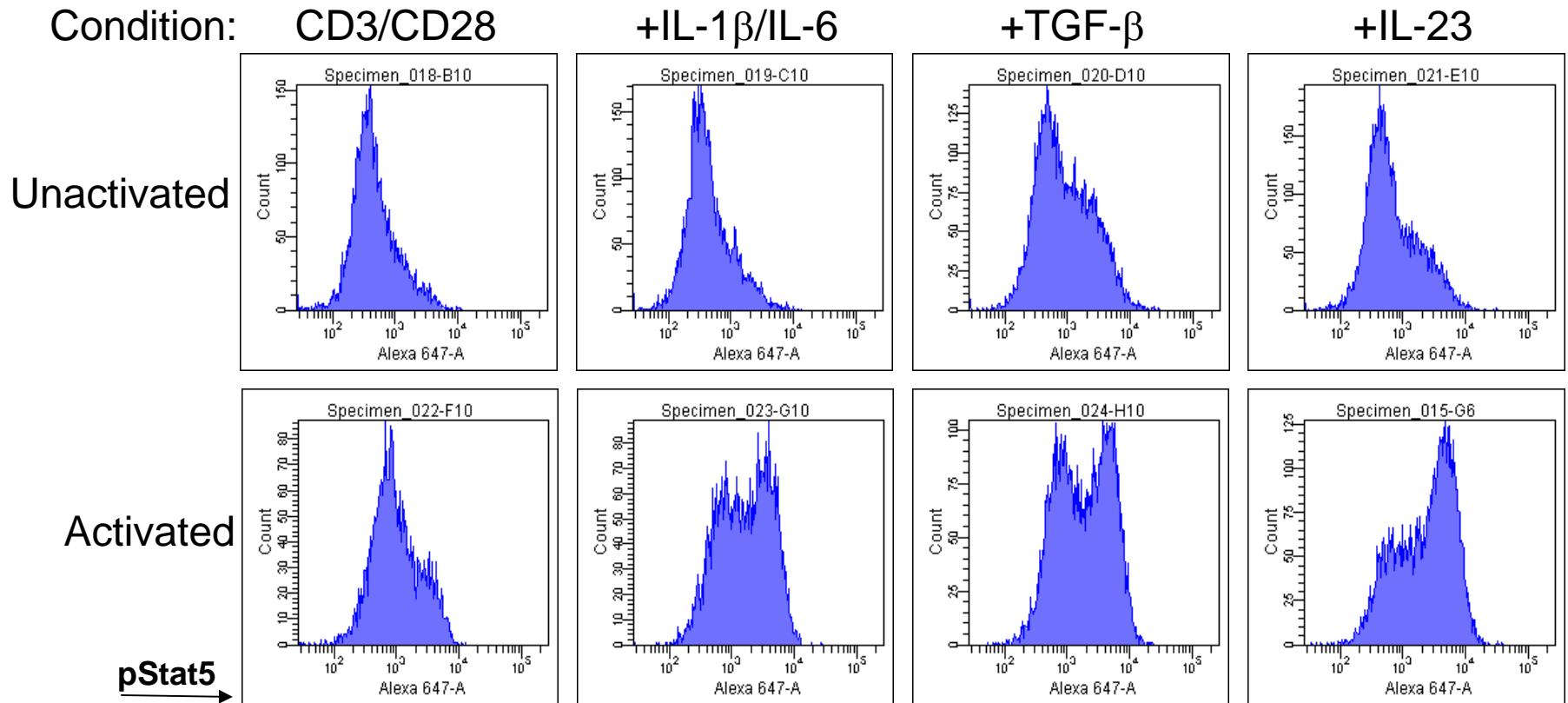
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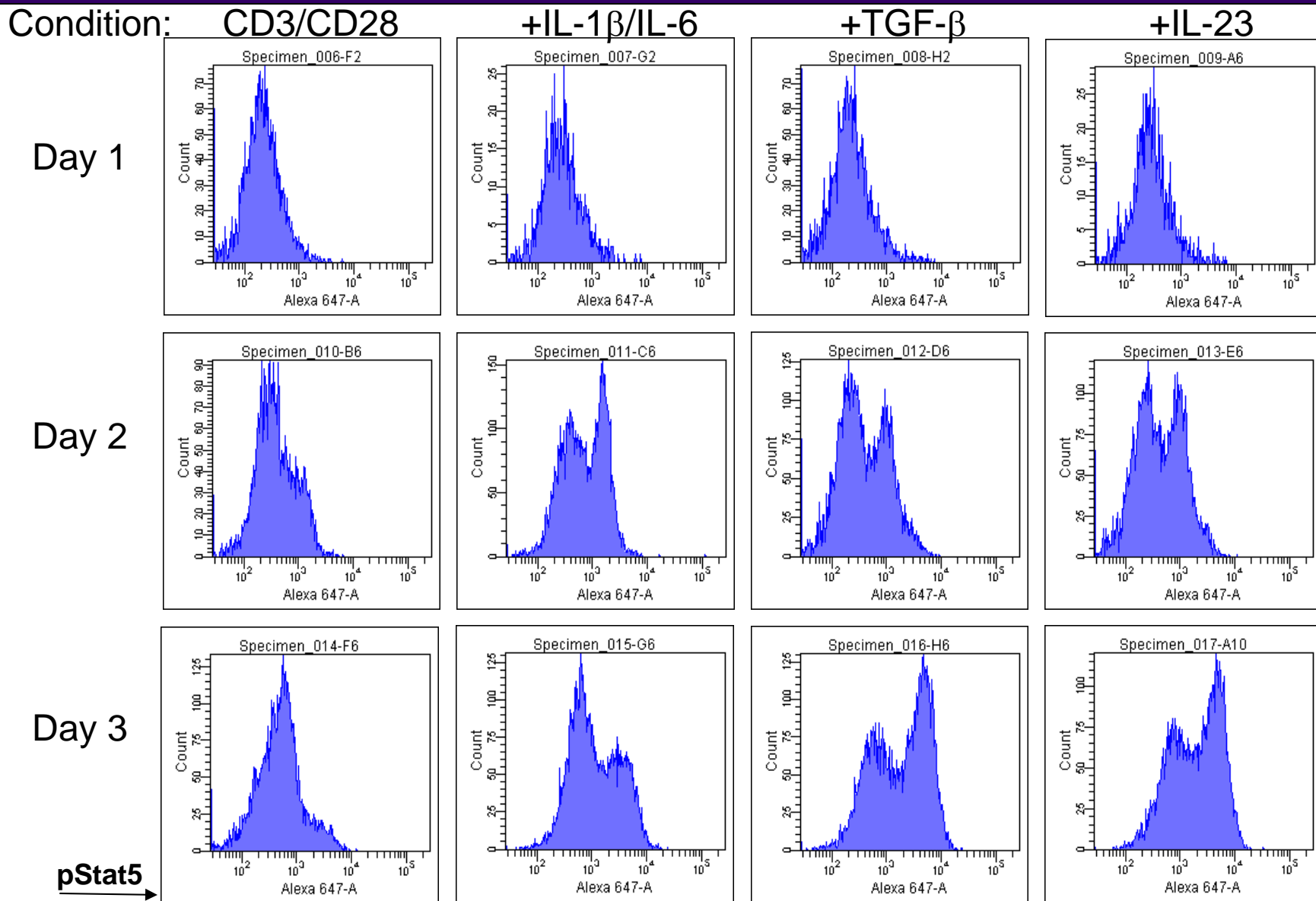
pStat5 detection on day 4

Unactivated: Cells were cultured, harvested, and stained with phosphospecific Stat5 antibody.

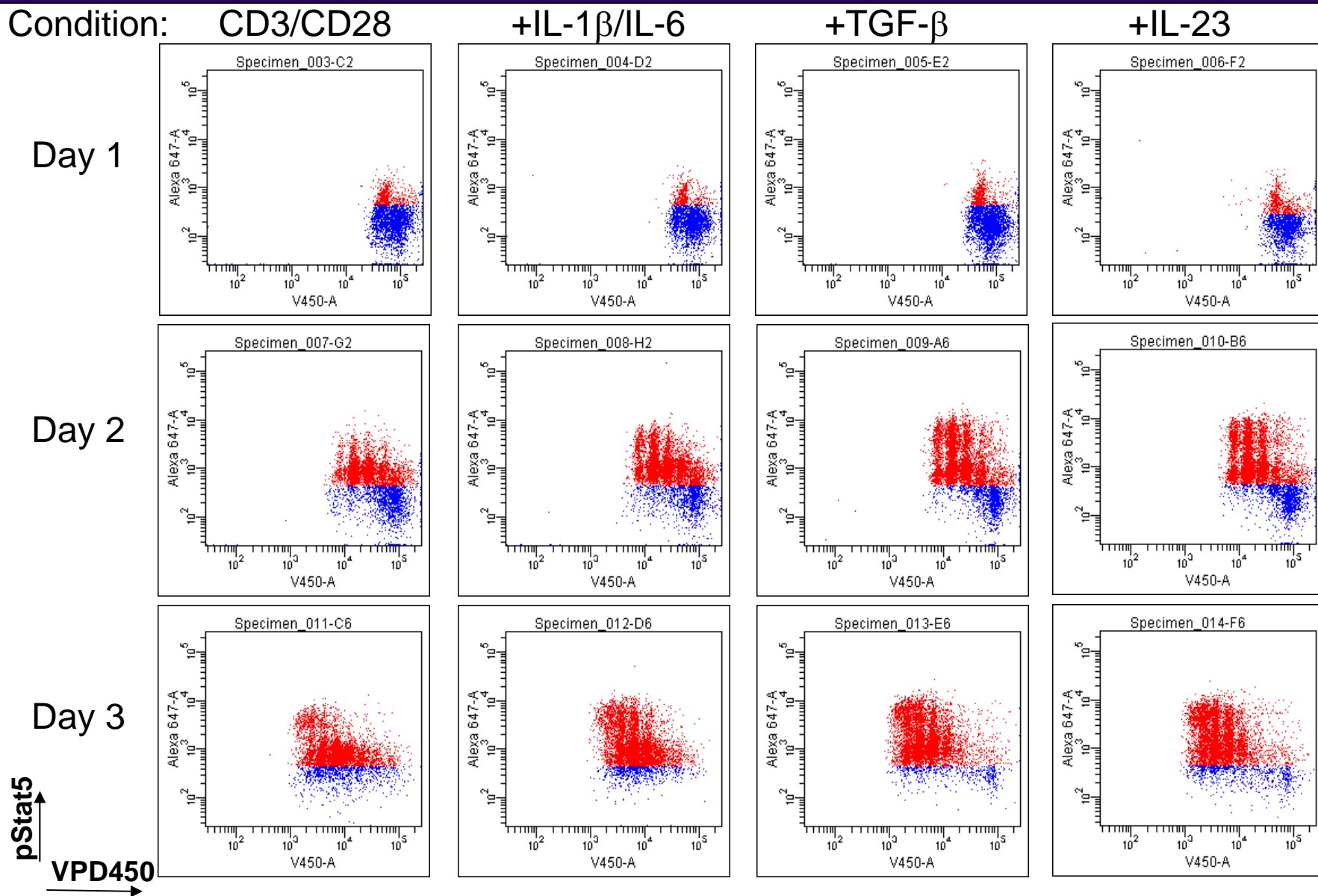
Activated: Cells were cultured and activated with PMA/Ionomycin for 5 hours and then stained with phospho-specific Stat5 antibody.



pStat5 in activated cells over time



pStat5 in proliferating cells



Conclusions

- Cells proliferated equally well under all four polarization conditions.
- In vitro cultures showed that TGF- β was important for polarization of CD4 cells towards Th17.
- Initial cultures show co-expression of IL-2 and IL-17 that later become independent of each other.
- Detection of secreted cytokines (by CBA) correlated with the intracellular staining.
- Cytokine production by proliferating cells resulted in increased phosphorylation of the signal transducer Stat5.



Acknowledgments

- Jeanne Elia
- Xiao-Wei Wu
- Ravi Hingorani
- Jacob Rabenstein
- Erika O'Donnell



Questions

E-mail research applications at
ResearchApplications@bd.com

