

Simultaneous correlation of cytokine production with Treg and Th17 cell proliferation

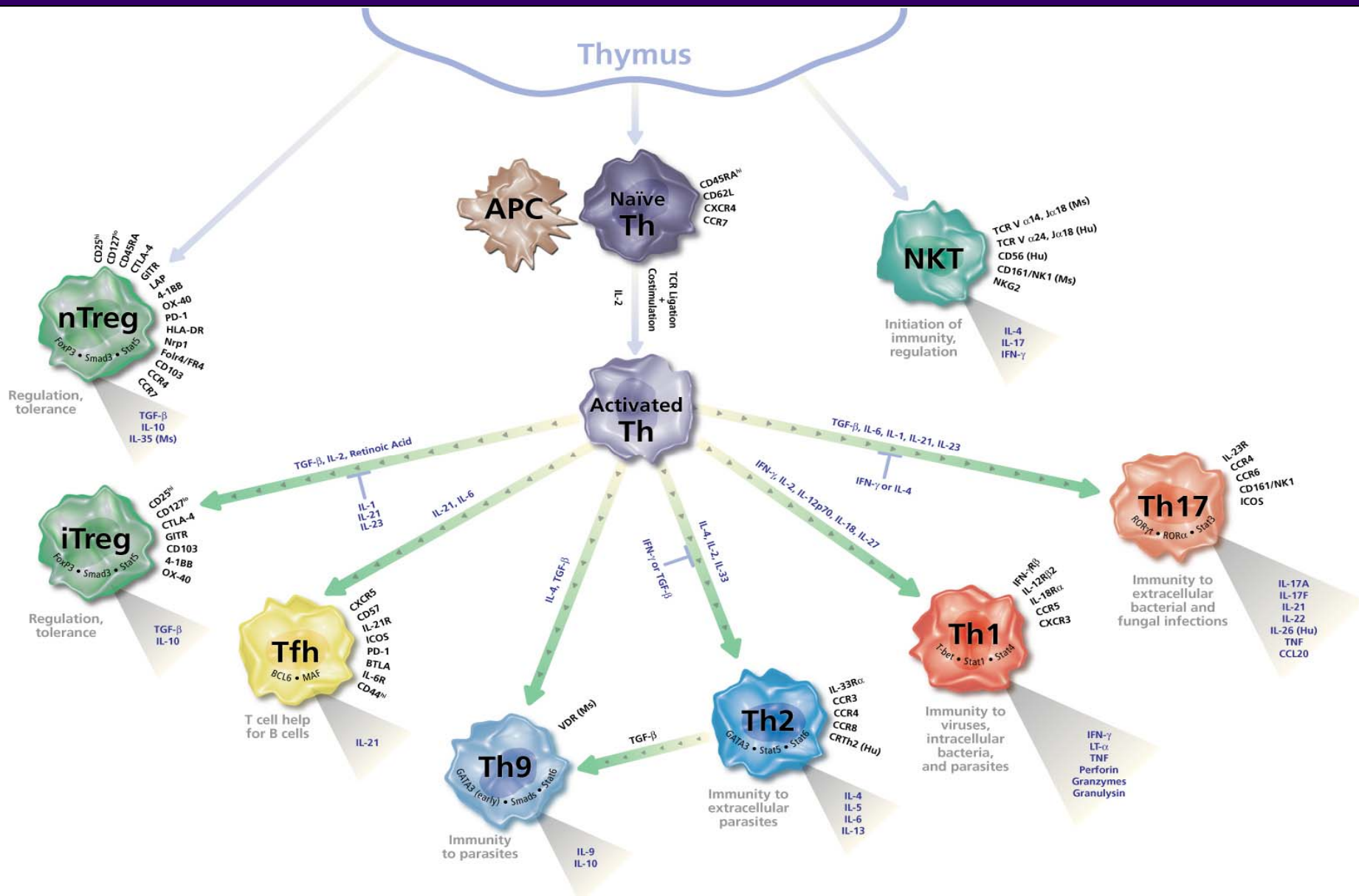
Jurg Rohrer, PhD
Director, R&D
BD Biosciences



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

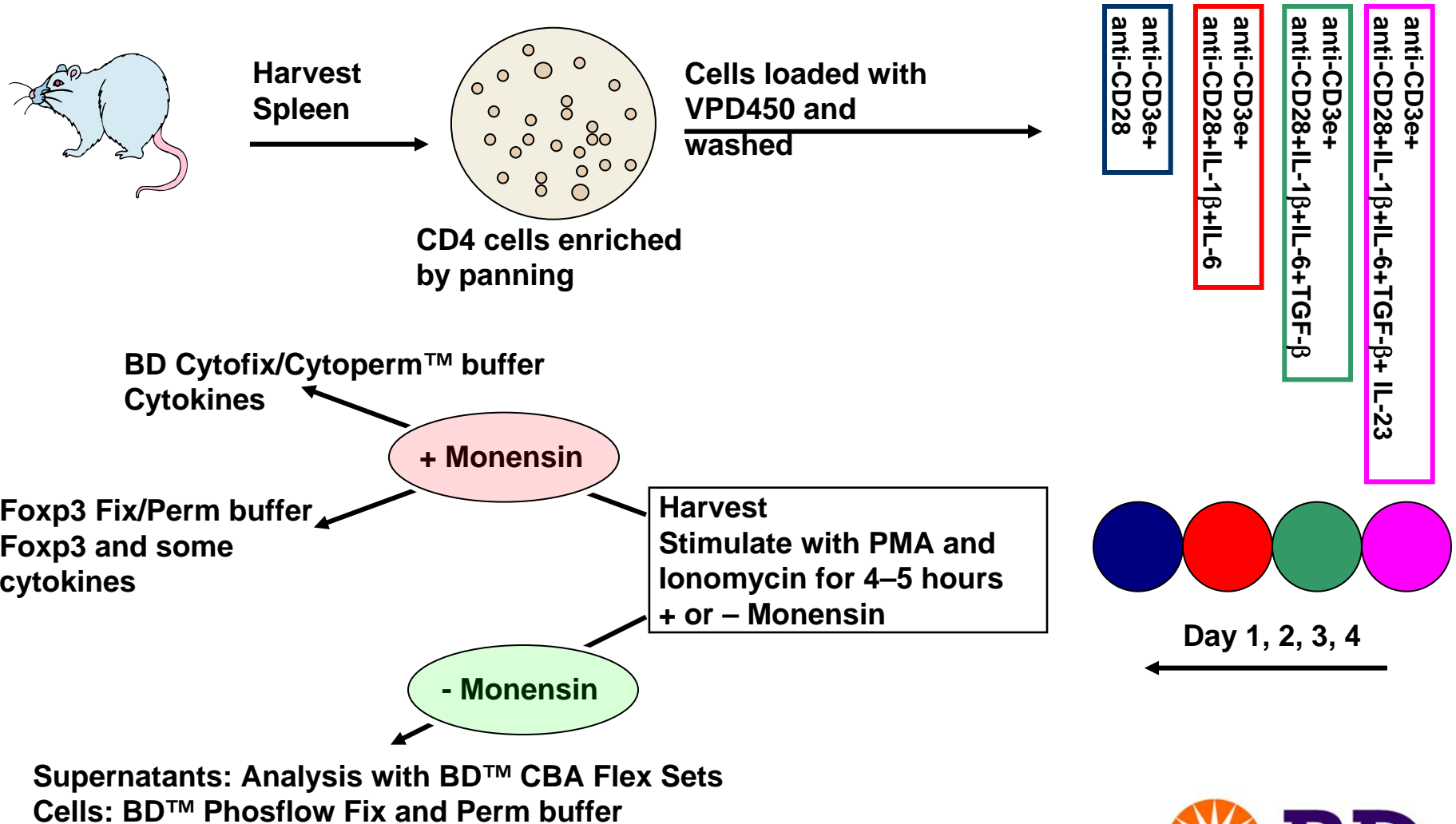


Experimental model

- 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

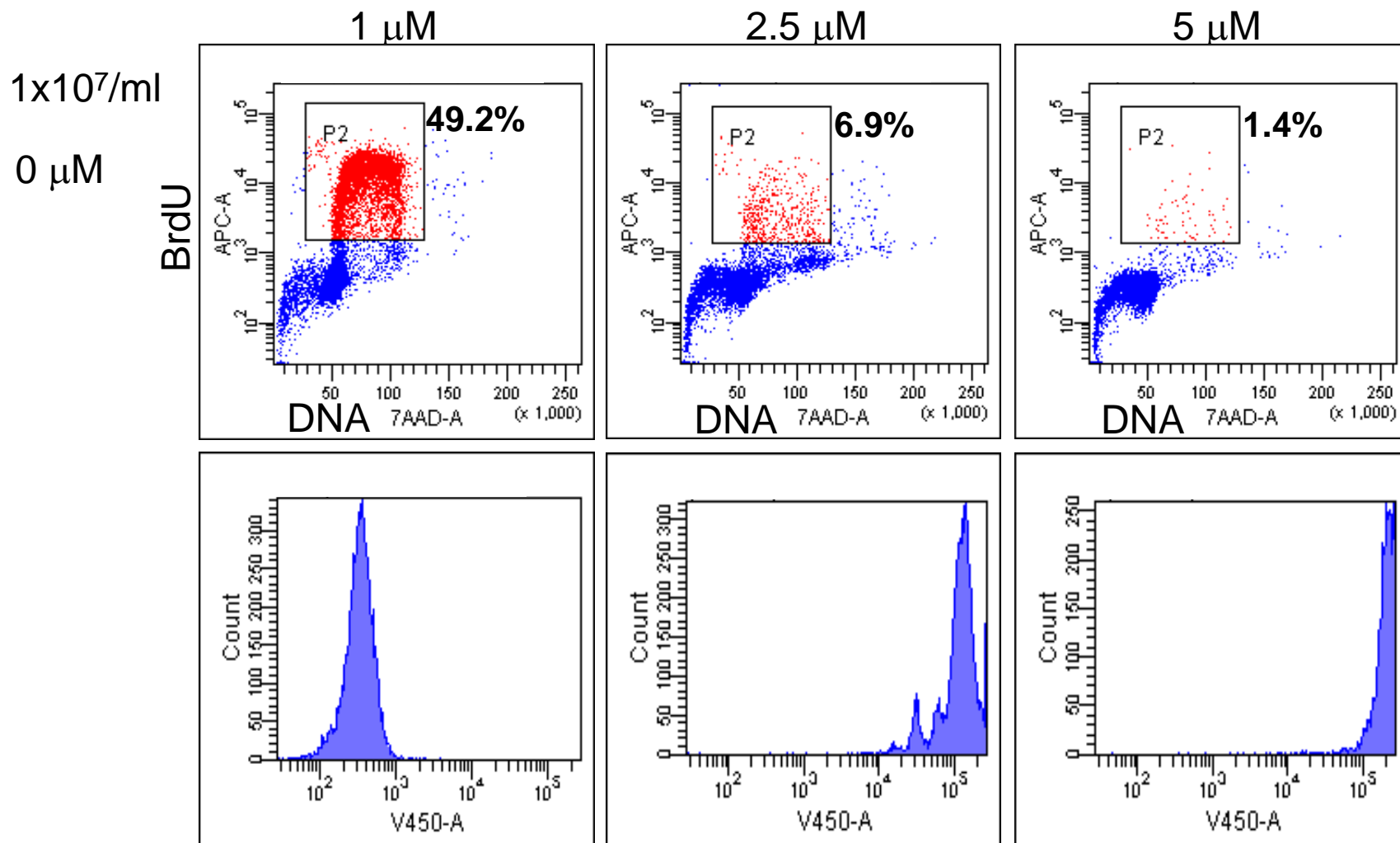
Reacts with cell
components to give
VPD450 adducts
retained inside cells



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]

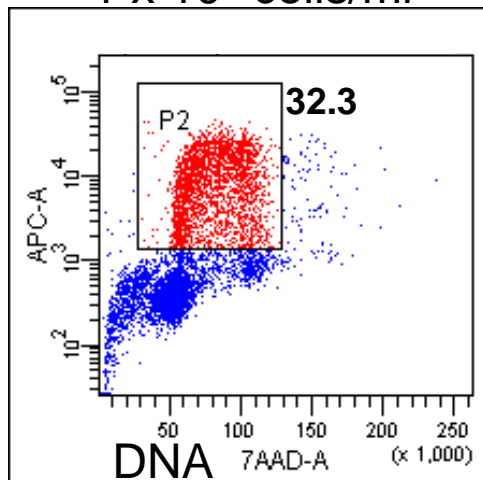


Spleen CD3/28 Day 2 - [Cell]

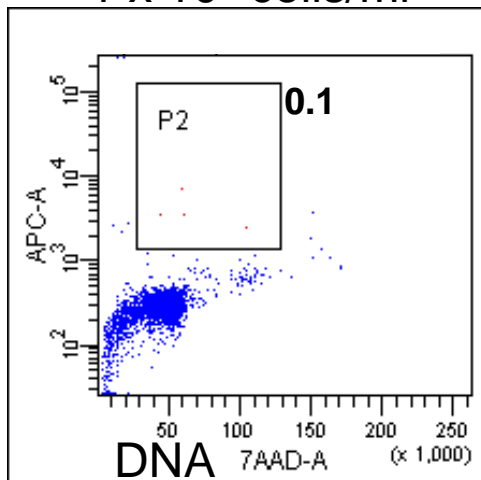
1 μ M

BrdU

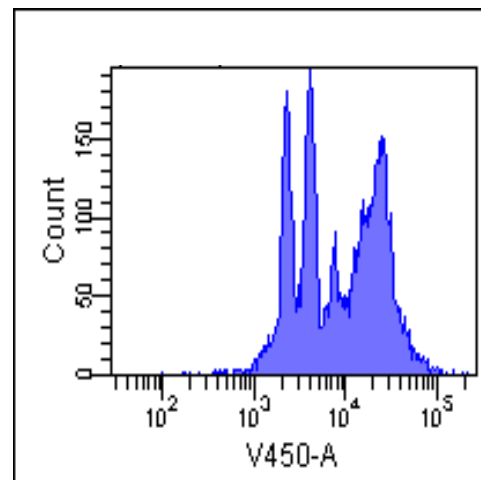
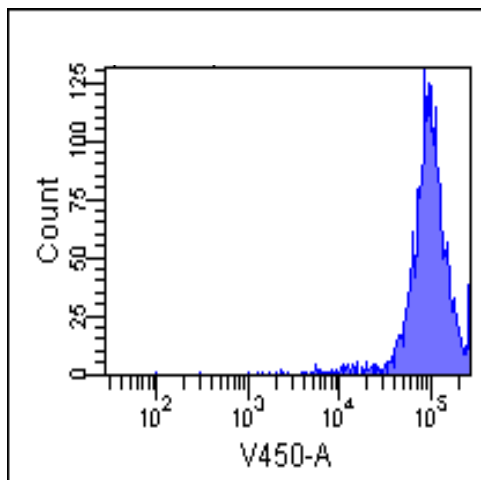
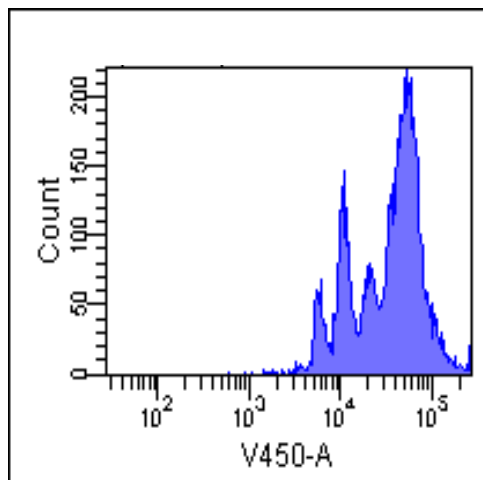
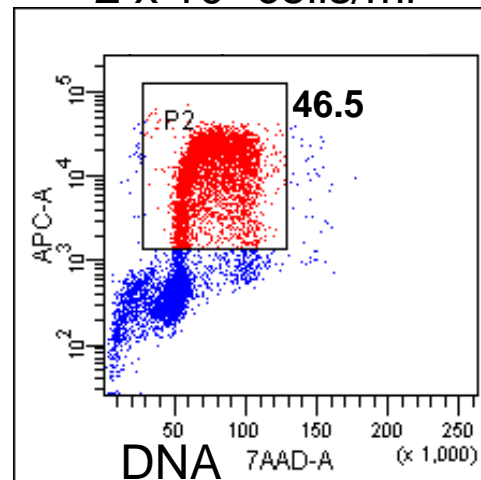
1 x 10⁷ cells/ml



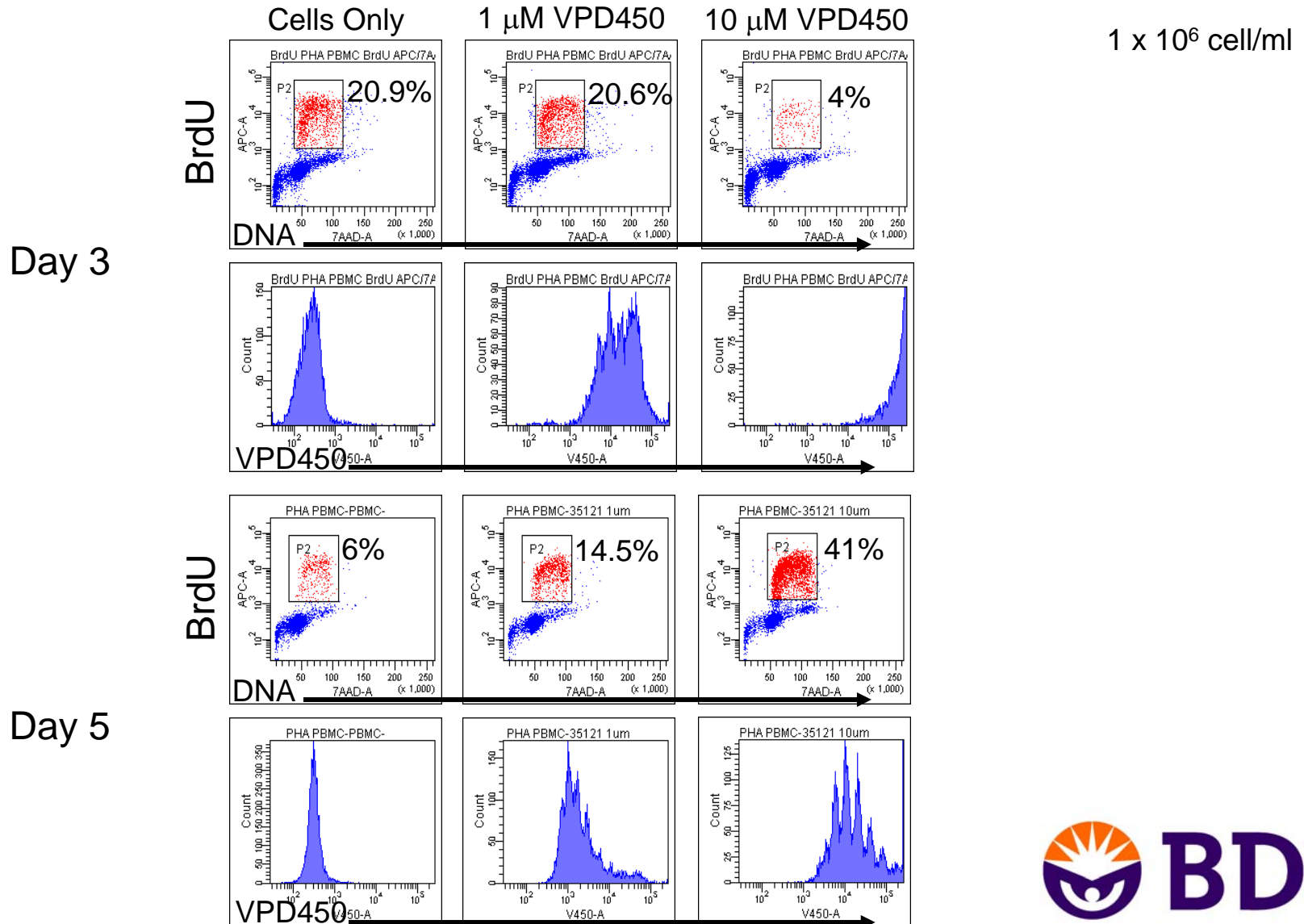
1 x 10⁶ cells/ml



2 x 10⁷ cells/ml



Human PBMC PHA Stimulation [VPD450]



VPD450 histograms

Condition:

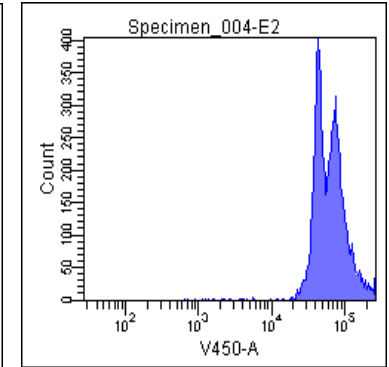
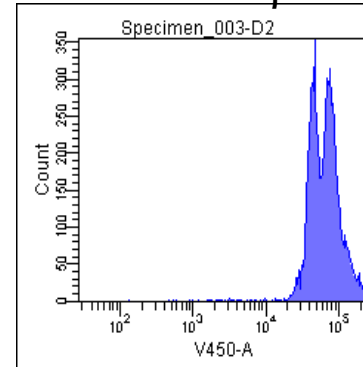
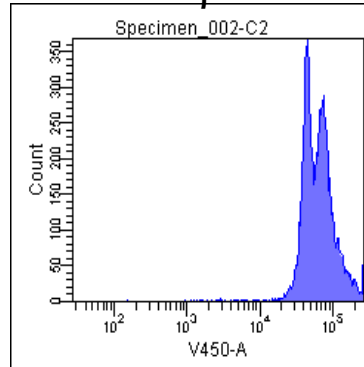
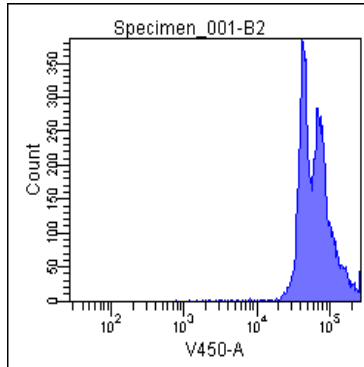
CD3/CD28

+IL-1 β /IL-6

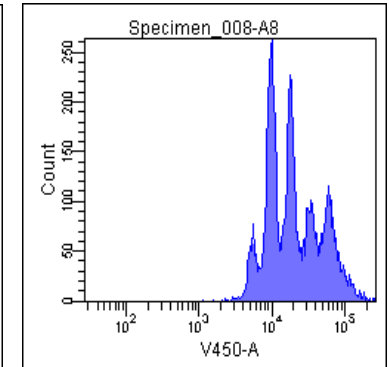
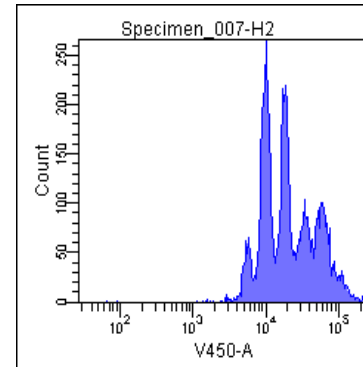
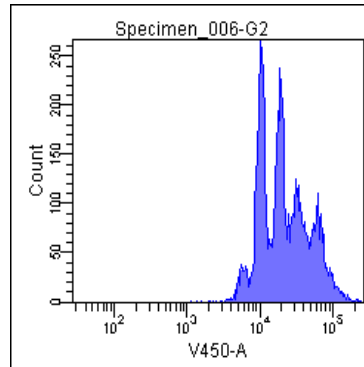
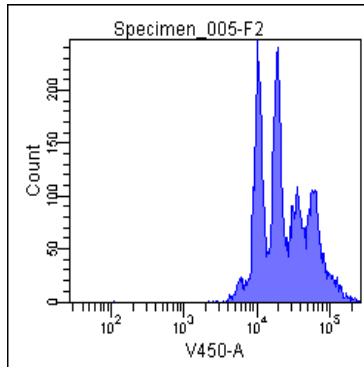
+TGF- β

+IL-23

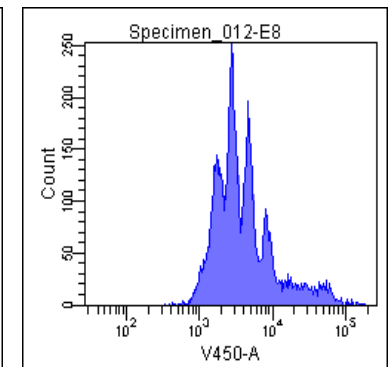
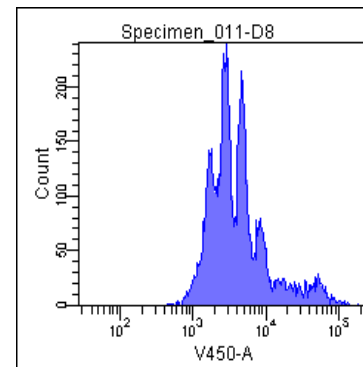
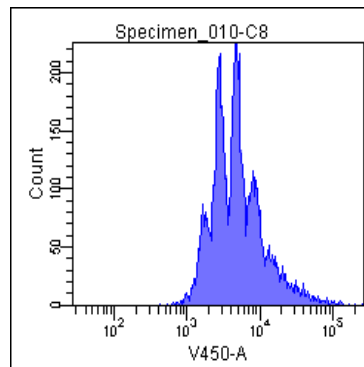
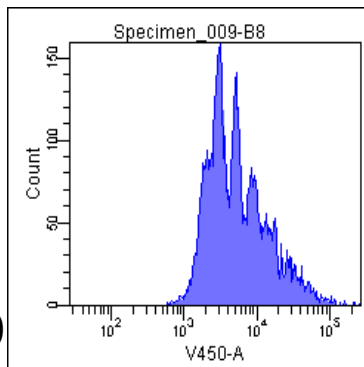
Day 1



Day 2



Day 3

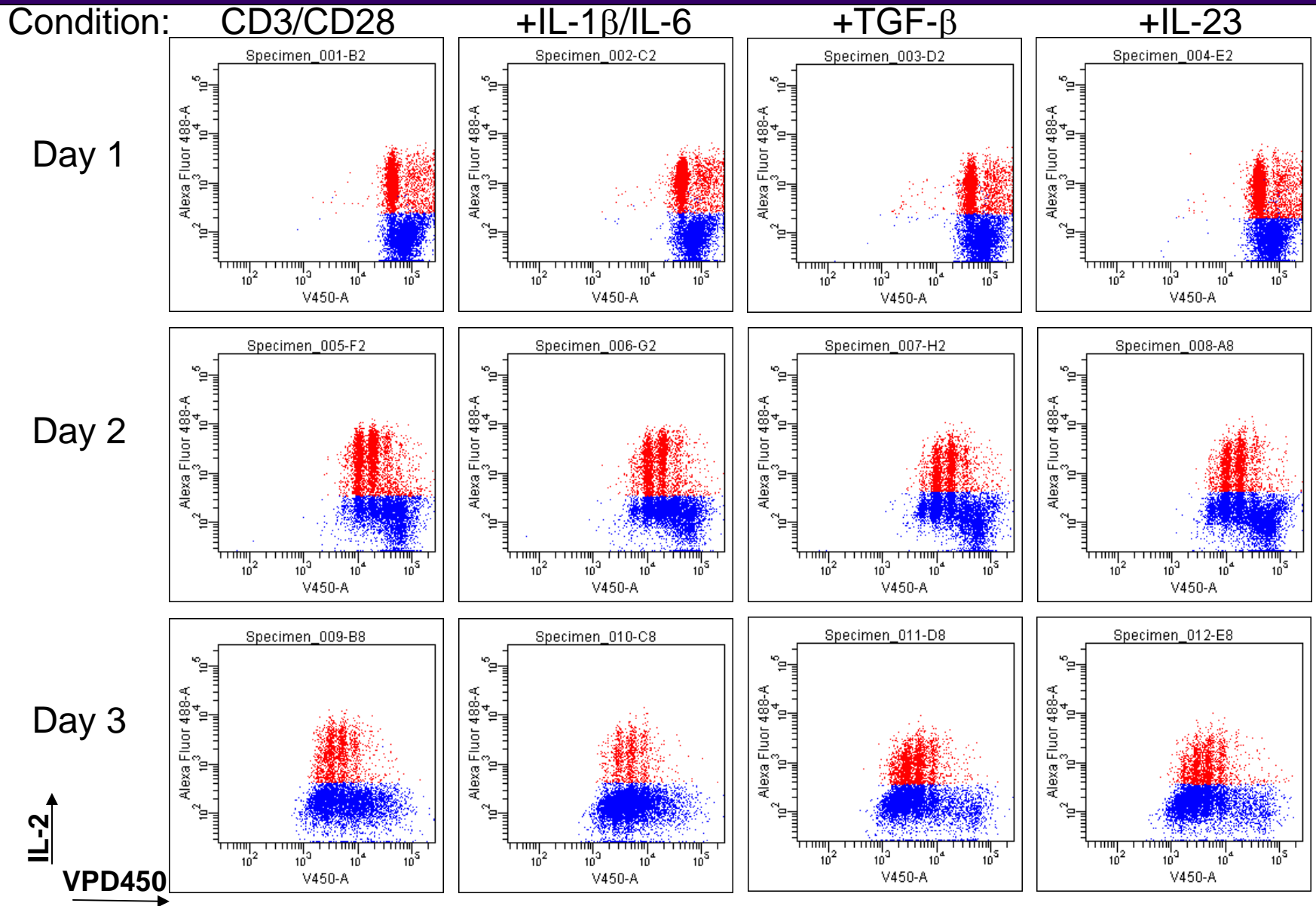


VPD450

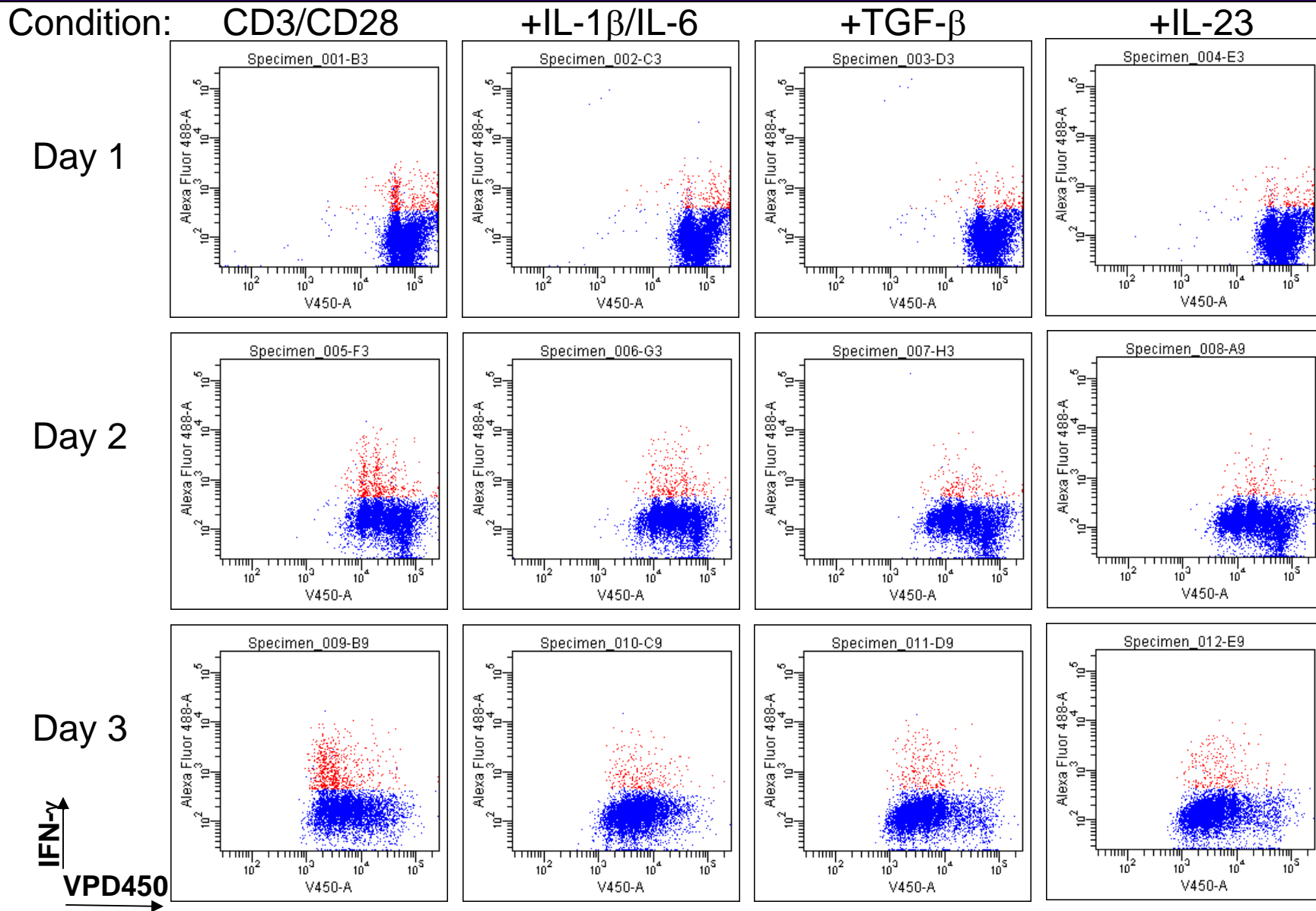
Which conditions for which cytokines

- 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?

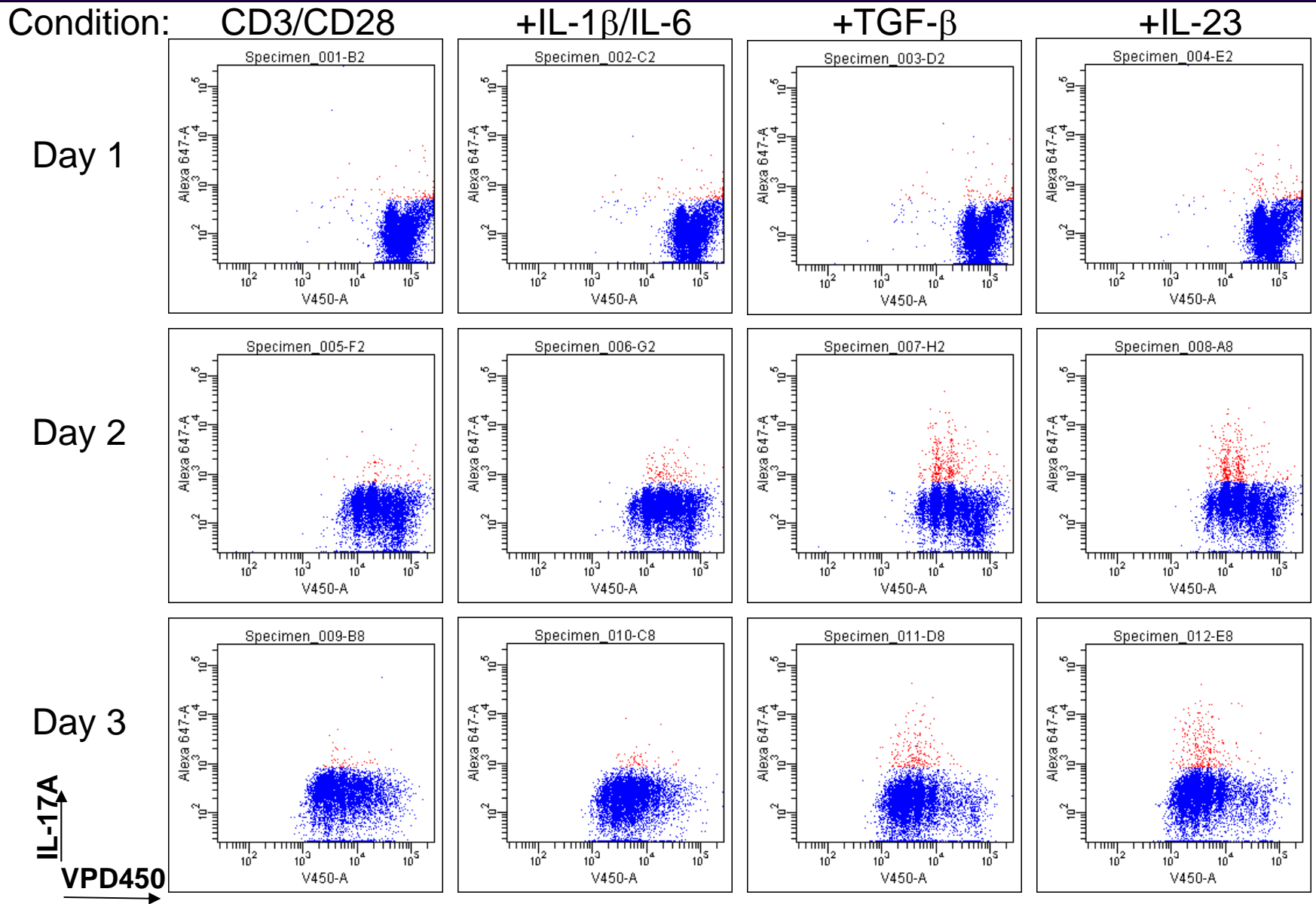
VPD450 vs IL-2 data



VPD450 vs IFN- γ data



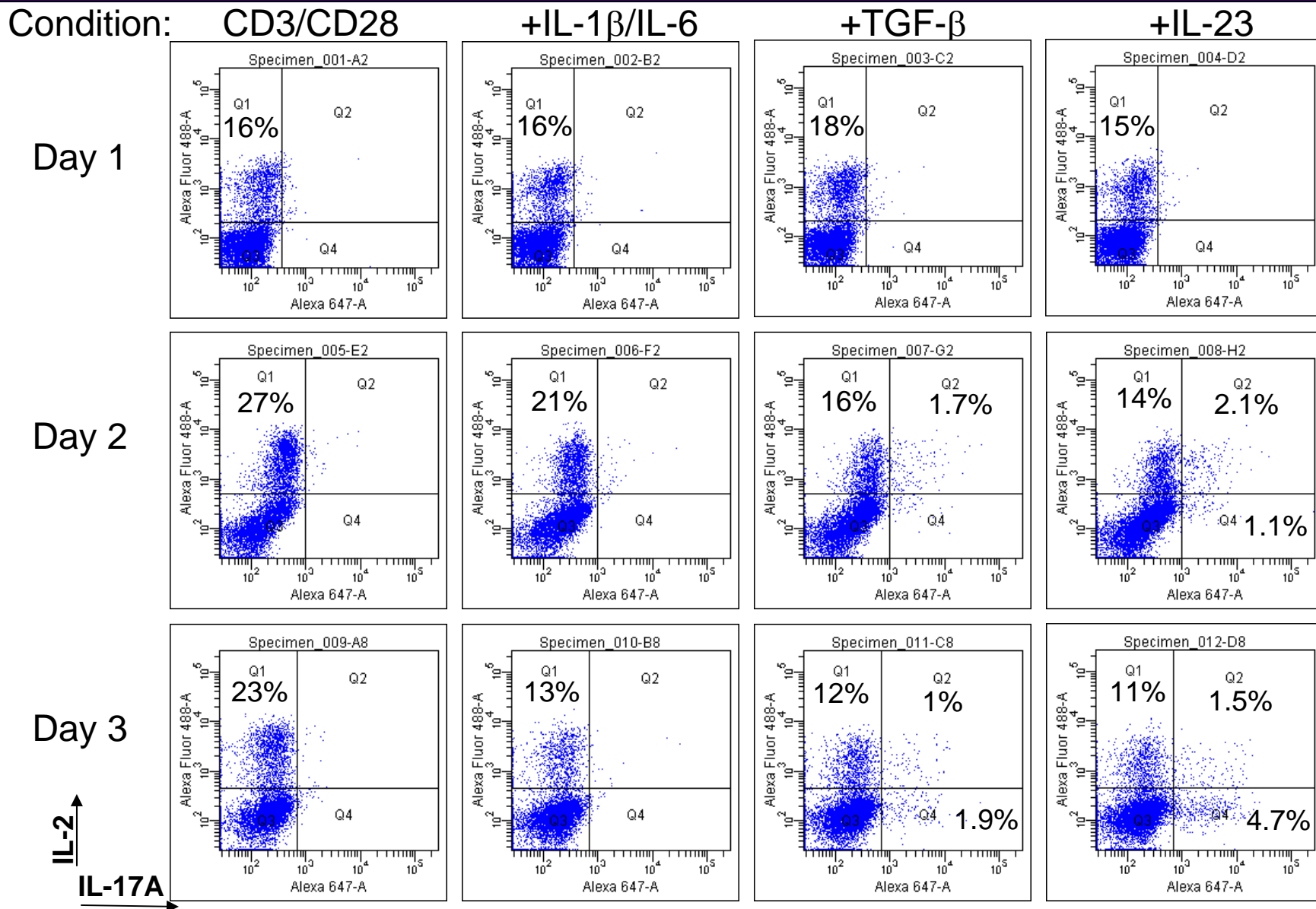
VPD450 vs IL-17A data



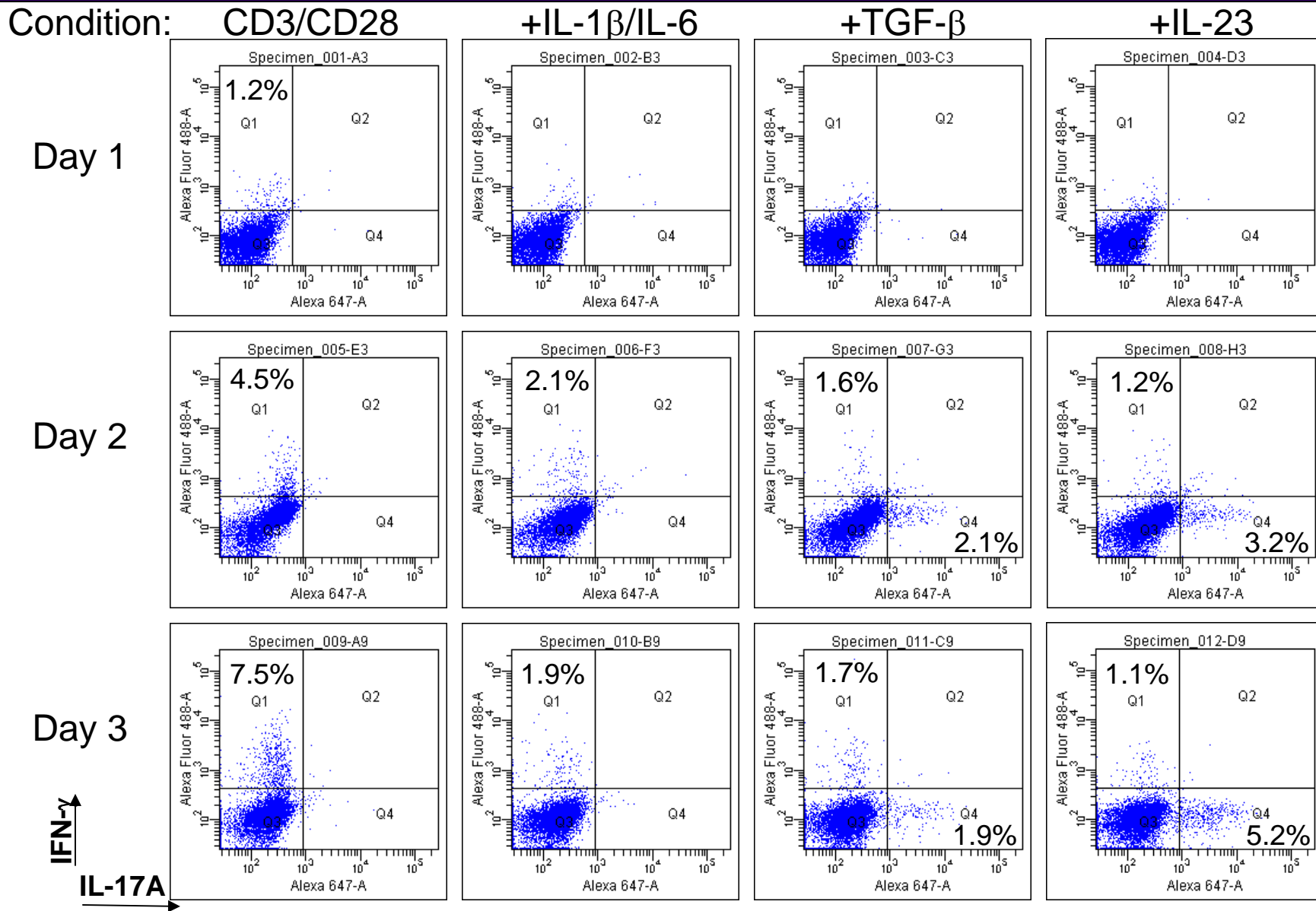
Cytokine co-expression

- 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?

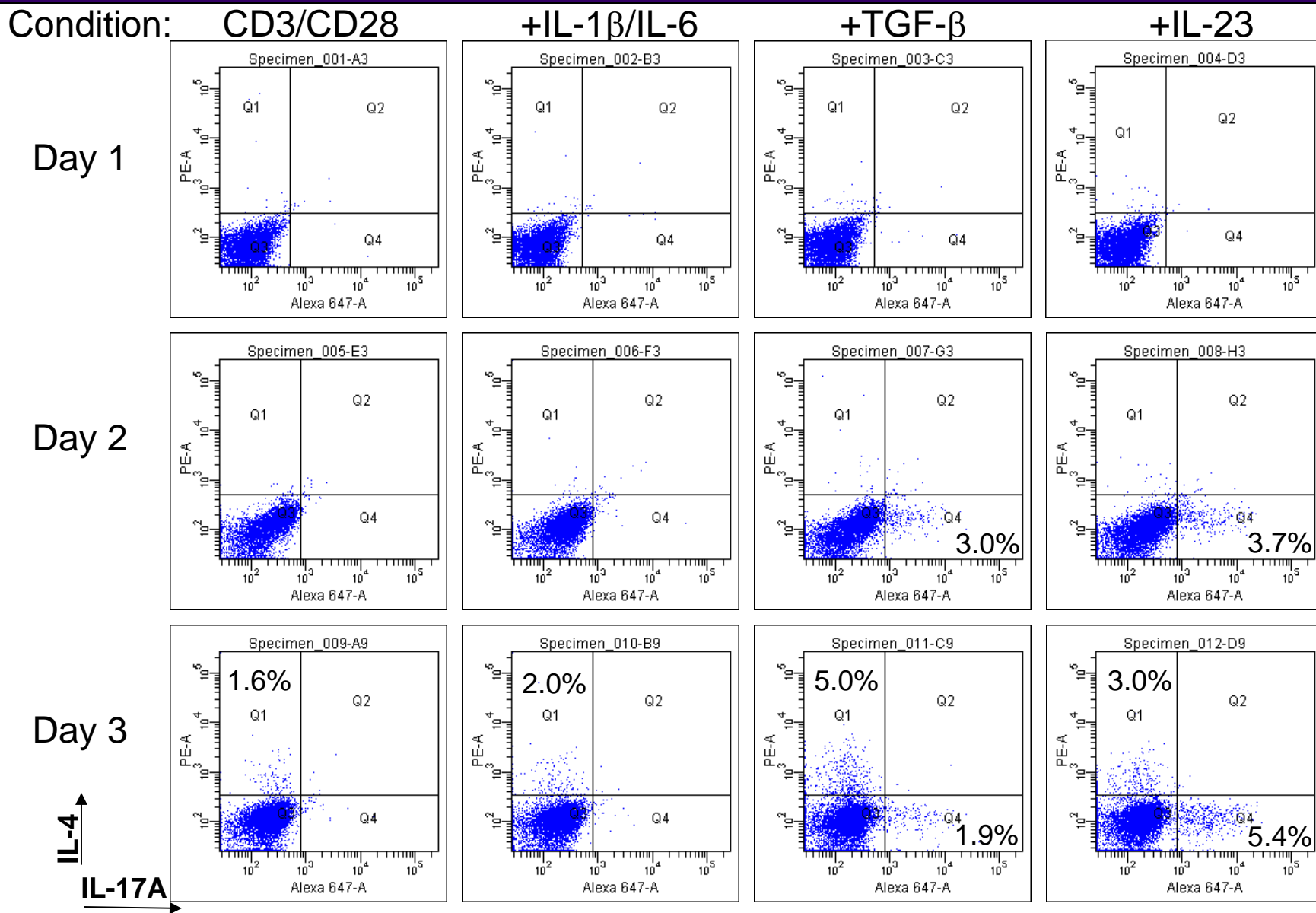
Co-expression of IL-17A vs IL-2



Co-expression of IL-17A vs IFN- γ



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

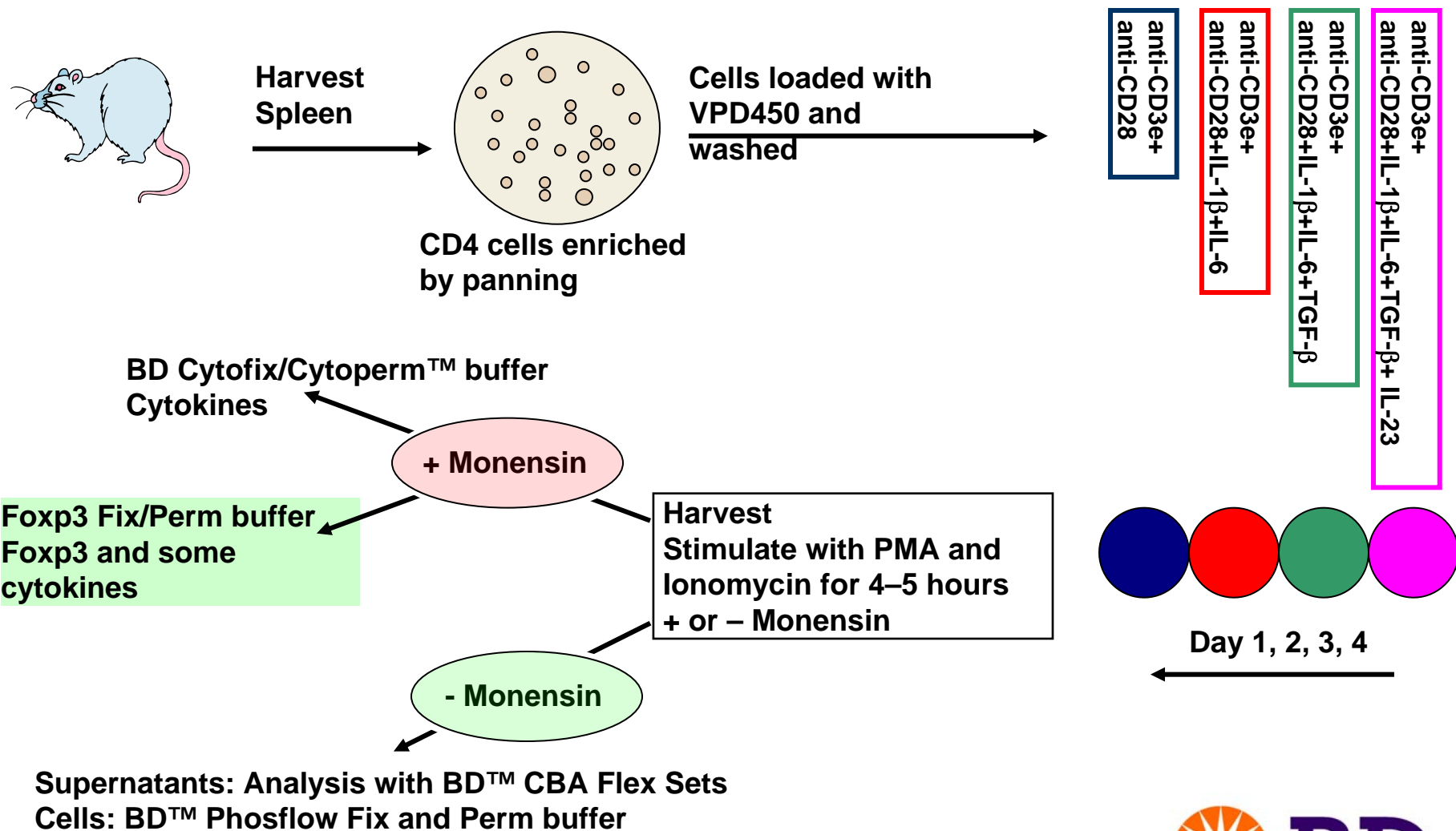


Tracking FoxP3

- 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?

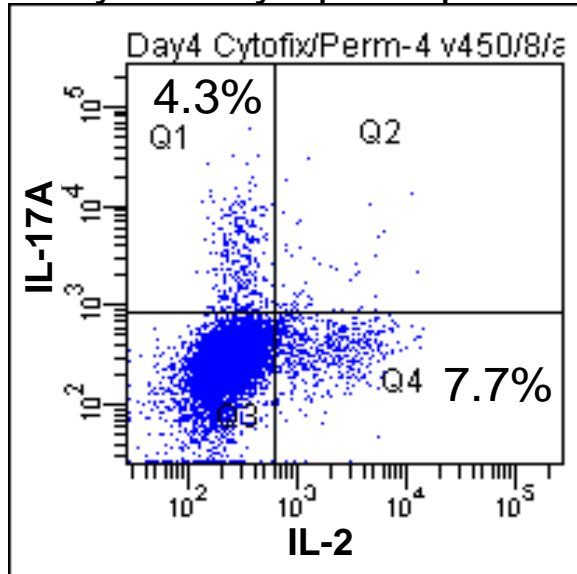


Experimental setup

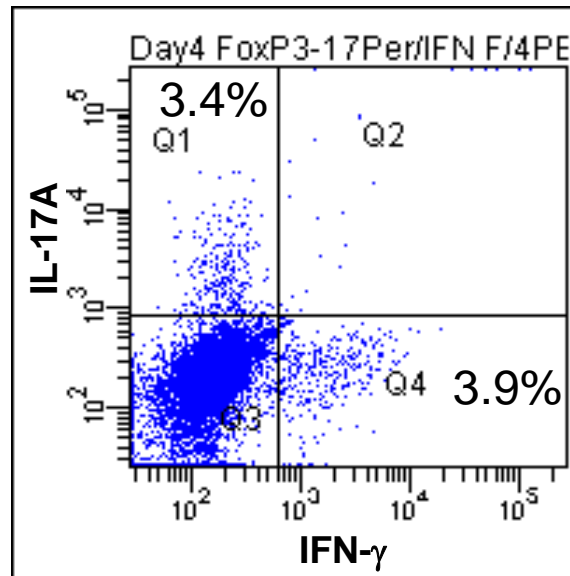
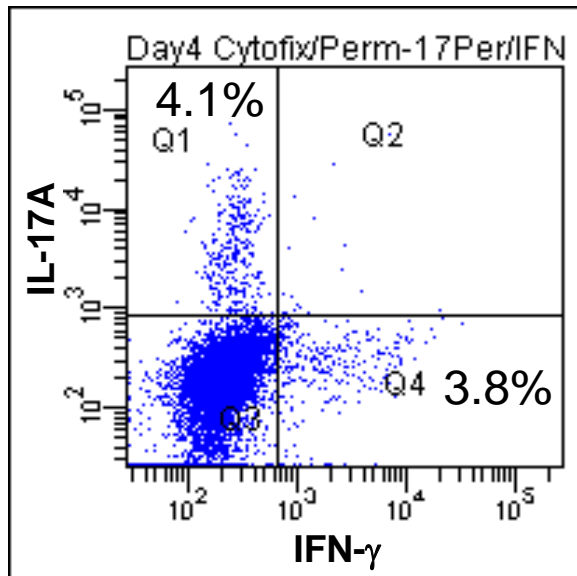
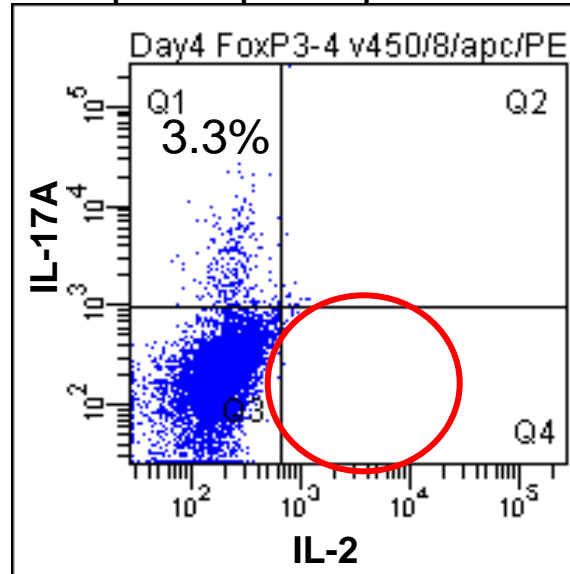


Comparison of two fix/perm protocols

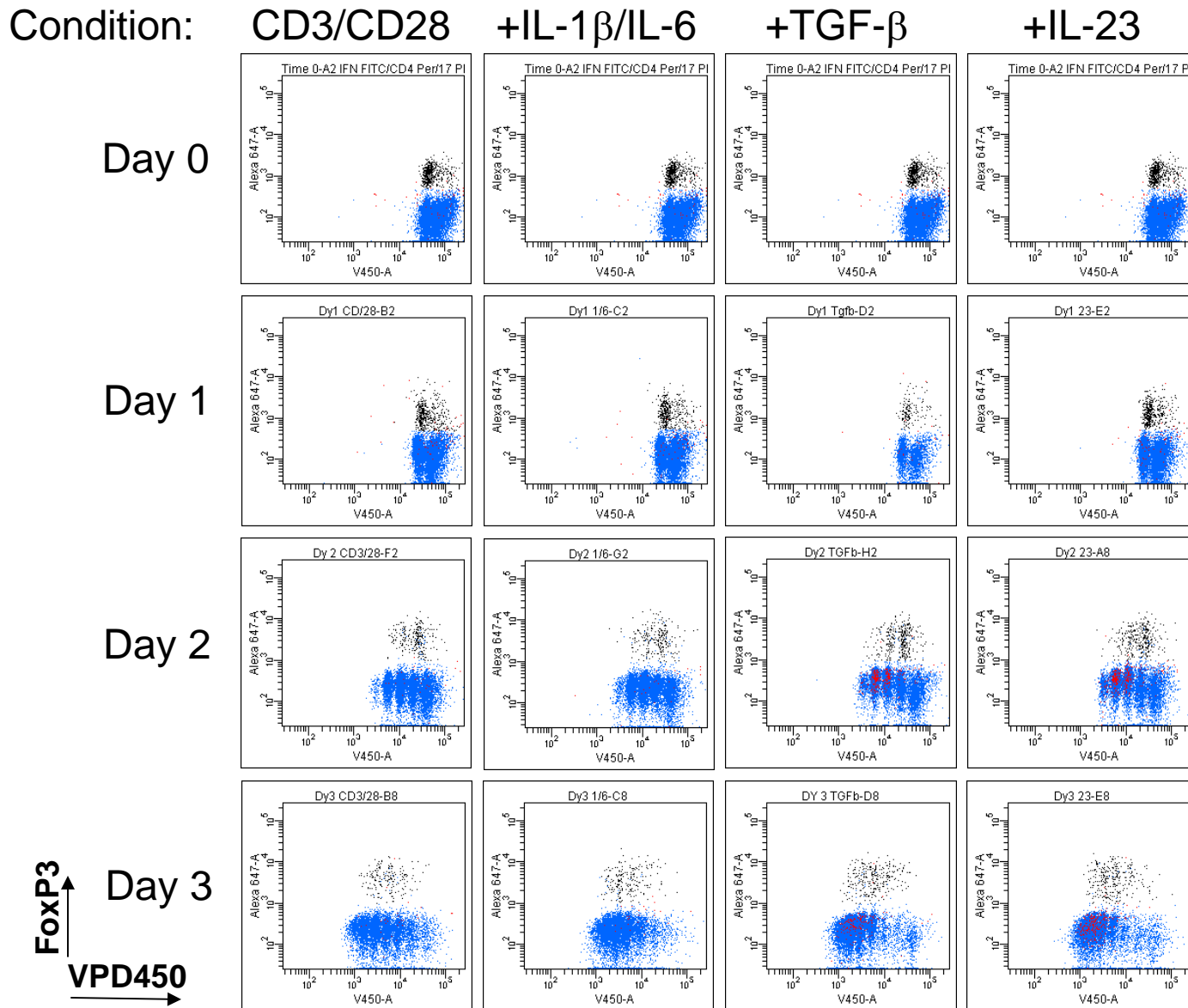
BD Cytfix/Cytoperm protocol



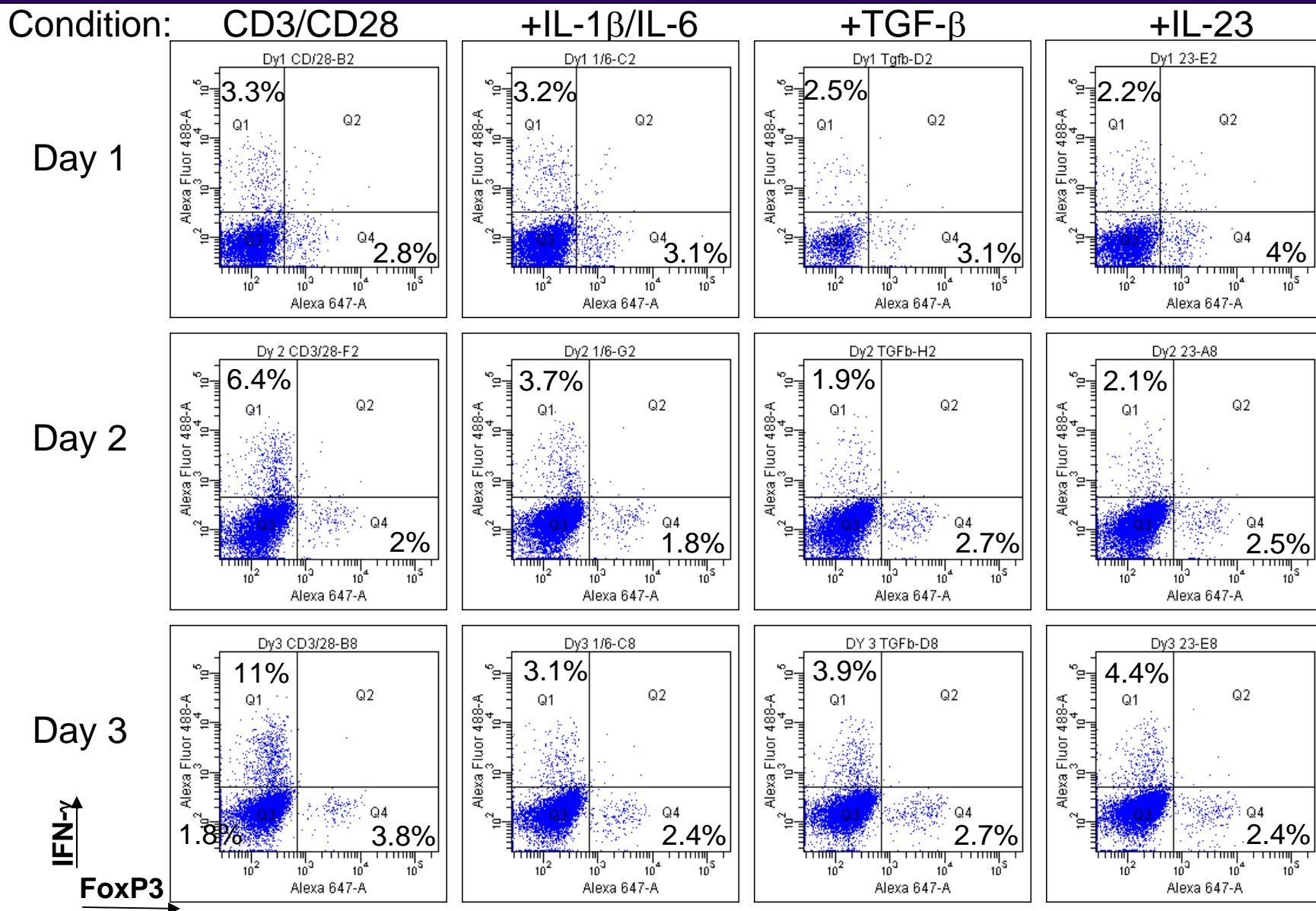
Foxp3 fix/perm protocol



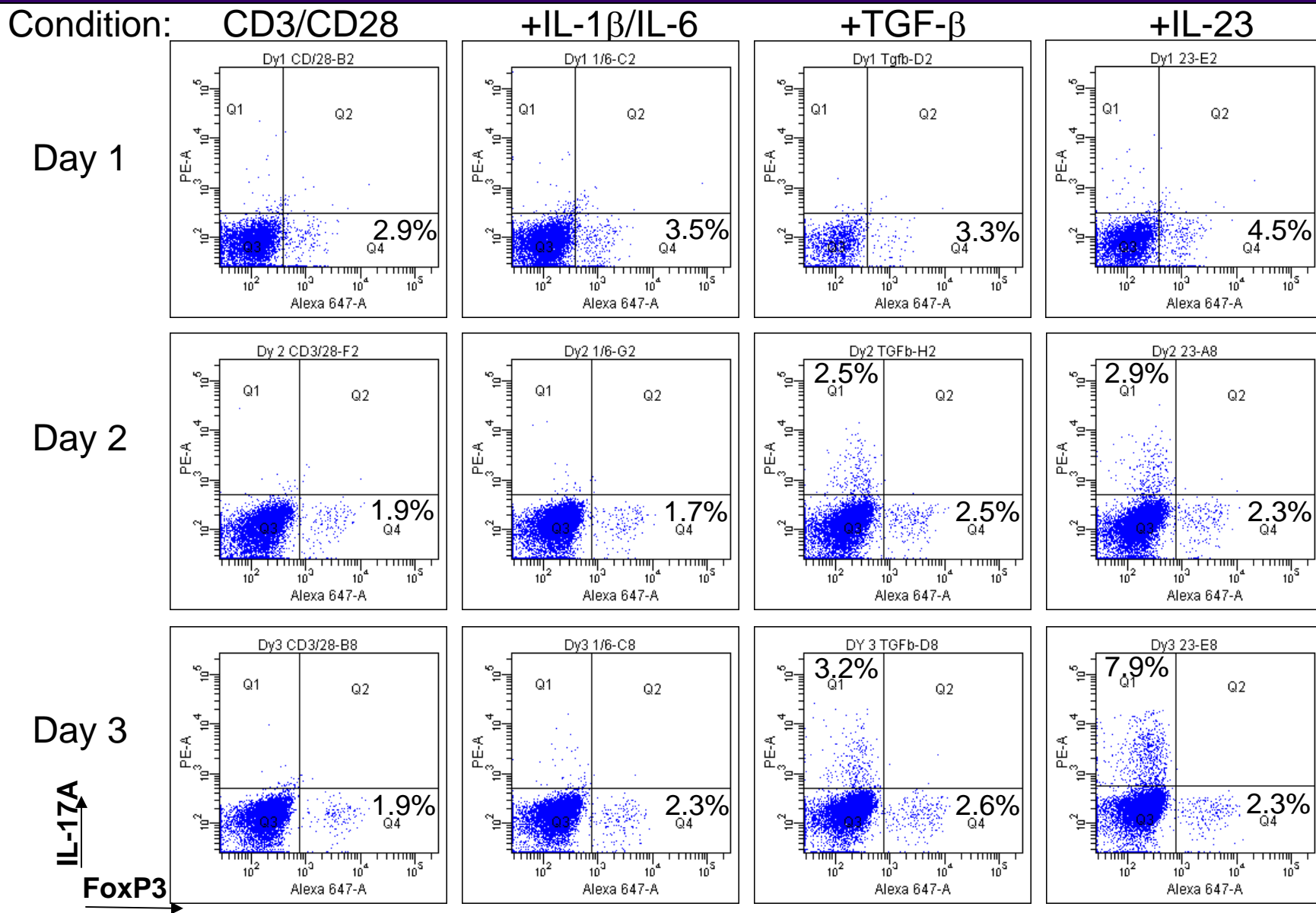
Proliferation of Treg and Th17 cells



Co-expression of *Foxp3* vs *IFN- γ*



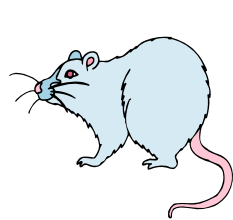
Co-expression of Foxp3 vs IL-17A



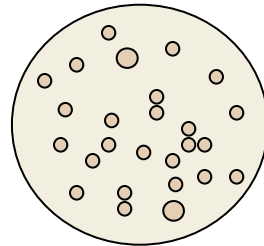
Cytokines in culture supernatants

- 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

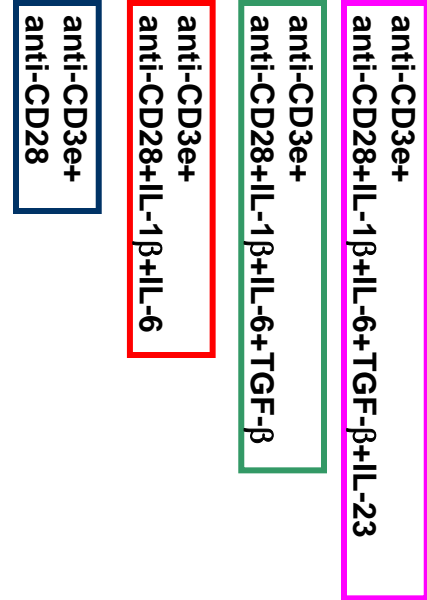


Harvest spleen



CD4 cells enriched by panning

Cells loaded with VPD450 and washed



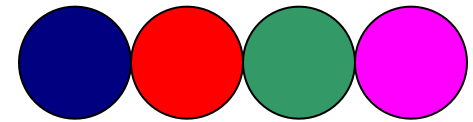
BD Cytofix/Cytoperm buffer
Cytokines

+ Monensin

Foxp3 Fix/Perm buffer
Foxp3 and some cytokines

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

- Monensin

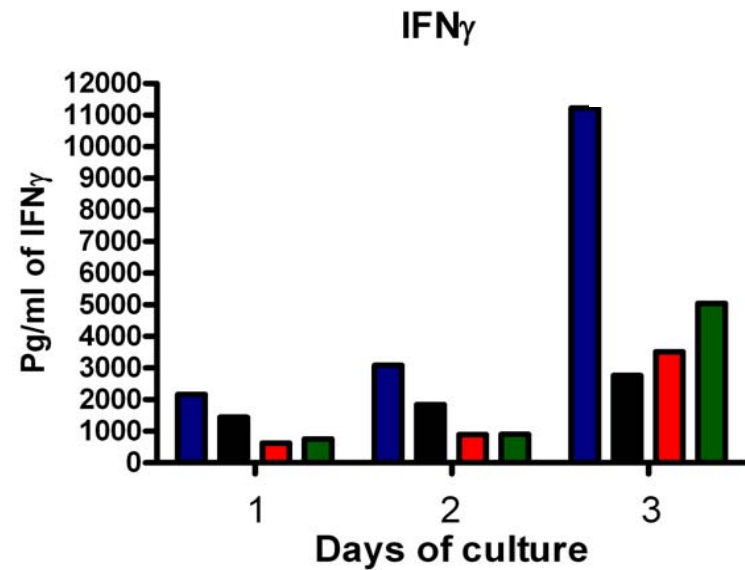
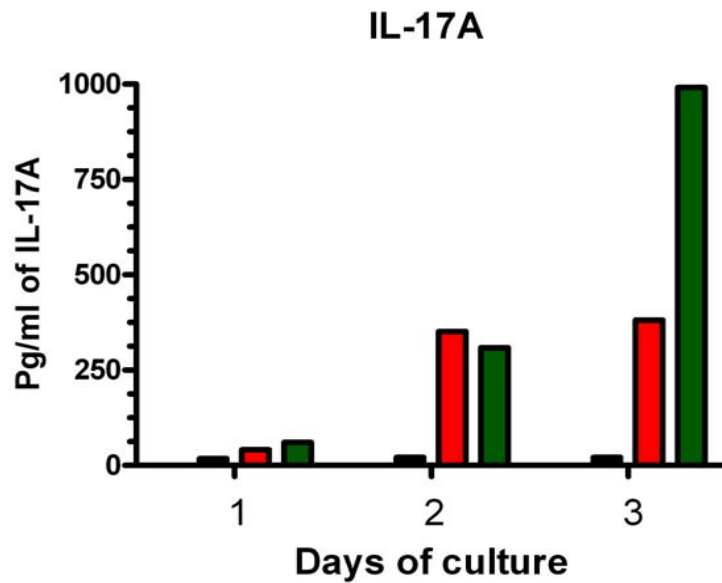


Day 1, 2, 3, 4

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

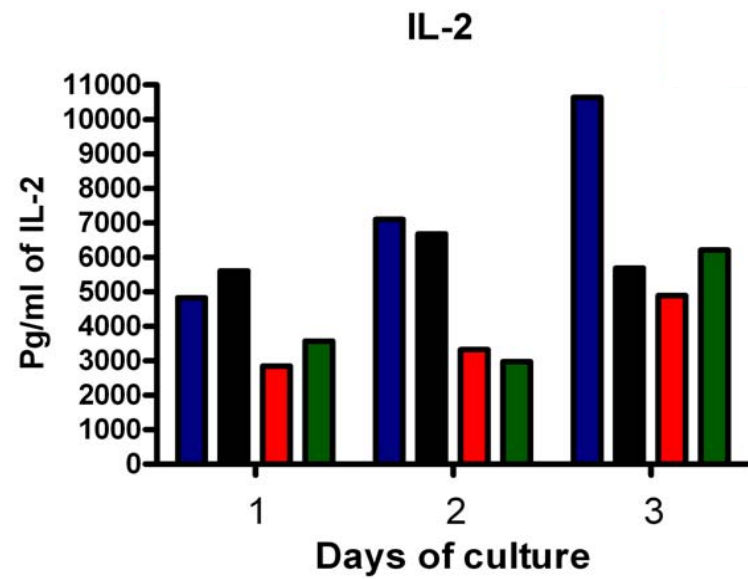
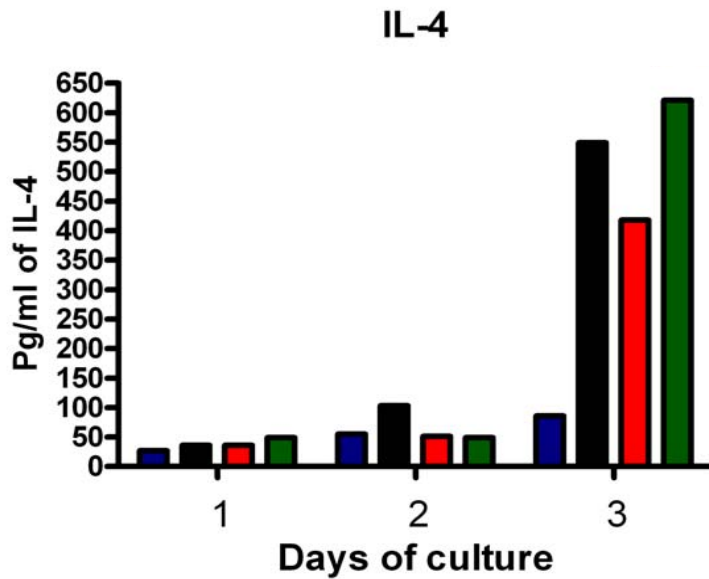


IL-17A and IFN- γ production



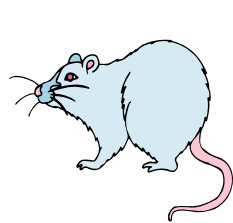
- CD3 /CD28
- CD3 /CD28/IL1 β /IL-6
- CD3 /CD28/IL1 β /IL-6/TGF β
- CD3 /CD28/IL1 β /IL-6/TGF β /IL-23

IL-4 and IL-2 production

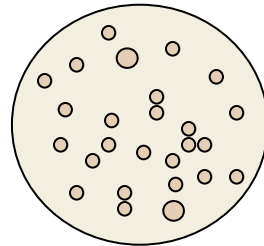


- CD3 /CD28
- CD3 /CD28/IL1 β /IL-6
- CD3 /CD28/IL1 β /IL-6/TGF β
- CD3 /CD28/IL1 β /IL-6/TGF β /IL-23

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

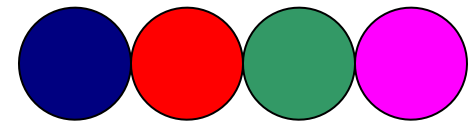
BD Cytofix/Cytoperm buffer
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Harvest
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+ or – Monensin

- Monensin



Day 1, 2, 3, 4

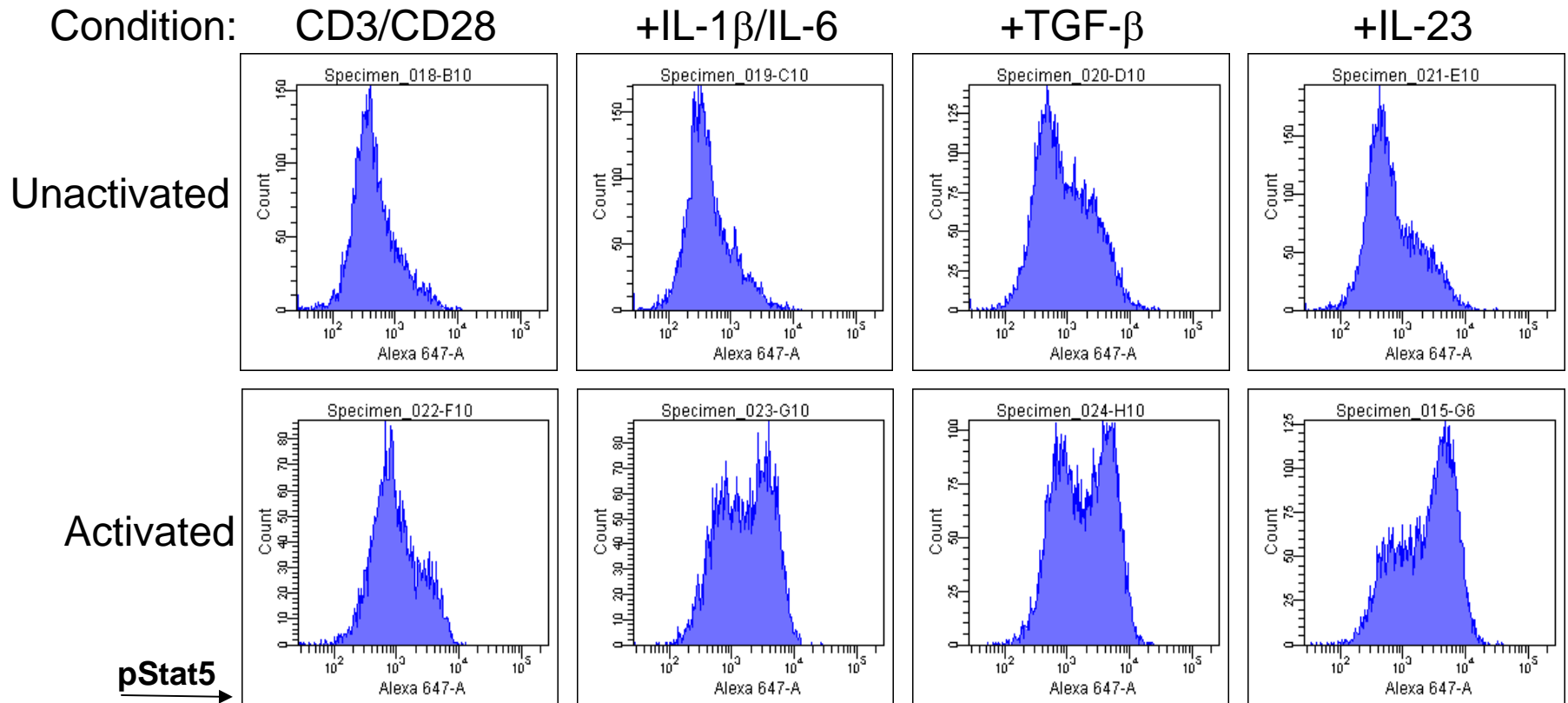
Supernatants: Analysis with BD CBA Flex Sets
Cells: BD Phosflow Fix and Perm buffer



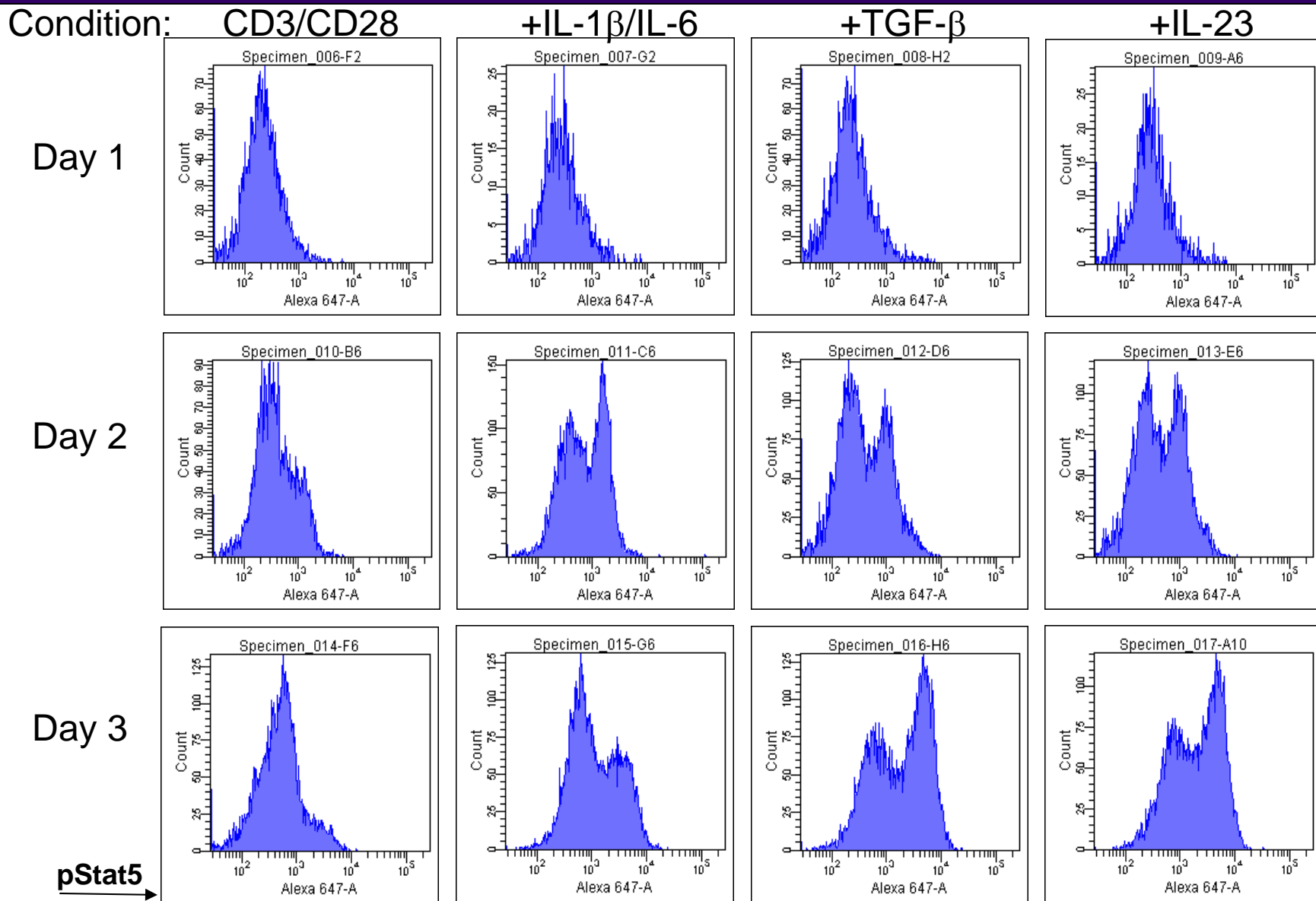
pStat5 detection on day 4

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

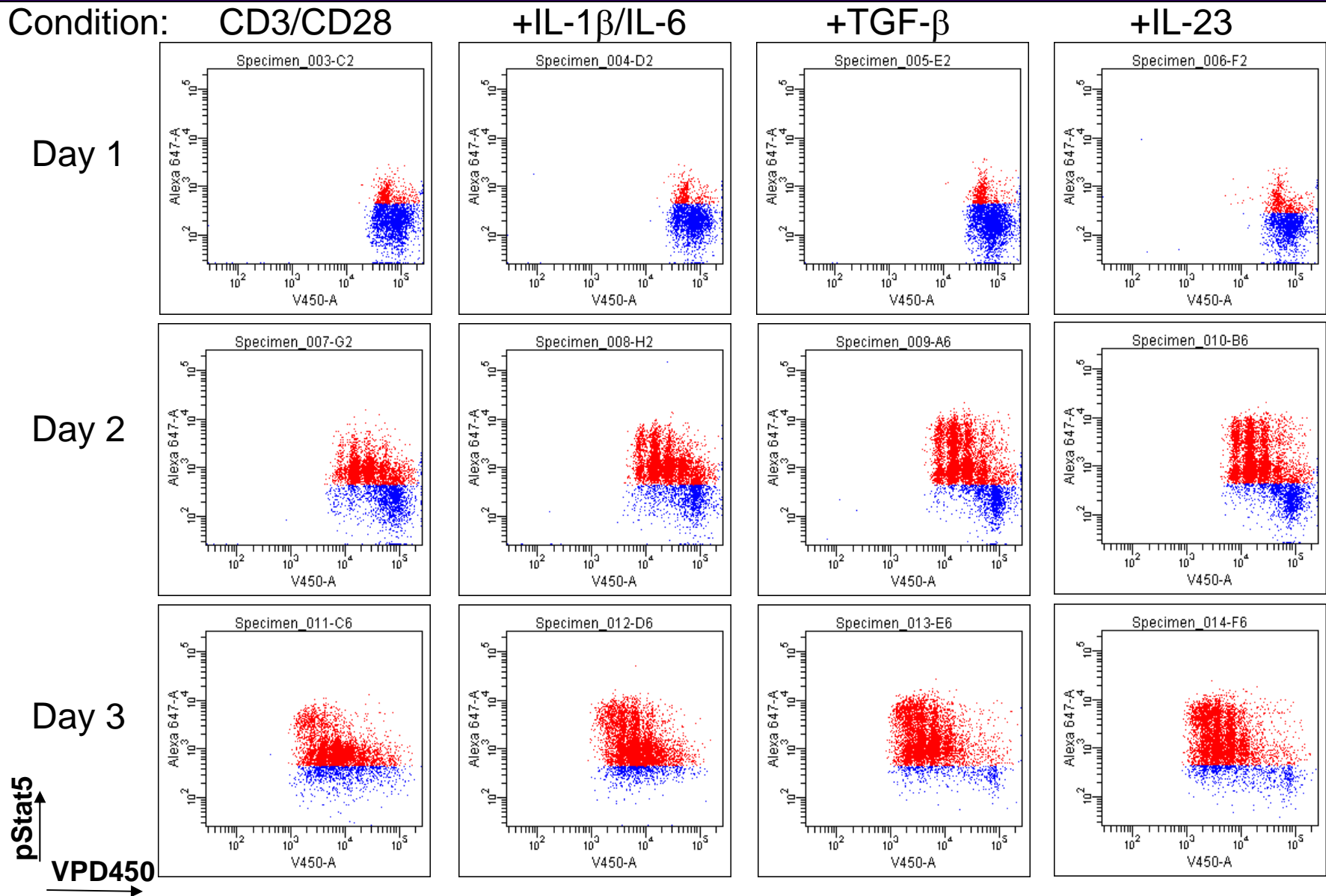
Activated: Cells were cultured and activated with PMA/Ionomycin for 5 hours and then stained with phospho-specific Stat5 antibody.



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

