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Study of the immune activation in HIV and its association with chronic inflammation

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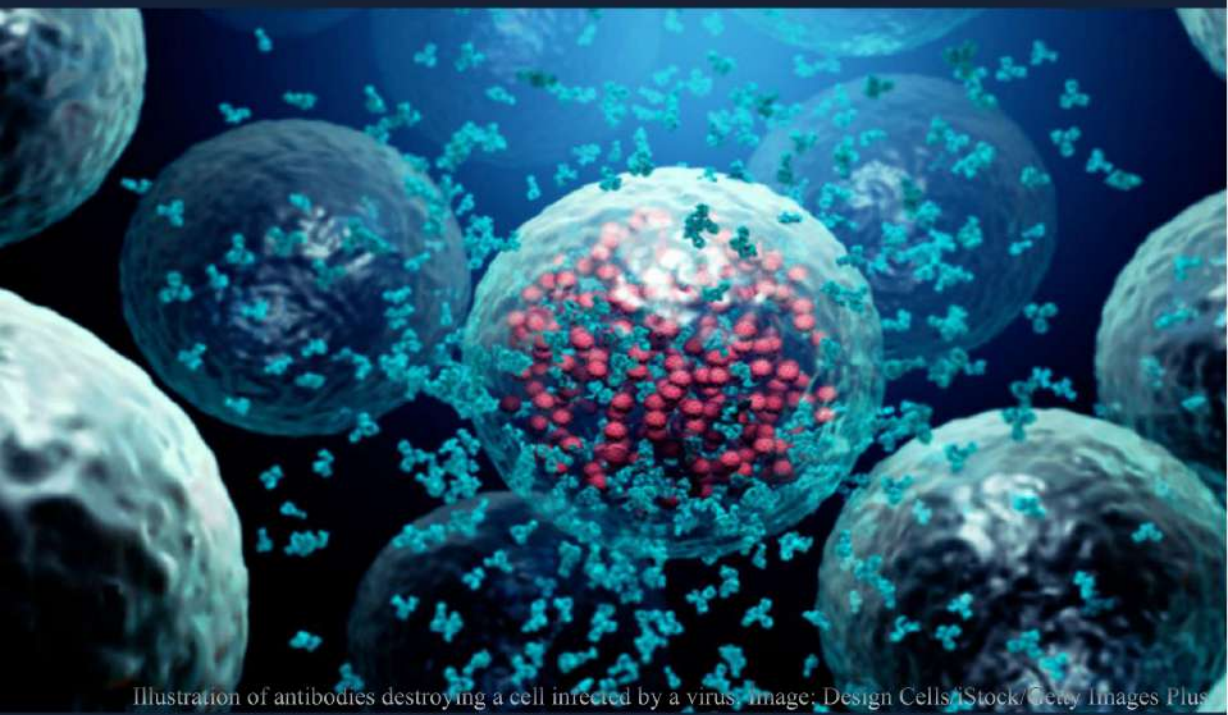

Illustration of antibodies destroying a cell infected by a virus. Image: Design Cells/iStock/Getty Images Plus

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DOCTORAL THESIS

Study of the immune activation in HIV and its association with chronic inflammation.

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Thank you all for contributing to the completion of this research.

Abbreviations

ART: Antiretroviral Therapy

CDC: Centers for Disease Control and Prevention

CMV: Cytomegalovirus

CTL: Cytotoxic T Lymphocyte

EBV: Epstein-Barr Virus

FBS: Fetal Bovine Serum

FDC: Fixed-Dose Combination

HIV: Human Immunodeficiency Virus

HSV: Herpes Simplex Virus

ICIs: Immune Checkpoint Inhibitors

IFN-γ: Interferon gamma

IL: Interleukin

INRs: Immunological non-responders

IRIS: Immune Reconstitution Inflammatory Syndrome

MHC: Major Histocompatibility Complex

MSM: Men who have Sex with Men

NK: Natural Killer

PBMC: Peripheral Blood Mononuclear Cells

PEP: Post-Exposure Prophylaxis

PLWH: People Living With HIV

PrEP: Pre-Exposure Prophylaxis

SIV: Simian Immunodeficiency Virus

Tcm: Central Memory T cells

TCR: T Cell Receptor

Tem: Effector Memory T cells

Temra: Terminally Differentiated Effector Memory T cells

TNF-α: Tumor Necrosis Factor alfa

Tregs: Regulatory T Cells

Index

Abstract

Introduction: HIV compromises the immune system, resulting in immunosuppression. Although antiretroviral therapy (ART) successfully suppresses HIV replication, persistent immune activation and chronic inflammation of unclear origin are associated with an increased risk of non-communicable diseases in individuals living with HIV. PrEP users, while protected from HIV infection, exhibit similar lifestyles and sexual behaviors to those of HIV-positive individuals. Investigating the immunophenotypic profiles of PrEP users offers valuable insights into the influence of HIV and lifestyle factors on the development of chronic diseases. This study aims to investigate the immunophenotypic changes in T-cell subsets, with a focus on γδ T cells, in HIV-positive individuals before and after ART, PrEP users, and healthy controls (HC). **Methods**: Peripheral blood mononuclear cells (PBMCs) were collected from 14 ART-naïve HIV-infected males, and after 48 weeks of ART, 10 PrEP users, and 11 healthy controls. T-cell differentiation, activation, and exhaustion markers were analyzed using multicolor Spectral flow cytometry. **Results**: ART significantly improved CD4+ and CD8+ T-cell counts but did not fully restore markers of differentiation, activation, and exhaustion. PrEP users exhibited heightened immune activation, especially in CD8+ T cells and γδ T-cell subsets, reflecting characteristics of immune aging. A marked depletion of Vδ2+ cells along with expansion of Vδ1+ cells was observed in both HIV-positive and PrEP groups. Additionally, elevated levels of terminally differentiated effector memory (Temra) γδ T cells in PrEP users suggest potential accelerated immunosenescence driven by repeated antigen exposure. **Conclusion**: Our findings reveal persistent immune dysregulation in T cells among HIV-positive individuals on ART and PrEP users, which may contribute to chronic inflammation and accelerated immunosenescence. Notably, γδ T-cell subsets appear to play a significant role in driving inflammation in both groups, underscoring the need for further investigation into their functional impact and therapeutic potential.

Resumen

Introducción: El VIH afecta el sistema inmunológico, causando inmunosupresión. Aunque la ART suprime eficazmente la replicación del virus, la activación inmune persistente y la inflamación crónica de origen desconocido aumentan el riesgo de enfermedades no transmisibles en personas con VIH. Los usuarios de PrEP, aunque protegidos contra el VIH, comparten estilos de vida y conductas sexuales similares a las personas seropositivas. Estudiar sus perfiles inmunofenotípicos proporciona valiosa información sobre la influencia del VIH y los factores de estilo de vida en enfermedades crónicas. Este estudio analiza cambios inmunofenotípicos en células T, con énfasis en células γδ T, en personas seropositivas antes y después de la ART, usuarios de PrEP y controles sanos. **Métodos:** Se separaron células mononucleares de sangre periférica (PBMC) de 14 hombres infectados con VIH que antes y después de ART, 10 usuarios de PrEP y 11 controles sanos. Se analizaron los marcadores de diferenciación, activación y agotamiento de células T utilizando citometría de flujo espectral multicolor. **Resultados:** La ART mejoró significativamente los recuentos de células T CD4+ y CD8+, pero no logró restaurar completamente los marcadores de diferenciación, activación y agotamiento. Los usuarios de PrEP mostraron una mayor activación inmune, especialmente en células T CD8+ y subconjuntos de células γδ T, reflejando características del envejecimiento inmune. Se observó una marcada depleción de células Vδ2+ junto con una expansión de células Vδ1+ en ambos grupos, los seropositivos y los usuarios de PrEP. Además, los niveles elevados de células γδ T de memoria efectora terminalmente diferenciadas (Temra) en usuarios de PrEP sugieren una inmunosenescencia acelerada potencialmente impulsada por la exposición repetida a antígenos. **Conclusión:** Nuestros hallazgos revelan una disfunción inmune persistente en las células T de personas seropositivas bajo ART y usuarios de PrEP, lo que podría contribuir a la inflamación crónica y la inmunosenescencia acelerada. Notablemente, los subconjuntos de células γδ T parecen desempeñar un papel significativo en la conducción de la inflamación en ambos grupos, destacando la necesidad de investigaciones adicionales sobre su impacto funcional y potencial terapéutico.

INTRODUCTION

1 Introduction

1.1 The human immunodeficiency virus (HIV) infection

HIV infection has remained a significant global public health issue since its discovery four decades ago, despite international advancements in prevention, testing, and treatment efforts (1). Human immunodeficiency virus type 1 (HIV-1) is a retrovirus transmitted through body fluids and secretions, but with a notable diversity influenced by policy, social and cultural contexts. In industrialized countries, HIV epidemics typically exhibit a lower overall burden but are highly concentrated within specific high-risk populations, particularly men who have sex with men (MSM) and transgender women who have sex with men. After infection, HIV-1 invades host cells by binding to CD4 receptors and one of two major chemokine co-receptors, CCR5 or CXCR4. The virus integrates into the host genome, establishing reservoirs in long-lived cells such as memory CD4+ T cells and tissue-resident macrophages, which are key barriers to eradication (1).

At the time of infection, the immune system responds rapidly with natural killer (NK) cells that destroy infected cells and secrete cytokines to limit viral spread. Adaptive immunity follows, with CD8+ T cells targeting and killing infected cells. However, the virus evades these responses through immune escape and exhaustion, impairing immune function. Without antiretroviral therapy (ART), unchecked viral replication depletes CD4+ T cells, leading to immune system collapse, chronic inflammation, and eventual progression to acquired immunodeficiency syndrome (AIDS), typically within 2–10 years. The availability and efficacy of ART (Figure 1) has significantly reduced the fear associated with AIDS, which this may encourage high-risk sexual practices, contributing to greater exposure to HIV (2–6).

1.2 Antiretroviral therapy

To date, more than twenty ART drugs at eight different classes were proved by the United States Food and Drug Administration (FDA) for HIV inhibition, targeting different stages of the HIV life cycle (7) (Figure 4). Currently, first-line ART regimens normally involve at least two drugs from nucleoside reverse transcriptase inhibitors (NRTIs) and integrate with at least one drug from non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs), or protease Inhibitors (PIs) (Table 1).

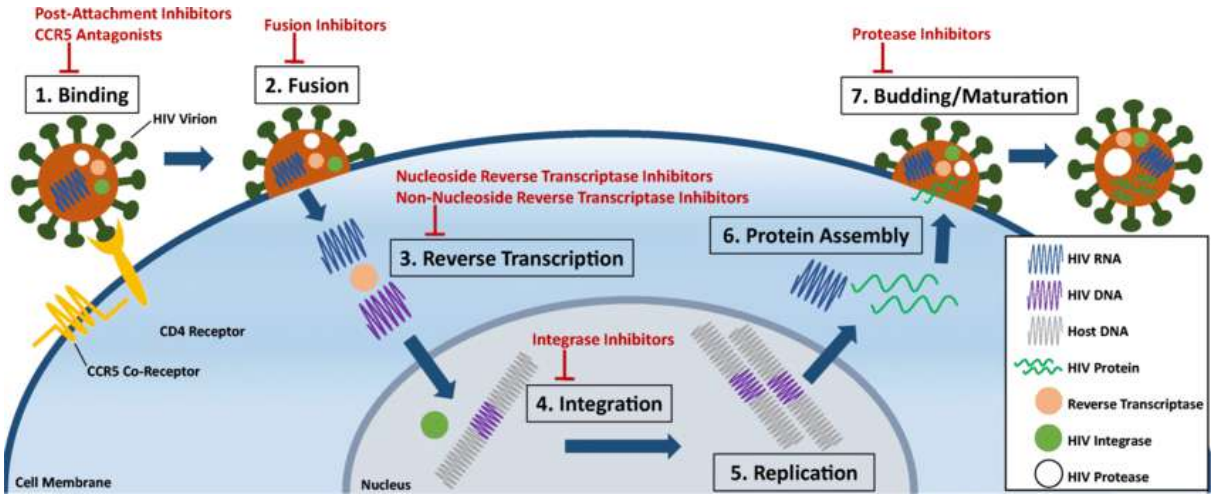


Figure 1. Impact of antiretroviral therapy (ART) drugs on the HIV life cycle. Source: J Neuroimmune Pharmacol. 2021 March; 16(1): 113–129. (7)

Table 1. Principal ART Drug classes, mechanism of action and examples

Class	mechanism of action	Examples
Entry Inhibitors (EIs)	**Prevent HIV from binding to CD4+ T-lymphocytes (includes post-attachment inhibitors and CCR5 antagonists)**	**Ibalizumab-uiyk (IBA), Maraviroc (MVC)**
Fusion Inhibitors (FIs)	**Prevent fusion of HIV with T-lymphocyte membrane and entry into the cell**	**Enfuvirtide (T-20)**
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	**Block reverse transcriptase by competitive inhibition, preventing transcription of viral RNA to DNA**	**AZT, Abacavir (ABC), Emtricitabine (FTC), Lamivudine (3TC), Tenofovir Alafenamide (TAF), Tenofovir Disoproxil Fumarate (TDF)**
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	**Block reverse transcriptase by non-competitive inhibition, preventing transcription of viral RNA to DNA**	**Doravine (DOR), Efavirenz (EFV), Etravirine (ETR), Nevirapine (NVP), Rilpivirine (RPV)**
Integrase Inhibitors (INSTIs)	**Prevent replication by blocking HIV integrase, which inserts viral RNA into host DNA**	**Dolutegravir (DTG), Raltegravir (RAL), Bictegravir (BIC)**
Protease Inhibitors (PIs)	**Prevent HIV from becoming active by inhibiting protease, which cleaves viral proteins for assembly and budding**	**Atazanavir (ATV), Darunavir (DRV), Fosamprenavir (FPV), Lopinavir (LPV), Ritonavir (RTV), Saquinavir (SQV), Tipranavir (TPV)**
Pharmacokinetic Enhancers (PKEs)	**Enhance the effectiveness of ART medicines**	**Cobicistat (COBI)**

Source: J Neuroimmune Pharmacol. 2021 March ; 16(1): 113–129. (7)

Despite its high efficacy in viremia control, ART is not curative; the virus persists in latent reservoirs within host cells. Consequently, a strict and lifelong regimen of ART is essential to HIV control (1). For optimal viral suppression and adherence, fixed-dose combination (FDC) therapies consolidate all components of an antiretroviral regimen into a single dosing unit. Atripla™ is known as the first approved once-daily fixed-dose antiretroviral drug for HIV-1

infection treatment, and contains three active ingredients: Efavirenz (EFV), Emtricitabine (FTC), and Tenofovir Disoproxil Fumarate (TDF), greatly simplified HIV treatment regimens and enhanced patient adherence (8).

Another commonly used fixed-dose combination (FDC) is Truvada, which combines tenofovir and emtricitabine. It is widely recognized as an antiretroviral-based prevention intervention recommended for HIV-negative individuals prior to sexual exposure as pre-exposure prophylaxis (PrEP) (9,10). Additionally, antiretroviral-based prevention also involves post-exposure prophylaxis (PEP), which applicates ART following work-related HIV exposure (11), as well as interventions to prevent vertical transmission in HIV-positive pregnant women, aiming to reduce the risk of mother-to-child transmission during pregnancy, labor, and breastfeeding (12).

While ART has transformed HIV infection from a fatal disease to a chronic, manageable condition, studies have evidenced that people living with HIV (PLWH) experience increasing morbidity and mortality due to non-AIDS chronic disease (1,13,14). These include cardiovascular disease (1,13,15–17), metabolic disorders (1,18–21), pulmonary disease (22), renal disease (1,13), osteoporosis (13), and neurological alterations (1,13,23,24). In the chronic phase of HIV infection, the immune system is persistently activated, leading to an accelerated decline in immune competence and contributing to the immunopathogenesis of HIV. This chronic immune activation is a multifactorial condition, driven by the direct effects of HIV infection, coinfections, persistent elevation of pro-inflammatory mediators, and microbial translocation. These factors collectively contribute to immune dysregulation, systemic inflammation, and the development of non-AIDS-related diseases (13).These findings highlight the necessity for comprehensive care strategies that address long-term complications of HIV infection with the goal of improving the quality of life and clinical outcomes for PLWH.

1.3 Individuals on Pre-exposure prophylaxis (PrEP)

In comparison to the extensive efforts to combat HIV infection, the utilization of PrEP as a preventive measure has been available for approximately a decade (25). In Spain, the National Health System funds PrEP for MSM and transgender women who meet at least two of the following requirements during last year (26):

1. Having had more than 10 different sexual partners
2. Having had condomless anal sex
3. Having used drugs in the context of unprotected sex
4. Having taken post-exposure prophylaxis (PEP) on several occasions
5. Having had at least one bacterial STI

Initial clinical researches on PrEP treatment centered on its safety and efficacy within target populations (27,28) and has since shifted toward examining its influence on the sexual behaviors of users (25). However, its impact at the immunological level remains largely unexplored.

Previous studies showed that PrEP has no capacity to impact HIV-specific immune responses in HIV-1 exposed seronegative individuals, which suggests that there is no immunologic priming provided by PrEP (29). On the other hand, increased immune activation and signs of neuronal injury were found in some PrEP users (30). Moreover, significant reduction in cervical immune cells was found in HIV-seronegative, HSV-2-seropositive women during PrEP, while CD4 and CD8 cells expressed increased levels of activation markers in peripheral blood (31).

These findings shed light on an unexplored aspect of the immune system in PrEP users: the prescription criteria for PrEP indicate that these individuals share lifestyles and behaviors similar to those of people living with HIV (PLWH), resulting in repeated exposure to the virus. Although protected from HIV infection, studying the immunophenotypic profiles of PrEP users

provides critical insights into the interplay between HIV exposure, lifestyle factors, and the development of chronic diseases.

1.4 CD4 and CD8 T cells in HIV infection

During the HIV infection, CD4+ and CD8+ T cells respond rapidly and undergo activation to eliminate the virus. However, persistent exposure of T cells to HIV results in a chronic activation of both CD4+ and CD8+ T cells, further evolving into a T-cell dysfunctional state: known as exhaustion (32,33) (Figure 2).

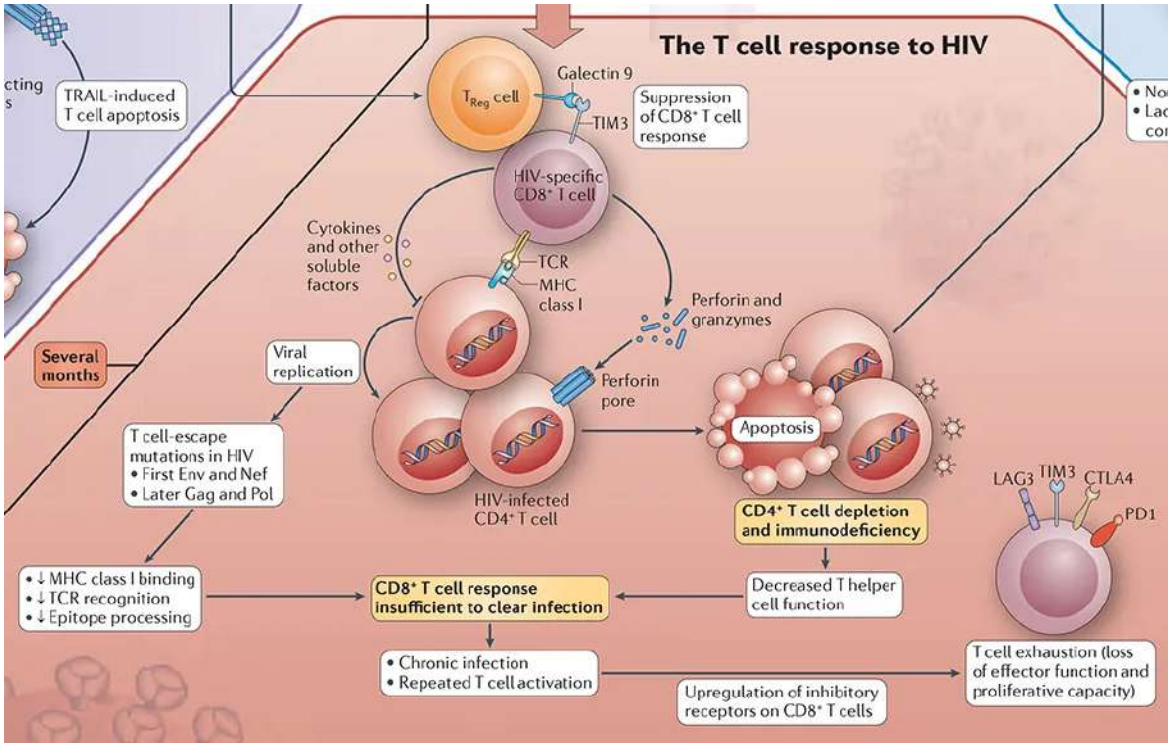


Figure 2. T Cell Dysfunction in HIV Infection. Source: Adapted from the Immune Response to HIV wallchart, created by Nature Reviews Immunology in partnership with STEMCELL Technologies

HIV mainly targets CD4 T cells by binding to CCR5 and CXCR4 surface receptors, resulting in massive CD4+ T-cell depletion and progression to AIDS. Systemic chronic immune activation is considered now as the driving force behind CD4+ T-cell loss even when the viral replication is successfully suppressed by ART(32). In PLWH successfully treated with ART, CD4+ T-cell differentiation still persists compromised (34) and the expression of activation markers (CD38 and HLA-DR) remains elevated (35). Highly activated CD4+ T cells and higher IFN-γ and IL-7 serum levels were observed in immune reconstitution inflammatory syndrome (IRIS) in HIV-infected patients on ART who had profound lymphopenia (36), suggesting a CD4+ T cell mediated mechanism of immune activation with remarkable loss of CD4 T cells. Inspiring from natural host of SIV infection, better preservation of CD4+ T-cell homeostasis may be critical for the preservation of normal immune competence (32).

In PLWH on ART, impaired CD4 T-cell function leads to abnormal CD8 T-cell immunity. Studies have observed that even after long-term ART, patients often exhibit elevated CD8+ T cell counts (37). These elevated CD8 + T cell counts are associated with a higher risk of virological failure as well as AIDS and non-AIDS events (38–40). CD8 T-cell expansion and activation are induced by multifactorial mechanisms including cytokine and chemokine stimulation, and by HIV-specific or non-specific antigens such as CMV and EBV. Cytokine stimulation and inflammation play a crucial role in the expansion of CD8+ T cells, even though the underlying mechanism is not fully clear (40). Similar to CD4+ T cells, CD8+ T-cell differentiation in ART-treated HIV infected patients is only partially restored (41), and ART fails to normalize the level of activation markers expressed on CD8+ T cells (35). Interestingly, HIV-specific CD8+ T cells mediate HIV control which is key for the suppression of viral replication in elite controllers, viremic controllers and long-term non-progressors (42,43).

Elevated activation and altered homeostasis of both CD4 and CD8 T cells eventually lead to T-cell exhaustion and senescence in PLWH, despite ART (33,41). In exhausted T cells, the increased expression of immune checkpoint inhibitors (ICIs) leads to loss of function and diminished proliferation. In the case of CD4 T cells, cytokine and chemokine secretion such as

IL-2, IFN-γ and TNF-α declined (33). T-cell exhaustion in PLWH is highly HIV-specific which is established from the early stage of infection, with no relation to CMV infection, and can contribute to the loss of viral control in those HIV controllers (33). Durable ART significantly reduces excessive activation and exhaustion of T cells in PLWH, however, the timing of ART initiation is crucial for effective restoration (33,41,44).

1.5 γδ T cells

In recent years, there has been growing interest in a distinct subset of unconventional T cells—γδ T cells—which differ from the well-known αβ T cells through their unique γ and δ loci involved in T cell receptor (TCR) rearrangement in the thymus and play a crucial role in immune responses.

1.5.1 γδ T-cell development and TCR rearrangement

T cells are a subset of lymphocytes that integrate into adaptive immunity, with their antigen specificity determined by the T cell receptor (TCR). The diversity and specificity of TCRs are established through a highly regulated process known as TCR gene rearrangement, which occurs during T cell development in the thymus. This mechanism enables the generation of a vast repertoire of TCRs, allowing for specific recognition during T cell response.

Conventional T cells, also called αβ T cells, are characterized by their α chain (light chain) and β chain (heavy chain). They differentiate into CD4+ T cells if recognize antigens presented by major histocompatibility complex (MHC) class II molecules or CD8+ T cells if recognize antigens presented by MHC class I molecules.

γδ T cells are unconventional due to the low abundance in the peripheral T cell population and distinct role in immunity. γδTCRs consist of two chains, the γ chain (light chain) and δ chain (heavy chain), which are analogous to the α and β chains found in conventional αβ T cells. Both αβ T cells and γδ T cells develop from T-cell precursors that migrate from the bone marrow to the thymus. Interestingly, the genes encoding the α and δ chain of T cell receptors are located on the same chromosomal region. During TCR gene rearrangement, successful α-chain gene rearrangement results in the deletion of the δ chain gene locus (45). This process ensures the mutual exclusivity of αβ and γδ T cell receptor expression, committing the developing T cell to either the αβ or γδ lineage. Because of the unique structure of the γδTCR, the activation of γδ T cells is not MHC-dependent, enabling both innate and adaptive immunity and broader spectrum of immune functions(46).

During the double-negative (DN) stages (Figure 3), TCRγ and TCRδ rearrange before TCRβ recombination, leading to the expression of the γδ TCR/CD3 complex on the plasma membrane. At this stage, much like the pre-TCR, the γδ TCR can self-oligomerize and trigger intracellular signaling pathways (46,47). γδ-selection is mainly dependent on the Syk-mediated signaling. However, the underlying mechanism of γδ-selection has been still unclear and debated (47).

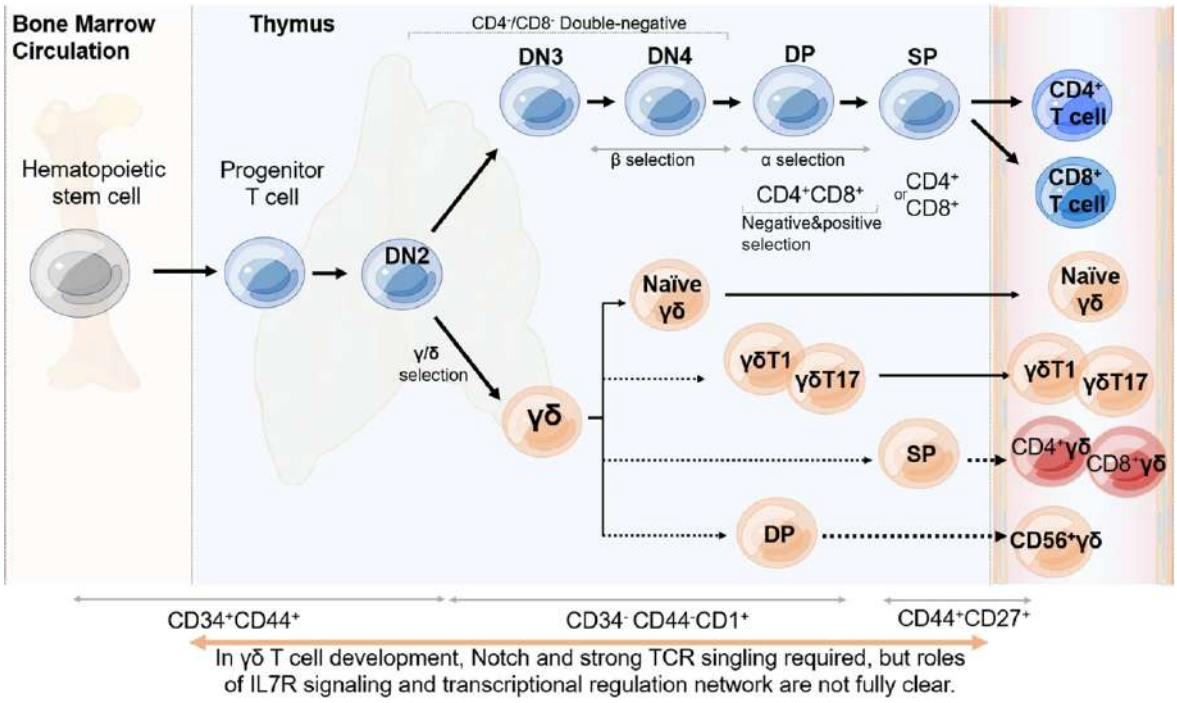


Figure 3. The possible mechanisms of human γδ T development and fate decision in thymus. Abbreviatures: DN, doble negative; DP, doble positive; SP, single positive. Source: Signal Transduction and Targeted Therapy (2023)8:434 (46)

Once γδ T cells complete their maturation journey in the thymus, some of them leave the organ with functional effector pre-programmed, and enter the bloodstream, migrate to barrier tissues. γδ T cells account for approximately 1–5% of total T cells in peripheral blood but are predominantly enriched in peripheral mucosal barriers, such as the skin, lungs and gut tissues (46–49).

In humans, Vδ1+ and Vδ2+ are the two most well-known subsets of γδ T cells, classified based on their V(D)J recombination genes. Vδ1+ T cells are generated in the human thymus a few months after birth, then mainly resident in the gut epithelium, skin, spleen, and liver, with only a small proportion detectable in circulating blood. In contrast, the Vδ2+ subset, which predominantly pairs with the Vγ9 TCR, is the most abundant γδ T-cell population in peripheral blood mononuclear cells (PBMCs), comprising approximately 90% of circulating γδ T cells.

These cells develop during early fetal stages, which supports their innate-like immune response (46,50) (Figure 4).

While Vδ2+ T cells exhibit a highly conserved pairing of Vγ9Vδ2 TCRs, Vδ1+ T cells have more diverse TCRs, with greater flexibility in pairing with various Vγ chains. Additionally, Vδ1+ clonotypes tend to be private and unique to individuals. These features, along with their capacity for rapid clonal expansion, endow Vδ1+ cells with adaptive immune characteristics and memory-like functionality(46).

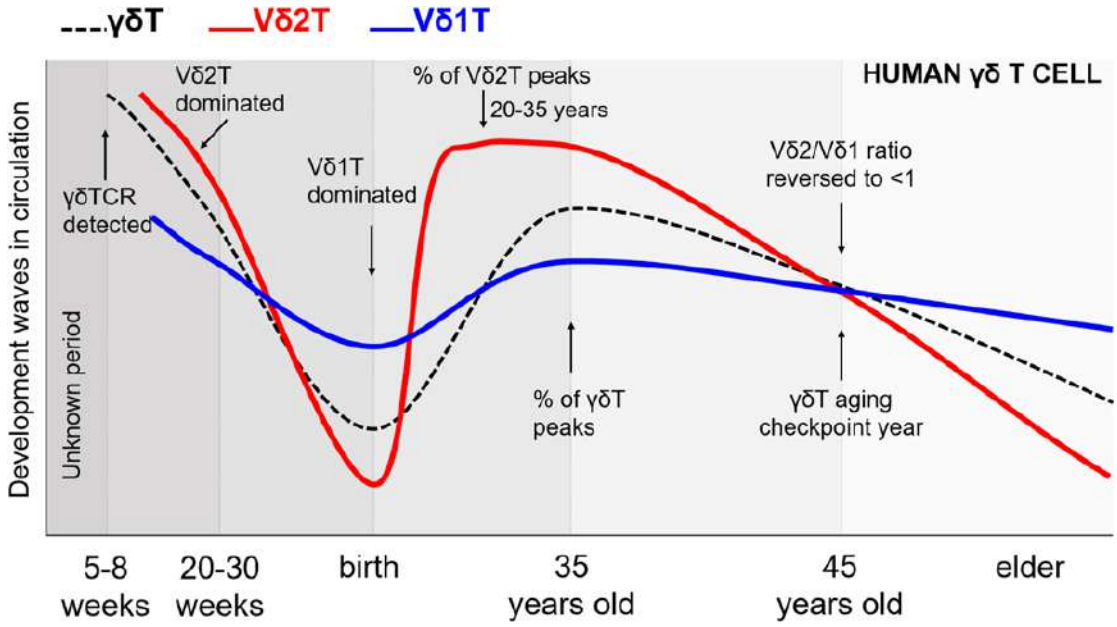


Figure 4. Development waves of total γδT, Vδ2T, and Vδ1T cell populations in circulation from embryonic stages through adulthood and into old age in healthy individuals. Source: Signal Transduction and Targeted Therapy (2023)8:434 (46)

1.5.2 γδ T-cell activation and function

Despite the exact mechanisms by which the pre-TCR and γδTCR direct differentiation into the αβT and γδT lineages are still unclear; it is likely that γδ-selection in most naturally generated γδ T cells does not depend on a cognate γδTCR ligand in the thymus. γδ T-cell activation,

which is independent of MHC, is driven directly by phosphoantigen recognition, with incorporation of co-stimulatory signals such as CD28 and NKG2D for enhanced cytotoxic and cytokine capacities. Moreover, γδ T cells can be activated by cytokines like IL-12, IL-15 and IL-23, which promote their proliferation and functional responses even in the absence of TCR engagement (46,48,51).

The γδTCR chains alone are insufficient to classify the immune function of γδ T cells, as each subset of γδ T cells is highly context-dependent and could be modulated by their immediate environment. According to their production of cytokine and chemokine, γδ T cells can be either anti-tumor (IFN-γ producing Tγδ1 cells) or pro-tumor/inflammatory (IL-17 producing Tγδ17 cells) in cancer patients (46,51). The functional variability observed in γδ T cells suggests that they can be both either protective or injurious (46,49).

1.5.3 γδ T-cell in HIV infection

The unique γδTCR makes the antigen recognition of γδ T cells independent of presentation by MHC molecules, allowing them to recognize a wide variety of peptide or non-peptide antigens. Specifically, direct recognition of lipopolysaccharides (LPS), lipoteichoic acid (LTA), via pattern recognition receptors, and phosphoantigens via the TCR, granting rapid immune response against pathogens in infectious diseases (46,49,50). Once activated, γδ T cells immediately release cytotoxic molecules to eliminate intracellular infections. In addition, γδ T cells produce various anti-infection cytokines, including IFN-γ, IL-17, and IL-22 to recruit and activate other immune cells including neutrophils, dendritic cells, macrophages, and NK cells to eradicate pathogens. Furthermore, γδ T cells can also interact with other immune cells through cell-to-cell contact, leading to optimal clearance of infection (46,49).

In the HIV infection context, both Vδ2+ and Vδ1+ cells present potential anti-HIV functions, although their contribution in natural control is still unclear (50). *Ex vivo* models showed that

both Vδ1+ and Vδ2+ cells from HIV-infected individuals performed direct recognition and elimination of HIV-infected cells, mediating NK cell-like direct cytotoxicity (50). Moreover, *ex vivo* analysis of PBMCs also showed that both Vδ1+ and Vδ2+ cells from ART-treated individuals were capable of degranulation and cytokine expression following CD16 cross-linking, suggesting these cells retain the ability to mediate antibody-dependent cellular cytotoxicity (ADCC) for robust anti-HIV effector functions (50,52) (Figure 5).

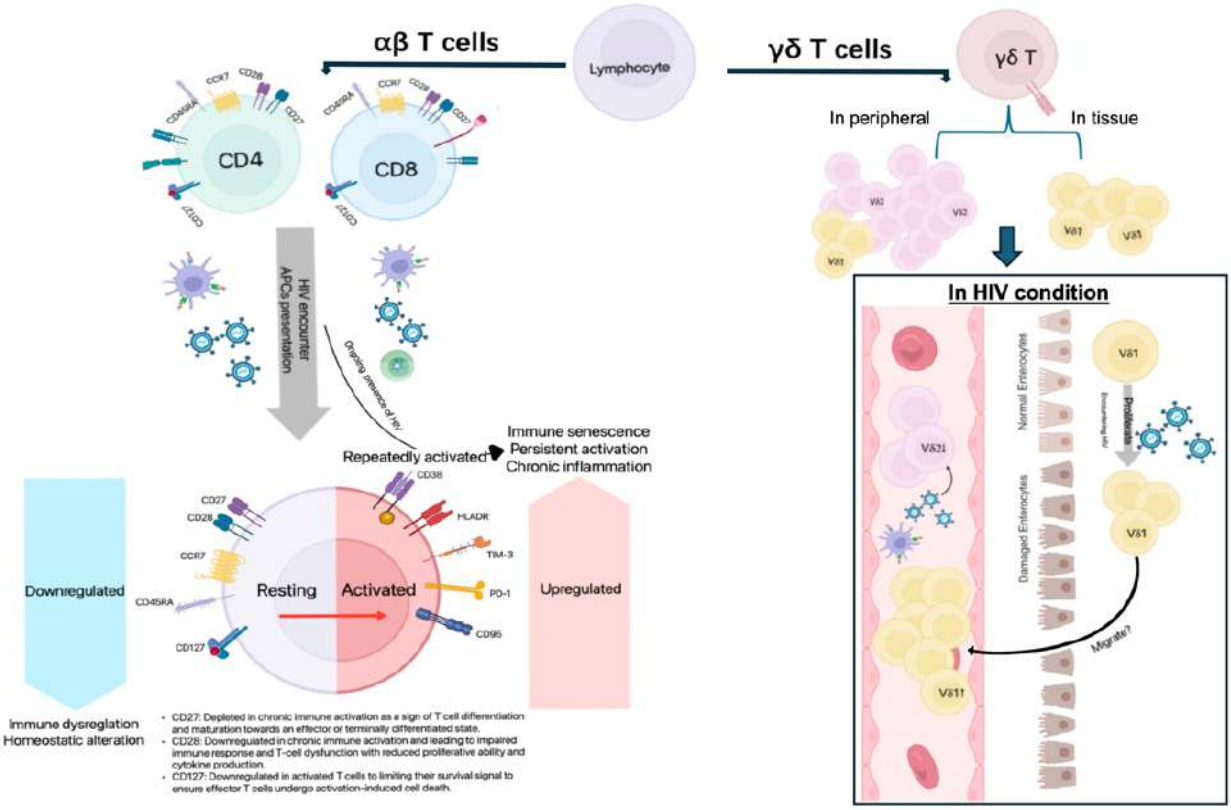


Figure 6. Potential immunophenotypic changes in HIV infection.

In healthy individuals, up to 90% of γδ T cells express the Vδ2 chain in peripheral blood. However, significant depletion of Vδ2+ cells have been described in infectious diseases, such as HIV (Figure 6), chronic hepatitis B, and SARS-CoV-2 infection, as well as in non-infectious chronic conditions such as cancer (46,50,53–55).

In contrast to Vδ2+ cells, Vδ1+ cells are dramatically expanded in peripheral blood, leading to an inverse Vδ1:Vδ2 T cell ratio in HIV infected patients compared to healthy individuals(46,50,54,55). Moreover, ART fails to restore this inverse Vδ1:Vδ2 T-cell ratio (52,55). An increased frequency of peripheral Vδ1+ cells has also been described in HIV controllers (56). Furthermore, peripheral Vδ1+ cells in HIV-infected individuals, not only those naïve untreated patients and chronically treated by ART, but also those elite and viremic controllers, exhibit higher activation level (CD38+HLA-DR+) and predominantly a terminally differentiated phenotype (CD45RA+CD27-) (56). Therefore, there are increasing interests of considering the potential immunotherapeutic role of γδ T cells for current interventions in HIV eradication strategies (50,57).

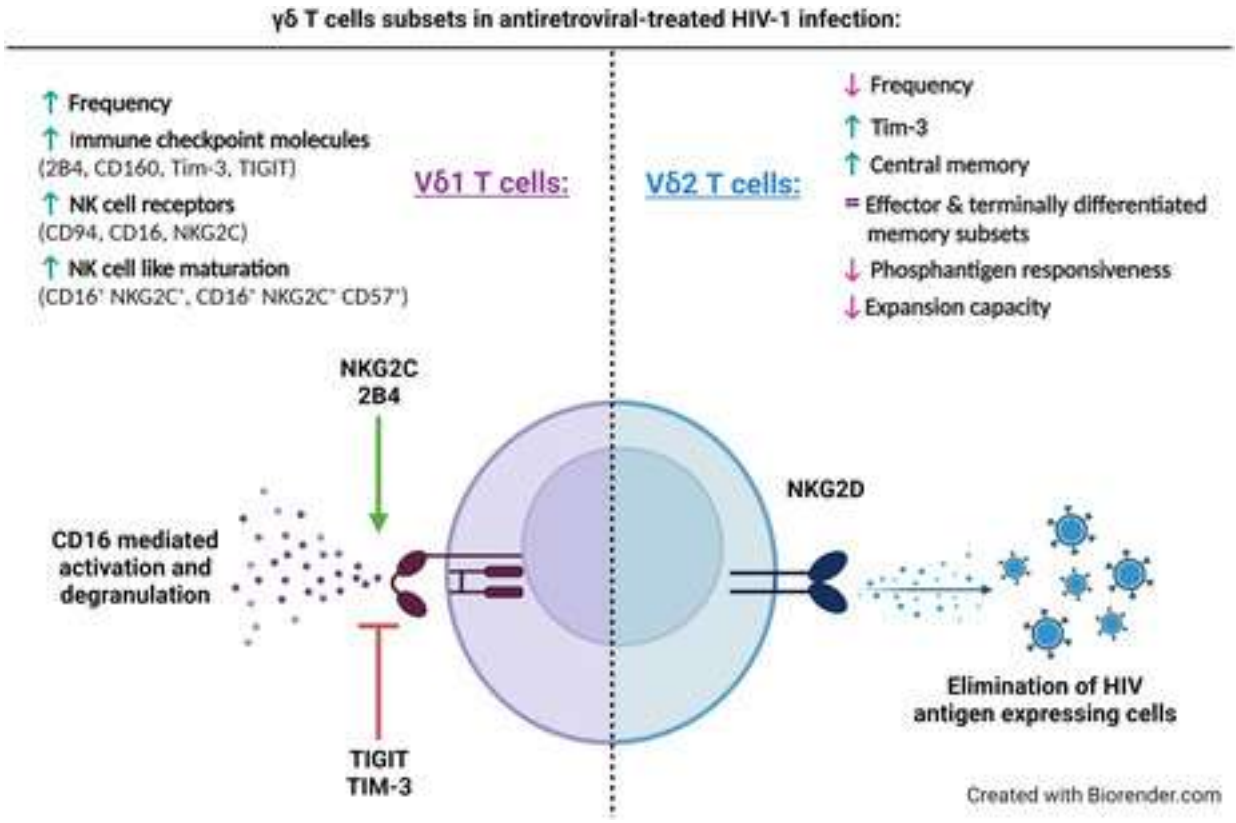
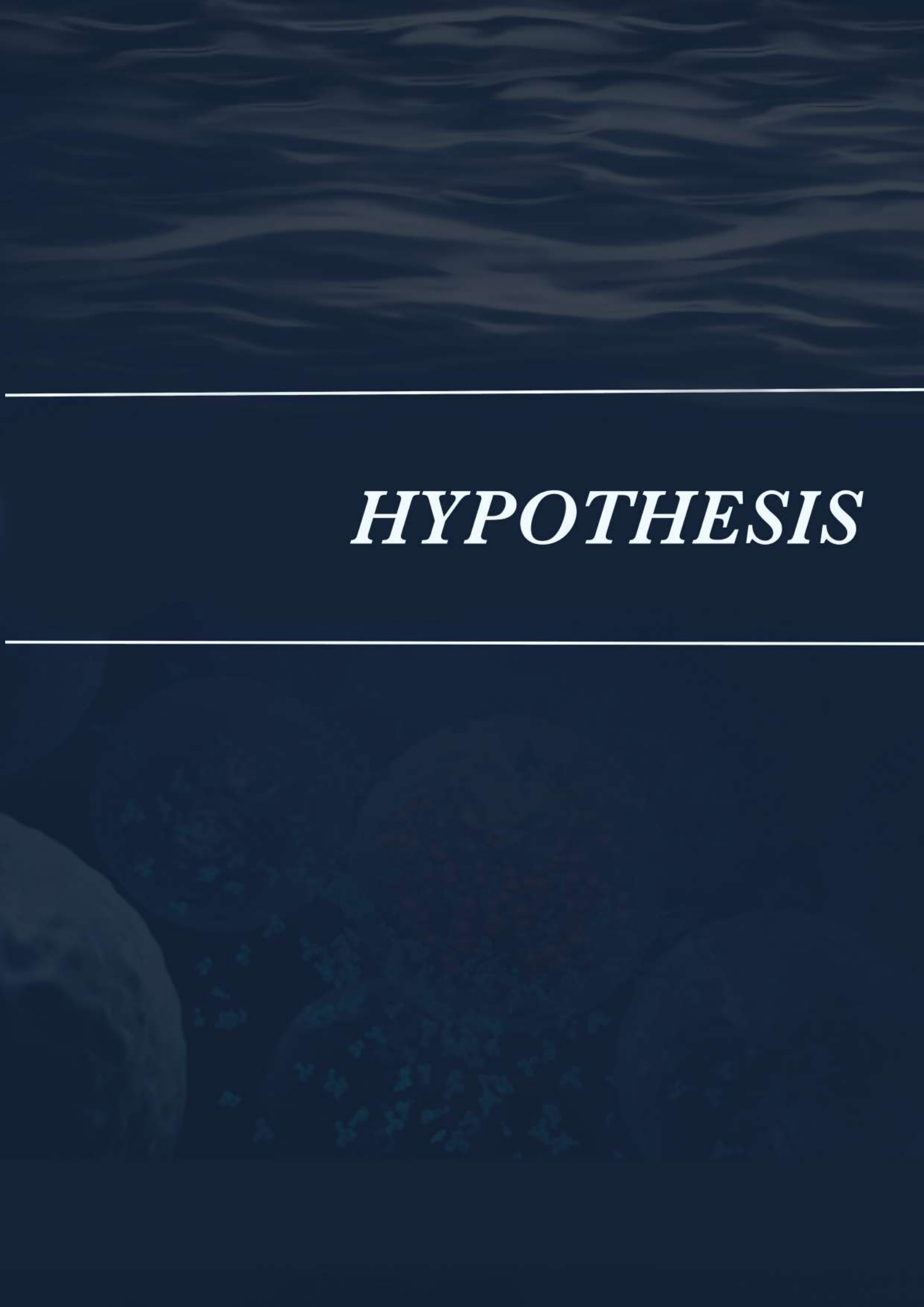


Figure 5. ADCC and NK cell receptor mediated function of γδ T cells in ART-treated HIV-1 infection. Source: Clinical & Translational Immunology 2024; e1486. doi: 10.1002/cti2.1486 (52)



HYPOTHESIS

2 Hypothesis

Persistent immunophenotypic changes of T cells due to HIV infection contribute to prolonged immune activation and chronic inflammation in chronically HIV infected individuals, despite antiretroviral treatment.

The immunophenotypic changes due to HIV infection are exacerbated in immunological non-responders (INRs) leading to impaired immune activation.

OBJECTIVES

3 Objectives

A) The main objective is to analyze the immunophenotypic changes in HIV+ individuals before and after 48 weeks of ART. It comprises the following points:

1 To describe the clinical characteristics of HIV infected individuals and HIV-negative controls recruited in the VIHNAI Cohort.

2 To assess immunophenotypic features in peripheral blood mononuclear cells (PBMCs) in HIV+ individuals before and after initiation of ART, and in HIV- individuals. To characterize differences in the expression of differentiation, activation, and exhaustion markers in the proportions of T-cell subpopulations: CD4, CD8, and γδ T cells.

B) Secondary objectives:

1) To explore of the immunophenotypic profile in individuals on PrEP.

2) To compare all previously assessed parameters between HIV+ immunological responders and HIV+ INRs.

MATERIALS & METHODS

4 Materials and methods

4.1 Study design and patients

A prospective study (VIHNAI cohort) was performed from February 2020 to July 2023. A group of 14 naïve men with HIV infection were selected from the Infectious Diseases Department at Hospital del Mar (Barcelona, Spain), along with two comparison groups of sex-, age-, comorbidities-, and body mass index (BMI)-matched non-infected HIV individuals: a control group who were apparently healthy and a PrEP group who shared the same environment, thereby similar lifestyle and behavior to HIV-infected individuals and were treated with PrEP (tenofovir and emtricitabine). All recruited individuals who met inclusion criteria were included in the VIHNAI cohort.

Patients with a history of co-infection with chronic hepatitis B, hepatitis C, advanced liver disease, neoplasia, chronic kidney disease, and chronic inflammatory diseases were excluded. All participants were visited at recruitment time (baseline) and after 48 weeks on ART in HIV+ participants, where demographic and anthropometric variables, full medical history, and blood samples were collected.

HIV+ individuals were classified according to Centers for Disease Control and Prevention (CDC) classification, with last review on September 21, 2023: A) Asymptomatic HIV infection; B) HIV infection with symptoms; C) HIV infection with AIDS-defining opportunistic infections. 1: CD4+ T-cell counts > 500/µL; 2: CD4+ T-cell counts between 200/µL and 400/µL; 3: CD4+ T-cell counts < 200/µL

The study protocol was approved by the Parc de Salut Mar Ethics Committee (2019/8619/I) in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization. Participants gave written informed consent. Privacy and confidentiality of subjects were respected at all times.

4.2 Sample collection.

From each participant, peripheral blood samples and intestinal biopsies were obtained at baseline and after 48 weeks on ART. Blood samples were used to isolate peripheral blood mononuclear cells (PBMCs).

PBMCs were isolated from whole blood by BD Vacutainer® CPT™ (Cell Preparation Tube) (BD, Franklin Lakes, NJ) according to the manufacturer's instructions, by centrifugation at 1800 × G for 20 minutes at room temperature within two hours of sampling. After centrifugation the CPT tubes were gently inverted several times, the PBMCs-containing plasma was harvested in a 50mL conical tube, washed twice in 1 X Dulbecco phosphate-buffered saline (DPBS; Gibco, Grand Island, NY) and counted for viability using 0.4% Trypan Blue (Sigma, St. Louis, MO) by TC20 Automated Cell Counter (Bio-Rad Laboratories, Inc.). The PBMCs were re-suspended in complete RPMI (Sigma, St. Louis, MO, USA) supplemented with foetal bovine serum (FBS) and Penicillin-Streptomycin, and with 10% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) for cryopreservation in liquid nitrogen until used.

4.3 Cytometry study

4.3.1 Panel design

This panel was developed on a Cytek® Aurora (Cytek Biosciences, Fremont, California) equipped with 4 lasers and 48 detectors. The panel has been designed with the support of the expert group from Cytek Biosciences Spain and CRG/UPF Flow Cytometry Unit (Barcelona, Spain), to achieve the most adequate combination of antibodies and fluorochromes with good compatibility and minimal spread to detect 19 specific markers (Table 2 and Figure 7). PD-1 BV421, CD28 BV650, CD27 BV711, CCR7 BV785, CD3

Spark Blue 550, TIM3 PE, CD127 PE/Dazzle 594, CD25 PE-Cy5, TCRγδ PE/Fire 700, CD95 PE-Cy7, FoxP3 Alexa Fluor 647, HLA-DR APC/Fire 810, and Viability ViaDye Red were obtained from BioLegend (San Diego, CA); CD45RA cFlour V450, CD8 cFlour V547, CD4 cFlour V610, CD38 cFlour R720, from Cytek (Fremont, CA); Vδ1-TCR VioBright 515 and Vδ2-TCR PerCP-Vio 700, from Miltenyi Biotec (Bergisch Gladbach, Germany).

Spectral signatures of the selected fluorochromes were visualized and analyzed using the Cytek Spectrum Viewer (Figure 8). The Similarity Index and the Complexity Index of the selected fluorochromes were calculated according to the Similarity™ & Complexity™ Indices from the Cytek Biosciences data set (Figure 9).

Table 2. Markers analyzed in Cytek® Aurora

Marker	Fluorochrome	Ab Clone	Purpose
PD-1	BV421	NAT105	Co-inhibitory molecule
CD45RA	cFluor V450	HI100	Subset Differentiation
CD8	cFluor V547	SK1	T-cell Lineage
CD4	cFluor V610	SK3	T-cell Lineage
CD28	BV650	CD28.2	Subset Differentiation
CD27	BV711	M-T271	Subset Differentiation
CCR7	BV785	G043H7	Subset Differentiation
Vδ1-TCR	VioBright 515	REA173	T-cell Lineage
CD3	Spark Blue 550	SK7	T-cell Lineage
Vδ2-TCR	PerCP-Vio 700	REA771	T-cell Lineage
TIM3	PE	F38-2E2	Co-inhibitory molecule
CD127	PE/Dazzle 594	A019D5	Subset Differentiation
CD25	PE-Cy5	BC96	Subset Differentiation
TCRγδ	PE/Fire 700	B1	T-cell Lineage
CD95	PE-Cy7	DX2	Subset Differentiation
FoxP3	Alexa Fluor 647	259D	Subset Differentiation
CD38	cFlour R720	HIT2	Activation
Viability	ViaDye Red	-	Viability
HLA-DR	APC/Fire 810	L243	Activation

Abbreviatures: Ab, mouse anti-human antibodies monoclonal

Panel® Design

	Violet			Blue			Yellow/Green			Red		
Emission	405			488			561			640		
	Marker	Rec. Dil.	Fluor	Marker	Rec. Dil.	Fluor	Marker	Rec. Dil.	Fluor	Marker	Rec. Dil.	Fluor
395												
420	PD-1		BV421									
440												
450	CD45RA		cFluor V450									
480												
500				V51-TCR		VioBright 515						
520												
550	CD8		cFluor V547	CD3		Spark Blue 550						
570							TIM-3		PE			
580												
600	CD4		cFluor v610				CD127		PE/Dazzle 594			
660	CD28		BV650									
680							CD25		PE-Cy5	FoxP3		Alexa Fluor 647
690							TCR gd		PE/Fire 700			
700	CD27		BV711	V52-TCR		PerCP-Vio 700						
730										CD38		cFluor R720
750										VD		ViaDye Red
780	CCR7		BV785				CD95		PE-Cy7			
800										HLA-DR		APC/Fire 810

Figure 7. Antibodies used and their corresponding wavelength

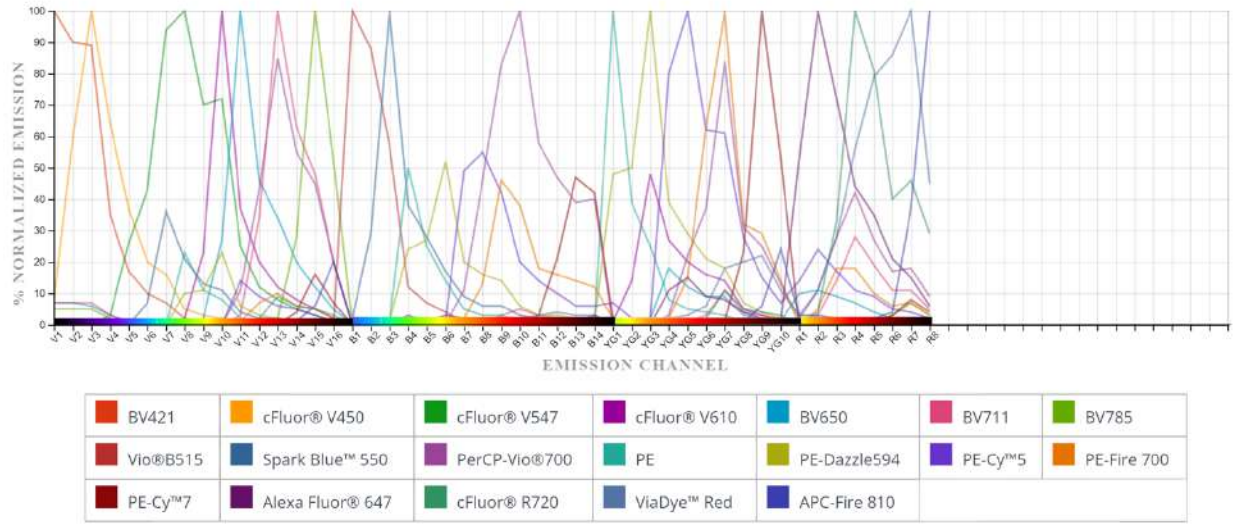


Figure 8. Spectro viewer used in our panel. Source: Cytek

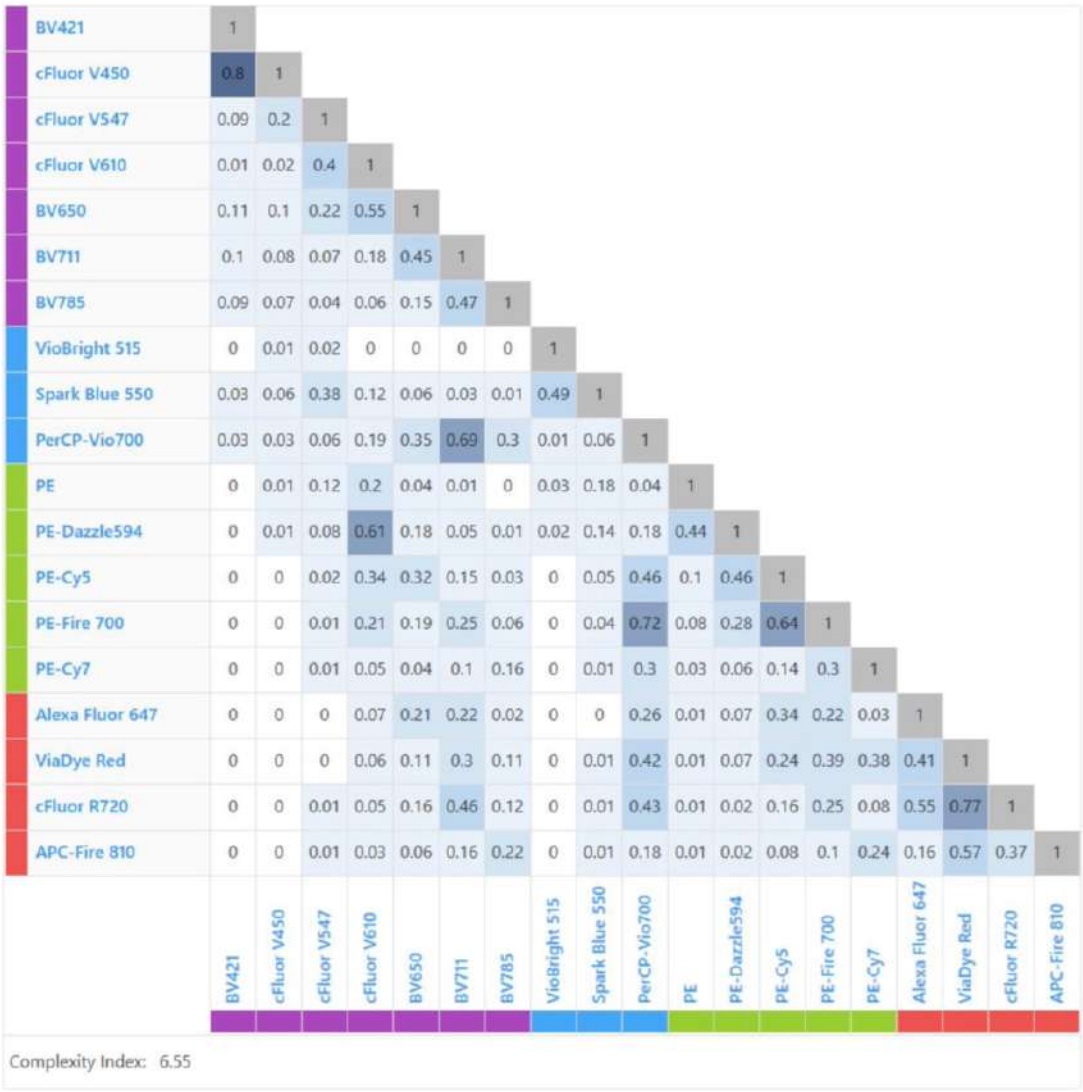


Figure 9. Similarity index of panel

4.3.2 Titration of antibodies

All used antibodies were titrated to determine the optimal concentration to remove the staining artefacts and to obtain the most favorable resolution of markers. Antibodies titration started at 25%, 50% and 100% of the concentration recommended by manufacturer's instructions. Cytometry tubes of 100,000 PBMCs were incubated with different concentration of antibodies diluted in staining buffer (eBioscience™, USA) to a final volume of 100µl for 30 min at 4°C

in the dark. Titration were performed under the same conditions as the full stain panel. Stain index was calculated for all used antibodies to define the optimal titres.

Stain Index (SI) equation = (MdnFI*pos* – MdnFI*neg*) / (2 × SD*neg*)

4.3.3 Single staining

Single staining with one specific antibody was performed using 300,000 PBMCs. One tube with 500,000 PBMCs was used for non-staining control. Cryopreserved cells were recovered quickly in a 37°C water bath and resuspended with complete RPMI (Sigma, USA) with 10% FBS. All single staining tubes and non-staining tube were treated and fixed under the same condition of full staining panel. Cells were incubated with an optimal concentration of antibodies titrated in the previous step; the incubation was for 30 min at 4°C in the dark. Intracellular staining was performed after permeabilization and fixation of cells. Fluorescence Minus One (FMO) Controls were applied on doubtful antibodies. Ultra CompBeads (BD Biosciences, USA) were also tested for single staining. The final decision was made by comparing the brightness achieved using single staining cells versus beads to obtain an optimal single-color control used for unmixing panel.

4.3.4 Immunofluorescence staining

Purified PBMCs were thawed at 37°C, washed fresh complete RPMI medium, and resuspended with the master mix of antibodies for all surface markers in Brilliant Stain buffer (BD Bioscience, USA) to a final volume of 100 µl. Then, cells were incubated at 4°C in the dark for 30 min. After two washes with 1mL Cell Staining Buffer (eBioscience™, USA), cells were fixed and permeabilized for 40 min using the FoxP3 / Transcription Factor Staining Buffer Set (eBioscience™, USA) according to the manufacturer´s instructions. Later, cells were washed

twice with 1mL of 1: 10 Permeabilization Buffer (eBioscience™, USA) in PBS (Gibco, USA). Intracellular staining was done with cells resuspended in 1:10 Permeabilization Buffer with FoxP3 antibody added to a final volume of 100 µl and incubated for 30min at 4°C in the dark. After two washes with 1mL 1: 10 Permeabilization Buffer, cells were resuspended in Cell Staining Buffer to a final concentration of 1,000,000 cells/mL and stored at 4°C protected from light for at least 15min before acquisition.

4.3.5 Fluorescence compensation and spectral unmixing

Spectral unmixing is used to identify the fluorescence signal for each fluorophore used in a given experiment.

PBMCs used as single-color controls were analyzed in the SpectroFlo instrument software (Cytek, Version 2.1.1) to detect at least 5% of cells with positive signal. Spectral unmixing was calculated using these results.

4.3.6 Manual gating

Initially, the PBMCs were identified based on the FSC and SSC parameters. Live cells were then selected using Viability ViaDye Red. T cells were selected by CD3+ marker and analyzed to gate T helper (CD4+) subset; cytotoxic T cells (CD8+) subset and unconventional γδ T cells (TCRγδ+). CD4+ T cells were analyzed to gate Regulatory T cells (Tregs) as CD25+CD127low (or CD25+FoxP3+CD127low). Then Tregs were further classified into Treg naive (CD45RA+FoxP3^{low}); FoxP3+ non-treg cells (CD45RA-FoxP3^{low}) and Treg effector (CD45RA-FoxP3^{high}). γδ T cells were analyzed to gate Vδ1 T cells (Vδ1+) and Vδ2 T cells (Vδ2+), CD127 and CD27 were used to identify γδ naïve T cells (CD27+CD127+). CCR7 and CD45RA marker were used to gate T-cell subsets (γδT cells, CD4+ and CD8+) into naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-)

and terminally differentiated effector memory (CD45RA+CCR7-). T-cell activation status was analyzed by estimating CD38, HLA-DR, CD25 and CD127 expression. PD-1 and TIM3 were used to evaluate T-cell exhaustion.

4.4 Data analysis

Flow cytometry data were processed using FlowJo v10.10.0 (FlowJo, LLC, Ashland, USA) and SpectroFlo® (Cytek Biosciences, Fremont, USA). Statistical analyses were conducted in GraphPad Prism v10 (GraphPad, Boston, USA). Correlations were assessed using Spearman's rank correlation test with two-tailed post-tests. For paired data analysis, paired t-tests and Wilcoxon matched-pairs signed rank tests were employed, while unpaired t-tests and Mann–Whitney U-tests were used for unpaired data. A p-value of <0.05 was considered statistically significant; otherwise, results were classified as not significant (ns).

RESULTS

5 Results

5.1 Clinical characteristics of the VIHNAI Cohort.

This is an observational study with prospectively collected clinical and demographic data and blood samples from 14 HIV-1-infected individuals (HIV+), 10 PrEP individuals (PrEPs), and 11 healthy controls (HC group). HIV+ patients were follow-up during 48 weeks on ART.

Clinical and demographic characteristics of the participants are outlined in Table 3. There were no significant differences in age and BMI among studied groups. HIV+ patients showed an elevated prevalence of smoking while PrEP individuals presented higher drug use compared to HC group. Moreover, the proportion of EBV and CMV co-infections is higher among HIV-infected individuals and PrEP users, respectively, compared to the HC group. No differences showed in Toxoplasma gondii and Treponema pallidum co-infection between the three groups.

Exploring baseline data of immunological and inflammation parameters (Table 4), HIV+ patients achieved significant improvements in CD4 count, CD8 count, CD4/CD8 ratio, and D-dimer levels after 48 weeks on ART. However, despite ART, significant differences in CD8 count and CD4/CD8 ratio between HIV+ patients and HC group group persisted after 48 weeks. The CRP levels in the HIV group did not improve after 48 weeks on ART and remained elevated compared to HC group. Interestingly, PrEP users exhibited significantly higher CD8 counts, IL-6 levels and fibrinogen levels compared to the healthy control group. Moreover, the fibrinogen levels in PrEP group even surpassed those of HIV+ patients before treated with ART.

Table 3. Baseline characteristics of the healthy controls (HC), PrEP users and HIV infected individuals (HIV+). Main demographic, medical, and HIV infection characteristics of the three groups are included.

	HC N=11	**PrEP N=10**	**HIV+ N=14**	***p*-Value**
Age (years)	32 (30-43)	40.50 (31–49.5)	38 (35.5–46.25)	NS
BMI (kg/m2)	25.93±4.89	24.72±2.76	23.93±1.72	NS
Smoking (*n*, %)	1 (9.09%)	2 (20%)	7 (50%)	**0.042**[a]
Alcohol (*n*, %)	2 (18.18%)	6 (60%)	5 (35.71%)	NS
Cocaine (*n*, %)	0	1 (10%)	4 (28.57%)	NS
THC (*n*, %)	2 (18.18%)	4 (40%)	1 (7.14%)	NS
Designer drugs (*n*, %)	0	6 (60%)	3 (21.43%)	**0.004**[b]
CMV co-infection (*n*, %)	6 (54.55%)	10 (100%)	13 (92.86%)	**0.035**[b]
EBV co-infection (*n*, %)	7 (63.64%)	8 (88.89%)	13 (100%)	**0.031**[a]
Toxoplasma gondii (n, %)	2 (18.18%)	3 (30%)	4 (28.57%)	NS
Treponema pallidum (*n*, %)	0	1 (10%)	2 (14.29%)	NS
Nadir CD4 count (per mL)	-	-	367.5 (250.3–635)	-
Baseline viral load (per mL)	-	-	149753 (41889–371632)	-
Ever met AIDS criteria (*n*, %)	-	-	1 (7.14%)	-

Abbreviatures: BMI, Body Mass Index; THC, Tetrahydrocannabinol; NS, non-significant. Quantitative variables are shown as median (interquartile range). *p*-value was obtained from ordinary one-way ANOVA statistical analysis for normally distributed data and Kruskal-Wallis nonparametric test for nonparametric data. Outliers identified by graphpad were treated as missing. Categorical variables were analyzed by Fisher´s exact test.

[a]: Statistically different between HC and HIV+ individuals.

[b]: Statistically different between HC and PrEP users.

Table 4. Immunological and inflammation parameters in routinely laboratory analysis of healthy controls (HC), PrEP users and HIV+ group on baseline (W0) and 48 weeks after ART (W48).

	HC	**PrEP**	**HIV W0**	**HIV W48**	***p-value***				
	N=11	**N=10**	**N=14**	**N=14**					
					HC vs PrEP	HC vs HIV W0	HC vs HIV W48	PrEP vs HIV W0	HIV W0 vs HIV W48
CD4 count/ml	807.4±145.7	974±217	537.2±292.9	828.4±364.9	NS	**0.01**	NS	**0.0006**	**<0.0001**
CD8 count/ml	487.3±186.8	756.6±296.7	1221±449.9	728.4±328.5	**0.026**	**<0.0001**	**0.0002**	**0.01**	**0.038**
CD4/CD8 ratio	1.74±0.75	1.30±0.49	0.43±0.26	0.93±0.47	NS	**<0.0001**	**0.0032**	**<0.0001**	**<0.0001**
IL-6	<1.5	1.675 (1.49-2.00)	2.21 (1.49-2.71)	2.08 (1.50-3.54)	**0.026**	**0.015**	**0.0067**	NS	NS
IL-5	<3	<3	<3	<3	NS	NS	NS	NS	NS
CRP	0.07 (0.04-0.21)	0.08 (0.05-0.27)	0.17 (0.10-0.53)	0.27 (0.10-0.42)	NS	**0.009**	**0.0032**	NS	NS
D-Dimer	304.5 (269-630)	293.5 (239-449.8)	390 (264.5-466.5)	269 (211-360)	NS	NS	**0.028**	NS	**0.027**
Fibrinogen	338.5±45.85	401.7±72.48	331.6±74.71	355.1±75.63	**0.032**	NS	NS	**0.032**	NS

Abbreviatures: CRP, C-Reactive Protein. Quantitative variables are shown as median (interquartile range). *p*-value was obtained from Mann-Whitney test comparing nonparametric data, T-test was performed for comparing parametric data. Paired analysis (paired T-test for parametric data, Wilcoxon test for nonparametric data) conducted to compare baseline HIV+ (HIV W0) vs 48 weeks on ART (HIV W48). Outliers identified by graphpad were treated as missing. NS represents no statistical significance.

5.2 Identification and distribution of T cell subsets

T-cell subsets were identified from PBMCs as cells expressing CD3, CD4, CD8, TCRγδ markers (Figure 10). CD45RA and CCR7 markers were used to identify, respectively, memory subsets of CD4+ and CD8+ T cells. Within γδ T cells, Vδ1 and Vδ2 were used to identify Vδ1+ and Vδ2+ subpopulations.

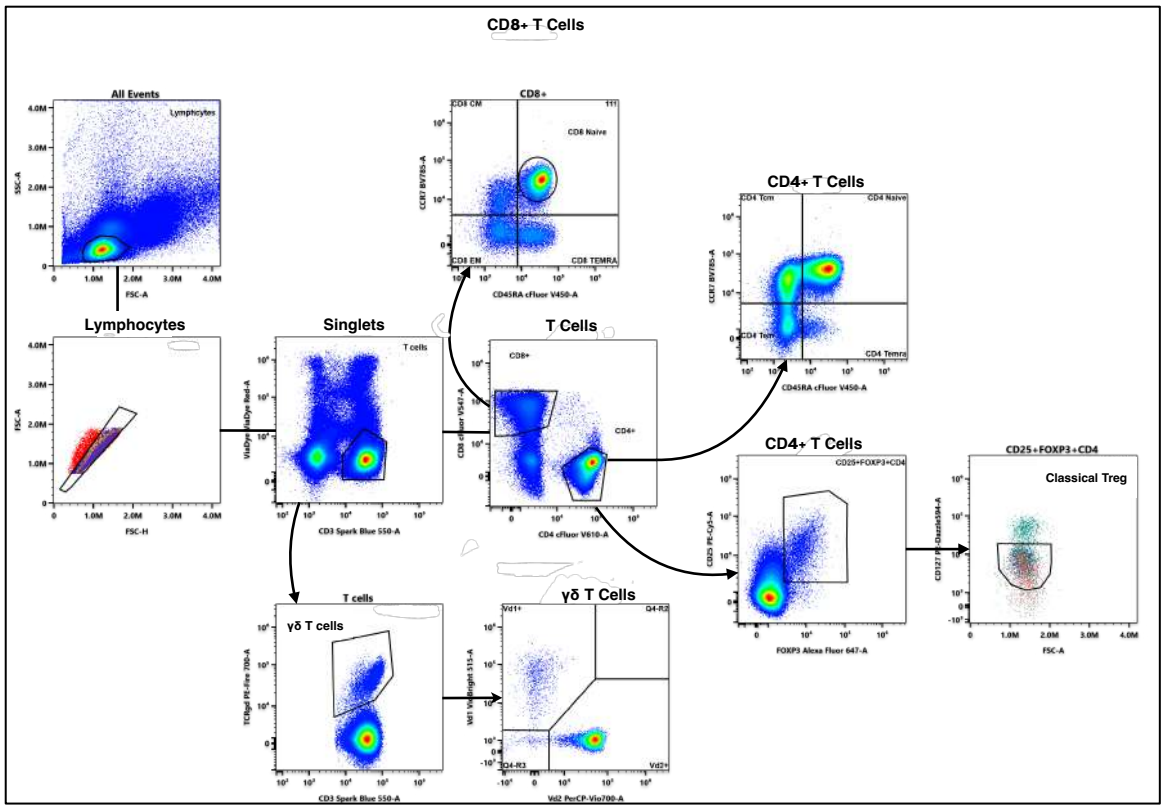


Figure 10. Flow cytometry gating strategy used to define cell populations from the PBMCs. T cells (CD3+), CD8+ T cells (CD3+CD8+), CD4+ T cells (CD3+CD4+), γδ T cells (CD3+TCRγδ+), Vδ1 T cells (CD3+TCRγδ+Vδ1+), Vδ2 T cells (CD3+TCRγδ+Vδ2+); CCR7 and CD45RA marker were used to gate T-cell subsets (CD4+ and CD8+) into naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-) and terminally differentiated effector memory (CD45RA+CCR7-).

Heatmap results show a different distribution of the studied T cell subsets among groups at baseline (Figure 11.a) with a clear alteration of all studied cell populations in HIV+ individuals compared to control and PrEP individuals (Figure 11.a, b). Partially cell restoration was observed in HIV+ patients after 48 weeks on ART compared to baseline.

5.2.1 CD+4 T cell analysis

As is expected, all CD4+ memory populations were decreased at W0. HIV+ HIV+ at baseline presented significant lower percentage of CD4+ T cells than healthy controls (33.47±9.80% vs 56.40±7.84%, $p<0.0001$) and individuals on PrEP (56.26±7.58%, $p<0.0001$) (Figure 12). After ART, total CD4+ T cells were largely restored (50.73±11.1%, $p<0.01$), reaching to the level observed in HC group.

Exploring CD4+ memory T cells in detail, no significant recovery was observed in any of the CD4+ memory subsets in response to ART. No important changes were observed in CD4+ naïve cells and CD4+ Tem cells.

Significant depletion of CD4+ Tcm cells in HIV+ and PrEP was observed at baseline compared to HC. After 48 weeks of ART, CD4+ Tcm cells remined lower proportion compared to HC (Figure 12). Regarding the CD4+ Temra cells, Significant differences only showed in comparison between PrEP users and HIV+ individuals both at baseline and W48.

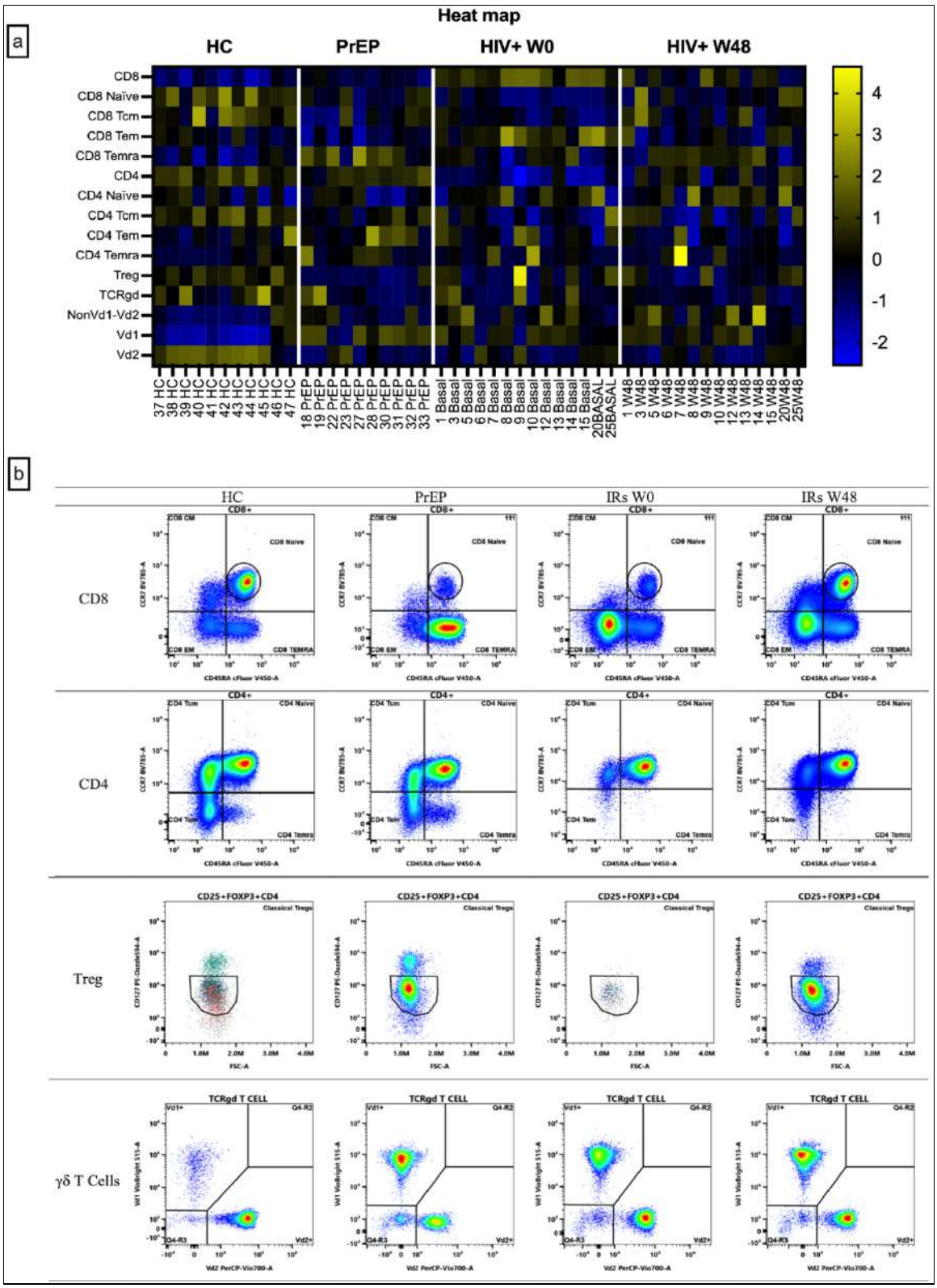


Figure.11: Overall description and distribution of T cell subsets. Heat map showing transformed frequencies of T-cell subpopulations across healthy controls (HC, n=11), PrEP users (n=10), HIV+ individuals before ART (HIV+ W0, n=14), and after 48 weeks of ART (HIV+ W48, n=14). Data were normalized per cluster (rows) with a mean of zero and standard deviation of one. Blue indicates under-representation, while yellow indicates over-representation. b) Representative flow cytometry plots of T-cell subpopulations by group, obtained through manual gating.

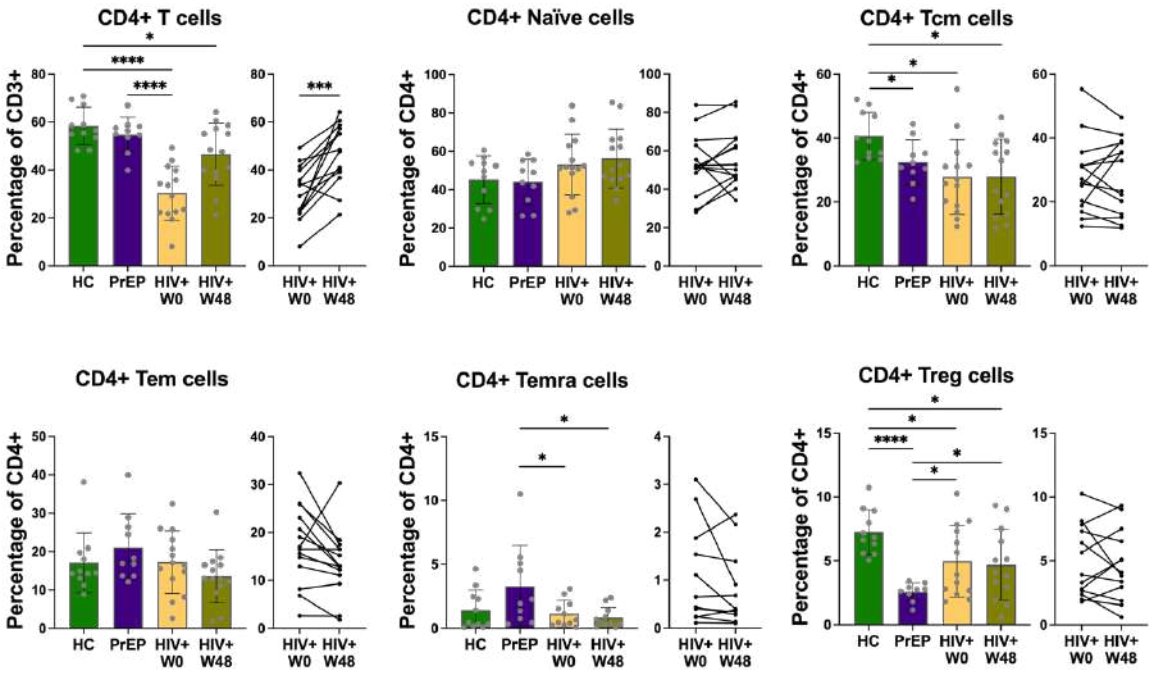


Figure 12. CD4+ T cell subpopulation analysis. Percentages of CD4+ T cell subpopulations in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+W48; n=14), obtained by manual gating. Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test statistical analysis was performed in normally distributed data and Mann-Whitney nonparametric test was performed for nonparametric data. Data represent individual values (connected dots) before (week 0) and after 48 weeks on ART. Paired t-test statistical analysis was performed in normally distributed data and Wilcoxon matched-pairs signed rank test was performed for nonparametric data; Outliers identified by GraphPad were treated as missing; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$; if not indicated, *p* value is not significant.

The proportion of regulatory T cells (Treg) in HIV+ at baseline was lower than HC group and. No significant improvement was observed in response to 48 weeks of ART (Figure 12). Therefore, significant depletion of Treg cells in HIV+ persisted after 48 weeks on ART compared to HC. PrEP exhibited lowest proportion of Treg among groups, which was even lower than those levels observed in HIV+ both at W0 and W48.

5.2.2 CD8+T cell analysis

Next, CD8+ T cell subset was explored in detail comparing among HIV + patients (HIV+) at baseline (W0), after 48 weeks on ART (W48), PrEP and HC group.

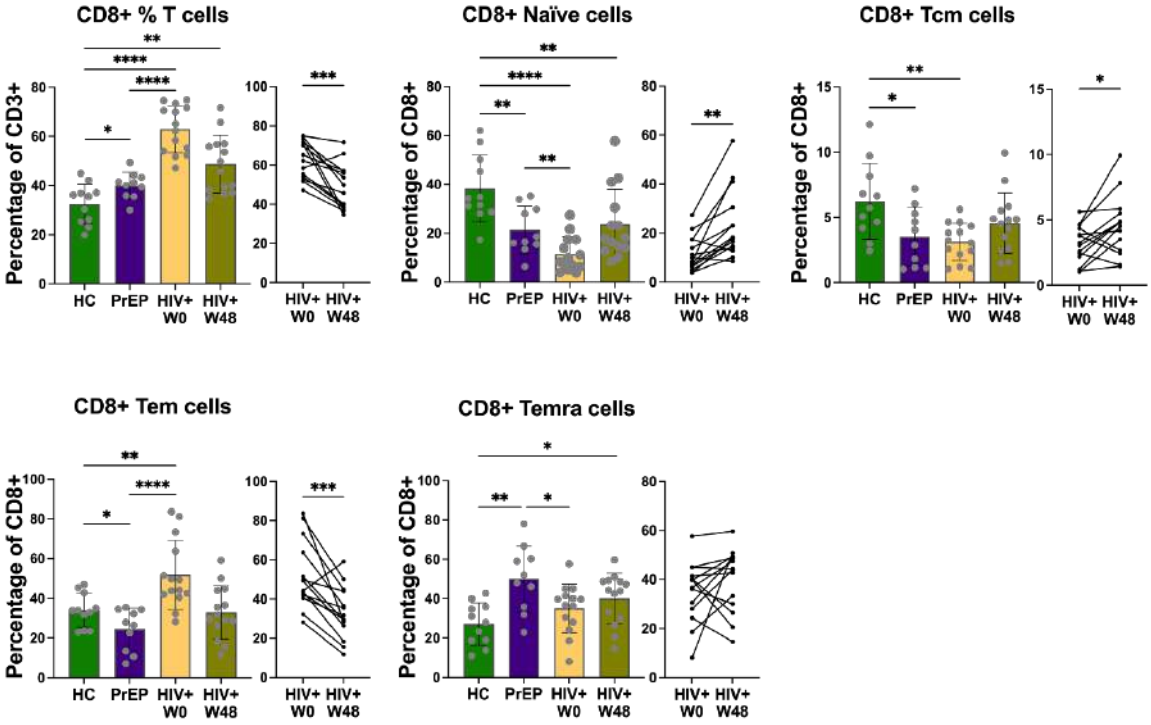


Figure 13. CD8+ T cell subpopulation analysis. Percentages of CD8+ T cell subpopulations in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+W48; n=14), obtained by manual gating. Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test statistical analysis was performed in normally distributed data and Mann-Whitney nonparametric test was performed for nonparametric data. Data represent individual values (connected dots) before (week 0) and after 48 weeks on ART. Paired t-test statistical analysis was performed in normally distributed data and Wilcoxon matched-pairs signed rank test was performed for nonparametric data; Outliers identified by GraphPad were treated as missing; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$; if not indicated, *p* value is not significant.

The percentages of total CD8+ T cells significantly increased in HIV+ compared to HC and PrEP group (Figure 13). After 48 weeks on ART, a significant decrease of CD8+ percentages was detected, however, failed to reach values similar to those of HC

($p<0.05$). CD8 Naïve cells declined significantly in HIV+ and PrEP group compared to HC. CD8 Naïve cells were successfully restored by 48 weeks of ART but failed to achieve comparable values of HC. In the case of Tcm cells, similar tendencies of alteration were observed among groups.

Similar significant changes were observed in CD8+ Tem cells in HIV+ at baseline and PrEP users. After 48 weeks on ART, the proportion of CD8+ Tem cells was recovered and reached values observed in HC.

Finally, CD8 Temra cells in HIV+ were comparable to levels of HC at baseline, then significantly higher than HC after 48 weeks on ART.

Interestingly, significant differences were observed between HC and PrEP groups in total CD8+ T-cell population and all its subpopulations. Especially, CD8 Temra cells from PrEP users were twice the level of those in HC group.

5.2.3 γδ T cell analysis

Important impacts were observed in γδ TCR+ T cell population, which depleted in HIV+ at baseline and further declined at w48 compared to HC despite statistical significance only was observed between HIV+ at W48 and HC group.

Altered balance between peripheral Vδ1+ and Vδ2+ cells were showed, particularly the Vδ2+ subset. Vδ1+ cells remarkably increased in PrEP, HIV+ at both baseline and w48, while significant depletion of Vδ2+ cells was observed among PrEP, HIV+ at both baseline and w48 compared to HC (Figure 14). γδ T cell that either expressed Vδ1 nor Vδ2 were observed higher in PrEP, HIV+ at both baseline and w48 compared to HC.

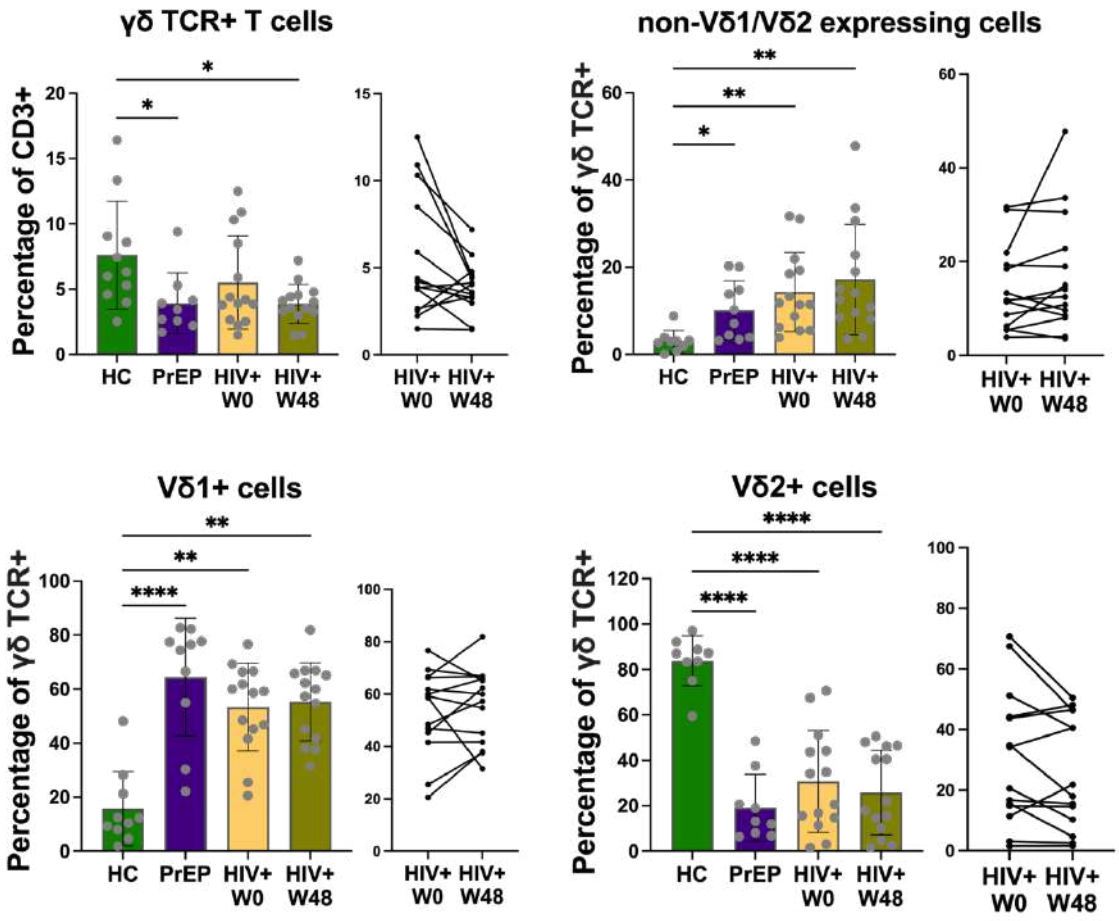


Figure 14. γδ T cell subpopulation analysis. Percentages of γδ T cell subpopulations in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+W48; n=14), obtained by manual gating. Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test statistical analysis was performed in normally distributed data and Mann-Whitney nonparametric test was performed for nonparametric data. Data represent individual values (connected dots) before (week 0) and after 48 weeks on ART. Paired t-test statistical analysis was performed in normally distributed data and Wilcoxon matched-pairs signed rank test was performed for nonparametric data; Outliers identified by GraphPad were treated as missing; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$; if not indicated, *p* value is not significant.

5.3 T cell homeostasis monitoring by analysis of Naïve/Memory T cell ratio

To better understand the homeostatic alteration of T cells in HIV+ individuals, T cell Naïve/Memory ratios from PBMCs were analyzed. As expected, both Naïve/Memory ratios for CD8+ and CD4+ were importantly impacted by HIV infection (Figure 15).

The Naïve/Memory CD8 T cell ratio was significantly reduced in HIV+ at baseline compared to HC group. This ratio was not restored (0.44) after 48 weeks on ART.

Of note, the Naïve/Memory ratio for CD8+ cells observed in PrEPs (0.3) was intermediate between HIV+ at baseline (0.1) and HC group (0.6). After ART, naïve/memory CD8 T cell ratios were comparable in HIV+ and PrEPs.

Unlike the CD8+ T cells, the Naïve/Memory CD4 T cell ratio was similar in HIV+ individuals compared to HC and PrEP. No significant differences showed despite this ratio was increased after 48 weeks of ART (Figure 15).

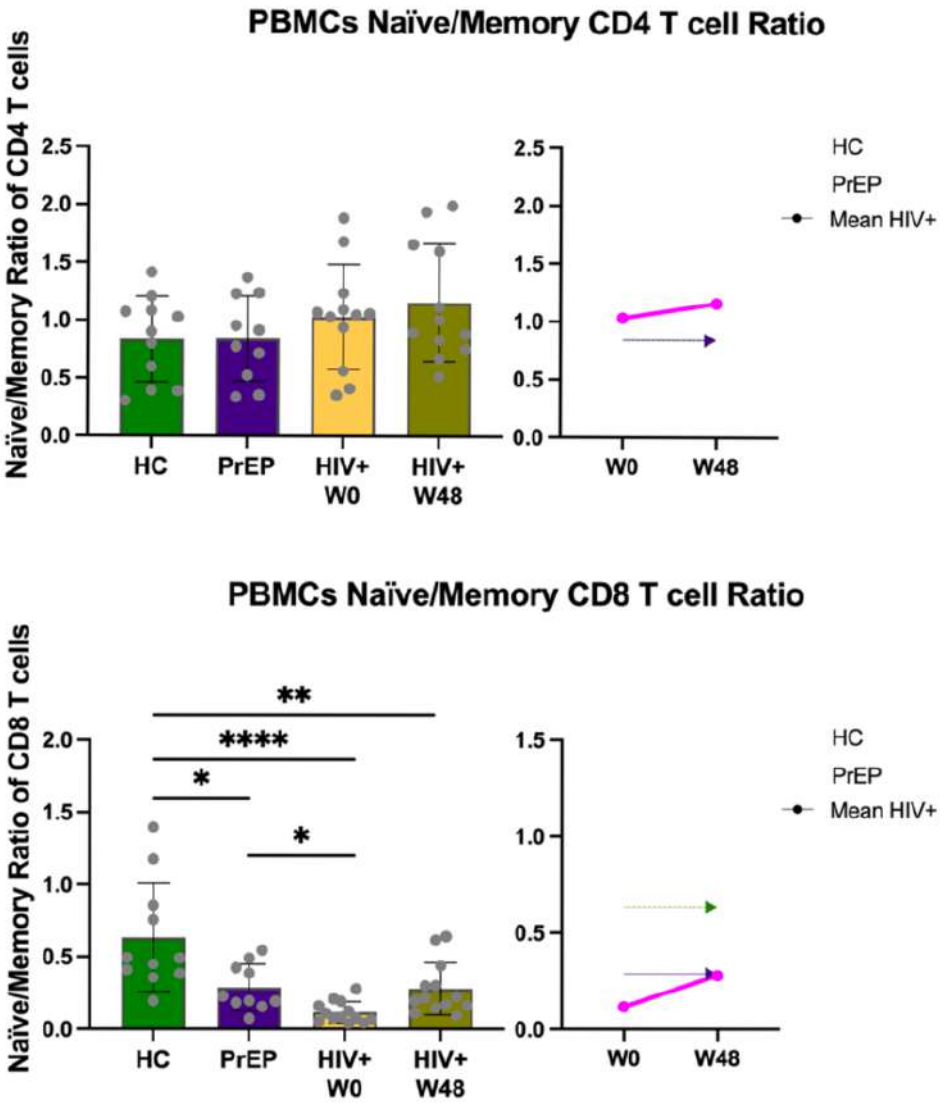


Figure 15. Naïve/Memory ratio for CD8+ and CD4+ T cells in PBMCs and changes in HIV+. Naïve/Memory ratio for CD8+ and CD4+ T subpopulations in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+W48; n=14), obtained by manual gating. Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test statistical analysis was performed in normally distributed data and Mann-Whitney nonparametric test was performed for nonparametric data. Data represent individual values (connected dots) before (week 0) and after 48 weeks on ART. Paired t-test statistical analysis was performed in normally distributed data and Wilcoxon matched-pairs signed rank test was performed for nonparametric data; Outliers identified by GraphPad were treated as missing; $^{*}P<0.05$, $^{**}P<0.01$ and $^{****}P<0.0001$; if not indicated, *p* value is not significant.

5.4 Impact of One-Year ART on T Cell Activation: Comparisons Across Healthy Controls, PrEP Users, and HIV+ patients

5.4.1 CD38 and HLADR expression on CD4+ cell

Regarding CD4+ cell activation, similar to CD8+ cell activation, remarkably immune activation was observed in HIV+ group compared to HC and PrEP, but no important differences were observed in HIV+ before and after ART (Figure 16a and 16c). Activated CD4+ T cells were predominantly CD38+, this subpopulation was significantly increased in HIV+ individuals both at baseline and at W48 compared to HC and PrEP group. CD4+HLADR+ presented a similar trend like CD4+CD38+ T cells, despite significant difference was only detected between HC and HIV+ at W48 (Figure 16. b).

The proportion of CD4+HLADR+CD38+ T-cell subset in HIV+ individuals at W0 was 7.9 fold higher than HC group (6.21±0.53% vs 0.78±0.13%, $p<0.001$) (Figure 16). Despite significant reduction after 48 weeks on ART, this population remained significantly higher than HC (2.95% (IQR: 1.34-5.96) vs 0.76% (IQR: 0.72- 0.90), $p<0.01$).. Similar results were observed in comparison between HIV+ and PrEP group.

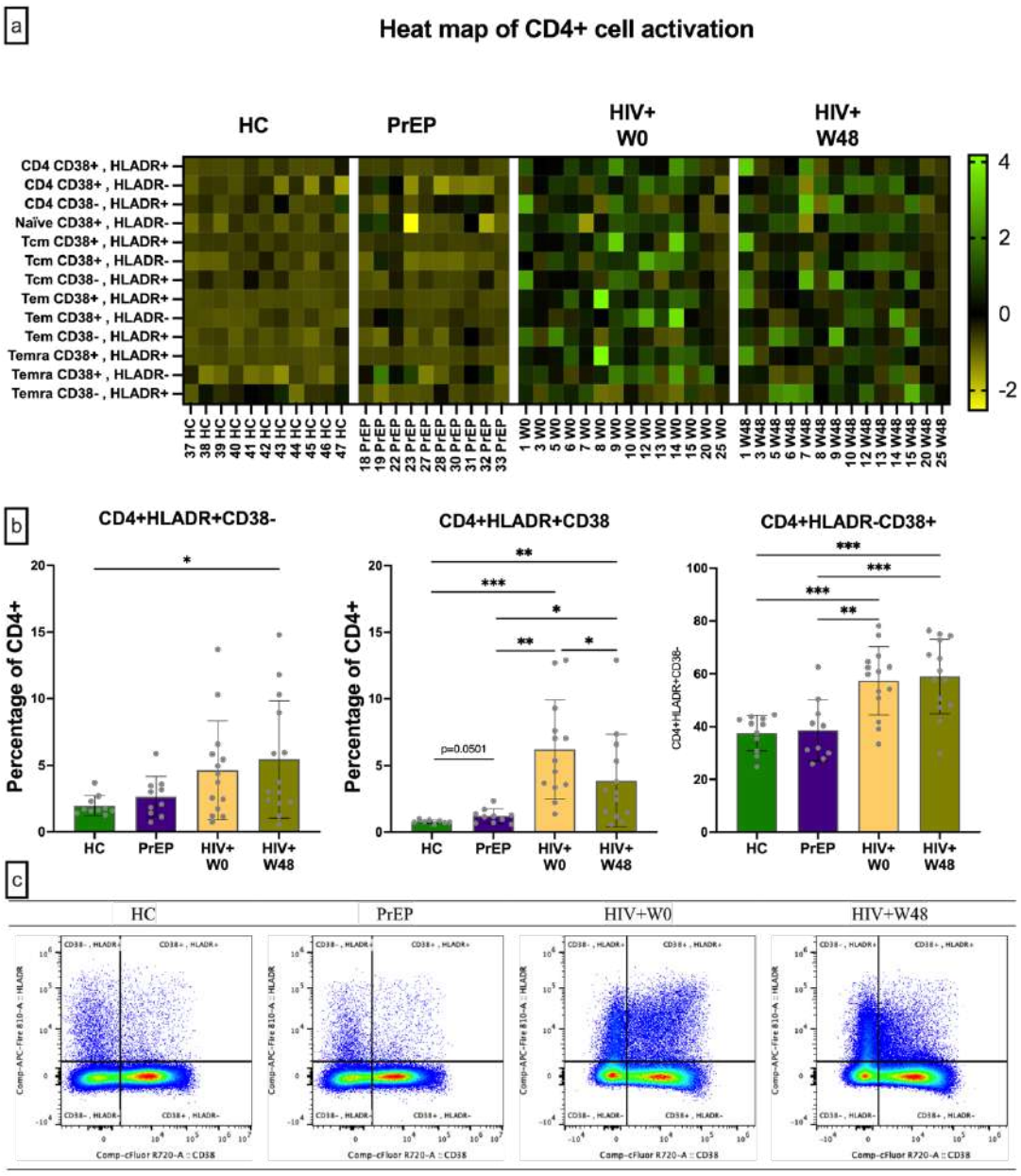


Figure 16: Activation marker comparisons across healthy controls, PrEP Users, and HIV+ in total CD4+ T cells. a) Heat map of activation markers in CD4+ T-cell subpopulations across healthy controls (HC, n=11), PrEP users (n=10), HIV+ before ART (HIV+ W0, n=14), and after 48 weeks of ART (HIV+ W48, n=14). Data were normalized per cluster to a mean of zero and standard deviation of one. Yellow indicates under-representation, and green indicates over-representation. b) Percentages of CD38 and HLA-DR expression on total CD4+ T cells. Data represent individual values (dots) with mean ± SEM. Statistical tests include Paired t-test/Wilcoxon signed rank test for dependent data and T-test/Mann-Whitney for independent data. Significance levels: ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001. Outliers identified by GraphPad were excluded. c) Representative flow cytometry plots obtained by manual gating.

5.4.2 CD38 and HLADR expression on CD8 + cells

To evaluate the activation levels of the CD8+ subpopulation, the expression of CD38, HLA-DR, and the co-expression of both (CD38+HLA-DR+) were measured in HIV+ individuals at both baseline (W0) and 48 weeks (W48) on ART and were compared to healthy controls and PrEP users (Figure 17).

Heatmap shows an altered activation profile of CD8+ cells in HIV+ individuals both at baseline and after 48 weeks on ART (Figure 17a). In this case, no evident differences were detected between PrEPs and HC group.

The CD8+HDLAR+ subpopulations tended to increase in HIV+ individuals despite ART, with higher increases in HIV+ at W48 (Figure 17b). Similarly, the CD8+CD38+ subpopulations in HIV+ was significantly elevated at W0 and further increased at W48 compared to HC.

In contrast, the proportions of CD8+CD38+HLA-DR+ T cells were significantly increased in HIV+ individuals at baseline while 48 weeks of ART significantly reduced this proportion. Despite the reduction, CD8+CD38+HLA-DR+ T cells persisted significantly higher in HIV+ at W48 than HC and PrEP group.

Interestingly, significant higher level of CD8+ T cells were CD38+HLA-DR+ in PrEP group than the level observed in HC. Meanwhile, CD8+ T cells expressing either HLA-DR or CD38 were comparable between PrEP and HIV+ individuals.

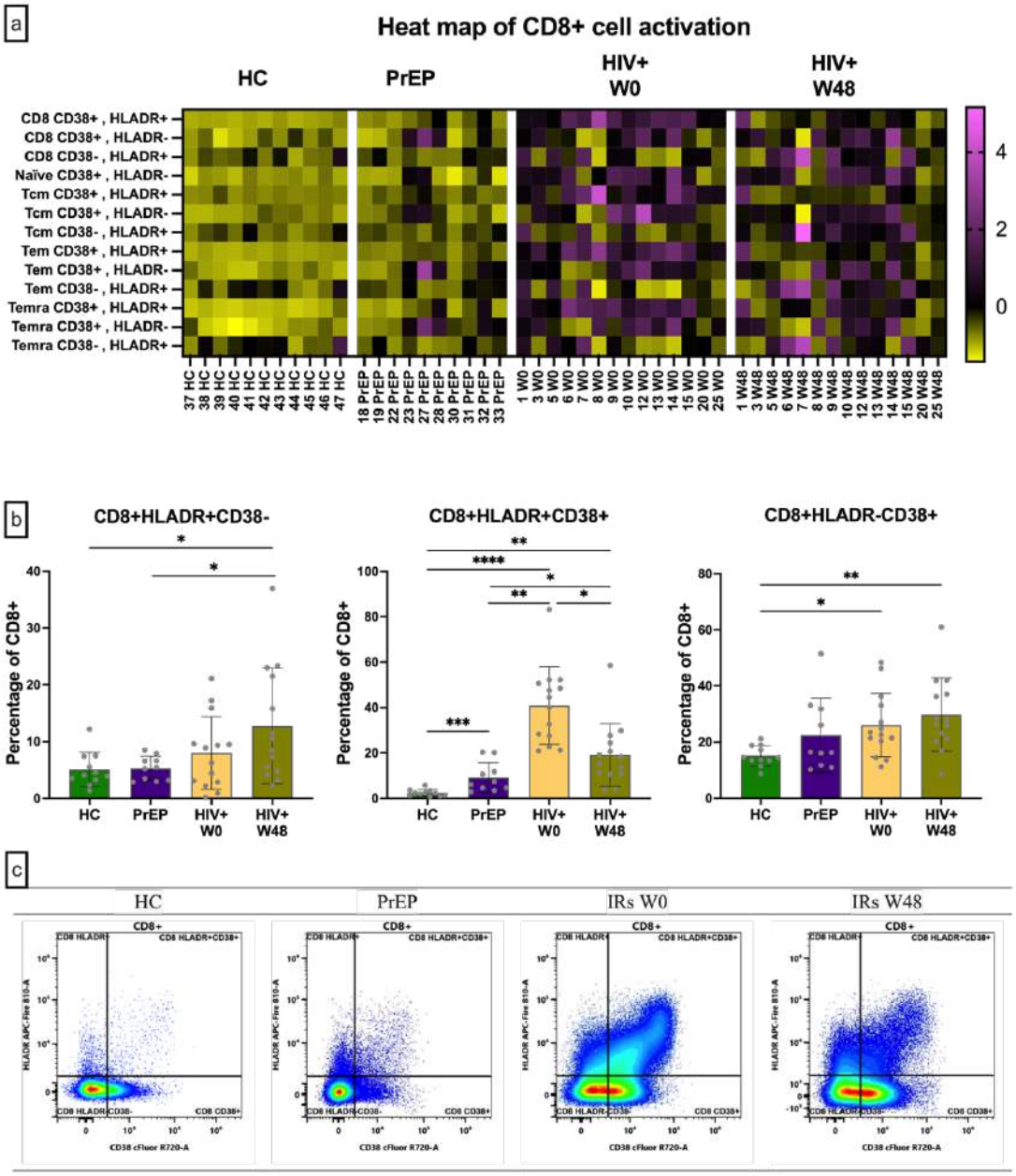


Figure 17. Activation marker comparisons across healthy controls, PrEP Users, and HIV+ in total CD8+ cells. a) Heat map of activation markers in CD8+ T-cell subpopulations across healthy controls (HC, n=11), PrEP users (n=10), HIV+ individuals before ART (HIV+ W0, n=14), and after 48 weeks of ART (HIV+ W48, n=14). Data were normalized per cluster to a mean of zero and standard deviation of one. Yellow indicates under-representation, and pink indicates over-representation. b) Percentages of CD38 and HLA-DR expression on total CD8+ T cells. Data are represented as individual values (dots) with mean ± SEM. Statistical tests include Paired t-test/Wilcoxon signed rank test for dependent data and T-test/Mann-Whitney for independent data. Significance levels: $*P<0.05$, $**P<0.01$, $***P<0.001$, and $****P<0.0001$. Outliers identified by GraphPad were excluded. c) Representative flow cytometry plots obtained by manual gating.

5.5 Impact of One-Year ART on T Cell Activation: Comparisons Across Healthy Controls, PrEP Users, and HIV+ patients

The percentages of PD-1 and TIM-3 expression were assessed to evaluate the exhaustion levels among CD8+ and CD4+ T-cell lineage.

5.5.1 Exhaustion marker expression on total CD4+ cells

Remarkably, CD4+TIM-3+ T cells were significantly lower in HIV+ individuals at baseline (W0) compared to HC group (0.42±0.24% vs. 1.05±0.42%, $p<0.001$) and PrEP users (1.34±1.08%, $p<0.01$) (Figure 18). After 48 weeks of ART, CD4+TIM-3+ T cells significantly increased but remained significantly lower than those in HC group and PrEP users.

The percentages of TIM-3 and PD-1 co-expressing CD4+ T cells in HIV+ individuals were comparable to those in HC group and PrEP users at baseline. However, after a significant reduction at 48 weeks of ART, these levels were significantly lower than those observed in HC group and PrEP users.

PD-1-expressing CD4+ T cells were higher across groups at W0, though the differences were not statistically significant.

All three subsets mentioned above were elevated in PrEP users compared to HC group, but the differences were not statistically significant.

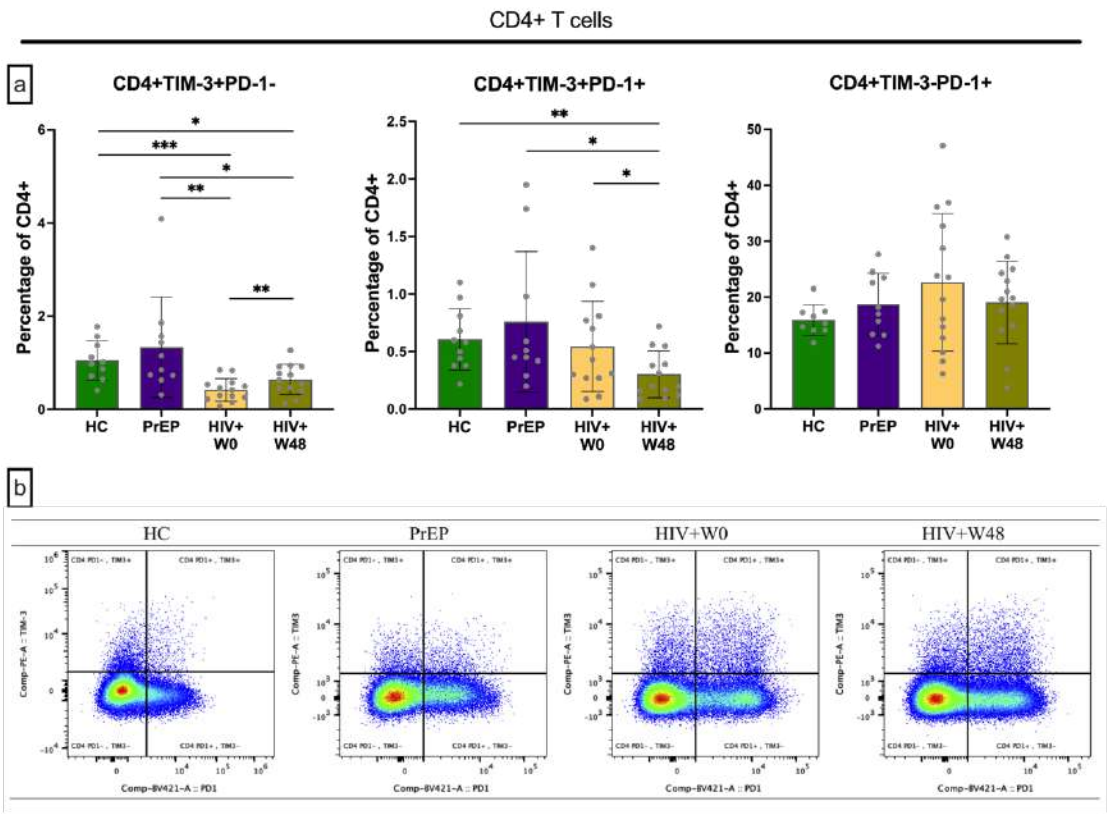


Figure 18. a) Exhaustion marker comparisons across healthy controls, PrEP Users, and HIV-infected Individuals on CD4+ cells. Percentages of expression of TIM-3 and PD-1 on CD4+ cell-subpopulation in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEPs; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+ W48; n=14), obtained by manual gating. Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). According to the normality of data Paired t-test/ T-test were performed for dependent data; Wilcoxon matched-paired signed rank test /Mann Whiteney were performed for independent data. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$; if not indicated, p value is not significant. Outliers identified by GraphPad were treated as missing. b) Representative flow plots of TIM-3 and PD-1 on CD4+ cell-subpopulation by group.

5.5.2 Exhaustion marker expression on total CD8+ cells

Significantly elevated proportion of CD8+TIM-3+PD-1+ and CD8+ PD-1+ were observed in all HIV+ individuals compared to HC group at baseline. After 48 weeks on ART, the proportions of CD8+TIM-3+PD-1+ and CD8+PD-1 cells were reduced significantly. No significant differences observed among groups while CD8+TIM-3+

T cells significantly increased after 48 weeks of ART. PrEPs showed comparable expression of all these markers to the HC group (Figure 19).

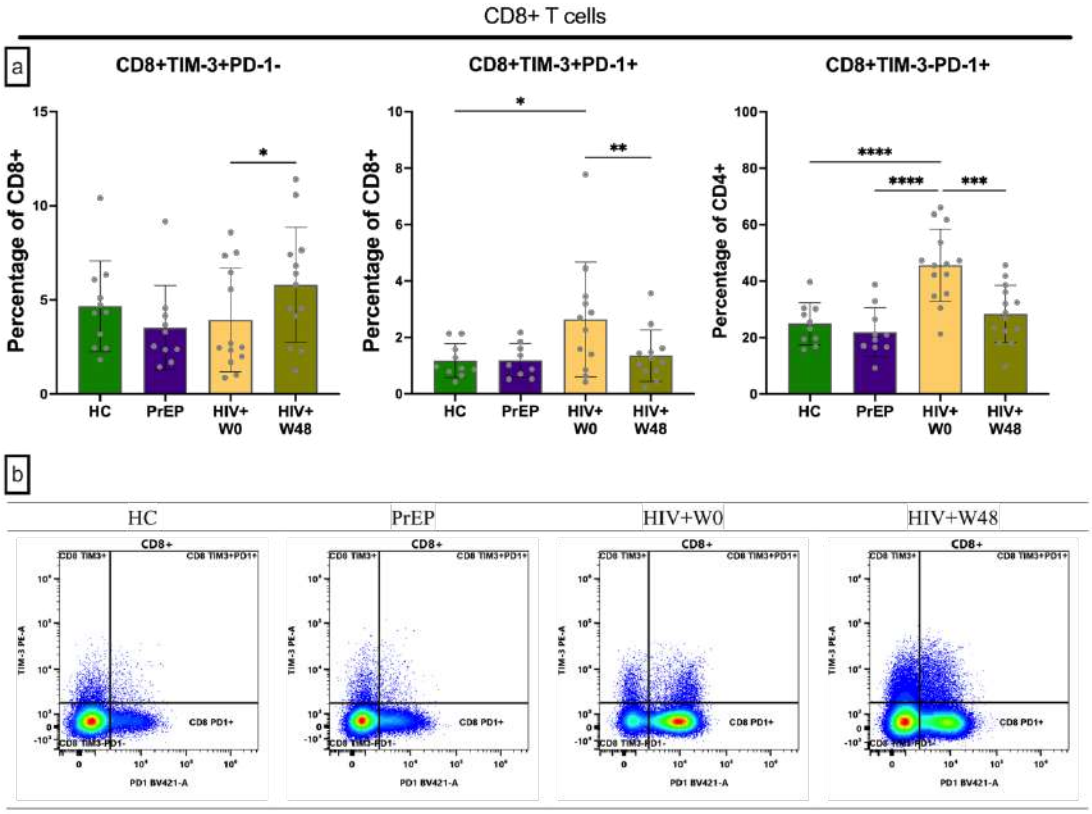


Figure 19. a) Exhaustion marker comparisons across healthy controls, PrEP Users, and HIV-infected Individuals on CD8+ cells. Percentages of expression of TIM-3 and PD-1 on CD8+ cell-subpopulation in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEPs; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+ W48; n=14), obtained by manual gating. Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). According to the normality of data Paired t-test/ T-test were performed for dependent data; Wilcoxon matched-paired signed rank test /Mann Whiteney were performed for independent data. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$; if not indicated, p value is not significant. Outliers identified by GraphPad were treated as missing. b) Representative flow plots of TIM-3 and PD-1 on CD8+ cell-subpopulation by group.

5.6 Immunophenotyping γδ T cells in PrEP, HIV+ W0 and HIV+ W48 individuals

To explore immunophenotypic characteristics of γδ T cells in PrEP group and in HIV+ individuals before and after ART, manual gating of γδ T cells and its subpopulation were obtained from Flowjo®.

5.6.1 Immune activation of γδ T cells in PrEP, HIV+ W0 and HIV+ W48 individuals

Selected γδ T cells were evaluated by measuring CD27 and CD45RA to determine the differentiation and homeostasis status.

The representative flow plots (Figure 20) show higher levels of CD45RA+ expressed γδ T cells (more terminally differentiation) both in PrEPs and HIV+ individuals at baseline and after ART compared to HC which this subpopulation is the minority. Peripheral γδ T cells (blue plots) predominantly expressed Vδ1+ (red plots) in PrEP and HIV+ individuals, whereas Vδ2+ (orange plot) was the main subset observed in HC group (Figure 20).

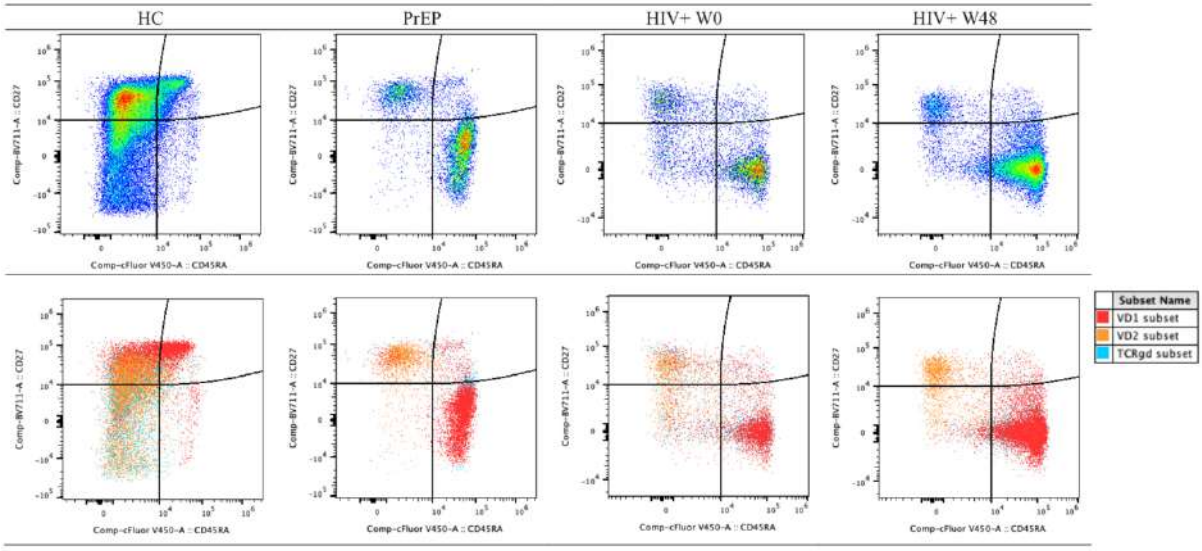


Figure 20. Representative flow plots of memory subset studied in γδ TCR+ T cells across healthy controls, PrEP Users, and HIV-infected Individuals.

Naïve γδ T cells were defined as CD27+CD45RA+, showed no significant differences among groups (Figure 21). Meanwhile, significant decrease of Tcm (labelled as TCRγδ +CD27+) T cells was observed in PrEPs and HIV+ at both W0 and W48 compared to HC group ($p<0.01$). As expected, γδ Tem (CD27-CD45RA-) cells were elevated in HIV+ individuals at baseline, induced by HIV, and declined after 48 weeks on ART, although their levels remained higher than those observed in HC and PrEP individuals ($p<0.05$). Interestingly, γδ Temra (CD27-CD45RA+) cells were significantly increased in PrEP group and HIV+ at both W0 and W48 compared to HC ($p<0.01$).

The homeostatic changes in Vδ1 cells in HIV+ and PrEP individuals closely mirrored the trends observed in γδ TCR cells (Figure 22). Significant loss of resting Vδ1+ T cells (CD27+CD45RA+/-) was observed in HIV+ individuals at both baseline and 48 weeks of ART. Meanwhile, effector memory phenotypic Vδ1+ cells (CD27-CD45RA+/-) were increased in HIV+ individuals at both baseline and 48 weeks of ART. Here, ART failed to restore homeostatic alteration in Vδ1+ cells except Tem Vδ1+ cells. Interestingly, a similar alteration in Vδ1+ cells was observed in PrEP individuals compared to HC group, despite the absence of HIV.

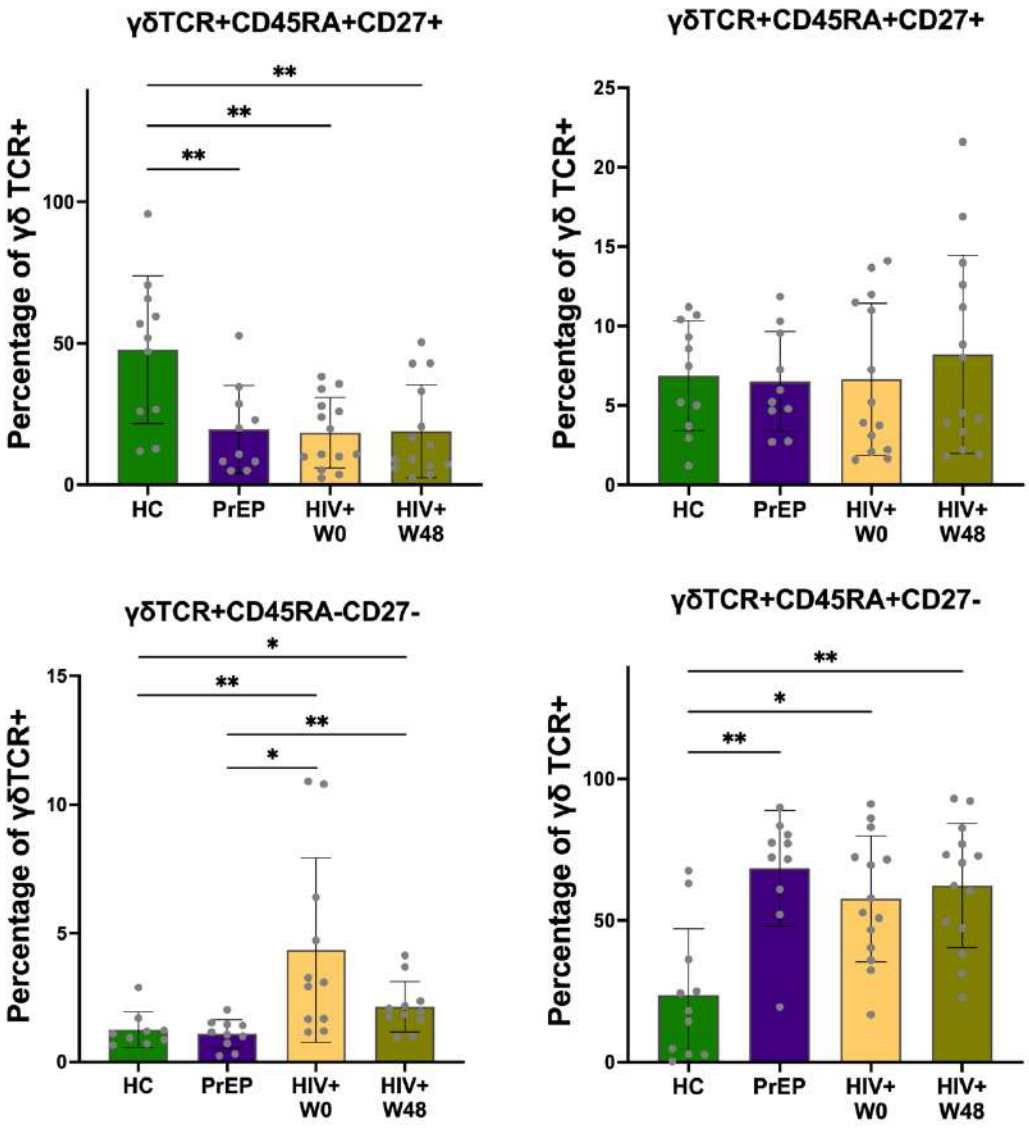


Figure 21. Comparison of memory subset studied in γδ TCR+ T cells across healthy controls, PrEP Users, and HIV-infected Individuals. Percentages of expression of CD45RA and CD27 on γδ TCR+ T cell-subpopulation in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+ W48; n=14), obtained by manual gating. a) Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test and paired T-test statistical analysis was performed in normally distributed data and Mann-Whitney test and Wilcoxon matched-paired signed rank test was performed for nonparametric data; ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; if not indicated, p value is not significant. Outliers identified by GraphPad were treated as missing.

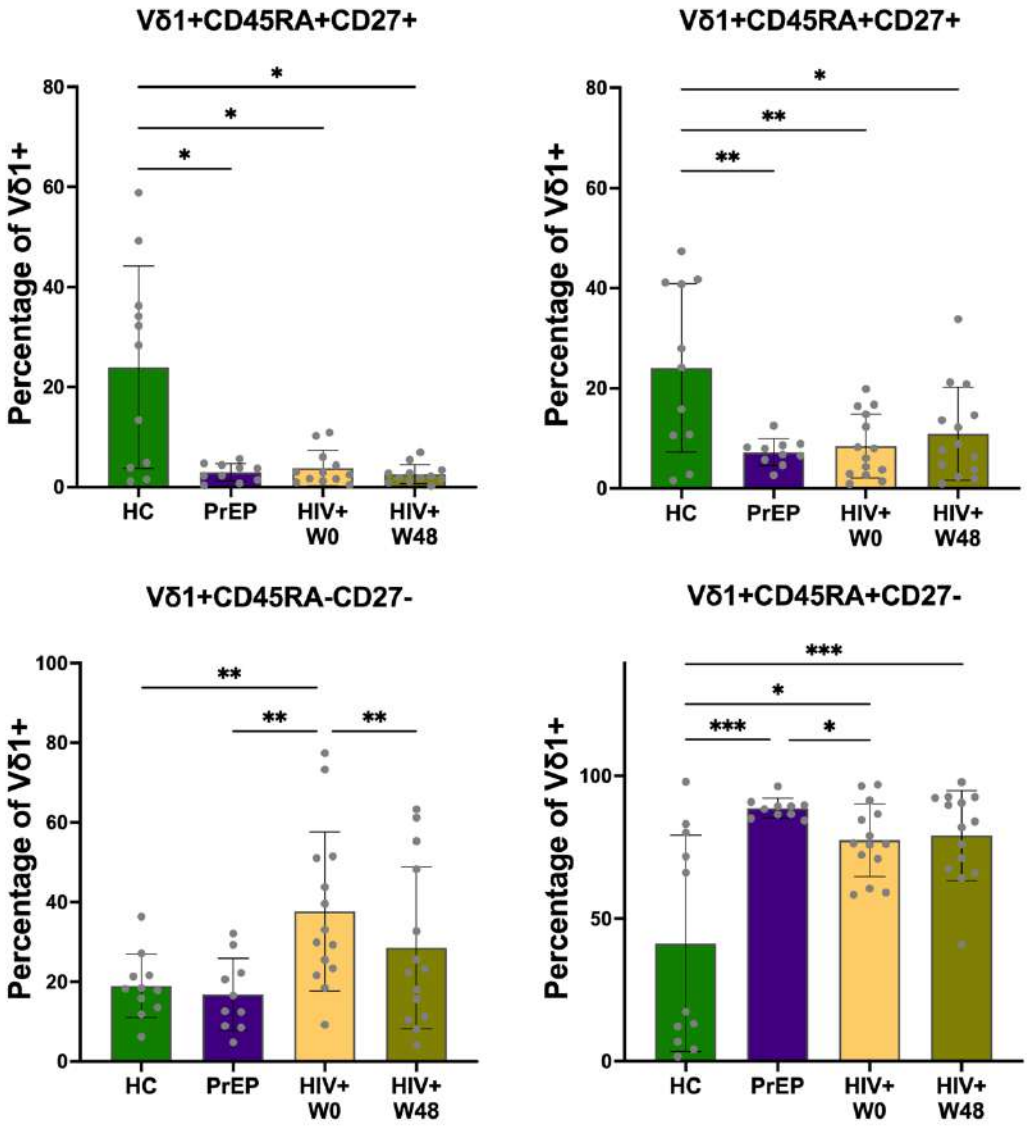


Figure 22. Comparison of memory subset studied in Vδ1+ T cells across healthy controls, PrEP Users, and HIV-infected Individuals. Percentages of expression of CD45RA and CD27 on Vδ1+ T cell-subpopulation in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+ W48; n=14), obtained by manual gating. a) Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test and paired T-test statistical analysis was performed in normally distributed data and Mann-Whitney test and Wilcoxon matched-paired signed rank test was performed for nonparametric data; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$; if not indicated, p value is not significant. Outliers identified by GraphPad were treated as missing.

In contrast to Vδ1+ cells, differentiation and homeostasis in Vδ2+ cells were less affected by HIV (Figure 23), despite the significant reduction of this population after the infection (Figure 11.a and 14). Even though no differences were found between HIV+ and HC individuals, ART seems to affect Tcm, Tem and Temra cells compared to HIV+ naïve cells. Moreover, PREP individuals showed the most altered levels of all subpopulations with a behavior similar to the ART effect.

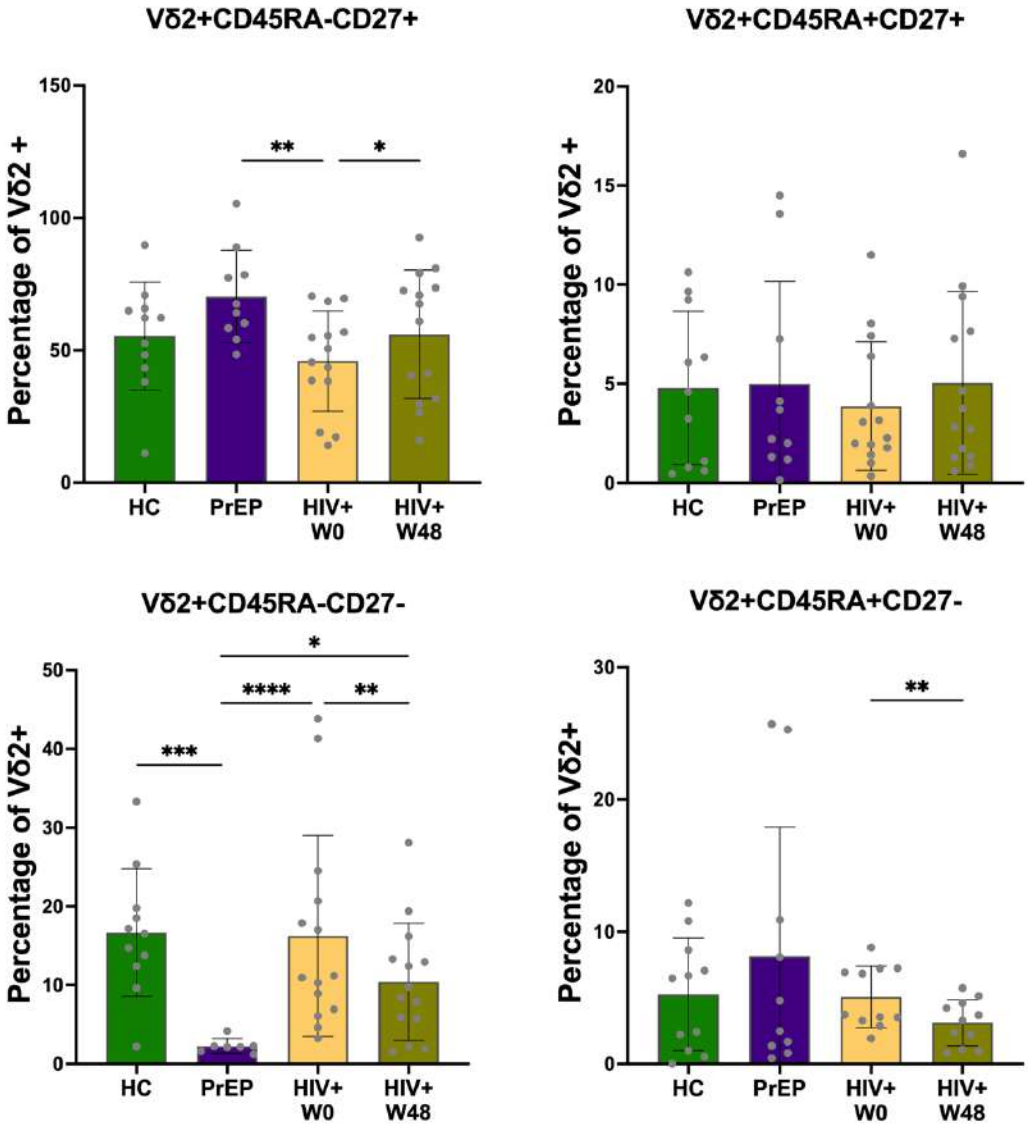


Figure 23. Comparison of memory subset studied in Vδ2+ T cells across healthy controls, PrEP Users, and HIV-infected Individuals. Percentages of CD45RA and CD27 expression on Vδ2+ T cells across healthy controls (HC, n=11), PrEP users (n=10), HIV+ before ART (HIV+ W0, n=14), and after 48 weeks of ART (HIV+ W48, n=14), obtained by manual gating. Data are shown as individual values (dots) with mean ± SEM. Statistical tests include T-test, paired T-test, Mann-Whitney, and Wilcoxon signed rank test. Significance levels: ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, ∗∗∗∗P<0.0001; non-significant values are not indicated. Outliers were excluded.

5.6.2 Immune activation of γδ T cells in PrEP, HIV+ W0 and HIV+ W48 individuals

Activated γδ T cells were defined by HLADR+CD38-, HLADR-CD38+, and HLADR+CD38+in HIV+ individuals (Figure 24). Regarding the total γδ T cells (γδ TCR+), HIV+ and PrEP individuals showed higher levels compared to HC group individuals. Moreover, ART treatment is not able to reduce this activation correlating with a chronic inflammation described in these individuals (Table 5). Similar pattern of activation in Vδ1+ γδ T cells were detected.

Regarding Vδ2+ γδ T cells, higher activation levels were detected in HIV+ individuals compared to HC and similar to Vδ1+ γδ T cells, ART does not totally restore the activation levels. Alternatively, Vδ2+CD38+ cells were significantly lower in PrEP group compared to HC group.

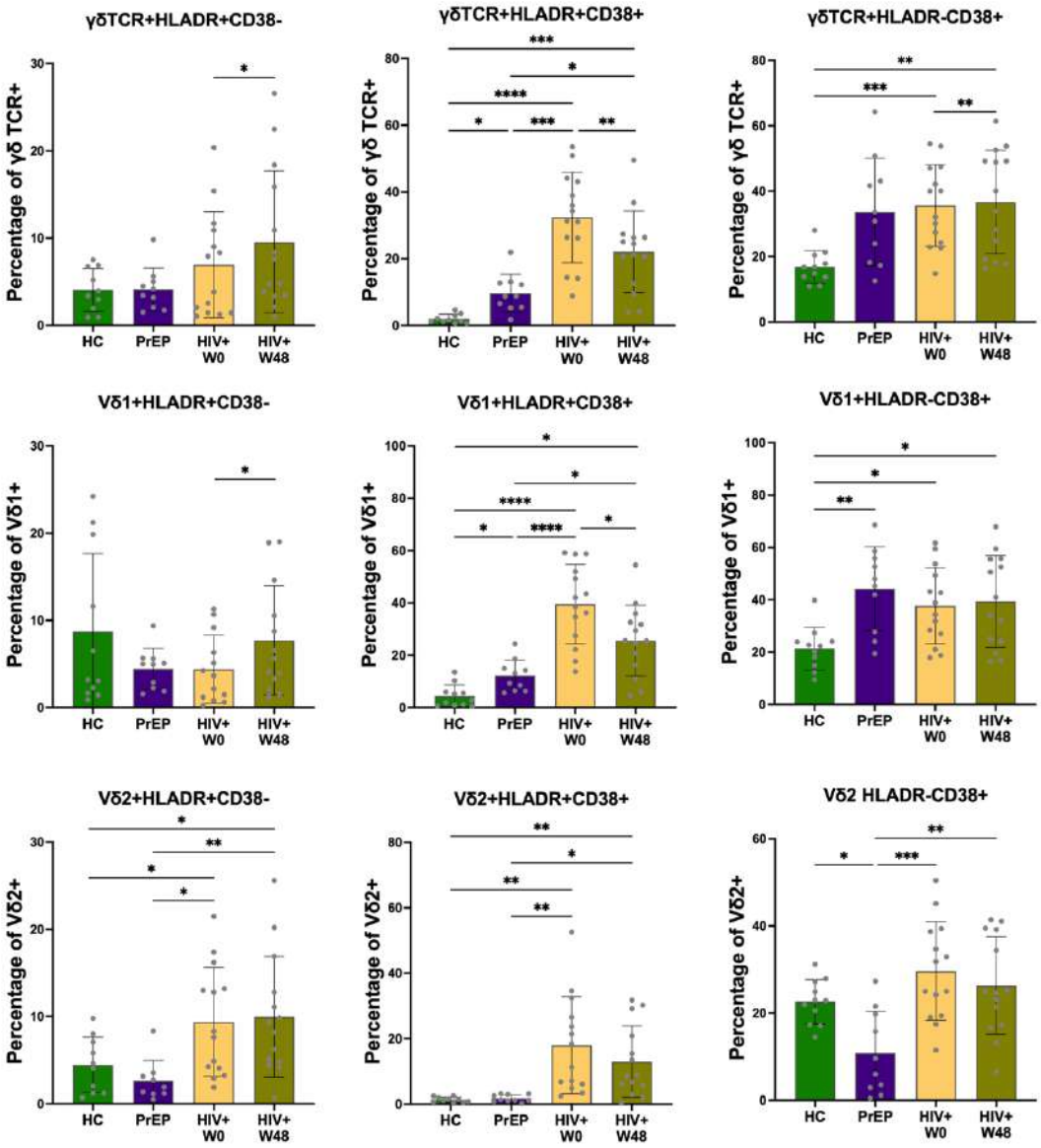


Figure 24. Comparison of activation markers in γδ T cells across healthy controls, PrEP Users, and HIV-infected Individuals. Percentages of expression of HLADR and CD38 on γδ T cell-subpopulation in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+ W48; n=14), obtained by manual gating. a) Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test and paired T-test statistical analysis was performed in normally distributed data and Mann-Whitney test and Wilcoxon matched-paired signed rank test was performed for nonparametric data; ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; if not indicated, p value is not significant. Outliers identified by GraphPad were treated as missing.

5.6.3 Exhaustion of γδ T cells in PrEP, HIV+ W0 and HIV+ W48 individuals

To explore γδ T cells exhaustion in HIV+ individuals both before and after ART, as well as in PrEP users, the expression levels of TIM-3 and PD-1 were evaluated (Figure 25).

Similar to activation levels in γδ T cells, higher exhausted cells were detected in the total γδ T cells mainly due to the higher exhaustion levels in Vδ1+ γδ T cells in both HIV+ and PrEP individuals compared to HC.

Regarding Vδ2+ γδ T cells, PrEP and HIV+ also showed higher levels of exhausted cells compared to HC but in this case, exhaustion levels in Vδ2+ cells were significantly increased after ART.

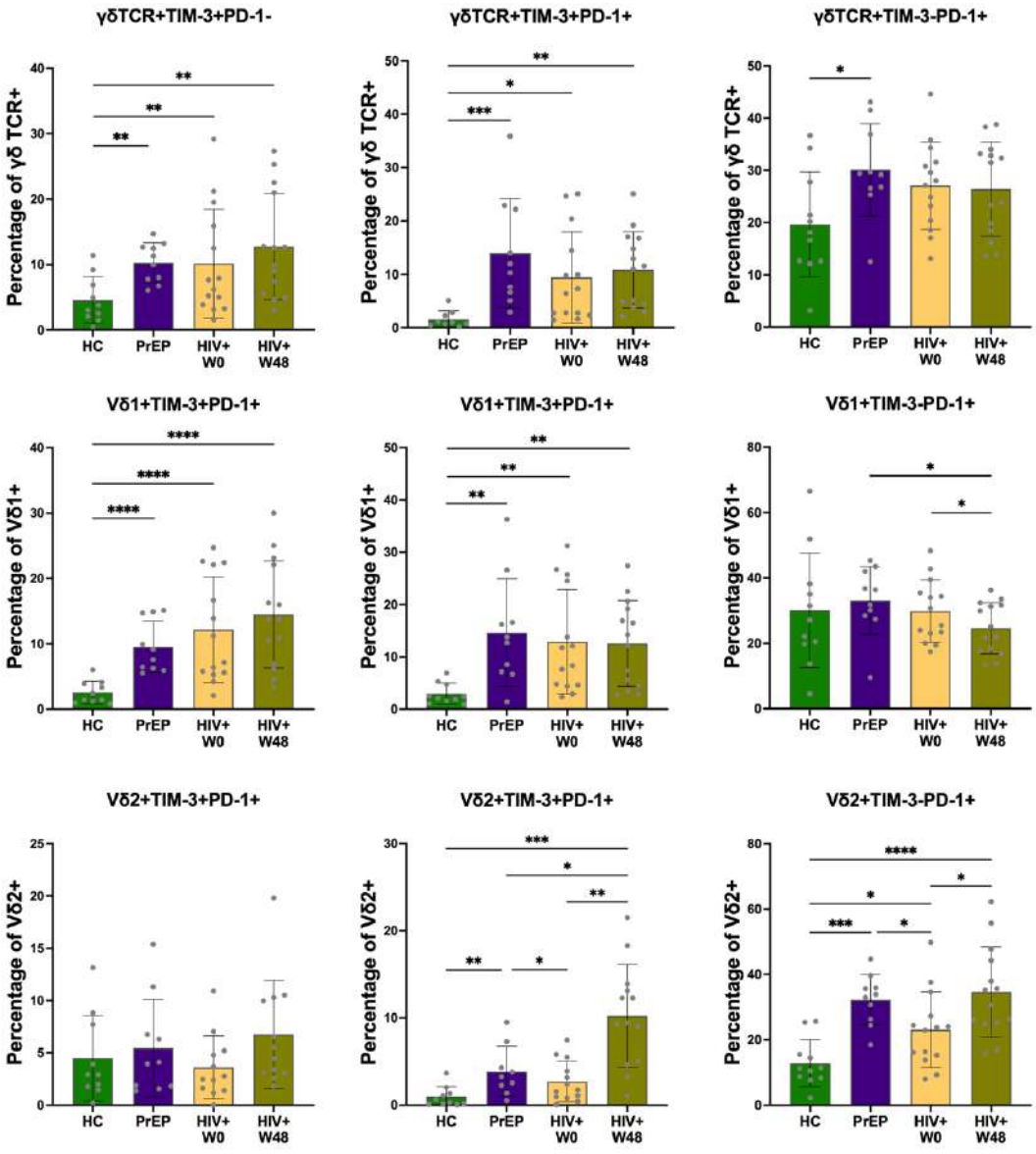


Figure 25. Comparison of exhaustion markers in γδ T cells across healthy controls, PrEP Users, and HIV-infected Individuals. Percentages of expression of TIM-3 and PD-1 on γδ T cell-subpopulation in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+ W48; n=14), obtained by manual gating. a) Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test and paired T-test statistical analysis was performed in normally distributed data and Mann-Whitney test and Wilcoxon matched-paired signed rank test was performed for nonparametric data; ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; if not indicated, p value is not significant. Outliers identified by GraphPad were treated as missing.

5.6.4 Differentiation, activation and exhaustion characteristics in non-Vδ1/Vδ2 γδ T-cell subpopulation

Unexpectedly, significant elevations were observed in a subpopulation that was neither Vδ1+ nor Vδ2+ (γδTCR+Vδ1-Vδ2-) in PrEP and HIV+ individuals compared to HC (Figure 14), its activation and exhaustion characteristics were further explored in this section.

In γδTCR+Vδ1-Vδ2- cells, predominantly high portion of terminally differentiated cells were observed in PrEP and HIV+ individuals (Figure 26).

The characteristics of expressing activation markers in non-Vδ1/Vδ2 γδ T-cell subpopulation were highly homogenous with the total γδ T cells and Vδ1+ T cells (Figure 24 and Figure 27). However, no elevation of Tem subset in this population was observed in HIV+ individuals.

Similarly, the exhaustion marker expression in non-Vδ1/Vδ2 γδ T-cell subpopulation shared consistent trends that were observed in total γδ T cells (Figure 25 and Figure 27).

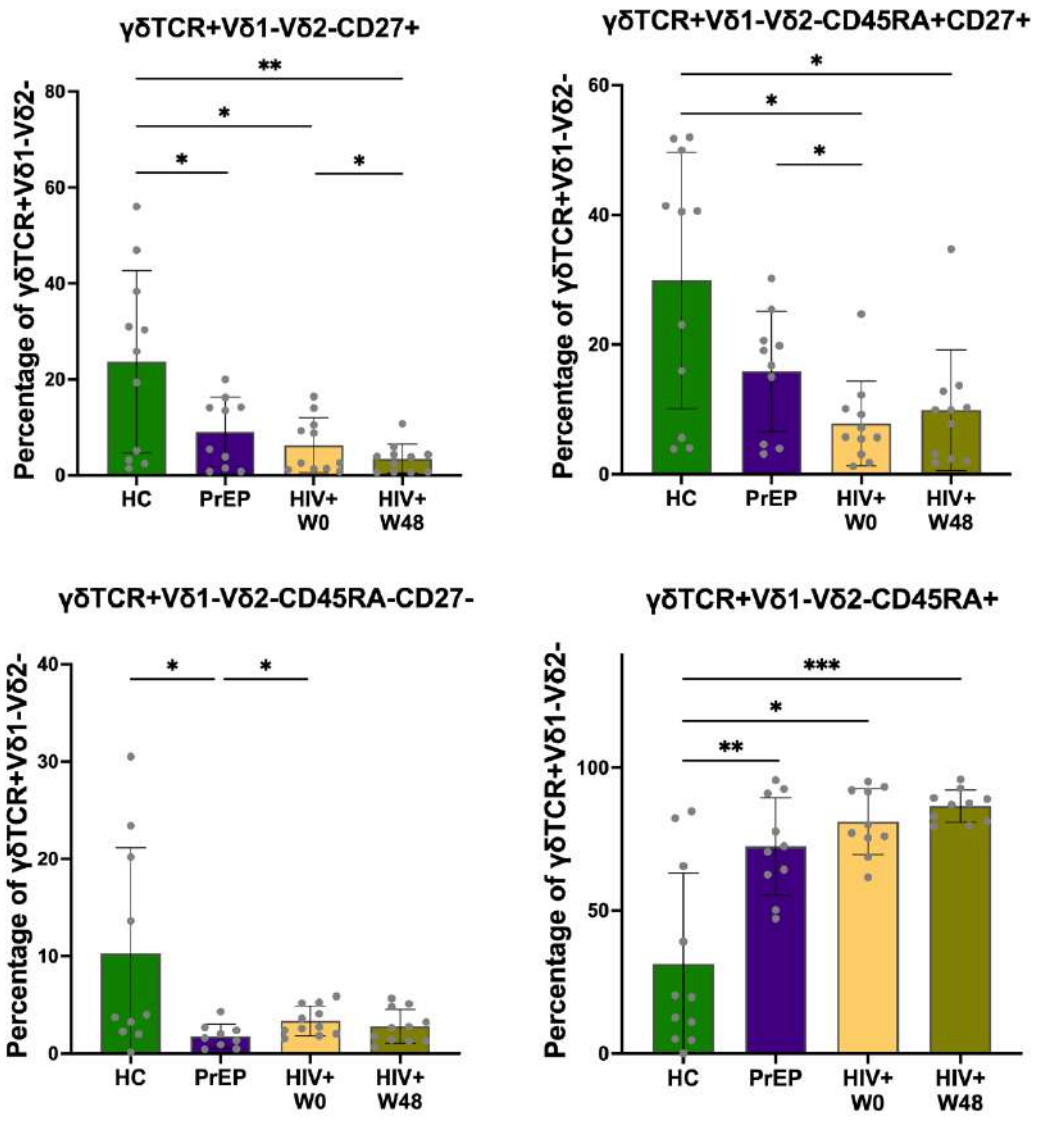


Figure 26. Comparison of CD27 and CD45RA expression in non-Vδ1/Vδ2 γδ T cells across healthy controls, PrEP Users, and HIV-infected Individuals. Percentages of expression of CD27 and CD45RA on γδ T cell-subpopulation in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+ W48; n=14), obtained by manual gating. a) Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test and paired T-test statistical analysis was performed in normally distributed data and Mann-Whitney test and Wilcoxon matched-paired signed rank test was performed for nonparametric data; ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; if not indicated, p value is not significant. Outliers identified by GraphPad were treated as missing.

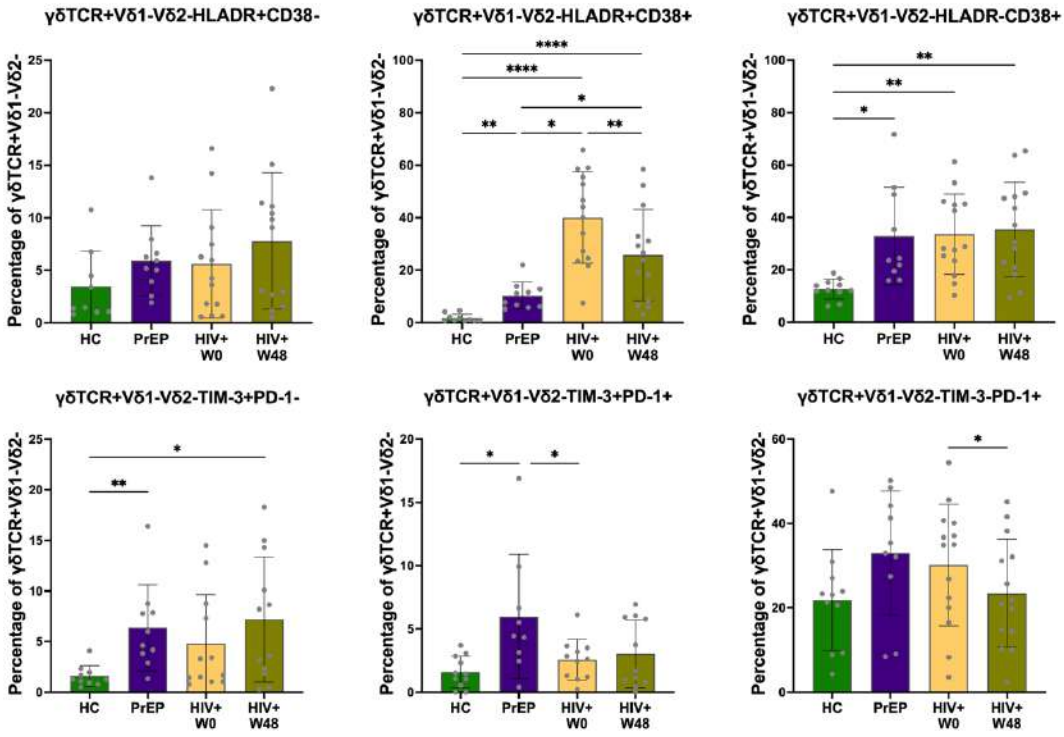


Figure 27. Comparison of activation and exhaustion markers in non-Vδ1/Vδ2 γδ T cells across healthy controls, PrEP Users, and HIV-infected Individuals. Percentages of expression of TIM-3 and PD-1 on γδ T cell-subpopulation in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), HIV+ before ART (HIV+ W0, n= 14) and HIV+ on 48 weeks of ART (HIV+ W48; n=14), obtained by manual gating. a) Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test and paired T-test statistical analysis was performed in normally distributed data and Mann-Whitney test and Wilcoxon matched-paired signed rank test was performed for nonparametric data; ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; if not indicated, p value is not significant. Outliers identified by GraphPad were treated as missing.

5.7 Exploring the immunophenotypic characteristics in potencial immunological non-responders (INRs).

After 48 weeks on ART, four out of 14 HIV+ individuals had CD4 counts below 500 cells/uL. Although immunologic non-responders (INRs) are typically identified after at least two years on ART, and with specific thresholds varying between studies, these four individuals were considered potential INRs in the present study. Those individuals who reached 500 cells/uL of CD4 counts were referred as immunologically responders (IRs) (Table 5).

When IRs individuals were analyzed separately, CD4+ and CD8+ T cells showed a partially restoration. Alternatively, CD4+ and CD8+ T cells in INRs were more impaired compared to IRs despite no statistical differences (Figure 31,32,33, and 34).

Regarding γδ T cells, both IRs and INRs showed important impairments in all analyzed subpopulations. Surprisingly, significant increased effector γδ T cells, effector Vδ1+ cells and Vδ2+HLADR+CD38+ cells were observed in INRs at W0 compared to those levels observed in IRs at W0 (Figure 35, 36 and 37).

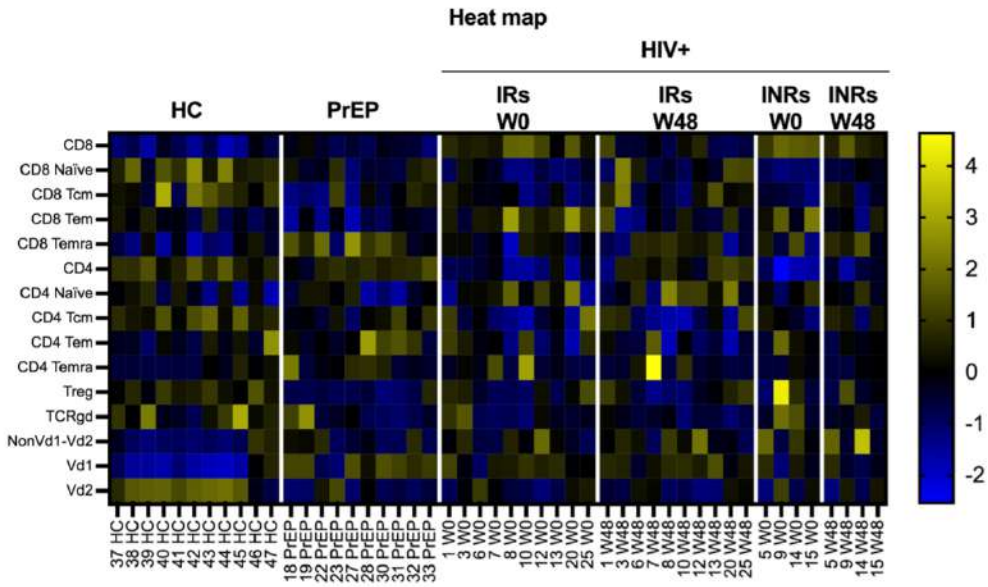
.

Table 5. Immunological and inflammation parameters of healthy controls (HC) and immunologically non-responders (INRs) at baseline (W0) and 48 weeks after ART (W48).

	HC N=11	**INRs W0 N=4**	**INRs W48 N=4**	***p-value***		
				W0 vs W48	HC vs W0	HC vs W48
CD4 count/ul	1069 (901–1199)	255.5 (130.5-379.0)	422 (318-466)	NS	**0.005**	**0.005**
CD8 count/ul	709 (685–1008)	980.5 (666.75-1356.5)	762 (637.25-988.0)	NS	NS	NS
CD4/CD8 ratio	1.27 (1.04-1.54)	0.25 (0.15-0.37)	0.57 (0.33-0.73)	NS	**0.005**	**0.005**
IL6	1.77 (1.49-2.00)	2.10 (1.50-2.70)	3.97 (1.86-5.55)	NS	NS	NS
IL5	<3	<3	<3	NS	NS	NS
CRP	0.08 (0.04-0.33)	0.15 (0.10-0.36)	0.54 (0.34-0.70)	NS	NS	**0.048**
D-Dimer	292 (149-350)	361.5 (217.5-3300.8)	268 (209-911)	NS	NS	NS
Fibrinogen	386 (315-449)	302 (264.5-378.5)	430.5 (349.0-451.3)	NS	NS	NS

Abbreviatures: CRP, C-Reactive Protein. Quantitative variables are shown as median (interquartile range). *p-value* was obtained from Mann-Whitney test comparing HC vs INRs W0/W48 and Wilcoxon test to compare W0 vs W48. Outliers identified by GraphPad were treated as missing. NS represents no statistical significance.

a)



b)

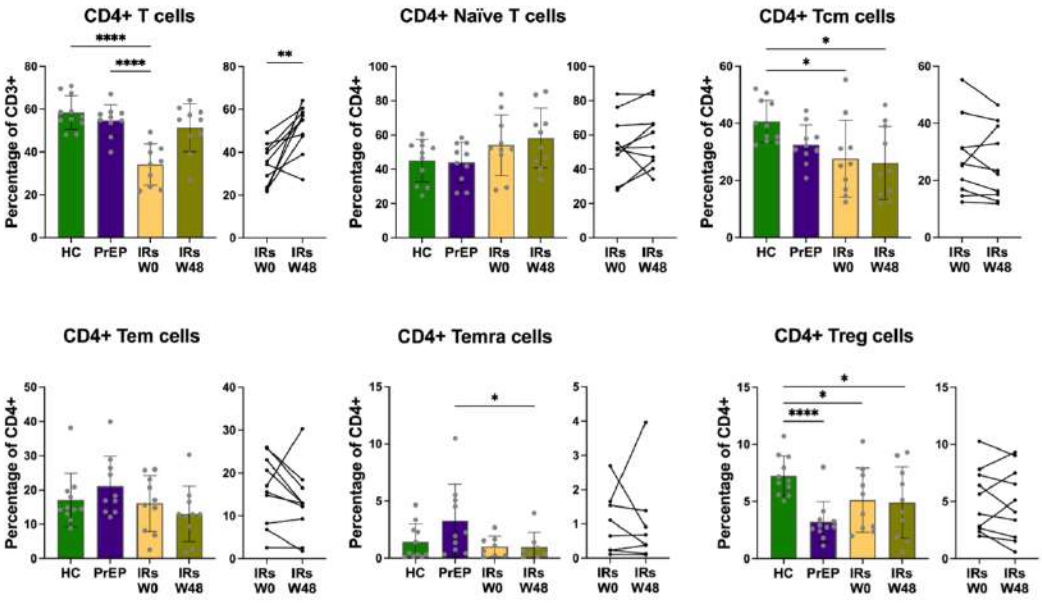


Figure 28. a) Heat Map of T-cell distribution obtained by Flow cytometry and analyzed with graphpath. The heat map represents transformed cell frequencies in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEPs; n=10), IRs before ART (IRs W0, n= 10) and IRs on 48 weeks of ART(IRs W48; n=10), INRs before ART (INRs W0, n= 4) and INRs on 48 weeks of ART(INRs W48; n=4) that were subsequently normalized per cluster (rows) to mean of zero and standard deviation of one. The color of the heat map varies from blue indicating relative under-representation to yellow indicating relative over-representation. b) Percentages of CD4 T cell memory subsets in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), IRs before ART (IRs W0, n= 10) and IRs on 48 weeks of ART (IRs W48; n=10), obtained by manual gating. Data are shown as individual values (dots) with mean ± SEM. Statistical tests include T-test, paired T-test, Mann-Whitney, and Wilcoxon signed rank test. Significance levels: ∗P<0.05, ∗∗∗∗P<0.0001; non-significant values are not indicated. Outliers were excluded.

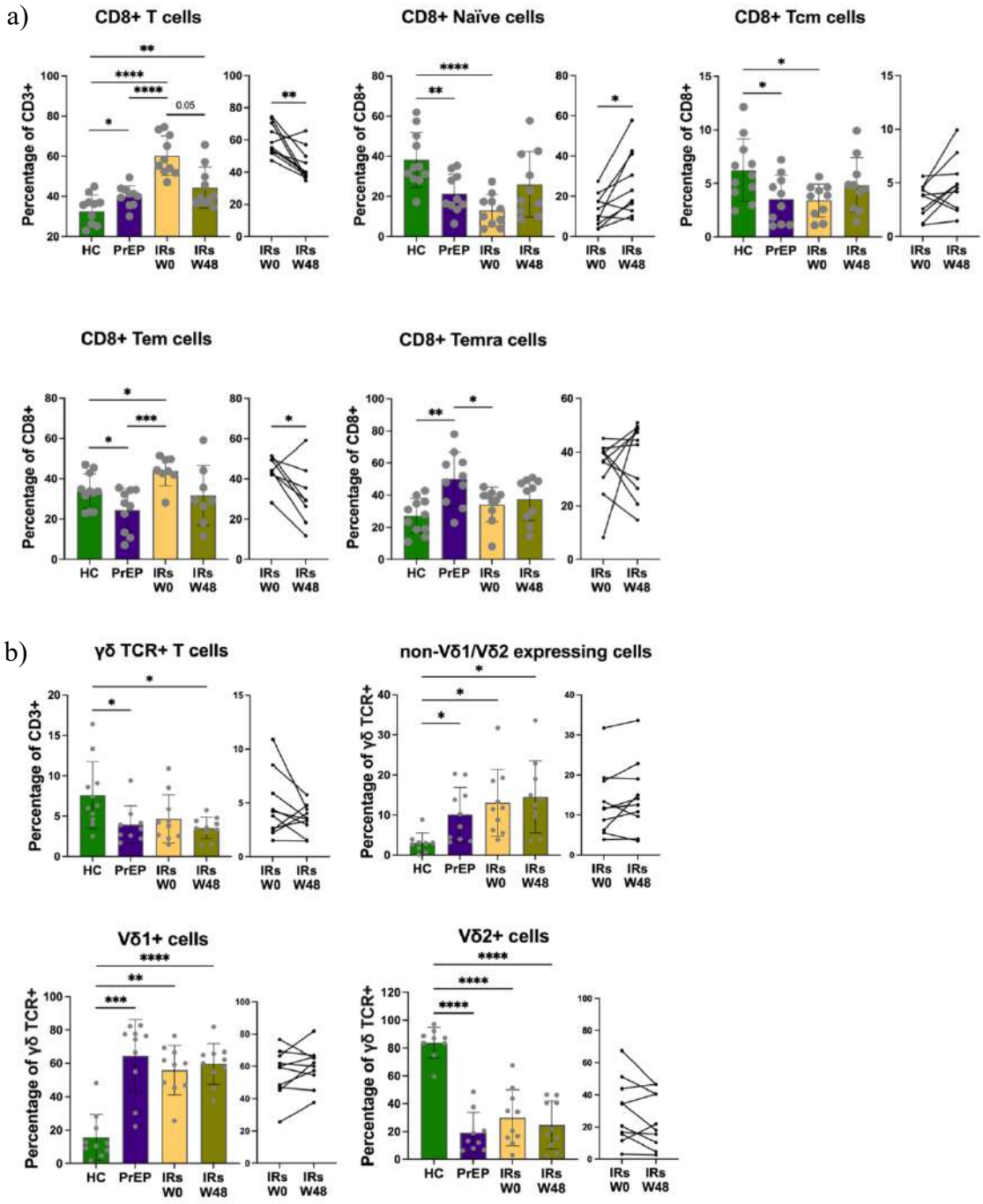


Figure 29. Percentages of CD8 and γδ T cell memory subsets in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEP; n=10), IRs before ART (IRs W0, n= 10) and IRs on 48 weeks of ART (IRs W48; n=10), obtained by manual gating. Results were grouped according to T cell subset: a) CD-8+, b) γδ TCR+ Data are shown as individual values (dots) with mean ± SEM. Statistical tests include T-test, paired T-test, Mann-Whitney, and Wilcoxon signed rank test. Significance levels: ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, ∗∗∗∗P<0.0001; non-significant values are not indicated. Outliers were excluded.

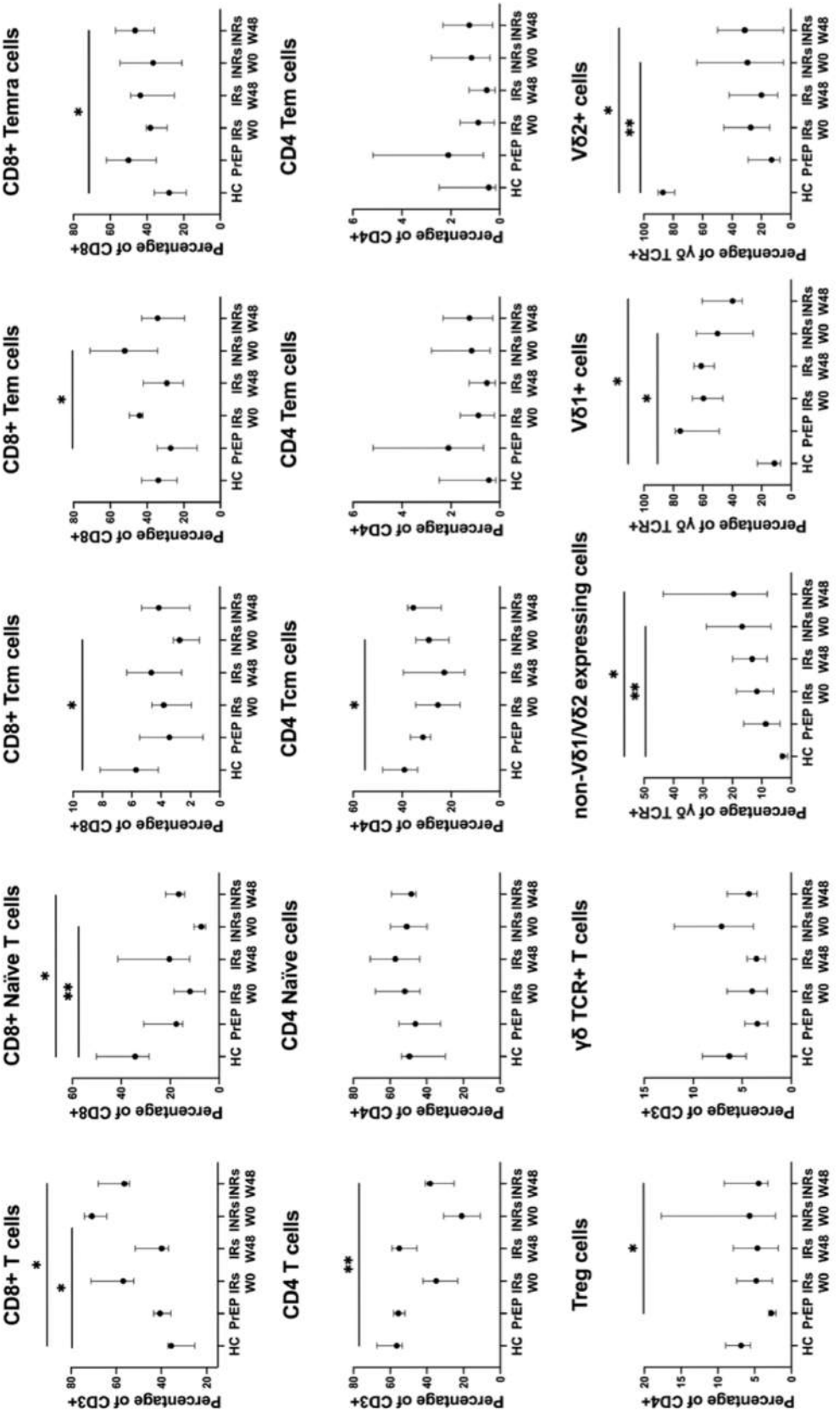


Figure 30. One-Year comparative efficacy of ART in HIV+ across T cell subpopulations in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEPs; n=10), IRs before ART (IRs W0, n= 10) and IRs on 48 weeks of ART(IRs W48; n=10), INRs before ART (INRs W0, n= 4) and INRs on 48 weeks of ART(INRs W48; n=4), obtained by manual gating. Data are shown as mean (dot) ± SEM. Statistical tests include T-test, paired T-test, Mann-Whitney, and Wilcoxon signed rank test. Significance levels: *P<0.05, **P<0.01; non-significant values are not indicated. Outliers were excluded.

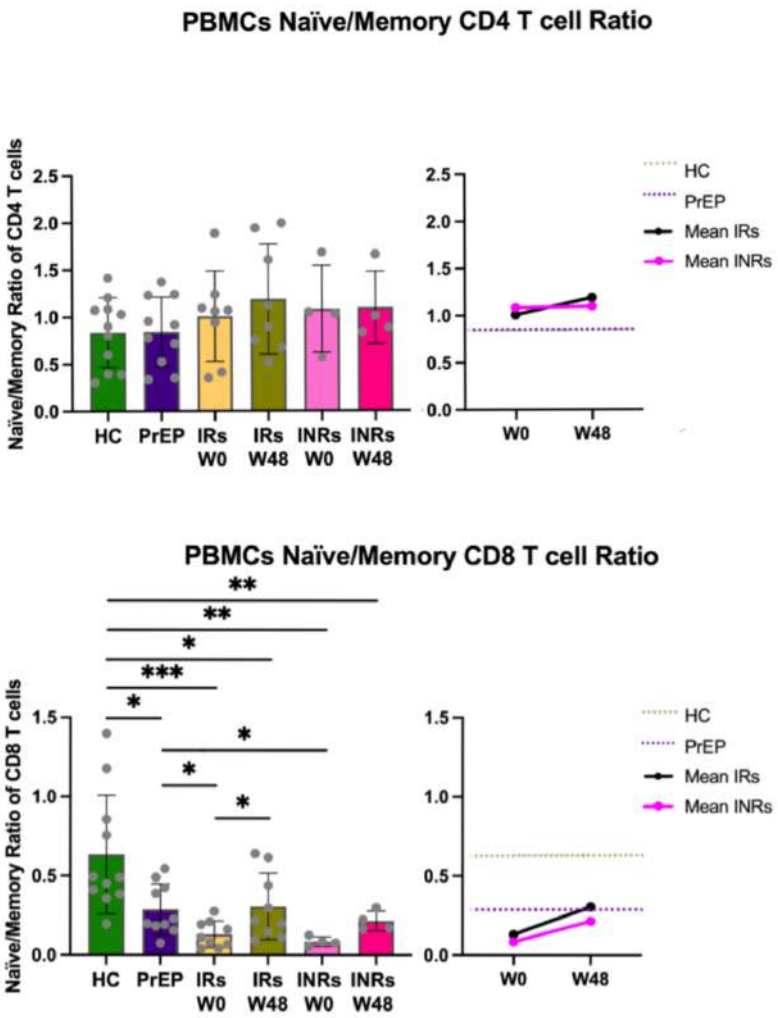


Figure 31. Naïve/Memory ratio for CD8+ and CD4+ T cells in PBMCs and changes in HIV+. Naïve/Memory ratio for CD8+ and CD4+ T subpopulations in healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEPs; n=10), IRs before ART (IRs W0, n= 10) and IRs on 48 weeks of ART(IRs W48; n=10), INRs before ART (INRs W0, n= 4) and INRs on 48 weeks of ART(INRs W48; n=4), obtained by manual gating. Data represent individual values (dots), mean (centre bar) ± SEM (upper and lower bars). T-test statistical analysis was performed in normally distributed data and Mann-Whitney nonparametric test was performed for nonparametric data. Data represent individual values (connected dots) before (week 0) and after 48 weeks on ART. Paired t-test statistical analysis was performed in normally distributed data and Wilcoxon matched-pairs signed rank test was performed for nonparametric data; Outliers identified by GraphPad were treated as missing; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; if not indicated, *p* value is not significant.

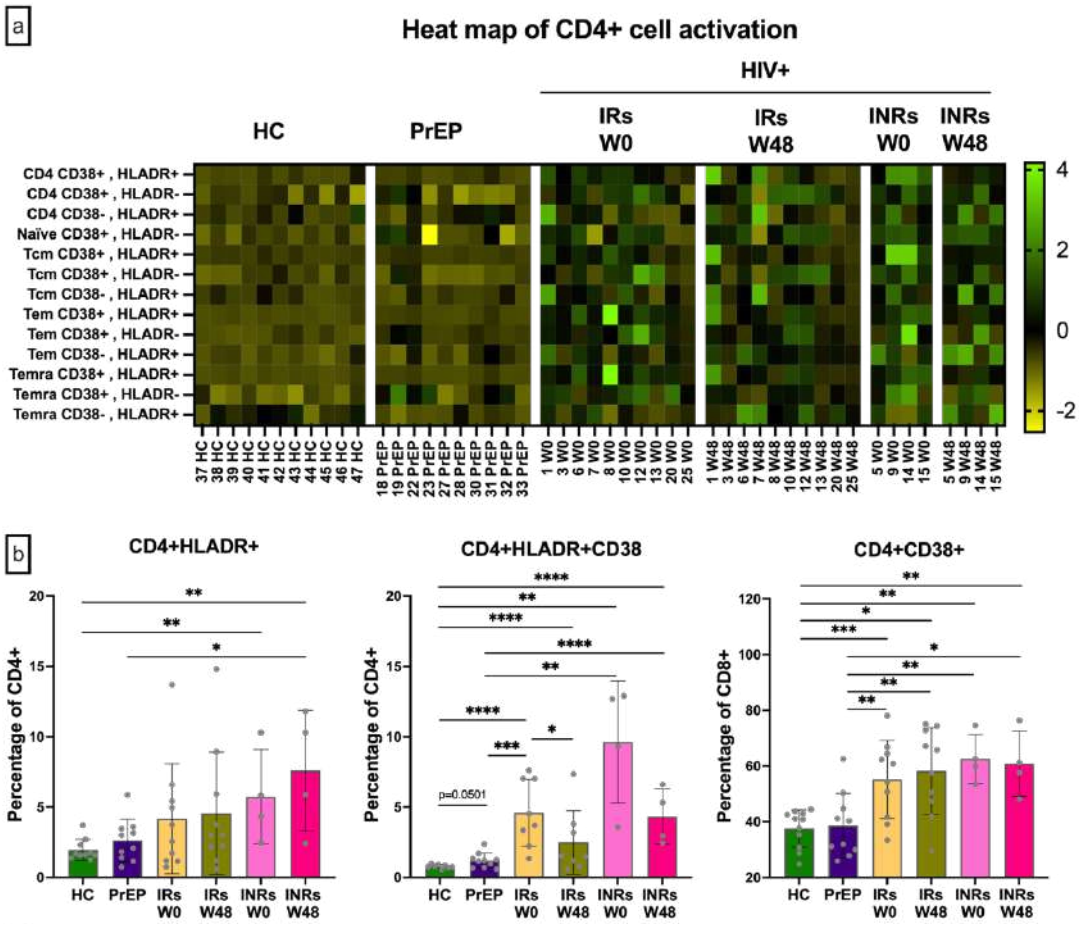


Figure 32. Activation marker comparisons across healthy controls, PrEP Users, and HIV+ in total CD4+ T cells. a) Heat map of activation markers in CD4+ T-cell subpopulations across healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEPs; n=10), IRs before ART (IRs W0, n= 10) and IRs on 48 weeks of ART(IRs W48; n=10), INRs before ART (INRs W0, n= 4) and INRs on 48 weeks of ART(INRs W48; n=4), obtained by manual gating. Data were normalized per cluster to a mean of zero and standard deviation of one. Yellow indicates under-representation, and green indicates over-representation. b) Percentages of CD38 and HLA-DR expression on total CD4+ T cells. Data represent individual values (dots) with mean ± SEM. Statistical tests include Paired t-test/Wilcoxon signed rank test for dependent data and T-test/Mann-Whitney for independent data. Significance levels are denoted as ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; non-significant *p-value* are not indicated. Outliers identified by GraphPad were excluded.

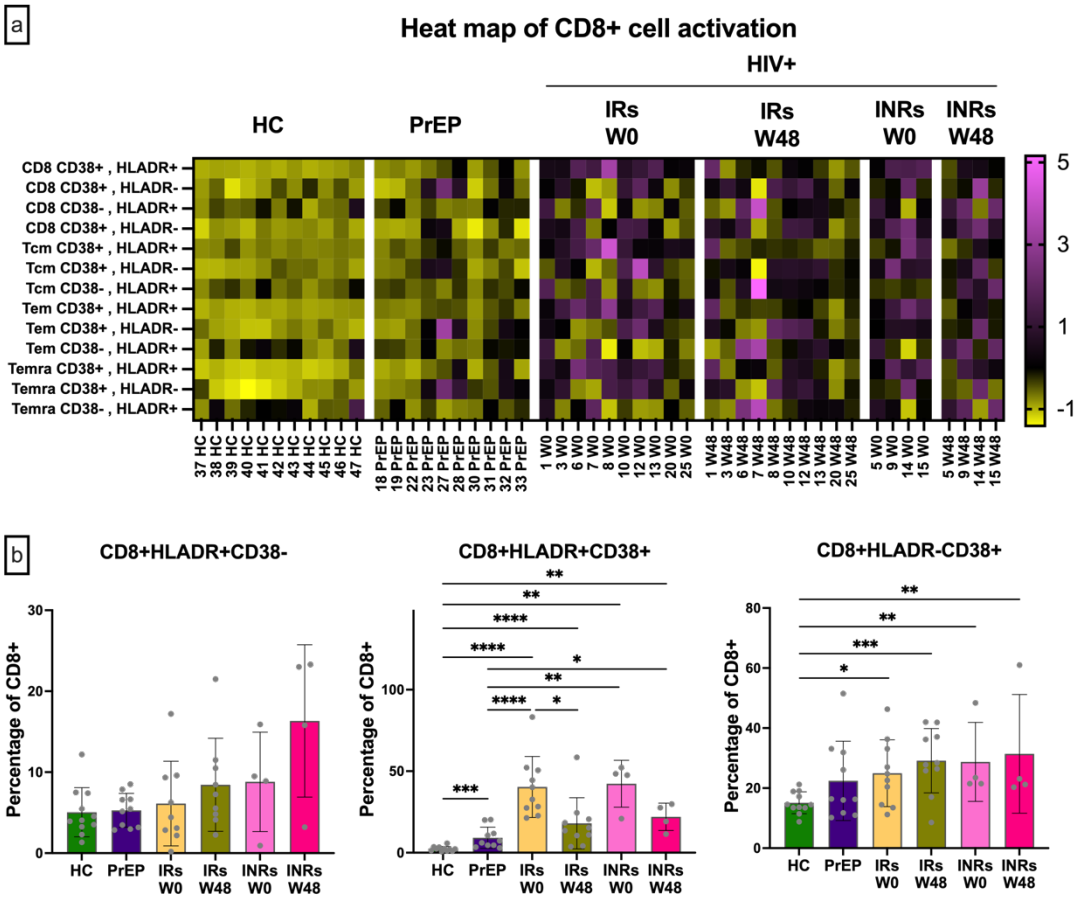


Figure 33. Activation marker comparisons across healthy controls, PrEP Users, and HIV+ in total CD8+ T cells. a) Heat map of activation markers in CD8+ T-cell subpopulations across healthy controls (HC; n=11), Pre-exposure prophylaxis taking individuals (PrEPs; n=10), IRs before ART (IRs W0, n= 10) and IRs on 48 weeks of ART(IRs W48; n=10), INRs before ART (INRs W0, n= 4) and INRs on 48 weeks of ART(INRs W48; n=4), obtained by manual gating. Data were normalized per cluster to a mean of zero and standard deviation of one. Yellow indicates under-representation, and green indicates over-representation. b) Percentages of CD38 and HLA-DR expression on total CD8+ T cells. Data represent individual values (dots) with mean ± SEM. Statistical tests include Paired t-test/Wilcoxon signed rank test for dependent data and T-test/Mann-Whitney for independent data. Significance levels are denoted as ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; non-significant *p-value* are not indicated. Outliers identified by GraphPad were excluded.

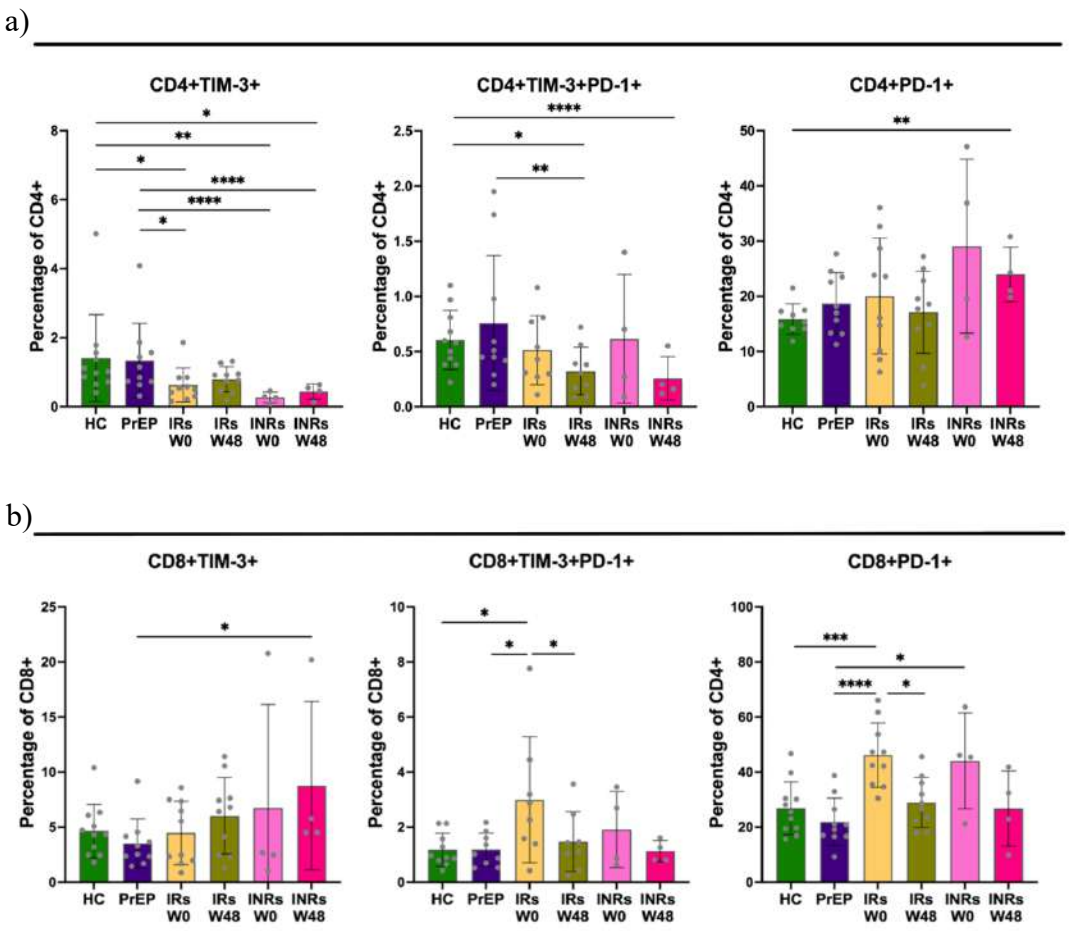


Figure 34. Exhaustion marker (TIM-3 and PD-1) comparisons were analyzed across healthy controls (HC; n=11), Pre-exposure prophylaxis users (PrEP; n=10), IRs before ART (IRs W0; n=10) and after 48 weeks of ART (IRs W48; n=10), and INRs before ART (INRs W0; n=4) and after 48 weeks of ART (INRs W48; n=4), obtained by manual gating. Results are grouped according to T-cell subsets: a) CD4+ and b) CD8+. Data are presented as individual values (dots) with mean ± SEM. Statistical analyses include paired T-test/Wilcoxon signed-rank test for dependent data and T-test/Mann-Whitney test for independent data. Significance levels are denoted as ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; non-significant *p-value* are not indicated. Outliers identified by GraphPad were excluded.

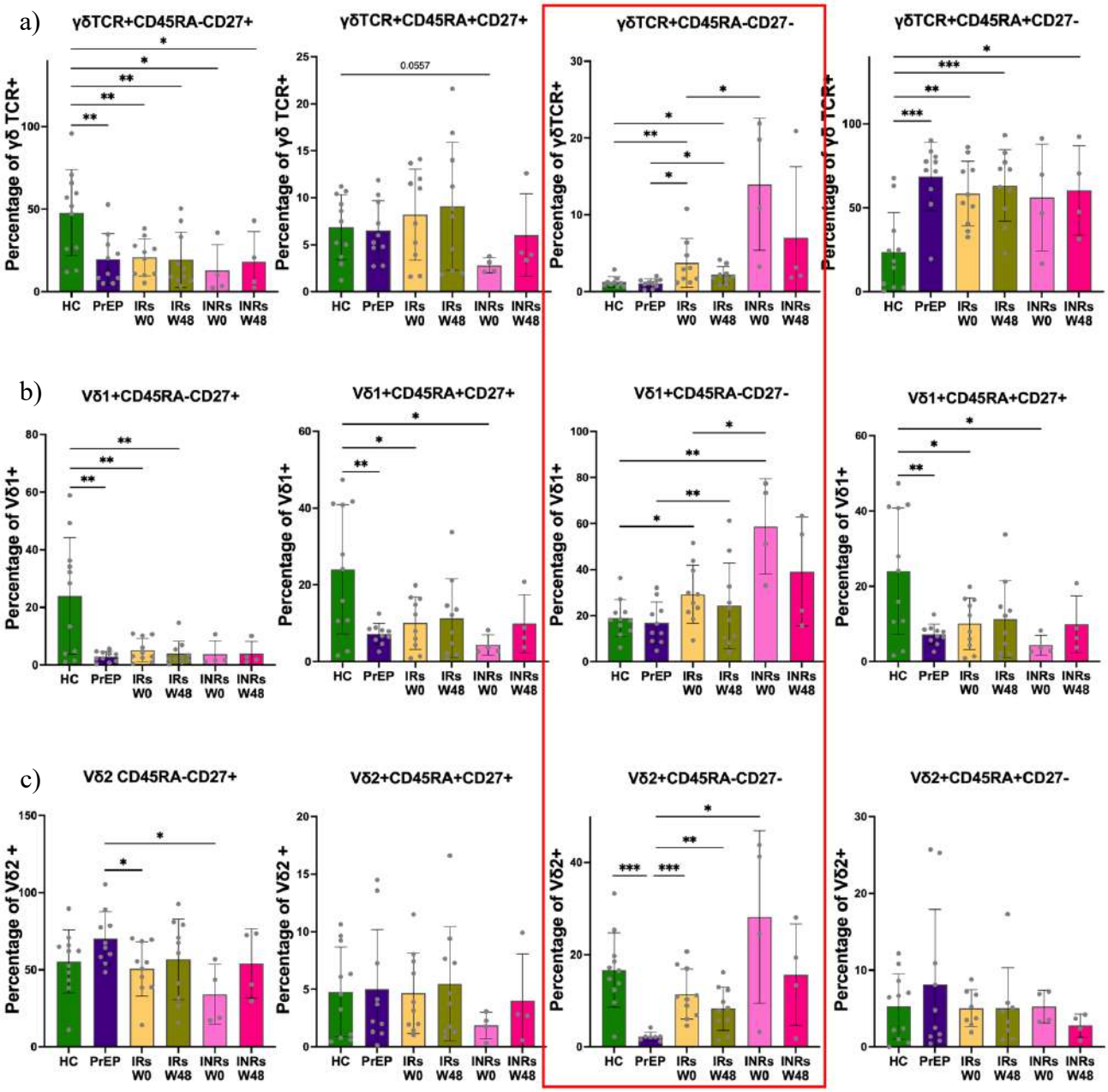


Figure 35. Percentages of CD45RA and CD27 expression on γδ T cells were analyzed across healthy controls (HC, n=11), Pre-exposure prophylaxis users (PrEP, n=10), IRs before ART (IRs W0, n=10), IRs after 48 weeks of ART (IRs W48, n=10), INRs before ART (INRs W0, n=4), and INRs after 48 weeks of ART (INRs W48, n=4), obtained by manual gating. Results are grouped by T-cell subsets: a) γδTCR+, b) Vδ1+, and c) Vδ2+. Data are displayed as individual values (dots) with mean ± SEM. Statistical analysis includes paired T-test/Wilcoxon signed rank test for dependent data and T-test/Mann-Whitney for independent data. Significance levels are denoted as ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001; non-significant *p-value* are not indicated. Outliers identified by GraphPad were excluded.

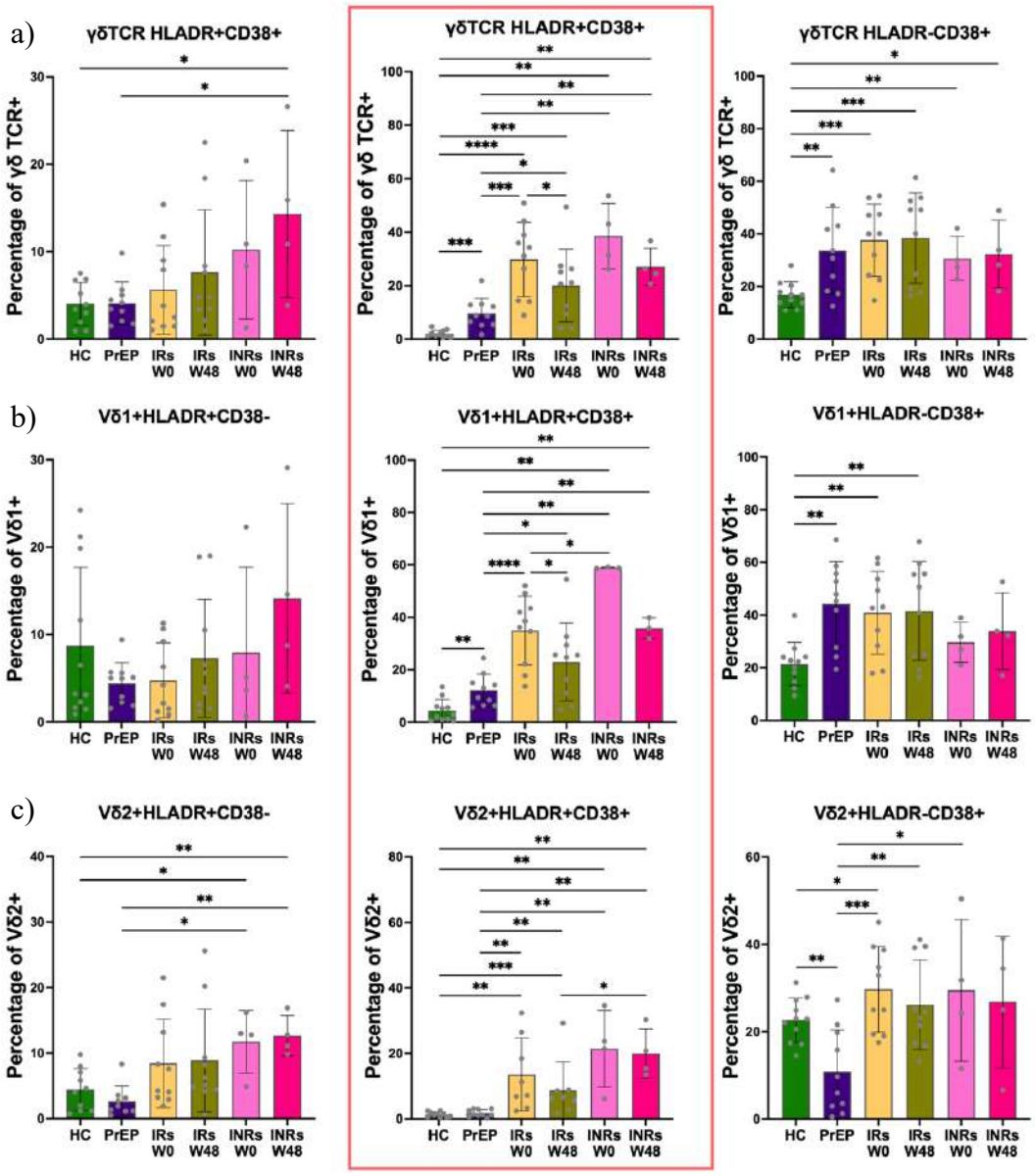


Figure 36. Percentages of HLA-DR and CD38 expression on γδ T cells were analyzed across healthy controls (HC, n=11), Pre-exposure prophylaxis users (PrEP, n=10), IRs before ART (IRs W0, n=10), IRs after 48 weeks of ART (IRs W48, n=10), INRs before ART (INRs W0, n=4), and INRs after 48 weeks of ART (INRs W48, n=4), obtained by manual gating. Results are grouped by T-cell subsets: a) γδTCR+, b) Vδ1+, and c) Vδ2+. Data are displayed as individual values (dots) with mean ± SEM. Statistical analysis includes paired T-test/Wilcoxon signed rank test for dependent data and T-test/Mann-Whitney for independent data. Significance levels are denoted as ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001; non-significant *p-value* are not indicated. Outliers identified by GraphPad were excluded.

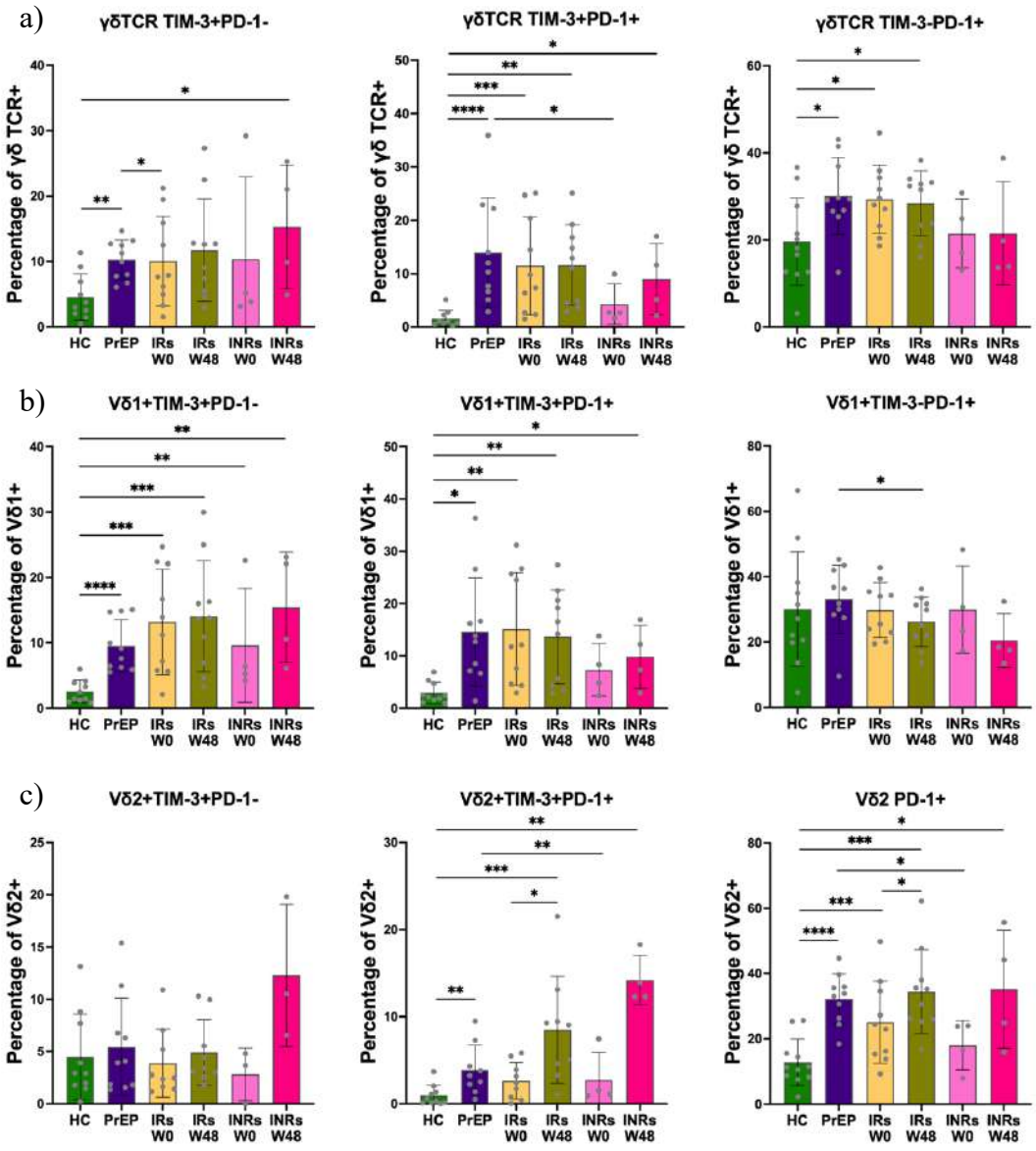


Figure 37. Percentages of TIM-3 and PD-1 expression on γδ T cells across healthy controls (HC, n=11), Pre-exposure prophylaxis users (PrEP, n=10), IRs before ART (IRs W0, n=10), IRs after 48 weeks of ART (IRs W48, n=10), INRs before ART (INRs W0, n=4), and INRs after 48 weeks of ART (INRs W48, n=4), obtained by manual gating. Results are grouped by T-cell subset: a) γδTCR+, b) Vδ1+, and c) Vδ2+. Data are presented as individual values (dots) with mean ± SEM. Statistical tests include paired T-test/Wilcoxon signed rank test for dependent data and T-test/Mann-Whitney for independent data. Significance levels: ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001, and ∗∗∗∗P<0.0001. Non-significant *p-value* are not indicated. Outliers identified by GraphPad were excluded.

DISCUSSION

6 Discussion

Ending the HIV and AIDS epidemic remains a key target of the WHO in the 2020s. While the widespread implementation of ART as a prophylaxis intervention has significantly slowed the rise in HIV infections, improving the cost and accessibility of ART is essential for achieving better control of the epidemic. Furthermore, clinicians face emerging challenges related to the increased risk of noncommunicable diseases in PLWH who have achieved viral suppression through ART. The underlying mechanisms and causes of this phenomenon remain unclear.

In this study, we applied a 19-color full-spectrum flow cytometry panel specifically designed for an in-depth analysis of T-cell differentiation, activation, and exhaustion states, enabling a comprehensive immunological monitoring of disease progression in a cohort of HIV-infected individuals before and after 48 weeks of ART. Then, transversal comparisons were conducted between HIV+ individuals, PrEP users and healthy controls. We identified the persistent disruptions in T-cell mediated immune response in HIV+ individuals after 48 weeks of ART. Specifically, ongoing immunophenotypic alterations in the homeostasis, activation, and exhaustion of γδ T cells highlight the critical role of this subpopulation in immune system reconstitution. Additionally, individuals receiving PrEP, who shared similar lifestyles and sexual behaviors as HIV+ individuals, exhibited altered immunophenotypic profiles resembling those of HIV+ individuals. These findings suggest that repeated exposition to infections can influence immune system health.

In the present study, ART successfully restored the absolute count of CD4 T cell, however, failed to restore the proportion of CD4+ cells in T-cell population, despite significant improvement achieved after 48 weeks on ART. Firstly, the proportion of CD4 among T cells is dependent of CD8 T-cell proportion since higher CD8 T-cell proportion can contribute to low CD4 T-cell percentage. Secondly, 48 weeks of follow-

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up is potentially inadequate to evaluate ART efficacy. Studies indicate that a 48-week follow-up period may be insufficient to fully assess the effects of antiretroviral therapy on immune system reconstitution, particularly concerning CD4+ T-cell recovery in individuals with chronic HIV infection. Research has shown that CD4+ T-cell counts can continue to increase beyond the first year of ART, with the most rapid recovery occurring in the initial twelve months, followed by more gradual increases over time. Additionally, absolute CD4+ T-cell counts increased from a median of 180 cells/μL at the initiation of ART to 576 cells/μL after five years, indicating that significant immune restoration can extend beyond 48 weeks (58). Besides, the incomplete recovery of Tcm CD4+ cells may contribute to the partial restoration of the CD4+ T-cell population (59). A previous study performed by Petkov S et al. (2022) involving HIV-1-infected individuals who initiated early ART and had a median treatment duration of 60 months, similarly, reported unsatisfactory recovery of Tcm CD4+ T cells. In that study, they identified compromised modulation of genes involved in the differentiation process from CD4+ naïve to Tcm cells in HIV-1+ patients receiving early ART (34). This impaired mechanism may explain the decreased levels of Tcm CD4+ T cells observed after 48 weeks of ART in our study.

Treg cells are identified that relatively increased among CD4+ T cells in HIV infection (60). However, the definition of Treg cells varies across studies, which commonly involves CD4, CD25, and FoxP3 as core markers (60,61), in combination with or without additional markers such as CD127 or other molecules associated with Treg cell function (60). In our study, we used CD4+CD25+FoxP3+CD127^{low} to identify Treg cells and we found decreased level of Treg cells after 48 weeks of ART, which contrasts with findings from other studies. A previous study demonstrated a relative increase of Treg frequency within CD4+ compartment compared to healthy controls (62), even when a stricter (CD4+ CD25^{high}, CD127^{low}, and FoxP3^{high}) was used. In that study, a group of subjects with at least one year of highly active antiretroviral therapy (HAART) was included. This group consisted of both males and females, with a median age of 50

years and, notably, a median CD4 count of 208 cells/μL. In this case, the percentage of Tregs among CD4+ T cells was negatively correlated with CD4 count (62). In comparison, the highest median CD4 count among studied groups (689 cells/μL [265–1622]) was lower than the mean CD4 count observed in HIV+ individuals analyzed in our study (828.4±364.9 cells/μL). These findings suggest a relative increase and absolute decrease of Treg frequency with progressive HIV disease (62). Collectively, differences in the definition of Treg cells, sampling characteristics, CD4 levels, and the duration of ART may contribute to the observed discrepancies between studies. Interestingly, Petkov S et al. (2022) (34) also revealed downregulation of Treg signature genes in HIV-1+ individuals with early ART, indicating impaired Treg formation and differentiation.

The percentage of CD8+ T cells showed better improvement after 48 weeks of ART, likely due to a decline in their cytotoxic activities as a direct response to viral suppression by ART. However, in our analysis, the total CD8+ T-cell proportion in HIV+ individuals remained persistently lower, with a reduced population of naïve cells and an increased proportion of Temra cells by the end of the study. Similarly, persistent elevation of CD8 T-cell proportion was observed in several studies (58,63). The restoration of the CD8 T cell population by ART is challenging due to the continuous exposure to HIV and other pathogens such as CMV and Epstein-Barr virus, which are commonly observed as co-infections in HIV infected individuals. Moreover, CD8 T-cell elevation in treated HIV infection may be maintained by gut mucosal dysfunction and microbial translocation (63). As a result, ongoing T cell activation may lead to a persistence in CD8+ Temra cells expanding and eventually, becoming exhausted. CD8+ Temra cells were described as a NK cell-like phenotype CD8+ T cell subpopulation that show high production of proinflammatory cytokines and cytotoxicity, and the accumulation of CD8+ Temra cells would lead to immunosenescence in aging population (64,65).

Persistent activation of CD4 and CD8 subset in PLWH on ART has been described in several studies (35,41,66,67). These studies described a loop where persistent activation of CD4 and CD8 T cells contributed to T-cell exhaustion, chronic inflammation and, eventually, the immune senescence in PLWH despite ART (35,64,68–71). Chronic viral replication exposure, microbial translocation induced by compromised gut barrier integrity, co-infections, and immune dysregulation are well-known factors that may involve in persistent immune activation in PLWH.

6.1 Immunosenescence in HIV+ individuals

Immunosenescence refers to the age-associated decline in immune system function, characterized by changes in the innate and adaptive immune systems. It results in increased susceptibility to infections, reduced vaccine efficacy, and sharp risk of chronic inflammation and age-related diseases. Regarding the T cell compartment, T-cell senescence is characterized by reduced naïve T-cell production, accumulation of highly differentiated antigen-experienced T cells, and a restricted T-cell receptor repertoire (72,73). Therefore, the accelerated immunosenescence in HIV+ untreated individuals can also be reflected by low Naïve/Memory T cell ratio and potentially contribute to immune aging in HIV+ individuals on ART (68).

In HIV chronic infections, persistent antigenic stimulation leads to sustained T-cell activation, resulting in the overproduction of inflammatory cytokines, continuous proliferation, and cellular stress. Over time, this prolonged activation causes T-cell exhaustion, where T cells lose their ability to proliferate, produce cytokines, and mediate effective immune responses. Exhausted T cells exhibit reduced functionality and express inhibitory receptors such as PD-1 and TIM-3. This state of exhaustion further contributes to immunosenescence, characterized by the loss of naïve T cells and

the accumulation of terminally differentiated, senescent T cells, such as TEMRA cells. In our study, the CD4+ T-cell count, a primary clinical parameter for assessing immune reconstitution in PLWH on ART, was successfully restored after 48 weeks of treatment. However, analysis of T-cell differentiation, activation, and exhaustion suggests that relying solely on CD4+ T-cell count is insufficient to monitor immune dysregulation and dysfunction in PLWH on ART.

6.2 γδ T-cell compartment in HIV+ individuals

γδ T cells with both innate-like and adaptive-like function play a crucial role in control of HIV infection (46,74). A significant alteration of γδ T-cells in the PBMC fraction was observed in our study, which is characterized by Vδ2+ T-cell depletion and Vδ1+ T-cell expansion. Circulating Vδ2+ T cell depletion started in an early stage in HIV infection (75) has long been described in HIV-infected patients with or without ART (75–80). This depletion is frequently correlated with the elevation of Vδ1+ T cells, manifesting an inverse Vδ1+/ Vδ2+ ratio similar to CD4+/CD8+ ratio in HIV-infected patients. One potential mechanism is the binding of HIV envelope protein gp120 to integrin α4β7 and CCR5 on Vδ2+ T cells, leading to activation-induced cell death (AICD) (46). Additionally, a previous study demonstrated that Vδ2+ T cells are infected by HIV and induced into reservoirs, even though they are primarily CD4-negative. Then, 6 days *in vitro* stimulation with IL-2 alone or IPP and IL-2 upregulated CD4 receptor expression on the surface of Vδ2+ cells from uninfected donors, indicating that HIV-induced immune activation might facilitate their susceptibility to infection *in vivo*. Notably, in 14 out of 18 aviremic patients, latent HIV was recovered from purified Vδ2 cells (81). Unlike Vδ2+ T cells, Vδ1+ T cells may play a more adaptive role in immunity mediating their MHC-like protein recognition, which is generally upregulated in transformed cells and virus infected cells (46). The expansion

of Vδ1+ subset in PBMCs is potentially attributed to a compensatory mechanism aimed at offsetting the loss of Vδ2+ T cells. Moreover, Vδ1+ T cells may survive from HIV-virus mediated death because the lack of the CCR5 receptor during the HIV infection (46,82). Interestingly, declined frequency of mucosal Vδ1+ T cells in gut was observed in HIV+ individuals while circulating Vδ1+ T cells increased (83), indicating the migration of Vδ1 Tcells from gut to peripheral blood is possibly a one of the mechanism of circulating Vδ1+ T cells expansion.

In fact, the susceptibility of γδ T cells to be infected by HIV was described in the early 2000s (82). Researchers found upregulated CD4 expression and downregulated CCR5 and proviral sequences were detected in purified γδ T cells separated from PBMCs from HIV-seropositive individuals. Then, the susceptibility was further investigated by infecting γδ T cells from HIV-seronegative individuals with laboratory isolate of HIV-1 that expresses green fluorescent protein (GFP). Blocking assays using an anti-CD4 monoclonal antibody demonstrated that HIV entry into γδ T cells was likely CD4-dependent (82). This early study opened a sight of that γδ T cells are vulnerable facing the HIV infection, despite there were no analysis among subpopulations of γδ T cells, is naturally to consider that Vδ1+ T cells are more likely candidates infected by HIV since the frequency of Vδ2+ T cells is depleted in HIV-seropositive individuals. A recent abstract provided a new sign of that upregulated CD4 on Vδ1+ cells following TCR stimulation, and activated Vδ1+ cells were amenable to *in vitro* infection in a CD4-dependent manner both in pheriopheral blood and tissue. Surprisingly, using droplet digital PCR (ddPCR) they observed that Vδ1+ cells from PBMCs were enriched in total HIV DNA in HIV+ individuals on ART compared CD4 T cells (84).

This distortion of γδ T cells in HIV infection is induced by a complicated mechanism. Chronic inflammation, dysregulation of CD4 T cells, and CMV co-infection may aggravate alteration of γδ T-cell subset. In fact, γδ T cells display significant heterogeneity, including variation across ethnic groups. Healthy West Africans exhibit

significantly higher frequencies of Vδ1+ γδ T cells, dominating their γδ T cell repertoire, unlike in Caucasians, with no evidence of antigen-driven expansion, highlighting population-level immune differences (85).

6.3 Differentiation of γδ T cells in HIV+ individuals

In our study, a significant dysregulation in the differentiation of γδ T cells was observed, which was primarily dependent to Vδ1+ T-cell dysregulated homeostasis with a dramatically expansion of Temra cells, while the Vδ2+ cell differentiation was likely less affected in HIV+ on ART. However, a previous study indicated that in chronic infection can elevate Temra Vδ2+ cells in HIV+ patient on ART (86). However, individuals on ART in this study were no comparable with those analyzed in our study. First, HIV+ on ART individuals were 20% female and were recruited in acute phase of infection, some of them started ART during the follow-up, but authors did not clarify the infection stage when the ART started, either the duration of ART. A recent review similarly concluded that the distribution of Vδ2+ memory subset is more likely controversial, which variate across different studies (74).

Even though Vδ1 subset is more likely considered as an adaptive-like player (46), current studies are investigating its cytotoxic role by the production of perforin and granzyme-B, or its immunoregulatory role by analyzing the IL-17 and IFN-γ (46). Here, we studied memory subset of Vδ1 T cells by measuring the surface CD45RA and CD27 expression. Similar elevated Temra proportion and naïve and Tcm Vδ1+T-cell decreased in PBMCs from chronic HIV-infected patients was observed in a previous study (83), despite only three out of 14 studied subject were treated with ART.

6.4 Activation of γδ T cells in HIV+ individuals

Persistent activation among γδ T cells and Vδ1+ and Vδ2+ subset in HIV+ on ART was showed in previous studies (74). Current studies on activation levels of γδ T cells are more based on its NK receptor (NKR)-mediated activation which is the most common mechanism of activation in γδ T-cell response in HIV infection (46,51,74), then receptors that associated with NKR function were more explored, including CD16, CD107a, CD158a, CD158b and NKG2A (74,87). Here we analyzed activation receptors (CD38 and HLA-DR) to determine the activation levels of γδ T-cell subset HIV+ individuals before and during the ART. Despite few studies have explored these receptors, similar elevated activation was observed in a previous study (86). Additionally, Vδ1 T cells from HIV+ patients demonstrate a preactivated phenotype, characterized by the expression of the activation marker CD38 and an enhanced capacity for *in vitro* expansion when cultured with IL-2. This preactivated state suggests a constant *in vivo* stimulation that makes these cells more responsive to activation and proliferation signals (88). In the present study, we provided a comprehensive look of how Vδ1 and Vδ2 cells functionally differ from each of them in chronic HIV infection suppressed by ART: both Vδ1 and Vδ2 cells presented upregulated CD38 and HLA-DR co-expression as a marker of high activation. However, Vδ1+ cells are more likely enriched with CD38+HLA-DR- phenotype, suggesting its main contribution to cytotoxicity, chronic immune activation, and inflammation. On contrary, Vδ2 cells from HIV+ individuals on ART were more CD38-HLADR+ compared to controls, indicating its potential antigen-presenting role in immune regulation.

6.5 Exhaustion of γδ T cells in HIV+ individuals

Similar to the analysis of activation receptors on γδ T cell, inhibitory receptors (IR) on γδ T cells are also limitedly studied in HIV. Vδ1+ cells from HIV-infected individuals with one to two years of ART showed significantly higher TIM-3 expression (but not PD-1) compared to those observed in healthy control (87). Notably, Riley M. F. Pihl et al. recently reported that the inhibitory receptor TIGIT is almost exclusively expressed on Vδ1+CD45RA+CD27− (Temra) cells (89). Combined with their previous findings, this highlights TIGIT+ γδ T cells as putative drivers of inflammation in ART-suppressed people with HIV (PWH) (90). These results suggest a strong correlation between the expansion of Vδ1+CD45RA+CD27− cells and inflammation in ART-suppressed PWH. Despite the heterogeneity in marker selection to analyze exhaustion levels on γδ T cells across different studies, our results suggested a potential contributory role of γδ T cell subset in chronic inflammation in HIV infection on ART.

6.6 HIV+ immunologically non-responder individuals

After 48 weeks of ART, four patients were identified with suboptimal CD4 count recovery in laboratory analyses. These patients were afterwards analyzed separately as potential candidates for progression toward INRs. Interestingly, when patients with clinically optimal CD4 recovery were analyzed independently, their immunophenotype still displayed persistent abnormalities in T-cell differentiation, activation, and exhaustion, indicating that ART may not fully restore immune homeostasis after 48 weeks. By contrast, potential INRs exhibited even poorer recovery and more severe immunophenotypic impairments. Despite the small sample size limiting statistical power, our findings underline the practical utility of CD4 counts as a reliable clinical marker for capturing profound immune dysregulation in HIV+ individuals on ART.

6.7 γδ T compartment in INRs

Interestingly, baseline analysis of γδ T cells showed a significant higher proportion of effector memory subsets in total γδ and Vδ1 T cells among INRs compared to IRs, with a similar trend observed in Vδ2 cells, albeit without statistical significance. Additionally, co-expression of CD38 and HLA-DR was significantly elevated in total γδT, Vδ1+, and Vδ2+ cells in INRs. Notably, Vδ1+ HLA-DR+CD38+ cells were significantly elevated at baseline in INRs, while Vδ2+ HLA-DR+CD38+ cells showed further increases after 48 weeks of ART, surpassing levels observed in IRs at 48 weeks. These findings suggest distinct γδ T cell activation and differentiation patterns in INRs which potentially contributing to suboptimal immune recovery. Higher activation level observed in Vδ2+ from INRs on ART further highlighted the intimate association between CD4 T-cell recovery and Vδ2+ functionality in HIV infection. Meanwhile, our results suggest that the effector cells within the total γδ T-cell subset or the Vδ1+ subset alone may serve as predictors of whether HIV patients can achieve effective immune recovery prior to initiating ART.

6.8 Immune system impairment in individuals on pre-exposure prophylaxis

PrEP is strongly recommended for individuals engaged in high-risk sexual practice or for inject drugs consumers, both of whom are considered highly vulnerable to HIV acquisition. Current studies on PrEP users primarily focus on the efficacy of the treatment, adherence to prescribed regimens, side-effects and the psychosocial and economic impacts. A previous study reported alterations in the immune system of HIV-exposed uninfected infants, characterized by a lower CD4/CD8 ratio, reduced

percentages of naïve cells in both CD4 and CD8 T cell subsets, and elevated levels of CD8+CD38+ cells (91). Building on these findings, we are particularly interested in investigating potential immune system changes in individuals on ART. Here we provided the first comprehensive immunophenotypic profiling in individuals on PrEP. Surprisingly, similar to HIV+ individuals, mainly on ART, T cells from individuals on PrEP exhibited unexpectedly high levels of activation and exhaustion marker expression, particularly in CD8+ and γδ T cells, despite the absence of HIV. Our findings reveal that individuals on PrEP presented an immune profile with signs of accelerated aging, which may ultimately lead to immunosenescence. We hypothesize that highly active sexual activity with several sexual partners leads to multiple antigen exposures playing a decisive role in driving the immune pattern found in our studies showing dysregulation of the immune system. All of this might lead to accelerated immune aging and significant immune impairments, characterized by heightened activation and exhaustion of T cells. Notably, both Vδ1 and Vδ2 γδ T cell subsets demonstrate increased susceptibility to HIV infection due to the upregulation of CD4, despite their primary CD4-negative nature (81,84). Furthermore, the expansion of circulating Vδ1 cells may reflect their migration from barrier tissues, such as the gastrointestinal tract or mucosal sites, likely triggered by local inflammation or tissue damage. Additionally, highly active sexual behaviors without adequate protection can result in repeated exposure to multiple antigens. This chronic antigen stimulation drives sustained immune activation and disrupts immune homeostasis, further contributing to immunosenescence and increasing susceptibility to HIV infection. A long-term cohort study is essential to further investigate immunosenescence markers, T cell functionality, and their potential correlation with the failure of PrEP.

6.9 Limitations

The limitations of this study should be acknowledged. First, the 48-week ART duration may not be sufficient to fully assess the effects of ART on immune recovery, and longer follow-up is essential. Second, our study exclusively recruited male participants, which limits the generalizability of our findings to the broader HIV population. Moreover, the small sample size may reduce the statistical power and hinder subtle differences or trends. For the INR group, although our results demonstrated more severe immune dysregulation, the small sample size limits the statistical power of these findings. Future studies with larger and more diverse cohorts are recommended to validate these results and enhance their applicability. As a strength, our small cohort of HIV+ individuals is highly homogeneous in terms of age (relatively young people), sex, and concomitant diseases and infections. We excluded chronic inflammatory-related diseases that can affect the immune system and mask the inflammatory effect due to HIV infection. Hence, the immune impairment observed in our study can be attributed mainly to HIV infection and secondly to the lifestyle. Third, while we provided a comprehensive description of immunophenotypic changes in the studied population, the mechanisms driving these changes remain unclear and require further investigation. Specifically, future studies should focus on γδ T cell functionality using techniques such as cell sorting and crosslinking to elucidate their role. For the PrEP population, our cross-sectional study design does not provide evidence to confirm early immune aging, emphasizing the need for a long-term longitudinal cohort. An *in situ* immunohistochemistry study to determine whether there is a direct correlation between tissue γδ T cell and their dysregulation in peripheral blood.

Nevertheless, this study contributes to broadening the insights of how HIV infection, along with environmental and lifestyle factors, distinctly impair the immune system—an alteration that has been previously attributed to HIV itself.

CONCLUSIONS

7 Conclusions

- **Immune recovery with ART**: While 48 weeks of ART significantly improved CD4+ T cell counts, persistent abnormalities in T cell activation, differentiation, and exhaustion indicate that ART is insufficient or needs more time to fully restore immune homeostasis in HIV+ individuals. These unresolved abnormalities, particularly within γδ T cells, may potentially contribute to chronic inflammation and impaired immune recovery.

- **Immunological impairment in individuals on PrEP**: PrEP users exhibited an elevated immune activation, with significant alterations observed particularly in CD8+ T cells and γδ T-cell functionality, and potential signs of accelerated aging. These findings underscore the need for long-term studies to evaluate the immunological consequences of lifestyle and its role in shaping γδ T cell responses.

- **Characteristics of INRs**: Potential INRs demonstrated more severe immune dysregulation compared to those with optimal CD4 recovery, with significant alterations in γδ T cell activation and differentiation. However, the small sample size limits the statistical power, necessitating larger studies to confirm these observations.


FUTURE LINES

8 Future Lines

- **Spectral Flow Cytometry for Immune Monitoring**

Spectral flow cytometry enables comprehensive and efficient analysis of T-cell subsets, offering great potential for monitoring immune reconstitution and detecting chronic inflammation markers in HIV patients undergoing ART.

- **Persistent T-Cell Activation and Chronic Inflammation**

Persistent T-cell activation drives chronic inflammation in ART-treated HIV patients. Future studies should focus on its underlying mechanisms to identify therapeutic targets and improve patient outcomes.

- **γδ T Cells in Immune Dysregulation**

γδ T cells regulate immune responses and may serve as key indicators of immune dysregulation in ART and PrEP populations. Long-term studies are essential to understand their role in chronic inflammation and immunosenescence.

- **γδ T Cells as Targets in HIV Pathogenesis**

γδ T cells, as barrier tissue-resident cells and potential targets of HIV, may contribute to gut epithelial damage and systemic inflammation in HIV patients. Investigating their harboring of HIV RNA and immune activation will provide insights into HIV pathogenesis and therapeutic opportunities.

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