The role and impact of Nef and Nef inhibitor in HIV-induced impairment of CD4 T-cells

by

Deepika Mahesh

B. Tech Biotechnology, Sri Venkateswara College of Engineering, 2022

Submitted to the Graduate Faculty of the

School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2024

UNIVERSITY OF PITTSBURGH

SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Deepika Mahesh

It was defended on

April 19, 2024

and approved by

Dr. Priscila Mayrelle Da Silva Castanha, Assistant Professor, Dept. of Infectious Diseases and Microbiology

Dr. Cynthia M McMillen, Assistant Professor, Dept. of Infectious Diseases and Microbiology

Thesis Advisor: Dr. Robbie B. Mailliard, Associate Professor of Medicine, Department of Medicine

The role and impact of Nef and Nef inhibitor in HIV-induced impairment of CD4 T-cells

Deepika Mahesh, MS

University of Pittsburgh, 2024

This study, utilizing *in vitro* models, aims to examine the effects of Nef and its inhibitors on the impairment of CD4 T cells induced by HIV. The primary objectives are to uncover the mechanism by which Nef impacts CD4 T cells and to assess the therapeutic viability of Nef inhibitors. Using the CEM-T4 cell line, we investigated the impact of Nef on CD4 expressions. Our findings demonstrate that Nef causes a decrease in CD4 expression. However, using the Nef inhibitor B9 at different concentrations, we observed an improvement in CD4 expression. These results suggest that Nef inhibitors can potentially mitigate the harmful effects of Nef on CD4 T cells. Our study delved into the effects of Nef on HIV-infected human primary CD4 T cells isolated from peripheral blood mononuclear cells (PBMCs), exploring various strains of HIV and administering the Nef inhibitor B9. Our findings highlight the promising potential of Nef inhibitors as a therapeutic intervention. Treatment with the Nef inhibitor effectively reduced viral replication and Nef protein expression levels. Additionally, we assessed the cytokine responses induced by HIV infection and its modulation by Nef inhibitors. Treatment with the Nef inhibitor resulted in a cytokine response similar to that of uninfected cells, indicating its ability to mitigate the immune response triggered by HIV infection. This underscores the need for further investigation into the mechanisms underlying HIV-induced immune dysregulation. This research provides insights into the potential use of Nef inhibitors as therapeutic interventions, particularly in modulating immune responses within PBMCs. The knowledge gained from this may contribute to a deeper

understanding of HIV pathogenesis and develop novel strategies for combating this global health challenge.

Table of Contents

List of Figures

Preface

First and foremost, I extend my heartfelt gratitude to Dr. Mailliard for entrusting me with this project and for taking me in and helping me. With your guidance and mentorship, I've acquired a wealth of new skills and techniques that will undoubtedly shape my future career in science.

I am deeply grateful to my lab mates, whose unwavering support and camaraderie have made even the most challenging days manageable and instrumental in my academic success. A special thanks to my roommates, whose warmth and companionship transformed our place into a true home away from home. Their presence and support have been a source of solace in this journey. I am thankful for Shreya Teredesai and Shreya Venkatasubramanian, who have been pillars of strength and unwavering sources of encouragement and support and have sustained me through the highs and lows of graduate school. To all my friends, old and new, thank you for the constant support and encouragement.

Words cannot adequately express my gratitude to my family for their unwavering support, sacrifice, and belief in me. Their encouragement, love, and understanding have been the cornerstone of my success. Thank you for standing by me, believing in my dreams, and empowering me to pursue them. I am grateful for your enduring support and unwavering faith in my abilities.

1.0 Introduction

The human immunodeficiency virus (HIV), discovered years ago, has had a profound impact on the world. It is the cause of acquired immunodeficiency syndrome (AIDS), a condition that severely weakens the immune system, making individuals susceptible to opportunistic infections and cancers. HIV-1 is the most common subtype and, in combination with its less frequent relative HIV-2, infects over 30 million people worldwide. As of 2020, according to the World Health Organization (WHO) (1), there are an estimated 37.7 million individuals worldwide living with HIV, indicating that the prevalence of HIV remains a significant public health concern. The virus belongs to the *Retroviridae* family, known for its ability to integrate into host genomes, leaving ancient viral sequences in mammalian DNA. HIV's ability to persistently infect humans is a significant concern as it can evade innate and adaptive immune responses (2-5).

HIV primarily spreads through unprotected sexual contact, sharing contaminated needles, and from mother to child during childbirth or breastfeeding. Despite extensive efforts, there is no cure for HIV. However, active current antiretroviral therapy (ART) has revolutionized treatment by effectively suppressing viral replication, thereby preventing disease progression and transmission. These drug cocktails primarily target viral enzymes, including reverse transcriptase and protease, though issues such as drug toxicity and the emergence of drug-resistant strains persist. Moreover, while ART can suppress viral replication to undetectable levels in most individuals, it does not eliminate the virus. HIV persists in long-lived cells, posing a significant challenge to eradication efforts (6-9) .

1.1 HIV Biology

1.1.1 Genome Structure

The HIV genome structure is integral to understanding the virus's replication and pathogenesis. It comprises two identical single-stranded RNA molecules enclosed within the virus particle's core. Upon infection, the viral RNA undergoes reverse transcription, leading to the generation of proviral DNA. This process involves the degradation of the RNA and integration of the double-stranded HIV DNA into the human genome. The DNA genome is flanked at both ends by long terminal repeat (LTR) sequences (7, 10, 11).

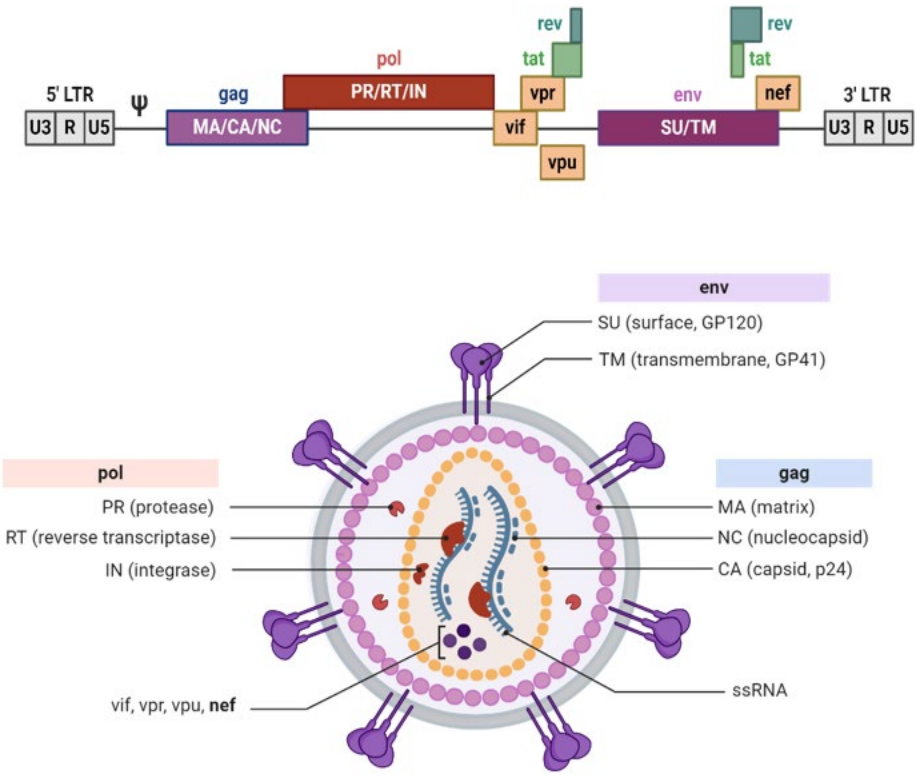

Figure 1: HIV Virion and Genome Structure ***(Created with Biorender)***

The 5' LTR region serves as the promoter for the transcription of viral genes. Following this region is the gag gene reading frame, which encodes essential structural proteins (10, 12-16). These include the matrix protein (MA, p17), capsid protein (CA, p24), nucleocapsid (NC, p7), and a smaller nucleic acid-stabilizing protein. The pol reading frame follows, encoding viral enzymes crucial for replication, including protease (PR), reverse transcriptase (RT), RNase H, and integrase (IN). Adjacent to the pol gene is the env reading frame, from which two envelope glycoproteins, gp120 (surface protein, SU) and gp41 (transmembrane protein, TM), are derived. In addition to structural proteins, the HIV genome also codes for several regulatory proteins. These include Tat and Rev, necessary for initiating HIV replication, and regulatory proteins, including Nef, Vif, Vpr, and Vpu, which impact viral replication, budding, and pathogenesis. Notably, HIV-2 codes for Vpx instead of Vpu, contributing to its reduced pathogenicity (10, 12-15, 17, 18). This genome structure provides the blueprint for HIV's complex life cycle and its interactions with host cells, highlighting potential targets for therapeutic intervention.

1.1.2 Replication Cycle

The lifecycle of HIV-1 is intricate and can be segmented into two primary phases: early and late. The early stage commences with the virus adhering to the host cell and concludes with integrating its genetic material into the host's DNA. This phase encompasses crucial steps such as viral attachment, fusion, reverse transcription, nuclear import, integration, and transcription. Once inside the host cell, the viral RNA undergoes reverse transcription, yielding proviral DNA that integrates into the host genome. This integrated viral DNA, termed provirus, acts as a scaffold for viral gene expression (19-22) .

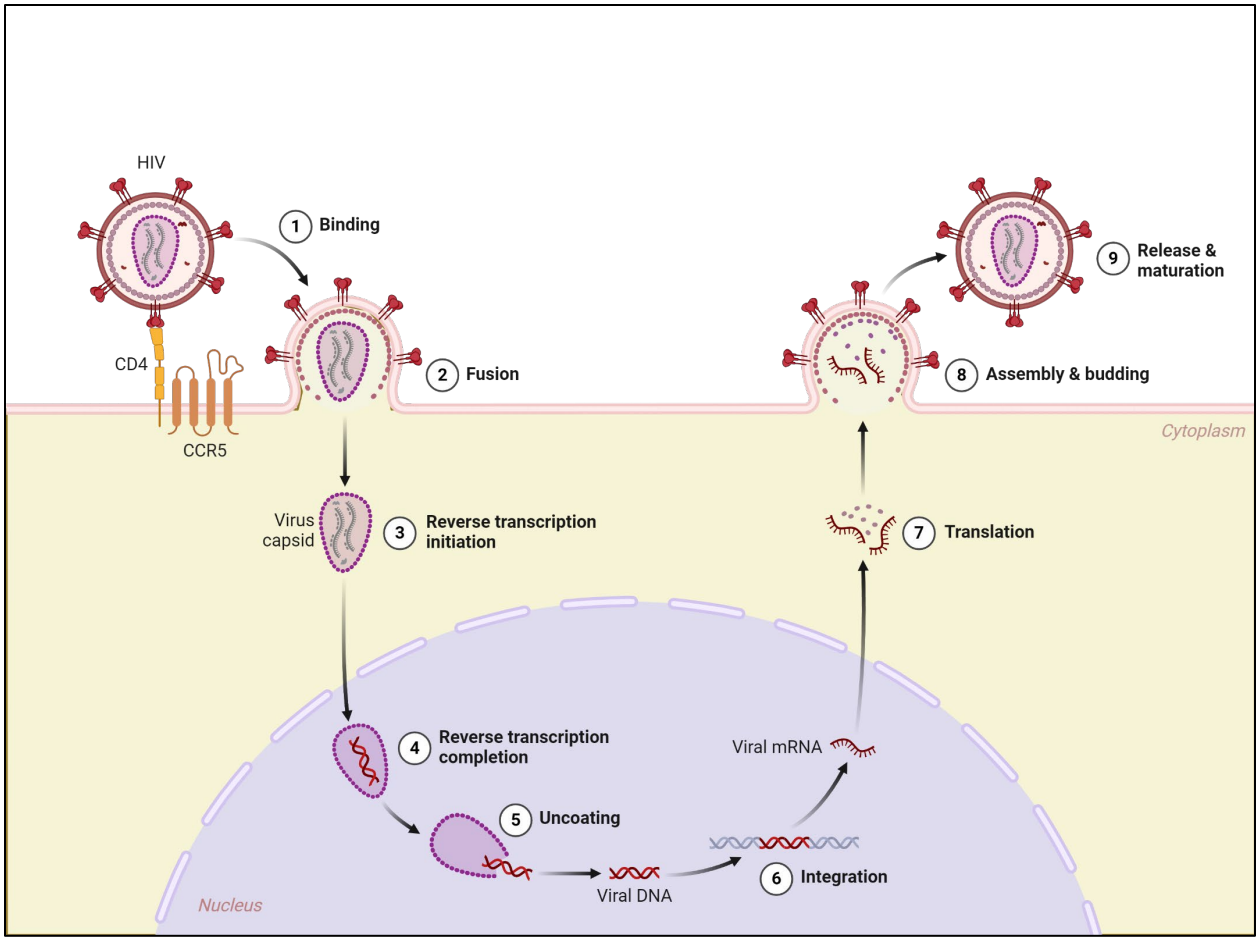

Figure 2: HIV Replication Cycle *(Created with Biorender)*

The final phase of the HIV-1 life cycle is of utmost importance, as it involves the translation of viral polyproteins to produce crucial proteins like Gag, Gag-Pol, Tat, and Rev. These proteins play a vital role in the assembly and maturation of the virus, with viral proteins and RNA being brought together at the plasma membrane, forming immature virions. Through budding, fully infectious viral particles are released from the host cell by interacting with viral proteins and cellular factors (23-25). HIV-1 encounters several obstacles throughout its replication cycle, including the high mutation rate of its reverse transcriptase enzyme, the need for rapid adaptation to the host's conditions, and the potential for resistance to antiretroviral medications. Moreover,

HIV-1 can form latent reservoirs within enduring cells, evade the host's immune defenses, and infect diverse cell types beyond CD4 T cells, including macrophages and dendritic cells (6, 26-28).

1.1.3 Host-Cell Interactions

The interactions between HIV and host cells are intricate and multifaceted, with significant implications for infection and disease progression. CD4 T cells, macrophages, and dendritic cells serve as primary targets for HIV due to their expression of receptors, including CD4, CCR5, or CXCR4, which facilitate viral entry. Once the virus enters host cells, it initiates replication, resulting in the depletion of CD4 T cells and gradually weakening the immune system. HIV targets activated and resting CD4 T cells, establishing reservoirs of infected cells distributed throughout the body (29, 30). Macrophages and dendritic cells play crucial roles in immune surveillance and antigen presentation. However, HIV can target these cells, with infected macrophages acting as viral reservoirs while contributing to chronic inflammation and tissue damage associated with HIV infection. Additionally, HIV can exploit dendritic cells to aid viral dissemination and evade immune detection. This compromises their ability to initiate effective antiviral responses by presenting viral antigens to CD4 T cells (31-36).

In individuals with chronic HIV-1 infection, the B cell compartment is also significantly impacted. This includes non-specific polyclonal B cell activation, decreased B cell proliferative responses, loss of naïve and resting memory B cells, and higher percentages of atypical or exhausted B-cells (37). Even with effective ART, these effects are not fully reversible, especially if treatment is delayed. Although HIV does not typically replicate within B cells *in vivo*, it can bind to these cells through interactions involving the complement receptor CD21, leading to

immune complex formation and potentially facilitating virus cell-to-cell transmission. HIV virions or viral proteins can directly affect B cells by inducing inflammatory cytokine secretion, suppressing B-cell class switch recombination, and stimulating B cells via factors released by infected macrophages. Indirect effects of ongoing HIV replication include immune-cell activation, CD4 T-cell depletion, increased serum levels of IL-7, and the release of systemic mediators such as lipopolysaccharide (LPS), B-cell-activating factor (BAFF), TNF, interferon-α (IFNα), IL-6, and IL-10. These interactions contribute to the dysregulation of B cell function and homeostasis in HIV infection (35, 37-42).

To maintain persistent infection, HIV employs various immune evasion tactics, including the downregulation of major histocompatibility complex (MHC) molecules. These molecules are essential for antigen presentation. However, viral proteins, including Nef and Vpu, facilitate the degradation and sequestration of MHC molecules within cells. This hinders the presentation of viral antigens to cytotoxic T-cells (32, 33, 43). Moreover, HIV evades neutralizing antibodies through mutations in its envelope glycoproteins. This reduces the efficacy of antibodies in blocking viral entry. The production of non-neutralizing antibodies further shields infected cells from immune clearance, promoting viral persistence. HIV-induced immune exhaustion in T cells, characterized by functional impairment and upregulation of inhibitory receptors, significantly hampers the immune system's ability to control viral replication. Chronic inflammation and persistent antigen stimulation contribute to T-cell exhaustion. Furthermore, HIV-induced apoptosis of infected and bystander cells, including CD4 T cells and macrophages, exacerbates immune dysfunction and disease progression (31, 32, 44-46).

1.2 CD4 T-Cells

CD4 T cells, commonly known as T helper (Th) cells, are indispensable components of our adaptive immune system. These cells play a vital role in orchestrating immune responses against pathogens, thus contributing significantly to our body's ability to fend off infections. Emerging from common lymphoid progenitors in the bone marrow, they undergo maturation in the thymus before dispersing to various lymphoid organs and peripheral tissues. Upon encountering antigens presented by antigen-presenting cells (APCs) via their T cell receptors (TCRs) and major histocompatibility complex class II (MHC-II) molecules, CD4 T cells become activated and differentiate into diverse effector Th subsets (47-51). This differentiation process is influenced by the cytokine milieu and specific transcription factors present in their surroundings. Various subsets of Th cells have been identified, each characterized by distinct functions and cytokine profiles.

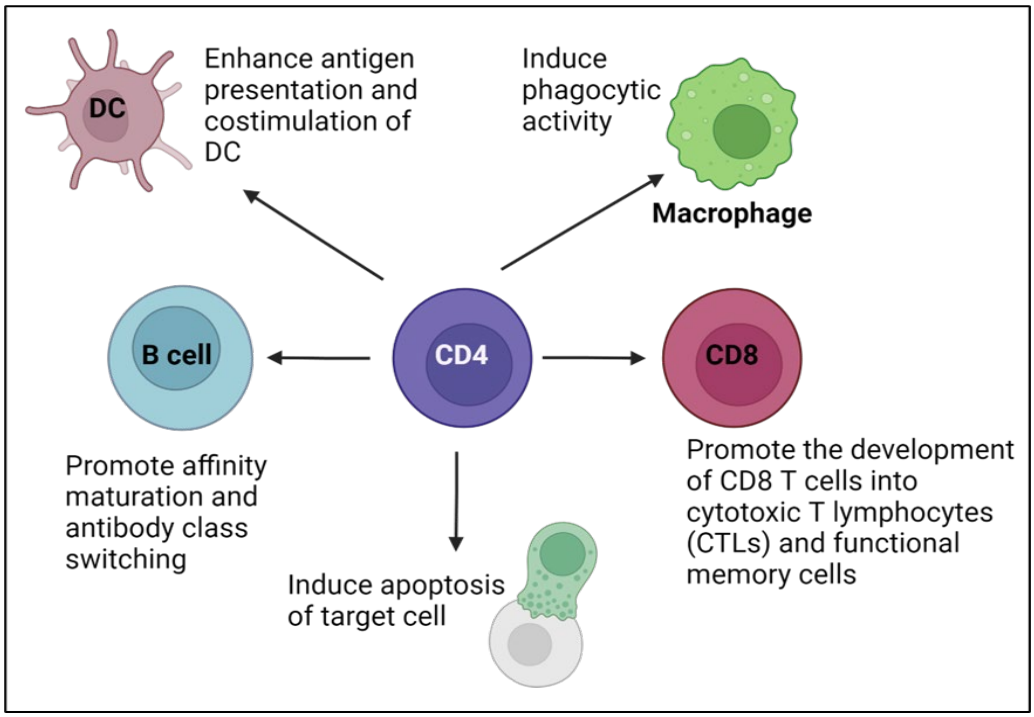

Figure 3: CD4 T Cell Functions ***(Created with Biorender)***

1.2.1 Types of CD4

Th1 cells primarily produce interferon-gamma (IFN-γ) and mediate cellular immune responses against intracellular pathogens such as bacteria and viruses. They activate macrophages to enhance their microbicidal activity, promoting the clearance of intracellular pathogens. Th2 cells produce cytokines such as interleukin-4 (IL-4), IL-5, and IL-13, vital for promoting humoral immune responses against extracellular parasites and allergens (52, 53). Th2 cells stimulate B cells to produce antibodies, facilitate eosinophil recruitment, and enhance mast cell activation. Th17 cells secrete IL-17 and IL-22 and play a critical role in mediating immune responses against extracellular bacteria and fungi. They are involved in the recruitment of neutrophils and the induction of antimicrobial peptide production by epithelial cells, protecting barrier tissues such as the intestines, lungs, and skin (47-50, 54-56).

T follicular helper (Tfh) cells help B cells within germinal centers of secondary lymphoid tissue organs, promoting the generation of high-affinity antibodies, affinity maturation, and class switch recombination (57, 58). They express the transcription factor Bcl6 and produce IL-21, essential for B cell differentiation and antibody production (50). Regulatory T Cells (Tregs) are crucial for maintaining immune tolerance and preventing autoimmunity (59, 60).They suppress immune responses by secreting anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta (TGF-β) (61). Tregs express the transcription factor Foxp3, which is essential for their suppressive function. Additionally, other Th cell subsets have been identified, including Th9 cells (IL-9 producers), Th22 cells (IL-22 producers), and follicular regulatory T cells (Tfr), each with unique roles in immune regulation and host defense (47-50, 62).

1.2.2 HIV Impact on CD4 T Cells

HIV primarily targets and destroys CD4 T cells by utilizing surface CD4 as the primary binding/entry receptor, leading to a gradual decline in their numbers as infection progresses. HIV infection not only selectively reduces the overall population of CD4 T cells, but it also impairs their ability to proliferate and mount effective immune responses, even in the early stages of infection. This impairment involves dysregulation of cell cycle progression and activation (63-65). Despite acquiring an activated phenotype, HIV-infected CD4 T cells often fail to progress through the cell cycle properly, resulting in a proliferation defect. Moreover, the HIV envelope protein can interact with CD4 and co-receptors on uninfected CD4 T cells, leading to their death and further depletion. This bystander cell-killing mechanism appears to contribute significantly to the overall loss of CD4 T cells during HIV infection. Additionally, co-receptors CCR5 and CXCR4 are crucial, as they facilitate HIV's ability to infect and deplete CD4 T cells, with different tropic strains targeting distinct subsets of CD4 T cells (32, 64, 66, 67).

1.3 Nef Protein

The Nef protein, a critical component encoded by primate lentiviruses such as HIV-1 and HIV-2, serves as a multifunctional adaptor protein pivotal in the pathogenesis of HIV infection (68). Structurally, Nef consists of a myristoylated core domain flanked by flexible N-terminal and C-terminal regions. This facilitates its association with cellular membranes, including the plasma and perinuclear membrane complexes (69, 70). Moreover, Nef is detected within virion particles, indicating its presence in various cellular compartments and potential involvement in multiple

stages of the viral life cycle (70, 71). Functionally, Nef lacks enzymatic activity but exerts diverse cellular effects by interacting with numerous host factors. Four primary activities of HIV-1 Nef have been extensively characterized. Firstly, Nef downregulates cell surface levels of CD4, the primary receptor for HIV entry, by promoting its internalization and subsequent degradation via the endosomal/lysosomal pathway. This activity is crucial for overcoming the effects of high cellular CD4 expression. This expression of CD4 on the surface of HIV-infected cells is not only detrimental to the infectivity of the viral particles produced, but it may also serve to limit the number of viral infections that can occur within a single cell. Thus, it facilitates the production of infectious particles (68, 72-79).

Nef downregulates cell surface levels of primary histocompatibility complex class I (MHC-I) molecules, impairing antigen presentation to cytotoxic T lymphocytes (CTLs). By diverting MHC-I from the default pathway to lysosomal degradation, Nef enables infected cells to evade destruction by the immune system during active viral replication (16, 75, 80, 81). Despite this evasion mechanism, the immune response to HIV antigens remains active, suggesting a complex interplay between Nef-mediated immune evasion and host immune surveillance. Nef mediates cellular signaling and activation by interacting with kinases such as Pak2 and Hck. Nef's activation of Pak2 enhances viral replication by potentially regulating the actin cytoskeleton function, although the precise mechanisms remain controversial (82-85). Similarly, Nef activates Hck, a myeloid lineage-specific tyrosine kinase, which may contribute to cellular transformation and modulation of transcription factors like Stat3. Nef enhances the intrinsic infectivity of HIV-1 virions by overcoming barriers to infection and promoting efficient viral entry and replication. Mechanisms underlying this enhancement include disruption of the actin cytoskeleton in target

cells, downregulation of unknown cellular proteins that inhibit viral infectivity, and protection of the viral core from post-fusion degradation (72-78, 86, 87).

1.4 Nef Inhibitor B9

The Nef inhibitor B9 represents a significant breakthrough in HIV/AIDS research, offering a promising avenue for antiretroviral therapy. B9 belongs to the diphenyl hydroxypyrazolodiazene class of compounds and binds directly to recombinant Nef protein. Functionally, B9 inhibits multiple crucial Nef activities, including enhancement of viral infectivity, replication, and downregulation of MHC-I molecules on the cell surface (88, 89). Its inhibitory effects extend to Nef-dependent activation of kinases like Itk and Hck, suggesting a broad spectrum of action against Nef-mediated pathogenic mechanisms (89-91).The synthesis and characterization of over 200 analogs of B9 are reported to optimize its pharmacological properties and enhance its efficacy.

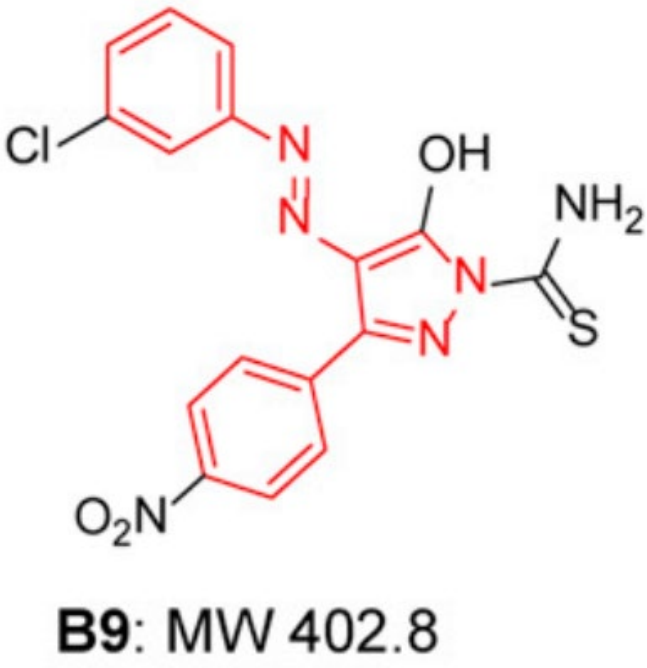

Figure 4: B9 Nef Inhibitor Structure (89)

While structurally diverse, these analogs retain the core hydroxypyrazole scaffold essential for Nef interaction and antiretroviral activity (90, 92). Several analogs exhibit nanomolar to picomolar binding affinity to Nef and demonstrate potent inhibition of Nef-mediated activities, including HIV-1 replication enhancement and MHC-I downregulation (92-96).

One of the key impacts of B9 and its analogs lies in their ability to restore MHC-I expression on the surface of HIV-infected CD4 T cells. By reversing Nef-induced MHC-I downregulation, these inhibitors enhance the immune recognition of infected cells by cytotoxic T lymphocytes (CTLs). This restoration of MHC-I expression facilitates the activation of autologous HIV-specific CD8 T cells, leading to the efficient clearance of latently infected CD4 T cell reservoirs in vitro (89-92).

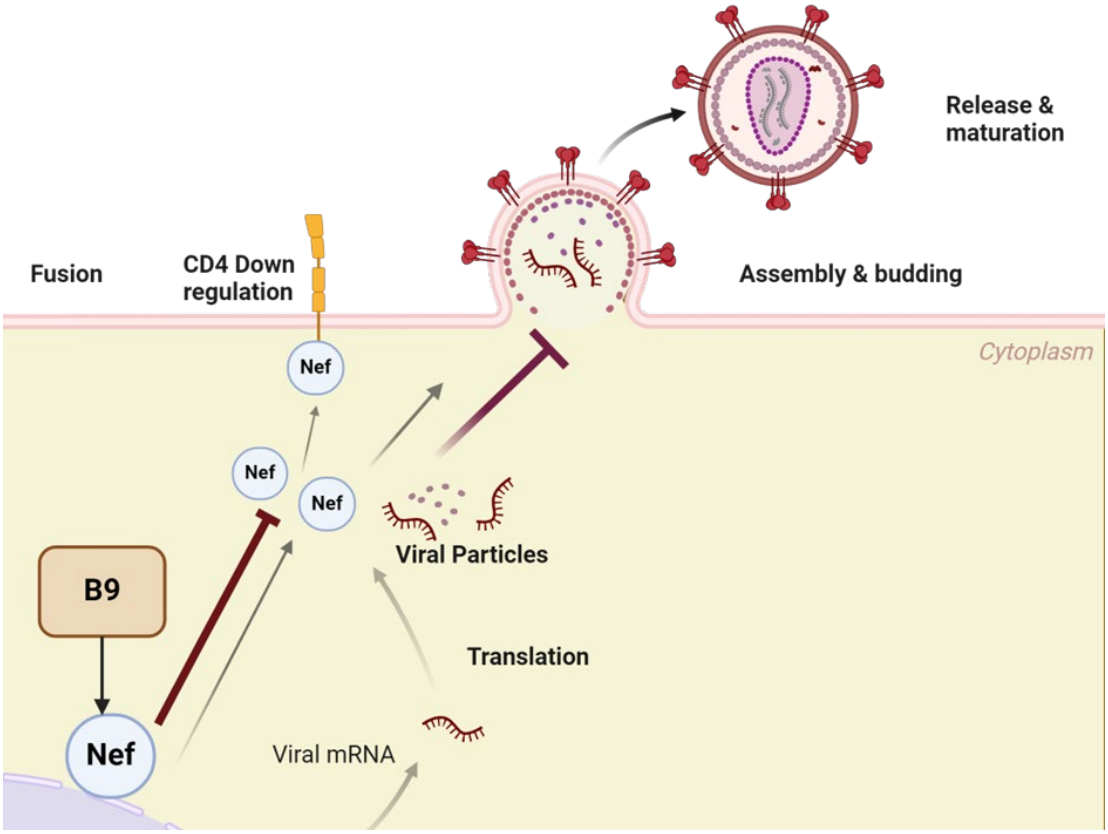

Figure 5: Effect of B9 Nef Inhibitor on Nef ***(Created with Biorender)***

This finding suggests a potential strategy for targeting persistent viral reservoirs and enhancing immune-mediated control of HIV infection. B9 disrupts Nef homodimerization, a crucial step for Nef-mediated pathogenicity. By preventing Nef homodimer formation, these inhibitors may interfere with downstream signaling pathways involved in viral replication and immune evasion. Moreover, computational docking studies indicate a common binding site for Nef inhibitors, supporting their efficacy against a broad range of HIV-1 subtypes (91-96).

2.0 Specific Aims

Antiretroviral therapy (ART) has been effective in managing HIV and improving the quality of life for those living with the virus. However, multidrug-resistant strains of HIV pose a persistent challenge. Previous experiments using a BLTS-humanized mouse model demonstrated that Nef homodimers play a crucial role in HIV viremia and immune dysregulation. More specifically, Nef enhances HIV replication and associated immune dysregulation. Disrupting Nef dimerization activity correlated with reduced viremia outcomes and less severe impacts on T cell subsets. *I hypothesize that the Nef inhibitors can reduce Nef mediated immune dysfunction in HIV infected CD4 T cells*. A deeper understanding of the mechanisms underlying Nef-mediated immune dysregulation and exploring the potential of Nef inhibitors could contribute to developing novel strategies to combat multidrug-resistant HIV strains and enhance the effectiveness of current HIV therapies.

To test and validate my hypothesis, I propose the following aims:

AIM 1: Determine the effect of Nef and Nef inhibitors in CEM T-4 cell lines independent of HIV infection:

A. Induce Nef expression on CEM T4 cell line to examine its impact on CD4 T cells.
B. Perform transcriptional analysis on cells to understand the cellular impacts of Nef
C. Treat Nef-expressing cells with B9 Nef inhibitor to determine the impact on CD4 expression.

AIM 2: Assess the effect of Nef dimerization mutants and Nef inhibitor on viral replication and cell death in HIV-infected human primary CD4 T cells.

A. Treat HIV-infected CD4 T cells with B9 Nef inhibitor to investigate and compare virus replication and cell viability to HIV WT infection.
B. Treat HIV-infected CD4 T cells with Nef dimerization mutants to compare and investigate cell viability and virus replication.
C. Treat HIV-infected CD4 T cells with Nef dimerization mutants and B9 Nef inhibitor to compare and investigate immune modulation.

3.0 Materials and Methods

3.1 CD4 T Cell Isolation and Activation

The study utilized human peripheral blood cell samples from two healthy adult donors. To isolate CD4 T cells, magnetic anti-CD4 Micro Beads (Miltenyi Biotec # 130-096-533) were used following the product's instructions. PBMCs were re-suspended, washed with PBS buffer, and cultured with Micro Beads. The cell suspension was loaded onto a MACS Column in the magnetic field of a MACS Separator, where the magnetic bead labeled CD4 T cells were retained. After removing the column from the magnetic field, the bead-labeled CD4 cells were collected. Following the manufacturer's instructions, the T cells were stimulated with anti-CD3/CD28 activating Dynabeads (Human T-Activator Beads; Thermofisher, Cat# 11131D, USA). The CD4 T cells were suspended in 5mL Complete T cell Growth Medium (RPMI-1640 containing 20% heat-inactivated FBS, 50 IU/ml IL-2, and 50 µg/ml gentamicin) in a T75 Flask. Pre-washed Dynabeads were added at the bead-to-cell ratio 1:1 and incubated in a humidified CO_2 incubator at 37℃ for 72 hours.

3.2 Infection of Activated CD4 T-Cells

The isolated and activated CD4 T-Cells were then infected with various strains of high titer MT2 (25,500 IU) HIV in equal titers (wild-type HIV (WT), a Nef-deleted mutant (ΔNef), or a Nef-dimerized mutant (Y115D). Infected cells were incubated in complete T cell Growth Medium

(RPMI-1640 containing 20% heat-inactivated FBS, 50 IU/ml IL-2, and gentamicin (50 µg/ml) in a 12-well plate and maintained in a humidified CO_2 incubator at 37℃ for 24 hours. Mock cells were uninfected cells maintained under similar conditions.

3.3 Inhibitor Study

The effect of B9 (Cat# 5006530001, Sigma Aldrich) was evaluated in CEM-T4 cells and CD4 T cells isolated from PBMCs. CEM-T4 cells with a Tet-induced expression of GFP and Nef GFP were induced using doxycycline and cultured in CEM Growth Medi. 1 x 10^6 cells were plated in a 12-well culture plate in a final culture volume of 1.5 ml in the presence of 6 µM, 1 µM, 0.3 µM, 0.1 µM or the DMSO carrier solvent alone as a control. The cells were then incubated in a humidified CO_2 incubator at a temperature of 37°C in a CEM growth medium for 8 days; the cells were monitored, and activity was assessed every 24 hours using flow cytometry. CD4 T cells were infected with 25500lU of high titer MT2 HIV (wild-type HIV, a Nef-deleted mutant (ΔNef), or a Nef-dimerized mutant (Y115D)). 1 x 10^6 cells were plated in a 12-well culture plate in a final culture volume of 1.5 ml in the presence of 1 µM, 0.3 µM, or the DMSO carrier solvent alone as a control. The cells were then incubated in a humidified CO_2 incubator at a temperature of 37℃ in a T cell Growth Medium for a period of 7 days. The cells were monitored, and activity was assessed on day 3 and day 7 using flow cytometry and MTT Assay. The supernatant was collected, viral replication was assessed using p24 ELISA, and cytokine content was measured by the Cytokine Bead Array Flow Cytometry Assay.

3.4 MTT Assay

To assess the cytotoxic effect of HIV infection on CD4 T cells, we utilized a colorimetric assay. CD4 T cells, which were infected with HIV and treated with B9, were monitored for 7 days. On days 3 and 7 of incubation, cell activity was assessed using the MTT Assay. CD4 T Cells ($1x10^5$) were plated in a 96-well plate.10 µL of a 12-mM MTT stock solution was added to each well, including a negative control with MTT stock solution added to the medium alone. The plate was incubated for 2–5 hours at 37°C, after which 50 µL of DMSO was added to each well. The plate was incubated for 10 minutes at 37°C, and the absorbance was read at 540 nm using an ELISA microplate reader. The data obtained was analyzed using GraphPad Prism (GraphPad Software).

3.5 p24 ELISA

To quantify viral replication during HIV infection and the impact of the B9 Nef inhibitor, supernatant collected from infected cells on day 3 and day 7 was analyzed using the HIV-1 Gag p24 Quantikine ELISA Kit (CAT# DHP240B). The supernatants were tested following the assay protocol described in the kit.

The Standard was reconstituted, resulting in the production of a stock solution containing 5000 pg/mL of the standard protein. The standard solution was mixed thoroughly to maintain homogeneity. 900 µl of Calibrator Diluent RD5-26 (diluted 1:4) was pipetted into the designated tube labeled 500 pg/mL. Subsequently, 500 µl of the same diluent was pipetted into each of the

remaining tubes. The tube containing the 500 pg/mL standard served as the high standard, while the Calibrator Diluent RD5-26 (diluted 1:4) acted as the zero standard (0 pg/mL).

The plate was prepared by adding 100 µl of Assay Diluent RD1-124 to each well. Then, 100 µl of the standard, control, or sample was added to each respective well, and the plate was securely covered with the adhesive strip provided. It was incubated at 37°C for 2 hours. Following the incubation period, the plate was washed using an ELISA plate washer. After washing, 200 µl of the HIV-1 Gag p24 Conjugate was added to each well, followed by covering the plate with a fresh adhesive strip. Incubation was conducted for a duration of 2 hours at 37°C. The aspiration and washing process was repeated.

A volume of 200 µl of Substrate Solution was added to each well, which was incubated in the dark for 30 minutes at 37°C. 50 µl of Stop Solution was added to each well, prompting a color change from blue to yellow. Optical density determination of each well was promptly conducted within 30 minutes using a microplate reader set to 450 nm. The resulting data was analyzed using GraphPad Prism (GraphPad Software).

3.6 Flow Cytometry

To assess Nef expression in CEM T4 cells, infected cells, and cells treated with B9 inhibitor, 1×10^6 cells were seeded into a 12-well culture plate. The CEM T4 cells were analyzed every 24 hours for a period of 8 days, and the CD4 cells infected were analyzed on day 7 post-infection. The cells were then collected by centrifugation and washed with 1 mL of 1% FBS in PBS (FACS buffer). Subsequently, single-cell suspensions were stained with 2 µl of a LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific CAT# L34957) and

incubated for 20 minutes. Then, the cells are washed using 150 µl of wash buffer. Then 2 µl fluorochrome-conjugated antibodies targeting specific cell markers (anti-human CD3-BioLegend Cat. No. 300312, anti-human CD3-BioLegend Cat. No. 563852, anti-human CD4-BioLegend Cat. No. 317410, anti-human CD8-BioLegend Cat. No. 300906, mouse anti-human CD4- BD Pharmingen™ Cat. No. 561030) and 48 µl of FACS wash buffer is added to the cells and incubated for 20 minutes. Then, the cells are washed 2 times with 150 µl of FACS wash buffer. The stained cells were transferred to round-bottom tubes using 2% PFA and analyzed using flow cytometry. A flow cytometer BD LSR Fortessa™ cell analyzer - flow cytometer (BD Biosciences) was used for data acquisition. The acquired data were then analyzed using FlowJo software. Initially, leukocytes were selected based on forward and side scatter characteristics. Subsequent analysis of various human lymphocyte populations and subsets was gated on human leukocytes.

3.7 Cytokine Bead Array Assay

The isolated and activated CD4 T-Cells were infected with various strains of high titer MT2 (25500 IU) HIV in equal titers and subjected to inhibitor B9. The culture supernatants were quantified for soluble immune checkpoint molecules using the LEGENDplex™ HU Essential Immune Response Panel (13-plex) w/VbP (BioLegend, catalog # 740930). The supernatants were tested following the assay protocol described in this manuscript. Data were analyzed using the LEGENDplex™ Data Analysis Software (BioLegend, PC version 8) and GraphPad Prism (GraphPad Software).

The standard was prepared by reconstitution of the lyophilized Human Essential Immune Response Panel Standard Cocktail with 250 µL of assay buffer. Following a resting period of 10

minutes at room temperature, the standard was transferred to appropriately labeled polypropylene microfuge tubes. Serial 1:4 dilutions were performed to obtain standard concentrations ranging from the top standard (C7) to the lowest standard (C0), utilizing assay buffer as the 0 pg/mL standard (C0).

50 µl of the sample was diluted with an equal volume (50 µl) of Assay Buffer in a V bottom 96 well plate. Mixed beads were vortexed for 30 seconds before adding 25 µl to each well, ensuring a total volume of 75 µl per well. The plate was sealed with a plate sealer, covered with aluminum foil, and incubated for 2 hours at 37°C. The supernatant was swiftly discarded by inversion and flicking of the plate. Plate washing was performed by dispensing 200 µl of 1X Wash Buffer into each well, followed by incubation for one minute. Detection Antibodies (25 µL) were added to each well, and the plate was sealed, covered with aluminum foil, and incubated for 1 hour at 37°C. Without washing the plate, SA-PE (25 µL) was added directly to each well, followed by sealing, foil covering, and shaking for 30 minutes at 37°C. Plate washing and bead resuspension steps were repeated to reduce background noise further and ensure optimal assay performance. 1X Wash Buffer (150 µL) was added to each well, and beads were resuspended by pipetting. Samples were read on a flow cytometer for subsequent data analysis.

3.8 Single-Cell Sequencing Analysis

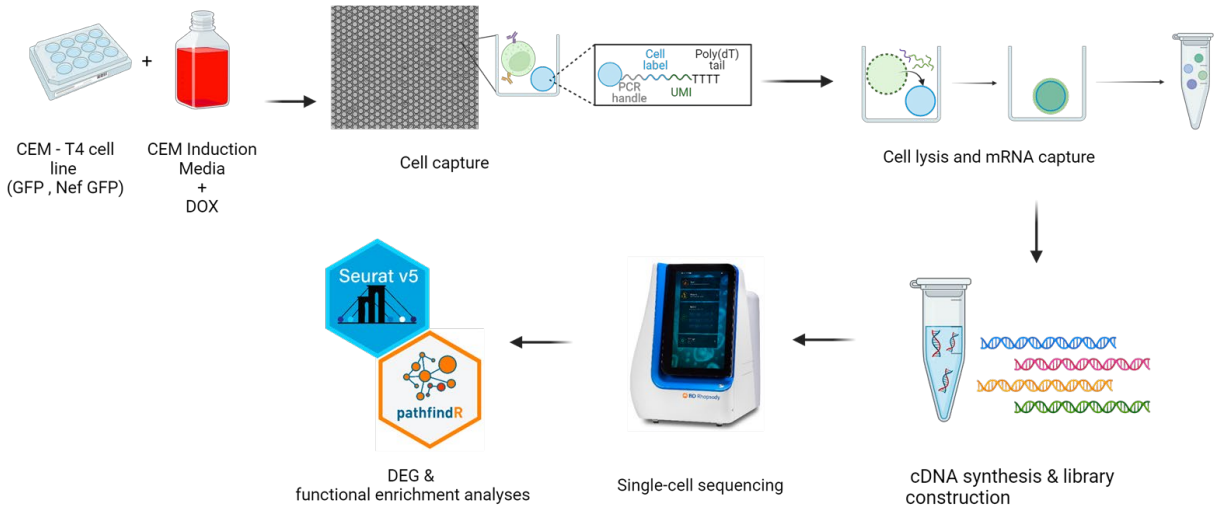

Figure 6: Single-Cell Sequencing Methodology ***(Created with Biorender)***

CEM-T4 cell lines expressing either GFP or Nef-GFP under control of a tetracycline-responsive promoter were obtained from Dr. Thomas Smithgall. The cells were then treated with 25 ng/mL doxycycline for 24 hours to induce transgene expression as described previously (97). After confirmation of eGFP expression by flow cytometry, the cells were stained with condition-specific oligomer-conjugated multiplexing antibodies. The cells were then loaded onto a BD Rhapsody cartridge with capture beads, lysed, and mRNA was captured according to manufacturer protocols. The captured mRNA was then reverse-transcribed into cDNA, and Illumina-compatible libraries were constructed from the cDNA. These libraries were sent to Emory for paired-end sequencing on an Illumina NovaSeq 6000 to obtain high-throughput transcriptomic data. Emory gave the data in the form of FastQ R1 and R2 files. The R1 files contained the identifications of

the tags, and R2 contained the details of the bioproducts. Upon receiving the sequencing data, it was run through the BD Whole Transcriptome Analysis Pipeline on the Seven Bridges Genomics platform that performed QC filtering cell calling. The resulting data were analyzed using the Seurat package version 5.0.3 in R Studio version 4.3.2 (98). Differential expression gene (DEG) analysis and functional enrichment analysis were conducted using the Seurat package in R. Principal component analysis (PCA) was performed to reduce the dimensionality of the data and identify major sources of variation between samples. Uniform Manifold Approximation and Projection (UMAP) was used to visualize high-dimensional transcriptomic data in two dimensions with the scPubr package, allowing for the identification of distinct cell clusters (99). Volcano plots were generated using the Enhanced Volcano package to visualize the relationship between gene expression fold change and statistical significance, highlighting genes that were significantly differentially expressed between experimental conditions (100). The pathfindR package version was used to perform pathway analysis (101). Additionally, dot plots were created to visualize the expression levels of specific pathways or gene sets, enabling the assessment of pathway activity across different experimental conditions.

4.0 Results

4.1 AIM 1: Effect of Nef and Nef Inhibitors in CEM T-4 Cell Lines Independent of HIV Infection

4.1.1 Downregulation of CD4 in Nef GFP Upon Induction with DOX

A CEM-T4 cell line with Tet-induced expression of GFP and Nef GFP was used to look at the effect Nef has on CD4 surface protein expression. The cells were cultured in CEM growth media and activated with 25 ng/ml DOX for stimulation. The cultures were then maintained for 48 hours, with data collected at various time points (1, 2, 4, 8, 24, and 48 hours).

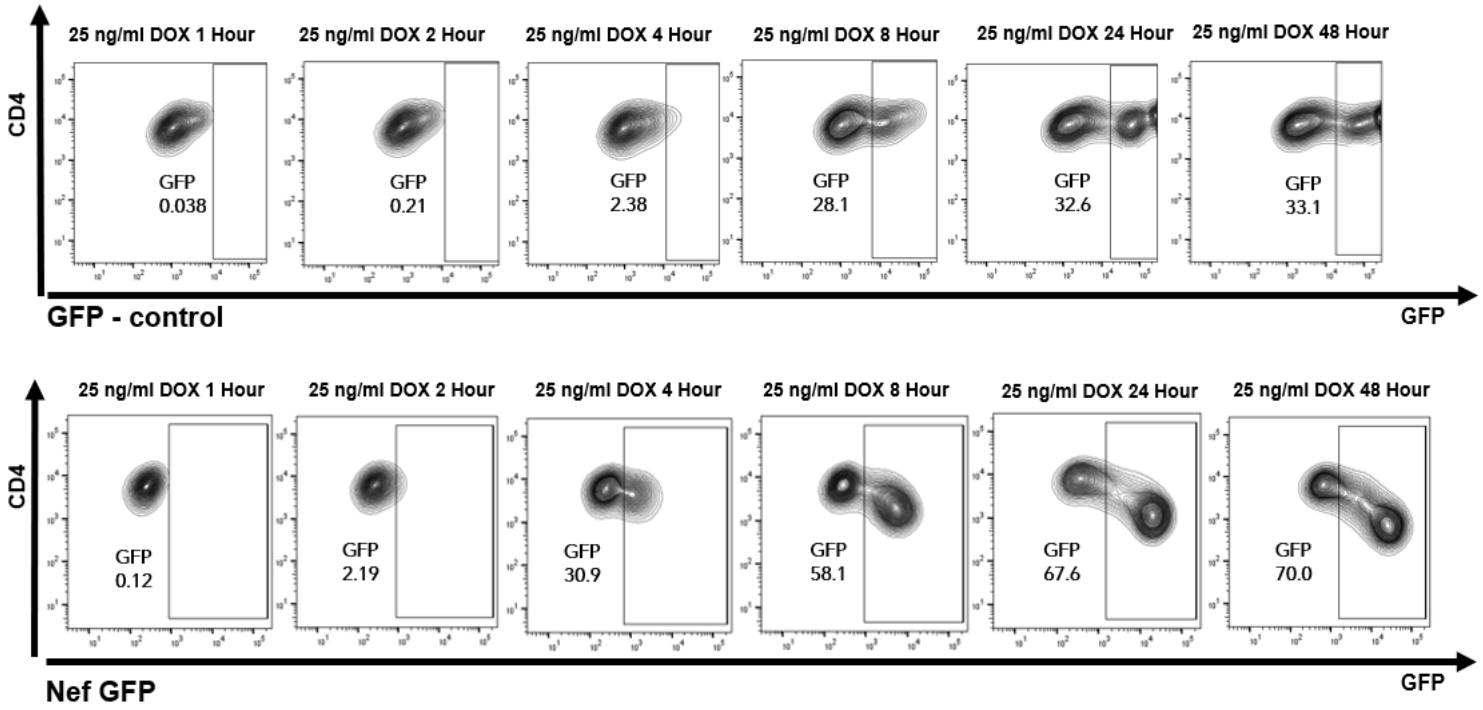

Figure 7: Downregulation of CD4 Expression by Nef Upon Induction of Nef GFP with DOX

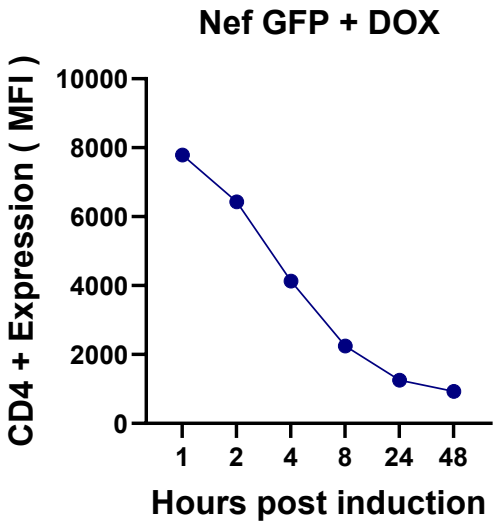

Figure 8: Relative MFI of CD4 Expression Downregulation by Nef Induced by Dox in Nef GFP

The flow cytometric dot plots in Figure 7 depict the downregulation of CD4 surface protein expression in CEM-T4 cells in the DOX-induced Nef GFP-expressing cells. The CD4-positive cells induced to express GFP were gated on, and the relative expression and downregulation of CD4 were determined. Figure 8 shows the summarization of the downregulation of CD4 expression over 48 hours recorded as mean fluorescence intensity (MFI). Our findings indicate that upon induction with DOX, Nef expression led to a notable downregulation of CD4 starting within hours post-induction.

4.1.2 CD4 Expression in Nef GFP Improves in the Presence of Nef Inhibitor

Flow cytometric analysis was performed to look at the effects of the Nef inhibitor B9 on CD4 expression levels and Nef downregulation in CEM T4 cell lines expressing both GFP and Nef GFP. The CEM T4 cells were cultured in CEM growth media and stimulated with 25ng/ml DOX for 24 hours to induce Nef expression. Subsequently, different concentrations of the Nef

inhibitor B9 (6 μM, 1 μM,0.3 μM, and 0.1 μM) were added to the cells, and the cultures were maintained for 8 days. Cell samples were collected at various time points (0, 24, 48, 72, 96, 120, 144, 168, and 192 hours) to investigate the effects of B9 on the cell lines and the potential restoration of CD4 expression.

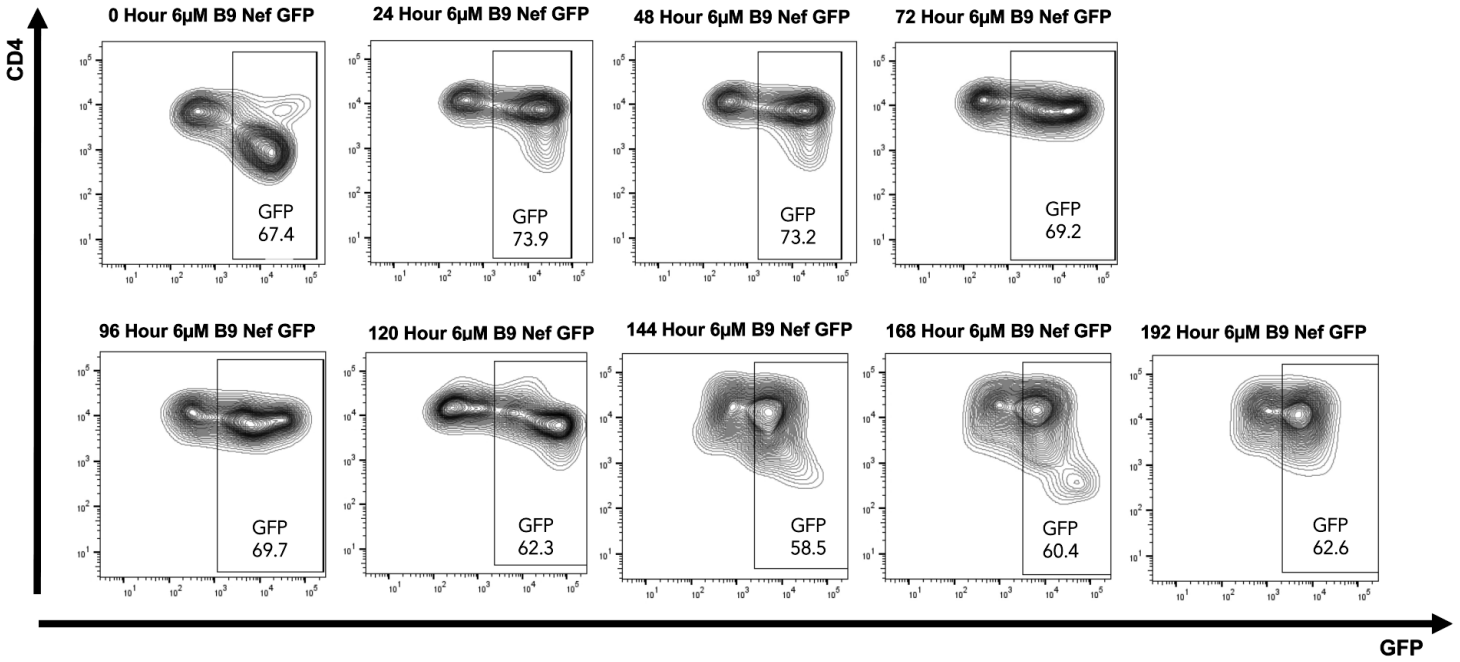

Figure 9: CD4 Expression in Dox-Induced Nef GFP Increases with B9 Treatment

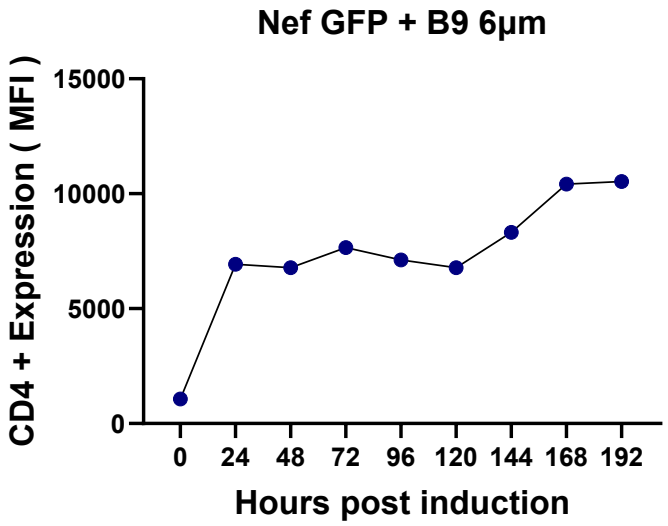

Figure 10: Relative MFI of CD4 Expression in HIV-Infected T Cells Increases with B9 Treatment

Flow cytometric analysis was performed to examine CD4 downregulation in Nef GFP-expressing cells following induction. The flow cytometric dot plots depicted the relationship between CD4 T cells and GFP expression. A gate was applied around the CD4-positive cells, and the percentages of Nef downregulation were determined by assessing GFP fluorescence against Nef expression. Figure 9 shows the analysis of the experiment presented in the form of a flow cytometric dot plot, which demonstrates the reverse downregulation of CD4 T cells in response to Nef GFP expression upon induction in the presence of the Nef Inhibitor at 6 µM. The highest concentration had shown the highest correction of CD4 compared to the other concentrations. Figure 10 shows a summarization of the effects on the CD4 expression in the presence of a B9 Nef inhibitor as MFI for 192 hours. The results of the analysis indicate that B9 can restore the expression of CD4, which was brought down by Nef at high concentrations.

4.1.3 Multiomic Analysis of Nef GFP and GFP Induced by Doxycycline

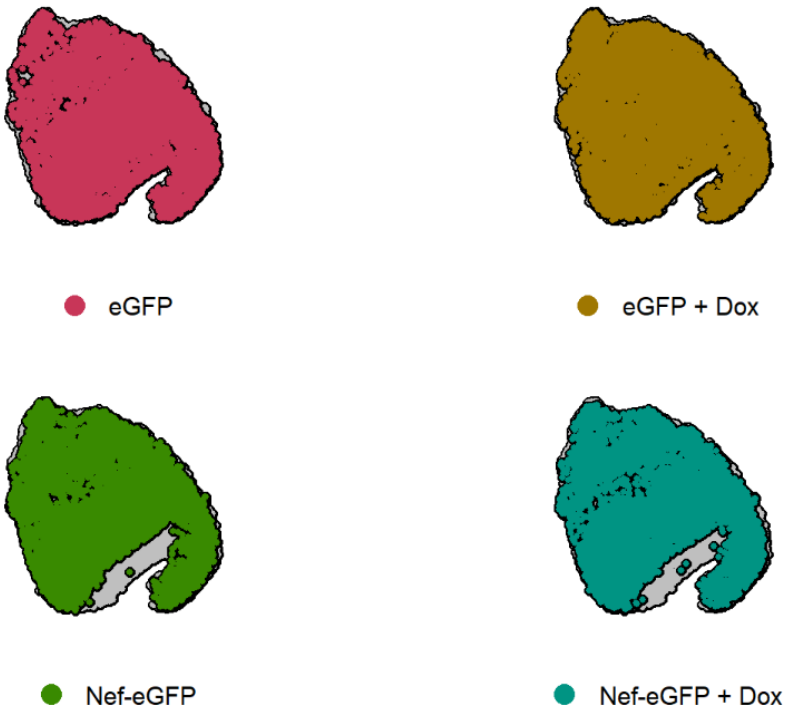

Figure 11: Uniform Manifold Approximation and Projection (UMAP) of Nef GFP and GFP Shows No Distinct Clusters

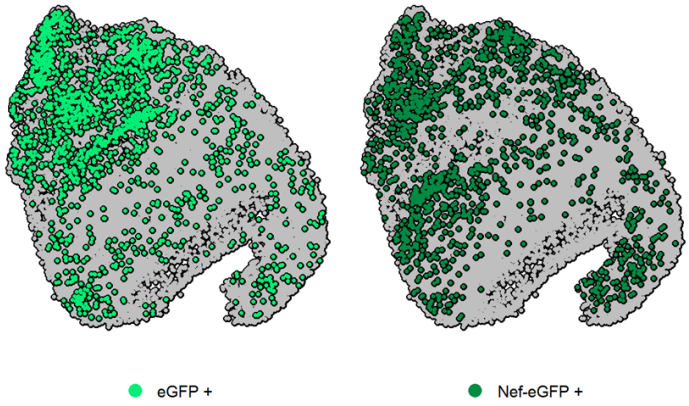

Figure 12: Uniform Manifold Approximation and Projection (UMAP) of Nef GFP and GFP Upon Induction Shows Similar Yet Distinct Expressions of Genes

The CEM T4 cell lines were induced to express GFP and Nef GFP with exposure to 25 ng/ml doxycycline, and subsequent single-cell RNA-seq analysis was conducted to examine their transcriptional landscapes. The analysis, depicted in Figure 11 using UMAP, did not reveal notable differences among the clusters, suggesting limited heterogeneity across the samples. Overall, this suggests that the major cellular impact of Nef may not be due to its influence on transcriptional activity. However, upon closer examination, it was observed that induced cells exhibited distinct gene expression patterns from each other (Figure 12), suggesting that Nef expression may, in part, be influencing certain genes.

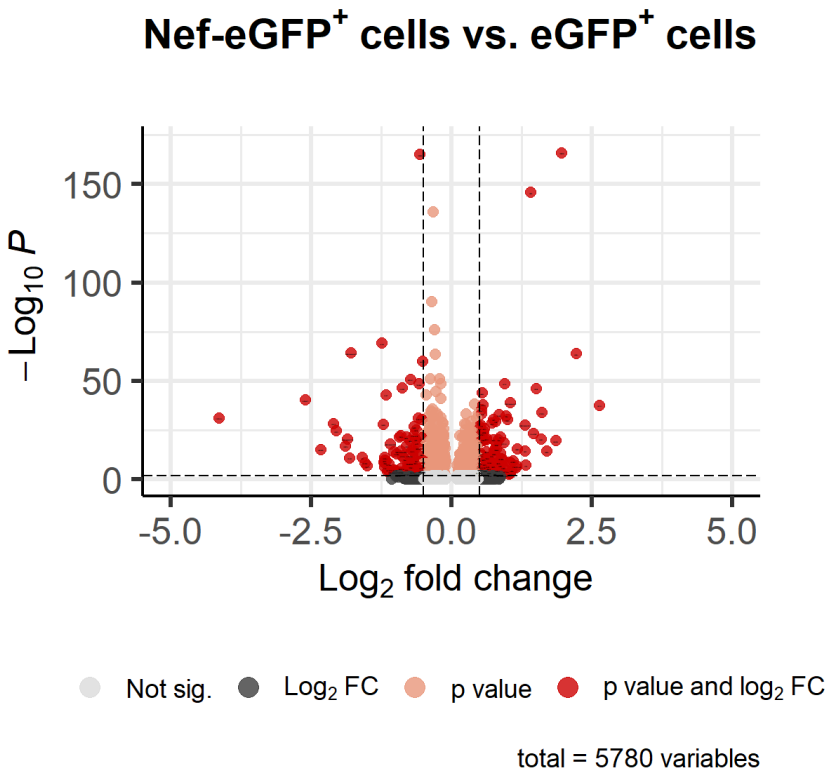

Figure 13: Volcano Plot Depicting Differential Expression of Genes in CEM T4 Cells Induced to Express Nef GFP vs GFP-Induced

To further explore the effects of Nef expression, Figure 13 illustrates a volcano plot, with red dots on the right representing genes with higher expression levels in Nef GFP-expressing cells, while red dots on the left represent genes with higher expression levels in GFP-expressing control cells. The Y-axis represents − log10 P values, indicating statistical significance, and the X-axis shows log2 fold change values, indicating the magnitude of change in gene expression. The analysis showed that the Nef GFP cells, induced by doxycycline-activated Nef expression, showed significant expression of the Nef GFP gene. The volcano plot shows certain changes in genes in the Nef GFP-induced cells. This suggests that while the overall transcriptional landscape may not show distinct differences, there are specific genes whose expression is significantly altered in

response to Nef expression. Additionally, genes that exhibited statistically significant changes in expression levels in response to Nef expression were identified and analyzed using a DOT plot.

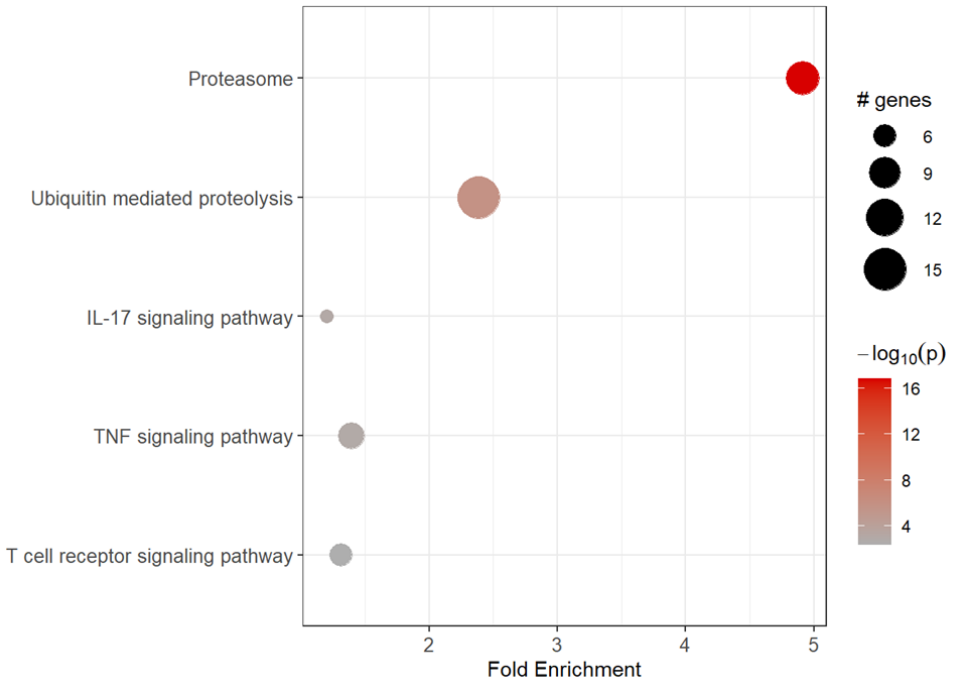

Figure 14: Gene Expression Pathways Most Affected by Nef Upon Induction Determined by scRNA-Seq Analysis

The dot plot analysis in Figure 14 of Nef-GFP expression in the CEM T4 cell lines induced with doxycycline revealed significant alterations in key cellular pathways. The size of expression shows the number of cells expressing the genes, and the increasing intensity of the color shows the significance of the genes expressed. This analysis indicated a large and bright red representation of the Upregulation of Proteasome Pathway and Ubiquitin-Mediated Proteolysis pathways, suggesting their heightened activity in cells expressing Nef-GFP. The dot plot also showed that Nef causes changes to the IL-17, TNF-alpha, and T-cell signaling Pathways expression levels. These pathways are crucial for maintaining cellular homeostasis and antigen

presentation and may be augmented to accommodate the high metabolic demands of expressing Nef-GFP proteins. Overall, these findings suggest that the expression of Nef-GFP in CEM T4 cells induces substantial changes in their transcriptional landscape, favoring pathways involved in protein degradation while suppressing other immune functions.

4.2 AIM 2: Effect of Nef Dimerization Mutants and Nef Inhibitors in Viral Replication and Cell Death in Human Primary CD4 T Cells

4.2.1 Nef Inhibitor B9 Reduces Viral Replication in Primary CD4 Cells

In this experiment, CD4 T cells were isolated from peripheral blood mononuclear cells (PBMCs) using CD4 capture beads and activated using anti-CD3/CD28 activation beads. After activation, the primary cells were infected with the HIV-WT strain 3 days after stimulation. They were then treated with a Nef inhibitor, specifically the Nef dimerization inhibitor B9, to observe its impact on viral infectivity and replication in primary T cells.

The levels of infectivity and replication were measured using an enzyme-linked immunosorbent assay (ELISA) that quantified p24 content. A mock-uninfected control group was also included for comparative analysis. The cells were treated with varying concentrations of the Nef inhibitor B9 (1 μM and 0.3 μM), and all experiments were conducted in quadruplicate to ensure statistical robustness. Figure 15 shows the results obtained from the p24 ELISA on Day 7. The analysis shows a gradual decrease in viral replication in the infected cells as the concentration of the Nef inhibitor B9 increased.

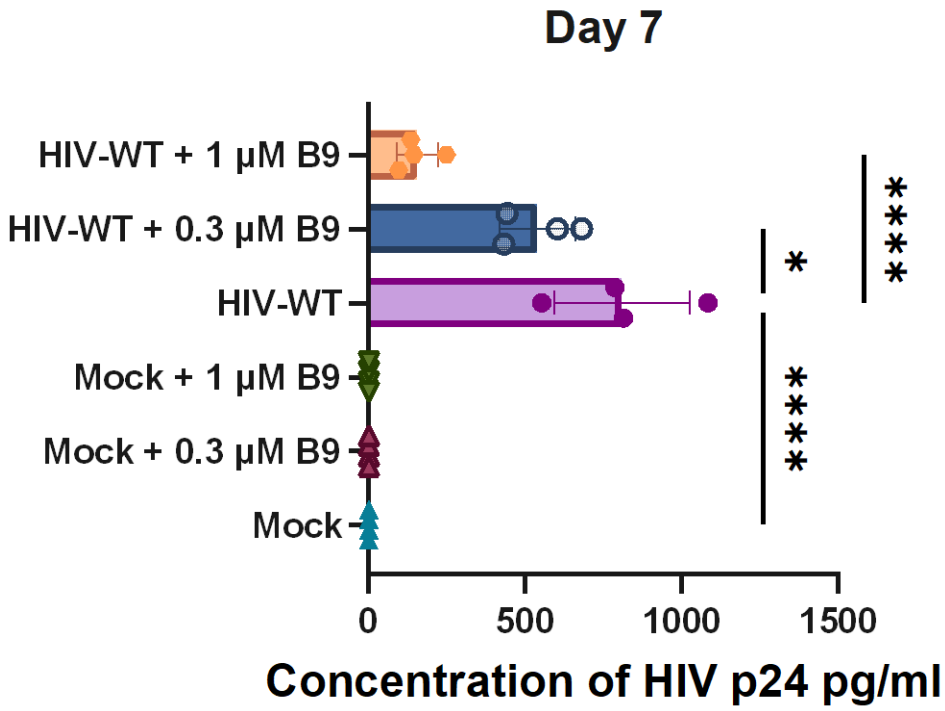

Figure 15: Nef Inhibitor B9 Decreases Viral Replication in HIV-Infected Primary CD4 T-Cell Day 7 Post-Infection (N=4) [* = P≤ 0.05, **** = P≤ 0.0001]

4.2.2 Nef Inhibitor B9 Shows Reduced Cytotoxic Effects in Primary Cells

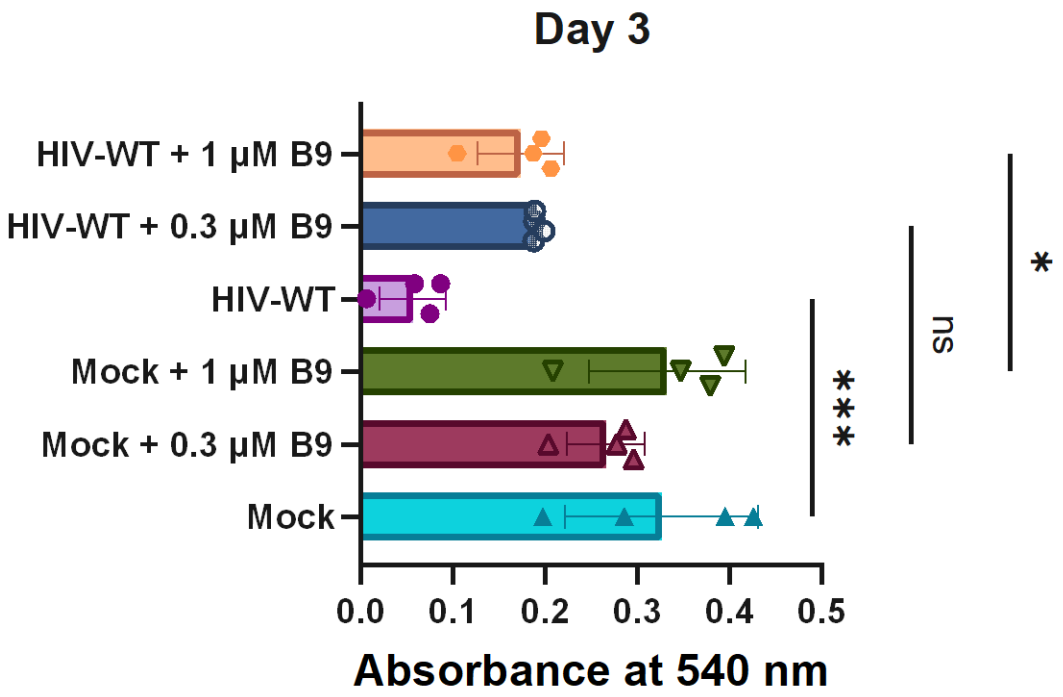

Figure 16: Nef inhibitor B9 Shows Improved Cell Viability in HIV-Infected Primary Cells and Cells Treated with B9 Day 3 Post-Infection (N=4) [Not Significant (ns) = P>0.05, * = P≤ 0.05, *** = P≤ 0.001]

Here, we examined the effects of Nef dimerization inhibitor B9 on cytotoxicity and cell viability in interaction with primary CD4 T cells infected with the HIV-WT strain. We used an MTT assay focused on CD4 T cells isolated and activated from PBMCs and infected with HIV-WT three days post-stimulation to determine cell viability.

After infecting the cells with the HIV-WT strain, we treated them with different concentrations of the Nef inhibitor B9 (1 µM and 0.3 µM). The cells were treated with MTT assay, and data was collected at OD 540 nm. We observed discernible trends in cell viability over time. Specifically, we evaluated the cell viability on days 3 and 7 post-infection. Figure 16 shows the

MTT analysis of the cells on Day 7 and found that the uninfected cells treated and untreated with B9 had much higher cell viability than the infected cells. The infected cells treated with B9 showed significantly better viability than the untreated cells when compared to uninfected cells. This showed that the B9 did not show much cytotoxic effects in primary cells.

4.2.3 Reduced Expression of Nef in HIV-WT Infected PBMCs with Increasing Concentration of Nef Inhibitor

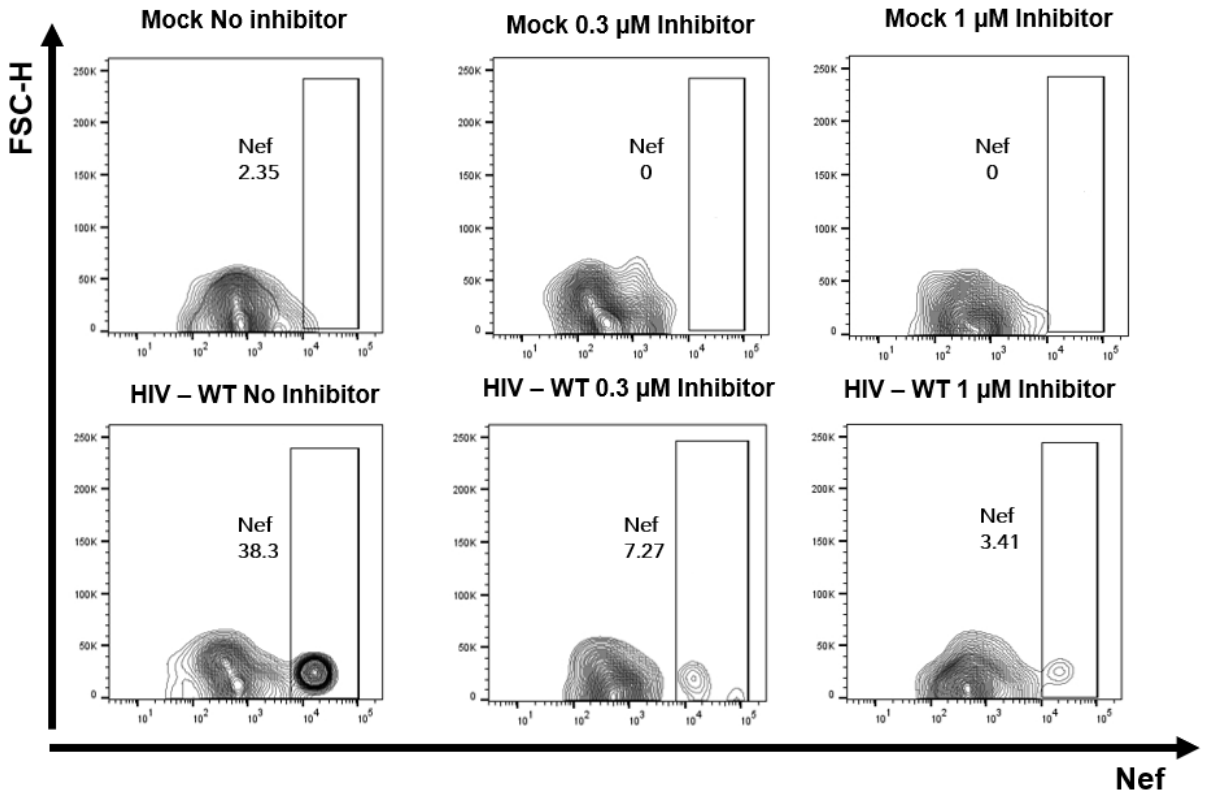

Figure 17: B9 Nef Inhibitor Reduced Nef Expression in CD4 T Cells in Primary Cells

To investigate the efficacy of the B9 Nef inhibitor in decreasing Nef expression at the cellular level, we utilized flow cytometry to analyze Nef protein expression in primary cells treated with varying concentrations of B9. Our experiment involved culturing CD4 T cells isolated from

peripheral blood mononuclear cells (PBMCs) in IL2 growth media, followed by activation with CD3/CD28 beads for stimulation. The cells were infected 3 days post-stimulation with the HIV-WT strain and treated with different concentrations of the Nef inhibitor B9 (1 μM and 0.3 μM). The culture was maintained for 7 days. Flow cytometry was performed to look at protein expression and asses if the inhibitor was able to reduce the Nef expression on day 7. Uninfected cells were used as a control to examine the inhibitor's effects on CD4 T-cells.

A gate was delineated around the CD4 cells to analyze the inhibitor's impact on viral replication. Figure 17 shows the contour plot showing that Nef expression is reduced in infected cells with increasing concentrations of Nef inhibitor treatment. To ensure statistical reliability, all experiments were conducted in quadruplicate.

4.2.4 Decreased Viral Infectivity and Replication in CD4 T Cells in the Presence of Nef Inhibitor and Nef Dimerization Mutants

We conducted a study comparing Nef dimerization mutants' impact with Nef inhibitor B9 on viral infectivity and replication in CD4 T cells derived from peripheral blood mononuclear cells (PBMCs). The quantification of viral infectivity and replication was based on p24 levels, using an enzyme-linked immunosorbent assay (ELISA). A mock-infected control group was included in the study to establish a baseline.

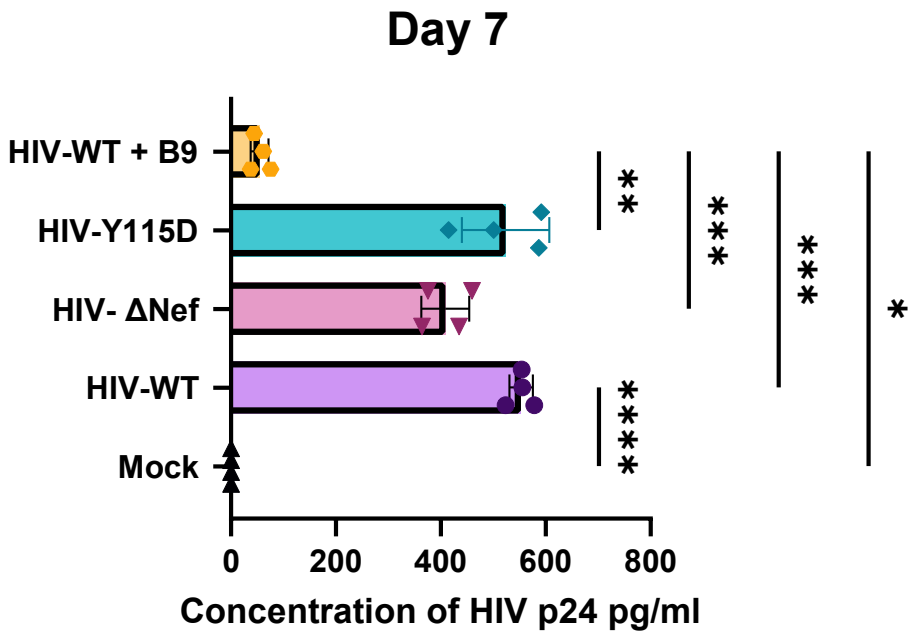

Figure 18: Nef Dimerization Mutants Show Increased Viral Replication Compared to Nef Inhibitor B9 in CD4 T Cells on Day 7 Post-Infection (N=4) [* = P≤ 0.05, ** = P≤ 0.01, *** = P≤ 0.001, **** = P≤ 0.0001]

First, CD4 T cells were isolated from PBMCs and then activated through stimulation. Afterward, we infected these cells with various strains of HIV, including HIV, HIV-ΔNef, and HIV-Y115D, all with equal virus titers. 24 hours after infection, we introduced 1pg/ml, the optimized concentration from the inhibitor study, of the Nef dimerization inhibitor B9 to the HIV-WT-infected cells. The data analyzed on day 7 post-infection is shown in Figure 18. We observed that HIV-WT had the highest viral replication, followed by the Nef dimerization strain Y115D and the Nef-deleted strain. Additionally, the cells treated with the inhibitor showed reduced viral replication. To ensure statistical reliability, all experiments were conducted in quadruplicate. Compared to the Nef dimerization strains, the B9 Nef inhibitor showed significantly reduced replication.

4.2.5 Nef Dimerization Mutants Have Decreased Cell Viability in CD4 T Cells Compared to Nef Inhibitor

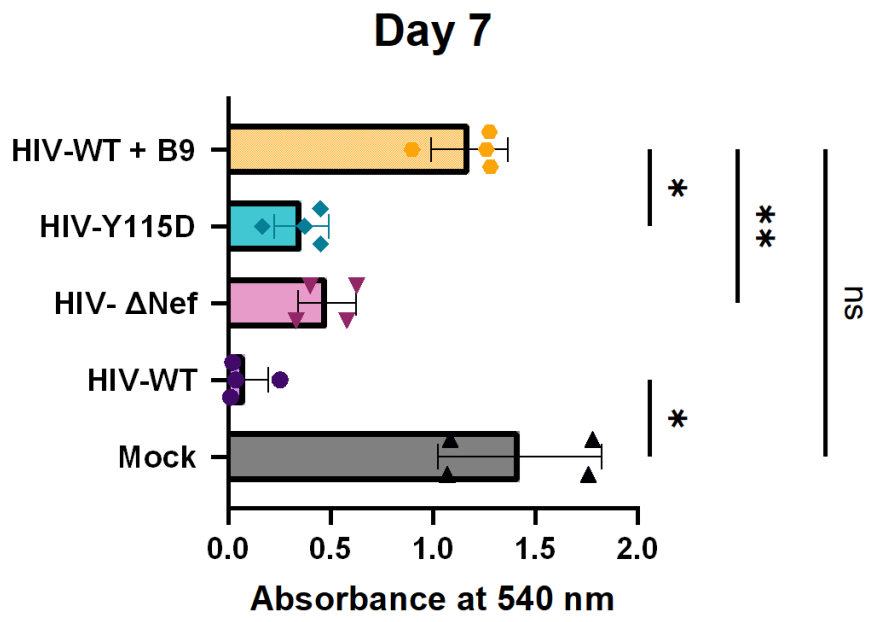

Figure 19: Nef Dimerization Mutants Show Decreased Cell Viability Compared to B9 Nef Inhibitor in CD4 T Cells 7 Days Post-Infection (N=4) [Not Significant (ns) = P>0.05, * = P≤ 0.05, ** = P≤ 0.01]

We conducted a study to determine how Nef dimerization mutants affect cell viability in CD4 T cells isolated from peripheral blood mononuclear cells (PBMCs) compared to Nef inhibitor B9. To assess cell viability, we used a colorimetric method known as the MTT assay. Additionally, we included a mock-infected control group to establish a baseline for comparison. First, we isolated CD4 T cells from PBMCs and stimulated them for activation. Next, we infected these 3 days' post-stimulation cells with various strains of HIV, including HIV, HIV-ΔNef, and HIV-Y115D, all with equal virus titers. 24 hours after infection, we introduced the Nef dimerization inhibitor B9 at a concentration of 1pg/ml to the HIV-WT-infected cells.

Figure 19 displays the data we analyzed 7 days after infection. We observed that HIV-WT had the lowest cell viability, followed by the new dimerization strains. However, the cells treated with inhibition showed better viability compared to infected cells. To ensure statistical reliability, we conducted all experiments in quadruplicates.

4.2.6 Cytokine Responses of B9 Inhibitor Similar to Uninfected Cells

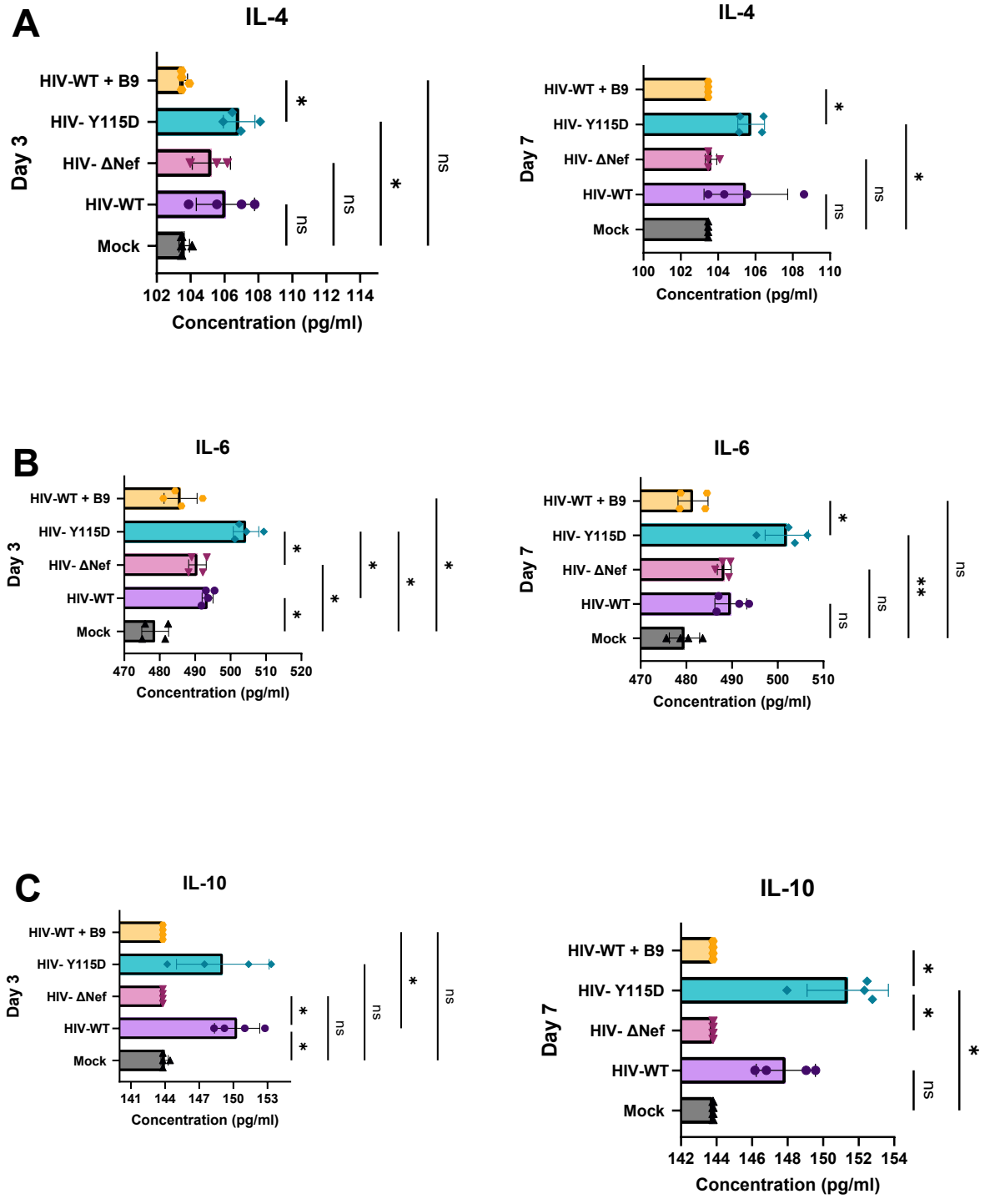

Figure 20: Cytokine Profiling of Nef Dimerization Mutants and Nef Inhibitor B9 in CD4 T-Cells Day 3 and 7 Post-Infection (A) IL-4 (B) IL-6 (C) IL-10 (D) IL-17 A (E) TNF-α (F) IFN-γ (N=4) Infected Cells Show Higher

Cytokine Expression [Not significant (ns) = P>0.05, * = P≤ 0.05, ** = P≤ 0.01, *** = P≤ 0.001, **** = P≤ 0.0001]

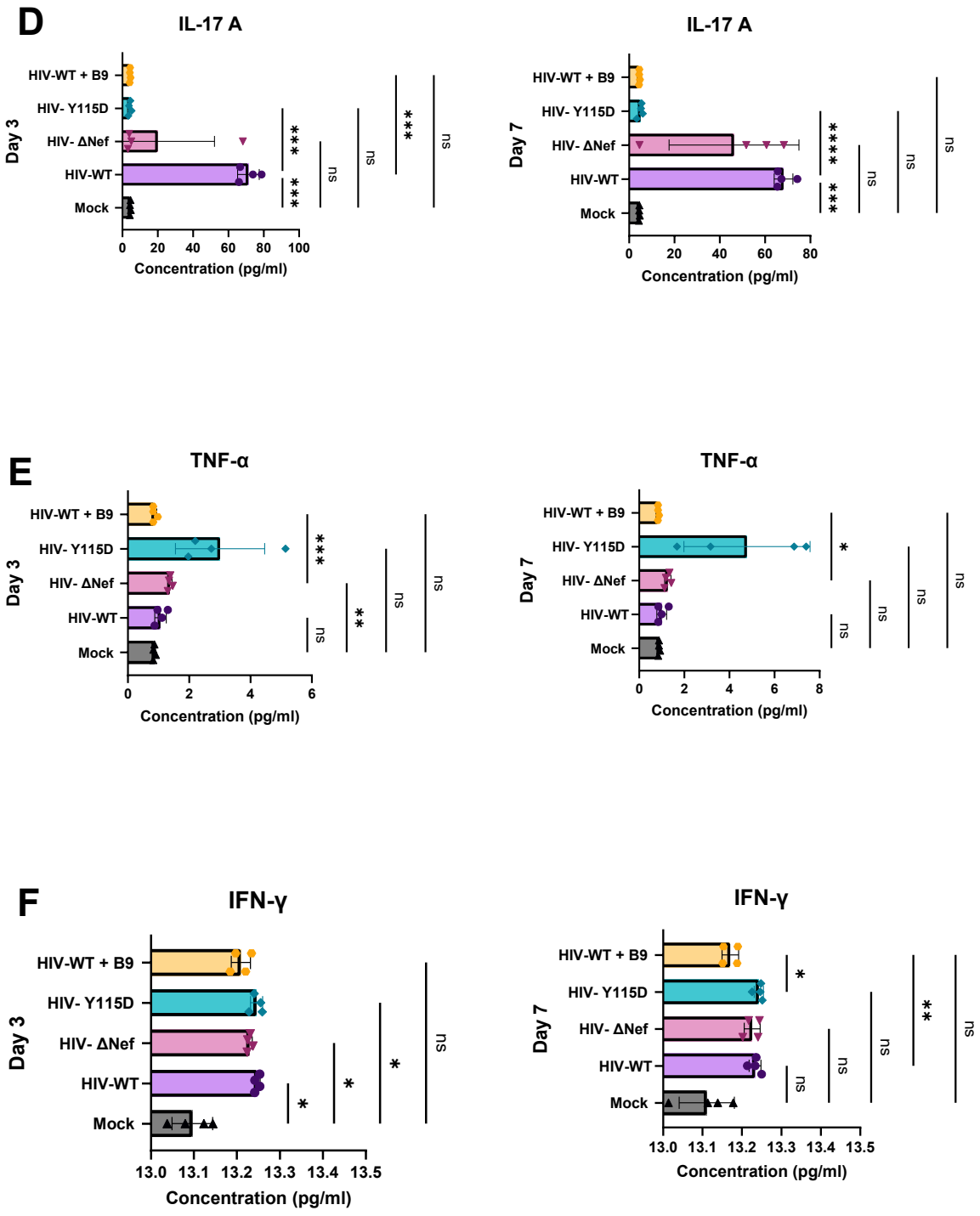

Figure 20: Cytokine Profiling of Nef Dimerization Mutants and Nef Inhibitor B9 in CD4 T-Cells Day 3 and 7 Post-Infection (A) IL-4 (B) IL-6 (C) IL-10 (D) IL-17 A (E) TNF-α (F) IFN-γ (N=4). Infected Cells Show

Higher Cytokine Expression [Not significant (ns) = P>0.05, * = P≤ 0.05, ** = P≤ 0.01, *** = P≤ 0.001, **** = P≤ 0.0001] (cont.)

To evaluate the cytokine profiles of CD4 T cells in response to Nef dimerization mutants and the B9 inhibitor, CD4 T cells were isolated from PBMCs, cultured in IL-2 growth media, and activated with CD3/CD28 beads for stimulation. Subsequently, CD4 T cells were infected 3 days post-stimulation with various strains of HIV, including HIV-WT, HIV-ΔNef, and HIV-Y115D, all at equal virus titers.

Upon infection, 1μM of the Nef dimerization inhibitor B9 was added specifically to the HIV-WT infected cells. The cultures were then maintained for a duration of 7 days. To assess the immune response, culture supernatants were collected on both day 3 and day 7 and analyzed using the LEGENDplex™ Human Immune Checkpoint assay. The results obtained showed in Figure 20 that in the presence of the B9 inhibitor, the cells exhibited a response similar to those of uninfected cells. Il-4, IL-6, and IFN-γ levels are shown to be expressed slightly higher in infected strains when compared to uninfected or cells treated with B9. The higher cytokine levels in the infected strains compared to the B9-treated and uninfected cells likely indicate an active immune response to the HIV infection. The elevated levels of cytokines in HIV-Y115D and HIV-WT indicated that Nef expression might be necessary for such a response to occur.

5.0 Conclusions and Discussion

Antiretroviral therapy (ART) is a critical component in the fight against HIV-1, significantly reducing the negative impact of the virus. However, despite its effectiveness, the development of multidrug resistance poses a significant challenge to HIV management. Multidrug resistance in HIV-1 can occur through various mechanisms, including genetic mutations that reduce the efficacy of antiretroviral drugs. This resistance can stem from two sources: transmitted drug resistance, where drug-resistant strains are passed between individuals, and acquired drug resistance, which occurs due to suboptimal adherence to ART. There has been an increase in transmitted drug resistance, surpassing the threshold of concern outlined by the World Health Organization. This undermines the efficacy of first-line ART regimens and limits treatment options for those newly diagnosed. Moreover, challenges like poor adherence to ART and continued viral replication in the presence of drugs contribute to the accumulation of resistance mutations, leading to the emergence of multidrug-resistant HIV-1 strains. This issue is particularly pressing in settings with limited access to newer antiretroviral agents and resistance testing, exacerbating the urgency to find a viable alternative (102-104).

The HIV-1 Nef protein plays a diverse role in enhancing viral infectivity, replication, and immune evasion while also contributing to broader immune dysregulation, leading to immunodeficiency and AIDS progression. Overall, Nef's complex effects on ART efficacy and the development of drug resistance underscore its significance. Its ability to sustain viral replication, evade immune surveillance, and interact with ART to disrupt cellular processes may all contribute to the challenges of achieving sustained viral suppression and preventing the emergence of drug-resistant HIV strains (74, 105, 106). Targeting Nef functions represents a potential strategy to

improve the effectiveness of ART or explore alternative treatment approaches. My thesis aims to establish the efficacy of Nef inhibitors on CD4 T-cells and to understand the effects of Nef on these cells. To achieve this, I proposed the following aims: 1) Investigating the effect of Nef and Nef inhibitors on CEM T-4 cell lines independent of HIV infection, and 2) Examining the effect of Nef dimerization mutants and Nef inhibitors on viral replication and cell death in CD4 T-cells in human primary cells.

Prior research shows that Nef is crucial for the establishment and maintenance of latent HIV-1, which can lead to co-morbidities. We have discovered Nef inhibitors that could promote HIV latency reversal, restoration of adaptive immunity, and reservoir reduction. Inhibitors have the potential to restore cell-surface MHC-I expression in latently HIV-infected CD4 T cells, allowing for recognition by CD8 T cells and enhancing HIV-infected CD4 T cell killing. Given that no FDA-approved drugs currently exist for this purpose, it is crucial to develop antiretrovirals that target Nef. In order to better understand the effects of Nef inhibitors, experiments were conducted using a CEM T4 cell line (107-111). To gain insight into Nef's ability to downregulate CD4 T-cells, I utilized a CEM T4 cell line with TET-induced expression of Nef GFP and GFP. This not only allowed me to focus on the effects of Nef expression but also the induction to express Nef, which mimics the expression of Nef during infection. Subsequently, I treated the cells with an inhibitor to evaluate if the B9 Nef inhibitor restored the CD4 expression. Doxycycline was used to induce expression on the cell line at a concentration of 25ng/ml. Over 48 hours (0, 2, 4, 8, 24, and 48h), it was observed that CD4 expression was downregulated by Nef starting at 8 hours post-induction. Then, I treated the cells with different concentrations of B9 post-induction (0.1, 0.3, 1, and 6μM) and observed the inhibitor's effects for 8 days. The data was analyzed by flow cytometry to see the expression of Nef and CD4 T cells. It was observed that Nef could mostly restore the

CD4 expression in the CEM T4 cell line at the highest concentrations and maintained till day 5, after which there is presumed cell death, consistent with previous studies performed at a lower concentration (112).

Single-cell RNA sequencing analysis was used to compare the transcriptomes of CEM T4 cells with Tet-induced expression of GFP and Nef GFP 24 hours post-induction. This analysis was selected based on the downregulation of CD4 observed through flow cytometry results. The first step was to perform principal component analysis (PCA), a form of dimensionality reduction. PCA groups similar data points together without explicitly indicating the differences, providing researchers with an overview of the similarities within the dataset. After PCA, uniform manifold approximation and projection (UMAP) was employed to condense the data into a more interpretable form. UMAP provides a bird's-eye view of the dataset, illustrating the heterogeneity within the clusters. However, the different conditions did not cluster separately, suggesting that the observed patterns were not solely attributable to the effects of Nef. Subsequently, differential gene expression analysis was conducted between cells expressing Nef GFP with and without doxycycline induction. Despite some cells not expressing Nef GFP in the presence of doxycycline, several significant genes were identified, with Nef GFP being one of them. The analysis revealed differences in gene expression levels, with red indicating higher expression and blue indicating lower expression.

Moreover, dot plots were employed to visualize gene expression levels, with darker dots indicating higher expression levels and larger dots signifying more cells expressing that gene. The dot plot analysis revealed an increase in the Proteasome Pathway and Ubiquitin-Mediated Proteolysis, indicating heightened protein degradation and turnover within the cells. These pathways are vital for maintaining cellular equilibrium and antigen presentation and may be

boosted to accommodate the metabolic demands associated with GFP and Nef-GFP protein expression. On the other hand, the dot plot for IL-17, TNF-alpha, and T Cell Signaling Pathways showed decreased expression levels. These pathways are crucial for immune responses, inflammation, and T-cell activation. The observed decrease suggests Nef expression may hinder normal T-cell signaling and mitigate inflammatory responses within these cells, inducing substantial changes in their transcriptional landscape, favoring pathways involved in protein degradation while suppressing normal immune functions. including cell signaling, apoptosis, protein processing, immune response, and DNA repair (113-115).

The statistical significance of these expression patterns underscores their biological relevance, indicating that the observed changes in pathway activity are not random fluctuations but meaningful alterations induced by Nef expression. This provides insight into how the HIV Nef protein manipulates cellular processes to support viral replication and evade immune surveillance. However, it is important to note that single-cell RNA sequencing analysis provides insights primarily at the mRNA level and may not fully capture post-transcriptional regulatory mechanisms. Nef performs its functions post-transcriptionally, and since we are looking at a cancer cell line, the expression observed in terms of RNA may not all be caused by Nef. Therefore, it is likely that PBMCs would have less background activity and reveal greater transcriptional differences caused by Nef. Nonetheless, these analyses offer valuable insights into the transcriptional changes associated with Nef expression in CEM T4 cells (116, 117).

Previous studies utilized the bone marrow-liver-thymus-spleen (BLTS) humanized mouse model to explore the interactions between HIV Nef and the human immune system. These studies showed that Nef deletion hindered HIV replication in BLTS-humanized mice. Additionally, the use of dimerization-defective Nef mutants, based on previous X-ray crystal structures,

demonstrated that Nef dimerization promotes robust HIV replication in vivo. The research also indicated that Nef dimers enhance T cell-mediated immune activation, checkpoint inhibitor expression, and dysregulation of various immune signaling pathways in vivo. However, despite the valuable insights gained from the BLTS humanized mouse model, certain limitations existed.

While this model incorporates human innate and adaptive immune cells, as well as primary and secondary lymphoid tissues, it may not fully replicate the complexities of the human immune system (93, 118) . In order to comprehensively understand Nef's impact on CD4 T-cells, I proposed utilizing a peripheral blood mononuclear cell (PBMC) model for Aim 2 of the study. This model offers a more physiologically relevant human cell model than the previously utilized BLTS mouse model. Additionally, it allows for a more controlled and detailed examination of Nef's impact on CD4 T-cells, providing an opportunity to dissect the specific mechanisms underlying Nef-mediated immune dysregulation at the cellular level (119, 120) (116, 117) .

The B9 Nef inhibitor is shown to rescue cell-surface MHC-I expression in latently HIV-infected, donor-derived CD4 T cells, enabling recognition by autologous CD8 cytotoxic T lymphocytes (CTLs) and enhancing HIV-infected CD4 T cell killing in cell lines (74, 121). To evaluate the effects of B9 Nef inhibitor on PBMCs, I experimented to assess cell viability and viral replication. I infected PBMCs with HIV-WT and treated them with varying concentrations of the inhibitor (1 μM and 0.3 μM) for 7 days. On day 7, I utilized p24 ELISA to measure viral replication and MTT assay to analyze cell viability. As the concentration of the inhibitor increased, viral replication was notably reduced compared to infected cells, indicating its effectiveness in hindering HIV replication. Additionally, the inhibitor did not compromise PBMC viability, affirming its potential safety for host cells. This observation was in stark contrast to the lower viability observed in infected PBMCs, highlighting the negative impact of HIV infection on

cellular health. Flow cytometric analysis also showed a decrease in Nef expressions with increasing concentrations of Nef inhibitor.

Earlier research indicates that homodimerization is crucial for Nef-mediated amplification of HIV infectivity, replication, and immune dysregulation in tissue culture models (74, 121). In this study, we present proof that proposes that Nef homodimers play a part in intensifying HIV viremia and associated T cell activation. To examine the effects of various strains of HIV (HIV-WT, HIV-ΔNef, HIV-Y115D) on CD4 T-cells, I isolated CD4 cells from PBMCs and stimulated them using the anti-CD3/CD28 beads in an IL-2 rich growth media to activate T-cell proliferation. Following this, the cells were infected with a high titer virus of equal MOI, and virus replication was measured on day 7 using p24 ELISA. The results showed that the wild-type HIV strain had the highest virus replication, followed by Nef deleted and dimerized strains. The various strains exhibited different levels of viral replication, with the wild-type strain exhibiting the highest replication, followed by Nef deleted and Nef dimerized strains. The level of disruption in Nef dimerization activity was consistent with the viremia outcomes. High concentrations of an inhibitor significantly reduced viral replication across all strains, highlighting the critical role of Nef in viral replication. CD4 T-cells treated with the inhibitor showed enhanced viability compared to those infected with different HIV strains, indicating a protective effect of the inhibitor on cell viability.

Infection with HIV can significantly impact cytokine responses, leading to an imbalance in cytokine production that contributes to the disease's pathogenesis by weakening cell-mediated immunity. A cytokine assay was conducted to assess the cytokine responses during treatment with an inhibitor. The assay involved infecting CD4 T cells with different strains and treating them with the B9 inhibitor. On days 3 and 7, the supernatants were collected and analyzed. The results also

indicated elevated levels of inflammatory and immunosuppressive cytokines with respect to HIV-Y115D and HIV-WT in the infected strains compared to those treated with the B9 inhibitor and uninfected cells. Notably, these strains express Nef, while the other strain under observation does not, and the inhibitor successfully blocks Nef's effects. These findings suggest that the presence of Nef in HIV infection triggers a robust immune response, resulting in increased cytokine production. The heightened levels of these cytokines in the infected strains indicate that the virus actively engages the immune system, leading to an inflammatory response. This aligns with existing research indicating that HIV infection can modulate cytokine profiles, with cytokines like IFN-γ, IL-4, and IL-6 often remaining elevated in HIV-infected individuals (122-124). The results showed that B9-treated infected cells exhibited cytokine responses similar to uninfected cells. This could suggest that B9 helps normalize immune function despite the presence of HIV by inhibiting Nef's effects on T cell activation and immune checkpoint expression. Targeting Nef dimerization with inhibitors like B9 could be a viable strategy for reducing the pathogenic effects of HIV on the immune system. Such inhibitors could potentially improve immune system function in HIV-infected individuals, leading to better treatment outcomes and a higher quality of life.

Although we are able to get a better understanding of the potential of Nef inhibitor as a possible alternative treatment to ART or the impact Nef has on the immune response, there are certain limitations to this study that need to be addressed. Using a cancer cell line like CEM T4 cells for single-cell RNA sequencing analysis may not fully represent the physiological context of Nef-mediated effects on immune cells. To ensure the translational significance of our results, future investigations should consider employing primary cells or alternative models that better mimic the in vivo immune microenvironment. Furthermore, broadening our exploration beyond a singular Nef inhibitor, such as B9, is imperative. While B9 has shown promise, investigating a

diverse array of Nef inhibitors could furnish a more holistic understanding of Nef-targeted therapy. This approach might unveil novel inhibitors with heightened efficacy or alternative mechanisms of action, thereby enriching our therapeutic arsenal against HIV. Lastly, it's pertinent to recognize the limitations inherent in solely relying on supernatant analysis to assess cytokine responses. This methodology may overlook nuanced cellular-level changes in cytokine production and signaling cascades. By incorporating advanced techniques like intracellular cytokine staining or single-cell cytokine profiling, researchers can glean deeper insights into the intricate interplay between Nef, immune responses, and cytokine dynamics within diverse cell subsets.

In essence, by addressing these considerations, we not only fortify the robustness and clinical relevance of our findings but also lay the groundwork for future investigations aiming to unravel the complexities of Nef-mediated immune modulation and devise more effective therapeutic strategies against HIV.

6.0 Future Directions

Future research initiatives could implement several crucial and innovative strategies to overcome the limitations that our study has highlighted.

One step would be incorporating intracellular cytokine staining assays to directly assess cytokine expression profiles within infected cells treated with Nef inhibitors. By examining cytokine production at the cellular level, rather than relying solely on supernatant analysis, we can better understand how Nef modulates immune responses and cytokine signaling pathways.

Another would be to explore a broader range of Nef inhibitors, including more potent or innovative compounds such as PROTACS (proteolytic-targeting chimeras). The efficacy and mode of action of these inhibitors should be evaluated, and primary cells like PBMCs should be used to determine their impact on Nef-mediated immune dysregulation and HIV replication dynamics, opening up new avenues of exploration in our field.

Next, I would perform single-cell RNA sequencing analysis on primary immune cells, such as CD4 T cells from PBMCs, to delineate the transcriptional landscape associated with Nef expression and inhibitor treatment. This approach would offer a more physiologically relevant perspective on Nef-mediated effects and provide deeper insights into gene expression patterns and regulatory networks.

By integrating these methodologies into future investigations, we cannot only overcome the limitations of our current study but also pave the way for significant advancements in our understanding of Nef-mediated immune dysregulation in HIV infection. This comprehensive approach holds the potential to deepen our insights into HIV pathogenesis and significantly inform

the development of more effective therapeutic strategies against the virus, inspiring a new wave of research in the field.

7.0 Public Health Significance

The investigation of B9, a Nef inhibitor, and its impact on the impairment of CD4 T cells has significant public health implications in various areas. The comprehension of Nef's role in HIV pathogenesis is crucial for devising practical treatment approaches. Nef is known to enhance viral replication and foster immune evasion, making it indispensable in the progression of HIV/AIDS.

By scrutinizing how B9 affects Nef-mediated immune dysregulation, researchers can acquire insights into the mechanisms of HIV pathogenesis and open doors for innovative therapeutic interventions. Combining Nef inhibitors with existing antiretroviral therapies could be a new approach to HIV/AIDS management. By reducing Nef-mediated immune dysregulation, these inhibitors may facilitate immune restoration and clearance of latent viral reservoirs, bringing us closer to a functional HIV cure.

Furthermore, examining B9's effects on CD4 T cells provides insights into enhancing immune function in HIV-infected individuals. B9's ability to restore MHC-I expression on HIV-infected CD4 T cells suggests that Nef inhibitors have the potential to improve immune surveillance and clearance of infected cells. This could lead to better treatment outcomes and a higher quality of life for those living with HIV.

Additionally, as Nef inhibitors are not yet FDA-approved, understanding their mechanisms and effects on immune regulation is pertinent for exploring them as an alternative to ART. This avenue holds promise for individuals facing challenges with traditional ART regimens, offering new hope for effective HIV treatment strategies. Understanding Nef's role in immune evasion is essential for creating targeted therapeutic interventions.

By unraveling the complexities of Nef-mediated immune modulation, researchers can identify potential intervention targets and develop innovative strategies to combat HIV/AIDS, one of the significant public health challenges that continue to prevail.

Bibliography

1.Organization. WH. *HIV data and statistics* 2023 [Available from: https://www.who.int/teams/global-hiv-hepatitis-and-stis-programmes/hiv/strategic-information/hiv-data-and-statistics#:~:text=An%20estimated%2025.6%20million%20%5B21.6,%25%5D%20had%20suppressed%20viral%20loads.

2.Martinez-Picado J, Deeks SG. Persistent HIV-1 replication during antiretroviral therapy. Curr Opin HIV AIDS. 2016;11(4):417-23.

3.Hockett RD, Kilby JM, Derdeyn CA, Saag MS, Sillers M, Squires K, et al. Constant mean viral copy number per infected cell in tissues regardless of high, low, or undetectable plasma HIV RNA. J Exp Med. 1999;189(10):1545-54.

4.Hatano H, Delwart EL, Norris PJ, Lee TH, Dunn-Williams J, Hunt PW, et al. Evidence for persistent low-level viremia in individuals who control human immunodeficiency virus in the absence of antiretroviral therapy. J Virol. 2009;83(1):329-35.

5.Mens H, Kearney M, Wiegand A, Shao W, Schonning K, Gerstoft J, et al. HIV-1 continues to replicate and evolve in patients with natural control of HIV infection. J Virol. 2010;84(24):12971-81.

6.Engelman A, Cherepanov P. The structural biology of HIV-1: mechanistic and therapeutic insights. Nat Rev Microbiol. 2012;10(4):279-90.

7.Zhu P, Liu J, Bess J, Jr., Chertova E, Lifson JD, Grise H, et al. Distribution and three-dimensional structure of AIDS virus envelope spikes. Nature. 2006;441(7095):847-52.

8.Shafer RW, Vuitton DA. Highly active antiretroviral therapy (HAART) for the treatment of infection with human immunodeficiency virus type 1. Biomed Pharmacother. 1999;53(2):73-86.

9.Vlahov D, Galai N, Safaeian M, Galea S, Kirk GD, Lucas GM, et al. Effectiveness of highly active antiretroviral therapy among injection drug users with late-stage human immunodeficiency virus infection. Am J Epidemiol. 2005;161(11):999-1012.

10.German Advisory Committee Blood SAoPTbB. Human Immunodeficiency Virus (HIV). Transfus Med Hemother. 2016;43(3):203-22.

11.Liu J, Bartesaghi A, Borgnia MJ, Sapiro G, Subramaniam S. Molecular architecture of native HIV-1 gp120 trimers. Nature. 2008;455(7209):109-13.

12.Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature. 1998;393(6686):648-59.

13.Rizzuto CD, Wyatt R, Hernandez-Ramos N, Sun Y, Kwong PD, Hendrickson WA, et al. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science. 1998;280(5371):1949-53.

14.Rossi E, Meuser ME, Cunanan CJ, Cocklin S. Structure, Function, and Interactions of the HIV-1 Capsid Protein. Life (Basel). 2021;11(2).

15.Clements JE, Zink MC. Molecular biology and pathogenesis of animal lentivirus infections. Clin Microbiol Rev. 1996;9(1):100-17.

16.Schaefer MR, Wonderlich ER, Roeth JF, Leonard JA, Collins KL. HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells. PLoS Pathog. 2008;4(8):e1000131.

17.Cenker JJ, Stultz RD, McDonald D. Brain Microglial Cells Are Highly Susceptible to HIV-1 Infection and Spread. AIDS Res Hum Retroviruses. 2017;33(11):1155-65.

18.Novikova M, Zhang Y, Freed EO, Peng K. Multiple Roles of HIV-1 Capsid during the Virus Replication Cycle. Virol Sin. 2019;34(2):119-34.

19.Ajasin D, Eugenin EA. HIV-1 Tat: Role in Bystander Toxicity. Front Cell Infect Microbiol. 2020;10:61.

20.Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature. 1984;312(5996):763-7.

21.Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA, Axel R. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell. 1986;47(3):333-48.

22.Schwartz S, Felber BK, Benko DM, Fenyo EM, Pavlakis GN. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J Virol. 1990;64(6):2519-29.

23.de Marco A, Muller B, Glass B, Riches JD, Krausslich HG, Briggs JA. Structural analysis of HIV-1 maturation using cryo-electron tomography. PLoS Pathog. 2010;6(11):e1001215.

24.Moore JP, Trkola A, Dragic T. Co-receptors for HIV-1 entry. Curr Opin Immunol. 1997;9(4):551-62.

25.Krishnan L, Matreyek KA, Oztop I, Lee K, Tipper CH, Li X, et al. The requirement for cellular transportin 3 (TNPO3 or TRN-SR2) during infection maps to human immunodeficiency virus type 1 capsid and not integrase. J Virol. 2010;84(1):397-406.

26.Shcherbatova O, Grebennikov D, Sazonov I, Meyerhans A, Bocharov G. Modeling of the HIV-1 Life Cycle in Productively Infected Cells to Predict Novel Therapeutic Targets. Pathogens. 2020;9(4).

27.Cosnefroy O, Murray PJ, Bishop KN. HIV-1 capsid uncoating initiates after the first strand transfer of reverse transcription. Retrovirology. 2016;13(1):58.

28.Burdick RC, Li C, Munshi M, Rawson JMO, Nagashima K, Hu WS, et al. HIV-1 uncoats in the nucleus near sites of integration. Proc Natl Acad Sci U S A. 2020;117(10):5486-93.

29.Martin-Moreno A, Munoz-Fernandez MA. Dendritic Cells, the Double Agent in the War Against HIV-1. Front Immunol. 2019;10:2485.

30.Ugur M, Mueller SN. T cell and dendritic cell interactions in lymphoid organs: More than just being in the right place at the right time. Immunol Rev. 2019;289(1):115-28.

31.Masenga SK, Mweene BC, Luwaya E, Muchaili L, Chona M, Kirabo A. HIV-Host Cell Interactions. Cells. 2023;12(10).

32.Govindaraj S, Babu H, Kannanganat S, Vaccari M, Petrovas C, Velu V. Editorial: CD4+ T cells in HIV: A Friend or a Foe? Front Immunol. 2023;14:1203531.

33.Perot BP, Garcia-Paredes V, Luka M, Menager MM. Dendritic Cell Maturation Regulates TSPAN7 Function in HIV-1 Transfer to CD4(+) T Lymphocytes. Front Cell Infect Microbiol. 2020;10:70.

34.Manches O, Frleta D, Bhardwaj N. Dendritic cells in progression and pathology of HIV infection. Trends Immunol. 2014;35(3):114-22.

35.Scagnolari C, Antonelli G. Type I interferon and HIV: Subtle balance between antiviral activity, immunopathogenesis and the microbiome. Cytokine Growth Factor Rev. 2018;40:19-31.

36.Robb ML, Eller LA, Kibuuka H, Rono K, Maganga L, Nitayaphan S, et al. Prospective Study of Acute HIV-1 Infection in Adults in East Africa and Thailand. N Engl J Med. 2016;374(22):2120-30.

37.Fauci SMAS. B cells in HIV infection and disease. Nature Reviews Immunology. 2010.

38.Sheetal Kaw SA, 1 , 2 Nikolaos Tsopoulidis, 1 Katharina Morath, 1 Bahar M Coban, 1 , 2 Ralph Hohenberger, 3 Olcay C Bulut, 2 , 4 Florian Klein, 5 , 6 Bettina Stolp, 1 and Oliver T Facklercorresponding author 1 , 2. HIV-1 infection of CD4 T cells impairs antigen-specific B cell function. The EMBO Journal. 2020.

39.Townsley SM, Donofrio GC, Jian N, Leggat DJ, Dussupt V, Mendez-Rivera L, et al. B cell engagement with HIV-1 founder virus envelope predicts development of broadly neutralizing antibodies. Cell Host Microbe. 2021;29(4):564-78 e9.

40.Van de Wijer L, van der Heijden WA, Ter Horst R, Jaeger M, Trypsteen W, Rutsaert S, et al. The Architecture of Circulating Immune Cells Is Dysregulated in People Living With HIV on Long Term Antiretroviral Treatment and Relates With Markers of the HIV-1 Reservoir, Cytomegalovirus, and Microbial Translocation. Front Immunol. 2021;12:661990.

41.Wu L, KewalRamani VN. Dendritic-cell interactions with HIV: infection and viral dissemination. Nat Rev Immunol. 2006;6(11):859-68.

42.Abbas F, Cenac C, Youness A, Azar P, Delobel P, Guery JC. HIV-1 infection enhances innate function and TLR7 expression in female plasmacytoid dendritic cells. Life Sci Alliance. 2022;5(10).

43.Olwenyi OA, Johnson SD, Pandey K, Thurman M, Acharya A, Buch SJ, et al. Diminished Peripheral CD29hi Cytotoxic CD4+ T Cells Are Associated With Deleterious Effects During SIV Infection. Front Immunol. 2021;12:734871.

44.Abbas W, Herbein G. T-Cell Signaling in HIV-1 Infection. Open Virol J. 2013;7:57-71.

45.Okoye AA, Picker LJ. CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure. Immunol Rev. 2013;254(1):54-64.

46.Courtney AH, Lo WL, Weiss A. TCR Signaling: Mechanisms of Initiation and Propagation. Trends Biochem Sci. 2018;43(2):108-23.

47.Zhu X, Zhu J. CD4 T Helper Cell Subsets and Related Human Immunological Disorders. Int J Mol Sci. 2020;21(21).

48.Kervevan J, Chakrabarti LA. Role of CD4+ T Cells in the Control of Viral Infections: Recent Advances and Open Questions. Int J Mol Sci. 2021;22(2).

49.Rishi Vishal Luckheeram, 2 Rui Zhou, 1 , 2 , 3 Asha Devi Verma, 4 and Bing Xia 1 , 2 , 3 , 5 ,*. CD4+T Cells: Differentiation and Functions. Clinical and Development Immunology. 2012.

50.Demetra S. M. Chatzileontiadou, † Hannah Sloane,1,† Andrea T. Nguyen,1 Stephanie Gras,1,2 and Emma J. Grant1,*. The Many Faces of CD4+ T Cells: Immunological and Structural Characteristics. Int J Mol Sci. 2021.

51.Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. Nat Rev Immunol. 2006;6(2):127-35.

52.Del Prete G. Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. Allergy. 1992;47(5):450-5.

53.Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature. 2006;441(7095):890-3.

54.Sokol CL, Chu NQ, Yu S, Nish SA, Laufer TM, Medzhitov R. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. Nat Immunol. 2009;10(7):713-20.

55.Gaffen SL. Structure and signalling in the IL-17 receptor family. Nat Rev Immunol. 2009;9(8):556-67.

56.Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126(6):1121-33.

57.Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular Helper T Cells. Annu Rev Immunol. 2016;34:335-68.

58.Roco JA, Mesin L, Binder SC, Nefzger C, Gonzalez-Figueroa P, Canete PF, et al. Class-Switch Recombination Occurs Infrequently in Germinal Centers. Immunity. 2019;51(2):337-50 e7.

59.Raffin C, Vo LT, Bluestone JA. T(reg) cell-based therapies: challenges and perspectives. Nat Rev Immunol. 2020;20(3):158-72.

60.Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelmann K, Wohlfert EA, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. Nature. 2014;513(7519):564-8.

61.Kanamori M, Nakatsukasa H, Okada M, Lu Q, Yoshimura A. Induced Regulatory T Cells: Their Development, Stability, and Applications. Trends Immunol. 2016;37(11):803-11.

62.Takayanagi H. Osteoimmunology in 2014: Two-faced immunology-from osteogenesis to bone resorption. Nat Rev Rheumatol. 2015;11(2):74-6.

63.Hazenberg MD, Hamann D, Schuitemaker H, Miedema F. T cell depletion in HIV-1 infection: how CD4+ T cells go out of stock. Nat Immunol. 2000;1(4):285-9.

64.Cummins NW, Badley AD. Mechanisms of HIV-associated lymphocyte apoptosis: 2010. Cell Death Dis. 2010;1(11):e99.

65.Finkel TH, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, Monks C, et al. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat Med. 1995;1(2):129-34.

66.Tsao LC, Guo H, Jeffrey J, Hoxie JA, Su L. CCR5 interaction with HIV-1 Env contributes to Env-induced depletion of CD4 T cells in vitro and in vivo. Retrovirology. 2016;13:22.

67.Li W, Qin Z, Nand E, Grunst MW, Grover JR, Bess JW, Jr., et al. HIV-1 Env trimers asymmetrically engage CD4 receptors in membranes. Nature. 2023;623(7989):1026-33.

68.Greenberg ME, Bronson S, Lock M, Neumann M, Pavlakis GN, Skowronski J. Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. EMBO J. 1997;16(23):6964-76.

69.Douglas JL, Gustin JK, Viswanathan K, Mansouri M, Moses AV, Fruh K. The great escape: viral strategies to counter BST-2/tetherin. PLoS Pathog. 2010;6(5):e1000913.

70.Kirchhoff F. Immune evasion and counteraction of restriction factors by HIV-1 and other primate lentiviruses. Cell Host Microbe. 2010;8(1):55-67.

71.Kestler HW, 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, et al. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell. 1991;65(4):651-62.

72.Wonderlich ER, Leonard JA, Collins KL. HIV immune evasion disruption of antigen presentation by the HIV Nef protein. Adv Virus Res. 2011;80:103-27.

73.Basmaciogullari S, Pizzato M. The activity of Nef on HIV-1 infectivity. Front Microbiol. 2014;5:232.

74.Foster JL, Garcia JV. HIV-1 Nef: at the crossroads. Retrovirology. 2008;5:84.

75.Buffalo CZ, Iwamoto Y, Hurley JH, Ren X. How HIV Nef Proteins Hijack Membrane Traffic To Promote Infection. J Virol. 2019;93(24).

76.Dekaban GA, Dikeakos JD. HIV-I Nef inhibitors: a novel class of HIV-specific immune adjuvants in support of a cure. AIDS Res Ther. 2017;14(1):53.

77.Jia X, Singh R, Homann S, Yang H, Guatelli J, Xiong Y. Structural basis of evasion of cellular adaptive immunity by HIV-1 Nef. Nat Struct Mol Biol. 2012;19(7):701-6.

78.Mohamed MM. Monocytes conditioned media stimulate fibronectin expression and spreading of inflammatory breast cancer cells in three-dimensional culture: A mechanism mediated by IL-8 signaling pathway. Cell Commun Signal. 2012;10(1):3.

79.Lama J, Mangasarian A, Trono D. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. Curr Biol. 1999;9(12):622-31.

80.Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature. 1998;391(6665):397-401.

81.Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat Med. 1996;2(3):338-42.

82.Foster JL, Garcia JV. Role of Nef in HIV-1 replication and pathogenesis. Adv Pharmacol. 2007;55:389-409.

83.Briggs SD, Lerner EC, Smithgall TE. Affinity of Src family kinase SH3 domains for HIV Nef in vitro does not predict kinase activation by Nef in vivo. Biochemistry. 2000;39(3):489-95.

84.Ye H, Choi HJ, Poe J, Smithgall TE. Oligomerization is required for HIV-1 Nef-induced activation of the Src family protein-tyrosine kinase, Hck. Biochemistry. 2004;43(50):15775-84.

85.Jolly C, Kashefi K, Hollinshead M, Sattentau QJ. HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. J Exp Med. 2004;199(2):283-93.

86.Campbell EM, Nunez R, Hope TJ. Disruption of the actin cytoskeleton can complement the ability of Nef to enhance human immunodeficiency virus type 1 infectivity. J Virol. 2004;78(11):5745-55.

87.Carl S, Greenough TC, Krumbiegel M, Greenberg M, Skowronski J, Sullivan JL, et al. Modulation of different human immunodeficiency virus type 1 Nef functions during progression to AIDS. J Virol. 2001;75(8):3657-65.

88.Mujib S, Saiyed A, Fadel S, Bozorgzad A, Aidarus N, Yue FY, et al. Pharmacologic HIV-1 Nef blockade promotes CD8 T cell-mediated elimination of latently HIV-1-infected cells in vitro. JCI Insight. 2017;2(17).

89.Emert-Sedlak LA, Narute P, Shu ST, Poe JA, Shi H, Yanamala N, et al. Effector kinase coupling enables high-throughput screens for direct HIV-1 Nef antagonists with antiretroviral activity. Chem Biol. 2013;20(1):82-91.

90.Duette G, Hiener B, Morgan H, Mazur FG, Mathivanan V, Horsburgh BA, et al. The HIV-1 proviral landscape reveals that Nef contributes to HIV-1 persistence in effector memory CD4+ T cells. J Clin Invest. 2022;132(7).

91.Emert-Sedlak LA, Moukha-Chafiq O, Shi H, Du S, Alvarado JJ, Pathak V, et al. Inhibitors of HIV-1 Nef-Mediated Activation of the Myeloid Src-Family Kinase Hck Block HIV-1 Replication in Macrophages and Disrupt MHC-I Downregulation. ACS Infect Dis. 2022;8(1):91-105.

92.Emert-Sedlak LA, Loughran HM, Shi H, Kulp JL, 3rd, Shu ST, Zhao J, et al. Synthesis and evaluation of orally active small molecule HIV-1 Nef antagonists. Bioorg Med Chem Lett. 2016;26(5):1480-4.

93.Staudt RP, Alvarado JJ, Emert-Sedlak LA, Shi H, Shu ST, Wales TE, et al. Structure, function, and inhibitor targeting of HIV-1 Nef-effector kinase complexes. J Biol Chem. 2020;295(44):15158-71.

94.Emert-Sedlak LA, Shi H, Tice CM, Chen L, Alvarado JJ, Shu ST, et al. Antiretroviral Drug Discovery Targeting the HIV-1 Nef Virulence Factor. Viruses. 2022;14(9).

95.Iyer PC, Zhao J, Emert-Sedlak LA, Moore KK, Smithgall TE, Day BW. Synthesis and structure-activity analysis of diphenylpyrazolodiazene inhibitors of the HIV-1 Nef virulence factor. Bioorg Med Chem Lett. 2014;24(7):1702-6.

96..

97.Emert-Sedlak LA, Tice CM, Shi H, Alvarado JJ, Shu ST, Reitz AB, et al. PROTAC-mediated degradation of HIV-1 Nef efficiently restores cell-surface CD4 and MHC-I expression and blocks HIV-1 replication. Cell Chem Biol. 2024;31(4):658-68 e14.

98.Hao Y, Stuart T, Kowalski MH, Choudhary S, Hoffman P, Hartman A, et al. Dictionary learning for integrative, multimodal and scalable single-cell analysis. Nat Biotechnol. 2024;42(2):293-304.

99.Generating publication ready visualizations for Single Cell transcriptomics using SCpubr. [Internet]. 2022.

100.Blighe K RS, Lewis M. 2022. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. . EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package version 1140. 2022.

101.Ulgen E OO, Sezerman OU. . pathfindR: An R Package for Comprehensive Identification of Enriched Pathways in Omics Data Through Active Subnetworks. Front Genet 10:858

2019.

102.Cheney L, Guzik H, Macaluso FP, Macian F, Cuervo AM, Berman JW. HIV Nef and Antiretroviral Therapy Have an Inhibitory Effect on Autophagy in Human Astrocytes that May Contribute to HIV-Associated Neurocognitive Disorders. Cells. 2020;9(6).

103.Ding Y, Chen M, Wang J, Yang Y, Feng Y, Wang L, et al. Increase in HIV-1-transmitted drug resistance among ART-naive youths at the China-Myanmar border during 2009 ~ 2017. BMC Infect Dis. 2021;21(1):93.

104.Al-Omairi O, Elgalib A, Al Kindi H. HIV Drug Resistance among Patients Failing Therapy at a Tertiary Center in Oman: A Case Record Review. Oman Med J. 2019;34(6):490-5.

105.Gorry PR, McPhee DA, Verity E, Dyer WB, Wesselingh SL, Learmont J, et al. Pathogenicity and immunogenicity of attenuated, nef-deleted HIV-1 strains in vivo. Retrovirology. 2007;4:66.

106.Dirk BS, Pawlak EN, Johnson AL, Van Nynatten LR, Jacob RA, Heit B, et al. HIV-1 Nef sequesters MHC-I intracellularly by targeting early stages of endocytosis and recycling. Sci Rep. 2016;6:37021.

107.Chelvanambi S, Bogatcheva NV, Bednorz M, Agarwal S, Maier B, Alves NJ, et al. HIV-Nef Protein Persists in the Lungs of Aviremic Patients with HIV and Induces Endothelial Cell Death. Am J Respir Cell Mol Biol. 2019;60(3):357-66.

108.Donoso M, D'Amico D, Valdebenito S, Hernandez CA, Prideaux B, Eugenin EA. Identification, Quantification, and Characterization of HIV-1 Reservoirs in the Human Brain. Cells. 2022;11(15).

109.Ferdin J, Goricar K, Dolzan V, Plemenitas A, Martin JN, Peterlin BM, et al. Viral protein Nef is detected in plasma of half of HIV-infected adults with undetectable plasma HIV RNA. PLoS One. 2018;13(1):e0191613.

110.Imamichi H, Smith M, Adelsberger JW, Izumi T, Scrimieri F, Sherman BT, et al. Defective HIV-1 proviruses produce viral proteins. Proc Natl Acad Sci U S A. 2020;117(7):3704-10.

111.Shi H, Tice CM, Emert-Sedlak L, Chen L, Li WF, Carlsen M, et al. Tight-Binding Hydroxypyrazole HIV-1 Nef Inhibitors Suppress Viral Replication in Donor Mononuclear Cells and Reverse Nef-Mediated MHC-I Downregulation. ACS Infect Dis. 2020;6(2):302-12.

112.Shu ST, Emert-Sedlak LA, Smithgall TE. Cell-based Fluorescence Complementation Reveals a Role for HIV-1 Nef Protein Dimerization in AP-2 Adaptor Recruitment and CD4 Co-receptor Down-regulation. J Biol Chem. 2017;292(7):2670-8.

113.Naamati A, Williamson JC, Greenwood EJD, Marelli S, Lehner PJ, Matheson NJ. Functional proteomic atlas of HIV infection in primary human CD4+ T cells. eLife. 2019;8.

114.Medh RD, Webb MS, Miller AL, Johnson BH, Fofanov Y, Li T, et al. Gene expression profile of human lymphoid CEM cells sensitive and resistant to glucocorticoid-evoked apoptosis. Genomics. 2003;81(6):543-55.

115.Matthias LJ, Yam PTW, Jiang X-M, Vandegraaff N, Li P, Poumbourios P, et al. Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1. Nature Immunology. 2002;3(8):727-32.

116.Das S, Rai A, Merchant ML, Cave MC, Rai SN. A Comprehensive Survey of Statistical Approaches for Differential Expression Analysis in Single-Cell RNA Sequencing Studies. Genes (Basel). 2021;12(12).

117.Dal Molin A, Baruzzo G, Di Camillo B. Single-Cell RNA-Sequencing: Assessment of Differential Expression Analysis Methods. Front Genet. 2017;8:62.

118.Zou W, Denton PW, Watkins RL, Krisko JF, Nochi T, Foster JL, et al. Nef functions in BLT mice to enhance HIV-1 replication and deplete CD4+CD8+ thymocytes. Retrovirology. 2012;9:44.

119.Homann S, Tibroni N, Baumann I, Sertel S, Keppler OT, Fackler OT. Determinants in HIV-1 Nef for enhancement of virus replication and depletion of CD4+ T lymphocytes in human lymphoid tissue ex vivo. Retrovirology. 2009;6:6.

120.Sudderuddin H, Kinloch NN, Jin SW, Miller RL, Jones BR, Brumme CJ, et al. Longitudinal within-host evolution of HIV Nef-mediated CD4, HLA and SERINC5 downregulation activity: a case study. Retrovirology. 2020;17(1):3.

121.Poe JA, Smithgall TE. HIV-1 Nef dimerization is required for Nef-mediated receptor downregulation and viral replication. J Mol Biol. 2009;394(2):329-42.

122.Watanabe D, Uehira T, Suzuki S, Matsumoto E, Ueji T, Hirota K, et al. Clinical characteristics of HIV-1-infected patients with high levels of plasma interferon-gamma: a multicenter observational study. BMC Infect Dis. 2019;19(1):11.

123.Keating SM, Jacobs ES, Norris PJ. Soluble mediators of inflammation in HIV and their implications for therapeutics and vaccine development. Cytokine Growth Factor Rev. 2012;23(4-5):193-206.

124.Ayatollahi Mousavi SA, Asadikaram G, Nakhaee N, Izadi A. Plasma Levels of IFN-gamma, IL-4, IL-6 and IL-17 in HIV-Positive Patients With Oral Candidiasis. Jundishapur J Microbiol. 2016;9(2):e32021.