Measuring Dendritic Cells

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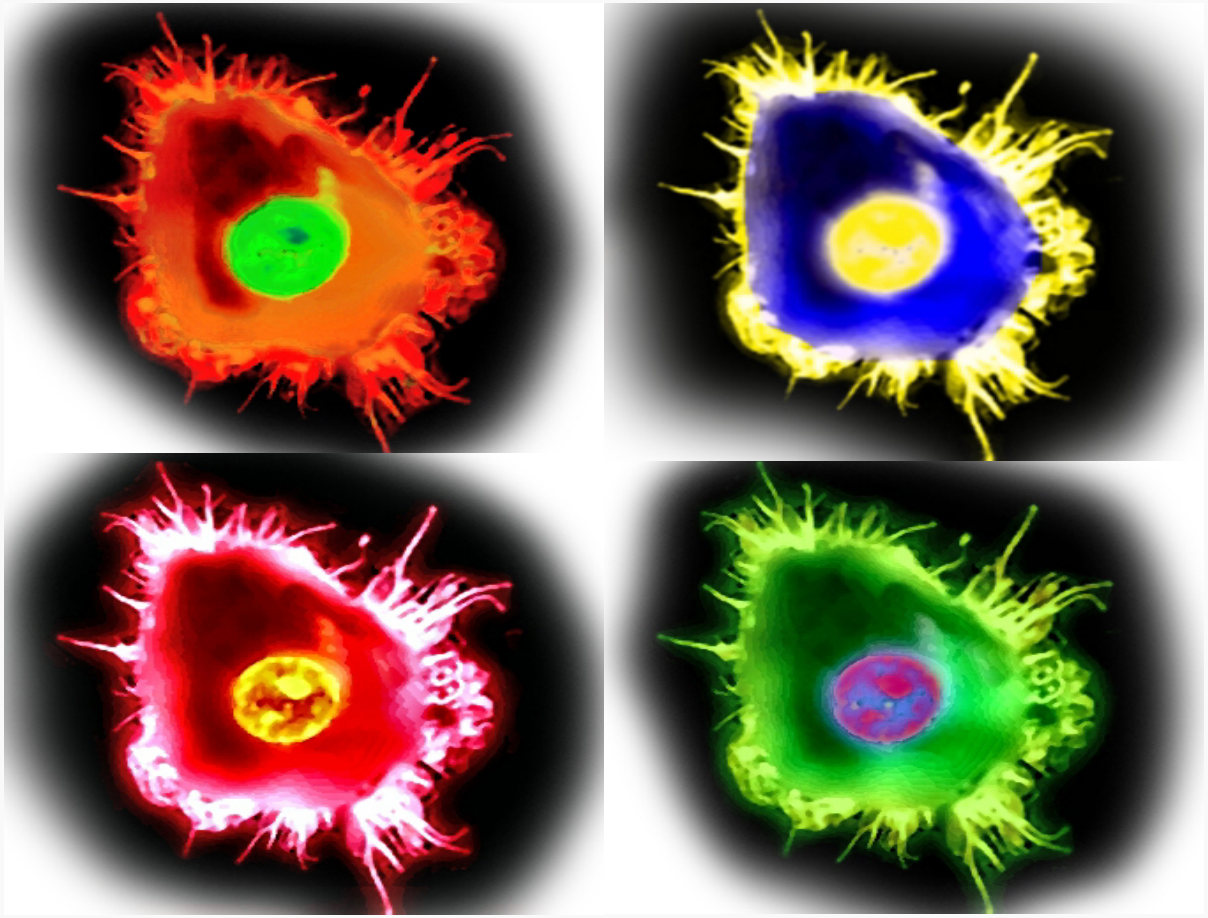


Measuring DC

- Rare event detection
- The basics of DC measurement
- Applications
  - Cancer
  - Asthma
  - DC trafficking in an animal model

I. Rare Event Detection

- Key elements
- Lower limit of detection
- Fluorochromes
- How many cells to acquire?
- Data analysis: Log-normal model

DCs in the Peripheral Blood

- Find the needle
- Determine that it really is a needle
- Make measurements to determine what kind of a needle it is

Key Elements

- Event frequency
  - Inherent property of sample
  - Enrichment possible
- Signal to noise ratio
  - Minimize noise
    - Nonspecific binding (1% mouse serum)
    - Cellular autofluorescence (dump gate, green or red excitation, quenching dyes)
    - Doublets (ratio of peak height/integral or peak height/width)
    - Sporadic mechanical or electrical noise (time parameter)
    - Dead cells (vital dyes)
  - Maximize signal
    - Best fluorochrome for most critical determination
    - Optimal antibody concentration

Know Your Own Limit (of Detection)

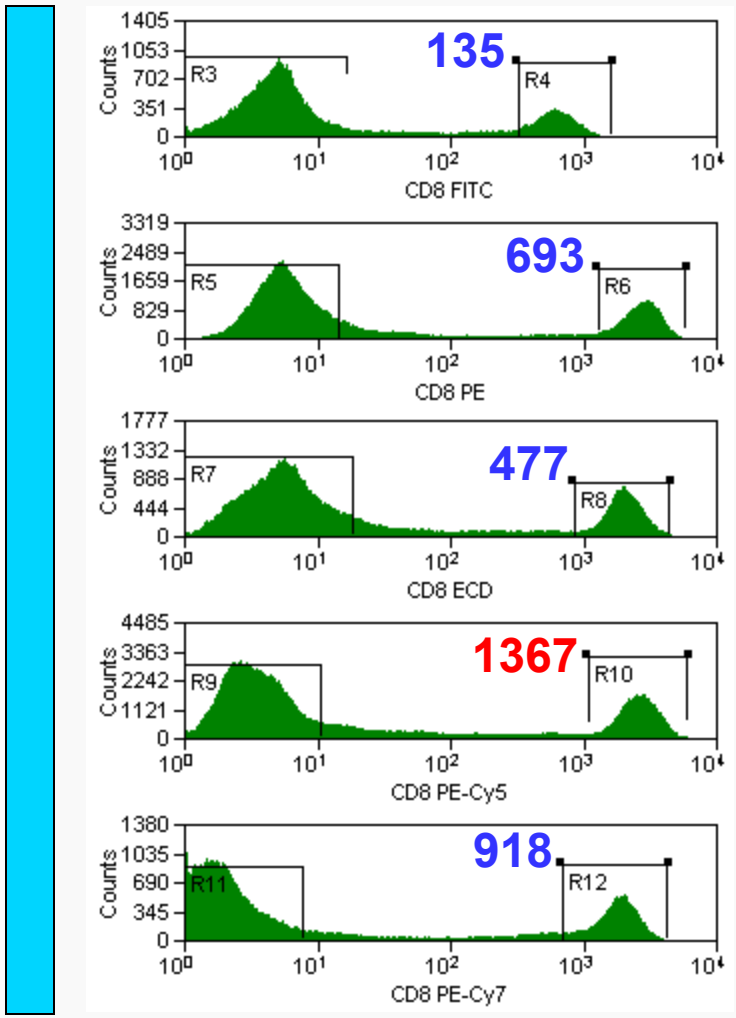
- Limit of detection

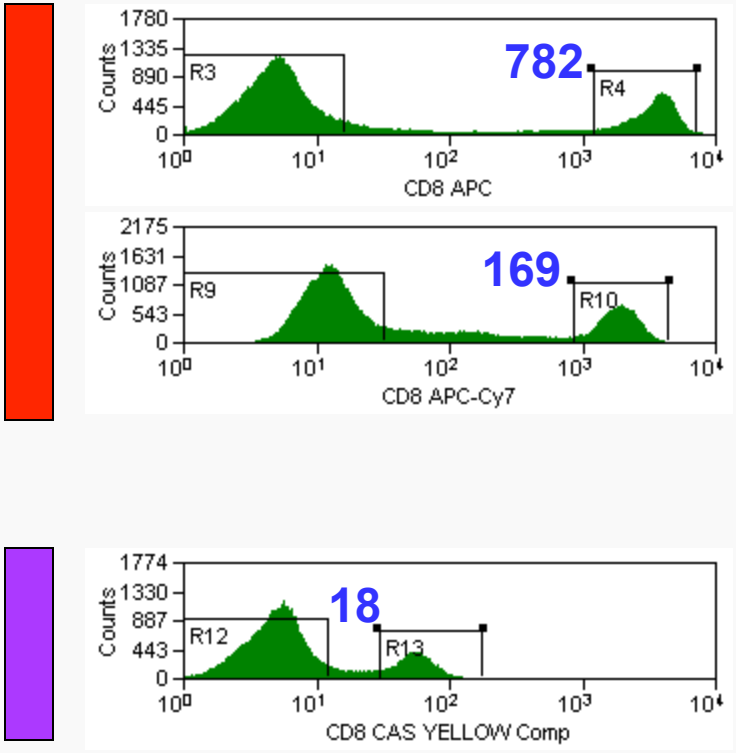
  Frequency of false positives in appropriate negative control (FMO isotype control, FMO isoclonic control, TMer binding of MHC disparate cells, known negative sample)
- Calculate upper 95^{th} or 99^{th} percentile of the frequency false positive in a series of negative controls
- Caution: Rare events are log normally distributed. Use arithmetic means and you will get the wrong answer!

Pull the Noise From the Signal

- Dump channel
- Unique location in multiparameter space
- Use the best fluorochrome for the most critical measurement
  - PE has high quantum efficiency
  - Red line used to excite APC and APC tandems excites less cellular autofluorescence
  - Green line can be used for PE and PE tandems

For a reagents available in several fluorochromes choose the one with the best **signal to noise** ratio for your critical measurement



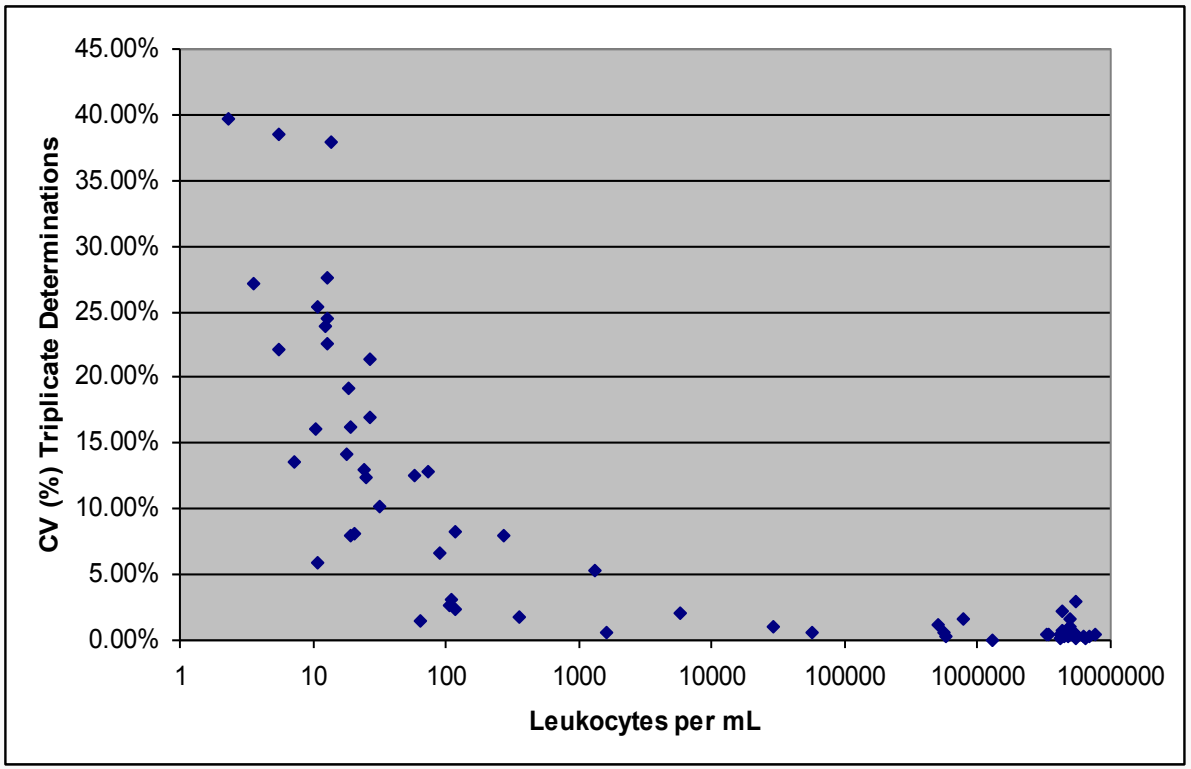


How Many Cells to Acquire

- Short answer: The rarer the event the more cells required
- Long answer: Depends on
  - Event frequency
  - Tightness of event cluster in multiparameter space
- You can determine the number empirically by determining the precision of replicate determinations
- No matter how many events you acquire, the limit of detection is governed by the signal to noise ratio

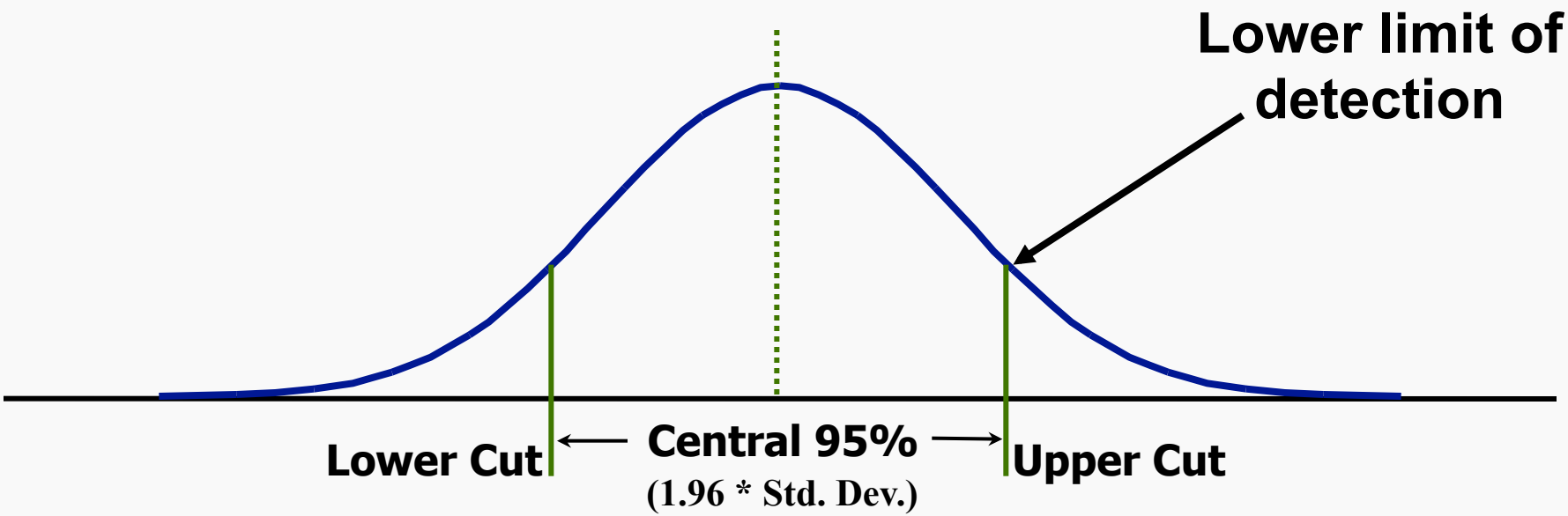
Precision of Replicate Determinations

All events in three 5 mL aliquots of leukocyte depleted platelet product were acquired



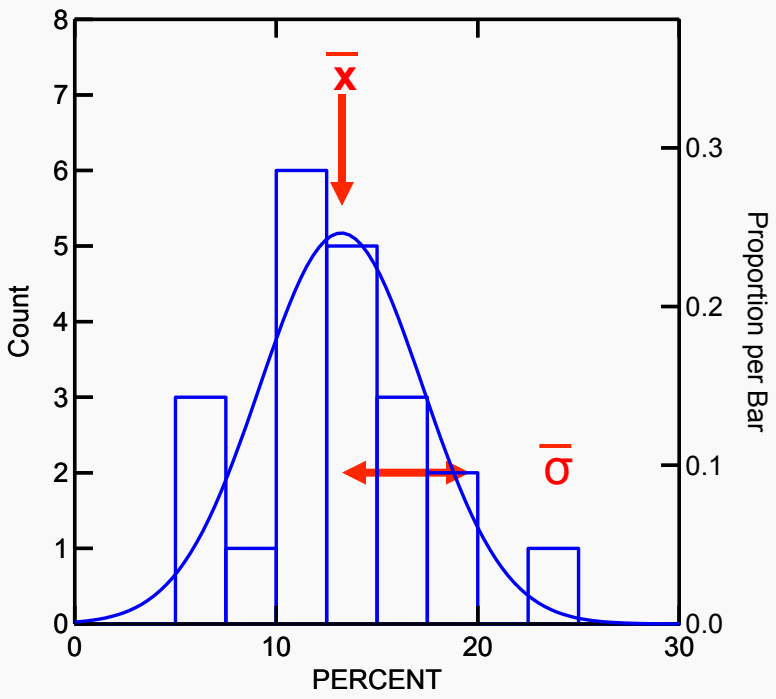
Detection of leukocytes in filtered platelet components
Donnenberg et al Transfusion, 2000.

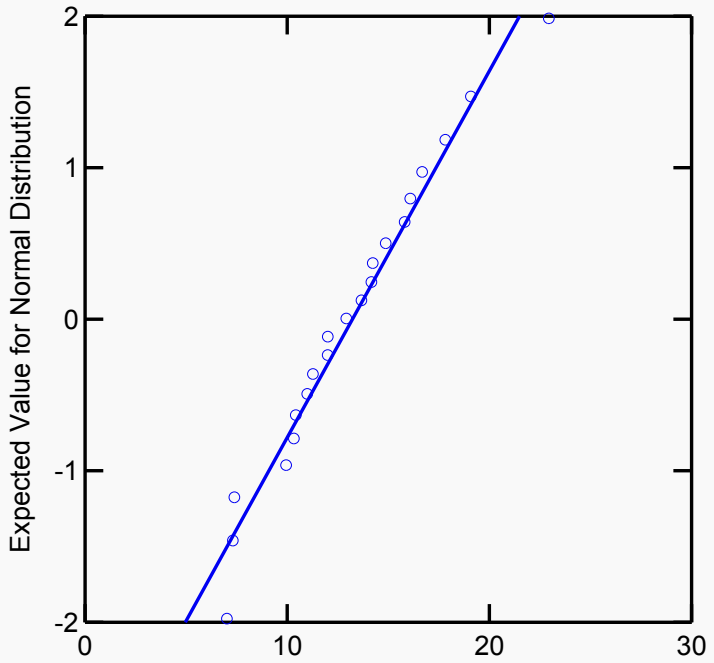
Predictions of the Normal Model



Negative Control Group (Percent positive)

CD4+ Percent is Normally Distributed

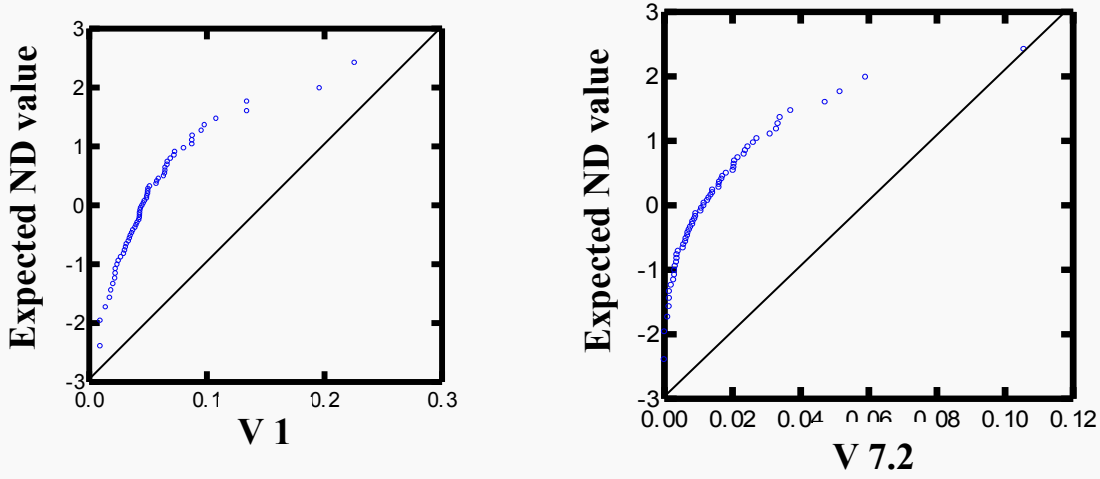




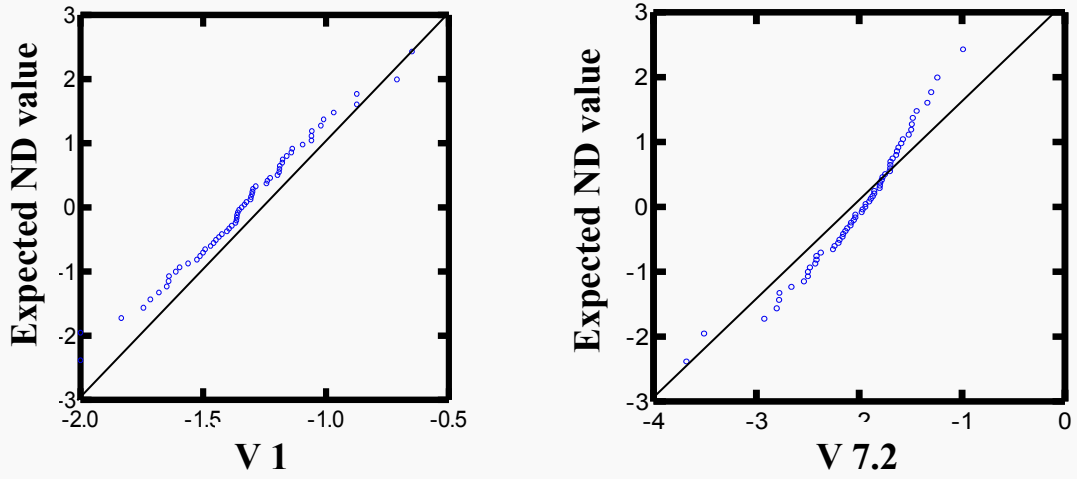
Percent CD4+ →

Vβ usage is log-normally distributed

A. Linear Scale



B. Log Transformed



Conclusion

Failure to use log transformed data results in:

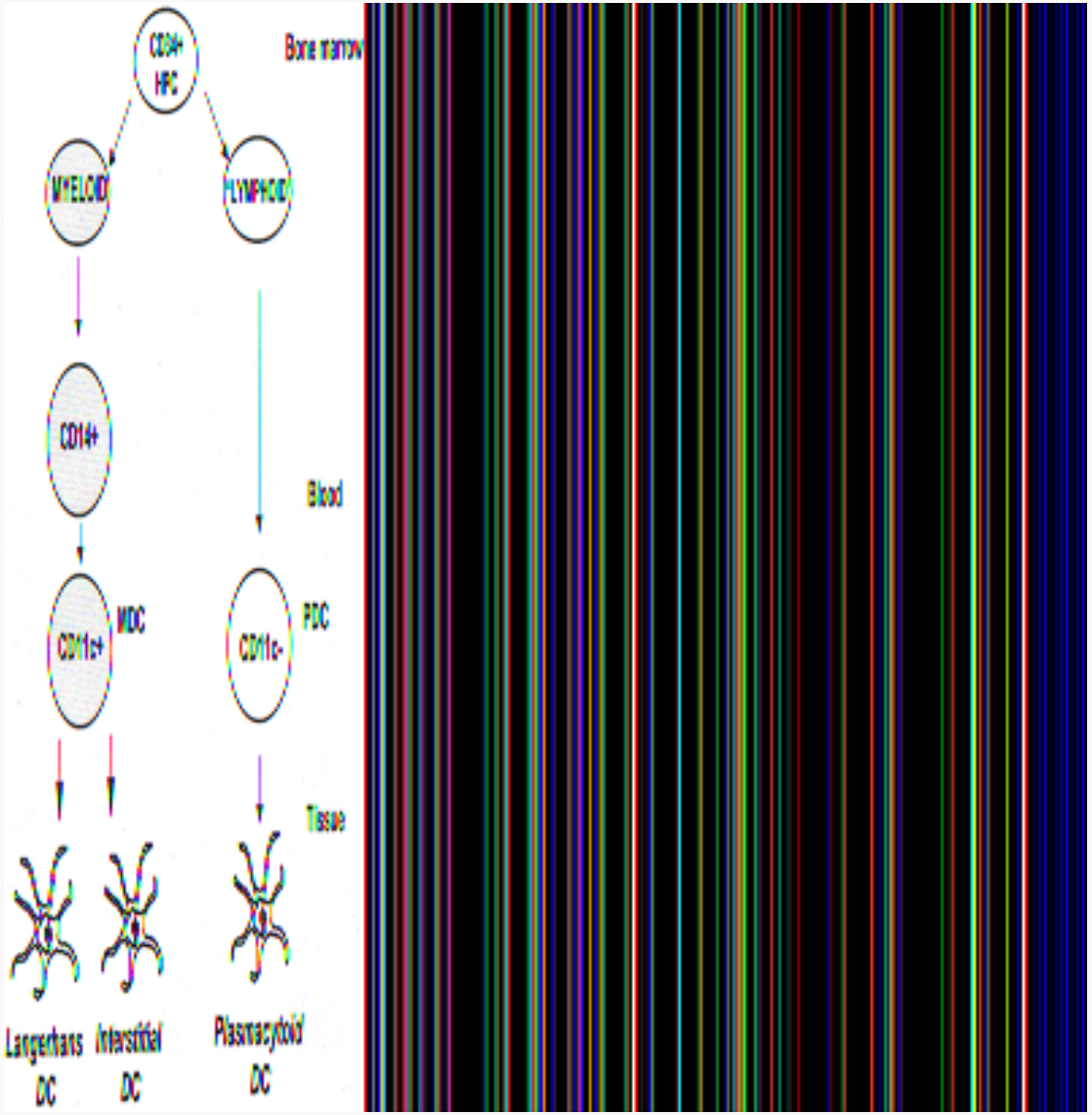
- Underestimate of the lower limit of detection
- Overestimate in percent positive
- A larger CV and a corresponding loss of power to detect significant differences between groups using parametric tests

II. Basics of DC Measurement

- The DC Differentiation Tree
- Immunophenotypic Markers
- Gating Strategies

Dendritic Cells

- DC are potent APC (acquisition, processing and presentation of Ag to induce MHC-restricted T cell-mediated IR)
- Involved in tolerance induction and regulation of immune reactivity
- Differentiate from myeloid (DC1) and lymphoid (DC2) precursors which give rise to mature DC


CD34+
HPC
Bone marrow
M+ELCID
LYMPHOID
CD14+
Blood
CD11c+
MDC
CD11c-
PDC
Tissue
Langerhans
DC
Interstitial
DC
Plasmacytoid
DC

Banchereau

DC Markers of Mice and Men

Species	Subset	Phenotype
Murine	Myeloid DC	$CD11c^+CD11b^+B220^-CD8\alpha^-$
	"Lymphoid-related" DC	$CD11c^+CD11b^-B220^-CD8\alpha^+$
	Plasmacytoid DC	$CD11c^+CD11b^-B220^+CD8\alpha^\pm$
	Liver-derived DC	$CD11c^+CD11b^-B220^+DEC205^+$
Human	Monocytoid DC (DC1)	$HLA\text{-}DR^+CD11c^+CD123^{lo}$
	Plasmacytoid DC (DC2)	$HLA\text{-}DR^+CD11c^-CD123^{hi}$
	Langerhans Cells	$HLA\text{-}DR^+CD11c^+CD1a^+$
	B cell-like DC[a]	$HLA\text{-}DR^+CD19^+CD20^+$
	Tonsil interdigitating DC	$HLA\text{-}DR^{hi}CD11c^+$
	Tonsil interdigitating DC	$HLA\text{-}DR^{hi}CD11c^-CD13^+$
	Tonsil interdigitating DC	$HLA\text{-}DR^{mod}CD11c^-CD123^-$
	Thymic DC[b]	$HLA\text{-}DR^{mod}CD11c^-CD123^{hi}$
	Thymic DC[b]	$HLA\text{-}DR^{mod}CD11c^+CD123^-$
	Thymic DC[b]	$HLA\text{-}DR^{hi}CD11c^+CD123^-$

Toby

Functional Cell Surface Markers

Antigen uptake receptors

DEC-205, MMR
Langerin, BDCA-2
DC-SIGN, ASGP-R
FCg-R, HSP-R, $\alpha_v\beta_5$

Maturation receptors

TLRs
TNF-Rs

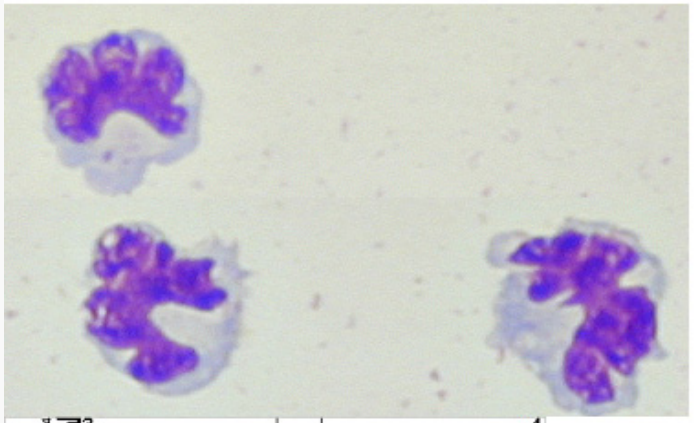
T cell adhesion &
costimulatory molecules

DC-SIGN
CD86 + MHC clusters

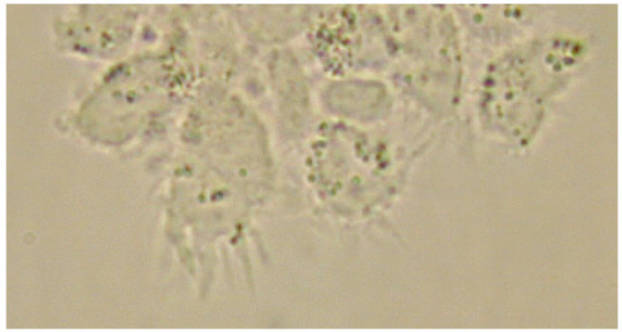
Steinman

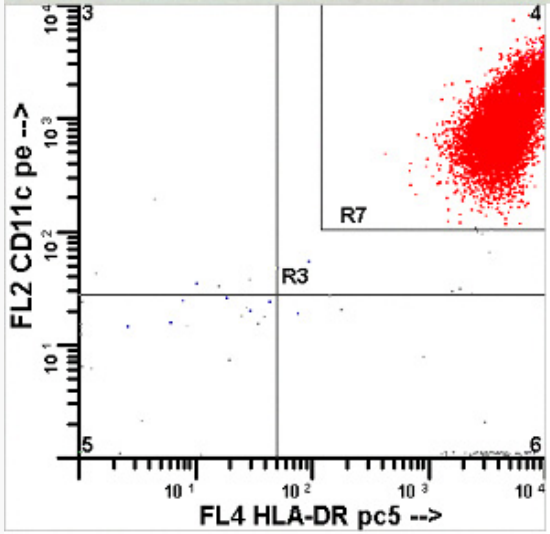
Immunomagnetic Isolation of DC1 using BDCA1

BDCA1+ Day 0

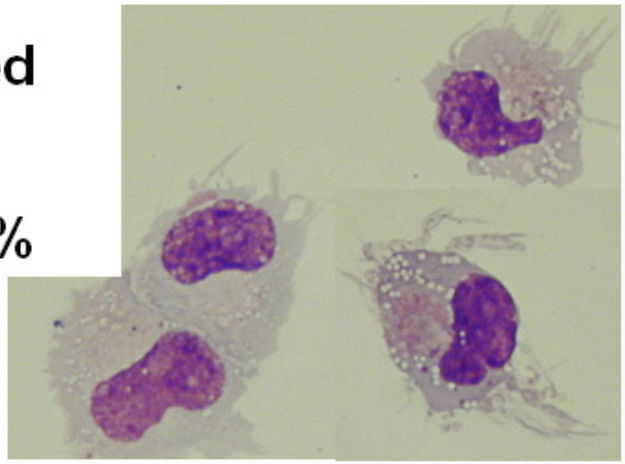


+IL-4 and GM-CSF Day 3



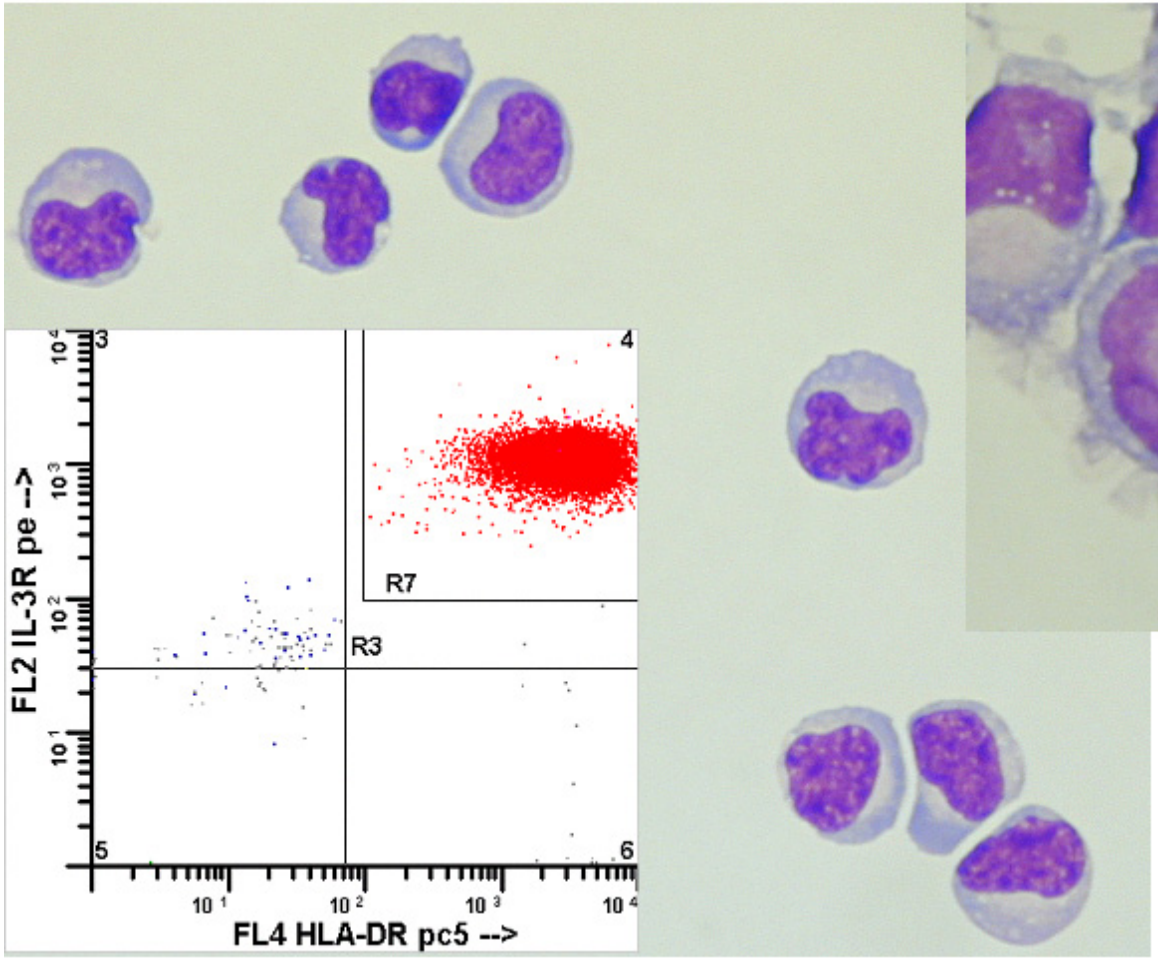


- Start 680 000
- Recovered 172 000
- Purity 66%

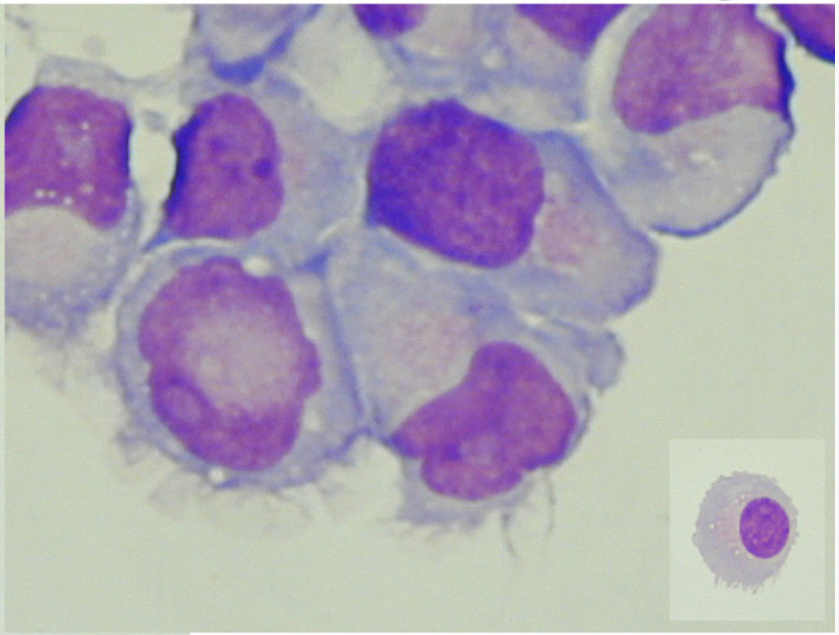


Immunomagnetic Isolation of DC2 using BDCA4

BDCA4+ Day 0

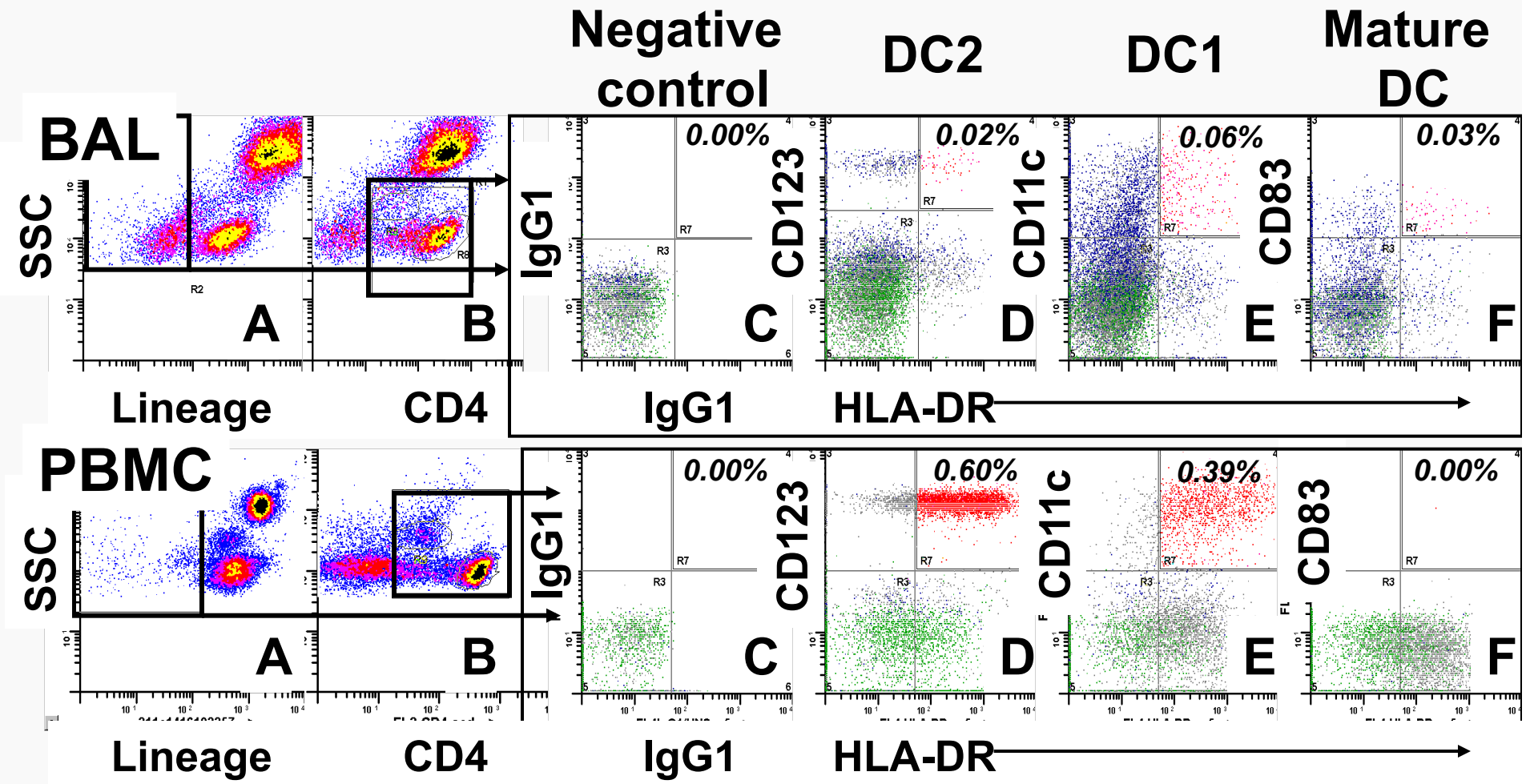


BDCA4+IL-3 Day 3

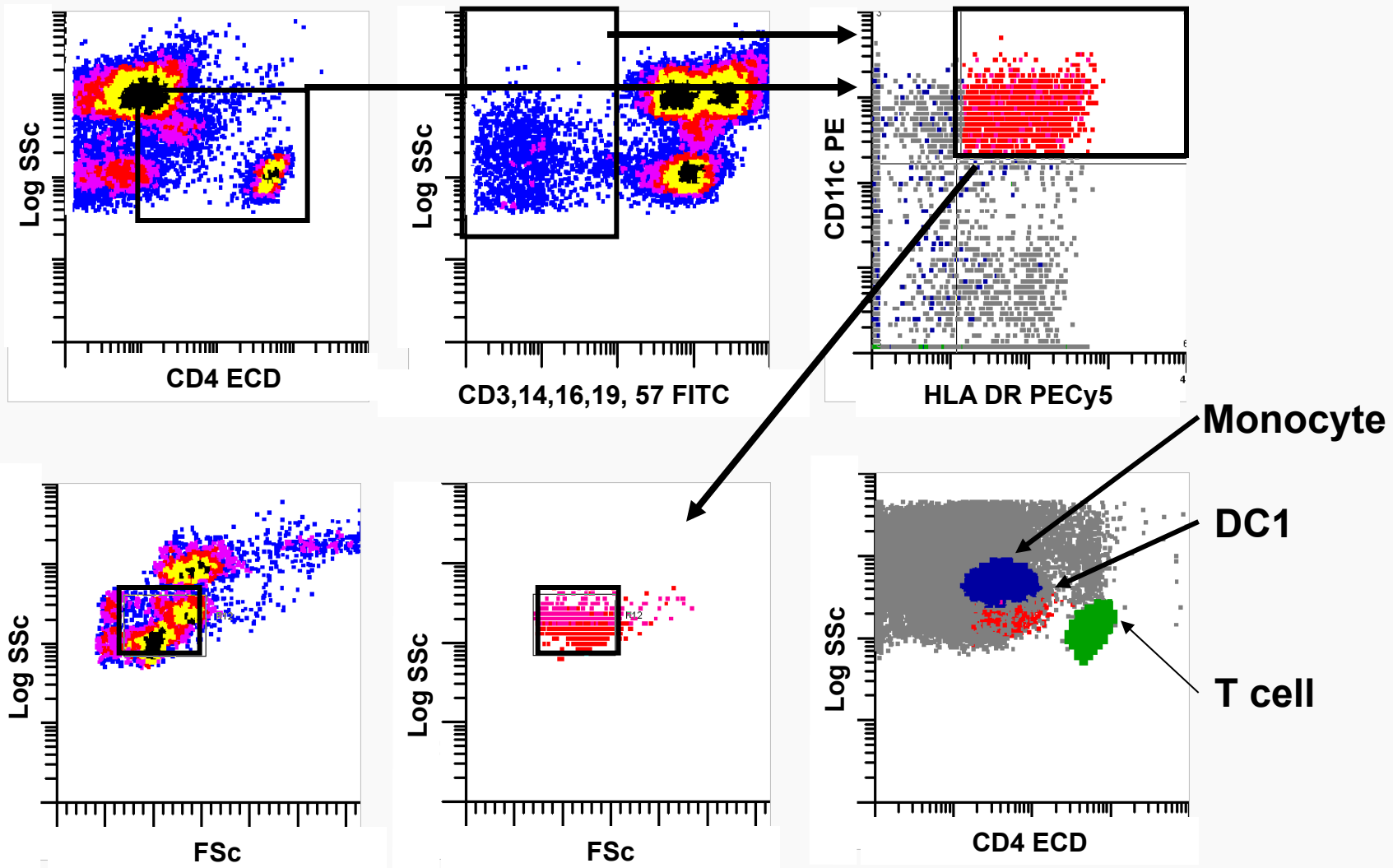


- Start 600 000
- Recovered 460 000
- Purity 89%

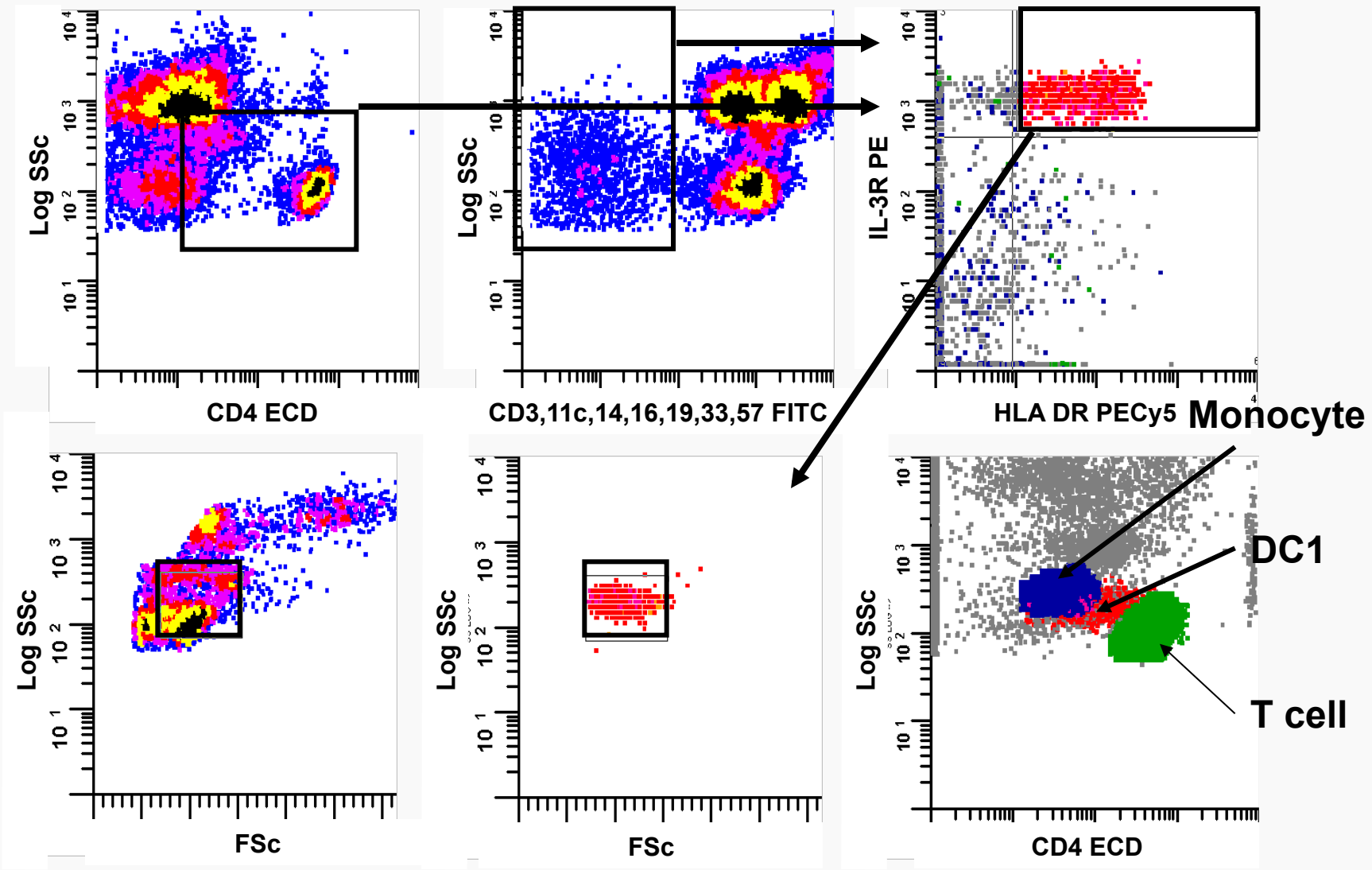
DC in Normal Controls



Dim CD4 Expression on DC1



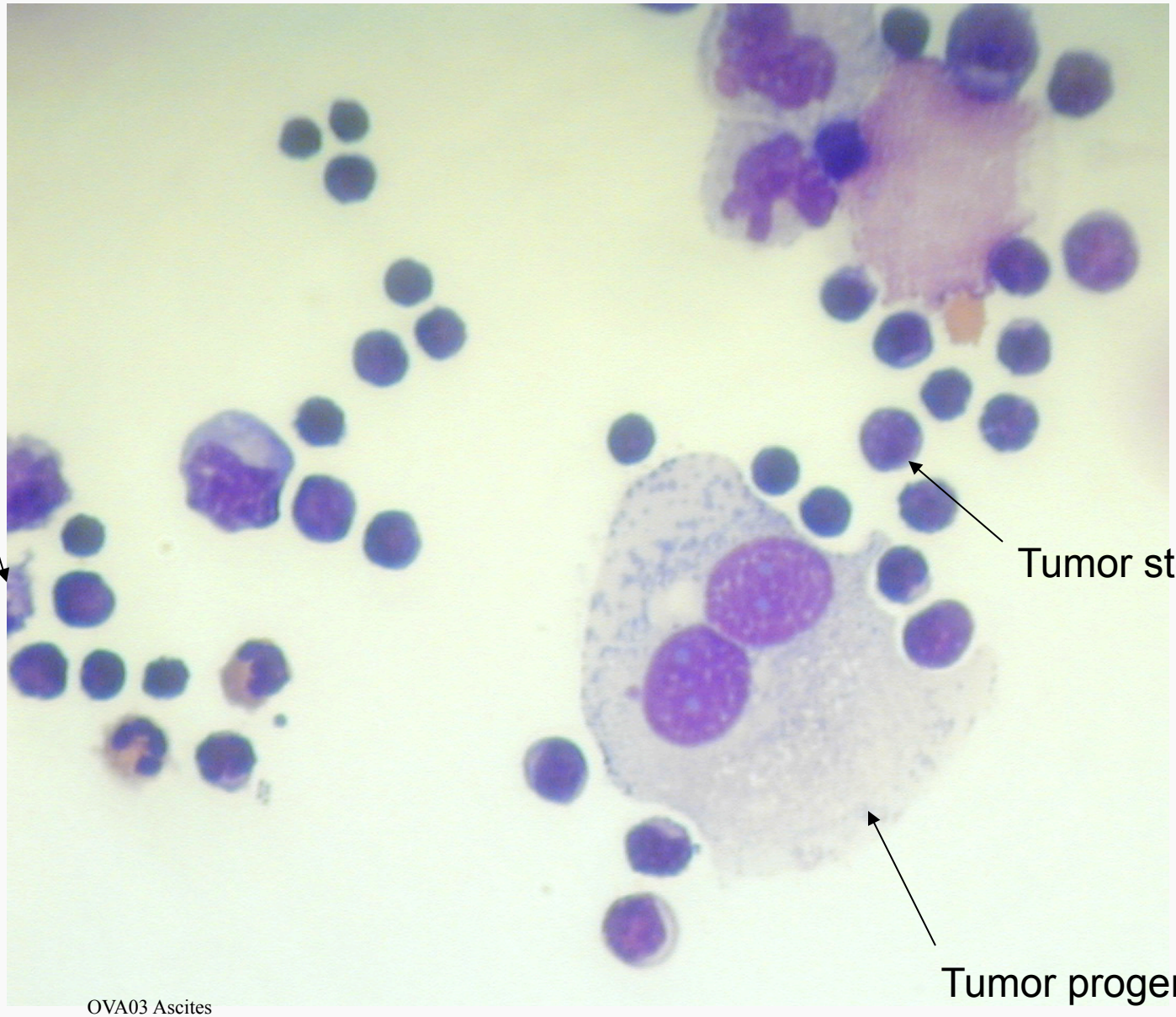
Intermediate CD4 Expression on DC2



III. Applications

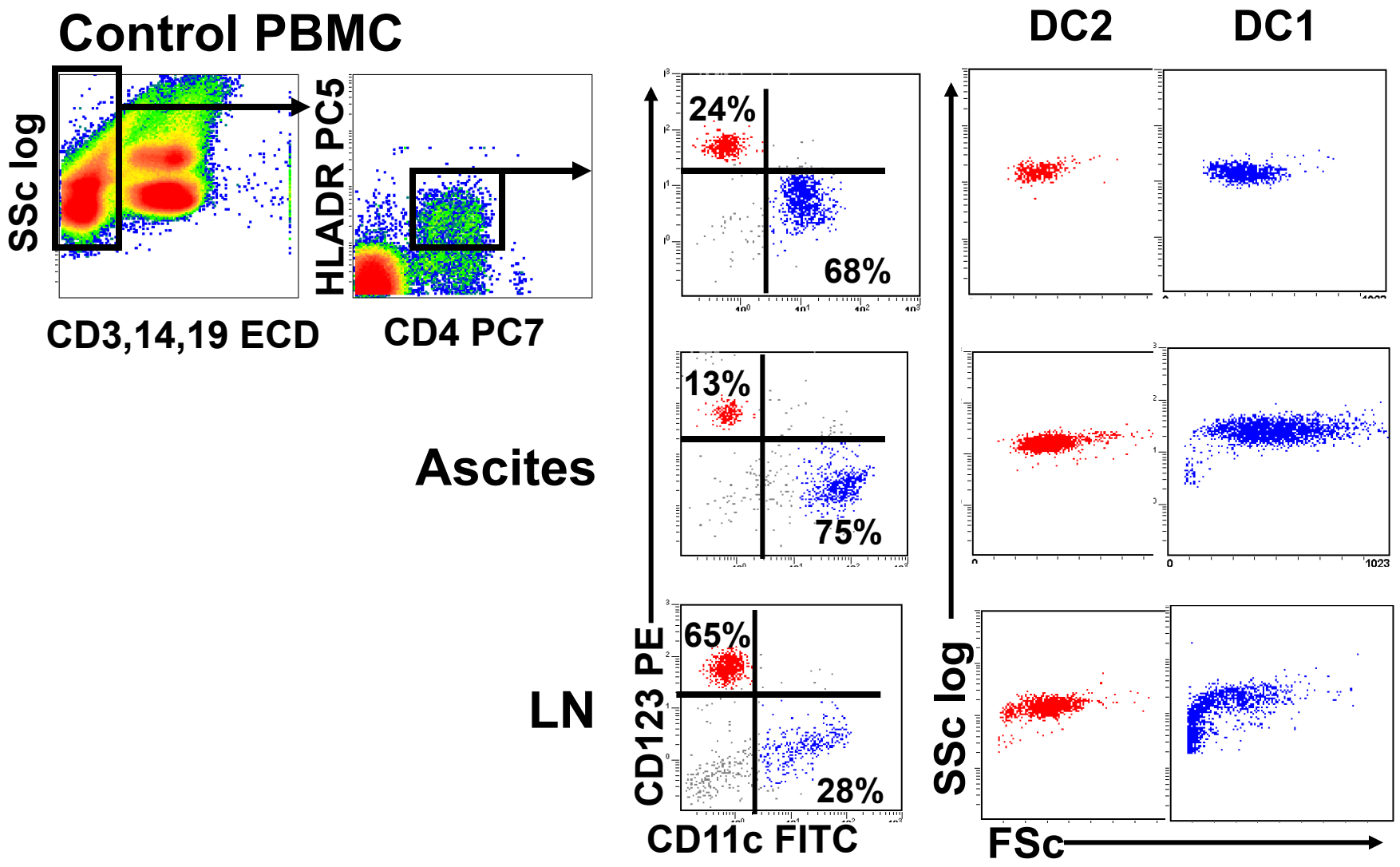
- DC subsets in cancer
- Lung DC in asthma
- DC trafficking in an animal model

Malignant Ovarian Ascites

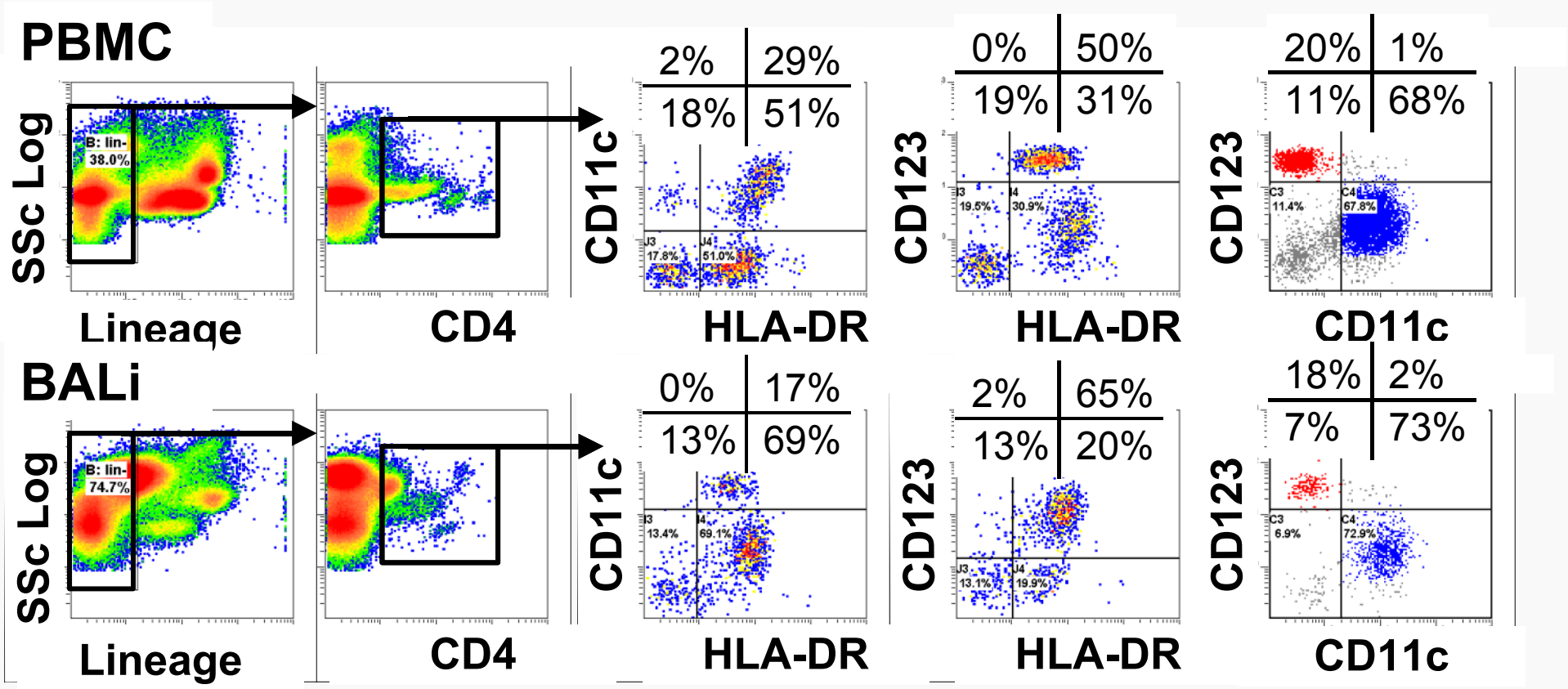


OVA03 Ascites

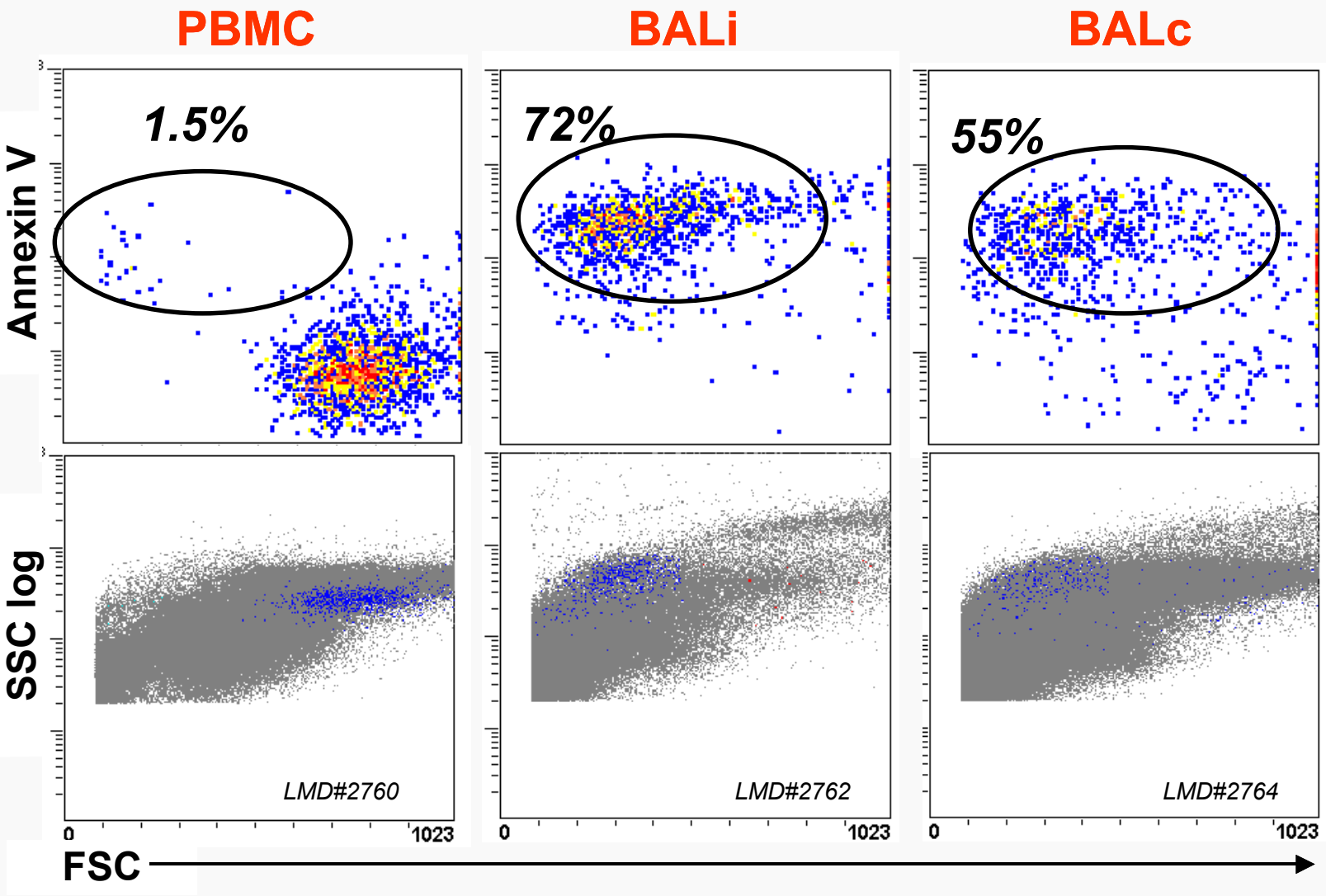
DC1 and DC2 in Ovarian Ca



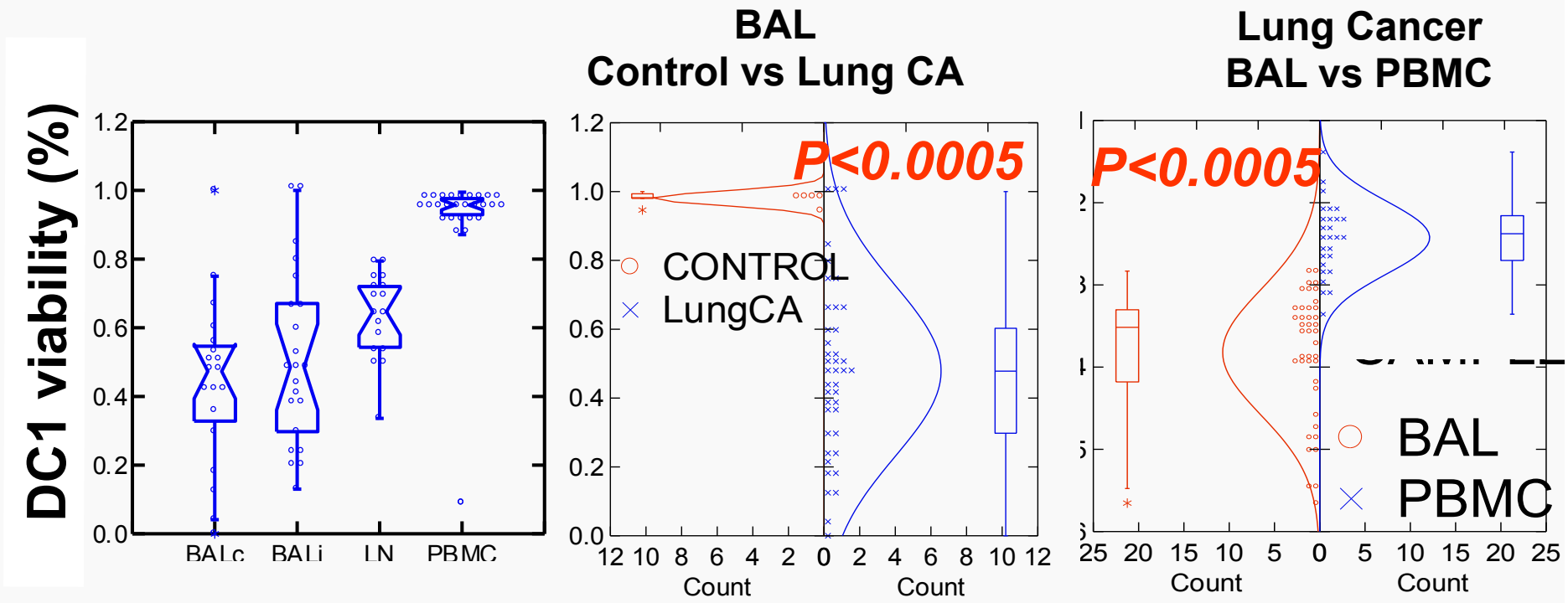
DC1 and DC2 in Lung Ca



Preferential Apoptosis of DC1 in Lung Cancer BALs



DC1 Viability



Conclusions

- DC are readily observed in malignant ascites of Ovarian CA and in BAL from Lung Ca patients.
- The relative proportion is similar to peripheral blood (DC1>DC2)
- Peri-tumor DC1 but not DC2 spontaneously apoptose in Ovarian and Lung CA
- In Lung Ca, DC1 in the lung contralateral to the tumor also have elevated apoptosis
- Preferential induction of DC1 apoptosis may represent a tumor survival mechanism (Th2 polarization)

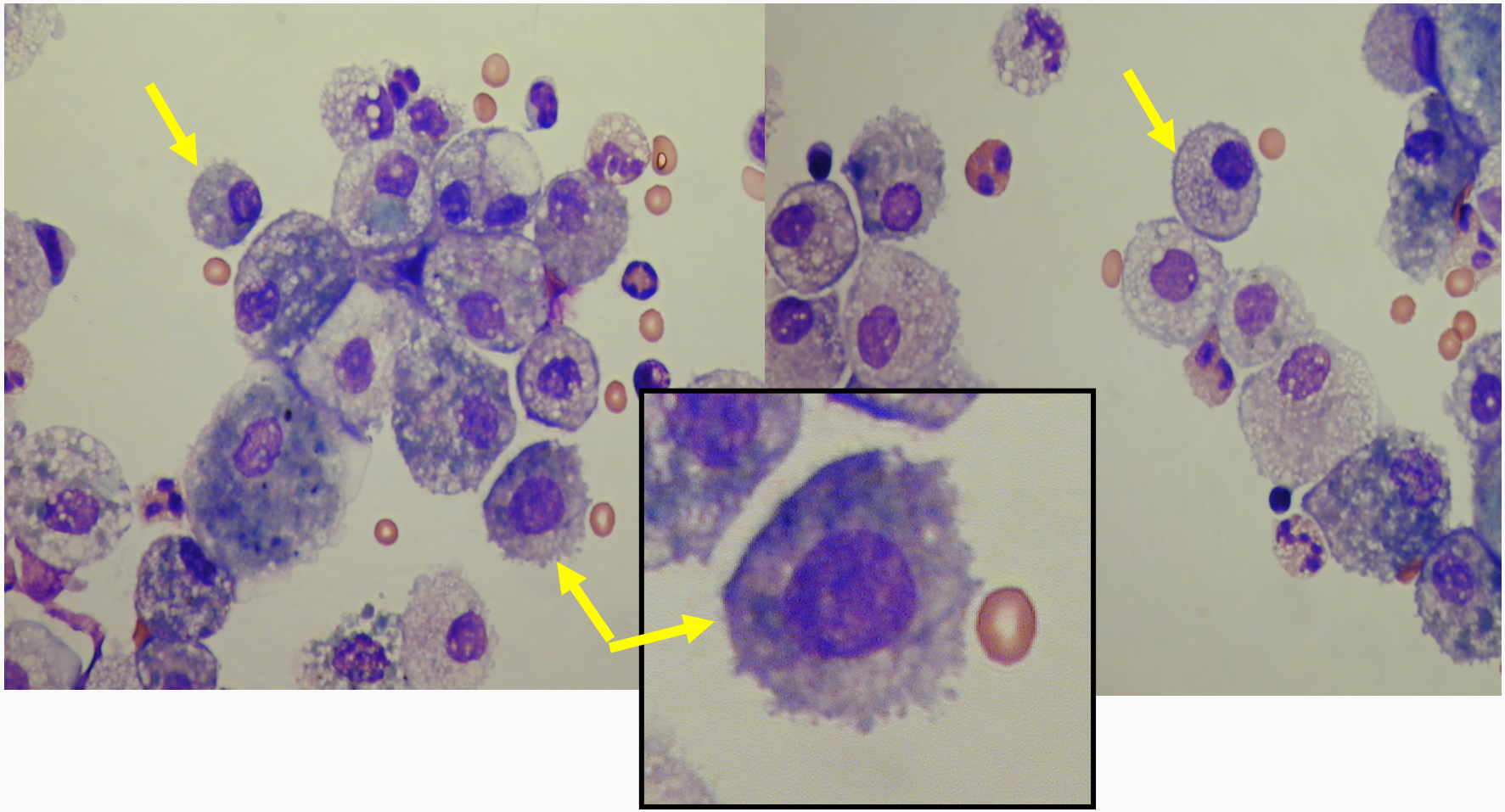
Dendritic Cells in Asthma

- DC are present at the interface between host and environment and can sample and process inhaled antigens
- DC express FcεRI and thus capture IgE. This increases the efficiency of processing inhaled allergens
- DC present processed allergens to naive and memory $CD4^+$ T cells
- Antigen dose (low), antigen exposure (chronic), costimulatory signals (e.g. CD80/CD86-CD28, CD30-CD30L, CD40-CD40L) and environmental cytokines (IL-4 from mast cells) all favor Th-2 polarization
- DC subsets?

Patients

- 5 healthy volunteer subjects
- 5 atopic asthma patients before and after challenge with *m Farinae*
- BAL before and 3 days after antigen challenge

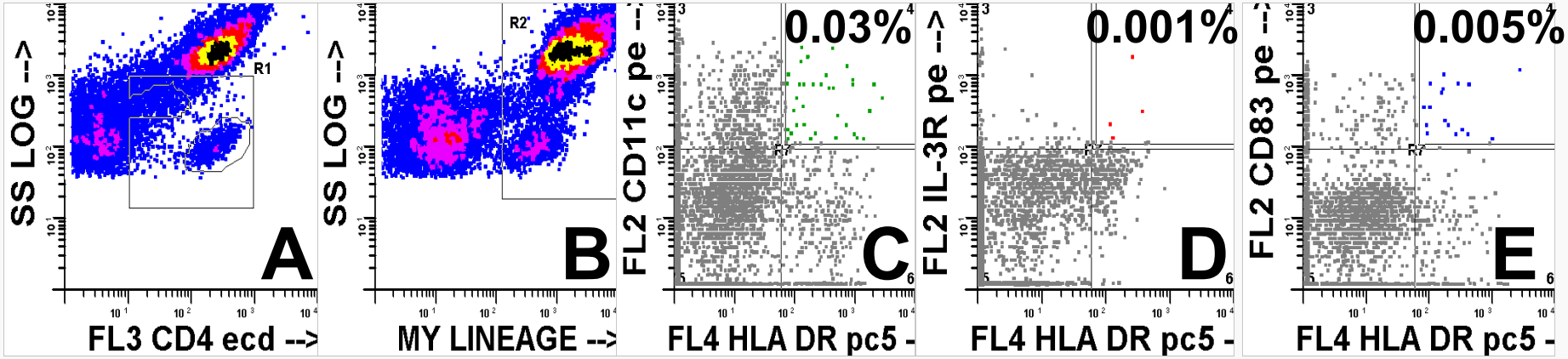
BAL Composition



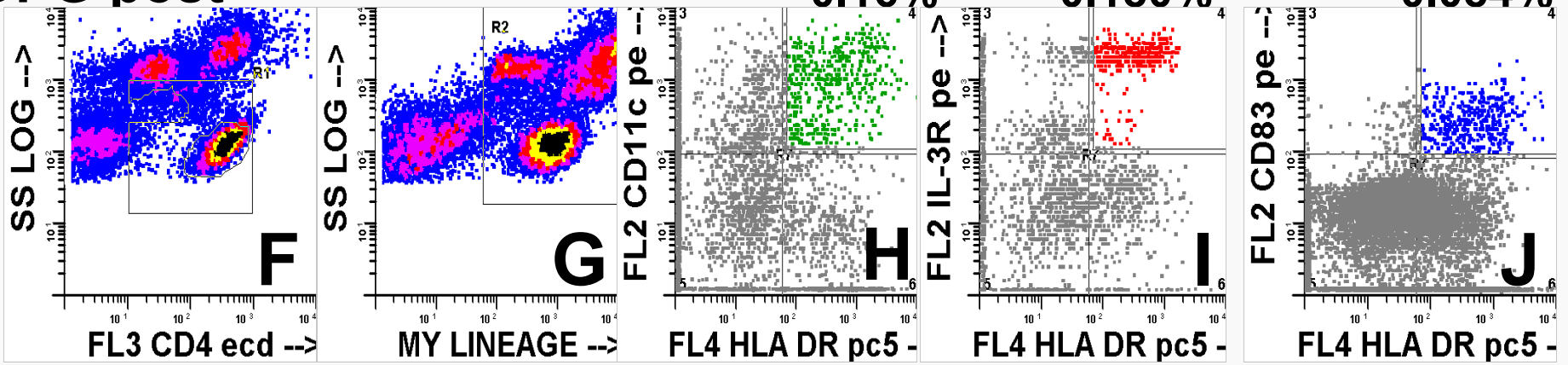
BAL014

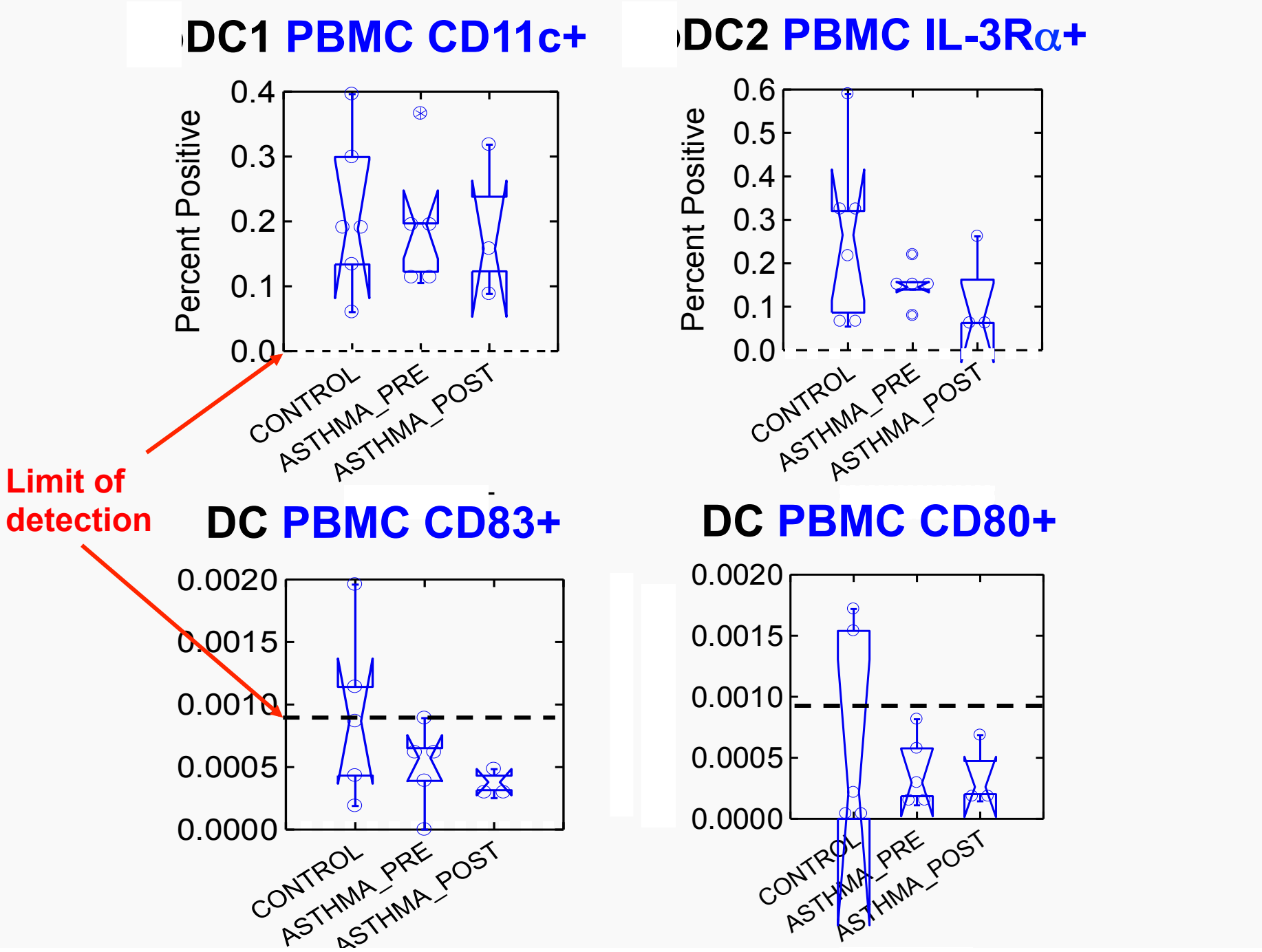
DC1, DC2 and mature DC in BAL: Asthma Pre/Post Ag challenge

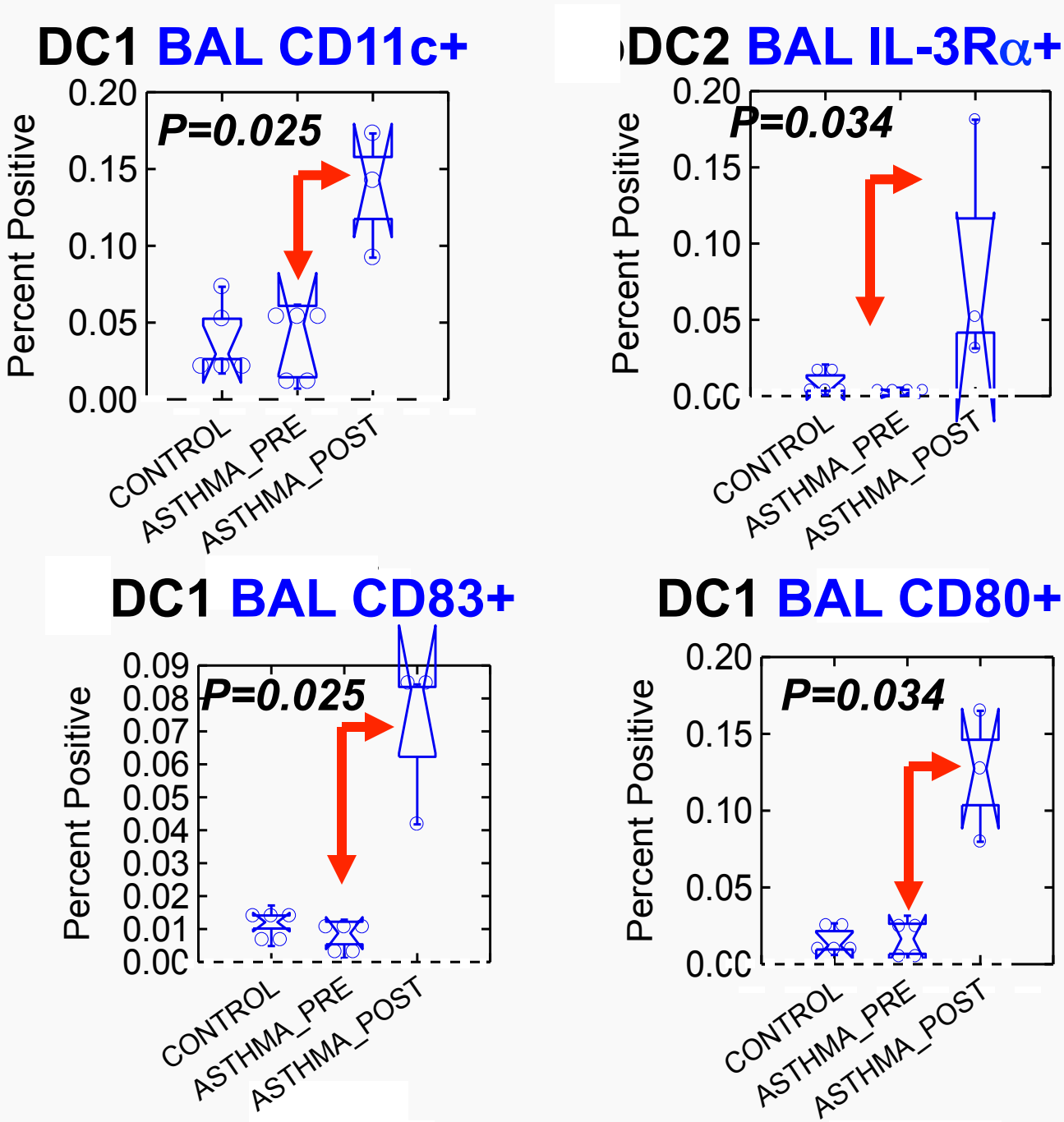
DPG pre



DPG post




DC1 PBMC CD11c+
Percent Positive
0.4
0.3
0.2
0.1
0.0
CONTROL
ASTHMA_PRE
ASTHMA_POST
DC2 PBMC IL-3Rα+
Percent Positive
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CONTROL
ASTHMA_PRE
ASTHMA_POST
Limit of detection
DC PBMC CD83+
0.0020
0.0015
0.0010
0.0005
0.0000
CONTROL
ASTHMA_PRE
ASTHMA_POST
DC PBMC CD80+
0.0020
0.0015
0.0010
0.0005
0.0000
CONTROL
ASTHMA_PRE
ASTHMA_POST


DC1 BAL CD11c+
P=0.025
Percent Positive
0.20
0.15
0.10
0.05
0.00
CONTROL
ASTHMA_PRE
ASTHMA_POST
DC2 BAL IL-3Rα+
P=0.034
Percent Positive
0.20
0.15
0.10
0.05
0.00
CONTROL
ASTHMA_PRE
ASTHMA_POST
DC1 BAL CD83+
P=0.025
Percent Positive
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
CONTROL
ASTHMA_PRE
ASTHMA_POST
DC1 BAL CD80+
P=0.034
Percent Positive
0.20
0.15
0.10
0.05
0.00
CONTROL
ASTHMA_PRE
ASTHMA_POST

SUMMARY

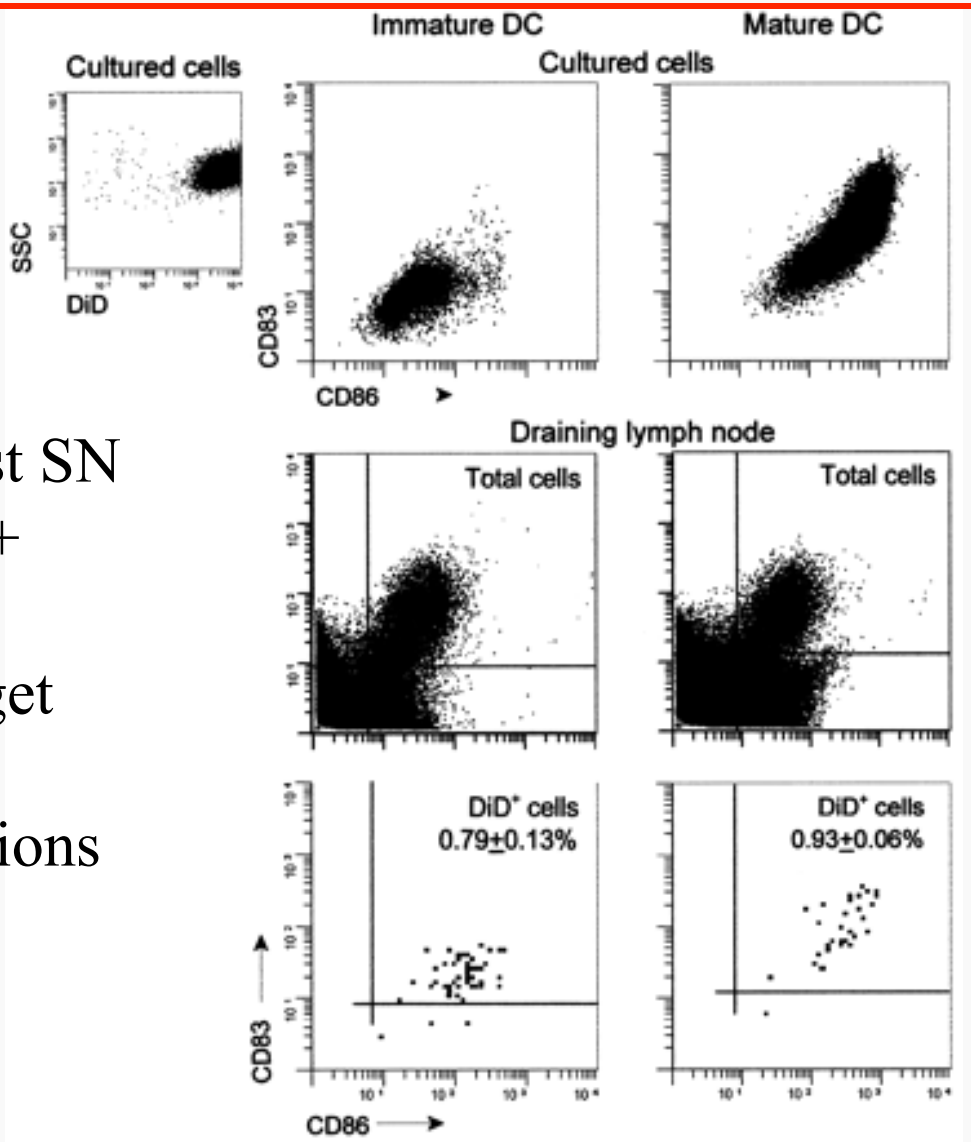
- No difference in peripheral DC in asthma and control subjects
- Both groups had detectable DC1 and DC2 (DC1>DC2), but no mature DC in the peripheral circulation
- No difference in BAL DC in asthma (pre challenge) and control subjects: both had DC1>>DC2. Neither had populations of mature DCs
- After antigenic challenge asthma patients had increased DC1 and mature myeloid DC ($CD83^{+}$)

Protocol

- Rhesus macaque
- Monocyte-derived DC cultured with IL-4, GM-CSF, CD40L
  - Immature 3 days
  - Mature 7 days
- Injected subcutaneously
- 36 hours later, draining LN removed and assayed for DiD+ DC
- 1.5 x 10^6 LN cells assayed in triplicate

Injected DCs mature on route to draining LN

- Dump Gate
- DiD/red laser: highest SN
- Cultured cells to set + gate
- Contralateral LN to get LLD
- Triplicate determinations to measure SD



Conclusions

- DC measurement is a rare event problem
- DC subsets and function can be measured by multiparameter flow cytometry
  - Apoptosis
  - Expression of costimulatory and adhesion molecules
  - Cytokine secretion
- DC biology is important in cancer, allergy, autoimmunity, infectious disease, transplantation, vaccines

Acknowledgements

- Vera Donnenberg
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  - E Michael Meyer
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