Development and Characterization of Novel Dendritic Cell (DC)-Targeting Vaccine Against Human Immunodeficiency Virus (HIV)-1 Envelope Conserved Elements (CEs)

By

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Abstract

Development of the human immunodeficiency virus type-1 (HIV-1) vaccine is an effective and powerful prevention method of the halting pandemic of the acquired immunodeficiency syndrome (AIDS). Dendritic cell (DC)-based HIV immunotherapeutic vaccine is very promising at optimizing the HIV-specific immune responses. Since the Ebola virus glycoprotein (EboGP) has strong DC-targeting ability, we hypothesized that the infusion of the highly conserved elements (CE) of HIV envelope glycoprotein including the Membrane-Proximal External Region (MPER), with the DC-targeting domains of EboGP (EboGPΔM), can direct these epitopes to the DCs/Macrophages and more efficiently elicit immunes responses in the host.

It is known that the mucin-like domain of Ebola GP is highly glycosylated, less conserved and dispensable for EBOV infection. In this study, we have replaced the mucin-like domain of Ebola GP with 9 arranged highly conserved elements (9CE) or MPER of HIV envelop glycoprotein to generate plasmids encoding EboGPΔM-9CE, EboGPΔM-MPER to test our hypothesis. To investigate whether these fusion proteins are able to efficiently enter DCs/Macrophages, we co-transfected 293T cells with HIV vector (ΔRI/ΔE/Gluc), HIV packaging plasmid (Δ8.2), and EboGPΔM-9CE and/or EboGPΔM-MPER plasmids to generate virus-like particles (VLPs) and used to infect human monocytes and Macrophages. Our results have shown that EboGPΔM-9CE- and/or EboGPΔM-MPER- VLPs can efficiently target a human monocytic cell line (THP-1) and monocyte-derived macrophages (MDMs). Also, we investigated the immunogenicity of EboGPΔM-9CE-and/or EboGPΔM-MPER and/or HIV Env(M)VLPs in *in vivo* study in BALB/C mice and evaluated the potential T cell- and B cell-mediated immune responses of the VLPs based vaccines.

The results have demonstrated that fusion of EboGP with 9CE or MPER can be successfully expressed in the cells and also can be incorporated into the pseudotyped VLPs at various levels. Also, the EboGPΔM-9CE and/or EboGPΔM-MPER-VLPs can efficiently target THP-1 and MDMs. The immunogenicity studies in the rodent animal model(s) demonstrated that both EboGPΔM-MPER- and EboGPΔM-9CE-VLPs induced more robust responses in comparison to HIV-Env-VLPs. Interestingly, the EboGP-MPER fusion protein-pseudotyped VLPs induced significantly higher anti-HIV MPER antibodies than native HIV Env VLPs in mice. Furthermore, this study revealed that EboGPΔM-MPER has induced not only a more effective immune response to MPER but also enhance the immune response against other HIV components, such as HIV Gag. Overall, the study present here has provided strong evidence for the development of a potent DC-targeting vaccine approach against HIV infection.

Dedication

To my beloved family, Majid, Mojgan, and my lovely sister, Negin.

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Table of Contents

Abstract v

Acknowledgments iv

List of Abbreviations viii

List of Figures and Tables x

Chapter 1: Introduction 1

1.1. Vaccine and Immunity 1

1.1.2. Virus-like particle vaccine 2

1.1.3. Innate immune response 2

1.1.3.1. The role of antigen-presenting cells (APCs) in HIV-1 infection *4*

1.1.4. Adaptive immunity 5

1.1.4.1. Humoral immune response 5

1.1.4.2. T cell-mediated immune response 6

1.2. Human Immunodeficiency Virus Type 1 (HIV-1) 8

1.2.1. General description of HIV -1 History 8

1.2.2 HIV-1 epidemiology 9

1.2.3. HIV-1 classification 10

1.2.4. HIV-1 genome organization and structure 10

1.2.5. HIV-1 structural proteins 14

1.2.6. HIV-1 enzymatic proteins 14

1.2.7. HIV-1 accessary proteins 15

1.2.8. Regulatory Proteins 15

1.2.9. **HIV-1 Replication cycle** 16

1.2.10. **T cells in HIV infection** 19

*1.2.10.1. CD4+ T cell***s** 19

1.2.10.2. CD4+ T cells in HIV Infection 20

1.2.10.3. CD8+ T cells in HIV Infection 21

1.2.11. HIV-1 genome diversity 22

1.2.12. HIV- Conserved Regions 23

1.2.13. Conserved Region in HIV-1 Env 23

1.2.14. Broad neutralizing antibody targets gp120 28

1.2.15. HIV Env gp41 28

1.2.16. Membrane-proximal external region (MPER) in HIV Env 30

1.2.17. Broad Neutralizing antibodies target MPER 30

1.3. Ebola virus 33
1.3.1. Ebola virus virology 33
1.3.2. Ebola glycoprotein 33
1.3.3. Ebola GP in viral entry 34
1.3.4. The role of Mucin-like domain of Ebola GP during viral replication 35
1.3.5. The affinity of Ebola GP for targeting APCs 37
Chapter 2: study rationale, hypothesis, and objectives 38
2.1. Study Rationale 38
2.1.1. HIV Env conserved regions in HIV vaccine design 38
2.1.2. The important roles of targeting antigen presenting cells in enhanced humeral and cellular immune responses 39
2.1.3. Modifications of the EBOV glycoprotein for the fusion of HIV-1 Env conserved epitopes 39
2.2. Hypothesis 40
2.3. Objectives 40
Chapter 3: Material and methods 41
3.1. Plasmid constructs 41
3.2. Recombinant proteins 42
3.3. Antibodies 42
3.4. Cell culture and macrophage preparation 42
3.5. Cell transfection 43
3.6. HIV virus-like particle (VLP) preparation 43
3.7. Western Blots 44
3.8. Gaussia luciferase Assay and NF-κB activity luciferase reporter assay 44
3.9. *In vivo* study and Mice immunization experiment 45
3.1. Anti-HIV and anti-EboGP antibody measurements by Enzyme-linked Immunosorbent Assay (ELISA) 45
3.11. Bacteria transformation and maxi preparation 46
3.11.1. Bacteria transformation 46
3.11.2. Minipreparation 47
3.11.3. Max preparation 48
Chapter 4: Results 50
4.1. Construction of the HIV Env conserved regions expression plasmids 50
4.2. Expression of EboGPΔM-9CE or EboGPΔM-MPER fusion proteins in the 293 T cells ... 57
4.3. The incorporation of EboGPΔM-9CE and/or EboGPΔM -MPER in VLPs 59

4.4. Investigation of dendritic cell and macrophages targeting ability of EboGPΔM-MPER, EboGPΔM-9CE or HIV Env peudotyped VLPs 63

4.5. Investigation of the ability of EboGPΔM-MPER, EboGPΔM-9CE-VLPs to induce anti-HIV antibodies in mice 68

Discussion 74

Future direction 78

Reference 79

LIST OF ABBREVIATIONS

ADCC - Antibody-dependent cell cytotoxicity

AIDS - Acquired immunodeficiency syndrome

APC - Antigen presenting cell

CD - Cluster of differentiation

CDC - Complement-dependent cytotoxicity

CL - Containment level

CTL - Cytotoxic T lymphocyte

DC - Dendritic cell

DC-SIGN - Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DNA - Deoxyribonucleic acid

EBOV - Ebola virus

EHF - Ebola hemorrhagic fever

ELISA - Enzyme-linked immunosorbant assay

ELISPOT - Enzyme-linked immunospot

Env - HIV Envelope protein

GP - EBOV glycoprotein

HAART - Highly active antiretroviral therapy

HESN - Highly-exposed, HIV-seronegative

HIV - Human immunodeficiency virus

HTLV-III - human T-lymphotropic virus-III

IFN - Interferon

IgG - Immunoglobulin G

IL - Interleukin

LAV - Lymphadenopathy-associated virus

LD50 - Lethal dose 50%

LTNP - Long-term nonprogressor

mAb - Monoclonal antibody

MHC - Major histocompatibility complex

MLD - Mucin-like domain

nAb - Neutralizing antibody

NK - Natural killer

NNRTI - Non-nucleoside reverse transcriptase inhibitor

NPC1 - Niemann-Pick type C1

NRTI - Nucleoside reverse transcriptase inhibitor

PCR - Polymerase chain reaction

RNA - Ribonucleic acid

RT - Reverse transcriptase

SIV - Simian immunodeficiency virus

ssRNA - Single-stranded RNA

TCR - T cell receptor

TIM-1 - T-cell immunoglobulin and mucin domain 1

TGF - Transforming growth factor

TNF - Tumor necrosis factor

VE - Vaccine efficacy

List of Figures and Tables

Figure 1.1. HIV Virion……..……..12

Figure 1.2. HIV genome…….……13

Figure 1.3. The life cycle of HIV-1…….18

Figure 1.4. The schematic representation of HIV envelope glycoprotein trimer structure….25

Figure 1.5. Schematic representation of HIV gp41 regions……28

Figure 1.6. MPER conformation and neutralizing antibody targeting sites……31

Figure 1.7. A schematic structure of EBOV GP…….35

Figure 3. 1. Schematic representation of ELISA assay process…..……45

Figure 4. 1. Schematic representation of the conserved regions (CE) in the envelope……..53

Figure 4. 2. Schematic diagram of the EboGP- 9CE or- MPER plasmids…….54

Figure 4. 3. The expression of EboGPΔM, HIV gp120 (M), EboGPΔM-9CE, and EboGPΔM-MPER in the cell lysate……..56

Figure 4. 4. The incorporation of EboGPΔM-9CE and/or EboGPΔM -MPER in VLPs……59

Figure 4.5. Investigation of dendritic cell and macrophages targeting ability of EboGPΔM-MPER, EboGPΔM-9CE or HIV Env pseudotyped VLPs……..……64

Figure 4. 6. Investigation of the ability of EboGPΔM-MPER, EboGPΔM-9CE-VLPs to induce anti-HIV antibodies in mice……69

Table 4. 1. HIV-1 GP 120 consreverd epitopes……..50

1. Introduction

1.1.Vaccine and Immunity

1.1.1. History of vaccine

Vaccines are one of the most successful and cheapest protective measures against many critical infectious diseases. Currently, the history of vaccines dates back to over 300 years ago[1]. In 1796, Edward Jenner observed that dairy farmers and milkmaids who naturally acquired lesions from cowpox infection were subsequently immune to smallpox epidemics. Accordingly, he administered pus or scabs from lesions obtained from a milkmaid infected with the bovine disease to another person to confer immunity to smallpox, and this was called arm-to-arm inoculation[2]. Later, he termed this procedure "vaccination" derived from *vacca*, the Latin term for the cow. In the late 19th century, Robert Koch provided evidence that microorganisms were the etiological agents of infectious diseases, like viruses, bacteria, fungi, and parasites in human illness. In 1885, Louis Pasteur developed the first human rabies virus vaccine by using a dead or attenuated virus that imitated the infectious agent to induce immunity to naïve individuals without causing disease [3]. As of these discoveries, multiple vaccines have been developed. These vaccines successfully eradicated smallpox and other previously lethal diseases, such as poliomyelitis, measles, mumps, and rubella, which affected millions of lives in the 20^{th} century [4]. Recently, many new advanced technologies have rapidly been developed that optimize the mimicry of pathogens to induce immunity as well as large-scale production[5].

Vaccines are generally divided into live and non-live (inactivated) groups. Live vaccines contain attenuated replicating strains of the related pathogenic organism, and non-live vaccines refer to vaccines that contain only components of a pathogen or killed whole microorganisms. In recent years, in addition to the 'traditional' live and non-live vaccines, several other vaccine

platforms have been developed, including viral vectors, nucleic acid-based RNA and DNA vaccines, and virus-like particles[6].

1.1.2. Virus-like particle vaccine

In the 1980s, the concept of VLPs derives from the discovery of subviral particles in the blood of patients infected with Hepatitis B (HBV). When plasma-derived subviral particles were administered to healthy individuals, the protection against HBV was conferred to healthy people, thereby introducing the first VLP-based vaccine [7,8]. Later, this vaccine was replaced by a recombinant version, which is safer. This achievement of this vaccine motivated scientists to research VLP vaccines. As a result, a vaccine for the human papillomavirus (HPV) is authorized today for human use[8]. Virus-like particles are self-assembled particles that mimic the natural virus structure. VLPs are not infectious since they do not contain the viral genome. VLPs represent the repetitive organized structure and particulate nature; thus, they are very efficiently uptaken by antigen-presenting cells leads to the stimulation of both arms of the immune response, humoral and cellular[9]. These unique VLP features make them appealing as an alternative to the existing vaccines and will be the subject of intensive research and advanced vaccine technology platforms [10].

1.1.3. Innate immune response

The human immune system consists of two arms, innate and adaptive immune responses, which work closely together to improve the host's ability to combat infections and malignancies[11]. Both arms of the immune system are dependent on leukocytes' activities, also known as white blood cells. The innate arm of the immune system is the first line of defence in the host by acting general, quick, and persistent mechanisms of resistance. Physical barriers, such as mucous layers and skin, prevent the entry of most pathogens into the host. Moreover, the skin has an acidity nature, which inhibits the growth of many microorganisms, while mucous gel and cilia on mucous membranes trap and transport pathogens out from the host body[12].

Physiologic barriers are another component of innate immunity. For instance, the normal body temperature range inhibits the growth of pathogens, while a rise in temperature during a fever can further inhibit pathogen growth. Lysozyme, an enzyme found in mucous secretions, can degrade and lyse bacteria by hydrolyzing the bacterial cell wall peptidoglycan[13]. Interferon molecules are released by virus-infected cells and elicit antiviral effects by binding to uninfected cells in proximity[14]. The complement cascade is another component of the innate immune system, which can activate complement proteins by three different pathways. First, it produces large numbers of activated complement proteins. These complement proteins bind to pathogens, opsonizing with IgG or IgM antibodies for engulfment by phagocytes bearing receptors for a complement. Second, some complement proteins have small fragments that act as chemoattractants to recruit more phagocytes area of the complement activation and also to activate these phagocytes. Third, the terminal complement components create pores in the bacterial membrane that kill the pathogen[15].

Cells of the innate immune system can recognize conserved molecular patterns conserved of many pathogens. Pattern recognition receptors (PRRs) present on innate cells are not specific to a specific antigen, but they recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns[16]. Recognition of these PAMPs occurs through PRRs, such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors, which recognize components such as nucleic acids peptidoglycan, flagellin, and lipopolysaccharide common to many pathogens[17]. After recognition of PAMPs, PRRs initiate a cascade that leads to cytokine release, complement activation, opsonization, and phagocyte activation [18]. Moreover, granulocytes include neutrophils, eosinophils, basophils, and mast cells, play essential roles in the innate immune response. Neutrophils are the first cells that arrive at the inflammation area and are highly active phagocytes, while eosinophils have a key role in phagocytosis and the destroy of parasites[19]. Basophils are present in the

blood, mast cells, tissues and secrete cytokines such as IL-4 and histamine, which mediate allergic responses[20]. Dendritic cells (DCs), and mononuclear phagocytes, such as monocytes and macrophages, act as a bridge between innate immunity and adaptive immunity. These cells are involved in antimicrobial and cytotoxic effects, phagocytosis, cytokine production, and antigen presentation [12].

Natural killer (NK) cells have a robust cytolytic function and lyse target cells by secreting perforin and granzyme. Also, they can regulate immunity by producing cytokines and induce apoptosis of target cells by coupling death-inducing receptors [17]. NK cells have receptors termed Killer immunoglobulin-like receptors or KIR on the surface. The genes encoded KIR are highly polymorphic[21]. HLA class I (HLA-I) molecules are known as ligands for the NK cell receptor of KIR[22]. NK cells interact with HLA-I by recognizing infected or dysfunctional host cells due to a low level of surface HLA I expression; thereby, antiviral effector functions such as exocytotic lytic activity and induction of apoptosis are initiated [23]. Recently, it has been shown that there is a significant association between the expression of KIR and slower HIV-1 disease progression, which suggested that cells that express KIR (NK cells and CD8+ T cells) are vital in HIV-1 disease control[24].

1.1.3.1. The role of antigen-presenting cells (APCs) in HIV-1 infection

Antigen-presenting cells (APCs) such as DCs and macrophages play a crucial role in host innate immunity, particularly HIV-1. They act as a bridge between innate and adaptive arms of host immunity mostly by mediating adaptive immune cells activation, differentiation, and proliferation process. Also, APCs link innate and adaptive immunity through antigen processing and presentation, providing co-stimulatory receptor pathways and secretion of cytokines that are necessary for adaptive immune response activation, differentiation process into effector cells, and clonal expansion [25,26]. Since DCs and macrophages express CD4 receptors in combination with chemokine receptor type 5 (CCR5) or C-X-C chemokine

receptor type 4 (CXCR4) coreceptors on their surface, they are also considered as targets for HIV-1 infection[27,28]. Moreover, during HIV-1 infection, PRR, such as TLR7/8, present on APCs detect viral particles through PAMPs like HIV-1 ssRNA. PAMPs bind to cognate PRR. Subsequently, a cascade of intracellular signalling is activated, resulting in cytokine, chemokines, and anti-microbial peptides production[29,30].

1.1.4. Adaptive immunity

Lymphocytes play the most significant role in adaptive immunity. The main disadvantage with adaptive immunity is that responses are much slower than innate immune responses. However, the most considerable advantage of adaptive immunity can generate memory cells, induce a more rapid response, and improve the pathogen's resistance [31]. The adaptive immune response is antigen-specific immunity and is comprised of T cell-mediated immune response and the humoral immune response, which is mediated by B cells and antibodies, respectively[32]. B cells produce antibodies rapidly in response to the antigen. They permeate to the body fluids through blood circulating, where they are able to interact specifically with the foreign peptide. Antibodies can bind to the pathogen and inactivates them by inhibiting their ability to bind to receptors on host cells. Also, antibody binding marks invading pathogens for destruction by making it easier for recognizing by phagocytic cells of the innate immune system [11].

1.1.4.1. Humoral immune response

HIV-specific humoral responses are stimulated after B cells antigen binding and subsequently differentiate into plasma B cells to produce antibodies. Plasma antibodies are detected about 1-3 months after infection, referred to as seroconversion. Neutralizing antibodies (nAbs) against HIV-1 mainly recognize the viral Env protein (gp120 and gp41 subunits) and are found in most infected individuals. Many vaccine design efforts have focused on inducing broadly neutralizing antibodies (nAbs), mainly targeted against HIV gp120. Most broadly nAbs block

entry of HIV into host cells by targeting the receptor binding site, thereby it prevents the interaction of gp120 with CD4, blocking the binding of gp120 with co-receptors. HIV-1 specific neutralizing antibodies, including those targeting regions in the gp41 subunit, have been demonstrated to protect against HIV infection upon passive transfer in macaques and humanized mice [33]. Vaccinees also showed a higher level of gamma immunoglobulin (IgG)3 isotype antibodies involved in mediating antibody-dependent cell cytotoxicity (ADCC). These responses deteriorated rapidly from 79% to 0% from 26 weeks to year 1, associated with the rapid decrease in vaccine efficacy observed after one year. Thus, high levels of non-neutralizing gp120-binding antibodies that mediate ADCC may be a better correlate of protection than nAbs [34]. However, there are still numerous challenges regarding the stimulation of protective anti-HIV antibody responses by vaccination. An efficient HIV-1 vaccine would induce antibodies that can overcome both rapid mutations of HIV in different clades with up to 35% differences in amino acid sequence and glycan shielding of the HIV envelope[35,36].

1.1.4.2. T cell-mediated immune response

In cell-mediated immune responses, T cells react directly against a foreign antigen. The T cell destroys a virus-infected host cell with viral antigens on its surface; thereby, the infected cells are eliminated before the virus has had a chance to replicate. Moreover, T cells release signal molecules that activate macrophages to destroy the invading pathogens they have phagocytosed[11]. During HIV-1 infection, both humoral and cell-mediated adaptive immune responses are stimulated after exposure to HIV-1 particles[37].

T lymphocytes (T cells), the main mediators of cell-mediated immunity arm, have unique T cell receptors (TCRs) on their surface, which specifically recognize cognate antigens presented by either major histocompatibility complex (MHC)-I or MHC-II[25]. Unlike B lymphocytes, T lymphocytes can only interact with antigens that have been presented by antigen-presenting

cells. Upon receptor binding with its cognate antigen, T cells proliferate and differentiate into effector cells. Naïve CD8+ T cells differentiate into cytotoxic T lymphocytes (CTLs), while naïve CD4+ T cells differentiate into T helper cells or regulatory T cells[25]. CTLs, which express CD8 co-receptor on their surface, recognize antigenic peptides presented by MHC-I, expressed on all nucleated cell types, excluding mature erythrocytes[38]. CTLs directly destroy cells infected with intracellular pathogens, such as viruses or malignant cells that express their cognate antigen[39]. Upon receptor binding and co-stimulation, CTLs stimulate caspases cascades in the target cell, which induces apoptosis and activates nucleases that ultimately destroy host cell and foreign DNA[40].

T helper cells express CD4 on their surface and recognize antigenic peptides presented by MHC-II. MHC-II is mainly expressed on the professional antigen-presenting cells, such as macrophages, DCs, and B cells[38,41]. Upon binding to the cognate antigens on target cells, helper T cells induce the essential signals that impact the activity of different cell types. For instance, Th1 cells release IFN-γ, promoting the activation of infected macrophages to destroy engulfed intracellular pathogens[26,41]. Th2 cells release IL-4, IL-5, and IL-13, which promotes the ability of antigen-stimulated B cells to secrete antibodies that has isotype class switching.

Furthermore, Th2 cells promote switching to IgE antibodies to control the clearance of extracellular multicellular parasites[41]. Th17 cells produce IL-17 and IL-6, making fibroblasts and epithelial cells produce chemokines that recruit neutrophils to sites of infection to mediate the destruction of extracellular pathogens. Follicular T helper (TFH) cells also contribute to the antibody-mediated response by producing cytokines, such as IFN-γ, which induces class switching in B cells[42]. Regulatory T cells (Treg) produce TGF (transforming growth factor)-β and IL-10 to limit the activity of lymphocytes and prevent autoimmune diseases[43].

1.2.Human Immunodeficiency Virus Type 1 (HIV-1)

1.2.1. General description of HIV -1 History

Acquired Immune Deficiency Syndrome (AIDS) was identified as a new disease in the year 1981 for the first time when the mortality rate among young homosexual men increased due to unusual opportunistic infections and rare malignancies [44]. The Human immunodeficiency virus (HIV) infection causes an infection that gradually destroys a particular type of white blood cells called CD4+ lymphocytes. Lymphocytes help defend the body against foreign substances. Thus, when HIV damages CD4+ lymphocytes, the body becomes more vulnerable to other infectious organisms [45].

Human immunodeficiency virus (HIV) belongs to the *Retroviridae* family and the genus *Lentivirus* and causes AIDS. Most of the *Retroviridae* family virus has high mutation frequency[46]. *Retroviridae* family viruses were found in various other species, including mice, cattle, cats and primates[46].

The first human retrovirus was first discovered in 1980, called the human T-cell leukemia virus (HTLV-1) [47]. In the subsequent period, the acquired immunodeficiency syndrome (AIDS) was rapidly spread in various parts of the world, like Western Europe, Australia, United States [48]. The hypothesis that an infectious agent, which is most likely a virus, was responsible for AIDS was suggested in 1982 [49]. In 1983, a complex and unusual retrovirus called HTLV-3 was firstly explained by Dr. Luc Montagner [50]. At the same time, the same retrovirus was isolated from the patients and named by different groups, including lymphadenopathy-associated virus (LAV), AIDS-related virus (ARV)[51,52]. HTLV-3 was renamed HIV-1 and proved to be the etiologic virus responsible for AIDS disease[53].

In total, HIV infections may be caused by one of two retroviruses, HIV-1 or HIV-2. HIV-1 causes most HIV infections in the world, but HIV-2 causes many HIV infections in West Africa. HIV-1 originated in Central Africa during the beginning of the 20th century when a closely related chimpanzee virus first infected people. The HIV-1 global epidemic began in the late 1970s [54].

HIV-1 is predominantly acquired via sexual contact with an infected individual; however, it can transmit by other routes, including exposure to blood, using contaminated injection needles and mother-to-child-transmission (MTCT) [55].

1.2.2. HIV-1 epidemiology

In 1989, the global burden of HIV was reported, with over 400,000 cases. (World Health Organization, 1989). In 2018, globally, it was estimated that 37.9 million people were living with HIV, and 770,000 related deaths were recorded that year (WHO, 2018). Global trends in HIV infection show an overall increase in the prevalence of HIV infections and significant declines in AIDS-related mortality, mainly due to the benefits of antiretroviral treatment. Sub-Saharan Africa has the highest burden of HIV infection, accounting for more than 70% of the global spread of infection. HIV prevention approaches in sub-Saharan Africa can affect the spread of HIV in the world. Regardless of substantial development in extensive use of antiretroviral therapy (ART), sub-Saharan Africa accounted for 74% of the 1.5 million AIDS-related mortalities in 2013[56].

One of the valuable successes has been developing antiretroviral therapy (ART) that reduces viral load and brings back the immune function. ART can both treat people and prevent new HIV infections. Furthermore, ART has a significant impact on the transmission of HIV and could help to control the HIV epidemic [57].

In 89 studies of adult patients in ART program in sub-Saharan Africa, with a follow up of 6 months to 2 years, the median proportions of patients with viral loads below 1000 copies/mL, 400 copies/mL and 50 copies/mL were 85%, 79% and 69%, respectively[58]

1.2.3. HIV-1 classification

HIV-1 is subdivided into the groups M, N, O and P [59]. Group M is the most predominant and transmittable of the HIV-1 group. It has been divided into subtypes called A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K are currently discovered. HIV-1 subtypes are also called clades. These clades have phylogenetically linked the strains of HIV-1 from one another with approximately the same genetic distance. Also, some other subtypes are linked geographically or epidemiologically. Genetic variation within a subtype can be 15 to 20%, while variation between subtypes usually accounts for 25 to 35% [60][61].

HIV-2 is divided into the group's A–H. A and B are the most prevalent groups and possibly the only pathogenic ones. It has been explained that the presence of these groups in humans is that the separate zoonotic transmission of a virus of each group to humans and further transmission among humans by sexual contact. [62]

1.2.4. HIV-1 genome organization and structure

The mature HIV virion is round in shape, approximately 100 nm in diameter. It is surrounded by a lipid bilayer envelope glycoproteins (Env) derived from the cell membrane. The envelope contains 72 knobs which consist of trimers of the Env proteins. On the surface of each mature HIV-1 virion, Env glycoproteins are formed from surface protein gp120 and the transmembrane protein gp41. The gp120 trimers surface protein (SU) are anchored to the membrane by the trimers of the transmembrane protein gp41 (TM) and show knobbed spike structure [63]. Gp120 and gp41 form a heterodimer through binding to each other noncovalently[64]. The HIV-1 virion central core consists of Gag structural proteins, including

p24(Capsid), p17(Matrix) and p7(Nucleocapsid). The capsid protein (CA) p24 forms the cone-shaped core; the p17(MA) constitutes the inner surface of the viral envelope, and the nucleocapsid protein p7 (NC) is located in the core as a viral RNA binding partner [65]. Inside of the core, some other viral enzymes and accessory proteins are located that associated with the viral genome **(Figure 1. 1.1)** [66,67]. HIV-1 genome is a 9.8 kilobase pairs (kbp) positive single-stranded RNA molecule. As shown in **(Figure 1. 1. 2)**, it contains 9 open reading frames (ORFs) and several structural landmarks. Three of them respectively encode Gag, Pol and Env polyprotein precursors. The structural proteins encoded by Env coat the virion, and Gag constitutes the inner core [68].

The Env precursor gp160 is encoded by the Env gene. Gp 160 is cleaved by endoprotease furin into two subunits, gp120 and gp41. Gag precursor p55 is further cleaved by protease (PR) to p17, p24, p9 and p7. The Gag and Pol together encode a Gag-Pol polyprotein by translational frameshifting and proteolytically processed into PR, Reverse transcriptase (RT), and Integrase (IN). Of these proteins encoded by the HIV-1 genome, six are non-structural proteins, including four accessory proteins and two regulatory proteins, such as regulator of expression of virion protein (Rev), transactivator (Tat), negative factor (Nef), virion infectivity factor (Vif), viral protein R (Vpr) and viral protein U (Vpu) [105]. Moreover, the HIV-1 genome forms several secondary structures responsible for regulating viral replication by affecting various steps of the viral life cycle from reverse transcription initiation to viral RNA packing, including packing signals (pis), internal ribosome entry site (IRES), transfer RNA mimics, cis-regulatory elements, ribosomal frameshift motifs, Tat-acting region (TAR), dimer initiation site (DIS), primer binding site (PBS) and a polypurine tract (PPT) [69,70].

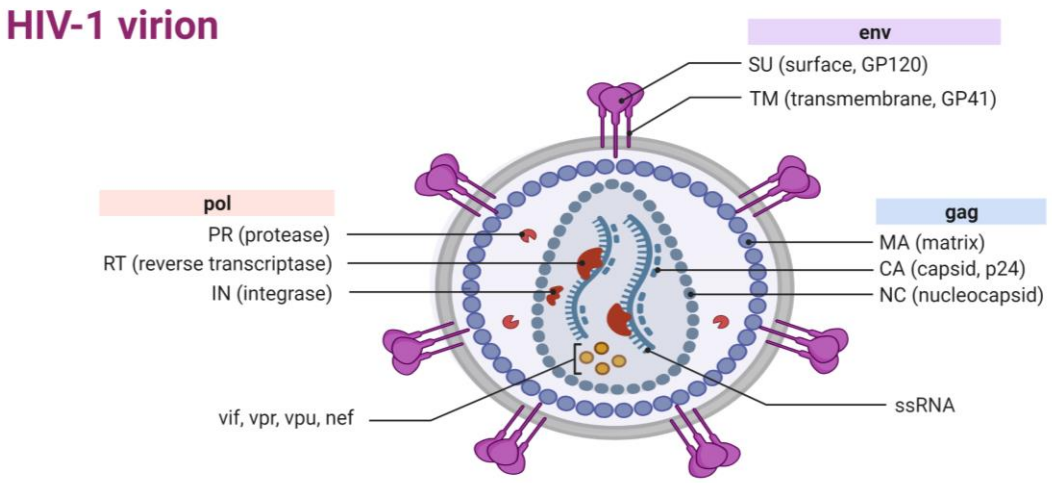


Figure 1. 1. HIV-1 virion structure and the organization of Gag, Env and Pol. (adapted from Ka-To et al., 2013)

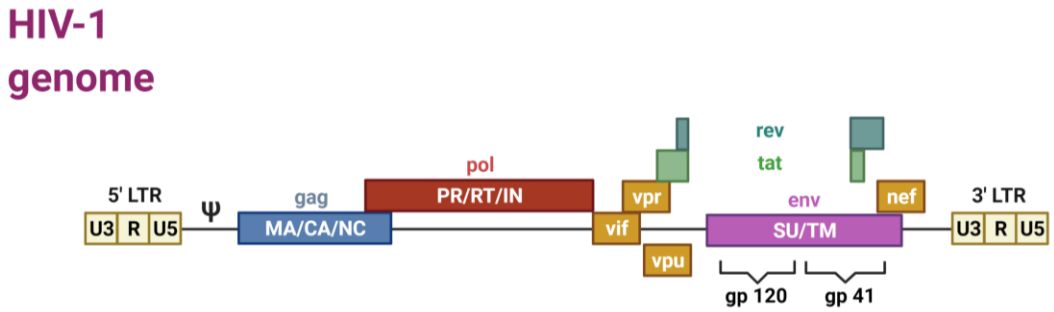


Figure 1.2. Schematic representation of HIV-1 genome structure. The genome contains structural genes (gag, pol and env), accessory genes(nef, vpr, vif, vpu), and regulatory genes (tat, rev). (Adapted from "Landmarks of the HIV genome" from www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html)

1.2.5. HIV-1 structural proteins

HIV-1 Env glycoprotein consists of the complex of gp120 and gp41 complex. Gp120 has mediated virus entry by binding to the cell surface receptor. Thus, gp120 is the main target for developing neutralizing antibody treatments or vaccine strategies[71]. However, the variable region of gp120 shows a high mutation rate and plays a vital role in escape immune recognition[72]. Gp41, as the transmembrane glycoprotein, mediates the fusion between virus and cell membrane after viral attachment [71]. Gag encoded the 55 kDa precursor (Pr55-Gag) protein and is further cleaved to generate MA, CA, p2, NC, p1 and p6. These structure proteins play an essential role in various steps of HIV-1 replication. The matrix protein (MA) is a 17kDa protein. As the N-terminal domain of Gag, MA contributes to the intracellular Gag move to the plasma membrane during the viral assembly. Since MA is located beneath the lipid bilayers, it has essential role to support the virion envelope structure.

Furthermore, because MA is a part of reverse transcription complex (RTC) and pre-integration complex (PIC), it also involves nuclear migration, nuclear import and integration[73–75]. CA is present in the viral core of HIV-1 virion and responsible for providing structural support to the HIV-1 core and protects RNA viral genome. Also, the interactions between CA domains facilitate Gag multimerization and the formation of immature virion in the viral assembly process[76]. During the uncoating step, CA also has been known to manipulate the capsid stability and disassembly to regulate the core uncoating[77]. NC is a nucleic acid-binding protein and selects genomic RNA and delivers them into progeny virus by binding to the packaging signal (Ψ) of genomic RNA [78].

1.2.6. HIV-1 enzymatic proteins

During the HIV-1 maturation, three enzymatic proteins, PR, RT and IN, are encoded through auto-activating and cleaving 160kDa Gag-Pol polyprotein (Pr160-Gag-Pol) by HIV-1 PR. PR as a homodimer aspartic protease, its principal activity is cleaving viral precursor proteins.

In addition, PR also contributes to the viral cytotoxicity effect by targeting some host cellular proteins, like cytoskeletal proteins, Bcl-2[79]. RT is an asymmetric heterodimer that consists of two subunits 66kDa and 51kDa. The primary function of RT is to catalyze the reverse transcription activity of HIV-1 genomic RNA into cDNA. Besides, RT has some other functions, including DNA-dependent DNA polymerase, RNase H, strand transfer and strand displacement synthesis [79]. IN as a tetramer, enzymatic proteins play an essential role in promoting the integration of the viral DNA into host cell chromosomal DNA. In addition to integration, IN involves reverse transcription, nuclear import and post-integration steps[80].

1.2.7. HIV-1 accessary proteins

Accessory proteins are critical for HIV-1 replication, targeting CD4+ T cells and macrophages and HIV-1 viral infection. Vpr is a 14kDa and multifunctional accessory protein that has functions in viral replication, such as HIV-1 nuclear import, cell cycle arrest, apoptosis. Firstly, Vpr makes cell cycle arrest to upregulate the viral transcription from increasing long terminal repeat (LTR) activity, which contributes to enhancing HIV-1 progeny virus production. Besides, the CD4+ T lymphocytes apoptosis mediated by Vpr is associated with the patient immune suppression and progress AIDS disease pathogenesis[81]. Vpu has two critical functions in favouring the HIV-1 life cycle. First, it downregulates the HIV-1 induced CD4 receptor by mediating the proteasomal degradation of new CD4 molecules synthesized in the endoplasmic reticulum (ER). Second, it facilitates the progeny virions production from infected cells by antagonizing Tetherin, interferon (IFN)-regulated host restriction factor [82]. Nef is 25-35kDa protein. Nef downregulates the chemokine receptor of CD4, CXCR4 and CCR5 receptors to avoid super infection [83].

1.2.8. Regulatory Proteins

Tat and Rev, as two critical regulatory proteins, Tat can interact with the TAR (trans-activating responsive) element and enhance the steady stage levels of all the viral transcripts. However,

Rev is responsible for transporting spliced, or unspliced HIV-1 genomic RNA via a cis-acting element called the Rev response element [84].

1.2.9. HIV-1 Replication cycle

The life cycle of HIV-1 can be summarized into 7 major steps including: 1) binding and entry, 2) fusion, 3) reverse transcription (RT), 4) nuclear entry and integration, 5) replication, 6) assembly, and 7) budding[85]. As shown in **Figure 1.3**, each step is briefly introduced. The first step in the HIV-1 life cycle is binding and entry into the host cell. Viral Env gp120 glycoprotein binds to CD4 receptors on the surface of CD4+ T lymphocytes or macrophages, and conformational changes are induced that increase the exposure of co-receptor binding domain of gp120 [86]. This conformational change plays a role in further interaction between target cell CCR5 or CXCR4 chemokine coreceptors (for R5- or X4- tropic strains) and the gp120-CD4 complex[87]. This stable co-receptor binding triggers the N-terminal end of the Env gp41 subunit to penetrate the target cell membrane, resulting in a conformational change in the gp41 subunit that brings the HIV Env and target cell membrane in closer proximity[88].

The second step of the HIV life cycle is binding the viral envelope and host cell membrane that results from the insertion of gp41 into the host cell membrane. Then, the viral core is delivered and uncoated into the host cell cytoplasm to release the viral RNA into the cytosol [89]. After fusion and uncoating the viral core in the cytoplasm, the HIV RT converts the viral RNA into double-stranded DNA through a reverse transcription process and forms the reverse transcription complex (RTC). The RTC combines with the proviral DNA to forms the pre-integration complex (PIC). PIC facilitates the transportation of viral cDNA into the host nucleus, where the DNA is joined into the host cell chromosome through viral integrase and cellular cofactors [34, 36]. After integrating the virus genome into the DNA of a host cell, the proviral cDNA can either undergo usual active transcription or enter the latency phase. Active transcription of the HIV provirus is initiated and accelerated by viral Tat. Also, other host

transcription factors such as NF-kB play an essential role in initiating the transcription of viral mRNA [90]. The transcribed viral mRNA is then moved out of the nucleus, and host cell ribosomes in the cytoplasm translated viral mRNA to polyproteins. In this stage, viral protein Rev facilitates the transport of viral mRNA from the nucleus to the cytoplasm [40]. Then, p17 (MA) activates the assembly of virion on the inner surface of host cell membranes by binding to the membrane and directing virions to the cell membrane, mediated by the Gag polyprotein and Gag-Pol-Pro polyprotein [91]. The virus core assembly occurs from necessary events, including gag-pol protein interaction with the plasma membrane, the placement of Env on the viral membrane, genome packaging of Vif, Vpr and Nef, and the viral genomic RNA into spherical immature virions[92,93]. Upon budding immature virions from the host cell membrane, the polyprotein Gag-Pol is cleaved by viral protease (PR) into the structural proteins MA, CA, NC and viral enzymes IN, RT and PR [43]. This proteolysis is essential for altering the immature virion into its mature infectious form, which is capable of initiating another replication cycle in a new target cell[92].

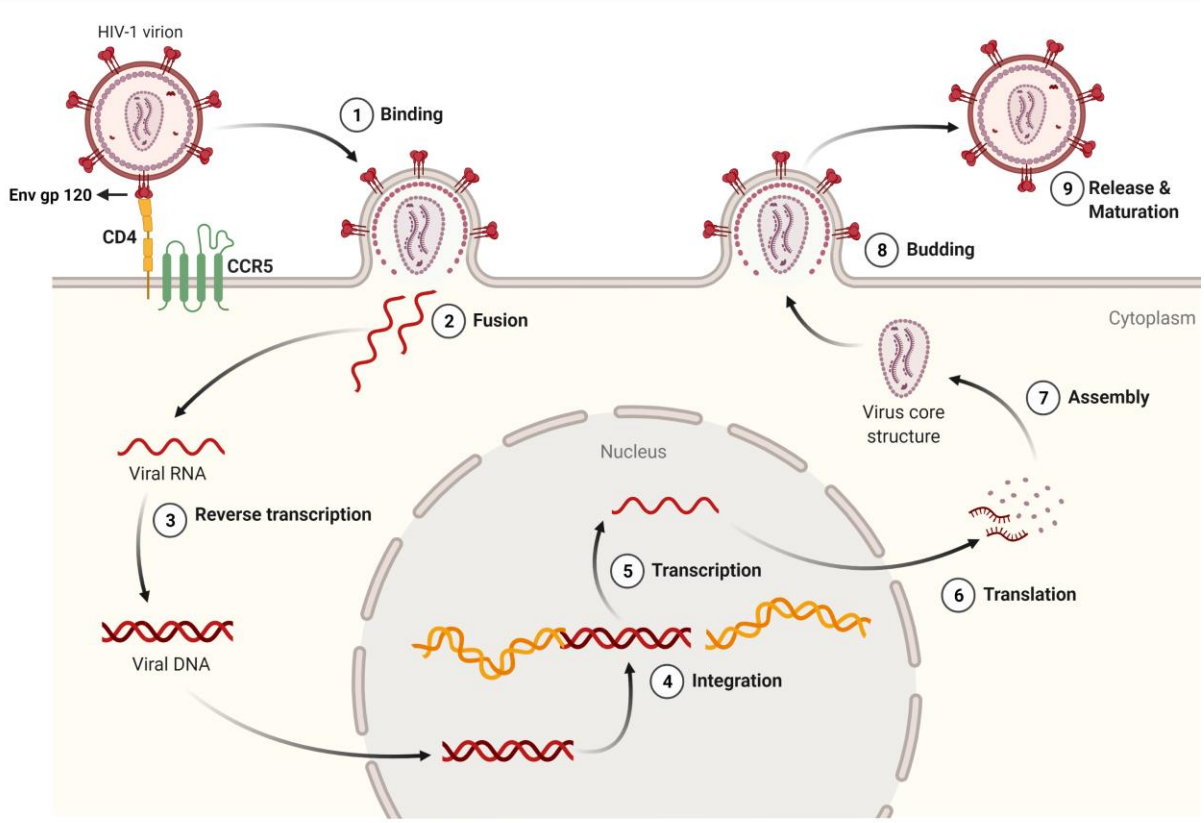


Figure 1.3. The life cycle of HIV-1. After HIV-1 approaches target cells, the interaction between HIV-1 envelope glycoprotein and CD4 receptor/coreceptors (CCR5/CXCR4) initiates the membrane fusion. Following that, the viral core uncoating occurs. Subsequently, the viral genomic RNA is released into the cytoplasm and convert to through viral DNA reverse transcriptase. The viral DNA is then moved into the nucleus and inserted into the host genome. Then, the following stimulation by viral protein Tat, viral mRNAs are produced from the nucleus and moved out into the cytoplasm, which is subsequently translated into viral proteins. After processed by cellular enzymes, assembly occurs on the inner side of the plasma membrane, where viral genome and proteins assemble into immature progeny virus. After budding, the polyprotein Gag and Gag-pol are further cleaved, and the immature progeny virus becomes mature. (adapted from Kirchhoff, 2013)

1.2.10. T cells in HIV infection

1.2.10.1. CD4+ T cells

The host cells use innate and adaptive immunity to detect and respond to infections. CD4+ T cells play a vital role in the stimulation of adaptive host immune responses in different ways. First, it acts as a bridge between innate and adaptive immune responses through recognition and binding of pathogen-derived peptides or vaccine immunogens presented by the major histocompatibility complex (MHC) II of antigen-presenting cells (APCs) results in the elimination of infections[94]. Innate signalling processes several pathways such as recognition of PAMPs (Pathogen-Associated Molecular Patterns) through PRRs (pattern recognition receptors), the secretion of pro-inflammatory cytokines and chemokines, and the expression of co-stimulatory molecules including CD40, CD80, and CD86 on APCs can all serve as necessary secondary signals result in naive CD4+ T cells activation[25]. After encountering CD4+ T with cognate antigen and co-stimulatory signals from innate immune cells, activation and differentiation of naive CD4+ T cells into effector T helper cells (Th cells) initiate, that can mediate the humoral and adaptive immunity in the host [95,96]. These effector CD4+ T cells can be categorized into three main types, including Th type-1 (Th1), Th type-2 (Th2) or Th type-17 (Th17) cells, based on their phenotype. Th1 cells produce the cytokines interferon (IFN)-γ, and tumour necrosis factor (TNF)-β. These cytokines make Th1 more effective in protecting against both intracellular infections caused by viruses, bacteria, and micro-organisms that grow in macrophages and eliminate cancerous cells. Th2 cells produce interleukin (IL-4), -5, -10 and -13. These interleukins trigger antibody production and target parasites through B cell activation to protect against parasites that are susceptible to IL-4-switched immunoglobulin (Ig)E secretion, IL-5-induced eosinophilia, and IL-3- and IL-4-stimulated mast cell proliferation and degranulation[97]. The third group is known as Th17 cells, which secrete IL-17, IL-17F, IL-6, IL-22, and TNF-α and have a significant role in both

tissue inflammation and activation of neutrophils to defence against extracellular bacteria[97]. During HIV infection, the changes in immune response and cytokine secretion resulting from HIV-induced immune activation increase vulnerability to activation-induced cell death by apoptosis[98].

1.2.10.2. CD4+ T cells in HIV Infection

CD4+ T cells are the main target of HIV infection, and gradual depletion of CD4+ T lymphocytes is a distinguishing character of HIV infection. HIV-1 preferentially targets and replicates within activated CD4+ T cells through gp120 subunit of HIV-1 Env, which mainly binds to cluster-of-differentiation (CD) 4 markers on CD4+ T cells, then it binds to co-receptor CCR5 or CXCR4 chemokine co-receptors on the surface of host cells. The expression of metabolic and activation markers, Ki67 and CD69, in CD4+ T cells indicates the proliferation capacity and HIV-1 cellular entry observed in plasma and endocervical CD4+ T cells[99,100]. HIV-1 infection impairs the function of CD4+ T cells. CD4+ T cell's destruction and gradual loss result from the release of viral progeny at the plasma membrane, the cytopathic effects of HIV-1 virions, and other mechanisms. Env expressing in the infected cells can induce syncytia formation and bystander apoptosis in uninfected CD4+ T in contact with Env expressing cells through gp41-hemifusion and autophagy [101]. The CD4+ T cells depletion, specifically T helper cells and HIV-1 specific memory CD4+ T cells, mainly occur within the lamina propria of the gastrointestinal tract and induces local inflammation and HIV-associated chronic immune activation, severely affecting the integrity of the gastrointestinal mucosa and host defences in the gut [102,103]. The impaired cellular immunity caused by CD4+ T cell depletion leads to microbial translocation and increases the level of microbial products in plasma, contributing to the persistent systemic immune activation[98]. The host immune responses are completely usurped by HIV-1 virions, unlike other pathogens. This persistent cycle of immune cell activation, infection, and CD4+ T-cell depletion contributes to immune dysfunction or T-

cell exhaustion, eventually leading to immunodeficiency and increase the susceptibility of HIV- infected people to a wide range of opportunistic infections. This can pose a significant challenge for HIV-1 vaccine development progress since vaccine immunization can also stimulate immune activation and proliferation, further replenishing the numbers of target cells[104].

1.2.10.3. CD8+ T cells in HIV Infection

CD8+ T lymphocytes have a crucial role in mediating host cellular immune responses, limiting HIV-1 replication, and postponing the ultimate disease progression[105]. Activation of resting CD8+ T cells is similar to CD4+ T cells activation involving antigen-specific TCR (T cell receptor) and MHC-co-receptor binding, as well as cytokine signalling [106]. In the early stage of infection, the TCR of CD8+ T cells stimulation leads to recognition and binding of processed foreign peptides presented by MHC class I molecules of infected host cells and APCs [107]. The activated Th1 cells produce pro-inflammatory cytokines like IL-2, IL-12, IL-21, and type I IFN. Also, innate immune cells like macrophages promote the activation and CD8+ T cells differentiation as well as the programming contraction phase of the effector T cell pool after infection[108–110]. Activated CD8+ T cells are capable of reacting and eliminate pathogens throughout the infection by differentiating into cytotoxic effector T cells and long-lived antigen-specific memory CD8+ T cells [111].

Moreover, short-lived Cytotoxic T lymphocytes (CTLs) destroy infected host cells by releasing perforin and granzyme molecules that induce direct cell death or CTL-mediated apoptosis [112]. CTLs are also known to produce chemokines such as MIP-1α, MIP-1β, and RANTES as well as antiviral cytokine-like IFN-γ expression [113,114]. In the late stage of infection, about 90-95% of CTLs go through a contraction phase leading to rapid apoptosis. Still, a long-

term antigen-specific memory CD8+ T cells pool is established and rapidly proliferates upon re-exposure to an antigen [115,116]. In terms of HIV-1 infection, multiple studies revealed the vital role of HIV-specific CD8+ T cell responses in controlling and infection and limiting disease progression. An early in vitro study in the late 1980s showed the evidence of CD8+ T cell suppression during HIV-1 replication by viral transcription suppression [117]. Also, in two 9 separate studies on the NHP/SIV model, loss of CD8+ T cells resulted in a significant increase in SIV replication during acute and chronic infection stages [118,119]. In HIV-infected individuals, early control of primary viral replication during acute infections coincides with the emergence of CD8 T cell responses [81, 82]. In both HIV-infected long-term non-progressors (LTNPs) and elite controllers (ECs), long-term viral load control was related to the higher frequency and polyfunctionality of CD8+ T cells, including potent cytotoxic responses and production of IFN-γ, TNF-α, MIP-1β, and IL-2 [120]. Overall, these studies suggest that further understanding of cellular immune response during HIV-1 infection is necessary to develop strategies for controlling and treating infection.

1.2.11. HIV-1 genome diversity

One of the significant challenges of HIV-1 virions is genetic diversity, which is one of the major obstacles to eradication and vaccine development. HIV-1 has a high replication rate. Consequently, the rate of error-prone reverse transcriptase and recombination events may increase during virus replication. In error-prone reverse transcriptase, mutations take place more frequently into the viral genome by the host innate immune effectors, such as the APOBEC nucleic acid editing enzymes that present mutations into viral genomes[121,122]. In retroviral recombination, HIV reverse transcriptase can use two copies of the co-packaged viral genome, which are genetically different, leading to the shuffling of mutations between viral genomes in the particle's population[123]. Within the host, virus mutant forms tend to follow mutation pathways to escape from antiretroviral drug and immune pressures resulting in the

distribution of HIV-1 strain worldwide [124,125]. Moreover, HIV-1 mutations allow the virus to escape from the CTL response, which plays a vital role in controlling HIV infection and leads to rapid disease progression to AIDS. Generally, the emergence of escape mutations during the immune response is advantageous for the virus but is related to costs to viral fitness[126].

1.2.12. HIV- Conserved Regions

Some regions in the HIV-1 genome have a high degree of conservation and limited opportunities for escape mutations associate with viral replication[126]. It has been shown that protective CTL responses have targeting ability against conserved epitopes that occurs in the onset of acute infection since early acute infection. CTL responses target variable epitopes with rapid viral escape. Due to the CTL retargeting, targeting of conserved epitopes in chronic infection is limited. At this time, significant depletion of HIV-1-specific CD4+ helper T lymphocytes has occurred, and CTLs become hypofunctional[127]. Therefore, an early focus of CTL targeting on highly sequence constrained epitopes appears to be necessary since the low rate of immune evasion[128]. Another vaccine study also demonstrated that HIV-1 conserved regions have higher immunogenicity, and vaccination with conserved regions guides immunodominance to the conserved epitopes compared to the full-length protein vaccination[127]. This feature enlightens the role of humeral immune responses related to the natural control of viral replication in infected cells[129].

1.2.13. Conserved Region in HIV-1 Env

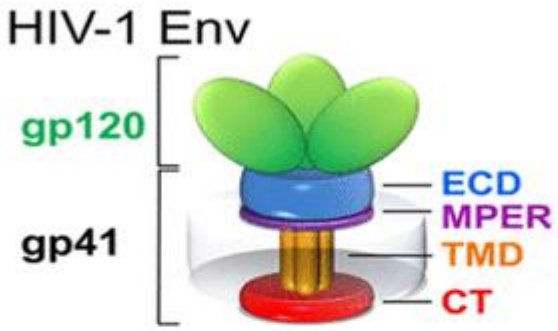
As described previously, Env in the HIV-1 genome is translated to a gp160 polyprotein, following by cleavage by host enzymes in the Golgi Body to form two subunits, gp120 (SU) and gp41 (TM) (**Figure 1. 4 A**). Gp120 has noncovalent interactions with gp41 subunit ectodomain. The noncovalent interaction in the gp120-gp41 complex is embedded in the

plasma membrane by CTLA-4 (Cytotoxic T-lymphocyte associated protein-4) mediated secretory pathway and incorporated into viral progeny[130].

In HIV-1 Env gp120 subunits, there are many sugar chain that is problematic for HIV-1 vaccine design. [131]. The gp120 glycoprotein consists of five conserved subdomains (C1–C5) and five hypervariable glycosylated loops (V1–V5) that contribute to the pathogenicity of HIV-1 [131](**Figure 1.4 B**). The variable epitopes have significant diversity in sequence as a result of high replication rates, recombination, and mutations[132]. This variability in HIV-1 epitopes is a major challenge of the virus to escape from the host immune response[133]. The conserved elements (C1-C5) of gp120 form a "core" of the protein which compromises the gp41-interactive inner domain and the highly glycosylated outer domain linked by the bridging sheet[134]. The internal domain contains three α-helixes (α_0, α_1 and α_5) and a seven-stranded β sandwich (β_0, β_1, β_3, β_5-β_7, and β_{25}). The loop excursions are originating from the sandwich form three separate topological layers, such as layer 1(α_0, β_0 and β_3), layer 2(α_1, β_1 and β_5) and layer 3(α_5, β_7 and β_{25})[135]. These layers play a crucial role in stabilizing the interaction between gp120 and CD4 complex (**Figure 1. 4 C**) [136]. The conserved regions located in the inner domain contain several residues of the CD4 binding site (CD4bs) in C1, C3 and C4 regions. Another CD4 binding site interacts with gp120 by presenting its phenylalanine 43 in a cavity (the Phe43 cavity), which is located between the inner and outer gp120 domains[137]. Moreover, the essential gp41-interactive region is located in the inner domain. It is composed of a seven-stranded β sandwich proximal to the gp41 interface[138]. The bridging sheet is surface-exposed on the outer domain of gp120, including the loop V_1, loop V_2, β_2, β_3, β_{20} and β_{21}[139]. The region consisting of the V_1/V_2 has a great variety in length, sequence, and glycosylation. The average length of V_1/V_2 is about 80 amino acids, with a possibility of high variations in length derived from the two regions, one in the middle of V_1 and the other near

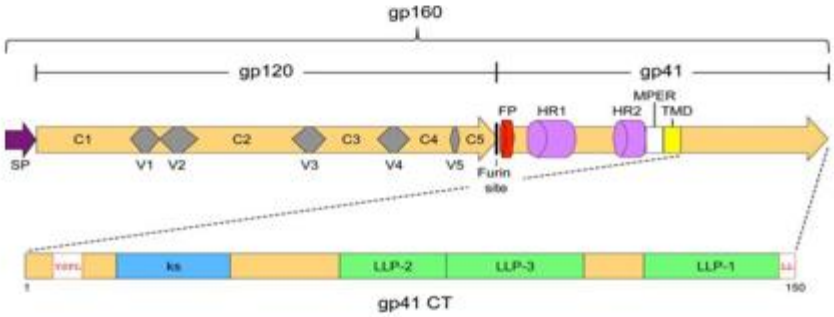
the C-terminal end of V_2[140]. The V_1/V_2 loop masks the CD4 receptor binding site, and may participate in viral escape from immune system[140].

A



(Adopted from Alessandro Piai et al., 2017, Journal of the American Chemical Society)

B



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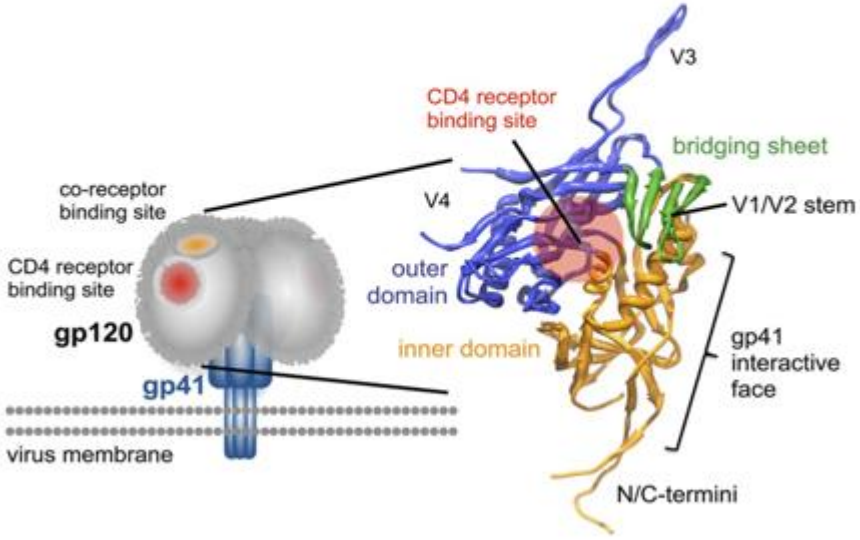


(Adopted from Miklos Guttman et al., 2012, J. Virology)

Figure 1.4. The schematic representation of HIV envelope glycoprotein trimer structure. A) HIV-1 envelope glycoprotein trimer is comprised of a noncovalently linked gp120 and gp41 heterodimer as a result of a cleavage of the viral gp160 precursor protein. B) The domains of Env. Precursor gp160 is cleaved into gp120 and gp41 subunits by cellular furin. Gp120 contains five conserved regions (C1-C5) and five variable regions (V1-V5), and gp41 contains extracellular domain (including FP (fusion peptide), HR1 (heptad repeat 1), HR2 (heptad repeat 2), and MPER (membrane-proximal external region), a transmembrane domain and cytoplasmic tail (including internalization signal YSPL and three LLPs). C) Crystal structure of gp120. The 4-stranded β-sheet subdomain termed the bridging sheet consists of two strands from the outer domain and the V1/V2 loop from the inner domain. The position of the CD4 binding site on gp120 is demonstrated. Gp120 is believed to interact with gp41 primarily through interactions involving the inner domain and N-/C-terminal extensions.

1.2.14. Broad neutralizing antibody targets gp120

The conserved regions construct the core of the protein and are necessary for binding to the CD4 receptor on target cells. To elicit broadly neutralizing antibodies (NAbs), at least one or more conserved epitopes should be recognized to overcome the extensive antigenic variation of HIV-1 Env gp120[141]. Unfortunately, few conserved epitopes on the envelope protein are accessible and easy to recognize for specific antibody binding and neutralization[142]. These conserved epitopes have been hidden either by glycosylation pattern or conformational masking [143]. Therefore, these epitopes are major targets of HIV-1 neutralizing antibodies. It has been shown that one-third of neutralizing activity of subtypes B and C neutralizing antibodies in polyclonal sera targeting the CD4 binding site (CD4b)[144,145].

Three monoclonal antibodies, such as 2G12, IgG1b12, 447-52D target gp120 that have been extensively described in their neutralizing activities. Among these anti-gp120 MAbs, 2G12 recognizes a unique epitope in a carbohydrate-rich region on the outer domain involving the C3-V4 region, but it is not directly associated with receptor binding sites on the protein. while IgG1b12 targets the CD4 binding site and 447-52D recognizes the V3 loop of gp120 [146,147].

1.2.15. HIV Env gp41

HIV gp41 can be divided into 3 major domains: 1) the extracellular domain (ectodomain) (residues 512–683 by standard HIV-1 HXB2 gp160 numbering), 2) the transmembrane domain (MSD, 683–707), and 3) the cytoplasmic domain (708–856). The major functions of the gp41 protein are mediated by the extracellular domain, which can be further subdivided into the following five functional regions: a fusion peptide (FP, 512–534) followed by the N-terminal heptad repeat (NHR/HR1, 542–591), the loop region (593–622) and the C-terminal heptad repeat (CHR/HR2, 623–661) and finally the membrane-proximal external region (MPER 662–683)(**Figure 1.5**) [64]

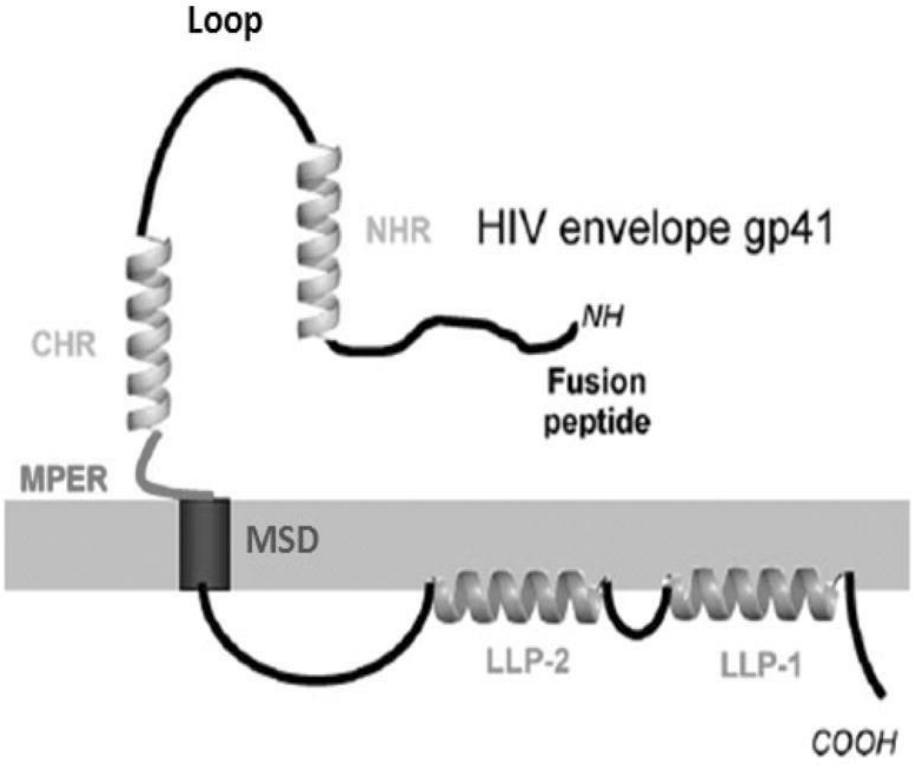


Figure 1.5. Schematic representation of HIV gp41 regions. NHR: N Heptad Repeat, CHR: C Heptad Repeat, MPER: Membrane Proximal Ectodomain Region, MSD: Membrane Spanning Domain, LLP1, LLP2: Lentiviral Lytic Peptide 1 and 2. (Adopted from Garg et al., 2011)

1.2.16. Membrane-proximal external region (MPER) in HIV Env

Adjacent to the loop domain is the C-heptad repeat (CHR) domain that terminates hydrophobic sequences, followed by the MPER domain[64]. One of the most conserved regions in HIV-1 Env gp41 known as the membrane-proximal external region (MPER). MPER has an approximately 24-residue hydrophobic region (residues 660–683), located in the terminal residues of the transmembrane domain (TMD), and has a key role in the fusion of virus and cellular membrane and infectivity [148]. Mutations within the MPER region of gp41 can inhibit viral entry or block it during pore expansion due to its critical role for envelope incorporation into the virion. The function of MPER appears to be associated with five highly conserved tryptophan residues that make up about 25% of the region since even single tryptophan substitutions interfered with Env incorporation and infectivity[149]. It is reported that MPER is a target for most bNAbs. Thus, it is considered an ideal target for vaccine design[150].

1.2.17. Broad Neutralizing antibodies target MPER

The significance of MPER has been recognized by containing epitopes for several monoclonal antibodies (mAbs), such as 2F5, 4E10, Z13,10E8, Z13e1, m66.6, CH12[149]. Among these antibodies, 2F5, 4E10, and 10E8 display broadly neutralizing activity in the host. Several studies revealed that sera from long-term nonprogressors (LTNPs) with <50 copies of HIV RNA/ml plasma level had weak neutralizing activity. Compared to patients with higher viremia levels, LTNPs made weak NAb responses that have been attributed to a low antigenic stimulation of B cells[151–153]. 2F5 targets the conserved sequence ELDKWA, while 4E10 and Z13 are mapped to the sequence NWF(D/N) IT in the C-terminus of the 2F5 epitope (**Figure 1.6**)[154].

The antibody binds to the specific peptide sequence, which is functionally necessary for the antibody. However, this binding is not sufficient to achieve MPER-dependent antibody neutralization. For instance, the Z13e1 antibody overlaps the epitopes bound by 4E10 with

similar affinities but showing significantly low neutralization potency than 4E10[155]. Hydrophobic residues seem to be necessary for MPER antibodies' neutralization capability [156] and interact directly with membrane lipids, while some residues are essential for binding to the peptide epitope. The mechanism of binding neutralizing antibodies follows a two-step, encounter-docking model. First, the antibody binds to the lipid membrane through its long hydrophobic residues and concentrates all around the MPER epitope. Upon conformational changes occur, the antibody attaches to the pre-hairpin intermediate of Env gp41[157]. The mechanism enables the easy antibody approach to the epitope, overcomes the insufficient exposure of MPER, and takes advantage of its position in close proximity feature to the viral membrane[157].

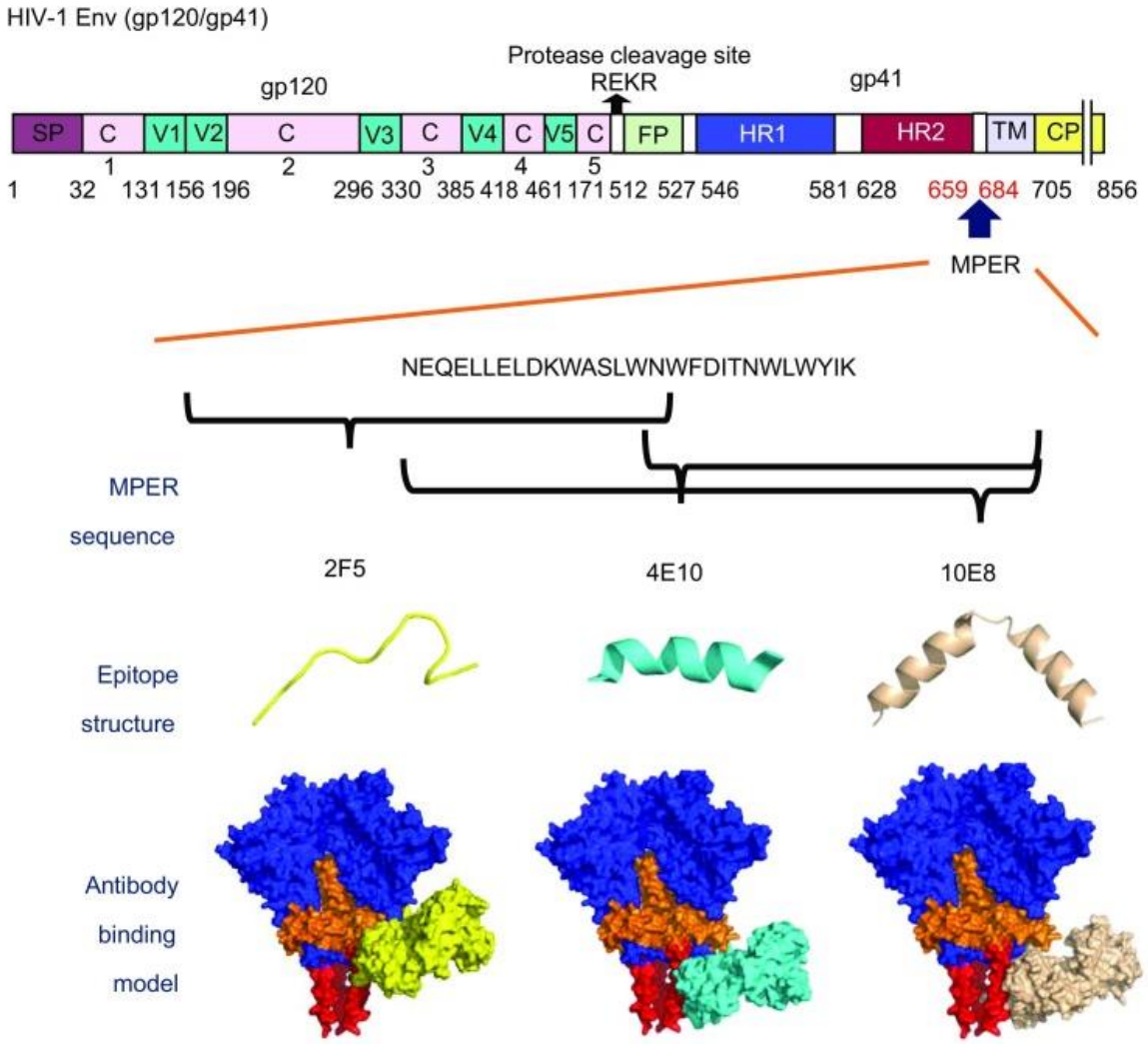


Figure 1.6. MPER conformation and neutralizing antibody targeting sites. MPER conformation in the envelope glycoproteins of HIV-1 and MPER binding to antibodies sites. As C-terminus of gp41 subunit ectodomain, gp41 MPER bridges the extracellular domain and transmembrane region of Env. The crystal structure of 2F5 Fab in complex with its epitope peptide shows that its epitope forms a β turn conformation, whereas the epitope of 4E10 forms an α helical conformation. Similar to 4E10, 10E8 forms two α helixes at N- and C-terminus of MPER, respectively (Adopted from Liu et al., 2018).

1.3.Ebola virus

1.3.1. Ebola virus virology

Ebola virus (EBOV) belongs to the *Ebolavirus* genus of the *Filoviridae* family. EBOV has a 19 kb length includes seven genes. The EBOV non-segmented negative-sense ssRNA genome contains short extragenic regions, including signals necessary for major virus replication, transcription initiation[158]. Each of the seven genes within the EBOV genome is flanked by highly conserved transcription start and stop signals separated by intergenic segments consisting of 4–7 non-conserved nucleotides in length[158]. EBOV genome encodes seven structural proteins. From 3′ to 5′ end, it is as follows 3′-NP, VP35, VP40, GP, VP30, VP24, L-5′. The VP (35) is RNA-dependent RNA polymerase cofactor, VP(40) is a matrix protein, GP 1,2 is spike glycoprotein, VP(30) is a transcriptional activator, VP (24) is the second protein matrix, and (L) is RNA polymerase enzyme[159]

Similar to the other filoviruses, EBOV is an enveloped virus. The length of a filamentous virus is about 80 nm to 14,000 nm. The structure of EBOV may appear as circular and "6"-shaped particles [160]. The NP-coated RNA genome is located within the EBOV filament. Moreover, L links with the polymerase cofactor, VP (35), which is located on the genomic complex, along with VP (24), and the transcription factor, VP (30). All around the viral genomic complex is surrounded by VP(40), which keeps the morphology of the virus, and GP spikes, which are expressed on the surface of the virus [161–163].

1.3.2. Ebola glycoprotein

EBOV GP contains seven repeats of adenosine nucleotides synthesized as a single polypeptide. This polypeptide is cleaved by a furin-like protease to produce a heterodimer conformation, GP1 and GP2 subunits, which associates together by a disulphide bond[164,165]. GP1 has a

significant role in cell receptor binding. It has three domains, including a receptor-binding domain (RBD), a glycan cap, and a mucin-like domain, a non-structural heavily O-glycosylated region. GP2 contains a transmembrane domain and is responsible for viral and membrane fusion. The surface of envelope GP is covered by N and O-linked glycans, which play a role in virus attachment. It is also crucial for shielding the GP from neutralizing antibody recognition[164].

The EBOV cell entry pathway is mediated by cysteine proteases, termed cathepsins. Cathepsins active within the cellular endosomal/lysosomal pathway in order to cleave EBOV GP1, remove both glycan cap and mucin domains, expose amino acid residues that increase receptor binding affinity and virus infectivity[164,166]

1.3.3. Ebola GP in viral entry

The EBOV glycoprotein (GP) has a trimeric structure consists of three monomers of GP1 and GP2. These components constitute a viral envelope, which has a crucial role in viral entry[165]. EBOV GP1 is essential for cell membrane binding, and receptor interaction and GP2 is necessary for membrane fusion[167].

There are some other attachment factors on the surface of target cells that have been shown to contribute to mediate GP1 viral attachment, including dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and T-cell immunoglobulin and mucin domain 1 (TIM-1), which results in the virus entry into cell endosomes through micropinocytosis-like internalization pathway [168]. Cellular cysteine proteases, such as cathepsins B and L, activate in the low pH condition of the vesicles. Subsequently, they remove the heavily glycosylated regions of GP1, which leads to the conformational change in GP and interaction with the Niemann-Pick type C1 (NPC1) receptor[169]. As a result of conformational changes, the hydrophobic fusion loop of GP2 is exposed, which leads to the for

viral and cellular membranes fusion to allow the EBOV entry into the cytoplasm[170]. Hence, the EBOV-GP is considered a potential target for therapeutic strategies to interrupt virus entry and inhibit the propagation of EBOV[171].

1.3.4. The role of Mucin-like domain of Ebola GP during viral replication

One critical factor contributing lethal infectivity of EBOV is the mucin-like domain (MLD). This domain consists of approximately 150 amino acids and many N- and O-linked glycosylation sites and is a distinguished character of filoviral GPs[172,173]. MLD is located in the GP_1 subunit of the EBOV (GP), rich in proline, serine, and threonine residues are heavily glycosylated domain and is one of the most variable epitopes in the EBOV GP(**Figure 1.7**)[173]. The expression of MLD constitutes one of several mechanisms by which EBOV successfully escapes and disrupts the activation of the host immune system. This heavily glycosylated domain of the EBOV (GP) arranges a steric shield over proteins at the cell surface like a glycosylated umbrella. This steric shield inhibits the detection of viral GP from neutralization by the adaptive immune response and has the functional effect in preventing interactions with CD8 T cells[172,174].

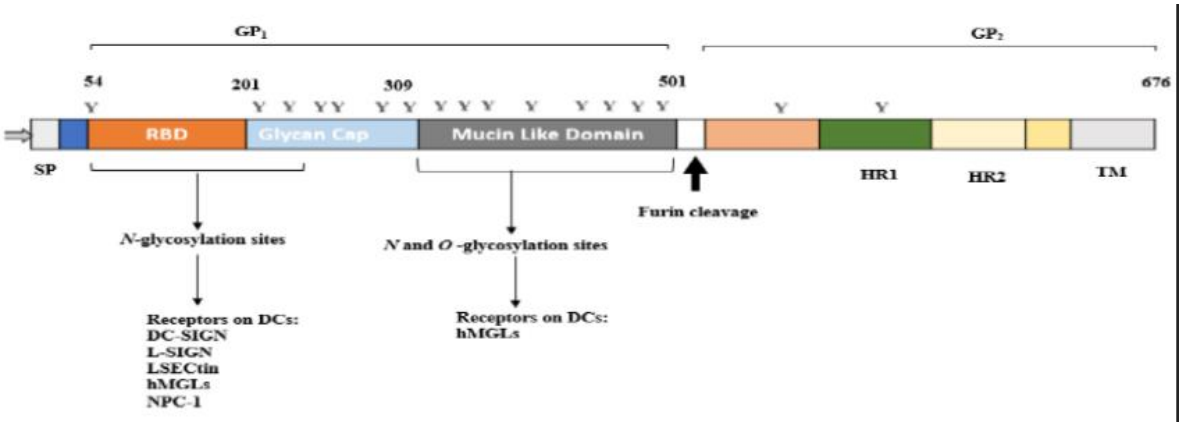


Figure 1.7. A schematic structure of EBOV GP, showing GP1,2. GP1 consists of RBD, glycan cap, and MLD, while GP2 contains the HR1 and HR2. Y denotes the N-glycosylation sites. Receptors on DCs have an affinity with the N-linked glycans on GP1, indicating that the binding sites of EBOV GP with DCs are on the RBD. In contrast, the glycan cap contributes to its binding because of the presence of N-glycosylation sites. The receptors on DCs for GP1 include DC-SIGN, L-SIGN, LSECtin, hMGLs, and NPC-1. Although N-glycosylation sites are present on the MLD, MLD is dispensable, and its absence has a role in the efficiency of cell entry of EBOV GP[175–178]. (Adopted from Olukitibi et al., 2019)

1.3.5. The affinity of Ebola GP for targeting APCs

Antigen-presenting cells (APCs), such as macrophages and dendritic cells, are the main targets of EBOV infection[179].

C-type lectin receptors (CLRs) are essential for body immunity since most of them are expressed by different leukocytes and play various host defence roles, including cell-cell adhesion, host-pathogen adhesion, and antigen uptake[180]. Unlike HIV-1 GP, EBOV GP has different receptors on DCs and macrophages, making them an efficient stimulator for APCs. CLRs are present on the DCs that can interact with N- and O-linked glycans on GP1 (RBD, MLD, and glycan cap) and leads to facilitate viral entry[178].

EBOV GP has three major receptors on the DCs: dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN), CLEC4G/LSECtin human macrophage galactose-type C-type lectins (hMGLs, CD301). Briefly, the N-glycan moieties and N-acetylgalactosamine present on the EBOV GP1 are essential features for the binding to these CLRs on the surface of macrophages and DCs. Moreover, other modifications in the binding sites on the EBOV GP can affect the binding efficiency of EBOV with both lectin receptors and other cellular factors to facilitate the stimulation of APCs[178]. Another possible receptor on DCs has been known to have an affinity for EBOV GP. This receptor is a hydrophobic Neimann-Pick C1 (NPC-1) receptor-binding pocket. It has been shown that EBOV GP could bind with the endosomal (NPC-1) receptor on DCs, firstly by interacting electrostatically with the NPC-1 through the hydrophilic crest on GP1. At the same time, hydrophobic trough exposure on GP1 facilitates specific cognate interactions due to their ability to migrate to lymph nodes to encounter DCs [178,181].

Chapter 2: study rationale, hypothesis, and objectives

2.1. Study Rationale

2.1.1. HIV Env conserved regions in HIV vaccine design

HIV Env is a highly glycosylated protein containing two 'conserved' and 'variable' regions. The variable regions consist of immunodominant epitopes. These epitopes are highly targeted by the immune system, which has resulted in the generation of immune escape variants[182]. The strategy to decrease this diversity is to lead immunity to the conserved sequences; therefore, we selected Env amino acids 521 to 606 to provide better coverage of HIV-1 genome sequences. Broadly neutralizing antibodies (bNAbs) have demonstrated protective effects against HIV-1 in primate studies and recent human clinical trials. Therefore, the major focus is on increasing broadly neutralizing antibodies (bNAbs), preventing viral entry by binding to conserved regions on the HIV envelope glycoprotein trimer, the only entry compound for HIV. The HIV-1 envelope glycoprotein consists of the surface moiety gp120, which is bound to the transmembrane protein gp41. The primary structure of gp120 can be subdivided into 5 relatively conserved regions and 5 variable loops (C1-V1-V2-C2-V3-C3-V4-C4-V5-C5, from amino to carboxy-terminus). These bnAbs targets six distinct sites on the HIV-1 envelope glycoprotein (Env) spike, including the CD4-binding site (CD4bs), V2 apex, N332/V3 base supersite, silent face, gp120-gp41 interface (including fusion peptide), and membrane-proximal external region (MPER). Furthermore, MAbs directed at gp120 epitopes or at gp41 epitopes have been shown to neutralize HIV-1 isolates of different clades[183]. In this project, we arranged 9 regions in HIV gp120 Env that are highly conserved throughout HIV M Group sequences (Env CE) and defined based on stringent conservation, functional importance.

2.1.2. The important roles of targeting antigen-presenting cells in enhanced humeral and cellular immune responses

APCs, such as DCs and Macrophages, have a vital role in the innate immune response, which presents viral antigens upon infection and activates naïve lymphocytes to stimulate cytolytic and memory responses. Since HIV infection and depletion of CD4+ T cells lead to the immunodeficiency associated with AIDS, an effective vaccine should direct viral antigens to APCs to stimulate innate immunity while sparing CD4+ T cells to prevent immunosuppression[25]. Therefore, targeting vaccines to DCs and macrophages is highly effective for vaccine design. Moreover, the generation of broadly neutralizing antibodies with great breadth and potency against HIV env can prevent HIV infection in different ways, including blocking receptor interaction, preventing membrane fusion, and enhancing decay of Env spikes [184].

2.1.3. Modifications of the EBOV glycoprotein for the fusion of HIV-1 Env conserved epitopes

Mucin-like domain (MLD) of EBOV GP has 150 amino acids, which is associated with cell cytotoxicity, causing rounding of cells and detachment from the extracellular matrix. Also, MLD mediates the downregulation of β1 integrin and MHC-I on the cell surface. EBOV GP directs the virus to many APC subsets. Thereby it is possible that the cytotoxicity of these cells mediated by the MLD could be involved in innate immune dysfunction and subsequently dysregulate the optimal adaptive immune responses [172,174,179,185]. Thus, the MLD of EBOV GP can be deleted (EboGPΔM) for insertion into the vaccine platform. Furthermore, our preliminary data revealed that fused HIV Env with EboGPΔM could efficiently target human DCs and macrophages. Thus, all these results have demonstrated the feasibility and potential of this newly developed EboGPΔM fusion vaccination technology as a new approach to improving immunogen delivery and enhancing vaccine efficacy. [186]

2.2. Hypothesis

The replacement of the mucin-like domain of Ebola GP with highly conserved elements (9CE), membrane-proximal external region (MPER) of HIV envelop glycoprotein can elicit an effective anti-HIV humoral and T cell-mediated immune responses in the host compare to HIV Env (M).

2.3. Objectives

The objectives are addressed in the following aim:

1. Generation of EboGPΔM-9CE, EboGPΔM-MPER and/or HIV Env (M) plasmids and investigation of their DC/macrophages targeting ability.
2. Generation of VLP vaccine expressing EboGPΔM-9CE, EboGPΔM-MPER and/or HIV Env(M) and investigate their ability to induced immune responses in mice.

Chapter 3: Material and methods

3.1. Plasmid constructs

HIV Gag-Pol expressing plasmids (CMVin-Gag/Pol), HIV-1 RT/IN/Env tri-defective proviral plasmid containing a gene encoding for secreted Gaussia luciferase (G-Luc) at the position of *nef* (ΔRI/ΔE/Gluc), the HIV Env glycoprotein is expressing plasmids pLET-Lai (X4-trophic) and pLET-JRFL (M-trophic) used in this study were previously described [187]. The codon-optimized (opt) Zaire Ebolavirus glycoprotein (EboGP) expressing plasmid (pCAGGS-optEboGP) was previously described[186]. To construct the mucin-like domain deleted EboGP plasmid (pCAGGS-optEboGPΔM), Two-step PCR was used to amplify the optEboGPΔM gene (5' primer, 5-AATTCGAGCTCGCCACCATG; ΔM primer-3', 5-TCTAGAGTAGGGCCCTCCTTCCTCGGAACGGATTT; ΔM primer-5', 5-AGGGCCCTACTCTAGAAACACCATCGCAGGTGTT; 3' primer, 5-TGCTAGCTCGAGCATGCTCAGA). Then the amplified optEboGPΔM gene was cloned into a pCAGGS vector using *Apa*I and *Xba*I sites.

HIV-1 gp120 9CE and HIV-1 gp41 MPER were designed based on the HIV Sequence Compendium database published by Los Alamos National Laboratory [188] and chemically synthesized by GenScript Inc (Piscataway, NJ, USA). The synthesized HIV Env 9CE was cloned into a pUC57 (2710bp) vector. To generate pCAGGS-optEboGPΔM-9CE, the gene sequence encoding 9CE and/or MPER was PCR and inserted into pCAGGS-optEboGPΔM at *Apa*I and *Xba*I sites. To amplify HIV Env 9CE, gel purifying (Qiagen) the fragment and replacing the construct in the deleted mucin-like domain of EboGP.

3.2. Recombinant proteins

Recombinant HIV1 gp41 protein(abcam, Cat# ab49070), p24 recombinant protein (Cat#13126), HIV-1 CN54 gp140 Protein (Cat#12064) were obtained from the NIH AIDS Research and Reference Reagent Program. The purified recombinant EboGP without the transmembrane domain (TM) (IBT Biosciences, Cat# 0501–015).

3.3. Antibodies

The Anti-HIV-1 gp120 monoclonal antibody (ID6) (Cat#11784), and Anti-HIV-1 gp41 Monoclonal Antibody 2F5, 4E10, and 10E8 (Cat#1475; Cat# 10091; Cat#12865), Anti-Gagp24 antibody (Cat# 13449)were obtained from the NIH AIDS Research and Reference Reagent Program. The virus stocks were measured for Gagp24 levels by anti-p24 ELISA Kit (obtained from the AIDS Vaccine Program of the Frederick Cancer Research and Development Center) and ready for infection experiments. Anti-Ebola Glycoprotein antibody, mouse MAb Monoclonal antibody (MAb) (Cat#40368-MM07), was purchase from the Sino Biological Company.

3.4. Cell culture and macrophage preparation

Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin. THP-1 cell line was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 unit/ml penicillin and 100 μg/ml streptomycin. All the cell lines were incubated at 37 ºC in a 5% CO2 incubator. To obtain the THP-1 derived Macrophages (MDMs), THP-1 cells were plated in a 24-well culture plate at a concentration of $5x10^5$cells/mL in complete media, were treated with a final concentration of 100 nM Phorbol

12-Myristate 13-Acetate (PMA; Sigma Aldrich, USA) for 48 h at 37°C and 5% CO2 before infection. PMA was used to stimulate the differentiation of THP-1 cells into macrophage-like cells.

3.5. Cell transfection

The day before transfection, 1.8×10^6 HEK 293T cells were plated into a 10 cm plate and incubated with an adequate amount of DMEM medium. The day after, the old medium was replaced with fresh DMEM without FBS 2 hours before transfection. The plasmid was diluted with PBS to 0.1 µg/µl, and PEI (Sigma Aldrich, USA) was diluted with PBS to 0.1 µg/µl. Both were mixed and incubated for 20 minutes at 37 ℃. Then, the mixture was added into the cells and shake gently. After 3 hours, the DMEM medium was replaced with a fresh complemented DMEM medium and incubated for 48 hours for further analysis.

3.6. HIV virus-like particle (VLP) preparation

To produce EboGPΔM-MPER-, EboGPΔM-9CE- and/or HIV Env (M)-VLPs, HEK 293T cells were co-transfected with the helper packaging plasmid pCMVΔ8.2 encoding for the HIV-1 Gag-Pol, and the HIV vector ΔRI/ΔE/Gluc[188,189], HIV Env (M tropic), EboGP expressing MPER or 9CE plasmids, as indicated in (**Figure 4. 4. a**). After 48 h of post-transfection, supernatants were collected and centrifuged at 3000 rpm for 30 min to clear cell debris. VLP particles were pelleted by ultra-centrifugation at 35000 rpm for 90 minutes and re-suspended in ice-cold Endotoxin-free PBS (EMD Millipore Corp). The virus stocks were quantified for HIV Gagp24 levels and stored in -80°C for both *in vitro* infection and/or *in vivo* immunization experiments.

The quantification of virus stocks was determined by Gag-p24 measurements using an HIV-1 p24 ELISA (p24 enzyme-linked immunosorbent assay) Kit (purchased from the

AIDS Vaccine Program of the Frederick Cancer Research and Development Center, Ft. Detrick, MD).

3.7. Western Blots

To test the incorporation and protein expression of EboGP and other viral protein in VLP particles, the purified VLPs (as adjusted by HIV Gagp24) were lysed in RIPA buffer and directly loaded onto a 12% or 10% SDS-PAGE gel. The proteins were transferred from the gel onto the nitrocellulose membrane overnight. Then, the membrane blocked by 5% milk in PBS and was washed three to five times with PBS containing 0.05% TW-20 and incubated with the with Mab Anti-EboGP, anti-HIVgp120(ID6), MAb anti-HIV-1 gp41 (2F5), or anti-HIVp24 antibodies primary antibody (dilution 1:1000) and relevant secondary antibody (dilution 1:5000). Following three washes in PBS, the target protein expression was visualized by the reaction of the secondary antibody and ECL substrate.

3.8. Gaussia luciferase Assay and NF-κB activity luciferase reporter assay

For measuring Gaussia luciferase activity at various time points after infection as indicated, supernatants were collected from the cell cultures. A 50 µl of GAR-1 reagent (Targeting systems) was added to 10 µl of sample and was mixed well and then measured in the luminometer machine (Promega, USA) [190].

The VSV-G pseudotyped lentiviral particles (Cignal Lenti vector) expressing a reporter firefly luciferase gene under the control of a minimal (m) CMV promoter and tandem repeats of the NF-κB transcriptional response element (TRE) (Cat# 336851, QIAGEN, Hilden, Germany) were used to transduce THP-1 cells. Following transduction, the THP-1 cells were cultured under puromycin selection to generate a homogenous population of the THP-1-NF-κB sensor cell line. Then, the THP-1-NF-κB sensor cells were treated with different EboGP-pseudotyped

HIV VLPs for 24 hours and were lysed and subjected to luciferase assay to monitor NF-κB signalling activity upon various VLP treatments [190].

3.9. *In vivo* study and Mice immunization experiment

Female BALB/c mice aged 4–6 weeks were purchased from the Central Animal Care Facility, the University of Manitoba (with the animal study protocol approval No. 16-012/1 (AC11159)). The mice (four to five mice per group) were injected subcutaneously with 100 ng (HIVp24) of VLPs in 100 µl endotoxin-free PBS on days 0, 21, and 56, and blood samples were obtained on days 21,34 and 76. Collected blood was allowed to clot for 30 min at room temperature and was centrifuged at 8,600 x g for 5 min, and the sera were kept at -20°C for further analysis.

3.10. Anti-HIV and anti-EboGP antibody measurements by Enzyme-linked Immunosorbent Assay (ELISA)

To investigate HIV Env and Gag-specific antibodies in sera, ELISA plates (NUNC Maxisorp, Thermo scientific) were coated with 100 µl of HIV-1 CN54 gp140 Protein(Cat#12064), Recombinant HIV1 gp41 protein(abcam, Cat# ab49070), recombinant EboGP without the transmembrane domain (TM) (IBT Biosciences, Cat# 0501–015) or HIV-1 p24 recombinant proteins (1µg or 0.5µg/ml) (NIH AIDS Reagent Program, Cat# 11784, Cat#13126) in 0.05M carbonate-bicarbonate buffer of pH 9.6 overnight at 4°C. The plates were washed twice with PBST and blocked with 1% BSA in PBS for 1 hrs at 37°C. 100µl of diluted mouse serum samples (1:50) in PBS was added and incubated for 2 hrs at 37°C. Plates were washed three times, and peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (GE Healthcare) was added and incubated for 1 h at 37°C. The plates were washed, and 3',3',5',5' Tetramethylbenzidine (TMB) (Mandel Scientific) was added and incubated for 15 min at room

temperature in the dark, then we added stop solution 1N HCL and measured the absorbance at 450nm (**Figure 3. 1**).

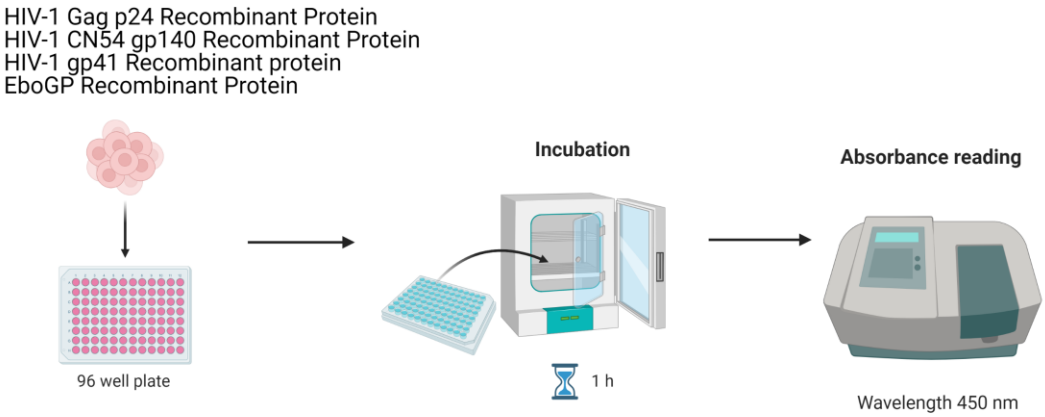


Figure 3. 1. Schematic representation of ELISA assay process**.**

3.11. Bacteria transformation and maxi preparation

3.11.1. Bacteria transformation

The competent bacteria AG1 was taken out of -80°C and thawed on ice. 100ng of DNA was added to 60 µL of competent AG1 bacteria in a 1.5mL microtube and mixed gently by flicking several times. Then the mixture of the competent cell and DNA were incubated on ice for 20-30 minutes, followed by heat shock to improve DNA uptake by bacteria. Each transformation

tube was placed into a 42°C water bath for 90 seconds and then on ice for 2 minutes. 1 ml liquid LB medium (Lysogeny broth medium) was added into the mixture, and the tube shake at 37℃ for 30 minutes. Finally, the heat-shocked bacteria were cultured on LB solid plate with a specific concentration of antibiotics in accordance with the bacterial resistance and incubated at 37℃ overnight.

3.11.2. Minipreparation

Liquid LB agar was prepared, and after autoclave, ampicillin antibiotic with correct concentration was added. By using a sterile toothpick, a single colony from your LB agar plate was selected and dropped into the liquid LB agar with 100 µg ampicillin/mL and swirled. The bacterial culture at 37°C for 12-18 hr in a shaking incubator. The mini preparation was done with QIAprep® Miniprep Kit protocol in the following process:

1. Bacterial cultured was spun by centrifugation at >8000 rpm for 3min.
2. Bacterial pellet resuspended in 300µl buffer P1.
3. 300 µl buffer P2 was added and vigorously inverted 4–6 times and incubated at room temperature (15–25°C) for 5 min.
4. 300 µl buffer N3 was added mix thoroughly by vigorously inverting 4–6 times.
5. Centrifuge the mixture at 14,000–18,000 x g for 10 min
6. Apply the supernatant from the previous step to the QIAGEN-tip and centrifuge for 1 min.
7. Wash the QIAGEN-tip twice with 2 ml Buffer QC
8. Elute the DNA with 500 µl elution buffer.
9. After 5 min, centrifuged for 1 min was done.

3.11.3. Max preparation

A single colony was selected from transformation LB agar plates using an inoculation loop and added to flasks containing 300 mL LB-Lennox broth supplemented with 100 µg ampicillin/mL. The bacterial cultures were allowed to incubate at 37 °C overnight on the shaker.

To isolate DNA plasmids from bacteria, the protocol for QIAfilter Plasmid Maxiprep Kit on the QIAGEN (QIAGEN, ON, Canada) was followed. Bacterial cultures were added to 500 mL Falcon tubes and centrifuged at 7,000 x g at 4 °C for 20 minutes. The supernatant was discarded into buckets containing bleach. The cell pellets obtained for each construct were pooled together and suspended in 10 mL Buffer P1. To lyse the bacterial cells, 10 mL Buffer P2 was added and incubated at room temperature (15–25°C) for 5 minutes. To neutralize the lysing reaction, 10 mL of chilled buffer P3 was added and the lysate incubated on ice for 20 minutes. The tubes were centrifuged at 7,000 x g for 30 minutes to pellet the cell debris. The supernatant containing the DNA was added to QIAfilters equilibrated with 15 mL Buffer QBT to allow DNA to bind to the column. The DNA was washed by adding 30 mL Buffer QC and allowing it to flow through the column. To elute the DNA, 15 mL Buffer QF was added to the column, and the flow-through was added to a clean 50 mL Nalgene Oak Ridge Round-bottom tube (Thermo Fisher Scientific, MA, USA). Subsequently, 10 mL of 100% isopropanol was added and mixed well to precipitate the DNA. To pellet the DNA, tubes were centrifuged at 13,000 x g at 4 °C for 30 minutes. The supernatant was gently discarded, and the pellet was washed gently by adding 20 mL 70% ethanol. The tubes were centrifuged at 13,000 x g at 4 °C for 30 minutes, the supernatant was discarded, and the pellet was dried at room temperature. The dried DNA pellet was resuspended in 800 µL of Buffer EB. Plasmid preparations were stored at -20 °C for further use.

Statistical analysis

Statistical analysis of the level of HIV GP 41, HIV GP 140, HIV p24 and Ebo GP ELISA was performed by using unpaired t-test by GraphPad Prism 6.01 software.

Chapter 4: Results

4.1. Construction of the HIV Env conserved regions expression plasmids

HIV Env is a highly glycosylated protein containing two 'conserved' and 'variable' regions. The variable regions consist of immunodominant epitopes. The immune system highly targets these epitopes, which has result in the generation of immune escape variants[182]. The strategy to decrease this diversity is to lead immunity to the conserved sequences. The HIV-1 envelope glycoprotein consists of the surface moiety gp120, which is bound to the transmembrane protein gp41(**Figure 4. 1. a**).

Env is the only protein present on the surface of the viral particle. HIV Env mediates viral entry activated by the exposure and interaction between the CD4 primary receptor and the cognate CD4 receptor binding site (CD4bs) on the gp120 subunit. This interaction induces many conformational changes, which has result in the formation of the chemokine coreceptor binding site on gp120, which leads to cell– viral membrane fusion and virus entry[191,192]. CD4bs is one of the most conserved epitopes targeted by neutralizing Abs (NAbs) on the virus surface, and effective and broadly neutralizing CD4bs-directed Ab responses are detected during the chronic stage of HIV infection[192].

The principal structure of gp120 can be subdivided into 5 relatively conserved regions and 5 variable loops (C1-V1-V2-C2-V3-C3-V4-C4-V5-C5, from amino to carboxy-terminus) (**Figure 4.1.a**). The conserved domain 1 (C1) in the N-terminal region of HIV gp120 plays a critical role in envelope function that has been investigated through alanine-scanning mutagenesis following by characterization of the mutagenic effects on virus entry; the expression, processing, and incorporation of the envelope; and the association of gp120 with gp41 [2]. Furthermore, C2, C3, C4, C5 on gp120 recognized as CD4 binding sites could bind with CD4bs monoclonal antibodies, including IgG1b12, HG16, VRC01, VRC03 [193]. CD4bs

antibodies have the ability to block the interaction of gp120/CD4 because they could recognize the conserved regions of gp120, thus, neutralizing the virus to prevent the proliferation of the infection and control the infection[86]. In this project, we arranged 9 regions in HIV gp120 Env that are highly conserved throughout HIV M Group sequences (Env CE) and defined based on stringent conservation, functional importance (**Figure 4.1.c**). The HIV gp120 9CE plasmid expressing protein compromises 9CE of HIV gp120 with 191 amino acids (**Table 4.2**). The epitopes are linked together via GPGPG linker for efficient immune presentation. GPGPG linker was introduced as a spacer between adjacent epitopes, minimizing junctional epitopes and augmented proteasome processing and increasing immunogenicity[194].

Conserved epitopes (CE)	**Length of Amino acids (aa)**	**Position**

C1	14	aa 34-48
C1	17	aa 66-83
C1	21	aa 107-128
C2	26	aa 202-228
C2	26	aa 241-267
C3	24	aa 364-388
C4	15	aa 413-428
C4	12	aa 447-459
C5	36	aa 469-505

Table 4. 1. HIV-1 GP 120 conserved epitopes. The table demonstrates the epitopes, numbers of copies and amino acid length, and the position of HIV gp120 9CE plasmids. Each epitope is joined by the GPGPG linker.

Moreover, the membrane-proximal external region (MPER) is a highly conserved region of the envelope glycoprotein (Env) gp41 subunit located near the viral envelope surface, and it plays a vital role in membrane fusion (**Fig.4.1. b**). This segment contains a tryptophan-rich motif

located next to the virus membrane, covering the last C-terminal residues of the gp41 ectodomain (aa 660–683) and links the extracellular matrix domain of Env with the TM domain. MPER conserved tryptophan residues and the isoleucines at positions 635 and 642 are strongly conserved across HIV strains, and the isoleucine at position 646 is conserved as isoleucine or leucine [195–197].

The MPER of Env is a significant target for broadly neutralizing antibodies (bNAbs) in infected individuals, including 2F5, 10E8, and 4E10, shown in chronically HIV-infected patients [198,199]. As shown in Figure 4.1.b, 2F5 targets the linear sequence 662ELDKWA667 in the N-terminal moiety of the MPER, where the central core 664DKW666 is essential for neutralization (**Figure 4.1.b**). The 2F5 has a high potency and efficiency in neutralizing around 57–67% of the viral isolates [200,201]. The 4E10 targets the distal region of conserved tryptophan-rich residues located C-terminal to the 2F5 epitope with the sequence of 671NWFDIT676 and is expanded toward C-terminal residues. 4E10 displays a moderate potency, but it has a breadth against 98–100% of the viral isolates[201,202]. Also, the 10E8 antibody broadly neutralizes by interacting with the MPER of the HIV membrane via its light chain in the HIV-1 isolates. Thus, it is a purpose of vaccine design to reproduce since it shows a promising treatment for HIV infection by passive immunization[203]. 10E8 and 4E10 target the similar helical epitope (C- terminal MPER residues 671–683), but their binding modes are different [204]. 10E8 binds with the viral membrane via its light chain and connects with MPER in a vertical direction in the HIV-1 membrane during neutralization[204]. In this project, MPER with 39 amino acids in length has been synthesized as a trimer. The first monomer is from HIV-1 Subtype A and/or C, the second monomer is subtype B and/or D, and the third monomer is subtype A and/or C.

Since the Ebola virus (EBOV) can specially target the antigen-presenting cells (APCs), including DCs and monocyte-derived macrophages (MDMs) through the EBOV glycoprotein

in (EboGP)[205]. It has also been shown that EboGP could stimulate human DCs to enhance both innate and adaptive immune responses through activation of NF-κB and MAPK signalling pathways[206].

Interestingly, EboGP has a mucin-like domain (MLD) region that is highly glycosylated, and the residue spans from 309 to 501 (**Figure 4. 2. b**). EboGP MLD also plays several functions during EBOV infection. Deleting this MLD region has been previously shown to enhance EboGP-mediated lentiviral vector entry in epithelial cells[207]. Based on the above information, our lab has developed an MLD-deleted EboGP (pCAGGS-EboGPΔM) (**Figure .4. 2. a**). Moreover, our previous results clearly demonstrated that an EboGPΔM could efficiently target the MDDCs and MDMs [208].

Therefore, in my project, we used EboGPΔM expressing plasmid as a backbone and inserted MPER or 9CE gene into the MLD position (**Figure 4. 2. c and d**). We named the two expressing plasmids as pCAGGs-EboGPΔM-9CE, pCAGGs-EboGPΔM-MPER.

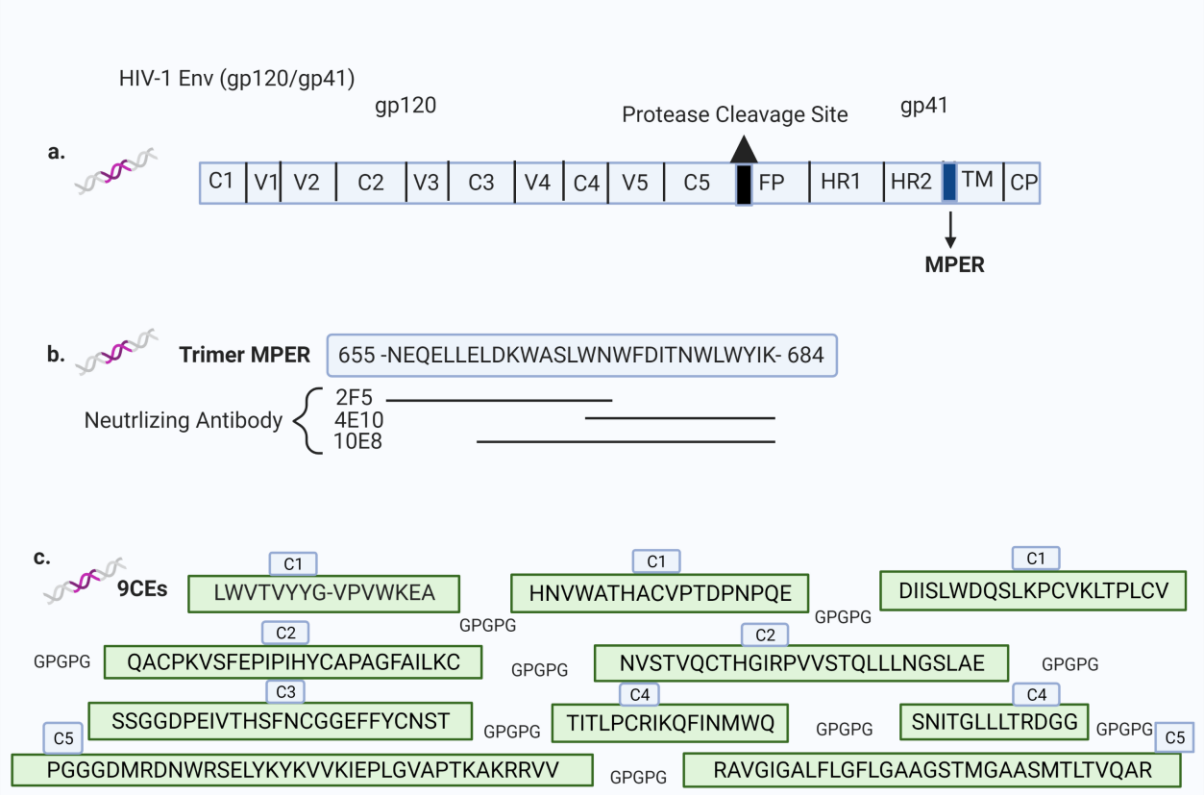


Figure 4. 1. a) Schematic representation of the conserved regions (CE) in the envelope glycoproteins of HIV- 1 (gp120/gp41). **b)** MPER in HIV-1 gp41 and sequences of MPER binding to neutralizing antibodies**. c)** **9** CE identified within HIV-1 Env gp120 protein are linked to each other through GPGPG linkers with the co-axial arrangement.

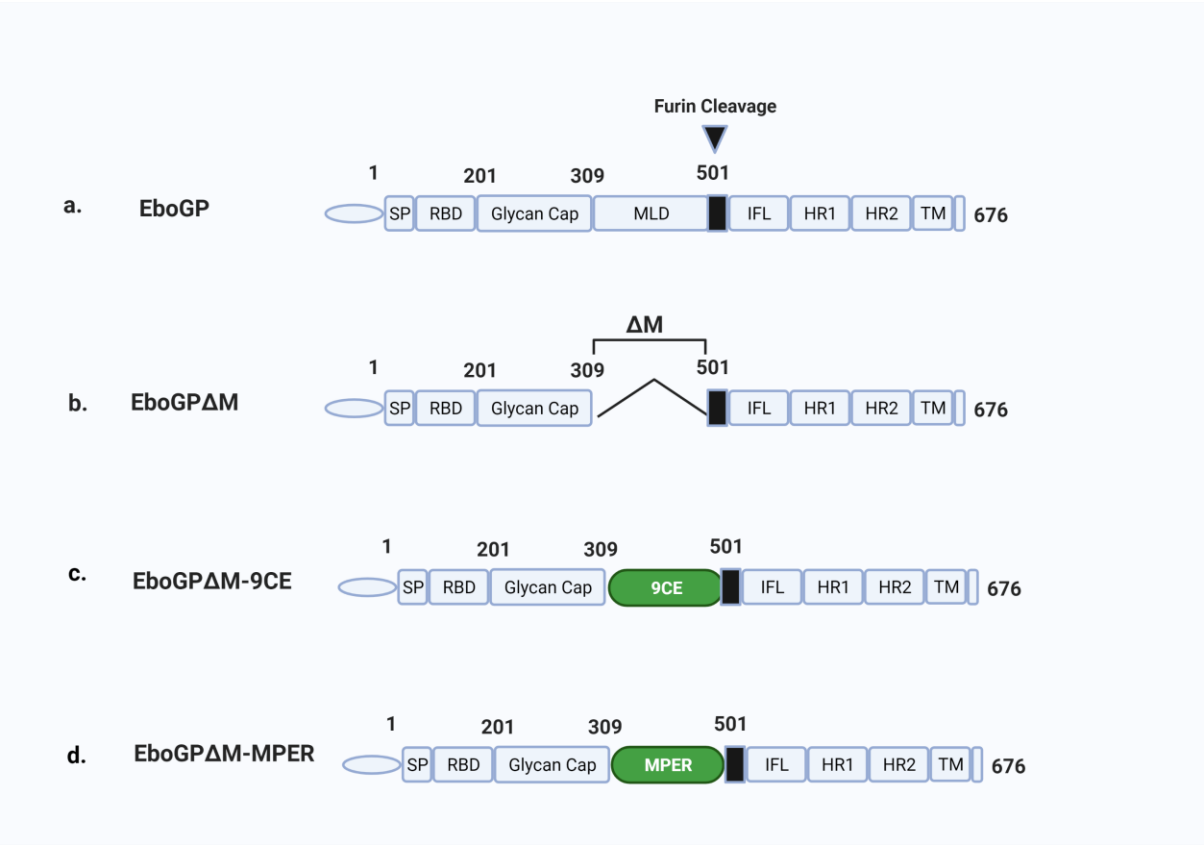


Figure 4. 2. Schematic diagram of the EboGP- 9CE or- MPER plasmids. EboGPΔM contained a deletion of the MLD region. The 9CE or MPER was inserted into the MLD position of EboGPΔM. SP: signal peptide; RBD: receptor binding domain; MLD: mucin-like domain; IFL: internal fusion loop; HR1, HR2: heptadrepeat1/2; TM: transmembrane domain.

4.2. Expression of EboGPΔM-9CE or EboGPΔM-MPER fusion proteins in the 293 T cells

To check the expression of EboGPΔM-9CE and/or -MPER in 293 T cells, pCAGGS-EboGPΔM-9CE or EboGPΔM-MPER was transfected into 293TN cells. Meanwhile, pCAGGS-EboGPΔM and a wild type HIV-Env (M tropic or M) (HIV-1 envelope glycoprotein plasmid pLET-Lai (X4-tropic) was transfected as a positive and negative control, respectively. The expression of EboGPΔM, 9CE or MPER in EboGPΔM-9CE, EboGPΔM-MPER, EboGPΔM, or HIV Env (M) in cells was first examined by Western blot using a mouse anti-EboGP antibody (**Figure 4. 3. a**). As expected, EboGPΔM was detected around 60 kDa in EboGPΔM-9CE, EboGPΔM-MPER and EboGPΔM, but not in HIV Env (M). EboGPΔM-9CE was found to locate around 100 kDa. For the EboGPΔM-MPER, we detected three protein bands range from 75 to 90 kDa in transfected cell lysate (**Figure 4. 3. a, upper lane**). However, by using MAb anti-HIV-1 gp41 (10E8) and MAb anti-HIV-1 gp41 (4E10) that specifically targeting MPER (**Figure 4. 1. b**), we were able to confirm that EboGPΔM-MPER protein was located around 75 kDa (**Figure 4. 3. b, upper and middle lane**). Furthermore, by using anti-HIV gp120 antibody (ID6), we can detect HIV gp120, but not EboGPΔM-9CE and MPER (**Figure 4. 3. a, lower panel**). Interestingly, EboGPΔM-9CE and EboGPΔM-MPER can be recognized by anti-human HIV-1 IgG antibody (**Figure 4. 3. b, lower panel**), isolated from HIV-1 infected donors and determined all HIV- proteins. Overall, our data suggest that EboGPΔM-MPER, EboGPΔM-9CE, or HIV Env (M) can be expressed efficiently in the 293 T cells.

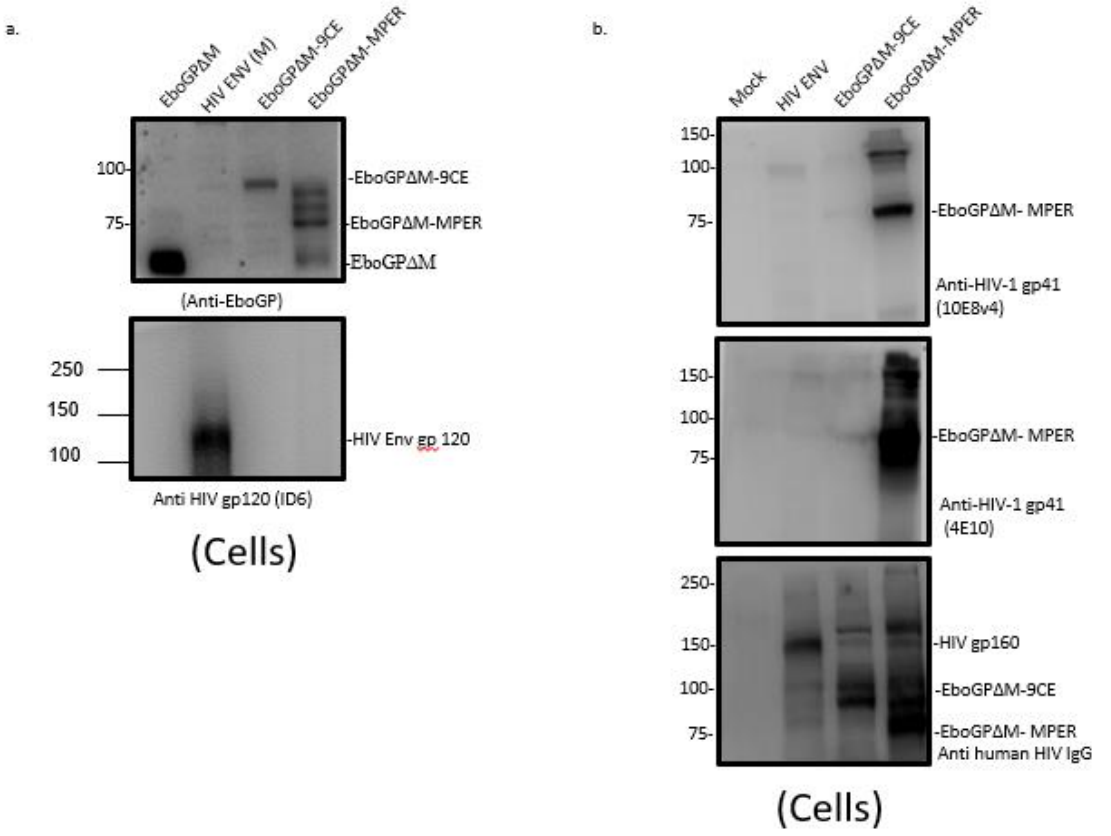


Figure 4. 3. a, b) The expression of EboGPΔM, HIV gp120 (M), EboGPΔM-9CE, and EboGPΔM-MPER in the cell lysate were detected by WB. The 293T cells were transfected by HIV Env(M), EboGPΔM, EbovGPΔM-9CE or EboGPΔM-MPER. Cells were lysed and analyzed by western blot with anti-HIV gp120 (ID6), anti-EboGP, Anti-HIV-1 gp41 (10E8v4), and Anti-HIV-1 gp41 (4E10) antibody.

4.3. The incorporation of EboGPΔM-9CE and/or EboGPΔM -MPER in VLPs

To generate HIV-based virus-like particles (VLPs) bearing EboGPΔM -9CE or EboGPΔM -MPER, the EboGPΔM-9CE or EboGPΔM -MPER expression plasmids were co-transfected with an HIV-based lentiviral vector in the envelope, pol and nef genes have been deleted GLuc(ΔRI/ΔE/GLuc), and a packaging plasmid (pCMVΔR8.2) in 293 T cells (**Figure 4. 4. a**). In parallel, HIV-1 Env (M) or EboGPΔM were included as a control. A ΔRI/ΔE/GLuc encodes the Gaussia luciferase (GLuc) gene in the nef position. GLuc is a bioluminescent enzyme that can be secreted into the cell culture supernatant, using as an indicator of viral replication by direct measurement of GLuc activity in the supernatant. After 48 hrs of transfection, the pseudotyped VLPs were concentrated by ultracentrifugation. To examine the incorporation levels of EboGP in VLP particles, the purified VLPs were lysed and analyzed by SDS-PAGE and Western blot with various antibodies. Using mouse anti-EboGP antibody, EboGPΔM or EboGPΔM9-CE can be clearly detected in VLPs (**Figure 4. 4. b, upper panel**), while EboGPΔM-MPER was detected by MAb anti-HIV-1 gp41 (2F5) (**Figure 4. 4. b, third panel**). The anti-EboGP antibody could not detect EboGPΔM -MPER on VLPs due to the amphiphilic feature of MPER. VLPs pellets were resuspended in ice-cold PBS. According to the amphiphilic feature of MPER and the topology of MPER in a membrane, hydrophobic residues remain buried into the membrane, whereas the most polar residues are exposed to the solvents[196]. However, the EboGPΔM-MPER protein expression in VLP lysate was clearly detected above 75kDa in the membrane probed with the MAb anti-HIV-1 gp41 (2F5) (**Figure 4. 4. b, third panel**).

2F5 antibody has a hydrophobic residue, which initially interacts with the viral membrane and leads to the binding of a membrane-embedded MPER monomer[156]. Furthermore, the presence of HIV Env on purified VLPs was confirmed by using anti-HIV gp120 (ID6) antibody

and anti-HIV-1 gp41 (2F5) (**Figure 4 .4. b, second and third panel**). Meanwhile, the HIV-1 capsid Gagp24 protein was detected in all the VLPs by rabbit anti-p24 antibodies (**Figure 4. 4. b, last panel**). Strangely, the p24 level of EBOGPΔM-MPER in VLPs is much less than the p24 levels of Env-VLPs, EBOGPΔM-VLPs and EBOGPΔM-9CE-VLPs. This finding was also confirmed by checking the viral production using the HIV p24 ELISA method (**Figure 4. 4. c**). One possible reason might be due to the expression of EBOGPΔM-MPER that appears to inhibit HIV viral production and/or viral gene transcription.

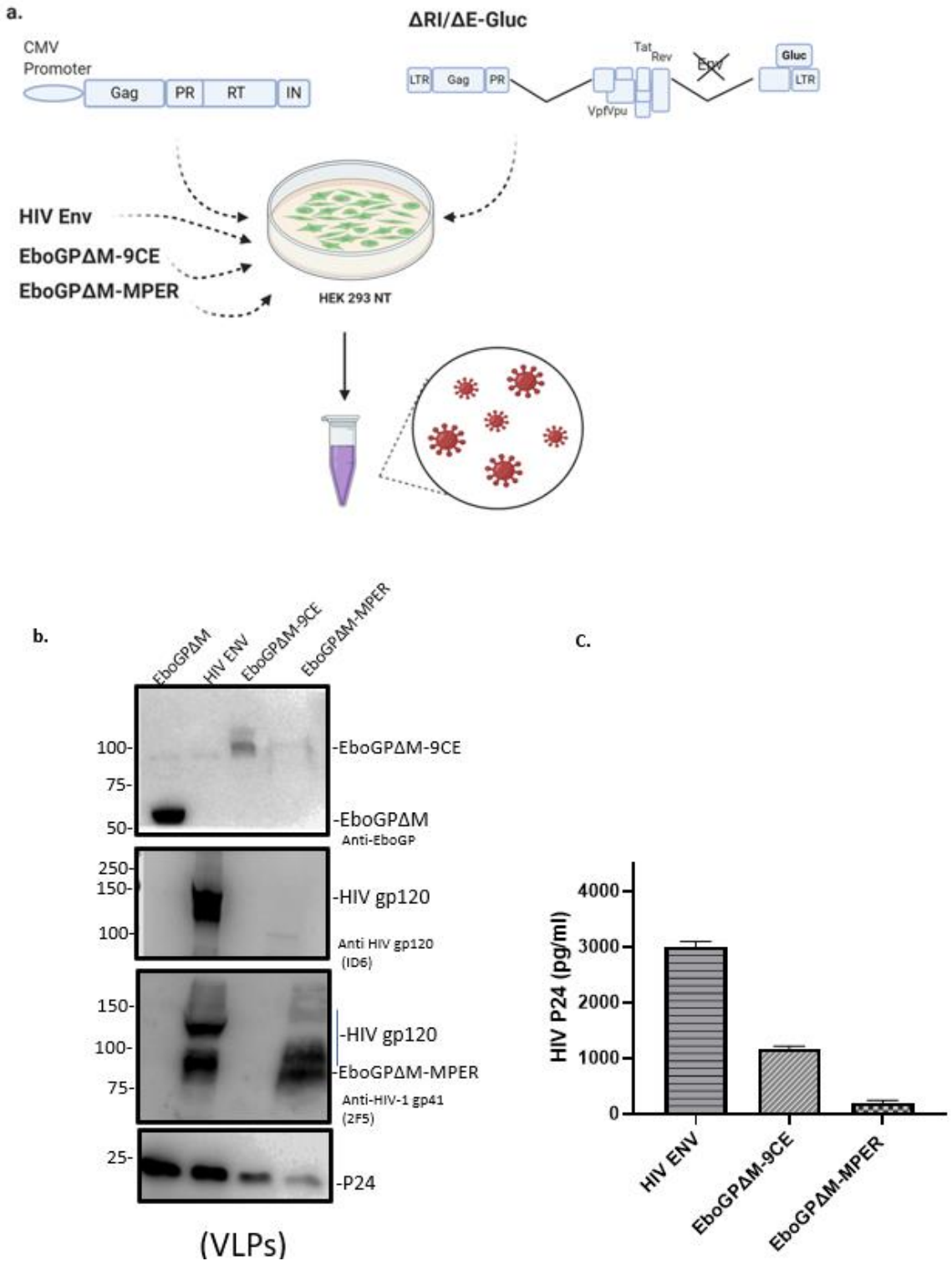

a.
ΔRI/ΔE-Gluc
CMV
Promoter
Gag
PR
RT
IN
LTR
Gag
PR
Tat
Rev
Vpf
Vpu
Env
Gluc
LTR
HIV Env
EboGPΔM-9CE
EboGPΔM-MPER
HEK 293 NT
b.
EboGPΔM
HIV ENV
EboGPΔM-9CE
EboGPΔM-MPER
100-
75-
50-
-EboGPΔM-9CE
-EboGPΔM
Anti-EboGP
250-
150-
100-
-HIV gp120
Anti HIV gp120
(ID6)
150-
100-
75-
-HIV gp120
-EboGPΔM-MPER
Anti-HIV-1 gp41
(2F5)
25-
-P24
(VLPs)
c.
HIV P24 (pg/ml)
4000
3000
2000
1000
0
HIV ENV
EboGPΔM-9CE
EboGPΔM-MPER

Figure 4. 4. a) 293 T cells were transfected by HIV-1 ΔRI/ΔE/Gluc+, CMV-Gag-Pol and HIV Env, EbovGPΔM, EbovGPΔM-9CE or EboGPΔM-MPER. The supernatant containing VLPs were collected, purified, lysed and analyzed by WB with anti-HIV Env, anti-EboGP antibody or anti-HIVp24 antibody. **b)** The presence of EboGPΔM, HIVgp, EboGPΔM-9CE and EboGPΔM-MPER in the pseudotyped HIV VLPs were detected by WB. **c)** VLP particles were subjected to ELISA assay to measure HIV p24 level. The data is representative of results obtained from three samples.

4.4. Investigation of dendritic cell and macrophages targeting ability of EboGPΔM-MPER, EboGPΔM-9CE or HIV Env pseudotyped VLPs

After demonstrating the ability of EboGPΔM-9CE and EboGPΔM-MPER can be incorporated into HIV-based VLPs. I further investigate whether EboGPΔM-9CE or EboGPΔM-MPER can efficiently target monocytes and macrophages. THP-1 cell is a human monocytic cell line derived from an acute monocytic leukemia patient [209], and THP-1 derived Macrophages were used for this analysis. To differentiate THP-1 to Macrophages (MDMs), THP-1 cells were plated in a 24-well culture plate at a concentration of $5x10^5$cells/mL in RPMI complete medium, were treated with a final concentration of 100 nM Phorbol 12-Myristate 13-Acetate (PMA) for 48 h at 37°C and 5% CO2 before infection to induce differentiation of THP-1 cells into macrophage-like cells. (**Figure 4. 5. a and b**). Briefly, equivalent amounts (normalized with HIV p24 levels) of EboGPΔM-MPER, EboGPΔM-9CE or HIV Env (M) pseudotyped GLuc+ VLPs were used to infect the cell lines. The entrance ability of the pseudotyped virus was detected by GLuc activity in the culture medium since the GLuc protein was expressed and released in the cell culture supernatant during viral replication. The supernatants were collected, and the Glu activities were checked at different time points after infection for THP-1 and MDMs (**Figure 4. 5. a and b**). As expected, the result showed that all VLPs are able to target monocytes. As depicted in **Figure 4. 5. a,** the infectivity level of THP-1 cells with EboGPΔM-MPER-, EboGPΔM-9CE- and/or HIV Env (M)-VLPs increased gradually after 3 days of infection. EboGPΔM-MPER- and EboGPΔM-9CE-VLPs can efficiently target THP-1 and produced high GLuc activity than that of H Env (M)-VLPs. The infectivity level of THP-1 cells with both EboGPΔM-MPER-, EboGPΔM-9CE- VLPs was about 10-fold higher than HIV Env(M)-VLPs.

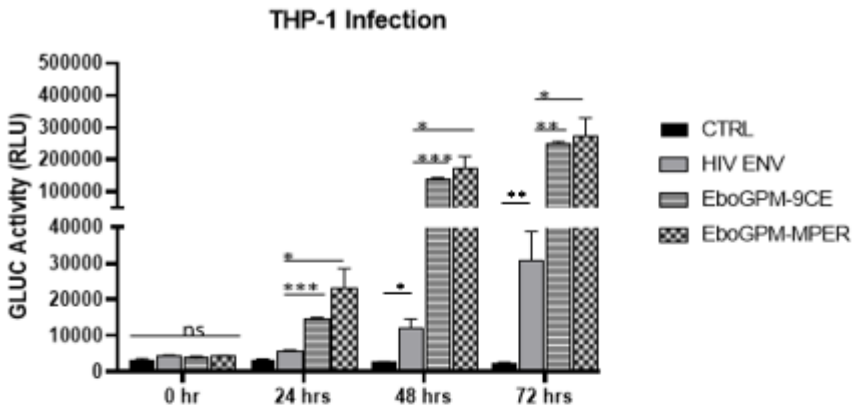
Furthermore, the infectivity level of the EboGPΔM-MPER-, EboGPΔM-9CE- or HIV Env (M) VLP were analyzed in THP-1 derived macrophages. As depicted in **Figure 4. 5. b,** similar to

THP1 cells, the ability of the EboGPΔM-MPER- or EboGPΔM-9CE VLPs to target the macrophages to cause infection was more significant than HIV Env (M) VLP. However, EboGPΔM-MPER VLP had better infectivity ability in THP-1 derived macrophages than in THP-1 cells. Cells infected with EboGPΔM-MPER-VLPs demonstrated high GLuc activity at about 25000, 200000, and 300000 RLU after 24, 48, and 72 hrs post-infection, respectively. The level of THP-1 derived macrophage infection with EboGPΔM-9CE-VLPs was approximately at 210000, 190000, and 220000 RLU after 24, 48, and 72hrs post-infection, respectively. Also, in both THP-1 and THP-1 derived macrophages, EboGPΔM-MPER VLPs had higher infectivity than EboGPΔM-9CE -VLPs. Interestingly, the GLuc activity level of cells infected with HIV Env(M)-VLPs without EboGPΔM remained below 30000 during the three-day post-infection (**Figure 4. 5. b**). Overall, these results clearly revealed that incorporating EboGPΔM into HIV virions could efficiently target and allow fusion into THP-1 and MDMs.

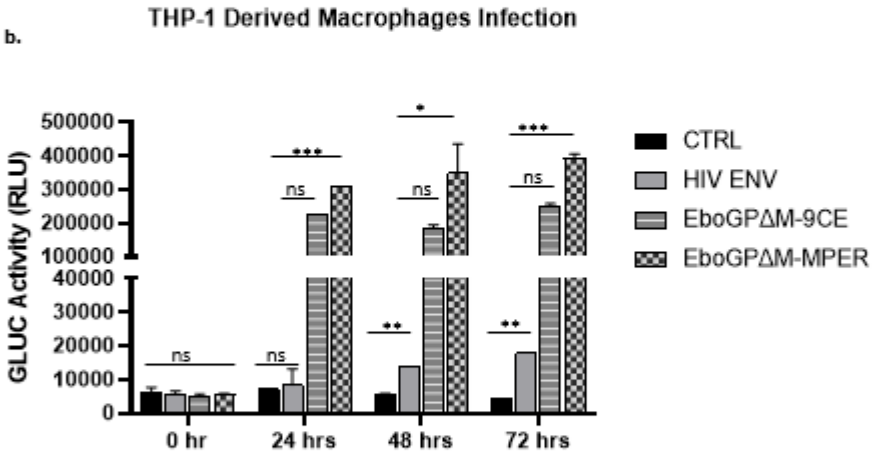
We then check the possible mechanism(s) responsible for the stronger ability of EboGP to stimulate immune responses by investigating whether EboGP-VLPs and HIV Env (M) -VLPs could stimulate the NF-κB signalling pathway. Previous studies have reported that EboGP could trigger NF-κB signalling pathways [210]. Therefore, using an NF-κB-Cignal Lenti luciferase report system which contains the lentiviral vector with a reporter firefly luciferase gene, we generated a THP-1-NF-κB sensor cell line. The lentiviral vector is under the control of tandem repeats of the NF-κB transcriptional response element (TRE) and CMV promoter. Briefly, THP-1 cells were transduced with NFκB-Cignal Lenti particles to generate THP-1-NF-κB sensor cells, as previously described[186]. Then, the THP-1-NF-κB sensor cells were treated by equivalent amounts (adjusted by P24) of EboGPΔM-MPER, EboGPΔM-9CE or HIV Env (M)-VLPs; after 8, 12, and 24 hrs, the luciferase activity was detected in the cells by lysing the cells and measuring the activation levels of the NF-κB signalling pathways in the cell lysis

(**Figure 4. 5. c**). The results showed that All VLPs are able to transiently stimulate NF-kB pathways in the cells at 8 hours after treatment. However, EboGP-VLPs induced higher levels of Luc activity than HIV ENV (M)-VLPs in THP-1-NF-κB sensor cells 8 hrs after stimulation (**Figure 4. 5. c**), suggesting that EboGP has a stronger ability to stimulate NF-κB pathways in the early stage of infection.

a.


THP-1 Infection
GLUC Activity (RLU)
500000
400000
300000
200000
100000
40000
30000
20000
10000
0
ns
*
***
*
*
***
**
*
**
0 hr
24 hrs
48 hrs
72 hrs
CTRL
HIV ENV
EboGPM-9CE
EboGPM-MPER

b.


THP-1 Derived Macrophages Infection
GLUC Activity (RLU)
500000
400000
300000
200000
100000
40000
30000
20000
10000
0
ns
ns
***
ns
*
ns
***
ns
**
**
0 hr
24 hrs
48 hrs
72 hrs
CTRL
HIV ENV
EboGPΔM-9CE
EboGPΔM-MPER

c.

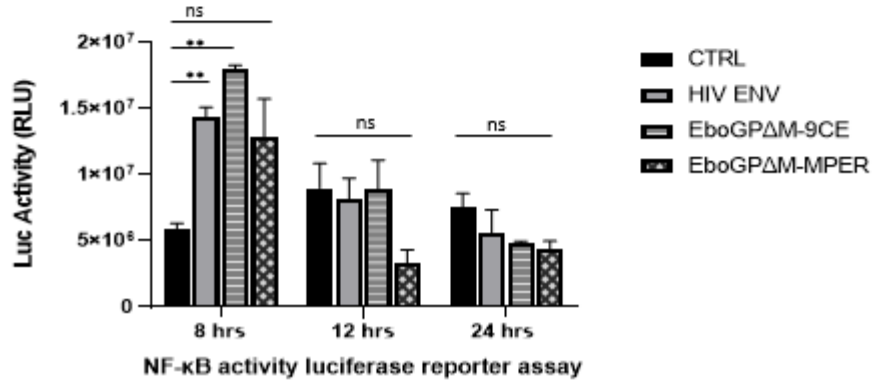

Luc Activity (RLU)
2×10⁷
1.5×10⁷
1×10⁷
5×10⁶
0
ns
**
**
ns
ns
8 hrs
12 hrs
24 hrs
NF-κB activity luciferase reporter assay
CTRL
HIV ENV
EboGPΔM-9CE
EboGPΔM-MPER

Figure 4. 5. a, b. THP1 or THP1 derived macrophages were infected by equal amounts (p24 levels) of EboGPΔM-MPER, EboGPΔM-9CE and/or HIV Env pseudotyped VLPs. At different time points post-infection, the supernatants were collected and subjected to GLuc activity. **c.** THP-1-NF-κB sensor cells were treated by equivalent amounts of HIV Env, EbovGPΔM-9CE or EboGPΔM-MPER VLPs for 8, 12, and 24 hrs, and activation of NF-κB signalling was detected by measurement of the Luc activity. Statistical significance between the three groups was determined using an unpaired t-test, and significant *p* values are represented with asterisks, *≤0.0120, **≤0.0048, ***0.0007. The data is representative of results obtained from four samples.

4.5. Investigation of the ability of EboGPΔM-MPER, EboGPΔM-9CE-VLPs to induce anti-HIV antibodies in mice

Our *in vitro* data showed that the EboGPΔM-MPER, EboGPΔM-9CE plasmids incorporated into VLPs are able to target monocytes, including THP-1 and MDMs, efficiently. This suggests that these vaccine candidates can efficiently stimulate the immune response against HIV-1 by targeting the macrophages in mice. Therefore, we investigate the ability of EboGPΔM-MPER, EboGPΔM-9CE VLPs to induce immune responses in an animal model using mice.

For this *in vivo* study, we used a BALB/c mice strain. This strain is currently in use in filovirus laboratories. This mouse model has been used for the analysis of Ebola virus infection for more than 15 years. It is a well-established model for the Ebola virus study. Also, EBOV has a record of 95% lethality in BALB/c mice [211]. Moreover, these mice are genetically potent to produce HIV-specific cytotoxic T cell responses related to HIV protection in humans[212].

A 16 female mice aged 4-6-weeks mice were purchased. The mice were grouped into four (4). Each group has 4 mice and immunized subcutaneously with 100 ng of EboGPΔM-MPER, EboGPΔM-9CE, or HIV Env-VLPs on days 0. Four mice were injected with 100 µl endotoxin-free Phosphate Buffered Saline (PBS) as the control group. All mice were boosted on days 21 and 56 with 100 ng of EboGPΔM-MPER, EboGPΔM-9CE or HIV Env-VLPs (**Figure 4. 6. a**). At days 21, 34, and 76 of post-immunization, the mice blood was collected, as described in Materials and Methods, and the anti-HIVgp41, -EboGP, -HIVp24, -HIVgp140 -specific humoral immune response were measured by corresponding ELISA. Meanwhile, we monitored the body weight of all groups of immunized mice. The body weight of EboGPΔM-MPER, EboGPΔM-9CE and/or HIV Env-VLPs groups was not statistically different between groups, and all groups of immunized mice remained healthy (**Figure 4. 6. b**). This suggests the biosafety of the candidate vaccines. In order to quantify humoral immune responses induced

by the EboGPΔM-MPER, EboGPΔM-9CE and/or HIV Env (M)-VLPs total anti-HIV gp41 IgG ELISAs in serum harvested from immunized mice at days 21, 34, and 76 post-immunization was tested by ELISA method. In brief, the antigen ELISA was performed using a 96 flat bottom plate coated with HIV gp41, HIV gp140, EboGP, HIV Gagp24, followed by incubated mice sera in 1:50 dilution for 2 hours. The anti-mouse IgG-HRP was added in 2% skim milk and incubated for 1 hr at 37℃, and subsequently, plates were washed, and 50µl of TMB substrate was added per well and was incubated for 20 min. Plates were read at 650 nm. The result revealed that at 21 days, the induced humoral immune was low in all vaccinated mice. However, after the first boost, the immune response had a slight increase, and after the second boost, it reached a high level. As expected, EboGPΔM-9CE cannot induce an anti-HIV GP41 IgG response. Interestingly, the mice injected with EboGPΔM-MPER-VLPs demonstrated significantly more robust antibody responses against HIV gp41 as compared to EboGPΔM-9CE and/or HIV Env VLPs (**Figure 4. 6. c**). The mice receiving EboGPΔM-9CE-VLPs had a slight increase as well. This study suggests that EboGPΔM-MPER-VLPs can elicit a higher anti-HIV GP41 immune response than HIV Env.

We further test the antibody against HIV gp140 from immunized mice (**Figure 4. 6. d**) by measuring the immune response against HIV gp140 Ag, including both HIV gp120 and HIV gp41. We expected that the immune responses in mice injected with EboGPΔM-9CE-VLPs would be higher since the HIV gp140 contain both 9CE epitopes and MPER epitope. Interestingly, the mice vaccinated with EboGPΔM-MPER-VLPs induced the highest level of the immune response. The mice immunized with HIV Env (M)-VLPs induce only a moderate level of antibody. However, EboGPΔM-9CE-VLPs could not induce antibodies against gp140. The possible explanation for higher immunogenicity of EboGPΔM-MPER may be due to the MPER recognition sites by several bnAbs, such as 2F5. MAb 2F5, among the antibodies, has

the broadest heterologous HIV-1 neutralizing activity, and it can bind to epitopes buried in the membrane[213].

Since the VLPs we used were based on HIV Gag protein, we test whether the mice can stimulate the immune response against viral protein p24. As depicted in **Figure 4. 6. e**, EboGPΔM-MPER-VLPs induced more robust antibody responses against HIVGagp24 than that of HIV Env-VLPs significantly. Consistent with the above results, the mice injected with EboGPΔM-9CE-VLPs did not considerably increase the anti-p24 antibody, suggesting anti-HIV immune response induced by EboGPΔM-9CE-VLPs are very weak.

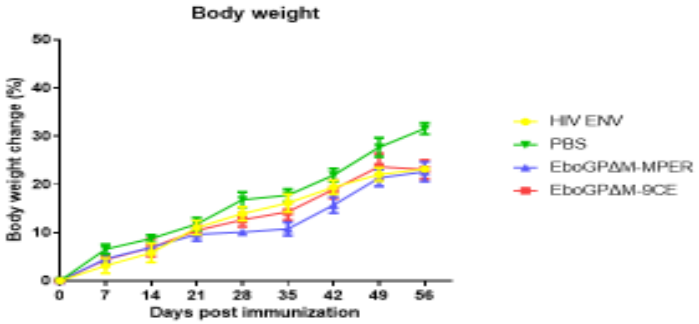
Finally, we check the anti-EboGP humoral immune responses induced by EboGPΔM-9CE-VLPs or EboGPΔM-MPER-VLPs. Interestingly, the level of anti-EboGP humoral immune increased approximately 3-fold after second post-immunization in the mice injected with EboGPΔM-9CE -VLPs, while the mice injected with EboGPΔM-MPER and/or HIV Env (M)-VLPs had a slight rise about 0.5 nm after post-immunization during 76 days. This result suggests that, unlike EboGPΔM-MPER-VLPs, EboGPΔM-9CE-VLPs induced higher anti-EboGP antibody. Overall, VLPs-associated EboGPΔM- MPER fusions are able to induce significantly higher anti-HIV MPER antibodies than native HIV Env VLPs in mice. Moreover, EboGPΔM- MPER not only induce a more effective immune response to MPER but also enhance the response against other HIV component, such as HIV Gag.

.

a.


Primary immunization
1st boost
2nd boost
Days
0
21
34
56
76
Blood collection
Mice sacrificed; blood collected

b.


Body weight
Body weight change (%)
50
40
30
20
10
0
0
7
14
21
28
35
42
49
56
Days post immunization
HIV ENV
PBS
EboGPΔM-MPER
EboGPΔM-9CE

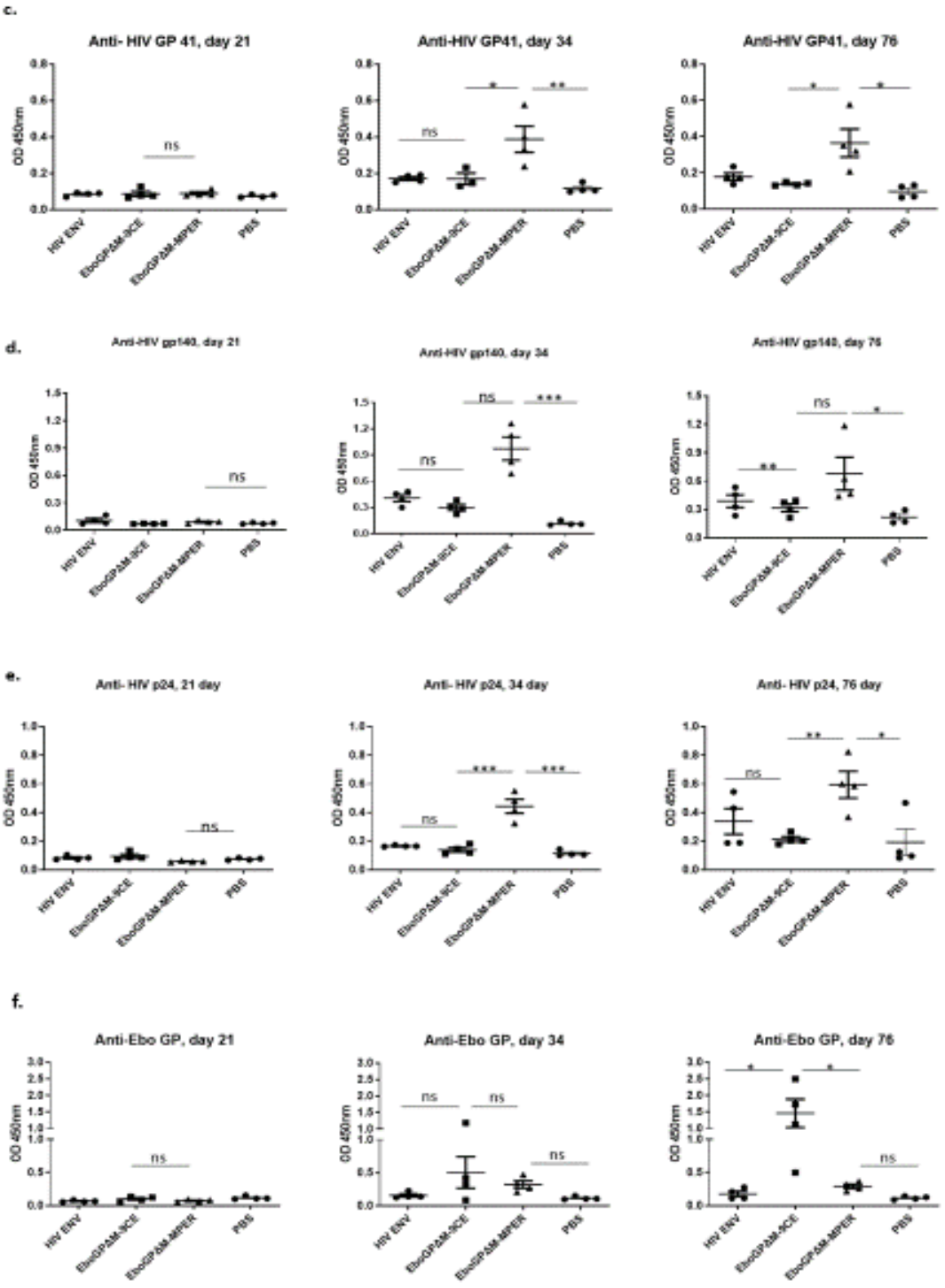

c.
Anti- HIV GP 41, day 21
Anti-HIV GP41, day 34
Anti-HIV GP41, day 76
d.
Anti-HIV gp140, day 21
Anti-HIV gp140, day 34
Anti-HIV gp140, day 76
e.
Anti- HIV p24, 21 day
Anti- HIV p24, 34 day
Anti- HIV p24, 76 day
f.
Anti-Ebo GP, day 21
Anti-Ebo GP, day 34
Anti-Ebo GP, day 76
OD 450nm
HIV ENV
EboGPΔM-9CE
EboGPΔM-MPER
PBS
ns

Figure 4. 6. a) Schematic of the EboGPΔM-MPER, EboGPΔM-9CE and/or HIV Env pseudotyped VLPs immunization protocol used in this study. The BALB/c mice were injected subcutaneously with 100 ng of VLPs, as indicated. Mice were boosted with the same amounts of VLPs at the day 21 and 56, and sera were collected at days 21, 34, and 76 days of post-immunization. **b)** Mice body weights were measured weekly in which 100% body weight was set at day 0. The levels of anti-HIV GP41 **(c)**, anti-HIV GP140 **(d)**, anti-HIVp24 **(e)**, anti-EboGP **(f)** antibodies in the sera of immunized BALB/c mice were detected by corresponding ELISAs. The data is representative of results obtained from four samples.

Discussion

The AIDS epidemic remains a major global health challenge, and the expectation was to develop a vaccine efficiently. Most current viral vaccines push the body's immune system to induce neutralizing antibodies (NAbs) that are able to suppress the infection and/or viral load[214].

Some of HIV vaccines using native Env as an antigen. However, it has a low efficacy to confer immunity against AIDS due to the low levels and breadth of the HIV-1 neutralizing antibodies, poor quality of the anti-HIV T-cell responses, and high immune responses to immunodominant non-protective HIV epitopes. Moreover, developing an effective HIV vaccine has been the most significant challenge due to the high genome mutation rate and recombination during viral replication [215]. Therefore, the development of a vaccine targeting only conserved epitopes of HIV-1 strain has been developed. HIV conserved epitopes have been tested widely in clinical studies. For instance, Moyo et al.,2018, showed efficient induction of T cells to the HIV-1 conserved epitopes of the HIV-1 proteome [216]. Moreover, another study showed that intramuscular administration of HIV conserved region DNA vaccine had no adverse systemic toxicological changes in the BALB/c[217].

Highly conserved regions of HIV-1 viral proteins, such as the HIV-1 envelope, are essential in vaccine design. The HIV-1 Env glycoprotein, gp120 and gp 41 has a key role in the virus replication cycle and contains conserved and functional domains. HIV-1 Env glycoprotein candidate vaccines induce high levels of Env-specific antibodies. These antibodies are able to neutralize a minority of HIV-1 strains. However, in the sera of some HIV-1-infected individuals, potent and broadly reactive neutralizing antibodies were detected. After analyzing these sera, some information about the viral epitopes targeted by neutralizing antibodies has been identified [218]. These epitopes are the several regions on gp120 subunits, including glycan-containing regions at the base of variable regions 1 and 2 (V1V2), the base of V3, and

the five conserved epitopes (C1 to C5) within the CD4 binding site (CD4bs) of gp120. The conserved epitope on the gp41 subunit is termed membrane-proximal region (MPER)[202,219–221]. Induction of broadly neutralizing Ab (bNAb) responses against the conserved HIV-1 envelope (Env) CD4 binding site (CD4bs) in gp120 and MPER in gp41 by vaccination are essential targets for vaccine development[222,223].

In this study, we designed 9 regions in HIV gp120 Env (Env 9CE) and defined them based on stringent conservation, functional importance. Moreover, we have chosen a highly conserved region (MPER) that contains epitopes recognized by several reported broadly neutralizing antibodies such as 2F5, 4E10, 10E8 detected MPER Abs in HIV 1 positive sera[141].

Furthermore, DC-based immunotherapeutic vaccines are a promising strategy to address the inadequate and insufficient immunity conferred by earlier tested HIV vaccines due to their capability to work along with the host immune system to optimize the adaptive response against antigens[186].

This project aimed to develop a promising vaccine targeting DCs to increase HIV-specific immune responses by pseudotyping HIV virions with EboGP. We aimed to develop a platform in which both EboGP and HIV Env were expressed on the non-replicating virus-like particles (VLPs). Our previous study demonstrated that incorporating EboGP in *trans* by non-replicating HIV VLPs could increase their ability to target human DCs and macrophages. In contrast, removing the mucin-like domain (MLD) region of EboGP further increased this effect [208,224]. A possible concern about this concept is that DCs are the primary targets for HIV-1 infection, resulting in exhaustion and dysfunction in their ability to contribute to the stimulation of T-cell proliferation. However, the mechanism of DC dysfunction during HIV-1 infection is not understood. Some studies suggest that DCs in HIV-infected people are able to keep their ability to stimulate allogeneic T-cell responses and secrete cytokines upon stimulation with Toll-like receptor 7/8 agonists. HIV VLPs expressing EboGP *in trans* is are

not capable of replicating. Thus, the probability of DCs depletion or dysfunction caused by this vaccine would be significantly low[225,226]. In my research project, we hypothesized that fusion of highly conserved epitopes of HIV-1 Env gp120 and gp41, 9CE and MPER, respectively, into the deleted mucin-like domain of the EboGP, can induce a robust humoral and cellular immune response.

First, we confirmed that the incorporation and expression of EboGP into HIV VLPs significantly target monocytes /macrophages compared with HIV Env (M) *in vitro*.

Second, we have demonstrated that EboGPΔM-9CE and EbpGPΔM-MPER-pseudotyped HIV VLPs induced significantly higher NF-κB signalling in THP-1 cells compared with HIV Env (M)-VLPs suggests that the DCs and macrophages targeting ability and immunostimulatory effects of EboGP-9CE and EbpGPΔM-MPER could enhance the ability of monocytes/macrophage to enhance the adaptive immune response, which is consistent to the findings in our previous report [208].

Interestingly, our animal study showed that EboGPΔM-MPER-VLPs could significantly induce robust host immune responses in the mouse model compared to HIV-Env (M)-VLPs. It was not only against HIV gp140 and gp41 but also HIV gag-p24. It suggests the great potential of further development of EboGPΔM-MPER-VLPs as an anti-HIV vaccine candidate. At this point, we still do not know whether EboGPΔM-MPER-VLPs could induce broader and more potent neutralizing antibody levels. It is definitely deserved further investigation.

Meanwhile, we observed that EboGPΔM-9CE-VLPs did not induce higher anti-HIV antibodies than that induced by native HIV Env VLPs in mice. One possible explanation may be that the conformational structure changes of conserved epitopes in fusing protein on the VLP surface may affect its conformation, disrupting its anti-GP120 in natural form. At this point, it will be valuable to test the sera could recognize the EboGPΔM-9CE. It has been shown that single amino acid changes in the conserved epitopes and generally hydrophobic regions of the gp120

glycoprotein have resulted in disrupted recognition and targeted by the neutralizing antibodies. Also, in some cases, it has result in the generation of neutralization escape mutants[227]. One hydrophobic element is the ring-like structure formed by two disulphide bonds, which link C3 and C4. Also, The hydrophobic C2 epitope spanning from arginine 252 to asparagine 262 contributes to the two neutralizing antibodies, 17b and 48d[227,228].

As depicted in **Figure 4. 4. b**, a significant reduction in the level of Gagp24 expression in the EboGPΔM-MPER-VLPs determined by western blotting. This result suggests that EboGPΔM-MPER expression might block transcription and subsequently expression HIV-1 genes, including Gag p24. Thus, EboGPΔM-MPER based vaccine may also play a role as a negative regulator against the replication of HIV-1. In addition, our *in vivo* study, EboGPΔM-MPER-VLPs, elicited robust antibody responses against the Gagp24, which are detectable at high titers after the second VLP immunization, indicating that EboGPΔM-MPER based vaccine has a robust stimulating activity to induce broad anti-HIV immunity.

Overall, the enhanced HIV-specific immune responses increased by the EboGP-pseudotyped HIV VLPs suggest its capability to increase HIV-specific immune responses as a novel DC-based vaccine strategy.

Future direction

Since HIV-1 conserved epitopes are the target of neutralizing antibodies, the neutralizing ability in the mice sera will be investigated in a future experiment. Neutralizing antibodies against HIV-1 Env effectively prevents infection with chimeric SIV–HIV (SHIV) viruses in macaques. Also, a series of monoclonal antibodies with the potency to neutralize HIV-1 have been isolated from HIV-1 infected patients[229,230]. Neutralization breadth acquisition is necessary for vaccine development since it can avoid immune escape caused by envelope variants[231]. One of the advantages of neutralization assay could that each pseudovirus has a well-defined clonal sequence and that large panels of diversity in HIV-1 Env's could be constructed rapidly, resulting in better representing the global HIV-1 genetic diversity. Moreover, by screening large panels of HIV-1 sera, we can identify the individual donors and choose the best neutralizing antibody that will be able to neutralize the majority of HIV-1 strains[232].

Measuring the spenocytes cytokine and chemokine level might be essential to compare the induction of systemic and mucosal inflammatory immune responses to the different types of vaccine immunogens. Analysis of the inflammatory responses to the immunogens in other mucosal sites like vaginal secretion could also be helpful. Moreover, it would also be informative to investigate which specific cell types produce certain types of cytokines and chemokines at each time point.

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