Preservation of Functionality, Immunophenotype and Recovery of HIV RNA from PBMC's Cryopreserved for >20 years

John Zaunders, Wayne B Dyer, Kazuo Suzuki, Angelique Levert, Mitchell Starr and Andrew Lloyd,

St Vincent's Hospital, Sydney
Sydney Blood Bank (LifeBlood),
Immunovirology Research Network (IVRN).

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ST VINCENT'S CENTRE FOR APPLIED MEDICAL RESEARCH

Speaker disclosures

- Kazuo Suzuki is a named inventor on a patent for the Double R assay for intracellular HIV RNA transcripts

- John Zaunders is a named inventor on a patent for the OX40 assay for antigen-specific CD4 and CD8 T cells

PBMC Biobanking at Centre for Applied Medical Research, SVH, Sydney

- **Major commitment for NSW State Reference Lab for HIV, at AMR**

  - **Senior Scientist and 4 full-time staff for trials and biobanking**
  - **Twenty -80°C Freezers**
  - **Four Vapour Phase Liquid Nitrogen tanks**

  - **Sydney Blood Bank HIV Research PBMC's**

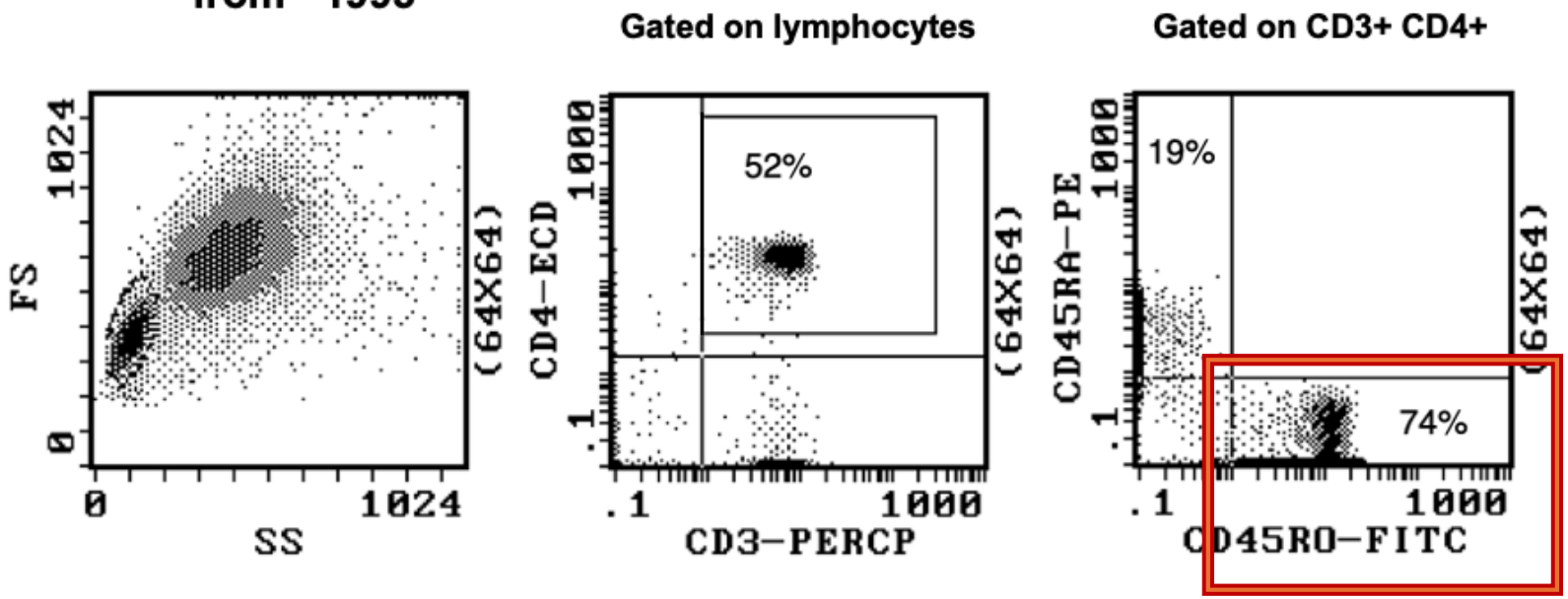
Sydney Blood Bank Cohort (SBBC) – attenuated *nef*-deleted HIV-1

- 1992 - 5 asymptomatic HIV+ transfusion recipients
- single HIV+ donor, D36, who was also a long-term non-progressor
- normal CD4 T cell counts, after 8-10 years of infection
- Sequenced virus from the SBBC had *nef* / 3'-LTR deleted HIV-1

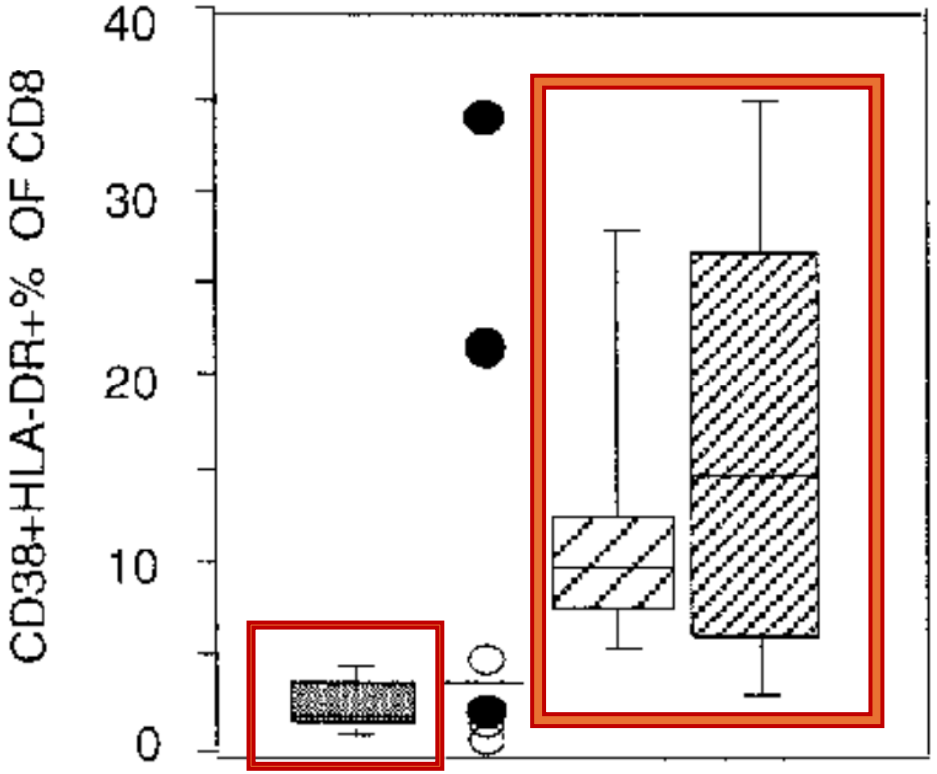
- 1995/6 - Studied SBBC CD4 and CD8 T cells using fresh blood samples:
- Four colour flow: CD45RA/RO, CD28- and activated CD38+/HLA-DR+
- Compared with age-matched and transfusion-matched HIV <u>neg</u> controls
- Also, compared with other <u>viraemic, untreated</u> HIV+ subjects

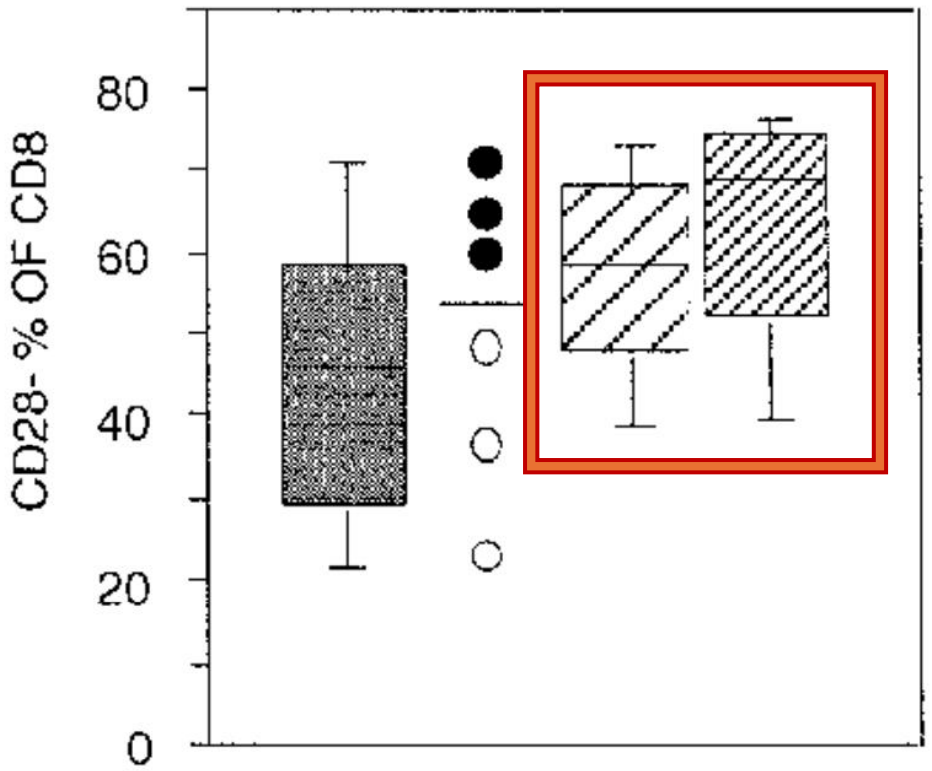
Coulter XL flow cytometer

Representative histograms from ~1995



Original Fresh Blood CD8 T cell subsets from 1995/6





CONTROL HIV NEGATIVE COHORT

TA-LTNP COHORT

SA-LTNP COHORT

○ SBBC (undetectable plasma HIV RNA)

● SBBC (detectable plasma HIV RNA)

PBMC cryopreserved in liquid N_2 from 3 Cohorts

- 1995 Old Controls HIV-uninfected from Blood Bank Study n = 20
- (mean age 60)

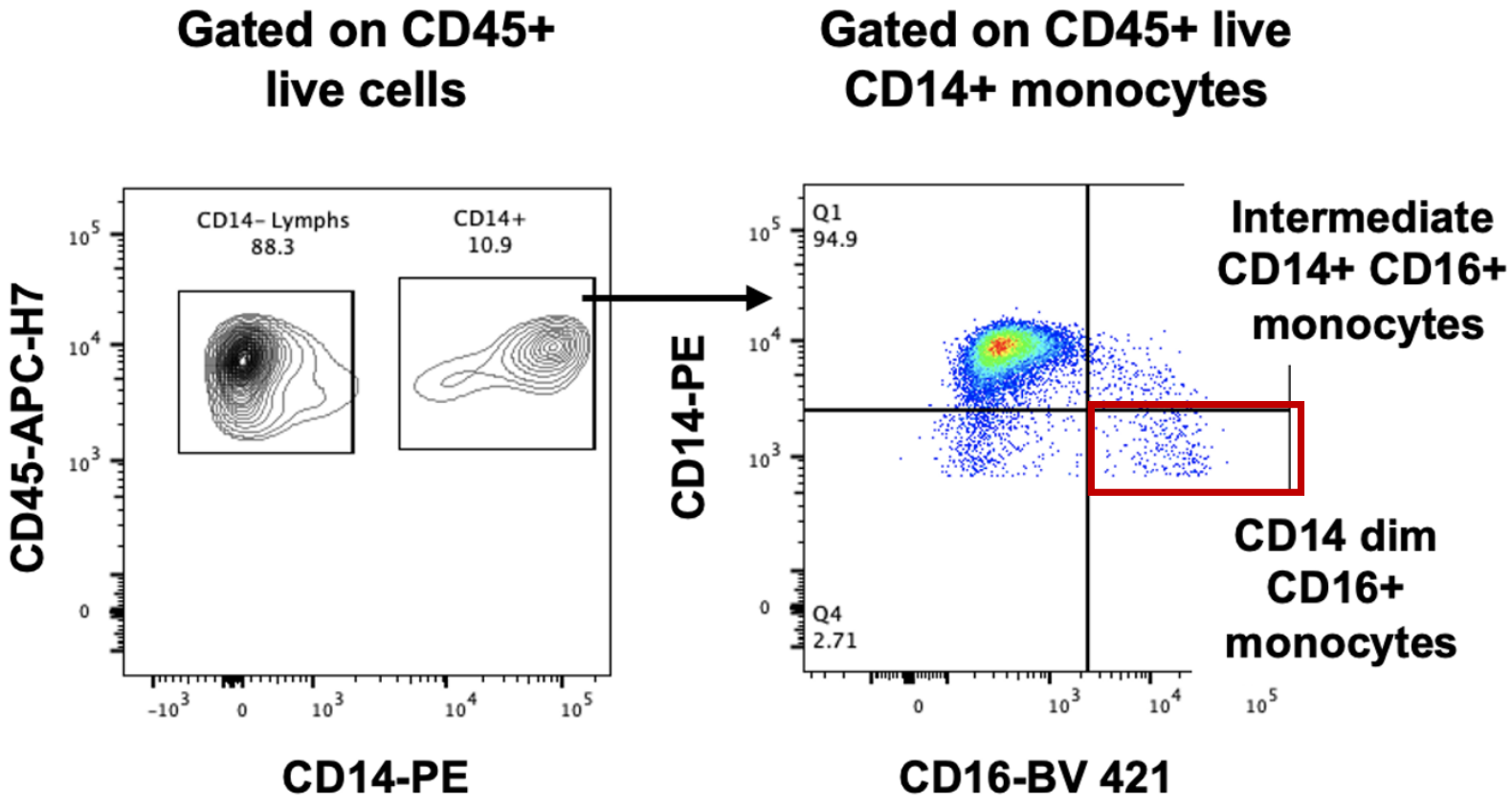
- 1995 Old HIV+ Untreated, viraemic transfusion-acquired and sexually-acquired HIV-infected subjects n = 12 (mean age 47)

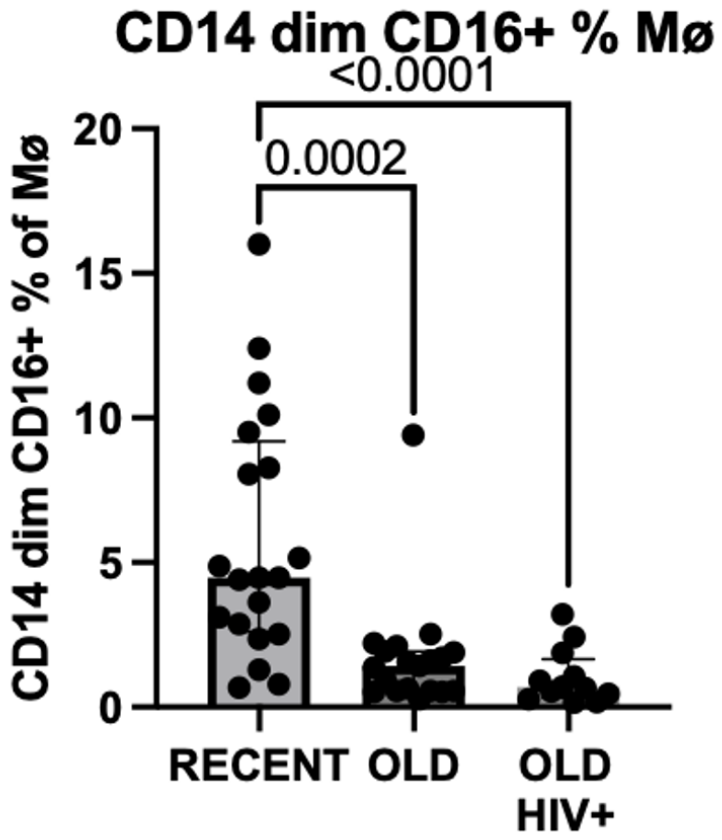
- 2020 Recent PBMC Anonymous healthy staff donors from 2020 n = 20
- (mean age 43) IVRN QAP for PBMC storage

- Thawed, treated with DNase 1 (0.1mg/ml) 15 min RT, ~90% viability

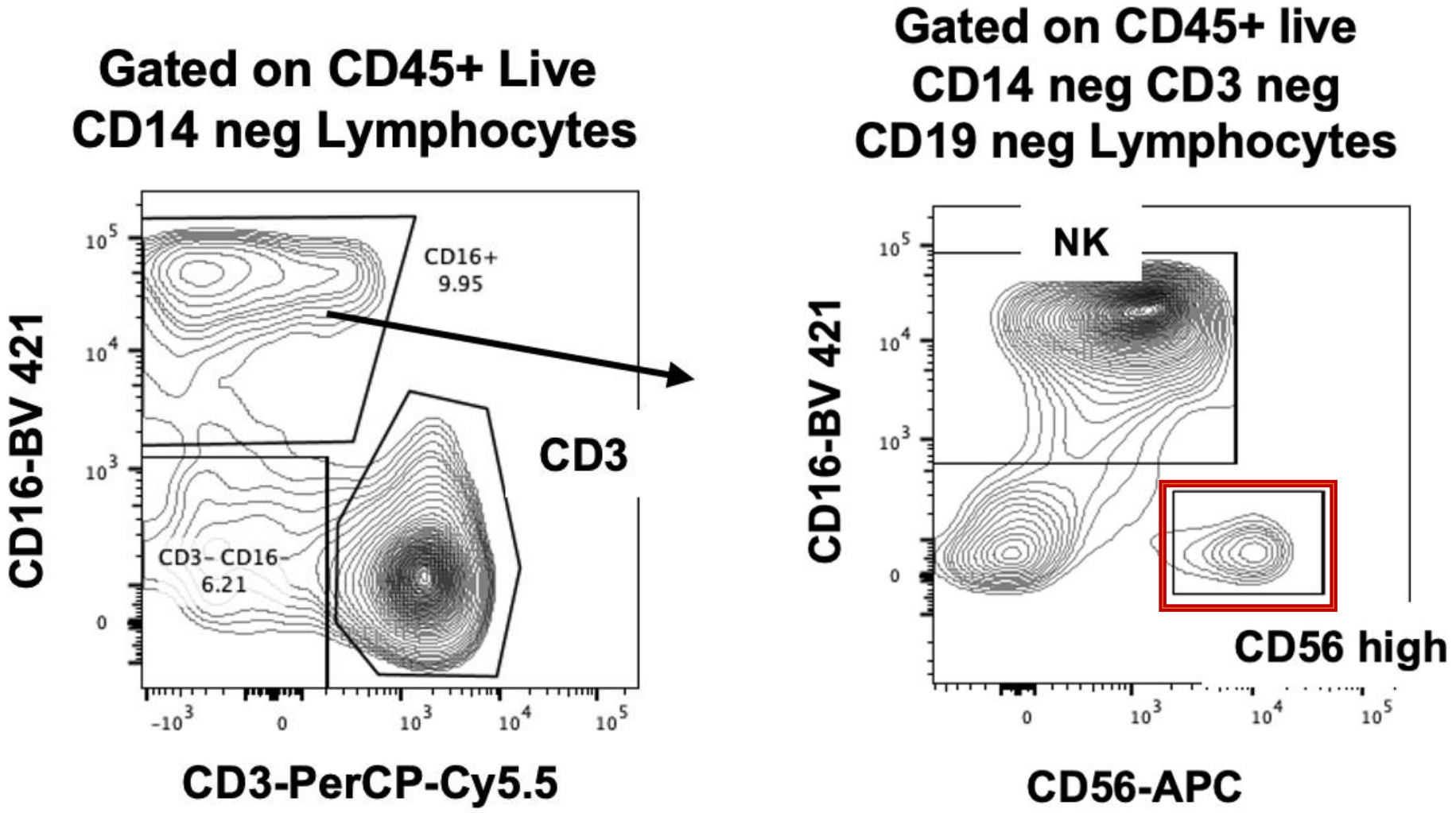
- Stained with two 18-colour panels (PBMC subsets and T cell subsets)
- analysis on a 5-laser Fortessa
- T cell subsets in fresh blood samples from staff controls n = 16 (mean age 46)

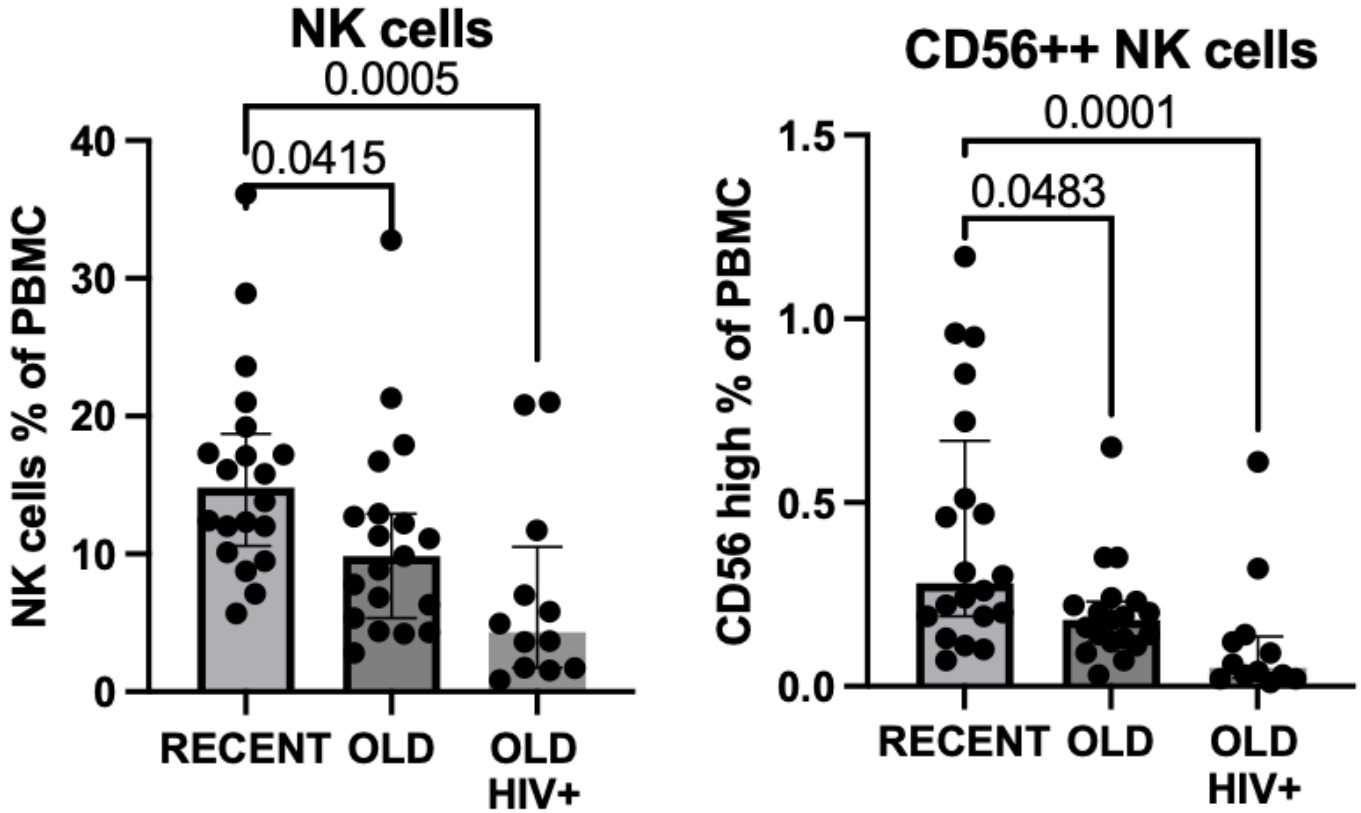
PBMC subsets – Monocyte subsets



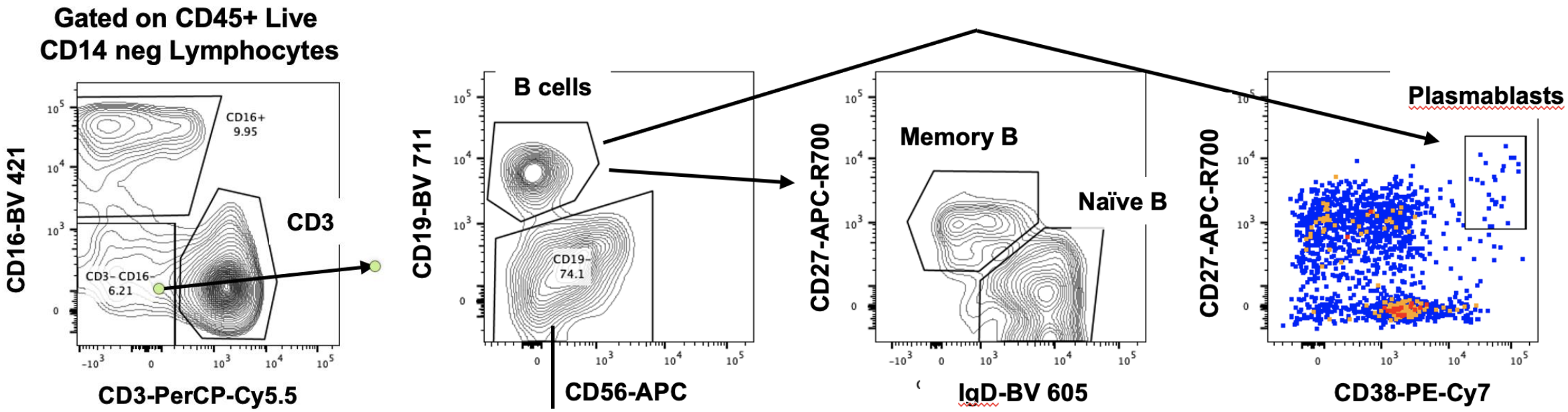


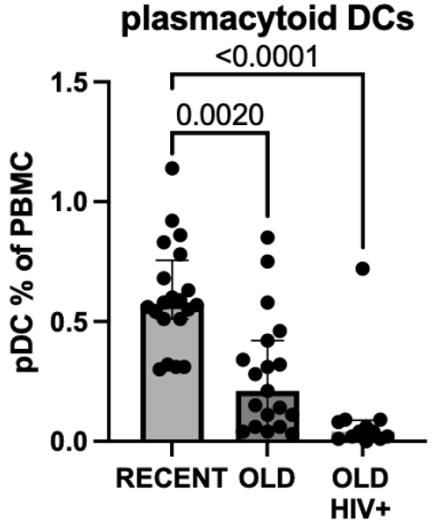
PBMC subsets – NK cell subsets



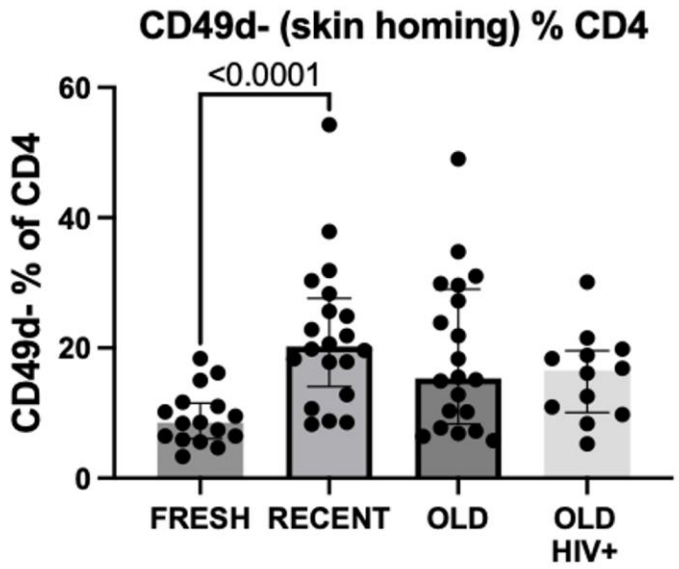

NK cells
0.0005
0.0415
NK cells % of PBMC
40
30
20
10
0
RECENT
OLD
OLD HIV+
CD56++ NK cells
0.0001
0.0483
CD56 high % of PBMC
1.5
1.0
0.5
0.0
RECENT
OLD
OLD HIV+

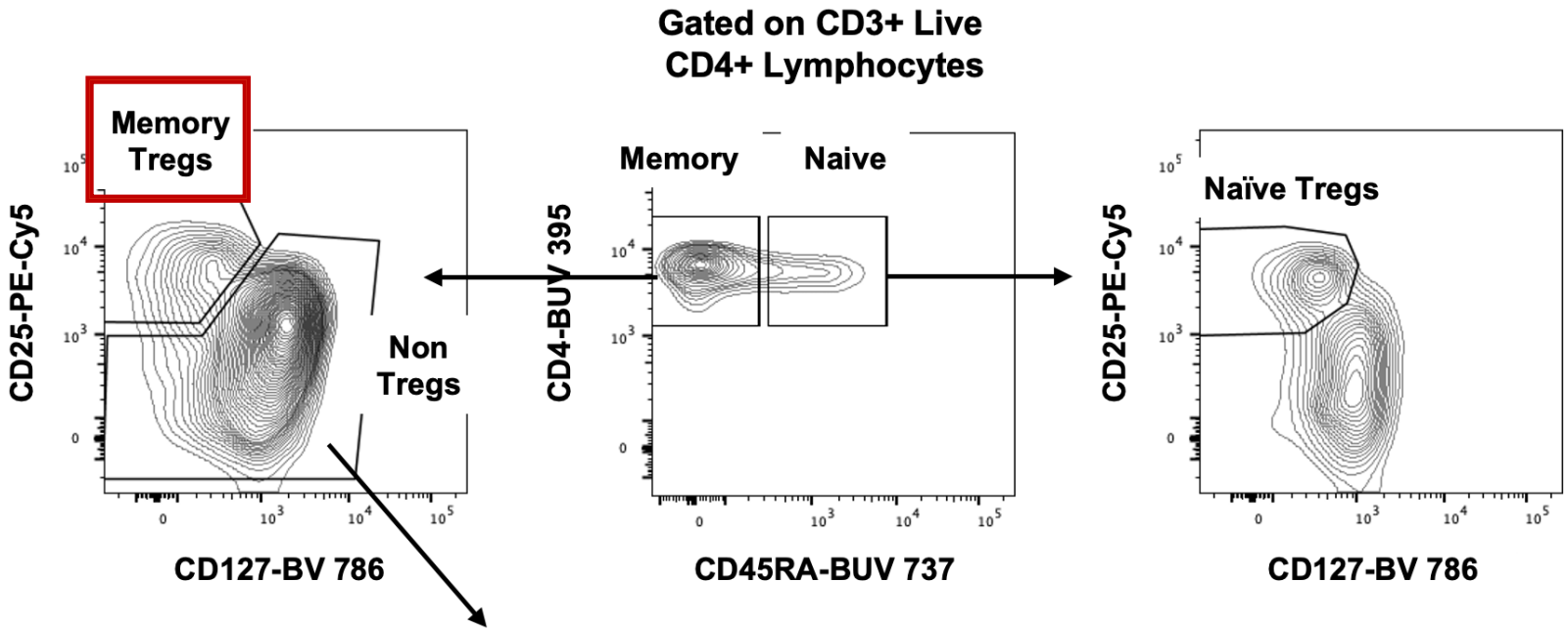
18-colour PBMC subsets – B cells, Basophils, mDC and pDC

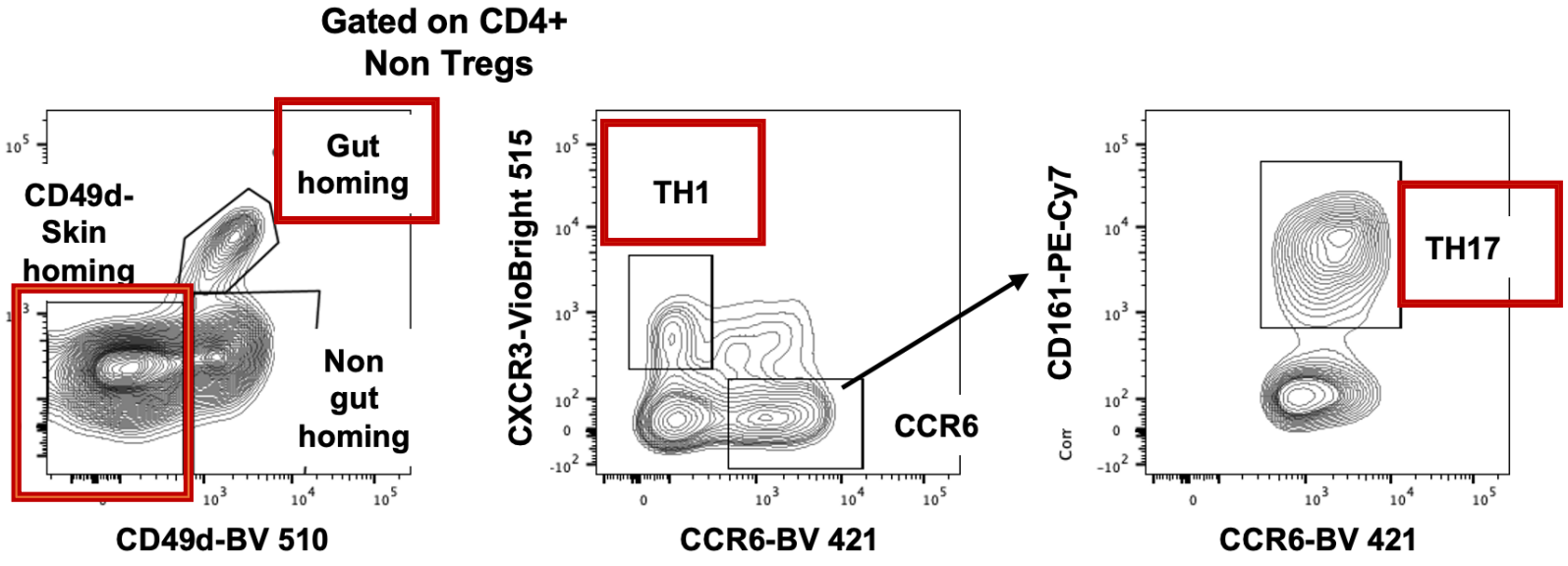




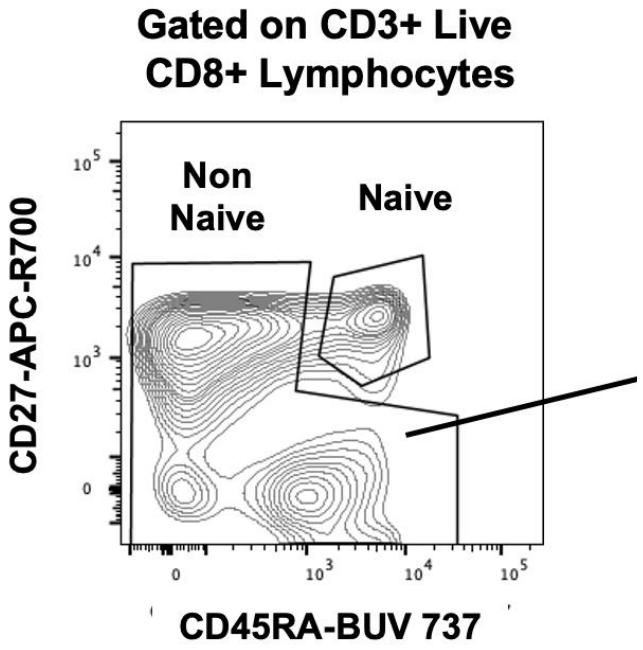
CD4 T cell subsets



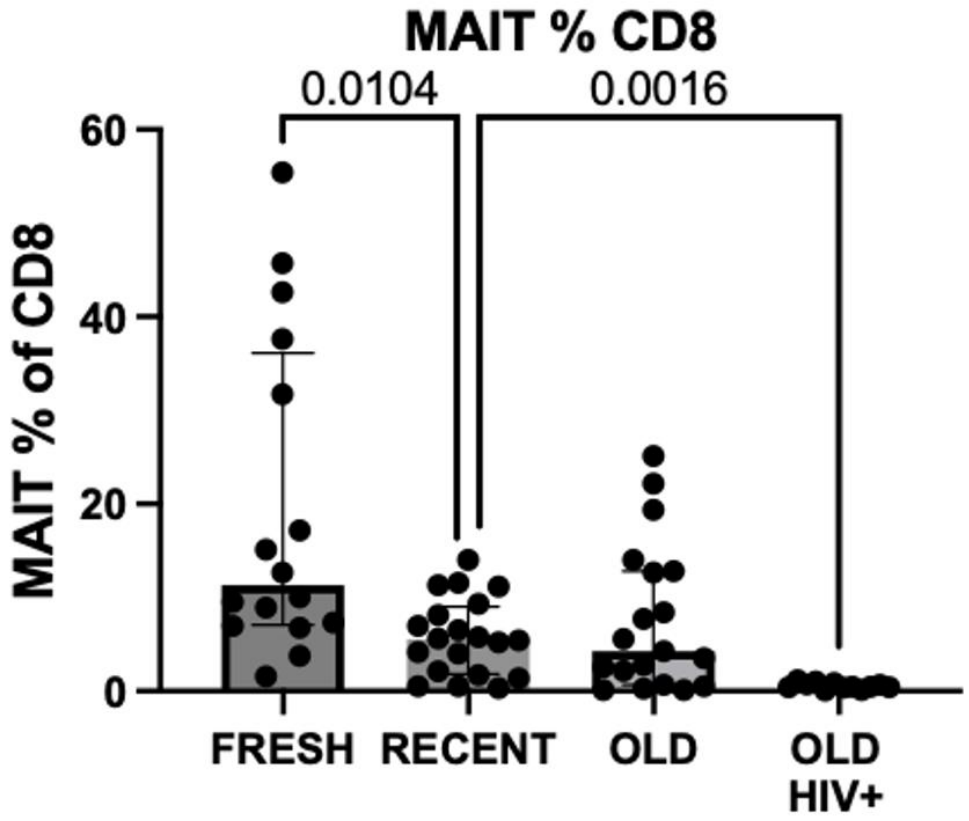


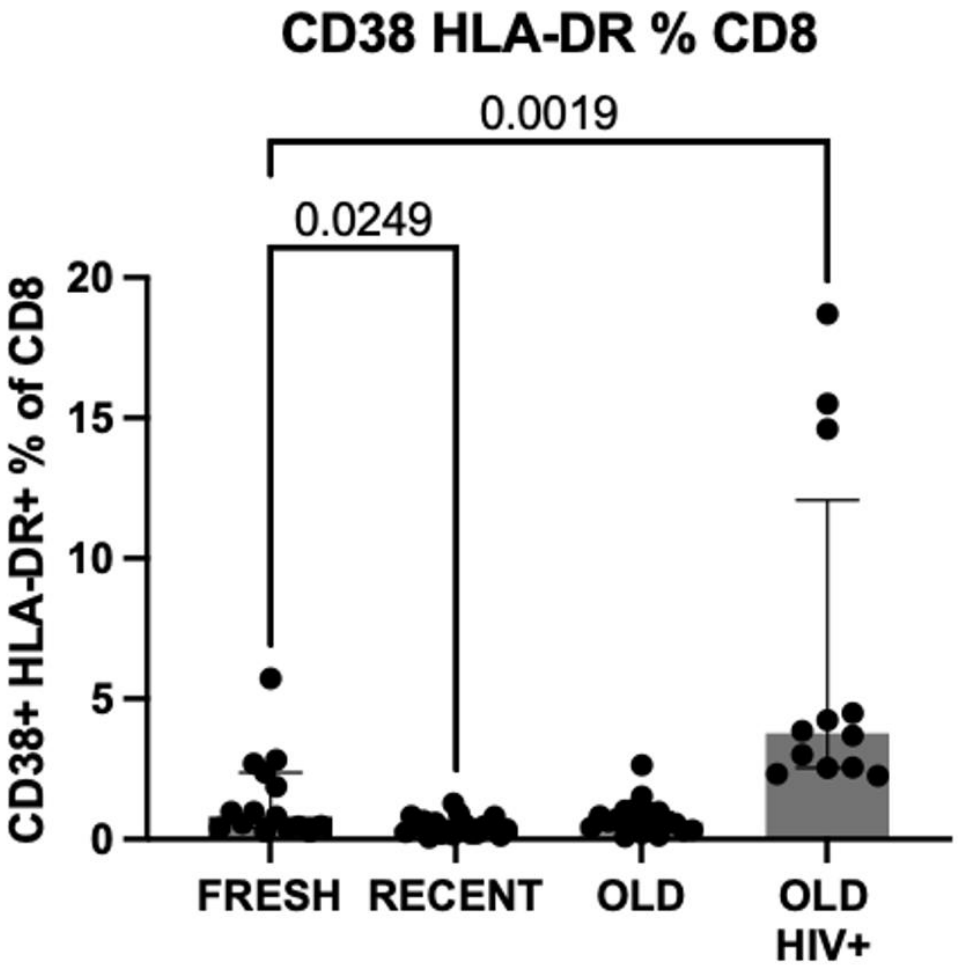


CD8 T cell subsets

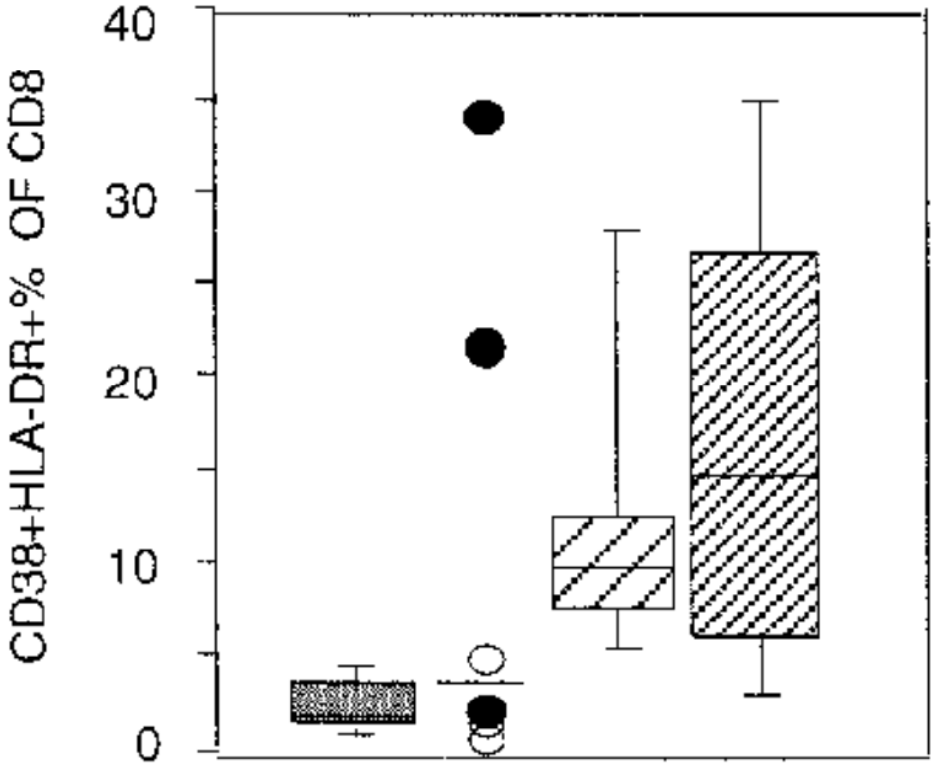


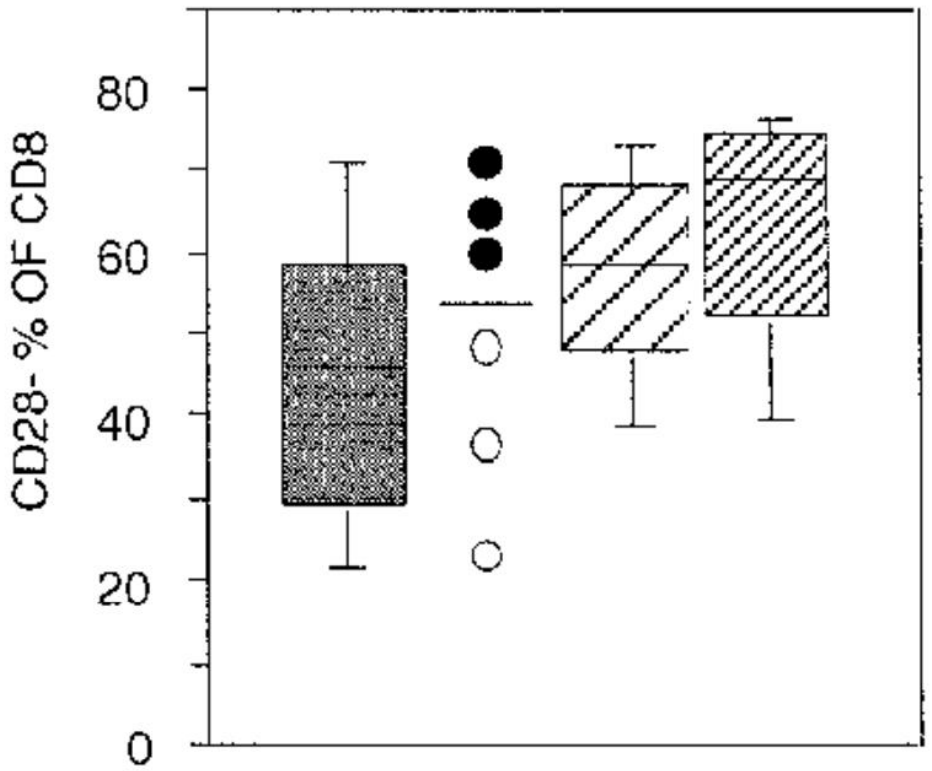
CD8 T cell subsets – MAIT cells and activated CD8 T cells





Original Fresh Blood CD8 T cell subsets from 1995/6





CONTROL HIV NEGATIVE COHORT

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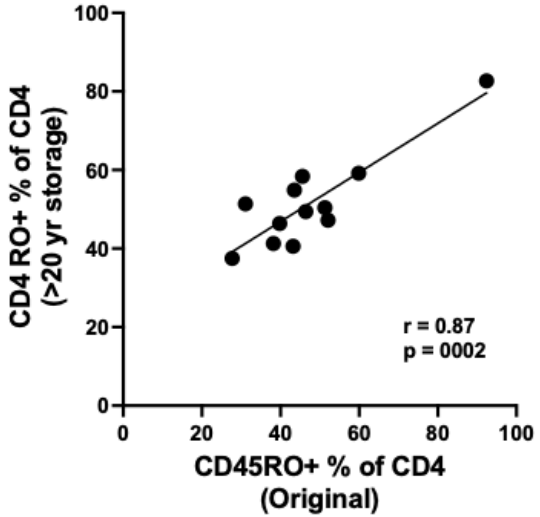
SBBC (undetectable plasma HIV RNA)

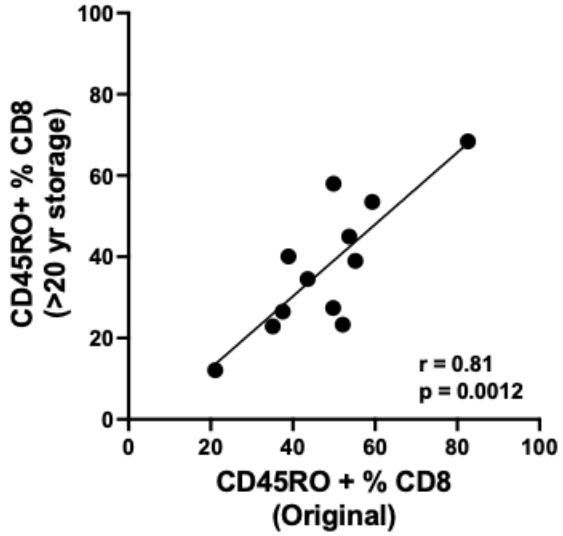
SBBC (detectable plasma HIV RNA)

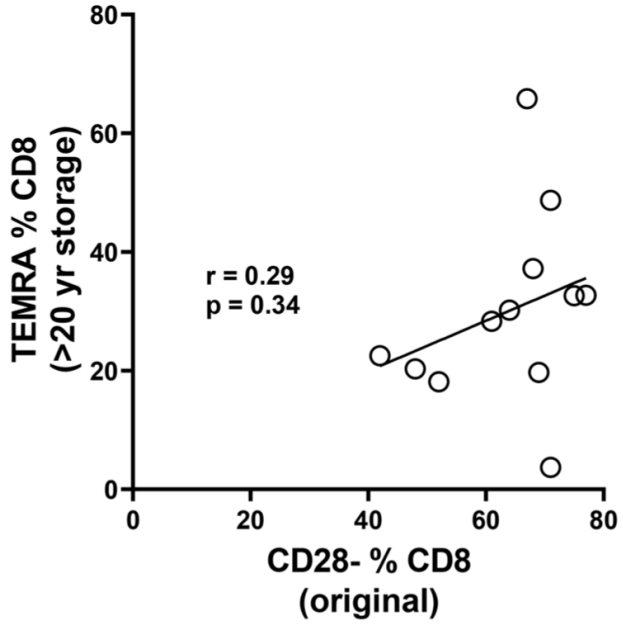
CD4 and CD8 T cell subsets – Thawed OLD HIV+ cells vs original fresh blood

CD45RO+ CD4 T cells



CD45RO+ CD8 T cells



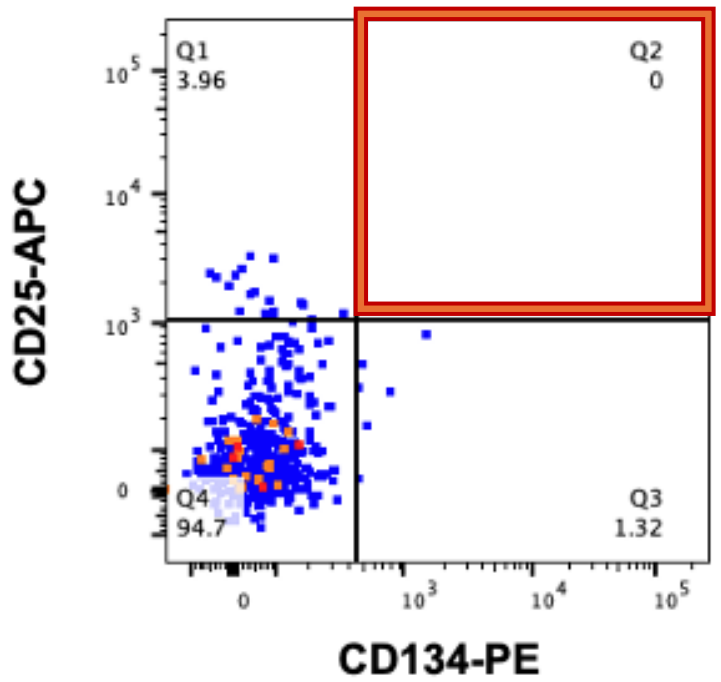


OLD HIV+ CD8 T cells

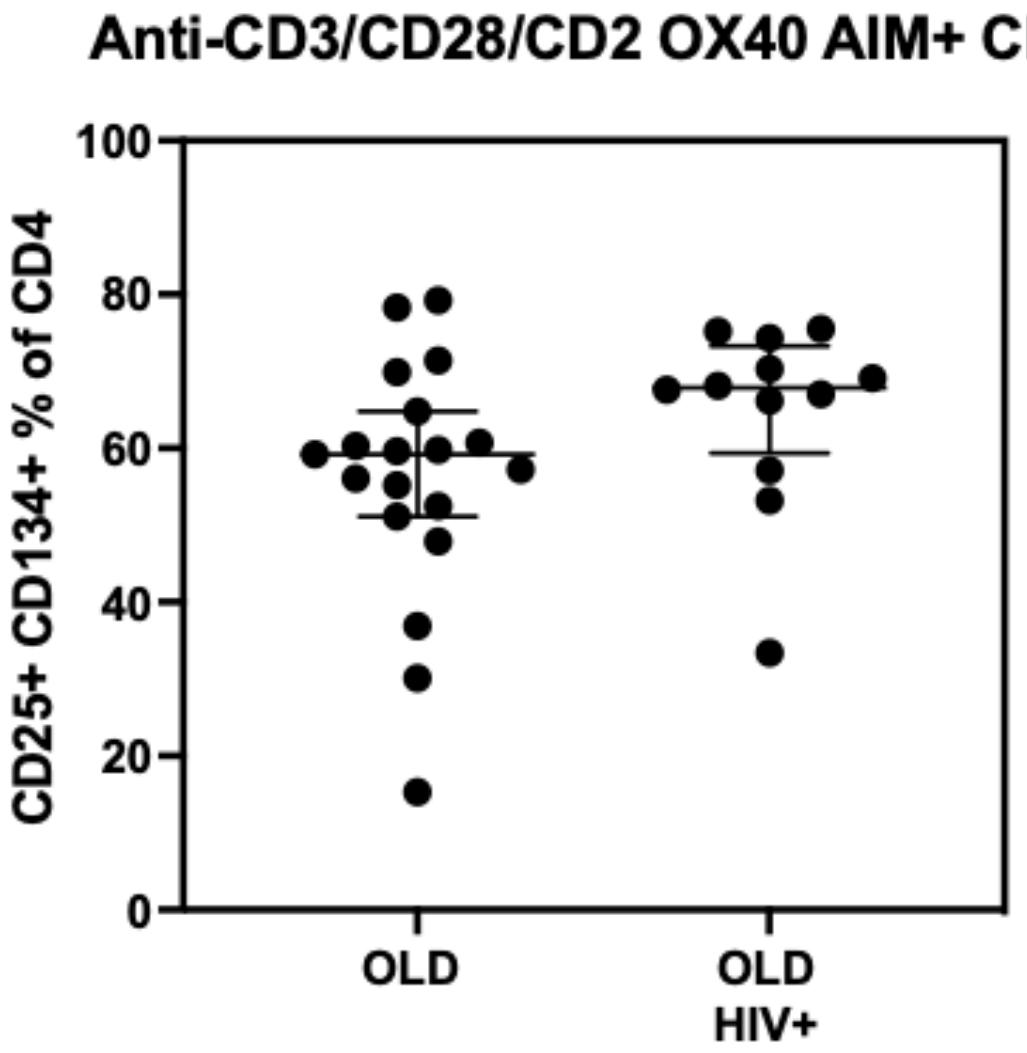
CD25+ CD134+ (OX40) Activation Induced Marker (AIM) assay of mitogen- and antigen-specific CD4 T cells

Gated on CD3+ CD4+ Lymphocytes

No stimulation background (negative control)



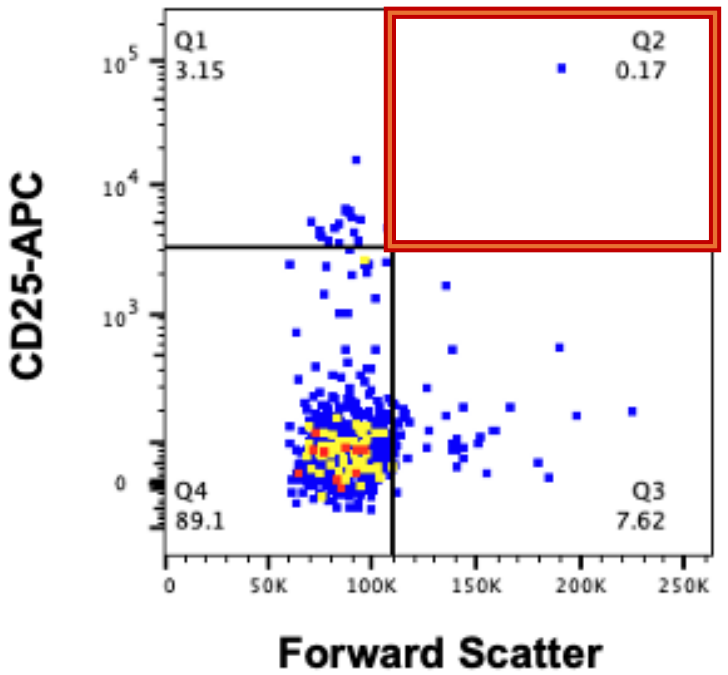
Polyclonal and antigen specific CD4 T cell responses
OX40 AIM (activation induced marker) 48 hour assay



Day 7 proliferation assay of mitogen- and antigen-specific CD4 T cells

Gated on CD3+ CD4+ Lymphocytes

No stimulation background (negative control)



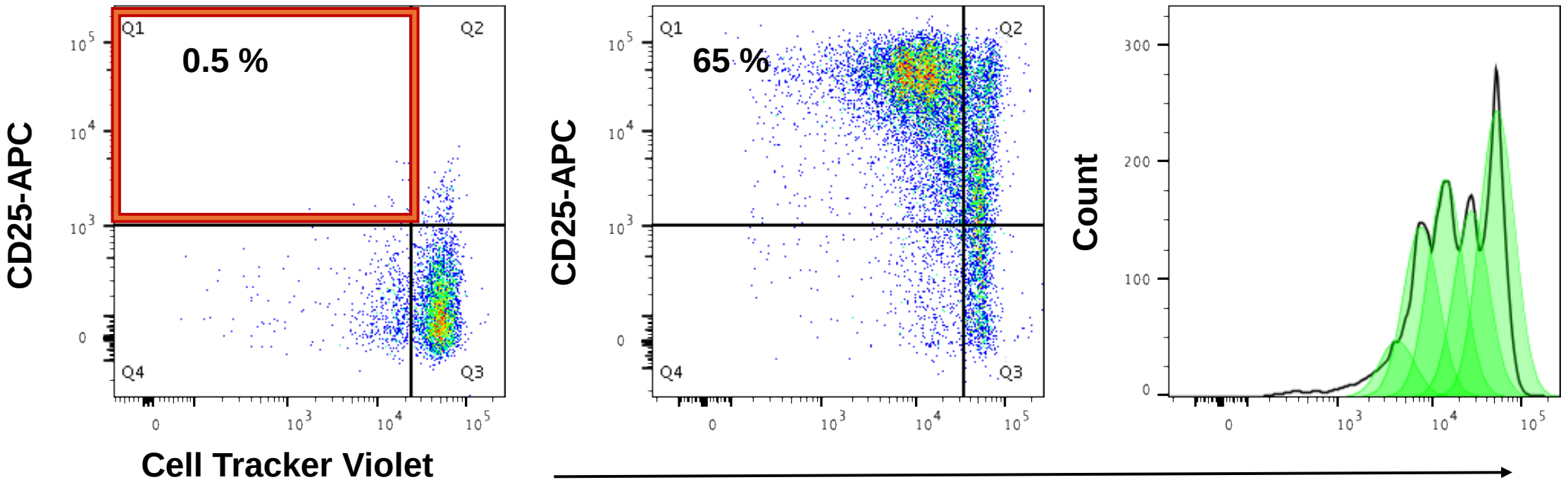
Optimal polyclonal T cell stimulation of PBMC *via* TCR *in vitro*

Day 4

Gated on CD4
Unstimulated

Gated on CD4
+ 3/28/2

Cell divisions
+ 3/28/2



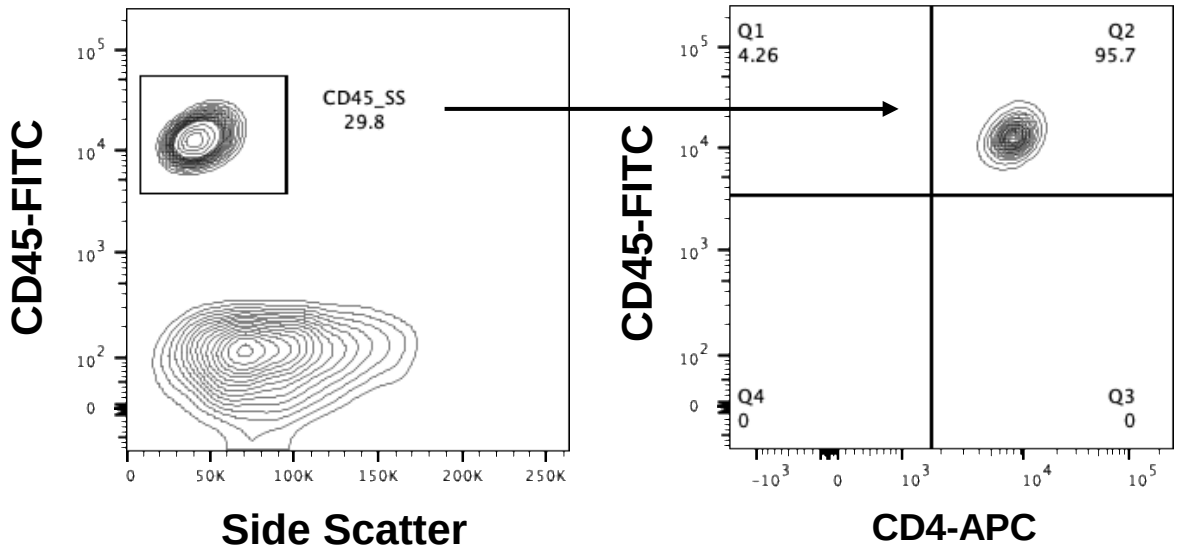
Polyclonal TCR cell stimulation of isolated CD4 T cells

Patient's ACD Blood

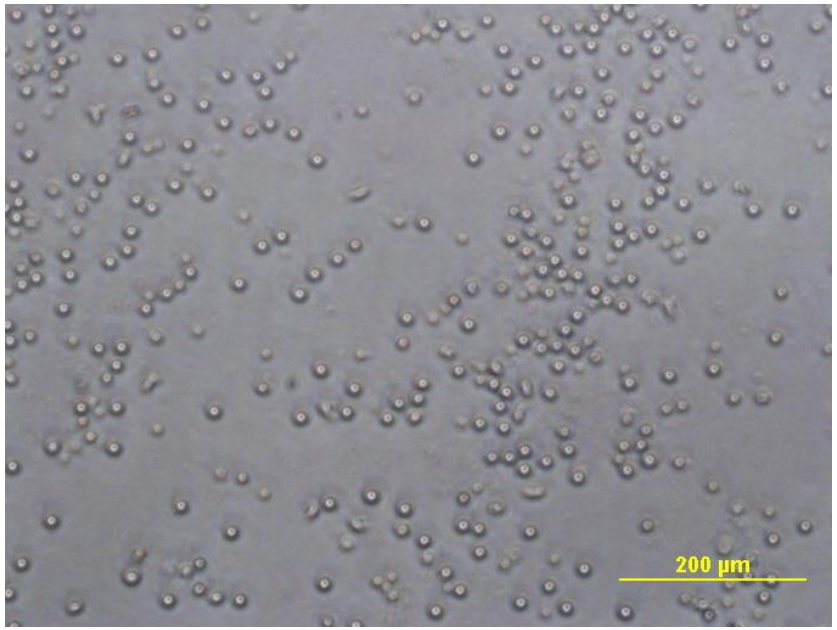
+ RosetteSep CD4 Enrichment Cocktail

Ficoll →

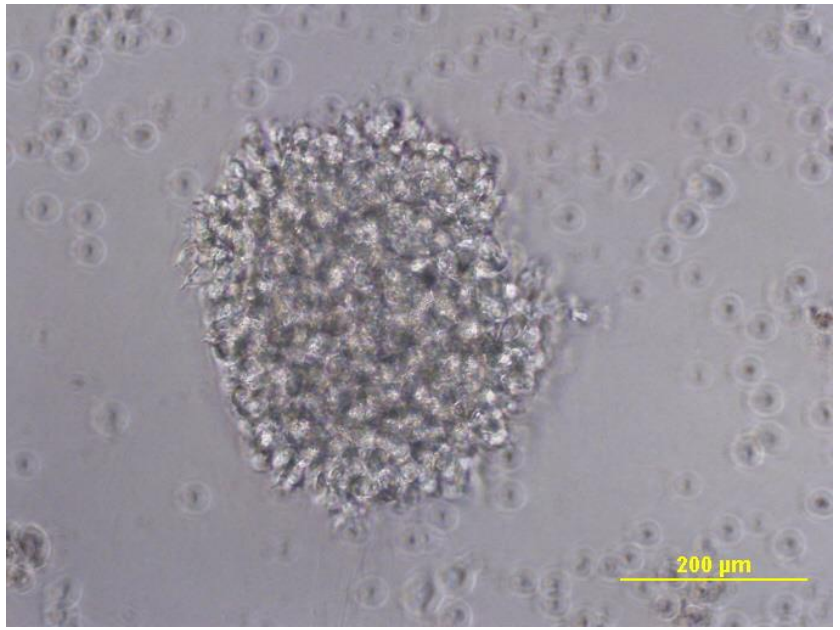
Isolated CD4 cells 96% purity



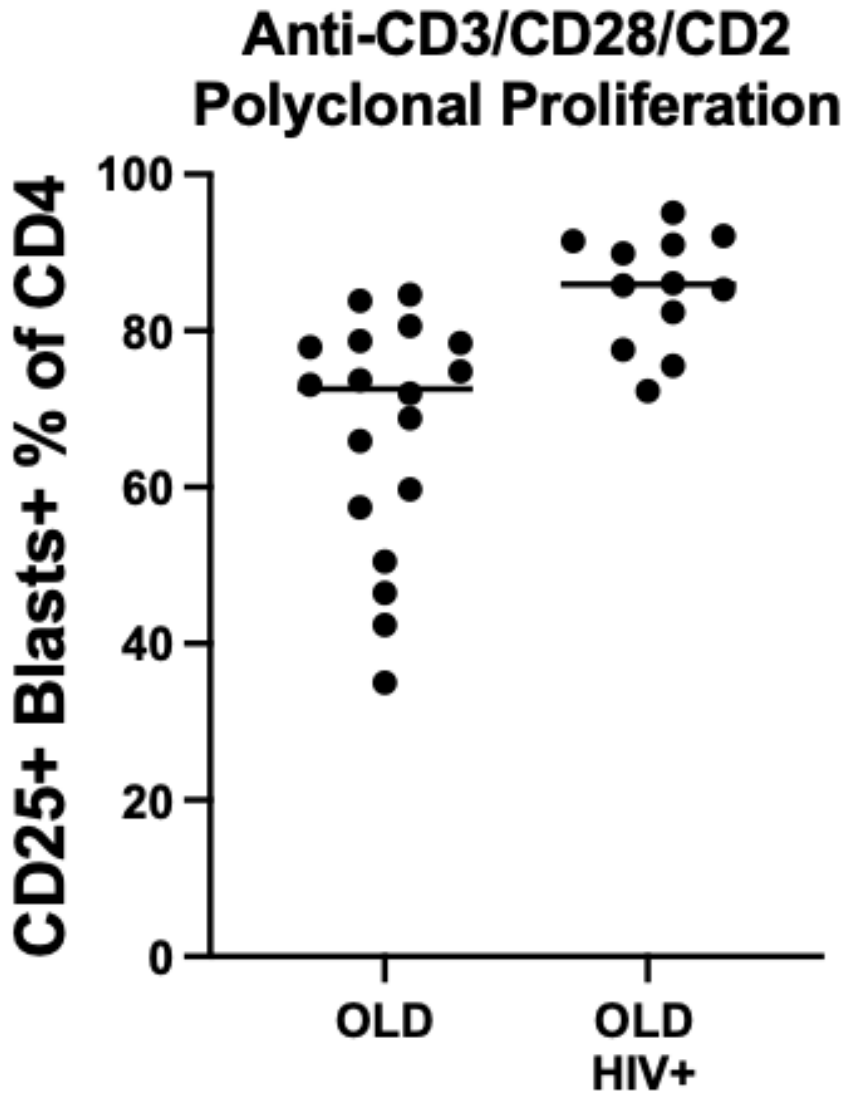
Cultured for 3 days + IL2



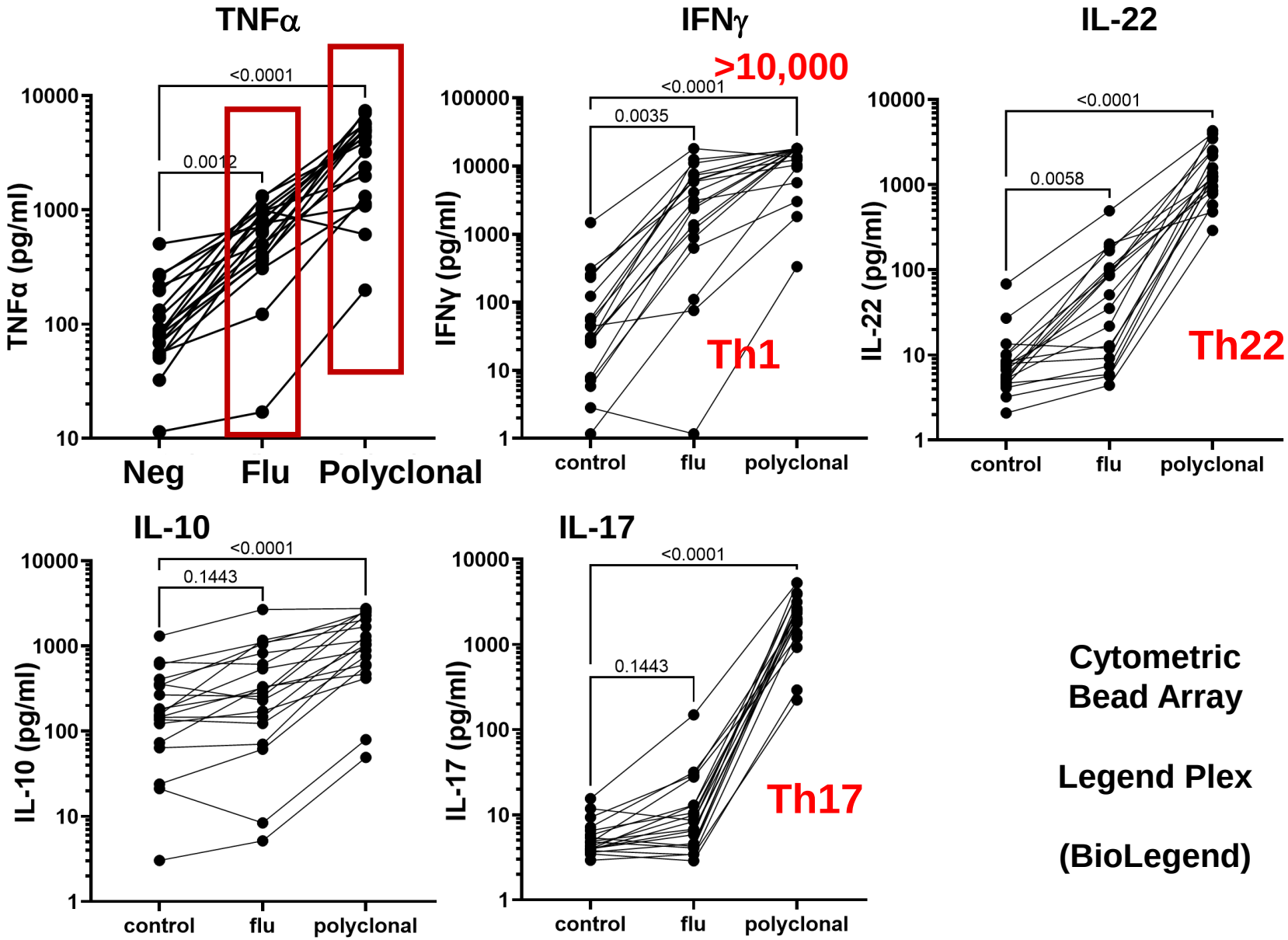
Cultured for 3 days + IL2 + Anti-CD3/CD28/CD2



Polyclonal and antigen specific CD4 T cell responses
7 day proliferation assay



Cytokine production in 48hr supernatants from Old HIV Uninfected Controls' PBMC cultured with Flu or with anti-CD3/CD28/CD2

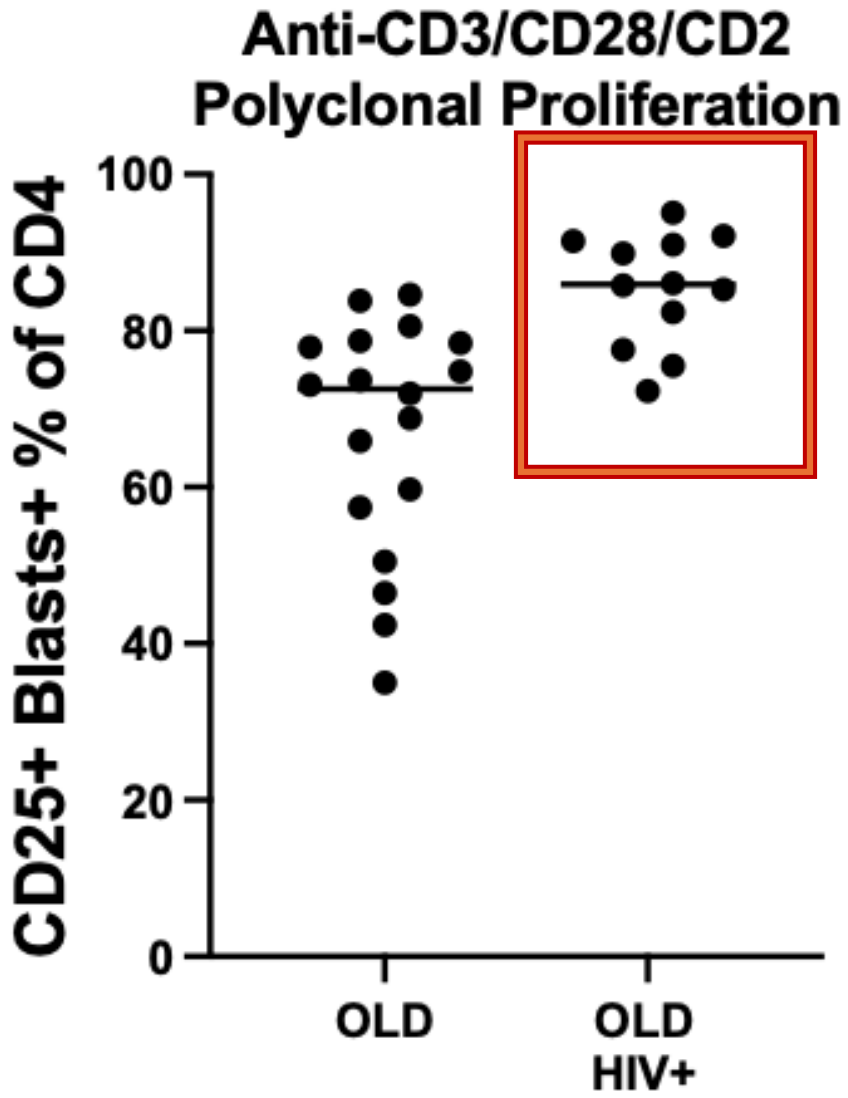


Cytometric Bead Array

Legend Plex

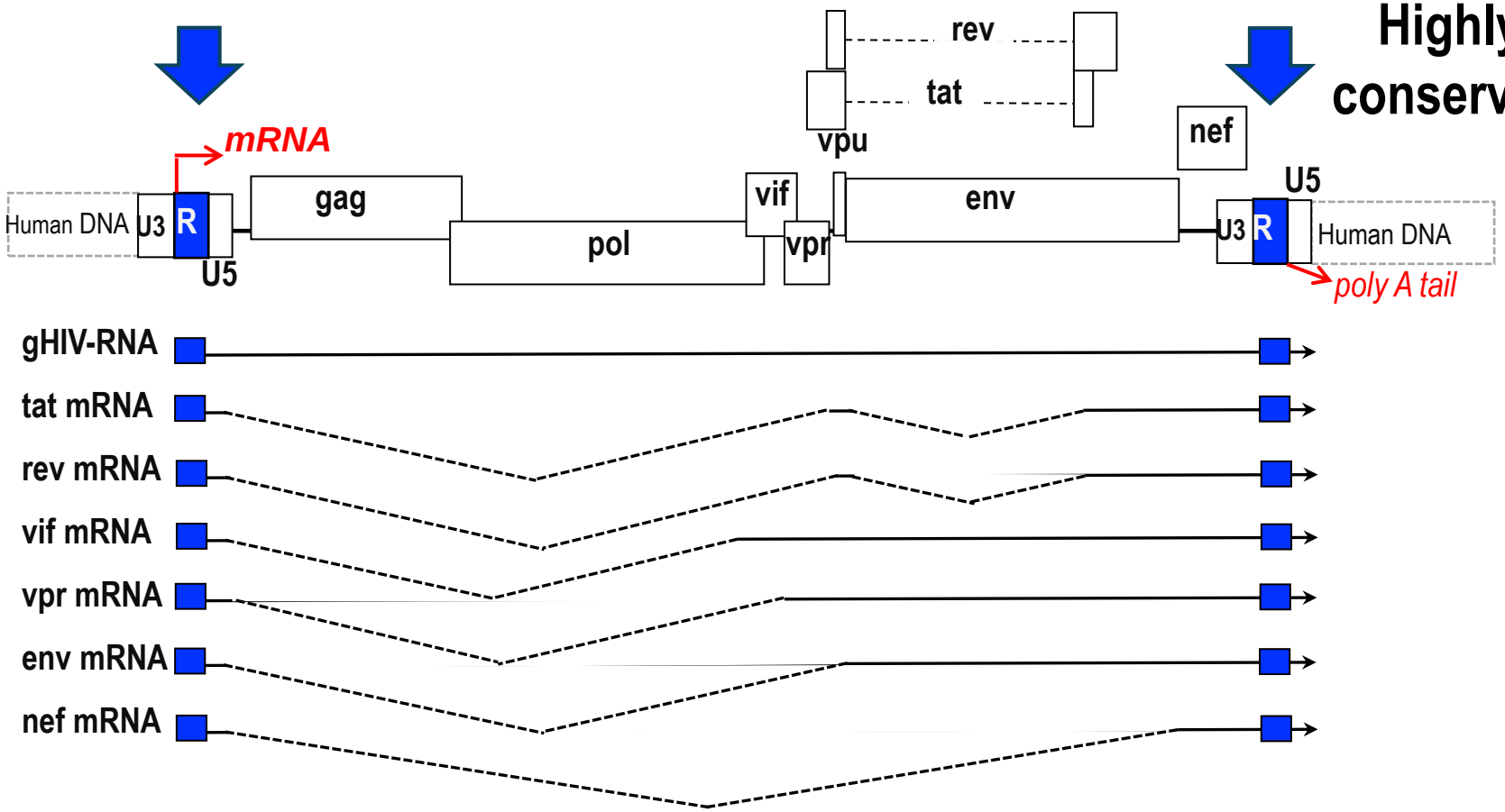
(BioLegend)

Polyclonal and antigen specific CD4 T cell responses
7 day proliferation assay



Extract RNA to measure cell-associated HIV RNA

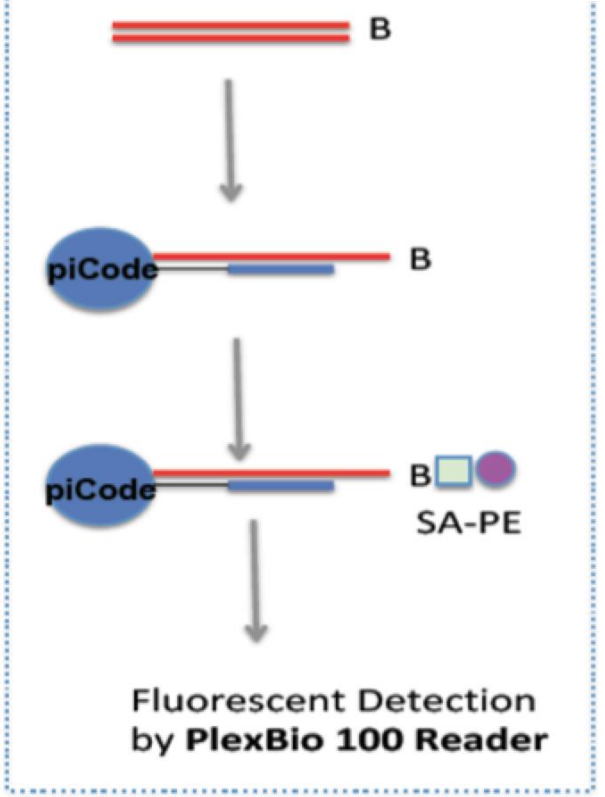
Double-R assay of HIV-1 DNA and HIV-1 RNA transcripts



Highly conserved

IntelliPlex assay

πCode



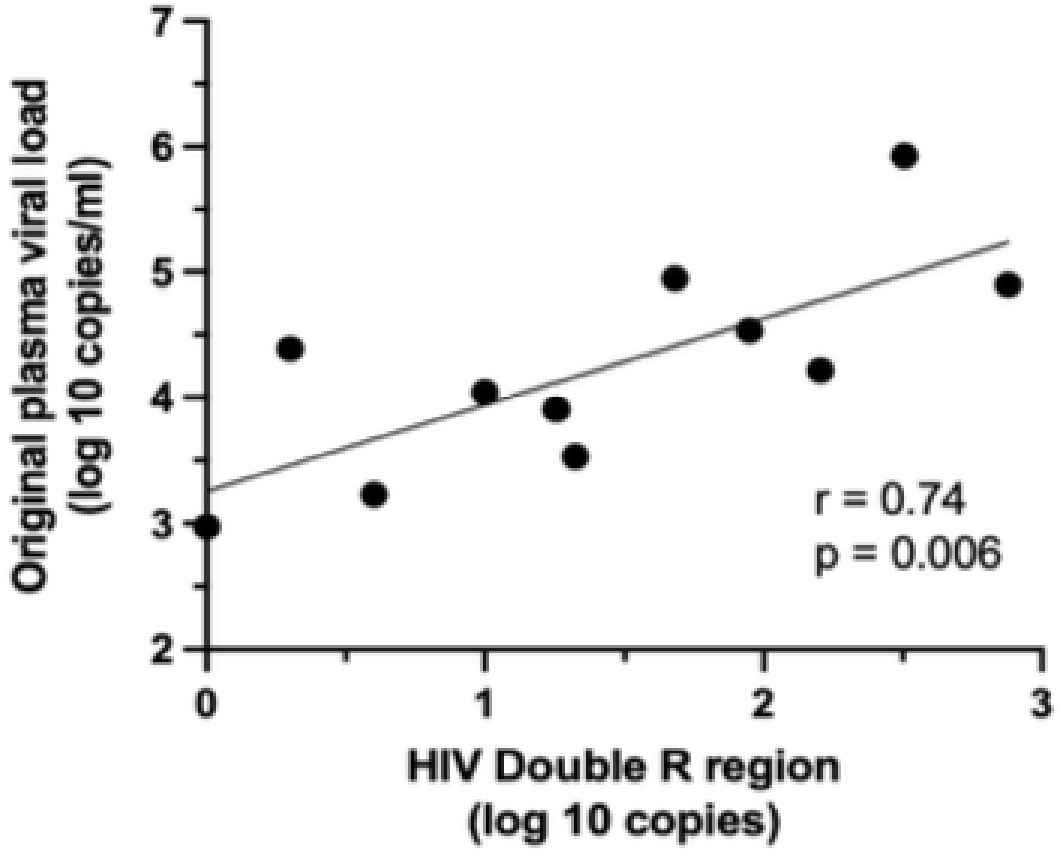
Suzuki et al *J.AIDS HIV Treatment* 2019

πCode assay >27x more sensitive than Real-Time PCR analysis

Old HIV+ donor PBMC optimally stimulated in vitro with anti-CD3/CD28/CD2

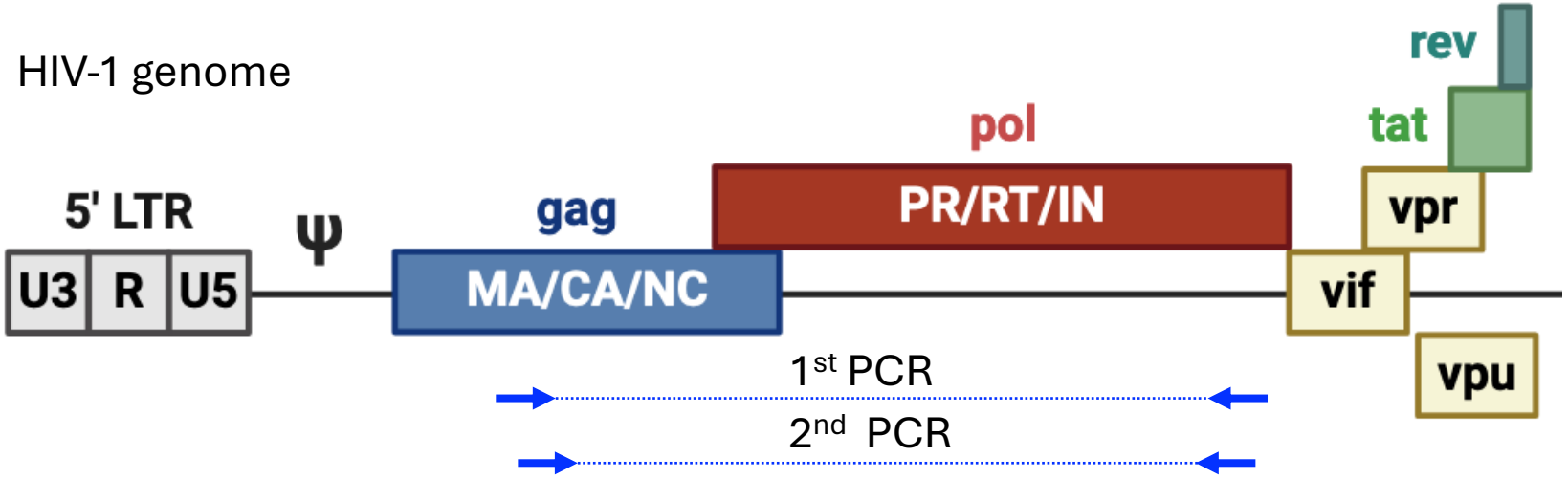
RNA extracted after 7 days

Transcripts vs donor's original plasma viral load



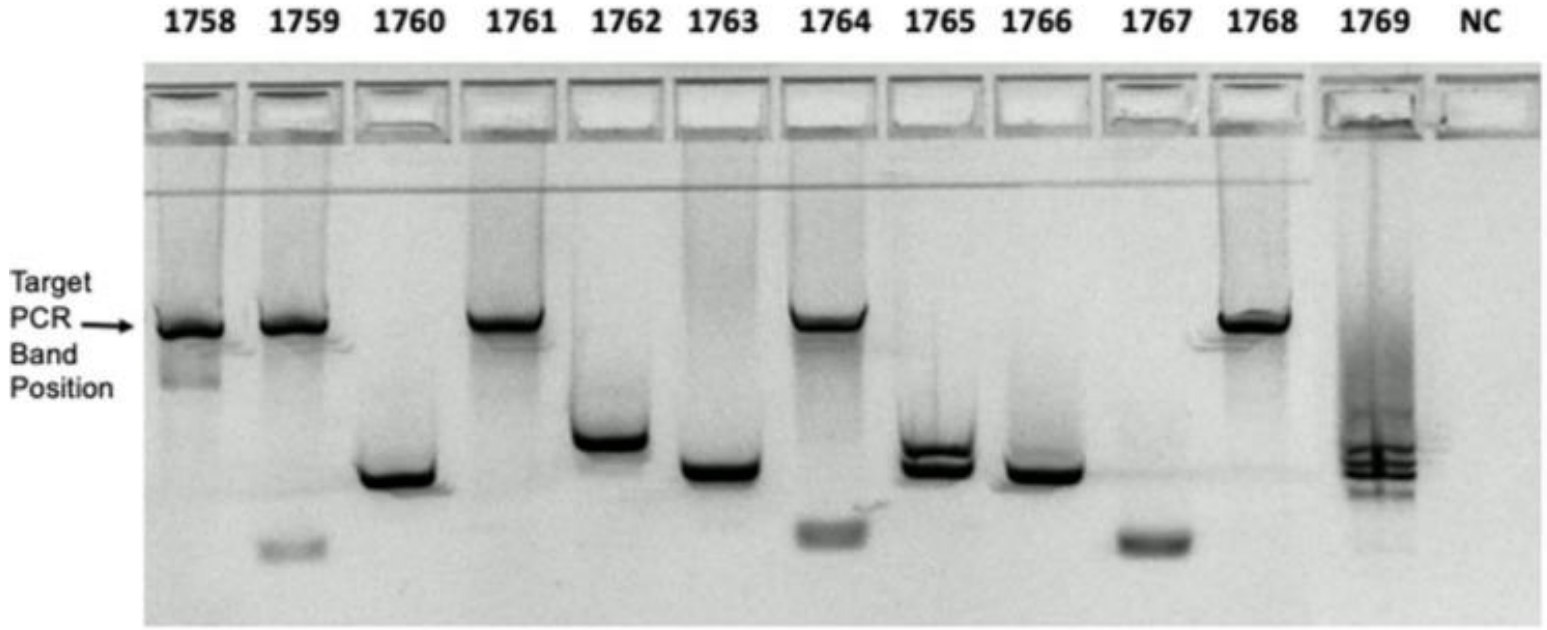
Recovery of HIV RNA after > 20 yr storage

Nested PCR of extracted RNA from cultured CD4 at day 7



4kbp product

Recovery of HIV RNA after > 20 yr storage: 4kbp product and sequencing



Oxford Nanopore sequencing of 4kbp product

Sample ID	CA	PR	RT	IN	Drug resistance interpretation
N64 (ID.1758)	none	none	M184V	none	emtricitabine (FTC), lamivudine (3TC)
N65 (ID.1759)	none	none	D67E,T69ins,T215Y	none	zidovudine (AZT), lamivudine (3TC)
N66 (ID.1761)	none	none	none	none	none
N67 (ID.1764)	none	none	none	none	none
N68 (ID.1768)	none	none	none	none	none

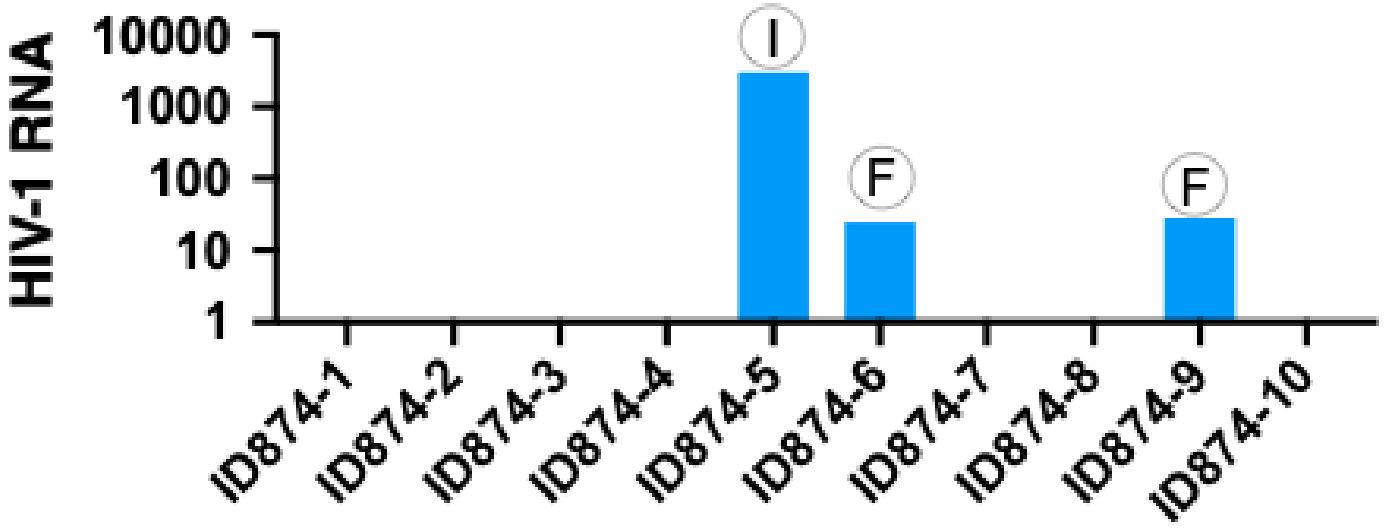
CA: Capsid, PR: Protease, RT: Reverse transcriptase, IN: Integrasae

Conclusions

- PBMC's stored for over 20 years still contained all well defined immune subsets of cells:
  - Naïve and memory B cells and plasmablasts
  - Basophils, DC subsets, NK cell and monocyte subsets
  - CD4 and CD8 naïve and memory subsets
- Unique opportunity to compare to original flow analysis when fresh:
  - Naïve and memory and CD28- cells correlated very well
  - Activated CD4 & CD8 cells in HIV+ patient samples did not survive well
- Stored PBMC'S showed polyclonal and recall antigen-specific functions:
  - OX40 AIM assays
  - Proliferation assays
  - Cytokine assays
- After 7 days of optimal stimulation for HIV + patient PBMC samples:
  - Production of HIV RNA transcripts from latent infection
  - Enough RNA was produced for long PCR products for sequencing.

Future Studies

- PBMC's stored for over 20 years are important:
  - Since late 1990's, most HIV+ patients have been on ART
  - Slow progressors, long-term non-progressors or Elite Controllers
  - Especially Elite Controllers
    - have undetectable plasma viremia without ART
    - Regarded as benchmark for functional cure
- Elite Controllers have undetectable cell-associated HIV RNA in PBMC *ex vivo*
  - Need multiple cultures of stimulated CD4 T cells to detect any CA-HIV-RNA



Acknowledgements

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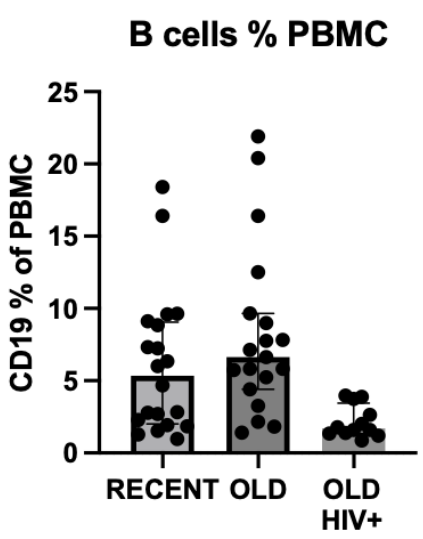
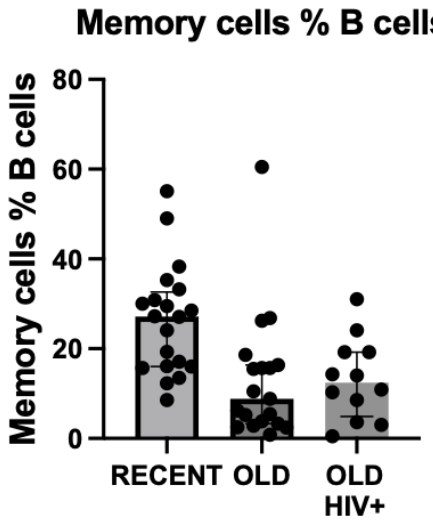
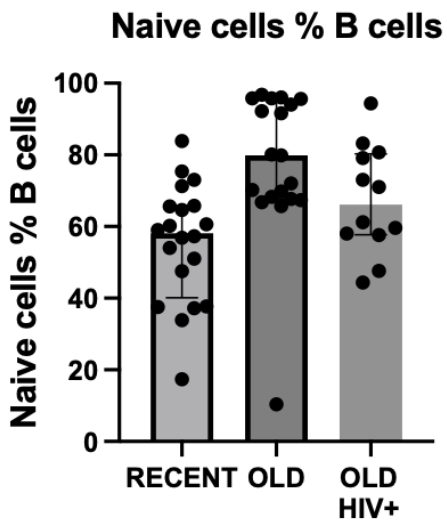
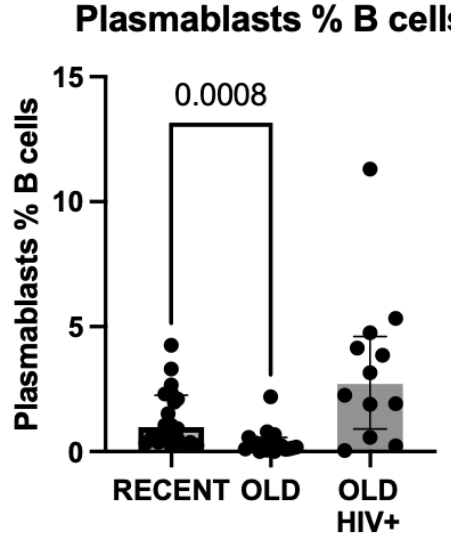
Support
Immunovirology Research Network (IVRN)
Denka Inc., Tokyo, Japan

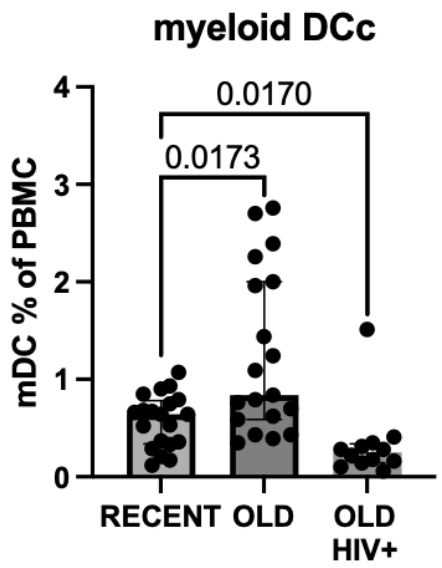
PBMC subsets – B cells, Basophils, mDC and pDC

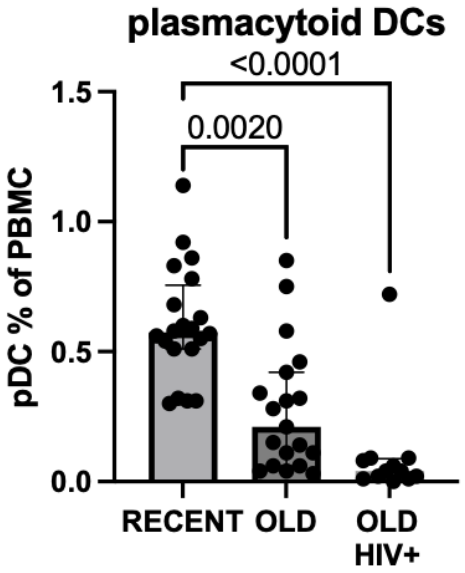


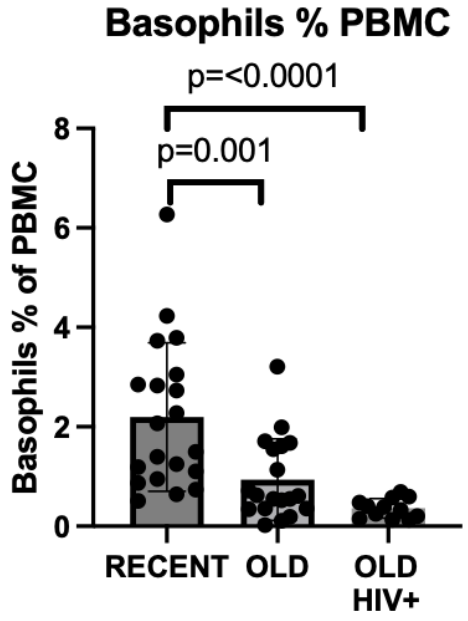


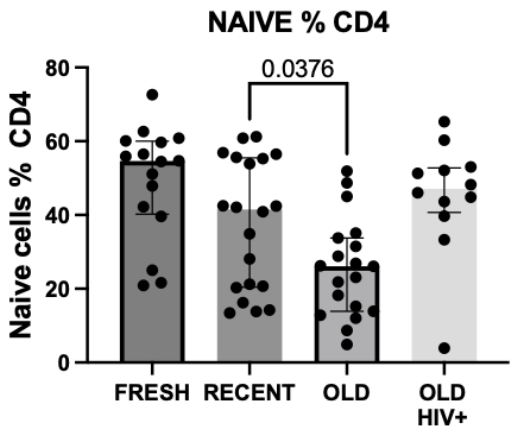


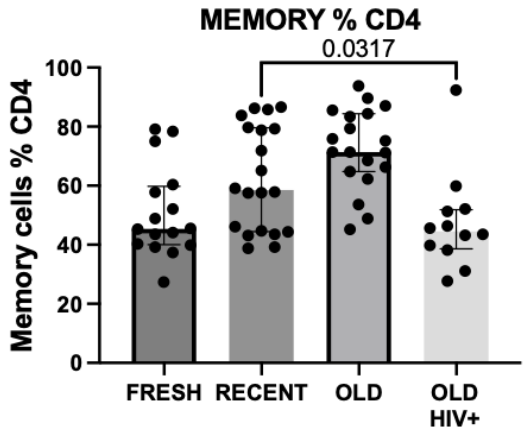


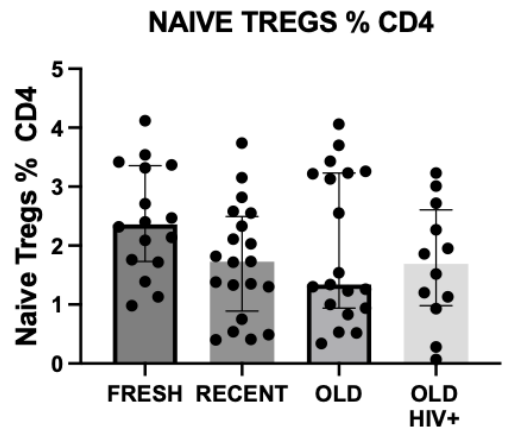


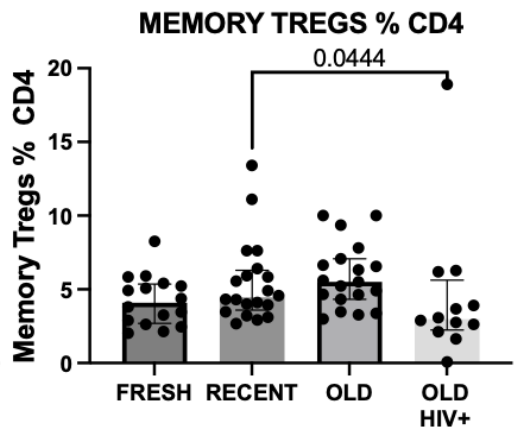


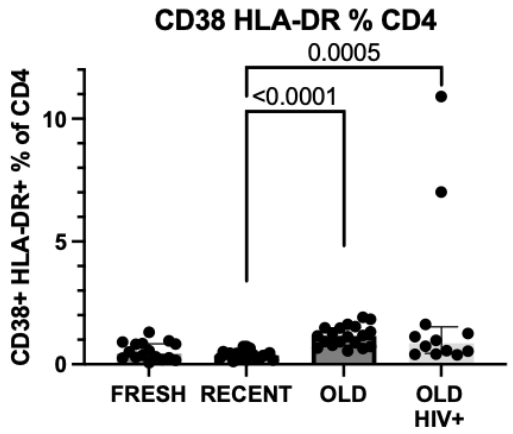


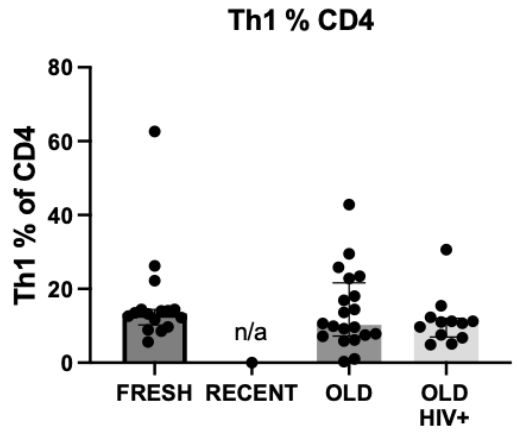

NAIVE % CD4
Naive cells % CD4
0.0376
FRESH
RECENT
OLD
OLD HIV+

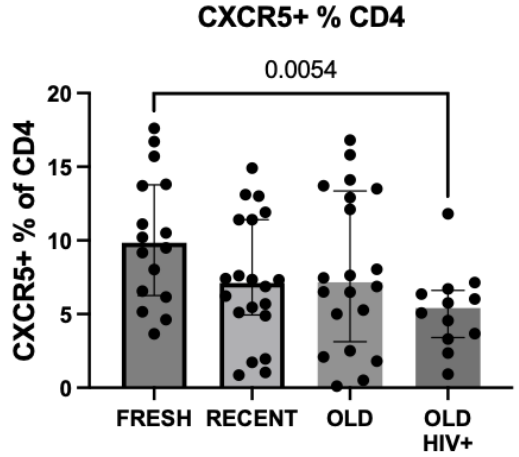

MEMORY % CD4
Memory cells % CD4
0.0317
FRESH
RECENT
OLD
OLD HIV+

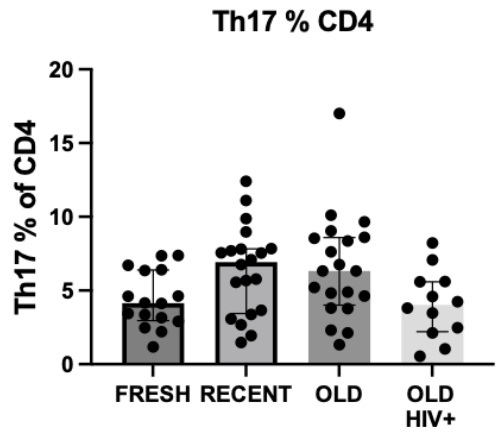

NAIVE TREGS % CD4
Naive Tregs % CD4
FRESH
RECENT
OLD
OLD HIV+

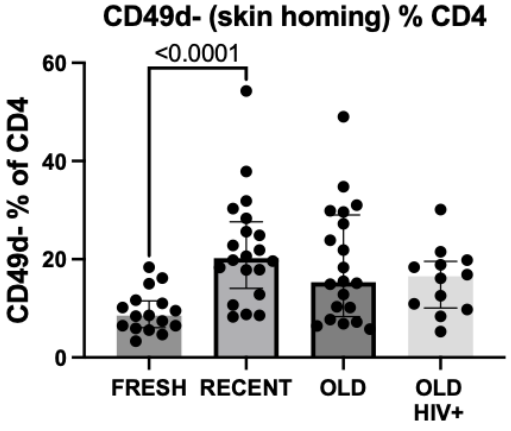

MEMORY TREGS % CD4
Memory Tregs % CD4
0.0444
FRESH
RECENT
OLD
OLD HIV+

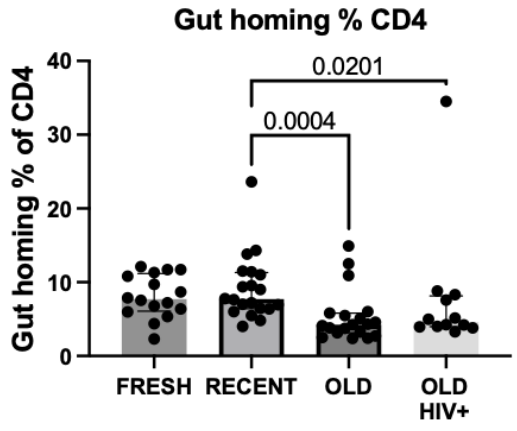

CD38 HLA-DR % CD4
CD38+ HLA-DR+ % of CD4
0.0005
<0.0001
FRESH
RECENT
OLD
OLD HIV+

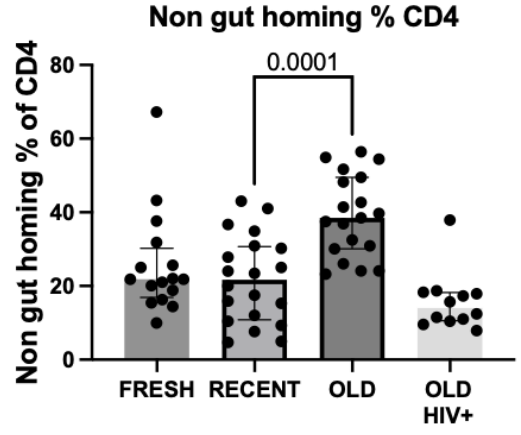

Th1 % CD4
Th1 % of CD4
n/a
FRESH
RECENT
OLD
OLD HIV+

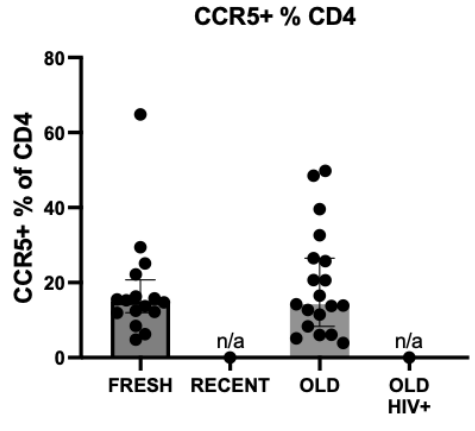

CXCR5+ % CD4
CXCR5+ % of CD4
0.0054
FRESH
RECENT
OLD
OLD HIV+


Th17 % CD4
Th17 % of CD4
FRESH
RECENT
OLD
OLD HIV+


CD49d- (skin homing) % CD4
CD49d- % of CD4
<0.0001
FRESH
RECENT
OLD
OLD HIV+


Gut homing % CD4
Gut homing % of CD4
0.0201
0.0004
FRESH
RECENT
OLD
OLD HIV+


Non gut homing % CD4
Non gut homing % of CD4
0.0001
FRESH
RECENT
OLD
OLD HIV+


CCR5+ % CD4
CCR5+ % of CD4
n/a
n/a
FRESH
RECENT
OLD
OLD HIV+

PBMC subsets – CD8 T cell subsets