

# *Simultaneous correlation of cytokine production with Treg and Th17 cell proliferation*

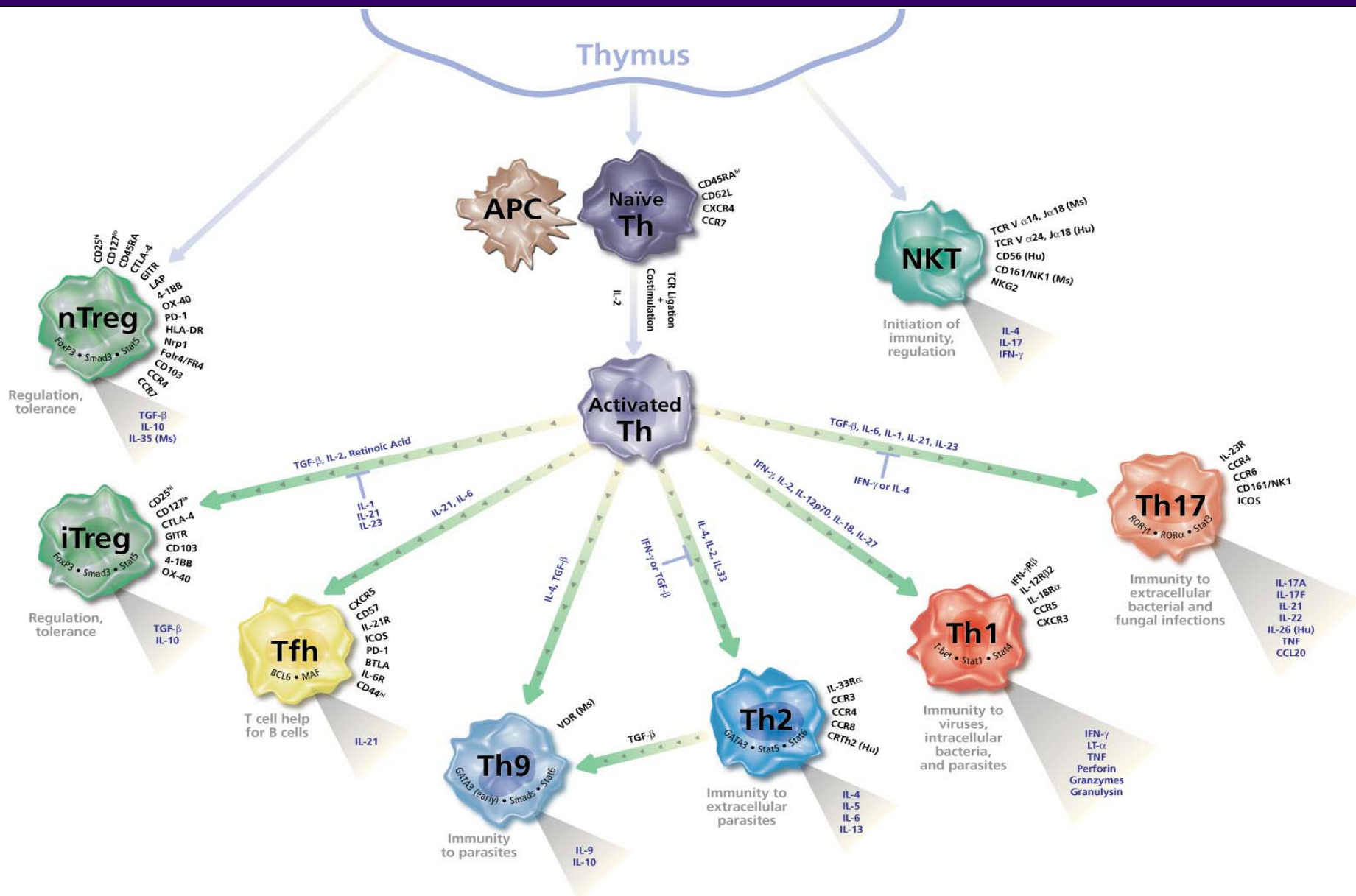
Jurg Rohrer, PhD  
Director, R&D  
BD Biosciences



# *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- $\beta$ , IL-6, IL-21, IL-1 $\beta$ , 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 $\beta$ , IL-2 and RA
- Produce TGF $\beta$  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

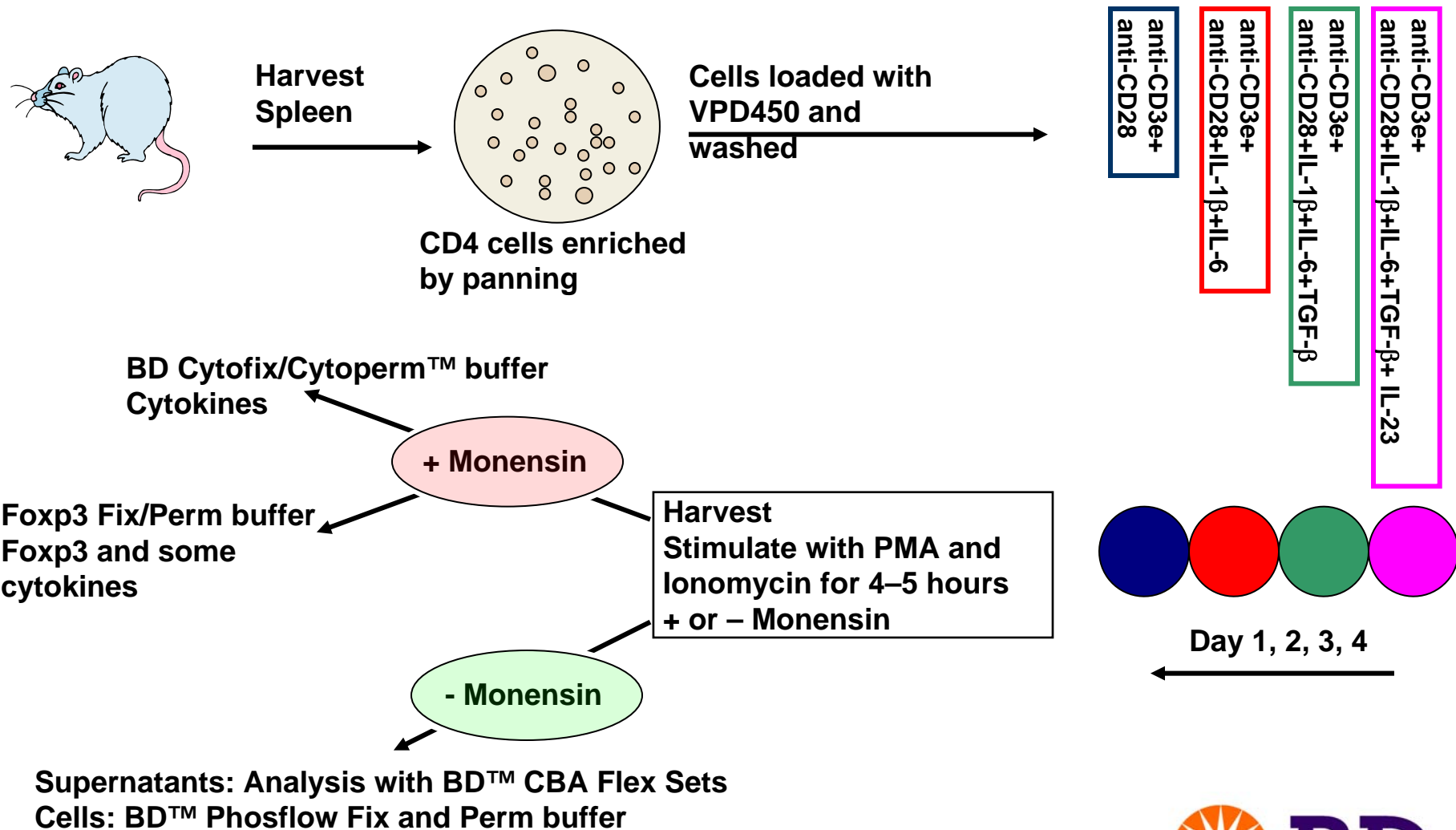


# *Experimental model*

- Enrich Balb/c splenocytes by positive selection via CD4<sup>+</sup> panning
- Load isolated cells with VPD450 1 $\mu$ M, 10 minutes
- Set up cultures as follows:
  - CD3/CD28
  - CD3/CD28/IL-6/IL-1 $\beta$
  - CD3/CD28/IL-6/IL-1 $\beta$ /TGF $\beta$
  - CD3/CD28/IL-6/IL-1 $\beta$ /TGF $\beta$ /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- $\gamma$  (IFN- $\gamma$ )



# Experimental setup



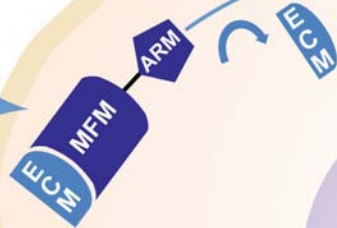
# Fluorescein Diacetate Derivative

## VPD450 Dye



Non-Fluorescent

Enters cells, esterases cleave ECM to give fluorescent product



Reacts with cell components to give VPD450 adducts retained inside cells

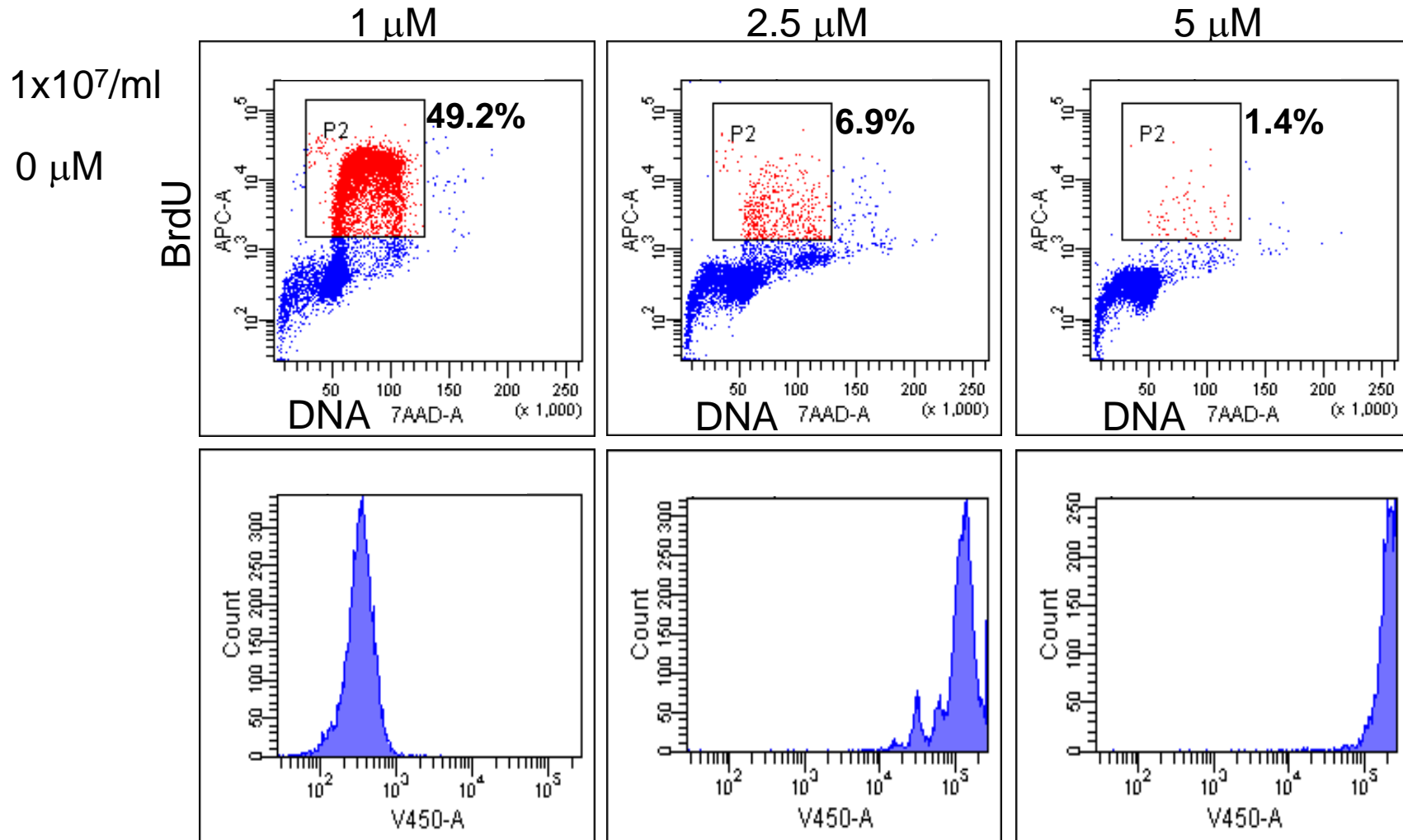


Fluorescent and Cell-retained

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



# Spleen CD3/28 Day 2 - [VPD450]

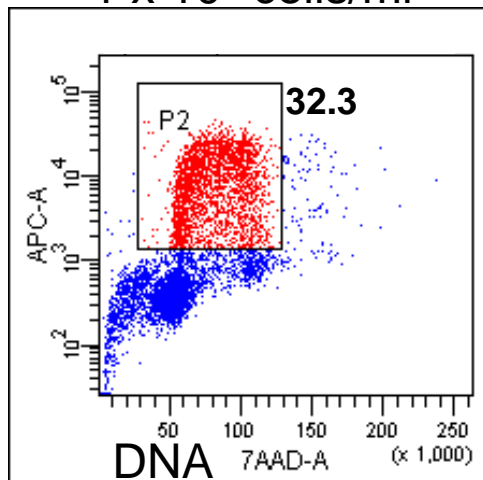


# Spleen CD3/28 Day 2 - [Cell]

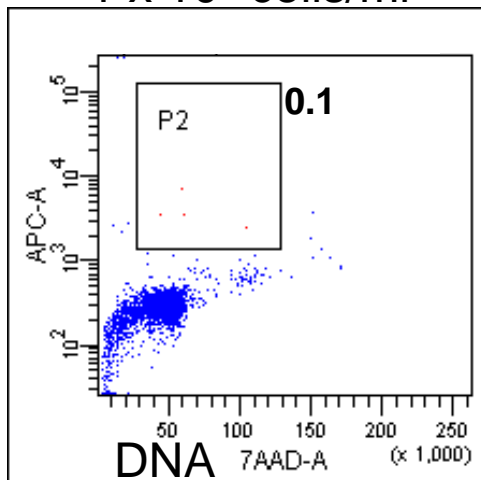
1  $\mu$ M

BrdU

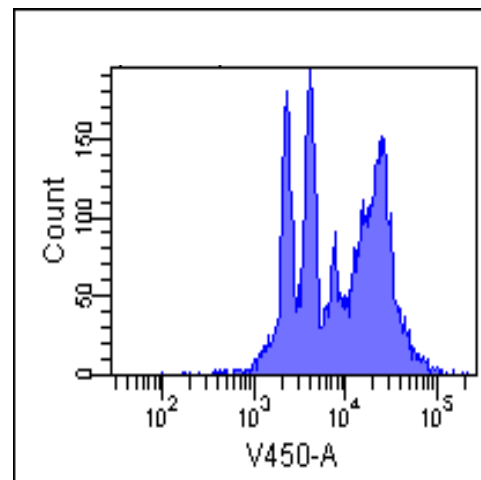
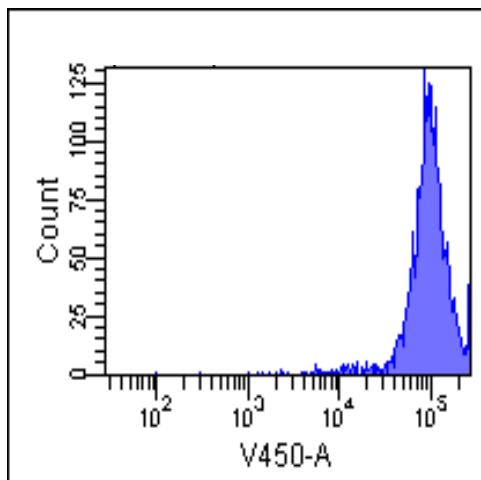
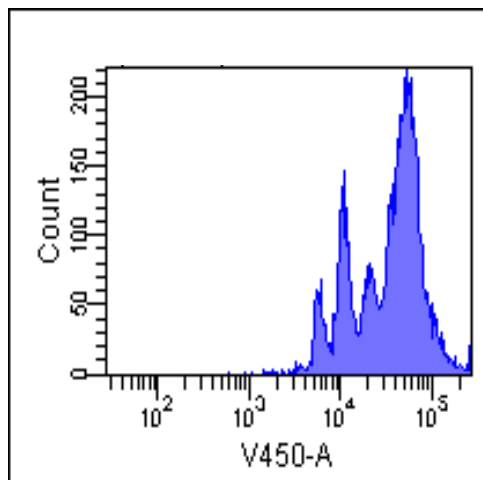
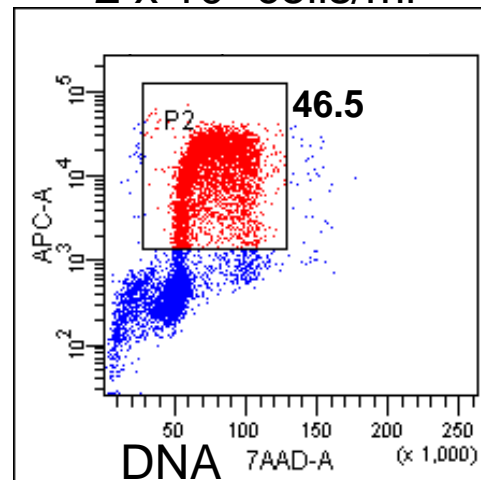
1 x 10<sup>7</sup> cells/ml



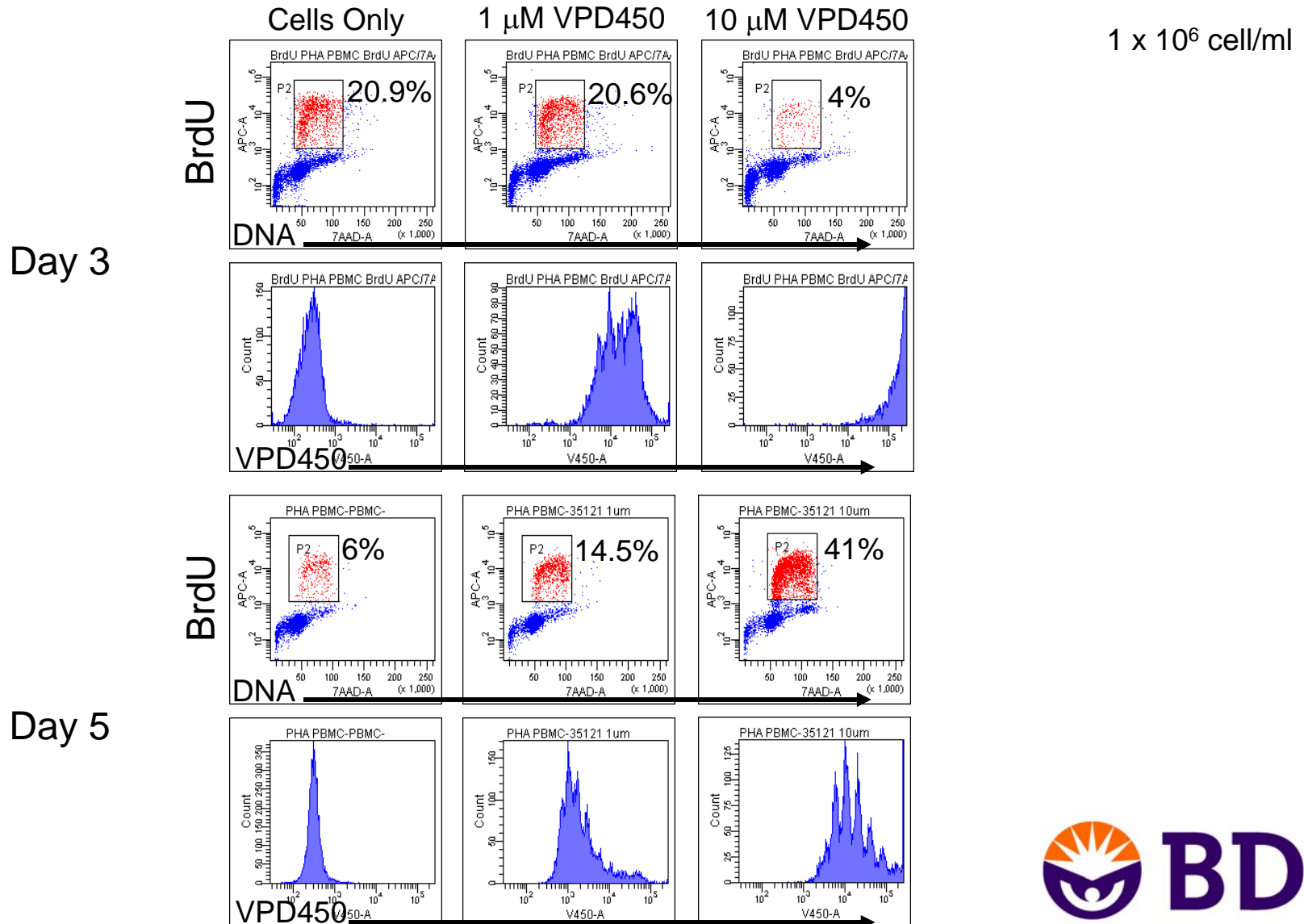
1 x 10<sup>6</sup> cells/ml



2 x 10<sup>7</sup> cells/ml



# Human PBMC PHA Stimulation [VPD450]



# VPD450 histograms

Condition:

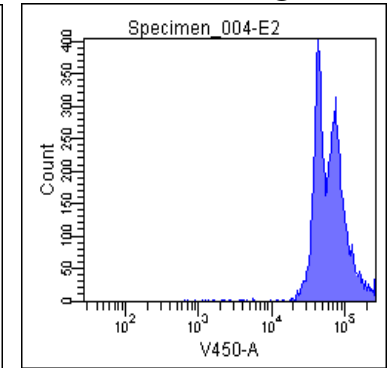
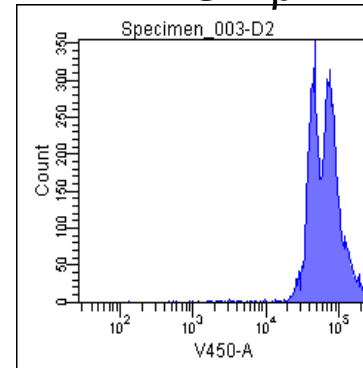
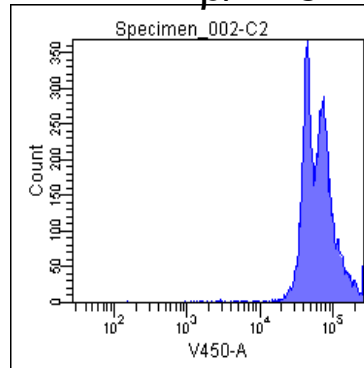
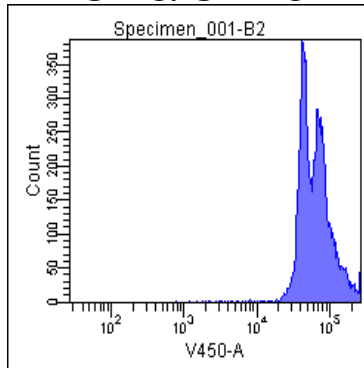
CD3/CD28

+IL-1 $\beta$ /IL-6

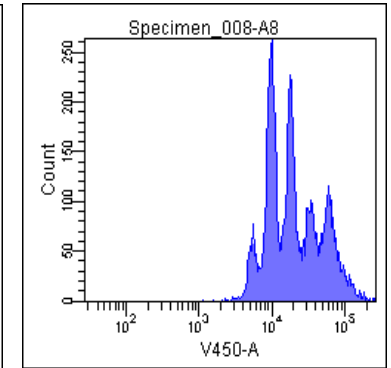
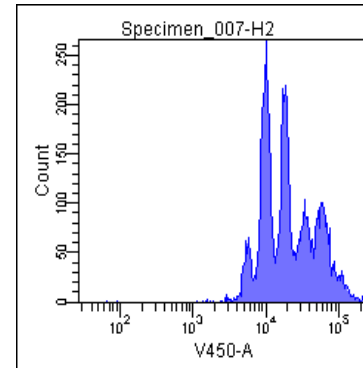
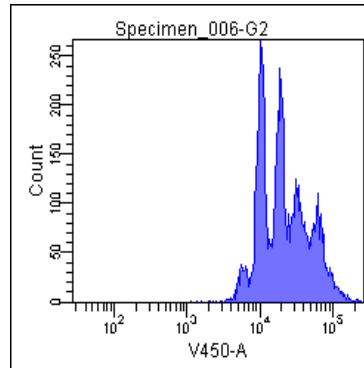
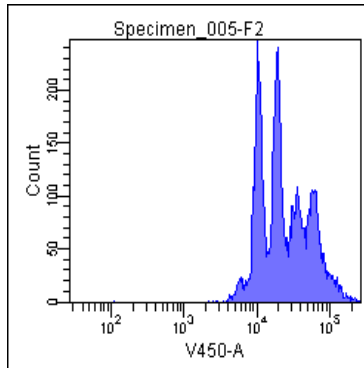
+TGF- $\beta$

+IL-23

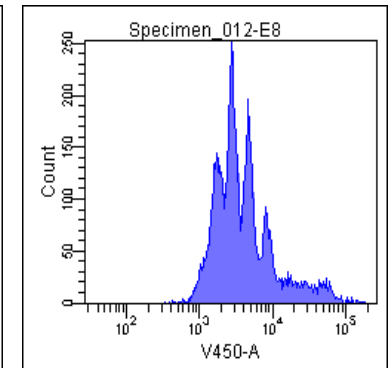
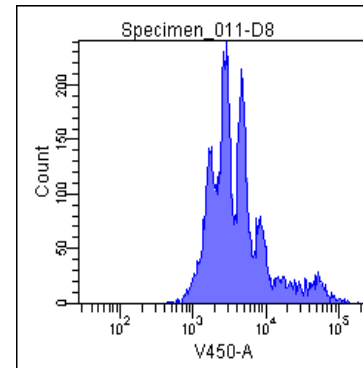
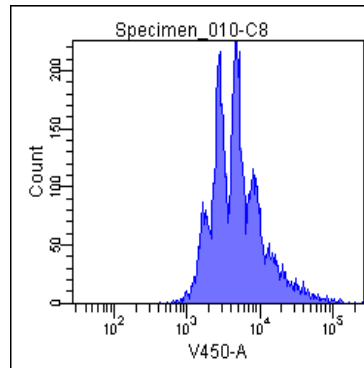
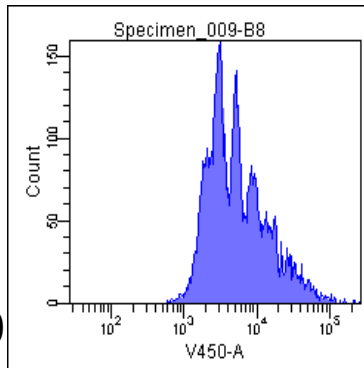
Day 1



Day 2



Day 3

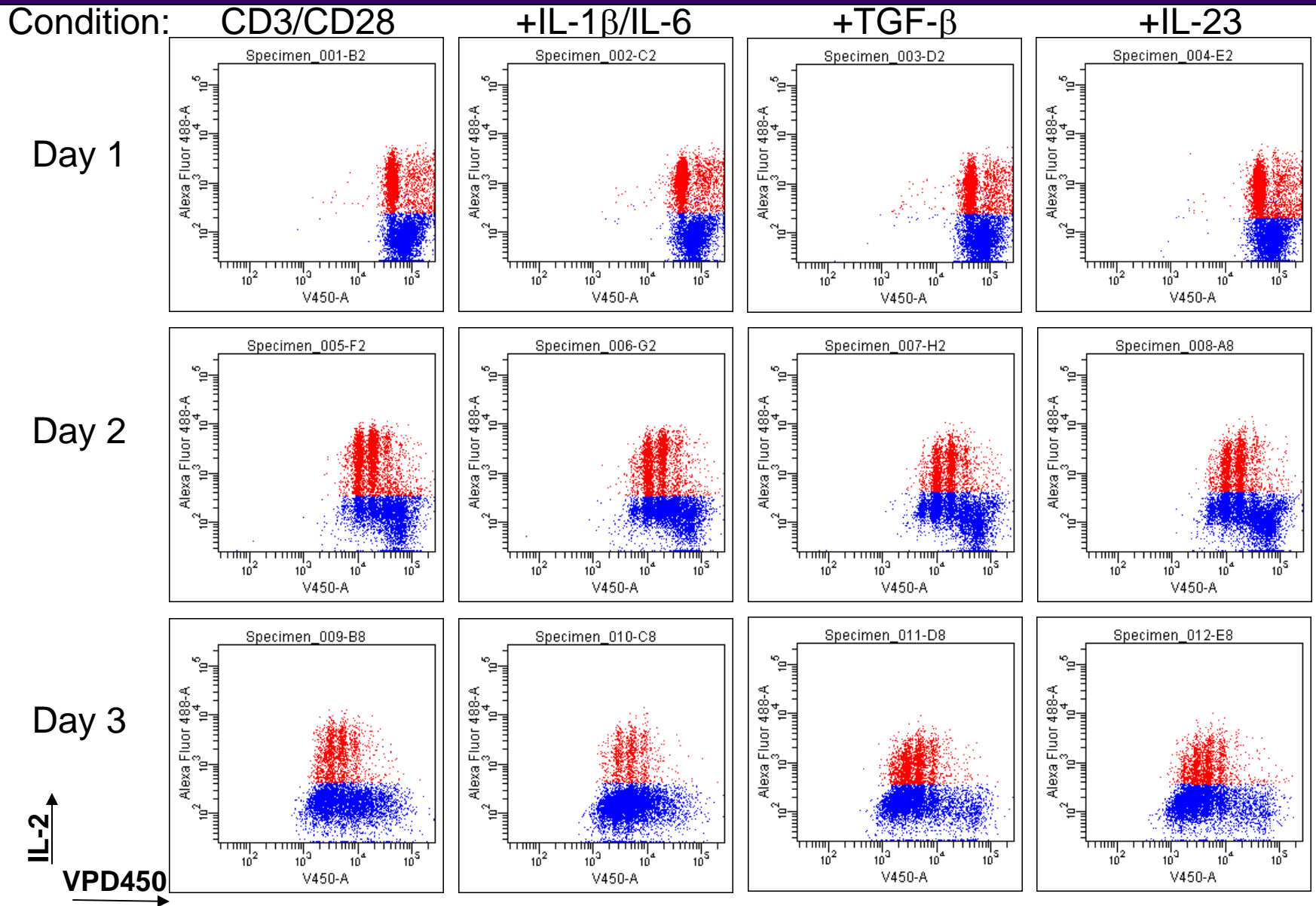


VPD450

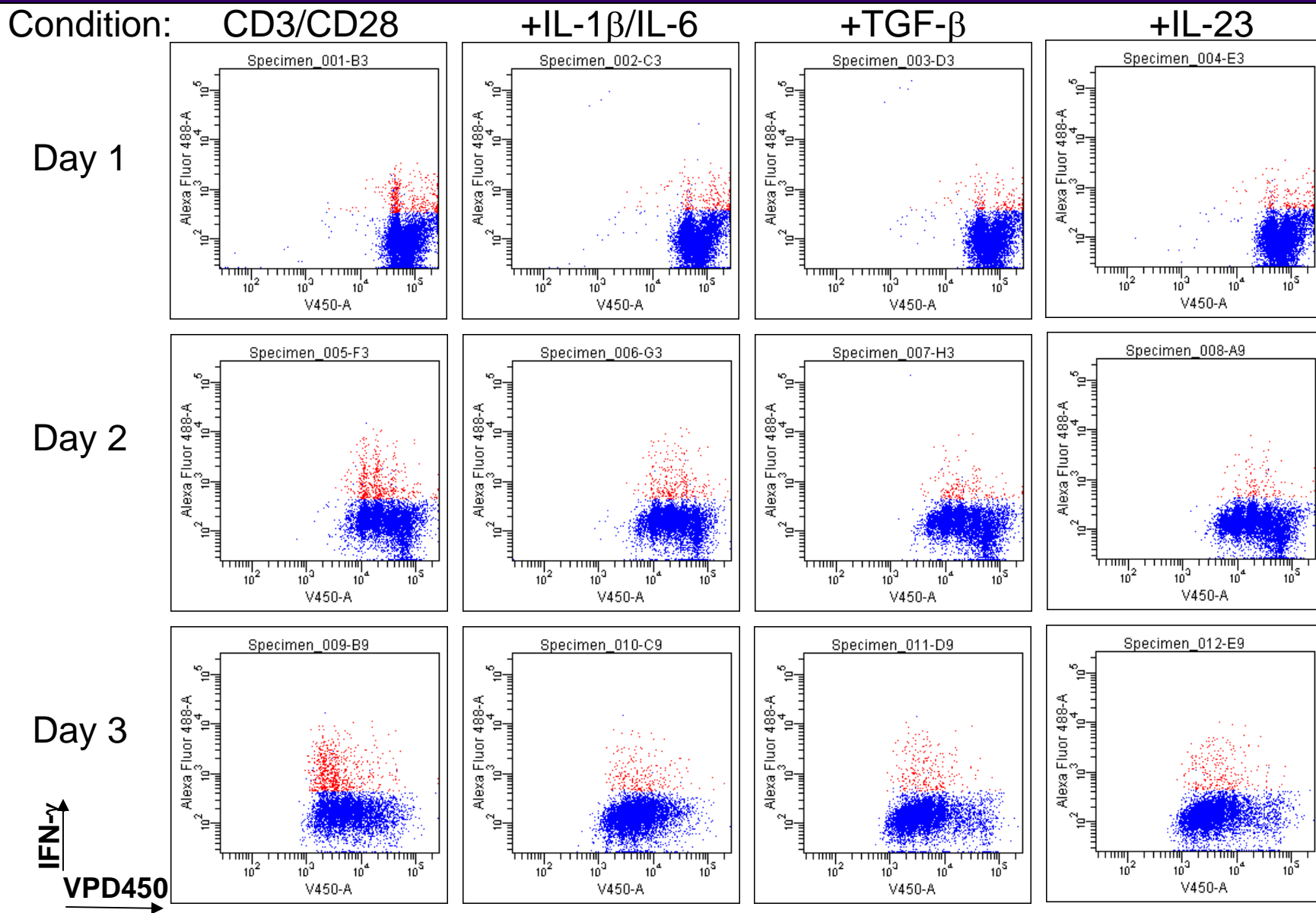
# *Which conditions for which cytokines*

- 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?

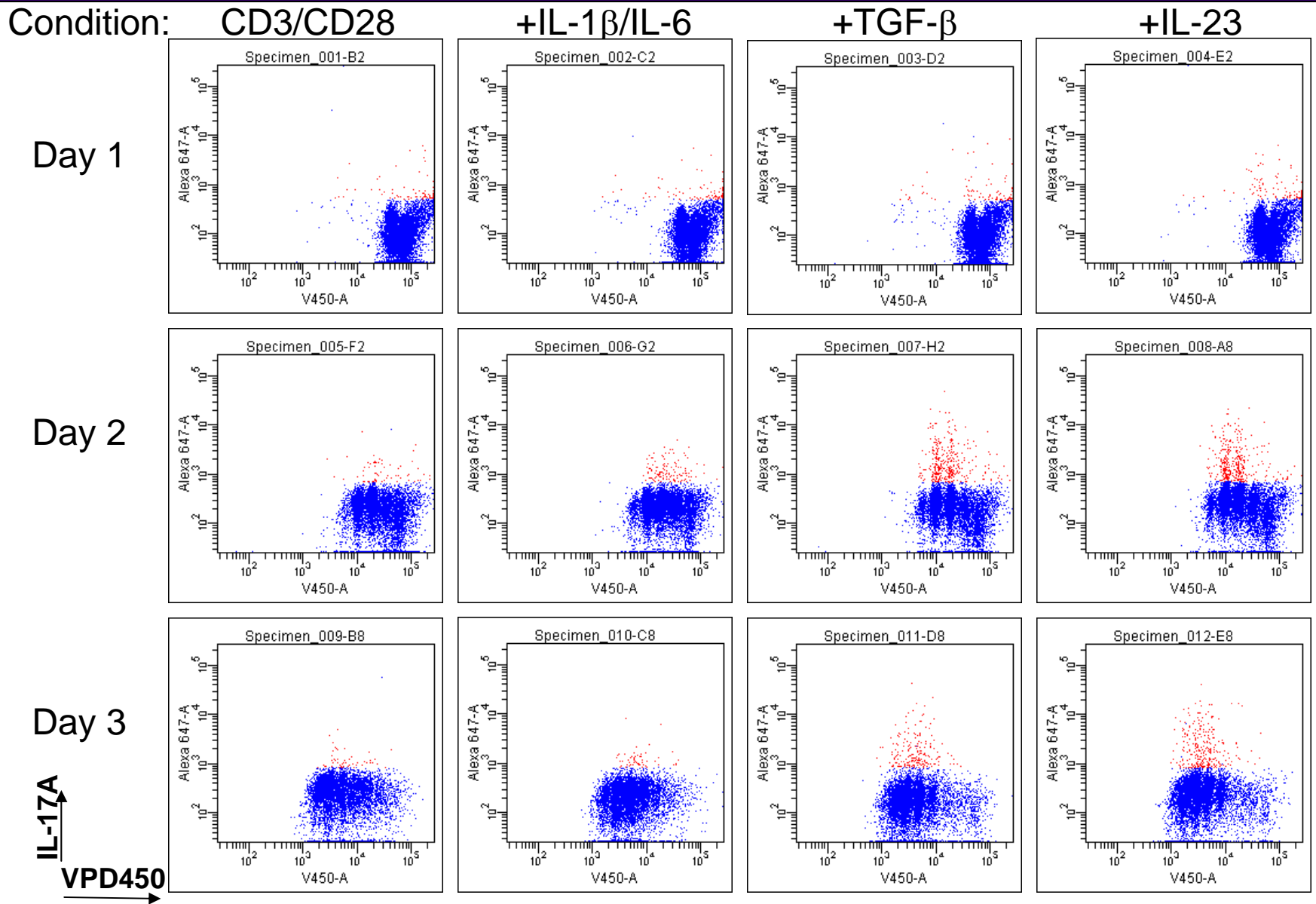
# VPD450 vs IL-2 data



# VPD450 vs IFN- $\gamma$ data



# VPD450 vs IL-17A data

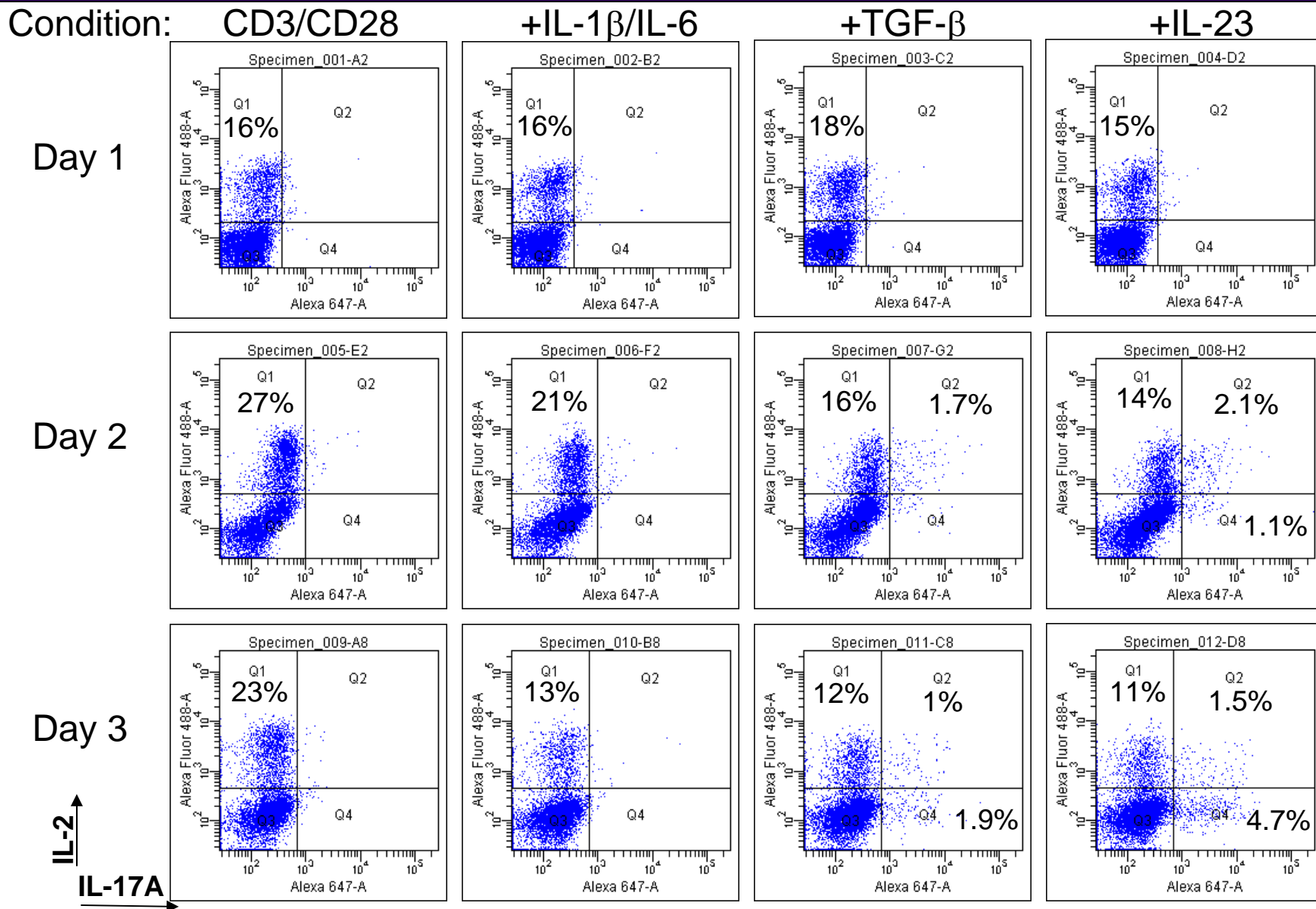




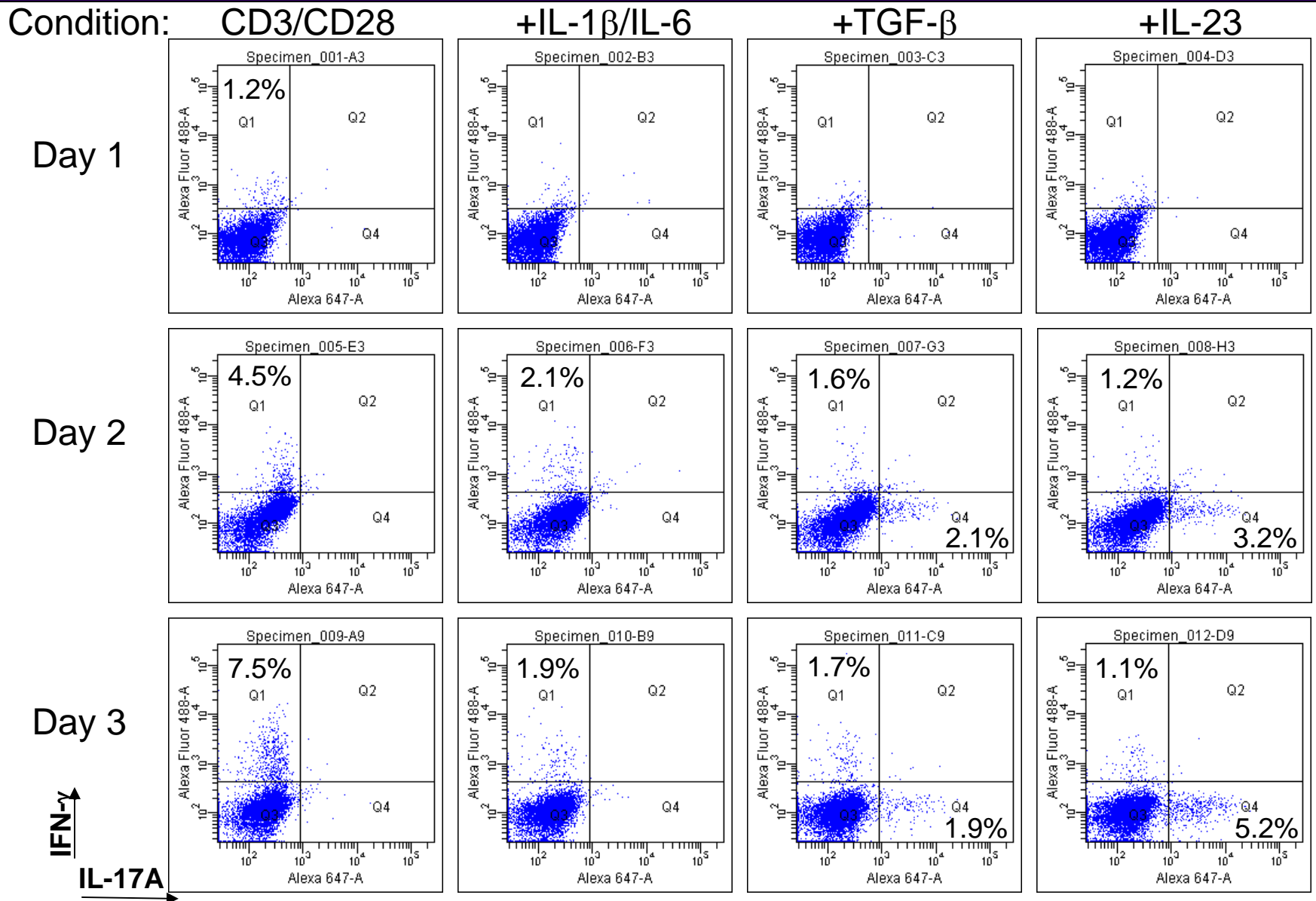
# *Cytokine co-expression*

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

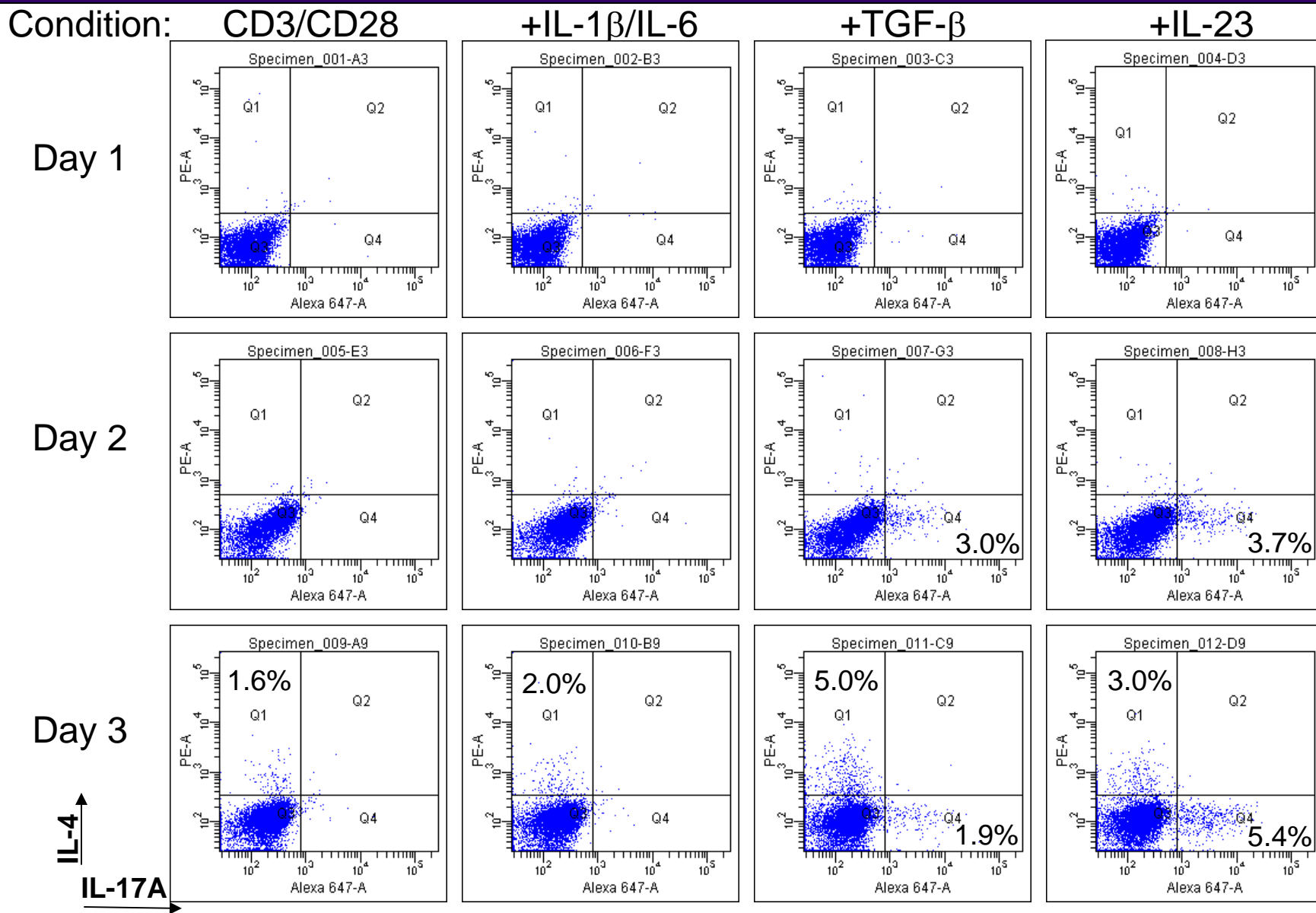
# Co-expression of IL-17A vs IL-2



# Co-expression of IL-17A vs IFN- $\gamma$



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

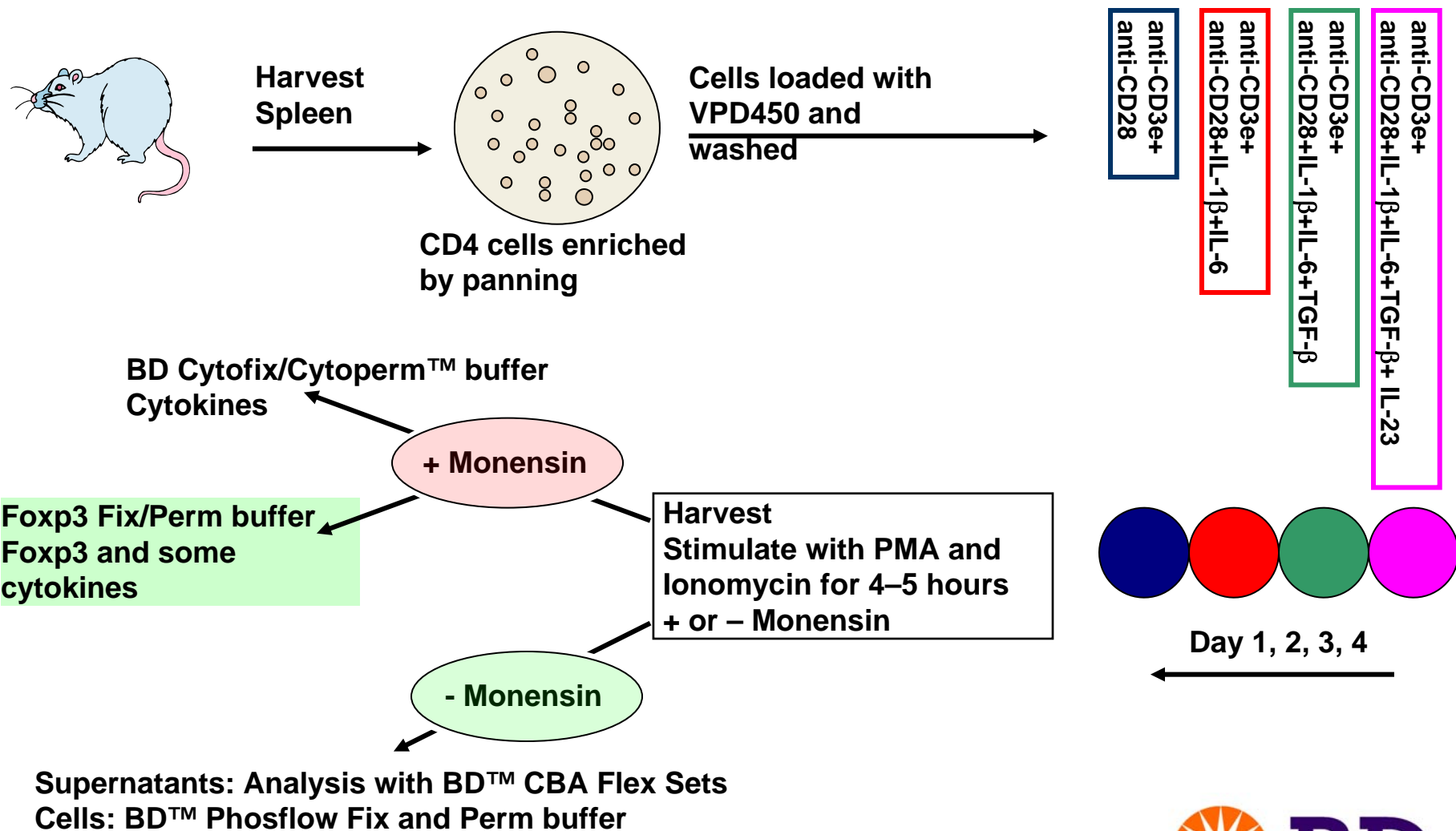


# *Tracking FoxP3*

- 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- $\gamma$  expression decreases.
- What are the FoxP3+ cells doing?

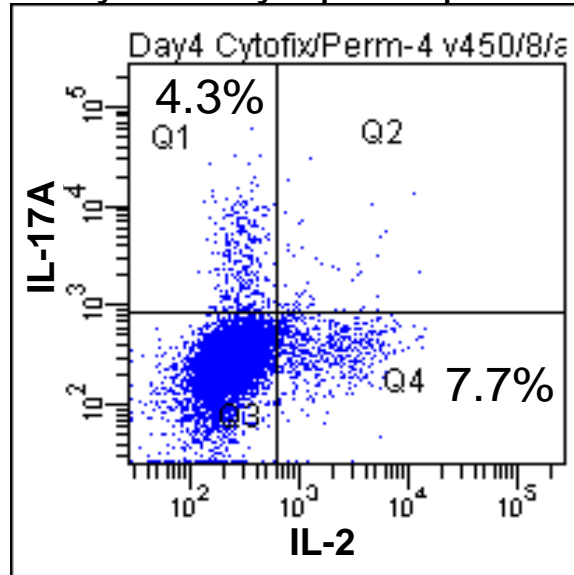


# Experimental setup

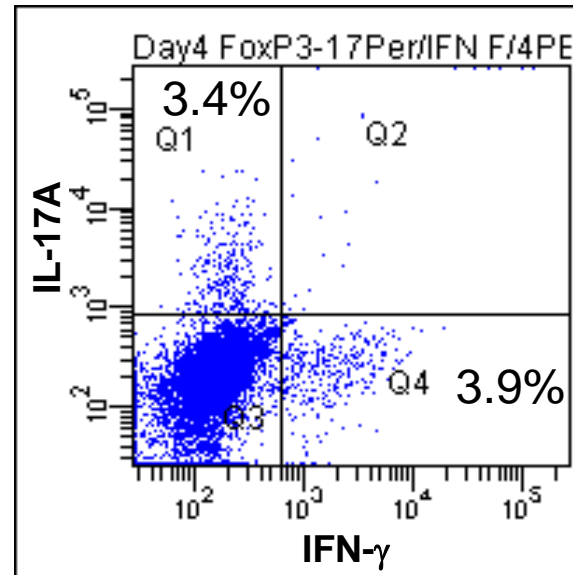
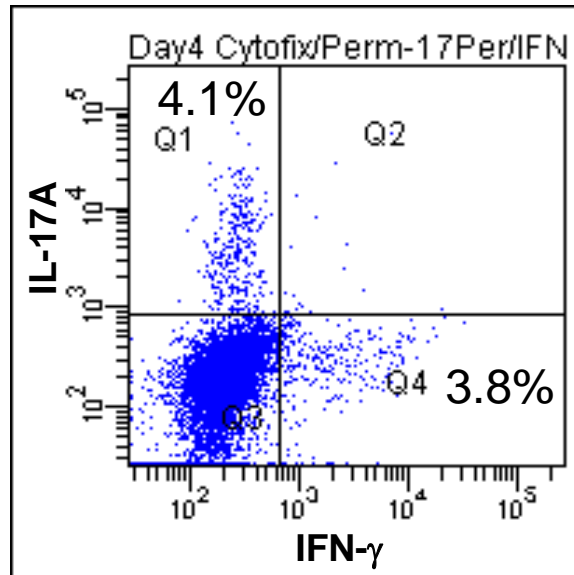
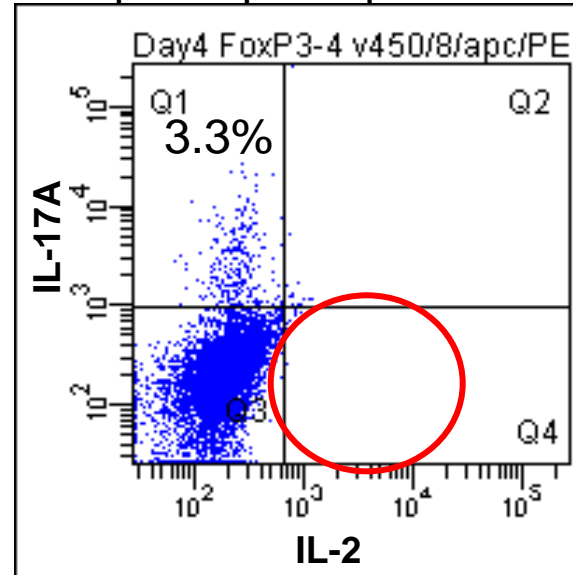


# Comparison of two fix/perm protocols

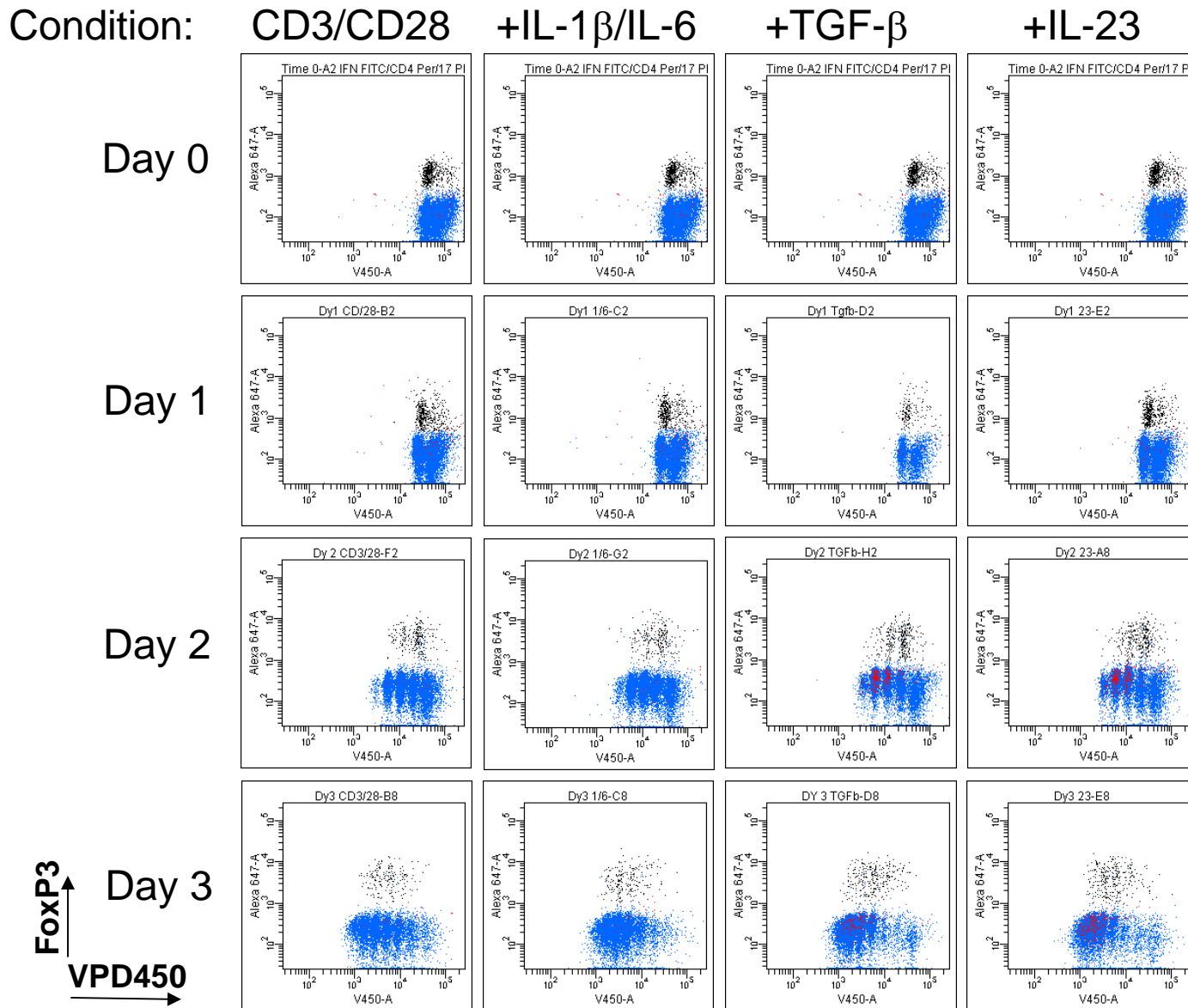
BD Cytfix/Cytoperm protocol



Foxp3 fix/perm protocol

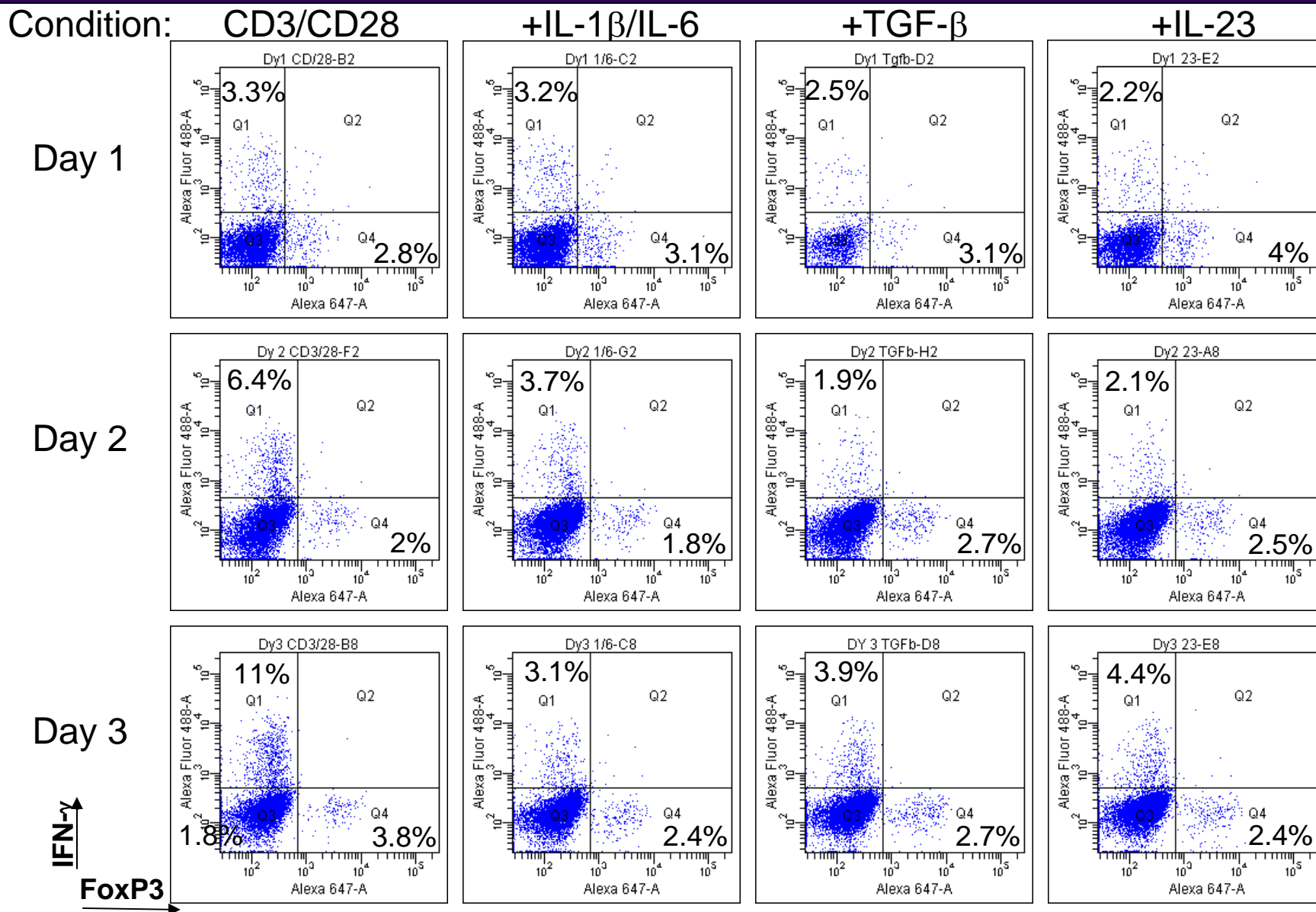


# Proliferation of Treg and Th17 cells

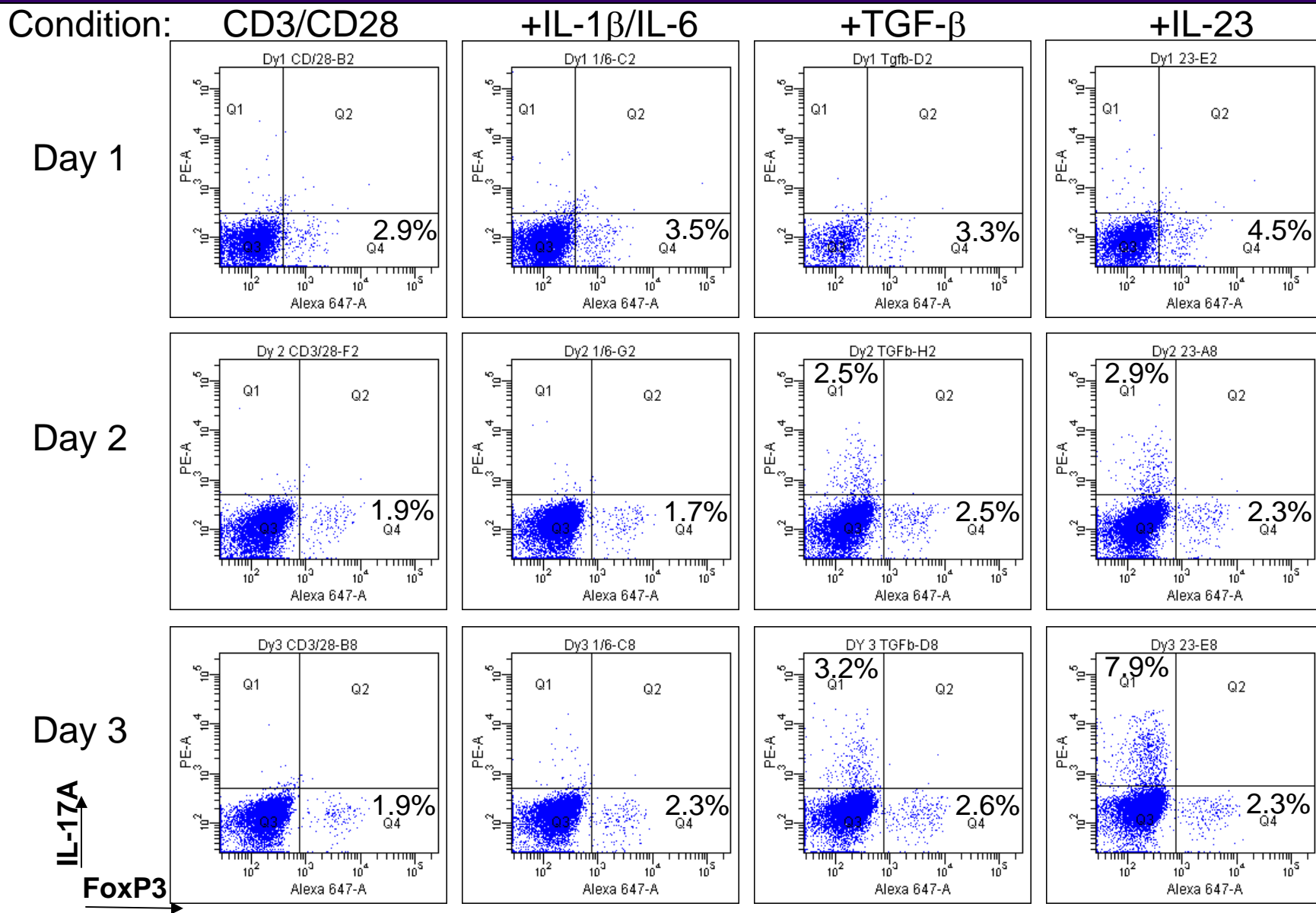




# Co-expression of *Foxp3* vs *IFN- $\gamma$*



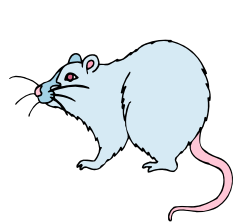
# Co-expression of *Foxp3* vs *IL-17A*



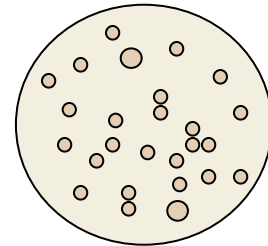
# *Cytokines in culture supernatants*

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

# Experimental setup

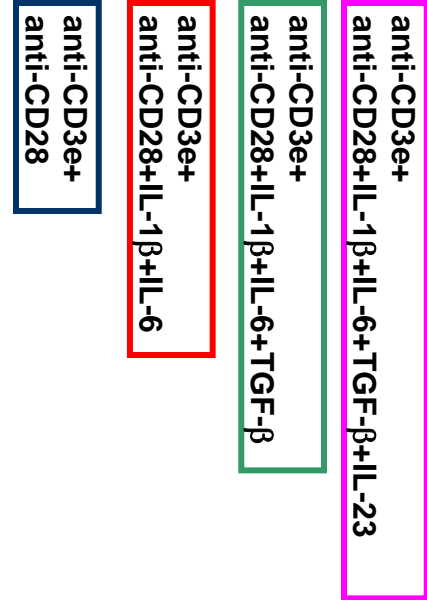


Harvest spleen



CD4 cells enriched by panning

Cells loaded with VPD450 and washed



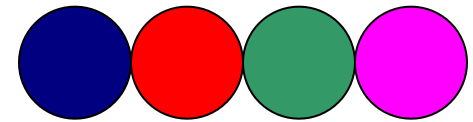
BD Cytofix/Cytoperm buffer  
Cytokines

+ Monensin

Foxp3 Fix/Perm buffer  
Foxp3 and some cytokines

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

- Monensin

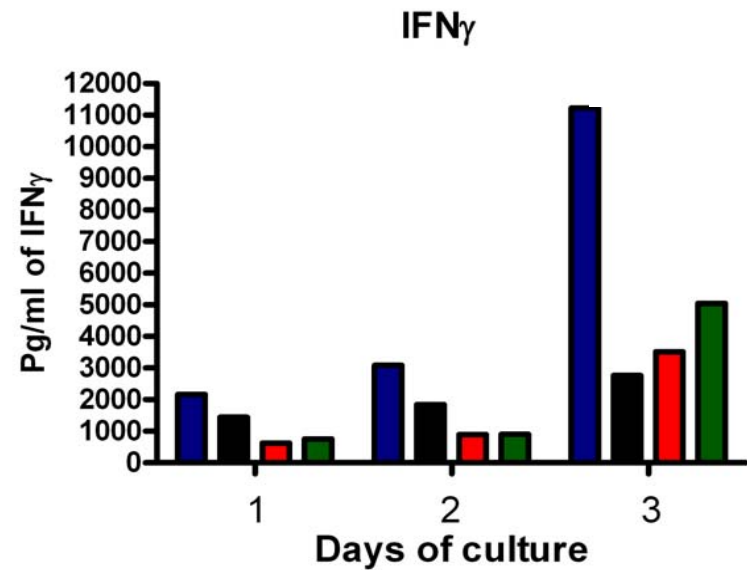
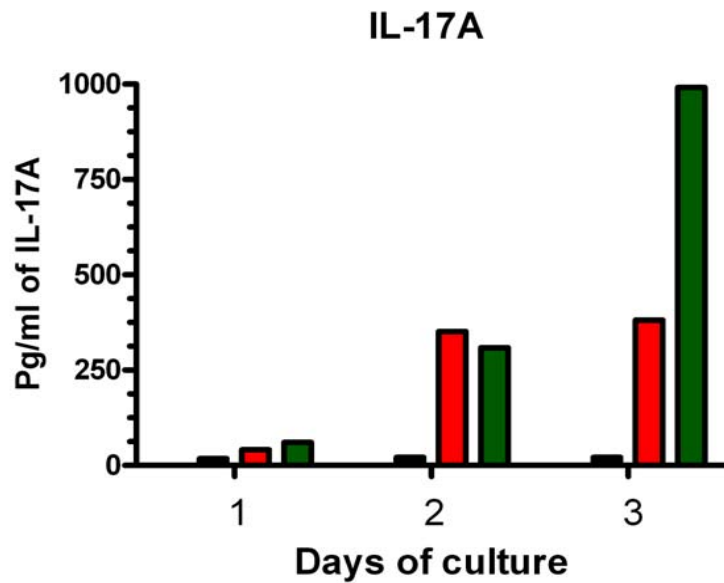


Day 1, 2, 3, 4

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

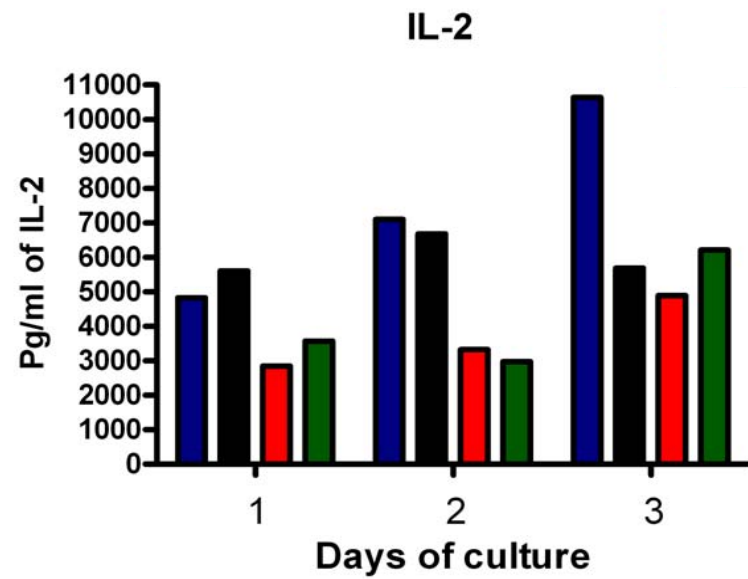
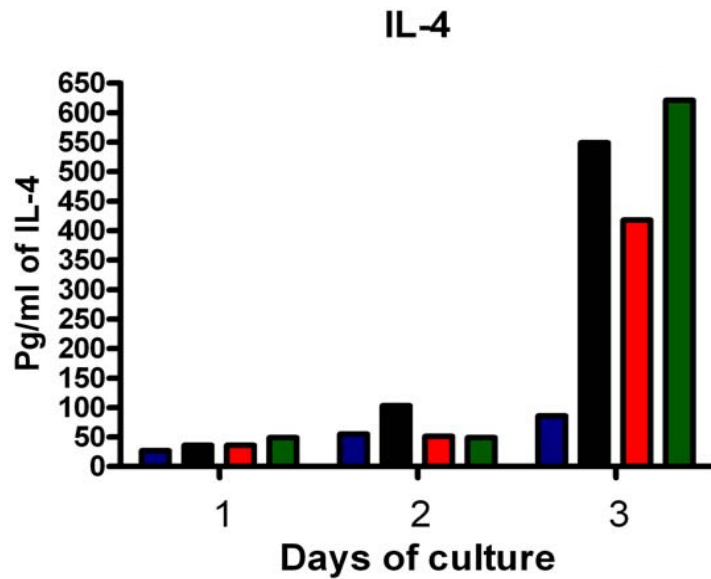


# IL-17A and IFN- $\gamma$ production



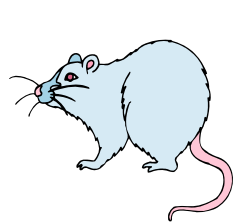
- CD3 /CD28
- CD3 /CD28/IL1 $\beta$ /IL-6
- CD3 /CD28/IL1 $\beta$ /IL-6/TGF $\beta$
- CD3 /CD28/IL1 $\beta$ /IL-6/TGF $\beta$ /IL-23

# IL-4 and IL-2 production

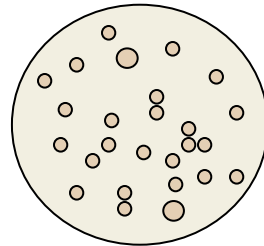


- CD3 /CD28
- CD3 /CD28/IL1 $\beta$ /IL-6
- CD3 /CD28/IL1 $\beta$ /IL-6/TGF $\beta$
- CD3 /CD28/IL1 $\beta$ /IL-6/TGF $\beta$ /IL-23

# Experimental setup

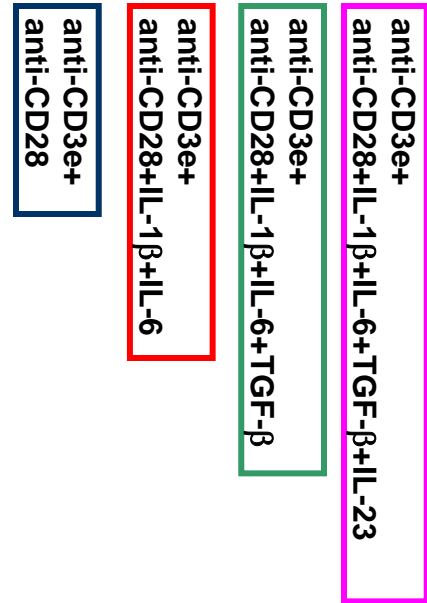


Harvest spleen



CD4 cells enriched by panning

Cells loaded with VPD450 and washed

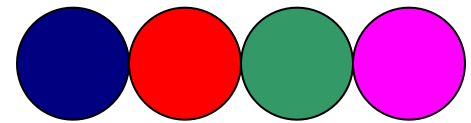
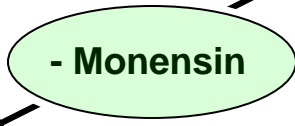


BD Cytofix/Cytoperm buffer  
Cytokines



Foxp3 Fix/Perm buffer  
Foxp3 and some cytokines

Harvest  
Stimulate with PMA and Ionomycin for 4–5 hours  
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Day 1, 2, 3, 4

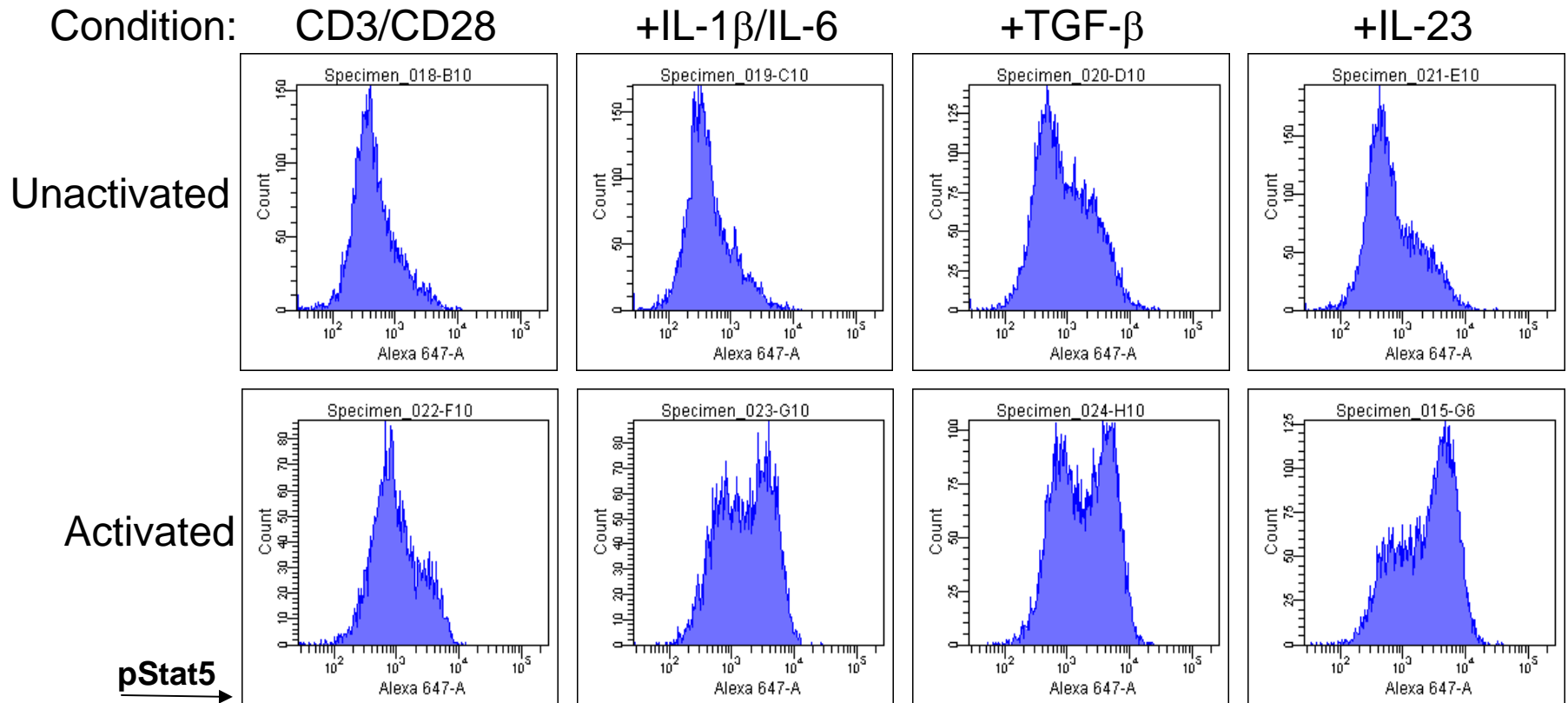
Supernatants: Analysis with BD CBA Flex Sets  
Cells: BD Phosflow Fix and Perm buffer



# *pStat5 detection on day 4*

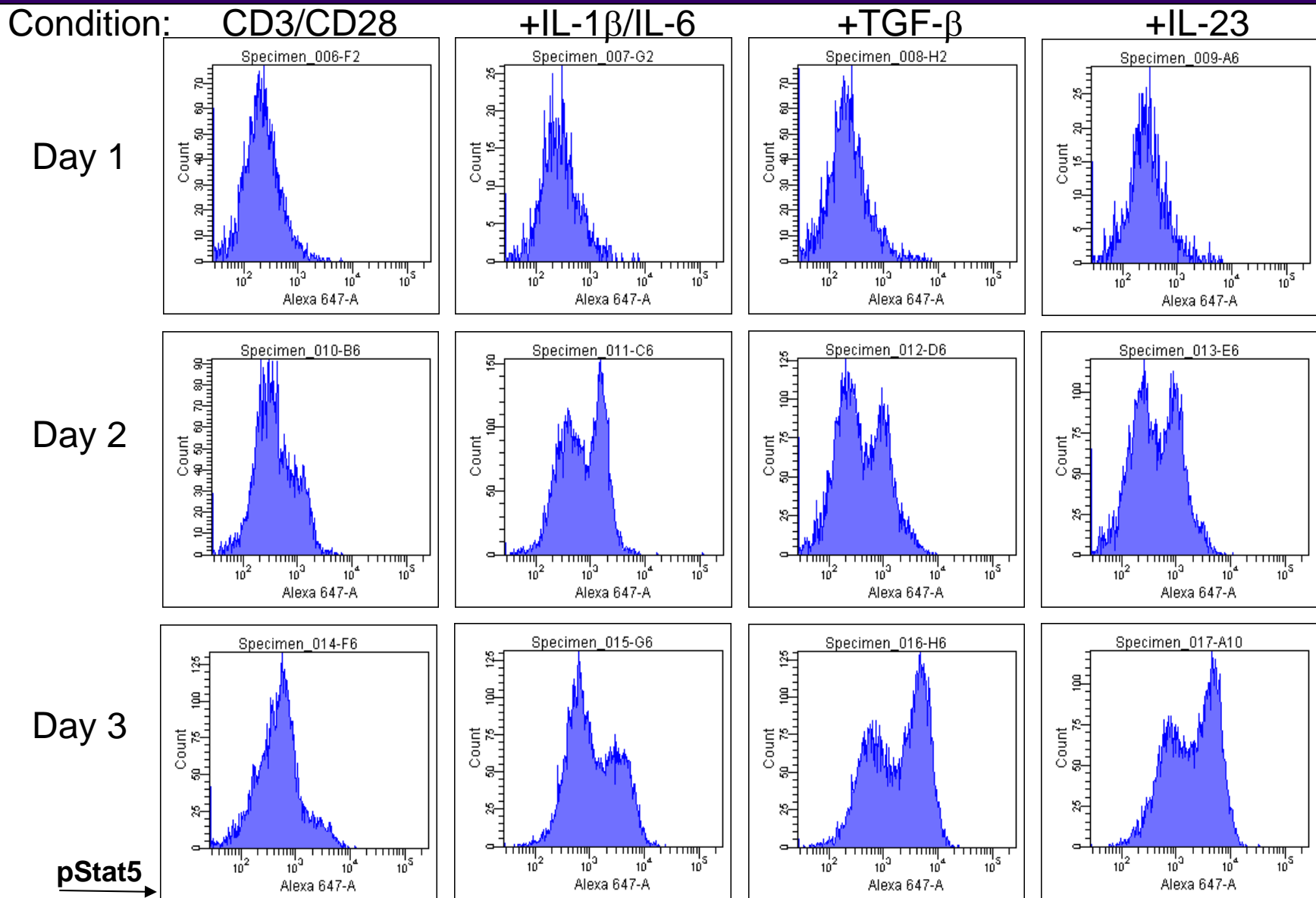
**Unactivated:** Cells were cultured, harvested, and stained with phosphospecific Stat5 antibody.

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

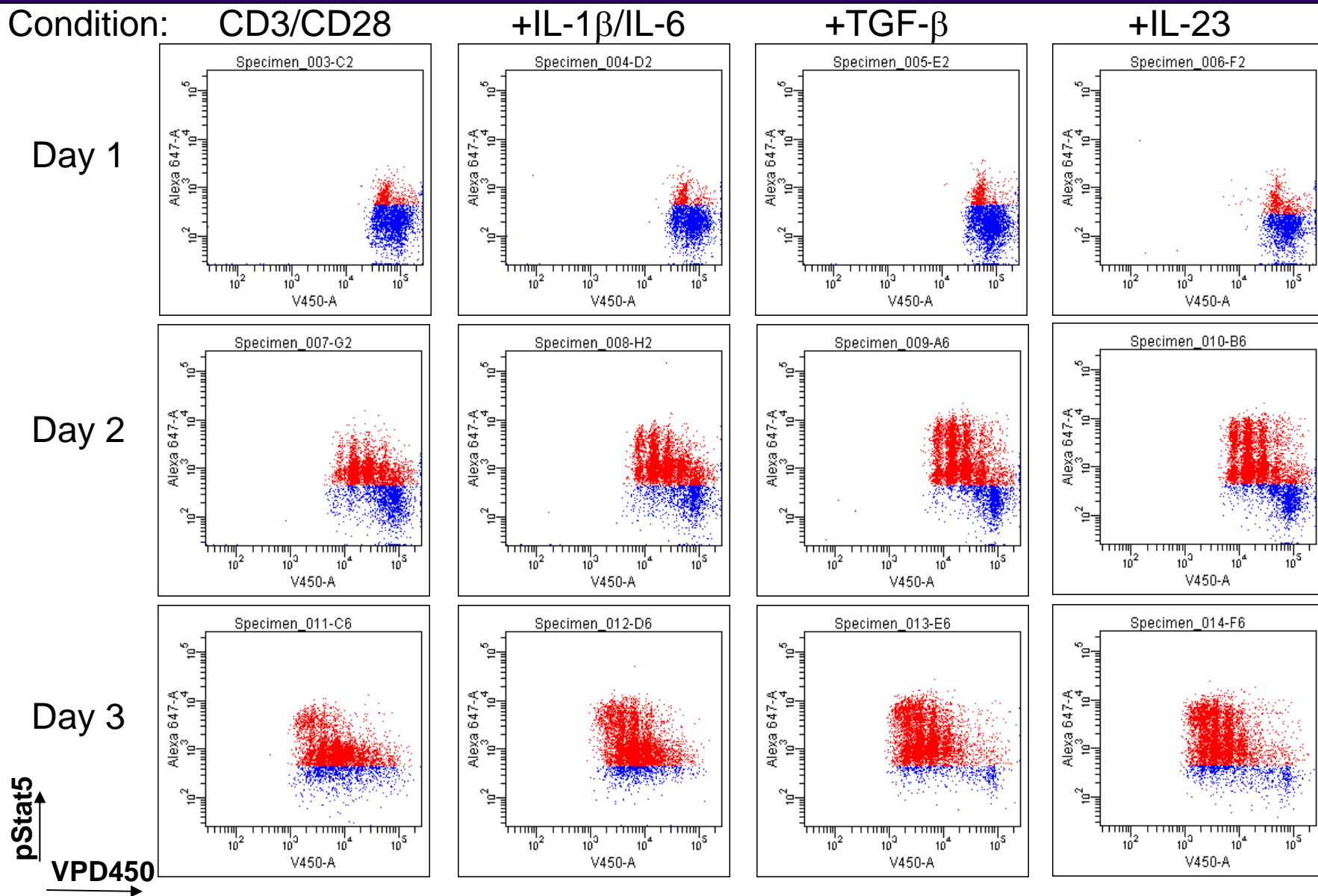




# *pStat5 in activated cells over time*



# *pStat5* in proliferating cells



# Conclusions

- Cells proliferated equally well under all four polarization conditions.
- In vitro cultures showed that TGF- $\beta$  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](mailto:ResearchApplications@bd.com)

