No cell is an island: the importance of immune cell doublets in flow cytometry and single cell RNA sequencing

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2019 WACD Meeting
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La Jolla Institute FOR IMMUNOLOGY | Life Without Disease.®

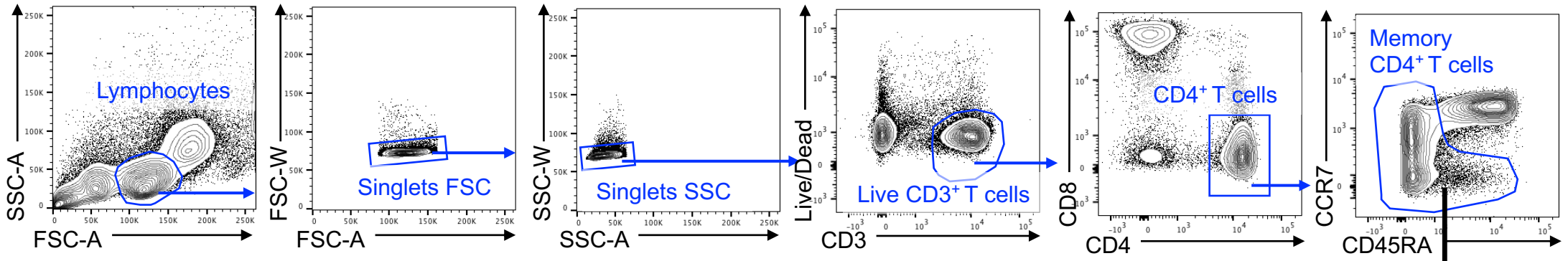
A 74-gene signature of latent TB in memory CD4 T cells

Frozen PBMC

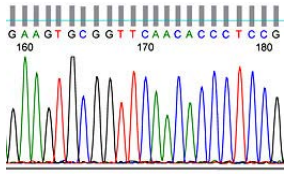
n=29 TB negative
n=30 Latent TB

- Various ethnicity
- 2 geographic locations: San Diego, Peru

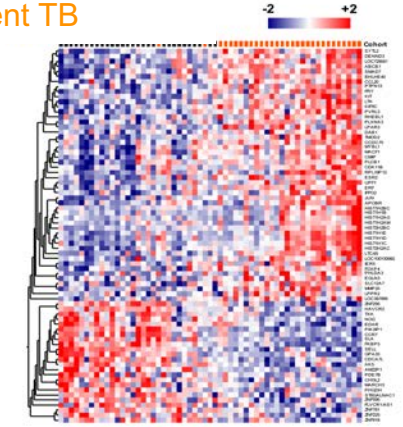
Memory CD4 T cells sorting

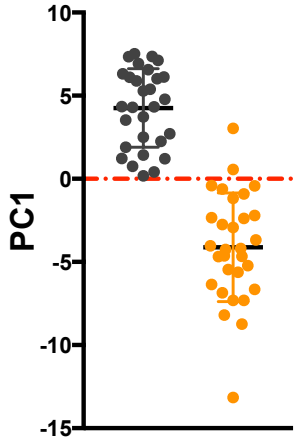


RNA Sequencing



Controls
Latent TB

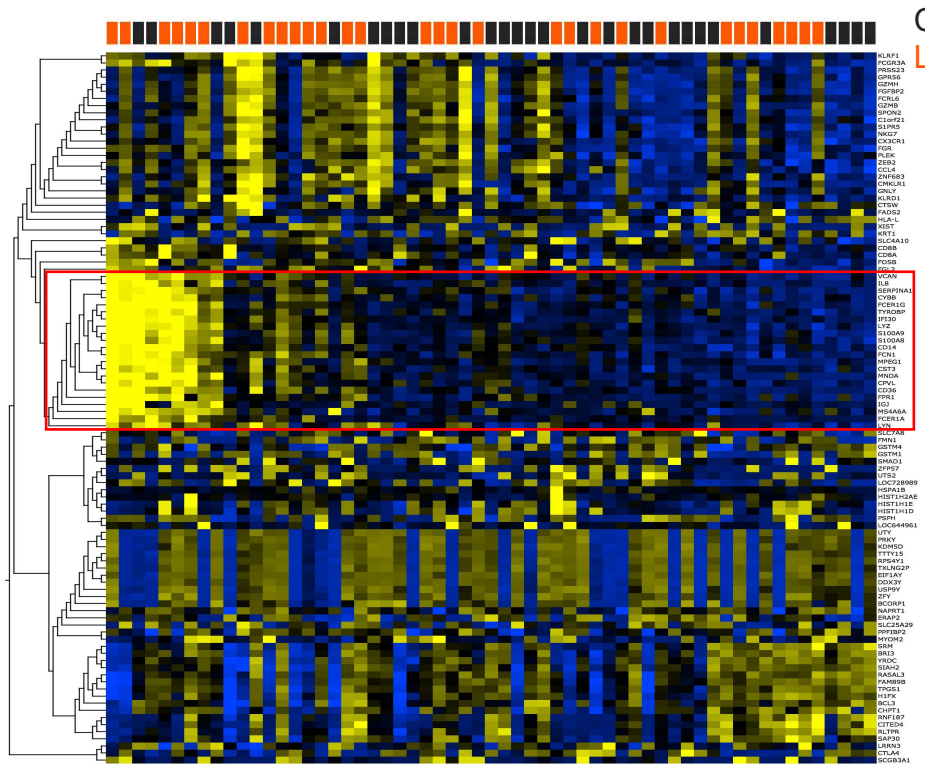




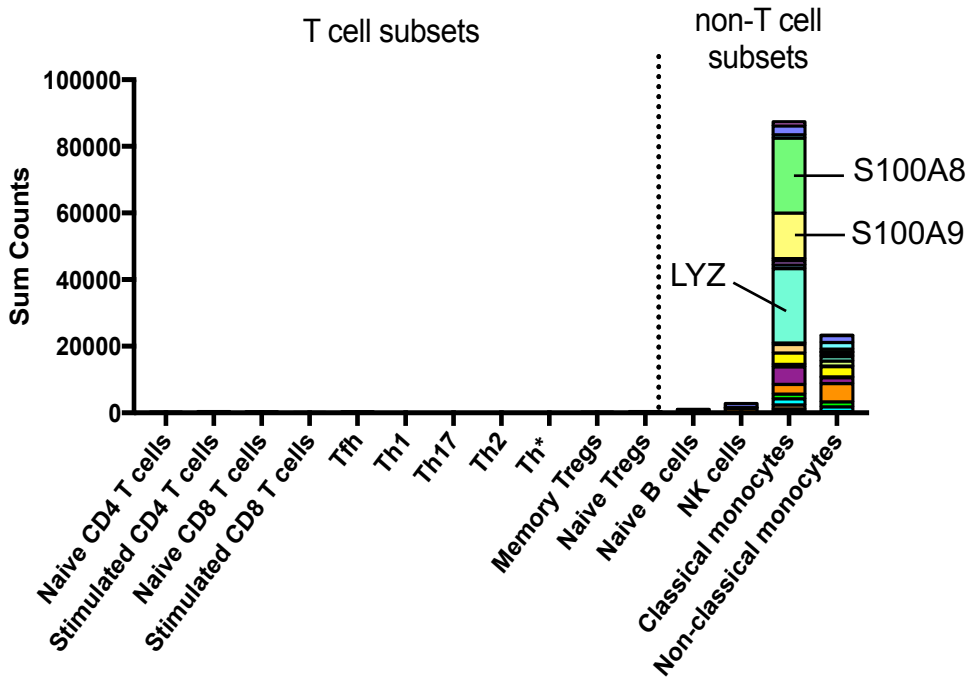
PC1 < 0
Specificity: 100% (29/29)
Sensitivity: 93% (28/30)

Burel et al, *J Immunol.* 2018

Unexpected detection of monocyte gene signature in sorted CD4$^+$ memory T cells from healthy and latent TB subjects

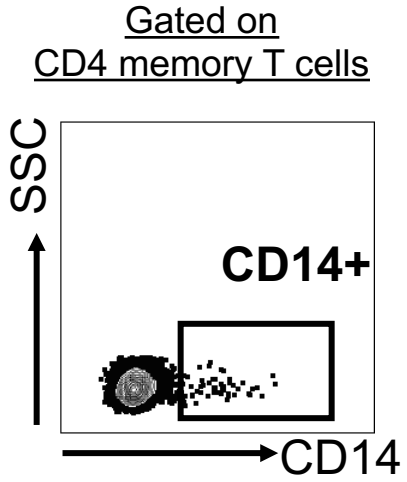


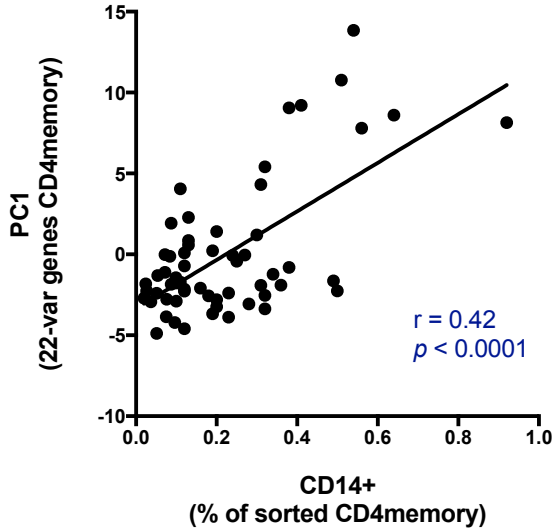
CD14
CD36
CPVL
CST3
CYBB
FCER1A
FCER1G
FCN1
FPR1
IFI30
IGJ
IL8
LYN
LYZ
MNDA
MPEG1
MS4A6A
S100A8
S100A9
SERPINA1
TYROBP
VCAN

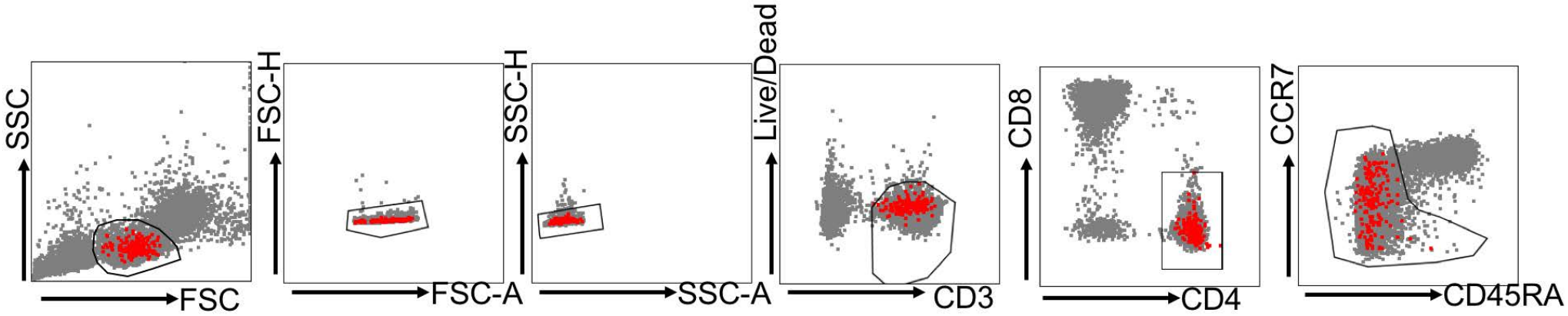


Burel et al, *eLife* 2019

The monocyte gene signature correlates with a population of CD14+ cells contained within CD4^{+} memory T cells

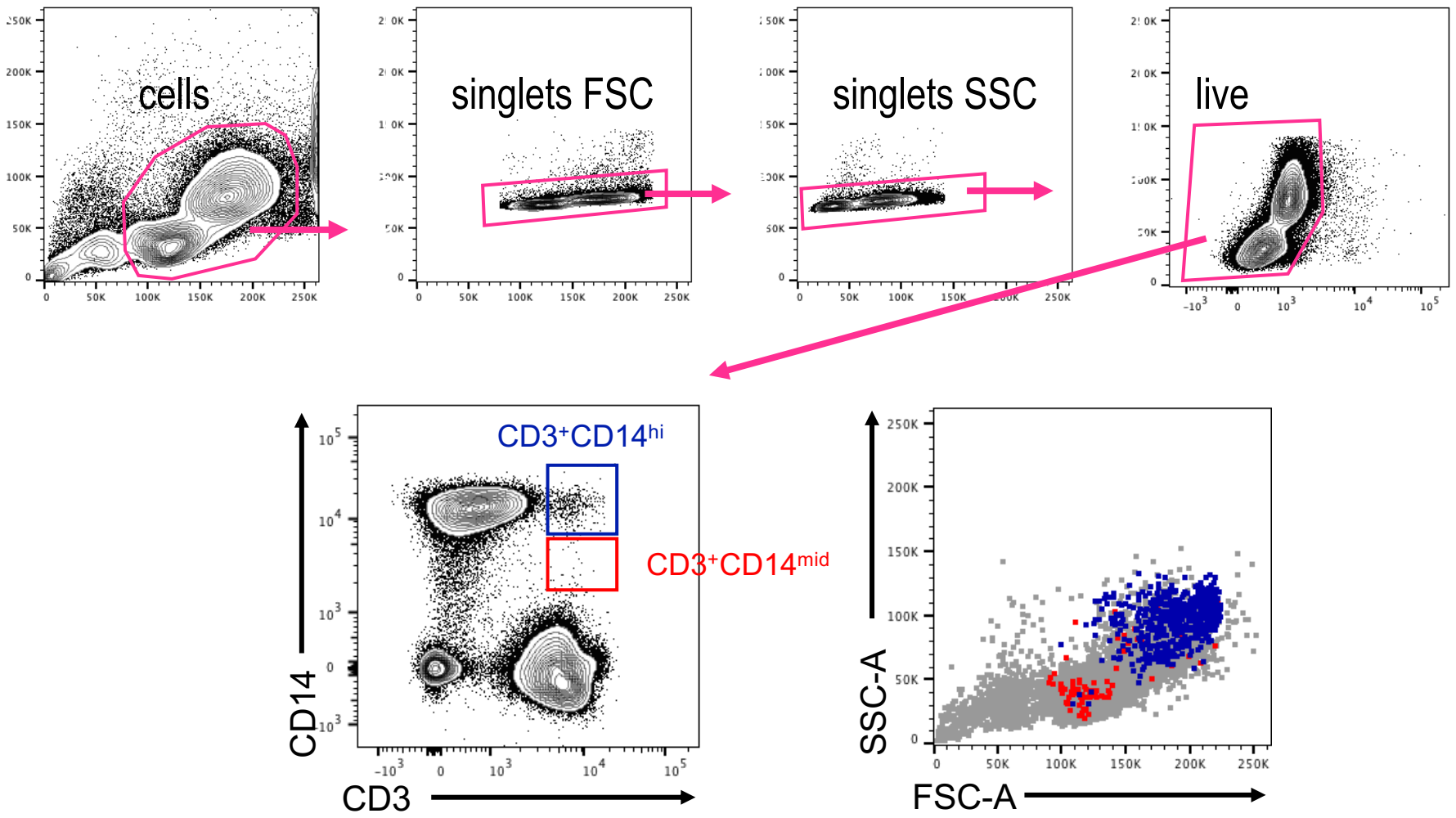




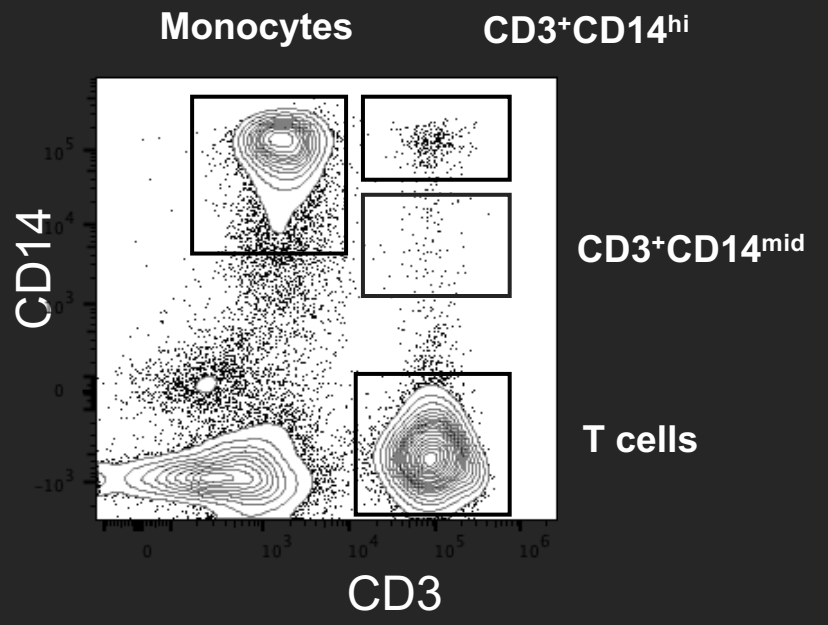


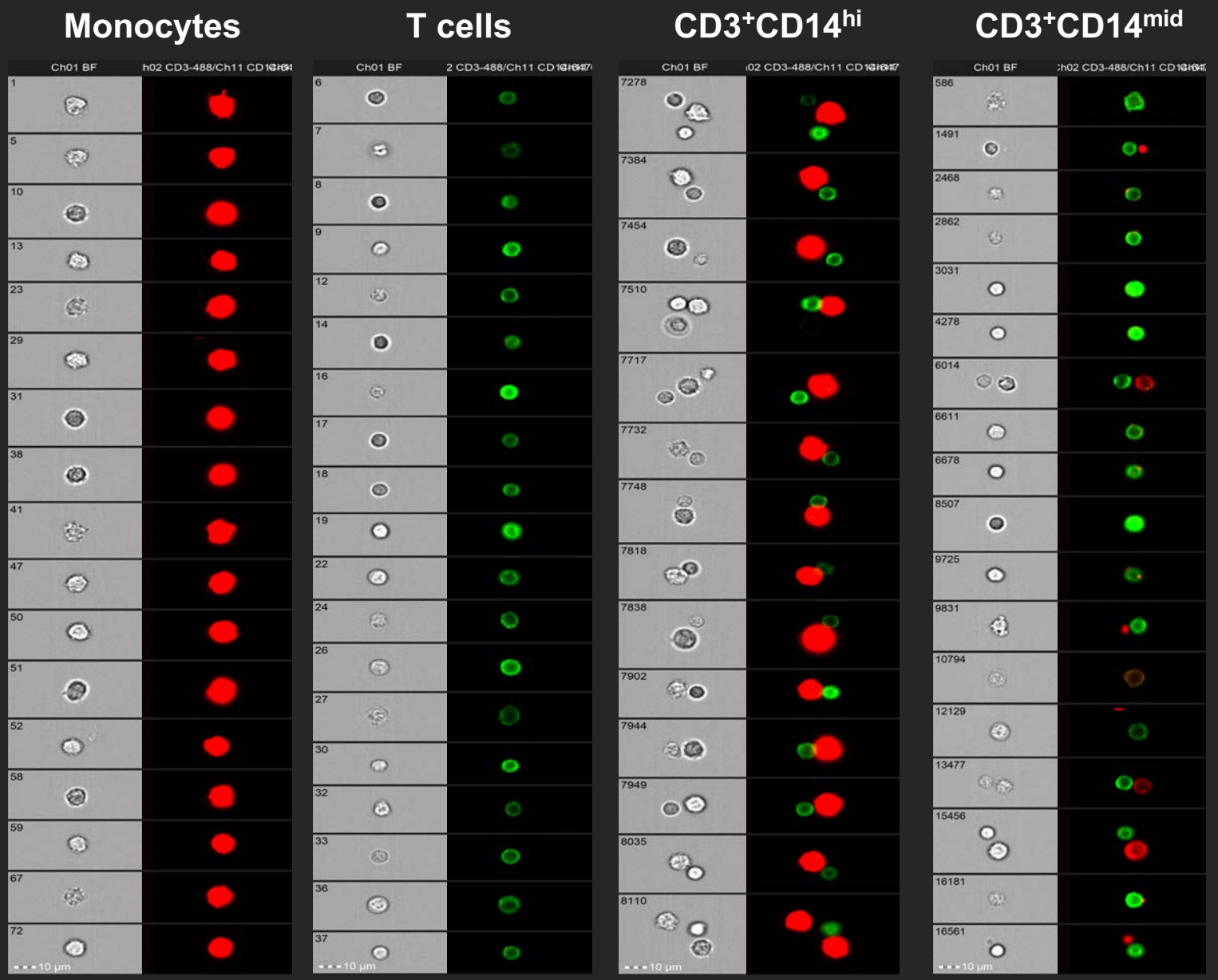
CD14+ memory CD4 T cells

$CD3^+CD14^+$ cells can be divided in two subpopulations



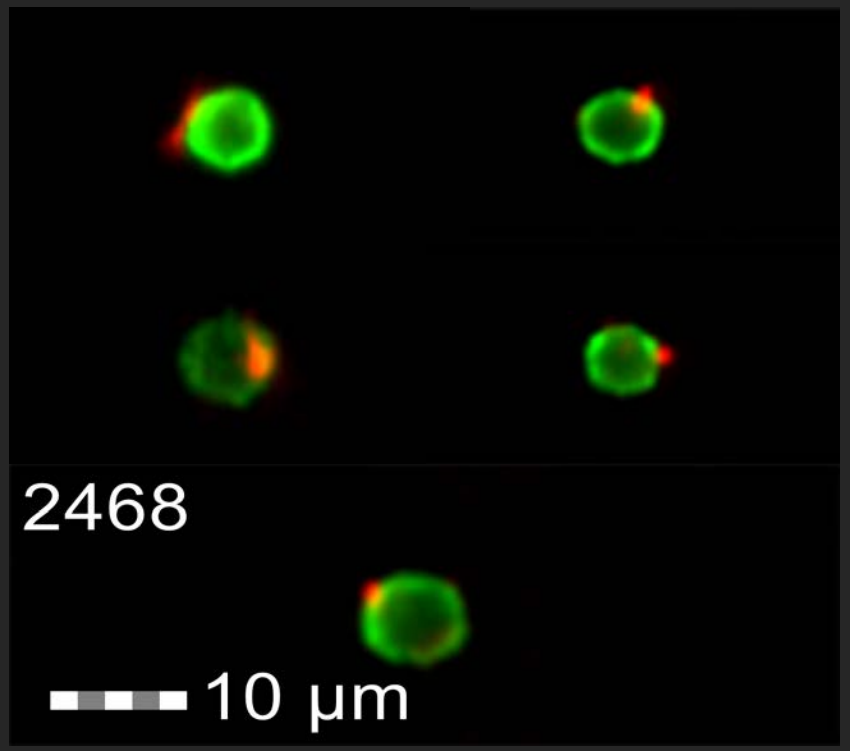
Imaging flow cytometry analysis of $CD3^{+}CD14^{+}$ cells





CD3+CD14^{mid} cells are T cells associated with monocytes fragments

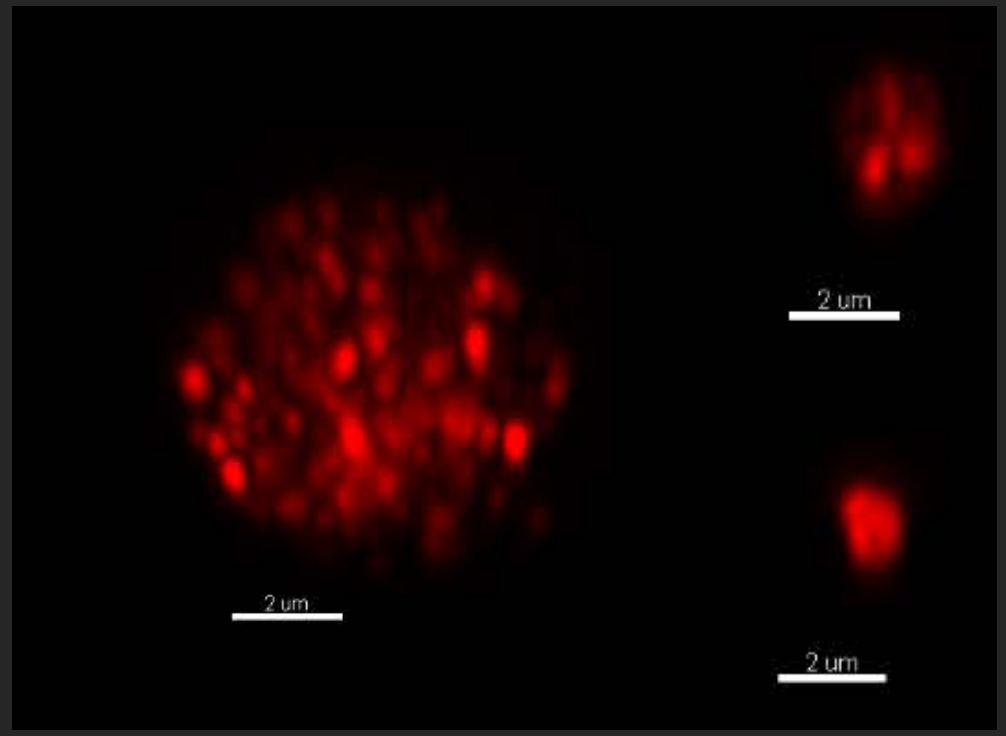
Imaging flow cytometry



Confocal microscopy

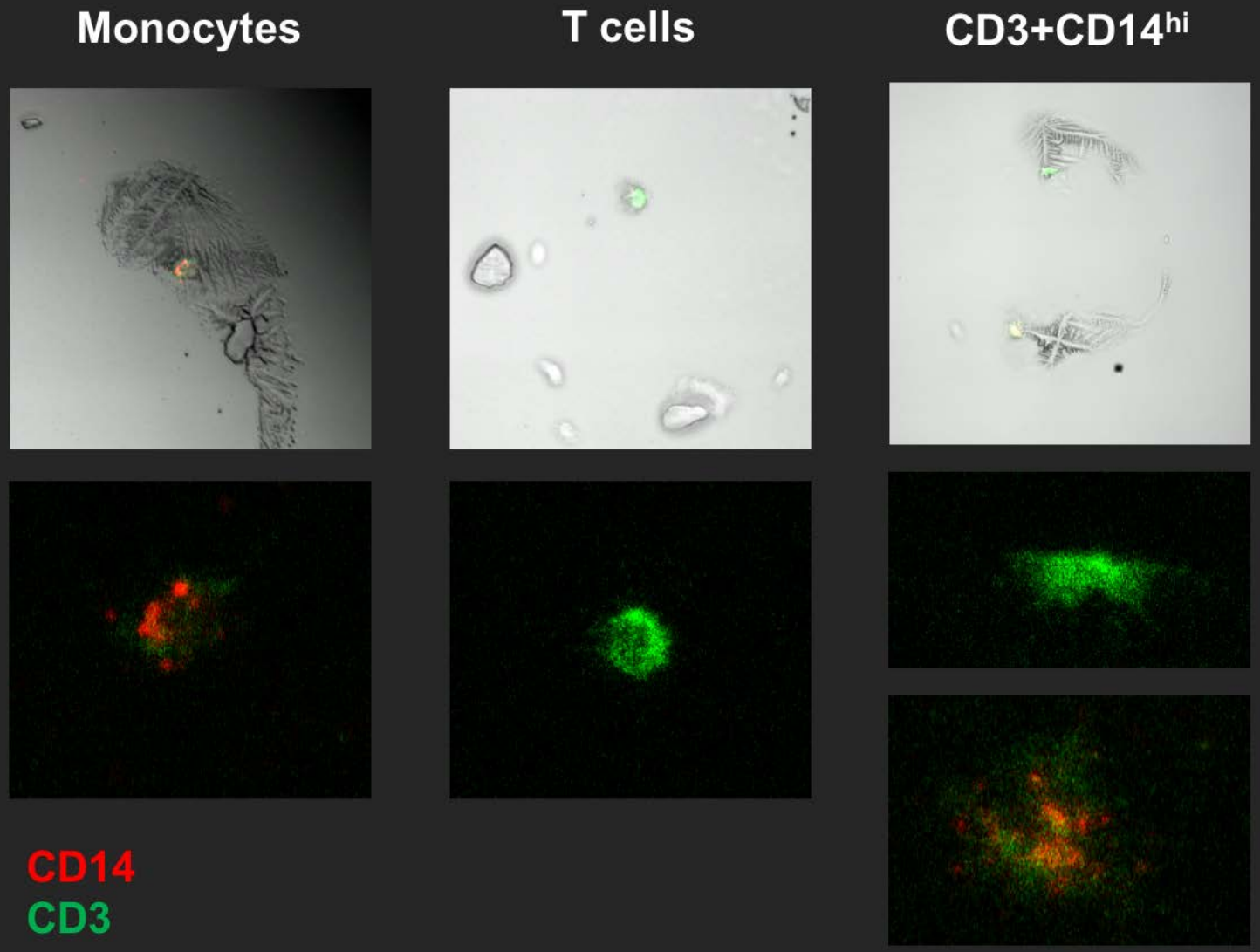
Intact monocyte

Fragments?

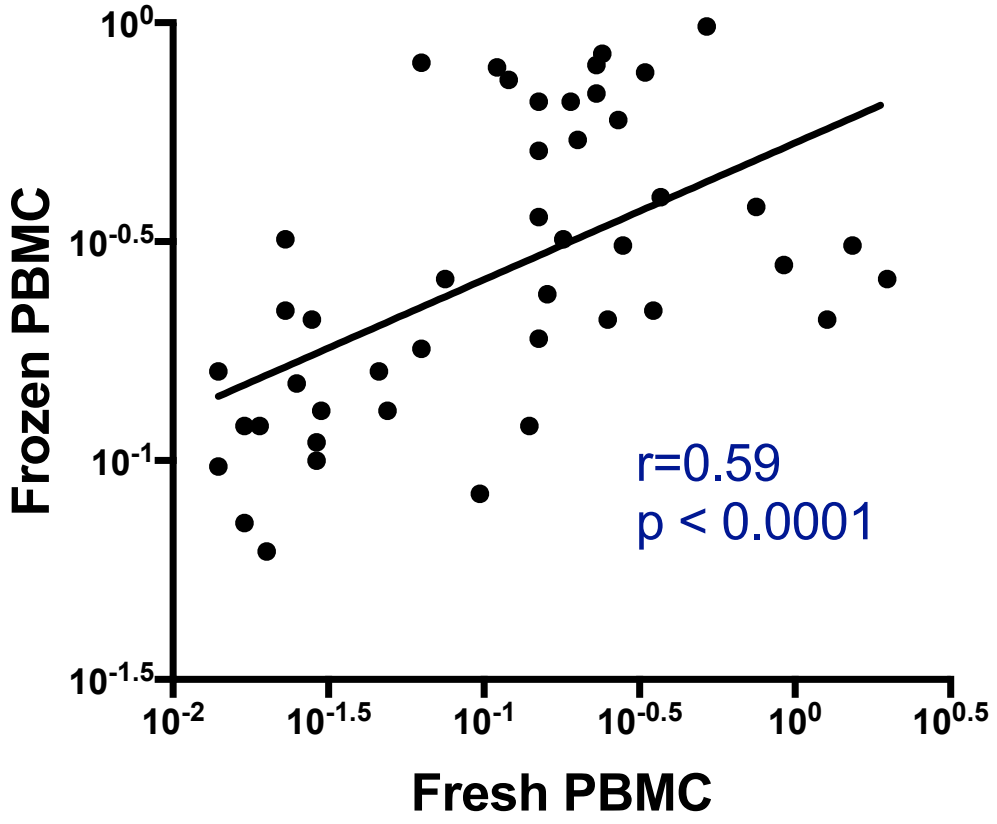


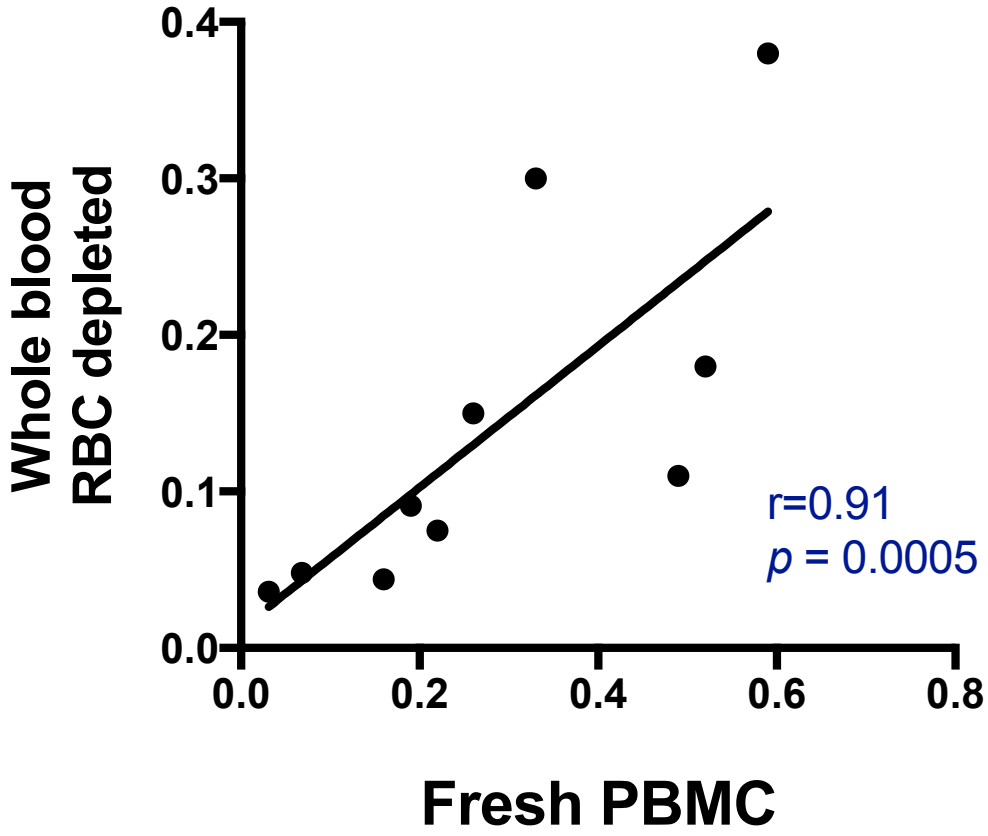
CD14
CD3

Microscopic evaluation of single cell sorts confirm the existence of Tcell:Monocyte complexes



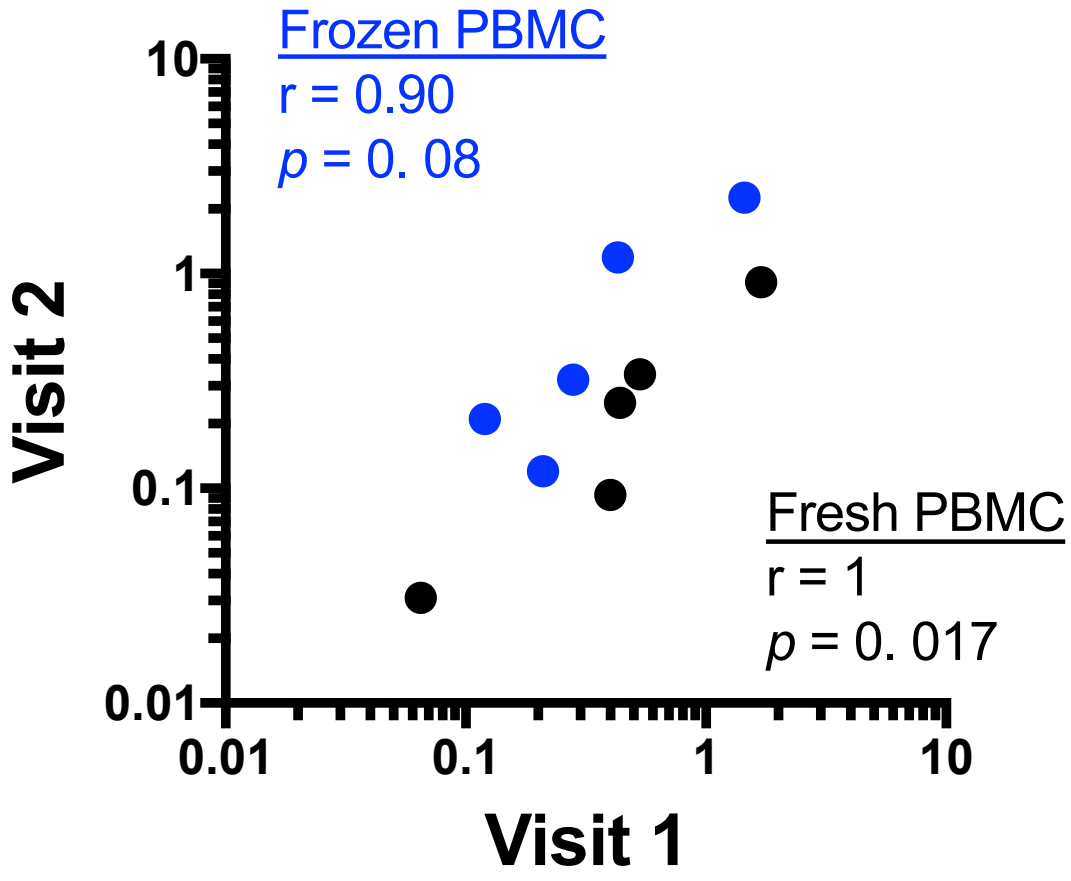
Ex vivo sample manipulation do not impact on the frequency of T cell:monocyte complexes



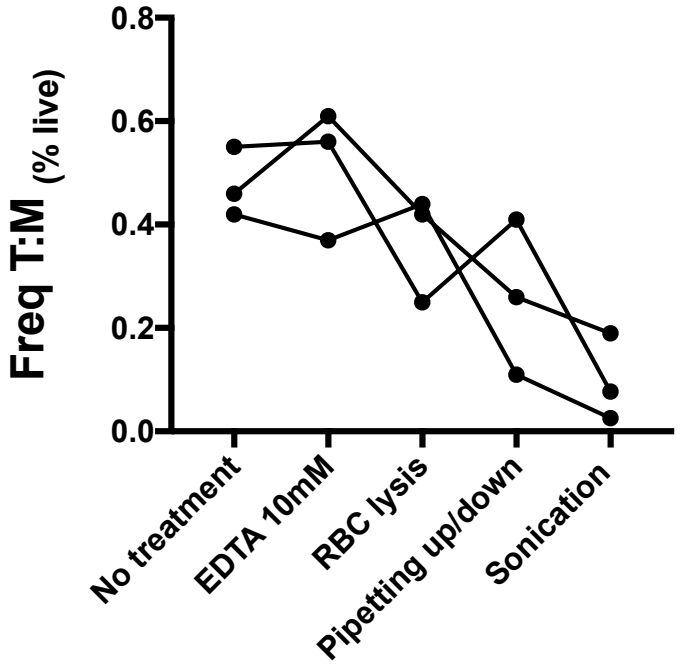


The frequency of Tcell:monocyte complexes is stable over time in healthy individuals

5 Healthy individuals
PBMC collected 1 week apart



T cell:monocyte complexes can be disrupted by physical and chemical manipulation

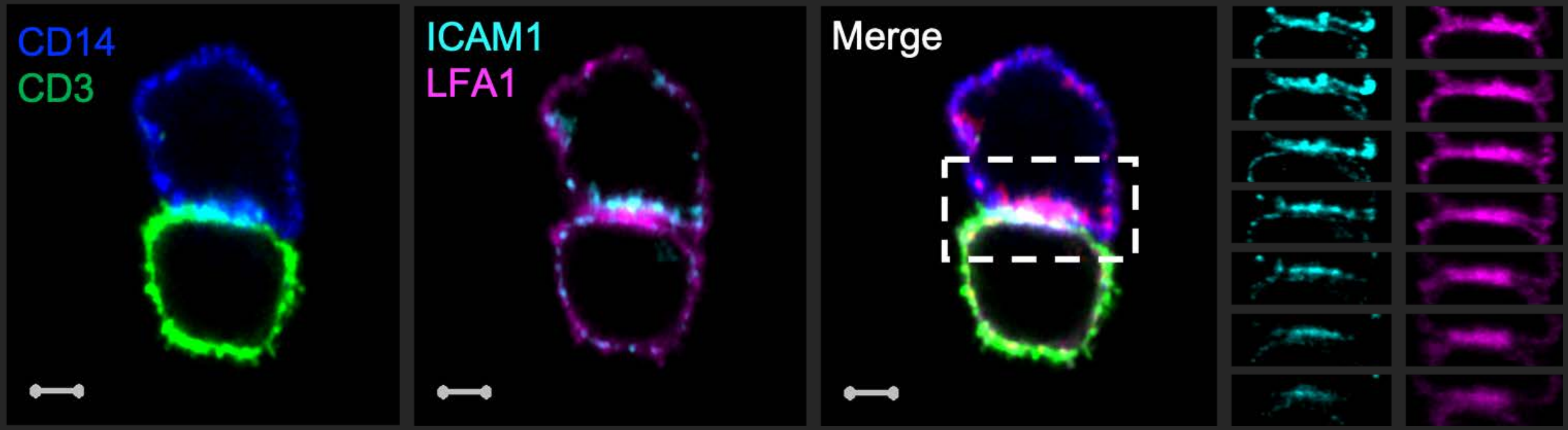


Condition	Pre staining treatment	Post staining treatment
No treatment	None	None
EDTA 10mM	None	Final resuspension in staining buffer at 10mM EDTA (instead of 2mM)
RBC Lysis	Incubate 10min at RT with 4mL 1X RBC lysis reagent, wash twice in staining buffer	None
Pipetting up/down	None	Vigorous pipetting up and down for 30sec after final resuspension in staining buffer
Sonication	None	Sonication for 2min 30sec at 42 kHz

$CD3^{+}CD14^{+}$ T cell:monocyte complexes present ‘immune synapses like’ structures

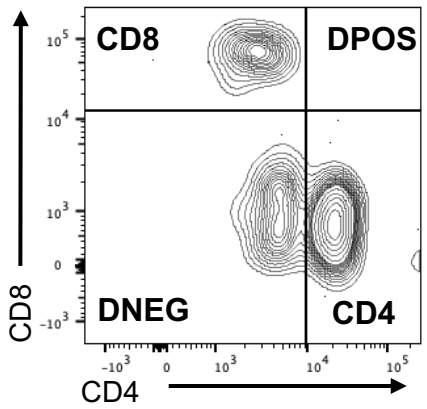
Yunmin Jung

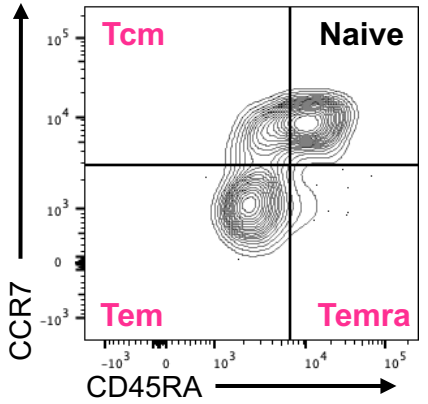
- Upon T cell activation, LFA-1 and ICAM-1 polarize at the immune synapse

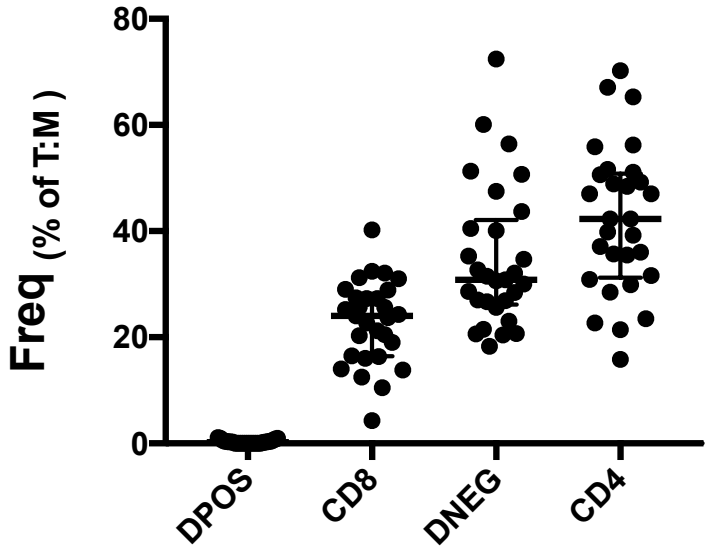


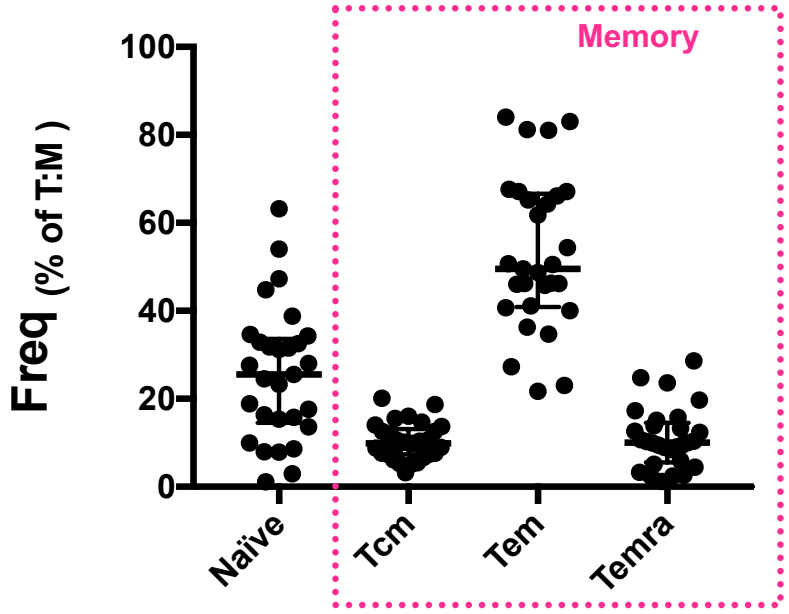
- Polarization of LFA-1 or ICAM-1 in intact Tcell:Monocyte complexes was observed in 29% (thirty out of 105) doublets analyzed

All T cell subsets can be found in a complex with monocyte

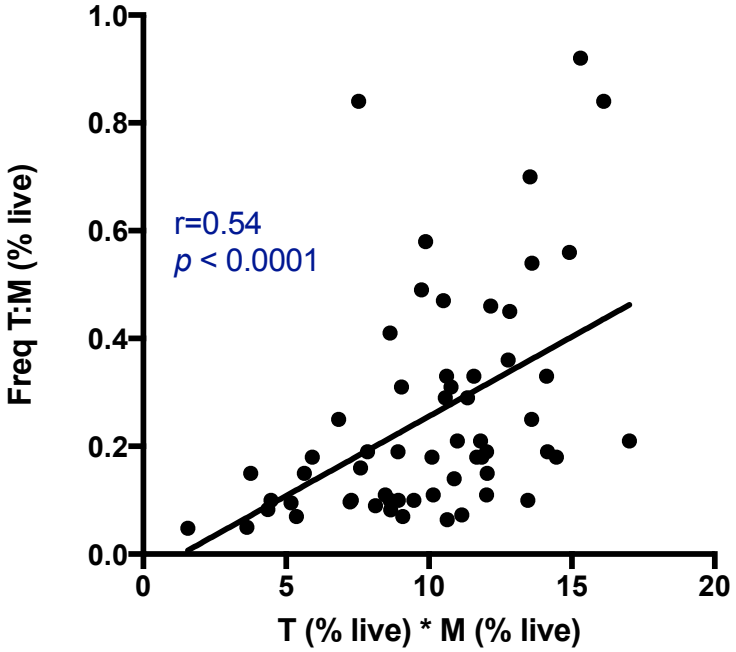








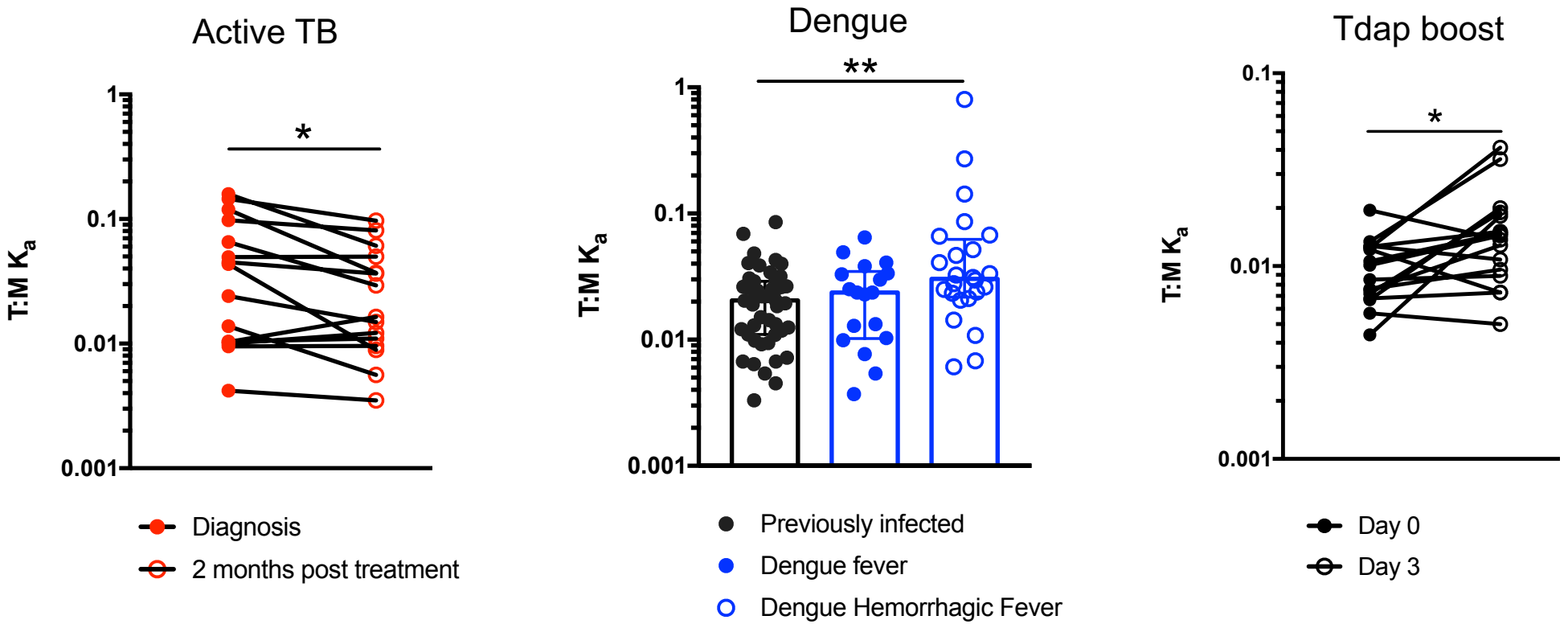
The constant of association Ka as a readout for the likelihood of T cell:monocyte complexes formation



$$\text{T:M } K_a = \frac{\text{Freq T:M }_{(\%\text{ live})}}{\text{Freq T }_{(\%\text{ live})} * \text{ Freq M }_{(\%\text{ live})}}$$

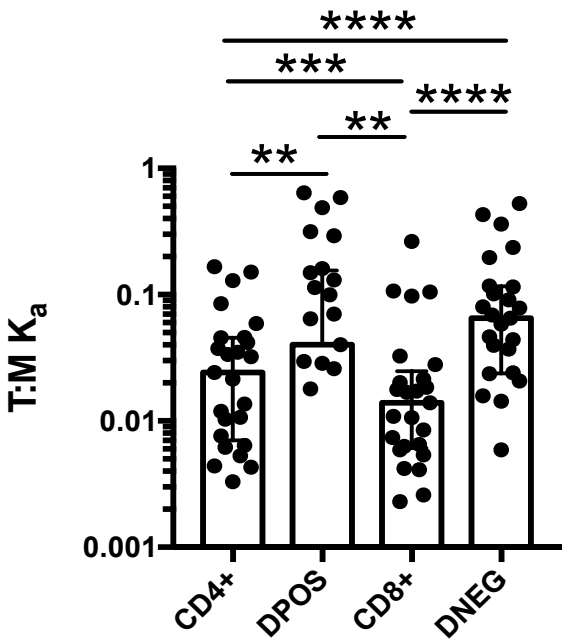
T = $CD3^+CD14^-$ T cells
M = $CD3^-CD14^+$ Monocytes
T:M = $CD3^+CD14^+$
K_a = constant of association

Ka of T cell:monocyte complexes varies with immune perturbations



The T cell subset in preferential association with a monocyte varies with the nature of the immune perturbation

Active TB
(diagnosis)

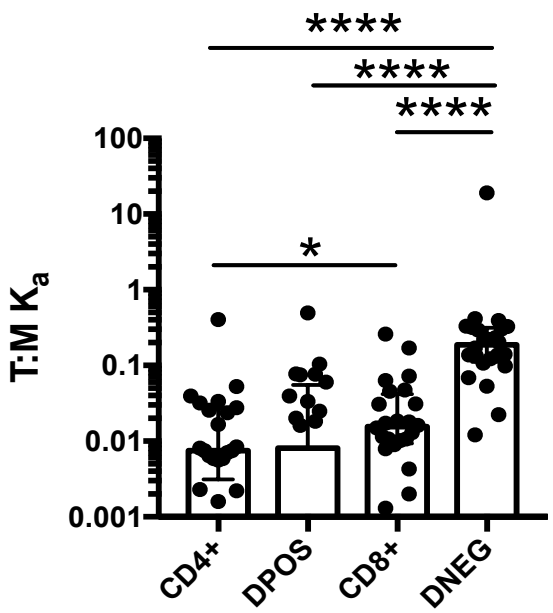


Bacteria

Intracellular & extracellular

CD4 > CD8

Dengue fever
(DHF)

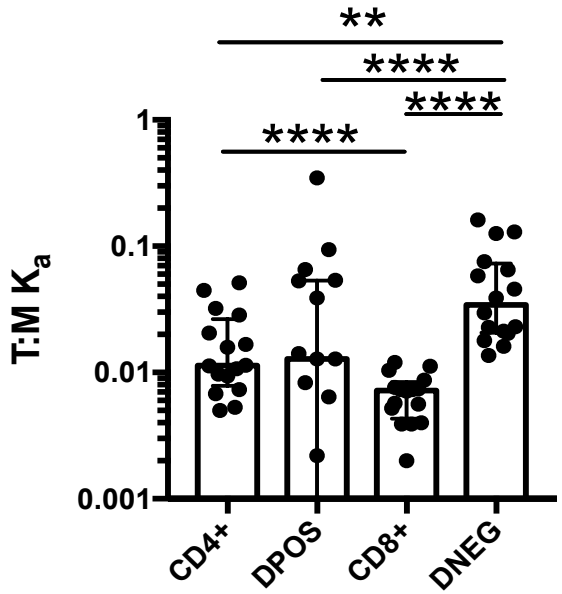


Virus

Intracellular

CD4 < CD8

Pertussis
(D3 post boost)



Acellular vaccine

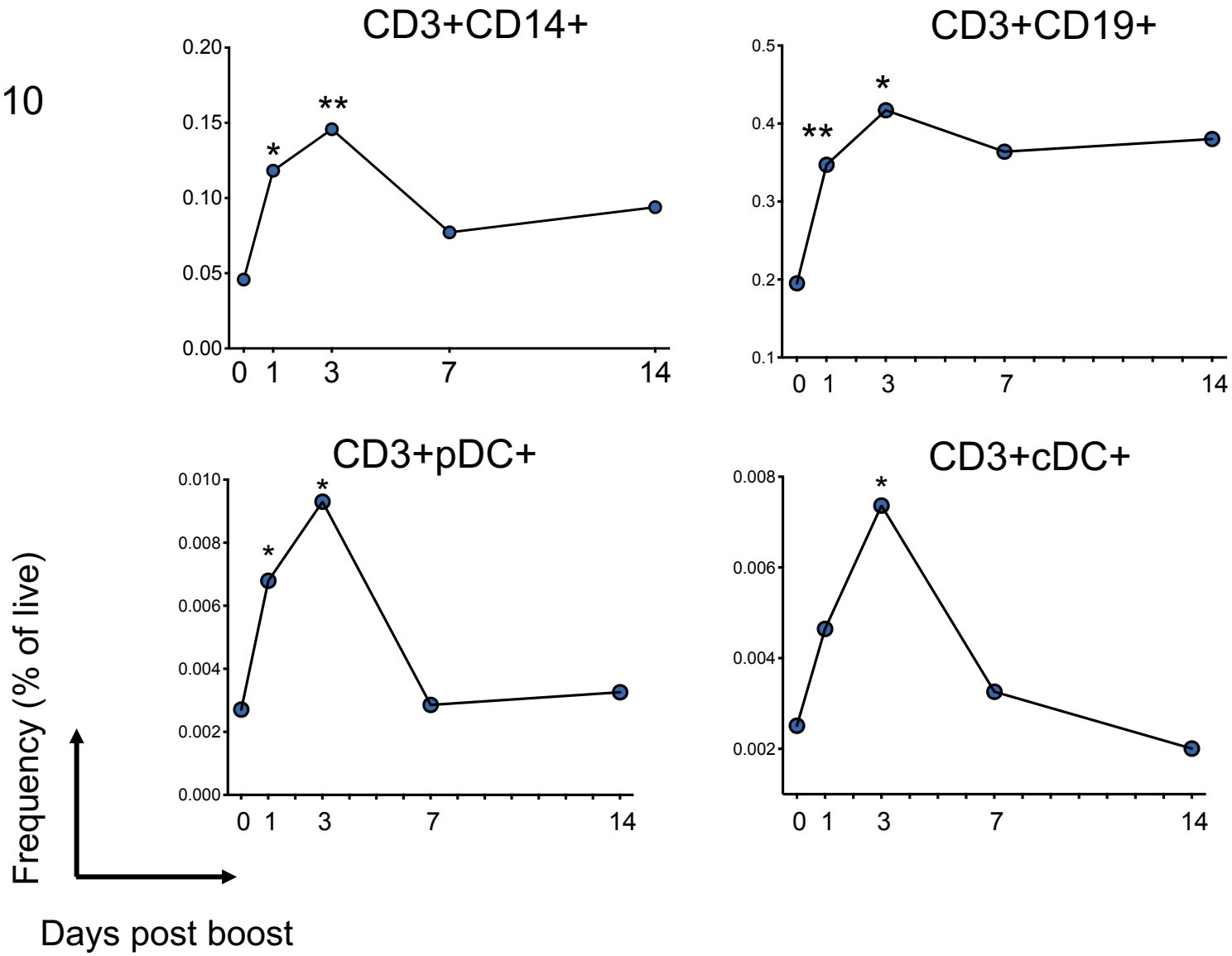
Extracellular

CD4 >> CD8

- The Ka of each T cell subset with monocytes follows expected CD4 vs CD8 polarization of immune responses for each perturbation model
- CD4-CD8- double negative T cells display the highest Ka with monocytes, regardless of the immune perturbation

Generalization to any T cell-APC?

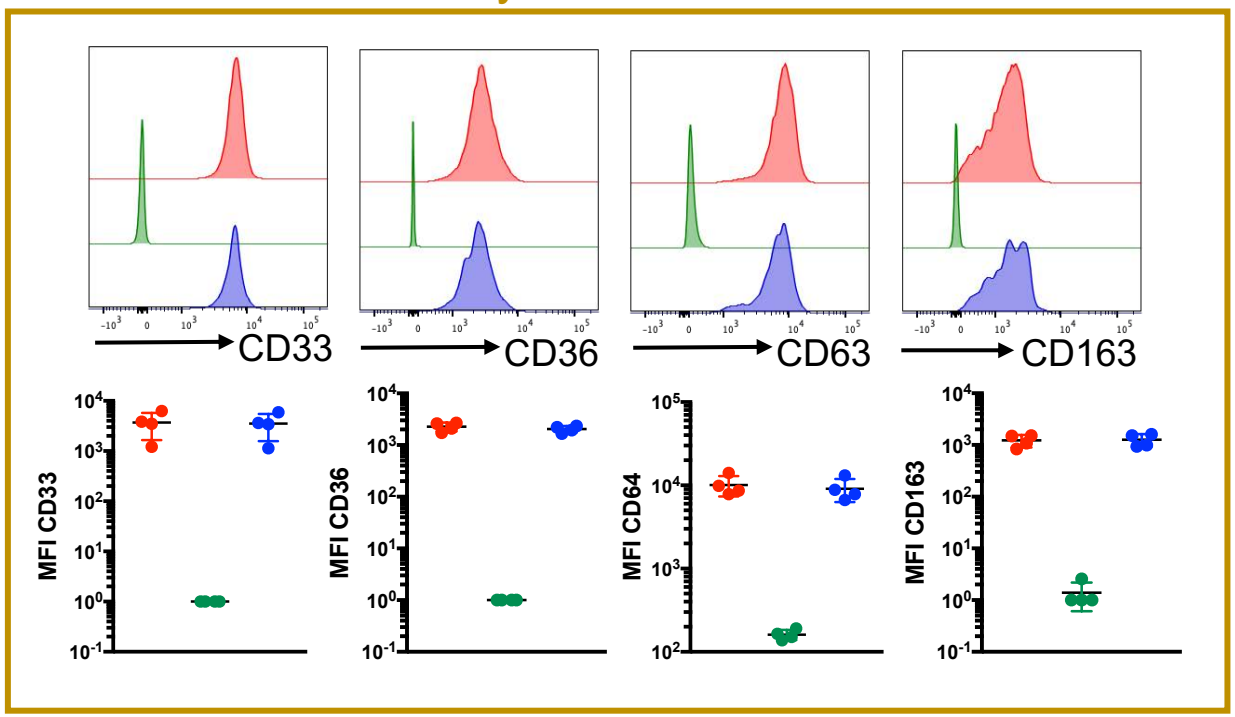
- Tdap boost vaccination, n=10
- Gated on live singlet cells



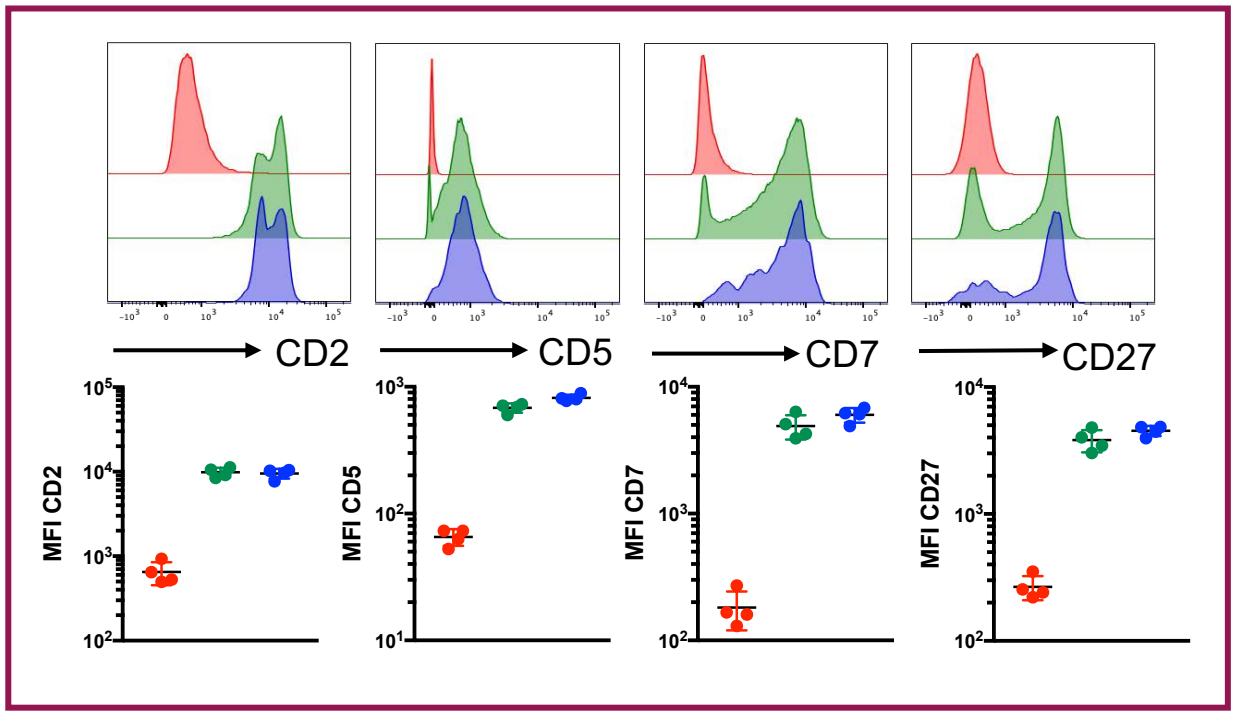
What are the implications of these doublets ‘hiding’ within the singlet gate for single-cell technologies?

Flow cytometry analysis of CD3+CD14+ cells

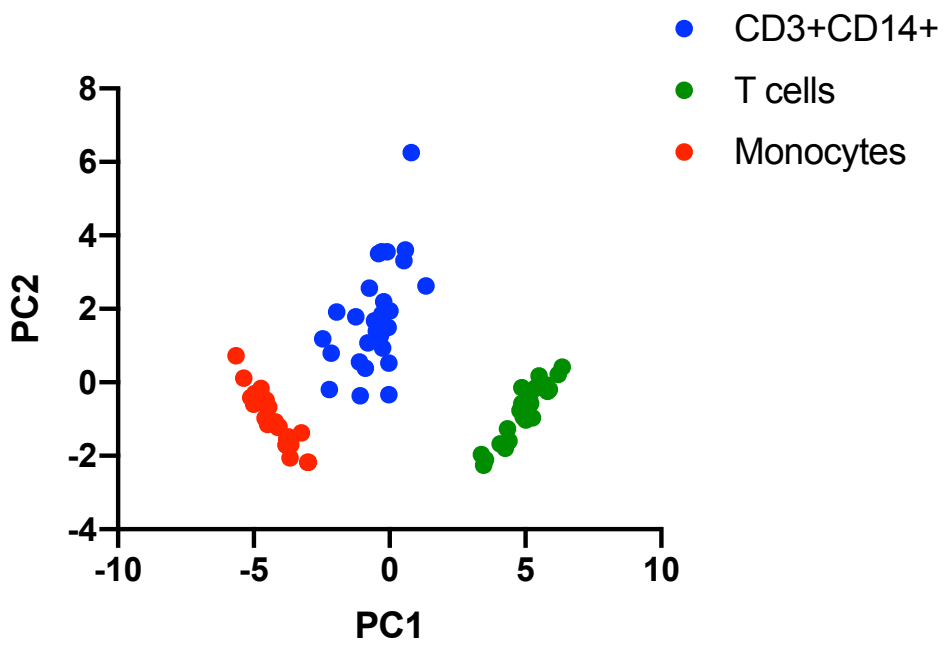
Monocyte canonical markers

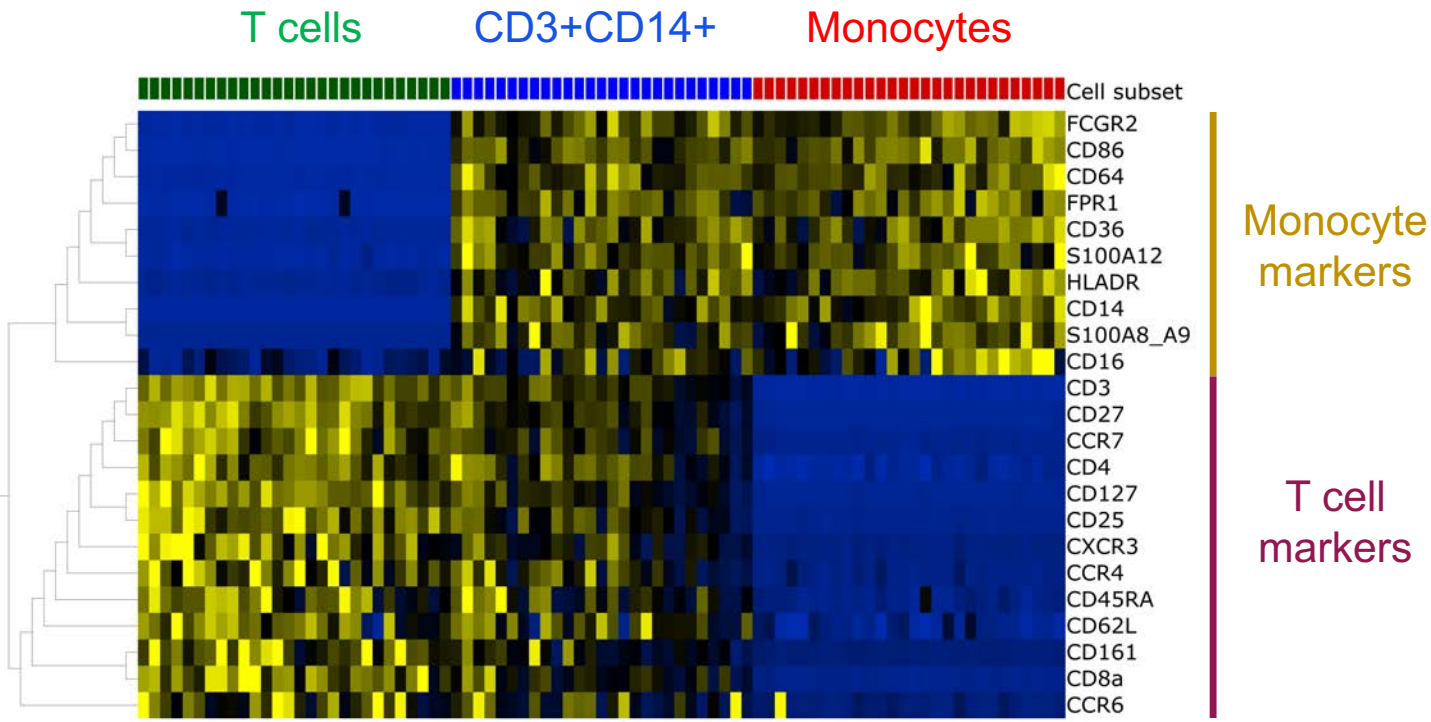


T cell canonical markers

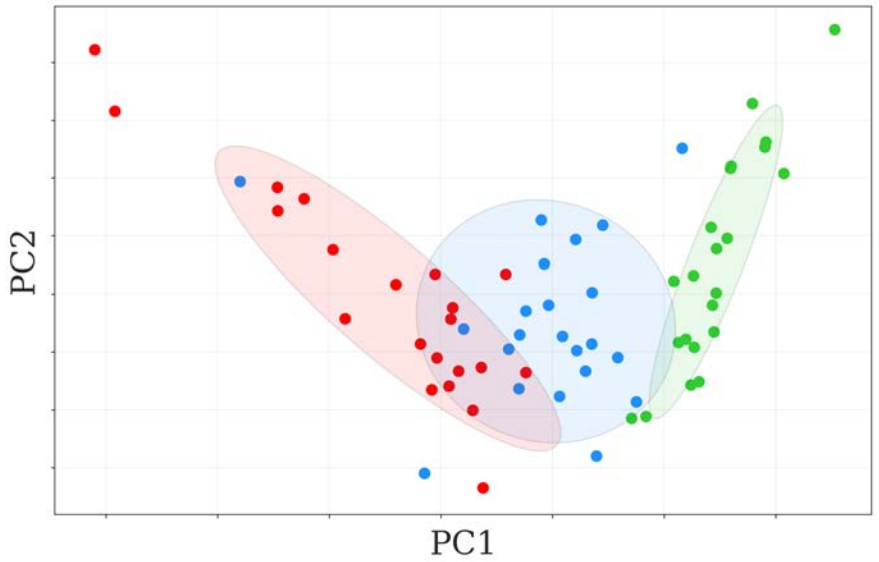


CyTOF analysis of CD3+CD14+ cells

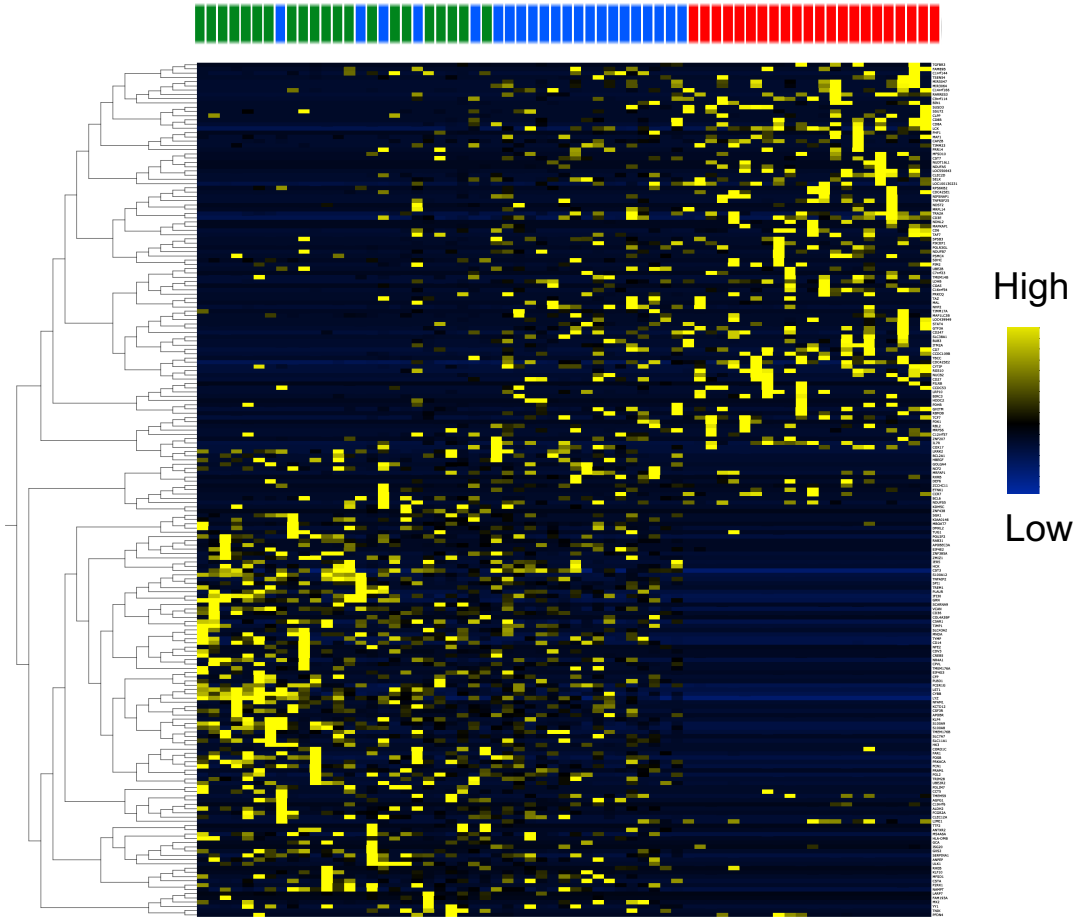




Single-cell RNAseq analysis of CD3+CD14+ cells



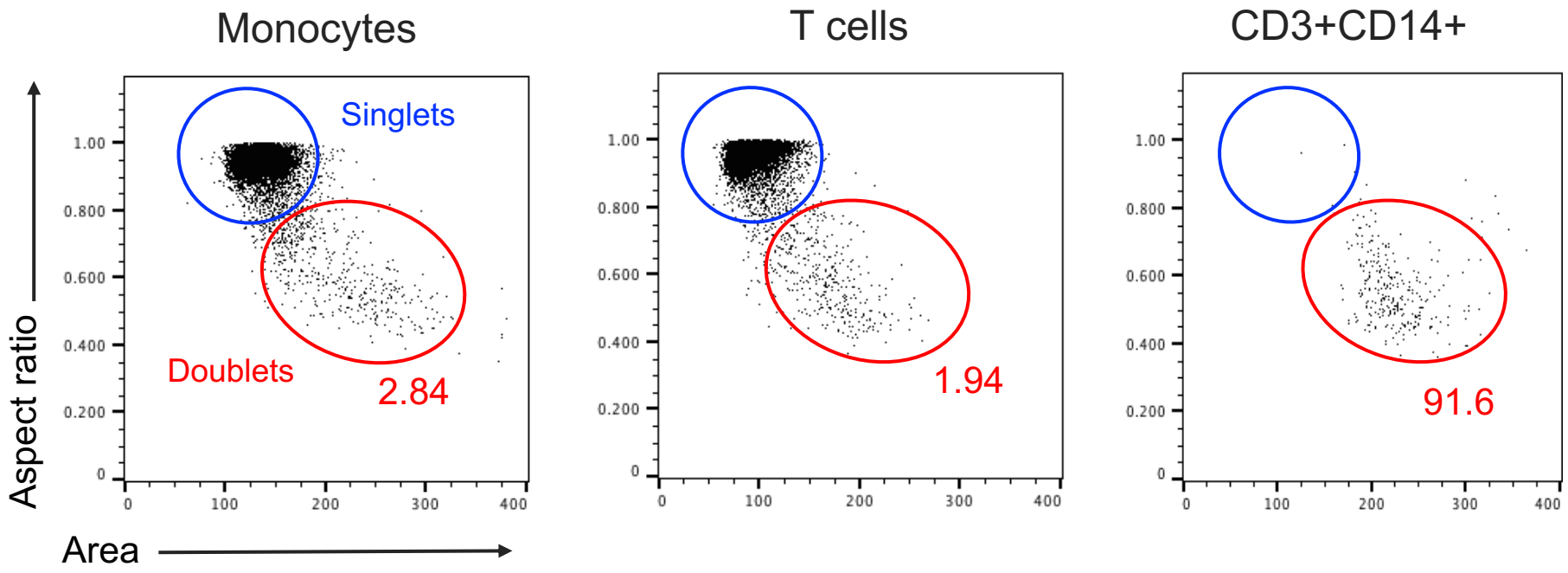
Monocytes
T cells
CD3+CD14+



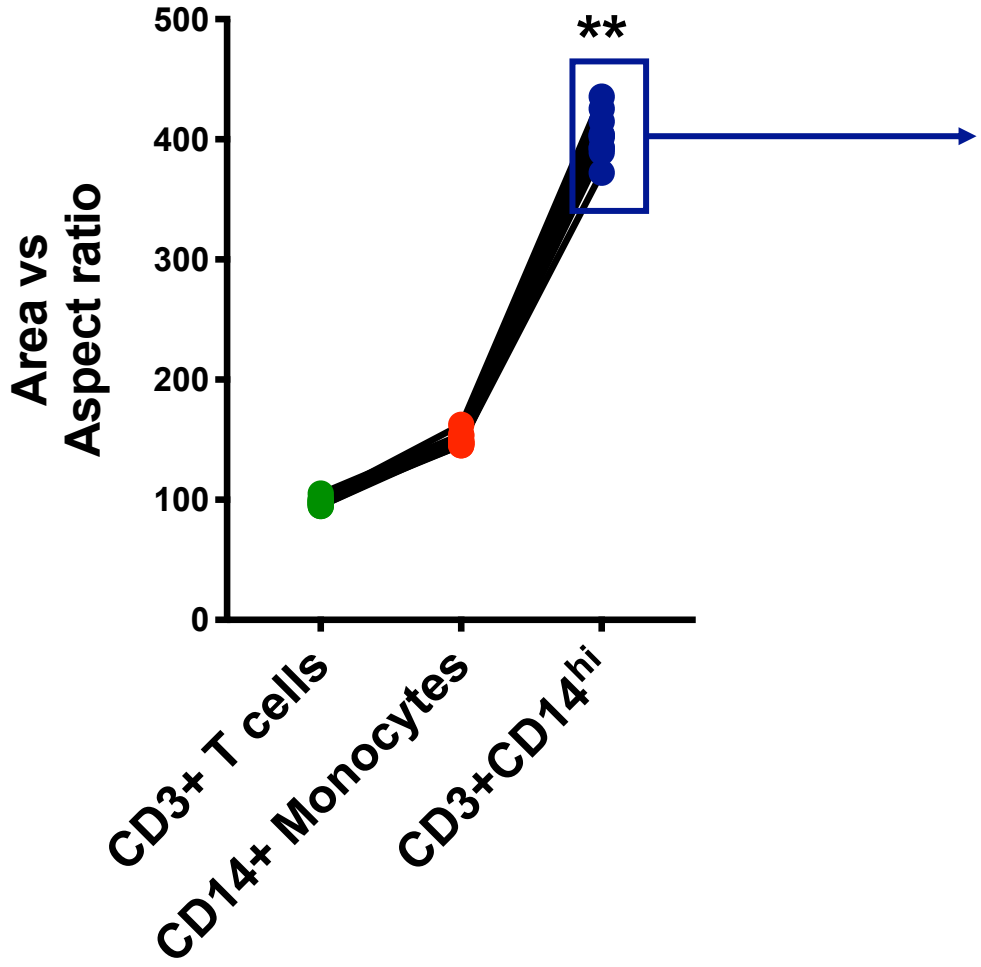
How can we discriminate
cell:cell complexes from singlets?

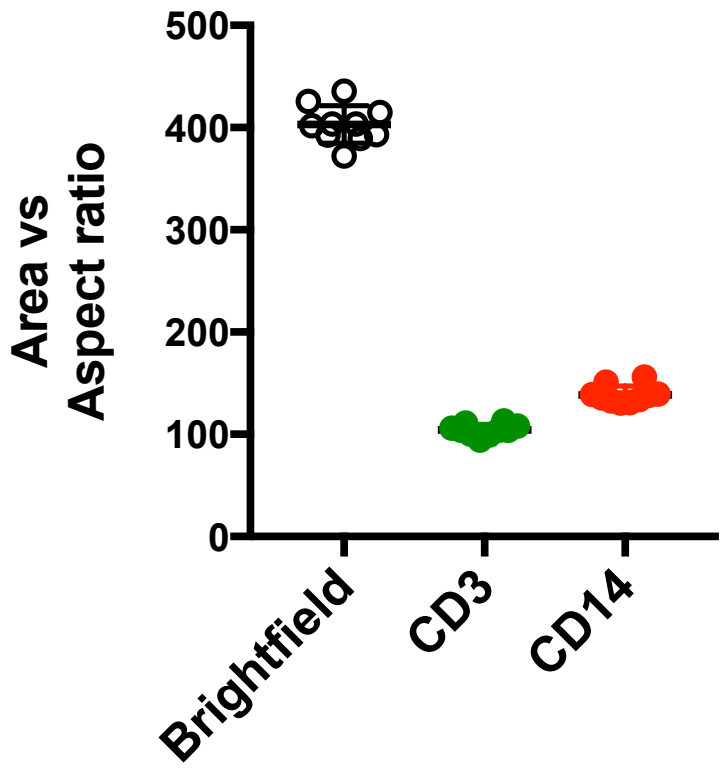
Imaging flow cytometry parameters can discriminate CD3+CD14+ complexes from singlets

Brightfield parameter



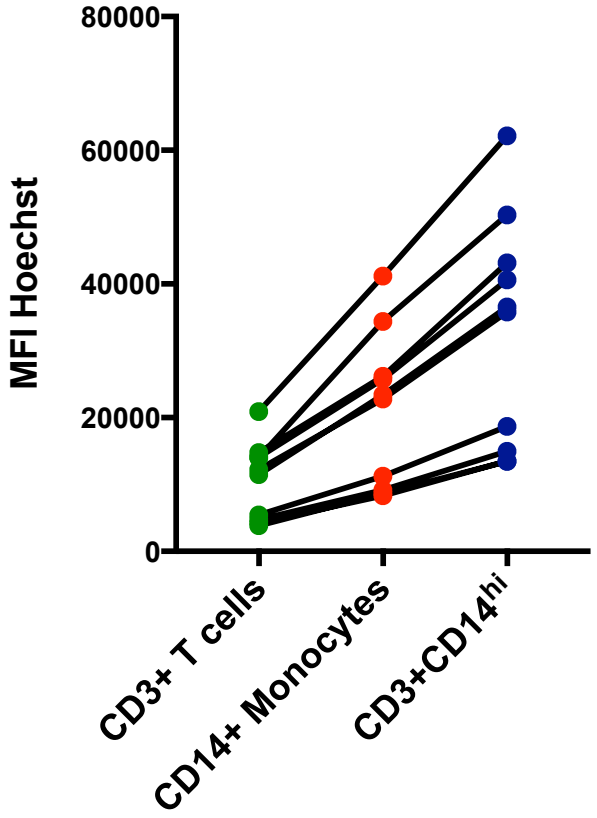
Imaging flow cytometry parameters can discriminate CD3+CD14+ complexes from singlets

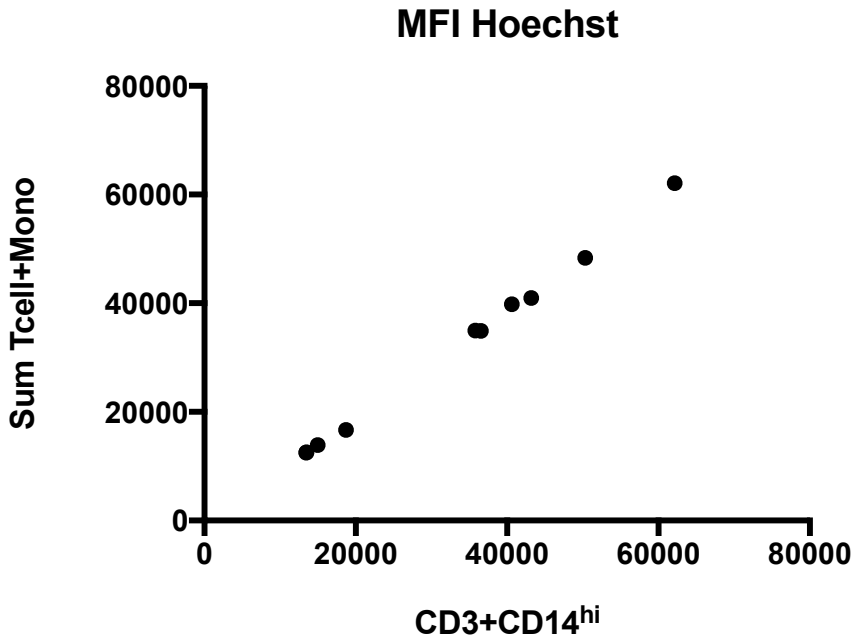




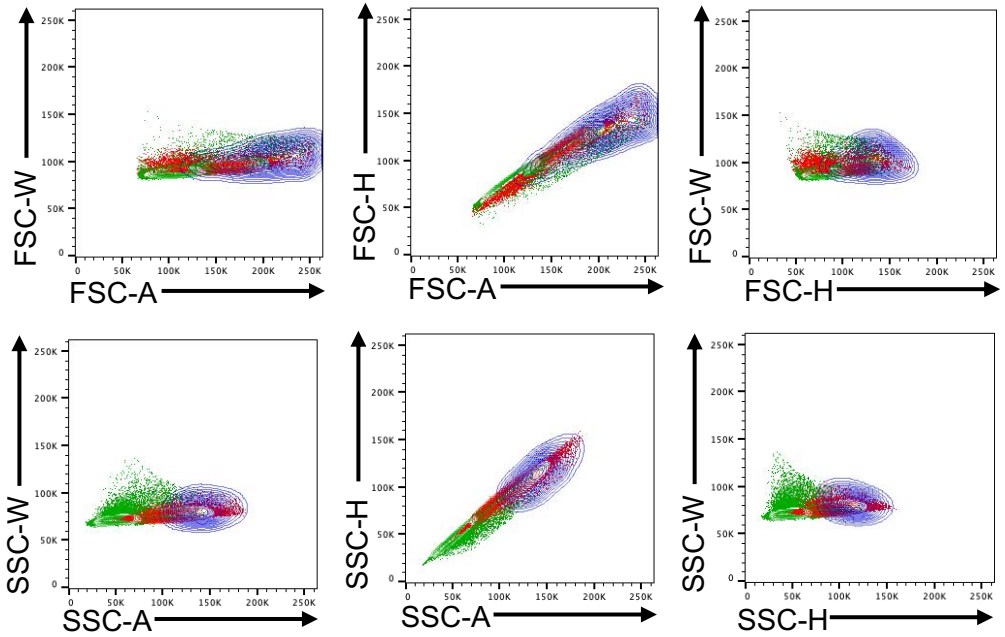
Hoechst MFI in CD3+CD14+ cells is the sum of singlet T cells and singlets Monocytes MFIs

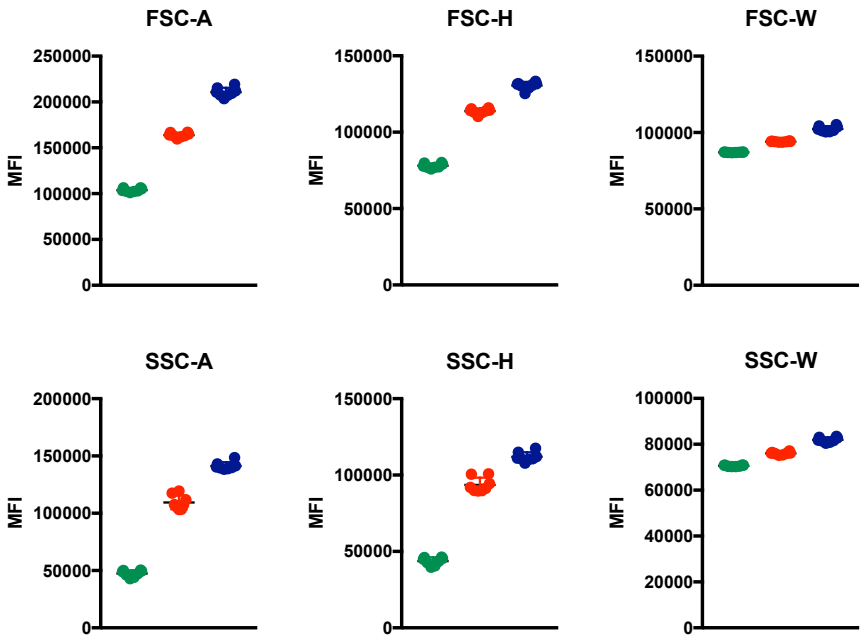
CD3
CD14
Hoechst

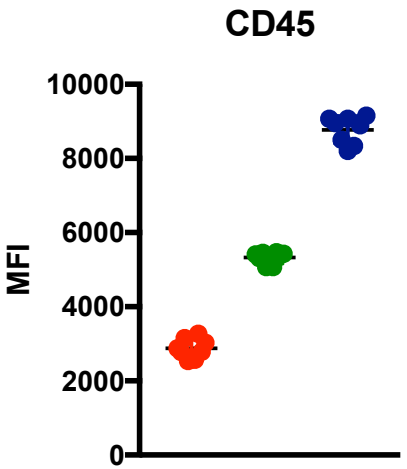




Non-Imaging flow cytometry parameters can partially discriminate CD3+CD14+ complexes from singlets



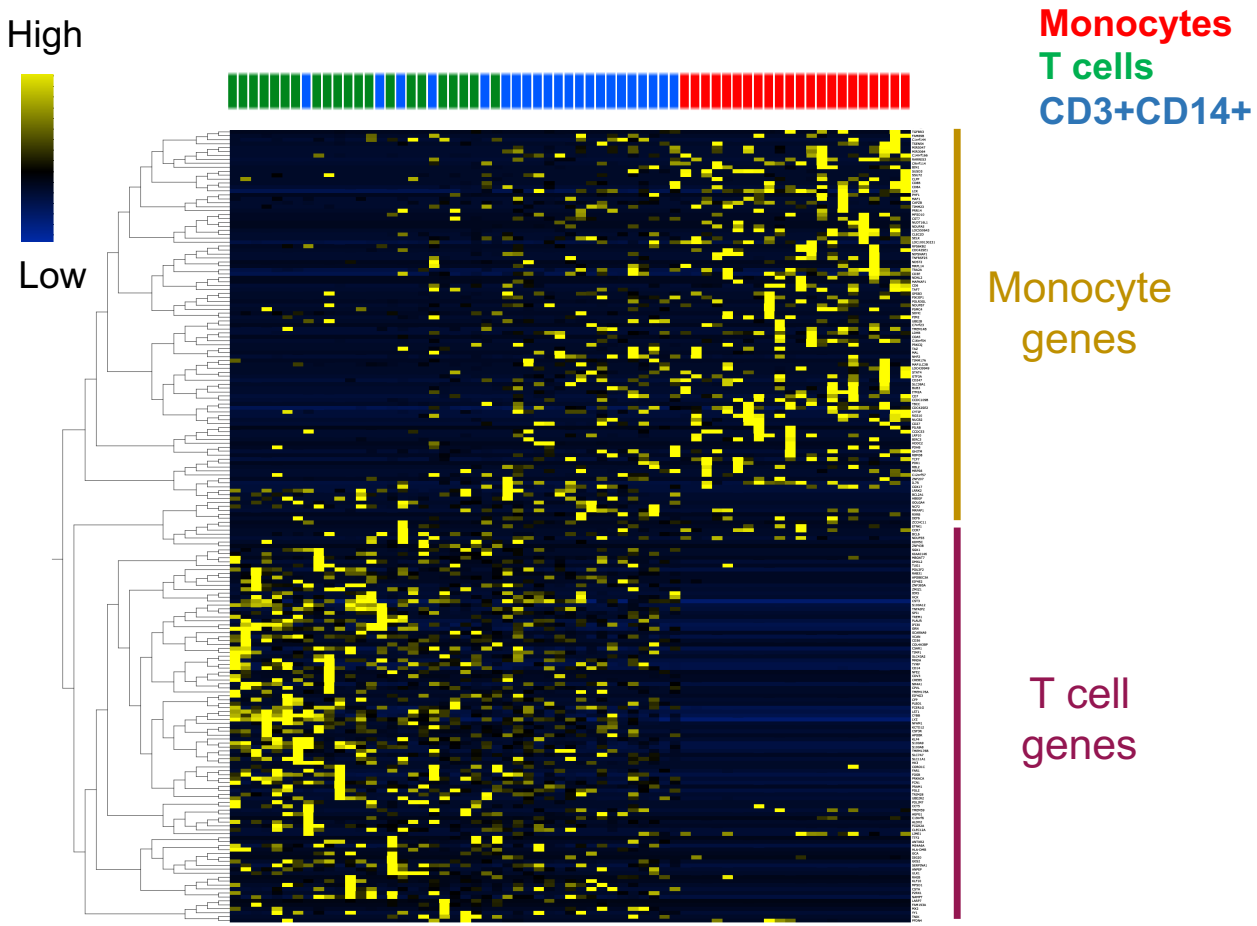


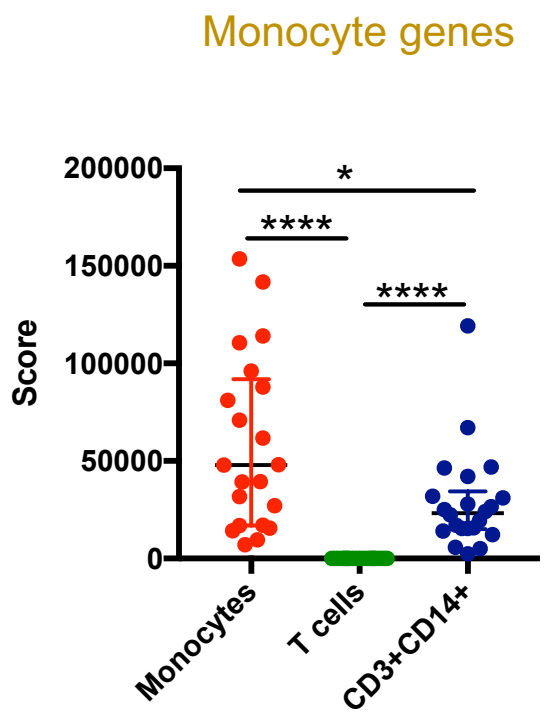


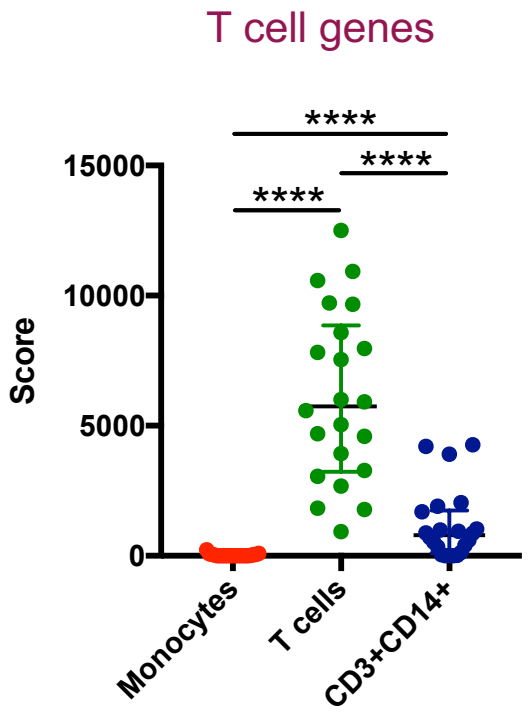
- Monocytes
- T cells
- CD3+CD14+ (T:M complexes)

Single-cell RNA sequencing signature of cell:cell complexes

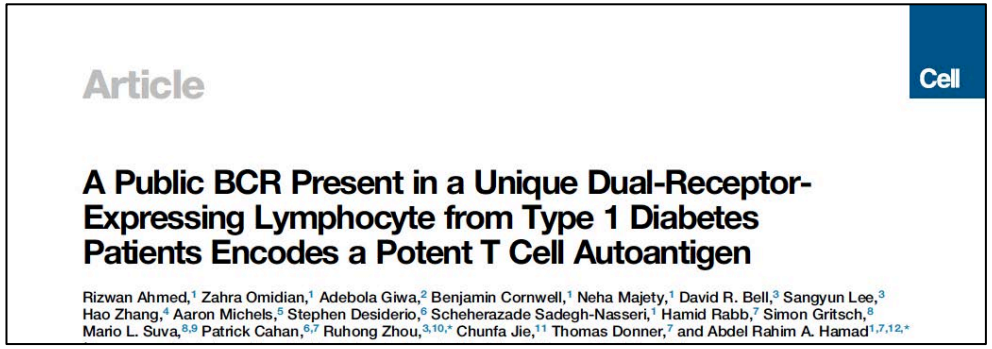
- RNAseq is not absolute quantification such as fluorescence intensity in flow cytometry
- TPM: relative quantity of mRNA







The case of DE cells (Ahmed et al., *Cell* 2019)

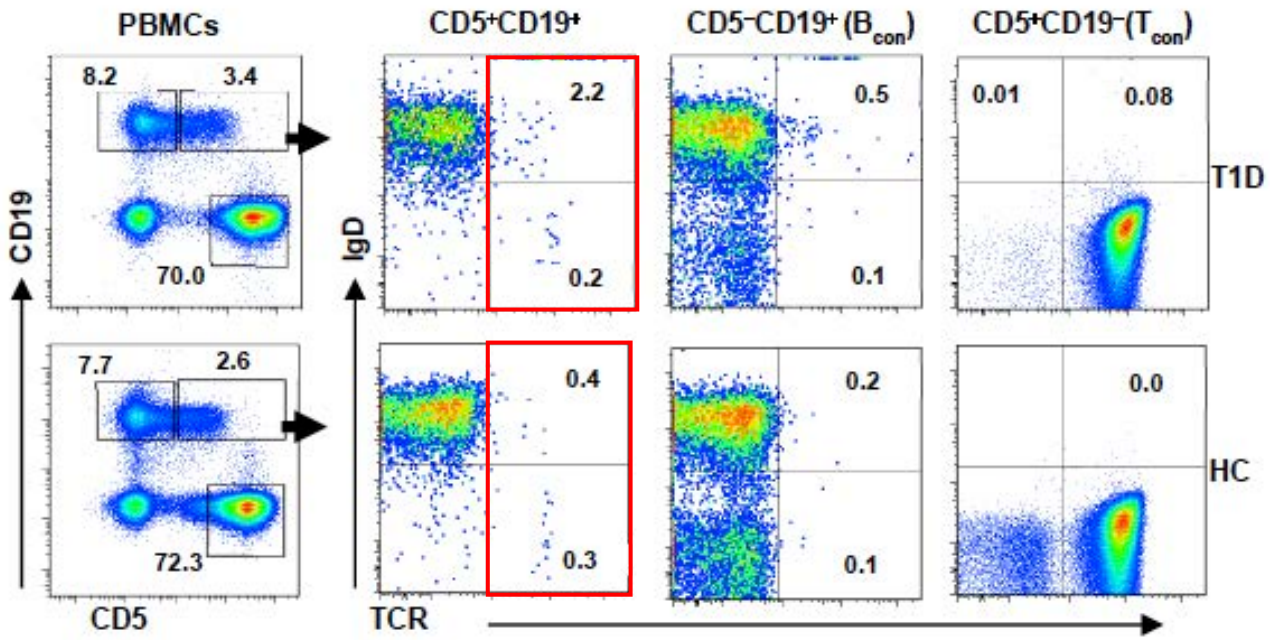

Article

Cell

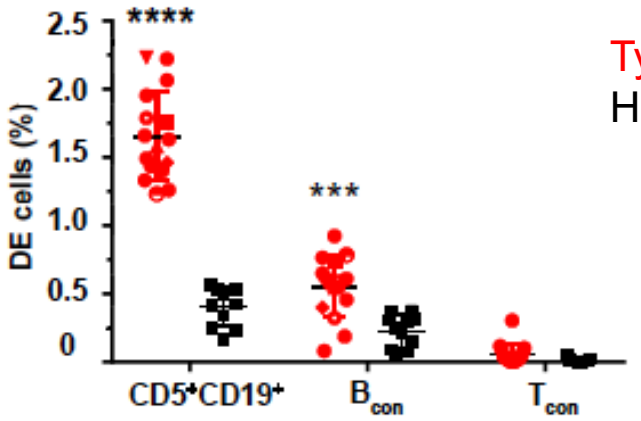
A Public BCR Present in a Unique Dual-Receptor-Expressing Lymphocyte from Type 1 Diabetes Patients Encodes a Potent T Cell Autoantigen

Rizwan Ahmed,[1] Zahra Omidian,[1] Adebola Giwa,[2] Benjamin Cornwell,[1] Neha Majety,[1] David R. Bell,[3] Sangyun Lee,[3] Hao Zhang,[4] Aaron Michels,[5] Stephen Desiderio,[6] Scheherazade Sadegh-Nasseri,[1] Hamid Rabb,[7] Simon Gritsch,[8] Mario L. Suva,[8,9] Patrick Cahan,[5,7] Ruhong Zhou,[3,10,*] Chunfa Jie,[11] Thomas Donner,[7] and Abdel Rahim A. Hamad[1,7,12,*]

- In type 1 diabetes patients, novel cell population with both T cell and B cell lineage specificities: DE cells
- DE cells encode for a potent T cell autoantigen that might be involved in diabetes pathogenesis

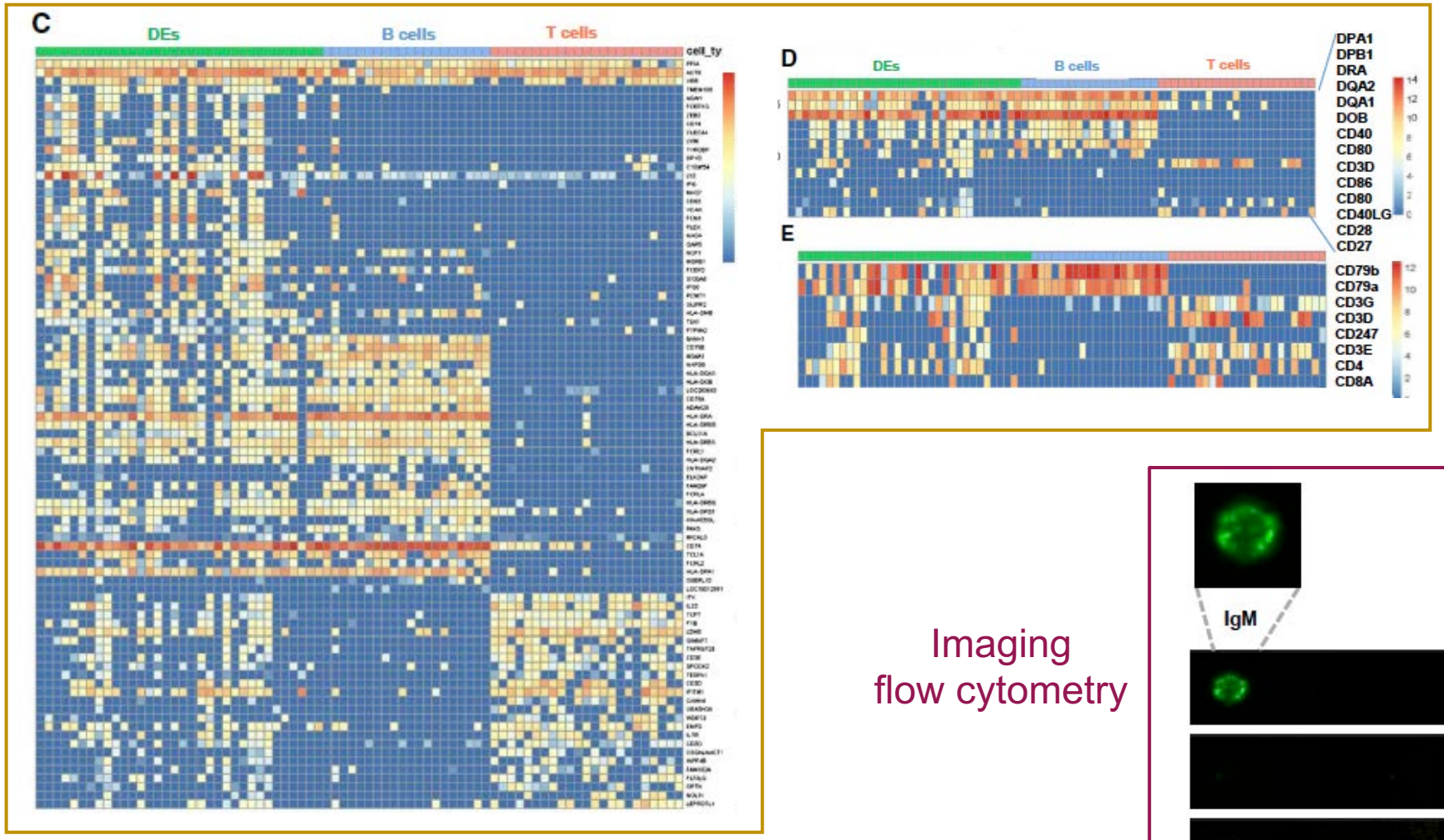


DE cells



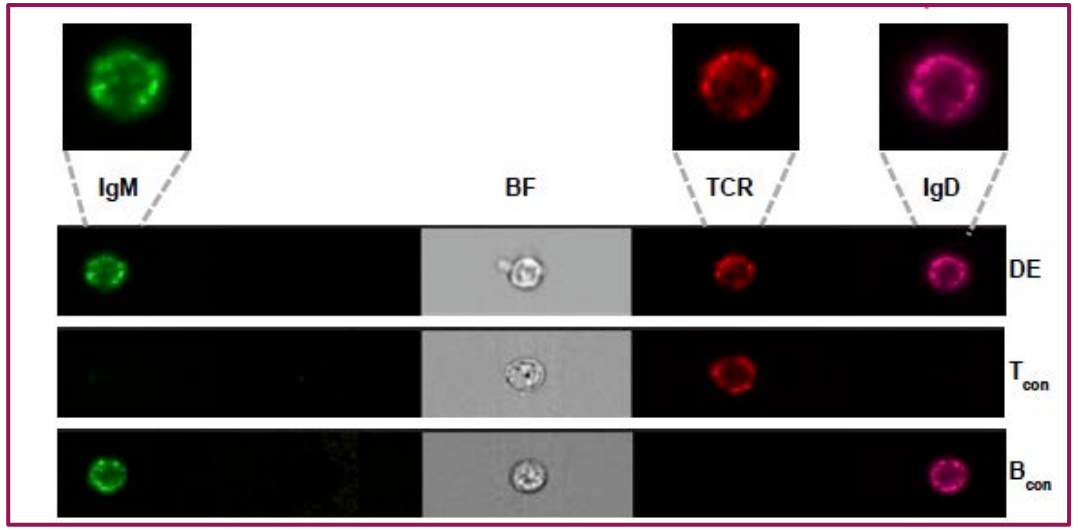
Type 1 diabetes
Healthy

DE cells are expressing both T cell and B cell lineage markers at protein and mRNA level

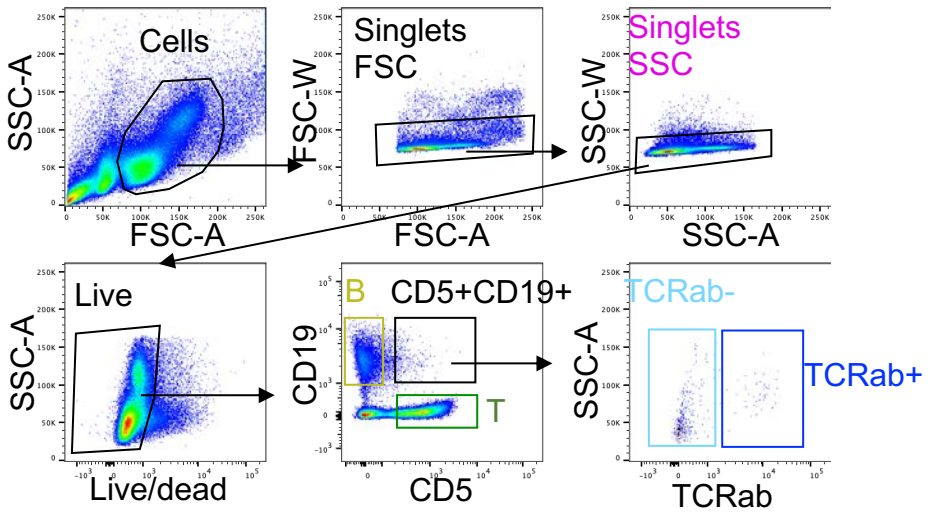


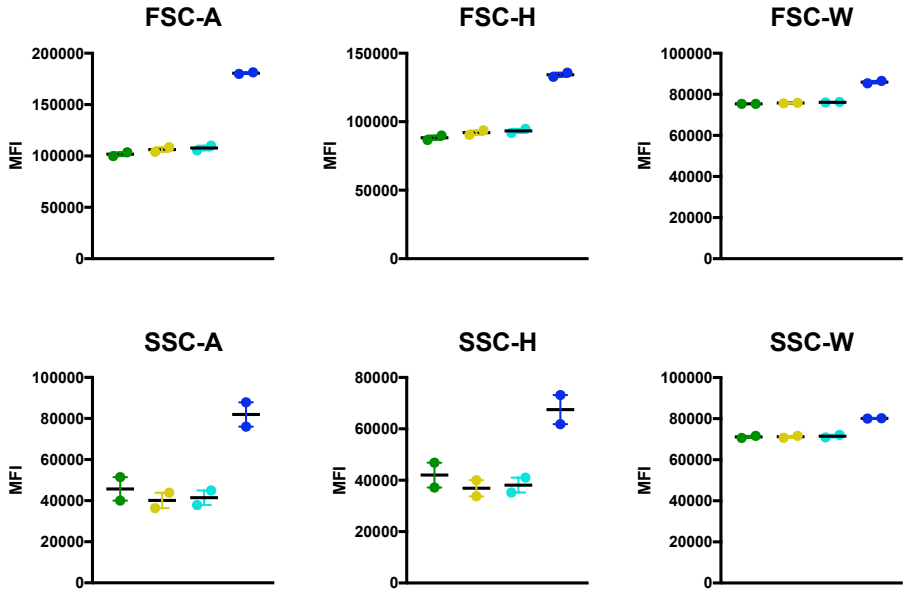
Single-cell sort RNAseq

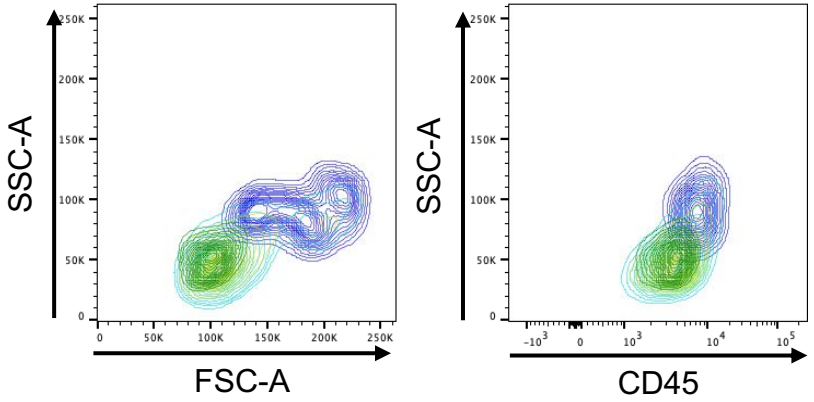
Imaging flow cytometry

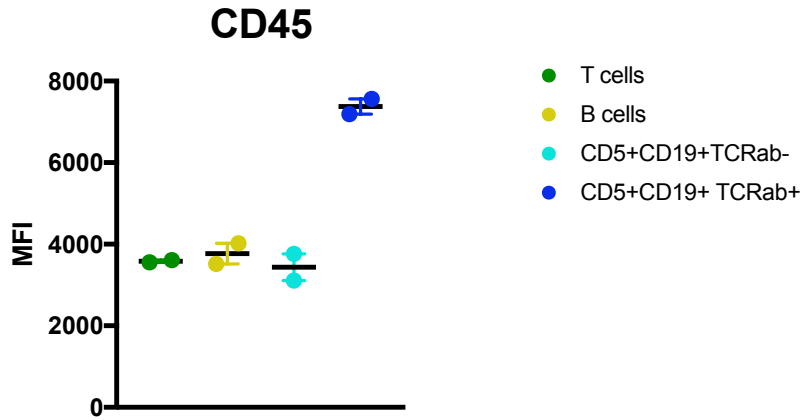


Our in-house analysis show DE cells in healthy PBMC have a flow cytometry signature of cell:cell complexes

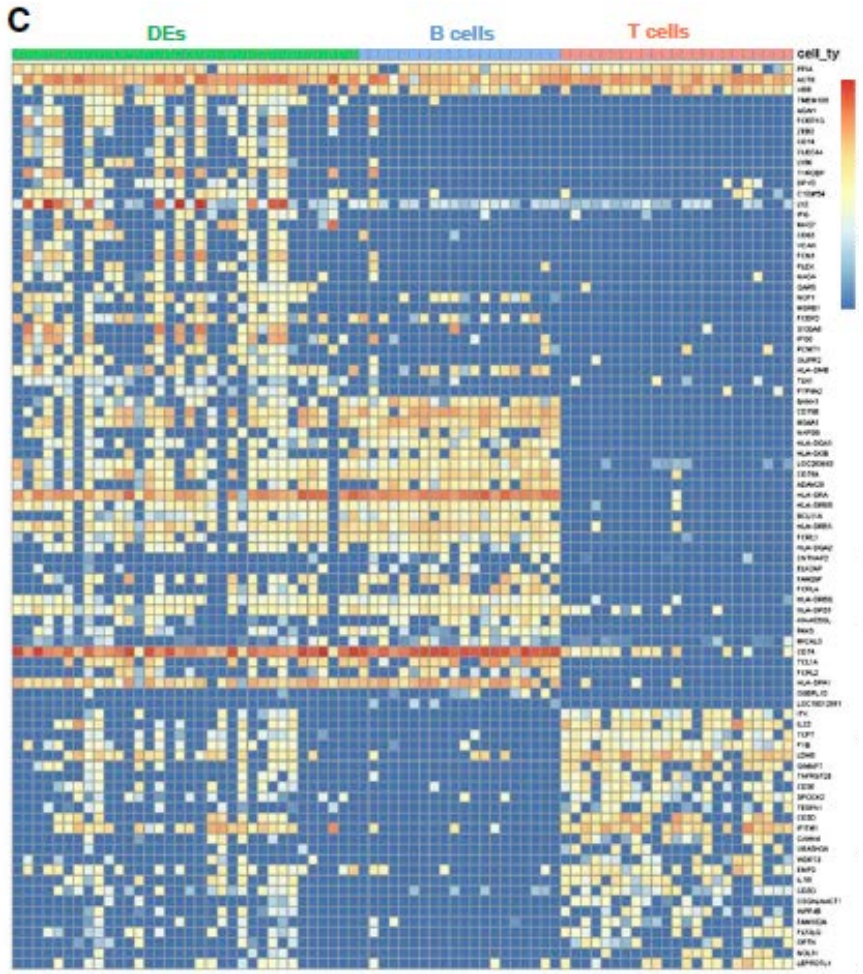




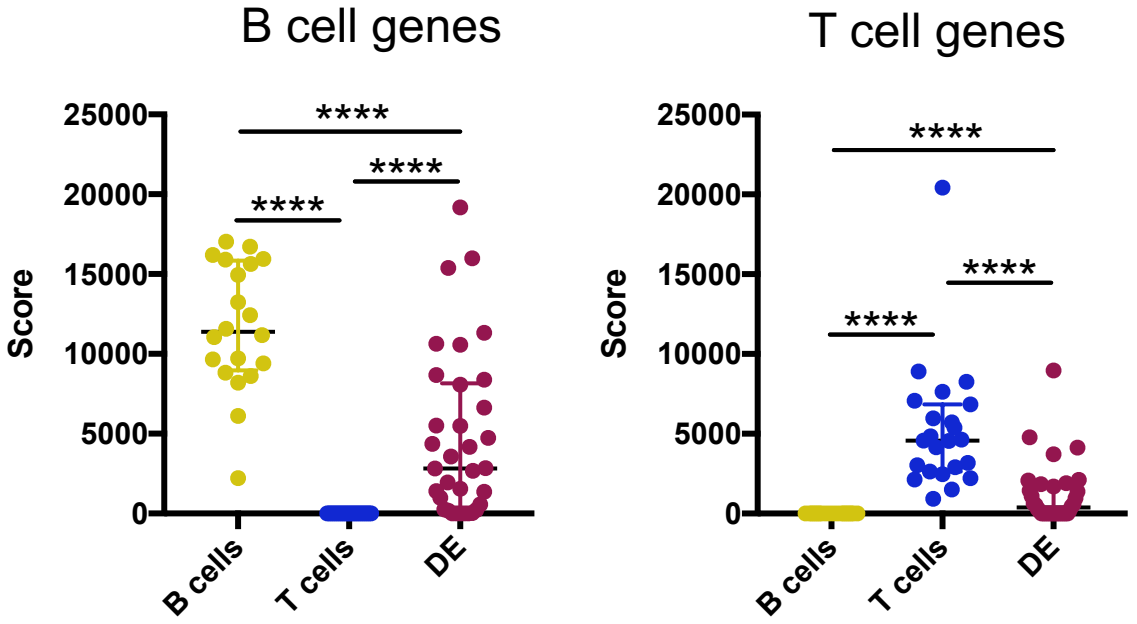


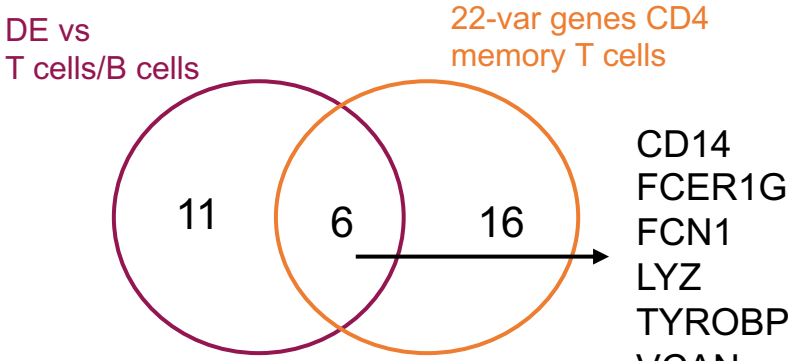


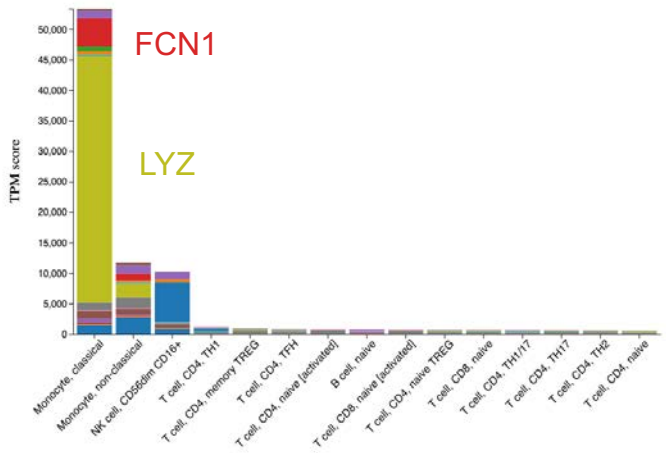
scRNAseq data of DE cells in Ahmed et al. has a signature of cell:cell complexes



GEO accession number: [GSE129112]

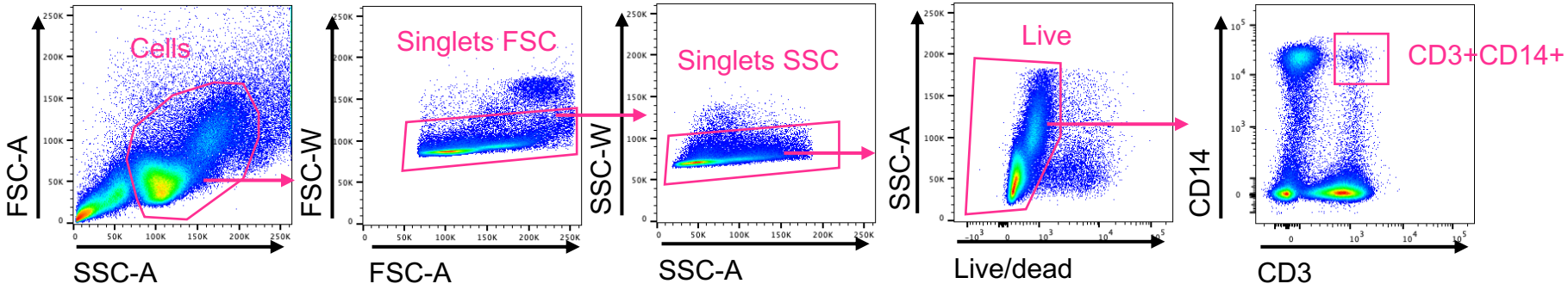






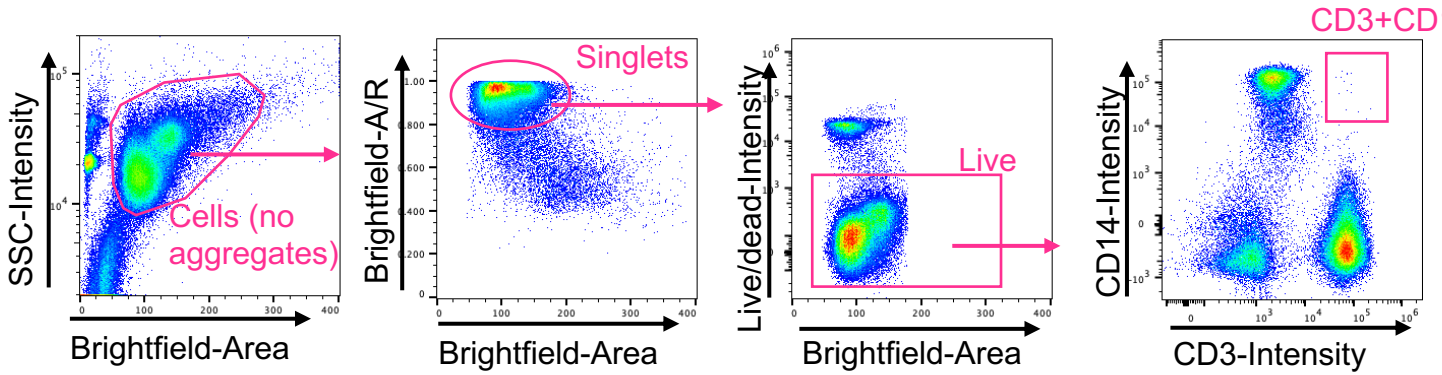
The challenge of comparing imaging and non-imaging flow cytometry gating

Non-imaging flow cytometry

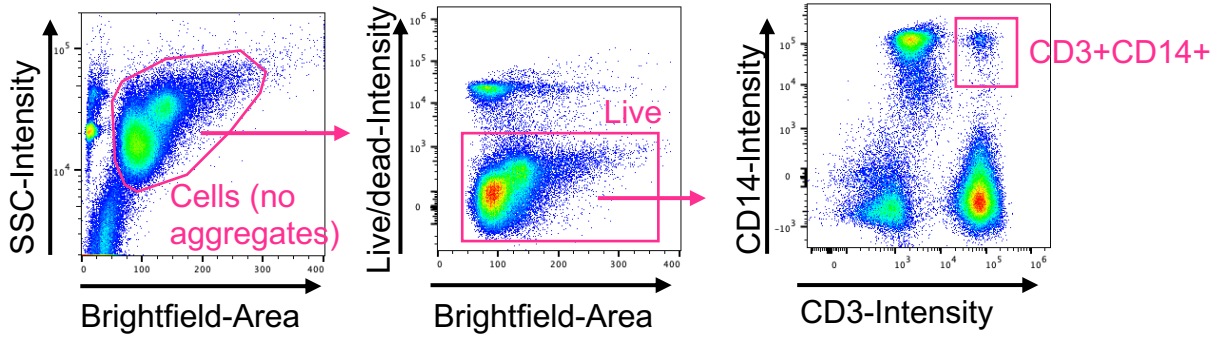


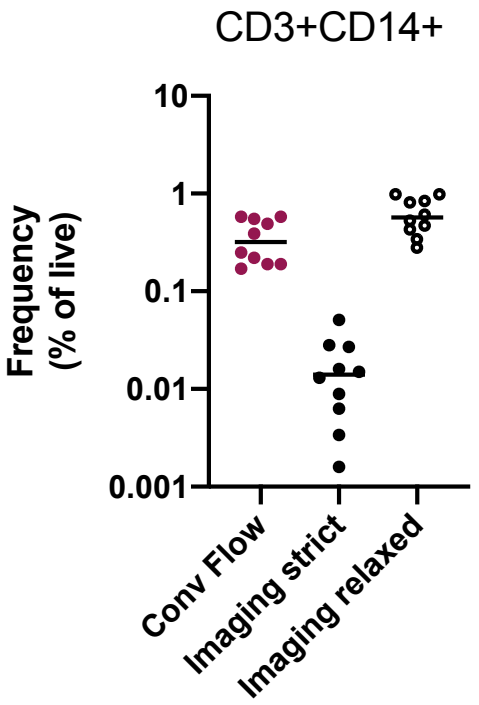
Imaging flow cytometry

‘Strict’ gating



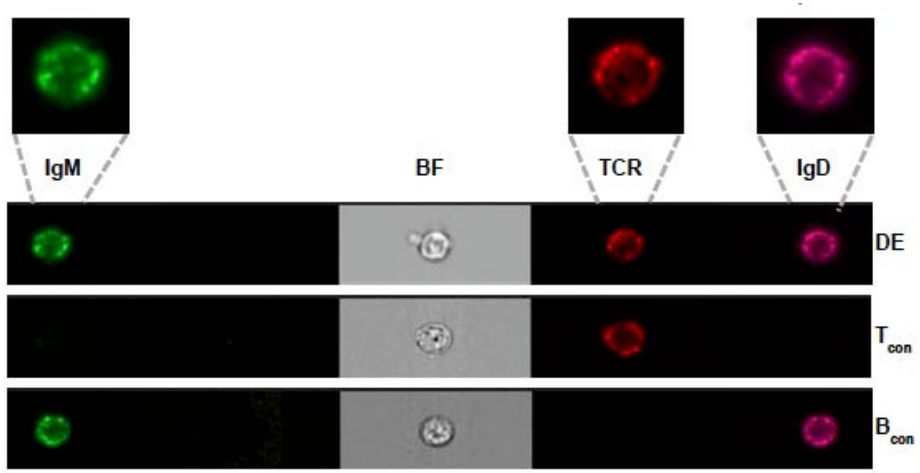
‘Relaxed’ gating





Gating strategy to identify DE cells in imaging flow cytometry

DE cells, Imaging flow cytometry (Ahmed et al.)



"Gating strategy involved selecting focused cells on the basis of gradient RMS and an aspect ratio that was consistent with single events and devoid of debris or multi-cellular events (doublets)."

It seems the 'strict' gating was applied in the Ahmed study…

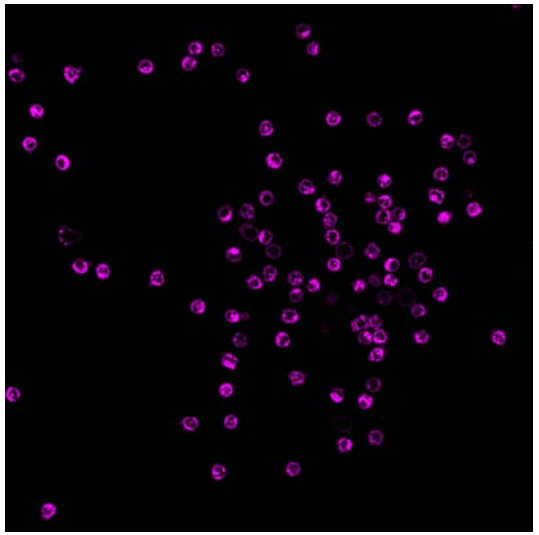
- Dual-expressing cells exist, but they might represent only a small proportion of sorted DE cells!

Our recommendations for cases of suspicious dual-expressing cell populations in flow cytometry

1. Check for signatures of doublets in flow cytometry and single-cell RNAseq data
2. Perform imaging flow cytometry with appropriate gating strategy
3. Direct microscopy analysis of sorted cells

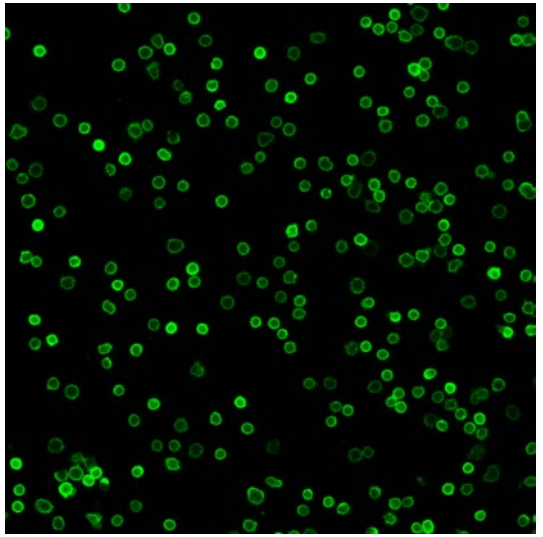
CD14
CD3

CD14+CD3-
Monocytes



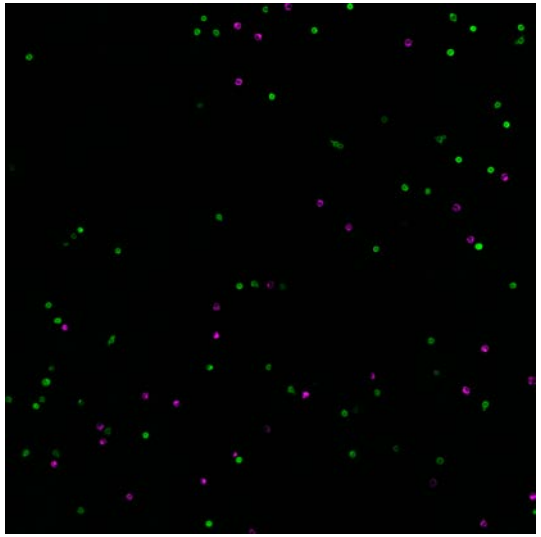
- 100% CD14+

CD14-CD3+
T cells



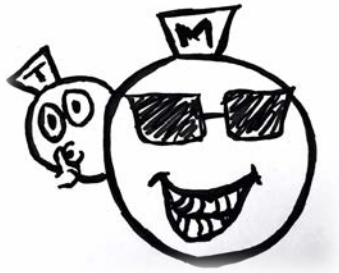
- 100% CD3+

CD3+CD14+
T:M complexes

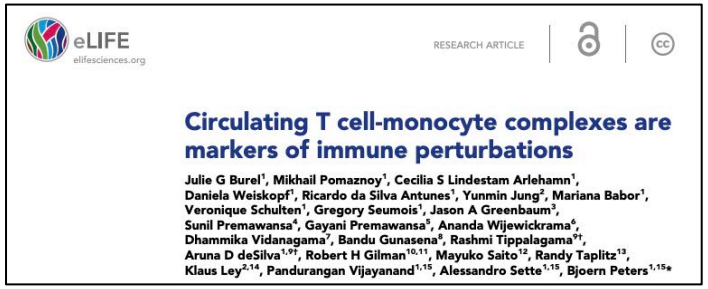


- 51% CD14+
- 49% CD3+
- <1% CD3+CD14+

Conclusions: one cell can hide another!



- The majority of CD3+CD14+ cells detected in the live singlet gate of human PBMC are not technical artefacts but circulating Tcell:monocyte complexes that reflect *in vivo* immune cell:cell interactions.
- The frequency of T cell:monocyte complexes correlates with clinical parameters and its T cell phenotype varies with the nature of immune perturbation
- This phenomenon might be generalized to any T cell:APC interaction.


eLIFE elifesciences.org RESEARCH ARTICLE

Circulating T cell-monocyte complexes are markers of immune perturbations

Julie G Burel[1], Mikhail Pomaznoy[1], Cecilia S Lindestam Arlehamn[1], Daniela Weiskopf[1], Ricardo da Silva Antunes[1], Yunmin Jung[2], Mariana Babor[1], Veronique Schulten[1], Gregory Seumois[1], Jason A Greenbaum[3], Sunil Premawansa[4], Gayani Premawansa[5], Ananda Wijewickrama[6], Dhammika Vidanagama[7], Bandu Gunasena[8], Rashmi Tippalagama[9†], Aruna D deSilva[1,9†], Robert H Gilman[10,11], Mayuko Saito[12], Randy Taplitz[13], Klaus Ley[2,14], Pandurangan Vijayanand[1,15], Alessandro Sette[1,15], Bjoern Peters[1,15*]

Burel et al, *eLife* 2019

- Overall, these results reveal that **a significant proportion of cell:cell doublets in flow cytometry are biologically meaningful**, and thus, the conventional wisdom in flow cytometry to avoid studying cell doublets should be re-visited
- **Thorough analysis checking for doublets signatures need to be systematically reported in single-cell techniques such as flow cytometry or single-cell RNA sequencing**

Acknowledgments

La Jolla Institute FOR IMMUNOLOGY | Life Without Disease.®

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