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Understanding the landscape of lymph node reservoirs during ART through single cell analysis

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Disclosures and cautionary statements

- MRB consults for Interius Biotherapeutics, Capstan, Merck, Gilead \bullet
- Entirety of data and concepts in this presentation are unpublished, preliminary, and may be updated as we accumulate more data Skepticism, ideas, and novel interpretations welcome.
- Vincent Wu, PhD.



• The work in this talk was performed by Jayme Nordin, PhD student, and



The virologic state of the HIV reservoir is heterogenous



To compound the issue, the cellular state of the HIV reservoir is heterogenous



The heterogeneity of the HIV reservoir necessitates single cell analysis but has several challenges



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High CD4+ T-cell heterogeneity (including in context of HIV)

Multiple including Chomont et al., Nat Med 2009; Szabo et al., Nature Comm 2019; Gálvez et al., mBio 2021; Wu et al., JCI Insight 2020, Wu et al., Nature Immuno 2022

Latent infection + rarity of infected cells

Chun et al., Nature 1997; Finzi et al., Science 1997; Eriksson et al., PLoS Pathog 2013

No known surface markers for infected cells (without relying on vRNA or viral protein)

Cohn et al., Nature Med 2018; Liu et al., Sci Transl Med 2020, Wu et al., Nature Immuno 2022



Nearly all the HIV reservoir during ART is in tissues

What is the reservoir?



000	000	
000	000	
000		
000	000	
000	000	
000	000	
000	000	
000	000	00
000	000	00

Before Therapy		After Therapy	
35.9%	LN	0.53%	0000
62.3%	Gut	98.0%	0000
0.23%	Spleen	0.28%	0000
0.04%	Brain	0.38%	0000
0.12%	Kidney	0.01%	0000
0.03%	Heart	0.0002%	0000
1.13%	Lungs	0.73%	0000
0.24%	Liver	0.07%	0000

Estes et al., Nat Med 2017

Blood - well studied, though varies somewhat depending on the publication

Tissues: We know a lot is in lymph nodes and gut lymphoid structures and tissues.



Estimated 2.5 x 10⁹ viral DNA+ cells in lymphoid tissue during ART (70 Kg human; T. Shacker, U. Minnesota)



Study Goal: to define HIV reservoir composition in LN of ART-treated PLWH

- Determine the phenotypic, transcriptomic, and epigenetic profile of HIV-infected memory CD4+ T cells in lymph nodes of ART-PLWH (DOGMAseq)
- Determine what are the predominant infected cell subsets in ART LN... is it Tfh cells?
 - Tfh cells have been shown in a variety of studies to be the dominant infected population in lymph nodes in viremic infection, but this mostly assumed in ART
- Determine whether the provirus in HIV infected LN CD4+ T cells during ART is quiescent or transcriptionally active, and potential differential characteristics thereof

Samples (n = 26) used for lymph node (LN) studies

ART

Participant ID	Date of sample collection (mm/dd/yy)	Days on ART	pVL (copies/mL)
A002	6/20/16	476	<40
A024*	9/2/16	784	Undetectable
A170	6/13/18	770	Undetectable
A213	10/29/18	7326	Undetectable
A262	7/15/19	1197	Undetectable
A304	11/23/22	1035	Undetectable
A025*	2/9/15	489	Undetectable
A303	9/21/15	355	Undetectable
A127	11/10/17	749	<40
A256	3/30/15	1027	<40

\mathbf{M}	ith		†	H	
VV		UU			

Participant ID	Date of sample collection (mm/dd/yy)	Days on ART	pVL (copies/mL)
U287*	2/24/20	-	Undetectable
U294*	7/7/21	-	Undetectable
U190	7/18/18	-	Undetectable
U191	7/19/18	-	Undetectable

Chronic

Participant ID	Date of sample collection (mm/dd/yy)	Days on ART	pVL (copies/mL)
C028	9/12/16	-	25,951
C087	6/12/17	-	1,771,593
C098	7/21/17	-	1,019,989
C102	7/27/17	-	1,692,571
C107	8/30/17	-	6,326,452
C111	9/4/17	-	552,134
C229	1/28/19	-	3,882,624
C242	3/27/19	-	715,509
C282	1/29/20	-	3,087,470
C101*	7/26/17	-	1,935,095
C235*	5/14/19	-	259,708
C250*	2/14/19	-	1,067,647

*Inguinal LN. All others are cervical LN. Collaboration with CIENI (Mexico)



DOGMAseq: single cell assessment of protein+RNA+DNA composition



live sorted memory CD4+ T cells for analysis

Targeted ~26667 cells for each chronic LN sample

Targeted ~56000 cells for each ART LN sample

Total cells passing QC: ART: 294,465 Chronic: 163,061 w/o HIV: 102,234

Definition of LN memory CD4 + CD3 DN T cell subsets

Clusters (HIV- & Chronic & ART)



- T follicular helper (Tfh)
 - Three subsets
- T regulatory cells (Treg)
 - Three subsets
- Resident memory T cells (Trm)
 - Two subsets
- Effector memory T cells (Tem)
 - Five subsets
- Central/early memory T cells (Tcm, Tscm)
 - Eight subsets
- Naive T cells
- CD3 DN subsets (three)

Detection of viral DNA+ and RNA+ cells



UMAP plots show aggregation of chronic+viremic+w/o HIV samples



1. The lymph node proviral DNA reservoir landscape is very heterogeneous and differs between ART and viremic PLWH

	Chronic	ART
CD4 Tscm/early memory activated -	0.15%	
CD4 Tscm/early memory -	·····• 5.89%	·····• 7.46%
CD4 Trm NR4A1/NR4A2 hi		● 2.69%
CD4 Trm #1 -	···• 1.57%	•••••••••••••••••••••••••••••••••••••••
CD4 Treg activated -		·• 0.9%
CD4 Treg #2 -	·····• 2.98%	·····• 5.07%
CD4 Treg #1-	••••• 9.62%	·····• 5.67%
CD4 Tn -	···• 1.64%	···• 1.79%
CD4 Th2/non-resident -	● 3.65%	•••••• 8.06%
CD4 Th17 -	····• 1.86%	·····• 5.37%
CD4 Th1 Tem activated -		• 0.3%
CD4 Th1 Tem #1 -	•••••••••••••••••••••••••••••••••••••••	··• 1.19%
CD4 Th1 Tem -	·• 0.89%	··• 1.19%
CD4 Tem NR4A1/NR4A2 hi -	·····• 4.4%	••••••• 15.52%
CD4 Tem #2 ·	0.07%	·····• 7.46%
CD4 Tem #1 -	··• 1.12%	···• 1.79%
CD4 Tcm/non-resident #2 -	·····• 7.46%	••••••• 8.36%
CD4 Tcm/non-resident #1 -	···• 1.72%	·····• 3.58%
CD4 Tcm -	•••••••••••••••••••••••••••••••••••••••	···• 1.79%
CD4 T early memory -	• 0.3%	• 0.6%
CD4 PRDM1+ GC-Tfh -	● 3.06%	····• 2.09%
CD4 IKZF1+ Tcm -	·····• 7.38%	·• 0.9%
CD4 GC-Tfh #2 -	• 0.75%	·····• 5.97%
CD4 GC-Tfh #1 -	•••••••••••••••••••••••••••••••••••••••	·• 0.9%
CD4 CD127+ Tem -	·• 0.82%	·····• 4.48%
CD3 DN γδ-	• 0.52%	
CD3 DN IL10+ -	• 0.37%	
(5 10 15 C	5 10 15
	% of HIV DNA+	cells per stage

2. Tfh cells comprise a large, but not dominant, proportion of the HIV DNA+ cells in viremic PLWH

Chronic

CD4 Tscm/early memory activated	0.15%
CD4 Tscm/early memory -	·····• 5.89%
CD4 Trm NR4A1/NR4A2 hi	
CD4 Trm #1 -	···• 1.57%
CD4 Treg activated	
CD4 Treg #2 -	·····• 2.98%
CD4 Treg #1-	•••••• 9.62%
CD4 Tn -	···• 1.64%
CD4 Th2/non-resident -	● 3.65%
CD4 Th17 -	····• 1.86%
CD4 Th1 Tem activated -	
CD4 Th1 Tem #1 -	••••• 15.29%
CD4 Th1 Tem	·• 0.89%
CD4 Tem NR4A1/NR4A2 hi	·····• 4.4%
CD4 Tem #2 ·	0.07%
CD4 Tem #1 -	·• 1.12%
CD4 Tcm/non-resident #2 -	•••••• 7.46%
CD4 Tcm/non-resident #1 -	···• 1.72%
CD4 Tcm -	••••••• 13.12%
CD4 T early memory	• 0.3%
CD4 PRDM1+ GC-Tfh ·	● 3.06%
CD4 IKZF1+ Tcm ·	•••••• 7.38%
CD4 GC-Tfh #2 ·	• 0.75%
CD4 GC-Tfh #1·	•••••• 15.36%
CD4 CD127+ Tem ·	• 0.82%
CD3 DN γδ-	• 0.52%
CD3 DN IL10+ -	• 0.37%
(5 10 15

Proportion of all HIV DNA+ cells

Tfh cells comprise a large proportion (~20%) of the proviral DNA+ cells in viremic LN

CD4 GC-Tfh #2 • 0.75% CD4 GC-Tfh #1 · ·····• 15.36% CD4 PRDM1+ GC-Tfh ⋅ · · · · • 3.06%









GC-Tfh definition by single cell quantification by single cell analysis is directly correlated to flow cytometry





Chronic

HIV-



3. The lymph node proviral DNA reservoir landscape during ART is not dominated by Tfh cells

CD4 Tscm/early memory activated	
CD4 Tscm/early memory	·····• 7.46%
CD4 Trm NR4A1/NR4A2 hi	● 2.69%
CD4 Trm #1	•••••••••••••••••••••••••••••••••••••••
CD4 Treg activated	• 0.9%
CD4 Treg #2	·····• 5.07%
CD4 Treg #1	·····• 5.67%
CD4 Tn	···• 1.79%
CD4 Th2/non-resident	•••••• 8.06%
CD4 Th17	·····• 5.37%
CD4 Th1 Tem activated	• 0.3%
CD4 Th1 Tem #1	··• 1.19%
CD4 Th1 Tem	··• 1.19%
CD4 Tem NR4A1/NR4A2 hi	15.52%
CD4 Tem #2	·····• 7.46%
CD4 Tem #1	···• 1.79%
CD4 Tcm/non-resident #2	•••••• 8.36%
CD4 Tcm/non-resident #1	·····• 3.58%
CD4 Tcm	···• 1.79%
CD4 T early memory	• 0.6%
CD4 PRDM1+ GC-Tfh	····• 2.09%
CD4 IKZF1+ Tcm	• 0.9%
CD4 GC-Tfh #2	·····• 5.97%
CD4 GC-Tfh #1	• 0.9%
CD4 CD127+ Tem	·····• 4.48%
CD3 DN γδ	
CD3 DN IL10+	
	5 10 15
_	

ART

Proportion of all HIV DNA+ cells

Tfh cells only comprise ~9% of the proviral reservoir (vs. 20% in viremia)

> CD4 PRDM1+ GC-Tfh ····• 2.09% ····• 5.97% CD4 GC-Tfh #2-CD4 GC-Tfh #1-• 0.9%

> > HIV DNA+ RNA-



Data shown by M. Pardons, oral presentation 1.2, confirm this



4. Central/early non-resident memory populations comprise a large proportion of the lymph node proviral DNA reservoir

CD4 Tscm/early memory activated CD4 Tscm/early memory ······ 7.46% CD4 Trm NR4A1/NR4A2 hi 2.69% CD4 Trm #1 6.87% CD4 Treg activated $\cdot \bullet 0.9\%$ CD4 Treg #2 5.07% CD4 Treg #1 5.67% CD4 Tn ···• 1.79% CD4 Th17 5.37% CD4 Th1 Tem activated • 0.3% CD4 Th1 Tem #1 · · ● 1.19% CD4 Th1 Tem ↓ · • 1.19% CD4 Tem NR4A1/NR4A2 hi CD4 Tem #2 7.46% CD4 Tem #1 ····● 1.79% CD4 Tcm/non-resident #1 ·····• 3.58% CD4 Tcm ····● 1.79% CD4 T early memory • 0.6% CD4 PRDM1+ GC-Tfh• 2.09% CD4 IKZF1+ Tcm ↓ • 0.9% CD4 GC-Tfh #2 5.97% CD4 GC-Tfh #1 **○** 0.9% CD4 CD127+ Tem 4.48% CD3 DN γδ CD3 DN IL10+ 15 10 **Proportion of all HIV DNA+ cells**

ART

Central/early memory populations comprise a large proportion (36%) of the proviral DNA reservoir:

CD4 Tscm/early memory -	·····• 7.46%
CD4 Th2/non-resident -	•••••• 8.06%
CD4 Th17 -	·····• 5.37%
CD4 Tcm/non-resident #2 -	•••••• 8.36%
CD4 Tcm/non-resident #1 -	·····• 3.58%
CD4 Tcm -	···• 1.79%
CD4 T early memory -	• 0.6%
CD4 IKZF1+ Tcm-	• 0.9%





5. NR4A1/NR4A2 hi effector memory CD4+ T cells are a large component of the proviral DNA reservoir

CD4 Tscm/early memory activated CD4 Tscm/early memory ······ 7.46% CD4 Trm NR4A1/NR4A2 hi 2.69% CD4 Trm #1 6.87% CD4 Treg activated ↓ • 0.9% CD4 Treg #2 5.07% CD4 Treg #1 5.67% CD4 Tn ···• 1.79% CD4 Th17 5.37% CD4 Th1 Tem activated • 0.3% CD4 Th1 Tem #1 ··• 1.19% CD4 Th1 Tem ↓ • 1.19% CD4 Tem NR4A1/NR4A2 hi 15.52% CD4 Tem #2 ·····• 7.46% CD4 Tem #1 ····● 1.79% CD4 Tcm/non-resident #2 ······ 8.36% CD4 Tcm ····● 1.79% CD4 T early memory • 0.6% CD4 PRDM1+ GC-Tfh• 2.09% CD4 IKZF1+ Tcm ↓ • 0.9% CD4 GC-Tfh #2 5.97% CD4 GC-Tfh #1 ·● 0.9% CD4 CD127+ Tem 4.48% CD3 DN γδ CD3 DN IL10+ 15 10 **Proportion of all HIV DNA+ cells**

ART

NR4A1/NR4A2 hi Tem cells comprise ~18% of the proviral DNA reservoir:

CD4 Trm NR4A1/NR4A2 hi	·····• 2.69%
CD4 Tem NR4A1/NR4A2 hi	• • • • • • • • • • • • • • • • • • • •

 NR4A1/NR4A2 hi Tem subsets are 8-10% of total lymph node CD4+ cells in the tissues

CD4 Trm NR4A1/NR4A2 hi CD4 Tem NR4A1/NR4A2 hi 0 10 20 30 Percent of cells in tissue Tissue CLN ILN



6. The viral reservoir during ART can be transcriptionally active, but is low frequency

ART

•••••• 6.38%
·····• 5.32%
•••••• 6.38%
····• 2.13%
·····• 3.19%
•••••• 8.51%
·····• 5.32%
·····• 4.26%
·• 1.06%
·• 1.06%
····• 2.13%
•••••• 10.64%
·····• 4.26%
•••••• 6.38%
•••••• 8.51%
·····• 5.32%
·• 1.06%
·• 1.06%
·• 1.06%
·····• 4.26%
·• 1.06%
•••••• 10.64%
5 10 15
Proportion of all HIV/ DNA+ col

1 RNA+ cell = ~1%



Total LN cells measured= 294,465 94 vRNA+ cells 0.03% of total cells vRNA+

7. Tfh cells are a minor component of the transcriptionally active reservoir during ART

ART

CD4 Tscm/early memory activated	
CD4 Tscm/early memory -	·····• 6.38%
CD4 Trm NR4A1/NR4A2 hi	·····• 5.32%
CD4 Trm #1 -	·····• 6.38%
CD4 Treg activated -	····• 2.13%
CD4 Treg #2 -	·····• 3.19%
CD4 Treg #1	•••••• 8.51%
CD4 Tn -	
CD4 Th2/non-resident -	·····• 5.32%
CD4 Th17 -	·····• 4.26%
CD4 Th1 Tem activated -	·• 1.06%
CD4 Th1 Tem #1-	·• 1.06%
CD4 Th1 Tem	····• 2.13%
CD4 Tem NR4A1/NR4A2 hi	·····• 10.64%
CD4 Tem #2 -	·····• 4.26%
CD4 Tem #1 -	·····• 6.38%
CD4 Tcm/non-resident #2 -	•••••• 8.51%
CD4 Tcm/non-resident #1 -	·····• 5.32%
CD4 Tcm -	
CD4 T early memory -	·• 1.06%
CD4 PRDM1+ GC-Tfh -	·• 1.06%
CD4 IKZF1+ Tcm -	·• 1.06%
CD4 GC-Tfh #2 -	·····• 4.26%
CD4 GC-Tfh #1 -	·• 1.06%
CD4 CD127+ Tem -	·····• 10.64%
CD3 DN γδ·	
CD3 DN IL10+ -	
CD3 DN -	
	5 10 15
	Proportion of all HIV RNΔ+ cells
	1 DNA = -10/
	I RINAT CEII = ~1%

 Tfh comprise a small component (6.4%) of the viral RNA+ reservoir in ART

8. NR4A1/NR4A2 hi effector memory CD4+ T cells comprise a large proportion of the transcriptionally active reservoir during ART

ART

CD4 Tscm/early memory activated	
CD4 Tscm/early memory -	•••••••••••••••••••••••••••••••••••••••
CD4 Trm NR4A1/NR4A2 hi	·····• 5.32%
CD4 Trm #1 -	·····• 6.38%
CD4 Treg activated	····• 2.13%
CD4 Treg #2 ·	·····• 3.19%
CD4 Treg #1	••••••• 8.51%
CD4 Tn -	
CD4 Th2/non-resident -	·····• 5.32%
CD4 Th17 -	·····• 4.26%
CD4 Th1 Tem activated	·• 1.06%
CD4 Th1 Tem #1·	·• 1.06%
CD4 Th1 Tem ·	····• 2.13%
CD4 Tem NR4A1/NR4A2 hi	·····• 10.64%
CD4 Tem #2 -	·····• 4.26%
CD4 Tem #1•	•••••••••••••••••••••••••••••••••••••••
CD4 Tcm/non-resident #2 ·	••••••• 8.51%
CD4 Tcm/non-resident #1 ·	·····• 5.32%
CD4 Tcm ·	
CD4 T early memory -	·• 1.06%
CD4 PRDM1+ GC-Tfh ·	·• 1.06%
CD4 IKZF1+ Tcm ·	·• 1.06%
CD4 GC-Tfh #2·	·····• 4.26%
CD4 GC-Tfh #1·	·• 1.06%
CD4 CD127+ Tem·	·····• 10.64%
CD3 DN γδ·	
CD3 DN IL10+ ·	
CD3 DN ·	
(5 10 15
	Proportion of all HIV RNA+ cells
	$\frac{1}{1} \mathbf{RN} \mathbf{A} + \mathbf{coll} = \mathbf{a} 10$

 Largest proportion of the viral RNA+ reservoir is CD4 NR4A1/NR4A2+ cells

CD4 Trm NR4A1/NR4A2 hi ······ 5.32% CD4 Tem NR4A1/NR4A2 hi ······ 10.64%





What are NR4A1/NR4A2 hi Tem CD4+ T cells?

- (CD69 low). Appears resting (low for HLA-DR, ki67, CD71)

• Phenotypic characteristics: central memory (CD28+ CD127hi CD45RO+ CD27+) non-resident

DNIA profiles Tap DEC NIDIAA (NIUr77) Jup Eas NIDIAA alas tap DECa I auraat hala DNIA

What are NR4A1/NR4A2 hi Tem CD4+ T cells?

- (CD69 low). Appears resting (low for HLA-DR, ki67, CD71)
- compared to all other clusters

 - -
 - Migratory profile, with high KLF2 and high CD69 RNA (but low CD69 protein) -



Phenotypic characteristics: central memory (CD28+ CD127hi CD45RO+ CD27+) non-resident

RNA profile: Top DEG NR4A1 (Nur77). Jun, Fos, NR4A2 also top DEGs. Lowest bcl2 RNA

- NR4A1 expression requires T cell receptor triggering, lasts 3-18hrs. Not bystander activation. Context dependent functions: transcription factor and/or facilitator of apoptosis (binds to Bcl2)

What are NR4A1/NR4A2 hi Tem CD4+ T cells?

- (CD69 low). Appears resting (low for HLA-DR, ki67, CD71)
- compared to all other clusters

 - -
 - Migratory profile, with high KLF2 and high CD69 RNA (but low CD69 protein)



Shows evidence of very recent activation... BUT APPEARS PHENUTYPICALLY TO BE \bullet ART.

• Phenotypic characteristics: central memory (CD28+ CD127hi CD45RO+ CD27+) non-resident

RNA profile: Top DEG NR4A1 (Nur77). Jun, Fos, NR4A2 also top DEGs. Lowest bcl2 RNA

- NR4A1 expression requires T cell receptor triggering, lasts 3-18hrs. Not bystander activation. Context dependent functions: transcription factor and/or facilitator of apoptosis (binds to Bcl2)

RESTING MEMORY; may be a major reason for viral rebound propensity upon ART interruption. Very likely also contributes to dissemination of infected cells across the body in both viremia and

Conclusions

- - This discrepancy may be due to depletion of cells with active viral replication in the LN at the time of ART initiation; dead cells can't form a reservoir!
- While Tfh cells are a major infected cell type in viremic PLWH, other populations dominate in ART PLWH.
- In ART LN, transcriptionally active infected cells are observed, indicating a continual effort for the virus to replicate
- Very recently antigen-specific activated CD4+ T cells harbor a disproportionately high level of virus during ART
- It is possible that other lymph nodes, of which there are >600 in humans, may have a different reservoir composition, especially mucosal draining lymphoid tissues

The HIV reservoir in lymph nodes of viremic vs ART PLWH differs in composition



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BEAT-HIV DELANEY COLLABORATORY



