The tool kit to investigate HIV reservoir

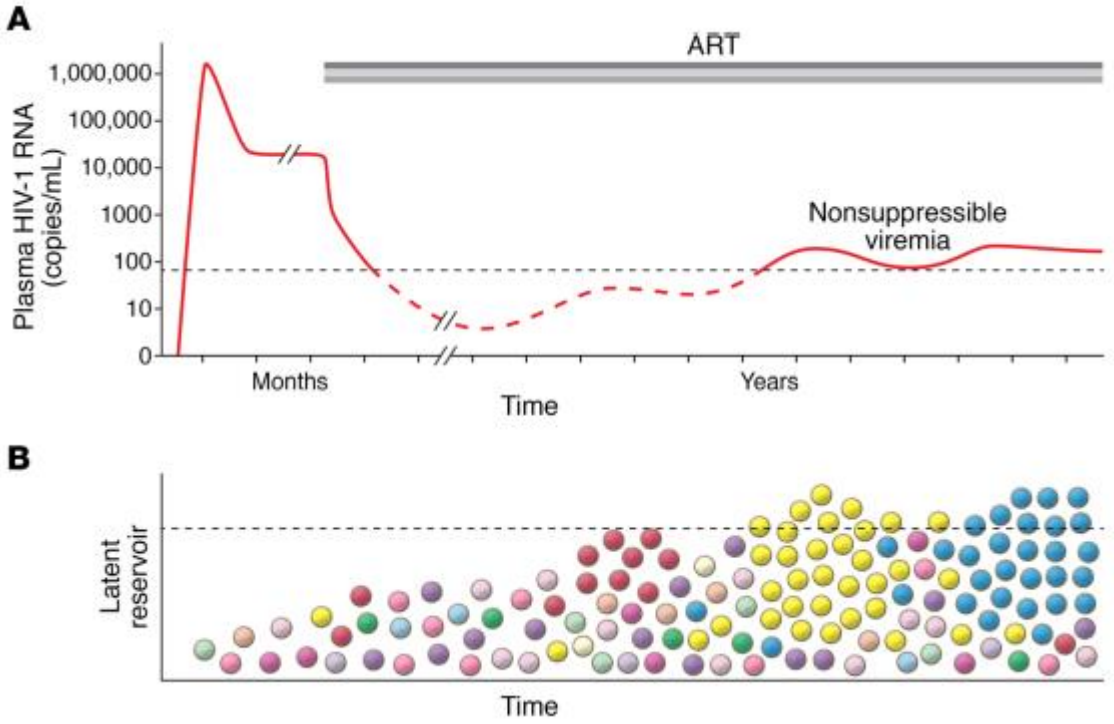
Vicenti Ilaria, Phd Biotechnologies

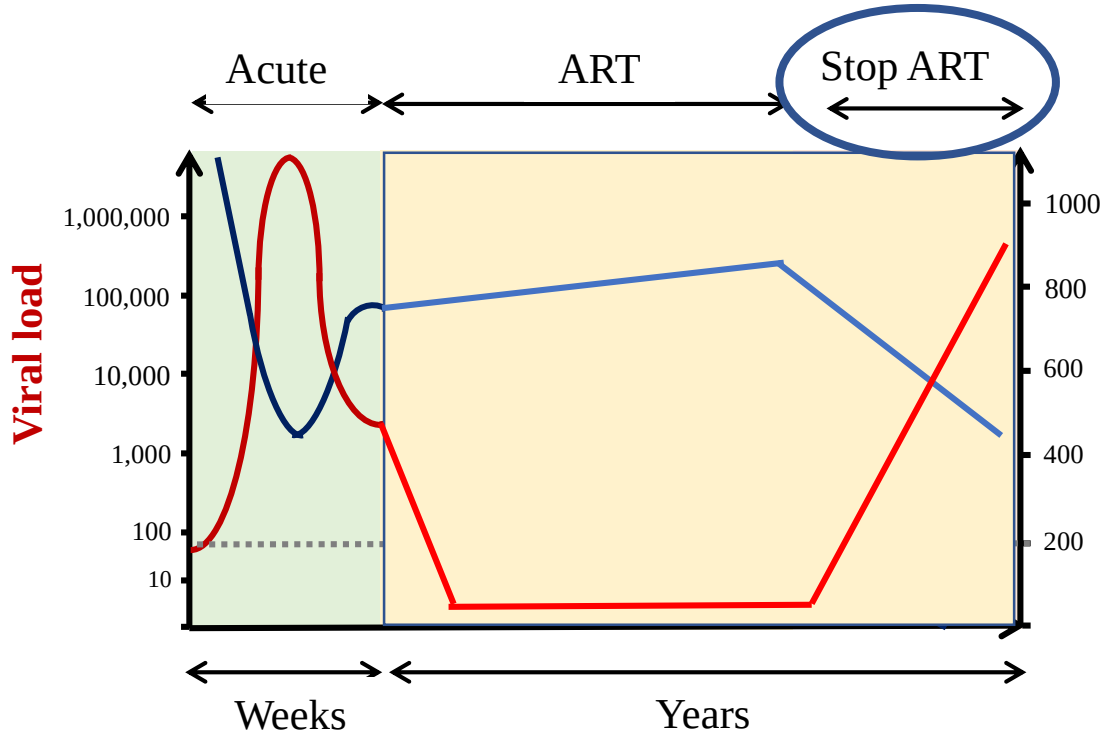
Department of Medical Biotechnologies

UOC Microbiologia e Virologia

Università degli Studi di Siena

HIV-1 persistence





Siliciano J.Clin.Invest. 2020

- Antiretroviral therapy (ART) successfully halts HIV-1 replication and prevents the infection of new cells in blood and tissue
- Very low levels of viremia (residual viremia) persists in the range of 1–2 copies/mL of plasma, and HIV viral load typically rebounds with cessation of treatment
- Lifelong persistence of HIV-1: The slow-decaying reservoir has an estimated half-life of 44 months, consequently the eradication would require 73.4 years on ART

Targeting the HIV reservoir

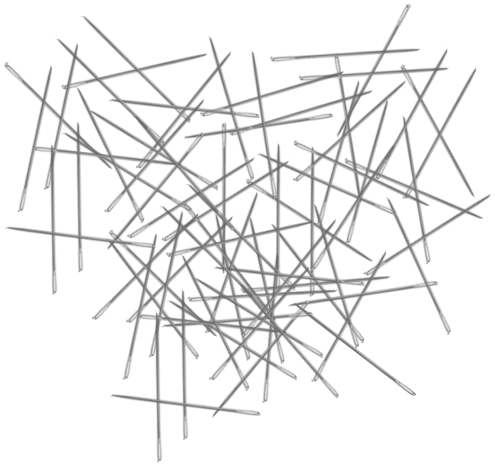
HIV reservoir: Cells infected with replication-competent HIV in the blood and at various anatomical sites that persist despite long-term ART

Infected cells are very rare!

~1 in 10,000 cells is infected

'Needle in a haystack'

A lot of viruses are defective!



Defective viruses

Intact viruses

Ratio Provirus
Intact/inducible = 40/1

Rebounding viruses

~95% of the viruses are defective

The reservoir has a high genetic diversity

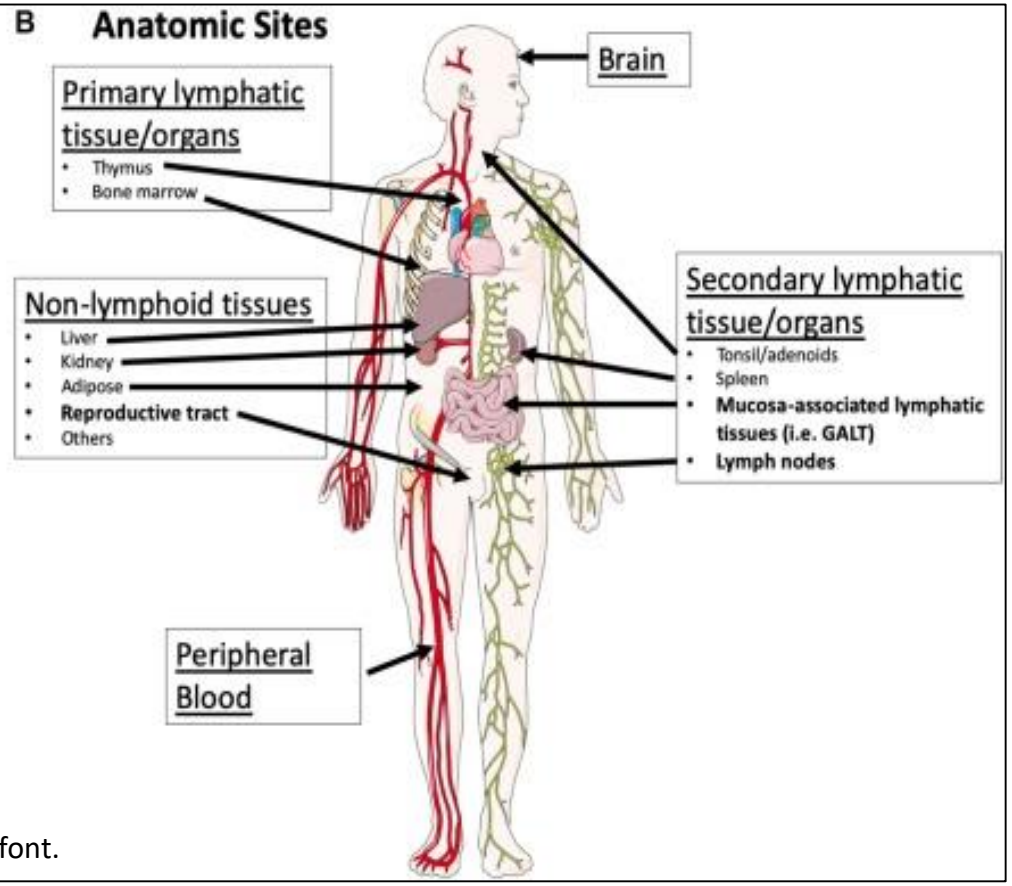
Adapted by Linos Vanderckove EMHH 2020

Barrier to eradication: compartmentalization of reservoirs

A Cell Types

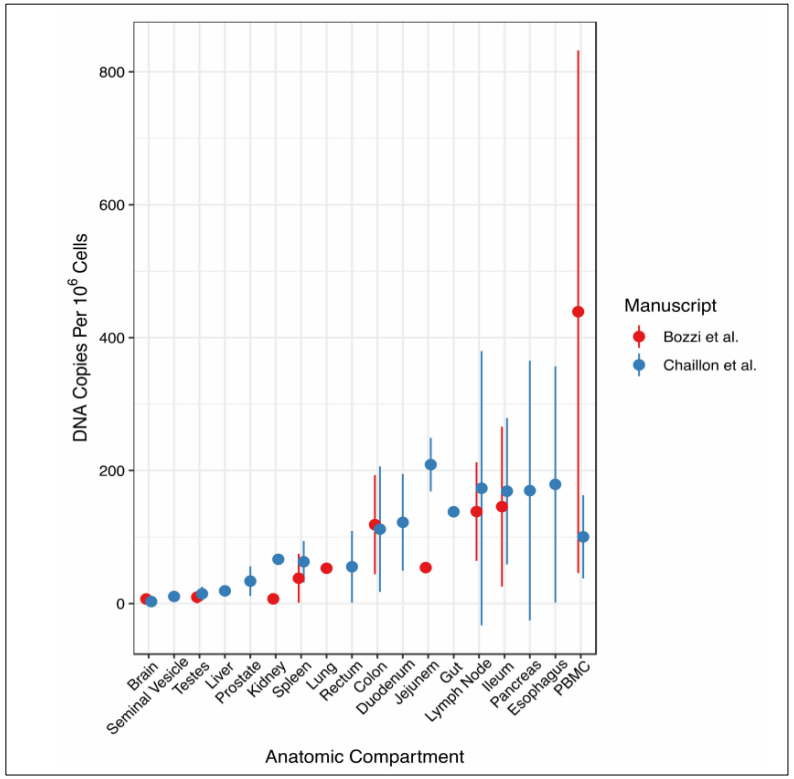
CD4 T cells		Other Cell Types
Differentiation Status	Functional Polarization	
T_{NA}	**Tfh**	Macrophages:
T_{SCM}	Th1	• **Urethral**
T_{CM}	Th2	• Microglia
T_{TM}	Th9	• Lung
T_{EM}	Th17	• Skin
T_{TD}	Treg	• Gut
T_{MM}		• Others
T_{RM}		Mast cells
γδ		Renal epithelium
		Astrocytes
		Hematopoietic progenitors

B Anatomic Sites



Replication competent reservoir is indicated in bold.
Potential replication competent reservoir is in regular font.

Falcinelli, Front. Microbiol. 2019



Lau, Viruses 2021

Tissue and cell types more frequently analysed:

- Blood-> (i) all cellular populations, (ii) PBMC, (iii) total CD4+ T cells, (iv) different memory cell subsets
- Lymph node biopsies-> different CD4+ T cell subsets
- GALT cells
- Macrophages (limited number of studies)

Why is it important to measure the viral reservoir?

HIV-1 cure strategies

almost 100 clinical trials are current underway;
https://www.treatmentactiongroup.org/cure/trials/

Clinical management of HIV-1 infection

Currently based only on total HIV-1 DNA

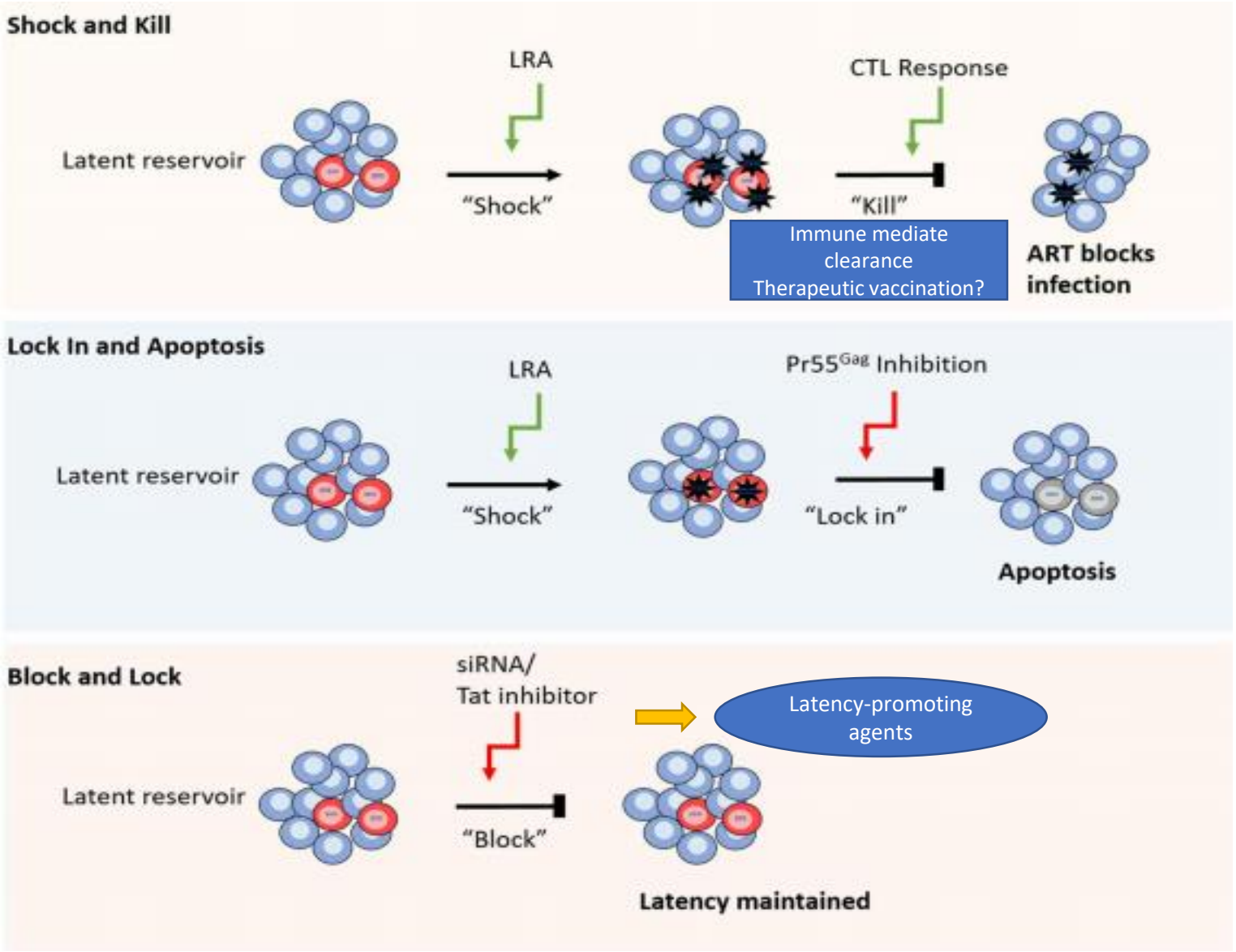
HIV-1 reservoir

Understanding HIV persistence

Defectivity, inducibility and clonality index

Drug intensification and de-escalation studies

Strategies for HIV-1 cure



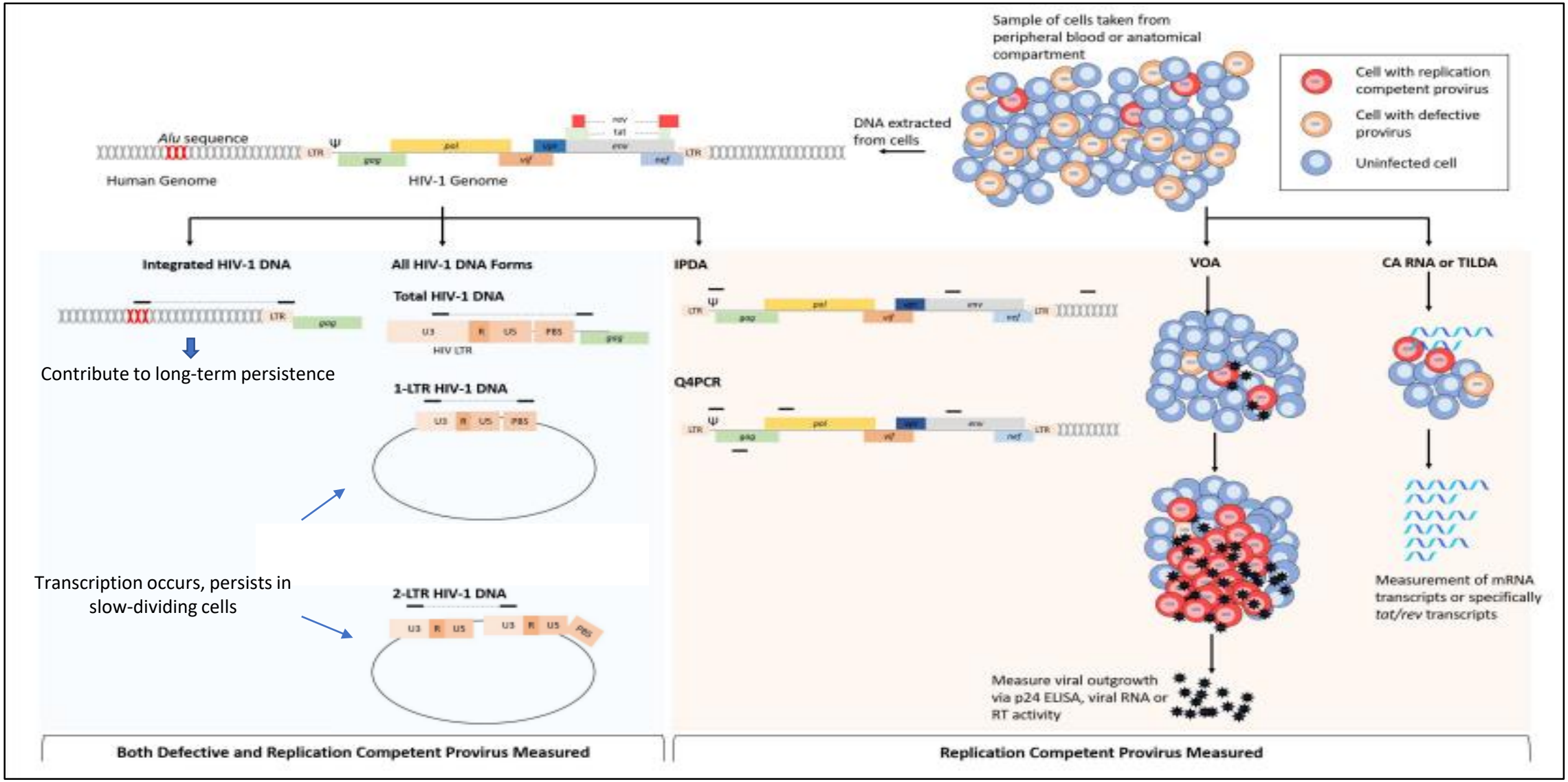
Sterilizing cure, eradication of reservoir

Critical: To determine whether remnants of viral genomes are expressed and biologically active

Functional cure, control of HIV-1 infection without the requirement of ART

Thomas, Front. in Microb. 2020

Comparison of assays to measure the HIV reservoir



Thomas et al., Frontiers in Cellular and Infection Microbiology, 2020

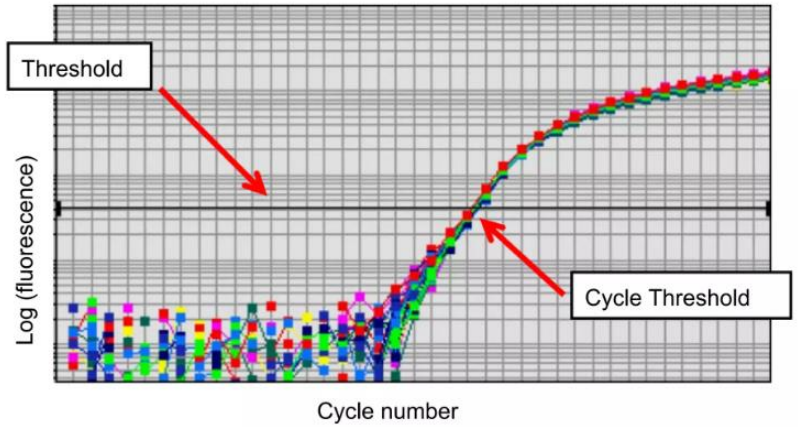
Quantification of total HIV-1 DNA

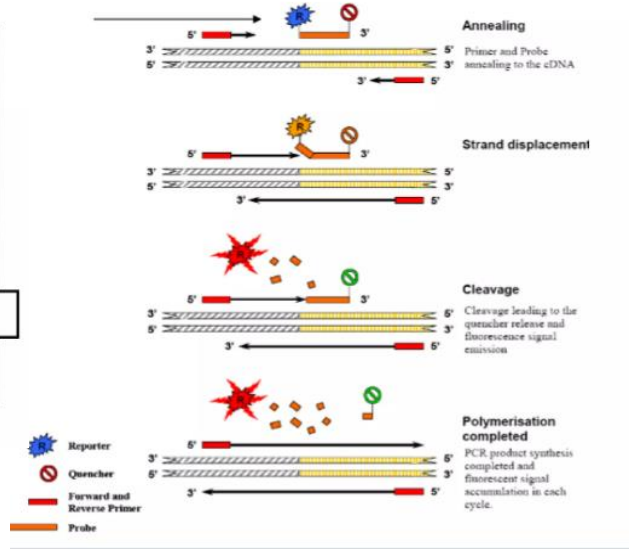
Quantification of all HIV-1 DNA forms

- Fast and relatively inexpensive molecular marker to measure the size of reservoir thus suitable for diagnostic application
- Only assay possible when a limited number of cells is available
- Overestimation of HIV-1 DNA reservoir (infected cell frequency 2-3 logs higher than qVOA) (Eriksson et al., Plos Pathog. 2013)
- Useful to predict viral rebound following treatment discontinuation (Williams et al., Elife 2014)
- Real time PCR limitations (i.e. quantification relative to a standard or amplification efficiency) could be mitigated by the use of digital droplet PCR platforms

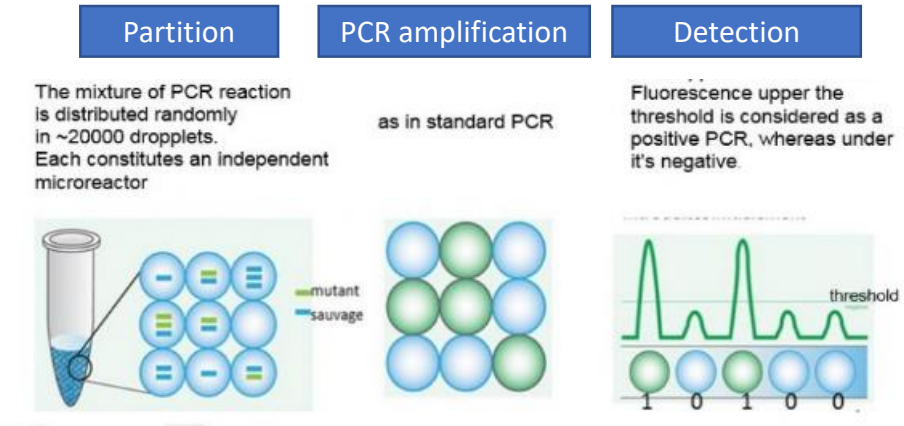
From quantitative real time PCR (qPCR) to digital droplet PCR (ddPCR)

qPCR: quantification is relative to a standard





ddPCR: absolute quantification is determined based on Poisson distribution of positive reactions



Adavantages of qPCR:
- Fast and relatively inexpensive
- Small sample volume
- Low technical skills

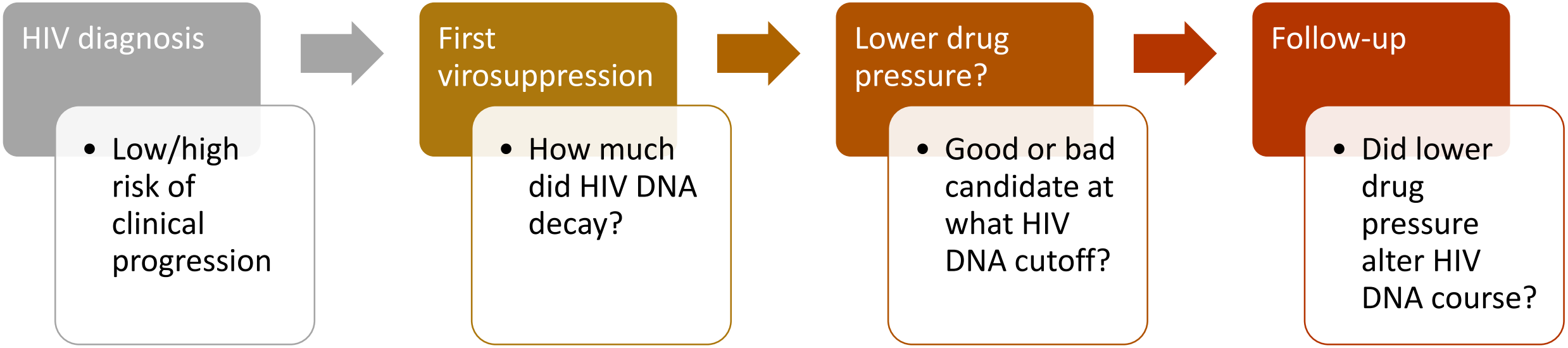
Disdvantages of qPCR:
- Quantification relative to a standard, so prone to bias
- Highly specific and prone to error from primer/template mismatches

Advantages of ddPCR:
- No need to rely on references or standards (absolute quantification)
- More tolerant to PCR inhibitors

Disdvantages of ddPCR:
- More expensive and less widely available than qPCR
- More prone to false-positives
- Setting thresholds to distinguish truly positive and negative partition is difficult
- Requires expert and trained personnel
- Highly specific and prone to error from primer/template mismatches

How to use HIV DNA quantification?

We need reference cut-offs!
However, each patient may serve as his/her control over time



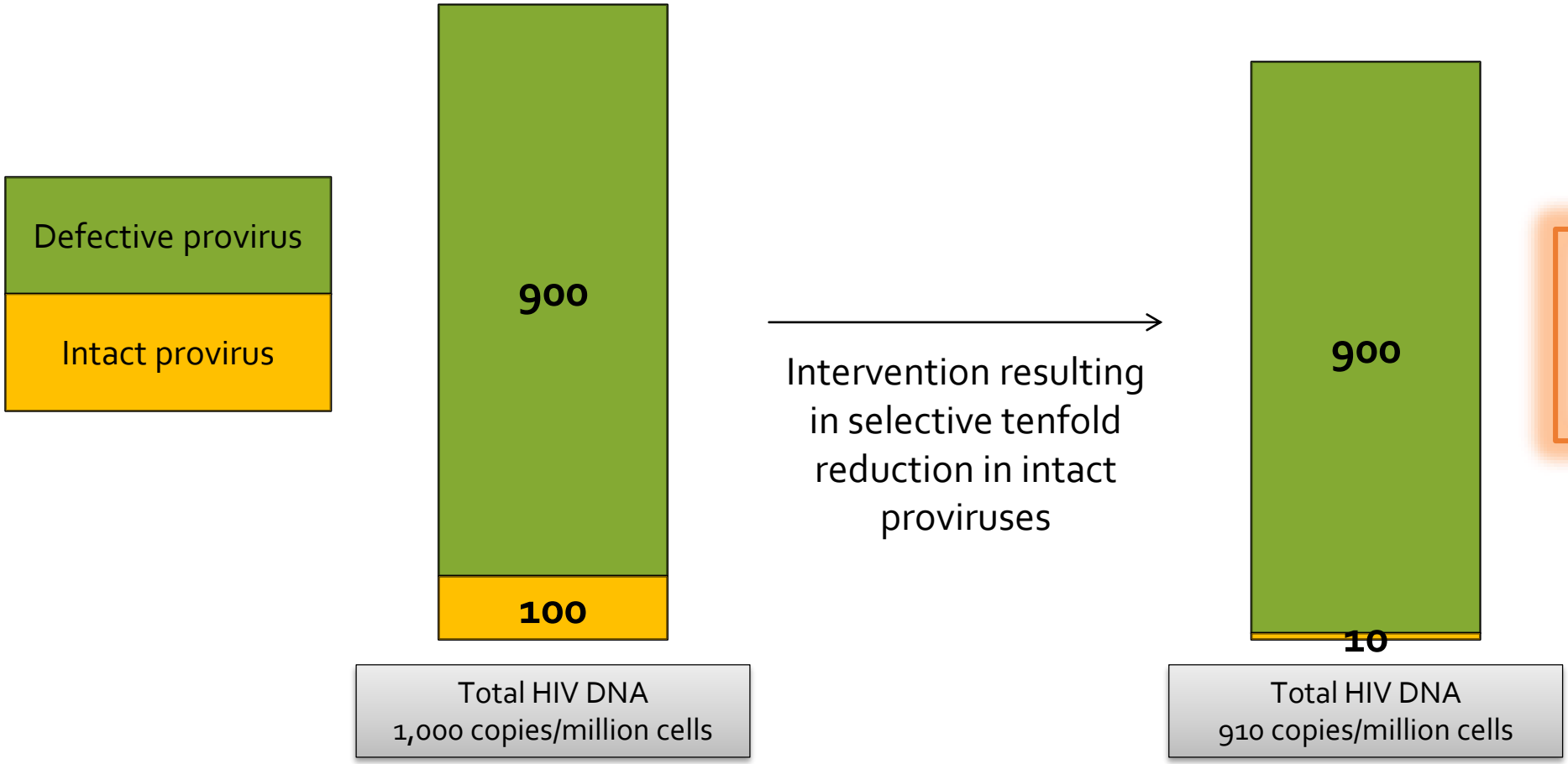
e.g. 2000
Copies/million PBMCs

e.g. 10-fold

e.g. 200
Copies/million PBMCs

e.g. continued decrease

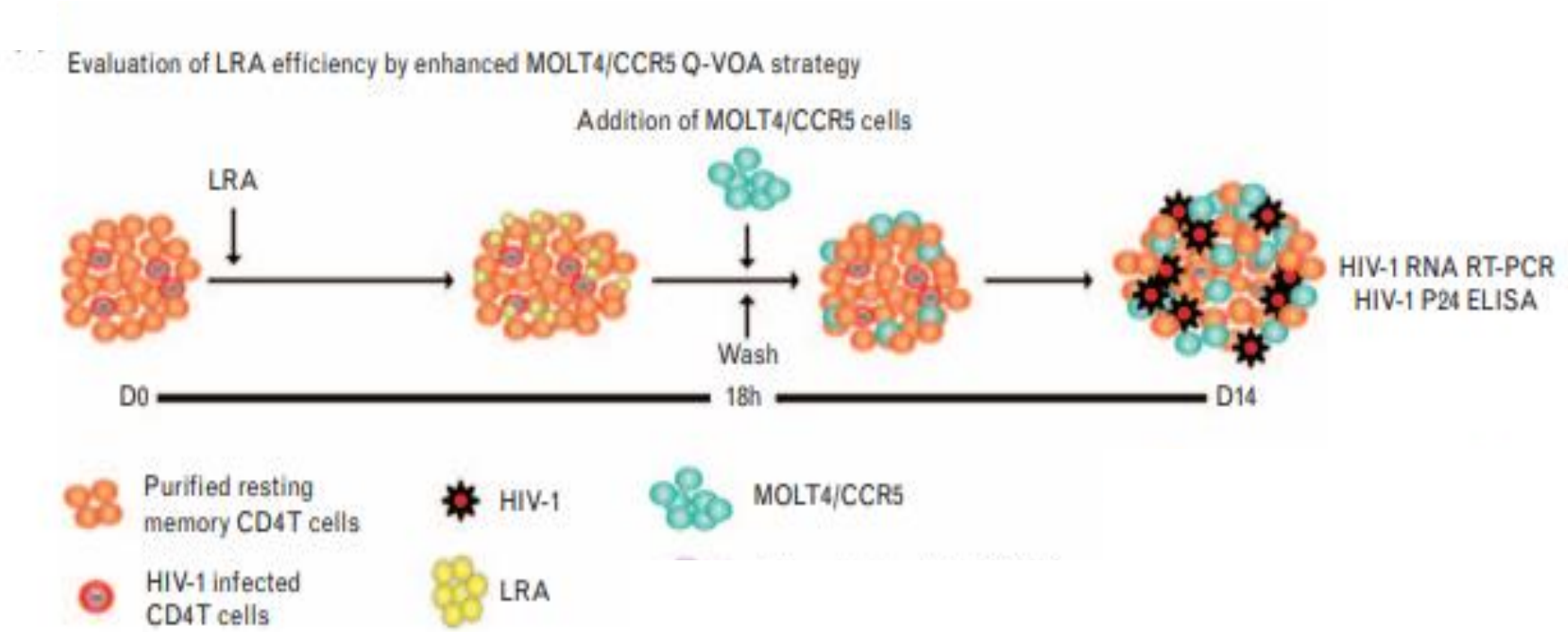
Standard HIV DNA PCR would not detect a *selective* decrease in *intact* provirus burden



To evaluate cure strategies the defective provirus must be excluded from reservoir measurement

The intervention was 90% effective, but this effect goes undetected!

qVOA (quantitative Viral Outhgrowth assay): Gold standard



<u>Advantages</u>:

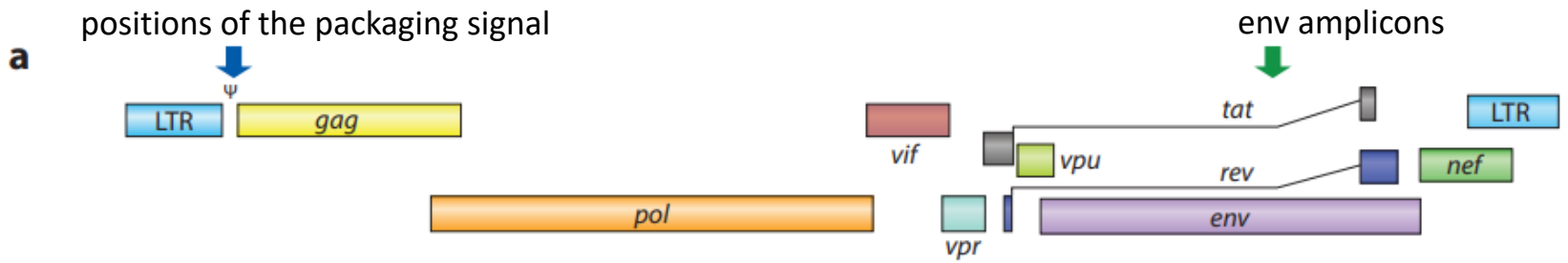
- Provides a estimation of the frequency of cells carrying replication competent virus

<u>Disadvantages</u>:

- Requires large blood volume
- Time-consuming (2-3 weeks) and requirement of BSL-3 facilities
- Not all intact provirus is detected (underestimation 25-60- fold)

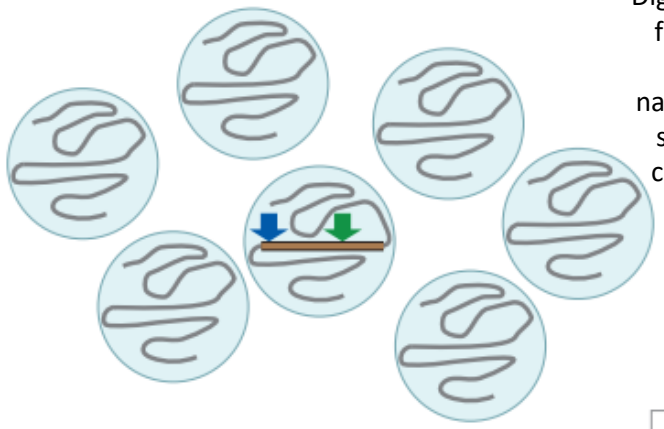
Banga, COHA 2016

Measuring intact vs. defective HIV proviruses: Intact Proviral DNA Assay (IPDA)



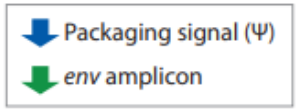
Major classes of HIV-1 defective proviruses found in treated PLWH (more than 90%). Deletions are indicated in white and G→A hypermutation with black lines.

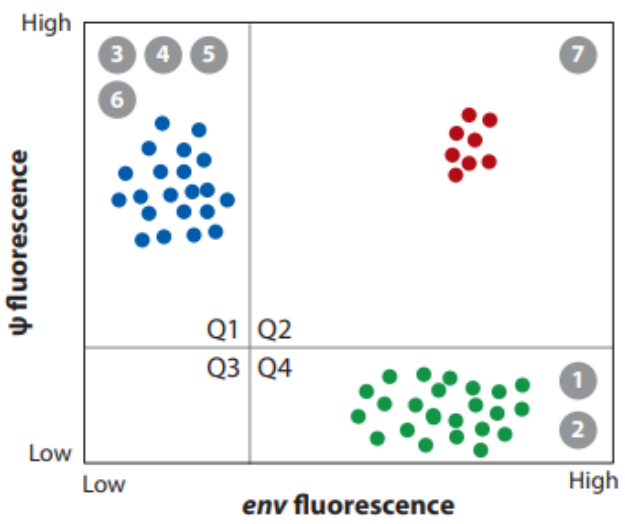
1. Proviruses with small deletions in ψ
2. Proviruses with large 5’ deletions
3. Proviruses with large 3’ deletions
4. Proviruses with very large internal deletions
5. Hypermutated proviruses
6. Proviruses with deletions and hypermutation
7. Intact proviruses lacking overt fatal defects



Multiplex PCR reactions

Digital droplet PCR: DNA from infected cells is suspended into nanoliter-sized droplets such that no droplet contains more than a single provirus.





The red dots are double positive events detected with both primer sets (intact provirus)

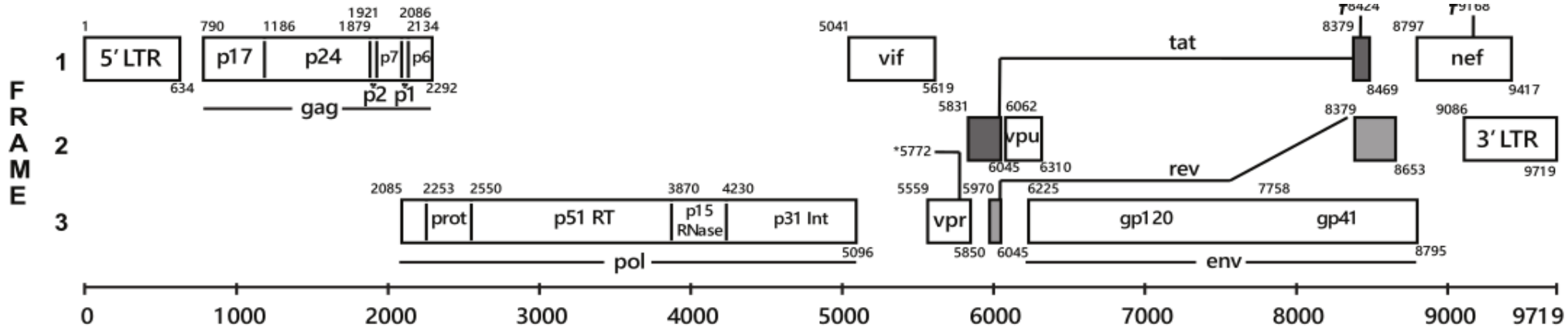
A separate multiplex PCR targets two regions of the human *RPP30* gene spaced at the same distance as the Ψ and *env* amplicons to provide cell number quantification and DNA shearing correction

Siliciano and Siliciano, Annual Reviews 2022

High throughput assays available to quantify intact virus

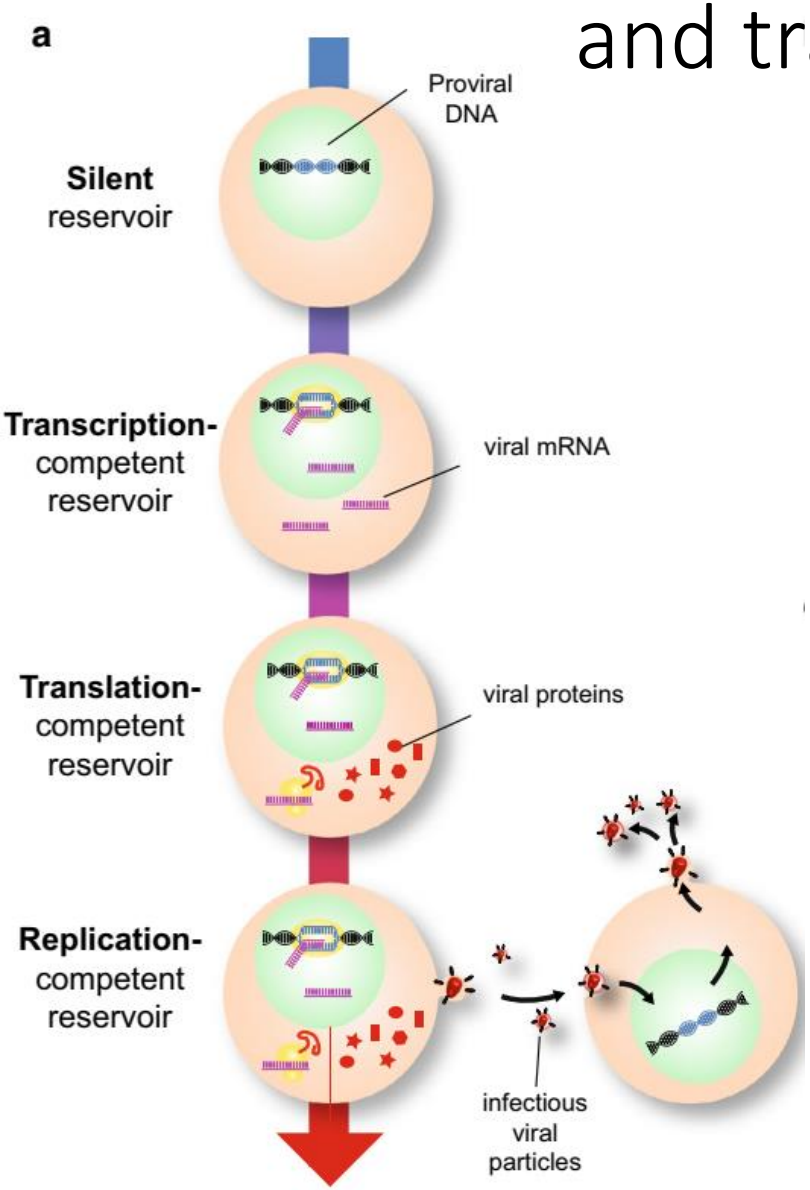
Advantages: IPDA requires a relatively small number of cells, is labour- and cost-effective

Disadvantages: Suffers from sequence polymorphisms and does not provide an inducibility index

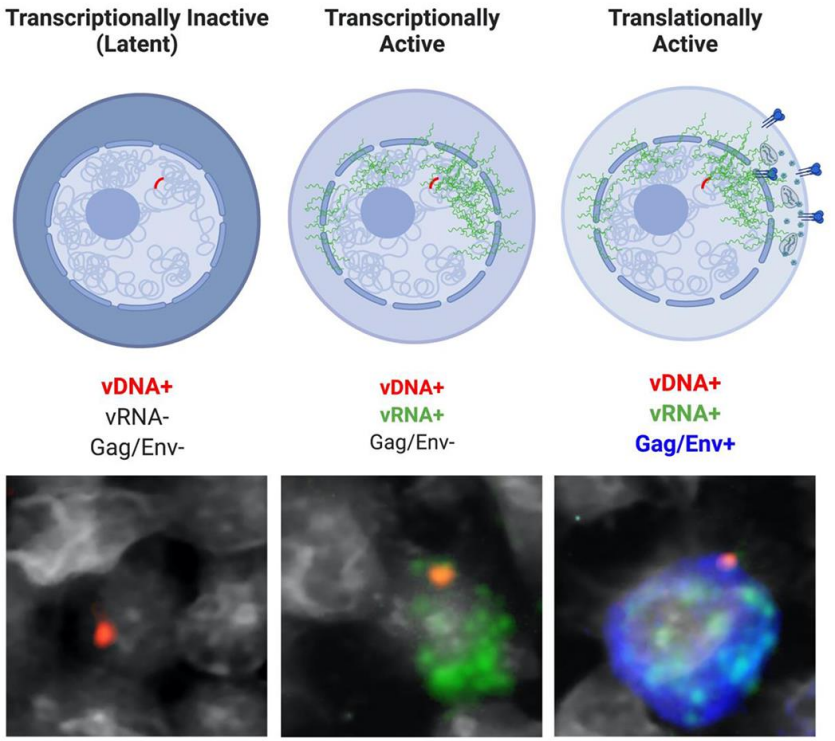


In quadruplex quantitative PCR the frequency of cells encoding intact and defective proviral HIV DNA is confirmed by near full length sequencing

Beyond the replication-competent HIV reservoir: transcription and translation-competent reservoirs



Baxter, Retrovirology 2018



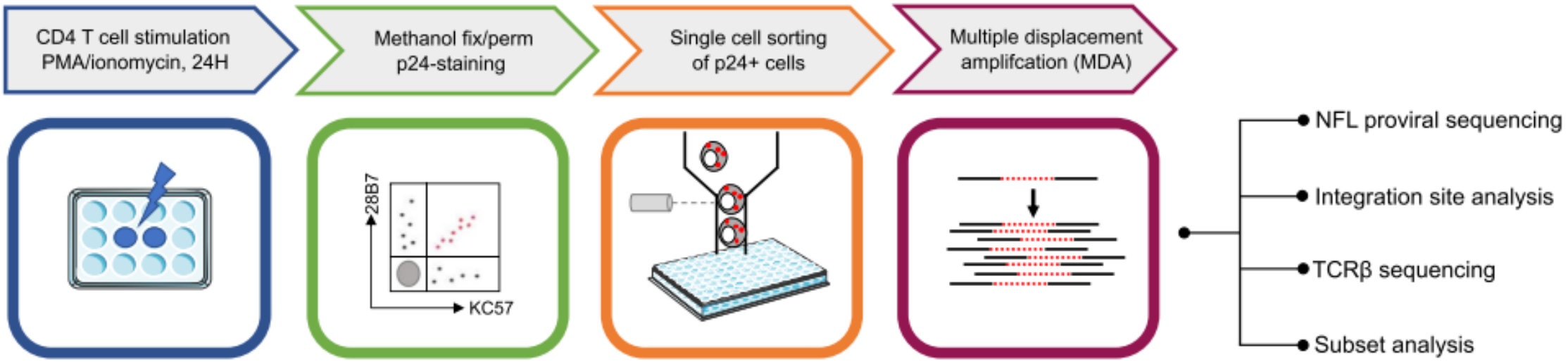
Fluorescent micrographs obtained by in situ spatial analysis with single-cell resolution

Busman-Sahaya, 2021 Current Opinion HIV and AIDS

- The study of HIV reservoir has moved from analysis of **replication-competent** virus to a comprehensive breakdown of the steps of HIV persistence and expression
- Cellular reservoirs that may not contain fully replication-competent virus, but are able to produce HIV **mRNAs** and **proteins**, are clinically relevant for the improved morbidity and mortality during long-term ART
- New assay combines the analysis of Cell Associated RNA (CAR) with protein translation

Single-cell analysis of inducible virus

Frequency of cells undergoing HIV transcription and/or translation after stimulation at the single-cell level



Advantages:

- Single-cell insight into HIV transcription and/or translation
- Phenotypic characterization of individual cells and clade independence
- Relative cost effectiveness

Disadvantages:

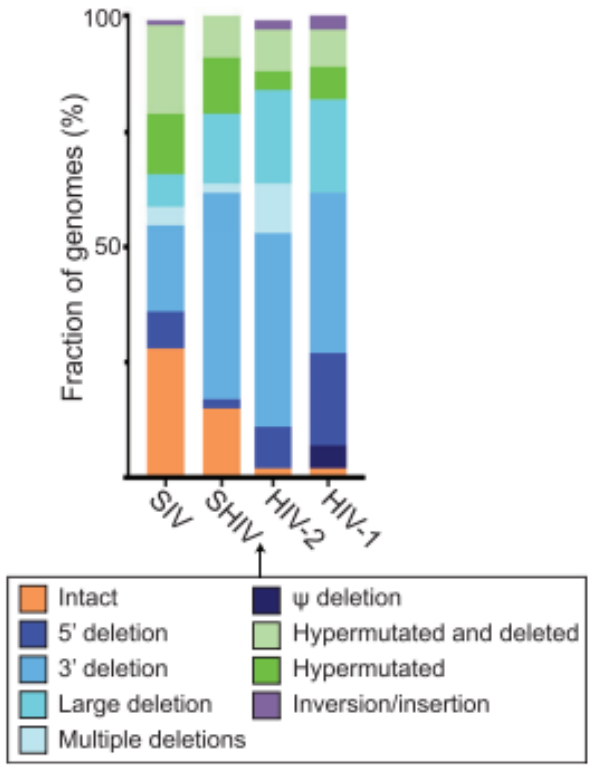
- Lack of measurement of replication competence

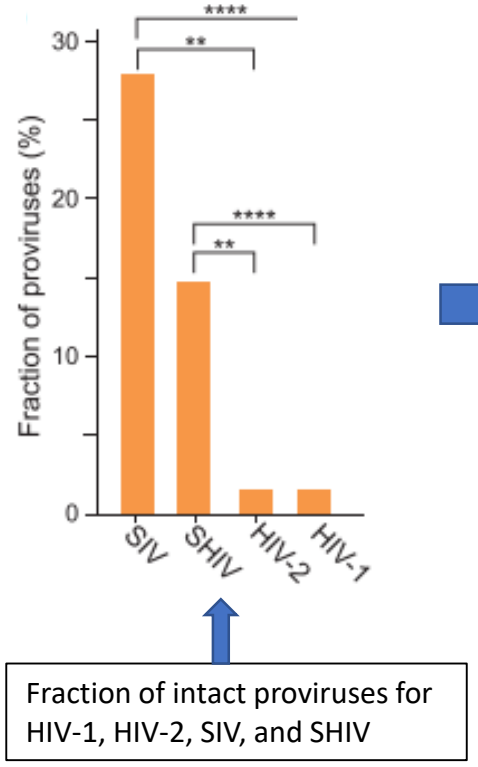
Cole, Nature Communications 2021

HIV-1 Dynamic in reservoir: Defective and intact virus

The proviral landscape in treated PLWH is dominated by defective sequences (Imamichi PNAS 2016, Bruner Nat Med 2016, Hiener 2017 Cell.Rep. and other) and this has been also observed in HIV-2 infection.

The frequency of defective SIV and SHIV genomes in ART treated rhesus macaques is lower than in humans

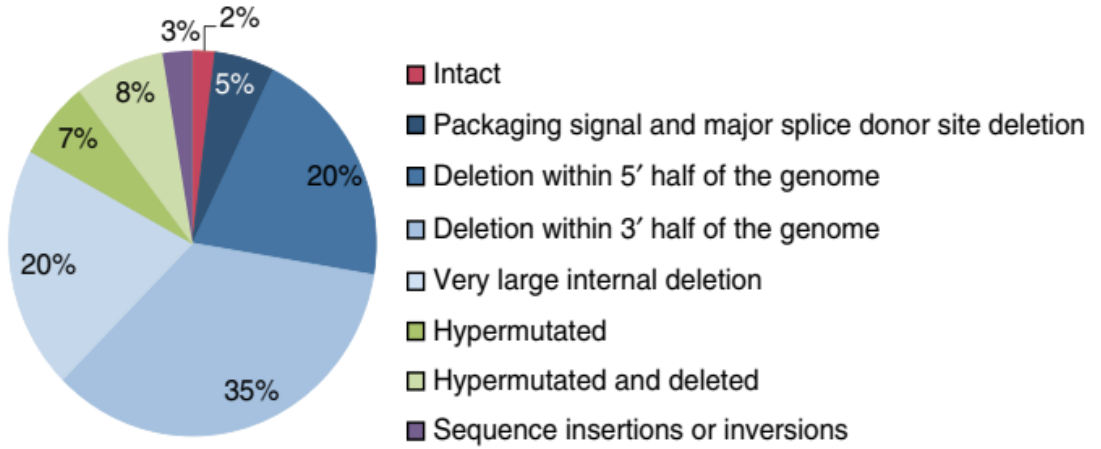




Fraction of intact proviruses for HIV-1, HIV-2, SIV, and SHIV

Bender, Cell Host Microbiome 2019

Characterization of defective HIV-1 virus in chronically treated subjects



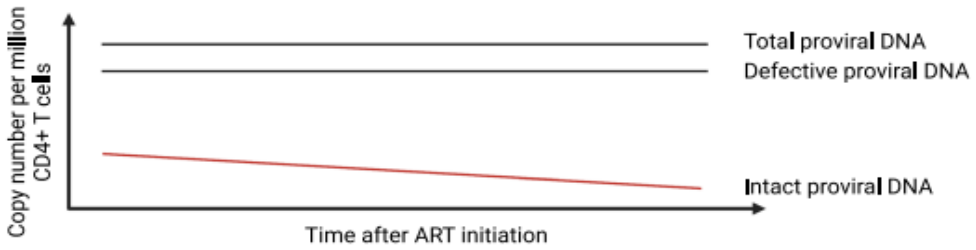
n = 10 individuals, 152 sequences

Possible outcome measure
DEFECTIVITY INDEX

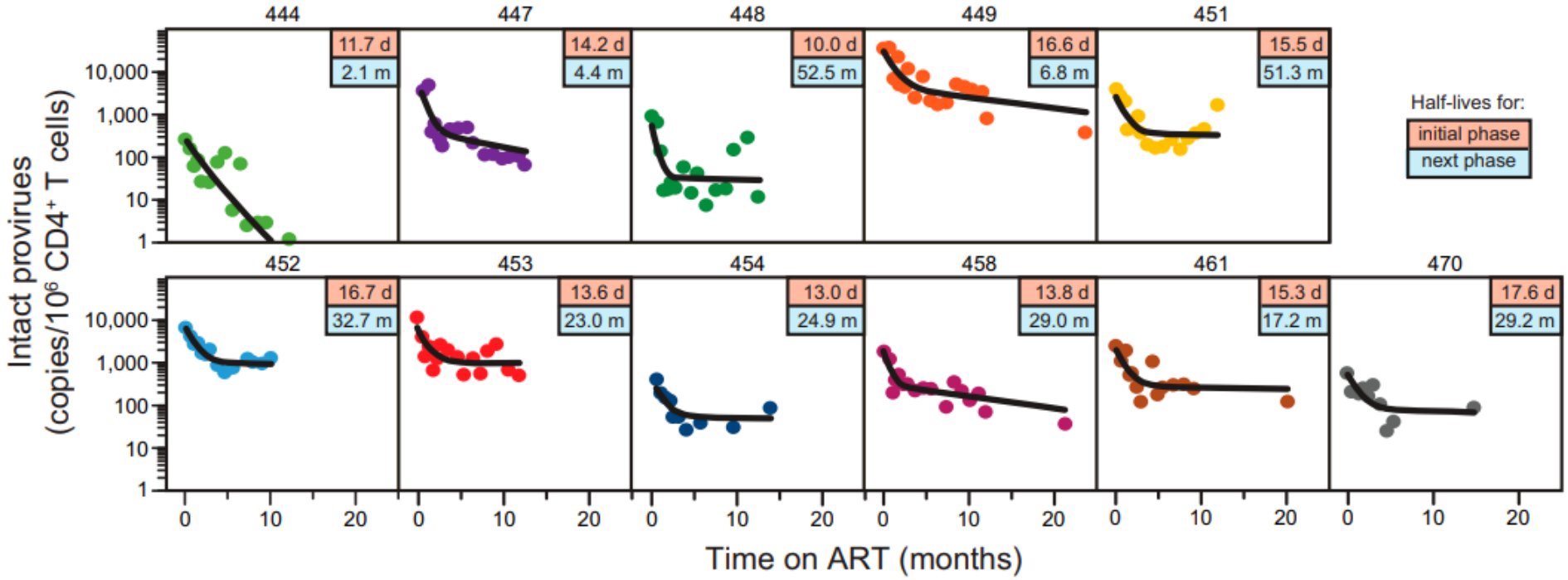
Ratio between defective and intact proviruses

Bruner, Nat Med 2016

Biphasic decay of circulating CD4+ T cells with intact proviruses



➡ Gradual decline in the intact proviral reservoir

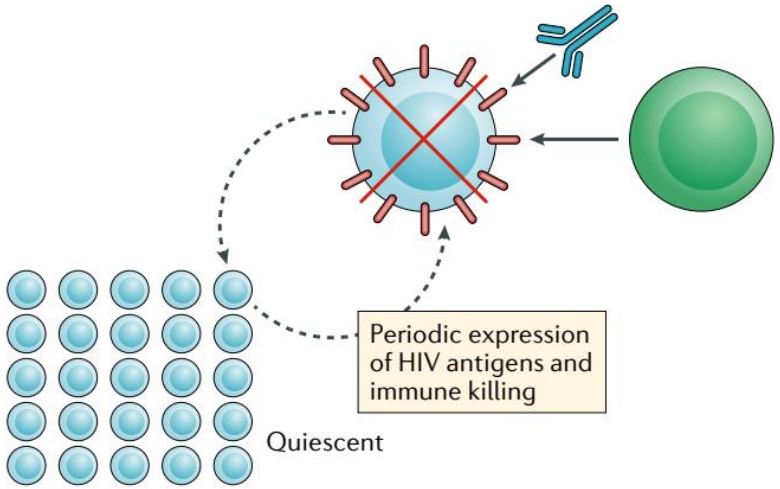


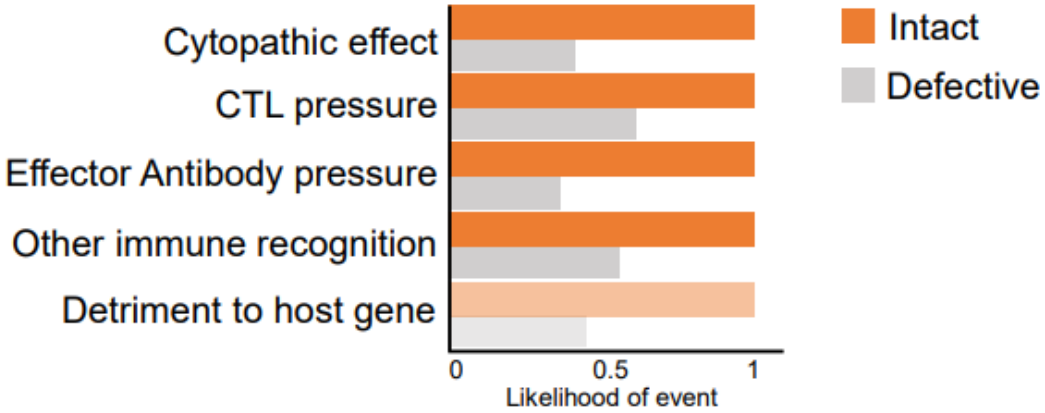
IPDA measurements (circles) and the best-fit biexponential decay model (black line) are shown for 11 participants for whom samples were obtained throughout the first year of ART. Boxed values in the upper right of each panel are the half-lives for the initial phase (top, in days) and the next phase (bottom, in months) based on the model

White et al., PNAS 2022

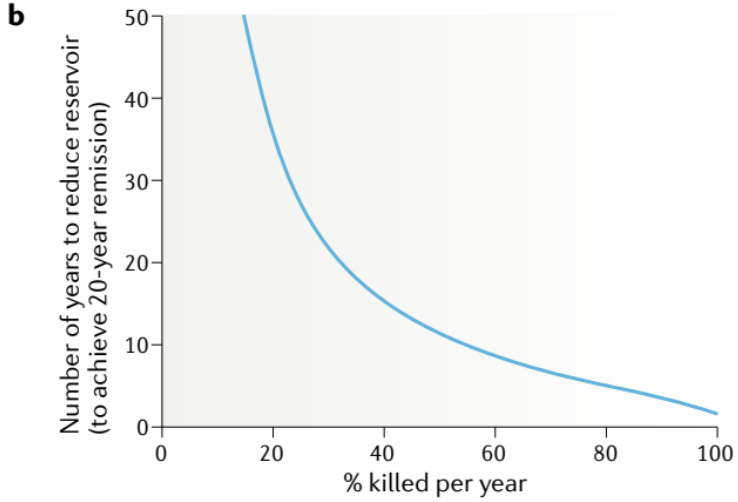
Reactivation of latent HIV infection during suppressive ART and reduction of the reservoir

Latent cells may periodically express HIV antigens and thus be susceptible to immune killing. Defective proviruses are less likely to be **negative selected**





Depending on the proportion of cells that express antigen and are killed each year, it will take different amounts of time until the reservoir is notably reduced.



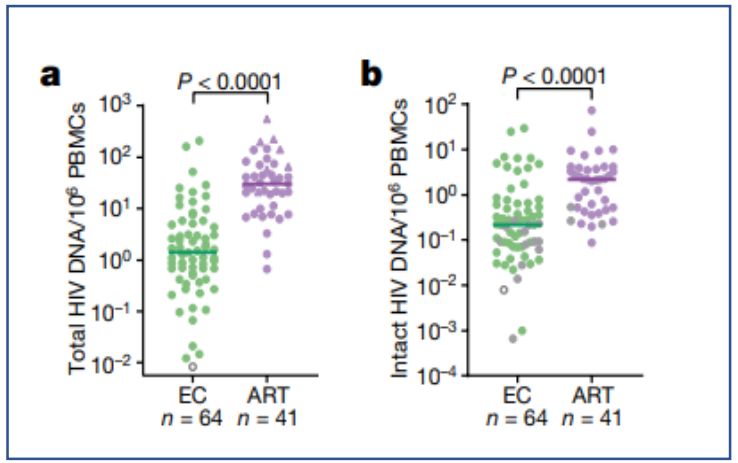
At any given time, most proviruses are silent. Selection takes time!

Davenport, Nat Rev Immunol 2018, Cho, 2022

Dynamics of the HIV reservoir in Elite Controller

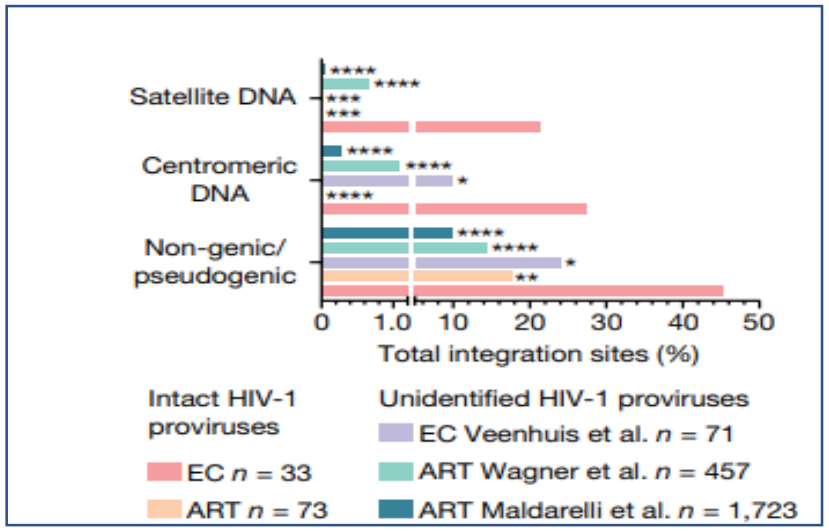
Elite Controller: Rare PLWH who control viral replication without ART.

- Selection against cells with active proviruses integrated into active genomic regions thorough strong antiviral immune responses
- Enrichment of cells with intact proviruses integrated into chromosomal region non permissive for transcription such as poorly transcribed centromeric region

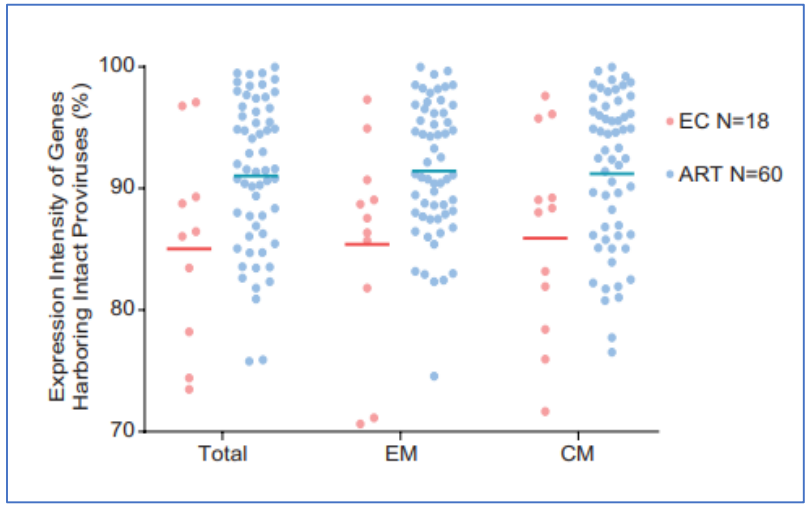


(a) The number of proviral amplification products, including intact and defective was **lower** in EC

(b) Frequencies of proviral sequences with intact genomes that did not contain defined lethal sequence defects were also **lower** in EC



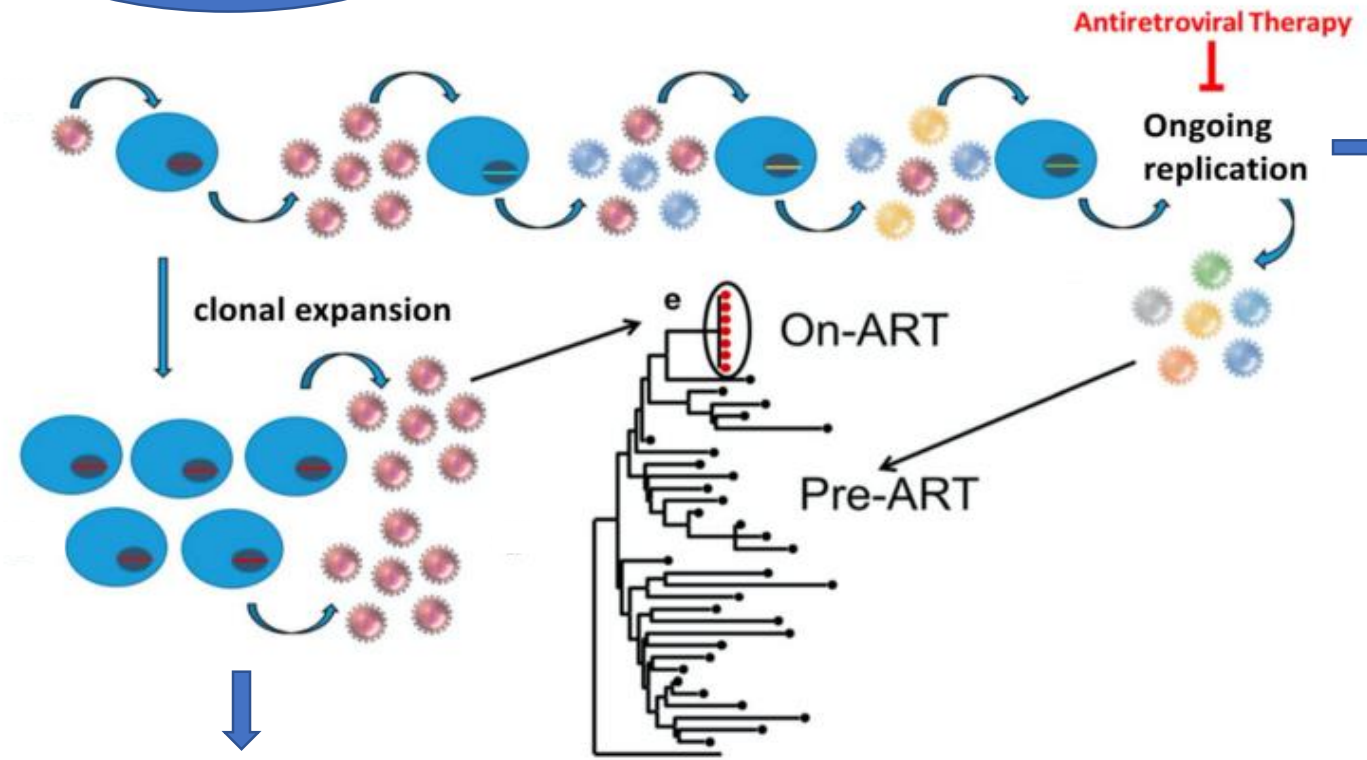
Increased frequency of genome-intact proviral sequences integrated in centromeric satellite DNA in EC



Lower expression of host genes that contain intact proviral sequences in EC

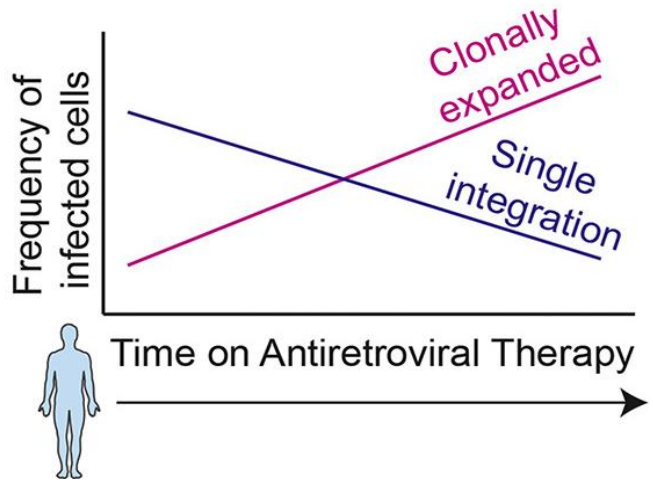
Mechanism of HIV persistance

ISA: Integration site analysis

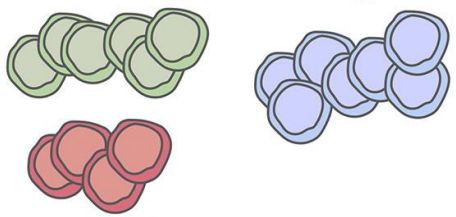


Unique single integration site

ART does not block viral replication in sanctuary sites
Presence of 2-LTR, accumulation of genetic changes in viral genome



Clones of infected cells



Identical Integration site from different cells

HIV-1 reservoir is maintained by cell division rather than by virus replication. Low level viremia due to occasional reactivation.

Possible outcome measure
CLONALITY INDEX
Ratio between clonally expanded proviruses and single integrants

Cohn, Cell 2015; Coffin, Viruses 2021

Methodologies allowed a deeper study of infected CD4+ T cells and their fate. However, it is not yet possible to investigate the total HIV reservoir!!!

Forces driving changes in proviral landscape

Elimination

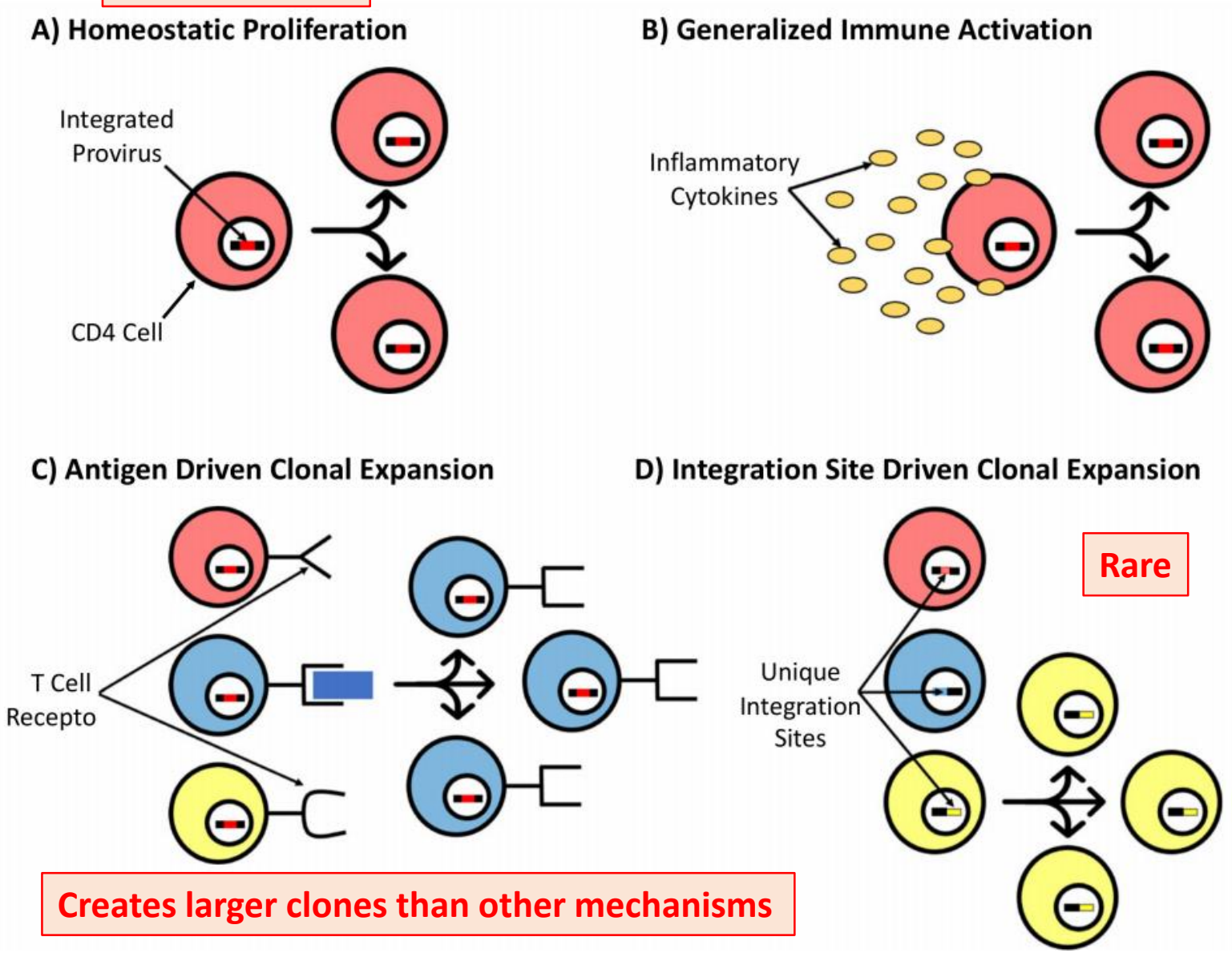
CD4+ T cell

Clonal expansion (about 40% of infected cells)

Persistence as single cell

Lau, Viruses 2021

More frequent

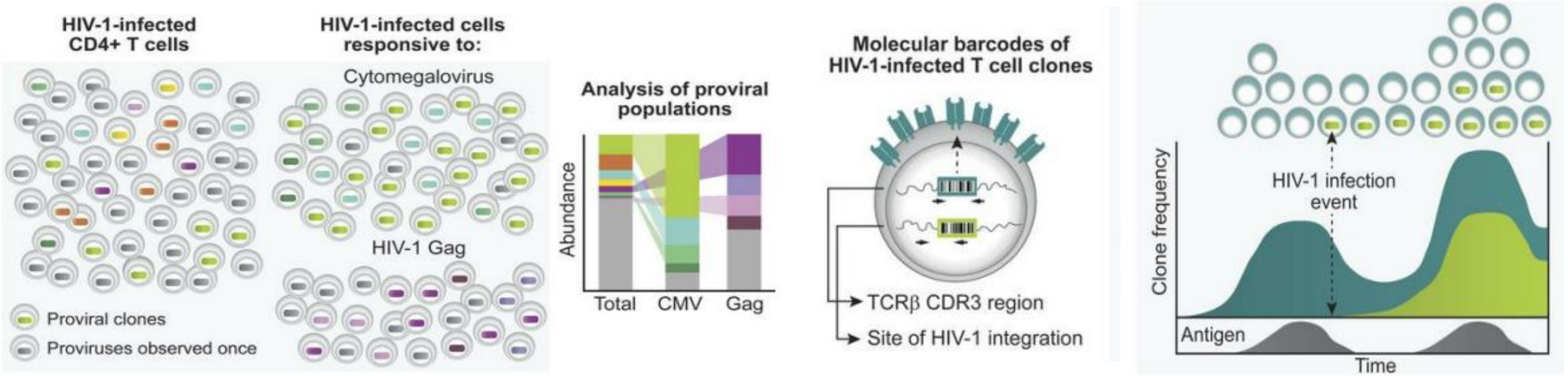


Creates larger clones than other mechanisms

Antigen-driven clonal expansion

CMV lysates, gag peptides and control CD3/28 antibodies were used to stimulate PBMCs from patients co-infected with HIV and CMV

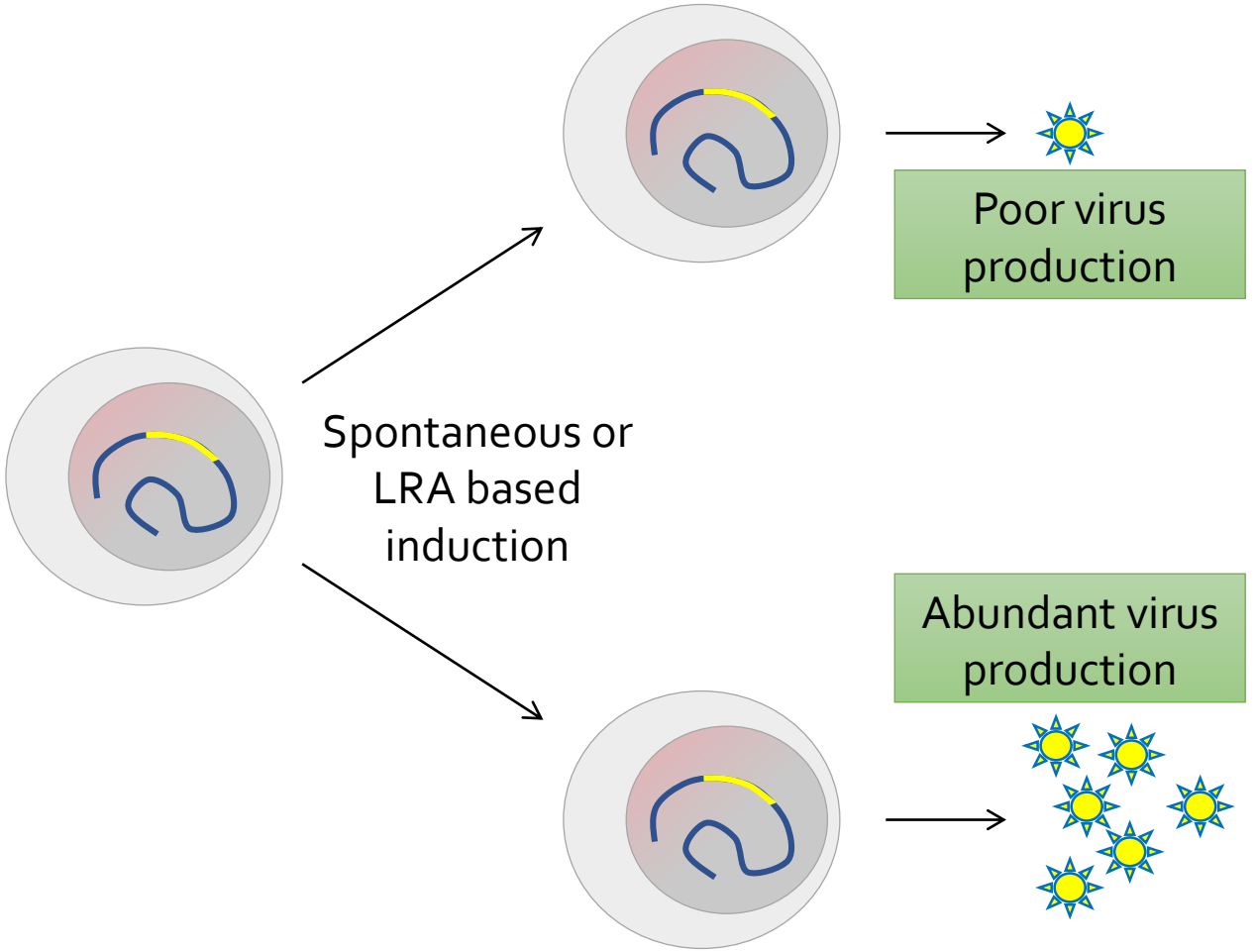
The provirus integration site, the T cell receptor β-chain (TCRβ) rearrangement, and the specific stimulating antigen (HIV Gag or CMV) was estimated simultaneously in expanded clones



Recurrent antigen exposure influence clonal selection and expansion in patients who are both HIV and CMV positive. These findings are consistent with report of viral blips being more common during the winter

Simonetti, JCI 2021

How dormant/inducible is the reservoir?



Candidate to ART deintensification or ART discontinuation

qVOA, system based on induction

Possible outcome measure
INDUCIBILITY INDEX

Extent of virus production upon induction with reference agents

Candidate to shock and kill based eradication pilot

Low inducibility possible cause:

- Induction but the transcriptional machinery is halted by epigenetic changes
- Induction but the transcriptional machinery is halted by host factor

Take home messages

- **HIV-1 reservoir features:**
  - The HIV reservoir composition and shape is determined by multifactorial events
  - Both intact and defective proviruses are present in a low frequency of infected cells, but the decay of intact virus is faster
  - Clonal expansion is mostly driven by immune stimuli
  - Some intact proviruses may persist thanks to a deeper state of latency, in part due to their surrounding genomic features
- **Toolkit to investigate reservoir improvement and limits**
  - **Total HIV DNA** remains a useful diagnostic tool, but a major effort must be performed to update the laboratory methods shifting from qPCR to ddPCR
  - Novel feasible markers upcoming to follow the dynamics of **defective/intact virus (IPDA)**
    - Requires a relatively low amount of blood but needs further standardization
  - **Ex vivo inducibility of latent provirus** and **provirus clonality** appealing but technically demanding (large blood volume, biosafety)
  - Accessing non-blood compartments remains a major challenge

Thank you for your attention!